Developing molecular diagnostics for the detection of strawberry viruses (follows BS07003)

Dr Fiona Constable
Victorian Department of Primary Industries (VICDPI)

Project Number: BS10002

BS10002

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the strawberry industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the Department of Employment, Economic Development and the Victorian Strawberry Industry Certification Authority.

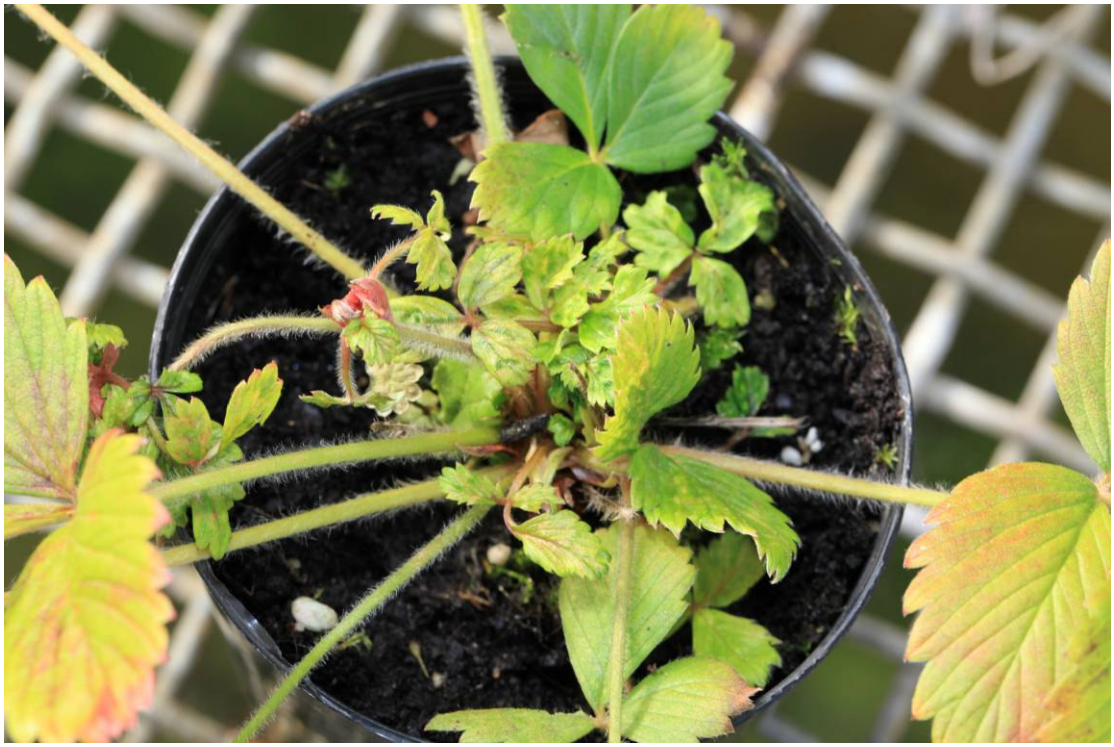
ISBN 0 7341 2685 9

Published and distributed by:
Horticulture Australia Ltd
Level 7
179 Elizabeth Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399

HAL

Horticulture Australia

BS10002: Developing molecular diagnostics for the detection of strawberry viruses (continues BS07003)



Final report for Horticulture Australia project BS10002

31 August 2011

Prepared by:

Fiona Constable, Chris Bottcher, Geoff Kelly, Narelle Nancarrow, Mirko Milinkovic and Brendan Rodoni

Department of Primary Industries Knoxfield

and

Lien Ko and Denis Persley

Department of Employment, Economic Development and Innovation

Victoria
The Place To Be

HAL
Know-how for Horticulture™

Queensland Government

ACKNOWLEDGMENTS

This project has been funded by HAL using voluntary contributions from Victorian Strawberry Industry Certification Authority (VSICA), Department of Employment, Economic Development and Innovation (DEEDI) and matched funds from the Australian Government. We would like to acknowledge and thank VSICA, Horticulture Australia, the Victorian Department of Primary Industries and Department of Employment, Economic Development and Innovation for their support. Many thanks also to Mark Whattam (Australian Quarantine Inspection Service) and Dr. Peter Merriman (VSICA) for their input and helpful discussions, which made this research successful. We would also like to thank the following overseas researchers who provided helpful discussions and information for the tests that were developed: Dr. R Martin, Dr. W Jelkmann and Dr J Thompson.

MEDIA SUMMARY

Certified strawberry runners are supplied through the Victorian and Queensland strawberry certification schemes to most strawberry fruit growers across Australia. These strawberry runners are certified as high health planting material and have been derived from high quality nucleus plants. The nucleus collection is indexed annually for the major strawberry viruses including *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) and Strawberry necrotic shock virus (formally thought to be *Tobacco streak virus*), as well fungal diseases. It is a fundamental requirement of the certification scheme that these pathogens are not detected in any strawberry plant within the nucleus collection. A strawberry nucleus collection has been maintained for the Victorian strawberry industry for nearly 50 years and these high health plants have contributed greatly to increased yields for strawberry growers due to the exclusion of the major pathogens from industry.

Scientists from DPI-Knoxfield have developed rapid and sensitive molecular diagnostic tests for the detection of endemic viruses of strawberries that are tested for in Australian certification schemes. 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.

In the past, detection of these viruses has relied on the use of the biological indexing method of petiole grafting onto sensitive indicator species. While this method is reliable and sensitive it is labour intensive, expensive and is time consuming taking 6-8 weeks for results based on symptom expression. Additionally, it can only be reliably done in the spring and early summer months of each year. Adoption of the molecular based tests, such as PCR will offer 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.

A “dummy nucleus” of strawberry cultivars that were inoculated with a range of viruses was established and maintained under the same conditions as the Victorian strawberry nucleus plants. These plants were tested monthly for viruses during three growing seasons to determine the reliability of the PCR tests that were developed by DPI. The results indicate that there is a seasonal effect on detection of some viruses in strawberries and spring (October-December) and autumn (March-May) are the best times for virus detection in Victoria.

In November 2010 a replicate “dummy nucleus” was also established in Queensland and the plants were tested monthly until July 2011. The results indicated that virus detection was variable under these conditions and there was no single month in which all viruses were reliably detected. It is therefore recommended that under these climatic conditions that two PCR tests are conducted each year in December - January and again in July.

Molecular tests were validated for eight high priority pests and diseases that pose a quarantine risk to the local Strawberry industry and must be tested for during post entry quarantine (PEQ) including: *Xanthomonas fragariae* (angular leafspot), *Phytophthora fragariae* var. fragariae (Strawberry red stele), *Arabis mosaic nepovirus* (ArMV), *Raspberry ringspot nepovirus* (RpRSV), *Tomato ringspot nepovirus* (ToRSV), *Tomato black ring nepovirus* (TBRV) *Strawberry latent ringspot sadwavirus* (SLRV) and *Tomato bushy stunt tombusvirus*, (TBSV).

The protocols for both endemic viruses and quarantine pathogens have been incorporated into a pathogen-testing manual that can be used by pathologists and industry in Australia. The protocols combine traditional biological indexing with the new molecular methods to improve the stringency of strawberry virus testing for the Australian strawberry industry. The molecular tests have also been incorporated into DPI-Knoxfield's fee for service unit (Crop Hygiene – Crop Health Services) and will support the Australian strawberry certification schemes. The technology has also been transferred to Queensland pathologists.

TECHNICAL SUMMARY

The supply of high-health, certified strawberry runners throughout Australia is dependent on the collections of nucleus plants maintained in Victoria and Queensland. These certification schemes have been operating for many years and have contributed greatly to increased yields and quality of fruit for strawberry growers by excluding these pathogens. Both the Victorian and Queensland collections are tested annually in spring using a biological indexing method of petiole grafting candidate tissue onto sensitive indicator species for several virus associated diseases. Biological indexing is reliable and sensitive only if done in spring or early summer, is labour intensive, expensive and time consuming, taking 6-8 weeks to generate a result.

Advances in molecular techniques have been published for 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 BS04004, "Developing molecular diagnostics for the detection of strawberry viruses" molecular based RT-PCR tests for these viruses were adopted from international, peer-reviewed literature and developed on positive control plants, maintained 12 months of the year in glasshouse conditions. The molecular tests are rapid, cost effective and sensitive when compared to biological indexing.

The aim of HAL project BS07003 and this project (BS10002) was to conduct a transitional validation phase of the molecular diagnostic assays to assist their integration and implementation into pathogen testing protocols for production of certified strawberry runners. In December 2008 a "dummy nucleus" of virus infected varieties was created and maintained and handled in a similar manner to the current Victorian nucleus collection. To create the "dummy nucleus", two strawberry varieties (3 replicates of each) were inoculated with three virus combinations. The nucleus plants from the 2008/09 season were used to generate daughter plants that became the nucleus plants in 2009/10. The 2009/10 nucleus plants were also used to generate daughter plants that became the "dummy nucleus" plants in 2010/11. In each year the "dummy nucleus" plants were tested by biological indexing and PCR to give confidence that the molecular tests would reliably detect strawberry viruses in plants that were grown in the same conditions in which the Victorian nucleus collection is maintained. In 2010/11 the ""dummy nucleus" was also replicated in Queensland and these strawberry plants were maintained in a similar manner to the Queensland nucleus collection. These plants were also tested by biological indexing and the molecular methods developed in this project.

The 2008/09 "dummy nucleus" was tested for viruses from December 2008, just prior to graft inoculation, until May 2009 and again from October 2009 until May 2010. The 2009/10 "dummy nucleus" was tested from October 2009 – May 2010 and the 2010/11 "dummy nucleus" was tested from November 2010-May2011. PCR testing of the 2008/09 Victorian

"dummy nucleus" collection showed that viruses may not be reliably detected in strawberry plants during the first six months post-inoculation. Reliable detection was only achieved during the following season. PCR testing revealed that the rate of virus transmission from mother to daughter plants can reach 100% for most viruses. The results of virus testing of both the 2008/09 and 2009/10 Victoria dummy nucleus plants during the 2009/10 season and the Victorian 2010/11 nucleus collection during the 2010/11 season suggest that spring (October-December) is the most reliable time for virus detection in Victoria. In some years autumn (March-May) may also be a reliable time for virus detection by molecular methods.

During the 2010/11 season the "dummy nucleus" collection in Victoria was replicated in Queensland using virus infected plants produced from the 2009/10 Victorian "dummy nucleus" collection. Plants in the Queensland "dummy nucleus" collection were also tested monthly for viruses using the molecular based protocols for endemic viruses. Unlike the Victorian results there did not seem to be a trend for the timing of detection of all virus species and no single month or season appears to be adequate for the detection of all virus species. Based on the results of the virus testing of the 2010/11 Queensland dummy nucleus July 2011 was the best time for the detection of SPaV and SmoV while SNSV, SCV and SMYEV were most frequently detected in December 2010, January 2011 and February 2011 respectively. Continued testing of the 2010/11 Queensland dummy nucleus from August-October 2011 and a further season of similar research may assist in forming a recommendation for the timing of detection by PCR, until then it may be useful to perform molecular virus testing in December-January and again in July in Queensland.

The results of three years of monthly PCR testing during the growing season indicated that strawberry virus detection by molecular methods in the third season after an infection event was no more efficient than the second season. Also, molecular indexing for strawberry viruses can be conducted in spring and autumn of the same season in Victoria. Consequently molecular indexing in spring and autumn of the first year and again in spring of the second year in combination with biological indexing conducted in spring of both years could be used to virus index new varieties prior to their introduction into the Victorian nucleus collection. This regime improves the stringency of strawberry virus testing while reducing the time before introduction into the nucleus collection from three years to two years. This will 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.

The results indicated that biological indexing is less reliable for virus detection than PCR techniques as many of the inoculated indicators that were expected to show symptoms were symptomless. PCR testing revealed that multiple viruses can be transmitted and that it was difficult to associate symptoms with specific viruses. However, the PCR tests were also not 100% reliable because there were only a few months in which viruses were detected in all known infected plants. This highlights the importance of using a combination of both molecular and biological tests for certification. Biological indexing remains useful for the

detection of strains of viruses which may not be detected using the PCR test that were developed and for other viruses for which tests have not been developed.

Molecular tests were validated for eight high priority pests and diseases that pose a quarantine risk to the local Strawberry industry and which are tested for during post entry quarantine (PEQ) including: *Xanthomonas fragariae* (angular leafspot), *Phytophthora fragariae* var. fragariae (Strawberry red stele), *Arabis mosaic nepovirus* (ArMV), *Raspberry ringspot nepovirus* (RpRSV), *Tomato ringspot nepovirus* (ToRSV), *Tomato black ring nepovirus* (TBRV) *Strawberry latent ringspot sadwavirus* (SLRV) and *Tomato bushy stunt tombusvirus*, (TBSV).

Based on the research of this project the previous diagnostic protocols for the production of pathogen tested strawberries in Australia have been updated to include validated molecular tests for virus detection. Specifically the following pathogen testing regimes for the introduction of new strawberry varieties into the Victorian nucleus collection and for varieties maintained in the Victorian nucleus collection have been recommended:

The following pathogen testing regime is recommended for new varieties before introduction into the Victoria nucleus collection:

1. Pathogen testing is conducted over a two year period.
2. All plants must be tested for virus associated diseases using biological indexing in spring of each year and the inoculated indicator plants must be free of symptoms which are indicative of virus infection.
3. All plants must be tested for viruses using molecular indexing in spring (October-December) and autumn (April-May) in both years of screening and none of the prescribed viruses should be detected.

The following pathogen testing regime is recommended for varieties maintained in the Victorian nucleus:

1. Plants that are continually maintained in the nucleus collection for two years or more will be pathogen tested for the specified viruses by biological indexing in spring every second year. In the alternate year they will be indexed in spring by molecular methods.
2. New varieties must undergo molecular indexing for viruses in spring during their first year in the nucleus collection.

The protocols have been incorporated into a pathogen testing manual for the Victoria strawberry runner certification program. With further research this manual can be adapted for the pathogen tested strawberry runner program in Queensland also using the results of our research.

Based on the results of this project similar testing regimes could also be adopted for Queensland except that:

1. Three years of molecular and biological indexing may be required for new varieties entering a Queensland nucleus collection;
2. Molecular indexing should be carried out twice in each year in December-February and again in July;

3. Molecular indexing may be required in each year for plants maintained in a nucleus collection;
4. Biological indexing could be done biennially.

The incorporation of the protocols into Australia's certification programs and PEQ will ensure the production of high quality pathogen-tested planting material using world's best practice diagnostic capabilities for strawberry certification. This diagnostic capability will be made available to the Australian strawberry industry on a fee-for-service basis through commercial diagnostic laboratories such as Crop Health Services, (Victorian DPI).

CHAPTER 1

INTRODUCTION

Strawberry certification schemes supply certified runners throughout Australia. The strawberry runners are certified on the basis of their high health status and are derived from collections of nucleus–plants maintained in high security facilities. Nuclear collections are tested annually for virus-associated diseases including Strawberry mild yellow edge, Strawberry crinkle, Strawberry mottle, Strawberry vein banding, Strawberry necrotic shock and Pallidosis diseases of strawberries using biological indexing (Whattam 1994). The nucleus collections are also tested for fungal and bacterial diseases. The Victorian Strawberry Certification Authority (VSICA) certification scheme has been operating for nearly 50 years in Victoria and together with other certification schemes in operation in Australia they have contributed greatly to increased yields and quality of fruit for strawberry growers by excluding these pathogens.

The nucleus collections are tested for virus associated diseases using biological indexing methods of petiole grafting candidate tissue onto sensitive indicator species (Frazier 1974). In a temperate climate this method is only reliable and sensitive during the spring and early summer. Biological indexing is also labour intensive, expensive and time consuming, taking 6-8 weeks to generate a result. Molecular indexing via the polymerase chain reaction (PCR) offers the Australian strawberry industry a more rapid and cost effective method of virus 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. Recent advances in molecular techniques have been published overseas for the detection of most of the viruses that infect strawberry plants (Thompson et al 2003; Tzanetakis et al 2003; Thompson and Jelkmann, 2003).

HAL project BS04004 demonstrated the experimental application of PCR for the detection of strawberry viruses including *Strawberry crinkle cytorhabdovirus* (SCV), *Strawberry mottle sadwavirus* (SMoV), *Strawberry mild yellow edge potexvirus* (SMYEV), *Strawberry vein banding caulimovirus* (SVBV), Strawberry necrotic shock virus (SNSV) and *Strawberry pallidosis associated crinivirus* (SPaV) and *Beet pseudos yellows crinivirus* (BPYV), which are both associated with pallidosis disease (Constable et al 2007). The PCR tests that were developed are rapid, cost effective and sensitive when compared to biological indexing. The tests were developed on positive control plants that were maintained 12 months of the year under glasshouse conditions. The results indicated that the viruses were most reliably detected by PCR during May-October under glasshouse conditions. These tests were partially validated by surveying strawberry plants from Queensland, Western Australia and Victoria and the results indicated that the tests can detect viruses in a field situation. Project BS04004 identified that a transitional phase was required to complete the implementation of the PCR tests for detection of the endemic strawberry viruses into a national standard for certification of strawberry runners in Australia. This would ensure that a diagnosis based on a PCR test is

as reliable as biological indexing and could be used by industry as part of the routine certification of strawberry runners.

In HAL project BS07003 a transitional phase was established in which the PCR tests for endemic viruses were further validated and adapted using a virus infected “dummy nucleus”. During BS07003 the “dummy nucleus” was routinely tested for viruses during the growing season and it was demonstrated that viruses may not be reliably detected in a nucleus plant until the season after an infection event occurred. It was also demonstrated that virus detection was most reliable in spring (October and November) and autumn (March until May) when virus infected plants were maintained in a similar manner to the nucleus collection. The aim of this project was to continue the validation of the protocols for another growing season to verify these results. Current certification scheme rules require three years of negative biological indexing conducted in spring before a new cultivar is included in a nuclear collection and made available to industry. If several PCR tests can be conducted on a candidate plant within the one growing season, it may be possible for a new variety to be incorporated into a nucleus collection in less than three years.

The validated molecular assays, developed in BS04004, BS07003 and this project (BS10002), are the recommended protocols for virus detection and as a part of this project they have been summarised in a draft pathogen-testing manual that will form the basis of a national certification standard. This draft manual will be submitted to the strawberry industry for consideration.

New varieties of strawberries are imported into Australia in tissue culture and these plants are grown and tested for the presence of fungi, bacteria and viruses using a range of biological indexing and visual observations. The Australian strawberry industry has identified a number of high priority pests and diseases that pose a quarantine risk to the local industry including *Xanthomonas fragariae* (angular leafspot), *Phytophthora fragariae* var. fragariae (Strawberry red stele), *Arabis mosaic virus* (ArMV), *Raspberry ringspot virus* (RpRSV) *Tomato ringspot virus* (ToRSV) and *Tomato black ring virus* (TBRV) of the genus nepovirus and by *Strawberry latent ringspot Sadwavirus* (SLRSV), *Tomato bushy stunt tombusvirus* (TBSV) and *Strawberry latent C rhabdovirus* (SLCV). Initial screening for the presence of key exotic pathogens of strawberries by PCR could be used in PEQ as a first line of defence and it may be possible to combine both the molecular and biological tests in an indexing strategy to increase biosecurity during the strawberry PEQ process. PCR tests have been developed overseas for the detection of *X. fragariae, P. fragariae var. fragariae*, ArMV, RpRSV, ToRSV, TBRV, SLRSV, TBSV and SLCV. Preliminary screening using molecular based tests would increase the efficiency of PEQ for strawberries and would also aid AQIS to make a rapid decision about the health status of the imported plant material. In BS07003 molecular assays for the detection of these pathogens were identified. This project will also demonstrate the capacity of PCR to detect exotic strawberry pathogens by validating them using field collected

strawberry samples. The outputs will be summarised as a series of recommendations for consideration by the Australian Quarantine Inspection Service and Biosecurity Australia.

Efficient pathogen testing protocols in PEQ and in the certification programs will 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.

Aim

This project will update and enhance the capability of Australian diagnostic laboratories to accurately and efficiently detect economically significant viruses of strawberries that are tested for annually using biological indexing in Australian strawberry certification schemes.

Research activities will centre around three objectives:

1. Complete the validation of the molecular based diagnostic assays for the detection of endemic strawberry viruses and integrate these tests into standard operating procedures for the strawberry runner certification scheme; at a national level;
2. Identify and validate appropriate protocols for the detection of quarantineable viruses, angular leafspot and red stele in strawberries;
3. Develop a pathogen testing manual for the strawberry industry.

CHAPTER 2

THE INTEGRATION OF THE MOLECULAR BASED DIAGNOSTIC PROTOCOLS FOR THE DETECTION OF ENDEMIC STRAWBERRY VIRUSES INTO THE STANDARD OPERATING PROCEDURES FOR THE STRAWBERRY RUNNER CERTIFICATION SCHEME.

Introduction

HAL project BS04004 demonstrated the experimental application of PCR for the detection of strawberry viruses including *Strawberry crinkle cytorhabdovirus* (SCV), *Strawberry mottle sadwavirus* (SMoV), *Strawberry mild yellow edge potexvirus* (SMYEV), *Strawberry vein banding caulimovirus* (SVBV), Strawberry necrotic shock virus (SNSV) and *Strawberry pallidosis associated crinivirus* (SPaV) and *Beet pseudos yellows crinivirus* (BPYV), which are both associated with pallidosis disease (Constable et al 2007). The PCR tests that were developed are rapid, cost effective and sensitive when compared to biological indexing. The tests were developed on positive control plants, maintained 12 months of the year in glasshouse conditions. Our results indicated that the viruses were most reliably detected by PCR during May-October under glasshouse conditions. These tests were partially validated by surveying strawberry plants from Queensland, Western Australia and Victoria and our results indicated that the tests can detect viruses in a field situation. However, project BS04004 identified that a transitional phase was required to complete the implementation of the PCR tests for detection of the endemic strawberry viruses into a national standard for certification of strawberry runners in Australia because the tests were developed on virus infected plants maintained under different conditions to the strawberry nucleus collection.

In BS07003 a “dummy nucleus” of virus infected varieties was established, which was maintained and handled in a similar manner to the current strawberry nuclear collection held at DPI, Knoxfield. During BS07003 and the current project this “dummy nucleus” was tested by biological indexing and PCR over several growing seasons from December 2008, just after inoculation, until May 2011. In 2009/10 and 2010/11 the “dummy nucleus” plants comprised of daughter plants generated from the previous season’s virus-infected mother plants. The aim of this experiment was to provide confidence that the molecular tests will detect viruses in strawberry plants maintained under the same conditions experienced by the nucleus collection currently held in Victoria.

Materials and methods

Establishment of the “dummy nucleus”

In December 2008 two varieties were inoculated with three virus combinations (5 replicates of each variety-virus combination) to produce a primary “dummy nucleus” collection of 30 plants. The virus infected plants used for graft inoculation were maintained in the glasshouse at DPI Knoxfield and included positive control CV12 infected with SMoV, SMYEV and SPaV; CV20 infected with SPaV, SCV and SMYEV; and CV22 infected with SPaV and SNSV. The inoculated plants were maintained in the screen house for the remainder of the growing season and until May 2010, the end of the next growing season. During January 2009 – April

2009 a total of 135 daughter plants were produced from the inoculated 2008/09 mother plants, and all of these plants formed the virus infected "dummy nucleus" collection in 2009/10. During January 2010 – April 2010 a total of 190 daughter plants were produced from the 2009/10 mother plants, and 100 of these formed the Victorian virus infected "dummy nucleus" collection for the 2010/11 season. The remaining 90 daughter plants were used to form the Queensland virus infected "dummy nucleus" collection for the 2010/11 season.

Maintenance of the "dummy nucleus" plants

Victoria: In each season (2008/09, 2009/10, 2010/11) the Victorian dummy nucleus plants were grown in 4.5L pots containing pasteurised general potting media (Debco) with a medium-term, slow-release fertiliser and maintained in a screen house during the growing season. In 2008/09 the plants were grown in the screen house from December 2008 until May 2009, in the 2009/10 and 2010/11 season the plants were maintained from September until May. The mother plants were used to produce daughter plants that would become the "dummy nucleus collection in the following season. The daughter plants were initially established in 0.1L pots containing pasteurised general potting media (Debco) with a medium-term, slow-release fertiliser and were also maintained in a screen house during the same season. The daughter plants were placed into cold storage at -2℃ between June and August of each season. At the beginning of September all of the plants were removed from cold storage and re-potted as mother plants as described above.

Queensland: In 2009/10 daughter plants were 2010/11 a "dummy nucleus" collection was also established in Queensland. The Queensland "dummy nucleus" plants were grown in 200 mm pots containing steam sterilised Power Blend potting media (composted bark and 10% sand, pH 5.8 from Growing Media Qld. Pty. Ltd.) with fortnightly application of Aquasol liquid fertiliser. Pots were maintained in a glasshouse at a constant temperature of 28℃ and additional lighting from mid-December 2010 to mid-April 2011 (Grolux fluorescent tubes).

Sampling

Each sample for PCR testing consisted of 2-3 leaves, with petioles attached.

2008/09 season: The original 30 plants of the 2008/09 Victorian "dummy nucleus" collection were tested in December 2008 for SCV, SMoV, SMYEV, SPaV, BPYV, SNSV and SVBV prior to inoculation to determine their virus status prior to graft inoculation of viruses. After grafting the inoculated mother plants were tested monthly from four weeks post inoculation in January 2009 until May 2009.

2009/10 season: Virus testing was conducted from October 2009 until May 2010. All of the 30 2008/09 Victorian "dummy nucleus" plants were tested for SCV, SMYEV, SPaV, BPYV and SNSV and SVBV in each month. The plants in the 2009/10 Victorian "dummy nucleus" were tested only for the viruses that had been detected in the mother plants from which they were derived in the previous year: 101/135 were tested for SMYEV, 89/135 were tested for SPaV, 25/135 were tested for SCV, 19/135 were tested for SNSV and 5/135 were tested for BPYV.

Although SMoV was not detected in any mother plant in the 2008/09 “dummy nucleus”, all of the 2009/10 Victorian “dummy nucleus” plants (135/135) were tested for this virus.

2010/11 season: Virus testing of the Victorian “dummy nucleus” was conducted from November 2010 until May 2011. All of the 100 2010/11 Victorian “dummy nucleus” plants were tested for SMoV, SCV, SMYEV, SPaV, BPYV and SNSV in each month. Virus testing of the Queensland “dummy nucleus” was conducted from November 2010 until July 2011. All of the 90 2010/11 Queensland “dummy nucleus” plants were tested for SMoV, SCV, SMYEV, SPaV and SNSV in each month.

Biological indexing of the “dummy nucleus”

Victoria: In December 2009 30 plants of the 2009/10 the “dummy nucleus” collection (Table 2) were leaf grafted onto two plants each of the strawberry virus indicators UC-4, UC-6 and UC-10. During the 2010/11 season the indicators plants were not sufficiently advanced to graft each of the indicator varieties at once. Consequently, 24 plants of the 2010/2011 “dummy nucleus” collection (Table 2) were grafted onto UC-4 indicators in December 2010, UC-6 indicators in January 2011 and UC-10 indicators in February 2011. Two leaves on two plants of each indicator were grafted with leaflets collected from the “dummy nucleus” plants (total of four grafts per indicator variety). The grafted indicators were gown in a mist bed for 1-2 weeks after grafting and then moved to a bench and maintained in the glasshouse under normal daylight conditions, at 18-24℃, The indicator plants were observed for symptom development over three months.

In April 2010 two leaves, including petioles, were collected from 21 (Table 2) of the grafted indicator plants of which six UC-4, five UC-6 and four UC-10 indicators were symptomatic of virus infection and two UC-4 and four UC-6 indicators which did not have symptoms. In April 2011 two leaves, including petioles, were collected from 24 grafted indicator plants including eight UC-4, 11 UC-6 and five UC-10 indicators (Table 2) and these were tested for SCV, SMoV, SMYEV, SPaV, BPYV, SNSV and SVBV. The remaining indicators were not tested due to time constraints.

Queensland: Graft inoculation was carried out in January 2011. Leaves were collected from groups of dummy nucleus plants that had originated from the same 2010/11 mother plant and pooled for inoculation of UC-4, UC-6 and UC-10 indicator plants. The nucleus plant-indicator combination is listed in table 5. Depending on the number of candidate leaflets available two leaves on two or three plants of each indicator were grafted with leaflets collected from the “dummy nucleus” plants (total of 4-6 grafts per indicator variety). Two positive control plants that were known to be affected by virus associated diseases were also used to inoculate UC-4, UC-6 and UC-10 indicator plants: three leaves of two plants each of UC-4, UC-6 and UC-10 were grafted with the positive control plants (Table 5). Grafting success rate was determined after 2 weeks of growing in a glasshouse under natural temperature and light conditions. Unsuccessful grafts were repeated at the end of January 2011. The grafted indicators were maintained in a glasshouse under natural temperature and light conditions

and six weeks after grafting, symptom development on the indicators was assessed. In April 2011 petiole samples were collected from the 48 grafted indicator plants for molecular testing.

Nucleic acid extraction

Total nucleic acid was extracted from 0.3g of strawberry petiole tissue using a modified lysis buffer (MacKenzie et al 1997) and the QIAxtractor (Qiagen). Briefly, 100-300mg of strawberry tissue was ground in 3ml of the modified lysis buffer and 1ml of each homogenate was transferred into separate wells of pre-racked 1.1ml strip minitubes arranged in standard 96-well format (Pathtech), each containing 100 µl of 20% N-lauroylsarcosine. The samples were incubated to 65℃ for 15 minutes. The rack of tubes was centrifuged at 1500 rpm for 5 minutes to clarify the liquid and 400 µl of each sample was transferred to another rack of 1.1ml strip minitubes. The second rack of tubes was placed in the QIAxtractor and 200 µl of 100% ethanol was added to each sample and the samples were mixed by aspiration. Four hundred microlitres of the mixed samples were then added to the 96-well 800 µl long drip Unifilter capture plate (Whatman) and the samples drawn through each well for 5 minutes at a vacuum pressure of 45 Kpa. Five hundred microlitres of propenol wash buffer (Sigma) was loaded onto each well of the capture plate and allowed to incubate for one minute before being drawn through at 50 Kpa for five minutes. The samples were then washed twice with 500 µl of 100% ethanol at 45 Kpa for five minutes. A further vacuum step was done at 40 Kpa for ten minutes to remove all traces of ethanol. The capture plate was then transferred robotically to a 96 well elution plate (Qiagen) and 200 µl of molecular grade water was added to each well of the capture plate and allowed to incubate at room temperature for 2 minutes before being drawn through to the elution plate for ten minutes at 45 Kpa to elute the RNA.

If the nucleic acid extracted from the strawberry petiole samples using the QIAxtractor was not of sufficient quality to be used in RT-PCR, RNA extraction was done using the RNeasy® Plant Mini Kit (QIAGEN Pty Ltd, Doncaster, VIC Australia) as described previously (MacKenzie et al 1997).

RT-PCR

The one-step RT-PCR protocols developed and recommended in the previous HAL project BS04004 were used for the detection of the NADH dehydrogenase ND2 subunit (ndhB gene), which is used to determine the quality of the extracted RNA, and for the detection of SCV, SMoV, SMYEV, SPaV, BPYV, SNSV and SVBV (Constable et al 2007).

Gel electrophoresis

After amplification, 10 µl from each PCR reaction was subjected to electrophoresis in a 1-2% agarose gel using 0·5 × 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).

Results

PCR

Virus testing of the plants used to establish the 2008/09 "dummy nucleus" indicated that 13/15 plants of Variety 2 (V2) were infected with SMYEV in December 2008 prior to inoculation (Table 1, Figure 1). SMYEV was not detected in Variety 1 (V1) and no other viruses were detected in any of the 30 plants. In January 2009, four weeks post inoculation, SCV was detected in 1/15 plants of V2, SPaV in 1/15 plants of V1 and in 11/15 plants of V2 and SMYEV was detected in 3/15 plants of V1 and 14/15 plants of V2 (Figure 1). By May 2009 all "dummy nucleus" plants (V1 + V2, total 30 plants) were infected with at least one virus: BPYV was detected in 1/30 plants, SNSV was detected in 5/30 plants, SCV was detected in 7/30 plants, SPaV was detected in 21/30 plants and SMYEV was detected 23/30 plants (Table 1). SVBV and SMoV were not detected.

Table 1. The total number of plants of each variety and the combined total for the 2008/09 "dummy nucleus" that were positive for BPYV, SPaV, SCV, SMoV, SNSV and SMYEV in 2008/09 and 2009/10

Virus	Total number of plants inoculated with each virus		Total number positive plants – 2008/09			Total number positive plants – 2009/10		
	Variety 1	Variety 2	Variety 1	Variety 2	Total	Variety 1	Variety 2	Total
BPYV	0	0	0/15	1/15	1/30	0/15	1/15	1/30
SPaV	15	15	9/15	12/15	21/30	14/15	15/15	29/30
SCV	5	5	3/15	4/15	7/30	6/15	5/15	11/30
SNSV	5	5	3/15	2/15	5/30	5/15	6/15	11/30
SMYEV	10	10	8/15	15/15*	23/30	8/15	15/15	23/30
SMoV	5	5	0/15	0/15	0/15	0/15	0/15	0/15

*SMYEV was detected in 13/15 plants of V2 prior to inoculation

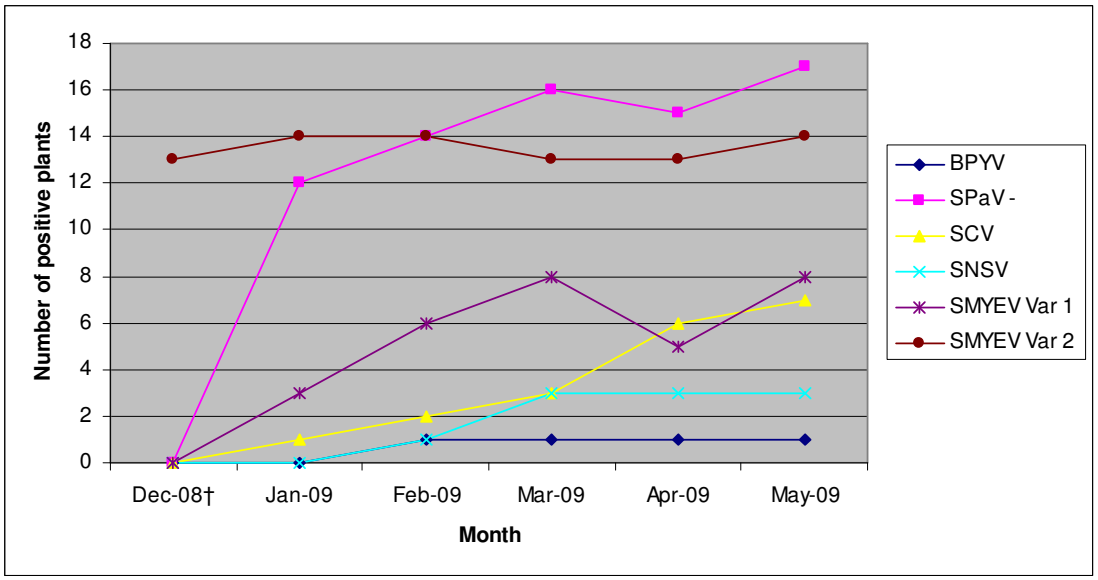


Figure 1. The number plants in the 2008/09 "dummy nucleus" that tested positive for BPYV, SPaV, SCV, SNSV and SMYEV in December 2008, prior to graft inoculation, and monthly from January 2009 - May 2009 after graft inoculation. For each virus except SMYEV these are the combined results for both varieties (30 plants). The results for SMYEV in V1 and V2 (15 plants each) are shown separately because 13/15 plants of V2 were known to be infected with SMYEV prior to inoculation.

Virus testing of the 2008/09 "dummy nucleus" plants continued in the 2009/10 season. By May 2010 the number of plants in which SPaV, SNSV and SCV were detected increased to 29/30, 11/30 and 11/30 respectively but the number of plants infected with BPYV and SMYEV did not change (Table 1). The number of plants which tested positive for each virus was variable throughout the season (Figure 2). The lowest number of positive results was obtained in October for SCV, in February for SMYEV, in October and February for SNSV and in November and February for SPaV. The highest number of positive results was obtained in December, January and April for SCV, November for SNSV, January and May for SPaV and December and May for SMYEV.

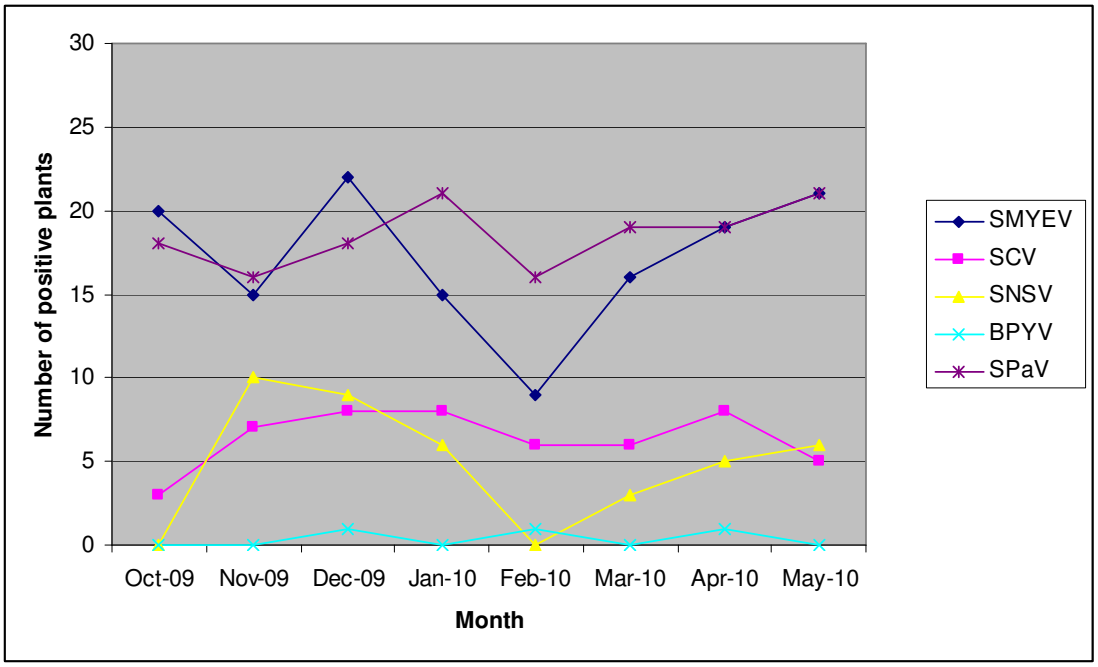


Figure 2. The number plants in the 2008/09 "dummy nucleus" that tested positive for each virus in the 2009/10 season from October 2009 - May 2009.

The number of plants in the 2009/10 "dummy nucleus" collection that were expected to be infected with BPYV, SCV, SMoV, SMYEV, SNSV and SPaV due to vertical transmission of the viruses from the mother plants was determined from the number of daughter plants that were produced from the 2008/09 "dummy nucleus" mother plants. In the 2009/10 "dummy nucleus", the number of plants that were expected to be infected with each virus was: 101 plants infected with SMYEV, 89 plants infected with SPaV, 25 plants infected with SCV, 19 plants infected with SNSV and 5 plants infected with BPYV. Each virus was detected by PCR at least once in each of the plants in which it was expected to occur during October 2009 until May 2010 indicating that the rate of vertical transmission of each virus from mother to daughter plants was 100%. SMoV was not detected in the 2008/09 "dummy nucleus"" and it was assumed that none of the plants in 2009/10 "dummy nucleus"" were infected with this virus. However SMoV was detected in 11/20 of the indicators grafted with plants from the 2009/10 "dummy nucleus" (Table 2) which prompted testing of all of the 2009/10 "dummy nucleus" plants for this virus. During 2009/10 SMoV was detected in a total of 46/135 plants.

The number of plants in the 2009/10 "dummy nucleus" collection which tested positive for each virus varied throughout the 2009/10 season (Figure 3). The lowest number of positive results was obtained in November for SCV, SPaV and SNSV, in January for SMYEV, in March for SMoV and BPYV was not detected in November, March or May. The highest number of positive results was obtained in March for SMYEV, April for SNSV, April and May for SCV, May for SMoV and SPaV and in December, February and April for BPYV.

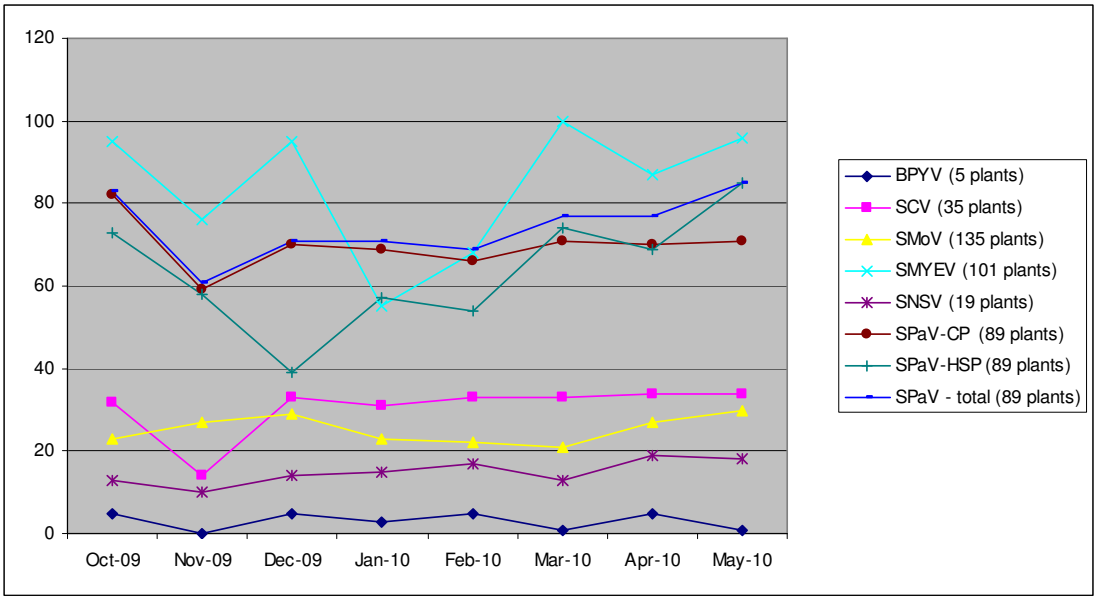


Figure 3. The number plants in the 2009/10 "dummy nucleus" that tested positive for each virus season from October 2009 - May 2010. The numbers in brackets against each virus acronym in the legend are the total number of plants that tested positive for each virus in the 2009/10 season. SPaV-CP refers to the results obtained for detection of the SPaV coat protein gene using the CP5'/CPn731R primer pair. SPaV-HSP refers to the results obtained for detection of the SPaV heat shock protein gene using the SP44F/SP44R primer pair.

The number of plants in the 2010/11 Victorian and Queensland "dummy nucleus" collections expected to be infected with BPYV, SCV, SMoV, SMYEV, SNSV and SPaV due to vertical transmission of the viruses from the mother plants was determined from the number of mother plants in the 2009/10 "dummy nucleus" infected with each virus and the number of daughter plants that were produced for the 2010/11 "dummy nucleus" from each mother plant. Although some of the Queensland "dummy nucleus" plants died throughout the season all plants were included in this calculation. The number of plants in the 2010/11 Victorian and Queensland "dummy nucleus" collections that were expected to be infected with each virus is given in Table 2. In both collections SCV, SMoV, SMYEV, SNSV and SPaV were not detected in all of the plants that were expected to be positive, based on the virus profile of the mother plant from which they were produced. In addition SCV, SMoV, SMYEV, SNSV and SPaV were detected in some plants which were not expected to be positive.

Table 2 The number of plants in the 2010/11 Victorian and Queensland "dummy nucleus" collections in which each virus was expected to be detected, the number of plants in which each virus was detected and the number of plants from which unexpected positive and negative results were obtained for each virus. The total number of plants in the 2010/11 Victorian and Queensland "dummy nucleus" collections that were tested for each virus was 100 and 89 respectively.

Virus	2010/11 – Victoria				2010/11 – Queensland			
	Expected number of positive plants	Number of positive plants detected	Unexpected positive results	Unexpected negative results	Expected number of positive plants	Number of positive plants detected	Unexpected positive results	Unexpected negative results
BPYV	0	0	0	0	Not Tested			
SCV	50	36	5	19	32	56	27	3
SMoV	42	25	6	23	28	80	56	4
SMYEV	69	74	13	8	61	65	10	6
SNSV	33	17	4	20	14	33	26	7
SPaV	82	80	2	4	70	81	14	3

The number of plants in the Victorian 2010/11 collection which tested positive for each virus was variable throughout the season although the overall trend for the detection of each virus was a small increase in the number of positive plants from November to December 2010 followed by a decline to March or April 2011 and then a small increase in the number of positive plants in May (Figure 4). The highest number of positive results was obtained in November 2010 for SNSV and December 2010 for SCV, SMoV, SMYEV and SPaV. The lowest number of positive results was obtained in January 2011 for SCV, May 2011 for SMoV and April 2011 for SMYEV, SNSV and SPaV.

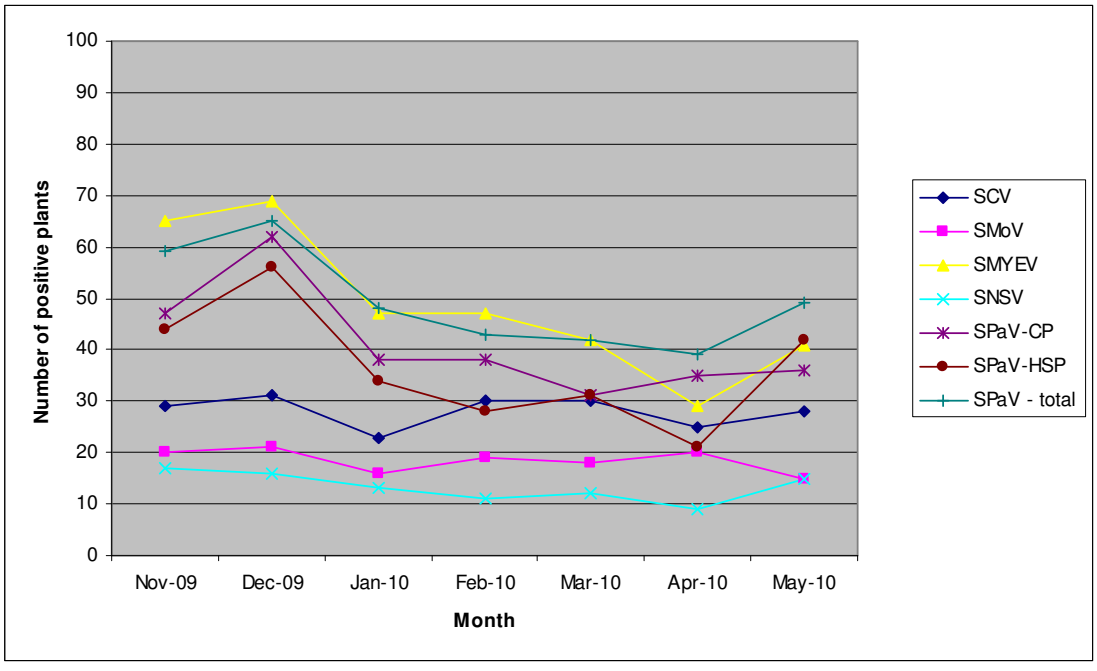


Figure 4. The number plants in the Victorian 2010/11 "dummy nucleus" that tested positive for each virus season from November 2010 - May 2011. The total number of plants tested for each virus was 100. SPaV-CP refers to the results obtained for detection of the SPaV coat protein gene using the CP5'/CPn731R primer pair. SPaV-HSP refers to the results obtained for detection of the SPaV heat shock protein gene using the SP44F/SP44R primer pair.

The number of plants in the 2010/11 Queensland "dummy nucleus" collection which tested positive for each virus was also variable throughout the season (Figure 5). The highest number of positive results was obtained in December 2010 for SNSV January 2011 for SMYEV, February for SCV and July for SMoV. When the results for the two PCR tests for SPaV are combined the highest number of positives was obtained in July 2011. The lowest number of positive results was obtained in March 2011 for SCV, December 2010 for SMoV, June 2011 for SMYEV and SPaV and February 2011 and July 2011 for SNSV.

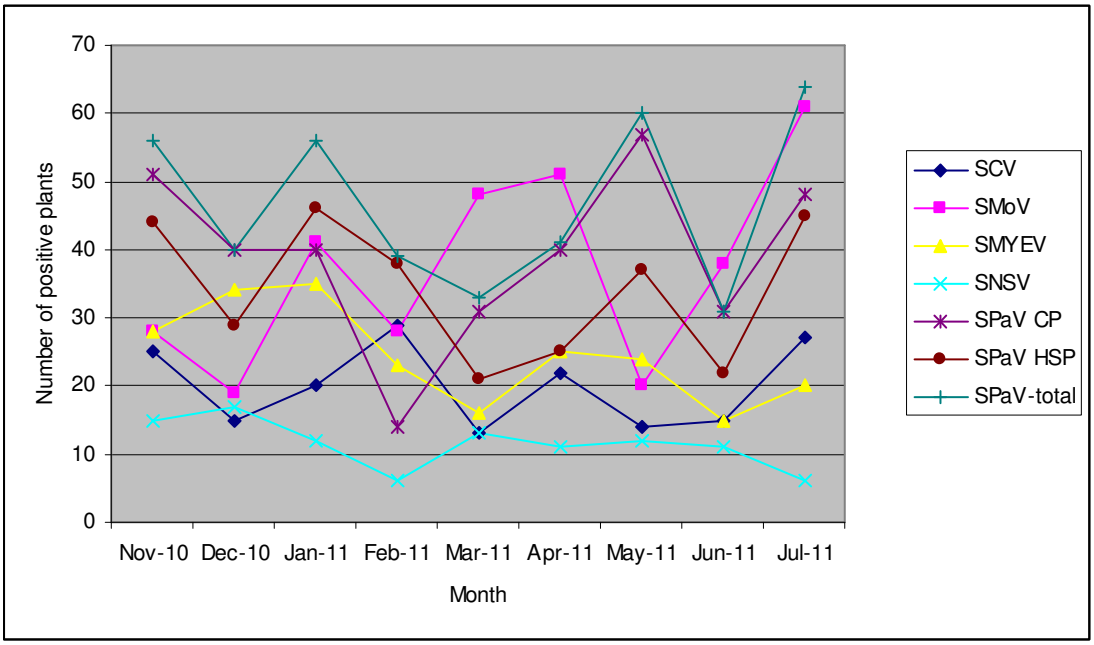


Figure 5. The number plants in the Queensland 2010/11 "dummy nucleus" that tested positive for each virus season from November 2010 - July 2011. Some plants died during the season and only the plants that were tested every month (73) are included in this graph. SPaV-CP refers to the results obtained for detection of the SPaV coat protein gene using the CP5'/CPn731R primer pair. SPaV-HSP refers to the results obtained for detection of the SPaV heat shock protein gene using the SP44F/SP44R primer pair.

Biological indexing

Leaf tissue from a selection of the 2009/10 (Table 3) and the 2010/11 (Table 4) "dummy nucleus" plants were grafted onto UC-4, UC-6 and UC-10 indicators and the indicators were observed for symptoms that may be associated with virus infection. Symptoms that were observed on the UC indicators included distortion, twisting, crinkling, flecking, mottling and chlorosis of the leaves and stunting of the plants.

2009/10 Victorian "dummy nucleus": Symptoms were most frequently observed on UC-4 indicators and 40/72 indicators were symptomatic. Only 7/72 UC-6 indicators and 23/72 UC-10 indicators displayed symptoms and all the symptomatic indicators had distortion of leaves. Based on the virus infected plant material with which each indicator was grafted, symptoms were expected in all UC-4 and all UC-10 indicators and in 24/72 UC-6 indicators.

Twenty one grafted indicators, including six symptomatic and two symptomless UC-4 indicators, six symptomatic and three symptomless UC-6 and four symptomatic UC-10

indicators, were selected for virus detection by PCR (Table 3). At least one virus and frequently multiple viruses were detected in all symptomatic and symptomless UC-4 and UC-6 indicators. Symptoms were expected in one of each of the symptomless UC-4 and UC-6 indicator plants because the viruses that were detected in each plant are associated with diseases that are expressed in these indicators. Two or more viruses were detected in two of the symptomatic UC-10 indicators plants only BPYV was detected in one of the symptomatic UC-10 indicators and viruses were not detected in the fourth symptomatic UC-10 indicator plant (Table 3).

2010/11 Victorian "dummy nucleus": Symptoms were most frequently observed on UC-6 indicators and 20/48 indicators were affected. Symptoms were observed on 13/48 UC-4 indicators and only 6/48 UC-10 indicators displayed symptoms (Table 4). Based on the virus infected plant material with which each indicator was grafted symptoms were expected in all UC-4 and all UC-10 indicators and in only 10/48 UC-6 indicators. Examples of the symptoms that were observed in UC-4, UC-6 and UC-10 indicators are shown in figure 1, figure 2 and figure 3 respectively.

Twenty four indicators, including eight UC-4, 11 UC-6 and five UC-10 indicators were selected for virus detection by PCR (Table 4). At least one virus and frequently multiple viruses were detected in all UC-4 indicators that were tested and of these 4/8 UC-4 plants had symptoms and 4/8 plants were symptomless (Table 4). Symptoms were expected in 3/4 of the symptomless UC-4 indicators that were infected with SNSV or SMoV in addition to BPYV and SPaV. At least one virus and frequently multiple viruses were detected in 10/11 UC-6 indicators that were tested and of these nine plants displayed symptoms although based on the viruses that were detected symptoms were not expected to be observed. Symptoms were expected in the one UC-6 indicator that was symptomless as it tested positive for SCV as well as SPaV and BPYV. The one UC-6 indicator in which viruses were not detected displayed symptoms of chlorosis and twisting of leaves. Two or more viruses were detected in 2/5 UC-10 indicators plants which also displayed symptoms. Viruses were not detected in 3/10 UC-10 indicator plants that were symptomless.

2010/11 Queensland "dummy nucleus": Symptoms were observed on four UC-4 indicators, two UC-6 indicators and one UC-10 indicator that were graft inoculated with plants from the Queensland "dummy nucleus". Viruses were detected by RT-PCR in 12/14 UC-4 indicators, 12/14 UC-6 indicators and 13/14 UC-10 indicators inoculated with the Queensland "dummy nucleus" plants and included the indicator plants that were symptomatic. Of the indicators inoculated with the Queensland "dummy nucleus" plants SMoV was detected in 15/42 inoculated indicators, SMYEV was detected in 10/42 inoculated indicators, SPaV was detected in 27/42 inoculated indicators and SNSV was detected in 22/42 inoculated indicators,. SCV was not detected in any indicator. Symptoms were not observed but viruses were detected in each of the UC-4, UC-6 and UC-10 indicators that were graft inoculated with positive control plants.

Table 3. The virus infected 2009/10 "dummy nucleus" plants that were tested by biological indexing on UC-4, UC-6 and UC 10 indicators in 2009/10, the presence or absence of symptoms on each indicator and results of PCR testing of 21 indicator plants for SMYEV, SCV, SPaV, SNSV, BPYV and SMoV

Plant identification†	Replicate	UC-4 symptoms	Virus detected	UC-6 symptoms	Virus detected	UC-10 symptoms	Virus detected
V1/CV12 2008/09 Mother plant SMYEV SPaV	**R1**	-	nt[#]	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV12/R1 2009/10 nucleus plant SMYEV SPaV	**R1**	D*	nt	-	nt	-	nt
	R2	D	nt	-	nt	-	nt
V1/CV12/R2 2009/10 nucleus plant SMYEV SPaV	**R1**	CD	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV12/R3 2009/10 nucleus plant SMYEV SPaV	**R1**	-	nt	-	SMYEV BPYV SPaV	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV12/R 2009/10 nucleus plant SMYEV SPaV	**R1**	D	nt	-	nt	-	nt
	R2	D	nt	-	nt	-	nt
V1/CV12/R5 2009/10 nucleus plant SMYEV SPaV	**R1**	CD	nt	-	SMYEV BPYV	-	nt
	R2	CD	SMYEV BPYV SPaV	-	nt	-	nt
V1/CV20 2008/09 Mother plant SCV SPaV	**R1**	DS	nt	CDT	SMoV, SCV. SPaV BPYV	D	nt
	R2	-	nt	CDT	nt	-	nt
V1/CV20/R1 2009/10 nucleus plant SPaV SCV, SMoV	**R1**	CDM	SMoV, SCV. BPYV	CDT	nt	CD	SMoV, SCV. SPaV BPYV
	R2	CDMR	nt	-	nt	D	nt
V1/CV20/R2 2009/10 nucleus plant SPaV SCV, SMoV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV20/R3 2009/10 nucleus plant SPaV SCV, SMoV	**R1**	DMS	SMoV, SCV. SPaV BPYV	CDT	SMoV, SCV. SPaV BPYV	-	nt
	R2	DMS	nt	-	nt	DST	nt
V1/CV20/R4 2009/10 nucleus plant SPaV SCV, SMoV	**R1**	DMS	nt	CDT	SMoV BPYV	-	nt
	R2	DMS	nt	-	nt	-	nt
V1/CV20/R5 2009/10 nucleus plant SPaV SCV, SMoV	**R1**	DMS	nt	-	nt	-	nt
	R2	CMST crinkle	SMoV SCV	-	nt	CDST	NEGATIVE

Plant identification†	Replicate	UC-4 symptoms	Virus detected	UC-6 symptoms	Virus detected	UC-10 symptoms	Virus detected
V1/CV22 2008/09 Mother plant SNSV SPaV	**R1**	DS	nt	-	nt	-	nt
	R2	DS	nt	-	nt	-	nt
V1/CV22/R1 2009/10 nucleus plant SNSV SPaV	**R1**	DS	nt	-	nt	CD	nt
	R2	DS	nt	-	nt	-	nt
V1/CV22/R2 2009/10 nucleus plant SNSV SPaV	**R1**	CDM	nt	-	nt	D	nt
	R2	-	nt	-	nt	D	nt
V1/CV22/R3 2009/10 nucleus plant SNSV SPaV	**R1**	DST	nt	-	nt	-	nt
	R2	DST	nt	-	nt	-	nt
V1/CV22/R4 2009/10 nucleus plant SNSV SPaV	**R1**	CDMT	nt	-	nt	DS	nt
	R2	CDM	nt	-	nt	CD	nt
V1/CV22/R5 2009/10 nucleus plant SNSV SPaV	**R1**	-	nt	-	nt	DS	nt
	R2	-	nt	-	nt	DS	nt
V2/CV12 2008/09 Mother plant SMYEV SPaV	**R1**	-	nt	-	nt	D	nt
	R2	-	nt	-	nt	-	nt
V2/CV12/R1 2009/10 nucleus plant SMYEV SPaV	**R1**	D	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV12/R2 2009/10 nucleus plant SMYEV SPaV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV12/R3 2009/10 nucleus plant SMYEV SPaV	**R1**	CDRST	nt	-	SMoV, SCV. SPaV BPYV	-	nt
	R2	CDRST	nt	-	nt	-	nt
V2/CV12/R4 2009/10 nucleus plant SMYEV SPaV	**R1**	DMST	SMoV, SMYEV, SPaV, BPYV	-	nt	CDS	nt
	R2	DMST	nt	-	nt	CD	nt
V2/CV12/R5 2009/10 nucleus plant SMYEV SPaV	**R1**	-	nt	-	nt	-	nt
	R2	-	SPaV	-	nt	-	nt
V2/CV20 2008/09 Mother plant SMYEV, SCV SPAV	**R1**	CDM	nt	CDT	SMoV, BPYV	-	nt
	R2	CDM	nt	-	nt	-	nt
V2/CV20/R1 2009/10 nucleus plant SMYEV, SCV SPAV	**R1**	CDM	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt

Plant identification†	Replicate	UC-4 symptoms	Virus detected	UC-6 symptoms	Virus detected	UC-10 symptoms	Virus detected
V2/CV20/R2 2009/10 nucleus plant SMYEV, SCV SPAV	**R1**	CDM	nt	-	nt	CD	nt
	R2	CDM	nt	-	nt	CDS	nt
V2/CV20/R3 2009/10 nucleus plant SMYEV, SCV SPAV	**R1**	-	nt	-	nt	D	BPYV
	R2	D	nt	-	nt	-	nt
V2/CV20/R4 2009/10 nucleus plant SMYEV, SCV SPAV	**R1**	-	nt	CDT	SMoV, SCV. SPaV BPYV	-	nt
	R2	CD	nt	-	nt	CDT	nt
V2/CV20/R5 2009/10 nucleus plant SMYEV, SCV SPAV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV22 2008/09 Mother plant SMYEV SNSV SPAV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV22/R1 2009/10 nucleus plant SMYEV SNSV SPAV	**R1**	CDMT	nt	-	nt	DS	NEGATIVE
	R2	CDMT	nt	-	nt	D	nt
V2/CV22/R2 2009/10 nucleus plant SMYEV SNSV SPAV	**R1**	D green petals	SMYEV, SPaV, BPYV	-	nt	-	nt
	R2	-	SMoV, SMYEV, SPaV, BPYV	-	nt	D	nt
V2/CV22/R3 2009/10 nucleus plant SMYEV SNSV SPAV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV22/R4 2009/10 nucleus plant SMYEV SNSV SPAV	**R1**	-	nt	-	SNSV, SMYEV, SPaV, BPYV	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV22/R5 2009/10 nucleus plant SMYEV SNSV SPAV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	D	nt

†Indicator plants were inoculated with the 2008/09 mother plant or with the daughter plants that were derived from that mother plant. V1 = variety 1, V2 = variety 2, C12, C20 and CV22 are the viruses sources used for inoculation of the 2008/09 “dummy nucleus” plants. R1-R5 are used to identify individual 2009/10 plants derived from the one 2008/09 mother plant. The virus acronyms indicate the viruses that had been detected by RT-PCR in the plants used to inoculate the indicators.

*Abbreviations of symptom descriptions are: - = no symptoms observed, C= chlorosis, D = distortion, R = crinkle, M = mottling, S = stunting and T = twisting.
#nt = not tested by PCR for virus.

Table 4. The virus infected 2010/11 Victorian "dummy nucleus" plants that were tested by biological indexing on UC-4, UC-6 and UC 10 indicators in 2010/11, the presence or absence of symptoms on each indicator and results of PCR testing of 21 indicator plants for SMYEV, SCV, SPaV, SNSV, BPYV and SMoV

Plant identification †	Replicate	UC-4 symptoms	Virus detected	UC-6 symptoms	Virus detected	UC-10 symptoms	Virus detected
V1/CV12/R 1 SMYEV SMoV	**R1**	-	nt	CMST	nt	-	nt
	R2	-	nt	CMST	BPYV, SMYEV, SMoV, SNSV, SPaV	-	nt
V1/CV12/R 2 SMYEV SMoV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV12/R 3 SMYEV SMoV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV12/R 4 SMYEV SMoV	**R1**	CST	nt	-	nt	-	nt
	R2	CFRS	nt	-	nt	-	NEGATIVE
V1/CV20/R1 SCV SMYEV SPaV	**R1**	-	nt	CT	BPYV	CS	nt
	R2	-	nt	-	nt	-	nt
V1/CV20/R2 SCV SMYEV SPaV	**R1**	C	nt	-	nt	ST	nt
	R2	FT	nt	-	nt	-	nt
V1/CV20/R3 SCV SMYEV SPaV	**R1**	CS	nt	-	BPYV,SCV, SPaV	-	nt
	R2	CFRST	BPYV, SCV, SMoV, SNSV, SPaV	-	nt	-	nt
V1/CV20/R4 SCV SMYEV SPaV	**R1**	-	nt	T	SMoV	CST	BPYV, SCV, SMoV, SMYEV, SPaV
	R2	-	nt	-	nt	-	nt
V1/CV22/R1 SMYEV SNSV SPaV	**R1**	-	BPYV,SNSV, SPaV	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV22/R2 SNSV SPaV	**R1**	-	nt	CT	nt	-	nt
	R2	-	nt	-	nt	-	nt
V1/CV22/R3 SNSV SPaV	**R1**	-	BPYV,SNSV, SPaV	CT	nt	-	nt
	R2	-	BPYV, SMoV, SPaV	-	nt	-	nt
V1/CV22/R4 SNSV SPaV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt

Plant identification †	Replicate	UC-4 symptoms	Virus detected	UC-6 symptoms	Virus detected	UC-10 symptoms	Virus detected
V2/CV12/R1 SMYEV SMoV	**R1**	-	nt	CST	SMYEV	RST	SMoV, SMYEV
	R2	-	nt	-	SMYEV	-	nt
V2/CV12/R2 SMYEV SMoV	**R1**	CFRST	nt	-	nt	-	nt
	R2	CFRST	BPYV	-	nt	-	nt
V2/CV12/R3 SMYEV SMoV	**R1**	CFRST	nt	CMRST	nt	-	nt
	R2	CFRST	BPYV, SMoV	CMRST	nt	-	nt
V2/CV12/R4 SCV SMYEV SMoV	**R1**	CFRST	nt	CMST	BPYV, SMoV, SMYEV	CRST	nt
	R2	CFRST	nt	-	nt	-	nt
V2/CV20/R1 SMYEV, SNSV SPAV	**R1**	-	nt	CT	BPYV, SMYEV, SNSV, SPaV	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV20/R2 SMYEV, SNSV SPAV	**R1**	-	nt	CT	BPYV	-	NEGATIVE
	R2	-	nt	CST	nt	-	nt
V2/CV20/R3 SMYEV, SNSV SPAV	**R1**	-	nt	CT	NEGATIVE	-	nt
	R2	-	nt	CMT	nt	-	nt
V2/CV20/R4 SMYEV, SNSV SPAV	**R1**	-	BPYV	-	nt	-	NEGATIVE
	R2	-	nt	-	nt	-	nt
V2/CV22/R1 SMYEV SNSV SPAV	**R1**	-	nt	CT	nt	-	nt
	R2	-	nt	CST	nt	-	nt
V2/CV22/R2 SMYEV SNSV SPAV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	-	nt	-	nt
V2/CV22/R3 SMYEV SNSV SPAV	**R1**	CST	BPYV, SMYEV, SMoV, SNSV, SPaV	CMRS	nt	-	nt
	R2	-	nt	CRS	BPYV	-	nt
V2/CV22/R4 SMYEV SNSV SPAV	**R1**	-	nt	-	nt	-	nt
	R2	-	nt	CMT	nt	ST	nt

†Indicator plants were inoculated with the 2010/11 "dummy nucleus" plants V1 = variety 1, V2 = variety 2, C12, C20 and CV22 are the viruses sources used for inoculation of the original 2008/09 "dummy nucleus" plants from which these 2010/11 "dummy nucleus" plants have been derived. R1-R4 are used to identify individual 2010/11 plants derived from the one mother plant. The virus acronyms indicate the viruses that had been detected by RT-PCR in the plants used to inoculate the indicators.

*Abbreviations of symptom descriptions are: - = no symptoms observed, C= chlorosis, D = distortion, R = crinkle, M = mottling, S = stunting and T = twisting.
#nt = not tested by PCR for virus.

Table 5. The virus infected 2010/11 Queensland "dummy nucleus" plants that were tested by biological indexing on UC-4, UC-6 and UC 10 indicators in 2010/11, the presence or absence of symptoms on each indicator and results of PCR testing of the indicator plants for SMYEV, SCV, SPaV, SNSV and SMoV

Nucleus plant candidate-indicator combination			Virus detected in the indicator*					Presence of symptoms
Plant identification†	**Virus detected in the nucleus plant**	**Indicator**	**SCV**	**SMoV**	**SMYEV**	**SNSV**	**SPaV**	
V1C12 R3-1	SCV, SMoV, SMYEV, SPaV	UC-4		+	+		+	
V1C12 R1-3/ V1C12 R1-5§	SCV, SMoV, SMYEV, SPaV	UC-4			+			symptoms
V1C20 R1-5/ V1C20 R4-4§	SCV, SMoV, SMYEV, SPaV	UC-4		+	+			
V1C20 R1-5	SCV, SMoV, SMYEV, SPaV	UC-4		+				
V1C20 R5-5/ V1C20 R4-5§	SCV, SMoV, SMYEV, SPaV	UC-4			+	+		symptoms
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4				+	+	
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4		+	+	+	+	symptoms
V1C22 R2-3/ V1C22 R1-1§	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4				+	+	
V2C12 R3-1	SCV, SMoV, SMYEV, SPaV	UC-4				+		
V2C12 R1-2	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4				+		
V2C20 R2-4	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4				+		mild symptoms
V2C20 R4-3	SCV, SMoV, SMYEV, SPaV	UC-4						
V2C22 R2-2	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4						
V2C22 R3-4	SCV, SMoV, SMYEV, SNSV, SPaV	UC-4		+				
V1C12 R1-3	SCV, SMoV, SMYEV, SPaV	UC-6		+	+			symptoms
V1C12 R1-5	SCV, SMoV, SMYEV, SPaV	UC-6						
V1C20 R5-5	SCV, SMoV, SMYEV, SPaV	UC-6					+	
V1C20 R5-2	SCV, SMoV, SMYEV, SPaV	UC-6				+	+	
V1C20 R4-4	SCV, SMoV, SMYEV, SPaV	UC-6				+	+	
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-6			+	+	+	mild symptoms
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-6					+	
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-6						
V2C12 R2-3	SMoV, SMYEV, SNSV, SPaV	UC-6				+	+	
V2C12 R1-1	SMoV, SMYEV, SNSV, SPaV	UC-6				+	+	
V2C20 R4-3	SCV, SMoV, SMYEV, SPaV	UC-6				+	+	
V2C20 R2-4	SCV, SMoV, SMYEV, SNSV, SPaV	UC-6					+	
V2C22 R3-4	SCV, SMoV, SMYEV, SNSV, SPaV	UC-6				+	+	

Nucleus plant candidate-indicator combination			Virus detected in the indicator*					Presence of symptoms
Plant identification†	**Virus detected in the nucleus plant**	**Indicator**	**SCV**	**SMoV**	**SMYEV**	**SNSV**	**SPaV**	
V2C22 R2-3	SMoV, SMYEV, SNSV, SPaV	UC-6		+				
V1C12 R1-3	SCV, SMoV, SMYEV, SPaV	UC-10		+			+	
V1C12 R1-5	SCV, SMoV, SMYEV, SPaV	UC-10					+	
V1C20 R1-4	SCV, SMoV, SPaV	UC-10		+		+	+	symptoms
V1C20 R4-4	SCV, SMoV, SMYEV, SPaV	UC-10		+			+	
V1C20 R5-2/ V1C20 R5-5§	SCV, SMoV, SMYEV, SPaV	UC-10					+	
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-10				+	+	
V1C22 R1-1	SCV, SMoV, SMYEV, SNSV, SPaV	UC-10		+			+	
V1C22 R1-4	SCV, SMoV, SNSV, SPaV	UC-10		+	+	+	+	
V2C12 R1-3	SCV, SMoV, SMYEV, SPaV	UC-10		+	+	+	+	
V2C12 R2-3	SMoV, SMYEV, SNSV, SPaV	UC-10			+	+	+	
V2C20 R1-3	SMoV, SMYEV, SNSV, SPaV	UC-10				+	+	
V2C20 R4-3/ V2C20 R2-4§	SCV, SMoV, SMYEV, SNSV, SPaV	UC-10		+		+	+	
V2C22 R2-2/ V2C22 R3-4§	SCV, SMoV, SMYEV, SNSV, SPaV	UC-10		+		+	+	
V2C22 R3-4	SCV, SMoV, SMYEV, SNSV, SPaV	UC-10						
Positive control		UC-4		+	+	+	+	
Positive control		UC-4		+	+	+	+	
Positive control		UC-6		+	+	+	+	
Positive control		UC-6		+	+	+	+	
Positive control		UC-10		+		+	+	
Positive control		UC-10		+	+	+	+	

†Indicator plants were inoculated with the 2010/11 “dummy nucleus” plants V1 = variety 1, V2 = variety 2, C12, C20 and CV22 are the viruses sources used for inoculation of the original 2008/09 “dummy nucleus” plants from which these 2010/11 “dummy nucleus” plants have been derived. R identifies the mother plant 2009/10 from which the 2010/11 nucleus plants were derived. The virus acronyms indicate the viruses that had been detected by RT-PCR in the plants used to inoculate the indicators.

§Two nucleus plants were grafted to the same indicator due to lack of material and because they were derived from the same 2008/09 nucleus mother plant.

* + = virus detected.

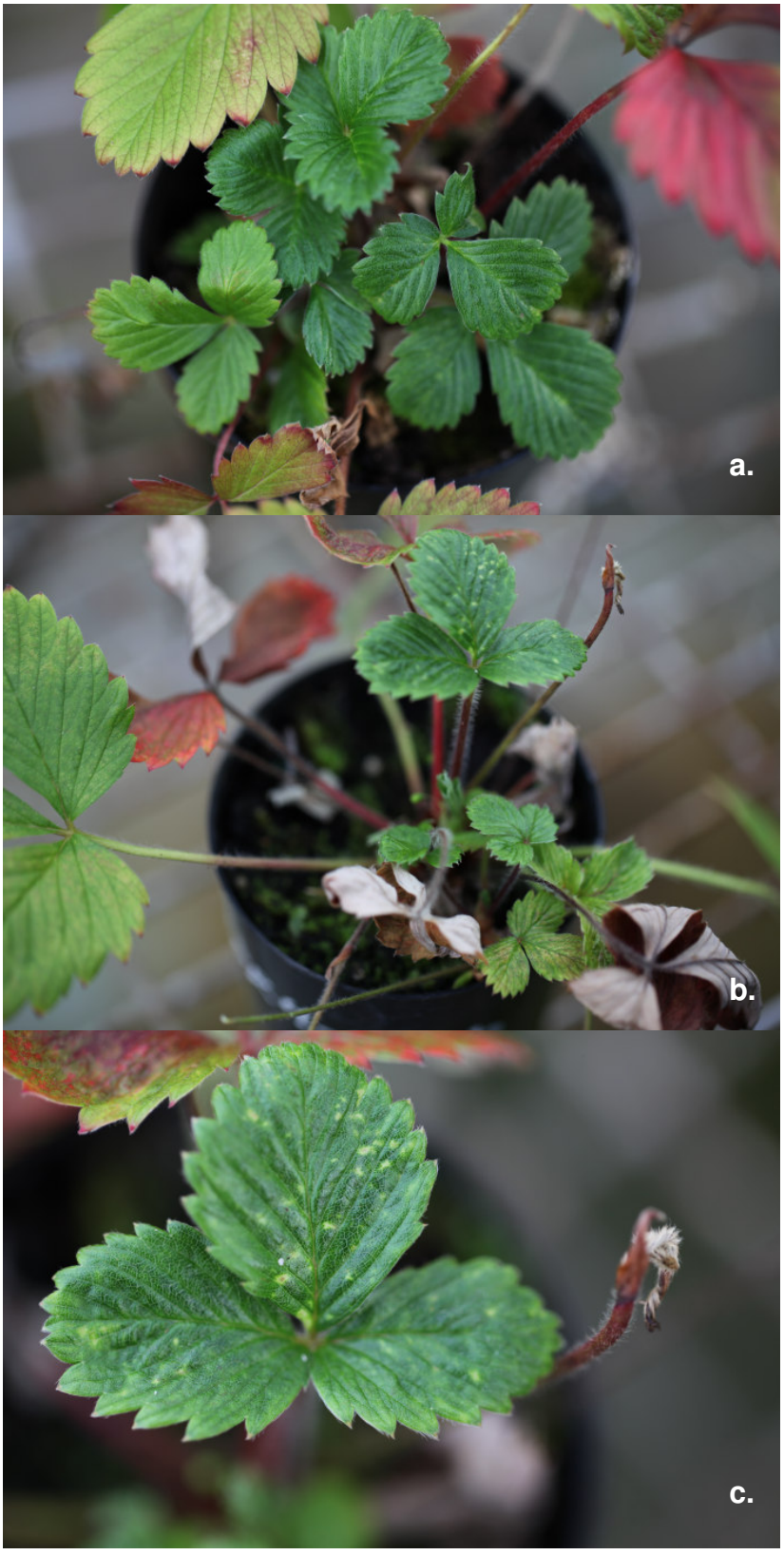


Figure 1. Examples of symptoms on UC-4 indicators after inoculation with virus infected candidate plants.

a) UC-4 inoculated with V2C22 R3. Note the twisting of leaves and mild chlorosis which is more noticeable along the veins. BPYV, SMYEV, SMoV, SNSV, and SPaV were detected by RT-PCR in this plant.

b) UC-4 inoculated with V1C20 R4. Note the twisting chlorosis and flecking of leaves.

c) a close up image of flecking that was observed in UC-4 inoculated with V1C20R4. BPYV, SCV, SMoV, SNSV, SPaV were detected by RT-PCR in this plant

(M.D. Jones)

Figure 2. Examples of symptoms on UC-6 indicators after inoculation with virus infected candidate plants.

a) UC-6 inoculated with V2/CV12/R1. Note the smaller twisted leaves at the crown. SMYE was detected by RT-PCR in this plant.

b) UC-6 inoculated with V1/C12/R1. Note the stunting of the plants and with small leaves and chlorosis at the margins. BPYV, SMYEV, SMoV, SNSV and SPaV were detected by RT-PCR in this plant.

c) UC-6 inoculated with V2/C12/R1. In addition to stunting a smaller chlorotic leaves some of leaves were slightly twisted. BPYV, SMoV and SMYEV were detected by RT-PCR in this plant.

d) UC-6 inoculated with V2/C12/R1. This plant was more severely affected compared to the other plants. SMYEV was detected by RT-PCR in this plant.

(M.D. Jones)

Figure 3. Examples of symptoms on UC-10 indicators after inoculation with virus infected candidate plants.

a) UC-10 inoculated with V2/C12/R1- Note the crinkled, twisted leaves which are smaller and chlorotic. SMoV and SMYEV were detected by RT-PCR in this plant.

b) UC-10 inoculated with V1/C20/R4. Note the smaller, twisted chlorotic leaves. BPYV, SCV, SMoV, SMYEV and SPaV were detected by RT-PCR in this plant.

(M.D. Jones)

Discussion

The results of this project suggest that PCR techniques can be reliably used for virus detection in strawberry plants maintained under the same conditions as the VSICA nucleus collection. Molecular detection of viruses in nucleus plants maintained in Queensland under glasshouse conditions at 28℃ was less reliable than in Victoria, suggesting that there is an environmental impact on the successful detection of strawberry viruses. However the rate of virus detection in Queensland and in Victoria using molecular methods was much greater than traditional biological indexing method of petiole grafting and symptom observation.

Victoria:

Our results indicate that there is a seasonal effect on detection of some viruses in strawberries and spring and early summer (October-December) are the best times for virus detection in Victoria. In some years autumn (March-May) may also be a good time for virus detection by molecular methods. These results are supported by our previous research (Constable et al 2007) in which it was found that strawberry viruses were most reliably detected by PCR during May-October under glasshouse conditions.

A 100% efficiency of detection by PCR was rarely obtained for any of the viruses at any time of the year. However over 95% of plants in both the 2008/09 and 2009/10 Victorian “dummy nuclei” in at least one month during the 2009/10 season and 100% of plants in the Victorian 2010/11 “dummy nucleus” tested positive for viruses in at least one month during the 2010/11 season. In each month only 2-3 leaves were sampled from each plant and it is also possible that uneven distribution of the viruses in the plant may have affected PCR efficiency throughout the year. Virus testing might be improved by sampling more than 2-3 leaves of the candidate plant for PCR detection of viruses.

Although the number of plants in which viruses were detected continued to increase over six months after inoculation and at least one of BPYV, SCV, SMYEV SNSV and SPaV had been detected in each of the 30 2008/09 “dummy nucleus” plants, the results showed that strawberry viruses may not be reliably detected in strawberry plants during the first six months after an infection event. Reliable detection was only achieved during the following season. It is likely that the viruses were not sufficiently translocated throughout the plants or did not reach sufficiently high titres to allow reliable detection until the following season. This highlights the need for continued and active indexing using diagnostic testing of strawberry plants within a nucleus collection for viruses, as there is a risk that new infection events may go un-detected for some time.

The detection of SNSV and SCV in 2008/09 Victoria “dummy nucleus” plants, which were not knowingly inoculated with these viruses, in the following season, may indicate spread within the 2008/09 “dummy nucleus” collection after inoculation. Similarly more plants than were expected tested positive for SCV, SMoV, SMYEV, SNSV and SPaV in both the 2010/11 Victoria and Queensland “dummy nucleus” collections. SNSV is transmitted by thrips and

pollen, SPaV is transmitted by glasshouse whiteflies and SCV, SMoV and SMYEV are aphid transmitted (Martin and Tzanetakis 2006). No infestations of aphids or thrips occurred in the "dummy nucleus" collections during the experiment and transmission of SNSV, SCV, SMoV and SMYEV by an insect vector seems unlikely. It is possible that some flowers were unnoticed and were not removed to prevent SNSV transmission by pollen. It is also possible that these viruses occur at levels below detection in some of the positive control plants that were used to inoculate the 2008/09 "dummy nucleus" plants.

Traditionally new varieties must have returned three negative biological indexing tests over three years before they could be introduced into the Victorian nucleus collection (Whattam 1994). Our results indicated that strawberry virus detection by molecular methods in the third season after an infection event was no more efficient than the second season in Victoria. Additionally, molecular indexing for strawberry viruses can be conducted in spring and autumn of the same season in Victoria. Consequently molecular indexing in spring and autumn of both years in combination with biological indexing conducted in spring of both years could be used to test new varieties prior to their introduction into the Victorian nucleus collection. This regime introduces more stringent virus testing measures for new varieties because they undergo five separate tests for viruses yet improves market access to varieties by allowing the testing to be conducted in a shorter time frame.

During the 2010/11 project the "dummy nucleus" collection in Victoria was replicated in Queensland and was tested monthly for viruses using the molecular based protocols for endemic viruses. Unlike the Victorian results there did not seem to be a trend for the timing of detection of all virus species and no single month or season appears to be adequate for the detection of all virus species. None of the individual virus species was detected in all of the plants that it was known to infect in any one month. Based on the results of the virus testing of the 2010/11 Queensland dummy nucleus July was the best time for the detection of SPaV and SmoV while SNSV, SCV and SMYEV were most frequently detected in December, January and February respectively. A further season of similar research may assist in forming a recommendation for the timing of strawberry virus detection by PCR, until then it may be useful to perform molecular virus testing in December-January and again in July in Queensland.

Our results show that the transmission rate of viruses from infected mother plants to daughter plants can be as high as 100%. This rate of transmission could lead to a rapid spread of viruses via runner production and would have an impact on quality and yield throughout the strawberry industry. This finding reinforces the need for continual and active testing of strawberry nucleus plants on an annual basis. It may also be valuable to test the next generation of strawberry derived from the original mother plants in case infection of the mother plants occurred during daughter plant production and was not detected.

UC-4 indicators can be used for detection of crinkle, mottle, mild yellow edge and necrotic shock diseases associated with SCV, SMoV, SMYEV, and SNSV respectively (Frazier 1974;

Martin and Tzanetakis 2006). UC-6 is used for detection of strawberry vein banding disease associated with *Strawberry vein banding virus* (SVBV) and crinkle disease (Frazier 1974; Martin and Tzanetakis 2006). UC-10 is used for detection of Pallidosis disease, which is thought to be associated with SPaV and BPYV and mild yellow edge disease (Frazier 1974; Martin and Tzanetakis 2006). Consequently in Victoria symptoms were also expected to be observed on all inoculated UC-4 and UC-10 indicators in both seasons and 24 and 10 UC-6 indicators in 2009/10 and 2010/11 seasons respectively due to the viruse known to infect the candidates with which they were grafted. Symptoms were also expected to be observed on all UC-4, UC-6 and UC-10 indicators plants that were inoculated with the 2010/11 Queensland "dummy nucleus plants and the positive controls. However, the results suggest that biological indexing was also not 100% reliable especially as symptoms were not always observed on the virus inoculated UC-4, UC-6 and UC-10 indicators that were expected to show symptoms. In Victoria symptoms were only observed on 56% of the virus inoculated UC-4 indicators, 30% of the virus inoculated UC-6 indicators and 32% of the virus inoculated UC-10 indicators in 2009/10 that were expected to show symptoms. Similarly during the 2010/11 growing season symptoms were only observed on 40% of the virus inoculated UC-4 indicators, 30% of the virus inoculated UC-6 indicators and 13% of the virus inoculated UC-10 indicators in Victoria and 12.5% (2/16) each of the UC-4 and UC-6 virus infected indicators and 6% (1/16) of the virus infected UC-10 indicators in Queensland that were expected to show symptoms. In Victoria, occasionally symptoms were observed on only one of the two replicate indicators used for each candidate and as such it is recommended that duplicate indicators should be used in biological indexing to reduce the risk of false negative results. The results are supported by previous researchers who observed that there was a risk of false negative results using graft indexing methods which, at that time, was assumed to be due to the erratic transmission of viruses (Cropley 1958, Mellor and Fitzpatrick, 1961). These researchers also recommended that multiple indicators be used for each candidate variety and if a negative result was returned the tests should be repeated several times before a plant can be considered "virus-free" (Cropley 1958, Mellor and Fitzpatrick, 1961).

In Victoria and Queensland viruses were detected in indicators that were symptomless and this result suggests that the absence of symptoms on indicators is not indicative of the absence of viruses in the candidate plant. These results show that biological indexing may not be 100% reliable for detection of viruses in strawberries and virus testing using biological indexing alone may lead to false negative results due to latent virus infections. It is recommended that biological indexing be used in conjunction with molecular indexing for strawberry viruses in Australia.

Traditionally, biological indexing in Queensland has been performed in the months September - December when grafting successes have been highest. It may be that the lower reliability of the biological indexing compared to molecular indexing in Queensland is associated with timing of the graft inoculation experiment and symptom development on the biological indicators may be better during September-December compared to January-March. It may

also be that molecular indexing reliability is also improved in September-December and PCR testing of the dummy nucleus during August to October 2011, to complete the full cycle of 12 months of testing will be continued during this time to test this hypothesis.

As observed in previous research projects (BS04004, Constable et al 2007) a range of symptoms affected the various indicators. The symptoms observed included mottling, chlorosis, twisting and distortion of the leaves and stunting of the plants. On one plant the petals were smaller than expected and pale green. The symptoms that were observed did not always match the symptoms that were expected in each indicator variety based on reports in the literature (Frazier 1974; Mellor and Fitzpatrick 1961; Martin and Tzanetakis 2006). Interestingly symptoms were observed in 16/38 UC-6 indicator plants in which symptoms were not expected in Victoria based on the viruses with which they were inoculated. The PCR results showed that at least one of SCV, SMYEV, SNSV and SPaV were transmitted to the Victorian indicators and in most indicators that were tested multiple viruses were transmitted. The PCR results indicated that some of the indicators were infected with multiple viruses and it is possible that the combination of detected viruses induced the symptoms that were observed. Multiple virus transmission can affect symptom expression (Mellor and Fitzpatrick, 1961) but the effect of multiple virus infections on symptom expression in individual indicator varieties is unclear and further work is required to assist in the interpretation of biological indexing results. It is also possible that undetected viruses were present in either the candidate plants or the indicator might also contribute to the symptoms that were observed.

In some cases viruses that were not expected in the indicators were detected by RT-PCR and it is possible that these viruses are present in the candidate plants at levels below the detection ability of the PCR tests. Alternatively it is possible that some viruses were transmitted between indicators after inoculation. For example, in 2009/10 and 2010/11 BPYV was detected in some indicators in which it wasn't expected as it was not detected in some in the specific candidate plants that were inoculated in 2009/10 or in 2010/11. It is possible that infection by BPYV was due to transmission of the virus to the grafted indicators by the greenhouse whitefly (GHWF, *Trialeurodes vaporariorum*), which was occasionally found on the grafted indicators. If glasshouse transmission of BPYV did occur, this highlights the need to control the presence of GHWF at all stages of the certification scheme and within the glasshouse containing the inoculated and un-inoculated indicator plants, especially as this insect is also a vector of SPaV (Martin and Tzanetakis 2006).

Some of the symptoms observed on the indicators might be attributed to specific viruses, such as mottling associated with SMoV infection (Frazier 1974; Greber 1979). However some symptoms might be attributed to several viruses, e.g. chlorosis associated with SCV on UC-4 or UC-6, SMYEV on UC-4 or UC-10 or SMoV and SNSV on UC-4 or twisting and distortion associated with SCV UC-4 or UC-6 or SMoV and SNSV on UC-4 (Frazier 1974; Greber 1979). Stunting could be due to multiple virus infection. Consequently biological indexing can only be used to indicate the possible presence of a virus but may not be used to differentiate

between viruses, especially if mixed infections occur. It is recommended that if disease is observed on an indicator a virus infection can be suspected but that virus testing should be supported by direct PCR testing of the mother plants in the nucleus collection.

In Victoria candidate plants could be reliably tested directly by PCR and these tests could replace biological testing of the strawberry nucleus plants for specific viruses. Detection by molecular indexing may be less reliable in Queensland. However, molecular indexing is more reliable than biological indexing in both Victoria and Queensland. However, biological indexing, particularly in PEQ, remains useful for the detection of strains of viruses which may not be detected using the PCR tests that were developed in this project and for other viruses such as *Strawberry latent C virus* for which tests have not yet been developed. Consequently it is recommended that biological indexing remain a part of the pathogen testing regime and that it should be used in conjunction with molecular methods for strawberry virus detection. Importantly neither molecular nor biological indexing was 100% reliable in Victoria and Queensland on continual active testing for viruses is required to increase the chance of virus detection and maintain the high health status of the certified runner programs in both states.

Conclusions:

Based on the results of this research it is recommend that the molecular protocols validated in the project be adopted for the detection of strawberry viruses and used in combination with biological indexing during the production of pathogen tested strawberry runners.

The following pathogen testing regime is recommended for new varieties before introduction into the Victoria nucleus collection:

1. Pathogen testing is conducted over a minimum two year period.
2. All plants must be tested for virus associated diseases using biological indexing in spring of each year and the inoculated indicator plants must be free of symptoms which are indicative of virus infection.
3. All plants must be tested for viruses using molecular indexing in spring (October-December) and autumn (April-May) in both years of screening and none of the prescribed viruses should be detected.

The following pathogen testing regime is recommended for varieties maintained in the Victorian nucleus:

1. Plants that are continually maintained in the nucleus collection for two years or more will be pathogen tested for the specified viruses by biological indexing in spring every second year. In the alternate year they will be indexed in spring by molecular methods.
2. New varieties must undergo molecular indexing for viruses in spring during their first year in the nucleus collection.

The adoption of these recommendations will 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.

It is difficult to recommend a single time for molecular indexing of plants grown in Queensland based on the results reported in this chapter. However, when compared with the results of biological indexing, molecular indexing is much more reliable for strawberry virus detection in Queensland. Based on the results of this project similar testing regimes could also be adopted for Queensland except that:

1. Three years of molecular and biological indexing may be required for new varieties entering a Queensland nucleus collection to reduce the risk of a false negative results due to the lower reliability of the PCR tests in this environment compared to Victoria;
2. Molecular indexing should be carried out twice in each year in December-February and again in July;
3. Molecular indexing may be required in each year for plants maintained in a nucleus collection;
4. Biological indexing could be done biennially.

Testing of the 2010/11 Queensland "dummy nucleus" will continue through August-October 2011 to determine if the reliability of the molecular tests improves during this time. Further work is required to validate testing regimes for Queensland.

CHAPTER 3

IDENTIFICATION AND VALIDATION OF MOLECULAR PROTOCOLS FOR THE DETECTION OF QUARANTINABLE VIRUSES, ANGULAR LEAFSPOT AND RED STELE IN STRAWBERRIES

Introduction

The Australian strawberry industry has identified a number of high priority pests and diseases that pose a quarantine risk to the local industry and must be tested for during post entry quarantine (PEQ) and include: *Xanthomonas fragariae* (angular leafspot), *Phytophthora fragariae* var. fragariae (Strawberry red stele), *Arabis mosaic nepovirus* (ArMV), *Raspberry ringspot nepovirus* (RpRSV), *Tomato ringspot nepovirus* (ToRSV), *Tomato black ring nepovirus* (TBRV) *Strawberry latent ringspot sadwavirus* (SLRV), *Tomato bushy stunt tombusvirus*, (TBSV) and *Strawberry latent C rhabdovirus* (SLCV). During PEQ these pathogens are tested using traditional methods such as visual inspection, Enzyme-Linked ImmunoSorbent Assay (ELISA), biological indexing and isolation onto selective media. There have been significant advances in molecular diagnostics for plant pathogens in recent years and tests for most of these exotic strawberry pathogens have been developed overseas.

As a part of BS07003 an extensive literature review and a thorough bioinformatics analysis was conducted and international experts were consulted to identify the most appropriate molecular diagnostic tests that can be used to detect these pathogens. In 2011 field surveys were conducted in Victoria and Queensland to validate the identified molecular tests under local conditions. This validation process was required to ensure that the tests did not detect nucleic acids of plant hosts or other organisms that occur in Australian strawberry plants.

The tests that were identified for validation in this project include:

- Detection of ArMV using the M2/M3 primers (Wetzel et al 2002);
- Detection of RsRSV using the primer pair RpRSV-F1/RpRSV-R1 (Ochoa-Corona et al 2006);
- Detection of SLRSV using the primer pairs SLRSV-5D/SLRSV-3D (Faggioli et al 2002) and SLRSV-F/SLRSV-R (Postman et al 2004);
- Detection of TBRV using the primer pair P1/P2 (LeGall et al 1995), 2MP5/2MP2 (Jończyk et al 2004) and TBRV-70F/ TBRV-70R A (Harper et al 2011);
- Detection of ToRSV using the primer pair D1/U1 (Griesbach 1995) and first round and nested PCR pairs ToRSVf1/ToRSVr1 and ToRSVf2/ToRSVr2 (Martin et al 2008);
- Detection of Tombusviruses using the primer pairs TomCPF/TomCPR (Russo et al 2002) and TBSVGralF1/ TBSVGralR1 (Harris et al 2006);
- Detection of *Xanthomonas fragariae* strains using the primer pair 241A/241B, 245A/245B Pooler et al (1996) and a nested PCR assay using the 245A/245B primer

pair in the first round and the nested primer pair 245.267/245.5 (Zimmermann et al 2004);

- Detection of *Phytophthora fragariae var. fragariae* using a nested assay with the first round primer pair DC6/ITS4 and the nested primer pair DC1/B5 (Bonants et al 2004).

No molecular test is available for SLCV nor is any genetic information available to aid in the design of a RT-PCR assay and in BS07003 it was recommended that biological indexing continue to be used for detection of this virus and that this method could be supplemented by electron microscopy (Constable et al 2010).

Methods

Sampling

Sampling (whole plants or leaf and petiole samples) began in February 2011 and was completed in March 2011. A total of 69 samples were collected from the DPI Victoria positive control collection (22/569) and nurseries and home gardens in Victoria (29/69) and Queensland (18/69). Samples included commercial cultivars of *Fragaria × ananassa* (46/69), *F. vesca* or *F. virginiana* (22/69), and *Fragaria* X *Potentilla* hybrids (1/69).

Nucleic acid extraction

From each sample, 0.3 mg of tissue was collected from the base of the petioles from all samples. Some root tissue was included from nine of the samples. Total nucleic acid was extracted from this tissue as described in Chapter 2.

RT-PCR and PCR amplification

Primers for the detection of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA; Thompson et al 2003) were used to determine the quality of the RNA in the total nucleic acid extracts (Table 3.1). Primers developed for bacterial phylogenetic studies (Weisberg et al 1991) were used in PCR to determine the quality of DNA in the total nucleic acid extracts (Table 3.1). The final concentration for the housekeeping primers in the one step RT-PCRs and PCR was 0.2 µM. The PCR primers used to detect each exotic and endemic pathogen, the type of assay used and the references containing specific cycling conditions for each pathogen are given in Table 3.1. The final concentration for each pathogen-specific primer for all PCR and one step RT-PCR reactions was 0.4 µM. The positive controls that were used are listed in Table 3.2.

The SuperScript™ One-Step RT-PCR System (Invitrogen) was used for detection of NAD mRNA and viruses. One step RT-PCR was conducted according to the manufacturer's instructions except that 0.4µl of SSIII/Taq enzyme mixture was used per RT-PCR reaction and the total reaction volume was 20 µl.

Table 3.1 List of quarntinable strawberry pathogens, the type of PCR test used for detection, the primers used and their annealing temperature, region amplified, expected product size and reference for each test used to detect exotic strawberry pathogens in Australia.

Pathogen	Test	Primer name	Orientation	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
ArMV	Specific RT-PCR	M2	F	(C/T)T(A/G)GATTTTAGGCTCAATGG;:	42℃	movement protein gene	290bp	Wetzel et al 2002
		M3	R	TG(C/T)AA(A/G)CCAGG(A/G)AAGAAAAT;				
RsRSV	Specific RT-PCR	RpRSVF1	F	TGTGTCTGGTTTTGATGCT	61℃	Partial RNA-2	385bp	Ochoa-Corona et al 2006
		RpRSVR1	R	GAGTGCGATAGGGGCTGTT				
SLRSV	Specific RT-PCR	SLRSV-F	F	CCTCTCCAACCTGCTAGACT	61℃	Partial coat protein gene	487bp	Postman et al 2004
		SLRSV-R	R	AAGCGCATGAAGGTGTAACT				
TBRV	Specific RT-PCR	P1	F	ATGGGAGAAGTGCTGG	42℃	Partial RNA-2	TBRV 332bp	Le Gall et al 1995
		P2	R	AATCTTTTTGTGTCCAACA				
		TBRV-70F	F	GCTCGTAACAGTTGCGGAGATAT	60℃	Partial RNA-2	72 bp	Harper et al 2011
		TBRV-70R	R	TGTCCACACTGTCATGGGA				
ToRSV	Specific RT-PCR	U1	F	GAC GAA GTT ATC AAT GGC AGC	55℃	RNA-1 region	450 bp	Griesbach 1995.
		D1	R	TCC GTC CAA TCA CGC GAA TA				
	Specific two-step RT-PCR and nested PCR	ToRSVrt	R	GCGAAAACAACGTCCTTGC	50℃	RdRP gene of RNA 1		Martin et al 2008
		ToRSVf1	F	CCGTTAGCAGCTTCCAAAAG	56℃		512bp	
		ToRSVr1	R	GTCCTCATGGAACCTTTCTC				
		ToRSVf2	F	GGTTATCCAGCCTTAAGCAAG	56℃		435 bp	
		ToRSVr2	R	CGGTAGGCTATGACAACTAC				
TBSV	Specific RT-PCR	TomCPF	F	CCG CCG TAG CAT GAC CAA GTA	55℃	Partial coat protein gene	1000 bp	Russo et al 2002.
		TomCPR	R	CCA TGA ACT GGT CTT GTT CAA				
	Specific RT-PCR	TBSVGralF1	F	AAGGGTAAGGATGGTGAGGA	61℃	polymerase; read through protein	590bp	Harris et al 2006
		TBSVGralR1	R	TTTGGTAGGTTGTGGAGTGC				
Phytophthora fragariae var. fragariae	Nested PCR	DC6		GAGGGACTTTTGGGTAATCA	57℃	Ribosomal DNA	1300bp	Bonants et al 2004
		ITS4		TCCTCCGCTTATTGATATGC				
		DC1		ACTTAGTTGGGGGCCTGTCT	65℃		750bp	
		B5		TGAGATCCACCCGCAGCA				
Xanthomonas fragariae	Specific single PCR and nested PCR	245A		CGCGTGCCAGTGGAGATCC	57℃	Unknown genomic DNA	300bp	Pooler et al (1996) ; Zimmermann et al 2004
		245B		CGCGTGCCAGAACTAGCAG				
		245.267		GGTCCAGTGGAGATCCTGTG			286bp	
		245.5		GTTTTCGTTACGCTGAGTACTG				

Platinum® *Taq* DNA Polymerase (Invitrogen) was used for PCR detection of the 16S rDNA and fungal and bacterial pathogens. PCR was conducted according to the manufacturer's instructions except that the total reaction volume was 20 µl and 0.8 units of Platinum® *Taq* DNA Polymerase was used in each reaction. For nested PCR 1 µl of the first round PCR reaction mixture was added to the PCR mixture containing the second primer pair. Water controls, without nucleic acid in the RT-PCR or PCR mix, were included in each test.

After amplification, 10 µL of each PCR was run on a 2% agarose gel in 0.5×. Tris-borate-EDTA (0·045 M Tris-borate, 1 mM EDTA, pH 8·0) running buffer, stained with ethidium bromide and visualised on a UV transilluminator. DNA Molecular Weight Marker X (Roche Diagnostics) was used to estimate amplicon size.

Table 3.2. A list of positive controls acquired from other researchers, the type of material provided and their origins

Pathogen	Type of material	Origin
ArMV	Total nucleic acid, *Vitis vinifera* isolate PV-0045	DSMZ – Deutsche Smmlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany.
RSRPV	Total nucleic acid *Vitis vinifera* isolate PV-0429	DSMZ – Deutsche Smmlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany.
SLRSV	Total nucleic acid isolate PV0247 (original host unknown)	DSMZ – Deutsche Smmlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany.
TBRV	Total nucleic acid *Rubus idaeus* isolate PV-0191 and Pelargonium isolate PV-0521	DSMZ – Deutsche Smmlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany.
ToRSV	Total nucleic acid Pelargonium isolate PV0049	DSMZ – Deutsche Smmlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany.
Tombusviruses – *Pear latent virus*	partial clone of cDNA encompassing the coat protein ligated into pGEM-T vector	Dr. M. Russo Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy.
Phytophthora fragariae var. fragariae	110bp synthetic positive control incorporating first round and nested PCR primers	Geneworks, SA, Australia
Xanthomonas fragariae	Total nucleic acid	Dr. Stephen Doughty, DPI, Victoria.

Results and discussion

ArMV, RpRSV, SLRV, TBRV, ToRSV, TBSV, *X. fragariae* and *P. fragariae* var. *fragariae* were not detected in any sample.

The RT-PCR and PCR tests for ArMV (Wetzel et al 2002), RsRSV (Ochoa-Corona et al 2006), SLRSV (Postman et al 2004), ToRSV (Griesbach 1995), *X. fragariae* strains (Pooler et al 1996; Zimmermann et al 2004) and *P. fragariae var. fragariae* (Bonants et al 2004) were reliable and generally specific for the target organism as very little non-specific detection of non-target nucleic acid were observed.

Subsequent bioinformatics analysis indicated that the SLRSV-5D/SLRSV-3D primer pair (Faggioli et al 2002) may not detect as many isolates of SLSRV as the SLRSV-F/SLRSV-R primer pair (Postman et al 2004) and it was decided that only the latter primer pair would be validated in this study.

For detection of ToRSV the specific nested PCR test developed by Martin et al (2008) was also trialled. Multiple non-specific bands of various sizes, including products similar to the expected size were observed in the nested PCR assay of many of the individual samples that were tested. Multiple non-specific bands were also observed in the first round reactions of the ToRSV RT-PCR test. Additionally this test did not detect the ToRSV positive control that was used in the first round RT-PCR assay or the nested PCR assay. These results indicate that this molecular assay is not reliable and it is not recommended for the detection of ToRSV.

Two tests were validated for detection of TBSV (Russo et al 2002; Harris et al 2006) and both were reliable for the detection of this virus in strawberries as no non-specific detection of non-target nucleic acid was observed. The bioinformatics analysis indicated that both primer pairs are likely to detect *Tombusvirus* species other than TBSV and if a positive result is obtained sequencing is required to confirm the identity of the virus that is detected. The bioinformatics analysis also indicated that the TBSVGralF1/ TBSVGralR1 primers (Harris et al 2006) will detect more *Tombusvirus* species or isolates and that there were less base mismatches between the primers and the virus isolates compared to the TomCPF/TomCPR primers (Russo et al 2002). However the analysis indicated that the TomCPF/TomCPR (Russo et al 2002) may detect some *Tombusvirus* isolates, including TBSV strains, that the TBSVGralF1/ TBSVGralR1 primers (Harris et al 2006) may not. Therefore both assays should be used for detection of TBSV in strawberries.

Three primer pairs for the detection of TBRV were identified for validation in this study including P1/P2 (LeGall et al 1995), 2MP2/2MP5 (Jończyk et al 2004) and TBRV-70F/TBRV70R (Harper et al 2011). Bioinformatics analysis indicated that all three primer pairs were likely to detect isolates of TBRV. Due to time constraints and costs it was decided to validate only two of the three primer pairs. The P1/P2 primer pair (LeGall et al 1995) was chosen as it had been validated for TBRV detection in Australian grapevines (Constable et al 2010). The TBRV-70F/TBRV70R primer pair (Harper et al 2011) was also chosen as it was the most recently developed primer pair developed specifically for TBRV detection. No non-specific detection of non-target nucleic acid was observed in samples that were tested with either of the RT-PCR assays that were validated (Le Gall et al 1995; Harper et al 2011) for TBRV detection of this virus in strawberries. Two TBRV positive controls (DSMZ TBRV

isolates PV0191 and PV0521, Table 1) were used during the validation of these assays and the P1/P2 primers (LeGall et al 1995) detected both isolates but the TBRV-70F/TBRV70R (Harper et al 2011) only detected isolate PV0521. The P1/P2 primers generated a slightly smaller amplicon in isolate PV0191 compared to isolate PV0521, which might be associated with genetic variation in the region of RNA-2 that was amplified. The RNA-2 sequence of TBRV-ED isolate has a 38 base deletion in this region (Genbank accession Nos. X808310) compared to the TBRV-MJ isolate (Genbank accession Nos. AY157994) and a similar deletion in the isolates used as positive controls in the PCR assays may account for the smaller PCR product that was observed for TBRV isolate PV0191. It is recommended that both assays be used for TBRV detection in strawberries because of the genetic variability in the region to which the P1/P2 primers were designed and the inability of the TBRV-70F/TBRV70R to detect some strains of TBRV.

The three primer pairs developed by Pooler et al (1996) were designed from regions of the *X. fragariae* genome identified during random amplified polymorphic DNA (RAPD) PCR. The 245A/245B primers (Pooler et al 1996) are most commonly used in a single PCR for detection of *X. fragariae*. The use of these primers is recommended for the reliable detection of *X. fragariae* from bacterial ooze of symptomatic plants and cultures of the bacterium. The additional primer pairs, 241A/214B and the 295A/295B, that were developed by Pooler et al (1996) can be used independently in a single PCR and may be used in multiplex PCR with the 245A/245B primer pair and for confirmation of infection of the bacterium. The use of these additional primers is suggested in the SPHDS protocol developed for detection and identification of *X fragariae* (Noble 2009). The 241A/214B and the 295A/295B primer pairs were not specifically validated in this project as 241A/214B are not as sensitive as the other primer pairs (Pooler et al 1996) and the product produced by the 295A/295B is less frequently observed than the products produced by the other primer pairs (Noble 2009). If a negative result is obtained but infection of the bacterium is suspected or if symptomless plants require active testing the use of the nested PCR for detection of *X. fragariae* is recommended as this has been shown to be more sensitive than single PCR (Zimmerman et al 2004).

All of the 69 samples were tested for *P. fragariae var. fragariae* using a nested assay with the first round primer pair DC6/ITS4 and the nested primer pair DC1/B5 (Bonants et al 2004), although only 9/69 samples contained root tissue. Consequently the nested PCR assay for detection of *P. fragariae var. fragariae* cannot be considered fully validated. However our results indicate that this test is unlikely to amplify *Fragaria* sp. genomic DNA that might cause a false positive result as no non-specific amplicons were generated from any sample. Testing of more root samples is required to validate this test.

Overseas researchers were contacted to obtain nucleic acid of *P. fragariae var. fragariae* as a positive control for PCR however no response was obtained. Consequently a synthetic 110bp oligonucleotide that incorporated the DC6/ITS4 first round primers and the nested primer pair DC1/B5 was used. The amplicons produced by the synthetic positive control in the first round

PCR (110bp) and the nested PCR (70bp) were smaller than the expected size and the use of the synthetic positive control may be useful in reducing the risk of false positive results due to contamination, especially in a nested PCR assay. If contamination by the synthetic positive control occurs it can be easily detected because the amplicon is not the correct size.

Conclusions

For detection of exotic pathogens the following molecular assays are recommended:

- ArMV detection using the M2/M3 primers (Wetzel et al 2002);
- Detection of RsRSV detection using the primer pair RpRSV-F1/RpRSV-R1 (Ochoa-Corona et al 2006);
- SLRSV detection using the primer pair SLRSV-F/SLRSV-R (Postman et al 2004);
- TBRV detection using the primer pair P1/P2 (LeGall et al 1995) and TBRV-70F/TBRV70R (Harper et al 2011);
- ToRSV detection using the primer pair D1/U1 (Griesbach 1995);
- Tombusvirus (TBSV) detection using the primer pairs TomCPF/TomCPR (Russo et al 2002) and TBSVGralF1/ TBSVGralR1 (Harris et al 2006);
- Detection of *Xanthomonas fragariae* strains using the primer pairs 245A/245B (Pooler et al 1996) in single PCR when bacterial ooze is present on plant tissue or from cultures of bacteria and a nested PCR assay using the primer pair 245A/245B in the first round and the nested primer pair 245.267/245.5 for active testing of symptomless plants or if the bacterium is suspected but not detected by the single PCR (Zimmermann et al 2004);
- *Phytophthora fragariae var. fragariae* detection using a nested assay with the first round primer pair DC6/ITS4 and the nested primer pair DC1/B5 (Bonants et al 2004). This test requires further validation on root and soil samples.

Adoption of these tests will improve the efficiency of the Australian PEQ for strawberries and may reduce the existing waiting period for release of new varieties. This will also improve our ability to respond in case of an incursion by providing more accurate and sensitive identification of strawberry pathogens. The tests will improve the ability of the Australian strawberry certification schemes to determine the pathogen status of selected strawberry varieties prior to inclusion and during regular maintenance and pathogen testing of the high health programs.

CHAPTER 4

DEVELOPMENT OF A PATHOGEN TESTING MANUAL FOR THE STRAWBERRY INDUSTRY

In Australia certified strawberry runners are supplied through the Victorian Strawberry Certification Authority (VSICA) and the Queensland Strawberry Runner Certification Scheme. The aim of these certification schemes is to ensure provision of high quality planting material by reducing the threat of spread of pathogens and pests that impact significantly on the health of strawberry plants, affecting the yield and quality of fruit and runner propagation material. The nucleus collections held by each scheme are indexed annually for the major strawberry diseases associated with viruses, bacteria and fungi that are known to occur in Australia. These pathogen tested schemes have been operational in Australia for nearly 50 years and have contributed greatly to increased yields for strawberry growers due to the exclusion of these pathogens from industry.

The provision of high health certified strawberry runners has traditionally been supported by the best available diagnostic tools for pathogen and pest detection. In Australia 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 (Whattam 1994). 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. Plants are also tested for *Strawberry necrotic shock virus* (SNSV, formally thought to be a strain of *Tobacco streak virus*, TSV) using herbaceous indexing. Plants in the nucleus collection are visually inspected on a routine basis for evidence of other diseases and actively tested by baiting and culturing for *Phytophthora* sp.

Biological indexing by graft inoculation of strawberry petioles for virus associated diseases was first described in 1956 (Bringhurst and Voth 1956) and was considered an improvement upon the traditional stolon grafting technique (Harris and King 1942) because it reduced the risk of introducing a latent virus into the candidate plants (Miller, 1958; Mellor and Fitzpatrick, 1961). The petiole graft inoculation method was further improved with the introduction of new indicator clones for better detection of strawberry virus associated diseases (Frazier 1974) and it is these clones that are still used today by most pathogen testing programs worldwide, including Australia (Whattam 1994).

Until recently, biological indexing has been the only method available for the detection of most of the virus associated diseases of strawberries. Biological indexing is labour intensive, expensive, time consuming as it takes 6-8 weeks to return a result (Bringhurst and Voth 1956) and can only be reliably done in the spring and early summer months of each year. However, there have been significant advances in methods for detection of strawberry viruses especially with the evolution of molecular biology techniques such as polymerase chain reaction (PCR). The HAL project BS04004 demonstrated the capacity of molecular protocols to specifically detect Australian strawberry virus isolates including *Strawberry crinkle*

cytorhabdovirus (SCV), *Strawberry mottle sadwavirus* (SMoV), *Strawberry mild yellow edge potexvirus* (SMYEV), *Strawberry vein banding caulimovirus* (SVBV), Strawberry necrotic shock virus (SNSV) and *Strawberry pallidosis associated crinivirus* (SPaV) and *Beet pseudos yellows crinivirus* (BPYV), which are both associated with pallidosis disease (Constable et al 2007). The PCR tests that were developed are reliable, sensitive rapid and cost effective when compared to biological indexing.

In HAL project BS07003 validation of the molecular assays for detection of SCV, SMoV, SMYEV, SVBV, SNSV, SPaV and BPYV using field collected strawberry samples from Victoria, South Australia, Western Australia and Queensland indicated that they were reliable for strawberry virus detection under Australian conditions (Constable et al 2010). A transitional phase was established during HAL project BS07003 in which the PCR tests were further validated and adapted for the detection of endemic viruses using a virus infected "dummy nucleus". The "dummy nucleus" was routinely tested for viruses during the growing season and it was demonstrated that viruses may not be reliably detected in a nucleus plant by molecular methods until the season after an infection event occurred. It was shown that virus detection of strawberry plants maintained in a screen house in Victoria was most reliable in spring (October –December) or autumn (March –May). It was also demonstrated in BS07003 and the current project that biological indexing is less efficient than molecular methods for detection of virus associated diseases (Constable et al 2010, Chapter 2). However neither of the methods are 100% efficient for virus detection and biological indexing remains a useful method for the detection of virus species and strains that may be not be detected during molecular indexing. Combining both molecular and biological indexing into an updated virus testing regime for the production of pathogen tested runners is recommended (Figure 1).

In the current project the results indicated that virus detection by molecular indexing in the third season after an infection event was no more efficient than the second season (Chapter 2). Also molecular indexing can be conducted in spring and autumn in Victoria and it is possible to gather three sets of negative results from molecular virus indexing over two growing seasons (Constable et al 2010, Chapter 2). Consequently it is recommended that a combination of molecular and biological indexing be conducted over two years for new varieties prior to their introduction into the nucleus collection. This change introduces more stringent pathogen testing measures for new varieties yet improves market access to varieties by allowing the testing to be conducted in a shorter time frame.

Molecular diagnostic tests to detect seven high priority pests and diseases that pose a quarantine risk to the local industry including were also validated: *Xanthomonas fragariae* (angular leafspot), , *Arabis mosaic virus*, *Raspberry ringspot virus*, *Tomato ringspot virus* and *Tomato black ring virus* of the genus *Nepovirus*, *Strawberry latent ringspot Sadwavirus* and *Tomato bushy stunt Tombusvirus*. Although not fully validated we are confidant that the molecular test for *Phytophthora fragariae* var. fragariae (Strawberry red stele) is a suitable

diagnostic protocol that can be used for its detection under Australian conditions. These validated protocols will be a valuable addition to the pathogen-testing manual and can be used by AQIS to screen strawberry plants that are imported into Australia.

An outcome of the research conducted in HAL projects BS04004 and BS07003 and this project is an improved world's best practice, diagnostic capability for the detection of economically significant viruses of strawberries that can be used with confidence by the Australian strawberry industry for the production of high health strawberry runners and by AQIS during PEQ. The output from this research is a draft pathogen-testing manual that incorporates the endemic and exotic protocols developed in these projects. The protocols in the manual will form the basis of a national certification standard to support the production of pathogen tested strawberry runners in Australia.

This manual has been written specifically for VSICA and will be submitted to VSICA for consideration. The manual could be adapted to the needs of the Queensland runner program using the results of this project and the following modifications should be considered:

1. Three years of molecular and biological indexing may be required for new varieties entering a Queensland nucleus collection;
2. Molecular indexing should be carried out twice in each year in December-February and again in July;
3. Molecular indexing may be required in each year for plants maintained in a nucleus collection;
4. Biological indexing could be done biennially.

Further work is required to validate these regimes for Queensland.

The "Technical manual" for the pathogen indexing of strawberry runners that was developed for VSICA is attached in appendix 1.

SCOPE OF THE MANUAL

- The manual is a technical document for "certification" programs, AQIS, and other users who wish to produce high health strawberries using industry approved standards.
- This manual is based on world's best practice validated diagnostic tests for strawberry pathogens and incorporates existing and new technology (driver for change).
- Through research and development, protocols have been identified which are efficient and increase biosecurity for the Australian strawberry industry and these protocols have been incorporated into the manual
- The format of the manual is prescriptive and designed for easy interpretation of the methodologies/techniques and interpretation of the results of the various diagnostic tests.
- The protocols in the manual allow the various stakeholders/users to adapt a standardized approach to pathogen testing strawberries including certification schemes located in Victoria and Queensland and Post Entry Quarantine (PEQ).

- The intention of this manual is that the protocols will be integrated into the conditions/standards of strawberry runner certification schemes such as VISCA

- This manual will become part of a standards based approach to strawberry runner certification in Australia

OVERVIEW OF INDEXING PROCEDURES

This procedure for pathogen testing of the strawberry nucleus is based on world's best practice validated diagnostic tests for strawberry pathogens and incorporates existing and new technology (Whattam 1994, Constable et al 2007, Constable et al 2010, Constable et al 2011).

1. Plants entering the nucleus collection must have returned two seasons of negative pathogen testing results which included two years of biological indexing in spring of each year and molecular indexing in the spring and autumn of both years. If all tests are negative the plants can be included into the nucleus collection.
2. Nucleus mother plants are tested every two years by biological indexing in spring and in the alternate year they are tested in spring by molecular indexing.
3. Nucleus mother plants are removed from cold storage at the beginning of September, and are established and maintained in a screen house with insect and pollen proof mesh or an insect proof glasshouse during September until May.
4. Nucleus plants must be inspected daily for symptoms of disease and for insect pests during the growing season.
    - Pathogens and insects must be treated as soon as is practical.
5. Pathogen testing for viruses by biological indexing and/or molecular indexing is conducted during October and November. Sampling and diagnostic testing is carried out according to the protocols in this manual:
    - Varieties that are continually maintained in the nucleus collection for two or more years will be pathogen tested for specified viruses by biological indexing every second year. In the alternate year they will be tested by molecular methods.
    - Varieties that are in their first year of incorporation in the nucleus collection must be indexed using molecular methods.
    - All plants will be tested by biological indexing onto *Chenopodium quinoa* for detection of *Tobacco streak virus* and nepoviruses in each year.
6. Biological indicators, including *Fragaria* species and *C. quinoa*, will be maintained and propagated by a diagnostic laboratory in an approved glasshouse according to the protocols outlined in this manual.
7. Biological indexing by graft inoculation of *Fragaria* sp. biological indicators and rub inoculation of *C. quinoa* will be carried by a diagnostic laboratory in glasshouse conditions in October and November according to the protocols in this manual.
8. Molecular indexing of prescribed pathogens will be carried out in October and November according to the protocols described in this manual. Testing will be done by a diagnostic laboratory.
    - Repeat molecular indexing for viruses can be carried out in April and May if required.
9. Fungal and bacterial culturing will be conducted in October and November by a diagnostic laboratory. Sampling and testing will be carried out according to the protocols outlined in this manual.
10. If negative pathogen testing results are obtained the daughter plants can be certified as pathogen tested and used as nucleus or foundation plants in the following year.

11. If a positive result is obtained for any prescribed pathogen, all mother plants, runners and daughter plants of the affected variety will be removed immediately from the collection and placed in an isolated facility.
    - If a virus is suspected the affected plants will be re-tested using biological and molecular methods for confirmation.
    - If a virus is suspected, all mother plants of each variety will be tested using molecular methods.
    - If a fungal or bacterial infection is detected a follow up samples with be re-submitted to the diagnostic laboratory for confirmation using culturing techniques and other methods if available (as determined by the diagnostic laboratory).

FIGURE 1. SUMMARY OF INTEGRATION OF BIOLOGICAL AND MOLECULAR INDEXING PROCEDURE FOR INDEXING OF STRAWBERRY RUNNERS

Stage	BIOLOGICAL		MOLECULAR	
Pre-nucleus stock under quarantine or locally sourced. Indexing by 3 rounds negative tests.	Two rounds of testing	Year 1 - Spring Year 2 - Spring	Four rounds of testing	Year 1 - Spring and autumn Year 2 - Spring and autumn
Annual Indexing of the Nucleus Mother plants	Every two years	Year 2 - Spring	Every two years	Years 1 - Spring
Annual procedure	Preparation of indicator plants	Propagation begins in July	Sampling from pre-nucleus stock or nucleus mother plants	October-November
	Sampling from pre-nucleus stock or nucleus mother plants	October-November	Sample preparation- nucleic acid extraction	October-November
	Grafting to indicator plants – includes candidates and positive controls	October-November	PCR reaction with specific primers to detect part of the pathogen genome	October-November
	Recording presence of symptoms compared to positive control plants	January-February	Recording the size of a PCR (DNA) product that indicates presence of pathogen	October-November Results gained within 10 working days of sample collection

REFERENCES

Bonants PJM, van Gent-Pelzer MPE, Hooftman R, Cooke DEL, Guy DC and Duncan JM. 2004. A combination of baiting and different PCR formats, including measurement of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. *European Journal of Plant Pathology* 110, 689-702.

Bringhurst RS and Voth V. 1956 Strawberry virus transmission by grafting excised leaves. Plant Disease Reporter. 40, 596-600.

Constable F, Bottcher, C, Kelly G, Lines R, Milinkovic M, Persley D and Rodoni B. 2007. Molecular diagnostics for the detection of strawberry viruses. Final report for Horticulture Australia project BS04004.

Constable F, Bottcher, C, Kelly G, Nancarrow N, Milinkovic M, Persley D and Rodoni B. 2010. Developing molecular diagnostics for the detection of strawberry viruses (Follows BS04004). Final report for Horticulture Australia project BS07003.

Constable F, Bottcher, C, Kelly G, Nancarrow N, Milinkovic M, Ko L, Persley D and Rodoni B. 2011. Developing molecular diagnostics for the detection of strawberry viruses (continues BS07003). Final report for Horticulture Australia project BS10002.

Cropley R. 1958. Erratic transmission of strawberry viruses by grafting excised leaves. Annual Report of the East Malling Research Station for 1957. pp124-125.

Duncan JM. 1976. The use of bait plants to detect *Phytophthora fragariae*. *Transactions of the British Mycological Society* **65,** 338-340.

Faggioli F, Ferretti L, Pasquini G and Barba M. 2002. Detection of *Strawberry latent ring spot virus* in leaves of olive trees in Italy using a one-step RT–PCR. *Journal of Phytopathology* **150**, 636–639.

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

Greber RS. 1979. Virus diseases of Queensland strawberries and the epidemiological effects of the strawberry runner approval scheme. *Queensland Journal of Agricultural and Animal Sciences.* **36,** 93-103.

Griesbach JA.1995 Detection of *Tomato ringspot virus* by polymerase chain reaction. *Plant Disease* **79,** 1054-1056

Harper SJ, Delmiglio C, Ward LI and Clover GRG. 2011. Detection of Tomato black ring virus by real-time one-step RT-PCR. Journal of Virological Methods 171, 190–194.

Harris RV and King E. 1942. Studies in strawberry diseases. The use of *Fragaria vesca* L. as an indicator of yellow edge and crinkle. Journal of Pomological and Horticultural Science. 19, 227-242.

Harris R, Ochoa-Corona R, Lebas B, Timudo O, Stewart F and Alexander B. 2006. Broad detection and diagnosis of viruses of the genus *Tombusvirus* by RT-PCR coupled to single strand conformation polymorphism analysis. In: Proceedings of the 7th Australasian Plant Virology Workshop, Rottnest Island, Perth, Australia, p. 48.

Jończyk M, Borodynko N and Pospieszny H. 2004. Restriction analysis of genetic variability of Polish isolates of *Tomato black ring virus*. *Acta Biochimica Polonica* **51**, 673-681.

Le Gall O, Candresse T and Dunez J. 1995. Transfer of the 31 non-translated region of *Grapevine chrome mosaic virus* RNA-1 by recombination to *Tomato black ring virus* RNA-2 in pseudorecombinant isolates. *Journal of General Virology* **76**, 1285-1289.

MacKenzie, DJ, McLean, MA, Murkerji, S and 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.

Martin RR and Tzanetakis IE. 2006. Characterization and recent advances in detection of strawberry viruses. *Plant Disease*. **90**, 384-396.

Martin RR, Pinkerton JN and Kraus J. 2008. The use of collagenase to improve the detection of plant viruses in vector nematodes by RT-PCR. *Journal of Virological Methods* **155**, 91–95

Mellor FC and Fitzpatrick RE. 1961. Strawberry viruses. Canadian Plant Disease Survey. 41, 218-255.

Miller PW. 1958. Comparative efficiency of excised leaf-petiole grafts and stolon grafts for transmitting certain strawberry viruses. Plant Disease Reporter. 42, 1043-1047.

Mohan SB. 1988. Evaluation of antisera raised against *Phytophthora fragariae* for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA). *Plant Pathology* **37,** 206- 216

Moltmann E and Zimmermann C. 2005. Detection of *Xanthomonas fragariae* in symptomless strawberry plants by nested PCR *OEPP/EPPO Bulletin* **35,** 53–54

Noble D. 2009. Diagnostic Methods for Strawberry Angular Leaf Spot *Xanthomonas fragariae* *http://www.padil.gov.au/pbt | Strawberry Angular Leaf Spot - Xanthomonas fragariae*

Ochoa-Corona FM, Lebas BS, Tang JZ, Bootten TJ, Stewart FJ, Harris R, Elliott DR and Alexander BJR. 2006. RT-PCR detection and strain typing of *Raspberry ringspot virus*. Proceedings of the XXth International Symposium on Virus and Virus-like Diseases of Temperate Fruit Crops and XIth International Symposium on Small Fruit Virus Diseases, Antalya, Turkey.

Pooler MR Ritchie DF and Hartung JS. 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology* **62,** 3121–3127.

Postman JD, Tzanetakis IE, and Martin RR. 2004. First report of *Strawberry latent ringspot virus* in a *Mentha* sp. from North America. *Plant Disease* 88, 907.

Russo M, Vovlas C, Rubino L, Grieco F and Martelli, GP. 2002. Molecular characterization of a tombusvirus isolated from diseased pear trees in southern Italy. *Journal of Plant Pathology* **84,** 161-166.

Thompson JR and Jelkmann W. 2003. The detection and variation of strawberry mottle virus. *Plant Disease*. **87,** 385-390.

Thompson JR, Wetzel S and Jelkmann W. 2004. Pentaplex RT-PCR for the simultaneous detection of four aphid-borne viruses in combination with a plant mRNA specific internal control in Fragaria spp. *Acta Horticulturae*. **656,** 51-56.

Tzanetakis IE, Wintermantel WM and Martin RR. 2003. First report of beet pseudo yellows virus in strawberry in the United States: a second crinivirus able to cause pallidosis disease. *Plant Disease*. **87,** 1398.

Wetzel T, Jardak R, Meunier L, Ghorbel A, Reustle GM and Krczal G. 2002. Simultaneous RT/PCR detection and differentiation of arabis mosaic and grapevine fanleaf nepoviruses in grapevines with a single pair of primers. *Journal of Virological Methods* **101**, 63–69

Whattam M. 1994. Strawberries: The production of pathogen tested nucleus plants. Technical manual. Institute for Horticultural Development, Victoria, Australia.

Weisberg, WG, Barns, S, Pelletier, DA and Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703.

Zimmermann C, Hinrichs-Berger J, Moltmann E, and Buchenauer H. 2004. Nested PCR (polymerase chain reaction) for detection of *Xanthomonas fragariae* in symptomless strawberry plants. *Journal of Plant Disease and Protection* **111**, 39–51.

Victorian Strawberry Industry Certification Authority (VSICA)

TECHNICAL MANUAL

Pathogen Indexing of Strawberry Runners

SCOPE

- This manual specifies the indexing protocols that are used to annually test the strawberry plants held in VSICA's nucleus facility for infection by selected plant pathogens.

- The manual introduces new molecular methods, based on world best practice and nine years of research and development, which improve the sensitivity and efficiency of pathogen testing operations.

- Conventional biological methods are integrated with molecular tests as Standard Operating Procedures (SOP) for use as a single robust protocol for commercial use.

- The new indexing protocols will be incorporated in the Conditions of the Victorian Strawberry Runner Certification Scheme (Scheme).

- Tissue sampling details have been included in the manual to accommodate the recent changes in location of the VSICA nucleus from DPI Knoxfield to Toolangi. Samples will be taken from varieties in the Holding Area and Nucleus House at Toolangi and tested at DPI laboratories at La Trobe University.

- The manual will harmonise diagnostic tests used by organisations involved in screening strawberry germplasm for growers in Australia. It is anticipated that this will include offshore Approved Source laboratories, AQIS - post entry quarantine and VSICA.

- Harmonisation of diagnostic protocols for screening of endemic and exotic strawberry pathogens will improve biosecurity, increase efficiency and reduce time delays and costs for the Australian strawberry industry.

INTRODUCTION

VSICA is an independent not-for-profit business that was established for early stage multiplication of varieties of pathogen tested (PT) strawberry runners.

This prescriptive manual integrates contemporary and modern molecular diagnostic testing for use in a commercial operation to support the certification of pathogen tested strawberry runners (Figure 1). It has relevance for other businesses that are involved in vegetative multiplication of high health strawberry runners.

Robust pathogen testing is a vital part of VSICA's business as it manages many PT varieties on behalf of industry clients. Germplasm is held in a secure facility known as the Nucleus and for strawberry cultivars to qualify for admission, candidate varieties must return negative results from three rounds of diagnostic testing. This can take up to three years to complete by conventional methods of biological indexing (Whattam 1994). In addition to the requirement for entry, the Nucleus mother plants are also tested on an annual basis for specified plant pathogens.

Failure to detect a specified infective agent will reduce productivity and quality benefits that PT material normally confers. For this reason tight security protocols are used to manage the Nucleus facility to minimise risk of introducing disease.

One traditional method of detecting viruses in strawberries involves use of grafting candidate tissue onto indicator plants that express characteristic symptoms of disease. The appearance of characteristic symptoms of disease on the indicator is indicative of the presence of virus in the sample. This method of biological testing has been an important certification tool in the production of pathogen tested strawberries for many years (Whattam 1994).

Although biological indexing is considered reliable it is labour intensive and protracted procedure that takes up to three months to complete. The development of new molecular techniques using the polymerase chain reaction (PCR) has provided opportunities to improve the sensitivity and efficiency of pathogen detection (Constable et al 2007, Constable et al 2011). PCR uses complex biochemistry for recognition and multiplication of unique segments of the genome of each of the target pathogens. Nine years of R&D supported by VSICA and Horticulture Australia have developed PCR into a robust method that can be used for the detection of strawberry viruses. Molecular methods detect specific virus species rather than symptoms associated with a virus or mixture of viruses in biological indexing.

It is recommended that both biological and molecular methods should be used for indexing (Constable et al 2007, Constable et al 2011). Consequently the commercial application of molecular testing has been incorporated into this manual for strawberry pathogen testing. The integration of molecular with biological methods provides benefits for Industry because it increases security and reduces risk of undetected infection.

FIGURE 1. SUMMARY OF INTEGRATION OF BIOLOGICAL AND MOLECULAR INDEXING PROCEDURE FOR INDEXING OF STRAWBERRY RUNNERS

Stage	BIOLOGICAL		MOLECULAR	
Pre-nucleus stock under quarantine or locally sourced. Indexing by 3 rounds negative tests.	Two rounds of testing	Year 1 - Spring Year 2 - Spring	Four rounds of testing	Year 1 - Spring and autumn Year 2 - Spring and autumn
Annual Indexing of the Nucleus Mother plants	Every two years	Year 2 - Spring	Every two years	Years 1 - Spring
Annual procedure	Preparation of indicator plants	Propagation begins in July	Sampling from pre-nucleus stock or nucleus mother plants	October-November
	Sampling from pre-nucleus stock or nucleus mother plants	October-November	Sample preparation- nucleic acid extraction	October-November
	Grafting to indicator plants – includes candidates and positive controls	October-November	PCR reaction with specific primers to detect part of the pathogen genome	October-November
	Recording presence of symptoms compared to positive control plants	January-February	Recording the size of a PCR (DNA) product that indicates presence of pathogen	October-November Results gained within 10 working days of sample collection

CURRENT AND FUTURE INDEXING REQUIREMENTS FOR PLANT PATHOGENS SPECIFIED IN THE CONDITIONS OF THE VICTORIAN STRAWBERRY RUNNER CERTIFICATION SCHEME (SCHEME)

Current

The current manual (Whattam 1994) used by The Victorian Strawberry Runner Certification Scheme (VSICA) prescribes protocols to be used by VSICA in the production of certified runners. This includes biological methods of indexing for specified plant pathogens.

Currently they comprise five viruses:

- *Strawberry mottle sadwavirus* (SMoV)
- *Strawberry crinkle cytorhabdovirus* (SCV)
- *Strawberry mild yellow edge potexvirus* (SMYEV)
- *Strawberry vein banding caulimovirus* (SVBV)
- *Tobacco streak virus* (TSV)

In addition indexing is conducted for Strawberry pallidosis disease and three fungal pathogens:

- species of *Phytophthora* (crown and root rot)
- *Verticillium dahliae* (verticillium wilt)
- *Gnomonia comari* (leaf blotch)

The new manual incorporates molecular assays to detect seven specific viruses including:

- *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) associated with Pallidosis disease
- Strawberry necrotic shock virus associated with Strawberry necrotic shock disease, which was formally thought to be caused by *Tobacco streak virus.*

Future

Molecular diagnostic tests are also presented in the manual for exotic pathogens of strawberries. These are being considered for inclusion in the Scheme and include:

- *Xanthomonas fragariae* (angular leafspot)
- *Phytophthora fragariae* var. fragariae (Strawberry red stele)
- *Arabis mosaic nepovirus* (ArMV)
- *Raspberry ringspot nepovirus* (RpRSV)
- *Tomato ringspot nepovirus* (ToRSV)
- *Tomato black ring nepovirus* (TBRV)
- *Strawberry latent ringspot sadwavirus* (SLRV)
- *Tomato bushy stunt tombusvirus*, (TBSV).

Additional note:

The new manual will need adapting to meet the needs of strawberry production in a subtropical environment where plants are exposed to different plant pathogens that cause debilitating disease.

OVERVIEW OF INDEXING PROCEDURES

This procedure for pathogen testing of the strawberry nucleus is based on world's best practice validated diagnostic tests for strawberry pathogens and incorporates existing (Whattam 1994) and new (Constable et al 2007, Constable et al 2010, Constable et al 2011) diagnostic protocols for detecting strawberry pathogens.

1. Plants entering the nucleus collection must have returned two seasons of negative pathogen testing results which included two years of biological indexing in spring of each year and molecular indexing in the spring and autumn of both years. If all tests are negative the plants can be included into the nucleus collection.
2. Nucleus mother plants are tested every two years by biological indexing in spring and in the alternative year they are tested in spring by molecular indexing.
3. Nucleus mother plants are removed from cold storage at the beginning of September, and are established and maintained in a screen house with insect and pollen proof mesh or an insect proof glasshouse during September until May.
4. Nucleus plants must be inspected daily for symptoms of disease and for insect pests during the growing season.
    - Pathogens and insects must be treated as soon as is practical.
    - If a vector of a prescribed pathogen is detected it is recommended that the exposed plants are tested for the pathogen.
5. Pathogen testing for viruses by biological indexing and/or molecular indexing is conducted during October and November. Sampling and diagnostic testing is carried out according to the protocols in this manual:
    - Varieties that are continually maintained in the nucleus collection for two or more years will be pathogen tested for specified viruses by biological indexing every second year. In the alternate year they will be tested by molecular methods.
    - Varieties that are in their first year of incorporation in the nucleus collection must be indexed using molecular methods in the first year.
    - All plants will be tested by biological indexing onto *Chenopodium quinoa* for detection of *Tobacco streak virus* and nepoviruses in each year.
6. Biological indicators, including *Fragaria* species and *C. quinoa*, will be maintained and propagated by a diagnostic laboratory in an approved glasshouse according to the protocols outlined in this manual.
7. Biological indexing by graft inoculation of *Fragaria* sp. biological indicators and rub inoculation of *C. quinoa* will be carried by a diagnostic laboratory in glasshouse conditions in October and November according to the protocols in this manual.
8. Molecular indexing of prescribed pathogens will be carried out in October and November according to the protocols described in this manual.
    - Repeat molecular indexing for viruses can be carried out in April and May if required.
9. Fungal and bacterial culturing will be conducted in October and November. Sampling and testing will be carried out according to the protocols outlined in this manual.
10. If negative pathogen testing results are obtained the daughter plants can be certified as pathogen tested and used as nucleus or foundation plants in the following year.
11. If a positive result is obtained for any prescribed pathogen, all mother plants, runners and daughter plants of the affected variety will be removed immediately from the collection and placed in an isolated facility.
    - If a virus is suspected the affected plants will be re-tested using biological and molecular methods for confirmation.

- If a virus is suspected all mother plants of each variety will be tested using molecular methods.
- If a fungal or bacterial infection is detected a follow up sample will be re-submitted to the diagnostic laboratory for confirmation using culturing techniques and other methods if available (as determined by the diagnostic laboratory).

REFERENCES

Constable F, Bottcher, C, Kelly G, Lines R, Milinkovic M, Persley D and Rodoni B. 2007. Molecular diagnostics for the detection of strawberry viruses. Final report for Horticulture Australia project BS04004.

Constable F, Bottcher, C, Kelly G, Nancarrow N, Milinkovic M, Persley D and Rodoni B. 2010. Developing molecular diagnostics for the detection of strawberry viruses (Follows BS04004). Final report for Horticulture Australia project BS07003.

Constable F, Bottcher, C, Kelly G, Nancarrow N, Milinkovic M, Ko L, Persley D and Rodoni B. 2011. Developing molecular diagnostics for the detection of strawberry viruses (continues BS07003). Final report for Horticulture Australia project BS10002.

Whattam M. 1994. Strawberries: The production of pathogen tested nucleus plants. Technical manual. Institute for Horticultural Development, Victoria, Australia.

SECTION 1: PREPARATION OF NUCLEUS PLANTS FOR INDEXING

VSICA is responsible for the maintenance of all plants in the nucleus collection.

The protocols for the general maintenance of the nucleus collection are not given in this manual however there some requirements that must be followed to ensure enough plant material is obtained from each variety for biological indexing in spring (October-November).

- To ensure adequate production of leaf material for pathogen testing, the nucleus mother plants must be removed from cold storage at the beginning of September and re-potted.
- A minimum of two additional daughter plants must be produced for destructive testing for the detection of *Phytophthora* sp. in March-April.
- All mother and daughter plants will be grown on raised benches and be easily accessible for visual inspection and sample collection.
- All nucleus and daughter plants must be maintained in a healthy, vigorous state.
- Nucleus mother and daughter plants should be inspected daily for evidence of disease and insect pests.
- Nucleus mother and daughter plants should be inspected daily for flowers and flower buds
    - Remove buds as soon as they appear to reduce the risk of virus transmission via pollen.
- All daughter plants that will be used to form the nucleus collection for the subsequent year will be placed into cold storage at -2℃ in June for a minimum of 8 weeks.

SECTION 2: PREPARING FACILITIES FOR INDEXING

2.1 MAINTAINING SECURITY OF AREAS WHERE PLANTS ARE GROWN AND INDEXED

This protocol applies to areas for uninoculated and inoculated biological indicator plants and the virus positive control plants and follows the procedures outlined by Whattam (1994).

The diagnostic laboratory is responsible for the maintenance of the uninoculated and inoculated biological indicator plants and the virus positive control plants.

- The uninoculated indicator plants, the inoculated indicator plants and the virus positive control plants will each be maintained in a separate secure glasshouse or isolated compartment within a glasshouse facility.
- Dedicated, isolated and signed secure areas are required for uninoculated indicators, inoculated indicators and positive controls.
- Where necessary, additional signs inside each area should identify the type of plant present and specify the handling requirements of these plants.
- Access to the facility will be controlled and limited to specified personnel.
- The facility will be enclosed with polythene or glass.
- All windows and vents will be screened with an insect proof mesh with a maximum aperture of 0.6mm to prevent entry of insects.
- The facility will have tight fitting doors and vents that remain rigid at all times.
- For controlled access the facility will have an insect proof anteroom with tight fitting doors at the entrance of the anteroom and the facility.
- The anteroom should be large enough to permit entry of a person and plant material with one door being shut at all times.
- The anteroom will have a foot bath utilizing an effective disinfectant:
    - The footbath must be replenished once a week or sooner if required.
    - All people are required to disinfest their shoes in this foot path before entering the facility containing the indicator plants.
- The facility should have a concrete floor and should not flood.
- The facility will be maintained at 18-25℃.
- Appropriate plant and glasshouse hygiene measures shall be maintained at all times:
    - Regular cleaning to remove dirt and rubbish on benches and floors.
    - Disinfection of cutting tools.
- The facility and the anteroom shall be maintained free of weeds, lichen and moss.
- The facility will have good ventilation and low humidity to discourage powdery mildew infections.
- Yellow sticky insect traps shall be appropriately installed in the facility at a minimum rate of one per 15 square meters of planted area and one shall be installed in the anteroom:
    - Sticky traps will be inspected 2-3 times per week.
    - Sticky traps will be replaced every two weeks.
    - If potential pathogen vectors are detected in the facility they will be sent to a diagnostic laboratory for identification.
    - If pathogen vectors are detected on the sticky traps or on plants, all plants within the facility shall be treated with an appropriate insecticide.
- If an insect vector is detected, the infested plants will be removed from the facility and destroyed.

- The remaining plants will be tested for the pathogen transmitted by the vector.
- A backup of *Fragaria* indicators will be maintained in tissue culture or in an alternative glasshouse.
- Employees of the diagnostic laboratory and visitors must not enter the facility after they have been working amongst/visiting field grown plants on the same day unless they have changed into clothing that has not been exposed to field conditions and ensured that any exposed body parts are not harbouring potential insect vectors of viruses and phytoplasmas such as whiteflies, thrips, aphids or leaf or plant hoppers.

2.2 PROPAGATION AND MAINTENANCE OF BIOLOGICAL INDICATORS

Fragaria vesca var semperflorens (Alpine), cultivars of *F. vesca* and *F. virginiana* and *Chenopodium quinoa* are used as biological indicator plants for pathogen detection (Frazier 1974, Whattam 1994, Martin and Tzanetakis 2006). Alpine is used for *Phytophthora* sp. baiting and *F. vesca, F. virginiana* and *C. quinoa* present characteristic symptoms of virus disease that are used to differentiate between viruses. Traditionally *F. vesca* UC-4, *F. vesca* UC-6 and *F. virginiana* UC-10 are used in combination with Alpine and *C. quinoa* in Australia (Whattam 1994).

2.2.1 *FRAGARIA* INDICATOR PLANTS

- Four indicator varieties are required for biological indexing including *Fragaria vesca* var *semperflorens* (Alpine), *F. vesca* UC-4, *F. vesca* UC-6 and *F. virginiana* UC-10.
- All indicator plants must be free of virus prior to inoculation.
- Biological indexing requires two indicators each of Alpine, UC-4 UC-6 and UC-10 per nucleus variety therefore this number plus 30 additional plants of each indicator type will be produced each season to ensure there are ample plants for inoculation and as mother plants are required for the following year.
- Production of indicator plants will commence no later than July prior to graft inoculation in October/November to ensure that there are adequate numbers of each indicator cultivar with at least three fully expanded leaves per plant.
- *Fragaria* indicator plants will be maintained in the secure facility all year.
- *Fragaria* indicator plants to be used as mother plants will be grown in pasteurised standard-type potting mix with a medium-term, slow-release fertiliser (e.g. Osmocote®) in 1.1 L pots.
- As the mother plants produce runners with daughter plants, each daughter plant will be trained directly into separate 0.36 L pots containing a standard-type potting mix with a medium-term, slow-release fertiliser (e.g. Osmocote®).
- New and sterile 0.36 L pots will be used.
- Each pot must be clearly labelled with the indicator variety.
- All mother and daughter plants will be grown on raised benches and be easily accessible for inspection.
- Plants will be adequately spaced on benches to physically isolate plants and aide visual inspection.
- *Fragaria* indicator plants must be maintained in a healthy, vigorous state.
- The *Fragaria* indicator plants will be inspected daily for evidence of disease and insect pests:
    - Pathogens and insects must be treated as soon as is practical.
    - If the plants are infected with a prescribed pathogen they must be destroyed.
- All *Fragaria* indicator plants must not be allowed to flower during the growing season:

- Inspect all plants daily for flower buds and remove buds as soon as they appear.

2.2.2 *FRAGARIA VESCA* VAR *SEMPERFLORENS* (ALPINE) INDICATORS FOR BAIT TESTING

- All Alpine indicator plants will be grown from seed that has been produced from a virus free source.
- The seed can be produced by the diagnostic laboratory from fruiting plants that have not been exposed to pathogens.
- Seed will be planted into a sterile 0.36L pot with a sterile seed raising mix with fertiliser a minimum of eight weeks prior to baiting for *Phytophthora sp.*
- When the seed has reached the two leaf stage they will be pricked out and individual seedlings will be potted into a sterile 0.36L pot containing a standard-type potting mix with a medium-term, slow-release fertiliser (e.g. Osmocote®).
- Two Alpine indicator plants will be produced for each candidate variety.
- Plants must be grown at 18-25℃ before and after inoculation.

2.2.3 *CHENOPODIUM QUINOA* INDICATOR PLANTS

- All *C. quinoa* indicator plants will be grown from seed that has been produced from a virus free source.
- The seed can be produced by the diagnostic laboratory from fruiting plants that have not been exposed to pathogens.
- Seed will be planted into a sterile 0.5L pot with a sterile seed raising mix with fertiliser.
- When the seed has reached the two leaf stage (approx. 10 days) they will be pricked out and three seedlings will be potted into a 0.25L pot containing a standard-type potting mix with a medium-term, slow-release fertiliser (e.g. Osmocote®).
- One pot of three *C quinoa* seedlings will be produced for each candidate variety.
- One of the three seedlings will be removed from the pot at the time of inoculation.
- Plants must be grown at 18-25℃ before and after inoculation.

2.3 PROPAGATION AND MAINTENANCE OF VIRUS POSITIVE CONTROL PLANTS

- The virus isolates for use as positive controls for biological and molecular indexing are maintained in plants of *Fragaria* sp.
- The virus positive control plants are maintained in a separate facility to the uninoculated and inoculated *Fragaria* indicators plants to reduce the risk of virus transmission between plants.
- All virus positive control plants must be maintained in a healthy, vigorous state.
    - If a new positive control plant is required and new plant may be propagated from a runner or material from the original plant will be petiole grafted to sensitive *F. vesca* UC-4 and UC-6, *F virginiana* UC-10 and *F. vesca* var *semperflorens* (Alpine) plants to transfer the virus isolate to the new host plant.
    - Some virus isolates may also be transferred from the original host to a new host by rub inoculation onto the same *Fragaria* species and clones. This method is the same as used for biological indexing on *Chenopodium quinoa* which is described in section 3.2.2.2.

- Newly inoculated plants are isolated on a glasshouse bench and labelled to identify the original source and disease or virus with which they were inoculated and date of inoculation.
- The plants will be tested by molecular methods to determine confirm the transmission of virus.
- The plants will be labelled with the viruses that are detected.

- Inspect virus positive control plants daily for evidence of disease and insect pests
  - Pathogens and insects must be treated as soon as is practical.
- All virus positive control plants must not be allowed to flower during the growing season.
- Inspect all plants daily for flower buds and remove buds as soon as they appear.

2.4 LABORATORY FACILITIES

- Government diagnostic laboratories used for diagnostic testing will have the necessary facilities for biological and molecular testing; and which use good laboratory practice.

Molecular laboratories:

- Three laboratories are required for molecular indexing:
  .1. Nucleic acid extraction laboratory – gel electrophoresis can be done in this laboratory.
  .2. Molecular laboratory for PCR reaction set up – no plant material or nucleic acid extracted from plant material shall enter this laboratory.
  .3. Molecular laboratory for the addition of nucleic acid to the PCR reactions.
- The equipment used is specific to each laboratory and must not be removed.
- If three laboratories are not available PCR reaction set up can be done in a PCR workstation in the same laboratory where nucleic acid is extracted:
  - The pipettes, boxes of tips and bags of tubes used in this work station must not be used for other purposes.
  - The workstation must have filter system in place to reduce contamination through aerosols and an ultraviolet lamp for irradiation of the interior of the cabinet to degrade potential contaminating nucleic acid.

Fungal and Bacterial culturing laboratories:

- All cultures of fungi and bacteria are located in a quarantine incubator for growth and identification.

Disposal of samples and cultures:

- All infective material, laboratory materials that have been in contact with samples or their extracts and fungal and bacterial cultures should be destroyed by steam sterilisation or accredited waste disposal facility.

SECTION 3: DIAGNOSTIC PROTOCOLS

3.1 PATHOGEN TESTING REQUIREMENTS

3.1.1 PATHOGEN TESTING REQUIREMENTS FOR NEW VARIETIES BEFORE INTRODUCTION INTO THE NUCLEUS

- All new varieties will be tested during two growing seasons for pathogens associated with prescribed pathogens (Table1).
- All plants must be inspected regularly throughout each growing season for visual symptoms that indicate infection by viruses, bacteria or fungi.
- All plants will be tested for virus associated diseases using biological indexing in spring of each year and the inoculated indicator plants must be free of symptoms which are indicative of virus infection.
- All plants will be tested for viruses using molecular indexing in spring and autumn in each year of screening and none of the prescribed viruses (Table 1) should be detected.
- All plants will undergo culturing for bacterial and fungal pathogens (Table 1) in each year and none of the prescribed bacteria or fungi should be detected.

3.1.2 PATHOGEN TESTING REQUIREMENTS FOR VARIETIES MAINTAINED IN THE NUCLEUS

- All plants must be inspected regularly throughout each growing season for visual symptoms that indicate infection by viruses, bacteria or fungi.
- Plants that are continually maintained in the nucleus collection for two years or more will be pathogen tested for the specified viruses (Table 1) by biological indexing every second year. In the alternate year they will be indexed by molecular methods.
- Plants entering the nucleus collection for the first year will undergo molecular indexing for viruses.
- All plants will undergo culturing for fungal pathogens in each year.

3.1.3 PRESCRIBED PATHOGENS REQUIRING TESTING FOR THE NUCLEUS COLLECTION

- Plants in the nucleus collection must be tested on an annual basis for the virus, fungal and bacterial pathogens listed in Table 1. The methods which may be used for the detection of each pathogen are also listed is this table.
- Details about the biology of the endemic pathogens are given in Appendix 1.

Table 1. A list of prescribed pathogens that plants in the nucleus collection must be tested for and the methods that may be used for detection of each pathogen.

Pathogen	Detection method			
	Visual inspection	Culturing	Biological	Molecular
Virus				
Beet pseudo-yellows crinivirus (BPYV)			**GI**[2]	**RT-PCR**[4]
Strawberry crinkle cytorhabdovirus (SCV)			**GI**	**RT-PCR**
Strawberry mild yellow edge potexvirus (SMYEV)			**GI**	**RT-PCR**
Strawberry mottle sadwavirus (SMoV)			**GI**	**RT-PCR**
Strawberry necrotic shock ilarvirus (SNSV)			**HI**[3]**, GI**	**RT-PCR**
Strawberry pallidosis associated crinivirus (SPaV)			**GI**	**RT-PCR**
*Strawberry vein banding caulimovirus (*SVBV)			**GI**	**PCR**[5]
Tobacco streak ilarvirus (TSV)			**HI**	**RT-PCR**
Bacteria				
Xanthomonas arboricola pv. *fragariae*[1]	**Yes**	**Yes**[7]		
Xanthomonas fragariae[1]	**Yes**	**Yes**[7]		**PCR**[7]
Rickettsia-like-organisim	**Yes**			**PCR**[7]
Phytoplasmas	**Yes**			**PCR**[7]
Fungi				
Colletotrichum species	**Yes**	**Yes**		
Gnomonia comari	**Yes**	**Yes**		
Phytophthora sp. including *P. fragariae* f.sp. *fragariae* [1]	**Yes**	**Yes**	**B**[6]	**PCR**[7]

[1] These are quarantine pathogens that are not known to occur in Australia or have been detected and eradicated - active surveillance by visual inspection will improve the biosecurity of the nucleus collection.

[2] GI = Biological indexing by graft inoculation onto the susceptible *Fragaria vesca* cvs UC-4 and UC-6 and *F. virginiana* cv UC-10 indicators

[3] HI = Biological indexing by rub inoculation onto *Chenopodium quinoa* (herbaceous indexing)

[4] RT-PCR = Detection of pathogen RNA by reveres transcription (RT) PCR

[5] PCR = Detection of pathogen DNA by polymerase chain reaction (PCR)

[6] B = Detection of pathogen by baiting susceptible *F. vesca* subsp. vesca forma *sempeflorens* 'Alpine'

[7] Culturing and /or PCR for *X. arboricola* pv *fragariae*, *X. Fragariae,* Rickettsia-like-organisim and phytoplasmas and PCR for *P. fragariae* f.sp. *fragariae* is not mandatory but may be requested by the VSICA for pathogen detection or used by the diagnostic laboratory for confirmation of infection.

3.2 PATHOGEN TESTING METHODS

3.2.1 VISUAL INSPECTION FOR DISEASE

Prescribed pathogens for which visual inspection is required include several bacteria and fungal pathogens. Table 2 lists the pathogens for which visual inspection of the nucleus is required, the diseases that they are associated with and the alternative methods of detection which can be used to confirm infection.

- Nucleus plants will be inspected regularly during the growing season for evidence of disease and pests.
- If a prescribed pathogen is suspected the facility manager will send the entire plant or a subsample to a diagnostic laboratory for diagnosis.
    - Alternatively the certifying body (e.g. VSICA) may request a diagnostician to visit the facility to observe the disease and sample the plant if required.
    - As a guide the photographs in appendix 2 can be used to assist and initial identification.
- Pot-grown strawberry plants that are not adequately fertilised may exhibit nutrient deficiencies that resemble virus infection, e.g. chlorosis and necrosis.
- Infection by viruses, *Xanthomonas arboricola* pv. *fragariae Xanthomonas fragaria* and *P. fragariae* f.sp. *fragariae* should be confirmed by molecular methods by the diagnostic laboratory. The methods are detailed in appendix 2.
- Infection by *Colletotrichum* sp., *G. comari* and other *Phytophthora* sp can be confirmed by culturing by the diagnostic laboratory.

Table 2. A list of pathogens and associated diseases for which the nucleus collection requires visual inspection and alternative methods for the detection and identification of the pathogens.

Pathogen	Associated disease	Alternative test
Bacteria		
Phytoplasmas	Lethal yellows, strawberry green petal, little leaf, witches' broom	PCR
Rickettsia-like-orgnasim	Lethal yellows	PCR
Xanthomonas arboricola pv. *fragariae*[1]	Bacterial leaf blight	Culturing
Xanthomonas fragariae[1]	Angular leaf spot	Culturing, PCR
Fungi		
Colletotrichum species	Anthracnose (black spot), Crown rot, root necrosis, black leaf spot	Culturing
Gnomonia comari	Fruit rot and leaf blotch	Culturing
Phytophthora sp. *P. fragariae* f.sp. *fragariae*	Crown rot, leather rot, root rot,	Culturing,
	Red stele (*P. fragariae* f.sp. *fragariae)*	PCR(*P. fragariae* f.sp. *fragariae* only*)*

3.2.2 ACTIVE PATHOGEN TESTING FOR VIRUSES, BACTERIA AND FUNGI

- Active testing for viruses and fungi is conducted in October-November.
- Fungal and virus testing can also be conducted in March-April.
- Active bacterial testing is not a requirement but may be requested.
- It is the responsibility of VSICA to inform the diagnostic facility of the number of varieties that require indexing by biological and molecular methods in June before indexing is conducted.
- The diagnostic laboratory will inform VSICA at the beginning of October of the likely timing of sample collection.
- One week's notice should be given to the diagnostic laboratory of the date of sample collection for graft inoculation.
- It is the responsibility of VSICA to co-ordinate sample collection to ensure that the samples arrive in a timely manner to the diagnostic laboratory.
    - VSICA may ask the diagnostic laboratory to collect samples on their behalf.
    - If desired VSICA may choose to collect the samples.
    - The sampling methods are detailed in section 3.2.2.1.
- It is the diagnostic laboratory's responsibility to co-ordinate diagnostic testing and ensure all samples undergo pathogen testing in a timely manner:
    - The diagnostic laboratory must ensure that adequate numbers of indicator plants are ready for pathogen testing in October/November.
    - The diagnostic laboratory must ensure that all consumable items are prepared and available for molecular indexing and fungal and bacterial culturing in October/November.
    - The pathogen testing methods are outlined in section 3.2.
- A record will be kept by the diagnostic laboratory that states:
    - The identifying code or name for the sample.
    - The date and time that the sample was received at the diagnostic laboratory.
    - The tests that have been ordered for each sample.
    - The date that each sample was tested by the required pathogen tests.
    - The results of diagnostic testing.

3.2.3 SAMPLING NUCLEUS PLANTS AND TRANSPORT OF SAMPLES TO DIAGNOSTIC FACILITIES FOR PATHOGEN TESTING

Generic sampling guidelines for active pathogen detection

- VSICA will ensure no quarantine or other regulations that impede transport from Toolangi to the diagnostic facility.
- A record will be kept by VSICA and the diagnostic laboratory that states:
    - The identifying code or name for the sample and the variety from which it was collected.
    - The date and time that the sample was collected.
    - The date and time that the sample arrived at the diagnostic laboratory.
    - The specific diagnostic tests that have been ordered.
- The samples will be accompanied with a list of the samples, the date they were collected and the pathogen testing that is required for each sample.

- Personnel entering the nucleus facility for the purpose of sampling will wear dedicated clothing to reduce the risk of introduction of pest and pathogens.
- Hands will be washed and cutting tools will be sterilised before use and between taking samples from separate varieties.
- The samples from each variety will be placed in a separate zip-lock bags that is clearly labelled with a code or name that is traceable by VSICA to the variety that is being tested:
    - Remove as much air as possible from the zip-lock bags.
    - The code or name will be used to identify the sample during all diagnostic testing and reporting.
- The bagged samples will be placed into an ice-box on ice at approximately 4℃ after sampling and during transport to the diagnostic facility.
- The samples will be logged to the diagnostic laboratory's database immediately upon arrival.
- Samples that are not used immediately upon arrival at the diagnostic facility will be stored in a cool room at 4℃.
- Each variety must be tested separately:
    - Tissue samples from up to 3 plants of one variety may be bulked for testing providing that they have been derived from a single original source plant when introduced to the nucleus collection.
    - If a variety consists of four or more mother plants, groups of 2 or 3 plants will be tested as separate samples.
    - Where the same variety was derived from a different source plant it must be treated as a separate variety.
- Do not collect samples on a Friday.

Sampling for virus detection

- Sampling will begin in October or as soon as the nucleus plants have at least three fully expanded leaves.
- A minimum of 12 leaves is required from each variety for indexing and at least three leaves must be collected from each plant.
- The leaf samples will include the entire petiole with the leaflets attached and must be sampled from the apical crown region.
- The samples will be submitted to the virologist of the diagnostic laboratory as soon as they are logged to the diagnostic laboratory's database.
- Any samples that are not used for grafting or are not used immediately upon arrival will be stored at 4℃ until required.
- For biological indexing on *Fragaria* indicators the leaf samples of the candidate plants must be collected on the day that they will be graft inoculated and transported as quickly as possible to the diagnostic laboratory:
    - The leaf samples should arrive no later than midday on the day of graft inoculation.
    - As soon as the samples arrive they should be placed into separate labelled containers of water to ensure adequate hydration prior to grafting.
    - Samples from a maximum of five varieties will be collected in one day for biological indexing by grafting.
    - After grafting the candidate samples must be kept for use in herbaceous indexing and fungal and bacterial culturing.

- For molecular indexing and herbaceous indexing on *C. quinoa* the samples must be used as soon as possible after removal from the host:
    - Leaf samples used for molecular indexing will be sub-sampled for nucleic acid extraction within 24 hours of arrival.
    - A second leaf subsample will be collected for herbaceous indexing at the same time.
    - If samples require storage before testing, the leaves must be kept whole, all surface water removed and the material stored in a plastic bag at 4°C for no more than 7 days.
    - Samples that have partially decayed or become mouldy should not be tested, and further samples must be collected.

Sampling for detection of fungi

- For fungal baiting and/or culturing, three root tip cuttings, 0.5cm in length, will be collected from each plant of each variety in October-December:
    - The root samples will be tested as soon as possible after collection.
    - If samples require storage before testing, the root pieces must be kept whole, all surface water removed and the material stored in a plastic bag at 4°C for no more than 24 hours.
    - Samples that have partially decayed or become mouldy should not be tested, and further samples must be collected.
- Alternatively two entire daughter plants grown specifically for the detection of fungi can be collected in March or April.
- Subsamples of the roots and bases of the leaf petioles collected for virus testing can be used to actively test for specific fungi using molecular methods if required.

Sampling for detection of bacteria

- For bacterial testing by culturing or molecular methods, subsamples of the petioles and leaflets will be collected from the samples taken for molecular or biological indexing of viruses:
    - Subsamples will be collected by the diagnostic laboratory during pathogen testing.
    - The subsamples will be labelled with a code or name that is traceable by VSICA to the variety that is being tested.
    - Depending upon the extraction technique used it may be possible to use the same nucleic acid that is used for the molecular indexing of viruses to test for bacteria using PCR. The nucleic acid extractions that can be used for testing of bacteria by molecular methods are detailed in appendix 3.

3.2.4 PATHOGEN TESTING PROTOCOLS

3.2.4.1 BIOLOGICAL INDEXING

Biological indexing by graft inoculation of *Fragaria* sp. onto sensitive *Fragaria* biological indicators and rub inoculation of *C. quinoa* will be carried by a diagnostic laboratory in glasshouse conditions in October and November according to the protocols in this manual.

Graft indexing of *Fragaria* biological indicators

- Graft inoculation of *Fragaria* indicators will be conducted in October-November.
- Each *variety* must be tested by leaf-grafting onto two replicate indicator cultivars of *Fragaria vesca* 'UC-4' and 'UC-6' and *Fragaria virginiana* 'UC-10'. The specific virus-disease-indicator combinations ate listed in Table 3.
- Vigorous indicator plants for graft assays are required.
- The indicator plants are ready for grafting when they have two or more fully expanded leaves.
- Two actively growing trifoliate leaves of each indicator plant will be grafted.
- Clearly label the pot of each indicator that will be grafted with a particular sample/variety with the code or name assigned to the sample by the VSICA and the date of grafting.
- The method of graft inoculation is described in detail in appendix 2.
- Inoculated indicator plants will be grown in a mist bed or covered with a plastic bag to create a humid environment for 1-2 weeks until a graft union has formed.
- The indicator plants must be maintained in a vigorous state of growth before and after grafting and must be grown under moderate temperatures and light intensities.
- A single plant each of UC-4, UC-6 and UC-10 must be left ungrafted of each day of grafting and subjected to the same horticultural practices and environmental conditions as the inoculated plants.
- Each indicator variety must be grafted with virus positive controls containing BPYV, SCV, SMoV, SMYEV, SNSV, SPaV and/or SVBV.
- Check the graft union on each indicator two weeks after inoculation. At least one graft per indicator plant must have survived. If both grafted leaflets have not survived the graft must be repeated.
- The inoculated indicator plants will be held in a secure facility as described in section 2.2.1 of this manual.
- The indicator plants will be examined twice per week for symptom expression over a four month period.

Table 3. Recommended *Fragaria* indicator cultivars for graft indexing for specific virus*

Viruses	Disease	*Fragaria* indicator cultivar
Beet pseudo-yellows crinivirus	Pallidosis	*Fragaria virginiana* 'UC-10'
Strawberry crinkle virus	Crinkle	*Fragaria vesca* 'UC-4' and 'UC-6'
Strawberry mild yellow edge-associated virus	Mild yellow edge	*Fragaria vesca* 'UC-4' and *Fragaria virginiana* 'UC-10'
Strawberry mottle sadwavirus	Mottle	*Fragaria vesca* 'UC-4'
Strawberry necrotic shock ilarvirus	Necrotic shock	*Fragaria vesca* 'UC-4' and *Chenopodium quinoa*
Strawberry pallidosis-associated virus	Pallidosis	*Fragaria virginiana* 'UC-10'
Tobacco streak ilarvirus	Necrotic shock	*Fragaria vesca* 'UC-4' and *Chenopodium quinoa*
Strawberry vein banding virus	Vein banding	*Fragaria vesca* ' 'UC-6'
Nepovirus	Stunting degeneration, death	*Chenopodium quinoa (all sp)* and *Fragaria virginiana* 'UC-10' (ToRSV only)

*Mixed virus infections may confound the symptoms that are observed on each indicator

Herbaceous indexing

- Herbaceous indexing by rub inoculation of *Chenopodium quinoa* indicators will be conducted in October-November.
- Each variety in the nucleus collection must be tested by rub inoculation onto two replicate *C. quinoa* indicator plants in each year. The specific virus-disease-indicator combinations are listed in Table 3.
- The indicator plants are ready for grafting when they have two or more fully expanded leaves.
- Clearly label the pot of each indicator that will be rub inoculated with a particular sample/variety with the code or name assigned to the sample by the VSICA and the date of inoculation.
- Remove one of the three *C. quinoa* plants from each pot:
    - Select *C. quinoa* indicator plants at the four- to six-leaf stage of growth with at least two fully expanded leaves.
- Two fully expanded leaves of each indicator plant will be rub inoculated.
- The method of herbaceous indexing by rub inoculation is described in appendix 2.
- The *C. quinoa* indicator plants must be maintained in a vigorous state of growth before and after grafting and must be grown under moderate temperatures and light intensities.
- A single pot containing two *C. quinoa* plants must be left uninoculated and subjected to the same horticultural practices and environmental conditions as the inoculated plants.
- A single pot containing two *C. quinoa* plants must be inoculated with a SNSV virus positive control and subjected to the same horticultural practices and environmental conditions as the inoculated plants.
- The inoculated indicator plants will be held in a secure facility as described in section 2.1 for a minimum of eight weeks.
- The indicator plants will be examined twice per week for symptom expression.

Bait testing for *Phytophthora* sp.

- Bait testing using *Fragaria vesca* var semperflorens (Alpine strawberries) for detection of *P. fragariae* by will be conducted by the diagnostic laboratory in October-November or March-April.
- Sampling of root cuttings will be conducted as described in this section.

When biological indexing is completed all inoculated indicator plants may be destroyed.

3.2.4.2 MOLECULAR INDEXING

Molecular indexing will be carried out by a diagnostic laboratory in October and November according to the protocols in this manual.

Sub-sampling for molecular detection of pathogens.

- Leaf samples should be processed as soon as possible after arrival:
    - If samples cannot be processed immediately they should be stored as described in section 3.2.2.1.
- A 0.5g tissue is required for nucleic acid extraction and will consist of the base of the petioles from at least two leaves per plant from each variety:
    - Remove the stipules if they are attached.
    - Do not use leaflet tissue.
    - **Return all unused tissue to the sample bag as it will be required for biological indexing on *C. quinoa* and bacterial and fungal culturing.**

Nucleic acid extraction

- Nucleic acid extraction is required for molecular indexing for the prescribed viruses, fungi and bacteria.
- Nucleic acid extraction is carried out in the nucleic acid extraction laboratory.
- RNA and/or DNA will be carried out according to the protocols detailed in appendix 3:
    - If there are more that eight samples a QiaExtractor® robot may be used extract total nucleic acid if available from each sample.
    - If the QiaExtractor® is not available or if there are eight samples or less RNA and/or DNA will be extracted using the Qiagen RNeasy® and DNeasy® kits respectively.
- All vials containing samples or extracts from plant samples should be labelled with the sample code, the date and initials of diagnostic scientist or a code that can be traced to the sample information date of extraction and name of the diagnostic scientist.

Housekeeping PCR for nucleic acid quality

- Prior to molecular indexing for pathogens all nucleic acid extracts will be subject to "housekeeping" RT-PCR and PCR assays to ensure that the nucleic acid is of adequate quality for pathogen detection by molecular methods and does not result in a false negative result. The protocol is detailed in appendix 3:
- The housekeeping PCR requires:
    - A positive control: RNA of known good quality.
    - A no template control: Sterile distilled water.

- The housekeeping RT-PCR or PCR assay will only be considered valid if:
    - the positive control produces the correct size product.
    - no bands are produced in the no template control.
- If a positive result is obtained the extract can be tested for the specified pathogens.
- A negative result indicates that nucleic acid has failed to amplify and is not of sufficient quality for pathogen detection:
    - Failure of the samples to amplify with the housekeeping primers suggests that the nucleic extraction has failed, compounds inhibitory to PCR are present in the nucleic acid extract or the nucleic acid has degraded.
    - Dilution or re-extraction of the nucleic acid may be required.
    - Do not use this nucleic acid for pathogen detection.
- If a negative result is returned the extract can be diluted 1/5 and 1/10 and the dilutions re-tested using the housekeeping assay.
    - If a positive result is returned the diluted nucleic acid extract can be tested for the specified pathogens.
    - If the diluted nucleic acid extracts return a negative result the sample must be re-extracted and the new extract must be tested using the housekeeping RT-PCR and/or PCR assay.

RT-PCR and PCR assays

- The viruses, bacteria and fungi of *Fragaria sp.* that can be detected by molecular methods are listed in Table 4.
- Each variety in the nucleus collection must be tested biennially for BPYV, SCV, SMoV, SMYEV, SNSV and SPaV using molecular methods detailed in this manual. In the alternate year the variety is tested for virus associated diseases by graft inoculation onto *Fragaria* indicator plants.
    - These tests must be conducted even if the nucleus plants are symptomless.
- Molecular testing for other viruses, bacteria and fungi is not mandatory but may be requested by the VSICA.
- All PCR tests must include:
    - Positive control: Nucleic acid extracted directly from the pathogen or from an infected plant.
    - No template control: Sterile distilled water.
    - (Optional) Negative control: Nucleic acid extracted from uninfected plant.
- The diagnostic laboratory will ensure that positive control for each of the specified viruses is available before molecular indexing is conducted.
- Specific protocols for the molecular detection of each pathogen are detailed in appendix 3.
- The results of molecular indexing will be examined by a qualified diagnostician to verify the results and determine that a positive result in a test sample is the same molecular size as the positive control for that pathogen.

Table 4. The pathogen for which molecular methods are available for detection and identification, the type of nucleic acid extraction that can be used and the assay which is used to detect the pathogen

Pathogen	Nucleic acid extraction	Assay
Pathogens that require mandatory biennial molecular testing		
Beet pseudo-yellows crinivirus	QiaExtractor or RNeasy	Specific RT-PCR
Strawberry crinkle virus	QiaExtractor or RNeasy	Specific RT-PCR
Strawberry mild yellow edge-associated virus	QiaExtractor or RNeasy	Specific RT-PCR
Strawberry mottle sadwavirus	QiaExtractor or RNeasy	Specific RT-PCR
Strawberry necrotic shock ilarvirus	QiaExtractor or RNeasy	Specific RT-PCR
Strawberry pallidosis-associated virus	QiaExtractor or RNeasy	Two specific RT-PCR tests
Strawberry vein banding virus	QiaExtractor or DNeasy*	Specific PCR
Pathogens for which molecular tests are available but use of this method is optional		
Arabis mosaic virus	QiaExtractor or RNeasy	Specific RT-PCR
Raspberry ringspot virus,	QiaExtractor or RNeasy	Specific RT-PCR
Strawberry latent ringspot virus	QiaExtractor or RNeasy	Specific RT-PCR
Tobacco streak virus	QiaExtractor or RNeasy	Specific RT-PCR
Tomato black ring virus	QiaExtractor or RNeasy	Specific RT-PCR
Tomato ringspot virus	QiaExtractor or RNeasy	Specific RT-PCR
Tomato bushy stunt virus	QiaExtractor or RNeasy	Specific PCR
Xanhtomonas.fragariae	QiaExtractor or DNeasy	Specific PCR
Phytophthora. fragariae var. *fragariae*	QiaExtractor or DNeasy	Specific PCR
Phytoplasmas	QiaExtractor or DNeasy	Specific PCR

* SVBV is a DNA virus but can be detected in nucleic acid extracted using the RNeasy kit

3.2.4.3 FUNGAL AND BACTERIAL AND CULTURING

- Where necessary fungal and bacterial cultures are stored on appropriate media and assigned an identifying number.
- Fungal and bacterial cultures are identified by a qualified taxonomist using appropriate illustrated reference material.

Fungal culturing

- Fungal culturing for *Colletotrichum sp*, *Gnomonia comari* and *Phytophthora sp* is mandatory.
- Fungal culturing will be conducted each year using the roots samples and a portion of the petioles of the leaf samples collected form the varieties in the nucleus collection using protocols described in appendix 2.

- If *Phytophthora sp., Colletotrichum sp*. or *G. comari* are detected the VSICA will be informed and the nucleus collection and the facility will be treated as required to ensure the eradication of the fungi:
    - Repeat testing may be required to ensure the fungi have been eradicated

Bacterial culturing

- Bacterial culturing is not mandatory but may be requested by the VSICA.
- Protocols for culturing depend upon the bacteria requiring isolation and will be determined by the diagnostic laboratory and are not given in this manual.

If available other methods, as determined by the diagnostic laboratory, may be used to detect and identify specific bacteria.

3.2.5 INTERPRETATION OF PATHOGEN TESTING RESULTS

Interpretation of biological indexing

- Pathogen detection by biologic indexing is based on symptom expression.
- Biological indexing will only be considered positive if:
    - Symptoms typical of a prescribed pathogen (viruses or *P. fragariae)* are detected on an indicator during the observation period.
    - Symptoms for virus resemble those observed on indicators inoculated with virus positive controls.
    - Indicators inoculated with the virus positive controls express symptoms indicative of virus infection.
    - Mixed virus infections could lead to symptoms which are difficult to interpret and PCR should be used to verify the presence of virus in the indicator and in the candidate. A negative result indicates that the pathogen was not detected.
- Images of virus infection in the *Fragaria* biological indicators are given in appendix 2
- A result from biological indexing is considered negative if symptoms of a prescribed pathogen are not detected during the observation period.

Interpretation of molecular indexing for pathogens

- The PCR tests will only be considered valid if:
    - the positive control produces the correct size product.
    - no bands are produced in the no template control and the negative control (if used).
- A sample result from valid molecular indexing is considered negative for a pathogen if the PCR assay does not return a PCR product of the expected size.
- A sample result from valid molecular indexing is considered positive for a pathogen if the PCR assay returns a PCR product of the expected size.

Interpretation of fungal and bacterial and culturing

- Fungal and bacterial culturing is considered negative if colonies do not exhibit typical growth and structural morphology of the fungus does not match that associated with the prescribed pathogens.
- Fungal and bacterial culturing is considered positive if colonies do exhibit typical growth and structural morphology of the fungus does not match that associated with the prescribed pathogens.

3.2.6 DETECTION OF QUARANTINE PATHOGENS

The following pathogens (Table 5) requiring testing during post entry quarantine. If available active testing of these pathogens in plants of the nucleus collection can be requested by VSICA.

- Some of the diagnostic tools for endemic pathogens outlined in this manual will detect pathogens of quarantine significance to strawberries.
- If a pathogen of quarantine significance is detected in the nucleus collection the diagnostic laboratory is bound by law to inform the appropriate authorities.
- The protocols for the detection of each pathogen are detailed in appendices 2 and 3.

Table 5. Pathogens requiring testing during post entry quarantine and the methods used:

Pathogen	Detection method			
	Visual inspection	Culturing	Biological	Molecular
Virus				
Arabis mosaic virus			**HI**[1]	**RT-PCR**[2]
Raspberry ringspot virus			**HI**	**RT-PCR**
Strawberry latent ringspot virus			**HI**	**RT-PCR**
Tomato bushy stunt virus			**HI**	**RT-PCR**
Tomato black ring virus			**HI**	**RT-PCR**
Tomato ringspot virus			**HI**	**RT-PCR**
Bacteria				
Xanthomonas arboricola pv. *fragariae*	**Yes**	**Yes**		
Xanthomonas fragariae	**Yes**	**Yes**		**PCR**[3]
Phytoplasmas	**Yes**			**PCR**
Ralsotonia solonacearum	**Yes**	**Yes**		
Rhodococcus fascians	**Yes**	**Yes**		
Candidatus Phlomobacter fragariae	**Yes**			
Fungi				
Phytophthora fragariae f.sp. *fragariae*	**Yes**	**Yes**	**B**[4]	**PCR**

[1] Biological indexing by rub inoculation onto *Chenopodium quinoa* and *Cucumus sativus* (herbaceous indexing)
[2] Detection of pathogen RNA by reverse transcription (RT) PCR
[3] Detection of pathogen DNA by polymerase chain reaction (PCR)
[4] Detection of pathogen by baiting susceptible *F. vesca* subsp. vesca forma *sempeflorens* 'Alpine'

SECTION 3: RECORDING RESULTS

A laboratory notebook or database must be kept to record all experimental data, in such a way that it is verifiable by other people.

- If a notebook is used it must have consecutively numbered fixed pages.
- All activities must be recorded in the laboratory notebook or database on a daily basis:
    - the date at the start of each session.
    - underline, sign and date at the completion of each session.
- No gaps or blank pages should be left in the laboratory notebook.
- Photographs can be stuck into the book or attached to the database to show results. The image in the book must refer to the location of the original image if it is stored electronically.
- Record all experimental data, results and observations in your laboratory notebook or database.
- Protocols can be written out or typed and pasted into the rear of the book or a separate protocol laboratory notebook or database.
- If using a separate Protocol book the specific location of that protocol must be noted.

SECTION 4: REPORTING RESULTS

- Records including the date of examination, the identity of the sample and any specific details of the sample, the tests that were conducted, the results of testing, including images were appropriate, and the name of diagnostician are retained on confidential file.
- When biological indexing is completed the diagnostic laboratory will inform VSICA via a written report which includes:
    - A list of the samples that were tested using the code or label that is traceable by VSICA to the plants that were tested.
    - The tests that were used to detect pathogens in each sample.
    - The results that were obtained.
- The report will be transmitted to VSICA by an appropriate science manager.
- **If a positive result is obtained during pathogen testing the diagnostic laboratory will inform VSICA immediately.**

SECTION 5: ACTION BY THE DIAGNOSTIC LABORATORY FOLLOWING POSITIVE PATHOGEN TESTING RESULTS

VSICA will be informed immediately of any positive results by an appropriate science manager.

Biological indexing

- If symptoms of a prescribed pathogen are observed on any indicator plant the infection must be verified in both the affected indicator plant and the variety that was inoculated:
    - The affected symptomatic indicator plant will be tested for pathogens using molecular methods as described in this manual if available.
    - Images of virus infection in the *Fragaria* biological indicators are given in appendix 2.
- If *P. fragariae* infection is suspected the affected indicator plants and the variety that was screened will be tested by PCR and culturing protocols detailed in sections 3.2 and appendix 3 to confirm infection.
- If a pathogen is detected in the indicator plant but not the nucleus plant using molecular (all pathogens) and culturing (for *P fragariae*) methods the tests must be repeated on all plants in question.

Molecular indexing for pathogens

- If a positive result is obtained VSICA must be informed and the result must be verified in the affected variety:
    - The original or second PCR product will be sequenced to verify its relationship to the pathogen and ensure that a false positive result was not obtained.
- If a pathogen is not detected in any of the re-tested nucleus plants of the affected variety the molecular tests must be repeated on all plants in question.
- If molecular indexing returns a PCR product similar but not identical to the expected size and the result is considered “strong” the PCR product may be sequenced by the diagnostic laboratory to determine its origin.

Fungal and bacterial and culturing

- If a positive result is obtained VSICA must be informed and the result must be verified in the affected variety:
    - Samples will be collected from each of the plants of the candidate nucleus variety and tested separately for the prescribed pathogens using molecular methods and culturing as described in this manual.

APPENDIX 1 – DESCRIPTION OF ENDEMIC PATHOGENS

STRAWBERRY VIRUSES

Twenty viruses have been reported to infect strawberries and seven of these are associated with diseases that are indexed for in Australian certification schemes. These viruses include *Strawberry crinkle cytorhabdovirus*, *Strawberry mottle sadwavirus*, *Strawberry mild yellow edge potexvirus*, *Strawberry vein banding caulimovirus*, *Strawberry pallidosis associated crinivirus* and *Beet pseudos yellows crinivirus*, which are both associated with pallidosis disease, and Strawberry necrotic shock virus (formally thought to be *Tobacco streak virus*). Although the diseases associated with these viruses have been reported in Australia only SNSV (formally TSV) was detected using serological methods (Greber, 1979) and the remaining viruses have only been identified by symptoms on susceptible commercial strawberry cultivars or indicator plants.

Strawberry crinkle virus

Strawberry crinkle virus (SCV) causes strawberry crinkle disease, which was first described in 1932 from commercial strawberry varieties in the USA (Zeller and Vaughan, 1932). A similar disease was reported in the UK in 1934 (Oligivie et al 1934). Crinkle disease has since been reported from many strawberry growing countries in Europe, UK, Asia, Africa, North America, South America, and Oceania.

SCV is a member of the genus *Cytorhabdovirus* in the family *Rhabdoviridae*. Virus particles are bacilliform, 74–88 nm in diameter and 163–383 nm in length with surface projections (Posthuma et al 2000). The SCV genome consists of a single negative-sense single stranded RNA molecule of 14,547 bases and seven open reading frames (ORFs) on the complementary RNA sequence (Schoen et al 2004).

Hosts

The natural host range of SCV is limited to *Fragaria* species. However it has been experimentally transmitted by mechanical inoculation or by infectious aphids to *Physalis pubescens, P. floridana, Nicotiana occidentalis, N. glutinosa* and *N. clevelandi* (Sylvester et al 1987; Richardson and Sylvester, 1988).

Symptoms

In some commercial strawberry cultivars SCV can reduce yield and affect fruit quality and this effect can be more severe when it occurs in combination with other viruses, such as SMoV and SMYEV (Frazier et al 1987). Other commercial cultivars may not be affected by SCV (Sylvester et al 1976).

Transmission

SCV is transmitted by the aphids *Chaetosiphon fragaefolii* Cockerell and *C. Jacobi* Hille Ris Lamberts (Vaughan, 1933). The virus can also be moved in infected runners.

Detection

SCV is detected using biological indexing by grafting onto the sensitive indicator plants *Fragaria vesca* cvs UC-4, UC-5, UC-6, and *F. vesca* var. *semperflorens* cv. Alpine. Diagnostic symptoms include petal-streak, petiole lesion symptoms, chlorotic to necrotic irregular spots on veins, epinasty, crinkling, distortion and uneven expansion of leaflets. Detection by molecular (RT-PCR) methods has been reported (Posthuma et al 2002; Thompson et al 2003; Klerks et al 2004; Mumford et al 2004; Posthuma et al 2001; Thompson et al 2004).

Strawberry mottle virus

Strawberry mottle disease is associated with *Strawberry mottle virus* (SMoV), which is widespread and can be found wherever strawberries are grown (Martin and Tzanetakis 2006). Many strains of SMoV infect strawberry plants asymptomatically, however virulent strains may reduce vigour and yield by up to 30% (Mellor and Krczal, 1987).

SMoV is a member of the genus *Sadwavirus*, which is yet to be assigned to a family. Virus particles are isometric and have a diameter of 37 nm (Yoshikawa and Converse, 1991). The SMoV genome is bipartite, linear, positive-sense, single-stranded RNA. RNA 1 and RNA 2 are 7,036 nt and 5,619 nt in length respectively (Thompson et al 2002).

Hosts

The natural host range of SMoV is limited to *Fragaria sp*. However it has been experimentally transmitted by mechanical inoculation or by infectious aphids to the herbaceous indicators *Chenopodium amaranticolor*, *C. quinoa*, *C. urbicum, C. murale Cyamopsis tetragonoloba*, *Fragaria chiloensis*, *Gomphrena globosa*, *Nicotiana occidentalis, N. benthamiana, Physalis exocarpa, Potentilla adscherica*, *P. Canadensis and P. sterilis* (Frazier 1968; Cieslinska and Stankiene 2005).

Symptoms

Symptoms are not obvious in commercial strawberry cultivars however SMoV can reduce vigour and yield depending on the severity of the strain (Mellor and Krczal, 1987). This effect can be more severe when it occurs in combination with other viruses, such as SCV and SMYEV (Frazier et al 1987).

Transmission

SMoV is naturally transmitted in a semi-persistent manner by the strawberry aphid *Chaetosiphon fragaraefolii* (Frazier and Sylvester, 1960). Other aphid species including *C. thomasi, C. minor, C. jacobi, Aphis gossypii* may also be vectors (Mellor and Krczal, 1987). The virus can also be moved in infected runners.

Detection

SMoV is detected via biological indexing by grafting onto the sensitive indicator plants *F. vesca* cvs UC-4 and UC-5. Diagnostic symptoms include mottle, vein clearing and stunting. Detection by molecular (RT-PCR) methods has been reported (Thompson and Jelkmann, 2003; Thompson, et al 2003; Thompson, et al 2004; Cieslinska, 2004; Yang et al 2005; Zhang et al 2006; Chang, et al 2007).

Strawberry mild yellow edge virus

Strawberry mild yellow edge virus (SMYEV) was shown to cause Strawberry mild yellow edge disease by inoculation of *Fragaria vesca* 'Alpine' seedlings with a SMYEV full length infectious clone (Lamprecht and Jelkmann, 1997). Symptoms produced on the *F. vesca* 'Alpine' indicator plants were indistinguishable from control plants inoculated with a naturally occurring isolate of SMYEV. SMYE disease was first reported in California in 1922 (Horne 1922) and in Europe in 1933 (Harris 1933) and has since been reported from many strawberry growing countries in Europe, UK, Asia, Africa, North America, South America, and Oceania.

SMYEV is a member of the genus *Potexvirus* in the family *Flexiviridae*. SMYEV particles are filamentous and flexuous with a length of 482 nm and a width of 13 nm. The genome is a linear positive-sense, single-stranded RNA molecule, 5966 nt in length (excluding the 3′ poly A-tail), and consists of five open reading frames (Jelkmann et al 1990; Jelkmann et al 1991).

Some evidence, including the presence of isometric particles, the size and pattern of dsRNA and the RT-PCR amplification of cDNA from infected plants using *Luteovirus* specific primers, indicates that SMYE disease might also be associated with a *Luteovirus*, (Yoshikawa et al 1984; Martin and Converse, 1985; Spiegel et al 1986; Spiegel, 1987; Montasser et al 2002; Hadidi et al 2003). Interestingly, the SMYE *Potexvirus* is not aphid transmissible from infected strawberry plants inoculated with the full length infectious clone but the SMYE disease is persistently transmitted in the field by the strawberry aphid (Lamprecht and Jelkmann, 1997). This evidence suggests that a helper virus may be required for SMYEV aphid transmission in the field and may explain the presence of the *Luteovirus* in field infected plants. The presence of a *Luteovirus* associated with SMYEV is difficult to confirm without specific tests such as ELISA or PCR.

Host

The natural host range of the SMYE *Potexvirus* is limited to *Fragaria sp*. It has been detected in *F. chiloensis* in remote areas of Chile where cultivated strawberries are not grown (Hepp and Martin 1991). The experimental host range of the SMYE *Potexvirus* includes *Chenopodium murale*, *C quinoa* and *Rubus rosifolius* (Lamprecht and Jelkmann, 1997).

Symptoms

Strawberry cultivars sensitive to SMYE *Potexvirus* develop dwarfing, marginal chlorosis, leaf distortion, and small fruit. Yield loss is also observed on some cultivars. Some varieties decline when infected with SMYE *Potexvirus* in combination with other viruses (Martin and Tzanenkais 2006).

Transmission

SMYEV is naturally transmitted in a semi-persistent manner by the strawberry aphid *Chaetosiphon fragaraefolii* ,*C. thomasi* and *C. minor* (Krczal, 1980; Converse et al 1987; Frazier 1975). The virus can also be moved in infected runners.

Detection

SMYE *Potexvirus* is detected via biological indexing by grafting onto the sensitive indicator plants *F. vesca* cvs UC-4, UC-5 and *F. vesca* var. *semperflorens* 'Alpine'. Diagnostic symptoms include chlorotic flecks in young leaves, gradual loss of vigour, chlorotic mottling, interveinal necrosis of older leaves (King and Harris 1942; Martin and Tzanenakis, 2006). SMYE *Potexvirus* can be detected by ELISA and RT-PCR (Jawee and Adams 1995; Quail et al 1995; Thompson, et al .2003; Thompson, et al. 2004; Yang et al 2005; Zhang et al 2006.; Chang, et al 2007).

Strawberry vein banding virus

Strawberry vein banding disease was first reported in the USA in 1955 (Frazier, 1955). It is now known to occur in Europe, Asia, North America, South America, and Oceania. *Strawberry vein banding virus* (SVBV) has been shown to be the cause of strawberry vein banding disease by inoculation of *Fragaria vesca* 'UC-5' plants with a SVBV full length infectious clone (Mahmoudpour 2003).

SVBV is a member of the genus *Caulimovirus* in the family *Caulimoviridae*. Virus particles are isometric and 40 to 45 nm in diameter (Petrzik et al 1998). The SVBV genome is linear double-stranded DNA approximately 7876 nucleotides in length and consists of seven open reading frames.

Hosts

The natural host range of SVBV is limited to *Fragaria sp*. It has been experimentally transmitted to *Sanguisorba minor* (Martin and Tzanetakis 2006).

Symptoms

Symptoms in *F. vesca, F. virginiana, F. chiloensis and F. x ananassa* include yellow vein banding, discontinuous banding, streaking and spotting of older leaves and curling of leaflets (Martin and Tzanetakis 2006). SVBV infections have also been associated with reduced runner production, yield, and fruit quality in sensitive commercial cultivars.

Transmission

SVBV is transmitted by several aphids including: *Acyrthosiphon pelargonii*, *Amphorophora rubi*, *Aphis idaei*, *A. rubifolii*, *Aulacorthum solani*, *Chaetosiphon fragaefolii*, *C. jacobi*, *C. tetrarhodum*, *C. thomasi*, *Macrosiphum rosae*, *Myzus ascalonicus*, *M. ornatus*,, *M. persicae*. However *Chaetosiphon* spp. are the most efficient vectors (Frazier 1960; Miller and Frazier, 1970). The virus can also be moved in infected runners.

Detection

SVBV is detected via biological indexing by grafting onto the sensitive indicator plants *F. vesca* clone UC-6 and *F. virginiana* clone UC-12 (Frazier, 1974). Symptoms include yellow vein banding, discontinuous banding, streaking and spotting of older leaves and twisting of leaflets.

SVBV can be detected by ELISA and RT-PCR (Honetslegrova et al 1995; Mraz et al 1997; Petrzik et al 2002; Vaskova and Spack, 2002; Thompson, et al .2003; Mahmoudpour, 2004; Thompson, et al. 2004; Vaskova and Spack, 2004; Vaskova et al, 2004; Chang, et al 2007).

Strawberry pallidosis associated virus

Pallidosis is a disease of strawberry first reported in California and Australia in 1957 (Frazier and Stubbs 1969). It has since been reported from Canada and Peru (Craig, 1981; Wintermantel et al 2006). *Strawberry pallidosis-associated virus* (SPaV) is one of two viruses associated with pallidosis disease (Tzanetakis et al 2004c; Tzanetakis et al 2006).

SPaV is a member of the genus *Crinivirus* in the family *Closteroviridae*. Virus particles are filamentous and flexuous and have two lengths (250 - 450 nm). The genome is a linear, positive sense, ssRNA, divided into two molecules that are separately encapsidated and both are needed for infectivity. RNA 1 is 8067 nucleotides long and encodes at two open reading frames (ORFs). RNA 2 is 7979 nucleotides long and encodes 8 ORFs (Tzanetakis et al 2005).

Host

The natural host range of SPaV is limited to *Fragaria sp*.

Symptoms

Commercial cultivars are usually symptomless when infected by SPaV alone. However reduced runner production and reduced root growth was reported from the cv. 'Northwest' grown in glasshouse conditions (Converse and Volk 1990). When SPaV occurs in combination with other viruses it may have significant impact on yield (Mullin et al 1975).

Transmission

SpaV is transmitted by the greenhouse whitefly *Trialeurodes vaporariorum* (Tzanetakis et al 2006). It may also be transmitted in runners.

Detection

Pallidosis disease is detected via biological indexing by grafting onto sensitive *F. virginiana* indicator plants 'UC-10'and 'UC-11'. Symptoms include distortion and marginal chlorosis of leaves and dwarfing (Fulton, 1987). SPaV can be detected by RT-PCR (Tzanetakis et al 2004).

Beet pseudos yellows virus

Beet pseudos yellows virus (BPYV) is also associated with Pallidosis disease in strawberries (Tzanetakis at al 2003). BPYV is a member of the genus *Crinivirus* in the family Closteroviridae. Virus particles are filamentous and flexuous and are approximately 1000 nm long (Yamashita et al 1979). The genome is a linear, positive sense, ssRNA, divided into two molecules that are separately encapsidated. RNA 1 is 8007 nucleotides long and encodes at least two open reading frames (ORFs). RNA 2 is 7904 nucleotides long and encodes 8 ORFs (Tzanetakis and Martin 2004).

Hosts

BPYV has an extensive host range and infects members of several plant families including: Chenopodiaceae, Compositae, Curciferae, Cucurbitaceae; Geraniaceae, Linaceae, Malvaceae, Portulaceae, Rosaceae, Ranunculaceae, Solonaceae, Umbilliferae and Urticaceae (Johnstone, 1987; Wisler et al 1998; Tzanetakis and Martin 2004). BPYV has been detected in strawberry plants in the USA, Peru and Australia and been reported in other hosts in Europe, UK, Asia, North America and Oceania.

Symptoms

Commercial cultivars are usually symptomless when infected by BPYV alone. However reduced runner production and reduced root growth was reported from the cv. 'Northwest' grown in glasshouse conditions (Converse and Volk 1990). When SPaV occurs in combination with other viruses a significant impact on yield may occur (Mullin et al 1975).

Transmission

BPYV is transmitted by the greenhouse whitefly *Trialeurodes vaporariorum* (Wisler et al 1998). It may also be transmitted in runners.

Detection

Pallidosis disease is detected via biological indexing by grafting onto sensitive *F. virginiana* indicator plants 'UC-10'and 'UC-11'. Symptoms include distortion and marginal chlorosis of leaves and dwarfing (Fulton, 1987). BPYV can also be detected by RT-PCR (Tzanetakis et al 2004).

Strawberry necrotic shock virus

Strawberry necrotic shock disease was first reported in 1956 (Frazier et al 1962). The disease was associated with *Tobacco steak virus* (TSV) the type member of the *Ilarvirus* genus. However, later studies using immunodiffusion tests, northern blot analysis and RT-PCR for detection of TSV did not return typical results, indicating that the strain from strawberry was distinct (Fulton, 1967; Stenger et al 1987; Tzanetakis et al 2004). When the RNA 3 and part of RNA 2 of the virus was isolated from SNSV affected plants and cloned, molecular analysis indicated that the virus had approximately 70% nucleotide similarity with TSV (Tzanetakis et al 2004). The virus associated with strawberry necrotic shock disease is Strawberry necrotic shock virus (SNSV).

SNSV is a tentative member of the genus *Ilarvirus* in the family *Bromoviridae*. Virus particles are isometric to bacilliform and have a diameter of 19-27.46-36 nm. The genome consists of three segments of linear, positive-sense, single-stranded RNA. RNA1 is 3429 nt, RNA 2 is 2876 nt and RNA 3 is 2245 nt long. The three segments are distributed among three particle types of different size.

Hosts

SNSV has been detected in *Fragaria* sp and is very closely related to strains infecting *Rubus*.

Symptoms

Symptoms are rarely seen on commercial cultivars but SNSV can reduce yield by 15% and runner production by 75% (Johnson et al 1984). The disease is named for the symptoms produced when graft inoculating infected material onto *F. vesca* var. *semperflorens* 'Alpine'), which shows a passing necrotic shock reaction followed by recovery (Stace-Smith et al 1987).

Transmission

SNSV is seed transmitted (Johnson et al 1984). It may also be transmitted in runners.

Detection

SNSV is detected via biological indexing onto *Chenopodium quinoa*, which shows chlorotic local lesions and tip necrosis. It can also be detected by grafting onto sensitive *F. vesca* 'UC-4' indicator plants. Symptoms include severe necrotic reaction in newly formed leaves 6-14 days after grafting. Subsequent young leaves appear normal and no further symptoms develop (Martin and Tzanetakis 2006). SNSV can also be detected by RT-PCR (Tzanetakis et al 2004).

Other viruses

The remaining 14 viruses listed in Table1.1 have not been reported to infect strawberry in Australia and several are considered of quarantine significance including: *Raspberry ringspot virus*, *Strawberry latent C virus*, *Strawberry latent ringspot virus*, *Tobacco ringspot virus*, *Tomato black ring virus*, and *Tomato ringspot virus* These viruses are not discussed in this

manual however an excellent review has been published that gives further details of all viruses reported to infect strawberry plants (Martin and Tzanetakis, 2006.)

FUNGAL AND FUNGUS-LIKE PATHOGENS

Strawberries can be affected by numerous fungal and fungus-like pathogens that are associated with crown, root and fruit rots and leaf diseases. While plants within the scheme are passively monitored for evidence of fungal pathogens, several are actively tested for within the nucleus collection, and include *Phytophthora* species, *Verticillium* species and *Gnomonia comari*,.

Phytophthora sp.

Phytophthora species are fungus-like organisms in the Oomycetes (water moulds) of the Kingdom Straminipila (Dick 2001). They spread through planting material but may also be found in soil and trash from previous crops. Spread through the soil occurs in moist conditions. *Phytophthora* species can cause significant economic losses in many plant species including strawberries. Several species which occur in Australia, including *P. bisheria, P. cactorum, P. citricola, P. parasitica,* and *P. megasperma* can cause root rot strawberries. These *Phytophthora* species also cause crown rot of strawberries. *P. cactorum* is most commonly associated crown and root rots and also causes leather rot of the fruit. *Phytophthora fragariae* f.sp. *fragariae,* which causes Red stele disease, has not been reported in Australia and is a quarantine pathogen.

Symptoms of crown rot include plant stunting and small leaves. Young leaves wilt and within a short period plants collapse. A brown discolouration may be observed in the crown vascular tissue when infected plants are cut open.

Characteristic symptoms of red stele include red discoloration in the root zone of plants. Young roots initially rot at the tip and diseased plants become stunted and eventually die. Red stele was reported once in Australia, however affected plants were eradicated. There was also doubt cast over the cause of the symptoms the diagnosis was based on visual examination only. As a consequence *P. fragariae* f.sp. *fragariae* remains a quarantine pathogen and that must be actively tested for in PEQ. Imported strawberry plants are screened for red stele by two methods i) visual assessment and ii) the baiting technique (Duncan. 1976).

***Verticillium* sp.**

Verticillium is a fungal genus in the division Ascomycota. They infect and cause disease in a broad range of plant species. Two *Verticillium* species have been reported to infect strawberry and include *V. dahliae* and *V. albo-atrum..* Both species are soil borne and they overwinter in soil or plant debris as dormant mycelium or microsclerotia. The microsclerotia can persist in soil for many years. The fungi invade the xylem tissue and become systemic and can be transmitted through planting material. They can also be spread by water, wind, in crop or weed debris, or in soil.

Both *Verticillium* sp. cause wilt disease in strawberries. The severity of symptoms differs depending on the susceptibility of the cultivar and can be confused with symptoms associated with other pathogens, such as *Phytophthora*.

Infected plants are often stunted, flattened, have small chlorotic leaves and appear wilted. The outer and older leaves of infected plants may droop, wilt and turn dry. Leaf margins become reddish yellow or dark brown and a similar discolouration can occur between the veins. Few, if any, new leaves develop. New leaves that do form are stunted and may wilt and curl up along the mid vein. Runners and petioles may develop brown or blue-black streaks or blotches. New roots that grow from the crown may be dwarfed with blackened tips. Crown and root tissue decay and brown streaks may be observed when they are cut open.

***Gnomoniosis* and *Gnomonia* species.**

Gnomoniosis and *Gnomonia* are also fungal genera in the division Ascomycota. The literature refers to two species of *Gnomonia* that can infect strawberry plants and include *G. comari* and *G. fragariae*. Recently it was been suggested that the taxonomy of *G. comari* be revised based on molecular analysis as well as biological traits (Sogonov et al 2008). It was suggested that *G. comari* occurring on strawberry be revised to *Gnomoniopsis fructicola. G. comari* has been retained and for the fungus occurring on other hosts. Previously published descriptions including *G. fragariae* f. *fructicola* Arnaud and *Gnomonia fructicola* (Arnaud) Fall may also refer to *G. fructicola*. Sogonov et al (2008) suggested that the name *G. fragariae* Kleb. be retained for the fungus causing leaf blotch, root rot and petiole blight of strawberry in Europe. This is also supported by sequence analysis of partial LSU rRNA gene and the total ITS region which showed that *G. fragariae* belongs outside of the *Gnomoniaceae* in the *Sydowiellaceae*. (Moročko and Fatehi 2007)

G. fructicola is found world wide and *G fragariae* has only been reported in Europe. It is possible that some references to both pathogens in the literature are confused (Maas 1998). Biologically they can be differentiated from one another because *G fragariae* causes root rot and *G. fructicola* does not (Morocko et al 2006). Also *G. fructicola* has been reported on fruit and associated with stem end rot while *G. fragariae* has not (Maas, 1998). Morphologically ascospores of *G. fragariae* are larger than those and *G. fructicola* and have filiform appendages (Maas, 1998).

Although *G. fructicola* is considered a “weak” pathogen of strawberries it is associated with leaf blotch, dry necrosis of sepal tips, petiole blight, stem end rot, fruit rot, decline and death of strawberry plants and. rotting of strawberry runners during cold storage (Alexopoulos and Cation 1948; van Adriechem and Bosher, 1958, Shipton 1967; Bolay 1972; Gubler and Feliciano 1999). It has also been shown to cause root rot and stunting of plants when *G. fructicola* and the root lesion nematode *Pratylenchus penetrans* occur together on the roots of strawberry plants (Kurppa and Vrain, 1989).It was also shown that the nematode could transport the conidiospores through the soil and may be a means of transmission of the fungus. The conidia and ascospores of *G. fructicola* are also spread by water splash during humid conditions (Bolton, 1954). The fungus overwinters on plant trash in the field and perithecia can occur in early spring on overwintered leaves.

G. fragariae is a serious pathogen in Europe as it can causes root rot and petiole blight and can cause a severe decline of field grown strawberry plants (Morocko et al 2006). There is very little information available of the spread of *G. fragariae* although the ascospores are highly infectious and are likely to be spread by water splash.

APPENDIX 2 – BIOLOGICAL INDEXING PROTOCOLS

1. **Graft indexing**
2. **Herbaceous indexing**
3. **Fungal baiting**
4. **Fungal culturing**
5. **Photos of symptoms**

1. Graft indexing of *Fragaria* biological indicators

Biological indexing by graft inoculation of *Fragaria* sp. biological indicators will be carried by a diagnostic laboratory in glasshouse conditions in October and November according to the following protocol.

Equipment

- 2 of each of the indicator plants *Fragariae vesca* UC-4, *F. vesca* UC-6 and *F. virgininiana* UC-10
- stericrepe tape, parafilm or grafting tape.
- Scalpel blades
- Scissors
- Beakers
- Labels
- cotton wool
- 70% ethanol
- distilled water
- mist bed or plastic bags

- The indicator plants must be maintained in a vigorous state of growth before and after grafting and must be grown under moderate temperatures and light intensities.
- A single plant each of UC-4, UC-6 and UC-10 must be left ungrafted each day and subjected to the same horticultural practices and environmental conditions as the inoculated plants.
- Each indicator variety must be grafted with virus positive controls containing BPYV, SCV, SMoV, SMYEV, SNSV, SPaV and/or SVBV.
- Inoculated indicator plants will be grown in a mist bed or covered with a plastic bag to create a humid environment for 1-2 weeks until a graft union has formed.

Method

1. Use a separate sterile scalpel blade for each variety.
2. Clearly label the pot of each indicator that will be grafted with a particular sample/variety with the code or name assigned to the sample by the managing organisation and the date of grafting.
3. Prune each of each of the indicator plants to two young actively growing tri-foliate leaves (Fig. 1a).
4. Make a 1-2 cm vertical incision down the middle of the petiole with the same sterile scalpel blade. (Fig. 1b).
5. Trim away the two outside leaflets from two of the candidate leaf samples leaving only the centre leaflet and the petiole.
    - Return all unused tissue to the sample bag as it will be required for biological indexing on *C. quinoa* and bacterial culturing.
6. Cut the candidate petiole into a wedge shape, trim the attached leaflet blade to 1cm and insert the candidate petiole in the split of the indicator plant petiole. (Fig. 1c).
7. Bind the graft firmly with self-adhesive medical tape (e.g. stericrepe), parafilm, grafting tape or similar(Fig. 1d).

8. Place the indicators into a mist bed or cover the pots with a plastic bag for one week.
9. Repeat this process for each of the remaining indicator plants.
10. Check the graft union on each indicator two weeks after inoculation:
    - Grafts are successful if they are still alive after two weeks.
    - At least one graft per indicator plant must have survived.
    - If both grafted leaflets have not survived the graft must be repeated.
11. Grafted plants are examined twice weekly for symptoms over a four month period:
    - Symptoms are often strongly expressed in the first few leaves to emerge and expand after inoculation.
    - Virus expression should occur within 2 -10 weeks.
    - Figures 2-8 are examples of symptoms that may be observed after graft inoculation of *Fragaria* indicators.

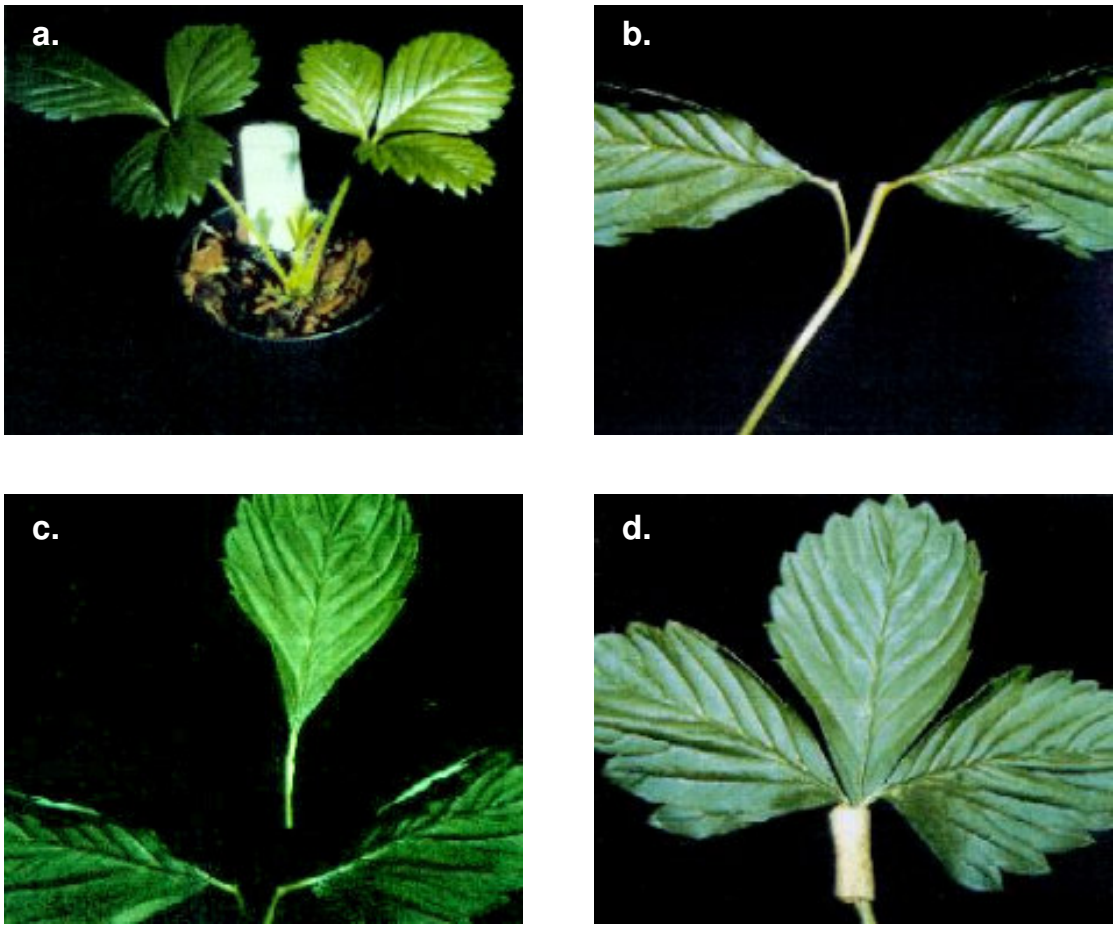


Figure 1. Diagram of petiole grafting for biological indexing using *Fragaria* indicators. (Images courtesy of Mark Whattam, AQIS)

a) Indicators are pruned to two young actively growing leaves;

b) the centre leaflet of the indicator is removed and an incision is made in the petiole 1-2cm long;

c) the petiole of the centre leaflet of the candidate plant is trimmed into a wedge shape and inserted into the cut petiole of the indicator;

d) the graft is tightly taped.

Table 3. Recommended *Fragaria* indicator cultivars for graft indexing for specific virus*

Viruses	Disease	*Fragaria* indicator cultivar	Symptoms on Indicator
Beet pseudo-yellows crinivirus	Pallidosis	*Fragaria virginiana* 'UC-10'	marginal leaf chlorosis, stunting
Strawberry crinkle virus	Crinkle	*Fragaria vesca* 'UC-4' and 'UC-6'	Deformed leaves and distorted petioles, leaflets with chlorotic spots, leaflets may be uneven in size, distorted, and crinkled, distorted petioles, small leaves. Necrotic lesions on runners, petioles, and petals may occur
Strawberry mild yellow edge-associated virus	Mild yellow edge	*Fragaria vesca* 'UC-4' and *Fragaria virginiana* 'UC-10'	cupped leaflets, chlorotic margins, reduced vigour
Strawberry mottle sadwavirus	Mottle	*Fragaria vesca* 'UC-4'	Symptoms may depend upon the strain: leaf mottle, to severe stunting and distortion, to plant death
Strawberry necrotic shock ilarvirus	Necrotic shock	*Fragaria vesca* 'UC-4' and *Chenopodium quinoa*	On UC-4 symptoms appear 6 to 14 days after grafting: some strains cause a severe necrotic reaction on the first 1-3 leaves that develop after inoculation. After the initial severe reaction subsequent leaves appear normal, and no further symptoms develop
			On *C. quinoa*, local necrotic lesions, systemic mottle or necrosis
Strawberry pallidosis-associated virus	Pallidosis	*Fragaria virginiana* 'UC-10'	marginal leaf chlorosis, stunting
Tobacco streak ilarvirus	Necrotic shock	*Fragaria vesca* 'UC-4' and *Chenopodium quinoa*	On UC-4 symptoms appear 6 to 14 days after grafting: some strains cause a severe necrotic reaction on the first 1-3 leaves that develop after inoculation. After the initial severe reaction subsequent leaves appear normal, and no further symptoms develop
			On *C. quinoa*, local necrotic lesions, systemic mottle or necrosis.
Strawberry vein banding virus	Vein banding	*Fragaria vesca* ' UC-6'	Three types of symptoms may be observed depending on the strain: vein banding along primary and secondary veins (most intense in the first few leaves that are produced after grafting), leaf curl or necrosis. Symptoms of necrosis may develop on mature leaves
Nepovirus	Stunting degeneration, death	*Chenopodium quinoa (all sp)* and *Fragaria virginiana* 'UC-10' (ToRSV only)	Depends upon the virus species: Chlorotic or necrotic local lesions, systemic chlorosis and deformation, necrosis systemic chlorotic mottle or apical necrosis

*Mixed virus infections may confound the symptoms that are observed on each indicator

Herbaceous indexing

Biological indexing by rub inoculation of *C. quinoa* will be carried by a diagnostic laboratory in glasshouse conditions in October and November according to the following protocol.

Equipment

- *Chenopodium quinoa* and *Cucumis sativus* (PEQ only) indicator plants (3 plants per indicator)
- Chilled extraction buffer (0.05M phosphate buffer pH 7.0 containing 0.1% sodium sulphite)
- Homex grinder and bags or mortar and pestle
- Distilled water
- 300µm carborundum powder
- Labels

Method

1. Grind sample leaf tissue at a rate of 1g tissue/5ml Phosphate buffer (0.05M sodium phosphate pH 7.0 containing 2% poly vinyl pyrrolidine – PVP) using a mortar and pestle:
    - a Homex bag and the Homex grinder or a mortar and pestle can be used to grind the sample.
2. Label the pot with the sample code with which the plants will be inoculated.
3. To identify the inoculated leaves at a later time, pierce two young fully expanded leaves, preferably opposite leaves, to be inoculated on each plant with a pipette tip (or similar).
4. Lightly dust the two pierced leaves with carborundum powder.
5. Dip a gloved finger or a cotton swab in the tissue/phosphate buffer slurry and apply the slurry to the pierced leaves of the indicator plants, gently rubbing the leaf 5-6 times from the petiole towards the leaf tip while supporting the leaf below with the other hand.
    - change gloves between or cotton swabs between samples to prevent cross contamination.
    - To improve virus transfer, indicator plants may be placed in the dark 12hrs prior to inoculation.
6. Rinse inoculated leaves with water after the slurry has been applied.
7. Grow plants in a glasshouse (18-25°C) for 4-8 weeks.
8. Observe the inoculated indicator plants daily for symptom development:
    - Symptoms may begin to appear 4-5 days after inoculation:
        - Symptoms of *Strawberry necrotic shock virus* (SNSV) or *Tobacco streak virus* (TSV) include: necrotic local lesions, systemic necrosis.
    - Symptoms of Nepoviruses include:
        - *Arabis mosaic virus* (ArMV)* - local lesions, systemic chlorotic mottling.
        - *Raspberry ringspot virus* (RpRSV)* - chlorotic or necrotic local lesions, systemic chlorotic mottle or apical necrosis.
        - *Tomato ringspot virus* (ToRSV)* - chlorotic local lesions, systemic apical necrosis
        - *Tomato black ring virus* (TBRV)*- necrotic local lesions, systemic chlorotic mottling, necrosis.
    - Symptoms of *Strawberry latent ringspot sadwavirus* (SLRVS)* include: chlorotic or necrotic local lesions, systemic chlorosis and deformation, necrosis or faint chlorotic mottle.

- Symptoms of *Tomato bushy stunt virus* (TBSV)* include chlorotic local lesions, rarely systemic .

- Dead tissue appearing on the inoculated leaf within the first few days of inoculation indicates excessive pressure has been applied during rub inoculation – the inoculation must repeated on uninoculated indicators.

*ArMV, RpRSV, ToRSV, TBRV, SLRSV and TBSV are quarantine pathogens.

Fungal baiting for *Phytophthora* sp.

1. Bait testing using *Fragaria vesca* var semperflorens (Alpine strawberries) for detection of *Phytophthora* sp. including *P. fragariae* by will be conducted by the diagnostic laboratory in October-November or in March-April.
2. In October-November a subsample of the roots of the mother plants is collected or in March-April a subsample of the roots of two daughter plants is collected.
3. A subsample of the root sample is used to inoculate the soil of two Alpine indicator plants per nucleus variety.
4. The subsample of root cutting will be sterilised in 1.0% sodium hypochlorite (NaOCl) with agitation for two minutes followed by three rinses of sterile distilled water.
5. The root cuttings of a variety will be mixed with pasteurised soil-less media and placed into a one 0.36L pot.
6. The roots of the Alpine seedlings should be `nicked' with a scalpel blade to increase the likelihood of infection by the pathogen.
7. Two Alpine seedlings will be placed into the soil-less media containing the root cuttings and the plants will be well watered.
8. The Alpine plants will be observed twice weekly for characteristic wilting symptoms during 6 weeks.
9. After six weeks the Alpine plants are lifted from the soil and the root cortex is examined for signs of red stele discoloration:

   Symptoms of *Phytophthora* sp. (not *P. fragariae*) include:

   - Wilted leaves.
   - Rotting of the crown tissue.

   Symptoms of *P. fragariae* include:

   - Wilted leaves.
   - Plants are stunted, lack vigour and eventually die.
   - The stele of young roots, when cut lengthwise, is reddish in colour.

Fungal culturing

Equipment

- Sterile scalpel blades
- Forceps
- Microscope
- Sterile water
- 10% sodium hypochlorite
- Containers for dipping and washing
- Incubator at 20-25℃

- Lima bean agar plates for *Phytophthora* sp. culturing made according to the following protocol:
    1. Boil 220 g of lima beans in 1L of water for 30 minutes.
    2. Strain through cheesecloth and make up to 1L with water.
    3. Add 1g of glucose and 17g of agar per litre.
    4. Autoclave at 121° for 20 minutes.
    5. When cool enough to handle pour approx. 20ml into sterile Petri dishes.
    6. 2ppm beta-sitosterol can be added before autoclaving for oospore production if required.
- Potato dextrose agar plates:
    - Commercial PDA is made according to the manufacturer's instructions or bought as pre-poured plates.

Method

1. Root samples and petiole samples are surface sterilised by dipping into 10% sodium hypochlorite for 3 minutes followed by three washes in sterile water.
2. The root and petiole samples will plate onto separate lima bean agar (PFR) and potato dextrose agar (PDA) and incubated at 20-25℃ in a secure temperature controlled incubator.
3. The plates will be observed three times per week for development of colonies of the prescribed fungi.
4. Microscopy techniques will be used to determine the identity of the fungi that have been isolated from the plants.
5. If required fungal colonies may be plated onto selective media for further identification as determined by the diagnostic facility.

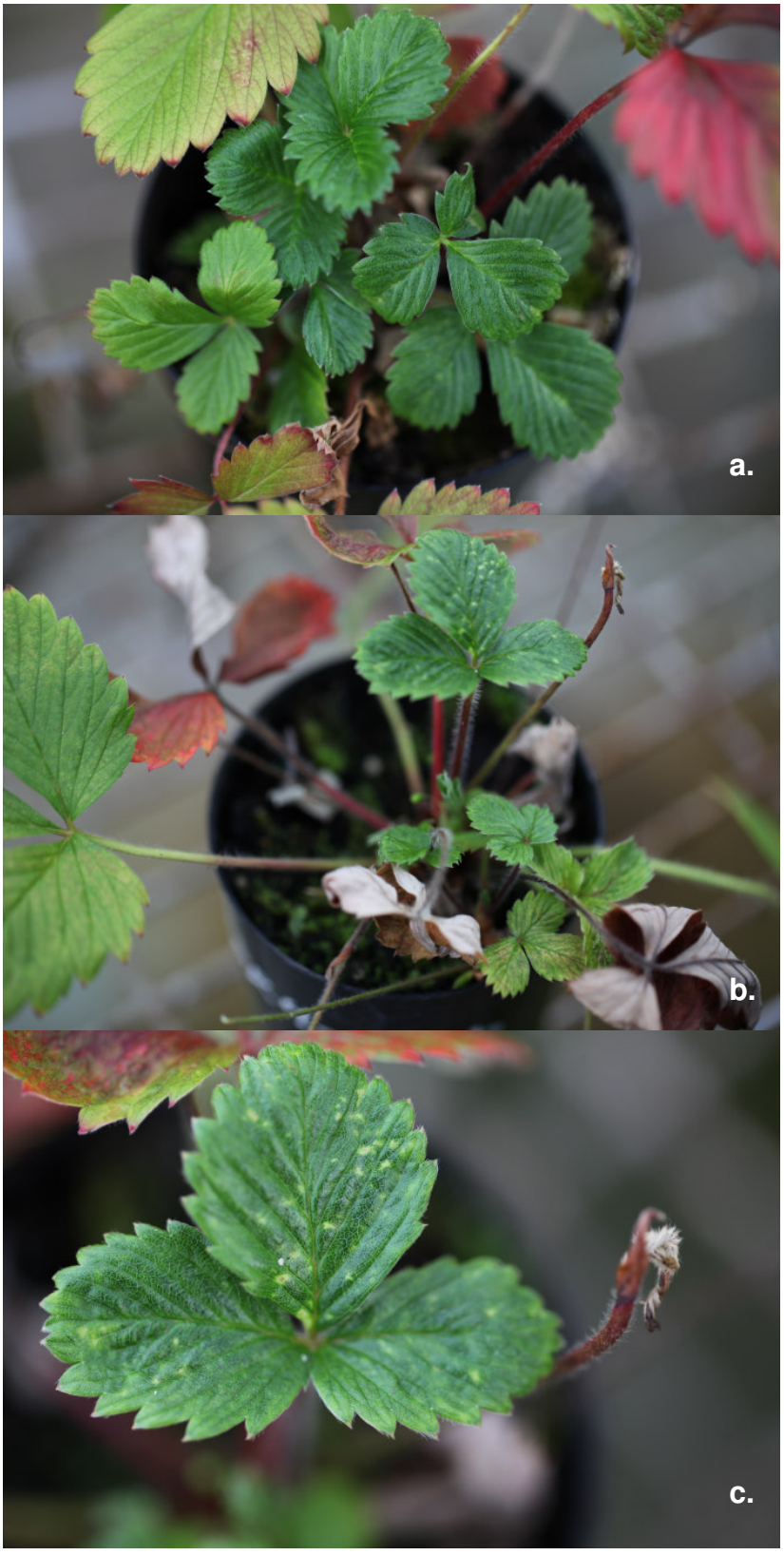


Figure 2. Examples of symptoms on UC-4 indicators after inoculation with virus infected candidate plants.

a) UC-4 inoculated with V2C22 R3. Note the twisting of leaves and mild chlorosis which is more noticeable along the veins. BPYV, SMYEV, SMoV, SNSV, and SPaV were detected by RT-PCR in this plant.

b) UC-4 inoculated with V1C20 R4. Note the twisting chlorosis and flecking of leaves.

c) a close up image of flecking that was observed in UC-4 inoculated with V1C20R4. BPYV, SCV, SMoV, SNSV, SPaV were detected by RT-PCR in this plant.

(M.D. Jones)

Figure 3. Examples of symptoms on UC-4 indicators after inoculation with virus infected candidate plants (Lien Ko, DEEDI).

a) UC-4 inoculated with V1C12 R1. Note the twisting of leaves, severe chlorosis and necrosis along the veins. SMYEV was detected by RT-PCR in this plant.

b) a close up image of the affected leaves.

Figure 4. UC-4 indicators inoculated with V2C12 R1 which was infected with SMYEV and SMoV. The viruses infecting these plants are unknown

a) Note the twisting of leaves, severe chlorosis and stunting.

b) A close up. of mottling and flecking on a leaf of a runner of the infected plant

(M.D. Jones)

Figure 5. Examples of symptoms on UC-6 indicators after inoculation with virus infected candidate plants.

a) UC-6 inoculated with V2/CV12/R1. Note the smaller twisted leaves at the crown. SMYE was detected by RT-PCR in this plant.

b) UC-6 inoculated with V1/C12/R1. Note the stunting of the plants and with small leaves and chlorosis at the margins. BPYV, SMYEV, SMoV, SNSV and SPaV were detected by RT-PCR in this plant.

c) UC-6 inoculated with V2/C12/R1. In addition to stunting a smaller chlorotic leaves some of leaves were slightly twisted. BPYV, SMoV and SMYEV were detected by RT-PCR in this plant.

d) UC-6 inoculated with V2/C12/R1. This plant was more severely affected compared to the other plants. SMYEV was detected by RT-PCR in this plant.

(M.D. Jones)

Figure 6. Examples of symptoms on UC-6 indicators after inoculation with virus infected candidate plants. (Lien Ko, DEEDI).

a) UC-6 inoculated with V1C22 R1. Note the twisting of leaves and chlorotic mottling. SMYEV, SNSV, and SPaV were detected by RT-PCR in this plant.

b) UC-6 inoculated with V1C22 R1. Note the twisting of leaves.

Figure 7. Examples of symptoms on UC-10 indicators after inoculation with virus infected candidate plants.

a) UC-10 inoculated with V2/C12/R1- Note the crinkled, twisted leaves which are smaller and chlorotic. SMoV and SMYEV were detected by RT-PCR in this plant.

b) UC-10 inoculated with V1/C20/R4. Note the smaller, twisted chlorotic leaves. BPYV, SCV, SMoV, SMYEV and SPaV were detected by RT-PCR in this plant.

(M.D. Jones)

Figure 8. Examples of symptoms on UC-10 indicators after inoculation with virus infected candidate plants. (Lien Ko, DEEDI).

a) and b) UC-10 inoculated with V1/C20/R1 Note the mottled and, twisted leaves and stunting. SMoV, SNSV and SPaV were detected by RT-PCR in this plant.

APPENDIX 3 – MOLECULAR DIAGNOSTIC PROTOCOLS

1. **EXTRACTION PROTOCOLS**

   1.1. Extract RNA for RT-PCR using the MacKenzie buffer and the RNeasy® Plant Mini Kit.

   1.2. Extract DNA for PCR using the DNeasy® Plant Mini Kit.

   1.3. Extract total nucleic acid using the QiaExtractor.

2. POLYMERASE CHAIN REACTION

   2.1. PCR materials and equipment.

   2.2. RT-PCR and PCR reaction set up.

   2.3. Cycling conditions for RT-PCR and PCR.

   2.4. Gel electrophoresis.

   2.5. Interpretation of results.

1. EXTRACTION PROTOCOLS

1. Extract RNA for RT-PCR using the MacKenzie buffer and the RNeasy® Plant Mini Kit (section 1.1).
2. Extract DNA for PCR using the DNeasy® Plant Mini Kit (Section 1.2).
3. Extract total nucleic acid for RT-PCR or PCR using the QiaExtractor – this method can be used if sample numbers exceed eight (Section 1.3).
4. All nucleic acid extraction is done in a nucleic acid extraction laboratory.
5. After extraction all nucleic acid extracts that are not used immediately must be stored in a freezer at -18°C.

1.1 RNA EXTRACTION PROTOCOL USING GUANIDINE THIOCYANATE BUFFER AND THE RNEASY® PLANT MINI KIT (Qiagen, MacKenzie et al 1997).

Materials and equipment

1. QIAGEN RNeasy® Plant mini kit
2. 1.5 ml centrifuge tubes or 2 ml screw cap centrifuge tubes
3. 20-200 µl and 200-1000 µl pipettes
4. 20-200 µl and 200-1000 µl sterile filter pipette tips
5. Autoclave
6. Balance
7. Bench top centrifuge
8. Distilled water
9. Ice machine
10. Freezer
11. Homex grinder and bags or mortar and pestle
12. Scalpel handle
13. Sterile scalpel blades
14. Vortex
15. Water bath at 60°C
16. Buffers

Guanidine thiocyanate buffer for total nucleic acid extraction (MacKenzie et al 1997)

Reagent	Final concentration	Amount needed for 1L
Guanidine thiocyanate	4M	472.64 g
3M Sodium Acetate	0.2M	66.67 ml
0.5M EDTA pH 8.0	25mM	50 ml
PVP-40	2.5%(w/v)	25 g

Make up to volume with sterile distilled water. Store at room temperature. Just before use, add 1% (v/v) β- mercaptoethanol.

17. Reference: MacKenzie, DJ, McLean, MA, Murkerji, S and Green, M. 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease* **81(2),** 222-226.

Method:

1. With a sterile scalpel, cut 500 mg of tissue from the petioles of the plant sample and place in a grinding bag (containing mesh to assist homogenisation) or mortar.
2. Add 1980 µl of extraction buffer to the bag or mortar.
3. In a fume hood, add 20 µl of β-mercaptoethanol to the bag or mortar.
4. Place bag in Homex homogeniser and grind sample. If using a mortar, grind in the fume hood with a pestle until a slurry has formed.
5. In a fume hood, transfer 1 ml of the sample to a 1.5 ml centrifuge tube.
6. In a fume hood, add 100 µl of 20% Sarkosyl (lauryl sarcosine sodium salt) to the tube and shake to mix.

7. Place the tube in a 70˚C water bath and incubate the samples for 15 minutes. Agitation in water bath is preferable but not necessary.
8. Transfer a maximum of 750 µl of the plant extract onto the lilac QIAshredder™ column. Close the tube and centrifuge at maximum speed for 2 minutes.
9. Mix 450 µl of the flowthrough with 225 µl of ethanol (96-100%), mix by pipetting and transfer all the mixture to the pink RNeasy® mini spin column, which has been placed in a 2 ml collection tube. Close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
10. Discard flow-through and replace column back into the same 2 ml collection tube.
11. Add 700ul of QIAGEN buffer RW1 to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
12. Discard flow-through and place column into a new 2 ml collection tube.
13. Add 500ul of QIAGEN buffer RPE (wash buffer) to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
14. Discard flow-through and replace column back into the same 2 ml collection tube.
15. Add another 500 µl of QIAGEN buffer RPE (wash buffer) to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
16. Discard flow-through and replace column back into the same 2 ml collection tube. Centrifuge at maximum speed for 1 minute.
17. Place the pink RNeasy® mini spin column in an appropriately labelled 1.5 ml centrifuge tube. Add 200 µl of RNase-free sterile water directly to the filter (don't apply down the side of the tube), close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 1 minute to elute the RNA.

1.2. MODIFIED DNA EXTRACTION PROCEDURE USING THE DNEASY® PLANT MINI KIT (QIAGEN, GREEN ET AL 1999).

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit
2. 1.5 ml centrifuge tubes
3. 20-200 µl and 200-1000 µl pipettes
4. 20-200 µl and 200-1000 µl sterile filter pipette tips
5. Autoclave
6. Balance
7. Bench top centrifuge
8. Distilled water
9. Ice machine
10. Freezer
11. Mortars and pestles or Homex grinder and bags
12. Scalpel handle
13. Sterile scalpel blades
14. Vortex
15. Water bath at 60°C
16. Buffers

CTAB extraction buffer for DNA extraction (Green et al 1999)

Reagent	Final concentration	Amount needed for 1L
CTAB (cetylmethylammonium bromide)	2.5%	25 g
Sodium Chloride	1.4 M	56 g
1M Tris, pH 8.0 (sterile)	100 mM	100 ml
0.5M EDTA, pH8.0 (sterile)	20 mM	40 ml
PVP-40	1%	10g

Make up to volume with sterile distilled water. Store at room temperature. Just before use, add 0.2% 2-mercaptoethanol (v/v) to the required volume of buffer

95% Ethanol

17. Reference: Green MJ, Thompson DA and MacKenzie DJ, 1999. Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Disease* **83,** 482-485

Method

1) Pre-heat Qiagen buffer AE buffer to 65°C.
2) Weigh approximately 500 mg of leaf petioles.
3)
   a) Place the material in a sterile mortar with 5 ml of CTAB extraction buffer containing 0.2% 2 – mercaptoethanol and grind thoroughly with the sterile pestle.

or

   b) Place the material in a grinding bag (containing mesh to assist homogenisation) with 5 ml of CTAB extraction buffer containing 0.2% 2 – mercaptoethanol. Place the bag in Homex homogeniser and grind the sample.

4) With a sterile plastic transfer pipette transfer 500ul of extract to a 1.5 ml centrifuge tube and add 4 µl of RNase A (Supplied with the DNeasy® kit), cap tube and incubate at 65°C for 25-35 minutes, mixing gently several times.

5) Add 162 µl of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.

6) Pour extract into a QIAshredder™ column and centrifuge at maximum speed for 2 minutes.

7) Transfer 450 µl of flowthrough from QIAshredder™ column to a 1.5 ml centrifuge tube containing 675µl QIAGEN buffer AP3. Mix by pipetting.

8) Transfer 650 µl of extract onto a DNeasy® spin column and centrifuge at 10000rpm for 1 minute.

9) Discard flow-through and add the rest of the sample to the column and centrifuge at 8000 rpm for 1 minute.

10) Place DNeasy® column in a new 2 ml collection tube and add 500 µl of QIAGEN buffer AW (wash buffer) and centrifuge at 8000 rpm for one minute.

11) Discard flowthrough and add another 500 µl of QIAGEN buffer AW and centrifuge at maximum speed for 2 minutes.

12) Discard flowthrough and collection tube. Ensure that the base of the column is dry (re-centrifuge the column if it remains a little wet) and place in an appropriately labelled 1.5 ml centrifuge tube. Add 200 µl of pre-warmed AE buffer directly to the filter (don't apply down the side of the tube) and centrifuge at 8000 rpm for 1 minute. Discard column and store DNA in at -18°C.

1.3. NUCLEIC ACID EXTRACTION USING THE QIAXTRACTOR

Materials and equipment

1. QIAxtractor
2. 96 Square-Well,1.2 ml, lysis plate
3. Adhesive plastic film to cover Lysis plate and unused wells of the capture plate
4. 3mL transfer pipettes
5. 96-well 800 µl long drip Unifilter capture plate (Whatman)
6. Caps or adhesive foil to cover the elution plate
7. 96 well elution plate (Qiagen)
8. 20-200 µl and 200-1000 µl pipettes
9. 20-200 µl and 200-1000 µl sterile filter pipette tips
10. Autoclave
11. Balance
12. Bench top centrifuge
13. Distilled water
14. Freezer
15. Homex grinder and bags or mortar and pestle
16. Scalpel handle
17. Sterile scalpel blades
18. Vortex
19. Water bath at 70°C
20. Buffers:

Guanidine thiocyanate buffer for total nucleic acid extraction (MacKenzie et al 1997)

Reagent	Final concentration	Amount needed for 1L
Guanidine thiocyanate	4M	472.64 g
3M Sodium Acetate	0.2M	66.67 ml
0.5M EDTA pH 8.0	25mM	50 ml
PVP-40	2.5%(w/v)	25 g

Make up to volume with sterile distilled water. Store at room temperature. Just before use, add 1% (v/v) β- mercaptoethanol.

Propanol Wash buffer for the Corbett X-Tractor

Reagent	Final concentration	Amount needed for 700ml
Ethanol	25%	175ml
2-Propanol	25%	175ml
1M Tris-Cl, pH 8	10mM	7ml
NaCl	100 mM	4.09g

Dissolve salts in water first before adding alcohols then make up to 700mL with dH_2O. Store at room temperature.

Ethanol (absolute)

Total nucleic acid extraction using the QIAxtractor and guanidine thiocyanate extraction buffer

1. With a sterile scalpel, cut 500 mg of tissue from the petioles of the plant sample and place in a grinding bag (containing mesh to assist homogenisation) or mortar.
2. Add 5 ml of Guanidine thiocyanate extraction buffer to the bag or mortar.
3. In a fume hood, add 30 µl of β-mercaptoethanol to the bag or mortar.
4. Place bag in Homex homogeniser and grind sample. If using a mortar, grind in the fume hood with a pestle until a slurry has formed.
5. In a fume hood, transfer 1 ml of the homogenate to one well of pre-racked 1.1ml strip minitubes arranged in standard 96-well format (Pathtech), each containing 100 µl of 20% N-lauroylsarcosine containing 100 µl of 20% N-lauroylsarcosine sodium salt (Sarkosyl).
6. Aspirate the sample to mix to mix.
7. Place the tube in a 70˚C water bath and incubate the samples for 15 minutes. Agitation in water bath is preferable but not necessary.
8. Close the tube and centrifuge at maximum speed for 2 minutes.
9. Transfer a maximum of 500 µl of the plant extract into one well of pre-racked 1.1ml strip minitubes arranged in standard 96-well format.
10. Place the second lysis plate in the QIAxtractor.
11. Add ethanol, propenol wash buffer and RNase-free sterile water tot e appropriate tubs and program the QIAxtractor to do the following:
    a. Add 500 µl of 100% ethanol to each sample.
    b. Mix the samples by aspiration.
    c. Transfer 500 µl of the mixed samples to the 96-well 800 µl long drip Unifilter capture plate (Whatman).
    d. Apply a vacuum pressure of 70 Kpa for 5 minutes to draw samples through the filter.
    e. Transfer 500 µl of the 500 µl propanol wash buffer to the capture plate.
    f. Apply a vacuum pressure of 50 Kpa for 5 minutes to draw through wash buffer.
    g. Repeat 11.e and 11.f
    h. The capture plate was then washed twice under vacuum with (Sigma) at 50 Kpa for 5 minutes.
    i. Transfer 500 µl of 100% ethanol to the capture plate.
    j. Apply a vacuum pressure of 45 Kpa for five minutes to draw through ethanol wash.
    k. Apply a vacuum pressure of 40 Kpa for 5 minutes to remove all traces of ethanol.
    l. Transfer the capture plate to a 96 well elution plate (Qiagen).
    m. Transfer 200 µl of RNase, DNase free water to each well of the capture plate.
    n. Incubate at room temperature for 2 minutes.
    o. Apply a vacuum pressure of 45 Kpa for 5 minutes to elute the RNA.
    p. Transfer the capture plate to its original position.
12. Remove the elution plate and seal tubes with caps or adhesive foil.
13. Store at -18℃.

MacKenzie, DJ, McLean, MA, Murkerji, S and Green, M. 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease* **81(2),** 222-226.

2 POLYMERASE CHAIN REACTION

2.1 LABORATORY REQUIREMENTS

- To reduce the risk of contamination and possible false positive results, particularly when nested PCR is used for pathogen detection, RT-PCR and PCR reactions must be set up in a different laboratory to where nucleic acid extractions are routinely done.
- RT-PCR and PCR reagent stocks and RT-PCR and PCR reaction set up must be done in a separate clean molecular laboratory or PCR workstation with dedicated pipettes, PCR tubes and tips that have not been exposed to nucleic acid extracts.
- Use a separate pipette for the addition of nucleic acids to the PCR reactions.
- Do not add nucleic acid to reactions in the same clean room or PCR workstation in which PCR stocks are handled.
- The RT-PCR and PCR assays developed in this project have been validated using the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase kit (Invitrogen) and the Platinum® *Taq* DNA Polymerase kit (Invitrogen) respectively. The use of different kits has not been validated and cannot be guaranteed for pathogen detection.

2.2 PCR MATERIALS AND EQUIPMENT

1. PCR reagents t
   - SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase kit (Invitrogen)
   - Platinum® *Taq* DNA Polymerase kit (Invitrogen)
2. Primers for the pathogen being tested (Table 1)
3. Positive controls (Table 2)
4. PCR grade water
5. 0-2 µl, 2-20 µl, 20-200 µl and 200-1000 µl pipettes
6. 0-2 µl, 2-20 µl, 20-200 µl and 200-1000 µl sterile filter pipette tips
7. 200 µl yellow pipette tips
8. 1.5 ml centrifuge tubes to store reagents
9. PCR tubes (volume depends on thermocycler)
10. Bench top centrifuge– with adapters for small tubes
11. Freezer
12. Ice machine
13. Gel tank, casting plate and combs
14. Power pack
15. Latex or nitrile gloves
16. Thermocycler
17. DNA molecular weight marker
18. UV transilluminator and camera
19. Buffers:

5 × TBE - 1L

Reagent	Final concentration	Amount needed for 1L
Tris-Cl	445mM	54.0 g
Boric acid	445mM	27.5 g
0.5M EDTA pH 8.0	10mM	20 ml

Make up to volume with distilled water. Store at room temperature. Dilute 1/10 for gel electrophoresis.

1% agarose gel – 100ml

1. Add 1g agarose per 100 ml 0.5 × TBE
2. Melt the agarose in a microwave
3. Add 10 µl Ethidium bromide (1 mg/ml) per 100 ml agarose gel.

6X gel Loading Buffer:

Bromophenol Blue	0.25% (w/v)
Glycerol	30% (w/v)

Make up to volume with sterile distilled H_2O.
Use 1 µl of loading buffer to 5 µl PCR sample.

Table 1. The list of endemic and exotic pathogens of *Fragaria sp.* tested for in Australia, the type of PCR test, the primers used, the annealing temperature, the region amplified, expected product size and reference for each test.

Pathogen	Test	Primer name	orientation	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Housekeeping gene assays								
RNA - NADH dehydrogenase mRNA	One step RT-PCR	AtropaNad2.1a AtropaNad2.2b	F R	GGACTCCTGACGTATACGAAGGATC AGCAATGAGATTCCCCAATATCAT	55℃	NADH dehydrogenase ND2subunit	188bp	Thompson et al 2003.
DNA -16S rRNA gene	PCR	FD2 RP1	F R	ACG GTT ACC TTG TTA CGA CTT		6S rRNA gene	1400-1500 bp	Weisberg et al 1991.
Endemic viruses								
Beet pseudo yellows virus	One step RT-PCR	BP CPm F BP CPm R	F R	TTCATATTAAGGATGCGCAGA TGAAAGATGTCCACTAATGATA	55℃	Coat protein	334pb	Tzanetakis et al 2003.
Strawberry crinkle virus	One step RT-PCR	SCVdeta SCVdetb	F R	CATTGGTGGCAGACCCATCA TTCAGGACCTATTTGATGACA	60℃	Polymerase	345bp	Thompson et al 2003.
Strawberry mottle virus	One step RT-PCR	SMoVdeta SMoVdetb	F R	TAAGCGACCACGACTGTGACAAAG TCTTGGGCTTGGATCGTCACCTG	50℃	Non-coding region	219bp	Thompson et al 2003.
Strawberry mild yellow edge	One step RT-PCR	SYEupstcp1a SYEPolyTb	F R	CCGCTGCAGTTGTAGGGTA TTTTTTTTTTTTTTTAAGAAAAAGAAAAACAAAC	50℃	Coat protein	913bp	Thompson and Jelkmann 2004..
Strawberry vein banding virus	One step RT-PCR	SVBVdeta SVBVdetb	F R	AGTAAGACTGTTGGTAATGCCA TTTCTCCATGTAGGCTTTGA	55℃	Coat protein	422bp	Thompson et al 2003.
Strawberry pallidosis virus	One step RT-PCR	SP 44 F SP 44 R	F R	GTGTCCAGTTATGCTAGTC TAGCTGACTCATCAATAGTG	52℃	Heat shock protein 70 homolog	517bp	Tzanetakis et al 2004a.
	One step RT-PCR	CP5' CPn731R	F R	AGCTAGAACAAGGCAAGTC GCCAATTGACTGACATTGAAG	52℃	Coat protein	752bp	Tzanetakis et al 2004a.
Strawberry necrotic shock	One step RT-PCR	SNSV CPbeg F SNSV CPend R	F R	GAGTATTTCTGTAGTGAATTCTTGGA ATTATTCTTAATGTGAGGCAACTCG	55℃	Coat protein	823bp	Tzanetakis et al 2004b.
Exotic pathogens								
Arabis mosaic virus	Specific RT-PCR	M2 M3	F R	(C/T)T(A/G)GATTTTAGGCTCAATGG;: TG(C/T)AA(A/G)CCAGG(A/G)AAGAAAAT;	42℃	movement protein gene	290bp	Wetzel et al 2002.

Pathogen	Test	Primer name	orientation	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Raspberry ringspot virus	Specific RT-PCR	RpRSVF1 RpRSVR1	F R	TGTGTCTGGTTTTGATGCT GAGTGCGATAGGGGCTGTT	61℃	Partial RNA-2	385bp	Ochoa-Corona et al 2006.
Strawberry latent ringspot virus	Specific RT-PCR	SLRSV-F SLRSV-R	F R	CCTCTCCAACCTGCTAGACT AAGCGCATGAAGGTGTAACT	61℃	Partial coat protein gene	487bp	Postman et al 2004.
Tomato black ring virus	Specific RT-PCR	P1 P2	F R	ATGGGAGAAGTGCTGG AATCTTTTTGTGTCCAACA	42℃	Partial RNA-2	TBRV 332bp	Le Gall et al 1995.
	Specific RT-PCR	TBRV-70F TBRV-70R	F R	GCTCGTAACAGTTGCGGAGATAT TGTCCACACTGTCATGGGA	60℃	Partial RNA-2	72 bp	Harper et al 2011.
Tomato ringpsot virus	Specific RT-PCR	U1 D1	F R	GAC GAA GTT ATC AAT GGC AGC TCC GTC CAA TCA CGC GAA TA	55℃	RNA-1 region	450 bp	Griesbach 1995.
Tomato busy stunt virus	Specific RT-PCR	TomCPF TomCPR	F R	CCG CCG TAG CAT GAC CAA GTA CCA TGA ACT GGT CTT GTT CAA	55℃	Partial coat protein gene	1000 bp	Russo et al 2002.
	Specific RT-PCR	TBSVGralF1 TBSVGralR1	F R	AAGGGTAAGGATGGTGAGGA TTTGGTAGGTTGTGGAGTGC	61℃	Polymerase read through protein	590bp	Harris et al 2006.
Phytophthora fragariae var. fragariae	Nested PCR	DC6 ITS4 DC1 B5	F R F R	GAGGGACTTTTGGGTAATCA TCCTCCGCTTATTGATATGC ACTTAGTTGGGGGCCTGTCT TGAGATCCACCCGCAGCA	57℃ 65℃	Ribosomal DNA	1300bp 750bp	Bonants et al 2004.
Xanthomonas fragariae	Specific single PCR and nested PCR	245A 245B 245.267 245.5	F R F R	CGCGTGCCAGTGGAGATCC CGCGTGCCAGAACTAGCAG GGTCCAGTGGAGATCCTGTG GTTTTCGTTACGCTGAGTACTG	57℃ 57℃	Unknown genomic DNA	300bp 286bp	Pooler et al 1996 ; Zimmermann et al 2004

Table 2. A list of positive controls used for RT-PCR and PCR detection of housekeeping genes and endemic and exotic pathogens, the type of material provided and their origins.

Pathogen	Type of material	Origin
NADH dehydrogenase mRNA	RNA of known good quality	RNA extracted from any host tissue
16S rRNA gene	DNA of known good quality	DNA extracted from any host tissue
Beet pseudo yellows virus	RNA of known good quality	RNA of the appropriate virus extracted from any host tissue. A cloned fragment of the virus may also be used.
Strawberry crinkle virus	RNA of known good quality	
Strawberry mottle virus	RNA of known good quality	
Strawberry mild yellow edge	RNA of known good quality	
Strawberry vein banding virus	RNA of known good quality	
Strawberry pallidosis virus	RNA of known good quality	
Strawberry necrotic shock	RNA of known good quality	
Arabis mosaic virus	Total nucleic acid, *Vitis vinifera* isolate PV-0045	DSMZ – Deutsche Smmlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany. A cloned fragment of the virus may also be used.
Raspberry ringspot virus	Total nucleic acid *Vitis vinifera* isolate PV-0429	
Strawberry latent ringspot virus	Total nucleic acid isolate PV0247 (original host unknown)	
Tomato black ring virus	Total nucleic acid *Rubus idaeus* isolate PV-0191 and Pelargonium isolate PV-0521	
Tomato ringspot virus	Total nucleic acid Pelargonium isolate PV0049	
Tombusviruses – *Pear latent virus*	Partial clone of cDNA encompassing the coat protein ligated into pGEM-T vector	Dr. M. Russo Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy.
Phytophthora fragariae var. fragariae	110bp synthetic positive control incorporating first round and nested PCR primers	Geneworks, SA, Australia
Xanthomonas fragariae	Total nucleic acid	Dr. Stephen Doughty, DPI, Victoria.

2.3 RT-PCR AND PCR AMPLIFICATION OF PATHOGENS

- Set up all PCR and RT-PCR reactions on ice.
- Prior to the addition to RT-PCR or PCR reactions all nucleic acid must be fully defrosted and mixed thoroughly to reduce the risk of false negative results.
- Table 3 gives the components and concentrations for one generic one-step RT-PCR reaction using the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase kit (Invitrogen):
    - 12.5 µl reactions are used for the RNA housekeeping PCR.
    - 20µl reactions are used for virus detection.
- Table 4 gives the components and concentrations for one generic PCR reaction using the Platinum® *Taq* DNA Polymerase kit (Invitrogen):
    - 20µl reactions are used for the DNA housekeeping assay and pathogen detection.
- For more than one reaction, make a master mix for the number of reactions required plus one extra reaction and aliquot the amount of each reaction required into individual labelled tubes.

- Prior to pathogen detection conduct a housekeeping RT-PCR or PCR to determine if the nucleic extract is of sufficient quality for pathogen detection.
- The cycling conditions for the housekeeping assays and pathogens is given in table 5
- Run the PCR products on a gel as described in section 2.4.

Table 3. The volumes of components required for one RT-PCR reaction for the detection of Housekeeping RNA (12.5 µl reaction) or viruses (20 µl reaction) using the Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase.

Reagent	Volume for a 12.5 µl reaction – Housekeeping RT-PCR	Volume for a 20 µl reaction – Pathogen RT-PCR
Sterile (RNase, Dnase free) water	4.5µl	6µl
10 µM forward primer	0.25µl	0.8µl
10 µM reverse primer	0.25µl	0.8µl
2 × reaction mix (Contains buffer, dNTPs and $MgCl_2$)	6.25µl	10µl
SuperScript™ III RT/Platinum® Taq mix	0.25µl	0.4µl
RNA template	1µl	2µl
Total reaction volume		20 µl

Table 4. The volumes of components required for one 20 µl PCR reaction for the detection of Housekeeping DNA or DNA pathogens using the Invitrogen Platinum® *Taq* DNA Polymerase kit.

Reagent	Volume
Sterile (RNase, Dnase free) water	18.05µl
10 × reaction buffer	2µl
50 mM $MgCl_2$	0.75µl
10 mM dNTP mixture	0.5µl
10 µM forward primer	0.8µl
10 µM reverse primer	0.8µl
Platinum® Taq (5 units/µl)	0.08µl
DNA template or control	2µl
Total reaction volume	20 µl

Table 5. The PCR cycling conditions used for the detection of housekeeping mRNA and DNA and strawberry pathogens.

Housekeeping or pathogen assay	Pre-cycling conditions - 1cycle		PCR – cycling conditions -35 cycles[1]			Post-cycling conditions - 1cycle	
Step	**Reverse transcription**	*Initial denaturation*	**Denaturation**	**Annealing**	**Elongation**	**Final elongation**	**Hold**
Temperature	**48℃**	**94℃**	**94℃**	**See table 2 for temperatures**	**94℃**	**72℃**	**23℃**
NADH dehydrogenase mRNA	20 minutes	2 minutes	40 seconds	40 seconds	40 seconds	5 minutes	indefinite
16S rRNA gene	NA[2]	2 minutes	45 seconds	30 seconds	30 seconds	5 minutes	indefinite
Beet pseudo yellows virus	45 minutes	2 minutes	1 minute	40 seconds	40 seconds	5 minutes	indefinite
Strawberry crinkle virus	45 minutes	2 minutes	1 minute	40 seconds	40 seconds	5 minutes	indefinite
Strawberry mottle virus	45 minutes	2 minutes	1 minute	40 seconds	40 seconds	5 minutes	indefinite
Strawberry mild yellow edge	45 minutes	2 minutes	1 minute	40 seconds	1 minute	5 minutes	indefinite
Strawberry vein banding virus[3]	NA	2 minutes	1 minute	40 seconds	40 seconds	5 minutes	indefinite
Strawberry pallidosis virus- both primer pairs	45 minutes	2 minutes	30 seconds	30 seconds	1 minute	5 minutes	indefinite
Strawberry necrotic shock	45 minutes	2 minutes	30 seconds	30 seconds	1 minute	5 minutes	indefinite
Arabis mosaic virus	45 minutes	2 minutes	20 seconds	20 seconds	30 seconds	5 minutes	indefinite
Raspberry ringspot virus	45 minutes	2 minutes	30 seconds	30 seconds	30 seconds	5 minutes	indefinite
Strawberry latent ringspot virus	45 minutes	2 minutes	30 seconds	30 seconds	30 seconds	5 minutes	indefinite
Tomato black ring virus – P1/P2 primers	45 minutes	2 minutes	30 seconds	30 seconds	30 seconds	5 minutes	indefinite
Tomato black ring virus -TBRV-70F/TBRV70R primers	45 minutes	2 minutes	30 seconds	30 seconds	30 seconds	5 minutes	indefinite
Tomato ringspot virus	45 minutes	2 minutes	30 seconds	30 seconds	30 seconds	5 minutes	indefinite
Tomato bushy stunt virus - TomCPF/TomCPR primers	45 minutes	2 minutes	45 seconds	45 seconds	90 seconds	5 minutes	indefinite
Tomato bushy stunt virus - TBSVGralF1/TBSVGraR1 primers	45 minutes	2 minutes	35 seconds	35 seconds	45 seconds	5 minutes	indefinite
Phytophthora fragariae var. fragariae - first round and nested PCR	NA	2 minutes	30 seconds	30 seconds	60 seconds	5 minutes	indefinite
Xanthomonas fragariae - first round and nested PCR	NA	2 minutes	60 seconds	60 seconds	60 seconds	5 minutes	indefinite

[1] Except *Strawberry necrotic shock virus* which has 40 cycles
[2] NA = not applicable
[3] *Strawberry vein banding virus* can be detected using the Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase.

2.4 GEL ELECTROPHORESIS OF PCR PRODUCTS

1. Use gloves for all steps.
2. Prepare a 1% agarose gel in 0.5 x TBE buffer. Dissolve the agarose in the buffer by heating in a microwave, swirl to mix and check to ensure all agarose has dissolved.
3. When the gel solution is cool enough to be held, add 5µl of 10mg/ml ethidium bromide to 50 ml 1% agarose and mix. Avoid bubbles – do not stir vigorously.
4. Tape both ends of the gel tray or place the gel tray into a horizontal gel castor and pour the agarose mixture. Remove any bubbles present; add a comb and leave to set.
5. Once the agarose has set remove the tape from the gel tray or remove the gel tray from gel castor, remove the comb from the agarose gel and place the agarose gel in the electrophoresis tank.
6. Add 0.5 x TBE buffer until the agarose gel is just covered.
7. Add 6 × loading buffer to the DNA sample at a rate of 1µl loading buffer to 5µl of the DNA sample and mix thoroughly.
8. Make a well plan in your laboratory book, including standards, and load DNA samples in the wells of the agarose gel accordingly.
9. Run gel at 100V for 30-60 minutes depending of the length of the agarose gel or until the bromophenol blue has reached 1cm from the end of the agarose gel.
10. View under UV illumination.

APPENDIX 4 – REFERENCES CITED IN THIS MANUAL

van Adriechem, M.C.J. and Bosher, J.E. 1958. Leaf blotch and petiole blight of strawberry caused by Gnomonia fructicola. Plant Disease Reporter 42, 772–775.

Alexopoulos, C.J. and Cation, D. 1948. Stem-end rot of strawberries. Phytopathology 38: 698–706.

Bolay, A, 1972. Contribution a la connaissance de Gnomonia comari Karsten (syn. G. fructicola [Arnaud] Fall). Etude taxonomique, phytopathologique et recherches sur sa croissance in vitro. Berichte der Schweizerischen Botanischen Gesellschaft 81: 398–482.

Bonants PJM, van Gent-Pelzer MPE, Hooftman R, Cooke DEL, Guy DC and Duncan JM. 2004. A combination of baiting and different PCR formats, including measurement of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. *European Journal of Plant Pathology* 110, 689-702.

Bringhurst RS and Voth V. 1956 Strwaberry virus transmission by grafting excised leaves. Plant Disease Reporter. 40, 596-600.

Chang, L., Zhang, Z., Yang, H., Li, H. and Dai, H. 2007. Detection of strawberry RNA and DNA viruses by RT-PCR using total nucleic acid as a template. Journal of Phytopathology. 155, 431-436.

Cieslinska, M. 2004. Detection of strawberry mottle virus (SMoV) using RT-PCR - comparison of two RNA extraction methods. Journal of Fruit and Ornamental Plant Research. 12, 17-22.

Cieslinska, M. and Stankiene, J. 2005.The reaction of herbaceous plant species to infection with Strawberry mottle virus (SMoV) isolates occurring in Poland and Lithuania. Phytopathologia Polonica. 37, 91-97.

Constable F, Bottcher, C, Kelly G, Lines R, Milinkovic M, Persley D and Rodoni B. 2007. Molecular diagnostics for the detection of strawberry viruses. Final report for Horticulture Australia project BS04004

Constable F, Bottcher, C, Kelly G, Nancarrow N, Milinkovic M, Persley D and Rodoni B. 2010. Developing molecular diagnostics for the detection of strawberry viruses (Follows BS04004). Final report for Horticulture Australia project BS07003.

Constable F, Bottcher, C, Kelly G, Nancarrow N, Milinkovic M, Ko L, Persley D and Rodoni B. 2011. Developing molecular diagnostics for the detection of strawberry viruses (continues BS07003). Final report for Horticulture Australia project BS10002.

Converse, R.H., Martin, R.R. and Spiegel, S. 1987. Strawberry mild yellow-edge. In: Virus diseases of small fruits (Ed. by Converse, R.H.), pp. 25-29. Agriculture Handbook No. 631. US Department of Agriculture, Washington DC, USA.

Converse, R. H. and Volk, E. 1990. Some effects of pallidosis disease on strawberry growth under greenhouse conditions. Plant Disease. 74, 814-816.

Craig, D. L. 1981. Strawberry cultivar reaction to pallidosis disease. Canadian Plant Disease Survey. 61, 41-42.

Dick, M. W. 2001. Straminipilous Fungi: Systematics of the Peronosporomycetes Including Accounts of the Marine Straminipilous Protists, the Plasmodiophorids and Similar Organisms. Kluwer Academic Publishers, Dordrecht, Boston.

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

Duncan J.M. 1976. The use of bait plants to detect Phytophthora fragariae. Transactions of the British Mycological Society 65, 338-340.

Faggioli, F., Ferretti, L., Pasquini, G. and Barba, M. 2002. Detection of Strawberry latent ring spot virus in leaves of olive trees in Italy using a one-step RT–PCR. Journal of Phytopathology 150, 636–639.

Frazier, N. W. 1955. Strawberry vein banding virus. Phytopathology. 45, 307-312.

Frazier, N. W. 1960. Differential transmission of four strains of Strawberry vein banding virus by four aphid vectors. Plant Disease. Reporter. 44, 436-437

Frazier, N. W. and Sylvester, E. S. 1960.. Half-lives of transmissibility of two aphid-borne viruses. Virology 12, 233-244.

Frazier, N. W. 1966. Pseudo mild yellowedge: A new strawberry virus disease in the Fragaria virginiana indicator clone M-1. Phytopathology 56, 571-572.

Frazier, N. W. 1968. Transmission of strawberry mottle virus by juice and aphids to herbaceous hosts. Plant Disease. Reporter. 52, 64-67.

Frazier, N. W. and Stubbs, L. L. 1969. Pallidosis-a new virus disease of strawberry. Plant Disease Reporter. 53, 524-526.

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

Frazier, N. W. 1975.Strawberry mild yellow-edge in North Carolina. Plant Disease Reporter. 59, 41-42.

Frazier, N. W., Jorgensen, P. S., Thomas, H. E., and Johnson, H. A., Jr. 1962. Necrotic shock – A virus disease of strawberries. Plant Disease Reporter. 46, 547-550.

Frazier, N.W., Sylvester, E.S. and Richardson, J. 1987. Strawberry crinkle virus. In: Viruses Diseases of Small Fruits (Converse, R.H. ed.). Washington, DC: United States Department of Agriculture, pp. 20–25.

Fulton, R. W. 1967. Purification and some properties of Tobacco streak and Tulare apple mosaic viruses. Virology 32, 153-162.

Fulton, J.P. 1987. Strawberry pallidosis. Pgs. 55-56 in: Virus Diseases of Small Fruits. R.H. Converse, (ed.) U.S. Dep. Agric. Agric. Handb. 631.

Greber, R.S. 1979. Virus diseases of Queensland strawberries and the epidemiological effects of the strawberry runner approval scheme. Queensland Journal of Agricultural and Animal Sciences. 36, 93-103.

Griesbach, J.A.1995 Detection of Tomato ringspot virus by polymerase chain reaction. Plant Disease 79, 1054-1056

Gubler, W.D. and Feliciano, A. 1999. Occurrence of leaf blotch and stem-end rot of strawberry in the Central Coast California. Plant Disease 83, 199.

Jończyk M, Borodynko N and Pospieszny H. 2004. Restriction analysis of genetic variability of Polish isolates of Tomato black ring virus. Acta Biochimica Polonica 51, 673-681.

Hadidi, A. Montasser, M. S. Levy, L. Goth, R. W. Converse, R. H. Madkour, M. A. and Skrzeckowski, L. J. 1993. Detection of potato leafroll and strawberry mild yellow-edge luteoviruses by reverse transcription-polymerase chain reaction amplification. Plant Disease 77, 595-601.

Harper SJ, Delmiglio C, Ward LI and Clover GRG. 2011. Detection of Tomato black ring virus by real-time one-step RT-PCR. Journal of Virological Methods 171, 190–194.

Harris RV and King E. 1942. Studies in strawberry diseases. The use of Fragarra vesca L. as an indicator of yellow edge and crinkle. Journal of Pomological and Horticultural Science. 19, 227-242.

Harris, R. V. 1933. The strawberry “yellowedge” disease. Journal of Pomology and Horticultural Science. 11, 56- 76.

Harris, R., Ochoa-Corona, R., Lebas, B., Timudo O, Stewart F and Alexander B. 2006. Broad detection and diagnosis of viruses of the genus Tombusvirus by RT-PCR coupled to single strand conformation polymorphism analysis. In: Proceedings of the 7th Australasian Plant Virology Workshop, Rottnest Island, Perth, Australia, p. 48.

Hepp, R.F. and Martin, R.R. 1991. Occurrence of strawberry mild yellow-edge associated virus in wild Fragaria chiloensis in South America. Acta Horticulturae. 308, 57-60.

Honetslegrova, J. Mraz, I. and Spak, J. 1995. Detection and isolation of strawberry vein banding virus in the Czech Republic. Acta Horticulturae. 385, 29-32.

Horne, W. T. 1922. Strawberry troubles. Calif. Agric. Exp. Stn. Rep. 1921-22:122- 123

Hughes KJD, Inman AJ and Cooke DEL. 2000. Comparative testing of nested PCR-based methods with bait-plant tests for detecting Phytophthora fragariae var. fragariae in infected strawberry roots from fruit crops in the UK. Bulletin OEPP 30, 533-538.

Jawee, A. and Adams, A. N. 1995. Serological detection of strawberry mild yellow edge-associated virus. Acta Horticulturae. 385, 98-104.

Jelkmann, W., Maiss, E. and Martin, R.R. 1991. The nucleotide sequence and genome organization of strawberry mild yellow edge associated potexvirus (SMYEAV). Journal of General Virology 73, 475-479.

Jelkmann, W., Martin, R.R., Lesemann, D.E., Vetten, H.J. and Skelton, F. 1990. A new potexvirus associated with strawberry mild yellow edge disease. Journal of General Virology 71, 1251-1258.

Johnson, H. A., Jr., Converse, R. H., Amorao, A., Espejo, J. I., and Frazier, N. W. 1984. Seed transmission of Tobacco streak virus in strawberry. Plant Disease. 68, 390-392.

Johnstone, G. R. 1987. Beet pseudo yellows closterovirus. In Plant viruses online; Descriptions and lists from the VIDE database.

Kaden-Kreuziger, D., Lamprecht, S., Martin, R. R. and Jelkmann, W. 1995. Immunocapture polymerase chain reaction assay and ELISA for the detection of strawberry mild yellow edge associated potexvirus. Acta Horticulturae. 385, 33-38.

King, M. E. and Harris, R V. 1942. Studies in strawberry virus diseases. V. The use of Fragaria vesca L. as an indicator of yellow-edge and crinkle. Journal of Pomology. 19, 227-242.

Klerks, M. M., Lindner, J. L., Vaskova, D., Spak, J., Thompson, J. R., Jelkmann, W. and Schoen, C. D. 2004. Detection and tentative grouping of Strawberry crinkle virus isolates. European Journal of Plant Pathology. 110, 45-52.

Krczal, H. 1980. Transmission of the strawberry mild yellow edge and strawberry crinkle virus by the strawberry aphid Chaetosiphon fragaefolii. Acta Phytopathologica Academiae Scientiarum Hungaricae 15, 97-102.

Kurppa S, and Vrain TC. 1989. Effect of Pratylenchus penetrans on the infection of strawberry roots by Gnomonia comari. Journal of Nematology 21: 511–516.

Lamprecht S. and Jelkmann W. 1997. Infectious cDNA clone used to identify strawberry mild yellow edge associated potexvirus as causal agent of the disease. Journal of General Virology 78, 2347–2353.

Le Gall O, Candresse T and Dunez J. 1995. Transfer of the 31 non-translated region of Grapevine chrome mosaic virus RNA-1 by recombination to Tomato black ring virus RNA-2 in pseudorecombinant isolates. Journal of General Virology 76, 1285-1289.

Maas JL. 1998. *Compendium of Strawberry Diseases, Second Edition*. American Phytopathology Society, St. Paul, Minnesota

MacKenzie, DJ, McLean, MA, Murkerji, S and 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.

Mahmoudpour, A. 2003. Infectivity of recombinant strawberry vein banding virus DNA. Journal of General Virology 84, 1377-1381.

Mahmoudpour, A. 2004. Diagnosis and quantification of Strawberry vein banding virus using molecular approaches. Acta Horticulturae. 656, 69-74.

Martin, R.R. and Converse, R.H. 1985. Purification, properties and serology of strawberry mild yellow-edge virus. Phytopathologische Zeitschrift 114, 21-30.

Martin RR and Tzanetakis IE. 2006. Characterization and recent advances in detection of strawberry viruses. Plant Disease. 90, 384-396.

Mellor, F.C. and Krczal, H. 1987. In: Viruses Diseases of Small Fruits; ed. R.H. Converse. U.S. Dep. Agric. Hdbk No. 631, p, 10

Miller, P.W. and Frazier, N.W. 1970. In: Virus diseases of small fruits and grapevines, a handbook (Ed. by Frazier, N.W.), pp. 8-10. University of California, Berkeley, California, USA.

Mohan SB. 1988. Evaluation of antisera raised against Phytophthora fragariae for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA). Plant Pathology 37, 206- 216

Mohan SB. 1989. Cross-reactivity of antiserum raised against Phytophthora fragariae with other Phytophthora species and its evaluation as a genus-detecting antiserum. Plant Pathology 38, 352–363

Moltmann E and Zimmermann C. 2005. Detection of Xanthomonas fragariae in symptomless strawberry plants by nested PCR OEPP/EPPO Bulletin 35, 53–54

Montasser, M. S., Al-Awadhi, H. and Hadidi, A. 2002. Identification of luteovirus nucleotide sequences in mild yellow-edge diseased strawberry plants. Plant Pathology Journal. 18, 1-5.

Moročko I and Fatehi J. 2007. Molecular characterisation of strawberry pathogen *Gnomonia fragariae* and its genetic relatedness to other *Gnomonia* species and members of *Diaporthales*. *Mycological Research* **111**: 603–614.

Moročko I, Fatehi J and Gerhardson B. 2006. *Gnomonia fragariae*, a cause of strawberry root rot and petiole blight. *European Journal of Plant Pathology* **114**: 235–244.

Mraz, I., Petrzik, K., Franova-Honetslegrova, J. and Sip, M. 1997.Detection of strawberry vein banding virus by polymerase chain reaction and dot blot hybridization. Acta Virologica. 41, 241-242.

Mullin, R. H., Frazier, N. W. and Schlegel, D. E. 1975. Meristem culture of Fragaria chiloensis infected with strawberry pallidosis. Plant Disease Reporter. 59, 268.

Mumford, R. A., Skelton, A. L., Boonham, N., Posthuma, K. I., Kirby, M. J. and Adams, A. N. 2004. The improved detection of Strawberry crinkle virus using real-time RT-PCR (TaqManReg.). Acta Horticulturae. 656, 81-86.

Noble D. 2009. Diagnostic Methods for Strawberry Angular Leaf Spot *Xanthomonas fragariae http://www.padil.gov.au/pbt | Strawberry Angular Leaf Spot - Xanthomonas fragariae*

Ochoa-Corona FM, Lebas BS, Tang JZ, Bootten TJ, Stewart FJ, Harris R, Elliott DR and Alexander BJR. 2006. RT-PCR detection and strain typing of *Raspberry ringspot virus*. Proceedings of the XXth International Symposium on Virus and Virus-like Diseases of Temperate Fruit Crops and XIth International Symposium on Small Fruit Virus Diseases, Antalya, Turkey.

Ochoa-Corona FM, Lebas BS, Tang JZ, Bootten TJ, Stewart FJ, Harris R, Elliott DR and Alexander BJR. 2006. RT-PCR detection and strain typing of Raspberry ringspot virus. Proceedings of the XXth International Symposium on Virus and Virus-like Diseases of Temperate Fruit Crops and XIth International Symposium on Small Fruit Virus Diseases, Antalya, Turkey.

Olsson CHB. 1995. Diagnosis of Phytophthora infections in raspberry and strawberry plants by ELISA tests. Journal of Phytopathology 143, 307–310

Oligivie, L., Swarbrick, T. and Thompson, C.R. 1934. A note on strawberry disease resembling the American crinkle. Long Ashton Res. Station Report For 1933, 96–97.

Petrzik,K., Benes,V., Mraz,I., Honetslegrova-Franova,J., Ansorge,W. and Spak,J. 1998. Strawberry vein banding virus--definitive member of the genus Caulimovirus. Virus Genes 16, 303-305

Petrzik, K., Kubelkova, D. and Mraz, I. 2002. Strawberry vein banding virus detection by double antibody sandwich ELISA with antibodies to recombinant coat protein. Acta Virologica 46, 125-126.

Pooler MR Ritchie DF and Hartung JS. 1996. Genetic relationships among strains of Xanthomonas fragariae based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. Applied and Environmental Microbiology 62, 3121–3127.

Postman JD, Tzanetakis IE, and Martin RR. 2004. First report of *Strawberry latent ringspot virus* in a *Mentha* sp. from North America. *Plant Disease* 88, 907.

Posthuma, K. I.;,Adams, A. N., Hong, Y. and 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.

Posthuma, K. I., Adams, A. N. and Hong YiGuo. 2000. Strawberry crinkle virus, a Cytorhabdovirus needing more attention from virologists. Molecular Plant Pathology 1, 331-336.

Posthuma, K. I., Hong, Y. G. and Adams, A. N. 2001. Molecular detection of Strawberry crinkle virus. Acta Horticulturae. 551, 75-79.

Prentice, I. W., and Harris, R. V. 1946. Resolution of strawberry virus complexes by means of the aphid vector Capitophorus fragariae Theb. Annals of Applied Biology. 33, 50-53.

Quail, A. M., Martin, R. R., Spiegel, S., and Jelkmann, W. 1995. Development of monoclonal antibodies specific for strawberry mild yellow edge potexvirus. Acta Horticulturae. 385, 39-45.

Richardson, J. and Sylvester, E.S. 1988. Successful juice inoculation of the aphid–vectored strawberry crinkle virus. Californian Agriculture. 42, 6–7.

Russo M, Vovlas C, Rubino L, Grieco F and Martelli, GP. 2002. Molecular characterization of a tombusvirus isolated from diseased pear trees in southern Italy. Journal of Plant Pathology 84, 161-166.

Schoen, C. D., Limpens, W., Moller, I., Groeneveld L., Klerks, M. M., and Lindner, J. L. 2004. The complete genomic sequence of Strawberry crinkle virus, a member of the Rhabdoviridae. Acta Horticulturae. 656, 45-50.

Shipton PJ, 1967. A fruit rot of strawberries caused by Zythia fragariae. Plant Pathology 16: 123–125

Sogonov MV, Castlebury LA, Rossman AY, Mejía LC and White JF. 2008. Leaf-inhabiting genera of the Gnomoniaceae, Diaporthales. Studies in Mycology 62: 1–79.

Spiegel, S. 1987. Double-stranded RNA in strawberry plants infected with strawberry mild yellow-edge virus. Phytopathology 77, 1492-1494.

Spiegel, S., Cohen, J. and Converse, R.H. 1986. Detection of strawberry mild yellow-edge virus by serologically specific electron microscopy. Acta Horticulturae 86, . 95.

Stace-Smith R., Converse R.H. and Johnson, H.A. 1987. Tobacco streak virus in strawberry.In: Converse RH (ed) Virus diseases of small fruit. USDA ARS Agriculture Handbook No 631,Washington, DC

Stenger, D. C., Mullin, R. H. and Morris, T. J. 1987. Characterization and detection of the Strawberry necrotic shock isolate of Tobacco streak virus. Phytopathology 77, 1330-1337.

Sylvester, E.S., Frazier, N.W. and Richardson, J. 1976. Strawberry crinkle virus. CMI/AAB Descriptions of Plant Viruses, no. 163.

Sylvester, E.S., Richardson, J. and Stenger, D.C. 1987. Use of injected Macrosipum euphorbiae aphids as surrogate vectors for transfer of strawberry crinkle virus to Nicotiana species. Plant Disease. 71, 972–975.

Thompson JR and Jelkmann W. 2003. The detection and variation of strawberry mottle virus. Plant Disease. 87, 385-390.

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

Thompson,J.R., Leone,G., Lindner,J.L., Jelkmann,W. and Schoen,C.D. 2002. Characterization and complete nucleotide sequence of Strawberry mottle virus: a tentative member of a new family of bipartite plant picorna-like viruses. Journal of General Virology. 83, 229-239.

Thompson, J. R., Wetzel, S., Klerks, M. M., Vaskova, D., Schoen, C. D., Spak, J. and 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.

Thompson JR, Wetzel S and Jelkmann W. 2004. Pentaplex RT-PCR for the simultaneous detection of four aphid-borne viruses in combination with a plant mRNA specific internal control in Fragaria spp. Acta Horticulturae. 656, 51-56.

Tzanetakis IE, Wintermantel WM and Martin RR. 2003. First report of beet pseudo yellows virus in strawberry in the United States: a second crinivirus able to cause pallidosis disease. Plant Disease. 87, 1398.

Tzanetakis, I. E. and Martin, R. R. 2004. Complete nucleotide sequence of a strawberry isolate of beet pseudoyellows virus. Virus Genes. 28, 239-246.

Tzanetakis, I. E., Mackey, I. C., and 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., and 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., and Martin, R. R. 2004c. Two criniviruses are associated with the strawberry pallidosis disease. Acta Horticulturae. 656, 21-26.

Tzanetakis, I. E. and Martin, R. R. 2005a. First report of strawberry as a natural host of Apple mosaic virus. Plant Disease. 89, 431.

Tzanetakis, I. E., and Martin, R. R. 2005.b Fragaria chiloensis cryptic virus: A new strawberry virus found in Fragaria chiloensis plants from Chile. Plant Disease. 89, 1241.

Tzanetakis, I. E., and Martin, R. R. 2005c.Strawberry latent virus: A potential link between plant and insect viruses. (Abstr.) Phytopathology 95, S105.

Tzanetakis, I. E., Reed, J. and Martin, R. R. 2005. Nucleotide sequence, genome organization and phylogenetic analysis of Strawberry pallidosis associated virus, a new member of the genus Crinivirus. Archives of Virology. 150, 273-286.

Tzanetakis, I. E., Wintermantel, W. M., Cortez, A. A., Barnes, J. E., Barrett, S. M., Bolda, M. P. and Martin, R. R. 2006. Epidemiology of strawberry pallidosis-associated virus and occurrence of pallidosis disease in North America. Plant Disease. 90, 1343-1346.

Vandroemme J, Baeyen S, Van Vaerenbergh J, De Vos P. and Maes M. 2008. Sensitive real-time PCR detection of Xanthomonas fragariae in strawberry plants Plant Pathology 57, 438–444

Vaskova, D. and Spak, J. 2002. Improved diagnostic tools for the certification of strawberry propagation material - the use of PCR and NASBA for detection of Strawberry Vein Banding Virus (SVBV). Plant Protection Science. 38: Special 1, 24-27.

Vaskova, D. H. and Spak, J. 2004. Molecular detection and variability of Strawberry vein banding virus. Acta Horticulturae 656, 33-38.

Vaskova, D., Spak, J., Klerks, M. M. Schoen, C. D, Thompson, J. R. and Jelkmann, W. 2004. Real-time NASBA for detection of strawberry vein banding virus. European Journal of Plant Pathology. 110, 213-221.

Vaughan, E.K. 1933. Transmission of strawberry crinkle disease of strawberry. Phytopathology, 23, 738–740

Weisberg, WG, Barns, S, Pelletier, DA and Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703

Weller SA, Beresford-Jones NJ, Hall J, Thwaites R, Parkinson N and Elphinstone JG. 2007. Detection of Xanthomonas fragariae and presumptive detection of Xanthomonas arboricola pv. fragariae, from strawberry leaves, by real-time PCR. Journal of Microbiological Methods 70, 379–83.

Wetzel T, Jardak R, Meunier L, Ghorbel A, Reustle GM and Krczal G. 2002. Simultaneous RT/PCR detection and differentiation of arabis mosaic and grapevine fanleaf nepoviruses in grapevines with a single pair of primers. Journal of Virological Methods 101,63–69

Whattam, M.J. 1994. Production of pathogen tested nucleus stock for the Victorian Strawberry Runner Certification Scheme. Vic. Dep. Ag. 1-27.

Wintermantel, W. M. Fuentes, S. Chuquillanqui, C. And Salazar, L. F. 2006. First report of Beet pseudo-yellows virus and Strawberry pallidosis associated virus in strawberry in Peru. Plant Disease. 90, 1457.

Wisler, G. C. Duffus, J. E. Liu, H. Y. And Li, R. H. 1998. Ecology and epidemiology of whitefly-transmitted closteroviruses. Plant Disease. 82, 270-280.

Yang H.Y., Zhang Z.H., Du GuoDong; Dai H.Y. and Gao X.Y. 2005. RT-PCR detection of strawberry mottle virus based on internal control. Acta Phytopathologica Sinica. 35, 116-122.

Yamashita, S., Doi, Y., Yora, K. and Yoshino, M. 1979.Cucumber yellows virus: its transmission by the greenhouse whitefly, Trialeurodes vaporariorum (Westwood), and the yellowing disease of cucumber and muskmelon caused by the virus. Annals of the Phytopathological Society of Japan. 45, 484-496.

Yoshikawa, N. and Converse, R. H. 1991. Purification and some properties of strawberry mottle virus. Annals of Applied Biology 118, 565-576.

Yoshikawa, N., Ohki, S.T., Kobatake, H., Osaki, T. and Inouye, T. 1984. Luteovirus-like particles in phloem tissue of strawberry mild yellow edge virus infected plants. Annals of the Phytopathological Society of Japan 50, 659-663.

Yoshikawa, N., Inouye, T. and Converse, R. H. 1986. Two types of rhabdovirus in strawberry. Annals of the Phytopathological Society of Japan. 52, 437-444.

Zeller, S.M. and Vaughan, E.K. 1932. Crinkle disease of strawberry. Phytopathology, 22, 709–713.

Zhang Z. H., Yang H.Y., Dai H.Y.; Li H. and Xiao M. 2006. Detection of Strawberry mottle virus and Strawberry mild yellow edge virus by multiplex RT-PCR. Acta Horticulturae Sinica. 33, 507-510.

Zimmermann C, Hinrichs-Berger J, Moltmann E, and Buchenauer H. 2004. Nested PCR (polymerase chain reaction) for detection of Xanthomonas fragariae in symptomless strawberry plants. Journal of Plant Disease and Protection 111, 39–51.