

**Virus identification and development of  
long-term management strategies for the  
rhubarb industry**

Dr John Thomas  
The Department of Agriculture, Fisheries and  
Forestry, QLD

Project Number: VG05053

## **VG05053**

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**FINAL REPORT**  
**HAL PROJECT VG05053**  
**(completion 31 May 2011)**

Virus identification and development of  
long-term management strategies for  
the rhubarb industry

John Thomas *et al.*

Agri-Science Queensland

a service of the Department of Employment, Economic Development and Innovation  
(formerly the Department of Primary Industries and Fisheries, Queensland)

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**Purpose of report:** The purpose of this report is to present the final results of all activities conducted under HAL Project VG05053 ‘Virus identification and development of long-term management strategies for the rhubarb industry’. The report provides a summary of project findings, a description of technology transfer activities, and recommendations arising from the outcomes of the project. The overall objective of this project was to devise a strategy for the control of rhubarb decline disease through 1) knowledge of the viruses present and their epidemiology, 2) production of virus-free planting material via tissue culture, and 3) formation of a national grower group to represent industry.

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## 2 GLOSSARY

|           |                                                               |
|-----------|---------------------------------------------------------------|
| bp        | base pairs                                                    |
| CMV       | <i>Cucumber mosaic virus</i>                                  |
| cDNA      | complementary DNA                                             |
| CLRV      | <i>Cherry leafroll virus</i>                                  |
| DAS-ELISA | double antibody sandwich ELISA                                |
| DEEDI     | Department of Employment, Economic Development and Innovation |
| ELISA     | enzyme-linked immunosorbent assay                             |
| EM        | transmission electron microscopy                              |
| NSW       | New South Wales                                               |
| nt        | nucleotides                                                   |
| PCR       | polymerase chain reaction                                     |
| RDaV      | Rhubarb decline associated virus                              |
| RT-PCR    | reverse transcription PCR                                     |
| TSWV      | <i>Tomato spotted wilt virus</i>                              |
| TuMV      | <i>Turnip mosaic virus</i>                                    |
| WA        | Western Australia                                             |

### 3 MEDIA SUMMARY

Rhubarb (*Rhuem rhabarbarum*) is an herbaceous perennial and is a cool season crop valued for its long thick, red stems. These stalks are used raw or cooked in sauces, pies and desserts and have good levels of sugars, fibre, protein, potassium, calcium, magnesium and vitamin. In Australia, rhubarb is grown as a specialty crop in all States, mostly near to the major cities. During the winter months (April to October), production centres on south-east Queensland, predominantly from the Tamborine district, and Western Australia. Summer production is centred on the southern States.

Rhubarb is propagated each year by division of 2-3 year old crowns. Seed is generally not used as it does not produce true-to-type plants and produces plants of inferior quality and often with green stems. In recent years rhubarb crops in areas of Queensland and NSW have developed severe yield decline, and leaf mosaic and necrosis symptoms.

This project

- determined that viruses were associated with the disease symptoms
- showed in a national survey that only Western Australian and Tasmanian crops were virus free
- detected up to seven different viruses in rhubarb
- described for the first time, worldwide, Rhubarb decline associated virus, which is closely linked to the disease syndrome
- developed detection assays for all viruses
- established a tissue culture and acclimatisation protocol for a number of rhubarb cultivars, including elimination of virus from infected plant lines
- showed that tissue cultured plants performed as well as conventional planting material in the field
- demonstrated that virus infection can lead to rapid yield decline in the field
- demonstrated that re-infection of virus free plants in the field can be very rapid if care is not taken to limit this reinfection.

The project has clearly shown that virus infection causing yield losses is common in rhubarb in some districts, especially Mt Tamborine (Qld), the Sydney basin (NSW) and Mornington Peninsula (Vic). Virus-free and acclimatised tissue culture plants can be produced under commercial conditions.

All the elements for a grower managed clean planting material are now available.

## 4 TECHNICAL SUMMARY

Rhubarb (*Rhuem rhabarbarum*) is an herbaceous perennial and is a cool season vegetable crop valued for its long thick, red stems. In Australia, rhubarb is grown as a specialty crop in all States, mostly near to the major cities. During the winter months (April to October), production centres on south-east Queensland, predominantly from the Tamborine district, and Western Australia. Summer production is centred on the southern States.

Rhubarb is vegetatively propagated each year by division of 2-3 year old crowns. Seed is generally not used as it does not produce true-to-type plants and produces plants of inferior quality and often with green stems. In recent years rhubarb crops in areas of Queensland and NSW have developed severe yield decline, and leaf mosaic and necrosis symptoms. Initial examinations revealed a range of virus particle types in infected plants, suggesting that a viral cause for the disease was likely.

The project had the following aims:

- Develop detection tests for the newly discovered viruses in rhubarb
- Conduct surveys to determine the extent of infection with each of the viruses in Australian crops
- Investigate insect vectors and alternative hosts of the virus
- Investigate tissue culture procedures for producing virus-free rhubarb
- Devise a strategy for the control of rhubarb decline disease through knowledge of virus epidemiology and availability of virus-free planting material
- Formation of a national grower group to represent industry

Initial surveys showed that virus infections were common in crops at Mt Tamborine (Qld), Mornington Peninsula (Vic) and the Sydney Basin (NSW). Up to seven viruses were found in crops from these districts, including:

- Turnip mosaic virus
- Cucumber mosaic virus
- Tomato spotted wilt virus
- Cherry leafroll virus
- Rhubarb decline-associated virus (RDaV - a novel virus)
- A novel totivirus
- Unidentified 30 nm isometric particles

RDaV was shown to be a closterovirus. An estimated 90% (14,642 nt) of its genome was sequenced, and shown to encode 10 open reading frames, with the same gene organisation as Carrot yellow leaf closterovirus. In phylogenetic analysis of the RdRp and the HSP70 homologue, RDaV grouped with definitive closteroviruses. It was in the same clade as Mint virus 1, its nearest relative, with which it shared ca 54% nt identity across the genome. RDaV was transmitted by the aphid *Aphis gossypii*, and also symptomlessly infected the weed curled dock (*Rumex crispus*), also in the family Polygoneaceae.

Detection assays have been developed for all identified viruses, but production of an antiserum to RDaV to enable the use of ELISA has proven difficult, with only moderate success with five different antisera prepared against expressed coat protein or synthetic peptides.

A national growers' group was convened, and met in Sydney in October 2008. At this forum, representative cultivars were selected for tissue culture and acclimatisation under commercial conditions.

Tissue culture and acclimatisation protocols were established for six commercial rhubarb lines. Rhubarb could be micropropagated to provide 3-4 X multiplication rate per subculture cycle and successfully acclimatised (over 90% of plants surviving). Using these methods thousands of plants were produced in a commercial laboratory and acclimatised in a commercial nursery. All plants established and grew well in commercial plantings and no off-types were identified. Virus-free tissue cultured rhubarb plants were produced through meristem culture, with or without heat therapy (33°C, 12 hr day, 30°C night), and this material was used to examine reinfection rates under commercial growing conditions.

Mixed infections were frequent in the field and it was difficult to definitively associate symptoms with a particular virus(-es). However, field reinfection trials and survey data showed that RDaV was most consistently associated with severe virus symptoms. Natural reinfection rates were high when virus-free material was planted within an infected commercial crop. After 20 months, 89% of plants were infected with RDaV and 34% with CMV (the latter always as mixed infections), though only 47% of these infected plants showed symptoms. Individual infected plants took up to 15-20 months to express symptoms.

The project has clearly shown that virus infection causing yield losses is common in rhubarb in many districts, and that RDaV is closely associated with these losses. Virus-free and acclimatised tissue culture plants can be produced under commercial conditions.

All the elements for a grower managed clean planting material scheme are now available. It is recommended that the industry adopts the use of virus-free planting material in areas affected by rhubarb decline, and that replanting takes into consideration the high reinfection rates likely if older, infected crops, and the weed, curled dock, are allowed to remain nearby at the time of replanting.

## 5 TECHNICAL REPORT

### 5.1 Introduction

Rhubarb (*Rheum rhabarbarum*) is an herbaceous perennial of the botanical family Polygonaceae. Rhubarb is a cool season crop valued for its long thick, red stems, which grow from the crown up to 75cm. These stalks are used raw or cooked in sauces, pies and desserts and have good levels of sugars, fibre, protein, potassium, calcium, magnesium and vitamin C.

In Australia, rhubarb (*Rheum rhaponicum*) is grown as a specialty crop in all States, mostly near to the major cities. During the winter months (April to October) the Australian market relies heavily on rhubarb produced in the cooler areas of south-east Queensland, predominantly from the Tamborine district, with about 25 ha, and to a lesser extent from Western Australia. The rhubarb industry in Western Australia is concentrated in and around the Perth Metropolitan area. Sales from one property at Gin Gin dominate the market in the state, and represent >90% of the entire industry. This property sometimes also sells rhubarb to eastern Australia. The property is dedicated to rhubarb production and grows little else. It has about 10 ha of rhubarb but picks only what is needed so as to avoid 'flooding' the market with this product. In the past, there were several more sizeable rhubarb producers but most of them have stopped producing rhubarb or now grow only a very small amount. Summer production is centred on the southern States. Queensland and Western Australia have an estimated annual production of 840 tonnes and 575 tonnes, respectively. Production Figures for other States are not available.

Rhubarb is propagated each year by division of 2-3 year old crowns. Seed is generally not used as it does not produce true-to-type plants and produces plants of inferior quality and often with green stems. The main characteristics sought in plants are high yield, straight erect thick evenly coloured red stalks of uniform thickness and length and ease of harvesting. Growers have carefully selected their own superior lines over a number of years and generally bulk up their planting material for own use only. Bulking is slow but has generally satisfied the crops cycle of between 3-6 years. Lines often retained by growers for decades. Such a propagation system favours the accumulation of virus infections, and overseas a number of viruses have been detected in these crops.

In recent years rhubarb crops in areas of Queensland and NSW have developed severe yield decline, and leaf mosaic and necrosis symptoms (Figure 1). A preliminary investigation revealed a number of viruses in affected plants, including cucumber mosaic virus, a possible potyvirus, a previously unknown closterovirus and at least two additional isometric viruses. No work has previously been done on rhubarb viruses in Australia.

Most research on rhubarb viruses has been undertaken in Europe, where virus infection has had a significant impact on yield (Walkey and Cooper 1972). The viruses encountered there were *Turnip mosaic potyvirus* (TuMV), *Arabis mosaic nepovirus* (ArMV) and *Cherry leafroll nepovirus* (CRLV) and less frequently

*Cucumber mosaic cucumovirus* (CMV) and *Strawberry latent ringspot nepovirus* (SLRSV) (Novak and Lanzova 1982; Verhoyen and Gorp 1972; Walkey *et al.* 1982).



Figure 1. Rhubarb decline-affected plant (right) compared with a healthy plant (left).

TuMV has also been described from rhubarb in North America (Robertson and Ianson 2005; Stace-Smith and Jacoli 1967). Additionally, an alphacryptovirus, Rhubarb temperate virus, has been described from Japan (Natsuaki *et al.* 1983). In Australia, the nepoviruses have been recorded, but have a very restricted known distribution, all having been recorded from Tasmania only, in hops (ArMV), *Sambucus* (CLRV) and *Rosa* (SLRSV), while CMV and TuMV occur in all States (Büchen-Osmond *et al.* 1988).

If this rhubarb decline disease is left unchecked it may quickly make rhubarb farming unviable in some districts, and some farms are currently faced with this prospect. The extent of virus infection in the rhubarb industry is not yet known but appears to be widespread. For disease control, there is a need to identify the causal viruses and subsequently the insect vectors and alternative host plants. The incidence of these viruses in crops outside the Tamborine district is unknown, but due to the use of vegetative planting material, it is likely that they will be widespread.

Production of disease free planting material of the superior industry selections will be fundamental to maintaining a viable rhubarb industry. However, currently there is no ready source of clean planting material, no means to rapidly multiply healthy planting material, and no indexing tests for the contaminating viruses. Virus elimination through tissue culture has been achieved in the UK (Walkey 1968; Walkey and Matthews 1979), and preliminary experiments at DPI&F showed that rhubarb could be established in tissue culture here.

The current project had the following aims:

- Develop detection tests for the newly discovered viruses in rhubarb
- Conduct surveys to determine the extent of infection with each of the viruses in Australian crops

- Investigate insect vectors and alternative hosts of the virus
- Investigate tissue culture procedures for producing virus-free rhubarb
- Devise a strategy for the control of rhubarb decline disease through knowledge of virus epidemiology and availability of virus-free planting material
- Formation of a national grower group to represent industry

The overall outcome of the project will be to have a viable rhubarb industry, with the capacity to produce virus free-planting material through an industry-run program.

The technical report will present results in the following order:

- Identification and characterisation of viruses in Australia rhubarb
- Surveys of Australian rhubarb crops
- Establishment of a rhubarb tissue culture and acclimatisation procedure
- Field evaluation of tissue cultured rhubarb



## **5.2 Viruses associated with rhubarb decline**

### **5.2.1 Identifying rhubarb viruses in Australian crops**

#### **5.2.1.1 Introduction**

The virus status of rhubarb in Australia has not previously been investigated. Previously reported viruses infecting rhubarb overseas include *Cherry leafroll virus* (CLRV), *Turnip mosaic virus* (TuMV), *Cucumber mosaic virus* (CMV), *Arabidopsis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRV) and a Rhubarb temperate virus. Samples were collected in all states to identify and survey for viruses infecting rhubarb crops in Australia.

#### **5.2.1.2 Materials, methods and results**

##### Plant samples

Individual diagnostic samples of rhubarb with virus-like symptoms were collected as described below. The viruses present were identified, and specific assays developed or adapted to enable subsequent surveying of crops nationally.

##### Method for viral miniprep and electron microscopy (EM)

Viral minipreps were performed as per (Geering *et al.* 2000). Essentially 2 g leaf tissue was ground in miniprep buffer, then clarified by filtration and low speed centrifugation. Virus particles were pelleted through a sucrose pad, resuspended in 100 µL of 10 mM potassium phosphate buffer pH 7.0 and deproteinated through a chloroform cleanup step. Extracts were placed on nitrocellulose-coated copper grids, negatively contrasted with 1% ammonium molybdate pH 6.8 and viewed in a Hitachi H7000 transmission electron microscope.

##### Method for randomly amplified cDNA, and cloning and sequencing of PCR amplicons

Randomly amplified cDNA was synthesised and the amplicons cloned and sequenced essentially as described by (Gambley and Thomas 2001). Sequencing was conducted by AGRF (Brisbane, Australia) and Macrogen (Seoul, South Korea).

##### Cherry leafroll virus (CLRV)

A rhubarb sample showing general chlorosis of the leaves was obtained from Summertown, South Australia on 19 April 2007. Viral minipreps were prepared and examined by transmission electron microscopy (EM). Angular, empty and full isometric particles 25-30 nm in diameter, and suggestive of nepoviruses, were observed. Randomly amplified cDNA was prepared from the miniprep, and the amplicons cloned and sequenced. BLAST analysis (Altschul *et al.* 1997) revealed matches with nepoviruses and, where they were available, most closely with CLRV (GenBank S84126) with 91.5% identity over 433 nt, covering part of the 3' untranslated region probably of RNA 1). The virus culture has been stored in the DEEDI Plant Virus Collection as isolate 2005 and the 433 bp sequence, lodged on GenBank under Accession GQ370453.

#### Turnip mosaic virus (TuMV)

Viral minipreps were prepared from three individual samples of rhubarb with a variety of virus-like symptoms, from a crop at Port Macquarie, NSW, on 23 June 2005. Symptoms included poor vigour, mild mottle, small chlorotic spots and reddish necrotic spots. Several types of filamentous and isometric virus-like particles were observed by EM, among them some resembling potyviruses. Randomly amplified cDNA was produced from a viral miniprep, cloned and sequenced. BLAST analysis of the nucleotide sequence of one clone revealed 97.9% identity to TuMV isolate HZ6 (GenBank AB252119) across 915 nt of the NIa-Pro and NIb protein coding regions. The presence of TuMV in the three original samples was confirmed by ELISA (BioRad TuMV double conjugate ELISA kit). The Port Macquarie isolates were deposited in the DEEDI Plant Virus Collection as accessions 1885-1887 and the partial TuMV sequence of isolate 1886 lodged with GenBank (Accession GQ370454).

#### Cucumber mosaic virus (CMV)

One of the viruses with isometric particles 25-30 nm in diameter, from DEEDI Plant Virus accession 1886, was also identified by sequence analysis of the randomly amplified cDNA from the viral miniprep. BLAST analysis of the nucleotide sequence of 13 clones revealed a contig of 11 clones with 99.1% identity to CMV isolate TC (GenBank EF640931) in a 956 nt region of the 2a gene located on RNA 2, one clone with 98.7% identity to a 612 nt region of the CMV 1a gene on RNA 1 (isolate NAK, GenBank AJ304404), and one clone with 98.4% identity to a 876 nt region including the coat protein gene on RNA 3 (GenBank AB006813). The presence of CMV in the original sample was confirmed by ELISA (BioRad CMV ELISA kit). These sequences were lodged with GenBank (Accessions GQ374453-GQ374455).

#### Tomato spotted wilt virus (TSWV)

Samples of rhubarb (cv. unknown) from a crop at Pozieres, near Stanthorpe, Queensland, were obtained on 15 March 2007, and displayed symptoms of chlorotic spots, red ringspots, or a chlorotic mosaic along central leaf veins. Tospovirus-like particles, but no other virus-like particles, were observed by EM of leaf sap, and infection by TSWV was demonstrated by ELISA (BioRad TSWV ELISA kit). Samples positive by ELISA were confirmed using a TSWV-specific PCR, performed essentially as described by Thomas *et al.* (Thomas *et al.* 2004) and were deposited in the DEEDI Plant Virus Collection as isolates 2029-2031 and 2133-2135.

#### Rhubarb decline associated virus (RDaV)

A viral miniprep was prepared for a rhubarb sample showing chlorotic and necrotic spotting that was collected on 24 September 2002 from Mt Tamborine, Queensland. Closterovirus-like particles were observed by EM (Figure 2). Randomly amplified cDNA was produced from the viral miniprep, cloned and sequenced. BLAST analysis of the nucleotide sequence of one clone revealed 73% similarity to Grapevine rootstock stem lesion associated virus (GenBank AF314061), a closterovirus. The rhubarb virus appeared to be a previously undescribed virus.

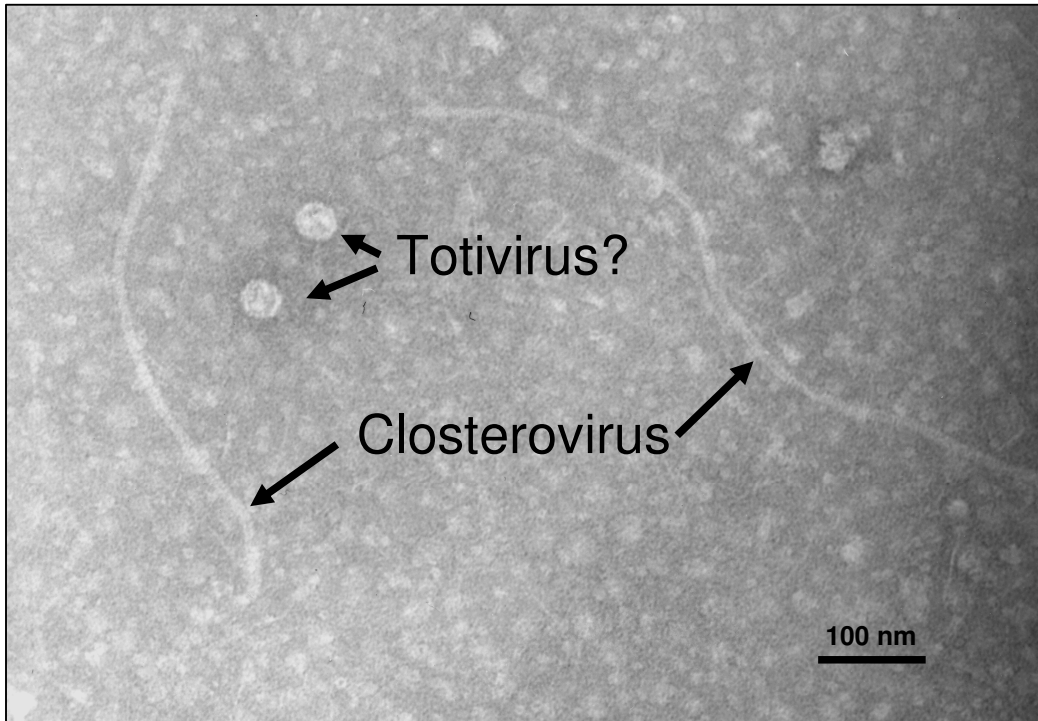


Figure 2. Closterovirus and totivirus-like particles from a viral miniprep, negatively stained in 1% ammonium molybdate, and viewed in an Hitachi H7000 electron microscope.

#### Unidentified isometric particles

Isometric virions ca 30 nm and ca 35 nm in diameter have also been observed in a number of rhubarb samples, including plants that tested negative for the other isometric viruses identified above. Rhubarb temperate virus is a tentative member of the genus *Alphacryptovirus* with 30 nm isometric particles. Attempts to obtain antiserum to this virus from Japan were unsuccessful, and as no cultures and no sequence information are available, it cannot be ascertained whether the unidentified 30 nm isometric particles detected in this project are the same/similar virus.

However, another viral sequence was obtained from the randomly amplified cDNA from the viral miniprep of DEEDI Plant Virus accession 1886 and BLAST analysis (tblastx) of the 993 nt sequence revealed sections of 34-55% identity to the coat protein region of the totivirus Black raspberry virus F (GenBank EU082131). This appeared to be a previously undescribed virus. Totiviruses have ca 40 nm diameter virions, and the larger isometric particles detected in this work (ca 35 nm, Figure 2) may be those of a totivirus.

#### **5.2.1.3 Discussion**

Rhubarb crops were found to be infected with up to seven viruses. Three of these viruses (CLRv, CMV, TuMV) have previously been reported to infect rhubarb, although these are new reports for Australia. This appears to be the first record worldwide of TSWV infecting rhubarb. Additionally, at least three novel viruses have been identified; Rhubarb decline virus (RDaV), a putative totivirus (cryptic virus) and another unidentified isometric virus(es).

Characterisation of RCV was undertaken as part of this project and is described below. Further sequencing was undertaken to investigate the totivirus sequence described above.

## **5.2.2 National survey for rhubarb viruses**

### ***5.2.2.1 Introduction***

A national survey of rhubarb crops was undertaken to determine the distribution of rhubarb decline disease, and the range of viruses present in Australian rhubarb. Viruses to be indexed for were selected on the basis of those previously recorded from rhubarb, or those detected in initial screening (Section 5.2.1 above).

### ***5.2.2.2 Materials and methods***

#### **Sample collection and virus indexing for WA crops**

Between July 2005 and June 2006, 613 samples were collected from rhubarb crops at four sites (Gin Gin, Gwelup, Trigg and Hamilton Hill) in the Perth metropolitan area, Western Australia.

Samples from Western Australia were collected as follows. Each sample collected consisted of the youngest shoot from an individual plant. Random shoot samples were collected every 5 paces within an individual rhubarb planting; 100 were taken per planting unless numbers of plants were too few for this. Plants with potential viral symptoms were sampled separately or dug up and then re-planted in the glasshouse to monitor symptoms over approximately 5 months.

#### ***Gin Gin property***

Rhubarb plantings ranged from 1 to 5 years in age. Five cultivars were being grown. Cvs “Balcatta Red”, “Sydney Crimson” and “South Australia Red” constituted a historical collection of old cultivars which the family that own the property had grown for 25, 20 and 15 years respectively. Only small amounts were present and rhubarb is no longer cut from these old cultivars. The growers cut only two newer rhubarb cultivars (A and B) which are from seedlings that they selected themselves. Re-planting with crowns of these two cultivars is normally done at 3 year intervals ‘A’ was the predominant cultivar. There were several 1-5 years-old plantings each of A and B. Occasionally they were present together in mixtures within the same planting. In general, the rhubarb plants of cvs ‘A’ and ‘B’ looked very vigorous and healthy, and were well cared for. The small historical cultivar plantings were rather less well tended.

The samples collected at Gin Gin were as follows; “South Australian Red” (50 random samples), “Balcatta Red” (12 random samples), “Sydney Crimson” (25 random samples). “Cultivar A”, 2 and 3 years old (100 random samples of each), 5 years old (10 random samples), “Cultivar B”, 2 years old (100 random samples), 5 years old (70 random samples).

Potentially symptomatic plants were found only in “Cultivar B” and were mostly paler, shorter, mottled and /or had rugose leaves. However, there were very few of these. Those collected were; 2 year old, 11 samples; 5 year old, 5 samples. Of these,

four 5 year old plants, and one 2 year old plant were dug up and re-planted in the glasshouse.

All 488 rhubarb samples from Gin Gin were tested by ELISA using the full range of nine different anti-viral antibodies (see below). Samples from the five potentially symptomatic plants of rhubarb cv. 'B' that were potted and grown on in the glasshouse, were tested by PCR using primers specific for RDaV. Leaf material from five potentially symptomatic plants of rhubarb cv. 'B' was sent to Dr J. Thomas of DEEDI for EM examination. There were four samples from 2 year old plants with symptoms of mottle (sample 1), yellow blotching on older leaves (sample 2), pallor and mottle (sample 3), and ruffled leaf margins (sample 4) and an additional sample from a 5 year old plant of cv. 'B' planted in the glasshouse.

The following numbers of symptomless weeds were also collected; sowthistle (75), wild radish (25).

#### *Gwelup property*

This property only maintains a small plot of about 150 rhubarb plants, cultivar unknown. There was still a problem of wilting and death of plants over summer so propagation from crowns was frequent and most plants were apparently <2 years old. About 25% of these plants were potentially symptomatic and showed leaf rugosity, leaf yellowing and/or plant dwarfing. No aphids or thrips were noticed on the rhubarb plants. In general, weeds were well controlled. Some flower and vegetable plants being grown on the property had virus-like symptoms.

The rhubarb samples collected included 100 random samples, and, in addition, one sample each from 11 plants showing one or other of the different potential symptom types mentioned in the previous paragraph. In addition, five potentially symptomatic rhubarb plants were dug up and planted in the glasshouse. One sample each was collected from seven plants with virus-like symptoms of snapdragon, ranunculus, lisianthus, aster, statice, onion and pea.

All 116 rhubarb (16 symptomatic) and seven flower or vegetable samples were tested by ELISA using the full range of nine different virus antibodies (see below).

Samples from the five potted rhubarb plants were tested by PCR using primers specific for the rhubarb closterovirus. Leaf material from seven potentially symptomatic rhubarb plants from Gwelup was sent to Dr J. Thomas of DEEDI for EM examination. Six were directly from the field and one was from a plant transplanted into the glasshouse which subsequently showed typical viral symptoms consisting of a few chlorotic blotches or rings associated with faint chlorotic spotting and vein clearing in young leaves (see following section).

#### *Additional properties*

Seven samples were collected from a property at Trigg growing a few plants of cv. "Waldin Crimson", and two from another property at Hamilton Hill growing an unknown cultivar. No symptoms were noted at either site. No virus was detected in any of these nine samples each from a different plant by ELISA using the nine different virus antibodies.

DAS-ELISAs were conducted for the following viruses: CLRV, TuMV, CMV, TSWV, *Arabid mosaic virus* (ArMV), *Impatiens necrotic spot virus* (INSV), *Strawberry latent ringspot virus* (SLRV), and a general tospovirus assay (serogroups I, II, and III). Indirect ELISAs were conducted for a general potyvirus assay.

Positive control virus-infected leaf tissue available at South Perth (CMV, TuMV and TSWV; plus *Bean yellow mosaic virus* (BYMV) for general potyvirus tests) or purchased from a commercial supplier overseas (ArMV) was used in ELISA with all antibodies, except for INSV, CLRV and SLRV. With the INSV, CLRV and SLRV antibodies, the concentrations of conjugate and globulin used in ELISA were those recommended by the supplier.

A PCR assay (Section 5.2.2.2) was used to screen samples for the presence of RDaV in 12 samples with potential viral symptoms.

For 10 samples with symptoms suggestive of viral infection, viral minipreps were prepared and visualised by electron microscopy (Section 5.2.1.2).

#### Sample collection and virus indexing (all other States)

Between July 2005 and September 2007, 250 samples were collected from rhubarb crops from the following locations. A single young, fully-expanded leaf was collected per plant.

- 5 sites at Mt Tamborine and 2 sites at Pozieres, Queensland
- 4 sites in the Sydney Basin and 1 site at Pt Macquarie, New South Wales
- 3 sites on Mornington Peninsula, Victoria
- 1 site at Moriarty, Tasmania
- 1 site in Summertown, South Australia

Symptomatic leaves were generally collected when present in the crops, and where not present, random samples were collected.

Samples were ground 1:10 in CMV extraction buffer (0.05M citrate buffer, pH 8.0, 0.5mM EDTA, 1% skim milk, 0.5% monothioglycerol, 0.05% Tween20), briefly centrifuged and the supernatant applied as antigen to the ELISA plate or diluted 1:10 in sterile distilled water for use as template in RT-PCR assays.

Previously determined positive controls from the DEEDI Plant Virus Collection were run along side test samples in each assay. Seedling rhubarb was used as the healthy control.

Testing for TuMV, CMV and TSWV was conducted using a TuMV DAS-ELISA kit (DSMZ, Germany), a CMV DAS-ELISA kit (Sediag, France) and a TSWV DAS-ELISA kit (Sediag, France), respectively.

Testing for RDaV was conducted using a one-step RT-PCR assay. Template was prepared as a 1:10 dilution in water of leaf tissue ground 1:10 in CMV extraction buffer. The 25  $\mu$ L RT-PCR mix contained 2  $\mu$ L template, 1  $\times$  PCR reaction buffer (Invitrogen), 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ M of each dNTP, 0.1% TritonX100, 10 mM DTT, 0.8  $\mu$ M each of primers Rhub7.F1 (5'-GAGGATGCTCGGGAAGTG-3') and Rhub7.R1 (5'-CGAGTAGATCGGGCAACG-3'), 10 U RNaseOUT (Invitrogen), 50 U Superscript III reverse transcriptase (Invitrogen) and 1.25 U *Taq* DNA

polymerase (Invitrogen). Reactions were heated at 80°C for 10 min, then chilled on ice before the remainder of the reaction components were added. The reactions were then run on a Bio-Rad thermocycler (iCycler or C-1000) according to the following program: 50°C for 45 min, 94°C for 3 min, 35 cycles of 94°C for 20 sec, 59°C for 30 sec and 72°C for 30 sec, followed by a final extension of 72°C for 3 min. The 259 bp product was analysed by electrophoresis in a 1.5-2% agarose gel in 0.5 × Tris-borate-EDTA buffer, and visualised by staining with ethidium bromide (Sambrook *et al.* 1989). Stained gels were photographed using a GelDoc (Bio-Rad).

Testing for CLRV was conducted using a one-step RT-PCR assay similar to that for RDaV. Primers were CLRV1 (5'-TTGGCGACCGTGTAACGGCA-3') and CLRV2 (5'-GTCGAAAGATTACGTAAAAGG-3') (Grieco *et al.* 2000) and the reaction program was as for RDaV but with an annealing temperature of 55°C. The 431 bp product was analysed by electrophoresis and visualised as for RDaV.

Viral minipreps (Section 5.2.1.2) were performed for many of the survey samples and these were visualised under the EM.

### 5.2.2.3 Results

A summary of viruses detected, by district, is shown in Table 1. No virus infections were detected in Western Australian or Tasmanian rhubarb, but at least one virus was found in rhubarb from all other districts and States. Detailed survey results are presented below and in Tables 3-5.

Table 1. Viruses detected in rhubarb-growing districts, given as the number of positive detections per number of plants tested.

| State           | District             | RDaV <sup>A</sup> | CMV <sup>B</sup> | TuMV <sup>B</sup> | TSWV <sup>B</sup> | CLRV <sup>A</sup>    | Small isometric <sup>C</sup> | Large isometric <sup>C</sup> |
|-----------------|----------------------|-------------------|------------------|-------------------|-------------------|----------------------|------------------------------|------------------------------|
| QLD             | Mt Tamborine         | 11 / 27           | 10 / 27          | 0 / 27            | 0 / 14            | 0 / 27               | 9 / 27                       | 4 / 27                       |
|                 | Stanthorpe           | 0 / 7             | 0 / 18           | 0 / 18            | 7 / 18            | 0 / 6                | 0 / 4                        | 0 / 4                        |
| NSW             | Pt Macquarie         | 3 / 4             | 1 / 4            | 3 / 4             | 0 / 4             | 0 / 4                | 1 / 4                        | 3 / 4                        |
|                 | Sydney Basin         | 22 / 52           | 12 / 52          | 20 / 52           | 9 / 52            | 0 / 52               | 6 / 9                        | 4 / 9                        |
| VIC             | Mornington Peninsula | 12 / 78           | 2 / 78           | 0 / 78            | 18 / 78           | 20 / 57              | 17 / 30                      | 26 / 30                      |
| TAS             | Moriarty             | 0 / 13            | 0 / 13           | 0 / 13            | 0 / 13            | nt <sup>D</sup>      | nt                           | nt                           |
| SA              | Summertown           | 0 / 2             | 0 / 2            | 0 / 2             | 0 / 2             | 1 / 2                | 1 / 2                        | 0 / 2                        |
| WA <sup>E</sup> | Perth metro          | 0 / 12            | 0 / 613          | 0 / 613           | 0 / 613           | 0 / 613 <sup>B</sup> | 0 / 10                       | 0 / 10                       |

<sup>A</sup> tested by RT-PCR

<sup>B</sup> tested by ELISA

<sup>C</sup> tested by viral miniprep checked under the electron microscope

<sup>D</sup> nt = not tested

<sup>E</sup> Samples from WA also tested negative by ELISA for ArMV, CLRV, INSV, SLRV, potyviruses in general and tospovirus serogroups groups I, II and III.

Shaded areas indicate a positive detection of a particular virus

## WA crops

### *Gin Gin*

No virus whatsoever was detected in any of 488 rhubarb or 100 weed samples tested by ELISA using the full range of nine different anti-viral antibodies. In the five potentially symptomatic plants of rhubarb cv. 'B' potted and grown on the glasshouse, the potentially abnormal appearance disappeared, no viral symptoms remained after 5 months, no virus was detected by PCR using primers specific for RDaV, and no virus particles were observed by EM.

### *Gwelup*

No virus was detected in any of the 100 random or 16 symptomatic rhubarb samples tested by ELISA using the full range of nine different virus antibodies.

In the five potentially symptomatic plants of rhubarb potted and grown on the glasshouse, their abnormal appearance largely disappeared, but some virus-like symptoms remained >5 months later in four of them. These symptoms consisted of a few chlorotic or necrotic blotches, rings or ringspots occasionally spreading along veins in old leaves of all four symptomatic plants. This was associated with faint chlorotic spotting and vein clearing in young leaves of two of the plants. No virus was detected in these five rhubarb plants by ELISA using the eight different virus antibodies, by PCR using primers specific for RDaV, and no virus particles observed by EM.

In the potentially symptomatic alternative host samples, CMV was detected in ranunculus, lisianthus, and pea, and TSWV in aster. In addition, onion and ranunculus reacted positively with the general potyvirus antibody, and aster with the general tospovirus antibody. None of the range of viruses tested for by ELISA using the eight different virus antibodies were detected in snapdragon or statice.

### *Trigg and Hamilton Hill*

No symptoms were evident at either site. No virus was detected in any of these nine samples by ELISA using the nine different virus antibodies.



### Crops in eastern States

#### *Mornington Peninsula, Victoria*

CLRV, RDaV and TSWV were the most commonly detected viruses (Table 2), and were mostly associated with symptomatic plants.

Table 2. Survey results for the Mornington Peninsula.

| Grower | Cultivar | Symptoms |     | Viruses detected <sup>B</sup>      |
|--------|----------|----------|-----|------------------------------------|
|        |          | No       | Yes |                                    |
| A      | cv A1    | 10       |     | 1xCMV                              |
|        |          |          | 2   | NVD                                |
| B      | cv B1    | 7        |     | L                                  |
|        |          |          | 9   | 4 x TSWV, 1 x RDaV, 5 x CLRV L, S  |
|        | cv B2    | 10       |     | NVD <sup>A,D</sup>                 |
|        |          |          | 6   | 4x RDaV, 3 x TSWV, 6 x CLRV, L, S, |
| C      | cv C1    | 5        |     | NVD <sup>A,D</sup>                 |
|        | cv C2    |          | 2   | 1 x CLRV <sup>A</sup>              |
|        | cv C3    |          | 2   | NVD <sup>A</sup>                   |
|        | cv C4    | 4        |     | NVD <sup>A</sup>                   |
|        | cv C5    |          | 5   | 2 x RDaV, 2 x TSWV <sup>A</sup>    |
|        | cv C6    |          | 5   | 4 x RDaV, 3 x TSWV, 5 x CLRV, L, S |
|        | cv C7    |          | 5   | 2 x RDaV, 3 x TSWV, 5 x CLRV, L, S |
|        | cv C8    | 4        |     | 1 x TSWV <sup>A,D</sup>            |
| TOTAL  |          | 40       | 36  |                                    |

<sup>A</sup> = no EM tests done

<sup>B</sup> = sometimes as mixed infections

<sup>C</sup> S = small isometric virions (ca 30 nm), L = large isometric virions (ca 35 nm)

<sup>D</sup> = not tested for CLRV

#### *Sydney Basin and Port Macquarie, NSW*

TuMV, RDaV and TSWV were the most commonly detected viruses (Table 3), and were mostly associated with symptomatic plants.

Table 3. Survey results for the Sydney basin and Port Macquarie

| Grower         | Cultivar           | Symptoms |     | Viruses detected <sup>B</sup>                         |
|----------------|--------------------|----------|-----|-------------------------------------------------------|
|                |                    | No       | Yes |                                                       |
| D <sup>E</sup> | cv D1 (planting 1) | 1        |     | NVD                                                   |
|                |                    |          | 10  | 8 x TSWV, 5 x RDaV, 4 x TuMV, 2 x CMV, L              |
|                | cv D1 (planting 2) | 3        |     | NVD, <sup>A</sup>                                     |
|                |                    |          | 6   | 1 x CMV, 1 x TuMV, 1 x RDaV, L, S                     |
| E <sup>E</sup> | cv E1              | 9        |     | 1 x CMV, 1 x TuMV, S                                  |
|                |                    |          | 7   | 4 x TuMV, 3 x CMV, 4 x RDaV, L, S                     |
| F <sup>E</sup> | cv F1              |          | 15  | 11 x TuMV, 6 x CMV, 1 x TSWV, 13 x RDaV, <sup>D</sup> |
| G <sup>F</sup> | cv G1              |          | 4   | 4 x RDaV, 3 x TuMV, 1 x CMV, L, S                     |
| TOTAL          |                    | 13       | 42  |                                                       |

<sup>A</sup> = no EM tests done

<sup>B</sup> = sometimes as mixed infections

<sup>C</sup> S = small isometric virions (ca 30 nm), L = large isometric virions (ca 35 nm)

<sup>D</sup> = not tested for CLRV

<sup>E</sup> Sydney Basin

<sup>F</sup> Port Macquarie

*Mt Tamborine and Stanthorpe, Queensland*

RDaV, CMV and TSWV were the most commonly detected viruses (Table 4), and were mostly associated with symptomatic plants.

Table 4. Survey results for Mt Tamborine and Stanthorpe.

| Grower         | Cultivar                | Symptoms |     | Viruses detected <sup>B</sup> |
|----------------|-------------------------|----------|-----|-------------------------------|
|                |                         | No       | Yes |                               |
| H <sup>E</sup> | cv H1                   | 18       |     | NVD <sup>A</sup>              |
| I <sup>E</sup> | cv I1                   | 19       |     | NVD <sup>A</sup>              |
|                |                         |          | 7   | 7 x TSWV                      |
| J <sup>F</sup> | cv Big Red (planting 1) | 1        |     | NVD                           |
|                |                         |          | 2   | 2 x CMV, 2 x RDaV, L, S       |
|                | cv Big Red (planting 2) | 4        |     | 3 x CMV, 2 x RDaV, S          |
|                | cv Success              | 6        |     | NVD                           |
| K <sup>F</sup> | cv Big Red              | 2        |     | 1 x CMV, 1 x RDaV, S          |
|                |                         |          | 1   | 1 x RDaV, 1 x CMV, L, S       |
|                | cv Emma                 | 1        |     | NVD                           |
|                | cv Prostrate            |          |     | NVD                           |
| L <sup>F</sup> | cv Big Red              | 2        |     | NVD                           |
|                |                         |          | 3   | 1 x CMV, 2 x RDaV, S          |
| M <sup>F</sup> | cv Big Red              |          | 3   | 3 x CMV, 3 x RDaV, L, S       |
| TOTAL          |                         | 53       | 16  |                               |

<sup>A</sup> = no EM, RDaV or CLRV tests done

<sup>B</sup> = sometimes as mixed infections

<sup>C</sup> S = small isometric virions (ca 30 nm), L = large isometric virions (ca 35 nm)

<sup>D</sup> = not tested for CLRV

<sup>E</sup> Stanthorpe

<sup>F</sup> Mt Tamborine

*South Australian and Tasmanian crops*

No viruses were detected in 44 plants from Tasmania, though these plants were not indexed for CLRV and were not examined by EM. In one of two plants from Summertown, South Australia, CLRV, large and small isometric particles were detected, though the plants showed no distinctive symptoms.

**5.2.2.4 Discussion**

Viruses were widespread in many cultivars and selections of rhubarb in the eastern mainland states. Virus symptoms were very variable and mixed infections common, making it very difficult to equate particular symptoms to individual viruses. Overall, symptomatic plants were generally shown to be virus infected (79%) and those that were negative often had mild mottle symptoms or trace of necrotic spotting, both potentially due other factors such as nutritional imbalance. Symptomless plants from the virus endemic areas were generally virus-free (89%). In areas with higher levels of symptomatic plants, the predominant viruses varied. In Port Macquarie and the Sydney basin, RDaV and TuMV were most common (CLRv not detected), in Mt Tamborine, RDaV and CMV (TuMV, TSWV and CLRv not detected) and in the Mornington Peninsula, RDaV, TSWV and CLRv. RDaV appears to be most consistently associated with severe disease symptoms.

Some cultivars were virus-free on farms where other cultivars had many infected, symptomatic plants, indicating that selection of healthy-looking crowns or use of virus-free seedlings may make a practical contribution to crop health.

The role of the large and small isometric virions is unknown and they do not appear to be closely associated with disease. The larger particles are morphologically distinct from all others known to infect rhubarb. The smaller particles are similar in size to those of CLRV and other nepoviruses, and CMV, but have been observed in plants that were indexed free of these other viruses.

Plantings in Western Australia have predominantly originated from seedlings, presumed to be virus-free, and isolation from other crops has probably allowed this and the Tasmanian crop to remain virus-free.

### **5.2.3 Characterising the unidentified isometric particles**

#### ***5.2.3.1 Introduction***

Putative totivirus sequence was obtained from DEEDI Plant Virus Collection isolate 1886 (Section 5.2.1.2) and large and small unidentified isometric particles were observed in the viral minipreps of a number of survey samples (Section 5.2.2.3). Further viral sequencing was undertaken and efforts made to link sequence information to particle size. Totivirus particles are ca 40 nm in diameter, while alphacryptoviruses, of which Rhubarb temperate virus is a possible member, have 30 nm virions.

#### ***5.2.3.2 Materials and methods***

##### Virus samples

DEEDI Plant Virus Collection isolate 2756 was collected from the Mornington Peninsula, Victoria and displayed symptoms of a very diffuse mottle. The isolate tested positive for CLRV and negative for RDaV by RT-PCR and CMV, TuMV and TSWV by ELISA. Electron microscopy of a viral miniprep (Section 5.2.1.2) of this isolate revealed the presence of both large and small isometric particles.

Isolate 2757 was collected from Mt Tamborine, Queensland and displayed necrotic spots and mottle. A viral miniprep was prepared and large and small isometric particles as well as closterovirus particles were observed by electron microscopy.

Further samples were used which were observed to contain only the small or only the large category of isometric particles. Isolate 2629 was collected from Mt Tamborine, Queensland and contained the large isometric particles. Two samples from the Sydney Basin, NSW contained the large isometric particles (isolates 2641 and 2801). One isolate (2630) from Mt Tamborine, Queensland contained the small isometric particles, and two further isolates (2639 and 2802) from the Sydney Basin, NSW contained the small isometric particles. Three rust-infected rhubarb leaves from Mt Tamborine were also tested by RT-PCR. Isolate 1886 and healthy seedling rhubarb were used as positive and negative controls respectively.

##### RT-PCR linking totivirus sequence with isometric particle size

Primers were designed against the original putative totivirus coat protein sequence obtained from isolate 1886 using Primer3 (<http://primer3.sourceforge.net/webif.php>).

cDNA was synthesised from viral minipreps with random hexamers and Superscript III (Invitrogen) as per the manufacturer's instructions. PCR was undertaken with CP-F1 (5'-TGGCATGATAACCACGTCTC-3'), CP-R1 (5'-CTGCCAAGCATCATC AACAC-3') and *Taq* DNA polymerase (Invitrogen) as per the manufacturer's instructions and amplified using the following program: 94°C for 1 min, 35 cycles of 94°C for 20 sec, 58°C for 30 sec and 72°C for 30 sec, followed by a final extension of 72°C for 3 min. The 323 bp product was analysed by electrophoresis in a 1.5-2% agarose gel in 0.5 × Tris-borate-EDTA buffer, and visualised by staining with ethidium bromide (Sambrook *et al.* 1989). Stained gels were photographed using a GelDoc (Bio-Rad).

### Sequencing

To enrich for potential totivirus sequence from isolate 2756, dsRNA extraction was performed on the viral miniprep using a buffered phenol treatment followed by ethanol precipitation. The dsRNA was resuspended in RNase-free water for cDNA synthesis described below.

A large scale viral miniprep (20g tissue) was performed for isolate 2757. To remove CMV particles which were known isometric particles from samples from Mt Tamborine, Queensland, the preparation was incubated with CMV antiserum (CMV-T strain, 5/8/80) for 30 min and then loaded onto a 10-40% sucrose gradient in 10 mM potassium phosphate buffer and centrifuged at 35 000 rpm for 1 h at 5°C using a SW55 Ti rotor (Beckman Instruments). The supernatant was collected, leaving behind the lowest 3 mm of solution which was also likely to contain immuno-detected CMV virions. Remaining virus particles, including totivirus particles, in the supernatant were pelleted by centrifugation at 50 000 rpm for 45 min at 5°C using a Type 75 Ti rotor (Beckman Instruments). The pellet was resuspended in 50 µL of 10 mM potassium phosphate buffer and used as template for cDNA synthesis.

Randomly amplified cDNA was produced (Section 5.2.1.2) and amplicons were purified using a QIAex II Gel Extraction Kit (Qiagen) and cloned and sequenced. Sequences were analysed by the tblastx algorithm (NCBI) and contiguous sequences were joined using the ContigExpress package within VectorNTI Advance v10 and v11 (Invitrogen). The BlastX package within VectorNTI Advance v11 (Invitrogen) was used to perform the nucleotide identity analysis.

### **5.2.3.3 Results**

#### Linking totivirus sequence with isometric particle size

Samples in which large or small isometric particles were observed by electron microscopy were assayed by RT-PCR with coat protein primers designed against the original putative totivirus sequence from isolate 1886 (Figure 3). No amplification was observed for most samples, however weak amplification was observed from one sample with large isometric particles from the Sydney Basin, NSW.

Rust-infected rhubarb leaves from Mt Tamborine, Queensland were tested with the same RT-PCR assay, however no amplification was observed from these samples (data not shown).

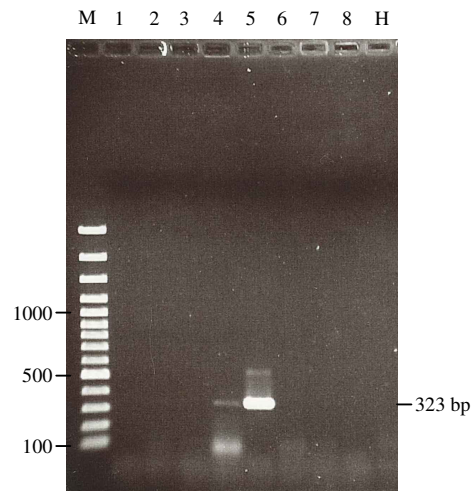


Figure 3. Totivirus PCR against samples containing large and small isometric particles, as observed by electron microscopy. Lanes 1-5, samples with large particles (1, isolate 2757; 2, isolate 2629; 3, isolate 2641; isolate 2801; 5, isolate 1886); lanes 5-8, samples with small isometric particles (5, isolate 1886; 6, isolate 2630; 7, isolate 2639; 8, isolate 2802); H, healthy control; M, 100 bp DNA ladder (Fermentas).

### Sequencing

Twenty-one fragments were sequenced from the dsRNA extraction of isolate 2756. Except for two fragments (A and B), these sequences were assembled into five contiguous sequences (Contigs; Figure 4). The original sequence obtained from isolate 1886 was incorporated into the 5' end of Contig 1, with 100% identity across the overlapping 103 nt. Database analysis by tblastx of all seven sequences revealed 29-65% amino acid identity to the totivirus Black raspberry virus F (BRV-F; GenBank EU082131).

Only one putative totivirus sequence, Fragment C, was obtained from isolate 2757. Blast analysis revealed a best match of 56% amino acid identity to the RdRp region of a putative mycovirus *Magnaporthe oryzae* virus 1 (GenBank AB176964).

However, mapping of these eight sequences to the BRV-F genome as an assembly template revealed that at least three species of RNA are present in isolate 2756 (Figure 4). Nucleotide identity between Contig 1 and Fragment A was calculated by BlastX (Invitrogen) as 57%. Nucleotide identities between Contigs 1-4 and Fragments B and C ranged between 33 and 58% (Table 5).

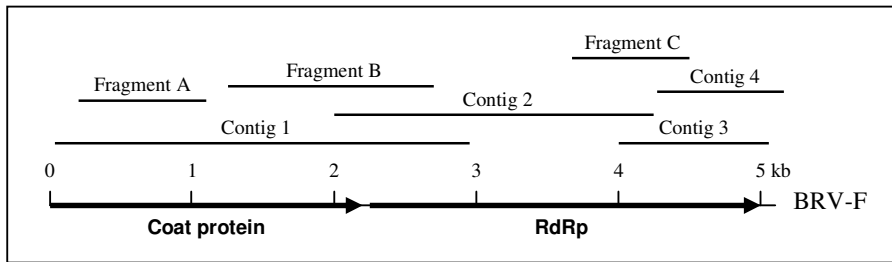


Figure 4. Totivirus sequences from random PCR of rhubarb sample, mostly from isolate 2756. The 5' end of Contig 1 originates from isolate 1886. Fragment C originates from isolate 2757. BRV-F, Black raspberry virus F (GenBank EU082131).

Table 5. Nucleotide identities between overlapping regions of totivirus sequences (see Figure 3), mostly from isolate 2756, from the Mornington Peninsula, Victoria.

|                         | Contig 1 <sup>A</sup> | Contig 2 | Contig 3 | Contig 4 |
|-------------------------|-----------------------|----------|----------|----------|
| Contig 1 <sup>A</sup>   |                       |          |          |          |
| Contig 2                | 33                    |          |          |          |
| Contig 3                |                       | 48       |          |          |
| Contig 4                |                       |          | 58       |          |
| Fragment B              | 46                    | 50       |          |          |
| Fragment C <sup>B</sup> |                       | 38       | 49       | 38       |

<sup>A</sup> Includes sequence from isolate 1886 at the 5' end

<sup>B</sup> DEEDI Plant Virus Collection isolate 2757, from Mt Tamborine, Queensland

#### 5.2.3.4 Discussion

Both large (ca 35 nm) and small (28-30 nm) isometric particles were observed in many rhubarb survey samples from NSW, Vic and QLD. Alphacryptoviruses, with Rhubarb temperate virus as a possible member, have ca 30 nm diameter particles, while betacryptoviruses and totiviruses have particles ca 40 nm in diameter. From isolates 1886 and 2756, multiple species of totivirus/cryptic virus RNA were revealed by alignment of randomly generated sequences. Primers designed against Contig 1 amplified sequence from a sample from the Sydney Basin, NSW in which large isometric particles were observed.

Although most totiviruses currently identified are of fungal origin, a number of plant derived totiviruses have recently been described (Roossinck, 2010). At this point, the possibility that the totivirus-like sequences are derived from a fungal endophyte cannot be ruled out. However, visible fungal infections were not present on many samples containing these sequences, and the sequences were not amplified from pustules of the rust fungus *Puccinia phragmitis* often found on virus-infected rhubarb.

By contrast, the sequence obtained from 2757 displayed high similarity to a putative mycovirus, Magnaporthe oryzae virus 1, and hence may be of fungal rather than plant origin.

Further work is required to clarify the number of distinct viruses with isometric particles that are present, and to link specific viral sequences to particle types. However, it appears that the presence of these viruses is not closely associated with the rhubarb decline disease.

## **5.2.4 Characterisation of Rhubarb decline virus**

### ***5.2.4.1 Introduction***

As demonstrated above (Section 5.2.2), RDaV appeared to be a previously unrecognised virus, and was also closely associated with rhubarb decline disease. For this reason, the virus was characterised to establish its identity, to determine some of molecular and biological properties including possible insect vectors, and to develop diagnostic assays.

### ***5.2.4.2 Materials and methods***

#### **Virus samples**

Rhubarb sample DEEDI Plant Virus Collection isolate 2785 originated from Mt Tamborine, Queensland and displayed small chlorotic spots and reddish necrotic spots on the leaves. Electron microscopy (Section 5.2.1.2) of this sample revealed closterovirus filamentous particles as well as large and small isometric virions, and was positive for RDaV and CMV by RT-PCR (Section 5.2.2.2) and ELISA (Section 5.2.1.2) respectively.

A rhubarb sample from Port Macquarie, NSW (isolate 1886) displaying typical rhubarb decline symptoms, including poor vigour, mild mottle, small chlorotic spots and reddish necrotic spots was selected for study. Electron microscopy of a viral miniprep of this sample revealed several types of filamentous particles suggestive of closteroviruses and potyviruses and also isometric virus-like particles.

#### **Double stranded (ds) RNA analysis**

dsRNA was extracted from 5 g leaf tissue (or green bark for CTV) as per Dale et al. (1986) from isolate 2785, CMV (isolate 207), Potato virus X (PVX; isolate 249) and Citrus tristeza virus (CTV; obtained from D. Hailstones, EMAI), with the latter three extracts being used as known size standards. ds RNA extracts were analysed by electrophoresis in a 0.6% agarose gel in 0.5 × Tris-borate-EDTA buffer, and visualised by staining with ethidium bromide (Sambrook *et al.* 1989). Stained gels were photographed using a GelDoc (Bio-Rad). The sizes of bands from isolate 2785 were determined by comparison with the known size standards.

#### **Sequencing the closterovirus genome**

A viral miniprep was prepared from isolate 1886 (Section 5.2.1.2). Randomly amplified cDNA was produced from the viral miniprep, and amplicons were cloned and sequenced. Sequences were analysed by BLAST (NCBI) and contiguous sequences were joined using the ContigExpress package within VectorNTI Advance v10 and v11 (Invitrogen). Specific primers were designed using the program Primer3 (<http://primer3.sourceforge.net/>) to allow amplification of the intervening regions for contigs to be joined. Fresh cDNA was synthesised from the viral miniprep using random hexamers and Superscript III (Invitrogen) as per the manufacturer's instructions. High fidelity amplifications were performed using Phusion DNA polymerase (Finnzymes) as per the manufacturer's instructions. The products were analysed by electrophoresis, visualised by staining with ethidium bromide and

photographed (Section 5.2.2.2). Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) and cloned and sequenced.

Rapid Amplification of cDNA Ends (RACE) was performed to obtain the 5' and 3' ends of the closterovirus genome. The 5' and 3' RACE System for Rapid Amplification of cDNA Ends version 2.0 Kit (Invitrogen) was used for 5' RACE. Essentially, randomly primed cDNA was synthesised from a viral miniprep of isolate 1886 and purified using a QIAquick PCR Purification Kit (Qiagen). The 3' ends of the cDNA were C-tailed with *TdT* (Terminal deoxynucleotidyl transferase; Invitrogen) and used as template for the nested PCRs, first with the Abridged Anchor Primer (AAP; 5'-GGCCACGCGTCGACTAGTACGGIIGGGIIGGGIIG-3') and specific primer RCV-R10 (5'-TCTGCACTTTCCACATCTCG-3'), and then with the AAP and specific primer RCV-R11 (5'-TCGTCACAGAGTGGGTCGTA-3'). Amplicons were cloned and sequenced (Section 5.2.1.2). For 3' RACE, the Poly(A) Tailing Kit (Ambion) was used to polyadenylate RNA from the viral miniprep and cDNA was synthesised from this RNA using primer Poty1 (5'-GGATCCCCGGGTTTTTTTTT TTTTTTTTV-3') and Superscript III (Invitrogen) as per the manufacturer's instructions. PCR with Taq DNA polymerase (Invitrogen) and primers Poty1 and RCV-R4 (5'-CGCTTTCTACCTGCGTTTTTC-3') was performed as per the manufacturer's instructions. Amplicons were cloned and sequenced.

Genome analysis (ORF determination and translation) was conducted using VectorNTI Advance v11 (Invitrogen). Protein molecular weights were calculated from the translated sequence using Protein Molecular Weight Calculator (<http://www.sciencegateway.org/tools/proteinmw.htm>). Phylogenetic analysis was performed using *MEGA* version 4 (Tamura *et al.*, 2007).

#### Aphid transmission

Approximately 200 aphids (*Aphis gossypii* Glover) were allowed to feed overnight on a field rhubarb leaf infected with RDaV and CMV. Approximately 20 aphids were transferred to each of eight virus-free rhubarb seedlings housed in individual insect cages. After one week, plants were sprayed with the insecticide Piramor and then maintained aphid-free in the glasshouse. Six weeks after the transmission attempt, the youngest fully expanded leaf on each plant was indexed by RT-PCR for RDaV and ELISA for CMV as described above for the virus surveys.

#### Antibody production for ELISA assay development

##### *Expression of major coat protein (pET-SUMO)*

cDNA synthesised with RCV\_CP\_R (5'-TCATTTCCCTATTTTCGTACAAG-3') and Superscript III (Invitrogen) as per the manufacturer's instructions. High fidelity amplifications to obtain the complete major coat protein were performed using primers RCV\_CP\_F (5'-ATGGCTGAATTGACTCCTCA-3') and RCV\_CP\_R with Phusion DNA polymerase (Finnzymes) as per the manufacturer's instructions. The amplicons were A-tailed and ligated into the pET-SUMO vector (Invitrogen) as per the manufacturer's instructions, transformed into Top10 chemicompetent cells (Invitrogen) and single colonies were assessed for vector construction by PCR and sequencing. Plasmid minipreps were prepared with a QIAprep Spin Miniprep Kit (Qiagen) and BL21 electrocompetent cells transformed for protein expression. Single colonies were used to prepare expression cultures in which protein expression was induced with 1 mM IPTG and the products assessed by SDS-PAGE and Coomassie



Brilliant Blue R250 or SimplyBlue (Invitrogen) staining. Protein was purified from the inclusion bodies using Talon resin (Clontech) and 150 mM imidazole and diluted to 1 mg/mL in PBS for immunization.

#### *Commercial expression of the major coat protein*

The cloned major CP gene from above was ligated into a number of expression vectors for small scale trial expressions at the Protein Expression Facility, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland. The pOPIN vector backbone was used, and the CP linked to a 6xHis tag alone or with the fusion proteins maltose binding protein (MBP), glutathione S-transferase (GST) or Thioredoxin (Trx). The best yields of soluble expressed protein were with the MBP and GST constructs. Large scale (1L) expressions were then undertaken with these two constructs. The extracts were concentrated with Amicon centrifugal concentrators with a 10 kDa MW cut-off, and then further purified by affinity chromatography.

#### *Commercial preparation of a synthetic peptide derived from the RDaV major CP sequence*

At Genscript (USA), the Jameson and Wolf (1988) algorithm was used to predict antigenic determinants on the RDaV major CP. A 14 amino acid peptide (shaded grey in Figure 5) was predicted to have maximum antigenicity and was synthesised. An extra C was added to the c-terminus to aid conjugation (CIHALQPTKDNKESV).

Figure 5. Predicted amino acid sequence of the RDaV major CP (sequence used for synthetic peptide shaded).

```
MAELTPQPETKVDSFSVNDPTAVIDAVELEQLKNAFGKFMVTKYNAKAEVDFHLGF  
LLFTYSVLTTSSKKISTVGEISRYTLSGVTYSIKHEDIQAFVSNENATKNFINPLRAF  
AKSFSAFYLRFHKTHKRYYP LNHRALDLGIPA EYGYLAADFLTDVESLSVEEQAVLL  
HGR IHALQPTKDNKESVYNLYEIGK
```

#### *Antibody production and testing*

Rabbit polyclonal antisera were prepared to the expressed coat proteins at the Institute for Medical and Veterinary Services, Adelaide, South Australia. For each of the RDaV CP fusion proteins, a series of three injections was given at three weekly intervals, and the serum obtained two weeks after the final injection. Doses at each injection were; SUMO-RDaV CP fusion protein (500 µg), MBP-RDaV CP fusion protein (120 µg) and GST-RDaV CP fusion protein (110 µg).

Rabbit polyclonal antisera against the synthetic CP peptide were prepared at Genscript. Two separate rabbits were each given a series of 4 injections, after which serum was collected.

Antisera were tested against RDaV from sap extracts or viral minipreps in a plate-trapped antigen ELISA, essentially as described by Geering and Thomas (1999). Sap samples or virus minipreps (above) of healthy and RDaV-infected rhubarb were prepared as 1:10 dilutions in carbonate coating buffer and reaction volumes for all but the substrate step were 50 µL.

#### Alternative host survey

To test for potential alternative hosts of RDaV, 11 weed species growing in and around a RDaV-infected rhubarb crop at Mt Tamborine, Queensland were sampled in winter (12/6/2008) and spring (23/10/2008) and indexed as pools of up to 10 plants for CMV (winter only) and RDaV by ELISA and RT-PCR respectively (Section 5.2.2.2). Species tested were *Rumex crispus* (curled dock), *Bidens pilosa* (cobbler's pegs), *Capsella bursa-pastoris* (shepherd's purse), *Chenopodium album* (fat hen), *Fumaria muralis* (common rampion-fumitory), *Gnaphalium pensylvanicum* (cudweed), *Lamium amplexicaule* (dead nettle), *Modiola caroliniana* (red flowered mallow), *Sonchus oleraceus* (milk thistle), *Stellaria media* (chickweed) and *Trifolium repens* (white clover).

#### 5.2.4.3 Results

##### dsRNA analysis

dsRNA species consistent with the size of the CMV components were obtained from isolate 2785, together with additional species of ca. 1.5, 2, 2.7, 6.5 and 16.5 kbp (Figure 6). The latter was assumed to represent the dsRNA form of the full length genome of RDaV, a suspected closterovirus. The origin of the two smaller was uncertain.

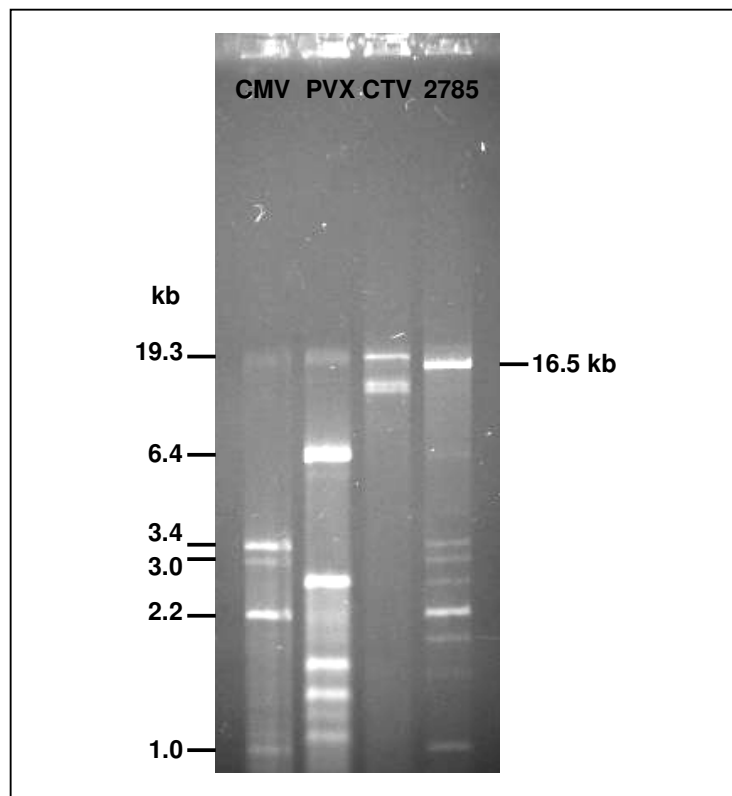


Figure 6. Double stranded RNA analysis of isolate 2785, which is infected with RDaV. Size standards were CMV (3389, 3035, 2197 and 1027 bp), PVX (6435 bp) and CTV (19296 bp). RDaV was calculated to be 16500 bp in size. Other smaller bands not attributed to the mixed CMV infection were of sizes 6500, 2700, 1950 and 1550 bp.

### The closterovirus genome

The genome of RDaV is at least 14 642 nt in length, and was constructed from 112 sequenced fragments from isolate 1886 (Figure 7). Initially, 37 random sequences were assembled in to five contigs spread throughout the closterovirus genome. Specific primers were used to join contigs, and 5' and 3' RACE used to extend outwards from the known sequence to the ends of the genome. The 3' end appears to have been obtained, but on the basis of comparison with genomes of other closteroviruses, and the dsRNA pattern obtained for RDaV, it is likely that around 1,000 bp remains to be sequenced at the 5' end. Sequence of this RDaV isolate, from accession 1886, was identical to the partial sequence obtained from the isolate in which RDaV was initially observed (Section 5.2.1).

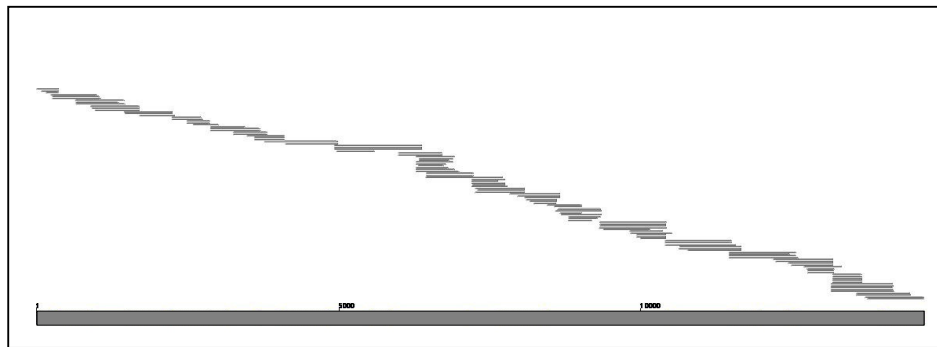


Figure 7. The RDaV partial genome of 14 642 bp was constructed from 112 overlapping sequenced fragments. All nucleotide positions were covered by 2-14 clones.

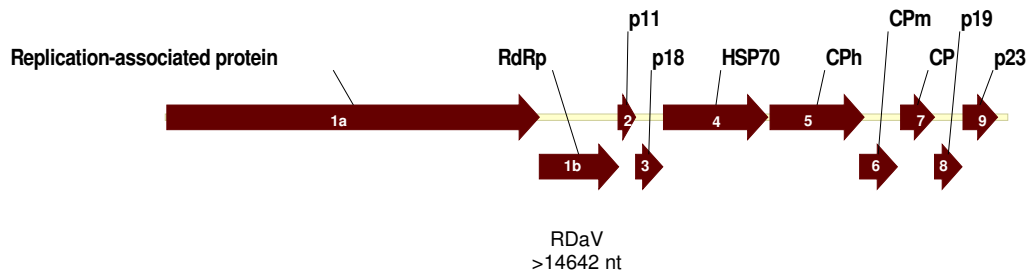


Figure 8. Putative ORFs of RDaV.

Table 6. Putative ORFs and proteins of RDaV and their database matches.

| ORF | Putative function                       | Position (nt) | Size (kDa) | Closest BLAST match         | GenBank      | Identity (%) |
|-----|-----------------------------------------|---------------|------------|-----------------------------|--------------|--------------|
| 1a  | Protease/<br>methyltransferase/helicase | 1-6487        | >243       | Carrot yellow<br>leaf virus | YP_003075964 | 39           |
| 1b  | RdRp                                    | 6486-7871     | 55         | Mint virus 1                | YP_224091    | 70           |
| 2   | unknown                                 | 7861-8160     | 11         | Mint virus 1                | YP_224092    | 30           |
| 3   | unknown                                 | 8160-8639     | 18         |                             |              |              |
| 4   | HSP70                                   | 8653-10464    | 66         | Mint virus 1                | AAX98727     | 51           |
| 5   | CPh                                     | 10509-12140   | 62         | Mint virus 1                | YP_224094    | 48           |
| 6   | CPm                                     | 12058-12720   | 24         | Mint virus 1                | YP_224095    | 52           |
| 7   | CP                                      | 12774-13364   | 22         | Mint virus 1                | AAX98728     | 38           |
| 8   | unknown                                 | 13361-13852   | 19         |                             |              |              |
| 9   | unknown                                 | 13855-14466   | 23         | Carrot yellow<br>leaf virus | YP_003075973 | 25           |

Analysis of the RDaV genome identified 10 putative ORFs (Figure 8; Table 6). The 5' was not obtained, but the 3' UTR was 176 nt in length. The gene number and order is the same as that of Carrot yellow leaf closterovirus. ORF 1a encodes a putative replication protein of >243 kDa with methyl transferase and helicase motifs. ORF 1b, the RdRp (55 kDa) is translated with a putative +1 frame shift. Following ORF 1b are p11, p18, HSP70 (66kDa), CPh (62 kDa), CPm (24 kDa), CP (22 kDa), p19 and p23.

Phylogenetic analysis of the HSP70 of members of the *Closteroviridae* showed that RDaV was most closely related to Mint virus 1 (54.3% identity at the amino acid level) and both grouped in a clade with definitive closteroviruses (Figure 9). Greatest aa similarity for ORFs 1b, 2, 4-7 was with Mint virus 1, and for ORFs 1 and 9 with Carrot yellow leaf virus (Table 6). ORFs 3 and 8 did not share any significant similarities to other viral proteins.

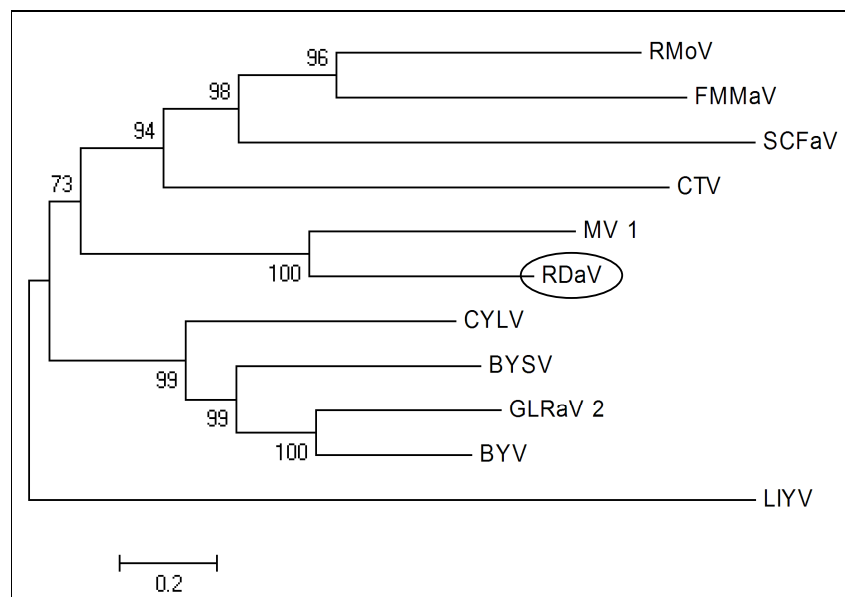


Figure 9. Phylogenetic analysis of the heat shock protein 70 of members of the *Closteroviridae*. Acronyms and GenBank numbers: BYV, *Beet yellows virus*, NP\_041872; BYSV, *Beet yellow stunt virus*, AAC55662; CTV, *Citrus tristeza virus*,

NP\_042864; CYLV, *Carrot yellow leaf virus*, YP\_003075968; FMMAV, Fig mild mottle associated virus, ACU57193; GLRaV 2, *Grapevine leafroll associated virus 2*, AAR21242; LIYV, *Lettuce infectious yellows virus* (Crinivirus), NP\_619695; MV 1, Mint virus 1, AAW32895; RMoV, Raspberry mottle virus, YP\_874188; SCFaV, Strawberry chlorotic fleck associated virus, ABI23185. The LIYV protein was used as an outgroup. Bootstrap values are shown as percentage value and only the nodes over 60% are labelled. The bar represents 0.2 amino acid changes per site.

Antibody production

No virus-specific reactions were noted for the antisera against the MBP-RDaV CP and GST-RDaV CP fusion proteins, or the synthetic peptides. However, for the SUMO-RDaV CP fusion protein, a weak reaction was noted against infective sap extracts, and a stronger reaction against the infective miniprep. A<sub>405</sub> absorbance values were:

|                    |       |
|--------------------|-------|
| Buffer control:    | 0.290 |
| Healthy sap:       | 0.775 |
| Diseased sap:      | 1.268 |
| Healthy miniprep:  | 0.257 |
| Diseased miniprep: | 1.247 |

Aphid transmission

RDaV was transmitted to five of the eight virus-free rhubarb seedlings, and infection confirmed by RT-PCR (Figure 10). Plants were grown on in the glasshouse and after approximately three years occasional leaf symptoms such as vein yellowing and chlorotic spotting were observed in some of the RDaV-positive plants.

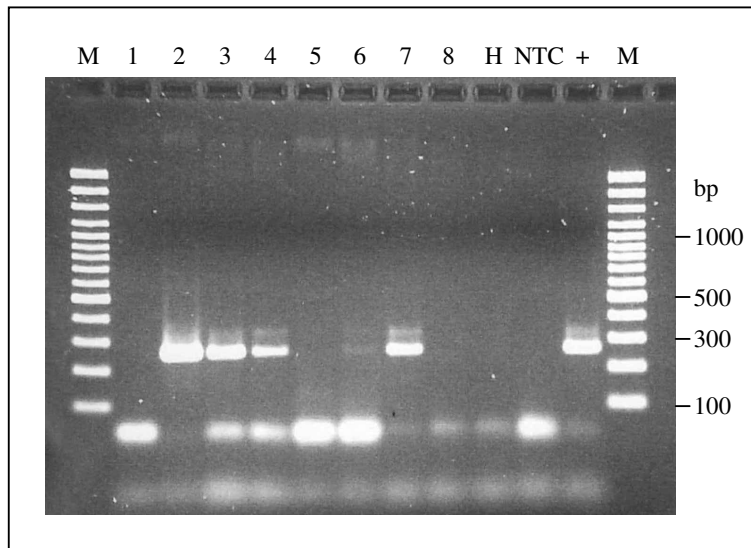


Figure 10. RDaV RT-PCR to index rhubarb seedlings six weeks after aphid transfer in the aphid transmission test. M, 100 bp Plus DNA ladder (Fermentas); lanes 1-8, inoculated seedlings 1-8; H, healthy seedling rhubarb; NTC, no template negative control; + positive control. Expected band size was 259 bp. Plants 2, 3, 4, 6 and 7 were positive for RDaV.

### Alternative host survey

Eleven weed species around the rhubarb crop at Mt Tamborine, Queensland were tested for CMV and RDaV to identify alternative host(s) of RDaV. Four species (*Capsella bursa-pastoris*, *Chenopodium album*, *Sonchus oleraceus* and *Stellaria media*) tested positive for CMV in winter. However, only samples of *Rumex crispus* (curled dock; Polygonaceae), were positive for RDaV in each season (Table 7). One plant of 200 was positive in winter, while higher incidence was observed in spring (9 of 20 pools of 10 samples each). Using the formula of Gibbs and Gower (1960), the proportion of infected plants in the pooled samples was 5.8%. All infected dock plants were symptomless.

Table 7. Weed species tested for CMV in winter and RDaV in winter and spring to identify alternative hosts of RDaV.

| Weed species                                      | Winter |      | Spring |
|---------------------------------------------------|--------|------|--------|
|                                                   | CMV    | RDaV | RDaV   |
| <i>Rumex crispus</i> (curled dock)                | -      | +    | +      |
| <i>Bidens pilosa</i> (cobble's pegs)              | -      | -    | -      |
| <i>Capsella bursa-pastoris</i> (shepherd's purse) | +      | -    | -      |
| <i>Chenopodium album</i> (fat hen)                | +      | -    | -      |
| <i>Fumaria muralis</i> (common rampion-fumitory)  | -      | -    | -      |
| <i>Gnaphalium pensylvanicum</i> (cudweed)         | -      | -    | -      |
| <i>Lamium amplexicaule</i> (dead nettle)          | -      | -    | -      |
| <i>Modiola caroliniana</i> (red flowered mallow)  | -      | -    | -      |
| <i>Sonchus oleraceus</i> (milk thistle)           | +      | -    | -      |
| <i>Stellaria media</i> (chickweed)                | +      | -    | -      |
| <i>Trifolium repens</i> (white clover)            | -      | -    | -      |

#### **5.2.4.4 Discussion**

Closteroviruses are aphid-transmitted viruses that are predominantly phloem-limited, occur in low concentrations in plants, and are often not mechanically transmissible. Although some members of the genus, e.g. *Citrus tristeza* virus, cause economically very important diseases that are well characterised and well studied, others are more enigmatic. They often occur as mixed infections, and are difficult to associate with specific symptoms. RDaV falls into the latter category. It was shown to be a novel closterovirus, most closely related to Mint virus 1 (MV1), with a 50.8% nucleotide identity across the sequenced genome. The genome of RDaV comprises 10 ORFs, six of which are most closely related to MV1, two to Carrot yellow leaf virus (CYLV), and two of which appear unrelated to other plant virus proteins. The genome organisation most closely resembles that of CYLV.

A phylogenetic analysis of the HSP70 gene also showed that RDaV was most closely related to MV1, and fell clearly within the clade of aphid-transmitted closteroviruses. Consistent with this grouping, RDaV was shown to be transmitted by the aphid *Aphis gossypii*. Curled dock (*Rumex crispus*), a member of the Polygonaceae along with rhubarb, was shown to be a symptomless field host of RDaV. This species is a common weed in the Mt Tamborine growing area of Queensland. In a spring survey, 5.8 % of random plant samples were estimated to be infected, indicating that this species could be a reservoir of infection.

Obtaining a specific antiserum to RDaV proved to be very challenging. No specific reaction in ELISA was obtained with the antiserum to the synthetic CP designed peptide, nor to two of the three bacterially expressed coat protein constructs. A virus-specific reaction was obtained with the antiserum against the SUMO-RDaV fusion protein, though the reaction was weak with sap extracts compared with partially purified virus minipreps. Further assessment and refinement of the antiserum is required before it can form the basis of a routine diagnostic assay.

## **5.3 Optimising micropropagation of rhubarb**

### **5.3.1 Initiation of rhubarb plants into tissue culture**

#### **5.3.1.1 Introduction**

Rhubarb (*Rheum rhabarbarum*) is a herbaceous perennial of the botanical family Polygonaceae. Rhubarb does best in cool climates but also grows well in sub-tropical environments except when subjected to extreme heat. Temperature above 25°C slows growth and reduces red colour of the stalks and may cause problems in plant establishment.

Rhubarb is propagated by subdividing “crowns” formed during the preceding season. Divisions are made by cutting the crown between the “bud” or “eyes” (that are growing points) and leaving a piece of storage root attached to each bud. The crowns are grown deep in soil heavily amended with organic matter and usually divided after the rhubarb plant has grown for 3-4 seasons. Because growers use their own plants and because they can be symptomless infected with virus the grower has no way to produce disease free plants. Micro-propagated European selections of rhubarb plants have been successfully used under European conditions for several decades. Tissue culture from virus indexed rhubarb plants provides a way to produce disease free plants. However initiation of clean tissue cultures is a problem with rhubarb since rhubarb tissue culture requires aseptic culture and the growing points are deep in the soil filled with microorganisms. The following experiment examines the effectiveness of a standard sterilisation method and following sections describe a series of experiments conducted over time aiming to improve efficiency of initiating clean rhubarb buds into tissue culture.

#### **5.3.1.2 Materials and methods**

Rhubarb explants were of the cultivar “Big Red” collected from Mt Tamborine, Queensland. Twenty-nine plants of “Big Red” were collected to evaluate effectiveness of the standard sterilisation process described below. Buds were extracted and sterilised 3-5 days after plant harvest when the crowns were washed with chlorinated water to remove dirt and debris, old leaf bases and dead tissue were removed. Leaves were carefully removed so as not to damage buds inside leaf bases. Apical growing points and buds were excised from the crown and rinsed under running tap water for 20 minutes then placed into sterilized jars. In total 326 buds were extracted and sterilised using the following common sterilisation method used in plant tissue culture. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Bleached and excess tissue was removed aseptically to leave a growing point between 1-7 mm the variation due to the size of the original bud and the amount of tissue removed. The bud was then placed into Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel with 1 mL/L Plant Preservative Media ((PPM) which is a plant tissue culture biocide product). pH was adjusted to 6.0 prior to autoclaving. Cultures were placed in the dark for 5 days at 25°C and then cultured at 25°C, under fluorescent lights at a photon flux density of



60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent) for 16 hour day and 8 hour night.

### 5.3.1.3 Results and discussion

The standard sterilisation method successfully allowed an average of 51% of buds to be established in tissue culture. There was a lot of variability in the success of buds that could be initiated between crowns resulting in standard deviation of 23%. The variation in successful initiation of buds into tissue culture is shown in the frequency histogram below. The histogram (Figure 11) indicates a high establishment success rate of between 30 to 70% of buds successfully initiated. Bacterial contamination was the limiting factor in initiation of rhubarb buds as would be expected when sourcing plant material growing in direct contact with soil. Even with a large variation in initiation success the average success rate of 51% provides a reasonable efficiency.

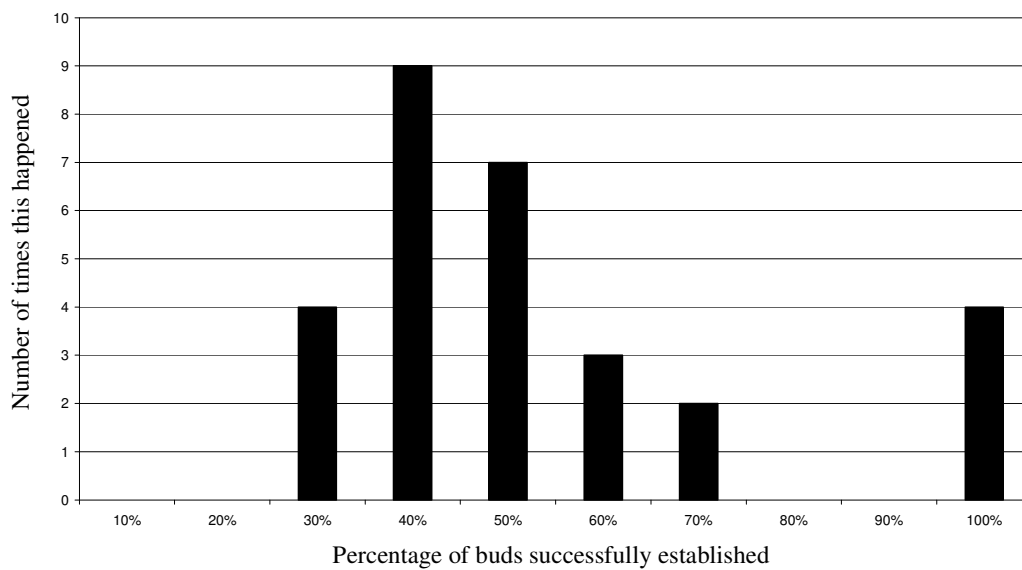


Figure 11. Frequency of rhubarb initiation success.

Further research was undertaken to determine if the rate of initiating clean buds could be improved.

## 5.3.2 Evaluation of a range of alternative sterilisation methods

### 5.3.2.1 Introduction

Further sterilisation methods were investigated to determine if bacterial contamination could be reduced and higher levels of bud establishment than the standard method described previously could be achieved. Several treatments described to have improved initiation in a range of other crops were compared (Constantine 1997) were compared with the standard method using sodium hypochlorite to determine if initiation rate could be improved.

### 5.3.2.2 *Materials and methods*

Three additional methods were investigated for sterilisation of rhubarb buds used in culture initiation. These were 1. Sodium dicloroisocyanurate (Na Dcc) 300 mg/L a.i. for 20 mins, 2. Sodium dicloroisocyanurate (Na Dcc), 300 mg/L a.i. for 48 hours, 3. Control - standard method using 2.0% a.i sodium hypochlorite and 4. Chlorine dioxide sterliant (CDs).

*Treatment 1:* 15 buds were excised from the crown and rinsed under running tap water for 20 minutes then placed into sterilized jars. Buds were surface sterilised in 300 mg/L NaDcc for 20 minutes, without rinsing. Buds were cut back to remove bleached tissue and placed into (Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytigel supplemented with 5 mL/L Plant preservation media (PPM). pH was adjusted to 6.0 prior to autoclaving. They were placed in culture room at 25°C±2, under 16 hour day and 8 hour night, at a photon flux density of 60 µmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent).

*Treatment 2:* 15 buds were excised from the crown and rinsed under running tap water for 20 minutes then placed into sterilized jars. Buds were surface sterilised in 300 mg/L NaDcc for 48 hours in solution and then placed into (Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytigel supplemented with 5 mL/L PPM. pH was adjusted to 6.0 prior to autoclaving. They were placed in culture room at 25°C±2, under 16 hour day and 8 hour night, at a photon flux density of 60 µmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent).

*Treatment 3* (standard method): 125 buds were excised from the crown and rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Buds were cut back to remove bleached tissue and placed into (Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytigel supplemented with 1 mL/L PPM. pH was adjusted to 6.0 prior to autoclaving. They were placed in culture room at 25°C±2, under 16 hour day and 8 hour night, at a photon flux density of 60 µmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent).

*Treatment 4:* This method used a two-part solution mixed together: Solution A: 30% Aqueous Lactic Acid with Solution B: 1.9% Aqueous solution of 80% sodium chlorite thereafter referred to as CDs. 1 part A and 1 part B plus 4 parts sterile water was mixed together with the addition of 1 drop of Tween 80 per 10 mL of CDs. The combined solution was left to stand 20 minutes before use. 15 apical growing points and buds were excised from the crown and rinsed under running tap water for 20 minutes then placed into sterilized jars. Growing points were sterilised in prepared CDs solution for 20 minutes, without rinsing, followed by initiation into MS basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L BAP and 2.5 g/l phytigel. pH was adjusted to 6.0 prior to autoclaving. They were placed in culture room at 25°C±2, under 16 hour day and 8 hour night, at a photon flux density of 60 µmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent).

### 5.3.2.3 Results and discussion

There was no significant differences between treatments although treatment 1 (Sodium dicloroisocyanurate (Na Dcc) 300 mg/L a.i. for 20 mins) had higher mean survival and Chlorine dioxide sterilant had the lowest (Figure 12).

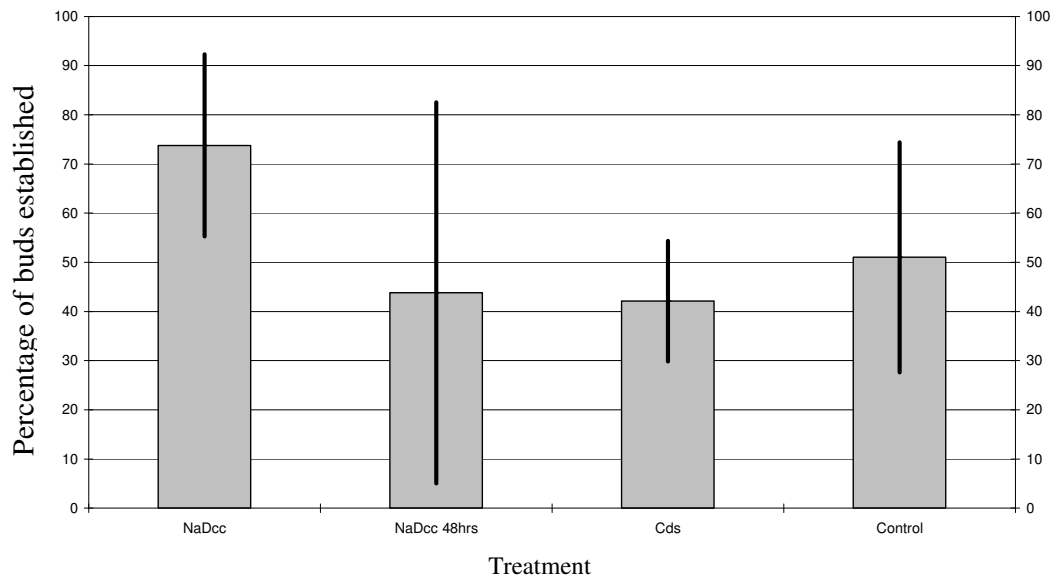


Figure 12. Comparison of sterilisation treatments for initiation of rhubarb growing points.

There was a lot of variability in all treatments. The “standard” treatment using sodium hypochlorite produced similar results to the other treatments and will be used in further rhubarb culture establishment. The decision to use sodium hypochlorite as the standard treatments was based on consideration of the practical and commercial application, the benefits of initiation methods, low cost, low toxicity and readily available chemical access. However further evaluation of the use of Sodium dicloroisocyanurate may be warranted if increased problems with contamination and establishment of cultures were encountered.

### 5.3.3 Effect of time of extraction of buds after harvesting crown on culture establishment success

#### 5.3.3.1 Introduction

Anecdotal evidence of previous experience initiating buds from soil grown crops suggested that buds taken from plants several days post harvest had higher success rate of establishment with reduced bacterial contamination. Earlier experimentation with a range of sterilisation methods failed to reduce bacterial contamination at initiation. Therefore this study evaluated whether time of bud extraction after plant harvest would affect the success of establishing clean buds into tissue culture.

### **5.3.3.2 Materials and methods**

Thirty rhubarb crowns from visually healthy plants were harvested from a commercial rhubarb farm at Mt Tamborine on October 9, 2007. Stems and leaves were removed from the crowns and soil was washed away. The crowns were spaced apart and left to air dry in a clean bench at room temperature until crowns were taken to extract buds for culture initiation. At 1, 3, 6, 10, 13 and 17 days after harvest 5 crowns were taken for each treatment and all buds from those crowns then extracted to determine if time was a factor in bacterial contamination and/or survival of buds initiated into culture.

At the appropriate treatment time, apical growing points and buds were excised from 5 of the crowns and rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Buds were cut back to remove bleached tissue and placed into (Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytagel supplemented with 1 mL/L PPM. pH was adjusted to 6.0 prior to autoclaving. The buds were placed in culture room at 25°C±2 and grown in darkness for 5 days and then grown under 16 hour day and 8 hour night, at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent).

### **5.3.3.3 Results and discussion**

The number of buds per crown ranged between 2 to 10 buds with a mean of 4.3 buds per crown. There was no significant difference on success of bud establishment between treatments with an average of 52.95% and median of 55.8% of buds surviving using the standard sterilisation method. This compares well to our earlier work using the standard sterilisation method on buds extracted 3-5 days after plant harvest where an average of 39.32% or median of 44.44% buds survived.

There was a reduction in contamination in buds extracted 10 days after crown (Figure 13). Highest contamination was observed in buds extracted one day after crown harvest where all buds were contaminated, and even by day 6 contamination remained high.

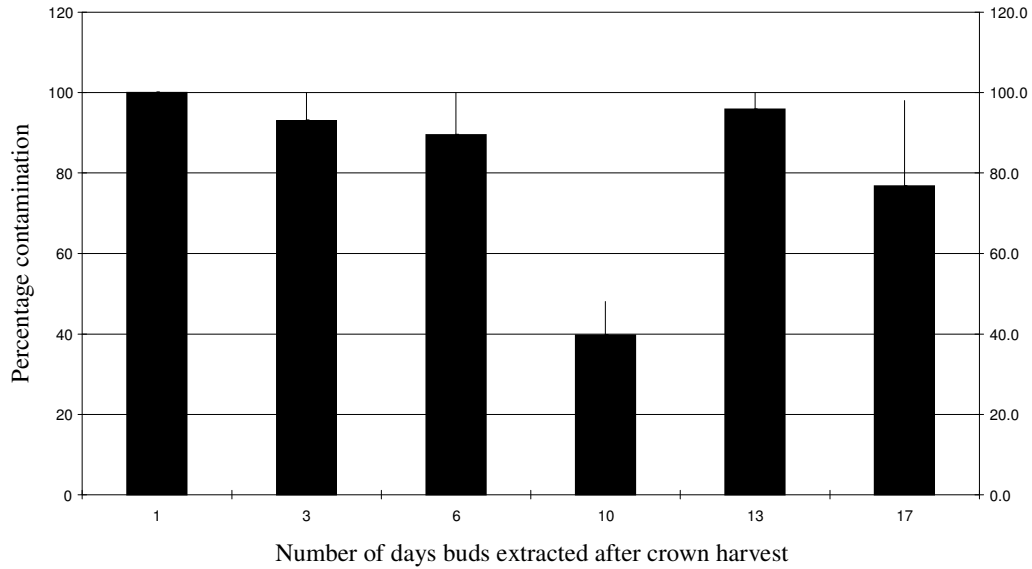


Figure 13. Affect of time of bud extraction on contamination of buds

The rhubarb buds remain viable a long time after crown harvest with buds extracted 17 days after crown harvest remaining viable (Figure 14). Considering the balance between contamination and survival it is recommended that buds be extracted one to two weeks after crown harvest providing the crowns could be cleaned and left to dry in a clean vented room at room temperature. Initiating buds immediately after harvest should be avoided due to high contamination.

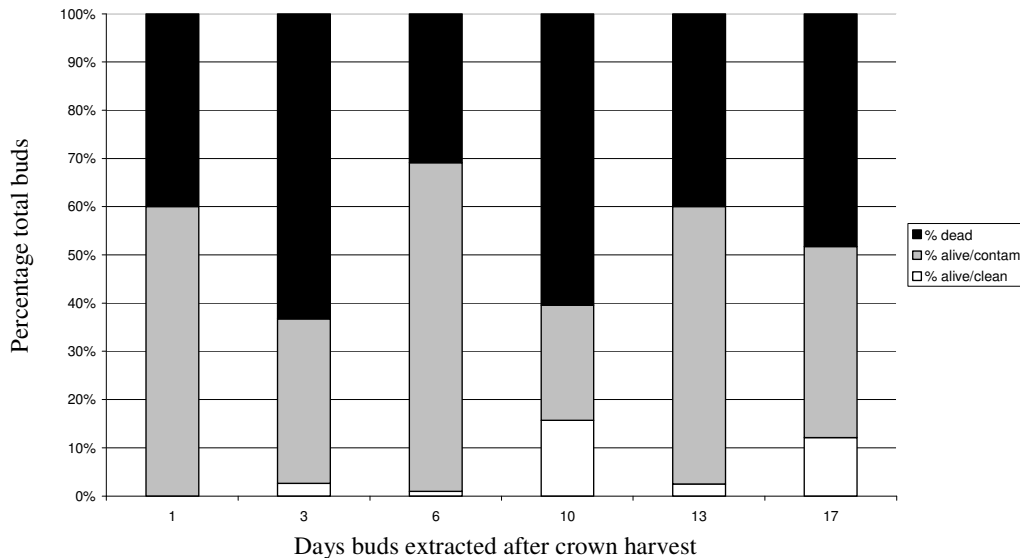


Figure 14. Fate of rhubarb buds extracted at different times after crown harvest.

### 5.3.4 Effect on bud size on survival

#### 5.3.4.1 *Introduction*

To improve initiation success of rhubarb this experiment was conducted to determine how bud size affected survival and contamination during initiation of cultures.

#### 5.3.4.2 *Materials and methods*

Forty-nine buds from 8 crowns were extracted sterilised and put into culture media. At the time they were placed into culture media the buds were measured and categorised into three size groups: 1. 6 mm or longer, 2. 2-5 mm long and 3. less than 2 mm long.

One week after crown harvest, buds were excised from the crowns and rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Buds were cut back to remove bleached tissue and placed into Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytigel supplemented with 1 mL/L PPM. pH was adjusted to 6.0 prior to autoclaving. The buds were placed in culture room at 25°C±2 and grown in darkness for 5 days and then grown under 16 hour day and 8 hour night, at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent).

#### 5.3.4.3 *Results and discussion*

While there was a large variation in the bud size in the different crowns of rhubarb the majority of buds fell into the 2-5mm range (Figure 15).

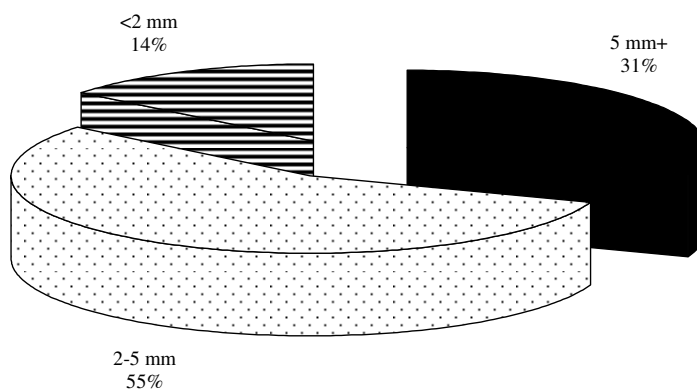


Figure 15. Proportion of different size buds in the rhubarb crown.

Overall 49% of all the buds survived. This was in line with results described in previous experiments (40% and 54% described previously). However when the size of buds was considered, there was a significant difference in survival. Buds 6 mm or larger had a significantly higher survival rate than other sizes averaging 89% survival

(Figure 16). Significantly more buds 2-5 mm survived (38%) compared to buds less than 2 mm where none survived.

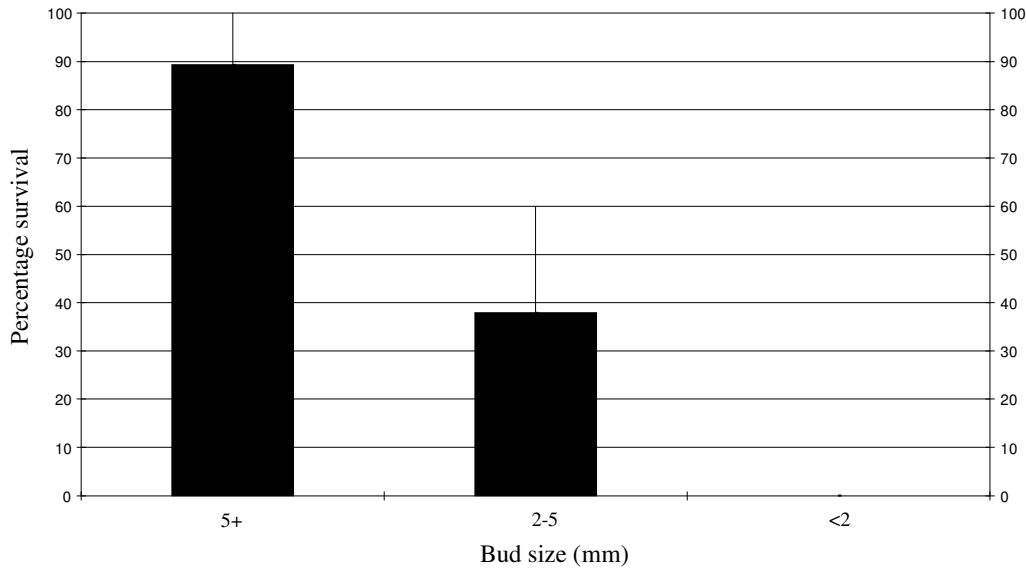


Figure 16. Survival of buds considering size.

Considering the survival rate of the buds attention should be focussed on sterilising buds larger than 2mm optimum survival was achieved using buds larger than 5mm.

### **5.3.5 Use of vented containers improves rhubarb tissue culture production**

#### ***5.3.5.1 Introduction***

Preliminary culture of rhubarb micropropagated in sealed containers indicated that the plants appeared to be susceptible to ethylene showing symptoms of leaf yellowing and senescence over time. After two weeks the healthy plants would begin to yellow and required subculture within four weeks or plants would suddenly die. This experiment was initiated to determine if vented containers could improve the quality of rhubarb tissue culture plantlets during micropropagation cycle.

#### ***5.3.5.2 Materials and methods***

Identical polycarbonate containers (125 mL) with venting with 0.2 micron surgical tape (Millipore) or without venting were used. In each container was placed a single healthy rhubarb explant produced from a virus free “Big Red” selection. The plantlets were placed into containers containing 50 mL of Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel. Cultures grown at 25°C, under fluorescent lights at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent) for 16 hour day and 8 hour night. There were 10 replicates per treatment each in three blocks. Plants were subcultured onto fresh media every 4 weeks and total fresh weight of explant, number of shoots (multiplication), and colour (scale 1. pale brown, 2. pale yellow-green, 3. light green, 4. dark green) were recorded. Preliminary split-plot analyses were used to compare results across all subcultures and Analysis of variance was used to compare treatments using Genstat.

### 5.3.5.3 Results and discussion

Plants in vented containers had a significantly greater increase in fresh weight (1.75 g) than plants in sealed containers (1.40 g) ( $P < 0.05$ ) (Figure 17).

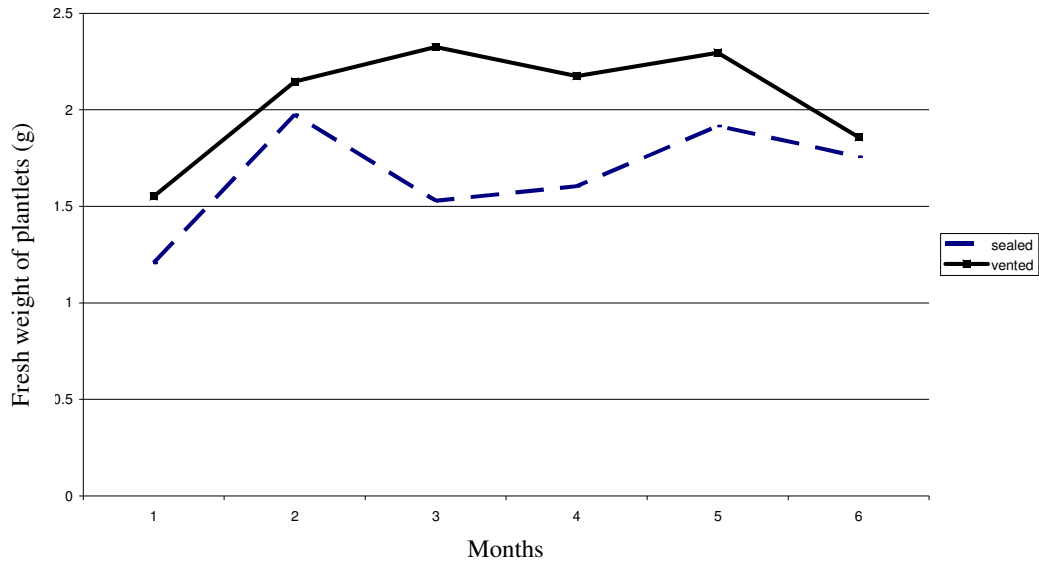


Figure 17. Rhubarb fresh weight in vented or sealed containers.

There was no significant difference in multiplication rate between treatments ( $P > 0.05$ ) though the multiplication rate for plants in vented containers (4.2) was higher than the rate for sealed tubes (3.9; Figure 18). The multiplication rate was significantly higher ( $P < 0.05$ ) in subcultures 5 and 6 (5.2 and 5.0) than in earlier subcultures (3.8, 4.0, 3.0 and 3.3).

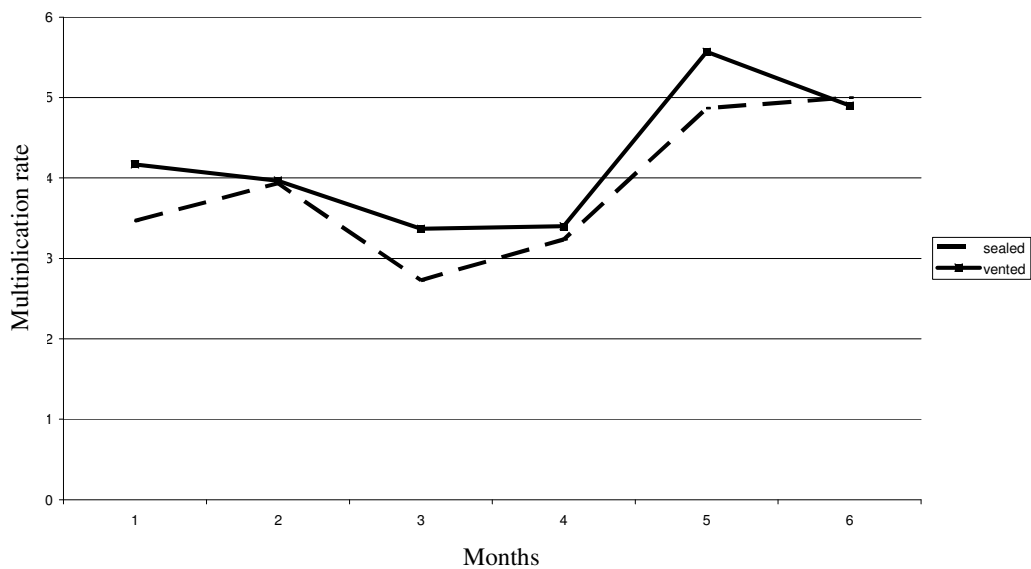


Figure 18. Multiplication rate over 6 subculture cycles.



There was no significant difference in health between treatments as noted by colour, with both treatment producing healthy plantlets (Figure 19).

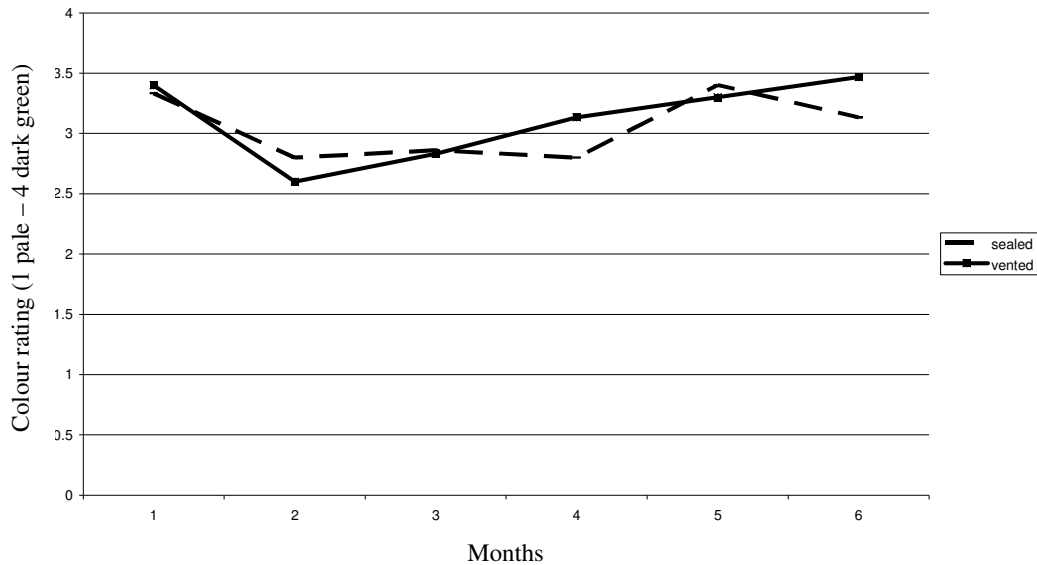


Figure 19. Colour rating of rhubarb in sealed or vented containers.

Vented containers overall produced better plants with significantly bigger/heavier plantlets and a trend to increased multiplication. Vented containers were subsequently adopted as best practice for micropropagation of rhubarb.

### 5.3.6 Identifying effective rate of cytokinin for multiplication of rhubarb

#### 5.3.6.1 *Introduction*

Commercial micropropagation of rhubarb requires consistent and high rates of multiplication. Research was undertaken using the selection ‘Big Red’ that would form the basis of the field trial virus infection research. This is the main commercial cultivar grown in the virus affected area of Queensland. A standard Murashige and Skoog media was used in vented containers, which was previously shown to improve *in vitro* performance.

#### 5.3.6.2 *Materials and methods*

Buds were initiated into culture 3-5 days after plant harvest the crowns were washed with chlorinated water to remove dirt and debris, old leaf bases and dead tissue were removed. Leaves were carefully removed so as not to damage buds inside leaf bases. Apical growing points and buds were excised from the crown and rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in

sterile distilled water. Bleached and excess tissue was removed aseptically to leave a growing point between 1-7 mm the variation due to the size of the original bud and the amount of tissue removed. The bud was then placed into containers with vents closed with 0.2 micron surgical tape (Millipore) containing Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel with 1 mL/L Plant Preservative Media ((PPM) which is a plant tissue culture biocide product). Cultures were placed in the dark for 5 days at 25°C and then cultured at 25°C, under fluorescent lights at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent) for 16 hour day and 8 hour night.

Twenty healthy established plantlets were multiplied on media containing the cytokinin Benzyl Amino Purine at 2.5 mg/L and were monitored for multiplication over 7 subculture cycles of 4 weeks.

Twenty healthy established plantlets plantlets were multiplied on media containing the cytokinin Benzyl Amino Purine at double the previous level at 5.0 mg/L and were monitored for multiplication over 4 subculture cycles of 4 weeks.

### 5.3.6.3 Results and discussion

Interestingly the lower level of cytokinin (2.5 mg/L BAP) provided a higher multiplication rate in the cultivar “Big Red” than when it was micropropagated on media containing the higher rate (5.0 mg/L BAP

Figure 20). Both dose rates provided very uniform plants with low levels of variation. BAP applied at 2.5 mg/L induced multiplication rate of over 3 times each subculture period that would be in the range of commercial viability. BAP applied at 5 mg/L induced multiplication that would not be commercially viable for the “Big red” selection.

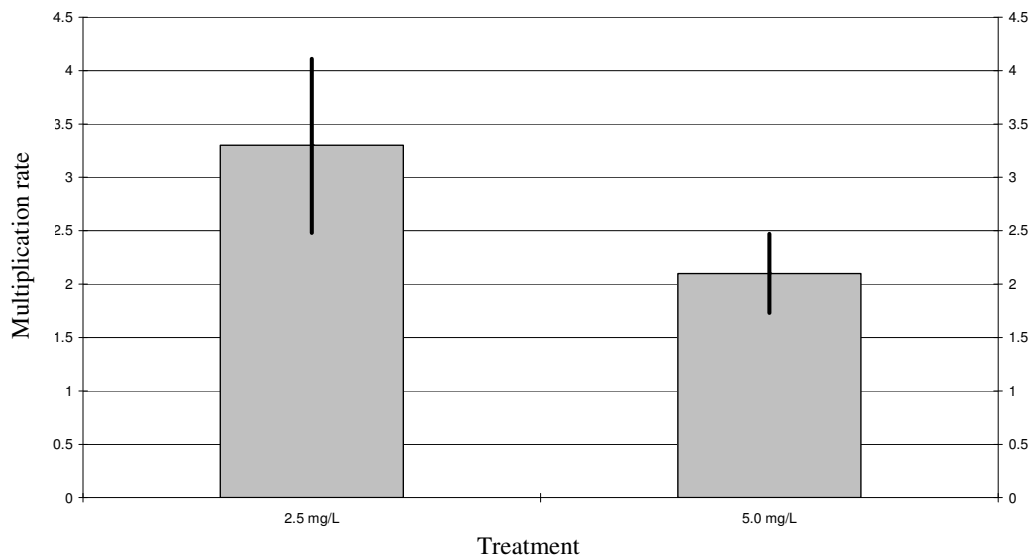


Figure 20. Effect of increased cytokinin Benzyl Amino Purine on multiplication.

The 2.5 mg/L BAP provided a consistent multiplication rate of 3.3 times increase every 4 weeks which is an acceptable commercial production level. Reduced level of cytokinin offers advantages in reduced cost and also best practice considering somaclonal variation. Lowest levels of growth regulators may reduce the incidence of off-types produced by stress on the plants or by induction of adventitious buds that may otherwise lie dormant. Further work in this project used 2.5 mg/L BAP for effective multiplication.

### **5.3.7 Can rhubarb micropropagation be improved using silicon amendments**

#### ***5.3.7.1 Introduction***

Silicon has been used to improve performance in banana (Hamill et al. 2010). A commercially registered agricultural soluble fertiliser called Kasil and containing Potassium Silicate was evaluated as an amendment to tissue culture media. Kasil is extremely alkaline and requires the addition of a large amount of hydrochloric acid to reduce pH in the media to levels that support plant growth. MES is a biological buffer that maintains pH in the range of 4-6 and was added as additional treatment to see if culture performance could be improved.

#### ***5.3.7.2 Materials and methods***

There were 8 treatments consisted of 2 factors: MES at 2 levels, 0 and 0.01 M, and Kasil at 4 levels, 0, 1.5, 5 and 10 mL/L amended to culture media (Table 8). The plantlets were placed into vented containers with 0.2 micron surgical tape (Millipore) containing 50 ml of Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel. Cultures grown at 25°C, under fluorescent lights at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent) for 16 hour day and 8 hour night. Plants were subcultured onto fresh media every 4 weeks. This experiment compared 8 treatments replicated 6 times in a completely randomised design. Colour was rated on a 1-5 scale with 1- brown, 2- yellow, 3- yellow green, 4- pale green, 5- dark green. Overall plant health was rated on a 1-4 scale: 1- dead, 2- brown/pale green, 3- pale green, 4- vigorous/dark green.

Table 8. Treatment combinations of buffer and silicon amendment concentrations

| Treatment Code | MES  | Kasil (mL/L) |
|----------------|------|--------------|
| 1              | 0    | 0            |
| 2              | 0    | 1.5          |
| 3              | 0    | 5            |
| 4              | 0    | 10           |
| 5              | 0.01 | 0            |
| 6              | 0.01 | 1.5          |
| 7              | 0.01 | 5            |
| 8              | 0.01 | 10           |

Data were analysed using analysis of variance and means compared using the protected least significant difference procedure. In the analysis the Kasil factor was split into a linear component and remaining variation. All testing was carried out at P=0.05.

### 5.3.7.3 Results and discussion

There was a significant effect of MES level, with 0 MES treatments (adjusted back transformed mean 0.9 g) significantly heavier than 0.01 MES treatments (adjusted back transformed mean 0.5 g; Table 9; Figure 21). An examination of the two-way Table of means showed that 0.01 MES plants were consistently lighter than 0 MES plants and that the log transformed weight of 0.01 MES plants increased linearly with amount of Kasil applied. Plants with 0 MES were heavier, with the largest average weight being for treatments receiving Kasil at 5 mL/L (adjusted back transformed mean 1.3 g).

Table 9. Influence of MES / Potassium Silicate (Kasil) combination media amendments on the final fresh weight of rhubarb plantlets.

| MES  | Kasil (mL/L) | Mean (g) |    |
|------|--------------|----------|----|
| 0    | 0            | 0.590    | bc |
| 0    | 1.5          | 0.785    | ab |
| 0    | 5            | 1.287    | a  |
| 0    | 10           | 0.750    | ab |
| 0.01 | 0            | 0.407    | c  |
| 0.01 | 1.5          | 0.492    | bc |
| 0.01 | 5            | 0.437    | bc |
| 0.01 | 10           | 0.780    | bc |
| SEM  |              | 0.1027   |    |

Same letters are not significantly different a  $p=0.05$  SEM=1.01

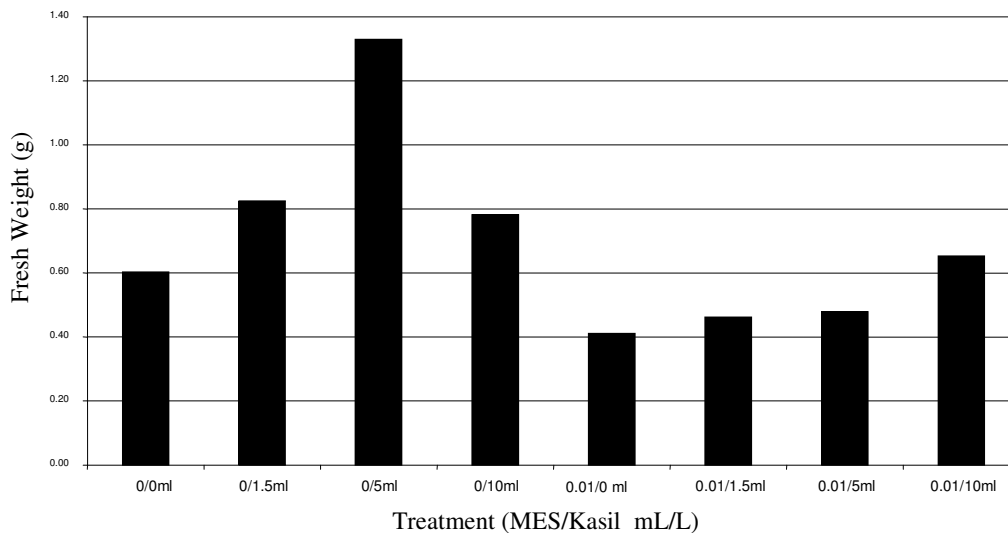


Figure 21. Influence of MES / Potassium Silicate (Kasil) combination media amendments on fresh weight of rhubarb plantlets.

There were no significant differences in multiplication rate, with the highest means corresponding to 0 MES with 1.5 and 5 mL/L Kasil (5.3 and 5.0, respectively; Figure 22).

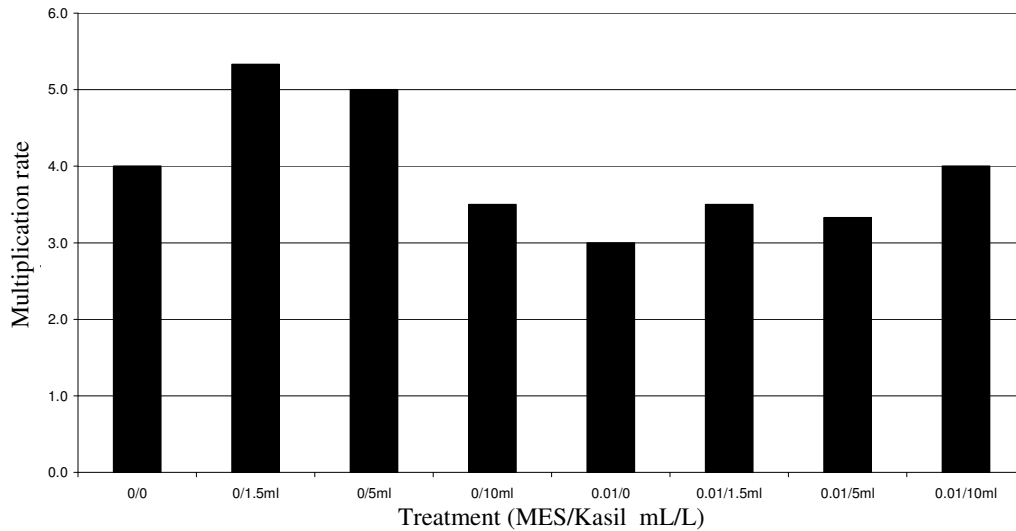


Figure 22. Effect of combination of MES / Potassium silicate (Kasil) media amendment on rhubarb multiplication rate.

Colour ratings declined linearly with increasing Kasil levels, with MES treated values lower than those without MES (Table 10; Figure 23). Optimum colour was shown in plants cultured without Kasil and with 1.5 and 5.0 mL/L Kasil alone.

Table 10. Effect of combination of MES / Potassium silicate (Kasil) media amendment on colour ratings.

| MES  | Kasil (mL/L) | Colour rating <sup>A</sup> |
|------|--------------|----------------------------|
| 0    | 0            | 4.5 ab                     |
| 0    | 1.5          | 4.7 a                      |
| 0    | 5            | 4.3 ab                     |
| 0    | 10           | 3.7 bc                     |
| 0.01 | 0            | 4.8 a                      |
| 0.01 | 1.5          | 4.0 abc                    |
| 0.01 | 5            | 3.3 cd                     |
| 0.01 | 10           | 2.5 d                      |
| SEM  |              | 0.32                       |

<sup>A</sup> 1-brown, 2- yellow, 3- yellow green, 4- pale green, 5- dark green

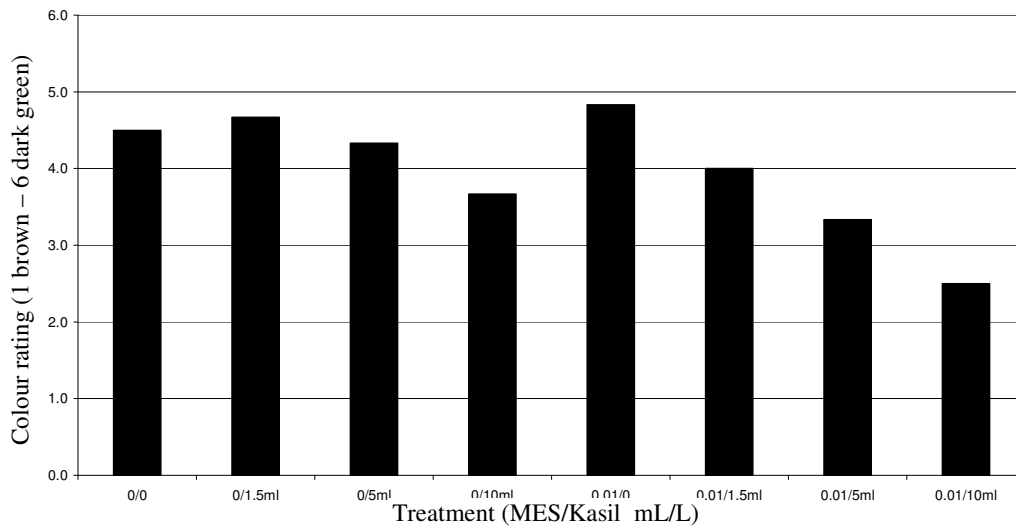


Figure 23. Effect of combination of MES / Potassium silicate (Kasil) media amendment on colour of rhubarb plantlets.

There were no significant differences in health ratings in all treatments except for 10 mL/L potassium silicate with MES where plants were much more unhealthy (Table 11; Figure 24).

Table 11. Effect of combination of MES / Potassium silicate (Kasil) media amendment on health of rhubarb plantlets.

| MES  | Kasil (mL/L) | Health <sup>A</sup> |   |
|------|--------------|---------------------|---|
| 0    | 0            | 3.6                 | a |
| 0    | 1.5          | 3.8                 | a |
| 0    | 5            | 3.5                 | a |
| 0    | 10           | 3.5                 | a |
| 0.01 | 0            | 4.0                 | a |
| 0.01 | 1.5          | 3.7                 | a |
| 0.01 | 5            | 3.3                 | a |
| 0.01 | 10           | 2.3                 | b |
| SEM  |              | 0.27                |   |

<sup>A</sup> 1- Dead, 2- brown/pale green, 3- pale green, 4- vigorous /dark green

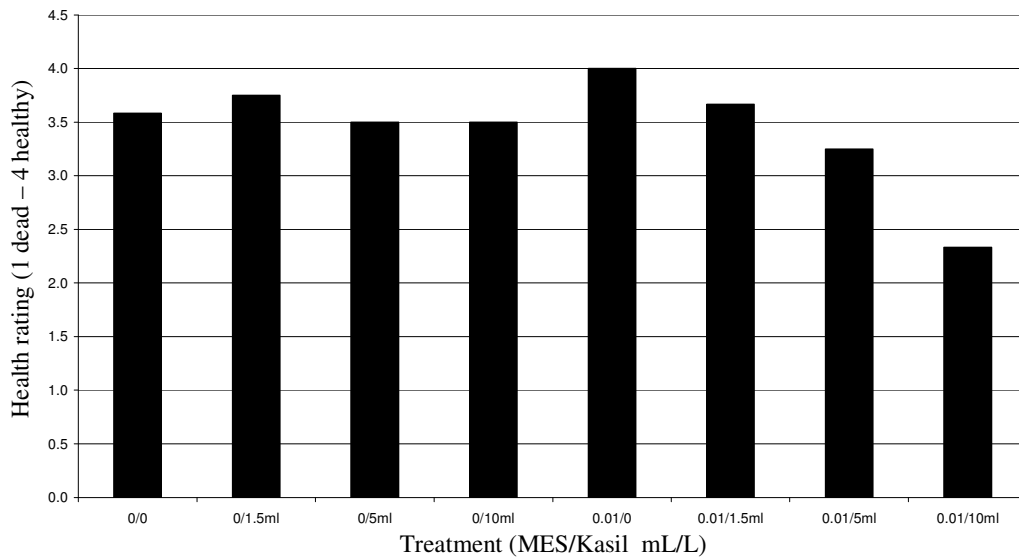


Figure 24. Effect of combination of MES / Potassium silicate (Kasil) media amendment on health rating of rhubarb plantlets.

The addition of potassium silicate did not increase multiplication of rhubarb with or without the buffer MES although the highest multiplication rate was with potassium silicate at 1.5 or 5 mL/L. Plants did not grow as well on MES amended media or with 10 mL/L potassium silicate. The fresh weight of rhubarb was significantly higher on media with 5 mL/L potassium silicate added compared to other treatments. Value of using potassium silicate to improve multiplication of cultivar “Big Red” is not warranted since standard media induced acceptable levels of multiplication (4 times) but may have application with other rhubarb cultivars.

## **5.4 Optimising methods of acclimatisation**

### **5.4.1 Evaluating the level of solidifying agent phytigel to optimise *in-vitro* rooting and survival.**

#### **5.4.1.1 Introduction**

Subsequent research in this project has developed rhubarb micropropagation to commercially viable levels; however transfer success from rooting media to potting media is inconsistent. Several problems have been identified at the transfer process, the principal one being removal of plantlets from tissue culture tubs is difficult and time consuming due to fragile roots and their breakage. Root breakage is particularly prominent if roots are long and tangled. Plants with damaged roots are more susceptible to fungal or bacterial attack and large losses can occur. The objective of this work was to improve root development and the deflasking and weaning process, so as to improve production efficiency and plant survival. This experiment investigated effect of phytigel concentrations in the rooting media to evaluate the best level to ensure transfer success and survival.

#### **5.4.1.2 Materials and methods**

##### In-vitro development

The parent stock culture derived from the cultivar 'Big Red', were initially grown and multiplied *in vitro* on a medium of 4.43 g/L Ms (Murashige and Skoog 1962) salts, with the addition of 2.5 mg/L benzyl amino purine (BAP), 20 g/L sucrose and solidified with 2.5 g/L phytigel. Four hundred healthy rhubarb plantlets were selected for their uniformity in size and divided between treatments and replicates.

To study the effects of phytigel concentration on plant growth and transfer ease, four treatments, each using one hundred healthy, well-developed rhubarb plantlets per treatment, were subcultured onto Ms salts, with the addition of 0.5 mg/L of indole acetic acid (IAA) to promote root development, 20 g/L sucrose and either respectively of 1.0, 1.5, 2.0 or 2.5 g/L phytigel. The pH of the medium was adjusted to 6.0 prior to autoclaving. The media was sterilised by autoclaving at 121°C for 20 minutes. At time of culture into rooting medium, excess leaves were removed from rhubarb plantlets leaving two to three leaves at the tip of a shoot. These were cut back to approximately 1.5 cm and placed into a 500 mL tub containing approximately 100 mL rooting media (ten plants per tub). Tubs were then placed randomly in a growth room at 28°C±2, under 8 hour light at a photon flux density of 60 μmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent).

##### Transfer and acclimatisation

Plantlets were grown *in-vitro* for three weeks, after which time plants were removed from culture tubs into a water-filled container and the roots gently washed to remove media. At this stage plants were assessed for their development, and transferred into 42 cell seedling trays filled to approximately 3 mm from the top with an equal mix of vermiculite/perlite. The seedling trays were watered, covered with plastic vented propagation covers (Hortico) and placed in a high humidity glasshouse at 27°C. After one week, plants were hardened off by gradual opening of cover vents and at two weeks covers were completely removed.



Approximately two months after transfer into seedling trays, three plants were randomly selected from each treatment and destructively sampled to measure total plant weight, leaf and root weight, leaf number and length, colour and overall appearance (disease, discolouration, vigour etc), and survival. After transfer into the glasshouse, no disease was detected from all treatments and replicates. A replication of the experiment was performed one week after the initial start.

#### 5.4.1.3 Results and discussion

##### Plant growth *in-vitro*

Although plantlets were selected for uniformity in size and appearance, the average mean start plant weight was significantly different between treatments (Table 12,  $P < 0.05$ ), however this difference had little effect on the plant weight at transfer.

Phytigel concentration had a significant effect on plant weight and root numbers. Plants cultured into medium with 1.0 g/L phytigel (treatment 1) had significantly higher mean plant weight of 0.55g, compared to the other three treatments (Table 12,  $P < 0.05$ ). Root induction was observed approximately one to two weeks after culture into the rooting mediums. Root number declined as phytigel concentration increased. The media with 1.0 g of phytigel was the most effective in promoting root formation, averaging 7.7 roots per plant (Table 12,  $P < 0.05$ ). All medium treatments produced plants without roots. The percentage of plantlets with roots and mean root number per plant were similar for media treatments 1 to 3. Treatment 4, with the highest concentration of phytigel (2.5 g/L), had fewer plants with roots at 70.5% and fewer roots per plant at 4.3. Treatment 2 had the highest percentage of plants with roots at 86.8% (Table 12,  $P < 0.05$ ).

Table 12. Effects of phytigel concentration on plant weight, root numbers and root percentage of *in vitro* rhubarb plantlets from starting weight till transfer (mean weight  $\pm$ SE). Values followed by the same letter are not significantly different from each other.

| Treatment            | Start fresh weight (g)          | Fresh Weight at transfer (g)  | Root Breakage (%)  | % with roots | Mean root number            |
|----------------------|---------------------------------|-------------------------------|--------------------|--------------|-----------------------------|
| 1 (1.0 g/L phytigel) | 0.063 $\pm$ 0.002 <sup>a</sup>  | 0.55 $\pm$ 0.021 <sup>b</sup> | 14.5 <sup>a</sup>  | 84.0         | 7.7 $\pm$ 0.39 <sup>a</sup> |
| 2 (1.5 g/L phytigel) | 0.074 $\pm$ 0.002 <sup>bc</sup> | 0.40 $\pm$ 0.015 <sup>a</sup> | 23.7 <sup>ab</sup> | 86.8         | 7.3 $\pm$ 0.37 <sup>a</sup> |
| 3 (2.0 g/L phytigel) | 0.069 $\pm$ 0.002 <sup>c</sup>  | 0.40 $\pm$ 0.016 <sup>a</sup> | 39.0 <sup>b</sup>  | 84.5         | 7.1 $\pm$ 0.37 <sup>a</sup> |
| 4 (2.5 g/L phytigel) | 0.065 $\pm$ 0.002 <sup>d</sup>  | 0.33 $\pm$ 0.013 <sup>c</sup> | 37.5 <sup>b</sup>  | 70.5         | 4.3 $\pm$ 0.32 <sup>b</sup> |

##### Plant performance after transfer

Plantlets sampled two months after planting showed no significant difference in mean fresh plant weight comparing all treatments ( $P < 0.05$ ), which ranged between 3.09g, the lowest for Treatment 3, and 3.80g the highest for Treatment 2 (Table 13). Treatment 2 produced significantly higher mean root weight at 1.36g, nearly twice the weight of that obtained by Treatment 3 at 0.70 (Table 13 and Figure 24).

Table 13. Effects of phytigel concentrations on fresh plant weight, root weight, ease of removal and survival of rhubarb plants, two months after transfer into potting medium (mean weight  $\pm$ SE). Values followed by the same letter are not significantly different from each other.

| Treatment | Fresh Plant Weight (g)       | Fresh Root Weight (g)         | Mean Ease of Removal rating (1-4) | Survival (%)                 |
|-----------|------------------------------|-------------------------------|-----------------------------------|------------------------------|
| 1         | 3.21 $\pm$ 0.23 <sup>a</sup> | 0.72 $\pm$ 0.08 <sup>a</sup>  | 4.0 $\pm$ 0.06 <sup>a</sup>       | 56 $\pm$ 0.035 <sup>a</sup>  |
| 2         | 3.80 $\pm$ 0.26 <sup>a</sup> | 1.36 $\pm$ 0.17 <sup>bc</sup> | 3.8 $\pm$ 0.06 <sup>b</sup>       | 74 $\pm$ 0.032 <sup>b</sup>  |
| 3         | 3.09 $\pm$ 0.20 <sup>a</sup> | 0.70 $\pm$ 0.06 <sup>a</sup>  | 2.5 $\pm$ 0.06 <sup>c</sup>       | 64 $\pm$ 0.034 <sup>ab</sup> |
| 4         | 3.39 $\pm$ 0.23 <sup>a</sup> | 0.97 $\pm$ 0.12 <sup>ac</sup> | 2.6 $\pm$ 0.06 <sup>c</sup>       | 66 $\pm$ 0.034 <sup>ab</sup> |

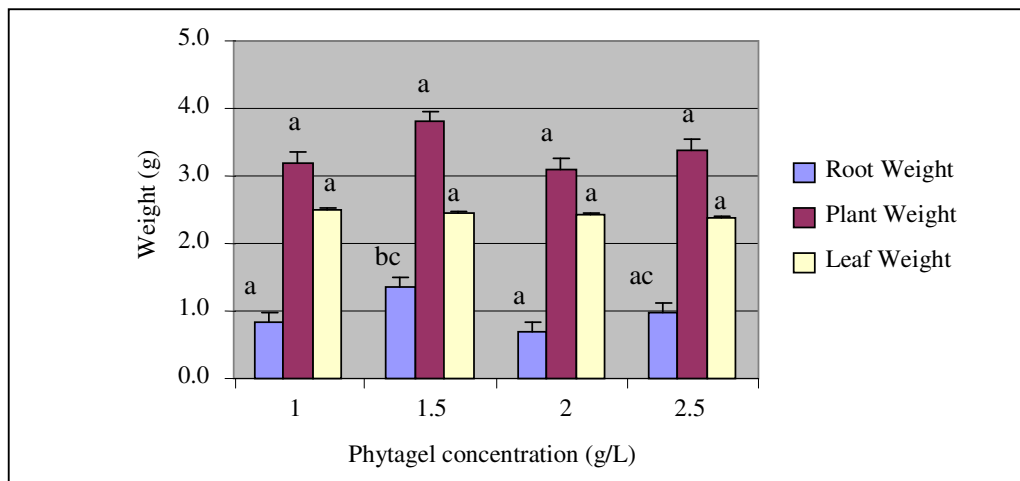


Figure 24. Effects of phytigel concentration on mean fresh plant, root and leaf weight, two months after transfer. Vertical bars are  $\pm$ S.E. Values followed by the same letter are not significantly different from each other.

#### Ease of removal

There were significant differences between phytigel treatments for ‘Ease of removal’ rating (Table 13 and Figure 25,  $P < 0.05$ ). The soft media of Treatment 1 containing 1.0g phytigel rated the best in ‘Ease of Removal’. Treatment 1 also had the least root breakage at removal at 14.5% (Table 12). However, Treatment 1 proved difficult to work with during culture due to its soft consistency. Plantlets cultured in rooting medium with 1.5 g/L phytigel were easy to remove, but some root loss did occur, nevertheless Treatment 2 showed significant better survival percentage (Table 13,  $P < 0.05$ ). Plantlets in Treatments 3 and 4 were difficult to remove and suffered the most root breakage at approximately 40% breakage.

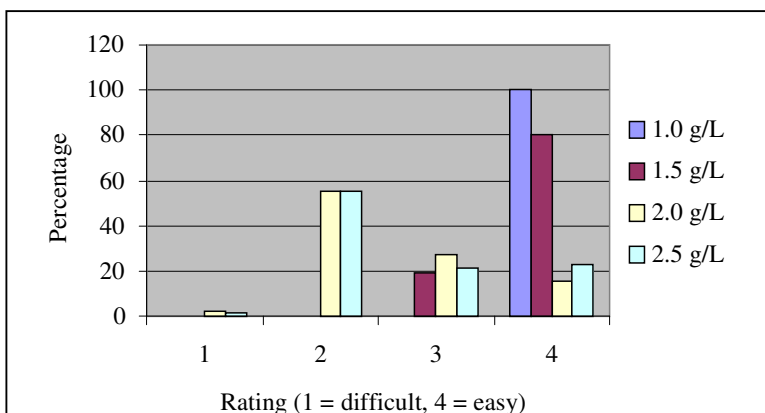


Figure 25. Ease of removal rating for rhubarb from rooting media.

### Plant survival

Two months after transfer plantlet survival for all treatments was investigated, including those with and without roots at transfer (Figure 26; Figure 27). Treatment 2 had the highest survival percentage of 74, as compared to Treatments 1, 3 and 4 with 56%, 64%, and 66% respectively.

Plant survival from plants with and without roots was examined. Figure 26 shows that plantlets transferred into potting media with roots had a maximum survival rate of just over 90% for Treatment 2 and that there were no significant differences between Treatments 1, 3 and 4 (Table 13,  $P < 0.05$ ). Of the plants with no roots at planting Treatment 3 had a significantly higher survival rate of over 60%, compared to 1, 2 and 4 at approximately 40%.

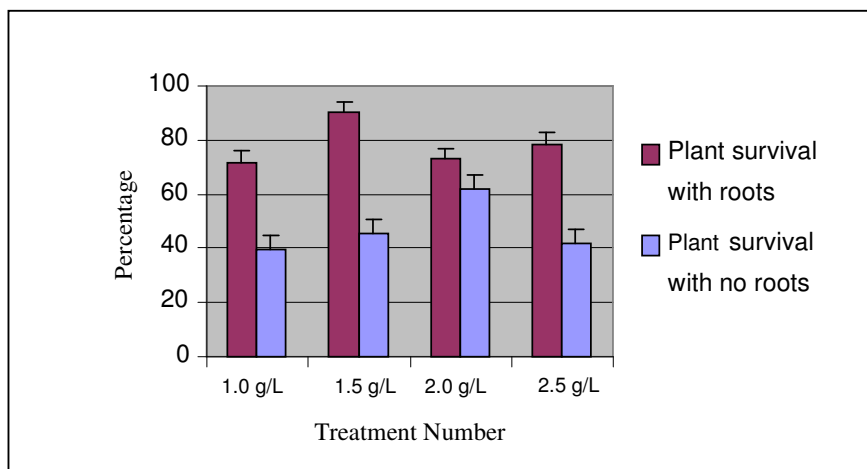


Figure 26. Plant survival with and without roots.

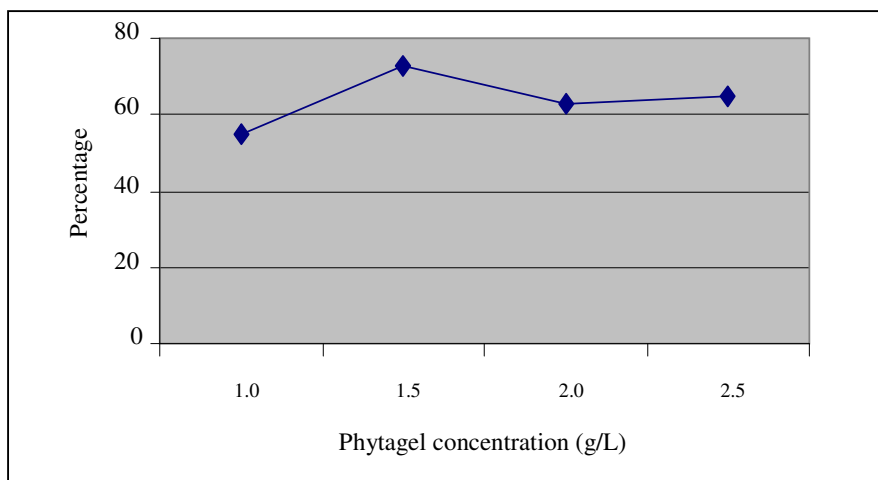


Figure 27. Overall plant survival combined

#### 5.4.1.4 Discussion

Phytigel concentration significantly affected plant weight and root number *in vitro*. Plant weight was significantly better for plants grown in media with 1.5 and 2.0 g/L phytigel. Root number declined as phytigel concentration increased. However, plant survival was affected more by the transfer and acclimatisation process than effects of rooting medium demonstrated by the significant difference in survival percentages shown in Table 13.

Plantlet age, vigour, environmental conditions or other factors could be responsible for the variation in survival rates. Selecting healthy disease-free plantlets for subculturing and healthy strong plantlets for transfer was an important factor in the development of the rooting protocol. Weak plants have less vigour for root development and growth and are more susceptible to pathogens. Plants are more susceptible to pathogens for the first few weeks after transfer. Therefore, when taking plants out of tissue culture tubs and planting into potting substrate, it is important not to damage plant roots and tissue.

To obtain a measure of transfer efficiency and understand the subsequent affect of transfer on acclimatisation, results from the 'Ease of Removal' rating, root breakage and survival were compared. The firm consistency media (2.0 and 2.5 g/L phytigel) were the most difficult for plant removal as Phytigel stuck to roots and high root loss occurred. The softer consistency media (1.0 and 1.5 g/L phytigel) sped the transfer process by allowing plants to come freely from the media, without the need to remove caked-on phytigel and with less root breakage.

### 5.4.2 Determining optimum duration for *in vitro* for rooting of rhubarb for best growth and survival

#### 5.4.2.1 Introduction

Due to virus disease problems currently affecting rhubarb production and lack of disease free planting material, the use of tissue culture as a means of supplying clean planting material to virus affected rhubarb farms will allow rhubarb growers access to superior planting material. The use of commercial laboratories and nurseries to supply

the large quantities of rhubarb plants required can ensure the sustainable production of rhubarb. Although multiplications of rhubarb and virus elimination by micro-propagation are well established, acclimatisation and subsequent survival of plantlets after transfer from *in vitro* condition to glasshouse conditions has been inconsistent.

Subsequent research in this project identified a suitable rooting medium for root development and transfer ease. This experiment aimed to determine if duration of plants in rooting media could affect ease of transfer and acclimatisation and if so identify the duration that improved success of plantlet establishment. Four treatment durations of either 1, 2, 3 or 4 weeks were compared.

#### **5.4.2.2 Materials and methods**

##### *in vitro* development

The parent stock culture derived from the cultivar 'Big Red', were initially grown and multiplied *in vitro* on Murashige and Skoog (Murashige and Skoog 1962) media, with the addition of 2.5 mg/L benzyl amino purine (BAP), 20 g/L sucrose and solidified with 2.5 g/L phytigel. pH was adjusted to 6.0 prior to autoclaving.

Four hundred healthy rhubarb plantlets were selected for their uniformity in size and divided between treatments and replicates.

Four treatments, each using one hundred healthy, well-developed rhubarb plantlets per treatment, were subcultured onto Ms salts, with the addition of 0.5 mg/L of indole acetic acid (IAA) to promote root development, 20 g/L sucrose and 1.5g/L phytigel. The pH of the medium was adjusted to 6.0 prior to autoclaving. The media was sterilised by autoclaving at 121°C for 20 minutes. At time of culture into rooting medium, excess leaves were removed from rhubarb plantlets leaving two to three leaves at the tip of a shoot. These were cut back to approximately 1.5 cm and placed into a 500 mL tub containing approximately 100 mL rooting media (ten plants per tub). Tubs were then placed randomly in a growth room at 28°C±2, under 8 hour light at a photon flux density of 60 µmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent).

##### Transfer and acclimatisation

Plantlets were grown *in vitro* for one, two, three or four weeks, after which time plants were removed from culture tubs into a water-filled container and the roots gently washed to remove media. At this stage plants were assessed for their development, and transferred into 42 cell seedling trays filled to approximately 3 mm from the top with an equal mix of vermiculite/perlite. The seedling trays were watered, covered with plastic vented propagation covers (Hortico) and placed in a high humidity glasshouse at 27°C. After one week, plants were hardened off by gradual opening of cover vents and at two weeks covers were completely removed.

#### **5.4.2.3 Results and discussion**

As expected mean plant weight significantly increased with increased time *in vitro*. Plants one week in rooting media had the lowest mean plant weight of 0.14 g increasing to 0.74 g for four weeks (Table 14, *P* < 0.05). At one week in rooting medium, plants had just formed very small initial roots.

Leaf weight was significantly different for all treatments (Table 14,  $P < 0.05$ ). The best mean root and leaf weights at transfer were produced by plants subjected to three and four weeks in the rooting media. It is interesting to note that most root development occurred from one to two weeks in media where mean root number increased from 1.39 to 5.17, an increase of over 250%, and then slowed to approximately 25% for three and four weeks in media to 6.26 and 6.96 respectively.

Table 14. Effects of duration *in vitro* until transfer (mean fresh weight  $\pm$ SE). Values followed by the same letter are not significantly different from each other.

| Time <i>in-vitro</i> | Start fresh plant weight (g) | Plant fresh weight at transfer (g) | Root fresh weight at transfer (g) | Leaf fresh weight at transfer (g) | Mean root number at transfer |
|----------------------|------------------------------|------------------------------------|-----------------------------------|-----------------------------------|------------------------------|
| 1 week               | 0.05 $\pm$ 0.002 a           | 0.14 $\pm$ 0.007 a                 | 0.01 $\pm$ 0.00 ab                | 0.14 $\pm$ 0.01 a                 | 1.39 $\pm$ 0.13 a            |
| 2 weeks              | 0.06 $\pm$ 0.002 ab          | 0.34 $\pm$ 0.013 b                 | 0.09 $\pm$ 0.01 a                 | 0.26 $\pm$ 0.02 b                 | 5.17 $\pm$ 0.28 b            |
| 3 weeks              | 0.06 $\pm$ 0.002 b           | 0.57 $\pm$ 0.020 c                 | 0.18 $\pm$ 0.02 b                 | 0.41 $\pm$ 0.03 c                 | 6.26 $\pm$ 0.28 c            |
| 4 weeks              | 0.06 $\pm$ 0.002 ab          | 0.74 $\pm$ 0.027 d                 | 0.16 $\pm$ 0.02 ab                | 0.54 $\pm$ 0.04 d                 | 6.96 $\pm$ 0.30 c            |

One month after transfer into potting media, plant development was noticeably better for plants which were in rooting media for three weeks as shown in Table 15 and Figure 28. In this period of time, leaf weight, root weight and leaf number were better than the plants subjected to the other time period treatments.

Table 15. Effects of time period *in-vitro* on plant, one month after transfer (mean fresh weight  $\pm$ SE). Values followed by the same letter are not significantly different from each other

| Time <i>in-vitro</i> | Plant weight (g)              | Leaf weight (g)               | Root weight (g)               | Plant Height                   | Leaf Number       |
|----------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|-------------------|
| 1 week               | 2.26 $\pm$ 0.12 <sup>ab</sup> | 1.81 $\pm$ 0.09 <sup>ab</sup> | 0.45 $\pm$ 0.05 <sup>a</sup>  | 12.27 $\pm$ 0.29 <sup>ab</sup> | 4.2 <sup>a</sup>  |
| 2 weeks              | 2.54 $\pm$ 0.14 <sup>a</sup>  | 2.05 $\pm$ 0.11 <sup>a</sup>  | 0.49 $\pm$ 0.04 <sup>a</sup>  | 11.32 $\pm$ 0.27 <sup>a</sup>  | 5.0 <sup>b</sup>  |
| 3 weeks              | 1.91 $\pm$ 0.09 <sup>b</sup>  | 1.60 $\pm$ 0.08 <sup>b</sup>  | 0.30 $\pm$ 0.03 <sup>b</sup>  | 13.41 $\pm$ 0.34 <sup>c</sup>  | 4.9 <sup>bc</sup> |
| 4 weeks              | 1.99 $\pm$ 0.11 <sup>b</sup>  | 1.58 $\pm$ 0.08 <sup>b</sup>  | 0.41 $\pm$ 0.04 <sup>ab</sup> | 13.36 $\pm$ 0.37 <sup>bc</sup> | 4.5 <sup>ac</sup> |

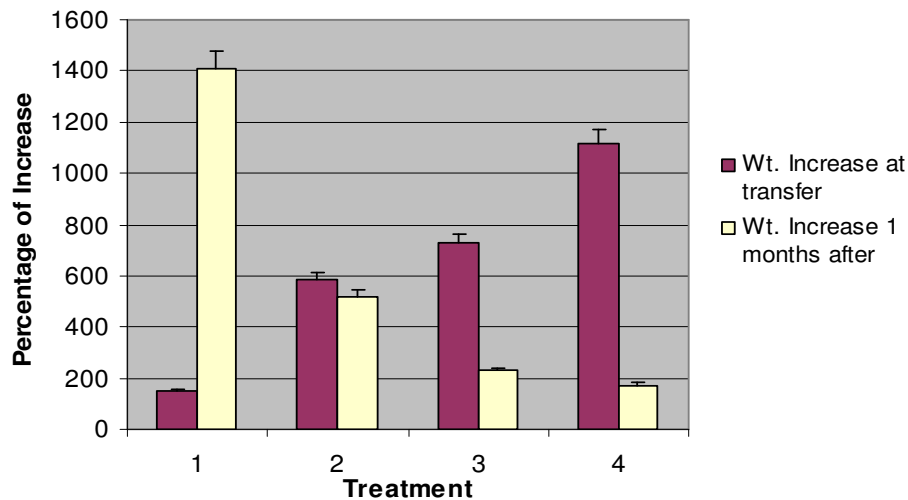


Figure 28. Comparisons of plant weight at transfer and at one month after growing *in vitro* for 1 to 4 weeks.

Data taken at time of transfer showed plants subjected to 1 week *in vitro* had the highest percentage of short roots (less than 5 mm) and the lowest mean root number at 1.5. This allowed easy transfer from containers, plus no root breakage was recorded due to the short roots (Table 16). Roots lengths progressively increased with time *in vitro* and as expected simultaneously so too did root damage. Plants *in vitro* for 1 week had only 1 plant with roots >50 mm, while those *in-vitro* for 4 weeks had the highest number at 120 (data not shown). Plants *in vitro* for 4 weeks suffered 35% root damage as compared to 0% for plants *in vitro* for 1 week.

Table 16. Effects of time *in-vitro* on plant root number, breakage at transfer, survival of plants with and without roots and plant survival one month after transfer. Values followed by the same *letter* are not significantly different from each other.

| Time <i>in-vitro</i> | Mean root number | % with broken roots | Survival % of plants with no roots | Survival % with roots | Total Plant Survival (%) |
|----------------------|------------------|---------------------|------------------------------------|-----------------------|--------------------------|
| 1 week               | 1.5 a            | 0.0 a               | 71.5                               | 87.4                  | 76.0 a                   |
| 2 weeks              | 5.2 b            | 5.0 a               | 61.5                               | 82.1                  | 81.9 a                   |
| 3 weeks              | 6.3 c            | 16.5 b              | 61.5                               | 80.7                  | 91.9 b                   |
| 4 weeks              | 7.0 c            | 35.0 c              | 40.0                               | 87.4                  | 74.4 a                   |

#### Plant survival

Plants with roots performed better than those without roots, with approximately 80% survival. However there were treatment effects on survival percentage from plants without roots. Plants that were only 1 week *in vitro* and had no roots had the highest survival rate at 71.5%. Plants 4 weeks *in vitro* had the lowest at 40%. This suggests that with increasing time *in vitro*, plantlets that have not induced root growth have a decreasing chance of surviving acclimatisation.

From the results in Table 16, Treatment 2 shows overall better growth. However from the data acquired one month after transfer, Treatment 3 out performs Treatment 2 as the most successful treatment with 91.9% survival success; this is significantly better than the other treatments ( $P < 0.05$ ).

Plantlets in the rooting media for one week were easier to remove, compared to those in the rooting media for four weeks due to their longer and often tangled roots. Minimal or no root and tissue damage means no requirement for further sterilisation or fungicides. Root development slowed considerable after three weeks, when leaf development increased. This suggests that leaf growth becomes prominent over root growth and that the rooting media plays less of a role in plant development and photosynthesis plays an increasing role. This highlights the advantage of transfer at three weeks.

For the large scale production of rhubarb plantlets, high survival rates can be achieved by using the protocol described in this report, using 1.5 g/L phytagel in the MS medium (determined in the previous report) and transferring plants at three weeks. This protocol produces sufficient plant growth, improves the deflasking and transfer procedure, reduces root damage, improves production efficiency, and reduces production costs.

### **5.4.3 Identifying effective rate of the auxin Indole Acetic Acid for rooting of rhubarb.**

#### ***5.4.3.1 Introduction***

To ensure best root development it was necessary to determine whether the auxin Indole Acetic Acid (IAA) was required for root development and if so what amount was required for best development. This was required so that rhubarb plants produced commercially would survive acclimatisation and grow well.

#### ***5.4.3.2 Materials and methods***

The rhubarb selection “Big Red” sourced from Mt Tamborine, Queensland for use for this experiment. Leaves from all crowns provided were indexed for virus. All plants were found to be virus free. All crowns were initiated into culture with apical growing points and buds were excised from the crowns and rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Buds were cut back to remove bleached tissue and placed into (Murashige and Skoog basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytigel (MSM). pH was adjusted to 6.0 prior to autoclaving. The buds were placed in culture room at 25°C±2 and grown in darkness for 5 days and then grown in vented containers under 16 hour day and 8 hour night, at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent). When cultures were multiplying uniform individual plantlets were grown on MSM the following a range of different types of media to compare root initiation and development and survival after deflasking and acclimatisation.

The trial compared 5 doses of IAA (0, 0.25, 0.5, 0.75 and 1 mg/L). Five tubs with each tub containing 10 plants were used for each treatment. Start fresh weight (g) was recorded when uniform explants were placed into various culture treatments. Plantlets were grown *in vitro* for three weeks, after which time plants were removed from culture tubs into a water-filled container and the roots gently washed to remove media. At this stage (3 weeks), plants were assessed for their development by recording fresh weight (g), number and fresh weight of roots, length of roots (small 10 mm or less, medium 11-20 mm, large more than 20 mm), if there was secondary branching of roots and number of leaves at 3 weeks and transferred into 42 cell seedling trays filled to approximately 3 mm from the top with an equal mix of vermiculite/perlite. The seedling trays were watered, covered with plastic vented propagation covers (Hortico) and placed in a high humidity glasshouse at 27°C. After one week, plants were hardened off by gradual opening of cover vents and at two weeks covers were completely removed.

Six weeks after transfer into seedling trays, three plants were randomly selected from each of the 5 tubs per treatment (15 plants) and destructively sampled to measure total plant weight, leaf and root weight, leaf number. Eight weeks after transfer into seedling trays when all plants were well established survival was measured. After transfer into the glasshouse, no disease was detected from all treatments and replicates.



Data were divided into a number of groups according to the type of measurement and number of plants assessed. These data were analysed by analysis of variance, with treatments as IAA levels and experiments, tubs within experiments and plants within tubs as blocking effects. The treatment effect was assessed against variation between tubs within experiments. The treatment effect was also split into polynomial effects – linear, quadratic and lack of fit.

Survival was measured after plants had been deflasked and acclimatised at 11 weeks as a binomial variable (yes/no) on all plants. Analysis was using generalised linear models for a binomial distribution with a logit link. Terms fitted were experiment and IAA level. The significance of experiment was tested by dropping it from this model. Experiment was significant ( $P=0.002$ ). Similarly the significance of IAA level was tested. It was not significant ( $P=0.214$ ).

#### 5.4.3.3 Results and discussion

There was no difference of starting fresh weights for any treatment. At three weeks there was no difference in number of leaves (average 3.0).

At week one, there was no difference in number of plants with roots between treatments, by week two onwards the control (0 IAA) and 0.75 mg/L IAA treatments had significantly more plants with roots with 1 mg/L IAA treatment having the least (Figure 29).

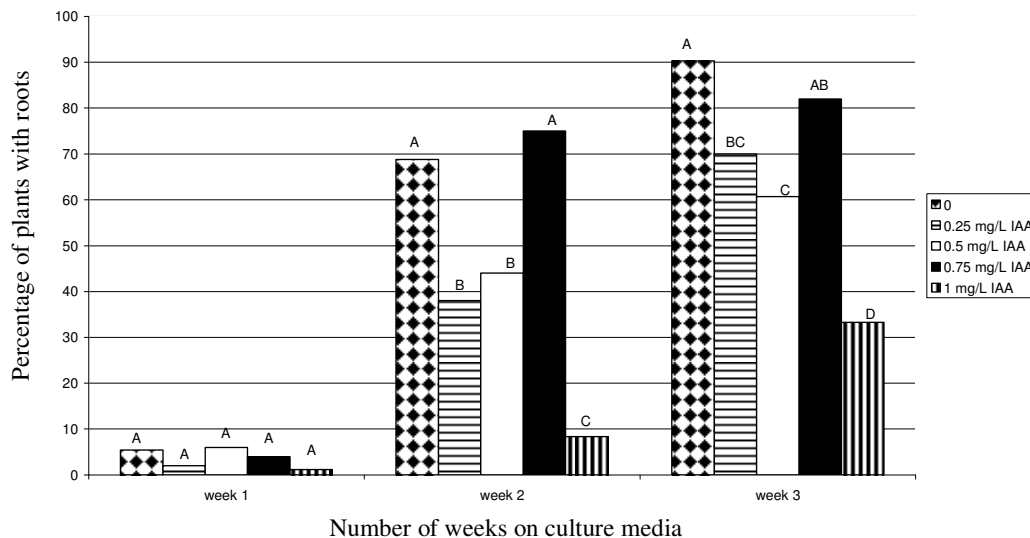


Figure 29. Percentage of rhubarb plants with roots over three weeks when cultured on media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P<0.05$ )

At three weeks on various culture treatments 0.75 mg/L and 1 mg/L IAA treatments produced plants with the most fresh weight and the control (0 IAA) the lowest (Figure 30).

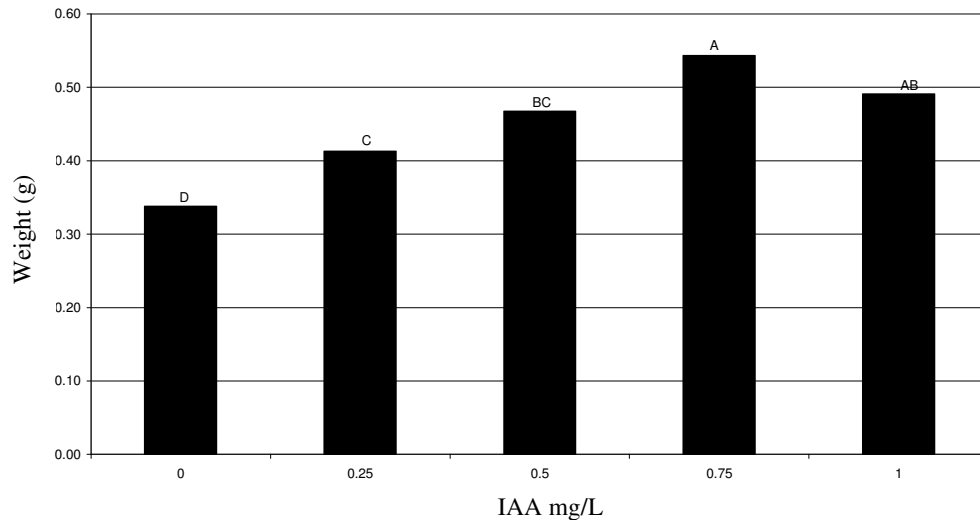


Figure 30. Total plant fresh weight of rhubarb grown for three weeks on media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

The total plant root weight varied between treatments by three weeks, with 0.75 mg/L IAA having significantly more root weight and 1 mg/L IAA least root weight with other treatments the same (Figure 31).

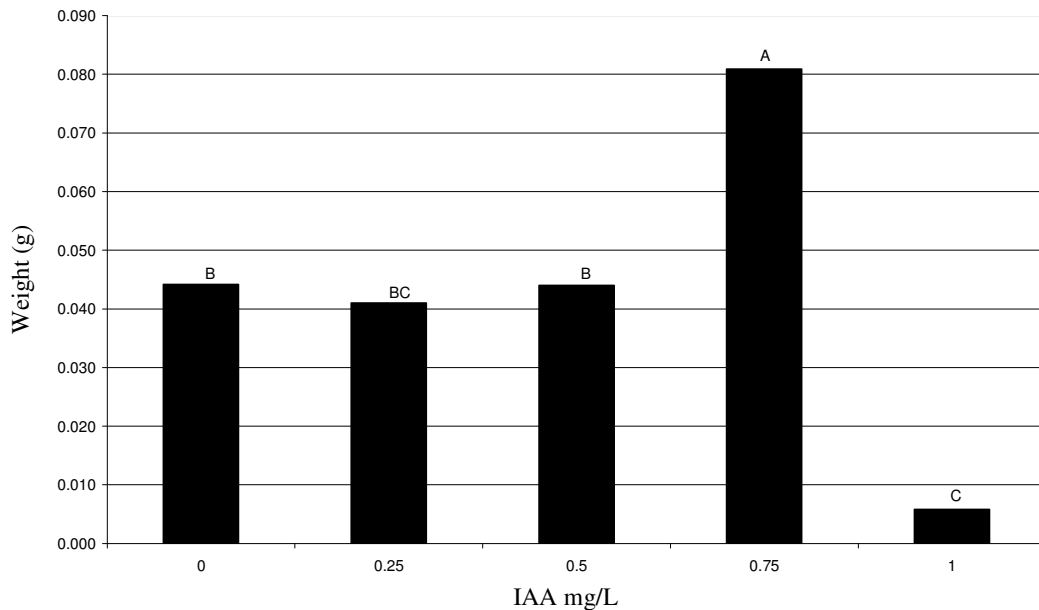


Figure 31. Fresh weight of rhubarb roots after three weeks on media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

After three weeks in culture there were also differences in root development. The control (0 IAA) and 0.75 mg/L IAA treatments had the longest roots (Figure 32).

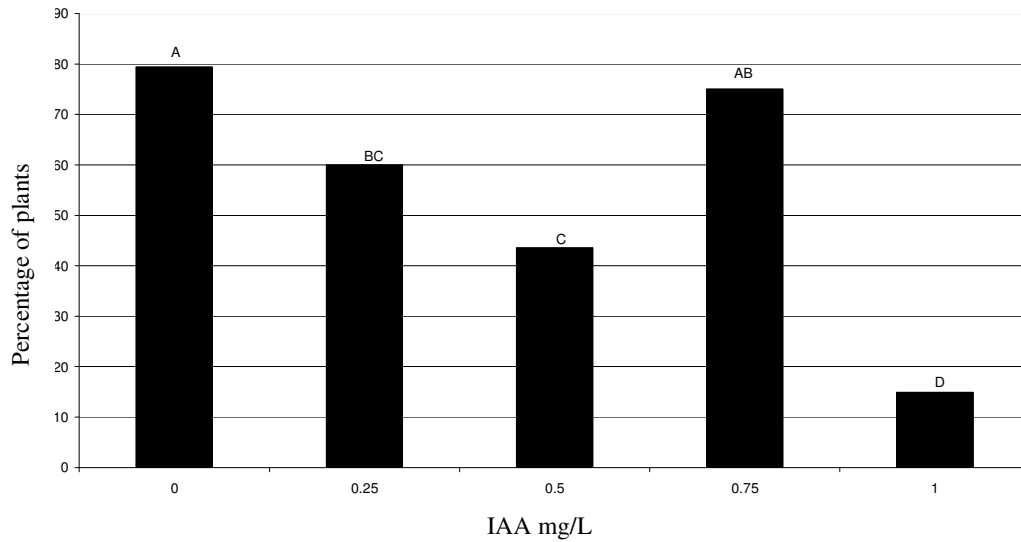


Figure 32. Percentage of rhubarb plants with roots longer than 10 mm after three weeks on media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

By three weeks in culture the control (0 IAA) and 0.75 mg/L treatments produced significantly more plants with secondary root branching (Figure 33).

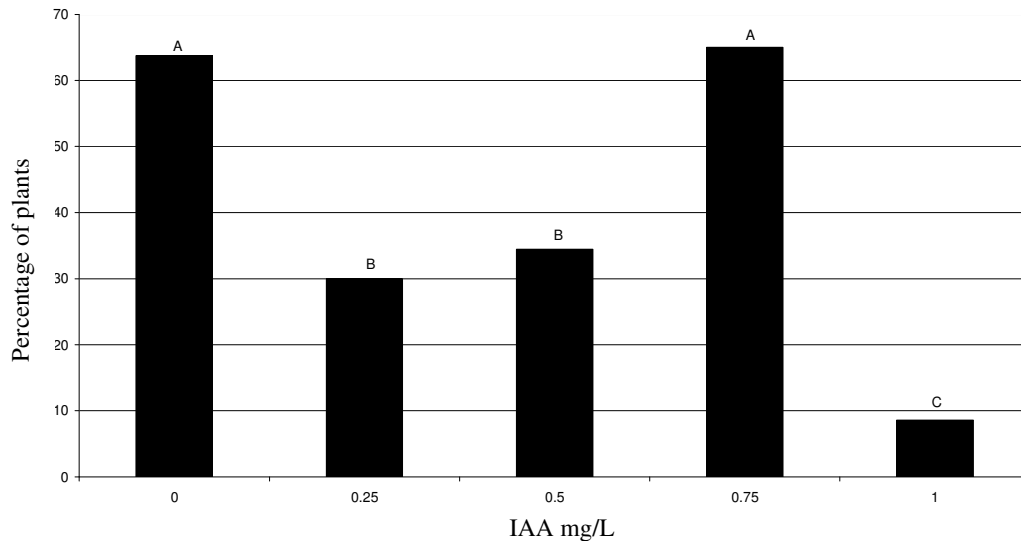


Figure 33. Percentage of rhubarb plants with secondary root development after three weeks on media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

At the end of the three week culture period the 0.75 mg/L IAA treatment showed consistently best results in fresh plants and root weight, number of plants with roots and size and development of roots. The control treatment (0 IAA) also produced

similar root development as the 0.75 mg/L IAA treatment but produced plants with lower plant and root fresh weight.

The aim of this research was ultimately to determine which treatment produced plants that would survive and grow after the acclimatisation process, which is a critical step in the tissue culture cycle. When plants were established at 11 weeks there was no difference in plant height between treatments. There was some variation of number of leaves produced with significantly less in the 0.25 mg/L IAA treatment (4.2 leaves) the other treatments were similar and ranged from 4.6 to 5.3 leaves with the highest treatment being the 1mg/L IAA followed by the control treatment.

The fresh plant weight was highest in the 0.75 and 1 mg/L IAA treatments (Figure 34).

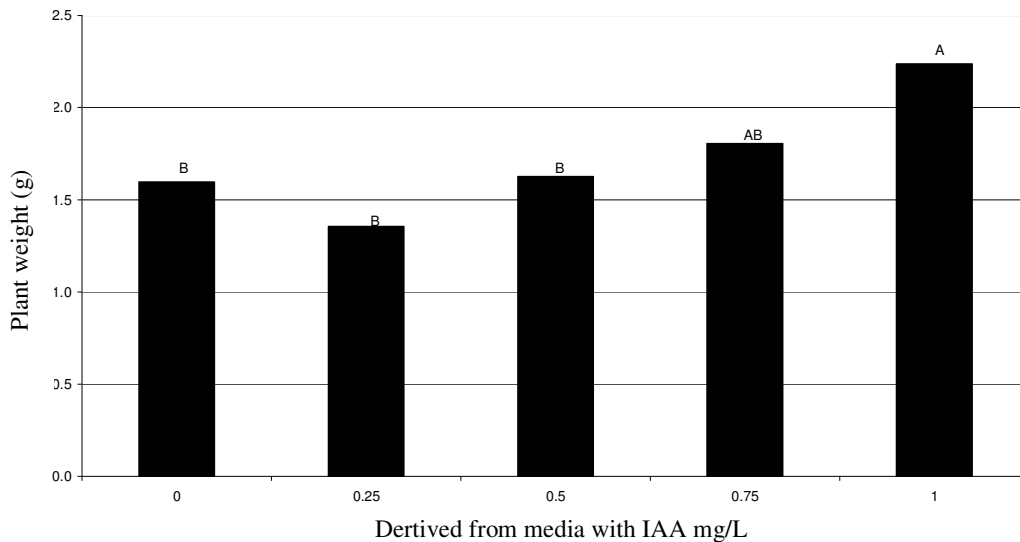


Figure 34. Rhubarb plant fresh weight at 11 weeks derived from tissue culture on various Indole Acetic Acid treatments. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

By 11 weeks the fresh root weight was highest in the 0.75 and 1 mg/L IAA treatments (Figure 35).

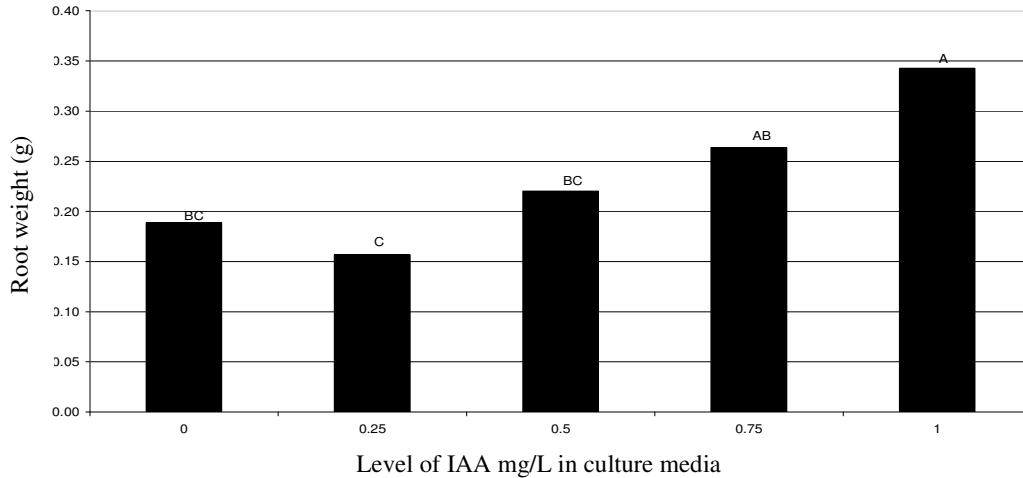


Figure 35. Fresh weight of rhubarb roots acclimatised at 11 weeks derived from media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

There was no significant difference in survival after acclimatisation of plants derived from any of the culture treatments (Figure 36).

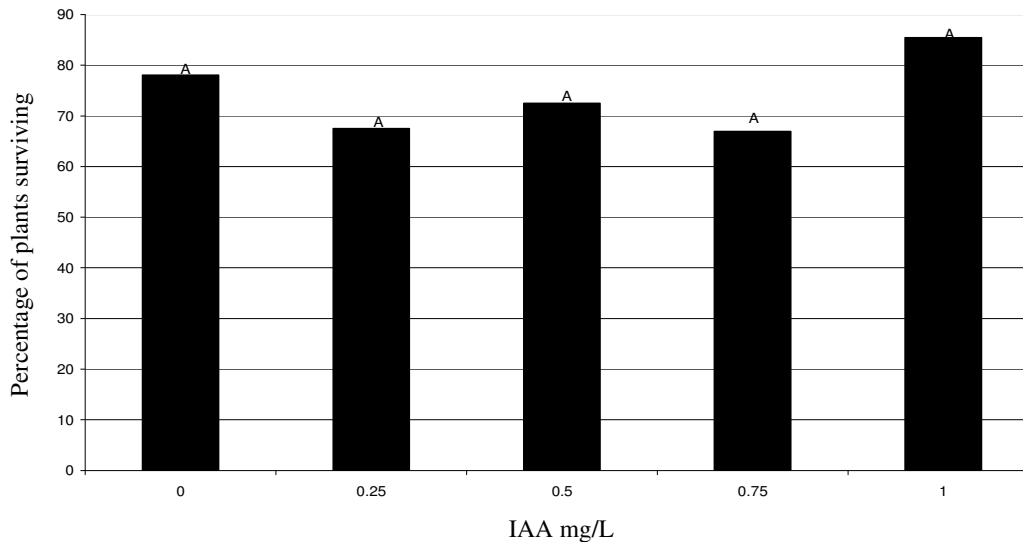


Figure 36. Survival of acclimatised plants at 11 weeks after previous culture in media amended with Indole Acetic Acid. Same letters indicate no significant difference, different letters indicate significant difference ( $P < 0.05$ )

Best root development was achieved on media with 0.75 mg/L IAA or no IAA and poorest root development seen in media with 1.0 mg/L IAA. However by 11 weeks when plants had acclimatised highest fresh and root weight was produced in plants grown on 1 mg/L IAA followed by 0.75 mg/L IAA. Development of roots on explants during the culture period was not essential for subsequent growth or survival of plants. Survival was the same for all treatments and was highest in the 1 mg/L IAA,

the treatment that produced the plants with fewest and smallest roots during the culture process. All treatments produce plants that survived at a acceptable range from 67% to 85%. No IAA was needed for plants to produce good roots and high survival but highest plant fresh weight was achieved when plants were cultured with 1 mg/L IAA and although not significantly better this treatment also gave the highest number of surviving plants (over 80%). It is recommended that IAA at 1 mg/L be used for rooting and acclimatisation of rhubarb.

#### **5.4.4 Investigation of multiplication rate across a subset of Australian rhubarb varieties.**

##### ***5.4.4.1 Introduction***

There is little known about the range of varieties used across Australia and little known about how they would perform in tissue culture. This experiment sourced rhubarb varieties from across Australia and compared their performance in tissue culture. Considering that these varieties may be needed in commercial production to manage disease, it is important that we understand if they can be readily micropropagated or if they are recalcitrant in tissue culture. The Murashige and Skoog (1962) procedure was used to successfully micropropagate “Big Red” the cultivar used in the previous research. This Murashige and Skoog medium and modifications of it were applied and compared to optimise multiplication of a subset of Australian rhubarb selections.

##### ***5.4.4.2 Materials and methods***

To obtain rhubarb material for this experiment a request went out to rhubarb representatives from all the Australian rhubarb producing states asking for access to their rhubarb varieties. Growers were asked to select plants that were healthy in appearance to have increased chance of obtaining virus free plants. Table 17 lists the selections that were provided for further research.

Table 17. Rhubarb selections used for further tissue culture micropropagation research.

| State of origin | Rhubarb selection   |
|-----------------|---------------------|
| Tasmania        | Burgess’s selection |
| South Australia | Driver’s Cherry Red |
| New South Wales | Shelley’s selection |
| Victoria        | Arnott’s Cherry Red |
| Queensland      | Eden’s Big Red      |
| Queensland      | Success             |

Leaves from all crowns provided were indexed for virus. All plants were found to be virus free. All crowns were initiated into culture with apical growing points and buds were excised from the crowns and rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds were removed from the running tap water they were placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Buds were cut back to remove bleached tissue and placed into (Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytagel .

pH was adjusted to 6.0 prior to autoclaving. The buds were placed in culture room at 25°C±2 and grown in darkness for 5 days and then grown in vented containers under 16 hour day and 8 hour night, at a photon flux density of 60 µmol m<sup>-2</sup> s<sup>-2</sup> (Philips TLD 36W/33 cool white fluorescent). When cultures were multiplying, individual plantlets were grown on a range of different types of media to compare multiplication and identify which media was best for specific rhubarb selections (Table 18). The experiments concentrated on improving Shelley’s and Success rhubarb selections since all other cultivars performed well on standard media. In some cases due to lack of “Success” plants (due to the low multiplication rate) this cultivar could not be included in all treatments.

Table 18. Media for multiplication of rhubarb selections.

| Media              | Description                                                                                                              |
|--------------------|--------------------------------------------------------------------------------------------------------------------------|
| MSM                | Murashige and Skoog basal media with 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytagel Standard Media |
| 2 MSM              | MSM but with 5 mg/L BAP                                                                                                  |
| 0.5 MSM            | Half strength basal MSM but with 20 g/L sucrose, 2.5 mg/L BAP                                                            |
| MSM K silicate     | MSM with 2.5mg/L BAP, 5 mL/L potassium silicate                                                                          |
| MSM silicate/IAA   | MSM with 2.5mg/L BAP, 5 mL/L potassium silicate, 0.18 mg/L Indole Acetic Acid (IAA)                                      |
| MSM K sulphate     | MSM with 2.5mg/L BAP, 1.3 g/L potassium sulphate                                                                         |
| MSM K sulphate/IAA | MSM with 2.5mg/L BAP, 1.3 g/L potassium sulphate, 0.18 mg/L IAA                                                          |

Data have been analysed in GenStat using loglinear models. This assumes that number of subcultures follows a Poisson distribution. Number of subcultures produced is analysed, with number of subcultures, transformed by log base e, as an offset. Thus predictions, assuming an offset of 0 (equivalent to number of subcultures = 1), when back-transformed, correspond to multiplication. Significant difference testing between variety means and media means used the LSD procedure.

#### 5.4.4.3 Results and discussion

Rhubarb selections were initiated and multiplied on the standard media that produced high levels of multiplications with healthy plants for the Cultivar ‘Big Red’ that was used in earlier research. There were significant differences in multiplication rate between the rhubarb selections when produced on standard media (Table 19). Most of the selections multiplied at a commercially acceptable level and were similar or better than “Big Red” the cultivar used in this research. Two of the selections (Shelley’s selection and Success) performed poorly and multiplied at a rate that would not support commercial production. Success was particularly slow to multiply. Success is a cultivar of particular interest since no virus infected plants have been found even when they have been growing surrounded by infected plants.

Table 19. Multiplications of rhubarb selections on MSM media (standard).

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Burgess Selection          | 3.1 <sup>ab</sup>   |
| Cherry Red RA              | 3.0 <sup>a</sup>    |
| Cherry Red DD              | 2.9 <sup>b</sup>    |

|                      |                  |
|----------------------|------------------|
| Big Red              | 2.8 <sup>b</sup> |
| Shelley's selection, | 2.2 <sup>c</sup> |
| Success              | 2.0 <sup>c</sup> |

Variety means not followed by a common letter are significantly different (P<0.05).

Further research to improve multiplication of Success is warranted to investigate its use in management of the virus. Further research was undertaken with different types of media to try to improve the multiplication rate. The next media used twice the amount of cytokinin that is used to induce multiplication.

When the rhubarb selections were grown with double cytokinin multiplication rate was improved in Cherry Red RA, Burgess selection, Shelley's selection and Success (Table 20). However as found in the earlier experiment multiplication was lower than at 2.5 mg/L "Big Red" at 5 mg/L BAP level. Multiplication rate of Shelley's selection and Success while it increased with 5 mg/L BAP was still low and multiplication rate was variable.

Table 20. Multiplications of rhubarb selections on 2 MSM media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Cherry Red RA              | 3.5 <sup>a</sup>    |
| Burgess Selection          | 3.5 <sup>ab</sup>   |
| Cherry Red DD              | 3.1 <sup>bc</sup>   |
| Shelley's                  | 2.9 <sup>c</sup>    |
| Success                    | 2.7 <sup>c</sup>    |
| Big Red                    | 2.6 <sup>c</sup>    |

Variety means not followed by a common letter are significantly different (P<0.05).

When rhubarb selections were cultured on MSM at half strength basal media with 2.5mg/L BAP multiplication was reduced significantly for Burgess selection. The performance of the Cherry Red RA, Shelley's and Success on 0.5 MSM was similar to that of MSM which is consistent with the same amount of cytokinin being used in both treatments (Table 21).

Table 21. Multiplication of rhubarb selections on 0.5 MSM media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Cherry Red RA              | 3.1 <sup>a</sup>    |
| Success                    | 2.1 <sup>b</sup>    |
| Shelley's                  | 2.1 <sup>b</sup>    |
| Burgess Selection          | 1.9 <sup>b</sup>    |

Variety means not followed by a common letter are significantly different (P<0.05).

Addition of potassium silicate to MSM improved multiplication of Burgess selection, Cherry Red RA and Shelley's selection and success to similar levels achieved by increasing cytokinin to 5 mg/L as found in the 2MSM treatment (Table 22).

Table 22. Multiplication of rhubarb selections on MSM K silicate media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Burgess Selection          | 3.6 <sup>a</sup>    |
| Cherry Red RA              | 3.5 <sup>a</sup>    |



|           |                   |
|-----------|-------------------|
| Shelley's | 2.6 <sup>ab</sup> |
| Success   | 2.4 <sup>b</sup>  |

Variety means not followed by a common letter are significantly different (P<0.05).

MSM amended with potassium silicate plus IAA improved multiplication of Burgess selection and Cherry red to give similar results to that of MSM with double cytokinin (5 mg/L BAP). Multiplication of Success was not improved and multiplication was very variable (Table 23).

Table 23. Multiplication of rhubarb selections on MSM silicate/IAA media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Burgess Selection          | 3.5 <sup>a</sup>    |
| Cherry Red RA              | 2.8 <sup>a</sup>    |
| Success                    | 2.2 <sup>a</sup>    |

Variety means not followed by a common letter are significantly different (P<0.05).

Rhubarb selections were grown on MSM with potassium sulphate (Table 24) which improved multiplication to similar levels as 2 MSM (5 mg/L BAP) for Cherry Red RA and Cherry Red DD. This treatment improved multiplication of Big Red but greatly improved multiplication of Shelley's selection to the level found when grown on MSM with 5 mg/L BAP.

Table 24. Multiplication of rhubarb selections on MSM K sulphate media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Cherry Red RA              | 3.6 <sup>a</sup>    |
| Big Red                    | 3.1 <sup>a</sup>    |
| Cherry Red DD              | 3.1 <sup>a</sup>    |
| Shelley's                  | 3.0 <sup>a</sup>    |

Variety means not followed by a common letter are significantly different (P<0.05).

When plants were grown with both potassium sulphate and IAA (Table 25) the plants multiplied similarly to when they were grown without IAA. In this experiment Success was included and multiplication was improved but not as much as on MSM with 5 mg/L BAP.

Table 25. Multiplication of rhubarb selections on MSM K /IAA sulphate media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Cherry Red RA              | 3.3 <sup>a</sup>    |
| Cherry Red DD              | 3.1 <sup>a</sup>    |
| Success                    | 2.5 <sup>a</sup>    |

Variety means not followed by a common letter are significantly different (P<0.05).

When comparing performance of the individual rhubarb selections across the various media it was found that there was a wide range for multiplication (Table 26). The rhubarb selection "Big Red" which was used in the virus research and development of effective micropropagation methods performed in the median range. Burgess selection and Cherry Red RA with 4 times multiplication rate were most amenable to micropropagation for all media and would perform well in commercial micropropagation. Cherry Red DD (multiplication rate 3.2) and Big Red

(multiplication rate 3.0) while lower still multiplied at a level that would be commercially viable. Shelley's selection (multiplication rate 1.9) and Success (multiplication rate 1.6) generally performed poorly.

Table 26. Performance of rhubarb selections averaged across all tested media.

| Rhubarb variety selections | Multiplication rate |
|----------------------------|---------------------|
| Burgess selection          | 4.0 <sup>a</sup>    |
| Cherry Red RA              | 4.0 <sup>a</sup>    |
| Cherry Red DD              | 3.2 <sup>b</sup>    |
| Big Red                    | 3.0 <sup>b</sup>    |
| Shelley's selection        | 1.9 <sup>c</sup>    |
| Success                    | 1.6 <sup>c</sup>    |

Variety means not followed by a common letter are significantly different (P<0.05).

However when Shelley's selection and Success were cultured on the MSM treatment with higher levels of the cytokinin BAP (5 mg/L) the multiplication rate increased to over 2.7 times multiplication which is a rate approaching commercial viability.

MSM media amended with extra cytokinin (5 mg/L BAP), potassium silicate or potassium sulphate all induced highest levels of multiplication when all varieties were considered (Table 27).

Table 27. Overall effect of media on multiplication rate considering all varieties.

| Media type                       | Multiplication rate |
|----------------------------------|---------------------|
| MSM K sulphate (2.5mg/L BAP)     | 3.8 <sup>a</sup>    |
| 2MSM (5mg/L BAP)                 | 3.7 <sup>a</sup>    |
| MSM K silicate-(2.5mg/L BAP)     | 3.5 <sup>a</sup>    |
| MSM K sulphate/IAA (2.5mg/L BAP) | 3.1 <sup>ab</sup>   |
| MSM (2.5mg/L BAP)                | 2.3 <sup>b</sup>    |
| 0.5 MSM (2.5mg/L BAP)            | 2.2 <sup>b</sup>    |
| MSM silicate/IAA (2.5mg/L BAP)   | 2.1 <sup>b</sup>    |

Media means not followed by a common letter are significantly different (P<0.05)

But while Table 27 describes effect on multiplication on all the rhubarb generally, the combination of each specific selection on the various media should be considered to determine the best media and this when developing micropropagation methods. Table 28 summarised the media that induced the highest average multiplication for each selection. The results in Table 28 support the general multiplication rate effects described in Table 27.

Table 28. Best media for multiplication of each selection.

| Rhubarb variety selections | Media                                                            | Multiplication rate |
|----------------------------|------------------------------------------------------------------|---------------------|
| Burgess Selection          | MSM with 2.5 or 5mg/L BAP,<br>MSM K silicate with or without IAA | range 3.1 to 3.6    |
| Cherry Red RA              | MSM 5mg/L BAP,<br>MSM K sulphate with or without IAA             | range 3.3 to 3.6    |
| Cherry Red DD              | MSM with 2.5 or 5mg/L BAP,<br>MSM K sulphate with or without IAA | range 2.9 to 3.1    |

|                     |                                       |                  |
|---------------------|---------------------------------------|------------------|
| Big Red             | MSM with 2.5 BAP,<br>MSM K sulphate   | range 2.8 to 3.1 |
| Shelley's selection | MSM with 5mg/L BAP,<br>MSM K sulphate | range 2.9 to 3.0 |
| Success             | MSM with 5mg/L BAP,<br>MSM K sulphate | range 2.5 to 2.7 |

---

In summary it was shown that all Australian rhubarb selections except Shelley's and Success selections multiplied in a range that would support commercial production. However multiplication of Shelley's selection and Success was significantly improved when multiplied on MSM media with 5 mg/L BAP or with MSM 2.5 mg/L BAP and K sulphate. Further research to optimise media should result in improved multiplication; for example combining 5 mg/L BAP with potassium sulphate may improve multiplication. The results of multiplication on a range of media applied to this subset of Australian rhubarb selections demonstrate that they are suitable for commercial micropropagation.

#### **5.4.5 Summary of key points for optimum micropropagation and deflasking procedure for *in vitro* rhubarb plantlets**

- Extract and initiate buds from crowns 5-10 days after harvest. Harvest and wash crown and leave to dry in a room at room temperature prior to extraction of buds.
- Aim to use buds 2 mm or larger; best results are obtained using buds larger than 5 mm.
- Initiate using sodium hypochlorite method. Wash crowns with chlorinated water to remove dirt and debris, old leaf bases and dead tissue are removed. Carefully remove leaves so as not to damage buds inside the leaf bases. Apical growing points and buds excised from the crown are rinsed under running tap water for 20 minutes then placed into sterilized jars. After buds are washed in running tap water, place them in distilled water containing sodium hypochlorite at 2% a.i. with a few drops of surfactant Tween 80. Shake gently 15 minutes. Rinse buds 4 times in sterile distilled water. Aseptically remove bleached and excess tissue. Place bud into Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel with 1 mL/L Plant Preservative Media ((PPM) which is a plant tissue culture biocide product). Adjust pH to 6.0 prior to autoclaving. Place cultures in the dark for 5 days at 25°C and then cultured at 25-27°C, under fluorescent lights at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent) for 16 hour day and 8 hour night.
- Subculture plants every three weeks.
- Use vented containers for micropropagation.
- For multiplication choose identify level of benzyl amino Purine BAP (cytokinin) for multiplication. Use Murashige and Skoog (MS) basal medium, with the addition of 20 g/L sucrose, 2.5 mg/L benzyl amino purine (BAP), 2.5 g/L phytigel. pH was adjusted to 6.0 prior to autoclaving. The buds were placed in culture room at 25°C±2 and grown in darkness for 5 days and then grown in vented containers under 16 hour day and 8 hour night, at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent). Add 2.5-5 mg/L BAP.
- For rooting use 1 mg/L Indole Acetic Acid (Auxin) for best plant acclimatisation and survival to over 80%.
- For rooting use of Phytigel at 1.5 g/L to further increase survival of plants (over 90% survival after acclimatisation).
- The optimum rooting culture cycle was 3 weeks in rooting media for highest survival with over 90% of plants surviving acclimatisation.

Rhubarb could be micropropagated to provide 3-4 times multiplication rate per subculture cycle and successfully acclimatised (over 90% of plants surviving). Using these methods thousands of plants were produced in a commercial laboratory and acclimatised in a commercial nursery. All plants established and grew well in commercial plantings and no off-types were identified.

## **5.5 Combining high temperature with tissue culture growth to eliminate Rhubarb decline associated virus (RDaV).**

### **5.5.1.1 Introduction**

This experiment was undertaken to determine if the RDaV could effectively be eliminated in plants produced from buds taken from infected plants by combining tissue culture with heat treatments. Virus can be readily propagated via micropropagated plants. In the case that virus free rhubarb plants could not be obtained it will be essential to have a means to remove virus from infected plants prior to initiating tissue cultures from them. This experiment used high culture temperature combined with multiplication and fast removal of small buds that were subsequently established at high temperature to produce second generation buds and so on to eliminate virus. Virus elimination through the conventional tissue culture process was also explored.

### **5.5.1.2 Materials and methods**

All plants used from the cultivar 'Big Red' collected from Mt Tamborine, Queensland and 3-5 days after plant harvest the crowns were washed with chlorinated water to remove dirt and debris, old leaf bases and dead tissue were removed. Leaves were carefully removed so as not to damage buds inside leaf bases. Apical growing points and buds were excised from the crown and rinsed under running tap water for 20 min then placed in distilled water with containing sodium hypochlorite at 2% a.i. to which a few drops of surfactant Tween 80 was added and gently shaken for 15 minutes. The buds were then rinsed 4 times in sterile distilled water. Bleached and excess tissue was removed aseptically to leave a growing point between 1 and 7 mm (the variation due to the size of the original bud and the amount of tissue removed). The bud was then placed into Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel with 1 mL/L Plant Preservative Media (PPM) which is a plant tissue culture biocide product). pH was adjusted to 6.0 prior to autoclaving. Cultures were placed in the dark for 5 days at 25°C and then cultured at respective temperatures, under fluorescent lights at a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-2}$  (Philips TLD 36W/33 cool white fluorescent) for 12 hour day and 12 hour night.

The first experiment was to identify the highest temperature that rhubarb plants could not only survive but also produce buds. Plantlets that had been established from crowns indexed and found to be virus free were use for the temperature growth response experiment. The temperatures were 30°C/30°C day night, 32°C/30°C day night, 35°C/30°C day/night and 40°C/30°C day night.

For the second experiment, after appropriate high temperature had been identified, 11 individual plantlets from 3 accessions initiated from crowns that displayed virus symptoms and tested positive to RDaV by RT-PCR (Section 5.2.2.2). Buds from the confirmed infected crowns were treated at high temperature of 33°C 12 hour day and 30°C night. All plantlets were subcultured onto fresh media in a 3 weekly cycle so that new buds were removed from the parent plants as soon as the bud was of a size that would remain viable when excised from the parent plant. When buds were produced from explants growing at high temperature in the virus elimination they were labelled as first generation, and the buds taken off them from those original buds

in the next subculture labelled second generation and those buds grew into plantlets where buds were taken off them labelled third generation The control consisted of 5 plantlets from 4 accessions initiated from positively confirmed infected crowns that were cultured under standard culture conditions of 25°C 12 hour day and 20°C night. All plants were cultured on Murashige and Skoog media (Murashige and Skoog 1962) with 20 g/L sucrose, 2.5 mg/L Benzyl Amino Purine (BAP) and 2.5 g/L phytigel with 1 mL/L Plant Preservative Media ((PPM) which is a plant tissue culture biocide product). pH was adjusted to 6.0 prior to autoclaving.

### 5.5.1.3 Results and discussion

Plants did not survive at 35°C or 40°C culture temperatures (Table 29).

Table 29. Temperature growth response for micropropagated rhubarb.

| Date    | First subculture         |                   |          | Second subculture |                   |                | Comments                                  |
|---------|--------------------------|-------------------|----------|-------------------|-------------------|----------------|-------------------------------------------|
|         | Treatments               | no. of plants sub | Survival | Date second sub   | no. of plants sub | Multiplication |                                           |
| 14.9.07 | 1 = 40°C day, 30°C night | 55                | 0        |                   |                   |                |                                           |
| 14.9.07 | 2 = 30°C day and night   | 55                | 54       | 4.10.07           | 55                | 72             | Plants starting to yellow on some leaves  |
| 20.9.07 | 3 = 35°C day, 30°C night | 65                | 0        |                   |                   |                | Limp and yellowing                        |
| 4.10.07 | 4 = 32°C day, 30°C night | 55                | 55       | 22.10.07          | 55                | 95             | Third sub from plants from 30°C treatment |

Rhubarb plants grew and produced buds when cultured at 30°C day and night or 32°C day and 30°C night. 33°C day and 30°C was chosen for the virus elimination treatment.

No virus was found in plants from the standard micropropagation or high temperature elimination treatments when tested directly from leaves produced in culture. Because tissue culture plants often reduce the level of virus in the plants (low-titre) the tissue culture plants from control treatments as well as all plants produced under high temperature over several generations was deflasked and grown in a glasshouse. The majority of plants were tested for virus one to two months after glasshouse establishment but no virus was detected (Table 30). Therefore all plants were grown for two years and retested for virus.

After two years growth in the glasshouse all plants, including those produced under standard micropropagation process, remained free of RDaV by indexing and did not show any visual virus symptoms. RDaV was removed from infected plants during conventional micropropagation as well as high temperature treatments.

Table 30. Results of valid tests (positive and negative controls as expected) for RDaV.

| Line code | Generation |    |     | Planting date | Tested 23/07/08 | Tested 22/09/10 |
|-----------|------------|----|-----|---------------|-----------------|-----------------|
| 63        | CONTROL    |    |     | 17/06/08      | Not tested      | No virus        |
| 69.2      | CONTROL    |    |     | 29/08/08      | Not tested      | No virus        |
| 81.2      | CONTROL    |    |     | no date       | Not tested      | No virus        |
| 90        | CONTROL    |    |     | 17/06/08      | Not tested      | No virus        |
| 90.4      | CONTROL    |    |     | 26/06/08      | Not tested      | No virus        |
| 90.2      | A          | 2  | 1st | 3/06/08       | No virus        | No virus        |
| 90.2      | B          | 7  | 2nd | 3/06/08       | No virus        | No virus        |
| 90.2      | B          |    | 2nd | 4/07/08       | No virus        | No virus        |
| 90.2      | C          |    | 3rd | 4/07/08       | No virus        | No virus        |
| 90.3      | A          | 1  | 1st | 3/06/08       | No virus        | No virus        |
| 90.3      | B          |    | 2nd | 29/08/08      | No virus        | No virus        |
| 90.3      | C          | 13 | 3rd | 3/06/08       | No virus        | No virus        |
| 90.4      | A          | 3  | 1st | 3/06/08       | No virus        | No virus        |
| 90.4      | B          | 6  | 2nd | 3/06/08       | No virus        | No virus        |
| 90.4      | C          |    | 3rd | 26/06/08      | No virus        | No virus        |
| 90.4      | C          |    | 3rd | 29/08/08      | No virus        | No virus        |
| 117.1.1   | B          | 11 | 2nd | 17/06/08      | No virus        | No virus        |
| 117.1.1   | C          |    | 3rd | 10/09/08      | No virus        | No virus        |
| 117.1.2   | B          | 12 | 2nd | 3/06/08       | No virus        | No virus        |
| 117.1.2   | C          |    | 3rd | 29/08/08      | No virus        | No virus        |
| 117.1.3   | B          | 10 | 2nd | 17/06/08      | No virus        | No virus        |
| 117.1.3   | C          |    | 3rd | 26/06/08      | No virus        | No virus        |
| 117.1.3   | C          |    | 3rd | 19/08/08      | No virus        | No virus        |
| 117.1.4   | A          | 5  | 1st | 3/06/08       | No virus        | No virus        |
| 117.1.4   | B          |    | 2nd | 29/08/08      | No virus        | No virus        |
| 117.1.4   | C          |    | 3rd | 10/09/08      | No virus        | No virus        |
| 69.2.1    | A          | 4  | 1st | 3/06/08       | No virus        | No virus        |
| 69.2.1    | A          |    | 1st | 3/06/08       | No virus        | No virus        |
| 69.2.1    | B          |    | 2nd | 17/06/08      | No virus        | No virus        |
| 69.2.1    | C          |    | 3rd | 26/06/08      | Not tested      | No virus        |
| 69.2.1    | C          |    | 3rd | 29/08/08      | Not tested      | No virus        |
| 69.2.2    | B          | 9  | 2nd | 3/06/08       | No virus        | No virus        |
| 69.2.2    | C          | 14 | 3rd | 3/06/08       | No virus        | No virus        |
| 69.2.3    | C          | 15 | 3rd | 17/06/08      | Not tested      | No virus        |

## **5.6 Field performance of tissue cultured rhubarb**

### **5.6.1 Yield of conventionally propagated rhubarb compared with tissue cultured rhubarb plants in a commercial field trial.**

#### **5.6.1.1 Introduction**

The aim of this research was to understand if virus-free tissue cultured plantlets could be used in development of a commercially viable method for managing virus on affected farms. It was important that the tissue cultured plants would produce a similar or better yield and quality in the same growth cycle as conventional rhubarb to ensure consistent financial returns to the grower with minimum change to farm practice. This experiment was undertaken to determine if tissue cultured rhubarb plants could be planted at the same time as conventional rhubarb material derived from crowns to produce the same yield with the same quality stems, and thus provide a satisfactory alternative planting material.

#### **5.6.1.2 Materials and methods**

The cultivar, used was selection “Big Red” known to be highly susceptible to virus infection and one of the main cultivars grown in the Queensland region affected by the new virus. Conventional planting material consisted of vegetatively propagated plants split from rhubarb crowns were sourced from clean plants. The tissue culture plants were produced using methods developed in this project and from conventional plants that had indexed negative to all virus and had shown no symptoms. The trial was located on a farm at Mt Tamborine which grew the trial using standard commercial practice.

The trial block was established in the middle a large farm block. Plants were established in 5 random rows and 5 plants of each of the treatments (tissue culture or conventional) planted at random positions within each of the 5 rows. In total there were 25 each of tissue culture and conventional plants. All plants indexed negative to virus at commencement of the trial. Data on total plant yield, number of stems and length of stems was collected over 5 harvests over two years. Data was analysed using Genstat.

#### **5.6.1.3 Results and discussion**

All plants were planted at a similar time and grew rapidly. There was no difference in total yield between either the tissue culture plants or the conventional plants (Figure 37), and no significant difference in proportions of plants with virus symptoms or virus infections between treatments (data not shown).



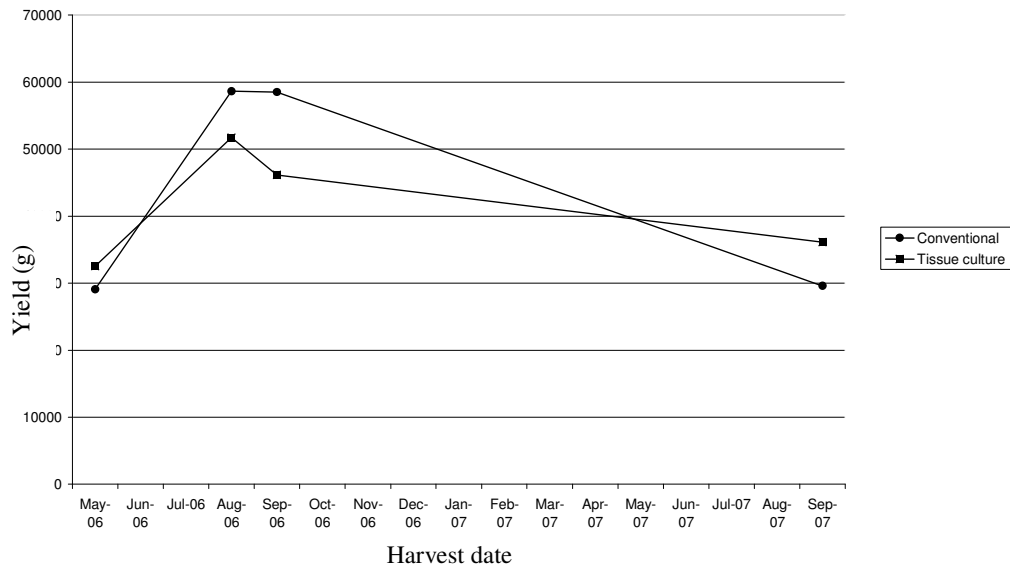


Figure 37. Yield of conventional vs tissue culture rhubarb. n = 25.

Mean plant fresh weight over the trial period was 1160 g for conventionally propagated plants and 1301 g from tissue culture plants.

There was also no significant difference in the number of stems produced in either treatment. Average number of stems was 11.04 stems from conventional plants and 11.44 stems from tissue cultured plants.

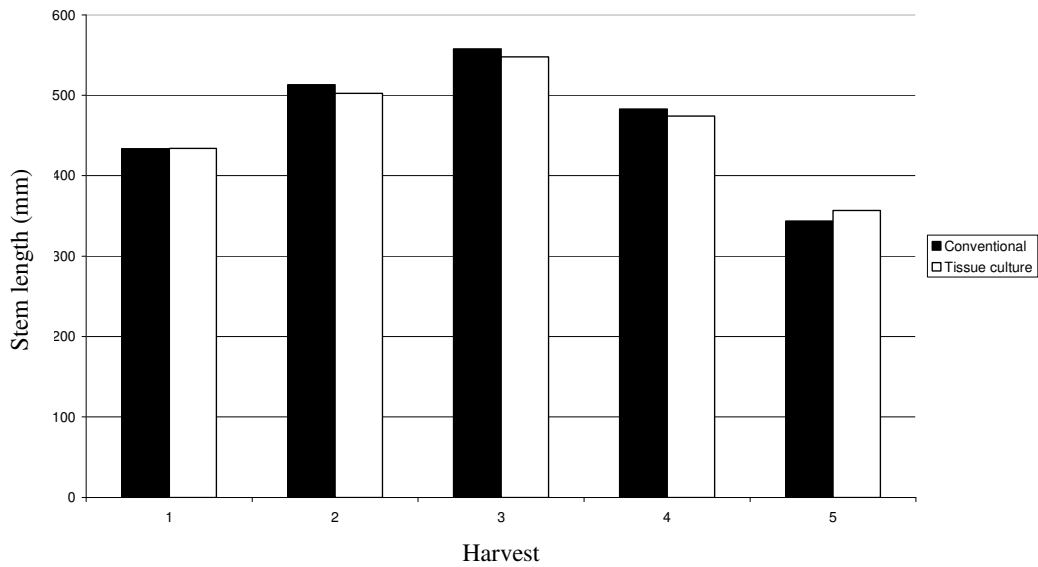


Figure 38. Comparison of rhubarb propagation method on stem length between conventional and tissue cultured plants over five harvests.

Average stem length was the same for conventional (466 mm) and from tissue culture derived plants (463 mm) as described in Figure 38.

In summary there was no significant difference between treatments ( $P>0.05$ ) for fresh weight or number of stems or stem length. The row effect was not significant and for all parameters there was no significant difference between treatments ( $P>0.05$ ). All plants appeared normal and there were no off-types. Virus free tissue culture plants can be grown on a rhubarb farm with little change to current commercial practice to obtain a similar high quality yield as from conventional plants.

## **5.6.2 Re-infection rates and symptom expression in virus-free tissue culture and conventional planting material**

### ***5.6.2.1 Introduction***

Susceptible, virus-free planting material is subject to re-infection if it is planted near a source of infection, and the insect vectors are present. This trial was designed to investigate the rate of re-infection in a commercial situation. It also provided the opportunity to look at symptom development in recently infected plants, and any differences in symptom expression between tissue cultured and conventional planting material.

### ***5.6.2.2 Materials and methods***

The Queensland cultivar “Big Red” was used for this trial. It is one of the main cultivars grown in Mt Tamborine region of Queensland and is known to be highly susceptible to virus infection. Virus-free conventional planting material was sourced from plants from a Mt Tamborine farm, while the tissue culture plants were produced from the same source, using methods developed in this project. All plants were symptomless and indexed negative for RDaV and CMV at the time of planting.

Fourteen plots, each of 20 virus-free tissue culture plants, were randomly planted within a virus-infected commercial block at Mt Tamborine on 14/1/2006. There were some transplanting losses, and in total 236 tissue cultured plants were assessed. Twenty five plants derived from conventional crowns, planted as part of a yield comparison trial, were also assessed. Plants were rated for symptom expression at 2, 5, 8, 15 and 20 months after planting. All plants were indexed for CMV and RDaV (Section 5.2.2.2) at the same dates, except at 5 months, where the 25 conventional plants and a subsample of 25 tissue culture plants only were tested. Genstat was used to analyse distribution of virus and symptoms and chi-square test was applied to assess whether the incidence of virus or symptoms varied between treatments.

### ***5.6.2.3 Results and discussion***

Results are summarized in Figure 39. No symptoms were expressed four months after planting for the conventional material. At 2 months, 3% of tissue culture plants showed symptoms and the first virus infections were recorded. Conventional planting material had a higher percentage of symptomatic plants than tissue culture material throughout the trial. At 8 months, 24% of plants from conventional planting material showed symptoms compared with 12% of plants from tissue culture. By 20 months,

76% of plants from conventional planting material showed symptoms, significantly more than the 37% of plants from tissue culture which expressed symptoms.

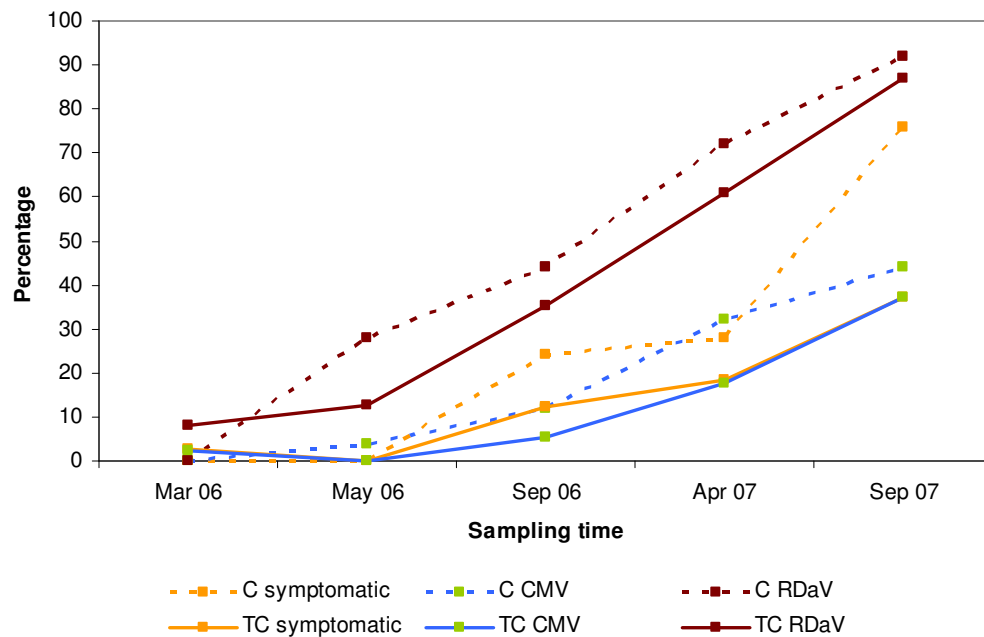


Figure 39. Reinfection rates and proportion of symptomatic plants, comparing tissue culture and conventional planting material.

Over the trial the incidence of virus was similar for the two treatments (conventional and tissue culture) and for the presence of either virus (CMV or RDaV). However, tissue culture plants tended to have a little less infection than the conventional planting material.

There was a much higher incidence of RDaV than of CMV at all sampling dates for both treatments. Rhubarb plants were rapidly infected by RDaV with 89% of all plants infected within 20 months of planting. This compared to the much lower final incidence of infection by CMV (34%) at the same time. All virus-infected plants contained RDaV, and a proportion of these plants (43%) also contained CMV.

Numbers of symptomatic plants were always fewer than those of virus-infected plants. For example, 8 months after planting out, 94 plants were virus-infected, but only 35 (37%) displayed symptoms. At 20 months, 207 plants were virus-infected, but only 98 (47%) displayed symptoms. Individual plants took 15-20 months to display symptoms after becoming infected with RDaV (with or without CMV).

The proportion of symptomatic plants was a little higher in conventional material than in tissue cultured plants, but not significantly so, during most of the trial. The low number of replicates in the conventional treatment may have contributed to this apparent difference. Unexpectedly, at the last sampling date, the level of symptom expression in the conventional material rose markedly, as was twice that of tissue cultured material (76% vs 37%).

The rapid infection process resulting in ca 90% crop infection within less than two years and the low level of symptom expression means that growers cannot reliably select plants that are free from virus by visual inspection alone. Since rhubarb crops are planted from crowns grown the previous season the growers will have difficulty avoiding replanting crops with already infected planting material. Planting with virus infected material combined with the rapid reinfection rates explains why crop loss is so fast and severe from apparently healthy plants. The use of virus-free tissue cultured plants offers a way to break the infection cycle if all conventional material is removed and virus-free plants are used to replant very few years.

### **5.6.3 Rhubarb field re-infection trial comparing silicate amendment of conventionally propagated plants with tissue cultured plants**

#### ***5.6.3.1 Introduction***

A second field experiment was established to determine how quickly virus could spread when introduced to a clean block and to determine if amendments could be applied to reduce virus infection or improve rhubarb growth. Calcium silicate had been shown by other authors to inhibiting insect feeding (Hua and Han 2010, Ameida et al 2009, Reynolds et al. 2009, Gomes 2008) which may lead to reduced spread of virus. Conventional material with low levels of RDaV and CMV was planted with virus free tissue culture plants treated with calcium silicate and a control, calcium carbonate in order to understand how quickly the virus would spread within the small block and how the virus affected overall yield.

#### ***5.6.3.2 Materials and methods***

The rhubarb trial block was located on Maroochy Research Station, Nambour in an area that does not produce rhubarb. The experiment used the variety “Big Red”. Thirty plants derived from crowns sourced from a commercial farm at Mt Tamborine were used for the conventional treatment. The plants symptomless, but had infection levels of 43% and 13% for RDaV and CMV, respectively. The small plants were provided from divided crowns and potted into pasteurised potting mix (50:50 peat:sand) and grown in a shadehouse. At the same time 90 virus free tissue cultured plants were produced using methods developed in this project and grown in the shadehouse and timed so that the plant size of tissue plants matched those of conventional plants. The border rows of block also planted with conventional plants sourced from crowns that from an infected farm and provided an additional source of virus infection for the trial sourced in the same manner as the conventional plant treatments. All plants were approximately 30 cm tall at time of planting on 22/5/2007 and none showed severe virus symptom.

A replicated trial using conventional clean plants and three treatments of tissue culture derived plants was planted in the middle of the border rows. The treatments were conventional plants, tissue culture plants, tissue culture with 50g calcium silicate (CaSO<sub>2</sub>) per plant applied to soil surface and tissue culture with calcium carbonate CaCO<sub>3</sub> (calcium control) 165 g per plant applied to soil surface. The design of the experiment was a randomised block, consisting of 3 blocks and 4 treatments, with

duplicate plots of each treatment in each block, resulting in a total of 24 plots. There were 5 plants in each plot and thus a total of 120 plants. Yield data were recorded on 3 dates (17/08/07, 10/10/07, and 11/09/08). Data recorded were stem length, number of stems per plant, stem diameter at 7.5cm from base, total weight of stems per plant. Data were analysed both combined across dates and separately for each of the three dates.

For the analysis across dates data measured on a plant basis were analysed using residual maximum likelihood methods, with fixed effects of treatment, date and their interaction and random effects of blocks, plots within blocks, plants within plots, and measurements per plant. Where a variance component estimate was negative, this term was dropped from the model and data reanalysed. In all cases the transformed analyses are preferred over the non-transformed ones. The constant 11 was chosen to add to weight values before log transformation as it is half the minimum nonzero value (Yamamura, 1999). For the analysis within dates data measured on a plant basis were analysed using residual maximum likelihood methods, with treatment as a fixed effect and random effects of blocks, plots within blocks, and plants within plots. Where a variance component estimate was negative, this term was dropped from the model and data reanalysed. For the analysis across dates data measured on a stem basis were analysed using residual maximum likelihood methods, with fixed effects of treatment, date and their interaction and random effects of blocks, plots within blocks, plants within plots, and stems within plants. Where a variance component estimate was negative, this term was dropped from the model and data reanalysed. For the analysis within dates data measured on a stem basis were analysed using residual maximum likelihood methods, with treatment as a fixed effect and random effects of blocks, plots within blocks, plants within plots, and stems within plants. Where a variance component estimate was negative, this term was dropped from the model and data reanalysed. Number of plants out of five (in a plot) was tabulated to produce zero total weight and analysed percentage non-yielding plants using generalised linear models assuming a binomial distribution with logit link. Effects considered were treatments, dates, their interaction and blocks. Non-significant terms were dropped from the model. The interaction was not significant for either analysis.

### ***5.6.3.3 Results and discussion***

#### Yield assessment

The type of planting material whether tissue culture or conventionally derived produced similar total plant yield. Treatment added to the tissue cultured plants likewise had no effect on total plant yield (Table 31). The yield shown in Table 31 indicated that the trend was for the tissue culture plants to have a higher yield than conventional plants (Figure 40).

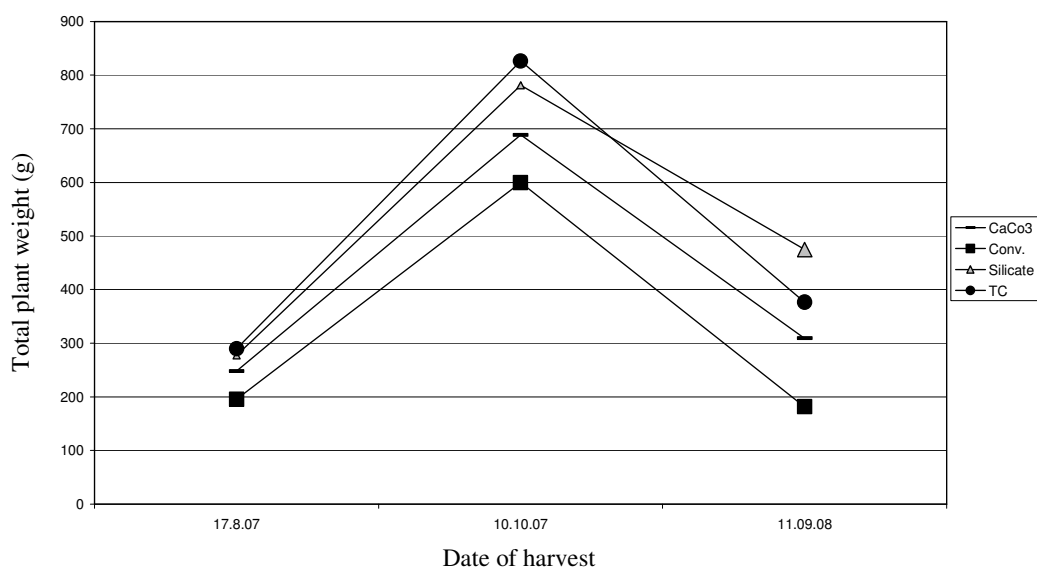


Figure 40. Total plant yield from different rhubarb treated plants over a two year field trial.

Table 31. Average plant total yield for each treatment, combined harvests.

| Treatment              | Average plant total yield (g) |
|------------------------|-------------------------------|
| TC + Calicum carbonate | 415.3 <sup>a</sup>            |
| Conventional           | 325.6 <sup>a</sup>            |
| TC + Calcium Silicate  | 511.1 <sup>a</sup>            |
| TC                     | 497.6 <sup>a</sup>            |

Means with same subscript are not significantly different at the P = 0.050 level, SEM=57.2

The crop performed quite differently over the two years and at the different harvest dates (Table 32). The plants yielded significantly more yield in October 2007 than August two months earlier or September the following year. However it must be considered that increased virus infection may have contributed to reduction in yield over time.

Table 32. Ranked means of average plant total yield at harvest, combined treatments.

| Date     | Mean total plant yield (g) |
|----------|----------------------------|
| 10.10.07 | 728.8 <sup>a</sup>         |
| 11.09.08 | 473.1 <sup>b</sup>         |
| 17.8.07  | 254.4 <sup>c</sup>         |

Means with same subscript are not significantly different at the P = 0.050 level.

Stems weight was less in the conventional treatment (Table 33) and highest in the calcium silicate treatment.

Table 33. Ranked means of stem weight, combined dates.

| Treatment             | Mean stem weight (g) |
|-----------------------|----------------------|
| TC + calcium silicate | 78.32 <sup>a</sup>   |

|                        |                    |
|------------------------|--------------------|
| TC                     | 77.22 <sup>a</sup> |
| TC + calcium carbonate | 68.18 <sup>a</sup> |
| Conventional           | 56.39 <sup>b</sup> |

Means with same subscript are not significantly different at the P = 0.050 level, LSD=10.51.

The average stem weight was greatest in October in the first year of the trial (Table 34) which contributed to total yield.

Table 34. Ranked means of stem weight, combined treatments.

| Date     | Mean stem weight (g) |
|----------|----------------------|
| 10.10.07 | 81.48 <sup>a</sup>   |
| 11.09.08 | 71.26 <sup>b</sup>   |
| 17.8.07  | 57.35 <sup>c</sup>   |

Means with same subscript are not significantly different at the P = 0.050 level, LSD=6.42

The length of the conventional stems was significantly shorter in the conventional plants over the life of the trial (Table 35). The calcium silicate had the longest stems. But it must be considered that conventional plants had higher rate of virus infection which would have contributed to plant growth.

Table 35. Average length of stems for each treatment, combined harvests.

| Treatment              | Mean stem length (mm) |
|------------------------|-----------------------|
| TC + calcium carbonate | 342.0 <sup>a</sup>    |
| Conventional           | 302.9 <sup>b</sup>    |
| TC + calcium silicate  | 359.6 <sup>a</sup>    |
| TC                     | 348.7 <sup>a</sup>    |

Means with same subscript are not significantly different at the P = 0.050 level.

The stems length was longer in the second year of the trial (Table 36), which is contrary to the yield results where total plant yield was highest in the first year of the trial and indicates reduced yield may have been attributed to increased virus in the crop.

Table 36. Average stem length at harvest, combined treatments.

| Date     | Mean stem length (mm) |
|----------|-----------------------|
| 17.8.07  | 267.6 <sup>c</sup>    |
| 10.10.07 | 355.1 <sup>b</sup>    |
| 11.09.08 | 392.2 <sup>a</sup>    |

Means with same subscript are not significantly different at the P = 0.050 level.

There was no significant difference in average number of stems for all treatments (Table 37). Calcium silicate had the highest average number of stems and conventional the least.

Table 37. Average stem number, combined harvests.

| Treatment              | Mean stem number   |
|------------------------|--------------------|
| TC + calcium carbonate | 5.900 <sup>a</sup> |
| Conventional           | 5.067 <sup>a</sup> |

|                       |                    |
|-----------------------|--------------------|
| TC + calcium silicate | 6.144 <sup>a</sup> |
| TC                    | 5.978 <sup>a</sup> |

Means with same subscript are not significantly different at the P = 0.050 level.

There was a large difference in the number of stems produced over the life of the trials, with significantly more stems produced in October of the first year (Table 38).

Table 38. Average stem number, combined treatments.

| Date     | Mean stem number   |
|----------|--------------------|
| 10.10.07 | 8.958 <sup>a</sup> |
| 17.8.07  | 4.325 <sup>b</sup> |
| 11.09.08 | 4.033 <sup>b</sup> |

Means with same subscript are not significantly different at the P = 0.050 level, LSD=0.7985.

The stems were significantly thicker in the tissue culture alone or tissue culture provided with calcium silicate (Table 39).

Table 39. Average stem diameter, combined harvest dates.

| Treatment              | Mean stem diameter (mm) |
|------------------------|-------------------------|
| TC + calcium carbonate | 16.33 <sup>b</sup>      |
| Conventional           | 16.06 <sup>b</sup>      |
| TC + calcium silicate  | 17.63 <sup>a</sup>      |
| TC                     | 17.63 <sup>a</sup>      |

Means with same subscript are not significantly different at the P = 0.050 level.

The stems were significantly thinner at the first harvest but were similar for subsequent harvests (Table 40).

Table 40. Average stem diameter, combined treatments.

| Date     | Mean stem diameter (mm) |
|----------|-------------------------|
| 17.8.07  | 14.48 <sup>b</sup>      |
| 10.10.07 | 18.03 <sup>a</sup>      |
| 11.09.08 | 18.22 <sup>a</sup>      |

The increase in total plant weight in October of the first year was attributed to plants producing significantly more stems that were individually heavier than other dates but not taller or thicker.

#### Virus infection

The rate of virus infection in the treatments was investigated.

At planting of the trial in May 2007, no plants showed any virus symptoms but by August 2007 over 40% of conventional plants showed symptoms. No symptoms were seen in the tissue cultured plants until July 2008 (Figure 41).



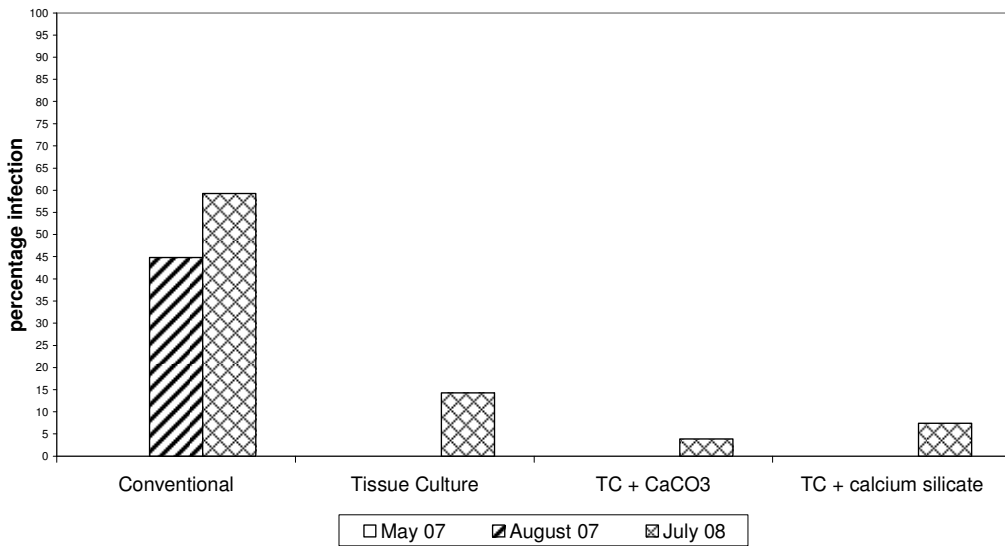


Figure 41. Percentage of rhubarb plants in each treatment showing virus symptoms.

Indexing of plants for CMV showed that in May 2007 conventional material was already symptomless infected with around 12% affected. This level of infection in the conventional plants remained fairly constant over the 16 months of the trial reaching 15%. Of the tissue culture treatments, only the standard tissue culture alone treatment was infected by CMV with 3% of plants (one plant) infected within 16 months of exposure to infected plants (Figure 42).

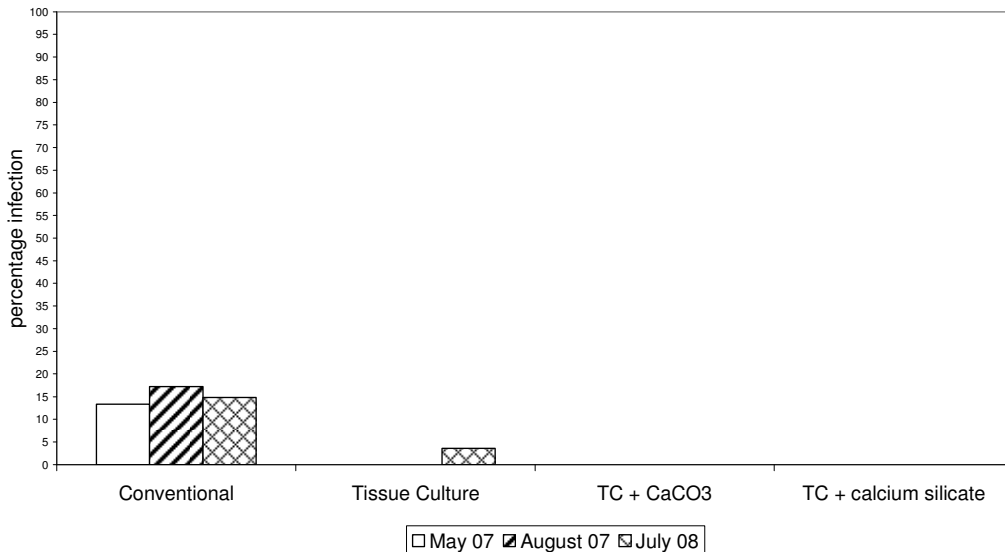


Figure 42. Percentage of rhubarb plants in each treatment infected by CMV.

At commencement of the trial 42% of conventional plants were symptomless infected with RDaV (Figure 43). The level of infection in conventional plants rose by

approximately 20% within 3 months and 74% of plants were infected after 15 months. Tissue culture plants showed no infection in the first year of the trial but were infected by 15 months. Level of infection of the tissue culture plants was highest in the standard tissue culture treatment and the tissue culture with calcium carbonate control having between 29 or 31% plants infected respectively. The lower percentage of plants infected (15%) in the in tissue culture plants treated with calcium silicate may indicate a treatment effect reducing the level of infection. The possible mechanism is unknown and could be either by reducing feeding time of aphids or by inducing plant systemic resistance.

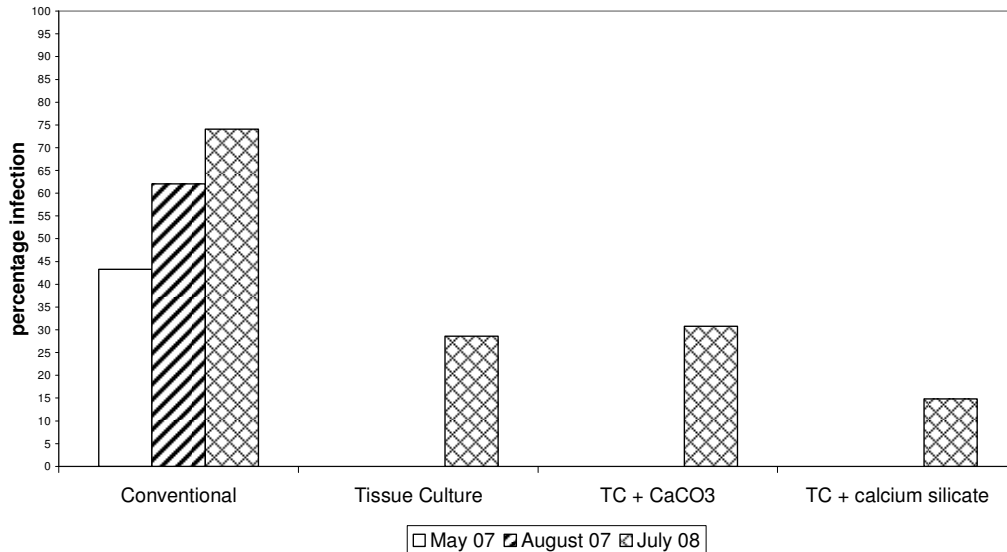


Figure 43. Percentage of rhubarb plants in each treatment infected by RDaV.

RDaV produced the higher infection rates and was associated with many more of the symptomatic plants of the virus symptoms. At planting, no virus symptoms were seen in the conventional planting material. But three months later, in August 2007, ca 45% of plants showed symptoms and ca 60% had symptoms by July 2008 (14 months). Infection by CMV over the period of the trial increased from 13% to only 17%. By comparison RDaV had increased from 44 to 74%.

Due to effects of virus infection, there were some plants that did not produce a marketable yield (Table 41). The incidence of the non-yielding plants was investigated considering treatments and harvest dates. As predicted there was a high incidence of non-yielding plants at the end of the trial when infection by RDaV had infected 75% of conventional plants and between 15 to 31% of tissue cultured plants. Fifteen months after planting, 33% of total plants no longer produced commercial yield due to virus infection.

Table 41. Percentage of non-yielding plants

| Date     | Percentage of plants |
|----------|----------------------|
| 17.8.07  | 1 <sup>b</sup>       |
| 10.10.07 | 1 <sup>b</sup>       |
| 11.09.08 | 33 <sup>a</sup>      |

Across the treatments, significantly more plants in the conventional treatment had zero yield (21%) than in other treatments (7-11%) and there were significantly more plants with zero yield by the end of the trial (Figure 44). Only 3% of conventional plants failed to produce marketable yield in the first year of the trial and all tissue culture plants produced marketable yield. By the next season, at the end of the trial (16 months) 57% of the conventional plants (by then 75% infected by RDaV) failed to produce harvestable yield. By comparison, 20-33% of the plants in the tissue culture treatments to produce marketable yield.

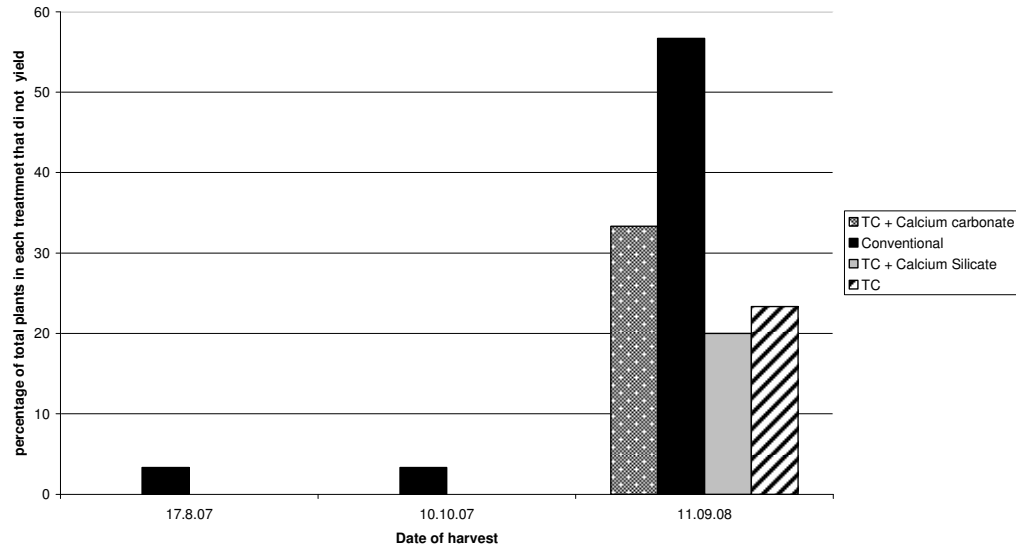


Figure 44. Percentage of non-yielding rhubarb plants in each treatment infected by RDaV over time.

This trial confirms the findings of the commercial field trial in showing that RDaV spreads rapidly in the field and that symptom expression levels lag considerably behind virus infection levels. Similarly, CMV spread was much slower than that of RDaV. The results of this trial demonstrate the severe effects of the newly recognised RDaV and help explain the significant yield decline which has resulted in farms being unable to continue to produce marketable rhubarb two to three years after first onset of virus symptoms. At commencement of the trial an average 10% of plants across all treatments were symptomlessly infected with RDaV. Sixteen months later, at the end of the trial, 37% of all plants were infected and 33% of the entire crop failed to produce marketable yield.

## **5.7 National growers group**

A national growers' representative group was convened, and met in Sydney on October 17<sup>th</sup>, 2008 at the Horticulture Australia Ltd. Office.

Attendees were:

Growers: Greg Xerri (NSW), Ross Arnott (Vic), Greg Rankin (Vic), Francis Tropeano (Vic), Tim Burgess (Tas), Sam Grima (NSW), David Driver (SA), Maurice Grubisa (WA).

Researchers: Liz Minchinton (Vic), Sharon Hamill (Qld), Jenny Jobling (NSW), Len Tesoreiro (NSW), Anowarul Bokshi (NSW)

HAL: Lucy Keatinge.

Sharon Hamill presented research results that were used as the basis of further discussion at the workshop.

At this forum, it was decided that additional funding should be sought to include rhubarb cultivars from across Australia in further research investigation tissue culture performance. The cultivars were determined on a voluntary basis by growers willing to provide their selections. Additional rhubarb cultivars amenable to tissue culture would then be able to be used in trial production through a commercial tissue culture and acclimatisation nursery system.

## **6 RECOMMENDATIONS**

The essential components of a grower-managed clean planting material scheme, based on virus-free tissue cultured plants, have been established. Some refinements and modifications as suggested below, would improve the efficiency and economy of this system.

### **6.1 Virus research**

- Although molecular assays for the detection of RDaV, a critical component of rhubarb decline disease, are available, serological assays such as ELISA would present a far more “user-friendly” option for a grower-managed scheme. A number of antisera were prepared during this project, but none were found to be suitable at present. Further investigation of serological assays or refinement of antiserum production to RDaV to allow the development of an ELISA test is recommended.
- Further characterisation of novel totiviruses and other unidentified viruses with small isometric particles is necessary to determine their role, if any, in rhubarb decline disease. Although RDaV was considered to have a major role in disease induction, these other uncharacterised viruses were common, and it was not possible to investigate the possibility of synergistic reactions in this project.

### **6.2 Tissue culture research**

- The research in this project has identified the key steps required to produce a range of different Australian rhubarb selections at commercially viable production efficiencies for both tissue culture laboratory and nursery. Following the recommendations in this report micropropagation of rhubarb can be conducted on a commercial basis.
- One hindrance to commercial rhubarb micropropagation continues to be the high level of contamination of buds during initiation into culture despite different approaches to sterilising starting material. This is because the crown of the rhubarb which grows deep in soil is the part used for culture and it is difficult to completely remove bacteria from the plant tissue. Laboratories lacking expertise in initiating buds from parts of plants grown deep in soil may experience difficulty with successful initiation. In our research, plants were taken from commercial farms from plants grown in the field. Commercial laboratories may consider growing stock rhubarb plants in well drained potting mix under clean nursery conditions to reduce contamination loss at initiation.

### **6.3 For the industry**

All the elements for a grower managed clean planting material scheme are now available. It is recommended that the industry adopts the use of virus-free planting material in areas affected by rhubarb decline, and that replanting takes into

consideration of the high reinfection rates likely if older, infected crops, and the weed, curled dock, are allowed to remain nearby at the time of replanting.

Growers who wish to use tissue culture will need to contact a commercial laboratory well in advance. It will take 1 to 2 years to produce plants in commercial quantities and growers will need to supply the plants for culture initiation. When using tissue cultured plants on a regular basis the production of plantlets from each mother plant should be limited to ensure high quality and reduce off-types caused by somaclonal variation. This means cultures need to be initiated each year rhubarb plants are required by the grower. Growers should also contact nurseries who are experienced in deflasking tissue cultured plants as they will have the correct facilities and experience to ensure maximum survival of plantlets.

## **7 TECHNOLOGY TRANSFER**

### **7.1 Articles**

Research findings arising from the project were presented at international conferences as follows.

- Conference presentation by John Thomas:  
Kathy Parmenter, Sharon Hamill, John Thomas, Brenda Coutts, Roger Jones (2006). Viruses associated with rhubarb decline disease. 7th Australasian Plant Virology Workshop, 8-11 November 2006, Rottnest Is, Western Australia, Australia.
- Conference poster:  
Sharon Hamill, Michelle Paynter, Kathy Parmenter, John Thomas & Janet Giles (2008). Micropropagated virus free Rhubarb enables industry to overcome a new severe virus decline disease in Australia. The sixth International Symposium on *In Vitro* Culture and Horticultural Breeding , International Society of Horticultural Science, Brisbane , Australia , August 24-28, 2008
- Conference presentation by Kathy Parmenter:  
Kathy Parmenter, Sharon Hamill, John Thomas (2008). Viruses associated with rhubarb decline disease. 8th Australasian Plant Virology Workshop, 19-22 November 2008, Rotarua, New Zealand.
- Conference poster:  
Parmenter, K. S. and Thomas, J. E. (2009). Tomato spotted wilt virus infection of rhubarb. The IXth International Symposium on Thysanoptera and Tospoviruses, 31 August-4 September 2009, Gold Coast, Queensland, Australia.
- Conference presentation by Sharon Hamill:  
Sharon Hamill, Michelle Paynter, Kathy Parmenter and John Thomas (2009). Using micropropagated virus free rhubarb plants to manage a new severe rhubarb virus disease. Conference of the Australian Branch of the International Association of Plant Biotechnology. 24-26 November, 2009
- Conference presentation by John Thomas:

Kathy Parmenter, John Thomas, Sharon Hamill, Michelle Paynter (2011). Rhubarb decline-associated virus, a novel closterovirus from rhubarb (*Rheum rhabarbarum*) from Australia. The 4th Conference of the International Working Group on Legume and Vegetable Viruses, 17-20 May 2011, Antequera, Málaga, Spain.

Several research papers are currently in preparation.

## **7.2 Visits/Meetings**

- Field surveys of properties in the Mt Tamborine district were conducted on 26 July 2005 to five sites by John Thomas, Kathy Parmenter and Vegetable IDO and to four sites on 9 March 2006 by John Thomas and Kathy Parmenter.
- Roger Jones and Brenda Coutts visited properties in the Perth district on 25 October 2005 to collect field survey samples.
- A field survey of two growers' properties in the Stanthorpe district was conducted by John Thomas and Kathy Parmenter on 15 March 2007.
- A Growers' Day attended by John Thomas, Sharon Hamill, Kathy Parmenter and six local rhubarb growers was held on 27 March 2007 at Mt Tamborine, Queensland. Presentations were made by John and Sharon and an information flyer was distributed on the day (Appendix 1) and this flyer was posted to a further five rhubarb growers from the Queensland and northern NSW production areas.
- John Thomas conducted field surveys on 5 July 2007 at three properties on the Mornington Peninsula, Victoria.
- Field surveys of two growers' properties in the Sydney Basin, NSW were conducted by John Thomas on 13 March 2006.
- Len Tesoriero collected field survey samples from one property in the Sydney Basin, NSW on 18 September 2007.
- Sharon Hamill and Len Tesoriero, along with rhubarb growers and a HAL representative, attended the Rhubarb Workshop held at the Horticulture Australia Ltd office in Sydney on 17 October 2008.

## **7.3 Reports**

- None/NA

## **7.4 Commercialisation/IP**

- None/NA

# **8 ACKNOWLEDGEMENTS**

- Rhubarb growers, particularly Charlie Eden, Daryl Franklin, Rod Harker, Fred Mancy, Daphne Falconer, John Burgess, Jeff McMahon, Nola Stumm, Dawn St

John, David Driver, Greg Xerri, Emmanuel Xerri, Sam Grima, Ross Arnott, Francis Tropeano, P. Cochrane.

- Field and laboratory assistance by Chris Botcher (Vic DPI), Visnja Steele, Merran Neilsen, Geoff Dun and Emma Ballard (ESP, DEEDI), Karen Eccleston, Emily Rames and Leisa Bradburn (MRS, DEEDI).

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# Virus Identification and Development of long-term management strategies for the Rhubarb industry

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### DECLINE DISEASE OF RHUBARB

In the last five years, rhubarb crops in the Tamborine area of south east Queensland have developed severe decline. Affected plants have yield reductions, stunting and leaf mottle and necrosis. Surveys and virus assays have shown decline to be widespread and occurring at a high incidence (near 100% in some crops). There is a high correlation between decline and the presence of viruses in plants, with most plants being infected by two or more viruses. There are no previous records of viruses from rhubarb in Australia. Virus decline has now also been identified in NSW. Of the viruses detected, only *Cucumber mosaic virus* and *Turnip mosaic virus* have previously been recorded from rhubarb, and then in overseas crops only. *Tomato spotted wilt virus*, a novel closterovirus and two novel spherical viruses have also been found.



Necrotic spots (left) and mosaic (right) symptoms of infection of rhubarb.



Healthy (left) and diseased (right) rhubarb.

The role of each virus in decline and their possible synergistic or additive effects has yet to be determined, though the closterovirus seems to be most commonly associated. Further work is required to characterise the viruses and determine their mode of transmission and alternative hosts and ways the virus can be managed on the farm.

### VIRUS FREE TISSUE CULTURE PLANTLETS

The first phase of the rhubarb project has been development of a reliable micropropagation method to rapidly produce virus-free rhubarb plantlets. Apparently healthy plants were selected in the field and indexed for freedom from viruses. The rhubarb lines that were multiplied via tissue culture were tested twice more prior to field establishment and again indexed negative for virus.

Rapid multiplication has been achieved using Murashige & Skoog (MS) media with the addition of 2.5 mg/L Benzyl amino purine. Vented containers were needed to maximise multiplication and to prevent browning, senescence or occasional death of cultures. Rooting rate of 90% has been achieved within two weeks using basal MS with the addition of IAA 0.5mg/L. Optimum rooting has been achieved by reduction of solidifying agents in the media to promote better root development and to facilitate maximum plant survival at acclimatisation.

We have developed micropropagation processes to a commercially viable level but the acclimatisation from tissue culture to nursery initially proved to be problematic. Now by increasing light and a reduction in water combined with improved root development media used in the final tissue culture step we can successfully acclimatise 90% of tissue cultured rhubarb plants to achieve a commercially viable level of plant establishment. The current research is

looking to develop best media in the final root stage that promotes maximum plant survival.

The final factor that may pose problems to commercial tissue culture production is high levels of contamination at establishment, which has previously resulted in commercial failures. Future work will aim to develop an efficient protocol to provide clean explants for culture initiation.

To date all work has been carried out using a superior long dark red stemmed selection. Future work will evaluate another selection that has reportedly shown little or no virus infection, but which has been difficult to initiate into culture. Newly initiated cultures of selections will be used at intervals, to avoid off-types that can be produced by somaclonal variation, especially after prolonged periods in tissue culture.



Tissue cultured rhubarb plantlets on agar.

The development of commercial rhubarb micropropagation and successful acclimatisation means we can progress to the next phase in the development of a clean plant scheme for the Australian rhubarb industry.

In parallel to the *in vitro* research has been the establishment of virus-free tissue culture rhubarb plants in the field. Data is being collected on yield of tissue culture versus conventional material and to date has demonstrated that tissue culture material can be planted and harvested at similar times to conventional planting material. Research into weed control at planting may need to be investigated. To date no major problems have been identified.

Further work is needed to manage the virus in the field. Additional trials will be established to understand the rate of infection by the aphid vector and rate of yield decline after infection.

#### NEXT STEPS

The next step will be evaluation and demonstration of the commercial production of virus free tissue culture rhubarb plants via a commercial nursery. Several thousand tissue culture virus free rhubarb plants have been produced for nursery acclimatisation and will be provided to growers for their evaluation. This process will assess the efficiency of commercial nursery produced plants and make tissue culture material available for grower assessment. The aim is to encourage grower adoption of virus-free plants and ownership of a clean plant scheme.

If plants are successfully acclimatised via the commercial nursery then commercial tissue culture laboratories will be approached to produce rhubarb plantlets for growers to purchase. Laboratories will be assisted by provision of clean starter rhubarb cultures to aid successful production in these early stages.



Hardened-off tissue cultured plantlets in seedling trays.

In tissue culture clean plant schemes growers usually need to order twelve months in advance of supply so that laboratories and nurseries can obtain enough starter material to produce plants. To ensure virus freedom, each of the plants used to initiate cultures will need to be virus tested. We hope that soon growers will have access to a clean rhubarb plant scheme so that they can regularly replant with virus-indexed material and produce good quality stems for market.