INVESTIGATION OF RHUBARB VIRUS DISEASES – A SCOPING STUDY

John Thomas and Sharon Hamill
Department of Primary Industries and Fisheries

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Project Leader: Dr John Thomas
Senior Principal Plant Virologist
Department of Primary Industries and Fisheries
Horticulture and Forestry Science
80 Meiers Road
INDOOROOPILLY QLD 4068
Phone: 3896 9371
Fax: 3896 9533
Email: john.thomas@dpi.qld.gov.au

Project Scientist: Sharon Hamill
Principal Scientist
Department of Primary Industries and Fisheries
Emerging Technologies
PO Box 5083, SCMC
NAMBOUR QLD 4560
Phone: 5444 9639
Fax: 5441 2235
Email: sharon.hamill@dpi.qld.gov.au

SUMMARY

In the last five years, rhubarb crops in the Tamborine area of south east Queensland have developed severe decline. Affected plants have yield reduction, stunting, leaf mottle and necrosis. Surveys and virus assays have shown decline to be widespread and occurring at a high incidence (up to near 100%). There was a high correlation between decline and the presence of viruses in plants, with most plants infected by two or more viruses. There are no previous records of viruses from rhubarb in Australia. Of the viruses detected, only *Cucumber mosaic virus* has previously been recorded from the species, and in overseas crops. The novel viruses found were a closterovirus, two spherical viruses and a probable potyvirus. The role of each in decline and their possible synergistic or additive effects has yet to be determined. Further work is required to characterise the viruses and determine their mode of transmission and alternative hosts.

This study has demonstrated that it is feasible to use tissue culture to produce rhubarb free from the virus complex. The tissue cultured plants could be acclimatised and hardened ready for field planting. Plants produced by this process could form the nuclear material for a virus- free planting material scheme.

Further work is required to refine the tissue culture system, particularly the contamination of initial explants and optimising rooting and acclimatisation of plantlets.

Introduction

Rhubarb (***Rheum rhabarbarum)*** is a herbaceous perennial of the botanical family Polygonaceae and is believed to have originated from ***R. rhaponticum*** (Siberia) and ***R. undulatum*** (Asia) (Sutherland 1995). Rhubarb is a cool season crop valued for its long thick red stems, which grow from the crown up to 75 cm in length. These stalks are used raw or cooked in sauces, pies and desserts. The stalks have good levels of sugars, fibre, protein, potassium, calcium, magnesium and vitamin C.

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, and to a lesser extent from Western Australia. Summer production is centred on the southern States. Queensland and Western Australia each grow about 25 ha of rhubarb, and annual production is estimated as 840 tonnes from Queensland and 575 tonnes from Western Australia.

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 plants are often of inferior quality with green stems. The main characteristics sought by growers are high yield, straight erect, evenly–coloured red stalks of uniform thickness and length and ease of harvesting. Growers have carefully selected their own superior lines over several years and usually bulk up planting material for own use only. Bulking is slow but has generally satisfied the crop cycle of between three and six years.

Over the past 3-5 years rhubarb crops in the Mount Tamborine area have developed severe yield decline, and leaf necrosis and mosaic symptoms. These symptoms strongly suggested that virus infection was involved in the decline. No work has been done on virus infection of rhubarb in Australia. Most research on viruses infecting

the crop has been done in Europe where several have been found including *Turnip mosaic virus, Arabis mosaic virus, Strawberry latent ringspot virus, Cherry leafroll virus* and *Cucumber mosaic virus* (Tomlinson and Walkey 1967; Walkey *et al.*1981).

The extent of virus infection in rhubarb at Mount Tamborine and other locations is unknown. There is a need to determine this and identify the viruses present, their vectors and alternative host plants as a prelude to recommending disease management strategies.

Production of disease free planting material of the superior industry selections will be fundamental to maintaining a viable industry. However, there is currently no ready source of clean planting material, no means of rapid multiplication nor indexing procedures to determine virus freedom.

The aims of the scoping study were to:

1) Develop detection tests for viruses in rhubarb in Queensland.
2) Conduct surveys to determine the extent of virus infection.
3) Investigate tissue culture procedures for rhubarb.
4) Use this preliminary data to develop a full research proposal.

Materials and Methods

Virus survey, detection and character isolation

Plants with symptoms of decline were collected from commercial crops at Mount Tamborine. Symptoms on individual plants were noted and photographs taken of representative samples.

Samples were examined using a range of techniques applied to the detection and characterisation of plant viruses. These included electron microscopy, serological tests (ELISA and ISEM), sap inoculation to test plants, PCR (polymerase chain reaction) and partial purification of isolated viruses.

Tissue culture experiments

Initiation, multiplication, rooting and acclimatisation of Rhubarb tissue culture explants.

Nine visually healthy rhubarb plants were selected from a rhubarb farm located at Mount Tamborine (C. Eden). The material was obtained on the basis that it would be available for evaluation by industry. Leaves were sent for virus testing and all tested negative after electron microscopic examination of partially purified minipreps. The plants were taken to Maroochy Research Station for tissue culture research. However, they had been maintained at the Tamborine farm for approximately six months prior to commencement of this scoping study. Since rhubarb is grown in heavy soil, bacterial contamination was considered to be one of the biggest obstacles to initiation of rhubarb.

Plants were washed under running tap water for 45 minutes and lateral and apical buds extracted prior to surface sterilisation in 1% sodium hypochlorite for 10 minutes with Tween 80 then rinsed four times in sterile distilled water.

Buds were inoculated and were cultured as described by Walkey (1968) and Walkey and Matthews (1979) onto Murashige and Skoog media (1962) containing 2.5 mg/L Benzyl amino purine and 1 mg/L Indole amino acid with 20 g/L sucrose. Plants were cultured under 12 hour light at 24°C and were sub-cultured onto fresh media every four weeks.

Plants were grown at three temperatures to compare performance 10, 16 and 24°C.

Plants produced via micropropagation were inoculated onto Murashige and Skoog media as described above without the cytokinin BAP but including the 1 mg/L of auxin IAA to induce roots.

Rooted explants were used in preliminary acclimatisation experiments using combinations of sand, peat and perlite.

Subsequent experiments investigated soil less potting mix with plants sown directly into speedling trays.

In vitro virus elimination/transmission studies

Leaves taken from field plants used to establish cultures tested negative for virus infection after electron microscope examination of miniprep extractions. These tested plants were then conventionally propagated so that there were three plants produced from the mother and grown in separate pots. Some of the plants were initiated into culture and the remainder maintained in an aphid proof glasshouse to ensure there was no subsequent infection. After the plants were established in culture, leaves from the *in vitro* plants and leaves from plants remaining in the glasshouse were retested.

Results

Virus surveys and virus detection and characterisation

Declining plants were stunted with reduced leaf area, shortened petioles and gave reduced yields. Leaves on affected plants were mottled with varying degrees of necrotic and chlorotic spotting and veinal necrosis. The range of symptoms were common in the crops examined, with incidences ranging from 10-20% to near 100% in the most common cultivar "Big Red".

At least five viruses have been detected in diseased plants with more than one virus usually present in the one plant with decline symptoms. Serological tests with antibodies to *Arabis mosaic virus* and *Cherry leaf roll virus,* both of which are common in rhubarb in Europe, were negative. *Cucumber mosaic virus* was identified by serology, PCR and host range. The other viruses detected included a virus with long filamentous particles which gave a positive PCR reaction with closterovirus group specific primers in PCR tests. Cloning and sequencing of partial genome fragments has shown that this virus is previously undescribed Two viruses with spherical particles of different sizes have also been frequently detected in infected plants by electron microscopy of partially purified preparations from leaf samples. A probable potyvirus is also present in some samples. Most plants had multiple virus infection. For example, of 10 samples examined, five had three or more viruses and at least two viruses were detected in all 10 samples.

Tissue culture

Initiation of rhubarb plants

Bacteria contamination was high in initial rhubarb explants. Ninety buds from seven mother plants were extracted and sterilised and used to initiate cultures. 67% of meristems were contaminated with bacteria, however a sufficient number of "clean" buds could be obtained from each mother plant for subsequent multiplication.

Multiplication

Rhubarb explants that were initiated free from bacteria were used in the multiplication experiments (Fig 1).

Newly initiated cultures took three months to establish. Average multiplication rate during the establishment phase was 1.1±0.33.

Multiplication commenced in the third month of culture 1.67±1.0 and was extremely variable between cultures. At commencement of the multiplication phase, three temperature regimes were compared, 10, 16 and 24°C. The multiplication and appearance of the plantlets was better at 16 and 24°C (1.83±0.82, 1.52±0.64) compared with the growth and appearance of plants at 10°C (1.25±0.38).

Plants were multiplied further at 24°C and well established cultures reached maximum multiplication of 4.5±0.1 when subcultured on a four weekly schedule. These rates of multiplication are considered high and are believed favourable for commercial multiplication.

Virus studies on tissue cultured plants

Field mother plants labelled 3 and 4 respectively tested negative for virus by electron microscopy and were potted on and multiplied into several individually potted plants. Some of the plants were initiated into tissue culture and the others were maintained in an aphid proof glasshouse.

Plants were retested approximately one year after the initial virus screen. Leaves from plants produced in tissue culture and from the plants that had been maintained in the glasshouse were examined for virus by electron microscopy using mini-prep extractions (Table 1).

Table 1. Virus assay of tissue culture plants

Mother plant ID	Plants in TC, code	Plants in glasshouse	Virus result
3	Rhu 7.1 (apical bud)		No Virus detected
3		Mother No. 3-1	Spheres ~40nm
3		Mother No .3-2	No Virus detected
4	Rhu 5.1 (apical bud)		No Virus detected
4	Rhu 6.1 (apical bud)		Spheres ~40nm
4		Mother No. 4-1	Spheres ~40nm

Plants that contained virus were destroyed. Only tissue culture plants 7.1 and 5.1 were maintained in culture and multiplied for the rooting and acclimatisation experiments.

These two accessions (7.1 and 5.1) were retested for a third time seven months later. No virus was detected by electron microscopy using miniprep extractions.

Root initiation

Plants were taken from multiplication and placed onto rooting media (Fig 2). Roots did not form after four weeks in culture but after the second cycle on rooting media roots began to form. One hundred percent of roots could be initiated on the *in vitro* plants after three consecutive cycles on the rooting media and did not prove to be a limiting factor (Fig 3.).

Plant acclimatisation

Preliminary studies compared acclimatisation in 100% vermiculite, 100% perlite and 100% pasteurised sand. Fifty plants per treatment were established in a glasshouse at 27°C in March 2005. Plantlets were gently removed from culture containers into water at room temperature. The roots were gently washed to remove agar and planted into germination trays. Plants were watered and immediately covered with a plastic vented propagation cover (Hortico) which was covered with 70% shade cloth. Plants were watered every second day and after two weeks the plastic cover was removed but plants remained under shade.

Plants were assessed for survival, root development (1 = none, 2 = some & new, 3 = well developed) and stem colour (1 = green, 2 = pink, 3 = dark red)

Table 2. Survival, root and stem colour development after acclimatisation on various media

Treatment	100% perlite	100% vermiculate	100% pasteurised sand
Survival	80.0%	78.0%	80.0%
Root development	2.30±0.56	2.50±0.49	2.49±0.68
Stem colour	2.25±0.73	1.41±0.54	1.58±0.54

Based on the preliminary acclimatisation results another experiment was performed using speedling trays so that individual explants could be potted directly into cells to save double handling and minimise root trauma at transplanting. Based on the preliminary results all three media treatments gave high survival rates but sand and vermiculite produced the best root development while perlite provided best stem colour development (Table 2). This experiment was to determine feasibility under more realistic/commercial conditions to produce plants until ready for field.

300 plants were grown in a shade house without air conditioning in May 2005. A potting media consisting of 1:1 perlite: vermiculite was placed into ten speedling trays (Hortico), each with 30 places. The media was watered until sufficiently wet and rhubarb explants with good root development were rinsed gently in water to remove agar from root system and planted directly into cells. Trays were covered with plastic propagation covers (Hortico) and covered with shade cloth. Trays were watered every second day. Covers were removed after two weeks and there was 100% survival. After three weeks plants were moved to a shade house with more light. Plants were provided with slow release fertiliser (Osmocote) and periodically fertilised with liquid fertiliser (Peters Professional 1g/L). Plant survival was evaluated after eight weeks and surviving plants were vigorous with dark green leaves and dark red stems. 72.31±7.89 of plants survived ready to field transplant stage (Fig 4).

Discussion

Surveys and virus detection and characterisation studies have shown that at least five viruses are present in rhubarb plants at Mt Tamborine showing decline symptoms.

Most of these viruses have yet to be positively identified and several are probably previously undescribed. There was a high correlation between virus presence in plants and decline symptoms. The extent of virus infection in rhubarb in this important production area appears to be high with from 10 to 20% up to near 100% of plants showing decline symptoms. It is also likely that the incidence in areas outside Tamborine is also high due to vegetative propagation of the crop. The contribution of each virus to decline needs investigation as does possible additive and/or synergistic effects of multiple virus infection.

There are no previous published reports of viruses infecting rhubarb in Australia. In this study, *Cucumber mosaic virus* (CMV) was the only virus found that has been previously found in rhubarb overseas.

For disease control, it is necessary to identify the causal viruses and subsequently the insect vectors or other means of transmission and the likely alternative host plants. For example, CMV is transmitted in a non –persistent manner by many aphid species and has a very wide host range among crop, ornamental and weed species. Similar data on the other viruses detected is necessary to provide rational recommendations on isolation requirements for propagation blocks, probable infection rates and likely efficacy of insecticides in limiting transmission.

The results from this scoping study indicate that it is feasible to use tissue culture to produce Australian rhubarb free from the virus complex. The rhubarb tissue culture plants could be acclimatised and were hardened ready for field planting. These plants would be suitable to start a mother block as a source of clean planting material. The virus results indicate there was inconsistent transmission of the virus and that virus can be transmitted in the tissue culture process. Whether the plants were free from virus from either starting with a virus free mother plant (or bud) or from the tissue culture process itself is not known. Whether some but not all buds on an infected plants contain virus is also unknown.

There is a need to refine the tissue culture processes, particularly initial explant contamination and there were some culture limitations at rooting and acclimatisation. Further comparison of tissue culture produced plants compared to conventional planting material will also need to be addressed. However this study demonstrates proof of concept showing that rhubarb can be multiplied as virus- free plants that can be acclimatised and field ready.

On the basis of this scoping study, a full proposal for work on viral decline and production of virus free planting material in Australian rhubarb crops was prepared and submitted to Horticulture Australia.

The expected outcomes from this work are
a) means of determining the health status of potential field planting material by using the virus assays developed in the project;
b) field control of rhubarb decline disease through a knowledge of the identity and epidemiology of the causal viruses and
c) superior yield and quality of rhubarb from crops produced from virus- free planting material.

If rhubarb decline remains unchecked it has the potential to make rhubarb production in Queensland, and probably other production areas, uneconomic.