Foreword

In 1989 a joint venture project between Daratech (the commercial arm of the Victorian Department of Agriculture) and Kamvale (a group of private investors) was established to find a replacement crop for the tobacco growers of Victoria. The entity Biofresh Australia Pty. Ltd. was formed, with a focus on field trials of growing mint for the production of mint oil, and secondary interest in research into virus elimination of garlic and the establishment of a tissue culture multiplication scheme and the field planting of a high health garlic with increased bulb weight to produce a higher yielding crop.

After negotiations in 1993 the investor component retained the Biofresh Australia Pty. Ltd. name and continued solely with the garlic interests of the project. A successful application to RIRDC the same year saw the establishment of a project into the research and development of an elite garlic through virus elimination and extensive pathogen testing and several generations of tissue culture, screenhouse plantings and finally field plantings to produce a virus-tested garlic seed crop.

The project provided a limited selection of high yielding virus-tested cultivars capable of commercial production as quality seed stock and, in addition, the potential for the drying and processing of a pharmaceutical quality garlic powder based on the levels of alliin (considered to be the bioactive compounding) in samples tested and incorporated into another RIRDC funded project.

The report has in fact identified the opportunities and markets of this product as not primarily the fresh produce market (as initially thought), but as elite seed stock more suited for the establishment of an internationally sourced high alliin content garlic powder for the pharmaceutical or complimentary medicine markets.

This project was funded from RIRDC Core Funds which are provided by the Federal Government and is an addition to RIRDC’s diverse range of over 450 research publications, forms part of our New Plants Products R&D program, which aims to facilitate the development of new industries based on plants or plant products that have commercial potential for Australia.

Most of our publications are available for viewing, downloading or purchasing online through our website:
- downloads at www.rirdc.gov.au/reports/Index.htm

Peter Core
 Managing Director
 Rural Industries Research and Development Corporation
Acknowledgements

- Agriculture Victoria - Scientists and staff at Institute for Horticultural Development, Knoxfield, Sunraysia Horticultural Centre, Irymple and Crop Health Services, Knoxfield.

- Cleangrow Laboratories.

- Dr. Brian Hanger, Botanist.

- Roger & Raelene Schmitke.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HRDC</td>
<td>Horticultural Research and Development Corporation</td>
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<tr>
<td>RIRDC</td>
<td>Rural Industries Research and Development Corporation</td>
</tr>
<tr>
<td>AG-VIC</td>
<td>Agriculture Victoria</td>
</tr>
<tr>
<td>AGGA</td>
<td>Australian Garlic Growers Association</td>
</tr>
<tr>
<td>IPS</td>
<td>Institute Plant Sciences, Burnley</td>
</tr>
<tr>
<td>CHS</td>
<td>Crop Health Services</td>
</tr>
<tr>
<td>SHC</td>
<td>Sunraysia Horticultural Centre</td>
</tr>
<tr>
<td>IHD</td>
<td>Institute of Horticultural Development</td>
</tr>
<tr>
<td>AGIA</td>
<td>Australian Garlic Industry Association</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>GMM</td>
<td>Garlic Multiplication Medium</td>
</tr>
<tr>
<td>GRM</td>
<td>Garlic Rooting Medium</td>
</tr>
<tr>
<td>RRM</td>
<td>Rose Rooting Medium</td>
</tr>
<tr>
<td>NRM</td>
<td>Native Rooting Medium</td>
</tr>
<tr>
<td>GTCC</td>
<td>Garlic Tissue Culture Collection</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometers</td>
</tr>
</tbody>
</table>

Rough Bulbing Stalks become branched and develop cluster of small bulbs instead of one large bulb.

“actively growing” a culture showing small side shoots coming from the base of a healthy in-vitro plantlet.
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Executive Summary

PROJECT TITLE:
Research and development and production of virus-tested garlic, garlic seed and garlic by-products.

OBJECTIVES:
- To develop tissue culture protocols to achieve rapid and reliable multiplication of a selection of virus-tested garlic cultivars.
- To develop methodology for the production and maintenance of screenhouse based “mother” stock through to field based, high health “foundation” planting.
- To achieve commercial scale production of the virus-tested material for the garlic industry to supply our domestic requirements.
- To develop education and marketing programs to ensure that the industry adopts the use of the high health product on a large scale.

BACKGROUND:
The driving force behind this project was the interest expressed in the late 1980’s by the Australian Garlic Growers Association to Daratech (the commercial arm of the Victorian Department of Agriculture) into the research and development of virus-tested garlic seed stock. Because of its expected production of a higher yielding crop and its high health status, potential existed to offer it as a commercially viable alternative crop for the tobacco growers of Victoria. Yields with traditional garlic crops were decreasing annually, and this laboratory based multiplication scheme of virus-tested plantlets could enable an expected yield increase of between 50-80%.

RESEARCH:
A heat treatment elimination of the viruses most affecting Australian garlic was performed at Institute for Horticultural Development, Knoxfield on a selection of garlic cultivars. This material, initiated in tissue culture, were subjected to a series of subdivisions on a multiplication media at a tissue culture facility. The virus-tested plantlets were then planted for one season in an insect proof screenhouse at Sunraysia Horticultural Centre, Irymple. The harvested single to four cloved mini-bulbs underwent two further seasons of field plantings to produce their full bulb weight. Pathogen testing was carried out by Crop Health Services during all stages of the project.

RESULTS:
- Virus elimination of the three viruses most commonly affecting the growth of Australian-grown garlic.
- Formulation of a garlic multiplication medium and the large scale multiplication of virus-tested garlic in tissue culture.
- Produced high health “mother” stock of virus-tested plantlets.
- Produced a commercial crop of virus-tested garlic seed stock.
- Increased the harvest yield by in excess of expected range of 50-80% (i.e. planted one tonne of virus-tested garlic seed and harvested 20 tonnes of bulbs).
- Developed a set of protocols for the production of pathogen tested garlic, including tissue culture, planting and pathogen testing protocols.
- For the first time, developed an Australian-grown garlic product whose origin can be traced directly to a certified laboratory.
- By-product measured high levels of alliin (considered the bioactive compound in garlic).

IMPLICATIONS FOR INDUSTRY:
Biofresh is able to provide the industry with virus-tested garlic seed stock that has significantly higher yields than traditional garlic seed. Returns of greater than 20 tonnes per hectare have been obtained and results in increased gross margins to garlic farmers.

From preliminary research, our virus-tested garlic has a high level of alliin. This, coupled with the substantially higher yield, means that the alliin quantities per square metre of crop, places our garlic in an attractive position for translating our virus-tested garlic commercial crop in the high quality garlic powder for the pharmaceutical and complimentary medicines industries.
1. Introduction

1.1 Background to the Project

This product was initially instigated as a joint venture between Daratech Pty. Ltd. and Kamvale Pty. Ltd. equal equity owners in the company Biofresh Australia Pty. Ltd., which was formed in 1989. Great interest was shown by the Victorian and Australian Garlic Growers Association (AGGA) for investigation into the research and development of virus-tested garlic. The HRDC commenced some funding for the project in 1989/90 under the title of “Development of methods for the rapid detection and elimination of virus diseases of garlic and the establishment of a tissue culture based pathogen tested scheme”.

Earlier experimentation had found that Australian garlic is infected with three common viruses: onion yellow dwarf (OYD), garlic yellow streak (GYS) and garlic mosaic (GM). These viruses were eliminated from some 10 cultivars/varieties of garlic.

Biofresh developed an ELISA test for the detection of these viruses in garlic. This work was carried out at the Institute Plant Sciences, Burnley (IPS) under the guidance of Dr. Robert Sward. The development of the tissue culture protocols for the rapid multiplication was also commenced at IPS, though numbers produced were less than projected. It was of paramount importance to the success of this scheme that a project be funded to continue investigation focussing on 4 varieties, but expanding work on the parameters of light, temperature, basal medium recipe (garlic multiplication medium - GMM) and sub-culturing regime along with improved techniques for meristem dissection. With a small quantity of virus-tested material sent to Cleangrow Laboratories at Tooradin, Victoria, a commercial tissue culture facility and nursery, a program was designed to further research and develop a repeatable and reliable large scale tissue culture multiplication.

An aphid proof cage was installed on the commercial property of Mr. Phillip Ward (President of AGGA) in Swanpool, Victoria to provide a protected environment for the transfer of virus-tested plantlets from the nursery to propagate our ‘mother stock’ of first generation virus-tested bulbs.

For optimum growth and maximum crop yields, research and development into many factors including nutrient requirements, regimes and the elimination of “rough bulbing” was required. The production of sufficient high health ‘virus-tested’ garlic seed through our developed tissue culture and growing protocols would provide for growers to increase their yields per hectare by 50-70% or between 16-20 tonnes per hectare in the first year of planting.
2. Objectives

The project was designed to provide a commercial advantage to Australian garlic growers for both the domestic and export markets by:-

- developing tissue culture protocols to achieve rapid and reliable multiplication of a selection of virus-tested garlic cultivars;

- developing the methodology for the production and maintenance of screenhouse-based “mother” stock through to field based, high health “foundation” planting;

- achieving commercial seed production of the virus-tested material for the garlic industry to supply our domestic requirements; and

- developing education and marketing programs to ensure that the industry adopts the use of the high health product on a large scale.
3. Methodology

3.1. Tissue Culture

A range of experiments were to be conducted to significantly improve the performance of the tissue culture material in order to produce acceptable, reliable and repeatable multiplication rates.

Although initial laboratory trials had identified a media suited to initiation of shoot tips and some multiplication, there were difficulties when the program was subjected to large scale production. Cultures grew unevenly, there was an increased level of dormancy, multiplication rates deteriorated with the presence of prematurely formed bulbs and a lack of vigour in the deflasked plantlets.

It was intended to use the skills of a commercially operating tissue culture facility under the guidance of botanist Dr. Brian Hanger to investigate these problems in order to formulate protocols for the successful large scale production of virus-tested plantlets from tissue culture - *Generation Zero (G0)*.

Equal increment dose response experiments followed by regression analysis of the data were done to determine :-

(a) the optimum auxin: cytokinin ratio of shoot multiplication media; and
(b) the optimum NH₄/NO₃ nitrogen source of the shoot multiplication media.

Further experiments recorded the difference in shoot numbers at different light/dark (L/D) regimes varying from 10L/14D to 16L/8D, and at two different temperatures (i.e. 20°C and 25°C).

The effect of shortening the time between sub-culturing dates from 4 to 2 weeks was investigated in order to achieve greater multiplication rates.

Since there was limited virus-tested material at the onset of these trials, the preliminary testing model was carried out using a virus-infected cultivar (Printinor) while numbers of virus-tested material were being bulked up.

Further, the presence of prematurely formed bulbs (microbulbs) in culture, although representing a reduction in multiplication rates, were investigated through experiments with varying levels of sucrose in the media and various temperature and light regimes. This opportunity to produce in-vitro bulbs instead of plantlets could eliminate problems of deflasking and growing-on of plantlets.

3.2 Screenhouse and in Field Planting

Hardening-off of deflasked plantlets through acclimatisation in both hot house then screenhouse environments was investigated with relevance to method of watering, temperature and humidity in order to produce a more uniform plantlet. The overall vigour and bulb size of these plantlets would determine the eventual number of cloves formed per bulb. From experiments carried out at Loxton Research Centre (SA) over 1987-91 and work currently being done by researchers at Ag Vic, it has been shown that for optimum growth of garlic bulb production, a specific range of nitrogen, potassium, phosphorus (N:P:K) is required and that these levels are different at various growth stages of the garlic plant.
At the initial stage of this project, an aphid proof screenhouse was erected at Toolangi Research Station and in the final year of the project at Sunraysia Horticultural Centre to monitor soil preparation, nutrient levels through a fertigation program and undergo pathogen testing at 2 stages during planting conducted by Crop Health Services for the production of virus-tested “mother stock” - *Generation One (G1)*.

The subsequent season would see the harvested bulbs field planted in isolation of any other allium crop at an operational garlic farm in northern Victoria. This was transferred to the Toolangi Research Station after the first season and finally to a commercial garlic farm in Waikerie, South Australia.

Skilful and careful practices were developed for planting, growing and harvesting to ensure the material stays free of a range of pathogens including viruses, soil-borne fungal and bacterial diseases, as well as avoiding infestations of damaging insects such as mites and aphids. Production of a crop following these grower guidelines including pathogen testing would produce high health virus-tested garlic ‘foundation’ seed stock - *Generation 2 (G2)*.

Crop Health Services, Institute for Horticultural Development, Knoxfield provides a comprehensive range of tests on a fee for service scale for the diagnosis of pests and diseases. Large scale testing of screenhouse and field samples are conducted by electron microscope and ELISA testing as well as culturing for soil-borne and bacterial diseases.

The successful methodology should produce the formulation of a range of protocols for initiation and maintenance of ‘nucleus stock’, tissue culture multiplication, pathogen testing and field planting of virus-tested garlic seed stock.

### 3.3 Initiation and Maintenance

Virus-tested cultivars are initiated from pathogen tested field material and are maintained by IHD Knoxfield. The tissue culture plantlets are held in 2 separate quality controlled locations and sub-cultured onto fresh media as required by growing conditions. Sub-samples are deflasked and grown in the glasshouse and re-indexed to ensure their virus-tested status. Upon at least 4 months prior notice from Biofresh, IHD supplies 10 tubs of actively growing cultures of each of the specified cultivars held to the nominated tissue culture multiplication facility.

As any new cultivars are introduced to the program of virus elimination and initiation, they will be added to the nucleus stock supply.
4. Discussion of Detailed Results

4.1 Nucleus Stock Maintenance

Program for the maintenance of a tissue culture collection of virus-tested garlic cultivars including the initiation of new material every season to provide a high health ‘nucleus’ stock. This collection is maintained by Crop Health Services, Knoxfield.

4.2 Flow Chart 1
Maintenance of Biofresh’s Garlic Tissue Culture Collection (GTCC)

- The GTCC is renewed from cloves collected from pathogen tested fieldgrown material and from new varieties included upon request from Biofresh.

- Garlic cloves are collected from the field in October/November of each year and tested for range of bacterial, fungal, viral and nematode pathogens.

- When tested pathogen “free” cloves undergo meristem extraction under aseptic conditions and placed onto multiplication media.

- Garlic plantlets are bulked up in tissue culture until 10 to 12 vials of actively growing material is obtained. Material is then routinely subcultured to maintain this number.

- IHD is notified in June/July by the nursery contractor of the number of cultures required per variety and time of delivery.

- The material supplied is actively growing with at least 4 individual shoots growing from the original meristem. The tissue culture tubes are to be brand new, free from any contamination and sealed with parafilm tape.

- The collection is renewed each season from cloves collected from pathogen tested fieldgrown material and from new varieties included upon request from Biofresh.
4.3 Large Scale in-Vitro Multiplication

4.3.1 Study A - Medium Formulation

Dr. Brian Hanger set out trials using different nutrient levels to investigate the effect of medium formulation in multiplication rates.

In a page by Takayuki Nagakub et al in Plant Cell Tissue and Organ Culture 32: 175-183, 1993 there were indications that the organic phosphorus levels rather than hormones were important for multiplication. It was noted that the ratio of potassium nitrate to ammonium chloride in the medium has an effect on multiplication rates.

Single shoots of the garlic cultivar “Printanor”, our working material, were planted onto a control medium (GMM) and then onto treatment mediums with varying nitrate -N to ammonium -N ratios. In total, there were five treatments and a control each consisted of ten tubs with ten shoots in each tub.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nitrate -N only</td>
</tr>
<tr>
<td>2</td>
<td>Nitrate-N to Ammonium-N 11.2:1</td>
</tr>
<tr>
<td>3</td>
<td>Nitrate-N to Ammonium-N 6.1:1</td>
</tr>
<tr>
<td>4</td>
<td>Nitrate-N to Ammonium-N 3.6:1</td>
</tr>
<tr>
<td>5</td>
<td>Nitrate-N to Ammonium-N 2.7:1</td>
</tr>
<tr>
<td>Control - GMM</td>
<td>Nitrate-N to Ammonium-N 2.1:1</td>
</tr>
</tbody>
</table>

Examination of the tubs over a 5 week period showed that as the nitrate to ammonium ratio decreased (i.e. from pure nitrate, through 11.2:1 to 2.7:1) in the medium:

(i) number of shoot clumps in a tub increased;
(ii) root growth generally improved;
(iii) die back of leaf tips decreased;
(iv) less purpling of leaf tips; and
(v) overall improvement in appearance of the tissue.

The shoots on the medium without any ammonium-N present appeared the most stressed with shoot purpling and tip die back, restricted root growth and no multiplication. These results are in opposition to those of the abovementioned literature. The best medium being that of the control.

This line of investigation was continued with further experiments using slightly modified media where the total nitrogen concentration was kept constant whilst varying the ratio of nitrate-N to ammonium-N.

Ten samples (units) were placed in each tub. A sample consisted of either a single well developed shoot or a small cluster of shoots. All tissues were trimmed to 2-3cm long. Some 8 weeks later, the garlic plantlets were sub-cultured back onto the same treatments. Clumps were broken up into either single shoot or a small cluster of shoots. When inspected 6 ½ weeks later, there was no way of differentiating from appearance which tubs had received the single shoots.
TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Tubs</th>
<th>Mean No. of Single Shoot Units in a Tub</th>
<th>SD of Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>5.2</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>3.5</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>3.4</td>
<td>1.7</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>7 (Control) *</td>
<td>10</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>8 (Control) *</td>
<td>7</td>
<td>3.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Ammonium chloride used instead of ammonium nitrate.*

Treatment 5 was superior to all other treatments and gave the most consistent results. The lower the number of single shoot units the better, as the material is in a higher multiplication phase. This confirms the indications of our previous study that plant health, root growth and shoot multiplication tend to improve as the nitrate: ammonium ratio decreased from nitrate only through the ratios 11.2:1 to 2.7:1.

It could be that each garlic cultivar has a specific nitrate: ammonium ratio for optimum multiplication.

### 4.3.2 Study B - Frequency of Sub-Culturing

In 1995, further advancement in trials was hampered by a disappointing number of tissue cultures supplied due to the presence of the resistant bacterial infection ‘white ghost’ in all lines from IHD. All those showing singulation were deflasked while the remainder were replaced on multiplication medium to try and build up the numbers. Sub-culturing was performed every 2-3 weeks to try and keep the material in a multiplication phase.

There appears to be a tendency to singulate as the generations in culture increase. A sub-culturing every 14 days was established as the optimum to improve the multiplication rate.

The following conclusions were made :-

1) Frequent sub-culturing (every 14 days) enhances multiplication rate and maintains the tissue in a strong and healthy state.
2) Tissue held in culture for long periods tends to lose vigour. New material should be initiated each season.
3) Holding plantlets for excessive periods without sub-culturing does not induce bulblet formation.
4) In the multiplication stage, focus must be on plants which have gone into multiple shoot production. Do not segregate into single shoots. Divide large clumps of multiple shoots into smaller clumps.
5) Strong growing single shoots on GMM tend to remain as single shoots.
6) Plantlets can be cold stored for at least 6 months and remain viable.
7) Plantlets with a developed root system can be successfully established out of cultures, but need acclimatisation.
8) Plantlets, provided they have root systems, can be planted up without removal from the agar media base.
9) Once removed and established out of culture, it is important to maintain a good level of nutrition to enable the plantlets to respond into active growth.

4.3.3 Study C - Plantlets With Stronger Shoot Growth

The cultures were generally excellent, characterised by clusters of short shoots with healthy, fine, thin leaves. The majority of the 10,000 plantlets produced were placed on rooting medium which induced root development but failed to strengthen shoot growth. These were then transferred into cell trays and held in a heated screenhouse and mist irrigated to prevent any moisture stress during acclimatisation. A small sample were placed directly into a cold screenhouse to determine if this had any influence on survival rates. With time, the majority of plants died. Only those plantlets which were singulated and relatively large at the time of transplant into the cells, showed any signs of surviving.

Successful establishment of the plantlets is very much dependant on shoot size. Those with the very fine leaves of less than 1-3cm generally fail to survive particularly if still part of a clump. Single plants with shoots 4cm plus had a lower mortality rate and once potted up and transferred to the cold screenhouse, most grew on to bulb up at the end of the season.

The tissue culture of garlic falls into three distinct stages of development:

1) The rapid multiplication in plant numbers by the induction of large numbers of shoot clusters;
2) The preparation of the tissue ready to remove from culture, ie to move out of the shoot cluster mode to the production of either strong, robust rooted single plantlets or small bulblets; and
3) The growing on of the plantlets or bulblets out of culture to complete their life cycle in the production of bulbs for future planting.

Over the course of our studies with the tissue culture of garlic, significant advancement has been made in each of these stages, especially in the formulation of the preferred multiplication growing medium. However, the key to a successful tissue culture program is in the integration of the three distinct stages.

Our work has repeatedly shown that once the garlic has been programmed to produce multiple shoots, it is extremely reluctant to switch its growth pattern back to the production of strong, singulated plants suitable for deflasking.

Although our results have produced some excellent plantlet material there is still not a constantly reliable vigorous number for large scale production of all varieties. Certain cultivars performed better then others. The leading performers, along with new varieties of high performance virus-infected material which are currently being put through virus elimination, will form the basis of extensive trials. It is felt that with the aid of some further work in improving handling techniques, that a selection of virus-tested cultivars can be provided for large scale production of sustainable plantlets which will produce 3-4 cloves from their first planting season.

4.3.4 Study D - Inducing Bulb Formation In Culture

It is known from work with other plants that the growth and development of plantlets in culture can be manipulated by varying the concentration of sucrose in the medium. This has been used
on potatoes to produce mini-tubers. Higher sucrose content of the medium has an osmotic effect on the plants, making conditions more stressful and could with garlic plantlets, initiate bulb formation in culture. We know that each garlic variety behaves differently in culture, some readily produce small bulbs whereas others do not. Printanor falls into the latter category.

Procedure:

Garlic rooting media were prepared with different sugar concentrations:

(i) Zero,     (iv) 30g/L      (iii) 100g/L

Everything else remains unchanged.

Code 'PS'    ‘Printanor’, growing as single shoots from single shoot explants
Code 'PC'    ‘Printanor’, single shoots separated out from shoot clusters
Code 'PM'    ‘Printanor’, growing as shoot clusters derived from the sub culture of shoot clusters

After repeated sub-culturing over the months on the garlic multiplication medium, the PS and PC cultures remained mostly single plantlets with good shoot growth, whereas the PM cultures were generally clusters of multiple shoots with short shoot growth.

After transfer to the sugar treatments, the number of tubs (each tub contained 15 plantlets or clusters) were as follows:

<table>
<thead>
<tr>
<th>Sugar Level</th>
<th>0g/L</th>
<th>30g/L</th>
<th>100g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Code 'PS'</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Code 'PC'</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Code 'PM'</td>
<td>13</td>
<td>13</td>
<td>16</td>
</tr>
</tbody>
</table>

Total number of tubs 28 28 31

The tubs were placed in the culture room for two weeks and then 10 tubs of each sugar treatment moved to the cold room for two to three months storage. The remaining tubs held in the culture room. The plantlets were not sub cultured for the duration of the study (three months).

Results:

Tubs were inspected after three months on the sugar treatments.
4.3.5 Plantlets in culture room

Best survival of the plantlets occurred at the 30g/L sugar level. Single plants had made strong growth with dense root mat. There was some basal stem swelling indicative of early bulb production. However, shoot clusters continued to grow as clusters with minimal root production and no swelling at base of shoots.

At the 100g/L sugar level, most plants had died back. Nevertheless, growth characteristics were similar to the 30g/L sucrose treatment. However, the single shoots appeared to have the strongest indications of bulb formation of all treatments.

At the zero sucrose level, most plantlets were dead and had made very little additional growth.

4.3.6 Plantlets in cold room

The general appearance of the plantlets confirmed the results of the treatments with plantlets held in the culture room. The exception was that there was minimal plant senescence and death because of reduced metabolic and growth rates at lower temperatures.

Conclusions:

(1) It confirmed that we are working with two distinct types of plants which are physiologically different from each other, ie single strong growing shoots and shoot clusters.
(2) Garlic plantlets are unable to sustain growth without the presence of sucrose in the medium.
(3) There was a visual indication that the highest sugar concentration enhanced swelling at the base of the single plantlets as a prerequisite to bulb formation.

A further study is needed with a wider range in sugar concentrations but working only with single shoots. Again, growing the plantlets in the culture and cold rooms.

4.3.7 Study E - Effect of Temperature

This study was conducted with well established single plantlets. These were transferred to a garlic rooting medium (GRM), held in the growth room for one week and then placed in the cold room. Over the following months, three tubs of plantlets were periodically removed from the cold room and the plantlets from two tubs transplanted to pots of potting mix and placed in a screenhouse under natural environmental conditions. The third tub was transferred to the growth room and plantlets grown on to determine if the cold treatment has led to bulb formation on completion of their life cycle.

The following treatments were established:

1. Plantlets held at all times with no cold treatment.
2. Plantlets held three months in cold treatment, ie until late October.
3. Plantlets held four months in cold treatment, ie until late November.
4. Plantlets held five months in cold treatment, ie until late December.
5. Plantlets held six months in cold treatment, ie until late January.

**These were potted up the same time as the three month cold treated plants.
On removal from culture, the plantlets removed after three and four months in culture were initially held in a warm greenhouse for two weeks before transfer to the unheated greenhouse. Plantlets removed later from the cold room went straight into the unheated greenhouse. The pots were watered as required and plantlets acclimatised well under these conditions.

Results:

1. There were minimal problems encountered in transferring single plantlets from culture to potting mix. There was almost 100% success rate.
2. Plantlets transferred directly without cold treatment made very little growth and failed to bulb up normally after 6 months.
3. Plants taken out of culture after 3 months made the best growth of all treatments. This is in part because they had the longest time span in the greenhouse to complete their life cycle.
4. All plantlets out of the cold room bulbled up, even if out of the cold room for only one month. Very small and weaker plantlets also produced bulbs.
5. Irrespective of the time out of culture, all plantlets seemed to complete their life cycle at the traditional time for garlic.

Conclusions:

1. We now have a viable method for the production of bulbs from tissue culture plantlets.
2. A period of at least 3 months in the cold room and enables plantlets to be planted up in the spring to complete a normal growth cycle by mid-summer.
3. It would be interesting to study the growth cycle of cold stored plantlets potted during the summer and autumn as it may be possible to have a year round bulb production using cold stored plantlets held in culture. Further work would involve testing the viability of bulbs produced by this method when planted at the normal time in the season for field grown garlic.

4.3.8 Study F - Hydroponics

A small number of plantlets including some as shoot clumps were successfully acclimatised and grown in a hydroponic system under shade house conditions.

Two types of plantlets were investigated:

1. Single shoot plantlets which had been stored while in culture in the cold room for a number of months.

   On transfer to the hydroponic system in spring, they went through a normal growth cycle and produced small bulbs which were harvested. These bulbs were held dry on a table at room temperature for 3 months and although small, they remained firm and appeared fully viable when planted into the perlite.

   All started to produce roots within 10 days. Only one bulb failed to grow, and this loss was caused by localised waterlogging.

2. Plantlets out of tissue culture which had not received the cold treatment and planted up in spring.

   These behaved differently in that they did not complete their life cycle. No bulbs were produced, and they remained vegetative and continued to grow throughout the autumn
period to produce shoot clusters. After one month, plant number (ie number of distinct shoots) had increased to 27, and multiplication continued and after 2 months, had reached 48 plants; still with no signs of bulbing.

Therefore, there could be a distinct benefit in working with plantlets from tissue culture in spring which have not been exposed to cold temperatures. By maintaining a good level of nutrition in the growing medium, this pilot study showed that plants keep growing more shoots.

At this point, approximately half the number of plants were removed from the hydroponic container, divided into single plants and replanted into another container (perlite as the growing medium) and spaced to ensure normal bulb development. About two thirds of the root system was removed at the time of shoot division and transplanting.

The appearance of the transplanted plants a month later indicated successful establishment, although the oldest leaves had yellowed.

All hydroponic plants were held in a shade house under natural climatic conditions and maintained on a low level of nutrition. As a consequence the bulbs were small in size when harvested. The yields are summarised in the following table:

<table>
<thead>
<tr>
<th>Treatment Rate</th>
<th>No. of Units</th>
<th>No. of Cloves</th>
<th>Multiplication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Planted</td>
<td>Harvested</td>
<td></td>
</tr>
<tr>
<td>Non chilled plantlets, replanted</td>
<td>18 shoots</td>
<td>84</td>
<td>4.7</td>
</tr>
<tr>
<td>non chilled plantlets, undisturbed</td>
<td>27 shoots</td>
<td>89</td>
<td>3.3</td>
</tr>
<tr>
<td>chilled plantlets, replanted bulbs</td>
<td>12 bulbs</td>
<td>73</td>
<td>6.1</td>
</tr>
</tbody>
</table>

This showed that hydroponics may be useful when working with small numbers. The extended growing period through summer and autumn increased plant numbers. The shoot clusters then can be broken up into individual rooted plants and handled the same way as seedlings for autumn planting. Once the non chilled plants went through normal winter conditions they bulbled up as normal.

The tissue culture plantlets from three tubs yielded 246 cloves in 18 months out of culture. Best multiplication was with the non-chilled plantlets because of the extended time during summer and autumn for continued multiplication. However, these are very difficult to successfully establish out of culture as our work has shown.

**General Concluding Remarks**

(i) The future priority area of research must be in breaking the multiplication mode to which the plants have been programmed so that strong single plants can be produced while in culture.
(ii) Because there is always the possibility that only small delicate plantlets are available for acclimatisation and growing on out of culture, different growing on procedures need to be devised and trialed to improve establishment rates and minimise plant loss, in-vitro bulb formation may provide to results for some cultivars.

4.3.9 Study G - Breaking The Extreme Multiplication Cycle

Some cultures were displaying enhanced multiplication with the 14 day cycle of sub-culturing, with the plantlets displaying a “grass” like appearance. Upon planting into cell trays filled with sterilised seedling mix, they were very fine shoots and showed little evidence of any root system. The cell trays were placed in an environmentally controlled greenhouse and the plantlets acclimatised under mist. Most failed to survive without producing bulbs. The collapse could not be attributed to any disease or insect pests. A limiting of the production of strong viable plantlets was of major concern. In an attempt to break this extreme multiplication cycle, ten tubs of plantlets were set aside for a temperature study using a cold room regime as set out below.

<table>
<thead>
<tr>
<th>Tub No.</th>
<th>Date Into Cold Room</th>
<th>Date Out of Cold Room</th>
<th>No. of Cold Weeks</th>
<th>General Appearance of Shoots at Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23 August</td>
<td>11 September</td>
<td>3</td>
<td>Singles</td>
</tr>
<tr>
<td>2</td>
<td>23 August</td>
<td>11 September</td>
<td>3</td>
<td>Clusters</td>
</tr>
<tr>
<td>3</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
<tr>
<td>4</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
<tr>
<td>5</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Singles/Clusters</td>
</tr>
<tr>
<td>6</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
<tr>
<td>7</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
<tr>
<td>8</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
<tr>
<td>9</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
<tr>
<td>10</td>
<td>1 August</td>
<td>11 September</td>
<td>6</td>
<td>Clusters</td>
</tr>
</tbody>
</table>

This study was aimed at switching the plantlets back to a normal type of growth pattern and making them responsive to rooting hormones. Plantlets were sub-cultured repeatedly for up to five months onto hormone-free media. After the third month, samples of the shoots were placed onto different rooting media to see if roots could be readily induced.
TABLE 5

<table>
<thead>
<tr>
<th>DATE</th>
<th>ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 July</td>
<td>Transferred to garlic rooting medium (GRM) with auxins</td>
</tr>
<tr>
<td>28 July</td>
<td>Transferred to GRM (without auxins)</td>
</tr>
<tr>
<td>28 July</td>
<td>Sub-cultured to GRM (without auxins)</td>
</tr>
<tr>
<td>11 September</td>
<td>Sub-cultured to GRM (without auxins)</td>
</tr>
<tr>
<td>26 September</td>
<td>Sub-cultured to GRM (without auxins)</td>
</tr>
<tr>
<td>14 October</td>
<td>Sub-cultured to GRM (without auxins)</td>
</tr>
<tr>
<td>29 October</td>
<td>Sub-cultured to either - GRM (without auxins) - NRM (Natives Rooting Medium 10g/L IBA and half strength salts) - RRM (Rose Rooting Medium 0.5g/L NAA and 5g/L IAA)</td>
</tr>
</tbody>
</table>

Results:

1. Over the entire period of this study, all shoots failed to produce any substantially strong root growth, therefore this problem had not been overcome.
2. After transfer to hormone free medium, the clumps became easier to divide into single or small shoot clusters. They still retained their cluster appearance in most tubs, although the ‘grassy’ appearance was disappearing, being replaced with stronger shoots. Therefore, the treatments were having some influence.
3. Best shoot growth occurred on RRM, followed by NRM and the poorest on GRM (hormone free). However, the most significant event was the formation of tiny ‘mini bulbs’ mainly on the GRM. These were very dark green spherical bodies, distinct entities within clusters and 2-4mm in diameter. They later appeared in all cultures, but not in all tubs. They are easy to harvest from the culture media and probably occurred in response to the cold treatment of the plantlets 2 months earlier. Their viability and importance for large scale production potentially presents a new direction in investigation.

4.4. Screenhouse Results

With our first season, the problems with acclimatisation of the plantlets out of tissue culture meant that there was limited quantity of material for this stage of planting. Due to the fragile character of the plantlets, it was decided to replant them in groups of 3-4 in 20cm pots in a high health aphid proof screenhouse erected at Cleangrow Laboratories, Tooradin.

(a) TOORADIN

IHD, Knoxfield supplied small quantities of starter cultures of each culture to Clean Grow Laboratories. These were multiplied in tissue culture over a number of months to produce in excess of 10,000 cultures for planting out, as per Table 6 below.
TABLE 6

<table>
<thead>
<tr>
<th>Number of Pots</th>
<th>Approx. Number of Plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofresh 1 (B1)</td>
<td>81</td>
</tr>
<tr>
<td>Biofresh 2 (B2)</td>
<td>136</td>
</tr>
<tr>
<td>Biofresh 3 (B3)</td>
<td>50</td>
</tr>
<tr>
<td>Biofresh 4 (B4)</td>
<td>312</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>579</strong></td>
</tr>
</tbody>
</table>

*NOTE: The Biofresh 4 cultures were in an "active multiplication mode" and did not singulate during the Rooting Medium Stage. We did not attempt to singulate when deflasking (because of the difficulty) nor when potting into 200mm. pots (because of space limitations). Biofresh 4 plantlets were in clusters of 3 - 6 with 10 clusters per pot. It was expected that each cluster would produce 3 - 6 bulblets.

The plantlets were grown in pots in an insect proof greenhouse where the temperature was maintained at 18 degrees at night and held at 25 degrees during the day.

Depending on the size of the cultures the material was planted in either pots or small cell trays in June-July. 2-3,000 plantlets were planted 10 to a pot in 20cm. diameter pots and these grew well through to maturity. 6-8,000 small cultures were deflasked and planted as clumps in small cell trays in sterilised soil. However, a large proportion of these failed to establish and grow. The low rate of survival appears to be directly related to the size of the initial plant material.

Harvested bulbs were collected and cool stored with the following modifications made for the ensuing season’s screenhouse plantings:

(i) direct soil planting into two isolated screenhouses to be performed at Ag Vic Toolangi Research Station and a garlic farm in Waikerie, South Australia. These two sites enabled a comparison in soil composition and temperature, climatic and environment effects.

(ii) depending on the variety, Australian garlic seed is usually planted in late February through March. As we are using a plantlet not a clove, a plant date up to 4 weeks later (end April) would be acceptable as this would be in alignment for the time of emergence of the traditional seed.

(iii) Attempt only to plant material that is singulated, acclimatised and at a height of 4-5cm with some root system, to achieve maximum multiplication within harvested bulbs.

(b) Table 7 below reflects the results at harvest at the two planting sites.
TABLE 7

<table>
<thead>
<tr>
<th>Variety</th>
<th>Toolangi Screenhouse (VIC)</th>
<th>Waikerie Screenhouse (SA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Plantlets</td>
<td>Harvest Wt. (kg)</td>
</tr>
<tr>
<td>B1</td>
<td>2830</td>
<td>4.0</td>
</tr>
<tr>
<td>B2</td>
<td>325</td>
<td>0.5</td>
</tr>
<tr>
<td>B3</td>
<td>180</td>
<td>0.25</td>
</tr>
<tr>
<td>B4</td>
<td>1310 discarded</td>
<td></td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>4645</strong></td>
<td><strong>4.75 kg</strong></td>
</tr>
</tbody>
</table>

Conclusions:

(i) The strike rate at Waikerie was approximately 90-95%. The plantlets maintained their high health status and grew well. Although bulbs were small, they contained a mix of single to 4 cloved bulbs. With this multiplication rate, full bulb weight potential could be reached in a further 1-2 seasons. Toolangi was trialed with the higher proportion of material yet far underperformed Waikerie with disappointing results. Plantlets grew slowly, were less vigorous and due to yellowing of leaf tips, looked less healthy.

(ii) It appeared the soil and environmental conditions of the Sunraysia district were more conducive to producing optimum ‘mother stock’. 1999 saw the relocation of this phase of the project to Sunraysia Horticultural Centre, Mildura (SHC).

(iii) A further refinement occurred in discontinuing production of B4 due to its poor performance and the commencement of the virus elimination of 2 new varieties for introduction into the scheme.

(iv) The establishment of SHC as the provider of virus-tested garlic mother stock is further supported by another RIRDC funded project investigating growth markers, nutrient and fertiliser supply and alliin levels which is currently set up at the same facility.

4.5 Field Planting Results

In our first season, the virus-tested cloves were obtained from two sources. Mature plants originally ex-Burnley Institute of Plant Services (IPS) grew to 30-50cm high and looked very healthy and plants originally ex-Cleangrow tissue cultures also grew well varying in size from 5-25cm.

As the plants dried off, checks were made and most looked to have produced good quality bulbs with 8-12 good sized cloves per bulb. The material was harvested and bulbs were sorted with respect to size as per Table 8.
## TABLE 8

<table>
<thead>
<tr>
<th>Variety</th>
<th>No. of Cloves Planted</th>
<th>No. of Bulbs Harvested</th>
<th>No. of Bulbs/Percentage of Crop</th>
<th>Average No. of Cloves/Bulbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XL</td>
<td>L</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>Biofresh 2 (B)</td>
<td>275</td>
<td>256</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>75</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Biofresh 1 (B)</td>
<td>52</td>
<td>47</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>11</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Biofresh 3 (B)</td>
<td>48</td>
<td>45</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>18</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Biofresh 2 (C)</td>
<td>25</td>
<td>23</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Biofresh 1 (C)</td>
<td>37</td>
<td>37</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

**NOTE:**

(B) - Ex Burnley 1993  
(C) - Ex Cleangrow 1993  
XL - Extra Large > 80mm diam.  
L - Large 40-80mm diam.  
M - Medium 25-40mm diam.  
S - Small < 25mm diam

In commercially grown crops, the accepted industry norm for the percentage of crop to size ratio is:

XL : L : M : S  
10 : 40 : 40 : 10

The above ex-Burnley varieties indicate 8-14% increase on the norm, while the ex-Cleangrow varieties are behaving as per previous year’s results and a further generation of growth should provide us with relevant results.

The harvested material was hung and dried 3-4 days in the screenhouse enclosure and then 3-4 weeks under the shelter of a garage allowing for full shade with continuous airflow. The bulbs were placed in onion bags and remained in the garage throughout the summer (considered standard practise). When the material was inspected prior to sorting for the next season’s planting, it was found to be totally hollow with only the outer scale leaves intact. The bulbs literally “cooked” and totally dehydrated under the extremely high storage temperatures. Post harvest storage must become a critical component of the growing protocol to be prepared.
Two further seasons of field planting at Toolangi Research Station produced disappointing results. Logistical problems caused a delay in planting dates. There were problems with frost, fungal infection, water stress and Fusarium.

Although bulbs appeared to be much more regular in shape with 8-10 cloves, good weight and well formed large perimeter cloves. Unfortunately, what also became apparent was the appearance of a darkened area within the bulbs representing the presence of Fusarium and water stained pockets just under the surface of the bulbs’ outer layer. Close to harvest, there were many days with temperatures well into the 20°s and 30°s, providing good conditions for the bulbing up of the garlic. Concurrently high humidity levels with one day of 42°C followed by severe rainfall conditions that caused the rampant spread of Fusarium. As they opened around the top of the bulb, water lodged into the cavities created.

Such an attack was very unfortunate as the bulbs harvested showed good weight, shape and clove formation. Overall multiplication rates, yield and the non-detection of pathogenic viruses were all in line with expectations.

26.5kg of material was sent to Waikerie, South Australia. It was felt that the environment, soil and climatic conditions along with the experience of a commercial garlic grower could advance this stage. A total harvest of 171.8kg provided good looking material and good multiplication rates of 3 varieties (B1, B2 & B3).

After a second season of planting, the results were quite dramatic.

<table>
<thead>
<tr>
<th>Seed Source</th>
<th>Variety</th>
<th>Planted Wt (kg)</th>
<th>Harvest Wt (kg)</th>
<th>Multiplication Rate</th>
<th>Variety Av. Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toolangi</td>
<td>B1</td>
<td>45.0</td>
<td>418</td>
<td>9.29</td>
<td></td>
</tr>
<tr>
<td>Waikerie</td>
<td>B1</td>
<td>6.0</td>
<td>1561</td>
<td>28.5</td>
<td>18.9</td>
</tr>
<tr>
<td>Toolangi</td>
<td>B2</td>
<td>30.0</td>
<td>631</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>Waikerie</td>
<td>B2</td>
<td>54.6</td>
<td>851</td>
<td>23.1</td>
<td>22.0</td>
</tr>
<tr>
<td>Toolangi</td>
<td>B3</td>
<td>141.0</td>
<td>2142</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>Waikerie</td>
<td>B3</td>
<td>36.8</td>
<td>1574</td>
<td>29.8</td>
<td>22.5</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td><strong>360.2kg</strong></td>
<td><strong>7177kg</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Overall, 360kg of virus-tested seed was planted and 7177kg (dry weight) was harvested, thus yielding an average multiplication rate of 19.9

(i.e. plant 1 tonne of virus-tested seed per hectare and harvest 20 tonnes).

Conclusions:

i) The multiplication rate of 20 validates the increased yields of 50-100% obtainable using virus-tested seed stock.

ii) Two growing seasons provide bulbs of approximately 15 cloves. These are at full bulb weight and suitable as commercial seed stock.

iii) Sufficient data has been collected to commence the preparation of grower guidelines for planting virus-tested garlic seed stock.

iv) The methods trialed could now be applied to a commercial crop of virus-tested garlic.

4.6 Pathogen Testing
Pathogen testing was carried out by Crop Health Services at IHD Knoxfield twice per season at all facilities used during the course of this project. Officers performed site inspections and supplied written reports.

For each generation, sufficient plant material was tested to give 95% confidence that less than 5% of plants could be infected.

Report comments were given on general hygiene and conditions of the facility, general plant health based on visual findings along with the results of Electron Microscopy and ELISA testing using commercial kits for Onion Yellow Dwarf Virus, Leek Yellow Stripe Virus, Shallot Latent Virus and Potyvirus General. Fungal, Bacterial, Nematode and Arthropod disease symptoms were expressed using standard diagnostic tests as per ISO 9002 manuals.

There were two major considerations that appeared as a result of the passage of testing over the term of this project: -

(i) the presence of fusarium oxysporum in deflasked plantlets had affected final product;
(ii) the field generations were not separated in field plantings.

In order to maintain high health status of this virus-tested material: -

1) If a crop consists of a mix of field generations or mix of seed source, then every sub group should be separated by a minimum of 500m from each other and any other allium crop.
2) The higher the health status of deflasked plantlets, the higher the multiplication rate of the crop.
3) The elite garlic virus-tested germplasm must be re-initiated each season.
4.7 Flow Chart 2

**Pathogen testing of Biofresh Australia Garlic Material**

Biofresh Australia has 3 field grown crops, of mixed generations, requiring pathogen testing

Tissue Culture Facility Victoria

Sunraysia Horticultural Centre Mildura

Garlic Grower Waikerie, S.A.

Grower of G0

Grower of the G1 generation

Grower of G2, G3 (G4 and G5) generations

Pathogen testing undertaken in August and November

G0 generation tested

- ELISA used to test for Onion Yellow Dwarf virus, Yellow Stripe Virus, Shallot Latent virus and general potyvirus.
- Electron microscope used to scan for a range of virus particles
- Fungal, bacterial and nematode balting undertaken.

G1 - G5 generation tested

- ELISA used to test for Onion Yellow Dwarf virus, Yellow Stripe Virus, Shallot Latent virus and general potyvirus.
- Electron microscope used to scan for a range of virus particles
- Fungal, bacterial and nematode balting undertaken.

Results forwarded to each grower and Biofresh Australia upon completion of testing.

4.8 Relevance and Implications to Industry
Existing garlic growers adopting this high health garlic seed stock will benefit immensely from the achievement of higher yielding crops. Our research supports a conservative increase in yields by as much as 50-80% when compared to the current output using virus infected material. This increase is translated into increased profits as this is achieved with minimal addition to the fixed farming costs per hectare. This increase in yield bringing with it increased revenue returns will cause a large number of new entrants to come into the field as garlic growers. This could be of major significance in the Sunraysia district as this region offers the infrastructure for supply of both mother and foundation seed stocks.
### TABLE 10

**BIOFRESH AUSTRALIA PTY. LTD.**

**GROSS MARGIN WORKSHEET - ONE HECTARE - PROJECTED NEW GROWER**

<table>
<thead>
<tr>
<th>INCOME</th>
<th>HARVEST YIELD</th>
<th>16.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>HARVEST YIELD CURED</td>
<td>16.0 TONNES</td>
<td></td>
</tr>
<tr>
<td>SEED REQUIRED</td>
<td>1.2 TONNES</td>
<td></td>
</tr>
<tr>
<td>SELLING PRICE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PER KG</td>
<td>TOTAL</td>
<td></td>
</tr>
<tr>
<td>CONTRACT SALES</td>
<td>16.0 tonnes</td>
<td>$3.00</td>
</tr>
<tr>
<td>COST OF PRODUCTION</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAND PREPARATION</td>
<td>COST/HR</td>
<td>TOTAL COST</td>
</tr>
<tr>
<td>TRACTOR</td>
<td>RIP 2.0 HRS/HA</td>
<td>$13.75 per hour</td>
</tr>
<tr>
<td>DISC (x 2)</td>
<td>1.0 HRS/HA</td>
<td>$13.75 per hour</td>
</tr>
<tr>
<td>BROADCAST</td>
<td>0.3 HRS/HA</td>
<td>$13.75 per hour</td>
</tr>
<tr>
<td>CULTIVATION</td>
<td>1.5 HRS/HA</td>
<td>$13.75 per hour</td>
</tr>
<tr>
<td>FERTILISER</td>
<td>2 IN 1 1,500 KG/HA</td>
<td>$288.00 per tonne</td>
</tr>
<tr>
<td>PLANTING</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRACTOR</td>
<td>SOWING 4.0 HRS/HA</td>
<td>$13.75 per hour</td>
</tr>
<tr>
<td>MECHANICAL CLOVE SEPARATION</td>
<td>CONTRACT</td>
<td>$200.00 per tonne</td>
</tr>
<tr>
<td>GROWING</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRACTOR</td>
<td>BOOMSPRAY (x 6) 0.2 HRS/HA</td>
<td>$13.75 per hour</td>
</tr>
<tr>
<td>FERTILISER</td>
<td>UREA 350 KG/HA</td>
<td>$425.00 per tonne</td>
</tr>
<tr>
<td>WEED CONTROL</td>
<td>HERBICIDE (x 3) 2.0 LT/HA</td>
<td>$13.75 per litre</td>
</tr>
<tr>
<td>HERBICIDE</td>
<td>1.0 KG/HA</td>
<td>$47.00 per kg</td>
</tr>
<tr>
<td>HERBICIDE</td>
<td>1.0 LT/HA</td>
<td>$88.00 per litre</td>
</tr>
<tr>
<td>PEST CONTROL</td>
<td>INSECTICIDE 0.7 LT/HA</td>
<td>$20.00 per litre</td>
</tr>
<tr>
<td>IRRIGATION</td>
<td>7.5 ML/HA</td>
<td>$12.92 per ml</td>
</tr>
<tr>
<td>MISCELLANEOUS LABOUR</td>
<td>(For entire season) 180 HOURS</td>
<td>$16.00 per hour</td>
</tr>
<tr>
<td>HARVESTING</td>
<td>WET WEIGHT 20.0 TONNES</td>
<td>CONTRACT</td>
</tr>
<tr>
<td>CASUAL LABOUR</td>
<td>1.5 HRS/TONNE</td>
<td>$16.00 per hour</td>
</tr>
<tr>
<td>TRANSFER TO STORAGE</td>
<td></td>
<td>$32.00 per tonne</td>
</tr>
<tr>
<td>TOPPING &amp; TAILING</td>
<td></td>
<td>$0.18 per kg</td>
</tr>
<tr>
<td>DISTRIBUTION</td>
<td>DRY WEIGHT 16.0 TONNES</td>
<td></td>
</tr>
<tr>
<td>FREIGHT &amp; HANDLING</td>
<td>FACTORY</td>
<td>ALL BULBS 16.06 TONNES</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIRE OF BINS 32 CONTRACT</td>
</tr>
<tr>
<td>TOTAL COST OF PRODUCTION</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COST OF SEED STOCK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GROSS MARGIN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 11

**Comparison Between Grower Production of Virus-Tested Seed Stock and Traditional Seed Stock - Per Hectare**

#### Virus-Tested Seed Stock

<table>
<thead>
<tr>
<th>Year</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Seed Stock - Tonnes</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Seed Stock - $21 Per Kg</td>
<td>$25,200</td>
<td>$25,200</td>
<td>$25,200</td>
<td>$15,120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grower's Own Stock</td>
<td>1.2</td>
<td>1.2</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grower's Stock - Cost</td>
<td>NIL</td>
<td>NIL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cost of Growing - Per Hectare</td>
<td>$14,000</td>
<td>$14,000</td>
<td>$14,000</td>
<td>$14,000</td>
<td>$14,000</td>
<td>$14,000</td>
</tr>
<tr>
<td>Cost of Production</td>
<td>$39,200</td>
<td>$14,000</td>
<td>$39,200</td>
<td>$14,000</td>
<td>$39,200</td>
<td>$29,120</td>
</tr>
<tr>
<td>Wet Harvest - Tonnes - Yield 16%</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Drying Loss - Tonnes - 20%</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Dry Harvest - Tonnes</td>
<td>14.8</td>
<td>16.0</td>
<td>16.0</td>
<td>14.8</td>
<td>15.0</td>
<td>15.3</td>
</tr>
<tr>
<td>Harvest Revenue - $3 Per Kg</td>
<td>$44,000</td>
<td>$48,000</td>
<td>$44,000</td>
<td>$48,000</td>
<td>$44,000</td>
<td>$45,600</td>
</tr>
<tr>
<td>Gross Margin Per Hectare</td>
<td>$4,800</td>
<td>$34,000</td>
<td>$4,800</td>
<td>$34,000</td>
<td>$4,800</td>
<td>$16,480</td>
</tr>
<tr>
<td>Return Per Tonne</td>
<td>11%</td>
<td>71%</td>
<td>11%</td>
<td>71%</td>
<td>11%</td>
<td>35%</td>
</tr>
<tr>
<td>(Planted 1.2 Tonne Per Ha)</td>
<td>$4,000</td>
<td>$28,000</td>
<td>$4,000</td>
<td>$28,000</td>
<td>$4,000</td>
<td>$13,600</td>
</tr>
</tbody>
</table>

#### Traditional Seed Stock

<table>
<thead>
<tr>
<th>Year</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Seed Stock - Tonnes</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Seed Stock - $4 Per Kg</td>
<td>$5,000</td>
<td>$5,000</td>
<td>$5,000</td>
<td>$3,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grower's Own Stock</td>
<td>1.2</td>
<td>1.2</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grower's Stock - Cost</td>
<td>NIL</td>
<td>NIL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cost of Growing - Per Hectare</td>
<td>$9,000</td>
<td>$9,000</td>
<td>$9,000</td>
<td>$9,000</td>
<td>$9,000</td>
<td>$9,000</td>
</tr>
<tr>
<td>Cost of Production</td>
<td>$14,000</td>
<td>$9,000</td>
<td>$14,000</td>
<td>$9,000</td>
<td>$14,000</td>
<td>$12,000</td>
</tr>
<tr>
<td>Wet Harvest - Tonnes - Yield 8%</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Drying Loss - Tonnes - 20%</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Dry Harvest - Tonnes</td>
<td>6.8</td>
<td>8.0</td>
<td>8.0</td>
<td>6.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Harvest Revenue - $3 Per Kg</td>
<td>$20,000</td>
<td>$24,000</td>
<td>$20,000</td>
<td>$24,000</td>
<td>$22,000</td>
<td>$22,000</td>
</tr>
<tr>
<td>Gross Margin Per Hectare</td>
<td>$6,000</td>
<td>$15,000</td>
<td>$6,000</td>
<td>$15,000</td>
<td>$8,000</td>
<td>$10,000</td>
</tr>
<tr>
<td>Return Per Tonne</td>
<td>30%</td>
<td>63%</td>
<td>30%</td>
<td>63%</td>
<td>36%</td>
<td>44%</td>
</tr>
<tr>
<td>(Planted 1.2 Tonne Per Ha)</td>
<td>$5,000</td>
<td>$12,500</td>
<td>$5,000</td>
<td>$12,500</td>
<td>$6,700</td>
<td>$8,340</td>
</tr>
</tbody>
</table>

Assumed that Virus-Tested Seed will be replaced each second year due to virus infection & lower yield.
Similarly, Traditional Seed will be replaced each second year due to lower yield.
Drying losses have been estimated by Professional Growers.
Yield on Virus-Tested Seed has been achieved in two consecutive years.
Additional cost of production of Biofresh Seed is due to the larger harvest & higher packaging costs.
The flow-on benefits to the rural industry in terms of labour requirements, plant and machinery, transport and distribution and most importantly technical advice, intellectual property and the potential for a certified growing scheme, cannot be precisely quantified, but is fully supported by the Australian Garlic Industry Association.

Biofresh intends to extend the sale and distribution of virus-tested seed to not only domestic markets but also to overseas’ growers and value-added markets.

Biofresh also intends to provide consultancy and advisory services on a commercial basis to both government and to private growers in other countries.

Clinical trials have shown that garlic has important cardioprotective benefits, including the ability to decrease platelet aggregation, lower total plasma cholesterol and reduce blood pressure. The most encouraging results have occurred in the area of cholesterol reduction. The compound responsible for these benefits is allicin, one of a number of compounds responsible for garlic’s characteristic flavour and odour. Allicin is present in garlic bulbs in the form of the bioactive compound alliin which is converted to allicin when the bulb is cut or crushed.

Australian garlic bulbs have been shown to produce high levels of this compound. Due to the increased bulb weight and higher crop yields obtainable with virus-tested seed, the product would provide advantages for pharmaceutical processing procedures, as less bulbs are required to achieve the desired bioactive output.

Samples of our virus-tested plantlets were invited into a RIRDC funded project testing a range of Australian varieties of garlic for their alliin levels. The results in Table 14 indicate alliin in mg per gram of fresh bulb weight.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alliin (mg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toolangi - Biofresh 4</td>
<td>15.4</td>
</tr>
<tr>
<td>Toolangi - Biofresh 2</td>
<td>15.1</td>
</tr>
<tr>
<td>Waikerie - Biofresh 2</td>
<td>14.4</td>
</tr>
<tr>
<td>Waikerie - Biofresh 3</td>
<td>12.9</td>
</tr>
<tr>
<td>Waikerie - Biofresh 4</td>
<td>12.2</td>
</tr>
<tr>
<td>Toolangi - Biofresh 1</td>
<td>12.0</td>
</tr>
<tr>
<td>Toolangi - Biofresh 3</td>
<td>11.3</td>
</tr>
<tr>
<td>Waikerie - Biofresh 1</td>
<td>9.9</td>
</tr>
</tbody>
</table>

To put these values into context, we have recorded levels between 1 and 20 mg/g in the two seasons that the project has been conducted. Prior to this, the highest recorded alliin levels in literature (world-wide) was in the order of 15-16 mg/g (FW). A level of 10 mg/g has been set as the minimum alliin level for economic extraction of the compound by the pharmaceutical consultant. Hence the values recorded here are quite high and certainly worth investigating further.

There is currently a multi-million dollar international business in the production and sale of hypolipidaemic (cholesterol lowering) drugs and other treatments. Treatment generally consists of dietary and lifestyle intervention (for up to six months) prior to courses of drugs being prescribed. There is a significant opportunity for garlic supplements to become part of this first line treatment program. Garlic has long been cited as having a range of health benefits and scientists have been investigating the biochemical pathway linked to the production of the specific cholesterol lowering compound, allicin. Garlic has the added benefit of having few known side effects and hence it should be possible to initiate prophylactic treatment earlier than other first line hypolipidaemic therapies.
There is strong competition from the large volumes of imported Chinese fresh garlic on the domestic market. To date, there is only a small pharmaceutical market for garlic in Australia, with almost all the pharmaceutical quality garlic powder being imported. Recently, several international companies have shown interest in sourcing Australian grown garlic and its higher alliin levels and our increased yields of virus-tested garlic provide an opportunity to enter this arena.

The further development of this program will require additional research to include the development of post harvest and storage protocols along with optimum drying and processing of the garlic to minimise alliin loss, and to produce a standardised alliin powder could create a new value-added Australian industry.

Due to this import competition of fresh produce and the opportunity for high quality garlic powder, Biofresh is keen to pursue the value-added market.

4.9 Intellectual Property

Biofresh has developed and owns the industrial property rights in an ELISA (Enzyme Linked Immunosorbent Assay) test for the detection of three viruses commonly affecting Australian garlic crops.

Biofresh has produced protocols for the production of pathogen tested garlic. This document includes the protocols for initiation of ‘nucleus stock’, rapid scale tissue culture multiplication, screenhouse planting of ‘mother stock’, pathogen testing and growing guidelines for ‘foundation stock’ and commercial scale farming of virus-tested garlic.

The development of these protocols provides the foundation for the establishment of a Quality Assurance Program and implementation of a Certified Growers Scheme for the production of high health, high quality, high alliin virus-tested garlic, garlic seed and garlic by-products. These would be imperative for Biofresh to advance into the value-added arena of the pharmaceutical and complimentary medicines.
5. Protocols for the Production of Pathogen Tested Garlic

5.1 Biofresh Australia Pty. Ltd.

Biofresh Australia is a privately owned Australian company which has been at the forefront of research and development technology in the garlic industry since 1989. The company has worked closely with Agriculture Victoria and stakeholders and the Rural Industries Research and Development Corporation have funded most of the work.

5.2 Biofresh’s Aim

To produce high health pathogen tested garlic seed material for sale to commercial growers and material for use in the pharmaceutical industry.

5.3 Supply and Maintenance of Tissue Culture Plantlets

Crop Health Services at Agriculture Victoria, Knoxfield

5.3.1 Supply of Tissue Culture Plantlets

- By March each year, provide 10 tubes of actively growing plantlets with a minimum of 4 shoots per tube, in agar, for further multiplication at Cleangrow.

- The cultivars to be multiplied are to be nominated by Biofresh by December of previous year.

- Plantlets to be derived from fully pathogen tested material.

**Current Varieties**

- Biofresh 1 - Biofresh derivative of Mexican variety
- Biofresh 2 - Biofresh derivative of Schumex variety
- Biofresh 3 - Biofresh derivative of California variety

5.3.2 Maintenance of Cultivars in Tissue Cultures

- Cultivars to be maintained in tissue culture to be nominated by Biofresh each January.

- Nucleus stock of the Biofresh cultivars will be maintained *in-vitro* under the following conditions:
  - On a sold, sterile Murashige and Skoog based medium in sterile sealed (Refer Appendix 6) autoclavable containers.
  - The tissue cultured plantlets are to be held under hygienic conditions in a suitable growth room or cabinet, which is regularly cleaned and disinfected.
- All subdivisions and transfer of plantlets to be done in a Laminarflow cabinet using aseptic (Refer Appendix 7: Aseptic Technique and Appendix 8: Meristem Initiation Technique).

- Each tissue culture vial to be clearly labelled with the variety, code and date of last sub-culturing.

- A laboratory manual must be kept listing date of each sub-culturing, numbers of each variety sub-cultured and any material discarded due to contamination.

- The number of cultures maintained must be sufficient to ensure that a cultivar is not lost as a result of natural attrition. Cultures of each cultivar will be maintained at Agriculture Victoria, Knoxfield.

- Agriculture Victoria, Knoxfield will notifyBiofresh forthwith of the loss of any cultivar.

5.3.3 Tissue Culture Laboratory

The cultures must be held in a dedicated controlled environment room which maintains a temperature of 17-22°C with a light regime of 16 hours light and 8 hours of darkness. Agriculture Victoria, Knoxfield will ensure that :-

- Except when access is required, these rooms shall be kept locked at all times.

- The rooms shall be checked each working day to ensure that the correct temperature and light regimes are maintained.

- Floors, benches and any areas where dust can accumulate must be wiped down with a disinfectant weekly. Growth rooms and the tissue culture laboratory must be kept clean and tidy at all times.

- Growth rooms or cabinets to be disinfected every 6 months.

- Only authorised, trained staff to be allowed access to the rooms and to sub-culturing of Biofresh’s material.

- A laboratory manual to be kept to check off each area when it is cleaned.

At least once a year the cultures must be fully pathogen tested and transferred onto fresh medium. All culture containers must be identified with two self-adhesive labels detailing the cultivar, line and date.

5.3.4 Initiation of New Cultivars Into Biofresh Scheme

Pathogenic virus elimination in new cultivars as selected by Biofresh will follow the same procedure as varieties currently in the scheme.

5.4 Pathogen Testing

Pathogen testing to be conducted as follows on generations nominated by Biofresh grown at designated Tissue Culture Facility (G0), in screenhouse (G1) and field plantings (G2 to G5) :-
G0  Once - August
G1  Twice - August and October
G2, G3, G4, G5  Twice - August and October

For each of these generations, sufficient plant material will be tested to give 95% confidence that less than 5% of plants could be infected (Refer Appendix 1 - Sampling Numbers).

A non-aligned systematic sampling method will be used to collect leaf and bulb material and pooled 1 : 5 (sample/leaves) and tested as 1 sample.

Plants to be tested for viruses and other pathogens as described below.

5.4.1 Virus Testing

G0  Electron microscopy, and ELISA testing using four commercial ELISA (Enzyme Linked Immunosorbent Assay) kits for the following viruses:
- Onion Yellow Dwarf Virus
- Leek Yellow Stripe Virus
- Shallot Latent Virus
- Potyvirus General

G1  Electron microscopy and ELISA as for G0

G2  ELISA as for G0

G3  ELISA as for G0

G4  ELISA as for G0

G5  Electron microscopy and ELISA as for G0

Protocols for ELISA testing technique can vary, depending upon the manufacturer and the specific virus. However, the method provided in Appendix 3 provides a standard method as outlined in Crop Health Services ISO 9002 manual.

Where ELISA based tests are not available when particles are detected, sap inoculations will be carried out onto appropriate herbaceous indicators.

Electron Microscopy. Using a Model H - 600S Hitachi Electron Microscope EM grids will be viewed at 50,000-60,000 X magnifications.

Carbon coated copper grids will be dipped in a plant extract/PTA buffer slurry in order to catch virus particulars.

The following virus particles will be scanned for:

*Onion yellow dwarf potyvirus* (*OYDV*-G)
Virions filamentous; not enveloped; usually flexuous; with no clear modal length; of 772 and 823 nm. Axial canal obscure. Basic helix obscure.

*Shallot yellow stripe potyvirus* (*SYSV*)
Virions filamentous; usually flexuous; of 750 nm.
Leek yellow stripe potyvirus (LYSV-G)
Virions filamentous; not enveloped; usually flexuous; with a clear modal length; of 820 nm. Axial canal obscure. Basic helix obscure.

Onion mite-borne latent virus (OmbLV-G)
Virions filamentous; not enveloped; usually flexuous; with a clear modal length; of 775 nm. Axial canal obscure. Basic helix obscure.

Shallot latent carlavirus (SLV-G)
Virions filamentous; not enveloped; usually straight (or slightly curved); with a clear modal length; of 650 nm. Axial canal obscure. Basic helix obscure.

Tobacco rattle tobravirus (TRV)
Virions rod-shaped; not enveloped; usually straight; with a clear modal length; of 46-114 nm (T), or 180-197 nm(B); 22 nm wide. Axial canal obvious; 4 nm in diameter. Basic helix obvious; pitch of basic helix 2.5 nm.

Arabis mosaic nepovirus (ArMV)
Virions isometric; not enveloped; 25-27 nm in diameter (in negative stain); angular in profile; without a conspicuous capsomere arrangement.

Tomato black ring necrovirus (TNV)
Virions isometric; not enveloped; 26 nm in diameter; angular in profile; without a conspicuous capsomere arrangement.

Lettuce necrotic yellow cytorhabdovirus (formerly rhabdovirus) (LNYV)
Virions rhabdo- or bullet-shaped; enveloped; with a clear modal length; of 227 nm (in negative stain); or 360 nm (in tissue sections); 52 nm wide (in tissue sections), or 66 nm wide (in negative stain). Axial canal obvious. Basic helix obscure; pitch of basic helix 4.5 nm.

Tobacco mosaic tobamovirus (TMV)
Virions rod-shaped; not enveloped; usually straight; with a clear modal length; of 300 nm; 17 nm wide. Axial canal obvious; 2 nm in diameter. Basic helix obvious; pitch of basic helix 2.3 nm.

5.4.2 Fungal, Bacterial, Nematode & Arthropod Testing
Bulb material will be removed from the samples collected for virus testing and submitted to the Diagnostics laboratory, Crop Health Services, for testing.
5.4.3 Fungal Testing

Culturing onto Potato Dextrose Agar and Water Agar media will test for the following fungal diseases. The method provided in Appendix 4, provides a standard method as outlined in Crop Health Services ISO 9002 manual.

- White root rot - *Scelerotium cepivorum*
- Pink rot - *Pyrenochaeta terrestris*
- Neck rot - *Botrytis allii*
- Penicillium decay - *Penicillium spp*
- Downey mildew - *Peronospora destructor*
- Black moulds - *Fusarium spp*
- Alternaria blotch
- Stemphylium blight

5.4.4 Bacterial Testing

Culturing onto Kings “B” Agar and Nutrient Agar media will test for the following bacterial diseases. The procedure for this is provided in Appendix 5, which is the current method used by Crop Health Services under ISO 9002 guidelines.

- Bulb bacterial rots
- Xanthomonas blight

5.4.5 Nematode Testing

Bulb material will be removed from the samples collected for virus testing and submitted to the Diagnostics laboratory, Crop Health Services, for direct microscope analysis for the presence of Stem and Bulb nematode (*Ditylenchus dipsaci)*.

5.4.6 Reporting

Results will be forwarded within 14-21 days of sampling.

Records and reports will be kept by Crop Health Services on database and laboratory workbook.

5.5 Bulk-Up of In–Vitro Virus-Tested Plantlets

5.5.1 Supply of tissue culture plantlets

By 1 July in each year: Agriculture Victoria, Knoxfield will provide 10 tubes of actively growing plantlets of each variety selected with a minimum of 4 shoots per tube, in agar, for further multiplication at a nominated tissue culture facility. The plantlets are to be derived from fully pathogen tested material.

5.5.2 Tissue Culture Laboratory Requirements

- Keep tissue culture laboratory clean and tidy at all times.
- Dust must not be allowed to accumulate.
- Wash floors and wipe benches regularly with an appropriate disinfectant (e.g. Biogram or Rephen).
- Staff should change outdoor footwear to laboratory slippers or overshoes.
- Staff should wear clean, regularly laundered laboratory coats, which are used exclusively in the laboratory area.
- Staff should wash hands on entering the laboratory to avoid contamination of plantlets.
- Contaminated cultures should be removed and sterilised immediately they are detected and then restrict access to only authorised staff and supervise visitors.
- Store sterile, unused culture tubes containing growth medium in a cool room or refrigerator.
- Smoking should not be permitted to avoid virus contamination.

5.5.3 Multiplication Procedure

*Type of material suitable for multiplication* - Cultures are “quarantined” for 4 weeks, i.e. allowed to incubate (untouched/unopened) in the control room, to allow for the detection of any contaminants being brought in with the samples.

*Multiplication rates expected per line* - Expected rates will vary according to the variety being multiplied. An acceptable range is 2-4 with a sub-culturing frequency of every 14 days.

*Actual procedure for multiplying tissue culture plants* - As per Appendix 7.

*Growth room environmental parameters* - Controlled regime of light and temperature, currently using 16/6 light/dark and 30°C.

5.5.4 Media

*Media ingredients* - As per Appendix 6.

*Media preparation technique* - Follow same procedure in Appendix 6.

*Media storage* - After sterilising is complete, place in cool room and keep no longer than one month.

5.5.5 Care of Tissue Cultured Plantlets

- Maintain in sterile sealed containers on a sterile growth medium.
- Hold under hygienic conditions in a suitable growth room or cabinet which is regularly cleaned and disinfected.
- All subdivision and transfer of *in-vitro* plantlets must be done in a laminar flow contamination control cabinet using aseptic techniques.
- Clearly label all containers by variety, code and date.

5.6 Procedure for the Transferral of Garlic Cultures into Plug Trays

5.6.1 Deflasking

Cultures which have been subdivided into garlic rooting medium (GRM) (i.e. without auxins to cause multiplication) for the last two cycles and are quite singulated are suitable for deflasking.
5.6.2 Method of Transferring

Using forceps, remove cultures with visible roots and implant into all trays with potting mix to just cover any bulb or root system. Perform task in clean conditions and at 18°C and 80% humidity. Place cell trays in aphid proof propagation house at 21-22°C and 85% humidity with overhead mist system.

- Tissue cultured plantlets, ready for transplanting, should only be removed from their tubes using sterile forceps.
- Label all plant containers clearly with cultivar and planting date, etc.
- Pots, boxes, trays or troughs must be raised as high as practicable above the floor level (preferably one metre high benches).
- Staff should minimise contact with plants whilst watering during growing period.
- Care must be taken to provide optimum watering regimes - over-watering must be avoided, particularly in the cooler months.
- All plants should be carefully examined at least once a week and any unthrifty, off-type or diseased plants removed as soon as they are detected to avoid contamination of other plants.
- Appropriate disease and insect control programs must be implemented and CHS notified of any problems.
- A sub-sample of each variety or line should be submitted to CHS at IHD Knoxfield for detailed pathogen testing twice during the season (early and mid season).
- Water supply should be filtered, chlorinated or UV-treated to minimise the risk for water-borne pathogens.

5.6.3 Growing Media

- Use an open well drained potting mix such as pine bark/sand or perlite/peat.
- Media should be steam sterilised at a temperature of 60°C for one hour or until the core temperature reaches this temperature for one hour. Live steam may be used, but care should be taken to avoid nutritional disorders.
- An appropriate fertiliser program must be used to ensure adequate plant growth.
- Pots, boxes, trays or troughs must be new or disinfected just prior to use.
- Use only new containers in the propagation of garlic transplants.

5.7 Hardening-Off of In–Vitro Transplants in Screenhouse

- Plantlets that have been acclimatised for 2 weeks in a propagation house at 21-22°C and 85% humidity are transferred to an aphid proof screenhouse.
- Plantlets remain in cell trays.

5.7.1 Screenhouse Requirements

- All structures must be aphid proof.
- Structures must be situated at least 500 metres away from any non pathogen tested Allium crop (garlic, onion, leek, shallot, etc.)
- Access must be through a headhouse or an anteroom with both outer and inner doors which seal adequately to exclude insects.
- A foot bath with regularly changed disinfectant (weekly minimum), must be located in the headhouse or anteroom at the entrance to the screenhouse.
- Staff are forbidden to enter houses after field work, unless their clothing is changed and footwear is disinfected.
- Hands should be either washed or surgical gloves worn before handling plants or bulbs.
- Smoking is forbidden.
- Polyhouse and glasshouse floors must be concreted, regularly cleaned and have adequate drainage.
- Plant debris or potting mix must not be allowed to accumulate on the floors or benches.
- Prior to planting all washable surfaces must be thoroughly hosed with water and then drenched with a hospital disinfectant or a solution of sodium hypochloride.
- Soil in screenhouses should be fumigated prior to each season’s planting and should be kept weed free throughout the season.
- Garlic material in plugtrays must be maintained on raised benches or tables to minimise any risk of contamination from the floor.
- The facility shall have a concrete floor and be entered through a headhouse or through a double door entrance porch or “airlock”. A footbath shall be located at the entrance within the “airlock”. All vents into the facility shall be covered with aphid proof mesh.
- The facility’s floor and benches shall be washed down and surface sterilised monthly.
- The facility must be checked monthly for the appearance of any holes in the aphid proof mesh covering or structure.
- A weekly diary must be kept recording details from each inspection, and any actions taken.

5.7.2 Harvesting, Storage & Transport

- After pulling garlic bulbs, ensure that they left to dry off in aphid proof conditions for at least 7 days to allow skins to mature before storage.
- Examine bulbs closely for damage and disease after drying off, and immediately remove any that are defective in any way.
- Store bulbs at 4°C in a dedicated cool room, or in a well ventilated storage area where there is no risk of aphid infection.
- Pack bulbs for storage in new or disinfected open mesh onion bags.
- To transport bulbs, place onion bags in new cardboard boxes or jute bags to prevent contamination.
- Ensure bulbs are clearly marked with variety, line (if applicable) and number.
- Keep adequate records for all laboratory and glass/poly/screenhouse operations.

5.7.3 Plant Maintenance

- Plans will be monitored weekly for the presence of any pests and diseases, with necessary chemical control measures taken immediately.
- Active monitoring for insect pests will involve the placement of at least 1 yellow sticky insect trap in each facility used.
- Plants will be maintained so that they do not exhibit any prolonged symptoms of either nutrient or water stress.
- Diseased foliage will be removed during each inspection.
- A weekly diary will be kept of observations made during each inspection, and actions taken as a result of pest/disease outbreak.
5.8 Bulk-up Of G1 in Screenhouse (Shc Mildura)

5.8.1 Size Selection
- Test soil for nutrient content. Soil pH should be between 6 and 7.
- Test soil for presence of pathogenic nematodes. Thorough sampling will ensure more meaningful results.
- No possibility of any volunteer *Allium* spp. plants.
- Must be situated at least 500 metres away from any non pathogen tested Allium crop.

5.8.2 Ground Preparation Prior to Establishment of Screenhouse
- Till ground to fine tilth.

5.8.3 Option to Apply Straight Superphosphate Directly into the Soil
- Apply at the rate of 200kg/hectare (calculate on a m$^2$ basis) and incorporate into soil.

5.8.4 Bed Formation
- Form beds to suit cage size and set up.

5.8.5 Cage Set-Up
- Cage must be constructed with aphid proof material with fully enclosed “double door anteroom” entry.
- Doors must be adequately sealed to exclude insects. Hoop structure (25m x 7m will house approximately 10,000 plantlets).
- Disinfectant foot bath to be placed in the anteroom and disinfectant changed regularly (at least weekly). The foot bath is to prevent the introduction of soil-borne pathogens on footwear into the cage.
- Either overhead watering system or one able to give complete coverage of plants at full growth height (min. 500mm above bed height).
- Hang insect traps. Best placed at ends of cage at highest points. Traps to be evaluated once a month.
- Spray to kill aphids and thrips before bringing in plant material.
- Install tensiometers at 15cm depth.

5.8.6 Pre-Plant Fungicide & Herbicide Treatments
- Apply fungicide to beds at the rate as per label to prevent White Rot (*Sclerotium cepivorum*) and Fusarium Rot.
- Next, spray with pre-emergence herbicide to beds at the rate as per label and water in.
- Submerge plantlet trays in a bath of fungicide using the same rate as above. Dip trays for 2 minutes, then drain.
- Change dip periodically if large number of trays need to be dipped.

5.8.7 Planting Regime
- Plant to the depth of plug (cell tray). 100mm between row spacings and 50mm between plant spacings.
- After planting, water in for about 2 hours.
5.8.8 Planting Time
- Plant one month after normal field planting time for each variety (i.e. B1 - 1 March in field → 1 April in cage).

5.8.9 Irrigation Frequency
- Soil to be kept moist throughout growing season. Can use probe moisture meter to help keep moisture content around 30%.

5.8.10 Fungicide
- Application of contact fungicide every 2 weeks for the growing life of the crop, at rate as per label, as a preventative for mildews and Stemphyllium sp.

5.8.11 Fertiliser
- Carry out leaf analysis soon after plant is well established.
- Spray each 4 weeks until 7-8 weeks before expected harvest date (same date as if planted in field) with Zinc, Manganese, Magnesium Sulphate, Calcium Nitrate, according to leaf analysis.
- Each element to be applied separately.

5.8.12 Harvesting
- As soon as tops start to “lay over”, start harvesting.
- The plantlets may require continual harvesting every few days or they will become a problem with their visual detection as tops disintegrate and watering continues.

5.8.13 Drying
- As plants dry, place harvested bulbs in onion type bags and hang in the screenhouse cage. As the process is completed, move bags to a weather protected enclosure at ambient temperature.

5.8.14 Crop Monitoring
- Monitor sticky traps at least twice a week.
- Monitor pressure of sprinklers and water distribution at least once a week.
- Monitor tensiometer readings every 2 days. Reading should be kept below 20.
- Monitor for diseases at least twice a week.
- Monitor weeds at all times and remove by hand if necessary.

5.9 Field Planting for Production of Virus-Tested Garlic

5.9.1 General Information
Garlic (Allium sativum) is a member of the onion and leek family (Alliaceae). It has many reputed medical benefits and is widely used in may communities as a fresh or processed herb. The mature garlic bulb consists of modified storage leaves. It contains 6 to 30 cloves, which are held together by outer skins. Garlic grows in temperate and sub-tropical climates.
5. 9.2 Climate

The best quality garlic grows in temperate climates. The temperate climate varieties require cool winters for clove initiation, followed by increasing hours of daylight in spring to give the best bulbing. Climatic variations in different districts can affect the degree of bulbing.

5. 9.3 Soils

Garlic grows best on light sandy soils, which are conducive to easy harvesting. The light sandy soils play an important part in producing a cured garlic product free of stains. Stains can come from the soil or from fungal and bacterial infections.

Poor results may be obtained on waterlogged, heavy or hard soils which restrict root growth.

Suitable soils have a pH of 6 to 7, although garlic grows well above this range. Careful nutrition management may be necessary to produce good quality garlic.

There should be a rotation of at least 2 years between crops in the garlic and onion family to prevent build-up of soil-borne diseases.

5. 9.4 Storage of Seed Prior to Planting

- Seed stock to be stored in well ventilated bins in a well ventilated shed at ambient temperature.

5. 9.5 Site Selection

- Site should be selected 12 months prior to planting.
- Site should be at least 500 metres away from any existing Allium crop.
- Soil pH should be between 6 to 7 - correct through the application of fertiliser if outside this range. Unsuitable if below 5.5.
- Encourage weed growth to reduce seed population by ploughing to bring seed to surface 3-4 times over the 12 months (i.e. fallow for 12 months).

5. 9.6 Watering System

Garlic needs to be kept constantly moist until maturity. During germination and hot weather, adequate moisture is particularly important. During these times, ensure that the moisture level is maintained very closely. Moisture levels near maturity must be lowered and the soil allowed to dry as the garlic plant starts to senesce.

- Ensure the watering system is of a good design and highly efficient to provide total and uniform coverage.
- Watering system can be linked to a moisture probe to trigger off watering as required.
- Do not water from late afternoon til 1:00 a.m.. This is to try and avoid moisture build-up overnight which will prevent spore build-up and germination.
- On days of 24°C mean temperature followed by late afternoon to midnight rain, override moisture sensors and water at 5:00 a.m. for 20 minutes on each zone. This is to minimise conditions which will allow fungal pathogen spores to settle and germinate.
- On days designated for early morning (daylight) fertigation, manually override the automatic watering system the night before to stop system. Re-set system in the evening after fertigation.

5. 9.7 Ground Preparation
- Till ground to fine tilth.
- Apply straight superphosphate and sulphate of potash directly into the soil at the appropriate rates per hectare, simultaneously.
- Incorporate fertiliser deep into the soil.
- If no January rain, irrigate to stimulate weeds.

5. 9.8 Bed Preparation

- At the end of February, commence watering and 3-4 cultivations to prepare a fine seed bed.

5. 9.9 Aphid Monitoring

- Visually monitor for aphids around perimeter of crop.
- Special attention should be given to areas facing the prevailing winds.
- Monitor weeds and native vegetation around crop.
- Sow wind-break crops (e.g. Triticale) which can also act as an insect trap, especially on the prevailing wind side of the garlic crop.

5. 9.10 Pre-Plant Preparation of Cloves

- In the morning, set up the cracker and grader and line up bins of seed stock to increase their temperature.
- Process only one day’s planting material. This is best achieved by running cloves through the machine once, late in the day, repeating process in the evening and again the next morning ready for planting.
- Keep cloves graded into 4-5 classes, as planter can be pre-set for each size.

5. 9.11 Fungicide Treatment of Cloves

- Apply a registered fungicide for the control of White rot in garlic. Contact Agriculture Victoria’s Chemical Standards Branch on (03) 9210 9379 or your local chemical retailer for details.

5. 9.12 Planting

Garlic does not produce viable seed and must be planted from cloves.

- Using mechanical planter - flat bed plant - 1300mm wide tractor beds, with four running rows, 300mm between each row and 100mm between plant, aiming for 40 cloves planted per running metre.
- Plant depth should be approximately clove size, no deeper than 50mm. Cloves need only a very thin covering of soil to germinate. Once the garlic starts to bulb, the plant must be deep enough in the soil for the bulb to be always covered with soil.
- Keep very moist for 2 weeks after planting, manually programming watering systems, making sure top 50mm is wet.
- Spray weeds with a registered herbicide, at the recommended rate, at the very first sign of garlic emergence, about 6-7 days after planting.
- When plants are approximately 50mm high, inter-row cultivate to hill soil up around plants.

5. 9.13 Weed Control

Garlic has a long growing period, giving weeds plenty of time to establish. Young garlic plants do not compete well with winter weeds and yields can be seriously affected if action is not taken.

- Spray out broad leaf and narrow weeds, using a registered herbicide at the recommended rate, after “hill up”, and repeat as necessary (approx. 2-3 times).
- Any persistent narrow leaf weeds should be sprayed out with an appropriate registered herbicide at the recommended rate. Herbicide rate can be increased to a maximum rate as plant growth increases for most persistent weed kill.
- Must use a non-ionic (vegetable) wetting agent with either of the narrow-leaf herbicide sprays.
- Use a broad spectrum pre-emergence herbicide after initial broad and narrow leaf treatments, as a preventive measure against further weed emergence.

5. 9.14 Fertilising - At About Fifth Leaf Stage

Garlic does not require large amounts of nitrogen in the early stages of growth. Plants benefit from applications of single superphosphate and potassium in the early growth stage and nitrogen applications later in the growth cycle. The actual amounts of fertiliser required will depend on a rate of factors and leaf analyses are the most effective method of determining nutrient requirements.

This is the fertiliser program currently used by Biofresh’s commercial crop grower, Roger Schmitke in Waikerie, South Australia. This program may not necessarily be appropriate for a
wide range of growers, but is put forward as an example of a fertiliser regime currently used for growing garlic.

**Suggested Nitrogen Program**

- Weekly application: 15kg/hectare from mid-June to mid-July by way of ammonium nitrate (30%N) through the sprinklers.
- Continue the treatment, using the following increments:
  - 20kg/hectare - mid-July to end July
  - 30kg/hectare - August
  - 40kg/hectare - September
- Take leaf analysis at the fifth leaf stage before any application of minor nutrients.
- Other than the above nitrogen program, make adjustments to any other nutrients by a single application. If there is a large derivation from acceptable range, then repeat leaf analysis after adjustment.
- From first week in May, commence a monthly application of Zinc, Manganese and Magnesium through to the first week in September, adding Calcium Nitrate from August (i.e. at this increased growth state). Application through boom spray with the addition of low biurette urea 1kg per hectare per spray, and a wetting agent.
- Apply Zn Mg (combination Zinc & Magnesium) powder at rate 2kg/hectare and Mn (Manganese) 4kg/hectare together with wetting agent (Agral) and low biurette. Apply from tractor pulled boom spray.
- Apply Calcium Nitrate 10kg/hectare and wetting agent. Spray in separate application due to its “clogging” characteristics.

**5. 9.15 Diseases**

- The fungal diseases Downy Mildew caused by *Peronospora destructor*, and Purple Blotch caused by *Alternaria porri* are just two that can cause significant losses in garlic crops. It is important to carefully monitor the crop and apply preventative sprays, before the occurrence of conditions that are conducive to a high risk of disease outbreak.

**5. 9.16 Insect - Pest Program**

*Aphids*

Continual monitoring of aphids is essential. Aphids are carriers of a number of virus diseases, which in combination can reduce plant vigour and therefore yields. These virus diseases are carried over in the garlic cloves.

- Monitor around the perimeters - if any aphids are detected, spray the entire crop through the boom spray with a registered insecticide at a rate as per the label (be careful of beneficials).
- Must spray extended borders of crop.
- Weed control around perimeters is critical. If possible, keep a 2 metre barren border.
- Selection of a cereal (i.e. Triticale) crop along perimeter to act as both wind break and possible “insect catch” crop.

*Thrips*
Thrips are a major pest of garlic. Mild infestations can severely reduce plant growth and vigour. Evidence of thrips damage can be hard to find. It may only be evident as lack of plant health and vigour. Severe infestations can produce small white patches on the leaves of the plants.

- Use an integrated pest management control program. If on visual monitoring the thrips population warrants treatment (i.e. increasing juvenile numbers and suitable conditions for their multiplication), then spray with a registered insecticide at the recommended rate.

Other Pests

- Cutworms. Use registered products as per labels.
- Red Legged Earth Mites.
- Two Spotted Mites.
- Snails.
- Nematodes. Pre-plant soil test is highly recommended.
- Birds.

5. 9.17 Harvesting

Soil moisture at the time of harvesting can have an impact on the degree of damage to the garlic. For best results, the soil should be friable with medium moisture content. If the soil is too dry, clods are formed on heavy soils. When the soil is too wet, it adheres to the bulbs and drying becomes prolonged and difficult and increased levels of microbial spoilage can occur. Garlic can go mouldy with the wet soil in as little as 24 hours.

Mature garlic crops near to harvest are always at risk during very hot weather, as there is reduced leaf cover for protection from sunburn. The situation is more critical if the bulbs have been lifted and are lying on the surface ready for pick-up. If too hot, the bulbs can sunburn and/or cook before they are gathered, resulting in a slimy rot. Garlic bulbs, either lifted or not, closely follow the surrounding air temperature and during the hottest part of the day, core temperatures can exceed the air temperatures by as much as 5 degrees. Harvesting garlic with high bulb temperatures should be avoided as it will adversely affect the quality through a greater likelihood of bruising.

- When the top of leaves start to dry back and stalk becomes very soft at ground level.
- A prime indicator if plant is ready for harvest is that if on cutting horizontally through the bulb, it separates as would a crisp apple opening.
- A mature plant will have a minimum of 3-5 skins surrounding the bulb.
- Lift plants using carrot harvester, reducing excess foliage and stacking straight into well ventilated bins.
- Stack bins, containing approx. 4 horizontal layers of plants, in a dry, shady, very well ventilated condition (i.e. covered, but open sided shed).
- Leave plants to dry until you can with ease physically twist the stalk and separate from the bulb at a distance of about 25cm from the bulb.
- Once stalks are removed, move the plants into a controlled environment (i.e. shed that is air-conditioned to 25°C and 72% humidity where they can stay for up to 6 months.

5. 9.18 Processing of Bulbs

- Mechanically top and tail and brush bulbs, then put into 0.5 tonne wooden bins. Topping and tailing too soon can promote storage rots.
- For seed purposes - keep in a well ventilated shed at ambient temperature.
WARNING! Do not hold seed garlic bulbs in carton for more than 1 week after delivery. The carton of seed garlic should be opened upon receipt and left in well ventilated shaded conditions in an area that cannot be invaded by insects or moths.

- It is important that seed stock be acclimatised to local conditions of planting. Growers should purchase their seed stock, allowing for a period of at least 8 weeks for seed acclimatisation to their planting conditions. This is essential to maximise and optimise yield results.
- Growers should be aware that during this period, there may be some loss in weight due to a loss in moisture depending on their storage conditions and this should be taken into consideration when calculating seed quantities required for area to be planted.
6. Appendices

Appendix 1: Pathogen Testing - Sampling Numbers

Sample size required to achieve a given probability of detection, for a range of population sizes and incidences. The incidence also equals the upper confidence limit (at confidence equal to the probability of detection) when no samples are diseased.

<table>
<thead>
<tr>
<th>Population Size</th>
<th>.0001</th>
<th>.0005</th>
<th>.001</th>
<th>.005</th>
<th>.01</th>
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<td>564</td>
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## Appendix 2: Garlic Viruses (Allium Sativum)

<table>
<thead>
<tr>
<th>VIRUS/GROUP COMMON VIRUSES OF GARLIC</th>
<th>PARTICLE SIZE &amp; SHAPE</th>
<th>HERBACEOUS INDICATORS (SAP TRANSMISSION)</th>
<th>INSECT VECTORS</th>
<th>SEROLOGICAL INDEXING</th>
<th>REFERENCE</th>
<th>CMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Onion yellow dwarf potyvirus - garlic strain (OYDV-G)</td>
<td>775 nm flexuous filament</td>
<td>none suitable</td>
<td>aphid spp.</td>
<td>Specific as for ELISA\decoration. (Agdia potyvirus mab = no reaction in ELISA)</td>
<td>Van Dijk (1993A)</td>
<td>158</td>
</tr>
<tr>
<td>2. Shallot yellow stripe potyvirus (SYSV): SYSV&lt;sub&gt;com&lt;/sub&gt; = common strain SYSV&lt;sub&gt;vir&lt;/sub&gt; = virulent strain</td>
<td>775 nm flexuous filament</td>
<td>none suitable. (infects OYDV-resistant onion cultivars)</td>
<td>aphid spp.</td>
<td>Weak or no reaction with OYDV antiserum</td>
<td>Van Dijk (1993A)</td>
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<tr>
<td>3. Leek yellow stripe potyvirus - garlic strain (LYSV-G)</td>
<td>800-820 nm flexuous filament</td>
<td>C.quinoa-CLL, 11 days C.amarant-CLL, 3 weeks</td>
<td>aphid spp.</td>
<td>Specific AS for ELISA\decoration. (Agdia potyvirus mab = +/- in ELISA)</td>
<td>Van Dijk (1993A)</td>
<td>240</td>
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<td>4. Onion mite-borne latent virus - garlic strain (OMbLV-G) [potyvirus or closterovirus?]</td>
<td>715-775 nm highly flexuous filament</td>
<td>C.murale-CLL or NLL</td>
<td>erophyid mites: (Aceria tulipae)</td>
<td>No specific antiseras.</td>
<td>Van Dijk et al. (1991)</td>
<td></td>
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<tr>
<td>5. Garlic (common) latent carlavirus (GLV or GCLV)</td>
<td>700 nm straight or slightly flexuous filaments</td>
<td>C.quinoa-CLL\NLL\GR C.amarant-CLL\NLL\GR N.occidentalis(P1) -CLL\SR</td>
<td>aphid spp.</td>
<td>Decoration by GCLV (Germany)</td>
<td>Van Dijk (1993B)</td>
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<tr>
<td>6. Shallot latent carlavirus - garlic strain (SLV-G) = GLV-Japan</td>
<td>650 nm straight or slightly flexuous filaments</td>
<td>C.quinoa-CLL\NLL\GR C.amarant-CLL\NLL\GR N.occidentalis(P1) -CLL\SR</td>
<td>aphid spp.</td>
<td>Decoration by SLV (Netherlands)</td>
<td>Van Dijk (1993B) Lee et al (1979)</td>
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</table>
### Appendix 2 (Cont...)

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Diameter</th>
<th>Shape</th>
<th>Host</th>
<th>Nematode</th>
<th>Detection</th>
<th>Reference</th>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>Tobacco Rattle Tobravirus (TRV)</td>
<td>50-115 nm + 190 nm straight tubular rods</td>
<td>C. amarant-NLL, Phaseolus vulgaris-NLL (pinpoint lesions - 3 days)</td>
<td>nematodes: (Trichodorus spp.)</td>
<td>Can be unreliable due to non-coated infectious forms</td>
<td>Van Dijk (1993B)</td>
<td>12 346</td>
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<tr>
<td>Arabis Mosaic Nepovirus (ArMV)</td>
<td>30 nm isometric</td>
<td>C. amarant-CLL, SCM C. quinoa-CLL, SCM</td>
<td>nematodes: (Xiphinema spp.)</td>
<td>ELISA, decoration</td>
<td>Van Dijk (1993B)</td>
<td>16</td>
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<tr>
<td>Tomato Blackring Nepovirus (TBV)</td>
<td>30 nm isometric</td>
<td>C. amarant-CLL+NLL + SN/SCM C. quinoa - CLL/NLL + SN/SCM</td>
<td>nematodes: (Longidorus spp.)</td>
<td>ELISA, decoration</td>
<td>Van Dijk (1993B)</td>
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<tr>
<td>Tobacco Necrosis Necrovirus (TNV)</td>
<td>30 nm isometric</td>
<td>Phaseolus vulgaris - LL* C. amaranticolor - LL* (* varies with strain)</td>
<td>Soil-borne (Olpidium brassicae)</td>
<td>Antisera available (ELISA &amp; decoration?)</td>
<td>Van Dijk (1993B)</td>
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<tr>
<td>Lettuce Necrotic Yellow Rhabdovirus (LNYV)</td>
<td>227 x 68 nm bacilliform &amp; enveloped</td>
<td>N. glutinosa - M or NLL+SVY</td>
<td>Aphid spp.</td>
<td>-</td>
<td>Sward (1990)</td>
<td>26 343</td>
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<tr>
<td>Tobacco Mosaic Tobamovirus (TMV) - crucifer strain</td>
<td>300 nm straight tubular rod</td>
<td>C. amarant - NLL+SR C. quinoa - NLL+SR</td>
<td>-</td>
<td>-</td>
<td>Li et al. (1988)</td>
<td>151</td>
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</tbody>
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#### Key

- LL = local lesions
- CLL = chlorotic local lesions
- NLL = necrotic local lesions
- GR = green rings surrounding necrotic lesions when leaves turn yellow
- SR = systemic reaction
- SN = systemic necrosis
- SCM = systemic chlorotic mottle
- SVY = systemic vein yellowing
- M = mosaic
Appendix 3: ELISA - Enzyme Linked Immunosorbent Assay

EQUIPMENT AND/OR MATERIAL NEEDED

- Polystyrene plates (96 well plates)
- ELISA kit
- Grinder
- Incubator
- 5 x PBS buffer
- PBS Tween buffer
- Coating buffer
- Extraction buffer
- Conjugation buffer
- Substrate buffer

REFERENCES

DEFINITIONS AND/OR BACKGROUND INFORMATION

- The ELISA technique is an extremely sensitive, rapid and specific test for detection of plant viruses and is adaptable for very large scale indexing.
- The technique is based upon the reaction of protein (virus coat) with its antibody.
- An enzyme chemically attached to the antibody reacts with substrate to produce a colour change.

METHOD

- Determine number of samples.
- Label the plate indicating the test undertaken and the date.
- Prepare coating buffer with the appropriate coating antisera according to the instructions provided with the ELISA kit.
- Dispense required volume of coating buffer in the required number of wells (as per ELISA kit).
- Place an empty plate on the coated plate and put them both into a clear snap-lock plastic bag.
- Incubate the coated plate as indicated on the kit.
- Meanwhile, obtain control positive and health samples from the glasshouse.
- Weigh samples.
- Grind control healthy sample first and control positive sample test.
- Grind samples with extraction buffer diluting to 1:10.
- Wash plate with PBS Tween washing buffer as indicate on the kit.
- After the last washing, tap the plate firmly but carefully upside down on bench so as to get rid of excess wash buffer.
- Add 2 wells per sample of the required volume of sample extraction as well as control positive, healthy and buffer.
- Cover the plate (as instructed above).
- Incubator overnight at 4°C.
- Next day, tip out the sample extraction from the plate.
Appendix 3 (Cont…)

- Wash carefully with tap water and then wash with PBS Tween (as instructed above).
- After the last washing, tap the plate firmly but carefully upside down on bench so as to get rid of excess wash buffer.
- Add required conjugate antisera from the kit in required volume of conjugate buffer.
- Dispense required volume of the above preparation in wells.
- Cover the plate (as instructed above).
- Incubate the plate as indicated on the kit.
- Tip out the content of plate and follow as instructed above.
- Just before use, dilute 1 substrate tablet/5ml substrate buffer.
- Add required volume of the above preparation and incubate for 30 minutes or more until the control positive clearly changes the colour at room temperature and the background colour reaction is not excessively high.
- Stop reaction with NaOH or sulphuric acid as instructed on the kit.
- Measure the optimum density on Titertek software at required nm (nanometers) once the colour has developed.
- Complete worksheet and final report.
Appendix 4: Standard Method for the Isolation of Fungi from Plant Tissue

EQUIPMENT AND/OR MATERIAL NEEDED:

- Scalpel
- Forceps
- 0.5% Sodium hypochlorite solution
- Crucible
- Beaker
- Potato Dextrose Agar (P.D.A.) plates for routine isolations
- Water Agar (W.A.) plates for routine isolations
- Incubator

REFERENCES

DEFINITIONS AND/OR BACKGROUND INFORMATION

- This method of recovery is used when there is no obvious fungal development such as hyphae, spores, fruiting bodies, etc. If these structures are present, different or additional techniques can be selected.
- The PDA plates may be used with or without the addition of an antibiotic such as achromycin.
- Standard aseptic technique should be followed.
- Prior to washing and undertaking isolations, the specimen should be examined under a dissecting microscope to observe/identify fungal structures which may be present in/on host tissue and assist in the selection of tissue for isolations.
- Only use recently collected material which has not dried out or severely rotted.
- In most cases, regardless of the host or symptoms (i.e. canker, rot, spot, vascular infection or die back), the tissue selected should be from where diseased tissue meets healthy (i.e. the active “front” of the infection).
- Young, recently affected areas should be selected, avoiding dead tissue which usually contains many saprophytic organisms.
- In most cases, the external tissue over the canker or rot is removed and discarded. This is not possible with leaf infections or other very thin plant material where surface/sub-surface tissue must be selected.
- Following the isolation procedure, any fungus recovered which requires further examination can be sub-cultured and maintained in pure culture.

METHOD

- Gently wash off any adhering soil or other extraneous matter from the plant tissue.
- Where feasible or applicable, remove and discard surface tissue from the selected area.
- Using scalpel, cut small sections of affected tissue (approx. 5mm x 2mm) mainly from the margin of the lesion or from an area where vascular/root discouloration is obvious.
- Place sections into a crucible and place the crucible into a beaker.
- Add enough 0.5% solution of sodium hypochlorite to cover all the sections and ensure they remain well covered/saturated for 2 ½ minutes (or other specified time).
- Remove crucible containing sections and place on a tissue (or similar) to drain excess hypochlorite.
- Using sterile forceps, aseptically transfer sections to agar plates (approx. 6/plate and 4 plates of media, unless otherwise specified). Do not place sections too close together or to near the edge of the plate.
- Incubate plates (inverted) at approx. 20-24°C.
Appendix 5: Standard Method for the Isolation of Bacteria from Plant Tissue

EQUIPMENT AND/OR MATERIAL NEEDED

- Inoculating hoop
- Scalpel
- Sterile water
- Petri dishes
- Kings B media (for routine isolations, otherwise choose appropriate selective media)

REFERENCES

DEFINITIONS AND/OR BACKGROUND INFORMATION

- Only use recently collected material which has not rotted or dried out in transit.
- In most cases, regardless of the host or symptoms (i.e. canker, rot, spot, vascular infection or die back), the tissue selected should be from where diseased tissue meets healthy (i.e. the active “front” of the infection).
- Young, recently affected areas should be selected, avoiding dead tissue which usually contains many saprophytic organisms.
- In most cases, the external tissue over the canker or rot is removed and discarded. This is not possible with leaf infections or other very thin plant material where surface/sub-surface tissue must be selected.
- The use of aseptic technique is necessary at all time.
- When streaking out bacterial suspensions or colonies, the surface of the agar plate should be dry.
- Following the isolation procedure, any colony of interest can be sub-cultured and grown on in pure culture for further biochemical testing.

METHOD

- Gently wash off any adhering soil or other extraneous matter from the plant tissue.
- Where feasible, aseptically remove and discard surface tissue from the selected area.
- Cut out small pieces of tissues (approximately 5mm x 3mm). Wipe with 75% alcohol or place in 75% alcohol for 2 minutes.
- Transfer to sterile water.
- Using a sterile scalpel, cut small sections of tissue from the appropriate area. Usually 4-5 slivers about 5mm long is sufficient.
- Place this tissue in 4-5 drops of sterile water in a petri dish and chop or macerate very finely. The amount of tissue and water should be well balanced ensuring there is not too much or too little water. Repeat the above procedure at least 4 times.
- Leave these suspensions for 15-30 minutes to allow the bacteria to diffuse into the water.
- Streak one loopful (from each suspension) onto the appropriate agar plate (usually Kings B). To obtain single colonies, use the dilution streak method.
- Incubate plates (inverted to catch condensation in the lid) usually at 24°C and examine daily.
Appendix 6: Method for the Preparation of Solid Multiplication Media

EQUIPMENT AND/OR MATERIAL NEEDED

- M & S medium with sucrose
- Mm 2iP stock solution
- 1mM NAA stock solution
- Adenine stock solution
- Na Orthophosphate diH2O stock solution
- Gelrite
- Distilled water
- Large beaker
- Electronic balance
- Plastic weighing dish
- Automatic pump dispenser
- Polycarbonate - autoclavable - tubes with screw cap lids
- Magnetic stirrer and flea
- pH meter
- Autoclave

REFERENCES

DEFINITIONS AND/OR BACKGROUND INFORMATION

- Solid media is used for the rapid multiplication of garlic tissue culture plantlets.
- To make up 1 litre of media, the following amounts are required per ingredient:

  M & S medium with sucrose 34.6g
  3mg 2iP stock solution (1Mm S/S) 14.76ml
  0.3mg NAA stock solution 1.61ml
  Adenine stock solution 1 ml
  Na Orthophosphate diH2O stock solution 13ml
  Gelrite 3.5gm
  Distilled water 1 litre

METHOD

- Measure out 1 litre of distilled water and pour into large beaker.
- Place a flea inside the beaker and place onto a magnetic stirrer.
- Turn magnetic stirrer on and stir medium.
- Add each ingredient (except Gelrite) and allow to dissolve.
- Adjust pH with 1M HCL until a pH of 5.7 is reached.
- Add Gelrite to medium, it is important that medium remains well agitated at Gelrite will not entirely dissolve.
- Whilst medium is being stirred, dispense 20ml into each polycarbonate tube using the automatic pump dispenser.
- Place caps on each tube firmly.
- Place tubes in Autoclave and sterilise @ 121°C for 20 minutes.
- After sterilising is complete, place in cool room and keep for no longer than 1 month.
Appendix 7: Standard Method for Culturing Garlic Material Under Aseptic Conditions

EQUIPMENT AND/OR MATERIAL NEEDED
- Laminar flow cabinet
- 70% Ethanol
- Scalpel and blade
- Bunsen Burner
- Large forceps
- Initiation media
- Dedicated culture room

REFERENCES

DEFINITIONS AND/OR BACKGROUND INFORMATION
- This is the routine sub-culturing of material in tissue culture using standard aseptic culturing techniques.
- Material used should be actively growing with one to several newly formed bulbs growing from the original bulb.
- Media should be made up within 2 weeks of use.
- Cultures are observed weekly for the emergence of any fungal or bacterial growth.

METHOD
- Place garlic tissue culture plantlets, initiation media tubes, forceps and scalpel in laminar flow.
- Flame forceps and scalpel, swab down garlic tissue cultures, initiation media tubes and laminar flow with 70% ethanol.
- Remove plantlets from culture. Plantlets should appear as one to several small bulbs, with some root formation in the media and some older, often dead, mature leaves.
- Dissect culture into single bulbs, remove dead leaves and half of the remaining leaf material and roots.
- Place each bulb into their own tube containing initiation media.
- Label date and variety.
- Flame forceps and scalpel, swab down cultures, initiation media and laminar flow with 70% ethanol before dissecting next culture.
Appendix 8: Standard Method for the Initiation of Garlic Meristem into Tissue Culture

EQUIPMENT AND/OR MATERIAL NEEDED

- Laminar flow cabinet
- 100% Ethanol
- 70% Ethanol
- Scalpel and blades
- Bunsen Burner
- Large forceps
- Initiation media
- 50ml beaker
- Culture room

REFERENCES

DEFINITIONS AND/OR BACKGROUND INFORMATION

- Cloves identified for inclusion into tissue culture will have tested negative for Shallot Latent Carlavirus, Leek Yellow Stripe Potyvirus, Onion Yellow Dwarf Potyvirus and general potyvirus using a commercially available ELISA (Enzyme Linked Immunosorbent Assay) kit.
- Grids prepared from leaf material, originating from these cloves will be scanned under an Electron Microscope for a range of virus particles, with none being observed.

METHOD

- Select garlic cloves for inclusion into tissue culture.
- Peel off outer leaves and cut away the large fleshy storage leaves. This will expose the meristem material which look like a small (about 1cm long) green tipped triangle on top of the existing basal plate.
- Carefully remove about 1-2mm of the basal plate. This is to remove any area exposed to pathogens such as Fusarium sp. whilst keeping enough of the basal plate for shoot development.
- Flame forceps and scalpel, swab down initiation media tubes and laminar flow with 70% ethanol.
- Surface sterilise garlic meristem by placing into a beaker containing 100% ethanol, for 12-15 minutes depending on its size.
- Flame forceps.
- Place meristem into initiation media, label and store in dedicated culture room.
- Cultures will be observed weekly for the emergence of any fungal or bacterial growth.