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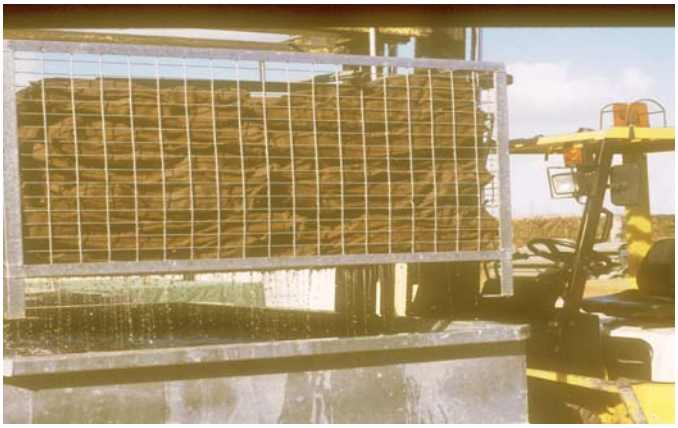
Australian Government

Grape and Wine Research and Development Corporation

An investigation of the role of endogenous and exogenous bacteria in grapevine propagation and development.



Vines bundled for hot water treatment.



Hot water treatment tank with bundles

FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **MU01/01**

Principal Investigator: **Dr Mary Cole**

Research Organisation: **Monash University**

Date: **3rd February 2006**

An investigation of the role of endogenous and exogenous bacteria in grapevine propagation and development

A final report to the Grape and Wine Research Development Corporation

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Abstract

There is a perception in the vine nursery industry that the HWT process sterilises both the surface and the wood of cuttings, but results from this research show clearly that many of the micro organisms associated with grapevines, particularly bacteria, are quite robust, not always killed by the high temperature of HWT and sometimes freely sporulating fungi are actually dispersed during the process. It is possible that micro organisms associated with grapevine cuttings may contribute either directly as opportunistic pathogens, or indirectly via toxic metabolites, to the poor performance or failure of cuttings or newly grafted vines, particularly if nursery conditions are stressful and favour the development of the micro organisms over the cuttings.

In this research, neither graft healing or root numbers were affected by HWT, but scion shoot growth was apparently promoted by HWT.

Recommendations from the research, and the experience of the authors, suggest that it would be prudent for nurseries to avoid the use of water in propagation, particularly if it is not treated to control water borne micro organisms. The practice of subjecting cuttings to a pre HWT hydration period should be abandoned, as should the practice of holding cut buds in water. Further commercial trials to assess the value of post HWT cooling of cuttings should be undertaken with a view to also abandoning this practice. It would also be prudent for nurseries to allow saturated cuttings to drain sufficiently to reduce surface moisture before placing them in cold storage to reduce the chances of the proliferation of epiphytic micro organisms.

Executive summary

There is a perception in the vine nursery industry that the HWT process sterilises both the surface and the wood of cuttings, but results from this research show clearly that many of the micro organisms associated with grapevines, particularly bacteria, are quite robust, not always killed by the high temperature of HWT and sometimes freely sporulating fungi, eg *Penicillium italicum,* are actually dispersed during the process. It is possible that micro organisms associated with grapevine cuttings may contribute either directly as opportunistic pathogens, or indirectly via toxic metabolites, to the poor performance or failure of cuttings or newly grafted vines, particularly if nursery conditions are stressful and favour the development of the micro organisms over the cuttings.

The effects of the changes to the population of surface micro organisms on cuttings that result from HWT protocols are also unknown. It is possible that the changes may alter the interaction of the surface micro organisms with other introduced micro organisms such as Botrytis.

Since little is known of the effects of these organisms on graft healing, root initiation and the long term health of vines when they infect the wood it would seem prudent to take steps to prevent the introduction of these organisms to the vine tissue by reducing the exposure of cuttings to contaminated water. Previous work has demonstrated that the supposed benefits of hydration are questionable and if cuttings are taken from well managed and adequately watered mother vines this step in the HWT protocol can be eliminated. Previous work also indicates that it may also be possible to eliminate the period in the cool down tank, although a commercial scale trial would need to be carried out to confirm this.

Recommendations from this research, and the experience of the authors, suggest that it would be prudent for nurseries to avoid the use of water in propagation, particularly if it is not treated to control water borne micro organisms. The practice of subjecting cuttings to a pre HWT hydration period should be abandoned, as should the practice of holding cut buds in water. Further commercial trials to assess the value of post HWT cooling of cuttings should be

undertaken with a view to also abandoning this practice. It would also be prudent for nurseries to allow saturated cuttings to drain sufficiently to reduce surface moisture before placing them in cold storage to reduce the chances of the proliferation of epiphytic micro organisms.

It is to be hoped that the results of this research increase the awareness in the nursery industry of the potential of HWT protocols to introduce micro organisms to the tissue of the treated cuttings. Although the benefits of HWT in controlling crown gall, phytoplasmas and Petri disease most likely outweigh any detrimental effects of these organisms since they have not been identified as causing disease in grapevines, it would be prudent for nurseries to reduce the exposure of cuttings and young rooted vines to potential contamination from untreated water. It is recommended that nurseries actively treat the water they use, particularly rainwater and water sourced from irrigation supplies, and that hydration be eliminated from the protocols.

In this research, neither graft healing or root numbers were affected by HWT, but scion shoot growth was apparently promoted by HWT. The effects of HWT on the physiology of dormant *Vitis vinifera* cuttings are poorly understood and it is therefore difficult to speculate on the probable cause of the increased scion shoot length in the HWT material. However, the results add to the body of evidence that the short term effects of HWT that cause retarded growth early in the growing season do not persist and are not observable by the end of the first growing season.

Nurseries do not always treat the water they use in propagation processes and frequently soak cuttings and pre cut buds as a means of preventing dehydration, a process that may also add to the micro organism load of the water. Consequently, it seems most likely that these organisms gain entry to vine tissue during propagation, although there are also limited opportunities for micro organisms to gain entry to mature grapevines via exposure of wounds to irrigation water and rain splash. Therefore, it is strongly recommended that grapevine nurseries use only treated water of potable quality and that consideration be given to stopping the practice of soaking cuttings and precut buds in order to improve grafting success and nursery profitability

through reduced wastage, while improving the quality of planting material available to the grape and wine industries.

The experiences of the authors over several years with failed cuttings and planting material adds weight to the hypothesis that some of these bacteria and some fungi that are not normally associated with disease symptoms may contribute to the poor performance of cuttings and planting material if the host is stressed by poor handling, propagating and storage practices, particularly if water saturated tissue is subjected to low oxygen environments and temperatures favourable to fungi and bacteria.

Background

There have been numerous reports since to mid to late 1990's of failure of young vineyards in Australia and overseas, particularly in the USA and Europe. These reports have coincided with the growth of the wine industry and the increased plantings that have taken place since the early 1990's. There has been a general shortage of good quality planting material and inferior vines that would have previously been discarded have sometimes been planted. In some cases the planting material has been apparently healthy when planted, but failed to establish. Some of these failures have been attributed to hot water treatment, or the presence of P. *chlamydospora,* however the researchers are aware of a number of instances where the cause of failure could not be attributed to either of these causes. Dissection of these failed vines revealed brown streaking in the xylem tissue, but the symptoms did not completely correspond with those that normally indicate *P. chlamydospora* infection. However the creamy, aromatic bacterium was frequently isolated from symptomatic tissue.
During hot water treatment (HWT) trials (SAR99/4) and collection of young grafted material for the trunk diseases project (CRV99/12), symptoms of brown streaking in the xylem have been observed in poor quality nursery stock in association with poorly healed graft unions and poor establishment and vigour in the vineyard.

Failures of large numbers of young vines from unknown causes have been reported from many areas including Riverland, Sunraysia, NE Victoria, Corawa region of NSW and Tasmania and result in delayed and diminished production and significant financial loss in the order of $50 000 to $100 000. In many of these instances brown streaking in the xylem has been observed. The xylem streaking is similar to the symptoms of *Phaeomoniella* (previously *Phaeoacremonium*), but *Phaeomoniella* is rarely isolated.

A creamy, extremely gelatinous and highly aromatic bacterium has been isolated consistently from transverse stem sections of affected scions and rootstocks. The frequent isolation of this bacterium from poor quality nursery stock and vines that have failed or performed poorly in the

field, but not from apparently healthy material, suggests that it may be pathogenic either in its own right, or a synergist with other xylem infecting pathogens.

This bacterium has also been isolated from grape skins and it is thought that because it is strongly aromatic it may affect flavour and aroma development in wine. It is, therefore, reasonable to assume that this organism is widespread on the phylloplane of vines.

The Australian wine industry requires sound, healthy planting material that is long lived requires minimum inputs of resources and labour and is highly productive. Knowledge of the epidemiology and pathogenicity of this bacterium is an important element in the development of parameters for the production of high quality grapevine planting material that performs well in the field and requires minimum inputs for optimum production of high quality grapes and wine.

Results from this project will be integrated into nursery and vineyard management protocols and promulgated widely through established communication channels including VINA, AVIA, grower associations, seminars, conferences and industry magazines and journals.

Much of the work that has been done on endogenous bacteria in grapevines has concentrated on crown gall *(Agrobacterium vitis*) and Pierce's Disease (*Xylella fastidiosa*) and it is difficult to find references to other endogenous bacteria in grapevines. However Boubals and Mur (1982) describe symptoms of browning in the xylem tissue of young grafted vines that had failed to thrive. They concluded that the symptoms were caused by an unidentified bacterium that was thought to be similar, but not identical to *Xanthomonas ampelina*.

Boubals and Mur (1982) also expressed concerns that this bacterium was the cause of graft failure and failure of vines in the field resulting in significant financial losses. They also suggested that although this was a newly reported problem the disease had probably existed unrecognised for some time. The use of copper based fungicides and careful nursery hygiene was recommended to control the problem.

Bell *et al*. (1995) extracted more than 100 isolates of bacteria from grapevine sap the majority of which were Gram-negative with Pseudomonas and Enterobacter predominating, although other genera were

also represented. The authors speculate that some of these isolates are likely to be pathogenic although they were obtained from vines that were largely asymptomatic. They also speculated that propagation techniques that expose wounds to contamination may favour the entry of bacteria into grapevines.

The Australian wine industry operates in a highly competitive global market that demands a high quality, value for money, product.
The failure of large numbers of vines in new plantings delays return on capital and increases the direct and indirect costs of winegrape production, potentially threatening the long term viability of the Australian wine industry. It is essential to resolve the cause of vine failure and the trunk disease syndrome in established vines and to provide industry with economical and practical management protocols for nurseries and vineyards to prevent losses and improve the efficiency of grape and wine production.

- During hot water treatment trials and collection of material for the trunk diseases project, symptoms of browning in the xylem similar to those of *Phaeomoniella chlamydospora* were observed in cuttings, young grafted vines and mature vines. Although these symptoms were similar to those caused by *P. chlamydospora,* the discolouration in the xylem was paler and there was less evidence of the black tar-like ooze associated with *P. chlamydospora*. *P. chlamydospora* was rarely isolated from these vines, but a creamy, extremely gelatinous and highly aromatic bacterium was regularly isolated. Pure cultures of the bacterium could be isolated from individual xylem vessels in transverse stem sections.

In the laboratory an isolate of the bacterium appeared to strongly inhibit *Trichoderma sp.*, a fungus that is increasingly used as a biocontrol agent for fungal pathogens, including Botrytis, in both the vine nursery industry and in general viticulture. Many bacteria among the Pseudomonads and *Bacillus* spp. are known to have biological control potential against fungal pathogens while others are pathogens themselves.

- Own-rooted vines produced from affected cuttings grow poorly in the field nursery and develop into poor quality rootlings that fail to establish in the vineyard. Grafted vines that are affected show incomplete healing of the graft union and dark streaking of the xylem extending into both the scion and rootstock. These vines also fail, or establish poorly, in the vineyard requiring increased resources, particularly water and fertiliser, but still resulting in lower production.

This bacterium has also been isolated from mature vines with symptoms of esca from *which P. chlamydospora* and *Phellinus spp*. are routinely isolated. The apparent purity and volume of the organism in the xylem vessels suggests that it must be having an impact on the viability of the rootlings. Whether this is a primary, secondary or synergistic role is not known, however, its presence does not necessarily indicate the presence of another pathogen, fungal or bacterial.

Relevant publications/references.

Bell C.R., Dickie G.A., Harvey W.L.G. and Chan J.W.Y.F. (1995)Endophytic bacteria in grapevine. Can. J. Microbiol. **41**:46-53.

Boubals D. and Mur G. (1982) Problemes poses par les bacteries lors de la multiplication de la vigne, **In:**Federation Francaise des Syndicats de Producteurs de Plants de Vigne *Ille colloque international sur la moltiplication de la vigne*, Bordeaux 2,3,4 septembre 1982.

Mur G. and Boubals D.(1982) La bacteriose des greffes-soudes. Progress Agricole et Viticole, 15-16, 342-348.

Project Aims and Performance targets

- Understand the role of this bacterium in the poorly performing planting material and in vine decline.
- Understand the role of this bacterium in the whole trunk diseases syndrome and its interaction with other trunk diseases.
- Integrate the results into propagation and vineyard management protocols to improve the quality of planting material and health and productivity of vineyards.
  Develop and maintain communication links with nurseries, growers and researchers through publications, seminars and workshops.

Outputs and Performance Targets

Outputs	Performance Target
1.Clarify the contribution of the bacterium in the decline of planting material that performs poorly in the field.	1.Management strategies for control of the bacterium in propagation, particularly at the hot water treatment stage.
2.Clarify the role of the bacterium in established vines showing the trunk disease syndrome.	2.Management strategies that maintain the health of vines in the field.
3.Integrate the results into nursery and vineyard management protocols.	3.Industry acceptance of integrated, practical management strategies to improve the quality of planting material and the performance of vines in the field.
4.Communicate findings to nurseries, growers and researchers.	4.Scientific papers, reports, seminars and workshops.

Relationship to industry priorities.

- Nurseries and growers are very keen to prevent further planting failures and decline of mature vines that affect grape production and quality and reduce and delay return on capital investment. There is strong motivation for adoption of new management procedures by industry provided that the procedures are both practical and economical to implement. Potential for adoption by industry and associated risks.

- Failure of new vines in the field incurs losses for both the grower and the nursery. Failure of young vines delays production in new vineyards for at least one year and replanting increases establishment costs. Nurseries frequently incur the cost of replacing failed vines and suffer loss of reputation that may result in a reduction in business, threatening the viability of their enterprise and the jobs of their employees. The development of protocols that result in the production and maintenance of healthy, productive vineyards will reduce the cost of establishing and maintaining vineyards and prevent vine failures caused by endogenous diseases. It is difficult to accurately asses the potential losses incurred when vines fail or unproductive, however the cost of replacing failed vines is between $2,000 and $7,000 per hectare not including lost production. Assessment of benefits to industry and associated costs.

Industry Support and ‘In Kind’ Contributions

Hutchison Vineyards Pty. Ltd.: specimens, samples and field trials.

Orlando Wyndham Group Pty. Ltd.: propagation facilities, specimens and field trials.

Boulevarde Nurseries Pty. Ltd.: propagation and tissue culture facilities and specimens.

Australian Vine Improvement Association: support and assistance with communication with industry.

Vine Industry Nursery Association: support and assistance with communication with industry.

Adro Grafted Vines: propagation facilities, specimens.

Coastal Ridge Nursery: specimens and field trials.

Collaborators

Mr Ian Pascoe, Agriculture Victoria, principal researcher, GWRDC, CRCV Trunk Diseases Project.

Mr Graeme Fletcher, Agriculture Victoria/VAMVVIA

Mr Paul Wright, Nursery Manager Orlando Wyndham Group Pty. Ltd.

Dr Jim Hardie, CRCV

Mr John Crocker, SARDI

Professor Graham H Fleet

Outcomes from grant:

Workshops

"Making Every Stick Count"

Merbein: 7th & 8th June 2004

9th & 10th June 2005

Griffith: 20th & 21st October 2002

A total of 60 delegates attended the workshops from Vic, NSW, SA, WA, Tasmania, New Zealand and California.

Publications

H. Waite & Cole M. 2003 'Are bacteria involved in grapevine trunk diseases?' National Grapegrowers magazine Autumn

Waite, H. 2005 'Hot water treatment: cooling the confusion', Australian and New Zealand Grapegrower and Winemaker, April 2005 pp.34-36.

Waite, H., Cole, M., Jaudzems, G. & Faragher, J. 2004, 'Recent advances in grapevinr propagation research', Australian and New Zealand Grapegrower and Winemaker Annual Technical Issue.

Personal contact

Inquiries from USA
Canada
Ukraine
New Zealand
All states of Australia.

Conferences

3rd IWGTD 2003, Christchurch, New Zealand

Paper: Waite, H. & May, P. 'The effects of Hot Water Treatment, hydration and order of nursery operations on cuttings of *Vitis vinifera* cvs.

Poster: Waite, H., Crocker, J., Fletcher, G., Wright, P. & delaine, A., 'Implications of hot water treatment and other nursery practices in the production of quality grapevine planting material'

4th IWGTD 2005, Stellenbosch, South Africa

Paper: Waite, H. Faragher, J. & Jaudzems, G. **'**The Effects Of Hot Water Treatment On Grapevine Cutting Physiology and Cell Ultrastructure'

Poster: Waite, H., Beggs, S., Dark, H. Kwak, P. & Murrells**, G., '**The effects of different post hot water treatment cool down protocols on dormant cuttings of *Vitis vinifera cvs.'*

ICCS 2006, Christchurch, New Zealand

Discussion group: Waite H., Hot water treatment.

Appendix 1

The Effects of Hot Water Treatment and Propagation Protocols on Populations of Exogenous and Endogenous Microorganisms Associated With Dormant Cuttings of Grapevines.

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Introduction

A survey of micro organisms associated with cuttings collected from source blocks used by the vine nursery industry (Waite & Cole unpub.) identified a variety of exogenous and endogenous micro organisms that are cosmopolitan inhabitants of the phyllosphere and decaying leaf litter (Hall *et al*. 1989; Dix & Webster 1995) including a creamy translucent bacteria and yeast complex (including *Pseudomonas* spp.and *Trichosporon* spp.), *Rhizopus* sp., *Penicillium* spp., *Cladosporium* sp., *Cephalosporium* sp. (*Acremonium* sp.), *Trichoderma* sp., *Alternaria* sp., *Rhizopus* sp., *Rhodotorula* sp. and *Cephalosporium* sp. It was thought that the presence of these organisms in the xylem tissue as well as on the surfaces of cuttings may be a result of accidental contamination of wounds made during pruning or propagation. Although they are not recognized as plant pathogens, several species including *Penicillium* spp. and *Enterobacter* spp. have been isolated from poorly performing cuttings and rootlings and it is thought that these organisms might contribute to the loss, or poor performance of cuttings or young grafted vines in the nursery and vineyard. The incidental contamination of graft unions with bacteria and fungi is known to be detrimental to graft healing (Hartmann *et al*. 1990) and propagation practices in vine nurseries expose cuttings to water for relatively long periods of time, particularly during the hot water treatment process, providing ample potential for micro organisms in the water or on the surfaces of the cuttings to move into the cut ends of the vines and wounds made during grafting and disbudding. Although the practices of hydrating cuttings for varying periods of time prior to HWT and cooling cuttings immediately post HWT

by plunging them into cold water for an equivalent period are widely regarded by the nursery industry as essential elements of successful propagation, experimental evidence indicates that hydration is not necessary and that the rapid cooling of cuttings in cold water might also be able to be eliminated, although this needs to be tested in commercial scale trials (Crocker *et al.* 2002; Waite *et al*. 2005).

In order to determine the contribution nursery practices make to the population of micro organisms on and in the tissue of grapevine propagating material and to establish the HWT protocols most likely to result in high quality propagating and planting material, cuttings of *Vitis vinifera* cv. Shiraz PT23 grafted to the rootstock 1103 Paulsen were sampled for endogenous and exogenous micro organisms at each stage of the propagation cycle.

Method and Materials

One hundred and twenty cuttings each of *Vitis vinifera* cv. Shiraz PT23 and the rootstock 1103 Paulsen were collected from registered source blocks in the Sunraysia district of north western Victoria in the first week of July 2003 and hot water treated and cool stored at the Victorian and Murray Valley Vine Improvement Association (VAMVVIA) accredited facility at Irymple. The subsequent stages of the propagation cycle were completed in an accredited vine nursery also located at Irymple.

The stages in the propagating cycle for the production of grafted rootlings are:

1) Collection of scion and rootstock cuttings from registered mother vine source blocks.
2) Hydration of scion and rootstock cuttings (10 minutes in chlorinated water).
3) Hot water treatment of scion and rootstock cuttings at 50^{o}C for 30min.
4) Post HWT cooling of scion and rootstock cuttings (immersion in cold water for 30min.).
5) Cold storage of scion and rootstock cuttings.
6) Disbudding of rootstock cuttings to prevent sprouting.
7) Union of scion and rootstock cuttings to form a new rootling.
8) Finished rootling 12 months after propagation.

At each of the first 5 stages of the propagation process 10 scion and 10 rootstock cuttings were randomly selected and sampled for exogenous and endogenous micro organisms. Only rootstock cuttings were sampled following disbudding since scion varieties are obviously not disbudded. Following grafting, 10 grafted vines were randomly selected at each stage for sampling. Sampling of exogenous micro organisms on cuttings was done by wiping a sterile swab dipped in sterile water over a 100mm section of the cutting surface 50mm either side of the middle node of each selected cutting. The swab was wiped over sterile PDA (potato dextrose agar) plates and incubating at 25^{o}C for 48 hours. Sampling of endogenous micro organisms was done by debarking the cuttings and taking 4 wood slivers from the interior of the cutting 10-15mm from the base. The wood slivers were transferred to sterile PDA plates and incubated at 25^{o}C for 48 hours. Following grafting and callusing exogenous micro organisms were sampled by swabbing a section of the cutting surface 50mm either side of the graft union. Endogenous micro organisms were sampled by taking 4 wood slivers from the graft union for each sampled vine. When the young vines were 12 months old external swabs were taken in the same manner as at callusing and wood slivers were taken from either side of the graft union. Micro organisms were identified by microscopic examination of the incubated plates.

Results and Discussion

Exogenous Microorganisms

Table 1: Frequency per 10 samples of microorganisms isolated from the surfaces of cuttings and grafted vines at each stage of the propagation cycle

	Creamy bacterial complex	*Enterobacter cloacae*	*Penicillium italicum*	*Penicillium digitatum*	*Clado-sporim sp.*	*Asper gillus sp.*	*Rhizopus sp.*	*Fusarium sp*	*Alternari a sp.*	*Tricho-derma sp.*	*Rhodot orula sp.*	Black yeast	*other*
Paulsen-collection	9	2	5	1	10			1	8		8	6	6
Shiraz-collection	10	1			10			1	9		2	5	10
Paulsen-post hydration	10	7	10	4	7	2		1	4		10	5	6
Shiraz-post hydration	9	2	5	1	10				10		3	7	*8*
Paulsen-post HWT	7	2	10	1	1		10				9		5
Shiraz-post HWT	7	6	9	2	4		8		1		7	1	8
Paulsen-post cool down	6	1	8	3	7		10				9		9
Shiraz-post cool down	8	3	9	2	10		7		1		8	1	9
Paulsen-post storage	8		10	4	1		8				5		4
Shiraz-post storage	8		10	4			9				2		9
Paulsen-post disbudding	10	2	10	3			10				4		2
Post callusing	9		3	2			8			9			
12 months	9	4	4	2	10				1	1	10		10

The occurrence of the creamy translucent bacteria (Figs 1 & 2) and yeast complex isolated from the surfaces of cuttings and the tissue was unaffected by nursery processes in this experiment (Table 1) and although individual species may have been affected, preliminary microscopic examination of the colony morphology indicated that there was little variation in the mix of species throughout the propagation cycle. However the occurrence of *Enterobacter cloacae* on the surface of cuttings, and to a lesser extent in the tissue declined after the post HWT cool down stage and during cold storage and reappeared by the time the vines had been exposed to the nursery environment for 12 months. The yeast *Rhodotorula* sp., which was present on the surface of all material until callusing was not found on the material when it was removed from the callusing boxes. The nursery routinely inoculates callusing boxes with a commercial strain of *Trichoderma harzianum* an antagonistic fungus (Dix & Webster 1995) used as a biocontrol for *Pythium* spp. and other pathogenic fungi including botrytis in callusing boxes and it is likely that the *Rhodotorula* sp. was suppressed by the *T. harzianum.* However after 12 months exposure in the nursery environment the rootling surfaces were repopulated. *T. harzianum* also appeared to suppress *Penicillium italicum* on the surface of the grafted vines in the callusing phase. The frequency of isolation and number of colonies per plate were much reduced and the population did not recover to the same degree as the *Rhodotorula* during the 12 months growing period in the nursery indicating that the competitive effect of *T. harzianum* persisted since there were most likely high levels of wind borne *Penicillium* inoculum from surrounding citrus orchards. Although the frequency of isolation of *P. digitatum* on the surface of callused vines was not affected by *T. harzianum* the number of colonies per sample plate was also much reduced. These results add to the body of evidence indicating that *T. harzianum* is an effective antagonist against a variety of pathogenic or potentially pathogenic microorganisms (Agrios 2005).

Surface populations of *Cladosporium* sp. diminished after HWT, but increased after immersion in the cool down tank. It is possible that the increase was a result of contamination of the water in the cool down tank either as a result of inadequate water treatment or exposure to

wind borne spores since the tanks at VAMVVIA are not enclosed in a shed, but sit on a concrete pad in the open air. The frequency of isolation of *Cladosporium* sp. declined again after cold storage and did not recover until the vines had been exposed to the open air in the nursery for 12 months. A possible explanation for this observation is that *Cladosporium* sp. is susceptible to cold stress, or that the spores become susceptible to cold after exposure to moisture as in the cool down tank.

The frequency of isolation of *Alternaria* sp., *Fusarium* sp. and an unidentified melanising yeast from cutting surfaces were also much reduced by HWT. The populations of these organisms, particularly *Fusarium* sp., did not recover even after 12 months in the nursery, a not unexpected result since *Fusarium* spp. are soil borne plant pathogens (Agrios 2005) and there was no opportunity for repopulation since the material was grown on in soil-less potting mix.

By contrast, Rhizopus was only isolated following the HWT phase, indicating that it was either a contaminant in the HWT and was not suppressed by the high temperatures or that spores were present on the surfaces of the cuttings and the HWT enabled them to break dormancy and thus grow in culture.

Endogenous Microorganisms

Although the populations of endogenous micro organisms isolated from the cuttings in this experiment generally paralleled the populations isolated from the cutting surfaces there were some differences (Table 2). The black yeast that was isolated from the surfaces of cuttings at cutting collection and after hydration was not isolated from the wood and the *Rhodotorula* sp. was only isolated from 1 wood sample at collection but occurred more frequently (Table 2) in the wood after 12 months in the nursery. This suggests that contamination of graft unions that may not have been fully sealed by callus tissue is likely to have occurred in the nursery once the protective wax had broken down, enabling irrigation run off from the leaves to trickle down the shoot and penetrate the graft union, carrying microbial contaminants with it. A similar scenario could also explain the reappearance of *Cladosporium* sp. and *Alternaria* sp. in the

wood after 12 months since both these organisms appeared to be suppressed by HWT and during cold storage. The isolation of *Trichoderma* sp. from the wood at 12 months is also likely to be a result of the same process. However the organism may have also gained entry through the graft union during callusing since the nursery concerned inoculates callusing boxes with Trichodex, a commercial preparation of *Trichoderma harzianum*.

The pattern of isolation of both the *Penicillium* spp. from the wood was similar to the isolation pattern from the cutting surfaces, although the actual frequency of isolation was lower. The processes of hydration and HWT resulted in an increased frequency of isolation from both the surfaces and the wood of cuttings indicating that *Penicillium* spp. are not killed by the HWT process and that both the hydrating tanks and the HWT tank are sources of contamination for these organisms, most likely as a result of being exposed to wind borne contaminants, or contamination of the water by previous batches of cuttings.

As was the case with exogenous populations, the occurrence of the creamy bacteria and yeast complex isolated form the wood of the cuttings was unaffected by any of the nursery operations. As with the exogenous population, preliminary microscopic examination of colony morphology indicated that the mix of species remained unchanged.

Fig 1. Cane slithers for incubation. **Fig 2.** Bacterial ooze from infected cane



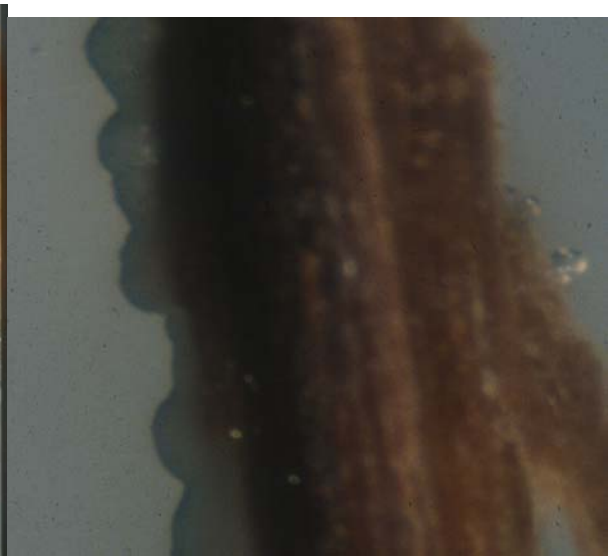


Table 2: Frequency per 10 samples of micro organisms isolated from the wood of cuttings and grafted vines at each stage of the propagation cycle

	Creamy bacterial complex	*Enterobacter cloacae*	*Penicillium italicum*	*Penicillium digitatum*	*Clado-sporim sp.*	*Rhizopus sp.*	*Fusarium sp*	*Alternaria sp.*	*Tricho-derma sp.*	*Rhodotorula sp.*	*other*
Paulsen-collection	8	5	1		4			2		1	8
Shiraz-collection	2	2			9			3			9
Paulsen-post hydration	10	6	1		4						5
Shiraz-post hydration	10	1			7			6			6
Paulsen-post HWT	9	3	3		1	6					4
Shiraz-post HWT	7	3	5	2		2	1	1			2
Paulsen-post cool down	9	1	5		1			1			6
Shiraz-post cool down	10	5	7	1	5			1			3
Paulsen-post storage	7	1	5	2							8
Shiraz-post storage	9	1	3	1		2					8
Paulsen-post disbudding	10	4	1	1							10
Post callusing	3	1	6	1		8					6
Paulsen -post 12 months	10		2					1	4	1	8
Shiraz-post 12 months	8	4	3	3	5	1		1	3	5	7

Conclusion and Recommendations

Although there is a perception in the vine nursery industry that the HWT process sterilizes both the surface and the wood of cuttings it is clear from the results of this experiment and published literature that many of the micro organisms associated with grapevines, particularly bacteria, are quite robust, not always killed by the high temperature of HWT and sometimes, as in the case of *Penicillium italicum*, actually dispersed during the process. Since little is known of the effects of these organisms on graft healing, root initiation and the long term health of vines when they infect the wood it would seem prudent to take steps to prevent the introduction of these organisms to the vine tissue by reducing the exposure of cuttings to contaminated water. Previous work has demonstrated that the supposed benefits of hydration are questionable and if cuttings are taken from well managed and adequately watered mother vines this step in the HWT protocol can be eliminated. Previous work also indicates that it may also be possible to eliminate the period in the cool down tank, although a commercial scale trial would need to be carried out to confirm this. The elimination of both these steps in the HWT process would result in considerable savings in labour and equipment for nurseries. However the elimination of these 2 processes would not entirely eliminate the opportunity for contamination of wounds in cuttings since active antimicrobial treatment of the water in the HWT tank is difficult to achieve and the water is used more than once because of the expense and time taken to heat the large tanks of water. The practice by some nurseries of raising the temperature of the HWT tank to 70^{o}C at the end of the day may control some of the contaminating micro organisms but is unlikely to eliminate them entirely.

The effects of the changes to the population of surface micro organisms on cuttings that result from HWT protocols are also unknown. It is possible that the changes may alter the interaction of the surface micro organisms with other introduced micro organisms such as botrytis. However the scope of this experiment did not cover the ecology of the isolated micro organisms and the effects of HWT protocols on the ecology of these micro organisms can only be speculated on.

It is to be hoped that the results of this experiment increase the awareness in the nursery industry of the potential of HWT protocols to introduce micro organisms to the tissue of the treated cuttings. Although the benefits of HWT in controlling crown gall, phytoplasmas and

Petri disease most likely outweigh any detrimental effects of these organisms since they have not been identified as causing disease in grapevines, it would be prudent for nurseries to reduce the exposure of cuttings and young rooted vines to potential contamination from untreated water. It is recommended that nurseries actively treat the water they use particularly rain water and water sourced from irrigation supplies and that hydration be eliminated from the protocols.



Bundles in the nursery



Bundles removed from HWT tank

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Appendix 2. The Effects Of Selected Microorganisms On Root Initiation, Graft Healing And Shoot Growth In *Vitis Vinifer* Cv. Shiraz PT23 Grafted To 1103 Paulsen Rootstock

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Introduction

There have been sporadic reports for a number of years from the grapevine nursery industry in Australia of poorly healed graft unions in bench grafted vines, a problem that result in the loss or poor performance of planting material in the nursery and the vineyard (Hartmann *et al.* 1990). These problems have often been attributed to hot water treatment (HWT) (50^{o}C for 30 minutes followed by an equivalent cooling period in cold water) a technique used to control trunk diseases including Petri disease and crown gall. However when dissected, the internal tissue of affected vines frequently showed brown streaking in the xylem extending away from the graft union in both the rootstock and scion similar to that seen in mature vines affected by fungal pathogens such as *Phaeomoniella chlamydospora,* but these pathogens were not always isolated from symptomatic vines.

However a creamy translucent bacterial and yeast complex including 3 bacteria species, *Pseudomonas putida*, *Rahnella aquatilus* and *Enterobacter intermedius* and a yeast, *Trichosporon pullulans* was regularly isolated from symptomatic young grafted vines and the wood of mature grapevines affected by fungal pathogens such as *P. chlamydospora,* particularly one year old rootlings that failed in the nursery or field and from which no identified grapevine pathogens were isolated. These organisms are sometimes found in digestive tracts and mucous membranes of humans where they can be opportunistic pathogens in immune suppressed individuals (Tortora *et al*. 2001). They are normally water and soil borne and are often isolated from water contaminated with storm water, sewage or animal faeces (WHO 2004). The isolation of these organisms from grapevines indicates that the vines are likely to have been exposed to untreated water, probably during propagation and to a

lesser extent from contaminated irrigation water coming in contact with wounds made during harvesting or pruning.

Many nurseries use untreated water before and after HWT for soaking cuttings and precut buds during the propagation process as a means of compensating for, or preventing dehydration of the propagating material, a practice widely regarded as beneficial by nurseries but found to be of limited value by Crocker *et al*. (2002). Although these organisms have not been reported to cause disease in grapevines, it was thought that they may interfere with graft healing if the cut surfaces were contaminated in the grafting process, or behave as opportunistic pathogens in stressed material, particularly as they were often isolated from vines or cuttings that had suffered environmental stress through hot water treatment, excessive soaking, or poor storage or growing conditions. It was thought that these organisms, may have been accidentally introduced into cutting tissue via contaminated water through wounds made during propagation, particularly the graft union, but also the cut ends and disbudding wounds on rootstocks.

In order to shed some light on the role of water borne contaminants in grapevine propagation the effects of 3 of the isolates, the bacteria *Rahnella aquatilus* and *Enterobacter intermedius* and the yeast, *Trichosporon pullulans,* on graft healing, scion shoot growth and root initiation in cuttings of the rootstock 1103 Paulsen grafted to the Shiraz clone PT23 (+/-HWT) were evaluated.

Method and Materials

Bench grade cuttings (straight, not less than 400mm long and 7-12mm in diameter) of the rootstock 1103 Paulsen and Shiraz PT23 were obtained from the Victorian and Murray Valley Vine Improvement Association (VAMVVIA) in mid June 2004. Half of each of the 1103 Paulsen and the Shiraz PT23 cuttings were hot water treated at 50^{o}C for 30 minutes, immediately plunged into cold water at ambient temperature for an equal amount of time and allowed to drain overnight. The cuttings were not soaked in water prior to HWT. The following day all the cuttings were placed in polythene bags with several small holes and stored in the VAMVVIA cool room at 1-2^{o}C until mid October.

The cuttings were removed from the cool room the day prior to grafting and the rootstock cuttings were divided into 4 groups of 60 HWT cuttings and 4 groups of 60 non HWT cuttings. The following day HWT and non HWT Shiraz PT23 cuttings were cut into 1 bud segments with a cleaned and sterilized wedge grafting tool for grafting to the rootstock cuttings. The base of the rootstock cuttings and the cut ends of the Shiraz PT23 buds were inoculated by dipping in a 10^6 solution of either *Rahnella aquatilis*, *Enterobacter intermedius* or *Trichosporon pullulans* for 5 minutes. The controls were dipped in sterile water for 5 minutes. Graft unions were held together with rubber grafting ties as per standard bench grafting practice. The grafted vines were then packed into polystyrene boxes with moist vermiculite and incubated at 27oC (+/_0.5^{o}C) for 2 weeks. The cuttings were removed from the callusing boxes and tops dipped into paraffin wax to cover the graft and prevent dehydration, potted into cardboard plant bands and grown on in a glasshouse until fully established and then hardened off in a shade house.

The young vines were assessed for graft healing and root and shoot development when fully dormant in late May 2005 after a full season's growth. Roots were assessed by counting the number of permanent roots that had formed. Root mass was not assessed since it was not possible to remove all the roots intact from the pots as some roots had penetrated other pots and the base of the boxes. Shoots were measured from the base of the shoot to the end of the lignified tissue and graft unions were assessed by calculating the percentage of callus that had united and held the stock and scion together around the graft union. Callus that had formed but not united the stock and scion was not included in the calculation since the quality of a graft union and the quality of the vine are measured by the degree of union between stock and scion. Data were analyzed by 1 and 2 way ANOVA and regression analysis where appropriate using the Minitab statistical package.

Results and Discussion

Root Numbers

There was no significant effect ($P<0.05$) of either HWT or the microorganisms on root numbers in this experiment (mean root number =4.5). Although HWT is known to affect root initiation in some scion varieties (Waite unpub. 2002), particularly when cuttings are taken from water stressed mother vines (Crocker *et al*. 2002) the assessments were made early in

the propagation cycle and focused on scion varieties as own rooted cuttings rather than rootstock varieties which are generally reported by the nursery industry to be less susceptible to HWT than scion varieties. Bell *et al.* (1995) reported isolating large numbers of bacteria (84 strains) from the tissue of apparently healthy grapevines, but also suggested that some species, particularly *Erwinia* spp. that have been reclassified to *Enterobacter* spp. may prove to be phytopathogenic. Bell *et al*. (1995) also speculated that the manner of propagating grapevines by inserting cuttings directly into the soil is a likely route of infection. It is interesting therefore to note that in the experiment reported here none of the microorganisms used to inoculate the bases of cuttings affected the capacity of the cuttings to develop roots.

Graft Healing

Poor performance of vines in the field has been linked to incompletely formed, poor quality graft unions. Hartmann *et al.* (1990) state that incomplete healing of graft unions impedes the movement of water and photosynthates resulting in poor performance or death of the scion. In this experiment graft healing was significantly affected by *R. aquatilis* and *E. intermedius*, particularly *R. aquatilis*, but not by *T. pullulans*, or HWT, or any interactions between the microorganisms and HWT. Both *R. aquatilus*, and *E. intermedius* suppressed callus development thus impeding the complete healing of the graft union.

Table 1. Means of graft healing expressed as the percentage of healing around the graft union inoculated with selected microorganisms.

Treatment	Control	*R. aquatilis*	*E. intermedius*	*T. pullulans*
% Graft healing	70^{a}	36^{c}	54^{b}	61^{ab}

Figures with the same superscripts are not significantly different ($P<0.05$)

Scion Shoot Growth

Although HWT is frequently reported to suppress shoot extension in scion varieties early in the growing season, shoot growth in HWT material generally equals shoot growth in untreated material by mid summer when treated and untreated material become indistinguishable from one another (Waite *et al*. 2001). In this experiment the final shoot length of the scion at the conclusion of the first season of growth was significantly longer ($P<0.05$) in the HWT group compared to the non HWT group.

Table 2. Means of scion shoot length in cm in 1 year old rootlings +/-HWT measured from base to the end of lignified tissue.

Treatment	-HWT	+HWT
Scion shoot length	12.51[a]	15.63[b]

Different superscripts indicate significantly different figures (P<0.05)

There was also a significant effect (P<0.05) of the microorganisms on scion shoot length, but no interaction between HWT and the microorganisms. All 3 organisms suppressed shoot development compared to the controls. This result is interesting since it might be expected that poor graft healing would be the most probable cause of the reduction in shoot length. However *T. pullulans* affected shoot length but not graft healing indicating that *T. pullulans* had a more direct effect on shoot growth other than impedance of the conducting tissue at the graft union.

Table 3. Means of scion shoot lengths in cm in 1-year-old rootlings inoculated with selected microorganisms measured from base to the end of lignified tissue.

Treatment	Control	*R. aquatilis*	*E. intermedius*	*T. pullulans*
Scion shoot length	19.61[a]	10.24[b]	13.08[b]	13.34[b]

Figures with the same superscripts are not significantly different (P<0.05)

Regression analyses of in all 4 treatments indicated that the relationship between graft healing and shoot length was weak in the group inoculated with sterile water and moderately weak in the groups inoculated with the selected microorganisms, indicating that factors other than the quality of the graft union including the microorganism infection and possibly soil moisture, rootstock scion combination, climate and nutrition also affect shoot growth (Düring 1994).

Table 4. Results of regression analyses of scion shoot length versus graft healing in 1-year-old rootlings inoculated with selected microorganisms.

Treatment	Control	*R. aquatilis*	*E. intermedius*	*T. pullulans*
R^2 value	0.296	0.551	0.466	0.481

Conclusion and Recommendations

In this experiment neither graft healing or root numbers were affected by HWT, but scion shoot growth was apparently promoted by HWT. The effects of HWT on the physiology of dormant *Vitis vinifera* cuttings are poorly understood and it is therefore difficult to speculate on the probable cause of the increased scion shoot length in the HWT material. However the results of this experiment add to the body of evidence that the short term effects of HWT which cause retarded growth early in the growing season do not persist and are not observable by the end of the first growing season.

The results of this experiment also demonstrate that some microorganisms not normally recognized as grapevine pathogens have a negative impact on the production of sound and healthy grafted vines by impeding the healing of graft unions. The organisms tested in this experiment were originally isolated from poorly performing vines (*T. pullulans*) and aymptomatic (*R. aquatilis*) and symptomatic rootlings (*E. intermedius*) and are normally associated with untreated water. Nurseries do not always treat the water they use in propagation processes and frequently soak cuttings and pre cut buds as a means of preventing dehydration, a process which may also add to the microorganism load of the water. Consequently it seems most likely that these organisms gain entry to vine tissue during propagation, although there are also limited opportunities for microorganisms to gain entry to mature grapevines via exposure of wounds to irrigation water and rain splash. Therefore it is strongly recommended that grapevine nurseries use only treated water of potable quality and that consideration be given to stopping the practice of soaking cuttings and precut buds in order to improve grafting success and nursery profitability through reduced wastage, while improving the quality of planting material available to the grape and wine industries.

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Microorganisms Associated With Grapevine Propagating Material and Nursery Processes.

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Introduction

There have been continuing reports from the grapevine nursery industry in Australia of microbial contamination of graft unions in bench grafted vines and proliferation of surface contaminants in stored propagating material, problems that result in the loss or poor performance of planting material in the nursery and the vineyard (Hartmann *et al.* 1990). It was thought that the source of the contaminating organisms may be the cuttings themselves and/or water used in nurseries during propagation. It was also thought that some nursery processes, particularly those involving water, favour the dispersal and growth of opportunistic microorganisms that are not normally associated with disease in grapevines. Organisms commonly isolated from affected material in include a group of translucent creamy bacteria, *Phaeomoniella chlamydospora*, *Penicillium spp.* botrytis and water borne organisms not normally associated with grapevines including *Enterobacter intermedius* (Waite & Cole unpub.).

Although propagation protocols vary from nursery to nursery, there are several processes that are common to the majority of nurseries that potentially expose cuttings to contamination, including the practice of soaking cuttings in water that may or may not have been treated for varying lengths of time prior to propagation (commonly 1 to 24 hours). Some nurseries also hold precut scion buds in water during the grafting process to prevent poor graft healing caused by dehydration of the cut surface. Both these practices are likely to result in microbial contamination of wounds by exogenous organisms on the cutting bark even if clean water is used. However water that has been treated and is actively antimicrobial may inhibit the callusing process and graft healing (Hartmann *et al.* 1990).

Hot water treatment (HWT) to control surface and endogenous pathogens is also practiced by some nurseries and Vine Improvement Associations that supply cuttings to nurseries.

Cuttings are given a pre treatment soaking in cold water for up to 24 hours followed by immersion in hot water at 54^{o}C for 5 minutes or 50^{o}C for 30 minutes and then plunged into cold water again for equal amounts of time to facilitate rapid cooling. The water used in HWT plants is normally of potable quality, but is not sterile. Even if the water is treated with active chlorine before it is used, the chlorine in the HWT tank is soon dissipated by the heat or inactivated on the surfaces of the cuttings and other organic material such as leaf litter introduced with the cuttings. All these processes have the potential to add to the external and internal microorganism load of cuttings whether the source of the contamination is the cuttings themselves, or water, air, surfaces, tools or equipment used in propagation.

The work reported here endeavoured to identify the common microorganisms associated with cuttings collected from source blocks used by the vine nursery industry and those associated with HWT and to determine if there were any similarities and differences between the microorganism populations associated with cuttings and those isolated from water and thus elucidate possible sources and means of contamination. This experiment also sought to identify microorganisms from populations associated with grapevine cuttings that although not recognized as grapevine pathogens, may contribute to the loss or poor performance of cuttings or young grafted vines in the nursery and vineyard.

Materials and Methods

Propagating Material

Cuttings were collected from 18 source blocks in the Sunraysia district of north western Victoria on the 29th and 30th of May 2002. In order to sample as wide a range of source material as possible, cuttings were taken from 7 scion blocks and 11 rootstock blocks varying in size from as few as 5 individual vines to several hectares. In the larger blocks 1 cutting was taken from a vine in every 5th panel and in the smaller blocks, a cutting was taken from every 8th or 5th vine depending on the size of the block. Where there were very small numbers of vines, every vine was sampled. Cuttings from each block were then randomly divided into bundles of 5 cuttings and 1 cutting was then randomly selected for sampling.

Sampling of exogenous microorganisms was done by swabbing a 200mm section of the cutting surface 100mm either side if the middle node of each selected cutting and wiping over

sterile PDA (potato dextrose agar) plates and incubating at 25°C for 48 hours. Following external swabbing, the swabbed section of each cutting was cut into 4 equal portions and a wood sliver taken from each section. The wood slivers were cultured on sterile PDA plates and incubated at 25°C for 3 days. Microorganisms were identified by microscopic examination of the incubated plates.

Water Samples

Samples of water (1L) were taken from the hydrating tank, hot water tank and cool down tank at a hot water treatment facility in the Sunraysia district of north west Victoria. The water samples were filtered through a 0.22µmembrane which was then transferred to sterile PDA plates and incubated at 25°C for 48 hours. Microorganisms were identified by microscopic examination of the incubated plates.

Results and Discussion

Microorganisms Associated With Propagating Material

There most common microorganisms found on cutting surfaces were a group of translucent creamy coloured bacteria, a translucent yellow bacterium (*Enterobacter cloacci*), a yeast (*Rhodotorula glutinis)* and 3 species of other fungi, *Penicillium italicum, Penicillium digitatum* and a *Cladosporium sp.* (Table 1).

The most widespread of all the microorganisms isolated found on all varieties and source areas sampled were *P. italicum* and the *Cladosporium* sp. *Penicillium italicum* was particularly abundant and the numerically dominant organism on the majority plates. Although it was not tested in this survey, the most likely source of the *Penicillium* inoculum found on the cutting samples is air borne spores from the citrus orchards that are dispersed throughout the Sunraysia district. Both *P. italicum* and *P. digitatum* are common wind borne fruit decaying pathogens in citrus orchards where they can be found sporulating on the abundant fallen and damaged fruit (Hall *et al*. 1989; Mayers & Persley 1982). *P. digitatum* and *P. italicum* are also able to grow at quite low temperatures (0-7°C) and cause spoilage of citrus fruit and other food products in storage (Pitt & Hocking 1997). Anecdotal evidence also indicates that infection of stored grapevine planting material by *Penicillium spp*. may contribute to failure or poor performance. During an investigation by the author of a previous

case of failed grapevine planting material an inspection of one-year-old dormant vines from the same source held in cold storage conditions similar to those of the failed material (approx. 5^{o}C) revealed an infection of a *Penicillium sp.* (probably *P. italicum*) so abundant that it completely covered every vine. It was concluded that in this case, the *Penicillium* infection was a contributing factor in the failure of the vines.

Cladosporium spp. particularly *Cladosporium herbarum,* are very abundant and widespread wind borne environmental saprophytes, or weak parasites, frequently found on the phylloplane and in dead organic matter (Hall *et al*. 1989; Dix & Webster 1995). *C. herbarum* is exclusively saprophytic (Carlile *et al*. 2001). Like a number of other fungi commonly found on the phylloplane the spores of *C. herbarum* are protected from ultraviolet damage by the melanin in their walls which enables them to survive the relatively exposed and somewhat hostile conditions of leaf and stem surfaces (Carlile & Watkinson 1994). The manner of spore dispersal in *Cladosporium* that is favoured by dry conditions explains the widespread occurrence of *Cladosporium* on grapevines in the Sunraysia district where the climate is dry and drip irrigation is favoured over sprinkler irrigation.

Rhodotorula spp., particularly *R. mucilaginosa* and *R. glutinis*, are also common inhabitants of the phyllosphere and are occasionally implicated in the spoilage of fresh fruit. Like the *Penicillium spp., Rhodotorula spp*. are able to grow at temperatures well below the usual storage temperatures of 1-2^{o}C for grapevine cutting material and survive heat treatments of food processing and could be a contributing factor in the deterioration of cuttings in cold storage in some instances (Pitt & Hocking 1997).

Of the less common fungi isolated from the surfaces of the cuttings the most abundant were *Alternaria* sp., *Rhizopus* sp., *Cephalosporium* sp. (*Acremonium* sp.) and *Trichoderma* sp. (Table 1). *Alternaria, Rhizopus* and *Cephalosporium* species are widely distributed fungi found on the surfaces of numerous plant species. *Alternaria* spp. are able to grow in low oxygen atmospheres (Pitt & Hocking 1997) and are associated with post harvest diseases in fruit (Beattie *et al.* 1989). *Rhizopus stolonifera*, a widely distributed species frequently found on plant surfaces and most likely the species isolated in this experiment, is a necrotroph that gains entry to plants through wounds and breaks down the tissue as it advances through the host (Carlile & Watkinson 1994).

Identification of the creamy translucent bacterial complex isolated from the exterior of the cuttings was problematic, but the majority of endophytic bacteria isolated from grapevines are gram negative species, predominantly *Pseudomonas* spp. and *Enterobacter* spp. (Bell et al. 1995). *Agrobacterium vitis* is also relatively common, particularly in rootstocks (Burr *et al.* 1989). Endophytic isolates identified in the course of this study have included *Pseudomonas putida*, *Agrobacterium* spp. and *Enterobacter* spp. *Agrobacterium vitis* is the causative agent of grown gall a disease that causes the formation of soft tumours of disordered tissue on the trunks and roots of grapevines (Burr *et al.* 1989). As the name suggests, *Enterobacter* spp. are common inhabitants of the gut in warm-blooded animals and are not normally pathogens unless the immune system of the host is suppressed. *Enterobacter* spp. are widespread and commonly found in sewage and soil and water contaminated with sewage (Tortora *et al*. 2001). The presence of *Enterobacter* spp. in and on grapevine propagating material suggests that the material has been exposed to water contaminated with soil or sewage, probably as irrigation water or untreated water used in propagation. *Pseudomonas* spp., some of which are plant pathogens, are widely distributed in soil and water and plant material (Agrios 2005). Bell *et al.* (1995) isolated at least 7 *Pseudomonas* spp. from grapevines in the Annapolis Valley in Nova Scotia and concluded that grapevines, like many other plant species are asymptomatic hosts of a wide range of bacteria and suggest that one of the reasons for such a wide range of endophytic bacteria is the common method of striking vine cuttings by inserting the cut ends directly into the soil. Since grapevine cuttings are frequently left lying on the ground before they are transported to the nursery and are often exposed to soil and untreated water during the propagation process it is not surprising that these organisms are frequently associated with grapevine propagating material.

The range of microorganisms isolated from the internal tissue of the cuttings reflected those isolated from the surfaces of the cuttings, but at much lower frequencies (Table 2) tending to confirm the hypothesis that microorganisms from the phyllosphere gain entry to grapevine tissue through wounds made during pruning and propagation.

The microorganisms isolated from the water samples (Table 3) included the group of creamy translucent bacteria, *P. italicum, P. digitatum*, a *Cladosporium* sp., a *Rhodotorulua* sp. and a *Fusarium* sp. (Table 3) all of which were found in or on the cutting samples indicating that

water used in HWT plants is a potential source of contamination. It is interesting to note that although neither *Penicilium* spp. or *Cladosporium* were found in the HWT tank, *P. italicum* and *Cladosporium* were found in the cool down tank. It is possible that the high temperature of the HWT tank controls these organisms, but that recontamination occurs via wind or dust borne spores since the HWT plant is operated in the open air and surrounded by unmade road surfaces. Regular sampling of water and cuttings before and after treatment would need to occur over the period of operation of the HWT plant for this to be confirmed.

Table 3. Microorganisms isolated from the hot water treatment plant

Tank	Translucent bacteria	*Penicillium italicum*	*Penicillium digitatum*	*Cladosporium* sp.	*Fusarium* sp.	*Rhodotorula* sp.	Other yeasts and bacteria
Hydrating tank	present	present	present	present	absent	present	present
HWT tank	present	absent	absent	absent	present	present	present
Cool down tank	present	present	absent	present	absent	present	present

The frequent association of the epiphytic microorganisms including *Penicillium* spp., *Rhodotorula* spp. and *Alternaria* spp., identified in this investigation with post harvest decay of other crops (Beattie *et al.* 1989; Carlile & Watkinson 1994) suggests that some of these organisms may also contribute to post harvest decay in stored grapevines and grapevine cuttings if storage conditions favour their growth. The surfaces of cuttings held in storage are frequently saturated as a result of hydration and HWT and consequently there is enough moisture to enable spores to germinate if the cool room is not well managed and storage temperatures are favourable to germination and growth of microrganisms.

The role of these endophytic bacteria in grapevines is uncertain (Bell *et al.* 1995). However the presence of the creamy translucent bacterial complex, *P. italicum, P. digitatum*, a *Cladosporium* sp., a *Rhodotorulua* sp. and a *Fusarium* sp. in water used in the HWT plant suggests that there are frequent opportunities for these organisms to gain entry to the vine tissue through wounds made during propagation. The experiences of the authors over several years with failed cuttings and planting material adds weight to the hypothesis that some of these bacteria and some fungi that are not normally associated with disease symptoms may contribute to the poor performance of cuttings and planting material if the host is stressed by poor handling, propagating and storage practices, particularly if water saturated tissue is subjected to low oxygen environments and temperatures favourable to fungi and bacteria. In the experience of the authors, commercial cool room temperatures are often higher than the recommended 1-2^{o}C and frequently fluctuate with the opening and closing of the door. In the light of this evidence, and the evidence of Crocker *et al.* (2002) that indicates hydration is not

necessarily beneficial to cutting development, and the evidence of Waite *et al.* (2005) that the practice of rapidly cooling HWT cuttings in water could be discontinued, the practice of hydrating cuttings, holding buds in water and other practices that expose cuttings to water must be questioned.

Recommendations

In the light of the evidence presented here and the experience of the authors it would be prudent for nurseries to avoid the use of water in propagation, particularly if it is not treated to control water borne microorganisms. We recommend that the practice of subjecting cuttings to a pre HWT hydration period be abandoned, as should the practice of holding cut buds in water. Further commercial trials to assess the value of post HWT cooling of cuttings should be undertaken with a view to also abandoning this practice. It would also be prudent for nurseries to allow saturated cuttings to drain sufficiently to reduce surface moisture before placing them in cold storage to reduce the chances of the proliferation of epiphytic microorganisms.

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Table 1. Identity and frequency of epiphytic microorganisms isolated from grapevine cuttings expressed as a ratio of infected to uninfected culture plates.

ID number	Variety	Transluce nt bacteria	*Entero-bacter cloacae*	*Pen. italicum*	*Pen. digitatum*	*Clado-sporim* sp.	*Asp.* sp.	*Rhizo* sp.	*Fusarium* sp.	*Alt.* sp.	*Trichoder ma* sp.	*Cephalospor ium* sp.	*Rhodotorul a* sp.	Black yeast	other
1	140 Ruggeri			1	1	0.25	0.25			0.25					0.5
5	140 Ruggeri	1		0.2	0.9	0.8				0.2	0.7		0.6	0.7	
10	140 Ruggeri	0.29	1	1	0.71	0.43		0.43		0.29			0.29	0.14	
14	140 Ruggeri		0.57	1	0.43	1				0.29			0.29	0.86	
2	Kober 5BB	1	0.88	1	1	0.5				0.38			0.25	1	
6	1103 Paulsen	1	0.2	0.2	0.8	1				0.4			0.6	0.57	
12	1103 Paulsen	0.5	0.63	0.63	0.38	0.88		0.13		0.25		0.25	0.25		0.88
8	Ramsey	0.14	0.71	1		0.71		0.29					0.43	0.57	
9	Ramsey	0.25	0.75	1	0.38	0.88		0.13		0.13			0.5	0.5	
15	Ramsey		0.5	1	1	0.75				0.25			1	1	0.86
11	99 Richter	0.33	0.33	1	1	1									0.13
3	Semillon	0.92	0.46	0.88	0.77	0.46				0.12	0.19		0.12	0.73	0.15
4	Colombard	1	0.6	1	0.9	0.6									
7	Pinot Noir	1	0.7	0.7	0.73	0.9			0.03	0.47	0.1	0.1	0.7	0.23	0.07
13	Chardonna y	0.25	0.38	1	0.88	0.75		0.5	0.25	0.13			0.25	0.63	0.57
16	Chardonna y	0.04	0.13	0.57	0.22	0.48		0.04			0.09		0.39	0.74	0.83
17	Sauvignon Blanc	1	0.5	1		0.25							0.25		0.75
18	Sauvignon Blanc	1	0.43	0.75		0.25					0.25		0.25		

Table 2. Identity and frequency of endophytic microorganisms isolated from grapevine cuttings expressed as a ratio of infected to uninfected culture plates.

ID number	Variety	Translucent bacteria	*Enterobacter cloacae*	*Penicillium italicum*	*Penicillium digitatum*	*Cladosporium* sp.	*Aspergillus* sp.	*Rhizopus* sp.	*Fusarium* sp.	*Alternaria* sp.	*Trichoderma* sp.	*Cephalosporium* sp.	*Rhodotorula* sp.	Black yeast	other
1	140 Ruggeri	0.75			0.5	0.5	0.25	0.75	0.25	0.25	0.25				0.25
5	140 Ruggeri	0.09	0.09					0.09		0.09		0.18			0.18
10	140 Ruggeri		0.29									0.29			0.29
14	140 Ruggeri										0.14				0.14
2	Kober 5BB	0.25	0.25	0.13	0.25	0.13		0.13			0.5				0.5
6	1103 Paulsen	0.2	0.2					0.2				0.2			
12	1103 Paulsen														0.5
8	Ramsey			0.14		0.03				0.29					0.14
9	Ramsey		0.13												0.88
15	Ramsey				0.13										0.13
11	99 Richter			0.33											
3	Semillon	0.05		0.09	0.18		0.05	0.14		0.05	0.14	0.14			0.36
4	Colombard	0.09	0.09					0.09			0.09	0.09			
7	Pinot Noir	0.13													0.13
13	Chardonnay	0.13													0.38
16	Chardonnay	0.09	0.3	0.13		0.04				0.17					0.48
17	Sauvignon Blanc	1	0.5	1		0.25							0.25		
18	Sauvignon Blanc	1	0.75	0.5		0.25					0.25		0.25		0.75

THE INFLUENCE OF EXOGENOUS AND ENDOGENOUS BACTERIA IN YOUNG GRAPEVINE DECLINE

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Introduction

There have been numerous reports since the mid to late 1990's of failure of young vines in Australia and overseas particularly in the USA and Europe. These reports have coincided with the growth of the wine industry resulting in a general shortage of quality material. Some of these failures have been attributed to hot water treatment, or the presence of *Phaeomoniella chlamydospora,* however the researchers are aware of a number of instances where the cause of failure could not be attributed to either of these causes (Figure 1). Bacteria thought to be involved with graft failure and those species isolated from grapevine sap



Figure 1. *P.chlamydospora* infected



Figure 2. Bundles of canes being removed from HWT

Materials and Methods

Vines that show decline in the first year and nursery stock where the graft has failed have been cut transversely into pieces approx. 1 cm in length. These have been surface sterilised and placed cut-end onto Nutrient Agar. Stem sections from cuttings and rootlings that have been subjected to 30 mins at 50^{o}C hot water treatment (HWT) (Figure 2) and then held in cool rooms before planting have been treated similarly as have xylem samples from mature vines with visual symptoms of *P. chlaymdospora*.

Results

A gelatinous, highly aromatic bacterium was regularly isolated from these samples, but *P. chlamydospora* was rarely isolated. Microscopic examination showed the bacterium oozing from individual vessels in xylem tissue

DISCUSSION

This bacterium is isolated consistently from transverse stem sections of affected scions and rootstocks and mature vines all showing browning of the xylem tissue normally associated with *P. chlaymdospora* but where *P. chlaymdospora* is rarely isolated. The frequent isolation of this bacterium from poor quality nursery stock and vines that have failed or performed poorly in the field, but not from apparently healthy material, suggests that it may be pathogenic whether in its own right, or a synergist with other xylem infecting pathogens. Preliminary investigations from the hot water treatment project [SAR 99/4] suggest that both unchlorinated cold water bathes and HWT tanks could be a potential source of distribution of this bacterium from the phylloplane to the internal

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MONASH UNIVERSITY

Appendix 5: OUTCOMES & RECOMMENDATIONS FROM THE PROJECT

- Since little is known of the effects of the bacteria and fungi isolated during this research on graft healing, root initiation and the long term health of vines when they infect the wood it would seem prudent to take steps to prevent the introduction of these organisms to the vine tissue by reducing the exposure of cuttings to contaminated water.
- The practice by some nurseries of raising the temperature of the HWT tank to 70^{o}C at the end of the day may control some of the contaminating micro organisms but is unlikely to eliminate them entirely.
- It would be prudent for nurseries to reduce the exposure of cuttings and young rooted vines to potential contamination from untreated water.
- It is recommended that nurseries actively treat the water they use particularly rain water and water sourced from irrigation supplies and that hydration be eliminated from the protocols.
- The results of this experiment add to the body of evidence that the short term effects of HWT that cause retarded growth early in the growing season do not persist and are not observable by the end of the first growing season.
- It is strongly recommended that grapevine nurseries use only treated water of potable quality and that consideration be given to stopping the practice of soaking cuttings and precut buds in order to improve grafting success and nursery profitability through reduced wastage, while improving the quality of planting material available to the grape and wine industries.
- In the light of the evidence presented here and the experience of the authors it would be prudent for nurseries to avoid the use of water in propagation, particularly if it is not treated to control water borne microorganisms.
- We recommend that the practice of subjecting cuttings to a pre HWT hydration period be abandoned, as should the practice of holding cut buds in water. Further commercial trials to assess the value of post HWT cooling of cuttings should be undertaken with a view to also abandoning this practice.

- It would also be prudent for nurseries to allow saturated cuttings to drain sufficiently to reduce surface moisture before placing them in cold storage to reduce the chances of the proliferation of epiphytic microorganisms.

APPENDIX 6: STAFF…….

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Appendix 7: INTELLECTUAL PROPERTY ……

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