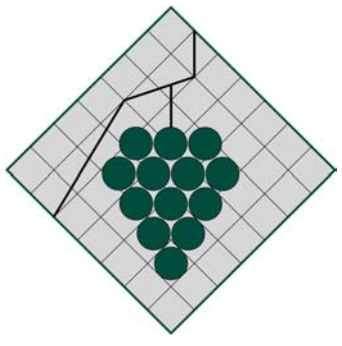


Phomopsis/grapevine disease interaction; a molecular investigation

FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CRV 96/2**

Principal Investigator: **Dr D Melanson**

Research Organisation: **Cooperative Research Centre for Viticulture**
Date: **1999**

FINAL REPORT - 1999

Project Title	*Phomopsis/grapevine* disease interaction; a molecular investigation
Project Code	CRV 96/2
Organizations	SA Research and Development Institute (SARDI) Cooperative Research Centre for Viticulture (CRCV) -
Location	Waite Precinct, Glen Osmond, SA

Principal Investigator Dr Dara L Melanson,
(Formerly Whisson and formerly with SARDI)

Current Address Oregon State University
Columbia Basin Agricultural Research Centre
P.O. Box 370, Pendleton, OR 97801 USA

Telephone: 1-541-278-4410 Fax: 1-541-278-4188

E-mail: dara.melanson@orst.edu

Research Staff Miss Belinda Brant
Technical Officer Grade 1

Funding agencies Grape and Wine Research and Development Corporation CRC for Viticulture
-50% final year only

Summary

Phomopsis viticola, which causes Phomopsis cane and. leaf spot disease, is now recognized as a widespread pathogen of grapevine in Australia. Studies overseas have demonstrated that this fungus is responsible for both seasonal decreases in yield and gradual decline of grapevine productivity and subsequent yield losses. Difficulties in controlling the disease can, in part, be attributed to a lack of understanding about the biology and taxonomy of *Phomopsis.* This includes fundamental knowledge of whether toxins are involved in symptom expression, extent of genetic and 'pathogenic variation, whether the fungus is systemic or not, rate and location of growth, induction of pycnidia and perithecia, whether the new inflorescences are infected and its effect on next year's growth. In addition, in Australia, two distinct taxa of *Phomopsis,* taxon 1 and taxon 2 (Merrin *et al,* 1995), commonly infect grapevines, and two others occur but are rare. Taxon 2 causes the same symptoms displayed by *P. viticola* in other parts of the world, which include lesions and bleaching of canes and small dark spots on leaves that are surrounded by yellow halos. However, taxon 1 infection only becomes apparent in late winter as bleached canes with tiny black spots. No symptoms are displayed on the green shoots or leaves during the growing season.

Whereas some information on the biology of taxon 2 (synonymous with *P. viticola* overseas) is available from previous studies conducted here and overseas, very little is known about taxon 1. Part of the problem is the difficulty in monitoring infection and growth of the fungus in green tissue, which is a symptomatic, with symptoms only visible in late winter as pycnidia or perithecia on bleached canes. In addition, the bleaching symptom may be confused with that caused by organisms other than *Phomopsis* and confirmation of the disease requires lengthy incubation of the canes to produce characteristic spores. These factors make the infection process, disease development, yield loss and long-term health and productivity of an infected grapevine difficult to monitor. In order to develop timely and effective control strategies with reduced chemical input it is imperative to have a good understanding of the biology of the pathogen and the effect of the disease on the productivity of the grapevine. Little was known about *Phomopsis* taxon 1 until the work of Merrin *et al* (1995) and Ms. Reiny Scheper, a former PhD student with the CRCV. The first characterized the morphology and distribution of the fungus and Ms. Scheper identified a molecular marker specific to taxon 1 and learned more about the life cycle with the discovery of a sexual phase (perithecia). The research reported here expanded knowledge of both taxa but mainly concentrated on taxon 1 because of its a symptomatic nature and lack of information on its biology. The research resulted in the development of a DNA-based diagnostic assay for both taxa, which has the potential to be developed into a commercial test. In addition, a taxon 2 specific molecular marker was identified which could be used to investigate more about the biology of this fungus in Australia.

Studies on infection and colonization of *Phomopsis* taxon revealed that the fungus grows subcuticularly as a mass of hyphae and does not invade underlying cells. This provided essential information on how much tissue to remove for a DNA-based molecular test using the taxon 1 specific marker. It indicated that only thin scrapings of the upper cell layers of the cane need to be removed, avoiding the woody centre. For taxon 2 infections, the same region could be taken, as well as areas of leaf with dense spotting. A quick and reliable procedure for extracting DNA from both woody and green grapevine canes was developed that was suitable for detection of *Phomopsis* in a slot blot test. Approximately 0.1 g of cane, scrapings provided 0.2 to 2 µg DNA depending on the age of the cane, whereas less than 0.05 to 1.5 µg DNA was extracted from single buds.

A taxon 2 specific DNA sequence was identified which is moderately repeated and detected genetic variation amongst the taxon 2 isolates tested. Both the taxon 1and 2 clones were used as probes in slot blot assays to identify infected canes. Because both clones are specific and recognize repeated sequences in the genomes this facilitates detection using this type of assay. Canes suspected of being infected with taxon 2 were positively identified with the appropriate clone whereas those without symptoms or suspected of taxon 1 infection were not. Similarly, the taxon 1 probe did not give a signal for DNA from taxon 2 infected canes. However, while the taxon 1 probe positively identified canes suspected of taxon 1 infection, a number of unburst buds that tested positive were collected from canes, which did not display the bleaching symptom typical of *Phomopsis* taxon 1 infection. This indicates that symptoms may not be a reliable indicator of the disease. In addition, using Southern blot analysis, the taxon 2 clones detected genetic variation between isolates of taxon 2 that originated from five different regions of Australia. Five distinct patterns were detected, which corresponded to the five regions. More isolates and enzyme/probe combinations need to be tested to confirm this association, but if further studies show a strong correlation of RFLP pattern to geographical region it may be possible to track the movement of both the disease and grapevine material.

The presence in Australia of two or more types of Phomopsis that infect grapevine has resulted in confusion over the taxonomy of *Phomopsis viticola.* In a preliminary attempt to resolve this, the taxon specific clones were used in a slot blot assay to determine whether there was any homology to DNA from Portuguese and Californian isolates of *Phomopsis.* Positive results were obtained for both taxon 1 and 2 isolates from Portugal using the appropriate clones, whereas no signal was obtained for the Californian grapevine or rose isolates. This indicates that taxon 1 and 2 *Phomopsis* found in Australia could be same as those found in Portugal and may, then, have originated from Europe. The lack of homology with *Phomopsis* of grapevine in California indicates that another type, or possibly species, of *Phomopsis* infects grapevines in the USA. However, since only one sample of grapevine *Phomopsis* DNA was provided, additional DNA extracts or isolates need to be obtained to verify this. Molecular genetic studies using the taxon 2-specific clone together with other markers, such as micro satellites, should be able to resolve this and provide information on the evolution of the fungus.

The field trials of the efficiency of fungicide treatments were inconclusive. Additional research on fungicide sensitivity of conidia of both taxa and ascospores of taxon 1 is required. Moreover, the question of whether taxon 1 infection causes yield loss has not been resolved. Field trials established in the Adelaide Hills showed there was no significant difference between the percent budburst of infected versus control (visibly not infected) of spur and cane pruned vines. Similarly, shoot length did not vary considerably between treatments. However, a number of unburst buds that tested positive in the DNA-based slot blot assay were collected from canes, which did not show bleached symptoms typical of *Phomopsis* taxon 1 infection. This indicates that symptoms may not be a reliable indicator of the disease and could be the reason why the field results were not conclusive.

In conclusion, a molecular assay was developed that provides a sensitive method for detecting *Phomopsis* in grapevine tissue independent of visual assessment of symptoms and should be incorporated into a testing service available to growers. In addition, both the taxon 1and 2 clones together with traditional pathological techniques should be used to obtain more information about the evolution of these fungi. Such information is invaluable to the development of more effective disease control with reduced chemical input.

Background

Phomopsis viticola is now recognized as a widespread pathogen of grapevine in Australia. Formerly considered a minor problem, the disease has become more evident in recent years, especially in the cooler wetter grape growing regions, concomitant with the vast increase in vineyard plantings. There is some confusion on the taxonomy of *Phomopsis* of grapevine in Australia, as two taxa have been found to commonly Infect grapevine. *Phomopsis* taxon 2 causes severe symptoms attributed to *P. viticola* in other parts of the world, and includes leaf distortion, stunting of shoots, reduced budburst and infection of mature berries. Reduced bunch set with subsequent losses in yield of 20-38% have also been recorded overseas (Pseicht and Pearson, 1989). Taxon 1 infection, which occurs mainly in the cooler regions, is asymptomatic during the growing season and only manifests itself in late winter as bleached canes with tiny black spots. Since *Phomopsis* does not have the dramatic disease effects demonstrated by other diseases such as powdery mildew it has not, until recently, been perceived as a significant problem. However, in the USA it is now recognized that the disease caused by *P. viticola* (= taxon 2) can cause both a seasonal decrease in yield and an overall decline in grapevine productivity over time. The short and long term effects of taxon 1 infection are unknown.

As yet, very little is known about the biology of *Phomopsis* taxon 1. Part of the problem in studying the disease is the difficulty in monitoring infection and growth in the green tissue, which can be a symptomatic, with symptoms only visible in late winter as pycnidia on bleached canes. Such latent infection is known for other *Phomopsis* species (Daykin and Milholland, 1990; Igoe, *et al,* 1995; Velicheti *et al,* 1993; Williamson *et al,* 1991). Since the bleaching, symptoms may be caused by organisms other than *Phomopsis;* confirmation of disease diagnosis requires incubation of canes to induce exudation of characteristic spores, which can take 4-6 weeks. These factors make the infection process, disease development, yield loss and long-term health and productivity of an infected grapevine difficult to monitor. In order to develop timely and effective control strategies with reduced chemical input it is imperative to have a good understanding of the biology of the pathogen and the effect of the disease on the productivity of the grapevine.

Objectives

1. To examine, histologically, the mode of entry of *Phomopsis* taxon 1 into the grapevine shoots and follow its colonization through the stem.
2. To use *Phomopsis-specific* DNA probes to identify the taxon and growth of the fungus in young shoots during the symptomatic phase.
3. To develop a sensitive and accurate DNA-based assay to identify *Phomopsis* infected grapevine canes.
4. To assist Dr. Wicks, SARDI, in field trials to determine the effect of different fungicide treatments on the yield of grapes and development of the disease in the following season.
5. To optimise conditions for the maintenance and long-term storage of isolates of *Phomopsis* and to establish a culture collection.

<u>New (modified) Objective (November 1997)</u>

1. To identify a molecular marker specific to *P. viticola* taxon 2.

Introductory Technical Information -problem/research need

At the present time identification of *Phomopsis* relies on symptoms such as bleaching which can be caused by a number of pathogens. Lesions produced by taxon 2 infection may also be difficult to diagnose. In order to confirm the disease, canes must be incubated for periods of up to three weeks in order to produce characteristic spores. The entire process is lengthy and can be inaccurate, which makes on-going field studies difficult and does not provide adequate or timely feedback to the grower. The advent of molecular techniques in conjunction with traditional technologies now makes it possible to investigate the *Phomopsis/grapevine* interaction in a more precise and direct manner. In particular, these tools are especially useful in monitoring a symptomatic or latent infections demonstrated by *Phomopsis* and other pathogens. An improved understanding about *Phomopsis* biology will help to predict disease and this will enable a more strategic approach to management, resulting in reduced chemical use with greater effectiveness. Therefore, the application of *Phomopsis* specific DNA markers will provide (1) the means to determine whether this fungus is responsible for delayed bud, burst, bud death and subsequent yield loss and (2) a tool for rapid identification of diseased vines and planting material.

Research Methodology

The methodology was changed from the original proposal due to the addition of a new research objective.

(A) Histological examination of *Phomopsis* infection and colonization of grapevine stems

Micropropagated grapevines were established as previously described (Evans et al, 1997) and the lower portion of each stem was inoculated with a 2 µl suspension (1 x 105 conidia/ml) of taxon 1 conidia prepared from a single cirrhus that had formed on a PDA (potato dextrose agar) plate. The plantlets were returned to the incubator for 5 days. Growth of the fungus inside the tissue could then be followed from the point of inoculums. Stems were hand-sectioned at various positions above the inoculation points and the pieces stained using KOH-aniline blue fluorescence (Hood and Shew, 1996). The purpose of this experiment was to determine which cells were colonized by taxon 1 and how the fungus spreads up the grapevine stem during the a symptomatic phase of its life cycle. It also provided information on what and how much cane tissue to remove for a DNA-based detection assay.

(B) DNA extraction from grapevine canes

Several methods of extracting DNA from grapevine canes were explored and assessed for yield and purity, as well as ease of use. Total DNA was extracted from thin slivers of cane removed with a scalpel blade. Approximately 0.1 g of cane tissue was ground to a fine powder in liquid nitrogen and brushed into a buffer modified from Rezaian and Krake (1987) and Thomas *et al* (1993). Contaminating proteins and polysaccharides were removed and the DNA was, precipitated with ethanol, dried and suspended in Tris-EDTA (TE) buffer. It was essential to develop an efficient method of extracting good quality DNA in order to accommodate large numbers of samples that could be used to detect the fungus in a molecular assay.

(C) Development of a *Phomposis* taxon 2 specific DNA marker

Total DNA from a taxon 2 isolate was digested with a restriction enzyme and ligated into a plasmid vector. The ligated DNA was used to transform competent bacterial cells using established techniques and colonies containing recombinant DNA were selected and the clones tested for specificity and copy number. One clone, which was moderately repeated and showed specificity to taxon 2 DNA, was selected for further analysis of taxon 2 isolates from different regions of Australia and its possible use in a DNA-based diagnostic assay. The development of a taxon 2 specific DNA probe was requested by the former Director of the GWRDC as it could be used in other GWRDC and CRCV funded projects to monitor the effectiveness of chemical treatments. It would also complement the research previously carried in this laboratory, which resulted in the identification of a taxon 1 specific marker (Ms. Reiny Scheper, former Pill student, CRCV and University of Adelaide).

(D) Detection of Phomopis taxon 1 and 2 in grapevine canes and Southern blot analysis of DNA extracted from taxon 2 isolates obtained from different regions in Australia

DNA was extracted from one-year-old grapevine canes, which displayed symptoms typical of taxon 1 infection (bleaching) and taxon 2 infection (bleaching and cracking). DNA was also extracted from buds suspected of being infected with taxon 1 *Phomopsis.* Approximately 100ng DNA of each sample was loaded into individual slots of the slot blot apparatus. DNA extracted from pure cultures of taxon 1 and 2 *Phomopsis,* as well as DNA extracted from grapevine and a number of fungi commonly found on grapevine were included as controls. This included DNA from *Botrytis cinerea, which* displays symptoms commonly confused with those produced by *Phomopsis* taxon 1. One membrane included DNA from twelve Portuguese isolates, one isolate from California and a *Phomopsis* isolate from rose. The DNA was fixed onto the membranes and hybridised with the taxon 1 and 2 specific probes according to established procedures. These experiments were conducted to determine whether *Phomopsis* could be detected in grapevine canes or buds, and whether it could be developed into a DNA- based diagnostic assay for the disease.

Isolates of taxon 2 were obtained from different regions of Australia and the DNA extracted for Southern blot analysis using the specific clone. The DNA was digested with a restriction enzyme and the fragments were separated on an agarose gel. The DNA was transferred onto a membrane and hybridised with the taxon 2 clones to detect differences in banding patterns between isolates. This was a preliminary experiment to determine whether there was genetic variation in isolates from different regions as taxon 2 is not known to have a sexual phase and would also provide an indication of the potential for pathogenic variation.

(E) Optimisation of maintenance and long term storage conditions for P. viticola isolates and the establishment of a culture collection

Various preparation methods and storage conditions were tested and include (a) drying of spores on porcelain beads used to store bacteria or yeast, (b) storage on an agar slope under mineral oil or water and (c) storage in 15% glycerol at -80°C. This was carried out to ensure a ready supply of isolates that would not change genetically thorough continued subculture on agar.

(F) Field trials to determine the effect of different fungicide treatments on grape yield and disease development in the following season

The Technical Assistant, Ms Belinda Brant, assisted Dr Trevor Wicks in applying various fungicide treatments at specified times to vineyards with known *Phomopsis* infection. Development of the disease was assessed in late winter using bleached canes and pycnidia as the indicator and in spring from the percentage budburst (including delayed). The results were compared to untreated canes. Details of the experiments may be found in progress and final reports prepared by Dr Wicks.

Detailed Results

(A) Mode of infection and colonization of *Phomopsis* taxon 1

Studies on the mode of infection and colonization of *Phomopsis* taxon 1 using micro propagated grapevine plants revealed that conidia (asexual stage) penetrated the stem directly and the fungus grew as a mass of coralloid hyphae beneath the cuticle. No symptoms were visible until pycnidia subsequently developed on the upper internodes of the plantlet. The taxon 1 fungus did not penetrate the xylem of the micro propagated grapevine plants. This mode of growth also appears to be the case for grapevines naturally infected with taxon 1. However, while *Phomopsis* taxon 2 also grows subcuticularly in the cane, it produces fissure-like lesions, which could either be due to invasion of the underlying tissue or to toxins released by the fungus. In addition to the severe symptoms on canes, taxon 2 also produces small dark spots on leaves that are surrounded by yellow haloes. We isolated the fungus more readily from these spots than the cane lesions.

(B) DNA extraction from grapevine cane

An investigation of the most appropriate method for extracting DNA from both woody and green grapevine canes resulted in the development of an inexpensive ethanol-containing buffer which does not require the use of phenol. The procedure is easy to do, only takes about 30 minutes and is carried out in small volumes using Eppendorf tubes, making it suitable for a diagnostic assay. However, subsequent tests showed that sufficient contaminants co-precipitate with the DNA to prevent binding of the problem the hybridisation step, quenching the signal. It was found that re-precipitation of the DNA with ethanol after re-dissolving the sample in 2 M NaC1 improved the signal 2.6 fold. Approximately 0.1 g of cane scrapings was routinely used to extract DNA. This provided 0.2 to 2 µg total DNA, depending on the maturity of the cane, and 0.1 µg DNA was loaded per slot, although the membrane is capable of binding 10 times this amount. Therefore it is possible to detect very low levels of this fungus by loading more of the sample. Unburst buds varied considerably in weight (0.005 to 0.2 g) as did the amount of DNA extracted (less than .0.05 to 1.5 µg) and the entire sample was loaded.

(C) Development of a taxon 2 specific DNA marker

A plasmid clone containing a 0.67 kb insert was identified as being specific to *Phomopsis* taxon 2. The clone hybridised to DNA from all taxon 2 isolates tested and did not show homology to DNA prepared from taxon 1 isolates, grapevine, *Botrytis cinerea, Uncinula necator, Aspergillus nidulans,* an unknown pycnidia-forming fungus from grapevine canes, *Hanseniaspora uvarum* (most abundant yeast on grapes) or *Saccharomyces cerevisiae.* In addition, the DNA sequence is moderately repeated in the taxon 2 genome and detected RFLPs (restriction fragment length polymorphisms) in DNA prepared from isolates from different regions of Australia.

(D) Detection of *Phomopsis* in DNA Devine tissue and Southern blot analysis of taxon 2 isolates

The taxon 1-specific clone, pT1P180, was used to detect the *Phomopsis* taxon fungus in cane samples obtained from several vineyards in Western Australia and South Australia using the slot blot assay. A dilution series of taxon 1 DNA was used as a control. No signal was obtained for DNA prepared from taxon 2 isolates, grapevine, *Botrytis, Aspergillus,* yeasts, powdery mildew or an unknown pycnidia forming fungus on grapevine. In addition, the probe readily detected the fungus in single buds that had not burst by the time the shoots were 30 cm long. Similarly, the taxon 2-specific probe, pT2P25, identified taxon 2 infection in cane samples obtained from the Coonawarra and Padthaway. The taxon 2 clone was also used to analyse pure cultures of this fungus obtained from McLaren Vale, Coonawarra and Padthaway in South Australia, Mildura in Victoria, Griffith, Wagga Wagga and Mudgee in NSW and southern Queensland. RFLP analysis of the DNA using the pT2P25 clone and the restriction enzyme, *Eco*RI, revealed five distinct banding patterns. These patterns were, associated with the geographical regions where the isolates originated.

Twelve isolates of *Phomopsis* were obtained, with an AQIS permit, from Dr Alan J.L. Phillips, Departamento de Fitopatologia, Estação Agronómica Nacional, Quinta do Marques, 2780 Oeiras, Portugal. The isolates were identified according to current concepts of *Phomopsis viticola,* and types 1 and 2, according to Merrin *et al.* (1995). The isolates were subcultured and DNA extracted. In addition, Dr Nancy Irelan, E. & J. Gallo Winery, Modesto, California, USA, provided two samples of DNA extracted from *Phomopsis* in California, one from rose-wood and the other from grape-wood. The isolates were identified by D. Fogle at the California Department of Food and Agriculture. In a slot blot assay the taxon 2-specific probe hybridised strongly to DNA prepared from all eight Portuguese isolates identified by Dr. Phillips as type 2, including three isolates identified as atypical, but not to DNA from the four type 1 isolates. The probe did not hybridise to DNA prepared from *Phomopsis* of rosewood or grape-wood obtained from California. Similarly, the taxon 1- specific probe hybridised to DNA prepared from the Portuguese type 1 isolates but not to the Californian isolates.

(E) Maintenance and long-term storage of *Phomopsis* isolates

Experiments to determine the most efficient method of storing cultures of *Phomopsis* long-term showed that Isolates can be easily maintained on an agar slope covered with mineral oil at either room temperature or 4°C (caps loose) or by placing a piece of agar containing young mycelia in sterile distilled water and storing at room temperature (caps tight). Cultures were able to be recovered after 9 months to two years storage and generally took 3 to 6 days to start growing. In addition, *Phomopsis* was recovered from a commercial product, 'Protect' bacterial preserver beads. In this case a single cirrhus was added to the fluid containing the beads and allowed to stand before removing most of the fluid and storing at -80°C. After 9 months a bead was removed, placed on agar and incubated to promote fungal growth. The fungus was also recovered from a suspension of conidia (single cirrhus) in Luria Broth diluted 1:1 with 30% glycerol (final, 15%). Isolates took a minimum of 6 days to start growing.

(F) Field trials to determine the efficacy of fungicide treatments

Miss Brant assisted Dr Trevor Wick's research group in the evaluation of fungicides for the control of *Phomopsis* cane and leaf blight. Detailed results are presented in reports prepared by Dr. Wicks but, essentially, the results were inconclusive.

Discussion

The major aims of this project were to learn more about the infection and colonisation process of *Phomopsis* taxon 1 and develop a DNA-based diagnostic assay to detect *Phomopsis* in grapevine canes. In order to fulfill this objective it was necessary to determine where and how the fungus colonizes the cane, in order to target what part of the cane should -be sampled and how much. In addition, an investigation on the best method of extracting DNA from grapevine cane was required to obtain high yields of good quality DNA that could be used in a slot blot assay. One of the problems with a slot blot system is that contaminants in the DNA preparation may interfere with subsequent detection by preventing hybridisation of the probe to the DNA sample fixed on the membrane (slot). A *Phomopsis* taxon 1-specific clone, previously identified by Ms Reiny Scheper as part of a CRC for Viticulture PhD scholarship, was assessed in the current project for its suitability as a probe in the assay. However, a specific probe was not available for taxon 2. Because this fungus is prevalent in the warmer regions of Australia and considered to be more destructive, a major change in the project, to develop a taxon 2-specific DNA marker, was undertaken in 1997.

Studies on infection and colonization of *Phomopsis* taxon 1 revealed that the fungus grows subcuticularly as a mass of hyphae and does not invade underlying cells. This provided essential information on how much tissue to remove for a DNA-based molecular test using the taxon 1 specific marker. It indicated that only thin scrapings of the upper cell layers of the cane need to be removed, avoiding the woody centre. For taxon 2 infections, the same region could be taken, as well as areas of leaf with dense spotting.

A quick and reliable procedure for extracting DNA from both woody and green grapevine canes was developed that was suitable for detection of *Phomopsis* in a slot blot test. Approximately 0.1 g of cane scrapings provided 0.2 to 2 µg DNA depending on the age of the cane, whereas less than 0.05 to 1.5 µg DNA was extracted from single buds. While the DNA extracted from cane was suitable for a slot blot assay of the fungus, it could not be used for RFLP (restriction fragment length polymorphism) analysis because it could not be digested with restriction enzymes. Several methods of purifying the DNA were investigated and we found that commercial kits designed to remove DNA from agarose gels are suitable. However, because the procedures involve centrifugation through a column or adsorption on beads, the DNA is sheared. This may not provide the clarity required for RFLPs but may be suitable for RAPD-PCRs, etc. (not attempted).

The taxon 2 specific sequence together with the previously identified taxon 1 specific sequence were successfully used as probes in slot blot assays to identify infected canes. Because both recognize repeated sequences in the genomes this facilitated detection using this type of assay. Canes suspected of being infected with taxon 2 were positively identified with the appropriate clone whereas those without symptoms or suspected of taxon 1 infection were not. Similarly, the taxon 1 probe did not give a signal for slots containing DNA from taxon 2 infected canes. However, while the taxon 1 probe positively identified canes suspected of taxon 1 infection, a number of unburst buds that tested positive were collected from canes which did not display the bleaching symptom typical of *Phomopsis* taxon 1 infection. This indicates that symptoms may not be a reliable indicator of the disease.

The taxon 2 clones also showed that there was genetic variation between isolates of taxon 2 collected from five different regions of Australia. Five distinct patterns were detected, which corresponded to the five regions. Although more isolates and probe/enzyme combinations need to be tested, the analysis showed that the Queensland and NSW isolates were closely related and the McLaren Vale isolates were least related to all others. The geographical identity of taxon 2 may be indicative of clonal reproduction, as a sexual phase has not been -observed for this fungus. In contrast, taxon 1 has a sexual phase and displays a high level of genetic variation (Scheper *et al,* 2000; R. W.A. Scheper, thesis in progress). If further studies I show a strong correlation of RFLP pattern to geographical region it may, not only be possible to track movement of the disease but also movement of grapevine material (cuttings, etc.).

Confusion over the taxonomy of *Phomopsis viticola* and the presence in Australia of two or more types of *Phomopsis* that infect grapevine (Merrin *et al.,* 1995) prompted an attempt to compare Australian isolates with those from overseas. DNA prepared from twelve Portuguese isolates of taxon 1 and 2 were detected by the corresponding taxon 1 and 2 specific Australian clones whereas no signal was obtained for the one grapevine isolate from California or the rose isolate, also from California using the slot blot technique. The results indicate that *Phomopsis* taxon 2 in Australia could be the same species as found in Portugal and is different from the Californian grapevine *Phomopsis.* However, since only one sample of grapevine *Phomopsis* DNA was provided from the USA, additional DNA extracts or isolates need to be obtained to verify this. It also appears that taxon 1 isolates in Australia could be the same species as those found In Portugal. It suggests that *Phomopsis* infecting grapevines in Australia may have originated from Europe. Molecular genetic studies using the taxon 2-specific clone together with other markers, such as micro satellites, should be able to resolve this and provide information on the evolution of the fungus.

The lack of homology with *Phomopsis* of grapevine in California indicates that another type, or possibly species, of *Phomopsis* infects grapevines in the USA. While two taxa are common in Australia, two others have been isolated from grapevine (Merrin *et al.,* 1995) and in Taiwan another *Phomopsis* of grapevine has been identified (Kuo and Leu, 1998). In addition, there are reports that a number of species of *Phomopsis* are not host restricted, infecting a number of unrelated host genera. This non-host specificity is supported by data from a molecular phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacer sequences (ITS1and ITS2) of *Phomopsis* isolates from diverse plant hosts and geographic regions (Rehner and Uecker, 1994).

Previous research on the efficacy of fungicide treatments in the field (Nair, GWRDC project DAN 94/1) determined that spring applications of Delan, Mancozeb, Tilt and Shirlan gave the best control in terms of yield, while sulphur provided some control and copper had no effect. In addition, since mancozeb and a number of other chemicals have been found to inhibit the viability of pycnidia *in situ,* it is possible that dormant sprays could lower the carry over of disease to the new season (Castillo-Pando *et al,* 1997). Further research needs to be conducted to improve the efficiency of such dormant sprays and more information on fungicide sensitivity of conidia of both taxa and ascospores of taxon 1 is required. Moreover, the question of whether taxon 1 infection causes yield loss has not been resolved. Field trials established in the Adelaide Hills showed there was no significant difference between the percent budburst of infected versus control (visibly not infected) of spur and cane pruned vines. Similarly, shoot length did not vary considerably between treatments. However, a number of unburst buds that tested positive in the DNA-based slot blot assay were collected from canes which did not show bleached symptoms typical of *Phomopsis* taxon 1 infection. This indicates that symptoms may not be a reliable indicator of the disease and could be the reason why the field results were not conclusive.

Implications and recommendations

1. The molecular assay provides a sensitive method for detecting *Phomopsis* in grapevine tissue independent of visual assessment of symptoms and should be incorporated into a testing service.

2. Given the severity of symptoms caused by *Phomopsis* taxon 2 further studies on the genetics and potential for the development of more aggressive pathotypes and fungicide resistance should be carried out using molecular and traditional pathological techniques.

3. Additional work needs to be carried out on the host range of taxon 1 and whether fungicide resistant strains are evolving. The information is essential in order to develop a more strategic approach disease control.

4. Additional work needs to be carried out on the taxonomy of *Phomopsis* given the anomalies of morphology and symptoms and the possibility of a lack of host specificity.

Intellectual property and technical information

The *Phomopsis* taxon 2 specific clone that was developed as a result of this research should be used in a DNA-based diagnostic test which would be made available to the growers and nurseries. This would reduce spread of the disease from region to region, ensure that re-planting material is free of disease and reduce costs of controlling the disease and replacing diseased vines.

References.

Castillo-Pando, M.S., NaIr, N.G., Emmett, R. W. and WIcks, T.J. 1997. Inhibition In pycnidial viability of *Phomopsis viticola* on canes in situ as an aid to reducing inoculum potential of cane and leaf blight disease of grapevines. Australasian Plant Pathology 26:21-25 and Correction (1997) Australasian Plant Pathology 26:206.

Daykin, M.E. and Milholland, R.D. 1990. Histopathology of blueberry twig blight caused by *Phomopsis vaccinii.* Phytopathology 80:736-740.

Evans, K.J., Whisson, D.L. and Scott, E.S. 1996. An experimental system for characterizing isolates of *Uncinula necator.* Mycological Research 100:675-680.

Hood, W.E. and Shew, H.D. 1996. Applications of KOH-aniline blue fluorescence in the study of plant-fungal interactions. Phytopathology 86:704-708.

Igoe, M.J., Peterson, N.C. and Roberts, D.L. 1995. A *Phomopsis* canker associated with branch dieback in Colorado blue spruce in Michigan. Plant Disease 79:202-205.

Kuo, K. and Leu, L-S. 1998. *Phomopsis vitimegaspora:* A new pathogenic *Phomopsis* from vines. Mycotaxon 66:497-499.

Merrin, S.J., Nair, N.G. and Tarran, J. 1995. Variation in *Phomopsis* recorded on grapevine in Australia and its taxonomic and biological implications. Australasian Plant Pathology 24:44- 56.

Pseicht, J. W. and Pearson, R.C. 1989. Time of infection and control of *Phomopsis* fruit rot of grape. Plant Disease 73:829-833.

Rehner, S.A. and Uecker, F.A. 1994. Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis.* Canadian Journal of Botany 72:1666-1674

Rezaian, M.A. and Krake, L.R. 1987. Nucleic acid extraction and virus detection in grapevine. Journal of Virological Methods 17:277-285.

Scheper, R. W.A., Crane, D.C., Whisson, D.L. and Scott, E.S. 2000. The *Diaporthe* teleomorph of *Phomopsis* taxon 1 on grapevine. Mycological Research, 104:226-231.

Thomas, M.R., Matsumoto, S., Cain, P. and Scott, N .S. 1993. Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification. Theoretical and Applied Genetics 86: 173-180

Velicheti, R.K., Lamison, C., Brill, L.M. and Sinclair, J.B. 1993. Immunodetection of *Phomopsis* species in a symptomatic soybean plants. Plant Disease 77:70-73.

Williamson, P.M., Sivasithamparam, K. and Cowling, W.A. 1991. Formation of subcuticular hyphae by *Phomopsis leptostromiformis* upon latent infection of narrow-leafed lupins. Plant Disease 75: 1023-1025.

APPENDIX: (Whisson = Melanson)

Conferences

D.L. Whisson, B. Brant, B.E. Stummer and R. W.A. Scheper (Aug 1998) Detection of *Phomopsis* in grapevine canes using molecular and classical techniques. Poster presentation at the 7th International Congress of Plant Pathology, Edinburgh.

D.L. Whisson, B. Brant, B.E. Stummer and R. W.A. Scheper (Oct 1997) Detection of *Phomopsis* in Grapevine Canes using Molecular and Classical Techniques. Poster (different from above) presented at the 11th Biennial Australian Plant Pathology Society Conference, Perth and the Inaugural South Australian Viticulture Technical Conference, "Staying Ahead" (Sept 1997) Waite Precinct, Urrbrae.

B. Brant, D.L. Melanson and R.W.A. Scheper (Sept 1999) *Phomopsis:* molecular detection on grapevine. Oral presentation at the 12th Biennial Australasian Plant Pathology Society Conference, Canberra.

Industry papers

D.L. Whisson, B. Brant, R. Scheper and B. Stummer (1998) Detection of *Phomopsis viticola* in grapevine cane. The Australian Grapegrower and Winemaker 417:73-75.

D.L. Whisson (1998) Phomopsis - the state of play'. Australian Viticulture -practical vineyard management. Vol 2, No.4:28-31.

D.L. Whisson, B. Brant, B.E. Stummer and R.W.A. Scheper (April/May 1998) Finding *Phomopsis* through molecular, classical techniques. GrapeGrowers, page 44.

Public presentation

Update on the current research on *Phomopsis* in the Adelaide Hills. Presented at The Adelaide Hills Grapegrowers' and Winemakers' Association Viticultural Seminar, 12 February 1998, Lenswood Research Centre.

Workshop and demonstration

Research - to - Practice™: IPM Viticulture: Research to Practice Training Workshop, 25 September 1997, The Old Mill, Hahndorf. Section 'Pest by Pest IPM' - *Phomopsis.*

Phomopsis viticola. Waite Open Day, February, 1997/98

Refereed journals

R. W.A. Scheper, D.C. Crane, D:L. Whisson d E.S. Scott (2000) The *Diaporthe* teleomorph of *Phomopsis* taxon 1 on grapevine. Mycological Research, 104:226-231.

Paper for refereed journal in preparation

D.L. Melanson, B. Brant, R. Scheper and B. Stummer (2000) Molecular detection of two Phomopsis that infect grapevine in Australia. Plant Disease, in preparation. (Delayed due to serious car accident and critical injuries sustained by D.L. Melanson)

Milestones

DATE	RESULTS
September, 1997	Technical Officer employed
	Glasshouse work and tissue culture of grapevines commenced
February, 1998	Grapevine plants and tissue culture plantlets inoculated
September, 1998	Inoculated samples processed, microscopic/histological analysis in progress
	Molecular detection of the fungus in experimental material in progress System for storing *P. viticola* isolates optimised
February, 1999	Field trials near completion
	Molecular and histological detection of fungus in grapevine stems completed
June 1999	Final report completed

All milestones were met, including the new milestone to develop a taxon 2-specific probe. In addition, Dr Melanson and Miss Brant conveyed results to the industry through industry journals and presentations as well as the scientific community at conferences and the proposed paper. Finally, the research was conducted with the cooperation of vineyard owners in the Adelaide Hills, advisers/consultants and researchers from SA and other states.