Phomopsis taxon 1 on grapevine: pathogenicity and management

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This thesis is dedicated to my sisters,
Michelle and Jodie Brant
who I miss dearly
but have given me the strength to achieve
my dreams.

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Abstract

In Australia, Phomopsis cane and leaf spot on grapevine is attributed to two taxa of *Phomopsis* on grapevine, named *Phomopsis* taxon 1 and taxon 2. *Phomopsis* taxon 2 causes symptoms similar to those observed worldwide, such as leaf spots and deep lesions on canes, but berry rot is rare in Australia. In Australia, yield loss is attributed to girdled shoots and weakened canes. In comparison, *Phomopsis* taxon 1 infection is assumed to be less damaging, although it has been suggested that taxon 1 causes failure of buds to burst, delayed bud burst and stunting of shoots. Currently, fungicides are applied to control Phomopsis cane and leaf spot in Australia, but these may be unwarranted if vineyards are infected solely by taxon 1. The pathogenicity of *Phomopsis* taxon 1 was examined in relation to symptom expression and bud loss. *Phomopsis* taxon 1-specific DNA probe, pT1P180, and taxon 2-specific probe, pT1P25, were used to detect taxon 1 and taxon 2 in infected buds, canes and shoots in glasshouse and field experiments.

Pathogenicity experiments in a glasshouse confirmed that the eleven isolates of *Phomopsis* taxon 1 examined did not cause leaf or shoot symptoms associated with Phomopsis cane and leaf spot. It was confirmed that taxon 2 is more virulent than taxon 1. Both taxa induced bleaching on cane but there was no evidence that taxon 1 caused bud death. Microscopic studies showed that taxon 1 colonised the epidermis of green grapevine shoots but not the vascular tissue.

To investigate the effect of taxon 1 on grapevine productivity, budburst, bunch count, shoot length and bleaching of canes were assessed in four vineyards in South Australia over three seasons. Taxon 1 was detected in unburst buds, canes and shoots using the taxon 1-specific DNA probe, pT1P180. This probe detected the fungus in both healthy and unhealthy

buds, and in bleached and non-bleached canes. There was little evidence that taxon 1 caused delayed budburst or bud death. Also, there was no evidence that taxon 1 caused stunted shoots or poor fruitfulness in the vineyards.

The existing DNA probe, pT1P180, was not suitable for use in a rapid diagnostic test, therefore a new taxon 1 DNA library was constructed and clones evaluated for specificity to taxon 1. Most of the clones were low copy and revealed simple banding patterns in Southern hybridisation experiments. Of the 17 isolates screened, 17 phenotypes were identified from hybridisation with nine putative taxon-specific probes. DNA from taxon 1 was amplified by PCR and two fragments, 420 bp and 900 bp, were cloned into the pGEM® T-easy vector. The clones were not suitable for use in a diagnostic assay using slot blot analysis but sequence data are available for the development of taxon-specific primers.

The studies suggest that *Phomopsis* taxon 1 is an endophyte, whereby infection does not cause harm to the grapevine. To clarify the situation in the viticultural industry, it is recommended that the terms *Phomopsis* taxon 1 and *Phomopsis* taxon 2 be replaced with the common names Diaporthe and Phomopsis, respectively. The taxa can be distinguished by monitoring for symptoms of Phomopsis cane and leaf spot during the growing season, or by examination of infected grapevine material sent to a diagnostic facility. This study suggests that chemical control is not warranted for control of Diaporthe on grapevine.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for photocopying and loan when deposited in the University Library.

Signed: **Date:** 18/11/02

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- Adelaide Hills Growers' Association information session, November 15, 2000, Macclesfield, SA.
- Cooperative Research Centre for Viticulture (CRCV) Research to Practice Integrated Pest Management (IPM) Pest and Disease™ workshop. November 14, 1999, McLaren Flat, SA.
- IAMA grower information session. 5 November 1999, McLaren Vale, SA.

Abbreviations

Amp	ampicillin
ANGIS	Australian National Genomic Information Service
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
bp	base pair
°C	degree celsius
CIAP	calf intestinal alkaline phosphatase
CLA	carnation leaf agar
cm, mm, nm, μm	centimetre, millimetre, nanometre, micrometre
CTAB	hexadecyltrimethylammonium bromide
cv	cultivar
dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytosine 5′-triphosphate
dGTP	2′-deoxyguanosine 5′-triphosphate
dNTP	2'-deoxynucleotide triphosphatases
dTTP	2′-deoxythymidine 5′-triphosphate
ddH_20	double distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediamine *tetra* acetic acid
g	acceleration due to gravity
g, mg, μg, ng	gram, milligram, microgram, nanogram
IBA	indole-3-butyric acid
IPTG	*iso*-propyl-β-D-thiogalactopyranoside
ITS	internal transcribed spacers
K_2HPO_4	di-potassium hydrogen orthophosphate
kb	kilo base
LSD	least significant difference
LB	Luria Bertani
M, mM	molar, millimolar
$MgCl_2$	magnesium chloride
min	minute
ml, μl	millilitre, microlitre
NaCl	sodium chloride
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
OD	optical density
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PVP	polyvinylpolypyrrolidone
®	registered trade mark
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNAse A	ribonuclease A
RO	reverse osmosis
Sarkosyl	n-lauroyl-sarcosine
SE	standard error
SD	standard deviation
SDS	sodium dodecyl sulphate
SCAR	sequence-characterised amplified region
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
UV	ultra violet
X-Gal	5-bromo-4-choloro-3-indolyl-β-galactopyranoside

Chapter 1

General Introduction

1.1 Introduction

Phomopsis cane and leaf spot, caused by the fungus *Phomopsis viticola*, is recognised as a widespread disease of grapevine in Australia. The identification of two major taxa of *Phomopsis* on grapevine (Merrin *et al*., 1995) has cast doubts on the correct taxonomic classification of the fungus, and whether or not each taxon actually affects the productivity of grapevine.

One of the main concerns associated with the disease is the lack of knowledge of the effects of *Phomopsis* taxon 1 on grapevine. In some areas of Australia, taxon 2 causes considerable damage to canes through the development of lesions, scarring and eventual breakage of the cane. In comparison, taxon 1 infection is assumed to be less damaging, although there have been suggestions that taxon 1 causes failure of buds to burst, delayed bud burst and stunting of shoots (Scheper *et al*., 1997a). Because of this uncertainty, some growers who suspect taxon 1 to be present in the vineyard regard this fungus to be as damaging as taxon 2.

This chapter focuses on *Phomopsis* taxon 1 and taxon 2 of grapevine. In particular, it concentrates on the effect of *Phomopsis* taxon 1 on budburst and symptom expression. Detection and identification of *Phomopsis* using molecular markers is assessed, as is the application of new techniques in the detection and monitoring of *Phomopsis* in asymptomatic

tissue. The establishment of molecular diagnostics is considered an important component in studies of the pathogenicity of *Phomopsis* taxon 1 and disease management.

1.2 The genus *Phomopsis*

The genus *Phomopsis* belongs to the order Sphaeropsidales (family: Sphaeropsidaceae), within the Deuteromycotina (Fungi Imperfecti), defined by the production of conidia in pycnidia (Alexopoulos and Mims, 1979). It is estimated there are over 40 species (Von Arx, 1970) and 400 species names (Sutton, 1980) within this genus.

Detailed classification of fungi requires knowledge of both the sexual and asexual stages. In the case of *Phomopsis* (anamorph), the teleomorph is believed to be *Diaporthe* (Alexopoulos and Mims, 1979; Von Arx, 1970). *Diaporthe* belongs to the division Ascomycotina (ascus producing), class Pyrenomycetes. Pyrenomycetes are distinguished by the production of perithecia. The Diaporthales (or Sphaeriales) consist of saprobes and plant parasites, mainly occurring on bark and wood (Hawksworth *et al*., 1983). *Phomopsis* and *Diaporthe* appear to be classified largely by host specificity and reproductive stages, but classification of species remains confusing.

Many taxa have been described solely on morphological characteristics and, for some taxa, more than one *Phomopsis* species has been recorded on the same host. At least 65 species of *Phomopsis* are plant pathogens, including *P. viticola* (cane and leaf spot of grapevine), *P. citri* (stem end rot of citrus), *P. cucurbitis* (black rot of cucumber) and *P. phaseoli* (stem canker of soybean) (Rehner and Uecker, 1994).

1.2.1 Anamorph of *Phomopsis* species

One of the features used to distinguish *Phomopsis* is the production of two types of asexual spores (pycnidiospores), alpha (α)- and beta (β)-conidia (Sutton, 1980). An intermediate form, the C-type, has been reported (Muntanola-Cvetkovic *et al.*, 1996). Only one in four species of *Phomopsis,* however, is known to produce β-conidia, making identification difficult (Rehner and Uecker, 1994). Spores are produced within the pycnidia, the walls of which are densely lined with a basal layer of conidiophores. There are two types of conidiophores: 1) straight, apically pointed, bearing hyaline α-conidia and 2) short and straight, bearing hyaline β-conidia. A combination of α-conidia and β-conidia is exuded from the pycnidium as a long curl (cirrhus), which can contain thousands of spores (Pine, 1958).

Alpha-conidia are unicellular, hyaline, fusiform, aseptate with two large oil drops (Cayley, 1923) termed guttules (Pine, 1958). Some species may contain more than two guttules, with *P. terminaliae* having four. The conidia have a central nucleus and contain mitochondria with numerous long cristae. The size of the α-conidia varies slightly, with most species in the range 5-12 μm long x 2 μm wide. However, *P. stipata,* has α-conidia of 16.5-25 μm x 2 μm (Hawksworth *et al.*, 1983).

Reddick (1914) first described the long, slender β-conidia as paraphyses, as the term has been for similar bodies in *Fusicoccum* species. Shear (1911), however, called them scoleospores to distinguish them from α-conidia. These slender, curved spores were identified as being similar to the stylospores of *Diaporthe* as described by Nitschke, (1867). Beta-conidia vary considerably in length, but most commonly are in the 12-30 μm range (Uecker, 1988).

Previously, β-conidia were not known to germinate (Alexopoulos and Mims, 1979), but recently Sergeeva *et al.*, (2001) reported the development of mycelium from β-conidia of

Phomopsis on grapevine. This is the first report of germination of β-conidia, although Pine, (1958) observed that β-spores developed lateral protrusions. It is unknown whether or not these protrusions were, in fact, germ tubes. In stained preparations, Cayley (1923) found no definite nucleus in β-conidia. The role and function of β-conidia is unclear.

The proportion of α- to β-conidia varies with the amount and type of nutrients. Pine, (1958) found that production of β-conidia differed significantly with a change in the level of carbon and, at very high levels, α-spores showed a considerable change in appearance, so much so that the original identity was lost. Beta-conidia are produced first in the young pycnidium, followed by α-conidia, yet only the α-spores are discharged (Cayley, 1923). Reddick (1914) also observed β-conidia in the pycnidium long after α-conidia were discharged.

1.2.2 Teleomorph of *Phomopsis* species

The genus *Diaporthe* is characterised by perithecia formed within a stroma embedded in the host, usually having an elongated neck (Alexopoulos and Mims, 1979). Perithecia, such as those of *D. perniciosa*, form in the deeper layers of the stroma or in the cortical tissues, either below, or by the side of the pycnidia. The necks of the perithecia arise from the same pore (ostiole) as the α-conidia, but only after conidia are exuded and the cavity wall empty (Cayley, 1923). The length of the perithecial neck depends upon environmental conditions, therefore, is not a reliable character to distinguish between species (Brayford, 1990). Within the perithecia, the wall lining is covered with paraphyses which provide a nutritive tissue for developing asci. Within the ascus, eight hyaline, 2-celled ascospores are typically produced. The ascospores do not vary greatly in size, most fall within the range 8-20 µm x 2-8 µm (Wehmeyer, 1933). At maturity, the perithecia release ascospores through the ostiole.

About 650 species of *Diaporthe* have been reported (Wehmeyer, 1933), some of which produce damaging phytotoxins e.g. *D. toxica. Diaporthe* states have been described for approximately 20% of all *Phomopsis* species (Rehner and Uecker, 1994). It has been suggested that pycnidial stages develop on living bark, whilst the perithecia develop on plant debris or dead plant parts (Wehmeyer, 1933; Cayley, 1923). This is evident for *D. helianthi* (Vukojevic *et al*., 1995) and *D. perniciosa* (Cayley, 1923).

1.3 Phomopsis cane and leaf spot disease of grapevines

1.3.1 Nomenclature of *Phomopsis viticola*

In 1880, Saccardo first described *P. viticola* as the casual agent of the grapevine disease Phomopsis cane and leaf spot. Although the fungus was originally named *Phoma viticola* (Saccardo, 1882), it was not until the genus *Phomopsis* was erected in 1909 that Saccardo transferred the name to *Phomopsis viticola.* The disease was first reported as "necrosis" (commonly known as "dead arm") in 1909, but the pathogen isolated from diseased grape material was named *Fusicoccum viticolum* (Reddick, 1909). In 1911, Shear investigated dead arm disease and found what he believed to be the ascogenous stage, *Cryptosporella viticola.* Although the cultures produced pycnidia almost identical to *F. viticolum,* Shear was unable to grow the perfect stage from the imperfect.

The disease was widely recognised as being caused by *F. viticolum* (teleomorph *C. viticola*), but Grove in England in 1917 realised Saccardo had already recorded *P. viticola* as the casual agent of the disease. In 1937, Goidanich re-examined the morphological and cultural characteristics of *F. viticoccum* and *P. viticola*, and recognised both to be the same species. Based upon the presence of two pycnidiospore types, as well as Saccardo's first description fitting that by Reddick, the older name *P. viticola* takes precedence. Further

evidence was given by the fact that Saccardo's 1915 description was based upon material collected in the same region in which Reddick was working (Pine, 1958).

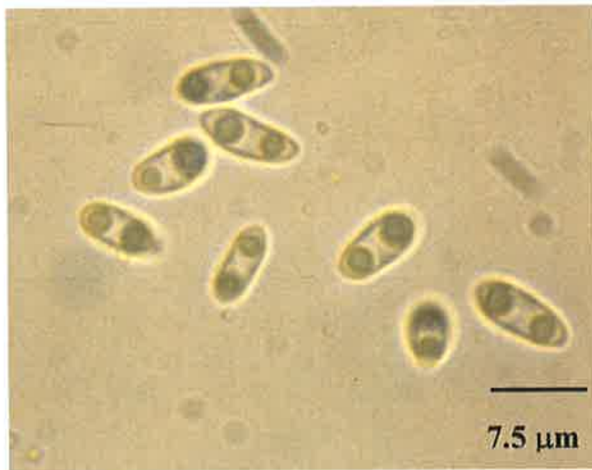
In Australia, plant disease records of New South Wales in 1935 listed *Cryptosporella viticola* (Reddick) Shear, as the cause of dead arm of grapes. The ascogenous stage of the fungus was also incorrectly reported in California (Hewitt, 1935) and Ontario (Coleman, 1928a) before Goidanich made his observations. In addition, confusion of the casual agent resulted from the name "dead-arm" which was used to describe similar diseases caused by *Sphaeropsis malorum* (Chamberlain *et al*., 1963) and *Eutypa armeniacae* (Reddick, 1914). Moller and Kasimatis (1981) showed that *E.lata* caused dead arm disease, previously attributed to *P. viticola*. Soon after, *P. viticola* was distinguished as the causal agent of Phomopsis cane and leaf spot (Cucuzza and Sall, 1982).

The taxonomy of *P. viticola* has become more complex with the report of four distinct taxa of *Phomopsis* in Australia in 1993 (Merrin *et al*., 1995). The taxa were distinguished primarily on the basis of variation in host response, pectic enzyme profile and optimum temperature for spore germination. Of the four taxa, *Phomopsis* taxon 1 fits the taxonomic description of *P. viticola* by Saccardo, but α-conidia of taxon 2 are not bi-guttulate. Six taxa associated with Phomopsis cane and leaf spot of grapevine were characterised by Mostert *et al*. (2001) and it was found that taxon 4 was most likely a *Libertella* sp. (anamorph of *Eutypa*). Differences were confirmed by separate groupings obtained in phylogenetic analyses. In Australia, only taxon 2 is known to cause the damaging symptoms of Phomopsis cane and leaf spot. In past research, however, the α-conidia of taxon 1 have been identified from infected material showing symptoms of the disease (Lal and Arya, 1982; Pine, 1958), except in cases where it was believed to be *Fusicoccum viticolum* (Shear, 1911). Questions arise as to whether the taxon 2 denoted by Merrin *et al*. (1995) is actually a taxon of

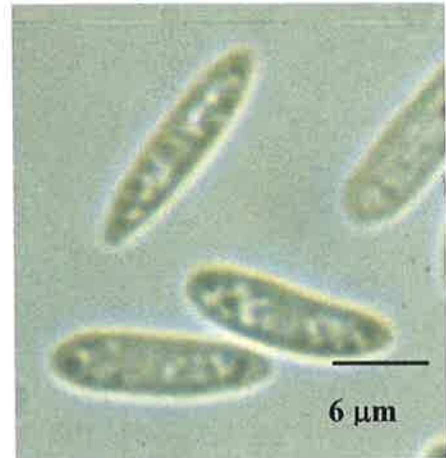
Phomopsis, or perhaps another genus altogether. The presence of β- and α-conidia may not constitute satisfactory evidence to classify taxon 2 as *P. viticola.*

Several features are used to distinguish the four taxa of *Phomopsis*. *Phomopsis* taxon 1 produces biguttulate α-conidia 4.8-7.2 μm x 1.4-2.2 μm (Figure 1.1a), white mycelium and pycnidial production occurs after 13 days *in vitro* (Nair and Tarran, 1994). In comparison, taxon 2 α-conidia are larger (8-12 μm x 2-3 μm) and not biguttulate (Figure 1.1b), resembling the description of *F. viticolum* (Shear, 1911). Growth of taxon 2 *in vitro* is slower than that of taxon 1 and cultures display prominent growth rings (Merrin *et al.*, 1995). Taxon 3 and taxon 4 are rare, with taxon 3 producing α-conidia of 6.2-8.8μm x 1.5-2.2 μm, and taxon 4 producing only β-conidia.

Diaporthe viticola was first described on *V. vinifera* by Nitschke (1867). Scheper *et al.* (2000) identified *D. viticola* as the teleomorph of *Phomopsis* taxon 1 in Australia. In controlled conditions, the irregularly shaped necks of the perithecia grew to 3 mm long, 90-110 μm in diameter with apical ostiole (Figure 1.2a). The perithecia contain numerous asci (40-58 × 7-9μm), with eight 2-celled ascospores (9.5-15 μm × 2.5–4 μm, Figure 1.2b, Scheper *et al.*, 2000). However Phillips (1999) examined *Diaporthe* species from grapevines from Australia and Portugal and determined that the name *D. perjuncta* was more suitable than *D. viticola*, but he did not propose a name for the anamorph. It is difficult to determine whether either author has correctly classified the *Diaporthe* species, given that morphology can be influenced by growth conditions. The teleomorph of taxon 2 of *Phomopsis* has not been discovered. In the present study, the terms *Phomopsis* taxon 1 and taxon 2 will be used and the perfect stage denoted as *D. perjuncta.*

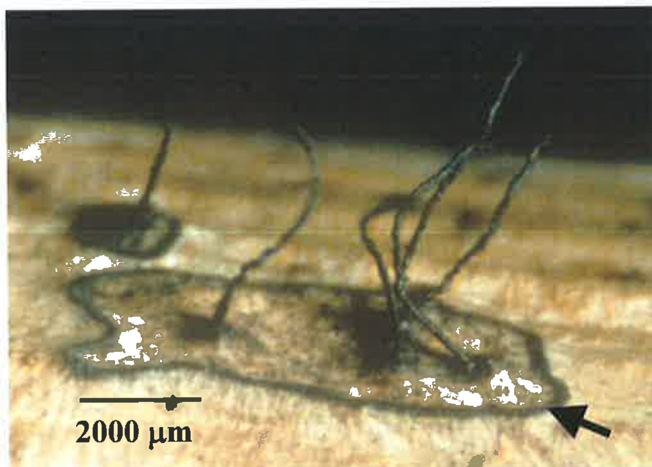


(a)

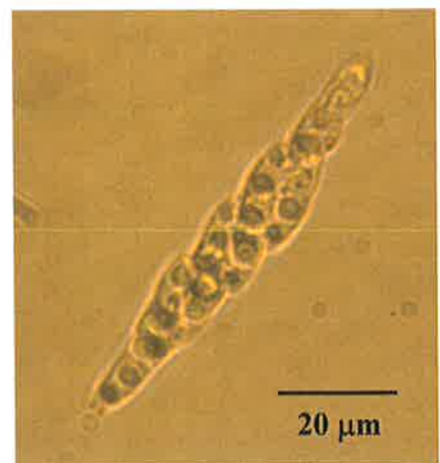


(b)

Figure 1.1. Alpha-conidia of **(a)** *Phomopsis* taxon 1 and **(b)** *Phomopsis* taxon 2.



(a)



(b)

Figure 1.2. (a) Perithecia of *Diaporthe perjuncta* surrounded by zone-lines (arrow) on the outer surface of grapevine cane. **(b)** Ascus, containing eight ascospores, from the perithecia.

1.3.2 Host range and distribution of the disease

Phomopsis viticola has been reported to infect most cultivars of *V. vinifera.* Although it has been suggested that some cultivars are more susceptible to the disease than others (Hewitt and Pearson, 1990). Coleman, 1928a reported that there was no single cultivar free from the disease. A study of susceptibility was carried out in 1973-1974 (Baltovski, 1980) and cultivars Cardinal, Afus-ali and Sultana showed a high percentage of vines infected. Further evidence is required, however, to exclude the influence of rainfall, canopy density and initial incidence of disease on susceptibility. In addition to cultivars of *V. vinifera,* the North American species *V. rupestris* (Galet and Morton, 1990), *V. labrusca* (Pscheidt and Pearson, 1989), *V. aestivales* and *V. rotundifolia* are known hosts (Uecker, 1988), as is *Ampelocissus quinequefolia.* There are no reports of infection on East Asian grapevine varieties. Although the classification of *Phomopsis* species has been based on host association, many have a broad host range (Rehner and Uecker, 1994). It is unknown if *P. viticola* is capable of infecting hosts unrelated to *Vitis* species.

Phomopsis cane and leaf spot disease is widely distributed throughout the viticultural areas of the world. The disease has been reported in the United States of America (Reddick, 1909), Portugal (Phillips, 1998), Canada (Coleman, 1928a), India (Lal and Arya, 1982), England (Grove, 1917), Italy (Goidanich, 1937), South Africa (Mostert *et al.*, 2000), Australia (Noble *et al.*, 1935), France, Africa and Asia (Hewitt and Pearson, 1990). In Australia, the disease is most common in the cooler districts of southern Australia, with records in most states except for Queensland and Northern Territory (Emmett and Wicks, 1994).

1.3.3 Disease cycle and epidemiology

The different taxa of *Phomopsis* of grapevine, recently identified, are not represented in previous lifecycle diagrams (Emmett and Wicks, 1994; Gubler and Leavitt, 1992). In 1997, Scheper *et al.* revised the disease cycles to distinguish between *Phomopsis* taxon 1 and taxon 2 (Figure 1.3). The basic epidemiology of *Phomopsis* of grapevine, however, can be described without reference to different taxa.

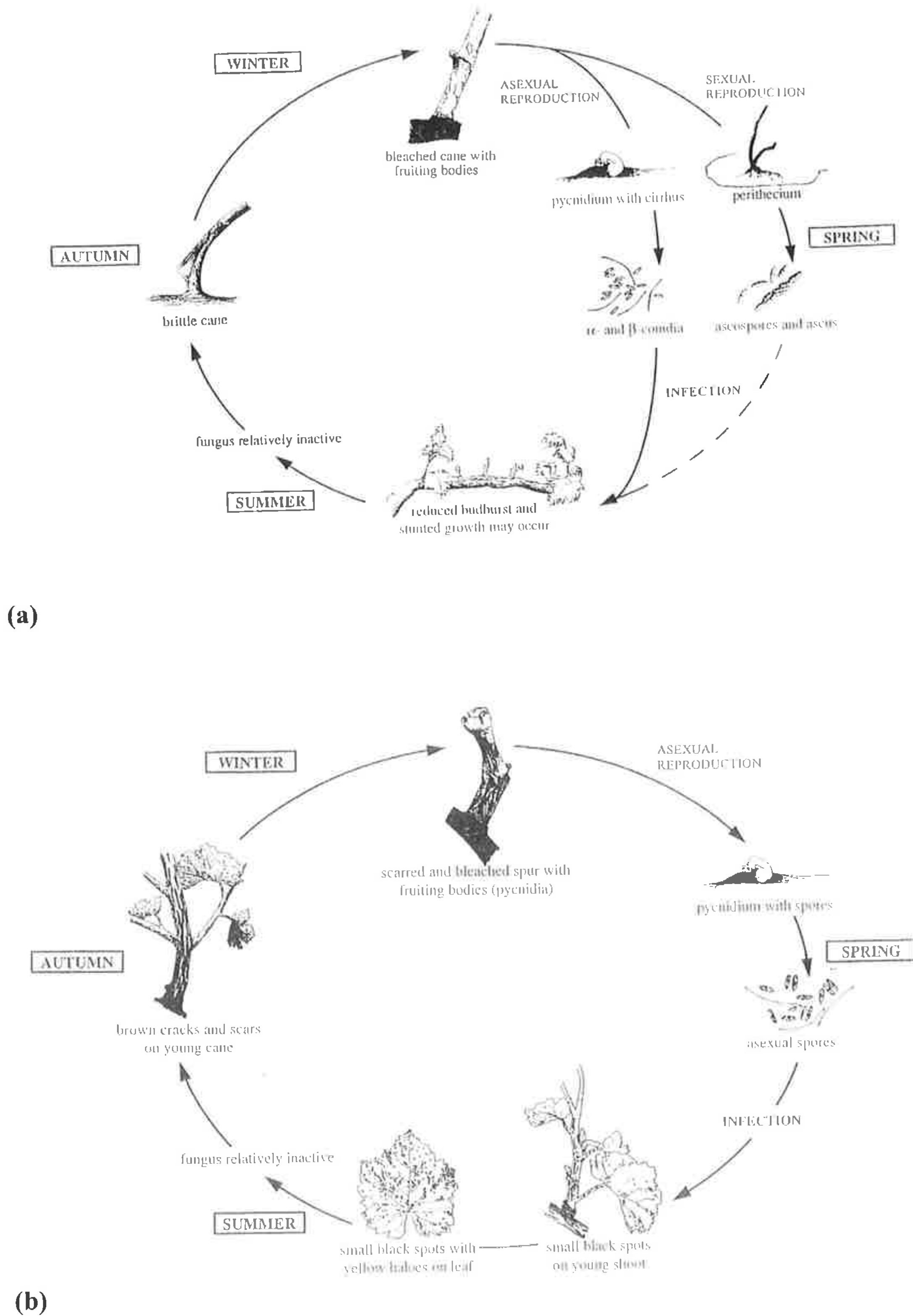
1.3.3.1 Overwintering

Phomopsis viticola overwinters as mycelium and pycnidia in bark (Hewitt and Pearson, 1990). The formation of pycnidia is a two-stage process. First the pycnidial wall is formed, then the conidiophores differentiate within the pycnidia. Pycnidium formation begins in autumn with the onset of cool weather, increasing through winter. Jailloux and Bugaret (1987) reported that mycelium overwinters in dormant buds and Mostert *et al.* (2000) isolated *Phomopsis* mostly from buds and nodes, indicating that these are probably important sites for survival.

1.3.3.2 Infection

In spring, the mature pycnidia erupt through the periderm of cane and other diseased tissue to provide the primary source of inoculum. When wet, spores are exuded in a long creamy/yellow cirrhus from the ostiole at the top of the pycnidium (Figure 1.4). A single cirrhus often contains several thousand α- and β-conidia (Reddick, 1914). The conidia are washed onto neighbouring vines or rain-splashed to other tissue areas. Infection occurs in the

Figure 1.3. Revised disease cycles of the two major *Phomopsis* taxa associated with Phomopsis cane and leaf spot of grapevine in Australia. **(a)** *Phomopsis* taxon 1 and **(b)** *Phomopsis* taxon 2 (Scheper *et al.*, 1997b).



presence of free water. The emerging hyphae can then penetrate through leaf stomata and cane lenticels, with young, succulent green shoots most susceptible to infection (Willison *et al.*, 1964). Injury from feeding by insects and mites was believed to assist colonisation of the grapevine by the fungus (Pine, 1959), however, this apparent correlation was most likely due to natural openings on the surface (Willison *et al.*, 1964).

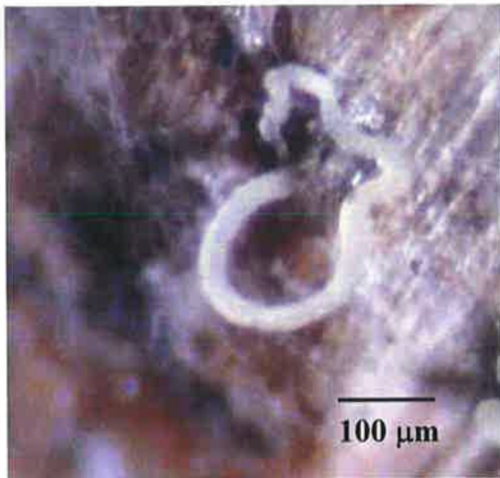


Figure 1.4. Cirrhus, containing pycnidiospores, of *Phomopsis* taxon 1 on the outer bark of grapevine cane.

Growth of mycelium permits spread of the fungus from diseased to healthy portions of the vine. Growth of the mycelium is concentrated in the cortex of shoots, petioles, tendrils and cluster stems, generally three-four cell layers beneath the epidermis. The fungus is able to develop pycnidia at new sites throughout the plant (Pine, 1958), however, the incidence and severity of disease depends on the amount of inoculum resulting from infection in the previous year (Willison *et al.*, 1964).

The fungus is found most frequently in the parenchyma cells of the host (Coleman, 1928a). Preliminary investigations by Melanson *et al.* (2002) showed that *Phomopsis* taxon 1 grows subcuticularly in the stem. Although the xylem is not affected by the presence of the fungus, the phloem becomes narrow and lignification is reduced. There is a reduction of starch production in colonised parenchyma cells or those near the invasion site, and an

accumulation of yellow-brown "wound gum", which gives infected areas a distinguishable brown colour (Reddick, 1914; Coleman, 1928a). It was thought that death of the shoot was caused by mechanical blocking of the vessels by the fungus. Cayley (1923), however, found that death is actually due to destruction of the phloem and cortical tissues, and blocking of the medullary rays. Symptoms of Phomopsis cane and leaf spot appear 21-30 days after natural infection (Hewitt and Pearson, 1990).

The fungus spreads mostly within the vine, rather than from vine to vine, therefore, spread within the vineyard is localised. Long-distance spread may occur from contamination of vineyard machinery or via propagating material such as budwood, cane cuttings and nursery stock (Hewitt and Pearson, 1990). In spur pruning, the basal nodes are retained from the previous year, thus if *Phomopsis* is present in the vine, a source of inoculum is provided for the new shoots. Thus, infection is greatest in the basal four internodes.

The fungus remains relatively inactive in summer but, with the onset of cool weather, activity resumes. In cool climates, the fungus may remain active throughout the growing season (Emmett and Wicks, 1994), but generally the most active growth of *Phomopsis* occurs during autumn and spring.

1.3.3.3 Favourable conditions

The development of Phomopsis cane and leaf spot disease is greatly influenced by weather conditions, inoculum density and host growth stage. Environmental conditions must be favourable for development and subsequent spread of the disease. Extensive studies have been undertaken to determine the conditions suitable for growth of the fungus and disease development.

Prolonged periods of rain and cold weather aid in the development of the disease. Pycnidium production requires cool temperatures. At least 10 hours of rain, combined with relatively low temperatures, are required for spores to be produced and a further 8-10 hours of moist conditions for infection to occur (Emmett *et al.*, 1992). Spores require water to germinate, and infection has been found to occur within a few hours in free water or 100% humidity (Hewitt and Pearson, 1990). The optimum temperature for spore germination and fungal growth is 23°C, with a minimum of 15°C and maximum of 35°C (Patil *et al.*, 1981). An increase beyond the optimum temperature restricts fungal growth (Emmett and Wicks, 1994). Berry infection is favoured by 20-30-hour wet periods during flowering. High levels of moisture during flowering are unlikely to occur in most viticultural regions in Australia, therefore bunch infection is rarely seen.

The inoculum density of the pathogen greatly affects the severity of the disease. Diseased material left from the previous season provides a source of inoculum. Production of pycnidia and infective α-conidia is greater during cool, weather, therefore, inoculum density is correlated with weather conditions. Because of the build up of inoculum, the disease becomes more severe with each successive cool wet spring (Hewitt and Pearson, 1990).

Symptoms are found mainly on the first three-four internodes of new growth due to pycnidia arising on 1-year-old wood. Young, developing shoots are most susceptible at budburst. As the shoot grows, the internodes further from the inoculum source are less likely to become infected (Merrin *et al.*, 1995). The effect of *Phomopsis* infection on the internal structure of the bud is unknown.

1.3.4 Symptom expression and effect on productivity

The first described symptoms of Phomopsis cane and leaf spot included failure of shoots to develop, dwarfed foliage, stunted shoots and very small leaves on one arm on the vine (Reddick, 1909). These observations were not truly characteristic of the disease, as symptoms caused by *P. viticola* were confused with those due to other pathogens in the same vine.

Phomopsis taxon 2 has been associated with yield losses of up to 30 % in Macedonia (Baltovski, 1980) and the United States of America (Pscheidt and Pearson, 1989). Although reports in Australia have indicated 20-38 % yield loss (Nair and Tarran, 1994), it is unknown whether this figure is reflective of *Phomopsis* infection only or a combination of other factors affecting grapevine. Limited information is available on the pathogenicity of *Phomopsis* taxon 1 on grapevine, however, studies by Mostert *et al.* (2000) suggested that taxon 1 may be endophytic, causing no damage to the host.

1.3.4.1 Symptoms caused by **Phomopsis** *taxon 1*

Phomopsis taxon 1 is distinguished from taxon 2 by having α-conidia with two guttules. This is important to consider because most reports of Phomopsis cane and leaf spot disease do not mention the isolation of taxon 1 spores from infected material. Thus symptoms described are more likely to result from other pathogens or taxon 2 infection. In Australia, *Phomopsis* taxon 2 is assumed to be the most damaging, and the effects of taxon 1 on grapevine are unclear.

In winter, vines infected with *Phomopsis* taxon 1 are speckled with pycnidia on bleached cane (Figure 1.5). As pycnidia mature, they erupt through the epidermis layer of the cane, causing the bark to lift up (Pine, 1958). The cane admits air underneath the epidermal

layer, thus giving the surface a white sheen appearance (Hewitt and Pearson, 1990). Bleaching caused by *Phomopsis* is extremely white, unlike the grey-powdery appearance of *Botrytis* infected cane. Pycnidia of *Phomopsis* taxon 1 resemble those of taxon 2, therefore only assessment of spore morphology can distinguish the two.

Figure 1.5. Bleaching of grapevine spur caused by infection of *Phomopsis* taxon 1. Pycnidia are visible as small black spots (arrow).

A unique feature of taxon 1 is the presence of the sexual stage (*D. perjuncta*). Perithecia form after the pycnidia cease to exude conidia. When perithecia are present, the canes have either narrow black zone-lines or black marks (Scheper *et al.*, 1997b). Zone-lines consist of swollen, melanized hyphae that originate at the junction between colonies (Muntanola-Cvetkovic *et al.*, 1996). Although was assumed that perithecia are produced only on canes with zone-lines (Scheper *et al.*, 2000), perithecial development has been observed on cane without zone-lines.

The effect of taxon 1 infection on productivity is unknown. In Australia, studies of the effect of taxon 1 on yield showed no correlation between yield loss and infection (Emmett *et al.*, 1998). There have been suggestions that taxon 1 cause failure of buds to burst, delayed

bud burst and stunting of shoots (Scheper *et al.*, 1997a; Brant *et al.*, 1999) but no evidence has been reported. Additionally, since taxon 1 does not produce symptoms on green shoots or tissue (Rawnsley and Wicks, 2000), the effect on shoot growth and vigour is also undetermined.

1.3.4.2 Symptoms caused by **Phomopsis** *taxon 2*

Most reports of Phomopsis cane and leaf spot show the casual pathogen to weaken canes, reduce yield, lower quality of fruit and kill nursery stock (Hewitt and Pearson, 1990). Generally, these losses in production are caused by severe infections by *Phomopsis* taxon 2.

Leaf symptoms are one of the first signs that *Phomopsis* taxon 2 is present within the vine. Leaves develop tiny, dark brown, necrotic lesions, approximately 1 mm in diameter, surrounded by a yellow margin (Figure 1.6). Where these are numerous, the leaves are distorted and some leaf sections killed (Emmett *et al.*, 1998). Severely infected leaves are often stunted and fall prematurely (Gubler and Leavitt, 1992).

Infected woody areas on basal portions of the cane are bleached. It is difficult to distinguish between the two taxa based on bleaching and the presence of pycnidia, as they show remarkable similarity. As new shoots develop, infected young shoots in the first four to six internodes develop chlorotic spots with dark centres (Figure 1.7a). Tissues become disorganised and collapse, resulting in the development of dark, longitudinal lesions (Figure 1.7b; Pine (1959). The lesions may occupy most of the shoot surface, eventually causing cracks of the epidermis and cortex of shoots (Gubler and Leavitt, 1992). Cracks may heal during the growing season to form rough tissue, but severe lesions will cause the cane to become brittle and break off. Yield loss occurs as a result of reduced bunch set, reduction of the cluster count and reduction of the next year's cropping level (Tassie and Freeman, 1992).

Figure 1.6. Leaf spots, characterised by a necrotic region approximately 1 mm in diameter surrounded by a yellow halo, caused by *Phomopsis* taxon 2 on a grapevine leaf.

Although fruit rot is rare in Australia, fungal infections of the pedicel or rachis are most likely to cause yield losses in cool climates. The fungus advances into the berry from a lesion on the pedicel, and produces pycnidia in the epidermis of the infected fruit (Hewitt and Pearson, 1990). Pycnidia from infected fruit has been identified as being similar to those of *Phomopsis* taxon 1 based upon the presence of α-conidia containing two guttules, and the presence of β-conidia (Lal and Arya, 1982). However, other reports of fruit rot (Pscheidt and Pearson, 1989; Baltovski, 1980; Erincik and Madden, 2001) have been associated with *Phomopsis* taxon 2, therefore it is unclear if both taxa cause fruit infection. Fruit symptoms are generally not extensive, with bunch infections localised on one vine (Gubler and Leavitt, 1992).

(a)

(b)

Figure 1.7. Shoot lesions on grapevine shoots produced by *Phomopsis* taxon 2. **(a)** Early lesion development on lower internodes. **(b)** Severe longitudinal lesions resulting in cracking of the epidermis (arrow).

1.3.4.3 Confusion of Phomopsis symptoms with those caused by other factors

Hail damage can cause bleaching of cane similar to infection by both *Phomopsis* taxon 1 and taxon 2 infection. Severely damaged tissue may develop callus where the hail has struck, and this may resemble immature lesions of taxon 2.

Cold injury can cause tissue malformation within the expanded bud. Such malformed leaves could be confused with symptoms due to *Phomopsis* taxon 2 (Pool, 1990), although they are also commonly confused with symptoms of eutypa dieback. Early descriptions of the Phomopsis cane and leaf spot were commonly confused with eutypa dieback, caused by *Eutypa lata* (syn. *E. armeniacae*), whereby infection of the vine was characterised by wedge-shaped discolouration of the xylem and dying of a single arm (Reddick, 1909). Other characteristic features of eutypa dieback thought to be caused by *P. viticola* include pruning wound cankers, severe dwarfing and discolouration of leaves and dwarfing of the internodes. Both *E. lata* and *P. viticola* produce pycnidia on cane and small necrotic spots on leaves. Moller and Kasimatis (1981) confirmed that only *E. armeniacae* is capable of inducing the pruning wound cankers and chlorotic, stunted spring foliage.

The presence of pycnidia on bleached cane is commonly assumed to be a result of *Phomopsis* infection. Other fungi such as *Botryosphaeria* spp. and *Botrytis cinerea* can produce fruiting bodies which resemble pycnidia or cause symptoms similar to those of Phomopsis. Black spot, caused by *Elsinoe ampelina*, induces small, brown leaf spots which are often confused with necrotic spots of taxon 2 infection. The lesions caused by black spot increase in size and the centre falls out giving a shot-hole appearance. Additionally, the deep cankers on stems can also be similar to those caused by taxon 2 (Emmett *et al.*, 1992). In the field, it is often difficult to distinguish between pycnidia produced by various fungi until observed under a microscope.

Mite damage on leaves is one of the symptoms most often confused with Phomopsis infection. The bud mite, *Colomerus vitis*, feeds on internal primordia of the grapevine bud, causing the expanded leaves to become stunted or wrinkled, and they often display small chlorotic spots which are often confused with leaf symptoms of *Phomopsis* taxon 2 (Goodwin, 1977). Spots caused by sucking insects are also similar to Phomopsis leaf symptoms, but these are mainly associated with veins and also, shoot symptoms will be absent (Emmett and Wicks, 1994).

Although there is no evidence to associate failure of buds to burst and Phomopsis infection, growers often attribute poor budburst to *Phomopsis* taxon 1. The observation may be based upon heavy infestation of bud mite which can cause bud death. Details of bud mite will be discussed in section 1.5.3.

1.4 The grapevine bud

The bud comprises a main bud (primary), secondary and tertiary bud (Figure 1.8). Although Perold (1927) used the term "eye", to distinguish the entire bud from a single bud, the term "compound bud" is more widely accepted (Pratt, 1990). The term "bud" will be used to differentiate between singular buds within the compound bud.

Every leaf axil forms two buds; the first forms a short lateral shoot during autumn which is seldom fertile, usually fails to lignify, and drops off in winter. Once the shoot abscises, a prominent scar remains and the first leaf of this shoot is reduced to a prophyll (Mullins *et al.*, 1992). In the axil of this prophyll, the primary bud of the second compound bud develops (Pratt, 1990). The primary bud grows, produces six to ten leaf primordia, and contains two inflorescence primordia. Brown scales and woolly threads cover the bud, which protect it against damage by cold in winter. The brown woolly threads remain visible at the

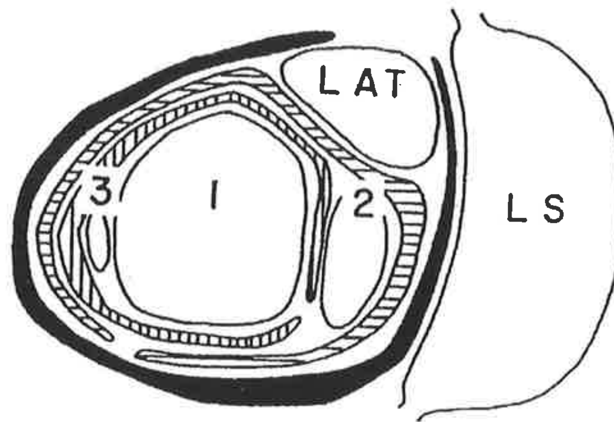
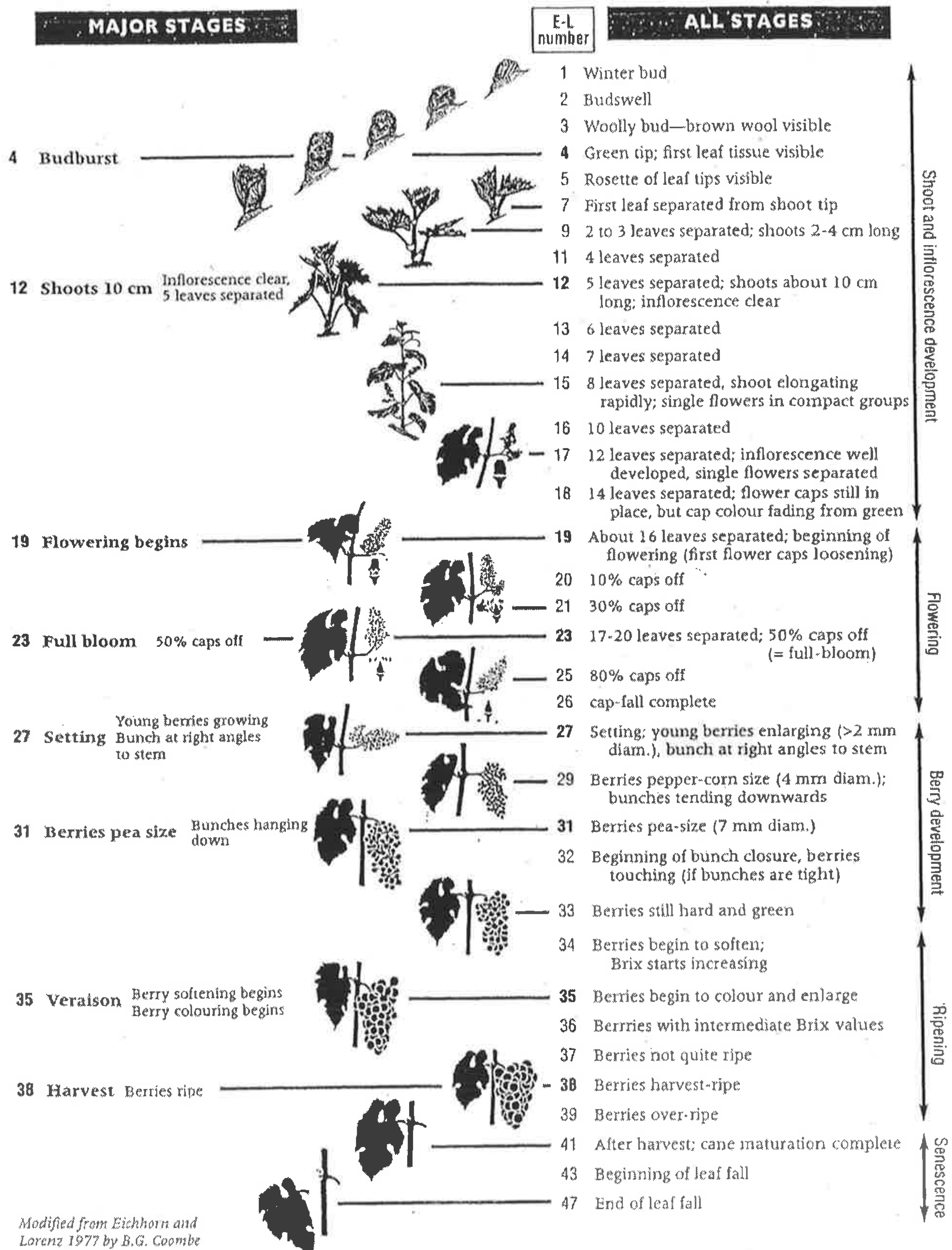


Figure 1.8. Transverse section through a compound bud of grape, showing relative positions of the leaf scar (LS), lateral shoot scar (LAT) and the three dormant buds: the primary bud (1); the secondary bud (2); the tertiary bud (3). (Reprinted from Pratt, 1990)

base of the young shoot and "wool" often covers the first internode. The primary bud develops into the new spring shoot (Mullins *et al.*, 1992), whilst the secondary and tertiary buds remain small and seldom contain inflorescence primordia. If the shoot of the primary bud is damaged or killed by frost, the secondary bud may develop a shoot to compensate for the loss, but is often unable to bear fruit.

Budburst (also termed "bud break") is defined as the stage of bud development where green tissue is visible (Pratt, 1990; Wolpert, 1992). In Australia, vines are dormant in winter months from June to late August, with bud burst and new growth commencing between late August and late September (Davidson, 1994). The phenology of grapevine was illustrated by Baggiolini (1952), with a more elaborate description by Eichhorn and Lorenz (1977). In 1995, Coombe proposed a new descriptive system of grapevine development encompassing the previous descriptions, called the Modified E-L system (Figure 1.9). The system is useful

Figure 1.9. Phenological stages of grapevine recommended by Eichhorn and Lorenz (1977) and modified by Coombe (reprinted from Coombe, 1995).



for identifying key stages of grapevine growth; budburst, flowering, veraison and ripening (Coombe, 1995).

Although, in Australia, there is no chilling requirement to break bud dormancy (Antcliff and May, 1961), it has been established that the number of newly burst buds is related to mean daily temperatures (Antcliff and Webster, 1955; Moncur *et al.*, 1989). The timing of bud burst is determined 1 month before actual bud burst, with terminal buds breaking earliest and basal buds latest (Antcliff and May, 1961). In addition, buds producing fruitful shoots tend to burst before shoots producing no fruit.

1.5 Bud abnormalities

The effect of *Phomopsis* taxon 1 on budburst is unknown, although it has been suggested that taxon 1 infection may cause a reduction in the number of buds to burst (Whisson *et al.*, 1998). It is difficult to distinguish whether bud failure, or death of the bud, is caused by physiological factors, pests or pathogens. For example, cane pruning often results in poor budburst in the mid-portion of the cane as a result of apical dominance. Bud abnormalities can be divided into lack of bud burst, or death of whole, or part, of the bud (Dry, 1986).

1.5.1 Failure of buds to burst

There are a number of possible factors that cause budburst failure. Many buds look normal during the dormant period, and swell, but never develop into shoots. Weather conditions, such as extreme cold, cause vine injury, especially in spring where budburst may be followed by cool days and nights. In theory, vines can withstand temperatures as low as –12°C (Dry, 1986), but young shoots and woolly buds can be killed in temperatures of –1°C to –3°C (Rogier, 1999). Additionally, basal buds are more susceptible to frost injury than buds at

nodes (Wolpert and Howell, 1984). The extent of damage can depend on the grape cultivar, e.g. Chardonnay buds burst early and are more likely to be affected by spring frosts, compared to Cabernet Sauvignon which bursts almost 2 weeks later. Bud injury can be detected while vines are still dormant by sectioning buds and observing internal tissue. Shoot primordia appear dark brown rather than pale green, and shoot growth will be sparse and irregular in damaged buds (Pool, 1990). Extreme injury may result in death of the entire bud, but more often, the primary bud only is killed allowing secondary buds to develop at a later stage.

Hail damage can destroy buds by direct physical damage, or indirectly by affecting the shoot. Severe hail may break shoots, leaves and portions of the internode resulting in failure of the buds to develop or causing the internal section of the bud to drop out (Pool, 1990). On shoots, irregular, raised callus usually develops around hail injury, which may surround the dormant bud. After hail, buds may appear healthy but the primary bud may be dead. Subsequent shoot growth from these buds is from the secondary or tertiary buds (Dry, 1986). In addition to destruction of buds and shoots by hail, injured shoots can become infected by pathogens.

Although cold injury and hail damage can cause obvious damage to the bud, bud failure can also be attributed to poor maturity of canes during the previous season. The basal parts of the spur or cane must be well lignified for good bud burst (Antcliff and May, 1961). This involves correct timing of pruning, application of fertilizers and overall good vineyard management.

1.5.2 Bud necrosis

Bud necrosis involves death of the primary bud within the compound bud. The condition has been associated with rapid shoot growth, low tissue carbohydrate levels and canopy shade (Vasudevan *et al.*, 1998). Externally, those buds with a necrotic primary axis resemble normal buds, therefore microscopic examination is required to identify bud necrosis correctly. Often the secondary buds compensate for loss of the primary bud by developing a new shoot, but these buds generally have low fruitfulness. Physiological stress can induce primary bud necrosis and, in some varieties, natural primary bud necrosis is high in nodes one to six on canes (Dry, 1986).

1.5.3 Bud mite

Three forms of mite on grapevine are recognised by the characteristic injury they cause; the bud mite, blister mite (*Colomerus vitis*) and the rust mite (*Calepitrimerus vitis*) (Davidson, 1994; Bernard *et al.*, 2000). Although regarded as a minor pest, heavy infestations inhibit normal vine growth. Both the blister and rust strains of mite affect leaves, but the bud mite is confined within the buds (Barnes, 1992).

The bud mites live, breed and feed within grape buds for the majority of their life (Bernard *et al.*, 2000). The mites live at the base of the outer bud scales, and feeding causes a blister-like growth on inner surfaces. Although mites can be present in all buds on the cane, infestation is more prevalent in the lowest two to three buds (Forster *et al.*, 1999). Spur-pruned canes, therefore, display uniform symptoms along the vine, whereas cane-pruned vines display symptoms near the basal internodes. Bud mite infestation is only evident once shoot symptoms appear but these are obscured as the foliage develops (Forster *et al.*, 1999). Usually budburst takes place, but young shoots are characterised by short basal internodes,

scarification of bark and flattened or "zigzag" shoots (Barnes, 1992; Bernard *et al.*, 2000). In severe cases of bud infestation, the entire bud may be killed. Symptoms can be confused with boron deficiency, frost damage, powdery mildew, eutypa dieback and *P. viticola* infection (Forster *et al.*, 1999).

Microscopic observation is required to detect bud mite early in the season. Sampling from basal buds on weakened canes during the dormant season is useful, but detection involves dissecting the buds to expose mites under the bud scales. Control of bud mite is difficult as the bud scales protect the mites. Control is best achieved by a spray of wettable sulphur or lime sulphur at bud burst when mites move into the expanding bud (Bernard *et al.*, 2000; Ludvigsen, 2000). In addition, predatory mites have been shown to be effective as they are able to feed on mites within the developing buds (Forster *et al.*, 1999).

1.6 Management of Phomopsis cane and leaf spot

One of the concerns associated with Phomopsis cane and leaf spot is the difficulty of differentiating *Phomopsis* taxon 1 and taxon 2 on dormant canes. In Australia, it is known that taxon 2 is more damaging, but the same control practices are used regardless of the taxon present in the vineyard. Because the effects of taxon 1 on grapevine are unknown, the use of chemicals for control of the disease may be unnecessary. The methods used for control of Phomopsis cane and leaf spot primarily target infection by taxon 2.

1.6.1 Cultural practices

The use of pathogen-free propagating material reduces the risk of introducing *Phomopsis* in a new vineyard, or when re-working vines. In addition, Chinosol® is recommended as a dip for suspect planting material to avoid the introduction of *Phomopsis*. If vines are infected with

Phomopsis, pruning practices should be implemented to remove diseased canes, and these canes should be avoided when developing new vine frameworks (Emmett *et al.*, 1992). The Diseased prunings should be removed from the vineyard and burnt to prevent carry-over of the disease into the next season (Hewitt and Pearson, 1990).

Pruning methods influence the development of the disease, and level of primary inoculum. Spur pruning is regarded as being less effective in the management of *Phomopsis*, as most infections occur on the basal part of the shoot. Cane pruning minimises the potential for new infections and spread of the disease. In comparison, mechanical and hedged-pruning retains more infected wood on the vine than hand pruning. Consequently, hedged vineyards are at a higher risk of disease due to higher inoculum levels (Pscheidt, 1989). *Phomopsis* was initially proposed as a wound parasite, requiring a wound site to penetrate the grapevine (Reddick, 1909), but Willison *et al.* (1964) showed that the fungus can penetrate the cuticle and cell wall directly. Pruning wounds, therefore, are not important infection sites and chemical control should be aimed at minimising primary infection on shoots.

1.6.2 Chemical control

One of the most effective control methods involves the application of a protectant (pre-infection) fungicide, such as Delan® (active compound dithianon), to dormant canes. A successful spray regime involves one application at 50% budburst and another application 2 weeks later, to prevent conidia infecting newly-developed shoots. This provides sufficient control unless favourable conditions persist, whereby further sprays may be required (Emmett and Wicks, 1994).

In the past, applications of eradicant chemicals, such as phenyl mercuric chloride (Taylor and Mabbitt, 1961), dinito-o-cresol, or sodium arsenite (Hewitt, 1935), were applied

during late dormancy (2 weeks before budburst). Although the eradicant fungicides suppressed pycnidial activity (Cucuzza and Sall, 1982), they were toxic to vines with improper timing (Moller and Kasimatis, 1981) and showed high levels of human toxicity. The use of these chemicals is now prohibited.

The effects of several chemicals, such as benomyl, fluazinam, mancozeb and 8-hydroxyquinoline sulphate, on the viability of pycnidia were tested in Australia. The chemicals inhibited the germination of conidia *in situ* on dormant canes (Castillo-Pando *et al*., 1997). Although benomyl was shown to be effective, regular use of the fungicide should be avoided as other pathogens, such *B. cinerea*, may develop resistance to this chemical. Application of dormant and foliar sprays have achieved the best control of the disease (Pine, 1957). New research has shown the most effective management of *Phomopsis* is achieved with a dormant spray of Shirlan® followed by applications of Delan® (Nair *et al*., 1998).

Alternatively, lime-sulphur applied to dormant grapevines cane reduced the production of cirrhi by *Phomopsis* and thus decreased the incidence of infection (Gadoury *et al*., 1994). However, lime sulphur is an unpleasant chemical and modern fungicides are more effective and cheaper to use. Although folpet is not registered for use in Australia, it was reported that the application of a mixture of fosetyl-Al + folpet to the developing bud inhibited sporulation by 95%, with the effect lasting up to 2 months after treatment (Jailloux and Bugaret, 1987). Fosetyl-Al exerts a strong inhibitory effect on the sporulation of *Phomopsis* and residue of the contact fungicide, folpet, persists on the buds.

1.7 Detection of *Phomopsis* by traditional methods

Because symptoms of Phomopsis cane and leaf spot often resemble those caused by other pathogens, or damage caused by adverse weather, detection is often difficult in the field. In order to achieve control of the disease, early identification of the pathogen is essential. Standard taxonomic methodologies for identification of *Phomopsis* taxa are based on morphological characteristics and assessment of disease symptoms. Cultural characteristics, such as mycelial pigmentation, and the formation, shape and size of conidia are often very variable, and are influenced by incubation conditions (Moricca *et al.*, 1998). In addition, the methods are often time-consuming and tedious, and require mycological expertise (Toth *et al.*, 1999).

Regardless of the detection method, material must be sampled from the host. The best time to identify *Phomopsis* is on dormant canes during or after pruning, when canes exhibit bleaching associated with pycnidium production. After collection of infected grapevine material, canes are placed at 15°C in moist conditions in darkness, which simulates suitable conditions for the production of cirrhi. If dormant canes are assessed, the incubation method may result in spore production within 1 week. Canes collected late in the growing season may have fewer sporulating pycnidia, therefore more time (up to 8 weeks) will be required to allow production of cirrhi (Whisson *et al.*, 1998)

Once cirrhi have been isolated from the cane, the taxon can be distinguished by morphological differences. Spores isolated from pycnidia are placed on potato dextrose agar (PDA) or similar growth medium and incubated at 16°C/22°C light/dark daily cycle to stimulate mycelial growth and further pycnidial production. Cultural characteristics may differ between taxa, but are insufficient for discriminating isolates or if they are similar species (Uddin and Stevenson, 1998). Mycelium of *Phomopsis* taxon 1 is more uniform in

morphology than taxon 2, white to cream in colour, and grows as a sparse to dense mat with aerial hyphae. While taxon 1 colonies grow relatively quickly, pycnidia do not generally form for several months and may require host tissue in the media to accelerate pycnidial production. In comparison, taxon 2 cultures may be cream to light brown in colour, flat, dense and compact, rarely colonising the entire Petri dish. Many isolates are irregular in shape, with many concentric rings where pycnidia are produced after 7-12 days.

Symptoms are used traditionally for rapid recognition of Phomopsis cane and leaf spot in the vineyard. Besides the appearance of bleaching and pycnidia, symptoms on green tissue are characteristic of taxon 2 infection. The appearance of lesions on cane, green shoots and leaves aid in visual identification of the disease. Diseased material is collected and plated on agar to promote mycelium growth. The fungus is isolated readily from the area surrounding the lesion, and not from the centre of the lesion itself. Identification during late spring and summer is of value only to establish control measures for the following season.

Grapevines infected with *Phomopsis* taxon 1 may display black, zone lines which arise from either vegetative compatibility (Brayford, 1990), or which are associated with the teleomorph, *D. perjuncta.* On cane incubated in favourable conditions, areas displaying zone lines often develop perithecia (Scheper *et al.*, 1997b). Zone lines have not been reported for cane infected with taxon 2, nor has the presence of a sexual stage.

1.8 Molecular methods used for identification and detection of plant pathogens

In recent years, the development of molecular techniques has increased the sensitivity and reliability of diagnostic tests. It can be argued that experienced diagnosticians can identify a disease by visual symptoms caused by the causal pathogen more quickly than any molecular test, however by this time the pathogen has already caused considerable damage to the host.

In practice, molecular tests can detect the pathogen in the host plant before visual symptoms appear (Fox, 1997). Although a number of molecular methods have been established for a range of fungal pathogens, techniques are continually being developed to make the procedures even more rapid and sensitive. The development of DNA-based molecular markers and Polymerase Chain Reaction (PCR) assays has improved the reliability, time and effectiveness of rapid detection of pathogens, in comparison to traditional methods, which are labour-intensive and time-consuming (Zhang *et al.*, 1997). PCR-based diagnostic assays are widely used, but other molecular methods, such as restriction fragment length polymorphisms (RFLPs) and slot blot hybridisation provide suitable methods for detection and characterisation of pathogens in infected material. In order to determine which method is best suited for use as a diagnostic technique, the type of infected material and the ease of obtaining high quality DNA must be considered.

1.8.1 Extraction of DNA

The procedure used for extraction of DNA is crucial for detection of pathogens in infected material. In general, all procedures involve the break down of cell walls, disruption of cell membranes, protection of the DNA from endogenous nucleases, and minimal thawing time of the sample to avoid degradation of the DNA (Rogers and Bendich, 1988).

The relatively small genome size of *V. vinifera* (0.50 pg/C), compared to many other perennial plant species, should facilitate molecular genetic studies (Lodhi *et al.*, 1994), but the extraction of nucleic acids is difficult due to a number of natural inhibitors. Grapevine tissue contains high amounts of polyphenols and polysaccharides which can interfere with endonuclease digestion of DNA and amplification by PCR (Kim *et al.*, 1990). In the living cell, phenolics are separated from DNA by compartmentalization in vacuoles that occupy a large part of plant cells. When cells are disrupted, the polyphenols become oxidised, causing

darkening of tissue homogenates and irreversibly react with protein and organelles (Couch and Fritz, 1990; Rezaian and Krake, 1987). The presence of these contaminants in DNA preparations often makes the sample viscous and renders DNA unsuitable for restriction digestion and amplification of the DNA (Lodhi *et al.*, 1994).

A number of standard methods have been devised for the extraction of DNA (Murray and Thompson, 1980; Raeder and Broda, 1985) but many of the techniques are time-consuming and tedious. Modifications of standard procedures are common to improve efficiency (Lodhi *et al.*, 1994; Maguire *et al.*, 1994; Zhu *et al.*, 1993) and commercially available kits, such as Dneasy® (Qiagen, Germany), have been developed to reduce the time required and allow the use of smaller quantities of starting material. For example, Green and Thompson (1999) showed that by using the Dneasy® kit, as many as 36 samples can be processed and ready for amplification of the DNA in less than 2 hours. However, these kits are mainly based on small-scale extraction of DNA and large-scale preparations can be expensive.

Factors which influence the extraction of DNA from grapevine include the nature of the cell wall (thickness and composition), secondary metabolites, type of tissue infected (e.g. phloem) and the type of infection (systemic or localised) (Thomson and Dietzgen, 1995). The developmental age of the grapevine affects the ease of cell disruption e.g., woody cane is tougher than young tissue, making it harder to break down during grinding. This is important to consider for the detection of *Phomopsis*, as the fungus exists in dormant vine material.

1.8.2 DNA hybridisation

Hybridisation involves the formation of a bonded complex between a target nucleic acid and a complementary probe. A probe consists of a labelled DNA sequence having a strong and detectable interaction with the target nucleic acid. They may represent either the complete

target sequence, or only part of it (Randles *et al.*, 1996). Probes can be low copy, indicating one or a few copies of each sequence are present, or multicopy, whereby dispersed repetitive sequences are recognised in high numbers (Curtis and Barnes, 1989). Species-specific DNA probes are useful for discriminating between strains of pathogens. Only probes capable of hybridising to all isolates of a given species are suitable for developing species–specific probes (Xu *et al.*, 1999).

Phomopsis taxon-specific probes, pT1P180 and pT2P25, were developed for the rapid identification of *Phomopsis* taxon 1 and taxon 2, respectively, in diseased vines (Melanson *et al.*, 2002). These probes represent sequences that are present specifically in the genome of *Phomopsis* taxon 1 and taxon 2, thus can be used to distinguish between the taxa, regardless of symptom expression. The highly sensitive nature of taxon-specific probes makes them ideal for early detection of a pathogen in the host plant (Koopmann *et al.*, 1994).

There are a few disadvantages with the use of DNA probes as a diagnostic tool. Nucleic acids must be extracted from an infected sample, bound to a filter, and hybridised with a radioactively-labelled probe (Fox, 1997). The procedure is, therefore, time-consuming and requires technical expertise. The development of non-radioactive digoxigenin (DIG) labelling eliminates the use of radioactive isotopes, but does not appear as sensitive and the procedure is just as time-consuming (Koopmann *et al.*, 1994). A suitable probe must allow a high level of sensitivity and must be specific to the target organism.

1.8.3 Polymerase chain reaction (PCR)

PCR, developed in 1985 (Edel, 1998), has quickly become the most widely used molecular technique in molecular biology. PCR is used to amplify a segment of DNA that lies between two regions of a known sequence (Sambrook *et al.*, 1989). PCR enables small amounts of specific DNA fragments to be amplified (10^6-fold amplification) during a repetitive series of

thermal cycling. A typical amplification reaction includes a sample of the target DNA, a thermostable polymerase, two oligonucleotide primers, deoxynucleotide triphosphatases (dNTPs), reaction buffer and magnesium (Sambrook and Russell, 2001). PCR amplification allows for the detection of picogram quantities of low copy DNA sequences and thus is more sensitive than direct probing.

Specific primers used in PCR allow for the detection of small quantities of fungal DNA in infected host material and have applications for epidemiological studies (Zhang *et al.*, 1997; Xu *et al.*, 1999). Development of specific primers requires the knowledge of sequences of at least a part of the target DNA region (Edel, 1998). Suitable sequences may be derived from amplification and direct sequencing of ribosomal DNA (rDNA) and, in particular, from the internal transcribed spacers (ITS1 and ITS2) of rDNA. For example, ITS sequences have been used to develop PCR-based assays for the detection of many plant pathogens in host plants including *Diaporthe phaseolorum* and *Phomopsis longicolla* in soybean tissue and seeds (Zhang *et al.*, 1997), *Phaeomoniella chlamydospora* in grapevine (Groenewald *et al.,* 2000) and *Verticillium* spp. in potato (Robb *et al.,* 1994). ITS sequences are useful in phylogenetic studies at the specific and generic levels.

Random amplified polymorphic DNA (RAPD) approaches can be used to generate molecular markers. RAPDs are based on the PCR amplification of genomic DNA using arbitrary primers. Specific banding patterns generated by RAPD markers can be analysed to differentiate and identify fungi (Edel, 1998). The bands can be cloned and sequenced, and these sequence-characterised amplified regions (SCARs) used to design specific primers for detection assays. This approach was first applied by Paran and Michelmore (1993) to distinguish downy mildew resistance genes in lettuce and has since been used for the detection of a range of pathogens, including *Aphanomyces euteiches* in peas (Vandemark *et al.,* 2001) and *Fusarium culmorum*, *F. graminearum* and *F. avenaceum* in cereals (Schilling

et al., 1996). The main advantage of this approach is that previous knowledge of DNA sequences is not required, so that any random primer can be tested for its ability to amplify fungal DNA.

One advantage of PCR is its ability to amplify picogram quantities of DNA in the presence of diverse contaminants (Cenis, 1992). The presence of host DNA, however, may influence the amount of fungal DNA detected by PCR-based diagnostic assays. For example, Moricca *et al.* (1998) showed that a minimum of 1 ng of *Fusarium oxysporum* f.sp. *vasinfectum* was detected in infected tissue of cotton, yet 50 pg of mycelium was detected following isolation. PCR diagnostic assays are reliable in detecting the pathogen from plants exhibiting different degrees of disease severity, even those without symptoms, indicating that PCR is robust and sensitive for early monitoring of disease (Moricca *et al.*, 1998).

Although PCR has proved to be highly efficient for analysis of various types of DNA, the investigation of DNA from grapevine is much more difficult (Edwards *et al.*, 1991). The presence of contaminating materials in DNA isolated from grapevine inhibits the amplification by PCR, rendering the procedure unsuitable for direct sampling from vines (Eastwell *et al.*, 1995). In this regard, the success of PCR is directly correlated to the method of DNA extraction. Another disadvantage is the sensitivity of PCR which may create false positives (Randles *et al.*, 1996). Regardless of the many modifications of extraction procedures, there is still a need for clean, rapid and easy preparation techniques for the use of woody grapevine in PCR. For example, woody tissue is used in the diagnosis of *Phomopsis* taxa from bleached canes (Melanson *et al.*, 2002). Other problems associated with PCR include the need for technical expertise, minimal technical error and replicable results between laboratories.

1.8.4 Southern hybridisation – dot, slot and RFLP analysis

Although PCR has become the focal point of most newly developed detection systems, Southern hybridisation methods have been widely used for the identification and detection of plant pathogenic fungi. Southern blotting involves the transfer of DNA from an agarose gel to a nitrocellulose or nylon filter, which is subsequently hybridised with a probe (Southern, 1975). The filter is washed to remove unbound nucleotides and autoradiography locates bands complementary to the probe. Dot and slot blot hybridisation involves fixing DNA directly to a nylon filter via an apparatus with a fixed pattern, hybridising with a labelled DNA probe and exposure to X-ray film to detect a single band (Sambrook and Russell, 2001).

Restriction fragment length polymorphism (RFLP) analysis by southern hybridisation reveals differences in banding patterns resulting from digestion of DNA with restriction enzymes (Koopmann *et al.*, 1994). Comparison of RFLP's provides an estimate of the type, level and distribution of DNA sequence polymorphism among the sample of alleles (Aquaro *et al.*, 1992). For example, the *Phomopsis* taxon 2-specific probe, pT2P25, identified five distinct banding patterns among isolates according to geographical region (Melanson *et al.*, 2002).

RFLPs have a wide use in genetic studies, but have limited future in routine diagnostic applications. They require pure DNA to allow complete digestion by the restriction enzyme, thus DNA of sufficient quantity and quality must be extracted. Other disadvantages associated with Southern hybridisation using RFLPs is they require much more time and involve the use of radioactive procedures.

The dot and slot blot nucleic acid-based techniques have the ability to detect and quantify fungi in the host tissue. This is achieved by placing a known quantity of DNA on the filter whereby the strength of the signal is proportional to the specific activity of the

probe. A probe bound to a high number of complementary DNA sequences will provide a strong signal, whereas low sequence number provides a weak signal. Slot blots provide sufficient information for distinguishing *Phomopsis* taxa by the taxon-specific probes (Melanson *et al.*, 2002). The only difference between dot and slot blotting, is the later focuses the applied DNA samples in a thin line instead of a circle. Unlike RFLP analysis of digested DNA by Southern transfer, a single band only is detectable but provides quantitative information by comparison with the known amount of target DNA.

The methods employed in dot and slot blot hybridisation are generally less time-consuming than Southern transfer, but still require extraction of DNA from the infected plant material and use of radioactive probes. In comparison, PCR can detect smaller amounts due to the amplification of target DNA using specific primers and DNA polymerase. For example, dot-blot hybridisation detected less than 0.16 ng of the pathogen, *Sporisorium reiliana*, in infected maize, yet when isolated from the host, 1.6 pg of fungal DNA was detected by PCR (Xu *et al.*, 1999). Thus PCR has been shown to be 20 –100 times more sensitive than Southern hybridisation.

Dot and slot blots are suitable for use in pathogen detection systems for grapevine because many samples can be simultaneously tested. Samples extracted from infected material can be transferred directly on to the filter and the location of the fungus in the plant can be determined. The success of slot blot hybridisation is demonstrated by the detection of *Phomopsis* taxon 1 and taxon 2 in grapevine buds (Melanson *et al.,* 2002) and *Gaeumannomyces graminis* in naturally infested soil (Herdina *et al.*, 1997).

1.9 Summary and objectives

This review of literature on Phomopsis cane and leaf spot highlights the need for more information on the pathogenicity of the fungi associated with the disease. The confusion, which exists between taxa of *Phomopsis* and other fungal species, needs to be clarified

The main issue to be addressed is the effect of taxon 1 on grapevine. In Australia, studies of the effect of *Phomopsis* taxon 1 showed no correlation between yield loss and infection (Nair *et al.*, 1998) but it has been suggested that infection may result in failure of buds to burst, delayed bud burst and stunting of shoots (Brant *et al.*, 1999; Scheper, 2001). A number of factors affect the development of the bud, including bud necrosis, bud mite and physiological aspects, hence it is possible that *Phomopsis*-infected vines may be under a variety of influences. Additionally, taxon 1 does not produce symptoms on green shoots or tissue, so the effect on shoot growth and vigour is also undetermined. Because of this uncertainty, it is essential to determine the pathogenicity of *Phomopsis* taxon 1 in order to establish whether or not control strategies are warranted.

Development of molecular detection techniques is crucial for quick and correct diagnosis of the disease, especially as the symptoms of Phomopsis cane and leaf spot are easily confused with those of other pathogens and damage cause by environmental factors. Molecular markers based on PCR and RFLP have been shown to be effective for detecting a wide range of fungal pathogens. Such markers are critical for early detection of the disease, particularly before symptoms appear as, by that stage, the plant has been infected for several weeks. Probes have been developed for the identification of *Phomopsis* taxon 1 and taxon 2, but the development of PCR-based assays would make the diagnostic procedure quicker, more reliable and remove the need for lengthy procedures required in more conventional hybridisation methods.

The objectives of this study were to; (1) assess the pathogenicity of *Phomopsis* taxon 1 on grapevine and clarify its role in failure of buds to burst and subsequent bud loss, (2) exclude bud mite and other fungal pathogens as a cause of bud loss, and to determine if bud mite or other bud abnormalities are associated with the effects of *Phomopsis* in the bud and (3) assess the efficacy of the *Phomopsis* taxon 1-specific DNA probe, pT1P180, and, if necessary, develop an alternative molecular diagnostic assay for detection of *Phomopsis* taxon 1 in grapevine. The information gained from this study will help to elucidate the pathogenicity of *Phomopsis* in grapevine and will contribute knowledge essential in the establishment of effective management strategies.

Chapter 2

General materials and methods

In this section, materials and methods which were commonly used in these studies are described. Modifications of a particular method are specified in the relevant chapter.

2.1 Collection and establishment of *Phomopsis* isolates

Canes with distinct bleaching were collected from vineyards with suspected *Phomopsis* infection during July-September. Canes were placed in a sealed container containing paper towel moistened with water and incubated at 15°C in darkness for at least 1 week. Creamy/white cirrhi, which developed after this time, were isolated from pycnidia under a dissecting microscope using a sterile, fine point needle. Each cirrhus was placed in 100 µl of sterile ddH_2O in a 0.5 ml Eppendorf® tube. A 10 µl suspension was placed on a glass slide and *Phomopsis* spp. were identified based on the morphology of the conidia (Merrin *et al.*, 1995). Conidia isolated from cirrhi were transferred to PDA (Difco, USA) in a 9-cm Petri dish and incubated at 22°C in light (Philips TLD 18W/33 3F, 380 nm–780 nm and near ultraviolet, 380 nm-400 nm) for 12 hours and 16°C in the dark for 12 hours. Mycelium, from germinating conidia, was transferred from the margin of an expanding colony to a new PDA plate and maintained in the above conditions. The isolates used in the study are listed in Table 2.1; these include isolates collected from infected grapevines in South Australia (SA) and those obtained from colleagues in other states of Australia and other countries.

Table 2.1. Origin and source of *Phomopsis* and other fungi used in this study.

Isolate No.	Site	Species	Source	Date isolated
A223.1	Ashton Hills, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	31/10/99
A223.2	Ashton Hills, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	31/10/99
A19	Ashton Hills, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	25/10/00
A200	Ashton Hills, SA	*Diaporthe perjuncta*	cane, *V. vinifera*	25/10/00
H307	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	1/8/97
H308	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	7/10/99
H309	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/10/99
H310	Hargrave Winery, Summertown, SA	*Phoma epicoccina*	cane, *V. vinifera*	1/11/99
HR9T11.1	Hargrave Winery, Summertown, SA	?	bud, *V. vinifera*	2/3/00
HR11T9	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
HR12T3	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
HR13T14.2	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
HR13T15	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
HR12T2.1	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
HR12T2.2	Hargrave Winery, Summertown, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	23/5/00
L405	Hillstowe Winery, Hillstowe, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	1/10/96
L406	Hillstowe Winery, Hillstowe, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	1/10/96
L407	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L408	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L409	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L410	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L411	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L412	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L413	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L414	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L415	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	28/8/00
L416	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/6/01
L417	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/6/01
L418	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/6/01
L419	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/6/01
L420	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/6/01
L421	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	5/7/01
L422	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	5/7/01
L423	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	5/7/01
L424	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	5/7/01
L425	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	5/7/01
L426	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	19/10/01
L427	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	19/10/01
L428	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	19/10/01
LR12T20	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
LRT10T6	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 2	bud, *V. vinifera*	2/3/00
LR9T16	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
LR11T20.1	Lenswood Centre, Lenswood, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	2/3/00
B500	Barratt Winery, Summertown, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	9/10/97
B501	Barratt Winery, Summertown, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	20/10/99
B502	Barratt Winery, Summertown, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	7/10/99
C600	Coonawarra, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/97
C603	Coonawarra, SA	?	cane, *V. vinifera*	1/2/97
C608	Coonawarra, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/97
C609	Coonawarra, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/97

Isolate No.	Site	Species	Source	Date isolated
50B	Coonawarra, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	1/8/94
51C.1	Coonawarra, SA	*Diaporthe viticola*	cane, *V. vinifera*	1/8/94
P712	Southcorp, Padthaway, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	18/1/99
P713	Southcorp, Padthaway, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	3/3/99
P716	Southcorp, Padthaway, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	3/3/99
J1	Mt. Jagged, Southern Fleurieu, SA	?	*Rubus fruticosus*	19/10/99
J2	Mt. Jagged, Southern Fleurieu, SA	?	*Rubus fruticosus*	20/10/99
J3	Mt. Jagged, Southern Fleurieu, SA	*Seimatosporium* sp.	cane, *V. vinifera*	1/11/99
J4	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
J5	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
J6	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
J7	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
J8	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
J9	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
JR5T16	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	bud, *V. vinifera*	17/11/99
JR4T10 (B3)	Mt. Jagged, Southern Fleurieu, SA	?	bud, *V. vinifera*	17/11/99
JR7T4	Mt. Jagged, Southern Fleurieu, SA	?	bud, *V. vinifera*	17/11/1999
D/SA/25	Santo Tirso, Portugal	*Phomopsis* taxon 1	cane, *V. vinifera*	1/1/98
P/CA/15/20/1	Oeiras, Portugal	*Phomopsis* taxon 1	cane, *V. vinifera*	1/1/98
P/SE/1	Almeirim, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/3/98
P/BU/2/1	Santo Tirso, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
P/BU/5/1	Santo Tirso, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
P/CA/17/7/1	Oeiras, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
P/CA/6/8/1/1	Oeiras, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
P/CA/11/31/2	Oeiras, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
P/BU/2/3	Santo Tirso, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
GD/SA/1/9	Oeiras, Portugal	*Phomopsis* taxon 1	cane, *V. vinifera*	1/1/98
P/CA/3/2/1	Oeiras, Portugal	*Phomopsis* taxon 2	cane, *V. vinifera*	1/1/98
DM/SA/12	Montemor-o-Novo, Portugal	*Phomopsis* taxon 1	cane, *V. vinifera*	1/4/98
UQ4423	Applethorpe R.C., QLD	*Phomopsis* taxon 2	unknown	1/5/98
UQ4424	Applethorpe R.C., QLD	*Phomopsis* taxon 2	unknown	1/6/98
UQ4683	Brisbane, Queensland	*Phomopsis* taxon 2	unknown	1/12/98
900.16	Best Winery, Lake Boga, Vic	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/99
901.18	Boulton, Vinefera, Vic	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/99
902.4	O'Briens, Robinvale, Vic	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/99
902.15	Robinvale, Vic	*Phomopsis* taxon 2	cane, *V. vinifera*	1/2/99
M827	Mudgee, NSW	*Phomopsis* taxon 2	cane, *V. vinifera*	1/7/98
M830.1	Mudgee, NSW	*Phomopsis* taxon 2	cane, *V. vinifera*	1/7/98
M831.1	Wagga, NSW	*Phomopsis* taxon 1	cane, *V. vinifera*	1/7/98
M832.4	Mudgee, NSW	*Phomopsis* taxon 2	cane, *V. vinifera*	1/7/98
M833.2	Wagga, NSW	*Phomopsis* taxon 1	cane, *V. vinifera*	1/6/98
M834.2	Wagga, NSW	*Phomopsis* taxon 1	cane, *V. vinifera*	1/6/98
M838.4	Wagga, NSW	*Phomopsis* taxon 1	cane, *V. vinifera*	1/6/98
M850	Griffith, NSW	*Phomopsis* taxon1	cane, *V. vinifera*	1/9/98
M851	McLaren Vale, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	1/11/98
M851.1	McLaren Vale, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	1/11/98
M860	Vasse Felix, WA	*Phomopsis* taxon 1	cane, *V. vinifera*	1/9/98
T101	Langhorne Creek, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	25/10/00
T100	Terraces Winery, McLaren Vale, SA	*Phomopsis* taxon 2	cane, *V. vinifera*	16/10/99

Isolate No.	Site	Species	Source	Date isolated
T32	Mt. Jagged, Southern Fleurieu, SA	*Phomopsis* taxon 1	cane, *V. vinifera*	24/7/01
M861	Mt. Jagged, Southern Fleurieu, SA	*Botrytis* sp.	cane, *V. vinifera*	24/7/01
M862	Mt. Jagged, Southern Fleurieu, SA	*Aspergillus* sp.	cane, *V. vinifera*	24/7/01
I1	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	leaf, *V. vinifera*	19/11/01
I2	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	leaf, *V. vinifera*	20/11/01
I3	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	leaf, *V. vinifera*	21/11/01
I4	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	leaf, *V. vinifera*	22/11/01
I5	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	stem, *V. vinifera*	23/11/01
I6	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	stem, *V. vinifera*	24/11/01
I7	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	stem, *V. vinifera*	25/11/01
I8	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	stem, *V. vinifera*	26/11/01
I9	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	stem, *V. vinifera*	27/11/01
I10	Garrett Ingoldby, McLaren Vale, SA	*Phomopsis* taxon 2	stem, *V. vinifera*	28/11/01
DAR69458	Yarra Valley, NSW	*Phomopsis* taxon 3	*V. vinifera*	2/1992

New South Wales (NSW), Queensland (QLD), South Australia (SA), Victoria (VIC), Western Australia (WA).
Isolates 50B, 51.1C supplied by R.W.A. Scheper.
Portugese isolates supplied by A.J.L. Phillips.
Cane material from NSW supplied from M. Castillo-Pando.
DAR isolate derived from NSW Agriculture.
? = unknown fungal species.

Selected cultures were grown on PDA slopes in McCartney bottles at 22°C/16°C light/dark daily cycle. After 2 weeks, mycelium was covered with sterile mineral oil to a depth of 3 cm and the cultures stored at room temperature in darkness. Alternatively, approximately 10 mycelial plugs (1 cm × 1 cm) were cut from a PDA plate and placed in 10 ml sterile ddH_20 in a McCartney bottle. Isolates were kept at 4°C for long term storage.

2.2 Molecular techniques

2.2.1 Extraction of total nucleic acid from grapevine material

Total DNA (genomic and mitochondrial) was extracted from a range of grapevine tissue, including buds, lignified cane, green shoots and leaves. Whole dormant buds were removed from the cortex with a scalpel and, in most cases, processed intact. Outer tissue of green

shoots was removed with a scalpel by scraping the blade along the surface to obtain fine slivers. A vegetable grater was used to remove lignified tissue from 1-year-old cane to the pith region. All grapevine samples were weighed, packaged in aluminium foil, quick frozen in liquid nitrogen and stored at -70°C until required as described by Melanson *et al.* (2002).

Approximately 50 mg of bud, or 100 mg of cane or shoot tissue was ground to a fine powder with a mortar and pestle in liquid nitrogen. The material was suspended in ten volumes of pre-heated (65°C) CTAB extraction buffer (Doyle and Doyle, 1980) for green shoots or SEAPS extraction buffer (Melanson *et al.*, 2002) for bud or cane material (see Appendix A). The suspension was incubated for 20 min at 65°C and extracted using an equal volume of chloroform/*iso*-amylalcohol (24:1). Where possible, the suspension was mixed on a benchtop rotor for 10 min, otherwise it was mixed by hand. The mix was centrifuged for 15 min in a bench top centrifuge at 14 000 *g*, the aqueous phase treated with RNAse A to a final concentration of 0.1 mg/ml and incubated at 37°C for 15 min. The solution was centrifuged at 14 000 *g* for 15 min, followed by a further extraction with an equal volume of chloroform/*iso*-amylalcohol (24:1). DNA was precipitated by the addition of a 0.67 volume cold isopropanol, except for green shoot samples, to which 0.1 volume of 10 M ammonium acetate (pH 5.2) was added to a final concentration of 1 M. Nucleic acids were precipitated at -20°C for a minimum of 1 hour, followed by centrifugation at 14 000 *g* for 20 min. DNA pellets were washed in 500 µl of 70% ice-cold ethanol, centrifuged at 14 000 *g* for 5 min, vacuum-dried and resuspended in 20-100 µl Tris-EDTA (TE) depending on pellet size.

The amount of DNA in each sample was estimated by running aliquots on a 1% agarose gel in Tris-Acetate-EDTA buffer (TAE; see Appendix A) and visualising the bands under UV light following ethidium bromide staining. The quantity of DNA was estimated by comparison with a known quantity of lambda DNA digested with *Hin*dIII.

2.2.2 Extraction of DNA from mycelium

A mycelial plug from a 7-14 day-old culture of *Phomopsis* was placed in a 9-cm Petri dish containing 20 ml potato dextrose broth (PDB; Difco, USA) and incubated at room temperature in the dark for a maximum of 5 days. The original mycelial plug was discarded and fresh mycelium harvested on a sterile 8 µm, 47 mm diameter MF-Millipore® nitrocellulose membrane filter (Millipore, Bedford, MA) in a Buchner funnel attached to a vacuum flask. The mycelium was rinsed three times with ddH_2O, weighed and frozen at –70°C until required.

The sample was ground to a fine powder with a mortar and pestle in liquid nitrogen and ten volumes of mycelial extraction buffer modified from Raeder and Broda (1985) (see Appendix A) were added to form a slurry. The solution was extracted with an equal volume of chloroform/*iso*-amylalcohol (24:1) and mixed on a benchtop rotating platform for 10 min to create an emulsion. The mix was centrifuged for 15 min in a bench top centrifuge at 14 000 *g*, the supernatant then transferred to a new tube and RNAse A added to a final concentration of 0.1 mg/ml. After incubation for 15 min at 37°C, the solution was centrifuged at 14 000 *g* for 15 min. The supernatant was removed, transferred to a new tube and extracted as above with an equal volume of chloroform/*iso*-amylalcohol (24:1). DNA was precipitated from the aqueous phase by the addition of 0.4 volume of 4 M ammonium acetate (pH 5.2) and 0.6 volume of cold isopropanol for 1-2 hours at -20°C, followed by centrifugation at 14 000 *g* for 20 min. The DNA pellet was washed in 500 µl 70% cold ethanol containing 10 mM magnesium acetate for 10 min, centrifuged for 5 min as above, vacuum-dried and resuspended in 20-100 µl 1× TE buffer depending on pellet size (see Appendix A). The amount of DNA in each sample was estimated by running aliquots on a 1% TAE agarose gel and visualising the bands under UV light following ethidium bromide staining. The quantity

of DNA was estimated by comparison with a known quantity of lambda DNA digested with *Hind*III.

2.2.3 Slot blot transfer

DNA samples obtained from grapevine buds, canes and shoots was adjusted to 100 ng total DNA in a total volume of 200 μl ddH_2O. DNA from purified *Phomopsis* taxon 1, *Phomopsis* taxon 2, other grapevine pathogens and grapevine tissue and ddH_20 (controls) were included on each slot blot (details provided in relevant chapters). Samples were adjusted to a final volume of 400 μl with the addition of 200 μl of 0.8 M NaOH, 20 mM EDTA solution (final concentration 0.4 M NaOH, 10 mM EDTA) and denatured at 100°C for 10 min. DNA was transferred to a 9.5 × 12.5 cm positively charged nylon membrane (Roche Diagnostics) with a Bio-Dot SF apparatus (Bio-Rad, USA), according to the manufacturer's instructions. After rinsing briefly with 2 × SSC (see Appendix A), DNA was fixed to the membrane using a Bio-Rad® GS Gene Linker™ UV chamber (Bio-Rad Inc., USA) at 150 mJ. Membranes were sealed in clear plastic sheets and stored at 23°C before use.

2.2.4 Southern DNA transfer for RFLP analysis

Approximately 200-500 ng of total DNA was digested with a restriction enzyme (Roche Diagnostics, Germany) overnight at 37°C. Complete digestion was confirmed by running an aliquot on a 1% agarose gel in TAE buffer and visualising the smear under UV light following ethidium bromide staining. The gel was immersed immediately in denaturation solution (1.5 M NaCl, 0.5 M NaOH) and placed on a shaking platform for 30 min at room temperature, followed by immersion in neutralising solution (1 M Tris-HCl, 1.5 M NaCl, pH

7.4) for a further 30 min. DNA was transferred to a positively charged nylon membrane (Roche Diagnostics) by the Southern transfer method (Southern, 1975). The location of wells was marked on the membrane by placing a 6B pencil through the agarose to the membrane then discarding the gel. Membranes were rinsed briefly in 2 × SSC and fixed as above (section 2.2.3).

2.2.5 Preparation of *Phomopsis*-specific probes

The *Phomopsis* taxon 1-specific probe pT1P180 and the *Phomopsis* taxon 2-specific probe pT2P25 (Melanson *et al.,* 2002) were recovered from storage at –70°C by streaking colonies of *Escherichia coli* strain JM109 containing the recombinant plasmids (pUC19) onto Luria-Bertani agar (LB, see Appendix A) containing ampicillin (Amp) to a final concentration of 100 μg/ml. A single colony was transferred to 10 ml LB broth containing 20 μl Amp (50 mg/ml) and incubated with constant shaking (200 rpm) at 37°C. Bacteria were harvested by centrifugation of 2 ml aliquots at 14 000 *g* for 15 min and plasmid DNA was prepared using the Wizard® Plus SV Miniprep DNA purification system (Promega, USA) as recommended by the manufacturer.

Purified plasmid DNA was digested with restriction enzyme *Pst*I (Roche Diagnostics) in a total volume of 100 μl. Complete digestion was checked by running an aliquot of 5 μl on a 1% agarose gel in TAE buffer and visualising the bands under UV light following ethidium bromide staining. The quantity of DNA was compared with a known quantity of lambda DNA digested with *Hin*dIII. Two wells of an electrophoretic comb were taped together to construct a large well in 1% TAE agarose to accommodate a total volume of 40 μl of digested plasmid DNA. The fragments were separated at 70 volts for approximately 1.5 hours and visualised as above. Fragments of the appropriate size (3.6 kb and 570 bp, *Phomopsis* taxon 1

and *Phomopsis* taxon 2-specific DNA probes, respectively) were isolated from the agarose gels, weighed and purified using the Geneclean II kit (Bio-101 Inc.) according to the manufacturer's recommendations. Two separate elutions were made to obtain the final suspension of DNA, to ensure complete removal of DNA from the Geneclean II spin column. DNA concentration was determined by comparison with a known quantity of lambda DNA digested with *Hin*dIII on 1% agarose gel in TAE buffer.

2.2.6 Hybridisation methods

Nylon membranes, between two nylon mesh sheets, were immersed in 2 × SSC and transferred to a 30-cm long Hybaid® bottle (Boehringer Mannheim, Germany). Excess 2 × SSC was removed, then membranes were prehybridised in 19.8 ml prehybridisation solution (see Appendix A) and 200 μl denatured sonicated herring sperm DNA (Roche Diagnostics) for a minimum of 6 hours at 65°C in a Hybaid® rolling oven (Boehringer Mannheim). During this time, a disposable chromatography column (Bio-Rad) was prepared with Biogel P60 50-100 microns polyacrylamide mesh (Bio-Rad) and equilibrated with TEN buffer (pH 8; see Appendix A). The prepared column was inserted into a 15 ml disposable plastic tube containing a 0.5 ml Eppendorf® tube inside a 1.5 ml Eppendorf® tube, both without lids. The TEN buffer was eluted by centrifugation in a swinging bucket rotor at 1 600 *g* for 4 min and centrifugation repeated until 100 μl TEN buffer was collected.

The components and protocol of the Megaprime DNA labelling system (Amersham Pharmacia Biotech, England) were used in most hybridisation experiments. Approximately 25-50 ng of *Phomopsis*-specific probe was used in the labelling reaction containing 5 μl random primer, 10 μl labelling buffer, 30 μCi ^{32}P-dCTP, 1 unit Klenow DNA polymerase and sterile ddH_20 to a final volume of 50 μl. The reaction mixture was incubated for 30 min

at 37°C, followed by the addition of 2 μl of 0.5 M EDTA and 50 μl of TEN buffer to give a total volume of 102 μl. The reaction mixture was centrifuged in a swinging-bucket rotor for 4 min at 1 000 *g* and approximately 100 μl of ^{32}P-labelled probe was collected in the 0.5 ml Eppendorf tube®. The ^{32}P-labelled probe was denatured for 5 min at 100°C and membranes hybridised in 9 ml hybridisation solution (see Appendix A), 700 μl sterile ddH_20 and 10 μg/ml denatured herring sperm DNA at 65°C for 16-20 hours.

Membranes were washed successively for 20 min in 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS; 0.5 × SSC, 0.1% SDS and, if necessary, 0.2 × SSC, 0.1% SDS for 20 min with gentle agitation. Membranes were air-dried briefly, placed between plastic sheets and exposed to X-ray film (X-Omat, Kodak, USA) to obtain an auto-radiographic image.

Before re-hybridisation, the membrane was stripped of all ^{32}P-labelled DNA by immersion in 0.4 M NaOH at 45°C for 30 min in a shaking water bath, followed by a wash in 0.1 × SSC, 0.5 M SDS, 1 M Tris (pH 8) for 15 min. Membranes were dried briefly on paper towel and sealed between two polyethylene sheets until required.

Chapter 3

Pathogenicity of *Phomopsis* taxon 1 on grapevine

3.1 Introduction

In 1995, it was reported that two types of *Phomopsis* on grapevine, termed *Phomopsis* taxon 1 and *Phomopsis* taxon 2, were associated with the disease Phomopsis cane and leaf spot (Merrin *et al.*, 1995). Although the pathogenicity of *Phomopsis* taxon 2 has been demonstrated (Emmett *et al.*, 1998; Gubler and Leavitt, 1992), the role of taxon 1 is poorly understood. *Phomopsis* taxon 2 causes symptoms such as leaf spots and deep lesions on canes, however, both taxon 1 and taxon 2 cause bleaching of cane in winter. Taxon 1 infection is assumed to be less damaging, although it has been suggested that taxon 1 causes failure of buds to burst, delayed bud burst and stunting of shoots (Scheper *et al.*, 1997).

Mostert *et al.* (2001) reported that *Phomopsis* taxon 1 (as *Diaporthe perjuncta*) was not pathogenic to grapevine. This assumption, however, was based on the production of lesions on green shoots by one isolate only and with limited replication. In an earlier experiment, isolates of *Phomopsis* taxon 1 and taxon 2 from grapevine could not be distinguished in pathogenicity experiments based on the development of lesions on shoots (Mostert *et al.*, 2000). Furthermore, it has been observed that *Phomopsis* taxon 2 is isolated mostly from buds and nodes (Mostert *et al.*, 2001), whereas little is known about *Phomopsis* taxon 1 infection of grapevine. Many growers continue to use fungicides to control Phomopsis cane and leaf spot, regardless of which type of *Phomopsis* is present in the

vineyard. Chemicals are applied because both taxa cause bleaching of canes and there is limited information on the pathogenicity of *Phomopsis* taxon 1.

The objective of the research described in this section was to determine if *Phomopsis* taxon 1 is pathogenic on grapevine and, if so, to compare the susceptibility of a range of cultivars to infection by *Phomopsis* taxon 1 and 2 using excised grapevine shoots in the glasshouse. To examine the effect of *Phomopsis* taxon 1 on bud burst, the following inoculation experiments were undertaken on; (1) lateral buds and leaves, (2) dormant buds and (3) premature leaf scars and wounds below dormant buds. Preliminary investigations by Melanson *et al.* (2002) indicated that *Phomopsis* taxon 1 infects subcuticularly in tissue cultured plantlets. Therefore, in the present study, histological studies were undertaken to assess fungal colonisation of mature grapevine stems.

3.2 Materials and methods

3.2.1 Production of conidia

Carnation leaf agar (CLA) was used to produce conidia of *Phomopsis* taxon 1 *in vitro*. Fresh carnation leaves were cut to approximately 1 cm in length, surface sterilized in 0.5 M sodium hypochlorite (NaOCl) for 30 sec, followed by three 3-min rinses in ddH_2O and sterilised in foil packages by autoclaving at 121°C for 20 mins. Packages were quick frozen in liquid nitrogen and stored at –70°C until required.

Six to eight pieces of sterile carnation leaf were placed on the surface of 1.2 % water (ddH_20) agar (Technical grade 3, Oxoid, England) in each 9 cm Petri dish before the agar solidified. A mycelial plug (*ca* 1 × 1 cm) from a *Phomopsis* isolate on PDA was placed in the centre of the CLA plate and incubated at 22°C in light (Philips TLD 18W/33 3F, 380 nm–780

nm and near ultraviolet, 380nm-400nm) for 12 hours and 16°C in the dark for 12 hours for 14-21 days (see section 2.1).

3.2.2 Preparation of spore suspension

Cirrhi produced by each isolate were removed from CLA and conidia observed at × 400 magnification to confirm the taxon of *Phomopsis.* Due to the small number of pycnidia present on the plates, individual cirrhi were removed with a sterile fine needle (Williamson *et al.*, 1995). Several cirrhi, collected from the same isolate, were immersed in sterile ddH_20 until the suspension was cloudy in appearance. Spore suspensions were prepared in McCartney bottles and the concentration adjusted to 10^4-10^6 conidia/ml using a haemocytometer.

It was observed that pycnidia of some isolates were produced on the underside of the carnation leaf as well as the upper surface and edges, therefore, in some instances, the entire leaf piece was placed in 1 ml sterile ddH_20 water to obtain conidia. The suspension was gently shaken by hand to release conidia from the cirrhi (Shivas, 1994) and the concentration of conidia adjusted as above.

To assess conidial germination, five 10 µl droplets of spore suspension were placed separately on 1.2 % water agar (Technical grade 3 agar, Oxoid, in ddH_20) and incubated overnight at 22°C/16°C with a 12 hour light/dark daily cycle, as described in section 2.1. Preliminary investigations showed maximum germination of conidia was achieved after 16 hours. Germination of conidia was determined by viewing the conidia on the agar plate at × 400 magnification with a compound microscope.

3.2.3 Isolates used in pathogenicity experiments

Isolates of *Phomopsis* used in the study were chosen based on origin, abundant growth of mycelium, similar stage of development at the time of inoculation and adequate quantity of conidia for preparation of spore suspensions. In some cases, it was not possible to use the same isolate in all experiments due to poor mycelial development, failure to produce conidia, mite infestation or contamination of pure cultures. Table 3.1 lists the isolates of *Phomopsis* taxon 1 and taxon 2 assessed in this study.

3.2.4 Experiment 1 - inoculation of shoots with mycelium

To compare symptoms caused by *Phomopsis* taxon 1 and taxon 2, green excised shoots were inoculated with mycelium of the fungus in the glasshouse. Green 6-month-old shoots (cv. Sultana), 30 cm long, 0.5 cm-1.2 cm diameter, were pruned from healthy grapevine in the Coombe vineyard of the Waite Agricultural Research Institute. Sultana has been reported to be highly susceptible to *Phomopsis* infection (Baltovski, 1980). All leaves and tendrils were removed. Shoots were swabbed with 70% ethanol and placed in 500 ml plastic jars containing 200 ml tap water (Figure 3.1).

Eight shoots were inoculated with one of nine treatments; *Phomopsis* taxon 1 isolates A223.1, H307, L406, J5; *Phomopsis* taxon 2 isolates C603, P712, 902.4, M851; and an uncolonized PDA plug (control). Shoots were wounded 10 cm from the top of the shoot by removing the cortex with a 4-mm diameter metal cork borer.

Table 3.1. Isolates of *Phomopsis* taxon 1, taxon 2 and taxon 3 used in pathogenicity experiments, cultivars inoculated and corresponding inoculation method.

Isolate	Taxon	Site	Expt 1 cv. Sultana Shoot mycelium	Expt 2 cv. Sultana Shoot mycelium	Expt 3 Various cv. Shoot mycelium	Expt 4 cv. Shiraz Bud mycelium	Expt 5 cv. Chardonnay Bud & leaf conidia	Expt 6 cv. Shiraz Bud conidia	Microscopy
A223.1	1	Ashton, SA*	✓	✓	✓	✓			✓
A223.2	1	Ashton, SA*		✓					✓
H307	1	Hargrave, SA*	✓	✓			✓		
L406	1	Hillstowe, SA	✓	✓				✓	
J4	1	Mt Jagged, SA*		✓					
J5	1	Mt Jagged, SA*	✓	✓					✓
L427	1	Lenswood, SA*		✓					
L424	1	Lenswood, SA*		✓					
T100	1	McLaren Vale, SA		✓					
T101	1	Langhorne Ck, SA		✓					✓
M860	1	Vasse Felix, WA		✓					
51C.1	1	Coonawarra, SA					✓		
M831.1	2	Wagga, NSW		✓					✓
M851	2	McLaren, SA	✓		✓				✓
LR10T6	2	Lenswood, SA					✓	✓	
C603	2	Coonwarra, SA	✓						
P712	2	Padthaway, SA	✓						
902.4	2	Robinvale, Vic	✓			✓			
DAR69458	3	Yarra Valley, NSW		✓					

*denotes sites used for 3-year field trial (see chapter 4)

A mycelial plug, taken from the margin of an expanding 8-day-old *Phomopsis* colony grown on PDA, was cut with the cork borer and inserted into the wound. The cork borer was washed in 70% ethanol between treatments. Each wound was sealed with Parafilm™ and shoots maintained in a glasshouse at 25°C with natural light. The water was replaced every second day. Lesions (minus diameter of initial wound) were measured 14 days after inoculation.

Four shoots were randomly taken from each treatment for re-isolation of the fungi. Pieces of stem tissue were removed from the margin of the lesion and cut into approximately 5 x 5 mm and surface sterilised with 0.5% NaOCl (Milton, Australia) for 30 sec followed by three successive 1-min rinses in sterile ddH_20. Sections were plated on PDA and incubated for several days at 16°C/22°C daily cycle as described previously.

Figure 3.1. Excised shoots (cv. Sultana) inoculated with mycelium of *Phomopsis* or agar (control).

In addition, DNA was extracted, using the CTAB DNA extraction procedure (section 2.2.1), from the four shoots used for re-isolation of the fungi. The lesions, or discoloured area around the wound site, were removed with a scalpel to approximately 40 mm in length, or to a maximum weight of 0.1 g. This DNA and purified DNA from *Phomopsis* taxon 1 and taxon 2, *Aspergillus* sp., *Botrytis cinerea* and grapevine DNA was used in slot blot analysis (section 2.2.3). The membrane was radioactively labelled with *Phomopsis* taxon 1 and taxon 2-specific probes, pT1P180 and pT2P25, respectively as described in section 2.2.5.

3.2.5 Experiment 2 – assessment of pathogenicity of *Phomopsis* taxon 1

The inoculation method described in section 3.2.4 was also used to assess the virulence of a number of *Phomopsis* taxon 1 isolates. Green 6-month-old shoots (cv. Sultana), 30 cm long, 0.5 mm-1.0 mm in diameter, were pruned from healthy grapevine in the Coombe vineyard of the Waite Agricultural Research Institute and prepared as described in section 3.2.4.

The *Phomopsis* isolates chosen were based on their similar growth rates of mycelium in culture and to represent different vineyards known to be infected by *Phomopsis* taxon 1. Eleven *Phomopsis* taxon 1 isolates (A223.1, A223.2, H307, L406, J4, J5, L427, L424, T100, T101, M860), one *Phomopsis* taxon 2 isolate (M831.1), a *Phomopsis* taxon 3 isolate (DAR69458) and an uncolonized PDA plug (control), were used to inoculate the shoots (Table 3.1). A total of 112 shoots was inoculated using eight replicate shoots per treatment. Shoots were wounded and inoculated as described in section 3.2.4, however the inoculum was taken from 7-day-old *Phomopsis* cultures on PDA. Two shoots were selected randomly from each treatment for re-isolation of the fungi. Incubation, assessment and re-isolation of the fungi from the margin of the lesions were conducted as detailed in section 3.2.4, but detection of *Phomopsis* using hybridisation analysis was not attempted.

3.2.6 Experiment 3 - susceptibility of grapevine cultivars to *Phomopsis* taxon 1 and taxon 2

A range of grapevine cultivars was assessed for susceptibility to *Phomopsis* taxon 1 and taxon 2 infection. Green 6-month-old shoots of four red grape varieties (cvs Shiraz, Grenache, Merlot and Cabernet Sauvignon) and three white grape varieties (cvs Semillon, Chardonnay and Sauvignon Blanc) were collected to assess the susceptibility of selected cultivars to infection by *Phomopsis* taxon 1. The selection of cultivars was based on availability of material. Green shoots, 30 cm long, 0.5 mm-1.0 mm diameter, were collected from the Coombe vineyard and Alverstore vineyard of the Waite Agricultural Research Institute in February and prepared as described in section 3.2.4.

Eight replicate shoots of each cultivar were inoculated with *Phomopsis* taxon 1 isolate A223.1, *Phomopsis* taxon 2 isolate M851 or a sterile PDA plug (control). A total of 168 shoots was inoculated with 24 shoots per treatment. Shoots were wounded and inoculated as described in section 3.2.4, however the inoculum was taken from 14-day-old *Phomopsis* cultures. Incubation, assessment and re-isolation of the fungi from the margin of the lesions in the seven cultivars were conducted as detailed in section 3.2.4, but DNA was not assessed. In addition, three excised shoots of Semillon were selected for re-isolation of isolate A223.1 from both necrotic lesions and 20-40 mm distant from the point of inoculation where no disease symptoms were observed. Seven 5 × 5 mm pieces, necrotic or symptomless, were cut from the three Semillon shoots and plated onto PDA as described section 3.2.4. Data were transformed to give a normal sampling distribution and log transformation values were subjected to ANOVA using Statistix® analytical software.

3.2.7 Experiment 4 – inoculation of dormant buds with mycelium

The main objective of this study was to examine the effect of *Phomopsis* taxon 1 on budburst. Two inoculation methods using mycelium were devised to facilitate rapid colonisation of the bud either by (1) penetration through a wound site or (2) penetration through a prematurely-created leaf scar.

Thirty disease-free certified dormant grapevine cuttings (cv. Shiraz) were obtained from the Vine Improvement Centre, Monash, SA and stored at 4°C before use. Cuttings were submerged in RO water for 24 hours at 4°C, then in 25 L RO water containing 10 ml sodium hypochlorite for a further 24 hours. The residual sodium hypochlorite was removed by soaking the cuttings in RO water for 24 hours. The base of each cutting was dipped in an aqueous solution of 2 g/L indole-3-butyric acid (IBA; Sigma, USA) for 40 sec. Cuttings were transferred directly into water-soaked rock-wool blocks (40 × 40 mm) and planted in 20 cm pots containing UC (University of California) potting mix and Osmocote® slow-release fertilizer (Yates, Australia). Grapevine plants were maintained in a glasshouse at 25°C with natural light and watered every day until required.

Prior to inoculation, green shoots were removed from the thirty 10-month-old grapevine plants. The remaining lignified cane, approximately 0.7 mm in diameter, consisted of dormant buds, which were prepared for inoculation by; (1) wounding below the bud with a scalpel or (2) removal of the leaf below the bud resulting in a premature leaf scar. Ten plants were used per treatment and 36 buds inoculated per treatment. The three treatments were; (1) *Phomopsis* taxon 1 isolate A223.1, (2) *Phomopsis* taxon 2 isolate 902.4 and (3) a PDA plug (control).

For the first method of inoculation, 16 dormant buds were selected among four of the plants per treatment. Due to variation in the number of buds per plant, the number of buds

inoculated was not the same on each plant. For example, plant one had two buds inoculated with *Phomopsis* taxon 1, plant two had six buds inoculated, plant three five buds inoculated and plant four had three buds inoculated, giving a total of 16 buds inoculated for the treatment. A wound (*ca* 5 mm wide, 3 mm deep) was made below each dormant bud with a scalpel. A mycelial plug of each isolate was taken from the margin of an expanding 7-day old *Phomopsis* colony on PDA and inserted into the wound.

The second method of inoculation was devised to recreate naturally-produced leaf scars on grapevine. Grapevines were at the stage of leaf abscission, therefore premature leaf scars beneath the bud were created by detaching the remaining petioles by hand (Uddin and Stevenson, 1997). Most leaf scar wounds exposed green tissue under the epidermis. Twenty buds were selected on six plants for inoculation with a mycelial plug of each *Phomopsis* taxon 1 or taxon 2 isolate as described above and all wounds sealed with Parafilm™. As for the first method, the number of buds inoculated was not the same on each plant.

For controls, 10 plants were inoculated with a sterile PDA plug following wounding by the two methods as described. In total, 36 buds were inoculated.

Plants were immediately placed in a shade house and maintained for at least 8 weeks until bud burst. During this time, the average daily temperature was 15°C day/7°C night. Plants were hand-watered when required. The percentage of buds burst, bleaching and development of pycnidia were recorded over 8 weeks, commencing at budburst. Four plants from each treatment were selected for re-isolation of the fungi. Data were subjected to general multi-factorial ANOVA in Statistix® analytical software.

3.2.8 Experiment 5 - inoculation of lateral buds and leaves with conidia

Grapevine plants were inoculated to determine the effect of *Phomopsis* taxon 1 infection on newly-developed buds and to clarify differences in symptoms caused by *Phomopsis* taxon 1 and taxon 2. Mature grapevine plants (cv. Chardonnay) were obtained from Catherine Hitch, SARDI. Plants were grown in UC potting mix in 20 cm black plastic bags in a growth room at 23°C under light for 14 hours and at 18°C in darkness for 10 hours. Plants were watered every day.

Suspensions of conidia of *Phomopsis* taxon 1 and taxon 2 were prepared and the viability of conidia determined as in section 3.2.2. Four treatments were applied; (1) *Phomopsis* taxon 1 isolate H307 at 10^4 α-conidia/ml, (2) *Phomopsis* taxon 1 isolate 51C.1 at 10^6 α-conidia/ml, (3) *Phomopsis* taxon 2 isolate LR10T6 at 10^6 α- and β-conidia/ml and (4) sterile ddH_20 as a control. It was not possible to use only α-conidia for isolate LR10T6, as cirrhi produced in culture comprised a high proportion of β-conidia.

Eight grapevine plants were used for each treatment. Plants were pruned 4 weeks prior to inoculation and, again, to two green shoots per plant 1 day before treatment. Each shoot had a lateral bud at the first node, and healthy leaves. On one shoot, the bud at the first node was inoculated, on the other the leaf at the first node was inoculated (Figure 3.2). The basal buds, or basal leaves, were not inoculated. The length of each shoot was recorded at the time of inoculation.

To provide the high humidity conducive for infection, clear plastic bags (65 × 40 cm) were sprayed inside with ddH_20 and placed over a garden stake to cover the individual shoots. Each bud was inoculated with 10 μl spore suspension or water for controls. The bag was tied with plastic-coated garden wire around the base of the shoot.

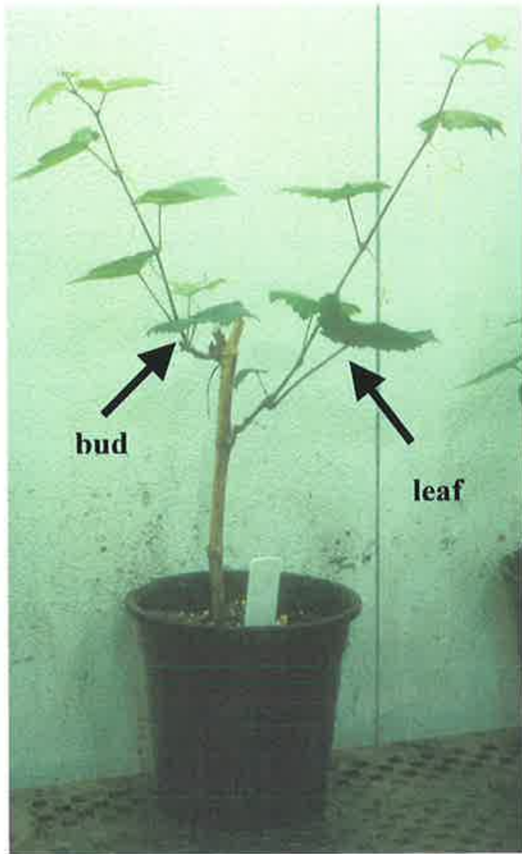


Figure 3.2. Grapevine as inoculated in experiment 5 (cv. Chardonnay). Bud inoculated on first shoot (arrow), a leaf inoculated on the other shoot (arrow) with conidia of *Phomopsis* taxon 1, *Phomopsis* taxon 2 or ddH_20 (control).

Leaves were inoculated by spraying a fine mist of spore suspension on both sides of the leaf using a Jet-Pak power unit (Wattyl, Australia). Contamination of surrounding plant material was minimised by inoculating the leaf in the plastic bag as above. A different spray nozzle was used for each treatment. The temperature inside the bags was measured with a temperature data-probe (Tinytalk II®, Hastings, Australia) over the next 48 hours after which the bags were removed.

Buds, leaves and internodes of inoculated shoots were rated for disease severity weekly for 8 weeks. A 0-4 scale was used based on the Barratt and Horsfall rating system and modified from Phillips (1998), as follows:

0 = no signs of disease

1 = few lesions covering no more than 25% of leaf or internode

2 = many lesions covering 25-50% of leaf or internode

3 = over 50 % leaf or internodal area severely scarred or necrotic

4 = death of shoot or bud (no shoots emerged).

The inoculated buds and leaves, in addition to non-inoculated tissue displaying symptoms such as leaf spot, were removed 8 weeks after inoculation. Leaf tissue was cut into 1 cm × 1 cm pieces and surface sterilised in 0.5 M NaOCl for 3 min followed by three 1-min rinses in ddH_20. Tissue was transferred to PDA and incubated at 22°C/16°C in a light/dark daily cycle for up to 6 days. In addition, woody cane of the original propagating material was placed in moist conditions at 15°C in darkness to induce production of pycnidia (see section 2.1).

DNA was extracted, using the SEAPS extraction method (section 2.2.1), from (1) buds inoculated with *Phomopsis* taxon 1 or control, (2) buds close to the inoculated leaves and (3) other buds not inoculated. In total, 96 samples were processed. DNA was not extracted from vines inoculated with *Phomopsis* taxon 2. Two slot blot membranes were prepared (section 2.2.3) using the total DNA as above and each slot included DNA from pure cultures of *Phomopsis* taxon 1, *Phomopsis* taxon 2 and grapevine DNA as controls. Total DNA was hybridised with the *Phomopsis*-specific DNA probes described in section 2.2.6. Data were subjected to ANOVA and regression analysis in Statistix® analytical software.

3.2.9 Experiment 6 - inoculation of dormant buds with conidia

Dormant cuttings of grapevine (cv. Shiraz), certified disease-free, were obtained from the Vine Improvement Centre, Monash, SA and treated as described in section 3.2.5, except that rock-wool blocks were uncovered when planted in 20 cm pots containing UC potting mix and 10 ml Osmocote® (Yates). Plants were maintained at 19°C/14°C in a 12 hour daily cycle in a

refrigerated glasshouse compartment to minimise the effects of natural temperature fluctuations in the glasshouse. After 1 week, pots were completely filled with UC potting mix. Plants were fertilised regularly with Nitrosol® (N,P,K: 12.2%, 2.9%, 8.5%, respectively, Garden King, Australia) and sprayed every 2-4 weeks with Triumph® insecticide (Yates) for control of mites and sap-sucking insects.

Eight dormant cuttings, consisting of three buds per cane, were used per treatment. Isolates were selected on the basis of conidium production. The four treatments were; (1) inoculation of buds with *Phomopsis* taxon 1 isolate L406, (2) inoculation of buds with *Phomopsis* taxon 2 isolate LR10T6, (3) inoculation of buds with sterile ddH_20 and (4) inoculation of the epidermis of the propagating cane with *Phomopsis* taxon 1 isolate L406. In total, 24 buds per treatment were inoculated with either fungus or water. Conidial suspensions were prepared and adjusted to 10^6 α-conidia/ml with a haemocytometer as in section 3.2.2. Three dormant buds on each vine were inoculated by placing a 50 µl droplet of conidial suspension onto the surface of the bud with a pipette. In most cases, the suspension was placed between the outer bud scales without disruption to the bud.

The second method of inoculation involved spraying the entire grapevine cutting with a fine mist of conidial suspension of *Phomopsis* taxon 1 using a Jet-Pak power unit (Wattyl). All plants were enclosed in clear plastic bags (30 × 40 cm) wetted with ddH_2O. The plants were inoculated in a growth room at 19°C/14°C in a light/dark daily cycle. A temperature data-probe was placed inside one of the bags to measure the temperature for the 48 hour dew period as in section 3.2.8. Each bud was assessed weekly for stage of development, shoot length and disease severity over 8 weeks. The Modified Eichhorn-Lorenz system was used to determine stage of development, such that:

1 = winter bud (dormant)

2 = bud swell

3 = woolly bud – brown bud visible

4 = green tip; first leaf tissue visible (budburst)

and thereafter 5 = shoot (modified from Hood and Shew, 1996).

A rating of "0" indicated death of a dormant or developing bud. Disease severity was scored according to section 3.2.8 and data analysed by ANOVA in Statistix™ analytical software.

3.2.10 Colonisation of grapevine tissue

To assess the colonisation of host tissue by *Phomopsis* taxon 1 and taxon 2, material from experiment 2 (section 3.2.5) and experiment 3 (section 3.2.6) was used. Three replicate excised shoots inoculated with either *Phomopsis* taxon 1 isolates A223.2, J5, T101 or *Phomopsis* taxon 2 isolate M831.1 (section 3.2.5) were selected. Excised shoots inoculated with PDA (control treatment) were used for comparison. At a later date, colonisation of Grenache shoots inoculated with *Phomopsis* taxon 1 isolate A223.1 and *Phomopsis* taxon 2 isolate M851 (section 3.5.6) was also assessed. Tissue samples were taken at the site of wounding and at intervals of 10 mm from the point of inoculation. Sections up to 40 mm from the margin of the lesion were assessed.

Fresh shoot tissue was cut to 10 mm sections and submerged in 25 ml of 1 M KOH in a McCartney bottle for a minimum of 30 min at room temperature, followed by three rinses in sterile ddH_2O for approximately 5 min each, modified from Hood and Shew (1996). Tissue was then hand-sectioned with a scalpel, either as a cross-section of the circumference of the shoot or a longitudinal section of the epidermis, and mounted on glass slides. Tissue was directly stained with 0.05% aniline blue (dye CI #42755, BDH, England) in 0.067 M K_2HPO_4 pH 9.0 and observed with a UV microscope at × 100 magnification (Olympus, Japan). The

microscope was equipped for epifluorescence microscopy with a 100 W high pressure mercury burner and BP495 exciter: barrier fluorescence filters.

3.3 Results

3.3.1 Production and germination of conidia

Fourteen days after inoculation, cream-coloured cirrhi were produced mainly on the edges of carnation leaf pieces, with zone lines appearing on some leaves after 10 days (Figure 3.3). One cirrhus, transferred to 100 µl sterile ddH_2O, yielded approximately 4.2×10^5 spores/ml. The percentage germination of α-conidia of *Phomopsis* taxon 1 isolates H307 and 51C.1 was 100% after incubation for 16 hours.

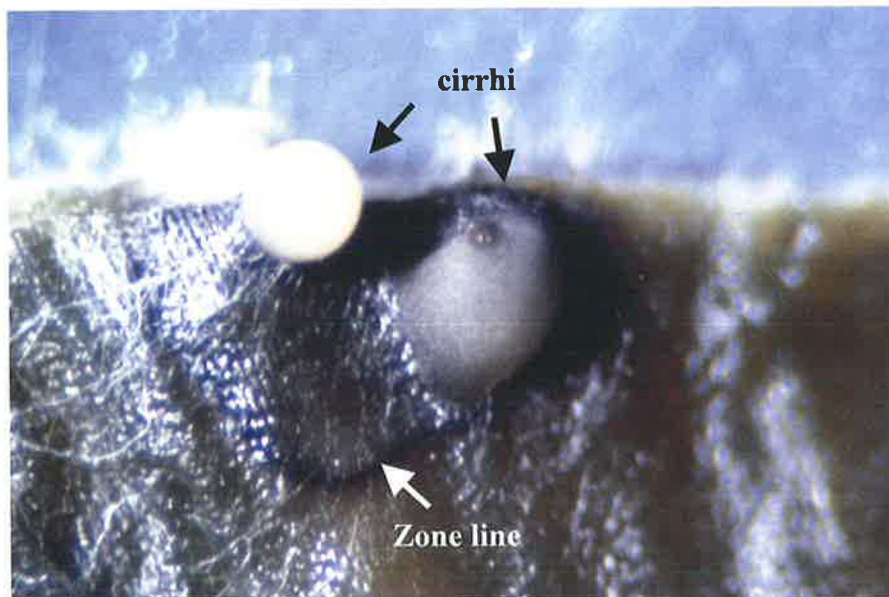


Figure 3.3. Cirrhi of *Phomopsis* taxon 1 isolate H307 (black arrows) on carnation leaf agar (CLA) produced 14 days after incubation. Note the zone line (white arrow) on the carnation leaf.

The rate of conidium production varied among isolates of *Phomopsis* taxon 1 and taxon 2, with taxon 2 isolates producing conidia more readily than those of taxon 1. Several isolates of *Phomopsis* taxon 2 were assessed for production of conidia, but only isolate LR10T6 produced conidia at the same time as *Phomopsis* taxon 1 isolate H307. Isolate LR10T6 produced a greater proportion (>60%) of β-conidia than α-conidia. Germination of β-conidia was not evident, however, 100% of the α-conidia germinated during 16 hours of incubation.

3.3.2 Experiment 1 – inoculation of excised shoots with mycelium

Browning or discolouration of tissue at the wound site was observed in most excised shoots 3 days after inoculation with *Phomopsis* taxon 1 or taxon 2. Isolates of *Phomopsis* taxon 1 caused minor brown, soft lesions around the inoculation site (Figure 3.4a) similar to those produced on shoots inoculated with PDA. The lesions did not resemble those caused by isolates of *Phomopsis* taxon 2 (Figure 3.4b).

Most shoots inoculated with *Phomopsis* taxon 1 remained green and turgid after 2 weeks. There was no significant variation among the isolates of *Phomopsis* taxon 1 (P=0.565). Isolate J5 produced pycnidia along the entire shoot of several replicate shoots and bleaching was evident. Pycnidia were also observed on shoots inoculated with *Phomopsis* taxon 1 isolates A223.1 and H307. Furthermore, a zone line developed around the wound site of an excised shoot inoculated with isolate H307. Tissue inoculated with isolates of *Phomopsis* taxon 2 appeared dark brown/black and developed into longitudinal, dry lesions that resembled characteristic symptoms of Phomopsis cane and leaf spot in the vineyard. *Phomopsis* taxon 2 caused lesions up to 41 mm long, however, the length of lesions produced by the four taxon 2 isolates was highly variable among replicates (Figure 3.5).

(a)

(b)

Figure 3.4. Lesions on green excised shoots (cv. Sultana) 2 weeks after inoculation. **(a)** Replicate shoots inoculated with *Phomopsis* taxon 1 isolate H307, **(b)** replicate shoots inoculated with *Phomopsis* taxon 2 isolate 902.4. Wound = 4 mm diameter.

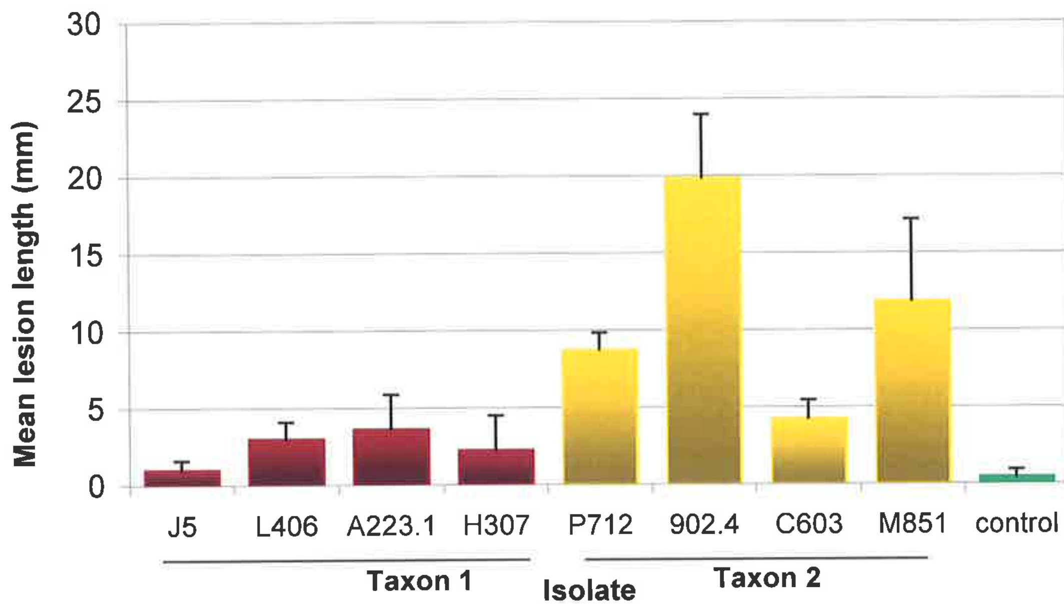


Figure 3.5. Average length of lesions (mm) produced on excised shoots (cv. Sultana) 2 weeks after inoculation with *Phomopsis* taxon 1, taxon 2 and PDA (control). Bars represent standard error.

Cracking of the epidermis was observed on 30% of the replicate shoots inoculated with three isolates except isolate C603.

Inoculation with *Phomopsis* taxon 2 produced many lesions with deep cracks (*ca* 0.5 mm deep) at the wound site. Pycnidia were also produced on shoots showing severe lesion development. For example, pycnidia were produced in seven of the eight replicate shoots inoculated with *Phomopsis* taxon 2 isolate 902.4. Immature pycnidia were also observed on shoots inoculated with isolate M851. Bleaching, or distinct pale discolouration of the epidermis, was evident on shoots inoculated with isolates 902.4 and M851. Overall, the health of shoots inoculated with *Phomopsis* taxon 2 was poor compared to shoots inoculated with taxon 1 and PDA (control).

No lesions developed on control shoots (Figure 3.6). The area surrounding the wound was dark green, however, this was most likely a direct result of tissue damage. The mean

length of lesions caused by *Phomopsis* taxon 1 infection was 2.5 mm compared to 11.2 mm following inoculation with taxon 2 (Table 3.2). The "lesion" lengths shown in Figures 3.4 and Table 3.2 refer to discolouration only. All shoots remained healthy and budburst had occurred on several shoots.

Figure 3.6. Lesion on green excised shoot (cv. Sultana) 2 weeks after inoculation with uncolonised potato dextrose agar (control).

Table 3.2. Average length of lesions (mm) produced on excised, green shoots 2 weeks after inoculation with *Phomopsis* taxon 1, taxon 2 and PDA (control). Data for all isolates are combined*.

Treatment	Average lesion length (mm)	Mean lesion length (mm)	Standard deviation
Phomopsis taxon 1	2.5	0-7.0	3.8
Phomopsis taxon 2	11.2	0-41.0	11.1
Control	0.4	0-1.5	0.6

*mean lesion length based on inoculation of eight replicate excised green shoots for each treatment. Each treatment included four isolates of *Phomopsis* taxon 1 or taxon 2 or potato dextrose agar (PDA, control).

Phomopsis taxon 1 was re-isolated on PDA from tissue taken from the margin of the brown areas on four replicate shoots inoculated with each isolate. The mycelium was white/cream and fast-growing. Pycnidia developed on stem tissue inoculated with isolate J5 after 3 weeks of incubation. Similarly, *Phomopsis* taxon 2 was re-isolated from the margins of lesions inoculated with isolates of the fungus. Although mycelium was slower-growing than mycelium of taxon 1 isolates, *Phomopsis* taxon 2 isolate 902.4 produced pycnidia and α-conidia were subsequently identified. The lesions which developed on the shoots inoculated with C603 differed from those produced by the other *Phomopsis* taxon 2 isolates. Pycnidia were produced on PDA, but no cirrhi or conidia were observed. It is possible that isolate C603 was no longer virulent after sub-culturing over time or was not, in fact, *Phomopsis.* The taxon 2-specific probe, pT2P25, did not hybridise to purified DNA of isolate C603, therefore the isolate was either not Phomopsis taxon 2 or poor transfer of DNA resulted in lack of hybridisation (Table 3.3).

Sections removed from the excised shoots inoculated with PDA (control) were shown to be infected with various fungi, including *Aspergillus* spp., *Penicillium* spp. and yeasts. No *Phomopsis* was detected.

Table 3.3. Detection of *Phomopsis* taxon 1 and taxon 2 in inoculated grapevine shoots by isolation onto PDA or by hybridisation of DNA with the taxon 1-specific probe, pT1P180 and the taxon 2-specific probe, pT2P25, in a slot blot assay*.

Isolate	*Phomopsis* taxon	PDA	Slot blot
J5	1	✓	✓
L406	1	✓	✓
A223.1	1	✓	✗
H307	1	✓	✗
P712	2	✓	✗
902.4	2	✓	✗
C603#	?	?	✗
M851	2	✓	✗

* Total DNA extracted from four replicate shoots of each treatment. Poor transfer of DNA to the membrane resulted in no or low hybridisation signals.
\# Morphology of isolate C603 did not resemble that of *Phomopsis* taxon 1 or taxon 2 on culture medium.

In some instances, it appeared that DNA did not bind to the nylon membrane during slot blot preparation, therefore results of hybridisation of the *Phomopsis*-specific probe to DNA obtained from shoots were inconclusive. This was further supported by the *Phomopsis* taxon 1-specific probe, pT1P180, showing no or very low hybridisation to purified DNA of *Phomopsis* taxon 1. Lack of DNA obtained from tissue samples prevented preparation of another slot blot membrane. Nevertheless, faint hybridisation signals were observed for shoots inoculated with *Phomopsis* taxon 1 isolates J5 and L406. In this experiment, the presence of *Phomopsis* in inoculated tissue was confirmed by conventional isolation onto PDA (Table 3.3).

3.3.3 Experiment 2 - assessment of pathogenicity of *Phomopsis* taxon 1

There was considerable variation observed between and within lesions on shoots inoculated with isolates of taxon 1 (Table 3.4). Shoots inoculated with *Phomopsis* taxon 1 isolates A223.1, J4, H307 and M860 produced minor lesions (< 5 mm) on all replicate shoots. In comparison, shoots inoculated with *Phomopsis* taxon 1 isolates A223.2, L427, T101 and J5 produced lesions ranging in length from 0 – 28 mm. Lesions greater than 20 mm long occurred on one replicate shoot only of each of these isolates, and did not appear to be in accordance with other observations. Re-isolation of the fungi from the excised shoot showed *Aspergillus* sp. and *Phomopsis* taxon 1 were present.

Data for symptoms on the excised green shoots (cv. Sultana) inoculated with 11 isolates of *Phomopsis* taxon 1 were combined and showed that 73% of shoots produced either no lesions or small lesions (<5 mm) resembling pale to dark brown rings around the wound area. This discolouration was indicative of damage to stem tissue at the time of wounding. The average lesion length on shoots inoculated with the isolates of *Phomopsis* taxon 1 was

4.2 mm (Figure 3.7). Overall, a minimum of three replicate shoots inoculated within each *Phomopsis* taxon 1 treatment did not produce lesions.

Table 3.4. Mean lesion length on excised green shoots (cv. Sultana) 2 weeks after inoculation with mycelium of 11 isolates of *Phomopsis* taxon 1, one isolate each of *Phomopsis* taxon 2 and *Phomopsis* taxon 3 and agar (control)[y].

Treatment	**Isolate**	**Mean lesion length (mm)** [z]	**Standard deviation**
Phomopsis taxon 1	T101	10.4 [b]	7.90
Phomopsis taxon 1	L427	6.6 [bc]	9.39
Phomopsis taxon 1	A223.2	6.1 [bcd]	7.77
Phomopsis taxon 1	J5	5.6 [bcde]	8.89
Phomopsis taxon 1	T100	4.3 [cde]	3.20
Phomopsis taxon 1	L406	3.3 [cde]	3.71
Phomopsis taxon 1	L424	3.1 [cde]	4.29
Phomopsis taxon 1	A223.1	3.0 [cde]	2.00
Phomopsis taxon 1	H307	2.0 [cde]	2.56
Phomopsis taxon 1	J4	0.7 [de]	1.03
Phomopsis taxon 1	M860	0.6 [de]	1.40
Phomopsis taxon 2	M831.1	23.0 [a]	12.57
Phomopsis taxon 3	DAR69458	0.1 [e]	0.35
Control	-	0.9 [cde]	1.13

[y] mean lesion length based on inoculation of eight replicate excised green shoots for each treatment
[z] means followed by different letters within a column are significantly different (P=0.05) based on LSD (least significant difference).

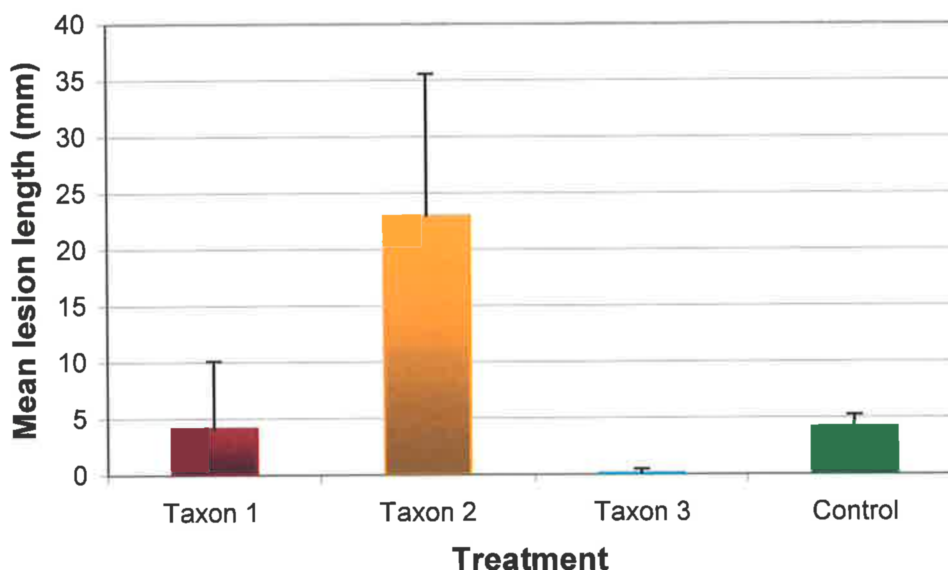


Figure 3.7. Mean lesion length (mm) on excised, green shoots (cv. Sultana) 2 weeks after inoculation with isolates of *Phomopsis* and agar (control). Mean length derived from 88 shoots inoculated with 11 *Phomopsis* taxon 1 isolates (data combined), eight replicate shoots inoculated each with *Phomopsis* taxon 2, *Phomopsis* taxon 3 and PDA (control). Bars represent standard deviation.

Pycnidia were observed on two replicate shoots inoculated with *Phomopsis* taxon 1 isolate L406.

Lesions did not develop on control shoots treated with a plug of PDA (Figure 3.8a). Wounds were generally dry, and cracking appeared at the margin of the wound in two replicates. Wounded tissue appeared dark green and shoots remained healthy during the assessment period.

Inoculation with the *Phomopsis* taxon 3 isolate DAR69458, did not cause lesion development and slight discolouration was observed on one shoot only (Figure 3.8a). Lesions produced on shoots inoculated with *Phomopsis* taxon 2 isolate, M831.1, were dark brown-black (Figure 3.8b) and significantly longer (mean of 23 mm, see Figure 3.7) than those caused by the other isolates and the control according to least significance difference (LSD)

(a)

(b)

Figure 3.8. Response of excised green shoots (cv. Sultana) 2 weeks after inoculation with isolates of *Phomopsis* taxon 1, *Phomopsis* taxon 2, *Phomopsis* taxon 3 and PDA (control). **(a)** Wounds inoculated with (from left) *Phomopsis* taxon 3 isolate DAR 69458, PDA (control) and *Phomopsis* taxon 1 isolate A223.2, **(b)** lesion on shoot inoculated with *Phomopsis* taxon 2 isolate M831.1. Wound = 4 mm in diameter.

at the 5% level. Variability was evident within the taxon 2 treatment. Five shoots inoculated with isolate, M831.1, produced lesions longer than 23 mm, with the longest lesion 41 mm. The other three shoots had lesions of 6, 8 and 14 mm long. *Phomopsis* taxon 2 isolates produced lesions that were wider (*ca* 5 mm wide) than lesions produced by isolates of *Phomopsis* taxon 1 (*ca* 1-2 mm wide). Slight bleaching was observed on one shoot inoculated with *Phomopsis* taxon 2 isolate M831.1, however, no pycnidia were present.

Phomopsis taxon 1 and *Phomopsis* taxon 2 were re-isolated from the margin of the lesions in the appropriate inoculated shoots, and also from tissue not displaying symptoms approximately 3 cm from the wound. Sections removed from the excised shoots inoculated with PDA (control) were shown to be infected with various fungi, including *Aspergillus* sp., *Penicillium* sp. and yeasts. *Phomopsis* was not recovered from dark green or discoloured tissue taken from the control shoots.

3.3.4 Experiment 3 – susceptibility of grapevine cultivars to *Phomopsis* taxon 1 and taxon 2

Cultivar, with the exception of Grenache, did not have a significant influence (P=0.2125) on the length of lesions produced after inoculation with *Phomopsis* taxon 1, taxon 2 and PDA (control). However, there were considerable differences in lesion length between treatments (P=0.0000). Most shoots inoculated with the *Phomopsis* taxon 1 isolate A223.1 showed no lesion development (Figure 3.9a) Slight discolouration was observed around the wound site, but this did not expand further. Lesions produced on excised shoots inoculated with taxon 1 were of similar length and severity on all cultivars, except Grenache. Lesions on Grenache were significantly longer (P=0.011) than those on shoots of other cultivars tested.

Phomopsis taxon 2 produced extensive dark brown/black, longitudinal lesions on all cultivars tested (Figure 3.9b). Lesions resulting from inoculation with the *Phomopsis* taxon 2

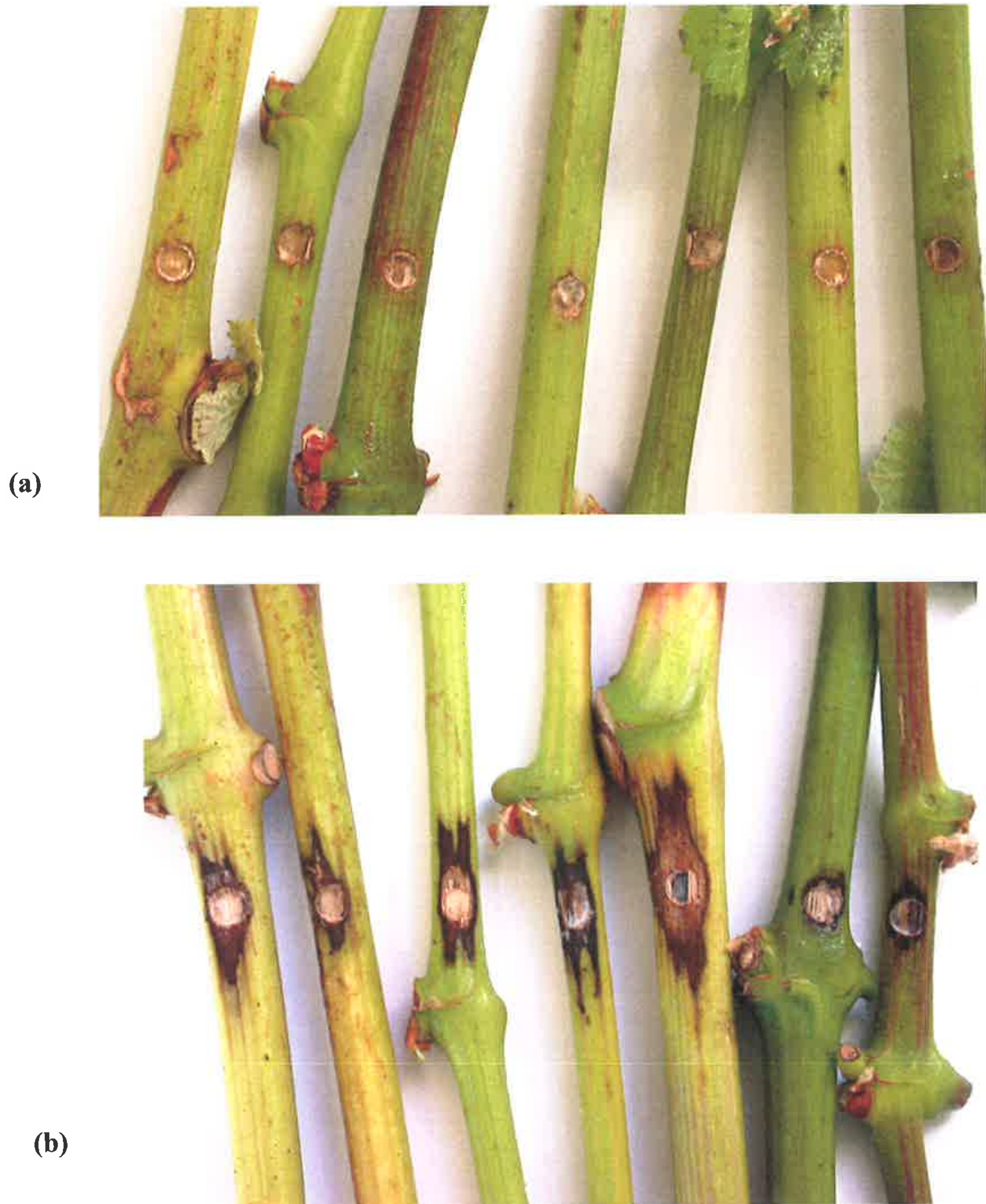


Figure 3.9. Lesion development on excised shoots of (from left) white cultivars: Semillon, Sauvignon Blanc, Chardonnay and red cultivars: Shiraz, Grenache, Cabernet Sauvignon, Merlot after inoculation with **(a)** mycelium of *Phomopsis* taxon 1 isolate A223.1 and **(b)** mycelium of *Phomopsis* taxon 2 isolate M851. Wound = 4 mm in diameter.

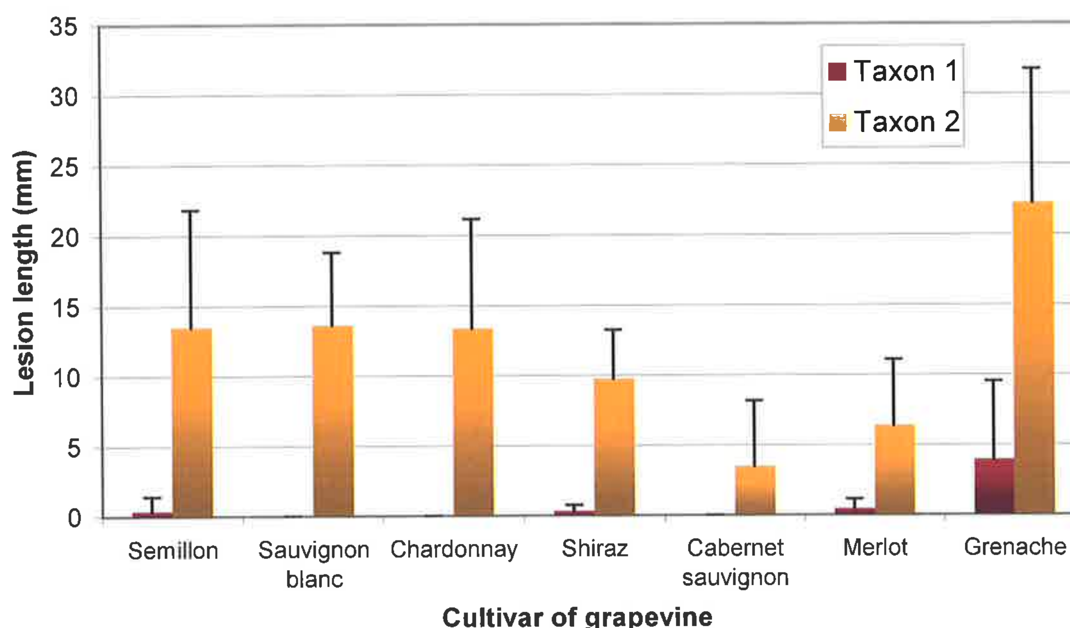


Figure 3.10. Mean lesion length produced on excised green shoots of seven grapevine cultivars 2 weeks after inoculation with *Phomopsis* taxon 1 isolate A223.1 and taxon 2 isolate M851. Values are represented by eight replicates per treatment. Shoots inoculated with PDA (control) did not produce lesions on the seven cultivars (with the exception of a 1 mm lesion produced on one replicate of Grenache). Bars represent standard deviation.

isolate M851 were similar on shoots of the white cultivars, with mean lesion lengths of 13.3 mm, 13.6 mm and 13.4 mm for Semillon, Sauvignon Blanc and Chardonnay, respectively (Figure 3.10). Greater variability was observed between red grape cultivars.

Lesions produced on Grenache shoots were longer and wider (*ca* 8 mm wide) than those on Shiraz, Cabernet Sauvignon and Merlot. In addition, bleaching caused by *Phomopsis* taxon 2 was observed on Grenache shoots only.

No lesions developed on shoots inoculated with agar (control), with the exception of slight discoloration observed in two replicate Grenache shoots. Control shoots remained green and healthy throughout the experiment. *Phomopsis* taxon 1 isolate A223.1 and

Phomopsis taxon 2 isolate M851 were re-isolated on PDA from the margin of the lesions. *Phomopsis* taxon 1 isolate A223.1 was isolated from necrotic regions on three Semillon shoots but no fungus grew from shoot tissue with no symptoms.

3.3.5 Experiment 4 – inoculation of dormant buds with mycelium

No difference was observed in budburst of dormant buds inoculated with mycelium inserted into a scalpel wound or into a premature leaf scar wound. Bud burst commenced on 19 September 2001, however, the timing of bud burst was extremely variable across all buds. Within 2 weeks, only nine buds had exposed green tissue. Final budburst percentage for each treatment was recorded 7 weeks after the commencement of budburst (Table 3.5). Since the number of buds inoculated per plant varied, true replicates did not exist for statistical analysis of the effect of the treatments on budburst. More buds burst on plants inoculated with *Phomopsis* taxon 1 isolate A223.1 than on those inoculated with *Phomopsis* taxon 2 or PDA (control). Likewise, more buds burst after inoculation with *Phomopsis* or PDA compared to non-inoculated buds. In general, budburst percentage was poor regardless of treatment.

Table 3.5. Percentage of budburst on grapevine plants (cv. Shiraz) inoculated with mycelium of *Phomopsis* taxon 1 isolate A223.1, *Phomopsis* taxon 2 isolate 902.4 and PDA (control) and without inoculation.

Taxon	Inoculation method*	% bud burst inoculated	% bud burst not inoculated	% total bud burst
Taxon 1	scalpel wound	50.0	9.5	47.6
Taxon 1	leaf scar	50.0	7.3	31.7
Taxon 2	scalpel wound	37.5	8.7	34.8
Taxon 2	leaf scar	30.0	22.2	35.6
Control	scalpel wound	43.7	8.7	39.1
Control	leaf scar	25.0	21.6	35.1

*Two methods of wounding prior to inoculation (1) wound made with scalpel beneath dormant bud and (2) prematurely-created leaf scar made by detaching the leaf petiole beneath dormant bud. Four plants were wounded with a scalpel and six plants had a premature leaf scar, however, the number of buds inoculated differed between plants. Inoculation commenced 20/7/01.

Analysis of variance for total number of buds burst per treatment indicated that there was no significant difference between treatments (P=1.000). Similarly, there was no significant difference between number of buds burst after inoculation of a scalpel wound or a prematurely-created leaf scar wound (P=0.1250).

In mid-October, all spurs inoculated with *Phomopsis* taxon 1 were notably bleached, particularly around the nodes and sites of inoculation (Figure 3.11). Pycnidia were evident on the bleached spurs. Similarly, 91.5% of spurs inoculated with taxon 2 were bleached, with 90% having pycnidia. Six buds, three of which were not inoculated, died on a plant 10 weeks after inoculation with *Phomopsis* taxon 2 isolate 902.4 at the premature leaf scar wound. In contrast, one spur inoculated with PDA appeared slightly bleached, but was this associated with extreme drying of the spur.

Figure 3.11. Bleaching and pycnidia on grapevine shoot 11 weeks after a dormant bud was inoculated with mycelium of *Phomopsis* taxon 1 isolate A223.1.

3.3.6 Experiment 5 – the effect of *Phomopsis* inoculation on lateral buds and leaves

At the time of inoculation, the length of the two shoots on each plant was measured; one shoot was used for bud inoculation, the other for leaf inoculation. The average shoot length was 26.2 cm (SD=8.96) and 17.7 cm (SD=6.01) for shoots inoculated on the bud and leaf, respectively. There was no significant difference between length of shoots used for bud and leaf inoculations (P=0.0681 and P=0.4446, respectively).

Disease development on inoculated plants over 8 weeks is shown in Table 3.6. There was no evidence that *Phomopsis* taxon 1 or taxon 2 caused failure of buds to burst as most buds in all treatments remained dormant (Eichhorn-Lorenz stage 01). As expected, stage of development varied significantly over time (P=0.000) in all treatments and replicates. One bud died 2 weeks after inoculation with *Phomopsis* taxon 2 isolate LR10T6 and another died after inoculation with water (control). By week 7, four buds inoculated with *Phomopsis* taxon 1 isolate H307 developed new shoots.

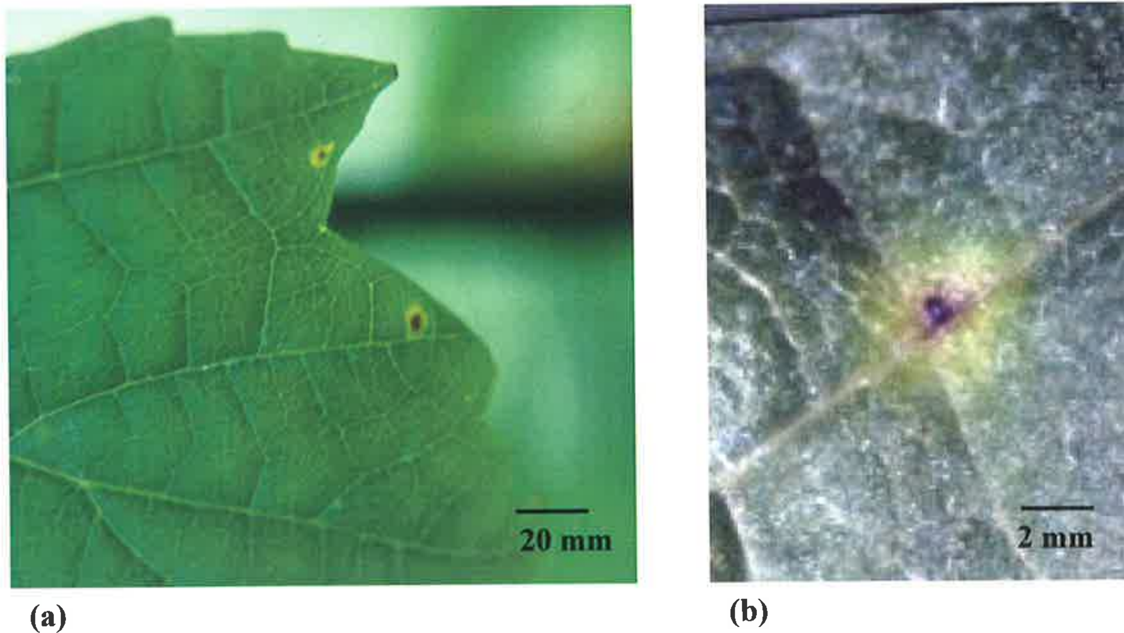
Small leaf spots developed on inoculated leaves within 3 weeks of inoculation with *Phomopsis* taxon 2 isolate LR10T6. The leaf spots comprised a brown necrotic area approximately 1 mm in diameter, surrounded by a pale yellow halo approximately 2-3 mm in diameter, characteristic of symptoms associated with Phomopsis cane and leaf spot (Figure 3.12a). After 4 weeks, several leaf spots were visible on three inoculated leaves and dark-coloured lesions were evident on petioles. The leaf spots, however, did not cover greater than 25% of the leaf area.

Plants inoculated with *Phomopsis* taxon 1 and ddH$_2$0 appeared healthy, although mite infestation was evident on most plants at week 5. Leaf spots caused by *Phomopsis* taxon 2 (Figure 3.12b) were clearly distinguishable from spots produced by mite feeding. Spots caused by mite feeding did not develop a necrotic lesion and were smaller and yellow in

Table 3.6. Stage of development and disease severity of shoots on which a bud or a leaf was inoculated with *Phomopsis* taxon 1 isolate H307 (10^4 conidia/ml), *Phomopsis* taxon 1 isolate 51C.1 (10^6 conidia/ml), *Phomopsis* taxon 2 isolate LR10T6 (10^4 conidia/ml) or water (control).

Treatment	Time after inoculation [a] (weeks)	Stage of bud development [b]	Disease severity [c]	
			Bud inoculated [d]	Leaf inoculated [d]
Taxon 1 10^4 conidia/ml	1	1.125	0	0
Taxon 1 10^6 conidia/ml	1	1	0	0.5
Taxon 2 10^6 conidia/ml	1	1	0	0
Control	1	1	0	0
Taxon 1 10^4 conidia/ml	2	1.5	0.25	0
Taxon 1 10^6 conidia/ml	2	1.25	0.25	0.5
Taxon 2 10^6 conidia/ml	2	1	0.75	0.25
Control	2	1	0	0
Taxon 1 10^4 conidia/ml	3	1.75	0.125	0
Taxon 1 10^6 conidia/ml	3	1.5	0.25	0.5
Taxon 2 10^6 conidia/ml	3	1	0.75	0.5
Control	3	1	0.625	0
Taxon 1 10^4 conidia/ml	4	1.75	0.125	0
Taxon 1 10^6 conidia/ml	4	1.875	0.125	0.625
Taxon 2 10^6 conidia/ml	4	1	0.75	0.75
Control	4	1	0.625	0
Taxon 1 10^4 conidia/ml	5	1.75	0	0
Taxon 1 10^6 conidia/ml	5	2.125	0	0.625
Taxon 2 10^6 conidia/ml	5	1	0.75	0.75
Control	5	1	0.625	0
Taxon 1 10^4 conidia/ml	6	2	0	0
Taxon 1 10^6 conidia/ml	6	2.125	0	0.625
Taxon 2 10^6 conidia/ml	6	1	0.75	0.75
Control	6	1	0.625	0
Taxon 1 10^4 conidia/ml	7	2	0	0
Taxon 1 10^6 conidia/ml	7	2.125	0	0.625
Taxon 2 10^6 conidia/ml	7	1	0.875	0.75
Control	7	1	0.625	0
Taxon 1 10^4 conidia/ml	8	2	0	0
Taxon 1 10^6 conidia/ml	8	2.125	0	0.625
Taxon 2 10^6 conidia/ml	8	1	0.875	0.75
Control	8	1	0.625	0

a = experiment commenced 1/6/2000.
b = stage of development whereby 0= death of bud, 1= winter bud (dormant), 2= bud swell, 3= woolly bud, 4= green tip (budburst) and thereafter 5= shoot.
c = eight replicates inoculated. Disease severity index modified from Phillips, 1998, whereby 0= no signs of disease, 1= few lesions covering no more than 25% leaf or internode, 2= many lesions covering 25-50% of leaf or internode, 3= over 50 % leaf or internodal area severely scarred or necrotic, 4= death of shoot or bud (no shoots emerged).
d = means within the treatments are not significantly different according to least significant difference (LSD) at the 5% level.



(a) (b)

Figure 3.12. (a) Lesions on leaf 21 days after inoculation of the leaf with *Phomopsis* taxon 2 isolate LR10T6 (10^6 conidia/ml). **(b)** Lesions displayed a necrotic centre, surrounded by a yellow halo.

colour. No difference in shoot vigour between the treatments was observed.

Phomopsis taxon 2 was re-isolated from one inoculated leaf displaying leaf spots. Attempts to isolate *Phomopsis* taxon 2 from other leaf spots on leaves were unsuccessful due to microbial contamination. *Phomopsis* taxon 1 and *Phomopsis* taxon 2 were not isolated from control plants. Other fungi, such as *Aspergillus*, *Botrytis* and *Rhizopus* spp., were isolated from tissues of the control plants.

The *Phomopsis* taxon 1-specific DNA probe, pT1P180, hybridised to DNA extracted from pure cultures of *Phomopsis* taxon 1. *Phomopsis* taxon 1 was not detected in DNA samples extracted from buds of inoculated grapevine plants possibly due to a low concentration of the fungi in buds.

3.3.7 Experiment 6 – assessment of dormant buds inoculated with *Phomopsis*

One week after inoculation of dormant buds with *Phomopsis* taxon 1, *Phomopsis* taxon 2, and ddH_20 (control), budburst commenced and many other buds were at a woolly bud stage of development. By week 2, 100% of buds that were located at the top of the propagated cane (bud 3) burst and most formed three to four fully developed leaves in all treatments. The third bud showed more advanced development than the first and second bud on the propagated cane. Most basal buds (bud one) remained dormant throughout the assessment period, regardless of treatment. As expected, the developmental stage of buds changed significantly over the assessment period (P=0.000), however, treatment did not have a significant effect on bud development (P=0.0674).

After 8 weeks, shoot length was recorded for those buds that produced shoots. Shoots developed from bud three were generally longer and healthier than those from other buds, regardless of treatment, therefore data for bud three only are shown (Figure 3.13). Buds inoculated with *Phomopsis* taxon 1 isolate L406 produced shoots that were significantly longer than those inoculated with *Phomopsis* taxon 2 isolate LR10T6 and controls (P=0.0256).

Shoots developing from canes inoculated with *Phomopsis* taxon 1 were not significantly different than those resulting from buds inoculated with *Phomopsis* taxon 2 (data not shown). These results indicated that *Phomopsis* taxon 1 did not cause delayed or reduced shoot growth in dormant Shiraz canes.

Three weeks after inoculation, spots were observed on one leaf developed from bud three inoculated with *Phomopsis* taxon 2 isolate LR10T6. Leaf spots were approximately 1 mm in diameter, and showed the characteristic yellow halo surrounding the brown area. In the following weeks, leaf spots did not develop on any other plants. However, by week 5, several buds inoculated with *Phomopsis* taxon 2 turned brown and died. Analysis of variance

indicated that disease severity, in terms of symptom expression and health of the plant, did not differ significantly (P=0.7337) between replicates or treatments at the 5% level. This may be due to the small sample size and death of buds following all treatments.

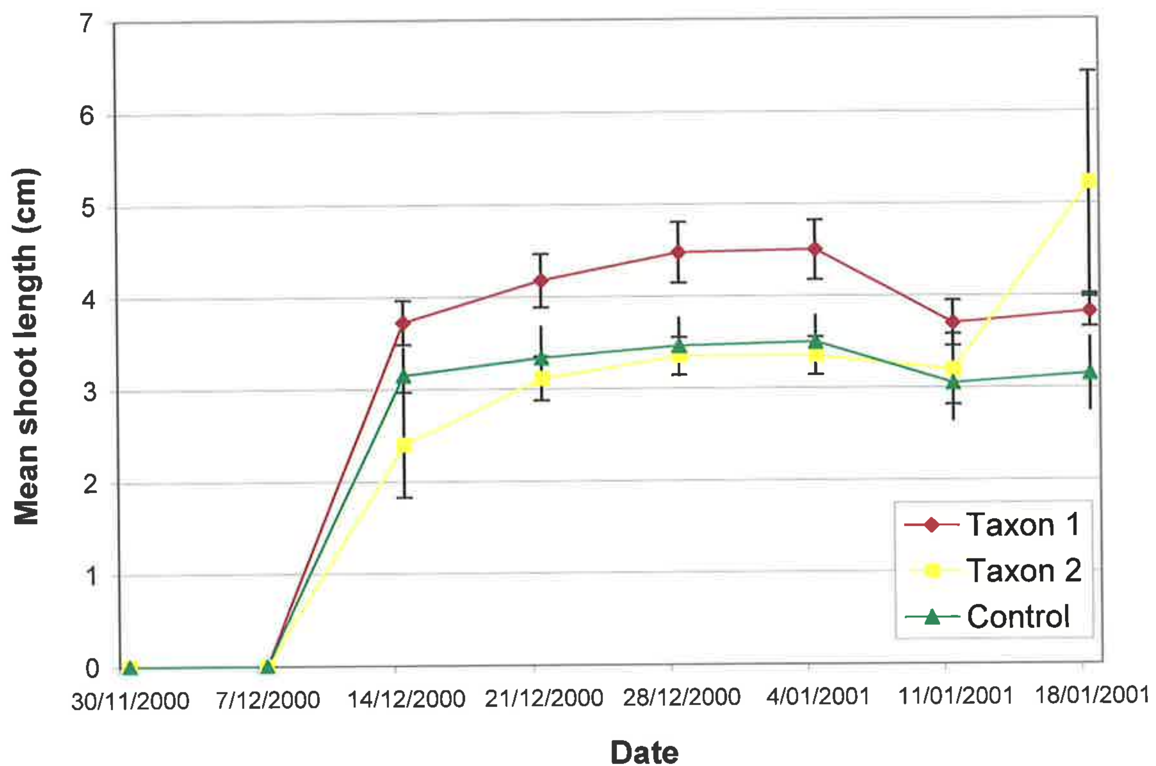


Figure 3.13. Shoot growth (cv. Shiraz) of bud three following inoculation of dormant buds with *Phomopsis* taxon 1 isolate L406, *Phomopsis* taxon 2 isolate LR10T16 and ddH_20 (control). Values represent the means of eight replicates per treatment. Bar represents standard error (P=0.0256, P<0.05). Plants were inoculated on 30/11/00.

3.3.8 Colonisation of grapevine tissue

Excised green shoots inoculated with *Phomopsis* taxon 1 showed tissue darkening at the site of inoculation (Figure 3.14a). Plant cells *ca* 10 mm away from the wound were healthy. Microscopic examination revealed hyphal growth between the cortex and epidermal layers (Figure 3.14b and c). Hyphae were observed growing through the intercellular spaces of the cortex, and did not appear to infect xylem and phloem tissue. Infected cells sometimes became necrotic and showed accumulations of brown polyphenols. Comparison of Figure 3.14b and 3.14c indicates that the darkening of host cells may be a hypersensitive response of the plant to *Phomopsis* taxon 1 isolate J5, as the fungus did not appear to invade the host cells.

In comparison, stem lesions caused by *Phomopsis* taxon 2 isolates M851 and M831.1 extended along the shoot, with the hyphae appearing as a subcuticular mass. However, the cortical cells seemed intact and hyphal growth was intercellular. At the site of inoculation, mycelial growth was abundant and cell arrangement was disrupted. Tissue which appeared healthy at 40 mm away from the site of inoculation was examined and hyphae were found to have grown under the cuticle (Figure 3.15a and b). Single hyphae were observed intercellularly, where they did not cause discolouration of cell tissue or cell death. No hyphae were observed in vascular cells or the pith.

Control shoots showed no evidence of fungal infection. Cells were disrupted at the wounding site and cortical cells darkened. Darkening of cells resembled that caused infection by *Phomopsis* taxon 1 at the point of inoculation. Callose deposits, which appeared bright yellow after staining with aniline blue, were observed in tissue inoculated with *Phomopsis* but were not evident in control shoots. They were observed near the wounding site and in the epidermal layer.

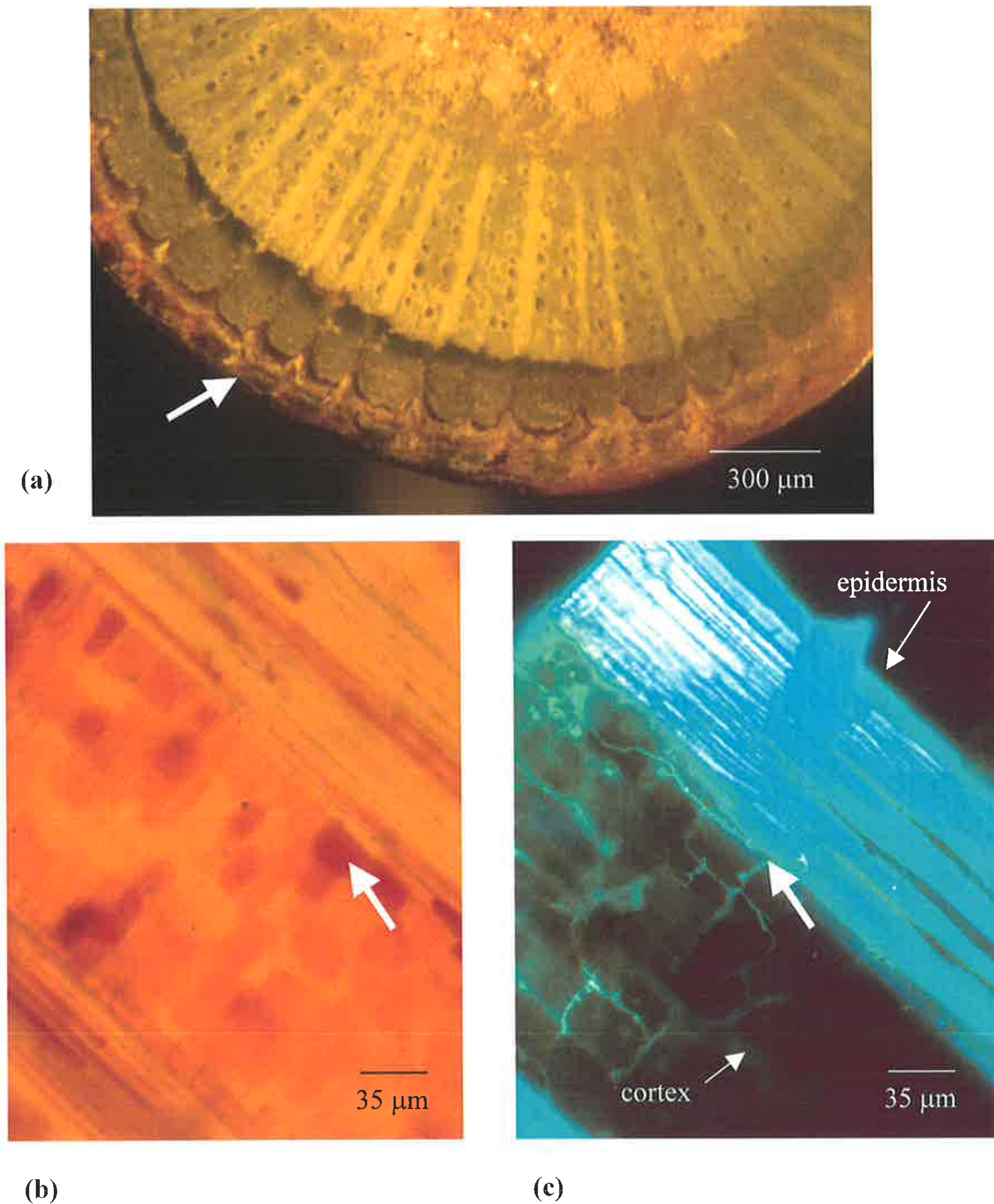


Figure 3.14. Excised green shoots (cv. Grenache) 3 weeks after inoculation with *Phomopsis* taxon 1 isolate J5. **(a)** Transverse section of stem from the margin of the lesion showing dark brown region of tissue (arrow) in epidermis (x 10). **(b)** Longitudinal section of shoot (unstained) showing darkened tissue at the margin of the lesion (arrow) (x 100). **(c)** Corresponding longitudinal section of shoot stained with 0.05% aniline blue in 0.067 M K_2HPO_4 showing hyphae confined to the cortex and epidermal layer (arrow) (x 100).

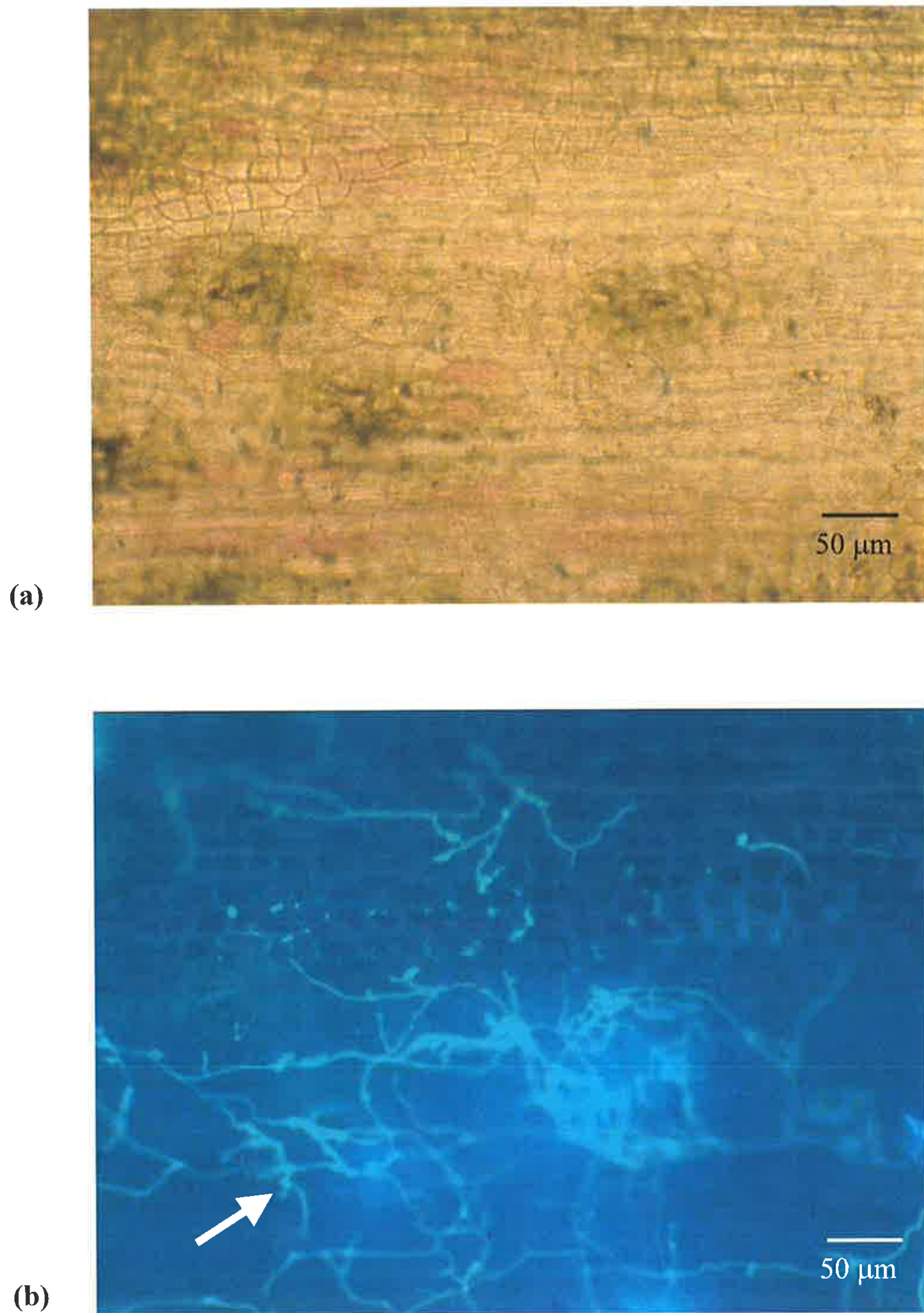


Figure 3.15. Excised green shoots (cv. Grenache) 3 weeks after inoculation with *Phomopsis* taxon 2 isolate M851. (a) Longitudinal section of healthy green shoot tissue (unstained) at the epidermis (x 100). (b) Corresponding longitudinal section of shoot stained with 0.05% aniline blue in 0.067 M K_2HPO_4 showing extensive hyphal growth confined to the epidermis (arrow, x 100).

3.4 Discussion

The discovery of two main taxa of *Phomopsis* associated with Phomopsis cane and leaf spot of grapevine in Australia (Merrin *et al.*, 1995) has caused confusion in the viticultural industry as to whether both of these *Phomopsis* taxa are pathogenic and need to be controlled. Since the discovery of the taxa, it has been shown that *Phomopsis* taxon 2 causes disease symptoms similar to those caused by the pathogen known worldwide as *Phomopsis viticola.* Symptoms include necrotic leaf spots surrounded by a yellow halo, and longitudinal lesions on green shoots resulting in cracking of lignified cane (Gubler and Leavitt, 1992; Hewitt and Pearson, 1990; Pine, 1959). These symptoms do not appear on vines infected with *Phomopsis* taxon 1, but confusion arises between the two taxa as both cause bleaching of dormant vines in winter. Studies by Scheper *et al.* (1997b) and Mostert *et al.* (2000) have shown that *Phomopsis* taxon 2 is more damaging than taxon 1 and the present study provides evidence that *Phomopsis* taxon 1 may be non-pathogenic when compared to virulent isolates of *Phomopsis* taxon 2. In particular, *Phomopsis* taxon 1 could not be shown to cause failure of buds to burst, delayed bud burst and stunting of shoots as suggested by Scheper *et al.* (1997).

Pathogenicity tests revealed that the 15 isolates of *Phomopsis* taxon 1 tested did not cause the leaf or shoot symptoms commonly associated with Phomopsis cane and leaf spot of grapevine. The *Phomopsis* taxon 1 isolates representing various viticultural regions of Australia, were shown to be less virulent than the five isolates of *Phomopsis* taxon 2 tested. Slight discolouration was observed at the point of inoculation with taxon 1 but it is likely that this was a response by the plant to wounding and the mass of colonising mycelium.

Microscopic studies showed that *Phomopsis* taxon 1 colonised the epidermis and cortex of the grapevine shoot. Hyphae were observed within and beneath the cuticle and in the intercellular spaces of the epidermis. *Phomopsis* taxon 1 caused darkening of cells at the

point of inoculation, but necrotic tissue was not observed beyond the inoculation area. In addition, the fungus was not recovered from healthy green inoculated shoots. Darkening of cells was restricted to the outer epidermal cells and there was no evidence of fungal colonisation of vascular tissue or the pith. Discolouration of cells after inoculation with *Phomopsis* taxon 1 was similar to cell damage observed for control shoots. *In vivo* experiments with excised shoots showed that *Phomopsis* taxon 1 did colonise grapevine, but further studies are required to show whether or not infection is not detrimental to plant growth. Browning of tissue is often caused by a hypersensitive reaction by the host plant (Deacon, 1997) or oxidation of phenolic compounds in the tissue in response to wounding.

Excised shoots inoculated with mycelium of *Phomopsis* taxon 2 developed dark brown/black longitudinal lesions similar to those produced by natural infection. Microscopic examination revealed intercellular hyphal growth through cortical cells, similar to infection by *Phomopsis* taxon 1. However, a large mass of subcuticular hyphae of taxon 2 was evident beneath necrotic lesions. Hyphae grew rapidly from the site of inoculation, with lesions observed up to 40 mm in length. Furthermore, the hyphae extended beyond necrotic tissue to areas without symptoms. Observations of longitudinal sections indicated that hyphal growth caused cell disruption by physically forcing the cells apart rather by intracellular growth. Eventual cracking of shoots infected with *Phomopsis* taxon 2 may, therefore, be attributed to the disruption of host cells. More isolates of *Phomopsis* taxon 2, a broader range of cultivars, more replicates and variations in environmental conditions require testing to confirm the effect of lesion development on plant function.

It has been reported that other *Phomopsis* species, such as *P. vaccinii*, cause dieback of shoots, resulting from necrosis and collapse of parenchyma cells in the cortex and vascular tissue after invading the cortex (Daykin and Milholland, 1990). In the present study, *Phomopsis* taxon 2 appeared to be restricted to the epidermal and cortical cells with no

growth inwards to the vascular bundle. The findings support an earlier report that *P. viticola* is not associated with dieback of grapevine (Moller and Kasimatis, 1981). In addition, hyphal masses were concentrated on proximal side of the inoculated point, with lesions formed longitudinally along the side of the shoot in either direction. Elongated lesions on internodes and necrotic leaf spots were produced 2 weeks after inoculation with *Phomopsis* taxon 2 as shown in earlier studies (Pscheidt and Pearson, 1989; Phillips, 1998).

Microscopic examination of cv. Grenache shoots inoculated with *Phomopsis* taxon 1 isolate J5 revealed that mycelial growth extended around the diameter of the shoot, indicating greater susceptibility to infection than for other cultivars tested. Longer lesions were produced on excised cv. Grenache shoots inoculated with *Phomopsis* taxon 2 than on any other cultivar tested. Gubler and Leavitt (1992) reported that Grenache was highly susceptible to Phomopsis cane and leaf spot in wet spring years. Grenache has also been shown to be highly susceptible to infection by *Eutypa lata* (Peros and Berger, 1994). The pathogenicity studies were more comprehensive than those conducted by Mostert *et al.* (2000) and showed that inoculation of excised green shoots is suitable for determining pathogen variability and cultivar susceptibility to infection by *Phomopsis*.

Koch's postulates were fulfilled by inoculation of grapevine plants and re-isolation of *Phomopsis* from inoculated shoots. Initial investigations showed that infection by *Phomopsis* taxon 1 was difficult to identify based on macroscopic observations, however, further studies revealed that inoculation with mycelium promoted bleaching of dormant cane. Bleaching is often associated with the disease, and it was shown that bleaching is induced by both *Phomopsis* taxon 1 or taxon 2. Furthermore, bleaching produced on plants in the glasshouse resembled bleaching symptoms displayed in the field at the same time of year. Bleaching, and the production of pycnidia, indicate that *Phomopsis* causes a physiological change in grapevine tissue. This can be associated with fungal colonisation of the epidermal cells, and

developing pycnidia forcing the layer of epidermal cells away from the cortex (Hewitt and Pearson, 1990).

There was no evidence in this study to support the assumption that *Phomopsis* taxon 1 causes bud death. Dormant buds inoculated with mycelium of *Phomopsis* taxon 1 and *Phomopsis* taxon 2, either through prematurely-created leaf scars or direct wounding, did not differ significantly in developmental stage to those of the control. In general, bud burst percentage was low in all treatments, and the effects of fungal colonisation on bud development were inconclusive. However, inoculation methods used in the study were effective in facilitating *Phomopsis* infection, because bleaching and leaf symptoms were observed. There have been reports of a *Phomopsis* sp. causing rapid development of peach shoot blight disease after inoculation of wounded dormant buds and breaking buds (Uddin and Stevenson, 1997). These findings may indicate that *Phomopsis* spp. on woody plants may readily invade the shoot through the young exposed tissue of the bud. It is possible that rapid development of buds on newly-propagated cane in the present study, did not allow sufficient time for *Phomopsis* to colonise and cause bud death in glasshouse conditions.

The results of tests in the glasshouse suggested that *Phomopsis* taxon 1 is endophytic. Endophytic fungi grow inconspicuously within tissue of functioning plants without causing symptoms or apparent injury to the host (Redlin and Carris, 1996). Evidence has been presented to show that *Phomopsis* taxon 1 does not cause disease in grapevines and could only be detected by staining, or when pycnidia erupt through the epidermis. In comparison, *Phomopsis* taxon 2 appeared to produce latent infections, which would persist until conditions are favourable for disease development. Many species of *Phomopsis* such as *P. citri* (Brown and Wilson, 1968), *P. leptostromiformis* (Cowling *et al.*, 2002), *P. longicolla* (Ellis *et al.*, 2002) and *P. phaseoli* (Cerkauskas *et al.*, 1983) cause latent infections. Many latent and endophytic fungi exist as subcuticular hyphae in the host plant, and occupy

intercellular spaces of the epidermis. As for infections by *Phomopsis viticola* (Pscheidt and Pearson, 1989), *P. occulta*, the causal agent of branch dieback in Colorado blue spruce (*Picea pungens* f. sp. *glauca*), causes necrotic lesions, yet the host continues to grow (Igoe *et al.*, 1995).

Further work is required to determine if darkening of plant cells associated with infection by *Phomopsis* taxon 2 is a result of a toxin produced by the fungus, or oxidation of phenolic compounds in grapevine tissue. It appears likely that cell disruption would eventually cause cracking of the epidermis, but it is unknown if the severity of necrotic lesions is related to environmental conditions or virulence of the *Phomopsis* isolate. For example, severe lesions developed on excised green shoots 2 weeks after inoculation with mycelium of *Phomopsis* taxon 2 in the glasshouse. The development of lesions may have been accelerated under these favourable conditions.

Pathogenicity experiments in a glasshouse confirmed that *Phomopsis* taxon 1 did not cause leaf or shoot symptoms. Both *Phomopsis* taxon 1 and taxon 2 are associated with bleaching of canes, but there was no evidence to suggest bud death is associated with colonisation by the fungi. Microscopic studies showed that taxon 1 colonised the epidermis of excised green grapevine shoots but not the vascular tissue. The results indicated that *Phomopsis* taxon 1 may be endophytic. Further studies, shown in chapter 4, were conducted to confirm the effect of taxon 1 on budburst and grapevine productivity in the field.

Chapter 4

The effect of *Phomopsis* taxon 1 on budburst in established vineyards

4.1 Introduction

Phomopsis cane and leaf spot has been reported to cause yield losses of up to 30% (Coleman, 1928b; Baltovski, 1980; Pscheidt and Pearson, 1989; Erincik and Madden, 2001). In most reports, yield reductions were greatest following infection of rachis and berries by *Phomopsis* taxon 2. Infection of fruit can occur early in the season and infections remain latent until berries begin to ripen (Erincik and Madden, 2001). Infected berries turn brown, shrivel and may abscise from the pedicel (Hewitt and Pearson, 1990). In Australia, however, berry rot is rare because climatic conditions late in the season are rarely favourable for *Phomopsis* infection. Instead, yield loss has been associated with girdled shoots and weakened cane (Emmett *et al.*, 1998). There is no information available on crop losses due to infection by *Phomopsis* taxon 1 of grapevine.

Pathogenicity experiments in a glasshouse (Chapter 3) confirmed that *Phomopsis* taxon 1 did not cause leaf or shoot symptoms commonly associated with Phomopsis cane and leaf spot. *Phomopsis* taxon 1 is difficult to identify in the field based on macroscopic observations, although bleaching of canes is often used to identify *Phomopsis* infection. However, diagnosis based on bleaching symptoms may be misleading because bleaching may be induced by *Phomopsis* taxon 1, *Phomopsis* taxon 2 and other pycnidium-producing fungi

such as *Botryosphaeria* spp. Growers in the Adelaide Hills, South Australia, have observed a large number of unburst buds on bleached cane (Melanson *et al.*, 2002).

Preliminary investigations by Melanson *et al.* (2002) showed that *Phomopsis* taxon 1 colonises buds and the fungus was detected in buds which failed to burst. *Phomopsis* taxon-specific DNA probes are useful to detect the fungus in grapevine material and in the absence of symptoms. The taxon-specific DNA probes, therefore, provide a tool to study the epidemiology of the fungus and whether *Phomopsis* taxon 1 is responsible for bud loss.

Based on field observations and growers' concerns, there was a need to clarify the effect of *Phomopsis* taxon 1 on grapevine health, and whether current control strategies for both *Phomopsis* taxa were warranted. The aims of the study were to; (1) determine if *Phomopsis* taxon 1 causes delayed budburst or bud death, (2) investigate the association between bleaching of canes and *Phomopsis* taxon 1 and (3) assess productivity of vineyards infected with *Phomopsis* taxon 1 in terms of bunch number.

4.2 Materials and methods

4.2.1 Selection of trial sites

Experimental sites were selected to monitor the effect of infection by *Phomopsis* taxon 1 on budburst over three seasons. Twelve commercial vineyards were visited in the Adelaide Hills, the McLaren Vale region and the Fleurieu Peninsula, SA in August 1999. All sites had a history of suspected *Phomopsis* infection but it was unknown if vines were infected by *Phomopsis* taxon 1 or taxon 2. One-year-old canes were visually examined for bleaching associated with *Phomopsis* infection and collected for diagnosis in the laboratory in August 1999 (see section 2.1). Furthermore, blackberry (*Rubus fruticosus*) canes displaying pycnidia and bleaching of the epidermis, found growing near vines at the Mt Jagged vineyard, were

similarly sampled to determine if *Phomopsis* taxon 1 was present. Final site selection was based on the identification of *Phomopsis* taxon 1 from sampled canes and the commencement of budburst in the vineyard. Several vineyards were deemed unsuitable because budburst commenced prior to establishing the experiment. Field trials were established at Hargrave vineyard in Summertown, Ashton Hills Winery, Ashton and Lenswood Horticultural Centre, Lenswood in the Adelaide Hills, SA (all cv. Chardonnay). A fourth trial site was established at Mt Jagged vineyard, Southern Fleurieu Peninsula, SA (cv. Shiraz, Figure 4.1). Vines at all sites were spur-pruned by hand. Climatic data from the Lenswood Horticultural Centre weather station for August – October in 1999, 2000 and 2001 were kindly supplied by R. Vickers, SARDI. Climatic averages for Lenswood Horticultural Centre weather station from 1967 to 2001 were obtained from the Bureau of Meteorology, Australia.

4.2.2 Experimental design

Phomopsis taxon 1 has been detected in canes that were not bleached (Melanson *et al.*, 2002) therefore, spurs were selected randomly in all vineyards. Samples were collected from five rows and 20 spurs selected per row (100 spurs per vineyard; see Appendix B). Spurs were individually tagged and numbered (R represents row and T for tag) in August of each trial season. Budburst percentage was counted as 'buds per spur' rather than 'nodes per spur' because individual buds were assessed for *Phomopsis*. It is known that more than one bud can occur per node. Buds which failed to burst were referred to as "unburst buds". The vineyards were maintained according to normal practices carried out by the viticultural manager.

Spurs were selected along the cordon of grapevines in the 1999 season, but in 2000 and 2001, spurs at the end of each cordon were selected to minimise differences in budburst which may have resulted from variation in nutrient flow within the vine.

Figure 4.1. Map of South Australia showing the four vineyard sites assessed for *Phomopsis* taxon 1; Ashton Hills Winery, Ashton; Hargrave vineyard, Summertown; Lenswood Horticultural Centre, Lenswood in the Adelaide Hills region and Mt Jagged vineyard, Southern Fleurieu Peninsula.

The presence of *Phomopsis* taxon 1 on each sampled spur was unknown until analysis of the samples in late November. In July and August of each year, canes were selected randomly throughout each vineyard and incubated as described in section 2.1 to promote production of pycnidia by *Phomopsis* taxon 1. The vineyard at Lenswood Horticultural Centre was initially selected as a control site, but *Phomopsis* taxon 1 and taxon 2 were isolated from cane material. As a result, spurs at the Lenswood vineyard were selected 2 weeks later than other sites in the Adelaide Hills in 1999. The observations of number of buds burst were compared to expected frequencies by application of the chi-square test in Statistix®.

4.2.3 Sampling of spurs for *Phomopsis* taxon 1

4.2.3.1 Year 1999

To assess whether *Phomopsis* taxon 1 was causing failure of buds to burst or delayed budburst, spurs were monitored at the four vineyard sites fortnightly from 20 August to 24 November 1999. Unburst buds at the Mt Jagged vineyard were collected prior to 24 November 1999 for preliminary assessment. Commencement of budburst was defined as the stage at which 50% of buds showed leaf emergence. Spurs were assessed for; (1) number of buds per spur, (2) number of buds burst and (3) bleaching. Shoot length on tagged spurs was also measured at each site in late October.

By late November, unburst buds on tagged spurs were collected to determine the presence of *Phomopsis* taxon 1. Initially, buds were collected in the field by cutting the individual bud away from the spur with a scalpel, but this method was unsafe and awkward. Subsequently, the spur was removed from the vine and taken to the laboratory for analysis. In

addition, one shoot was removed from the same spur from the lowest node position but closest to the unburst bud.

To determine the presence and location of *Phomopsis* taxon 1, each spur collected was divided into four sub-samples; (1) unburst buds, (2) canes, (3) first three internodes of shoots and (4) leaves (Figure 4.2). DNA was extracted from each sub-sample as described in section 2.2.1, and individual slot blot membranes were prepared for each vineyard site. Total DNA was hybridised with the *Phomopsis* taxon 1-specific probe, pT1P180 and the taxon 2-specific probe, pT2P25 (see section 2.2.5) and the presence of *Phomopsis* taxon 1 determined for the four sub-samples. The amount of *Phomopsis* taxon 1 DNA in total DNA extracted from grapevine was quantified using purified DNA from taxon 1 (controls).

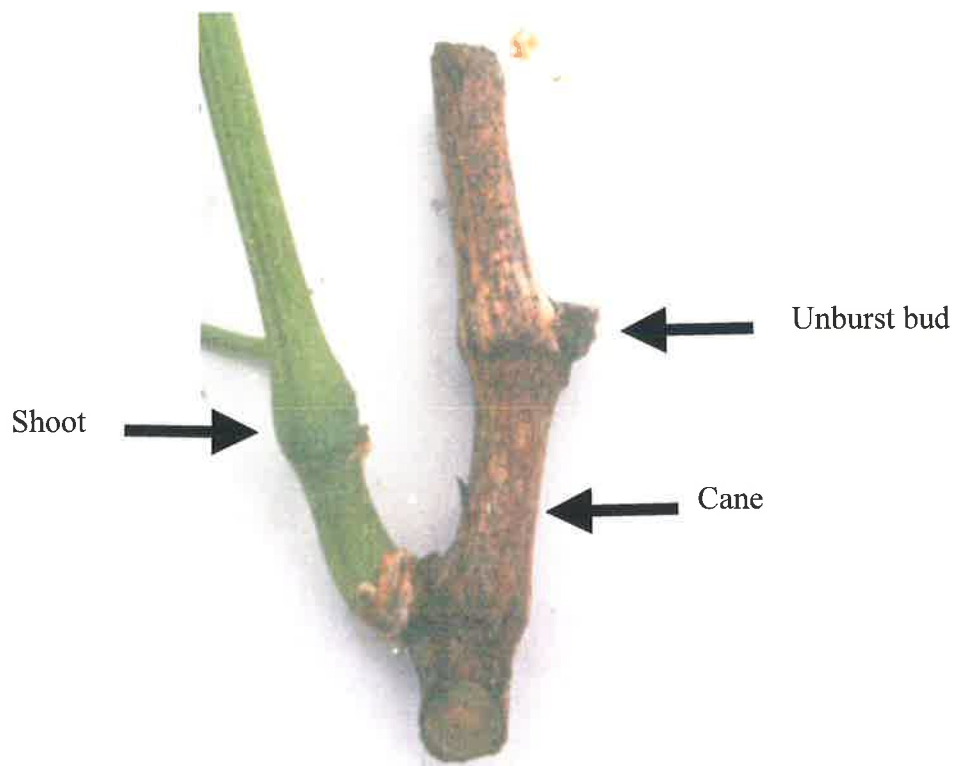


Figure 4.2. Sampled spur (cv. Shiraz) showing three of the four sub-samples for assessment of *Phomopsis* taxon 1 infection; unburst bud, cane and the first three internodes of a shoot. Leaves are not shown.

Ten unburst buds, from the total number of unburst buds collected at each site, were randomly selected for microscopic observation. The outer bud scales were pulled apart with forceps, and the buds were cut in half with a scalpel to expose the primordia. The health of the buds was scored as follows:

1 = healthy, tissue is green and the bud tightly compact

2 = moderately healthy, bud tissue slightly brown in colour

3 = unhealthy, tissue moist, brown in colour

4 = dead, bud is dry and brown.

After microscopic assessment of bud health, the buds were placed aseptically on PDA (Difco, USA) and fungi were subcultured after 2 days of incubation as described in section 2.1. Preliminary investigations indicated that surface-sterilisation was required. For three sites, buds were surface sterilised with 0.5% NaOCl (Milton, Australia) for 1 min, followed by three successive 1-min rinses in sterile ddH_20 prior to plating on PDA. The buds were removed from the agar and frozen at -70°C prior to extraction of DNA.

Shoots were removed from the spur and separated into the first, second and third internode. The basal three internodes were selected because *Phomopsis* is known to infect lower internodes of shoots (Hewitt and Pearson, 1990). Internode length was measured and DNA was extracted from shoot and cane as described in section 2.2.1.

Leaves with necrotic spots were selected from the spurs collected at the Mt Jagged vineyard. Leaf lesions were cut into approximately 5 mm × 5 mm pieces and surface sterilised for 3 min in 0.5% NaOCl (Milton) followed by three rinses in sterile ddH_20, then incubated as described in section 2.1. The remaining leaf sections were weighed and frozen at -70°C prior to DNA extraction (see section 2.2.1).

DNA samples from unburst buds, canes, shoots and leaves were adjusted to 100 ng total DNA and transferred to a slot blot membrane as described in 2.2.3. Each membrane

included purified DNA of *Phomopsis* taxon 1 isolate A223.1 or B500, purified DNA of *Phomopsis* taxon 2 isolate M827 or C608, 100 ng of DNA obtained from grapevine and ddH_20. Total DNA was hybridised with the *Phomopsis* taxon 1-specific probe, pT1P180 and the taxon 2-specific probe, pT2P180, as described in section 2.2.6.

4.2.3.2 Year 2000

To determine the effect of *Phomopsis* taxon 1 on budburst over a number of seasons, 100 spurs were selected in August 2000 at each of the four sites using rows similar to those assessed in 1999. Spurs were monitored monthly until 30 November as described in section 4.2.3.1, however, shoot length on each spur was not measured. In addition, bunch count per spur was recorded in late November to investigate productivity of vineyards infected with *Phomopsis* taxon 1. As in the previous year, the entire spur was removed for analysis of unburst buds, cane and first three internodes of the shoot for the presence of *Phomopsis* taxon 1, however leaves were not sampled in 2000. The three sub-samples were assessed as described in section 4.2.3.1, and DNA extracted, transferred to a slot blot membrane and hybridised with the taxon-specific probes, pT1P180 and pT2P25, as described in section 2.2.6.

As a control sample, 20 spurs with developed shoots were randomly collected from each site to determine if *Phomopsis* taxon 1 was also present in healthy cane. As for the treatment of sampled spurs in the 3-year-trial, DNA was extracted from the developed shoot and lignified cane of the healthy spur and hybridised as described in 2.2.6.

4.2.3.3 Year 2001

Field monitoring continued into the third season with 100 new spurs selected at each of the four sites in August 2001 and monitored fortnightly between 4 September and 6 November as described in section 4.2.3.1. Shoot length was measured on 6 November at Ashton and Hargrave vineyards only. Bunch count per tagged spur was recorded at every site in November and the unburst buds, canes and first three internodes of the shoots were assessed for *Phomopsis* taxon 1 infection as in section 4.2.3.1. Leaves were not sampled. As in 2000, 20 spurs with developed shoots were randomly collected from each site and analysed as in section 4.2.3.2. Lignified cane was placed at 15°C and assessed for the presence of *Phomopsis* taxon 1 as in section 2.1.

4.2.4 Collection of additional unburst buds at the Mt Jagged vineyard

In 2000, the viticultural manager at the Mt Jagged vineyard was concerned by a high number of unburst buds on weak spurs in a localised area of the vineyard (cv. Shiraz). For this reason, 23 unburst buds were collected from three rows on 24 October from 10 spurs at this site. The health of the buds was examined microscopically as described in section 4.2.3.1. DNA was extracted from the unburst buds using the SEAPS DNA extraction method as described in section 2.2.1, and spurs were incubated at 15°C in moist conditions to promote production of pycnidia (section 2.1). A slot blot was prepared containing 100 ng DNA from individual unburst buds, 100 ng of grapevine DNA, 50 ng of *Phomopsis* taxon 2 and a serial dilution of purified DNA from *Phomopsis* taxon 1 isolate A223.1 (1 ng, 3 ng, 6 ng, 12 ng, 25 ng and 50 ng). Total DNA was hybridised with the *Phomopsis* taxon 1-specific DNA probe, pT1P180, as described in section 2.2.6.

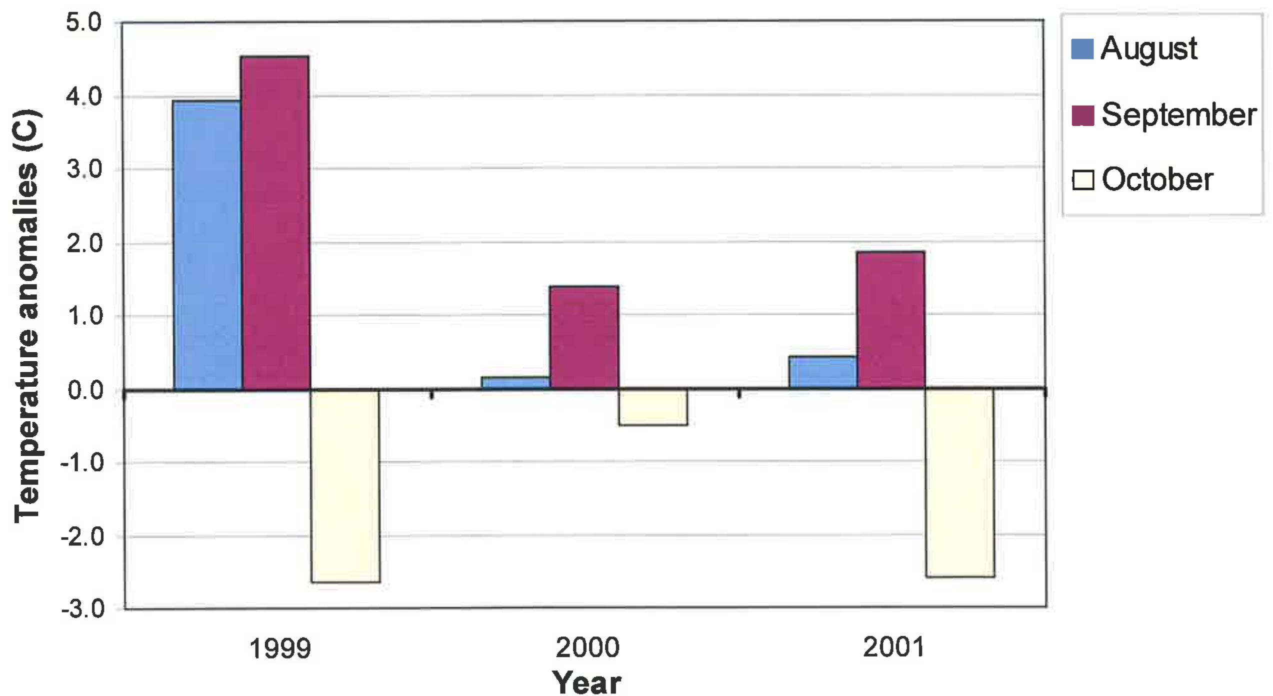
4.3 Results

4.3.1 Assessment of trial sites for *Phomopsis*

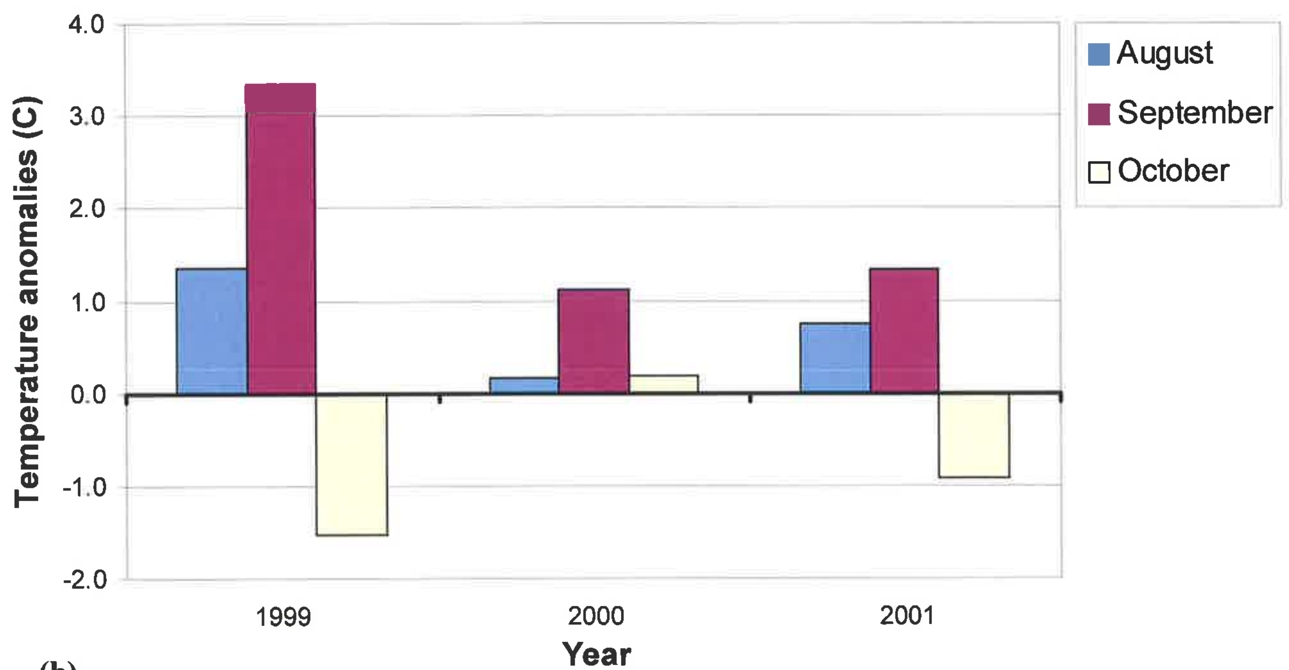
Most of the canes, collected from vineyards with suspected *Phomopsis* infection, showed numerous pycnidial structures which were subsequently shown to be produced by fungi other than *Phomopsis* taxon 1 or taxon 2. The fungi, identified by Michael Priest (NSW Agriculture), were *Seimatosporium lonicerae, Phoma epicoccin* and *Phoma macrostomum. Aspergillus niger* was also isolated from canes. *Phomopsis* taxon 1 was detected in Ashton Hills, Hargrave and Lenswood Horticultural Centre vineyards. Although canes from several vineyards in the McLaren Vale region were sampled, the canes were either not infected with *Phomopsis* taxon 1, or bud burst had commenced. Cane collected from the Mt Jagged vineyard showed the highest incidence of *Phomopsis* taxon 1. Blackberry (*Rubus fruticosus*) canes from the Mt Jagged vineyard produced cirrhi after 5 days incubation. Cirrhi were isolated and the fungus was identified based on conidial morphology as *Didymella applanata* (Williamson, 1991).

4.3.2 Climatic data for August, September and October 1999-2001

Temperature data were obtained to consider the effect of climatic conditions on timing of budburst. Figure 4.3 shows the anomaly mean temperature for Lenswood Horticultural Centre for the months of August, September and October in 1999, 2000 and 2001. Compared with the normal monthly temperatures in 1967-2001, August and September in years 1999, 2000 and 2001 were relatively warm. Temperatures in October were approximately 2-3°C below normal, except in 2000.



(a)



(b)

Figure 4.3. (a) Mean maximum and **(b)** minimum air temperature anomalies (°C) for August, September and October recorded by the Lenswood Horticultural Centre automatic weather station in 1999, 2000 and 2001 compared to the 1967-2001 normal monthly temperatures.

4.3.3 Budburst of sampled spurs

4.3.3.1 Year 1999

Budburst, as defined in section 4.2.3.1, commenced between 8 and 15 September 1999 at the four sites. Budburst was earlier than expected due to warm temperatures in August (see Figure 4.3). More than 65% of buds had burst within 2 weeks from the commencement of budburst (Figure 4.4). The total number of buds retained on 100 sampled spurs ranged from 243 to 303 buds at each vineyard, and from these buds, total budburst percentage was calculated.

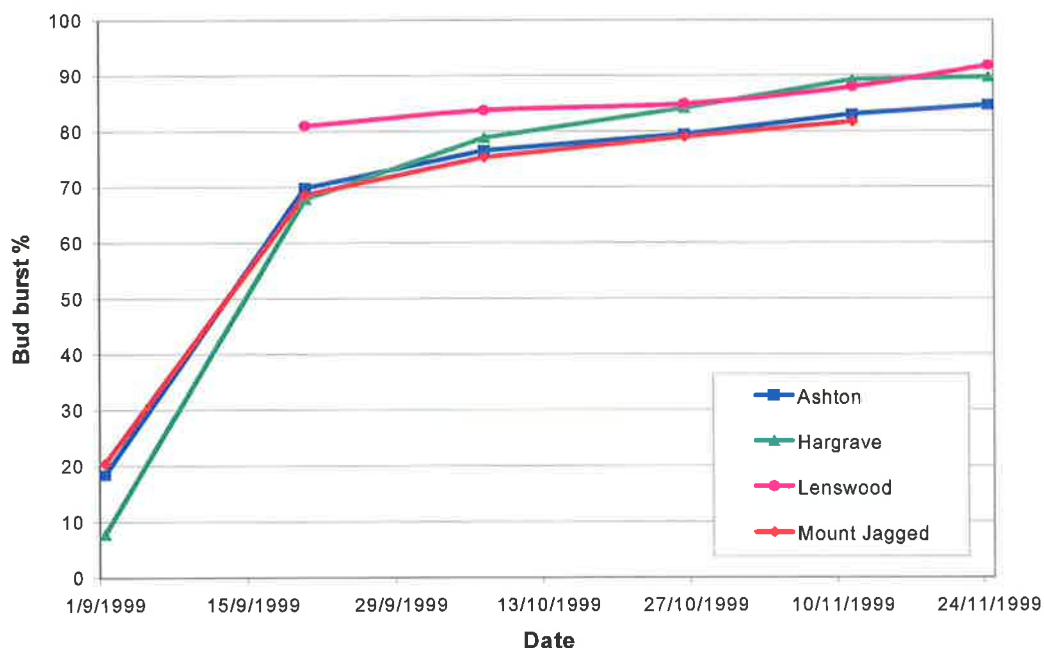


Figure 4.4. Percentage of buds burst from 100 spurs sampled at four vineyard sites in 1999. The Lenswood vineyard was assessed 2 weeks later than other sites and unburst buds were collected from the Mt Jagged vineyard prior to 24 November 1999.

At the Ashton vineyard, more buds were retained at pruning and more buds failed to burst than at other sites assessed (Table 4.1). Budburst percentage was highest at the Hargrave and Lenswood vineyards. Vines at Lenswood were robust and healthy. At the Hargrave vineyard, a high number of basal buds also burst, but these were not included in monitoring records. In comparison, basal buds failed to burst at Ashton Hills.

Vines at the Mt Jagged vineyard showed uneven budburst at the beginning of the season and this was typical throughout the vineyard block. At the time unburst buds were collected, 18 buds were missing. Missing buds was attributed to insect damage, weak bud development, possible physical damage or other factors unknown. Missing buds were considered for calculation of final budburst percentage. There was no significant difference (P=0.0524) between the total number of buds, and those that failed to burst at each site (Table 4.2, Appendix C).

Table 4.1 Total number of buds monitored, mean number of buds, total number of unburst buds collected and final budburst percentage on 24 November 1999 from 100 spurs sampled at four sites. Missing buds have been omitted from calculations of percentage buds burst.

Site	Total no. buds	No. buds/spur (mean)	No. unburst buds	Buds burst (%)
Ashton Hills	303	3.0	46	84.8
Hargrave	248	2.5	27	89.5
Lenswood	258	2.1	20	91.9
Mt Jagged	243	2.6	30	81.6

Table 4.2. Number of unburst buds and total number of buds burst at four vineyard sites on 24 November 1999. Contingency tables using chi-square analysis determined expected values and P=0.0524 indicated that the number of buds which failed to burst did not significantly differ at all sites (P>0.05). Missing buds have been omitted from statistical analysis.

Site	Outcome	No. unburst buds	No. buds burst
Ashton	Observed	46	257
	Expected	35	268
Hargrave	Observed	27	221
	Expected	29	219
Lenswood	Observed	20	238
	Expected	30	228
Mt Jagged	Observed	30	213
	Expected	28	215

4.3.3.2 Year 2000

Buds at the three vineyards in the Adelaide Hills were at woolly bud (Eichhorn-Lorenz stage 3) on 13 September 2000, with budburst following soon after. The average temperatures in August and September 2000 were cooler than the previous year (Figure 4.3). Budburst was assessed monthly in 2000, therefore approximately 50% budburst was estimated to have occurred at 24 September 2000 (Figure 4.5).

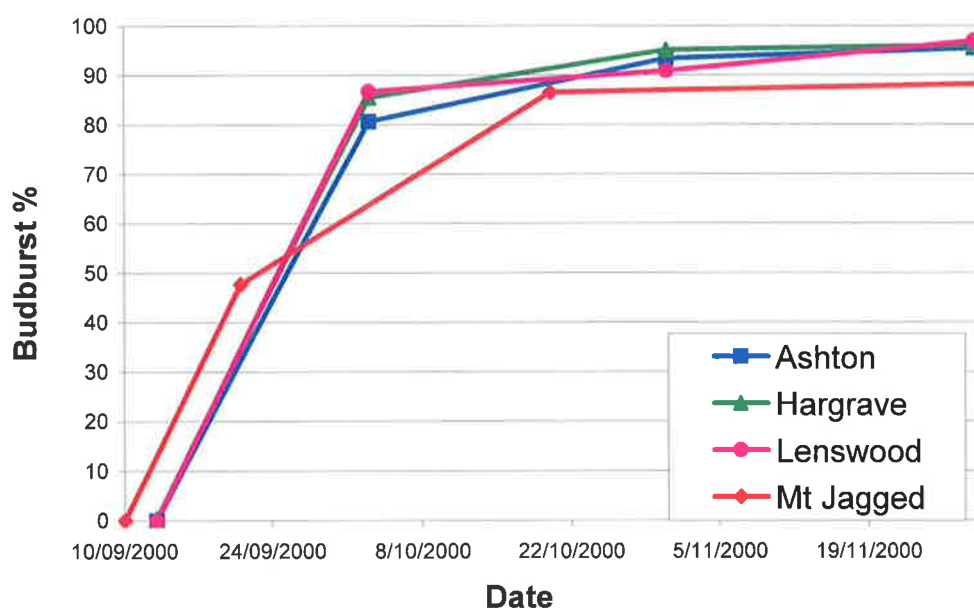


Figure 4.5. Percentage of buds burst from 100 sampled spurs at four vineyard sites in 2000.

Budburst was slightly earlier at the Mt Jagged vineyard than at sites in the Adelaide Hills.

Subsequently, high budburst resulted in fewer unburst buds collected in 2000 from spurs sampled at each site than in 1999. Comparison of the observed and expected number of unburst buds at each site in 2000, indicated a significant association between sites (P=0.007). More buds failed to burst at the Mt Jagged vineyard than expected by chance (Table 4.4), however there was no significant difference between observed and expected number of unburst buds at the vineyards in the Adelaide Hills (see Appendix C).

Table 4.3 Total number of buds, mean number of buds, total number of unburst buds collected and final budburst percentage from 100 spurs sampled at four sites in 2000. Missing buds have been omitted from calculations of percentage buds burst.

Site	Total no. buds	No. buds/spur (mean)	No. unburst buds	Buds burst (%)
Ashton Hills	240	2.4	8	95.4
Hargrave	206	2.1	7	96.1
Lenswood	198	2.0	6	96.9
Mt Jagged	238	2.4	24	88.2

Table 4.4 Number of unburst buds and total number of buds burst at four vineyard sites on 30 November 2000. Contingency tables using chi-square analysis determined expected values and P=0.007 indicated more buds failed to burst at the Mt Jagged vineyard than expected (P<0.05). Missing buds have been omitted from statistical analysis.

Site	Outcome	No. unburst buds	No. buds burst
Ashton	Observed	8	229
	Expected	12	225
Hargrave	Observed	7	199
	Expected	11	195
Lenswood	Observed	6	192
	Expected	10	188
Mt Jagged	Observed	24	210
	Expected	12	222

4.3.3.3 Year 2001

Budburst, as defined in section 4.2.3.1, commenced on 14 September at the three sites in the Adelaide Hills and on 28 September at the Mt Jagged vineyard, Southern Fleurieu (Figure 4.6). As in previous years, more buds were retained at pruning at Ashton Hills than at other sites. The same number of buds were sampled at the Hargrave and Mt Jagged vineyards. At the Mt Jagged vineyard, the number of buds was reduced from an average of 2.4 buds/spur in 2000 to 2.0 buds/spur in 2001 to reduce excessive shoot vigour. Budburst percentage was consistently highest at Lenswood over the three years. Late pruning (early September) at the Hargrave vineyard did not appear to have affected overall budburst percentage (Table 4.5).

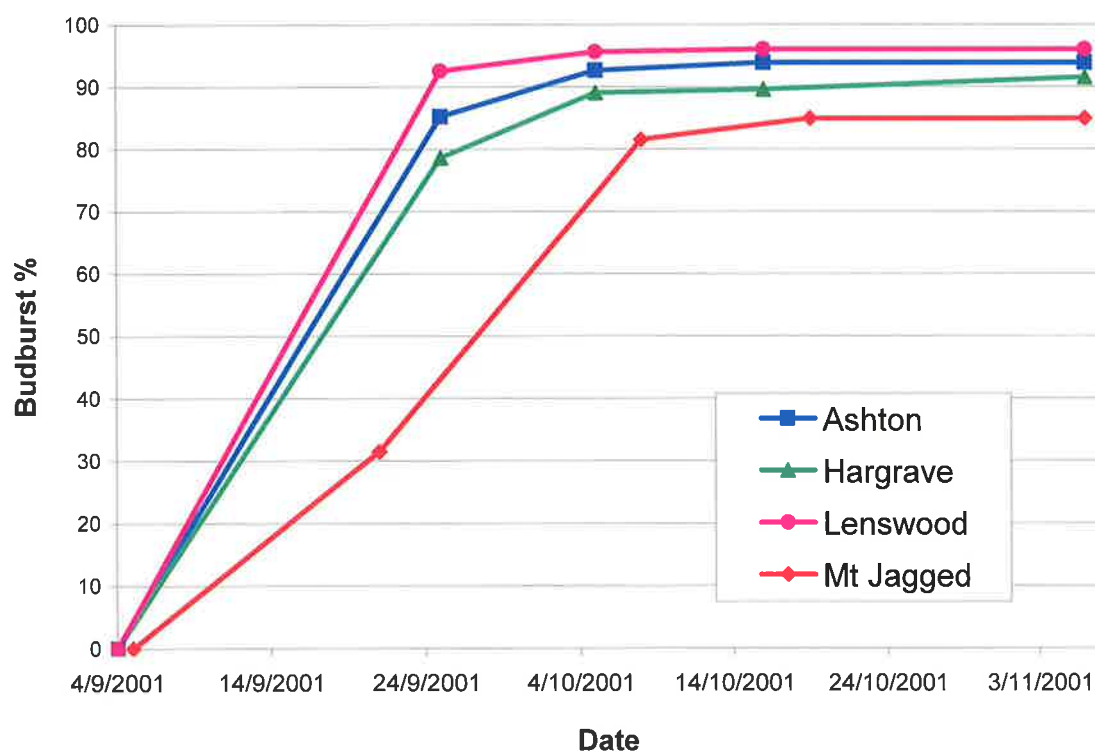


Figure 4.6. Percentage of budburst over time from 100 sampled spurs in five rows at each of the four vineyard sites assessed in 2001.

Table 4.5. Mean number of buds, total number of unburst buds collected and final budburst percentage on 6 November 2001 from 100 sampled spurs at four sites. Missing buds have been omitted from calculations of percentage buds burst.

Site	Total no. buds	No. buds/spur (mean)	No. unburst buds	Buds burst (%)
Ashton Hills	231	2.3	13	93.9
Hargrave	200	2.0	20	91.6
Lenswood	218	2.2	9	96.1
Mt Jagged	200	2.0	27	84.9

Table 4.6. Number of unburst buds and total number of buds burst at four vineyard sites in 2001. Contingency tables using chi-square analysis determined expected values and P=0.0011 indicated a significant number of buds failed to burst at the Mt Jagged vineyard (P<0.05). Missing buds have been omitted from statistical analysis.

Site	Outcome	No. unburst buds	No. buds burst
Ashton	Observed	13	218
	Expected	19	212
Hargrave	Observed	20	180
	Expected	16	184
Lenswood	Observed	9	209
	Expected	18	200
Mt Jagged	Observed	27	168
	Expected	16	179

Chi-square analysis showed there was a significant difference (P=0.0011) in the number of buds that failed to burst between the four sites in 2001 (Table 4.6, also see Appendix B). The most significant difference occurred at the Mt Jagged vineyard where 27 unburst buds were observed, 11 more than expected. As in 2000, the number of unburst buds at Ashton and Lenswood was less than the expected, indicating failure of buds to burst was not a feature of these vineyards.

4.3.4 Bleaching of sampled spurs

Bleached canes, often associated with *Phomopsis* infection, were observed at all sites (Table 4.7). *Phomopsis* taxon 1 was identified from the four vineyards (as described in section 2.1). Most bleaching was observed around the node. The incidence of bleaching was greater in 2000 than in 1999 at all sites. Bleaching was most severe at the Mt Jagged vineyard in all years. The association between bleaching and incidence of *Phomopsis* taxon 1 is presented in the following sections.

Table 4.7. Incidence of bleached canes on 100 sampled spurs at each of four vineyard sites in September 1999, 2000 and 2001.

	Incidence of bleached canes (%)		
Site	**1999**	**2000**	**2001**
Ashton Hills	25	62	8
Hargrave	23	48	4
Lenswood	28	51	33
Mt Jagged	46	88	45

4.3.5 Length of shoots developed on sampled spurs

4.3.5.1 Year 1999

Shoot length, measured on 26 October 1999, was highly variable within each of the four sites. Maximum shoot length was 106 cm, 89 cm, 97 cm and 120 cm at the Ashton, Hargrave, Lenswood and Mt Jagged vineyards, respectively. At the Ashton Hills vineyard, newly-developed shoots lacked vigour and approximately 10% of shoots were weak and broken. However, the cause of weakened shoots was unknown. Newly-developed shoots at the Hargrave vineyard were unhealthy and leaves showed symptoms of possible light brown apple moth infestation.

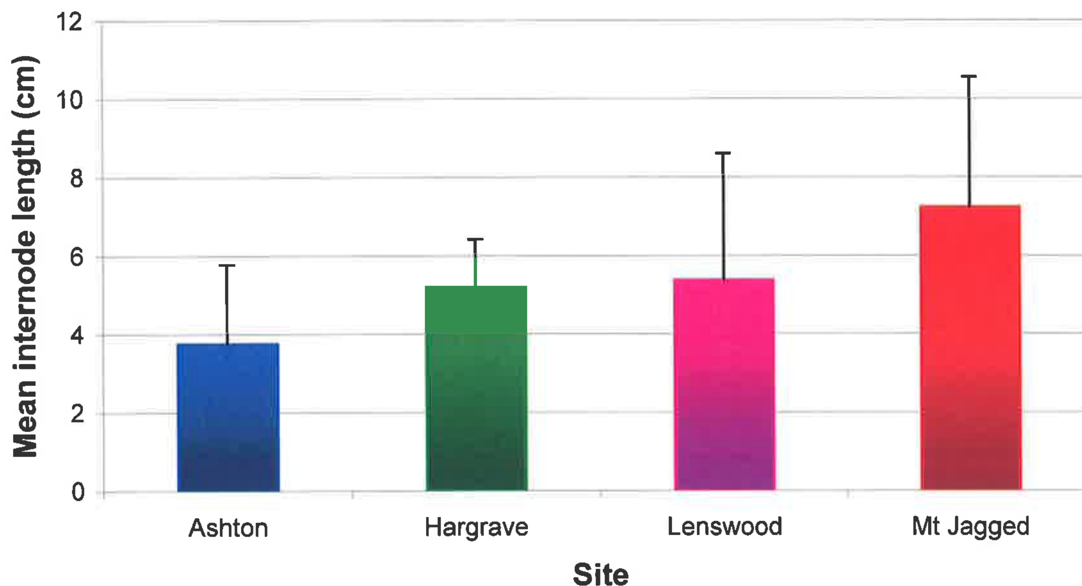


Figure 4.7. Mean length of the basal three internodes (cm) of shoots collected from spurs with unburst buds at four vineyard sites on 26 October, 1999. Bars represent standard deviation.

As *Phomopsis* on grapevine is known to cause symptoms predominantly on basal internodes, three internodes were assessed and used for extraction of total DNA (as described in section 4.2.3.1). The results showed that there was little variation in the length of internodes used for assessment (Figure 4.7).

Although growers in the Adelaide Hills have suggested that *Phomopsis* taxon 1 causes a reduction in shoot length, one-way analysis of variance showed there was no significant association between bleaching symptoms and shoot length in 1999 (P=0.6569, P=0.6547 and P=0.4293 for Ashton Hills, Hargrave and Lenswood, respectively). However, at the Mt Jagged vineyard, shoots on bleached spurs were significantly longer (P=0.0207) than on non-bleached spurs (Figure 4.8).

Shoot length was not measured in 2000.

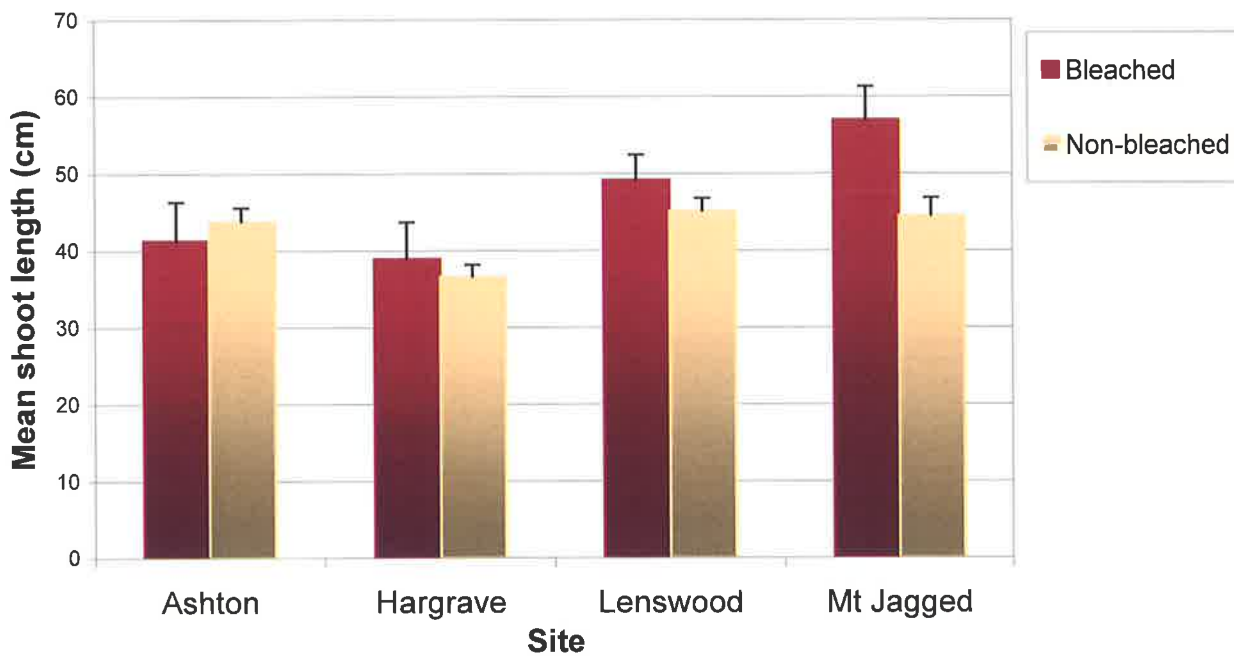


Figure 4.8. Mean shoot length (cm) on bleached spurs and non-bleached spurs recorded at four vineyard sites on 26 October 1999. Bars represent standard error.

4.3.5.2 Year 2001

Little variation was observed in length of shoots measured at two sites on 6 November 2001, approximately 8 weeks after budburst (Figure 4.9). The average length of shoots was 25.7 cm (SD=11.62) and 21.64 (SD=12.44) at Ashton Hills and Hargrave vineyards, respectively. Shoots at Hargrave were scarred and showed leaf symptoms associated with mite infestation and light brown apple moth damage. In addition, shoots appeared flattened. Shoots at Ashton Hills also showed signs of mite damage. Although in successive years, shoot length was measured 8 weeks after bud burst, a true comparison of length could not be made because shoot vigour is influenced by a number of factors including number of buds retained, vine capacity and climate.

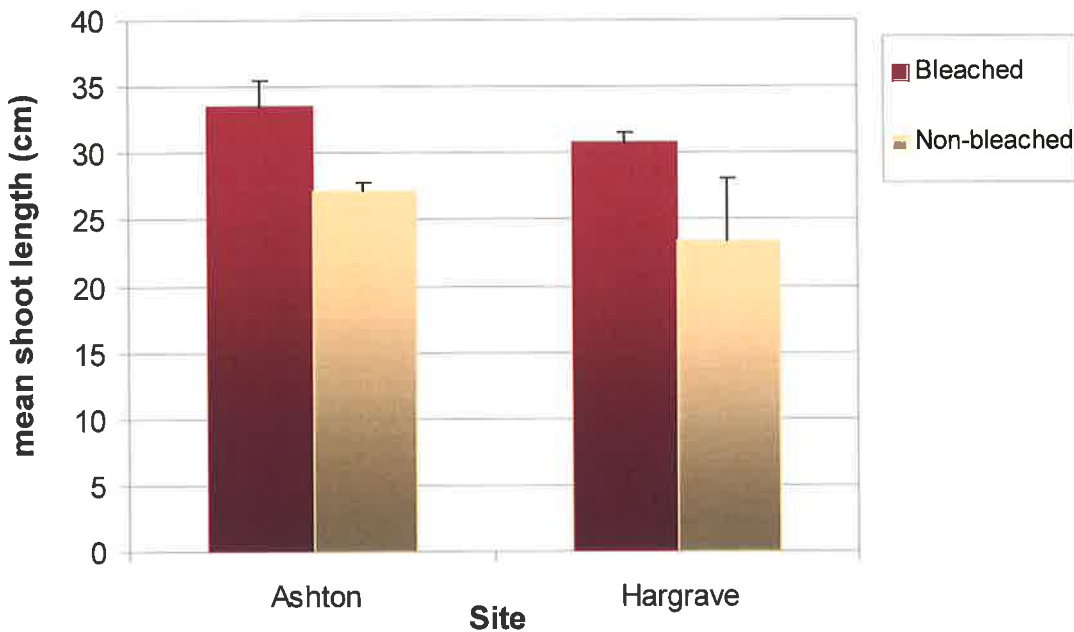


Figure 4.9. Mean shoot length (cm) on bleached spurs and non-bleached spurs recorded at Ashton Hills and Hargrave vineyards on 6 November, 2001. Bars represent standard error.

One-way analysis of variance indicated that shoot length did not differ significantly between shoots on bleached spurs and those on non-bleached spurs at both sites. There was no evidence to support the assumption that shoots were stunted on bleached spurs (whereby bleaching was often attributed to infection by *Phomopsis* taxon 1). However, bleaching was minimal in 2001, where 8% and 4% of sampled spurs were bleached at Ashton Hills and Hargrave, respectively. There was no obvious association between bleaching and shoot length.

4.3.6 Bunch count on sampled spurs

The numbers of bunches on the developed shoots were significantly different at the four sites in 2000 (P=0.0024) and 2001 (P=0.0011, Table 4.8). The greatest differences were observed between the Ashton Hills and Mt Jagged vineyards, where vines at Ashton Hills consistently produced most bunches. Bunch number was lowest at the Mt Jagged vineyard. Mt Jagged

vines were more vigorous than those in the Adelaide Hills vineyards, which may have contributed to poor bud fruitfulness.

There was no correlation between bunch number and bleaching on the sampled spurs using one-way analysis of variance (Table 4.9). Although bleaching was most severe at all sites in 2000, no effects on bunch number were evident.

Table 4.8. Mean bunch number on 100 sampled spurs at four vineyard sites in November 2000 and 2001. Means in columns followed by the same letter are not significantly different at $P<0.05$ according to least significant difference.

	Mean bunch number	
Site	**2000**	**2001**
Ashton Hills	1.27 [a]	1.62 [a]
Hargrave	1.09 [bc]	0.82 [c]
Lenswood	1.19 [ab]	1.45 [b]
Mt Jagged	1.02 [c]	0.57 [d]

Table 4.9. Mean number of bunches on bleached spurs and spurs not bleached from a sample size of 100 spurs at four vineyard sites in November (a) 2000 and (b) 2001. There were no significant differences between the means ($P<0.05$).

(a) 2000

Site	**Bleached spur**	**Non-bleached spur**	***P*-value**
Ashton	1.22	1.36	0.1579
Hargrave	1.04	1.11	0.5779
Lenswood	1.28	1.16	0.2074
Jagged	1.02	1.02	0.9939

(b) 2001

Site	**Bleached spur**	**Non-bleached spur**	***P*-value**
Ashton	1.86	1.61	0.3704
Hargrave	1.00	0.82	0.6081
Lenswood	1.40	1.52	0.4117
Jagged	0.74	0.51	0.1274

4.3.7 Detection of *Phomopsis* in unburst buds using conventional and molecular techniques

The number of unburst buds collected at each site is shown in Table 4.10. In 1999, ten unburst buds were randomly selected from the total collected sample at the Hargrave, Lenswood and Mt Jagged vineyards for microscopic examination and isolation of *Phomopsis* taxon 1 directly from the bud onto PDA (see section 4.2.3.1). Preliminary investigations were carried out on buds from Mt Jagged. Eight buds from the Mt Jagged vineyard were dead (bud health score 4), whereby the internal bud lacked green tissue and woolly hairs were observed only. The remaining two buds were healthy. After 2 days of incubation, mycelium that grew from buds was transferred to new PDA plates. After a further 7 days of incubation, cultures were contaminated with yeast and other fungi making it difficult to identify any *Phomopsis* taxon 1 that may have been present. Surface-sterilization of ten buds from Hargrave and Lenswood vineyards was shown to minimise growth of many contaminants. *Phomopsis* taxon 1 was isolated on PDA from 70% of unburst buds and 40% of unburst buds from the Hargrave and Lenswood vineyards, respectively.

Table 4.10. Number of unburst buds collected at each site in November 1999, 2000 and 2001.

	Number of unburst buds		
Site	**1999**	**2000**	**2001**
Ashton Hills	46	8	13
Hargrave	27	7	20
Lenswood	20	6	9
Mt Jagged	30	24	27
Total	123	45	69

Ten unburst buds from the Hargrave, Lenswood and Mt Jagged vineyards were assessed to compare the efficiency of culturing and hybridisation methods for the detection of

Phomopsis taxon 1. An extra ten buds were assessed from the Lenswood vineyard to determine if the quality of genomic DNA was affected after removal of unburst buds from PDA. The unburst buds weighed between 7 mg and 40 mg each. Total DNA extracted from the unburst buds after incubation on PDA was degraded compared to DNA extracted from fresh unburst buds as shown by the smears on the agarose gel in Figure 4.10a and b. It was possible that degradation of DNA would hinder hybridisation of the taxon-specific probes to DNA, but this did not interfere with specificity of the DNA probes. *Phomopsis* taxon 1 was detected in DNA obtained from both sources (Figure 4.11).

Slot blot analysis of DNA from the ten unburst buds removed from PDA revealed *Phomopsis* taxon 1 in 90% of unburst buds from Hargrave, 60% from Lenswood and 80% of unburst buds from the Mt Jagged vineyards. *Phomopsis* taxon 1 was detected in both healthy and dead buds, and from canes with and without bleaching (Table 4.11). In comparison, fungal contamination hindered the detection of *Phomopsis* taxon 1 by conventional methods. *Phomopsis* taxon 1 was not identified from unburst buds collected from the Mt Jagged vineyard by culturing, however, detection based on hybridisation of the taxon 1-specific probe, pT1P180, revealed 80% of these buds were infected by taxon 1. Likewise, *Phomopsis* taxon 1 was detected in more unburst buds from the Hargrave and Lenswood vineyards by hybridisation of DNA to the taxon 1-specific probe than by culturing. In general, hybridisation of the taxon-specific probe to DNA extracted from unburst buds detected between 20 ng and 300 ng of *Phomopsis* taxon 1 per 10 mg of grapevine tissue. For example, of the ten unburst buds used in both culturing and hybridisation of DNA to the taxon 1-specific probe, five unburst buds contained 20 ng of *Phomopsis* taxon 1 DNA (data not shown) and unburst bud R9T16 contained approximately 100 ng of taxon 1 DNA per 10 mg (Figure 4.11). Bud R9T16 was removed from bleached cane, however *Phomopsis* taxon 1 was detected in unburst buds from both bleached and non-bleached cane.

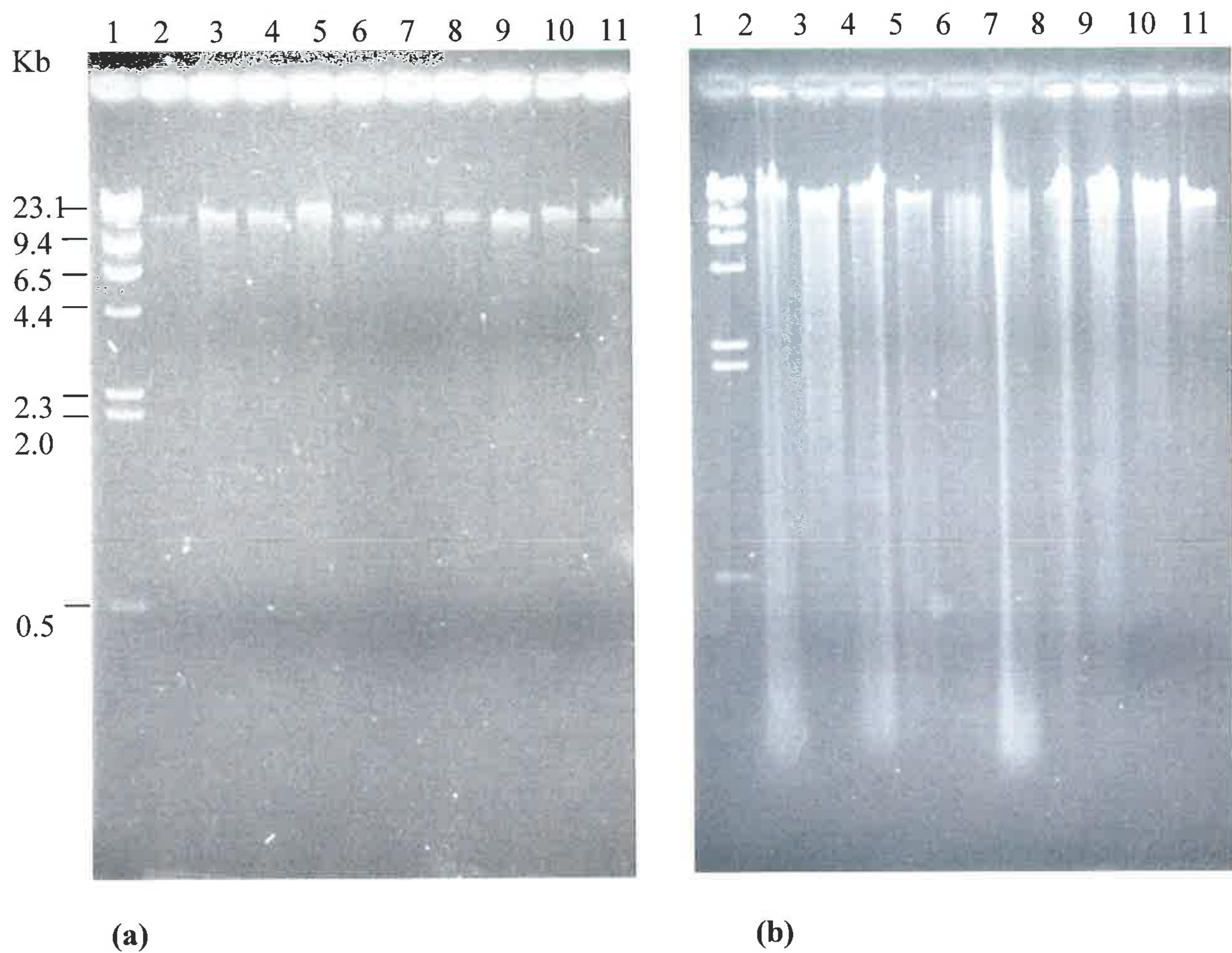


Figure 4.10. Total DNA extracted from 20 unburst buds of grapevine collected from Lenswood on November 1999. **(a)** Lane 1, 0.25 µg lambda DNA digested with *Hin*dIII; lanes 2-11, total DNA extracted from 10 unburst buds immediately after excision from the spur. **(b)** Lane 1, 0.25 µg lambda DNA digested with *Hin*dIII; lanes 2-11, total DNA extracted from 10 unburst buds after incubation for 2 days on PDA.

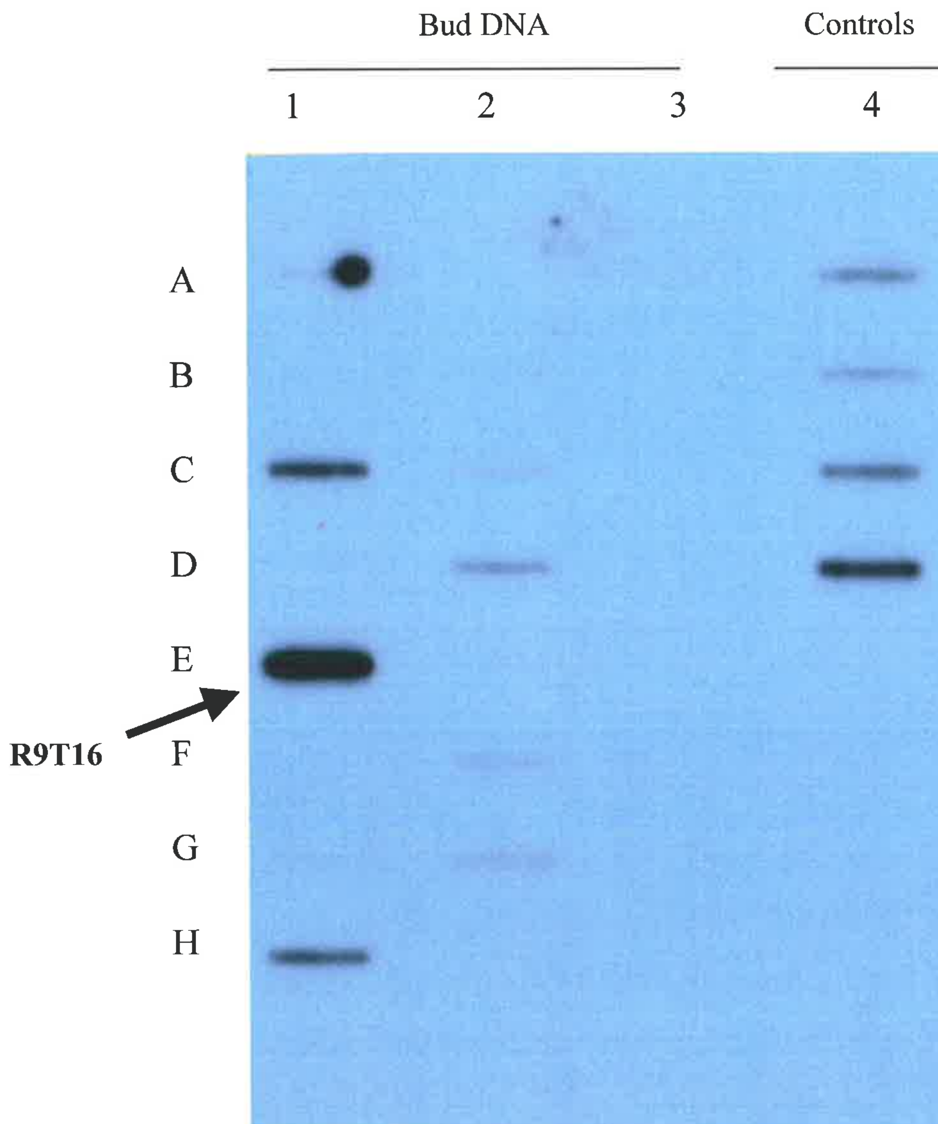


Figure 4.11. Detection of *Phomopsis* taxon 1 in unburst buds (cv. Chardonnay), collected from Lenswood vineyard in November 1999, by slot blot analysis (Slot L99) using the *Phomopsis* taxon 1-specific probe, pT1P180. Slots 1A-3D contain 100 ng total DNA obtained from unburst buds; slots 3E-3F contain ddH_20; slots 3G and 3H contain 100 ng grapevine DNA. Slots 4A-4D contain purified DNA from *Phomopsis* taxon 1 isolate A223.1, 5 ng, 10 ng, 20 ng and 40 ng, respectively. Slots 4E-4H contain purified DNA from *Phomopsis* taxon 2 isolate M827, 5 ng, 10 ng, 20 ng and 40 ng, respectively. Each slot represents an individual DNA sample. R9T16 (slot 1E, arrow) shows hybridisation of the taxon 1-specifc probe to DNA obtained from an unburst bud that was cultured on PDA.

Table 4.11. Ten unburst buds collected from spurs (bleached and non-bleached) at three vineyard sites in November 1999 assessed for health and isolation of *Phomopsis* taxon 1 from the bud (1) by culture on PDA and subsequently (2) by hybridisation of total DNA to the *Phomopsis* taxon 1-specific probe pT1P180.

Site	Bud sample [a]	Bleached cane	Bud health [b]	*Phomopsis* taxon 1	
				PDA [c]	DNA [d]
Hargrave	R9T3	✗	4	✓	✓
	R9T11	✗	4	✗	✗
	R10T18 (bud one)	✗	4	✗	✓
	R11T9	✓	4	✓	✓
	R12T2 (bud one)	✗	4	✓	✓
	R12T2 (bud two)	✗	4	✓	✓
	R12T3	✓	4	✓	✓
	R12T8	✗	4	✗	✓
	R13T14	✓	4	✓	✓
	R13T15	✗	4	✓	✓
Lenswood	R9T1	✗	1	✓	✓
	R9T2	✓	4	✗	✗
	R9T17	✗	2	✗	✗
	R10T6	✓	1	✓	✓
	R11T20 (bud one)	✗	1	✓	✓
	R11T20 (bud two)	✗	3	✗	✗
	R12T4	✗	1	✗	✓
	R12T8	✗	1	✗	✓
	R12T20	✗	1	✓	✓
	R13T7	✗	1	✗	✗
Mt Jagged	R4T10	✗	4	✗	✗
	R5T2	✗	4	✗	✓
	R5T5	✗	4	✗	✓
	R5T15	✗	1	✗	✓
	R6T20	✗	4	✗	✓
	R7T1	✗	4	✗	✓
	R7T7	✓	4	✗	✓
	R7T14	✓	4	✗	✓
	R8T4	✓	4	✗	✓
	R8T7	✓	3	✗	✗

[a] = Bud sample denoted by R=row and T=tag at each site.

[b] = Buds were scored according to the health of the bud whereby;

1 = healthy, tissue is green
2 = moderately healthy, bud tissue slightly brown
3 = unhealthy, tissue moist, brown in colour
4 = dead, bud is dry and brown.

[c] = Bud cut in half, placed on PDA and mycelium isolated 2 days after incubation. Pure cultures were prepared on PDA.

[d] = Bud removed from PDA after 2 days, total DNA extracted and hybridised with the *Phomopsis* taxon 1-specific probe, pT1P180, using slot blot analysis.

The taxon 2-specific probe, pT2P25, hybridised to DNA obtained from unburst bud R10T16, indicating that *Phomopsis* taxon 2 was present. Furthermore, mycelium that grew from the unburst bud R10T16 was transferred to a new PDA plate, and conidia were identified as those of *Phomopsis* taxon 2. The taxon 2-specific probe hybridised to DNA obtained from cane corresponding to the unburst bud R10T16, but taxon 2 was not detected in other samples collected from the Lenswood vineyard.

In 1999, 123 unburst buds were collected from four vineyard sites from a total sample size of 1052 buds. Total DNA hybridised with the taxon 1-specific DNA probe, pT1P180, revealed that 69 unburst buds were infected with *Phomopsis* taxon 1. Chi-square analysis showed there was a significant association (P=0.0023) between *Phomopsis* taxon 1 infection and the number of unburst buds in 1999. The association was significant at the Hargrave (P=0.0002) and Mt Jagged vineyards (P=0.0001) but not at the Ashton Hills (P=0.4867) and Lenswood vineyards (P=0.9402). Statistical analysis of the data to determine the effect of *Phomopsis* taxon 1 on budburst using the chi-square test did not produce reliable statistics because the presence of *Phomopsis* taxon 1 was not assessed for every bud (burst and unburst) at each site.

Table 4.12 summarises the number of unburst buds collected from each site over the three seasons and the presence of *Phomopsis* taxon 1 in these buds was determined by hybridisation of the taxon 1-specific probe, pT1P180, to all DNA samples. In 1999, 63% and 60% of the unburst buds collected at the Hargrave and Mt Jagged vineyards, respectively, were infected with *Phomopsis* taxon 1. Although the incidence of *Phomopsis* taxon 1 infection was high, it was difficult to confirm that *Phomopsis* taxon 1 caused failure of buds to burst because abnormal budburst was not observed in the four vineyards. In subsequent

Table 4.12. Number of unburst buds and corresponding number of unburst buds infected with *Phomopsis* taxon 1 from total number of buds assessed at four vineyard sites in 1999, 2000 and 2001. The presence of *Phomopsis* taxon 1 was determined by hybridisation of the taxon 1-specific probe, pT1P180 to DNA samples obtained from unburst buds. The incidence of *Phomopsis* taxon 1 infection (%) in unburst buds is denoted in the brackets.

(a) 1999

Site	Total no. buds	No. unburst buds	No. unburst buds infected with taxon 1*
Ashton	303	46	20 (43%)
Hargrave	248	27	17 (63%)
Lenswood	258	20	9 (45%)
Jagged	243	30	18 (60%)

(b) 2000

Site	Total no. buds	No. unburst buds	No. unburst buds infected with taxon 1
Ashton	240	8	4 (50%)
Hargrave	206	7	0 (0)
Lenswood	198	6	2 (34%)
Jagged	210	24	9 (37%)

(c) 2001

Site	Total no. buds	No. unburst buds	No. unburst buds infected with taxon 1
Ashton	231	13	1 (8%)
Hargrave	200	20	0 (0)
Lenswood	218	9	5 (56%)
Jagged	200	27	4 (15%)

*chi-square analysis showed a significant association between failure of buds to burst and *Phomopsis* taxon 1 infection (P=0.0023) among all sites in 1999, but there was no association observed in 2000 and 2001.

years, however, *Phomopsis* taxon 1 was not detected in unburst buds at the Hargrave vineyard, indicating that bud loss was not caused by taxon 1.

In 2000 and 2001, the incidence of *Phomopsis* infection decreased markedly compared to 1999 at all sites except the Lenswood vineyard. At the Lenswood vineyard, five buds of the nine unburst buds collected were infected with *Phomopsis* taxon 1. Adequate budburst was achieved at all vineyard sites and fewer buds failed to burst than in 1999. Over the three years, only 35 of 674 buds failed to burst at the Lenswood vineyard and, of these, 16 were infected with *Phomopsis* taxon 1. Likewise, of 774 buds monitored over 3 years at the Ashton vineyard, 67 did not burst and, of these, 25 were infected with *Phomopsis* taxon 1. This trend was observed at all sites thus, it was considered unlikely that *Phomopsis* taxon 1 affected budburst.

Budburst was random along the rows at all sites and unburst buds infected with *Phomopsis* taxon 1 were not localised in the vineyard (Figure 4.12). At Mt Jagged vineyard, row 7 showed the highest incidence of unburst buds infected with *Phomopsis* taxon 1, but taxon 1 did not affect budburst in neighbouring rows. Because continuous sampling was not carried out within the entire experimental area at each vineyard, statistical analysis could not be performed to determine the overall incidence of *Phomopsis* taxon 1.

4.3.8 Detection of *Phomopsis* taxon 1 in canes

In this section, the term 'cane' denotes the woody tissue of the sampled spur as described in section 4.2.3.1. An average of 1 μg total DNA was extracted from 0.1 g of cane. Hybridisation of DNA with the taxon 1 and taxon 2-specific probes, pT1P180 and pT2P25, respectively, using a slot blot assay revealed *Phomopsis* taxon 1 in canes at the four vineyards in all years. The taxon 1-specific probe, pT1P180, did not hybridise to DNA from *Phomopsis*

Ashton Hills Vineyard (cv. Chardonnay)

Hargrave Vineyard (cv. Chardonnay)

Lenswood Horticultural Centre (cv. Chardonnay) Block D

Mt Jagged Vineyard (cv. Shiraz)

Figure 4.12. Plan of experimental vineyard sites at Ashton Hills, Hargrave, Lenswood and Mt Jagged in 1999. The position of individual vines is denoted by a grid section. Spurs were sampled on vines designated 1= one spur, 2= two spurs and 3= three spurs. The unburst buds infected with *Phomopsis* taxon 1 are denoted in red. Missing vines are denoted in grey.

taxon 2, grapevine and other fungi associated with grapevine. By comparing the intensity of the hybridisation signals obtained from a dilution series of purified DNA of *Phomopsis* taxon 1, between 1 and 75 ng taxon 1 DNA was detected in samples containing approximately 100 ng of total DNA (data not shown). Dilution series of purified DNA from taxon 1 were included on slot blot membranes only where space permitted. The slot blot displayed in Figure 4.13 shows the hybridisation of the taxon 1-specific probe to DNA obtained from bleached and non-bleached canes from the Mt Jagged vineyard in 1999. Of the 30 unburst buds collected, 21 were infected with *Phomopsis* taxon 1. There was no correlation between infection by *Phomopsis* taxon 1 and bleached cane. For example, the hybridisation signal displayed in slot 4E resulted from DNA extracted from a non-bleached cane sample. In contrast, *Phomopsis* taxon 1 was detected in DNA extracted from a bleached cane sample (e.g. slot 2B).

Analysis of variance indicated that significantly more canes were infected with *Phomopsis* taxon 1 in 1999 than in other years (P=0.0069). In 1999, there was a high incidence of *Phomopsis* taxon 1 in canes at the Hargrave and Mt Jagged vineyards; however, in subsequent years taxon 1 was not detected (Table 4.13). Of the 100 spurs sampled having unburst buds in the first year at the Hargrave vineyard, 95% were infected with taxon 1 but not all canes were bleached (55%). At the Mt Jagged vineyard, 70% canes were infected, with 57% of these bleached. In addition, taxon 1 was not detected in bleached canes at the Mt Jagged vineyard in 2001. The results indicated that bleaching may be associated with factors other than infection by *Phomopsis* taxon 1.

In 2000 and 2001, canes were collected from spurs having all buds burst (control). DNA was extracted from cane and hybridised to the taxon 1-specific probe, pT1P180 and the taxon 2-specific probe, pT2P25. The incidence of *Phomopsis* taxon 1 was highly variable at the four sites in the 2 years (Table 4.14). *Phomopsis* taxon 1 was detected in 100% of spurs

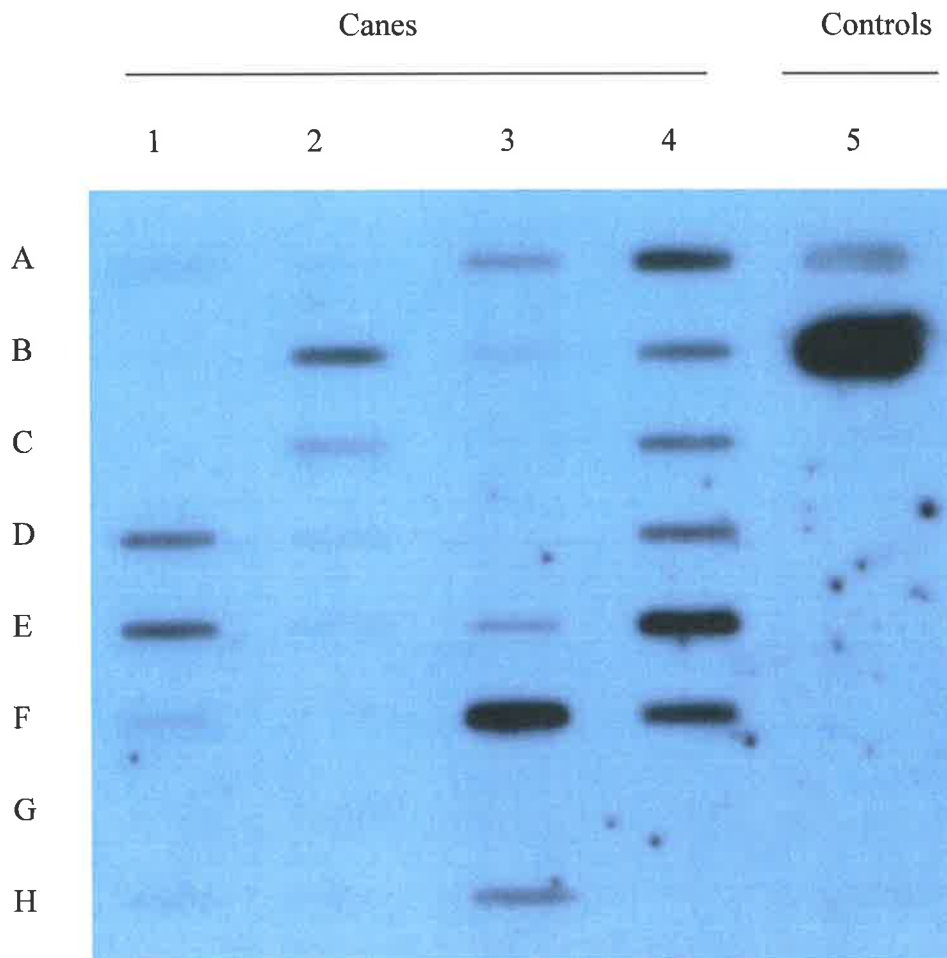


Figure 4.13. Detection of *Phomopsis* taxon 1 in cane (cv. Shiraz), collected from Mt Jagged vineyard in November 1999, by slot blot analysis (Slot MJ68) using the *Phomopsis* taxon 1-specific probe, pT1P180. Slots 1A-4F contain 100 ng DNA from cane; slots 4G and 4H contain ddH_20. Slots 5A and 5B contain purified DNA from *Phomopsis* taxon 1 isolate A223.1 50 ng and 100 ng, respectively. Slots 5C and 5D contain purified DNA from *Phomopsis* taxon 2 isolate M827 25 ng and 100 ng, respectively. Slot 5E contains 100 ng grapevine DNA; slot 5F, 100 ng *Aspergillus* sp. DNA; slot 5G, 100 ng *Botrytis cinerea* DNA and slot 5H, 100 ng *Saccharomyces cerevisiae* DNA. Each slot represents an individual DNA sample.

Table 4.13. Number of canes having unburst buds, bleaching and the incidence of *Phomopsis* taxon 1 as determined by the use of taxon 1-specific DNA probe, pT1P180, at four vineyard sites in 1999, 2000 and 2001. The incidence of *Phomopsis* taxon 1 infection (%) in canes having unburst buds is denoted in the brackets.

(a) 1999

Site	No. canes	No. bleached canes	No. canes infected with taxon 1
Ashton	40	8	12 (30%)
Hargrave	20	11	19 (95%)
Lenswood	16	7	10 (63%)
Jagged	30	17	21 (70%)

(b) 2000

Site	No. canes	No. bleached canes	No. canes infected with taxon 1
Ashton	8	4	6 (75%)
Hargrave	7	1	0 (0)
Lenswood	6	3	4 (67%)
Jagged	22	20	12 (55%)

(c) 2001

Site	No. canes	No. bleached canes	No. canes infected with taxon 1
Ashton	13	1	1 (8%)
Hargrave	20	2	0 (0)
Lenswood	9	5	7 (78%)
Jagged	26	13	0 (0)

where all buds burst at the Lenswood vineyard in 2000 and 80% of canes collected in 2001. Of the 20 canes collected at the Lenswood vineyard in 2001, two were infected with *Phomopsis* taxon 2. *Phomopsis* taxon 1 was detected in 30% and 50% of canes at the Mt Jagged and Ashton vineyards, respectively. At the Hargarve vineyard, *Phomopsis* taxon 1 was not detected. The results indicated that *Phomopsis* taxon 1 was present in both spurs with unburst buds and spurs with developed shoots.

Table 4.14. Incidence of *Phomopsis* taxon 1 in spurs having all buds burst in four vineyards in 2000 and 2001 as determined by hybridisation of DNA obtained from cane with the taxon 1-specific probe, pT1P180.

	Incidence of *Phomopsis* taxon 1 (%)	
Site	**2000**	**2001**
Ashton	30	50
Hargrave	0	0
Lenswood	100	80
Mt Jagged	10	30

4.3.9 Detection of *Phomopsis* taxon 1 in shoots

Green shoots were more vigorous at the Mt Jagged vineyard than at the other sites in 1999 and 2000, however shoots were not significantly longer than those at other sites. Shoots were collected from spurs having unburst buds. There were no significant differences in internode length at any of the sites or in the three seasons. An average of 2 μg of total DNA was extracted from 0.1 g of green shoot. In the first year, DNA was not obtained from nine of the 20 shoots collected at the Mt Jagged vineyard due to loss of DNA during preliminary experimentation with extraction methods. Hybridisation of the taxon 1-specific probe, pT1P180, to total DNA using a slot blot assay showed *Phomopsis* taxon 1 to have been in shoots at all sites. Comparison of hybridisation signals from total DNA to intensity of signals

from purified *Phomopsis* taxon 1 DNA revealed between 2 ng and 20 ng of taxon 1 DNA in 100 ng total DNA (Figure 4.14). The taxon 1-specific probe did not hybridise to purified DNA from *Phomopsis* taxon 2 and grapevine. The amount of *Phomopsis* taxon 1 DNA detected in shoots was less than was identified in unburst buds and canes.

The incidence of *Phomopsis* taxon 1 varied considerably among the four sites in the first year. Hybridisation of total DNA revealed that 70% of the 37 shoots collected from the Ashton vineyard in 1999 were infected with *Phomopsis* taxon 1 (Table 4.15). More green shoots than unburst buds were infected at this site. In the following years, however, there was little or no *Phomopsis* taxon 1 detected in shoots collected from the Ashton vineyard. The incidence of taxon 1 in shoots sampled from the Mt Jagged vineyard was low in all years and infection was not observed in 2001. A lack of hybridisation signals indicated that there was no, or an undetectably small amount, of *Phomopsis* taxon 1 present in the shoots.

Non-infected shoots were not significantly longer than shoots infected by *Phomopsis* taxon 1 in 1999. Insufficient assessments were made of shoots from spurs having either burst and unburst buds, and *Phomopsis* taxon 1 was not detected in 2000 and 2001, thus a chi-square test was not performed. The results were based on internode length of shoots from spurs having unburst buds. Statistical analysis of total shoot length (see section 4.3.5) and infection by *Phomopsis* taxon 1 was not valid, as all shoots in the four vineyards were not assessed. There was little evidence to suggest that *Phomopsis* taxon 1 is associated with poor shoot growth.

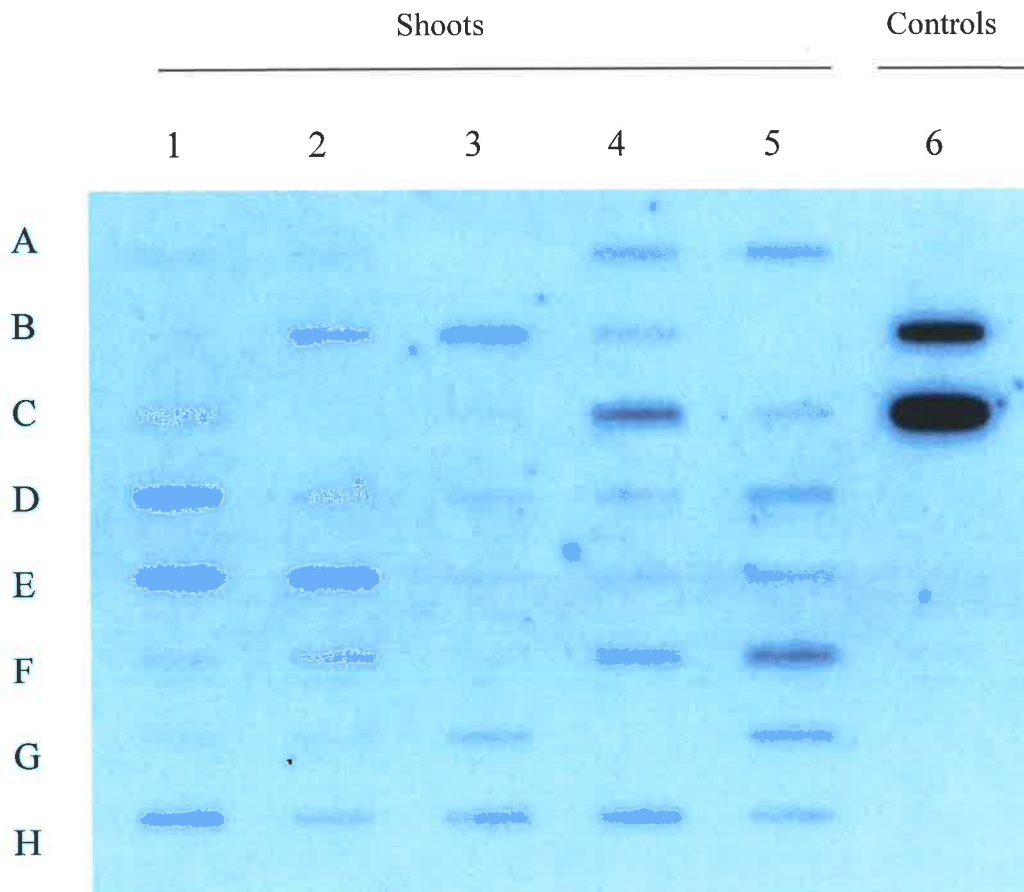


Figure 4.14. Detection of *Phomopsis* taxon 1 in green shoots (cv. Chardonnay), collected from Ashton Hills vineyard in November 1999, by slot blot analysis (Slot A169) using the *Phomopsis* taxon 1-specific probe, pT1P180. Slots 1A-5H contain 100 ng total DNA from green shoots on sampled spurs having an unburst bud, except slots 2C, 3A and 4G which contain ddH_20. Slots 6A-6C contain purified DNA from *Phomopsis* taxon 1 isolate A223.1, 1 ng, 12.5 ng and 25 ng, respectively. Slots 6D-6F contain purified DNA from *Phomopsis* taxon 2 isolate C608, 1 ng, 12.5 ng and 25 ng, respectively. Slot 6G contains 100 ng grapevine DNA and slot 6H contains ddH_20. Each slot represents an individual DNA sample.

Table 4.15. Incidence of *Phomopsis* taxon 1 in green shoots collected from sampled spurs having unburst buds at four vineyard sites in November 1999, 2000 and 2001. Number of shoots assessed is denoted in brackets.

	Incidence of *Phomopsis* taxon 1 (%)		
Site	**1999**	**2000**	**2001**
Ashton	70 (37)	12 (8)	0 (12)
Hargrave	15 (20)	0 (6)	0 (19)
Lenswood	43 (16)	0 (4)	0 (16)
Mt Jagged	5 (20)	9 (22)	0 (9)

4.3.10 Detection of *Phomopsis* taxon 1 in leaves

Leaf lesions were obtained from nine leaves at the Mt Jagged vineyard in 1999. The necrotic spots were approximately 1 mm in diameter, however, there was no distinct yellow halo. After 2 days of incubation, white, aerial mycelium was isolated from two leaves but sub-culturing to fresh PDA showed the fungus to be neither *Phomopsis* taxon 1 nor taxon 2. To verify these findings, mycelium was recovered from the agar and DNA extracted from mycelium as described in section 2.2.2. Hybridisation of total purified DNA with the taxon 1-specific probe, pT1P180, confirmed that *Phomopsis* taxon 1 was not detected.

4.3.11 Additional unburst buds assessed for *Phomopsis* taxon 1 at Mt Jagged

Microscopic examination revealed that 61% of the 23 unburst buds collected in a localised area of the cv. Shiraz block at the Mt Jagged vineyard, were dead (Table 4.16). Most buds were dry and lacked green tissue. When dead buds were cut from the spur, the tissue on the spur was dry and brown at the site of attachment. In comparison, green tissue was observed when healthy buds were isolated from the spur.

Table 4.16. Unburst buds collected from ten spurs (cv. Shiraz) from a localised region of the vineyard at Mt Jagged on 24 October 2000, assessed for health and presence of *Phomopsis* taxon 1 by use of the taxon 1-specific probe, pT1P180.

Spur	Bud sample	Bud health [a]	*Phomopsis* taxon 1 [b]
1	1	4	✗
1	2	4	✗
2	1	4	✗
2	2	4	✗
3	1	1	✗
3	2	4	✗
3	3	4	✗
3	4	3	✗
4	1	2	✗
4	2	4	✗
5	1	2	✗
5	2	1	✓
6	1	4	✗
7	1	4	✗
7	2	1	✗
7	3	4	✗
8	1	1	✗
8	2	4	✗
8	3	3	✗
8	4	4	✗
9	1	4	✗
10	1	2	✗
10	2	4	✗

[a] = Buds were scored according to the health of the bud whereby;
1 = healthy, tissue is green
2 = moderately healthy, bud tissue slightly brown
3 = unhealthy, tissue moist, brown in colour
4 = dead, bud is dry and brown.

[b] = Total DNA extracted and hybridised with the *Phomopsis* taxon 1-specific probe, pT1P180, using slot blot analysis.

The weight of unburst buds varied from 5 mg to 80 mg. The quantity of DNA from each bud averaged 200 ng per 10 mg of bud tissue. The quality of DNA was similar for healthy and unhealthy buds. The *Phomopsis* taxon 1-specific probe hybridised to DNA from one healthy bud. Comparison of the intensity of the hybridisation signal to purified DNA of taxon 1 showed 6 ng *Phomopsis* taxon 1 DNA in 100 ng total DNA from the unburst bud (data not shown). The results indicated that the healthy bud was infected by *Phomopsis* taxon 1. No signals were detected following hybridisation of the taxon 1-specific probes to DNA from other unburst buds, taxon 2 or grapevine DNA. *Phomopsis* taxon 1 was not detected in unhealthy buds.

4.4 Discussion

There has been a great deal of confusion concerning the casual agent of Phomopsis cane and leaf spot. It was shown in 1995 that two taxa are associated with the disease in Australia (Merrin *et al.*, 1995) and, since then, the morphology and genetic variation of both taxa have been investigated (Melanson *et al.*, 2002; Scheper, 2001). It is still uncertain if chemical control for both taxa is warranted because the pathogenicity of taxon 1 has not been confirmed. This study has shown that the pathogenicity of *Phomopsis* taxon 1 is difficult to verify when distinctive symptoms are not produced on grapevine. Bleaching of cane is used as a guide to assess if a vineyard is infected with *Phomopsis*, but the findings showed that *Phomopsis* taxon 1 is not wholly responsible for bleaching of canes. The results of the present investigation (see chapter 3) support the suggestion that *Phomopsis* taxon 1 may be endophytic, such that infection does not cause disease (Sinclair and Cerkauskas, 1996). Although 100% budburst is desired, this is rarely achieved. Percent budburst depends on a range of factors such as pruning level, climate and water stress (Baldwin, 1965) and, because

they often interrelate, it is difficult to isolate the impact of these factors on budburst. Poor budburst cannot be expressed as a quantifiable amount, since percent budburst is relative to management practices and history of the vineyard (M. McCarthy, personal communication). The present study showed that *Phomopsis* taxon 1 did not significantly inhibit budburst. However, this aspect was difficult to investigate because there was great variation in the number of unburst buds observed over 3 years at each of the four vineyards. The budburst percentage is largely dependent on the number of nodes left after pruning the previous winter (Tassie and Freeman, 1992). If too many buds are retained, the vine does not have enough capacity for all of the buds to burst. In the first year, more buds were retained at pruning at the Ashton vineyard than at other sites. The observation that basal buds did not burst at the Ashton vineyard, compared to other sites in the Adelaide Hills, suggest that the vines did not have the capacity for all buds to burst, thus budburst percentage was reduced. In the following years, fewer buds were left after pruning and > 93% budburst was achieved.

Observations and measurement of total shoot length and internode length indicated that vines at Mt Jagged were very vigorous. Shiraz vines have a spreading habit and vigorous shoot growth (Dry and Gregory, 1988). Vines with excess vigour have longer shoots, extensive lateral shoots and shading problems. Budburst in spring is affected by the growth of the vine in the previous season, therefore, failure of buds to burst can be associated with vigorous shoot growth. Vigorous vines produce dense canopies and, if shaded nodes in the canopy are retained at pruning, budburst is often low (Coombe, 1988). Shading also increases the incidence of fungal diseases. This may explain the severity of bleaching associated with pycnidium-producing fungi at the Mt Jagged vineyard in all years. Additionally, the vines sampled were located near a dam of 4 ha and high relative humidity may have contributed to fungal infection also. However, the results showed that bleaching was not always associated

with *Phomopsis* taxon 1. For example, in 2001, 45% of spurs sampled from the Mt Jagged vineyard were bleached but *Phomopsis* taxon 1 was not detected in any of these.

Budburst is hastened by warm temperatures during the latter part of winter (Antcliff and Webster, 1955; McIntyre *et al.*, 1982). This was evident in 1999, where early budburst occurred following above average temperatures in August. It is unlikely that fungal colonisation of the bud would cause delayed bud growth, as budburst is highly influenced by temperature. It is more likely that fungal infection would cause bud death as a result of colonisation of the host tissue, such has been reported for infection of raspberry by *Didymella applanta* and *Botrytis cinerea* (Rebandel, 1985).

In the present study, there was little evidence of *Phomopsis* taxon 1 causing delayed budburst or bud death. The number of unburst buds was greatest in 1999, but percent budburst was acceptable at the four sites. The highest level of infection in unburst buds was seen at the Hargrave vineyard. However, the suggestion that the fungi caused bud loss was not supported because only 17 unburst buds of 248 buds were infected with *Phomposis* taxon 1. Furthermore, *Phomopsis* taxon 1 was not detected in this vineyard in the following seasons.

The vineyard sites were selected based on apparent infection of *Phomopsis* taxon 1, but there was no significant effect on shoot growth or bunch number. In many instances, shoots were longer on bleached canes than non-bleached canes, although bleaching may be attributed to factors other than *Phomopsis* taxon 1. In 1999, infection of green shoots by *Phomopsis* taxon 1 varied considerably between sites, ranging from 5% to 70% of infected shoots at the Mt Jagged and Ashton vineyards, respectively. Bunch number was not significantly different on bleached and non-bleached canes.

Microscopic studies described in chapter 3 showed that *Phomopsis* taxon 1 colonised the epidermis of green grapevine shoots and, hence, it is unlikely that the fungus colonised

the bud from vascular tissue. Melanson *et al.* (2002) showed perithecia protruding from an infected bud, but the health status of the bud was not reported. In the present study, unburst buds were dry and brown, with the internal bud tissue completely missing. Mycelium was not observed in these buds, although *Phomopsis* taxon-specific DNA probes showed the presence of *Phomopsis* taxon 1 in both healthy and unhealthy buds. In the study of localised bud death in the Mt Jagged vineyard, unburst buds were not infected with *Phomopsis* taxon 1. It could be suggested that dead buds do not provide DNA of adequate quality for use in a slot blot assay or the fungus grows from the dead buds into new host tissue. However, *Phomopsis* taxon 1 was detected in DNA obtained from contaminated buds in culture and showed strong hybridisation signals.

These findings suggested that *Phomopsis* taxon 1 did not cause bud death, as budburst percentage appeared "normal" in vineyards infected with *Phomopsis* taxon 1. Examination of shoot length showed that *Phomopsis* taxon 1 did not affect shoot development. A control site was needed to identify "normal" budburst and shoot growth, however, *Phomopsis* taxon 1 was found in the four vineyards. Initially, the Lenswood site was selected as a control, but the vines were infected with both *Phomopsis* taxon 1 and taxon 2. *Phomopsis* taxon 2 was not detected in other sites. It was not feasible to remove and assess every sampled spur in the four commercial vineyards, but such data may have provided more information on the extent of *Phomopsis* taxon 1 infection. In the following chapters, studies undertaken to determine when buds are infected, and if bud death is associated with infection by *Phomopsis* taxon 1 or by external factors are reported.

Chapter 5

Phomopsis taxon 1 and other factors influencing the health of dormant buds

5.1 Introduction

Phomopsis taxon 1 has been reported to be associated with reduced budburst and stunted growth of new shoots, but the findings were primarily based on observations of bleached spurs (Scheper, 2001). It has since been shown that *Phomopsis* taxon 1 is not always responsible for bleaching of spurs and canes (Melanson *et al.*, 2002). Furthermore, experiments described in chapter 4 showed *Phomopsis* taxon 1 was not consistently associated with unburst buds, thus there may be other possible causes of bud loss.

Symptoms of *Phomopsis* leaf infection are often confused with those caused by mites. For example, leaf spots caused by *Phomopsis* taxon 2 are commonly confused with chlorotic lesions caused by mite feeding, and stunting of newly-developed shoots is often associated with both infection by *Phomopsis* and infestation by mites. Rust mite and bud mite are associated with bud loss and poor shoot development (see section 1.5.3). Buds infested with bud mite usually burst, but death of dormant buds has been known to occur (Bernard *et al.*, 2000). Rust mites, however, survive in the outer bud scales and are less likely to cause bud death (Smith and Schuster, 1963; Forster *et al.*, 1999).

It is not known if bud death may be caused by colonisation of the bud by *Phomopsis* in the current season, or if failure of buds to burst is associated with infection by the fungus in the previous season. Also, there are no reports of bud death and poor fruitfulness of buds

infected by *Phomopsis* taxon 1. It is important to identify if buds are infected by *Phomopsis* taxon 1 and, if so, whether the fungi causes budburst failure. This would provide information on the necessity and timing of chemical sprays for the control of *Phomopsis* taxon 1. Furthermore, the early detection of *Phomopsis* taxon 1 in dormant buds will provide information on the extent of infection before buds are retained at pruning for the new season and if new control strategies are required.

The objectives of the experiments reported in this chapter were to; (1) determine if mites or other agents contribute to bud death or influence infection of the bud by *Phomopsis* taxon 1, (2) determine if and when dormant buds are infected by taxon 1 and (3) examine if *Phomopsis* taxon 1 influences bud fruitfulness.

5.2 Materials and methods

5.2.1 Collection of dormant buds in autumn

To investigate if buds retained at pruning were infected with *Phomopsis* taxon 1, buds were collected from 6-month-old lignified shoots in autumn of 2000 and 2001. Twenty buds were collected from ten 6-month-old lignified shoots (cv. Shiraz) at the Mt Jagged vineyard, Southern Fleurieu Peninsula, on 18 April, 2000. At the time of collection, grapevine were at Eichhorn-Lorenz stage 41 (Coombe, 1995). Leaf fall had not commenced. Buds from the first three nodes were collected and weighed.

DNA was extracted from the buds using the SEAPS extraction procedure as described in section 2.2.1 and suspended in 20 µl Tris-EDTA. DNA transferred to a slot blot membrane included: 5 ng, 10 ng, 20 ng and 40 ng purified DNA of *Phomopsis* taxon 1 isolate A223.1; 50 ng and 100 ng purified DNA from *Phomopsis* taxon 2 isolate P712; 100 ng and 200 ng DNA obtained from grapevine and sterile ddH_20. Six-month-old woody cane infected with

taxon 1 was included on the membrane as a control sample. Total DNA was hybridised with the *Phomopsis* taxon 1-specific probe, pT1P180 and taxon 2-specific probe, pT2P25 as described in section 2.2.6.

In 2001, 40 buds were collected from 7-month-old lignified shoots at each of the vineyards at Ashton Hills, Hargrave and Lenswood Horticultural Centre (all cv. Chardonnay) on 30 May (see section 4.2.1, Figure 4.1). The health of the buds from each site was assessed microscopically at 16 X magnification as described in section 4.2.3.1. DNA was extracted from the buds at the three sites and DNA hybridised with the taxon-specific probes as previously stated.

5.2.2 Collection of dormant buds in winter

To assess the relationship between the health of dormant buds and infection by *Phomopsis* taxon 1 or mites, 50 buds (cv. Shiraz) were randomly selected in the Mount Jagged vineyard, on 10 August and 14 August in 2000 and 2001, respectively. Also, 50 buds were collected from the Hargrave vineyard on 28 August 2000. Entire spurs were removed from the cordon and buds removed in the laboratory using a scalpel. Buds were stored in 2 ml Eppendorf® tubes at 4°C until examination.

Buds were examined with a microscope (Olympus) at 16 X magnification for health status and the presence of bud and rust mites. The outer bud scales were pulled apart with forceps, and the woolly tissue removed from the side of the buds. Buds were cut longitudinally in half to expose the primordia. The presence of mites was recorded, as was damage to bud tissue caused by mite feeding. Feeding damage was typically observed between the leaf primordia and bud scales. The health of the buds was scored as described in

section 4.2.3.1, except that rating 2 was modified to include damage to outer bud scales by mite feeding.

After microscopic examination, DNA was extracted from 50 complete buds (including removed sections, such as woolly tissue and bud scales) from the Mt Jagged vineyard in 2000 using the DNeasy Plant Mini kit (Qiagen, Germany) according to the manufacturer's instructions. In 2001, DNA was extracted from 50 buds collected from the Mt Jagged vineyard using the SEAPS extraction method as described in 2.2.1. DNA samples were adjusted to 100 ng total DNA, or if 100 ng was not obtained, DNA as was available was transferred to a slot blot membrane. Each membrane included a dilution series of purified DNA of *Phomopsis* taxon 1 isolate A223.1, 100 ng purified DNA of *Phomopsis* taxon 2 isolate M827 and sterile ddH_20. Total DNA was hybridised with the taxon-specific probes as previously described in section 5.2.1.

5.2.3 Assessment of infection by *Phomopsis* taxon 1 and bud fruitfulness

Poor fruitfulness was observed in Shiraz vines at the Mt Jagged vineyard in 2000, therefore, buds were collected prior to pruning to assess the relationship between bud fruitfulness and infection by *Phomopsis* taxon 1. Fifty buds, from the first four nodes, were collected at random from the Mt Jagged vineyard (cv. Shiraz). Also, 50 buds were collected from the Hargrave vineyard (cv. Chardonnay) on 13 June 2001, but the history of fruitfulness in this vineyard was not known. Buds were dissected and assessed for bud fruitfulness by Simon Tolley and Murray Leake, Nepenthe Viticulture, Charleston, South Australia. Buds were placed in Eppendorf® tubes and stored at -70°C until required.

Total DNA was extracted using the SEAPS extraction protocol as described in section 2.2.1. Three slot blot membranes were prepared (section 2.2.3) with total DNA from each

bud (*ca* 200 ng). Each slot contained purified DNA from *Phomopsis* taxon 1 isolate A223.1 (12 ng and 25 ng), purified DNA from *Phomopsis* taxon 2 isolate 261.7 (50 ng), 100 ng DNA obtained from grapevine and ddH_20. Hybridisations were done as described in section 2.2.6.

5.2.4 Assessment of infection by *Phomopsis* taxon 1 and budburst

Many growers associate poor budburst with *Phomopsis* taxon 1 infection, although this is commonly based on observations of bleaching alone. Canes were collected from vines (cv. Shiraz) with a history of poor bud development from Moonlit Springs vineyard, Southern Fleurieu Peninsula, on 21 June, 2001. It was suspected *Phomopsis* taxon 1 was causing bud loss. Necrosis of the diaphragm, located at the axis of the node, was observed in most of the canes sampled but the cause of this phenomenon was not known.

Seven lignified canes were collected and cut longitudinally to examine necrosis of the diaphragm. Forty buds were isolated from the areas assessed for necrosis of the diaphragm and scored for bud health according to section 5.2.1. The presence of *Phomopsis* taxon 1 in buds was determined by hybridisation of total DNA with the taxon 1 and taxon 2-specific probes as described in section 2.2.6. Also, bleached canes were collected and incubated at 15°C as described in section 2.1. Statistical analysis was undertaken to assess if there was a correlation between *Phomopsis* taxon infection and bud health using analysis of variance in the Statistix® software programme.

5.2.5 Spurs inoculated with *Phomopsis* taxon 1 in the field

Vines (cv. Chardonnay) were inoculated with *Phomopsis* taxon 1 in autumn to assess if early infection by taxon 1 influenced budburst in spring. Spurs were inoculated on 15 May, 2001 with mycelium of *Phomopsis* taxon 1 isolate A223.1, *Phomopsis* taxon 2 isolate 902.4 and

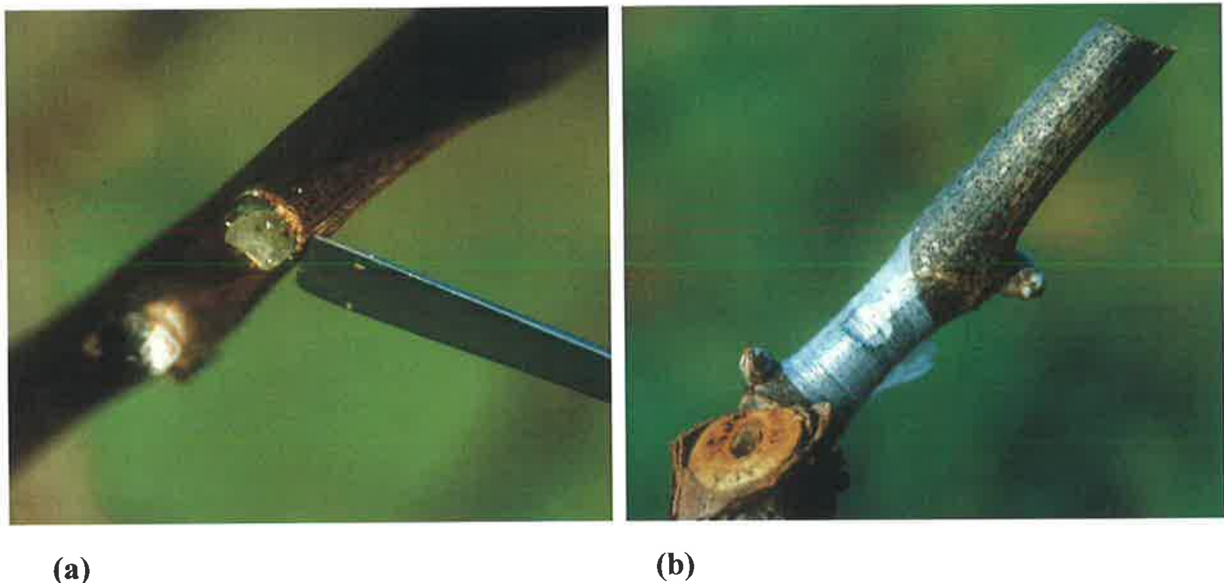
PDA (control) in row 10 at the Lenswood Horticultural Centre, SA. Isolates that appeared most pathogenic in pathogenicity experiment 1 (see section 3.2.4) were selected.

Spurs were pruned to two nodes per spur. At the time of inoculation, grapevines were at Eichhorn-Lorenz stage 43, whereby leaf fall had commenced. Thirty spurs were inoculated with either *Phomopsis* taxon 1, taxon 2 or PDA (control). The fungal treatments were arranged at opposite ends of the row (40 vines per row), with 30 spurs inoculated on eight vines. Ten spurs inoculated with PDA were located on the same vine as those inoculated with *Phomopsis* taxon 1 or *Phomopsis* taxon 2. Another ten spurs were inoculated with PDA on four vines in a separate panel of the row (Table 5.1).

Table 5.1. Vines (cv. Chardonnay) inoculated in row 10 at the Lenswood Horticultural Centre, SA on 15 May, 2001. Thirty spurs were inoculated with either *Phomopsis* taxon 1 isolate A223.1, PDA (control) or the *Phomopsis* taxon 2 isolate 902.4.

		ROW 10		
Block	**Vine no.**	**Control**	**Taxon 1**	**Taxon 2**
1	vine 1			
	vine 2			
	vine 3			
	vine 4			
2	vine 5	control	taxon 1	
	vine 6	control	taxon 1	
	vine 7	control	taxon 1	
	vine 8	control	taxon 1	
3	vine 9	control	taxon 1	
	vine 10	control	taxon 1	
	vine 11			
	vine 12			
4	vine 13			
	vine 14			
	vine 15			
	vine 16			
5	vine 17	control		
	vine 18	control		
	vine 19	control		
	vine 20	control		
6	vine 21			
	vine 22			
	vine 23			
	vine 24			
7	vine 25	control		taxon 2
	vine 26	control		taxon 2
	vine 27	control		taxon 2
	vine 28	control		taxon 2
8	vine 29	control		taxon 2
	vine 30	control		taxon 2
	vine 31			
	vine 32			
No. spurs inoculated		**30**	**30**	**30**

Each spur was wounded on the second internode between the first and second bud with a 4-mm diameter cork borer to expose the cambium (Figure 5.1a). A mycelium plug, taken from the margin of a 2-week-old colony on PDA, was inserted into the wound and then sealed with Parafilm™ (Figure 5.1b).



(a) **(b)**

Figure 5.1. Spurs (cv. Chardonnay) inoculated between the first and second bud with mycelium of *Phomopsis* taxon 1 isolate A223.1 on 15 May 2001 at the Lenswood Horticultural Centre vineyard. **(a)** Insertion of the mycelium plug into a 4-mm wound. **(b)** Wound was wrapped in Parafilm™ and spurs were collected from the vineyard after 22 weeks for assessment of *Phomopsis* infection.

Budburst and shoot length were measured each fortnight from 4 September to 31 October 2001 (as defined in section 4.2.3.1). Bleaching was recorded on 25 September 2001. All inoculated spurs were collected on 31 October 2001 and unburst buds and shoots removed in the laboratory. Spurs were incubated in moist conditions at 15°C for 4 days and examined for *Phomopsis* taxon 1, taxon 2 and zone-lines (see section 2.1). *Phomopsis* taxon 1 was re-isolated from pycnidia. Total DNA was extracted from unburst buds using the SEAPS

extraction procedure and transferred to a slot blot membrane, which included a dilution series of purified DNA from *Phomopsis* taxon 1 isolate A223.1, 50 ng DNA of *Phomopsis* taxon 2 isolate M827 and ddH_20. *Phomopsis* taxon-specific probes were used in hybridisation as described in section 2.2.6. Statistical analysis of budburst percentage, bleaching and shoot length among the three treatments was performed using analysis of variance in Statistix®.

5.3 Results

5.3.1 Dormant buds infected with *Phomopsis* taxon 1 sampled in autumn

Buds collected from the Mt Jagged vineyard in autumn 2000 varied in weight from 6 mg to 49 mg. The amount of DNA obtained from each bud varied considerably, whereby 16 ng to 400 ng of total DNA was extracted from 10 mg of bud tissue. Hybridisation of the taxon 1-specific probe, pT1P180, to total DNA showed 60% of buds collected were infected with *Phomopsis* taxon 1 (Figure 5.2). Comparison of hybridisation signals to those of purified taxon 1 DNA showed that between 3 to 10 ng of *Phomopsis* taxon 1 DNA was detected in buds containing approximately 100 ng of total DNA. Hybridisation of DNA with the *Phomopsis* taxon 2-specifc did not reveal taxon 2 in the buds sampled.

Phomopsis taxon 1 was detected in all buds obtained from the same spur (Table 5.2). For example, of the three lignified shoots collected from spur six, all buds isolated were infected by *Phomopsis* taxon 1. It is not known if infection occurred in early spring, or in autumn when conditions were favourable for colonisation by the fungus. The results, however, suggest buds retained at pruning may already be infected by *Phomopsis* taxon 1.

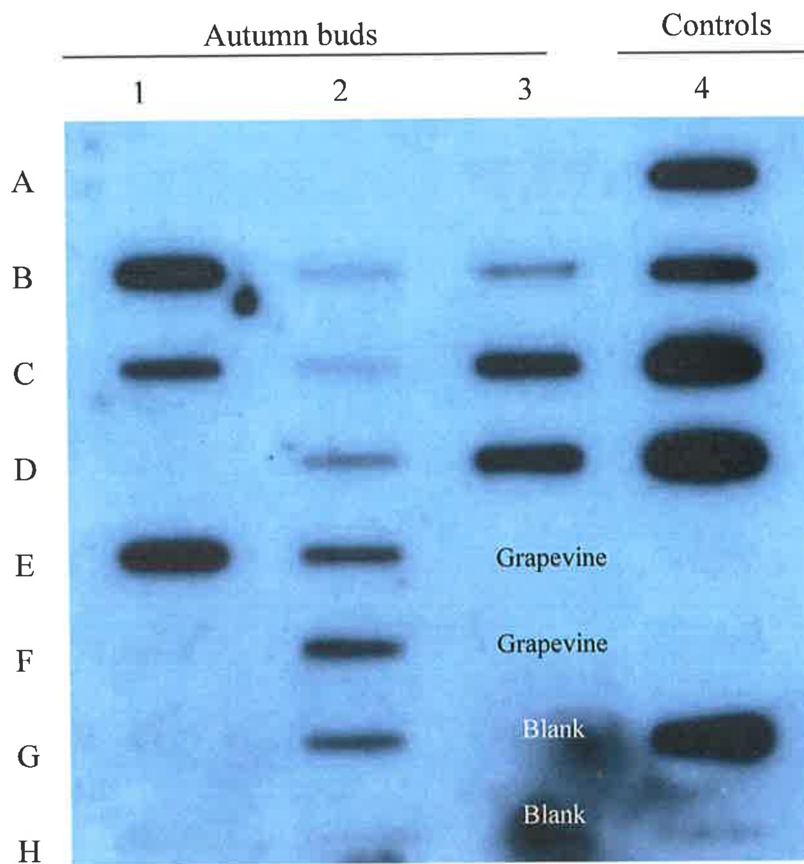


Figure 5.2. Detection of *Phomopsis* taxon 1 in dormant buds (cv. Shiraz) collected on 18 April, 2000 (autumn) from the Mt Jagged vineyard, by slot blot analysis (Slot M124) using the *Phomopsis* taxon 1-specific probe, pT1P180. Slots 1A-3D contain 100 ng of total DNA obtained from dormant buds. Slots 3E and 3F contain 100 ng and 200 ng DNA from grapevine. Slots 3G and 3H contain ddH_20. Slots 4A-4D contain 10 ng, 5 ng, 20 ng and 40 ng of purified DNA from *Phomopsis* taxon 1 isolate A223.1, respectively. Slot 4E and 4F contain 50 ng and 100 ng DNA from *Phomopsis* taxon 2 isolate P712. Slots 4G and 4H contain 100 ng of miscellaneous DNA extracted from cane infected with *Phomopsis* taxon 1 (for control purposes only). Each slot represents an individual DNA sample.

Table 5.2. Detection of *Phomopsis* taxon 1 in buds by hybridisation of DNA with the taxon 1-specific probe, pT1P180. Buds were collected from ten 6-month-old lignified shoots on various spurs (cv. Shiraz) at the Mt Jagged vineyard on 18 April, 2000.

Spur number	Shoot number	Bud position	*Phomopsis* taxon 1
1	1	1	✗
1	1	2	✗
2	2	1	✓
3	3	1	✓
3	3	2	✓
4	4	1	✗
4	4	2	✗
4	4	3	✗
5	5	1	✗
6	6	1	✓
6	6	2	✓
6	7	1	✓
6	7	2	✓
6	8	1	✓
6	8	3	✓
7	9	1	✗
7	9	2	✗
8	10	1	✓
8	10	2	✓
8	10	3	✓

All buds collected from the Hargrave and Lenswood vineyards in 2001 were healthy, whilst only four buds collected at the Ashton Hills vineyard were dead. Buds weighed from 3 to 31 mg. Mites were not observed. Mite feeding damage was not evident in the buds and macroscopic symptoms were not obvious on grapevines.

Slot blot analysis of DNA from the forty buds collected at each of the three sites in 2001 revealed *Phomopsis* taxon 1 in 35% and 2% of buds from the Ashton Hills and Hargrave vineyards, respectively (data not shown). The taxon 1-specific probe hybridised to 20 ng to 300 ng of *Phomopsis* taxon 1 DNA per 10 mg. However, no hybridisation signals were obtained on the slot blot prepared with total DNA from buds collected at the Lenswood

vineyard due to poor transfer of DNA to the membrane. This was observed during preparation of the slot blot. There was insufficient DNA to repeat the hybridisation experiment.

5.3.2 Dormant buds infected with *Phomopsis* taxon 1 sampled in winter

In 2000, rust mites were observed in the outer bud scales of seven of the 50 buds collected at the Mt Jagged vineyard. In most buds, feeding damage was evident when mites were observed. Mites were not observed in dead buds. Bud mites were not observed in any of the buds sampled.

Phomopsis taxon 1 was detected in six of the seven buds infested with rust mite. Although feeding damage was evident, buds infected with *Phomopsis* taxon 1 and infested with rust mite were relatively healthy. Although greater than 48% of the buds sampled were considered healthy (bud health rating 1), *Phomopsis* taxon 1 was detected in 68% of the buds collected from the Mt Jagged vineyard in 2000 (Table 5.3).

Table 5.3. Percentage of dormant buds (cv. Shiraz), collected in August (winter) in 2000 and 2001 at the Mt Jagged vineyard infected with rust mite, classified as dead, infected with *Phomopsis* taxon 1 alone and infected with *Phomopsis* taxon 1 plus rust mite.

	Year of bud collection	
Dormant buds	**2000**	**2001**
% with rust mites	14	0
% dead	30	18
% infected by *Phomopsis* taxon 1	68	10
% *Phomopsis* taxon 1 and rust mites	86	0

Hybridisation of the taxon-specific probe to DNA extracted from buds detected between 20 ng and 450 ng of *Phomopsis* taxon 1 DNA per 10 mg. The concentration of *Phomopsis* taxon 1 DNA in total DNA obtained from a dormant bud varied considerably between samples (Figure 5.3). Analysis of variance showed that there was no significant difference between the health of the buds infected by *Phomopsis* taxon 1 and uninfected buds (P=0.1917). The incidence of *Phomopsis* taxon 1 was greater in dormant buds collected in winter than was observed in unburst buds (45%) collected in spring of the same year (see section 4.3.7).

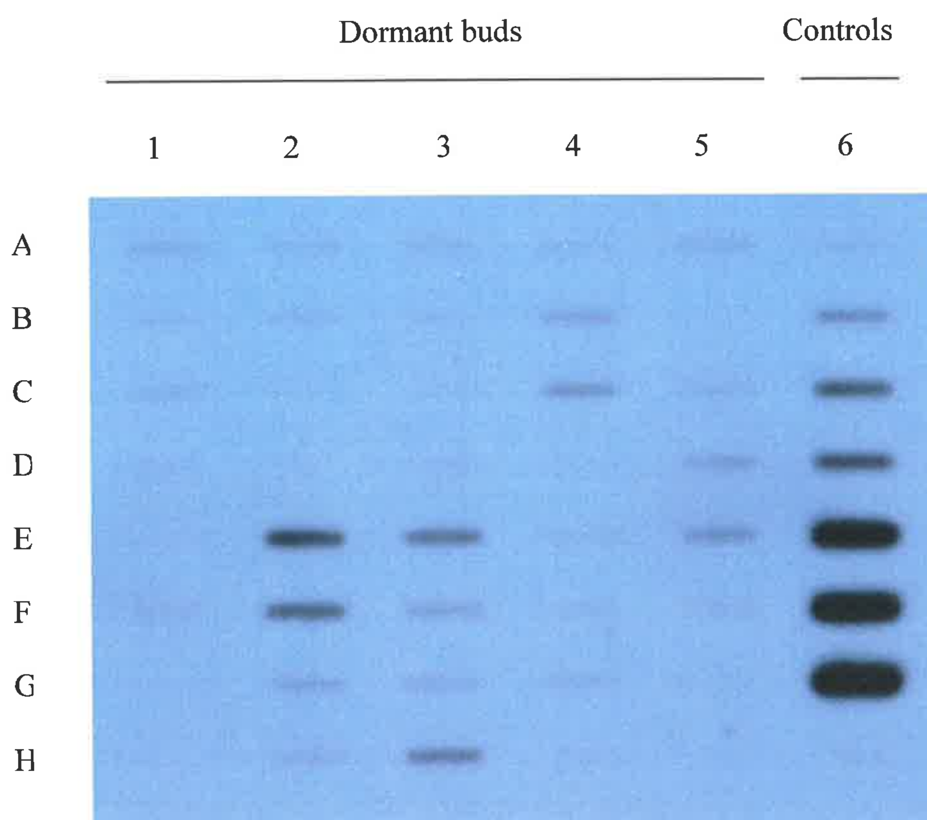


Figure 5.3. Detection of *Phomopsis* taxon 1 in dormant buds (cv. Shiraz) collected in August 2000 from the Mt Jagged vineyard, by slot blot analysis using the *Phomopsis* taxon 1-specific probe, pT1P180. Slots 1A-5H contain 100 ng of total DNA from dormant buds. Slots 6A-6G contain 1 ng, 3 ng, 6 ng, 12 ng, 25 ng, 50 ng and 100 ng purified DNA from *Phomopsis* taxon 1 isolate A223.1, respectively. Slot 6H contains ddH_20. Each slot represents an individual DNA sample.

In 2001, mites were not detected in the 50 dormant buds collected at the Mt Jagged vineyard, although nine buds were dead (18%, see Table 5.3). Dead buds were dry and brown, with woolly hairs evident only. The average bud health score was 2. Slight browning was observed in outer scales of some buds, but the green tissue healthy. *Phomopsis* taxon 1 was detected in 10% of the buds, but there was no correlation between dead buds and infection by *Phomopsis* taxon 1. Lower levels of *Phomopsis* taxon 1 infection were observed in winter of 2001 than in 2000. This finding was similar to the incidence of taxon 1 in unburst buds (see section 4.3.7, Table 4.12)

Greater than 95% of the dormant buds collected from the Hargrave vineyard in 2000 were healthy. Mites were not observed and *Phomopsis* taxon 1 was not detected by hybridisation with the taxon 1-specific DNA probe. Similarly, *Phomopsis* taxon 1 was not detected in unburst buds or canes collected in November 2000 (see sections 4.3.7 and 4.3.8).

5.3.3 Effect of *Phomopsis* taxon 1 on bud fruitfulness

Dissection of buds showed that bud fruitfulness was 50% and 69% at the Mt Jagged and Hargrave vineyards, respectively in 2001 (Table 5.4). At both sites, bud one (i.e. the first bud on the cane) showed poor fruitfulness and, at the Hargrave vineyard, this bud was significantly less fruitful than bud two (P=0.0289). At the Hargrave vineyard, bud two showed more primordia per bud than other buds on the cane. A few healthy buds did not contain primordia. Vines at the Mt Jagged vineyard had more dead buds than at the Hargrave vineyard, at which only one dead bud was present on the canes collected. Slot blot analysis revealed 86% of the buds collected at the Mt Jagged vineyard were infected with *Phomopsis* taxon 1. At this site, the incidence of *Phomopsis* taxon 1 was high in all buds assessed. *Phomopsis* taxon 1 was detected in 32% of buds collected from the Hargrave vineyard. Analysis of variance showed there was no significant difference in number of primordia in

buds infected with *Phomopsis* taxon 1 and buds not infected (P=0.8931 and P=0.7754 at the Mt Jagged and Hargrave vineyards, respectively).

Table 5.4. Assessment of number of primordia and presence of *Phomopsis* taxon 1 in buds collected from Mt Jagged, Southern Fleurieu (cv. Shiraz) and Hargrave, Adelaide Hills (cv. Chardonnay) on 18 June, 2001*. Bud dissection service provided by Nepenthe Viticulture, SA and *Phomopsis* taxon 1 diagnosed by slot blot analysis using *Phomopsis* taxon 1-specific DNA probe, pT1P180.

Mt Jagged	**Bud 1**	**Bud 2**	**Bud 3**	**Bud 4**	**Average**
% fruitful buds [a]	39	47	62	50	50
Mean no. primordia/bud	0.5	0.6	0.9	0.5	0.6
Buds dead [b]	4	4	4	0	3
% with *Phomopsis* taxon 1	72	94	85	100	88

Hargrave	**Bud 1**	**Bud 2**	**Bud 3**	**Bud 4**	**Average**
% fruitful buds [a]	35	82	60	100	69
Mean no. primordia/bud	0.4	0.8	0.6	1.0	0.7
Buds dead [b]	1	0	0	0	0
% with *Phomopsis* taxon 1	18	24	47	67	39

* 50 buds sampled at each site from 17 canes.
[a] % fruitful buds relates to the number of inflorescences primordia per bud. Each inflorescence primordia gives rise to a bunch
[b] *Phomopsis* taxon 1 was not associated with bud death (where P=0.4813 and P=0.5208 at the Mt Jagged and Hargrave vineyards, respectively)

Approximately 100 ng total DNA per 10 mg was extracted from each bud collected at each site. The amount of DNA obtained from dead buds did not differ from that obtained from healthy buds. Figure 5.4 shows hybridisation signals obtained on a slot blot membrane containing DNA from buds collected from the Mt Jagged vineyard hybridised with the taxon 1-specific probe pT1P180. Between 3 ng to 10 ng of *Phomopsis* taxon 1 DNA was

detected in 100 ng total DNA obtained from the buds. *Phomopsis* taxon 1 was detected in total DNA from dead buds (e.g. slot 2E) and from buds having one or more primordia (e.g. slot 1C). Death of buds, however, was not associated with *Phomopsis* taxon 1 infection, where P=0.4813 and P=0.5208 at the Mt Jagged and Hargrave vineyards, respectively.

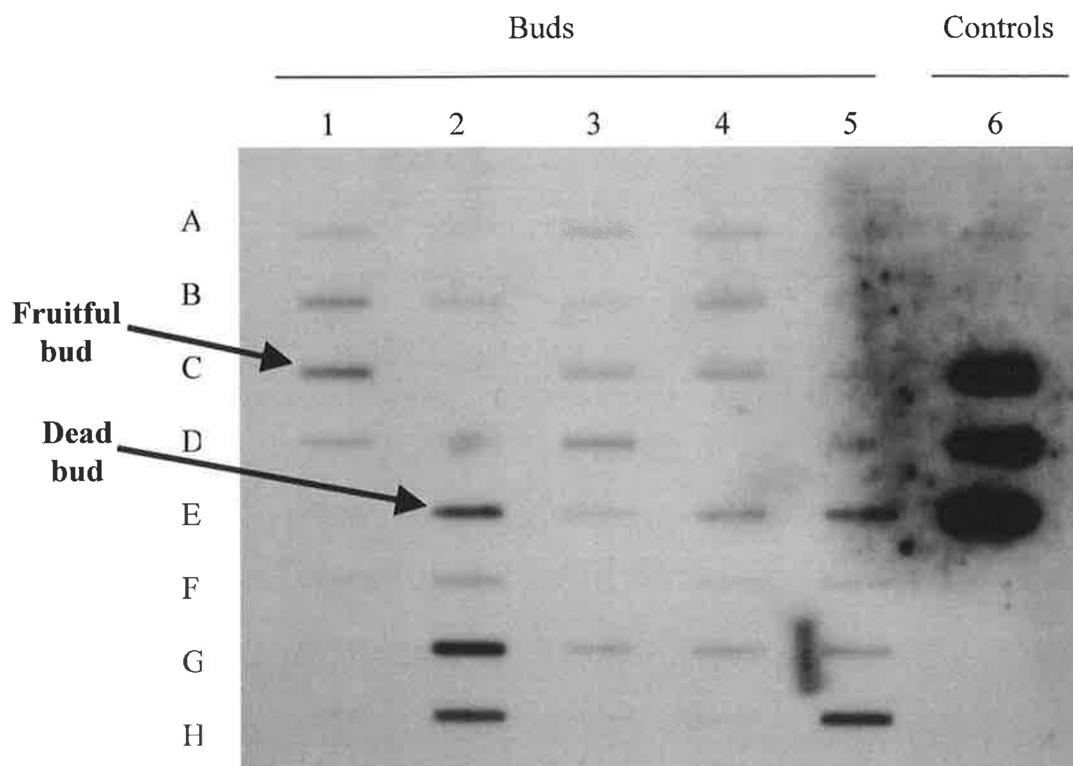


Figure 5.4. Detection of *Phomopsis* taxon 1 in a representative sample of buds assessed for bud fruitfulness (cv. Shiraz) from the Mt Jagged vineyard on 18 June, 2001, by slot blot analysis (Slot F140) using the *Phomopsis* taxon 1-specific probe, pT1P180. Slots 1A-5H contain approximately 200 ng of total DNA from buds. Slots 6A-6B contain 200 ng of total DNA from buds, slots 6C-6E contain 12 ng, 25 ng and 50 ng purified DNA from *Phomopsis* taxon 1 isolate A223.1, respectively. Slot 6F contains 50 ng purified DNA from *Phomopsis* taxon 2 isolate M827, slot 6G contains 50 ng DNA obtained from grapevine and slot 6H contains ddH_20. Each slot represents an individual DNA sample.

5.3.4 Assessment of infection by *Phomopsis* taxon 1 and poor budburst

Of the seven canes collected, necrosis of the diaphragm was observed in more than 61% of the canes (Table 5.5). The diaphragm, normally green in colour, was brown, dry and often cracked. Vascular tissue surrounding the diaphragm was green and turgid. In many cases, the necrotic areas extended into the compound bud and appeared to cause browning of the primary bud. Necrosis, however, was not associated with all dead buds. Slot blot analysis, using the taxon 1 and taxon 2-specific probes, revealed that *Phomopsis* taxon 1 was not present in any of the buds sampled.

Table 5.5. Percentage of nodes with necrotic diaphragms, dead buds and infection by *Phomopsis* taxon 1 from canes collected from Moonlit Springs vineyard, Southern Fleurieu on 21 June 2001 where bud loss and poor fruitfulness occurred in the previous season. Total sample size of 40 buds assessed.

	Percentage of nodes		
Cane	**Necrotic diaphragm**	**Dead buds**	***Phomopsis* taxon 1**
1	67	44	0
2	30	10	0
3	12	12	0
4	0	0	0
5	50	50	0
6	100	60	0
7	50	75	0
Average	61	33	0

Approximately 100 ng total DNA was extracted from each of the 40 buds (3 mg to 21 mg each). The quality of DNA, measured by gel electrophoresis, did not differ between healthy and dead buds. Hybridisation signals were strong for purified DNA from *Phomopsis* taxon 1 (controls) and there were no background signals. *Phomopsis* taxon 1 was not detected in bleached canes. The results suggest that other factors may be associated with bud loss at this site.

5.3.5 Bud development on spurs inoculated with *Phomopsis* taxon 1

Budburst was estimated to have occurred at 16 September 2001 at the Lenswood vineyard (as defined in section 4.2.3.1). Analysis of variance showed that the final budburst percentage on inoculated spurs did not differ significantly between the three treatments (P=0.3654, Figure 5.5).

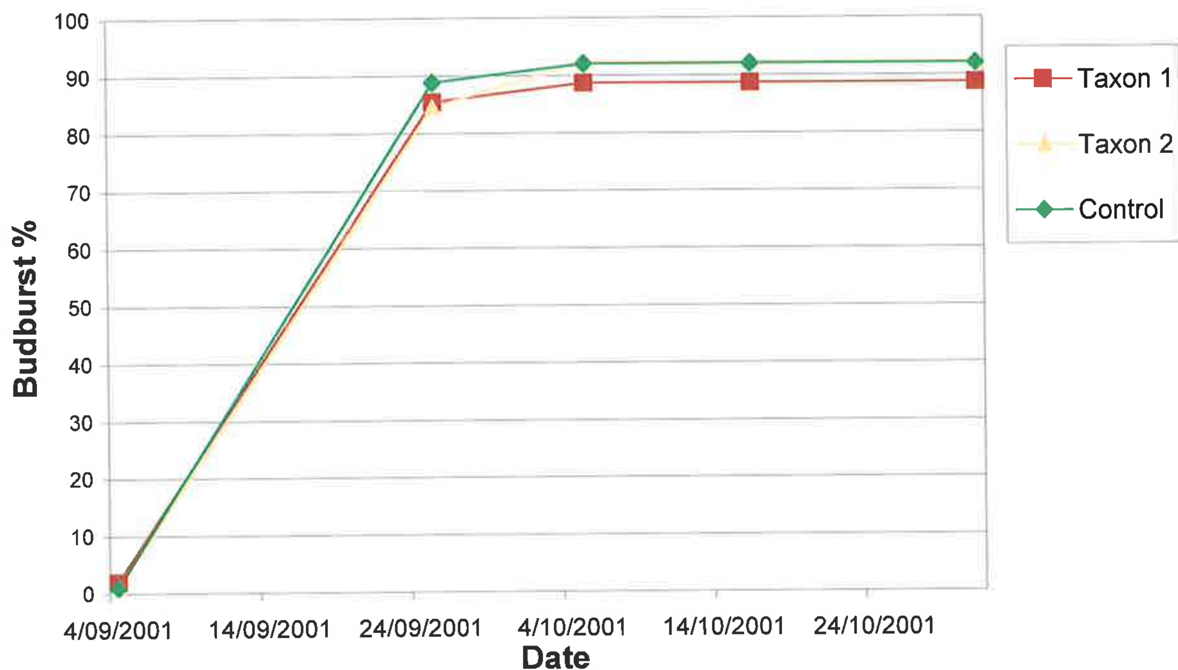


Figure 5.5. Percentage of buds bursting over time on spurs inoculated with *Phomopsis* taxon 1 isolate A223.1, *Phomopsis* taxon 2 isolate 902.4 and PDA (control) at Lenswood Horticultural Centre on 15 May, 2001. From estimation of time between sample dates, approximately 50% budburst occurred at 16 September 2001.

Five unburst buds were collected from spurs inoculated with *Phomopsis* taxon 1 and six unburst buds were collected from both taxon 2 and PDA (control) treatments. There was no significant difference (P=0.4992) between length of shoots developing on spurs inoculated with *Phomopsis* taxon 1, taxon 2 or PDA (Figure 5.6). The mean length of the shoots on 31 October, 2001 was remarkably similar between treatments, with shoots of 31.2 cm, 31.4

cm and 31.4 cm in length for the control, *Phomopsis* taxon 1 and taxon 2 inoculations, respectively. In the vineyard, no symptoms were evident on shoots from spurs inoculated with *Phomopsis* taxon 2 and budburst was not inhibited.

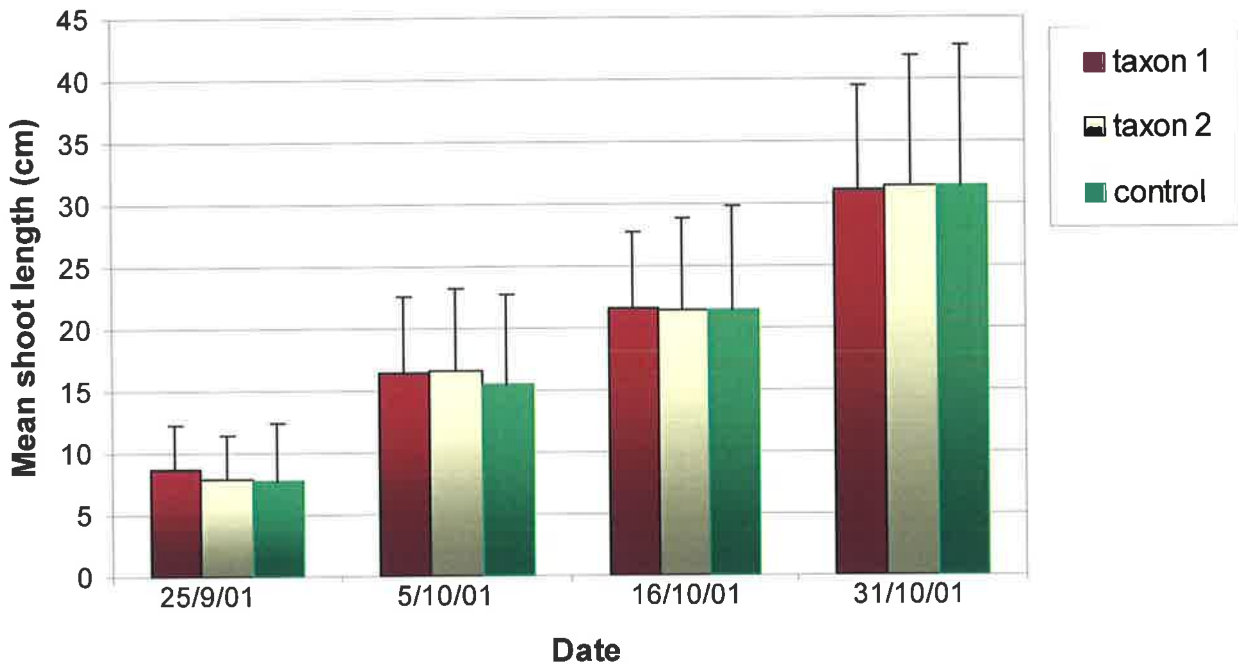


Figure 5.6. Length of shoots (cv. Chardonnay) developed over time on 30 spurs each inoculated with *Phomopsis* taxon 1 isolate A223.1, *Phomopsis* taxon 2 isolate 902.4 and PDA (control) at Lenswood Horticultural Centre on 15 May, 2001. Bars represent standard deviation.

Bleaching was observed on spurs inoculated with the three treatments (Table 5.6). Although the incidence of bleaching was not significant (P=0.0081), bleaching was higher in spurs inoculated with *Phomopsis* taxon 1 and taxon 2 than the controls. Bleaching was observed on spurs inoculated with PDA. After 4 days of incubation at 15°C, zone-lines were observed on spurs inoculated with *Phomopsis* taxon 1. Zone-lines were mostly associated with bleaching and indicated that taxon 1 was present. In addition, perithecia were observed

protruding from the tissue around the wounding site on spurs inoculated with taxon 1. After 4 to 7 days of incubation, conidia were isolated from pycnidia and identified at × 400 magnification.

Table 5.6. Spurs (cv. Chardonnay) inoculated with *Phomopsis* taxon 1 isolate A223.1, *Phomopsis* taxon 2 isolate 902.4 or PDA (control) at the Lenswood Horticultural Centre vineyard on 15 May 2001, assessed 22 weeks after inoculation for the presence of zone-lines, *Phomopsis* taxon 1 and taxon 2 on the basis of spore morphology.

	Percentage of spurs			
Treatment	**Bleached**	**Zone-lines**	**Taxon 1**	**Taxon 2**
Phomopsis taxon 1	38	33	87	0
Phomopsis taxon 2	49	0	20	67
PDA (control)	33	6	40	0

Phomopsis taxon 1 was also isolated from 40% of control spurs and 20% of spurs inoculated with *Phomopsis* taxon 2 (Table 5.6). This indicated a natural infection of *Phomopsis* taxon 1 in the vineyard. Of the 30 spurs inoculated with taxon 1 isolate A223.1, 87% were infected and no macroscopic symptoms were observed. *Phomopsis* taxon 2 was isolated from spurs inoculated with taxon 2 only.

After incubation in moist conditions at 15°C, woody tissue of spurs collected from the vineyard were not suitable for extraction of DNA. Unburst buds arising on canes subjected to inoculation treatments were stored at –20°C, but because buds thawed during storage, DNA was not extracted from the buds. Isolation of the fungi and the observation of perithecia after incubation, however, confirmed that *Phomopsis* taxon 1 and taxon 2 were present in inoculated spurs.

5.4 Discussion

Prior to this study, it was not known if *Phomopsis* taxon 1 affected grapevine productivity. In vineyards infected with *Phomopsis* taxon 1, taxon 1 was detected in some but not all unburst buds (chapter 4). This study revealed that *Phomopsis* taxon 1 was present in dormant buds in autumn and winter prior to budburst, but there was no correlation between bud death and infection by *Phomopsis* taxon 1. *Phomopsis* taxon 1 was detected in productive buds and there was no evidence that taxon 1 affected the development of inflorescence primordia in the buds. Vines inoculated with mycelium of *Phomopsis* taxon 1 and taxon 2 did not have a large number of buds that failed to burst. These findings suggest *Phomopsis* taxon 1 is not associated with poor budburst and bud death.

Application of the *Phomopsis* taxon 1-specific probes showed that 60% of the buds collected in autumn at the Mt Jagged vineyard were infected with *Phomopsis* taxon 1. The buds infected with *Phomopsis* taxon 1 originated from a small number of shoots, therefore, it is most likely that pruned spurs would have infected buds. Also, most buds infected with taxon 1 were from the first few nodes of the lignified shoot and are likely to be retained at pruning. The findings indicate that *Phomopsis* taxon 1 was already present in dormant buds early in the season and may overwinter in these dormant buds as reported by Hewitt and Pearson (1990). Most buds infected with taxon 1, however, were healthy and there was no evidence of early infection causing bud death. At pruning, spurs with weak buds are often removed and it would be difficult to distinguish buds infected with *Phomopsis* taxon 1 on the vines. Bleaching, often used as an indication of *Phomopsis* taxon 1 infection, has been shown to be a poor indicator of *Phomopsis* infection. *Phomopsis* taxon 1 was not detected in many buds collected in autumn at the Ashton Hills and Hargrave vineyards. The incidence of infection of buds in autumn correlated with infection levels in the spring of the same year. It

was not possible to detect *Phomopsis* taxon 1 in autumn buds and then to monitor budburst, as diagnosis required the removal of the buds.

The assessment of buds for *Phomopsis* taxon 1 in winter indicated that the fungus did not affect overall health of the bud. *Phomopsis* taxon 1 was detected in both healthy and unhealthy buds. At the Mt Jagged vineyard, taxon 1 was detected in more buds in winter than in unburst buds in spring of that year. This implied that although buds were infected, they still burst.

Vines at the four sites were initially shown to be infected by *Phomopsis* taxon 1, but in 2000 and 2001, taxon 1 was not detected in samples collected from the Hargrave vineyard. Nevertheless, bud death was still observed at the Hargrave site. *Phomopsis* taxon 1 could not be associated with bud death at this vineyard. Mites were considered as a possible cause of bud death, but there was little evidence to link bud death with infestation of the bud with mites.

Bud mites were not observed at any of the vineyards. Bud mites penetrate the inside of the bud where they feed, multiply and overwinter (de Klerk 1981; Barnes, 1992), whereas rust mites overwinter under outer bud scales of dormant buds and colonise new leaves (Duso and De Lillo, 1996). The percentage of buds infested by bud and rust mites is highest in late summer and early autumn and death of the entire bud may occur (Duso and De Lillo, 1996). In this study, rust mites were found in dormant buds in winter, but these buds were relatively healthy. *Phomopsis* taxon 1 was detected in 85% of buds colonised with rust mites but it is proposed that these buds were infected with *Phomopsis* prior to mite infestation. These findings suggest that bud death may be unrelated to *Phomopsis* taxon 1 or mites.

Based on the assessment of bud fruitfulness, *Phomopsis* taxon 1 did not reduce bunch number. Examination of buds from two vineyards showed that *Phomopsis* taxon 1 was not associated with bud death or low fruitfulness. In some instances, *Phomopsis* taxon 1 was

detected in buds having one or more inflorescence primordia but was not detected in dead buds. Bud dissection analysis correlated to final bunch number observed (section 4.3.6), whereby more bunches were observed in the Hargrave vineyard than at the Mt Jagged vineyard. There was no evidence to suggest that *Phomopsis* caused poor bunch count. These results further support the hypothesis that *Phomopsis* taxon 1 is not associated with bud death.

Natural infection by *Phomopsis* taxon 1 had occurred in vines at the Lenswood Horticultural Centre vineyard prior to establishment of the inoculation experiment because the fungus was isolated from both PDA and taxon 2-inoculated spurs. Nevertheless, budburst was not affected. The inoculation of spurs in May may not have provided sufficient time or suitable conditions for *Phomopsis* taxon 1 and taxon 2 to colonise dormant buds. However, bleaching of spurs and re-isolation of the fungi from spurs inoculated with taxon 1 and taxon 2 indicated that infection did occur. Newly-developed shoots and new buds are infected in spring by conidia of *Phomopsis* on grapevine (Emmett *et al.*, 1998; Hewitt and Pearson, 1990). If these buds are retained, *Phomopsis* may be detected in the following year. All inoculated spurs were removed, therefore, it was not possible to assess infection in the following season.

It is more likely that bud death, and subsequent reduction in the number of buds bursting, is associated with physiological and environmental controls of crop development. There are a number of factors that can influence grapevine growth, including vigour, supply of carbohydrate reserves (Vasudevan *et al.*, 1998), shading (May and Antcliff, 1963), overcropping and leaf area (Winkler, 1972). Management practices such as pruning level and shoot thinning ultimately affect grapevine growth. These variables are difficult to assess and often yield losses are wrongly attributed to pests and diseases, as is the case of infection by *Phomopsis* taxon 1.

These results showed that *Phomopsis* taxon 1 was not associated with bud death. At Moonlit Springs vineyard, necrosis of the diaphragm and bud loss were not caused by *Phomopsis* taxon 1. DNA probes were useful for the detection of *Phomopsis* in grapevine buds, but some problems were experienced with transfer of DNA to the nylon membrane during slot blot preparation. Co-purified substances in the extractions may have interfered with the binding of DNA to the membrane. Also, the taxon 1-specific probe, pT1P180 (Melanson *et al.*, 2002), required a long exposure time (7 days) to produce detectable hybridisation signals due to lack of sensitivity. For the purpose of this study, slot blot hybridisation and the taxon 1-specific probe were suitable for the detection of *Phomopsis* taxon 1 in grapevine buds and cane. Although this DNA probe provided an accurate assessment of *Phomopsis* taxon 1 infection, the test is not suitable for use in routine diagnostics. For this reason, alternative molecular markers specific to *Phomopsis* taxon 1 were developed.

Chapter 6

Development of *Phomopsis* taxon 1-specific molecular markers

6.1 Introduction

Conventional methods for detecting *Phomopsis* involve visual inspection or culturing from grapevine tissue. Because bleaching of cane is not a reliable indicator of *Phomopsis* infection, identification of conidia is important in the differentiation between taxon 1 and taxon 2. The use of molecular technology for the detection and identification of *Phomopsis* taxa assists in the understanding of the epidemiology of Phomopsis cane and leaf spot. *Phomopsis* taxon-specific molecular markers offer a reliable means of identification and may be useful for distinguishing genetic variability among the taxa (Melanson *et al.*, 2002). The probes, pT1P180 and pT2P25 for *Phomopsis* taxon 1 and taxon 2, respectively, have been used in a slot-blot assay to detect and quantify *Phomopsis* in grapevine tissue. However, because pT1P180 requires a prolonged exposure time to obtain adequate hybridisation signals, it is not suitable for use in a rapid diagnostic test.

Polymerase chain reaction (PCR) has been used for detection and characterisation of a number of fungal plant pathogens in grapevine, including *Phaeomoniella chlamydospora* (Groenewald *et al.*, 2000), *Uncinula necator* (Stummer *et al.*, 2000) and *Eutypa lata* (Lecomte *et al.*, 2000). PCR and the development of SCAR markers (see section 1.8.3) involves the use of species-specific primers to detect the presence of fungi in infected material and their development requires knowledge of the DNA sequence of the target pathogen. PCR amplification using pathogen-specific primers allow the detection of picogram amounts of the target pathogen in infected tissue. For example, Mazzaglia *et al.*

(2001) showed that primer specific PCR amplification is effective for detection of low levels of the endophytic fungus, *Biscogniauxia mediterranea* in asymptomatic tissue of oak. The development of a reliable PCR-based assay, however, can be hindered by inhibitors in grapevine tissue and, often, results cannot be replicated between different laboratories (Judelson and Tooley, 2000).

The objective of this study was to develop an alternative *Phomopsis* taxon 1-specific DNA probe, based on recombinant DNA techniques and PCR technology, for use in routine identification by slot blot hybridisation. Two strategies were used; (1) development of a *Phomopsis* taxon 1 specific genomic DNA library and (2) identification and isolation of a specific PCR fragment that could be used as a species-specific probe for *Phomopsis* taxon 1.

6.2 Materials and methods

6.2.1 Construction of genomic library of *Phomopsis* taxon 1

6.2.1.1 Preparation of insert and vector DNA

To develop *Phomopsis* taxon 1–specific clones for use in a diagnostic test, taxon 1 DNA was ligated into the plasmid vectors pBluescript or pUC19 using methods modified from Sambrook and Russell (2001). DNA was extracted from *Phomopsis* taxon 1 isolate B500 using the CTAB extraction protocol described in section 2.2.2. *Phomopsis* taxon 1 DNA (1 μg) was digested in a reaction containing 20 μl DNA solution, 30 units (10 units/μl) of the restriction enzyme *Pst*1 (Roche Diagnostics, Germany) and 0.1 volume of 10 X restriction enzyme buffer. The reaction mixture was made up to a total volume of 50 μl by the addition of sterile ddH_20 and incubated for 16 hours at 37°C. The DNA was precipitated with absolute ethanol and 15 mM sodium acetate, pH 4.8, for 1 hour at – 20°C. DNA was collected by

centrifugation (14 000 *g* for 15 min), washed twice with 70% ethanol for 10 min at 4°C and vacuum-dried. The sample was resuspended in 8 μl Tris-EDTA (TE) to a final DNA concentration of 0.1 μg/μl (see Appendix A).

DNA (2 μg) of the plasmid vectors pBluescript or pUC19 was digested with *Pst*I in a reaction mixture containing 60 units (10 units/μl) of *Pst*1 (Roche Diagnostics) and 0.1 volume of 10 X restriction enzyme buffer in a total volume of 50 μl by the addition of sterile ddH_20. The reaction mix was incubated for 16 hours at 37°C. DNA was precipitated with ethanol/sodium acetate and resuspended in TE, as above, to give a final DNA concentration of 0.1 μg/μl. The digested vector DNA was dephosphorylated with 2 μl 10 × phosphatase buffer (50 mM Tris-HCl, 0.1 mM EDTA pH 8.5) and 20 units of calf intestinal alkaline phosphatase (CIAP) for 30 min at 37°C, followed by 10 min at 65°C. The reaction was completed by the addition of an extra 10 units CIAP in a total volume of 50 μl. The DNA was then extracted once with an equal volume of phenol/chloroform (1:1) and once with chloroform/*iso*-amylalcohol (24:1). DNA was precipitated with 2 volumes of cold absolute ethanol and 0.5 volume 7.5 M ammonium acetate pH 5.4, washed with 70% ethanol, vacuum-dried and suspended in 20 μl sterile ddH_20. The concentration and approximate size of *Phomopsis* taxon 1 insert DNA, pBluescript and pUC19 vector DNA were estimated by analysing aliquots by gel electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA buffer (TAE, see Appendix A). Bands were visualised under UV light following ethidium bromide staining. The quantity of DNA was compared with a known quantity of *Hin*dIII-digested lambda DNA.

6.2.1.2 Preparation of competent cells

Escherichia coli strain JM101 was streaked on Luria Bertani (LB) agar (see Appendix A) and incubated overnight at 37°C. A single colony was selected and grown in 25 ml LB medium overnight (16-20 hours) at 37°C with shaking at 200 rpm. A 1 ml aliquot was transferred to 100 ml LB medium in a 500 ml glass flask and shaken at 200 rpm for approximately 3 hours at 37°C until the OD_{600nm} reached 0.45-0.55. Cells were chilled immediately in ice water for 2 hours, centrifuged at 2 500 g for 15 min at 4°C, then the pellet was resuspended in 20 ml ice-cold Trituration Buffer (TB) (see Appendix A). Competent cells were incubated on ice for 45 min, centrifuged at 1 800 g for 10 min and resuspended in 10 ml TB. Sterile 80% glycerol was gradually added to the suspension to give a final concentration of 15% (v/v). Competent cells were dispensed in 1 ml quantities to 1.5 ml Eppendorf tubes and immediately frozen in liquid nitrogen prior to storage at –70°C.

6.2.1.3 Ligation of genomic **Phomopsis** *taxon 1 DNA into a plasmid vector*

Phomopsis taxon 1 DNA (isolate B500) was transformed into the vectors pBluescript or pUC19 using methods modified from Sambrook and Russell (2001). DNA molar ratios for ligation reactions were calculated using the formula:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert required}$$

A series of ligation ratios was prepared, including 3:1, 2:1, 1:1, 1:2 and 1:3 (insert:vector). The ligation reaction consisted of a minimum 50 ng linearised dephosphorylated pBluescript or pUC19 DNA, 66.6 – 266.6 ng *Pst*1-digested *Phomopsis* taxon 1 DNA, 2 µl 10 × ligation buffer, 10 mM adenosine 5'-triphosphate (ATP, 10 mg/ml) and 0.25 units T4 DNA ligase

(0.5 units/μl) adjusted to a total volume of 20 μl in sterile ddH_20. The ligation reaction was mixed and incubated for 1 hour at 37°C.

6.2.1.4 Transformation of competent cells

Ligation products were transformed into competent cells of *E. coli* strain JM101 by transferring 10 μl of ligation reaction mixture to 200 μl competent cells. In addition, three control reactions were prepared, as in section 6.2.1.3, with the following modifications;

(1) *Pst* 1-digested/dephosphorylated pBluescript or pUC19 vector, ligation buffer and ATP, excluding *Phomopsis* taxon 1 DNA and T4 DNA ligase, to assess the number of background cells derived from the vector. No colonies expected.

(2) *Pst* 1-digested/dephosphorylated pBluescript or pUC19 vector, ligation buffer, ATP and T4 DNA ligase, excluding *Phomopsis* taxon 1 DNA, to assess CIAP treatment. No or a small number of colonies expected.

(3) 25 ng of super helical circular pUC19 DNA added directly to 100 μl of competent cells, to assess the efficiency of competent cells.

Transformation conditions involved incubation on ice for 30 min, heat shock without agitation for 60 sec at 42°C followed by the addition of 400 μl SOC medium (see Appendix A) and shaking at 37°C for 1 hour.

6.2.1.5 Selection of recombinant Escherichia coli *colonies*

A 100 μl aliquot of each transformation reaction was plated onto duplicated LB plates containing ampicillin (Amp, 50 mg/ml, Roche Diagnostics), 2% 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-Gal, Roche Diagnostics) and 0.1 M isopropyl-β-D-thiogalactoside

(IPTG, Roche Diagnostics) for colour selection (see Appendix A) and incubated at 37°C overnight. Recombinant colonies (containing inserts of *Phomopsis* taxon 1 DNA) were identified as white colonies on this medium. Non-recombinant colonies were blue.

White colonies containing recombinant DNA were transferred with a sterile wooden toothpick to a fresh LB agar plate containing 75 μg/ml Amp (LBAmp agar, see Appendix A), arranged in grid formation (Grunstein and Hogness, 1975; Sambrook and Russell, 2001). A blue, non-recombinant plasmid colony was included in the last grid position. The new plates containing the selected recombinant DNA were incubated overnight at 37°C. The following day, bacteria containing recombinant plasmids were transferred with a tooth-pick to four 132 mm diameter, 0.45 μm positively-charged nylon membranes (Amersham, UK), previously scribed with a grid pattern to accommodate 140 colonies, lying on LBAmp medium plates. Membranes were marked A-D and a blue non-recombinant colony was included in the last grid position. Replicate plates were prepared simultaneously on LBAmp agar and incubated overnight at 37°C.

The 16-hour-old colonies were lysed by placing nitrocellulose membranes, colony side up for 3 min on three pieces of Whatman 3MM paper which were saturated with 10% sodium dodecyl sulphate (SDS). DNA on the membranes was denatured by saturating membranes in 1.5 M NaCl, 0.5 M NaOH, for 5 min and transferred to fresh denaturation solution for a further 5 min. Colonies were neutralised three times for 5 min each in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5), followed by a 2-min wash in 2 X SSC (Sambrook and Russell, 2001) and fixed to the nitrocellulose membrane using a Bio-Rad® GS Gene Linker™ UV chamber at 150 mJ. Membranes were sealed in clear plastic sheets and stored at room temperature before use.

To evaluate the specificity of the clones to taxon 1, colony blots containing recombinant DNA were hybridised separately with 50 ng genomic DNA obtained from

grapevine, *Phomopsis* taxon 1 isolate H307 or *Phomopsis* taxon 2 isolate M827 as described in section 2.2.6. Membranes were exposed to X-ray film (X-Omat, Kodak, USA) for 7 days at –70°C to obtain an auto-radiographic image. The strength of each signal, arising from hybridisation of DNA to the recombinant clone, was scored as high, medium, low, very low and no signal.

6.2.2 Selection of *Phomopsis* taxon 1-specific clones

6.2.2.1 Isolation of recombinant plasmids

Clones showing a strong hybridisation signal and specificity to *Phomopsis* taxon 1, were selected for use as potential taxon 1-specific DNA probes. These clones were streaked onto LBAmp agar and incubated overnight at 37°C. A single colony was then transferred to 10 ml LB broth containing 40 mM Amp and incubated with shaking at 200 rpm for a minimum of 16 hours at 37°C. A 2 ml aliquot was transferred to an Eppendorf tube and cells were pelleted by centrifugation at 10 000 *g* for 2 min. Plasmid DNA was extracted using the Wizard® *Plus* SV Miniprep purification system (Promega) according to the manufacturer's recommendations. The plasmid DNA was suspended in 80 μl TE and the concentration of DNA was estimated by assessing a 5 μl aliquot by electrophoresis on a 1% agarose gel in TAE buffer as described in section 6.2.1.1. Approximately 1 μg of purified plasmid DNA was digested with *Pst*1 in a total volume of 30 μl as described in section 6.2.1.1. Digestion products were assessed by analysing a 5 μl aliquot by gel electrophoresis as previously stated. Due to incomplete digestion of plasmid DNA, DNA samples were further purified by one of two methods (a) extraction with phenol/choloroform or (b) Gene-clean II kit (Bio-101, USA) as follows. (a) The volume of each DNA sample was increased from 80 μl to 200 μl TE and

extracted with an equal volume of phenol/chloroform (1:1), followed by extraction with an equal volume of chloroform/*iso*-amylalcohol (24:1) as described in section 6.2.1.1. (b) Ten samples of plasmid DNA were purified using the Geneclean II kit (Bio-101, USA) according to the manufacturer's recommendations.

The quality and approximate concentration of DNA were assessed by digestion of plasmid DNA with restriction enzymes *Pst*I or *PVU*II (Roche Diagnostics) as described previously. Digested DNA, and 25 µl pUC19 *Pst*I-digested DNA, was analysed by gel electrophoresis and visualised under UV light following ethidium bromide staining. Based on preliminary comparison of the two purification methods, all plasmid DNA was then extracted using the Wizard® *Plus* SV Miniprep purification system (Promega) and subsequently purified using the Geneclean II kit (Bio-101, USA) according to the manufacturer's recommendations.

6.2.2.2 Analysis of recombinant plasmids for specificity to **Phomopsis** *taxon 1*

Recombinant plasmid DNA (2 µg) and 500 ng DNA from pUC19 vector was digested with *Pst*1 and transferred to a positively charged nylon membrane (Roche Diagnostics) by the Southern transfer method as described in section 2.2.4. To test the specificity of the plasmids to *Phomopsis* taxon 1, membranes (Southern blot 62 and 63) were hybridised with 50 ng genomic DNA from grapevine, *Phomopsis* taxon 2 isolate M827, *Phomopsis* taxon 1 isolate H307 or 25 ng DNA from pUC19 as described in section 2.2.6. Plasmid clones showing a signal after hybridisation to taxon 1 DNA only were chosen for further analyses. Taxon 1-specific clones were selected on the basis of insert size and strength of hybridisation signal.

To assess the specificity and sensitivity of the eight putative taxon 1-specific clones, 17 isolates of *Phomopsis* taxon 1 were selected for Southern blotting. Approximately 500 ng

of *Phomopsis* taxon 1, *Phomopsis* taxon 2 and grapevine DNA was digested with *Pst* I or *Eco*RI (Roche Diagnostics) in a total volume of 30 μl as described in section 6.2.1.1. Each digested sample was separated by gel electrophoresis on three replicate 1% agarose gels in TAE buffer and visualised by ethidium bromide staining. DNA was transferred to a positively-charged nylon membrane (Roche Diagnostics) overnight as described by Southern (1975) (see section 2.2.4). Lambda DNA digested with *Hin*dIII was included on each gel as a reference marker.

6.2.2.3 DNA labelling and hybridisation

Southern blots 79 and 80 contained approximately 200 ng of genomic *Phomopsis* taxon 1 and taxon 2 DNA digested with restriction enyme *Eco*R1, whereas Southern blot 81 contained taxon 1 and taxon 1 DNA digested with *Pst*I. The eight putative taxon 1-specific clones were digested with *PVU*II and 25 ng of DNA was labelled with [α-^{32}P] dCTP using 0.1 μg of the *PVU*II-specific oligonucleotides, P19S1 and P19S2, as primers in the reaction mix (see section 2.2.6 and Appendix A). Following hybridisation with the putative taxon 1-specific clones, membranes were exposed to X-ray film (X-Omat®, Kodak) to obtain an auto-radiographic image. In total, 16 selected clones were radio-actively labelled. Clones requiring a short exposure time to obtain strong hybridisation signals were deemed suitable for use as a diagnostic DNA probe

6.2.2.4 Storage of recombinant plasmids

White bacterial colonies containing *Phomopsis* taxon 1-inserts were transferred to 10 ml LBAmp broth and incubated with shaking at 200 rpm overnight at 37°C. A 1 ml aliquot from

the overnight culture was transferred to an Eppendorf tube containing 450 µl sterile LB:glycerol (1:1) and 0.45 µl Amp (50 mg/ml). Colonies were quick frozen in liquid nitrogen and stored at –70°C.

6.2.3 PCR amplification and primer selection

Nine randomly amplified polymorphic DNA (RAPD) primers; AM-01, AM-02, AM-6, AM-7, AM-10, AM-17, AM-18, AM-19, AM-20 from the Operon Technologies primer kit OPA and the R1 primer (5'-GTCCATTCAGTCGGTGCT-3', Weining and Langridge, 1991) were screened with *Phomopsis* taxon 1 and taxon 2 DNA. Initial screening of RAPD primers involved three isolates of *Phomopsis* taxon 1; A223.1, H307, L401; one isolate of *Phomopsis* taxon 2, P712 and an isolate of *E. lata,* M280. Primers were selected on the basis that amplified *Phomopsis* taxon 1 DNA produced a number of distinguishable bands. Suitable primers were used to amplify DNA from 18 isolates of *Phomopsis* taxon 1, eight isolates of *Phomopsis* taxon 2, one isolate each of *E. lata*, *Cryptovalsa* sp., *Phellinus punctata*, *Phellinus* sp., *Phaeomoniella chlamydosporum*, *Botryosphaeria ribis*, *B. cinerea, Phaeoacremonium aleophilium*, *U. necator, Saccharomyces cerevisiae* and *Hanseniaspora uvarum* and grapevine.

A serial dilution of DNA (from 1 ng to 50 ng) from *Phomopsis* taxon 1 isolate A223.1 was assessed in PCR reactions to determine the optimum DNA concentration for PCR amplification. Based on this preliminary experiment, 10 ng of genomic DNA was used in each PCR reaction. PCR amplification was carried out in a final volume of 25 µl. Each reaction contained 1.5 mM $MgCl_2$, 2.5 µl of 10 × thermophilic buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% Triton X-100), 100 µM each of dATP, dTTP, dGTP and dCTP (Roche Diagnostics), 25 pmol/µl of the R1 primer (5'-GTCCATTCAGTCGGTGCT-3'), 10 ng

template DNA and 1 unit of *Taq* DNA polymerase (Promega). DNA amplification was performed in a PTC-100 programmable thermocycler (MJ Research, Inc., USA) using a two-step programme of 94°C for 1 min, 6 cycles of 94°C for 30 sec, 40°C for 1 min and 1 min at 72°C; 94°C for 30 sec; 28 cycles 58°C for 1 min and 72°C for 1 min; and a cycle of 5 min at 72°C. A volume of 5 µl of each amplification product was analysed by electrophoresis on a 1.2%-1.5% agarose gel in TBE buffer (see Appendix A) and fragment size compared with a 100 bp DNA marker XIV (Roche Diagnostics).

PCR amplification products obtained from *Phomopsis* taxon 1 isolates J4, J6, L424 and *Phomopsis* taxon 2 isolates P712 and C608 were loaded on a 1.2% TBE agarose gel and run at 70 V for 1.5 hours for electrophoretic separation of distinct bands. Specific fragments of 420 bp and 900 bp, were identified for *Phomopsis* taxon 1 and a fragment of size 600 bp was identified for *Phomopsis* taxon 2. Each band was excised from the agarose gel and DNA was extracted using the *Jet*Quick gel extraction Spin Kit (Genomed, USA) according to the manufacturer's recommendations. DNA was re-suspended in two successive elutions of 30 µl of TE, and 20 µl TE, respectively. The concentration of DNA was analysed by electrophoresis on a 1.2% agarose gel in TBE buffer, including a standard 100 bp DNA size marker (Roche Diagnostics), as previously described. Purified DNA extracted from the agarose gel (final concentration of 250 ng/µl) were analysed by automated DNA sequencing (Flinders University of South Australia DNA Core Sequencing facility).

6.2.4 Cloning of PCR-amplified *Phomopsis* DNA

The *Phomopsis* taxon 1 fragments, 420 bp and 900 bp, and a *Phomopsis* taxon 2 fragment (600 bp) derived from PCR amplification using the R1 primer were ligated into 50 ng pGEM®-T easy vector (3015 bp) using the pGEM®-T Easy Vector system (Promega)

according to the manufacturer's recommendations. Five DNA molar ratios of 3:1, 2:1, 1:1, 1:2 and 1:3 (insert:vector) were calculated and prepared using the formula described in section 6.2.1.3. The total ligation reaction mixture included 5 µl 2 × rapid ligation buffer, and 3 units of T4 DNA ligase adjusted to a total volume of 10 µl in sterile ddH_20 in a 0.5 ml thin-walled PCR tube. The ligation reaction was mixed and incubated overnight (maximum 16 hours) at 4°C.

Ligation products were transformed into pre-prepared competent cells of *E. coli* strain JM101 (Promega) by transferring 10 µl of ligation reaction to 200 µl competent cells in a 50 ml Falcon® tube (Becton Dickinson Labware, USA). Three control reactions were prepared;

(1) positive control of 542 bp fragment from pGEM-luc DNA (control DNA, Promega) to test if ligation was successful,

(2) pGEM®-T Easy vector, excluding *Phomopsis* taxon 1 insert, to assess number of background colonies resulting from undigested vector,

(3) transformation control of 25 ng circular pUC19 DNA only to assess efficiency of competent cells.

Transformation conditions involved incubation of the mix on ice for 30 min, heat shock without agitation for 60 sec at exactly 42°C followed by the addition of 400 µl SOC medium (see Appendix A) and shaking at 37°C for 1.5 hours. A 100 µl aliquot of each transformation reaction was plated onto duplicated LBAmp plates (see section 6.2.1.5) for colour selection and incubated at 37°C overnight.

White recombinant colonies were selected as in section 6.2.1.5 and DNA was extracted using the Wizard® *Plus* SV Miniprep purification system (Promega) according to the manufacturer's recommendations. The plasmid DNA was suspended in 100 µl nuclease-free ddH_20 and the concentration of DNA was analysed by gel electrophoresis as described in

section 6.2.1.1. The presence of an insert was confirmed by digestion with restriction enzymes *Eco*R1 or *PVU*II (Roche Diagnostics) in a total volume of 50 µl as described previously. If partial or incomplete digestion occurred, plasmid DNA was further purified using the Geneclean II kit (Bio-101) as described in section 6.2.2.1.

6.2.5 Amplification of recombinant plasmid DNA with universal primers

Recombinant plasmid DNA was amplified to assess the presence of *Phomopsis* DNA. The PCR amplification was performed in a total volume of 50 µl containing approximately 50 ng of plasmid DNA, 250 µM of each dNTPs, 10 × thermophilic buffer, 25 mM $MgCl_2$, 0.4 µM of each universal primer M13F and M13R (Messing, 1983) (see Appendix A) and 0.2 units of *Taq* DNA polymerase (Promega). The reaction was performed in a PTC-100 thermocycler (MJ Research Inc., USA) using a two-step programme of: 95°C for 2 min, 34 cycles of 95°C for 30 sec, 55°C for 30 sec, and 55 sec at 72°C; and 5 min at 72°C. A volume of 5 µl of each amplification product was analysed by gel electrophoresis on a 1.2% agarose in TBE buffer as described in section 6.2.6.

6.2.6 Analysis of *Phomopsis* taxon-specific clones obtained by PCR amplification

The selected clones, derived from four amplicon fragments of taxon 1 isolate J6, were labelled pT1420-# whereby p=plasmid, T1=taxon 1, 420=insert size and # =white recombinant colony denoted by the grid on an LBAmp plate.

Southern membranes (Southern blots 79, 80 and 81) containing *Phomopsis* taxon 1 and taxon 2 DNA digested with either *Eco*R1 or *Pst*I (see section 6.2.2.2) were labelled with the four R1 clones of *Phomopsis* taxon 1 (pT1420-3, pT1420-4, pT1420-13, pT1420-18) as

described in section 2.2.6. A slot blot membrane was prepared using 100 ng of total DNA obtained from *Phomopsis* taxon 1-infected grapevine buds, canes and shoots as described in section 2.2.3. Each membrane included purified DNA of *Phomopsis* taxon 1 isolate A223.1, purified DNA of *Phomopsis* taxon 2 isolate P712, 100 ng of DNA obtained from grapevine and ddH_20. Total DNA was hybridised with each of the four R1 clones containing *Phomopsis* taxon 1, pT120-3, pT120-4, pT120-13, pT120-18, as described in section 2.2.6.

Undigested purified recombinant plasmid DNA (pT1420-3, pT1420-4, pT1420-13, pT1420-18) was analysed by automated DNA sequencing (Flinders University of South Australia DNA Sequencing Core facility,) in forward and reverse directions using the universal primers M13F and M13R. In addition, purified DNA (not amplified by PCR) of taxon 2-specific DNA probe, pT2P25 was sequenced. DNA sequence editing was conducted using the program Chromas version 1.45 (Technelysium, Australia). Bioinformatics analyses were conducted using BioManager.com provided by ANGIS. *Phomopsis* taxon 1 sequences were aligned with ClustalW and entered into GenBank to compare sequence similarity to database sequences using blastn and blastx programmes.

6.3 Results

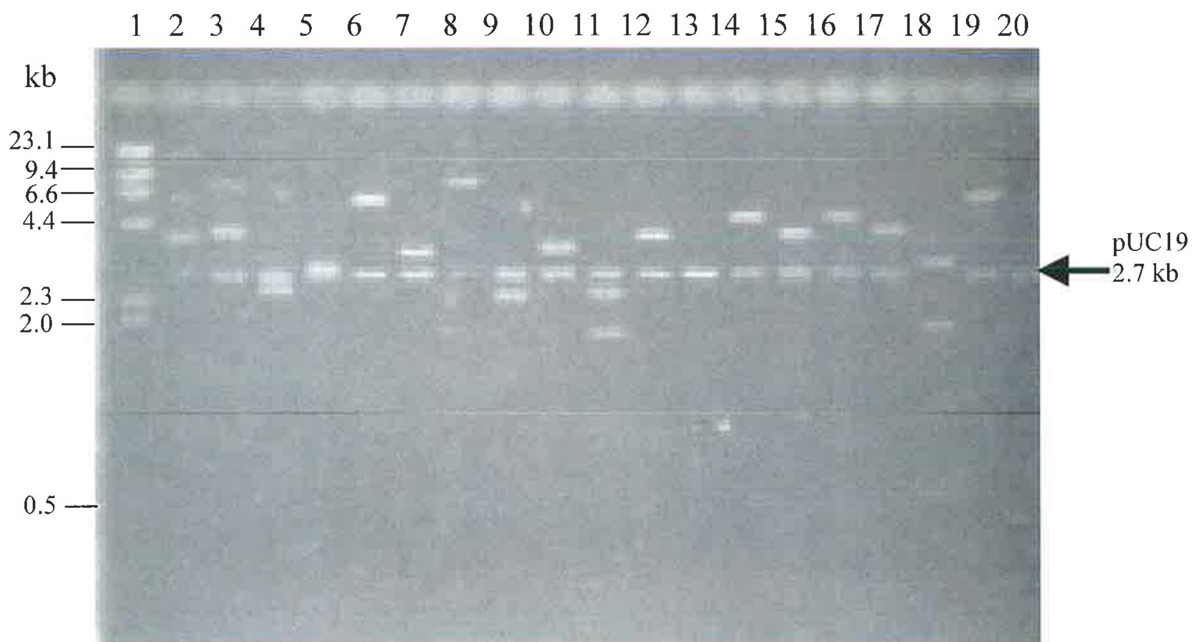
6.3.1 Analysis of *Phomopsis* taxon 1 genomic DNA library

The ligation reaction containing DNA molar ratios of 2:1 (*Phomopsis* taxon 1: pUC19 vector) produced 560 white recombinant colonies. Hybridisation experiments with labelled genomic DNA from *Phomopsis* taxon 1 showed that only 37 of the 560 colonies produced hybridisation signals that were considered high (Table 6.1). Some signals were observed after hybridisation with DNA from *Phomopsis* taxon 2 and grapevine, therefore these clones were not selected for further evaluation. A total of 66 clones was selected as hybridising to DNA

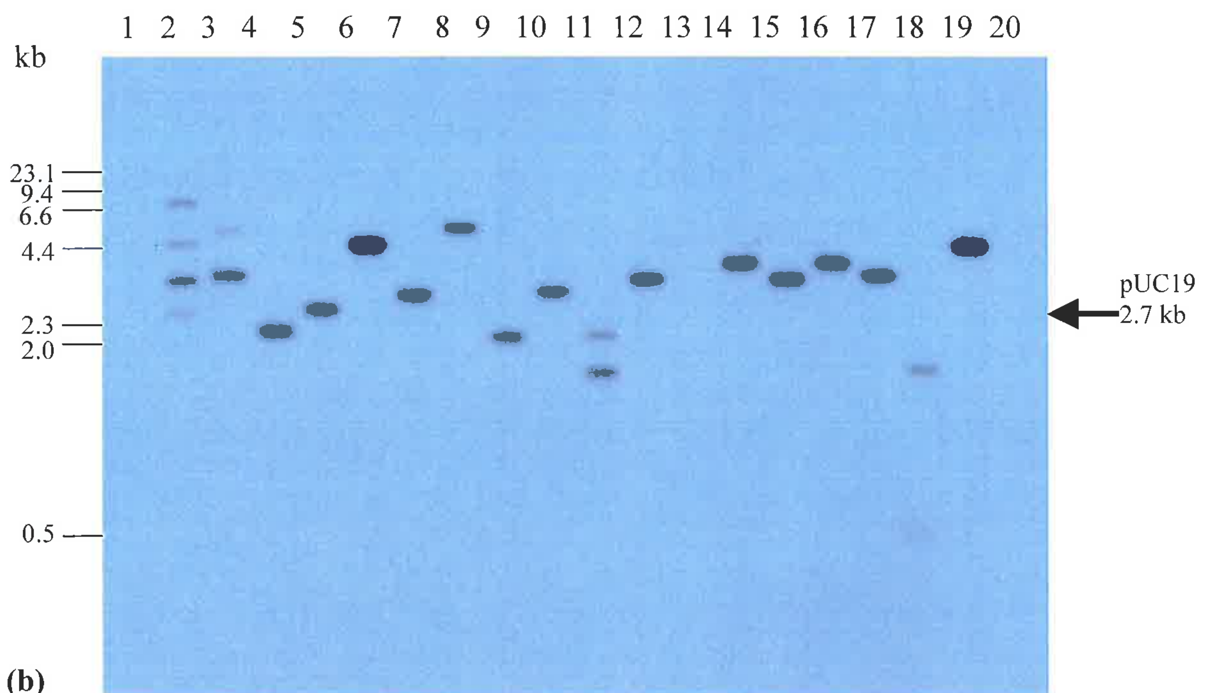
of *Phomopsis* taxon 1 but having no hybridisation signal to genomic DNA from *Phomopsis* taxon 2 and grapevine. This included nine clones showing a high signal and 57 showing a medium signal on colony blots. After hybridisation of recombinant plasmid DNA with genomic DNA from *Phomopsis* taxon 1 isolate H307 on a Southern blot, estimation of signal strength was based on exposure for 7 days at –70°C (Figure 6.1a and b).

Table 6.1. Number of colonies with corresponding hybridisation signal after radio-actively labelling with genomic DNA from *Phomopsis* taxon 1 isolate H307, *Phomopsis* taxon 2 isolate M827 and grapevine.

Genomic DNA	Number of colonies and corresponding hybridisation signal			
	High	**Medium**	**Low**	**Very low-none**
Phomopsis taxon 1	37	110	164	249
Phomopsis taxon 2	39	43	81	397
Grapevine	18	0	0	542



(a)



(b)

Figure 6.1. Example of *Phomopsis* taxon 1 recombinant plasmid DNA digested with restriction enzyme *Pst*I, extracted using the Wizard *SV* Plus Miniprep purification system (Promega) followed by purification with Geneclean II (Bio-101). Lane 1, lambda DNA marker digested with *Hin*dIII; lanes 2-19, *Phomopsis* taxon 1 clones digested with *Pst*I (B130, B147, C2, C3, C15, C30, C43, C47, C57, C78, C85, C112, C136, D11, D25, D39, D40, D43, respectively). Lane 20 contains pUC19 DNA digested with *Pst*I. Arrow indicates pUC19 vector DNA, 2.7 kb. **(a)** 1% TAE agarose gel electrophoresis of purified plasmid DNA digested with *Pst*I. **(b)** Corresponding autoradiograph after hybridisation with *Phomopsis* taxon 1 isolate H307 after 7 days exposure at –70°C.

Of the 66 clones radio-actively labelled with DNA from *Phomopsis* taxon 1 isolate H307, three clones did not contain inserts and 15 clones contained multiple inserts. No signals were obtained following hybridisation with DNA from grapevine or *Phomopsis* taxon 2 after 7 days exposure. Long exposure time and intensity of signal strength indicated that the taxon 1-specific clones were low copy and did not contain repetitive sequences. Based on hybridisation of DNA to *Phomopsis* taxon 1, clones were classified into two groups; those having one insert size of less than 2.7 kb (seven clones) or larger than 2.7 kb (22 clones) (Table 6.2).

6.3.2 Analysis of *Phomopsis* taxon 1-specific clones

Of the 66 clones screened (section 6.3.1), 11 taxon 1-specific clones; A99, A122, B6, B89, C2, C3, A61, A127, C47, C136 and B34, were chosen for further analysis. Clones were specific to *Phomopsis* taxon 1 and did not hybridise to DNA from *Phomopsis* taxon 2 or grapevine. The clones were digested with *PVU*II, and hybridised to Southern blots containing purified DNA from isolates of *Phomopsis* taxon 1, *Phomopsis* taxon 2 and grapevine. Hybridisation signals were scored after exposure for 7 days at –70°C. Nine clones hybridised to *Phomopsis* taxon 1 and the restriction fragment length polymorphisms (RFLPs) differentiated between the 17 isolates (Table 6.3). Seventeen phenotypes were identified among the 17 isolates. Clones A61 and A127 did not generate bands for any of the isolates tested.

Although hybridisation experiments using purified plasmid DNA indicated that the selected clones were taxon 1–specific, all clones did not hybridise to all 17 isolates. Longer exposure times may have revealed bands. It may be likely that some of the isolates did not contain DNA fragments present in the putative taxon 1-specific clones.

Table 6.2. Estimated insert size and intensity of signal of putative taxon 1-specific clones, digested with *Pst*I, after hybridisation to genomic DNA of *Phomopsis* taxon 1 isolate H307 [a].

Clone	Insert size (kb)	Intensity of signal
B89	2.0	medium
B6	2.5	low
C2	2.6	medium
A127	1.9	low
A99	2.6	low
C47	2.6	high
A122	1.8	low
C3	2.8	medium
B34	2.8	medium
B44	2.8	medium
C136	3.0	medium
D25	3.0	medium
A25	3.0	medium
D45	3.0	medium
D11	3.8	medium
D39	3.8	medium
B118	3.8	medium
C85	3.8	medium
A61	3.9	medium
C30	3.9	medium
A75	3.9	low
B32	4.1	medium
B51	4.1	medium
C30	4.0	medium
C57	4.0	medium
C15	6.0	medium
C43	6.8	medium
D43	6.0	medium
D44	3.4	low

[a] Estimation of copy number based on strength of the hybridisation signal after exposure for 7 days at −70°C. Clones did not hybridise to genomic DNA from *Phomopsis* taxon 2 isolate M827 and grapevine.

Table 6.3. The size and presence or absence of each restriction fragment length polymorphism (RFLP) after exposure for 7 days at –70°C in DNA from 17 isolates of *Phomopsis* taxon 1 digested with restriction enzymes *Pst*I or *Eco*R1 using 11 *Phomopsis* taxon 1-specific clones, whereby 0= no fragment and 1= restriction fragment present. Phenotypes are distinguished by the overall similarities in fragments among all clones tested.

									Phomopsis		taxon 1 isolate									
Probe	Enzyme	Band	Size (kb)	J5	J4	J6	H311	H308	H307	L407	L401	L402	L416	L417	M834.2	M838.4	A223.1	A223.2	A20	B500
A99	EcoRI	1	20	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
		2	12.8	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1
A122	EcoRI	1	19.5	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
		2	16.5	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
		3	9.0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		4	6.7	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
		5	4.3	0	0	0	1	1	1	0	1	1	0	0	1	1	1	0	0	1
		6	3.4	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0
		7	2.7	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1
B89	EcoRI	1	6.5	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
		2	5.0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
		3	4.3	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
C47	EcoRI	1	18.0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
		2	8.7	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1
B34	EcoRI	1	4.3	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0
B6	PstI	1	16.0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0
		2	8.0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	0
		3	6.7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		4	5.9	0	0	0	1	1	1	1	1	1	1	0	1	1	1	0	0	1
		5	2.9	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1
C2	PstI	1	7.9	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
		2	6.2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
		3	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
		4	4.5	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0
		5	4.2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		6	3.5	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0
		7	3.0	0	0	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1
		8	2.7	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1
C3	PstI	1	16.2	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0
		2	12.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
		3	8.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
		4	8.6	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
		5	5.0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
		6	3.3	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	0	0
		7	3.1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
C136	PstI	1	19.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
		2	12.8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
		3	9.4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		4	7.8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		5	6.7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		6	4.3	1	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1
Phenotype				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

One clone, A122, hybridised to at least one fragment from all 17 isolates of *Phomopsis* taxon 1. Table 6.3 shows that the RFLPs identified for each isolate and the probes resolved a single fragment in all *Phomopsis* taxon 1 isolates, ranging in size from 3.0 kb to 12.8 kb. Hybridisation of a range of taxon 1 isolates with clone C3 resulted in a band of 3.1 kb in 15 of the 17 isolates tested. Clones A99 and C47, both approximately 2.6 kb, identified one fragment (12.8 and 8.7 kb, respectively) in all taxon 1 isolates except J6 and L417, and in isolate L416, two fragments were generated. Similarly, clone B89 identified one band at 4.3 kb for all *Phomopsis* taxon 1 isolates, except for H308, L402 and L416 (Figure 6.2). In this example, two bands were generated for isolates H308 and L402 and no band for L416.

Clones B6, C2 and C3 generated multiple fragments containing five to eight bands in taxon 1 isolate A223.1. Eight bands were identified in isolate A223.1 after hybridisation with clone C2 (Figure 6.3b). With this clone, multiple bands were also generated in DNA of isolates L401 and A223.2. Isolates A223.1 and A223.2 originated from a single vineyard, but clone C2 did not generate similar banding patterns for these isolates (see Table 6.3). Clones A122, B6 and C136 also produced variations of banding patterns between isolates A223.1 and A223.2 (Table 6.3), indicating that genetic variation exists between the isolates examined.

Isolates L401 and A223.1 displayed identical banding patterns after hybridisation of DNA with clone C3, but overall the phenotype was different. Hybridisation of clones to DNA of taxon 1 isolate J6 resulted in only two bands, one each with clones A122 and B89. This may have been due to insufficient DNA loaded on to the gel (as shown by gel electrophoresis, Figure 6.3a) or longer exposure times may have been required to obtain similar hybridisation signals.

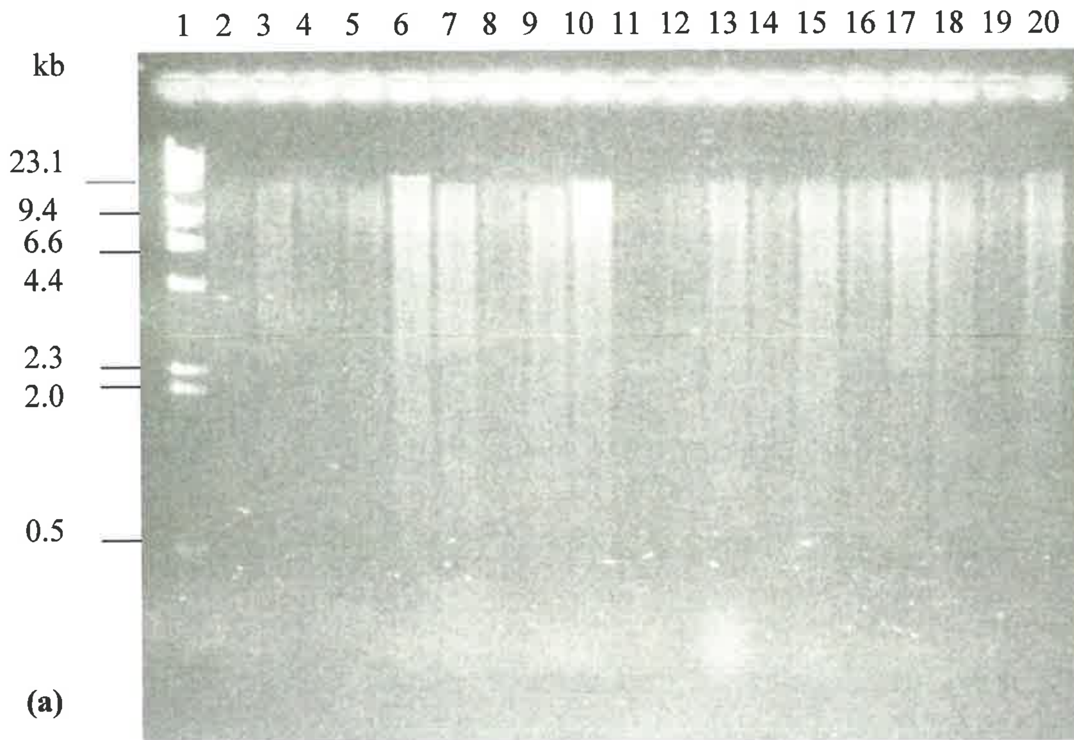


Figure 6.2. Southern blot (Southern 79) of DNA from *Phomopsis* taxon 1, *Phomopsis* taxon 2 and grapevine digested with restriction enzyme *Eco*RI. Lane 1, Lambda DNA digested with *Hin*dIII; lanes 2-18 DNA from *Phomopsis* taxon 1 isolates J5, J4, J6, H311, H308, H307, L407, L401, L402, L416, L417, M834.2, M838.4, A223.1, A223.2, A20, B500; lane 19, DNA from *Phomopsis* taxon 2 isolate C608 and lane 20 DNA from grapevine.

(a) 1% TAE agarose gel electrophoresis of genomic DNA digested with *Eco*RI.

(b) Corresponding autoradiograph after hybridisation using the putative taxon 1-specific probe B89.

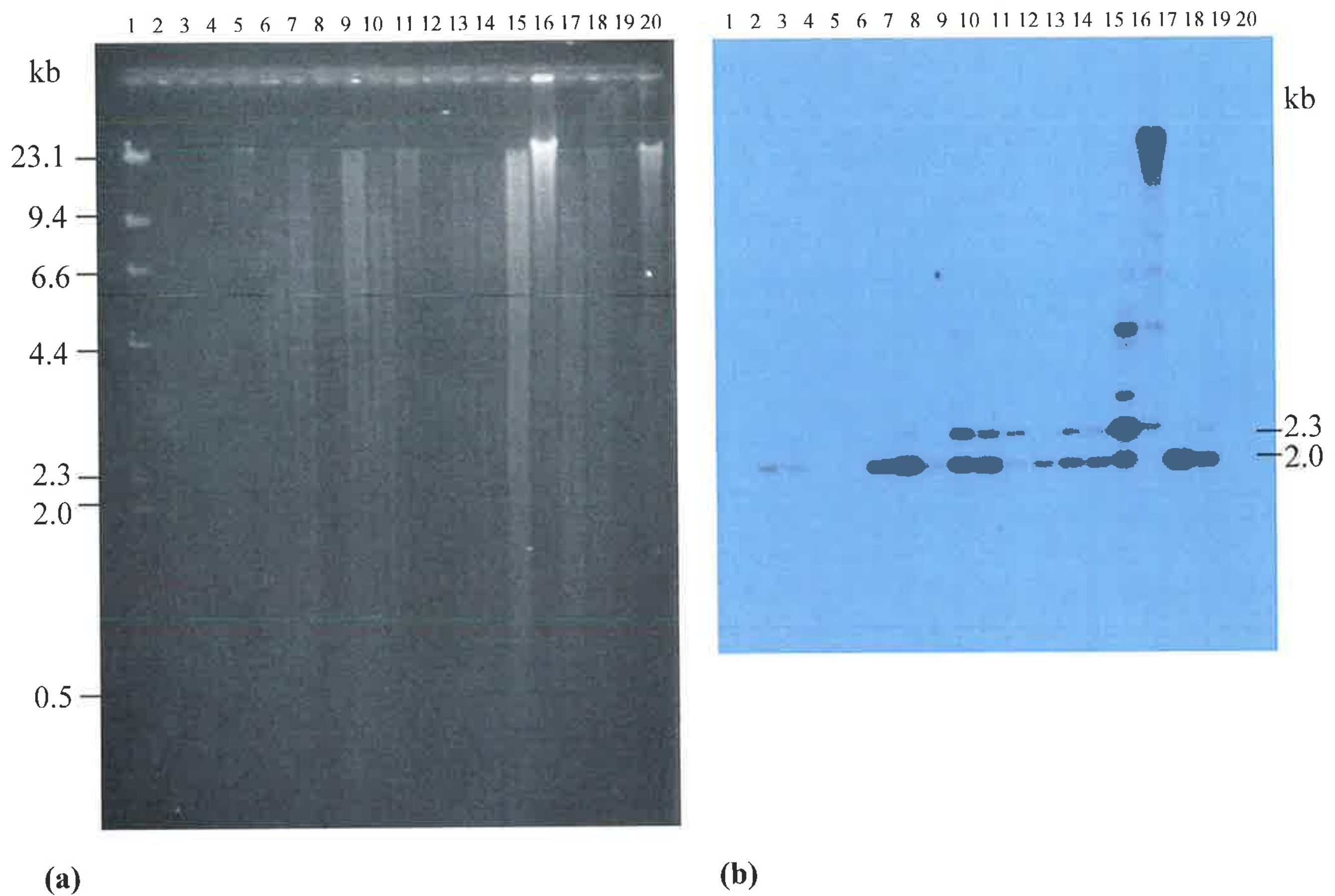


Figure 6.3. Southern blot (Southern 81) of DNA from *Phomopsis* taxon 1, *Phomopsis* taxon 2 and grapevine digested with restriction enzyme *Pst*I. Lane 1, Lambda DNA digested with restriction enzyme *Hin*dIII; lanes 2-18 DNA from *Phomopsis* taxon 1 isolates J5, J4, J6, H311, H308, H307, L407, L401, L402, L416, L417, M834.2, M838.4, A223.1, A223.2, A20, B500; lane 19, DNA from *Phomopsis* taxon 2 isolate C608 and lane 20 DNA from grapevine. Note that lanes 16 and 20 contain partially digested DNA.

(a) 1% TAE agarose gel electrophoresis of genomic DNA digested with *Pst*I.

(b) Corresponding autoradiograph after hybridisation using the putative taxon 1-specific probe C2.

Nine taxon 1-specific clones hybridised to one fragment in most of the 17 *Phomopsis* taxon 1 isolates after digestion of genomic DNA with either *Pst* or *Eco*R1. Clones A122, B6, C2, C3 and C136 identified multiple fragments in a number of taxon 1 isolates. These results indicated that the taxon 1-specific clones can be used to provide a multi-locus phenotype and detect polymorphisms between taxon 1 isolates.

6.3.3 Analysis of *Phomopsis* taxon-specific clones obtained by PCR amplification

Of the nine RAPD primers screened, three did not amplify *Phomopsis* DNA and the others did not result in clear, distinctive bands among isolates of *Phomopsis* taxon 1. Nevertheless, banding patterns differed between *Phomopsis* taxon 1 and taxon 2, with AM-18 generating considerably variability (data not shown). The lack of clearly defined amplified products between the taxon 1 isolates caused difficulties in interpreting the results. DNA of *E. lata*, however, was amplified in all reactions.

Preliminary investigations revealed that amplification of DNA using the primer R1 provided banding patterns which allowed for differentiation between isolates of *Phomopsis* taxon 1 and taxon 2. Two distinct fragments, 420 bp and 900 bp, were consistently amplified from DNA of a range of isolates of *Phomopsis* taxon 1. The R1 primer did not amplify these fragments when applied to *Phomopsis* taxon 2 DNA. Amplification of *Phomopsis* taxon 2 DNA with the R1 primer revealed a distinct fragment of approximately 600 bp. Figure 6.4 shows an example of amplicons generated when the R1 primer was used to amplify DNA of 17 isolates of *Phomopsis* taxon 1 and five isolates of *Phomopsis* taxon 2. Faint bands were observed for taxon 1 isolate L424 and the 900 bp fragment was not amplified in isolate M834.2.

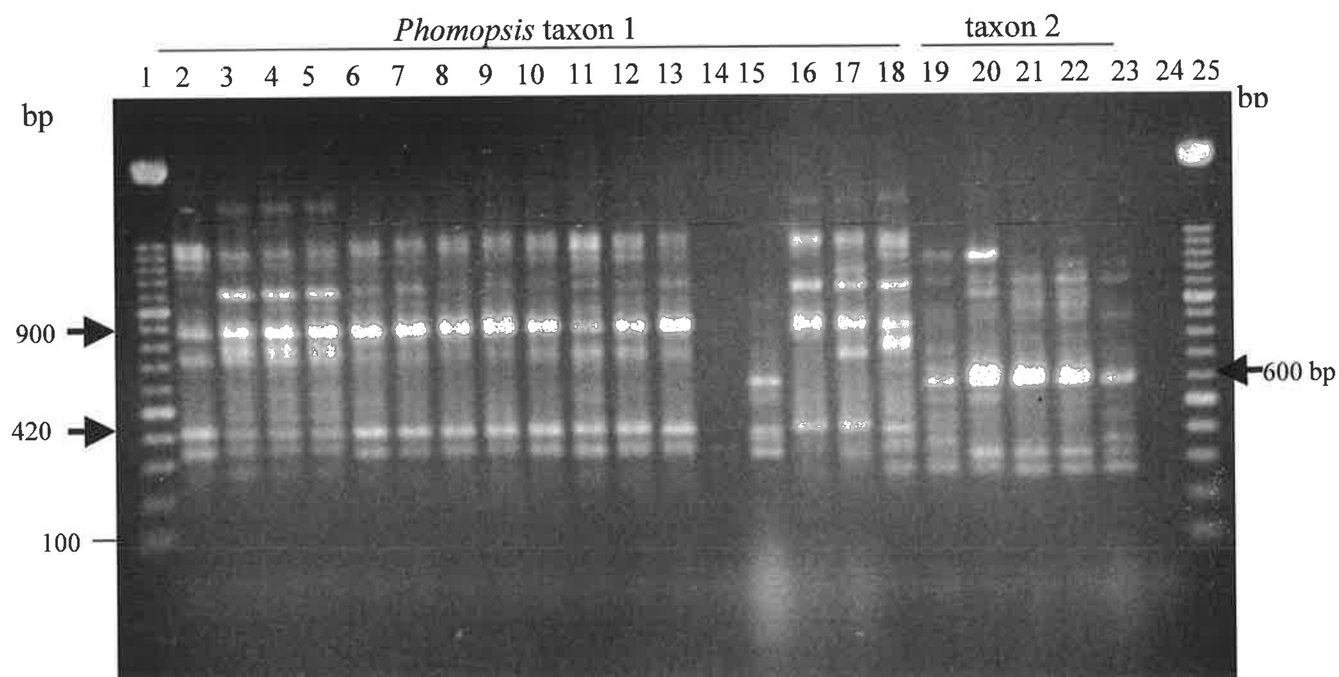


Figure 6.4. PCR amplification of genomic DNA of *Phomopsis* taxon 1 and *Phomopsis* taxon 2 using the R1 primer. PCR products were separated on a 1.5% TBE agarose gel. Lane 1, DNA marker XIV; lane 2-18 *Phomopsis* taxon 1 isolate A223.1, A223.2, A19, A20, H307, H308, H309, L417, J6, L401, L402, L403, L424, M834.2, M838.4, M831.1, J4; lanes 19-23, *Phomopsis* taxon 2 isolate P712, C608, UQ4424, M833.2 and C609; lane 24 ddH_20 and lane 25, 100 bp DNA marker XIV. Arrows indicate fragments for *Phomopsis* taxon 1 (420 bp and 900 bp) and *Phomopsis* taxon 2 (600 bp) which were subsequently isolated.

When used in PCR with DNA from a range of isolates of other fungal pathogens and yeasts associated with grapevine, the R1 primer did not result in amplification of the 420 bp fragment. However, amplification of DNA from a number of other fungi including *P. punctata* and *P. chlamydosporum*, resulted in bands of a size similar to 900 bp (Figure 6.5). In this gel, amplification of DNA with the R1 primer consistently revealed differences between *Phomopsis* taxon 1 and other fungi isolated from grapevine.

After isolation of the amplified fragments using the *Jet*Quick gel extraction kit (Genomed), PCR products of 420 bp and 900 bp, from *Phomopsis* taxon 1 isolates A223.1, J6, J4 and L424, and of 600 bp from *Phomopsis* taxon 2 isolates C609, P712 and C608 were directly sequenced. Unfortunately, direct DNA sequencing resulted in short sequences not suitable for analysis. It was necessary to sub-clone the PCR products to obtain sequence data.

Difficulties were experienced in obtaining recombinant DNA colonies, however, cloning was achieved after extensive purification of the PCR fragments using the Geneclean II kit (Bio-101). Specific fragments from *Phomopsis* taxon 1 and taxon 2 DNA were amplified and purified in a number of PCR-assays to ensure DNA was available. Although cloning of PCR products from five different *Phomopsis* isolates was attempted, 103 white colonies containing recombinant DNA were obtained from cloning of the 420 bp fragment amplified from *Phomopsis* taxon 1 isolate J6 at molar ratios of 2:1 and 3:1 (taxon 1:pGem-T Easy vector). Cloning using the 900 bp fragment purified by Gene-clean was not successful.

Digestion of the fragments (purified by Gene-clean) with *Eco*R1 followed by PCR amplification using the universal primers M13F and M13R confirmed the presence of the taxon 1 insert in six clones (pT1420-1, pT1420-3, pT1420-4, pT1420-13, pT1420-18, pT1420-31, Figure 6.6). The results indicated that the insert was approximately 650 bp, however, amplification of clones pT1420-3, and pT1420-4 showed a slightly larger fragment

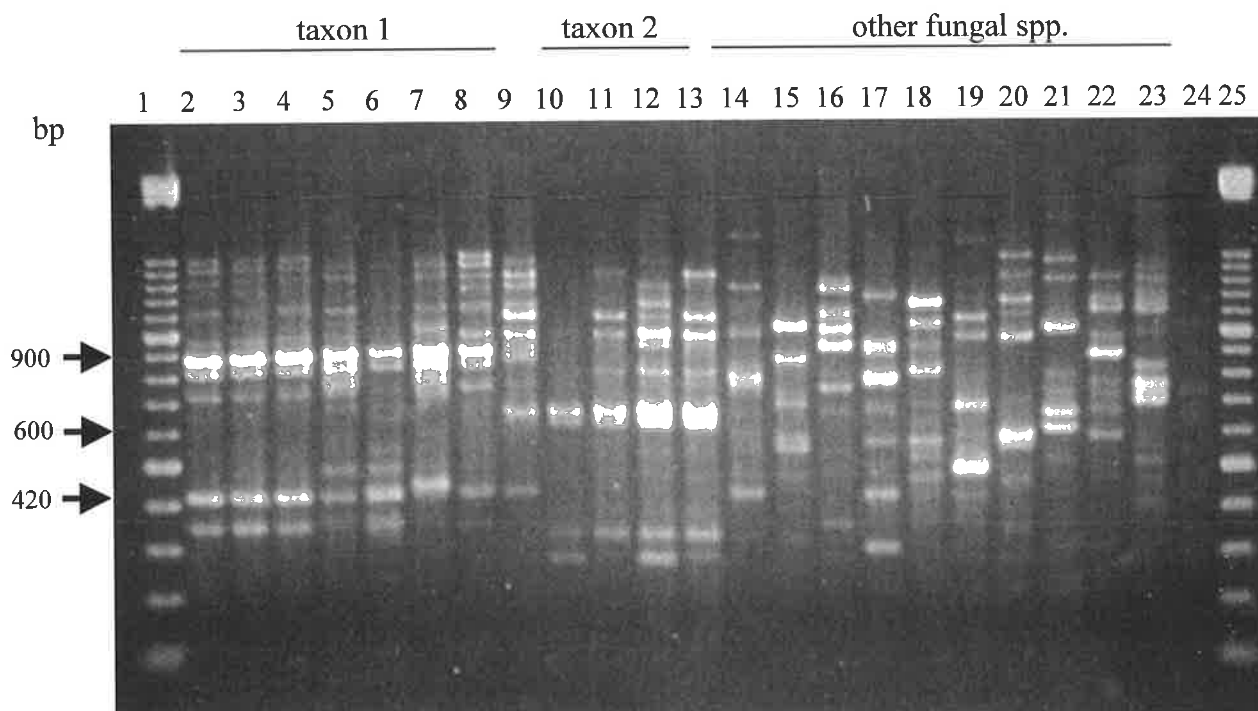


Figure 6.5. PCR amplification of DNA from *Phomopsis* taxon 1, *Phomopsis* taxon 2 and a range of other fungi associated with grapevine. PCR products were separated on a 1.5% TBE agarose gel. Lane 1, DNA marker XIV; lanes 2-9 *Phomopsis* taxon 1 isolate A223.1, H308, L401, A20, H307, J6, L424, I5; lanes 10-13, *Phomopsis* taxon 2 isolate M833.2, P712, C609, C608; lanes 14-22, *Eutypa lata*, *Cryptovalsa* sp., *Phellinus punctata*, *Phellinus* sp., *Phaeomoniella chlamydosporum*, *Botryosphaeria ribis*, *Phaeoacremonium aleophilium*, *Botrytis cinerea* and *Uncinula necator*; lane 23, grapevine DNA; lane 24, ddH_20 and lane 25, 100 bp DNA marker XIV. Arrows indicate isolated fragments for *Phomopsis* taxon 1 (420 bp and 900 bp) and *Phomopsis* taxon 2 (600 bp).

(700 bp) than in other clones tested (lanes three and four, Figure 6.6). This suggested that two fragments may have been cloned. After amplification, the fragments included vector sequences flanking the M13 primers and taxon 1 insert.

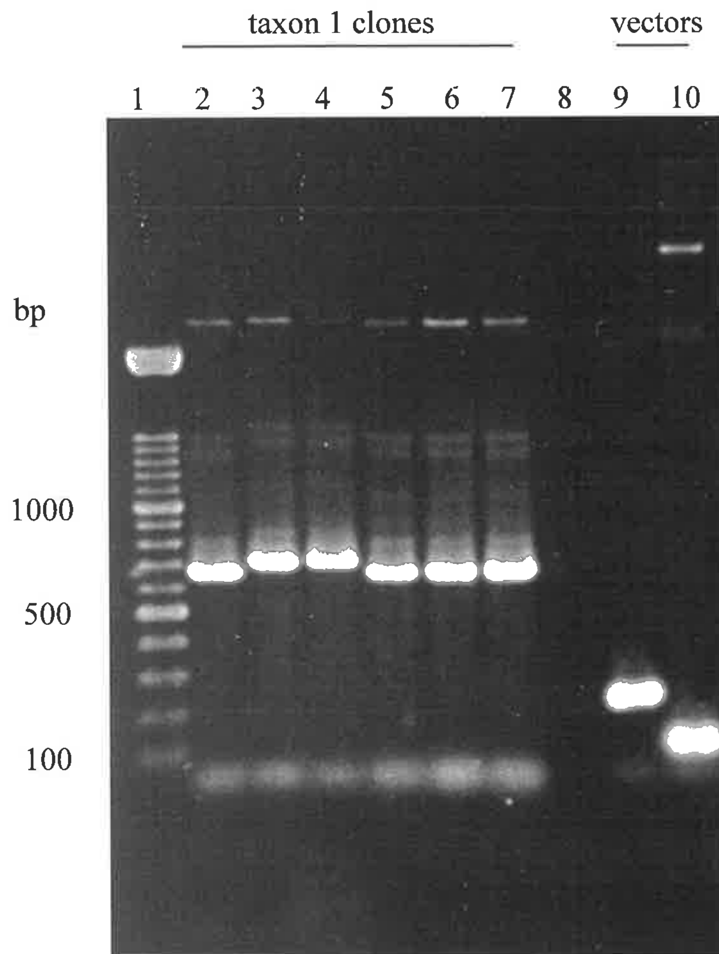


Figure 6.6. PCR amplification of six PCR-based taxon 1-specific clones obtained from *Phomopsis* taxon 1 isolate J6 using primers M13F and M13R. Amplification shows the presence of the taxon 1 insert in the pGEM-T Easy vector. Lane 1, DNA marker XIV, lanes 2-7, clones pT1420-1, pT1420-3, pT1420-4, pT1420-13, pT1420-18 and pT1420-31, lane 8, ddH_20, lane 9, undigested pBluescript vector and lane 10, undigested pUC19 vector (control).

Southern and slot hybridisation using three selected clones (pT1420-3, pT1420-4 and pT1420-18) containing the R1 amplicon, 420 bp, all yielded low signals to genomic DNA from isolates of *Phomopsis* taxon 1 and to grapevine tissue infected with *Phomopsis* taxon 1. A prolonged exposure time was required to obtain distinguishable hybridisation signals. After 7 days, Southern hybridisation with clone pT1420-4 revealed two polymorphic bands, 2.7 kb and 3.8 kb, in DNA from seven of the 16 isolates of *Phomopsis* taxon 1 digested with *Pst*I (Figure 6.7). This confirms that similar sequences are present more than once in the genome. Variation in the intensity of the hybridisation signals may have resulted from variable concentrations of DNA, therefore a longer exposure time may have revealed more bands.

Figure 6.7. Southern blot (Southern 81) of PCR-based putative *Phomopsis* taxon 1-specific clone pT1420-4 to *Eco*R1-digested genomic DNA from *Phomopsis* taxon 1. Lane 1, Lambda DNA digested with *Hin*dIII; lanes 2-17 DNA from *Phomopsis* taxon 1 isolates, J4, L418, H311, H308, H307, L405, L401, L402, L416, L417, M834.2, M838.4, A223.1, A223.2, A20, B500; lane 18, DNA from *Phomopsis* taxon 2 isolate C608 and lane 19, DNA from grapevine.

Hybridisation of the clone pT1420-3 to genomic DNA of *Phomopsis* taxon 1 resolved one fragment, of 19.6 kb, among 16 isolates. Clone pT1420-18 did not appear to hybridise to DNA of *Phomopsis* taxon 1 isolates. A long exposure time was required to obtain intense hybridisation signals on slot blots following radio-active labelling with the three clones (data not shown). Low hybridisation signals and poor banding profiles indicated that there were few copies of these sequences in the genome. The results showed that the PCR-based clones were specific to *Phomopsis* taxon 1 and did not hybridise to *Phomopsis* taxon 2 or grapevine DNA. Because clones showed poor sensitivity in slot blot analysis, it was deemed the clones were not suitable for use in a rapid diagnostic test and were not evaluated with other fungi.

Alignment of DNA sequence data showed the clones pT1420-3 and pT1420-4 were identical and of the same length (494 bp, Figure 6.8). Likewise, data sequence for clones pT1420-13 and pT1420-18 revealed identical sequences of 443 bp in length (Figure 6.9) but these differed from clones pT1420-3 and pT1420-4. Consensus analysis showed that the two pairs of sequences did not align and differences were observed in the proportion of base pairs (Table 6.4). In particular, clones pT1420-3 and pT1420-4 had a higher percentage of adenine (A) than pT1420-13 and pT1420-18. The four sequences of the *Phomopsis* taxon 1 clones were dissimilar to the sequence derived from the taxon 2-specific probe pT2P25 (see Appendix D). Protein and nucleic acid sequence analysis confirmed that no similarities were found among species that were available for comparison in the GenBank database.

Figure 6.8. Alignment of DNA sequences of *Phomopsis* taxon 1-specific clones pT1420-3 and pT1420-4. Identity shared with the consensus sequence is represented by ".". The position of the primer R1 (5'-GTCCAGCATTCAGTCGGTGCT-3') is indicated above the nucleotide sequence.

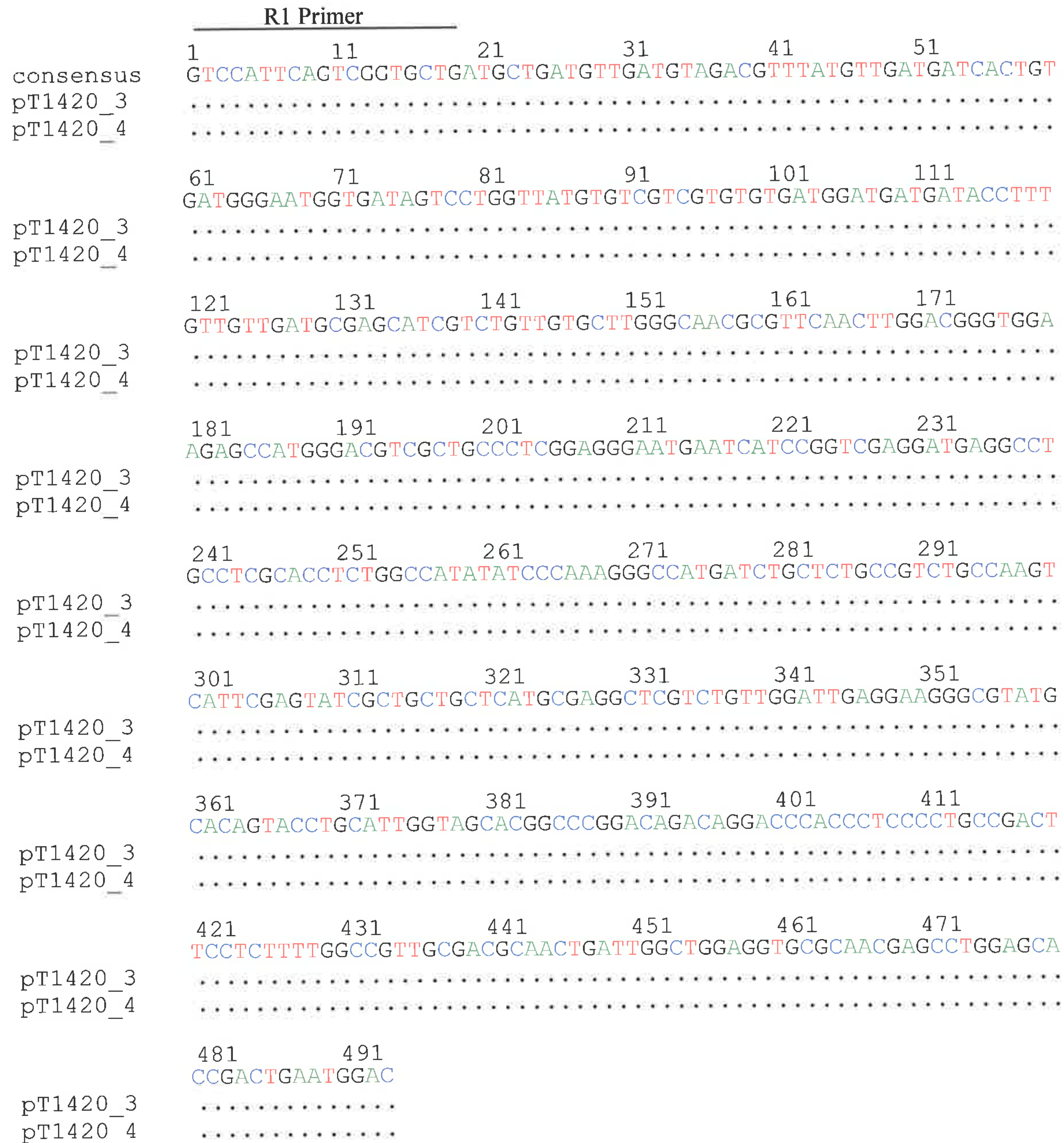


Figure 6.9. Alignment of DNA sequences of *Phomopsis* taxon 1-specific clones pT1420-13 and pT1420-18. Identity shared with the consensus sequence is represented by ".". The position of the primer R1 (5'-GTCCAGCATTCAGTCGGTGCT-3') is indicated above the nucleotide sequence.

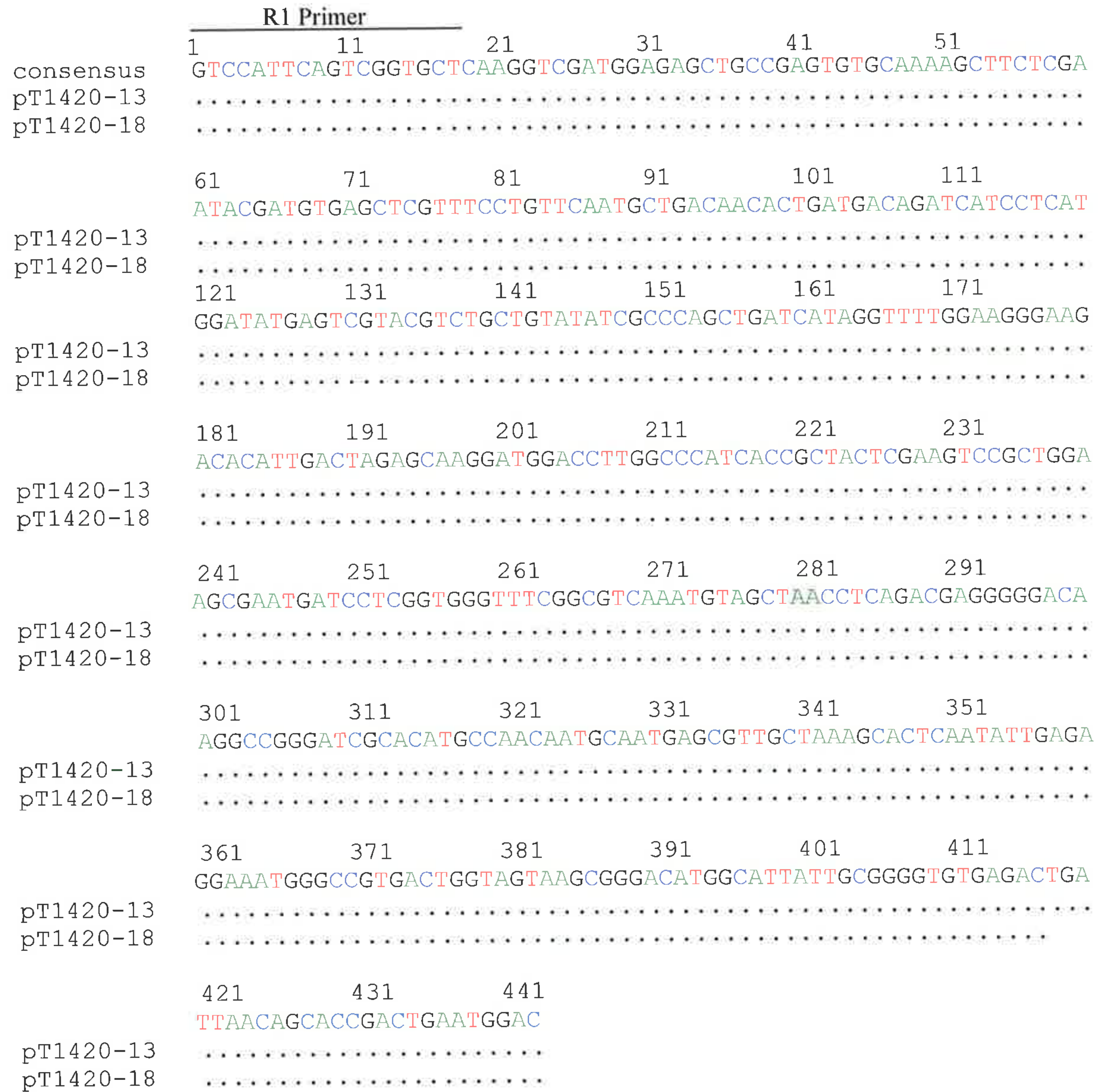


Table 6.4. Incidence of bases (%) in the sequences of four PCR-based *Phomopsis* taxon 1 clones, pT1420-3, pT1420-4, pT1420-13 and pT1420-18 derived from amplification using the R1 primer and DNA of *Phomopsis* taxon 2-specific probe pT2P25 (Melanson *et al.*, 2002).

Clone	% bases					Nucleotide
	A	C	G	T	G+C	Length
pT1420-3	18	24	31	26	55	494
pT1420-4	18	24	31	26	55	494
pT1420-13	26	22	29	22	52	443
pT1420-18	26	22	29	22	52	443
pT2P25	25	25	25	25	49	534

Amplification of the initial purified 420 bp fragment confirmed that two separate PCR products had been cloned into the pGem-T easy vector, as shown in Figure 6.10 (also see Figure 6.6). After purification with the Geneclean II kit, DNA was approximately 5 ng/ul, and thus difficult to visualise on the agarose gel. Sequence data confirmed two PCR products (494 and 443 bp) were cloned. The results showed that cloning of the 900 bp fragment amplified using the R1 primer and *Phomopsis* taxon 1 DNA was unsuccessful due to the presence of multiple PCR products.

6.4 Discussion

The objective of this work was to develop new DNA probes for the rapid detection of *Phomopsis* taxon 1 in grapevine tissue. The existing *Phomopsis* taxon 1-specific probe, pT1P180, had been used for the detection of *Phomopsis* taxon 1 in grapevine canes, shoots and buds (Chapters 3 and 4) but required prolonged exposure to obtain an intense hybridisation signal on slot blot membranes. The fragment size (3.6 kb) rendered pT1P180 unsuitable for obtaining sequence data without additional cloning. In addition, preliminary RFLP studies using pT1P180 indicated that the probe was low copy and, although it did

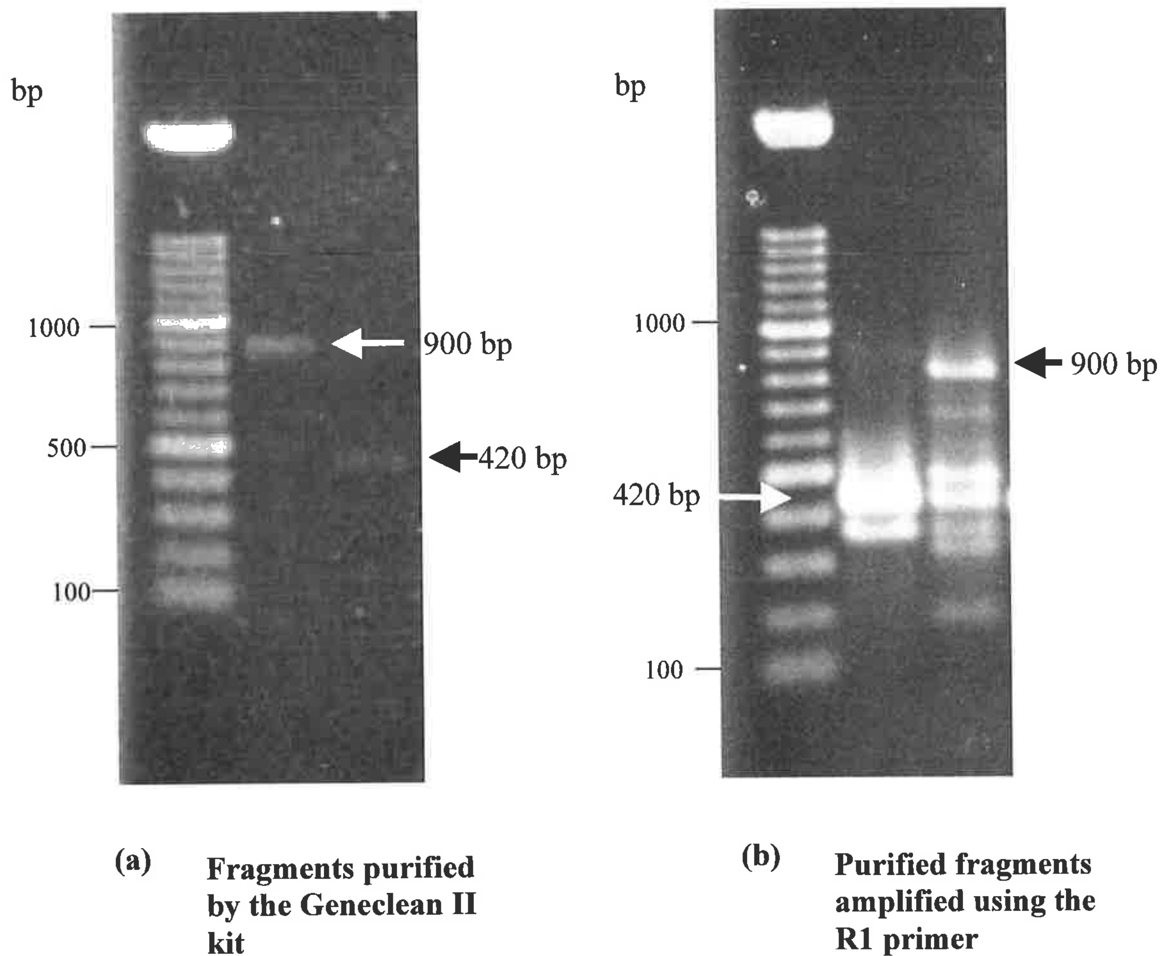


Figure 6.10. Gel electrophoresis (1% agarose in TAE buffer) of fragments 420 bp and 900 bp derived from *Phomopsis* taxon 1-specifc isolate J6.

(a) Fragments purified by Geneclean II kit (Bio-101) following amplification with the R1 primer. Lane 1, 100 bp DNA marker XIV, lane 2, 900 bp fragment and lane 3, 420 bp fragment.

(b) Purified fragments, 420 bp and 900 bp, used as templates in PCR amplification using the R1 primer. Amplification products clearly show two bands resulting from the template 420 bp and numerous bands amplified from the template 900 bp. Lane 1, 100 bp DNA marker XIV, lane 2, 420 bp fragment and lane 3, 900 bp fragment.

have high specificity to *Phomopsis* taxon 1, was not useful for DNA fingerprinting. The probe pT1P180 may be adequate for research studies but is unsuitable for use in a rapid commercial diagnostic test. In comparison, the *Phomopsis* taxon 2-specific probe, pT2P25, was found to be highly specific, highly repeated and detected genetic variation among taxon 2 isolates (Melanson *et al.*, 2002).

Putative taxon 1-specific low-copy clones were developed for *Phomopsis* taxon 1 and detected genetic variation among the taxon 1 isolates tested. Seventeen unique phenotypes were revealed in the 17 isolates of taxon 1 following hybridisation with nine putative taxon 1-specific probes. The clones developed in this study were specific to isolates of *Phomopsis* taxon 1 examined and did not hybridise to genomic DNA from taxon 2 or grapevine. Most of these clones resolved a single fragment in the taxon 1 isolates, however two or more polymorphic bands were consistently produced in six of the 17 isolates tested. This implied that the combination of the 11 clones used in this study detected genetic variability among some of the isolates.

Due to time constraints, the clones were tested on slot and Southern blots containing *Phomopsis* taxon 1, taxon 2 and grapevine only. Hybridisation experiments using other fungi associated with grapevine would confirm specificity of the clones to *Phomopsis* taxon 1. If further testing showed the clones to be specific to taxon 1, they have the potential to be used as species-specific probes, whereby a single DNA fragment can distinguish between *Phomopsis* taxon 1 and taxon 2. For example, DNA probes have been developed for differentiation of R-type and W-type isolates of the fungus *Pseudocercosporella herpotrichoides*, whereby the clones hybridise to a single *Eco*R1 fragment in R-type isolates (Nicholson and Rezanoor, 1994).

The RAPD primers tested in this study did not reveal a distinct fragment specific to *Phomopsis* taxon 1. Primer R1 generated different banding patterns between *Phomopsis*

taxon 1 and taxon 2. It was not necessary to digest genomic DNA with a restriction enzyme to obtain complex banding patterns as was conducted by Weining and Langridge (1991). One fragment, of 420 bp, which was common to all isolates of taxon 1 examined, was isolated and cloned following separation of PCR amplicons of *Phomopsis* taxon 1 isolate J6. However, upon amplification of this purified R1 clone, two fragments were identified. This was confirmed by the recognition of two distinct sequences in the analysis of sequence data of the recombinant plasmid. Amplification of the 900 bp fragment identified among PCR products of taxon 1, *P. punctata* and *P. chlamydosporum* confirmed multiple PCR products were amplified which may account for the unsuccessful cloning of this DNA fragment.

Direct sequencing of PCR products permits the rapid characterisation of sequences without the need for subcloning (Newton and Graham, 1994). In this study, direct sequencing was not successful due to contamination of the sample by other PCR-amplified products. Although PCR products were checked by agarose gel electrophoresis, direct sequencing is sensitive to the presence of low molecular weight primers and dNTPs (Sambrook and Russell, 2001). It is also possible that silica gel from the gel extraction kit was carried over to the purified sample and this has been known to hinder PCR amplification.

Southern hybridisation using the putative taxon 1-specific PCR-based low-copy DNA clone pT1420-3 detected one monomorphic band in the *Phomopsis* taxon 1 isolates tested. In general, the clones did not produce strong hybridisation signals to *Phomopsis* taxon 1 isolates by Southern and slot blot hybridisation. The long exposure time required to obtain intense hybridisation signals indicated the clones would not be suitable for use in a rapid diagnostic test as anticipated using slot blot analysis. However, the PCR-based probes were specific to isolates of *Phomopsis* taxon 1 examined and did not hybridise to taxon 2 or grapevine. The PCR-based probes did not identify complex banding patterns among the isolates tested, although clone pT1420-3 identified two bands among the taxon 1 isolates tested. The results

indicated that the clones were not a useful tool to differentiate among taxon 1 isolates by DNA fingerprinting.

The taxon 1-specific DNA probes were developed for use in a slot blot diagnostic test, however, hybridisation signals were similar to those obtained with the existing *Phomopsis* taxon 1 DNA probe, pT1P180. Radioactive labelling is sensitive enough to allow detection of sequences at the level of a few picograms (Koopmann *et al.*, 1994) but the results confirmed that the clones were low copy and not highly repetitive. Due to problems experienced with cloning and time constraints, taxon-specific primers could not be developed. However, extensive sequence information is available to facilitate the design of specific primers for use in a PCR-based diagnostic assay.

The development of a suitable PCR-based diagnostic test would involve considerable screening of DNA obtained from grapevine wood infected with *Phomopsis* and other fungi. It has been shown that amplification of DNA from woody tissue is often difficult (Rezaian and Krake, 1987). Methods have been devised to assist the identification of pathogens in woody tissue by PCR, such as modification of DNA extraction techniques (Maguire *et al.*, 1994; Zhang *et al.*, 1998; Labra *et al.*, 2001), addition of polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) to reduce polyphenols (Couch and Fritz, 1990) and dilution of DNA prior to amplification (Mazzaglia *et al.*, 2001). Although the new putative taxon 1- specific DNA probes were not suitable for use in a slot blot hybridisation test, development of a PCR-based assay could be used to distinguish between *Phomopsis* taxon 1 and taxon 2.

Chapter 7

General Discussion

7.1 Introduction

Studies on *Phomopsis* taxon 1 were undertaken to determine if the fungus is a pathogen on grapevine. Experiments were conducted to determine the pathogenicity of taxon 1 in relation to symptom expression and the effect on budburst and subsequent shoot growth. Experiments were performed over 3 years in glasshouse and field conditions and utilised taxon-specific DNA probes developed for the detection of *Phomopsis* taxon 1 and taxon 2 in grapevine buds and cane (Melanson *et al.*, 2002). Although Scheper (2001) reported that taxon 1 was associated with bud death and stunted growth of grapevines, these findings could not be verified. The results of this study suggest that *Phomopsis* taxon 1 is not a pathogen on grapevine and does not cause failure of buds to burst or bud death. Commercial vineyards infected with *Phomopsis* taxon 1 did not display poor budburst and shoot length was not adversely affected. Also bud fruitfulness and number of bunches were not influenced by *Phomopsis* taxon 1 infection. Pathogenicity experiments revealed that the 12 isolates of *Phomopsis* taxon 1 examined did not cause symptoms associated with Phomopsis cane and leaf spot. This investigation supports the suggestion by Mostert *et al.* (2000) that *Phomopsis* taxon 1 is endophytic in grapevines.

7.2 Endophytic growth of *Phomopsis* taxon 1

For the purpose of this study, an endophyte is defined as a fungus that does not cause injury to the host (Sinclair and Cerkauskas, 1996). The delimitation of pathogen and harmless endophyte is difficult as there are many fungal endophytes that might behave, in certain circumstances, as pathogens. Symptomless endophytes can be categorised into two ecological groups; those in grasses, in which the fungus and host plant form a relationship that is mutually beneficial, and the endophytes of trees and shrubs (Sinclair and Cerkauskas, 1996; Petrini, 1996). Most studies of endophytes of woody plants have focused on temperate forest plants, especially forest species. There is little or no information on endophytes colonising grapevine.

Phomopsis taxon 1 was detected in asymptomatic tissue by light microscopy and by using molecular methods. *Phomopsis* taxon 1 did not produce symptoms associated with Phomopsis cane and leaf spot, and microscopic studies revealed the fungus colonised the epidermis and cortex of the grapevine host but not the vascular tissue. In general, endophytic fungi do not colonise the vascular tissue of the plant host (Sinclair and Cerkauskas, 1996). Endophytes of the fungal genus *Neotyphodium* colonised vascular bundles in grasses following artificial inoculation but this did not occur in natural associations (Christensen *et al.*, 2001). The absence of hyphae in vascular bundles facilitates continued growth of the host and the fungus, forming a mutualistic symbiosis. Endophytic fungi grow within intercellular spaces of host tissue and there are no reports of intracellular growth (Bacon and De Battista, 1991). Endophytic fungi utilize nutrients from the host (Sinclair and Cerkauskas, 1996) but, as they establish an obligate biotrophic relationship with the host (Bacon and De Battista, 1991), are not known to kill their host (Agrios, 1988).

Many endophytes do not sporulate readily in culture on agar media (Bills, 1996) and sporulate preferentially on plant tissue. This was found to be true for *Phomopsis* taxon 1, whereas *Phomopsis* taxon 2 sporulated readily on a range of media. In a study of *Guignardia citricarpa*, which causes black spot disease of citrus, Baayen *et al.* (2001) showed that two strains were associated with the disease. Pathogenic strains were slow-growing, whilst morphologically similar non-pathogenic strains of *G. citricarpa* (also referred to as *Guignardia* sp.) were fast-growing on culture media. Non-pathogenic strains were identified as a separate species, *G. mangiferae*, an endophyte of woody plants. These findings are similar to the present study, where differences in morphology and pathogenicity have been observed between *Phomopsis* taxon 1 and taxon 2 on grapevine.

Because symptoms were not observed after colonisation of the host by *Phomopsis* taxon 1, the fungus may be considered as endophytic. Bleaching cannot be regarded as a symptom of taxon 1 infection because physical damage, extremes of weather, *B. cinerea* and some saprophytic pycnidium-producing fungi can also cause bleaching. Furthermore, *Phomopsis* taxon 1 was isolated from non-bleached spurs and healthy shoots, supporting the observation that taxon 1 displays asymptomatic growth. Endophytic colonisation does not result in the production of symptoms or disease, whereas fungi with a latent phase colonise for a period of time with minimal damage to the host before inducing symptoms (Sinclair and Cerkauskas, 1996). Thus *Phomopsis* taxon 1 can be considered as endophytic and taxon 2 as a latent pathogen. Many species of *Phomopsis* are classified as "latent-infecting" fungi including *P. longicolla*, the causal agent of Phomopsis seed decay in soybeans (Sinclair, 1993), and *P. leptostromiformis* in narrow-leafed lupins (Williamson and Sivasithamparam, 1994).

Endophytic fungi are able to infect and colonise many aerial plant parts, including stems, leaves and bark (Bills, 1996). Mostert *et al.* (2001) isolated *Phomopsis* mostly from

buds and nodes, indicating that these are likely to be important sites for survival. *Phomopsis* taxon 1 was also isolated from buds in the present study. Because there was no conclusive evidence that taxon 1 caused budburst failure or injury to shoots, colonisation of the bud by taxon 1 was assumed to be endophytic. In contrast, *Phomopsis* taxon 2 overwinters in grapevine buds (Pine, 1959; Hewitt and Pearson, 1990) and causes symptoms on newly developed shoots.

Although the incidence of taxon 2 is correlated with cool, wet spring weather (see section 1.4.3.3), it is unlikely that the pathogenicity of taxon 1 is dependent on environmental conditions. Infection, however, may be influenced by health of the plant. Studies of grass endophytes have shown that the degree of mutualism between the host and endophyte is dependent on the plant environment. When plants are grown under stressful conditions, such as low nutrient levels or low light intensity, growth of plants with endophytes is marginally delayed compared to uninfected plants (Helander *et al*., 1996). It is possible that those excised shoots that developed lesions in the present study were susceptible to infection due to removal from the host plant. It was proposed by Dorworth and Callan (1996) that endophytic fungi can become pathogenic when the host plant is weakened. These fungi may undergo active mycelial development in response to water stress in host organs (Boddy and Griffith, 1989). Endophytic fungi of aerial plant parts produce symptoms on the host after triggering by the appropriate ecological or physiological stimuli (Petrini, 1996).

It has been reported that non-pathogenic fungi can induce physiological reactions of the host plant such as browning, alteration of the cell wall and production of tyloses (Matta, 1971). Contact of such fungi with a plant, and also mechanical injury, can induce a metabolic change such as an increase of phenolics (Agrios, 1988; Matta, 1971). In this study, darkening of host cells and callose production in epidermal cell walls were observed in shoots inoculated with *Phomopsis* taxon 1. Thickening of cell walls may be a result of partial

digestion of the cell wall caused by enzymes produced by the fungus, or a host reaction to invasion (Whisson *et al*., 1992; Manners, 1982).

Some endophytic fungi influence the spread and establishment of pathogens in the host plant and thus can be possible biocontrol agents (Clay, 1988). A large number of genera of endophytic fungi were found to co-exist in bark with virulent strains of *Cryphonectria parasitica*, the casual agent of chestnut blight, in oak and endophytic fungi have been recovered from both healthy and cankered bark (Bills, 1996). Various endophytic fungi isolated from eggplant were found to suppress the pathogenic effect of *Verticillium dahliae* (Narisawa *et al*., 2002). The possible interaction of *Phomopsis* taxon 1 and taxon 2 was not assessed, although the fungi have been observed on the same vine. The potential use of *Phomopsis* sp. to combat *Phytophthora palmivora* in durian has been investigated in Queensland, Australia (Brown *et al*., 2001). *Phomopsis* sp. have been isolated from asymptomatic leaf tissue (K. Brown, personal communication) yet it may be that the fungi are latent until favourable environmental conditions promote development of symptoms in the host. In the present study, the detection of endophytic fungi has been based on diagnostic methods rather than on direct observation of the host-fungus interaction. Microscopic examination confirmed the location of *Phomopsis* taxon 1 in the host tissue but further studies are needed to assess if stress on the plant influences mycelial growth and the overall ability of the fungus to cause harm.

7.3 The application of molecular markers for the detection of *Phomopsis* taxon 1

The present study showed that traditional diagnostic techniques were not useful for the detection of *Phomopsis* in buds. Because the taxa of *Phomopsis* on grapevine cannot be distinguished by bleached cane, the existing taxon 1 and taxon 2-specific probes, pT1P180

and pT2P25, offered a reliable test of identification. It was envisaged that the development of a new taxon 1 genomic DNA library would provide suitable clones for rapid detection of *Phomopsis* taxon 1 in a slot blot assay, and allow the detection of genetic variability among isolates by RFLP analysis. However, the clones provided no obvious advantages for rapid detection over pT1P180. The large insert size of pT1P180 did not permit direct sequencing of the cloned insert, as efficiency of sequencing decreases with increasing length of the sequence (Slightom *et al.*, 1991). Further work is required to subclone the appropriate fragment within the clone and subsequently, to sequence the smaller fragments obtained.

Molecular markers have been used to detect *Phomopsis* spp. from different hosts and provide a better understanding of the genetic relatedness of these species. For example, Uddin and Stevenson (1998) revealed similarities in DNA fingerprinting and ITS sequences of isolates of *Phomopsis* spp. isolated from Asian pear and plum. They suggested that the isolates were closely related species and that the *Phomopsis* sp. that causes shoot blight of peach is not host specific. The taxon 1 and taxon 2-specific DNA probes, likewise, could be used in RFLP analysis to determine if *Phomopsis* taxa from grapevine colonise plants other than members of the Viticeae. In the present study, the concept of host specificity for *Phomopsis* taxon 1 on grapevine was not examined. Further work is required to determine if *Phomopsis* on grapevine can colonise a broad host range.

Molecular studies have been used to generate distinct groupings of *Phomopsis* spp. based on host association and geographic origin (Rehner and Uecker, 1994). Using ITS sequence analysis, isolates of *Phomopsis* spp. derived from diverse plant hosts and geographic origins were resolved into three distinct groups. These groups were distinguished as those from (i) shrubs and trees in North America, (ii) woody and herbaceous plants from tropical regions and (iii) herbaceous plants from temperate areas. The diversity of host taxa associated with each grouping supports the suggestion that some *Phomopsis* spp. are capable

of infecting more than one host. Therefore, it is possible that a number of species of *Phomopsis* can infect grapevine (Melanson *et al.*, 2002) and, in turn, taxon 1 and taxon 2 may infect other plant hosts in the vineyard.

The development of molecular markers and associated RFLP and slot blot analysis have shown that *Phomopsis* taxon 1 is clearly distinguishable from taxon 2 (Melanson *et al.*, 2002). There have been suggestions that taxon 1 and taxon 2 may be different species based on morphological and genetic differences (Mostert *et al.*, 2000; Melanson *et al.*, 2002). Although Mostert *et al.* (2001) and Phillips (1999) recently described *Phomopsis* on grapevine based on comparison of ITS sequences and morphological characteristics, the *Phomopsis* taxon-specific DNA probes could be used to obtain further information to distinguish the taxa. Mycelial compatibility studies (Scheper, 2001) suggested that taxon 1 and taxon 2 are genetically isolated, but investigation of the nature of sexual reproduction in taxon 1 was inconclusive. Phillips (1999) showed that *D. perjuncta*, the sexual stage of *Phomopsis* taxon 1, is self-fertile whereas no sexual stage has been identified for *Phomopsis* taxon 2. Further information is required to determine the genetic variability of a wider range of isolates from different geographical regions to confirm the differentiation into separate species.

Most reports of Phomopsis cane and leaf spot do not give taxonomic descriptions of the fungus isolated from infected grapevine, but it can be assumed that the symptoms described were due to *Phomopsis* taxon 2 (see section 1.4.2). It is possible, due to asymptomatic growth of taxon 1, that the fungus has not been identified in other parts of the world. Phomopsis cane and leaf spot is widespread in North America but there are no reports of the isolation of *Phomopsis* taxon 1 from infected tissue (W. Wilcox and M. Ellis, personal communication). The use of *Phomopsis* taxon 1 and taxon 2-specific DNA probes would thus be useful in the identification and distribution of the taxa in different viticultural regions.

Further studies using the taxon-specific DNA probes could provide information on the origin and evolution of *Phomopsis* populations on grapevine.

7.4 Management of *Phomopsis* on grapevine

Although this study confirmed that *Phomopsis* taxon 1 did not cause symptoms on green shoots and leaves, the symptom of bleaching still poses a problem in the identification of the two taxa on dormant canes. Bleaching can be caused by a number of agents, therefore cannot be used as a sole indicator of *Phomopsis* infection. In recommendations to viticulturists, monitoring is perhaps the most important tool in distinguishing between taxon 1 and taxon 2 in the vineyard. It is advisable that the vineyard is monitored regularly through the year. Bleached canes should be tagged and inspected for leaf spots or lesions after budburst. Symptoms of infection by *Phomopsis* taxon 2 can best be seen on newly-developed shoots in spring and summer as leaf spots and longitudinal lesions (see section 1.4.4.2). If these symptoms are not present on green shoots, then bleaching is likely to be associated with taxon 1 or other agents.

Also, bleached canes should be collected in winter and incubated in moist conditions (see section 2.1). If zone-lines and perithecia develop, it is likely that *Phomopsis* taxon 1 is present in the vineyard. Cane material should be sent to a diagnostic facility in winter for identification of *Phomopsis* by spore morphology.

Based on the findings of the present study, it is recommended that chemical control is not required for control of *Phomopsis* taxon 1 on grapevine. There is no evidence to warrant the use of chemicals in vines having endophytic fungi. This knowledge has important ramifications in the viticultural industry, whereby mancozeb and dithianon (e.g. Delan®) have been widely used for the control of Phomopsis infection, regardless of which taxon was

present in the vineyard (see section 1.6.2). Many of the chemicals are expensive but, as is the case for Delan®, are effective against other fungal pathogens. A range of fungicides commonly used for the treatment of Phomopsis cane and leaf spot have been evaluated in the laboratory (Castillo-Pando *et al.*, 1997) and in vineyards (Nair *et al.*, 1998). In these studies, it was difficult to evaluate the effectiveness against infection by *Phomopsis* taxon 1 because no symptoms were present. Furthermore, the vineyards known to have *Phomopsis* taxon 1 did not exhibit abnormal budburst and there were no significant differences in budburst between fungicide-treated and untreated vines (Nair *et al.*, 1998). It is recommended that Delan® is applied for the control of *Phomopsis* taxon 2 (see section 1.6.2). The reduction in the use of chemicals in vineyards with *Phomopsis* taxon 1 has economic and environmental advantages. The risk of the development of fungicide resistance by *Phomopsis* taxa to Delan® or mancozeb is low, but reduced chemical input minimises the amount of residues in crops and soil and the detrimental effects on natural biodiversity.

The study of the pathogenicity of *Phomopsis* taxon 1 was initiated by concerns expressed by growers that taxon 1 caused bud loss (Rawnsley and Wicks, 2000). Although every attempt was made to investigate the effect of the fungus on grapevine both in the glasshouse and field, the 3-year study may not have provided sufficient time to evaluate the influences of environmental stresses on the endophytic behaviour of the fungus. The virulence of a pathogen can be highly influenced by seasonal variability in climate, cultural practices and grapevine physiology (Agrios, 1988; Sinclair and Cerkauskas, 1996). However, if failure of buds to burst and bud death were associated with infection by *Phomopsis* taxon 1, this should have been evident by a greater number of dead buds. Budburst was not affected in the four vineyards assessed. The findings of experiments conducted in the glasshouse and in the field confirmed that *Phomopsis* taxon 1 was not associated with bud loss. Other factors

which may cause bud loss should be considered, such as pruning strategy, crop vigour, bud necrosis, application of fertilisers and irrigation (Winkler, 1972).

Questions have also been raised (Melanson *et al.*, 2002; Scheper, 2001) as to whether long-term asymptomatic growth of *Phomopsis* taxon 1 could result in a gradual decline of grapevine productivity. In general, growth of hyphae of endophytic fungi is synchronized with growth of aerial plant parts (Bacon and DeBattista, 1991). Following plant maturation, most or all hyphal extension and branching ceases. Hyphae remain viable for the entire life of the host, utilising nutrients absorbed from within the apoplast to remain metabolically active (Christensen, 2001). As *Phomopsis* taxon 1 did not colonise the vascular tissue, there was no evidence to suggest that infection restricts transportation of nutrients in the vine.

The present study showed that infection by taxon 2 caused characteristic symptoms of Phomopsis cane and leaf spot. Lesion development in some diseases can be attributed to toxins produced by the fungal pathogen. For example, *P. helianthi* produces a phytotoxin, termed phomozine, which causes physiological changes in infected sunflower plants and play a role in symptom development (Mazars *et al.*, 1991). Further studies are required to determine if lesions caused by *Phomopsis* taxon 2 on grapevine are a result of toxin production.

7.5 Revised terminology of *Phomopsis* taxon 1 and taxon 2

It is evident that there are differences between *Phomopsis* taxon 1 and taxon 2 (Phillips 1999, Mostert *et al.*, 2001; Scheper, 2001), however, the current terms cause confusion in the viticultural industry. Many growers and viticultural personnel use the name 'Phomopsis', with no reference to the fungi associated with the disease. This study has shown that *Phomopsis* taxon 1 is not pathogenic to grapevine and is not associated with most of the

symptoms associated with Phomopsis cane and leaf spot. *Phomopsis* taxon 1 has a teleomorph but a sexual stage is not known for taxon 2. Mostert *et al.* (2001) and Phillips (1999) support the name, *Diaporthe perjuncta,* for the teleomorph of *Phomopsis* taxon 1 (see section 1.4.1). The anamorph is unchanged until further evidence suggests otherwise.

From the findings of this study, and the existence of the teleomorph of taxon 1, it is recommended that the terms *Phomopsis* taxon 1 and taxon 2 are replaced with the common names Diaporthe and Phomopsis, respectively. Thus, *Phomopsis viticola* causes the disease known as Phomopsis cane and leaf spot, and *Diaporthe perjuncta* has no detrimental effect on grapevine. The widespread use of these terms and the knowledge that *Phomopsis* taxon 1 is innocuous to grapevine would enable growers to modify their management practices accordingly.

In conclusion, *Phomopsis* taxon 1 can be classified as an endophytic fungus on grapevine. This has implications for current control strategies, where chemicals are being applied regardless of the taxon present in the vineyard. It is recommended that chemical control is not required for control of *Phomopsis* taxon 1 on grapevine. The use of the term, *Diaporthe*, to describe taxon 1 infection would assist viticulturists and industry personnel to differentiate between the two taxa of *Phomopsis* in the application of appropriate control strategies. The use of molecular markers for the detection and identification of *Phomopsis* taxon 2 will improve our understanding of the epidemiology of Phomopsis cane and leaf spot in the future.

APPENDIX A

CTAB extraction buffer for green grapevine shoot material

CTAB	2% (w/v)
Tris-HCl	100 mM
EDTA	20 mM
NaCl	1.4 M

Sterilised above solution by autoclaving. On the day of use, the following were added:

2-mercaptoethanol	0.2% (v/v)
PVP-360	1%

5X Denhardts

Bovine Serum Albumin, Fraction V	2%
Ficoll, type 400	2%
Polyvinylpyrrolidone-360	2%
Fragmented herring sperm DNA	100 µg/ml
Sodium dodecyl sulphate (SDS)	0.1%

Prepared as a 5× solution and stored at -20 °C.

Hybridisation solution

SSC	4 X
Denhardt's	2 X
Fragmented herring sperm DNA	100 µg/ml
Sodium dodecyl sulphate	0.1%

LB (1 litre)

Bacto®- Tryptone	10 g
Bacto®- Yeast extract	5 g
NaCl	5 g

Adjusted to pH 7.0 with NaOH.

LB agar	*Add to above solution:*	
	Bacto®- Agar	15 g
LB Amp	*To LB agar:*	
	Ampicillin (50 mg/ml)	20 µl/10 ml
LB Amp for colour selection	*To LB agar:*	
	Ampicillin (50 mg/ml)	20 µl/10 ml
	X-Gal	2%
	IPTG	0.1 M

M13 Forward and M13 Reverse primers (Universal) (Messing, 1983)

M13 F = 5'-GTA-AAA-CGA-CGG-CCA-G-3'

M13 R = 5'-CAG-GAA-ACA-GCT-ATG-AC-3'

Mycelial extraction buffer

NaCl	500 mM
Sodium acetate	150 mM
Sarkosyl	2.5%
EDTA	20 mM

pH adjusted to 5.4.

Prehybridisation solution

SSC	4 X
Denhardt's	5 X
Fragmented herring sperm DNA	100 ug/ml
Sodium dodecyl sulphate	0.1%

***PVU*II specific primers**

S1 = 5'-ACAGCTATGACCATG-3'
S2 = 5'-TCCCAGTCACGACGT-3'

20 X SSC (1 litre)

NaCl	88 g
Sodium acetate	175 g

SEAPS extraction buffer for grapevine bud and lignified cane material
(Melanson *et al*., 2002)

NaCl	1.5 M
Sodium acetate	150 mM
Sarkosyl	2.5%
EDTA	50 mM
Polyvinylpyrrolidone-10 (PVP-10)	2.5%
Ethanol	20%

pH adjusted to 5.4.

SOC medium (1 litre)

Bacto®- Tryptone	20 g
Bacto®- Yeast extract	5 g
NaCl	0.6 g
KCl	0.19 g
$MgSO_4.7H_20$	1 M
$MgCl_2$	1 M
50% glucose	7 µl

TEN buffer

Tris-HCl	10 mM
EDTA	1 mM
NaCl	100 mM

Tris-Acetate-EDTA (TAE) buffer

Tris-HCl	40 mM
Sodium acetate	20 mM
EDTA	1 mM

Prepared as a 50 × solution. pH adjusted to 8.0.

Tris-EDTA (TE) buffer

Tris-HCl	10 mM
EDTA	1 mM

pH adjusted to 8.0.

Tritration Buffer (TB)

Piperazine-N, N'-bis [2-ethane-sulfonnic acid] (PIPES)	10 mM
$CaCl_2$	15 mM
KCl	250 mM
$MnCl_2$	55 mM

All components combined except $MnCl_2$., pH adjusted to 6.7 with KOH. $MnCl_2$, dissolved then added to solution. Solution filter sterilised through a 0.45 mm filter unit. Stored at 4 °C.

Appendix B

Plan of experimental vineyard at Ashton Hills winery. The position of individual vines is denoted by a grid section (green). Spurs were sampled on vines designated 1=one spur, 2=two spurs.

1999

Ashton Hills Vineyard (cv. Chardonnay)

Block	Vine	Row 56	Row 57	Row 58	Row 59	Row 60
Block 1	vine 7	1	2			
	vine 8					1
	vine 9		1	1	1	
	vine 10	1	1	1		
	vine 11	1			1	
	vine 12	1		1		2
Block 2	vine 13	1	1		1	
	vine 14					
	vine 15		1		1	
	vine 16	1				1
	vine 17	1	1	1	2	
	vine 18		1			
Block 3	vine 19			1	1	
	vine 20	2				2
	vine 21			1		2
	vine 22		1		1	
	vine 23	1		1		
	vine 24		2			1
Block 4	vine 25	1	1			
	vine 26	1		2	1	
	vine 27			1	1	2
	vine 28		1		1	
	vine 29	1				
	vine 30		2	1		1
Block 5	vine 31	1		2	2	1
	vine 32		1	2	1	1
	vine 33		1			1
	vine 34	2				1
	vine 35					1
	vine 36	1	1		1	
Block 6	vine 37	1		1	2	
	vine 38			1		1
	vine 39	1	1	1		
	vine 40	1		1	1	
	vine 41		1	1		1
	vine 42				1	1
Block 7	vine 43					
	vine 44				1	
	vine 45					
	vine 46					
	vine 47					
	vine 48					

2000

Ashton Hills Vineyard (cv. Chardonnay)

Block	Vine	Row 52	Row 51	Row 50	Row 49	Row 48
Block 1	vine 7					
	vine 8	1				
	vine 9					
	vine 10	1				
	vine 11			1		
	vine 12		1	1		1
Block 2	vine 13			1		
	vine 14	1				1
	vine 15	1			1	1
	vine 16					
	vine 17		1	1		1
	vine 18	1		1	1	
Block 3	vine 19		1			1
	vine 20		1			
	vine 21			1		
	vine 22	1				1
	vine 23	1	1		1	
	vine 24		1	1	1	1
Block 4	vine 25				1	
	vine 26	1		1		
	vine 27		1	1		1
	vine 28	1	1	1		1
	vine 29	1	1		1	1
	vine 30	1				
Block 5	vine 31					
	vine 32					
	vine 33		1	1		1
	vine 34			1		
	vine 35	1			1	
	vine 36	1			1	1
Block 6	vine 37	1	1	1		
	vine 38				1	
	vine 39		1	1		
	vine 40	1		1	1	
	vine 41		1			1
	vine 42					1
Block 7	vine 43	1		1	1	1
	vine 44	1			1	
	vine 45				1	1
	vine 46	1	1			
	vine 47		1	1		
	vine 48				1	
Block 8	vine 49	1				
	vine 50		1	1	1	1
	vine 51		1			1
	vine 52		1			
	vine 53				1	1
	vine 54		1	1	1	
Block 8	vine 55					1
	vine 56		1			
	vine 57			1		
	vine 58				1	
	vine 59				1	
	vine 60	1			1	

2001

Ashton Hills Vineyard (cv. Chardonnay)

Block	Vine	Row 52	Row 51	Row 50	Row 49	Row 48
Block 1	vine 7					
	vine 8		1			
	vine 9		1			
	vine 10	1				
	vine 11					
	vine 12			1		1
Block 2	vine 13		1			
	vine 14		2			1
	vine 15	1		1	1	
	vine 16			1		1
	vine 17		1	1		1
	vine 18			1	1	
Block 3	vine 19	1				1
	vine 20	1				
	vine 21		1	1		
	vine 22		1		1	
	vine 23					1
	vine 24				1	
Block 4	vine 25	1		1		1
	vine 26	1		1	1	
	vine 27		1	1		1
	vine 28	1		1		1
	vine 29	1			1	1
	vine 30					
Block 5	vine 31					
	vine 32	1				
	vine 33	1		1		1
	vine 34			1		
	vine 35		1			
	vine 36	1			2	1
Block 6	vine 37	1		1		
	vine 38				1	
	vine 39		1	1		
	vine 40		1	1	1	
	vine 41			1		1
	vine 42					
Block 7	vine 43				1	2
	vine 44				1	
	vine 45	1	1		1	1
	vine 46					
	vine 47	1	1			
	vine 48			1		
Block 8	vine 49				1	
	vine 50	1	1	1	1	1
	vine 51					
	vine 52		1			1
	vine 53	1				1
	vine 54		2	1	1	
Block 8	vine 55				1	1
	vine 56	1				
	vine 57		1	1		
	vine 58	1			1	
	vine 59				1	
	vine 60	1	1		1	
Block 8	vine 61					
	vine 62					
	vine 63	1				
	vine 64					
	vine 65					
	vine 66					

Plan of experimental vineyard at Hargrave. The position of individual vines is denoted by a grid section (green). Spurs were sampled on vines designated 1=one spur, 2=two spurs.

1999
Hargrave Vineyard (cv. Chardonnay)

Block	Vine	Row 13	Row 12	Row 11	Row 10	Row 9
Block 1	vine 6		1			
	vine 7				1	
	vine 8			1		
	vine 9		1		1	
	vine 10					
Block 2	vine 11		1		1	
	vine 12	1		2		1
	vine 13				1	1
	vine 14		2	1		
	vine 15	2				
Block 3	vine 16					
	vine 17					
	vine 18	1	1			2
	vine 19		1		1	
	vine 20	1			1	1
Block 4	vine 21		1	1		
	vine 22				1	1
	vine 23	1	1	1		
	vine 24	1	1		1	
	vine 25					
Block 5	vine 26			2		1
	vine 27				1	
	vine 28	1	1			
	vine 29			1		
	vine 30				1	
Block 6	vine 31	1	2	1	1	2
	vine 32				1	1
	vine 33	1		1	1	
	vine 34	1			1	1
	vine 35		1			1
Block 7	vine 36			1		1
	vine 37			1		
	vine 38	1	1		1	
	vine 39				1	1
	vine 40			1		
Block 8	vine 41	1	1			
	vine 42	1	1	1		1
	vine 43					
	vine 44				1	
	vine 45			1	1	
Block 9	vine 46			1		1
	vine 47		1			
	vine 48			2	1	
	vine 49		1	1		
	vine 50	1				1
Block 10	vine 51					1
	vine 52	1			1	
	vine 53		1			
	vine 54					
	vine 55					1
Block 11	vine 56					
	vine 57	1				1
	vine 58	1				
	vine 59					
	vine 60					
Block 12	vine 61					
	vine 62					
	vine 63	1				
	vine 64					
	vine 65					
Block 13	vine 66	1				
	vine 67					
	vine 68					
	vine 69					

2000
Hargrave Vineyard (cv. Chardonnay)

Block	Vine	Row 11	Row 10	Row 9	Row 8	Row 7
Block 1	vine 6					
	vine 7					
	vine 8		1			1
	vine 9		1			
	vine 10	1	1			
Block 2	vine 11					1
	vine 12		1	1	1	
	vine 13	1	1		1	
	vine 14					
	vine 15	1				1
Block 3	vine 16		1		1	1
	vine 17	1		1		
	vine 18	1		1		
	vine 19	1	1	1		
	vine 20				1	1
Block 4	vine 21					1
	vine 22	1	1	1		1
	vine 23				1	
	vine 24					
	vine 25	1				
Block 5	vine 26			1		
	vine 27			1	1	
	vine 28	1				1
	vine 29				1	
	vine 30		1	1		1
Block 6	vine 31					
	vine 32		1			1
	vine 33			1	1	
	vine 34	1	1			1
	vine 35			1		1
Block 7	vine 36	1				
	vine 37	1	1		1	1
	vine 38	1			1	
	vine 39				1	
	vine 40		1	1		
Block 8	vine 41	1				1
	vine 42			1	1	
	vine 43		1			
	vine 44	1			1	
	vine 45					
Block 9	vine 46		1	1		
	vine 47	1				
	vine 48			1	1	1
	vine 49	1	1	1		
	vine 50			1		
Block 10	vine 51	1	1			1
	vine 52					1
	vine 53				1	1
	vine 54	1				
	vine 55	1			1	
Block 11	vine 56		1	1		
	vine 57		1			
	vine 58				1	
	vine 59				1	1
	vine 60					
Block 12	vine 61		1			
	vine 62			1		
	vine 63			1		
	vine 64					
	vine 65			1	1	1
Block 13	vine 66				1	
	vine 67					
	vine 68					
	vine 69					

2001
Hargrave Vineyard (cv. Chardonnay)

Block	Vine	Row9	Row 8	Row 7	Row 6	Row 5
Block 1	vine 6	1		1		
	vine 7	1				
	vine 8			1	1	
	vine 9	1				
	vine 10					1
Block 2	vine 11			1		
	vine 12					
	vine 13	1				1
	vine 14			1	1	
	vine 15	1	1	1		
Block 3	vine 16		1			
	vine 17					
	vine 18		1	1		
	vine 19				1	1
	vine 20	1				1
Block 4	vine 21		1			
	vine 22		1	1	2	
	vine 23			1		
	vine 24	1	1		1	1
	vine 25		1	2		1
Block 5	vine 26		1			
	vine 27	1			1	
	vine 28	1		1		
	vine 29		1		1	2
	vine 30					
Block 6	vine 31	1			1	
	vine 32	1	1	1		
	vine 33		2		1	
	vine 34			1		1
	vine 35					1
Block 7	vine 36			1	1	1
	vine 37		1			
	vine 38					
	vine 39	1	1		1	1
	vine 40			1	1	
Block 8	vine 41					1
	vine 42	1	1	1	1	
	vine 43					
	vine 44		1			1
	vine 45			1	1	1
Block 9	vine 46					1
	vine 47		1			
	vine 48		1			1
	vine 49				1	
	vine 50	1		1	1	
Block 10	vine 51	1				
	vine 52					1
	vine 53					1
	vine 54		1			
	vine 55	1		1	1	
Block 11	vine 56	1				1
	vine 57			1	1	
	vine 58	1			1	
	vine 59					
	vine 60	1				
Block 12	vine 61	1				
	vine 62					
	vine 63		1			
	vine 64					
	vine 65					
Block 13	vine 66					
	vine 67					
	vine 68					
	vine 69					

Plan of experimental vineyard at Lenswood Horticultural Centre. The position of individual vines is denoted by a grid section (green). Spurs were sampled on vines designated 1=one spur, 2=two spurs.

1999

Lenswood Horticultural Centre (cv. Chardonnay) Block D

Block	Vine	Row 9	Row 10	Row 11	Row 12	Row 13
Block 1	vine 5					
	vine 6					
	vine 7					
	vine 8					
Block 2	vine 9		2	1		
	vine 10	1	1	1		1
	vine 11	1		1	2	1
	vine 12	1		1	2	
Block 3	vine 13	2		1	1	
	vine 14	1	1		1	1
	vine 15	1	1	1	1	1
	vine 16	1	2			1
Block 4	vine 17	1	1		1	2
	vine 18		1	2	1	1
	vine 19	1	2	1	1	
	vine 20			1	2	
Block 5	vine 21			1		
	vine 22	2	1	1	2	2
	vine 23			1		2
	vine 24	1	1	1		2
Block 6	vine 25	3	2		1	2
	vine 26		1		1	
	vine 27	1	1	2	1	
	vine 28				1	1
Block 7	vine 29			1	1	1
	vine 30	2			1	1
	vine 31		2	2		1
	vine 32	1	1	1		

2000

Lenswood Horticultural Centre (cv. Chardonnay) Block D

Block	Vine	Row 9	Row 10	Row 11	Row 12	Row 13
Block 1	vine 5				1	1
	vine 6	1			1	
	vine 7			1		1
	vine 8	1	1	1	1	1
Block 2	vine 9		1		1	
	vine 10	1	1	1	1	1
	vine 11	1		1		
	vine 12	1		1	1	1
Block 3	vine 13	1	1	1		
	vine 14		1	1	1	1
	vine 15		1	1		
	vine 16	1	1		1	1
Block 4	vine 17		1		1	
	vine 18	1	1	1	1	1
	vine 19	1	1	1	1	1
	vine 20	1	1	1	1	1
Block 5	vine 21			1	1	1
	vine 22	1	1			1
	vine 23				1	1
	vine 24	1	1		1	
Block 6	vine 25	1	1	1	1	1
	vine 26	1	1		1	1
	vine 27			1		1
	vine 28	1		1		
Block 7	vine 29	1	1	1	1	
	vine 30		1	1		1
	vine 31		1		1	1
	vine 32	1	1	1		1
Block 7	vine 33	1				
	vine 34		1	1		
	vine 35	1		1		1
	vine 36	1			1	

2001

Lenswood Horticultural Centre (cv. Chardonnay) Block D

Block	Vine	Row 8	Row 9	Row 11	Row 12	Row 13
Block 1	vine 5					
	vine 6		1	1		1
	vine 7		1	1	1	1
	vine 8		1			
Block 2	vine 9	1			1	1
	vine 10		1	1	1	1
	vine 11	1	1	1		1
	vine 12	1		1	1	1
Block 3	vine 13	1	1	1		1
	vine 14	1	1	1	2	
	vine 15		1			1
	vine 16	1	1	1	2	
Block 4	vine 17			1	2	1
	vine 18		2	1	1	
	vine 19	1	1	2	1	
	vine 20	1	1	1	1	1
Block 5	vine 21				1	
	vine 22	1	1	1	1	1
	vine 23	1	1	1		1
	vine 24			1		
Block 6	vine 25	1	1	1	1	
	vine 26	1		1		1
	vine 27	1	1		1	1
	vine 28	1	1	1	1	2
Block 7	vine 29	1	1			1
	vine 30	1				1
	vine 31	1		1		
	vine 32	1	1		1	1
Block 7	vine 33					
	vine 34					1
	vine 35	1				
	vine 36	1			1	

Plan of experimental vineyard at Mt Jagged. The position of individual vines is denoted by a grid section (green). Spurs were sampled on vines designated 1=one spur, 2=two spurs.

1999

Mt Jagged Vineyard (cv. Shiraz)

		Row 8	Row 7	Row 6	Row 5	Row 4
Block 1	vine 5	2				1
	vine 6			1		1
	vine 7			1	2	
	vine 8	2	1			1
Block 2	vine 9			2	1	1
	vine 10				1	2
	vine 11	1	1	1	1	
	vine 12					
Block 3	vine 13	1	1	2		2
	vine 14	1	1		1	
	vine 15	1			1	1
	vine 16		2			1
Block 4	vine 17		1	1	1	
	vine 18	1	1	1		
	vine 19			1	2	1
	vine 20	1		1	1	1
Block 5	vine 21		1	1		1
	vine 22		2	1	2	1
	vine 23	1		1		
	vine 24		1	1	1	
Block 6	vine 25	1	1	2		2
	vine 26	2	1		1	
	vine 27		1			2
	vine 28			1	1	
Block 7	vine 29	1		2		1
	vine 30		2			
	vine 31				1	1
	vine 32	1	1		1	
Block 8	vine 33	1	2		1	
	vine 34	2			1	
	vine 35	1				
	vine 36					

2000

Mt Jagged Vineyard (cv. Shiraz)

		Row 8	Row 7	Row 6	Row 5	Row 4
Block 1	vine 5					
	vine 6					
	vine 7					
	vine 8					
Block 2	vine 9					
	vine 10					
	vine 11	1		1		
	vine 12			1		1
Block 3	vine 13	1		1		1
	vine 14		1			1
	vine 15	1			1	
	vine 16	1		1		
Block 4	vine 17		1			1
	vine 18	1				1
	vine 19		1			
	vine 20	1			1	
Block 5	vine 21					
	vine 22		1	1	1	1
	vine 23	1		1	1	1
	vine 24	1	1	1		1
Block 6	vine 25	1	1	1	1	
	vine 26					1
	vine 27			1	1	
	vine 28		1			
Block 7	vine 29	1				
	vine 30	1			1	1
	vine 31		1	1	1	
	vine 32		1			
Block 8	vine 33		1		1	
	vine 34	1	1		1	1
	vine 35		1	1	1	1
	vine 36	1		1		1
Block 8	vine 37	1			1	1
	vine 38		1	1	1	
	vine 39	1	1	1		
	vine 40	1		1		1
Block 8	vine 41		1		1	1
	vine 42		1		1	
	vine 43			1		1
	vine 44	1	1		1	
Block 8	vine 45	1		1		1
	vine 46	1				
	vine 47		1			
	vine 48			1	1	1
Block 8	vine 49	1	1	1	1	
	vine 50		1			1
	vine 51			1	1	
	vine 52				1	

2001

Mt Jagged Vineyard (cv. Shiraz)

		Row 9	Row 8	Row 7	Row 6	Row 5
Block 1	vine 5					
	vine 6					
	vine 7	1				
	vine 8		1		1	
Block 2	vine 9			1		1
	vine 10					1
	vine 11	1	1		1	
	vine 12		1	1		1
Block 3	vine 13		1			
	vine 14	1			1	
	vine 15		1			1
	vine 16				1	
Block 4	vine 17		1	1		1
	vine 18	1			1	
	vine 19		1			
	vine 20	1	1	1	1	
Block 5	vine 21		1	1		1
	vine 22			1	1	
	vine 23					
	vine 24	1	1		1	1
Block 6	vine 25		1	1		1
	vine 26	1		1	1	
	vine 27	1	1	1		1
	vine 28		1		1	1
Block 7	vine 29					1
	vine 30	1	1	1		1
	vine 31					
	vine 32		1	1		1
Block 8	vine 33	1	1	1		1
	vine 34	1				
	vine 35	1	1		1	1
	vine 36	1		1	1	1
Block 8	vine 37				1	1
	vine 38					
	vine 39	1		1		
	vine 40		1	1	1	1
Block 8	vine 41	1			1	
	vine 42		1			1
	vine 43	1				
	vine 44			1	1	
Block 8	vine 45		1			
	vine 46	1		1	1	1
	vine 47					1
	vine 48	1		1	1	
Block 8	vine 49					
	vine 50	1				
	vine 51			1	1	
	vine 52	1		1		1

Appendix C

Chi-square analyses

Chi-square analysis of total number of buds burst (productive buds) and number of unburst buds (failed buds) at four vineyard sites in 1999 (see section 4.3.3.1).

```
CHI-SQUARE TEST FOR HETEROGENEITY OR INDEPENDENCE

Where        FAILBUD = Total number of buds that failed &
             PRBUD = Total number of buds burst

                         VARIABLE
CASE*               FAILBUD      PRBUD
                 +-----------+-----------+
Ash     OBSERVED |   46      |  257      |    303
        EXPECTED |   35.43   |  267.57   |
     CELL CHI-SQ |    3.16   |    0.42   |
                 +-----------+-----------+
Har     OBSERVED |   27      |  221      |    248
        EXPECTED |   29.00   |  219.00   |
     CELL CHI-SQ |    0.14   |    0.02   |
                 +-----------+-----------+
Lens    OBSERVED |   20      |  238      |    258
        EXPECTED |   30.17   |  227.83   |
     CELL CHI-SQ |    3.43   |    0.45   |
                 +-----------+-----------+
Jag     OBSERVED |   30      |  213      |    243
        EXPECTED |   28.41   |  214.59   |
     CELL CHI-SQ |    0.09   |    0.01   |
                 +-----------+-----------+
                    123         929         1052

OVERALL CHI-SQUARE      7.71
P-VALUE               0.0524
DEGREES OF FREEDOM         3

CASES INCLUDED 8    MISSING CASES 0
```

* Each case represents the sites Ash = Ashton Hills, Har = Hargrave, Lens = Lenswood, Jag = Mt Jagged

Chi-square analysis of total number of buds burst (productive buds) and number of unburst buds (failed buds) at four vineyard sites in 2000 (see section 4.3.3.2).

```
STATISTIX FOR WINDOWS

CHI-SQUARE TEST FOR HETEROGENEITY OR INDEPENDENCE

Where FAILBUD = Total number of buds that failed to burst
      PROBUDS = Total number of productive buds that burst


                              VARIABLE
CASE                   FAILBUD     PRODBUDS
                     +-----------+-----------+
Ash     OBSERVED |      8     |    229    |      237
        EXPECTED |    12.19   |   224.81  |
     CELL CHI-SQ |     1.44   |     0.08  |
                 +-----------+-----------+
Har     OBSERVED |      7     |    199    |      206
        EXPECTED |    10.59   |   195.41  |
     CELL CHI-SQ |     1.22   |     0.07  |
                 +-----------+-----------+
Lens    OBSERVED |      6     |    192    |      198
        EXPECTED |    10.18   |   187.82  |
     CELL CHI-SQ |     1.72   |     0.09  |
                 +-----------+-----------+
Jag     OBSERVED |     24     |    210    |      234
        EXPECTED |    12.03   |   221.97  |
     CELL CHI-SQ |    11.90   |     0.65  |
                 +-----------+-----------+
                       45          830           875

OVERALL CHI-SQUARE      17.16
P-VALUE                0.0007
DEGREES OF FREEDOM          3

CASES INCLUDED 8    MISSING CASES 0
```

Each case represents the sites Ash = Ashton Hills, Har = Hargrave, Lens = Lenswood, Jag = Mt Jagged

Chi-square analysis of total number of buds burst (productive buds) and number of unburst buds (failed buds) at four vineyard sites in 2001 (see section 4.3.3.3).

STATISTIX FOR WINDOWS

CHI-SQUARE TEST FOR HETEROGENEITY OR INDEPENDENCE

Where FAILBUD = Total number of buds that failed to burst
PROBUDS = Total number of productive buds that burst

CASE*		FAILBUD	PRODBUDS	
Ash	OBSERVED	13	218	231
	EXPECTED	18.89	212.11	
	CELL CHI-SQ	1.83	0.16	
Har	OBSERVED	20	180	200
	EXPECTED	16.35	183.65	
	CELL CHI-SQ	0.81	0.07	
Lens	OBSERVED	9	209	218
	EXPECTED	17.82	200.18	
	CELL CHI-SQ	4.37	0.39	
Jag	OBSERVED	27	168	195
	EXPECTED	15.94	179.06	
	CELL CHI-SQ	7.67	0.68	
		69	775	844

(VARIABLE: FAILBUD, PRODBUDS)

OVERALL CHI-SQUARE 15.99
P-VALUE 0.0011
DEGREES OF FREEDOM 3

CASES INCLUDED 8 MISSING CASES 0

* Each case represents the sites Ash = Ashton Hills, Har = Hargrave, Lens = Lenswood, Jag = Mt Jagged

Appendix D

Sequence data of *Phomopsis* taxon 2-specific probe, pT2P25 (Melanson *et al*., 2002).

```
  1   CTGCAGCAGGGAGGAAACTAACTGAGTGGCAGCCAGTATAGGATCCAACATGAATGTTTG
 61   CTTTGCTTTGCAGCCTCCGCCTGACCGAGGGATATGAAGTGGCAGGCGAAGATTAAGCT
121   GGCCTGTGTCTTATCTACCTACCTAGGTACCTAGGTATACGGCTTGACCACCTTATGCCC
181   CAACAAAACGTCATCAGATGGCATCACGTAGCCACAAAGACTACAAGCACGAGGCACCCC
241   CAGCCACGCAGTGCGTTTACCTGTACTGCTGCTGCTAGGCACCACTAAGTACTACTTACG
301   GCCCAACCTGGTACTCTTTGTGTTCCTCTAAAATTAAGAAGAGGTATTATTAGTATACTA
361   ACTAAAAATGTTACAGTAAGTCCTACTGTGGTGCCGTACTGAAAAAAGGGCACCCCTATC
421   AGGTATCTTTCGGCACgGGTGCTGGTTGGTCAGGACTTGACGAGGGCGCCCTTTTTTACC
481   CATGCCACCATAGTAAGTCTTGCAGGTGGGTGTGAGATTTTAGGTCCTGCTCAG
```

base	A	C	G	T	*G+C*
count	136	133	131	134	*264*
%	25.46	24.9	24.53	25.09	*49.43*

Length: 534 nucleotides

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