CHARACTERIZATION OF *UNCINULA NECATOR*, THE GRAPEVINE POWDERY MILDEW FUNGUS

KATHERINE J. EVANS
B.Agr.Sc. (Hons), The University of Melbourne,
MS, Purdue University

Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide

Department of Crop Protection
Faculty of Agricultural and Natural Resource Sciences

May, 1996.

I dedicate this thesis to the memory of my grandmother,

Gwendoline McCulloch

1904-1996.

TABLE OF CONTENTS

ABSTRACT

Powdery mildew of grapevine, caused by *Uncinula necator*, is a chronic yield and quality loss problem in all viticultural regions of the world. As little is known about the population structure of *U. necator,* molecular and phenotypic markers were developed for studies of genetic diversity in this economically important plant pathogen.

Methods were developed for (a) the clonal isolation of *U. necator*, (b) the maintenance of *U. necator* on micropropagated *Vitis vinifera* cv. Cabernet Sauvignon *in vitro*, (c) the mass production and collection of conidia and (d) extraction of DNA from conidia and infected grapevine tissue. Clonal lines of *U. necator* were established using single conidial chains from bulk isolates originating from diseased *V. vinifera* and *V. amurensis* leaves and berries from South Australia, Western Australia and Tasmania.

An *in vitro* mating system for *U. necator* was established for identifying mating type and for genetic studies. Cleistothecia, containing viable ascospores, formed on micropropagated grapevines *in vitro* following co-inoculation of two *U. necator* clonal lines of opposite mating type. Specific incubation conditions were required, however, for the initiation of cleistothecia. These findings are discussed in relation to potential mechanisms regulating sexual reproduction. Among 21 clonal lines from South Australia tested, ten lines were identified as the "plus" mating type and seven lines were identified as the "minus" mating type. The mating type of the remaining four clonal lines has not been confirmed. Six of the 17 lines were paired in all possible combinations, and cleistothecia formed in all pairings where they were expected to occur for a heterothallic ascomycete with two mating types.

Total *U. necator* DNA was cloned into the *Eco*RI or *Pst*I sites of the plasmid vector, pUC19. Cloned sequences were screened by using them as DNA probes for identifying restriction fragment length polymorphisms (RFLPs) among total DNAs from clonal lines of *U. necator*. Selected *U. necator* clones were evaluated for somatic stability and species-specificity. Three multiple-copy probes, pUnP14, pUnP27 and pUnE4, produced banding patterns that were stable for DNA extracted from different asexual generations of *U. necator* clonal lines. These probes did not hybridise to grapevine DNA and thus could be applied directly to DNA extracted from infected tissue. For medium to high copy probes, between 1

and 10 µg of DNA extracted from infected detached leaves or micropropagated leaflets was required to produce hybridisation signal strengths equivalent to that obtained using 100 ng of purified *U. necator* DNA. Clones pUnE4 and pUnP14 appeared to be specific to *U. necator* because they did not hybridise to DNAs from a range of higher fungi associated with the mycobiota of grapevine leaves and berries. A single, high-copy *Eco*RI fragment was detected by probe pUnE4 in all *U. necator* clonal lines tested.

Genetic diversity in the Australian *U. necator* population was investigated in a preliminary study of 35 *U. necator* clonal lines. Using the DNA fingerprinting probes, pUnP14 and pUnP27, 15 different genotypes were identified among 29 *U. necator* clonal lines. Genotypes which were detected multiple times originated from the same sampling site and/or from more than one viticultural region. Additional DNA markers may distinguish these genotypes further. Variation was also detected in micro-geographical samples, for example, both mating types and different genotypes of *U. necator* were detected on a single plant of *V. amurensis*.

RFLP analysis of the fingerprinting patterns produced by pUnP14 was used to group clonal lines sharing ≥ 80% restriction fragments. This procedure identified two distinct genetic groupings, lineage A and B, the existence of which was supported by RFLP analysis of the banding patterns obtained using probes pUnP27, pUnE21, and by R1, a cereal intron-exon splice junction primer which amplified multiple PCR products from total *U. necator* DNA. Of the 35 clonal lines, 11 were assigned to lineage A and 24 were assigned to lineage B. More variation occurred within lineage B when compared with lineage A. Additional DNA markers may re-define these genetic groupings and the variation within them.

This study, therefore, provides the methodology and preliminary information with which to investigate population biology of *U. necator*, including the relative importance of the sexual stage in Australia and other regions, and the impact of disease management strategies, for example fungicide applications, on population dynamics.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, 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 copy of my thesis, when deposited in the University Library, being available for loan or photocopying.

SIGNED: **DATE:** April 30, 1996.

ACKNOWLEDGMENTS

My sincere gratitude is extended to Dr Eileen Scott, Dr Dara Whisson and Professor Bob Symons for their advice, enlightening discussions, editing skills and general support throughout this project.

In addition, I thank

Dr Jim Hardie and the Cooperative Research Centre for Viticulture for supporting this work,

Dr Belinda Stummer for technical advice and instruction,

Dr Bob Emmett, Dr David Gadoury, Mr Peter Magarey and Dr Trevor Wicks for their advice and constructive criticism,

Ms Barbara Hall, Mr Peter Wood, Mr Ben Wagner and Mr Tony Somers for supplying diseased grapevine material,

Dr Martin Barlass and Dr Richard Cirami for supplying the first batch of grapevine cultures and cuttings,

Mr Reza Balali, Dr Michael Gillings and Dr Peter Langridge for supplying DNA from various fungi and PCR primers,

Dr Hugh Wallwork for supplying the basic design for the cyclone separator device,

Ms Jennie Groom and Ms Emily Shepherd for photographic assistance,

Ms Lynne Giles for statistical advice,

Ms Janine Lloyd, Mr Terry Feckner, Mr Gary Taylor, Ms Anke Johnsen, fellow graduate students and members of the Department of Crop Protection for their friendship, help and support,

Dr Rick Latin, Mr Ron Mayer, Mr John Thomson, Mr Doug Wilson and Mr Arthur Williamson for setting me on the path to higher learning,

and

my family for their encouragement, especially my parents, Jeanne and David Karmouche, and my dear, tolerant husband, Evan.

PUBLICATIONS AND CONFERENCE PROCEEDINGS

The following publications and conference proceedings were produced during the Ph.D candidature:

Evans, K. J., Whisson, D. L. & Scott, E. S. (1996). An experimental system for characterising isolates of *Uncinula necator*. *Mycological Research*, ~~in press~~. 100, 675-680.

Evans, K. J., Whisson, D. L. & Scott, E. S. (1995). Characterisation of *Uncinula necator*, the grapevine powdery mildew fungus. In *Scientific Programme and Abstracts, Tenth Biennial Conference, Australasian Plant Pathology Society, Lincoln University, New Zealand, 28-30 August, 1995*. p 53.

Evans, K. J., Scott, E. S., Whisson, D. L. & Stummer, B. E. (1996). Characterisation of *Uncinula necator*, the grapevine powdery mildew fungus. In *Proceedings of the Ninth Australian Wine Industry Technical Conference, Adelaide, South Australia, 16-19 July, 1995* (ed. C. S. Stockley, A. N. Sas, R. S. Johnstone & T. H. Lee), ~~in press~~. Winetitles: Adelaide, South Australia. p 190.

ABBREVIATIONS

ATP	adenosine triphosphate
cv.	cultivar
cvs	cultivars
diam.	diameter
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
g	acceleration due to gravity
h	hour
IPTG	isopropyl-β-D-thiogalactopyranosid
kbp	kilobase pairs
LB	Luria-Bertani
M	molarity
Mb	megabases
min	minute
N	normality
OD	optical density
s	second
SDS	sodium dodecyl sulphate
SSC	See Sambrook *et al.* (1989)
STE	See Sambrook *et al.* (1989)
uv	ultra violet
V	Volts
X-gal	5-bromo-4-chloro-2-indolyl-β-D-galactopyranoside

1.0 INTRODUCTION

Uncinula necator, the causal agent of powdery mildew of grapevine, is the most economically important fungal pathogen of cultivated grapevines throughout the world. Increased emphasis on the production of disease-free berries with minimal pesticide input provides a sound reason for exploring more efficient disease management strategies through a better understanding of *U. necator* epidemiology and population biology. Little is known about the population structure of *U. necator,* mainly because this ascomycete has been difficult to study genetically. The obligately biotrophic nature and lack of morphological, physiological, biochemical and genetic markers account for the limited knowledge of *U. necator* population genetics. The genetic interaction of *U. necator* with economically important *Vitis* spp. has not yet been characterized. However, the *Erysiphe (Blumeria) graminis-Hordeum vulgare* pathosystem has well defined virulence and resistance genes plus a range of molecular markers which have been used to track the movement of genotypes in Europe for many years (Wolfe & McDermott, 1994). *U. necator*, in common with *E. graminis*, is a member of the Erysphales and reproduces both asexually and sexually. Despite the differences in host biology, the similarities in these fungi make *E. graminis-H. vulgare* a useful model for studying genetic interactions and population biology of *U. necator.*

The reproductive strategy of *U. necator* enhances the likelihood of the appearance of new genotypes and their potential rapid proliferation. Bipolar heterothallism (two mating types) was characterized relatively recently among isolates from New York State (Gadoury & Pearson, 1991). In addition, ascospores appear to be the primary inoculum for powdery mildew epidemics in New York State vineyards (Pearson & Gadoury, 1987). The sexual stage of *U. necator* was first recorded in Australia in 1984 (Wicks, Magarey & Emmett, 1985), whereas it has been known in North America since 1834 (Kapoor, 1967) and since 1893 in Europe (Bulit & Lafon, 1978). While a functional sexual stage creates significant opportunity for variation, nothing is known about the impact of sexual reproduction on the genetic diversity of *U. necator.* Population biology studies of this fungus will (a) determine the relative importance of the sexual stage in viticultural regions within Australia and other continents, and (b) reveal the impact of disease management strategies, for example

fungicide applications, on population dynamics. In order to characterize a *U. necator* population and to understand the genetic bases for variation, molecular-genetic and phenotypic markers must be developed.

This thesis is divided into four parts: (i) a review of the literature, (ii) general materials and methods, (iii) results of experiments, and (iv) a general discussion of the findings and opportunities for future research. The review of the literature (Chapter 2) examines the epidemiology and management of grapevine powdery mildew in order to highlight areas for future research. The molecular and population genetics of plant pathogenic fungi are then reviewed in relation to the development and application of molecular markers for *U. necator*. A statement of the objectives of the research presented in this thesis follows the review of the literature.

2.0 LITERATURE REVIEW

The hardier plants were naturally tried out in the open, and those species of fungi which could stand the climate spread and multiplied faster than the rabbits in Australia.

E. C. Large, 1940.

2.1 INTRODUCTION

Powdery mildew of grapevine, caused by *Uncinula necator* (Schw.) Burr., is a chronic yield and quality loss problem in all viticultural regions of the world. *U. necator*, an obligately biotrophic ascomycete, can infect all green tissues of the grapevine, producing a distinctive, whitish-grey, powdery appearance (Figure 2.1). These disease symptoms are a direct result of the presence of superficial hyphae producing conidiophores and conidia on the surface of the host tissue (Figure 2.2). The fungus absorbs nutrients via haustoria which develop in host epidermal cells. Infected berries split when the expansion of the epidermis is restricted but the pulp continues to expand. Berry infections reduce grape yield, grape and wine quality (Pool *et al.*, 1984).

This review focuses on the causal organism of the disease, *U. necator*. Disease history, economic importance, epidemiology and management are reviewed in order to highlight areas for future research. The molecular genetics of plant pathogenic fungi is then examined in terms of the use of molecular-genetic and phenotypic markers to improve (a) knowledge of *U. necator* genetics and population biology and (b) the efficiency of disease management. The relative merits of the various approaches which may be used in the development of markers are discussed. The applications of these markers are then examined in the context of current priorities for research into disease management.

2.2 HISTORY AND ECONOMIC IMPORTANCE OF DISEASE

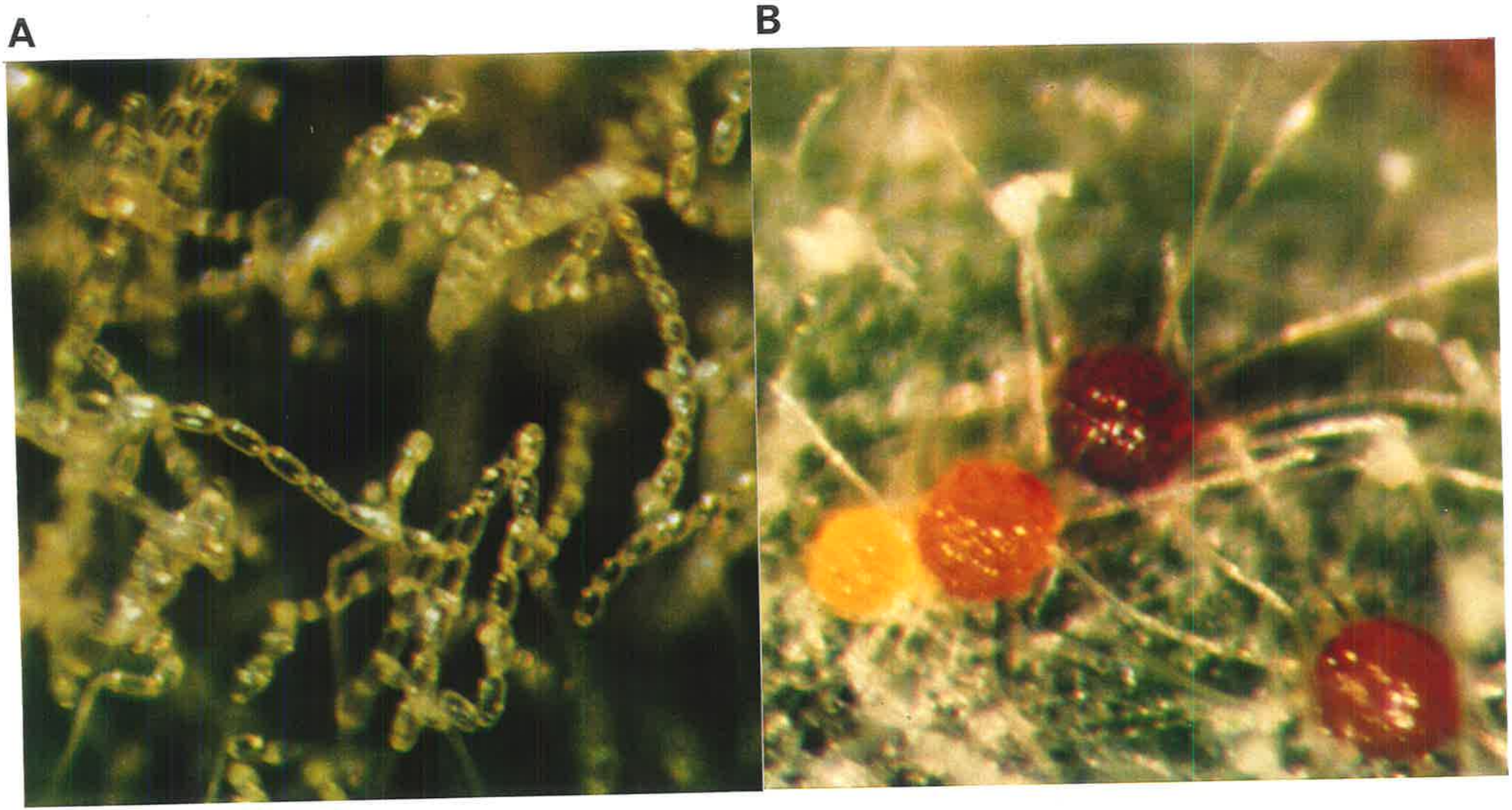
The history of this plant disease is well documented (Large, 1962; Bulit & Lafon, 1978; Yarwood, 1978; Pearson & Gadoury, 1992). Epidemics of grapevine powdery mildew in Europe from 1847-1854 marked the first recorded serious crop losses. By 1855 crop losses were reduced significantly by the widespread adoption of sulphur applications (Yarwood, 1978). Today, the disease occurs in most grape-growing areas of the world, and is still regularly and successfully controlled by sulphur. Although the fungus was first described in

Figure 2.1 Symptoms of powdery mildew disease on berries. These split berries have a whitish-grey, powdery appearance and cleistothecia are visible as black dots on the skin.

Figure 2.2 Reproductive structures of *U. necator.*

A. Chains of *U. necator* conidia produced using detached-leaf culture (refer to Section 3.1) following inoculation and incubation at 25°C for 12 days. Each conidium is approximately 25 µm long.

B. Immature cleistothecia of *U. necator*, ranging in colour from yellow to orange and brown. Mature cleistothecia are dark brown to black and 90-100 µm in diameter.


A
B

eastern North America by Schweinitz in 1834 (Kapoor, 1967), most native American species of *Vitis* are much less susceptible than cvs of *Vitis vinifera*. It is likely that *U. necator* originated in the Eastern and Central United States where the fungus and its wild vine hosts co-evolved for many years (Roy & Ramming, 1990; Pearson & Gadoury, 1992). How the disease was introduced to Europe and the rest of the world is unknown.

Whereas Schweinitz described the teleomorph, Berkeley named the anamorph *Oidium tuckeri* after the gardener, Tucker, who first observed the disease in England in 1847 (Large, 1962). Cleistothecia were observed in Europe in 1893 but it was not until the 1930s that evidence for the teleomorph and the anamorph being the same fungus was obtained (Yarwood, 1978). The disease occurs in all Australian grape-growing areas, where it has been present since at least 1866 (Emmett *et al.*, 1990). The presence of the sexual stage, however, was not recorded until 1984 (Wicks *et al.*, 1985). *U. necator* is heterothallic (Smith, 1970; Gadoury & Pearson, 1991) and plant pathologists speculate that the opposite mating type was introduced inadvertently by the importation of diseased grapevine material into Australia.

As in Europe, Australian grape production is based on cvs of the mildew-susceptible *V. vinifera* (Gregory, 1992). In 1994-95, total Australian grape production was 766,923 tonnes, with 75 percent used in wine production, 19 percent used for dried fruit production, and 6 percent were sold as table grapes or other products (Australian Bureau of Statistics (ABS), Catalogue No. 1329.0). This harvest was 153,236 tonnes lower than in the 1993-94 season because drought conditions affected many non-irrigated vines during the growing season. The total area of vines, however, increased 8 percent, to a record 72,707 hectares, reflecting the increased demand for Australian wine in overseas markets (ABS, Catalogue No. 1329.0).

There are many diseases and disorders of grapevine, the cumulative effect of which can have considerable impact on grape production (Emmett *et al.*, 1992c). The economic importance of a disease is measured in terms of direct crop losses (quantity and quality) and also the costs of disease management and research. In any one season, the incidence and severity of powdery mildew varies, depending on: (a) previous disease management, which influences primary inoculum levels; (b) weather conditions, which affect the rate and timing

of disease increase; (c) canopy and irrigation management, which govern the microclimate; and (d) the susceptibility of the cvs of grapevines grown.

For all grape products, crop quality is of major concern. Berry infections must be maintained at very low levels because wine made from 3% or more infected berries may have off flavours and greater acidity than wine made with healthy berries (Pool *et al.*, 1984). In areas producing table grapes, infected fresh fruit becomes unmarketable when covered with unsightly scarred skin. In addition, infected berries tend to split, providing entry sites for *Botrytis cinerea* and other bunch rot fungi.

Grapevines are a perennial crop and so consideration must be given to the effects of disease over more than one season. Plant pathologists have observed that a series of severe seasonal infections of powdery mildew reduces the vigour and productivity of grapevines (Emmett *et al.*, 1992c). Pool *et al.* (1984) conducted a crop-loss study on the *Vitis* interspecific hybrid, Rosette, in New York State, which supports these observations. Untreated vines were compared with vines sprayed with fungicides at various intervals over three seasons. At the end of the experiment, there was a 40% reduction in vegetative growth, as measured by the pruning weight, of untreated vines compared with vines sprayed throughout the season. This loss of vegetative growth was associated with a 65% reduction in the weight of grapes harvested per vine.

Studies of this nature have not been conducted in Australia, but yield reductions, resulting from disease in small plot trials, have been demonstrated when fungicide spray programmes were discontinued in any one season. Wicks *et al.* (1988) conducted small plot trials at various locations in South Australia to evaluate sterol-biosynthesis inhibiting fungicides. Powdery mildew developed in all treatments that were not sprayed with fungicide. At harvest, the percentage of untreated bunches with mildew ranged from 56 to 100% with up to 99% of the surface area of bunches covered in mildew. Bunch weights were significantly reduced compared with those from sprayed vines and many untreated bunches contained split and dehydrated berries. Emmett *et al.* (1992a) prepared disease progress curves for unsprayed vines which illustrate the potential for greater than 80% disease severity on berries and leaves if appropriate control measures are not applied.

The true cost of this disease to Australian grape production has not been fully quantified, but plant pathologists estimate that costs of disease management and yield losses

are 5% of the value of total grape production (R.W. Emmett, P.A. Magarey, R.D. Magarey and T.J. Wicks, personal communication). Management costs can be estimated to be at least the amount of fungicide used for powdery mildew control in a particular region. For example, in California in 1987, 68% of all pesticides used on grapes, as determined by kilograms of material applied, were fungicides for control of powdery mildew (Chellemi & Marois, 1992). The cost of control to Californian growers exceeded US$30 million in 1987 (Roy & Ramming, 1990). In addition, growers and taxpayers fund research and extension programmes in each region in an effort to improve the efficiency of disease management.

2.3 PATHOGEN AND HOST

U. necator belongs to the family *Erysiphaceae* and sub-family *Erysipheae* (Clare, 1964). Members of *Erysipheae* are obligate biotrophs with epiphytic hyphae connected to haustoria in host epidermal cells. The genus *Uncinula* is distinguised from other genera by its cleistothecial characters (Figure 2.2); namely, that the cleistothecial appendages are coiled at the apices. Pearson and Gadoury (1992) provide a good description of all structures of *U. necator* .

U. necator infects members of *Vitaceae* including the genera *Vitis*, *Parthenocissus* and *Ampelopsis* (Boeswinkel, 1980). There is limited evidence for pathogenic specialization in *U. necator*. Gadoury and Pearson (1991) did not detect pathogenic specialization among 35 isolates from various *Vitis* spp. inoculated onto *in vitro* plants or seedlings of the *Vitis* interspecific hybrid cv. Chancellor. Sixteen of these 35 isolates, however, did not infect *in vitro* plants of *Parthenocissus quinqefolia* and the remaining isolates had a latent period of 10 to 26 days on *P. quinqefolia* compared with 5 to 6 days on the Chancellor grapevines. Only three of the isolates sporulated on *P. tricuspidata*. From these experiments, it appears that isolates from *Vitis* spp. were best adapted to grow on *Vitis* spp., but some of the isolates were able to grow on *Parthenocissus* spp. Li (1993) inoculated five cvs of *V. vinifera* with four isolates of *U. necator* (origin unknown). There was no significant interaction (P=0.05) between the cvs and the isolates, although the sample size may have been too small to detect any interaction.

There are significant differences in susceptibility to powdery mildew within *Vitis* spp. For example, *V. vinifera* and Asiatic species are more susceptible than American

species such as *V. lambrusca* (Pearson & Gadoury, 1992). *V. lambrusca*, however, may be more susceptible to powdery mildew in North America than in Europe (Pearson & Gadoury, 1992). Cvs within *V. vinifera* also appear to vary significantly in susceptibility (Doster & Schnathorst, 1985; Stein *et al.*, 1985; Roy & Ramming, 1990; Li, 1993) although there are temporal and spatial inconsistencies in the relative susceptibilites reported in the literature (Pearson & Gadoury, 1992). The evaluation of host resistance is problematic when a small number of isolates of unknown pathogenicity and virulence is used in assays. Gadoury and Pearson (1991) illustrate this research problem by demonstrating substantial variation in the rate of colony expansion of 35 *Vitis* spp. isolates on *V. labruscana* cv. Catawba. This *Vitis* sp. is thought to be less susceptible to powdery mildew disease than is *V. vinifera.* Some of the *Vitis* spp. isolates, however, produced similar rates of colony expansion on *V. vinifera* cv. White Reisling and *V. labruscana* cv. Catawba.

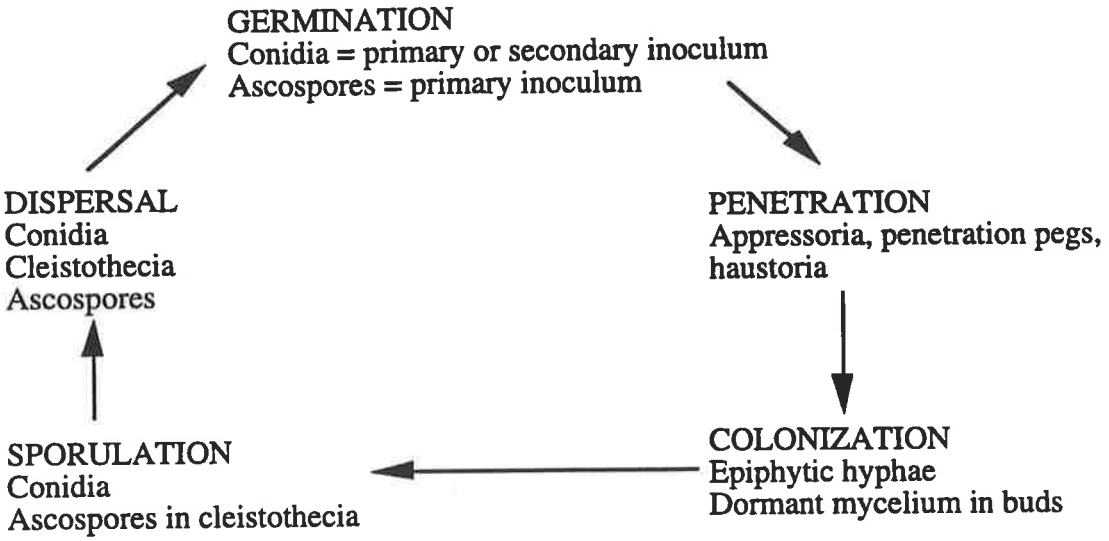
2.4 DISEASE EPIDEMIOLOGY

An important research objective of many plant pathologists is to understand disease epidemics under field conditions. An epidemic occurs when there is a change in disease intensity in a host population in time and space (Campbell & Madden, 1990). Epidemiology is the study of these changes, involving the pathogen, the host and the environment.

2.4.1 The disease cycle

The disease cycle can be separated into five phases: germination, penetration, colonization, sporulation and dispersal (Figure 2.3; Chellemi & Marois, 1992). The disease cycle may be completely asexual when both mating types are geographically isolated from each other. In this situation, the fungus probably overwinters by colonizing buds which become dormant (Sall & Wyrsinski, 1982; Pearson & Gärtel, 1985). Pearson and Gärtel (1985) speculated that bud infections occurred early in the growing season, based on the direct observation of *U. necator* hyphae in the interior of green axillary buds relatively early in the growing season. However, the time of bud infection with respect to the stage of bud development is unknown. Following bud break, the newly emerging shoots become covered with white mycelium and are called "flag shoots". Conidia produced from this mycelium are the primary inoculum for the first disease cycle of the growing season. The production of

Figure 2.3 The disease cycle of *U. necator*.


GERMINATION
Conidia = primary or secondary inoculum
Ascospores = primary inoculum
PENETRATION
Appressoria, penetration pegs, haustoria
COLONIZATION
Epiphytic hyphae
Dormant mycelium in buds
SPORULATION
Conidia
Ascospores in cleistothecia
DISPERSAL
Conidia
Cleistothecia
Ascospores

conidia from the primary infections initiates the secondary spread of the disease. The number of disease cycles per growing season depends on host susceptibility and environmental conditions.

In regions where cleistothecia form on diseased vines, the role and importance of the sexual cycle is often poorly understood. The exception is New York State, where cleistothecia are washed by late summer and autumn rains onto the bark of the vine where they overwinter (Pearson & Gadoury, 1987; Gadoury & Pearson, 1988). Ascospores are then discharged during spring rains, beginning after bud burst, and infect shoots growing near the bark. The resulting colonies produce conidia for the secondary spread of the disease. In New York State, *U. necator* has never been observed to overwinter as mycelium in dormant buds (Pearson & Gadoury, 1987), and ascospores are the primary inoculum for powdery mildew epidemics. In contrast, both flag shoots and cleistothecia can be found in Californian vineyards, and in European countries such as France, Italy, Austria and Germany (Diehl & Heintz, 1987; Hill *et al.*, 1995; W. D. Gubler and H. Denzer, personal communication). With the exception of the studies in New York State, there are no published reports on the relative occurrence of primary inoculum from cleistothecia and flag shoots in different vineyards, regions and years. Flag shoots develop in all South Australian viticultural regions and in the Sunraysia region of Victoria (Emmett *et al.*, 1990; Emmett *et al.*, 1992a; T.J. Wicks, personal communication). The hypothesis that flag shoots are the predominant source of primary inoculum in warm-climate regions such as those in Italy, California and Australia should be investigated further. In addition, the role and relative importance of sexual reproduction in Australia should be evaluated. Large numbers of cleistothecia have been recovered from the bark of vines in the Riverland and Sunraysia regions (Magarey *et al.*, 1993b), and ascospores released from cleistothecia under laboratory conditions infect detached leaves (Magarey *et al.*, 1993c). Indirect evidence that ascospore infections are occurring in the vineyard is based on early season observations of colonies on the underside of leaves near the bark. Direct evidence, however, that ascospores are a source of inoculum and infection in the field is lacking.

2.4.2 Host and environmental factors

Variation in host susceptibility was discussed in Section 2.3. In addition, there is variation in host tissue susceptibility. The incidence of infection and the rate of colonization and sporulation are inversely proportional to leaf and berry maturity (Delp, 1954; Doster & Schnathorst, 1985; Chellemi & Marois, 1992). When potted *V. vinifera* plants, grown in the field or in a greenhouse environment, were inoculated with *U. necator*, Delp (1954) observed 40% infection on 1 week-old leaves but only 10% infection on 2 month-old leaves after 2 days incubation in a laboratory nursery. Colony diameter was also reduced on the 2 month-old leaves, and no infection occurred on leaves more than 2 months old. Berries of *V. vinifera* cvs Muscat of Alexandria, Tokay, Carignane and Thompson Seedless are susceptible to infection until their sugar content reaches about 8 percent (Delp, 1954). Once infected, sporulation continues until the sugar content of the berries reaches 15 percent. Using *V. vinifera* cv. Chardonnay, Chellemi and Marois (1992) found a negative exponential relationship between berry susceptibility and accumulation of soluble solids, with fruit above 7° Brix rarely infected.

Given that a grapevine cultivar is susceptible and pathogen inoculum abundant, an epidemic occurs when the weather is favourable and remains that way for long enough for damage to result from disease. Disease development is likely to be governed by the most limiting environmental factors and, more realistically, the interactions of those limiting factors (Waggoner *et al.*, 1980). Table 2.1 summarizes some important environmental factors for disease development and their respective ranges and optima. Temperature ranks high on the list of factors limiting the rapidity of secondary disease cycles. In addition, the germination rate of conidia at a particular temperature may be influenced by the temperature during conidium formation (Fessler & Kassemeyer, 1995). Latent periods range from 5 days at 25°C to 32 days at 7°C (Delp, 1954). Germination of conidia is inhibited above 35°C (Delp, 1954). In summary, rapid disease development will occur at 25°C in the absence of free water, at high relative humidity and under diffuse light conditions. Conidia and epiphytic hyphae of powdery mildew fungi are killed by brief exposure to ultra-violet (uv) light from laboratory lamps (Mount & Ellingboe, 1968; Martin *et al.*, 1975) and so natural uv light may contribute to reduced disease incidence in non-shaded vines.

Table 2.1 A summary of environmental variables and their functional range and optima for various phases in the life cycle of *U. necator*.

Environmental variable	Pathogen response	Functional range	Optimum level or range	Reference
Temperature	Infection by conidia	7 - 31°C	24 - 26°C	Delp (1954) Doster & Schnathorst (1985)
Temperature	Latent period (inoculation to sporulation)	32 days at 7°C, 6 days at 30°C	5 days at 26°C	Delp (1954)
Temperature	Infection by ascospores	No infection at 5 and 31°C on *in vitro* plantlets	20 - 25°C on *in vitro* plantlets	Gadoury & Pearson (1990b)
Temperature	Ascospore discharge	≥4°C	10 - 32 °C with sufficient wetting	Diehl & Heintz (1987) Gadoury & Pearson (1990a)
Free Water	Germination of conidia	Germination poor on or in free water	No free water	Delp (1954) Sivapalan (1993)
Free Water	Germination of ascospores	Declines as relative humidiy decreases.	Free water and saturated atmospheres	Gadoury & Pearson (1990b)
Free Water	Ascospore discharge	≥2.5 mm rain	Free water	Diehl & Heintz (1987) Gadoury & Pearson (1990a)
Relative Humidity	Infection by conidia	<20% - 100%	40 - 100%	Delp (1954)
Relative Humidity	Asexual sporulation	30 - 100%	90 - 100%	Pearson & Gadoury (1992)
Light	Germination of conidia	Diffuse and bright light	Diffuse light	Pearson & Gadoury (1992)

U. necator has potential for rapid growth and reproduction. Chellemi and Marois (1992) estimated the maximum reproductive potential of an individual conidium of *U. necator* to be 2272 conidia per generation at 26°C, the corresponding doubling time being 0.56 days. More realistically, Pearson and Gadoury (1992) have recorded infection rates of up to 0.31 to 0.35 $unit^{-1}day^{-1}$ on *Vitis* interspecific hybrid cv. Rosette. Their method for calculating the infection rate, and thus the unit of measurement, was not defined.

Environmental factors influencing ascosporic infections are different from those affecting conidial infections. In New York State, ascospores are released between bud break and bloom, after at least 2.5 mm rainfall and when temperatures are above 4°C (Gadoury & Pearson, 1990a). Unlike conidial germination, ascospore germination is greatest in free water. In addition, Gadoury and Pearson (1988) examined the effect of a number of environmental factors on the initiation and development of cleistothecia *in vitro*. They found that the intiation of cleistothecia on a susceptible host depended solely on hyphal contact between compatible isolates. Cleistothecia also formed under various conditions of host tissue age, daylength and relative humidity. The subsequent development of cleistothecia was temperature-dependent, with rapid development between 20 and 25°C.

2.5 DISEASE MANAGEMENT

In order to develop an efficient disease management strategy, the construction of a disease progress curve (Campbell & Madden, 1990) is a basic step in the development and evaluation of control measures. Temporal "growth curve" analyses of grapevine powdery mildew epidemics often fit the logistic model. In the absence of management, grapevine powdery mildew can increase rapidly from very low levels of primary infection (Emmett *et al.* 1992a; Pearson & Gadoury, 1992; Emmett *et al.*, 1993a). Thus, management efforts are directed mainly at reducing the number and rapidity of secondary disease cycles. In addition, the onset of an epidemic can be delayed by reducing primary inoculum sources, for example, cleistothecia and/or dormant mycelium (Sall, 1980; Stapleton *et al.*, 1988; Gadoury *et al.*, 1994).

2.5.1 Chemical control

Chemical control methods are based on protecting grapevines from infection by repeated application of fungicides during the growing season. After an effective sulphur treatment was discovered for this disease in the 1850s, there were minimal efforts to find alternative control methods until the introduction of the demethylation inhibiting fungicides (DMI) in the 1960s (Scheinpflug & Kuck, 1987).

Grape growers worldwide rely heavily upon chemical control methods, and sulphur is still the most widely used fungicide. The main drawback of using sulphur is that its performance is temperature-dependent (Pearson & Gadoury, 1992). In addition, sulphur may taint wine if applied within 1 month of harvest and can leave undesirable residues on table grapes (Emmett *et al.*, 1992c). For these reasons, DMI fungicides such as triadimenol, propiconazol, fenarimol and flusilazol, are applied as protective sprays when sulphur applications would be less effective, for example in cool weather, or less desirable, for example close to harvest. Growers are generally advised to apply protective sprays beginning early in the season before disease becomes severe. For example, in the Sunraysia-Riverland district, a common spray programme for a highly susceptible cv., with disease carryover from the previous season, would involve applications of fungicide at 2, 4, and 6 weeks after budburst, with further sprays at 2 to 3-week intervals between berry set and berry softening (Emmett *et al.*, 1992b; Emmett *et al.*, 1993b).

Several new approaches to chemical control have been developed recently. Applications of lime-sulphur to dormant grapevines kill cleistothecia and delay the development of mildew epidemics in New York State (Gadoury *et al.*, 1994). This treatment is currently uneconomical. The use of the vapour phase of certain DMI fungicides is currently being explored as an alternative application method for these fungicides (Pearson *et al.*, 1994). The technique involves using fungicide-treated cheesecloth wicks or rope hung beneath the fruiting zone of the grapevine trellis. The benefits and timing of this method will need to be evaluated in terms of labour and management costs, residues on fruit and fungicide resistance strategies.

Consumers now demand reduced chemical residues in their food supply. The number of pesticide sprays required varies from one viticultural region to another, depending on climate and the types of pests and diseases. Magarey (1992) summarizes the

opportunities that Australian grape growers have to minimize unnecessary fungicide applications in the dry climate of inland viticultural areas. Most importantly, increased monitoring of vineyard microclimate enables researchers to correlate weather data with disease increase and spread, and to develop more efficient spray schedules. For example, weather conditions favourable for ascospore release can be used to predict the timing of primary infections in New York State (Pearson & Gadoury, 1987; Gadoury & Pearson, 1990a) and California (Reiger, 1995; W. D. Gubler, personal communication). In addition to weather-based spray schedules, researchers are testing the efficacy of fungicides which have less environmental impact, such as sodium bicarbonate, ammonium bicarbonate, oils and wetting agents (Magarey *et al.*, 1993a; Wicks *et al.*, 1995).

2.5.1.1 Fungicide resistance

Fungicide resistance is a term used to denote those individuals within a pathogen population that show a heritable, reduced sensitivity to a fungicide. "Field" resistance occurs when decreased fungicide efficacy is correlated with increased frequency of resistant strains. In general, the more non-specific a fungicide is in its mode of action, the less chance there is that a pathogen can become resistant to it. Sulphur, for example, is unlikely ever to give rise to resistance problems because it interferes with a variety of metabolic processes and would require multiple changes in the pathogen genome to counter its effects (Lyr, 1977). The DMI fungicides, however, specifically inhibit C-14-demethylation of 24-methylenedihydrolanosterol during sterol biosynthesis (Buchenauer, 1987). This single mode of action might suggest a high risk of resistance, but other factors contribute to the risk of resistance developing under field conditions. These factors include fungal biology, such as fitness of resistant strains and reproduction rate, as well as management factors. For example, the intensity of fungicide use determines the duration of exposure, in generations, of the target population.

Shifts in powdery mildew populations toward DMI field resistance have occurred from the early 1980s, especially in the case of cereal and cucurbit powdery mildews in Europe (Schepers 1983; Schepers, 1985; Limpert, 1987; Brown & Wolfe, 1991; Staub, 1991). Changes in sensitivity of various mildew populations to DMI fungicides have generally been gradual. Reduced sensitivity has been correlated with poor disease control or

relatively short persistence of action by a particular DMI fungicide (De Waard, 1994). While there have been few incidents of complete failure to control, prolonged use of DMI fungicides will continue to select for resistant individuals, which necessitates the implementation of strategies to delay shifts to higher levels of resistance (Köller & Scheinpflug, 1987; Staub, 1991). The genetic and biochemical mechanism of resistance in each field population is not well understood (Brent & Holloman, 1988).

Reduced sensitivity of *U. necator* populations to DMI fungicides, specifically triadimefon and triadimenol, was first reported in Portugal (Steva *et al.*, 1988; Steva *et al.*, 1989), followed by France (Cartolaro & Steva, 1990; Steva & Clerjeau, 1990; Steva *et al.*, 1992; Steva, 1994), Italy (Steva, 1994) and California (Gubler *et al.*, 1994). Gubler *et al.* (1994) also reports that DMI fungicide resistance appears to be more severe in vineyards in which cleistothecia are produced, which suggests that sexual reproduction may be increasing the frequency of fungicide resistant strains at a rate faster than in populations reproducing clonally. Debieu *et al.* (1995) found that the sterol compositions of triadimenol-sensitive (EC_{50}<0.5 mg l^{-1}) and resistant (EC_{50}>1 mg l^{-1}) strains of *U. necator* were similar. Therefore, altered sterol biosynthesis is not the mechanism for triadimenol resistance. This research, however, is a first step toward understanding the mechanism of triadimenol resistance in *U. necator*, which may include reduced fungicide uptake, detoxification of the fungicide or mutation of the target site of action.

In order to identify shifts in fungicide sensitivity in a population, base-line sensitivity data should be collected before a fungicide is introduced to a region. Unfortunately, the collection of these data for DMI fungicides has not occurred in any viticultural region. In addition, methods for assessing DMI fungicide sensitivity vary widely. Ideally, methods should be standardized across geographical regions. Fungicide sensitivity is often measured by exposing the fungal isolate to a series of fungicide doses, measuring the response, and then fitting a regression model from which the median effective dose (ED_{50}) is estimated. Brown (1991) argues that this type of analysis may be inappropriate for powdery mildew fungi because the fungicide dose, which is the independent or predictor variable in the regression model, may exhibit within-treatment variation as a result of applying the fungicide to the host substrate. Siebels and Mengden (1994) report an *in vivo* assay system which would overcome some of the shortcomings of current *in vitro* assays. Any shifts in DMI

fungicide sensitivity in Australian vineyards should be quantified soon so that appropriate management strategies are implemented.

2.5.2 Breeding for resistance, biological and cultural control

The long term objective of any disease management strategy is to develop commercial cvs that are less susceptible to disease. Powdery mildew resistance has been a low priority for grapevine breeders because of the high efficacy of chemical control and the need to maintain the strong varietal qualities of *V. vinifera* grapes. Cvs of *V. vinifera* originated from wild populations around the Mediterranean Basin and the Middle East about 4000 BC (Olmo, 1986). These wild populations were probably a complex of subspecies of *V. vinifera* with wide variation in morphological characters. In contrast, North America has over 23 species of wild grapes (Olmo, 1986). Only one of these wild grapes, *Muscadinia rotundifolia* Small (formerly *Vitis rotundifolia*), is known to be highly resistant to *U. necator* (Olmo, 1986). Attempts to produce commercially acceptable, resistant hybrids by making crosses with *V. vinifera* have so far been unsuccessful.

Pearson and Gadoury (1992) summarize the susceptibility of various *Vitis* species and cvs to powdery mildew. There are still many questions relating to the genetic nature of resistance to *U. necator*. Specific host resistance genes and pathogenic races have not been defined, although a functional sexual stage potentially provides an opportunity for a "gene-for-gene" type interaction (Flor, 1955). Polygenic and/or rate-limiting resistance has also not been quantified adequately. Li (1993) observed a continuous distribution of susceptibility within cvs of *V. vinifera*. Progenies produced by both selfing and hybridization of cvs showed a positive correlation to the mean level of resistance of the parent(s), suggesting the existence of minor resistance genes. Eibach *et al.* (1989) also examined the heritability of resistance to *U. necator* by examining the degree of resistance of progenies of *Vitis* interspecific crosses. They observed additive gene effects with regard to resistance to *U. necator*.

If suitable resistance genes are identified for use in a breeding programme, a major limitation for incorporating desirable genes will be the need to retain all the varietal traits of the dominant *V. vinifera* cvs, such as Chardonnay and Cabernet Sauvignon. Molecular techniques may eventually allow rapid incorporation of desirable genes into acceptable

genetic backgrounds. Researchers are currently developing protocols for the genetic transformation and regeneration of *V. vinifera* (N. Scott, personal communication) and successful regeneration of transformed *V. rupestris* has been reported (Martinelli & Mandolino, 1994). Given that many vines are in the vineyard for 30 years or more, any breeding programme must strive for durable resistance because a cv. cannot be replaced every time a new virulent strain appears in a vineyard.

Biological control agents are now being examined as management measures for many plant pathogens, including *U. necator*. The mycoparasites *Ampelomyces quisqualis* Ces. (Sundheim, 1982) and *Tilletiopsis* spp. (Knudsen & Skou, 1993) can colonize mycelial and reproductive structures of powdery mildew fungi, but they require free water for spore germination, and moderate temperatures and relative humidities greater than 90% for maximum effectiveness. When compared with natural parasitism of cleistothecia by *A. quisqualis* in New York State, increased parasitism occurred when this mycoparasite was applied to vines using colonized cotton-wick cultures (Falk *et al.*, 1995a). Increased parasitism was correlated to a reduction in the number of cleistothecia dispersed from the leaves to the bark. Thus, in New York State, there is the potential to reduce the number of overwintering cleistothecia following applications of *A. quisqualis*. In addition, field trials involving the release of *A. quisqualis* pycnidia in New York State vineyards showed that disease severity but not incidence could be reduced by this biocontrol agent, but only in a high rainfall season (Falk *et al.*, 1995b). A commercial formulation of *A. quisqualis* spores, AQ10, has been registered in California with the recommendation that it be applied early in the season before disease severity becomes severe (Arcamonte, 1995). Efficacy, environmental limitations and compatibility with fungicides and other management programmes need to be evaluated further.

Cultural methods of disease management are directed at altering microclimate. Canopy management and row orientation appear to be the best ways of changing microclimate conditions, namely relative humidity and light intensity. Training systems that produce an open canopy with good light penetration and air flow slow the development of disease and allow better coverage by fungicide sprays (Emmett *et al.*, 1994).

2.6 MOLECULAR AND POPULATION GENETICS OF PLANT PATHOGENIC FUNGI

The genetic structure of a natural population is defined as the amount and distribution of genetic variation within and between pathogen populations (McDonald & McDermott, 1993). Mechanisms which determine genetic structure include mutation, migration, genetic drift and natural selection (Smith, 1989). The term "gene flow" covers all mechanisms which result in the movement of genes or genotypes from one population to another, including sexual reproduction, migration, extinction and recolonization (McDermott & McDonald, 1993). All these factors shape the evolution of plant pathogen populations and information about their relative contribution may be used to decide how best to deploy sustainable disease management strategies. Molecular markers can be used to study these evolutionary mechanisms and will be examined using examples from various pathosystems, including the *Erysiphe (Blumeria) graminis-Hordeum vulgare* pathosystem, as models for studying *U. necator* genetics and population biology. The first step is to evaluate techniques for their potential in characterizing *U. necator* genotypes.

2.6.1 Intraspecific variation

Plant pathologists have identified intraspecific variation in natural populations of filamentous fungi, but the concept of "mycelial individualism" is still being developed (Rayner, 1991; Anderson & Kohn, 1995). Given that isolates of the powdery mildew fungi are morphologically identical, host colonization is mycelial and clonal reproduction significant, a definition of what constitutes an individual in a population is required. Several authors (Rayner 1991; Smith *et al.*, 1994) suggest calling fungal individuals *genets* (Kays & Harper, 1974). For filamentous fungi, a genet is a mitotic cell lineage which constitutes all fungal structures derived from an identical genetic source, for example mycelia and asexual propagules.

To identify and study the bases for variation, individuals, or genets, must be distinguished by markers which are stable and correlated to genetic differences. Before the development of molecular techniques, naturally occurring genetic markers for various pathogens included sexual compatibility and vegetative incompatibility genes, virulence genes and fungicide resistance genes. The genotype of the isolate is inferred from the phenotype, and the markers can only be used in pathosystems where they are easily

characterized. For example, the use of virulence genes requires a "gene-for-gene" interaction of the pathogen with its host (Flor, 1955), and a well characterized series of resistant cvs. Isoenzymes have also been used extensively to analyze variants of specific enzymes (isozymes) in fungi (Micales *et al.*, 1992). More recent molecular techniques, which detect naturally occurring DNA sequence polymorphisms, access both the coding and non-coding regions of the genome. DNA techniques are now being used in conjuction with traditional techniques because they provide abundant and selectively neutral genetic markers, which can be applied to all fungal cell types (Michelmore & Hulbert, 1987; Cooley, 1992).

Little is known about the genetic diversity of *U. necator*. Bipolar heterothallism (two mating types) was characterized relatively recently among New York isolates (Gadoury & Pearson, 1991) and there is no evidence for pathogenic specialization of *Vitis* spp. isolates on *Vitis* spp. (refer to Section 2.3). Benomyl resistant strains have been identified in New York State (Pearson & Taschenberg, 1980) and future research with DMI fungicides may provide additional fungicide resistance markers. Double stranded (ds) RNA species have been associated with *U. necator* isolates from New York State (Azzam *et al.*, 1991; Azzam & Gonsalves, 1991), which raises the possibility of using them as markers.

2.6.2 Genetic bases for variation

While mutation is an important source of variation, other mechanisms for generating variants, such as sexual and/or mitotic recombination, may apply in filamentous ascomycetes. All ascomycetes, including *U. necator*, are haploid in the vegetative phase and are capable of sexual reproduction, the genetic consequence being segregation and recombination. Mendelian genetic analysis is applicable in many of these fungi (Fincham *et al.*, 1979). For example, bipolar heterothallism, a common mating system in ascomycetes, is determined by a one-locus, two-allele gene (Fincham *et al.*, 1979 ; Glass & Kuldau, 1992).

When clonal reproduction predominates, a fungal population is still capable of significant variation and rapid evolution (Sebens & Thorne, 1985; Silander, 1985; Michelmore & Hulbert, 1987). In both sexually and clonally reproducing populations, there can be novel, non-Mendelian mechanisms of genetic change. For example, mitotic recombination can occur within heterokaryons following the fusion of two nuclei, crossing

over during mitosis and the separation of the diploid nucleus into its haploid components (Tinline & MacNeill, 1969). Menzies and MacNeill (1986) examined mitotic recombination in *E. graminis* f.sp. *tritici*. They observed fusion of germ tubes among conidia of two different races. In addition, non-parental genotypes were observed in progeny from 37 asexual crosses between races differing in virulence and/or fungicide resistance. Mitotic recombination has not been detected in *E. graminis* f.sp. *hordei* (Hermansen, 1980). In natural fungal populations the formation of heterokaryons appears to be an infrequent event and the prevalence of mitotic recombination in nature is unknown (Glass & Kuldau, 1992). Additional non-Mendelian mechanisms for genetic change include (a) cytoplasmically transmitted genetic elements, (b) chromosome polymorphisms and (c) transposable elements (for review see Mills & McClusky, 1990; Kistler & Miao, 1992).

2.6.3 Development of molecular markers

Given the limited number of existing and useful markers for *U. necator*, the development of molecular markers, especially DNA markers, will provide a level of precision not previously available. Three general techniques are employed for examining DNA sequence polymorphisms at the intraspecific level. These techniques are (1) Southern hybridization for analysis of restriction fragment length polymorphisms (RFLPs) and/or DNA fingerprints, (2) DNA amplification by the polymerase chain reaction (PCR), and (3) electrophoretic karyotyping (Michelmore & Hulbert, 1987; Cooley, 1992). Before selecting a specific method, genome organization, the source and quantity of fungal DNA, and the utility of a particular method at the intraspecific level (for review see Kohn, 1992) should be considered. The relative merit of any technique or approach should also be evaluated in terms of the research objectives, technical difficulty, sample turnover, labour requirements and costs of equipment and reagents.

2.6.3.1 Genome organization and evolution

Fungi contain DNA both in the nuclei and in the cytoplasm, with extrachromosomal DNA occurring in mitochondria and plasmid-like DNA (Taylor, 1986; Meinhardt *et al.*, 1990). The organization of eukaryotic genomes has been reviewed extensively (Tauz & Renz, 1984; Flavell, 1986; Epplen, 1988; Tauz, 1989); a fundamental feature is the occurrence of regions

of coding DNA, and non-coding DNA containing two classes of repeated DNA. The first class of repeated DNA is arranged in tandem arrays and the second class is dispersed throughout the genome. It is important to recognize these different types of sequences because, according to one theory of molecular evolution, different sequences have different rates of sequence divergence over time (Dover, 1987). The different divergence patterns are thought to be the result of "molecular drive", or the turnover of repeated sequences in the genome (Dover, 1987), as well as natural selection and genetic drift. Repeated sequences usually evolve more rapidly than single-copy sequences, by processes such as unequal crossing over, deletion, transposition and conversion (Dover, 1982; Flavell, 1986). For example, the components of the nuclear ribosomal sequences show different rates of evolution (for review see Hillis & Dixon, 1991). The coding regions for the 18S, 5.8S and 28S ribosomal genes in fungi are more highly conserved than the internal transcribed spacer (ITS) and the intergenic transcribed spacer (IGS) (White *et al.*, 1990). The choice of nucleic acid sequence for detecting DNA polymorphisms depends on the taxonomic level under investigation (Kohn, 1992) and is usually determined empirically.

The genome size and karyotype of *U. necator* is unknown. The total DNA per haploid genome of *Aspergillus nidulans* and *Neurospora crassa* is estimated to be 2.6 x 10^7 and 4.7 x 10^7 base-pairs, respectively (Timberlake, 1978; Orbach *et al.*, 1988). The DNA content of *U. necator* is likely to be of the same order of magnitude. Electrophoretic karyotyping has revealed ascomycete haploid chromosome numbers ranging from three to more than 15 (Mills & McCluskey, 1990; Skinner *et al.*, 1991). Skinner *et al.* (1991) categorized fungal chromosome sizes as "*Saccharomyces cerevisiae*-like" (<1 - 2 Mb) or "*Schizosaccharomyces pombe* - like" (>2 Mb). The genome of *E. graminis* appears to consist of seven or eight chromosomes with five of these chromosomes being very small (0.2-0.8 Mb, Borbye & Giese, 1992). Chromosome length polymorphisms are common in ascomycetes and have been observed in *Cladosporium fulvum* (Talbot *et al.*, 1991), *Cochliobolus heterostrophus* (Tzeng *et al.*, 1992), *Colletotrichum gloeosporioides* (Masel *et al.*, 1993), *Leptosphaeria maculans* (Plummer & Howlett, 1993), *Magnaporthe grisea* (Hamer *et al.*, 1989; Talbot *et al.*, 1993), *Nectria haematococca* (Miao *et al.*, 1991) and *Septoria nodorum* (Cooley & Caten, 1991).

2.6.3.2 DNA probes

A probe is a molecule having a strong and detectable interaction with a specific target. DNA probes are cloned sequences of DNA used to identify homologous regions of target DNA. When a DNA sequence is used to probe restriction enzyme-digested DNA, the number of bands observed following Southern hybridisation and autoradiography depends on the number of target sequences homologous to the probe and the number and intensity of restriction sites within these sequences (Michelmore & Hulbert, 1987). For RFLP analysis, single bands are called loci and the term "DNA profile" refers to genet-specific DNA patterns obtained using multiple, single-locus or low-copy probes. Medium to high copy probes, on the other hand, result in a banding pattern which is often called a "DNA fingerprint".

The most common types of DNA probes used to identify intraspecific differences within plant pathogenic ascomycetes are random sequences of unknown origin from total genomic DNA, nuclear DNA or mitochondrial DNA from the target species (Table 2.2). Mitochondrial DNA (mtDNA) is a useful marker for population and evolutionary studies because it is usually inherited maternally and therefore does not recombine during sexual reproduction (Michelmore and Hulbert, 1987). Usually fewer than ten isolates from diverse locations are needed to screen a DNA library for suitable probes. Conversely, a large number of probe and restriction-enzyme combinations may need to be screened in order to identify markers suitable for genetic analyses and gene flow studies. In addition, the efficiency with which a collection of random, genomic probes detects polymorphisms may vary widely. For example, McDonald and Martinez (1990a) tested 196 probe-enzyme combinations using a geographically diverse group of six *Mycosphaerella graminicola* isolates. RFLPs were detected using 145 probe-enzyme combinations. The number of random, genomic probes detecting RFLPs in other ascomycetes, when compared to the number screened, is reported infrequently and is likely to be lower than that reported for the genetically variable *M. graminicola.*

RFLPs and DNA fingerprinting methods can detect intraspecific variation on a very fine scale. McDonald and Martinez (1990b, 1991) characterized 93 isolates of *Mycosphaerella graminicola* from a single wheat field using RFLPs in the nuclear genome. Data from several different genomic DNA probes that hybridized to unique, unlinked loci were combined to generate DNA profiles. Twenty two different profiles were characterized

Table 2.2 Examples of DNA probes used for RFLP analysis of genomic DNA from various plant pathogenic ascomycetes.

Species	Number of isolates studied	Probe*	Number of probe/enzyme combinations	Research objective	Reference
Cryphonectria parasitica	39	G,U	1	a) marker development b) population structure studies	Milgroom *et al.* (1992a)
Cryphonectria parasitica	32	G,U	17	genetic diversity study	Milgroom *et al.* (1992b)
Erysiphe graminis f. sp. *hordei*	6	G,U	unknown	marker development	O'Dell *et al.* (1989)
Erysiphe graminis f. sp. *hordei*	28	G,U	11	a) marker development b) linkage analysis	Christiansen & Giese (1990)
Erysiphe graminis f. sp. *hordei*	97	G,U	1	gene flow study	Brown *et al.* (1990)
Magnaporthe grisea	18	G,U	1	a) identify clonal lineages b) correlate markers with pathotype	Levy *et al.* (1991)
Magnaporthe grisea	10	G,U	25	correlate markers with pathotype	Ko *et al.* (1993)
Mycosphaerella graminicola	6	G,U	196	marker development	McDonald & Martinez (1990a)
Mycosphaerella graminicola	93	G,U	12	epidemiological and genetic diversity studies	McDonald & Martinez (1990b)
Mycosphaerella graminicola	537	G,U	9	gene flow measurement	Boeger *et al.* (1993)
Ascochyta rabiei	6	O	16	marker development and pathotyping	Weising *et al.* (1991)
Leptosphaeria maculans	16	O	18	a) marker development b) pathotype differentiation	Meyer *et al.* (1992)
Various	11	O	30	marker development for separating species and strains	Meyer *et al.* (1991)
Colletotrichum gloeosporioides	8	M	2	a) marker development b) differentiate pathotypes and physiological races	Braithwaite & Manners (1989)

* G=genomic, U=unknown sequence, O = synthetic oligonucleotide, M = minisatellite.

among the 93 isolates. The same isolates were characterized with a single multiple-locus probe that recognized dispersed repeated sequences. The association between the profiles and the fingerprints was near unity, suggesting that the two methods are comparable for distinguishing individual isolates. The probability that any randomly chosen isolate would have had the same profile or fingerprint as any given isolate was estimated to be in the range 1.5×10^{-3} to 5.9×10^{-9}. Isolates with identical profiles or fingerprints were thus likely to be clonally related.

The advantage of using random, genomic probes for powdery mildew fungi is that sequences which are species specific can be used to identify variation using infected host tissue rather than separating fungal structures, for example conidia, from the host. Purified DNA, however, must be obtained in order to develop DNA probes. In order to clone *E. graminis* f. sp. *hordei* DNA, O'Dell *et al.* (1989) collected approximately 50 g of conidia from 8,000 infected barley seedlings. Their DNA yield was approximately 5 µg per g of frozen conidia. This example illustrates that the extraction of DNA from conidia is much less efficient than from mycelia of other fungi cultured axenically, where yields of 1.2-1.5 mg DNA per g of freeze dried mycelia are common (Raeder & Broda, 1985). DNA probes developed for *E. graminis* f. sp. *hordei* (O'Dell *et al.*, 1989; Christiansen & Giese, 1990; Giese *et al.*, 1990) include a fingerprinting probe, pEgE9 (E9, O'Dell *et al.*, 1989), which does not hybridize to cereal DNA. Repeated *E. graminis* sequences are detected using 8.0 µg of total DNA from infected cereal leaves. This probe is now used routinely to examine the population structure of *E. graminis* f. sp. *hordei* in Europe (Brown & Wolfe, 1990; Brown *et al.*, 1990; Brown *et al.*, 1991; Brown *et al.*, 1993; Brown & Simpson, 1994).

In addition to random probes, length variation in the tandem arrays of repeated DNA can be used for RFLP analysis. Depending on the length of the basic repeat units, these sequences are called minisatellites (between 10 and 40 nucleotides, Jeffreys *et al.*, 1985) or microsatellites (2 to 10 nucleotides, Epplen, 1988; Litt & Luty, 1989). Jeffreys *et al.* (1985) were the first to use probes that hybridized to variable number tandem repeat (VNTR) loci located in the minisatellite regions of human DNA. These human DNA probes also detect DNA polymorphisms in other species, including fungi (Braithwaite & Manners, 1989). Using core sequence information from VNTR probes, short oligonucleotide probes consisting of tandem repeats of core sequence can be synthesized and used as fingerprinting

probes (DeScenzo & Harrington, 1994). Synthetic oligonucleotides have now been tested as probes for several ascomycetes (Table 2.2). Intraspecific variation in these fungi has been identified using dinucleotide, trinucleotide or tetranucleotide repeats. In addition, a minisatellite element from bacteriophage M13 hybridizes to all species tested to date (Zimmerman *et al.*, 1989; Nicholson *et al.*, 1993) and thus may be useful as a fingerprinting probe for *U. necator*. If DNA is extracted from infected host tissue, then synthetic oligonucleotide probes will have little utility if restriction fragments cannot be distinghished as being specific to *U. necator*.

Some studies have involved heterologous probes developed from genomic libraries of a different species (Braithwaite & Manners, 1989; Braithwaite *et al.*, 1990). These probes may detect sufficient polymorphism and obviate the need for cloning the DNA of the species under study. Alternatively, they may be used to clone the corresponding sequence from the species under study. For example, Braithwaite *et al.* (1990) examined polymorphisms in ribosomal DNA (rDNA) of *Colletotrichum gloeosporioides* using an rDNA probe from *Aspergillus nidulans*. A length variation and an *Xba I* restriction site distinguished pathotype A isolates from pathotype B isolates. E9 or other *E. graminis* sequences should, therefore, be tested for homology with *U. necator* DNA.

2.6.3.3 DNA amplification

The amplification of DNA sequences by PCR is being used increasingly as a means to identify variation in fungi (for review see Arnheim & Erlich, 1992; Foster *et al.*, 1993; Annamalai *et al.*, 1995). One advantage of PCR is its ability to amplify picogram quantities of DNA (Cenis, 1992). A key component of the reaction is the oligonucleotide primers. The type of primer divides PCR markers into two groups; those known as sequence tagged sites (STSs, Olsen *et al.*, 1989) with primers designed from a known sequence and those based on arbitrary primers (Welsh & McClelland, 1990; Williams *et al.*, 1990). Given the lack of sequence information for *U. necator*, the development of STSs will require significant research input.

Random amplified polymorphic DNA

The PCR for randomly amplified polymorphic DNA (RAPD) analysis is conducted with a short, single primer (often a decamer) of arbitrary sequence (Welsh & McClelland, 1990; Williams *et al.*, 1990). The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as the presence or absence of a particular amplified band. Additional sequence polymorphisms may be detected using restriction enzyme digestion of amplified products (Ouellet & Seifert, 1993; Rafalski & Tingey, 1993).

An increasing number of plant pathogenic ascomycetes has been subjected to RAPD analysis, including *E. graminis* f. sp. *hordei* (Goodwin & Annis, 1991; Guthrie *et al.*, 1992; Haemmerli *et al.*, 1992; Nicholson *et al.*, 1993; Ouellet & Seifert, 1993; McDermott *et al.*, 1994). This technique has also been used for other plant pathogens, including *Peronospora parasitica* (Tham *et al.*, 1994), *Pseudocercosporella herpotrichoides* (Nicholson & Rezanoor, 1994), *Fusarium oxysporum* f.sp. *cucurbitae* (Crowhurst *et al.*, 1991), *Botrytis cinerea* (Van der Vlugt-Bergmans *et al.*, 1993), *Rhizoctonia solani* (Duncan *et al.*, 1993) and *Cronartium quercuum* f. sp. *fusiforme* (Doudrick *et al.*, 1993). In developing RAPD primers, significant numbers of primers may need to be screened before useful polymorphisms are detected (Ouellet & Seifert, 1993; Van der Vlugt-Bergmans *et al.*, 1993). Many DNA fragments may be amplified by a random primer, but a common number is between one to five fragments per isolate per oligonucleotide decamer. More than one primer may be needed to generate data for a DNA profile to distinguish genetically different isolates. Therefore, RAPD analysis can be similar to RFLP analysis using multiple single- or low-copy probes. Both RAPD and RFLP markers exhibit Mendelian inheritance (Waugh & Powell, 1992). Whereas RFLP markers are co-dominant, RAPD markers are dominant with alternate alleles being "null". It cannot be assumed that null alleles represent the same genetic state because different rearrangements of the genome can lead to the loss of a particular amplification product.

For obligate biotrophs, a major advantage of RAPD analysis is the small amounts of DNA required. For example, McDermott *et al.* (1994) obtained reproducible banding patterns for *E. graminis* f. sp. *hordei* using 1 - 5 ng of DNA from conidia. In addition, they reported that contaminating DNA must be greater than 5% of the DNA mixture before it is

detectable, but no data were presented to support this statement. Délye *et al.* (1995) reported a RAPD assay for *U. necator* using total DNA from mycelia and conidia scraped off grapevine leaves. Sixteen out of 95 primers screened against 13 *U. necator* strains, from France, Spain and Portugal, revealed DNA polymorphisms. There are constraints, however, associated with this approach. High DNA purity is required for RAPD analysis (Arnheim & Erlich, 1992; Cenis, 1992) and reaction conditions must be rigorously optimised and standardized for reproducible amplification of products (Devos & Gale, 1992; Duncan *et al.*, 1993). RAPD fingerprints may be reproduced in different laboratories and in different thermocyclers but only when the same reaction and thermocycle (temperature profile) conditions are used (Tommerup *et al.*, 1995). In contrast, RFLP probes are readily transferred from one laboratory to another.

There is much discussion within the literature on crop improvement about the relative merits of RAPDs and RFLPs as genetic markers (Rafalski *et al.*, 1991; Waugh & Powell, 1992; Rafalski & Tingey, 1993; Ragot & Hoisington, 1993; Hallden *et al.*, 1994; Santos *et al.*, 1994). The immediate advantages of RAPDs over RFLPs are the smaller quantities of DNA required and the rapidity of the technique. The whole analysis can be completed in one working day compared to at least 3 days for RFLPs, although much of the RFLP analysis time is "passive". Furthermore, there is no need to clone DNA to find a suitable probe. In the longer term, the benefit-cost ratio of each technique will vary depending on the research objective. Variables such as sample size, number of re-uses of Southern blots, or the cost of DNA polymerase must be factored into the benefit-cost equation (Ragot & Hoisington, 1993). In addition, RFLPs may reveal genetic variation in some cases where RAPDs do not, and vice versa.

Sequence tagged sites

As described previously, the eukaryotic genome is interspersed with simple, tandemly-repeated motifs. These microsatellites exhibit site-specific length variation (Olsen *et al.*, 1989; Beckman & Soller, 1990). Specific microsatellite repeats can be amplified using a pair of unique oligonucleotides flanking the microsatellite repeat. Microsatellite motifs often represent variable, polyallelic genetic loci (Litt & Luty, 1989; Beckman & Soller, 1990). Unlike VNTR loci in the human genome, which appear to be concentrated in the proterminal

regions of chromosomes, microsatellites are dispersed throughout the genome, in transcribed as well as non-transcribed sequences (Braaten *et al.*, 1988; Royle *et al.*, 1988).

Microsatellite sequences can be used as single primers in amplification reactions similar to RAPD reactions (Gupta *et al.*, 1994; Schlick *et al.*, 1994). The development of an STS marker, however, requires sequence information obtained from cloned DNA. Methods for identifying clones with microsatellite sequences and sequencing strategies are rapidly becoming more efficient, as reported by Thomas *et al.* (1993) and Thomas and Scott (1994), who developed STS markers to identify cvs of *V. vinifera*. These techniques will be applicable to *U. necator*, provided DNA amplification products are unique to *U. necator* when using DNA from infected host tissue. Rafalski and Tingey (1993) compare the properties of RFLP, RAPD and microsatellite systems for generating genetic markers. Microsatellite technology appears to have the highest development and start-up costs and this feature must be factored into the research budget. A benefit of this technique is that, unlike DNA probes that must be physically transferred between laboratories, specific primers can be generated within a single laboratory or research institution.

Another type of STS is located within rDNA regions. These regions contain both variable and conserved regions, allowing discrimination at the genus, species or intraspecific level (White *et al.*, 1990). At the intraspecific level, variation is most likely to occur in the noncoding regions of rDNA. That is, the ITS regions and the IGS sequence that separates ribosomal repeat units (Henrion *et al.*, 1992; Poupard *et al.*, 1993; Edel *et al.*, 1995). Sequences from both these regions can be amplified with primers designed using sequence information from the conserved region of rDNA in all fungal species (White *et al.*, 1990; Henrion *et al.*, 1992). If the amplifed ITS regions show no variation at the intraspecific level, then polymorphisms may be identified by restriction enzyme digestion of the amplified products or by direct sequencing. If the ITS region is sequenced, more specific primers for identifying strains or pathotypes may be designed (Poupard *et al.*, 1993; Bryan *et al.*, 1995). The IGS region has been used to differentiate strains within *Fusarium oxysporum* (Appel & Gordon, 1995; Edel *et al.*, 1995) and within *Laccaria* spp. The utility of these approaches for detecting intraspecific variation in *U. necator* would need to be determined empirically.

2.6.3.4 Extrachromosomal genetic elements

Extrachromasomal genetic elements such as dsRNAs and DNA plasmids may be useful as genetic markers if these elements are retained during asexual propagation. Double-stranded RNAs are widespread in plant pathogenic fungi and include a diverse collection of genetic elements such as mycoviruses, virus-like particles and unencapsidated dsRNA (for review see Nuss & Koltin, 1990). In some cases, the presence of dsRNA is correlated with phenotypic effects such as hypovirulence, for example in *Cryphonectria parasitica* (Chen *et al.*, 1994), or hypervirulence, as has been demonstrated in *Phytophthora infestans* (Tooley *et al.*, 1989). Where phenotypic effects are absent, the polymorphic nature of dsRNA profiles provides a useful genetic marker. For example, the electrophoretic patterns of dsRNA from 14 Australian isolates of *Colletotrichum gloeosporioides* revealed different patterns for Type A and Type B isolates (Dale *et al.*, 1988). Eight dsRNA species, ranging in molecular weight (relative molecular mass) from 0.95 x 10^6 to 6.6 x 10^6, were detected in conidia and cleistothecia of New York State isolates of *U. necator* (Azzam & Gonsalves, 1991; Azzam *et al.*, 1991). Isometric and rigid rod-like particles were observed in cleistothecia, which were squashed on a glass slide in water, stained with 1% uranyl acetate, and examined using transmission electron microscopy. The location of the particles within the cleistothecium is unknown. Further studies are needed to examine the distribution of these dsRNA species in structures of *U. necator* obtained from different vineyards and geographic regions.

Intraspecific variation with respect to fungal plasmid DNA may be due to (a) its presence or absence in total DNA, (b) variations in plasmid size, and/or (c) homology with related plasmids (Kinsey, 1985; Kistler & Leong, 1986; Hashiba, 1987; Giese *et al.*, 1990). Many linear plasmids found in fungi are maternally inherited and appear to be localized in the mitochondria (Tudzynski & Esser, 1985). Some plasmid-like DNAs are defective deletion variants of segments of the normal mitochondrial chromosome (Kinsey, 1985). Little is known about the function of plasmids because no phenotype has been associated with the presence of plasmid DNAs in most fungi. The exceptions are yeast plasmids, which may have killer and immunity functions, and plasmids of *Neurospora* and *Podospora anserina*, which confer senescence phenotype (Samac & Leong, 1989; Meinhardt *et al.*, 1990). Like dsRNA, stable variation in plasmids with no effect on phenotype can provide useful genetic markers. For example, strains of *Fusarium oxysporum* f.sp. *conglutinans* that infected

radish could be distinguished from strains infecting cabbage by the lack of DNA homology between similar-sized mitochondrial plasmids (Kistler & Leong, 1986). Giese *et al.* (1990) detected plasmid-like DNA in the mitochondria of certain isolates of *E. graminis* f.sp. *hordei* and, as the plasmid appeared to be inherited maternally, it may be a useful cytoplasmic marker. Plasmids have not yet been detected in *U. necator*.

2.6.4 Evaluating molecular markers

One of the prime objectives of developing molecular markers for *U. necator* is to distinguish genetically different individuals, or genets. For a marker or set of markers to be useful, two criteria must be satisfied. The first criterion is that the DNA profile or fingerprint must be variable enough to give unique patterns for each genetically different individual. The second criterion is that the mutation rate for each marker must not be so high that clonal identity changes too often relative to the time scale of interest; that is, somatic stability must be demonstrated. For example, tests for the genetic relatedness of strains may not be reliable if mutations occur at marker loci during successive asexual generations which occur during routine culture maintenance. Spontaneous mutations are known to occur frequently at certain DNA fingerprint loci (Wu & Magill, 1995). For example, the MGR583 clone of *Magnaporthe grisea* has homology with a known transposable element (Valent & Chumley, 1991). This feature of certain fingerprint clones may explain changes in fingerprint patterns over relatively few asexual generations.

For estimates of relatedness or genetic diversity, the criteria for usefulness of markers become more stringent. That is, it is desirable to have simple Mendelian inheritance of the markers, no linkage among loci, and identifiable alleles at each locus. For example, the calculation of genetic-diversity statistics such as Nei's (1973) statistic will be correct only if: (1) each DNA band represents only one genetic locus; (2) each locus is observed only as a single DNA fragment length; (3) none of the loci scored are genetically linked; (4) no locus is subject to natural selection and (5) no locus is linked to a gene which is affected by selection. Brown and Simpson (1994) used these criteria to test the validity of using the multi-copy DNA probe, E9, for quantitative genetic analyses of *E. graminis* f.sp. *hordei*. Some of the criteria listed above were not satisfied and they concluded that the probe should be used only for determining clonal identity. Similar genetic analyses of DNA fingerprints of

Phytophthora infestans (Goodwin *et al.*, 1992b) and *Cryphonectria parasitica* (Milgroom *et al.*, 1992a) illustrate that linkage between some fingerprint loci is likely to occur. DNA fingerprint similarity is often used to make inferences about population genetic structure. Lynch (1991) states that DNA-fingerprint data can provide approximations of measures of population structure, for example relatedness, and highlights issues concerning biased estimates of the conventional population statistics.

2.6.5 Applications of molecular markers for *U. necator*

Certain applications of molecular markers for *U. necator* have already been discussed, including clonal identification and the estimation of genetic diversity and population structure. In this section, these applications will be examined in more detail and will include the use of markers as techniques for studying population genetics, the genetic bases for variation and how this information can be used to determine appropriate disease management strategies.

2.6.5.1 Population structure

A population's structure is often greatly influenced by the prevailing mode of reproduction. Tibayrenc *et al.* (1991) propose that the mode of reproduction of microorganisms in nature can be determined only by population genetic information. By quantifying the frequency distribution of genotypes, using molecular techniques, evidence for clonal reproduction can be obtained by criteria such as: (a) genotype over-representation, for example a multi-locus genotype may be present in excess over other genotypes; (b) absence of recombinant genotypes; and (c) non-random association between loci (linkage disequilibrium). In the clonal model, individuals do not share a common gene pool, but consist of independently propagating clonal lineages. Therefore, a clonal lineage is a sub-set of the population in which all genotypes originate from the same clone (Anderson & Kohn, 1995). Evidence for clonal reproduction does not mean that sexual reproduction is absent but, rather, that it may be infrequent. For example, Förster *et al.* (1994) obtained evidence that races of *Phytophthora sojae* have arisen both by progressive mutation (clonal evolution) and by infrequent outcrosses. The rice blast fungus, *Magnaporthe grisea*, exists as a limited number of clonal lineages based on high similarity of DNA fingerprint data. Moreover, some of

these fingerprint profiles have been stable for longer than 30 years (Levy *et al.*, 1991). An extreme case of clonal colonization is that of a lineage of *Armillaria bulbosa* which was demonstrated to be about 1,500 years old and covered 15 hectares of land (Smith *et al.*, 1992). In a population reproducing sexually, segregation and recombination result in genotype frequencies which exhibit random associations between loci. Sexual reproduction is an important factor contributing to the genetic diversity of *Mycosphaerella graminicola* (McDonald & Martinez, 1991). As described in Section 2.6.3.2, large numbers of isolates were collected from a single wheat field over a 3-year period. During this time, there were no significant changes in allele frequencies for eight RFLP loci and there were no DNA fingerprints common across years (McDermott & McDonald, 1993).

One of the most important questions in biology is whether or not sexual reproduction is crucial in the evolutionary process (Ebert & Lorenzi, 1994). Theoretical geneticists hypothesize that sexual species adapt to environmental change more quickly than asexual species (Peck, 1994). In theory, sex ensures continued appearance of appropriate genotypes and clonal reproduction allows their rapid colonization and indefinite proliferation (Sebens & Thorne, 1985). *U. necator*, which can utilize sexual and asexual reproduction, appears to have considerable evolutionary potential. Theoretical models and population studies of model eukaryotes, using molecular markers, may eventually elucidate these unanswered questions.

The population dynamics of plant pathogenic fungi depend not only on the reproductive system but also on all gene flow mechanisms including migration, extinction and recolonization, and movement of genes via extrachromosomal genetic elements. Migration of new genotypes across continents can be monitored by using phenotypic and molecular markers. For example, the migration of *Phytophthora infestans* has been investigated using mating type, allozyme loci and DNA fingerprinting probes. A genetically diverse Mexican population appears to be the source of migrants, especially of the A2 mating type, to other regions of the world. Existing populations, representing a single clonal lineage, have been replaced by more diverse populations through sexual recombination events resulting from the introduction of the A2 mating type (Drenth *et al.*, 1994). Evidence for the evolution of more genetically diverse populations is based on the appearance of many

different DNA fingerprints. Unfortunately, there are no data on the genetic diversity of *U. necator* in Australia prior to the appearance of the opposite mating type.

Molecular and phenotypic markers have been used extensively to study the population genetics of the *E. graminis -H. vulgare* pathosystem (for review see Wolfe & McDermott, 1994). This pathosystem demonstrates the mechanisms of gene flow and selection in determining population structure. The movement of genotypes across Europe has been tracked extensively using variation in phenotype, specifically virulence and fungicide sensitivity, or DNA profiles. It appears that *E. graminis* genotypes are capable of long-distance migration by the dispersal of wind-borne conidia. In Europe, the pathogen appears to consist of a single gene pool with local differential selection causing distinct sub-populations. For example, following the introduction of a barley resistance gene, the pathogen population usually adapts quickly to overcome the resistance. The deployment of the *Mla13* resistance gene illustrates gene flow followed by selection. This gene was introduced into the former Czechoslovakia in the late 1970s. After 6 to 7 years the pathogen population became dominated by the virulence gene *Va13*. *Mla13* resistance was not introduced to Switzerland until 1987, but it only took 2 years for the population to become dominated by *Va13*. Using RAPD and virulence markers, the pathogen population in both the former Czechoslovakia and Switzerland were found still to be polymorphic (Wolfe *et al.*, 1992; Wolfe & McDermott, 1994). The more common clones, however, were identical in Switzerland and Czechoslovakia, indicating gene flow between the two countries.

In addition to the prevailing reproductive mode, the population structure of *U. necator* is likely to be influenced by a number of environmental factors. These include limits on pathogen colonization, such as the distribution of the host species and the fact that this fungus is subject to local extinction and recolonization events. Wind dispersal of conidia and/or ascospores may influence gene flow, as will selective pressures such as repeated use of DMI fungicides. All these factors can be used to model gene flow and assess the impact of new disease control strategies such as the introduction of host resistance genes.

2.6.5.2 Genetic and epidemiological studies

Molecular markers have a multitude of applications in experiments where it is necessary to track the fate of clonal lines or to examine the inheritance of genetic markers. In the case of

Cryphonectria parasitica, biological control depends on the transmission of a hypovirulence factor associated with dsRNA. In order to understand the population structure of this pathogen, Milgroom and Lipari (1993) developed mtDNA markers to track the dispersal of ascospores. A first step in their research was to obtain evidence that mtDNA markers are inherited maternally and, therefore, do not recombine during sexual reproduction. DNA fingerprints of both parental strains and ascospore progeny of sexual crosses were obtained using total digested DNA probed with a nuclear fingerprinting sequence and with purified mtDNA. The segregation of the DNA fingerprint fragments confirmed that outcrossing had occurred and the mtDNA RFLP patterns of the progeny were identical to that of the maternal parental isolate.

The above example illustrates the use of molecular markers in inheritance studies. Ecological studies also provide evidence for other types of genetic interactions. For example, Adachi and Tsuge (1994) used RFLP analysis of nuclear ribosomal RNA to track single spore-derived isolates within a single black spot lesion on a Japanese pear leaf. Their study provided evidence for the frequent occurrence of co-infection in single black spot lesions by different isolates of *Alternaria alternata,* a phenomenon which has also been noted in lesions infected with *Mycosphaerella graminicola* (McDonald & Martinez, 1990b). These events raise questions about the genetic interaction of these isolates through recombination, whether sexual or asexual.

The ability to track isolates of *U. necator* will have applications ranging from ecological to inheritance studies. Markers may be used to (a) follow the inheritance of DNA markers in sexual crosses or the inheritance of extrachromosomal genetic elements, (b) obtain evidence for mitotic recombination in asexual crosses, and (c) track the short or long distance migration of genotypes. Markers linked to certain phenotypes, for example virulence genes, allow the identification of genes and provide the tools for examining the genetic basis of change in phenotype. The ability to find neutral genetic markers linked to a phenotype, however, will depend on how tightly the linkage is maintained. That is, linkage may be tight within a clonal lineage but may not be maintained if recombination occurs. Where recombination is frequent, DNA markers may need to be located close to the target gene.

While molecular markers are useful for tracking clonal lineages or sexual progeny, a more complete understanding of pathogen evolution can be obtained by studying the genetic bases for variation. The first step in this process is to elucidate genome organization through the development of genetic maps. Genetic maps are being prepared for several plant pathogenic fungi, including *Bremia lactucae*, *Phytophthora infestans* and *Magnaporthe grisea* (Michelmore & Hulbert, 1987; Hulbert *et al.*, 1988; Skinner *et al.*, 1993). In the case of *Bremia lactucae*, DNA markers have been used to demonstrate somatic fusion as a mechanism for the generation of new pathotypes (Hulbert & Michelmore, 1988).

2.6.5.3 Pathogen evolution and disease management

Once genetic diversity has been identified within a pathogen population it is vital to understand how this diversity is created in order to understand the rate and nature of pathogen evolution. Knowledge of genome organization, mechanisms for genetic variation, population structures and gene flow all contribute to the overall understanding of pathogen evolution.

While knowledge of the genetic bases for variation in most plant pathogenic fungi is limited, significant advances in the knowledge of population structures are already providing clues as to which disease management strategies will be sustainable. For example, studies on the evolution of pathogenicity within clonal lineages, as defined by DNA fingerprint pattern, allozyme genotype and mating type, of *Phytophthora infestans* in the United States and Canada reveal the probable breakdown of existing North American sources of major-gene resistance (Fry *et al.*, 1993; Goodwin *et al.*, 1995). Given that recent *P. infestans* migrants from northwestern Mexico overcame all potato and tomato resistances tested, one scenario is that effective control of this disease using existing resistance genes will diminish (Goodwin *et al.*, 1995). The migration of *P. infestans* may also alter the epidemiology of the disease on tomatoes. Prior to migration, most US isolates did not infect tomato (Goodwin *et al.*, 1995). Wider distribution of new genotypes which infect tomato means that pathologists must consider the possibility of the spread of inoculum from potato to tomato crops.

Correlation of the population structure with the reproductive mode, for example, provides the theoretical bases for the disease management strategies which might result in

durable crop resistance and/or decreased frequencies of fungicide tolerant strains. In populations reproducing clonally, pathotypes are often less diverse within clonal lineages than between them and each clonal lineage is associated with a particular subset of pathotypes. Clonal lineages do not share a common gene pool and so a mutation in one lineage does not affect other lineages. Examples of plant pathogenic fungi to which this phenomenon appears to apply include *Magnaporthe grisea* (Levy *et al.*, 1991; Levy *et al.*, 1993; Xia *et al.*, 1993), *Melamspora lini* (Burdon & Roberts, 1995), *Phytophthora infestans* (Goodwin *et al.*, 1995) and *Rhynchosporium secalis* (Goodwin *et al.*, 1992a). In the case of *Phytophthora infestans* in the United States and Canada, mutation, selection and genetic drift have probably contributed to changes in pathogenic variation observed within clonal lineages where older lineages show more pathogenic variation than those that were introduced recently (Goodwin *et al.*, 1995). There is evidence to suggest, however, that mutation rates at pathogenicity loci are higher than at the molecular loci that define a clonal lineage, which emphasizes the point of not relying solely on molecular data for interpreting evolutionary events. In contrast, sexual recombination may lead to large numbers of pathotypes in a small geographical area (Goodwin *et al.*, 1992a); that is, individuals share a common gene pool and genetically distinct groups may be absent. How new pathotypes are created has impact on strategies for deployment of resistance genes. For example, if genetic variation occurs on a fine scale within individual crops then resistance genes may need to be deployed on a fine scale, by using cultivar mixtures. On the other hand, if there are are strong correlations between clonal lineages and pathotypes, one breeding strategy might be to choose combinations of resistance genes within a single host genotype. This strategy, however, may lead to selection for more complex pathotypes, capable of overcoming all resistance genes in a single host.

2.7 SUMMARY

This review of the literature on grapevine powdery mildew and the biology of *U. necator*, highlights the gaps in current knowledge with regard to the efficient management of this disease. In summary, effective chemical control methods are available but the threat of fungicide resistance, and regulatory and marketplace demands will eventually force growers to become less reliant on fungicides. The development of crop stage/weather-based spray

schedules based on epidemiological models is a first step to reduce unnecessary fungicide sprays. Sensitivity data for DMI fungicides are required so that strategies for delaying fungicide resistance can be implemented more effectively. The development of more efficient chemical technologies, cultural control methods and biological control agents may also allow a more integrated and sustainable approach to disease control. In the long term, studies on the genetic basis of powdery mildew resistance should continue so that durable resistance can be incorporated rapidly into accepted genetic backgrounds.

One issue to be resolved is the role of the sexual stage in disease development, including the relative importance of ascospores and conidia during primary infection events. If cleistothecia are a significant source of primary inoculum in Australia then methods for reducing numbers of viable cleistothecia should be investigated, and the subsequent effect on disease development established. In situations where the number of infections by ascospores is low, sexual reproduction could still be playing a role in the biology of *U. necator* by generating variants, specifically novel pathotypes and/or increased frequencies of fungicide tolerant strains via selection. Therefore, epidemiological studies, involving vineyard monitoring for cleistothecia, ascospores and flag shoots, will provide information for more fundamental research on fungal biology and ecology.

The development of efficient disease management strategies ultimately relies on the ability to quantify the changes in populations of plant pathogens associated with populations of plants. An important step in this process is to characterize the pathogen, using molecular-genetic and phenotypic markers, so that population biology can be studied. The development of molecular markers was reviewed in theoretical and practical terms, including technical requirements, cost-effectiveness and applicability to the *U. necator-Vitaceae* pathosystem. The relative usefulness of markers based on RFLP, RAPD, STS and extrachromosomal genetic-elements was evaluated, followed by a discussion of the appropriate use of DNA fingerprints and profiles in genetic studies. While the usefulness of molecular markers must be determined empirically, markers specific for *U. necator* should be identified among RFLP and STS markers. The latter approach requires more research input but may be the most efficient technique, once developed.

Potential applications of markers for *U. necator* were explored using examples from other pathosystems. These applications include studies of population structure, genetics and

epidemiology, the data from which can be combined to determine the rate and nature of pathogen evolution, and the likely long term success of alternative disease management strategies.

2.8 RESEARCH OBJECTIVES

Having defined current priorities for research through a critical evaluation of the literature, the objectives of the work presented in this thesis are:

1. to develop an experimental system for the molecular-genetic characterization of *U. necator*;
2. to develop an *in vitro* mating system for
   (i) identifying mating types of Australian isolates, and for
   (ii) conventional genetic analyses;
3. to develop RFLP markers for distinguishing genetically different isolates of *U. necator*, including probes which are specific to *U. necator*; and
4. to investigate the genetic diversity of *U. necator* in Australia using the DNA markers developed in this project.

These objectives form part of a long term research objective to understand the genetic bases for variation in *U. necator* and the rate and nature of the co-evolution of *U. necator* and members of *Vitaceae* in a changing environment.

3.0 General Materials and Methods

3.1 Isolation of clonal lines of *U. necator*

Diseased leaves or berries were collected from various Australian vineyards and transported directly to the laboratory. All diseased material came from non-phylloxera infested regions of South Australia, Western Australia and Tasmania. Diseased material was collected from non-phylloxera infested areas of New South Wales but conidia were non-viable after transportation by air to South Australia. Each disease sample came from a single block or row of vines, usually from several adjacent panels of vines.

A detached leaf technique was developed for the isolation of clonal lines of *U. necator* (Table 3.1). Clonal lines are defined as single conidial-chain isolates of *U. necator* which are cultured continuously on grapevine tissue. Leaves were collected from glasshouse grown *Vitis vinifera* cv. Cabernet Sauvignon, clone G9V3. Grapevines were propagated from hardwood cuttings obtained from Registered Source Areas (Riverland Vine Improvement Committee, Barmera, South Australia) and maintained free from powdery mildew using vapours of penconazole (Szkolnik, 1983). Cuttings with roots and dormant buds were planted into 20 cm diam. pots containing U. C. potting mixture (Baker, 1957). After 6 weeks, cuttings with shoots were fertilized fortnightly with a complete water soluble fertilizer (1.6 g l^{-1} Aquasol® (NPK 23:4:18), Hortico, Laverton North, Victoria). During the autumn and winter, active shoot growth was maintained by providing a 1 h photoperiod, from 00.00 to 01.00 h, from a string of 60 W incandescent bulbs 40 cm apart and located above the plants (K. Skene, personal communication).

For detached leaf culture, leaves were selected to fit into a 10 cm diam., 2 cm deep Petri plate containing 20 ml of 10 g l^{-1} distilled water agar (Bitek, Difco Laboratories, Michigan). All manipulations were done in a laminar-flow hood. Four sterile toothpicks were placed parallel to each other at 1.5 cm intervals on the agar surface. Leaves were washed in distilled water and a fresh cut was made to the basal end of the petiole before surface sterilization in 0.5 g l^{-1} sodium hypochlorite for 3 min. The leaves were then rinsed three times in sterile distilled water and placed on the bed of toothpicks, with the basal 2-4 mm of the petiole immersed in the agar. The upper surface of each leaf was dried completely

Table 3.1 Origin of clonal lines of *U. necator*.

Clonal line[c]	Source[a] Host cv. and organ	Location	Date of collection of diseased material
920101	Chenin Blanc leaves	Waite Campus, Adelaide Plains, SA	Nov. 1992
920102	Chenin Blanc leaves	Waite Campus, Adelaide Plains, SA	Nov. 1992
920103[c]	Chenin Blanc leaves	Waite Campus, Adelaide Plains, SA	Nov. 1992
920104	Chenin Blanc leaves	Waite Campus, Adelaide Plains, SA	Nov. 1992
920201	Chenin Blanc leaves	Waite Campus, Adelaide Plains, SA	Dec. 1992
920202	Chenin Blanc leaves	Waite Campus, Adelaide Plains, SA	Dec. 1992
920301	Chardonnay leaves	Loxton, Riverland, SA	Dec. 1992
920302	Chardonnay leaves	Loxton, Riverland, SA	Dec. 1992
920401	Chardonnay leaves	Loxton, Riverland, SA	Dec. 1992
920401	Chardonnay leaves	Loxton, Riverland, SA	Dec. 1992
930101[c]	Chardonnay berries	Block 1, Nuriootpa, Barossa Valley, SA	Jan. 1993
930102	Chardonnay berries	Block 1, Nuriootpa, Barossa Valley, SA	Jan. 1993
930103	Chardonnay berries	Block 1, Nuriootpa, Barossa Valley, SA	Jan. 1993
930104	Chardonnay berries	Block 1, Nuriootpa, Barossa Valley, SA	Jan. 1993
930201	White Grenache berries	Block 2, Nuriootpa, Barossa Valley, SA	Jan. 1993
930202[c]	White Grenache berries	Block 2, Nuriootpa, Barossa Valley, SA	Jan. 1993
930203	White Grenache berries	Block 2, Nuriootpa, Barossa Valley, SA	Jan. 1993
930301	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930302[c]	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930303	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930304	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930305	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930401	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930402	Crouchon leaves	Block 3, Nuriootpa, Barossa Valley, SA	Jan. 1993
930501	Chardonnay berries	Pridmore, McLaren Flat, SA	Jan. 1993
930502	Chardonnay berries	Pridmore, McLaren Flat, SA	Jan. 1993
930503	Chardonnay berries	Pridmore, McLaren Flat, SA	Jan. 1993
930504	Chardonnay berries	Pridmore, McLaren Flat, SA	Jan. 1993
930601	Chardonnay berries	Trot, McLaren Flat, SA	Jan. 1993
930602	Chardonnay berries	Trot, McLaren Flat, SA	Jan. 1993
930603	Chardonnay berries	Trot, McLaren Flat, SA	Jan. 1993
930701	cv. unknown, berries	Flinders University, Adelaide Plains, SA	Jan. 1993
930702	cv. unknown, berries	Flinders University, Adelaide Plains, SA	Jan. 1993
930703	cv. unknown, berries	Flinders University, Adelaide Plains, SA	Jan. 1993
930704	cv. unknown, berries	Flinders University, Adelaide Plains, SA	Jan. 1993
930801[c]	*V. amurensis* leaves	Home garden, Adelaide Plains, SA	Jan. 1993
930802	*V. amurensis* leaves	Home garden, Adelaide Plains, SA	Jan. 1993
930803	*V. amurensis* leaves	Home garden, Adelaide Plains, SA	Jan. 1993
930804	*V. amurensis* leaves	Home garden, Adelaide Plains, SA	Jan. 1993
930901[c]	Sauvignon Blanc berries	Morialta, Adelaide Hills, SA	Jan. 1993
930902	Sauvignon Blanc berries	Morialta, Adelaide Hills, SA	Jan. 1993
931001	Chardonnay berries	Block 1, Summertown, Adelaide Hills, SA	Feb. 1993
931002[c]	Chardonnay berries	Block 1, Summertown, Adelaide Hills, SA	Feb. 1993
931101	Chardonnay berries	Block 2, Summertown, Adelaide Hills, SA	Feb. 1993
931102	Chardonnay berries	Block 2, Summertown, Adelaide Hills, SA	Feb. 1993
931201[c]	Chardonnay berries	Block 3, Summertown, Adelaide Hills, SA	Feb. 1993
931202	Chardonnay berries	Block 3, Summertown, Adelaide Hills, SA	Feb. 1993
931301	Chardonnay berries	Block 4, Summertown, Adelaide Hills, SA	Feb. 1993
931302[c]	Chardonnay berries	Block 4, Summertown, Adelaide Hills, SA	Feb. 1993

Table 3.1 continued on the next page

Table 3.1 (continued)

Clonal line[c]	Source[a] Host cv. and organ	Location	Date of collection of diseased material
931303	Chardonnay berries	Block 4, Summertown, Adelaide Hills, SA	Feb. 1993
931401	Chardonnay berries	Block 2, Summertown, Adelaide Hills, SA	Mar. 1993
931402	Chardonnay berries	Block 2, Summertown, Adelaide Hills, SA	Mar. 1993
931501	Chardonnay berries	Block 3, Summertown, Adelaide Hills, SA	Mar. 1993
931502	Chardonnay berries	Block 3, Summertown, Adelaide Hills, SA	Mar. 1993
940101	Sauvignon Blanc berries	Yallingup, Margaret River, WA	Dec. 1994
940102[c]	Sauvignon Blanc berries	Yallingup, Margaret River, WA	Dec. 1994
940103	Sauvignon Blanc berries	Yallingup, Margaret River, WA	Dec. 1994
940104	Sauvignon Blanc berries	Yallingup, Margaret River, WA	Dec. 1994
940201	Flame Seedless berries	Block 1, Swan Valley Research Station, WA	Jan. 1994
940202	Flame Seedless berries	Block 1, Swan Valley Research Station, WA	Jan. 1994
940203	Flame Seedless berries	Block 1, Swan Valley Research Station, WA	Jan. 1994
940204	Flame Seedless berries	Block 1, Swan Valley Research Station, WA	Jan. 1994
940301[c]	Perlette berries	Block 2, Swan Valley Research Station, WA	Jan. 1994
940302	Perlette berries	Block 2, Swan Valley Research Station, WA	Jan. 1994
940303	Perlette berries	Block 2, Swan Valley Research Station, WA	Jan. 1994
940304	Perlette berries	Block 2, Swan Valley Research Station, WA	Jan. 1994
940401	CG 4320[b] berries	Block 3, Swan Valley Research Station, WA	Jan. 1994
940402[c]	CG 4320[b] berries	Block 3, Swan Valley Research Station, WA	Jan. 1994
940403	CG 4320[b] berries	Block 3, Swan Valley Research Station, WA	Jan. 1994
940404	CG 4320[b] berries	Block 3, Swan Valley Research Station, WA	Jan. 1994
940501[c]	cv. unknown, berries	Block 1, Lindemans, Coonawarra, SA	Jan. 1994
940502	cv. unknown, berries	Block 1, Lindemans, Coonawarra, SA	Jan. 1994
940503	cv. unknown, berries	Block 1, Lindemans, Coonawarra, SA	Jan. 1994
940504	cv. unknown, berries	Block 1, Lindemans, Coonawarra, SA	Jan. 1994
940601	Traminer berries	Block 2, Lindemans, Coonawarra, SA	Jan. 1994
940602	Traminer berries	Block 2, Lindemans, Coonawarra, SA	Jan. 1994
940603	Traminer berries	Block 2, Lindemans, Coonawarra, SA	Jan. 1994
940604	Traminer berries	Block 2, Lindemans, Coonawarra, SA	Jan. 1994
940701	Chardonnay berries	Home garden, Launceston, Tas.	Mar. 1994
940702[c]	Chardonnay berries	Home garden, Launceston, Tas.	Mar. 1994
940703	Chardonnay berries	Home garden, Launceston, Tas.	Mar. 1994
940704	Chardonnay berries	Home garden, Launceston, Tas.	Mar. 1994

[a] All clonal lines except 930801-930804 were obtained from *Vitis vinifera*.
SA = South Australia, WA = Western Australia, Tas. = Tasmania.

[b] Accession No. IC758274.

[c] Clonal lines used for quantification of conidial yields.

within the laminar-flow hood. The bed of toothpicks raised the leaf slightly above the agar surface, providing a dry leaf surface for colonization by *U. necator*.

A sterile artist's paint brush was used to brush conidia, from diseased leaves or berries, onto the detached leaf cultures described above. At least three detached leaf cultures were prepared to sample conidia randomly from the bulked disease material from each location. The lid of the Petri plate was replaced and the plate sealed with Parafilm®. The plates were incubated at a slight angle (approx. 10°) in an illuminated growth chamber (330 $\mu E\ s^{-1}\ m^{-2}$ from cool white fluorescent bulbs) with a 12 h photoperiod at 25°C. Individual chains of conidia, which were free from visible contamination by other microbes, were transferred from 10 to 12 day-old colonies to healthy detached leaf cultures using the method described by Gadoury & Pearson (1991). A single artist's paintbrush hair attached to a dissecting needle with petroleum jelly was used to transfer individual chains of conidia. Six individual chains of conidia were selected at random from colonies growing on the detached leaves and transferred to separate locations on a healthy detached leaf. This procedure was repeated at least twice. At least one out of every six single-conidial chains produced a sporulating colony. Ten to 12-day-old colonies, which were free from visible microbial contamination, were transferred individually, using a sterile artist's paint brush, either to a healthy detached leaf culture or directly to a micropropagated grapevine for culture maintenance *in vitro*. Conidia were transferred to micropropagated grapevines *in vitro* with greater than 99% of inoculations producing an infection without microbial contamination. Conidia of isolates maintained on detached leaf cultures were transferred to healthy detached leaf cultures every 14 days. Clonal lines isolated during the 1992/93 growing season were maintained as detached leaf cultures for 2-5 months prior to being transferred to micropropagated grapevines because plantlets were not established *in vitro* at that time.

3.2 CULTURE MAINTENANCE

Clonal *U. necator* lines were maintained *in vitro* on *V. vinifera* cv. Cabernet Sauvignon, clone CW44 (Figure 3.1). Micropropagated shoots were kindly provided by Dr M. Barlass. Shoots were cut into nodal segments and five segments placed upright into 50 ml of maintenance medium in 250 ml polycarbonate culture tubes with polypropylene breather lids (Disposable Products, South Australia). The maintenance medium consisted of MS medium

Figure 3.1 Dual culture of *U. necator* and *V. vinifera* cv. Cabernet Sauvignon. The culture container is 11 cm tall.

(Murashige & Skoog, 1962) with all nutrients at half strength, 15 g l^{-1} sucrose, 0.01 mg l^{-1} α - napthaleneacetic acid (NAA) and 7 g l^{-1} agar (Bitek, Difco Laboratories, Michigan). The pH of the medium was adjusted to 5.8 with 1 N sodium hydroxide before the addition of the agar and autoclaving. Cultures were maintained at 25°C with a 16 h photoperiod (250-450 μE s^{-1} m^{-2} from cool fluorescent bulbs). After 10 days, most nodal segments had developed small roots and one segment per tube was transferred to 70 ml of the same maintenance medium, minus NAA, in a 500 ml culture tube with a breather lid. After approximately 21 days, the plantlet, which had developed at least five expanded leaves, was inoculated with *U. necator* conidia. The four plantlets remaining in the 250 ml culture tube were cut into nodal segments, approximately 31 days after the previous transfer and placed on fresh maintenance medium containing NAA. This culture cycle was repeated each month to provide a continuous supply of plantlets for dual culture with *U. necator*. Every 8 - 10 weeks conidia were transferred aseptically to healthy *in vitro* plantlets by removing an infected leaf and brushing the conidia against a leaf of the plantlet being inoculated (Gadoury & Pearson, 1988).

3.3 Mass production and collection of conidia

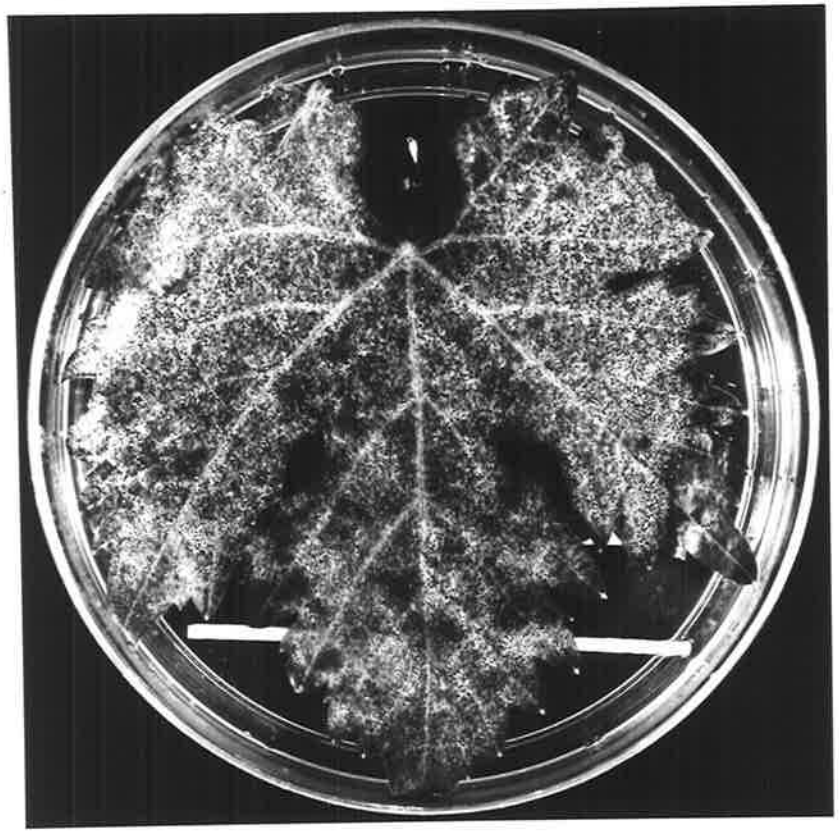
Conidia were mass produced by inoculating detached leaf cultures with aseptic conidia from infected micropropagated leaves by brushing the infected leaf over the leaf being inoculated. Following inoculation, conidia were harvested twice, after 12 and 16 days respectively, using a cyclone separator (Figure 3.2) connected to a vacuum pump (Tervet *et al.*, 1951; H. Wallwork, personal communication). The conidia were harvested inside a perspex hood that had been wiped with 70% ethanol to minimize contamination of the cultures and prevent cross contamination between isolates. The cyclone separator was rinsed with 70% ethanol and allowed to dry before each harvest. Conidia were collected directly into a 1.5 ml microfuge tube, frozen in liquid nitrogen, and stored at -70°C. Conidia harvested on different days were stored separately.

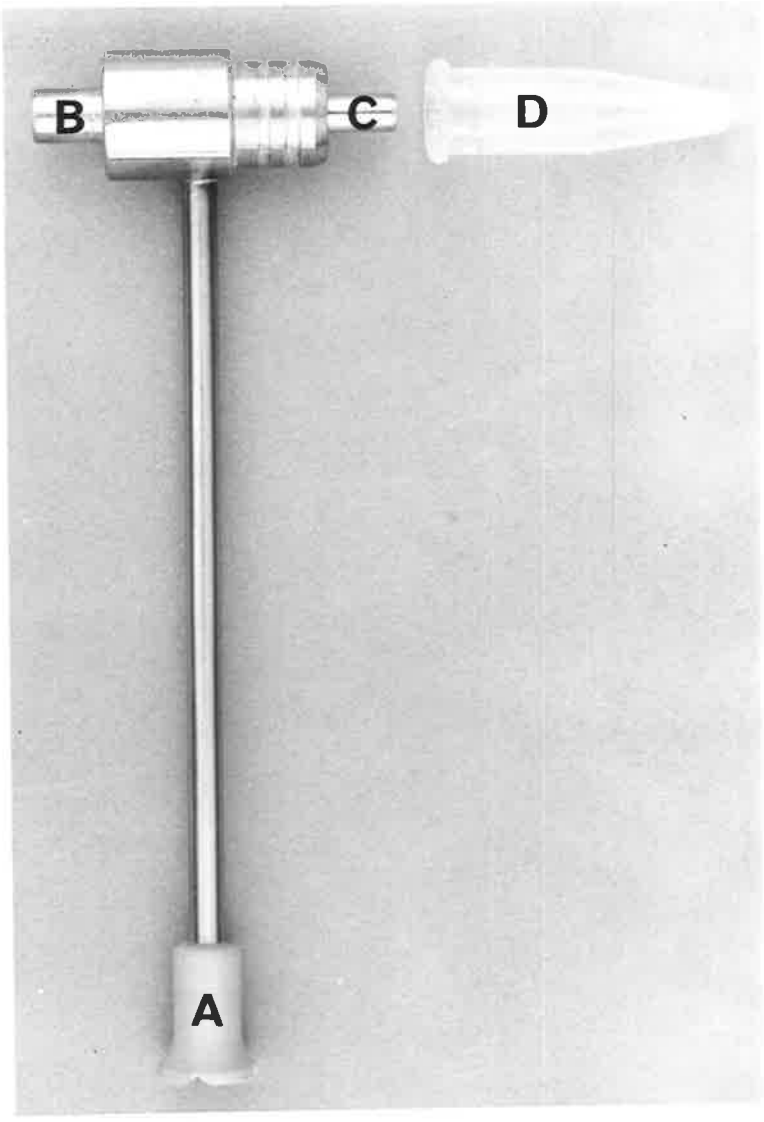
The yield of conidia from twenty detached leaf cultures was quantified for fourteen clonal lines of *U. necator* (marked by a "c" in Table 3.1). The mean yield (n=14) for the first harvest, at 12 days, was 70 mg conidia per 20 leaves (S.D. = 23.2), with yields ranging from 41-112 mg conidia per 20 leaves. The mean yield for the second harvest, at 16 days,

Figure 3.2 Mass production and collection of conidia of *U. necator*

Left. Sporulating colonies of *U. necator* on a detached grapevine leaf on water agar in a 10 cm diam. Petri plate, 12 days after inoculation.

Right. The cyclone separator device adapted for the collection of conidia into a 1.5 ml microfuge tube. **A**, The air intake is designed to prevent host tissue blocking the opening; **B**, Air is drawn by a vacuum pump connected with tubing (not illustrated); **C**, Conidia are separated from the air by a central tube extending below the point at which air enters the cyclone separator; **D**, The microfuge tube (length = 4 cm) is attached to the device with a piece of tubing (not illustrated) that encases both the microfuge tube and the arm of the device.




B
C
D
A

was 19 mg per 20 leaves (S.D. = 11.7), with yields ranging from 8-39 mg per 20 leaves. Variation in conidial yields appeared to be due to clonal line fitness and/or the health of the detached leaf. Conidia were harvested from sporulating colonies that were free from microbial contamination when examined at 50x magnification. Occasional contamination of the water agar by fungi was reduced by incorporation of 10 mg l^{-1} pimaricin. Conidia could be harvested a third time, up to 28 days after inoculation, but leaf senescence reduced the quantity of conidia collected to less than 10 mg per 20 leaves.

3.4 DNA EXTRACTION FROM CONIDIA

The DNA extraction method for conidia was modified from that of Whisson (1996) for *Erysiphe graminis* conidia and from that of Matthew, Herdina & Whisson (1995) for *Rhizoctonia solani* mycelia. A 700-900 µl volume of extraction buffer (refer to Appendix) containing 2 mg ml^{-1} predigested pronase was added to 30 to 40 mg frozen conidia. The conidia were suspended, pummeled with glass beads for 1 min and incubated according to the method of Whisson (1996).

The nucleic acids were extracted using phenol-chloroform. The volume of the final aqueous phase was increased to 500 µl with a solution containing 50 mM Tris-HCl, 20 mM EDTA, pH 8, and the nucleic acids were precipitated by the addition of 0.4 volumes (200 µl) of 4 M ammonium acetate, pH 5.2, and 0.6 volumes (300 µl) of cold isopropanol and placing the tube on ice for 2 h. The nucleic acid pellet, obtained after centrifugation at 15,800 ***g*** (Eppendorf centrifuge) for 20 min, was washed briefly with cold 70% ethanol containing 10 mM magnesium acetate, and dissolved in 50 µl of TE buffer (refer to Appendix). RNaseA was added to a final concentration of 50 µg ml^{-1} and incubated at 37°C for 30 min. The volume of the solution was then increased to 150 µl with TE buffer and extracted with chloroform/isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase by the addition of two volumes of cold absolute ethanol, overnight at -20°C. The solution was centrifuged as before for 20 min and the DNA pellet washed with cold 70% ethanol, 10 mM magnesium acetate for 30 min at 4°C, pelleted again, and dissolved in 20 µl TE buffer. The DNA solution was stored at -70°C until required.

The amount of DNA in each sample was estimated by running aliquots on a 1% agarose gel and visualizing the bands under uv light following ethidium bromide staining.

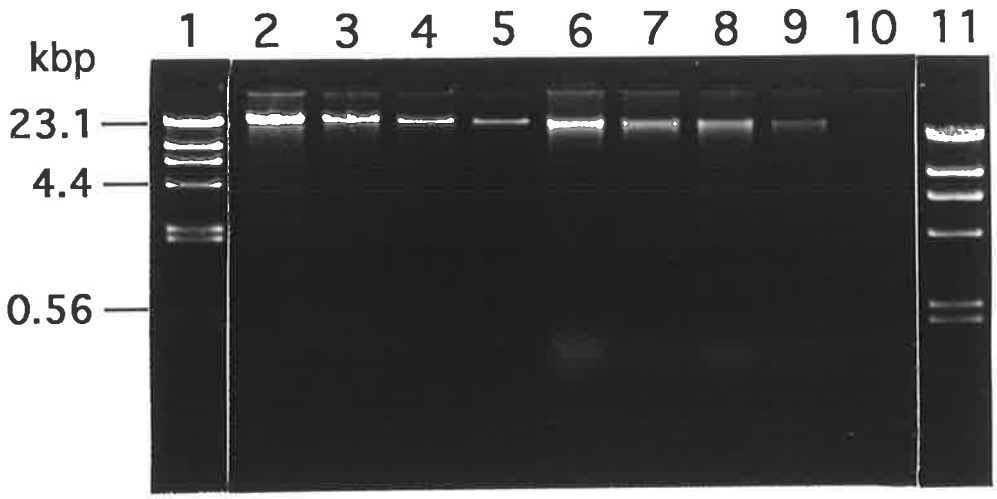
The intensity of the staining of *U. necator* bands was compared with that of known quantities of *Rhizoctonia solani* DNA and *Hin*dIII digested lambda DNA. DNA yields ranged from 170-280 ng per 30 mg of conidia (Figure 3.3), or 6-9 ng per mg conidia, which is similar to that reported by O'Dell *et al.* (1989) for *E. graminis* conidia. Using the large quantity of 20 g conidia they extracted approximately 5 ng per mg conidia. Furthermore, the acidic extraction buffer is simple and preliminary experiments have revealed that the pH of 5.2 produced DNA which was of higher quality than that obtained using an extraction buffer of pH 8. *U. necator* DNA could be digested by a range of restriction endonucleases including *Eco*RI, *Eco*RV, *Bam*HI, *Pst*I, *Sau*3A and *Taq*I (data not shown).

3.5 DNA EXTRACTION FROM MICROPROPAGATED GRAPEVINE LEAFLETS

The method for extracting DNA from infected micropropagated leaflets was modified from the procedure described in Section 3.4. Micropropagated leaflets with sporulating colonies of *U. necator* were harvested and frozen in liquid nitrogen. A quantity of 1.0 g of frozen tissue was ground to a fine powder in liquid nitrogen and suspended in seven volumes (7 ml) of cold extraction buffer (refer to Appendix) containing 100 mg of polyvinyl polypyrrolidone (PVPP) per g of tissue.

Two phenol-chloroform extractions were required to remove most of the protein and cell debris. For the first extraction, the aqueous phase was separated from the organic phase by centrifugation at 15,400 ***g*** for 30 min. The centrifugation time for the second phenol-chloroform extraction was reduced to 10 min. The aqueous phase was then extracted twice with chloroform/isoamyl alcohol (24:1). The nucleic acids were precipitated from the aqueous phase by the addition of 0.4 volumes of 4 M ammonium acetate, pH 5.2, and 0.6 volumes of cold isopropanol overnight at -20°C. The nucleic acid pellet, obtained after centrifugation at 15,400 ***g*** for 15 min, was washed with cold 70% ethanol containing 10 mM magnesium acetate, and dissolved in 200 µl of TE buffer. The yield of DNA was estimated by running aliquots on a 1% agarose gel and visualizing the bands under uv light following ethidium bromide staining. The intensity of the staining of bands was compared with that of known quantities of *Hin*dIII digested lambda DNA. This extraction procedure yielded at least 20 $\mu g\ g^{-1}$ leaf tissue. RNA was removed from the nucleic acid solution either during restriction enzyme digestion or as a separate step by adding RNase A to a final

Figure 3.3 Agarose gel electrophoresis of undigested total DNA from *U. necator* conidia. *Lane 1,* 0.5 µg lambda DNA digested with *Hin*dIII (fragment sizes: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kbp, respectively); *Lanes 2 to 5, Rhizoctonia solani* DNA, 200 ng, 100 ng, 50 ng and 25 ng, respectively; *Lanes 6 to 8, U. necator* DNA, 10µl of a 20 µl DNA solution prepared from 31.7 mg, 29.7 mg and 35.6 mg conidia of three different clonal lines, respectively; *Lane 9,* 5 µl taken from the same 20 µl solution as in lane 8; *Lane 10,* 1µl taken from the same 20 µl solution as in lane 8; *Lane 11,* 0.5 µg lambda DNA digested with *Hin*d III. This sample was loaded and run through the gel for a short distance before the other samples (Lanes 1 - 10) were loaded.


1 2 3 4 5 6 7 8 9 10 11
kbp
23.1
4.4
0.56

concentration of 0.1 mg ml^{-1} and incubating at 37°C for at least 30 min. The enzyme was removed by chloroform/isoamyl alcohol (24:1) extraction followed by precipitation of the DNA with two volumes of cold absolute ethanol, overnight at -20°C. The DNA was recovered by centrifugation, resuspended in TE buffer and stored at -20°C until required.

3.6 ELECTROPHORESIS, SOUTHERN TRANSFER, RADIOLABELLING AND HYBRIDISATION METHODS

For RFLP analysis, 10 or 80 ng of *U. necator* DNA was digested with one of a selection of restriction enzymes overnight at 37°C. The DNA fragments were fractionated by electrophoresis in 1% agarose gels, using TAE buffer (refer to Appendix), at 30 V for 5.5 - 6 h (0.5 cm thick, 10 cm-long gels) or 22 h (0.6 cm thick, 20 cm-long gels). Gels were stained with ethidium bromide to visualize the DNA before transfer to either 0.2 µM Biodyne A (Pall Corporation) or ZetaProbe Genomic Tested (Bio-Rad) membranes according to the method of Southern (1975). DNA was cross-linked to the nylon-backed nitrocellulose membranes using uv light from a GS Gene Linker (Bio-Rad). Between 25-100 ng of each DNA probe used in the Southern hyridisations was radiolabelled using 30-50 µCi α-^{32}P-dCTP by either (a) the random primer method (Feinberg & Vogelstein, 1983) using the components of the Megaprime DNA labelling system (Amersham) or (b) using denatured pUC19 specific primers and template DNA in a pUC19 specific oligolabelling buffer (refer to Appendix) at 37°C for 30-45 min. The labelled DNA was separated from unincorporated nucleotides either in a Bio-Gel P-60 column (refer to Appendix) or in Sephadex G-100 (medium grade suspended in TE buffer) in a 15 cm Pasteur pipette with a small quantity of glass wool in the neck. Radiolabelled probes were denatured by boiling for 5 min, cooled on ice, then added to the hybridisation solution. All membranes were pre-hybridised for a minimum of 4 h at 65°C, then hybridised for 20-24 h at 65°C in a rolling-bottle hybridisation oven. The membranes were separated by nylon mesh inside a 14 cm or 30 cm bottle containing 5 ml or 10 ml of hybridisation solution for the 10 cm and 20 cm gels, respectively. The Biodyne A membrane was used with the pre-hybridisation and hybridisations solutions listed in the Appendix. Following hybridisation, the membrane was washed twice at 65°C in 2x SSC, 0.1 % SDS for 20-30 min and once at 65°C in 0.5x SSC, 0.1 % SDS for 20-30 min. For the ZetaProbe Genomic Tested membrane, the ZetaProbe

Standard Protocol (Bio-Rad) for hybridisation and washing was used. Membranes were exposed to X-ray film (X-Omat, Kodak) at -70°C, inside a cassette containing intensifier screens.

4.0 DEVELOPMENT OF AN *IN VITRO* MATING SYSTEM

There were many pitfalls in Nature for those too quick to see what they wanted to see.

E. C. Large, 1940.

4.1 INTRODUCTION

The presence of cleistothecia in Australian vineyards is widespread and often associated with high disease intensity. Cleistothecia also occur on wild and ornamental species of *Vitaceae*, for example, *Vitis amurensis*. *U. necator* is heterothallic (Smith, 1970) and Gadoury and Pearson (1991) demonstrated the existence of two mating types among *U. necator* isolates from New York State. Although cleistothecia were first observed in Australian vineyards in 1984 (Wicks *et al.*, 1985), the existence of two mating types and heterothallism have not been confirmed in this continent.

Mating type is a useful phenotypic character in population studies. For example, prior to the 1970s only the A1 mating type of *Phytophthora infestans* was reported outside central Mexico, where both the A1 and A2 mating types were known to occur (Fry *et al.*, 1993). The A2 mating type is now reported to exist in all continents except Australia and Antarctica, and mating types have been used to track the migration and diversification of *P. infestans* populations. This phenotypic marker can also be used to obtain evidence for the prevailing reproductive mode, which is determined by population genetic information (refer to Chapter 2). For example, where genotype frequencies indicate asexual reproduction, supporting evidence would be a predominance of one mating type over another and/or tight linkages between certain genotypes and mating type (Goodwin *et al.*, 1992c; Anderson & Kohn, 1995). Conversely, where genotype frequencies indicate sexual reproduction and random mating in a particular region, both mating types are likely to be distributed evenly within the region (Goodwin *et al.*, 1992c; Drenth *et al.*, 1994). Among 35 *U. necator* isolates from New York State vineyards, 24 isolates of one mating type and 10 isolates of the other mating type were identified (Gadoury & Pearson, 1991). The population structure of *U. necator* in New York State is probably influenced by sexual reproduction because both mating types are distributed widely in this region and ascospores are the primary inoculum for powdery mildew epidemics (Pearson & Gadoury, 1987). It is not known how and when the second mating type was introduced to Australia and, unfortunately, the first mating type

and the structure of the Australian *U. necator* population were not characterized prior to that introduction. Population studies, however, will elucidate the frequencies of mating types and genotypes. A long term research objective is to develop DNA markers linked to mating type so that phenotypes can be characterized rapidly.

Another reason for identifying mating types in a population is to develop a mating system for genetic studies. Determination of the inheritance of Mendelian traits or cytoplasmically transmitted genetic elements requires the production of cleistothecia and viable ascospores under controlled conditions. For example, studies of the inheritance of genes for fungicide resistance require a reproducible mating system. The development of plant pathogen populations with increased frequencies of fungicide tolerant strains is generally monitored by field sampling and fungicide bioassays (De Waard, 1994). This approach usually involves establishment of "baseline" sensitivity data, preferably before the fungicide is introduced to the pathosystem. Butters *et al.* (1986) suggest an alternative approach to obtaining baseline data. Their method involves crossing two fungicide sensitive strains and estimating the fungicide sensitivity of the progeny. In the case of *E. graminis* f. sp. *hordei*, the progeny resulting from the pairing of ethirimol or triadimenol sensitive isolates had a greater range of fungicide sensitivity than was observed when monitoring baseline sensitivity in natural populations. Analysis of progeny phenotypes where the parental strains have different fungicide sensitivities can also be used to predict the likely development of resistance by determining the number of genes controlling the phenotype (De Waard, 1994).

Methods for producing cleistothecia of *U. necator in vitro* and for studying cleistothecium and ascospore biology were developed by D. M. Gadoury and R. C. Pearson (Pearson & Gadoury, 1987; Gadoury & Pearson, 1988, 1990a, 1990b, 1991). They reported that the initiation of cleistothecia appeared to require only hyphal contact between compatible isolates. Environmental factors such as temperature, day length, humidity, leaf age and host resistance did not affect cleistothecium initiation and, once initiated, only temperature and host resistance affected their growth. In addition, these researchers observed that cleistothecia dehisced naturally in free water after a relatively long maturation process, which was correlated to a decrease in the water potential of the ascospore cytoplasm and a decrease in the strength of the cleistothecium wall. In New York State, cleistothecial

development is correlated closely with crop phenology, and 75-100% of ascospores are released between grapevine bud-burst and bloom. The timing of ascospore release elsewhere is unknown, although Gubler *et al.* (1994) hypothesize that there are two ascospore-derived populations per year in certain Californian viticultural regions. Their hypothesis was based on the observation that viable ascospores were released from cleistothecia collected in late Summer and Autumn, and again in early Spring, after the cleistothecia had overwintered.

There are two methods for identifying mating type in populations not characterized previously and where reference isolates for mating type are unavailable. The first method involves obtaining cleistothecia from the field and deriving isolates from single ascospores. These single ascospore-derived isolates can then be paired, with the knowledge that one cleistothecium will contain ascospores of both mating types if the fungus is heterothallic (Nelson, 1996). The difficulty of this approach for *U. necator* would be obtaining cleistothecia of the appropriate maturity from the field. Assuming cleistothecia in Australia require a maturation process similar to that described for the New York State population then there would be a narrow window each year for obtaining single ascospore-derived isolates. The second method for identifying mating type involves crossing a number of clonal lines from a random sample of single conidial-chain-derived isolates from various locations. The assumption, in this case, is that both mating types are distributed evenly in the population.

In this study, two mating types were identified among a collection of 20 South Australian *U. necator* clonal lines, six of which were paired in matrix form to demonstrate heterothallism. Techniques for the production of cleistothecia and viable ascospores *in vitro* were evaluated, because cleistothecia formed only when the methods used by Gadoury and Pearson (1988) were modified. The implications of the differences between Gadoury and Pearson's experimental system and the one described in this study are discussed.

4.2 Materials and Methods

4.2.1 Identification of mating types

In order to identify mating types, 20 South Australian clonal lines (see Section 3.1, Table 4.1) were paired in all possible combinations. The experimental unit was a micropropagated grapevine, cv. Cabernet Sauvignon, clone CW44, in a 500 ml container 21 days after

Table 4.1. *U. necator* clonal lines used in matrix pairings described in Section 4.2.1.

Matrix cross number	Clonal line	Source	Date on which the clonal line was paired with all other clonal lines except those paired previously
1	920102	Adelaide Plains	October 10, 1993
2	920401	Riverland	October 10, 1993
3	930101	Barossa Valley	December 4, 1993
4	930202	Barossa Valley	December 4, 1993
5	930304	Barossa Valley	February 18, 1994
6	930402	Barossa Valley	February 18, 1994
7	930501	McLaren Flat	May 2, 1994
8	930504	McLaren Flat	May 2, 1994
9	930602	McLaren Flat	June 3, 1994
10	930702	Adelaide Plains	June 3, 1994
11	930704	Adelaide Plains	July 7, 1994
12	930801	Adelaide Plains	July 7, 1994
13	930803	Adelaide Plains	August 5, 1994
14	930804	Adelaide Plains	August 5, 1994
15	930901	Adelaide Hills	September 5, 1994
16	931002	Adelaide Hills	September 5, 1994
17	931102	Adelaide Hills	September 5, 1994
18	931201	Adelaide Hills	September 5, 1994
19	931302	Adelaide Hills	September 5, 1994
20	931502	Adelaide Hills	September 5, 1994

subculturing (refer to Section 3.2 for micropropagation techniques). Each plantlet had at least five expanded leaves and was approximately 8-10 cm tall. The pairing of two clonal lines will be referred to in the text as a "cross". For co-inoculation, aseptic conidia, on a sporulating leaflet, were brushed onto healthy leaflets at random locations, each leaflet receiving both clonal lines, and the same clonal line in each set of crosses was always applied first. The experiment was designed as a matrix of crosses, where sets of crosses were conducted at different times and with different batches of micropropagated grapevines. For example, the first set of crosses involved pairing clonal lines 920102 and 920401 with all other clonal lines listed in Table 4.1. The second set of crosses involved pairing clonal lines 930101 and 930202 with all other clonal lines except those paired in the first set of crosses, and so on. Each clonal line was paired with itself as a control for selfing. The matrix of crosses was conducted once and consisted of one micropropagated grapevine per cross.

The cultures were incubated at 25°C with a 16 h photoperiod (250-450 $\mu E\ s^{-1}\ m^{-2}$) and assessed *in situ*, using a Wild stereomicroscope at 50x magnification, for the appearance of cleistothecia at weekly intervals for the first 8 weeks, then at monthly intervals thereafter. If cleistothecia did not form by 60 days post-inoculation, then the whole culture tube was transferred to a glasshouse where the temperature and light intensity were 11-25°C and 1200-3300 $\mu E\ s^{-1}\ m^{-2}$, respectively. The cultures were assessed for the presence of cleistothecia as above, 4 weeks after being transferred to this new environment. At this time, the density of cleistothecia on a leaflet of the cross between clonal lines 930602 and 930504 was estimated by counting cleistothecia in four fields of view using a Wild stereomicroscope at 50x magnification.

4.2.2 Production of cleistothecia *in vitro*

In order to produce cleistothecia reproducibly and accelerate their formation *in vitro*, several environmental and/or cultural conditions were assessed, including different inoculation techniques. Clonal lines 930202 and 930801, representing the two putative mating types, were paired in all experiments and line 930202 was inoculated first unless specified otherwise. Other clonal lines were included in experiments as specified. A minimum of three replicates was included for each treatment. Various control treatments and controls for

selfing were included for each experiment as specified. All cultures were inspected for cleistothecium formation, *in situ* using a Wild stereomicroscope at 50x magnification, at regular intervals until they formed mature cleistothecia or until the host began to senesce. For the purposes of these experiments, mature cleistothecia are defined as those which were morphologically mature; that is, the cleistothecia were approximately 90-100 µm in diameter, dark brown to black, with appendages, and spherical with a basal concavity when dry. Therefore, the ascospores inside were not necessarily mature and viable. Cleistothecia that were morphologically immature were yellow, orange or light brown. A series of preliminary experiments is described below and the methods used in each experiment are summarized in Table 4.2.

Experiment 1

The objective of this experiment was to produce cleistothecia on detached leaves. If cleistothecia formed on detached leaves then this result would support the hypothesis that there were factors preventing the initiation of cleistothecia on the micropropagated-grapevine cultures. Detached leaf cultures were prepared and incubated as described in Section 3.1 with the exception that the agar contained either 10 mg l^{-1} pimaricin or 10 mg l^{-1} pimaricin plus 30 mg l^{-1} benzimidazole. Benzimidazole was added to the agar to determine whether or not it would delay leaf senescence. In addition to clonal lines 930202 and 930801, lines 931502, 930101 and 920103 (refer to Table 3.1, Chapter 3) were selected for various pairings. For clonal lines 930202 and 930801, each clonal line was paired with itself as a control for selfing. Four inoculation methods were also tested. For inoculation method 1 (I_1), conidia of each clonal line were brushed onto the leaf in one-quarter leaf sectors so that colonies of the two clonal lines were adjacent or diagonally opposite each other. In theory, the colonies of the two clonal lines should merge or overlap at their boundaries. For inoculation method 2 (I_2), conidia of one clonal line were brushed over the whole leaf before conidia of the second line were applied. Inoculation method 3 (I_3) was the same as I_2 except that the "second" clonal line was inoculated before the "first" clonal line. Inoculation method 4 (I_4) involved rubbing together two sporulating leaflets of each of the two clonal lines over the adaxial surface of the detached leaf so that the conidia settled at random on the leaf. The inoculation methods were not replicated within each cross because of the preliminary nature

Table 4.2 A summary of the methods used for experiments described in Section 4.2.2.

Experiment	Clonal lines paired	Host substrate	Inoculation method[a]	Time from inoculation to imposition of incubation conditions of each treatment[b]	Incubation conditions of each treatment	Subsequent incubation conditions	Other conditions imposed
1	930202 x 930801 931502 x 920103 931502 x 930101	detached leaf	I_1, I_2, I_3, I_4	zero days	25°C, 12 h photoperiod for 21 days	-	± benzimidazole in medium
2	930202 x 930801	21 day-old micropropagated grapevine in a 500 ml container	Refer to Section 4.2.1 (similar to I_2)	21 days	A. 25°C, 16 h photoperiod for 90 days B. ambient lab. temp., continuous light	20°C, continuous light -	A. Various: uv light, darkness, ABA, sucrose B. penconazole vapours
3	930202 x 930801	21 day-old micropropagated grapevine in a 500 ml container	Same as Experiment 2 or I_4	14 or 21 days	ambient lab. temperature continuous light	-	penconazole vapours
4	930202 x 930801	detached leaflet culture using (A) a 53 day-old or (B) a 28 day-old micropropagated grapevine	I_1	zero days	A. 25°C, 12 h photoperiod for 10 days B. 25°C, 12 h photoperiod for 21 days	20°C, continuous light	-
5	930202 x 930801	(A) 25 or (B) 33 day-old micropropagated grapevines in a 250 ml container	A. Conidia of each line brushed on either side of the midrib B. I_1.	A. 5 days B. 7 days	20°C, continuous light A. infected one-node segments or whole plantlets B. whole plantlets	-	-

[a] Refer to Experiment 1, Section 4.2.2 for description of inoculation methods.

[b] Cultures were incubated at 25°C with a 16 h photoperiod (250-450 $\mu E\ s^{-1}\ m^{-2}$), unless specified otherwise.

of the experiment. Following inoculation the cultures were incubated as described in Section 3.1.

Experiment 2

The objective of this experiment was to determine whether or not cleistothecia formed when the physiology of the micropropagated grapevine and/or fungus was altered using various "stress" treatments. Micropropagated grapevines were co-inoculated with clonal lines 930202 and 930801 as described in Section 4.2.1. Treatments were imposed 21 days after inoculation to allow for adequate colonization of leaflets. The treatments were:

1. uv light exposure for 10 s
2. uv light exposure for 30 s
3. uv light exposure for 60 s
4. vapours from wicks treated with a 0.1 $\mu g\ ml^{-1}$ solution of penconazole
5. vapours from wicks treated with a 0.01$\mu g\ ml^{-1}$ solution of penconazole
6. darkness for 7 days
7. untreated control (see below)
8. abscisic acid agar, 100 µM
9. abscisic acid agar, 10 µM
10. abscisic acid agar, 1 µM
11. abscisic acid agar, 0.1 µM
12. sucrose agar, 0.2 M
13. sucrose agar, 0.1 M
14. untreated control (see below)

Treatments 1-7 and 8-14 were conducted with different batches of micropropagated grapevines. The untreated control plantlets were co-inoculated with the two clonal lines and incubated at 25°C with a 16 h photoperiod (250-450 $\mu E\ s^{-1}\ m^{-2}$) . Although each clonal line was incubated as a maintenance culture alongside the untreated control plantlets, strict controls for selfing were not included in this experiment. The uv light was supplied from a germicidal uv lamp (245 nm) inside a biological safety cabinet, class II. The lids of the culture tubes were removed and the open end of the tube placed 15 cm from the uv lamp. The tubes were covered with aluminium foil for 2 days after exposure to uv light because it has been reported that visible light can reverse the effects of uv light (Buxton *et al.*, 1957). Similarly, the darkness treatment was imposed by covering the tubes with aluminium foil. Penconazole-treated wicks were prepared by dipping half a 5 cm-diam. sterile Whatman No. 1 filter paper in the appropriate dilution, in distilled water, of Topas® formulated as a 100 $g\ l^{-1}$ emulsifiable concentrate. Excess solution was drained from the wick which was then placed partly on the agar surface and partly on the wall of the tube. An abscisic acid stock

solution was prepared by dissolving 25 mg of (±) -*cis, trans*-abscisic acid (ABA, Sigma) in 2.5 ml of dimethyl sulphoxide (DMSO). This solution was diluted with distilled water to a final concentration of 2.38 mg ml^{-1} then filter sterilized. A 1 ml aliquot of this stock solution was added to 19 ml of 7 g l^{-1} molten, sterile distilled-water agar (Bitek, Difco Laboratories, Michigan) cooled to 50°C and poured into a 9 cm-diam. Petri plate. When set, the agar was chopped into small pieces and placed on top of the existing 70 ml of medium supporting the micropropagated grapevine. The final concentration of the ABA in 90 ml of medium was nominally 100 µM. The other ABA treatments were prepared by serial dilution of the ABA stock solution. Sucrose was applied to the agar medium in the same manner as described for the ABA treatments except that the sucrose was dissolved directly in the water agar at the appropriate final concentration.

All cultures were incubated at 25°C with a 16 h photoperiod (250-450 µE s^{-1} m^{-2}) except for the penconazole treatments which were placed in continuous light (100-160 µE s^{-1} m^{-2}) from fluorescent lamps on a laboratory bench. The latter treatments were separated from the rest as a precaution against penconazole vapours escaping through the breather caps and entering adjacent culture tubes. Co-inoculated plantlets which were not exposed to penconazole vapours were not included as untreated controls for the incubation conditions on the laboratory bench. This omission was rectified in Experiment 3.

Ninety days after co-inoculation, two plantlets from each of treatments 11-13 and from the untreated control (14) were transferred to an incubator illuminated continuously (550-900 µE s^{-1} m^{-2} from cool white fluorescent bulbs) at 20°C.

Experiment 3

A. The penconazole treatments of Experiment 2 (treatments 4 and 5) were repeated except that the treatments were imposed 14 days post-inoculation and the untreated control was incubated under the same continuous light conditions.

B. The treatment using the wicks treated with 0.01 µg ml^{-1} penconazole (treatment 5, Experiment 2) was repeated. In addition, two different inoculation methods were tested. The first inoculation method was the same as used in Experiment 2, and the second method involved rubbing together two sporulating leaflets of each clonal line above the open culture

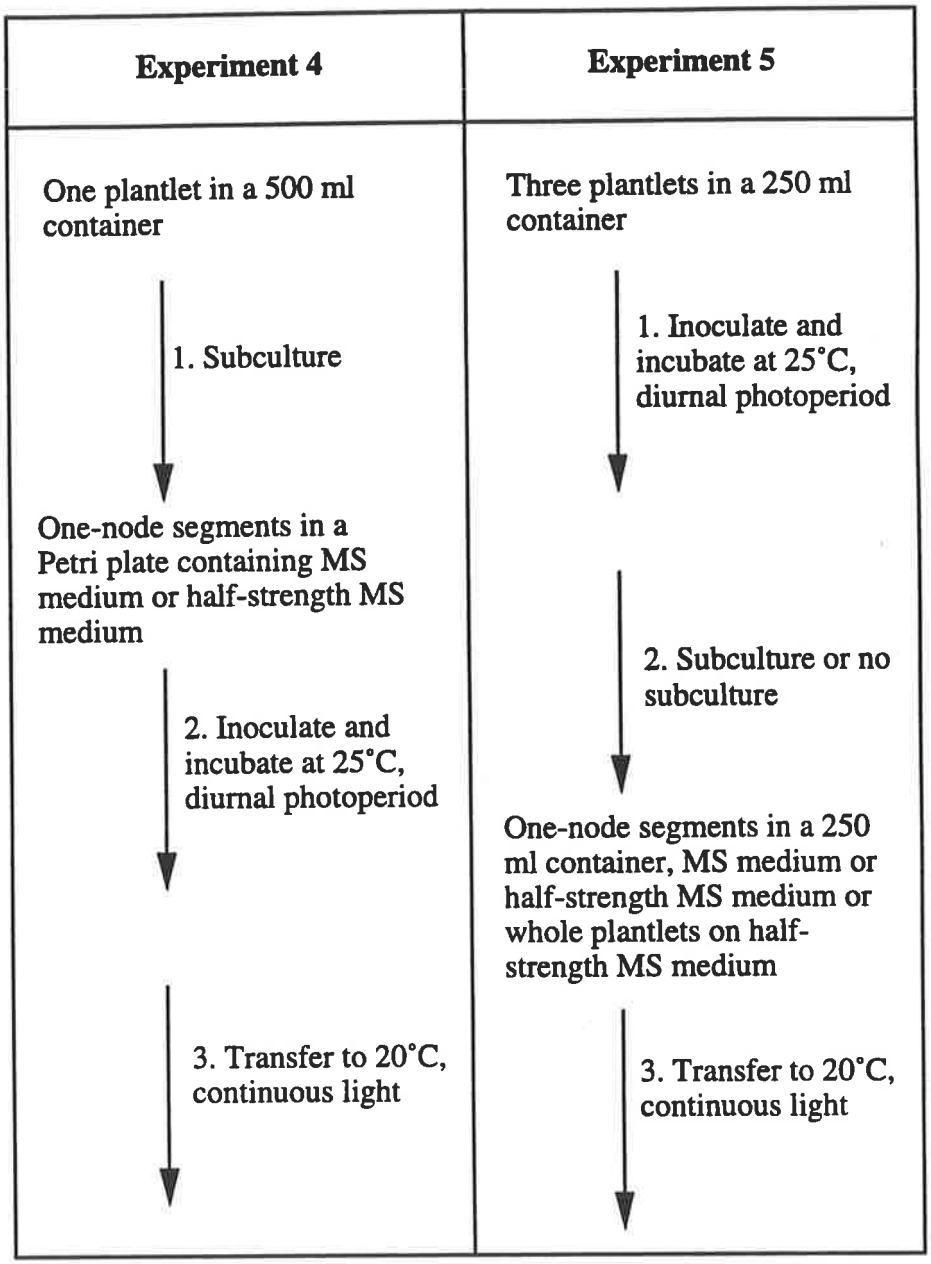
tube so that the conidia settled at random on the plantlet being inoculated (as in I_4 of Experiment 1). The treatments were imposed 21 days after inoculation, and treated and untreated plantlets, inoculated using both techniques, were incubated in continuous light (100-160 $\mu E\ s^{-1}\ m^{-2}$) from fluorescent lamps on a laboratory bench.

Experiment 4

The objective of this experiment was to develop a detached-leaflet culture using micropropagated grapevine leaflets as an alternative means of producing mature cleistothecia. The protocol for this experiment is presented, in simplified form, as a flow chart in Figure 4.1. Two variations of the protocol were assessed:

A. Cultures were prepared by cutting three one-node segments, each having one expanded leaflet, from a micropropagated grapevine in a 500 ml container, 53 days after subculturing. These three nodes were then inserted into 20 ml of MS medium (refer to Section 3.2) in a 2 cm-deep, 9 cm-diam. Petri plate, with the margins of the leaflets resting on the agar and the centre portion raised above the medium. The lid of the Petri plate was replaced and sealed with Parafilm®. Four plates were prepared then incubated in an illuminated growth chamber (330 $\mu E\ s^{-1}\ m^{-2}$ from cool white fluorescent bulbs) with a 12 h photoperiod at 25°C for 7 days to allow the leaflets to overcome any stress associated with subculturing. The leaflets were then co-inoculated with clonal lines 930202 and 930801 in quarter-leaflet sectors as described for Experiment 1, and the plates resealed with Parafilm® and incubated as before. If cleistothecia did not form within 10 days, the cultures were transferred to another incubator, illuminated continuously (550 $\mu E\ s^{-1}\ m^{-2}$ from cool white fluorescent bulbs) at 20°C. The density of cleistothecia on one leaflet was estimated 70 days post-inoculation by counting cleistothecia in four fields of view of a Wild stereomicroscope at 50x magnification. At 48 and 70 days post-inoculation, cleistothecia were scraped off a leaflet with a scalpel blade and placed on a microscope slide. The viability of ascospores was assessed by squashing cleistothecia in 10 $\mu g\ ml^{-1}$ fluorescein diacetate (FDA, Sigma) stain prepared by diluting a stock solution of 2 $mg\ ml^{-1}$ in acetone with distilled water. The asci and ascospores were examined by uv illumination with a Zeiss III RS microscope and FT 510 and LP 520 filters (FITC method). Viable ascospores fluoresced brightly.

Figure 4.1 Flow charts representing the protocols described for Experiments 4 and 5, Section 4.2.2.


Experiment 4
One plantlet in a 500 ml container
1. Subculture
One-node segments in a Petri plate containing MS medium or half-strength MS medium
2. Inoculate and incubate at 25°C, diurnal photoperiod
3. Transfer to 20°C, continuous light
Experiment 5
Three plantlets in a 250 ml container
1. Inoculate and incubate at 25°C, diurnal photoperiod
2. Subculture or no subculture
One-node segments in a 250 ml container, MS medium or half-strength MS medium or whole plantlets on half-strength MS medium
3. Transfer to 20°C, continuous light

B. The second variation of the protocol involved the use of one-node segments, each having one expanded leaflet, from a micropropagated grapevine in a 500 ml container, 28 days after subculturing. These one-node segments were transferred to half-strength MS medium (refer to Section 3.2) in a 2 cm-deep, 9 cm-diam. Petri plate. The leaflets were placed on autoclaved, 9 cm^2 pieces of flyscreen (Boral Cyclone) positioned on top of the medium. This flyscreen platform prevented most of the leaflet from touching the surface of the medium and was aimed at improving colonization of the leaflet by *U. necator*. The leaflets were inoculated immediately by picking up conidia of each clonal line with a sterile cotton bud and placing them in quarter sectors on the leaflet as described in Experiment 1. The plates were incubated in an illuminated growth chamber (330µE s^{-1} m^{-2} from cool white fluorescent bulbs) with a 12 h photoperiod at 25°C for 21 days prior to being transferred to the 20°C, continuous light incubator.

Experiment 5

This experiment was an extension of Experiment 4, in that several more culture types were tested as potential substrates for cleistothecium production. The protocol for this experiment is presented, in simplified form, as a flow chart in Figure 4.1. Again, two variations of the protocol were assessed:

A. In the first test, a culture consisting of three plantlets, growing in the same 250 ml container on 50 ml of half-strength MS medium containing NAA (refer to Section 3.2), was co-inoculated with clonal lines 930202 and 930801, 25 days after being subcultured. Leaflets were inoculated by brushing conidia of each clonal line on opposite sides of the leaflet midrib. Twelve of these co-inoculated cultures were incubated at 25°C with a 16 h photoperiod (250-450 µE s^{-1} m^{-2}) for 5 days to establish adequate leaflet colonization. Infected plantlets in six of these cultures were then cut into one-node segments and placed on 50 ml of MS medium or 50 ml of half-strength MS medium containing NAA in 250 ml polycarbonate containers. Four infected one-node segments were placed in each container and there were three containers per medium type. These cultures, containing the co-inoculated, one-node segments, were then transferred to another incubator which was illuminated continuously (550-900 µE s^{-1} m^{-2} from cool white fluorescent bulbs) at 20°C.

In addition, three containers of the original co-inoculated cultures, that had not been cut into one-node segments and sub-cultured, were also transferred to the continuous-light incubator. The remaining three cultures were not transferred to the continuous-light incubator.

B. In the second test, three plantlets growing on 50 ml of half-strength MS medium containing NAA in a 250 ml container were inoculated 33 days after being sub-cultured. The inoculation method involved picking up conidia of each clonal line with a sterile cotton bud and placing them in quarter sectors on the leaflet as described in Experiment 1. Six of these co-inoculated cultures were incubated at 25°C with a 16 h photoperiod (250-450 $\mu E\ s^{-1}\ m^{-2}$). After 7 days incubation, three of these cultures were transferred to the 20°C, continuous light incubator, and the other three cultures were maintained under the diurnal-light incubation conditions.

4.2.3 Demonstration of the heterothallic nature of *U. necator*

4.2.3.1 Inoculation and assessment of cleistothecium density

Leaflets of a micropropagated grapevine in a 500 ml container, 21 days after subculturing (refer to Section 3.2), were co-inoculated by picking up conidia of each clonal line with a sterile cotton bud and placing them in quarter-leaflet sectors as described in Experiment 1, Section 4.2.2. The sources of conidia were sporulating leaflets of dual *in vitro* cultures inoculated 36 days previously. Three clonal lines of the putative plus mating type, 930202, 930504 and 930704, and three of the putative minus mating type, 930801, 931502 and 930602, were paired in all possible combinations including pairings of the same clonal line ("selfs"). The co-inoculated cultures were incubated at 25°C with a 16 h photoperiod (250-450 $\mu E\ s^{-1}\ m^{-2}$) for 28 days to establish vigourous leaflet colonization. The cultures were then transferred to an incubator illuminated continuously (550-900 $\mu E\ s^{-1}\ m^{-2}$ from cool white fluorescent bulbs) at 20°C, and examined at weekly intervals for the presence of cleistothecia. Each cross was conducted using one micropropagated grapevine but the entire matrix of crosses was conducted three times using different batches of micropropagated grapevines. Therefore, each matrix of crosses was a replication, in time, of one experiment. Clonal lines were considered to be opposite mating types if cleistothecia formed in at least one replication of the experiment.

For the first replication only, the density of mature cleistothecia per cm^2 was estimated 98 and 126 days after inoculation. The density of cleistothecia per cm^2 for the second and third replicates was estimated at 124 and 111 days post-inoculation, respectively. For each leaflet, cleistothecia were counted in three fields of view using a Wild stereomicroscope at 50x magnification.

4.2.3.2 Assessment of viability of ascospores

For the first replicate only, the viability of ascospores was assessed by vital staining at 126 days post-inoculation, using cleistothecia produced from a cross of clonal lines 931502 and 930202. Vital staining of ascospores was also conducted at 159 days after inoculation, using cleistothecia harvested from a pooled sample of leaflets from various crosses. In addition, the infectivity of ascospores was assessed at this time.

For the assessment at 126 days post-inoculation, a leaflet with numerous cleistothecia was shaken vigo rously in 10 ml of distilled water and the suspension sieved through a tea strainer into an open Petri plate. Ten mature cleistothecia were picked up by their appendages using forceps and placed on a microscope slide. The viability of ascospores was assessed by vital staining (refer to Experiment 4, Section 4.2.2).

For the assessment at 159 days post-inoculation, 14 leaflets bearing cleistothecia from eight different crosses were pooled together because of the low yields of cleistothecia. The cleistothecia were harvested by placing the leaflets in ice-cold distilled water, which was then shaken vigo rously for 5 min. The liquid and debris were poured through two nested stainless steel sieves of 250 µM and 90 µM mesh aperture, respectively. The cleistothecia collected in the 90 µM sieve were washed onto a 5 cm-diam. Whatman No. 1 filter paper in a Buchner funnel and partially dried on the paper by vacuum. Two 1-cm^2 sections of filter paper bearing cleistothecia were transferred to a microscope slide and the viability of ascospores assessed by vital staining (refer to Experiment 4, Section 4.2.2).

Given that the culture conditions suitable for ascospore germination and subsequent colony development on detached leaves had not been optimized previously, the infectivity of ascospores was assessed by using various modifications of the detached leaf culture described in Section 3.1. The cultures were prepared as follows:

Condition 1: Three plates were prepared in which a semi-circle (radius = 2.5 cm) of filter paper bearing cleistothecia was placed on the surface of the water agar in the centre of the plate. A bed of four toothpicks was placed on either side of the filter paper. A wet, surface sterilized leaf was then placed on the toothpicks as described previously. The surface of the leaf, however, was not allowed to dry as described in Section 3.1, and the lid of the plate was replaced and resealed with Parafilm®. The target for any ascospore release was the wet, abaxial surface of the detached leaf.

Condition 2: One detached leaf culture was prepared as described in Section 3.1 except that the leaf was not dried after surface sterilization, as for condition 1. A semi-circle of filter paper bearing cleistothecia was placed in the lid of the Petri plate and moistened with sterile distilled water so that there was a thin film of water on the paper surface. The target for any ascospore release was the wet, adaxial surface of the detached leaf. A control for condition 2, consisting of a single detached leaf culture without cleistothecia and where the leaf was not dried after surface sterilization, was included. The lids of the plates were replaced and resealed with Parafilm®.

Condition 3: Cleistothecia from a semi-circle of filter paper were washed, using sterile distilled water, directly onto the adaxial surface of a dried detached-leaf culture. Therefore, the cleistothecia on the leaf surface were in droplets of water. The lid of the plate was replaced and resealed with Parafilm®.

The experiment comprised three plates of condition 1, and one plate each of condition 2, the control for condition 2, and condition 3. All plates were incubated for 48 h with a 12 h photoperiod (330 $\mu E\ s^{-1}\ m^{-2}$ from cool white fluorescent bulbs) at 25°C, then transferred to a laminar flow hood, the lids removed and the leaves dried for 30 min. After the leaves were dry, individual plates were treated as follows:

Condition 1: The leaf in the first plate was transferred to a bed of eight parallel toothpicks at 0.75 cm intervals on a fresh water agar plate. The lid was replaced but the plate was not sealed with Parafilm®. The leaf in the second plate was transferred to a bed of four toothpicks on a fresh water agar plate; the leaf rested upside down on the toothpicks with some areas of the leaf margin resting on the agar surface. This second plate and the third plate, in which the leaf remained *in situ*, were resealed with Parafilm®.

Condition 2: The filter paper bearing cleistothecia was removed from the lid of the Petri plate and the plate resealed with Parafilm®. The control plate was resealed with Parafilm®.

Condition 3: The lid was replaced but the plate was not resealed with Parafilm®.

All plates then incubated with a 12 h photoperiod (330 μE s^{-1} m^{-2} from cool white fluorescent bulbs) at 25°C, and the leaves examined at regular intervals for the development of *U. necator* colonies.

4.3 RESULTS

4.3.1 Identification of mating types

Cleistothecia did not form on any co-inoculated plantlets incubated at 25°C with a 16 h photoperiod for 60 days. Cleistothecia did form, however, on 21 out of 153 plantlets within 4 weeks of the plantlets being transferred to the glasshouse (Table 4.3). Cleistothecia did not form on plantlets inoculated with a single clonal line or on the co-inoculated plantlets which were maintained in the constant temperature conditions for up to 210 days post-inoculation. The glasshouse environment appeared to impose "stress" on the plantlets because leaflet margins often became necrotic and some plantlets died. Infected plantlets of the same age remaining in the constant temperature environment did not display these symptoms. In addition, the medium supporting many of the plantlets in the glasshouse eventually became contaminated by micro-organisms. Plantlets were scored for the presence of cleistothecia only if they were not contaminated. The estimated density of cleistothecia of all maturities (yellow, brown and black) on a leaflet of cross [930602 x 930504] at 87 days post-inoculation was in the range 640-1,170 cleistothecia per cm^2.

4.3.2 Production of cleistothecia *in vitro*

Experiment 1

Immature cleistothecia were first observed on the detached leaves of various crosses 10 days after inoculation. The final assessment was made 21 days post-inoculation when the leaves were beginning to senesce (Table 4.4). Benzimidazole did not appear to delay leaf senescence. Cleistothecia formed in at least one pairing of opposite mating types identified in Section 4.3.1. In addition, cleistothecia formed in cross [930103 x 931502], a cross which was not included in the matrix of crosses described in Section 4.2.1. Mature

Table 4.3 Cleistothecium formation on micropropagated *V. vinifera* cv. Cabernet Sauvignon inoculated with 20 clonal lines of *U. necator* paired in 210 combinations, 88 days after inoculation and 28 days after transfer of the culture containers to a glasshouse environment. The key to symbols is given below the matrix.

	1[a]	2[a]	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20	x	x	C	x	C	x	C	x	x	x	C	x	x	C	x	x	x	x	x	S
19	x	x	x	x	x	x	x	x	C	x	x	x	x	x	x	x	x	x	S	
18	x	x	x	x	x	x	C	x	x	x	x	x	x	x	x	x	x	S		
17	x	x	x	C	C	C	x	C	x	x	x	x	x	x	x	x	S			
16	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	S				
15	x	x	x	x	x	x	C	x	x	x	C	x	x	x	S					
14	x	x	x	x	x	x	x	x	x	x	x	x	x	S						
13	x	x	x	x	x	C	C	x	x	x	x	x	S							
12	x	x	C	C	C	x	x	x	x	C	x	S								
11	x	x	x	x	x	x	x	x	C	x	S									
10	x	x	x	x	x	x	x	x	x	S										
9	x	x	x	x	x	x	x	C	S											
8	x	x	x	x	x	x	x	S												
7	x	x	x	x	x	x	S													
6	x	x	x	x	x	S														
5	x	x	x	x	S															
4	x	x	x	S																
3	x	x	S																	
2	x	S																		
1	S																			

Matrix Key:

Numbers **1** to **20** refer to clonal lines listed in Table 4.1

[a] Crosses with clonal lines bearing this superscript were not transferred to the glasshouse at 60 days post-inoculation.

C = cleistothecia formed on leaflets within 28 days of transfer to the glasshouse or within 88 days post-inoculation.

x = cleistothecia did not form on leaflets within 28 days of transfer to the glasshouse or within 88 days post-inoculation.

S = a clonal line which was co-inoculated with itself ("Self"). Cleistothecia did not form on leaflets of any "Selfs".

Table 4.4 Cleistothecium formation 21 days after pairing various *U. necator* clonal lines on detached grapevine leaves on different agar media and using different inoculation methods.

	No benzimidazole in agar medium							Benzimidazole in agar medium						
	line 930801				line 931502			line 930801				line 931502		
	I_1	I_2	I_3	I_4	I_1	I_2	I_4	I_1	I_2	I_3	I_4	I_1	I_2	I_4
line 920103	-	-	-	-	C (45)	-	C (54)	-	-	-	-	C (63)	-	NC
line 930101	-	-	-	-	NC	C (36)	NC	-	-	-	-	C (54)	NC	NC
line 930202	C (63)	C (27)	C (36)	C (45)	-	-	-	C (54)	C (27)	C (27)	C (45)	-	-	-

I_n = Inoculation method (n=1, 2, 3 or 4). Refer to Section 4.2.2.

C = Cleistothecia formed on leaf. The number in parenthesis refers to the diameter in µm of the largest cleistothecium observed.

NC = No cleistothecia formed on leaf.

- = the cross was not conducted using the inoculation technique indicated.

cleistothecia did not form before the leaves senesced, however, and the largest observed was 63 μm in diameter. Cleistothecia did not form when the same clonal line was paired with itself nor did they form in five out of 18 pairings where they were expected. All inoculation methods, however, resulted in cleistothecium production in at least one pairing. The relative efficacy of each inoculation method cannot be assessed, however, because the methods were not replicated for each cross. Cleistothecia formed in small, separated areas on each detached leaf, regardless of inoculation method.

Experiment 2

Cleistothecia did not form on any of the co-inoculated plantlets where "stress" treatments were applied, except for treatment 5 in which the cultures were exposed to vapours from wicks treated with 0.01 μg ml^{-1} penconazole and incubated in continuous light. Immature cleistothecia were first observed on one leaflet 13 days after treatment. By 26 days post-treatment, mature and immature cleistothecia were observed on two leaflets of the same plantlet. One mature and one immature cleistothecium were observed on one leaflet of a second plantlet exposed to the same treatment. Cleistothecia were not observed on the third replicate plantlet of this treatment.

UV light followed by 2 days darkness (treatments 1-3) was phytotoxic, the symptoms being more severe as the exposure to uv light increased. At the lowest exposure (10 s uv light), most margins of the lower leaflets were necrotic and the uppermost leaflets were necrotic. All plantlets exposed to 7 days of darkness (treatment 6) died. ABA and sucrose (treatments 8-13) had no visible effects on the plantlets, except for the highest dose of ABA (nominally 100 μM) which appeared to be phytotoxic because the leaflets abscised at the stem end of the petiole.

All eight plantlets transferred to the 20°C, continuous-light incubator, 90 days after inoculation and 79 days after the previous treatment, had immature (yellow) and maturing (brown) cleistothecia on one or two leaflets, 14 days after being transferred to the new incubation conditions. Most cleistothecia were mature after 28 days in continuous light at 20°C.

Experiment 3

A. Cleistothecia formed on one leaflet on one out of three replicate plantlets which had been co-inoculated by brushing conidia of each clonal line separately onto leaflets and, 14 days later, were exposed to a penconazole wick (0.01 or 0.1 $\mu g\ ml^{-1}$) in continuous light. Several immature cleistothecia were observed 14 days after adding the 0.01 $\mu g\ ml^{-1}$ penconazole wick. By 28 days, this leaflet had six mature (black) cleistothecia and the leaflet of the plantlet treated with the 0.1 $\mu g\ ml^{-1}$ penconazole wick had seven mature cleistothecia. Cleistothecia did not form on any of the other replicate plantlets including the untreated control.

B. In a partial repetition of this experiment, in which each replicate plantlet was exposed to a 0.01 $\mu g\ ml^{-1}$ penconazole wick and two different inoculation methods were used, cleistothecia did not form on any of the replicate plantlets, including the untreated control.

Experiment 4

A. Successful infections were established on only two leaflets in two out of the four plates prepared using the nodal segments from 53 day-old micropropagated grapevines. Nine days after transferring these plates to the continuous light incubator numerous immature cleistothecia were observed on the two leaflets. Maturing (brown) and immature (yellow) cleistothecia were observed 12 days post-transfer and mature cleistothecia (black) were observed in increasing numbers between 17 and 21 days post-transfer. The highest density of cleistothecia on the two leaflets at 70 days post-inoculation was estimated to be 4,500 cleistothecia per cm^2; most of these cleistothecia were black. By this time the leaflet appeared chlorotic and dead. At 48 days post-inoculation, cleistothecia squashed in FDA contained up to four asci with up to four brightly fluorescing ascospores per ascus. These ascospores appeared to vary in maturity; some had granulated protoplasm and some asci contained unpartitioned protoplasm. At 70 days post-inoculation, however, the ascospores did not fluoresce under uv illumination following FDA staining of squashed cleistothecia and were light brown in colour when viewed by light microscopy.

B. Successful infections were established on only four leaflets in three plates out of the four prepared using the 28 day-old micropropagated grapevines. Cleistothecia were first observed on one leaflet 5 days after being transferred to the continuous light incubator. By 19 days post-transfer more immature cleitothecia had formed on this leaflet and on a second leaflet in the same plate, and older cleistothecia were brown or black.

Experiment 5

Twelve days after being transferred to the 20°C, continuous-light incubator, the subcultured, infected one-node segments showed symptoms of transfer "stress", in that some leaflets or margins of leaflets turned red. The plantlets which were not subcultured remained healthy after being transferred to the continuous light incubator. The one-node segments appeared to recover from the transfer "stress" and all treatments were assessed 30 days post-transfer. Two out of three containers for each treatment transferred to the continuous light incubator contained at least one leaflet which had black, brown and yellow cleistothecia. No cleistothecia developed on plantlets remaining in the 25°C, diurnal-light conditions.

The plantlets in the second test were slightly older and larger at the time of inoculation than those in the first test. Condensation occurred within the culture containers and only one or two leaflets were colonized by *U. necator*. Immature and maturing cleistothecia were observed on only one leaflet 20 days after being transferred to the continuous light incubator. Mature cleistothecia were evident on this leaflet 27 days post-transfer.

4.3.3 Demonstration of the heterothallic nature of *U. necator*

4.3.3.1 Cleistothecium incidence and density

The incidence of cleistothecium formation is shown in Tables 4.5 and 4.6. For the first replicate of the experiment, the first immature cleistothecia were observed 48 days after inoculation or 27 days after cultures were transferred to the 20°C, continuous light incubator. These immature cleistothecia were present on leaflets of crosses of clonal lines [930202 x 931502] and [930202 x 930801]. Immature cleistothecia were first observed on leaflets of the cross [930504 x 930801] at 56 days after inoculation and on leaflets of the cross [930704 x 930801] at 63 days after inoculation. Cleistothecia of all stages of maturity were observed

Table 4.5 Cleistothecium formation when six *U. necator* clonal lines were paired in all possible combinations on micropropagated *V. vinifera* cv. Cabernet Sauvignon. The cultures were incubated for up to 98 days in a 20°C incubator illuminated continuously from 28 days after co-inoculation. The key to symbols is given below the matrix.

Clonal line	930202	930504	930704	930801	931502	930602
930602	C^o C^e	C^o C^e	C^o C^e	NC^o NC^e	NC^o NC^e	NC^o NC^e
931502	C^o C^e	C^o C^e	C^o C^e	NC^o NC^e	NC^o NC^e	
930801	C^o C^e	C^o C^e	C^o C^e	NC^o NC^e		
930704	NC^o NC^e	NC^o NC^e	NC^o NC^e			
930504	NC^e NC^e	NC^o NC^e				
930202	NC^o NC^e					

C^o cleistothecia observed to form

C^e cleistothecia expected to form

NC^o cleistothecia not observed to form

NC^e cleistothecia not expected to form

Table 4.6 The leaflet position and density of mature cleistothecia on each leaflet in pairings of compatible *U. necator* clonal lines.

Cross	Leaflet position[a]	Mean (n=3) number of mature (black) cleistothecia per cm^2 at various days post-inoculation			
		1st replicate		2nd replicate	3rd replicate
		98 days	126 days	124 days	111 days
930202 x 930801	1	458	644	0	0
	3	279	433	0	0
	4	0	0	266	0
	5	391	491	0	0
	7	369	744	0	0
930202 x 931502	2	335	422	0	0
	4	491	645	0	0
	5	151	300	0	289
930202 x 930602	2	0	0	0	300
930504 x 930801	3	134	122[b]	0	0
930504 x 931502	3	101	100	0	0
930504 x 930602	-	0	0[c]	0	0
930704 x 930801	3	0	0	67	0
	5	101	122	0	0
930704 x 931502	3	67	133	0	0
	4	134	333	0	0
930704 x 930602	4	257	278	0	0

[a] counting from the base of the plantlet, only leaflets bearing cleistothecia are included.

[b] the leaflet was necrotic.

[c] immature cleistothecia were present at a mean density of 511 cm^{-2} on one leaflet.

on all the other pairings of putative opposite mating types, except [930202 x 930602] and [930504 x 930602], by 98 days after inoculation (see Table 4.6). Cleistothecia did not form on the former cross but immature and maturing cleistothecia were observed on the latter cross 126 days after inoculation. Both plantlets had only a few areas weakly colonized by *U. necator.*

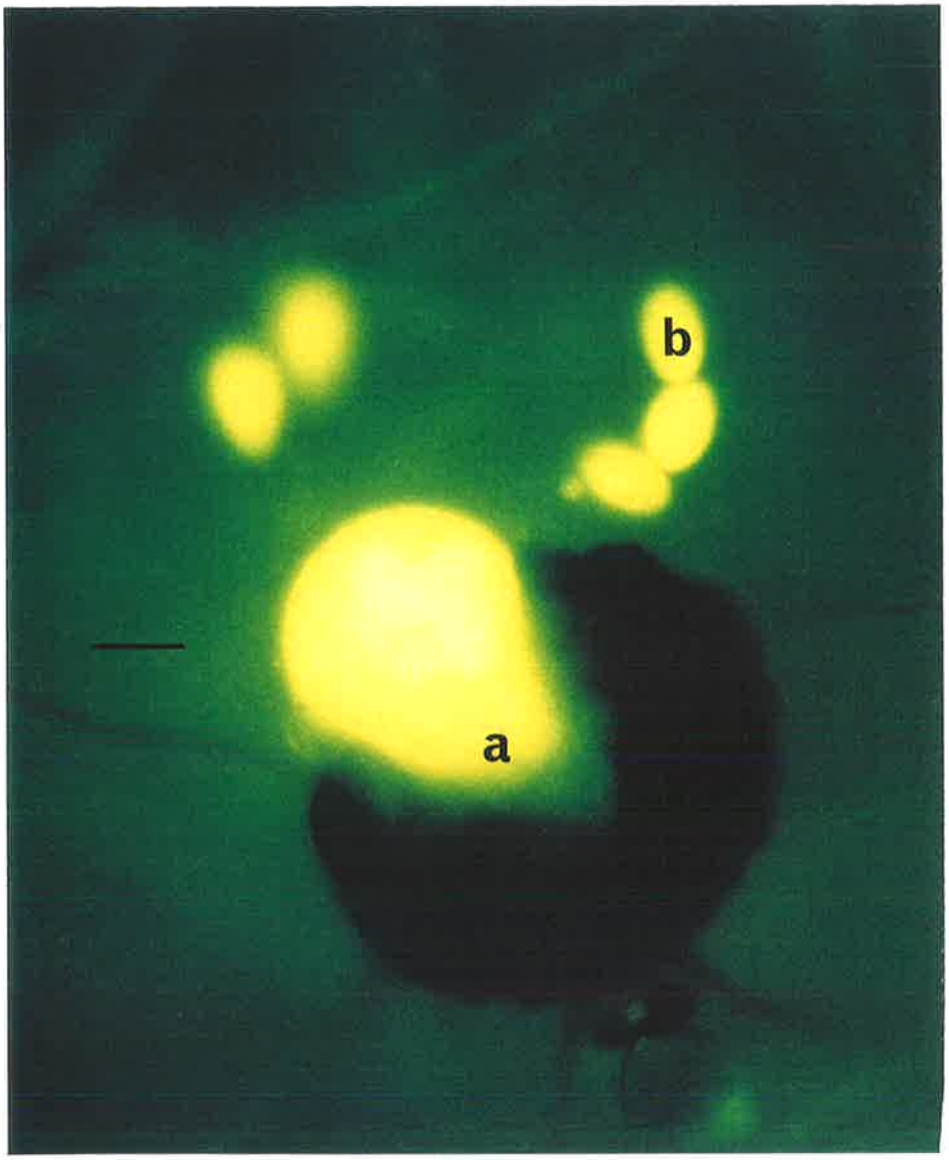
In the second replicate of the experiment, most plantlets were colonized poorly by *U. necator* and cleistothecia of all stages of maturity were observed 98 days post-inoculation on one leaflet only of crosses [930704 x 930801] and [930202 x 930801]. In the third replicate of the experiment, the plantlets were colonized poorly again and cleistothecia of all stages of maturity were observed 98 days post-inoculation on one leaflet only of crosses [930202 x 931502] and [930202 x 930602]. This third replicate was terminated at 111 days post-inoculation when the incubator stopped functioning.

Table 4.6 lists the number of leaflets producing cleistothecia per plantlet and the average number of mature cleistothecia per cm^2 leaflet. For the first replicate, the numbers of mature cleistothecia appeared to increase on some leaflets between 98 and 126 days after inoculation. By 126 days post-inoculation the plantlets were beginning to senesce.

4.3.3.2 Viability of ascospores

The ten cleistothecia selected from a leaflet of the cross [931502 x 930202] each contained at least two asci with three to four ascospores containing granulated protoplasm which fluoresced brightly when stained with FDA and examined by uv illumination (Figure 4.2). Approximately 4,700 cleistothecia were harvested from 14 leaflets, 159 days after inoculation; this estimate was based on counts of the cleistothecia on the filter papers. In the ascospore viability test each detached leaf culture was exposed to approximately 800 cleistothecia. The cleistothecia collected on the filter papers did not appear to have any conidia adhering to their surface when examined at 50x magnification. Attempts to obtain *U. necator* colonies from ascospores produced *in vitro* were successful using detached leaves and culture conditions 1 and 3. Thirteen days after cleistothecia were placed in the detached-leaf culture plates, three sporulating colonies of *U. necator* were observed on the abaxial surface of the leaf placed upside down after 48 h exposure to the cleistothecia in condition 1. At the same time, two sporulating colonies were also observed on the adaxial leaf surface of

Figure 4.2 A cleistothecium, harvested from a micropropagated *V. vinifera* cv. Cabernet Sauvignon 159 days after co-inoculation, and squashed in FDA stain. A brightly staining ascus (**a**) and ascospore (**b**) are visible using uv illumination. The scale bar represents 20 µm.


b
a

the culture in which the cleistothecia were washed directly onto the detached leaf (condition 3). The conidia from all colonies originating from ascospores produced typical *U. necator* colonies when inoculated onto healthy detached-leaf cultures.

4.3.4 Summary of results

By combining the results of all experiments in this study, mating types were assigned to 19 out of 21 *U. necator* clonal lines (Table 4.7). The six clonal lines used in the heterothallism experiment were designated as reference lines for mating type. Clonal line 920103 is classified putatively as a "plus" mating type because only immature cleistothecia formed when this line was paired with a "minus" mating type. Clonal line 920103 is a single-conidial chain isolate derived from line 920102. If lines 920102 and 920103 are the same clone, then line 920102 is putatively a "plus" mating type.

4.4 Discussion

Although heterothallism has already been identified in *U. necator* isolates from New York State (Gadoury and Pearson, 1987), reference isolates for mating type were not available in Australia. Therefore, mating types were assigned by first identifying potential reference isolates by crossing a number of clonal lines from a random sample of single conidial-chain-derived isolates from various locations. This approach was adopted because (a) cleistothecia were observed in many Australian viticultural regions, (b) a culture collection of clonal lines was being established for DNA genotyping and (c) methods for the production of cleistothecia *in vitro* had been reported previously (Gadoury and Pearson, 1988).

Methods similar to those used by Gadoury and Pearson (1988) were adopted initially but 190 pairings of Australian clonal lines failed to produce cleistothecia on micropropagated grapevines within 60 days when incubated at 25°C using diurnal-light patterns. When 153 of these co-inoculated cultures were moved to a glasshouse environment over the course of a year, 14 % of pairings produced cleistothecia. This result may not represent the maximum number of compatible pairings because (a) compatible lines may not have produced cleistothecia due to poor health of the host and/or (b) colonization by one of the two clonal lines may have been unsuccessful. In addition, the percentage of compatible pairings may

Table 4.7 The mating type or putative mating type of selected South Australian clonal lines of *U. necator* determined using the combined results of this study

Clonal line	Source	Mating type	
		Designation	Status[a]
920102	Adelaide Plains	plus	putative[b]
920103	Adelaide Plains	plus	putative[c]
920401	Riverland	unknown	-
930101	Barossa Valley	plus	confirmed[d]
930202	Barossa Valley	plus	reference line[f]
930304	Barossa Valley	plus	confirmed[d]
930402	Barossa Valley	plus	confirmed[d]
930501	McLaren Flat	plus	confirmed[d]
930504	McLaren Flat	plus	reference line[f]
930602	McLaren Flat	minus	reference line[f]
930702	Adelaide Plains	plus	confirmed[e]
930704	Adelaide Plains	plus	reference line[f]
930801	Adelaide Plains	minus	reference line[f]
930803	Adelaide Plains	minus	confirmed[d]
930804	Adelaide Plains	plus	confirmed[e]
930901	Adelaide Hills	minus	confirmed[d]
931002	Adelaide Hills	unknown	-
931102	Adelaide Hills	minus	confirmed[d]
931201	Adelaide Hills	minus	confirmed[e]
931302	Adelaide Hills	plus	confirmed[e]
931502	Adelaide Hills	minus	reference line[f]

[a] Mating type

putative:
- [b] clonal lines 920102 and 920103 are the same clonal line because line 920103 is a single conidial-chain isolate derived from line 920102.
- [c] cleistothecia did not mature on detached leaves.

confirmed:
- [d] confirmed in crosses with at least two other clonal lines of opposite mating type.
- [e] cleistothecia formed in one pairing only but with a clonal line the mating type of which was confirmed by pairings with clonal lines of opposite mating type.

reference clonal line:
- [f] based on the results of the matrix pairings presented in Table 4.5.

reflect a situation where some of the clonal lines may have been genetically identical and/or the mating types may have been unevenly distributed in the *U. necator* population sampled.

Gadoury and Pearson (1988) provide evidence that cleistothecia are formed after hyphal contact between compatible isolates in a wide range of environmental conditions. The Australian isolates of *U. necator,* when compared with isolates from New York State, may have different environmental requirements for cleistothecium formation. Alternatively, there may be an artefact in the *in vitro* system used in this study which prevented cleistothecium formation during incubation at 25°C with a diurnal photoperiod. There were a number of differences between the two experimental systems: a *V. vinifera* cultivar was used in this study instead of a *Vitis* interspecific hybrid; the plantlets were larger prior to inoculation; the MS medium was half-strength and contained no hormones; and long wavelengths of light were not provided by incandescent bulbs (D.M. Gadoury, personal communication). The growth conditions in this study were, however, highly favourable for asexual sporulation and large numbers of conidia were evident on colonized leaflets. At the time of inoculation, leaflets were still expanding on the growing shoot which may explain the fact that the vines did not appear to be heavily colonized until about 1 month after inoculation. The relative growth rates of the host and the fungus may be a major difference between the dual cultures reported here and those used by Gadoury and Pearson (1988), and may have delayed physical contact between compatible isolates in the present study. It does not, however, explain why the cultures did not form cleistothecia until they were moved to the glasshouse environment.

The results of Experiment 1 (Section 4.3.2), conducted using detached leaves, demonstrated that cleistothecia could be initiated within 14 days of co-inoculation of clonal lines. Senescence of the detached leaves, however, could not be delayed long enough for cleistothecia to mature. This suggests that there was, indeed, some artefact preventing cleistothecium initiation on micropropagated grapevines incubated in similar conditions.

Partial necrosis of leaflets in the co-inoculated *in vitro* cultures after transfer to the glasshouse environment suggests that the light quality and quantity and/or temperature was sub-optimal for the maintenance of healthy plantlets. Altered leaflet physiology may have altered the fungal physiology by imposing a nutrient or water stress. In addition, environmental conditions such as light quantity and quality may have altered fungal

physiology directly. Given that cleistothecia were produced when plantlets appeared to be stressed in the glasshouse environment, a series of preliminary experiments to identify potential environmental and/or cultural conditions required for the rapid initiation of cleistothecia was conducted.

Both the uv light and darkness (treatment 6) treatments were imposed as a potential means of suppressing asexual sporulation and inducing the sexual process of *U. necator*. Exposure of *U. necator* to uv light was based on the following hypothesis: if the majority of conidia and epiphytic hyphae were killed by brief exposure to uv light, then any surviving hyphae attached to haustoria may have responded to the "stress" by developing ascogenous hyphae. Although the effect of light quality and quantity on the asexual sporulation of *U. necator* is incompletely understood, the darkness treatment (treatment 6) was also tested as a potential method for suppressing conidium production. It appears, however, that both the uv light and darkness treatments were too harsh and the treatments should be modified by reducing exposure times.

The exposure of *U. necator* to penconazole vapours was an additional attempt to suppress condium production. Penconazole is a triazole fungicide, a group which is reported to have plant growth regulating properties (Asare-Boamah *et al.*, 1986). The vapours of this fungicide may have altered fungal physiology indirectly by altering leaflet physiology. For example, when Asare-Boamah *et al.* (1986) compared the foliage of bean seedlings drenched or not drenched in 10 $\mu g\ ml^{-1}$ triadimefon, the treated seedlings had reduced leaf area and transpiration, and an increase in shoot water potential 8 days after treatment. These events were correlated to a transient two-fold increase in endogenous abscisic acid (ABA), when compared to the untreated seedlings. The response of plants to a range of environmental stresses is often correlated to a rise in endogenous ABA, which allows the plant to exhibit increased resistance to the agent causing the stress (Boussiba *et al.*, 1975). Since exogenous application of ABA can sometimes mimic the resistance seen when endogenous levels rise, ABA was applied to the co-inoculated cultures to mimic the physiological reponse of the plantlet when exposed to an environmental stress. The application of exogenous ABA to micropropagated plantlets caused abscision of leaflets at the highest dose, but cleistothecia did not form on plantlets in this treatment; nor did they

form on plantlets growing on concentrated sucrose media (treatments 12 and 13), a treatment intended to reduce the water potential of the plantlets.

Cleistothecia formed in the dual cultures treated with penconazole vapours but there was insufficient evidence to determine if cleistothecium initiation was correlated to the penconazole vapours and/or the continuous light regime. Further experiments are required to determine if penconazole or other triazole fungicides alter host and/or fungal physiology and if the effect is correlated to cleistothecium production. Given that *U. necator* formed cleistothecia in the preliminary experiment on only one out of three replicate plantlets, additional experiments involving an expanded range of treatments and increased replication are required in order to identify the contributing factors.

In subsequent experiments, cleistothecia formed on various types of host material in the continuous light incubator at 20°C. On the one-node segments from micropropagated grapevines, the majority of cleistothecia matured within 21 days of inoculation. This result was comparable to those of Gadoury and Pearson (1988), who reported that the mean number of days from inoculation until 50% or more of the cleistothecia were mature was 25 days at 25°C. In the study presented here, the time between the appearance of immature cleistothecia and the observation of morphologically mature cleistothecia was less than 20 days at 25°C, irrespective of whether the dual cultures were on whole plantlets or single-node segments.

When compared with the incubation in diurnal light, three environmental conditions were different in the continuous-light incubator, namely; light regime, temperature and light intensity. Therefore, cleistothecium production may be correlated to one of these variables or to a combination of the variables. The difference in light intensity between the two incubators, however, did not appear to be a contributing factor because cleistothecia were observed to form on plantlets placed on a shelf of the continuous-light incubator receiving low light intensity (80-110 $\mu E\ s^{-1}\ m^{-2}$) in preliminary experiments (data not presented). A change in temperature from 25°C to 20°C also did not appear to influence cleistothecium initiation because cleistothecia were observed to form at 25°C in the continuous-light incubator (data not presented). The duration of the photoperiod, however, is likely to be a factor correlated to the initiation of cleistothecia in this *in vitro* system. In *Phaeoisariopsis personata*, continuous light as low as 60 $\mu E\ s^{-1}\ m^{-2}$ inhibited conidial production (Butler *et*

al., 1995). In addition, conidia and conidiophores of *U. necator* appeared to senesce in areas where immature cleistothecia were forming. The same observations have been made in the vineyard following the examination of colonized tissue; that is, asexual sporulation appeared to cease in areas where cleistothecia were forming. Sexual morphogens, which are lipid or protein factors produced by mated cultures, suppress asexual growth and stimulate sexual development in various ascomycetes including *Aspergillus nidulans*, *Nectria haematococca* and *Pyrenopeziza brassicae* (Ilott *et al.*, 1986; Champe & El-Zayat, 1989; Siddiq *et al.*, 1992; Dyer *et al.*, 1993; Nelson, 1996). If *U. necator* produces such a chemical factor, then cultural or environmental conditions may have suppressed its production under the diurnal-light incubation conditions. Further experiments are required to detemine cause and effect relationships and if the cessation of conidium production is a prerequisite for the initiation of cleistothecia. Therefore, the artefact delaying cleistothecia initiation in co-inoculated *in vitro* cultures should be examined further as a means of understanding the mechanisms regulating sexual reproduction.

The time taken for cleistothecium initiation appeared to be dependent on the type of host material, for example, whole plantlets, one-node segments or detached leaves, and the culture conditions used. In all conditions tested, the density of cleistothecia was greatest when cleistothecia formed early on heavily colonized leaflets which remained healthy as the cleistothecia matured. The time allowed for abundant colonization of co-inoculated leaflets, prior to imposing specific incubation conditions, varied from 5 to 21 days for one-node segments, and from 14 to 28 days for whole plantlets. Cleistothecia formed most rapidly on one-node segments from micropropagated grapevines, but it was difficult to obtain consistent infection on these leaflets. The use of 21-day-old micropropagated grapevines in 500 ml containers allowed a compromise between rapid cleistothecium formation and consistent, vigourous host colonization. These plantlets were pre-colonized prior to the initiation of cleistothecia because *U. necator* did not colonize the plantlets well if cultures were placed directly in the continuous-light incubator at the time of inoculation. Cleistothecia did, however, take at least 27 days to form after transfer to the continuous-light incubator. There was considerable variation between paired clonal lines in the time taken for cleistothecia to form and in the density of cleistothecia. The varying results may be related to variations in the rate of leaflet colonization and the time taken for physical contact between

compatible hyphae. Gadoury and Pearson (1991) reported densities of cleistothecia on micropropagated leaflets in the range 239-526 per cm^2 at 60 days. Using micropropagated grapevines in the 500 ml containers, cleistothecium densities ranged from 100 to 744 per cm^2 at 126 days post-inoculation, a range which overlaps that observed by Gadoury and Pearson (1991). The greatest density of cleistothecia, 4,500 per cm^2, was observed on the one-node segments in a Petri plate. The ascospores inside these cleistothecia, although viable at 48 days post-inoculation, appeared to have aged and lost viability by 70 days after inoculation.

Using micropropagated grapevines, viable ascospores were observed 126 days after inoculation or 78 days after the first immature cleistothecia formed and viable, colony-producing ascospores were recovered from cleistothecia approximately 33 days later. This result was consistent with those of Gadoury and Pearson (1991), who observed viable ascospores, by use of vital staining, from cleistothecia produced *in vitro* approximately 70 days after the cleistothecial initials developed. These researchers induced cleistothecia, produced *in vitro*, to discharge ascospores onto glass slides, after the cleistothecia had been placed in mesh bags on the vineyard floor during the winter for an unspecified duration. The percentage viability of ascospores within these cleistothecia, assessed using vital staining, was in the range 22 to 64 percent. The percentage germination of the discharged ascospores in water, however, ranged from 54 to 81 percent. In this study, the life cycle of *U. necator* was completed *in vitro* by demonstrating the infectivity of ascospores on detached leaves. The infection efficiency of ascospores appeared to be low because only five colonies developed from up to 4,700 cleistothecia. These five colonies may have developed, however, from more than five ascospores. Pearson and Gadoury (1987) reported an infection efficiency of 3-9% for ascospores discharged from cleistothecia collected on diseased leaves in the vineyard and overwintered in mesh bags in the vineyard. A time series experiment is required to determine when ascospores produced *in vitro* have the highest infection efficiency.

Heterothallism among South Australian clonal lines of *U. necator* was demonstrated by pairing three clonal lines of each putative mating type in all possible combinations. Cleistothecia formed in at least one out of three replicates of all nine pairings where they were expected to form. The apparent lack of compatibility of clonal lines that were expected

to form cleistothecia in some replicates was probably the result of poor host colonization and failure of the clonal lines to make hyphal contact. At the time of the second and third replications of the matrix pairings, the grapevines appeared to be losing vigour as a result of being propagated *in vitro* for at least 2 years. Establishment of new grapevine cultures *in vitro* from field or glasshouse material or modification of the culture environment may have improved the vigour of the dual cultures. Another factor influencing compatibility may be the fitness of the clonal lines. The fitness of clonal lines among the *U. necator* collection appeared to vary in terms of the rate of leaflet colonization and the area colonized. The aim of inoculating each clonal line in quarter-leaf sectors, therefore, was to allow each line to colonize the leaflet prior to physical contact with the other clonal line. This technique should minimize situations in which one clonal line colonizes the leaflet before the other clonal line becomes established. Variations in clonal line fitness and leaflet physiology may contribute to differences in the time taken for cleistothecia to form in different pairings of clonal lines.

All experiments in this study were conducted with *V. vinifera* cv. Cabernet Sauvignon but responses may be different on other cultivars and *Vitis* spp., including the *Vitis* interspecific hybrid used by Gadoury and Pearson (1988). In Australia, abundant cleistothecium production occurs on the ornamental *V. amurensis*. This *Vitis* sp. could be established *in vitro* and micropropagated for use in future experiments. Other factors worth exploring further are light quality, for example the influence of light of long wavelength (>700 nm), and inoculum concentration and method.

4.5 Summary and conclusions

In summary, two mating types were identified among 21 clonal lines of *U. necator* from South Australia. Ten clonal lines were designated as the "plus" mating type and seven clonal lines were designated as the "minus" mating type. The mating type of the remaining four clonal lines has not been confirmed. South Australian *U. necator* clonal lines were shown to be heterothallic and cleistothecia containing viable, infective ascospores were produced on micropropagated grapevines. Further experiments are necessary to determine the optimum conditions for the initiation of cleistothecia and the optimum time and method for the recovery of single, viable ascospores from cleistothecia produced *in vitro*.

Initiation of cleistothecia appeared to be prevented by an artefact of the dual culture system in which co-inoculated micropropaged *V. vinifera* cv. Cabernet Sauvignon were incubated using diurnal light patterns. The artefact may be related to the fact that growth conditions were highly favourable for asexual sporulation and appeared to be overcome by exposure to a continuous light regime. The differing periods of time between co-inoculation and initiation of cleistothecia in different *in vitro* culture systems suggest that leaflet physiology is playing a role in cleistothecium production and that specific environmental or cultural cues are required for the fungus to commence its sexual stage *in vitro*.

5.0 DEVELOPMENT AND EVALUATION OF RFLP PROBES AS MARKERS FOR *U. NECATOR*

All sciences start with recognizing the substances being studied.
R. L. Metzenberg, 1991.

5.1 INTRODUCTION

The development of DNA markers for *U. necator* will provide precise methods for studying the molecular and population genetics of this pathogen. The type of DNA marker for identifying genetic variation must be determined empirically. In Chapter 2, RFLP analysis was identified as a robust method for studying the genetic diversity of plant pathogenic ascomycetes. RFLP markers can be used (i) to identify genetically different individuals, (ii) to examine frequencies of genetic loci in population studies, and (iii) to map linkages to mating type, fungicide resistance, virulence or other genes of interest. For example, Christiansen and Giese (1990) used random clones from genomic libraries of *Erysiphe graminis* f. sp. *hordei* DNA for RFLP analysis and identified two groups of linked loci which contained both RFLP markers and virulence genes. The advantage of using cloned DNA of the species under study is that species-specific probes can be developed, thus allowing variation to be identified in DNA samples of mixed origin. In addition, clones can be transferred readily from one laboratory to another.

In this chapter, methods for cloning *U. necator* DNA and screening cloned sequences as potential RFLP probes are described. The homology of two *E. graminis* clones to *U. necator* DNA was also investigated. The potential for genetic change in *U. necator* clonal lines in culture, by mutation or by contamination from conidia of other clonal lines, was examined using selected *U. necator* probes. In addition, selected *U. necator* probes were evaluated for species specificity, and were used to determine the amount of infected grapevine leaf DNA required to produce the same hybridisation signal strength as was observed using purified *U. necator* DNA.

5.2 MATERIALS AND METHODS

5.2.1 Ligation of *U. necator* DNA into pUC19 vector

The procedure for the ligation of *U. necator* DNA into vector DNA, plasmid pUC19, was based on protocols described by Sambrook *et al.* (1989). Vector DNA was digested to

completion with the restriction enzymes *Eco*RI or *Pst*I. Approximately 5-6 µg of *Eco*RI digested pUC19 DNA was dephosphorylated in a reaction volume of 30 µl containing 24 units of calf intestinal alkaline phosphatase (CIP), according to the manufacturer's specification (Boehringer Mannheim). Following incubation at 37°C for 30 min, an additional 12 units of CIP was added to the reaction mixture and incubated for 30 min at 37°C. The reaction was stopped by the addition of EDTA, pH 8, to a final concentration of 10 mM and the solution heated at 65°C for 10 min. The solution was cooled on ice, increased to 200 µl with TE (refer to Appendix), extracted with phenol/chloroform and the DNA precipitated with two volumes of absolute ethanol overnight at -20°C. The DNA was recovered by centrifugation and resuspended in TE buffer.

Vector to insert (*U. necator* DNA) molar ratios were calculated prior to ligation (refer to Appendix). A quantity of total *U. necator* DNA (refer to Section 3.4), in the range 200 to 400 ng and from clonal line 920103, was digested to completion with 20 units of *Eco*RI or *Pst*I, at 37°C for 4 h. The digested DNA was extracted with phenol/chloroform and precipitated with two volumes of absolute ethanol overnight at -20°C. The DNA was recovered by centrifugation, resuspended in TE buffer and stored at -20°C until required for the ligation reaction. The ligation reaction for *Eco*RI digested vector and inserts was performed in a reaction volume of 30 µl containing 135 ng *U. necator* DNA, 190 ng dephosphorylated, linearized pUC19 vector, 1 unit of T4 DNA ligase and ligation buffer containing 1 mM ATP (Boehringer Mannheim). The reaction mixture was incubated at room temperature for 6 h then stored at -20°C until required. The ligation reaction for *Pst*I digested vector and inserts was the same, except that the reaction mixture contained 270 ng *U. necator* DNA and 60 ng of linearized pUC19 vector.

5.2.2 Transformation of competent cells of *Escherichia coli*

Competent cells of *Escherichia coli,* strain JM101, were prepared by inoculating 10 ml of Luria-Bertani (LB) broth with a single colony. The inoculated broth was incubated in an orbital shaker at 37°C overnight. A 1 ml sample of this overnight culture was added to 100 ml LB broth and incubated in an orbital shaker at 37°C until the OD_{600nm} was 0.2-0.3. Cells from 30 ml of this culture were recovered by centrifugation at 5,000 *g* for 5 min at 4°C and resuspended in 30 ml of 0.1 M magnesium chloride. The cells were pelleted

again, resuspended in 30 ml of 0.1 M calcium chloride and incubated on ice for 15 min. The cells were pelleted one more time and resuspended in 3 ml of a solution containing 0.05 M calcium chloride and 15 % glycerol. The competent cells were divided into 600 µl aliquots and stored at -70°C until required.

A 200 µl volume of competent cells was transferred immediately upon thawing to a sterile 10 ml polypropylene tube on ice. The cells were transformed with 40 ng of ligated DNA (refer to Section 5.2.1) that had been diluted to a volume of 20 µl with TE. The ligated DNA solution (20 µl) was added to the cells, mixed gently and incubated on ice for 20 min. Two control tranformation reactions were incubated in the same manner: the first control transformation reaction contained linearized pUC19 and no *U. necator* DNA, and the second contained closed circular pUC19 and no *U. necator* DNA. Following incubation, all cells were heat shocked at 37°C for 2 min then added to 5 ml of LB broth and incubated at 37°C for 1 h. The cells were pelleted by centrifugation at 680 ***g*** for 5 min, resuspended in 400 µl LB broth and four 100 µl samples were spread out on agar medium in each of four 9 cm-diam. Petri plates. The medium consisted of 3 ml of 0.7% distilled water agar (Difco Bacto), containing 50 µg ml^{-1} ampicillin, 20 µg ml^{-1} X-gal and 24 µg ml^{-1} IPTG, overlaid on 20 ml of LB agar containing 50 µg ml^{-1} ampicillin (LBamp agar). Following incubation overnight at 37°C, recombinant clones, containing inserts of *U. necator* DNA, were identified as white colonies; non-recombinant colonies were blue.

Recombinant colonies were transferred, in duplicate and in a grid pattern, to LBamp agar and to 0.45 µm nitrocellulose membranes (Scheicher & Scheull) placed on the surface of LBamp agar, and incubated overnight at 37°C (Sambrook *et al.*, 1989). A non-recombinant colony was included on each agar plate. Colonies growing on the LBamp agar were transferred individually to 1 ml of LB broth containing 15% glycerol, vortex-mixed briefly then stored at -70°C. Clones were numbered in chronological order using the code pUn*# where p = plasmid, Un = *Uncinula necator*, * = E for *Eco*RI or P for *Pst*I, and # = a number. The colonies on the nitrocellulose membranes were lysed and the DNA was fixed to the filters according to the method of Grunstein and Hogness (1975). Using this method, the membranes, colony-side up, were imbibed sequentially in the following solutions for 5 min each: 10% SDS once, denaturation solution (1.5 M sodium chloride, 0.5 N sodium hydroxide) twice, neutralising solution (1 M Tris, 1.5 M sodium chloride, pH

7.4) three times and 2x SSC once. The solutions were contained in Petri plates, each containing three Whatman filter papers so that a thin film of solution was present on the uppermost filter paper. Excess solution was removed from the nitrocellulose membranes by blotting them on paper towels between changes of solution. The cellular debris was wiped off each filter with non-absorbent cotton wool while immersed in 2x SSC. DNA was cross-linked to the filters using uv light from a GS Gene Linker (Bio-Rad).

Clones containing *U. necator* inserts were identified by hybridising total *U. necator* DNA to the DNA bound to the nitrocellulose membranes (colony blots). Radiolabelled *U. necator* DNA was prepared as follows: a partial digest of 60 ng of total *U. necator* DNA, from clonal line 920103, was carried out using 5 units of *Eco*RI in a reaction volume of 20 µl at 37°C for 1 h. The enzyme was removed from the solution by chloroform/isoamyl alcohol (24:1) extraction. DNA was precipitated from the aqueous phase with an equal volume of cold isopropanol, on ice for 4 h, then recovered by centrifugation and resuspended in 10 µl of TE buffer. Half of this DNA sample was radiolabelled by the random primer method (see Section 3.6). The labelled DNA was separated from unincorporated nucleotides in a Bio-Gel P-60 column, denatured and hybridised, in a volume of 10 ml, to the colony blots for 24 h, using the method described for Biodyne A membranes in Section 3.6. The membranes were then washed three times in 2x SSC, 0.1% SDS for 30 min at 65°C, to remove unbound DNA, and exposed to X-ray film (Kodak X-Omat) at -70°C. The resulting hybridisation signal from each clone was classified as high, medium, low or no signal.

5.2.3 Plasmid preparation

5.2.3.1 Crude plasmid preparation

An alternative procedure for assessing the copy number of cloned fragments was evaluated and compared with the colony lysis method. Plasmid DNA from 115 clones was prepared in a crude form, fractionated in an agarose gel, and prepared for Southern hybridisation using total *U. necator* DNA as the probe (refer to Section 5.2.2).

The method for crude plasmid preparation was modified from a procedure described by Holmes and Quigley (1981; J. N. Timmis, personal communication) and was intended to be a rapid technique for screening large numbers of clones. For each clone, a

single recombinant colony was removed with a sterile toothpick and the cells streaked in straight lines across one quarter of a 9 cm-diam. Petri plate containing LBamp agar, which was then incubated overnight at 37°C. The resultant bacterial cells were harvested using a sterile toothpick until a large clump of cells was visible. These cells were suspended in 100 µl TE buffer (refer to Appendix) to which 50 µl of a dye solution containing 5% SDS, 0.2% bromophenol blue and 25% glycerol was added. The mixture was incubated at 65°C for 30 min, vortex-mixed well, and the total DNA pelleted by centrifugation at 15,800 *g* for 30 min. The supernatant, containing the plasmid DNA, was removed and maintained at 65°C, along with all the other samples, until all the samples were loaded into wells of a 1.2% agarose gel. Following electrophoresis at 60 V for 3.5 h, the DNA in the gel was stained with ethidium bromide and photographed under uv illumination before being transferred to a ZetaProbe membrane and hybridised with total *U. necator* DNA. The hybridisation signal for each preparation was compared with the ethidium bromide staining intensity of the DNA in the agarose gel and each clone classified as having high, medium or low copy *U. necator* inserts. In addition, as an independent assessment, the copy number of approximately half the clones screened was estimated by Dr B. Stummer.

5.2.3.2 Small and large scale plasmid preparation

Plasmid DNA was isolated in order to estimate the size of a cloned insert and to screen cloned sequences as potential Southern hybridisation probes. Recombinant *E. coli* bacteria were recovered from a frozen glycerol culture (see Section 5.2.2) and a single colony transferred to 5 ml of LBamp broth and incubated in an orbital shaker at 37°C until the OD_{600nm} was 0.5-0.6. The cells were pelleted by centrifugation at 15,800 *g* for 5 min, resuspended in cold STE (see Sambrook *et al.*, 1989) and pelleted as before. The supernatant was removed and the cells were resuspended gently in 360 µl of cold sucrose buffer (refer to Appendix) followed by the addition of lysozyme, in sucrose buffer, to a final concentration of 2 mg ml^{-1}. After incubating the solution on ice and mixing occasionally for 10 min, RNase A was added to a final concentration of 0.1 mg ml^{-1}, followed by Triton buffer (refer to Appendix) to a final concentration of 0.5% Triton X-100. The solution was mixed very gently by inverting the tube and then incubated at room temperature for 10 min. The genomic DNA was pelleted by centrifugation at 15,800 *g* for

30 min. The supernatant was removed, extracted with phenol/chloroform and the plasmid DNA precipitated with absolute ethanol overnight at -20°C. The DNA was recovered by centrifugation and resuspended in TE buffer.

The above protocol was scaled upwards for obtaining large quantities of plasmid DNA. In large scale preparations, plasmid DNA was purified further by fractionating the DNA from other components in a caesium chloride gradient (Sambrook *et al.*, 1989).

5.2.4 Screening *U. necator* sequences as RFLP probes

Cloned *U. necator* sequences were screened to determine if they were suitable Southern hybridisation probes for distinguishing clonal lines. The copy number of clones referred to in this section is the copy number of *U. necator* inserts estimated by the colony lysis method. Fifteen high-copy clones were screened by hybridising each clone to a Southern blot of 10 ng of digested total DNA from each of two or three *U. necator* clonal lines (920103-Adelaide Plains, 930202-Barossa Valley, 931302-Adelaide Hills). These small quantities of *U. necator* DNA were used because of the limited amounts of DNA available. Total *U. necator* DNA was restriction enzyme digested with *Eco*RI, *Bam*HI or *Eco*RV in a 4 µl reaction volume and fractionated in a 10-cm long, 1% agarose gel. The DNA was transferred to a Biodyne A membrane and hybridised separately to 25 ng of selected clones that had been linearized with *Kpn*I and radiolabelled by the random primer method. The labelled DNA was separated from unincorporated nucleotides in a Bio-Gel P-60 column and hybridisation was carried out as described in Section 3.6. A control hybridisation using pUC19 DNA as the probe demonstrated that it did not hybridise to total *U. necator* DNA digested with *Eco*RI.

Eight medium or low-copy clones were evaluated during the second round of screening. The DNA inserts were excised by restriction enzyme digestion, fractionated in an agarose gel and recovered using a Geneclean kit (Bio 101). In this case, 80 ng of digested total *U. necator* DNA, from the same clonal lines as were used for screening high-copy probes, was fractionated in a 20-cm long, 1% agarose gel then transferred to a ZetaProbe membrane. Fifty ng of the *U. necator* insert DNA was radiolabelled by the random primer method and hybridised to the DNA on the membrane.

5.2.5 Evaluation of selected *U. necator* and *E. graminis* probes

Selected *U. necator* probes were screened against a larger number of *U. necator* clonal lines, the results of which will be presented mainly in Chapter 6. The putative high-copy clones pUnE4 and pUnE6 were examined in more detail to investigate the nature of the DNA polymorphism. Clone pUnE4 was linearized with *Kpn*I, radiolabelled and hybridised to DNA from 21 clonal lines of *U. necator*. Ten ng of total *U. necator* DNA from each clonal line was digested with *Eco*RI, fractionated through a 20 cm long, 1% agarose gel, transferred to a Biodyne A membrane and hybridised to the radiolabelled probe according to methods described in Section 3.6. Clone pUnE6 was hybridised to 10 ng of total *U. necator* DNA, undigested or digested with *Eco*RI, from five clonal lines.

The homology of two *Erysiphe graminis* clones, pEgE9 (O'Dell *et al.*, 1989) and pGEE381 (Christiansen & Giese, 1990), to *U. necator* DNA was evaluated. The *E. graminis* clones were digested with *Eco*RI and 25 ng of total, linearized plasmid DNA was radiolabelled by random priming. The probes were hybridised to 10 ng of *Eco*RI digested total DNA from two clonal lines of *U. necator*, and to 10 ng of *Hin*dIII or *Bam*HI digested total DNA and undigested total DNA from an Australian isolate of *E. graminis*. The DNA was fractionated in a 10-cm long, 1% agarose gel and transferred to Biodyne A membrane for Southern hybridisation. *E. graminis* conidia and probes were kindly provided by Dr D. Whisson. The probes originated from the research groups referenced above.

Clones identified as being useful for RFLP analysis were evaluated further to identify probes that detected only *U. necator* sequences in infected grapevine tissue. In addition, each clone was evaluated for its potential to detect genetic change in a *U. necator* clonal line. Clones were classified as being somatically stable if the hybridisation pattern for a clonal line was constant for DNA extracted from conidia sampled at intervals for at least 15 months.

Total DNA was extracted from healthy detached grapevine leaves and from detached leaves which were colonized by *U. necator* (refer to Section 3.3) using a method modified from that described by Lodhi *et al.* (1994). The modifications involved removing the RNase A by chloroform/isoamyl alcohol (24:1) extraction and precipitating the DNA with 2.5 M sodium chloride and 2 volumes of absolute ethanol for a second time in order to reduce polysaccharide contamination.

Clones pUnP27, pUnP14 and pUnE4 were hybridised to various amounts of *Eco*RI digested total DNA from infected grapevine leaves, either from detached-leaf culture or from micropropagated plantlets (see Section 3.5). Each DNA sample was fractionated in a 20-cm long, 1% agarose gel, transferred to a ZetaProbe membrane and hybridised to a probe that had been radiolabelled using pUC19 specific primers. The signal obtained by Southern hybridisation was used to estimate the quantity of infected grapevine DNA required to obtain a signal similiar to that obtained using purified *U. necator* DNA.

In order to test probe specificity further, clones pUnP14 and pUnE4 were hybridised to at least 10 ng of total DNA, undigested or digested with *Eco*RI, from *Aspergillus nidulans* isolate DAR35797, *Monilinia fructicola* isolate DAR27029, *Phomopsis viticola* isolate DAR27955, *Botrytis cinerea* isolate ICMP9433, *Penicillium digitatum* isolate N3, *Rhizoctonia solani* isolate RH124, *Saccharomyces cerevisiae* isolate BP10, and *Rhynchosporium alismatis*. With the exception of *Rhizoctonia solani* and *Rhynchosporium alismatis*, these higher fungi represent genera which are likely to be associated with the mycobiota of grapevine leaves and berries (Duncan *et al.*, 1995). The fungal DNAs were kindly provided by Dr M. Gillings and DNA from *U. necator* clonal line 920103 was included in the hybridisation as a control.

Clones pUnP27, pUnP14 and pUnE4 were also hybridised to DNA from conidia of different asexual generations of *U. necator* clonal lines 920103 and 931302. The different asexual generations consisted of conidia collected at various intervals over at least 15 months. Approximately 80 ng of total *U. necator* DNA from each sample of conidia was digested with *Eco*RI or *Bam*HI, fractionated in a 20 cm agarose gel and transferred to a ZetaProbe membrane. Ten µg of total grapevine DNA from healthy detached leaves was digested with *Eco*RI and fractionated on the same gel. Between 50 and 100 ng of pUnE4 and pUnP14 was radiolabelled using pUC19 specific primers (refer to Appendix), whereas the insert of clone pUnP27 was excised and radiolabelled by the random primer method.

5.3 RESULTS

5.3.1 Screening of *U. necator* sequences

A total of 867 putative recombinant (white) colonies were obtained following the transformation of *E. coli* cells with total *U. necator* DNA: 159 clones were obtained for *U.*

necator sequences ligated into the *Eco*RI site of pUC19, whereas 708 clones were obtained for sequences ligated into the *Pst*I site. Following lysis of colonies and binding of DNA to nitrocellulose membranes, total *U. necator* DNA hybridised to DNA from all the recombinant clones. The hybridisation signal was classified as high, medium or low (Table 5.1, Figure 5.1); no signal was detected from the DNA of non-recombinant (blue) colonies.

Two methods for classifying the copy number of clones, namely colony lysis and the crude plasmid preparation method, were compared using 115 clones (Table 5.2, Figure 5.2). Given that the classification was based on a visual assessment, the percentage agreement for the two methods was estimated to be 75%, 52% and 70% for the high, medium and low copy categories, respectively. Most re-classifications using the crude plasmid preparation method involved a change of one level only, for example, medium to high copy or medium to low copy. Two clones were re-classified two levels apart, that is from high copy, according to the colony lysis method, to low copy, according to the crude plasmid preparation method.

The size of *U. necator* inserts in both the *Pst*I and *Eco*RI cloning sites was estimated for 66 clones, where n = 42 for *Pst*I clones and n = 24 for *Eco*RI clones (Figure 5.3). Insert sizes ranged from 0.3 kbp to 13.9 kbp with 50% of inserts in the 0.2-2 kbp range and 85% of inserts in the 0.2-6 kbp range.

Figure 5.4 illustrates the restriction enzyme digestion of total *U. necator* DNA with *Eco*RV, following ethidium bromide staining. At least 16 distinct fragments are evident as brightly stained bands against a background smear of DNA. Clonal line 931302 (lane 3) had a 9.4 kbp fragment which appeared to be absent from lines 920103 and 930202 (lanes 1 and 2, respectively). When DNA polymorphisms were investigated using RFLP analyses, initial screening of 15 putative high-copy *U. necator* sequences revealed DNA polymorphisms between clonal lines 920103 and 930202 with two probe-enzyme combinations and between clonal lines 920103 and 931302 with 12 probe-enzyme combinations (for example, see Table 5.3, Figure 5.5). The majority of these polymorphisms occurred at one restriction site, often as a deletion in clonal line 931302 or 920103. There were several types of banding patterns produced by probes selected as being high copy number; that is, the pattern was either (a) one or two bands with a strong

Table 5.1. Classification of the hybridisation signal from *U. necator* cloned sequences when hybridised to total *U. necator* DNA following colony lysis.

pUC19 cloning site	Number of clones in each copy number category			
	High	Medium	Low	Total
*Pst*I	173 (24%)	99 (14%)	436 (62%)	708
*Eco*RI	59 (37%)	25 (16%)	75 (47%)	159

Table 5.2 Comparison of the colony lysis method with the crude plasmid preparation method for estimating the copy number of *U. necator* sequences.

Number of clones in each copy number category				
Crude plasmid preparation method	Colony lysis method			
	High	Medium	Low	Total
High	13 (75%)[a]	7	0	20
Medium	2	24 (52%)[a]	16	41
Low	2	14	38 (70%)[a]	54
Total	17	44	54	115

[a] An estimate of the percentage agreement, in parenthesis, between the two methods

Figure 5.1 Examples of the hybridisation signals detected by autoradiography following hybridisation of total *U. necator* DNA to DNA from recombinant bacterial cells lysed and bound on cellulose nitrate membranes. L, low copy clone; M, medium copy clone; H, high copy clone; C, non-recombinant colony (no signal).

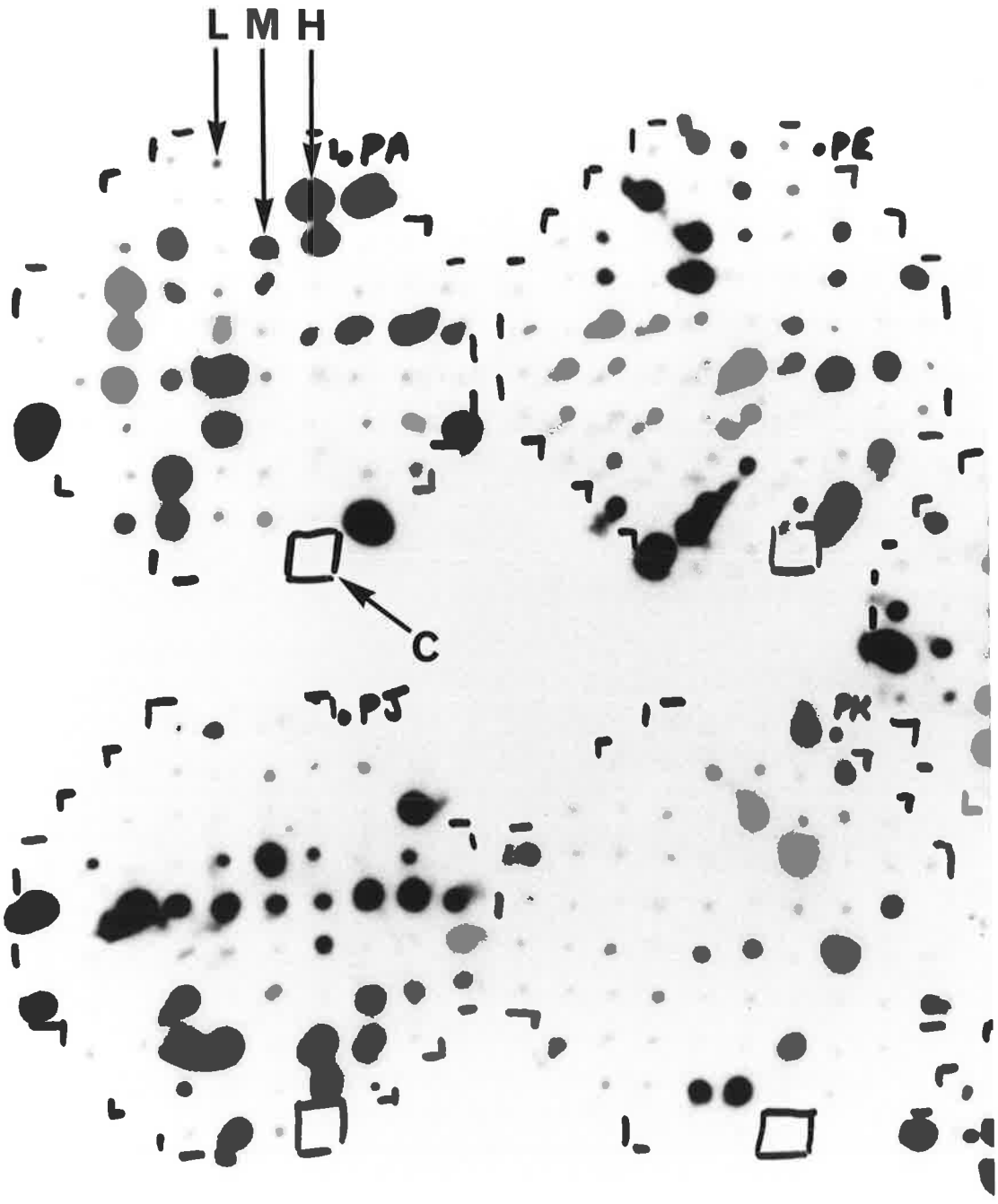

L M H
PA
PE
C
PS
PK

Figure 5.2 An example of crude plasmid preparations of *U. necator* clones and the corresponding autoradiograph following hybridisation with total *U. necator* DNA.

A. Crude plasmid DNA, containing *U. necator* inserts, fractionated in a 1.2% agarose gel and stained with ethidium bromide. L, low copy clone; M, medium copy clone; H, high copy clone. DNA size markers, in kbp, are shown to the left.

B. The corresponding hybridisation signals detected by autoradiography when total *U. necator* DNA was hybridised to the Southern blot. L, low copy clone; M, medium copy clone; H, high copy clone. The probe did not hybridise to pUC19 DNA (data not shown).

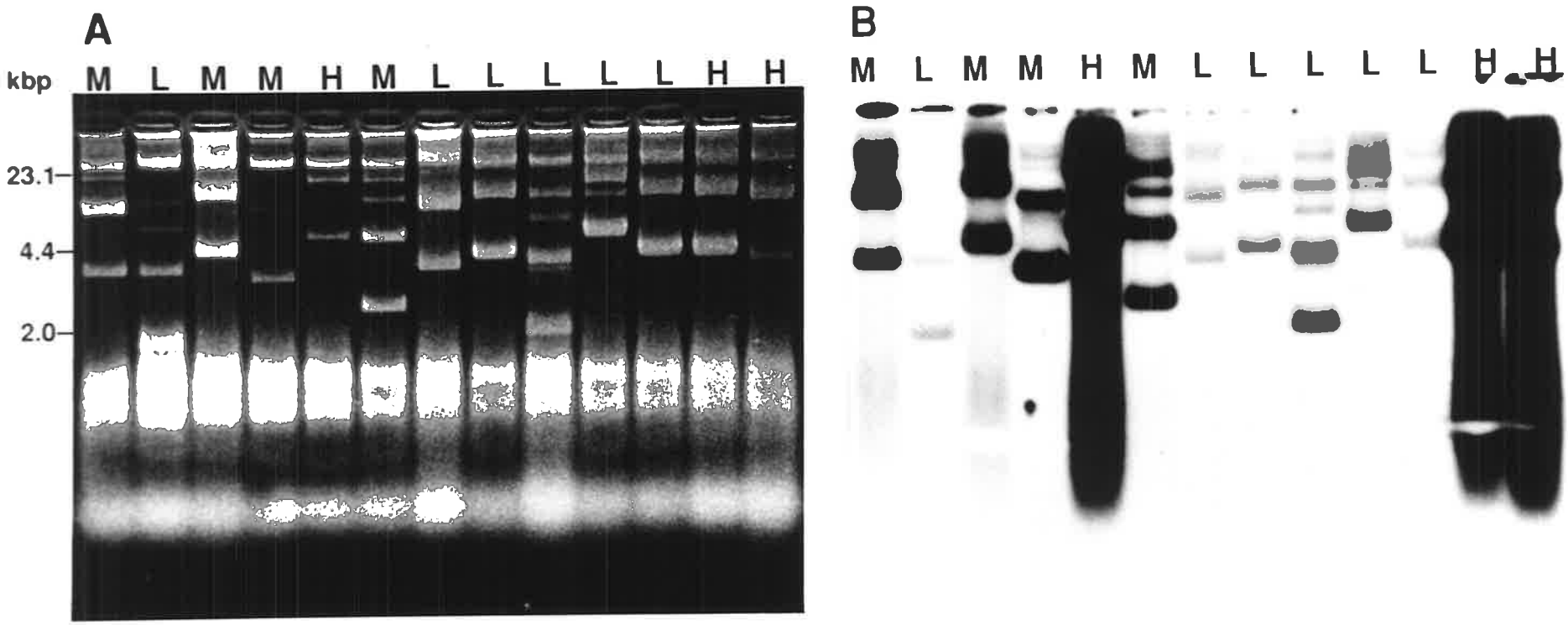

A
kbp
M L M M H M L L L L L H H
23.1
4.4
2.0
B
M L M M H M L L L L L H H

Figure 5.3 Estimated sizes of *U. necator* inserts in both *Pst*I and *Eco*RI cloning sites of pUC19 for 42 *Pst*I clones and 24 *Eco*RI clones.

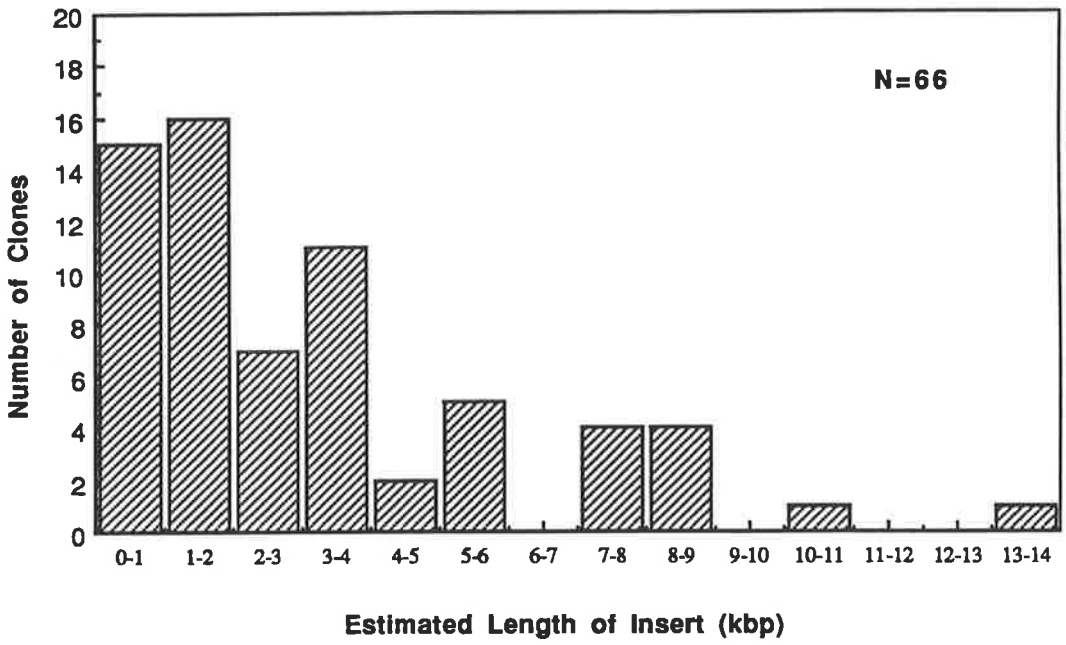

N=66
Number of Clones
0
2
4
6
8
10
12
14
16
18
20
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
Estimated Length of Insert (kbp)

Figure 5.4 Total *U. necator* DNA (approximately 100 ng lane^{-1}) digested with *Eco*RV and stained with ethidium bromide. DNA size markers, in kbp, are shown to the left. *Lane M,* 0.5 µg lambda DNA digested with *Hind* III; *Lane 1,* Line 920103 (Adelaide Plains); *Lane 2,* Line 930202 (Barossa Valley); *Lane 3,* Line 931302 (Adelaide Hills).

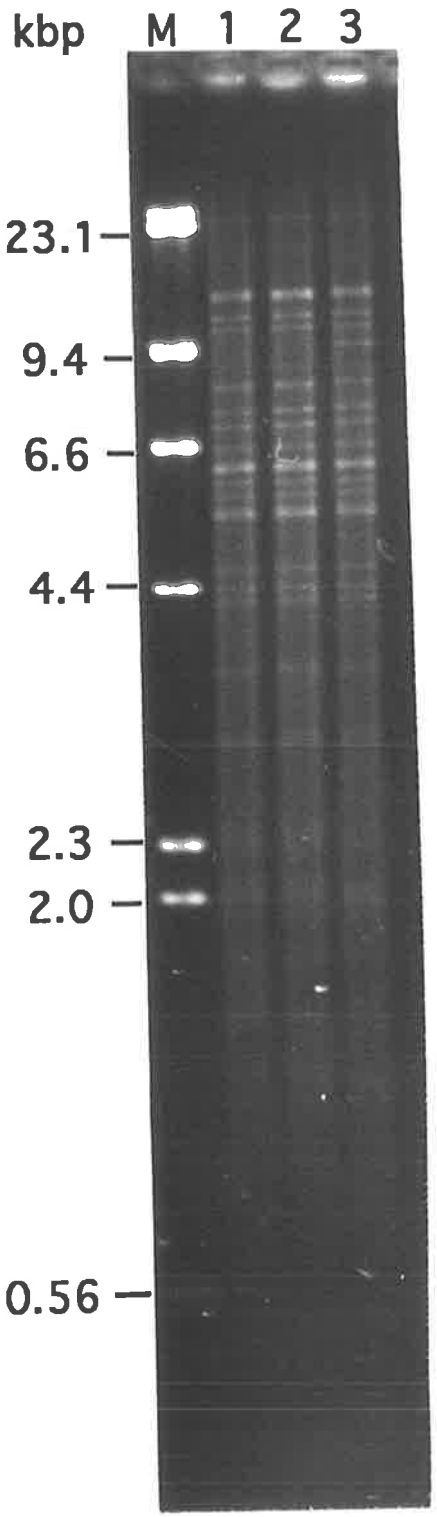

kbp
M
1
2
3
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Table 5.3 Putative DNA polymorphisms among clonal lines of *U. necator* following the screening of *U. necator* clones as Southern hybridisation probes for total *U. necator* DNA digested with various restriction enzymes. The insert of each probe originated from clonal line 920103.

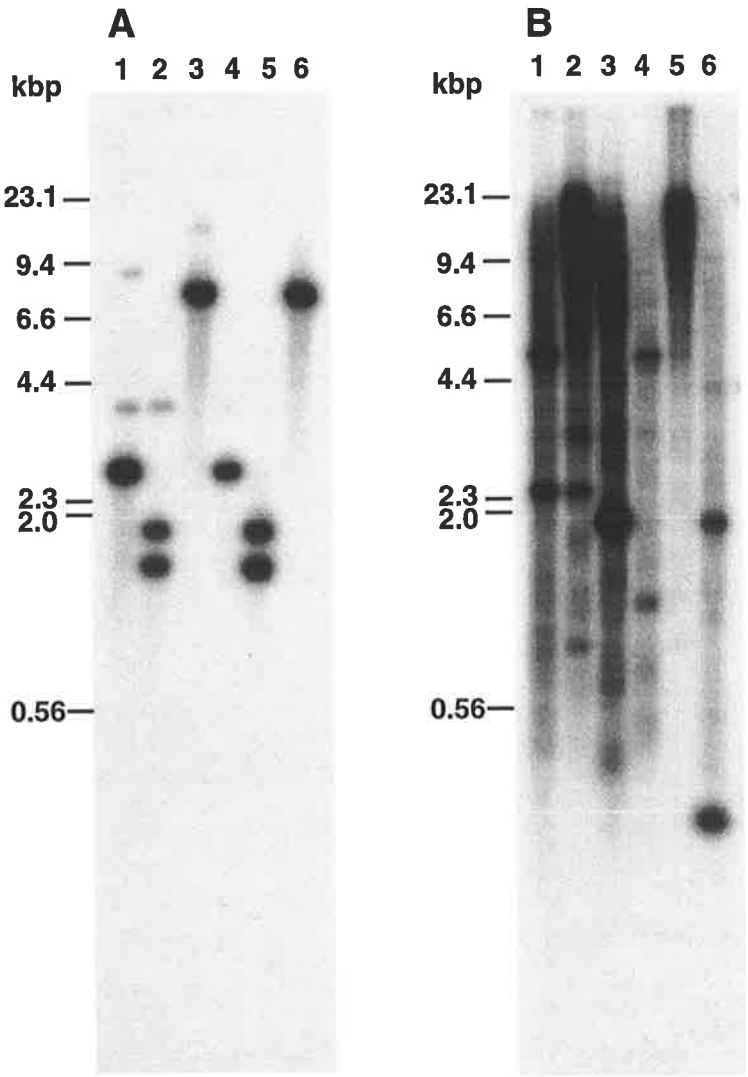
Probe	Insert size (kbp)	Copy number[a]	Clonal lines 920103 and 930202			Clonal lines 920103 and 931302		
			*Eco*RI	*Bam*HI	*Eco*RV	*Eco*RI	*Bam*HI	*Eco*RV
pUnP1	0.6	high	NP(1)	-	NP(2)	-	-	P(1)
pUnP2	2.3	high	NP, hr	-	NP, hr	P(1)[b], hr	-	NP, hr
pUnP5	0.8	high	-	-	-	P(1), hr	-	P(1)[b], hr
pUnP9	1.7	high	-	-	-	P(1), hr	-	P(1)[b], hr
pUnP24	3.6	high	NP(2), hr	P(1)[b], hr	P(1), hr	-	-	-
pUnP25	4.3	high	NP, hr	NP, hr	NP, hr	-	-	-
pUnE6	1.8	high	NP, hr	NP, hr	NP, hr	P(1)[b], hr	NP, hr	NP, hr
pUnE4	0.8	high	-	-	-	P(2)[b]	P(1)	NP(2)
pUnE7	2.1	high	NP(1)	NP(2)	NP(1)	-	-	-
pUnE10	3.5	high	NP(2)	NP(1)	NP(1)	-	-	-
pUnE13	3.4	high	NP(1)	-	-	NP(1)	-	-
pUnE21	0.3	high	-	-	-	P(1), hr	-	P(1)[b], hr
pUnE25	5.5	high	NP(2)	NP(1)	NP(1)	-	-	-
pUnE26	5.9	high	NP(2), hr	-	NP, hr	-	-	-
pUnE27	5.3	high	-	-	-	P(1)	NP(2)	NP(1)
pUnP13	1.4	medium	NP, hr	NP, hr	NP, hr	-	-	P, hr
pUnP14	2.6	medium	-	-	-	P(14)[c]	P[c]	P[c]
pUnP18	3.3	medium	-	-	-	P, hr	P, hr	P, hr
pUnP27	2.1	medium	-	-	-	P	P(17)	P
pUnE9	1.2	low	-	-	P, hr	P[c], hr	P[c], hr	P[c], hr
pUnE16	3.0	medium	-	-	P(1)[b]	-	-	NP(2)
pUnE17	0.9	medium	-	-	-	NP(1)	NP(1)	NP(2)
pUnE28	8.1	low	NP, hr	NP,hr	NP, hr	-	-	-

P(n) Polymorphic (number of polymorphic bands).
NP(n) Not Polymorphic (number of bands observed).
hr highly repetitive hybridisation pattern, separate fragments not always discernable.
a classified using the colony lysis method.
- probe-enzyme combination was not evaluated.
b polymorphism involved a deleted restriction site.
c clonal lines 920103 and 931502.

Figure 5.5 Southern hybridisations of pUnE7 **(A)** and pUnP9 **(B)** to total *U. necator* DNA (approximately 10 ng lane^{-1}), from various clonal lines, digested with *Eco*RI, *Bam*HI or *Eco*RV.

A. *Lane 1*, Line 920103, digested with *Eco*RI; *Lane 2*, Line 920103, digested with *Bam*HI; *Lane 3*, Line 920103, digested with *Eco*RV; *Lane 4*, Line 930202, digested with *Eco*RI; *Lane 5*, Line 930202, digested with *Bam*HI; *Lane 6*, Line 930202, digested with *Eco*RV. DNA size markers, in kbp, are shown to the left.

B. *Lane 1*, Line 920103, digested with *Eco*RI; *Lane 2*, Line 920103, digested with *Bam*HI; *Lane 3*, Line 920103, digested with *Eco*RV; *Lane 4*, Line 931302, digested with *Eco*RI; *Lane 5*, Line 931302, digested incompletely with *Bam*HI; *Lane 6*, Line 931302, digested with *Eco*RV. DNA size markers, in kbp, are shown to the left.


A
1 2 3 4 5 6
kbp
23.1
9.4
6.6
4.4
2.3
2.0
0.56
B
1 2 3 4 5 6
kbp
23.1
9.4
6.6
4.4
2.3
2.0
0.56

signal (for example, see Figure 5.5A), (b) a smear of multiple fragments, or (c) one or two bands with a strong signal against a background smear of fragments (for example, see Figure 5.5B). Two out of the six putative medium-copy probes, pUnP14 and pUnP27, produced fingerprinting patterns with 14 or 17 polymorphic fragments using the restriction enzymes *Eco*RI or *Bam*HI, respectively (Table 5.3). Examples of the multiple, distinct fragments produced by these two probes are illustrated in Figures 5.10B (pUnP14/*Eco*RI) and 5.11 (pUnP27 in combination with *Bam*HI or *Eco*RI), the polymorphic nature of which will be described in Chapter 6. The two putative low-copy probes, pUnE9 and pUnE28 (Table 5.3), produced banding patterns which were a smear of multiple fragments similar to the patterns observed using the putative high-copy probe pUnP9 (Figure 5.5B).

5.3.2 Evaluation of selected DNA probes

Clone pUnE4 detected a 2.6 kbp fragment in four clonal lines of *U. necator* (Figure 5.6, lanes 1, 8, 15 and 19, corresponding to clonal lines 920103, 930501, 930101 and 920401, respectively), which appeared to be absent from the other 18 lines screened. In addition, probe pUnE4 hybridised to a 0.8 kbp *Eco*RI fragment, which (a) was the size of the insert of clone pUnE4 obtained from DNA of clonal line 920103 (lane 1), and (b) produced a strong signal in all clonal lines tested. Probe pUnE4 also hybridised to a minor 1.8 kbp *Eco*RI fragment in some of the clonal lines.

Probe pUnE6 hybridised strongly to undigested, high molecular weight, total *U. necator* DNA of various clonal lines (Figure 5.7, lanes 1, 3, 5, 7 and 9) and less strongly to a 6.6 kbp fragment present in undigested total *U. necator* DNA from clonal lines 920103, 930501 and 930202 (Figure 5.7, lanes 1, 3 and 5). This 6.6 kbp fragment was not detected in undigested DNA from clonal lines 930304 and 931302 (Figure 5.7, lanes 7 and 9). Following digestion of total *U. necator* DNA with *Eco*RI, the hybridisation pattern produced by pUnE6 was a smear of multiple fragments (Figure 5.7, lanes 2, 4, 6, 8 and 10). This clone did not appear to hybridize to a 1.8 kbp *Eco*RI fragment in clonal line 920103 (lane 2), which is the size of the pUnE6 insert obtained from DNA of clonal line 920103.

The *E. graminis* multiple-copy clone pEgE9 hybridised strongly to DNA from an Australian *E. graminis* isolate (Figure 5.8A, lanes 3 and 4). Clone pGEE381 appeared to

Figure 5.6 Southern hybridisation of pUnE4 to *Eco*RI digested total *U. necator* DNA (approximately 10 ng lane^{-1}) from various clonal lines:
Lane 1, line 920103, sampled September 1, 1993; *Lane 2*, line 920103, sampled March 30, 1994; *Lane 3*, line 930702; *Lane 4*, line 930703; *Lane 5*, line 930801; *Lane 6*, line 930803; *Lane 7*, line 930804; *Lane 8*, line 930501; *Lane 9*, line 930504; *Lane 10*, line 930602; *Lane 11*, line 930901; *Lane 12*, line 931302; *Lane 13*, line 931401; *Lane 14*, line 931502; *Lane 15*, line 930101; *Lane 16*, line 930202; *Lane 17*, line 930304; *Lane 18*, line 930402; *Lane 19*, line 920401; *Lane 20*, line 940501; *Lane 21*, line 940102. The faint signal of the 1.8 kpb band was present in lane 1 when the exposure of the membrane to the X-ray film was increased. DNA size markers, in kbp, are shown to the left.

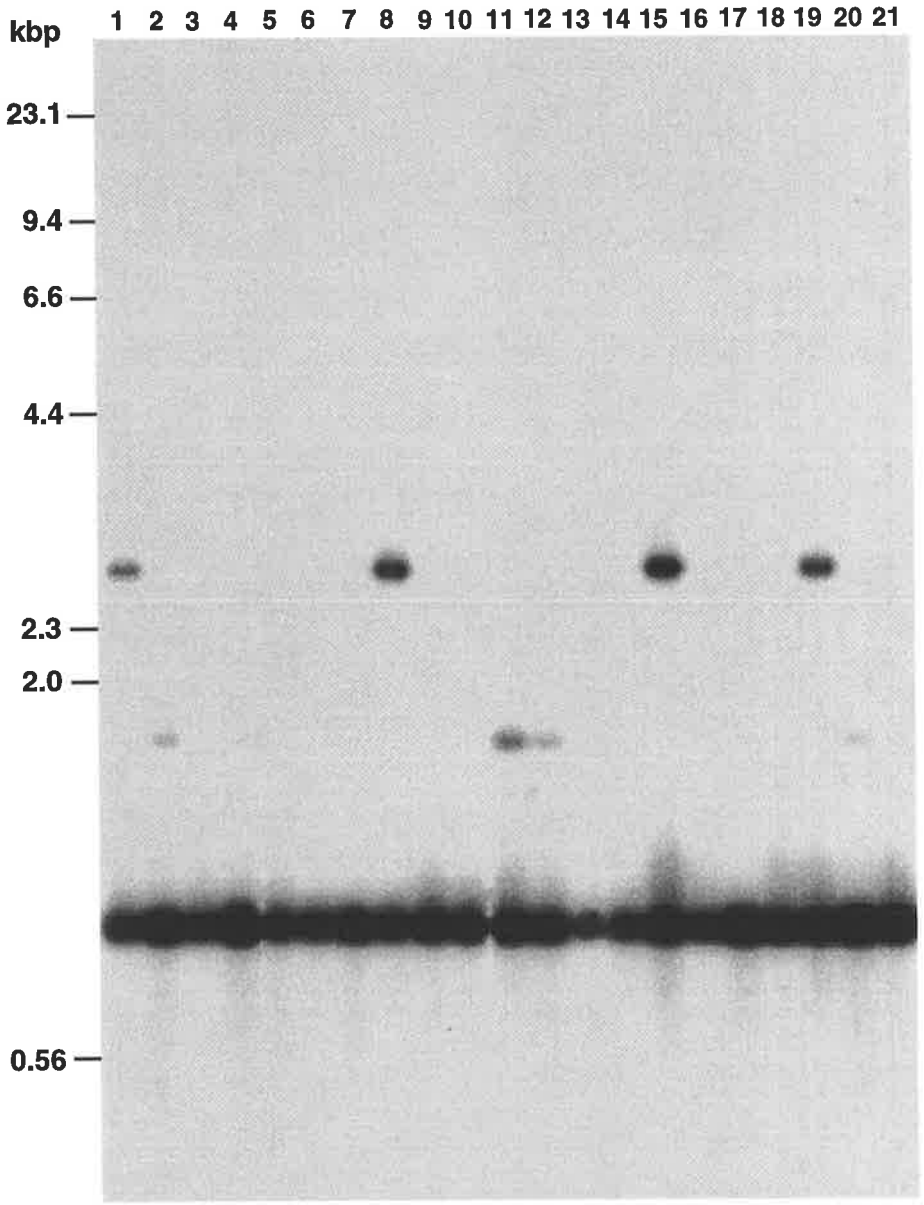

kbp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 5.7 Southern hybridisation of pUnE6 to total undigested or *Eco*RI digested *U. necator* DNA (approximately 10 ng lane^{-1}) from various clonal lines:

Lane 1, line 920103, undigested; *Lane 2*, line 920103, digested; *Lane 3*, line 930501, undigested; *Lane 4*, line 930501, digested; *Lane 5*, line 930202, undigested; *Lane 6*, line 930202, digested; *Lane 7*, line 930304, undigested; *Lane 8*, line 930304, digested; *Lane 9*, line 931302, undigested; *Lane 10*, line 931302, digested. DNA size markers, in kbp, are shown to the left.

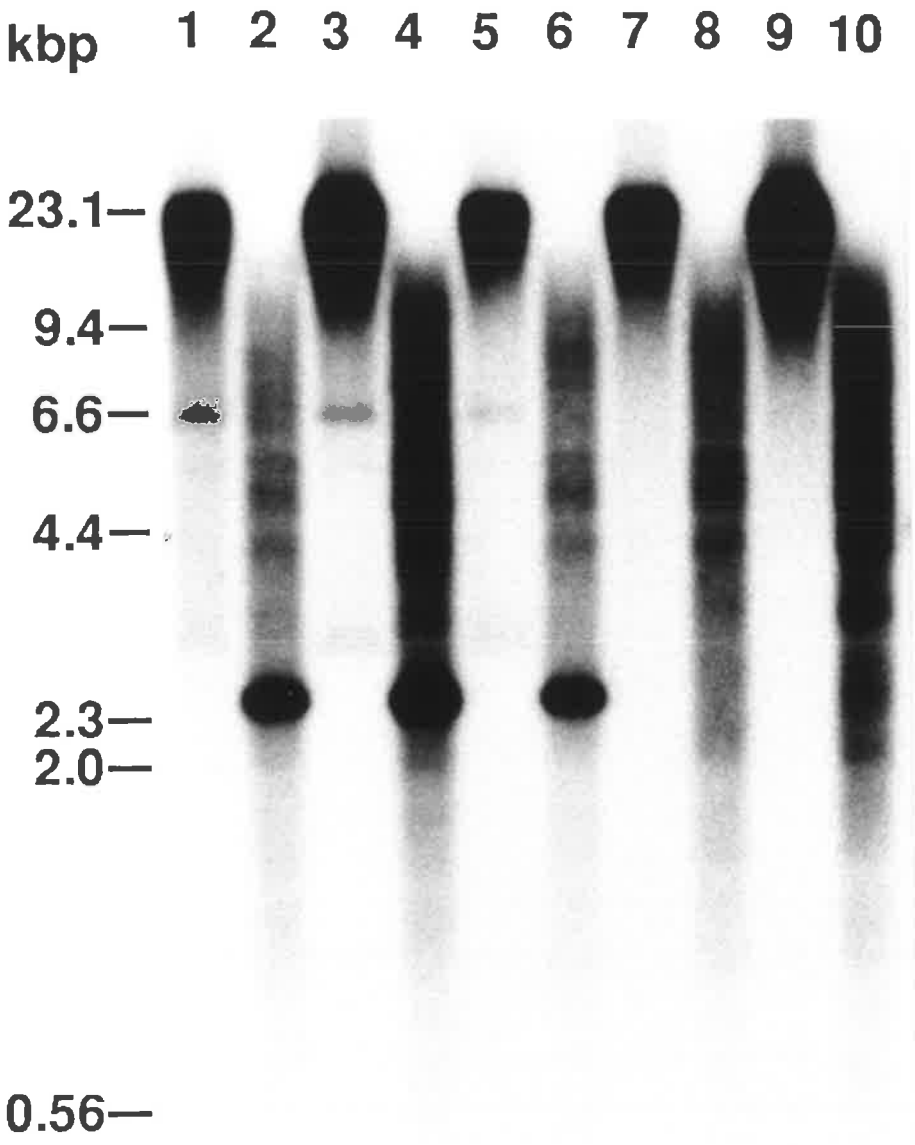

kbp
1
2
3
4
5
6
7
8
9
10
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 5.8 Southern hybridisations of *E. graminis* probes pEgE9 **(A)** and pGEE381 **(B)** to total DNA (approximately 10 ng lane^{-1}) from various sources: *Lane 1*, *U. necator* clonal line 920103, digested with *Eco*RI; *Lane 2*, *U. necator* clonal line 930202, digested with *Eco*RI; *Lane 3*, *E. graminis* DNA digested with *Hin*dIII **(A)** or *Bam*HI **(B)**; *Lane 4*, *E. graminis* DNA, undigested. DNA size markers, in kbp, are shown to the left.

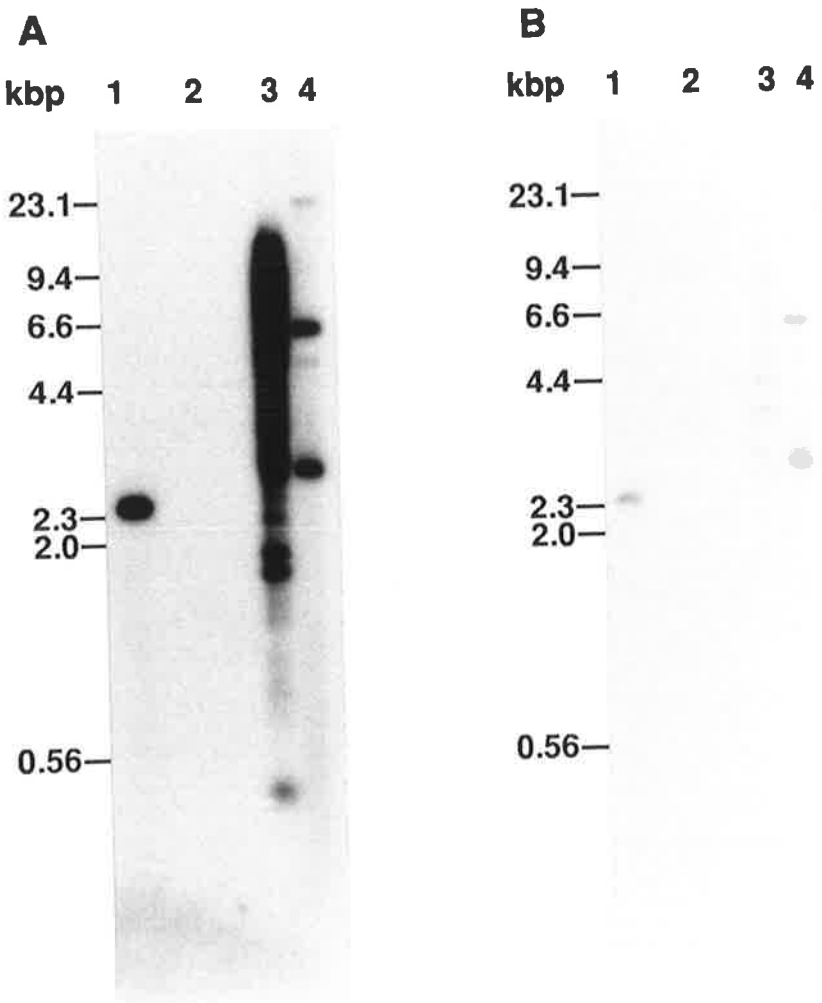

A
kbp 1 2 3 4
23.1
9.4
6.6
4.4
2.3
2.0
0.56
B
kbp 1 2 3 4
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 5.9 Southern hybridisations of pUnP27 **(A)** and pUnE4 **(B)** to *Eco*RI digested total DNA from healthy detached grapevine leaves or leaves infected with *U. necator* clonal line 920103:

Lane 1, 10 µg DNA from healthy grapevine leaves; *Lane 2*, 20 µg infected-leaf DNA; *Lane 3*, 10 µg infected-leaf DNA; *Lane 4*, 5 µg infected-leaf DNA; *Lane 5*, 2.5 µg infected-leaf DNA; *Lane 6*, 1.25 µg infected-leaf DNA. DNA size markers, in kbp, are shown to the left.

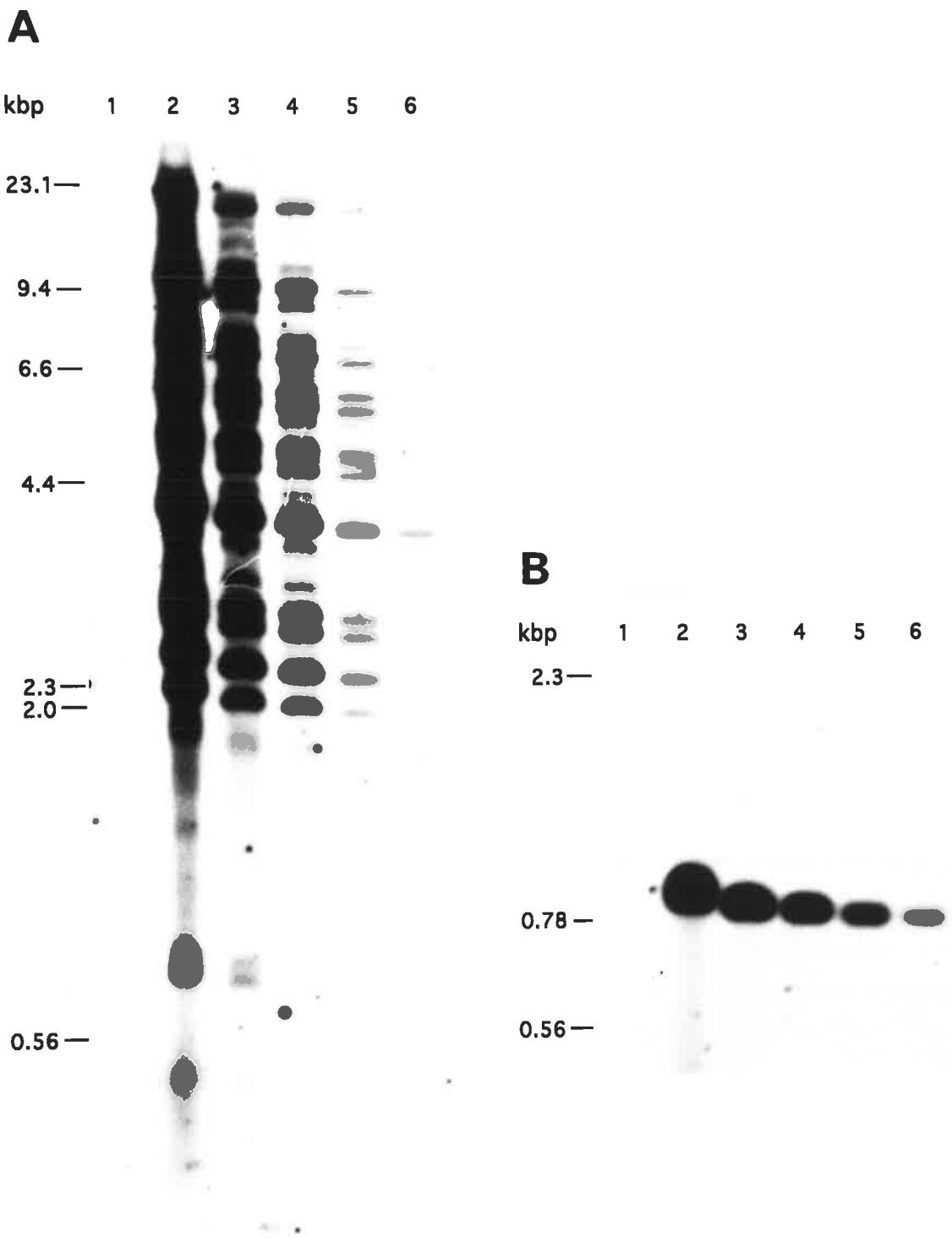

A
kbp
1
2
3
4
5
6
23.1
9.4
6.6
4.4
2.3
2.0
0.56
B
kbp
1
2
3
4
5
6
2.3
0.78
0.56

Figure 5.10 Agarose gel electrophoresis **(A)** and autoradiograph **(B)** of a Southern hybridisation of radiolabelled pUnP14 to *Eco*RI digested total DNA from micropropagated grapevine leaflets infected with *U. necator* clonal line 931502:
Lane 1, 1.25 µg infected- leaflet DNA; *Lane 2*, 2.5 µg infected-leaflet DNA; *Lane 3*, 5 µg infected-leaflet DNA; *Lane 4*, 100 ng of purified *U. necator* DNA from clonal line 930801. DNA size markers, in kbp, are shown to the left.

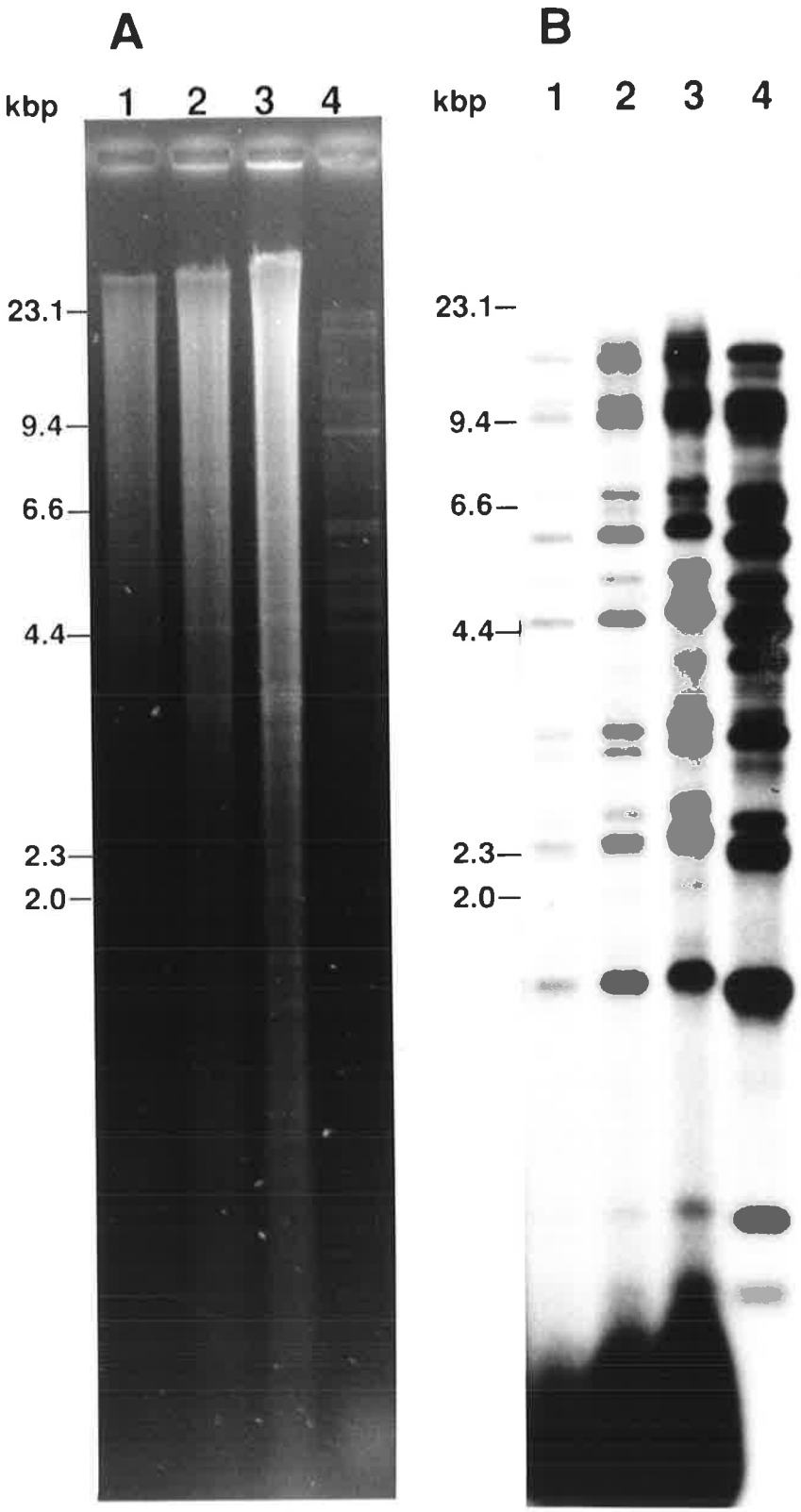
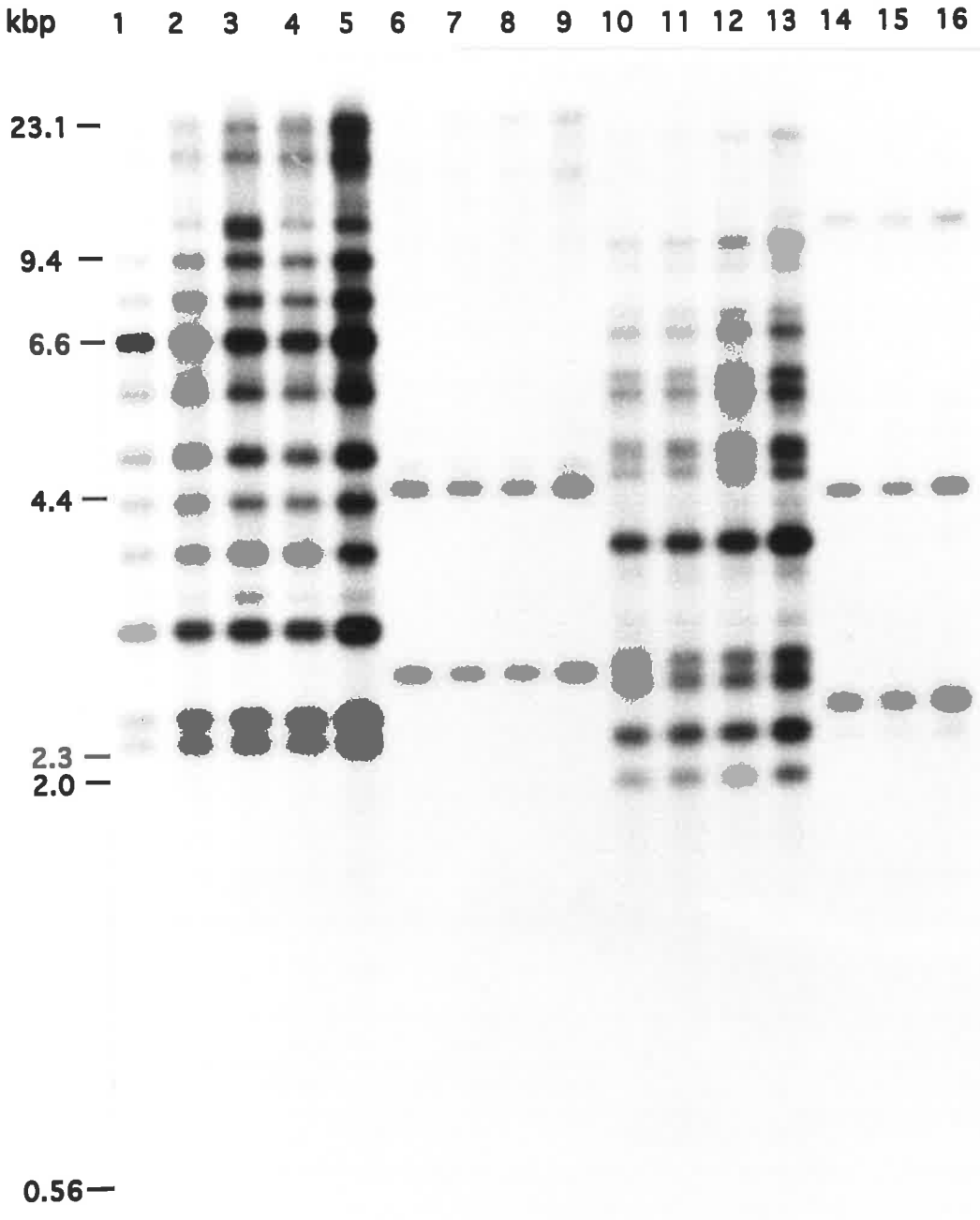

A
kbp
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0
B
kbp
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0

Figure 5.11 Southern hybridisation of pUnP27 to restriction enzyme digested total *U. necator* DNA (approximately 80 ng lane^{-1}) from different asexual generations. DNA size markers, in kbp, are shown to the left. Lanes are described in the table below:

Lane[a] on Figure 5.11	*U. necator* clonal line, restriction enzyme	Date of inoculation of *in vitro* culture from which conidia were mass produced
1	920102, *Bam*HI	December 4, 1993
2	920103[b], *Bam*HI	October 9, 1993
3	920103[b], *Bam*HI	December 4, 1993
4	920103[b], *Bam*HI	March 30, 1994
5	920103[b], *Bam*HI	December 31, 1994
6	931302, *Bam*HI	November 4, 1993
7	931302, *Bam*HI	February 18, 1994
8	931302, *Bam*HI	May 2, 1994
9	931302, *Bam*HI	March 3, 1995
10	920102, *Eco*RI	December 4, 1993
11	920103[b], *Eco*RI	October 9, 1993
12	920103[b], *Eco*RI	March 30, 1994
13	920103[b], *Eco*RI	December 31, 1994
14	931302, *Eco*RI	February 18, 1994
15	931302, *Eco*RI	May 2, 1994
16	931302, *Eco*RI	March 3, 1995

[a] The lane containing 10 µg of total DNA from healthy grapevine leaves is not shown because no hybridisation signal was detected.

[b] Clonal line 920103 is a single-conidial chain isolate derived from clonal line 920102.


kbp
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
23.1
9.4
6.6
4.4
2.3
2.0
0.56

hybridise weakly to the same isolate (Figure 5.8B, lanes 3 and 4), possibly because the radiolabelled probe had a low specific activity. Both *E. graminis* probes hybridised to a 2.9 kbp *Eco*RI fragment of *U. necator* clonal line 920103 (Figure 5.8A and B, lane 1) but no hybridisation signal was detected from DNA of *U. necator* clonal line 930202 (Figure 5.8A and B, lane 2).

Selected *U. necator* clones were evaluated for species specificity. Clones pUnP27, pUnP14 and pUnE4 did not hybridise to grapevine DNA (Figure 5.9A and B, lane 1; data for pUnP14 not presented) but each clone did hybridize to various amounts of total DNA, digested with *Eco*RI, from detached leaves or micropropagated leaflets infected with *U. necator* (Figure 5.9A and B, lanes 2-6, or Figure 5.10B, lanes 1-3). Ten µg of infected leaf DNA (Figure 5.9A, lane 3) produced a hybridisation signal that was at least as strong as the signal obtained using 100 ng of purified *U. necator* DNA (for example, see Figure 5.10B, lane 4). When probe pUnP14 was hybridised to *Eco*RI digested DNA from infected micropropagated leaflets (Figure 5.10B, lanes 1-3), purified *U. necator* DNA was included in the screen for direct comparison (lane 4). As for DNA from infected detached leaves, approximately 5 µg of DNA from infected leaflets (Figure 5.10B, lane 3) produced a hybridisation signal that was approximately half as strong as the signal obtained using 100 ng of purified *U. necator* DNA (Figure 5.10B, lane 4). In addition, the banding pattern observed using DNA from leaflets colonized with clonal line 931502 (Figure 5.10B, lane 3) was the same as the pattern observed using purified DNA from conidia of the same clonal line (data presented in Chapter 6).

Probes pUnE4 and pUnP14 did not hybridize to DNA from eight different species representing genera of the higher fungi. The data are not presented because hybridisation signals were detected by autoradiography only in the lanes containing total *U. necator* DNA.

When probes were evaluated for somatic stability, pUnP27 and pUnP14 produced consistent banding patterns for each asexual generation of clonal lines 920103 and 931302 over a period of at least 15 months. The data for pUnP27 are presented in Figure 5.11: using either *Bam*HI (lanes 1-9) or *Eco*RI (lanes 10-16), the banding pattern for clonal line 920103 (lanes 2-5 or 11-13) was the same for each asexual generation tested but was significantly different from that observed for clonal line 931302 (lanes 6-9 or 14-16). As

for clonal line 920103, line 931302 had the same banding pattern for each asexual generation tested with a particular restriction enzyme (lanes 6-9 or 14-16). Clonal line 920102 (lanes 1 and 10), from which 920103 was derived, had the same banding pattern as line 920103 for each restriction enzyme tested.

Probe pUnE4, detected a single, 4.2 kbp *Bam*HI fragment (Figure 5.12, lanes 1-9) and a single, 0.8 kbp *Eco*RI fragment (Figure 5.12, lanes 10-16) in all asexual generations of clonal lines 920103 (lanes 2-5 and 11-13) and 931302 (lanes 6-9 and 14-16) tested, over a period of at least 15 months. In this case, there were no differences in the hybridisation pattern among the clonal lines for each restriction enzyme tested. This hybridisation was compared with the previous hybridisation of pUnE4 to *Eco*RI digested total *U. necator* DNA (Figure 5.6) and it was noted that the 2.6 kbp *Eco*RI fragment and the minor 1.8 kbp *Eco*RI fragment, in one or both samples of clonal line 920103 shown in Figure 5.6 (lane 1, September 1, 1993 and lane 2, March 30, 1994), were absent in all samples of this same clonal line in Figure 5.12 (lanes 11-13, October 9, 1993 to December 31, 1994).

5.4 Discussion

Molecular characterization of *U. necator* required the development of efficient DNA extraction methods for both conidia and infected grapevine tissue, as described in Chapter 3. The high quality of total DNA extracted from *U. necator* conidia was confirmed by the successful ligation of this DNA into the plasmid vector, pUC19, and sufficient *U. necator* clones were obtained for DNA marker development. In these experiments, cloning into the *Pst*I site of pUC19 was more efficient than cloning into the *Eco*RI site of this plasmid vector. The average length of the inserts was smaller than the size used to calculate the vector:insert molar ratios, which illustrates the difficulty of estimating the mean size of fragments in digested total DNA prior to cloning. Therefore, cloning into the *Eco*RI site may have been more efficient using a different DNA ratio to achieve the same vector:insert molar ratio.

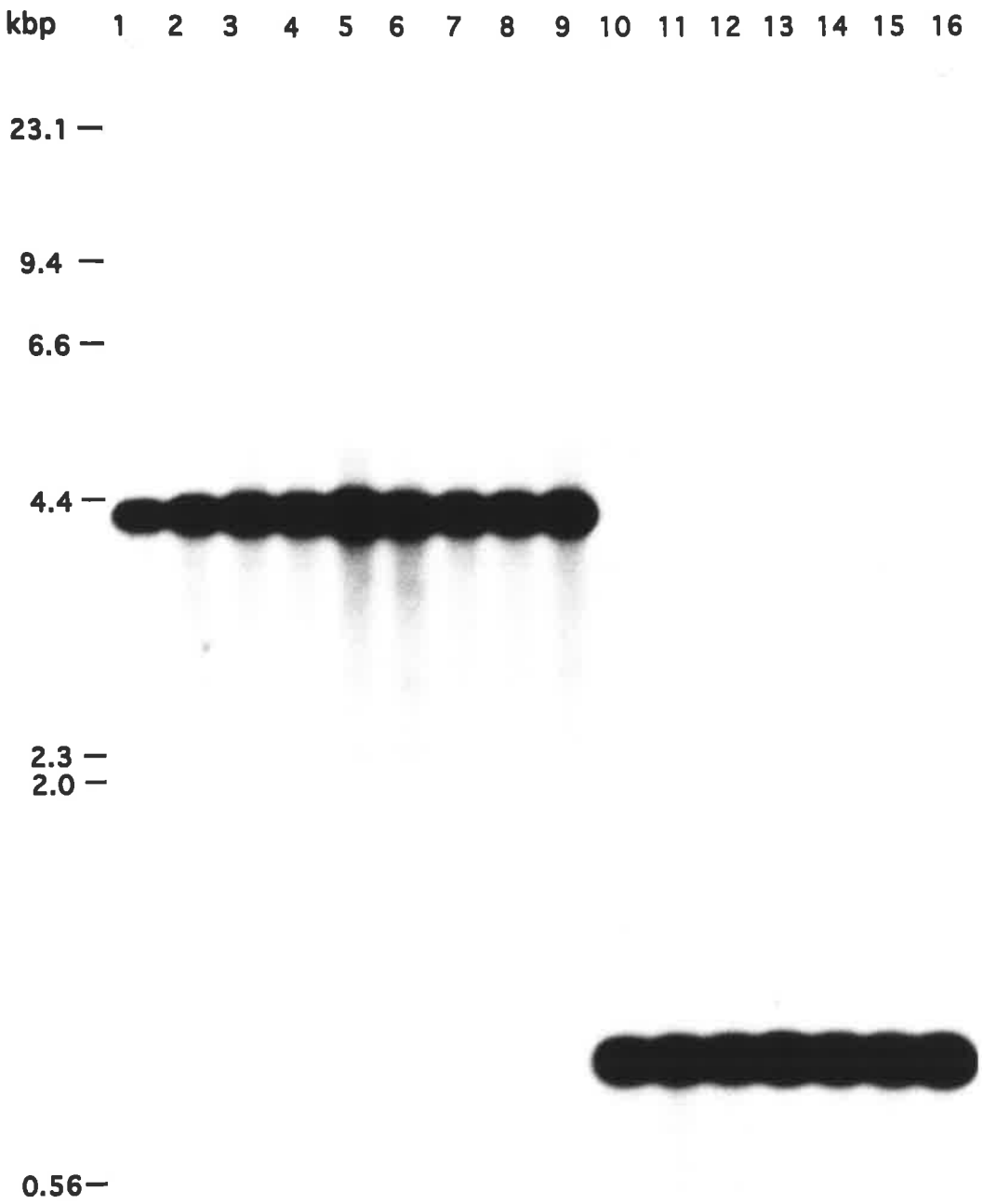
In order to identify clones representing dispersed repeated sequences, which were likely to be useful as fingerprinting probes, the copy number of cloned *U. necator* sequences was estimated. Such sequences were expected to have a medium to high copy number. Two methods for classifying the copy number were compared because the colony

Figure 5.12 Southern hybridisation of pUnE4 to restriction enzyme digested total *U. necator* DNA (approximately 80 ng lane^{-1}) from different asexual generations. DNA size markers, in kbp, are shown to the left. Lanes are described in the table below:

Lane[a] on Figure 5.12	*U. necator* clonal line, restriction enzyme	Date of inoculation of *in vitro* culture from which conidia were mass produced
1	920102, *Bam*HI	December 4, 1993
2	920103[b], *Bam*HI	October 9, 1993
3	920103[b], *Bam*HI	December 4, 1993
4	920103[b], *Bam*HI	March 30, 1994
5	920103[b], *Bam*HI	December 31, 1994
6	931302, *Bam*HI	November 4, 1993
7	931302, *Bam*HI	February 18, 1994
8	931302, *Bam*HI	May 2, 1994
9	931302, *Bam*HI	March 3, 1995
10	920102, *Eco*RI	December 4, 1993
11	920103[b], *Eco*RI	October 9, 1993
12	920103[b], *Eco*RI	March 30, 1994
13	920103[b], *Eco*RI	December 31, 1994
14	931302, *Eco*RI	February 18, 1994
15	931302, *Eco*RI	May 2, 1994
16	931302, *Eco*RI	March 3, 1995

[a] The lane containing 10 µg of total DNA from healthy grapevine leaves is not shown because no hybridisation signal was detected.

[b] Clonal line 920103 is a single-conidial chain isolate derived from clonal line 920102.


kbp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
23.1
9.4
6.6
4.4
2.3
2.0
0.56

lysis method was likely to be inaccurate if plasmids containing different sized *U. necator* inserts replicated at different rates. For example, a plasmid containing a large high-copy number insert may replicate at a slower rate than a plasmid containing a small low-copy number insert and so the DNA from each recombinant cell type may produce a similar signal when hybridised to total *U. necator* DNA. By doing a crude plasmid preparation, the quantity of target DNA could be controlled somewhat by relating the hybridisation signal to the intensity of ethidium bromide staining of the recombinant plasmid DNA in the agarose gel. The colony lysis and crude plasmid preparation methods gave the same result for the majority of classifications of copy number. For each classification method, different results occurred mostly in the medium copy number category. The two methods for classifying copy number were selected because they were conducted more rapidly than if total *U. necator* DNA had been hybridised to known concentrations of purified plasmid DNA. Rapid classification is required for examining large numbers of clones, especially in experiments to identify single copy sequences or to distinguish one type of clone, for example rDNA, from another using a subtractive hybridisation protocol. Landry and Michelmore (1985) used purified plasmid DNA in a dot blot procedure to identify low-copy clones of lettuce DNA. The advantage of their approach is that only a fraction of the plasmid DNA is used for the dot blot and the remainder is used as a probe for RFLP marker development.

*Pst*I is a methylation-sensitive restriction endonuclease (for methylated nucleotides C and A) and is often used in the construction of genomic libraries to reduce the cloning of non-coding DNA, which in many eukaryotic organisms is methylated (Tanksley *et al.*, 1987; Nelson & McClelland, 1991). When DNA was fractionated in an agarose gel and stained with ethidium bromide, it appeared that *U. necator* DNA was digested to completion with *Pst*I and a range of restriction endonucleases (refer to Section 3.4). In addition, 24% of *U. necator* sequences cloned into the *Pst*I site of pUC19 were putatively high copy in number (Table 5.1). This finding is in agreement with the research of Christiansen and Giese (1990), who cloned total *E. graminis* DNA into the *Pst*I site of pUC19 and obtained significant numbers of clones containing repetitive sequences. Therefore, repeated DNA in *E. graminis* and *U. necator* may be less frequently methylated than DNA in other organisms.

The presence of a 9.4 kbp fragment, detected by ethidium bromide staining, in *Eco*RV digested DNA from clonal line 931302, which was absent from lines 920103 and 930202 (Figure 5.4), was the first evidence of a DNA polymorphism among these *U. necator* clonal lines. The staining intensity of the fragment suggests that it is repeated in the genome and is likely to be either nuclear or mitochondrial in origin. This restriction site was not detected by Southern hybridisation of selected *U. necator* clones to *Eco*RV digested total DNA because none of the small number of clones screened contained the sequence homologous to the one observed in clonal line 931302.

DNA polymorphisms were detected among *U. necator* clonal lines when selected *U. necator* clones of putative high copy number were used in RFLP analyses. Christiansen and Giese (1990) discarded *E. graminis* clones containing high copy number sequences because the remaining clones provided a rich source of both multiple-copy and single-copy probes which produced polymorphic bands that were easy to score. Since the putative medium-copy clones of *U. necator* DNA, pUnP27 and pUnP14, also detected multiple, distinct fragments, it appears that high-copy clones, like those of *E. graminis*, may yield fewer probes useful for fingerprinting purposes.

The high-copy number probe pUnE4 initially appeared to hybridise to three *Eco*RI fragments in total *U. necator* DNA from various *U. necator* clonal lines. The sum of the 0.8 kbp fragment, giving the most intense signal, and the 1.8 kbp fragment, giving a weak signal, was 2.6 kbp, which was the size of the third fragment. Therefore, it is likely that the DNA in the lanes containing the 1.8 kbp and 2.6 kbp fragments was digested only partially. Additional evidence for this suggestion was that the strongly hybridising 0.8 kbp *Eco*RI fragment was evident in subsequent hybridisations using pUnE4, whereas the other fragments were absent. Furthermore, somatic stability of this 0.8 kbp fragment was demonstrated in clonal lines 920103 and 931302 over a 15-month period. Given that this fragment was present in high copy number in all clonal lines tested and that pUnE4 did not hybridize to DNA from a range of higher fungi, it may be possible to use this clone in a diagnostic test to detect *U. necator* in grapevine buds and/or other asymptomatic tissue. In this study, 1 µg of DNA from infected grapevine leaf tissue was required to detect *U. necator*, with certainty, using pUnE4 as a DNA probe. If the insert of pUnE4 were sequenced, it may be possible to develop PCR primers for the amplification of a *U.*

necator-specific product directly from smaller quantities of DNA from infected grapevine tissue. The origin of the pUnE4 sequence in the *U. necator* genome should also be determined because if it is located in the mtDNA, then its high-copy number may make it a useful cytoplasmic marker. This sequence is unlikely to be a conserved region of rDNA because pUnE4 did not hybridise to DNA extracted from other species of fungi.

The 6.6 kbp fragment identified by pUnE6, in undigested total DNA from certain *U. necator* clonal lines, could be an extrachromosomal genetic element, because it appears to be too small to be mitochondrial DNA. The presence, stability and origin of such an element, however, must be confirmed by further investigation using DNA from the same and additional *U. necator* clonal lines.

Both *E. graminis* probes, pEgE9 and pGEE381, detected an *Eco*RI fragment in one of the two *U. necator* clonal lines examined. This fragment was the same size for both probes which suggests that pEgE9 and pGEE381 have sequences in common and that the homology of these two clones should be investigated further. In addition, both probes detected distinct fragments, the origin of which is unknown, in the undigested DNA from *E. graminis* conidia. The utility of these probes as markers for *U. necator* should be determined by screening DNA from more *U. necator* clonal lines.

Clones pUnP27 and pUnP14 were useful fingerprinting probes for *U. necator* because both clones resulted in a banding pattern which could be scored easily. Neither probe hybridised to grapevine DNA, and total DNA extracted directly from infected grapevine leaves produced the same banding pattern as was observed using purified *U. necator* DNA. In addition, the hybridisation patterns were stable for different asexual generations of the same clonal line over at least 15 months. Approximately 100 times more DNA was required from both infected detached leaves and infected micropropagated leaflets to produce a hybridisation signal of the same strength as that produced by purified *U. necator* DNA. The extraction of DNA directly from infected micropropated leaflets reduces the need to maintain grapevines in the glasshouse for the mass production of conidia by detached leaf culture. Therefore, the time saving made by not mass producing conidia compensates for the larger amounts of DNA required for Southern hybridisation when using DNA from infected grapevine tissue. The *U. necator*-specific clones pUnP14 and pUnE4 may also be hybridised to μg quantities of DNA from diseased material

collected directly from the field, but only for studies which do not require the isolation of clonal lines of *U. necator*.

The function and location of pUnP27, pUnP14 and other sequences in the *U. necator* genome are unknown. The distribution of these sequences in the genome could be confirmed by segregation and linkage analysis and/or by hybridising the clones to chromosome-size fragments of DNA following pulsed-field gel electrophoresis (Mills & McCluskey, 1990). Like *E. graminis* f.sp. *hordei*, *U. necator* has a large amount of repetitive DNA (O'Dell *et al.*, 1989; Christiansen & Giese, 1990). In both *E. graminis* and *Magnaporthe grisea* certain dispersed repeated sequences have been found to have properties of retrotransposons or some type of transposable element (Hamer *et al.*, 1989; Rasmussen *et al.*, 1993; Skinner *et al.*, 1993). The sequences detected by pUnP27 and pUnP14 appeared to be stable over the time frame examined and there is no evidence so far to suggest that these sequences have properties of transposable elements, unless the element is inactive. The *U. necator* clones appear to be similar to those used for fingerprinting in fungi such as *Sclerotinia sclerotiorum* (Kohn *et al.*, 1991), *Mycosphaerella graminicola* (McDonald & Martinez, 1991) and *Cryphonectria parasitica* (Milgroom *et al.*, 1992). The fingerprinting probes developed for these ascomycetes detect moderately repetitive, dispersed sequences that possibly represent multigene families. Further analyses of the *U. necator* DNA sequences discussed above will elucidate their potential functions.

6.0 PRELIMINARY STUDIES ON THE GENETIC DIVERSITY OF *U. NECATOR* IN AUSTRALIA

The first experiment that anyone working in molecular evolution, taxonomist or breeder, conducts is an assessment of variability.

J. W. Taylor, 1986.

6.1 INTRODUCTION

Estimates of genetic diversity in pathogen populations form the basis of studies to determine population structures. Three sampling strategies may be used to determine the amount and distribution of genetic diversity. The first strategy involves sampling a small number of isolates from a wide geographical area. This approach has been adopted for molecular marker development and also for obtaining preliminary estimates of variability in studies of genetic diversity (refer to Table 2.2, Chapter 2). The second strategy involves sampling large numbers of isolates on a micro-geographical scale to determine base-line levels of variability. This approach was adopted by McDonald and Martinez (1990b) to estimate the genetic diversity among 93 isolates of *Septoria tritici* from a single wheat field. Their aim was to obtain accurate measurements of within-population variation in order to estimate variation among populations. The third strategy involves obtaining large numbers of isolates within several populations to survey the frequency of genotypes on a macro-geographical scale. Such knowledge can be used to test hypotheses about the migration of a pathogen, and the genetic relationships among current populations. For example, Goodwin *et al.* (1993) assessed isolates of *Rhynchosporium secalis* for genetic variability at eight isozyme loci; analysis of populations in Australia, Europe, Japan and the United States suggests that *R. secalis* has migrated from a common centre of origin.

In the case of the *E. graminis-H. vulgare* pathosystem, all three sampling strategies have been employed to study population biology. For example, six *E. graminis* isolates from barley grown at various locations in the United Kingdom were used to develop DNA markers, such as the DNA fingerprinting probe E9 (O'Dell *et al.*, 1989). Brown *et al.* (1990) subsequently obtained 97 isolates of *E. graminis* f. sp. *hordei* by trapping conidia from the air during 1 week in one location. These isolates were used to determine the genetic structure of the pathogen population, using the DNA marker E9 and markers for virulence and fungicide sensitivity. Furthermore, the pathogen in Europe was surveyed extensively

between 1985 and 1991 to estimate frequencies of DNA haplotypes, virulence and fungicide phenotypes, and to investigate the effect of gene flow and selection on the population structures (Limpert, 1987; Limpert *et al.*, 1990; Wolfe *et al.*, 1992). PCR-based markers, in conjuction with markers for virulence and fungicide sensitivity, are now being used in the analysis of *E. graminis* populations (Wolfe *et al.*, 1992; McDermott *et al.*, 1994).

The *U. necator* culture collection described in Chapter 3 consists of isolates obtained by macro-geographical and micro-geographical sampling, and was intended to provide material for preliminary studies on the variability of *U. necator*. In this chapter, an investigation of the level of genetic variation among 35 *U. necator* clonal lines, using three *U. necator* RFLP probes and a cereal intron splice-junction (ISJ) primer, is reported. Pairwise comparisons of genetic similarity were made in order to group clonal lines with similar electrophoretic patterns into putative lineages. A nomenclature for clonal line genotype is proposed, followed by a discussion of the frequency and macro- and micro-geographical distribution of genotypes. These preliminary studies are discussed in the context of future studies to estimate genetic diversity in time and space.

6.2 Materials and methods

6.2.1 Identification of *U. necator* genotypes using RFLP probes

Clones pUnP14, pUnP27 and pUnE21 (refer to Section 5.3) were used as Southern hybridisation probes to determine the genotypes of 35 *U. necator* clonal lines from South Australia, Western Australia and Tasmania. An 80 ng quantity of total *U. necator* DNA from each clonal line was digested with *Eco*RI, *Bam*HI or *Eco*RV, fractionated in a 20 cm-long, 1% agarose gel and transferred to a ZetaProbe membrane. The membranes were hybridised to 50 ng of each radiolabelled probe prepared using either pUC19-specific primers or by random priming of the *U. necator* insert DNA excised from the recombinant plasmid (refer to Section 3.6).

Banding pattern similarity among pairs of *U. necator* clonal lines was calculated for the probe-enzyme combination pUnP14/*Eco*RI, by the formula:

$$S_{xy} = 2n_{xy}/(n_x + n_y),$$

where n_{xy} is the number of shared fragments, and n_x and n_y are the number of fragments in *U. necator* clonal lines x and y (Lynch, 1990). Restriction fragments producing a strong

hybridisation signal were scored as being present (1) or absent (0) for each *U. necator* clonal line. Fragments producing a weak hybridisation signal were recorded but not used in the calculation of the similarity index. Genetic linkage among marker loci has not been determined and so the similarity index was not used to estimate genetic relatedness within this sample of *U. necator* clonal lines but rather as a preliminary attempt to allocate lines into hypothetical genetic groups or lineages. In this context, the term lineage is used as a hypothetical term because it implies that clonal lines in the same lineage have a common origin. Clonal lines sharing ≥ 80% fragments were considered to be members of the same lineage. Clonal lines with < 60% shared fragments were considered to be members of different lineages. Intermediate clonal lines, with 60-79% shared fragments for a single pairwise comparison, were assigned to a lineage if the majority of pairwise comparisons with other clonal lines in the lineage had ≥ 80% shared fragments. This method for assigning clonal lines to lineages was based on the fingerprint groups established arbitrarily by Xia *et al.* (1993) for *Magnaporthe grisea*.

Differences between *U. necator* clonal lines detected using the probe-enzyme combinations pUnP27/*Bam*HI and pUnE21/*Eco*RV were identified by inspection of banding patterns because the patterns were not as complex as those observed using pUnP14/*Eco*RI.

6.2.2 Determination of genotypes of *U. necator* clonal lines using primer R1

Amplification of *U. necator* DNA was performed in a FTS-1S Capillary Fast Thermal Sequencer (Corbett Research, Sydney). The reaction was carried out in a 20 µl volume containing 1-3 ng DNA template, 40 ng ISJ primer, R1 ($^{5'}$GTCCATTCAGTCGGTGCT$^{3'}$, Weining & Langridge, 1991), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100 and 1 unit Taq DNA polymerase (Promega). The template DNA and R1 primer were denatured by heating the reaction mixture at 95°C for 3 min then cooling the mixture on ice prior to the addition of Taq DNA polymerase. All reactions consisted of: (i) one cycle of 3 min at 94°C, 30 s at 45°C and 90 s at 72°C, (ii) four cycles of 15 s at 95°C, 15 s at 45°C and 90 s at 72°C, (iii) thirty cycles of 5 s at 94°C, 10 s at 58°C and 90 s at 72°C, and (iv) a final extension step of 5 min at 72°C. A ramp rate of 0.25°C s^{-1} was used between the annealing and primer extension

temperatures. PCR products were analysed on 3% agarose gels and visualised under uv light following ethidium bromide staining. The size marker used was pGEM® (Promega).

Differences between *U. necator* clonal lines using the R1 primer were determined by inspection of banding patterns.

6.2.3 Nomenclature for *U. necator* genotypes obtained using multiple markers

The nomenclature for a *U. necator* genotype is $a_{1-n}.b_{1-n}.c_{1-n}.....z_{1-n}$ where each letter corresponds to a marker and is an integer ranging from 1 to n, n being the maximum number of genotypes recorded for that marker. Where the marker is a phenotypic marker, the genotype is inferred from the phenotype. For example, if marker *a* detects eleven different genotypes, numbered 1 to 11, and marker *b* detects eight different genotypes numbered 1 to 8, then genotype 3.6 would refer to the third genotype detected by marker *a* and the sixth genotype detected by marker *b*. This nomenclature is a type of octal notation similar to the one described by Limpert and Müller (1994) for the designation of pathotypes of plant pathogens.

6.3 RESULTS

Probe pUnP14

Probe pUnP14 hybridised to 21 different *Eco*RI fragments of total *U. necator* DNA from the 29 clonal lines screened, ranging in size from 13.5 to 0.5 kbp (Table 6.1), and to 13-15 restriction fragments in each of these *U. necator* clonal lines (Table 6.2, Figure 6.1). Eleven different hybridisation patterns were identified among these 29 *U. necator* clonal lines when only the major bands were scored (Table 6.2). Two groups of hybridisation patterns were evident: two different hybridisation patterns were identified among the first group and nine different hybridisation patterns were identified among the second group. Therefore, clonal lines were separated into two groups or lineages, A or B, based on the percentage similarity of restriction fragment patterns for each pairwise comparison (Table 6.3). Within each lineage, clonal lines were assigned to a DNA fingerprint group for all clonal lines sharing the same restriction fragment pattern. Within lineage A, six clonal lines were assigned to DNA fingerprint group A_1 and one clonal line clonal line, 930202, which shared 96% of

Table 6.1 Restriction fragment sizes detected following Southern hybridisation of three *U. necator* DNA probes to restriction-enzyme digested total *U. necator* DNA from up to 35 *U. necator* clonal lines[a] screened.

Fragment Number	Fragment size (kbp) using the DNA probe-restriction enzyme combination specified		
	pUnP14/*Eco*RI	pUnP27/*Bam*HI	pUnE21/*Eco*RV
1	13.5	17.0	6.0
2	11.8	15.3	2.3
3	11.4	12.4	0.4
4	10.8	10.9	-
5	9.6	9.5	-
6	8.0	8.4	-
7	6.7	7.1	-
8	6.3	5.8	-
9	5.8	5.7	-
10	5.1	5.3	-
11	4.6	5.0	-
12	4.3	4.2	-
13	3.5	4.1	-
14	3.3	3.3	-
15	2.9	2.9	-
16	2.5	2.5	-
17	2.3	2.3	-
18	1.8	-	-
19	1.4	-	-
20	0.7	-	-
21	0.5	-	-

[a] Clonal lines are listed in Table 6.2.

Table 6.2 The presence or absence of each restriction fragment in DNA from various *U. necator* clonal lines using various probe-enzyme combinations.

U. necator clonal line	Source	Restriction fragment[a]		
		pUnP14/*Eco*RI	pUnP27/*Bam*HI	pUnE21/*Eco*RV
920103	Adelaide Plains	110011011^{m}11101^{m}11^{m}11100	11111111001101011	010
930702	Adelaide Plains	110011011^{m}11101^{m}1011100	11111111001101011	-
930703	Adelaide Plains	-	-	010
930704	Adelaide Plains	110011011^{m}11101^{m}11^{m}11100	11111111001101011	010
931002	Adelaide Hills	110011011^{m}11101^{m}11^{m}11100	11111111001101011	010
930202	Barossa Valley	110011011^{m}11101^{m}1111100	11111111001101011	-
930203	Barossa Valley	-	11111111001101011	1^{m}10
930302	Barossa Valley	110011011^{m}11101^{m}11^{m}11100	11111111001101011	-
930304	Barossa Valley	110011011^{m}11101^{m}1011100	11111111001101011	010
930305	Barossa Valley	-	11111111001101011	010
940702	Launceston	-	11111111001101011	010
930801	Adelaide Plains	10110110111010 01^{m}10111	00000000110000100	011
930802	Adelaide Plains	10110110111010 0010111	00000000110000100	-
930803	Adelaide Plains	10110110111010 01^{m}10111	00000000110000100	-
930804	Adelaide Plains	10110110110001 11^{m}10111	00000010110000100	-
930501	McLaren Flat	10110110110001 11^{m}10111	00000000110000100	011
930504	McLaren Flat	10110110110001 11^{m}10101	00010000110000100	-
930602	McLaren Flat	10110110110011 0010111	00000000110000100	011
930901	Adelaide Hills	10110110110011 0010111	00000000110000100	-
931101	Adelaide Hills	10110110110011 01^{m}10111	00000000110000100	-
931201	Adelaide Hills	10110110110011 01^{m}10111	00100100110000100	011
931302	Adelaide Hills	10110110111000 11^{m}10111	00000000110000100	011
931401	Adelaide Hills	-	00000000110000100	-
931502	Adelaide Hills	10110110110001 01^{m}10111	00000000110000100	-
930101	Barossa Valley	10110110110001 11110111	00000000110000100	1^{m}11
930402	Barossa Valley	10110110110001 1010111	00000000110000100	-
920302	Riverland	10110110110101 11^{m}10101	00000100110000100	011
920401	Riverland	10110110110011 0110111	00000000110000100	-
940501	Coonawarra	10110110110011 01^{m}10111	00000000110010100	011
940504	Coonawarra	-	00000000110010100	-
940604	Coonawarra	10110110110001 11^{m}10111	00000000110000110	011
940102	Margaret River	10110110110011 01^{m}10111	00000000110000100	-
940203	Swan Valley	10110110110011 0010111	00000000110000100	-
940301	Swan Valley	10110110110011 01^{m}10111	00000000110000100	011
940402	Swan Valley	10110110111000 11^{m}10111	00000000110000100	-

[a] Restriction fragments are ordered as in Table 6.1. Presence and absence of restriction fragments are denoted by 1 and 0, respectively.

[m] Minor band producing a weaker hybridisation signal than the other restriction fragments.

- The hybridisation pattern of the *U. necator* clonal line was not determined.

Figure 6.1 Southern hybridisation of pUnP14 to *Eco*RI digested total *U. necator* DNA (approximately 80 ng lane^{-1}) from various clonal lines: *Lane 1*, line 920103; *Lane 2*, line 930702; *Lane 3*, line 930704; *Lane 4*, line 930202; *Lane 5*, line 930304; *Lane 6*, line 930302; *Lane 7*, line 931002; *Lane 8*, line 940702; *Lane 9*, line 930801; *Lane 10*, line 930804; *Lane 11*, line 930501; *Lane 12*, line 930602; *Lane 13*, line 931302; *Lane 14*, line 930101; *Lane 15*, line 920302; *Lane 16*, line 940501; *Lane 17*, line 940604; *Lane 18*, line 940301. DNA size markers, in kbp, are shown to the left.

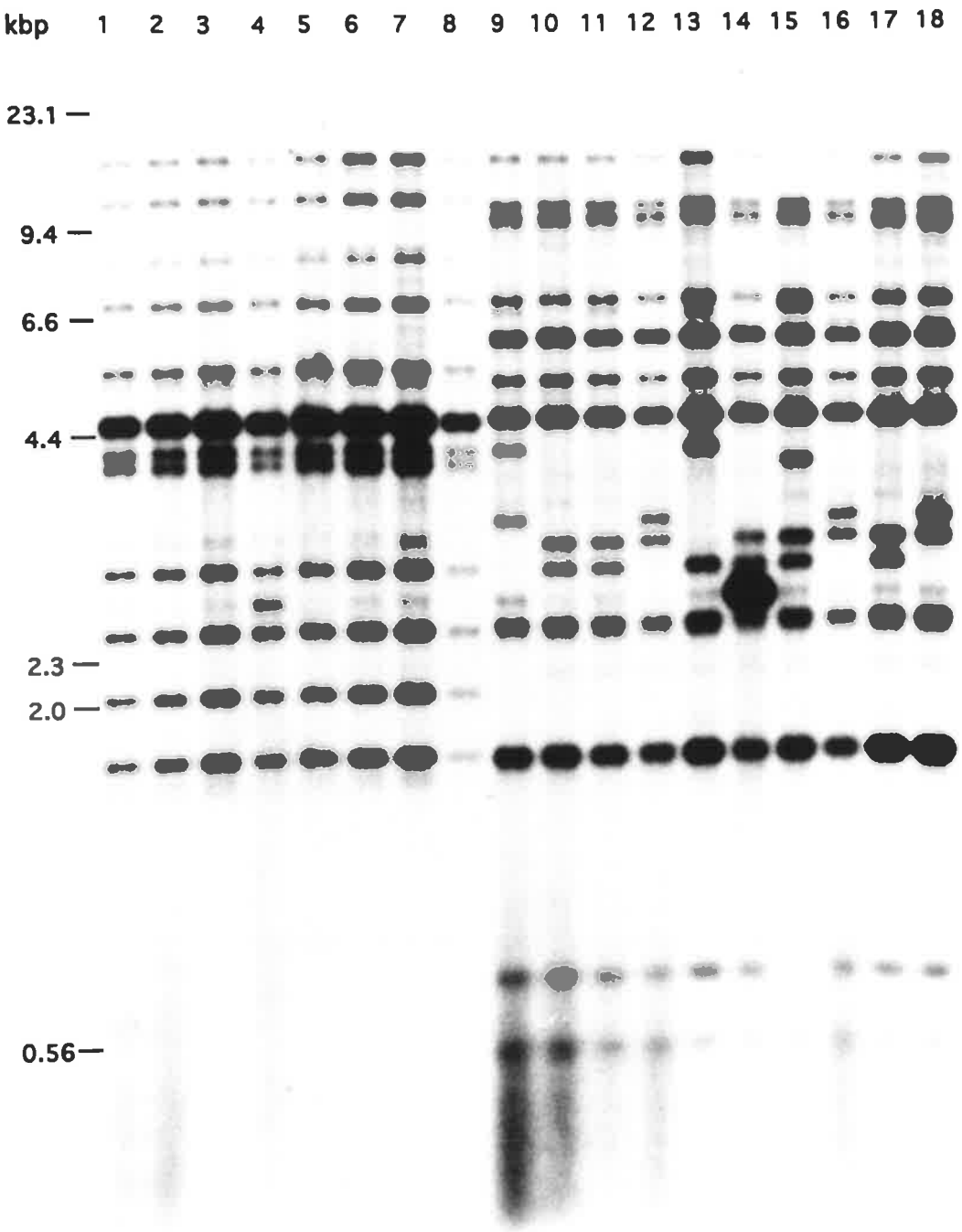

kbp
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Table 6.3 Hybridisation pattern similarity (S_{xy})[a] among pairs of *U. necator* clonal lines using the probe-enzyme combination pUnP14/*Eco*RI.

	Group[b]	A_1	A_2	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8	B_9
Group[b]	No.[c]	6	1	8	4	3	2	1	1	1	1	1
B_9	1	0.56	0.54	**0.85**	**0.92**	0.77	**0.85**	**0.96**	**0.88**	**0.89**	**0.81**	**1.00**
B_8	1	0.38	0.37	**0.96**	**0.89**	**0.89**	**0.81**	**0.85**	**0.92**	**0.93**	**1.00**	
B_7	1	0.46	0.44	**0.89**	**0.96**	**0.81**	**0.89**	**0.92**	**0.92**	**1.00**		
B_6	1	0.42	0.40	**0.96**	**0.96**	**0.88**	**0.88**	**0.92**	**1.00**			
B_5	1	0.50	0.48	**0.88**	**0.96**	**0.80**	**0.88**	**1.00**				
B_4	2	0.56	0.54	**0.85**	**0.92**	**0.92**	**1.00**					
B_3	3	0.48	0.46	**0.92**	**0.85**	**1.00**						
B2	4	0.48	0.46	**0.92**	**1.00**							
B_1	8	0.40	0.38	**1.00**								
A_2	1	**0.96**	**1.00**									
A_1	6	**1.00**										

a Calculated using the formula described in Section 6.2.1. Bold numbers indicate ≥ 80% similarity.

b DNA fingerprint groups. Groups with the same letter and subscript have the same DNA hybridisation pattern.

c Number of clonal lines in each group.

fragments with lines in A_1, was assigned to fingerprint group A_2. The similarity in hybridisation patterns for pairwise comparisons of clonal lines in lineage A versus lines in lineage B ranged from 37 to 56%. Within lineage B (22 clonal lines), 231 pairwise comparisons of banding patterns of *U. necator* clonal lines revealed 16.5% (n = 38 = the number of pairwise comparisons) with identical fragments, 46% (n = 107) with 90-99% shared fragments, 36% (n = 83) with 80-89% shared fragments and 1.5% (n = 3, B_3 compared to B_9) with 77% shared fragments. The two clonal lines involved in the last comparison were assigned to lineage B because pairwise comparisons with all other clonal lines in lineage B produced similarities ≥ 80%. In summary, nine DNA fingerprint groups were identified in lineage B (Tables 6.3, 6.4).

Probe pUnP27

Probe pUnP27 hybridised to 17 different *Bam*HI fragments, ranging in size from 17.0 to 2.3 kbp, of total *U. necator* DNA from the 34 clonal lines screened. (Table 6.1). Probe pUnP27 hybridised to three to five restriction fragments in 24 clonal lines and to 13 restriction fragments in ten clonal lines (Table 6.2, Figure 6.2). Therefore, two distinct banding patterns among the clonal lines were identified by this probe. As for the data obtained using probe pUnP14, clonal lines were separated into two lineages, A or B, based on the hybridisation patterns. Eight different hybridisation patterns were identified by pUnP27 among the 34 *U. necator* clonal lines when only major bands were scored (Table 6.2). This probe hybridised to the same 13 restriction fragments in all clonal lines (n=10) in lineage A. In lineage B, pUnP27 hybridised to the same three restriction fragments in each of 17 *U. necator* clonal lines, their hybridisation pattern being completely different (no shared fragments) from those in lineage A. Seven other clonal lines were assigned to lineage B because they shared the same three restriction fragments. In addition, these seven clonal lines assigned to lineage B had either (a) one or two fragments extra which were shared with lines in lineage A (lines 930804, 930504, 931201, 920302 and 940604), or (b) a fourth fragment which was absent in all other clonal lines tested (lines 940501 and 940504). In summary, seven DNA fingerprint groups were identified in lineage B using pUnP27. Clonal lines assigned to DNA fingerprint group B_1, using pUnP14, were assigned to three different DNA fingerprint groups by pUnP27; namely, B_{11}, B_{14} and B_{16} (Table 6.4).

Table 6.4 Lineage and DNA fingerprint group of *U. necator* clonal lines obtained using four DNA markers.

U. necator clonal line			Lineage[a] using DNA markers			
Number	Source[b]	Mating Type[c]	pUnP14/ *Eco*RI	pUnP27/ *Bam*HI	pUnE21/ *Eco*RV	Primer R1
920103	Waite Campus, Adelaide Plains	plus	A_1	A_{11}	A	A
930702	Flinders Uni., Adelaide Plains	plus	A_1	A_{11}	-	-
930703	Flinders Uni., Adelaide Plains	-	-	-	A	-
930704	Flinders Uni., Adelaide Plains	plus	A_1	A_{11}	A	A
931002	Block 1, S'town, Adelaide Hills	-	A_1	A_{11}	A	A
930202	Block 2, Nuri., Barossa Valley	plus	A_2	A_{11}	-	A
930203	Block 2, Nuri., Barossa Valley	-	-	A_{11}	A	-
930302	Block 3, Nuri., Barossa Valley	-	A_1	A_{11}	-	-
930304	Block 3, Nuri., Barossa Valley	plus	A_1	A_{11}	A	A
930305	Block 3, Nuri., Barossa Valley	-	-	A_{11}	A	-
940702	Home garden, Launceston	-	-	A_{11}	A	A
930801	Home garden, Adelaide Plains	minus	B_3	B_{11}	B	B
930802	Home garden, Adelaide Plains	-	B_3	B_{11}	-	-
930803	Home garden, Adelaide Plains	minus	B_3	B_{11}	-	-
930804	Home garden, Adelaide Plains	plus	B_2	B_{12}	-	-
930501	Pridmore, McLaren Flat	plus	B_2	B_{11}	B	-
930504	Pridmore, McLaren Flat	plus	B_5	B_{13}	-	B
930602	Trot, McLaren Flat	minus	B_1	B_{11}	B	-
930901	Morialta, Adelaide Hills	minus	B_1	B_{11}	-	-
931101	Block 2, S'town, Adelaide Hills	minus	B_1	B_{11}	-	-
931201	Block 3, S'town, Adelaide Hills	minus	B_1	B_{14}	B	-
931302	Block 4, S'town, Adelaide Hills	plus	B_4	B_{11}	B	B
931401	Block 2, S'town, Adelaide Hills	-	-	B_{11}	-	-
931502	Block 3, S'town, Adelaide Hills	minus	B_6	B_{11}	B	B
930101	Block 1, Nuri., Barossa Valley	plus	B_7	B_{11}	B	B
930402	Block 3, Nuri., Barossa Valley	plus	B_2	B_{11}	-	-
920302	Loxton, Riverland	-	B_9	B_{15}	B	-
920401	Loxton, Riverland	-	B_8	B_{11}	-	B
940501	Block 1, L'mans, Coonawarra	-	B_1	B_{16}	B	-
940504	Block 1, L'mans, Coonawarra	-	-	B_{16}	-	-
940604	Block 2, L'mans, Coonawarra	-	B_2	B_{17}	B	-
940102	Yallingup, Margaret River	-	B_1	B_{11}	-	-
940203	Block 1, Res. Stn, Swan Valley	-	B_1	B_{11}	-	-
940301	Block 2, Res. Stn, Swan Valley	-	B_1	B_{11}	B	B
940402	Block 3, Res. Stn, Swan Valley	-	B_4	B_{11}	-	-

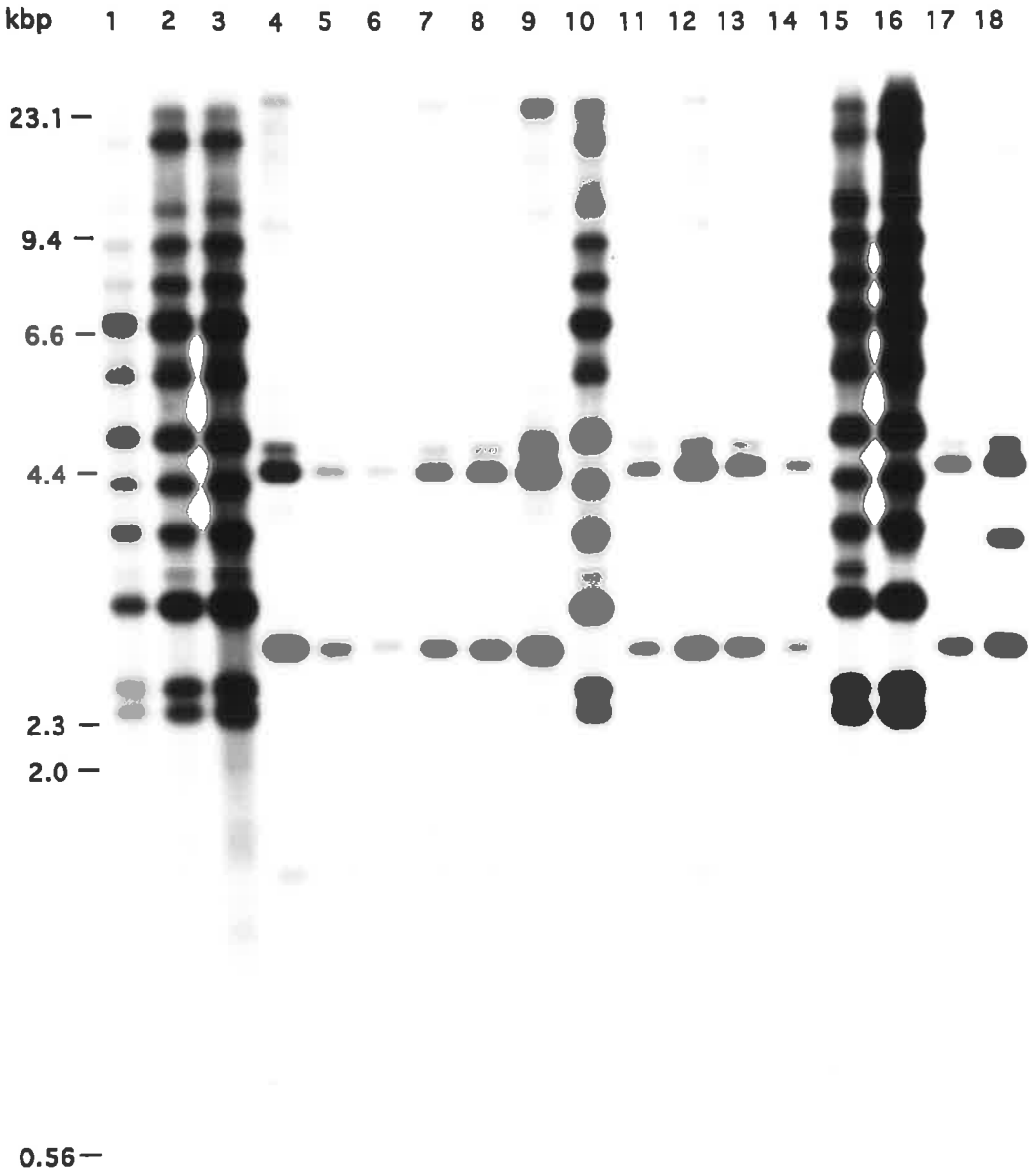
[a] A lineage is defined in Section 6.2.1.
For probe-enzyme combinations pUnP14/*Eco*RI or pUnP27/*Bam*HI, lineages with the same letter and subscript have the same DNA fingerprint for comparisons made within a column only.

[b] Abbreviations: S'town = Summertown, Nuri. = Nuriootpa, L'mans = Lindemans, Res. Stn = Research Station.

[c] Refer to Chapter 4 for the methods used to determine mating type.

- The mating type or lineage of the clonal line was not characterized.

Figure 6.2 Southern hybridisation of pUnP27 to *Bam*HI digested total *U. necator* DNA (approximately 80 ng lane^{-1}) from various clonal lines: *Lane 1*, line 920103; *Lane 2*, line 930702; *Lane 3*, line 930704; *Lane 4*, line 930801; *Lane 5*, line 930802; *Lane 6*, line 930803; *Lane 7*, line 930501; *Lane 8*, line 930602; *Lane 9*, line 930901; *Lane 10*, line 931002; *Lane 11*, line 931101; *Lane 12*, line 931302; *Lane 13*, line 931502; *Lane 14*, line 930101; *Lane 15*, line 930202; *Lane 16*, line 930304; *Lane 17*, line 920401; *Lane 18*, line 940501. DNA size markers, in kbp, are shown to the left.


kbp
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Similarly, clonal lines assigned to DNA fingerprint group B_2, using pUnP14, were assigned to three different genotypes by pUnP27; namely, B_{11}, B_{12} and B_{17}. Conversely, B_{11}, identified by pUnP27, was sub-divided into seven fingerprint groups by pUnP14; namely, B_1, B_2, B_3, B_4, B_6, B_7 and B_8.

Probe pUnE21

Probe pUnE21 hybridised strongly to two *Eco*RV fragments in total *U. necator* DNA, 2.3 and 0.4 kbp, against a smear of weak background signals in the 18 *U. necator* clonal lines screened (Tables 6.1 and 6.2, Figure 6.3). As for probes pUnP14 and pUnP27, probe pUnE21 identified two distinct hybridisation patterns among these 18 clonal lines, and these lines were assigned to two lineages, A or B (Table 6.4). In addition, clonal lines 930203 and 930101, which were assigned to different lineages, appeared to share a minor 6.0 kbp fragment (Figure 6.3, lanes 4 and 14).

Primer R1

Primer R1 amplified total *U. necator* DNA with products in the size range 390 to 1200 bp (Figure 6.4). This marker identified two banding patterns among the 13 *U. necator* clonal lines tested, with a perfect correlation to lineage A and B as defined by all the RFLP probes (Table 6.4). Clonal lines within lineage A had one major product stained with ethidium bromide, 490 bp in size, and lines within lineage B had three major products, 1100, 490 and 430 bp in size.

Pooled marker data

None of the DNA markers was correlated to mating type (Table 6.4). Five *U. necator* lines assigned to lineage A were, however, the plus mating type, whereas lines in lineage B were either the plus or minus mating type. Table 6.5 summarises the genotypes of 29 *U. necator* clonal lines. Genotypes 1.1, 3.2, 5.2, 4.2 and 6.2 were represented by six, six, three, two and two clonal lines, respectively. The remaining ten genotypes were represented by one clonal line per genotype.

Lineage A was observed in three South Australian viticultural regions (Barossa Valley, Adelaide Plains and Adelaide Hills) and in Launceston, Tasmania (Figure 6.5).

Figure 6.3 Southern hybridisation of pUnE21 to *Eco*RV digested total *U. necator* DNA (approximately 80 ng lane^{-1}) from various clonal lines: *Lane 1*, line 920103; *Lane 2*, line 930703; *Lane 3*, line 930704; *Lane 4*, line 930203; *Lane 5*, line 930304; *Lane 6*, line 930305; *Lane 7*, line 931002; *Lane 8*, line 940702; *Lane 9*, line 930801; *Lane 10*, line 931201; *Lane 11*, line 931302; *Lane 12*, line 930501; *Lane 13*, line 930602; *Lane 14*, line 930101; *Lane 15*, line 920302; *Lane 16*, line 940501; *Lane 17*, line 940604; *Lane 18*, line 940301. DNA size markers, in kbp, are shown to the left.

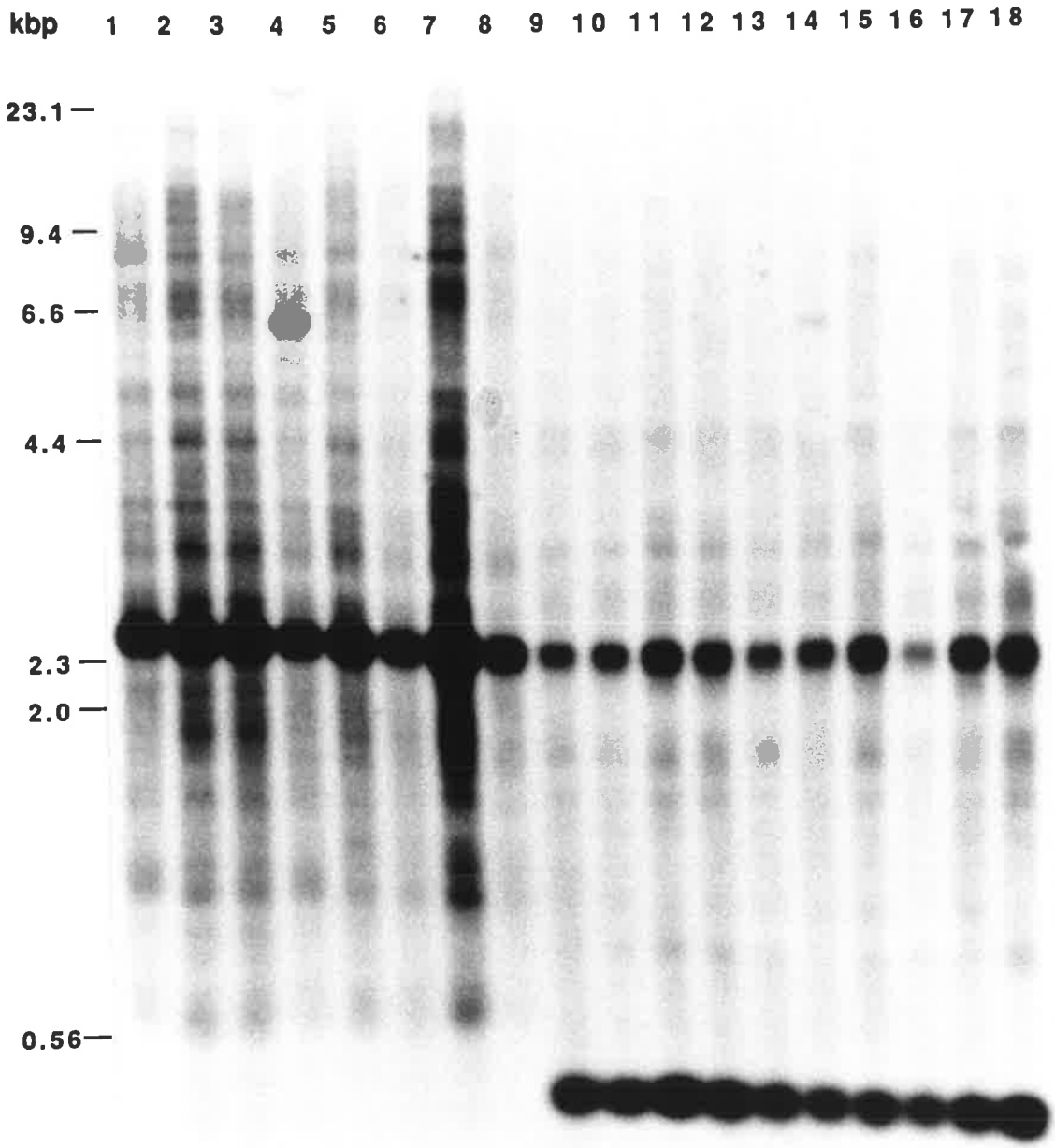

kbp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 6.4 Amplification of *U. necator* DNA with the ISJ primer, R1. The PCR products were fractionated on a 3% agarose gel: *Lane 1*, line 920103; *Lane 2*, line 930704; *Lane 3*, line 930202; *Lane 4*, line 930304; *Lane 5*, line 931002; *Lane 6*, line 940702; *Lane 7*, line 930504; *Lane 8*, line 931302; *Lane 9*, line 931502; *Lane 10*, line 930801; *Lane 11*, line 940301; *Lane 12*, line 920401; *Lane 13*, 930101. PCR products were not evident in the control reaction containing no *U. necator* DNA (data not shown). DNA size markers, in kbp, are shown to the left.

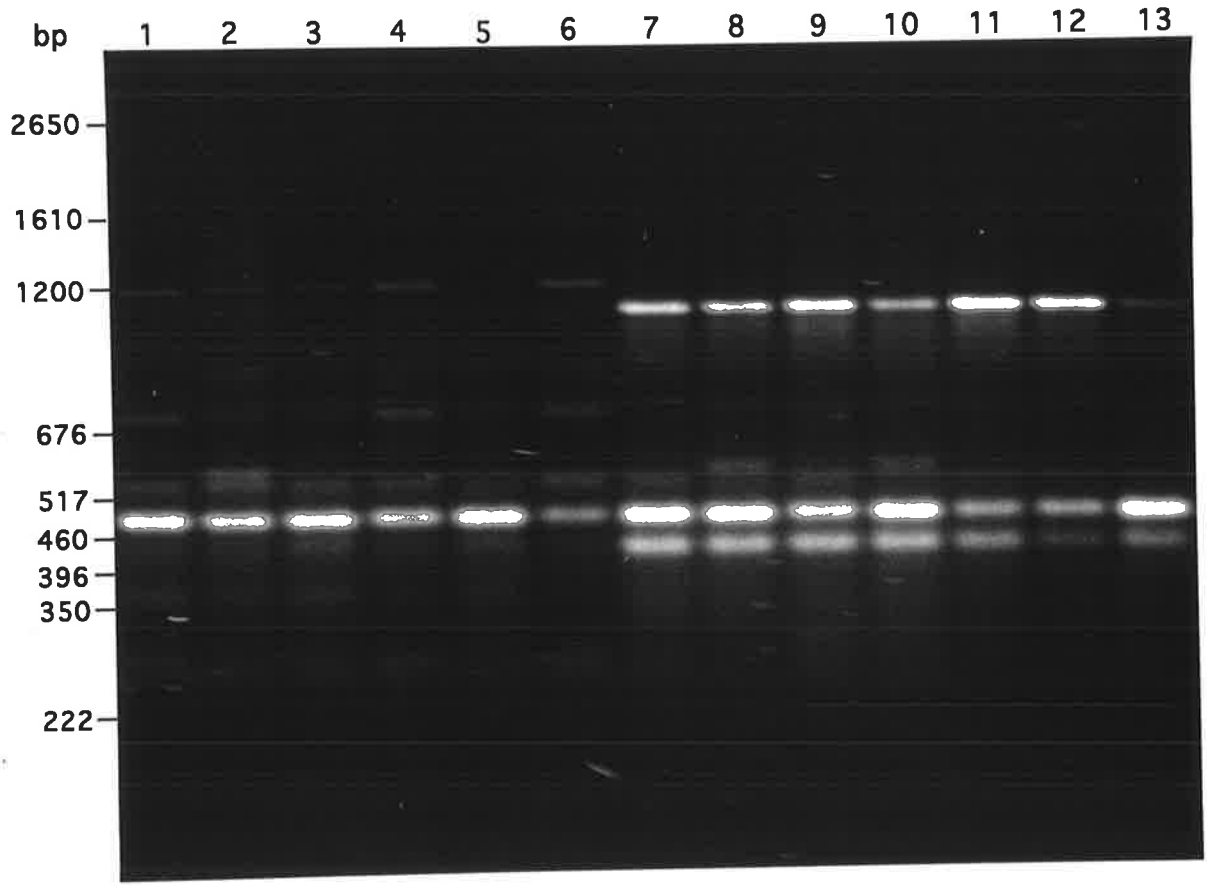
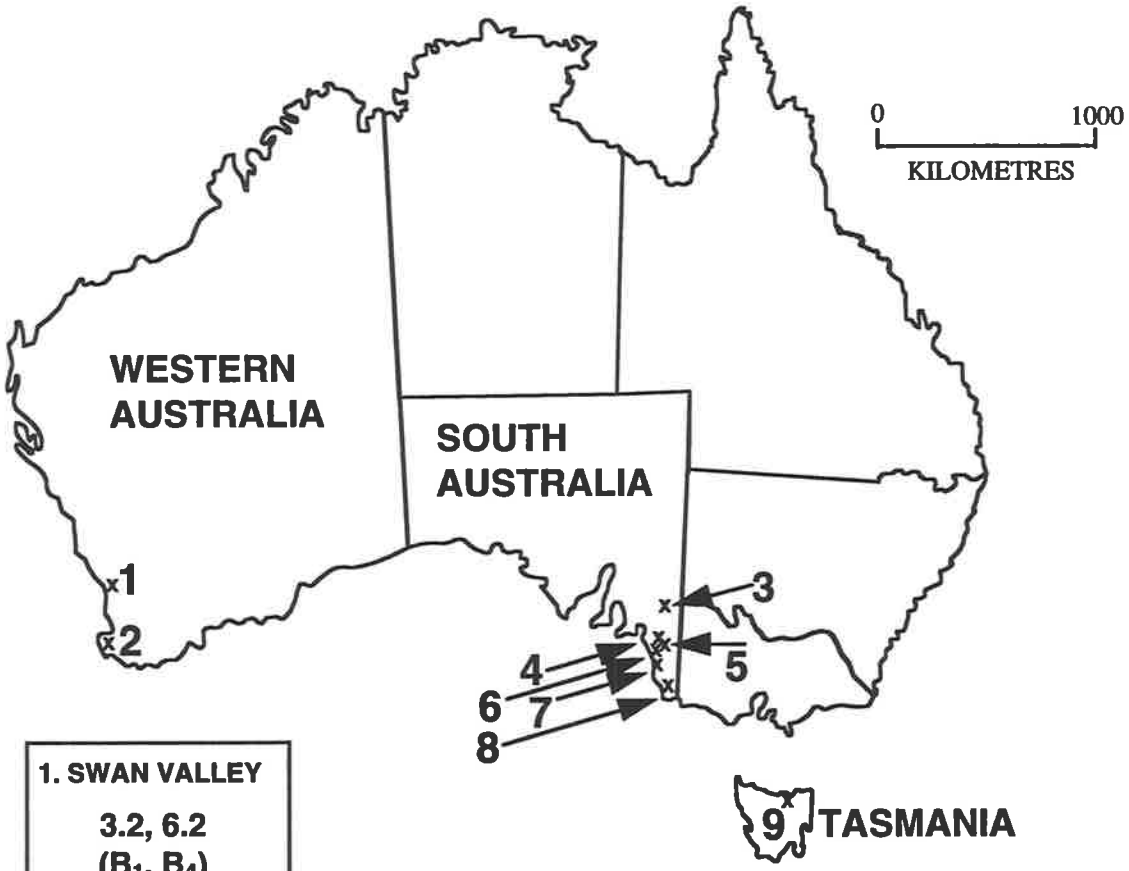

bp
1
2
3
4
5
6
7
8
9
10
11
12
13
2650
1610
1200
676
517
460
396
350
222

Table 6.5 Assignment of each *U. necator* clonal line to a lineage and genotype.

U. necator clonal line		Lineage	Genotype[a] using two DNA probes and mating type.
Number	Source		
920103	Waite Campus, Adelaide Plains	A	1.1.1
930702	Flinders University, Adelaide Plains	A	1.1.1
930704	Flinders University, Adelaide Plains	A	1.1.1
931002	Block 1, Summertown, Adelaide Hills	A	1.1.-
930202	Block 2, Nuriootpa, Barossa Valley	A	2.1.1
930302	Block 3, Nuriootpa, Barossa Valley	A	1.1.-
930304	Block 3, Nuriootpa, Barossa Valley	A	1.1.1
930801	Home garden, Adelaide Plains	B	5.2.2
930802	Home garden, Adelaide Plains	B	5.2.-
930803	Home garden, Adelaide Plains	B	5.2.2
930804	Home garden, Adelaide Plains	B	4.3.1
930501	Pridmore, McLaren Flat	B	4.2.1
930504	Pridmore, McLaren Flat	B	7.4.1
930602	Trot, McLaren Flat	B	3.2.2
930901	Morialta, Adelaide Hills	B	3.2.2
931101	Block 2, Summertown, Adelaide Hills	B	3.2.-
931201	Block 3, Summertown, Adelaide Hills	B	3.5.-
931302	Block 4, Summertown, Adelaide Hills	B	6.2.1
931502	Block 3, Summertown, Adelaide Hills	B	8.2.2
930101	Block 1, Nuriootpa, Barossa Valley	B	9.2.1
930402	Block 3, Nuriootpa, Barossa Valley	B	4.2.1
920302	Loxton, Riverland	B	11.6.-
920401	Loxton, Riverland	B	10.2.-
940501	Block 1, Lindemans, Coonawarra	B	3.7.-
940604	Block 2, Lindemans, Coonawarra	B	4.8.-
940102	Yallingup, Margaret River	B	3.2.-
940203	Block 1, Research Station, Swan Valley	B	3.2.-
940301	Block 2, Research Station, Swan Valley	B	3.2.-
940402	Block 3, Research Station, Swan Valley	B	6.2.-

[a] Nomenclature of the genotype is $a_{1\text{-}n}.b_{1\text{-}n}.c_{1\text{-}n}$ where $a_{1\text{-}n}$ is genotype 1 to 11 using probe-enzyme combination pUnP14/*Eco*RI, $b_{1\text{-}n}$ is genotype 1 to 8 using probe-enzyme combination pUnP27/*Bam*HI, $c_{1\text{-}n}$ is mating type 1 (plus) or 2 (minus). A dash (-) means that no data for that particular marker and clonal line were generated.

Figure 6.5 Geographical distribution of *U. necator* genotypes determined using probe-enzyme combinations pUnP14/*Eco*RI and pUnP27/*Bam*HI. The letters in parentheses are the DNA fingerprint groups determined using pUnP14/*Eco*RI.



1. SWAN VALLEY
3.2, 6.2
(B_1, B_4)

2. MARGARET RIVER
3.2
(B_1)

9. LAUNCESTON
(A)

4. BAROSSA VALLEY
1.1, 2.1, 4.2, 9.2
(A_1, A_2, B_2, B_7)

3. RIVERLAND
10.2, 11.6
(B_8, B_9)

6. ADELAIDE PLAINS
1.1, 4.3, 5.2
(A_1, B_2, B_3)

5. ADELAIDE HILLS
1.1, 3.2, 3.5, 6.2, 8.2
(A_1, B_1, B_4, B_6)

7. McLAREN FLAT
3.2, 4.2, 7.4
(B_1, B_2, B_5)

8. COONAWARRA
3.7, 4.8
(B_1, B_2)

Lineage B was found in all regions sampled except Launceston, where only one disease sample was obtained. B_1, the most common DNA fingerprint group using probe pUnP14, was found in three South Australian viticultural regions (Adelaide Hills, McLaren Flat and the Coonawarra) and two Western Australian regions (Swan Valley and Margaret River). All regions, except the Riverland and the Coonawarra, had genotypes in common with at least one other region.

On a finer geographical scale, differences in mating type, lineage or genotype among *U. necator* clonal lines were observed at seven out of eight vineyards or gardens where more than one clonal line was isolated (Tables 6.5, 6.6). For example, both mating types and different genotypes were detected from lines originating from a single plant of *V. amurensis*, an ornamental glory vine; that is, the mating type and genotype of clonal line 930804 was different from that of lines 930801, 930802 and 930803. At the Summertown vineyard, both mating types, both lineages and five different genotypes were sampled from Chardonnay vines within a 500 m radius. Two clonal lines, 931201 and 931502, which were different genotypes, originated from the same panel of vines. At the Nuriootpa vineyard, both lineages and four different genotypes were sampled from three blocks of vines. Both lineages and two different genotypes, were sampled within block 3 of this vineyard.

6.4 DISCUSSION

Using three DNA probes and one PCR primer, *U. necator* clonal lines were assigned to one of two broad groups, lineages A and B, based on hybridisation or amplification patterns. In addition, these lineages were determined using pairwise similarity (S_{XY}) comparisons using the probe-enzyme combination pUnP14/*Eco*RI. Lineage B was more common than lineage A, with 24 out of 35 clonal lines (69%) being assigned to this lineage. The perfect correlation between the four DNA markers for assigning *U. necator* clonal lines to lineages suggests that the most efficient marker can now be used for the routine separation of lineages. R1 appeared to be the most efficient marker for this purpose because at least 80-fold less template DNA was required for a PCR reaction and results were obtained within one working day, compared to at least 5 days required for RFLP analysis using DNA probes. The *U. necator*-specific DNA probes, however, can be used to target both purified

Table 6.6 Examples of micro-geographical variation in characters of selected *U. necator* clonal lines.

U. necator clonal line		Mating Type	Lineage	Genotype[a]
Number	Source			
930801	*V. amurensis* vine, Adelaide Plains	minus	B	5.2
930802	*V. amurensis* vine, Adelaide Plains	unknown	B	5.2
930803	*V. amurensis* vine, Adelaide Plains	minus	B	5.2
930804	*V. amurensis* vine, Adelaide Plains	plus	B	4.3
931002	Block 1, cv. Chardonnay, Summertown, Adelaide Hills	unknown	A	1.1
931101	Block 2, cv. Chardonnay, Summertown, Adelaide Hills	minus	B	3.2
931201	Block 3, cv. Chardonnay, Summertown, Adelaide Hills	minus	B	3.5
931502	Block 3, cv. Chardonnay, Summertown, Adelaide Hills	minus	B	8.2
931302	Block 4, cv. Chardonnay, Summertown, Adelaide Hills	plus	B	6.2
930101	Block 1, cv. Chardonnay, Nuriootpa, Barossa Valley	plus	B	9.2
930202	Block 2, cv. White Grenache, Nuriootpa, Barossa Valley	plus	A	2.1
930302	Block 3, cv. Crouchon, Nuriootpa, Barossa Valley	unknown	A	1.1
930304	Block 3, cv. Crouchon, Nuriootpa, Barossa Valley	plus	A	1.1
930402	Block 3, cv. Crouchon, Nuriootpa, Barossa Valley	plus	B	4.2

[a] Nomenclature of the genotype is $a_{1\text{-}n}.b_{1\text{-}n}$ where $a_{1\text{-}n}$ is genotype 1 to 11 using probe-enzyme combination pUnP14/*Eco*RI, and $b_{1\text{-}n}$ is genotype 1 to 8 using probe-enzyme combination pUnP27/*Bam*HI.

U. necator DNA and DNA from infected tissue, whereas the PCR reaction must be performed with purified *U. necator* DNA.

R1, an 18 base ISJ primer, is based on the consensus sequences for the intron-exon splice junctions of plants and generates products from the exon, or coding region (5' splice site, exon targeting, Weining & Langridge, 1991). This primer has also been used to amplify DNA from *Gaeumannomyces graminis* (Harvey, 1993) and *Rhizoctonia solani* (Duncan *et al.*, 1993; Whisson *et al.*, 1995). The amplification of *U. necator* DNA using R1 as a primer indicates that R1 has some homology with *U. necator* DNA and implies the existence of conserved sequences at the junctions to plant exons.

Whereas primer R1 and probe pUnE21 were useful for distinguishing lineages only, probes pUnP14 and pUnP27 were used to determine *U. necator* genotypes by combining the data from both probes. Probe pUnP14 detected the most variation among clonal lines and, as for probe pUnE21, it detected a small difference within lines assigned to lineage A. Using pUnP14 and pUnP27, 15 different genotypes were identified among 29 clonal lines. Five of these genotypes occurred multiple times and the same genotypes, as defined by pooled marker data using probes pUnP14 and pUnP27, were present in different viticultural regions. The probability that clonal lines with the same genotype are clones can be calculated. If two clonal lines share the same fingerprint pattern, then the probability (p) that they have identical restriction fragments by chance is the product of the frequencies of each fragment in the population, assuming each fragment is an allelle which is associated randomly in the population and the allele frequencies at each locus in that population are known (Jeffreys *et al.*, 1985; McDonald & Martinez, 1991). Expressed differently, p is the probability that two clonal lines, which are the progeny of different matings, share the same banding pattern. Therefore, if p is very low, then the two clonal lines are probably clones. Data on allele frequencies and linkage among marker loci have not been generated in this study and so the probability of clonal identity cannot be determined.

Although the number of *U. necator* clonal lines characterised was too small to determine correlations between genotypes and viticultural regions, a preliminary description of their geographical distribution was made. Larger sample sizes and additional markers may identify variation within these genotypes and would allow the identification of any correlation between genotype and viticultural region. In addition, both mating types were

widely distributed in the *U. necator* populations sampled. Mating type was not correlated to any genotype using the markers developed in this study although there was an association between the five clonal lines of lineage A and the plus mating type. A larger sample size and/or additional DNA markers are required to test the hypotheses that (a) clonal lines in lineage A are always the plus mating type, and (b) clonal lines in lineage A are a clonal lineage.

Both mating types and/or different genotypes were detected on the same vine or panel of vines, which indicates the potential for genetic recombination at these locations. Cleistothecia were observed at these sites and the null hypothesis for gametic equilibrium (random mating) should be tested by sampling many individuals at these sites. Detailed sampling of clonal lines in time and space at several locations would allow the estimation of (a) the frequency distribution of genotypes, and (b) the contributions of sexual and clonal reproduction to population structure (Tibayrenc *et al.*, 1991). For example, Milgroom *et al.* (1992a) estimated genotypic diversity and gametic disequilibrium among 39 isolates of *Cryphonectria parasitica* from a 25 x 25 m plot of forest of oak spp. using frequencies of DNA fingerprint loci. Six out of the 39 isolates had identical genotypes but when these six putative clones were removed from the analysis, the remaining isolates appeared to result from random mating.

The presence of two putative genetic groupings, lineages A and B, provides preliminary data for use in studies on the population structure and evolution of *U. necator*. It is likely that the Australian population originated from another continent and the two lineages may be the result of either the same or separate migration events. Comparison of the recent genetic structure of the Australian population with the structures on other continents will provide evidence for the origin of the pathogen. The timing and the number of the migration events, however, may not be apparent because of the lack of historical data. The potential for genetic interaction between the two lineages could be investigated by conducting sexual crosses in the laboratory. If each parent represents a different lineage, then any recombination in the progeny would suggest that recombination could occur in the field.

The term lineage is used rather than *clonal* lineage (Anderson & Kohn, 1995) because the latter term implies that each genotype within the lineage originated from the same clone and that the variation within a clonal lineage is a result of accumulated genetic changes over

time. An alternative hypothesis is that the genotypes within a lineage arose independently. While a common origin would explain the genetic similarities within a lineage, it cannot be assumed that this is the case without comparing data from historical and recent populations generated by sampling large numbers of individuals within and between populations, and characterizing the similarities among the genotypes and phenotypes obtained. Additional markers may redefine these genetic groupings and identify other mechanisms for variation, such as the transposition of extrachromosomal genetic elements.

7.0 GENERAL DISCUSSION

The directions taken by our future research efforts will depend, for the first time, on our interests and our needs, not on our capabilities.
R. L. Metzenberg, 1991.

Genetic variation in the Australian *U. necator* population has been identified for the first time. The experimental system developed in this study provided the essential techniques for developing molecular and phenotypic markers for *U. necator*. Mating types of Australian clonal lines, unknown previously, were identified, and viable cleistothecia and infective ascospores were produced *in vitro*. This is the first report of the development of RFLP probes specific to *U. necator;* these probes resulted in stable banding patterns when clonal lines were sub-cultured repeatedly over at least 15 months. In addition, the foundation for studies investigating the population biology of *U. necator* was established following the identification of two distinct genetic groups, designated A and B, and micro-geographical variation among 35 *U. necator* clonal lines from various Australian viticultural regions.

A discussion of the merits of the experimental system is warranted given the challenges associated with culturing this obligately biotrophic fungus. *U. necator* requires a constant supply of healthy grapevine tissue for its growth and the experimental system developed in this study provides the means for maintaining and manipulating large numbers of clonal lines. Techniques developed or modified from those reported previously include the clonal isolation of *U. necator*, the mass production and collection of conidia, and subsequent DNA extraction. The mass production of conidia using detached leaf cultures and the cyclone separator collection device allows conidia to be harvested from many different clonal lines. Conidia can be harvested from dual cultures on micropropagated plantlets, but manipulations are more time consuming compared with dual culture on detached leaves. The detached leaves provide a large surface area for sporulation whereas conidia need to be collected from many small leaflets of the *in vitro* plantlets to achieve the same yield. Detached leaf culture is a standard technique for the cereal powdery mildew pathogen, *E. graminis* (Yarwood, 1946; Wolfe, 1965; Jørgensen, 1988; McDermott *et al.*, 1994), but has not been described previously for the mass production of conidia of *U. necator*. Grapevines, being perennial, are propagated by cuttings. Leaves must be collected from young shoots which are pruned to maintain active growth. Grapevines with active

shoot growth can be maintained in the glasshouse for up to 1 year provided they are pruned carefully. The establishment of single conidial chain-derived colonies can be achieved by using detached leaves in double Petri plates (Quinn & Powell, 1981; Pearson & Gadoury, 1987; Gadoury & Pearson, 1991). The detached leaf technique described here is equally useful for this purpose and has the advantage of providing a sterilized leaf surface for the production of aseptic conidia. Aseptic conidia are required for (a) inoculation of micropropagated plantlets for maintenance of isolates, and (b) DNA extraction and amplification.

The use of micropropagated plantlets of *V. vinifera* enabled the production of aseptic inoculum of *U. necator* both for culture maintenance (Gadoury & Pearson, 1988) and for bulking conidia on detached leaves. In addition, dual cultures on plantlets can be maintained for up to 6 months before the plantlets senesce, although in this study conidia were transferred to healthy plantlets every 8-10 weeks in order to maintain vigourous dual cultures. Development of methods for the long term storage of *U. necator* conidia, for example by drying and/or cryopreservation, would reduce both the amount of time spent maintaining cultures and the number of asexual generations in culture during which mutation or contamination might occur. The long term storage of dual *in vitro* cultures at lower temperatures should also be investigated. These types of storage techniques are important for the maintenance of reference isolates from historical populations, which are used to confirm the characters of isolates in population studies.

A new procedure for the extraction of high quality DNA from micropropagated leaflets infected with *U. necator* is presented. This DNA extraction method involved simple modifications of the acidic extraction buffer used for conidia to minimize co-extraction of grapevine compounds, for example polyphenols and polysaccharides (Lodhi *et al.*, 1994), which contaminate the DNA preparation. If infected grapevine DNA is to be used for PCR reactions only, then there is scope to modify the DNA extraction procedure again. Rapid extraction methods similar to those used by Luck and Gillings (1995) for grapevine berries infected with *Botrytis cinerea* could be adopted. In their procedure, DNA is extracted from the berry using a commercial product known as Gene Releaser™ (Bioventures Inc.), a polymeric matrix which sequesters contaminants such as polyphenols (M. Gillings, personal communication).

An *in vitro* mating system for *U. necator* was developed in order to characterize the mating types of clonal lines. The characterization of mating type means that a DNA marker linked to this phenotype can now be developed. Such a marker would obviate the need to conduct the time consuming crosses required to determine mating type. The marker would need to be tightly linked to the mating type gene to prevent its dissociation during recombination. DNA markers would also be used for cloning and characterizing the mating type genes.

Given that viable cleistothecia and infective ascospores were produced *in vitro*, this mating system can now be refined as a technique for studying *U. necator* molecular genetics, with the objective of deriving single-ascospore colonies from controlled pairings of opposite mating types. A reproducible mating system is essential for genetic studies and linkage analysis, with immediate applications being (a) the determination of linkage among DNA fingerprint loci and other DNA marker loci (for example, Milgroom *et al.*, 1992a), (b) the estimation of base-line fungicide sensitivities of ascospore-derived progeny from a cross of two fungicide sensitive strains (for example, Butters *et al.*, 1986), and (c) the determination of the number of genes controlling fungicide sensitivity by analysis of progeny phenotypes in crosses of parental strains of different or similar fungicide sensitivity (for example, Peever & Milgroom, 1992). In addition, a reproducible mating system and techniques for preventing or delaying cleistothecium formation *in vitro*, as demonstrated in the experiments of Chapter 4, can be used to gain a more complete understanding of the mechanisms regulating sexual reproduction. If asexual sporulation is suppressed before or during cleistothecium formation, then the chemical factor/s responsible for suppression could be characterized; this may have practical implications for disease management (D. M. Gadoury, personal communication).

Genetic variation, identified by RFLP probes and primer R1, was observed at both the macro- and micro-geographical scale in the Australian population. The fingerprinting probes developed in this study were useful for identifying different genotypes, but their utility for estimating genetic diversity needs to be evaluated. In order to estimate genetic diversity, using formulae such as Nei's (1973) statistic, the loci identified by DNA markers must fulfil certain criteria, as described in Section 2.6.4. If the fingerprinting probes, pUnP14 and pUnP27, are found to be inappropriate for calculating diversity statistics, then

an alternative approach will be to screen cloned *U. necator* sequences further for potential single-locus or low-copy RFLP probes. Single loci often represent low copy DNA and so more target DNA is required during Southern hybridisation, which may present a practical difficulty in population studies with large sample sizes. The use of multiple, polymorphic single-locus probes, however, can provide sufficient data for estimating genetic diversity. Nei *et al.* (1983), for example, suggest that more than 30 loci should be used when estimating phylogenies from gene frequency data. Where DNA fingerprints are used for estimating genetic diversity, estimates obtained using data generated by DNA fingerprints and multiple single-locus RFLP probes should be compared because, in some cases, these estimates are very different (for example, McDonald & Martinez, 1991; Milgroom *et al.*, 1992b). McDonald and Martinez (1991) provide some explanations for these observed differences, which could be a consequence of limited numbers of loci sampled and/or differential rates of recombination or mutation at different loci.

The genetic diversity of *U. necator* populations, estimated with RFLP markers, should be compared with diversity estimates obtained using other molecular markers, when these are available. Délye *et al.* (1995) used RAPD primers to amplify DNA extracted from *U. necator* mycelia and conidia which had been scraped from infected grapevine leaves. Although the yield of DNA from mycelia is higher than from conidia (refer to Section 2.6.3.2), the practical difficulty of scraping mycelia from leaves is that it must be done with extreme care to prevent grapevine tissue contaminating the sample. It appears that the 16 RAPD markers developed by Délye *et al.* (1995) detected low rates of polymorphism among DNAs from European isolates of *U. necator*. They estimated that the genetic dissimilarity among 13 isolates was $\leq 5\%$, which may simply reflect a small sample size. These researchers suggest, however, that the low diversity is a consequence of a "founder" effect, whereby only a small number of genotypes entered Europe to give rise to the 1845-1852 epidemics. Given that *U. necator* has been present in Europe for at least 150 years, the pathogen population has had the opportunity to diversify through mutation and recombination. Evidence for this hypothesis is that variation in phenotypes for fungicide sensitivity has been reported in France, Portugal and Italy (Steva, 1994). As for the European population of *U. necator*, the Australian population probably also had a narrow genetic base initially. The greatest genetic dissimilarity identified between two Australian

clonal lines in this study, obtained using the fingerprinting probe pUnP14, was 63%. Such a difference may not have been present in the founding population if it consisted of only one or several closely related clones. An alternative hypothesis for the proposed low level of genetic diversity among European isolates is that the RAPD markers do not detect significant intraspecific variation. These issues highlight the importance of using a wide range of markers, both molecular and phenotypic, for estimating genetic diversity.

The isolation of clonal lines of *U. necator* in this study was analogous to sampling individuals from a population. Development of *U. necator*-specific PCR primers, which will amplify very small quantities of DNA, could eventually obviate the need to isolate clonal lines because DNA could be amplified from diseased material sampled directly from the field. Protocols for sampling the *U. necator* population directly from the field will need to be developed. It is likely that more than one genet can colonize an infected leaf or berry because cleistothecia, formed by the interaction of hyphae of two mating types, are observed on both these tissue types. Therefore, populations rather than individuals may need to be sampled and so analyses of population structure would have to be modified accordingly. If *U. necator*-specific sequences exhibit site-specific length variation in *U. necator* then this type of PCR primer can be applied in a manner analogous to RFLP markers. Total *U. necator* DNA has now been cloned into LambdaGEM®-11 vector (a derivative of EMBL3, Promega) in order to identify such sequences, specifically for the development of primers flanking microsatellites (B.E. Stummer, personal communication). The genotypes identified in this study can now be used as known variants in the development of these additional markers.

The presence of two putative lineages in the sample of 35 *U. necator* clonal lines provides preliminary data to address questions relating to the diversity and evolution of *U. necator* in Australia. Comparison of genotype frequencies in all Australian viticultural regions and in such regions worldwide would provide a clearer picture of genetic diversity and pathogen evolution. Genetic diversity is expected to be greatest in North America where the pathogen is thought to originate (Roy & Ramming, 1990; Pearson & Gadoury, 1992). Assuming *U. necator* in Australia was a migrant, then the two lineages may be the result of the same or separate migration events. Further studies would elucidate whether the lineages are evolving independently or if there is genetic interaction within and between the two lineages. An *in vitro* mating system will be required for such studies so that clonal lines

from each lineage can be paired and the genotypes of recombinant progeny investigated. The existence of these recombinant genotypes in the population would be evidence for genetic interaction between the lineages.

Estimates of the genetic diversity of *U. necator* should include samples from all members of *Vitaceae* because cleistothecia produced on ornamental vines and wild vines are a potential source of variants for vineyard colonization, and *vice versa*. The likelihood that ascospores and/or conidia from wild and ornamental *Vitaceae* cause disease in commercial *V. vinifera* should be investigated. The development of mtDNA markers may provide a tool for tracking ascospore migration within and among members of the *Vitaceae* and for estimating potential dispersal distances (Milgroom & Lipari, 1993). If *U. necator* mtDNA is inherited maternally, markers developed using such DNA would be useful for identifying individuals in a population because the DNA does not recombine during sexual reproduction. Thus, another application for the *U. necator* mating system will be to determine the inheritance of *U. necator* mtDNA. A significant quantity of conidia, however, will need to be collected in order to extract sufficient purified mtDNA for cloning sequences for use as a DNA probes (for example, Fukuda *et al.*, 1994; Lacourt *et al.*, 1994).

In conjunction with tracking ascospore migration, the movement of genotypes in general should be investigated in order to elucidate the source of primary inoculum within vineyards, and among vineyards and regions. "Flag shoots", for example, are rarely observed in the Adelaide Hills region, whereas cleistothecia are produced in abundance. Are ascospores a source of primary inoculum in these vineyards or do conidia immigrate from other regions? Powdery mildew disease is often evident in the McLaren Vale region, 30-40 km south-west of the Adelaide Hills region, at an earlier calendar date than in the Adelaide Hills region, which suggests that south-westerly winds may transport conidia from the McLaren Vale region to the Adelaide Hills region. Conidia or asexual spores of many fungi are known to be transported long distances (for example, Hirst *et al.*, 1967; Aylor *et al.*, 1982), especially those of *E. graminis* (Andrivon & Limpert, 1992). Movement of spores among regions is likely to be in the direction of the prevailing winds, although variable weather patterns may transport spores in any direction (Aylor *et al.*, 1982). Evidence for the movement of *E. graminis* genotypes between Czechoslovakia and Switzerland was described

in Section 2.6.5.1, and DNA markers could be used in a similar way to determine gene flow among *U. necator* populations.

Cleistothecium production appears to vary among vineyards and viticultural regions, and may be related to differences in disease intensity (Gadoury & Pearson, 1988), distribution of mating types and/or climate. A comparison of genotype and phenotype frequencies in vineyards producing large numbers of cleistothecia with those which do not, may provide insight into the way the pathogen is evolving. Similarly, genotype frequencies in vineyards in which cleistothecia are the only form of overwintering structure, for example, in New York State vineyards, could be compared with genotype frequencies from vineyards in which the only overwintering form of *U. necator* is thought to be mycelium in dormant buds (Pearson & Gärtel, 1985). The perennation of *U. necator* in grapevine buds is poorly understood and the development of molecular detection techniques will allow the efficient quantification of bud infection and a means of monitoring the perennation of particular genotypes. In the course of DNA marker development, the probe pUnE4 was identified as being suitable for the detection of *U. necator*. This probe could, therefore, be developed further as a PCR-based marker to investigate the bud perennation of *U. necator*. For example, infected buds could be identified to determine the bud development stage at the time of infection. Using glasshouse and field material, the timing of bud infections could be correlated with the stage of bud development and environmental data. Knowledge of bud infection windows in relation to crop growth could eventually result in (a) a model for the spatial and temporal appearance of "flag shoots" and (b) decision rules for timing fungicide sprays, thereby reducing carry-over of this disease from one season to the next.

The potential for the development of DMI fungicide resistance in *U. necator* populations was reviewed in Chapter 2. Where mixed reproductive modes exist, one important question to ask is whether or not sexual reproduction is contributing to accelerated selection for fungicide resistant strains. Gubler *et al.* (1994) conducted a time series experiment to monitor changes in triadimefon sensitivity in *U. necator* populations in California. Thirty ascospore-derived colonies obtained in the month of January from overwintering cleistothecia were assayed for triadimefon sensitivity. Another 30 ascospore-derived colonies were obtained from cleistothecia in the following Autumn (October). Asexual generations occurring in the intervening months were sampled four times and the

mean level of triadimefon sensitivity decreased during the growing season. The second ascospore population had a lower mean triadimefon sensitivity than the first ascospore population, which was equivalent to the triadimefon sensitivity of the conidial population at the time cleistothecia developed in the field (W.D. Gubler, personal communication).

A similar study to that reported by Gubler *et al.* (1994) could be conducted using DNA markers to determine population structures as selection pressures are applied. Examples of model studies include those reported by Brown and Wolfe (1990) and Brown *et al.* (1990) for *E. graminis* f. sp. *hordei*. In European populations of this barley pathogen, race-specific resistance is the dominant and continual selective force whereas selection by fungicides is intermittent. Fungicides, however, may be used on many barley varieties, which means that *E. graminis* isolates of different virulences would be selected for fungicide resistance. It is, perhaps, not surprising that Brown *et al.* (1990) demonstrated that *E. graminis* isolates with the same level of resistance to the triazole fungicide, triadimenol, were genetically diverse according to RFLP analyses. Furthermore, linkages between fungicide resistance and virulence genes have been identified (Brown & Wolfe, 1990), which suggests that asexual reproduction is maintaining well-adapted combinations of genes, at least for the duration of the growing season.

In the case of *U. necator*, where virulence phenotypes and pathotypes have not been identified, the correlation between the relative contribution of sexual reproduction to population structures and directional shifts in fungicide resistance could be quantified. Assessment of these variables and the quantification of disease progress would provide a clearer picture of the roles of sexual reproduction, the rate of asexual reproduction and selection in altering population structures. The rate of asexual reproduction is a function of pathogen fitness, cultivar susceptibility and environment (Campbell & Madden, 1990; Shaner *et al.*, 1992). Thus, fit isolates of *U. necator* colonizing highly susceptible cultivars have generation times as short as 5 days when the weather is favourable (Delp, 1954). Genetic recombination can introduce variations in fitness and/or fungicide sensitivity which may contribute to selection for increased frequencies of fungicide resistant strains. The combination of sexual reproduction and numerous asexual generations means that successful combinations of genes are linked for at least one growing season. If linkages between enhanced fitness and fungicide resistance are maintained through to the next growing season,

then directional shifts in fungicide resistance in the population are likely to occur as the number of fungicide applications and asexual generations increases (Staub & Sozzi, 1984; Köller & Scheinpfulg, 1987). Alternatively, if fungicide resistance is associated with reduced fitness, the mean sensitivity of the population would be expected to return to lower or even base-line levels once the selection pressure (fungicide applications) is removed. This phenomenon has been reported for the dicarboximide fungicides, which are used for the control of various diseases including bunch rot of grapes (Lorenz *et al.*, 1994).

Neutral genetic markers, which have no effect on phenotype, can be used to examine population structures and linkage disequilibria (Brown & Wolfe, 1990; Milgroom *et al.* 1992a). Such studies would provide information about the genetic structures at the start of the growing season, for example clonal structure or random mating, and also information regarding the fate of clonal lines during the course of the epidemic and from year to year. In France, primary infections of *E. graminis* infecting spring barley originate from conidia dispersed from winter barley crops, which in 1993 were infected with pathotypes which contained one to three virulence genes (Hoffstadt *et al.*, 1995). As the 1993 growing season of spring barley progressed, selection by race-specific resistance genes resulted in an increase in the frequency of complex pathotypes of *E. graminis*, which contained five to nine virulence genes. This study illustrates changes in population structure within a growing season caused by selection, but other causes of change include gene flow, for example, an influx of migrants, and recombination. In vineyards where ascospores of *U. necator* are a significant source of primary inoculum, genetically diverse populations may become established at the beginning of a growing season whereas subsequent selection for fitness and/or fungicide resistance could result in the population becoming less diverse as the growing season progresses. Any new disease management strategies, such as the release of grapevine cvs genetically engineered for resistance to *U. necator*, biological control agents and new fungicides should be assessed for their impact on the genetic structure of *U. necator* populations.

In summary, crop loss can occur by poor timing or application of fungicide sprays, and/or by altered frequencies of pathotypes or fungicide tolerant strains. The contribution of each factor cannot be defined clearly unless the pathogen population is quantified and characterized. Powdery mildew disease has traditionally been quantified using disease

progress curves with no knowledge of the underlying genetic structure of *U. necator*. The identification of genetic variation in the Australian *U. necator* population is the first step in determining population structures of this plant pathogen. Monitoring population dynamics allows the prediction of future events, especially the risk of crop loss from disease. The experimental system and the RFLP probes developed in this study provide a strong foundation from which to investigate the genetic bases for variation and pathogen evolution. Additional molecular markers for *U. necator* will be required to fulfil this objective. The existing set of *U. necator*-specific markers and the *U. necator*-specific PCR primers being developed will allow direct and efficient sampling of *U. necator* genotypes from *Vitaceae*. These markers will need to be screened for linkage to genes of interest, for example fungicide resistance and mating type genes, so that phenotypes can be characterized rapidly and their frequencies monitored. Knowledge of *U. necator* population dynamics will allow plant pathologists to assess which disease management strategies will be the most efficient and sustainable.

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APPENDIX

Bio-Gel P-60 column

Bio-Gel P-60 (Bio-Rad) polyacrylimide beads were suspended in TE buffer (refer to Section 3.4) containing 2% SDS and incubated overnight at 4°C to allow the beads to imbibe the buffer. A 0.5 ml eppendorf tube, with a basal hole made with a 27G needle, was placed inside a cap-less 1.5 ml eppendorf tube. The column was constructed in the smaller eppendorf tube and consisted of 100 µl of 50-100 mesh (wet) gel overlaid with 400 µl of 100-200 mesh (wet) gel. The column was placed in a swing-out rotor and centrifuged at approximately 90 rpm to remove the buffer. The column was washed twice with TE buffer containing 2% SDS, and the buffer eluted before the sample was loaded.

Denhardt's solution

bovine serum albumin, Fraction V	0.2 mg ml^{-1}
ficoll, type 400	0.2 mg ml^{-1}
polyvinyl pyrrolidone 360	0.2 mg ml^{-1}

Prepared as a 100 x solution and stored at -20°C.

DNA extraction buffer for conidia

sodium acetate	150 mM
EDTA	20 mM
sarkosyl	3%

pH adjusted to 5.2 with acetic acid.

DNA extraction buffer for micropropagated grapevine leaflets

sodium acetate	150 mM
EDTA	20 mM
sarkosyl	3%
sodium chloride	250 mM

pH adjusted to 5.4 with acetic acid.

Gel loading buffer

urea	0.4 M
sucrose	5%
EDTA, pH 7	5 mM
bromophenol blue	0.01%

Prepared as a 10 x solution and stored at -20°C.

pUC19 specific oligolabelling buffer

d(ATP, GTP, TTP)	20 µM
Tris-HCl, pH 7.6	50 mM
sodium chloride	50 mM
magnesium chloride	10 mM
acetylated DNAase-free bovine serum albumin, Fraction V	100 µg ml^{-1}

Prepared as a 2 x solution and stored at -20°C.

pUC19 specific primers

primer 1 = 5' ACAGCTATGACCATG 3'	4 ng μl^{-1}
primer 2 = 5' TMCCAGTMACGACGT 3'	4 ng μl^{-1}

Prepared as a 0.1 µg μl^{-1} solution of each primer and stored at -20°C. For radiolabelling, 1 µl of each primer stock solution in a total reaction volume of 25 µl was used.

Southern pre-hybridisation solution

SSC*	4 x
Denhardt's solution	5 x
sodium dodecyl sulphate	0.1%
denatured, fragmented herring sperm DNA	100 µg ml^{-1}

Stored at -20°C.
* prepared as a 20 x stock solution according to Sambrook *et al.* (1989).

Southern hybridisation solution

SSC*	4 x
Denhardt's solution	2 x
sodium dodecyl sulphate	0.1%
denatured, fragmented herring sperm DNA	100 µg ml^{-1}

Prepared as a 0.9 volume solution and stored at -20°C.
* prepared as a 20 x stock solution according to Sambrook *et al.* (1989).

Sucrose buffer

sucrose	15%
Tris-HCl, pH 8.5	50 mM
EDTA	50 mM

Tris-acetate (TAE) buffer

Tris	40 mM
sodium acetate	20 mM
EDTA	1 mM

pH adjusted to 7.8 with acetic acid.

Prepared as a 50 x solution and stored at 4°C.

Tris-EDTA (TE) buffer

Tris-HCl	10 mM
EDTA	1 mM

pH adjusted to 8.0.

Triton buffer

Triton X-100	5%
Tris-HCl, pH 8.5	50 mM
EDTA	50 mM

Vector:insert molar ratios for ligations

1. 50 μg ml^{-1} of pUC19 (2.678 kbp) has a molar concentration of 29.4 nM
2. The average length of *U. necator* DNA following *Eco*RI digestion was estimated to be 4 kbp (50 μg ml^{-1} = 19.7 nM)
3. The average length of *U. necator* DNA following *Pst*I digestion was estimated to be 9 kbp (50 μg ml^{-1} = 8.76 nM)

Ligation into dephosphorylated pUC19 *Eco*RI site

The DNA concentration ratio required to achieve a vector: insert molar ratio of 2:1 is:

{50 μg ml^{-1} : (14.4 nM x 50 μg ml^{-1})/19.7 nM = 37.3 μg ml^{-1}} = 1.34 : 1

Ligation into pUC19 *Pst*I site

The DNA concentration ratio required to achieve a vector: insert molar ratio of 1:2 is:

{50 μg ml^{-1} : (58.8 nM x 50 μg ml^{-1})/8.76 nM = 335.6 μg ml^{-1}} = 1 : 6.7