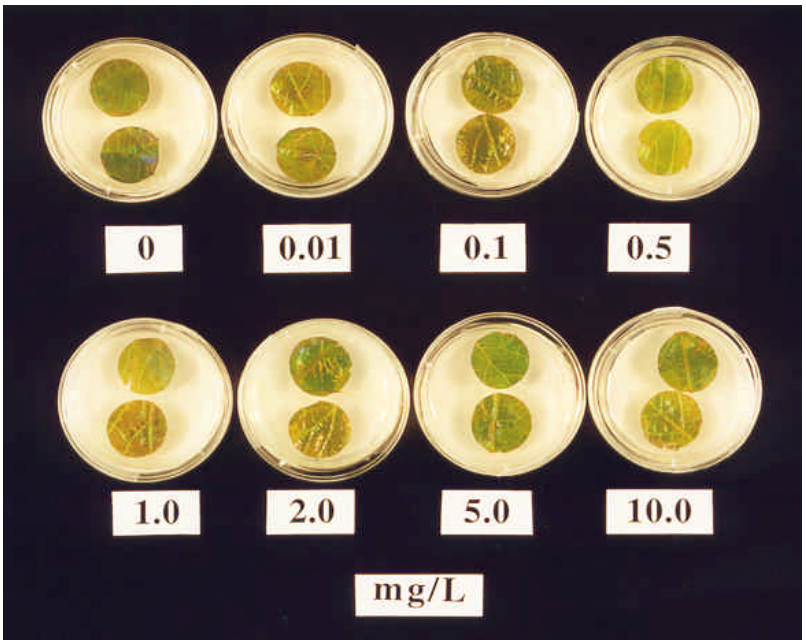
Detecting DMI resistant strains of the grapevine powdery mildew fungus using molecular probes



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GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Executive Summary

Powdery mildew has been estimated to cost the Australian Grape and Wine Industry approximately $30 million per annum. The disease is controlled in conventional vineyards mainly by sulphur and demethylation inhibiting fungicides (DMIs). DMIs have been used extensively for over 10 years in Australian vineyards and are being applied four times or more per season. DMIs inhibit one target enzyme in the fungus, therefore, a modification in one fungal gene can result in the development of a DMI resistant strain. Lack of disease control due to DMI resistance has been reported in Europe and the USA and this project was initiated in response to anecdotal reports of resistance in Australia. Sandra Savocchia began a Cooperative Research Centre for Viticulture (CRCV) PhD scholarship in February 1997 with the aim of determining whether or not DMI resistance was present in Australian strains of the powdery mildew fungus and, if warranted, to develop molecular probes for rapid detection of DMI resistance. When the first CRCV finished in June 1999, GWRDC funded the continuation of the project.

The grapevine powdery mildew fungus, *Uncinula necator*, grows only on green tissues of grapevines and closely related plants (e.g. glory vines), which makes cultivating it in the laboratory and assessing fungicide resistance difficult. By means of conventional bioassays, reduced sensitivity to triadimenol (e.g. Bayfidan), a commonly used DMI and, to a lesser extent, to fenarimol (e.g. Rubigan) was detected in Australian strains of the fungus. The bioassays involved (i) collecting infected leaves and berries from vineyards in which poor control by DMIs had been reported (to provide "selected strains") and from vineyards treated only with sulphur (to provide "unexposed strains"), (ii) establishing genetically uniform, single spore-derived strains of *U. necator* on grapevine leaves or tissue cultures in the laboratory, (iii) inoculating these single spore-derived strains onto discs which had been cut from disease-free grapevine leaves and soaked in fungicide solutions ranging in concentration from 0 – 10 mg/L, (iv) assessing growth of the fungus on the fungicide-treated leaf discs, relative to untreated controls and (v) calculating the concentration of fungicide which inhibited development of powdery mildew colonies on the leaf discs by 50% (EC_{50}). Overall, 60 strains (12 unexposed and 48 selected) were assessed for resistance to triadimenol and, of these, 34 (11 unexposed and 23 selected) were tested for resistance to fenarimol. Discriminating EC_{50} values, used to define individual strains as resistant, were calculated to be >0.42 mg/L for triadimenol and >0.12 mg/L for fenarimol. Resistance factors (RF) were calculated based on the ratio between the EC_{50} of the selected strain and the average EC_{50} of the unexposed strains, to give an indication of the degree of resistance.

The EC_{50} of the unexposed strains ranged from 0.03 to 0.12 mg/L to triadimenol and from 0.01 to 0.33 mg/ to fenarimol. EC_{50} values for the selected strains ranged from 0.01 to 3.81 mg/L for triadimenol and 0.01 to 0.98 mg/L for fenarimol. These figures represent a 13-fold increase in the mean EC_{50} values for triadimenol, in the selected compared to the unexposed populations, and a corresponding 2-fold increase for fenarimol. Therefore, in the vineyards where poor control of powdery mildew had occurred, strains of *U. necator* were found to be, on average, 13 times less sensitive to triadimenol and half as sensitive to fenarimol than strains from vineyards not treated with DMIs.

Strains of the powdery mildew fungus from vineyards in McLaren Vale, Langhorne Creek and the Adelaide Hills showed the greatest shift towards resistance to triadimenol (in decreasing order) whereas strains from Mildura, 40 % of which were obtained from Sunraysia Horticultural Centre (SHC), showed the smallest shift. The spray program used at SHC takes into account fungicide resistance management strategies, and this may account for the small shift observed.

A moderate level of cross-resistance between triadimenol and fenarimol was observed. All *U. necator* strains but one were more sensitive to fenarimol than to triadimenol. It is possible, therefore, that DMIs from different chemical groups could be used in rotation with each other and with other chemicals registered for use in the control of powdery mildew.

The bioassay is labour intensive and time consuming, and takes at least 12 days before a result is available. Therefore, the potential of DNA-based methods to provide a rapid and reliable assay for DMI resistance was evaluated. Strains characterised as sensitive and resistant to triadimenol in the bioassay were used to develop a DNA-based assay to detect DMI resistance.

Using PCR (polymerase chain reaction) and primers developed by French researchers (Délye and co-workers, INRA, France), a DNA fragment, containing part of the gene which encodes the target site enzyme of DMI fungicides, was amplified in Australian strains of *U. necator*. The resulting DNA fragments from triadimenol-sensitive and -resistant strains were then cloned and sequenced, and a single nucleotide mutation (A to T) was identified in DNA from the resistant strain but not from the sensitive strain. The same mutation has been reported in triadimenol-resistant European strains of *U. necator*. A different set of primers developed by the French researchers was tested for the ability to distinguish between DNA from resistant and sensitive strains, but proved to be inefficient. New PCR primers were, therefore, designed in this study, and used to amplify a diagnostic DNA fragment from DNA of triadimenol-resistant strains of *U. necator* (EC_{50} values ≥0.42 mg/L for triadimenol) but not from DNA of triadimenol-sensitive strains (EC_{50} values <0.42 mg/L). The PCR-based diagnostic assay was first developed using DNA obtained from spores of the fungus grown in pure culture, and was then applied to DNA from tissue-culture plantlets infected with *U. necator* in the laboratory and from infected leaves and berries collected from vineyards. The PCR-based diagnostic assay provided a rapid and reliable means of identifying resistance to triadimenol in the powdery mildew fungus, and was estimated to cost approximately A$10 per sample (excluding labour).

The inheritance of triadimenol resistance was examined by pairing sexually compatible triadimenol-sensitive and -resistant strains in the laboratory and analysing the progeny obtained from the resulting sexual spores (ascospores). The progeny strains were examined for (i) mating type, (ii) fungicide sensitivity (using the bioassay) (iii) the mutation (using the PCR assay) and (iv) DNA characteristics (using a DNA probe developed by Belinda Stummer that provides a "fingerprint" for individual strains). All progeny strains were of the same mating type. Five progeny were classed as triadimenol-sensitive and 22 as triadimenol-resistant. Based on the PCR assay, mating type and DNA bar code, all progeny were identical to the resistant parent. This suggested the possibility of self-fertility in the two strains of *U. necator* tested.

This study has provided a baseline measure of the sensitivity of Australian strains of *U. necator*, not previously exposed to DMIs, to triadimenol and fenarimol. Results have shown that, since the introduction of DMI fungicides into Australian vineyards, there has been a decrease in sensitivity to triadimenol and, to a lesser extent, fenarimol. This information coupled with the PCR-based diagnostic test for DMI resistance will be useful when developing disease management strategies that reduce and delay the development of resistance to DMI fungicides.

Ms Savocchia's research was supervised by Eileen Scott, Belinda Stummer and Robyn van Heeswijck of the University of Adelaide and by Trevor Wicks of the South Australian Research and Development Institute. Dara Melanson (Whisson), formerly of the South Australian Research and Development Institute, was a supervisor prior to her move to Oregon State University in July 1999.

Background to the research

Powdery mildew

Powdery mildew is the most economically important disease of grapevine world-wide. The disease is caused by *Uncinula necator*, a fungus which grows only on green grapevine tissue. Fungal growth (mycelium and spores) on the surface of leaves, foliage and developing berries results in a characteristic powdery grey/white appearance. In the absence of effective control measures, up to 100% of grape bunches can become completely covered in mildew. Infected berries may split, allowing secondary infection by a range of pathogens, including bunch rot organisms. Wine made with 3% or more mildewed berries may have off-flavours (Pool *et al.*, 1984) and table grapes scarred as a result of infection are unmarketable.

Disease management

Grape growers rely mainly on sulphur and demethylation inhibiting (DMI) fungicides for control of powdery mildew. In most regions of Australia, DMIs are recommended for early season control, when temperatures are sub-optimal for control with sulphur, and late in the season when sulphur may cause phytotoxicity or chemical residues may taint berries (Wicks *et al.*, 1997). They have also been recommended for eradication of established powdery mildew infections in December-January when early control has been inadequate (Wicks *et al.*, 1998).

The failure of DMIs to give adequate control of powdery mildew was reported by growers in south-eastern Australia in the mid-1990s. In response to these concerns, the first Cooperative Research Centre for Viticulture (CRCV) agreed to fund research on DMI resistance, and Sandra Savocchia was appointed to a PhD studentship in February 1997.

Resistance to DMI fungicides

DMIs are taken into the leaf, where they disrupt the synthesis of sterols in the powdery mildew fungus by inhibiting the C14α-demethylase enzyme. Sterols are components of the membranes of fungal cells, and inhibition of sterol synthesis causes breakdown of membranes and inhibits fungal growth. Because of the single-site activity of the DMIs, a mutation in a single fungal gene can result in a change at the site of action and a resistant strain can develop. A population of the fungus not previously exposed to DMIs comprises individuals ranging from highly sensitive to considerably less sensitive than the average individual, so exposure to a DMI will select the less sensitive individuals and shift the population towards the resistant end of the spectrum. Köller *et al.* (1991) assigned an operational definition of DMI resistance, based on the response of a DMI-treated population, such that, phenotypes are resistant if their frequencies have increased substantially at sites with unsatisfactory disease control in response to extensive use of a fungicide. "Practical resistance" occurs when the frequency of resistant types precludes commercially acceptable disease control (Erickson and Wilcox, 1997).

Resistance of *U. necator* to DMIs was first reported in Portugal in 1988 and soon after in France and Italy (Steva, 1994). In California, many growers abandoned sulphur when the DMI triadimefon (Bayleton®) was registered in 1982. Large-scale losses occurred in 1985, 1986, 1989 and 1990, and were shown, by means of bioassays, to be due to DMI resistance (Gubler *et al.*, 1994). Resistance to the DMIs, triadimefon, myclobutanil (Rally®, Myclos®) and fenarimol (Rubigan®), has been reported in California by Gubler *et al.* (1996) and Ypema *et al.* (1997) and to triadimenol (the active form of triadimefon), myclobutanil and fenarimol in New York State by Erickson and Wilcox (1997). These research groups used bioassays to measure sensitivity to DMIs. Most bioassays use discs punched

from leaves of vines grown in disease-free conditions; the discs are treated with the fungicide(s) of interest at a range of concentrations, placed on agar medium in sterile dishes and inoculated with the test strain(s) of the powdery mildew fungus. Growth of the fungus on the leaf discs is recorded, usually 10-14 days after inoculation, and the percentage of inhibition of fungal growth at each fungicide concentration is calculated relative to controls (without fungicide). The EC_{50} (concentration of fungicide which inhibits fungal growth by 50%) is then determined. Before being tested, genetically uniform, single spore-derived strains need to be established from the original sample, and the fungus must be bulked up, meaning that the bioassay can take up to 5 weeks. This is too slow to allow growers to make informed decisions about spray strategies.

Cross-resistance

The application of one type of fungicide may select for strains of fungi with reduced sensitivity not only to the fungicide applied but to other fungicides belonging to the same or different chemical groups also. This is known as cross-resistance. Cross-resistance to DMIs has been reported in populations of *U. necator* in North America (Gubler *et al.*, 1996; Erickson and Wilcox, 1997). Gubler *et al.* (1996) showed that sensitivity to triadimefon, myclobutanil and fenarimol increased gradually from the beginning to the end of the growing season, even though triadimefon was the only fungicide applied. They also found that resistance over-wintered in the cleistothecia (sexual reproductive structures) of the fungus. Cross-resistance has implications for disease management using DMIs, therefore, it is important to establish if it occurs in Australia.

Mechanisms of resistance

Resistance to DMIs has been associated with mutations in the gene which codes for the C14α-demethylase enzyme in various fungi, including *U. necator* (Délye *et al.*, 1997b). Délye and co-workers cloned the *U. necator* 14α-demethylase gene and found that strains with moderate to high levels of resistance to triadimenol had a single mutation, the substitution of the nucleotide adenine (A; in sensitive strains) by thymine (T; in resistant strains) at nucleotide 462 in the gene. This results in the substitution of the amino acid tyrosine (in the C14α-demethylase enzyme of sensitive strains) by phenylalanine (in resistant strains) (Délye *et al.*, 1997b).

Progress in this project (February 1997-June 1999) prior to GWRDC funding

Ms Savocchia modified the bioassay of Erickson and Wilcox (1997), and used it to assess 54 strains of *U. necator* for sensitivity to triadimenol. She found strains with EC_{50} values of 0.03-3.91 mg/L. Some strains showed tolerance of triadimenol at concentrations up to 52 times that of strains from vines not previously treated with this fungicide (Savocchia *et al.*, 1998). She had begun to assess specific PCR primers, developed by French researchers (Délye *et al.*, 1997a), for the ability to amplify the 14α-demethylase gene from representative resistant and sensitive Australian strains of *U. necator*.

Objectives

1. investigate the existence of cross-resistance between different DMIs
2. develop DNA markers which discriminate between DMI resistant and sensitive strains
3. determine pattern of inheritance of DMI resistance and of DNA markers
4. complete postgraduate research training and PhD thesis.

Introductory technical information

The project was initiated in response to concerns about DMI resistance expressed to Dr Trevor Wicks (SARDI), Dr Bob Emmett (AgVic) and other grapevine pathologists by grape growers in south-eastern Australia. The research has been actively supported by grape growers and state department of agriculture pathologists.

Poor control of powdery mildew may result from DMI resistance but it may, instead, be due to poor spray coverage, inappropriate timing of sprays and other management issues. It is important that growers can differentiate between these causes of control failure, so that they can make informed decisions about sustainable management of powdery mildew. A routine diagnostic service for DMI resistance would allow growers to obtain that information. The number of enquiries that we have received about such a service underlines the value growers place on this knowledge.

Methods

Bioassay for DMI resistance

Diseased leaves or berries were collected from various Australian vineyards or home gardens: from five sites with no previous exposure to DMI fungicides and nine with suspected practical resistance to these fungicides. The vineyards sampled, the number and type of sprays applied to control powdery mildew and the number of single-spore strains established from each, are listed in Table 1.

In total, 60 single spore-derived cultures of *U. necator* strains were tested for sensitivity to triadimenol; 12 were from vineyards or home gardens with no previous exposure to DMI fungicides (unexposed population) and 48 were from vineyards with suspected practical resistance to these fungicides (selected population). Of these 60 strains, 34 were tested for sensitivity to fenarimol, 11 belonging to the unexposed population and 23 belonging to the selected population.

In order to establish a constant supply of disease-free leaves for the mass production of conidia and fungicide resistance testing, grapevines were established and maintained in the glasshouse. Sulphur pots were used to prevent powdery mildew infection. *U. necator* strains were maintained on tissue-cultured grapevines and conidia of these strains were mass-produced in the laboratory by culturing on leaves detached from disease-free vines. Different concentrations of technical grade triadimenol and fenarimol were prepared immediately prior to each assay (10.0, 5.0, 2.0, 1.0, 0.5, 0.1, 0.01 mg/L).

The following method was adapted from Erickson and Wilcox (1997). Disease-free, immature, bright, shiny leaves were harvested from the third or fourth node of glasshouse-grown *V. vinifera* cv. Cabernet Sauvignon, clone LC10, and were surface-sterilised. Sufficient leaves were harvested to provide four discs per fungicide concentration for each strain being tested. In sterile conditions six to eight discs were cut from each leaf using a cork borer (11 mm in diameter). The leaf discs were randomised and placed into 90 mm x 14 mm Petri plates containing 4 ml of a given fungicide concentration (see figure on front cover). Sterile distilled water containing 0.05% Tween 20 (wetting agent) was used as the control treatment. Each disc was considered to be a replicate. After 30 min, leaf discs were removed from the fungicide solution and were allowed to dry for 5 min before being placed, right side up, in pairs, into 36 mm Petri plates containing 1% water agar amended with antibiotics (10 mg/L rifampicin, 5ug/L pimaricin and 150mg/L sodium ampicillin). Leaf discs were left for 3 h before being inoculated with *U. necator*.

The four replicate discs in two Petri plates were inoculated with a single strain of *U. necator*, as follows. Eight open Petri plates, containing two leaf discs each, were placed at the base of a galvanised iron settling tower. Conidia were dislodged from infected detached leaves, at the top of the tower, by gently tapping the lower surface of the leaf with a sterile paintbrush. The conidia were allowed to settle onto the leaf discs for 1 min before the tapping was repeated. The procedure was repeated once. Inoculated leaf discs were incubated in an illuminated growth chamber with a 12 h photoperiod at 25°C for 12 days.

After 12 days, powdery mildew infection and sporulation were examined and the percentage surface area colonised by the fungus was determined for each leaf disc. A score was assigned to each leaf disc and the mean of the four scores (discs) per fungicide concentration per strain was calculated. The relative growth data were plotted against the logarithm (log) of the appropriate transformed fungicide concentration and fitted to a negative logistic regression curve using Genstat 5 (Lawes Agricultural Trust, Hertfordshire, England). Resistance factor (RF) values were calculated for each strain exposed to a DMI as follows: $RF = EC_{50selected}/\text{Mean } EC_{50unexposed}$. The mean EC_{50} of unexposed strains was calculated separately for each fungicide tested. This value was used as a basis to calculate the EC_{50} of strains exposed to fenarimol and triadimenol.

The statistical distributions of EC_{50} values for each fungicide in the unexposed and selected populations were tested for normality using the Wilk-Shapiro/Rankit Plot procedure (Statistix for Windows Version 2.0 Analytical Software). The non-parametric Wilcoxon-Mann-Whitney two-sample tests and parametric Two-Sample T Test were used to determine if there were significant differences between the mean EC_{50} values of the unexposed population and the selected population for each fungicide and for individual regions. For each fungicide, a one-way ANOVA was used to determine variation between the unexposed and selected populations.

Correlation analysis was conducted between log EC_{50} values of unexposed and selected strains to measure the degree of cross-resistance between triadimenol and fenarimol. Also, a second method of statistical analysis was used to provide a comparison of strains in sensitivity categories that were classified as resistant based on their pronounced frequency increases (relative to unexposed populations). The relationships were displayed as a Venn diagram.

Development of new DNA markers for DMI resistance

The presence of the 14α-demethylase gene was confirmed in 54 single spore-derived strains using the French PCR primers flanking this gene. Of these, triadimenol-sensitive strain APf1 (EC_{50} = 0.06 mg/L; RF = 1) and triadimenol-resistant strain LCb6 (EC_{50} = 2.21 mg/L, RF = 34) were chosen for cloning and sequence analysis of the 14α-demethylase gene.

Genomic DNA extracted from 60 single spore-derived strains of *U. necator* maintained in the laboratory, eight field samples (bunches) infected with powdery mildew and six other fungi isolated from grapevine berries and leaves (provided by Belinda Stummer) was used to screen for the presence of the point mutation reportedly associated with DMI resistance. For laboratory cultures, DNA was extracted from 5 to 40 mg of conidia harvested from detached leaves as described by Evans *et al.* (1996). For *U. necator*-infected tissue culture plantlets and field-collected grape berries, four different methods were tested for extraction of genomic DNA. The DNEasy™ Plant Mini Kit (Qiagen) was shown to yield high quality DNA and was used for all subsequent experiments involving PCR amplification of DNA from infected grapevine tissue.

The PCR primers used are shown in Table 2. Each PCR was performed at least twice on two independent DNA samples. Primers C14 and C14R, reported by French researchers to correspond to sequences flanking the 14α-demethylase (CYP51) open reading frame of *U. necator* (Délye *et al.*, 1997a; GenBank accession no. U72657), were used to produce a 1756 bp DNA fragment by PCR. PCR was carried out in a 25 µl reaction mix containing 1X *Taq* DNA polymerase buffer (Promega), 2mM $MgCl_2$, 250 µM of each dNTP, 0.2 µM of each primer (C14 and C14R), 40-60 ng of template DNA and 1 unit of *Taq* DNA polymerase (Promega). The reaction mix was submitted to an initial denaturation at 94°C for 4 min, followed by 37 cycles of 1 min denaturation at 94°C, 2 min annealing at 65°C and 2 min extension at 72°C, with a final extension step of 5 min at 72°C. PCR products were analysed by gel electrophoresis.

The gene amplicons from strains APf1 and LCb6 were cloned in *E. coli* bacteria (JM109) using the pGEM®-T Easy Vector system (Promega technical manual part #TM042). Following transformation of competent cells, each culture was placed onto Luria-Bertani (LB) agar containing ampicillin, and incubated overnight at 37°C. Single putative recombinant (white) colonies were transferred to TYP broth containing ampicillin and incubated overnight at 37°C. Plasmid DNA was isolated using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega). The presence of a cloned insert was confirmed by restriction enzyme digestion or by PCR using universal primers, M13F and M13R (Messing, 1983) and gel electrophoresis.

The PCR products from each of strains APf1 and LCb6 were sequenced (Flinders University DNA Sequencing Service) in both directions (forward and reverse). In order to extend and complete the DNA sequence determination of the 1756 bp DNA fragment, additional PCR primers had to be designed to enable amplification of sub-fragments. Primers C14-1 and C14R-2 were, therefore, used to amplify by PCR a 1176 bp DNA fragment (nucleotides 257 to 1432 of the 14α-demethylase gene) and primers C14-3 and C14R-4 were used to amplify a 356 bp DNA fragment (nucleotides 64 to 419). Compilation and editing of the 14α-demethylase (CYP51) gene DNA sequence, obtained from the three PCR fragments, was carried out using the program SeqEd™ Version 1.0.3 (Applied Biosystems Inc.). DNA sequence analysis was carried out using programs of the GCG Sequence Analysis Software Package Version 8 (Genetics Computer Group, Madison, WI, USA) through the webANGIS interface (http://www.angis.org.au).

PCR amplification of a specific allele (PASA): the mutant allele

The PCR primer MUT1 was designed by French researchers to work only on those alleles of the 14α-demethylase (CYP51) gene exhibiting the A-to-T mutation at nucleotide 462 (Délye *et al.*, 1997b). Together with French primer U14DM it was used to test for amplification of a 476 bp DNA fragment which should occur only in the presence of the mutant allele in the template DNA. Initially, the PCR parameters described by Délye *et al.* (1997b) were tested, however, difficulty was encountered in replicating their results and in detecting the presence of the A-to-T mutation in DNA templates extracted from both conidia and infected grapevine tissue of the Australian samples. Therefore, the new primer MU3R (Table 2) was designed by extending MUT1 by three nucleotides at the 5'end and U14DM was replaced with primer MU4 (Table 2). MU4 and MU3R were designed to PCR amplify a 409 bp fragment of the 14α-demethylase gene only when the A-to-T mutation is present at nucleotide 462. Parameters varied to optimise the PCR included; primer, magnesium and DNA template concentrations; number of amplification cycles; addition of formamide and annealing temperature (Sommer *et al.*, 1992). The final optimised conditions were as follows; a 25 µl reaction mix containing 1X *Taq* DNA polymerase buffer (Promega), 1.5mM $MgCl_2$, 250 µM of each dNTP, 0.06 µM of each primer (MU4 and MU3R), < 2 ng of *U. necator* template DNA and 1 unit of *Taq* DNA polymerase (Promega). Initial denaturation was at 94°C for 3 min, followed by 35 cycles of 1

min denaturation at 94°C, 2 min annealing at 51°C and 2 min extension at 72°C, with a final extension step of 5 min at 72°C. Primers MU4 and MU3R were also tested for their ability to amplify the 409 bp fragment of the 14α-demethylase gene from DNA extracted from *U. necator* infected grapevine tissue.

Further optimisation of the PCR involved the development of a two-round, nested PCR procedure for amplification of a specific allele (PASA) based on the method of Sommer and Tautz (1989). In the first-round PCR, primers U14DM and C14R-2 were used to amplify a 1432 bp fragment of the 14α-demethylase gene (nucleotides 1 to 1432) from all DNA templates (mutant or wild-type). The size and the amount of first-round PCR product was determined by visual comparison with the SPP1/*Eco*RI molecular weight marker (GeneWorks, South Australia). In the second-round PCR, a defined amount of the first-round PCR product was added to a reaction mix containing three primers (MU4, MU3R and C14R-2) to PCR amplify (i) a DNA fragment encompassing the 14α-demethylase gene in all templates (mutant or wild-type; this acts as an internal control to verify the technical success of the PCR and the quality of the first-round DNA sample; (ii) a DNA fragment encompassing the A-to-T mutation in DNA templates containing this mutation but not in templates from DMI-sensitive strains. This PASA technique was first tested using single spore-derived strains of *U. necator* grown in the laboratory and then with diseased berries collected from the vineyard.

Inheritance of resistance and molecular markers

The mating type of 27 strains of *U. necator* was determined by pairing each with strains of known mating type, on tissue-cultured grapevines in the laboratory (Evans *et al.*, 1997a; Stummer *et al.*, 2000). Representative, sexually compatible, triadimenol-sensitive and -resistant strains, APf1 (mating type = 'Mat+', EC_{50} = 0.06 mg/L, RF = 0.9) and LCb6 (mating type = 'Mat-', EC_{50} = 2.21 mg/L, RF = 34), respectively, were chosen for the study of inheritance. Strains were paired on grapevines in a growth room and cleistothecia were raised to maturity as described by Gee *et al.* (2000). Cleistothecia were harvested and 11 ascospore-derived colonies, in total, were produced on detached leaves. From these, 27 progeny strains were established on tissue-cultured grapevine plantlets. The mating type of all progeny was determined by pairing each with two strains of known mating type. All progeny were analysed for sensitivity to triadimenol using the bioassay and PASA (see above). In addition, to detect genetic recombination all progeny were subjected to Southern analysis using the DNA probe, pUn122-11 (Stummer *et al.*, 2000). The "fingerprints" produced by hybridisation of the probe to the DNA were compared for the two parents and the progeny strains. Differences in the fingerprints were recorded by visual inspection.

Results

Fungicide sensitivity

Sixty single-spore strains were obtained and tested for sensitivity to triadimenol and 34 were tested for sensitivity to fenarimol. The origins mean EC_{50} values and resistance factor values for each strain and each fungicide are given in Table 3. Of the unexposed population, strains from the Barossa Valley and Adelaide Hills were most sensitive to triadimenol, whereas strains from the Riverland were most sensitive to fenarimol. The selected population showed a much greater range in sensitivity to each fungicide tested. For triadimenol, RF values ranged from 0.2 to 59, and for fenarimol, from 0.1 to 12.

The frequency distributions of the unexposed and selected populations for triadimenol and fenarimol are shown in Figure 1. The sensitivity distribution of the populations was continuous, as expected. However, according to the Wilk-Shapiro/Rankit Plot procedure, mean EC_{50} values for each fungicide

were not normally distributed, except for triadimenol within the unexposed population. Therefore, each population median was analysed for differences using the Two-Sample T Test. In addition, the Wilcoxon-Mann-Whitney two-sample test was carried out to determine if there were any significant differences between the median of the unexposed and selected populations for each fungicide (Tables 4 and 5).

According to both statistical tests, sensitivity distributions for triadimenol differed significantly ($P < 0.02$) between the unexposed and selected populations. Mean EC_{50} values of strains from the unexposed population ranged from 0.03 to 0.12 mg/L with a mean value of 0.07 mg/L and a median value of 0.06 mg/L (Table 6). In contrast, the mean EC_{50} values of strains from the selected population ranged from 0.01 to 3.81 mg/L with a mean value of 0.83 mg/L and a median value of 0.75 mg/L. The median EC_{50} values within the unexposed versus the selected population varied by a magnitude of 13. Only 18% of strains in the selected population were in the same range as the unexposed population.

According to the Wilcoxon-Mann-Whitney test, sensitivity distributions for fenarimol did not differ significantly ($P = 0.02$) between the unexposed and selected populations. Also, according to results from the T-tests there was no significant difference between unexposed and selected populations. Mean EC_{50} values of strains from the unexposed population ranged from 0.01 to 0.33 mg/L with a mean value of 0.08 mg/L and a median value of 0.05 mg/L (Table 6). In contrast, the mean EC_{50} values of strains from the selected population ranged from 0.01 to 0.98 mg/L with a mean value of 0.19 mg/L and a median value of 0.1 mg/L. The median EC_{50} values within the unexposed versus the selected population varied only by a magnitude of two. Of the strains in the selected population, 80% were in the same range as the unexposed population.

The same two statistical tests were carried out to analyse differences between the mean EC_{50} values of strains from the unexposed population and the selected population, with respect to triadimenol and fenarimol (Table 7). The effect of both triadimenol and fenarimol on the mean EC_{50} values obtained for the unexposed population did not differ significantly ($P > 0.05$). These values differed only by a factor of one. According to the Wilcoxon-Mann-Whitney two-sample test, for the selected population, a significant ($P < 0.05$) difference was observed between the two fungicides in that the mean EC_{50} values differed by a magnitude of four. However, results from the Two-Sample T Test suggest that no difference between the two fungicides was observed.

Specific EC_{50} values used to classify strains as resistant to triadimenol and fenarimol were calculated using the data presented in Figure 1. A strain was classified as resistant to a fungicide if its EC_{50} placed it in a category with decreased sensitivity relative to the unexposed population. For triadimenol, the percentage of *U. necator* strains in the selected population exceeded the percentage of strains in the unexposed population in the category with EC_{50} values between 0.15 and 0.6 mg/L (Figure 1). The mean EC_{50} of strains in this category was 0.42 mg/L. For fenarimol, the percentage of the selected population exceeded the percentage of the unexposed population in the category with EC_{50} values between 0.08 and 0.15 mg/L (Figure 1). The mean EC_{50} of strains in this category was 0.12 mg/L. Hence, the cut-off values used to define individual strains as resistant to triadimenol or fenarimol were >0.42 mg/L and >0.12 mg/L, respectively.

Cross-resistance

The degree of cross-resistance between triadimenol and fenarimol was estimated by two different methods (Erickson and Wilcox, 1997). In the first, data for all strains from both the unexposed and selected populations that were tested for reduced sensitivity to triadimenol and fenarimol were combined, the individual log EC_{50} values for each fungicide were regressed against the log EC_{50} values for the same strains for the remaining fungicide and examined for correlation (Figure 2a). Based on this analysis, there was a moderate correlation (r = 0.43) in EC_{50} values for triadimenol and fenarimol. This correlation was highly significant (P = 0.00003).

In the second method, strains in sensitivity categories that were classified as resistant based on their pronounced frequency increases (relative to unexposed populations) from vineyards exhibiting practical resistance were compared according to the method of Köller *et al.* (1997) and Erickson and Wilcox (1997). The specific EC_{50} cut-off values used to classify strains as resistant to triadimenol (> 0.42 mg/L) and fenarimol (> 0.12 mg/L) were used in this method. Based on this, 87% and 48% of the 23 selected strains tested were classified as resistant to triadimenol and fenarimol, respectively. Of the 20 strains classified as resistant to triadimenol, 43% were classified as cross-resistant to fenarimol. Only 4% of the 23 selected strains were resistant to fenarimol only and 9% were sensitive to both. These results are represented by a Venn diagram in Figure 2b.

Development of a new DNA assay for DMI resistance

A single 1756 bp fragment, comprising the 14α-demethylase (CYP51) gene, was PCR amplified from DNA extracted from conidia of 54 strains of *U. necator* using the French primers C14 and C14R. No amplification products were obtained with template DNA from other fungi tested. The complete DNA sequence was obtained for the 1756 bp fragment of the 14α-demethylase gene from strains APf1 and LCb6. Alignment of the DNA sequences revealed a single A-to-T mutation at nucleotide 462 in strain LCb6. The deduced amino acid sequences of the 14α-demethylase enzyme from APf1 and LCb6 were compared to those deduced from previously published 14α-demethylase gene sequences within the GenBank database. The APf1 sequence was shown to be identical to the sequence of a triadimenol-sensitive strain (FPE11) from France (Délye *et al.*, 1997a; GenBank accession no. U72657) and the sequence for LCb6 was identical to that for a triadimenol-resistant strain (PAZ11) from Portugal (Délye *et al.*, 1997b; GenBank accession no. U83840).

The French primers MUT1 and U14DM (Délye *et al.*, 1997b) were used in an attempt to amplify a 476 bp DNA fragment of the 14α-demethylase gene, specifically in strains containing the A-to-T mutation (triadimenol-resistant strains of *U. necator*). Délye *et al.* (1997b) reported successful PCR amplification using approximately 10 ng of template DNA, however, when this amount was used in our laboratory, no amplification was observed. Instead, amplification of the DNA fragment was obtained only when 50 to 60 ng of template DNA was used in the PCR. Following optimisation of PCR conditions, a 476 bp DNA fragment was successfully amplified from 30 of the strains displaying resistance factor (RF) values greater than 6. No amplification of the DNA fragment was observed in strains with RF values less than 6.

Since primers MUT1 and U14DM were not functioning with small amounts of template DNA, their suitability for PCR was examined. Both primers were found to have design faults that could have impaired the efficiency of the PCR. Accordingly, two new primers, MU3R and MU4, were designed, synthesised and the conditions required for the specific amplification of the mutant (A-to-T) allele were successfully determined using DNA extracted from conidia.

However, when the primers MU4 and MU3R and the optimised PCR protocol were used on DNA extracted from *U. necator*-infected grapevine tissue, the results were not reproducible. This could be attributed to variation in the amount of *U. necator* DNA in each sample of infected plant material. Quantifying the amount of *U. necator* DNA in each extract was not possible without prior analysis of each total DNA sample by Southern hybridisation or gel electrophoresis.

In order to avoid the problem with variable and indeterminate amounts of *U. necator* DNA in the DNA template samples, a nested PASA was developed (Figure 3). In the first-round PCR, primers U14DM and C14R-2 were used to amplify a 1432 bp DNA fragment of the 14α-demethylase gene from template DNA of both mutant and wild type strains of *U. necator* (Figure 4). The amount of DNA amplified in the first-round PCR was readily quantified after gel electrophoresis using visual comparison with a molecular weight marker. Approximately 125 to 1400 ng of DNA product was amplified from 5 to 10 ng of *U. necator*-infected grapevine DNA, respectively. No amplification was observed with DNA extracted from uninfected grapevine nor from *Cladosporium* sp., *Phomopsis viticola*, *Alternaria* sp., *Saccharomyces cerevisiae*, *Botrytis cinerea* and *Aspergillus niger*.

In the second-round PCR, primers MU4 and MU3R were successfully used to amplify a 409 bp DNA fragment only from those DNA templates containing the mutant (A-to-T) allele. In order to maximise the specificity of the second-round PCR, various amounts of first-round PCR product from sensitive (APf1) and resistant (LCb6) strains were tested. When 0.01 to 0.5 pg of first-round PCR product was used in the second-round PCR, only those template DNAs derived from strains with the mutant (A-to-T) allele produced the 409 bp amplification product. No amplification product was obtained from template DNA derived from the DMI sensitive strain.

In order to provide an internal positive control to demonstrate the technical success of the PCR, a third primer (C14R-2) was added to the second-round PCR. Either one or two amplification products were then produced depending on the presence or absence of the A-to-T mutation and, correspondingly, the DMI sensitivity of the strain (Figure 5). Primers MU4 and C14R-2 amplified a 1362 bp DNA fragment of the 14α-demethylase gene in all strains, while MU4 and MU3R amplified an additional 409 bp fragment only in resistant strains, indicating the presence of the mutant A-to-T allele of the 14α-demethylase gene. No amplification was observed with DNA from uninfected grapevine or other fungi isolated from grapevine after the second-round PCR, indicating the specificity of the primers for *U. necator* DNA.

Inheritance of resistance and molecular markers

All 27 progeny strains formed cleistothecia only when paired with a strain of the 'MAT+' mating type, thus all were deemed to be of the 'MAT-' mating type.

The sensitivities of the progeny strains to triadimenol, in terms of mean EC_{50} and RF values, are given in Table 8. A range in sensitivity to triadimenol was observed for progeny strains, with EC_{50} values ranging from 0.07 (A41c, A42c) to 2.21 (A51a) mg/L, with RF values of 1 and 34, respectively. These values lie between, or are identical to, the EC_{50} values determined for parental strains APf1 (0.06 mg/L) and LCb6 (2.21 mg/L). The sensitivity distribution of progeny strains was continuous, with a mean EC_{50} value of 0.91 mg/L and a median value of 0.82 mg/L. The median EC_{50} values for the progeny strains differed from APf1 and LCb6 by a magnitude of 3 and 14, respectively. Of the progeny, 7% were in the same category as sensitive parent, APf1, and 11% were in the same category as resistant parent, LCb6. The majority of the progeny were in the 0.6 to 1.05 mg/L EC_{50} category. Considerable variation was also observed between the progeny collected from the same

ascospore-derived colony. For example, the two single-spore progeny isolated from colony number five showed EC_{50} values of 0.07 and 1.72 mg/L.

The observed segregation ratio for resistance to triadimenol was 5 (sensitive): 22 (resistant) (1:4). This segregation ratio was significantly different from 1:1 ($\chi^2 = 8.33$, $p<0.05 = 3.84$), which, given that *U. necator* is haploid, is inconsistent with there being a single gene that controls response to triadimenol.

The nested PASA technique was used to determine the presence or absence of the A-to-T mutation in the 14α-demethylase gene associated with resistance to triadimenol. In the first-round PCR, primers U14DM and C14R-2 amplified a 1432 bp fragment in all progeny strains, as expected. The amount of DNA amplified in the first-round PCR, as determined by comparison to the molecular weight marker, was approximately 1 to 41 ng/µl of DNA from initial DNA template of 5 to 10 ng, respectively. In the second-round PCR, using primers MU4, MU3R and C14R-2, two DNA fragments were amplified in all progeny strains and parent LCb6, and only one fragment was amplified in parent APf1. Therefore, the 409 bp DNA fragment encompassing the A-to-T mutation was amplified from all but the sensitive parent APf1.

The DNA probe, pUN122-11, was used to detect genetic differences between the parental strains and the 27 progeny strains. The probe hybridised to nine fragments in triadimenol-sensitive strain, APf1, and to eight in triadimenol-resistant strain, LCb6. Three bands present in LCb6 are absent in APf1 and APf1 has one band that is absent in LCb6. The banding patterns obtained for all progeny strains were identical to triadimenol-resistant parent, LCb6, hence no genetic recombination could be detected.

Complete postgraduate research training and PhD thesis

The thesis was submitted on 18 September 2001. Papers reporting the research findings are in preparation. Progress in the research was delayed by (i) an inadequate supply of disease-free leaves for use in the bioassay, as a result of seasonal variation and damage to vines by mites in the glasshouse and (ii) difficulties encountered in amplifying DNA of *U. necator* using PCR primers reported by French researchers. This necessitated the design and synthesis of new primers, optimisation of PCR protocols and development of the nested PASA technique. Ms Savocchia has gained experience in a range of experimental techniques, and has benefited from interactions with industry personnel.

Discussion

Fungicide sensitivity distributions and cross-resistance

All strains available were tested for sensitivity to triadimenol, however, due to time constraints and the availability of resources, only a selection were tested for sensitivity to fenarimol. Fungicide sensitivity was evaluated in (i) a population of strains not previously exposed to DMIs and (ii) a population exhibiting practical resistance to DMIs. No *U. necator* strains were collected prior to the release of DMIs in Australian vineyards, therefore, strains representing the unexposed population were collected from vineyards in which sulphur was the only chemical used for the control of powdery mildew for the past 10 years. In contrast, the population exhibiting practical resistance to DMIs comprised strains collected from vineyards in which up to five DMI sprays were applied in one growing season.

A bioassay modified from Erickson and Wilcox (1997) was used to identify representative triadimenol-sensitive and -resistant strains for use in further studies and to determine the distribution of sensitivities among the unexposed and selected strains. This method also allowed the comparison of shifts in the

sensitivity of strains to each DMI tested and the examination of cross-resistance relationships. The mean EC_{50} values of the unexposed population were 0.07 and 0.08 mg/L for triadimenol and fenarimol, respectively. In comparison, Erickson and Wilcox (1997) reported EC_{50} values of 0.09 and 0.03 mg/L for triadimenol and fenarimol, respectively, in an assay of 77 *U. necator* strains from New York State. Nass (1991) reported EC_{50} values of 0.07 and 0.04 mg/L for triadimenol and fenarimol, respectively, in an assay of three *U. necator* strains from New Zealand. This type of bioassay, therefore, appears to give reproducible results across research groups.

The baseline sensitivities obtained for triadimenol and fenarimol showed a continuous distribution, with EC_{50} values ranging from 0.03 to 0.12 mg/L and 0.01 and 0.33 mg/L, respectively. For both triadimenol and, to a lesser extent fenarimol, strains from the selected population also showed a continuous distribution of sensitivities and were found to have decreased in sensitivity compared to the unexposed population. This indicates that the *U. necator* populations in vineyards where DMIs have been used have shifted toward resistance to this group of fungicides. This is the first documented case of DMI resistance in a grapevine powdery mildew population in Australia.

Distributions of sensitivity for triadimenol were significantly different for the unexposed versus the selected populations. EC_{50} values for the selected population ranged from 0.01 to 3.81 mg/L, with 82% of the population showing values greater than the highest EC_{50} value (0.12 mg/L) of the unexposed population. There was a 13-fold increase in the mean EC_{50} values for triadimenol in the selected versus the unexposed populations. This may represent the magnitude of shift that has occurred in the *U. necator* population since DMIs were first used in the DMI-treated vineyards. It appears that Australian populations of *U. necator* have not shifted as much as New York State populations, in which a 30-fold increase was observed for triadimenol (Erickson and Wilcox, 1997).

The McLaren Vale, Langhorne Creek, Adelaide Hills and Mildura populations had the largest shifts (in decreasing order of magnitude) from the unexposed population. Strains from Mildura, 40% of which were obtained from Sunraysia Horticultural Centre, showed the smallest shift towards resistance. This may reflect the spray program used at this site, which follows fungicide resistance management guidelines. Three vineyards at Langhorne Creek and Adelaide Hills exceeded the recommended number of DMI sprays per season, having received either four or five sprays in one season. This may explain the shift toward a more resistant population in these regions.

A much less pronounced shift was observed in the distribution of sensitivities to fenarimol and the difference between the unexposed and selected populations was not statistically significant. EC_{50} values for the selected population ranged from 0.01 to 0.98 mg/L, with only 17% of the population lying outside the highest EC_{50} value (0.33 mg/L) of the unexposed population. This 17% comprised three strains from Western Australia and one from Langhorne Creek. The observation that mean EC_{50} values for fenarimol in the selected population had shifted only by a factor of two is similar to the situation in New York State vineyards, for which Erickson and Wilcox (1997) reported a two-fold increase in median EC_{50} values, and Californian vineyards, for which Gubler *et al.* (1996) reported an overall one to three-fold increase for this fungicide.

The values calculated for classifying a strain as resistant to triadimenol (EC_{50} >0.42 mg/L) and fenarimol (EC_{50} >0.12 mg/L) were comparable to those obtained by Erickson and Wilcox (1997) (>0.56 and >0.18 mg/L for triadimenol and fenarimol, respectively).

The degree of cross-resistance between triadimenol and fenarimol was examined by two different methods, the advantages and limitations of which were discussed by Erickson and Wilcox (1997). Using correlation analysis, all strains examined in the bioassay were taken into account. This allowed for the maximum number of strains to be analysed, removing any bias when selecting strains. A positive correlation (r = 0.43) was found in sensitivities to triadimenol and fenarimol. Erickson and Wilcox (1997) found a slightly greater positive correlation of sensitivities to triadimenol and fenarimol (r = 0.56). Using the Venn diagram method, we observed a greater degree of cross-resistance than did Erickson and Wilcox (1997). For example, of the strains classified as resistant to triadimenol (87%), 43% of these were also classified as resistant to fenarimol, whereas Erickson and Wilcox (1997) found a slightly greater percentage of strains to be resistant to triadimenol (93%), and of these, only 17% were resistant to fenarimol. These differences can be explained by the fact that, in our study, the mean EC_{50} value for strains tested for fenarimol (0.19 mg/L) was greater than that found by Erickson and Wilcox (1997) (0.07 mg/L). The use of the Venn diagram gave a clear interpretation of results and the degree of cross-resistance. The Australian strains showed an overall moderate degree of cross-resistance between the two fungicides. For all strains except one, sensitivity levels to fenarimol were always greater than to triadimenol.

These findings have implications for managing powdery mildew and DMI resistance in the vineyard. It has been suggested that differences in cross-resistance patterns and in sensitivity shifts between DMIs may be due to the intrinsic activity of certain DMIs (Gubler *et al.*, 1996). They may also reflect differences in the genetic control of the variation in sensitivity to triadimenol and fenarimol, despite the known common mode of action, suggesting that there may be a difference in the selection process. To date, only a single gene is known to confer resistance to DMI fungicides in *U. necator*, however, multiple gene resistance has been confirmed in other plant pathogens (e.g. Peever and Milgroom, 1993). Other factors may also account for the difference observed in levels of resistance to triadimenol and fenarimol. Low application rates of DMIs have been found to increase the rate at which resistance evolves in *Venturia inaequalis* (the apple scab fungus) by selecting strains that withstand low doses (Köller and Wilcox, 1999). However, reduced doses had no effect on shifts in resistance in *Septoria tritici* (Shaw and Pijls, 1994). A study conducted in French vineyards showed that, for *U. necator*, the use of half the recommended application rate of triadimenol was more effective in selecting resistant strains within the population than were full application rates (Steva, 1994). In Australian populations of *U. necator*, the effects of using application rates, other than those recommended by the chemical manufacturer, on DMI resistance levels are unknown. There is a need for further studies in which different DMI rates are applied in field experiments and the *U. necator* population examined subsequently for sensitivity to various DMIs. Also, to obtain a more accurate representation of the sensitivity levels that exist in Australian populations of *U. necator*, there is a need to study more strains from a number of different vineyards within a region.

The results suggest that, in the event of poor control with one DMI, the use of an alternative DMI may provide adequate control of *U. necator*. This strategy has also been suggested for use in New York State by Erickson and Wilcox (1997). DMIs from different chemical groups could be used in rotation with each other and with other chemicals available to control powdery mildew. Field trials to monitor levels of resistance following application of different DMIs could be conducted in future studies. This may help reduce selection pressure for the development of practical resistance and, in turn, will provide a larger armoury to control powdery mildew effectively.

In summary, reduced sensitivity to triadimenol and, to a lesser extent, fenarimol was confirmed for *U. necator* strains collected from vineyards in various viticultural regions of Australia and partial cross-

resistance between these two DMIs was also found. It is important to note, however, that the bioassay for fungicide resistance is laborious and time consuming, requiring at least 12 days before a result becomes available. Also, there is a constant requirement for disease-free leaves, meaning that grapevine plants need to be maintained in a controlled environment all year round. Supplies of leaves fluctuate and may be compromised by mites and other pests. Therefore, to increase the number of strains assessed at any one time, a more rapid means of determining if resistant strains exist within a vineyard is required.

Development of a new DNA assay for DMI resistance

Although the French PCR primers C14 and C14R (Délye *et al.*, 1997a) were used to amplify a 1756 bp fragment in all 54 strains tested, confirming the presence of the 14α-demethylase gene, these primers were inefficient and required a relatively large amount of genomic DNA as template, even after the optimisation of PCR conditions in our laboratory. New primers (C14-1, C14R-2, C14-3 and C14R-4) were designed and used to obtain the complete sequence of the 14α-demethylase gene. The new primers required only 10 to 25 ng of genomic DNA as template for efficient amplification. Direct sequencing of the PCR products using custom-made primers proved to be more efficient than cloning of PCR fragments. This meant that there was no need to clone each PCR fragment to be analysed, hence, there was no need for mass-production of conidia, which is difficult for an obligate biotroph such as *U. necator*.

The 14α-demethylase gene sequences of triadimenol-sensitive strain APf1 and -resistant strain LCb6 were identical to those determined for DMI-sensitive and -resistant *U. necator* strains in Europe, respectively (Délye *et al.*, 1997a; 1997b). This suggests that the gene is highly conserved amongst *U. necator* strains from different regions. In comparing the gene sequences of APf1 and LCb6, a single point mutation (A-to-T) was identified at nucleotide 462. This point mutation was reported for triadimenol-resistant strains of *U. necator* exhibiting an RF value of greater than 5 (Délye *et al.*, 1997b) and in DMI-resistant strains of the barley powdery mildew fungus (Délye *et al.*, 1998). Although other point mutations have been identified within the 14α-demethylase gene of European strains of *U. necator* (Délye *et al.*, 1999), no further nucleotide differences were found when comparing the sequences of Australian strains APf1 and LCb6. This suggests that the point mutation at nucleotide 462 may be diagnostic for DMI-resistant strains.

PCR allele specific amplification (PASA) of the A-to-T mutation at nucleotide 462 was initially performed using the French primers MUT1 and U14DM (Délye *et al.*, 1997b) with DNA from 60 single spore-derived strains of *U. necator*. In spite of the requirement for relatively large quantities of conidia and DNA, these primers were used identify the A-to-T mutation in all 38 strains with RF values of at least 6. These results are similar to those obtained by Délye *et al.* (1997b), in that strains with RF values of at least 5 contained this mutation. The slight difference in RF values may be due to variation in the bioassay for fungicide resistance between laboratories. However, primers MUT1 and U14DM proved insufficiently specific for the mutant allele under our laboratory conditions, presumably due to the composition of the primers. Primers MU4 and MU3R were, therefore, designed for specific amplification of the mutant A-to-T allele in strains of *U. necator*. However, standard PCR using these two primers alone produced variable results when amplifying *U. necator* DNA from total DNA extracted from infected plant material, due to variation in the amount of fungal DNA present in the total preparation.

To overcome this difficulty, the amount of *U. necator* DNA to be used in PASA was quantified using a nested PCR protocol. Two rounds of PCR were carried out and the amount of DNA was quantified

after the first round. Primers U14DM and C14R-2 were used in the first-round PCR to specifically amplify a 1432 bp DNA fragment of the 14α-demethylase gene in all strains of *U. necator*. Amounts of the DNA fragment amplified varied with the original sample of infected tissue. Consequently, the use of a standard amount of template DNA in the second-round PCR was crucial for successful and specific PCR amplification of a 409 bp PCR product only from strains containing the mutant A-to-T allele. The optimum amount of first-round PCR product to be used in the second-round PCR was between 0.01 and 0.5 pg. A third primer (C14R-2) was included in the second-round PCR reaction mix, resulting in a 1432 bp PCR product from all *U. necator* strains, acting as a positive internal control. The mutant A-to-T allele was detected only in *U. necator* strains with RF values of 6 or greater.

In summary, it is possible to detect triadimenol-resistant strains of *U. necator* from infected tissue-culture plantlets and grapevine field samples by using a simple, total DNA extraction method (DNEasy™ Plant Mini Kit; Qiagen) and nested PASA. At the time this study was conducted, the cost of performing the PASA technique was estimated to be under $10 per sample (not including labour). The use of such an assay may allow the rapid detection of DMI resistance in powdery mildew-infected material collected in the vineyard, with minimal sample preparation. Information on the existence and distribution of DMI resistance in the vineyard within 3-4 days of submitting a sample for testing would allow the grower to implement control strategies aimed at minimising both the ineffective use of fungicides and unnecessary yield loss.

Inheritance of resistance and molecular markers

Cleistothecia formed by pairing triadimenol-sensitive strain APf1 and triadimenol-resistant strain LCb6 yielded infective ascospores. Few ascospore-derived infections were established on detached leaves, with only 11 discrete colonies formed and 27 single-spore progeny being established *in vitro*. This is consistent with previous reports of low infection efficiencies of ascospores of *U. necator* (Gee *et al.*, 2000; Pearson and Gadoury 1987).

All 27 progeny strains were of the 'MAT-' mating type, as was the triadimenol-resistant parent APf1. Therefore, the segregation ratio for mating type did not conform to the 1:1 ratio that is expected for a single major gene in a haploid organism. Similar results, in which one mating type predominates, have been observed for progeny generated by crossing different strains of *U. necator* (B. Stummer; unpublished).

Results for the segregation of triadimenol resistance suggested that the trait may be controlled by more than one gene. The EC_{50} values obtained for the progeny strains were continuously and approximately normally distributed between the two parents. Of the 27 progeny, only five were classed as being sensitive to triadimenol. The segregation ratio for triadimenol resistance was 1:4. It is also interesting to note that there was variation between progeny isolated from the same ascospore-derived colony. To date, numerous studies have been carried out to determine the genetic control of resistance to DMIs, however, results are often contradictory. Resistance to the DMI fungicides, flusilazole and myclobutanil, in *V. inaequalis* was found to be controlled by many genes (Sholberg and Haag, 1993), whereas earlier studies had shown triadimenol resistance in this fungus to be controlled by a single gene. The progeny from five crosses between triadimenol sensitive and resistant strains of *Pyrenophora teres* segregated into two discrete distributions in 1:1 ratios, however, when the progeny were tested for resistance to propiconazole, the phenotypes were continuously distributed and no single major locus appeared to be segregating (Peever and Milgroom, 1992). Similar results were observed by Brown *et al.* (1992) and Blatter *et al.* (1998) for the inheritance of triadimenol resistance in

Erysiphe graminis f. sp. *hordei.* In contrast, earlier studies by Hollomon *et al.* (1984) had suggested that triadimenol resistance in *E. graminis* f. sp. *hordei* was controlled by many genetic factors and, for this reason the powdery mildew fungi appear to have responded slowly to selection for resistance to DMI fungicides.

Analysis using nested PASA revealed the mutant A-to-T allele in all progeny strains. The presence of this mutation in progeny strains displaying RF values less than 6 can only be explained by variation in the bioassay for fungicide resistance or the possibility of a PCR artefact. In future studies, the DNA from inconsistent samples could be sequenced to confirm the presence/absence of the mutation. Thus far, the results suggest that recombination has not occurred, however, it may be that the resistant parent is a dominant type, or that self-fertilisation has occurred. Based on DNA fingerprinting, all progeny were identical to one another and to the resistant parent. This suggests that no recombination had occurred, strengthening the likelihood of self-fertilisation. Additional DNA probes should be used to test this observation. Due to time constraints, only one cross was performed. In future studies, pairing strains of *U. necator* with different levels of triadimenol resistance will be necessary to determine the inheritance of resistance to triadimenol. In addition, pairing strains characterised for resistance to fenarimol will determine if fenarimol resistance segregates in the same way as triadimenol resistance. A paper by Peever and Milgroom (1992) indicated that a single model of the inheritance of resistance to different DMIs may not be possible but, rather, patterns of inheritance may vary depending on the resistance phenotypes of the parents and the pathogen/DMI combination tested.

In summary, the segregation pattern of various markers has been analysed as a contribution towards determining the inheritance of triadimenol resistance in *U. necator*. All markers showed a segregation ratio that was significantly different from 1:1. In addition, no recombination was detected between the two parental strains, indicating the possibility of self-fertilisation, although this was contradicted by the continuous distribution in sensitivity to triadimenol observed among the progeny. An extensive analysis of sexual progeny is required to provide information on the heritability of triadimenol resistance and selection for fungicide resistant strains in the field, but is beyond the scope of this project.

Implications and recommendations

Resistance to triadimenol has been demonstrated in strains of the powdery mildew fungus obtained from Australian vineyards. This makes the rigorous adherence to fungicide resistance management strategies all the more important, in order to maintain the usefulness of this group of fungicides. It is important to maintain the efficacy of DMIs for as long as possible, as it has been found that resistance has developed rapidly to the strobilurin fungicides, recently registered for control of powdery mildew in the USA (D. Gadoury, Cornell University, personal communication).

The mutation-specific PCR primers developed in this study, when used in the nested PASA technique, have the potential to allow rapid diagnosis of DMI resistance in the grapevine powdery mildew fungus. With some additional development, and the formulation of a vineyard sampling strategy, a PCR-based diagnostic procedure could be available for use in routine diagnostic services. The identification of DMI resistance early in the growing season would alert the grower to the need to use alternative management strategies for powdery mildew. This action would extend the useful life of the DMI fungicides and minimise crop losses due to failure to control powdery mildew.

If there is a potential market for a rapid diagnostic test for DMI resistance, then we recommend that the nested PASA technique be considered for commercial development and sampling strategies for assessing the distribution of DMI resistance in vineyards be evaluated.

Information on the levels of resistance to triadimenol and to other DMIs should be incorporated into IPM Viticulture Research to Practice™ workshops and into AusVit™ to assist growers to make informed decisions about fungicide use.

Intellectual property

The molecular diagnostic technique is available for commercial development and application. Under current arrangements, all knowledge and intellectual property arising from this project during the term of the CRCV is licensed to CRCV Technologies Pty Ltd as the commercial agent of the Centre. Any benefits accruing to CRCV Technologies Pty Ltd from commercial applications of the intellectual property are eventually to be shared among the contributing parties at the direction of the CRCV according to total contribution (cash and in-kind) of each party in the project. Diagnostic services are offered through the University of Adelaide and SARDI in South Australia and similar services are available in other states. Adoption of a DMI resistance testing service by industry was considered likely to be high in the early stages of this project. This was supported by the numerous requests we received for DMI resistance testing.

Technical summary

A bioassay was modified from that of Erickson and Wilcox (1997) to detect DMI-resistant strains of *U. necator*. Baseline sensitivities to triadimenol and fenarimol were established for *U. necator* strains collected in Australian vineyards. In addition, strains were characterised as resistant or sensitive using this bioassay.

A PCR-based assay was developed to detect strains of *U. necator* showing reduced sensitivity to DMI fungicides. PCR primers were designed and used to amplify the mutant allele associated with DMI resistance in strains of the fungus shown in the bioassay to have a resistance factor of 6 or greater.

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Table 1. Origin of single spore-derived strains of *U. necator*.

Viticultural region	Location	Host cv./organ	Single-spore strain code[c]	Spray history[f]	Date collected
Adelaide Plains, SA	Fullarton, SA	*V. amurensis*/leaves	APD1[d]	Nil	14/1/93
Adelaide Plains, SA	Fullarton, SA	*V. amurensis*/leaves	APD2[d]	Nil	14/1/93
Adelaide Plains, SA	Waite Campus, Row 9	Chardonnay/leaves	APF1[e]	Nil	4/12/96
Barossa Valley	Nuriootpa, Block 3	Grenache Blanc/ berries	BNB2[d]	Nil for 10 years	7/1/93
Barossa Valley	Nuriootpa, Block 3	Crouchon/leaves	BNC1[d]	Nil for 10 years	7/1/93
Barossa Valley	Nuriootpa, Block 3	Crouchon/leaves	BNC2[d]	Nil for 10 years	7/1/93
Margaret River, WA	Yallingup	Sauvignon Blanc/ berries	MRA1[d]	Combined Bayfidan® (30ml/ha)/ sulphur	16/12/94
Swan Valley, WA	Swan Valley Research Station, Block 1	Flame Seedless/ berries	SVA1[d]	Bayfidan®, wettable sulphur	13/1/94
Swan Valley, WA	Swan Valley Research Station, Block 1	Flame Seedless/ berries	SVA2[d]	Bayfidan® 250 EC, wettable sulphur	13/1/94
Langhorne Creek, SA	Vineyard 1	Shiraz/leaves	LCA1	Tilt® EC, 100ml/300L, 3 sprays; Bayfidan® 250 EC, 1 spray	24/2/97
Langhorne Creek, SA	Vineyard 1	Shiraz/leaves	LCA2	Tilt® EC, 100ml/300L, 3 sprays; Bayfidan® 250 EC, 1 spray	24/2/97
Langhorne Creek, SA	Vineyard 1	Shiraz/leaves	LCA3	Tilt® EC, 100ml/300L, 3 sprays; Bayfidan® 250 EC, 1 spray	24/2/97
Langhorne Creek, SA	Vineyard 1	Shiraz/leaves	LCA4	Tilt® EC, 100ml/300L, 3 sprays; Bayfidan® 250 EC, 1 spray	24/2/97
Langhorne Creek, SA	Vineyard 1	Shiraz/leaves	LCA5	Tilt® EC, 100ml/300L, 3 sprays; Bayfidan® 250 EC, 1 spray	24/2/97
Langhorne Creek, SA	Vineyard 1	Shiraz/leaves	LCA6	Tilt® EC, 100ml/300L, 3 sprays; Bayfidan® 250 EC, 1 spray	24/2/97

Table 1. continued on the next page

Table 1. continued

Viticultural region	Location	Host cv./organ	Single-spore strain code[c]	Spray history[f]	Date collected
Langhorne Creek, SA	Vineyard 2	Chardonnay/berries	LCB1	Rubigan® 120 SC, 400ml/420L, 5 sprays	24/2/97
Langhorne Creek, SA	Vineyard 2	Chardonnay/berries	LCB2	Rubigan® 120 SC, 400ml/420L, 5 sprays	24/2/97
Langhorne Creek, SA	Vineyard 2	Chardonnay/berries	LCB3	Rubigan® 120 SC, 400ml/420L, 5 sprays	24/2/97
Langhorne Creek, SA	Vineyard 2	Chardonnay/berries	LCB4	Rubigan® 120 SC, 400ml/420L, 5 sprays	24/2/97
Langhorne Creek, SA	Vineyard 2	Chardonnay/berries	LCB5	Rubigan® 120 SC, 400ml/420L, 5 sprays	24/2/97
McLaren Vale, SA	Pridmore	Chardonnay/berries	MVa3[d]	Unknown	13/1/93
McLaren Vale, SA	Blewitt Springs	Chardonnay/ leaves	MVD1	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/ leaves	MVD2	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/ leaves	MVD3	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/berries	MVD4	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/berries	MVD5	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/berries	MVD6	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/berries	MVD7	Bayfidan® 250 EC, 3 sprays	24/2/97
McLaren Vale, SA	Blewitt Springs	Chardonnay/berries	MVD8	Bayfidan® 250 EC, 3 sprays	24/2/97
Adelaide Hills, SA	Summertown	Chardonnay,berries	AHD2[d]	Unknown	9/3/93
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF1	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF2	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF3	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF4	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF5	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF6	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF7	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 1	Chardonnay/berries	AHF8	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98

Table 1. continued on the next page

Table 1. continued

Viticultural region	Location	Host cv./organ	Single-spore strain code[c]	Spray history[f]	Date collected
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG1	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG2	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG3	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG4	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG5	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG6	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG7	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG8	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG9	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Adelaide Hills, SA	Eden Valley 2	Sauvignon Blanc/ berries	AHG10	Bayfidan® 250 EC, 100ml/ha, 4 sprays	19/2/98
Mildura, Vic	SHC[a]	Chardonnay/leaves	VMA1	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	SHC	Chardonnay/leaves	VMA2	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	SHC	Chardonnay/leaves	VMA3	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98

Table 1. continued on the next page

Table 1. continued

Viticultural region	Location	Host cv./organ	Single-spore strain code[c]	Spray history[f]	Date collected
Mildura, Vic	SHC	Chardonnay/leaves	VMA4	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	SHC	Chardonnay/leaves	VMA5	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	SHC	Chardonnay/leaves	VMA6	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	Red Cliffs	Chardonnay/berries	VMB1	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	Red Cliffs	Chardonnay/berries	VMB2	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	Red Cliffs	Chardonnay/berries	VMB3	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	Red Cliffs	Chardonnay/berries	VMB4	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	Red Cliffs	Chardonnay/berries	VMB5	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Mildura, Vic	Red Cliffs	Chardonnay/berries	VMB6	Bayfidan® 250 EC, 3 sprays and Sulphur	4/12/98
Riverland, SA	LRC[b]	Chardonnay/berries	RLD1	Nil for 10 years	23/12/97
Riverland, SA	LRC	Chardonnay/berries	RLD2	Nil for 10 years	23/12/97
Riverland, SA	LRC	Chardonnay/berries	RLD3	Nil for 10 years	23/12/97
Riverland, SA	LRC	Chardonnay/berries	RLD4	Nil for 10 years	23/12/97
Riverland, SA	LRC	Chardonnay/berries	RLD5	Nil for 10 years	23/12/97

[a] SHC: Sunraysia Horticultural Centre, Irymple. Trial Site: Row 10 and 22.

[b] LRC: Loxton Research Centre.

[c] Strains named according to viticultural region (upper case), vineyard site (lower case) and sample number. South Australia: AP, Adelaide Plains; BN, Barossa Valley; LC, Langhorne Creek; MV, McLaren Vale; AH, Adelaide Hills; RL, Riverland. Victoria: VM, Mildura. Western Australia: MR, Margaret River; SV, Swan Valley (Stummer *et al*., 2000).

[d] Single-spore strains established *in vitro* by K. Evans (1996).

[e] Single-spore strains established *in vitro* by B. Stummer.

[f] Number and type of sprays used in the season during which the strain was collected.

Table 2. Nucleotide sequence and application of the oligonucleotide primers used in this study.

Primer	Application	Nucleotide sequence (5'to 3')	Reference
C14	CYP51	TAA GGT AGT ATT GAG GCG GG	Délye *et al.*, 1997a
C14R	amplification	TTC TAA CCC TAA CAC CTG CC	Délye *et al.*, 1997a
C14-1	and DNA	CTC TTT TAC ATG CCC ATC TCC	Not published[a]
C14R-2	sequencing	CAT CAA CCG CAT CAT TTC CTA	Not published
C14-3		TTC ATG GTC ACA AGT ATC GCA	Not published
C14R-4		AAA TCT CTT CGG CGT TGA CAT	Not published
MUT1	PASA	AAT TTG GAC AAT CAA	Délye *et al.*, 1997b
U14DM		ATG TAC ATT GCT GAC ATT TTG TCG G	Délye *et al.*, 1997b
MU3R		TGG AAT TTG GAC AAT CAA	Not published
MU4		TCA CAA GTA TCG CAT TTT	Not published
M13F	DNA	GTA AAA CGA CGG CCA G	Messing (1983)
M13R	sequencing	CAG GAA ACA GCT ATG AC	

[a] Primers developed in the present study.

All primers were synthesised by GeneWorks (South Australia).

Table 3. Mean EC_{50} and RF values of *U. necator* strains not exposed and exposed to DMI fungicides. Standard error of the mean is represented in parentheses.

Viticultural region in Australia	Strain	Sensitivity to Triadimenol		Sensitivity to Fenarimol	
		Mean EC_{50}[a] (mg/L)	RF[b] Values	Mean EC_{50}[a] (mg/L)	RF[b] Values
Adelaide Plains (1), SA	APf1[c,e]	0.06 (0.18)	1	0.33 (0.10)	4
Adelaide Plains (2), SA	APd1[c,d]	0.06 (0.12)	1	0.21 (0.18)	3
	APd2[c,d]	0.05 (0.20)	1	0.11 (0.26)	1
Riverland, SA	RLd1[c]	0.09 (0.09)	1	0.05 (0.20)	1
	RLd2[c]	0.07 (0.15)	1	0.05 (0.26)	1
	RLd3[c]	0.12 (0.22)	2	0.01 (0.18)	0.1
	RLd4[c]	0.03 (0.23)	0.5	0.01 (0.19)	0.1
	RLd5[c]	0.10 (0.21)	1	0.02 (0.15)	0.2
Barossa Valley, SA	BNb2[c,d]	0.03 (0.20)	0.5	0.03 (0.15)	0.4
	BNc1[c,d]	0.06 (0.18)	1	0.05 (0.13)	1
	BNc2[c,d]	0.04 (0.19)	1	0.02 (0.13)	0.2
Margaret River, WA	MRa1[d]	0.83 (0.12)	13	0.55 (0.07)	7
Swan Valley, WA	SVa1[d]	1.73 (0.11)	27	0.98 (0.09)	12
	SVa2[d]	0.91 (0.10)	14	0.60 (0.12)	7
Langhorne Creek (1), SA	LCa1	2.32 (0.08)	36	0.21 (0.17)	3
	LCa2	0.80 (0.13)	12	0.05 (0.17)	1
	LCa3	0.80 (0.12)	12	0.10 (0.17)	1
	LCa4	0.68 (0.12)	10	0.04 (0.23)	0.5
	LCa5	1.27 (0.08)	20	0.06 (0.24)	1
	LCa6	1.18 (0.13)	18	0.15 (0.21)	2
Langhorne Creek (2), SA	LCb1	1.19 (0.13)	18	0.43 (0.10)	5
	LCb2	0.36 (0.18)	6	0.18 (0.15)	2
	LCb3	0.06 (0.11)	1	0.17 (0.10)	2

Table 3. continued on the next page

Table 3. continued

Viticultural region in Australia	Strain	Sensitivity to Triadimenol		Sensitivity to Fenarimol	
		Mean EC_{50}[a] (mg/L)	RF[b] Values	Mean EC_{50}[a] (mg/L)	RF[b] Values
	LCb4	0.12 (0.16)	2	0.01 (0.09)	0.1
	LCb5	0.07 (0.19)	1	0.05 (0.20)	1
	LCb6	2.21 (0.14)	34	0.17 (0.17)	2
McLaren Vale, SA	MVd1	0.88 (0.13)	14	0.05 (0.12)	1
	MVd2	1.79 (0.15)	28	0.05 (0.18)	1
	MVd3	1.41 (0.11)	22	NT	NT
	MVd4	1.41 (0.11)	22	0.1 (0.23)	1
	MVd5	0.93 (0.12)	14	NT	NT
	MVd6	3.81 (0.10)	59	0.1 (0.21)	1
	MVd7	0.75 (0.15)	12	NT	NT
	MVd8	1.09 (0.11)	17	NT	NT
	MVd9	1.06 (0.09)	16	NT	NT
Adelaide Hills (1), SA	AHd2[c,d]	0.04 (0.20)	1	NT	NT
Adelaide Hills (2), SA	AHf1	0.56 (0.17)	9	0.02 (0.15)	0.2
	AHf2	1.10 (0.21)	17	0.05 (0.11)	0.6
	AHf3	0.41 (0.17)	6	NT	NT
	AHf4	0.49 (0.14)	8	NT	NT
	AHf5	0.69 (0.13)	11	NT	NT
	AHf6	1.48 (0.14)	23	NT	NT
	AHf7	0.10 (0.15)	2	NT	NT
Adelaide Hills (3), SA	AHg1	0.67 (0.12)	10	NT	NT
	AHg2	0.75 (0.15)	12	NT	NT
	AHg3	1.08 (0.13)	17	NT	NT
	AHg4	0.43 (0.15)	7	NT	NT
	AHg5	1.07 (0.15)	16	NT	NT

	AHg7	0.48 (0.19)	7	0.12 (0.21)	1

Table 3. continued on the next page

Table 3. continued

Viticultural region in Australia	Strain	Sensitivity to Triadimenol		Sensitivity to Fenarimol	
		Mean EC_{50}[a] (mg/L)	RF[b] Values	Mean EC_{50}[a] (mg/L)	RF[b] Values
	AHg9	0.75 (0.15)	12	0.13 (0.27)	2
Mildura (1), Vic	VMa1	0.01 (0.28)	0.2	NT	NT
	VMa2	0.46 (0.22)	7	NT	NT
	VMa3	0.20 (0.13)	3	NT	NT
	VMa5	0.55 (0.24)	8	NT	NT
Mildura (2), Vic	VMb1	0.10 (0.10)	2	NT	NT
	VMb2	0.10 (0.20)	2	NT	NT
	VMb3	0.11 (0.20)	2	NT	NT
	VMb4	0.04 (0.29)	1	NT	NT
	VMb5	0.25 (0.25)	4	NT	NT
	VMb6	0.84 (0.24)	13	NT	NT

[a]Concentration inhibiting development of *U. necator* by 50%.

[b]RF calculated for strains exposed to DMIs as follows: $RF=(EC_{50selected}/\text{Mean } EC_{50unexposed})$.

[c]Strain not previously exposed to triadimenol.

[d]Single-spore strains established *in vitro* by K. Evans (1996).

[e]Single-spore strain established *in vitro* by B. Stummer.

(1), (2), (3) = separate vineyards within a viticultural region.

NT = not tested for sensitivity to fenarimol.

Table 4. Wilk-Shapiro statistic values of mean EC_{50} values for unexposed and selected populations of *U. necator* to triadimenol and fenarimol.

Fungicide	Unexposed	Selected
Triadimenol	0.9254[a]	0.8470[b]
Fenarimol	0.7104[b]	0.6789[b]

[a] Not significantly different at $P = 0.05$
[b] Significantly different at $P = 0.05$

Table 5. T-statistic and Mann-Whitney U-statistic for analysis of differences between unexposed and selected populations of *U. necator* to triadimenol and fenarimol.

Fungicide	T-statistic	Mann-Whitney U-statistic
Triadimenol	7.692[a]	6.586[a]
Fenarimol	1.446[b]	1.975[c]

[a] Significantly different at $P = 0.05$.
[b] Not significantly different at $P = 0.05$
[c] Not significantly different at $P = 0.02$

Table 6. Means, medians and range of EC_{50} values of sensitivity to triadimenol and fenarimol between unexposed and selected populations of *U. necator*. Standard deviations are represented in parentheses.

	Mean EC_{50} (mg/L)		Median EC_{50} (mg/L)		Range of EC_{50}'s (mg/L)	
Fungicide	**Unexposed**	**Selected**	**Unexposed**	**Selected**	**Unexposed**	**Selected**
Triadimenol	0.065 (0.02)	0.83 (0.7)	0.06	0.75	0.03-0.12	0.01-3.81
Fenarimol	0.081 (0.10)	0.191 (0.24)	0.05	0.10	0.01-0.33	0.01-0.98

Table 7. T-statistic and Mann-Whitney U-statistic for analysis of differences between triadimenol and fenarimol in the unexposed and selected populations of *U. necator*.

Population	T-statistic	Mann-Whitney U-statistic
Unexposed	-1.0[a]	1.02[a]
Selected	-4.5[a]	4.5[b]

[a] Not significantly different at $P = 0.05$
[b] Significantly different at $P = 0.05$

Table 8. Mean EC_{50} and RF values of *U. necator* progeny obtained by crossing triadimenol-sensitive strain APf1 and triadimenol-resistant strain LCb6. Standard error of the mean is represented in parenthesis.

Colony number[a]	Single-spore progeny or parental strain	Sensitivity to triadimenol	
		Mean EC_{50}[c] (mg/L)	RF[d] Values
N/A	APf1[b]	0.06 (0.18)	1
	LCb6[b]	2.21 (0.14)	34
1	A11a	1.74 (0.13)	27
	A11b	1.55 (0.18)	24
	A11c	0.36 (0.21)	6
2	A21a	0.66 (0.18)	10
	A21b	1.41 (0.15)	22
	A21c	0.53 (0.13)	8
3	A31a	0.32 (0.21)	5
	A31b	0.82 (0.19)	13
	A31c	0.86 (0.19)	13
4	A32	0.43 (0.21)	7
	A32a	0.52 (0.15)	8
5	A41b	1.72 (0.16)	26
	A41c	0.07 (0.24)	1
6	A42a	1.27 (0.26)	20
	A42b	0.30 (0.12)	5
	A42c	0.07 (0.25)	1
7	A43a	1.89 (0.15)	29
	A43b	1.24 (0.18)	19
	A43c	0.30 (0.12)	5
8	A51a	2.21 (0.16)	34
	A51b	0.82 (0.24)	13
9	A52a	1.86 (0.16)	29
	A52b	0.48 (0.16)	7
	A52c	0.89 (0.30)	14
10	A61a	0.63 (0.18)	10
	A61b	0.68 (0.26)	10
11	A62	1.06 (0.18)	16

[a]The discrete ascospore-derived colony from which single-spore strain were isolated.
[b]Triadimenol-sensitive (APf1) and triadimenol-resistant (LCb6) parents of the progeny listed.
[c]Concentration inhibiting development of *U. necator* by 50%.
[d]RF calculated for strains exposed to DMIs as follows: $RF=(EC_{50selected}/\text{Mean } EC_{50unexposed})$.
N/A = not applicable.