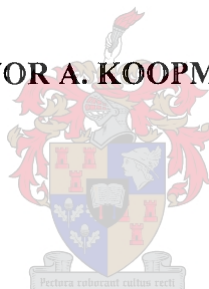


**GENETIC DIVERSITY IN *PLASMOPARA VITICOLA* IN SOUTH AFRICA**

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**Thesis presented in partial fulfillment of the requirements for the degree of Master  
of Science in Agriculture at the University of Stellenbosch**

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**March 2007**

## DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Date:.....06/03/07.....

## GENETIC DIVERSITY IN *PLASMOPARA VITICOLA* IN SOUTH AFRICA

### SUMMARY

Downy mildew, caused by the obligate pathogen *Plasmopara viticola*, is a very destructive grapevine disease. The asexual phase (sporangia) of the pathogen has long been viewed as the life cycle stage that is most important in causing expansion of epidemics. Contrarily, the role of the sexual phase (oospores) has primarily been viewed as only providing the initial primary inoculum of the epidemic at the start of the season. However, population genetic studies in Europe have challenged these long standing epidemiological views.

Downy mildew is mainly controlled through the application of fungicides, since no commercially acceptable resistant cultivars are available. The use of reliable high throughput *in vitro* resistance screening methods is very important for identifying new sources of resistance, as well as for mapping of quantitative trait loci (QTLs) involved in downy mildew resistance. Resistance screenings also require the use of effective long-term pathogen storage methods, since it allows the continual use of the same well characterised *P. viticola* isolates in different resistance screenings over seasons.

The first main aim of this study was to investigate the population genetic structure of *P. viticola* populations in South Africa in two vineyards. The second aim was to determine whether an *in vitro* leaf disk method is a reliable and reproducible resistance screening method for determining downy mildew resistance of grapevine seedlings. The third aim was to evaluate long-term storage techniques for *P. viticola* isolates.

The population genetic structure of *P. viticola* was investigated in two consecutive grape growing seasons in an organically managed and a conventional fungicide sprayed vineyard. The study showed that population differentiation between the two vineyards was low (0.004 and 0.016) in both growing seasons, suggesting one metapopulation. New genotypes (12% to 74%) contributed to the epidemic throughout the growing seasons in both years and vineyards. The epidemic in both years and vineyards were dominated by one or two genotypes, which contributed between 14% and

67% through asexual reproduction to the epidemic. The remaining genotypes showed low levels of asexual reproduction, with most genotypes never being able to reproduce asexually. Ten genotypes were able to survive asexually from one season to the next. Moreover, the predominant genotype in the organically grown vineyard during 2004/05 survived asexually to the next season, where it also dominated the epidemic.

Evaluation of an *in vitro* leaf disk method showed that the method was a reliable and reproducible method for screening the downy mildew resistance of the progeny of a Regent × Red Globe cross. Spearman correlation analyses revealed a moderate to high (0.64 to 0.82) correlation between three screening trails that were conducted over two growing seasons. However, the percentage seedlings that belonged to the different OIV 452 rating classes differed between the third (2005/06) and the first two (2004/05) resistance screening trials. This difference was statistically supported by one-way analysis of variance of rank means of these screenings, as well as Chi-square test of the screening × rating scale contingency table. This discrepancy indicates the importance of the inclusion of tolerant and sensitive reference seedlings, as well as the parents of the cross in each screening trial.

Evaluation of different long-term storage methods for *P. viticola* showed that the pathogen was best stored as lesions. Successful storage methods included the storage of whole leaves with sporulating lesions in sealed Petri dishes, or the storage of small leaf lesion fragments within 2 ml centrifuge tubes at -20 and -80°C. Viability testing of these storage methods after a period of 6 (leaves within Petri plates) and 17 months (lesions within centrifuge tubes) showed that the pathogen remained viable for these periods, although the viability of sporangia were reduced.



## GENETIESE DIVERSITEIT IN *PLASMOPARA VITICOLA* IN SUID-AFRIKA

### OPSOMMING

Donsskimmel, veroorsaak deur die verpligte patogeen *Plasmopara viticola*, is 'n vernietigende siekte op wingerd. Tot op hede is die ongeslagtelike fase (sporangiums) van die patogeen beskou as die belangrikste fase in die lewensiklus wat uitbreiding van epidemies veroorsaak. Hierteenoor is die rol van die geslagtelike fase (oöspore) hoofsaaklik gesien as die fase wat verantwoordelik is vir die verskaffing van die primêre inokulum van die epidemie, aan die begin van die seisoen. Populasie-genetika studies in Europa bevraagteken egter hierdie epidemiologiese sienswyses.

Donsskimmel word hoofsaaklik deur die toedien van fungisiedes beheer, aangesien geen kommersieel aanvaarbare weerstandbiedende kultivars beskikbaar is nie. Die gebruik van betroubare hoë deurset *in vitro* weerstandstoetse is baie belangrik vir die identifikasie van nuwe bronne van weerstand, sowel as vir die kartering van kwantitatiewe eienskap gene, betrokke by weerstand teen donsskimmel. Om weerstandstoetse volhoubaar uit te voer, word 'n effektiewe opbergingsmetode benodig om die patogeen oor lang periodes op te berg. Sodoende word verseker dat dieselfde *P. viticola* isolate in verskillende weerstandstoetse, oor verskillende seisoene, gebruik kan word.

Die eerste doelwit van hierdie studie was om die genetiese struktuur van *P. viticola* populasies in twee Suid-Afrikaanse wingerde te ondersoek. Die tweede doelwit was om te bepaal of 'n *in vitro* blaarskyf metode 'n akkurate en herhaalbare weerstandstoets vir die bepaling van donsskimmel weerstand van wingerdssaailinge is. Die derde doelwit was om langtermyn opbergingsmetodes vir *P. viticola* isolate te evalueer.

Die populasie genetiese struktuur van *P. viticola* is in twee opeenvolgende groeiseisoene in 'n organies-verboude en konvensioneel fungisied-behandelde wingerd ondersoek. Die studie het getoon dat populasie differensiasie tussen die twee wingerde in beide seisoene laag (0.004 en 0.016) was, wat een meta-populasie aandui. Nuwe

genotipes (12% tot 74%) het deur die seisoen tot die epidemie, in beide jare en wingerde, bygedra. In beide jare en wingerde, is die epidemie deur een of twee genotipes, wat tussen 14% en 67% deur ongeslagtelike voorplanting tot die epidemie bygedra het, gedomineer. Die oorblywende genotipes het lae vlakke van ongeslagtelike voorplanting getoon, met die meeste genotipes wat nooit in staat was om ongeslagtelik voor te plant nie. Tien genotipes was in staat om ongeslagtelik van een seisoen na die volgende te oorleef. Verder het die predominante genotipe gedurende 2004/05 in die organies-verboude wingerd ongeslagtelik na die volgende seisoen oorleef, waar dit ook die epidemie gedomineer het.

Evaluasie van die *in vitro* blaarskyf metode het getoon dat dit 'n betroubare en herhaalbare metode is vir die toets vir donsskimmelweerstand van saailinge van 'n Regent x Red Globe kruising. Spearman korrelasie analise het 'n gemiddeld tot hoë (0.64 tot 0.82) korrelasie tussen drie weerstandstoetse getoon, wat oor twee groeiseisoene uitgevoer is. Die persentasie saailinge wat tot die verskillende OIV 452 klassifikasieklasse behoort het, het egter tussen die derde (2005/06) en eerste twee (2004/05) weerstandstoetse verskil. Hierdie verskil is statisties deur eenrigting analise van variansie van rang gemiddeldes van hierdie weerstandstoetse ondersteun, asook deur die Chi-kwadraat toets van die weerstand x groepering skaal gebeurlikheidstabel. Hierdie teenstrydigheid dui op die belang van die insluiting van bestande en vatbare verwysing saailinge, asook die ouers van die kruising, in elke weerstandstoets.

Evaluasie van verskillende langtermyn opbergingsmetodes vir *P. viticola* het getoon dat die patogeen die beste as letsels gestoor word. Suksesvolle opbergingsmetodes sluit die berging van heel blare met sporulerende letsels in geseëde Petri bakkies in, of die opberg van klein blaarletsel deeltjies in 2 ml sentrifugebuisies by -20 en -80°C. Lewensvatbaarheidstoetse van hierdie opbergingsmetodes het getoon dat die patogeen na 6 (blare in Petri bakkies) en 17 maande (letsels in sentrifugebuisies) nog steeds lewensvatbaar was, hoewel die lewensvatbaarheid van sporangiums afgeneem het.

## ACKNOWLEDGEMENTS

I wish to express my sincere thanks to the following:

Thanks to my Heavenly Father for making all this possible and for things that no words can describe;

My study leaders, Dr. Adele Mcleod and Dr. Paul H. Fourie for guidance, advice incredible knowledge, unlimited time and patience throughout the duration of this study;

Dr. Celeste C. Linde for sharing her amazing knowledge;

Dr. Iwan F. Labuschagne for his support and encouragement;

Frikkie Calitz for statistical analysis;

The staff and fellow students at the Department of Plant Pathology, University of Stellenbosch, especially Andre Williams, Tamryn Mclean and Yared Tewaldemedhin for helping with field visits and sampling;

The National Research Foundation, Thrip, Deciduous Fruit Producers Trust and The Agriculture Research Council (ARC) for financial assistance;

Errol van Kerwel at Plant Biotechnology for taking care of greenhouse plants;

The Breeding and Evaluation Division at ARC Infruitec-Nietvoorbij, especially the stone and pome fruit people at Bien Donne, Simondium for their friendship and support;

Mauricell, thanks for your support and for always being there for me;

A very special thanks to my family, especially my mother for her unconditional love, constant encouragement and for always believing in me.

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# 1. EPIDEMIOLOGY AND CONTROL OF THE GRAPEVINE DOWNY MILDEW PATHOGEN *PLASMOPARA VITICOLA*

## INTRODUCTION

Downy mildew of grapevine is caused by *Plasmopara viticola* (Berk et Curt.) Berlese et de Toni, a heterothallic, diploid oomycete (Wong *et al.*, 2001). The pathogen is an obligate-biotroph which cannot be cultivated on artificial media, since hyphae develops only after contact with the host under favourable conditions (Barlass *et al.*, 1986; Riemann *et al.*, 2002). Downy mildew is of great economical importance, and is especially destructive in viticulture regions with warm, wet conditions during the growing season, including parts of Europe and North America (Lafon & Clerjeau, 1994). The disease can cause direct crop losses due to partial or total destruction of grape bunches, as well as indirect losses due to a reduction in photosynthetic foliage (Magarey *et al.*, 1994). In South Africa, downy mildew is a devastating disease especially during wet years in the Western Cape, and in the summer rainfall regions such as the Northern Cape, Eastern Cape and Kwazulu Natal regions (Marais, 1973).

Downy mildew symptoms appear mainly on leaves, although all green parts of the grapevine are susceptible. Foliar symptoms first appear on the upper side as small, pale yellow, circular oil spots. The spots are yellow in white grape varieties and red in some red grape varieties such as Ruby Red. After humid nights, a white downy felt of oomycete growth appears on the underside of the oil spots. Later and in hot weather, the centers of the oil spots dry out and become brown to red brown with a chlorotic outer ring (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994). Young shoots and bunches may also be attacked. Shoots turn brown and oily, where after fungal growth may appear on the infected parts. Infected shoots later become necrotic and die (Lafon & Clerjeau, 1994). Berries are susceptible until the pea berry stage and when infected, berries become brownish purple, shrink and fall off. Although berries become resistant after pea berry stage, bunch parts are still susceptible. The infected bunch parts turn brown and the berries attached to them

become shriveled and brown, dry out and may even fall off (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994).

Downy mildew is native to Northern America, from where it was accidentally introduced to Europe and the rest of the world. This introduction was indirectly caused in the late 1870s by the devastating root-feeding insect *Phyloxera* (*Daktulosphaira vitifoliae*), which threatened almost every vine in Europe and other grape growing countries. In 1878, growers were forced to pull up old vines and replace them with new *Vitis vinifera* vines, grafted onto phyloxera-resistant rootstocks from North America. These American rootstocks were contaminated with downy mildew, probably as oospores, which ultimately almost wiped out the entire European wine industry (Lafon & Clerjeau, 1994; Alexopoulos *et al.*, 1996). The industry was saved by the discovery of Bordeaux mixture (mixture of lime and copper) that brought the disease under control. This was also the first known fungicide, and the first known chemical used to control a plant disease (Alexopoulos *et al.*, 1996). Early in 1907, downy mildew was found for the first time in South Africa in Grahamstown in the Eastern Cape province (Dewar, 1907). The pathogen was only detected almost sixty years later, in 1968, in the largest grape growing region of South Africa, the Western Cape province (Marais, 1973).

Control of downy mildew epidemics is very important in order to prevent huge economical losses, especially due to bunch infections. The disease is mainly controlled through the application of correctly timed fungicide sprays, especially early in the season. Additionally, some viticulture practices may reduce, but will not control the severity of infection and facilitate disease control (Marais, 1973). Currently, the use of resistant cultivars is of limited value as a disease control strategy, since all *Vitis vinifera* varieties (Eurasian species) are considered susceptible to downy mildew and only differ in their level of susceptibility (Brown *et al.*, 1999b).



## EPIDEMIOLOGY OF *PLASMOPARA VITICOLA*

### Conventional view on the epidemiology of *P. viticola*

*Plasmopara viticola* can reproduce asexually through the production of sporangia that release zoospores, as well as sexually through the production of thick walled resistant oospores (Lafon & Clerjeau, 1994). Since the pathogen is heterothallic, both mating types (A1 and A2) are required for sexual reproduction to take place (Wong *et al.*, 2001). Historically, the life cycle of the pathogen has been viewed as consisting of one sexual cycle in autumn and many asexual cycles during the grapevine growing season (Magarey *et al.*, 1994). The asexual phase of *P. viticola* has long been viewed as the life cycle stage that is most important in causing expansion of epidemics. Furthermore, the migration of asexual spores is also commonly believed to be the way in which the pathogen spreads spatially, often over long distances in short time periods. Contrarily, the role of the sexual phase has been primarily viewed as providing the initial primary inoculum of the epidemic only (Lafon & Clerjeau, 1994).

Sexually reproduced oospores of the pathogen can overwinter in diseased leaves and other infected residues (bunches or shoots) on the ground for three to five years in a dormant phase (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994; Vercesi *et al.*, 1999). Alternatively, it has been hypothesised that in regions with mild winters, pathogen mycelium may be able to hibernate in grapevine buds or remaining leaves (Marais, 1973). However, inoculum for new infections during spring is thought to be exclusively provided by germinating oospores, which require a specific set of environmental parameters to germinate, called the 'three ten rule': at least 10 mm rain should fall and the temperature should be at least 10°C for a period of 24 hours (10:10:24), while grapevine shoots must be at least 10 cm long and the tissue must be covered with a water film to ensure a high probability for successful infection (Lafon & Clerjeau, 1994; Serra *et al.*, 1998; Gobbin *et al.*, 2003a). Germinated oospores produce zoospore releasing sporangia that are splashed by water and rain or carried by wind to the leaves and other susceptible host tissue, initiating the first infections referred to as primary infections (Lafon & Clerjeau, 1994).

Sporangia are formed at least 3 days after such a rainfall event (10:10:24), although it may take longer (Gilles, 2004).

Once a sporangium is produced by an oospore, certain specific conditions are required before host infection can take place. Sporangia are usually inactivated after several hours in sunlight, so that most infections would generally occur in the morning (Lafon & Clerjeau, 1994). Infection can only occur when the susceptible plant parts remain wet for 2 to 3 hours, since the pathogen needs free water for infection. (Magarey *et al.*, 1994). Furthermore, *P. viticola* can only infect the plant through active stomata and therefore only sections of plants with functional stomata are susceptible (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994). Plants are infected when biflagellate zoospores, released from sporangia, swim to the vicinity of the stomata where they encyst. These cysts germinate and form a germ tube, which penetrates the stomata and starts colonising the host by producing somatic hyphae (Lafon & Clerjeau, 1994).

Colonisation of host tissue by somatic hyphae results in the formation of oil spots or lesions, 5 to 15 days after infection depending on the weather conditions (20-25°C) and the age of the tissue. Primary infection levels are usually very low and only a few leaves in a vineyard row will have oil spot symptoms (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994; Riemann *et al.*, 2002). Following warm, wet or very humid nights, sporulation will occur. Sporangiohores with asexually produced sporangia, emerge through stomata of infected leaves and other grapevine tissue (e.g. berries) (Lafon & Clerjeau, 1994). The sporangia, which will cause the secondary infections, are distributed by rain and/or wind, and again the pathogen requires free water (2 to 3 hours) for germination and penetration of the stomata (Orlandini *et al.*, 1993; Magarey *et al.*, 1994). The sporangia's potential to form zoospores decrease with age especially during unfavourable conditions (Magarey *et al.*, 1994; Brown *et al.*, 1999a; Kast *et al.*, 1999). This is not the case with the somatic hyphae inside the plant tissue, since old oil spots may again produce sporangia. During favourable conditions (repeated rainfall or heavy dew in humid areas), the disease can spread extremely rapidly through many of these secondary cycles. Late in the growing season, sexual reproduction takes place when two compatible hyphae meet and form an oogonium and antheridium. Subsequently, fertilisation will take place resulting in the formation of an



oospore, which is the surviving structure in the winter (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994).

### **New views on the epidemiology of *P. viticola* as revealed through population genetic studies**

Recently, population genetic studies in Europe have challenged some conventional epidemiological views on *P. viticola* (Staurk-Urnau *et al.*, 2000; Gobbin *et al.*, 2003a, b, 2005, 2006a; Rumbou & Gessler, 2004, 2006). The most important epidemiological views that have been questioned is whether asexual spores are most important for expansion of epidemics, whether oospores only contribute to the epidemic at the start of the season, and lastly whether asexual spores can migrate over long distances (Gobbin *et al.*, 2005, 2006b).

The first population genetic study on *P. viticola* was conducted by Staurk-Urnau *et al.* (2000), who used randomly amplified polymorphic DNA (RAPD) markers for determining the genetic diversity of primary and secondary inoculum. These authors were the first to show that *P. viticola* populations have a high genetic variability at the beginning of the season, and that only a few genotypes dominate the epidemic.

The first population genetic study using microsatellite markers specific for downy mildew was conducted by Gobbin *et al.* (2003a). The clone library constructed for development of the microsatellite markers initially yielded 61 putative microsatellite loci, of which five (BER, ISA, REX, GOB and CES) were suitable for *P. viticola* population studies. Two of the microsatellite marker loci contained a (TC)<sub>n</sub> repeat (loci BER and ISA), two had a (TC)<sub>n</sub>(AC)<sub>n</sub> repeat (loci CES and REX) and one had a (CT)<sub>n</sub>(CTAT)<sub>n</sub> repeat (locus GOB). The amplicon sizes of all the loci were between 100 and 200 base pairs (bp), with the exception of GOB, which yielded amplicon sizes between 300 and 400 bp. These markers were used to determine the genetic structure of downy mildew populations in Europe (Gobbin *et al.*, 2003a, b, 2005; Rumbou & Gessler, 2004, 2006).

Population genetic studies in Europe using the aforementioned markers have found that oospore infections have a larger impact on the epidemiology of the disease than previously envisaged (Gobbin *et al.*, 2003b; Rumbou & Gessler, 2004). Oospore infections

were observed throughout the growing season for at least two months, resulting in a qualitative as well as quantitative effect on the epidemics. Oospores contributed between 40 to 80% of genotypes found throughout the year (Rumbou & Gessler, 2004; Gobbin *et al.*, 2005, 2006a; Gessler *et al.*, 2006). Therefore, it has been concluded that the contribution of oosporic infections to an epidemic has gone largely unrecognised prior to population genetic studies (Gobbin *et al.*, 2003b; Rumbou & Gessler, 2004). Recently, the importance of oosporic infections was also supported by the study of Kennely *et al.* (2005) in North America, who showed that if downy mildew free grapevine seedlings are exposed to naturally infested oospore vineyard soil in a location isolated from any natural downy mildew inoculum, infections of seedlings will take place throughout the season due to the season-long germination of oospores.

Historical views on the contribution and behaviour of asexual spores to epidemics have also been challenged by population genetic studies. These studies have shown that in each year the expanding force of the disease was due to the exceptional ability of one to three early genotypes to infect, multiply and dominate the population, causing 20 to 90% of the total lesions during the year. The rest of the genotypes only produced one to three lesions each, where after they disappear (Gobbin *et al.*, 2003b, 2005, 2006a, b; Rumbou & Gessler, 2004). Approximately three-quarters of the genotypes never gave rise to asexual progeny (Gobbin *et al.*, 2005). The reason for their failure to cause further infections might in part be due to the fact that sporangia are very sensitive to environmental conditions such as long-wavelength UV light (solar irradiation  $> 600 \text{ W/m}^2$ ) and high temperature (Rotem *et al.*, 1985; Mizubuti *et al.*, 2000). Population genetic studies also proved for the first time that *P. viticola* can survive between seasons as asexual spores or vegetative mycelium in warmer climates such as the Greek islands (Rumbou & Gessler, 2006).

Another epidemiological aspect that population genetic studies have questioned is whether long-distance dispersal of asexual spores occurs. Studies on the dispersal pattern of the predominant secondary genotypes revealed that they all had different dispersion patterns, which may be the result of the interaction of climatic conditions, cultivation systems, host variety and strain specific ability. The dispersal studies also showed that genotypes colonised plot areas at a relatively slow rate and that there is no long-range

secondary sporangial migration, suggesting that long-range secondary sporangial migration has been overestimated in the past (Gobbin *et al.*, 2005). Gobbin *et al.* (2006a) concluded that the migration distances of secondary sporangia of *P. viticola* are short (shorter than 20 m), and that only one or two dominant genotypes succeed to undergo asexual cycles and produce a high number of clones. Furthermore, it has been shown that barriers such as irregular topography, geographical barriers (mountains, sea), and the Mediterranean climate (long periods of heat and low humidity) limit the migration of some *P. viticola* populations in Europe (Rumbou & Gessler, 2006).

*Plasmopara viticola* populations in Australia and North America have also been investigated using the markers of Gobbin *et al.* (2003a), but only on a limited scale. In Australia, only five vineyards were analysed in one season (Hug *et al.*, 2006), whereas one vineyard was studied in two consecutive years in North America (Kenelly *et al.*, 2006). In these studies, it was also found that oospores contributed throughout the season to development of the epidemic, and that one genotype dominated the epidemic, similar to the situation in Europe (Gobbin *et al.*, 2003b, 2005; Rumbou & Gessler, 2004).

Recently, Delmotte *et al.* (2006a, b) developed seven additional microsatellite markers for *P. viticola*. The seven markers were tested on 1058 *P. viticola* lesions, from which 1008 lesions were from Europe and 50 lesions from American vineyards. The markers showed low level of polymorphisms that ranged from two to six alleles per locus.

Scherer & Gisi (2006) used amplified fragment length polymorphism (AFLP) markers in combination with the microsatellite markers of Gobbin *et al.* (2003a) to characterise 54 *P. viticola* isolates collected from seven regions in four European countries. Their study showed that in contrast to the microsatellite markers, the AFLP markers were able to show clustering of isolates collected from the same region in some instances (Scherer & Gisi, 2006).

## CONTROL OF *PLASMOPARA VITICOLA*

Downy mildew is a very destructive disease, requiring an integrated control strategy in order to prevent economical losses. The timely application of fungicides is the main method of disease control. In addition to chemical control, the correct cultural practices should also be used as part of an integrated disease management strategy (Marais, 1973). Cultural practices that should be adhered to include proper management of the vineyard location, row direction, canopy management, weed control, avoidance of vigorous growth, irrigation and fertilisation. Vineyards should be planted in well-drained soils with direct sunlight, and rows should be planted in the direction of the prevailing wind, which will facilitate good circulation and faster drying of the plant parts and fruit. Drip- or micro-irrigation must be used instead of overhead irrigation to keep the susceptible plant parts dry. These measures must be adhered to in order to avoid excess water and high humidity in the vineyard, which stimulate pathogen development (Marais, 1973; Lafon & Clerjeau, 1994). Host resistance can also be a very important control measure. However, downy mildew resistant cultivars with acceptable agronomical characteristics are currently unavailable (Lafon & Clerjeau, 1994). Therefore, active research fields currently include breeding and genetic engineering of cultivars with acceptable resistance levels, as well as studies on the elucidation of mechanisms contributing to resistance.

### **Chemical control**

Downy mildew is mainly controlled through the application of fungicides. Successful disease control depends on controlling primary infections, since the pathogen spreads rapidly during the explosive secondary cycle, which is also very difficult to control. The critical period for control is early in the season, especially from the time when the first shoots reach 10 cm in length until the pea berry stage. Infection during this stage may cause total crop loss (Magarey *et al.*, 1994).

Two strategies, a post-infection strategy and a preventive strategy may be used for fungicide applications (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994). The post-infection

strategy is when no chemical control is used until an infection period occurred. A systemic chemical product must be sprayed immediately after the infection period, before oil spots occur. Once oil spots are present, a systemic product must be sprayed after a warm humid night so that sporulation can be reduced and plant tissue can be protected against secondary infections. This strategy is only recommended for regions where unfavourable conditions for disease development occur for most of the season (Magarey *et al.*, 1994). The preventive strategy is conservative and less risky. This strategy requires all green parts of the vine to be protected with a registered contact fungicide. However, when wet conditions are forecast or experienced, vineyards should be sprayed with a systemic product. Vineyards must be sprayed at 14-day-intervals (wet conditions) and weekly during the critical period around flowering. Later in the season when plant growth slowed down, intervals may be extended to 3 weeks. Disease control is not only dependent on the application of correctly timed fungicide sprays, but also on obtaining good coverage of all the green parts of the plant with fungicide, especially when contact fungicides are sprayed (Magarey *et al.*, 1994).

Different groups of systemic- and contact fungicides are used in controlling downy mildew on grapes. Several groups of systemic fungicide groups are available, including valinamides, cinnamic acid amides (Reuveni, 2003; Gisi *et al.*, 2006), phenylamides, methoxyacrelates, acrylalines, cinnamic acid derivatives, imidazolinones, alkyl phosphanates, strobilurins and inorganic compounds. Contact fungicides include organic fungicides, N-trihalomethylthio's, copper-based groups and dithiocarbamates (Nel *et al.*, 2003).

Unfortunately, *P. viticola* has developed resistance towards several fungicides due to the intensive, continuous and exclusive use of these chemicals. Resistance of *P. viticola* has been reported for the phenylamides (Morton & Urech, 1988; Lafon *et al.*, 1994; Fourie, 2004), 1-3-ethylerea (Gullino *et al.*, 1997; Genet & Vincent, 1999), carboxylic acid amides and Quinone Outside Inhibitor (or strobilurin) fungicides (Wong & Wilcox, 2000; Collina *et al.*, 2005; Sierotzki *et al.*, 2005; Gisi *et al.*, 2006) during the past few years. Resistance to some of these fungicides developed shortly after their introduction into the market, for



example resistance towards metalaxyl (phenylamides) developed only 2 years after its introduction (Morton & Urech, 1998).

### **Disease forecasting models**

Disease control of downy mildew generally requires the use of fungicides several times during the growing season. Some of these applications are often unnecessary since they are applied when conditions are unfavourable for infection. Therefore, disease forecasting models, which forecast the main phases of the pathogen's life cycle and when infections are likely to take place, have the potential to be very useful in determining when grapevines are susceptible to downy mildew and when spray applications are necessary. The main aim of the forecasting models is to provide a decision-support system for effective and sustainable disease control, therewith reducing fungicide applications by better timing and omitting sprays when weather conditions are unfavourable for downy mildew development (Orlandini *et al.*, 1993; Madden & Ellis, 2000; Gilles, 2004).

A wide range of forecasting systems has been proposed for grapevine downy mildew. Most of these models make use of long- or short term weather data, to predict the favourable conditions for the different stages during the life cycle of the pathogen. These models make use of temperature, relative humidity, rainfall and leaf wetness to predict the risk of disease development (Lalancette *et al.*, 1988a, b; Madden & Ellis, 2000; Gilles, 2004). Additionally, some forecasting models such as the New-leaf appearance model, also calculate and take into account the rate at which new leaves develop during the growing season, in order to predict when fungicides should be applied (Gilles, 2004). Models like the EPI model (Potential state of infection model) and the Simpo-model, predict the primary infections by predicting oospore maturation and germination, whereas other models only predict the secondary infections and the survival of these structures (Gilles, 2004). Lalancette *et al.* (1988a) developed a model to describe the effect of light and relative humidity on sporulation of downy mildew on grapevine leaves.

The development of downy mildew forecasting models is often specific for a certain region and a certain host genotype. The use of these models could result in inaccurate predictions of downy mildew on other host genotypes. For example the Plasm model, which was developed and tested for sporulation and infection on a less susceptible American grapevine (Lalancette *et al.*, 1988a, b), will most likely not be accurate for predicting epidemics on more susceptible varieties. The use of forecasting models is furthermore complicated by the finding that the probability of error for predicting the disease increases when a combination of models are used. Therefore, it is more desirable to use one forecasting model that was developed for a specific region and host genotype (Gilles, 2004).

In South Africa, a forecasting model (Metos downy mildew early warning model) for downy mildew was developed during the 1995/06 grape season, and has subsequently been used by producers for predicting epidemics. The model makes use of hourly weather data to send out warnings as soon as environmental conditions are favourable for infections (Fourie & Vermeulen, 2000). Since certain shortcomings were identified within this forecasting model, some modifications were made to the model, which resulted in the development of a new downy mildew early warning model (DMEW). This model is more accurate and reliable, as well as being more user friendly. The DMEW model was found to be more effective than the previous model, causing 8% less disease with only one extra application of fungicides. However, there are still problems that need to be addressed within this model. Some of the main problems include the use of point weather information, and making use of historic and contemporary weather data to signal warnings of downy mildew infections. The model only provides current weather data, and cannot predict future weather conditions, resulting in a limited amount of time that is available for quick and effective control (Haasbroek & Vermeulen, 2004).

### **Host resistance**

Grape species and cultivars show various degrees of resistance to downy mildew. The *Vitis vinifera* species is more susceptible (S-species) to *P. viticola* than the American

(*V. cineria*, *V. riparia*, *V. rubra*, *V. candicans*) and Asian *Vitis* species. The American and Asian species further show different levels of resistance, ranging from moderately resistant (M-species) (*V. rupestris*, *V. amurensis*) to highly resistant (R-species) (*V. cineria*, *V. riparia*, *V. rubra*, *V. candicans*) (Dai *et al.*, 1995b; Brown *et al.*, 1999a, b; Merdinoglu *et al.*, 2003). The Muscadine grape (*Muscadinia rotundifolia*), also an American genus is totally resistant against the downy mildew fungus (Merdinoglu *et al.*, 2003). *Vitis vinifera* varieties differ in susceptibility, with varieties such as Chardonnay and Sultana being more susceptible than Cabernet Sauvignon and Semillon (Barlass *et al.*, 1986; Lafon & Clerjeau, 1994). Interspecific hybrids of *V. vinifera* and the North American species have yielded cultivars with both good wine-grape qualities as well as showing greater resistance to downy mildew (Lafon & Clerjeau, 1994).

**Host responses in resistant and susceptible *Vitis* species.** Resistance to downy mildew can be controlled by several genes as is found in Asian *Vitis* species, or it can be controlled by a single dominant gene such as is found in *V. rotundifolia* (Espino & Nesbitt, 1982). Single gene resistance is controlled at the stomatal level, whereas multiple gene resistance is controlled at the inter- and intracellular developmental level (Brown, *et al.*, 1999b). Interestingly, Kozma & Dula (2003) found that the Run 1 gene is responsible for the total resistance against powdery mildew and downy mildew resistance.

Grapevines make use of many host response mechanisms such as the hypersensitive response, structural barriers and phytoalexins that all contribute to their resistance phenotype. Trans-resveratrol is one of the phytoalexins in grapevines that is thought to be important in resistance to downy mildew, most likely due to its toxicity towards *P. viticola* sporangia (Dai *et al.*, 1995b; Maunch-Mani, 2002). Flavonoids are also thought to play an important role in resistance, as well as the lignification of cells (Dai *et al.*, 1995a, b; Maunch-Mani, 2002).

The most prevalent difference in the defense reactions to infections by *P. viticola* in highly resistant (R-species) and moderately resistant (M-species) grape species, is the speed at which the response reaction is launched. In R-species, a blue autofluorescence



(trans-resveratrol) can be detected only one day after infection, whereas in M-species it is only detected after two days and none can be detected in the S-species (Dai *et al.*, 1995a, b). It has also been shown that although the M-species produce other chemicals and mechanical barriers following infection, such as resveratrol, flavonoids and lignin, all these reactions occurred later in the M-species than in the R-species, where a rapid accumulation of flavonoids can be seen in the stomatal tissue and in the cells (Dai *et al.*, 1995b). In the M-species, the flavonoids accumulate at a later stage around the necrotic area, and although not as effective as in R-species, they still play an important role in limiting the spread of the disease (Dai *et al.*, 1994, 1995a, b). Hypersensitivity and necroses of guard cells occur after infection of R-species, causing limited growth or the death of the pathogen at an early stage (Dai *et al.*, 1995b).

**Methods used for evaluating host resistance.** The evaluation of different species and cultivars for resistance against downy mildew requires an effective and accurate screening method. A number of different techniques have been developed for this purpose, such as field (Brown *et al.*, 1999a, b), greenhouse and laboratory-based methods (Barlass *et al.*, 1986; Staudt & Kassemeyer, 1995; Song *et al.*, 1998; Liu *et al.*, 2003).

The evaluation of host resistance against downy mildew can broadly be divided into field evaluations and laboratory evaluations. Field evaluations involve the rating of susceptibility against natural field inoculum under field conditions (Brown *et al.*, 1999a, b). In general, only a limited number of plants can be screened in this manner, and it is also a time consuming process. On the other hand, laboratory-based techniques are capable of screening a larger number of plants in a relative short period of time. Such techniques include the use of *in vitro* plants (Barlass *et al.*, 1986), single leaf-node cuttings (Liu *et al.*, 2003), leaf discs (Staudt & Kassemeyer, 1995) and detached leaves (Song *et al.*, 1998) that are inoculated with *P. viticola*. In all of these techniques, a specific downy mildew concentration will be applied to leaves, or leaf discs, from the 5<sup>th</sup> to 6<sup>th</sup> internode either by spraying or droplet inoculation (Wiedemann-Mardinoglu *et al.*, 2003).

A few studies have been specifically aimed at investigating the correlation between field and laboratory evaluations. Brown *et al.* (1999a) found that the leaf disc and

greenhouse procedures (inoculation of young leaves on plants) gave the best predictors of downy mildew field resistance. Fischer *et al.* (2004) found that the use of either field resistance evaluations or the *in vitro* leaf disk method, was sufficient for mapping quantitative trait loci (QTLs) for resistance against downy mildew.

## METHODS USED FOR PROPAGATION AND STORAGE OF *PLASMOPARA VITICOLA*

*Plasmopara viticola* is an obligate parasite and cannot be grown on synthetic media in pure culture. It requires the presence of living host tissue and the provision of conditions favourable for infections and sporulation (Barlass *et al.*, 1986; Riemann *et al.*, 2002). Morel (1944) was the first to maintain downy mildew in the laboratory. He used the concept of dual culture where grapevine callus was inoculated with *P. viticola* spores *in vitro*, and maintained by sub-culturing the callus and re-inoculating it with the “cultured” pathogen spores. The method has been modified, using *in vitro* plantlets regenerated from the shoot apex of cultures. *In vitro* plantlets must attain a height of 50 mm and a minimum of three leaves before it can be inoculated with downy mildew (Barlass *et al.*, 1986). Downy mildew spores can also be propagated in Petri dishes on detached leaves of susceptible cultivars. The detached leaves are placed with the adaxial (upper) surface on moist filter paper, and are inoculated with 20 µl droplets of a sporangial suspension. The spore droplets have to be kept from drying out for at least 4 hours, before the leaves can be dried and incubated further (Staudt & Kassemeyer, 1995; Kast & Stark-Urnau, 1999).

The long-term storage of obligate oomycete pathogens such as *P. viticola* is difficult. Only a few reports have been published for the long-term storage of *P. viticola*, whereas more methods have been published for the storage of other obligate oomycetes such as *Peronospora* species. Dahmen *et al.* (1983) reported the storage of *P. viticola* sporangia for up to nine years in liquid nitrogen by using cryoprotectants such as skim milk-glycerol and 15% dimethyl sulfoxide (DMSO). Kortekamp *et al.* (1997) stored *P. viticola* sporangia for an unspecified period of time at -25°C after drying under vacuum at room temperature. Gulya *et al.* (1993) was also able to store air-dried sporangia of

*Plasmopara halstedii* that was fast-frozen, for 4.75 years in liquid nitrogen. Bromfield & Schmitt (1967) first reported the successful storage of blue mould on tobacco, caused by *Peronospora tabacina*, by freezing suspended conidia in 15% DMSO for up to 25 months in liquid nitrogen. Cohen & Kuc (1980) stored leaves with sporulating lesions of *Peronospora tabacina* successfully at  $-20^{\circ}\text{C}$  for 3 months. *Peronospora vicaciae*, causal agent of downy mildew on peas, was stored successfully as lesions for 3 to 6 months at  $-80^{\circ}\text{C}$  (Gill & Davidson, 2005).

## CONCLUSION

Downy mildew caused by *Plasmopara viticola*, remains one of the most destructive diseases of grapevines when conditions are favourable for disease development. The disease causes great economical losses due to direct losses incurred with the partial or total destruction of grape bunches, as well as indirect losses caused by lesions that reduce the available photosynthetic foliage. Currently, the disease is mainly controlled through the application of fungicides, which is expensive as well as harmful to the environment. Although resistant cultivars hold great promise for disease control, there are no commercially acceptable cultivars which are resistant to downy mildew.

The epidemiology of *P. viticola* has long been viewed as consisting of a primary oosporic infection at the start of the season that initiates the disease, with secondary asexual cycles driving the epidemic throughout the season. However, population genetic studies in Europe have found that oosporic infections contribute significantly to epidemic development throughout the season, and that only a few genotypes in each epidemic are able to reproduce asexually (Staurk-Urnau *et al.*, 2000; Gobbin *et al.*, 2003a, b, 2005, 2006a; Rumbou & Gessler, 2004, 2006).

The contribution of sexual and asexual spores to the development of downy mildew epidemics in South Africa is unknown. It is hypothesised that the epidemiology of *P. viticola* in South Africa with its milder winters and warmer summers could differ from that of European epidemics. Therefore, the first aim of this study was to investigate the

population genetic structure of *P. viticola* populations in two vineyards over two consecutive seasons, using microsatellite markers.

The evaluation of grapevine cultivars and lines for resistance against downy mildew requires the use of high throughput *in vitro* screening methods. Furthermore, resistance screening tests require long-term storage methods of the pathogen, which will allow new grapevine breeding accessions to be screened on an ongoing basis. Therefore, the second aim of this study was to determine if an *in vitro* leaf disk method was a reproducible technique over seasons for screening grapevine seedlings derived from a cross between Regent and Red globe for resistance to downy mildew. The third aim was to evaluate several long-term storage methods of *P. viticola*.

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## 2. POPULATION GENETIC STRUCTURE OF *PLASMOPARA VITICOLA* IN SOUTH AFRICA

### ABSTRACT

*Plasmopara viticola* populations in South Africa were studied for two consecutive grape growing seasons, in an organically managed and a conventional fungicide sprayed vineyard, which were separated by a distance of seven kilometres. The populations were genotyped with four published microsatellite markers (GOB, CES, ISA and BER), during three to four samplings in each season. Population differentiation between the conventional fungicide sprayed vineyard and organically managed vineyard was low (0.004 and 0.016) in both growing seasons, suggesting one metapopulation. However, differences in the relative contribution of the predominant and new genotypes to epidemics in the two vineyards suggested that fungicide applications may have selected for reduced pathogen diversity. In both years and vineyards, sexual (oosporic) reproduction or migration occurred throughout the year and contributed between 12% and 74% to the epidemic. In both years and vineyards, epidemics were dominated by one or two genotypes that each contributed between 14% and 67% to the epidemic through asexual reproduction. The remaining genotypes showed low levels of asexual reproduction, with most genotypes never being able to reproduce asexually. However, for some genotypes asexual reproduction was important, since it enabled survival of the genotype from one season to the next. In total, 10 genotypes were able to survive asexually from one season to the next. Moreover, the predominant genotype in the organically grown vineyard in the 2004/05 season survived asexually to the next season where it also dominated the epidemic, although its frequency declined at the end of the 2005/06 season. Clonality of the isolates that survived between seasons was confirmed by genotyping a subset of these isolates with newly published microsatellite markers (Pv7, P13, Pv14, Pv17 and PV31).

## INTRODUCTION

Downy mildew, one of the most destructive diseases of grapevines is caused by the diploid, obligate-biotrophic oomycete *Plasmopara viticola* (Berk et Curt.) Berlese et de Toni (Lafon & Clerjeau, 1994; Wong *et al.*, 2001). Oomycetes are an unique group of eukaryotes, which belong to the kingdom Chromista (Cavalier-Smith, 1981, 2000). Similar to most other oomycetes, *P. viticola* can reproduce asexually through the production of sporangia that release zoospores, as well as sexually through thick walled resistant oospores (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994). Sexual reproduction can only take place if both mating types (A1 and A2) are present, since *P. viticola* is heterothallic (Wong *et al.*, 2001).

*Plasmopara viticola* is native to Northern America, from where it was accidentally introduced to Europe and the rest of the world. This introduction was indirectly caused in the late 1870s by the devastating root-eating insect phyloxera (*Daktulosphaira vitifoliae*), which caused the replacement of old vines with new vines grafted onto phyloxera-resistant rootstocks from North America that contained downy mildew inoculum (Lafon & Clerjeau, 1994; Alexopoulos *et al.*, 1996). The introduction of *P. viticola* into Europe, most likely consisted of more than one introduction event from North America (Gobbin *et al.*, 2006a). In South Africa, downy mildew was reported for the first time in 1907 in Grahamstown in the Eastern Cape Province (Dewar, 1907). The pathogen was only reported for the first time sixty years later, in 1968, in the largest grape growing region of South Africa, the Western Cape Province (Marais, 1973).

The asexual phase (sporangia) of *P. viticola* has long been viewed as the life cycle stage that is most important in causing expansion of epidemics. The pathogen has many asexual cycles during the season under favourable environmental conditions. These cycles result in mass production of inoculum over short time spans, as well as long distance dispersal of sporangia. Contrarily, the role of the sexual phase (oospores) has been primarily viewed as only providing the initial primary inoculum of the epidemic at the start of the season (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994). However, *P. viticola* population genetic studies in Europe, using mainly microsatellite markers, but also randomly amplified polymorphic DNA (RAPD) markers, have recently challenged

these long standing epidemiological views (Gobbin *et al.*, 2003a, 2006a; Stark-Urnau *et al.*, 2000). These studies have shown that oosporic infections do not only contribute to primary infections, but that they can also contribute significantly (30% - 40%) to the development of epidemics throughout the growing season (Gobbin *et al.*, 2003b, 2006a, b; Rumbou & Gessler, 2004; Gessler *et al.*, 2006). Furthermore, it has been shown that only a few genotypes will significantly contribute through asexual reproduction to expansion of the epidemic, with most genotypes not producing a significant number of asexual progeny. Currently, it is hypothesised that long-distance migration of asexual spores are limited (Gobbin *et al.*, 2003a, b, c, 2005, 2006a, b; Rumbou & Gessler, 2004, 2006; Gessler *et al.*, 2006).

Microsatellite markers for genotyping *P. viticola* isolates have been developed independently by two research groups. The first set of markers was developed by Gobbin *et al.* (2003a), and consists of five markers containing a range of two to 101 alleles per locus. These markers have been used to study several European populations (Gobbin *et al.*, 2003a, b, c, 2005; Rumbou & Gessler, 2004, 2006), one North American population (Eugster *et al.*, 2003; Kennelly *et al.*, 2005, 2006) as well as five Australian populations (Hug *et al.*, 2006). Recently, Delmotte *et al.* (2006a) developed a second set of seven microsatellite markers that contains relatively low levels of polymorphisms, ranging from one to six alleles per locus. These markers have only been used to characterise *P. viticola* populations on a more limited scale in Europe (1008 lesions) and North American (50 lesions) (Delmotte *et al.*, 2006a, b).

In South Africa, the severity of downy mildew epidemics varies between years, due to substantial variation in climatic conditions (Marais, 1973). In most years, epidemics can be readily controlled due to low precipitation levels and high temperatures. However, in years with high rainfall and lower temperatures explosive epidemics occur that are difficult to control. The relative contribution of sexual and asexual reproduction to the development of epidemics in South Africa is currently unknown. This is an important epidemiological aspect of the disease that will influence management strategies. The epidemiology of the disease in South Africa is hypothesised to be different from that in Europe, since aside from unique climatic conditions in South Africa, the genetic background of South African populations may be different from

European populations. The origin of the founder population of current South African *P. viticola* populations is unknown.

The aims of this study were to (1) determine the relative contribution of sexual and asexual reproduction to the development of *P. viticola* epidemics in two South African vineyards in two consecutive growing seasons, (2) determine whether *P. viticola* genotypes can survive asexually or vegetatively from one season to the next and (3) to determine whether vineyard management, organic versus conventional fungicide treatment, will influence the *P. viticola* population structure. The study was conducted in one organically managed and one conventional fungicide managed vineyard in two consecutive growth seasons.

## MATERIALS AND METHODS

### Vineyard sites

Downy mildew lesions were collected in two 10 to 12 years old Red Globe vineyards. The one vineyard was an organically managed vineyard where only copper and compost teas were sprayed, whereas the other vineyard was a conventional fungicide managed vineyard that was sprayed with synthetic downy mildew fungicides (fosetyl-Al + mancozeb, iprovalicarb + propineb and potassium phosphate). The vineyards were situated in the Northern Paarl area (Western Cape province) approximately 7 km apart. The experimental blocks in the organically managed vineyard consisted of 12 rows each containing 30 vines. In the conventional fungicide managed vineyard there were 8 rows each containing 20 vines. Vine spacing was 2 m within rows and 3 m between rows in both vineyards. Weather data, which included hourly temperature, rainfall, leaf wetness and relative humidity, were recorded with a weather station in the organic grown vineyard. The Plant Plus (Dacom Plant Service, Netherlands) downy mildew forecasting software was used to estimate infection periods of the disease.

### Sampling

Sampling dates were based on predictions made by the Plant Plus downy mildew forecasting software. Downy mildew lesions (known as oil spots) were collected three



times during each of the 2004/05 and 2005/06 seasons, with the exception of the organically managed vineyard where there were four sampling times in 2005/06. Single lesions were sampled by removing a small section of each lesion with a sterilised scissor. The remainder of the lesion was left, allowing the genotype to keep on contributing to the epidemic. The sampled lesions were labelled according to the row, plant and leaf number in order to identify their exact location in the vineyard. The number of lesions collected at each sampling time was determined by the severity of infections, resulting in either a total (T) or partial (P) sampling strategy. A total sampling strategy was followed when the disease incidence was low and all lesions could be collected, whereas a partial sampling strategy was followed at high disease incidences, with only two lesions being sampled from every second vine.

### **DNA isolation**

DNA extraction was done using the Qiagen DNeasy kit (Qiagen Inc., Valencia, CA, USA). Each sampled lesion was placed in an Eppendorf tube with 0.5 g glass beads (2 mm diameter), and subsequently snap frozen in liquid nitrogen. Lesions were homogenised by shaking the tubes for 10 min at high frequency (30 1/s) using a Mixer Mill Type MM 301 (Retsch GmbH & Co. KG, Germany), equipped with a 2 × 12 tube (1.5 ml) adapter set. Immediately after homogenisation, extraction buffer (Gobbin *et al.*, 2003a) was added and tubes were placed at 65°C for 10 min., followed by centrifugation for 5 min at 20 000 g in order to precipitate cellular debris. The cleared supernatant (350 µl) was mixed with 525 µl of binding buffer AP3/E (Qiagen). The columns were each washed twice with 0.5 ml AW Buffer, followed by an ethanol wash and membrane drying according to manufactures instructions (Qiagen). DNA was eluted twice with 100 µl of sterile distilled water.

### **Microsatellite amplification**

All sampled lesions (Table 1) were genotyped using microsatellite markers (ISA, GOB, BER, CES) developed by Gobbin *et al.* (2003a). PCR amplification of ISA and BER was done in a multiplex reaction, whereas GOB and CES were each amplified in single reactions. PCR reactions consisted of a total volume of 10 µl, containing 5 µl

DNA solution (not quantified), 1x Supertherm Gold buffer (Southern Cross Biotechnology LTD, Cape Town, South Africa), and specific dNTPs, primer, Taq polymerase and  $MgCl_2$  concentrations for amplification of each of the different loci. The dNTPs concentration for the ISA and BER multiplex reaction was 0.3 mM, whereas the concentrations for CES and GOB amplifications were 0.4 mM and 0.3 mM, respectively. The final  $MgCl_2$  concentrations for the ISA and BER multiplex reaction was 1.9 mM  $MgCl_2$ , whereas the concentrations for GOB and CES were 1.9 and 1.8 mM  $MgCl_2$ , respectively. One unit Taq polymerase (Supertherm Gold, Southern Cross Biotechnology) was used for amplification of GOB, and 0.7 U Taq for CES amplification as well as for the ISA and BER multiplex reaction. Primer concentrations for amplification of the different loci were:  $BER_f$ : 0.4  $\mu M$ ,  $BER_r$ : 0.4  $\mu M$ ;  $ISA_f$ : 0.12  $\mu M$ ,  $ISA_r$ : 0.12  $\mu M$ ;  $CES_f$ : 0.14  $\mu M$ ,  $CES_r$ : 0.14  $\mu M$  and  $GOB_f$ : 0.4  $\mu M$ ,  $GOB_r$ : 0.4  $\mu M$ . Forward primers were all labelled with the following dyes:  $GOB_f$  and  $ISA_f$  with 6-FAM,  $CES_f$  with VIC and  $BER_f$  with PET (Applied Biosystems, Foster City, CA, USA). PCR was performed in a Gene Amp PCR system 9700 (Applied Biosystems) under the following conditions: 7 min at 95°C, 32 cycles of 30 s at 96°C, 30 s at 60°C and 50 s at 72°C with a final extension of 7 min at 72°C. Successful amplification was checked by running 5  $\mu l$  of each PCR product on a 2% agarose gel in 1x TBE buffer using a 100bp ladder prior to fragment analyses.

A subset of *P. viticola* oil spots, consisting of 64 lesions that were representative of ten specific genotypes obtained from different sampling times, seasons and vineyards (Table 3), were also genotyped using five (Pv7, P13, Pv14, Pv17 and PV31) of the seven microsatellite markers developed by Delmotte *et al.* (2006a). PCR amplification of the five markers consisted of two multiplex reactions (Pv17 along with Pv31, and Pv7 along with Pv13), whereas Pv14 were amplified in a single reaction. PCR reactions consisted of a total volume of 10  $\mu l$ , containing 5  $\mu l$  DNA solution (not quantified), 1x Supertherm Gold buffer, 0.7 U Supertherm Gold Taq, 0.3 mM dNTPs and specific primer and  $MgCl_2$  concentrations for amplification of the different loci. The  $MgCl_2$  concentration for the multiplex reactions was 2.5 mM, whereas the concentration for Pv14 amplification was 2 mM. Primer concentrations for amplification of both forward and reverse primers of Pv17, Pv31 and Pv13 were 0.1  $\mu M$ , whereas 0.2  $\mu M$  was used for Pv14 and 0.5  $\mu M$  for



Pv7 primers. Forward primers were all labelled with the following dyes: Pv7<sub>f</sub> and Pv14<sub>f</sub> with 6-FAM, Pv17<sub>f</sub> with VIC, Pv13<sub>f</sub> with PET and Pv31<sub>f</sub> with NED (Applied Biosystems). PCR was also performed in a Gene Amp PCR system 9700 (Applied Biosystems) for the multiplex reactions under the following conditions: 2 min at 94°C, 32 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C with a final extension of 2 min at 72°C. Amplification conditions for Pv14 were similar as for the multiplex reactions, except that the annealing temperature was 55°C. Successful amplification was checked on a 2% agarose gel.

Fragment analysis was performed on an ABI 3100 (Applied Biosystems) genetic analyser. PCR products of GOB (3.5 µl), CES (1.5 µl) and the ISA and BER multiplex (1.0 µl) amplifications were mixed with 8 µl dH<sub>2</sub>O. One microliter of this mixture was added to 0.5 µl Lizz 75-500 bp ladder and 10 µl HiDi (Applied Biosystems) in the sequencing plate. Fragment analysis of the PCR products of the Delmotte *et al.* (2006a) loci were analysed in a similar manner, except that 1 µl of PCR product was mixed with 6 µl of water. Data were visualised with Gene Mapper 3.0 (TM) (ABI PRISM®). For each locus, different fragment lengths were considered as different alleles. Combining the information provided by the scoring of the different loci, samples showing identical allele patterns were interpreted as clonal progeny, which were derived from the same oosporic infection. Different genetic profiles of oil spots were interpreted as individuals derived from independent oosporic infections.

Allele sizes of the South African populations were calibrated to the allele sizes of published European populations (Gobbin *et al.*, 2005). This was done by running PCR fragments from 30 European genotypes (DNA kindly provided by D. Gobbin, Institute of Integrative Biology, Swiss Federal Institute of Technology, Universitätstrasse 8092 Zürich, Switzerland) on the sequencer used in this study.

### **Data analyses**

All the analyses, except genotypic analyses, were done using clone-corrected data sets to prevent over-representation of alleles in frequently occurring clones. Analyses were done using POPGENE 3.2 software (Yeh & Boyle, 1997). The evenness of distribution of genotypes within a population was measured by normalising  $H'$  for

variable sample size by scaling the index by  $H'_{max}$ , which is the maximum genotypic diversity for a given sample size expressed here as the logarithm of the sample size  $\ln(n)$  (Nei, 1973). The mating structure of populations was also assessed by testing for Hardy-Weinberg Equilibrium within populations. Population differentiation ( $F_{ST}$ ) (Weir, 1997) between pair wise comparisons of all populations was conducted.

## RESULTS

### Weather conditions and development of epidemics

Downy mildew symptoms appeared at approximately the same time period (beginning to mid of October) in the two sampling seasons (2004/05 and 2005/06) after a period of high rainfall (36 mm and 60 mm per day) in September. In general, the 2004/05 season was more conducive to the development of downy mildew than the 2005/06 season. Within the survey period, there were a total number of 13 rain events in 2004/05 and only seven rain events in the 2005/06 season. The average daily temperatures were 18°C, 22.2°C and 24.1°C at the start (September-October), middle (November-December) and end (January-February) of the 2004/05 season, respectively, whereas temperatures were 18.1°C, 26.5°C and 31.2°C at the corresponding times in the 2005/06 season. The average daily relative humidity was 90%, 62% and 50% at the start (September-October), middle (November-December) and end (January-February) of the 2004/05 season, whereas in the 2005/06 season it was 75%, 50% and 36% at the corresponding time periods.

In the organic vineyard, the initiation of epidemics was slow at the beginning of both seasons, but as the year progressed it became more severe (Table 1). In both years, approximately 60% to 90% of the vines were heavily infected at the end of the season. However, no new lesions were present from the end of October to the end of November 2004 (rainfall of 47.8 mm) in the organically managed vineyard, even though 7.4 mm rain fell at the end of November.

In the conventional fungicide managed vineyard, the disease initially progressed at a rapid rate in 2004/05, but was readily controlled in the middle and at the end of the

season. Contrarily, in the 2005/06 season the epidemic was initially slow at the beginning and middle of the season, but became more severe at the end of the season.

### **Microsatellite amplification**

Amplicon sizes of loci ISA and BER were easily scored. However, locus CES was more difficult to score since amplicons had several stuttered bands, and in some instances the heterozygous amplicons were of similar sizes. All loci had two alleles per locus as in a typical diploid system, except GOB where a relatively high percentage of the genotypes (41%) contained three alleles. Three alleles at this locus were also detected in some European genotypes (16%), which were used for the calibration of allele sizes in our study.

Missing data could not be avoided, since some lesions were partially necrotic and the quality of the extracted DNA was not good enough for PCR amplification. Furthermore, half of the lesions from the first sampling time in 2004/05 in both vineyards could not be amplified due to the use of an incorrect DNA extraction buffer. Therefore, these two sampling dates were classified as being a partial sampling strategy (Table 1).

### **Population structure in the organically managed vineyard**

In total, 206 and 324 lesions were genotyped in the 2004/05 and 2005/06 growing seasons, respectively. The most diverse locus according to the number of alleles and Nei's gene diversity was GOB, followed by CES, ISA and BER (Table 2). The number of private alleles in the organic grown vineyard was low, only consisting of 3 alleles in each season (Table 2). The genotypic diversity was low ( $E_H = 0.22-0.29$ ) throughout the two seasons, except at the start of the 2005/06 season where it was moderate ( $E_H = 0.36$ ) (Table 1).

New genotypes comprised a large number (58 to 88%) of the total number of genotypes at each of the sampling times throughout the two seasons. These new genotypes contributed between 30% and 74% of the disease incidence (foliar lesions) at the different sampling times throughout the season (Table 1). In both seasons between 6% and 15% of the genotypes were able to reproduce asexually, i.e. produce more than 5 lesions (Table 1).

In both seasons, there were two predominant genotypes in the epidemic. In the 2004/05 season, these genotypes each caused approximately 15% of the epidemic, whereas in the 2005/06 season the predominate genotypes each contributed on average 21% and 11% of the disease incidence throughout the season (Table 1). Plotting of the asexual spread of the predominant genotypes in each of the growing seasons showed that the genotypes initially showed clonal multiplication at the initial infection site, followed by a plot-scale dispersal, with the number of lesions increasing over time. However, the exception was the 2005/06 predominant genotype G1 that initially had a plot-scale dispersion, but which subsequently showed a significant reduction in the number of lesions after the second sampling, until only two lesions were detected in the fourth sampling (Table 1, Fig. 1B). The spread of the predominant genotype G1 in the 2004/05 and 2005/06 seasons is represented in Fig. 1. The first infections caused by genotype G1 in the 2004/05 season was not detected (Fig. 1A), most likely due to the fact that not all lesions from the first sampling could be genotyped due to the incorrect DNA extraction buffer that was used.

#### **Population structure in the conventional fungicide managed vineyard**

In total, 183 and 157 lesions were genotyped in the 2004/05 and 2005/06 growing seasons, respectively. According to Nei's gene diversity, the most diverse loci were GOB and CES, followed by BER and ISA (Table 2). Only one private allele was present in each of the two seasons (Table 2).

The genotypic diversity was low in 2004/05 ( $E_H = 0.25 - 0.30$ ), and medium ( $E_H = 0.36 - 0.41$ ) in 2005/06, except at the end of the season when it declined ( $E_H = 0.18$ ) (Table 1). New genotypes constituted a relative large percentage (22% to 82%) of the total number of genotypes present at each sampling time throughout the season in both years, causing between 12% and 39% of the disease throughout the season (Table 1). The number of genotypes that were able to show some asexual reproduction, i.e. cause more than 5 lesions, was relatively low (3% to 28%) (Table 1).

Two genotypes dominated the epidemic in the 2004/05 growing season, each causing on average 27% and 16% of the overall disease incidence. In the 2005/06 season, only one genotype predominated the epidemic and contributed on average

through the season to 40% of the disease incidence (Table 1). The frequency of the other most frequently observed genotype in the 2005/06 season fluctuated over the sampling periods (Table 1). Plotting of the asexual spread of the two 2004/05 predominant genotypes showed an initial multiplication close to the first infection site, followed by a plot-scale dispersal with the number of lesions increasing over time. An example of the plot-scale dispersal patterns of one of the predominant genotypes in 2004/05 is shown in Fig. 2A. Contrarily, plotting of the spread of the one predominant genotype (G10) in the 2005/06 season showed that it had a random plot-scale dispersal without previous clonal multiplication close to the source (Fig. 2B).

### **Occurrence of genotypes between years and across the two vineyards**

In total, 10 genotypes were detected in both seasons (Table 3). One of the genotypes (G1), which was detected in both growing seasons in the organically managed vineyard, was also the predominant genotype in both seasons in this vineyard. Plotting of the location of genotype G1 on vines in the two different seasons showed that it initiated the epidemic from one focal point in each season, followed by a stepwise plot-scale dispersion (Fig. 1). However, in the 2005/06 season a significant reduction in the number of lesions caused by G1 was seen after the second sampling (Fig. 1B).

Six of the genotypes that were detected in the organic vineyard were also present in the fungicide sprayed vineyard in both seasons (Table 3). Interestingly, the predominant genotype G10 in the conventional fungicide sprayed vineyard in the 2005/06 season was also detected in the organic vineyard, but was present at only low frequencies in this vineyard (Table 3). Similarly, the predominant genotype G1 that was present in the organic vineyard in both seasons, was only present at a low frequency in the 2004/05 season and absent in the 2005/06 season in the conventional fungicide managed vineyard (Table 3). Furthermore, the predominant genotype G8 that was present in the 2004/05 season in the conventional fungicide managed vineyard was also present in the organically managed vineyard in both seasons, but remained at low levels (Table 3).

A subset of the lesions, containing the genotypes that were detected between vineyards as well as between seasons (Table 3), was further genotyped using the microsatellite markers of Delmotte *et al.* (2006a). These markers confirmed that the



lesions of each of the genotypes (G1 to G10) that survived from one season to the next were indeed clonal.

### **Population differentiation and Hardy-Weinberg Equilibrium**

Population differentiation ( $F_{st}$ ) between the organically managed and conventional fungicide managed populations was 0.004 and 0.016 in the 2004/05 and 2005/06 seasons respectively. In general, pairwise  $F_{st}$  values obtained for all the population comparisons were low. The highest  $F_{st}$  values (0.033-0.059) were obtained when populations from the first sampling in the organic vineyard in 2004/05 were compared with several populations present at different sampling times in the organic and fungicide treated vineyards in the 2005/06 season (Table 4).

Clone corrected populations sampled at different sampling times were all analysed for Hardy-Weinberg Equilibrium. The analyses showed that all loci were in Hardy-Weinberg equilibrium, except for locus BER at all sampling times and locus ISA at the last sampling time in the fungicide sprayed vineyard, and in the last two sampling times in the organic grown vineyard in the 2005/6 growing season (Table 5). Analyses of the expected and observed heterozygosity at loci ISA and BER suggested that there were undetected null alleles at these loci (Table 5).

## **DISCUSSION**

The majority of *P. viticola* population genetic studies have been conducted in unsprayed vineyards, with only four vineyards being treated with fungicides (Gobbin *et al.*, 2005; Rumbou & Gessler, 2006). Consequently, the effect of fungicide applications on the population structure of *P. viticola* is unknown. Therefore, this study is the first study attempting to address the effect of fungicide applications on the population structure of *P. viticola*.

Population genetic analyses showed that *P. viticola* populations from organic and fungicide treated vineyards belong to the same metapopulation because 1) there is low population differentiation ( $F_{st}$ ) between the conventional fungicide and organically managed populations ( $F_{st} = 0.004$ : 2004/5,  $F_{st} = 0.016$ : 2005/6), 2) the number of private alleles in the two vineyards was similar and low, and 3) genotypes were shared between

populations. Nonetheless, it is important to note that the predominant genotype in the organic vineyard was not the predominant genotype in the fungicide treated vineyard, even though it was present at low frequencies at all 2004/05 sampling times in this vineyard. Also, the predominant genotypes in the fungicide treated vineyards contributed much more to the epidemic than those in the organic vineyard, suggesting selection of specific genotypes. Furthermore, fewer new genotypes could be detected during the 2004/05 season in the fungicide treated vineyard, whereas genotypic diversity was also reduced dramatically towards the end of the growing season in 2005/06. Thus, although the population genetic structure of *P. viticola* supports one metapopulation, there are some indications that fungicide applications selected for reduced pathogen diversity. Alternatively, this could have been caused by different climatic conditions across vineyards and seasons. Further investigations are required in which the fitness of isolates from both vineyards under fungicide treatment conditions need to be compared.

In South Africa, oosporic (sexual) inoculum or migration plays an important role in the development of epidemics throughout the season. In seasons and vineyards, the contribution of oosporic infections or migration (identified as new genotypes detected at each sampling) to disease development varied from 12% to 74%, with the contribution being the lowest in the 2004/05 conventional fungicide managed vineyard. The importance of oosporic infections or migration prevailed up until the end of each growing season, causing between 15% and 44% of the disease incidence. Although in Europe the occurrence of new genotypes throughout the season, has been attributed to new oosporic infections (Eugster *et al.*, 2003; Rumbou & Gessler, 2004), more data is required in South Africa to determine the origin of new genotypes. The importance of oospore inoculum throughout the growing season, has also been shown through a field infection study. In this study, potted seedlings became infected throughout the season upon placement, at 2 to 5 day intervals, on naturally infected vineyard soil that was removed from the vineyard and placed in a downy mildew free region (Kennelly *et al.*, 2005, 2006).

The ability of genotypes to contribute to epidemics through asexual reproduction appears to be genotype specific in South Africa. In general, only one or two genotypes dominated epidemics in both vineyards and seasons. These dominant genotypes each caused a moderate to high amount of the disease (14% to 67%) throughout the growing

seasons. The reported amount of disease caused by predominant *P. viticola* genotypes, usually only one or two per epidemic, varies between vineyards and countries (Gobbin *et al.*, 2006b). However, there is a tendency in isolated mountain populations, Greek island and Australian populations for the predominant genotype to contribute to a significant amount of disease (40% to 94%), most likely due to a lower frequency of oosporic infections (Gobbin *et al.*, 2003b, 2005, 2006b; Hug *et al.*, 2006; Rumbou & Gessler, 2006). Alternatively, this could be attributed to most of the genotypes not being able to survive bottlenecks at the end of the growing season, leaving only one or two predominant genotypes that drive epidemic development. Although the relative contribution of one of the predominant genotypes was as high as 67% in one vineyard in South Africa, this was not true for all the epidemics.

In both analysed vineyards, only a small number of genotypes (3% to 28%) were able to show some low level of asexual reproduction, whereas most genotypes were never observed to reproduce asexually. Similar results were found in central Europe, Australia and in Greek island populations (Gobbin *et al.*, 2005; Hug *et al.*, 2006; Rumbou & Gessler, 2006). The limited number of *P. viticola* genotypes that was able to produce a significant number of lesions through asexual reproduction is surprising, considering that approximately  $2.4 \times 10^5$  *P. viticola* sporangia are produced on 1 cm<sup>2</sup> of an artificial infected lesion (Reuveni, 2003). Therefore, only a small number of sporangia ultimately seem to succeed in infecting the host. The reason for this failure may be linked to lower levels of asexual spore production under field conditions, a high sensitivity of sporangia to unfavourable environmental conditions such as long-wavelength UV light and high temperatures (Rotem *et al.*, 1985; Mizubuti *et al.*, 2000).

The tendency of only a limited number of genotypes being able to reproduce asexually, could also be due to the genetic background of these isolates, with most isolates not having the ability to successfully reproduce asexually. This would suggest that generation through sexual recombination of a genotype that is also able to reproduce asexually, is a rare event in *P. viticola*. Limited evidence for this can be found in a laboratory studies by Kast *et al.* (2001) and Kast (2004), which showed that *P. viticola* isolates differ in virulence and that not all field lesions are able to produce asexual spores under laboratory conditions. Similar observations were made in this study (unpublished

data). In other oomycetes such as *Phytophthora infestans*, it has indeed been shown that in some sexual crosses the progeny are able to infect, but are unable to produce asexual spores (Mayton *et al.*, 2000). Furthermore, in *P. infestans* and *P. cinnamomi* (soilborne oomycete) most of the progeny from a sexual cross were found to be significantly less virulent than the parental isolates (Mayton *et al.*, 2000; Linde *et al.*, 2001). Consequently, Mayton *et al.* (2000) concluded from their study on *P. infestans*, where laboratory and field inoculations were conducted, that most progeny were not as virulent and fit as the parental strains, and that these will most likely not survive as epidemiological important individuals.

The survival of *P. viticola* genotypes from one season to the next as asexual spores or as vegetative mycelium has only recently been reported for the first time in one Greek island population (Rumbou & Gessler, 2006). In South Africa, ten different genotypes survived between seasons in an asexual or vegetative form. Moreover, in the organically managed vineyard, the predominant genotype G1 in the 2004/05 season was able to survive as asexual spores or as vegetative mycelium to the next season (2005/06) where it also dominated the epidemic. It is interesting to note that the spread of the G1 genotype in the 2005/06 season also followed a step wise dispersal pattern with only two lesions being present at the start of the season. This suggests that only a small number of the G1 clones (16 lesions were present at the end of the season) were able to survive as asexual or vegetative mycelium from the 2004/05 season to the 2005/06 season. Survival of genotypes between seasons in South Africa might be due to the mild winters, where vegetative mycelium or sporangia may overwinter in grapevine buds or leaves that often remain hanging on the vines until the next season. Another hypothesis for the apparent survival of isolates between seasons could be that the microsatellite markers of Gobbin *et al.* (2003a) were unable to distinguish true clonal genotypes in South Africa, due to low levels of polymorphism in two of the tested loci (ISA and BER). However, additional genotyping of these isolates with new markers published by Delmotte *et al.* (2006a) confirmed that the specific genotypes were indeed clones. Furthermore, considering the polymorphisms found with both microsatellite markers, the estimated number of genotypes that could be detected are  $3.6 \times 10^5$  (number of allele combinations per locus =



$n(n + 1)/2$ , where  $n$  = number of alleles), which is a considerably higher number than what was detected in this study.

The South African *P. viticola* populations contained some genotypes that have three alleles at the GOB locus, similar to what has recently been reported in two Greek island populations (Rumbou & Gessler, 2006). However, unlike the Greek island populations, the South African genotypes did not contain three alleles at other loci such as CES and ISA (Rumbou & Gessler, 2006). The presence of three alleles at the GOB locus in some South African genotypes, as well as in two Greek island genotypes (Rumbou & Gessler, 2006) and in some European genotypes (this study) suggests that there is either a gene duplication at this locus, or that the isolates are triploid ( $3n$ ) or trisomic ( $2n + 1$ ). The presence of *P. viticola* isolates with elevated ploidy levels is not unexpected, since some evidence for elevated ploidy levels has also been found in other oomycetes including *P. infestans* and *P. cinnamomi*. In *P. infestans*, the presence of polyploids has been shown through DNA content measurements (Tooley & Therrien, 1991). Furthermore, in *P. infestans* as well as *P. cinnamomi*, microsatellite analyses have also identified genotypes with three alleles at some loci (Dobrowolski *et al.*, 2003; Lees *et al.*, 2006), suggesting elevated ploidy levels. However, the trisomic or triploid nature of these isolates still needs to be investigated through sexual inheritance studies. The presence of *P. viticola* isolates with possible elevated ploidy levels is important, since this could potentially not only influence the success with which isolates reproduce sexually, but also their ability to adapt to environmental conditions (Rumbou & Gessler, 2006). In plants, it has been found that cultivars differing in ploidy level are difficult to cross (Sanford, 1983). Furthermore, Dick (1972) hypothesised that in oomycetes the development of polyploidy and possibly aneuploidy would increase heterozygosity, which could compensate for the loss of evolutionary potential often associated with a fixed genotype. It is noteworthy that two of the most virulent and persistent clonal lineages (US-8 and US-11) of *P. infestans* in the USA are most likely aneuploids (trisomic,  $2n + 1$ ) (Goodwin *et al.*, 1992; Fry & Goodwin, 1997), supporting the hypothesis of Rumbou & Gessler (2006) that *P. viticola* isolates with elevated chromosome numbers might have an adaptive advantage. However, in our study only three of the ten isolates that survived between seasons had three GOB alleles (Table 3),



which does not completely support this theory. The generation of oomycete genotypes with elevated chromosome numbers might occur through the production of unreduced gametes, meiotic nondisjunction during sexual reproduction (Goodwin *et al.*, 1992) or zoospore fusion (Judelson & Yang, 1998).

The findings from this study, along with those of Rumbou & Gessler (2006) suggest that the population structure and epidemiology of *P. viticola* in warmer climates might be different than what is found in regions with more favourable environmental conditions for disease development. In regions with warmer climates, such as the Greek islands, South Africa and Australia, populations often experience bottlenecks during unfavourable climatic conditions during the growing season, most likely resulting in a strong selection for genotypes capable of surviving adverse environmental conditions. The survival of genotypes in an asexual or vegetative phase is specifically important for future control strategies. Asexual survival structures will most likely only need a minimum of 4 hours wetting period for infection early in the grape growing season. In contrast, if only sexual spores are the survival structure these structures are predicted to germinate only once the 10:10:24 ( ) rule is met. Consequently, downy mildew forecasting models that currently predict the initiation of the first epidemics on sexual spore germination, needs to be adjusted to include the initiation of epidemics from asexual or vegetative structures. Future studies should be aimed at characterising more *P. viticola* populations in South Africa as well as Australia, in order to determine the extent of asexual or vegetative survival of genotypes between seasons.. Specifically in Australia where the absence of oospores in a large number of vineyards (Killigrew & Sivasithamparam, 2005) suggests that epidemics might be dominated by highly adapted asexual clonal lineages that can survive from one season to the next in these vineyards.

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**Table 1.** Occurrence of *Plasmopara viticola* genotypes and genotypic diversity of populations collected at different sampling times in two consecutive growing seasons in an organic and conventional fungicide managed vineyard

Sample	Sampling date	Lesions analysed <sup>a</sup>	Lesions by predominant genotypes (%) <sup>b</sup>	Total no. genotypes	New genotypes (%) <sup>c</sup>	Genotypes causing >5 lesions (%)	Lesions by new genotypes (%) <sup>d</sup>	$E_H$ <sup>e</sup>
<b>Organic vineyard</b>								
2004/05	1 <sup>st</sup> - 04/10/04	16 (P)	0 (6)	5	100	0	100	0.28
	2 <sup>nd</sup> - 08/12/04	77 (T)	18 (20)	32	88	6	74	0.29
	3 <sup>rd</sup> - 08/02/05	113 (P)	14 (12)	53	58	9	30	0.25
2005/06	1 <sup>st</sup> - 06/10/05	11 (T)	18 (0)	8	100	0	100	0.36
	2 <sup>nd</sup> - 14/11/05	98 (T)	44 (7)	27	85	15	48	0.22
	3 <sup>rd</sup> - 28/11/05	112 (P)	20 (12)	51	69	6	42	0.24
	4 <sup>th</sup> - 30/01/06	103 (P)	2 (16)	49	69	10	44	0.26
<b>Conventional fungicide vineyard</b>								
2004/5	1 <sup>st</sup> - 04/10/04	64 (P)	14 (2)	31	100	3	100	0.26
	2 <sup>nd</sup> - 26/10/04	67 (T)	28 (22)	23	22	9	12	0.30
	3 <sup>rd</sup> - 08/12/04	52 (T)	39 (25)	18	33	28	15	0.24
2005/06	1 <sup>st</sup> - 06/10/05	13 (T)	23 (0)	6	100	0	100	0.41
	2 <sup>nd</sup> - 14/11/05	23 (T)	30 (17)	11	73	9	39	0.36
	3 <sup>rd</sup> - 28/11/05	121 (T)	67 (2)	17	82	18	28	0.18

<sup>a</sup> Lesions were collected using either a partial (P) or total (T) sampling strategy. During a total sampling strategy all the lesions were collected, whereas in the partial sampling strategy only two lesions were sampled from every second vine.

<sup>b</sup> Number of lesions containing the most predominant genotype, followed by the number of lesions caused by the second most dominant genotype in brackets.

<sup>c</sup> Number of genotypes that were not detected in the previous sampling, divided by the total number of genotypes detected for each specific sampling time.

<sup>d</sup> Number of lesions caused by new genotypes divided by the total number of lesions of each specific sampling time.

<sup>e</sup> Shannon's equitability calculated by  $H/H_{max}$ , where  $H_{max} = \ln N$  ( $N$  = number of individuals in the sample).

**Table 2.** Number of alleles, dominant alleles, private alleles and gene diversity of *Plasmopara viticola* populations sampled during different times in an organic and conventional fungicide managed vineyard in the 2004/05 and 2005/06 grape growing seasons

Sampling	Number of alleles (k)				Dominant allele size <sup>1</sup>				Private alleles				Gene diversity <sup>2</sup>				
	k <sub>ISA</sub>	k <sub>GOB</sub>	k <sub>CES</sub>	k <sub>BER</sub>	ISA	GOB	CES	BER	ISA	GOB	CES	BER	ISA	GOB	CES	BER	
<b>Organic vineyard</b>																	
2004/05	1 <sup>st</sup>	2	7	5	2	144.1	369/385.8	171	180.9					0.18	0.84	0.72	0.32
	2 <sup>nd</sup>	2	16	9	2	144.1	385.8	139	180.9		1			0.29	0.90	0.79	0.37
	3 <sup>rd</sup>	3	20	10	2	144.1	385.8	139	180.9	1	1			0.37	0.88	0.78	0.29
2005/06	1 <sup>st</sup>	2	6	4	2	144.1	385.8	171	180.9					0.38	0.80	0.65	0.12
	2 <sup>nd</sup>	2	13	8	2	144.1	385.8	139	180.9					0.38	0.90	0.74	0.18
	3 <sup>rd</sup>	2	13	7	2	144.1	385.8	139	180.9					0.43	0.89	0.67	0.21
	4 <sup>th</sup>	2	17	8	2	144.1	385.8	139	180.9		3			0.46	0.90	0.72	0.35
<b>Conventional fungicide vineyard</b>																	
2004/05	1 <sup>st</sup>	2	14	10	2	144.1	369.3	171	180.9					0.26	0.85	0.75	0.23
	2 <sup>nd</sup>	2	16	9	2	144.1	385.8	139	180.9					0.26	0.87	0.77	0.34
	3 <sup>rd</sup>	2	11	4	2	144.1	385.8	139	180.9			1		0.31	0.84	0.69	0.24
2005/06	1 <sup>st</sup>	2	7	5	2	144.1	369/385.8	139/165	180.9					0.28	0.82	0.74	0.38
	2 <sup>nd</sup>	2	12	4	2	144.1	385.8	139	180.9					0.30	0.90	0.64	0.24
	3 <sup>rd</sup>	2	14	8	2	144.1	385.8	139	180.9		1			0.24	0.86	0.81	0.51

<sup>1</sup> Allele sizes were calibrated according to a set of European *Plasmopara viticola* isolates (Gobbin *et al.*, 2003a).

<sup>2</sup> Nei's gene diversity.

**Table 3.** *Plasmopara viticola* genotypes that were able to survive between seasons (2004/05 and 2005/06) in an organic or conventional fungicide managed vineyard. The number of lesions of each specific genotype at the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> or 4<sup>th</sup> sampling time within each vineyard and year is shown. The predominant genotypes during 2004/05 were G1 and G7, and G1 and G6 during 2005/06 in the organic vineyard, whereas G8 and G10 were the predominant genotypes in the fungicide managed vineyard during the 2004/05 and 2005/06 seasons, respectively

Genotype	Organic 04/05			Organic 05/06				Fungicide 04/05			Fungicide 05/06		
	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>
G1	0	14	16	2	43	20	2	1	5	3	0	0	0
G2	0	2	10	1	2	1	0	0	0	0	0	0	0
*G3	1	2	3	0	1	1	0	0	0	0	1	0	0
*G4	0	0	6	1	3	0	0	0	0	0	0	0	0
G5	0	0	1	2	0	0	0	0	0	0	0	0	0
G6	0	3	3	0	6	12	15	0	0	0	0	0	0
G7	1	16	13	0	1	0	0	0	1	2	0	0	0
G8	0	3	0	0	0	0	1	2	19	20	1	0	0
*G9	0	2	2	0	5	9	1	1	5	5	1	4	2
G10	0	0	0	0	1	0	3	1	0	2	4	8	80

\* Genotypes with three alleles at the GOB locus.

**Table 4.** Estimates of pair-wise  $F_{st}$  averaged over four microsatellite loci of 13 sampling times in a fungicide sprayed and organically managed vineyards

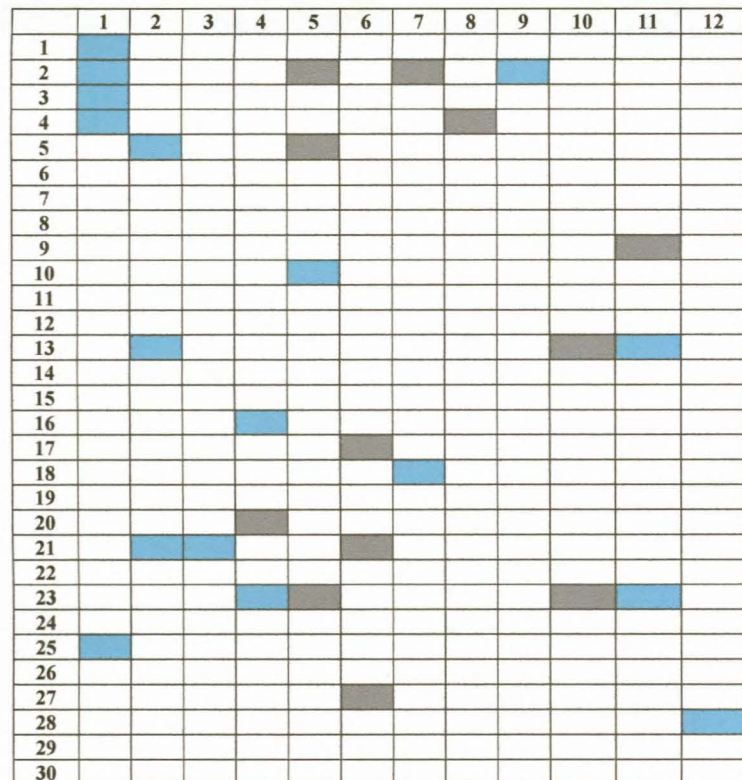
Sampling	Organic 04/05			Organic 05/06				Fungicide 04/05			Fungicide 05/06		
	1	2	3	1	2	3	4	1	2	3	1	2	3
Organic 04/05	1												
	2	0.028											
	3	0.026	0.004										
Organic 05/06	1	0.034	0.029	0.018									
	2	0.038	0.011	0.004	0.014								
	3	0.059	0.018	0.012	0.034	0.009							
	4	0.055	0.013	0.010	0.038	0.012	0.007						
Fungicide 04/05	1	0.015	0.017	0.012	0.018	0.018	0.038	0.037					
	2	0.013	0.010	0.009	0.021	0.014	0.031	0.029	0.003				
	3	0.038	0.009	0.008	0.028	0.012	0.019	0.016	0.019	0.014			
Fungicide 05/06	1	0.041	0.009	0.013	0.044	0.021	0.029	0.020	0.027	0.019	0.007		
	2	0.060	0.016	0.018	0.051	0.019	0.011	0.018	0.047	0.038	0.022	0.029	
	3	0.035	0.009	0.015	0.055	0.030	0.033	0.025	0.029	0.022	0.022	0.013	0.028

**Table 5.** Observed and expected heterozygosity of the different sampling times during the 2004/05 and 2005/06 seasons in an organically managed and conventional fungicide treated vineyard

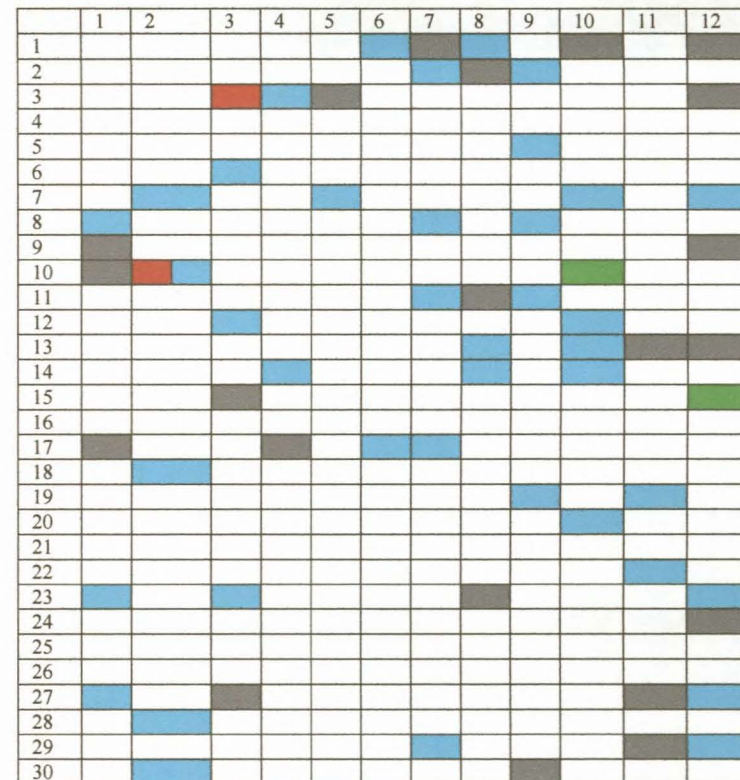
		Heterozygosity (observed/expected)			
		ISA	GOB	CES	BER
Organic 04/05	1	0.2000 / 0.2000	1.0000 / 0.9333	1.0000 / 0.8000	0.0000 / 0.3556*
	2	0.3548 / 0.2967	1.0000 / 0.9127	0.9355 / 0.8070	0.0968 / 0.3728*
	3	0.4773 / 0.3728	0.9773 / 0.8939	0.9773 / 0.7913	0.0909 / 0.3009*
Organic 05/06	1	0.5000 / 0.4000	1.0000 / 0.8500	0.8750 / 0.6917	0.1250 / 0.1250*
	2	0.5200 / 0.3927	0.9600 / 0.9135	1.0000 / 0.7592	0.0400 / 0.1837*
	3	0.6333 / 0.4401*	0.9333 / 0.9034	0.9000 / 0.6814	0.0333 / 0.2153*
	4	0.7333 / 0.4697*	0.9778 / 0.9081	0.9333 / 0.7308	0.0889 / 0.3586*
Fungicide 04/05	1	0.3077 / 0.2655	0.9231 / 0.8665	0.8462 / 0.7640	0.0385 / 0.2376*
	2	0.3000 / 0.2615	1.0000 / 0.8936	0.9500 / 0.7936	0.1000 / 0.3436*
	3	0.3889 / 0.3222	1.0000 / 0.8651	0.9444 / 0.7127	0.0556 / 0.2460*
Fungicide 05/06	1	0.3333 / 0.3030	1.0000 / 0.8939	1.0000 / 0.8030	0.1667 / 0.4091*
	2	0.3636 / 0.3117	1.0000 / 0.9394	1.0000 / 0.6710	0.0909 / 0.2468*
	3	0.2778 / 0.2460*	0.8889 / 0.8857	0.7778 / 0.8286	0.2222 / 0.5270*

\* Loci which were in Hardy-Weinberg disequilibrium.



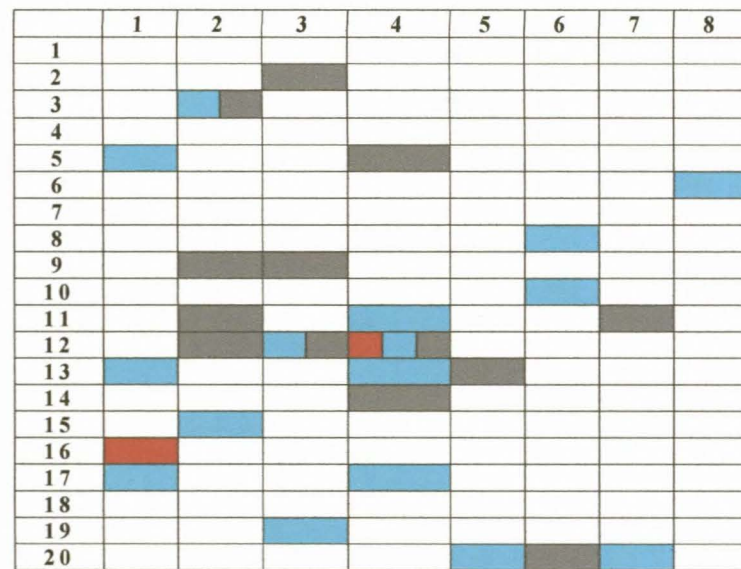


(A) 2004/05 season



(B) 2005/06 season

**Fig 1.** Schematic representation of the predominant genotype G1 in an organically managed vineyard where *Plasmopara viticola* populations were studied in the (A) 2004/5 and (B) 2005/6 growing seasons. Each square represents one vine, with vines being spaced 2 m within rows and 3 m between rows. The dispersion pattern of the predominant genotype G1 in each growing season is shown at three consecutive samplings times (1<sup>st</sup> = ■, 2<sup>nd</sup> = ■, 3<sup>rd</sup> = ■, 4<sup>th</sup> = ■).



(A) 2004/05 season



(B) 2006/05 season

**Fig 2.** Schematic representation of the predominant genotypes (A) G8 in the 2004/05 growing season and (B) G10 in the 2005/06 growing season in a fungicide managed vineyard where *Plasmopara viticola* populations were studied. Each square represents one vine, with vines being spaced 2 m within rows and 3 m between rows. The dispersion pattern of the two predominant *P. viticola* genotypes in each growing season is shown at three consecutive samplings times (1<sup>st</sup> = ■, 2<sup>nd</sup> = ■, 3<sup>rd</sup> = ■, 4<sup>th</sup> = ■).

### 3. RESISTANCE SCREENING OF GRAPEVINE SEEDLINGS AGAINST *PLASMOPARA VITICOLA* AND LONG-TERM STORAGE OF SPORANGIA

#### ABSTRACT

Downy mildew, caused by the obligate pathogen *Plasmopara viticola*, is a very destructive grapevine disease. The disease is mainly controlled through fungicide applications, since no commercially acceptable resistant cultivars are available. Therefore, the aim of this study was to determine the downy mildew resistance of *Vitis vinifera* seedlings derived from a cross between the cultivars Regent and Red Globe. Resistance of the seedlings was evaluated using an *in vitro* leaf disk method and the OIV 452-rating system. Resistance evaluation trials were repeated three times within and between seasons, specifically twice in 2004/05 using 167 and 149 seedlings, and once in 2005/06 using 104 seedlings. Spearman correlation analysis revealed a moderate to high (0.64 to 0.82) correlation between the three screenings trails, indicating that the screening method was reproducible within and between the two seasons. However, mean values obtained for ranks and infection classes differed significantly between the third (2005/06) and the first two (2004/05) resistance screening trials, emphasising the importance of including tolerant and susceptible reference seedlings as well as the parents of the cross in each screening trial. As the inoculum mixture used in the two seasons differed with regard to genotypic composition, this finding also emphasized the importance of using the same pathogen genotype population between different screenings over seasons in order to obtain consistent resistance screening results. Therefore, the second aim of the study was to evaluate a few long-term storage methods for *P. viticola*. The two main storage methods that were evaluated consisted of either storing the pathogen while still associated with the host (sporulating lesions), or storage of only pathogen spores (sporangia) at -20°C and -80°C. The storage methods were initiated at two time periods including May 2004 and January 2006, with viability analyses after 17 and 6 months, respectively. Viability testing of the pathogen from both storage periods revealed that the pathogen is best stored when still associated with host tissue, i.e. as lesions, either at -20 or at -80°C.

## INTRODUCTION

Downy mildew caused by *Plasmopara viticola* (Berk et Curt.) Berlese et de Toni is a sporadic, but destructive disease of grapevines in the Western Cape province of South Africa (Marais, 1973), and is mainly controlled through fungicide applications. The pathogen is an obligate parasite that cannot be cultivated on artificial media, since hyphae develops only after contact with the host (Riemann *et al.*, 2002). Therefore, cultivation of the pathogen requires living host tissue (grapevine) and favourable conditions for infection and sporulation (Barlass *et al.*, 1986; Wong *et al.*, 2001).

The spores produced by *Plasmopara viticola* can either be sexual (oospores) or asexual (zoospore released from sporangia) (Wong *et al.*, 2001). Oospores are formed in leaves at the end of the season, whereas sporangia are produced either from germinating oospores or from sporulating lesions. The asexual sporangia release zoospores, which are the infective propagules of the pathogen that require free water for infection and temperatures of approximately 20-25°C (Lafon & Clerjeau, 1994). The pathogen further requires a high relative humidity for sporulation (Lafon & Clerjeau, 1994; Magarey *et al.*, 1994).

Downy mildew is native to Northern America, and was first introduced to Europe and the rest of the world in the late 1870s where it almost wiped out the susceptible commercial vineyards (Alexopoulos *et al.*, 1996). Therefore, soon after the introduction of downy mildew into Europe a great effort was made at breeding varieties that are resistant to the pathogen. The American and Asian *Vitis* species has been shown to represent the best sources for resistance breeding (Dai *et al.*, 1995; Brown *et al.*, 1999a, b; Merdinoglu *et al.*, 2003). Recurrent selection and back crossing of these species have been used effectively to improve downy mildew resistance by selecting resistant seedlings with good fruit characteristics as parents for succeeding generations (Brown *et al.*, 1999b).

The evaluation of grapevine cultivars for resistance to downy mildew requires accurate and efficient screening methods. Both field evaluations and laboratory-based techniques have been used for this purpose. Laboratory-based techniques, for example leaf discs (Denzer *et al.*, 1995; Staudt & Kassemeyer, 1995), detached leaves and other *in*



*in vitro* culture methods (Barlass *et al.*, 1986) are advantageous since they allow rapid screening of large numbers of breeding progenies (Brown *et al.*, 1999a). The *in vitro* leaf disk method is the most widely used method (Denzer *et al.* 1995; Staudt & Kassemeyer, 1995), and has also been shown as a good predictor of field resistance (Brown *et al.*, 1999a; Fischer *et al.*, 2004).

The screening of new grapevine breeding accessions for resistance to downy mildew on an on-going basis requires the long-term storage of specific *P. viticola* isolates (genotypes). This is important since *P. viticola* isolates have been shown to differ in virulence (Kast *et al.*, 2000; Kast, 2004), which could affect screening results. The storage of specific isolates, therefore, is important to ensure more consistent results especially for mapping of quantitative trait loci (QTLs). Furthermore, due to the obligate nature of the pathogen, storage of isolates will provide back-up isolates should incubator failure result in the death of isolates. Currently, downy mildew sporangia are mainly maintained through transfer of sporangia from one leaf or plant to another on a regular basis (once every week) (Staudt & Kassemeyer, 1995; Kast & Stark-Urnau, 1999), which is labour intensive and furthermore not always successful.

A few attempts have been made at long-term storage of *P. viticola*, although more literature is available on the storage of other obligate oomycetes such as *Peronospora* species. Dahmen *et al.* (1983) reported the storage of *P. viticola* sporangia for up to nine years in liquid nitrogen by using cryoprotectants such as skim milk-glycerol and 15% dimethyl sulfoxide (DMSO). Bromfield & Schmitt (1967) first reported the successful storage of blue mold of tobacco, caused by *Peronospora tabacina*, by freezing suspended conidia in 15% DMSO for up to 25 months in liquid nitrogen. However, liquid nitrogen is not always accessible for all researchers, since it requires special equipment and a continual supply of liquid nitrogen. Kortekamp *et al.* (1997) were able to store *P. viticola* sporangia successfully at -25°C, but they, along with Dahmen *et al.* (1983), did not provide specific detailed protocols of their storage methods. The storage of *Peronospora* lesions at -20°C (Cohen & Kuc, 1980), -60°C (Viranyi, 1985) and -80°C (Gill & Davidson, 2005) has been found successful for storage of *Peronospora* from 6 to 12 months.

The first aim of the study was to evaluate seedlings derived from a cross between two *V. vinifera* cultivars Regent (resistant) and Red globe (susceptible), for downy mildew resistance using an *in vitro* leaf disc method. The phenotypic data of the screening tests will be used in future mapping studies on quantitative trait loci (QTLs) for resistance against downy mildew, in a collaborative project with the Agricultural Research Council (Infruitec-Nietvoorbij). The second aim of the study was to evaluate different long-term storage techniques for *P. viticola*, in order to maintain a characterised population of *P. viticola* isolates that can be used in all future downy mildew grapevine resistance screenings.

## MATERIALS AND METHODS

### Seedling growth conditions

One hundred and eighty seedlings derived from a cross between Regent (resistant parent) and Red globe (susceptible) were evaluated for downy mildew resistance. The seedlings were planted in a sand-bark mixture in 2 liter plastic bags. The plants were grown in a glasshouse at 25°C, and were watered regularly with no fungicides being applied. Red Globe vines (grafted on Richter 99) were also kept at similar glasshouse conditions, whereas leaves of Regent used in the resistance screenings, were obtained from field grown grapevines. Chardonnay (clone CY 133B) vines, crafted on Richter 99, which are highly susceptible to *P. viticola* were used for the production of *P. viticola* inoculum. These vines were planted in a sand-bark mixture in 5 liter plastic nursery bags and were maintained in the same manner as the other seedlings. The plants were kept free from powdery mildew (*Erysiphe necator*) by vapour action from four 10 × 30 cm cotton cloths that were dipped in formulation-strength penconazole (Topaz® EW 200g/L, Syngenta).

### *Plasmopara viticola* isolates

Grapevine leaves with downy mildew lesions were collected at four different vineyards in the Western Cape Province. The lesions were incubated in Petri dish moist chambers for 2 days at 20°C and a 12-hour light period, where after sporangia sporulating



from lesions were brushed into sterilised water using a small moist paint brush. The sporangial suspension was used to drop inoculate Chardonnay leaves within Petri dish moist chambers. The inoculated leaves were incubated for 7 days at 20°C and a 12-hour light period. A subset of the isolates that sporulated from the inoculated leaves was genotyped using four microsatellite markers (Gobbin *et al.*, 2003; Chapter 2). Subsequently, seven isolates each having a unique genotype and representing the four vineyards were selected for the resistance screenings. Different genotypes were used for the screening of seedlings between the 2004/05 and 2005/06 seasons, due to isolates from the 2004/05 being contaminated through successive transfers resulting in the loss of these isolates. The isolates were maintained on leaves in Petri dishes of the cultivar Chardonnay and were transferred once a week to new leaves.

### **Inoculum preparation**

Inoculum for resistance screenings was prepared by inoculating each of the seven *P. viticola* isolates onto surface sterilised (0.05% sodium hypochlorite for 2 minutes, followed by a triple rinse in sterilised distilled water) Chardonnay leaves. The inoculated leaves were incubated in Petri dish moist chambers for 7 days, at 20°C and a 12-hour light period. Sporangia of each isolate were brushed from the leaves into sterile distilled water and the spore suspension of each isolate was adjusted to  $1 \times 10^5$  sporangia/ml using a haemocytometer. Subsequently, equal volumes of each of the seven isolates were mixed and placed at 4°C for 30 min to allow water uptake by sporangia, and to induce zoospore release (personal communication A. Kortekamp, Institute for Phytomedicine, University of Hohenheim, Germany).

### ***In vitro* leaf disc assay**

Fully expanded leaves from the 5<sup>th</sup> and 6<sup>th</sup> nodes of shoots of each seedling were removed and surface-sterilised as described above. The sterilised leaves were dried on paper towels, before cutting leaf discs (16 mm in diameter) with a cork borer from the leaves. The leaf disks were floated upside down in 90 mm Petri dishes, each containing 20 ml of sterilised distilled water and 10 leaf disks. Each leaf disk was inoculated with a 20 µl droplet of inoculum ( $1 \times 10^5$  sporangia/ml). Each seedling genotype was replicated

twice, i.e. two dishes with ten leaf disks each. The inoculated leaf disks were left uncovered over night, to allow drying of droplets, followed by the closing of lids and incubation for seven days at 20°C and a 12-hour light period.

Seven days after inoculation, the leaf disks were evaluated using the OIV 452-rating system (Wiedemann-Merdinoglu *et al.*, 2003). This rating system consisted of a rating scale of 1-9, where 1 indicated highly resistant seedlings and 9 indicated highly susceptible seedlings. A description of the ratings used in the scale is as follows: 1 = very low (tiny necrotic spots, no sporulation nor mycelium); 3 = low (small patches < 1 cm in diameter, little sporulation or mycelium, sporulation lesion smaller than droplet); 5 = medium (little patches 1-2 cm in diameter, more or less strong sporulation, sporulation lesion same size as original droplet); 7 = high (vast patches, strong sporulation and abundant mycelium, sporulation lesion exceeding the size of droplet); 9 = very high (vast patches, strong sporulation and dense mycelium) (Fig. 1). Resistance evaluation trials were conducted twice in 2004/05 using 167 and 149 seedlings, and once in 2005/06 using 104 seedlings.

### **Statistical analyses**

As data consisted of values on a rating scale, Spearman rank order correlation coefficients were calculated between the different screening times for average ratings. In order to compare ratings from different screenings, ratings were also subjected to ranks before one-way analysis of variance was performed. Tukey's LSD (Least Significant Difference) was calculated at a 5% significance level to compare rank means. In order to test for independence between screenings, frequencies observed in a screening × rating scale contingency table were subjected to a Chi-Square test. All the above techniques were performed using SAS statistical software (SAS, 1999).

### **Evaluation of storage methods**

*Plasmopara viticola* lesions were stored using two methods based on the principle of either, (1) storage of the pathogen while still associated with the host tissue, i.e. sporulating lesions, or (2) removal of spores (sporangia) from lesions and storing these at

-20°C and -80°C. The two storage methods were initiated at two different time periods, one being in May 2004 and the other in January 2006.

Lesions stored in May 2004 were stored by first collecting downy mildew lesions from grapevine nurseries. These lesions were stored by (1) immersion of lesions in 1 ml of a 50% glycerol solution in 2.2. ml centrifuge tubes at -80°C, (2) placing lesions in 2.2. ml centrifuge tubes at -80°C, (3) collecting sporangia from leaves by brushing them from the leaves onto a Petri dish, which were left to dry at room temperature before brushing the sporangia into in a 2.2. ml centrifuge tube and storing the sporangia in tubes at -20°C (Kortekamp *et al.*, 1997; personal communication A. Kortekamp, Institute for Phytomedicine, University of Hohenheim, Germany). The viability of the stored lesions was assessed after 17 months in October 2005.

Lesions stored in January 2006, were stored by first collecting 10 single downy mildew lesions from vineyards, which were inoculated and incubated onto fresh Chardonnay leaves as described above. After 7 days of incubation, the leaves containing approximately 15 sporulating lesions were stored by placing the parafilm sealed Petri dish containing the leaf with sporulating lesions at (1) -80°C or at (2) -20°C. Pathogen storage according to the protocol of Kortekamp was also conducted as described above, except that tubes were stored at -20°C as well as -80°C. The viability of these lesions was tested after 6 months.

The viability of all the stored lesions were tested after 17 (May 2004 storage) or 6 months (January 2006 storage) by brushing sporangia from the stored lesions into 2 ml sterilised distilled water using a moist paint brush, or just adding water to stored sporangia (Kortekamp storage protocol). The harvested sporangia were placed for 30 min at 4°C, which ensured water uptake by sporangia and induced zoospore release. Subsequently, 20 µl sporangial and zoospore droplets were inoculated onto the underside of Chardonnay leaves in Petri plates containing wet filter paper. The Chardonnay leaves were obtained from the 5<sup>th</sup> and 6<sup>th</sup> nodes of plants, and were surface-sterilised as described above. After inoculation, the Petri plates were closed for 4 hours after which it was opened again to allow drying of droplets. Once the droplets were dried, the Petri plates were closed and incubated for 7 days at 20°C and a 12 hour day length. After 7

days, the percentage of droplets that yielded sporulating *P. viticola* colonies was calculated.

## RESULTS

### *In vitro* leaf discs assay

The same number of seedlings could not be screened in each of the three resistance screening tests, due to some seedlings not having healthy leaves and death of seedlings. Consequently, 167 and 149 seedlings were screened in 2004/05 and only 104 seedlings in 2005/06. Downy mildew sporulation on susceptible seedlings was visible 4 to 5 days after inoculation, with disease severity varying in the different accessions. Resistance screening of the two parents (Regent and Red Globe) were only included in screening trail 3 in 2005/06, resulting in a mean rating score of 3 and 7 for Regent and Red Globe, respectively. The majority of the seedlings were classified in a rating class of 5 and 7, whereas none of the seedlings received a rating of 1 (Fig. 2).

Resistance screenings of seedlings within and between years were comparable. Relatively high Spearman correlation coefficients (0.708 and 0.820) were found between screening 1 (2004/05) and the other two screenings, whereas the correlation between screening 2 (second trial in 2004/05) and screening 3 (2005/06) was medium (0.644) (Table 1). The correlation coefficients between the replicates within the screenings trails were very high (0.87 to 0.98; results not shown).

Analysis of variance of rank values indicated significant differences between screenings ( $P = 0.0005$ ; Table 2). Rank means and rating class means for screening 1 and 2 (2004/05) were similar, but differed significantly from those values obtained for screening 3 (2005/06) (Table 3). This difference was also depicted in the screening  $\times$  rating scale contingency figure (Figure 2) and proved to be significant by Chi-square test (Chi-square value 35.6348;  $P < 0.0001$ ). This was due to a higher percentage of the seedlings belonging to a rating of 3 and a lower percentage belonging to a rating of 9 in screening trial 3 (2005/06), when compared to the other two screenings (2004/05) (Figure 2).

### Evaluation of storage methods

Only one of the storage techniques used to store downy mildew lesions in May 2004 resulted in the recovery of the pathogen. The successful technique consisted of the placement of sporulating lesions taken directly from the field into 2.2 ml tubes and storing these at -80°C. The viability testing of these techniques showed that only 20% of the inoculated droplets showed active sporulation after incubation.

In 2005/06, a slight modification of the 2004/05 techniques was used, since resistance screening tests required the enumeration of isolates on leaves prior to storage. Therefore, the isolates were first enumerated on leaves in January 2006, and then directly stored in the Petri dish moist chamber in order to save time. The storage of sporulating leaves within parafilm sealed Petri dishes stored at -80°C and -20°C were both successful. Viability testing of these stored lesions resulted in only 30 to 40% of the inoculated droplets showing active sporulation following 7 days of incubation, with no difference being seen between the two techniques. The storage of *P. viticola* according to the protocol of Kortekamp was not successful in the current study.

### DISCUSSION

Resistance screenings of grapevine seedlings from a Regent × Red Globe cross showed that the seedlings varied in their resistance to downy mildew. The resistance evaluations revealed that most of the seedlings were susceptible, with an OIV 452 rating class of 7 or 9. Similar results were found by Brown *et al.* (1999b), who showed that more of the grapevine seedling progeny were susceptible than resistant from a cross between a resistant and a susceptible parent. None of the seedlings that were screened were classified in the rating class of 1, which indicates that there is no downy mildew single gene resistance (R-gene) present in the screened population.

The three resistance screenings were conducted within and between seasons, and were shown by Spearman correlation analyses to be reproducible. Therefore, the *in vitro* leaf disk method proved to be a reproducible technique, well suited for resistance evaluation. However, the percentage seedlings that belonged to the different OIV 452 ratings classes differed between the third (2005/06) and the first two (2004/05) resistance screening trials. This difference was statistically supported by one-way analysis of



variance of rank means of these screenings, as well as Chi-square test of the screening  $\times$  rating scale contingency table. The reason for this difference could be due to the fact that a number of the seedlings died, due to a natural downy mildew epidemic that occurred in the greenhouse between the screenings conducted in 2004/05 and 2005/06. This resulted in the elimination of 76 downy mildew seedlings that were mainly susceptible (35% from class 9, 25% from class 7, 24% from class 5 and 15% from class 3; results not shown) from the population. This difference might also be attributed to the different inoculum mixtures that were used in the two growing seasons, or the physiological state of leaves could have been different between the screenings. The difference between rating classes in experiments, emphasises the importance of the inclusion of tolerant and sensitive reference seedlings in each screening trial, as well as the parents of the cross. In this study, the parents of the cross was only included in one resistance screening trial.

The evaluation of long-term storage methods showed that the storage of *P. viticola* was best when the pathogen was still associated with the host tissue. *Plasmopara viticola* sporangia were still viable after storage of sporulating leaf lesions at -20 and -80°C, for periods of 6 to 17 months. The storage of lesions as small pieces within centrifuge tubes or as whole leaves within Petri dishes were both effective. Although the sporangia were still viable after storage at these conditions, the percentage inoculum droplets that sporulated were relatively low, indicating that the germination rate of the sporangia was reduced during storage. Dahmen *et al.* (1983) also found that *P. viticola* sporangia had a relative low germination rate of approximately 50% after storage, but that each of these sporangia were able to cause disease. This was also found for other obligate or semi-obligate oomycetes such as *Phytophthora infestans* and *Pseudoperonospora cubensis* (Dahmen *et al.*, 1983). The reduction in germination rate is most likely caused by damage during freezing and thawing (Cohen & Kuc, 1980; Dahmen *et al.*, 1983). It has been found that *P. viticola* is highly susceptible to rapid freezing especially when being directly frozen within liquid nitrogen. Dahmen *et al.* (1983) has found that the germination rate of stored sporangia can be greatly increased by thawing stored samples in a water bath of 20 or 40°C, instead of thawing samples at room temperature on a bench.



Earlier studies have mostly made use of cryoprotective solutions for successful storage of oomycetes in liquid nitrogen (Bromfield & Schmitt, 1967; San Antonio & Blount, 1973; Dahmen *et al.*, 1983), whereas more recent studies have investigated faster and easier storage methods that do not require the use of liquid nitrogen. These storage methods mostly involve the storage of obligate oomycetes as spores that are still associated with the host tissue, i.e. as sporulated lesions on leaves (Gulya *et al.*, 1993; Kortekamp *et al.*, 1997; Gill & Davidson, 2005). The research of this study has also shown that the storage of *P. viticola* lesions is a very fast and easy long-term storage method that is effective for storage of the pathogen. Although Kortekamp *et al.* (1997) stored air-dried *P. viticola* sporangia at -25°C and Gulya *et al.* (1993) stored *Plasmopara halstedii* sporangia in liquid nitrogen, this study was not able to obtain similar results.

The study has shown that the *in vitro* leaf disk method is suitable for screening large numbers of grapevine seedlings for resistance to *P. viticola*. The method was reproducible over experiments and seasons, and will be used in the future for all resistance screenings where the parents of crosses and other reference seedlings will also be included. Future studies will have to determine whether the results of this screening method are comparable with resistance evaluations conducted under field conditions. Resistance screenings will be made more feasible in the future through the use of the long-term storage method that was developed for storage of *P. viticola* isolates for 6 to 17 months. The storage method will assure that when conducting resistance screenings, the same isolates can be used over different seasons.

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**Table 1.** Spearman correlation coefficients from the average rating between the different resistance screenings trials. Grapevine seedlings were screened for resistance to downy mildew, using a rating system of 1 to 9, in three resistance screenings trials conducted in 2004/05 and 2005/06

	Screening 1 (2004/05)	Screening 2 (2004/05)	Screening 3 (2005/06)
Screening 1		0.709	0.820
Screening 2			0.644
Screening 3			

**Table 2.** Analysis of variance of rank values, which are based on averages of the OIV 452 rating of sporulation on floating grapevine leaf discs 7 days after drop-inoculation with a sporangial suspension of *Plasmopara viticola*. Rank values were obtained in three separate screenings of 167 and 149 seedlings (2004/05), and 104 seedlings (2005/06) derived from a cross between Regent (resistant parent) and Red globe (susceptible parent)

Criteria	df	SS	MS	F-value	P-value
Screenings	2	823436.62	411718.3	7.61	0.0005
Error	841	45519269.9	54125.17		
Residual	843	46342706.5			

**Table 3.** Means of rank values and rating class (calculated) obtained in three separate screenings of 167 and 149 seedlings (2004/05), and 104 seedlings (2005/06) derived from a cross between Regent (resistant parent) and Red globe (susceptible parent)

Resistance screening trial number	Rank Means <sup>x</sup>	Rating class mean
1 (2004/05)	423.84 a	5.84
2 (2004/05)	452.93 a	6.10
3 (2005/06)	370.31 b	5.35

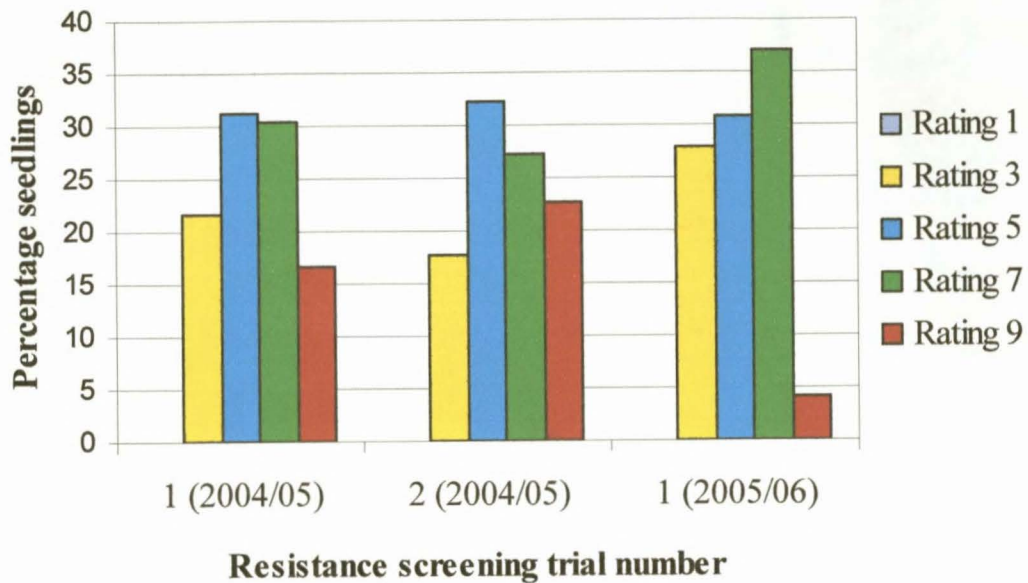
<sup>x</sup> Values in each column followed by the same letter do not differ significantly ( $P < 0.05$ ).





**Fig 1.** Grapevine leaf disks inoculated with *Plasmopara viticola* exhibiting the four different OIV 452-rating classes (3-9). A rating of 3 = low (small patches < 1cm in diameter, little sporulation or mycelium, sporulation lesion smaller than droplet); 5 = medium (little patches 1-2 cm in diameter, more or less strong sporulation, sporulation lesion same size as original droplet); 7 = high (vast patches, strong sporulation and abundant mycelium, sporulation lesion exceeding the size of droplet); 9 = very high (vast patches, strong sporulation and dense mycelium).





**Fig 2.** Percentage grapevine seedlings from a Regent  $\times$  Red Globe cross showing different levels (ratings) of resistance against *Plasmopara viticola*. The resistance trials were conducted three times, twice in the 2004/05 season and once in 2005/06 season. Resistance was screened using an *in vitro* leaf disk method and the OIV 452- IOV rating scale (1-9), where a rating of 1 indicates complete resistance and a rating of 9 indicates highly susceptible seedlings.