

Genetic characterization and fungicide resistance profiles of *Botrytis cinerea* in rooibos nurseries and pear orchards in the Western Cape of South Africa

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Declaration

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Summary

Botrytis cinerea Pers. Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] causes serious losses of over 200 crops worldwide, including rooibos seedlings and pears. This pathogen is characterized by morphological, physiological and genetic diversity. The genetic diversity and population structure have not been investigated for *B. cinerea* populations in South Africa. *Botrytis cinerea* collected from rooibos seedlings and in pear orchards in the Western Cape of South Africa were investigated in the present study. The study was done with the aid of microsatellite markers, the amplification of mating type alleles *MAT1-1* and *MAT1-2* and determination of resistance towards various fungicides. Population dynamics was inferred and a similar picture emerged in both production systems.

Botrytis cinerea annually causes severe losses of rooibos seedlings (*Aspalathus linearis*) in nurseries situated in the Clanwilliam region. Sampling was done in five nurseries and the cryptic species status of the isolates obtained was determined through restriction enzyme digestion of the *Bc-hch* gene. All but one (206 out of 207) of the isolates belonged to Group II or *B. cinerea* 'sensu stricto'. Analysis of the *B. cinerea* Group II population, using seven microsatellite loci, was performed to assess the genetic population structure. Total gene diversity (H) was high, with a mean of 0.67. Two of the nurseries populations' sample sizes were severely limited after clone correction, yet 100 genotypes were discerned among the 206 isolates genotyped. The percentage of maximal genotypic diversity (G) ranged between 16 and 68 for the five populations, with a total value of 17 for the 100 genotypes. One genotype, represented by 27 clones, was isolated from four nurseries. Relatively low but significant population differentiation was observed in total between nurseries (mean $F_{ST} = 0.030$, $P = 0.001$). The distribution of mating types *MAT1-1* and *MAT1-2* differed significantly from the ratio of 1:1 for the total population plus two of the nurseries' populations. Three nursery populations had an equal mating type distribution. The index of association (I_A) analyses suggests that the populations are asexually reproducing. Analysis of molecular variance (AMOVA) indicated that 97% of the total genetic variation is distributed within subpopulations. Fungicide resistance frequency against iprodione for 198 of the genotyped isolates displayed highly varying levels of resistance amongst the five nurseries. The mean total incidence of resistance towards iprodione was 43%, ranging from 0% to 81% for the five nurseries. Baseline sensitivity towards pyrimethanil yielded an average EC_{50} value of 0.096 mg/L.

Botrytis cinerea isolates were collected from pear blossoms (*Pyrus communis*) in four orchards. Two orchards in the Ceres area and two in the Grabouw area were sampled from. A total of 181 isolates were collected from the four orchards. Incidence of blossom infection in the orchards ranged from 3% to 17%. Overall, there was a high incidence of isolates that had only the *Boty* transposable element (74%) compared to those harbouring both (*Boty* and *Flipper*), simultaneously (*transposa*, 24%). One isolate examined had the *Flipper* element

only. Cryptic species status according to restriction enzyme digestion of the *Bc-hch* gene indicated that all the isolates belonged to Group II or *B. cinerea* 'sensu stricto'. Analysis of the Group II population, through the use of seven microsatellite loci, was performed to assess the genetic population structure. Total gene diversity (H) was high, with a mean of 0.69 across all populations. Although two of the subpopulations displayed a high clonal proportion, overall 91 genotypes were discerned among the 181 isolates. The percentage of maximal genotypic diversity (G) ranged between 18 and 33 for the four populations, with a total value of 14 for the 91 genotypes. One genotype, represented by 27 clones, was isolated from all orchards. Moderate, but significant population differentiation was present in total among orchards (mean $F_{ST} = 0.118$, $P = 0.001$). The distribution of the mating types, MAT1-1 and MAT1-2, did not differ significantly from a 1:1 ratio for the total population as well as the subpopulations. Index of association (I_A) analyses, on the other hand, suggests that the populations reproduce asexually. Analysis of molecular variance (AMOVA) indicated that 88% of the total genetic variation is distributed within subpopulations, 9% between subpopulations and only 3% between production areas. Fungicide resistance frequency against fenhexamid, iprodione and benomyl varied, with the highest levels of resistance present against benomyl and low levels of resistance seen towards iprodione and fenhexamid.

In conclusion, this study has shown that there exist within the studied populations of *B. cinerea*, obtained from rooibos nurseries and pear orchards, an adaptive capacity to overcome current means of control. The use of population genetics to further our understanding of how plant pathogens interact and spread throughout a given environment is of cardinal importance in aiding the development of sustainable and integrated management strategies. Knowledge of the dispersal of *B. cinerea* in the two studied cropping systems has shed light on the inherent risk that it poses, and this together with knowledge of the levels of resistance that occurs should serve as an early warning to help divert possible loss of control in future.

Opsomming

Botrytis cinerea Pers. Fr. [teleomorf *Botryotinia fuckeliana* (de Bary) Whetzel] veroorsaak ernstige verliese van meer as 200 gewasse wêreldwyd, insluitende rooibossaaillinge en pere. Hierdie patogeen word deur morfologiese, fisiologiese, asook genetiese diversiteit gekenmerk. Die genetiese diversiteit en populasie-struktuur van *B. cinerea* populasies wat in Suid-Afrika voorkom, is nog nie ondersoek nie. *Botrytis cinerea* verkryg vanaf rooibossaaillinge en in peerboorde in die Wes-Kaap van Suid-Afrika is ondersoek. Hierdie studie is met behulp van mikrosatellietmerkers, amplifikasie van die twee paringstipe gene (*MAT1-1* en *MAT1-2*), asook die bepaling van weerstandsvlakke teenoor verskeie swamdoders, uitgevoer. Populasie-dinamika is afgelei en 'n soortgelyke tendens is in beide produksie-sisteme waargeneem.

Botrytis cinerea veroorsaak jaarliks ernstige verliese van rooibossaaillinge (*Aspalathus linearis*) in kwekerye in die Clanwilliam-area. Monsters is in vyf kwekerye versamel en die kriptiese spesiestatus van die verkrygte isolate is deur restriksie-ensiem-vertering van die *Bc-hch* geen bepaal. Almal behalwe een (206 uit 207) isolaat het aan Groep II of *B. cinerea* 'sensu stricto' behoort. Analise van die *B. cinerea* Groep II populasie, deur middel van sewe mikrosatellietmerkers, is uitgevoer om die genetiese populasie-struktuur te bepaal. Totale geendiversiteit (H) was hoog, met 'n gemiddelde van 0.67. Alhoewel twee van die kwekerye se monstergrootte erg ingeperk is ná kloonverwydering, is daar nogtans 100 genotipes onder die 206 isolate wat geïsoleer is, waargeneem. Die persentasie van maksimale genotipiese diversiteit (G) het tussen 16 en 68, vir die vyf populasies, gewissel, met 'n totaal van 17 vir die 100 genotipes. Een genotipe, verteenwoordig deur 27 klone, is uit vier kwekerye geïsoleer. Relatief lae dog noemenswaardige populasie-differensiasie is in totaal tussen kwekerye waargeneem (gem. $F_{ST} = 0.030$, $P = 0.001$). Die verspreiding van die twee paringstipes (*MAT1-1* en *MAT1-2*) het beduidend verskil van 'n 1:1 verhouding vir die totale populasie, asook twee van die kwekerye se populasies. Die drie oorblywende kwekerye se populasies het egter 'n gelyke verdeling van die twee paringstipes getoon. Die indeks van assosiasie (I_A) analyses toon dat die populasies ongeslagtelik voortplant. Analise van molekulêre variasie (AMOVA) het aangedui dat 97% van die totale genetiese variasie binne die subpopulasies versprei is. Hoogs variërende vlakke van weerstand tussen die vyf kwekerye teenoor die swamdoder iprodion, is vir die 198 isolate wat getoets is, gevind. Die totale gemiddelde frekwensie van weerstand teenoor iprodion was 43%, wat tussen 0% en 81% vir die vyf kwekerye gevarieer het. Fondasie-vlak-sensitiwiteit vir pyrimethanil het 'n gemiddelde EC_{50} waarde van 0.096 mg/L opgelewer.

Botrytis cinerea isolate is ook vanuit peerbloeisels (*Pyrus communis* L.) vanuit vier boorde versamel, twee uit elk van die Ceres- en Grabouw-areas. In totaal is 181 isolate

vanuit die vier boorde versamel. Die frekwensie van bloeiselinfeksie het tussen 3% en 17% gewissel. Oor die algemeen was daar 'n hoë frekwensie van isolate wat slegs die *Boty* transponeerbare element teenwoordig gehad het (74%) in vergelyking met dié wat tegelykertyd beide (*Boty* en *Flipper*) teenwoordig gehad het. Een isolaat het slegs die *Flipper* element gehad. Bepaling van die kriptiese spesiestatus met behulp van restriksie-ensiëmvetering van die *Bc-hch* geen het aangedui dat alle versamelde isolate tot Groep II of *B. cinerea* '*sensu stricto*' behoort het. Analise van die Groep II populasie, deur middel van sewe mikrosatellietmerkers, is uitgevoer om genetiese populasie-struktuur te bepaal. Totale geendiversiteit (H) was hoog, met 'n gemiddelde van 0.69 oor alle populasies. Alhoewel twee subpopulasies 'n hoë klonale fraksie getoon het, is 91 genotipes tussen die 181 isolate wat verkry is, onderskei. Die persentasie van maksimale genotipiese diversiteit (G) het tussen 18 en 33 vir die vier populasies gewissel, met 'n totale waarde van 14 vir die 91 genotipes. Een genotipe, verteenwoordig deur 27 klone, was in al vier boorde teenwoordig. Gematigde dog beduidende populasie differensiasie was in totaal tussen boorde teenwoordig (gem. $F_{ST} = 0.118$, $P = 0.001$). Die verspreiding van die paringstipes (MAT1-1 en MAT1-2) het nie betekenisvol van 'n 1:1 verhouding vir die totale populasie, insluitende die subpopulasies, verskil nie. Indeks van assosiasie (I_A) analyses het egter aangedui dat die populasies ongeslagtelik voortplant. Analise van molekulêre variasie (AMOVA) het aangedui dat 88% van die totale genetiese variasie in subpopulasies te vinde was, 9% tussen subpopulasies en slegs 3% tussen produksie-areas. Frekwensie van swamdoder weerstandbiedendheid vir fenhexamid, iprodion en benomyl het gewissel, met die hoogste vlakke teenoor benomyl waargeneem, maar baie lae vlakke teenoor fenhexamid en iprodion.

Samevattend het hierdie studie getoon dat die populasies van *B. cinerea* wat in hierdie twee produksie-sisteme, op rooibossaailinge en in peer boorde, ondersoek is, 'n aanpasbaarheid toon om huidige metodes van beheer te oorkom. Die gebruik van populasie-genetika as 'n hulpmiddel om ons kennis van patogeen-interaksies en -verspreiding te verbreed, is van kardinale belang in die ontwikkeling van geïntegreerde en volhoubare beheermaatreëls. Kennis van die verspreiding van *B. cinerea* in die bestudeerde gewasproduksiestelsels, werp lig op die inherente risiko wat dié patogeen inhou. Dít, tesame met kennis van die weerstandsvlakke wat voorkom, kan as 'n vroegtydige waarskuwing dien ten einde moontlike verlies van beheer in die toekoms te help teenwerk.

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Chapter 1

A review of *Botrytis cinerea* causing grey mould of rooibos seedlings and calyx-end decay of pears with specific reference to cryptic speciation, population genetic structure and fungicide resistance

1.1 Introduction

Botrytis cinerea Pers. Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a haploid, filamentous, heterothallic plant pathogen that causes losses in over 200 commercial crops worldwide (Giraud *et al.*, 1997; Williamson *et al.*, 2007). The wide host range, varied morphology and genetic diversity of this fungus hinder attempts at control (Fournier *et al.*, 2002; Williamson *et al.*, 2007). *Botrytis cinerea* is a necrotrophic pathogen that causes host cell death of the tissues it infects, resulting in progressive decay (Staats *et al.*, 2005). It can survive as conidia and saprophytically as mycelium on decaying plant matter. For longer periods more resistant structures, called sclerotia, can survive in crop debris (Jones and Aldwinckle, 1997; Staats *et al.*, 2005; Williamson *et al.*, 2007).

Recently, *B. cinerea* has been recognized as consisting of a species complex. What was first observed as two groups of *B. cinerea*, *vacuina* and *transposa* (Giraud *et al.*, 1997) based on the presence or absence, respectively of two transposable elements (TE"s), was later in part confirmed to be two distinct cryptic species (Albertini *et al.*, 2002; Fournier *et al.*, 2003, 2005), which did not strictly coincide with the division set forth in 1997. The first, *B. „pseudocinerea“* or Group I, composed of *vacuina* isolates only, is characterized by low genetic diversity with a restricted host range. The second, *B. cinerea „sensu stricto“* or Group II, composed of *vacuina* and *transposa* isolates, is characterized by high gene diversity and a broader host range. It is considered to be endemic and the more pathogenic of the two species on grapevines (Fournier *et al.*, 2005; Martinez *et al.*, 2005).

The genetic diversity observed in this fungus has long been thought to be due to heterokaryosis (Beever and Weeds, 2004) and aneuploidy (Faretra and Pollastro, 1996). Sexual recombination is not traditionally considered the cause, since apothecia are rarely observed in the field (Giraud *et al.*, 1997). However, several recent studies have challenged this traditional view. High levels of genetic variation,

including evidence of sexual recombination, has been found within Group II in several countries on different hosts (Fournier and Giraud, 2007; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). Investigating the genetic diversity and population structure of any plant pathogen is important to assess the potential to adapt to control strategies such as fungicide applications (McDonald and Linde, 2002).

This review will include an overview of two diseases caused by *B. cinerea* on crops of economic importance in the Western Cape. The disease cycle and management strategies to control grey mould found on rooibos seedlings and calyx-end decay of pears is discussed. Furthermore, an overview is provided of *B. cinerea* in relation to cryptic species status, population genetic structure and chemical control.

1.2 *Botrytis cinerea* in rooibos nurseries

Rooibos [*Aspalathus linearis* (Brum.f) Dahlg.] is endemic in the Cederberg area, specifically in the areas surrounding the Cederberg- and Olifantsriver mountains. Of this area Niewoudtville is the northernmost point and Eendekuil the southernmost (Spies, 2005; Joubert *et al.*, 2008), representing an extremely localized area in the west of the Western Cape in South Africa. Rooibos has been used by the natives of this area for an extended period before the first export of 524 tons in 1955. It is only in recent years that this tea has gained popularity internationally with the amount being exported rising more than nine fold from 1993 to 2007. In 2010, a total of 14000 tons was produced, of which 6000 tons was exported (Johan Brand, Rooibos Ltd, pers. comm.). Tea prepared from rooibos is sought after for its medicinal properties which includes being an anticarcinogenic, antimutagenic, antioxidant and antispasmodic agent (Joubert *et al.*, 2008). With the growing green conscious mindset of today it can only be foreseen that the demand for this product will steadily increase. The fact that the growing of rooibos is restricted to such a small area, which corresponds to its natural habitat, and that attempts to establish it in other areas have failed, means all barriers to its cultivation must be broken down (Spies, 2005; Joubert *et al.*, 2008). The most important foliar disease of rooibos seedlings (Fig. 1; A) in South Africa is grey mould (causal agent *B. cinerea*) and in the worst case scenario can cause losses of up to 80% in a season if not managed properly (Sandra Lamprecht, pers. comm.).



Fig. 1. Rooibos seedling production in the Clanwilliam area of the Western Cape of South Africa with A; Example of an apparently healthy nursery B; Grey mould of rooibos seedlings in adjacent rows C; Severe *B. cinerea* infection of seedlings as seen from above D; and from the side.

1.2.1 Disease cycle

Grey mould of rooibos seedlings was first reported in 1994 (Sandra Lamprecht, pers. comm.). It was, however, probably present before that time since *B. cinerea* is such a ubiquitous plant pathogen. *Botrytis cinerea* attacks the seedlings at three to four months of age, infecting the lower parts of the stems and leaves, which in turn causes wilting and dieback of these parts (Fig. 1; C-D). Older seedlings are more susceptible to this disease than younger ones, and this could be due to several factors. The denser canopy creates a humid microclimate and along with less penetration of fungicides and light, creates conditions conducive to the spread of this disease (Spies, 2005). The presence of *B. cinerea* on plant material outside nurseries has been confirmed as well as the presence of airborne inoculum within nurseries (Spies, 2005). Inside the nursery *B. cinerea* has been observed on organic debris. It is also present on weeds such as wild radish (*Raphanus raphanistrum* L.) and devil's thorn (*Emex australis* Steinh.). It appears that the seeds of these two weeds are usually infected. These seeds can be present within the soil, or can be carried into the nurseries, where they then grow into infected plants (Spies, 2005).

1.2.2 Management and control

Cultural and chemical management strategies, are recommended for grey mould by Rooibos Ltd. (Rooibos Ltd., P.O. Box 64, Clanwilliam, 8135, South Africa) to its growers. Cultural management strategies include sowing approximately 15 g of seed per meter in an east-west orientation with 10 cm row spacing. These are all aimed at minimising the humidity around seedlings. Farmers are advised to water their seedlings in the morning so as to reduce the presence of free water on the plants at night. The final recommendation is to frequently remove weeds in the nurseries (Spies, 2005). Management of weeds inside and outside the nursery is crucial to limiting the spread of this fungus to healthy seedlings. Nursery locations are rotated each year, with the same seed bed only being planted once every four years. However, despite these cultural practices *B. cinerea* still remains a problem which requires the use of fungicides at regular intervals. Current fungicide recommendation is a once-off application of iprodione (dicarboximide) to seedlings at four weeks of age. This is followed by iprodione and pyrimethanil (anilinopyrimidine) applications

alternated weekly starting at 11 weeks of age until transplanting, at more than 20 weeks of age. Multiple fungicide applications are not ideal for the high demand for organically produced rooibos.

1.3 Calyx-end decay of pears caused by *Botrytis cinerea*

Postharvest decay of pears (*Pyrus communis* L.) causes economic losses each year due to loss of product and repacking costs after the culled fruit has been removed. Three types of postharvest decay of pears occur due to *B. cinerea* infection. These are stem-end-, puncture wound- and calyx-end decay (Jones and Aldwinle, 1997). For the export market it is especially important to limit the incidence of decay. Generally, levels of 2% infection or more in a random sample of pears reaching its destination leads to the rejection of the whole consignment (Cheryl Lennox, pers. comm.). Approximately 10 million cartons (12.5 kg each) were passed for export in 2006 and this number rose to approximately 15 million cartons in 2010. Taking this into account, the economic losses can be considerable. Calyx-end decay is an important cause of postharvest rot of „Packham’s Triumph” and „Forelle” pears and these cultivars represent 29% and 26% respectively, of the total hectares planted per cultivar in South Africa (Anon. 2010). The overview of the disease cycle will focus on inoculum sources responsible for blossom infection which leads to eventual calyx-end decay.

1.3.1 Disease cycle

Infection leading to calyx-end decay occurs at or around full bloom (Fig. 2). Natural infection of floral parts, especially styles and stamens has been shown (Sommer *et al.*, 1985). Preharvest levels of calyx infection are, however, a poor indicator of postharvest incidence of grey mould (Lennox and Spotts, 2004). In a histological study done by De Kock and Holz (1992) on artificially inoculated flowers they found that eventual calyx-end decay invariably spreads from mesocarp tissue adjoining the sepals in a radial manner into the vascular bundles. It was found that latent infection is initiated through spores that land on an anther and subsequently grows down the filament and then through the sepals into tissues at the top end of

the flower receptacle. It can also grow into the mesocarp that adjoins the sepals. Spores that landed on the stigma never progressed far enough down the styles to enter the carpel. Through the knowledge of the manner in which *B. cinerea* enters the flower components it was suggested that postharvest control of grey mould could be achieved by spraying at 75% -100% petal fall and again one month later, when floral tubes start to close (Sommer *et al.*, 1985; De Kock and Holz, 1992).

Propagules of *B. cinerea* can be found in a variety of sources pre- and post harvest and all may serve as a potential source of postharvest decay. These include the soil, plant litter, air and the packinghouse dump tank water (Lennox *et al.*, 2003). It is, however, taken for granted that the initial inoculum of the pathogen occurs in the orchard where it can be dispersed as wet/dry conidia that can land upon dry or wet fruit (Lennox *et al.*, 2003). These conidia can come from sclerotia that germinate when conditions are favourable (Spotts and Serdani, 2006). Sclerotia are melanized survival structures which encase the mycelium and protect against desiccation, UV-radiation and attack by microbes (Williamson *et al.*, 2007). Mycelium originating from sclerotia produces conidiophores along with conidia which serve as primary inoculum sources within the orchard. Sclerotia can also germinate carpogenically, developing into an apothecium through photomorphogenesis, which can then produce ascospores, another possible source of infection (Amselem *et al.*, 2011). However, the frequency of this event is debated as apothecia have rarely been observed in the field or in orchards (Giraud *et al.*, 1997; Spotts and Serdani, 2006). In Oregon in the United States, few sclerotia were found from the middle of summer until fall on naturally infected pear fruit on the orchard floor. Production of sclerotia increased dramatically from middle November to middle December and it was found that 90% of these sclerotia were viable the following season. The fruit on the orchard floor had by that time become so decomposed that the sclerotia were mixed in with the plant debris in the top layer of litter (Spotts and Serdani, 2006).

Mycelium within the orchard can also survive within dead host tissue, such as dead leaves, flowers and mummified fruits and are usually ideally situated to initiate infections through mycelium and conidia (Williamson *et al.*, 2007). Conidia generated at a primary source of infection follow a well-defined daily cycle that starts with initiation, production and dissemination which is regulated by variations in temperature and humidity (Williamson *et al.*, 2007). Typically, a rapid decline in humidity together with a rise in temperature in the early morning causes a twisting and drying event of the conidiophores which then eject their conidia into air currents

through gusty winds, as individuals or as small clumps (Williamson *et al.*, 2007). Rain splash dispersal up to 2 m also occurs and human mediated dispersal has also been pinned as a possible vector (Jarvis, 1980). Water dispersal postharvest in packinghouses plays a role where conidia are carried in the flumes. When a *B. cinerea* infection becomes established in a wound or calyx on the pear it can spread to adjacent fruit from the original infection quite easily. This is known as nesting (Jones and Aldwinckle, 1997). Therefore it is preferable to pack and ship the pears within three to six months, under controlled atmosphere conditions to lessen economic losses due to grey mould (Lennox and Spotts, 2004).



Fig. 2. A; Pear orchard in full bloom in the Western Cape of South Africa B; A bee pollinating a blossom C; A variety of weeds growing beneath a pear tree D; Latent grey mould infection emerging from a weed after two weeks in a moisture chamber with black sclerotia indicated by white circles (Photos A and B courtesy of W. Bester).

1.3.2 Management and control

Cultural methods to control grey mould of pears rely mainly on orchard sanitation (Spotts and Serdani, 2006), such as removal of fallen fruit and dead vegetative material from the orchard. Effective control is, however, only achieved in conjunction with the application of fungicides. Studies done by Lennox *et al.* (2004) found that culled pears from the Mid-Columbia region in Oregon and in Washington contained a high percentage of pears decayed due to *Botrytis* infections (2.26%). Their study revealed that puncture wound decay caused by *Botrytis* was significantly higher than stem-end and calyx-end decay (1.13%, 0.84% and 0.36%, respectively) (Lennox *et al.*, 2004). In a similar study done by Spotts *et al.* (1998) it was found that over a four year period the incidence of wounding on „D“ Anjou“ pear fruit was 2.9% during harvest and handling. Interestingly the incidence of wounding during handling on „Beurre Bosc“ pears was 4.3% if harvested by workers paid by the hour, but was 13.9% when the workers were paid by the number of bins harvested (Spotts *et al.*, 1998). These results indicate that delicate handling of the pears, during harvest and postharvest, is crucial to limiting at least the stem-end and puncture wound decay types. It was found that benomyl applications reduced the *B. cinerea* levels in the blossoms themselves, but had no effect on the eventual calyx-end decay in storage of „d“Anjou“ pears (Lennox and Spotts, 2004). Infection of the stems and puncture wounds can be limited by protecting the abscission zone of the stem and puncture wounds by application of a biological and / or chemical fungicide. Such a strategy should reduce the levels of losses in storage (Lennox *et al.*, 2003).

Current recommendations in South Africa to combat calyx-end infection of pears preharvest, is to apply a dicarboximide (iprodione or procymidone) at full bloom and/or 75% petal fall („Bon Chretien“, „Buerre Bosc“ and „Packham“s Triumph“ pears only). The benzimidazole, benomyl, is registered as a full bloom and/or 75% petal fall spray, but in practice is rarely used anymore. Iprodione is also registered for postharvest control („Packham“s Triumph“ only) and is used as a dip or drench treatment postharvest. This is not advisable if a dicarboximide fungicide has been used at bloom, since continued applications of a fungicide with the same active ingredient lead to the selection of resistant strains (Lennox and Spotts, 2003). Therefore, the use of different active ingredients should be encouraged. Effective preharvest treatments, chemically and biologically, should reduce losses caused by

calyx-end decay. However, this needs to be established through trials under South African conditions.

1.4 Cryptic speciation within *Botrytis cinerea*

One of the first studies which helped prove that *B. cinerea* was composed of two cryptic species and set the benchmark for *B. cinerea* population genetics was done by Giraud *et al.* (1997). They found that by using PCR-RFLP and *transposable* elements as markers, *B. cinerea* could be divided into two sympatric species. The transposable elements (TE's) that were characterized were *Boty*, a 6 kb gypsy like retrotransposon, and *Flipper*, a 1842 bp transposable element (Diolez *et al.*, 1995; Levis *et al.*, 1997). On the basis of the presence or absence of these TE's they divided their isolates into two groups. *Transposa* contained the transposable elements *Boty* and *Flipper*, and *vacuma* which did not contain any TEs. Strains that only contained the *Boty* element were also identified. The two groups were also different from one another in all the other markers tested (Giraud *et al.*, 1997). These findings were then confirmed by Giraud *et al.* (1999) by using different PCR-RFLP markers, found by SWAPP (sequencing with arbitrary primer pairs) in addition to the markers used in their previous study. They again found a high degree of genetic diversity. They tested whether there was a difference between the isolates from the 21 plants and their locations and found none. They did, however, find a significant difference in the TE types occurring on different hosts. For instance, *vacuma* strains were prevalent on green peas and *transposa* was more prevalent on bramble (Giraud *et al.*, 1999).

For various plant species, isolates taken from flowers, calyptras and stamens, *vacuma* prevailed (59%) in early season (Giraud *et al.*, 1999). This was also the case for vineyards in Champagne where *vacuma* strains was isolated just after flowering and made up more than 50% of the total *B. cinerea* population (Giraud *et al.*, 1997). In a study by Martinez *et al.* (2003) near Bordeaux they found that *vacuma* strains had the fastest mycelial growth rate and was present in 72.7% of isolates taken from blossoms versus 15.8% taken from leaves. It was suggested that this is an indication of greater saprophytic capability and that it explains the fact that *vacuma* isolates are more frequently collected from senescing floral parts. It was also established that there was a definite negative correlation between mycelial growth rate and virulence

in several studies. Consequently it was suggested that *vacuma* might follow a more ruderal lifestyle compared to *transposa* although there was no significant difference in their sporulating capability determined in these studies (Martinez *et al.*, 2003, 2005). This illustrates the idea that *vacuma* and *transposa* strains differ in their ability to infect different hosts, which then implies that the TE status might confer some traits. The study of Giraud *et al.* (1999) agreed with the findings that the two groups of *B. cinerea* occur in sympatry on different hosts.

Muñoz *et al.* (2002) developed a duplex PCR test to amplify the TE's and obtained similar results in their Chilean samples as observed by other authors. They found that *vacuma* and *transposa* isolates occurred and were sympatric in their populations. Isolates that contained only the *Boty* element were also detected as in France. There was great genetic diversity in their samples which indicates the absence of clonal reproduction. They also found significant differences on isolates collected from kiwifruit compared to blueberries, tomatoes and grapes. This again seems to indicate differential infection capabilities of the strains differing in TE type (Muñoz *et al.*, 2002). Taking this study and the work done by Giraud *et al.* (1997; 1999) into consideration it appears that *B. cinerea* has some form of host specialization despite the traditional view. Other studies also suggest this using various markers (Thompson and Latorre, 1999; Muñoz *et al.*, 2002; Fournier and Giraud, 2007; Karchani- Balma *et al.*, 2008; Váczy *et al.*, 2008).

In 2002 Albertini *et al.* found that among their *vacuma* isolates there were some that were resistant to fenhexamid and had increased sensitivity to 14 α -demethylase inhibitors (DMIs). They set about determining whether mutations at the target gene level (*CYP51*) were responsible for the increased sensitivity to DMIs in strains resistant to fenhexamid. The gene is highly polymorphic, with polymorphisms discriminating not between *transposa* and *vacuma* strains, but rather between *vacuma* isolates resistant to fenhexamid and sensitive *vacuma* isolates (Albertini *et al.*, 2002). This division of *B. cinerea* was further clarified by Fournier *et al.* (2003) who characterized and cloned the gene *Bc-hch* which is the homolog of the *Neurospora crassa* *het-c* vegetative incompatibility locus. By screening an 1171 bp fragment of the gene for polymorphism using the restriction enzyme *HhaI* they found two restriction digest patterns. The one allele they named *Bc-hch1* and the other *Bc-hch2*. When they tested fungicide resistance profiles of their isolates they found that fenhexamid resistance was strongly associated with the *Bc-hch1* allele and coincidentally that all these isolates were of the *vacuma* type. On the other hand all

the isolates that had the second allele were sensitive to fenhexamid and were of the *vacuma* or *transposa* type. They divided the isolates into two groups on the basis of these observations, Group I containing all the *vacuma* isolates resistant to fenhexamid and Group II, all those that were sensitive and of the *vacuma* or *transposa* type (Fournier *et al.*, 2003). *Vacuma* isolates resistant to fenhexamid has been named *B. „pseudocinerea”* and isolates that are sensitive and of the *vacuma* or *transposa* TE type, as *B. cinerea „sensu stricto”* (Fournier *et al.*, 2002).

Fournier *et al.* (2005) further illustrated that Group I and II are different cryptic species which cannot interbreed using multiple gene genealogies. They sequenced the polymorphisms in the *CYP51* and *Bc-hch* genes as done by Albertini *et al.* (2002) and Fournier *et al.* (2003), and included two other nuclear genes, β -tubulin and the 63R locus. Their results indicated that *B. cinerea* consistently clustered into two clades within these gene phylogenies, Groups I and II (Fournier *et al.*, 2005). Furthermore, Group I strains had significantly longer conidia when compared with Group II. Group I is also characterised by a narrower host range and constricted temporal distribution, being present mainly in spring on grapevine, whereas Group II are equally prevalent in spring and autumn (Fournier *et al.*, 2005). Group I isolates also had a lower genetic diversity compared with that of Group II, which undergoes regular recombination (Fournier and Giraud, 2007; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). Groups I and II have been reproductively isolated for a long time, with negligible gene flow and migration between the two groups (Fournier *et al.*, 2005). Low genetic diversity in Group I could be due to demographical processes such as a founder effect, strong bottleneck, selective sweep (Galtier *et al.*, 2000), the absence of sexual reproduction (Tibayrenc *et al.*, 1991) or genetic drift.

1.5 Population genetics of *Botrytis cinerea*

Due to changing agroecosystems a new understanding of pathogen epidemiology is needed to ensure efficient control of plant diseases (Gilligan, 2008). Advances in molecular methods used to infer population genetics have generated large datasets that pose analytical challenges, but in turn yield a wealth of information aiding in a better understanding of plant diseases (Linde, 2010). Knowledge of genetic structure and reproductive mode is of cardinal importance to assist in the development of control strategies specific to a plant pathogen. Due to

the movement of pathogen inoculum, improved control strategies should not be limited to one field or producer. Rather, conglomerates of regional growers working together will achieve better control than those trying to optimise yields within a given field (Gilligan, 2008).

Investigating plant diseases includes the study of selectively neutral genetic variation between and within plant pathogen populations, better known as population genetics (Hartl and Clark, 1997). Population genetics attempts to gauge the relative importance of evolutionary forces such as selection, recombination, genetic drift, migration and mutation, together with changes in gene frequencies in shaping genetic structure of populations (Linde, 2010). In plant pathogens, the interactions of these evolutionary forces are important. In combination with the specific life history traits of the pathogen (Barrett *et al.*, 2008), the pathogen's ability to adapt and rapidly overcome control measures such as fungicide treatments or resistance breeding, can be ascertained (McDonald and Linde, 2002). McDonald and Linde (2002) provide an excellent review on the different evolutionary forces. The present review will include evolutionary forces and population parameters that have been determined for *B. cinerea* populations in various agroecosystems.

Several techniques have been used to study the genetic diversity of *B. cinerea*. Restriction fragment length polymorphism (RFLP) of several genes (IGS, ATP synthase, ADP/ATP translocase and nitrate reductase) have been used to reveal the existence of genetic structure in *B. cinerea* populations sampled from grapevine (Giraud *et al.*, 1997). In a study done by Moyano *et al.* (2003), the use of random amplified polymorphic DNA (RAPD) and amplified-fragment length polymorphism (AFLP) were compared as means to study genetic diversity in *B. cinerea*. Polymorphisms were detected more frequently per primer using AFLP, although RAPD detected polymorphisms per loci more frequently. In conclusion they found that diversity was higher when determined with RAPD than AFLP and interpretations of genetic relationships between isolates could be done effectively (Moyano *et al.*, 2003). RAPD has been used to reveal differentiation between populations from grapes and tomato (Muñoz *et al.*, 2002). Microsatellite markers are, however, increasingly being used to understand the population structure of *B. cinerea* and other plant pathogens (Fournier *et al.*, 2002; Banke and McDonald, 2005; Koopman *et al.*, 2007; Hunter *et al.*, 2008; Scott and Chakraborty, 2008; Atallah *et al.*, 2010). Due to the high mutation rates of microsatellites, these markers are ideal tools for use in population genetics studies. They are expected to be more

sensitive to recent demographic and phylogeographic events than previously employed molecular markers such as RFLP and housekeeping DNA sequence loci (Linde, 2010). Fournier *et al.* (2002) characterized nine polymorphic microsatellite markers for *B. Cinerea*. Studies conducted using these markers have shown that Group II *B. cinerea* populations are characterized by a high genetic diversity (Fournier and Giraud, 2007; Isenegger *et al.*, 2008a, 2008b; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). In spite of the lack of visual confirmation of apothecia in the field (Giraud *et al.*, 1997; Spotts and Serdani, 2006) sexual reproduction has been proven, albeit indirectly, through the combination of several methods.

Calculation of the index of association ($I_A = (V_O/V_E) - 1$) indicates the relative importance of genetic recombination for a plant pathogen and is used to infer fungal population structure (Milgroom, 1996). The I_A is a generalized measure for linkage disequilibrium (Brown *et al.*, 1980; Maynard-Smith *et al.*, 1993; Haubold *et al.*, 1998). It gives information on whether two different individuals sharing an allele at one locus are more likely to share an allele at another locus (Karchani-Balma *et al.*, 2008). The index is computed as follows; for any two paired individuals, the number of loci with which they differ is calculated. The variance of this number is then compared with the expected variance if there were no linkage disequilibrium, or in other words, linkage equilibrium. The value is expected to be zero if the null hypothesis is met, which is that of complete panmixia, *i.e.* a randomly mating population, with no association between loci (Maynard-Smith *et al.*, 1993; Fournier and Giraud, 2007). This value increases as the linkage disequilibrium increases. Strictly speaking all other factors (*e.g.* genetic drift, selection, migration *etc.*) have to be excluded in order to conclude that non-random mating or asexual reproduction is leading to observed population structure (Milgroom, 1996). The observation of linkage equilibrium between microsatellite loci in some studies (Fournier *et al.*, 2005; Fournier and Giraud, 2007) is contrasted by others which indicate that microsatellite loci are in linkage disequilibrium (Ma and Michailides, 2005; Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). The study done by Ma and Michailides (2005) in California used microsatellite primed (MP) - PCR and it is suggested to have indicated mostly the presence/absence of the markers and not the length as determined using microsatellites, leading to the observed linkage disequilibrium (Fournier and Giraud, 2007). To account for the observed linkage disequilibrium seen in the study done by Isenegger *et al.* (2008b), they proposed that anthropogenic activity may be responsible or that the studied populations represented recent

founder events, which in turn resulted in disturbance of gene flow and genetic drift, thereby affecting their linkage disequilibrium analysis.

Further indirect evidence for sexual recombination is the fact that both mating types of *B. cinerea*, MAT1-1 and MAT1-2, are widespread in nature (Faretra *et al.*, 1988; Beever and Parkes, 1993; Faretra and Pollastro, 1993; Delcán and Melgarejo, 2002; Angelini *et al.*, 2010). Faretra *et al.* (1988) found that approximately 16% of field strains and 6% of ascospore progeny were homothallic (self fertile) and able to cross with reference strains carrying both mating types. The remainder of both types of sampled isolates was heterothallic (self sterile), and able to produce sexual progeny with reference isolates carrying one of the mating type genes (Faretra *et al.*, 1988). In the study of Delcán and Melgarejo (2002) they found 31% of the isolates to be homothallic and the remaining 69% were heterothallic. Mating types were widespread and both types of isolates present irrespective of sampling point, whether spatial or temporal (Delcán and Melgarejo, 2002). Pairs of homothallic ascospores were found in 7 out of 105 asci by Faretra and Pollastro (1996) and they concluded that homothallism in mono-ascospore isolates is not due to inclusion of multiple nuclei or through mutation of *MAT1-1* to *MAT1-2*, or vice versa. Comparison of the *MAT1* locus of *Sclerotinia sclerotiorum* and *Botrytis fuckeliana* has suggested that heterothallism evolved from homothallism in the family *Sclerotiniaceae* (Amselem *et al.*, 2011). Determination of mating type distribution within a pathogen, if possible, is the simplest way to ascertain the potential for sexual recombination and thus evolutionary potential. It is assumed that when a 1:1 ratio between mating types within a population is observed, they are randomly mating because there appears to be frequency dependant selection of each type.

Genotype diversity refers to the number and frequencies of unique individuals, or multilocus genotypes, within a population (McDonald and Linde, 2002). The presence of repeated multilocus genotypes within a population is one of the most obvious means of detecting asexual reproduction, although they cannot serve as definitive proof thereof (Halkett *et al.*, 2005). Fungi that undergo sexual reproduction are expected to have more genotype diversity than those reproducing asexually (Milgroom, 1996). The numbers and frequencies of clones within a population can vary as a result of directional pressures such as fungicide applications which favour resistant clones. Genetic drift (founder effects and bottlenecks) and genotype flow may also influence the genotype diversity within a population (McDonald and Linde, 2002). Clonal distribution and frequency of genotypes

between and within populations can be inferred based on microsatellite profile matches. One index of genotypic diversity (\hat{G}) is denoted by the following formula,

$$\hat{G} = 1 / \sum_{x=0}^n [f_x \cdot (x/n)^2]$$

where; n = sample size, f_x = number of distinct haplotypes observed x times within a population (Stoddard and Taylor, 1988). Values for \hat{G} can range between 1, when all individuals have the same genotype, to n (sample size) if each individual has a unique genotype (Milgroom, 1996). For comparison between populations, the index (\hat{G}) is divided by the number of samples to obtain the percentage of maximal genotypic diversity (G) (McDonald *et al.*, 1994).

Determination of migration and migration routes are important to assess the threat that a pathogen poses. The distance a pathogen is able to disperse gives an indication of its ability to colonise areas free of disease or novel fungicide resistant strains. The number of migrants exchanged between populations gives an indication of how panmictic, or randomly mating, populations are (Linde, 2010). Knowledge of gene flow (migration) is important especially in quarantine and disease management. Gene flow is a major evolutionary force in determination of genetic variation and thus differentiation between populations (Isenegger *et al.*, 2008b). Traditionally, migration rates are estimated from Wright's F_{ST} and is used to infer patterns of gene flow (Wright 1951; Slatkin and Barton, 1989). It is a measure of the genetic differentiation of a subpopulation relative to the total population due to non-random mating. Values can range between 0 (no population differentiation) and 1 (complete population differentiation, with no gene flow between populations). Thus a low F_{ST} value indicates that most of the diversity is found within the subpopulation and not much between the populations. Conversely, a high value indicates substantial genetic differentiation between the populations. In some studies, sampled populations of *B. cinerea* have shown low to moderate differentiation as a result of unrestricted gene flow, regardless of host plant of origin or geography (Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). Other studies have revealed some form of genetic differentiation between isolates sampled from different hosts/phenological stages (Giraud *et al.*, 1997; Muñoz *et al.*, 2002; Fournier *et al.*, 2007; Esterio *et al.*, 2011), although not always through the use of microsatellite markers developed by Fournier *et al.* (2002). The situation as to whether or not *B.*

cinerea truly displays some form of host specialization needs to be further investigated.

1.6 Chemical control of *Botrytis cinerea*

Control of *B. cinerea* is achieved through an integrated strategy involving cultural management practices along with fungicides from several classes. However, chemical control represents the most effective means of limiting disease outbreaks in agricultural systems. This may change if discretion is not applied in managing the use of the limited chemistries that are available to us. Fungicide resistance in *B. cinerea* has been reported worldwide, often with a high frequency of resistant phenotypes in studied populations (Leroux *et al.*, 1999; Yourman and Jeffers, 1999; Lennox and Spotts, 2003; Myresiotis *et al.*, 2007; Korolev *et al.*, 2009; Sun *et al.*, 2010; Zhao *et al.*, 2010; Leroch *et al.*, 2011). In the mid-1990s novel botryticides were released, expanding the spectrum for resistance management (Rosslenbroich and Stuebler, 2000). These included; pyrimethanil, mepanipyrim and cyprodinil (anilinopyrimidines), fenhexamid (hydroxyanilide) and fludioxonil (phenylpyrrole). Resistance as well as cross-resistance to these new actives has been reported in Europe (Leroux *et al.*, 1999; Myresiotis *et al.*, 2007; Korolev *et al.*, 2009, 2011; Sun *et al.*, 2010). Despite these observations, with the addition of these three chemistries for the control of grey mould, resistance in *B. cinerea* should be properly managed.

Benzimidazoles (e.g., benomyl, carbendazim, thiabendazole and thiophanate-methyl) were first introduced in the late 1960's (Lyr, 1995). The effectiveness of this chemical group was rapidly overcome by high level resistance development in strains (Ben^{R1}) of *B. cinerea* which displayed negative cross-resistance towards the *N*-phenylcarbamates (Chapeland *et al.*, 1999). Use of *N*-phenylcarbamates (e.g., diethofencarb) applied in conjunction with benzimidazoles was then recommended, but this led to positive cross-resistance to diethofencarb (Ben^{R2}). Changes in the amino acid composition of the β -tubulin protein due to point mutations result in reduced binding efficacy and hence resistance towards benzimidazoles (Yarden and Katan, 1993; Chapeland *et al.*, 1999; Banno *et al.*, 2008). Mutations at amino acid positions 198 and 200 of the β -tubulin gene *BenA*, confers resistance to benzimidazoles in the field for several plant pathogens (Koenraad *et al.*, 1992; Yarden and Katan, 1993; Albertini *et al.*, 1999; Ma and Michailides, 2005). Four

benzimidazole resistant phenotypes from fields have been identified (Yarden and Katan, 1993; Leroux *et al.*, 2002; Banno *et al.*, 2008).

Botrytis cinerea isolates resistant towards benzimidazoles and dicarboximides (e.g., iprodione, procymidone and vinclozolin) simultaneously, are widespread (Yourman and Jeffers, 1999; Kalamarakis *et al.*, 2000; Leroux *et al.*, 2002; Banno *et al.*, 2008). In South Africa, Fourie and Holz (1998) reported cross-resistance between dicarboximides and benzimidazoles in isolates collected from table grape vineyards (Fourie and Holz, 1998). Genetic analyses have confirmed that resistance towards dicarboximides is conferred by a single locus, *Daf1* (Faretra and Pollastro, 1991), which has been confirmed as the two-component histidine kinase or *Bos1* gene (Cui *et al.*, 2002). Up to date eight different mutations have been identified within the *Bos1* gene (Leroux *et al.*, 2002; Oshima *et al.*, 2002; Cui *et al.*, 2004; Banno *et al.*, 2008; Ma *et al.*, 2007; Fujimara, 2010), which has been shown to be associated with virulence (Ma *et al.*, 2007). A rapid method for genotyping the *BenA* and *Bos1* genes has been developed by Banno *et al.* (2008) and a fluorescent hybridization probe is used in real-time PCR in order to monitor fungicide resistance towards benzimidazoles and dicarboximides in field populations. Extension of this technique to the South African *B. cinerea* populations could provide a useful tool for rapid screening of resistance in field populations.

The hydroxylanilide fenhexamid is one of the only fungicides of the C-4-demethylation inhibitors used in agriculture. Three categories, HydR1, HydR2 and HydR3 classify resistance to fenhexamid (Leroux, 2004). *Botrytis „pseudocinerea”* or Group I strains belong to the HydR1 category and are naturally resistant to fenhexamid (Albertini *et al.*, 2002; Fournier *et al.*, 2003, 2005). A possible explanation proposed for this by Fournier *et al.* (2003) is that the cryptic species within *B. cinerea* have become adequately genetically differentiated to have fixed several private alleles, which happens to include the resistance alleles. These strains were detected before introduction of fenhexamid, although efficacy in the field does not appear to be affected (Suty *et al.*, 1999; Esterio *et al.*, 2007). This could be due to resistance in HydR1 strains only being displayed during mycelial growth (Fillinger *et al.*, 2008). *Botrytis cinerea* Group I strains are more susceptible to various classes of fungicides, including inhibitors of sterol Δ^{14} -reductase (e.g. fenpropidin and fenpropimorph) and demethylation inhibitors (DMIs) (Albertini *et al.*, 2002; Leroux *et al.*, 2002). Sequence variation within the genes *CYP51* and *erg27*, encoding eburicol 14 α -demethylase and 3-ketoreductase, respectively, could explain the DMI

hypersensitive and fenhexamid resistant phenotypes (Albertini *et al.*, 2002; Albertini and Leroux, 2004). These strains appear to metabolize fenhexamid more rapidly than strains of *B. cinerea* Group II type (Suty *et al.*, 1999). Indirect evidence shows the involvement of a cytochrome P450 mono-oxygenase (P450) in fenhexamid detoxification (Leroux *et al.*, 2000).

Botrytis cinerea Group II isolates contain the HydR2 and HydR3 resistance categories, which were found prior to registration of fenhexamid in Germany and Japan (Leroux *et al.*, 2002). These were moderately (HydR2) to highly (HydR3) resistant towards fenhexamid in mycelial growth studies, with only HydR3 isolates having resistance during germ tube elongation (Leroux *et al.*, 2002). The HydR3 isolates could further be divided into strains belonging to HydR3⁺ and HydR3⁻. This division corresponds with highly and weakly to moderately resistant isolates (Fillinger *et al.*, 2008), according to EC₅₀ values (mycelial growth) determined by these authors. Resistance in HydR3 was characterized as being conferred by mutations in the *erg27* gene, encoding a 3-ketoreductase (Albertini and Leroux, 2004; Fillinger *et al.*, 2008). Mutations at position 412 of the C terminus of the putative transmembrane domain conferred high levels of resistance towards fenhexamid (HydR3⁺). A range of point mutations corresponding to amino acid changes between positions 195 to 400 of the protein characterized the HydR3⁻ isolates (Fillinger *et al.*, 2008). In two HydR2 strains studied by Albertini and Leroux (2004) no mutations within the *erg27* gene was detected. The mechanism of resistance in HydR2 strains is not clear yet, but the involvement of a P450, different from that of resistance in HydR1 strains, is hypothesised (Leroux *et al.*, 2001).

Anilinopyrimidines in use today are cyprodinil, mepanipyrim and pyrimethanil. Three resistant phenotypes have been detected. The most resistant of these phenotypes (AniR1), only displayed resistance towards anilinopyrimidines (Chapeland *et al.*, 1999). One major gene (*Ani 1*) is proposed to be involved in conferring resistance in AniR1 strains (Fritz *et al.*, 2003). The proposed target site for the anilinopyrimidines has been the cystathione β -lyase in the methionine biosynthesis pathway, however no amino acid changes were detected in this protein (Leroux *et al.*, 2002; Fritz *et al.*, 2003) in a comparison of sensitive to resistant isolates. The exact mechanism of resistance towards anilinopyrimidines remain to be elucidated, but it is proposed to be similar to fenhexamid resistance (Leroux *et al.*, 2002; Fillinger *et al.*, 2008).

The absence of fitness penalties along with phenotypic stability has been illustrated for the anilinopyrimidines (Moyano *et al.*, 2003; Bardas *et al.*, 2008) and benzimidazoles, but not for dicarboximides (Yourman *et al.*, 2001). Resistance stability towards dicarboximides has only been illustrated for strains that are resistant towards benzimidazoles and dicarboximides, simultaneously (Yourman *et al.*, 2001). The absence of correlations between fitness and resistance has been reported in several plant-pathogen systems (Peever and Milgroom, 1994; Raposo *et al.*, 1996; Karaoglanidis *et al.*, 2001; Martinez *et al.*, 2005). These observations support the hypothesis that the observed fitness differences between sensitive and resistant isolates may in fact be due to differences in genetic background rather than a real fitness cost (Martinez *et al.*, 2005). Quantitative polygenic-controlled resistance is more frequently associated with fitness costs than qualitative monogenic-controlled resistance. Considering the fact that anilinopyrimidine and benzimidazole resistance are phenotypically stable traits which are monogenically controlled, disruptive selection is to be expected (Chapeland *et al.*, 1999; Bardas *et al.*, 2008). In the case of dicarboximide resistance, selection pressure will need to be continuously applied in order to maintain the resistance within a population. It has been shown through a sensitivity monitoring program that even with stable benzimidazole resistance in *Cercospora beticola*, the resistance frequency significantly declined after many years of cessation of benomyl application (Karaoglanidis *et al.*, 2003). Thus, even with predictions for durable and long-term resistance, the sensitivity of a given population should be continuously monitored to detect changes in resistance frequency. Existence of fitness costs necessitates management strategies such as alterations of fungicides with differing biochemical modes of action. If there are no fitness costs associated with the development of resistance, other management strategies such as mixtures, reduction in either spray dose or number of applications should be implemented.

In the past the main mechanisms of resistance were site-specific, resulting in reduced binding efficacy of the drug in question. What is becoming clear is that the frequency of isolation of *B. cinerea* displaying multidrug resistance (MDR) from European vineyards has risen sharply in the last decade (Leroux, 2004; Bardas *et al.*, 2010; Kretschmer *et al.*, 2009; Leroch *et al.*, 2011). These resistance mechanisms are often associated with constitutive overexpression of either the ATP-binding cassette (ABC) - or major facilitator superfamily (MFS) transporters (Esterio *et al.*, 2011). These transporters have low substrate specificity and confer MDR to

unrelated classes of fungicides (e.g. dicarboximides, phenylpyrroles and sterol biosynthesis inhibitors). It is considered to be MDR even if a single gene is responsible for the multiple resistances (Leroux *et al.*, 2010). Multidrug resistance is a well documented phenomenon in human pathogens, but has only recently started to emerge in plant pathogens. Resistance towards individual fungicides in MDR strains is as yet still much lower than those of the site-specific mutations that confer resistance toward specific fungicides (Kretschmer *et al.*, 2009).

Long term use of iprodione in rooibos nurseries, has led to resistance in *B. cinerea*. Research by Spies (2005) in the 2003/2004 growing seasons indicated that the mean resistance of isolates towards 1.0 mg/L iprodione collected inside and outside nurseries fluctuated from approximately 22% in March, to 15% in May before again rising to 23% in July. Reassessing the resistance levels of iprodione is needed and together with determination of the baseline sensitivity towards pyrimethanil would be invaluable to aid in decision making regarding the continued usage of these fungicides and application of alternate chemical management strategies in rooibos nurseries.

Little information is available regarding the status of *B. cinerea* fungicide resistance in South African pear orchards. As calyx-end decay results from infection during bloom in the orchard, it requires a preharvest management strategy (Sommer *et al.*, 1985; De Kock and Holz, 1992). However, the exact relationship between blossom infection and subsequent calyx-end decay has been unclear. Lennox and Spotts (2004) found that the mean incidence of calyx-end grey mould of „d'Anjou" pears was 1.2 and 0.2% in 1996 and 1997 in Oregon (USA), respectively. Together with these results it was found that application of benomyl in the orchard reduced levels of blossom infection, but did not have an effect on calyx-end grey mould of the fruit in storage. Determination of the incidence of infection of blossoms by *B. cinerea* within South African pear orchards, along with the respective levels of resistance towards various fungicides needs to be ascertained.

1.7 Aims of this study

Two crops cultivated in the Western Cape were identified to study populations of *B. cinerea* isolates. Grey mould on rooibos and calyx-end decay on pears are both economically important diseases. With both crops various questions arise about the status of *B. cinerea*. Such as which cryptic species occur in South Africa? What is the genetic structure and diversity of the *B. cinerea* populations? What level of fungicide resistance is found in the field?

The aims of the current study therefore include:

- to determine the cryptic species status of *B. cinerea*,
- to determine the population structure of *B. cinerea*,
- characterize the genetic diversity found within and among populations of *B. cinerea*,
- assess if recombination takes place among *B. cinerea* isolates in the field,
- to determine fungicide resistance levels of *B. cinerea* populations collected from rooibos nurseries and pear orchards.

The knowledge acquired by conducting this study will aid in understanding the dispersal patterns of *B. cinerea* in both rooibos nurseries and pear orchards. This will in turn contribute to improved management strategies for the control of these diseases.

1.8 References

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CHAPTER 2

Characterization of the genetic variation and fungicide resistance found among *Botrytis cinerea* populations from rooibos seedlings in the Western Cape of South Africa

2.1 Abstract

Botrytis cinerea annually causes severe losses of rooibos seedlings in nurseries situated in the Clanwilliam region in the Western Cape of South Africa. To genetically characterize the *B. cinerea* population, a total of 207 *B. cinerea* isolates were collected from diseased rooibos seedlings from five nurseries. The cryptic species status of these isolates was tested with restriction enzyme analysis of the *Bc-hch* gene and it was found that 206 (out of 207) of the isolates belonged to Group II of *B. cinerea*. Further analysis of *B. cinerea* Group II populations was performed to assess the genetic population structure, using seven microsatellite loci. Valuable insight was gained into the meta-population as well as the individual subpopulations. Total gene diversity (H) was high, with a mean of 0.67. Two of the populations' sample sizes were severely limited after clone correction, yet 100 genotypes were discerned among the 206 isolates genotyped. The percentage of maximal genotypic diversity (G) ranged between 16 and 68 for the five populations, with a total value of 17 for the 100 genotypes. Genotype flow was evident between nurseries which indicated the spread of clonal lineages, in agreement with the relatively low but significant population differentiation between nurseries (mean $F_{ST} = 0.030$, $P = 0.001$). The distribution of the mating type alleles *MAT1-1* and *MAT1-2* differed significantly from the ratio of 1:1 for the total population plus two of the subpopulations. The remainder had an equal mating type distribution, bar one isolate which was homothallic. The index of association (I_A) analyses suggests that the populations are asexually reproducing. Analysis of molecular variance (AMOVA) indicated that 97% of the total genetic variation is distributed within subpopulations. Fungicide resistance frequency against iprodione was also determined for 198 of the genotyped isolates, with highly varying levels of resistance between the five nurseries. The mean incidence of resistance towards iprodione was 44% in total, ranging from 0% to 81% for the five nurseries. Baseline sensitivity towards pyrimethanil was also determined with an average EC_{50} value of 0.096 mg/L. This study has shown that there exists within the studied population of *B. cinerea*, an adaptive

capacity to overcome current chemical means of control. Novel management strategies need to be implemented in order to ensure the sustainability of this important crop.

2.2 Introduction

Botrytis cinerea Pers. Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] was first reported on rooibos in 1994 (Sandra Lamprecht, pers. comm.), although it most likely was present before since it is a ubiquitous pathogen worldwide. It is the most important foliar disease of rooibos seedlings [*Aspalathus linearis* (Brum.f) Dahlg.] in South Africa and can cause losses of up to 80% in a season if not managed properly (Sandra Lamprecht, pers. comm.). *Aspalathus linearis* grows naturally in the Cederberg area, representing an extremely localized area in the west of the Western Cape in South Africa. Tea prepared from rooibos is sought after for its array of medicinal properties, which includes being an excellent antioxidant, antimutagenic, anticarcinogenic and antispasmodic agent (Joubert *et al.*, 2008). The production of rooibos has steadily increased due to international demand (Joubert *et al.*, 2008). Exports rose from 750 tons in 1993, to 6000 tons in 2010, in which a total of 14000 tons was produced (Johan Brand, Rooibos Ltd, pers. comm.).

Sowing of rooibos in nurseries takes place in autumn (April) and grey mould becomes a problem on the seedlings at about 2 to 4 months of age. This is due to a denser canopy which allows less light and fungicides to penetrate and which also creates a humid microclimate conducive to the spread of the disease. Even though cultural management practices are implemented such as sowing 15 grams of seed per meter in an east-west orientation with 10 cm row spacing, aimed at reducing the humidity around seedlings, the use of fungicides is heavily relied upon. Current fungicide recommendation is an initial once off application of iprodione to seedlings at 4-weeks-old. This is followed by iprodione and pyrimethanil applications alternated weekly starting at 11-weeks-old until transplanting, at more than 20 weeks of age. Multiple fungicide applications are not ideal for the high demand for organically produced rooibos. Due to the regular use of iprodione, resistance has been reported (Spies, 2005). In the 2003/2004 growing season, during June, the overall mean incidence of resistance towards iprodione at 1.0 mg/L was approximately 20% in four nurseries (Spies, 2005). Evaluating the current level of resistance towards iprodione

and determining the baseline sensitivity towards pyrimethanil will be invaluable to reassess management strategies.

Botrytis cinerea has recently been recognized as consisting of a species complex, comprising two distinct groups or cryptic species (Fournier *et al.*, 2005). The first, Group I, is characterized by low genetic diversity with restricted host and/or geographic ranges. The second, Group II, is characterized by high gene diversity and no apparent host specialization. Group II is considered to be *B. cinerea* 'sensu stricto' and the most widely occurring and most pathogenic (Fournier *et al.*, 2005; Martinez *et al.*, 2005).

High levels of genetic variation and sexual recombination have been found within Group II in France, Tunisia and Hungary (Fournier and Giraud, 2007; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008) on different hosts using microsatellite markers developed by Fournier *et al.* (2002). Investigating the genetic diversity and population structure of any plant pathogen is important to assess the adaptive potential in order to overcome control strategies such as fungicide applications (McDonald and Linde, 2002).

It is currently not known what the composition of the populations of *B. cinerea* occurring on rooibos seedlings in the Clanwilliam area is. Therefore, the objectives of this study were to assess: i) the cryptic species status of *B. cinerea* isolates collected from five rooibos nurseries in the Clanwilliam area in the Western Cape of South Africa; ii) the genetic structure and diversity of the five populations of *B. cinerea* through the use of seven microsatellite markers and iii) the fungicide sensitivity of the sampled isolates towards iprodione and baseline sensitivity of a subset of this population towards pyrimethanil.

2.3 Materials and methods

2.3.1 *Botrytis cinerea* sampling

Diseased 3-month-old rooibos seedlings were collected from five open-bed (direct sowing in field soil) nurseries in the Clanwilliam area within a 25 km radius (Fig. 1). Sampling was done randomly over the entire planted area of the nurseries (ranging in size from 0.25 to 1 ha) during the first 2 weeks of June in 2008. Infected

plant material with sporulating lesions was placed onto Petri dishes containing potato dextrose agar (PDA) (Difco Laboratories, Detroit, Michigan, USA), after which the cultures were purified by growing them on divided Petri dishes, one half containing PDA and the other water agar (Oxoid Ltd., Basingstoke, Hampshire, England) with a piece of sterile carnation leaf. The isolates were single spored and stored on PDA slants and in sterile distilled water in 9-mL McCartney bottles at 4°C at the Department of Plant Pathology, Stellenbosch University.

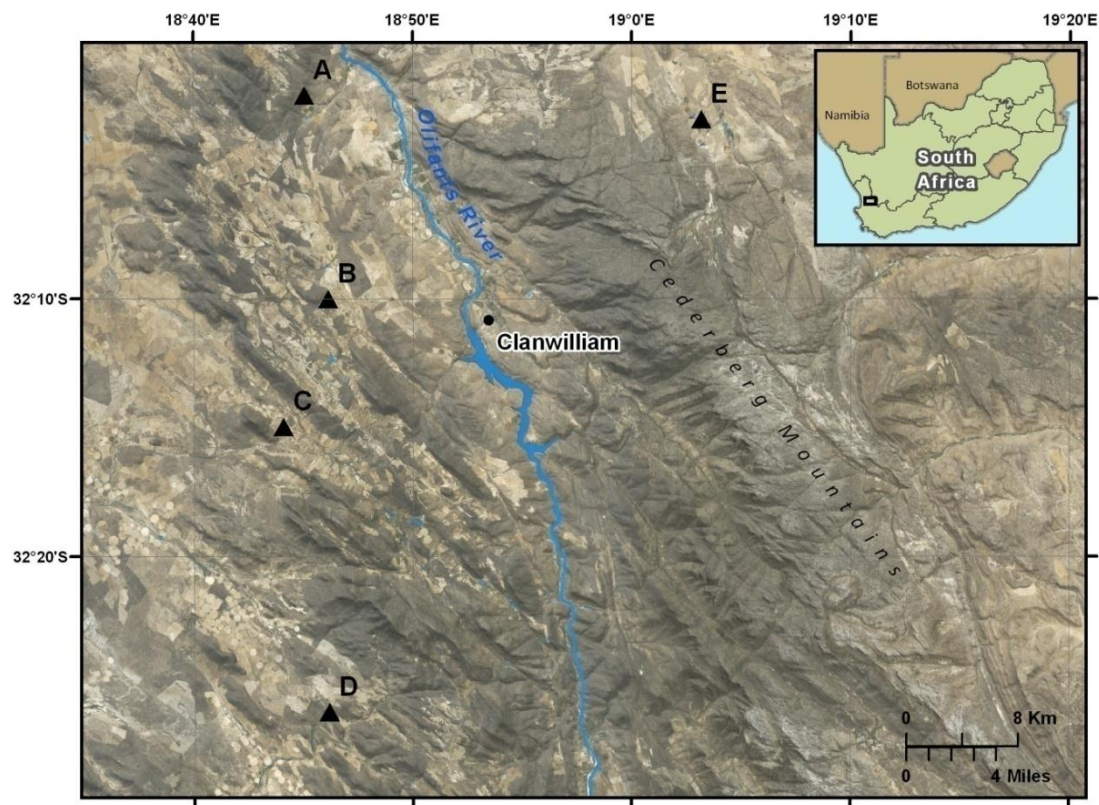


Fig 1. The location of the five rooibos nurseries (A-E) in the Clanwilliam production area from which *Botrytis cinerea* populations were sampled. Nurseries A to D is situated west of the Olifants river and nursery E is situated to the east in the Cederberg mountains.

2.3.2 DNA isolation

DNA was extracted from mycelial cultures after 2 weeks' growth on PDA Petri dishes at 25°C. Approximately 100 mg fungal tissue was used to extract DNA as described by Goodwin *et al.* (1992). The concentration of each DNA sample was

then determined on a NanoDrop® ND-1000 Spectrophotometer and diluted accordingly, to a final concentration of 25 ng/μL.

2.3.3 *Botrytis cinerea* Group I and II distinction

Primers 262 and 520L were used as described by Fournier *et al.* (2003) to amplify the Bc-*hch* gene, a homologue of the *hch* vegetative incompatibility locus of *Neurospora crassa*. PCR-RFLP profiles of the Bc-*hch* gene was generated through digestion with the restriction enzyme *Hha* I for 90 min at 37°C and visualized under UV-light on a 2.0% agarose gel by means of ethidium bromide staining.

2.3.4 Mating type determination

The distribution of two mating types, MAT1-1 and MAT1-2, within the populations was determined through the use of primers developed by van Kan *et al.* (2010). The primers HMG5 (5'-ATGTCTCTCTCCTCTCTCCG-3') and HMG3 (5'-GGAAAAGAATGTGTAGAGATCCTG-3') partially amplified the MAT1-2 HMG gene (approx. 1050 bp) and the primers MATalpha5 (5'-ATGACGGCTCCCTTCAAAACC-3') and MATalpha3 (5'-GGTGGTGAAGGGACATCTTC-3'), the MAT1-1 alpha gene (approx. 1100 bp). Two separate reactions were set up for each mating type locus. PCR reactions were performed in a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems), in a total reaction volume of 50 μL. Each reaction contained approximately 125 mg of fungal DNA, 0.4 μM of each primer, 0.24 mM of each dNTP, 1.5 mM MgCl₂, 1x reaction buffer and 1 U *Taq* polymerase. The PCR was performed using the following conditions; an initial denaturing step of 1 min at 94°C, 32 cycles of denaturing for 40 s at 94°C, annealing for 1 min 30 s at 58°C and extension for 1 min at 72°C. This was followed by final extension at 72°C for 10 min. In each PCR a positive control was included, the isolate SAS 56 for the MAT1-1 alpha gene - and SAS 405 for the MAT1-2 HMG gene amplification (kindly provided by Jan van Kan, Wageningen University, The Netherlands) (Faretra *et al.*, 1988).

2.3.5 Microsatellite amplification

A total of 206 Group II isolates were successfully genotyped using seven of the nine microsatellite markers developed by Fournier *et al.* (2002). Loci Bc9 and Bc4 were excluded due to the fact that Bc9 was only 70 bp from Bc10 (linked microsatellite loci) and Bc4 had low allele polymorphism (Fournier *et al.*, 2002). PCR amplifications were multiplexed except for Bc7. PCR reactions consisted of a total reaction volume of 25 μL , containing 1 μL DNA (25ng/ μL), 12,5 μL KAPA2G™ FastHotStartReadyMix (KapaBiosystems, Mowbray, Western Cape, South Africa) and the following primer (forward and reverse) concentrations for the multiplex: Bc1, 0.16 mM; Bc2, 0.8 mM; Bc3, 0.3 mM; Bc5, 0.4 mM; Bc6, 0.32 mM and Bc10, 0.12 mM. The single reaction had a primer concentration of 1.2mM for Bc7. The 5' ends of the forward primers were labeled fluorescently as follows: Bc1/Bc10, VIC; Bc3/Bc5, 6-FAM; Bc2/Bc7, PET and Bc6 with NED. Amplifications were performed using a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems). An initial denaturation step at 95°C for 1 min, was followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s and elongation at 72°C for 10 s. This was followed by a final elongation step at 60°C for 15 min.

PCR products were diluted 1 in 15 for the multiplex reaction, and left undiluted for the single reaction. A post-PCR clean-up was performed with a PCR product purification kit (MSB spin PCRapace, Invitex), after which the samples were mixed in a 1:1 ratio with Hi-Di formamide (Applied Biosystems) in a total volume of 10 μL , followed by addition of 0.45 μL GeneScan™-600 LIZ® Size Standard (Applied Biosystems). Fragments were separated in an automated single capillary genetic analyzer ABI3730x1 DNA sequencer (Applied Biosystems). Data was analyzed using the software Gene Mapper version 3.7 (Applied Biosystems). Only one allele was amplified for all loci, consistent with the haploid nature of *B. cinerea*.

2.3.6 Population genetics analyses

Clonal distribution and frequency of genotypes between and within populations was inferred based on microsatellite profile matches. An index of genotypic diversity (\hat{G}) denoted by the following formula,

$$\hat{G} = 1 / \sum_{x=0}^n [fx \cdot (x/n)^2]$$

was calculated within populations; n = sample size, fx = number of distinct haplotypes observed x times within a population. For comparison between populations, the index (\hat{G}) was divided by the number of samples to obtain the percentage of maximal genotypic diversity (G) (Stoddard and Taylor, 1988; McDonald *et al.*, 1994).

The software GenAlEx version 6.4 (Peakall and Smouse, 2006) was used to calculate Nei's gene diversity (Nei, 1973), the number of different multilocus genotypes (MLG), index of association (I_A), Wright's Fixation Index (F_{ST}) and to perform an analysis of molecular variance (AMOVA).

The index of association ($I_A = (V_O/V_E) - 1$) is a generalized measure for linkage disequilibrium (Brown *et al.*, 1980; Maynard-Smith *et al.*, 1993; Haubold *et al.*, 1998). It gives information on whether two different individuals sharing an allele at one locus are more likely to share an allele at another (Karchani-Balma *et al.*, 2008). The index is computed as follows; for any two paired individuals, the number of loci with which they differ is calculated. The variance of this number is then compared with the expected variance if there were no linkage disequilibrium, in other words linkage equilibrium. The value is expected to be zero if there is no association between loci and increases as the linkage disequilibrium increases (Maynard-Smith *et al.*, 1993; Fournier and Giraud, 2007). The null hypothesis of complete panmixia, *i.e.* I_A equal to zero, was tested by comparing the observed data set to 999 randomized data sets, in which infinite recombination were imposed, by randomly shuffling the alleles among individuals independently of each locus.

Wright's Fixation Index (F_{ST}) was calculated to determine the extent of population subdivision in pairwise comparisons, after 999 permutations. It is a measure of the genetic differentiation of a subpopulation relative to the total population due to non-random mating (Wright 1951; Slatkin and Barton, 1989). Values can range between 0 (no population subdivision) and 1 (complete population subdivision, with no gene flow between populations). Thus a low F_{ST} value indicates that most of the diversity is found within the subpopulation and not much between the populations. Conversely, a high value indicates substantial genetic differentiation between the populations.

To estimate the relative contribution of location on the genetic variation observed, a hierarchical analysis of molecular variance (AMOVA) was performed and

the significance of genetic variations were determined after 999 permutations in pairwise population comparisons.

And finally, to determine whether there is a significant correlation between geographic distance, expressed as the log of geographic distance in pairwise comparisons of populations, and genetic differentiations between all pairs of genotypes of subpopulations, a Mantel test was performed (Mantel, 1967). The significance of this comparison was determined after 999 permutations. A significant negative correlation is expected if the geographic distance affects the extent of genetic differentiation among populations, which would indicate isolation by distance.

All the tests were conducted on the five individual populations as well as the total *B. cinerea* population obtained. The values calculated for the I_A was done for individual populations only. Populations were clone-corrected prior to analyses to remove bias of over-representation of clones.

2.3.7 Screening for resistance towards iprodione

A total of 198 genotyped isolates were successfully screened for resistance to iprodione. Technical-grade iprodione (a.i. 98%, ACI Chemicals, Stellenbosch, South Africa) was dissolved in acetone to make a 1.0 g/L stock solution. Mycelial growth sensitivity was determined for three concentrations of amended PDA; 0 (control), 3.0 and 5.0 mg/L as per the methods described by Fourie and Holz (1998). In all cases the final amount of acetone in the medium was 0.1%, including the control plates. Each isolate had three replicates per concentration.

Plates were center-inoculated with 5-mm diameter mycelium plugs (mycelium facing downward) from actively growing colony margins of 3-day-old cultures. Radial growth of colonies was measured after 36 h incubation at 22°C in the dark. Isolates were regarded as sensitive when growth was observed on the control plates and no growth was observed at either concentration, ultra-low-level resistant (ULR) when able to grow at 3.0 mg/L and low-level resistant (LR) if able to grow at 5.0 mg/L. The resistance frequency expressed as a percentage of the number of isolates tested, was then determined for *Botrytis* populations from each nursery and the population as a whole.

2.3.8 Determination of baseline sensitivity towards pyrimethanil

Pyrimethanil has been used in the nurseries since 2003 (S. Lamprecht, pers. comm.). A subset of 30 isolates, representing the five nurseries was used to determine the baseline sensitivity. Technical grade pyrimethanil (a.i. 99.4%; Janssen Pharmaceutica N.V., Belgium) was dissolved in acetone to make a 1.0 g/L stock solution. Sensitivity towards pyrimethanil was tested on an L-asparagine-based agar medium (ASP-agar) (Hilber and Schüepp, 1996). The ASP-agar was amended with 0, 0.05, 0.1, 0.2, 0.3, 0.5, 1.0 and 2.5 mg/L of pyrimethanil. In all cases the final concentration of acetone was 0.1% in the medium, including the control plates.

Pyrimethanil amended ASP-agar plates were center-inoculated with 5-mm diameter mycelium plugs (mycelium facing downward) from actively growing colony margins of 3-day-old cultures. Each isolate was represented by three replicates per concentration. Radial growth of colonies was measured twice perpendicularly after four days incubation at 20°C in the dark. For each concentration, percentage inhibition relative to the control was calculated. The EC_{50} value (effective concentration inhibiting mycelial growth by 50%) was then determined for each isolate from the most appropriate non-linear regression model describing the inhibition \times concentration interaction using SAS Version 9.2 (SAS Institute Inc., Cary, North Carolina, USA) through a regression analysis of the log-inhibition.

2.4 Results

2.4.1 *Botrytis cinerea* Group I and II distinction

A total of 207 isolates of *B. cinerea* was collected from diseased rooibos seedlings from five nurseries. Only one isolate showed the diagnostic band of 601 bp and was thus characterized as belonging to Group I (Fig. 2). This isolate came from nursery E and was excluded from any further analyses. The remaining 206 isolates of *B. cinerea* were characterized by the diagnostic band of 517 bp as belonging to Group II.

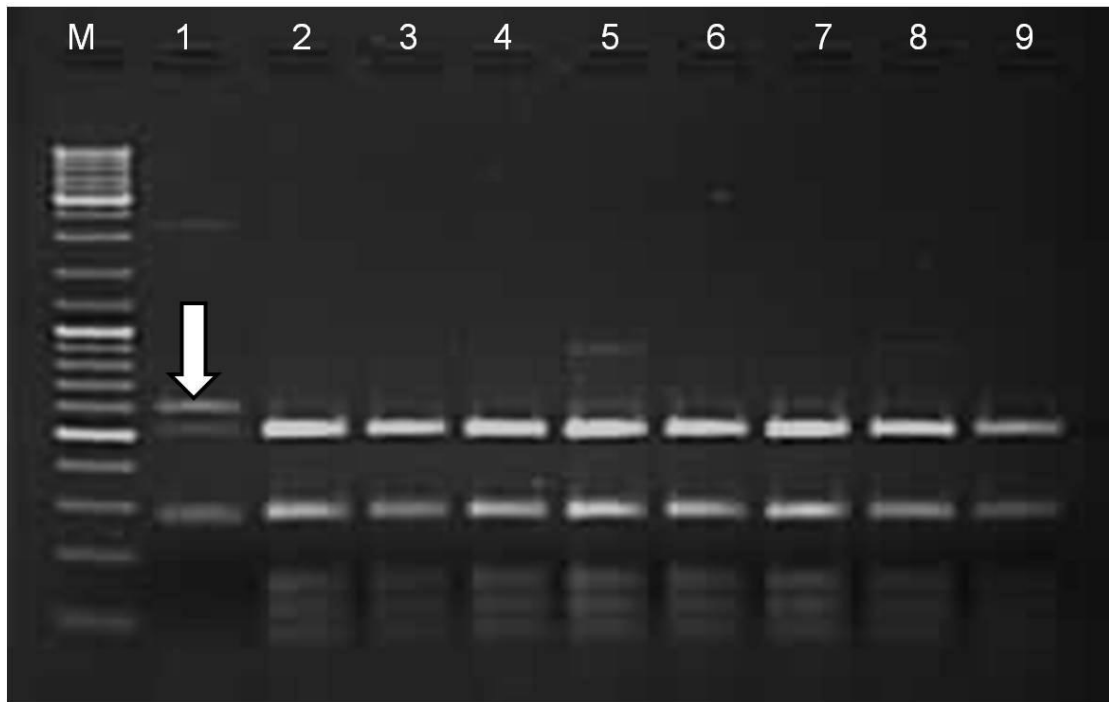


Fig 2. Example of gel electrophoretic profile of the *Bc-hch* locus digest with *HhaI*. Lane 1 illustrates the profile of *Botrytis cinerea* Group I (diagnostic band of 601 bp indicated by white arrow) and lanes 2-9 illustrates isolates belonging to Group II (diagnostic band of 517 bp). Lane M contained a 100 bp ladder (Promega).

2.4.2 Mating type determination

The amplification of the *MAT1-1* alpha gene yielded a product of approximately 1050 bp and the amplification of the *MAT1-2* HMG gene yielded a product of approximately 1100 bp (Fig. 3). Isolates were considered to be heterothallic if either the *MAT1-1* or *MAT1-2* gene was amplified, and homothallic if both were amplified for the same isolate (Fig. 3).



Fig 3. Example of gel electrophoretic profile of the mating type PCR amplification. Lane M contained a 100 bp ladder (Promega). The same sample, amplified either for *MAT1-1* (above) or *MAT1-2* (below), were loaded in lanes 1 to 13. Lanes 1-7, 10, 12 and 13, represent samples with the *MAT1-1* allele. Lanes 8, 9, 11 and 12 represent samples with the *MAT1-2* allele. Lane 12 contains the homothallic isolate (nursery B). Lane 14 contained the respective positive controls for each reaction, above (*MAT1-1*, isolate SAS 56, 1050 bp) and below (*MAT1-2*, isolate SAS 405, 1100 bp). Lane 15 contained the respective negative controls.

In total, 64% of the isolates had only the *MAT1-1* allele, 35.5% only the *MAT1-2* allele and one isolate sampled from nursery B had both (Table 1). A chi-square test was performed to determine the goodness of fit of this distribution compared to the expected mating type ratio of 1:1, for the total population ($\chi^2 = 17$, $P < 0.05$, $df = 1$) and sub-populations for which values ranged from ($\chi^2 = 0.05$, $P > 0.05$, $df = 1$) to ($\chi^2 = 23$, $P < 0.05$, $df = 1$) (Table 1). Overall, the distribution of the two mating types differed significantly from the 1:1 ratio assumed under random mating. Nursery A's population was comprised entirely of isolates containing the *MAT1-2* allele, and in nursery D two thirds of the isolates had the *MAT1-1* allele. The hypothesis of recombination, with the resultant equal distribution of *MAT1-1* and *MAT1-2* alleles, was thus rejected for nurseries A and D and accepted for nurseries B, C and E (Table 1).

Table 1. Distribution of mating types of *Botrytis cinerea* isolates.

Population	No. of isolates	MAT1-1	MAT1-2	MAT1-1 and MAT1-2	χ^2 ^A
Nursery A	23	23	0	0	23*
Nursery B	50	30	19	1	2.5 ^B
Nursery C	58	32	26	0	0.6
Nursery D	54	36	18	0	6*
Nursery E	21	11	10	0	0.05
Total	206	132	73	1	17* ^B

^A χ^2 value based on 1:1 ratio and 1 degree of freedom

^B Homothallic isolate excluded from χ^2 analyses

* Indicates mating type frequencies significantly different at $P < 0.05$

2.4.3 Genetic diversity

All microsatellites amplified in this study were polymorphic with the number of alleles amplified per locus ranging from 8 (Bc3, Bc5, and Bc10) to 13 (Bc6), thus giving sufficient discriminatory power to assess population dynamics. The mean number of alleles for all loci in the total population was 5.6 (Table 2).

2.4.3.1 Genetic diversity within populations

Among the 206 isolates genotyped, 100 multilocus genotypes (MLG) were found within the five South African populations on rooibos. Identical genotypes were assumed to be clones. The number of MLG within populations varied with the most in nursery C (32 out of 58) and the least in nursery A (4 out of 23) (Table 2). The percentage of maximal genotypic diversity ranged from 16 in nursery A to 68 in nursery E (Table 2). Mean gene diversity (H) determined for the clone corrected populations were high, with values ranging from 0.56 (nursery E) to 0.76 (nursery D) (Table 2). The overall mean gene diversity was high, with a value of $H = 0.67$ (Table 2). The mean number of alleles per locus ranged from 3.0 to 7.9 (Table 2). The index of association (I_A) was significantly different from zero for four of the five populations, with a maximum significant value of 2.346 for nursery D ($P = 0.001$), thus rejecting the hypothesis of random mating and indicating that there is a strong association between loci. In the total population, 63 genotypes were observed once, and 37 were observed at least twice ($x \geq 2$) or more (Table 3). Genotypes observed once varied between populations, with the most in nurseries B and D (20) and the least in nursery A (3) (Table 3).

Table 2. Genetic diversity indexes of *Botrytis cinerea* populations collected from five rooibos nurseries in South Africa.

Population	No. of isolates	MLG ^A	G ^B	H ^C	Mean no. of alleles per locus ^D	I _A ^E
Nursery A	23	4	16	0.59 (0.06)	3.0 (0.378)	5.878 (0.022)
Nursery B	50	29	46	0.74 (0.01)	6.6 (0.369)	1.991 (0.001)
Nursery C	58	32	41	0.72 (0.04)	7.9 (0.769)	1.887 (0.001)
Nursery D	54	21	27	0.76 (0.02)	6.4 (0.481)	2.346 (0.001)
Nursery E	21	14	68	0.56 (0.04)	4.0 (0.218)	1.754 (0.005)
Total	206	100	17	0.67 (0.02)	5.6 (0.367)	—

^A MLG: Multilocus genotypes

^B G: Percentage of maximal genotypic diversity (McDonald *et al.*, 1994)

^C H: Gene diversity (Nei, 1973)

^D Standard deviation shown in brackets

^E I_A: Index of association [P -values in brackets]

Table 3. Frequency distribution of *Botrytis cinerea* multilocus genotypes (MLG) within rooibos seedling populations in the Clanwilliam area of South Africa.

Number of genotypes observed x times						
x	Nursery A	Nursery B	Nursery C	Nursery D	Nursery E	Total ^A
1	3	20	18	20	14	63
2	1	6	6	7	2	15
3	1	2	4	0	1	8
4	0	3	4	0	0	6
5	1	0	0	2	0	5
6	0	0	0	0	0	1
7	0	0	0	0	0	1
10	1	0	0	1	0	0
27	0	0	0	0	0	1

^A Five nursery populations pooled together, only genotypes shared among populations recorded in total

2.4.3.2 Genetic diversity and differentiation between populations

The results of pairwise determination of population differentiation yielded low significant F_{ST} values (Table 4). The highest F_{ST} and correspondingly low Nm values were obtained in pairwise comparisons between nursery E with nursery B and D (F_{ST} = 0.080/0.064, P = 0.001/0.009, Nm = 5.8/7.3, respectively). Lower significant values were obtained in comparisons of nursery B and E to nursery C (F_{ST} = 0.046/0.049, P = 0.001/0.009, Nm = 10.3/9.8, respectively). Other comparisons yielded non-significant values (Table 4).

More direct evidence of genotype flow was found through the observation of distinct clonal genotypes shared between two or more populations. The most frequently occurring genotype, represented by 27 clones, was found in nurseries A (10x), B (4x), C (3x) and D (10x). These are all the nurseries occurring on the west side of the Cederberg (Fig. 1). Two other genotypes were shared by more than two nurseries. One, represented by 6 clones, was shared between nurseries C (1x), D (2x) and E (3x). The other, represented by 4 clones, was shared between nurseries B (1x), C (2x) and D (1x).

Table 4. Pairwise F_{ST} (above diagonal) with gene flow (Nm) values (below diagonal) among five *Botrytis cinerea* populations sampled from rooibos.

Nursery A	Nursery B	Nursery C	Nursery D	Nursery E	Population
-	0.000	0.015	0.000	0.125	Nursery A
∞	-	0.046*	0.000	0.080*	Nursery B
32.3	10.3	-	0.000	0.049*	Nursery C
∞	∞	∞	-	0.064*	Nursery D
3.5	5.8	9.8	7.3	-	Nursery E

* significant values ($P < 0.01$)

∞ arbitrary values due to no measurable population subdivision

Analysis of molecular variance (AMOVA) showed that 97% of the total variation was found within populations and 3% between them, with a total F_{ST} value of 0.030 ($P = 0.001$) (Table 5). A Mantel test showed no significant correlation between geographic distance and population differentiation, indicating no isolation by distance ($y = -7 \times 10^{-6}x + 6.124$, $R^2 = 0.031$, $P = 0.301$).

Table 5. Analysis of molecular variance (AMOVA) among *Botrytis cinerea* populations from rooibos nurseries.

Source	d.f.	SS	MS	Est. Var.	% ^A	Stat	Value	P-value
Between								
Populations	4	16.751	4.188	0.083	3			
Within Populations	95	250.669	2.639	2.639	97			
Total	99	267.420	6.827	2.721		F_{ST}	0.030	0.001

^A Percentage of total variation

2.4.4 Screening for resistance towards iprodione

The five populations sampled differed in their resistance profiles towards iprodione. The lowest incidence of resistance was found in nursery A, where none of the isolates were able to grow at the discriminatory dose of 3.0 mg/L iprodione. The highest resistance frequency was 81% in total (5% ULR, 76% LR) towards iprodione found in nursery E. Overall, the resistance frequency was 44% (including ULR and LR isolates tested) (Fig. 4).

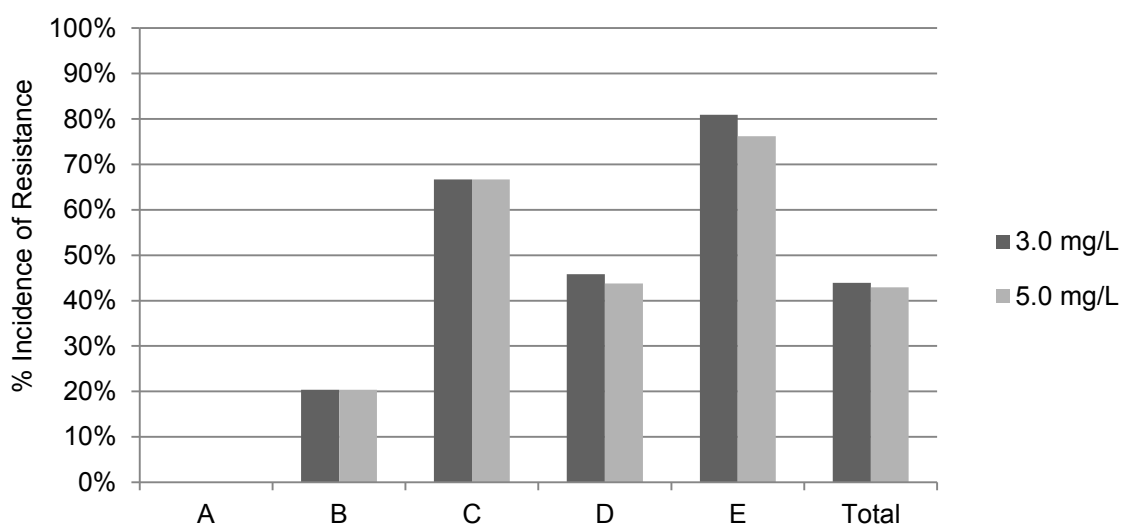


Fig. 4. Iprodione resistance frequency of 198 *Botrytis cinerea* isolates from five nurseries (A-E).

2.4.5 Baseline sensitivity towards pyrimethanil

Four regression models (Natural Growth, Modified Exponential, Gompertz and Logistic Model) were fitted to the data obtained, all of which gave very good statistical fits ($R^2 > 0.877$). The Logistic Growth Model consistently gave the highest correlation for isolates ($R^2 > 0.930$), except in the case of one isolate ($R^2 = 0.711$). This isolate was seen to be more resistant than the others and its mycelial growth was only inhibited by 23% relative to the control at 2.5 mg/L, the highest concentration tested. The EC_{50} value for this isolate could not be determined. Of the remaining 29 isolates, EC_{50} values for pyrimethanil ranged from 0.047 to 0.166 mg/L. The mean EC_{50} value for all the isolates was 0.096 mg/L with a standard deviation of 0.019.

2.5 Discussion

This is the first study to investigate the genetic structure and mating type distribution of *B. cinerea* populations isolated from rooibos seedlings. In this study one isolate only did not yield the 517 bp fragment after digestion of the *Bc-hch* locus, indicating that the majority of the isolates belong to *B. cinerea* Group II. Similarly, in Australia, South Asia, Bangladesh, Tunisia and Hungary sampled populations of *B. cinerea* consisted of only Group II (Isenegger *et al.*, 2008a; Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). *Botrytis cinerea* sampled in autumn from vineyards in France had 95% Group II isolates (Fournier and Giraud, 2007). Group I isolates are reported to be subject to temporal succession on grapevine at least, and have been underrepresented when sampled together with Group II isolates (Fournier *et al.*, 2005; Fournier and Giraud, 2007). Group I isolates are distinct and form a separate phylogenetic species from Group II (Fournier *et al.*, 2005).

The microsatellite markers used in this study was found to be sufficiently discriminatory in order to assess the level of diversity in the studied populations of *B. cinerea*. Genotype diversity varied widely between populations, and was lowest for nursery A. However, nursery E had a substantially higher diversity compared to the other nurseries. The genotypic diversity indicated that a considerable proportion of clonal lineages were present within the nurseries sampled. Out of the total of 206 isolates genotyped, 100 genotypes indicated a moderate to high level of clonality. The gene diversity of the population indicates a highly heterogenous population, with values obtained similar to the *B. cinerea* populations sampled from different hosts around the world (Alfonso *et al.*, 2000; Fournier and Giraud, 2007; Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008). Giraud *et al.* (1997) found an *H* value of 0.316 for one of their populations from grapes in France, stating that this value was atypically high for a presumed asexually reproducing population. The mean gene diversity for the total population sampled was 0.67, and thus this would indicate that the studied population of *B. cinerea* Group II is not an entirely asexually reproducing population, if compared to Giraud *et al.* (1997)

However, in the present study, the index of association (I_A) was in all cases different from zero, with significant *P*-values ($P < 0.01$) for four of the sampled populations. This indicates that there is no recombination occurring, in contrast to some studies (Fournier *et al.*, 2005; Fournier and Giraud, 2007; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008) and in agreement with others (Ma and Michailides, 2005; Isenegger *et al.*, 2008b). In the study done by Ma and Michailides (2005), microsatellite primed (MP) - PCR was used and this mostly revealed the presence/absence of the markers and not the length as determined in this study, which is more informative (Fournier and Giraud, 2007). However, Isenegger *et al.*

(2008b) used markers developed by Fournier *et al.* (2002) and also found that there was significant linkage disequilibrium in their studied populations on chickpeas. Possible reasons provided by them for the lack of genetic recombination were that there was no significant recombination occurring, or that the analyses were hampered by genotype flow and panmixia/random mating, which in turn caused linkage disequilibrium (Isenegger *et al.*, 2008b).

The reason for the observed linkage disequilibrium in this study could be due to the relatively recent cultivation of rooibos seedlings in this area, causing founder populations or genetic bottlenecks of *B. cinerea*. Indeed the seasonal cultivation of seedlings of this crop may be leading to establishment of founder populations each year from inoculum entering from the surrounding vegetation. The presence of *B. cinerea* on plant material outside nurseries has been confirmed as well as the presence of airborne inoculum in nurseries (Spies, 2005). Each nursery field is planted once every four years and this may contribute to the establishment of founder populations. This, in turn, could lead to a population comprised mostly of clones, as virulent genotypes adapted to a particular host can increase rapidly in a season. Well-adapted genotypes then disperse throughout the nursery environment, leading to loss of control. Also, human mediated genotype flow as indicated by shared genotypes among nurseries, result in a disturbance in the balance between gene flow and genetic drift, affecting linkage disequilibrium analyses. Host specificity can develop due to strong directional pressure on one or two genes, for example with fungicide applications (McDonald and Linde, 2002). The resistance of the studied populations towards iprodione differed greatly and ranged from nearly the entire population displaying low-level resistance (nursery E) to no resistance observed at all (nursery A). No link between fungicide resistance and genotype profiles was found in the present study. Resistance towards dicarboximides (e.g. iprodione) is conferred through a diverse range and combinations of point mutations within the osmosensing histidine kinase *Bos-1* gene (Leroux *et al.*, 2002; Oshima *et al.*, 2002; Cui *et al.*, 2004; Ma *et al.*, 2007). Considering this, no link is to be expected. Taking into account the direct evidence for genotype flow, spread of resistant isolates from nursery E to nursery A, for example, is not improbable. The determination of pyrimethanil's EC_{50} values suggests that a discriminatory dose of 1.0 mg/L to be used for routine resistance screening. This will aid in reassessing management strategies involving the application of pyrimethanil in the long term.

Up until now mating type characterization has been done through traditional mating experiments. PCR primer pairs have recently been developed to amplify the two mating type loci in *B. cinerea* (Angelini *et al.*, 2010; van Kan *et al.*, 2010; Amselem *et al.*, 2011). Using these primers, Angelini *et al.* (2010) found the isolates they investigated to be of either the

MAT1-1 or MAT1-2 type. This study establishes that the two mating types, MAT1-1 and MAT1-2, are common in the total *B. cinerea* Group II population obtained from rooibos nurseries in the Clanwilliam area of the Western Cape. The distribution of the mating types did not differ significantly from a 1:1 ratio for nurseries B, C and E, but differed significantly for the total population as well as nurseries A and D. Nursery A yielded only isolates of the MAT1-1 type. In general, it may be that there is sexual recombination occurring, which generate well-adapted genotypes, which then reproduce clonally (McDonald and Linde, 2002). This could lead to an overestimation of asexual reproduction. This premise is further supported by the fact that both mating types, MAT1-1 and MAT1-2 are widespread in nature. One homothallic isolate was found to occur in nursery B. Faretra *et al.* (1988) found that approximately 16% of field strains and 6% of ascospore progeny, respectively, were homothallic, *i.e.* self fertile and able to cross with reference strains carrying both mating types. The remainder of both types of sampled isolates was heterothallic, *i.e.* self sterile, and able to produce sexual progeny with reference isolates carrying one of the mating type genes (Faretra *et al.*, 1988). The fact that both mating types are represented in this study indicates that the possibility for sexual recombination does exist, in turn suggestive of the evolutionary potential of the *B. cinerea* occurring on rooibos seedlings.

In agreement with direct evidence for gene and genotype flow, the AMOVA revealed that 97% of variation was found within populations and 3% was represented by variance between nursery populations. The F_{ST} value for the total population was very low at 0.030 (P -value = 0.001). In comparisons between nursery populations, very low to moderate levels of differentiation was seen with only a few of them significant. The only significant comparison that can be interpreted with confidence is that of nursery B compared to nursery C (F_{ST} = 0.046) as the others are hampered by low sample sizes after clone correction. Yet, Karchani-Balma *et al.* (2008) study showed similar levels of population subdivision even with the added effect of growing systems (greenhouses or open systems) and geographic distance in various crops (grapevine, strawberry, tomatoes and faba bean). Slightly higher values (total F_{ST} = 0.101) were observed by Isenegger *et al.* (2008b) when they looked at *B. cinerea* Group II occurring on chickpea in Bangladesh. In France, Fournier and Giraud (2007) found F_{ST} values of 0.058 and 0.045 for geographically isolated populations of *B. cinerea* sampled from *Vitis* and *Rubus fruticosus* (bramble occurring next to vineyards), respectively. Host plant significantly restricted gene flow among different populations, but did not represent a complete barrier to gene flow between *Vitis*- and bordering *Rubus*-, isolated *Rubus* populations (F_{ST} = 0.10/0.13), respectively. Thus, the overall population can truly be considered as a single large panmictic population.

There was no identity according to geographic origin, with migration/gene flow preventing genetic differentiation. Indeed, the most frequently isolated genotype was found 27 times, interestingly only in the four nurseries on the west side of the Cederberg Mountains. This suggests a preferred migration path, and illustrates genotype flow to a large extent, possibly with the prevailing wind or exchange of nursery material. Nevertheless nursery E on the east side of this geographic barrier was not completely isolated in terms of genotype flow, sharing genotypes with the other nurseries except A. These results showed that the *B. cinerea* Group II populations were not significantly influenced by spatial considerations, at least at the studied scale. This is corroborated by the findings of other studies that showed *B. cinerea* populations on an assortment of crops worldwide, having high gene diversities and low population subdivision (Alfonso *et al.*, 2000; Moyano *et al.*, 2003; Ma and Michailides, 2005; Isenegger *et al.*, 2008a; Karchani-Balma *et al.*, 2008). Knowing this information is important in reassessing disease management strategies and their viability, as spread of resistant phenotypes is not inconceivable. A Mantel test showed that there was no significant isolation by distance according to spatial sampling and distance. Thus, the studied populations of *B. cinerea* are dispersed through anthropogenic activities, or represent recent founder populations.

To conclude, the studied populations of *B. cinerea* Group II occurring on rooibos seedlings in the Clanwilliam area in the Western Cape of South Africa were characterized by high levels of gene diversity and a predominantly asexual reproduction system. Yet there was admixture to a large extent and they represent a single large panmictic population. This together with no isolation by distance suggests that the South African *B. cinerea* populations on rooibos most likely represent a founder population. The level of linkage disequilibrium may contribute to a fitter population on the whole, keeping well-adapted combinations of genes together through asexual reproduction, and together with high levels of gene diversity, may lead to rapid evolution and spread of resistance to control measures such as fungicide applications. Farmers within an area should coordinate fungicide applications to ensure sustained control of *B. cinerea* populations. Regular monitoring of resistance would be necessary to know which fungicides would prove more effective at a given point in time.

2.6 References

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Chapter 3

Genetic population structure and fungicide resistance of *Botrytis cinerea* in pear orchards in the Western Cape of South Africa

3.1 Abstract

Botrytis cinerea isolates from pear blossoms (*Pyrus communis*) were characterized with two transposable elements (TEs) *Boty* and *Flipper*, seven microsatellite loci, the mating type ratio and resistance to benomyl, iprodione and fenhexamid. A total of 181 *B. cinerea* isolates from four orchards in two production areas in the Western Cape in South Africa were screened. The incidence of blossom infection in the four orchards ranged from 3% to 17%. Overall there was a higher incidence of isolates that had only the *Boty* element (74%) compared to those harboring both TEs (*transposa*, 25%). Only one isolate examined had only the *Flipper* element present. The cryptic species status according to vegetative-incompatibility alleles of the *Bc-hch* gene indicated that all the isolates belonged to *B. cinerea* Group II. Analyses of *B. cinerea* Group II populations, through the use of microsatellites, were performed to assess the genetic population structure. Insight was gained into the meta-population as well as the individual subpopulations. Total gene diversity (H) was high, with a mean of 0.69 across all populations. Although two of the subpopulations displayed a high clonal proportion, overall 91 genotypes were discerned among the 181 isolates genotyped. The percentage of maximal genotypic diversity (G) ranged between 18 and 33 for the four populations, with a total value of 14 for the 91 genotypes. Genotype flow was evident between orchards which indicated the spread of clonal lineages, in agreement with the moderate, but significant population differentiation among orchards (mean $F_{ST} = 0.118$, $P = 0.001$). Index of association (I_A) analyses suggests that the populations reproduce mostly asexually. Analysis of molecular variance (AMOVA) indicated that 88% of the total genetic variation is distributed within subpopulations, 9% between subpopulations and only 3% between production areas. Mating type distribution did not differ significantly from a 1:1 ratio, for the total population as well as the subpopulations. Fungicide resistance frequency against the three fungicides tested varied, with the highest levels of resistance present against benomyl and low levels of resistance seen towards iprodione and fenhexamid. Selection for isolates resistant towards benomyl was evident in one orchard, with corresponding population subdivision observed between this orchard's population and the other orchard populations. This study has shown that the studied population of *B. cinerea* has the potential to overcome current chemical control strategies.

3.2 Introduction

Calyx-end decay of pears (*Pyrus communis* L.) caused by *Botrytis cinerea* Pers. Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] result in economic losses each year in South Africa. Calyx-end decay results from early season infection of pear blossoms by *B. cinerea*. Following blossom infection, decay invariably spreads from the mesocarp tissue, which adjoins the sepals in a radial manner, into the vascular bundles (De Kock and Holz, 1992). This knowledge of the manner in which *B. cinerea* enters the flower components led to the suggestion that postharvest control of grey mould could be achieved by fungicide application at full bloom and/or 75% petal fall, followed one month later when the floral tubes start to close with another fungicide application (De Kock and Holz, 1992).

Propagules of *B. cinerea* (e.g. sclerotia, mycelium and asexual spores) in the orchard can be found in the soil, plant litter and in the air (Lennox *et al.*, 2003). Furthermore, sclerotia often overwinter in crop debris (Staats *et al.*, 2005; Williamson *et al.*, 2007) within the orchard. Sclerotia that germinate during early spring produces asexual spores, which can be dispersed as wet or dry conidia which serves as inoculum for fruit infection (Lennox *et al.*, 2003; Spotts and Serdani, 2006) or blossom infection. Therefore orchard sanitation is crucial as a preventative measure to limit inoculum build-up (Spotts and Serdani, 2006).

Botrytis cinerea has recently been recognized as consisting of a species complex, consisting of two distinct groups or cryptic species (Fournier *et al.*, 2005). These two cryptic species are divided as; Group I (*'pseudocinerea'*), containing all *vacuma* isolates resistant to fenhexamid, and Group II (*cinerea 'sensu stricto'*), naturally sensitive to fenhexamid, and being either of the *vacuma* or *transposa* type. These groups are cryptic species that cannot interbreed (Fournier *et al.*, 2003). Group I is characterized by low genetic diversity with restricted host and/or geographic ranges. Group II is characterized by high gene diversity and no apparent host specialization. Group II is considered to be *B. cinerea 'sensu stricto'* and the most widely occurring and most pathogenic (Fournier *et al.*, 2005; Martinez *et al.*, 2005). Group I isolates are sometimes found in sympatry with Group II *B. cinerea*, but at low frequency (Albertini *et al.*, 2002; Fournier *et al.*, 2003, 2005).

Transposable elements are often used to distinguish between isolates of *B. cinerea*. The transposable elements (TE's) that were characterized are *Boty*, a 6 kb long-terminal-repeat retrotransposon, and *Flipper*, an 1842 bp Fot1-like transposable element (Diolez *et al.*, 1995; Levis *et al.*, 1997). On the basis of the presence or absence of these TE's, Giraud *et al.* (1997) found *B. cinerea* could be divided into two sympatric species. *Transposa* contains the transposable elements *Boty* and *Flipper* and *vacuma* contains neither transposable element. In grapevine pathology studies in France, isolates taken from

flowering parts in vineyards were found to be predominantly of the *vacuma* type (Giraud *et al.*, 1997, 1999; Martinez *et al.*, 2003). It was suggested that this is an indication of greater saprophytic capability, explaining the fact that *vacuma* isolates are frequently collected from senescing floral parts. These observations thus supported the possibility of genetic differentiation between transposon types (Martinez *et al.*, 2003, 2005).

The genetic diversity observed in *B. cinerea* has long been thought to be due to heterokaryosis (Beever and Weeds, 2004) and aneuploidy (Faretra and Pollastro, 1996). Sexual recombination has not been considered as a contributor, since apothecia are rarely observed in the field (Giraud *et al.*, 1997). Only indirect indications of sexual recombination were found within Group II in France (Fournier and Giraud, 2007), Tunisia (Karchani-Balma *et al.*, 2008) and Hungary (Váczky *et al.*, 2008) on various hosts.

The resistance levels of *B. cinerea* from South African pear orchards towards the fungicides benomyl, iprodione and fenhexamid is currently unknown. Benomyl and iprodione is registered as a full bloom and 75% petal-drop spray in South Africa. However, in practice benomyl is rarely used in orchards and iprodione is used in postharvest applications only. Although fenhexamide is not registered for control of grey mould in South Africa, determining baseline sensitivity will aid in the characterization of possible *vacuma* isolates into Group I or II. Knowledge regarding the population dynamics together with the application of effective fungicides will aid in the integrated management of calyx end decay of pears.

The objective of this research was to investigate the population dynamics of *B. cinerea* in four pear orchards in two areas within the Western Cape of South Africa. The following aspects were investigated: i) the status of the cryptic species found; ii) the evolutionary potential of the pathogen as typified by measures of its genetic diversity, mode of reproduction and gene flow through amplification of seven microsatellite markers; iii) whether genetic differentiation as determined with microsatellites corresponded to transposon types; iv) the mating type distribution of the studied population; v) baseline sensitivity towards fenhexamid and vi) the fungicide resistance levels towards fenhexamid, iprodione and benomyl.

3.3 Materials and Methods

3.3.1 *Botrytis cinerea* sampling

In total, 3640 pear blossoms were sampled from four orchards (910 blossoms per orchard). Two orchards were in the Ceres area („Forelle“) and two in the Grabouw area („Packam’s Triumph“). All orchards were within a 50km radius (Fig. 1). The cultivars sampled represent the dominant cultivar in each region. Hierarchical sampling (McDonald *et al.*, 1999) was done in the spring (October) of 2009 to include 30 trees per orchard. Sampling in rows started with the fifth tree in a row, and three trees were sampled adjacent to each other. Thereafter one tree was skipped, the next tree sampled, then two trees were skipped and the next tree sampled. After this tree, 20 trees were skipped before sampling the sixth and final tree in the row. Similarly the adjacent row was sampled next, one row skipped, and the next row sampled. Two rows were then skipped before sampling the next and then skipping three rows before sampling the fifth and final row. From each tree 30 blossoms were collected, except for the 21st tree (row 4), from which 40 were taken. This was the intended intensive sampling point in each orchard. Representative samples of weeds were taken around each tree, if occurring.

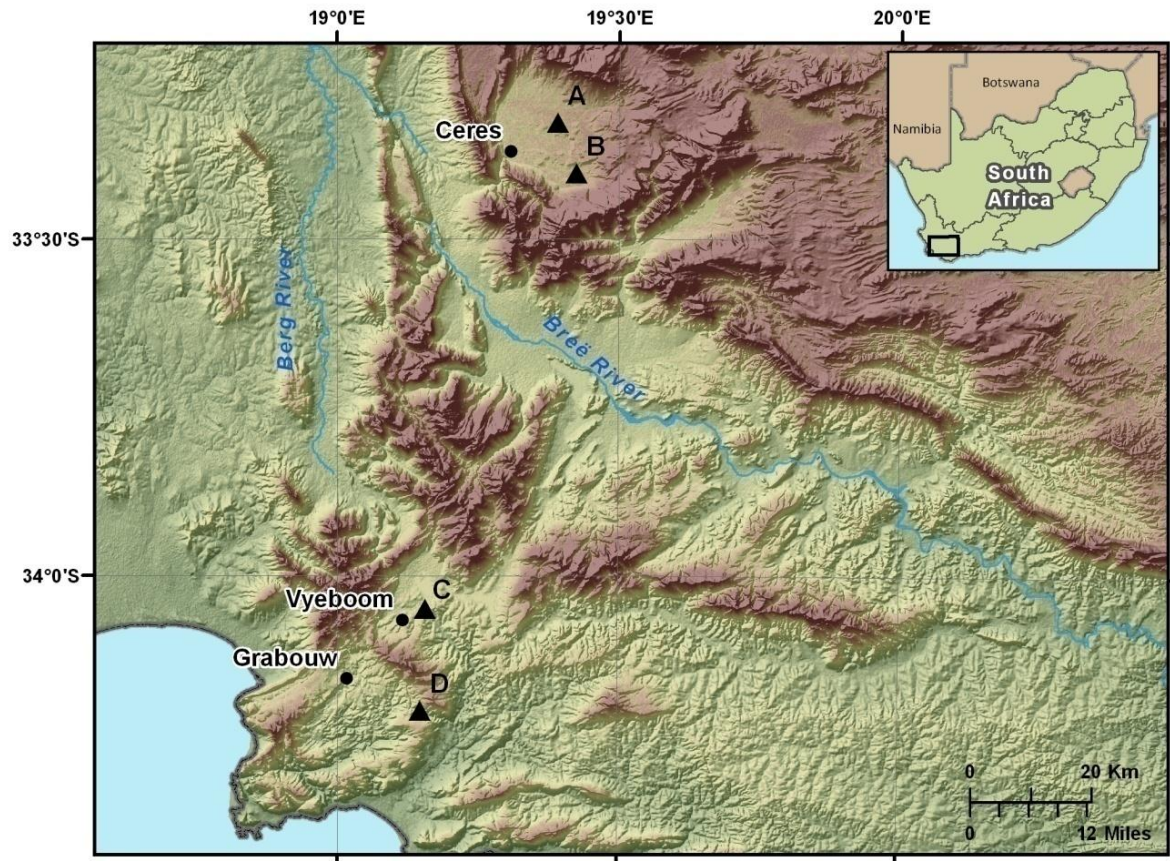


Fig 1. Location of four pear orchards (A-D) in the Western Cape of South Africa from which *Botrytis cinerea* populations were sampled. Orchards A and B is situated close to Ceres and orchards C and D, close to Vyeboom and Grabouw, respectively.

3.3.2 Processing of blossoms

Thirty blossoms per tree were sterilized for 30 seconds in 70% ethanol and divided into two groups of 15 blossoms each. Two incubation methods were used to maximise *B. cinerea* isolation. For the first method, three blossoms were incubated in a Petri dish containing Keressies medium (Keressies, 1990). Blossoms placed on Keressies medium had their petals removed and placed upside down on the surface of the medium *i.e.* with anthers and stamens touching the medium. The second method involved incubating five blossoms per plastic container containing a paper towel wetted with 2 to 3 ml of sterile distilled water. In the case of the 21st tree the flowers were divided into two groups (20 each). Half were placed individually on Petri dishes containing Keressies medium. The other half was placed in groups of four per plastic container. Petri dishes were not sealed as to induce sporulation of *B. cinerea* and plastic containers were opened once a week to ensure fresh air and growth

stimulation. These were left for approximately two weeks at room temperature, after which they were checked for growth of *B. cinerea*. Isolates were single spored and stored on PDA slants and in sterile distilled water in 9-mL McCartney bottles at 4°C at the Department of Plant Pathology, Stellenbosch University.

3.3.3 Processing of weeds

Weeds were sterilized by immersing plant material for 30 s in 70% ethanol, followed by 2 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol. After sterilization the plant material was immersed in 0.03% paraquat (WPK, Paraquat, 200 g/l [bipyridyl], WPK Agricultural, Cape Town, South Africa) for 30 s. The Paraquat facilitates latent infections to emerge (Biggs, 1995). Plant material was rinsed in dH₂O and left to air dry before being incubated in moisture chambers for at least two weeks. Vents were opened during the day to allow fresh air to circulate. Plant material was routinely checked and any *B. cinerea* sporulation observed were collected via single spore isolation and stored as described above.

3.3.4 DNA isolation

DNA was extracted from mycelial cultures after two weeks' growth on PDA Petri dishes at 25°C. Approximately 100 mg mycelium was used to extract DNA as described by Goodwin *et al.* (1992). The concentration of each DNA sample was determined on a NanoDrop® ND-1000 Spectrophotometer and diluted to a final concentration of 25 ng/μL.

3.3.5 *Botrytis cinerea* Group I and II distinction

The *Neurospora crassa* vegetative incompatibility locus homolog, *Bc-hch*, was amplified as described by Fournier *et al.* (2003) using the primers 262 and 520L. Restriction fragment length polymorphisms (RFLPs) were generated through digestion of the 1171 bp product with the restriction enzyme *Hha* I for 90 min at 37°C, resolved on a 2% agarose gel and visualized by ethidium bromide staining under a UV-light.

3.3.6 Detection of the transposable elements *Boty* and *Flipper*

The transposable element status of the isolates was determined, using the primer pairs BotyF4/BotyR4 and F300/F1550 for *Boty* (Ma and Michailides, 2005) and *Flipper* (Levis *et al.*, 1997), respectively. The PCR reactions were performed separately in a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems), in a total reaction volume of 40 µL as described by Ma and Michailides (2005). The PCR conditions were as follows; an initial preheat of 95°C for 3 min was followed by 40 cycles of 40 s denaturation at 94°C, 40 s annealing at 67°C and 58°C for *Boty* and *Flipper* respectively, and 1 min extension at 72°C followed by a final extension at 72°C for 10 min. PCR products were stained with ethidium bromide and visualised on a 1.5% agarose gel under UV-light. Each reaction was performed in duplicate.

3.3.7 Mating type determination

The presence/absence of the mating type genes, *MAT1-1* and *MAT1-2* within each isolate was determined through the use of primers developed by van Kan *et al.* (2010). The primers HMG5 (5'-ATGTCTCTCTCTCTCTCCG-3') and HMG3 (5'-GGAAAAGAATGTGTAGAGATCCTG-3') amplified a partial *MAT1-2* HMG gene (approx. 1100 bp). The MATalpha5 (5'-ATGACGGCTCCCTTCAAAACC-3') and MATalpha3 (5'-GGTGGTGAAGGGACATCTTC-3') primers were used to amplify the *MAT1-1* alpha gene (approx. 1050 bp). Separate reactions were set up for each mating type locus. PCR reactions were performed in a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems), in a total reaction volume of 50 µL. Each reaction contained approximately 125 mg of fungal DNA, 0.4 µM of each primer, 0.24 mM of each dNTP, 1.5 mM MgCl₂, 1x reaction buffer and 1 U *Taq* polymerase. The PCR was performed using the following conditions; an initial denaturing step of 1 min at 94°C, 32 cycles of denaturing for 40 s at 94°C, annealing for 1 min 30 s at 58°C and extension for 1 min at 72°C. This was followed by final extension at 72°C for 10 min. In each PCR a positive control was included, the isolate SAS 56 for the *MAT1-1* alpha gene - and SAS 405 for the *MAT1-2* HMG gene amplification (kindly provided by Jan van Kan, Wageningen University, The Netherlands) (Faretra *et al.*, 1988).

3.3.8 Microsatellite amplification

A total of 181 *B. cinerea* Group II isolates were successfully genotyped using seven of the nine microsatellite markers developed by Fournier *et al.* (2002). Loci Bc9 and Bc4 were excluded because Bc9 was only 70 bp from Bc10 (linked microsatellite loci) and Bc4 had low allele polymorphism (Fournier *et al.*, 2002). All microsatellite loci were multiplexed except for Bc7. PCR reactions consisted of a total reaction volume of 25 µL, containing 1 µL DNA (25 ng/µL), 12,5 µL KAPA2G™ FastHotStartReadyMix (KapaBiosystems, Mowbray, Western Cape, South Africa) and the following primer (forward and reverse) concentrations for the multiplex: Bc1, 0.16 mM; Bc2, 0.8 mM; Bc3, 0.3 mM; Bc5, 0.4 mM; Bc6, 0.32 mM and Bc10, 0.12 mM. The single reaction had a primer concentration of 1.2 mM for Bc7. The 5' ends of the forward primers were labeled fluorescently as follows: Bc1/Bc10, VIC; Bc3/Bc5, 6-FAM; Bc2/Bc7, PET and Bc6 with NED. Amplifications were performed using a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems). An initial denaturation step at 95°C for 1 min, was followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s and elongation at 72°C for 10 s. This was followed by a final elongation step at 60°C for 15 min.

PCR products were diluted 1 in 15 for the multiplex reaction, and left undiluted for the single reaction. A post-PCR clean-up was performed, after which the samples were mixed in a 1:1 ratio with Hi-Di formamide (Applied Biosystems) in a total volume of 10 µL, followed by addition of 0.45 µL GeneScan™-600 LIZ® Size Standard (Applied Biosystems). Fragments were separated in an automated single capillary genetic analyzer ABI3730x/ DNA sequencer (Applied Biosystems). Data was analyzed using the software Gene Mapper version 3.7 (Applied Biosystems). Only one allele was amplified for all loci, consistent with the haploid nature of *B. cinerea*.

3.3.9 Population genetics analyses

Clonal distribution and frequency of genotypes between and within populations was inferred based on microsatellite profile matches. An index of genotypic diversity (\hat{G}) denoted by the following formula,

$$\hat{G} = 1 / \sum_{x=0}^n [fx \cdot (x/n)^2]$$

was calculated within populations; n = sample size, fx = number of distinct haplotypes observed x times within a population. For comparison between populations, the index (\hat{G}) was divided by the number of samples to obtain the percentage of maximal genotypic diversity (G) (Stoddard and Taylor, 1988; McDonald *et al.*, 1994).

The software GenAlEx version 6.4 (Peakall and Smouse, 2006) was used to calculate Nei's gene diversity (Nei, 1973), the number of different multilocus genotypes (MLG), index of association (I_A), Wright's Fixation Index (F_{ST}) and to perform an analysis of molecular variance (AMOVA).

The index of association ($I_A = (V_O/V_E)-1$) is a generalized measure for linkage disequilibrium (Brown *et al.*, 1980; Maynard-Smith *et al.*, 1993; Haubold *et al.*, 1998). It gives information on whether two different individuals sharing an allele at one locus are more likely to share an allele at another (Karchani-Balma *et al.*, 2008). The index is computed as follows; for any two paired individuals, the number of loci with which they differ is calculated. The variance of this number is then compared with the expected variance if there were no linkage disequilibrium, in other words linkage equilibrium. The value is expected to be zero if there is no association between loci and increases as the linkage disequilibrium increases (Maynard-Smith *et al.*, 1993; Fournier and Giraud, 2007). The null hypothesis of complete panmixia, *i.e.* I_A equal to zero, was tested by comparing the observed data set to 999 randomized data sets, in which infinite recombination were imposed, by randomly shuffling the alleles among individuals independently of each locus.

Wright's Fixation Index (F_{ST}) was calculated to determine the extent of population differentiation in pairwise comparisons, after 999 permutations. It is a measure of the genetic differentiation of a subpopulation relative to the total population due to non-random mating (Wright, 1951; Slatkin and Barton, 1989). Values can range between 0 (no population differentiation) and 1 (complete population differentiation, with no gene flow between populations). Thus a low F_{ST} value indicates that most of the diversity is found within the subpopulation and not much between the populations. Conversely, a high value indicates substantial genetic differentiation between the populations.

To estimate the relative contribution of location on the genetic variation observed, a hierarchical analysis of molecular variance (AMOVA) was performed and the significance of genetic variations were determined after 999 permutations in pairwise population comparisons.

And finally, to determine whether there is a significant correlation between geographic distance, expressed as the log of geographic distance in pairwise comparisons of populations, and genetic differentiation between all pairs of genotypes of subpopulations, a Mantel test was performed (Mantel, 1967). The significance of this comparison was

determined after 999 permutations. A significant negative correlation is expected if the geographic distance affects the extent of genetic differentiation between populations, which would indicate isolation by distance.

All the tests were conducted on the four individual populations as well as the total *B. cinerea* population obtained. The values calculated for the I_A was done for individual populations only. All populations were clone-corrected prior to allele based analyses to remove bias of over-representation of clones.

3.3.10 Determination of baseline sensitivity towards fenhexamid

Fenhexamid is not registered for control of *B. cinerea* on pears in South Africa. A subset of 29 isolates, representing the four orchards was used to determine the baseline sensitivity. Commercial grade fenhexamid (Teldor 500 SC, Bayer CropScience, South Africa) was dissolved in sterile distilled water to obtain a 1.0 g/L stock solution. Sensitivity towards fenhexamid was tested on PDA medium. The PDA was amended with 0.0, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0 and 10 mg/L of fenhexamid.

Fenhexamid amended PDA plates were center-inoculated with 5-mm diameter mycelium plugs (mycelium facing downward) from actively growing colony margins of 3-day-old cultures. Each isolate was represented by three replicates per concentration. Radial growth of colonies was measured twice perpendicularly after 3 days incubation at 22°C in the dark. For each concentration, percentage inhibition relative to the control was calculated. The EC₅₀ value (effective concentration inhibiting mycelial growth by 50%) was determined for each isolate from the most appropriate non-linear regression model describing the inhibition × concentration interaction using SAS Version 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Based on these results, and work by Esterio *et al.* (2007), 1.0 mg/L and 10.0 mg/L fenhexamid was chosen as the discriminatory doses.

3.3.11 Screening for resistance towards fenhexamid, iprodione and benomyl

A total of 181 genotyped isolates were screened for resistance to iprodione and benomyl. A subset of 99 genotyped isolates (25 isolates per orchard and 24 isolates for orchard D) was screened for resistance against fenhexamid. Technical-grade iprodione (a.i. 98%, ACI Chemicals, Stellenbosch, South Africa), commercial grade benomyl (Benomyl, Bayer CropScience, South Africa) and fenhexamid (Teldor 500 SC, Bayer CropScience,

South Africa) was dissolved in acetone (iprodione) or sterile distilled water (benomyl, fenhexamid) to make 1.0 g/L stock solutions. Mycelial growth sensitivity was determined for discriminatory concentrations of iprodione (3.0 mg/L), benomyl (5.0 mg/L) (Fourie and Holz, 1998) and fenhexamid (1.0 and 10.0 mg/L) on amended PDA. In all cases, where applicable, the final amount of acetone in the medium was 0.1%, including the control plates. Each isolate had three replicates per concentration.

Plates were inoculated with 5-mm diameter mycelium plugs (mycelium facing downward) from actively growing colony margins of 3-day-old cultures. Radial growth of colonies was measured after 36 h incubation at 22°C in the dark. Isolates were regarded as sensitive when growth was observed on the control plates and no growth was observed on the fungicide-amended plates, and resistant when growth was observed on the fungicide-amended plates (Fourie and Holz, 1998; Esterio *et al.*, 2007). The resistance frequency expressed as a percentage of the number of isolates tested, was then determined for *Botrytis* populations from each orchard and the population as a whole.

3.4 Results

A total of 181 *B. cinerea* isolates were obtained from blossoms and weeds in four pear orchards. *Botrytis cinerea* isolated from blossoms is assumed to lead to eventual calyx end decay. *Botrytis cinerea* was found on all of the trees in orchard B and D and on approximately half the trees in orchard A and C. The highest number of infected flowers per tree out of 30 varied between orchards; from 3 in A, 4 in C to 8 in B and 9 in D. Orchard B and D both had high incidences (10% and 17%, respectively) of *B. cinerea* infection on blossoms sampled. Both orchards A and C had 3% blossom infection incidence.

Weeds were also infected with *B. cinerea* but the incidences on these were lower than on the blossoms and they represent approximately 14% of the total isolates sampled (Table 1). Three species were responsible for 65% of the total *B. cinerea* sampled from weeds. These were: *Hypochoeris radicata* ("Skaapslai"), *Plantago lanceolata* ("Oorpynhoutjie") and *Sonchus asper* ("Doringsydissel").

Table 1. Number of *Botrytis cinerea* isolates obtained from different weeds collected next to sampled pear trees.

Orchard	A	B	C	D	Total
Weeds					
<i>Plantago lanceolata</i>	2	3	0	0	5
<i>Bromus catharticus</i>	0	1	0	1	2
<i>Sonchus asper</i>	3	2	1	0	6
<i>Medicago polymorpha</i>	1	1	0	0	2
<i>Erodium moschatum</i>	0	1	1	0	2
<i>Hypochoeris radicata</i>	0	0	6	0	6
<i>Arctotheca calendula</i>	0	0	1	0	1
<i>Conyza bonariensis</i>	0	0	0	1	1
	6	8	9	2	26

3.4.1 *Botrytis cinerea* Group I and II distinction

All 181 isolates belonged to Group II *B. cinerea* as all yielded a 517 bp fragment after digestion of the Bc-*hch* locus with *HhaI*.

3.4.2 Transposable element characterization

According to the transposable element types, *Boty* and *Flipper*, three possible transposon types of *B. cinerea* were detected. Twenty-five percent (25.4%) of the isolates had both the *Boty* and *Flipper* elements (*transposa* type), 74% had only the *Boty* element (*boty*-only), and one (0.6%) had the *Flipper* element only (*flipper*-only) (Table 2). No isolates of the *vacuma* type, *i.e.* containing neither transposable element, were detected in this study. The incidence of *transposa* and *boty*-only isolates was similar in Ceres (23 and 77%, respectively) and Grabouw areas (28 and 71%, respectively). The one *flipper*-only isolate was detected from orchard D. At orchard level, there was one orchard in each region which had a higher incidence of *boty*-only isolates, with 84% for orchard A and 100% for orchard C, as well as a higher incidence of *transposa* isolates (Table 2).

Table 2. Number of *Botrytis cinerea* isolates of the different transposable element types from each orchard.

Orchard	No. of isolates	<i>Boty</i>	<i>Flipper</i>	<i>Transposa (Boty + Flipper)</i>
A	25	21	0	4
B	58	43	0	15
C	25	25	0	0
D	73	45	1	27
Total	181	134	1	46

3.4.3 Mating type determination

The amplification of the *MAT1-1* alpha gene yielded a product of approximately 1050 bp and the amplification of the *MAT1-2* HMG gene yielded a product of approximately 1100 bp (Fig. 3).



Fig 3. Example of gel electrophoretic profile of the mating type PCR amplification. Lane M contained a 100 bp ladder (Promega). The same sample, amplified either for *MAT1-1* (above) or *MAT1-2* (below), were loaded in lanes 1 to 15. Lanes 1-5 and 11-15 represent samples with the *MAT1-1* allele. Lanes 6-10 represent samples with the *MAT1-2* allele. Lane 16 contained the respective positive controls for each reaction, above (*MAT1-1*, isolate SAS 56, 1050 bp) and below (*MAT1-2*, isolate SAS 405, 1100 bp). Lane 17 contained the respective negative controls.

All isolates were heterothallic, containing either the *MAT1-1* or *MAT1-2* allele. In total, approximately 47% of the isolates had the *MAT1-1* allele and 53% the *MAT1-2* allele (Table 3). A chi-square test was performed to determine the goodness of fit of this distribution compared to the expected mating type ratio of 1:1, for the total population ($\chi^2 = 0.67$, $P > 0.05$, $df = 1$) and subpopulations for which values ranged from ($\chi^2 = 0.36$, $P > 0.05$, $df = 1$) to ($\chi^2 = 1.72$, $P > 0.05$, $df = 1$) (Table 3). The hypothesis of recombination, with the resultant equal distribution of *MAT1-1* and *MAT1-2* alleles, was thus accepted for all orchard populations including the total population (Table 3).

Table 3. Distribution and mating type frequencies of *Botrytis cinerea* isolates.

Population	No. of isolates	MAT1-1	MAT1-2	χ^2 ^A
Orchard A	25	15	10	1.0
Orchard B	58	24	34	1.72
Orchard C	25	14	11	0.36
Orchard D	73	32	41	1.11
Total	181	85	96	0.67

^A χ^2 value based on 1:1 ratio and 1 degree of freedom

3.4.4 Genetic diversity

All microsatellites amplified were polymorphic and the number of alleles amplified per locus ranged between 7 and 21, thus giving sufficient discriminatory power to assess population dynamics. The mean number of alleles for all loci was 6.2 (Table 4).

Table 4. Genetic diversity indexes of *Botrytis cinerea* populations collected from four pear orchards in South Africa.

Population	No. of isolates	MLG ^A	G ^B	H ^C	Mean no. of alleles per locus ^D	I _A ^E
Orchard A	25	10	32	0.75 (0.04)	5.8 (0.738)	3.856 (0.537)
Orchard B	58	35	32	0.79 (0.03)	9.1 (1.335)	2.340 (0.001)
Orchard C	25	7	18	0.62 (0.03)	3.3 (0.360)	5.306 (0.001)
Orchard D	73	39	33	0.61 (0.05)	6.4 (0.719)	1.144 (0.001)
Total	181	91	14	0.69 (0.02)	6.2 (0.571)	—

^A MLG: Multilocus genotypes^B G: Percentage of maximal genotypic diversity (McDonald *et al.*, 1994)^C H: gene diversity (Nei, 1973)^D Standard deviation shown in brackets^E I_A: index of association (*P*-values in brackets)

3.4.4.1 Genetic diversity within populations

Microsatellites were analysed for a total of 181 isolates, and 91 multilocus genotypes (MLG) were observed within the South African populations in pear orchards. Identical genotypes were assumed to be clones. The most MLGs were detected in orchard D (39 out of 73) and the least in orchard C (7 out of 25) (Table 4). The percentage of maximal genotypic diversity ranged from 18 in orchard C to 33 in orchard D (Table 4). Nei's measure of gene diversity (*H*) (per population) yielded high values ranging from 0.61 (orchard D) to 0.79 (orchard B), with a mean of 0.69 for the total population (Table 4). Orchards A and B had considerably higher gene diversity compared to C and D. Mean number of alleles per locus ranged from 3.3 in orchard C to 9.1 in orchard B. The index of association (*I_A*) differed significantly from zero for three of the four populations with values ranging from 1.144 (orchard D) to 5.306 (orchard C), rejecting the hypotheses for random mating and indicating strong associations between loci (Table 4). In the total population 66 genotypes were observed once and 25 were observed at least twice ($x \geq 2$) or more (Table 5). Genotypes that were observed once varied between populations and ranged between 7 (orchard C) and 36 (orchard D).

Table 5. Frequency distribution of *Botrytis cinerea* multilocus genotypes (MLG) within pear orchard populations in the Ceres and Grabouw areas of South Africa.

Number of genotypes observed x times					
Region	Ceres		Grabouw		
x	Orchard A	Orchard B	Orchard C	Orchard D	Total ^A
1	11	24	7	36	66
2	2	6	1	5	14
3	1	4	0	1	3
4	0	0	0	1	1
5	0	0	0	1	1
6	0	0	0	1	2
7	1	0	0	0	1
8	0	0	2	0	0
9	0	0	0	1	1
10	0	1	0	0	0
14	0	0	0	0	1
27	0	0	0	0	1

^A Four orchard populations pooled together, only genotypes shared among populations recorded in total

3.4.4.2 Genetic diversity and differentiation between populations

Results for the pairwise determination of population differentiation, F_{ST} , yielded moderate values, with not all comparisons being significant (Table 6). Only comparisons of orchard D to the other three orchards yielded significant values. Moderate F_{ST} and correspondingly low Nm values were obtained when orchard D was compared to orchard A ($F_{ST} = 0.120$, $P = 0.001$, $Nm = 3.6$), to orchard B ($F_{ST} = 0.134$, $P = 0.001$, $Nm = 3.2$) and to orchard C ($F_{ST} = 0.192$, $P = 0.001$, $Nm = 2.1$). Other pairwise comparisons yielded non-significant values (Table 6). A similar analysis of the two dominant transposable element type populations (*flipper*-only isolate excluded) indicated very low differentiation between them as populations, with an F_{ST} value of 0.044 (Table 7).

Table 6. Pairwise F_{ST} (above diagonal) with Nm values (below diagonal) among four *Botrytis cinerea* populations sampled from pear orchards.

Orchard A	Orchard B	Orchard C	Orchard D	Population
-	0.017	0.101	0.120*	Orchard A
29.6	-	0.036	0.134*	Orchard B
4.4	13.3	-	0.192*	Orchard C
3.6	3.2	2.1	-	Orchard D

* significant values ($P < 0.01$)

Analysis of molecular variance (AMOVA) revealed that 88% of the total variation was distributed within the populations, 9% between them and only 3% among regions with a total F_{ST} value of 0.118 ($P = 0.001$) (Table 7). Since all isolates sampled from the „Forelle“ came from Ceres and all those sampled from the „Packham’s Triumph“ came from Grabouw, it was impossible to separate the effect of cultivar from that of region in this study. A Mantel test showed no significant negative correlation between geographic distance and population differentiation, indicating no isolation by distance ($y = 0.803x + 3.872$, $R^2 = 0.126$, $P = 0.315$).

Table 7. Analysis of molecular variance (AMOVA) with geographic origin and transposable element types (*transposa* and *boty*-only) as grouping factors.

Source	d.f.	SS	MS	Est.Var.	% ^A	Stat	Value	P-value
Between Regions	1	15.006	15.006	0.080	3			
Between Populations	2	12.811	6.406	0.274	9			
Within Populations	87	230.666	2.651	2.651	88			
Total	90	258.484	24.063	3.006		F_{ST}	0.118	0.001
Between TE types	1	8.061	8.061	0.129	4			
Within TE types	88	247.506	2.813	2.813	96			
Total	89	255.567	10.873	2.942		F_{ST}	0.044	0.001

^A Percentage of total variation

More direct evidence of genotype flow was evident through the investigation of clonal genotypes shared between two or more populations. Repeated genotypes were observed several times within an orchard, especially within orchards B and D. The distribution of genotypes occurring at least thrice within an orchard was followed and together with the hierarchical sampling scheme presented as schematic diagrams (Figs. 4 to 7). The most frequently isolated genotype (X) was composed of 27 clones and isolated from orchards A, B and C (Figs. 4 to 6). The second most frequently isolated genotype (W) had 14 clones and was shared among orchards A, B and C (Figs. 4 to 6).

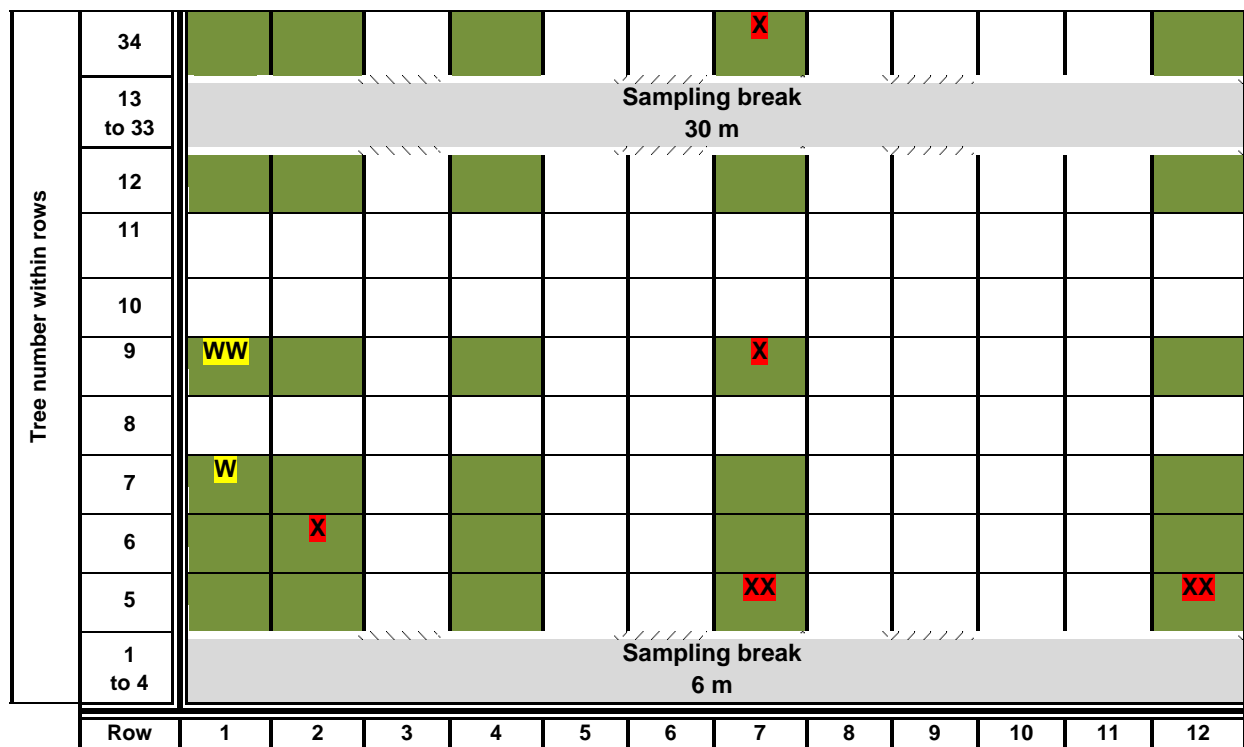


Fig 4. Orchard A with distribution of genotypes W and X. Trees 1.5 m apart and rows 4 m apart.

Sampled trees are indicated by green blocks.

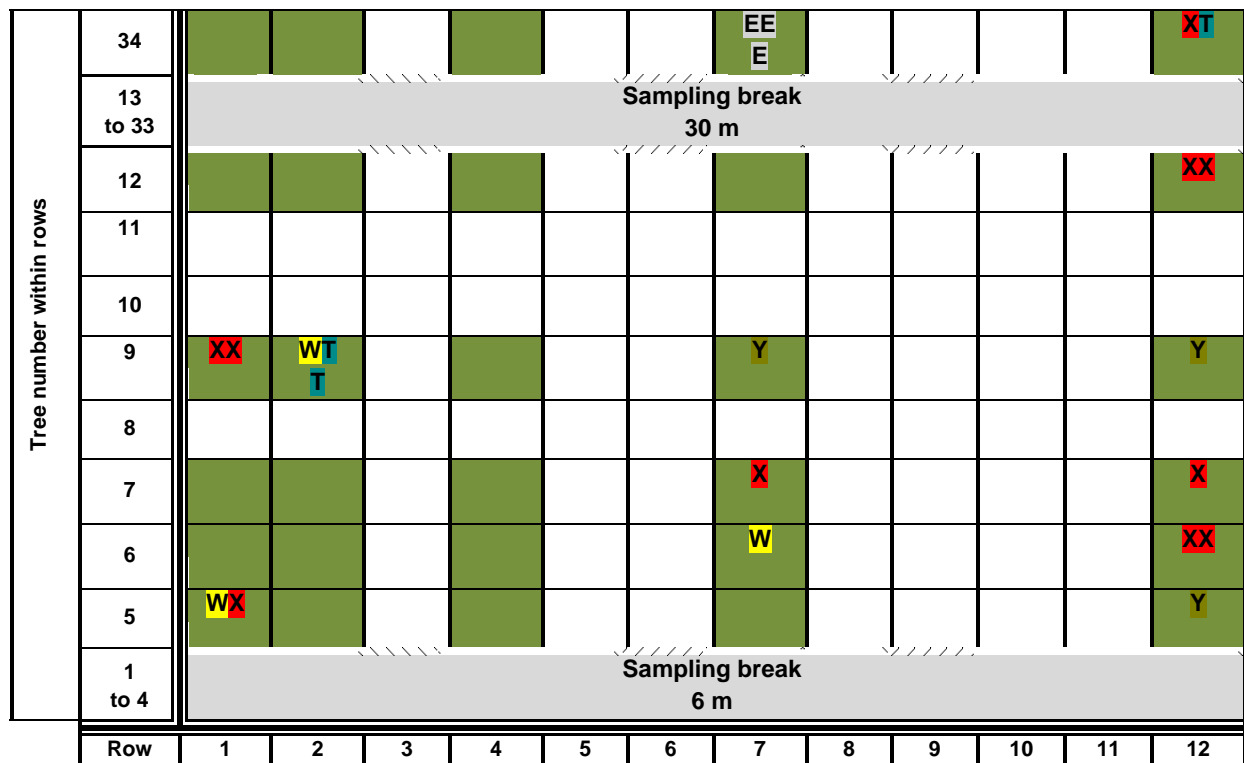


Fig 5. Orchard B with distribution of genotypes E, T, W, X and Y. Trees 1.5 m apart and rows 4.5 m apart. Sampled trees are indicated by green blocks.

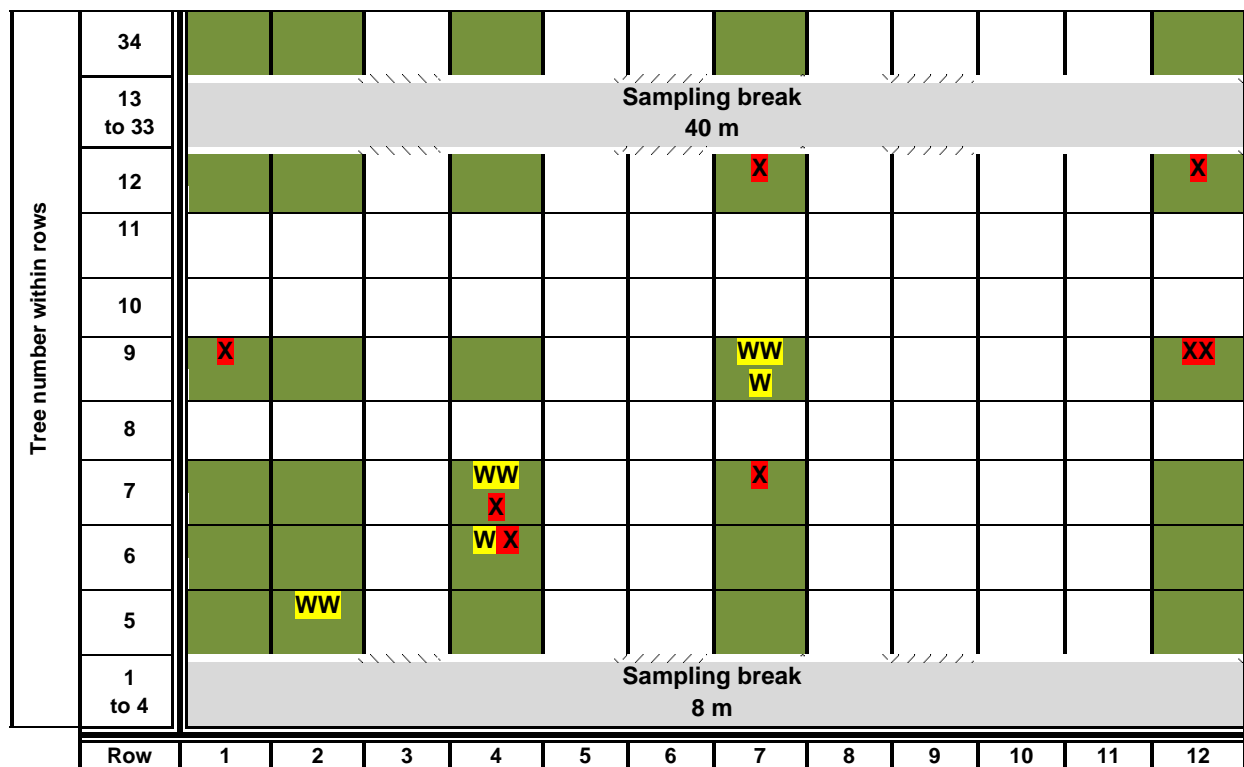


Fig 6. Orchard C with distribution of genotypes W and X. Trees 2.0 m apart and rows 4.5 m apart. Sampled trees are indicated by green blocks.

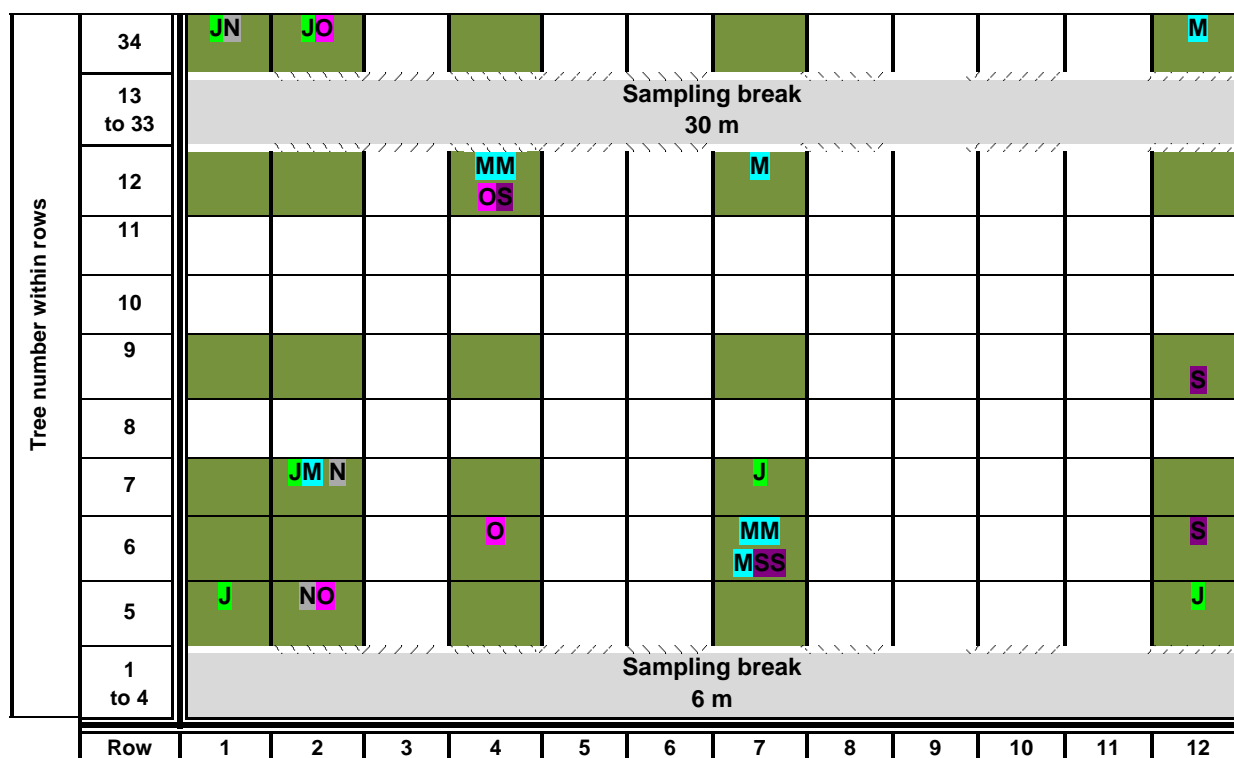


Fig 7. Orchard D with distribution of genotypes J, M, N, O and S. Trees 1.5 m apart and rows 4 m apart. Sampled trees are indicated by green blocks.

3.4.5 Baseline sensitivity towards fenhexamid

Four regression models (Natural Growth, Natural Growth – Ln, Allometric and the Logistic Model) were fitted to the data obtained, of which the Natural Growth Model consistently gave the best statistical fit ($R^2 = 0.884$). For the 29 isolates, EC_{50} values for fenhexamid ranged from 0.020 to 0.289 mg/L. The mean EC_{50} value for all the isolates was 0.048 mg/L with a standard deviation of 0.048.

3.4.6 Screening for resistance towards fenhexamid, iprodione and benomyl

3.4.6.1 Fenhexamid

Overall, low levels of reduced sensitivity towards fenhexamid were detected. Ten percent of the total population was resistant at 1.0 mg/L, of which the Ceres region represented 8%, with 2% in orchard A and 6% in orchard B. The remaining 2% was found in

the Grabouw and Vyeboom regions, divided equally between orchard C and D. One isolate was able to grow at 10.0 mg/L and came from orchard B (data not shown).

3.4.6.2 Iprodione

The four populations sampled yielded a very low incidence of resistance towards iprodione. Only three resistant isolates were found in the Ceres area, one from orchard A and two from orchard B. In the Grabouw area there was no incidence of resistance towards iprodione. These three isolates only amounted to 2% resistance in the total population.

3.4.6.3 Benomyl

Three of the four orchards had negligible resistance towards benomyl, with frequencies ranging from 0% to 4%. However, orchard D had a 78% incidence of resistant isolates (Fig. 8).

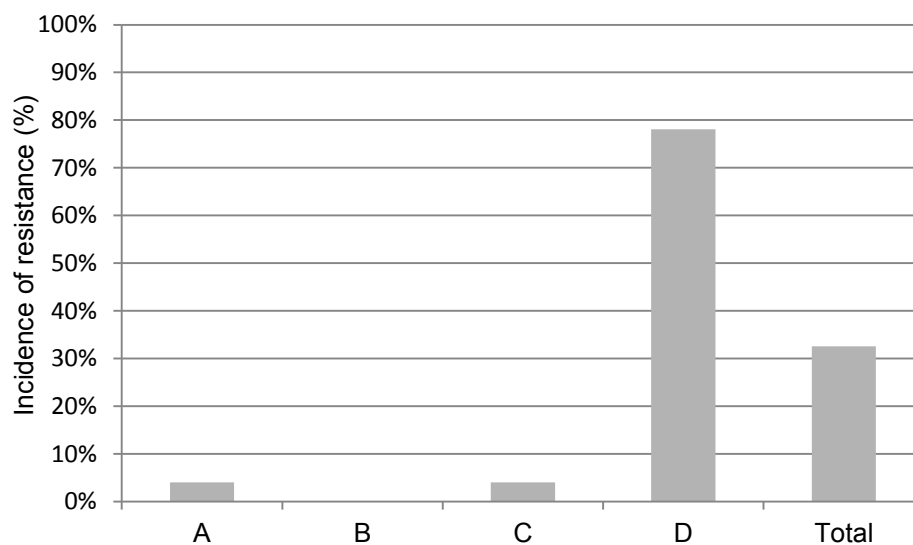


Fig. 8. Incidence of resistance of 181 *Botrytis cinerea* isolates in four orchards (A-D) towards 5.0 mg/L benomyl.

3.4.6.4 Multiple resistance

There were two isolates resistant to more than one fungicide. The one (from orchard A) showed resistance towards iprodione and benomyl. The other (from orchard D) was resistant towards benomyl and displayed reduced sensitivity towards fenhexamid (1.0 mg/L).

3.4.6.5 Fungicide sensitivity within genotypes

Genotypes isolated three or more times in all cases showed the same resistance pattern towards benomyl, being either sensitive or resistant. Genotypes that only had resistant clones were found exclusively in orchard D (J, M, N, O) (Fig. 7).

3.5 Discussion

This is the first study to fully evaluate the natural occurrence of *B. cinerea* on blossoms in pear orchards in South Africa. The extent of blossom infection varied between orchards with one orchard per region (orchards B and D) having a high incidence (more than 10%). Microclimatic conditions may have played a role in this regard as these two orchards are next to mountains, where rainfall is usually higher. This could be creating an environment conducive to the spread of this disease which would explain the higher incidence in blossom infection in orchards B and D. Orchard practices could also contribute to *B. cinerea* incidence. Orchards B and D were the two orchards that had the most vegetation beneath the trees, although this was not necessarily reflected in the incidence of *B. cinerea* obtained from weeds in these orchards. In the case of orchard D only two weed samples revealed latent *B. cinerea* infections.

Different weed species showed the highest infection of *B. cinerea* at regional and orchard level. *Plantago lanceolata* ("Oorpynhoutjie") had a high incidence of *B. cinerea* infections in Ceres and *Hypochoeris radicata* ("Skaapslaai") a high incidence in orchard C in Grabouw. However, it should be kept in mind that *B. cinerea* is a ubiquitous plant pathogen capable of infecting over 200 dicotyledonous plants and many monocotyledonous plants (Williamson *et al.*, 2007). More extensive sampling of weeds would be necessary to obtain an answer to which weed species are most prone to harbor *B. cinerea* infections. Minimizing vegetation as well as dead organic matter in the orchard as part of an integrated

management strategy will remain important to limit the spread of *B. cinerea* in orchards (Spotts and Serdani, 2006).

All of the isolates sampled in this present study belonged to *B. cinerea* Group II, according to the RFLP patterns of the *Bc-hch* locus (Fournier *et al.*, 2003, 2005). This finding reinforces what is found by other researchers across the world, where this cryptic species is also the predominantly occurring species (Fournier and Giraud, 2007; Isenegger *et al.*, 2008b; Váczy *et al.*, 2008; Esterio *et al.*, 2011). Genotypic diversity measures were similar for orchards A, B and D, but were substantially lower for orchard C. Overall genotypic diversity measures indicated the presence of clonal lineages within orchards, with one genotype per orchard occurring at least 7 times. Genotypes shared by orchards A, B and C may serve as an indication of how successful these clonal genotypes were in spreading between isolated orchards and regions. This observation provides direct evidence for genotype flow.

In the studied population no *vacuma* isolates were detected. Three other transposon types were however detected; *transposa*, *boty*-only and *flipper*-only. The majority of the isolates (74%) were of the *boty*-only type and the rest were of the *transposa* type (25%), bar one isolate, which only had the *Flipper* transposable element. The presence of *flipper*-only isolates was not observed in California (Ma and Michailides, 2005) and Chile (Muñoz *et al.*, 2002) and was strongly under-represented in France (Albertini *et al.*, 2002) and Hungary (Váczy *et al.*, 2008). However, in Bangladesh a high incidence of *flipper*-only isolates has recently been observed (Isenegger *et al.*, 2008a). The high incidence of *boty*-only isolates observed in this study agrees with the results of analysis of a population from India and Nepal (72% *boty*-only) (Isenegger *et al.*, 2008a). Analysis of whether there was differentiation between *transposa* and *boty*-only as grouping factors for genotypes yielded a very low F_{ST} value of 0.044 ($P = 0.001$). Thus, this result suggests that there is no restriction in gene flow between these transposon types. It has been found in previous studies that microsatellite variation does not conform to transposon types (Isenegger *et al.*, 2008a) and this could also explain the low differentiation seen between our transposon-type populations. The distinction between *transposa* and *vacuma* is, however, not a valid diagnostic tool to distinguish between cryptic species (Fournier *et al.*, 2005). In population genetic studies the presence of transposable elements is viewed as indirect proof that recombination is occurring. Loss of sex or exclusively asexual reproduction is predicted to lead to a loss of retrotransposons over time, as seen in bdelloid rotifers, an ancient asexually reproducing phylum (Arkhipova and Meselson, 2000). Transposable elements also provide a means to follow changes within a population, if there are multiple sampling events. This could aid in forming a clearer picture of the population dynamics over time.

In agreement with recent population studies conducted on Group II *B. cinerea* in different locations (Fournier and Giraud, 2007; Isenegger *et al.*, 2008a, 2008b; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008), our results showed a high genetic diversity for the 181 isolates genotyped in South Africa. Despite this, measures of the index of association consistently rejected the null hypothesis of complete panmixia (random mating) with I_A values significantly different from zero in three of the four populations (B, C and D). The I_A measure indicates the relative importance of genetic recombination and is used to infer fungal population structure (Milgroom, 1996). Genotypes shared between orchards and the fact that no isolation by distance was observed, suggests that dispersal is aided by anthropogenic activities. This results in disturbance of gene flow and genetic drift, leading to misleading estimates of the index of association (Isenegger *et al.*, 2008b).

Population differentiation was low to moderate among populations, indicating that the studied populations are not considerably influenced by geographic location or by cultivar, „Forelle“ or „Packham’s Triumph“. It was not possible to separate geographic - and host cultivar effect, but it is doubtful that the different cultivars had any effect at all. Significant, but moderate F_{ST} values were found only in comparisons of orchard D to orchards A, B and C. This is most likely due to selection as this population is the only population having high benomyl resistance. Other F_{ST} comparisons were low to moderate, but not significant. In plant species a high turnover rate has been correlated with low levels of differentiation, as extinction prone species tend to be able to disperse very efficiently (Godt and Hamrick, 1991; Broyles and Watt, 1993). Harvesting of pears counts as an extinction event since the *B. cinerea* isolates present within them are permanently removed from the orchard environment. Subsequent re-colonization once again takes place in spring when flowering commences, leading to a small “founder” effect within orchards each year.

Clonal genotypes’ presence within populations is consistent with asexual conidia that can spread readily within a field (Jarvis, 1980). The total area of an orchard sampled was approximately 2100 m² and thus it was not surprising that these clones could spread and establish throughout the orchard. This occurs through gusty winds, human mediated dispersal or rain splash dispersal (up to 2 m) (Jarvis, 1980). However, in the case of pear orchards, bees could act as a vector by dispersing *B. cinerea* conidia through pollination activity. The possible role of dispersal of *B. cinerea* through insect vectors (e.g. mites and bees) needs to be investigated through further studies.

Gene flow restricted population differentiation between orchards A and B, which are the closest geographically (approximately 8 km apart), in a natural basin formed by the surrounding mountains. The two orchards furthest from each other (A and D, 90 km apart) were moderately differentiated yet still shared genotypes. Whether these shared genotypes

are identical by descent or identical in state, in other words if it is in actual fact size homoplasy, remains an open question. However, with such a small sampling radius it is unlikely that these populations are evolving independently. Geography clearly does not influence the studied population to a large degree, as has been seen in other studies (Ma and Michailides, 2005; Fournier and Giraud, 2007; Karchani-Balma *et al.*, 2008).

PCR primer pairs have recently been developed to amplify the two mating type loci in *B. cinerea* (Angelini *et al.*, 2010; van Kan *et al.*, 2010; Amselem *et al.*, 2011). Up until now mating type characterization has been done through traditional mating experiments. Using PCR primers, Angelini *et al.*, (2010) found the isolates they investigated to be of either the MAT1-1 or MAT1-2 type. The present study established that the two mating types, MAT1-1 and MAT1-2, are common in pear orchards in the Western Cape. This is in agreement with what has been found on other hosts (Beever and Parkes, 1993; Faretra and Pollastro, 1993; Delcán and Melgarejo, 2002). In contrast to some previous studies, there was a slightly higher incidence of isolates carrying the MAT1-2 allele (Beever and Parkes, 1993; Faretra and Pollastro, 1993; Van Der Vlugt-Bergmans *et al.*, 1993). However, the observed mating type ratio did not differ significantly from the expected 1:1 distribution for the total population and subpopulations, under the hypothesis of random mating. Taking this into account, another explanation for the observed linkage disequilibrium could be that the studied populations of *B. cinerea* Group II has a mixed reproduction system, with infrequent sexual recombination occurring. However, apothecia have never been observed in orchards in South Africa.

The occurrence of resistance towards the three fungicides tested varied from low frequencies of resistance towards iprodione, moderate frequencies of resistance towards fenhexamid and mostly low frequencies of resistance towards benomyl except for orchard D where high resistance levels were found. These observations can for the most part be explained. Firstly, the dicarboximide iprodione is registered as a full bloom and/or 75% petal-drop spray in South Africa, but is rarely applied as such. It is used in the post-harvest treatment of the fruit as well, and the recommendation to farmers is not to use the same active ingredient postharvest. However, some farmers do apply it as it has a short withholding period. The records for the orchards in the Ceres region, from which the reduced sensitivity isolates towards iprodione were obtained, did not indicate any dicarboximide sprays. Iprodione is registered and used for the control of *B. cinerea* on other crops cultivated in the Ceres region namely on stone fruit trees and onions, from where iprodione resistant inoculum might have migrated.

Resistance to the fungicide fenhexamid is phenotypically linked to *B. cinerea* Group I isolates. A possible explanation for this proposed by Fournier *et al.* (2003) is that the two

cryptic species have become adequately genetically differentiated to have fixed private alleles, which happens to include the resistance alleles (HydR1). In the past it was used as a diagnostic tool to discriminate between *B. 'pseudocinerea'* and *B. cinerea 'sensu stricto'*. However, Group II isolates resistant to fenhexamid have been found, even prior to registration of the fungicide, in Germany and Japan (Leroux *et al.*, 2002). These were moderately (HydR2) to highly (HydR3) resistant towards fenhexamid in mycelial growth studies, with only HydR3 isolates having resistance during germ tube elongation (Leroux *et al.*, 2002). Esterio *et al.* (2007) found higher levels of resistance towards fenhexamid, based on EC₅₀ values, yet did not report failure of control through fenhexamid applications on grapevines. In our case 10% of the screened isolates displayed reduced sensitivity during mycelial growth at 1.0 mg/L and one isolate was resistant and able to grow at 10.0 mg/L. The natural resistance found towards fenhexamid within our Group II isolates indicates that care should be taken to prevent resistance build up, were this fungicide to be registered for use on pears.

Resistance towards benomyl was low in three of the four orchards except orchard D, where a high incidence of resistance (almost 80%) was observed. Benomyl is registered as a full bloom and/or 75% petal drop spray in South Africa, and legislation enforced its application up until 40 years ago. A minority of farmers today still apply one benomyl application per season at full bloom and/or 75% petal drop. The records for orchard D showed that benomyl was applied up until 2009. Thus there was constant selection for resistance within this orchard. This could explain the observation that clones in genotypes J, M, N and O occurring exclusively within orchard D were all resistant to benomyl. This suggests that this trait has become fixed and is able to persist, due to fungicide selection each year (Lyr, 1995), which in turn leads to successful spread of these genotypes within orchard D. The consequent movement of these highly adapted resistant genotypes to orchard C, which is geographically close, can easily be imagined when considering the direct evidence of genotype flow.

One isolate was found within orchard A which displayed cross resistance to benomyl and iprodione. In such a case the isolate would survive an orchard application of benomyl and postharvest application of iprodione. If such an isolate were to be re-introduced in an orchard, it would be able to multiply and start a source of dual resistance isolates. Considering this, together with the relatively high gene flow observed in this study, it is possible that this resistance gene or genotype could move to orchard D. However, resistance towards dicarboximides has been associated with fitness penalties and considering this, there will have to be selection within a given orchard to maintain the existence of such isolates (Yourman *et al.*, 2001; Fourie and Holz, 2003; Cui *et al.*, 2004).

In recent years, it has become clear that *B. cinerea* has been driven to evolve multidrug resistance (MDR) through fungicide applications. Simultaneous resistance towards chemically unrelated groups is obtained through active efflux of drugs, thereby conferring resistance against multiple fungicides (Myresiotis *et al.*, 2007; Kretschmer *et al.*, 2009; Bardas *et al.*, 2010). This trend together with results represented here should serve as a notice for the South African pear industry. The studied population of *B. cinerea* in pear orchards is essentially a single large panmictic one and the findings of this study stress the importance of integrated disease management at a regional level with constant assessment of fungicide resistance levels. Collaboration between producers and industry is essential to aid in the overall sustainable management of this disease in the future. Loss of chemical control is illustrated by the prolonged selection for benomyl resistance in orchard D. Integrated use of multiple chemical classes should be encouraged in this orchard, or ideally complete cessation of benomyl application.

In conclusion, the studied populations of *B. cinerea* occurring in pear orchards in the Ceres and Grabouw area in the Western Cape of South Africa are characterized by high levels of gene diversity and a predominantly asexual reproduction system. Yet there is population admixture to a large extent, evidenced through the spread of clonal genotypes, and the studied population represents a large panmictic population. This together with no isolation by distance suggests that the South African *B. cinerea* populations in pear orchards most likely represent founder populations. The level of linkage disequilibrium may contribute to keeping well-adapted combinations of genes together through asexual reproduction. This together with high levels of gene diversity and the possibility of sexual reproduction suggests a mixed reproduction system. Knowledge of the population genetics and resistance levels of *B. cinerea* during blossoming in pear orchards may in future aid in the development of more integrated management strategies.

3.6 References

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Chapter 4

Concluding discussion

Investigating the genetic diversity and population structure of any plant pathogen is important in order to assess the adaptive potential in order to overcome control strategies such as fungicide applications (McDonald and Linde, 2002). The genetic diversity and population structure of *Botrytis cinerea* from rooibos nurseries and pear orchards was investigated in the current study. These results together with the resistance levels have yielded valuable insights into the two metapopulations of *B. cinerea* sampled.

Analysis of the cryptic species status of *B. cinerea* isolates revealed that all but one (in nursery E) of the isolates yielded a 517 bp fragment after digestion of the Bc-*hch* locus. This indicates that the majority of the sampled isolates belong to *B. cinerea* Group II which is in agreement with what has been found in other studies (Isenegger *et al.*, 2008a; Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008; Váczy *et al.*, 2008). Group I is sometimes found in sympatry with Group II, however, it is seen as a separate phylogenetic species (Fournier *et al.*, 2005; Fournier and Giraud, 2007).

The microsatellite markers used in this study was found to be sufficiently discriminatory in order to assess the level of diversity in the studied populations of *B. cinerea* Group II. Taking into account the overall genetic diversity of the populations, it indicates a highly heterogenous population as a whole, with values obtained similar to *B. cinerea* populations sampled from different hosts around the world (Alfonso *et al.*, 2000; Fournier and Giraud, 2007; Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008). The genotypic diversity for the populations, from nurseries and orchards, indicated the presence of clonal lineages within all. Genotypes occurring at a high frequency within each population, with multiple clones per genotype, indicated the success that these particular genotypes had in terms of spreading disease in each cropping system. Also, the observation of genotypes shared between nurseries and between orchards, especially the genotype comprised of 27 clones, provided very direct evidence for genotype flow within each cropping system.

In the present study, the index of association (I_A) was in all cases different from zero. This indicates that the studied populations are reproducing asexually, in contrast to some studies (Fournier *et al.*, 2005; Fournier and Giraud, 2007; Karchani-Balma *et al.*, 2008, Váczy *et al.*, 2008) and in agreement with others (Ma and Michailides, 2005; Isenegger *et al.*, 2008b). Possible reasons for this observation provided by Isenegger *et al.* (2008b) is that

either there is no recombination occurring, or that the analyses were hampered by factors such as genotype flow and population admixture (Millgroom, 1996; Isenegger *et al.*, 2008b). In order to reject the hypothesis for random mating, *i.e.* sexual reproduction occurring, and accepting the alternative hypothesis of linkage disequilibrium (asexual reproduction) one has to strictly speaking discount all other factors (*i.e.* genotype flow, population admixture, selection and drift, *etc.*) (Millgroom, 1996). In this study, the observed genotype flow and population admixture may also have given rise to the observed I_A , leading to possibly false assumptions of linkage disequilibrium.

Further evidence for the observed linkage disequilibrium can be found in the way in which each of these cropping systems operates. The rotation of fields over a period of four years in rooibos nurseries and the relatively small timescale that blossom infection can occur within pear orchards, together with harvesting of the pears afterward, could be causing founder effects or bottlenecks within each cropping system at the point in time it was investigated. If this is the case, it may then in turn quickly lead to a population comprised of successful virulent genotypes within a season, which could be selected for through fungicide applications. It may be that there is sexual recombination occurring, which generate well-adapted genotypes, which then reproduce clonally (McDonald and Linde, 2002). This hypothesis is supported by the presence of both mating types within each investigated population, which suggests that sexual reproduction is possible. The characterization of the *B. cinerea* MAT loci through PCR-based methods, as done in this study, has the potential to become an important tool in population studies on *B. cinerea*, since traditional mating type studies is a time consuming endeavour.

In population genetics studies the presence of transposable elements is, however, an indirect proof that recombination is occurring. Loss of sex or exclusively asexual reproduction is predicted to lead to a loss of retrotransposons over time, as seen in bdelloid rotifers, an ancient asexually reproducing phylum (Arkhipova and Meselson, 2000). Transposable elements were only characterised for the *B. cinerea* Group II from pear blossoms, because distribution of TE types were linked to phenological stages of grapevines in the past. In previous studies, a high incidence of *vacuma* isolates was present on flowering parts of grapevine when compared to later phenological stages, such as bunch closure (Giraud *et al.*, 1997; Martinez *et al.*, 2005). However, these observations may in actual fact have been due to differences between Group I and II (Fournier *et al.*, 2005; Fournier and Giraud, 2007) and not between transposon types. In the studied population of *B. cinerea* Group II in pear orchards a high incidence of *boty*-only isolates (74%) was detected. This is in agreement with the composition of populations isolated from India and Nepal, where *boty*-only isolates made up 72% of the populations. *Transposa* isolates (25%)

represented the majority of the remainder of the isolates. Only one isolate belonged to the *flipper*-only type which is in agreement with other studies where this type has been severely underrepresented (Albertini *et al.*, 2002; Váczy *et al.*, 2008) or completely lacking (Muñoz *et al.*, 2002; Ma and Michailides, 2005). Analysis of molecular variance (AMOVA) of *transposa* and *boty*-only isolates as grouping factors yielded a low F_{ST} value of 0.044 ($P = 0.001$). This result suggests that there is no restriction in gene flow between these transposon types. It has been found in previous studies that microsatellite variation does not conform to transposon types (Isenegger *et al.*, 2008a) and this could also explain the low differentiation seen between the transposon-type populations. Transposable elements also provide a means to follow changes within a population, provided there are multiple sampling events. This could aid in forming a clearer picture of the population dynamics over time.

Genotypes shared between different populations within each cropping system and no isolation by distance observed in either, suggests that human mediated dispersal may be taking place or that the studied populations represent recent founder events. This results in disturbance of gene flow and genetic drift, again affecting the linkage disequilibrium analysis.

In agreement with direct evidence for gene and genotype flow, the AMOVA for both cropping systems sampled revealed the majority of the variation to be found within subpopulations which measured between 0.25 and 1 ha for each of the rooibos populations and approximately 0.21 ha for each of the pear populations. In the case of the pear population, there was the added effect of region factored into the calculation, yet this accounted for only 3% of the total variance seen. Overall, measures of population differentiation for the populations ranged from low (rooibos, $F_{ST} = 0.030$, $P = 0.001$) to moderate (pears, $F_{ST} = 0.118$, $P = 0.001$). There was no true identity according to geographic origin, with migration preventing genetic differentiation. These results show that the *B. cinerea* Group II populations are not significantly influenced by spatial considerations, at least at the studied scale. This study reinforces the findings of other studies which showed that *B. cinerea* populations worldwide are genetically diverse and characterized by low to moderate population differentiation (Alfonso *et al.*, 2000; Moyano *et al.*, 2003; Ma and Michailides, 2005; Isenegger *et al.*, 2008b; Karchani-Balma *et al.*, 2008).

No link between fungicide resistance and genotype profiles was found in the population sampled from rooibos. However, this was not the case for the population obtained from orchard D in the pear population. Here several multilocus genotypes (MLGs) were observed that were represented by only benomyl resistant clones. These isolates were, however, not shared with any other orchard population. This suggests that the resistance allele may have become fixed in these MLGs within orchard D, due to prolonged selection through the application of benomyl. This observation taken together with the genotype flow

seen overall could mean that one producer's possible malpractice is effectively detracting from options for the control of *B. cinerea* available to other producers in his region and even province. Existence of a few isolates resistant to two actives in the pear population possibly indicates the emergence of multidrug resistance (MDR) in this cropping system, as has been seen in Europe in recent years (Myresiotis *et al.*, 2007; Kretschmer *et al.*, 2009; Bardas *et al.*, 2010). This situation needs to be monitored on a regular basis. A rapid method for monitoring benzimidazole and dicarboximide resistance in field populations of *B. cinerea* has been developed and could possibly be extended for monitoring resistance in field populations in South Africa. Genotyping the *BenA* (β -tubulin) and *Bos1* (two-component histidine kinase) genes, mutations of which confers resistance to benzimidazoles and dicarboximides respectively, has been developed by Banno *et al.* (2008) and a fluorescent hybridization probe is used in real-time PCR in order to monitor fungicide resistance.

In conclusion, the observed levels of linkage disequilibrium overall may be contributing to keeping well-adapted combinations of genes together through asexual reproduction, with interspersed sexual recombination being most likely, despite no apothecia being observed in either cropping system to date. A mixed reproduction system poses a large risk in terms of possible rapid break-down of control measures. The integrated management of the disease, at orchard and regional level, is therefore underlined through the findings of the population dynamics of the studied populations of *B. cinerea* Group II on rooibos seedlings and in pear orchards. Knowledge of the population genetics and resistance levels of *B. cinerea* Group II in these two production systems will in future aid in the development of more integrated management strategies. For example, sanitation of orchards and nurseries should be more effective to prevent the buildup of inoculum, which could spread to other orchards, nurseries and regions. In addition, regular resistance- and genotype profile screenings would aid in determining and monitoring the spread of resistant isolates.

Future studies should include *B. cinerea* sampled from other cropping systems (e.g. grapevine, strawberry and tomato) to determine whether they represent genetically isolated or similar populations of *B. cinerea*. Preferably several samplings should be done over a period of time in each system to ascertain whether there are; I) changes in the temporal distribution of the different cryptic species, II) changes in the frequency of the different transposable element types occurring, III) changes in resistance frequency towards various fungicides and IV) changes in the distribution of mating types. This should provide a clear picture of the population dynamics of *B. cinerea* in South Africa. Regarding the study conducted on the population obtained from pears, the possible role of insect vectors, especially bees and mites, should be thoroughly investigated through further experiments.

Future work applicable to both populations studied, would be a more directed and intensive sampling of weeds in and around each nursery and orchard to elucidate the role of secondary hosts important to each particular environment.

4.1 References

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