INOCULUM ECOLOGY OF PETRI DISEASE FUNGI IN GRAPEVINES OF SOUTH AFRICA

by

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SUMMARY

Petri disease is among the important grapevine trunk diseases affecting lifespan and productivity of young vines worldwide. Infection result in poor vine stand in newly established vineyards and a general vine decline. Pathogens causing this disease are known invaders of susceptible pruning wounds. The knowledge of when aerial spore inoculum of these pathogens are released in vineyards has not been reported in South Africa, and this result in growers pruning without the knowledge of whether that would coincide with periods of high aerial spore concentration. This study aimed at investigating when aerial spores of Petri disease pathogens are released, and to determine their source of inoculum.

Knowledge regarding spore release in South African vineyards was determined for two seasons in 2012 and 2013. Spore traps were affixed to arms of infected vines in six vineyards and two rootstock mother blocks. Results showed the occurrence of Petri disease pathogens throughout the year and *Phaeomoniella chlamydospora* and *Pm. minimum* were trapped in all vineyards. A total of 14 *Phaeoacremonium* species were identified from the different blocks. Spore release was shown to coincide with pruning and suckering activities, however, there was no positive correlation between rainfall and spore release events.

The occurrence of Petri disease pathogens fruiting bodies was determined by surveying six vineyards and two rootstock mother blocks between 2012 and 2014. Dead wood from diseased vines were collected for microscopic examination. *Phaeomoniella chlamydospora* pycnidia were found in all vineyards and rootstock mother blocks surveyed. Perithecia of *Pm. minimum* were only found in vineyards of Stellenbosch P2 and B3, Rawsonville and a rootstock mother block in Slanghoek. Additionally, mating studies with isolates of *Pm. australiense* and *Pm. scolyti* were conducted *in vitro*. After seven and eleven months fertile perithecia of *Pm. australiense* and *Pm. scolyti* were observed, respectively. Crosses of both species corresponded to a heterothallic mating system. This study gives the first report of the occurrence of pycnidia of *Pa. chlamydospora* and perithecia of *Pm. minimum* in South African vineyards and rootstock mother blocks and also the first description of sexual morphs of *Pm. australiense* and *Pm. scolyti*.

The pathogenic status of 10 *Phaeoacremonium* species found in South African vineyards was studied. Fresh pruning wounds of a nine-year-old Cabernet Sauvignon vineyard were inoculated with 10^4 conidia/ml of each fungus per wound and assessed after 18 months. All inoculated isolates successfully colonized pruning wounds causing lesions significantly different from the negative control and were re-isolated at varying percentages ranging from 28.57% to 85.71%. The study confirmed the capability of all tested *Phaeoacremonium* species to infect grapevine pruning wounds and cause lesions.

The genetic diversity and mode of reproduction were assessed using microsatellite markers and also by determining the mating type distribution of aerial trapped spores of *Pm. minimum*. In total 320 *Pm. minimum* isolates were assessed with *Mat1-2* specific-primers. Both mating types of *Pm. minimum* were found in all eight vineyards. An equal distribution of *MAT1-1* and *MAT1-2* were found in six of the vineyards, but not in the Paarl A and Wellington populations. Primers for dinucleotide microsatellite loci were designed and 15 microsatellite loci were identified to be polymorphic and could thus be used to assess the genetic diversity of the *Pm. minimum* isolates. A total of 134 multilocus genotypes (MLGs) were observed of which 115 were observed once and 19 genotypes were observed either two or more times. The presence of the same MLG in a vineyard at different collection times, supports the presence of asexual reproduction, and the widespread distribution of MLGs is most probably due to infected nursery planting material. The total gene diversity (*H*) was high with a mean of 0.58 across all populations. Analysis of molecular variance indicated that 94% of the genetic variation was distributed within populations and only 6% between populations. High and significant population differentiation values were only obtained when Paarl Z was compared to Stellenbosch P2. This study confirms the importance of infected planting material that can distribute similar MLGs over long distances. Therefore, the management of Petri disease needs to focus on ensuring clean mother vines and nursery plants.

OPSOMMING

Petri-siekte is ‘n belangerike wingerdstamsiekte wat die leeftyd en produktiwiteit van jong wingerde wêreldwyd affekteer. Infeksie deur hierdie siekte veroorsaak dat nuwe wingerdaanplantings swak vestig tesame met ‘n algemene afname in die wingerd. Die patogene wat hierdie siekte veroorsaak is bekend vir hul vermoë om vatbare snoeiwonde te infekteer. Kennis rakende die periode waarin luggedraagde spoorinokulum van hierdie patogene vrygestel word, is nog nie in Suid-Afrika gerapporteer nie. Produsente snoei gevolglik sonder om te weet of dit saam met periodes sou val waarin die konsentrasies van luggedraagde spore hoog is. Die doel van hierdie studie was om vas te stel wanneer luggedraagde spore van Petri-siekte patogene vrygestel word, asook om die bron van die inokulum te bepaal.

Inligting aangaande die spoorvrystelling in Suid-Afrikaanse wingerde was vir twee seisoene in 2012 en 2013 versamel. Spoorlokvalle was aangebring op die stamme van geïnfekteerde wingerdstokke in ses wingerde en twee onderstokmoederblokke. Die resultate het aangedui dat Petri-siekte patogene reg deur die jaar voorkom en het ook *Phaeomoniella chlamydospora* en *Pm. minimum* in al die wingerde gevang. In totaal is 14 *Phaeoacremonium* spesies geïdentifiseer vanuit verskeie blokke. Daar is bevind dat spoorvrystelling in dieselfde periode voorkom as snoei- en suieraktiwiteite. Daar was egter geen positiewe korrelasie tussen die reënval en spoorvrystelling gevind.

Opnames was in ses wingerde en twee onderstok moederblokke gedoen tussen 2012 en 2014 om die voorkoms van die vrugliggame van Petri-siekte patogene vas te stel. Dooie hout van geïnfekteerde wingerde was ingesamel om te ondersoek op mikroskopiese vlak. Vrugstrukture van *Phaeomoniella chlamydospora* was gevind in al die wingerde en onderstokmoederblokke waarvoor opnames gedoen was. Vrugstrukture van *Pm. minimum* was slegs gevind in wingerde van Stellenbosch P2 en B3, Rawsonville en ‘n onderstok moederblok in Slanghoek. Daarbenewens is *in vitro* paringstudies ook uitgevoer met isolate van *Pm. australiense* en *Pm. scolyti*. Vrugbare geslagtelike vrugstrukture van *Pm. australiense* en *Pm. scolyti* is na sewe en elf maande onderskeidelik, waargeneem. Kruisings van beide spesies het met ‘n heterotalliese paringstelsel ooreengestem. Hierdie studie lewer die eerste verslag van die voorkoms van vrugstrukture van *Pa. chlamydospora* en *Pm. minimum* in Suid-Afrikaanse wingerde en onderstokmoederblokke, asook die eerste beskrywing van die geslagtelike vrugstrukture van *Pm. australiense* en *Pm. scolyti*.

Die patogeniese status van 10 *Phaeoacremonium* spesies wat in Suid-Afrikaanse wingerde voorkom was bestudeer. Vars snoeiwonde van ‘n nege-jaar-oue Cabernet Sauvignon wingerd was geïnokuleer met 10^4 kondida/ml van elke swam per wond en 18

maande later geëvalueer. Al die isolate wat geïnokuleer was, was suksesvol daarin om die snoeiwonde te koloniseer en letsels te vorm wat noemenswaardig verskil het van die negatiewe kontrole, en kon geherisoleer word teen persentasies wat gewissel het tussen 28.57% en 85.71%. Die studie het die vermoë van die *Phaeoacremonium* spesies wat ondersoek is, om wingerd snoeiwonde te infekteer en letsels te veroorsaak, bevestig.

Die genetiese diversiteit en tipe voortplanting is geëvalueer met behulp van mikrosatelliet merkers, asook om die paringstipe-verspreiding van die luggedraagde spore van *Pm. minimum* wat gevang is, vas te stel. In totaal is 320 *Pm. minimum* isolate geëvalueer met Mat1-2 spesifieke-inleiers. Beide paringstipes van *Pm. minimum* is gevind in al agt wingerde. ‘n Gelyke verspreiding van *MAT1-1* en *MAT1-2* is gevind in ses van die wingerde, maar nie in die Paarl A of Wellington populasies nie. Inleiers vir dinukleotied mikrosatelliet-lokusse is ontwerp en 15 mikrosatelliet-lokusse was gevind om polimorfies te wees en kon daarom gebruik word om die genetiese diversiteit van die *Pm. minimum* isolate te bepaal. ‘n Totaal van 134 multilokus genotipes (MLG’s) is waargeneem, waarvan 115 een keer voorgekom het en 19 daarvan twee of meer kere voorgekom het. Die voorkoms van dieselfde MLG in ‘n wingerd op verskillende versamelingstye ondersteun die voorkoms van ongeslagtelike voortplanting, terwyl die wye verspreiding van dieselfde MLG’s waarskynlik toegeskryf kan word aan besmette kwekery plantmateriaal. Die totale geendiversiteit (*H*) was hoog in alle bevolkings met ‘n gemiddeld van 0.58. Die ontleding van molekulêre variansie het daarop gedui dat 94% van die genetiese variasie binne-in bevolkings verspreid is en slegs 6% tussen bevolkings verspreid is. Hoë en noemenswaardige bevolkingsdifferensiasie-waardes was net gevind toe die Paarl Z en Stellenbosch P2 met mekaar vergelyk is. Hierdie studie bevestig die bydrae van besmette plantmateriaal wat soortgelyke MLGs oor lang afstande kan versprei. Die bestuur van Petri-siekte moet gevolglik daarop fokus om skoon moederwingerde en kwekeryplante te verseker.

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CHAPTER 1

REVIEW OF PETRI DISEASE OF GRAPEVINES WITH A FOCUS ON INOCULUM ECOLOGY

INTRODUCTION

Grapevine trunk diseases are a complex of wood diseases, which includes Botryosphaeria dieback caused by several species of Botryosphaeriaceae (Van Niekerk *et al*., 2004, 2010a), Petri disease caused by *Phaeomoniella* (*Pa.*) *chlamydospora* W. Gams, Crous, M.J. Wingf. & L. Mugnai and *Phaeoacremonium* spp. (Mugnai *et al*., 1999; Mostert *et al*., 2006a), esca caused by *Pa. chlamydospora*, *Phaeoacremonium* (*Pm.*) *minimum* Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous and wood rot basidiomycetes (Mugnai *et al.*, 1999, Fischer, 2006), Eutypa dieback caused by *Eutypa lata* (Pers.) Tul. and C. Tul. and *Eutypella* spp. (Munkvold *et al*., 1994; Trouillas and Gubler, 2004), and Phomopsis dieback caused by *Diaporthe* spp. (Van Niekerk *et al.*, 2005; Ùrbez-Torres *et al*., 2013).

Infection due to trunk disease pathogens causes a reduction in vine vigour and productivity (Munkvold *et al*., 1994; Wicks and Davies, 1999). These infections result in poor quality and quantity of grapes and wine and reduced lifespan of vineyards (Mugnai *et al*., 1999). Petri disease infection was reported to cause graft failure and poor establishment of vines which subsequently result in earlier replant of vineyards. In Australia, a 50% loss of newly planted vines attributed to Petri disease infection was reported (Pascoe and Cottral, 2000). The cost of replanting infected vineyards is substantial (Scheck *et al*., 1998a; Calzarano *et al*., 2001).

This review will focus on Petri disease and its ecology in vineyards. During the last two decades, intensive research has been undertaken to understand the epidemiology of Petri disease in different countries. These studies list the different hosts from which Petri disease pathogens have been isolated (Mostert *et al*., 2005; 2006a; Damm *et al*., 2008; Cloete *et al*., 2011; Gramaje *et al*., 2012; Úrbez-Torres *et al*., 2014), the distribution of the disease across grape growing regions (Mostert *et al*., 2005; 2006a; Damm *et al*., 2008; Cloete *et al*., 2011; Gramaje *et al*., 2012; Úrbez-Torres *et al*., 2014), mode of disease spread and infection (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Moyo *et al*., 2014; Agustí-Brisach *et al*., 2015), reproduction of the pathogens (Edwards *et al*., 2001a; Mostert *et al*., 2003; 2006a; Rooney-Latham *et al*., 2005a; Eskalen *et al*., 2005a; b) and also the pathogenic status of species within the *Phaeoacremonium* W. Gams, Crous & M.J. Wingf. genus and the symptoms they cause on grapevines (Halleen *et al*., 2007). However, knowledge of the life cycle of the Petri disease pathogens in vineyards is still not well documented in South Africa.

Petri disease

Petri disease was first reported by Petri (1912) in Italy, with infected vines showing stunted growth and dieback (Scheck *et al*., 1998b; Mugnai *et al*., 1999; Edwards *et al*., 2001b). In South Africa this disease was reported for the first time in 1994 (Ferreira *et al*., 1994). This disease is commonly found in vines 1−5 years old and was previously known as young grapevine decline and “black goo” due to the black gums that ooze out from xylem of infected vines when cross sections are made through rootstocks (Bertelli *et al.*, 1998; Scheck *et al*., 1998b; Ferreira *et al.*, 1999; Mugnai *et al*., 1999; Pascoe and Cottral, 2000).

Distribution of *Phaeomoniella chlamydospora* and host range

Phaeomoniella chlamydospora has been reported in Arkansas (Úrbez-Torres *et al*., 2012), Argentina (Crous and Gams, 2000; Gatica *et al*., 2001), Australia (Crous and Gams. 2000; Smetham *et al*., 2010), Brazil (Correia *et al*., 2013), California (Crous and Gams 2000), Chile (Diaz and Latorre, 2014), Europe (Crous and Gams, 2000), France (Smetham *et al*., 2010), Iran (Mohammadi *et al*., 2013), Italy (Crous and Gams, 2000), Michigan (Urbez-Torrez *et al*., 2013), Missouri (Urbez-Torrez *et al*., 2012), New Zealand (Crous and Gams, 2000), New York (Stewart *et al*., 2003), Pennsylvanica (Stewart *et al*., 2003), Slovakia (Kakalikova *et al*., 2006), South Africa (Ferreira *et al*., 1994), Switzerland (Casierie *et al*., 2009), United States (Gatica *et al*., 2001) and Uruguay (Abreo *et al*., 2001). The pathogen is most commonly associated with grapevines worldwide. More recently, the pathogen has also been reported to cause necrotic streaking of the vascular system on olives (*Olea europea*) (Úrbez-Torres *et al*., 2013).

Description of *Phaeomoniella chlamydospora* and its asexual morph

Phaeomoniella chlamydospora consist of branched mycelium, septate hyphae of up to 10 strands, tuberculate (wart size of up to 1 µm) to verruculate. Walls are green and the septa darker, becoming lighter towards the conidiogenous region, 2 to 4 µm wide. Conidiophores are micronematous, arising from aerial or submerged hyphae, erect, simple, cylindrical with an elongate-ampulliform to lageniform apical cells. Conidiogenious cells are light green to subhyaline, smooth, elongate-ampulliform to lageniform or subcylindrical, 8−20 µm long, with a terminal narrow funnel-shaped collarette, 0.5−2.0 µm long and wide. Conidia are subhyaline, oblong-ellipsoidal to obovate, permanently straight, (1.5−)3.0−4.0(−4.5)×1.0−1.5(−2.0) µm (Crous and Gams, 2000).

No sexual morph of *Pa. chlamydospora* has been reported yet, however the asexual morph has been described by Crous and Gams (2000). *Phaeomoniella chlamydospora* forms pycnidia with a brown conidiomata, globose that consist of conidiophores that are smooth, 1 to multiseptate, pale brown and subcylindrical. Conidiogenous cells monophialidic terminal

mostly sybcylindrical to oblong-ellipsoidal. Conidia are hyaline, oblong-ellipsoidal to obovate, permanently straight, sized (1.5−)20.0−2.5×1.0−1.5 µm.

Distribution of *Phaeoacremonium* species and host range

Species of *Phaeoacremonium* associated with Petri disease have been reported in all grape growing areas, from different substrates including woody hosts, human (Mostert *et al*., 2006a), soil (Rooney *et al*., 2001) and arthropods (Edwards *et al*., 2001a; Moyo *et al*., 2014). An updated summary of *Phaeoacremonium* species distribution has recently been published (Gramaje *et al*., 2015). Since Crous *et al*. (1996) described the genus *Phaeoacremonium* in 1996, 47 different species have been reported worldwide (Dupont *et al*., 1998; Dupont *et al*., 2000; Groenewald *et al*., 2001; Dupont *et al*., 2002; Mostert *et al*., 2006a; Damm *et al*., 2008; Essakhi *et al*., 2008; Graham *et al*., 2009; Gramaje *et al*., 2009a; b; 2012; Úrbez-Torres *et al*., 2014). However, three of these species have been reported only as a sexual morph, namely *Pm. vibratile* (F.r) D. Gramaje, L. Mostert & Crous, *Pm. aquanticum* (D.M. Hu, L. Cai & K.D. Hyde) D. Gramaje, L. Mostert & Crous and *Pm. leptorrhynchum* (Durieu & Mont.) D. Gramaje, L. Mostert & Crous. To date, 42 *Phaeoacremonium* species have been reported on woody hosts, 11 species have been isolated from human infections (Mostert *et al*., 2005) and three species have been isolated from soil as one of the substrate. A total of 28 species have been isolated from infected grapevines worldwide, of which 12 specieshave been associated with grapevines in South Africa, namely *Pm. alvesii* L. Mostert, Summerb. & Crous, *Pm. austroafricanum* L. Mostert, W. Gams & Crous, *Pm. fraxinopennsylvanicum* (T.E. Hinds) D. Gramaje, L. Mostert & Crous, *Pm. iranianum L*. Mostert, Gräfenhan, W. Gams & Crous, *Pm. krajdenii* L. Mostert, Summerb. & Crous, *Pm. minimum*, *Pm. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf., *Pm. scolyti* L. Mostert, Summerb. & Crous, *Pm. sicilianum* Essakhi, Mugnai, Surico & Crous*, Pm. subulatum* L. Mostert, Summerb. & Crous, *Pm. venezuelense L. Mostert, Summerb. & Crous* and *Pm. viticola* J. Dupont (Crous *et al*., 1996; Mostert *et al*., 2005; Mostert *et al*., 2006a; White *et al*., 2011).

Description of *Phaeoacremonium* species and its sexual morph

The *Phaeoacremonium* genus was classified in the order Calosphaeriales under the *Togniniaceae* family (Réblová *et al*., 2004; Mostert *et al*., 2006a). Calosphaeriales comprise of wood inhibiting perithecial ascomycetes that invade the wood and the periderm (Réblová *et al*., 2004). The link between *Phaeoacremonium* and its sexual morph was first confirmed by DNA phylogeny and *in vitro* mating studies and from moist incubated grapevine wood (Mostert *et al*., 2003; Pascoe *et al*., 2004; Rooney-Latham *et al*., 2005a). The asexual morph was known as *Phaeoacremonium* and the sexual morph as *Togninia*. However, the recent abolishment of dual nomenclature and the agreement to a single nomenclature for fungi has

accepted the name *Phaeoacremonium* over *Togninia* (Hawksworth, 2011; Hawksworth *et al*., 2011; Wingfield *et al*., 2012; Kirk *et al*., 2013; Gramaje *et al*., 2015).

Phaeoacremonium species form perithecia during sexual reproduction (Mostert *et al*., 2003). Mostert *et al*. (2006a) described these structures in detail in *in vitro* pairing assays by cross-inoculating *Phaeoacremonium* isolates on autoclaved grapevine shoots placed on water agar in Petri dishes. According to Mostert *et al*. (2006a), perithecia of *Phaeoacremonium* species were found occurring superficially or imbedded in grapevine wood and agar. Perithecia are globose to subglobose and dark brown to black in colour. They formed up to three necks which are either branched or not. The necks are between 275−880 µm long. Inside the perithecia, there are ascogenous hyphae which are ascus-producing or supporting structures, they form a zig-zag arrangement when viewed under a light microscope which Barr (1985) considered as a distinguishing feature of the *Phaeoacremonium* sexual morph. The asci develop on the croziers on the ascogenous hyphae and contain eight ascospores. Asci are clavate with bluntly obtuse bases without a stalk. Asci are released from the perithecia through the neck, followed by the ascospore discharge. The ascospores are on average 6.5 µm long and 2.5 µm wide, are hyaline and aseptate, may be allantoid, reniform, cylindrical or oblong-ellipsoidal. Paraphyses are septate, long, hyaline, broadly cellular, slightly constricted at the septa and tapered towards the end (Mostert *et al*., 2006a). Twelve *Phaeoacremonium* spp. sexual morphs have been described *in vitro* (Mostert *et al*., 2006a), with only three being reported in nature, namely *Pm. minimum* (Rooney-Latham *et al*., 2005a), *Pm. viticola* (Eskalen *et al*., 2005a) and *Pm. fraxinopennsylvanicum* (Eskalen *et al.*, 2005b).

In culture, *Phaeoacremonium* colonies are flat with entire margins, moderately dense, predominantly felty and sometimes woolly textured (Mostert *et al*., 2005; Mostert *et al*., 2006a). The colony colour varies from pale to deeply brown. Mycelia consist of branched, septate hyphae occurring singly or in bundles of 4 to 27. Some species have wart-like structures on the conidiophores that differ in density and size between different species. *Phaeoacremonium parasiticum* has the largest sized warts of about 3 µm. Conidiophores maybe long or short and frequently branched or unbranched. Phialides may arise directly from mycelia or integrated in conidiophores. Three types of phialides are found in *Phaeoacremonium*. Conidia vary in shape from oblong to ellipsoidal, to obovate to cylindrical to allantoid to reniform. These conidia can become guttulate after 7 to17 days on media (Mostert *et al*., 2005; Mostert *et al*., 2006a).

Previously, identification of *Phaeoacremonium* was through morphological characterization using keys described by Crous *et al*. (1996), Dupont *et al*. (2000) and Mostert *et al*. (2005). DNA phylogenetic studies of the internal transcribed spacers (ITS 1 and ITS 2) and the 5.8S rRNA gene as well as the partialβ-tubulin, actin and calmodulin genes of *Phaeoacremonium* successfully described an important number of novel species and

reassignment of former ones (Dupont *et al*., 2000; Groenewald *et al*., 2001; Mostert *et al*., 2003; 2005). New species are continuously reported because of advanced molecular identification techniques and isolations from new hosts.

Petri disease symptoms on grapevines

Petri disease pathogens infect the xylem tissue of the host plant and cause different symptoms which impairs normal plant growth and productivity (Feliciano and Gubler, 2001). *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. are considered latent pathogens, known to survive asymptomatically inside vines and become pathogenic during periods of stress, especially drought conditions (Bertelli *et al*., 1998; Ferreira *et al*., 1999; Eskalen *et al*., 2004; Aroca *et al*., 2006).

Different symptoms have been reported as a result of infection by Petri disease pathogens, which include external and internal symptoms. For the external symptoms, plants may show a general decline, stunted growth and dieback (Scheck *et al*., 1998b; Ferreira *et al*., 1999; Mugnai *et al*., 1999). Foliar symptoms including interveinal chlorosis and stunted leaves have also been reported from seedlings inoculated with *Pm. parasiticum*, *Pm. angustius*, *Pm. inflatipes* W. Gams, Crous & M.J. Wingf. and *Pm. venezuelense* (Aroca and Raposo, 2009). Internal symptoms include the presence of wood gummosis, which may be seen as droplets when a vine is cut transversely, or appear as black or brown streaks extending from a pruning wound or graft union when the vine is cut longitudinally (Mugnai *et al.,* 1999; Scheck *et al.,* 1998b; Groenewald *et al*., 2001). Discoloration of the vascular tissue is one of the characteristic symptoms associated with plants infected by vascular fungi (Sands *et al*., 1997; Harrington *et al*., 2000). The pathogen infection result in reduced xylem function causing blocked xylem vessels due to the formation of tyloses, production of gums or with physical structures of the pathogen (Ferreira *et al*., 1999; Edwards *et al*., 2007a; b; Sun *et al*., 2008). The plugging causes plant stress, poor uptake and translocation of water and minerals and ultimately dieback and plant death (Ferreira *et al*., 1999; Mugnai *et al*., 1999). The extent of streaking formation inside cuttings differ between rootstock varieties (Diaz *et al*., 2009) and there are also susceptibility differences between scion cultivars (Feliciano *et al*., 2004).

For a fungal pathogen to infect and cause disease symptoms, it must overcome the defence mechanisms developed by their host. This mechanism can either be physical (e.g. cuticle) or a chemical barrier, comprising of antifungal compounds (Shigo and Marx, 1977). Shigo and Marx (1977) described a model of “compartmentalization of decay in trees” (CODIT) which distinguished four different boundaries in injured trees. In this model, the occlusion of gums and tyloses was considered the least effective barrier. However, this is how grapevines were found to respond to Petri disease pathogens infection (Mugnai *et al*., 1999).

Phaeomoniella chlamydospora and *Pm. minimum* have shown the ability to break the polyphenolic compounds *in vitro* and trigger faster accumulation of tyloses and phenolic compounds in the colonized tissue (Mugnai *et al.,* 1999; Del Río *et al*., 2002; Diaz *et al*., 2009; Andolfi *et al*., 2011).

Pathogenicity of Petri disease pathogens

The pathogen status of Petri disease pathogens has been investigated together with the symptom expression and plant part infected. This has been extensively studied on pruning wounds, spurs and vine trunks, however, some researchers also tested the possibility of infection through the roots and berries. Pathogenic effects are evaluated by determining the ability of pathogens to cause wood discoloration in inoculated vine tissue (Feliciano *et al*., 2004; Eskalen *et al*., 2007) and the probability of re−isolating the inoculated pathogen to comply with Koch's postulates.

Different symptoms of Petri disease including dieback, stunted growth, reduced root weight and vine death have been found with artificial inoculation (Ferreira *et al*., 1999; Adalat *et al*., 2000; Feliciano *et al*., 2004; Aroca and Raposo, 2009). Inoculation trials were conducted to assess the pathogenicity status of different *Phaeoacremonium* species on detached grapevines in the glasshouse as well as on standing vines in the field. This included use of conidial suspensions to inoculate pruning wounds (Halleen *et al*., 2007), soaking grapevine cutting or seedlings (Eskalen *et al*., 2001; Gramaje *et al*., 2008; Aroca and Raposo, 2009) and to vacuum inoculate the vascular system of cuttings (Rooney and Gubler, 2001). Conidial suspensions were also inserted in grapevine trunks in the field and on grapevine nursery plants in the glasshouse (Halleen *et al*., 2007), soil drenching of potted grapevine cuttings with conidial suspensions (Aroca and Raposo, 2009) and rubbing of berries with carborundum dust containing conidia of *Pm. minimum* and *Pa. chlamydospora* (Gubler *et al*., 2004). All these methods enabled successful characterisation of the symptoms of Petri disease caused by *Pa. chlamydospora* and *Phaeoacremonium* spp. Recently sucker wounds were also shown as ports of entry for these pathogens in vineyards (Makatini, 2014).

Petri disease pathogen, *Pm. inflatipes* has been recovered from the soil in California (Rooney *et al*., 2001). Soil drenching of *Vitis vinifera* cv. Malvar and cv. Airen seedlings with conidial suspensions under glasshouse conditions confirmed the capability of *Pm. minimum*, *Pm. angustius*, *Pm. inflatipes*, *Pm. krajdenii, Pm. fraxinopennsylvanicum, Pm. parasiticum*, *Pm. scolyti*, *Pm. venezuelense* and *Pm. viticola* to infect seedlings through the root system (Aroca and Raposo, 2009). Inoculated seedlings showed significant vascular discoloration after five months of inoculation for all species and further reported reduced root weight for the *Pa. chlamydospora*, *Pm. fraxinopennsylvanicum* and *Pm. minimum* treatments. Foliar

symptoms such as interveinal chlorosis and stunted leaves, which progressively became dryer were evident after 10 weeks of inoculating grapevine shoots with *Pm. angustius*, *Pm. inflatipes*, *Pm. parasiticum* and *Pm. venezuelense* (Aroca and Raposo, 2009). There was no correlation between the level of foliar symptoms and intensity of vascular discoloration, thereby concluding that foliar symptoms cannot be used to estimate the level in internal discoloration (Aroca and Raposo, 2009). Pathogenicity studies conducted in South Africa with conidial suspensions and mycelial plugs confirmed five species as pathogens of grapevines, namely *Pm. minimum*, *Pm*. *krajdenii*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* (Halleen *et al*., 2007). Gubler *et al.* (2004) further showed the capability of *Pa. chlamydospora* and *Pm. minimum* to infect intact (in the field) and detached (in the laboratory) berries of grapevines. Their research with carborundum dust also demonstrated the possibility of berries to be injured and infected by blowing dust or sand. In this case, lesions were only found on detached berries which according to Gubler *et al*. (2004) could be attributed to the controlled conditions in the laboratory compared to vineyards. Infections were also reported on non-injured inoculated berries, thereby leading to question as to whether pathogens enter berries through lenticels, minute undetectable breaks in the cuticle or minute injuries caused by insects or other agents.

Toxins associated with Petri disease pathogens, *Pa. chlamydospora* and *Pm. minimum* comprises of different classes which have been identified from culture filtrates, namely α–glucans (pullulans) and 2 naphthalenone (scytolone and isosclerone) (Andolfi *et al*., 2011). Pullulans are known to cause severe symptoms on grapevine leaves and scytalones to cause chlorotic and necrotic spots, however, scytalones have also been shown to promote plant growth. *Phaeoacremonium minimum* toxic metabolites also included p-hydroxybenzaldehyde from culture filtrates that inhibited callus formation (Tabacchi *et al*., 2000). Other *Phaeoacremonium* species such as *Pm. minimum*, *Pm. angustius*, and *Pm. viticola* produce protease, lipase, amylase, cellulose, xylanase, pectinase, and urease activities which degrade plant cell wall components (Santos *et al*., 2006). Studies by Abou-Mansour *et al*. (2004) and Luini *et al*. (2010) indicated that toxins secreted by *Pa. chlamydospora*, especially the polypeptide fraction, strongly affects physiological processes, thereby leading to reduced plant response, cell death and tissue necrosis.

Epidemiology of Petri disease

Fruiting bodies as source of inoculum

Fruiting bodies are overwintering structures that form an important part of a disease cycle. Fruiting bodies of Petri disease pathogens have not been reported in South African vineyards,

however, pycnidia of other trunk disease pathogens such as *Diplodia mutila* (Fr.) Mont. and *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. have been found in South African vineyards (Van Niekerk *et al*., 2010b).

The sexual morph of *Pa. chlamydospora* has never been found in the field nor successfully induced in the laboratory. However, pycnidia of this pathogen have been reported in Australian vineyards. The pathogen has been shown to overwinter as pycnidia found on dead wood (Pascoe and Cottral, 2000; Edwards and Pascoe, 2001). The pycnidia were first reported on standing vines in Australia in deep cracks and crevices and on pruning wounds (Edwards and Pascoe, 2001). According to Edwards *et al.* (2001a) these cracks and crevices provide a protected humid environment suitable for the development of pycnidia. These authors were unable to conclude if the pycnidia were the primary source of *Pa. chlamydospora* aerial spores because conidia did not germinate on artificial media, therefore, pathogenicity tests with the pycnidia conidia were unsuccessful. Adalat *et al*. (2000) and Pascoe and Cottral (2000) successfully germinated conidia on artificial media, and regarded the pycnidia to be the primary source of inoculum of *Pa. chlamydospora* although they did not conduct inoculation studies to confirm that these spores are able to infect wounds. Finding of mycelia sporulating on infected wood surfaces suggest another means of inoculum production in vineyards (Edwards *et al*., 2003).

Perithecia of three *Phaeoacremonium* species have been reported in vineyards, namely *Pm. minima, Pm. fraxinopennsylvanica* and *Pm. viticola* (Rooney-Latham *et al*., 2005a; Eskalen *et al*., 2005 a; b; Baloyi *et al*., 2013). Surveys showed perithecia to form mostly on decayed xylem tissue of pruning wounds or inside deep cracks along trunks, cordons and spurs. Old spurs often left in the field for several years may provide an ideal habitat for the perithecia to survive from year to year (Rooney-Latham *et al*., 2005a). According to Rooney-Latham *et al*. (2005a), perithecia of *Pm. minimum* collected in vineyards were similar to those that were produced in the laboratory in terms of shape and size dimensions (Mostert *et al*., 2003; Mostert *et al*., 2006a) and are suspected to be a source of aerial spore inoculum.

Spore release of Petri disease pathogens

Spore release patterns of Petri disease pathogens were previously studied in France and California to determine when aerial spore inoculum occur in vineyards (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). This was conducted by affixing microscopic slides coated with petroleum jelly on vines showing decline symptoms. Larignon and Dubos (2000) reported only two Petri disease pathogens in French vineyards whereas Eskalen and Gubler (2001) recorded three pathogens in Californian vineyards trapped as aerial spores. Larignon and Dubos (2000) found aerial spores of *Pa. chlamydospora* to be present throughout the trapping

period, however, *Pm. minimum* was more commonly trapped during vegetative periods in France. In California, *Pa. chlamydospora* was also recorded throughout the year, together with *Pm. minimum* and *Pm. inflatipes*. Spore release occurred during winter and spring periods highlighting the risks associated with susceptible pruning as well as sucker wounds made during these periods (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). In Australia, Edwards *et al*. (2001b) did not trap any *Pa. chlamydospora* spores in vineyards using microscopic slides coated with petroleum jelly. Spore trapping studies using a volumetric spore trap in a South African vineyard also could not detect the release of any Petri disease pathogens (Van Niekerk *et al*., 2010b).

Spore release of *Pa. chlamydospora* and *Pm. inflatipes* correlated with rainfall periods in California (Eskalen and Gubler, 2001). However, there was no correlation between rainfall and spore release found in France (Larignon and Dubos, 2000). Spore counts and the number of species that release spores may vary between regions and time of year as described by Eskalen and Gubler (2001).

The influence of climate on Petri disease

Environmental conditions influence *Phaeoacremonium* ascospore release and therefore the probability that the spores land on susceptible wounds (Mugnai *et al*., 1999; Eskalen and Gubler, 2001; Rooney-Latham *et al*., 2005a; b, c). The importance of some environmental parameters on Petri disease epidemiology are highlighted below.

Temperature

Temperature has an important role in the survival and colonisation of spores in the field. *In vitro*, *Pa. chlamydospora* can grow between 10 to 35°C and *Pm. minimum* from 10 to 40°C (Crous *et al*., 1996; Valtoud *et al*., 2009). It is evident that both these species can survive and grow in a wide range of temperatures. Aerial spores were recorded throughout the year, irrespective of the growth season, able to infect winter and spring wounds (Larignon and Dubos, 2000; Eskalen and Gubler, 2001) and berries during summer months in California (Eskalen and Gubler, 2001). Rooney-Latham *et al*. (2005a) postulated that perithecia of *Pm. minimum* would most likely be formed during the dry summer months in California, being able to grow at high temperaturesand then release spores during rainfall periods.

Rainfall

Rainfall has been shown to be of great significance in disease spread as spores of *Phaeoacremonium* species were found to be water splashed (Rooney-Latham *et al*., 2005b). Moreover, spore release has been correlated with high rainfall periods in California (Eskalen *et al*., 2001). Hausner *et al*. (1992) speculated that wet conditions dissolves the ascus sack

layer of *Pm. minimum* perithecia and thereby, releasing ascospores. Ascospores are forcibly discharged from the perithecia after complete dehydration, sufficient remoistening then redrying (Rooney-Latham *et al*., 2005b).

Light

Light was suggested as a possible factor in the formation of perithecia of *Phaeoacremonium* species. Rooney-Latham *et al.* (2005b) reported the ability of *Phaeoacremonium* spp. to form perithecia *in vitro* when incubated in light opposed to those that were incubated in the dark. The importance of light was also shown by the necks of *Pm. minimum* which faced towards the surface of the cordon or trunk, suggesting phototropic sensitivity (Rooney-Latham *et al*., 2005a).

Other sources of inoculum

Infected grapevine propagation material

Several studies have indicated the importance of rootstock mother vines as the primary source of disease spread (Pascoe and Cottral, 2000; Zanzotto *et al*., 2001; Fourie and Halleen, 2002; Edwards *et al*., 2003, Halleen *et al*., 2003; Fourie and Halleen, 2004). In Spain, shared genotypes were reported in distant regions of 350 and 700 km apart, clearly indicating the long-range dispersal, possibly through infected material (Gramaje *et al*., 2012). This is in agreement with an earlier report of Smetham *et al*. (2010) who also reported long distance dispersal of the same haplotypes between vineyards which were 300 to 400 km apart. Pathogens are found to be already present in apparently healthy rootstock propagation material as latent pathogens (Morton, 1997; Larignon and Dubos, 1997; Mugnai *et al*., 1999; Fourie and Halleen, 2002; Halleen *et al*., 2003). Conidia and hyphal fragments of *Pa. chlamydospora* have been found in the pith region of rootstock canes, along the full length of canes (Feliciano and Gubler, 2001; Edwards *et al*., 2003), ultimately hypothesising that spores are carried in sap flow of infected mother plants, which causes the subsequent contamination (Edwards *et al*., 2003). *Phaeomoniella chlamydospora* was predominantly recovered from the rootstock section of both recently grafted and more mature vines (Sidoti *et al*., 2000; Ridgway *et al*., 2002; Halleen *et al*., 2003; Fourie and Halleen, 2004; Aroca *et al*., 2006; Gimènez-Jaime *et al*., 2006; Retief *et al*., 2006; Whiteman *et al*., 2007; Zanzotto *et al*., 2008). Scion material are not considered as a primary source of pathogen inoculum due to low pathogen incidence (Zanzotto *et al*., 2001; Halleen *et al*., 2003; Whiteman *et al*., 2007). The use of infected material result in lower survival rate of young vines in newly planted vineyards (Graham *et al*., 2009).

Fruit orchards close to vineyards

A number of *Phaeoacremonium* species have been reported from fruit trees and other woody hosts such as *Actinidia* spp., *Cydonia oblonga*, *Dactylis glomerata*, *Malus domestica*

Nectanda spp., *Olea europaea*, *Phoenix dactylifera*, *Prunus* species, *Pyrus malinae*, *Quercus agrifolia* and *Salix* spp. (Hausner *et al*., 1992; Mostert *et al*., 2005; Sánchez-Márquez *et al*., 2007; Damm *et al*., 2008; Prodi *et al*., 2008; Cloete *et al*., 2011; Carlucci *et al*., 2013; Nigro *et al*., 2013; Lynch *et al*., 2013; Mohammadi, 2014; Sami *et al*., 2014). In South Africa, *Pm. minimum*, *Pm. iranianum*, *Pm. fraxinopennsylvanicum*, and *Pm. viticola* have been isolated from *M. domestica* and *P. malinae* (Cloete *et al*., 2011). *Phaeoacremonium minimum* isolates from *M. domestica* successfully infected and caused discoloured lesions in grapevine shoots in detached shoot assays (Cloete *et al*., 2011). Arzanlou *et al*. (2013) reported on the capability of *Pm. minimum* isolates from *M. domestica*, *P. armeniaca* and *V. vinifera* to cause lesions in *M. domestica* shoots irrespective of the host of origin. *Phaeoacremonium* species have also been isolated from *Pistacia vera* trees (Mohammadi *et al*., 2015), *Cydonia oblonga* and *Crataegus monogyna* (Sami *et al*., 2014), which may also serve as reservoirs for Petri disease pathogens. The wide host range illustrate the importance of trees that commonly grow in the vicinity of vineyards that could harbour *Phaeoacremonium* species (Eskalen *et al*., 2007). Perithecia of *Pm*. *viticola* and *Pm. fraxinopennsylvanicum* were found on *Fraxinus latifolia* established near vineyards in California (Eskalen *et al.*, 2005b). This shows the potential of alternative hosts that are commonly grown in the vicinity of vineyards to serve as reservoir hosts and sources of inoculum (Eskalen *et al*., 2007; Arzanlou *et al*., 2013; Gramaje *et al*., 2015).

Pruning wounds as main ports of entry

Pruning occurs during the dormant season as vine maintenance with the aim of maintaining a desired form of a vine, producing fruit of the target composition, to select productive nodes, reduce crop load or regulate vegetative growth (Creasy and Creasy, 2009). Pruning wounds are regarded as the main entry ports for trunk pathogens to infect vines and cause disease. Petri disease pathogens have been isolated from lesions extending from pruning wounds (Ferreira *et al*., 1989; Adalat *et al*., 2000; Serra *et al*., 2008; Rolshausen *et al*., 2010). Spores of Petri disease pathogens could land on susceptible pruning wounds via water or the wind/air currents (Larignon and Dubos, 2000; Eskalen and Gubler, 2001), vectored by insects (Moyo *et al*., 2014) and also pruning shears (Augustí-Brisach *et al*., 2014). Inoculation studies with conidial suspensions of *Pa. chlamydospora* and *Pm. minimum* on pruning wounds have shown wounds to differ in their level of susceptibility depending on the time of wounding (Eskalen *et al*., 2007), with wounds made in the early dormancy being susceptible for longer periods than those made in late dormancy. In addition, Van Niekerk *et al*. (2011) reported a decline in wound susceptibility with increasing wound age, regardless of the time at which the wound was made, although the rate of decline differed between the two years of the study. Van Niekerk *et al*. (2011) then further postulated that wound repair processes differs between

seasons due to the difference in temperatures and total rainfall. In California, fresh pruning wounds were reported to remain susceptible to infection for up to four months after pruning, being more susceptible the first two months (Eskalen *et al*., 2007). Grapevines are pruned during the dormant season, which is during the rainy months in the Western Cape of South Africa, therefore exposing the wounds to possible aerial spore inoculum. It is therefore important to understand grapevine pruning wound susceptibility and Petri disease ecology in order to develop efficient control measures.

Genetic diversity of Petri disease pathogens

Genetic diversity studies of *Pa. chlamydospora* isolates has been carried out in different countries including New Zealand (Pottinger *et al*., 2002), France (Borie *et al*., 2002) and Australia (Smetham *et al*., 2010), with these researchers concluding that there is a low genetic variation between different isolates. The absence in genetic variation suggest the pathogen reproduce asexually, which explains the absence of sexual structures within vineyards. Mostert *et al*. (2006a) and Gramaje *et al*. (2012) reported same genotypes in isolates from different countries, and this was thought to be due to single introduction events from the same source of inoculum. Furthermore, Mostert *et al*. (2006b) reported genotype variation among isolates obtained from single vines in South African vineyards, thus indicating the possibility of multiple infections from different source of inoculums to occur within vineyards. A recent study also reported a small but significant variation among isolates from southern France and southern Australia using microsatellite markers (Smetham *et al*., 2010). Genetic variation was thought to be due to gene flow and mutation processes being high in *Pa. chlamydospora*, and not sexual reproduction. Borie *et al*. (2002) also suggested this to be due to parasexuality than sexual reproduction.

The genetic variation of *Pm. minimum* population has been studied in more details than that of other *Phaeoacremonium* species. These studies were done in Australia (Cottral *et al*., 2001), France (Péros *et al*., 2000; Borie *et al*., 2002), Italy (Tegli *et al*., 2000) and Spain (Gramaje *et al*., 2013; Martín *et al*., 2014). However, there have not been any genetic diversity studies conducted in South Africa on *Pm. minimum* populations. Different molecular techniques have been used to study genetic diversity of *Pm. minimum*. These includes amplified fragment length polymorphism (AFLPs), random amplified polymorphic DNA (RAPDs), inter-simple sequence repeat (ISSR), random amplified microsatellites (RAMS) and universal primed PCR (UP-PCR) (Perós *et al*., 2000; Tegli, *et al*., 2000; Cottral *et al*., 2001; Gramaje *et al*., 2013). Analyses revealed a considerable genetic diversity among populations suggestive of ongoing recombination to occur. Occurrence of different sources of inoculum within a population was shown by the presence of several different haplotypes.

Perithecia of *Pm. minimum* has been found in vineyards. Studies by Rooney-Latham *et al*. (2005a) and Mostert *et al*. (2003) confirmed that *Pm. minimum* has a heterothallic system. Isolates from different geographic and climatic regions were found to be sexually compatible. The occurrence of two different mating types of *Pm. minimum* isolates within vineyards showed the possibility of random mating to occur. Therefore, resulting in spread of different genotypes which could in time, increase the level of fungal recombination, generate new combinations of virulent genes that match corresponding resistance genes in their host or favour rapid adaptation of genes to changing environment, which will enable them to survive in different regions with different climatic conditions (Fisher, 1930; Anderson and Kohn, 1995). Previous studies reported a moderate genetic diversity of *Pm. minimum* populations, therefore suggesting that both asexual and sexual reproduction of this species occur in the same vineyard (Tegli, 2000; Gramaje *et al*., 2012).

Management of Petri disease

The management of trunk diseases is challenging due to the systemic infection and growth of the pathogens. In established vineyards, infection occurs through pruning wounds, which is an unavoidable cultural practice in vineyards. The period of wound susceptibility is long and could coincide with high aerial spore release (Eskalen *et al*., 2007). Periods of aerial spore release of Petri disease pathogens have not yet been identified in South African vineyards, and therefore specific recommendations to farmers with regard to high or low spore release periods cannot be made with confidence. There is therefore a critical need to study spore release in South African vineyards. Furthermore, different control and management strategies need to be investigated and implemented to effectively manage the spread of disease. Below are some methods which have been reported for Petri disease or other trunk diseases.

Sanitation within vineyards

Sanitation is a cultural practice recommended to reduce fruiting bodies which form on pruning debris and old wood within vineyards (Larignon and Dubos, 2000; Edwards and Pascoe, 2001). Grapevine spurs and trunks that become infected with Petri disease pathogens can be surgically removed to effectively remove the infected vine parts and prevent it from spreading to the entire vine as recommended for Eutypa infected vines (Sosnowski *et al*., 2011). However, these removed parts are usually left in the vicinity of vineyards where fruiting bodies could develop and produce spores. Sanitation practices could reduce the amount of ascospores produced in the next season and also prevent contamination of soil with conidia from mycelial fragments. Gubler *et al*. (2005) regards sanitation as the appropriate way to prevent the production of inoculum. Rather than leaving pruning debris on the vineyard floor, it can be composted. Lecomte *et al*. (2006) showed that composted pruning debris removed

from vines naturally infected and showing characteristic symptoms of Petri disease pathogens eradicated *Pa. chlamydospora* and *Pm. minimum.* Artificially inoculated pruning wood material was also prepared by inoculating autoclaved grapevine wood pieces with *Pa. chlamydospora*, and similar results were observed (Lecomte *et al*., 2006).

Cultivar resistance

No grapevine cultivar is resistant to any trunk disease, however, variability in the level of susceptibility has been observed. Thompson Seedless is considered as a susceptible scion cultivar compared to Grenache and Cabernet Sauvignon when inoculated with *Pm. minimum* (Feliciano *et al*., 2004). In Chili, Diaz *et al*. (2009) observed considerable difference between rootstock susceptibility with regard to streak formation inside the cuttings and reported cultivar Paulsen 1103 and 101-14 Mgt as less susceptible to infection by *Pa. chlamydospora* and *Pm. minimum* compared to SO4 (*V. berlandieri* x *V. riparia*). These results were in agreement with earlier research which did not report any resistant rootstock cultivar in California against *Pa. chlamydospora*, *Pm. inflatipes* and *Pm. minimum* (Eskalen *et al*., 2001). Aroca and Raposo (2009) reported variation in symptom expression between grapevine cultivars Malvar and Airen when seedlings were inoculated with conidial suspensions *of Pa. chlamydospora*, *Pm. minimum*, *Pm. angustius*, *Pm. inflatipes*, *Pm. krajdenii*, *Pm. fraxinopennsylvanicum*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. venezuelense* and *Pm. viticola*, using a vacuum-inoculation method. The unavailability of resistant cultivars therefore emphasis the need to adopt other preventative strategies to prevent infection of Petri disease pathogens.

Wound protection

By protecting pruning wounds infection of Petri disease pathogens can be prevented. An overview of chemical and biological pruning wound protectants is thus provided.

Chemical wound protection

There are currently no fungicides registered to be used as pruning wound protectants against Petri disease pathogens. However, Groenewald *et al*. (2000) tested the effect of 12 registered chemicals on *Pa. chlamydospora* mycelial growth. Among those chemicals tested, benomyl, fenarimol, kresoximmethyl, prochloraz manganese chloride and tebuconazole showed mycelial inhibition of *Pa. chlamydospora* at low concentrations (0.01-0.5 ppm). Jasper (2001) also found anilopyrimidine, benzimidazole, demethylation inhibitor, and quinone-outside inhibitor classes to be effective in reducing germination and mycelium growth of *Pa. chlamydospora.* The authors concluded that these fungicides may have potential to protect vines from Petri disease pathogens. Bioassays where pruning wounds of inoculated shoots were sprayed with fungicides showed efficacy of benomyl, pyraclostrobin, tebuconazole and thiophate-methyl against *Pa. chlamydospora* (Diaz and Latorre, 2013). Furthermore, these

fungicides provided effective control of *Pa. chlamydospora* when applied as a pre- and post-inoculation paste treatment on Cabernet Sauvignon pruning wounds in a field trial.

Biological control agents

Biological control agents are regarded as suitable alternatives to chemical control for long-term wound protection against trunk diseases. Kotze *et al*. (2011) evaluated potential biocontrol agents against a wide range of grapevine trunk disease pathogens including Petri disease pathogens. Control levels from 69% to 92% were obtained with isolate USPP T1 (*Trichoderma atroviride*) against *Phomopsis viticola*, *E. lata*, *Pa. chlamydospora* and *Botryosphaeria* species. *Trichoderma* has several modes of action, including competition, antibiosis, parasitism and also has the ability to activate host response (Kotze *et al*., 2011). More recently, the major secondary metabolite produced by *T. harzianum* and *T. atroviride* has been identified as 6-pentyl-α-pyrone, a compound that has shown inhibition of *Pa. chlamydospora* (Mutawila *et al*., 2016). The advantage of using *Trichoderma* as a biocontrol agent is that it grows faster than most trunk disease pathogens, which serve as an advantage to establish and compete for space and inhibit the pathogen growth (Kotze *et al*., 2011).

CONCLUSION

Understanding the biology of a disease is the first crucial step towards disease management as it contributes significantly to the development of sound management strategies relevant to that disease. The occurrence of Petri disease has been of great concern to grape and wine industries around the world for the past two decades. While researchers have embarked on different research aspects such as to understand the epidemiology of trunk diseases in South Africa, thorough understanding of Petri disease ecology is still lacking. Previous research reported on Petri disease pathogen surveys, their status as pathogens, and spread by propagation material. However, issues such as inoculum sources, spore release patterns and the type of reproduction occurring in South African vineyards has not been researched.

It is with this knowledge that fundamental disease management strategies can be developed. This study will investigate the life cycle of Petri disease pathogens by identifying inoculum sources within South African vineyards, how pathogens reproduce within vineyards, when spores are released, and provide an update on the pathogenic status of *Phaeoacremonium* species not previously found in South Africa or unknown world-wide. Therefore, this study will provide the grape and wine industries with new insights into the biology of Petri disease pathogens and will aim to highlight possible stages in the life cycle which could be targeted as part of an integrated strategy to combat this disease.

Rationale and scope of study

Petri disease has been studied for nearly 20 years locally. The disease is caused by *Pa. chlamydospora* and *Phaeoacremonium* spp., mainly *Pm. minimum*. Twenty-nine *Phaeoacremonium* species have been associated with grapevines worldwide of which twelve have been found in South Africa. Infection points have been identified and infected mother vines were shown to be responsible for the "spread" of the disease. Good progress has been made in grapevine nurseries to eradicate or reduce these infections although it is clear that infection levels can increase again once the nursery vines are established in vineyards.

Little attention has been given to the spread of the disease within vineyards, and no study has investigated the actual source of pathogen inoculum, the fruiting bodies that produce and release the spores. Crous and Gams (2000) described the formation of a Phoma-like synanamorph (pycnidium) when *Pa. chlamydospora* isolates were placed on carnation leaf agar and on infected canes incubated at 10°C. However, the only place where these pycnidia have ever been found in nature was in Australia where it occurred in deep cracks and crevices on grapevine cordons and trunks. The role thereof could not be determined since the conidia could not germinate. Fruiting bodies (perithecia) of only three of the 29 Phaeoacremonium species associated with grapevines worldwide, *Pm. minimum*, *Pm. fraxinopennsylvanicum* and *Pm. viticola* sexual morphs have thus far been found on grapevines in California (Eskalen *et al*., 2005a; b; Rooney-Latham *et al*., 2005c). *Phaeoacremonium fraxinopennsylvanicum* perithecia were also found on declining ash trees surrounding vineyards which might act as additional inoculum sources. *Togninia minima* perithecia were found in cracks and crevices associated with pruning wounds and cracks as a result of drying wood.

Aerial spores of *Pa. chlamydospora* and *Pm. minimum* have been trapped in French and Californian vineyards (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). Only one study thus far attempted to investigate spore release patterns in South Africa, although no Petri disease pathogens were detected in a Quest volumetric spore trap which were placed in a single vineyard in Stellenbosch from June to mid-September 2008. Absence of trapped aerial spores of Petri disease pathogens does not mean that spores were not released during this period. More vineyards should have been investigated over more than two seasons and the technique could possibly not have picked up the slower growing fungal colonies. Grapevine pruning wounds stay susceptible to infection for at least 4 weeks, although some studies even suggest 2 months. If spore release were found to be later in spring or early summer, sucker wounds might also become an important portal for infection and protection needed.

Knowledge of the origin of inoculum and the time of release is one of the most fundamental aspects of any disease, since effective control and/or management strategies throughout the entire process, from the propagation of plant material, the nursery to the establishment of a new vineyard and ultimately managing it into a productive vineyard with a long life span, rely heavily on this information.

Therefore, the above gaps were addressed as part of this study to better understand vine to vine spread of Petri disease pathogens occurring in South African vineyards.

Aims of the study

The aims of this study were to gain insight into the inoculum ecology of Petri disease pathogens and to understand the spread from infected vines to healthy vines inside mother blocks and vineyards. The objectives of this study were therefore:

i. To determine when the fungal spores are released by means of spore trapping studies;

ii. To determine if and where fruiting bodies of *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. occur on grapevines;

iii. To determine the pathogenicity of *Phaeoacremonium* species found in South African vineyards.

iv. Study the genetic diversity among *Phaeoacremonium minimum* isolates within and between vineyards.

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CHAPTER 2

ECOLOGY OF PETRI DISEASE PATHOGENS IN SOUTH AFRICAN VINEYARDS

ABSTRACT

Pruning wounds are known ports of entry for aerial spores of Petri disease pathogens. Petri disease is one of the important grapevine trunk diseases caused by xylem inhibiting fungi, *Phaeomoniella (Pa.) chlamydospora* together with species of *Phaeoacremonium*. However, knowledge regarding spore release in South African vineyards is unknown. Therefore, the aim of this study was to determine when spores of Petri disease pathogens are released in vineyards of the Western Cape Province. The research was conducted for two seasons from mid-May to end of November 2012 and from mid-March to end of November 2013. Spore traps consisting of microscope slides coated with petroleum jelly were affixed to arms of infected vines in six vineyards and two rootstock mother blocks. The slides were replaced weekly. In the laboratory, slides were washed with distilled water, then passed through 5 µm then 0.4 µm micro filters. The filters were backwashed with 1 ml sterile water and plated onto PDA-Chloramphenicol. The plates were grown at 25^{o}C and colonies resembling those of *Pa. chlamydospora* and *Phaeoacremonium* spp. were counted and subcultured for further molecular identification. Species of *Phaeoacremonium* were identified by amplification of the

partial beta-tubulin gene. For the most frequently occurring species, *Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum* Taqman probes and primers were developed to facilitate fast detection using real-time PCR. Results showed the occurrence of Petri disease pathogens throughout the year. *Phaeomoniella chlamydospora* and *Pm. minimum* were trapped in all vineyards. A total of 14 *Phaeoacremonium* species were identified from the different blocks and included, *Pm. alvesii Pm. australiense, Pm. griseo-olivaceum, Pm. griseorubrum, Pm. iranianum, Pm. inflatipes Pm. minimum, Pm. scolyti, Pm. sicilianum, Pm. subulatum, Pm. parasiticum, Pm. pruniculum, Pm. venezuelense* and *Pm. viticola*. Spore release was shown to coincide with pruning and desuckering activities, however, there was no correlation between total rainfall and spore release events. Occurrence of six different *Phaeoacremonium* species in rootstock mother blocks highlights the high risk of pathogen spread through infected nursery material. The high species diversity and frequency of release coinciding with traditional pruning and desuckering practices emphasizes the need to develop effective wound protection strategies to avoid infection of unprotected pruning wounds.

INTRODUCTION

Petri disease is an important trunk disease in grape and wine production worldwide (Ferreira *et al.*, 1994; Mugnai *et al.*, 1999; Pascoe, 1999; Chicau *et al.*, 2000; Gatica *et al.*, 2001). Petri disease is caused by *Phaeomoniella (Pa.) chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams and *Phaeoacremonium* species (Scheck *et al.*, 1998a; Mugnai *et al.*, 1999; Mostert *et al.*, 2006) and is usually associated with younger vines (1-5 years) in newly established vineyards (Del Río *et al.*, 2002; Halleen and Groenewald, 2005). Thirteen species of *Phaeoacremonium* have been isolated from symptomatic vines in South Africa, namely *Pm. alvesii*, *Pm. austroafricanum*, *Pm. fraxinopennsylvanicum*, *Pm. griseorubrum, Pm. iranianum*, *Pm. krajdenii*, *Pm. minimum*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. sicilianum*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* (Crous *et al.*, 1996; Groenewald *et al.*, 2001; White *et al.*, 2011). Infected vines show stunted growth, shortened internodes and dieback. Internal symptoms normally include black to brown spots and brown streaking in the xylem tissues (Ferreira, 1989; Scheck *et al.*, 1998a; Mugnai *et al.*, 1999). The black discoloration is a result of plant response to infection, which includes the formation of tyloses and phenolic compounds in the xylem tissue (Mugnai *et al.*, 1999; Lorena *et al.*, 2001; Del Río *et al.*, 2004) which prohibits proper water uptake in the host (Edwards *et al.*, 2007). This decreases the lifespan of infected vineyards and subsequently, replanting of young vineyards (Scheck *et al.*, 1998b; Ferreira *et al.*, 1999; Pascoe and Cottal, 2000). The pathogens associated with the disease are well-adopted endophytes that cause disease during stress conditions (Scheck *et al.*, 1998a; Ferreira *et al.*, 1999).

Petri disease pathogens spread within vineyards by means of aerial inoculum from reproductive structures (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). The asexual stage of *Pa. chlamydospora* (pycnidia) has been reported as the source of aerial inoculum and no sexual stage has been reported (Crous and Gams, 2000; Edwards *et al.*, 2001; Eskalen *et al*., 2002; Edwards and Pascoe, 2004). Since Mostert *et al.* (2003) confirmed the sexual stage of *Phaeoacremonium* spp. by *in vitro* pairings, five *Phaeoacremonium* sexual morph connections from isolates originating from grapevines were made, namely *Pm. austroafricanum, Pm. krajdenii, Pm. viticola, Pm. parasiticum* and *Pm. minimum*. However, only three *Phaeoacremonium* sexual morph have been found on grapevines in nature, namely *Pm. minimum* (Rooney-Latham *et al.*, 2005a), *Pm. viticola* and *Pm. fraxinopennsylvanicum* (Eskalen *et al.*, 2005a; b).

Vine infection could be through use of infected plant material (Bertelli *et al.*, 1998; Rego *et al.*, 2000; Halleen *et al.*, 2003; Aroca *et al.*, 2006; Zanzotto *et al.*, 2007), infected soil (Rooney *et al.*, 2001) or through pruning wounds which are considered the main ports of entry (Adalat *et al.*, 2000; Larignon and Dubos, 2000; Eskalen and Gubler 2001; Eskalen *et al.*, 2007; Serra *et al.*, 2008; Rolshausen *et al.*, 2010; Van Niekerk *et al.*, 2011). Spore trapping studies have shown the presence of aerial spore inoculum in vineyards, and high spore counts to coincide with high rainfall events (Eskalen and Gubler, 2001). This rainfall is of significance in forcible discharge of ascospores (Rooney-Latham *et al.* 2005b). Species diversity was shown to differ between countries and may be highly influenced by difference in climatic conditions between countries (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). However, aerial spores from adjacent orchards established near vineyards may serve as another source of aerial spore inoculum (Mostert *et al.*, 2006; Cloete *et al.*, 2011; Arzanlou *et al.*, 2013). Spores are dispersed over long distances as aerial inoculum or infected plant material (Halleen *et al.*, 2003; Cloete *et al.*, 2011; Arzanlou *et al.*, 2013) and poses a high risk of disease spread.

Petri disease pathogens enter the vines through susceptible pruning wounds (Larignon and Dubos, 2000; Eskalen *et al.*, 2007; Van Niekerk *et al.*, 2011). Wound stay susceptible for at least three weeks (Van Niekerk *et al*., 2011), although it could be as long as four months (Eskalen *et al*., 2007). Due to the long period of wound susceptibility, it is crucial that this cultural practice does not coincide with high spore release, as it will lead to a higher probability of new wound infections. However, in South African vineyards, spore release patterns of Petri disease pathogens and frequency is unknown. Attempts to quantify and correlate spore release of Petri disease causing-pathogens with rainfall using a volumetric spore trap was not successful in South Africa because Petri disease pathogens were overgrown by fast growing trunk disease pathogens (Van Niekerk *et al.*, 2010). The aim of this study was therefore, to

determine if spores of Petri disease pathogens are present in South African vineyards, when they are released and under which climatic conditions.

MATERIALS AND METHODS

Site selection

Six vineyards from Paarl, Stellenbosch, Durbanville and Rawsonville, and two rootstocks mother blocks in Slanghoek and Wellington known to be infected with Petri disease were selected for this study (Table 1). Within each vineyard, five vines or five rootstock mother plants were selected and marked.

Spore trapping

The protocol was adopted from Eskalen and Gubler (2001). Spore traps consisted of microscopic slides coated with Vaseline on both sides and was affixed with a wire and binder clips to the vine cordon on each of the five plants. The slides were arranged directly above old pruning wounds or cracks. One plant in each of the five vineyards also had an additional slide on the trunk placed halfway between the graft union and cordon.

The study was conducted over two seasons, from late May to the first week of December in 2012 and from mid-March to the first week of December in 2013. The slides were replaced weekly. The slides were placed individually in sterile Falcon tubes and immediately taken to the laboratory for processing. In the laboratory, each slide was washed in 5 ml sterile distilled water (dH_2O) to suspend the spores, then filtered through 5 µm and 0.45 µm microfilters. This was to separate larger sized spores from smaller sized spores. The filters were then backwashed with 1 ml dH_20 of which 200 µl was plated onto each of the three PDA-Chloramphenicol (PDA-C) agar plates. The plates were sealed with Parafilm and incubated at 25^{o}C for 6–8 weeks. The number of *Pa. chlamydospora* and *Phaeoacremonium* spp. colonies were monitored and recorded. *Phaeomoniella chlamydospora* colonies were morphologically identified according to Crous and Gams (2000). *Phaeoacremonium* species were subcultured for further identification. In cases where more than one colony of the same species occurred on the three dishes of the same plant, a representative sample was subcultured.

Collection of climate data

The mean weekly data per area obtained from the ARC-Nietvoorbij agrometeorological division weather stations were used in this study for analyses. Variables describing the weather conditions during the week of spore trapping included minimum temperatures, maximum temperatures, minimum relative humidity, and total rainfall.

Statistical analysis

Correlation of weather data variables and colony counts were determined by statistical analysis using Pearson's correlation analysis (Snedecor and Cochran, 1967) in XLSTAT 2014.4.06. For species occurring as predominant species, namely *Pa. chlamydospora*, *Pm. minimum* and *Pm. sicilianum*, a separate pooled data correlation analysis was done for all *Phaeoacremonium* species.

DNA extraction

Mycelia of two-week-old *Phaeoacremonium* cultures were scraped into 2 µl eppendorf tubes. An amount of 0.5 g glass beats was added to the tubes as well as 600 µl of CTAB extraction buffer (1M Tris, pH 7.5; 5 M NaCl; 500 mM EDTA, pH 8.0). The tubes were shaken for 6 min at 30 1 s^{-1} using a Mixer Mill type MM 301 (Retsch Gmbh & Co. KG, Germany) before incubating in a waterbath for 30 min at 65°C. After incubation 400 µl chloroform:isoamylalcohol (24:1) was added, followed by centrifugation at 13 100 rpm for 7 min. The watery supernatant was transferred to a new Eppendorf tube and 250 µl of 7.5 M ammonium acetate solution (pH 7) and 600 µl cold isopropanol were added. The samples were then incubated for 15 min at -20°C. The tubes were then centrifuged at 13 100 rpm for 15 min. The supernatant was discarded and 1 ml cold ethanol was added and samples centrifuged at 13 100 rpm for 5 min and supernatant was discarded. The pellet was dried on a bench for 24 hrs whereafter the pellet was dissolved in 100 µl sterile distilled H_2O and stored at 4°C.

PCR and sequencing

PCR was performed using T1 and Bt2b primers to amplify the partial beta-tubulin gene (Glass and Donaldson, 1995; O' Donnel and Cigelink, 1997). The reactions were performed using 0.65 units Biotaq polymerase, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.25 pmol of each primers, 1mg/ml bovine serum albumin (BSA) and 5 µl DNA solution. The amplification was performed on a GeneAmp PCR System 2700 (Applied system Biosystems, Foster City California). The cycling conditions were 5 min at 96°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, 1 min and 30 s at 72°C and a 7 min extension step at 72°C to complete the reaction. PCR product was separated by electrophoresis at 80 V on a 1% agarose gel stained with 0.5 µg/ml ethidium bromide in a 0.5 × TAE buffer (0.04 M Tris, 0.02 M glacial acetic acid and 1.27 mM, EDTA, pH 7.85). The gel was visualized under UV light using a GenGenius Gel Documentation and Analysis System.

PCR products were purified according to the manufacturer's instructions using a commercial kit (Nucleospin MSB PCRapace, Invitek Extract 2 in 1 Purification Kit, Machery-Nagel GmbH & Co., Germany). A sequencing PCR reaction was run in a total reaction volume of 10 µl. The reaction consisted of 5 × Buffer, 0.4 mM each of the primer. PCR was performed using the

following conditions; an initial denaturing step at 95ºC for 60 seconds, followed by 30 cycles of 10 s at 95ºC, 5 s at 50ºC and 4 min at 60ºC and a final step at 30 s at 60ºC. Fragments were separated on an ABI 3130*xl* genetic analyzer. Sequence data for both directions were analyzed using Geneious 3. 5. 6 and edited manually using sequence alignment editor v.2.0a11. Species identification was done using the megablast function of the NCBI's GenBank nucleotide database (www.ncbi.nlm.nih.gov).

Design of qPCR material design and cycling conditions

Primers (IDT, Coralville, IA) and "Taqman" hydrolysis probes (Life Technologies, Carlsbad, CA) were designed to bind within the beta-tubulin gene region and are listed in Table 2. Three different species-specific primer/probe sets were designed for the respective detection of *Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum* following the same strategy as used by Martín *et al.* (2012) for the detection of *Pm. minimum*. Although the *Pm. minimum* system from this study is very similar to that of Martín *et al.* (2012) there are sequence differences. Species-specific forward primers were designed, namely F_ibt_Paleo, F_ibt_Ppara and P_ibt_Psicil, for *Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum,* respectively, intended for use with the universal reverse primer R_IBT_uni that recognises all *Phaeoacremonium* spp. templates. Species-specific hydrolysis probes (Paleo, Ppara and Psicil, for *Pm*. *minimum*, *Pm. parasiticum* and *Pm. sicilianum,* respectively) were 5' labelled with unique fluorophores (FAM, VIC and JUN) that did not display excitation and emission overlap, allowing for multiplex experimental design. Furthermore, all hydrolysis probes carried a 3' QSY quencher (Table 2). *Phaeoacremonium minimum* detection assays were run individually and that for *Pm. parasiticum* and *Pm. sicilianum* in duplex. Reactions for *Pm. minimum* detection were run in a 20 µl final volume containing 2x KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA), 0.2 µM each of F_ibt_Paleo, R_ibt_uni and Paleo as well as 0.5 µl of template (genomic DNA 10x diluted in PCR grade water). Reactions were run in a CFX96 Touch™ cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using the following programme: 3 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 67°C. Reactions for *Pm. parasiticum* and *Pm. sicilianum* detection was run in duplex in a 20 µl final volume containing 2x KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA), 0.1 µM each of F_ibt_Ppara and F_ibt_Psicil, 0.2 µM each of R_ibt_uni, Ppara and Psicil as well as 0.5 µl of template (genomic DNA 10x diluted in PCR grade water). Reactions were also run in a CFX96 Touch™ cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using the following programme: 3 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 65°C. All assays included a non-template control as well as controls for *Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum*. All isolates not positively identified as either *Pm. minimum*, *Pm. parasiticum*

or *Pm. sicilianum* by means of the above mentioned assays were identified by means of sequencing of the beta-tubulin gene region.

RESULTS

Phaeoacremonium species identification

A total of 1532 representative *Phaeoacremonium* spp. were recorded for the 2013 spore trapping season. Among which, 919 were amplified with the *Pm. minimum* Taqman probe, 157 with *Pm. sicilianum* probe and 24 with the *Pm. parasiticum* probe. A total of 432 isolates which did not amplify with the Taqman probes were sequenced as described above. The results for the representative sequence results are presented in Table 5.

Species diversity in vineyards

Petri disease pathogens, *Phaeomoniella chlamydospora* and a number of *Phaeoacremonium* species were found in both seasons of spore trapping trials, in which, a total of nine species were trapped in 2012 and fourteen species in 2013 (Table 3). Pathogen diversity differed between blocks, ranging from two to six species within a block in 2012, to two to eight species in 2013. The least number of species within a vineyard were found in Wellington (rootstock mother block), with only two species trapped in both seasons. The highest number of species were recorded in Slanghoek and Rawsonville (six species in each during 2012) and Stellenbosch P2 and Rawsonville during 2013 (eight species). *Phaeomoniella chlamydospora* and *Pm. minimum* were trapped in all blocks in both seasons. Pathogens found in both seasons were *Pa. chlamydospora*, *Pm. minimum*, *Pm. australiense*, *Pm. parasiticum*, *Pm. pruniculum*, *Pm. sicilianum*, *Pm. scolyti*, *Pm. subulatum* and *Pm. viticola*. Pathogens only reported in 2013 were *Pm. alvesii*, *Pm. griseorubrum*, *Pm. inflatipes*, *Pm. iranianum*, and *Pm. venezuelense*. *Phaeoacremonium sicilianum*, *Pm. scolyti* and *Pm. subulatum* were only found in Rawsonville and Slanghoek. All the species found in the 2012 trapping period were trapped again in the 2013 season, except for *Pm. griseo-olivaceum.* In Stellenbosch B3, *Pm. pruniculum* was only trapped in 2012 and not in 2013, instead, *Pm. parasiticum* was reported in this block in 2013. However, *Pm. pruniculum* was reported in Stellenbosch P2 in 2013.

Spore release events

Spore release events were observed throughout the trapping period, with only a few weeks where no spore events recorded. In the 2012 season, spore trapping were carried out for 29-31 weeks in the vineyards and 27 and 28 weeks in the two rootstock mother blocks, respectively. The number of weeks with spore release events varied between blocks, ranging from 19-29 weeks. In Stellenbosch P2 spore release events were recorded in all 29 weeks in 2012, however, in Rawsonville, Slanghoek and Wellington it was for 25 weeks and in Paarl Z,

Paarl A, Stellenbosch B3, and Durbanville, it was 19, 23, 26, and 28 weeks, respectively (Table 4). A significant number of spore release events occurred during winter and spring pruning periods (Table 4). Spores were present in every week of trapping in Stellenbosch P2 and Durbanville in 2012 during the winter pruning period. There was a total of fourteen weeks of trapping during the spring pruning period, in which spores were trapped in all weeks in Stellenbosch P2. However, only seven spore release events were observed in Paarl Z. In the 2013 season, spore trapping occurred over 38 weeks in all vineyards, except in rootstock mother in Slanghoek (8 weeks) because the block was uprooted. Spore release events were recorded in each week of the 11 week winter pruning period in Stellenbosch B3, Durbanville, Paarl A and Rawsonville. In spring there was also only one week of no spore release in Stellenbosch B3, Durbanville and Rawsonville and two weeks in Paarl A. However, spore release occurred throughout the 14 weeks in Stellenbosch P2 during spring periods in 2012 and 2013. During the winter periods when rootstock cutting were harvested from mother blocks (early-June to mid-June), spore release events were recorded every week in Slanghoek in 2012 and Wellington in 2013.

Paarl A

In Paarl A, three pathogens were trapped in 2012, namely *Pa. chlamydospora*, *Pm. minimum* and *Pm. parasiticum* (Fig. 1). Spores were recorded from week 10/05/2012 to 27/11/2012. *Phaeoacremonium minimum* spore release was recorded for 14 out of 31 weeks from week 10/05/2012 to week 27/11/2012 and showed the highest total number spores released (525 spores) compared to the other two pathogens. Spore release peaks were recorded in weeks 24/07/2012 and 20/11/2012 at spore counts of 116 and 168 spores/week, respectively. There was a total of six consecutive weeks showing no spore release of *Pm. minimum*, in weeks 09/10/2012, 16/10/2012, 23/10/2012, 30/10/2012, 06/11/2012 and 13/11/2012. *Phaeomoniella chlamydospora* showed the highest number of spore release events of 19 out of 29 weeks, however, this was at very low spore counts of between 1 and 84 spores. The highest spore count of 84 spores was recorded in week 14/08/2012. *Phaeoacremonium parasiticum* was only recorded once, in week 27/11/2012 at spore counts of 23. Five pathogens were recorded in Paarl A in 2013, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. iranianum* and *Pm. viticola* (Fig. 2). *Phaeomoniella chlamydospora* was released at higher total spore counts (1922 spores) than other pathogens. Spores of *Pa. chlamydospora* were recorded for 27 out of 38 weeks from week 18/03/2013 to 02/12/2013. There were five weeks with spore release peaks of ≥ 100 spores/week, namely in weeks 12/08/2013 (139 spores), 30/09/2013 (201 spores), 07/10/2013 (101 spores), 02/12/2013 (103 spores) and the highest spore release peak of 785 spores in week 18/11/2013. *Phaeoacremonium minimum* showed the highest number of spore release events of 29 out of

38 weeks, from week 18/03/2013 to 02/12/2013. High spore release peaks of 105, 169, 250, 206, and 113 were recorded in weeks 08/04/2012, 27/05/2013, 18/06/2013, 01/07/2013 and 15/07/2013, respectively. There were four consecutive weeks of no spore release for *Pm. minimum*, namely in weeks 29/04/2013, 06/05/2013, 13/05/2013 and 20/05/2013. *Phaeoacremonium iranianum* spores were only released for 6 out of 38 weeks at spore counts between 2 and 80 spores/week in weeks 02/04/2013, 08/04/2013, 22/04/2013, 27/05/2013, 15/07/2013 and 22/07/2013. Spore release of *Pm. parasiticum* was recorded for only two weeks in weeks 22/04/2013 and 27/05/2013 at spore counts of 7 and 20, respectively, whereas that of *Pm. viticola* was recorded for just one week in week 22/04/2013 (1 spore in Paarl A, three pathogens were recorded in 2012, however, two additional pathogens, namely *Pm. iranianum* and *Pm. viticola* were recorded in 2013. *Phaeoacremonium minimum* was released at high spore counts in 2012 and *Pa. chlamydospora* in 2013.

Paarl Z

In 2012, three pathogens were recorded in Paarl Z, namely *Pa. chlamydospora*, *Pm. minimum* and *Pm. parasiticum* (Fig. 3). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of spores released (1820 spores) and the number of spore release events. Spores were released for 14 out of 30 weeks from week 22/05/2012 to 20/11/2012. High spore release peaks of 100 spores and higher were recorded for six weeks in weeks 05/06/2012 (145 spores), 12/06/2012 (473 spores), 31/07/2012 (115 spores), 07/08/2012 (100 spores) and 14/08/2012 (172 spores) and highest peak of 719 spores in week 19/06/2012. There was a period of five consecutive weeks without spore release events of *Pm. minimum*, namely 21/08/2012, 28/08/2012, 04/09/2012, 11/09/2012 and 18/09/2012. *Phaeomoniella chlamydospora* was recorded for 12 out of 30 weeks between 22/05/2012 and 06/11/2012. Two high spore release peaks were recorded in weeks 14/08/2012 (103 spores) and 28/08/2012 (205 spores). Spore release of *Pa. chlamydospora* were not recorded in consecutive weeks on three occasions, for four weeks (26/06/2012 to 17/07/2012), three weeks (04/09/2012 to 18/09/2012) and for another four weeks (13/11/2012 to 04/12/2012). *Phaeoacremonium parasiticum* was recorded for only three weeks at very low counts of 1, 8 and 9 spores/week in weeks 22/05/2012, 31/07/2012 and 20/11/2012. In 2013, five pathogens were trapped in Paarl Z, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. inflatipes* and *Pm. iranianum* (Fig. 4). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of spores released (6613 spores) and the number of spore release events. Spores of *Pm. minimum* were released for 32 out of 38 weeks from 08/03/2013 to 25/11/2013. High spore release peaks were recorded for 12 weeks in weeks 02/04/2013 (480 spores), 08/04/2013 (1340 spores), 22/04/2013 (1165 spores), 27/05/2013 (1071 spores), 04/06/2013 (500 spores), 18/06/2013 (151 spores), 01/07/2013 (257 spores),

08/07/2013 (150 spores), 15/07/2013 (525 spores), 29/07/2013 (200 spores), 12/08/2013 (121 spores) and 02/09/2013 (206 spores). The highest spore release peak was in week 08/04/2013 (1340 spores). *Phaeomoniella chlamydospora* spore release was recorded for 20 out of 38 weeks and spore release ranged between 1 and 87 spores/week. Although this pathogen was recorded from beginning to the last date of spore trapping, there were a number of occasions showing consecutive weeks of no spore release. This was for three weeks (22/04/2013 to 06/05/2013), for eight weeks (27/05/2013 to 15/07/2013) and for another three weeks (29/07/2013 to 12/08/2013). *Phaeoacremonium parasiticum* was recorded for seven weeks at counts ranging between 1 and 600 spores/week in weeks 18/03/2013, 26/03/2013, 08/04/2013 (highest peak), 29/04/2013, 27/05/2013, 29/07/2013 and 11/11/2013. *Phaeoacremonium iranianum* was released for only five weeks (week 08/04/2013, 15/04/2013, 29/04/2013, 27/05/2013 and 22/07/2013), and the highest spore release of 100 spores was recorded in week 15/04/2013. *Phaeoacremonium inflatipes* was recorded once in week 18/06/2013, this was at 110 spores/week. Five pathogens were trapped in 2013 compared to three in 2012. All pathogens trapped in 2012 were recorded again in 2013, together with two additional pathogens, namely *Pm. inflatipes* and *Pm. iranianum*. *Phaeoacremonium minimum* was the predominant pathogen in both seasons. More spore release peaks of ≥ 100 spores/week were observed in 2013 (15) compared to 2012 (9).

Stellenbosch B3

Four Petri disease pathogens were trapped in Stellenbosch B3 in 2012, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. pruniculum* and *Pm. viticola* (Fig. 5). Higher spore release events were observed between 22/05/2012 to 11/09/2012, although low incidences were recorded up to 04/12/2012. *Phaeoacremonium minimum* was the predominant pathogen based on the total number of spores released (1208 spores) and the number of high spore release events. Spore release of this pathogen was recorded for 17 out of 29 weeks from week 22/05/2012 to 27/11/2012. High spore release of ≥ 100 spores/week of *Pm. minimum* were recorded for five weeks, namely in weeks 26/06/2012 (153 spores), 10/07/2012 (615 spores), 07/08/2012 (104 spores), 21/08/2012 (112 spores), and 11/09/2012 (100 spores). The highest spore release event occurred in week 10/07/2013 at 615 counts. Between weeks 18/09/2012 and 04/12/2012, *Pm. minimum* was released only once at spore counts of 1 and 5, and *Pa. chlamydospora* for three weeks at spore counts of 1 and 53. *Phaeomoniella chlamydospora* spore release was recorded in 22 out of 29 weeks from week 22/05/2012 to 27/11/2012. However, there were no high spore release peaks of ≥ 100 spores/week. The highest spore release was 74 spores in week 07/08/2012. There was only two spore release events of *Phaeoacremonium pruniculum* in weeks 29/05/2012 and 03/07/2012 and one for *Pm. viticola* in week 29/05/2012. This was however at very low spore release counts of 1-3

spore counts/week. A total of four pathogens were recorded in 2013, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum* and *Pm. viticola* (Fig. 6). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of spores, with spore release recorded for 28 out of 38 weeks from week 26/03/2013 to 25/11/2013. High spore release peaks of ≥ 100 spores/week of *Pm. minimum* were recorded in week 02/04/2013 (147 spores), 08/04/2013 (433 spores), 27/05/2013 (998 spores), 22/07/2013 (220 spores), 29/07/2013 (100 spores) and 14/10/2013 (171 spores). The highest peak of *Pm. minimum* was in week 27/05/2013 wherein 998 spores were recorded. *Phaeomoniella chlamydospora* was recorded for 24 out of 38 weeks from week 26/03/2013 to 25/11/2013, however, only one week showing a high spore release peak of 129 spores was recorded in week 12/08/2013. The only *Pm. parasiticum* spore release event was recorded in week 08/04/2013 and *Pm. viticola* in weeks 08/04/2013 and 21/10/2013.

Four pathogens were recorded in both seasons, however, *Pm. pruniculum* was found in 2012, and *Pm. parasiticum* in 2013. *Phaeoacremonium minimum* was the predominant pathogen in both seasons. The highest spore release peak was recorded in 2013 and occurred end-May, whereas this occurred in early-July in 2012.

Stellenbosch P2

In 2012, a total of three Petri disease pathogens were trapped in Stellenbosch P2, namely *Phaeomoniella chlamydospora*, *Pm. minimum* and *Pm. parasiticum* (Fig. 7). Spore release occurred throughout the trapping period. *Phaeomoniella chlamydospora* was the predominant pathogen recorded in this season based on the total number of spores released (1798 spores), with spore release events recorded for 26 out of 29 weeks from week 22/05/2012 to 27/11/2012. However, there were only three weeks showing high spore release peaks of 751, 102 and 153 in weeks 19/06/2012, 07/08/2012 and 30/10/2012, respectively. The highest peak of 751 spores was recorded in week 19/06/2012. *Phaeomoniella chlamydospora* spores were released every week from 22/05/2012 to 27/11/2012, except in weeks 02/10/2012 and 20/11/2012. *Phaeoacremonium minimum* spore release were recorded for 26 out of 29 weeks, with high spore release peaks of more than 100 spores/week of this pathogen recorded in five weeks, namely 26/06/2012 (148 spores), 10/07/2012 (279 spores), 02/10/2012 (111 spores), 20/11/2012 (130 spores) and 27/11/2012 (150 spores). The highest peak was in week 10/07/2012 at 279 spores. *Phaeoacremonium parasiticum* spore releases were recorded for ten weeks in weeks 22/05/2012, 29/05/2012, 05/06/2012, 19/06/2012, 03/07/2012, 18/09/2012, 06/11/2012, 13/11/2012, 20/11/2012 and 04/12/2012. Although this was at very low counts (1 to 11 spores/week). The highest spore release peak of 11 spores was recorded in week 29/05/2012. In 2013, eight Petri disease pathogens were recorded in Stellenbosch P2. This included *Pa. chlamydospora, Pm. minimum*, *Pm. parasiticum*, *Pm. alvesii*, *Pm. pruniculum*, *Pm. iranianum*,

Pm. inflatipes and *Pm. griseorubrum* (Fig. 8). *Phaeomoniella chlamydospora* was the predominant pathogen based on the total number of spores released (2677 spores). Spores of *Pa. chlamydospora* were recorded for 32 out of 38 weeks, with eight weeks showing high spore release of more than 100 spores/week. This was in weeks 15/04/2013 (544 spores), 22/04/2013 (580 spores), 20/05/2013 (313 spores), 26/08/2013 (137 spores), 17/09/2013 (101 spores), 07/10/2013 (194), 11/11/2013 (248 spores) and 02/12/2013 (100 spores). The highest spore release peak of *Pa. chlamydospora* was 580 spores in week 22/04/2013. *Phaeoacremonium minimum* spore release was recorded for 30 out of 38 weeks at counts of between 1-164 spores/week. There were two weeks of high spore release of 164 and 108 spores in weeks 08/04/2013 and 29/07/2013, respectively. Spore release of *Pm. parasiticum* was recorded for 12 out of 38 weeks, with the highest spore release peak of 139 spores recorded in week 20/05/2013. Pathogens trapped at counts lower than 100 spore/week during 2013, included *Pm. alvesii*, *Pm. pruniculum*, *Pm. iranianum*, *Pm. inflatipes* and *Pm. griseorubrum*. Spore release of these pathogens were for either one or two weeks as follows, *Pm. alvesii* was released in weeks 15/04/2013 and 22/04/2013, *Pm. pruniculum*, in week 29/07/2013, *Pm. iranianum* in weeks 02/04/2013 and 22/04/2013, *Pm. inflatipes* in week 10/06/2013 and *Pm griseorubrum* in week 27/05/2013. A higher number of pathogens were trapped during 2013 than in 2012 season (eight vs three pathogens). All pathogens reported in 2012 were reported again in the 2013 trapping season. *Phaeomoniella chlamydospora* was the predominant pathogen in both seasons in Stellenbosch P2. However, there were more weeks of high spore release peaks of *Pa. chlamydospora* in 2013 than in 2012. Spore release *Pa. chlamydospora* and *Pm. minimum* were both trapped for 26 out of 29 weeks, however, the total number of spores counts for *Pa. chlamydospora* were higher than that of *Pm. minimum*.

Durbanville

Three Petri disease pathogens were recorded in Durbanville in 2012, namely *Pa. chlamydospora*, *Pm. minimum* and *Pm. griseo-olivaceum* (Fig. 9). *Phaeomoniella chlamydospora* was released at higher total spore counts (626 spores) and more spore release events (25 out of 30 weeks). Spore release of *Pa. chlamydospora* was recorded for 25 out of 30 weeks from week 22/05/2012 to 04/12/2012, although only one week showed a peak of ≥ 100 spores/week, namely 123 spores in week 28/08/2012. Spore release of *Pm. minimum* was recorded for 24 out of 30 weeks from week 29/05/2013 to 04/12/2012, with the highest peak recorded in week 21/08/2012 (122 spores). *Phaeoacremonium griseo-olivaceum* was recorded only once, in the last week of spore trapping (04/12/2012) however, spore release counts was very low (37 spores/week in 2013, four Petri disease pathogens were recorded in Durbanville, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum* and *Pm. inflatipes* (Fig. 10). *Phaeoacremonium minimum* was by far the predominant pathogen

showing the highest total number of spores released (4016 spores) and more spore release events. Spore release was recorded for 31 out of 38 weeks. High spore release peaks of ≥ 100 spores/week were recorded for nine weeks, 02/04/2013 (711 spores), 08/04/2013 (, 322), 22/04/2013 (423), 29/04/2013 (373 spores), 27/05/2013 (632 spores) , 04/06/2013 (100 spores), 24/06/2013 (750 spores), 01/07/2013 (141 spores) and 29/07/2013 (134 spores). The period between 05/08/2013 and 25/11/2013 was characterized by fewer spore release events and low spore counts. The highest spore release peak was 750 spores in week 24/06/2013. However, there were three consecutive weeks of no spore release recorded in weeks 17/09/2013, 23/09/2013 and 30/09/2013. *Phaeomoniella chlamydospora* was recorded for 28 out of 38 weeks between 18/03/2013 and 02/12/2013, however, the highest spore release was 90 spores/week (week 26/03/2013). *Phaeoacremonium parasiticum* was recorded for only four weeks at spore count range of 1-17 spores/week in weeks 18/03/2013, 26/03/2013, 22/04/2013 and 27/05/2013. *Phaeoacremonium inflatipes* was recorded in only two weeks at 4 and 50 spores/week in weeks 04/06/2013 and 01/07/2013, respectively. The predominant pathogens in 2012 was *Pa. chlamydospora*, whereas it was *Pm. minimum* in 2013. For both pathogens, spore release peaks of ≥ 100 spores/week were very low in 2012 (3 events), compared to 9 events in 2013. *Phaeoacremonium griseo-olivaceum* was only trapped in 2012, nonetheless, two additional pathogens were recorded in 2013, which were not trapped in 2012, namely *Pm. parasiticum* and *Pm. inflatipes*.

Rawsonville

In 2012, six pathogens were recorded in Rawsonville, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. sicilianum* and *Pm. subulatum* (Fig. 11). *Phaeoacremonium sicilianum* was the predominant pathogen based on the total number of spores released (5605 spores). *Phaeoacremonium sicilianum* was released for 18 out of 29 weeks from 22/05/2012 to 04/12/2012. Nine weeks showing spore release peaks of more than 100 spores/week were recorded in weeks 22/05/2012 (178 spores), 31/07/2012 (348 spores), 18/09/2012 (161 spores), 16/10/2012 (640 spores), 30/10/2012 (394 spores), 06/11/2012 (238 spores), 13/11/2012 (1838 spores), 20/11/2012 (1233 spores) and 04/12/2012 (401 spores). The highest *Pm. sicilianum* spore release peak was recorded in week 13/11/2012 with spore counts of 1838. *Phaeoacremonium scolyti* was released for 7 out of 29 weeks between 22/05/2012 to 23/10/2012, with four weeks showing spore release peaks of over 100 spores/week in weeks 12/06/2012 (158 spores), 17/07/2012 (700 spores), 18/09/2012 (177 spores) and 16/10/2012 (1100 spores). However, there was four occasions of weeks without spore release of *Pm. scolyti*, this was for three weeks (week 19/06/2012 to 10/07/2012), eight weeks (week 24/07/2012 to 11/09/2012), three weeks (week 26/09/2012 to 09/10/2012) and for six weeks (week 30/10/2012 to 04/12/2012). *Phaeoacremonium minimum* was recorded

for 18 out of 29 weeks, however, only two weeks of spore release peaks of ≥ 100 spores/week were recorded in weeks 21/08/2012 and 16/10/2012 at spore counts of 104 and 313, respectively. *Phaeomoniella chlamydospora* spores were released for 16 out of 29 weeks from week 22/05/2012 to 20/10/2012 with the highest spore release peak of 210 spores/week (18/09/2012). *Phaeoacremonium parasiticum* was recorded for three weeks in weeks 05/06/2012 (20 spores), 06/11/2012 (1 spore) and 04/12/2012 (25 spores) and *Pm. subulatum* was recorded at very low counts (16 spores) for only one week (26/06/2012In 2013, eight pathogens were trapped in Rawsonville, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. sicilianum*, *Pm. subulatum*, *Pm. australiense*, *Pm. scolyti* and *Pm. venezuelense* (Fig. 12). *Phaeoacremonium sicilianum* was the predominant pathogen showing the highest total number of spores (6219 spores) and high number of spore release events, 28 out of 38 weeks between weeks 18/03/2013 and 02/12/2013. High spore release peaks of ≥ 100 spores/week were recorded for 17 weeks, namely in weeks 18/03/2013 (365 spores), 26/03/2013 (333 spores), 15/04/2013 (665 spores), 22/04/2013 (100 spores, 29/04/2013 (305 spores), 06/05/2013 (339 spores), 13/05/2013 (350 spores), 20/05/2013 (608 spores), 24/06/2013 (460 spores), 01/07/2013 (233 spores), 22/07/2013 (150 spores), 29/07/2013 (255 spores), 26/08/2013 (230 spores), 23/09/2013 (100 spores), 07/10/2013 (626 spores), 25/11/2013 (110 spores) and 02/12/2013 (663 spores). The highest spore release of *Pm. sicilianum* was 665 spores in week 15/04/2013. There was six consecutive weeks in which *Pm. sicilianum* spores were released at high peaks of between 100 and 665 spores, from week 15/04/2013 to week 20/05/2013. There were however, occasions in which *Pm. sicilianum* spores were not recorded for consecutive weeks. This was for three weeks (29/09/2013, 09/09/2013 and 17/09/2013) and again for four weeks (weeks 14/10/2013, 21/10/2013, 28/10/2013 and 04/11/2013). *Phaeoacremonium minimum* was trapped for 21 out of 38 weeks from week 26/03/2013 to 25/11/2013. High spore release peaks of ≥ 100 spores/week were recorded for six weeks in weeks 26/03/2013 (152 spores), 08/04/2013 (251 spores), 22/04/2013 (100 spores), 06/05/2013 (118 spores), 20/05/2013 (400 spores) and 27/05/2013 (147 spores). The highest spore release was in week 20/05/2013 (400 spores). *Phaeoacremonium parasiticum* was recorded in high spore release peaks of ≥ 100 spores/week for three weeks, in weeks 15/04/2013 (614 spores), 22/04/2013 (100 spores) and 20/05/2013 (200 spores). Spore release events for this pathogen was recorded for only five weeks between 08/04/2013 and 28/10/2013. *Phaeomoniella chlamydospora* spore release was recorded for 25 out of 38 weeks, with only two weeks showing high spore release peaks of 295 and 225, in weeks 18/03/2013 and 07/10/2013, respectively. Some pathogens were trapped at very low counts, including *Pm. scolyti* in weeks 06/04/2013 (50 spores) and 27/05/2013 (5 spores), *Pm. subulatum* in week 10/06/2013 (13 spores) and *Pm. venezuelense* in week 13/05/2013 (1 spore). More pathogens were trapped in 2013 than in 2012. A total of

six pathogens were trapped in 2012, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. sicilianum*, *Pm. subulatum*, and *Pm. scolyti* and in 2013, all those pathogens and an addition of two more pathogens, which are *Pm. australiense* and *Pm. venezuelense*. *Phaeoacremonium sicilianum* was predominantly trapped in both seasons. *Phaeoacremonium scolyti* was released at higher counts in 2012 than in 2013. *Phaeoacremonium parasiticum* was trapped on only two occasions in 2012, and this increased to five weeks in 2013. The total number of spores released, spore release events and the number of peaks ≥ 100 spores/week recorded were also higher in 2013 than 2012. Spore release activity for *Pm. sicilianum* was high from July to November in 2012 whereas in 2013, higher spore release of *Pm. sicilianum* were from March to December 2013.

Slanghoek

A total of six Petri disease pathogens were reported in Slanghoek in 2012, namely *Pa. chlamydospora*, *Pm. minimum*, *Pm. australiense*, *Pm. scolyti*, *Pm. subulatum* and *Pm. sicilianum* (Fig. 13). *Phaeomoniella chlamydospora* was released at higher total spore counts (947 spores) than the other pathogens, however, there was only one week of ≥ 100 spores/week and that was 03/07/2012 with 587 spores. Spores were released for 19 out of 28 weeks, from week 05/06/2012 to 27/11/2013. *Phaeoacremonium minimum* was trapped for 22 out of 28 weeks from week 12/06/2012 to 04/12/2012. There were two spore release peaks of ≥ 100 spores/week in weeks 26/06/2012 (239 spores) and 10/07/2012 (121 spores). *Phaeoacremonium scolyti* was recorded for 10 out of 28 weeks, between weeks 19/06/2012 and 06/11/2012. The spore release peaks of ≥ 100 spores/week were recorded in weeks 19/06/2012 (146 spores) and 26/06/2012 (195 weeks) (Fig. 13). *Phaeoacremonium australiense* was only recorded in week 07/08/2012 at 100 spores. Two pathogens were recorded for Slanghoek in 2013, namely *Pa. chlamydospora* and *Pm. minimum* (Fig. 14). Spores were recorded for only eight weeks between 18/03/2013 and 06/05/2013 because the block was pulled out. *Phaeoacremonium minimum* was the predominant pathogen trapped within this period with spore release peaks in weeks 08/04/2013 (105 spores), 27/05/2013 (169 spores), 18/06/2013 (250 spores), 01/07/2013 (206 spores) and 15/07/2013 (113). *Phaeomoniella chlamydospora* was detected for only one week (18/03/2013) at very low counts.

Wellington

There were only two pathogens trapped in Wellington in 2012, namely *Pa. chlamydospora* and *Pm. minimum* (Fig. 15). *Phaeomoniella chlamydospora* was the predominant pathogen based on the total number of spores released (1281 spores) and the number of spore release events, with spores released for 23 out of 27 weeks from week 11/06/2012 to 04/12/2012.

Spore release peaks of 108, 188 and 578 were recorded in weeks 11/06/2012, 02/07/2012 and 27/08/2012, respectively. The highest spore release peak of *Pa. chlamydospora* of 578 spore counts was in week 11/06/2012. *Phaeoacremonium minimum* spore release were recorded for 20 out of 27 weeks, with only one week showing a spore release peak of 103 in week 09/07/2012. In 2013, *Pa. chlamydospora* and *Pm. minimum* were the only two pathogens trapped in Wellington (Fig. 16). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of spores (4061 spores) and the number of spore release events recorded for 26 out of 38 weeks between weeks 26/03/2013 and 02/12/2013. High spore release peaks of ≥ 100 spores/week of *Pm. minimum* were recorded for nine weeks, namely 22/04/2013 (352 spores), 27/05/2013 (800 spores), 04/06/2013 (109 spores), 01/07/2013 (174 spores), 15/07/2013 (800 spores), 29/07/2013 (220 spores), 23/09/2013 (627 spores), 04/11/2013 (178 spores) and 18/11/2013 (483 spores). The highest spore release peak was 800 spores occurring in two weeks, week 27/05/2013 and 15/07/2013. *Phaeomoniella chlamydospora* was recorded for 16 out of 38 weeks from week 15/04/2013 to 02/12/2013. High spore release of ≥ 100 spores/week of *Pa. chlamydospora* were recorded in only three weeks, namely 29/07/2013 (465 spores), 02/09/2013 (102 spores) and 02/12/2013 (159 spores). Petri disease pathogens, *Pa. chlamydospora* and *Pm. minimum* were the only pathogens recorded in both seasons. *Phaeomoniella chlamydospora* was the predominant pathogen in 2012 and *Pm. minimum* in 2013. Spore release peaks were more in 2013 (4 events for *Pm. minimum* and 9 events for *Pa. chlamydospora*) than in 2012 (3 events for *Pm. minimum* and 1 event for *Pa. chlamydospora*). There are periods of several consecutive weeks without spore release events, but it varies and cannot be predicted without sophisticated prediction models.

Spore release events during and after pruning periods

Pruning dates of each vineyard studied in 2012 and 2013 are listed in Table 1 and also indicated by the red arrow in each figure of spore release patterns (Fig 1 to 16). Spore release events occurred during the week of pruning in Paarl Z, Stellenbosch B3, Stellenbosch P2 and Durbanville in 2012. Spore release of either *Pa. chlamydospora* or *Pm. minimum*, or of both these pathogens were trapped during the week of pruning, and also in the subsequent three weeks after pruning. Spores of *Pa. chlamydospora*, *Pm. minimum* and *Pm. sicilianum* were also trapped within the weeks after pruning occurred in Rawsonville. No spores were released during the week of harvesting rootstock material in Slanghoek and Wellington, however, highest spore release of *Pm. minimum* were recorded in Wellington in the first week after pruning. In Slanghoek, the highest spore release of *Pm. minimum* and *Pm. scolyti* for this block were trapped in the fourth week after pruning.

In 2013, spore release was recorded in the same week that pruning occurred in all vineyards studied, with high spore release peaks of *Pm. minimum* observed in Paarl A, Stellenbosch P2, Durbanville and Wellington. *Phaeomoniella chlamydospora* together with *Pm. minimum*, *Pm. parasiticum Pm. iranianum*, *Pm. sicilianum*, *Pm. scolyti* and *Pm. inflatipes* were also released within the four weeks after pruning. Spores of *Pa. chlamydospora* and *Pm. minimum* were recorded in rootstock mother blocks within four weeks after rootstock harvest in Wellington.

Correlation of spore release events with weather data

There was no correlation between colony counts of *Phaeoacremonium* spp. and all the weather variables tested. Scatterplots, PCA, PLS and multiple regression analysis also showed no correlations between colony counts and weather variables. There was, however, a significantly moderate positive correlation between *Pm. sicilianum* counts and maximum temperatures (0.051; $P < 0.001$; $R^2 = 0.251$) and a weak correlation between colony counts and minimum temperatures (0.339; $P < 0.005$; $R^2 = 0.115$). There was also a negative correlation between *Pm. sicilianum* and minimum relative humidity (-0.476; $P < 0.0001$; $R^2 = 0.227$).

DISCUSSION

Petri disease is a serious disease in South Africa since the early 1990's (Ferreira *et al.*, 1994). Knowledge on the biology of Petri disease pathogens is important to develop sound management strategies. This study was conducted to understand when spores of Petri disease pathogens are released in vineyards of the Western Cape Province, and what risk they pose to pruned vines.

Aerial spores of *Pa. chlamydospora* together with *Phaeoacremonium* species were trapped in this study. A total of 14 *Phaeoacremonium* species, namely *Pm*. *minimum*, *Pm. australiense*, *Pm. alvesii, Pm*. *griseo-olivaceum*, *Pm. griseorubrum, Pm. inflatipes, Pm. iranianum, Pm. scolyti*, *Pm. sicilianum*, *Pm. subulatum, Pm. parasiticum*, *Pm. pruniculum, Pm*. *venezuelense* and *Pm. viticola* were detected in vineyards and rootstock mother blocks. Compared to similar spore trapping trials conducted in other countries, this is by far the highest species diversity recorded in vineyards. Only two Petri disease pathogens were detected in France (*Pa. chlamydospora* and *Pm. minimum*) (Larignon and Dubos, 2000) and three pathogens in California (*Pa. chlamydospora*, *Pm. minimum* and *Pm. inflatipes*) (Eskalen and Gubler, 2001). *Phaeoacremonium* species found in this study were among the 12 species previously reported from South African vineyards (Crous *et al*., 1996; Mostert *et al*., 2005; Mostert *et al*., 2006; White *et al*., 2011), accept for *Pm. krajdenii*, *Pm. austroafricanum and Pm. fraxinopennsylvanicum* which were not found in the current study.

Five *Phaeoacremonium* species only reported on *Prunus* spp. were trapped as aerial inoculum in our study, namely *Pm. australiense*, *Pm. griseorubrum*, *Pm. griseo-olivaceum* and *Pm. pruniculum* (Damm *et al*., 2008). Moreover, *Pm. inflatipes* is reported for the first time in South African vineyards, with its occurrence previously reported in vineyards of the USA (Rooney *et al*., 2001), Chile (Mostert *et al*., 2006) Iran (Mohammadi *et al.*, 2013) and Spain (Gramaje *et al*., 2009). Finding of aerial inoculum of species never reported on grapevines suggests a need to conduct pathogenicity studies to understand their role in vineyards.

Two pathogens were trapped in all vineyards and rootstock mother blocks, namely *Pa. chlamydospora* and *Pm. minimum.* For the fact that both *Pa. chlamydospora* and *Pm. minimum* fruiting bodies were found on grapevines (Baloyi *et al*., 2013; 2016), could be the reason they are the abundant in aerial inoculum. One of these two pathogens were the predominant species in all vineyards, except Rawsonville. In some blocks the predominant species also changed between the two seasons, for example *Pm. minimum* was high in Paarl A in 2012 but in 2013 season it was *Pa. chlamydospora*. These two pathogens are widely distributed in all grape growing regions (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Halleen *et al*., 2003; Mohammadi *et al*., 2012). Their ability to grow in a wide range of biotic conditions could be one of the reasons for their adaptive character and wide-spread occurrence (Whiting *et al*., 2001; Mostert *et al*., 2006). In Rawsonville, *Pm. sicilianum* was the predominant species in both seasons. *Phaeoacremonium sicilianum* was previously isolated from vineyards in Calitzdorp (White *et al*., 2011) and was also found in Italian (Essakhi *et al*., 2008) and Spanish vineyards (Gramaje *et al*., 2009).

Petri disease pathogens were detected throughout the trapping period, with occasional weeks of no spore release. Spore counts differed according to vineyards, sampling date and year of collection. This could be attributed to differences in inoculum density of fruiting bodies between vineyards or favorable microclimatic conditions. The periods of high spore release peaks (≥ 100 spores per week) were inconsistent between seasons and pathogens. *Phaeoacremonium minimum* spore release peaks were high between June and October in 2012 in the Paarl and Stellenbosch vineyards, and between March and October in all vineyards except Rawsonville in 2013. *Phaeomoniella chlamydospora* high spore release peaks were mostly between June to October in 2012 and only in the month of August in Stellenbosch B3, Paarl A and Paarl Z and in Durbanville in 2012. This was also observed between August and October 2013 in Durbanville and Rawsonville. *Phaeoacremonium sicilianum* peak releases were between May and December in 2012 and between April and December in 2013. Spore release of some pathogens were at very low counts in both seasons, which implicate that the pathogen might have just recently been introduced into the area or could be due to unfavorable conditions. The presence of aerial spores in autumn (March and

April) discourages recommendation of clean pruning practices without wound protection during this period, as spores are available to infect the large wounds made during this activity.

Spore release occurred during winter (June-August) and spring (September-November) pruning periods. Spore release events were recorded in every week of the winter pruning period in Stellenbosch P2 and Durbanville in 2012 and again in Stellenbosch B3, Paarl A, Durbanville and Rawsonville in 2013. Although this was not the case for every vineyard studied, spore release events occurred in most weeks during winter and spring pruning periods. Spore release events occurred every week in Durbanville and Rawsonville in 2012 and in Stellenbosch B3, Paarl A, Durbanville and Rawsonville in 2013. This emphasizes the high probability that wounds can become infected. Furthermore, winter and spring wounds remain susceptible for long periods (Eskalen *et al*., 2007; Van Niekerk *et al*., 2011; Makatini, 2014). In the current study, spore release events were observed during the four-week period after winter pruning when wounds are at their most susceptible for infection. During this period, pathogens such as *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. inflatipes*, *Pm. griseorubrum*, *Pm. iranianum*, *Pm. sicilianum*, *Pm. scolyti* and *Pm. subulatum* were recorded. This is in agreement with previous results of Eskalen and Gubler (2001) which showed spore release of *Pm. minimum* and *Pm. inflatipes* to coincide with winter and spring pruning periods in California (Eskalen and Gubler, 2001). The capability of *Pa. chlamydospora* to infect spring wounds under field conditions has been proved (Makatini, 2014). These results emphasize the fact that pruning activities in South Africa coincide with spore release events, which poses high chances of new wound infections. Therefore, wound protectants should be applied whenever pruning occurs, and specifically wound protectants that provide prolonged protection as a result of the long susceptibility period together with the availability of a diversity of Petri disease spores.

The occurrence of aerial spore inoculum of *Pa. chlamydospora* and *Phaeoacremonium* species in the rootstock mother blocks is a great concern, especially since these pathogens were trapped during the first week after pruning in which rootstock cuttings were removed and supplied to nurseries for propagation purposes. *Phaeomoniella chlamydospora* and *Pm. minimum* were found in both rootstock mother blocks in 2012. Use of infected rootstock mother plants and propagation material seriously impact trunk disease spread as infected asymptomatic shoots may be used for grafting and planted in newly established vineyards (Bertelli *et al.*, 1998; Fourie and Halleen, 2002; Halleen *et al.*, 2003; Fourie and Halleen, 2004). Results of Edwards *et al*. (2003) suggested *Pa. chlamydospora* spores to be carried in sap flow of infected mother plants which causes subsequent contamination of canes. Multiple pruning wounds serve as the port of entry to infect the rootstock stump, which will subsequently start producing infected shoots (Fourie and Halleen, 2002; 2004). There are to

date neither resistant rootstock cultivars nor wound protectant programs in South African rootstock mother blocks against Petri disease pathogens. The finding of six different Petri disease pathogens in the Slanghoek mother block illustrate the fact that different pathogens could possibly infect rootstock mother vines and spread in propagation material to vineyards in different regions. Initiation of wound protection research in rootstock mother blocks should be considered as a major priority in order to prevent mother plant infections and to improve the phytosanitary status of propagation material.

There was no correlation between colony counts and rainfall in this current study. This finding is similar to report of Larignon and Dubos (2000) who did not find correlation between *Pm. minimum* spore release and rainfall in California. However, earlier studies did successfully correlate spore release of *Pa. chlamydospora* and *Pm. inflatipes* with rainfall in France (Eskalen and Gubler, 2001). The increase in maximum temperatures resulted in increased colony counts for *Pm. sicilianum*. This could be the reason for the abundance of *Pm. sicilianum* in Rawsonville area, which is warmer than other areas at which spore trapping was conducted. The species has an optimum temperature requirement of 27ºC in culture (Essakhi *et al*., 2008), which could favour its adaptability in Rawsonville.

The total number of pathogens varied between vineyard and rootstock mother blocks. Stellenbosch P2, Rawsonville and the Slanghoek rootstock mother block had the highest species diversity (eight, eight and six species, respectively), compared to the two species found in the Wellington rootstock mother block. However, it was noteworthy that vineyards in the same area shared the same pathogens. For instance, in Stellenbosch, *Pm. parasiticum*, *Pm. pruniculum* and *Pm. viticola* aerial inoculum were trapped in Stellenbosch P2 and Stellenbosch B3. *Phaeoacremonium australiense*, *Pm. subulatum*, *Pm. scolyti* and *Pm. sicilianum* were trapped in Rawsonville and Slanghoek. *Phaeoacremonium iranianum* aerial inoculum was trapped in Paarl A and Paarl Z. These results suggests aerial spores of species to travel between vineyards that are in close proximity to each other. This can also be the case in vineyards established in close proximity to fruit orchards (Cloete *et al*., 2011). For example aerial spores of species previously reported on *Prunus* spp. were trapped inside vineyards in this study, namely *Pm. griseo-olivaceum* and *Pm. pruniculum* (Damm *et al.*, 2008). The status of these species as pathogens of grapevine needs to be determined.

These results therefore show the risk at which infected vineyards and orchards can serve as a source of aerial spore inoculum to healthy vineyards in close proximity. This is supported by the finding of *Pm. minimum* which was isolated from *Prunus* spp. (Damm *et al.*, 2008), *Olea europea* (Ùrbez-Torres *et al*., 2013), *Cudonia oblonga* (Sami *et al*., 2014), *Malus domestica* (Cloete *et al*., 2011), *Phoenix dactylifera* (Mohammadi, 2014) and *Salix* spp. (Hausner *et al.*, 1992). *Phaeoacremonium parasiticum* was also isolated from other hosts

including *Achtinidia chinesis* (Dí Marco *et al.*, 2004), *Aquilaria agalocha* and *Cupressus* spp. (Mostert *et al.*, 2006), *Prunus* spp. (Damm *et al.*, 2008) and *Olea europea* (Nigro *et al.*, 2013). According to Cloete *et al.* (2011) and Arzanlou *et al.* (2013) these species have the capability of forming lesions on grapevines irrespective of the host from which they were originally isolated. These results highlight the risk of establishing vineyards in close proximity to *Prunus* and other woody hosts. This was also mentioned by Eskalen *et al.* (2007) after finding *Pm. fraxinopennsylvanicum* and *Pm. viticola* sexual morphs on grapevines and ash trees that were in close proximity to vineyards in California. Further research on alternative hosts as inoculum sources of Petri disease pathogens should be investigated to better understand the host range of these pathogens. However, in practical terms the importance of effective pruning wound protection is even of higher priority because vineyards, orchards and other woody hosts are increasingly planted in close proximity to each other in almost all production areas of the world. Farming with single crops in large areas is not a viable option in most agricultural areas.

Inevitably, the common practice of establishing new vineyards in close proximity to older vineyards may lead to faster disease progression in young vines and early infection during wounding as spores are aerially dispersed. In this study spore traps were also placed in newly established vineyards adjacent to old blocks (data not shown). In these vineyards, pathogens trapped in old blocks were also trapped in young blocks, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. sicilianum* and other trunk disease pathogens. This emphasizes the need to adopt wound protection and control strategies as early as possible. According to Kaplan, *et al.* (2014) farmers in California delay adoption of preventative measures against Petri disease pathogens until 10−40% disease incidence. There are several ways in which Petri disease pathogens can be dispersed from one vineyard to another, namely arthropods which act as vectors (Moyo *et al*., 2014), aerial dispersed spores (Larignon and Dubos, 2000; Eskalen and Gubler, 2001), and pruning shears (Augustí-Brisach *et al.*, 2015). Most commonly, vineyards are infected from the start through the use of infected planting materials (Gramaje *et al*., 2013).

The purpose of this study was to determine which spores of Petri disease pathogens are found in South African vineyards and when they are released. To conclude these findings, 15 Petri disease pathogens were trapped as aerial spore inoculum in vineyards and rootstock mother blocks. The spore release events coincide with periods of pruning activities in vineyards and rootstock mother blocks. Interestingly, spores were available even during spring pruning and the late summer periods, a period some viticulturist believed to be free of spores and consequently a perfect time to conduct clean pruning practices where large wounds are created. The occurrence of spores extends to periods at which wounds are still susceptible. Therefore, wound protectants should be applied whenever vine wounding occurs irrespective

of the time of the year. Developing new wound protectant formulations should consider the length of wound susceptibility, as well as the constant availability of aerial spore inoculum. Spore release differs between years possibly due to variation in climatic conditions, and therefore, trapping over several consecutive seasons is suggested to better understand spore release patterns and to develop accurate spore release forecasting models. Further studies on sources of inoculum within vineyards and mother blocks are highly recommended in order to reduce aerial inoculum.

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Zanzotto, A., Autiero, F., Bellotto, D., Dal Cortivo, G., Lucchetta, G. and Borgo, M. 2007. Occurrence of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* in grape propagation materials and young grapevines. European Journal of Plant Pathology 119: 183–192.

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Table 1. Characterization of vineyards and rootstock mother blocks in which spore trapping studies were conducted in 2012 and 2013, and the pruning dates.

Vineyard	Cultivar	Age of vineyard	Pruning date	
			2012	2013
Paarl A	Red Muscadel	30	26/06/2012	27/08/2013
Paarl Z	Hanepoot	40	17/07/2012	08/07/2013
Stellenbosch B3	mixed cultivar	27	24/07/2012	27/07/2013
Stellenbosch P2	Pinotage	35	10/07/2012	27/07/2013
Durbanville	Sauvignon blanc	29	31/07/2012	18/06/2013
Rawsonville	Chenin blanc	24	24/07/2012	27/05/2013
Slanghoek	Ramsey	19	29/05/2012	06/05/2013
Wellington	Ramsey	17	04/06/2012	04/06/2013

Table 2. Primers and hydrolysis probes used in this study. Oligonucleotide modifications and template specificity are indicated.

Name	Sequence	Modifications	Specificity
(Primers)			
F_ibt_Paleo	GCTTCGACGTCCTCGA	none	*Pm. minimum*
F_ibt_Ppara	GCTTCGACGACCTCGA	none	*Pm. parasiticum*
F_ibt_Psicil	AGCTTCGAACCATCTCGA	none	*Pm. sicilianum*
R_ibt_uni	GCATTGGCCGGTCTG	none	universal
(Hydrolysis probes)			
Paleo	CAGAATCTACCCCAGATCATCGACCAGC	5'-FAM™, 3'-QSY®	*Pm. minimum*
Ppara	CGACTCTGACCCCAAAAGCATCGAC	5'-VIC®, 3'-QSY®	*Pm. parasiticum*
Psicil	CCTCGATATCGTCCTCAAAATGTCTCTCAGAC	5'-JUN®, 3'-QSY®	*Pm. sicilianum*

Table 3. Diversity of Petri disease causing pathogens collected from spore traps placed in six vineyards and two rootstock mother blocks in the Western Cape Province during 2012 and 2013. (*) Denotes species trapped in 2012, (*) Denotes species trapped in 2013.

	Paarl A[a]		Paarl Z[a]		Stellenbosch B3[a]		Stellenbosch P2[a]		Durbanville[a]		Rawsonville[a]		Slanghoek[b]		Wellington[b]	
Pathogens	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013[c]	2012	2013
Pa. chlamydospora	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Pm. minimum	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Pm. alvesii								*								
Pm. australiense												*	*			
Pm. griseorubrum								*								
Pm. griseo-olivaceum									*							
Pm. parasiticum	*	*	*	*		*	*	*		*	*	*				
Pm. pruniculum					*			*								
Pm. inflatipes				*				*		*						
Pm. iranianum		*		*				*								
Pm. sicilianum											*	*	*			
Pm. scolyti											*	*	*			
Pm. subulatum											*	*	*			
Pm. venezuelense												*				
Pm. viticola		*			*	*										
Total	3	5	3	5	4	4	3	8	3	4	6	8	6	2	2	2

[a] Paarl A, Paarl Z, Stellenbosch B3, Stellenbosch P2, Durbanville and Rawsonville were vineyards.
[b] Slanghoek and Wellington were rootstock mother blocks.
[c] Rootstock mother block was pulled out from week 13/05/2013, data from 13/05/2013 to 02/12/2013 not available.

Table 4. Number of weeks with spore release events in the 2012 and 2013 trapping seasons.

		Paarl A	Paarl Z	Stellenbosch B3	Stellenbosch P2	Durbanville	Rawsonville	Slanghoek[d]	Wellington[d]
2012									
	Winter pruning[a]	8 of 11	9 of 11	10 of 11	11 of 11	11 of 11	9 of 11	4 of 4	3 of 4
	Spring pruning[b]	10 of 14	7 of 14	12 of 14	14 of 14	13 of 14	13 of 14	N/A	N/A
	Total[e]	23 of 31	19 of 30	26 of 29	29 of 29	28 of 30	25 of 29	25 of 28	25 of 27
2013									
	Winter pruning[a]	11 of 11	9 of 11	11 of 11	10 of 11	11 of 11	11 of 11	N/A[c]	4 of 4
	Spring pruning[b]	12 of 14	14 of 14	13 of 14	14 of 14	13 of 14	13 of 14	N/A	N/A
	Total[e]	35 of 38	38 of 38	33 of 38	37 of 38	37 of 38	37 of 38	7 of 8	37 of 38

[a] Winter pruning refers to pruning from mid-June to end-August

[b] Spring pruning refers to pruning occurring from beginning of September to end-November

[c] Rootstock mother block was pulled out during the week of 13/05/2013

[d] Rootststock cuttings were harvested from early-June to mid-June

[e] Total number of spore trapping weeks for each vineyard and the total number spore release events

Table 5. Comparative analyses of sequence nucleotides and megablast based nucleotides of *Phaeoacremonium* species trapped in vineyards.

Species	GeneBank ref	Identities	Gaps
Pm. alvesii	EU883990.1	609/612 (99%)	0/612 (0%)
Pm. australiense	EU128073.1	683/683 (100%)	0/683 (0%)
Pm. griseorubrum	EU128075.1	747/750 (99%)	0/750(0%)
Pm. griseo-olivaceum	EU128097.1	671/676 (99%)	0/676 (0%)
Pm. inflatipes	GQ903719.1	575/582 (99%)	0/582 (0%)
Pm. iranianum	KJ200941.1	504/506 (99%)	0/506 (0%)
Pm. minimum	HQ605018.1	618/620 (99%)	0/620 (0%)
Pm. parasiticum	KF870482.1	694/695 (99%)	0/695 (0%)
Pm. pruniculum	EU128095.1	551/556 (99%)	0/556 (0%)
Pm. sicilianum	KM016927.1	600/603 (99%)	0/603 (0%)
Pm. subulatum	EU128092.1	616/618 (99%)	0/618 (0%)
Pm. scolyti	EU128090.1	744/751 (99%)	0/751(0%)
Pm. venezuelense	AY579318.1	441/442 (99%)	0/442 (0%)
Pm. viticola	EU128093.1	587/590 (99%)	0/590 (0%)

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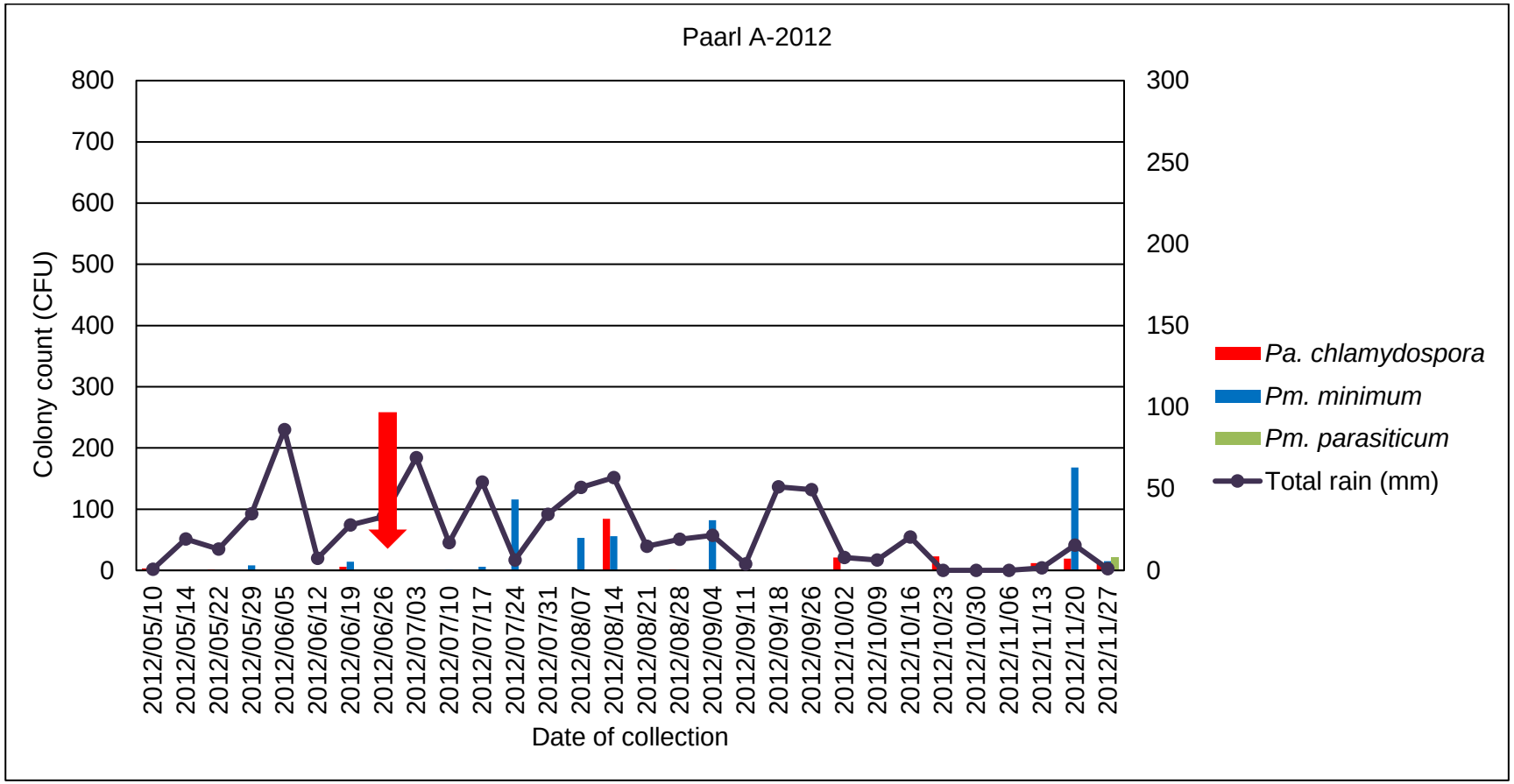

Figure 1. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (↓) indicates the date at which vines were pruned.

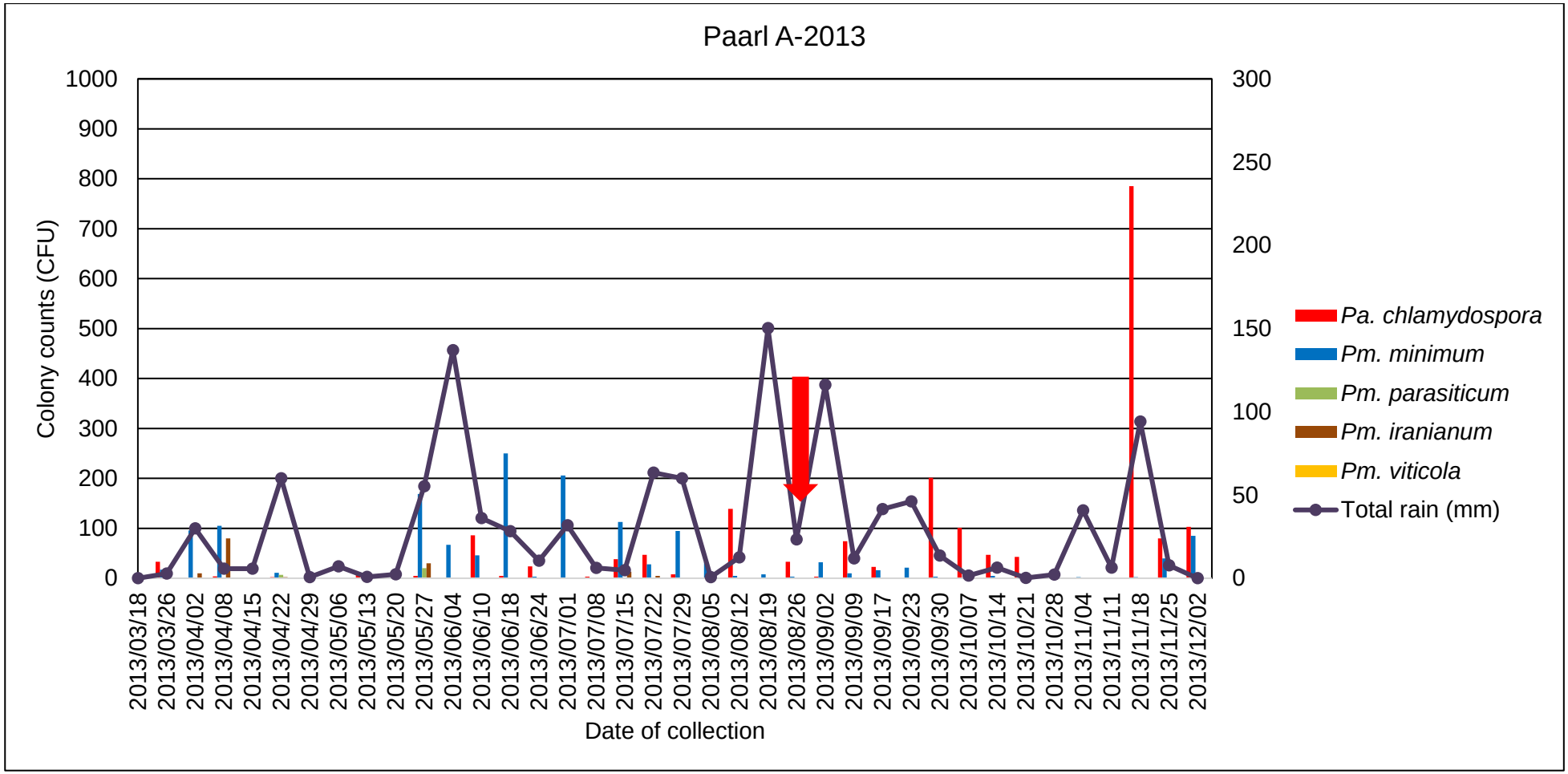

Figure 2. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2013. The red arrow (▬) indicates the date at which vines were pruned.

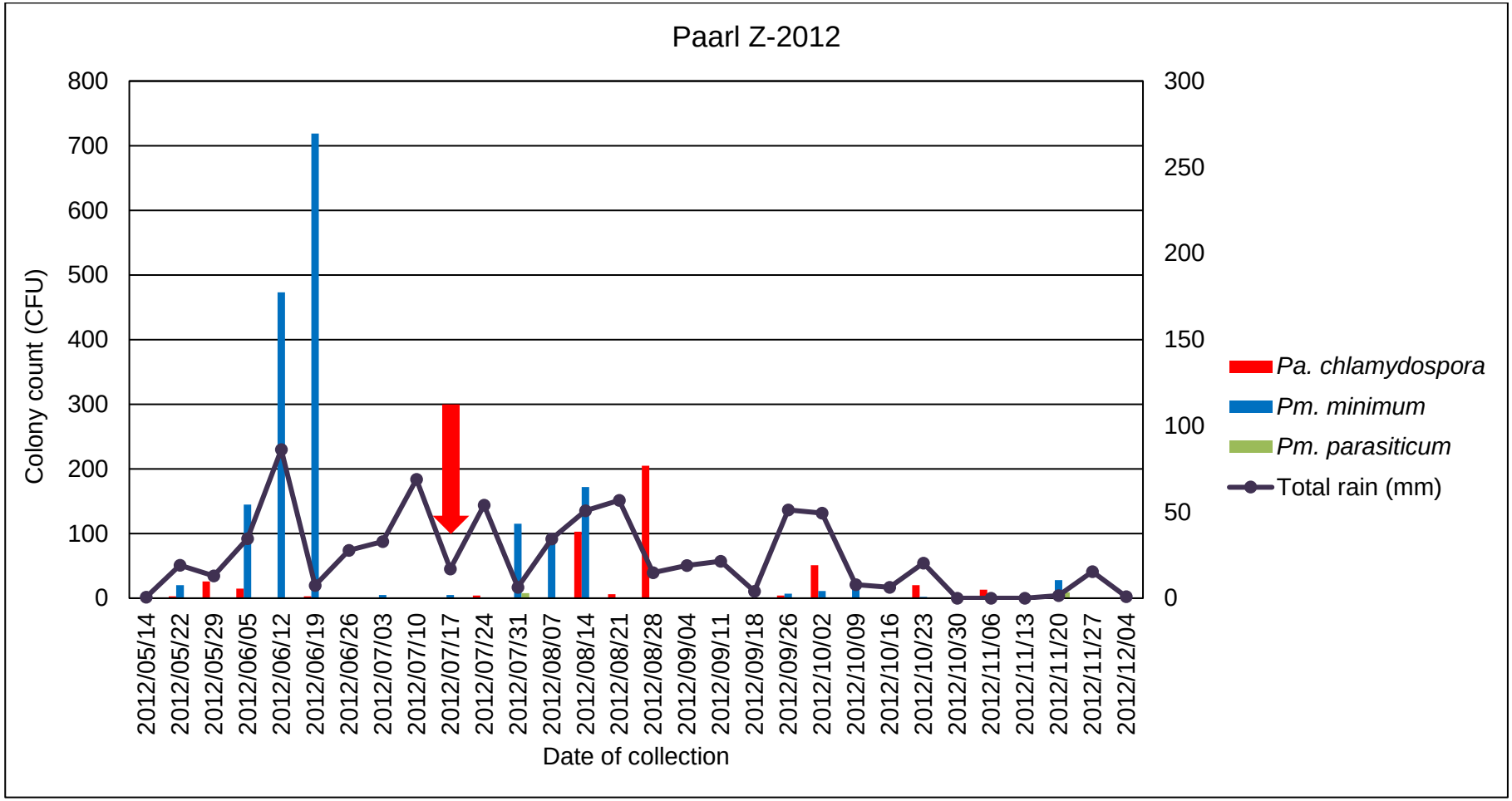

Figure 3. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which vines were pruned.

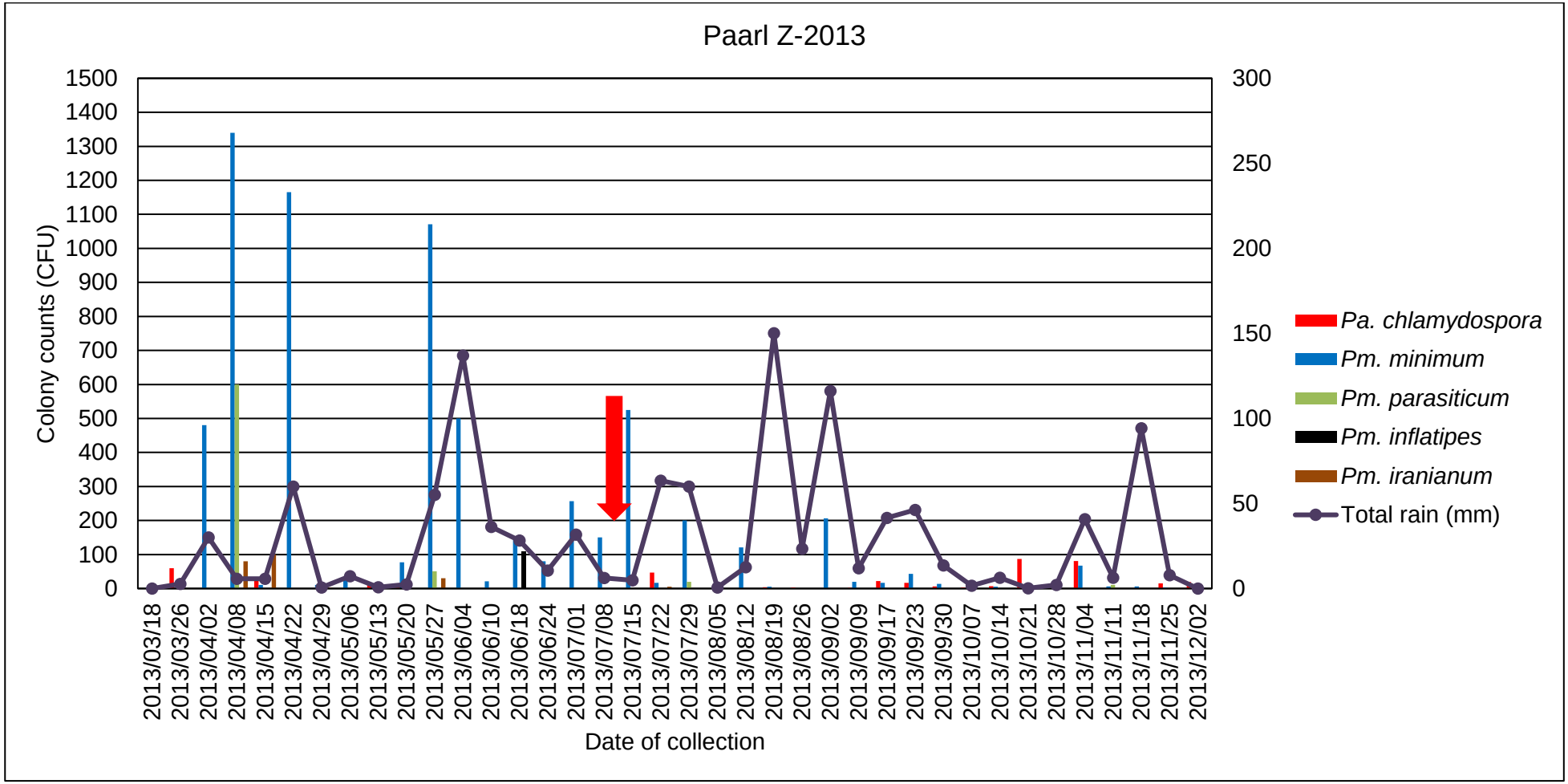

Figure 4. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2013. The red arrow (▬) indicates the date at which vines were pruned.

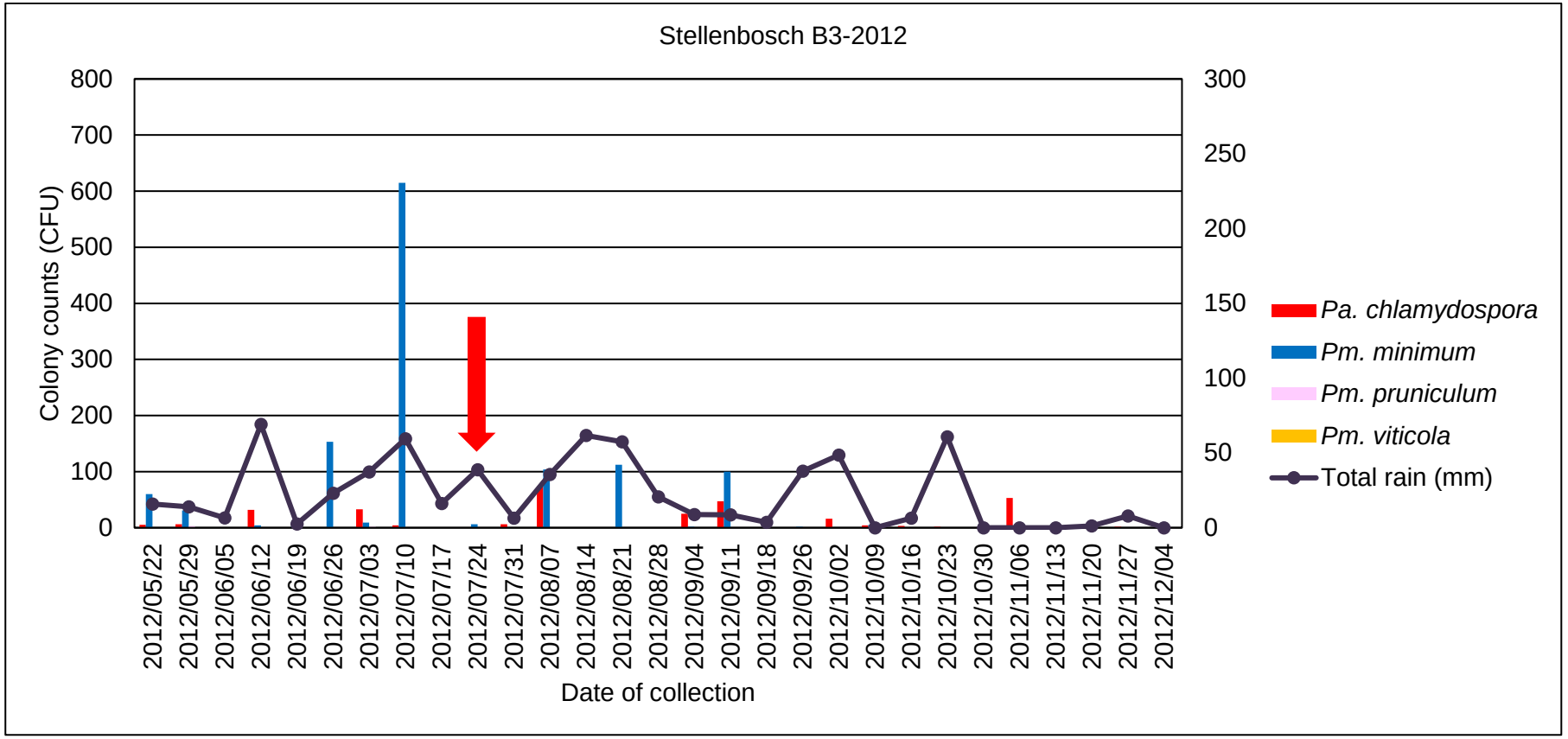

Figure 5. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which vines were pruned.

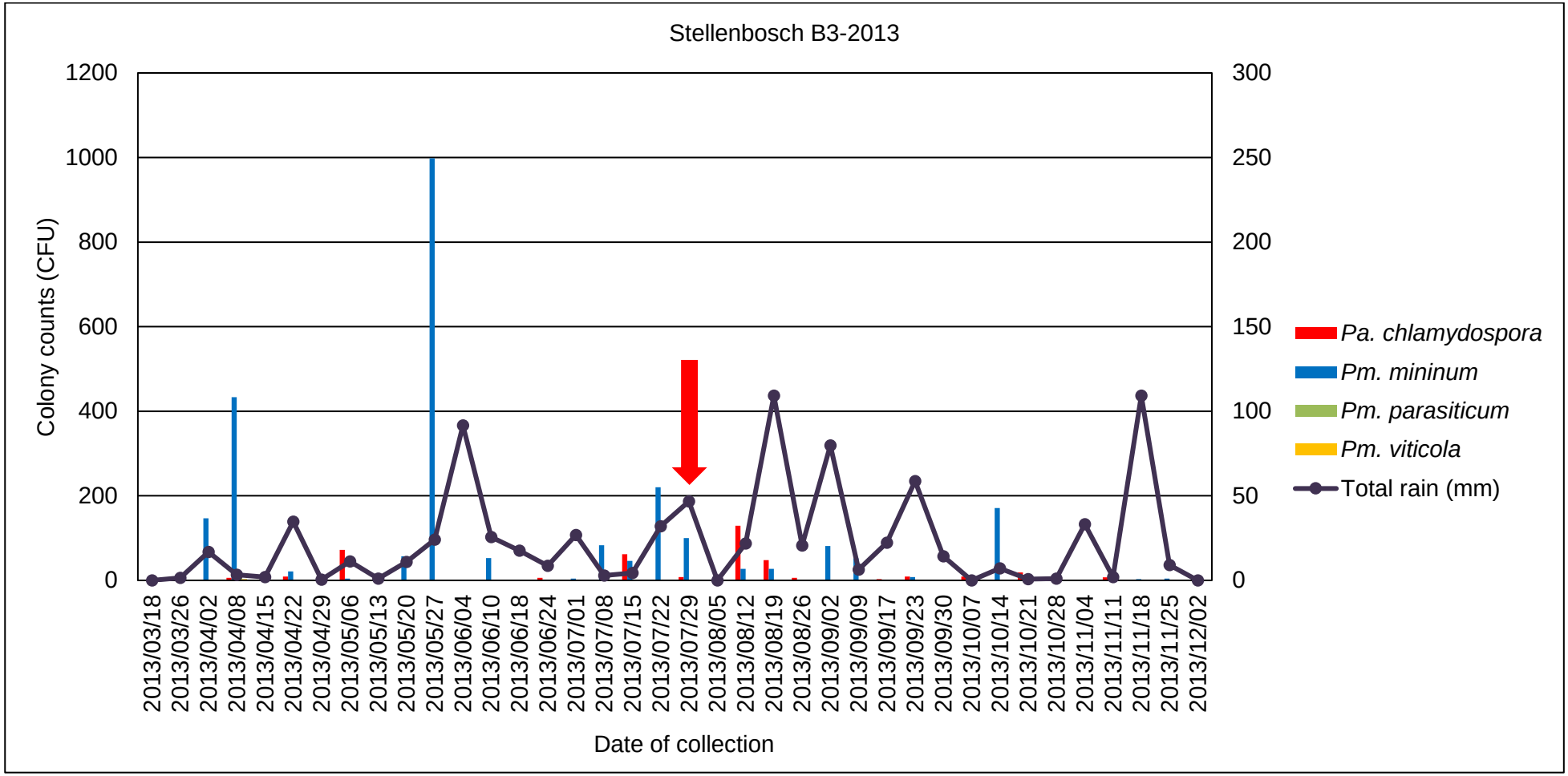

Figure 6. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2013. The red arrow (▬) indicates the date at which vines were pruned.

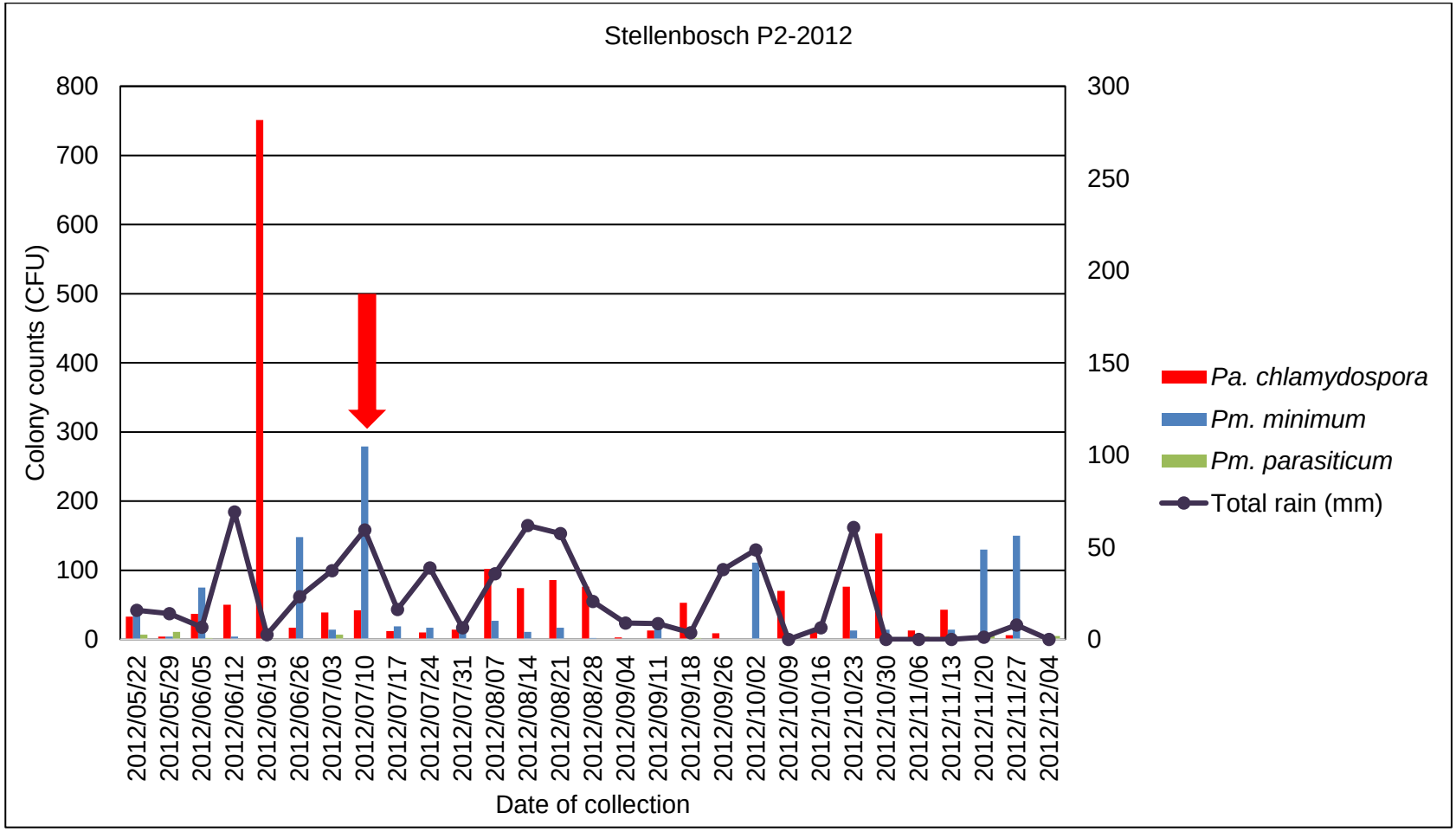

Figure 7. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which vines were pruned.

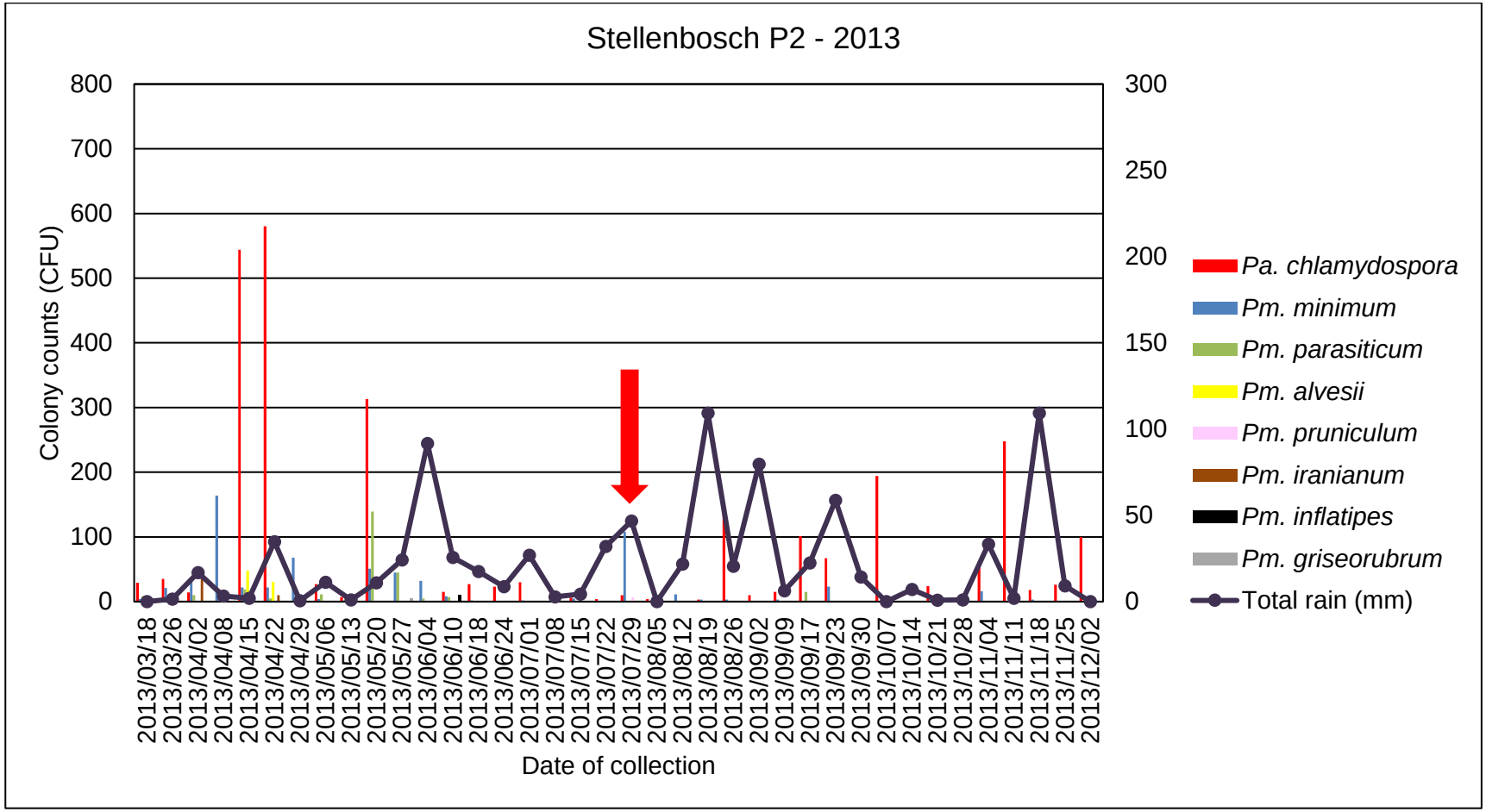

Figure 8. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week 2013. The red arrow (▬) indicates the date at which vines were pruned.

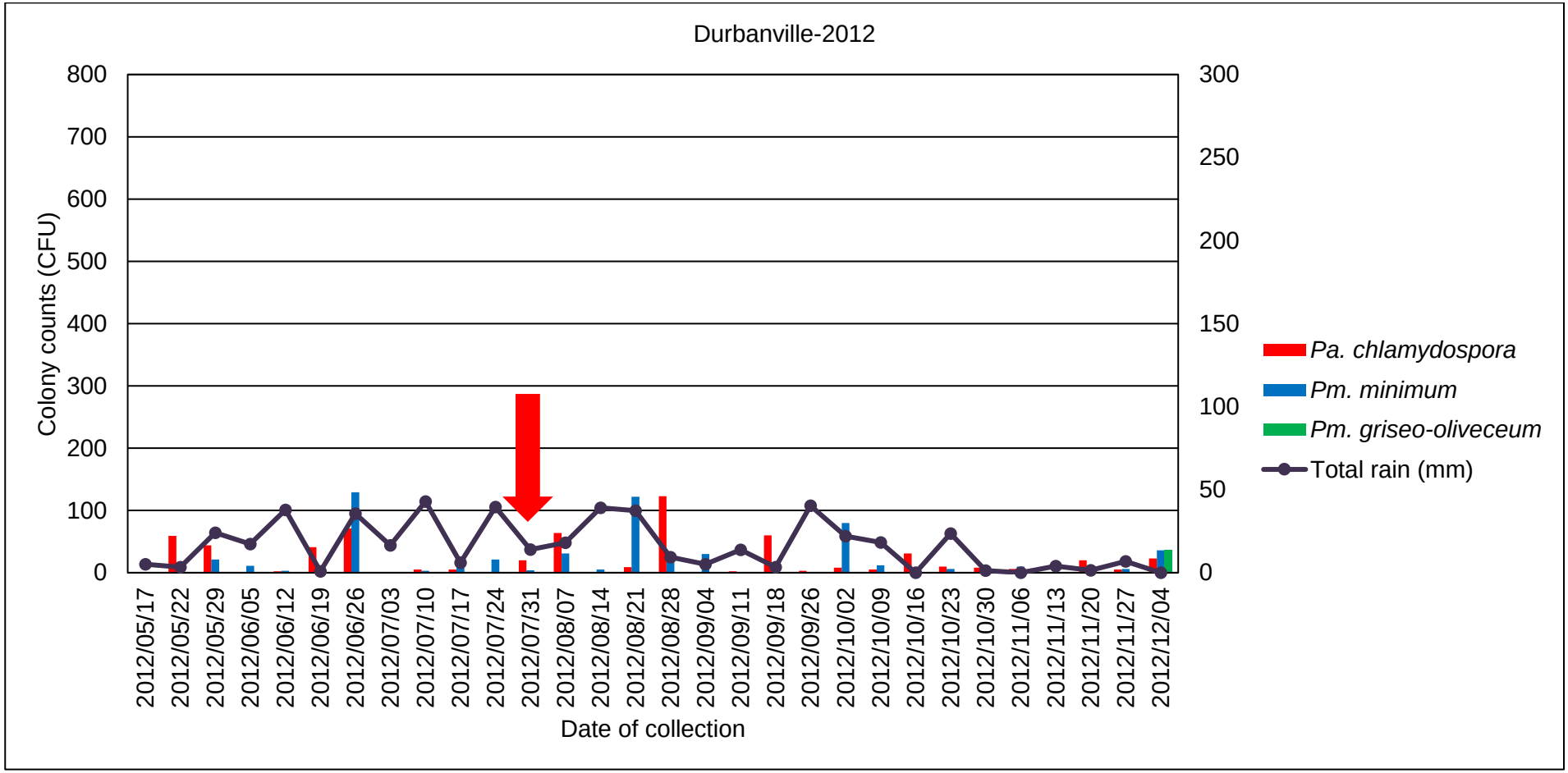

Figure 9. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which vines were pruned.

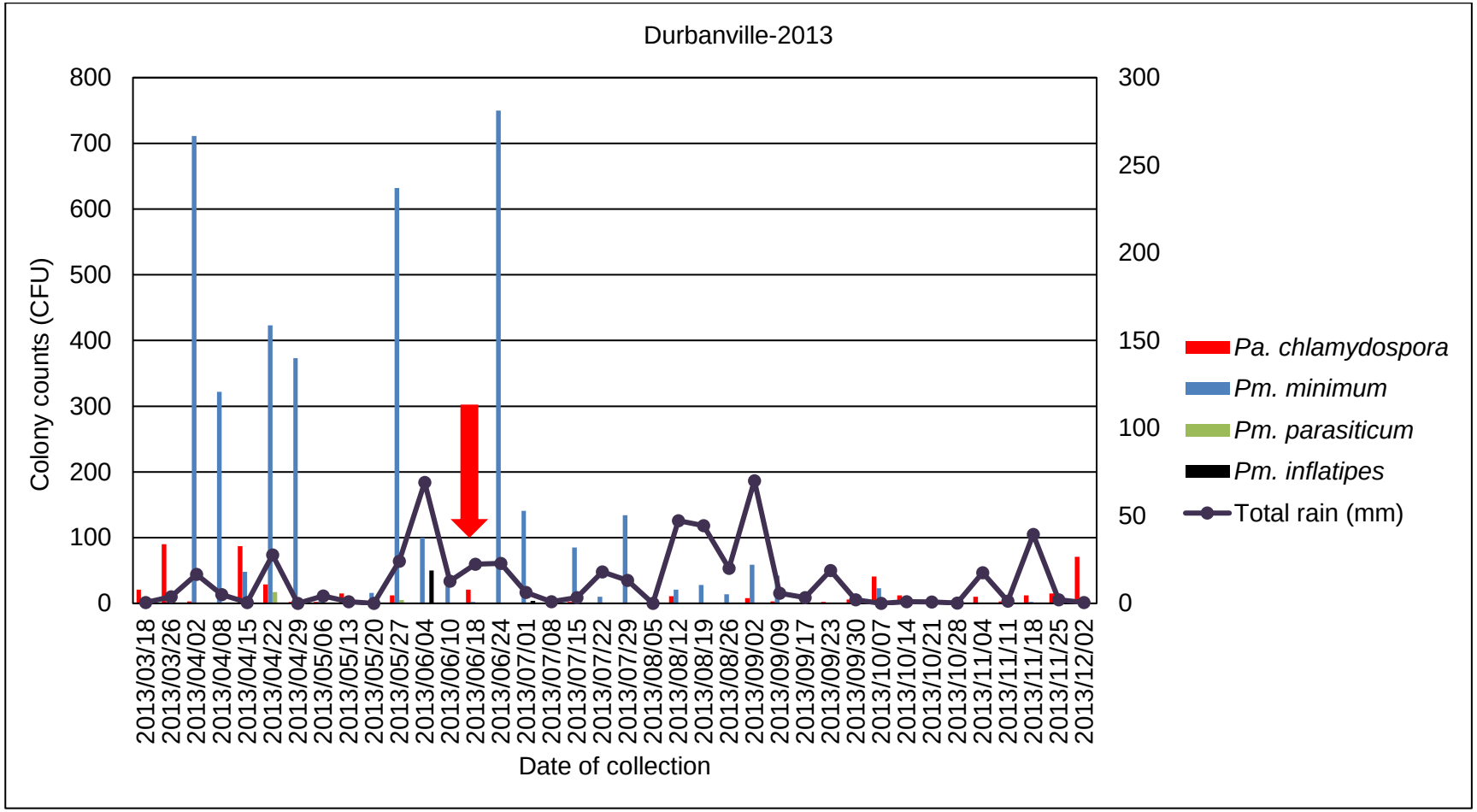

Figure 10. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2013. The red arrow (▬) indicates the date at which vines were pruned.

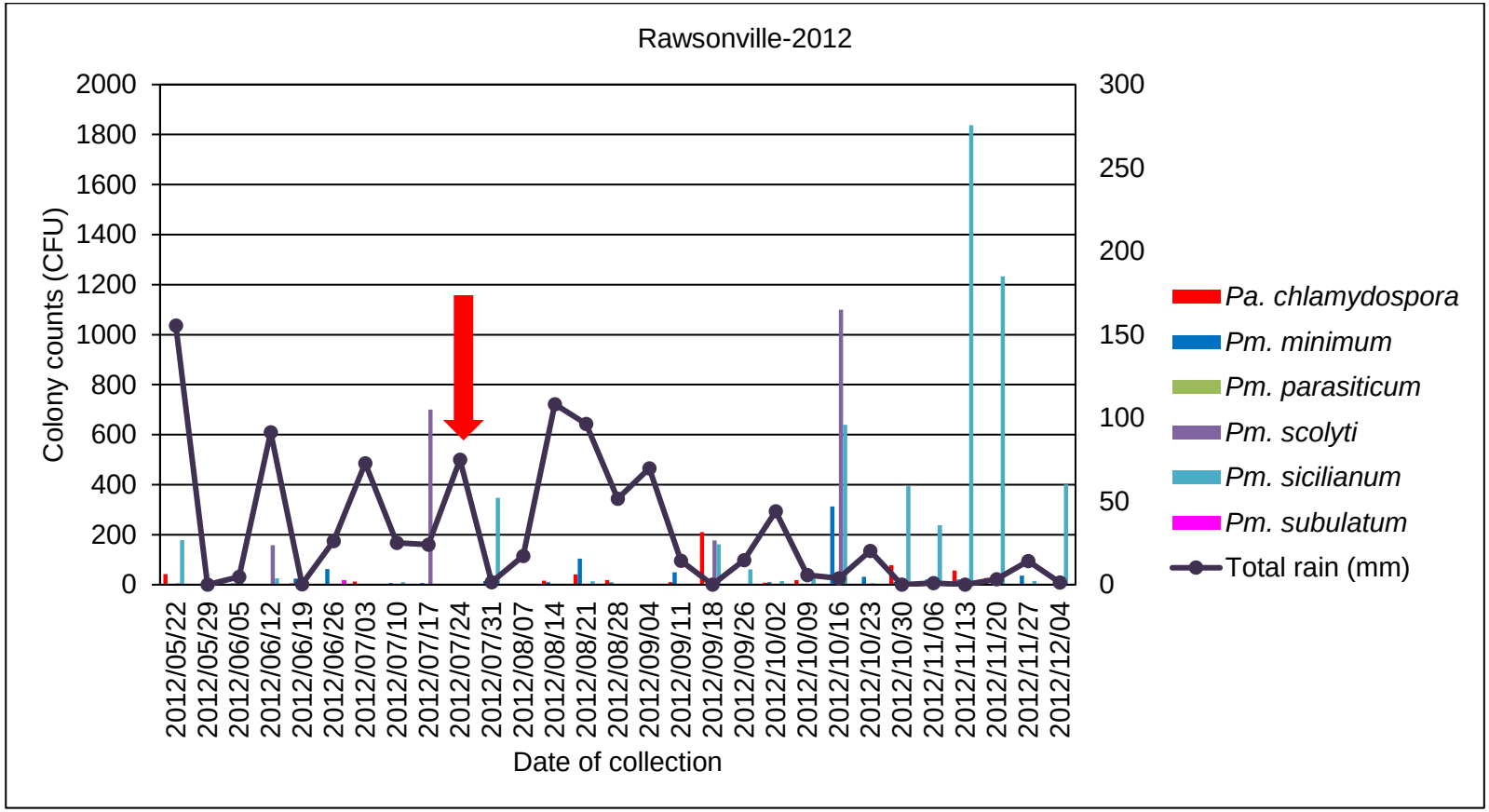

Figure 11. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which vines were pruned.

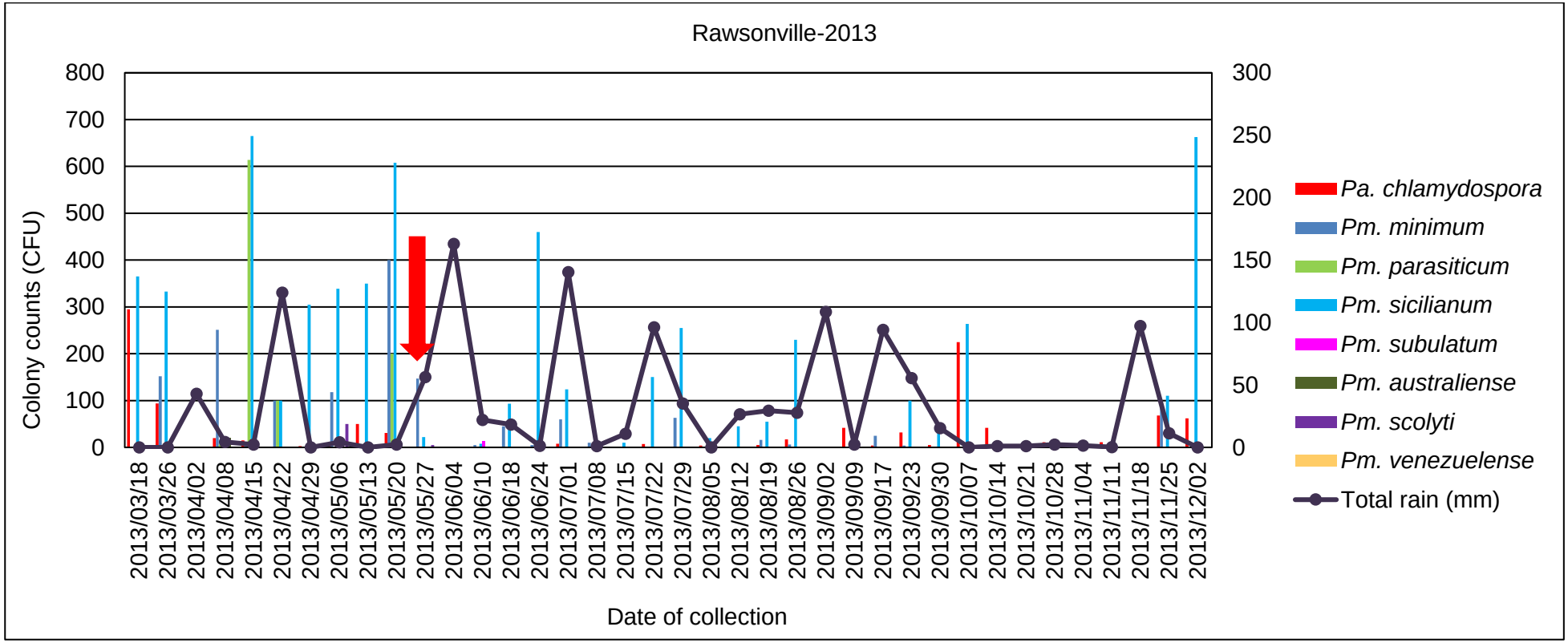

Figure 12. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2013. The red arrow (▬) indicates the date at which vines were pruned.

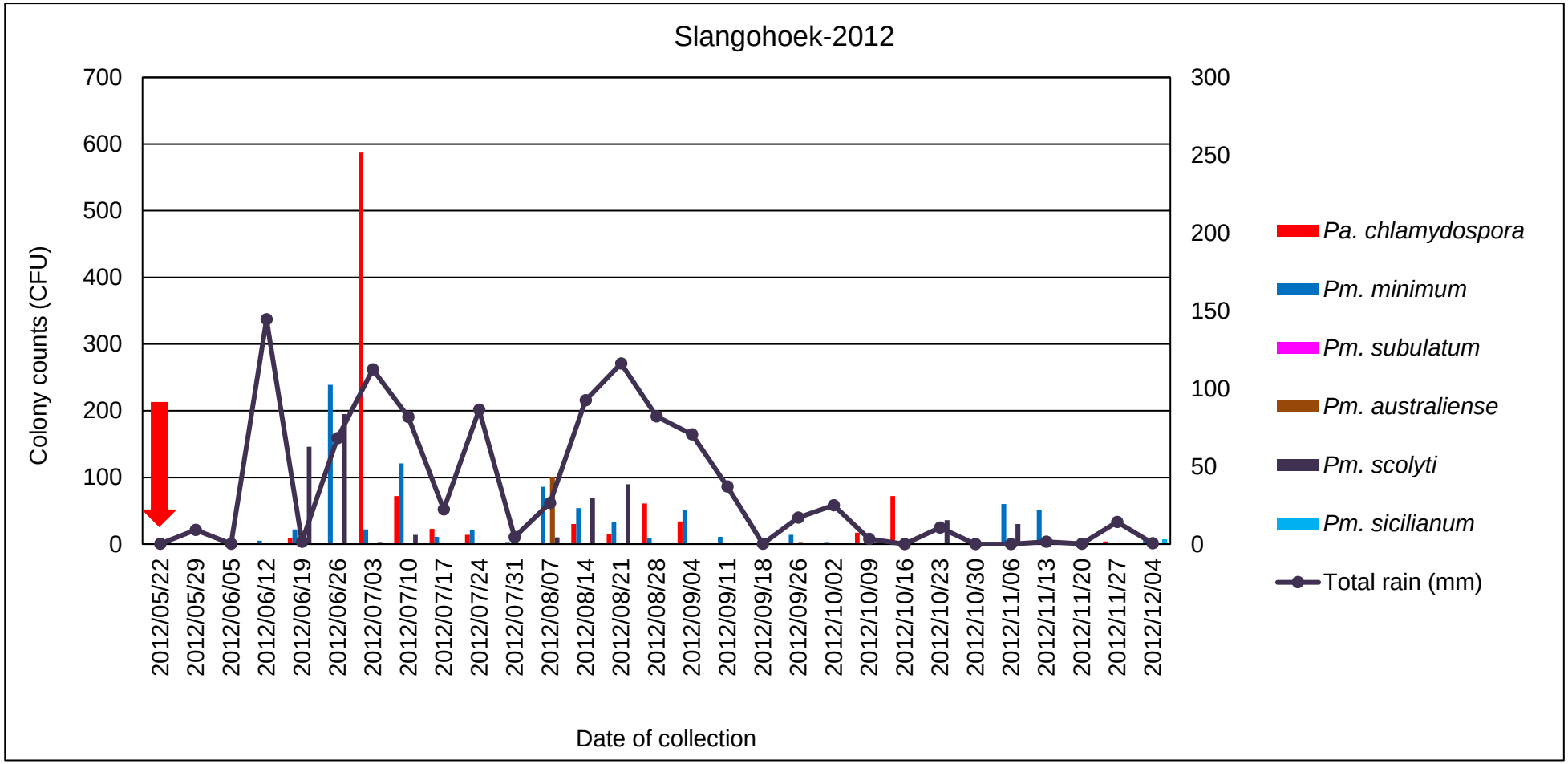

Figure 13. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which rootstock cuttings were harvested.

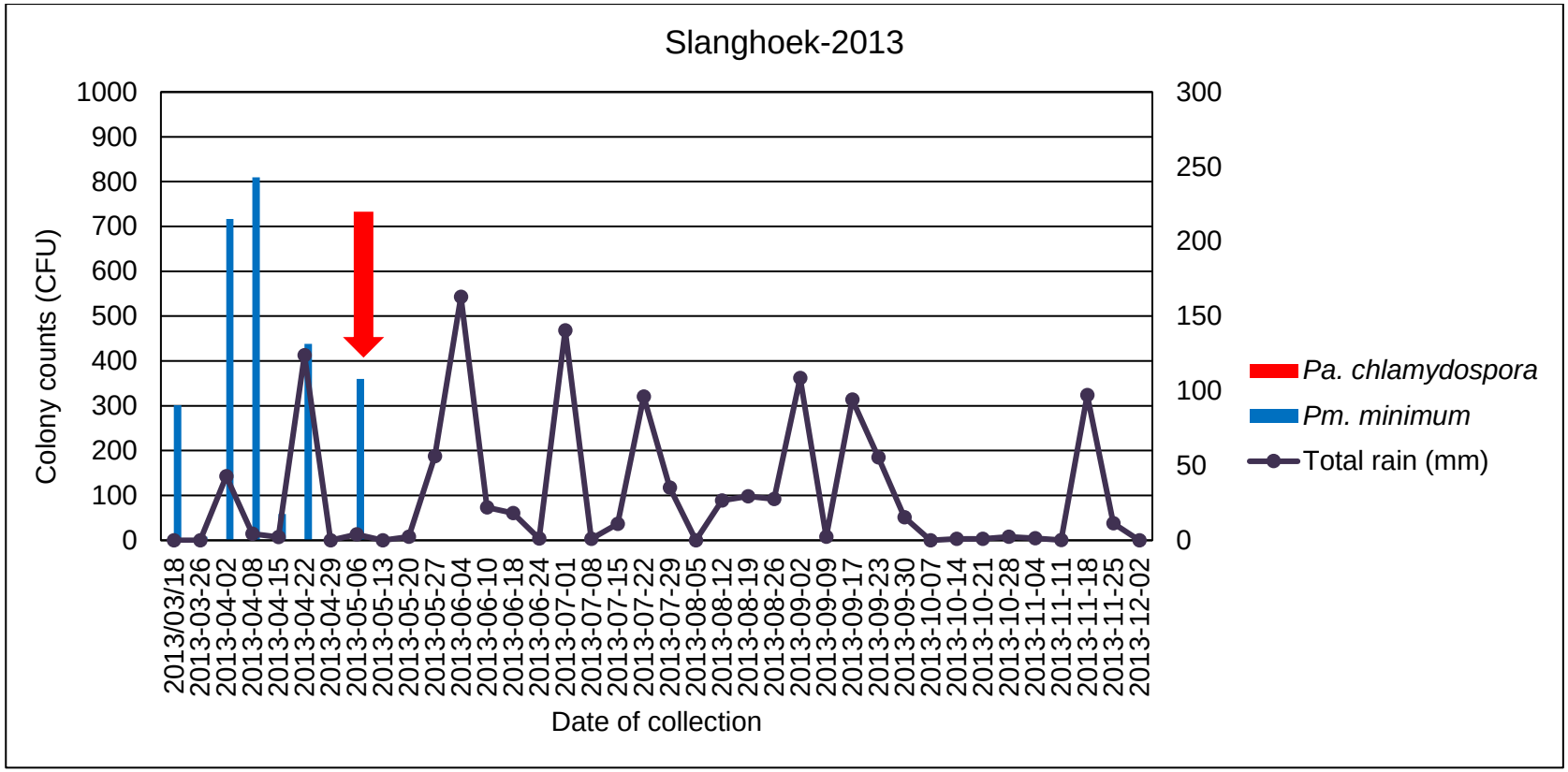

Figure 14. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which rootstock cuttings were harvested.

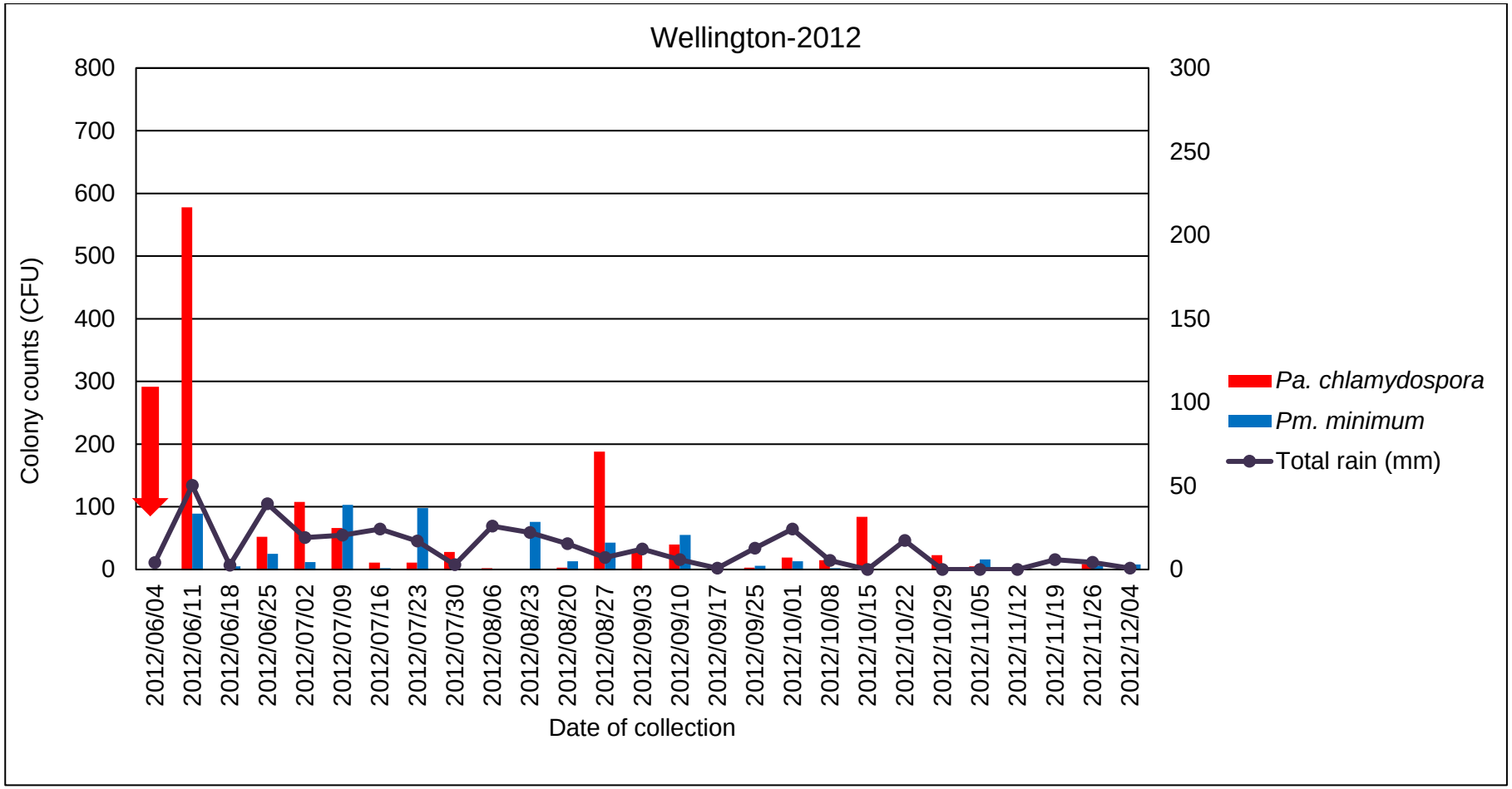

Figure 15. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which rootstock cuttings were harvested.

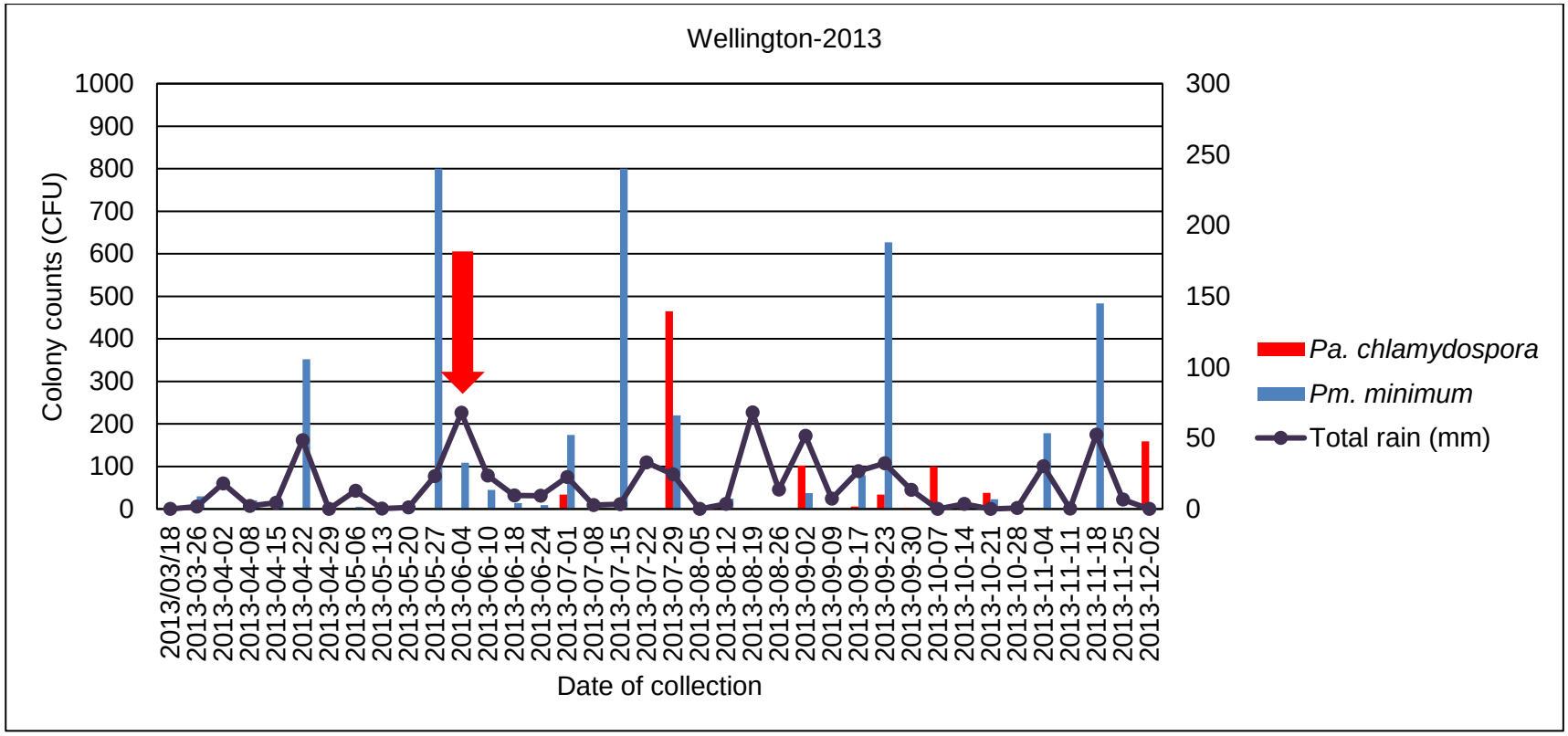

Figure 16. Total number of *Pa. chlamydospora* and *Phaeoacremonium* spp. CFU per week in 2012. The red arrow (▬) indicates the date at which rootstock cuttings were harvested.

CHAPTER 3

OCCURENCE OF FRUITING BODIES OF PETRI DISEASE PATHOGENS IN SOUTH AFRICAN VINEYARDS AND IN VITRO INDUCTION OF PHAEOACREMONIUM SEXUAL MORPHS

ABSTRACT

Pycnidia of *Phaeomoniella* (*Pa*.) *chlamydospora* and perithecia of *Phaeoacremonium* (*Pm*.) *minimum* have been found on diseased grapevines, but the occurrence of these fruiting structures have not been investigated on grapevines in South Africa. Six vineyards and two rootstock mother blocks in the Western Cape were surveyed between 2012 and 2014 for the presence of pycnidia of *Pa*. *chlamydospora* and perithecia of *Phaeoacremonium* species. Dead wood from diseased vines were collected for microscopic examination. Collected wood pieces were cut into smaller pieces and viewed under a dissecting microscope for the occurrence of fruiting bodies. Single conidial or ascospore isolates were made from pycnidia and perithecia resembling *Pa*. *chlamydospora* and *Phaeoacremonium* species, respectively. Species identity was determined by sequencing the internal transcribed spacers 1 and 2 and 5.8S rRNA gene for *Pa*. *chlamydospora* and the partial beta-tubulin gene for *Phaeoacremonium* species. *Phaeomoniella chlamydospora* pycnidia were found in all vineyards and rootstock mother blocks surveyed. Perithecia of *Pm. minimum* were only found in Stellenbosch P2 and B3, Rawsonville and a rootstock mother block in Slanghoek. Additionally, mating studies with isolates of two *Phaeoacremonium* species were conducted to induce the sexual state *in vitro*. Conidial suspensions of *Pm. australiense* and *Pm. scolyti* were placed onto sterilized dormant grapevine canes in Petri dishes containing water agar and placed at 25^{o}C. After seven and eleven months fertile perithecia of *Pm. australiense* and *Pm. scolyti* were observed, respectively. Crosses of both species corresponded to a heterothallic mating system. This study gives the first report of the occurrence of pycnidia of *Pa. chlamydospora* and perithecia of *Pm. minimum* in South African vineyards and rootstock mother blocks and also the first description of sexual morphs of *Pm. scolyti* and *Pm. australiense*.

INTRODUCTION

Petri disease, previously known as young vine decline or black goo, is a threat to newly planted vineyards worldwide (Ferreira *et al.*, 1994; Scheck *et al.*, 1998; Mugnai *et al.*, 1999). In severe cases up to 50% losses have been reported in vineyards in Australia (Pascoe and Cottral, 2000). *Phaeomoniella chlamydospora* W. Gams, Crous & M.J Wingf. & L. Mugnai together with *Phaeoacremonium* W. Gams, Crous & M.J. Wingf. species are pathogens causing Petri disease, with infection resulting in graft failure, stunted growth, dieback and gradual death in grapevines of one to five years of age (Crous *et al.*, 1996; Mugnai *et al.*, 1999; Mostert *et al.*, 2006a). The disease has been reported since 1912 in Italy, and was first reported in South African vineyards in 1994 (Ferreira *et al*., 1994). Although Petri disease has been intensively studied for over two decades, reports on the sexual and asexual fruiting bodies of its pathogens are still limited.

Phaeomoniella chlamydospora was initially classified in the *Phaeoacremonium* genus together with *Pm. minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous, however, morphological, cultural and molecular differences suggested the species to belong to a different genus (Dupont *et al*., 1998; Tegli, 2000). The genus *Phaeomoniella* was therefore, estabilised to accommodate *Pa. chlamydospora* (Crous and Gams, 2000). *Phaeomoniella chlamydospora* can form pycnidia *in vitro* (Crous and Gams, 2000; Pascoe and Cottral, 2000) and on grapevines in the field (Edwards and Pascoe, 2001; Edwards *et al.*, 2001). The field observations were on two vines in Australia where pycnidia of *Pa. chlamydospora* were found in either a cleft or deep crack in the cordons of the vines, one ten-year-old Pinot Noir vine and the other a seven-year-old Shiraz vine (Edwards and Pascoe, 2001; Edwards *et al.*, 2001). The asexual morph of both *Pa. chlamydospora* and *Phaeoacremonium* species are hyphomycetous with *Pa. chlamydospora* having only a pycnidial synanamorph (Crous and Gams, 2000; Crous *et al*., 1996).

The genus *Phaeoacremonium* was established in 1996, initially consisting of only six *Phaeoacremonium* species (Crous *et al*., 1996). To date 47 species have been identified within this genus, of which 28 species have been reported on grapevines. Thirteen of these species sexual morphs have been described (Mostert *et al.*, 2003; 2006a; Réblová and Mostert, 2007; Damm *et al.*, 2008; Réblová *et al*., 2015). Of these, eleven sexual morphs have been obtained with *in vitro* matings (Mostert *et al.*, 2006a). Recently the sexual morph state, *Togninia*, has been synonymized to *Phaeoacremonium* in line with the drive for a single nomenclature for fungi (Gramaje *et al.,* 2015). *Phaeoacremonium minimum* has a heterothallic mating system (Mostert *et al*., 2006a). In South Africa, *Pm. minimum* mating studies on grapevine canes *in vitro* showed a 1:1 mating type distribution which suggest random mating

to occur in vineyards (Mostert *et al*., 2003). Similar findings were reported in California and Spain (Rooney-Latham *et al*., 2005a; b; Gramaje *et al*., 2013).

Perithecia of some *Phaeoacremonium* species have been found in nature on grapevines, plums, beech, ash trees and deciduous trees, namely *Pm.* africanum (Damm, L. Mostert & Crous) D. Gramaje, L. Mostert & Crous, *Pm. griseo-olivaceum* (Damm, L. Mostert & Crous) D. Gramaje, L. Mostert & Crous, *Pm. vibratilis* (Fr.) D. Gramaje, L. Mostert & Crous, *Pm. minimum, Pm. viticola* J. Dupont*, Pm. fraxinopennsylvanicum, Pm. cinereum* Gramaje, Mohammadi, Banihash., Armengol & L. Mostert (Rooney-Latham *et al.*, 2005a; Eskalen *et al.*, 2005a; b; Réblová and Mostert, 2007; Damm *et al*., 2008; Réblová *et al*., 2015). The first report of perithecia of *Pm. minimum* in vineyards was made by Rooney-Latham *et al*. (2005b) in California and was also found on moist incubated grapevine wood in Australia and California (Pascoe *et al*., 2004; Rooney-Latham *et al*., 2005b). The perithecia of *Pm. minimum* developed superficially or partially embedded in wood tissue. It was mostly found in the decayed xylem tissues of old pruning wounds or inside deep cracks along the trunk, cordons and spurs (Rooney-Latham *et al.,* 2005a). Perithecia necks were mostly found facing towards the surface of the cordon or trunk, suggesting phototropic sensitivity (Rooney-Latham *et al*., 2005b).

Aerially dispersed conidia or ascospores of Petri disease pathogens are important sources of inoculum (Eskalen and Gubler, 2001). Spore trapping studies in vineyards have shown that *Pa. chlamydospora*, *Pm. minimum* and *Pm. fraxinopennsylvanicum* are aerially dispersed (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Eskalen *et al.*, 2005b). Eskalen and Gubler, (2001) correlated spore release of *Pa. chlamydospora* and *Pm. inflatipes* events with rainfall and concluded that these spores are dispersed as airborne inoculum following rainfall events in California. However, *Pm. minimum* was trapped throughout the trapping season, but was not correlated with rainfall (Eskalen and Gubler, 2001). Wet conditions are required to dissolve the asci sack layer and thereby releasing ascospores from the fruiting structures to infect fresh pruning wounds, as speculated by Hausner *et al*. (1992). Pruning wounds are the main ports at which Petri disease pathogens enter the vine (Adalat *et al.*, 2000; Larignon and Dubos, 2000; Eskalen and Gubler 2001; Rooney-Latham *et al.*, 2005b; Eskalen *et al.*, 2007; Serra *et al.*, 2008; Rolshausen *et al.*, 2010; Van Niekerk *et al.*, 2011). Pruning wounds remain susceptible to infection for up to four months depending on the time at which the wounds were made (Eskalen *et al.*, 2007; Van Niekerk *et al.*, 2011). In South Africa, high rainfall events are likely to coincide with winter pruning activities and thus increase chances of fresh wound infection, therefore exposing the wounds to high levels of aerial spore inoculum.

Perithecia form an important part of the disease cycle by producing and releasing ascospores during favorable conditions. A total of 15 Petri disease pathogens have been reported in South African vineyards as aerial inoculum in vineyards, however, the source of this inoculum is unknown. No Petri disease fruiting structures have been found in previous studies (Van Niekerk *et al*., 2010). Understanding the biology of these pathogens significantly contributes to development of appropriate control measures. The aims of this study were therefore to (i) survey vineyards in the Western Cape for the occurrence of fruiting bodies of Petri disease pathogens and (ii) conduct mating studies of *Pm. scolyti* and *Pm. australiense in vitro* to induce fruiting structures.

MATERIALS AND METHODS

Fruiting body survey

Wood collection sites

Wood samples were collected from six vineyards and two rootstock mother blocks in the Western Cape. These were in Paarl A, Paarl Z, Durbanville, Stellenbosch B3, Stellenbosch P2, Rawsonville, as well as two rootstocks mother blocks, one in Slanghoek and one in Wellington.

Wood collection

Trunks and cordons of old vines showing decline symptoms, old pruning wounds, or cracks and crevices were randomly sampled from vineyards and taken to the laboratory for microscopic examination during 2012, 2013 and 2014. Grapevine woods were cut into small pieces of 5–10 cm in length to expose wounds and crevices.

Microscopic examination

The cracks, crevices and surfaces of wood pieces were viewed under a dissecting microscope (Leica MZ95) for the presence of *Pa. chlamydospora* pycnidia and *Phaeoacremonium* perithecia. If found, the fruiting bodies were removed with a sterile scalpel and mounted on a microscope slide with a drop of dH_2O and examined with a Nikon Eclipse E600 compound microscope. Morphological features of pycnidia such as conidiophores, conidiogeneous cells and conidia were measured at 1000× magnification as described by Crous and Gams (2000). Morphological features of perithecia were compared to those described by Mostert *et al*. (2006a) with regards to the size in length and width of ascomata, neck, asci, ascospores and paraphyses. A total of 30 measurements at 1000× magnification of the asci, ascospores and 10 measurements of paraphyses were made. The 5^{th} and 95^{th} percentiles were defined for all the measurements. The content of the microscope slide was washed onto water agar (WA) with 1 ml of dH_2O spread out with a sterile glass rod and left slanted overnight in a laminar

flow cabinet. After 24 hours, the plates were viewed under a dissecting microscope and germinating single spores transferred to potato dextrose agar with chloramphenicol (PDA-C) plates.

Molecular identification

Species identification were done for cultures obtained from *Pa. chlamydospora* pycnidia as well as *Phaeoacremonium* perithecia.

DNA extraction

Mycelia of a 2 to 3 week-old culture were scraped into 2 ml Eppendorf tubes. An amount of 0.5 g glass beats were added to the tubes as well as 600 µl of CTAB extraction buffer (1 M Tris, pH 7.5; 5 M NaCl; 500 mM EDTA, pH 8.0) (Damm *et al*., 2008). The tubes were shaken for 6 min using a Mixer Mill type MM 301 before incubating in a waterbath for 30 min at 65^{o}C. After incubation 400 µl chloroform:isoamylalcohol (24:1) was added, followed by centrifugation at 13 100 rpm for 7 min. The watery supernatant was transferred to new Eppendorf tubes and 250 µl of 7.5 M ammonium acetate solution (pH 7) and 600 µl cold isopropanol were added. The samples were then incubated for 15 min at -20^{o}C. Thereafter the tubes were centrifuged at 13 100 rpm for 15 min. The supernatant was discarded and 1 ml cold ethanol was added and samples centrifuged at 13 100 rpm for 5 min and supernatant discarded. The pellet was dried on a bench for 24 hours where after the pellet was dissolved in 100 µl sterile distilled H_2O and stored at 4^{o}C.

PCR sequencing

Species identity was determined by sequencing the internal transcribed spacers 1 and 2 and 5.8S rRNA gene for *Pa*. *chlamydospora* (White *et al*., 1990) and T1 and Bt2b primers for *Phaeoacremonium* species (Glass and Donaldson, 1995; O' Donnel and Cigelink, 1997). The reactions were performed using 0.65 units Biotaq polymerase, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.25 pmol of each primers, 1 mg/ml bovine serum albumin (BSA) and 5 µl DNA in a total volume of 25 µl. The amplification was performed on a GeneAmp PCR System 2700 (Applied system Biosystems, Foster City California). The cycling conditions were 5 min at 96^{o}C, followed by 40 cycles of 30 s at 94^{o}C, 30s at 56^{o}C, 1 min and 30 s at 72^{o}C and a 7 min extension step at 72^{o}C to complete the reaction. Bands were separated by electrophoresis at 80 V on a 1% agarose gel stained with 0.5 µg/ml ethidium bromide in a 0.5 × TAE buffer (0.04 M Tris, 0.02 M glacial acetic acid and 1.27 mM, EDTA, pH 7.85). The gel was visualized under UV-light using a GenGenius Gel Documentation and Analysis System.

PCR products were purified according to the manufacturer's instructions using a commercial kit (Nucleopin Extract 2 in 1 Purification Kit, Machery-Nagel GmbH & Co.,

Germany). The Big dye system was used to perform DNA sequencing reactions. The reaction contained 5 × Buffer and 0.4 mM of each primer in a total reaction volume of 10 µl. The PCR was performed using the following conditions; an initial denaturing step at 95°C for 60 seconds, followed by 30 cycles of 10 s at 95°C, 5 s at 50°C and 4 min at 60°C and a final step at 30 s at 60°C. Fragments were separated on an ABI 3130*xl* genetic analyzer. Sequence data for both directions were analyzed using Geneious 3. 5. 6 (2007, Biomatters Ltd., Auckland, New Zealand) and edited manually using Sequence alignment editor v2.0a11 (Rambaut, 2002). Species identification was done using the megablast function of the NCBI's GenBank nucleotide database (www.ncbi.nlm.nih.gov).

Pathogenicity study conducted with conidia from pycnidia of Phaeomoniella chlamydospora

A 9-year-old Cabernet Sauvignon vineyard in Stellenbosch was used to test pathogenicity of conidia from *Pa. chlamydospora* pycnidia. Spore suspensions were inoculated on fresh pruning wounds (3 bud, 1-year-old spur pruned canes). One pruning wound on 20 vines was each inoculated with 100 µl conidial suspensions (10^3 spores/100 µl) and one pruning wound each on another 20 vines with 100 µl sterile water as negative control. The trial was evaluated after five months.

Mating studies

Phaeoacremonium isolates used

Phaeoacremonium australiense and *Pm. scolyti* were tested *in vitro* for their ability to form perithecia. These isolates were obtained from vineyards and alternative host surveys (isolates from wounds), as well as from vineyard spore trapping trials (Table 1). Twenty-six isolates were used for *Pm. australiense* and 20 for *Pm. scolyti.*

Preparation of spore suspensions

Single-spored conidial isolates were grown on PDA-C plates for two weeks at 25°C. A sterile glass rod was used to dislodge conidia from the surface of mycelia, and the suspension was prepared in 5 ml sterile dH_2O (Mostert *et al*., 2003). A 100 µl aliquot spore suspension of each isolate was pipetted onto autoclaved grapevine canes placed onto water agar plates. Each plate received 100 µl aliquots of each of two isolates and control pairings received 200 µl of the same isolate. Isolates were mated with each other in all possible combinations. The plates were sealed with parafilm and incubated at 25°C with approximately 10 hrs light and 14 hrs dark. Plates were closely monitored for the formation of perithecia at 2 week intervals.

Characterization of the perithecia

Methods characterizing perithecia were as described by Mostert *et al.* (2006a). Successful crosses were expected to form perithecia that produce a large number of ascospores which

readily germinate on culture media. Perithecia were mounted individually on microscopic slides and viewed with a light microscope. Thirty measurements were made to determine the size of the asci, ascospores and paraphyses. Ten measurements were made to determine perithecial size.

Statistical analyses

Random mating in populations was investigated with a chi-square (χ^2) test on mating type ratios with the null hypothesis that each of either *Pm. australiense* or *Pm. scolyti* isolates did not significantly differ from a 1:1 mating type ratio. This was obtained by comparing the number of actual and expected mating types of *Pm. australiense* and *Pm. scolyti.* The degrees of freedom were used in comparison with the tabulated probability to determine if the chi-square value was significant at 5% probability. The test was analyzed using excel statistical tool (www.xlstat.com).

RESULTS

Identification of species

Sequence identification

Cultures obtained from pycnidia were identified as *Phaeomoniella chlamydospora* (Baloyi *et al*., 2016). A megablast search on GenBank showed it to be 100% similar to *Pa. chlamydospora*, GenBank FJ530942 (Identities 590/590 (100%), gaps 0/590 (0%)). *Phaeoacremonium minimum* was identified from cultures made from perithecia. Sequence similarity of 100% was found with *Pm. minimum* sequences JQ691670.1, HQ605018.1, HQ605014.1 (Identities 647/647 (100%), gaps 0/647 (0%)).

Description of fruiting structures found in nature

Phaeomoniella chlamydospora *pycnidia*

Pycnidia were found on the bark surfaces in aggregations of 1-50. Conidiomata were black, pycnidial, globose, up to 175 µm in diameter. Conidiophores were pale-brown, 1-multiseptate, 8−24×1.5−3 µm, conidiogeneous cells (n=20) 1.5−3×1−2 µm and conidia were hyaline, oblong-ellipsoidal to obovate, (n=40) 1.5−2.5×1−1.5 µm. The morphological features of the pycnidia found in South African vineyards were similar to those described by Crous and Gams (2000).

Phaeoacremonium minima *perithecia*

Perithecia were globose to subglobose, black, and often embedded in the wood tissue but also present on the surface of the wood. The length of the necks was 250−300×47.5−55 µm. The asci were hyaline, 16−25×3.5−5 µm. Ascospores were hyaline, ellipsoid, 5−6×1.5−2 µm.

Measurements of *Pm. minimum* perithecia found in South African vineyards were similar to those described by Mostert *et al*. (2006a).

Fruiting body survey

Pycnidia

During the 2012, 2013 and 2014 field sampling surveys, a total of 1989 wood pieces with exposed wounds and cracks were visualized under a microscope for the occurrence of fruiting bodies. *Phaeomoniella chlamydospora* pycnidia were found in 54 (2.7%) of these samples, representing all vineyards surveyed (Table 2). The highest occurrence of *Pa. chlamydospora* pycnidia was observed in Rawsonville with *Pa. chlamydospora* pycnidia present in 28 (2.7%) samples. This was also the vineyard from which the most samples were taken. The least positive samples for *Pa. chlamydospora* pycnidia were found in Paarl A and Z, however, these were also the vineyards less sampled from. Pycnidia were commonly found in cracks, on wood surfaces and pruning wounds and rarely formed in crevices (Table 3).

Pathogenicity study conducted with of conidia from Phaeomoniella chlamydospora *pycnidia*

Discoloration in the form of brown-black streaks with a mean length of 87.6 mm (std. dev. 12.08 mm) was observed within the inoculated pruning wounds after five months of inoculation. These vascular streaks are identical to the ones associated with Petri disease. No streaks were observed in any of the control wounds ($P<0.0001$). *Phaeomoniella chlamydospora* was re-isolated from the entire length of the discoloured tissues from 90% of the inoculated wounds. No *Pa. chlamydospora* or any other fungal trunk pathogens were isolated from wounds treated with water.

Perithecia

Perithecia of *Pm. minimum* were reported in only 42 (2.1%) of the 1989 samples (Table 2). Perithecia was only found in Stellenbosch P2 and Stellenbosch B3, Rawsonville and Slanghoek. A total of 31 of the 42 samples were from the Slanghoek rootstock mother block. While Rawsonville had many *Pa. chlamydospora* pycnidia only one sample had perithecia. No perithecia were found in Paarl A, Paarl Z, Durbanville and Wellington. Perithecia found in South African vineyards were similar to *in vitro* perithecia dimensions described by Rooney-Latham *et al*. (2005a). Perithecia were found inside crevices and cracks and on pruning wound. These structures commonly occurred in cracks and crevices and occurred the least on pruning wounds (Table 3).

Mating studies

Mature perithecia of *Pm. australiense* were observed after 7 months. Of the 26 single conidial strains 17 belonged to one mating type and four belonged to the opposite mating type (Fig.

2). Isolate FH-P742 was the most fertile and was compatible with most isolates. The mating distribution was at a ratio of 17:4. There was no self-mating observed, suggesting the species to have a heterothallic mating system. Statistical analyses revealed a significantly skewed distribution of the two mating types (Table 4). All isolates of *Pm. australiense* obtained from Stellenbosch (quince), Slanghoek (vineyard spore traps), Franschhoek (persimmons), Durbanville (apples) and Vredendal (roses) were of the same mating type. Strains from Rawsonville (vineyard spore traps) were of the opposite mating type. *Phaeoacremonium australiense* obtained from spore traps in the Rawsonville vineyard was the most fertile, and positively mated with strains from Stellenbosch (quince, plums and roses), Slanghoek (vineyard spore trap), Franschhoek (persimmons) and also strains from the same block in Rawsonville.

Matings of *Phaeoacremonium scolyti* isolates formed protoperithecia after 4 months and these developed into mature perithecia after 11 months. Of the 20 isolates of *Pm. scolyti* used in this study, seven belonged to one mating type and six to the other and there was no successful crossing of seven isolates used (Fig. 3). There was no statistical difference between the distribution of the two mating types, therefore, agreeing with a 1:1 Mendelian segregation of mating types (Table 4). *Phaeoacremonium scolyti* has a heterothallic mating system. Both mating types occurred in each of the areas where isolates originated from, namely Rawsonville, Slanghoek and Wellington (Fig. 3).

Phaeoacremonium australiense sexual morph

Perithecia black, mostly aggregated and sometimes solitary, mostly subepidermal also on the surface of the epidermis; subglobose, (250–)273–395(–450) µm diam, and (200–)236–425(–500) µm tall. Wall consisting of two regions of *textura angularis:* outer region dark-brown, cells smaller and rounded than the inner layer, cells thick (individual cells not visible further outward), 20–54 µm thick; inner region hyaline (centrum) to pale brown, 6–12 cells and 12–35 µm thick. *Perithecial necks* black, 1–2 per perithecium, straight to curved, 250–260 (av. 132) µm long, 50–190 (av. 102) µm wide at base, and 20–50 (av. 25) µm wide at the apex, neck sometimes divided into two at the apex. *Paraphyses* hyaline, septate, cylindrical narrowing towards the tip, 35–110(av. 70) µm long, 3–4(av. 3) µm wide at the base, persistent. Asci appearing spicate when mature, hyaline, clavate, with bluntly rounded apices, tapering towards truncate base (15–)17–22×4–5.5(av. 19×5) µm. *Ascospores* aseptate, hyaline, oblong-ellipsoidal to allantoid with rounded ends, sometimes containing small guttules at the ends, biseriate 4–6×1–2 µm (Fig. 4).

PREM herbarium specimen number 61423.

Notes. Has a heterothallic mating system. Perithecia formation of *Pm. australiense* took 7 months. On average the necks of *Pm. australiense* are shorter as compared to most *Phaeoacremonium* perithecia, except *Pm. inconspicua*.

Phaeoacremonium scolyti sexual morph

Perithecia formed on wood and in agar, single but mostly in clusters, globose to subglobose, (260–)301–664(–675) µm tall and (250–) 268–578(–675) µm diam. Wall consist of two cell regions, with more small and round cells from outside and more oval towards the inner part of perithecium. Cells are dark brown from the outer part and becomes light brown on the inner region and the centrum is hyaline. The inner layer is 11–24 µm thick and the outer layer is 15–18 µm thick. The outer layer consists of 3–5 cells and the inner layer consist of 5–7 cells. *Perithecial necks* 1–2(–3) per perithecium or one neck that branch into two near apex or on neck base. Measure 250–1050 µm long, 60–210 µm wide at base, 20–115 µm wide at apex. *Paraphyses* hyaline, septate, cylindrical, narrowing and thread-like towards the tip, 42–82 (av. 58.5) µm long, 2–3 (av. 2.3) µm wide at the base and 2−4 (av. 2.8) µm at the apex, persistent. *Asci* appears spicate, hyaline, truncate at the apex, (16–)17.5–20.6(–21) µm. *Ascogenous hyphae* hyaline, branched, smooth-walled, remnant bases 24–69×1.5–2 µm. *Ascospores* aseptate, hyaline, allantoid with rounded ends, containing small guttules at the ends, biseriate (4–)4.5–6×1–2 µm (Fig. 5).

PREM herbarium specimen number 61422.

Notes. Has a heterothallic mating system. Formation of *Pm. scolyti* perithecia took 11 months. The perithecia length and diameter measurements of *Pm. scolyti* are higher than all recorded perithecia in this genus, except for *Pm. vibralitis* (Table 5).

DISCUSSION

This study reports on the occurrence of pycnidia of *Pa. chlamydospora* and perithecia of *Pm. minimum* on field grapevines in South Africa. Additionally, the sexual morphs of *Pm. australiense* and *Pm. scolyti* were induced *in vitro* with the mating of conidial isolates. Significant advances were made in the knowledge regarding the fruiting structures of Petri disease pathogens.

Pycnidia of *Pa. chlamydospora* was previously only reported in cracks and clefts of grapevines in Australia (Edwards *et al*., 2001). The present study further reports the structures to also form on wood surfaces and pruning wounds. Pycnidia of *Pa. chlamydospora* were found on vines in all eight vineyards investigated in the Western Cape Province of South Africa. They were, however, found at low frequencies with only 2.7% of samples investigated revealing pycnidia. The pycnidia are small and were sometimes found mixed with pycnidia of

other fungal species. The finding of pycnidia of *Pa. chlamydospora* elaborates the importance of them being part of the source of inoculum within vineyards. This study reports the capability of *Pa. chlamydospora* conidiospores from the pycnidia to infect vine pruning wounds, which was not reported previously (Edwards *et al*., 2001). The presence of pycnidia in rootstock mother blocks shows the likelihood of the mother plant to get infected by aerial spores from the pycnidia, and for mycelia or conidia to be transported into new shoots that are harvested for propagation purposes. Infected rootstock material has been considered the main source of spread of *Pa. chlamydospora* (Halleen *et al*., 2003). This was the reason for the development of molecular detection tools for rapid identification of *Pa. chlamydospora*, to prevent the use of infected planting materials in newly established vineyards (Ridgeway *et al*., 2002; 2005; Retief *et al*., 2006).

Low genetic variation has been reported among isolates of *Pa. chlamydospora*, therefore suggesting asexual reproduction to be the main mode of reproduction among South African isolates (Mostert *et al*., 2006b). Similar studies conducted in Spain revealed significant genetic variation among their isolates (Tegli, 2000) which Mostert *et al.* (2006b) suggested to be due to the parasexuality rather than sexual reproduction. There has not been any report on the sexual morph of *Pa. chlamydospora* reported anywhere in the world thus far. The source of conidia in the field is either from pycnidia or mycelia (Edwards *et al*., 2001).

Perithecia of *Pm. minimum* were discovered on dead parts of vines collected from four of the vineyards investigated in the Western Cape Province of South Africa. This is the second report, after California, of finding perithecia on grapevines in nature (Rooney-Latham *et al.*, 2005b). Perithecia with long necks developed on wood surfaces, in cracks and crevices examined. The perithecia were found in decayed xylem tissues of old pruning wounds or inside deep cracks along trunks, cordons, and spurs as previously suggested by Rooney-Latham *et al.* (2005b). The likelihood of finding them in deep cracks and crevices highlights the importance of suitable humid conditions for them to form as previously suggested by Rooney-Latham *et al*. (2005b), and this is supported by their development during high moist incubation (Pascoe *et al*., 2004; Rooney-Latham *et al*., 2005a). Comparatively few structures were found considering the amount of aerial spores that has been trapped in vineyards in the spore trapping study conducted in these same vineyards (Chapter 2). This could, however, be due to the fact that they form in deep cracks and crevices which were difficult to observe. In Rawsonville, more pycnidia were found as compared to perithecia. However, spore trapping studies conducted in 2012 and 2013 showed a higher occurrence of *Pm. minimum* aerial spore release than *Pa. chlamydospora*. In Slanghoek, *Pa. chlamydospora* was the predominant species, however, pycnidia of this species was found at low incidences. *Phaeoacremonium minimum* was trapped as the second highest species and this is comparable to the high

number of perithecia found during the surveys. This suggests the possibility of aerial spores trapped in Chapter 2, as ascospores.

Phaeoacremonium australiense had a skewed mating type distribution. Reports suggest a skewed mating type distribution to be because of genetic drift or selection mechanism occurring in that population and further indicate clonal reproduction to occur (Elliot, 1994). One mating type of *Pm. australiense* occurred in Stellenbosch, Constantia, Franschhoek, Durbanville and Rawsonville. The opposite mating type was only in Rawsonville. The difficulty of finding the opposite mating types within the same region may be the reason we did not find the sexual morph of this species in nature. Strains from different hosts were compatible, and emphasize the risk of establishing alternative woody hosts in the vicinity of vineyards as they may serve as inoculum reservoirs. More especially because *Pm. australiense* has been reported on *Prunus salicina* and *V. vinifera* in South Africa. This suggestion is also emphasized by previous studies which reported perithecia of *Pm. viticola* and *Pm. fraxinopennsylvanicum* on vines and ash trees established in close proximity. *Phaeoacremonium australiense* has only been isolated from *P. salicina* in South Africa (Damm *et al*., 2008), and from *V. vinifera* in Australia (Mostert *et al*., 2005) and Uruguay (Abreo *et al*., 2011).

Phaeoacremonium scolyti has a heterothallic mating system. The two mating types occurred in Rawsonville, Slanghoek and Wellington, which suggests the likelihood of sexual reproduction to occur. For the fact that both *Pm. australiense* and *Pm. scolyti* are heterothallic, occurrence of both mating types in one vineyard, or on one vine indicate the high chances in which perithecia could form. However, perithecia of these species have not been found in nature during the surveys. Furthermore, the occurrence of *Pm. australiense* aerial spore inoculum in Chapter 2 suggest that it could be conidiospores rather than ascospores. Nonetheless, movement of planting materials consisting of different mating types between areas could in future result on the overlap of the two mating types required for sexual reproduction to occur.

Formation of these perithecia requires specific conditions depending on the *Phaeoacremonium* species (Rooney-Latham *et al.*, 2005b). Mostert *et al.* (2003) observed *Pm. minimum* perithecia to form within 2–3 weeks in continuous light, whereas Rooney-Latham *et al.* (2005a) reported the formation after 4–5 weeks in 12 hr photoperiod assays *in vitro*. This emphasizes the importance of light in perithecia formation, although Hausner *et al*. (1992), contrarily found perithecia formation of *Pm. fraxinopennsylvanicum* not to be stimulated by light. In previous studies, authors reported the presence of both mating types of *Pm. minimum* on the same vine thus, indicating the high chances at which sexual reproduction

structures could form, and that the sexual state could readily form in the field under the suitable environmental conditions (Pascoe *et al.*, 2004; Mostert *et al.*, 2006a).

To date, perithecia of 13 *Phaeoacremonium* sexual morphs have been described including *Pm. australiense* and *Pm. scolyti* reported in the current study. The two sexual morphs described as in this study both have a heterothallic mating system. The perithecia took seven and 11 months to form for *Pm. australiense* and *Pm. scolyti*, respectively. Out of the 18 *Phaeoacremonium* species reported in South Africa, ten of them have had their sexual morph described, namely *Pm. africanum*, *Pm. austroafricanum*, *Pm. australiense, Pm. griseo-olivaceum*, *Pm. krajdenii*, *Pm. minimum*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. viticola* and *Pm. fraxinopennsylvanicum*.

The occurrence of *Pa. chlamydospora* and *Pm. minimum* fruiting structures suggest them to be a source of inoculum of aerial spores trapped within vineyards and rootstock mother blocks. Although spores trapped in Chapter 2 were not identified as ascospores or conidia, results on high genetic variation in *Pm. minima* isolates and low genetic variation in *Pa. chlamydospora* isolates (Tegli, 2000; Mostert *et al*., 2005) does support these findings. Currently, there is no chemical control of Petri disease that will prevent the formation of fruiting structures. This is possibly an area that could be explored in future. Castillo-Pando *et al*. (1997) showed the potential of some fungicides to be used as spray treatments to inhibit the viability of *Phomopsis viticola* pycnidia on grapevine canes. Such strategies together with sanitary practices such as composting infected pruning debris at temperatures of 75^{o}C could result in reduced inoculum from infected pruning debris (Lecomte *et al*., 2006).

Further studies on the mating type distribution and population genetics studies of *Phaeoacremonium* species are required to investigate the risk of recombination occurring in South African vineyards. Further studies are required to survey for perithecia of other *Phaeoacremonium* species which have not been found in South African vineyards.

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Table 1. Collection details of *Phaeoacremonium* isolates used for mating studies.

***Phaeoacremonium* species**	**Accession number**	**Location**	**Host/ Origin**	**Collector**
Pm. australiense	PMM 2277a	Stellenbosch	*Cydonia oblonga*	P. Moyo
	PMM 1842b	Stellenbosch	*Rosa* sp.	P. Moyo
	FH-P742d	Rawsonville	Spore trap in vineyard	M.A. Baloyi
	PMM 2277d	Stellenbosch	*Cydonia oblonga*	P. Moyo
	PMM 989b	Vredendal	*Rosa* sp.	P. Moyo
	FH-P742c	Rawsonville	Spore trap in vineyard	M.A. Baloyi
	FH-P314a	Rawsonville	Spore trap in vineyard	M.A. Baloyi
	PMM 989c	Vredendal	*Rosa* sp.	P. Moyo
	PMM 735c	Franschhoek	*Diospyros virginiana*	P. Moyo
	PMM 1826	Stellenbosch	*Vitis vinifera*	P. Moyo
	PMM 2231	Durbanville	*Malus domestica*	P. Moyo
	PMM 735a	Franschhoek	*Diospyros virginiana*	P. Moyo
	FH-P312a	Slanghoek	Spore trap in vineyard	M.A. Baloyi
	PMM 2231b	Durbanville	*Malus domestica*	P. Moyo
	PMM 758c	Stellenbosch	*Prunus domestica*	P. Moyo
	PMM 758e	Stellenbosch	*Prunus domestica*	P. Moyo
	PMM 758d	Stellenbosch	*Prunus domestica*	P. Moyo
	FH-P185c	Slanghoek	Spore trap in vineyard	P. Moyo
	PMM 758a	Stellenbosch	*Prunus domestica*	P. Moyo

	FH-P185b	Slanghoek	Spore trap in vineyard	M.A. Baloyi
	FH-P185a	Slanghoek	Spore trap in vineyard	M.A. Baloyi
	PMM 991a	Vredendal	*Rosa* sp.	P. Moyo
	PMM 1911a	Constancia	*Psidium guajava*	P. Moyo
	PMM 1911c	Constancia	*Psidium guajava*	P. Moyo
	PMM 1911	Constancia	*Psidium guajava*	P. Moyo
	FH-P314b	Slanghoek	Spore trap in vineyard	M.A. Baloyi
Pm. scolyti	FH-PS 2	Wellington	*Vitis vinifera*	F. Halleen
	FH-P30	Rawsonville	Spore trap in vineyard	M.A Baloyi
	FH-P31	Rawsonville	Spore trap in vineyard	M.A Baloyi
	FH-P267	Rawsonville	Spore trap in vineyard	M.A Baloyi
	FH-PS 54	Wellington	*Vitis vinifera*	F. Halleen
	FH-PS 58	Wellington	*Vitis vinifera*	F. Halleen
	FH-PS 59	Wellington	*Vitis vinifera*	F. Halleen
	FH-P67	Rawsonville	Spore trap in vineyard	M.A Baloyi
	FH-PS 72	Wellington	*Vitis vinifera*	F. Halleen
	FH-P85	Slanghoek	Spore trap in vineyard	M.A Baloyi
	FH-P86	Slanghoek	Spore trap in vineyard	M.A Baloyi
	FH-PS 91	Wellington	*Vitis vinifera*	F. Halleen
	FH-P122	Rawsonville	Spore trap in vineyard	M.A Baloyi
	FH-PS 163	Wellington	*Vitis vinifera*	F. Halleen
	FH-PS 157	Wellington	*Vitis vinifera*	F. Halleen

FH-P183	Slanghoek	Spore trap in vineyard	M.A Baloyi
FH-P188	Slanghoek	Spore trap in vineyard	M.A Baloyi
FH-P201	Rawsonville	Spore trap in vineyard	M.A Baloyi
FH-P208	Slanghoek	Spore trap in vineyard	M.A Baloyi
FH-P239	Slanghoek	Spore trap in vineyard	M.A Baloyi

Table 2. Total number of infected grapevine wood sampled collected between 2012 and 2014, and microscopically assesed for presence of pycnidia and perithecia of *Phaeomoniella chlamydospora* and *Phaeoacremonium* species, respectively.

Vineyards sampled	Total number of samples [a]	Number of samples with *Pa. chlamydospora* pycnidia	Number of samples with *Pm. minimum* perithecia
Stellenbosch P2	196	11	2
Stellenbosch B3	116	6	8
Paarl A	64	1	0
Paarl Z	20	1	0
Durbanville	230	3	0
Rawsonville	1023	28	1
Slanghoek	110	2	31
Wellington	230	2	0
Total	1989	54	42

[a] Grapevine wood piece cut into 5−10 cm in length.

Table 3. Summary of pycnidia of *Phaeomoniella chlamydospora* and perithecia of *Phaeoacremonium minimum* found in six vineyards and two rootstock mother blocks surveyed during 2012, 2013 and 2014.

Fruiting body	Vineyard	Date of collection	Total number of samples with fruiting bodies [a]	Locations where fruiting bodies were found				Number of fruiting bodies
				Wood surface	Crevices	Cracks	On pruning wound	
Pycnidia	**Stellenbosch - Block P2**	2012/07/23	1	1	-	-	-	-
		2014/06/06	8	1	5	1	1	3-50
		2014/08/07	2	2	-	-	-	2-5
	Stellenbosch - Block B3	2012/11/16	1	-	-	-	1	4
		2014/03/01	5	2	-	3	-	2-11
	Paarl - Block A	2012/05/24	1	-	-	1	-	-
	Paarl - Block Z	Unknown	nd [b]	1	-	-	-	3
	Durbanville - Block D	2012/06/18	-	3	-	-	-	3-5
	Rawsonville - Block K	2012/06/18	14	2	-	6	6	-
		2013/04/29	6	1	-	-	5	-
		2014/08/07	8	2	-	5	1	2-18
	Slanghoek - Block H	2013/10/07	-	2	-	-	-	-
	Wellington - Block O	Unknown	nd [b]	-	-	2	-	-
Perithecia								
	Stellenbosch - Block P2	2014/06/06	2	-	2	-	-	2-3 [a]
	Stellenbosch - Block B3	2012/06/18	1	-	-	1	-	nd [b]
		2014/03/01	7	2	2	3	-	2-56 [a]
	Rawsonville - Block K	2012/07/23	1	-	-	1	-	nd [b]
	Slanghoek - Block H	2012/06/18	9	1	1	7	-	nd [b]
		2012/07/23	3	1	2	-	-	nd [b]
		2012/10/22	4	3	1	-	-	nd [b]
		2013/06/18	15	5	5	3	2	nd [b]

[a] A sample was a 5−10 cm wood piece.

[b] nd = not determined.

Table 4. Distribution of mating types among conidial strains of two *Phaeoacremonium* species after being subjected to *in vitro* mating studies.

Species	Number of conidial strains	Mating type distribution	*P*-value [a]
Phaeoacremonium australiense	26	17:4	0.005
Phaeoacremonium scolyti	20	7:6	0.429

[a] Probability value calculated with a proportion test under the null hypothesis of a 1:1 ratio.

Table 5. Summary of perithecial, ascus and ascospore dimensions of 13 *Phaeoacremonium* sexual morphs.

Species	Perithecial dimensions (μm)	Neck length (μm)	Ascus dimensions (μm)	Ascospore shape	Ascospore dimensions (μm)
Pm. africana[a]	(270–)315–440(–460) tall (215–)270–395(–440) diam	550–1000	(16–)18–23(–24)×4–4.5	ellipsoidal to subcylindrical	(2.5–)3.5–4.5(–5.5)×1.5–2(–2.5)
Pm. argentinensis[b]	(142–)144–245 tall (113–)115–171 diam	390–1470	(12–)13–18(–20)×(3–)3.5–4	oblong-ellipsoidal to cylindrical	3–4×1–1.5
Pm. australiense[c]	(200–)236–425(–500) tall (250–)273–395(–450) diam	250-260	(15–)17–22×4–5.5	oblong-ellipsoidal to allantoid	4–6×1–2
Pm. austroafricana[b]	(88–)92–193(–201) tall (64–)66–175(–181) diam	490–1470	(16–)17–21–(–22)×4–5	reniform to oblong-ellipsoidal	3–5×1.5–2

Pm. fraxinopennsylvanica[b]	(181–)187–258(–270) tall (181–)185–252(–270) diam	390–1125	15–20×4(–5)	oblong-ellipsoidal to slightly curved	3.5–5×1
Pm. inconspicua[b]	142–196 tall 74–167 diam	83–113	20–30(–32)×6–8	allantoid or oblong-ellipsoidal	7–10×1.5–2
Pm. krajdenii[b]	(202–)203–284(–287) tall (197–)203–275 diam	220–440	(16–)18–22(–23)×4–5	allantoid to oblong-ellipsoidal	4–5(–6)×1–1.5
Pm. minima[b]	(200–)285–325(–400) tall (160–)250–285(–420) diam	800–1800	(17–)19–20(–27)×4–5	oblong-ellipsoidal or allantoid	(4–)5(–6.5)×1–2
Pm. novae-zealandiae[b]	(147–)158–196 tall (142–)144–177(–181) diam	220–1250	(15–)17–23×4–5	oblong-ellipsoidal	3–4×1–2
Pm. parasitica[b]	(216–)229–379(–409) tall (181–)199–345–(368) diam	215–810	(12–)14–18×(3.5–)4–5	allantoid	4–5×1–1.5
Pm. rubrigena[b]	(225–)234–354(–362) tall (172–)198–459(–470) diam	515–1300	(12–)16–19×4–4.5	allantoid or cylindrical	4–6×1–1.5
Pm. scolyti[c]	(260–)301–664(–675) tall (250–)268–578(–675) µm diam	250-1050	(16–)17.5–20.6(–21)	allantoid	(4–)4.5–6×1–2
Pm. viticola[b]	(211–)222–324(–328) tall 225–362(–377) diam	360–1030	(17–)18–24(–26)×(3–)3.5–4(–5)	oblong-ellipsoidal to reniform	3–5×1.5–2(–2.5)

Pm. vibratilis[d]	340–500(–700) tall 335–500(–670) diam	No neck	(20–)23–27×5–6	suballantoid to oblong	(5–)5.5–6(–7)×1–1.5

[a] Damm *et al*., 2008.

[b] Mostert *et al*., 2006a.

[c] Sexual morph described in this current study.

[d] Réblova and Mostert, 2007.

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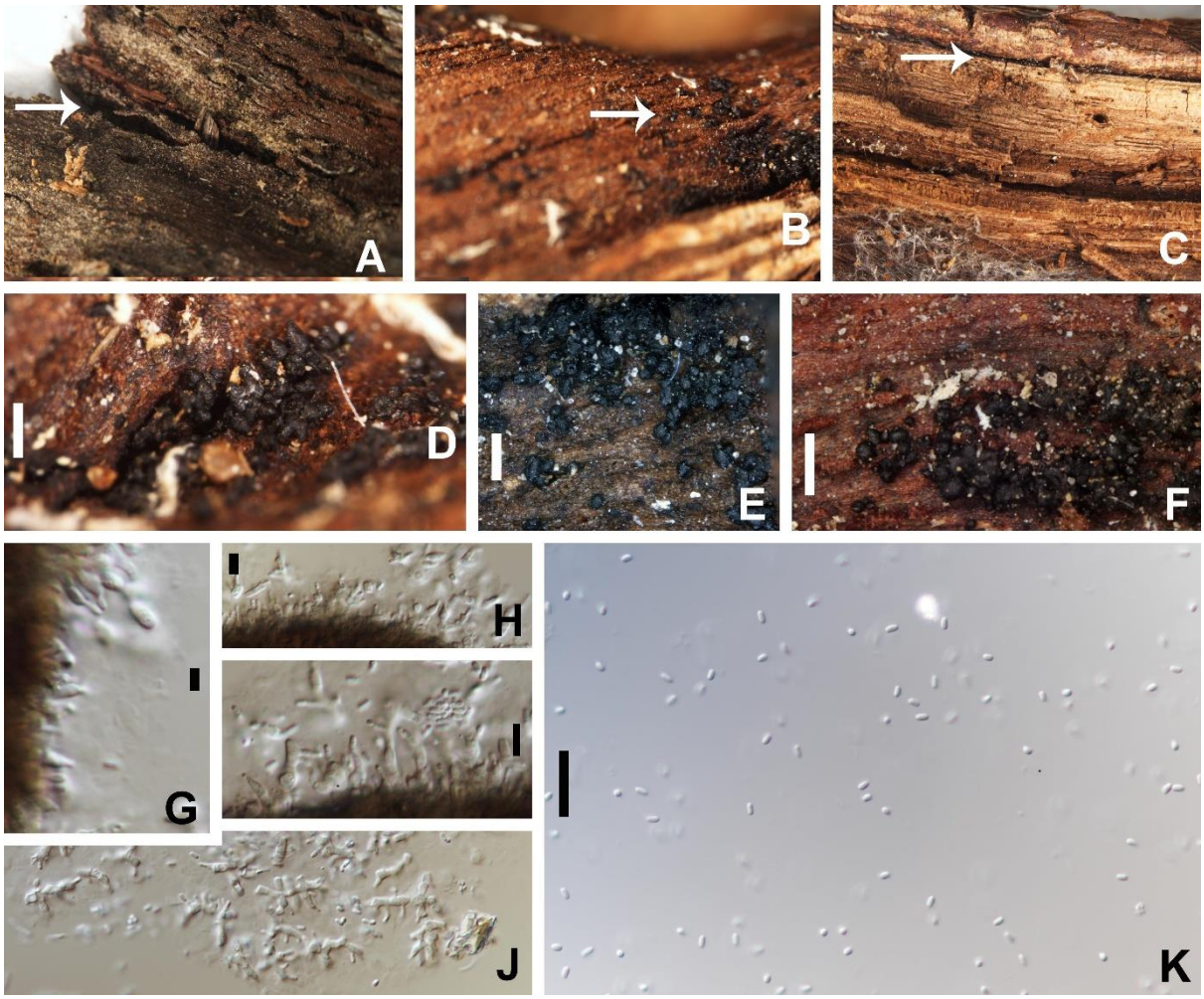

Figure 1. *Phaeomoniella chlamydospora* pycnidia on infected dead wood of *Vitis vinifera*. A−C. Cracks with pycnidia indicated by arrows. D−F. Pycnidia on the wood surface. G-J. Conidiophores. K. Conidia. Scale bar: D-F = 500 µm; G, I-K = 10 µm; H = 5 µm. Scale bar at G applies for I-J.

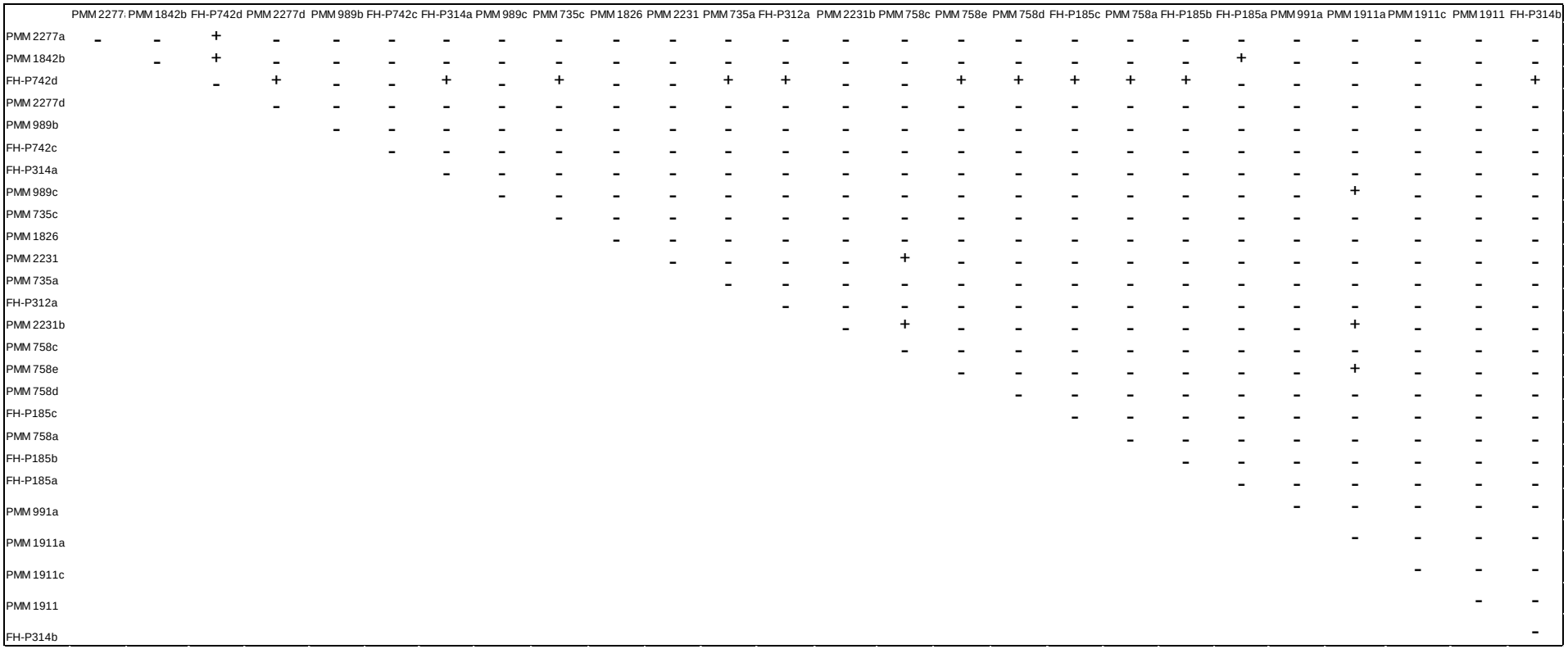

Figure 2. Schematic representation of the mating study with single conidial isolates of *Phaeoacremonium australiense.* A (-) means no perithecia formed, while (+) means perithecia formed and exuded fertile ascospores.

	FH-PS2	FH-P30	FH-P31	FH-P267	FH-PS54	FH-PS58	FH-PS59	FH-P67	FH-PS72	FH-P85	FH-P86	FH-PS91	FH-P122	FH-PS163	FH-PS157	FH-P183	FH-P188	FH-P201	FH-P208	FH-P239
FH-PS2	-	+	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-
FH-P30		-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FH-P31			-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
FH-P267				-		-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
FH-PS54					-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+
FH-PS58						-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
FH-PS59							-	-	-	-	-	-	-	-	-	-	-	+	-	-
FH-P67								-	-	-	-	-	-	-	-	-	-	-	-	-
FH-PS72									-	-	-	-	-	-	-	-	-	-	-	-
FH-P85										-	-	-	-	-	-	-	-	-	-	-
FH-P86											-	-	-	-	-	-	-	-	-	-
FH-PS91												-	-	-	-	-	-	-	-	-
FH-P122													-	-	-	-	-	-	-	-
FH-PS163														-	-	-	-	-	-	-
FH-PS157															-	-	-	-	-	-
FH-P183																-	-	-	-	-
FH-P188																	-	-	-	-
FH-P201																		-	-	-
FH-P208																			-	-
FH-P239																				-

Figure 3. Schematic representation of the mating study with single conidial isolates of *Phaeoacremonium scolyti.* A (-) means no perithecia formed, while (+) means perithecia formed and exuded fertile ascospores.

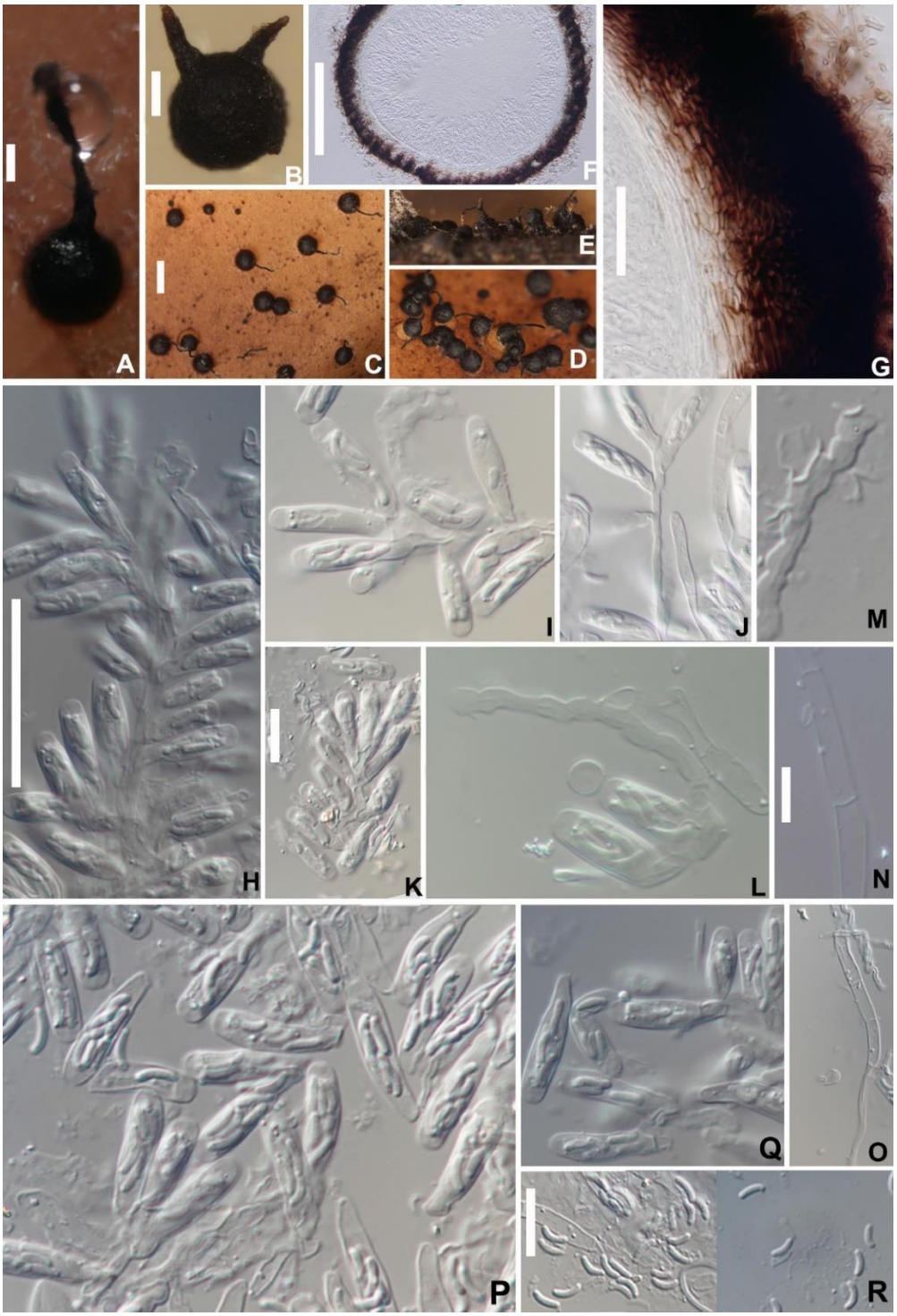

Figure 4. *Phaeoacremonium australiense* sexual morph formed *in vitro*. A-D. Perithecia on agar. E. Perithecia on canes of *Vitis vinifera*. F-G. Longitudinal section through perithecium. H-L. Ascogenous hyphae with asci attached. M. Remnant bases. N. Paraphyses. O. Paraphyses becoming thread like towards the tips. P-Q. Asci. R. Ascospores. Scale bars: A-B = 100 µm; C = 500 µm; G, K, N, O, P, R = 10 µm; H = 20 µm. Scale bar for C applies to D-E, bar for H applies for I, J, Q.

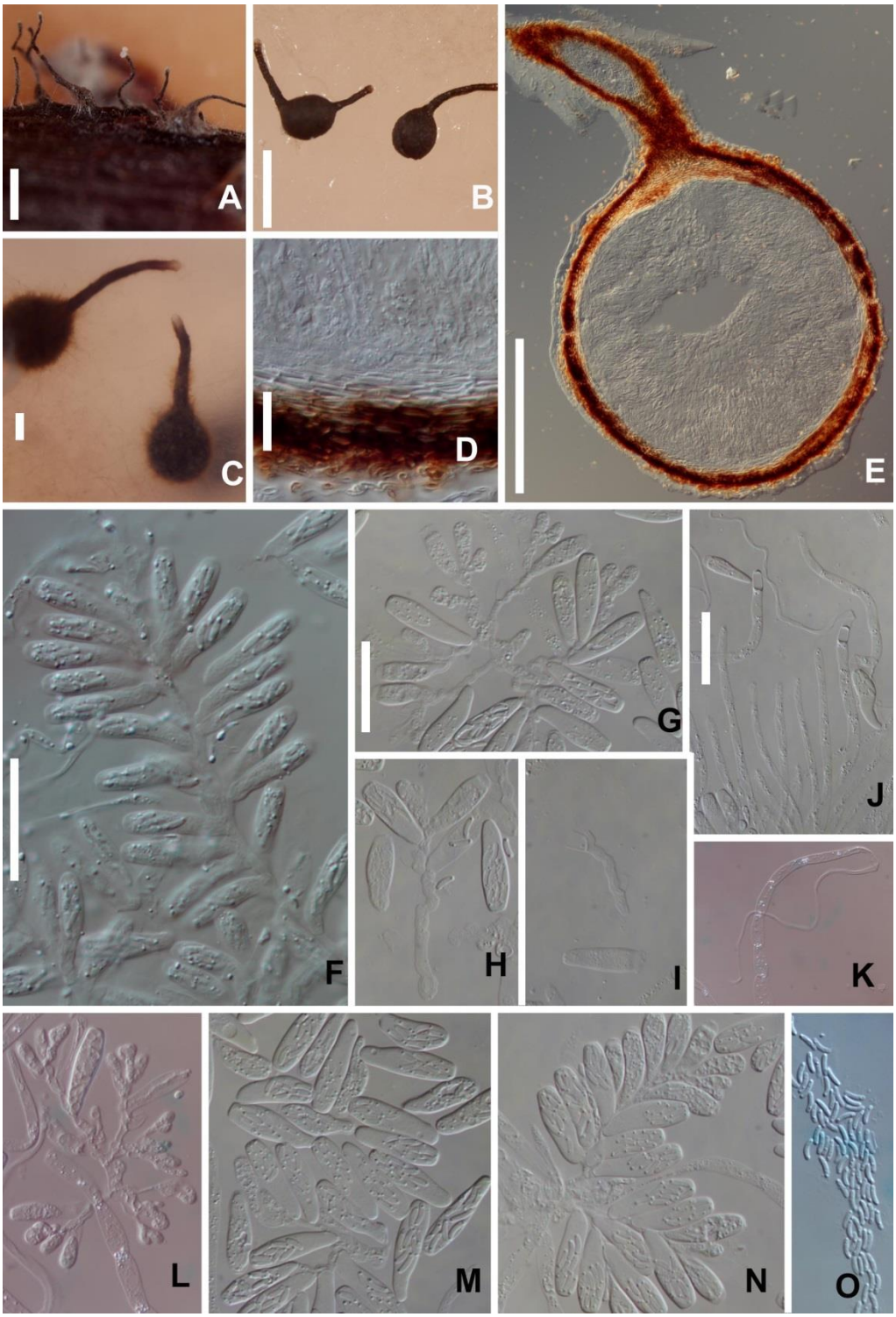

Figure 5. *Phaeoacremonium scolyti* sexual morph formed *in vitro*. A. Perithecia on canes of *Vitis vinifera*. B-C. Perithecia on agar. D. Perithecial wall. E. Longitudinal section through perithecia F-H. Ascogenous hyphae with asci attached. I. Remnant base. J-K. Paraphyses becoming thread-like towards the tips. L. Immature asci. M-N. Asci. O. Ascospores. Scale bars: A, C = 100 µm; B = 500 µm; D, E, G, K = 10 µm; F = 20 µm. Scale bar for G applies to H, I, L, M, N, O; bar for J applies for K.

CHAPTER 4

PATHOGENICITY OF PHAEOACREMONIUM SPECIES ASSESSED ON CABERNET SAUVIGNON VINES

ABSTRACT

Thirteen species of *Phaeoacremonium* have been isolated from grapevines in South Africa of which only six species have been confirmed as pathogens through pathogenicity tests conducted on field-grown grapevines. The aim of the current study was to determine the pathogenic status of four species of *Phaeoacremonium* found for the first time on grapevines during this study (Chapter 2), namely *Pm. australiense*, *Pm. griseo-olivaceum*, *Pm. scolyti* and *Pm. sicilianum* as well as six species previously found in South Africa of which the pathogenic status have not been determined, namely *Pm. austroafricanum*, *Pm. alvesii*, *Pm. fraxinopennsylvanicum*, *Pm. griseorubrum*, *Pm. iranianum* and *Pm. pruniculum*. *Phaeoacremonium parasiticum* was used as the positive control and sterile water as the negative control. Up to three isolates were used per species depending on availability of isolates. Fresh pruning wounds of a nine-year-old Cabernet Sauvignon vineyard at Nietvoorbij, Stellenbosch, South Africa were inoculated with 10^4 conidia/ml of each fungus per wound. Inoculated pruning wounds were removed after 18 months, cut longitudinally and lesion lengths measured. Samples were surface sterilized and wood tissue plated onto potato dextrose agar amended with chloramphenicol to determine re-isolation percentages. All inoculated isolates successfully colonized pruning wounds and proved to be pathogenic causing lesions significantly different from the negative control. All isolates were re-isolated at varying percentages ranging from 28.57% to 85.71%. *Phaeoacremonium griseo-olivaceum* STE-U 7859 produced the longest lesion (79.53 mm) and *Pm. iranianum* STE-U 6998 the shortest (62.00 mm). No significant differences in lesion lengths were observed between the species inoculated. There were also no significant differences between isolates of the same species, except in *Pm. pruniculum* where STE-U 5968 produced significantly longer lesions (77.27 mm) than STE-U 7857 (62.26 mm). The study confirmed the capability of all tested *Phaeoacremonium* species to infect grapevine pruning wounds and cause lesions. The study confirmed the importance of pruning wounds as ports of entry by these pathogens.

INTRODUCTION

Of the 47 *Phaeoacremonium* species known, 28 species have been isolated from grapevines (Gramaje *et al*., 2015; Ariyawansa *et al*., 2015). Species of *Phaeoacremonium* are known inhabitors of a wide range of woody hosts and have also been isolated from humans (Ajello *et al.*, 1974; Crous *et al.*, 1996; Mostert *et al.*, 2005; Mostert *et al.*, 2006a; Damm *et al.*, 2008; Gramaje *et al.*, 2012). Some of the species associated with grapevine have also been found from *Actinidia* spp., *Acquilaria* spp., *Cupressus* spp.,

Cydonia spp., *Dodonaea* spp., *Fraxinus* spp., *Malus* spp., *Nectandra* spp., *Prunus* spp., *Pyrus* spp., *Phoenix* spp., *Olea* spp., and *Quercus* spp. (Crous *et al*., 1996; Mostert *et al*., 2005; 2006b; Eskalen *et al*., 2005a; b; Damm *et al*., 2008; Prodi *et al*., 2008; Aroca and Raposo, 2009; Cloete *et al*., 2011; Gramaje *et al*., 2012; Lynch *et al*., 2013; Mohammadi, 2014; Sami *et al*., 2014; Carlucci *et al*., 2015).

Phaeomoniella (Pa.) chlamydospora (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams together with *Phaeoacremonium (Pm.)* spp. cause Petri disease (Scheck *et al.*, 1998; Mugnai *et al.*, 1999; Mostert *et al.*, 2006a) commonly found in young vines of approximately 1-5 years of age (Del Río *et al.*, 2002; Halleen and Groenewald, 2005). Species of *Phaeoacremonium* are well adapted endophytes, capable of becoming pathogenic during stressful conditions of the vine (Morton, 1995; Scheck *et al.*, 1998; Ferreira *et al.*, 1999). External decline symptoms include leave necrosis, shoot dieback, shortened internodes or in extreme cases the death of the vine. Internal symptoms include brown streaks in xylem tissue of a longitudinally cut infected vine, or the black/brown gummosis exuded on transversally cut wounds (Ferreira *et al*., 1999; Scheck *et al.*, 1998; Mugnai *et al.*, 1999; Groenewald *et al.*, 2001; Fourie and Halleen, 2004). *Phaeoacremonium* species are also associated with esca of grapevines. Esca is caused by the Petri disease pathogens together with wood rotting fungi of the Hymenochaetales (Mugnai *et al*., 1999; Cloete *et al*., 2015). The brown streaking found in both Petri diseased and esca grapevines is due to the accumulation of phenolic compounds and tyloses in infected tissue and result in clogging of the xylem vessels which impairs proper translocation of water and nutrients (Ferreira *et al*., 1999; Edwards *et al.*, 2007).

Petri disease pathogens infect grapevines primarily through susceptible pruning wounds (Larignon and Dubos, 2000; Eskalen *et al*., 2007; Van Niekerk *et al*., 2011). Sucker wounds have also been shown to be susceptible (Makatini, 2014). Fruiting bodies formed within vineyards release aerial spores during and following rainfall periods (Edwards and Pascoe, 2001; Rooney-Latham *et al.*, 2005a; Eskalen *et al.*, 2005a; b). Spore trapping studies conducted during the current study (Chapter 2) have shown the presence of high aerial spore numbers during pruning periods, which extends for the entire period of wound susceptibility including the desuckering periods. Previous studies conducted in France and California also showed available aerial spores during the pruning periods (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). Winter pruning activities in the Western Cape occurs in June, July and August, whereas desuckering activities which is the removal of unwanted shoots from vine trunk, cordon and spurs occurs in spring and early summer (September, October and November). Multiple wounds made on each vine during these periods emphasize the high risk of wounds being infected. Other modes of pathogen spread in vineyards have recently been reported namely, arthropods which vector Petri disease pathogens to pruning wounds (Moyo *et al.*, 2014), infected pruning shears (Agustí-

Brisach *et al.*, 2015) and the use of infected planting material (Halleen *et al.*, 2003; Fourie and Halleen, 2004).

Susceptibility of grapevines is affected by physiological wood response to wounding (Biggs, 1989; Biggs and Peterson, 1990) and the cultivar (Feliciano *et al*., 2004). After pruning, the tissue begins to desiccate and parenchyma cells accumulate phenolic compounds (Biggs, 1989). As a result, xylem vessels can be clogged with tyloses and/or polysaccharide gums. Vines produce phenolic compounds; exude sap flow or dries up at the wounded area as a natural defense mechanism which is dependent on temperature (Shain, 1979; Munkvold and Marois, 1995; Van Niekerk *et al.*, 2011). In California, pruning wound susceptibility was shown to last for up to 16 weeks, which would enable even slow growing pathogens to colonize and infect the wounds before it heals (Eskalen *et al.*, 2007). In California vines pruned early in the dormant season have a longer period of susceptibility to *Eutypa lata* than those pruned later in the dormant season (Petzoldt *et al.,* 1981; Munkvold and Marois, 1995; Chapuis *et al.,* 1998). In France, Larignon and Dubos (2000) showed wounds to be susceptible to *Pa. chlamydospora* infections for 9-12 weeks and 7-9 weeks to *Pm. minimum* in the early dormant season and for only two weeks in late winter pruning periods. Furthermore, Munkvold and Marois (1995) found that vines pruned towards the end of dormancy release exudes that contain carbohydrates, amino acids and organic acids which promoted rapid growth of microflora that competes with trunk disease pathogens. Other factors such as vine training method as in the case of bilateral cordon result in multiple wounds and thus more ports of entry which may allow multiple infections (Gu *et al.*, 2005). Pruning spurs to two buds have shown to promote rapid infection of the cordon, as lesions extended quicker into the vine cordons and trunks from the inoculated pruning wound (Eskalen *et al.*, 2007; Halleen *et al.*, 2007). To date no cultivar has been shown to be resistant to species of *Phaeoacremonium*, or any of the trunk disease pathogens.

Different methods have been used in testing pathogenicity of Petri disease pathogens including inoculation of wounded shoots with agar plugs, agar plugs inserted into trunks, inoculation of pruning wounds with conidial suspensions and inoculation of sand with conidial suspensions before planting (Adalat *et al.*, 2000; Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Rooney-Latham *et al.*, 2005b; Eskalen *et al.*, 2007; Halleen *et al.*, 2007; Serra *et al.*, 2008; Rolshausen *et al.*, 2010; Van Niekerk *et al.*, 2011). Adalat *et al.* (2000) tested the infection potential of *Pm. inflatipes* W. Gams, Crous & M.J. Wingf., *Pm. minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous and *Pa. chlamydospora* by inoculating sand with spore suspensions and showed the ability of all these pathogens to cause brown streaks in cultivars Chardonnay and Pinot Noir after six months of inoculation. Similar vascular discoloration was reported with *Pa. chlamydospora* and *Pm. minimum* on Cabernet Sauvignon, Grenache and Thompson Seedless pruning wounds (Feliciano *et al.*, 2004). *Phaeoacremonium krajdenii* L. Mostert, Summerb. & Crous*, Pm. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams,

Crous & M.J. Wingf., *Pm. subulatum* L. Mostert, Summerb. & Crous and *Pm. venezuelense* L. Mostert, Summerb. & Crous inoculated in trunks and pruning wounds in South African vines formed brown streaking of wood within 14 months (Halleen *et al.*, 2007). Virulence differed among different isolates, however, *Pa. chlamydospora* was found to be the most virulent from glasshouse and field trials, as compared to *Phaeoacremonium* species tested (Adalat *et al.*, 2000; Halleen *et al.*, 2007).

To date, 13 *Phaeoacremonium* species have been isolated from grapevines in South Africa, namely, *Pm. minimum*, *Pm. alvesii* L. Mostert, Summerb. & Crous, *Pm. austroafricanum* L. Mostert, W. Gams & Crous, *Pm. griseorubrum* L. Mostert, Summerb. & Crous, *Pm. iranianum* L. Mostert, Gräfenhan, W. Gams & Crous, *Pm. kradjenii*, *Pm. fraxinopennsylvanicum* (T.E. Hinds) D. Gramaje, L. Mostert & Crous, *Pm. parasiticum*, *Pm. scolyti* L. Mostert, Summerb. & Crous, *Pm. sicilianum* Essakhi, Mugnai, Surico & Crous, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* J. Dupont. Spore trapping studies conducted in South African vineyards revealed the occurrence of additional species previously isolated from other woody hosts within the vicinity of vineyards during periods of pruning activities, namely *Pm. australiense* L. Mostert, Summerb. & Crous, *Pm griseo-olivaceum* (Damm, L. Mostert & Crous) D. Gramaje, L. Mostert & Crous and *Pm. pruniculum* L. Mostert, Damm & Crous (Chapter 2). Of these *Pm. minimum*, *Pm. kradjenii*, *Pm. parasiticum*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* have been tested on grapevines in South Africa (Halleen *et al*., 2007). These findings raised concern as to whether all these *Phaeoacremonium* spp. are pathogens of grapevines. The current study was conducted to assess their status as pathogens on grapevine pruning wounds under field conditions.

MATERIALS AND METHODS

Isolates selection and inocula preparation

Phaeoacremonium species used in this study were previously recovered and identified in spore trap trials (Chapter 2) and disease incidence surveys (Table 1). The species tested included *Pm. australiense*, *Pm. austroafricanum, Pm. alvesii*, *Pm. fraxinopennsylvanicum, Pm. griseorubrum, Pm. griseo-olivaceum, Pm. iranianum, Pm. pruniculum, Pm. scolyti* and *Pm*. *sicilianum. Phaeoacremonium parasiticum* was used as the positive control (Halleen *et al.*, 2007) and sterile water was the negative control. Up to three strains per isolate were used depending on availability. Isolates were plated onto Potato Dextrose Agar with Chloramphenicol (PDA-C) at 25^{o}C for 2 weeks. The cultures were flooded with 20 ml sterile distilled, double autoclaved water. Conidia were dislodged from the mycelia using a sterile glass rod and the suspension was filtered with a double cheese cloth. The spore concentration was adjusted to 10^4/ml using a haemocytometer.

Pruning wound inoculation

The trial was conducted between August 2013 and March 2015 in a nine-year-old Cabernet Sauvignon vineyard at the ARC Infruitec-Nietvoorbij (Nietvoorbij Campus), in Stellenbosch. The trial was set up in a completely randomized block design (CRBD) with a total of 27 treatments replicated 15 times. Pruning wounds were the experimental unit. The vines were spur pruned to two buds. Five pruning wounds were made per vine and each vine received the same treatment. Due to sap flow, pruning wounds were not inoculated immediately but within 24 hrs of pruning with 20 µl conidial suspensions.

Trial evaluation

The trial was evaluated after 18 months from date of inoculation. Stubs with inoculated pruning wounds were removed and taken to the laboratory for isolations. The stubs were cut longitudinally to measure lesion lengths with a caliper. The stubs were then surface sterilized by immersing into 70% ethanol for 30 s, 1 min in 3.5% sodium hypochlorite and again for 30 s in 70% ethanol. Four pieces of small tissue sections (1×1×2 mm) were dissected with a sterile scalpel from the wound scar interface and plated onto PDA-C plates. Petri dishes were kept at 25^{o}C and closely monitored for any *Phaeoacremonium* spp. growth. Re-isolated pathogens were identified based on morphological characteristics and then verified by randomly selecting two representative isolates from each treatment for sequencing. DNA was extracted with CTAB buffer according to Damm *et al*. (2008). The partial beta-tubulin gene area were amplified with PCR using primers T1 and Bt2b (Glass and Donaldson, 1995; O' Donnel and Cigelink, 1997) as decribed by Mostert *et al*. (2006b). PCR products were cleaned using an MSB Spin PCRapase kit (Invitek, Germany) and cleaned products were sequenced with the same primers using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA). The products were then analyzed on an ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN). Sequences were compared to reference sequences of each species in megablast function of the NCBI's GenBank nucleotide database (www.ncbi.nlm.nih.gov) to confirm the identity. Mean values of lesion length were calculated and subjected to a one way analysis of variance (ANOVA) using SAS, Student 's t-test for least significant difference (LSD) was calculated at 5% probability level to separate means.

RESULTS

Mean lesion lengths of vascular discoloration caused by the 27 treatments and mean re-isolation percentages are given in Table 2. Inoculated pruning wounds had developed black to brown discoloration after 18 months from the date of inoculation (Fig. 1). All isolates were considered pathogenic as they produced lesion lengths significantly different to the negative control (P<0.0001), and some lesions extended beyond the inoculated pruning wound into the cordon. There was no

significant variation between species observed. *Phaeoacremonium griseo-olivaceum* (strain STE-U 7859) produced the longest lesion length of 79.53 mm. The shortest lesion length (62.00 mm) was produced by *Pm. iranianum* (STE-U 6998). Positive control wounds inoculated with *Pm. parasiticum* produced lesion lengths of 74.5 and 65.21 mm. All the inoculated isolates produced lesion lengths similar to *Pm. parasiticum*, a known pathogen, and therefore all these isolates can be regarded as pathogenic. There was no significant differences between isolates of the same species except for *Pm. pruniculum* where STE-U 5968 produced a significantly longer lesion (77.27 mm) than STE-U 7857 (62.26 mm).

All isolates were re-isolated from inoculated pruning wounds after 18 months. The most frequently re-isolated isolates were STE-U 7854 and STE-U 7855 (*Pm. scolyti*), STE-U 7860 and STE-U 7858 (*Pm. griseo-olivaceum*), STE-U 7879 (*Pm. sicilianum*), and STE-U 6987 (*Pm. fraxinopennsylvanicum)*, with re-isolation percentages that ranged from 78.57 to 84.62%. Two of the three *Pm. alvesii* isolates were re-isolated at the lowest re-isolation percentages of 28.57 and 35.71 % as compared to all tested isolates (Table 2).

DISCUSSION

The present study showed that all eleven inoculated *Phaeoacremonium* species are pathogenic and capable of causing vascular discoloration when inoculated onto grapevine pruning wounds. The ten *Phaeoacremonium* species with unknown pathogen status on grapevines all formed lesions not significantly different from *Pm. parasiticum*, a known pathogen of grapevines. Of the species tested *Pm. alvesii*, Pm. *fraxinopennsylvanicum*, *Pm. iranianum*, *Pm. scolyti* and *Pm. sicilianum* have been tested on grapevines in other grapevine producing countries (Gramaje *et al*., 2007; Aroca and Raposo, 2009; Gramaje *et al*., 2009; Mohammadi *et al*., 2012; Özben *et al*., 2012; Úrbez-Torrez *et al*., 2014; Mohammadi and Hashemi, 2015). All these species caused vascular discoloration on inoculated grapevine shoots, which is consistent with the findings of the current study. Furthermore, reduced root weight, chlorotic leaves, severe defoliation and wilting symptoms were observed from grapevine shoots inoculated with *Pm. fraxinopennsylvanicum* (Gramaje *et al*., 2007; Aroca and Raposo, 2009; Úrbez-Torrez *et al*., 2014). Low mean shoot weight was also reported in grapevine shoots inoculated with mycelial plugs of *Pm. iranianum* and *Pm. sicilianum* (Gramaje *et al*., 2009).

The lesions form as a result of oxidation and translocation of some breakdown products of plant cells attacked by fungal enzymes and is important in indicating severity of the infection (Agrios, 2005). Sparapano *et al.* (2000) identified several substances involved in Petri disease fungi, namely, phytotoxic compounds; pectic enzymes and lignin degrading enzymes. Research has shown *Pa. chlamydospora* and *Pm. minimum* to produce phytotoxins such as pullulans, scytalone and isosclerone (Andolfi *et al.*, 2011). Scytalone was suspected of intensifying the brown-black colour of infected wood.

Naphthaquinones were found to lower the resistance of infected plants to pathogenic fungi and enhance virulence factors inhibiting the plant defense reaction (Andolfi *et al.*, 2011).

Phaeoacremonium griseo-olivaceum and *Pm. pruniculum* have not been reported on grapevines anywhere in the world. *Phaeoacremonium griseo-olivaceum* was previously only reported from the sexual morph (perithecia) found on *Prunus* in South Africa (Damm *et al.*, 2008). This species was trapped as airborne spores in a vineyard from the Durbanville area in December 2012. Pathogenicity has only been tested on plums (*Prunus armeniaca*) and apricots (*P. salicina*) in South Africa (Damn *et al.*, 2008). Findings of these species as aerial inoculum (Chapter 2) and discovering their capability to cause symptoms in grapevines highlights the risk of establishing stone fruit orchards in close proximity to vineyards. Results from Chapter 2 indicate the possibility of Petri disease pathogens to be cross dispersed between orchards and vineyards and increase diversity of pathogens within a vineyard. This is in agreement with a study that showed that *Pm. minimum* isolated from apple trees could infect grapevine shoots (Cloete *et al.*, 2011). Aroca and Raposo (2009) also used isolates of *Pm. inflatipes* from *Quercus* and *Pm. fraxinopennsylvanicum* from *Fraxinus* spp. to show the capability of these species to infect and cause discoloration on grapevines. *Phaeoacremonium* species have a wide host range, which favors the survival and spread of pathogens as several woody hosts serve as reservoirs. Several surveys report on the occurrence of *Phaeoacremonium* on woody hosts including *Prunus* spp. (Damm *et al.*, 2008; Gramaje *et al*., 2012); *Fraxinus* spp. (Eskalen *et al.*, 2005b); *Malus* spp. (Cloete *et al.*, 2011), *Quercus* spp. (Aroca and Raposo, 2009), *Actinidia deliciosa* (Dí Marco *et al.*, 2000) and *Olea europea* (Carlucci *et al.*, 2015) which are commonly established within the vicinity of vineyards.

Eleven *Phaeoacremonium* species are known to infect humans, causing phaeohyphomycosis and other related symptoms. Nine of these also infect woody hosts, namely *Pm. alvesii*, *Pm. griseorubrum*, *Pm. inflatipes*, *Pm. krajdenii*, *Pm. minimum*, *Pm. parasiticum*, *Pm. rubrigenum* W. Gams, Crous & M.J. Wingf., and *Pm. venezuelense* (Gramaje *et al*., 2015). This include two species found to be pathogens of grapevines in this current study, namely *Pm. alvesii* and *Pm. griseorubrum.*

All species of *Phaeoacremonium* tested on pruning wounds in this study were successfully re-isolated, although this was at varying percentages between species and isolates. Similarly, previous pathogenicity studies re-isolated *Pm. scolyti* at high percentages (100%) when tested on grapevine cultivar Monastrell cuttings, however the mean lesion length was not the longest compared to other species tested (Aroca and Raposo, 2009).

There was no significant difference in virulence between isolates of *Pm. alvesii*, *Pm. australiense*, *Pm. austroafricanum*, *Pm. fraxinopennsylvanicum*, *Pm. griseorubrum*, *Pm. griseo-olivaceum*, *Pm. scolyti*, *Pm. sicilianum* and *Pm. iranianum*. However, there was a noteworthy difference

between isolate s of *Pm*. *pruniculum*. The most virulent isolate of *Pm. pruniculum* (STE-U 5968) was previously isolated from *Prunus* spp. in the Limpopo Province (Damm *et al.,* 2008) and was significantly different from the isolate found as airborne inoculum during spore trap studies in the Western Cape Province. The two isolates may be genetically different, therefore, resulting in different levels of virulence. Although only one isolate each of *Pm. austroafricanum* and *Pm. fraxinopennsylvanicum* could be included in this study, both isolates proved to be pathogenic.

Future studies identifying resistant cultivars as disease management strategies is worth investigating to reduce the level of infection in vineyards. Halleen *et al.* (2007) observed differences between isolates when potted nursery vines were inoculated with various *Phaeoacremonium* spp. and classed some species namely*, Pm. minimum*, *Pm. viticola* and *Pm. krajdenii* as non-pathogenic endophytes, based on the shoot lesions. When these species were inoculated in trunks and pruning wounds of mature field-grown vines, they were just as pathogenic as the others. Isolates used in the current study were from different locations and were only tested in one Stellenbosch vineyard which is climatically different from other regions where isolates used in this study were collected. The capability of isolates from all these regions to infect grapevine pruning wounds irrespective of where they were collected from highlights the high adaptive character of these pathogens. This therefore, shows the importance of movement of material between regions which can result in the introduction of new pathogenic species into areas where they have not been previously reported. This is in reference to species such as *Pm. sicilianum*, *Pm. australiense*, *Pm. griseo-olivaceum*, which were never reported in Stellenbosch vineyards but have shown the ability to cause infection on pruning wounds in this region.

As was reported by Halleen *et al*. (2007), in the current study many lesion lengths extended from the inoculated two bud spur into the cordons. This shows the risk at which vine cordons can be infected when pruned to two buds. Other pruning practices such as double pruning have previously reported to manage cordon infection by *Eutypa lata* through pruning wounds (Gubler *et al.*, 2005).

This study confirms pruning wounds as a port of entry for infection by *Pm. australiense*, *Pm. austroafricanum*, *Pm. alvesii*, *Pm. fraxinopennsylvanicum*, *Pm. griseorubrum*, *Pm. griseo-olivaceum*, *Pm. iranianum*, *Pm. pruniculum*, *Pm. scolyti* and *Pm. sicilianum*, irrespective of where they have previously been isolated. The importance of wound protection is thus emphasized by the capability of all 11 species to cause symptoms in inoculated wounds.

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Table 1. *Phaeoacremonium* species and isolates used in the pathogenicity study with the host and place of origin.

***Phaeoacremonium* species**	**Accession number**	**Host**[a]	**Place of origin**
Pm. australiense	STE-U 7863	Spore trap	Slanghoek
	STE-U 7862	Spore trap	Slanghoek
	STE-U 7861	Spore trap	Slanghoek
Pm. austroafricanum	LM 733	*Vitis vinifera*	
Pm. alvesii	STEU-6988	*Vitis vinifera*	Klawer
	STEU-6989	*Vitis vinifera*	Klawer
	STEU-7000	*Vitis vinifera*	De Rust
Pm. fraxinopennsylvanicum	STE-U6987	*Vitis vinifera*	Hermanus
Pm. griseorubrum	STE-U 7881	*Vitis vinifera*	Wellington
	STE-U 7882	*Vitis vinifera*	Wellington
	STE-U 7856	*Vitis vinifera*	Wellington
Pm. griseo-olivaceum	STE-U 7860	Spore trap	Durbanville
	STE-U 7859	Spore trap	Durbanville
	STE-U 7858	Spore trap	Durbanville
Pm. iranianum	STE-U 6998	*Vitis vinifera*	Calitzdorp
	STE-U 6999	*Vitis vinifera*	Calitzdorp
Pm. pruniculum	STE-U 5968	*Prunus salicina*	
	STE-U 7857	Spore trap	Stellenbosch
Pm. scolyti	STE-U 7854	Spore trap	Slanghoek
	STE-U 7855	Spore trap	Rawsonville
	STE-U 7876	Spore trap	Rawsonville
Pm. sicilianum	STE-U 7879	*Spore trap*	Rawsonville
	STE-U 7880	Spore trap	Rawsonville
	STE-U 7877	Spore trap	Rawsonville

[a] Isolates were obtained as aerial spore inoculum in vineyards.

Table 2. Mean lesion lengths and re-isolation percentages of *Phaeoacremonium* spp. inoculated on Cabernet Sauvignon pruning wounds.

Phaeoacremonium spp.	Mean lesion length (mm)	Mean re-isolation (%)	Accession number
Pm. griseo-olivaceum	79.53**a**	78.57	STE-U 7859
Pm. pruniculum	77.27**ab**	64.29	STE-U 5968
Pm. griseo-olivaceum	75.54**abc**	80.00	STE-U 7858
Pm. parasiticum	74.53**abc**	78.57	STE-U 7875
Pm. sicilianum	74.44**abc**	76.92	STE-U 7880
Pm. australiense	74.30**abc**	73.33	STE-U 7863
Pm. griseorubrum	73.27**abc**	69.23	STE-U 7881
Pm. australiense	73.06**abc**	42.86	STE-U 7861
Pm. griseorubrum	72.95**abc**	69.23	STE-U 7882
Pm. sicilianum	72.04**abc**	76.92	STE-U 7877
Pm. scolyti	71.59**abc**	85.71	STE-U 7854
Pm. scolyti	71.51**abc**	84.62	STE-U 7855
Pm. sicilianum	71.51**abc**	80.00	STE-U 7879
Pm. alvesii	70.91**abc**	35.71	STE-U 7000
Pm. iranianum	70.05**abc**	71.43	STE-U 6999
Pm. austroafricanum	67.97**abc**	69.23	LM 733
Pm. alvesii	67.54**abc**	28.57	STE-U 6988
Pm. griseo-olivaceum	67.33**abc**	84.62	STE-U 7860
Pm. scolyti	65.77**abc**	66.67	STE-U 7876
Pm. fraxinopennsylvanicum	65.65**abc**	78.57	STE-U 6987
Pm. parasiticum	65.21**abc**	57.14	STE-U 7878
Pm. alvesii	64.50**bc**	53.85	STE-U 6989
Pm. australiense	64.39**bc**	57.14	STE-U 7862
Pm. griseorubrum	64.17**bc**	73.33	STE-U 7856
Pm. pruniculum	62.26**c**	57.14	STE-U 7857
Pm. iranianum	62.00**c**	60.00	STE-U 6998
Control (sterile water)	14.46**d**	0	
LSD% (*P* = 0.05)	14.93		

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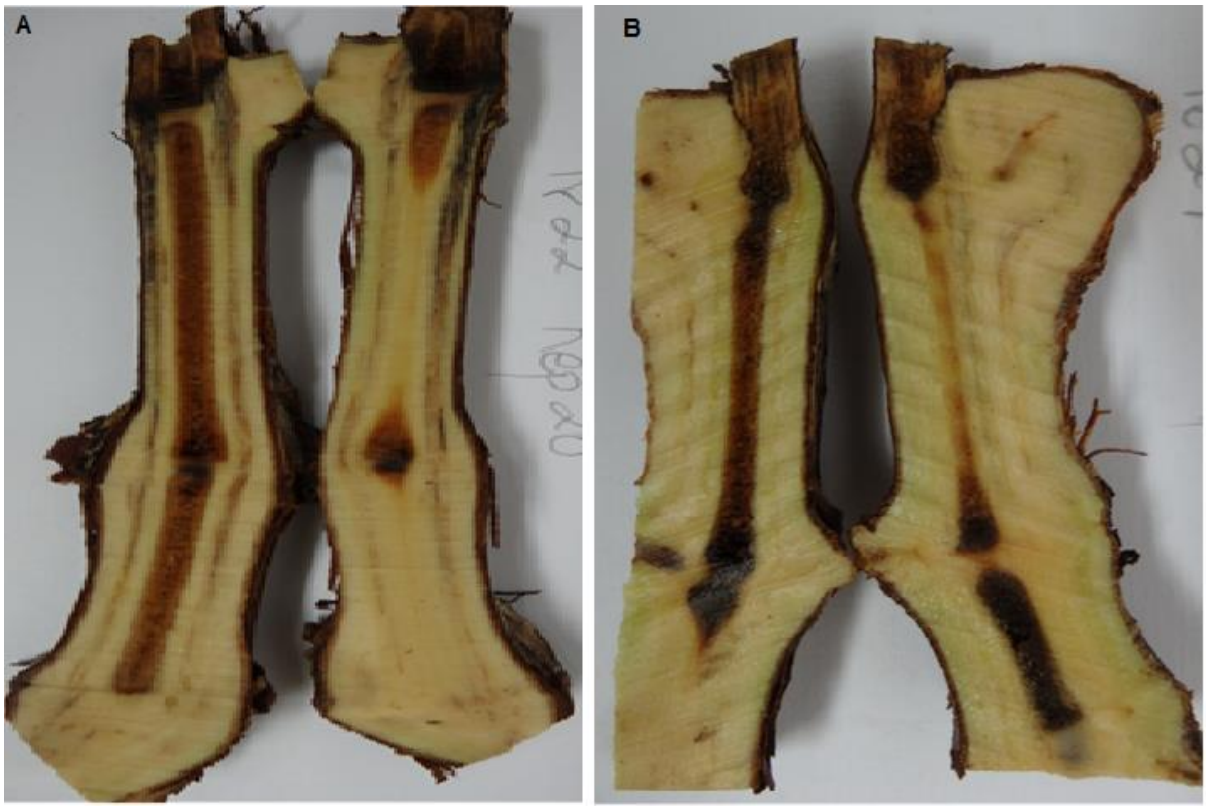

Figure 1. Vascular discoloration observed in Cabernet Sauvignon grapevines after 18 months of inoculation with *Phaeoacremonium* species (**A**) and untreated control (**B**).

CHAPTER 5

GENETIC DIVERSITY OF *PHAEOACREMONIUM MINIMUM*, ASSOCIATED WITH PETRI DISEASE AND ESCA OF GRAPEVINES IN SOUTH AFRICA

ABSTRACT

Phaeoacremonium (*Pm*.) *minimum* isolates were cultured from spore traps placed on individual vines from six vineyards and two rootstock mother blocks in the Western Cape of South Africa during 2013. In total 320 *Pm. minimum* isolates were collected. The mating type distribution was assessed with *Mat1-2* specific-primers. Both mating types of *Pm. minimum* (*MAT1-1* and *MAT1-2*) were found in all eight vineyards. An equal distribution of the two mating types was found occurring among isolates of *Pm. minimum* in six of the vineyards, but not in the Paarl A and Wellington populations. Primers for dinucleotide microsatellite loci were designed and 15 microsatellite loci were identified to be polymorphic and could thus be used to assess the genetic diversity of the *Pm. minimum* isolates. A total of 134 multilocus genotypes (MLGs) were observed of which 115 were observed once and 19 genotypes were observed either two or more times. The presence of the same MLG in a vineyard at different collection times, indicates asexual reproduction. The most common MLG occurred 77 times, being present in all of the eight vineyards. The widespread distribution of MLGs is most probably due to infected nursery plants stock. The total gene diversity (*H*) was high with a mean of 0.58 across all populations. Analysis of molecular variance indicated that 94% of the genetic variation was distributed within populations and only 6% between populations. High and significant population differentiation values were only obtained when Paarl A was compared to Stellenbosch P2. For fifteen other comparisons, 12 significant moderate PhiPT values were obtained signifying little genetic differentiation between the populations. The near to equal distribution of mating types is indicative of sexual reproduction, whereas the occurrence of isolates with the same MLG and mating type over time in a vineyard indicates asexual reproduction. This study confirms the importance of infected planting material that can distribute similar MLGs over long distances. Therefore, the management of Petri disease needs to focus on ensuring clean mother vines and nursery plants.

INTRODUCTION

Twenty-eight species of *Phaeoacremonium* has been reported from grapevines exhibiting Petri disease or esca (Gramaje *et al*., 2015). Of these *Phaeoacremonium* (*Pm*.) *minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous is the most commonly isolated species (Larignon

and Dubos, 1997; Mugnai *et al*., 1999; Groenewald *et al*., 2001; Berraf-Tebbal *et al*., 2011; Mohammadi *et al*., 2013; Úrbez-Torres *et al*., 2014).

The sexual morph of *Pm. minimum* has been found on grapevines in California and South Africa (Rooney-Latham *et al*., 2005a; Baloyi *et al*., 2013), and also on moist incubated wood collected from Australian vineyards (Pascoe *et al*., 2004). The finding of the sexual morph of *Pm. minimum* in vineyards confirms that sexual reproduction is possible in vineyards (Rooney-Latham *et al*., 2005a). Rooney-Latham *et al*. (2005a) found clusters of perithecia on dead vascular tissue or on decayed pruning wounds of grapevines in five Californian counties. In the current study (Chapter 3), perithecia of *Pm. minimum* were found in four of the eight vineyards investigated. *In vitro* matings with single conidial isolates confirmed *Pm. minimum* to have a heterothallic mating system (Mostert *et al*., 2003; Rooney-Latham *et al*., 2005b). Both mating types were found in a vineyard and also in the same vine (Mostert *et al*., 2003; Pascoe *et al*., 2004; Rooney-Latham *et al*., 2005b). Equal proportions of both mating types have been found among 21 *Pm. minimum* isolates from South Africa (Mostert *et al*., 2003) and 58 isolates from Spain (Gramaje *et al*., 2013), suggesting sexual reproduction is possible in those populations. The development of a specific primer for only the *Mat1-2* locus of *Pm. minimum* made it possible to establish the distribution of mating types with a multiplex PCR (Arzanlou and Narmani, 2014). The PCR included a positive control to confirm the presence of *Pm. minimum*. Out of 31 isolates, 19 were *Mat1-2* and 12 were of *Mat1-1* identity.

The genetic diversity of *Pm. minimum* populations has been studied using different molecular tools including amplified fragment length polymorphism (AFLPs), random amplified polymorphic DNA (RAPDs), random amplified microsatellites (RAMS), single nucleotide polymorphisms (SNPs) and universal primed PCR (UP-PCR) (Péros *et al*., 2000; Tegli, *et al*., 2000; Cottral *et al*., 2001; Gramaje *et al*., 2013; Abreo, 2015). With RAPDs and RAMS Tegli *et al*. (2000) showed that 15 *Pm. minimum* isolates from six regions in Italy had low levels of genetic diversity and low levels of genetic disequilibrium. Even though the results indicated that sexual reproduction might occur, higher number of isolates would be needed to confirm their conclusion. Using RAPDS lower levels of genetic diversity was obtained for 118 *Pm. minimum* isolates from southern and south-western France (Borie *et al*., 2002). The four most frequent genotypes were found in all the populations and represented 82% of all the isolates. In Spain, using UP-PCR to characterize a collection of 60 *Pm. minimum* isolates obtained from 11 grapevine growing provinces, 54 unique genotypes were observed (Gramaje *et al*., 2013). *Phaeoacremonium minimum* isolates from mainly Castilla y León in Spain were analysed with RAPDs and found that 27 of 36 isolates were unique haplotypes and for 17 isolates analysed further with AFLPs, all were unique haplotypes (Martín *et al*., 2014). They concluded to have found a high level of haplotype diversity indicative of sexual reproduction. A genetic diversity

study on a global scale used five SNPs (identified in the actin and betatubulin genes) to compare 66 isolates of *Pm. minimum* from nine countries (Abreo, 2015). The isolates from Italy, Spain and Uruguay had higher genetic and genotypic diversity than isolates from South Africa, Canada and Algeria. The *Pm. minimum* isolates Abreo (2015) included from South Africa included only three from grapevines and eight from stone fruit trees. The genetic diversity of *Pm. minimum* populations from grapevines in South Africa has not been previously determined on a wider scale and would aid understanding the reproductive mode of *Pm. minimum*, inoculum dispersal and geographic spread.

Microsatellites or simple sequence repeats (SSR) are tandem repeats of 1-6 nucleotide units spread along the genome. They are very effective DNA markers in population genetics, with a high multi-allelic nature, high reproducibility and co-dominant inheritance (Benson, 1999). The technique can effectively be used to assess gene flow, migration and gene diversity. SSRs show high polymorphism and specificity, and unlike RAPDs, they are very repeatable. This tool allows for precise discrimination even of closely related individuals and has an expected greater frequency (Kolpakov *et al*., 2003; da Maia *et al*., 2008). Recently the genome sequence of *Pm. minimum* has been made available (Blanco-Ulate *et al*., 2013) opening possibilities for the development of SSR markers that may be used to study this species' population genetics.

An investigation into the genetic diversity and mating type distribution of *Pm. minimum* on a wider scale, including vineyards and rootstock mother blocks in different regions, has not been done in South Africa. Since the sexual morph has been found in a few vineyards, the question still remains what the mode of dispersal of *Pm. minimum* is within and between vineyards. The objectives of this study were to: (i) identify polymorphic SSR loci for *Pm. minimum*; (ii) determine the genetic diversity of *Pm. minimum* populations obtained from vineyards and rootstock mother blocks and (iii) determine the mating type distribution in the *Pm. minimum* populations. This knowledge will improve our understanding of the mode of reproduction occurring in vineyards and rootstock mother blocks, the dispersal range of similar genotypes, all of which will contribute to developing improved managenent strategies to combat Petri disease and esca.

MATERIALS AND METHODS

Phaeoacremonium minimum isolates collection

A total of 320 *Pm. minimum* isolates were used in this study to analyze the genetic diversity and mating type distribution. The isolates were collected from March to December in 2013 with spore traps (microscope slides coated with petroleum jelly) as described by Eskalen and Gubler (2001) in Chapter 2. The spores were collected from six vineyards, namely

Stellenbosch P2 (35−year−old Pinotage), Stellenbosch B3 (27−year−old mixed cultivar block), Rawsonville (24−year−old Chenin blanc), Paarl A (30−year−old Red Muscadel), Paarl Z (40−year−old Hanepoot), Durbanville (26−year−old Sauvignon blanc), as well as two rootstocks mother blocks, namely Slanghoek (23−year−old Ramsey) and Wellington (17−year−old Ramsey), all situated in the Western Cape Province in South Africa. In Rawsonville and Paarl A, spore traps were also placed in a young vineyard established adjacent to the older vineyard. Spore traps were placed along the wire where the vine arm of these young vines will be trained. A total of 40 isolates from each vineyard were selected for this study except Rawsonville, which consisted of 42 isolates and Slanghoek of 38 isolates. The eight vineyards were divided into six regions, namely Paarl, Stellenbosch, Durbanville, Rawsonville, Slanghoek and Wellington.

DNA extraction

Pure cultures were obtained by growing isolates from spores collected with spore traps on Potato Dextrose Agar amended with Chloramphenicol (PDA–C). Conidial suspensions from each isolate were spread on PDA−C. Single spores were selected after 24 hrs of incubation at 25ºC, and transferred to fresh PDA−C. Plates were incubated for 2 weeks at 25ºC. Extraction of DNA from mycelia was done using CTAB extraction protocol as described by Mostert *et al*. (2006). The concentration of each DNA sample was determined on an ND-1000 spectrophotometer (Nanodrop), and adjusted to a final concentration of 30 ng/µl.

Mating type determination

The multiplex PCR protocol developed by Arzanlou and Narmani (2014) was followed to simultaneously identify *Pm. minimum* and the mating type of each isolate. The multiplex reaction consisting of a *Pm. minimum* species-specific primer set was used together with the *MAT1-2* gene-specific primer set. Primer sets PmaleoF (5'-CTCTGCGACGCGTCCCAGATTC-3') and PmaleoR (5'- TCGCGA TGGCCC ACTGCCTAC -3') identified *Pm. minimum* isolates (*c*. 500 bp). The primers PmaleoMat1-2F (5'- CCTATCGTCAAGGCAGCAGCTCATCC- 3') and PmaleoMat1-2R (5'- CTTCTCGTAGTGCTTGCGCTTGC- 3') amplified the *Mat1-2* locus (*c*. 230 bp). All PCR reactions were performed in a total reaction of 20 µl volume, containing 1 µl of genomic DNA (25-30 ng), 10 µl KAPA Taq Ready mix (KapaBiosystems, Mowbray, Western Cape, South Africa) and 0.4 µM of each primer. The PCR reactions were performed in a GeneAmp PCR System 2720 thermocycler (Applied Biosystems) under the following conditions: an initial stage of 96ºC for 5 min, followed by 40 cycles of 30 s at 94ºC, 30 s at 56ºC annealing, 60 s at 72ºC and an extension stage of 7 min at 72ºC. DNA amplicons were separated using electrophoresis at 80 V for 45 min in 1% ethidium bromide-stained agarose

gel and visualized under UV-light. Chi-square analyses were done to determine the goodness of fit of the mating type distribution compared to the expected mating type ratio of 1:1, for the total population and subpopulations.

SSR development

Genome sequence data for *Pm. minimum* strain UCR-PA7 was downloaded in unassembled "contig" format as made available by Blanco-Ulate *et al*. (2013). Thirty of the longest di-nucleotide repeats with flanking regions with high heterogeneity were tested by PCR amplification using genomic DNA from South African *Pm. minimum* isolates from a wide spatial and temporal distribution. Primers were designed to yield amplicons of roughly 200bp in size. The extent of amplicon size variation was investigated using high resolution gel electrophoresis ("Bioanalyzer", Agilent Technologies). Twenty of the apparently most heterogeneous loci were more accurately assessed for locus heterogeneity by fluorescent labelling of amplicons (VAM, VIC, NED and PET; Life Technologies) followed by high resolution (1bps) electrophoretic separation at the central analytical facility (CAF) Stellenbosch University (SU). SSR allele data was analysed using the software package GeneMapper® (Life Technologies) and GenAlEx v 6.5 (Peakall and Smouse, 2012) was used to determine the number of alleles per locus and whether it was polymorhpic.

SSR amplification

PCR was performed in a 20 µl final volume, in a multiplex containing 10 µl KAPA Taq Ready mix (KapaBiosystems, Mowbray, Western Cape, South Africa) and 1 µl DNA (30 ng/µl), 0.2 µl each of four forward labeled primer 5' end and 0.2 µl each of four reverse primers labeled with either one of the four fluorescent dyes, namely PET, NED, FAM and VIC to allow multiple reaction of products of the same size. Labeled primers were supplied by Life Technologies. The PCR reactions were performed under the following conditions: 5 min at 94^{o}C, followed by 35 cycles of 30 s at 94^{o}C, 30 s at 55^{o}C (annealing temperature), 30 s at 72^{o}C, followed by final extension stage for 5 min at 72^{o}C. Amplification were performed using a GeneAmp® PCR system 2720 thermocycler (Applied Biosystems). PCR product purification kit (MSB spin PCRapace, Invitek) was used for PCR post clean-up prior to electrophoresis, thereafter 2 µl of cleaned PCR product was 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). Electrophoresis was performed in an automated single capillary genetic analyser ABI3730*xl* DNA sequencer (Applied Biosystems) to separate fragments (all supplied by Applied Biosystems) at the central analytical facility (CAF) Stellenbosch University (SU). Data was analyzed using GeneMapper version 3.7 (Applied Biosystems).

Population genetics analyses

MLG diversity

Multilocus genotypes (MLGs) sharing the same alleles at all 15 loci were regarded as clones. The number of MLGs for the population was determined with GenAlEx v6.5. Clonal fraction was calculated as the occurrence and frequency of clones within population using the formula [(*N*-*G*)/*N*], where *N* is the sample size and *G* is the number of multilocus genotypes (MLGs) present. The expected number of MLGs after rarefraction (eMLG) was calculated to quantify genotypic diversity. The equitability in the distribution of sampling units was estimated by calculating the evenness index (E.5), with zero indicating no evenness and a value of 1 indicating equal abundance (Grunwald *et al*., 2003). Ewens-Watterson test was performed to test the neutrality of microsatellite markers. The observed F (sum of squares of allelic frequency) and limit (upper and lower) at 95% confidence regions were determined using POPGENE v1.31 to evaluate the selective neutrality of the polymorphic SSRs. The number of permutations for significance testing was set at 1000 for analyses.

Mode of reproduction

To test for recombination, the random association among SSR alleles were determined by calculation the index of association (I_A) and the standardized index of association $\bar{r}_d$. These indices estimate the degree of association of alleles at different loci within and among populations compared to that observed in a permutated data set. For physically unlinked loci, a value of zero is expected under random mating (linkage equilibrium). A value significantly larger than zero indicates linkage disequilibrium among loci, which may be achieved by no or infrequent sexual reproduction. P values were calculated after 1000 permutations in the R package Poppr (Kamvar *et al*., 2014).

Genetic diversity within and between populations

All other diversity indices for *Pm. minimum* isolates occurring in each population and for the population as a whole were computed using clone-corrected data to avoid over representation of clones. Nei's gene diversity (*H*) (Nei, 1973), the mean number of alleles, the number of private alleles were estimated using GenAlEx v6.5. Allelic richness of each population was estimated using the rarefaction method implemented in in R package Poppr, which estimate the mean number of alleles per locus.

Analysis of molecular variance (AMOVA) was used to test for differentiation within and among populations and regions. The PhiPT (analogue of F_{ST}) (Wright, 1951) was calculated to determine the extent of population differentiation in pairwise comparison after 999

permutations in GenAlEx v6.5. The PhiPT varied from zero to one, with zero meaning no variation between populations and one indicating completely differentiated.

A minimum spanning network (MSN) to assess the possible evolutionary relationships among MLGs from all the populations in vineyards and rootstock mother blocks, was constructed in the R package Poppr (Kamvar *et al*., 2014).

RESULTS

Mating type determination

The amplification of the *Mat1-2* locus yielded a product of approximately 230 bp, confirming the presence of the *Mat1-2* mating type (Fig. 1). The species-specific primers yielded a product of approximately 550 bp confirming the isolates to be *Pm. minimum* (Fig. 1). The presence of the 550 bp product and absence of a product at 230 bp indicated that the isolate is of the opposite mating type, *MAT1-1*. Both mating types of *Pm. minimum* were found in all eight vineyards. Chi-square analyses confirmed a distribution of near 1:1 ratio for *MAT1-1* and *MAT1-2*, therefore accepting the null hypotheses that both mating types are distributed at equal ratios in six vineyards, except for Paarl A and Wellington (Table 1). The majority (85%) of isolates in Paarl A were *MAT1-2* whereas in Wellington 68% of the isolates were *MAT 1-1*.

SSR development

Fifteen loci were found to display sufficient heterogeneity for analysis (Table 2). All microsatellite loci amplified were polymorphic and the number of alleles per locus ranged from two to 15 (Table 2). Also listed in Table 2 are the primers and fluorescent labels used for amplification as well as the observed size distribution within populations.

Population genetics analyses

MLG diversity

The 15 microsatellite loci were analyzed for 320 isolates of *Pm. minimum,* representing eight populations. A total of 134 MLGs were observed in South African vineyards. The clonal fraction was highest in Paarl Z (0.60) and lowest in Paarl A (0.30) (Table 3). In the total population, 115 of the genotypes were observed once and 19 genotypes were observed either two or more times (Table 4). The observed fixation indexes value of all 15 of the polymorphic microsatellite markers was within the 95% confidence interval of theoretical expectation, therefore accepting the null hypothesis of neutral selection for these loci, which indicates that they are unlinked.

Mode of reproduction

A non-random association of alleles was indicated by the significant I_A and $\bar{r}_d$. Linkage disequilibrium was high in all populations, and was highest in Paarl Z, Paarl A, Durbanville, Rawsonville and Wellington which suggest asexual reproduction to be prevalent in *Pm. minimum* populations in South Africa (Table 3).

Genetic diversity within and between populations

The mean number of alleles in all populations within all loci was 0.34 (Table 3). Mean gene diversity (*H*) ranged from 0.48 (Wellington) to 0.58 (Slanghoek) (Table 3). The AMOVA revealed that 6% of the genetic variation was distributed among populations and 94% within populations with a PhiPT of 0.063 (*P* = 0.001) (Table 7).

Results of the pairwise determination of population differentiation showed one case of significant high genetic differentiation and thirteen cases of significant moderate genetic differentiation (Table 5). Significant high genetic differentiation was found between Paarl A and Stellenbosch P2 (PhiPT = 0.159, *P* = 0.001). Significant moderate genetic differentiation was found between Paarl A and all other vineyards, except for Stellenbosch P2. Wellington had significant moderate genetic differentiation with Paarl Z, Stellenbosch B3, Stellenbosch P2 and Durbanville. Other significant moderate genetic differentiation values were observed between Slanghoek with Stellenbosch B3 and Stellenbosch P2, Stellenbosch P2 and Paarl Z as well as Stellenbosch P2 versus Rawsonville. All other pairwise comparisons were non-significant (Table 5).

Examination of shared MLGs showed direct evidence of genotype flow between two or more populations. The two most frequently isolated genotypes occurred 77 (genotype M) and 36 (genotype P) times each and was found in all vineyards. The third most frequently isolated genotype occurred 19 times and was isolated from six vineyards, namely Slanghoek, Rawsonville, Durbanville, Stellenbosch P2, B3 and Paarl A. The fourth most common genotype occurred 17 times in all vineyards except Paarl A. The MLG distribution in four of the populations is presented in Figs. 3 to 6. The most commonly occurring MLG, M, was found from spore traps of all five vines in Stellenbosch P2 (Fig. 3), Rawsonville (Fig. 5) and Durbanville (Fig. 6). It was also distributed from March to December in either one or more vineyards (Table 6). The distribution of clonal MLGs across vineyards and months is shown in Table 6. Certain months had a higher incidence of clonal MLGs. In April (12 MLGs), June (8 MLGs), July (9 MLGs), August (8 MLGs) and September (10 MLGs) higher number of MLGs were found than in March (3 MLGs), May (5 MLGs), October (4 MLGs), November (6 MLGs) and December (1 MLG) (Table 6). Some MLGs were only found in certain months, for instance

MLG O was found in September, October and November, MLG K was found in May and June only. Some MLGs were only found in one month, namely MLG L in April and MLG I in July.

Isolates assigned to the same MLGs had different mating types, especially in MLGs that were frequently encountered (M, P, B, N; Table 8), indicating that the 15 SSR loci were insufficient to discriminate among genotypes. Isolates of the same MLG collected from the same vine at different collection dates could indicate the presence of asexual reproduction. Isolates trapped from young vineyards established close to the older vineyards shared the same MLGs and mating type (Table 8). This suggests dispersal of the same genotypes is possible from an older vineyard to a younger vineyard since these isolates were obtained from aerial inoculum, however, similar genotypes from nursery material can't be ruled out.

MSN analyses divided the MLGs into two major groups (Fig. 2). On the left hand side of the figure are multiple nodes/genotypes representing isolates from more than one vineyard (genotypes that got shared widely). On the right hand side are nodes/genotypes comprised of a single colour, only present in specific vineyards. The nodes to the right harbor a lot of isolates from Paarl A, Rawsonville and Slanghoek. Interestingly, Wellington is situated between the two groups, indicating that this area where most of the grapevine nurseries are situated, might play a pivotal role in the distribution of genotypes. The widely distributed genotypes most probably came from plant material sourced from similar grapevine nurseries.

DISCUSSION

In this study the genome of *Pm. minimum* was mined for di-nucleotide microsatellite loci. Fifteen polymorphic loci were identified suitable for population genetic analyses. The genetic variation of 320 *Pm. minimum* isolates obtained from eight populations from six vineyards and two rootstock mother blocks in the Western Cape Province were assessed using these SSR makers. Additionally the mating type of the 320 isolates was determined with *Mat1-2* specific primers.

The *Mat1-2* specific primers revealed a near equal mating type distribution for all the *Pm. minimum* isolates as well as for six of the vineyards. Therefore, agreeing with the null hypotheses that *Mat1-1* and *Mat1-2* are equally distributed, except for Paarl A (30-year-old Red Muscadel) and Wellington (17-year-old Ramsey rootstock block). In the latter two vineyards, unequal mating type distributions could be due to a more abundant occurrence of asexual reproduction. Unfortunately, the distribution of *Pm. minimum* mating types within vineyards has been poorly studied. Gramaje *et al.* (2013) analysed too few isolates at intra-vineyard level. Other studies assessed mating type distribution of *Pm. minimum* isolates from various vineyards and always found an equal distribution (Mostert *et al*., 2003; Rooney-Latham *et al*., 2005b; Gramaje *et al*., 2013), except in the case of Iran where more *Mat1-2*

than *Mat1-1* isolates were found. On a regional level, Gramaje *et al*. (2013) also reported skewed mating type distributions of *Pm. minimum* isolates from Valencia and Zaragoza. The near to equal distribution of the mating types in six of the vineyards indicates that random mating and recombination most likely occur in these vineyards and rootstock mother block.

Phaeoacremonium minimum populations in South Africa were in linkage disequilibrium suggesting predominance of asexual reproduction with possibly infrequent sexual reproduction occurring in vineyards. The relatively low incidence of finding sexual fruiting bodies of this species in vineyards supports the finding (Baloyi *et al*., 2013).

The gene diversity (*H*) obtained over the total South African *Pm. minimum* population was 0.526 (Nei, 1973). Together with a high number of MLGs indicate that the *Pm. minimum* population in South Africa is genetically diverse. Using SNPs Nei's gene diversity for 11 *Pm. minimum* isolates from South Africa was low (Abreo, 2015). This might be due because too few isolates were investigated, and eight of the isolates were from *Prunus* trees. Comparing the genetic diversity of *Pm. minimum* effectively with that found in other countries, would require similar SSR analyses on world-wide populations of this pathogen.

Most genetic diversity was distributed within populations and among populations contributed another 6% of genetic variation. No significant differences were found in the genetic variation of *Pm. minimum* populations between different geographic regions in Italy (Tegli *et al.*, 2000), France (Péros *et al*., 2000); or in Spain (Gramaje *et al*., 2013).

Comparing different vineyards in South Africa, Paarl A was highly differentiated from Stellenbosch P2. Unique features of Paarl A and Stellenbosch P2 is that both these vineyards are situated next to fruit orchards. The rootstock mother block in Wellington again is surrounded by many different grapevine rootstock cultivars. *Phaeoacremonium minimum* has been reported as a pathogen of fruit trees usually found near vineyards (Cloete *et al*., 2011; Damm *et al*., 2008; Arzanlou *et al*., 2013), suggesting these hosts serve as source of different genotypes that can spread to vineyards. The surrounding variety of grapevine cultivars next to the Wellington rootstock mother block could serve as reservoirs of different genotypes which differentiate it from Paarl Z. Another reason could be due to insufficient time of genotypes to diverge (Milgroom and Lipari, 1995), since the Wellington rootstock mother block was the youngest vineyard investigated (17-years-old). These factors could all contribute to the *Pm. minimum* population of Paarl Z being different from the other three vineyards. The moderate to low genetic differentiation between populations of other vineyards may be due to sufficient unrestricted gene flow that allowed wide spread of genotypes to different regions or these vineyards were established from vines from the same nursery harbouring the same pathogen population, hence it looks like there is no population differentiation.

In the total South African *Pm. minimum* population investigated, 19 MLGs occurred more than once. The presence of the same MLG in a vineyard supports the presence of asexual reproduction. The most common MLG occurred 76 times, being present in all of the eight vineyards and also over the whole of the sampling period (March to December). Finding the same MLG in different vineyards is most probably from sourcing planting material from the same nurseries since asexual spores would not be able to disperse over such long distances. Ten MLGs found in rootstock mother blocks, were also found in one or more vineyards in which samples were collected. The role of infected grapevine planting material is well documented. These findings agree with previous studies that showed infected rootstock material as the source of pathogen spread to newly infected vineyards (Fourie and Halleen, 2002; 2004). One of the rootstock mother blocks (Wellington) was situated between two groups of MLGs as illustrated on the minimum spanning network diagram, linking both wide spread genotypes and genotypes that occur in one place. The same MLG, also having the same mating type, was often found from the same vine at different collection times. Since the isolates were not obtained from isolations from the vine, but from aerially dispersed spores, this finding supports aerially transmission of asexually produced spores.

A high number of unique MLGs (134 out of 320 isolates) were obtained among the *Pm. minimum* population in South Africa. Even higher levels of genotype/haplotype diversity were also found in *Pm. minimum* populations in Spain with 54 out of 60 isolates (UP-PCR) (Gramaje *et al*., 2013) and 27 out of 36 isolates (RAPDs) (Martín *et al*., 2014) having unique genotypes. In the present study the diversity of MLGs per vineyard ranged from 10 to 25. The presence of different genotypes in a vineyard indicates the exposure to several sources of inoculum and multiple infection events (Gramaje *et al*., 2013). Unique genotypes could also be the consequence of sexual recombination. According to the MSN analysis genotypes situated in one place were found more commonly in Paarl A, Rawsonville and Slanghoek. Interestingly in Rawsonville and Slanghoek perithecia of *Pm. minimum* were found (Chapter 3), both vineyards with a higher occurrence of unique genotypes.

In conclusion, populations of *Pm. minimum* in South African vineyards have both multiple unique MLGs as well as multiple isolates with the same MLG. The presence of both mating types in vineyards and also trapped from one vine indicate that the sexual morph is possible to form. The high number of similar MLGs indicates the presence of asexual reproduction. Similar MLGs are widespread occurring in multiple vineyards on almost every vine. This confirms the importance of infected planting material distributing similar MLGs over long distances. The management of Petri disease needs to focus on ensuring clean mother material and nursery plants. Also knowledge regarding the potential for *Pm. minimum* to

recombine in the field is important in the management of any single site inhibitor fungicides that could be used to control Petri disease.

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Table 1. Distribution of mating type frequencies of *Phaeoacremonium minimum* isolates.

Population	No. of isolates	*MAT1-1*	*MAT1-2*	X^2 [a]
Paarl A	40	6	34	21,05 [b]
Paarl Z	40	24	16	1,6
Stellenbosch B3	40	16	24	1,6
Stellenbosch P2	40	19	21	0,1
Durbanville	40	23	17	0,9
Rawsonville	42	17	25	1,52381
Wellington	40	27	13	6,5[b]
Slanghoek	38	16	22	0,947368
Total	320	148	192	1,65625

[a] X^2value based on 1:1 ratio and 1 d.f.

[b] Significant values ($P < 0.05$)

Table 2. Characteristics of 15 microsatellite loci for *Phaeoacremonium minimum* amplifications: locus name, core repeat, primer sequences, annealing temperature (Tm) and number and size range in base pair.

Loci	Repeat motif	Primer sequence (5'-3')	Tm (°C)	Size range (bp)	Total (n=320)	Number of alleles
M1	(CA)20	F:ACCTAGACGCTCGAATGTGC R:TTGGCTCGATGGGGTAGTAG	58	146-176	253	7
M2	(GT)20	F:AAGTCTTCTGATCGGCTCCA R:AGATCACAGCGGCTCTTTGT	57	163-193	221	9
M3	(AGG)16	F:AATTCCACCCTTCCTTTCGT R:GCCACTGACCCCTGTAACAT	55	192-196	308	3
M5	(TG)19	F:GACAGGCAGTGGCACAGAT R:CTGGAGAATATGGGGTCTCG	58	190-218	244	7
M6	(AC)17	F:AGAGACAGTGCGACCTCGAT R:TTTGTCTTCGGGTACCTTGC	59	185-201	251	6
M7	(AC)17	R:GTTCCATCCATCCACCATCT R:AACGCACAGGATCAGCAAG	55	199-231	264	7
M9	(GA)15	F:CATGATTGCAAAACGCAAAG R:TGTAAGCAGTGGCACAGGTC	53	169-199	145	6
M11	(CA)17	F:CGACGTTTGGCTTTCTTTGT R:GGGCTAAAGGAGATCAAGACA	55	183-191	259	5
M12	(CT)15	F:CCCATCCCATCTTTCATTTG R:CATGTGCAGCGATAGGAAAA	52	194-272	252	15
M20	(AG)14	F:CCATGTGCTTCTCTCCTTGA R:GCTAGTAACGCCACATTAGAA	55	139-169	256	6
M16	(AC)10(TC)10(AC)5	F:GCCAAATGTGGATTTGGATT R:GTTAATAAGCTGTGGAAGTG	53	213-251	253	9
M19	(AG)14	F:ACTTAAACAAAGCAGGTGTTG R:TCATCTGCAGGTATCTGTCTT	53	160-214	258	10
M27	(AG)12	F:CTCGACTGTAGACTGGAGAAC R:TACGTGATGGACGGAAGTTG	55	125-169	250	7
M22	(AC)13(AG)10	F:AGAGGGTCTTCAGGTACCTAC R:GACATCAAAAGGACGGCATG	56	208-234	248	8
M23	(AC)10-AT-(AG)2-GG-(AG)8	F:CCTTAGCTCTAGGAGTGCTG R:TGCCGACTGGATGTGATAC	55	161-163	255	2

Table 3. Genetic diversity indices of *Phaeoacremonium minimum* populations from eight vineyards in South Africa.

Population	No. of isolates	No. of MLG's [a]	Clonal Fraction	eMLG	E.5	*H* [b]	Mean number of alleles per locus	No. effective alleles	I_A	$\bar{r}_d$
Paarl A	40	28	0.30	21.24	0.55	0.533 (0.044)	4.2 (±0.33)	2.36 (±0.20)	4.20 (0.001)	0.303 (0.001)
Paarl Z	40	16	0.60	26.8	0.7	0.540 (0.030)	3.07 (±0.25)	2.29 (±0.14)	8.50 (0.001)	0.606 (0.001)
Stellenbosch B3	40	21	0.48	15.54	0.62	0.41 (0.045)	3.53 (±0.29	1.86 (±0.17)	5.60 (0.001)	0.417 (0.001)
Stellenbosch P2	40	24	0.40	20.34	0.54	0.37 (0.042)	3.67 (±0.33)	1.72 (±0.14)	4.60 (0.001)	0.334 (0.001)
Durbanville	40	22	0.45	26	0.61	0.44 (0.039)	2.73 (±0.23)	1.89 (±0.14)	6.10 (0.001)	0.474 (0.001)
Rawsonville	42	22	0.48	20.2	0.64	0.49 (0.038)	3.8 (±0.38)	2.09 (±0.41)	6.60 (0.001)	0.480 (0.001)
Slanghoek	38	26	0.32	23.05	0.68	0.54 (0.04)	3.8 (±0.33)	2.31 (±0.16)	5.50 (0.001)	0.397 (0.001)
Wellington	40	24	0.40	23.05	0.62	0.470 (0.050)	2.93 (±0.32)	2.08 (±0.17)	6.40 (0.001)	0.501 (0.001)
Total	320	134	0.58	22.76	0.27	0.47 (0.015)	0.34 (±0.12)	3.5 (±0.12)	5.84 (0.001)	0.42 (0.001)

[a]MLG: Multilocus Genotypes.

[b]H: Gene diversity (Nei, 1973), standard error shown in parentheses.

[c]Standard deviation shown in brackets.

[d]Standard error shown in brackets.

Table 4. Frequency distribution of *Phaeoacremonium minimum* microsatellite multilocus genotypes (MLGs) within eight vineyards in Western Cape.

No. of times observed	Paarl A	Paarl Z	Stellenbosch B3	Stellenbosch P2	Durbanville	Rawsonville	Slanghoek	Wellington	Total pop[a]
1	24	9	11	13	12	16	17	14	115
2	1	5	1	1	6	2	4	3	6
3	2	0	1	2	1	1	0	0	4
4	0	0	1	0	0	0	0	0	1
5	0	0	1	1	0	1	0	0	0
6	0	0	0	0	0	0	0	1	1
7	1	0	0	0	0	0	0	0	2
8	0	0	0	1	0	0	0	0	1
9	0	0	0	0	0	0	1	1	0
10	0	1	1	0	0	0	0	0	0
11	0	1	0	0	1	0	0	0	0
12	0	0	0	0	0	1	0	0	0
17	0	0	0	0	0	0	0	0	1
19	0	0	0	0	0	0	0	0	1
36	0	0	0	0	0	0	0	0	1
77	0	0	0	0	0	0	0	0	1

[a]Eight vineyards pooled together.

Table 5. Pairwise population differentiation, PhiPT (above diagonal) with *P* values (below diagonal) among eight *Phaeoacremonium minimum* populations sampled from vineyards (significant PhiPT values in bold print).

Population	Paarl A	Paarl Z	Stellenbosch B3	Stellenbosch P2	Durbanville	Rawsonville	Slanghoek	Wellington
Paarl A		**0.084**	**0.111**	**0.159**	**0.115**	**0.052**	**0.085**	**0.135**
Paarl Z	0.011[b]		**0.063**	**0.088**	0.023	0.023	0.027	**0.071**
Stellenbosch B3	0.005[b]	0.067		0.000	0.000	0.000	**0.054**	**0.095**
Stellenbosch P2	0.001[a]	0.032[b]	0.371		0,017	0.015	**0.071**	**0.092**
Durbanville	0.004[b]	0.195	0.344	0.185		0.000	0.039	**0.079**
Rawsonville	0.028[b]	0.194	0.379	0.178	0.346		0.018	**0.052**
Slanghoek	0.004[b]	0.143	0.034[b]	0.013[b]	0.063	0.166		0.039
Wellington	0.001[a]	0.05[b]	0.009[b]	0.012[b]	0.027[b]	0.061	0.068	

[a] Significant values ($P \leq 0.001$)

[b] Significant values ($P < 0.05$)

Table 6. Distribution of shared *Phaeoacremonium minimum* multilocus genotypes (indicated by a letters) in six vineyards and two rootstock mother blocks in the Western Cape Province.

Population	March	April	May	June	July	August	September	October	November	December
Paarl A		M	B,M	B,M	M	P	D,P	P	D	
Paarl Z		L,M,R		S	E,M,P,Q	E,M,Q	M,P	P	N,P	
Stellenbosch B3		B,G,M	M	B,M	B,E,M,N,P	D,E,J,M	M,N,P	F,M,P	N,O,P	
Stellenbosch P2		C,M,N,P		B,J,K,M	I,M,P	P	B,C,F,M,N	H	O	
Durbanville		L,M	B,M,Q	L,M,N	A,E,I,M	M,P,Q	E,P	M,O,P	N,O	
Rawsonville		B,M,P	M	M,N,G	B,M	N,P	B,D,M		B,O	
Slanghoek	B,M,R	B,J,K,L,M,N,P,Q,	M							
Wellington		M	H,K	E,L,M,N	M	C,N,P	E,M,O,P,Q	P	M,O,P	M
Total	3	11	5	9	8	8	10	5	6	1

Table 7. Analyses of molecular variance (AMOVA) with geographic origin as grouping factors.

Source	df	SS	MS	Est. Var.	%	Stats	Value	P value
Among Pops	7	66.307	9.472	0.252	6%			
Within Pops	175	653.764	3.736	3.736	94%			
Total	182	720.071		3.987	100%	PHiPT	0.063	0.001

Table 8. Isolates of four microsatellite multilocus genotypes (MLGs) that occurred 17 times or more, indicating the vine position and the mating type (*MAT* type).

MLG	Vineyard/Row/Vine Number	*MAT* type	Collection date
M (77)	Paarl A (R4)	2	16/07/2013
	Paarl A (R10)	2	08/04/2013
	Paarl A (R10)	2	02/07/2013
	Paarl A (R23)	2	28/05/2013
	Paarl A (R23)	2	25/06/2013
	Paarl A (R23)	2	16/07/2013
	Paarl A (AJ3)[a]	2	30/04/2013
	Paarl Z (R3V3)	2	16/07/2013
	Paarl Z (R3V3)	1	30/07/2013
	Paarl Z (R3V3)	1	13/08/2013
	Paarl Z (R3V3)	1	03/09/2013
	Paarl Z (R4V3)	2	03/09/2013
	Paarl Z (R4V5)	1	02/04/2013
	Paarl Z (R4V5)	1	23/04/2013
	Paarl Z (R4V5)	1	16/07/2013
	Paarl Z (R4V5)	1	13/08/2013
	Paarl Z (R4V5)	1	03/09/2013
	Stellenbosch B3 (R14P1)	1	23/04/2013
	Stellenbosch B3 (R14P1)	2	23/04/2013
	Stellenbosch B3 (R14P1)	2	21/05/2013
	Stellenbosch B3 (R14P13)	2	21/05/2013
	Stellenbosch B3 (R16)	1	02/04/2013
	Stellenbosch B3 (R16)	2	30/07/2013
	Stellenbosch B3 (R16)	1	01/10/2013
	Stellenbosch B3 (R21)	2	11/06/2013
	Stellenbosch B3 (R21)	2	06/08/2013
	Stellenbosch B3 (R21)	2	17/09/2013
	Stellenbosch P2 (R7)	2	30/04/2013
	Stellenbosch P2 (R9A)	1	11/06/2013
	Stellenbosch P2 (R11B)	1	18/06/2013
	Stellenbosch P2 (R16)	2	16/07/2013
	Stellenbosch P2 (R19)	2	04/06/2013
	Stellenbosch P2 (R19)	2	03/09/2013
	Stellenbosch P2 (R23)	2	16/04/2013
	Stellenbosch P2 (23)	2	30/07/2013
	Durbanville (R5)	1	28/05/2013
	Durbanville (R5)	1	02/07/2013
	Durbanville (R12)	2	16/04/2013
	Durbanville (R12)	1	04/06/2013
	Durbanville (R20)	1	23/04/2013
	Durbanville (R20)	1	13/08/2013

	Durbanville (R20)	1	08/10/2013
	Durbanville (R28)	2	23/04/2013
	Durbanville (R28)	1	25/06/2013
	Durbanville (R31)	2	30/04/2013
	Durbanville (R31)	2	14/05/2013
	Rawsonville (R8)	1	08/04/2013
	Rawsonville (R8)	2	17/09/2013
	Rawsonville (R9)	2	28/05/2013
	Rawsonville (R9)	1	08/04/2013
	Rawsonville (R9A)	1	11/06/2013
	Rawsonville (R24A)	1	11/06/2013
	Rawsonville (R24B)	2	09/07/2013
	Rawsonville (R36)	1	28/05/2013
	Rawsonville (R36A)	2	09/07/2013
	Rawsonville (R36)	2	03/09/2013
	Rawsonville (R42)	2	11/06/2013
	Rawsonville (R42)	2	28/05/2013
	Rawsonville (RJ37)[a]	2	28/05/2013
	Slanghoek (H1)	1	02/04/2013
	Slanghoek (H1)	1	02/04/2013
	Slanghoek (H1)	2	02/04/2013
	Slanghoek (H1)	1	07/05/2013
	Slanghoek (H1)	1	07/05/2013
	Slanghoek (H1)	2	23/04/2013
	Slanghoek (H3)	1	02/04/2013
	Slanghoek (H3)	1	16/04/2013
	Slanghoek (H5)	2	18/03/2013
	Wellington (W2)	2	02/07/2013
	Wellington (W4)	2	08/04/2013
	Wellington (W4)	1	23/04/2013
	Wellington (W4)	1	19/06/2013
	Wellington (W4)	2	16/07/2013
	Wellington (W4)	1	17/09/2013
	Wellington (W4)	1	03/12/2013
	Wellington (W5)	1	19/06/2013
	Wellington (W5)	2	16/07/2013
P (36)	Paarl A (R10)	2	06/08/2013
	Paarl A (R10)	2	15/10/2013
	Paarl A (R10)	1	05/11/2013
	Paarl A (R35)	1	10/09/2013
	Paarl Z (R3V3)	1	01/10/2013
	Paarl Z (R3V3)	1	05/11/2013
	Paarl Z (R4V3)	2	29/10/2013
	Paarl Z (R4V5)	2	05/11/2013
	Paarl Z (R4V5)	1	19/11/2013
	Paarl Z (R4V5)	2	26/11/2013

	Paarl Z (R5V4)	2	10/09/2013
	Paarl Z (R5V4)	2	01/10/2013
	Paarl Z (R5V4)	2	05/11/2013
	Paarl Z (R5V7)	2	16/07/2013
	Paarl Z (R5V7)	2	30/07/2013
	Stellenbosch B3 (R16)	2	16/07/2013
	Stellenbosch B3 (R16)	2	15/10/2013
	Stellenbosch B3 (R18)	2	10/09/2013
	Stellenbosch B3 (R18)	1	22/10/2013
	Stellenbosch B3 (R18)	1	26/11/2013
	Stellenbosch P2 (R19)	1	02/04/2013
	Stellenbosch P2 (R19)	2	02/07/2013
	Stellenbosch P2 (R19)	1	13/08/2013
	Durbanville (R5)	1	10/09/2013
	Durbanville (R12)	1	20/08/2013
	Durbanville (R28)	2	08/10/2013
	Rawsonville (R8)	2	20/08/2013
	Rawsonville (R36)	2	08/04/2013
	Rawsonville (R36)	1	06/08/2013
	Slanghoek (H2)	2	23/04/2013
	Wellington (W5)	2	13/08/2013
	Wellington (W4)	1	23/09/2013
	Wellington (W4)	1	22/10/2013
	Wellington (W5)	2	01/10/2013
	Wellington (W5)	2	19/11/2013
	Wellington (W5)	2	05/11/2013
B (19)	Paarl A (R4)	1	28/05/2013
	Paarl A (R10)	2	04/06/2013
	Paarl A (R10)	2	19/06/2013
	Stellenbosch B3 (R14P13)	1	08/04/2013
	Stellenbosch B3 (R14P13)	1	11/06/2013
	Stellenbosch B3 (R18)	2	09/07/2013
	Stellenbosch P2 (R7)	2	04/06/2013
	Stellenbosch P2 (R7)	2	23/09/2013
	Stellenbosch P2 (R7D)	1	11/06/2013
	Stellenbosch P2 (R9)	2	21/05/2013
	Stellenbosch P2 (11)	1	10/09/2013
	Durbanville (D12)	1	07/05/2013
	Rawsonville (R8)	1	26/11/2013
	Rawsonville (R24)	1	30/04/2013
	Rawsonville (R24)	2	30/07/2013
	Rawsonville (R42)	2	08/04/2013
	Rawsonville (R42)	2	03/09/2013
	Slanghoek (H4)	1	18/03/2013
	Slanghoek (H4)	1	16/04/2013
N (17)	Paarl Z (R4V3)	1	19/11/2013

Paarl Z (R5V7)	2	05/11/2013
Stellenbosch B3 (R14P1	1	23/09/2013
Stellenbosch B3 (R14P1	1	19/11/2013
Stellenbosch B3 (R21)	2	30/07/2013
Stellenbosch B3 (R21)	2	26/11/2013
Stellenbosch P2 (R19A)	1	10/09/2013
Stellenbosch P2 (R9D)	1	10/09/2013
Stellenbosch P2 (R23)	2	08/04/2013
Durbanville (R20)	2	25/06/2013
Durbanville (R20)	1	26/11/2013
Rawsonville (R24B)	1	11/06/2013
Rawsonville (R42A)	1	05/08/2013
Slanghoek (H1)	2	16/04/2013
Slanghoek (H1)	2	16/04/2013
Wellington (W2)	1	19/06/2013
Wellington (W5)	2	13/08/2013

[a] Isolates from young vines placed adjacent to the old vineyards.

LIST OF FIGURES

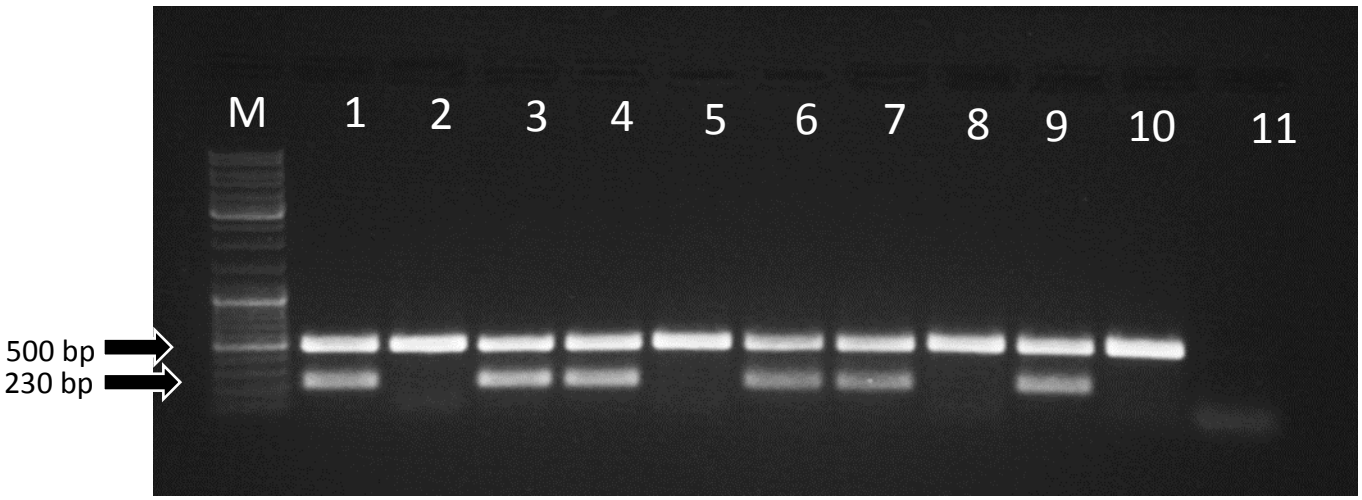

Figure 1: Example of a gel of the multiplex PCR for simultaneous amplification of *Phaeoacremonium minimum* and the *Mat 1-2* locus. The 500 bp fragment confirms Pm. minimum and the 230 bp fragment the presence of the *Mat 1-2* locus. Lanes 1, 3, 4, 6, 7 and 9 represent isolates of the *MAT1-2* mating type. Lane M contained a 100 bp DNA ladder (Promega) and lane 11 the negative control.

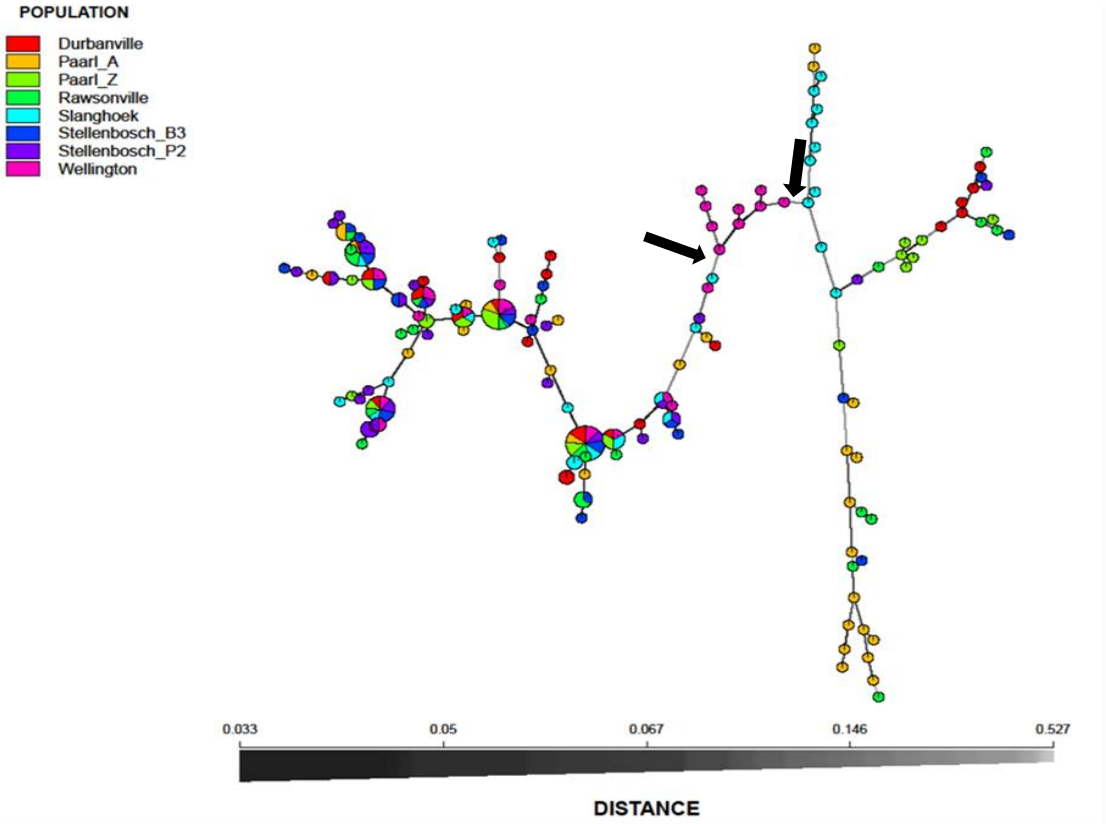

Figure 2. A minimum spanning network based on pairwise distances between 134 MLGs in the *Phaeoacremonium minimum* populations. Each node represents a different MLG. Node sizes and colors correspond to the number of individuals and population membership, respectively. Edge thickness and color are proportional to absolute genetic distance.

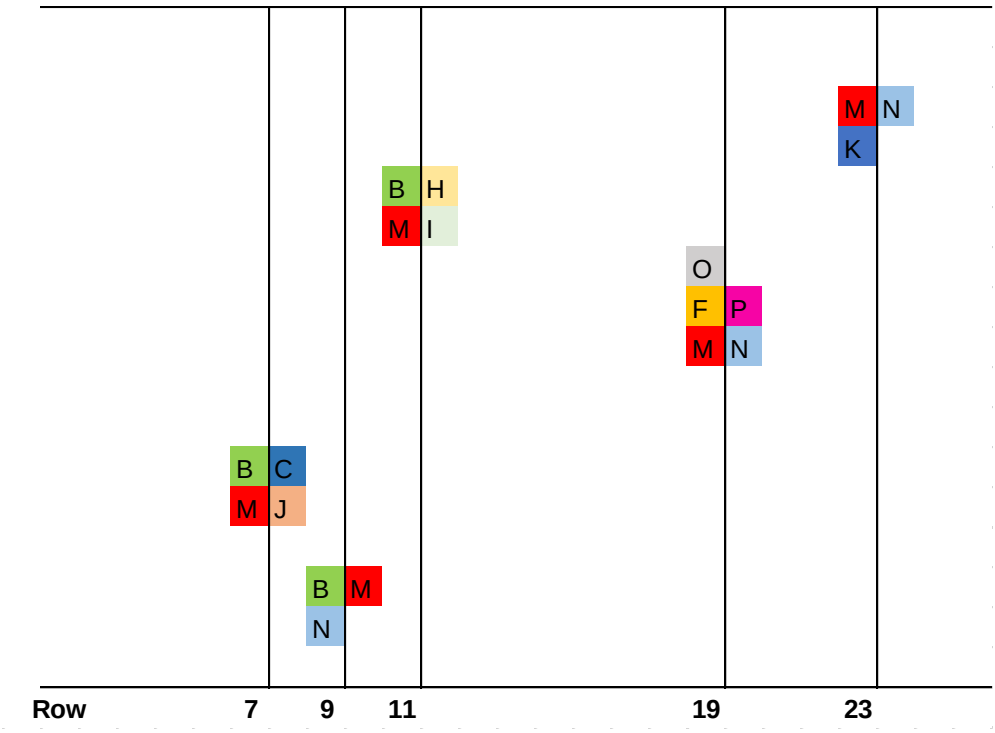

Figure 3. Vineyard layout of Stellenbosch P2 with distribution of shared multilocus genotypes of *Phaeoacremonium minimum* on five vines.

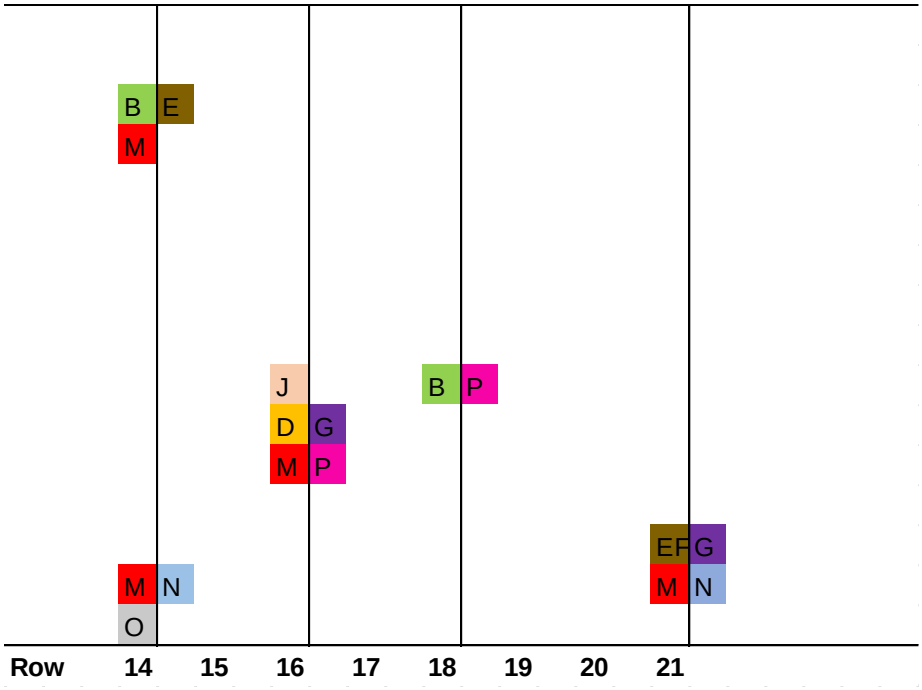

Figure 4. Vineyard layout of Stellenbosch B3 with distribution of shared multilocus genotypes of *Phaeoacremonium minimum* on five vines.

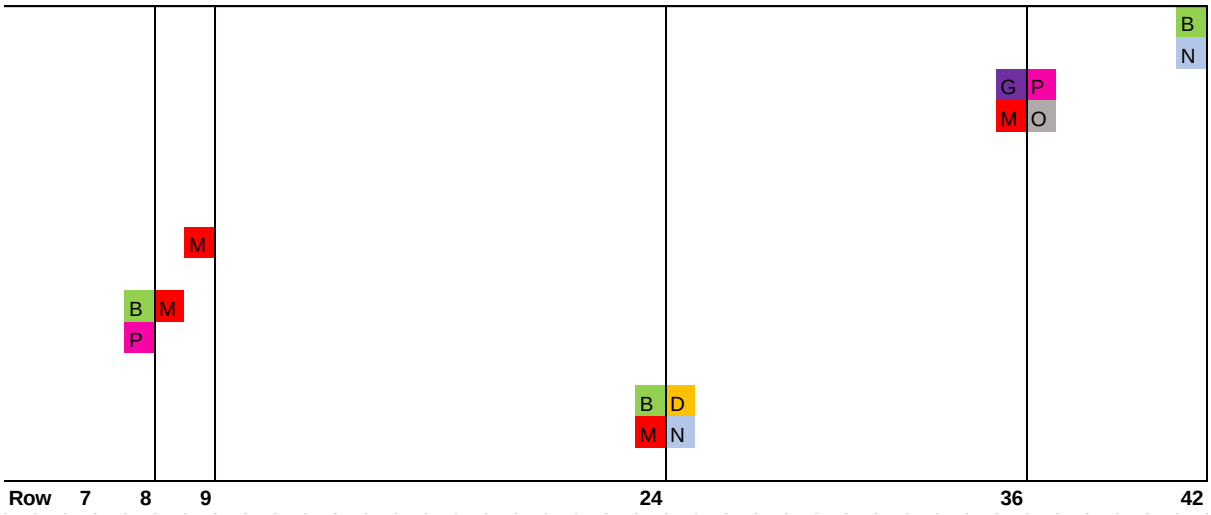

Figure 5. Vineyard layout of Rawsonville with distribution of shared multilocus genotypes of *Phaeoacremonium minimum* on five vines.

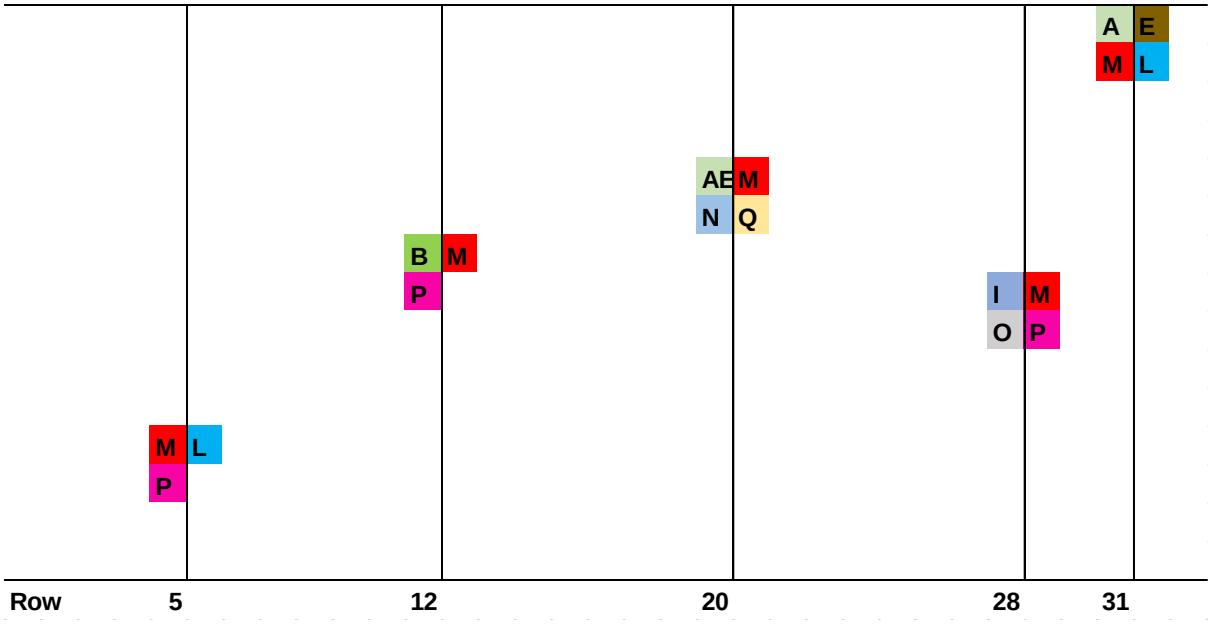

Figure 6. Vineyard layout of Durbanville with distribution of shared multilocus genotypes of *Phaeoacremonium minimum* on five vines.

CHAPTER 6

RESEARCH CONCLUSION, REMARKS AND RECOMMENDATIONS

The main aim of this study was to get a better understanding of the ecology of Petri disease pathogens found in vineyards of the Western Cape Province of South Africa. Spore traps were placed in vineyards to study the diversity of Petri disease pathogens and the period of spore release. Vineyard surveys were conducted to find *Phaeomoniella chlamydospora* pycnidia and *Phaeoacremonium* perithecia. Mating studies with selected *Phaeoacremonium* species were also conducted to induce and describe the sexual morphs. Futhermore the pathogenic status of selected *Phaeoacremonium* species occurring in South Africa were determined on pruning wounds of field-grown vines. Lastly the population structure of *Phaeoacremonium minimum* was assessed for genetic variation to understand the main mode of reproduction as source of inoculum.

Species diversity in vineyards

The current study reported a total of 15 Petri disease pathogens to occur as aerial spore inoculum in vineyards. This is the highest species diversity of Petri disease pathogens ever reported in a spore trapping trial compared to only two and three Petri disease pathogens reported in France and California, respectively (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). Like in other reports, *Pa. chlamydospora* and *Pm. minimum* were the most commonly occurring pathogens with either one of the two occurring as the predominant species in a vineyard. However, *Pm. sicilianum* was the predominant species in Rawsonville. This study serves as the first report on the occurrence of *Pm. inflatipes* in South African vineyards. The finding of some species in the current study only reported previously in *Prunus* spp., namely *Pm. griseo-olivaceum* and *Pm. pruniculum* raised the suspicion of them being capable of infecting grapevines. The potential role of fruit orchards as reservoirs for Petri disease pathogens has previously been reported (Cloete *et al*., 2011; Arzanlou *et al*., 2013). This study confirmed the possibility of cross contamination to occur between vineyards and fruit orchards which result in a high diversity of *Phaeoacremonium* species, whereas, establishing the two in close proximity is a common practice in the Western Cape Province. Eleven of the species trapped as aerial spore inoculum in this study were previously reported on other woody hosts especially in fruit orchards worldwide. This includes *Pm. alvesii*, *Pm. australiense*, *Pm. griseorubrum*, *Pm. inflatipes*, *Pm. iranianum*, *Pm. minimum*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. sicilianum*, *Pm. viticola* and *Pm. venezuelense*. The results highlight the importance of management strategies to prevent infections and reduce inoculum

in both vineyards and fruit orchards. Species of *Phaeoacremonium* were reported to occur in both vineyards and fruit orchards. These species were also capable of infecting and causing lesions in both vines and fruit trees (Cloete *et al*., 2011; Arzanlou *et al*., 2013). Therefore, management strategies such as sanitation and pruning wound protection should also take place in fruit orchards. As to whether all these species found can be effectively prevented by just one type of wound protectant is unknown and is worth investigating. Furthermore, older vineyard blocks that are about to be removed can serve as sources of inoculum when spores are released to newly established blocks. This is supported by the trapping of *Pm. minimum* isolates of the same genotype and mating type in old and newly established blocks next to each other. This highlight the need for farmers to adopt pruning wound protection practices in young vineyards to minimize infection of young vines, as well as sanitation practices in older vineyards to reduce inoculum.

Spore release of Petri disease pathogens

Aerial spores were available throughout the trapping periods, especially during winter and spring pruning periods in both years. The spore release peaks coinciding with standard pruning periods in South Africa indicate the constant availability of spores during periods of wounding. It is known that these pruning wounds are main ports of entry of Petri disease pathogens, and that pruning wounds remain susceptible for up to two months. This study recommends the development of pruning wound protectants best suited for use on dormant (winter pruning wounds) as well as active vines (spring pruning wounds) for effective protection from infection of Petri disease pathogens. The winter season in the Western Cape is characterized by rainy weather, a factor that should be taken into consideration when pruning wound protectants are developed.

Wounds made on rootstock mother plant stumps during harvesting of shoots poses a serious risk of new infections each season. Spores of Petri disease pathogens are present during this period to infect susceptible wounds. There are currently no wound protection programs developed to protect these wounds. Therefore, this study suggests future research on developing effective pruning wound protection programs to prevent infection occurring in rootstock mother blocks. This, together with currently available molecular techniques for rapid detection of Petri disease pathogens will consequently reduce the use of infected propagation materials and thus, reduce disease spread in newly planted vineyards.

Previous studies showed high spore release of *Pm. minimum* to coincide with high rainfall periods in Californian vineyards (Eskalen and Gubler, 2001). However, this study did not show any positive correlation between spore release peaks and rainfall. Further research

over a longer period and different climatic regions is recommended to confirm if spores of Petri disease pathogens are correlated with weather conditions in South Africa.

Spore release of species found in vineyards differed in frequency of spore release events and the amount of aerial spores recorded. This is in reference to *Pm. sicilianum* which was predominant in Rawsonville and was only found in this vineyard and Slanghoek. The areas of Rawsonville and Slanghoek and those in which this species was not reported are geographically separated by a mountain, and have warmer temperatures. It could be that *Pm. sicilianum* have long been introduced and established over time in Rawsonville, but have a restricted movement as a result of geographical constraint. The source of inoculum and the mode of reproduction for this species is unknown and is worth investigating. This can be achieved by developing molecular tools that will enable the determination of mating type gene distribution and to also develop microsatellite markers to predict the occurrence of sexual reproduction within this species and the source of inoculum.

Source of inoculum and mating studies

Phaeomoniella chlamydospora and *Pm. minimum* were the predominant species found in vineyards, except in Rawsonville where *Pm. sicilianum* was the predominant species. Intensive surveys for the occurrence of primary sources of inoculum for these pathogens were undertaken in the same vineyards in which spore trapping was conducted. This study reported for the first time in South African vineyards, the presence of pycnidia of *Pa. chlamydospora* and the sexual morph of *Pm. minimum*. The fruiting structures were found on the bark, in small cracks, pruning wound crevices, and inside deep cracks along the trunk of infected grapevines. Finding of fruiting bodies in cracks and crevices should be considered in future studies when developing management programs on inhibition of the formation of Petri disease fruiting bodies.

The fact that fruiting bodies were found on dead vine debris within vineyards and rootstock mother blocks, strongly emphasize the need to practice sanitation, by removing pruning debris from vineyards. This practice could reduce the number of fruiting bodies and aerial spore load produced on infected debris. Finding of perithecia was difficult and it could not be found in all the vineyards and rootstock mother blocks studied. However, the near equal mating type distribution previously reported (Mostert *et al*., 2003) shows that sexual reproduction is likely to occur in vineyards. The minute size of the perithecia and common habitat in deep cracks and crevices due to their high humidity requirement (Pascoe *et al*., 2004; Rooney-Latham *et al*., 2005) could be the reason for not finding them in some vineyards.

The number of perithecia of *Pm. minimum* were high in the Slanghoek rootstock mother block, which suggest that aerial spores released from these structures can infect

susceptible wounds made during shoot harvesting and infect the rootstock mother plant. The spores could be transported through the xylem vessels or through sap of the mother plant into xylem tissue of the shoots to be harvested. There is, therefore, a need to develop wound protect programs in rootstock mother blocks to prevent infection of the mother plant so as to increase their lifespan. The role of infected rootstock material as the main source of spread of *Pa. chlamydospora* is well known (Fourie and Halleen, 2004). The high number of perithecia found in Slanghoek indicates the high risk of spreading different mating types and new genotypes to areas where previously not reported.

The sexual morphs of *Pm. australiense* and *Pm. scolyti* were successfully induced *in vitro* in this study, and this is the first time the fruiting bodies of these two species were described. The period of perithecia formation was longer than that of previously described species (Mostert *et al*., 2006). Although these fruiting structures have not yet been found in nature, results from the current study indicate the capability of South African isolates to form perithecia. Both species have a heterothallic mating type system. For *Pm. australiense* successful crosses were from isolates of different vineyards. This could be the reason why perithecia of *Pm. australiense* were not found in nature. It is, however, a matter of time before mating types of *Pm. australiense* could overlap as a result of movement of infected plant materials. On the other hand, the sexual morph of *Pm. scolyti* is likely to form in Rawsonville, Slanghoek and Wellington where both mating types were found occurring in the same vineyard. Further studies are required to characterize the optimal conditions required for these structures to form in the laboratory, so as to shed a light on the conditions in which they are likely to form and mature in nature.

Phaeoacremonium sicilianum spores were caught in high numbers in Rawsonville, however, the fruiting structures of these species have not been found during the survey studies. Further studies on the source of inoculum for these species are recommended. The use of molecular tools will also enable the prediction of the type of reproduction occurring within this species and mating type distribution. Therefore, relevant molecular tools need to be developed to determine the mating type and genetic diversity of this species. Optimization of growth conditions such as light and darkness ratio and temperature ranges to induce the fruiting structures *in vitro* are also recommended. Although this method is time consuming, it could assist in the knowledge of the kind of structures to expect during field surveys.

Pathogenicity of *Phaeoacremonium* species

Spore trapping results showed a high diversity of *Phaeoacremonium* species which are present as aerial spore inoculum within vineyards and rootstock mother blocks. However, the pathogenicity status of a few of these species on grapevines is unknown. The present study

investigated the pathogenicity status of four *Phaeoacremonium* spp. found as aerial spore inoculum in vineyards (Chapter 2), and six species reported in South Africa during previous survey studies (White *et al*., 2011). All species tested namely, *Pm. alvesii*, *Pm. australiense*, *Pm. austroafricanum*, *Pm. fraxinopennsylvanicum*, *Pm. griseo-olivaceum*, *Pm. griseorubrum*, *Pm. iranianum*, *Pm. pruniculum*, *Pm. scolyti* and *Pm. sicilianum* are confirmed as pathogens of grapevines.

The current study therefore confirmed the pathogen status of *Pm. alvesii*, *Pm. australiense*, *Pm. fraxinopennsylvanicum*, *Pm. iranianum*, *Pm. scolyti* and *Pm. sicilianum* on grapevines as previously reported in other countries (Gramaje *et al*., 2007; Aroca and Raposo, 2009; Gramaje *et al*., 2009; Mohammadi *et al*., 2012; Özben *et al*., 2012; Úrbez-Torrez *et al*., 2014; Mohammadi and Hashemi, 2015). The two species previously reported as pathogens of *Prunus* spp., namely *Pm. griseo-olivaceum* and *Pm. pruniculum* were also pathogenic to grapevine pruning wounds. This finding emphasizes the role of *Prunus* orchards established close to vineyards to serve as reservoirs for *Phaeoacremonium* species.

Most inoculated species formed lesions which also extend beyond the inoculated two node spur, therefore, showing the quick rate at which infection can extend from wounds into vine cordons. Although double pruning is a standard practice in most vineyards around the Western Cape, aerial spore inoculum is available even during late winter pruning to infect susceptible wounds when final pruning is conducted. Therefore, the pruning wound made at the second node at this time should be protected against infection.

The study confirms pruning wounds as an important port of entry for Petri disease pathogens, which is consistent with results from previous research. This shows the importance of protecting pruning wounds whenever wounding occurs in the vineyards, because spores are available to infect wounds whenever pruning activities occur in South African vineyards.

Genetic diversity of *Phaeoacremonium minimum*

Populations of *Pm. minimum* in vineyards showed high gene diversity, with some of the populations moderately diverse from each other. The two mating types required for sexual reproduction to occur were present in near to equal numbers vineyards except for Paarl A and Wellington. The results indicate that both sexual and asexual reproduction occur in *Pm. minimum* populations. New genotypes could be generated through sexual reproduction and maintained by asexual reproduction.

Factors influencing the occurrence of both these reproduction modes in vineyards are unknown and are worth investigating. This could be influenced by prevailing weather conditions, especially because stressful conditions such as low temperatures or drought are

known to trigger sexual reproduction in fungi (Elliot, 1994). Future studies illustrating conditions that trigger preference in mode of reproduction will shed light as to when ascospores or conidiospores are likely to be released in vineyards.

In general few sexual structures of *Pm. minimum* were found in Western Cape vineyards. This, together with the I_A and $\bar{r}_d$ indices that indicate asexual reproduction, calls for the need to determine virulence levels of the high number of MLGs for *Pm. minimum* found in local vineyards (Gramaje *et al*., 2013). In addition, determining the response of isolates from these MLGs to developed control strategies such as fungicides and biological control agents for a sustainable disease management strategy are required.

This will aid the understanding of the role of these two modes of reproduction as far as infection and epidemiology of *Pm. minimum* is concerned. The source of asexual aerial spores occurring in vineyards is most probably from mycelia. This finding highlights that sanitation or specifically, removal of old pruning wound debris alone may not be the only means of minimizing spore production in the field. Therefore, other means of post disease infection management needs to be developed.

The role of infected plant material on disease spread is well known. This is further supported by the number of same MLGs found in vineyards and Slanghoek rootstock mother block, which was genetically differentiated to only a few of the vineyards. Future studies consisting of samples from a wide range of different rootstock mother block and vineyards can therefore be critical in confirming this finding.

Phaeoacremonium minimum has also been isolated from several woody hosts in South Africa. Therefore, future genetic diversity studies could include isolates from these woody hosts found in the vicinity of vineyards. This will provide a better understanding of the genetic differentiation of MLGs from these hosts and vines, and highlight characters which could be exchanged and hinder future disease management.

CONCLUSION

To conclude, aerial spore inoculum consisting of diverse *Pa. chlamydospora* and 14 species of *Phaeoacremonium* is present for prolonged periods in vineyards and rootstock mother blocks to infect susceptible pruning wounds. All these species are capable of infecting grapevines and causing typical symptoms of Petri disease. Future research is required to understand the sources of aerial spores occurring in vineyards and their correlation to prevailing weather conditions. Fruiting bodies do form in vineyards, and mating studies also confirm the capability of these structures to form for *Pm. australiense* and *Pm. scolyti*, however, more surveys are still required to see if they occur in nature. These surveys need to

be expanded to orchards established near vineyards as species of *Phaeoacremonium* trapped in this study have also been reported in orchards.

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