THE ROLE OF ARTHROPODS IN THE DISPERSAL OF TRUNK DISEASE PATHOGENS ASSOCIATED WITH PETRI DISEASE AND ESCA

PROVIDENCE MOYO

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

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DECLARATION

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ABSTRACT

Petri disease and esca are devastating grapevine trunk diseases and compromise the sustainability of viticulture world-wide. Despite being extensively studied, knowledge of inoculum sources and mechanisms of spread of the causal pathogens is limited. Arthropods have been suspected to play a role in the spread of Petri disease and esca pathogens. However, little information is known about the extent to which arthropods are associated with these pathogens. This study aimed to determine whether arthropods occurring within or on declining grapevines, are associated with trunk disease pathogens and to identify arthropods associated with pruning wounds. The potential of selected arthropods to act as vectors of trunk disease pathogens was also investigated.

Two vineyards exhibiting grapevine trunk disease infections were sampled weekly for two years for collection of arthropods. Arthropods were collected using pruning wound traps, visual searches as well as trunk and cordon traps. Fungal spores from surfaces of arthropods were collected in water. Samples were subjected to nested PCR using primers Pm1/Pm2 and Pch1/Pch2 to verify the presence of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora*, respectively. Water samples were also cultured and grapevine trunk disease pathogens obtained were identified by sequencing the internal transcribed spacers 1 and 2 and the 5.8S rRNA gene or the partial beta-tubulin gene. A total of 10 875 arthropod individuals, belonging to more than 31 families, were collected from declining grapevines. The most abundant arthropods included millipedes, ants, spiders and beetles. Portuguese millipedes and cocktail ants were associated with fresh grapevine pruning wounds. Thirty-three percent of the 5677 water samples analysed, contained propagules of pathogens associated with Petri disease and esca. Of these, 37 % were recovered from millipedes, 22 % from cocktail ants, 15 % from spiders and 10 % from beetles. All the major groups of grapevine trunk diseases were detected on the arthropods. *Phaeoacremonium* species were detected in 1242 samples while *Phaeomoniella chlamydospora* was identified from 855 samples. Other fungi isolated included members of the Botryosphaeriaceae, Diatrypaceae and Diaporthales.

The potential of grapevine sap as a food source for Portuguese millipedes and cocktail ants was investigated, *in vitro*. Millipede individuals were offered a choice between water and grapevine sap while ants in nests were presented with grapevine sap, tuna and water and monitored for ingestion of sap. Both taxa preferred grapevine sap over the other

food items, indicating close association with pruning wounds. Subsequently, the ability of both taxa to transmit a DsRed-transformed *Phaeomoniella chlamydospora* isolate to fresh pruning wounds of canes in polystyrene strips, floating in water, and potted vines was tested. Arthropods were exposed to the fungus for 24 hours and transferred to the base of the plants and canes and were removed after three days. Isolations after a month revealed that millipedes and ants were capable of transmitting the fungus onto wounds and cause infection. Millipede faecal pellets were also evaluated as potential sources of inoculum. Millipedes were fed on *Phaeomoniella chlamydospora* for 24 hours, surface sterilised and allowed to defaecate in sterile Petri dishes overnight. Faecal material was collected, macerated in water and plated onto potato dextrose agar. Propagules of *Phaeomoniella chlamydospora* survived passage through the gut of millipedes and were passed out in a viable state to form colonies of *Phaeomoniella chlamydospora*.

This study concludes that a wide variety of arthropods can be a source of inoculum of trunk diseases in vineyards. The results of the dissemination trial provides evidence that millipedes and ants are able to disseminate and infect vines with *Phaeomoniella chlamydospora.* It is therefore, highly likely that other grapevine trunk disease pathogens are transmitted in the same manner. This knowledge highlights the need for control of certain arthropods to be taken into consideration when managing grapevine trunk disease pathogens.

OPSOMMING

Petri siekte en esca is verwoestende wingerd stamsiektes en verhinder die volhoubaarheid van wingerdproduksie wêreldwyd. Hierdie siektes is al intensief bestudeer, maar kennis rakende die inokulum bronne en meganismes van verspreiding van die veroorsakende patogene is beperk. Arthropoda is al vermoed om ‘n rol te speel in die verspreiding van Petri siekte en esca patogene, maar weinig informasie is bekend oor die mate waartoe arthropoda geassosieer is met die patogene. Hierdie studie het ten doel gestel om die arthropoda wat op of in wingerdstokke wat terugsterf voorkom te identifiseer en te bepaal watter van die arthropoda geassosieer is met stamsiekte patogene. Daar is ook ten doel gestel om die arthropoda wat geassosieer is met vars snoeiwonde te identifiseer en ook die moontlike vektor status van die stamsiekte patogene deur arthropoda.

Arthropoda is weekliks vir twee jaar gekollekteer vanaf twee wingerde met stamsiekte infeksies. Snoeiwond lokvalle, visuele soektogte en stam- en kordon lokvalle was gebruik om arthropoda te vang. Swamspore van die oppervlak van die arthropoda is afgewas met water. Van hierdie water monsters is gebruik om dubbelvoudige polimerase ketting reaksies (PKR) te doen met die inleiers Pm1/Pm2 en Pch1/Pch2 om vir die teenwoordigheid van *Phaeoacremonium* spp. en *Phaeomoniella chlamydospora* onderskeidelik te toets. Die oorblywende water monster is gekweek op medium om die swamme teenwoordig te bepaal. Die wingerd stamsiekte patogene is verder geidentifiseer deur die DNS volgordes te bepaal van die interne getranskribeerde spasies 1 en 2 en die 5.8S rRNS geen of ‘n gedeelte van die beta-tubulien geen. In totaal is 10 875 arthropoda, wat behoort tot 31 families, gekollekteer vanaf wingerde wat terugsterf. Die mees algemene arthropoda was duisendpote, miere, spinnekoppe en kewers. Die Portugese duisendpote en die wipstert mier is geassosieer met vars wingerd snoeiwonde. Van die 5677 water monsters wat geanaliseer is, het 33% propagules van die Petri siekte of esca patogene gehad. Van hierdie was 37 % afkomstig vanaf duisendpote, 22 % van wipstert miere, 15 % van spinnekoppe en 10 % van kewers. Al die hoofgroepe van wingerd stampatogene is opgespoor op die arthropoda. *Phaeoacremonium* species is opgespoor in 1242 monsters en *Phaeomoniella chlamydospora* is gevind in 855 monsters. Ander swamme wat ook geisoleer is sluit lede van die Botryosphaeriaceae, Diatrypaceae en Diaporthales in.

Die potensiaal van wingerdsap as ‘n bron van voedsel vir Portugese duisendpote en wipstert miere is *in vitro* ondersoek. Duisendpoot invidue is ‘n keuse gegee tussen water en

wingerd sap terwyl mierneste ‘n keuse gehad het tussen water, wingerd sap en tuna. Die duisendpote en miere is gemonitor vir die inname van wingerdsap in die teenwoordigheid van die ander bronne. Beide die duisendpote en miere het wingerdsap verkies wat aandui dat hulle ‘n noue assosiasie met wingerd snoeiwonde het. Vervolgens is beide taksons getoets vir hul vermoë om ‘n DsRooi-getransformeerde *Phaeomoniella chlamydospora* isolaat te vektor na vars snoeiwonde op lote gemonteer op polistireen stroke wat in water dryf en op wingerd plante in potte. Die duisendpote en miere is blootgestel aan die swam vir 24 uur en oorgedra na die basis van die plante en lote en is weer verwyder na drie dae. Na ‘n maand is isolasies gedoen wat gewys het dat die duisendpote en miere die swam suksesvol kon oordra na die snoeiwonde en infeksie veroorsaak. Duisendpoot uitwerpsels is geëvalueer vir die potensiaal as inokulum bron. Duisendpote het gevoed op *Phaeomoniella chlamydospora* vir 24 uur, daarna oppervlakkig gesteriliseer en toegelaat om oornag uitwerpsels te maak in steriele Petri bakkies. Uitwerpsels was gekollekteer, fyngemaak in water en op aartappel dekstrose agar uitgeplaat. Propagules van *Phaeomoniella chlamydospora* het die verteringskanaal van die duisendpote oorleef en het tipiese kolonies op die agar gevorm.

Hierdie studie het vasgestel dat ‘n verskeidenheid van arthropoda ‘n bron van inokulum van stamsiektes in wingerd kan wees. Die resultate van die vektor proewe het gewys dat duisendpote en miere die vermoë het om *Phaeomoniella chlamydospora* te versprei na snoeiwonde wat die swam dan suksesvol geinfekteer het. Dit is daarom hoogs waarskynlik dat van die ander wingerd stamsiekte patogene ook versprei kan word op dieselfde manier. Hierdie kennis demonstreer dat die beheer van spesifieke arthropoda in ag geneem moet word in die bestuur van wingerd stamsiektes.

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CONTENTS

CHAPTER 1

A REVIEW OF PETRI DISEASE AND ESCA AND THE ROLE OF ARTHROPODS IN THE DISPERSAL OF PLANT PATHOGENIC FUNGI

1.1 Importance of Petri disease and esca

Petri disease and esca are important grapevine trunk diseases as they have become a limiting factor for grapevine production. Their incidence and severity have increased in the last two decades and they have been reported in all the major viticultural areas around the world (Mugnai *et al*., 1999; Chicau *et al*., 2000; Larignon and Dubos, 2000; Edwards *et al*., 2001b; Eskalen and Gubler, 2001; Fourie and Halleen, 2004a; Zanzotto *et al*., 2007; Gramaje *et al*., 2008). It has been suggested that the increase in the incidence and severity is a result of factors such as the changes in the nursery and vineyard management practices, lack of pruning wound protection and the deregistration of effective fungicides due to toxicity (Graniti *et al*., 2000). Both Petri disease and esca are an impediment to profitability and sustained production because they can shorten the productive life of vineyards dramatically (Rolshausen *et al*., 2010). They are responsible for graft failure, loss of vigor and productivity in established vines (Mugnai *et al*., 1999; Rumbos and Rumbou, 2001; Whiting *et al*., 2001). Vineyard managements costs can increase as a result of labour involved in remedial pruning and/or replanting in vineyards where there is poor vine establishment (Rumbos and Rumbou, 2001; Whiting *et al*., 2001; Rolshausen *et al*., 2010). The clusters of berries borne on esca-infected vines display dark spots on the epidermis, known as black measles (Reisenzein *et al*., 2000) and this can be a problem to table grape growers because such clusters are unmarketable (Rooney-Latham *et al*., 2005b). In addition, the wine produced from affected clusters can be of poor quality (Calzarano *et al*., 2001).

1.2 Etiology of Petri disease and esca

Petri disease is caused by a combination of *Phaeomoniella (Pa.) chlamydospora* (W. Gams, Crous and M.J. Wingf. and L. Mugnai) Crous & W. Gams and several *Phaeoacremonium (Pm.*) W. Gams, Crous & M.J. Wingf. species (Scheck *et al*., 1998; Mugnai *et al*., 1999; Groenewald *et al*., 2001). Twenty-five species of *Phaeoacremonium* have been isolated from declining grapevines (Table 1), but *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai, is the most common and widely distributed (Crous *et al*.,

1996; Mugnai *et al*., 1999; Mostert *et al*., 2006; Essakhi *et al*., 2008). *Togninia* Berl. teleomorphs have been described for seven of the 25 *Phaeoacremonium* species (Mostert *et al*., 2006). Perithecia of three of the *Togninia* species, have been found in nature namely, *T. minima* (Tul. & C. Tul.) Berl. (Rooney-Latham *et al*., 2005a), *T. fraxinopennsylvanica* (T.E. Hinds) Georg Hasner, Eyjolfsd. & J. Reid and *T. viticola* L. Mostert, W. Gams & Crous (Eskalen *et al*., 2005a, 2005b).

A new definition of esca has been proposed and the term 'esca' is to be used when referring to wood decay (its original meaning) caused by basidiomycete fungi and the term 'grapevine leaf stripe' (previously known as 'young esca') is to be used for the vascular disease caused by *Pa. chlamydospora* and *Pm. aleophilum*. The term 'esca-proper' will then be used when both esca and grapevine leaf stripe occur together in a vine (Surico, 2009). However, several other fungi, which are involved in other grapevine declines, have been isolated from esca-affected vines. Such fungi include species of the Botryosphaeriaceae, Diatrypaceace as well as Diaporthales (Fischer and Kassemeyer, 2003; Edwards and Pascoe, 2004; Calzarano and Di Marco, 2007; Péros *et al*., 2008; White *et al*., 2011). However, for the purpose of this study, the biology of the pathogens that cause grapevine leaf stripe will be discussed with regards to esca.

1.3 Symptoms of Petri disease and esca

External symptoms of Petri disease include stunted growth, shortened internodes, reduced vigor, shoot dieback as well as a general decline of young grapevines resulting in plant death (Fourie and Halleen, 2004a; Retief *et al*., 2005; Gramaje *et al*., 2008). The common internal symptom is the black/brown wood streaking with the presence of gummy masses in the xylem vessels which have been found to originate from wounds or the graft union. The gummy masses, also known as black goo (Fig. 1A), appear as minute black/brown spots in cross section and as black/brown streaks when viewed longitudinally. *Phaeomoniella chlamydospora* and species of *Phaeoacremonium* have been isolated from these black spots of declining grapevines (Mugnai *et al*., 1999; Del Rio *et al*., 2001; Whiting *et al*., 2001).

The term 'esca', in this context, will be used to include the occurrence of both the external and/or internal symptoms on vines. The leaves of esca-affected vines develop an interveinal foliar chlorosis or reddening resulting in a 'tiger stripe' pattern (Fig. 1B).

However, these leaf symptoms are discontinuous, not occurring every year on diseased vines (Redondo *et al*., 2001; Marchi *et al*., 2006). The berries on affected vines become shrivelled and discoloured with minute black spots known as 'black measles'(Fig. 1C) and dieback of the shoot tips occur (Mugnai *et al*., 1999; Reisenzein *et al*., 2000; Edwards and Pascoe, 2004). The severe form of esca, also known as apoplexy, is a sudden wilting of the entire plant including the clusters of berries. Apoplexy is thought to be favoured by hot summers, in particular when rainfall is followed by dry, hot weather (Mugnai *et al*., 1999; Peros *et al*., 2008). Internal symptoms of esca are similar to those of Petri disease but can be distinguished by white rot (Fig. 1D) in cross sections of trunks or cordons. The white rot is often surrounded by a thick black/brown margin (Mugnai *et al*., 1999; Koklu, 2000; Pollastro *et al*., 2000; White *et al*., 2011). White rot has been found in the trunk next to pruning wounds and extending into the basal parts of the plant, usually within a section of the internal tissue and can spread to the surface of the trunk (Fischer and Kassemeyer, 2003).

1.4 Sources and dispersal of Petri disease and esca pathogens

Pathogens that cause Petri disease and esca are thought to occur as latent infections in the tissues of grapevines and that they probably become pathogenic after vines are subjected to stress. Such stress include water deficit and improper planting procedures (Mugnai *et al*., 1999; Whiting *et al*., 2001; Retief *et al*., 2005; Rooney-Latham *et al*., 2005c; Di Marco and Osti, 2009).

Several inoculum sources for *Pa. chlamydospora* and *Phaeoacremonium* spp. have been identified. Rootstock mother vines and various propagation processes have been shown to be primary inoculum sources for these pathogens (Mugnai *et al*., 1999; Halleen *et al*., 2003; Edwards and Pascoe, 2004; Aroca *et al*., 2010). Isolations have shown that these pathogens are present in apparently healthy rootstock mother vines (Fourie and Halleen, 2004b) and cuttings (Halleen *et al*., 2003) as well as grafted nursery plants (Zanzotto *et al*., 2001). Mycelial growth of *Pa. chlamydospora* (Pascoe and Cottral, 2000) and spores of *Pm. aleophilum* and *Pm. inflatipes* W. Gams, Crous & M.J. Wingf. (Feliciano and Gubler, 2001) have been observed within xylem vessels of grapevines. These findings led to the hypothesis that infection by these pathogens occur via spores or hyphae from mother vines into canes (Fourie and Halleen, 2002; Edwards *et al*., 2003). Different propagation processes and stages which include grafting, callusing and hydration tanks have been found to be sources

of inoculum for Petri disease pathogens. In 2001, researchers in Italy investigated the occurrence of Petri disease fungi on grafted vines and reported detecting Petri disease pathogens and suggested that contamination occurred after grafting (Zanzotto *et al*., 2001). *Phaeomoniella chlamydospora* was later found to be present in hydration tanks, grafting tools, callusing media and soil in South African nurseries (Retief *et al*., 2006). *Phaeomoniella chlamydospora* and some species of *Phaeoacremonium* were also detected in hydration tanks, scissors, grafting machines as well as in peat used for root development in Spanish nurseries, using PCR-based detection techniques (Aroca *et al*., 2010). Retief *et al*. (2006) suggested that mycelium and conidia present on the surfaces of grapevine cuttings probably wash off into the water during hydration or it oozes from the xylem vessels into the water.

The potential of soil as a source of inoculum is shown through the recovery of Petri disease pathogens from soil in vineyards. *Phaeoacremonium inflatipes* was recovered from soil and standing water in Californian vineyards (Rooney *et al*., 2001). *Phaeomoniella chlamydospora* was detected, using molecular techniques, from soil samples collected from beneath rootstock mother vines known to be infected with the pathogen in South African (Damm and Fourie, 2005; Retief *et al*., 2006) and New Zealand (Whiteman *et al*., 2005) vineyards. *Phaeomoniella chlamydospora* has been suggested to be present in vineyard soils as mycelium, conidia, chlamydospores or fruiting structures (Retief *et al*., 2006). It has also been suggested that *Pa*. *chlamydospora* could be a soil-borne pathogen because of its ability to form chlamydospores in culture (Bertelli *et al*., 1998). The chlamydospores are also thought to form conidia that can germinate and penetrate roots of vines in nurseries and vineyards (Bertelli *et al*., 1998; Mugnai *et al*., 1999).

Diseased grapevine wood act as inoculum sources for Petri disease pathogens in vineyards. *Phaeomoniella chlamydospora* was recovered from plant sap and on the outer bark of diseased grapevines (Rooney *et al*., 2001). Pycnidia of *Pa*. *chlamydospora* have been found to survive on grapevine bark tissue (Edwards and Pascoe, 2001) and are thought to be the sources of *Pa*. *chlamydospora* spores in the vineyards (Eskalen and Gubler, 2001). *Phaeomoniella chlamydospora* was found sporulating inside cracks of diseased vines (Edwards *et al*., 2001a; Edwards and Pascoe, 2001). Perithecia of *Togninia* species have been found on dead vascular tissue in deep cracks of trunks and cordons and on decaying pruning wounds of affected grapevines in vineyards (Eskalen *et al*., 2005a, 2005b; Rooney-Latham *et al*., 2005a). Perithecia of *T*. *viticola* and *T*. *fraxinopennsylvanica* were also found

on ash trees located close to the vineyards in California (Eskalen *et al*., 2005a, 2005b). The presence of perithecia on ash trees illustrates the possibility of infected trees surrounding vineyards of being sources of inoculum (Eskalen *et al*., 2007a).

In vitro studies conducted in California showed that ascospores of *T. minima* are released from perithecia after precipitation (Rooney-Latham *et al*., 2005b). The asci emerged through the ostiole and either accumulated at the ostiole or contracted and forcibly discharged the ascospores. It was then speculated that the same mechanism of spore release occurs in vineyards and the ascospores that are forcibly discharged are then aerially dispersed and could land on fresh grapevine pruning wounds and cause infection. Spore trapping studies conducted in Californian vineyards showed that *Pa. chlamydospora* and *Pm. aleophilum* can be spread as airborne inoculum (Eskalen and Gubler, 2001). Spore release for *Pa. chlamydospora* occurred during and after rainfall in late winter and early spring and coincided with pruning and pruning wounds, but spore release for *Pm. aleophilum* was not always correlated with rainfall. Larignon and Dubos (2000) also found that the occurrence of *Pa. chlamydospora* was correlated with rainfall and rainfall plays an important role in the release of aerial inoculum in French vineyards.

The presence of spores of *T. minima* in the vineyards, in the absence of rainfall, could be explained by the ascospores that accumulate at the ostiole of perithecia of *T. minima* (Rooney-Latham *et al*., 2005a). These ascospores are then spread by irrigation practices or insects (Rooney-Latham *et al*., 2005a). Spores of *T. minima* were successfully isolated from drip irrigation puddles under grapevines in California, but were not isolated directly from irrigation water as it passed through the emitter (Rooney *et al*., 2001). This indicates that the water was contaminated after splashing over the vine. Insects may also contribute to the dispersal of ascospores because they are produced in a slimy droplet (Rooney-Latham *et al*., 2005a) which is ideal to stick or smear onto insects moving over the diseased wood. Evidence that aerial inoculum might not be the only mechanism of pathogen dispersal in the field is shown in recent studies in Italy (Michelon *et al*., 2007) and South Africa (Van Niekerk *et al*., 2010), which failed to trap spores of pathogens in the air using volumetric spore traps. The different spatial patterns of disease symptoms observed in vineyards affected by esca further supports the hypothesis that several mechanisms may be involved in the dispersal of pathogens. The different spatial patterns observed included a tendency for infected vines to be aggregated along rows (Mugnai *et al*., 1999; Pollastro *et al*., 2000), a random spatial pattern of infected vines (Reisenzein *et al*., 2000; Redondo *et*

al., 2001; Marchi *et al*., 2006) as well as both aggregation and random spatial patterns (Surico *et al*., 2000; Edwards *et al*., 2001b). These patterns can be attributed to different modes of dispersal which may include insects, propagation material, rain splash as well as air currents (Reisenzein *et al*., 2000; Surico *et al*., 2000).

1.5 Disease management

The eradication of Petri disease and esca fungi once they have colonised the grapevines is difficult (Mugnai *et al*., 1999; Di Marco *et al*., 2004). Several studies have shown that grapevines are infected during nursery propagation stages and therefore, propagation material and different nursery stages act as a source of inoculum for Petri disease and esca fungi (Zanzotto *et al*., 2001; Halleen *et al*., 2003; Aroca *et al*., 2010). In the vineyards, pathogens infect vines through pruning wounds (Eskalen and Gubler, 2001; Rolshausen *et al*., 2010) and therefore, these need to be protected. Various chemical, biological and cultural strategies have been studied to control Petri disease and esca of grapevines during the grapevine propagation process as well as in the vineyards. Disease management options are mostly preventative and limited to minimising infection risk (Hunt, 2004).

1.5.1 Chemical control

Chemical strategies in the control of Petri disease and esca in nurseries mainly involve drenches and dips of propagation material in fungicides at the various propagation stages (Fourie and Halleen, 2004a, 2006; Gramaje *et al*., 2009). A number of chemical products have been tested to prevent or reduce Petri disease and esca infection of woody tissues of grapevine propagation material but no chemical product has been registered yet (Jaspers, 2001; Gramaje *et al*., 2009). Soaking propagation material prior to cold storage or grafting in fungicides such as benomyl, carbendazim and captan has been shown to be effective in reducing Petri disease pathogens in nursery plants (Fourie and Halleen, 2004a, 2006). In 2007, California researchers reported that soaking dormant rootstocks and scions in ziram, thiram, thiophanate-methyl and lime sulphur, before grafting reduced incidence of *Pa. chlamydospora* and *Pm. aleophilum* in vines (Eskalen *et al*., 2007b). However, although some fungicides were found to be effective in controlling Petri disease fungi, some such as iprodione have been found to be poorly effective in reducing the germination and growth of Petri disease fungi (Jaspers, 2001; Gramaje *et al*., 2009). Petri disease and esca pathogens

are vascular pathogens that inhabit xylem vessels and the success of fungicide treatment in nurseries are therefore, limited by the inability of the fungicides to penetrate the wood tissue leading to poor efficacy (Waite and May, 2005).

Pruning wound protection with chemicals has also been studied. Eskalen *et al*. (2007b) examined the potential of fungicides such as thiophanate-methyl, cyproconazole, boron and pyraclostrobin in protecting pruning wounds in the field against *Pa. chlamydospora* and *Pm. aleophilum* and found that these fungicides were effective. Rolshausen *et al*. (2010) later also found boron to be effective in controlling Petri disease fungi when the fungicide was applied directly on pruning wounds. Sodium arsenite has been used as a preventative application with great effect (Mugnai *et al*., 1999). The chemical was applied as a foliar spray or painted onto the trunk or arms of infected vines. However, due to its toxicity and negative impact on the environment, it has been banned from most countries (Mugnai *et al*., 1999; Di Marco *et al*., 2000).

1.5.2 Biological control

Studies have been carried out on the potential application of *Trichoderma* Pers. (Schumach) spp. in bio-control of Petri disease and esca in nurseries and the field. *Trichoderma* formulations have been found to be suitable agents in the protection of pruning wounds against infection by Petri disease and esca fungi (Hunt *et al*., 2001; Di Marco *et al*., 2004; Kotze *et al*., 2011). Di Marco *et al*. (2004) reported that *Trichoderma* strains were able to reduce infection of grapevine cuttings and pruning wounds of potted vines by *Pa. chlamydospora*. The incidence of *Pa. chlamydospora* and *Phaeacremonium* species in grapevine rootstock material was reduced by soaking the rootstocks in *Trichoderma* formulations (Fourie and Halleen, 2004a). Kotze *et al*. (2011) reported that a *Trichoderma* isolate, USPP-T1, was effective in reducing the incidence of *Pa. chlamydospora* when tested on pruning wounds of field grapevines. The mechanisms used by *Trichoderma* include production of antibiotics, mycoparasitism, competition for nutrients and space with pathogenic fungi as well as stimulation of host resistance (Di Marco *et al*., 2004; Kotze *et al*., 2011).

1.5.3 Cultural control practices

Implementing traditional cultural practices remains essential in reducing the inoculum load and the spread of esca and Petri disease. Sanitary measures reduce inoculum originating from the vineyard and these include remedial pruning, which is cutting off dead arms below the diseased and discoloured wood, as well as uprooting dead or dying vines and removal of pruned material from the vineyard floor (Mugnai *et al*., 1999). However, the effectiveness of remedial pruning is dependent on whether all infected wood is removed ensuring that shoots used for training are not infected.

Pathogens have been isolated from apparently healthy vines and hence occur as latent pathogens in the host becoming pathogenic when vines are stressed (Mugnai *et al*., 1999; Whiting *et al*., 2001; Retief *et al*., 2005; Rooney-Latham *et al*., 2005c). Stress conditions such as improper soil preparation, fertilisation and irrigation, in vineyards can result in symptom expression of infected plants. Maintaining optimal fertilisation and irrigation, therefore, results in vines which are healthy. Vines that are grown in conditions where cultural practices are carried out according to the best recommendations are less susceptible to disease since they are not predisposed to stress conditions that favour pathogenic infections (Fourie *et al*., 2000).

Treating propagation material with hot water for 30 minutes at 50 °C followed by 30 minutes in cold water has been found to be effective in disinfecting shoots during the propagation process (Crous *et al*., 2001; Fourie and Halleen 2004a; Waite and May, 2005). However, there are conflicting reports on the effectiveness of hot water treatment. *In vitro* tests carried out by Whiting *et al*. (2001) found that *Pa. chlamydospora* and *Pm. inflatipes* were not killed by hot water treatment at 51 °C for 30 minutes and suggested that the treatment can not eliminate the pathogens in dormant canes. These findings were further confirmed by Rooney and Gubler (2001) who found hot water treatment at 51 °C for 30 minutes to be ineffective in the control of Petri disease pathogens in inoculated dormant wood. A study carried out by Gramaje *et al*. (2008) evaluated the effect of hot water treatment *in vitro* on mycelial growth and germination of Petri disease pathogens. In this study, the authors did not inoculate dormant grapevine wood. This was done in the study by Gramaje *et al*. (2009) which showed that hot water treatment at 53 °C for 30 minutes was effective in controlling Petri disease pathogens. Habib *et al*. (2009) found that treating

infected planting material at 50 °C for 45 minutes reduced the frequency of isolation of *Pa. chlamydospora* compared to untreated controls.

1.6 Dissemination of fungal pathogens by arthropods and development of plant disease

Arthropods facilitate the development of plant diseases in several ways. They can serve as agents of dispersal of the pathogens, be responsible for inoculation and ingression as well as allowing pathogens to over-season in and/or on their bodies (Leach, 1940; Agrios, 2005). A number of arthropods facilitate the entry of pathogens into plants through the wounds they make on plant parts, which can be either above or below ground. Arthropods feeding on plant parts predispose them to attack by pathogenic fungi by weakening the plant and creating wounds which can serve as ports of entry for fungi. Arthropods play a major role in the dispersal of fungal pathogens, although most transmission of fungi by arthropods is unintentional (Agrios, 2005). Spore dissemination occurs as a result of arthropods becoming contaminated with fungal reproductive propagules, either externally and/or internally, as they visit infected tissues. These can then transport the spores to uncolonised plants or plant parts (Agrios, 2005). Various arthropods have been implicated in the dispersal of different fungal pathogens including flies and springtails (Abbott, 2002; Lilleskov and Bruns, 2005), mites (Roets *et al*., 2011), beetles (Lilleskov and Bruns, 2005) and ants (El-Hamalawi and Menge, 1996). Arthropod morphology often help in spore acquisition as they have cuticular processes such as setae and microtrichia (fixed hairs consisting of very small pointed extensions of the cuticula) and body appendages such as legs, wings and antennae that serve as structures that can hook and distribute spores (Leach, 1940).

Ambrosia fungi are fungi which have obligate associations with arthropods (especially beetles in the Platypodidae and Scolytidae) and they are a source of food for these beetles (Batra, 1963; Cassar and Blackwell, 1996; Henriques *et al*., 2006). These fungi flourish inside tunnels made by the beetles, in dead trees, and are introduced into the plant when the beetles bore into xylem vessels and deposit the ectosymbiotic fungal spores (Cassar and Blackwell, 1996). In some instances, the fungi are transmitted in specialised glandular pouches called mycangia and usually only one fungal species is transmitted in the mycangium of female beetles (Cassar and Blackwell, 1996).

Oak wilt is caused by the fungal pathogen, *Ceratocystis fagacearum* (Bretz) Hunt., which enters xylem vessels of oak trees through fresh wounds (Juzwik *et al*., 2004; Agrios, 2005). Sap beetles (Coleoptera: Nitidulidae) are the primary spore vectors and are attracted to the aromatic volatiles produced by the growing fungus on the diseased trees (Juzwik *et al*., 2004). The beetles crawl over, and may tunnel into the mats as they feed on the fungal tissues, acquiring viable fungal propagules on their external surfaces, and internally as they ingest fungal material (Juzwik *et al*., 2004; Agrios, 2005). Successful disease transmission occurs when *C. fagacearum* infested sap beetles visit susceptible fresh wounds and fermented sap from older wounds on healthy oaks (Juzwik *et al*., 2004). The spores of the fungus have been shown to be able to survive for the entire period of hibernation of the beetles (Carter, 1973; Agrios, 2005).

Dutch elm disease kills elm trees by clogging their xylem vessels thereby, blocking movement of water from the roots to other parts of the tree (Agrios, 2005). It is caused by *Ophiostoma ulmi* (Buisman) Melin & Nannf. and *O. novo-ulmi* Brasier which are spread by bark beetles (Scolytidae) (Jacobi *et al*., 2007). The fungi colonise the wood of dying or dead elm trees which is also where the bark beetle larvae develop. The fungi sporulate in the larval tunnels and the newly emerged adult beetles leave the tunnels carrying thousands of sticky spores on their bodies (Agrios, 2005). New infections occur when the spores from beetles are deposited on the moist, freshly wounded tissues of a susceptible tree, usually as a result of burrowing activities of beetles (Agrios, 2005). Fungal spores produced in infected wood can be carried upward in the tree *via* xylem sap and may cause additional infections (Agrios, 2005; Purcell and Almeida, 2005).

Similar to beetles, fungal reproductive propagules can also be transported by other arthropods. *Botrytis cinerea* Pers.:Fr., a fungus which causes bunch rot of grapes, is vectored by larvae of the grape berry moth, *Lobesia botrana* Den. & Schiff. from infected to healthy grape berries (Fermaud and Le Menn, 1992). Argentine ants, *Iridomyrmex humilis* (Mayr), have been reported to vector *Phytophthora citricola* Sawada to healthy lauraceous trees, *Persea indica* (L.) Spreng and cause 73 % infection (El-Hamalawi and Menge, 1996). A wide variety of arthropods including beetles, springtails, oribatid mites and centipedes have also been found to carry the ectomycorrhizal fungus, *Tomentella sublilacina* (Ellis & Holw.), on their exoskeleton and internally (Lilleskov and Bruns, 2005). The corn earworm moth, *Helicoverpa zea* Boddie is a pest of sorghum and has also been shown to transmit *Claviceps africana* Frederickson, Mantle & de Millano and cause ergot infection on healthy

sorghum plants (Prom *et al*., 2003). Mites in the genus *Trichouropoda* Berlese were found to carry spores of the fungus *Gondwanamyces proteae* (M.J.Wingf., P.S.van Wyk & Marasas) G.J.Marais & M.J.Wingf. within pit mycangia at the base of their legs, vectoring spores in the flower heads of *Protea repens* (L.) L (Roets *et al*., 2011).

1.7 Fungal adaptations for arthropod dispersal

Fungi produce three general types of inoculum and these are vegetative mycelium, sclerotia and spores. Sclerotia and mycelium are poorly adapted to arthropod dissemination although the mycelium in plant parts eaten by arthropods can act as inoculum (Leach, 1940). The most common type of reproductive structure for fungi is spores, including asexual and/or sexual spores. Spore masses are often well adapted for arthropod dispersal (Leach, 1940). Several fungal pathogens produce their spores in sticky exudates that become hard when dry and can easily be dispersed by the wind. However, before the spore droplets are dry, arthropods can play an important role in their dissemination (Leach, 1940). Any arthropod that comes into contact with moist, sticky spores can potentially act as a vector of these. Such sticky spores readily adhere to the legs, wings, bristles and other body parts and could easily be brushed off (Leach, 1940; Abbott, 2002) when the arthropods move about. Ascospores which are wet and produced in sticky masses and are associated with arthropods for dispersal have a better chance of reaching an infection court than those that are dispersed by wind and/or water because they are usually transported directly to possible infection courts (Leach, 1940; Carter, 1973) to initiate infection. It is also suspected that characteristics of ascomycetes such as evanescent asci, loss of forcible ascospore discharge, as well as long necked perithecia are a result of selection for arthropod dispersal (Cassar and Blackwell, 1996).

The production of slimy droplets is common among hyphomycetes and ascomycetes, with the latter also often producing long necked perithecia (Abbott, 2002). For example, spores of the blue-stain fungi, *Ceratostomella* Sacc. species, are produced in sticky solutions under the bark making wind dissemination impossible (Leach, 1940). Sporulation of the blue-stain fungi occurs in tunnels and pupal chambers of bark beetles and the sporophores and perithecia point toward the centre of the tunnels such that the spore masses are in a perfect position for contaminating emerging arthropods (Leach, 1940). Hyphomycetous species such as *Graphium* Corda and *Leptographium* R.W. Davidson often produce large slimy droplets on top of elongated stalks which are tall enough to come into contact with

arthropods moving over the surface of the substratum they colonise (Abbott, 2002). Other species (*Trichoderma* Pers. and *Acremonium* Fr.) produce large numbers of small droplets at the apex of simple conidiophores whereas genera such as *Microascus* Zukal and *Chaetomium* Corda, extrude ascospores from the ascocarp neck in a droplet or cirrhi (spore masses) from where these can easily adhere to arthropods (Abbott, 2002).

Spores of several fungal species are resistant to digestive enzymes found in the guts of insects since they stay viable after ingestion. For example, spores of the cotton wilt pathogen, *Fusarium vasinfectum* G.F.Atk), were disseminated in the faecal pellets of grasshoppers that ingested infected plant material (Leach, 1940). Some ambrosia fungi are transmitted in the crop of the female beetle, *Xyleborus dispar* Fabricius, which regurgitates them to initiate a culture in a new brood channel (Carter, 1973). Spores of the fungus, *Claviceps paspali* Stev., can survive the passage through the intestinal tract of the green fly *Pyrellia coerulea* (Wied.) (Carter, 1973). Other fungal genera such as *Ceratocystis* Ellis & Halst., can also produce conidia coated with mucous to prevent them from being digested from within the guts of the beetles which disseminate them (Carter, 1973). El-Hamalawi and Menge (1996) found that faeces of garden snails (*Helix aspera* Muller), when fed avocado plants (*Persea americana* Mill.) infected with *Phytophthora citricola*, contained viable propagules of the fungus. Inoculation of wounds of healthy avocado plants with these faeces resulted in 77 % infection (El-Hamalawi and Menge, 1996). Researchers in Spain were able to isolate *Phytophthora citrophthora* (R.E.Sm. & E.H.Sm.) Leonian, a pathogen that causes Phytophthora branch canker in citrus, from faeces of naturally infested *Helix aspersa* snails (Alvarez *et al*., 2009). Faecal pellets of the millipede, *Harpaphe haydeniana* (Wood), were also found to contain the fungus, *Tomentella sublilacina*. Inoculation of healthy seedlings of the Bishop Pine, *Pinus muricata* D. Don., with the millipede faeces revealed that the spores of *Tomentella sublilacina* were still viable after passing through the gut of the millipede (Lilleskov and Bruns, 2005).

1.8 Potential dissemination of grapevine trunk disease pathogens by arthropods

An ideal vector should be able to acquire the pathogen and transport it to uncolonised tissues for initiation of the disease in plants (Leach, 1940; Purcell and Almeida, 2005). Many arthropods may carry plant pathogens but cannot transmit them to a particular part where infection may result and therefore are not vectors (Leach, 1940; Purcell and Almeida, 2005).

Limited information is known about the role arthropods play in the dissemination of Petri disease and esca pathogens. Edwards *et al*. (2001a) speculated about the possible role of arthropods in the dispersal of *Pa. chlamydospora*. Rooney-Latham *et al*. (2005a) stated that wood-boring insects might play a role in the dispersal of *T. minima*. To date only *Diplodia seriata* De Not. (‘*Botryosphaeria obtusa* (Schwein.) Shoemaker’), which is sometimes isolated from esca-affected vines, has been found on rove beetles collected from pruning wounds in a vineyard (Epstein *et al*., 2008).

Collembolans and mites were observed in the cracks and crevices on the bark of diseased grapevines (Edwards *et al*., 2001a) where pycnidia and sporulating hyphae of *Pa. chlamydospora* were found (Edwards *et al*., 2001a; Edwards and Pascoe, 2001). The sporulation of this pathogen in the same niche as the arthropods were found could promote effective, though incidental, dispersal of the pathogen to healthy vines. It is suspected that the collembolans and mites that carry fungal spores on their exoskeletons can deposit them onto open xylem vessels of pruned shoots (Edwards *et al*., 2001a). Furthermore, the sheltered nature of the cracks and crevices provides limited scope for other dispersal methods such as rain or wind and both the phialidic conidial heads and the pycnidial cirrhi of *Pa. chlamydospora* are presented in a way that they can readily come into contact with small arthropods (Edwards *et al*., 2001a).

The perithecia of *Togninia* have long necks (Rooney-Latham *et al*., 2005a; Mostert *et al*., 2003) and have been found within trunks and cordons of diseased vines. Perithecia of *Togninia* are phototropic, thus their necks were found oriented towards openings of cracks, pruning wounds and insect tunnels (Eskalen *et al*., 2005a, 2005b; Rooney-Latham *et al*., 2005a). *In vitro* tests to determine how ascospores of *T. minima* are released from perithecia showed that when perithecia were moistened, asci exude from the perithecial ostioles and accumulated in groups around the perithecial neck. This suggests that a similar mechanism of ascospore release occurs in infested vineyards following precipitation (Rooney-Latham *et al*., 2005b). The presence of perithecia clustered inside cracks and insect tunnels in grapevines supports the possibility of an association between insects and *T. minima* (Rooney-Latham *et al*., 2005a), and probable transmission of ascospores by insects. Similarly, the production of ascospores in slimy droplets favours the involvement of insects in their dispersal rather than aerial dispersal (Cassar and Blackwell, 1996).

In an investigation of Botryosphaeriaceae-related dieback of grapevines, Epstein *et al*. (2008) postulated that although pruning wounds were primarily infected with *D. seriata* conidia that are disseminated by windblown rain, some inoculum may be disseminated by other mechanisms. To test their theory, duct tape covered with a sticky substance, was placed over pruning wounds and from it, rove beetles (Staphilinidae) infested with *D. seriata* were recovered. Beetles have been found to vector numerous plant pathogens (Leach, 1940; Carter, 1973; Juzwik *et al*., 2004; Agrios, 2005) and therefore, the possibility of rove beetles acting as vectors of Petri disease and esca pathogens cannot be ruled out. Furthermore, *Pm. rubrigenum* W. Gams, Crous & M.J. Wingf. (a Petri disease fungus) was isolated from larvae of the oak bark beetles, *Scolytus intricatus* (Ratz.) (Scolytinae), and their galleries on oak trees, *Querus robur* L. and also from ash bark beetles, *Leperisinus fraxini* Panzer (Scolytinae) found under the bark of ash trees, *Fraxinus excelsior* L. (Kubatova *et al*., 2004).

The mere association of an arthropod with diseased plants or the presence of inoculum on or in the arthropod's body, however, does not establish that the arthropod is a vector of a pathogen. Any evidence that presents a certain arthropod as a vector of a plant pathogen should always meet Leach's rules for proof of arthropod transmission (Leach, 1940). These rules state that the: i) arthropod should have a close association with diseased plants, ii) arthropod should regularly visit healthy plants under conditions suitable for transmission of the disease, iii) arthropod should carry the pathogen in nature or following visitation to a diseased plant and iv) disease should be produced experimentally by arthropod visitation under controlled conditions.

1.9 Aims of this study

From the literature reviewed in this chapter it is evident that arthropods could be involved in the dissemination of Petri disease and esca pathogens on grapevines. The sporulation structures of Petri disease and esca fungi, which produce spore droplets either on conidiophores or on perithecial necks, hold the potential to be dispersed by arthropods. Spore droplets are ideal to stick onto the exoskeletons of arthropods crawling over and into the crevices or cracks of diseased tissues and possibly be deposited onto the exposed xylem vessels of healthy vines as the arthropods explore fresh pruning wounds.

The role possibly played by arthropods in the dissemination of these pathogens needs to be investigated and possible vectors identified. The objectives of this study, therefore, were to:

i) determine which arthropods are associated with declining grapevines and freshly made pruning wounds,

ii) determine whether pathogens associated with Petri disease and esca can be detected on the arthropods and

iii) determine whether arthropods can vector Petri disease pathogens to pruning wounds.

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Table 1. List of *Phaeoacremonium* species that have been found on grapevines and the respective teleomorphs described.

***Phaeoacremonium* species**	***Togninia* species**	**Reference**
Pm. aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	*T. minima* (Tul. & C. Tul.) Berl*	Crous *et al*. 1996
Pm. alvesii L. Mostert, Summerb. & Crous		Mostert *et al*. 2005
Pm. angustius W. Gams, Crous & M.J. Wingf.		Crous *et al*. 1996
Pm. armeniacum A.B. Graham, P.R. Johnst & B. Weir		Graham *et al*. 2009
Pm. australiense L. Mostert, Summerb. & Crous		Mostert *et al*. 2005
Pm. austroafricanum L. Mostert, W. Gams & Crous	*T. austroafricana* L. Mostert, W. Gams & Crous	Mostert *et al*. 2006
Pm. cinereum D. Gramaje, H. Mohammadi, Z. Banihasshemi, J. Armengol & L. Mostert		Gramaje *et al*. 2009
Pm. croatiense Essakhi, Mugnai, Surico & Crous		Essakhi *et al*. 2008
Pm. globosum A.B. Graham, P.R. Johnst & B. Weir		Graham *et al*. 2009
Pm. griseorubrum L. Mostert, Summerb. & Crous		Mostert *et al*. 2005
Pm. hispanicum D. Gramaje, J. Armengol & L. Mostert		Gramaje *et al*. 2009
Pm. hungaricum Essakhi, Mugnai, Surico & Crous		Essakhi *et al*. 2008
Pm. inflatipes W. Gams, Crous & M.J. Wingf.		Crous *et al*. 1996
Pm. iranianum L. Mostert, Gräf., W. Gams & Crous		Mostert *et al*. 2006
Pm. krajdenii L. Mostert, Summerb. & Crous	*T. krajdenii* L. Mostert, W. Gams & Crous	Mostert *et al*. 2005
Pm. mortoniae Crous & W. Gams	*T. fraxinopennsylvanica* (T.E. Hinds) Georg Hasner, Eyjolfsd. & J. Reid*	Groenewald *et al*. 2001

Table 1. Continued

***Phaeoacremonium* species**	***Togninia* species**	**Reference**
Pm. occidentale A.B. Graham, P.R. Johnst & B. Weir		Graham *et al*. 2009
Pm. parasiticum (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf.	*T. parasitica* L. Mostert, W. Gams & Crous	Crous *et al*. 1996
Pm. rubrigenum W. Gams, Crous & M.J. Wingf.	*T. rubrigena* L. Mostert, W. Gams & Crous	Crous *et al*. 1996
Pm. sicilianum Essakhi, Mugnai, Surico & Crous		Essakhi *et al*. 2008
Pm. scolyti L. Mostert, Summerb. & Crous		Mostert *et al*. 2005
Pm. subulatum L. Mostert, Summerb. & Crous		Mostert *et al*. 2005
Pm. *tuscanum* Essakhi, Mugnai, Surico & Crous		Essakhi *et al*. 2008
Pm. venezuelense L. Mostert, Summerb. & Crous		Mostert *et al*. 2005
Pm. viticola J. Dupont	*T. viticola* L. Mostert, W. Gams & Crous*	Dupont *et al*. 2000

* Indicates *Togninia* species which have been found in nature.

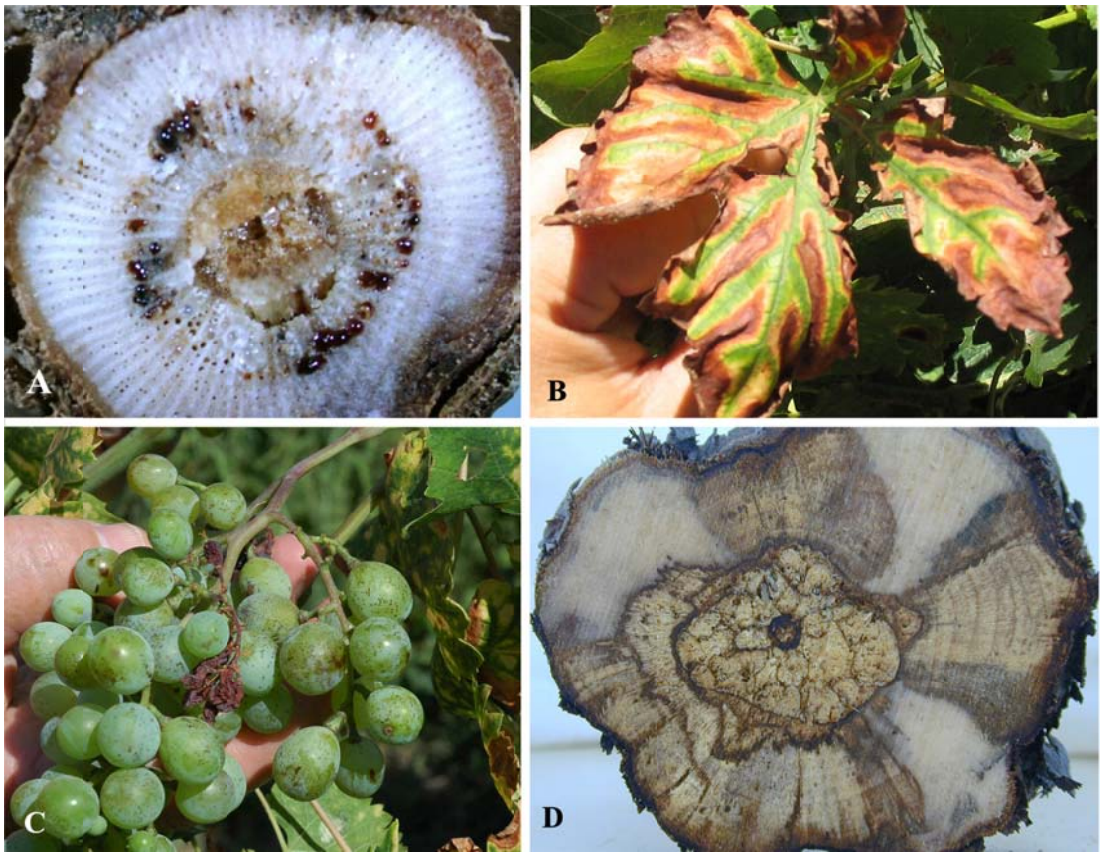

Figure 1. Symptoms of Petri disease and esca. **A.** Transverse section of a Petri diseased vine showing 'black goo'oozing from xylem vessels. **B.** Tiger stripe leaf symptoms of esca diseased vine. **C.** Black spots, also known as 'black measles' on grapes. **D.** White rot associated with esca diseased vines.

CHAPTER 2

ARTHROPODS AS CARRIERS OF PATHOGENS ASSOCIATED WITH PETRI DISEASE AND ESCA IN VINEYARDS

2.1 ABSTRACT

Petri disease and esca are devastating grapevine diseases found in all major grape growing countries and have been studied for several years. However, several questions remain uncertain regarding the epidemiology of the causal pathogens, specifically with regards to dissemination. Little information is known about the role of arthropods as possible spore vectors of these grapevine trunk disease pathogens. This study sets out to determine whether arthropods from declining grapevines are associated with trunk disease pathogens. Arthropods were collected from diseased vines, in two vineyards using trunk and cordon traps, visual searches and pruning wound traps, on a weekly basis over a two-year period. Fungal spores from the surfaces of arthropods were collected in water. Samples were subjected to nested PCR using primers Pm1/Pm2 and Pch1/Pch2 to verify the presence of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora*, respectively. Fungal taxa present in the water samples were also cultured. Grapevine trunk disease pathogens obtained from cultures were identified by sequencing the internal transcribed spacers 1 and 2 and the 5.8S rRNA gene or the partial beta-tubulin gene. A total of 10 875 arthropods, belonging to more than 31 families, were collected from declining grapevines and pruning wound traps. The most abundant arthropods included millipedes, ants, spiders and beetles. Thirty-three percent of the 5677 water samples analysed, contained propagules of Petri disease and esca pathogens. Of these, 37 % were recovered from millipedes, 22 % from cocktail ants, 15 % from spiders and 10 % from beetles. *Phaeoacremonium* species were detected in 1242 samples while *Phaeomoniella chlamydospora* was identified from 855 samples. Other fungi isolated included members of the Botryosphaeriaceae, Diatrypaceae and Diaporthales. Arthropod numbers differed significantly between the two vineyards and collection years. Statistical analyses revealed that precipitation had no significant influence on the number of arthropods that tested positive for pathogens while average temperature significantly influenced the numbers of pathogen-carrying millipedes, spiders and ants. There was also a positive linear relationship between the total number of arthropods and those that tested

positive for pathogens. Managing the spread of grapevine trunk disease pathogens may therefore, need to include management of a wide range of different arthropod taxa.

2.2 INTRODUCTION

Petri disease and esca are a significant threat to grape production in various countries (Chicau *et al*., 2000; Fischer, 2002; Rolshausen *et al*., 2010; Gramaje and Armengol, 2011; White *et al*., 2011). Poor establishment of vines, reduced vine vigour and premature decline caused by these diseases result in the reduction of grape yield and quality and ultimately, leads to significant financial losses. Petri disease is caused by a combination of *Phaeomoniella (Pa.) chlamydospora* (W. Gams, Crous and M.J. Wingf. and L. Mugnai) Crous & W. Gams and *Phaeoacremonium (Pm.*) species W. Gams, Crous & M.J. Wingf. (Scheck *et al*., 1998; Mugnai *et al*., 1999; Groenewald *et al*., 2001) while combinations of these fungi and basidiomycetes cause esca (Surico, 2009). However, fungi from the Botryosphaeriaceae, Diatrypaceace and the Diaporthales, associated with black dead arm, Eutypa dieback and Phomopsis dieback, respectively have also been isolated from esca-affected vines (Fischer and Kassemeyer, 2003; Edwards and Pascoe, 2004; Calzarano and DiMarco, 2007; Péros *et al*., 2008, White *et al*., 2011). Although these diseases have been studied over a number of years, several questions remained regarding the epidemiology of the causal pathogens.

Numerous inoculum sources of Petri disease fungi have been identified. Propagation material has often been shown to be a primary inoculum source for Petri disease pathogens (Mugnai *et al*., 1999; Edwards and Pascoe, 2004; Aroca *et al*., 2010). These pathogens were present in apparently healthy rootstock mother vines (Fourie and Halleen, 2004), cuttings (Halleen *et al*., 2003) and grafted nursery plants (Zanzotto *et al*., 2001). The potential of soil as a source of inoculum in vineyards was established by the recovery of *Pm. inflatipes* W. Gams, Crous & M.J. Wingf. from soil in Californian vineyards (Rooney *et al*., 2001) and *Pa. chlamydospora* from soil in South African vineyards (Damm and Fourie, 2005; Retief *et al*., 2006). In addition to propagation material and soil, infected grapevine wood may also serve as a significant inoculum source for Petri disease pathogens in vineyards. *Phaeomoniella chlamydospora* was recovered from the outer bark of diseased grapevines through washings in water (Rooney *et al*., 2001). Pycnidia of *Pa. chlamydospora* have been found on grapevine bark tissue (Edwards and Pascoe, 2001) and are thought to act as an

inoculum source of *Pa. chlamydospora* spores in the vineyards (Eskalen and Gubler, 2001). Perithecia of *Togninia minima* (Tul. & C. Tul.) Berl., *T. fraxinopennsylvanica* (T.E. Hinds) Georg Hasner, Eyjolfsd. & J. Reid and *T. viticola* L. Mostert, W. Gams & Crous have been found clustered on dead vascular tissues in deep cracks on the trunks and cordons, within insect tunnels and on the surfaces of decaying pruning wounds of affected grapevines (Eskalen *et al*., 2005a, 2005b; Rooney-Latham *et al*., 2005). This indicates the possibility of the involvement of arthropods in the dissemination of ascospores exuding from the necks of the perithecia. These pathogens have also been isolated from ash trees in close proximity to vineyards in California which indicates that these hosts may also be potential sources of inoculum (Eskalen *et al*., 2005a, 2005b, 2007).

The knowledge of how Petri disease and esca pathogens are dispersed from infected to healthy vines in established vineyards is limited. Spores of these pathogens have been collected from the air in vineyards in California (Eskalen and Gubler, 2001) and France (Larignon and Dubos, 2000) and can then penetrate vines through pruning wounds (Eskalen and Gubler, 2001; Rolshausen *et al*., 2010). The release of spores occurs mostly during winter and is linked with rainfall events (Eskalen and Gubler, 2001; Rooney *et al*., 2005; Serra *et al*., 2008). However, in recent studies where Petri disease fungi were present on pruning wounds, no airborne spores were detected (Michelon *et al*., 2007; Van Niekerk *et al*., 2010). It is thus, assumed that air currents are not the only dispersal mechanism of these pathogens in established vineyards. Some support for this can be found in the analyses of spatial distribution patterns of esca-diseased symptomatic grapevines, which showed that infected vines can either be aggregated along rows (Mugnai *et al*., 1999; Pollastro *et al*., 2000) or distributed in a random spatial pattern (Reisenzein *et al*., 2000; Redondo *et al*., 2001; Marchi *et al*., 2006) or both (Surico *et al*., 2000; Edwards *et al*., 2001b). The different spatial patterns of disease symptoms observed in these cases is attributed to various modes of dispersal of the causal pathogens, including air currents, insects and infected propagation material (Reisenzein *et al*., 2000; Surico *et al*., 2000).

One of the adaptations of fungi for arthropod dispersal is the production of spores in sticky exudates (to facilitate attachment to arthropod exoskeletons) (Leach, 1940). These become hard when dry and wind subsequently, can become an effective dispersal agent (Leach, 1940). Any arthropod that comes into contact with these sticky spores can potentially act as a vector. Such sticky spores readily adhere to the legs, wings, bristles and other arthropod body parts and can be easily brushed off (Leach, 1940; Abbott, 2002) when

these move about. Some ascomycetes produce long-necked perithecia with necks often oriented towards the centre of insect galleries in plant material. This provides an ideal position for attachment of spores to emerging insects (Abbott, 2002). *Togninia* species produce such long-necked fruiting bodies (oriented towards the centre of insect galleries and openings of the wood) within cracks of diseased grapevine wood (Rooney-Latham *et al*., 2005). Sticky droplets of ascospores are produced at the tips of the perithecia during wetter periods (Rooney-Latham *et al*., 2005). This suggests a strong possibility that the spores of taxa in this genus may be disseminated *via* arthropods under field conditions.

Arthropods may play a greater role in the spread of Petri disease and esca pathogens than is currently suspected. Evidence of possible entomochoric dispersal is shown in the isolation of *Phaeoacremonium rubrigenum* W. Gams, Crous & M.J. Wingf. from larvae and galleries of the oak bark beetle, *Scolytus intricatus* (Ratz.) (Scolytinae) and from the ash bark beetle (*Leperisinus fraxini* Panzer) (Scolytinae) (Kubatova *et al*., 2004). *Diplodia seriata* De Not., a pathogen sometimes isolated from esca-affected vines (Armengol *et al*., 2001) was also isolated from rove beetles collected from grapevine pruning wounds (Epstein *et al*., 2008). In addition, Edwards *et al*. (2001a) observed an association of collembolans and mites with sporulating structures of *Pa*. *chlamydospora*, produced in cracks and crevices of diseased grapevines in Australia, and suggested that these could play a role in the dissemination of this pathogen.

Little information is currently known about the extent of the association between arthropods and Petri disease and esca pathogens, even though arthropods are suspected to play a primary role in the spread of these. If arthropods are found to play a major role in disease transmission in grapevines, the management of arthropods must form part of an integrated disease management strategy. The objectives of the study were therefore, to identify arthropod taxa associated with both diseased grapevines (inoculum source) and fresh pruning wounds (the inoculation area) and to determine whether the reproductive propagules of Petri disease and esca pathogens are present on these. We also investigated whether the numbers of arthropods found to carry grapevine trunk disease pathogens are influenced by rainfall and temperature in the field.

2.3 MATERIALS AND METHODS

2.3.1 Selection of vineyards

Two wine grape vineyards with a known history of grapevine trunk diseases were selected in the Western Cape of South Africa; a 36-year-old Pinotage vineyard at Nietvoorbij in Stellenbosch and a 25-year-old Chenin blanc vineyard in Rawsonville. The region has a Mediterranean climate with cool, wet winters and warm, dry summers. The vineyards are 64 km apart and separated by the Du Toitskloof mountains.

2.3.2. Arthropod collection

Three collection techniques were used in order to maximize arthropod catches from selected vines and included trunk and cordon traps, visual surveys and pruning wound traps.

2.3.2.1 Trunk and cordon traps

Trunk and cordon traps, made from corrugated fiberboard, were placed on thirty vines per vineyard. Grapevines on which the traps were placed, were selected on the basis of poor growth and the presence of dead spurs or arms, typical of grapevine trunk disease infections. Selected vines also showed signs of arthropod activity like borer holes and the presence of crawling insects within cracks and crevices. Disease symptoms indicated the possible presence of inoculum of trunk disease fungi. Three traps were placed on each vine; one around the trunk (between the graft union and the cordons) and one on each of the cordons. Traps were 100 mm wide and held in place with a thin metal cord (Fig. 1A). Traps were monitored weekly from mid-May to the end of November, for two years (2010 and 2011), and all arthropods sheltering in these were collected. The sampling period roughly coincides with winter pruning and the removal of unwanted shoots (in spring and early summer) which result in many wounds. Arthropods collected were grouped into morphospecies and stored in sterile 14-ml McCartney bottles either separately or in groups of up to 20 individuals of the same arthropod morphospecies until further processing in the laboratory. Only individuals collected from the same plant were grouped per bottle, with the number of individuals placed together dependent on the size of individuals (the bigger the fewer per vial).

2.3.2.2 Visual surveys

Diurnal visual surveys for arthropods were conducted once a week in the same vineyards and over the same period as for the trunk and cordon traps. Surveys were conducted on a random number of diseased vines. Surveys were conducted by actively searching for arthropods at the surface, under loose bark and in the cracks and crevices on the trunks, arms and at the base of diseased vines. Collected arthropods were treated similarly as those collected from the trunk and cordon traps. In addition to the diurnal surveys, three active nocturnal surveys were also conducted in the Pinotage vineyard, just after pruning to assess possible arthropod activity on wounds at night.

2.3.2.3 Pruning wound traps

Thirty apparently healthy vines in close proximity to the vines with trunk and cordon traps were selected in both vineyards. One shoot from each vine was pruned in mid-June, another (on the same plant) 4 weeks later (July), and a third shoot was pruned in August. These times represented the three most commonly employed pruning periods (early, mid and late pruning). It also resulted in pathogen-susceptible pruning wounds over an extended (12-week) period. The shoots were pruned back to two buds before placement of pruning wound traps. Pruning wound traps were designed to collect arthropods that were attracted to the wounds. Traps were constructed from transparency paper (to avoid attracting arthropods due to color) that was cut in a circular shape (150 mm in diameter), folded into a funnel shape that was kept in position using metal staples. The bottom of the funnel was cut slightly larger than the width of the pruned shoot to which the trap was fastened, secured to the shoot using a drawing pin and painted with a sticky substance (Plantex, Chempac, South Africa) to trap arthropods that crawled onto wounds. The traps were placed over the pruning wound in such a manner that more than half the length of the shoot was covered and two thirds of the cup extended above the top of the pruned cane (Fig. 1B). Traps were replaced once a week for 4 weeks for each pruning time. Removed traps were inspected for arthropods using a stereomicroscope. Arthropods were collected and treated as previously described.

2.3.3 Arthropod identification

All arthropods collected were initially identified to the highest taxonomic level possible using general field guides and text books (Picker *et al*., 2004; Scholtz and Holm, 2008) and the help of specialists when available. The family and species names of a few arthropods were determined by Dr. Francois Roets (Department of Conservation Ecology and Entomology, University of Stellenbosch). Ants were identified by Dr. Pia Addison (Department of Conservation Ecology and Entomology, University of Stellenbosch) and spiders were identified by Prof. Ansie Dippenaar-Schoeman (Arachnology unit of the Agricultural Research Council-Plant Protection Research Institute).

2.3.4 Screening of arthropods for presence of Petri and other grapevine trunk disease pathogens

2.3.4.1 Washing of arthropods and identification of fungal pathogens using plating techniques

All collected arthropods (living) were aseptically removed from the glass bottles and placed into sterile eppendorf tubes filled with 2 ml sterile water. Samples were vortexed for 60 seconds to loosen any fungal spores present on their exoskeletons (Roets *et al*., 2006). Arthropods were removed and placed into 2-ml eppendorf tubes that contained 100 % ethanol and stored at room temperature for later identification. One milliliter of the water was transferred onto potato dextrose agar (PDA, Biolab, South Africa) amended with chloromycetin (250mg/L) in three Petri dishes and spread evenly with a sterile L-shaped rod. Petri dishes were incubated at 23 – 24 °C, exposed to approximately 12 hours day light and 10 hours of darkness and monitored for four weeks for growth of pathogens. Cultures containing grapevine trunk disease pathogens were hyphal-tipped and aseptically transferred onto fresh PDA dishes to create pure cultures. Identification was achieved using cultural and morphological characteristics as well as DNA sequencing of all Botryosphaeriaceae, Diaporthales and Diatrypaceae. *Phaeomoniella chlamydospora* was identified by its unique cultural and morphological characteristics (Crous and Gams, 2000). *Phaeoacremonium* species were also identified using cultural and morphological characteristics (Mostert *et al*., 2006) as well as DNA sequencing, however, due to the high numbers of cultures obtained, a representative number of the samples collected in a week were sequenced.

2.3.4.2 Identification of fungal pathogens obtained from water washings using molecular techniques

2.3.4.2.1 *DNA extraction from water*

The protocol described by Retief *et al*. (2005) was followed for extraction of DNA from the remaining 1-ml water samples obtained from the washing of arthropods. However, the protocol was modified in the amount of CTAB extraction buffer (2 % CTAB, 1 M Tris, pH 7.5; 5 M NaCl; 0.5 M EDTA, pH 8.0) added and the addition of glass beads. The 1-ml sample (left from arthropod washings) was first centrifuged (Spectrafuge 24D, Labnet international Inc., USA) for 10 minutes at 15 800 x g and the supernatant discarded. The pellet was re-suspended in 1 ml CTAB extraction buffer. Glass beads (0.5 g) were added to the solution and the tubes were shaken for 5 minutes at 30 $1s^{-1}$ frequency using a Mixer Mill type MM 301 (Retsch Gmbh & Co.KG, Germany) and incubated at 65 °C for 1 hour. After incubation, 400 µl of chloroform: isoamyalcohol (24:1) was added and tubes inverted to mix the contents and centrifuged at 15 800 x g for 15 minutes. The watery supernatant was transferred to new 1.5-ml eppendorf tubes where 50 µl of 7.5 M ammonium acetate (pH 7.0) and 600 µl of cold isopropanol were added and the tubes were incubated at -20 °C for 1 hour before being centrifuged at 15 800 x g for 10 minutes, and the supernatant discarded. Thereafter, 1 ml of cold 70 % ethanol was added to each tube and the tubes were incubated at -20 °C for 30 minutes, centrifuged at 15 800 x g for 5 minutes and the supernatant was discarded. DNA pellets were dried at room temperature overnight and 50 µl of double distilled water was added before storage at 4 °C.

2.3.4.2.2 *DNA extraction from pure cultures*

Mycelium from actively growing cultures was placed in 2-ml eppendorf tubes and 0.5 g of glass beads and 600 µl CTAB were added. The same equipment and protocol used for the DNA extraction from water samples was used. However, tubes were shaken for 7 minutes prior to incubation at 65 °C for 30 minutes. After incubation, 400 µl chloroform:isoamylalcohol (24:1) was added and the tubes mixed by inverting them ten times and centrifuged at 15 800 x g for 7 minutes. The supernatant was collected and 250 µl of cold ammonium acetate solution (7.5 M) and 600 µl cold isopropanol were added and mixed by inverting. Tubes were incubated for 15 minutes at -20 °C and centrifuged at 15 800 x g for 15 minutes. The supernatant was discarded and 1 ml of cold 70 % ethanol added

before centrifuging at 15 800 x g for 5 minutes. The supernatant was discarded and the pellets were left to dry at room temperature overnight. Pellets were dissolved in 100 µl double distilled water and stored at 4 °C.

2.3.4.2.3 *Detection of Petri disease fungi using nested PCR*

The primary PCR reaction amplified the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal RNA gene with the universal fungal primers ITS1 and ITS4 (White *et al*., 1990). The reaction contained 5 µl of DNA isolated from the water samples, 1 × PCR buffer, 2.5 mM $MgCl_2$, 1 µg/µl BSA, 0.2 mM of dNTPs, 0.25 mM of each primer and 0.65 U of Bioline Taq polymerase in a total reaction volume of 25 µl. Reaction conditions consisted of an initial denaturation step at 94 °C for 3 minutes followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C and a final extension step at 72 °C for 7 minutes.

Two separate secondary PCRs were conducted to detect *Pa*. *chlamydospora* with the species-specific primer pair Pch1/Pch2 (Tegli *et al*., 2000) and species of *Phaeoacremonium* with the genus-specific primer pair Pm1/Pm2 (Aroca and Raposo, 2007). The Pch1/Pch2 primer pair reaction volume of 25 µl, consisted of 1× PCR buffer, 1.5 mM $MgCl_2$, 1 µg/µl BSA, 0.2 mM dNTPs, 0.30 mM of each primer, 0.65 U of Bioline Taq polymerase and 1 µl of DNA. Reaction conditions consisted of an initial denaturation step at 94 °C for 3 minutes followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 40 s at 72 °C and a final step at 72 °C for 7 minutes. The concentration of the reagents for PCR using primer pair Pm1/Pm2 were the same except that 3 mM of $MgCl_2$, 0.8 mM of dNTPs and 0.5 mM of each primer were used. Reaction conditions consisted of an initial denaturation step at 94 °C for 5 minutes followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 50 s at 72 °C and a final step at 72 °C for 7 minutes. All PCR reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA).

PCR products were separated by electrophoresis on a 1 % (w/v) agarose gel in 1× TAE running buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.5). The gel was stained with ethidium bromide and visualised under ultraviolet (UV) light using a GeneGenius Gel Documentation and Analysis System (Syngene, UK) and a 100 bp ladder (Promega) was used as molecular size marker. The expected length of PCR product for the

Pch primers was 360 bp (Tegli *et al*., 2000) and for the Pm primers 415 bp (Aroca and Raposo, 2007).

2.3.4.2.4 *PCR from DNA isolated from fungal cultures*

The ITS1, ITS2 and the 5.8S ribosomal RNA gene was amplified as previously described for the identification of species in the Botryosphaeriaeceae, Diatrypaceaecae and the Diaporthales. The β-tubulin gene was amplified using the primers T1 (O' Donnel and Cigelink, 1997) and Bt2b (Glass and Donaldson, 1995) to identify species of *Phaeoacremonium*. Two µl of DNA were used in each PCR. A 25 µl reaction was carried out for the primers T1 and Bt2b and it consisted of 1 × Buffer, 1.5 mM $MgCl_2$, 1 µg/µl BSA, 0.2 mM dNTPs, 0.30 mM of each primer and 0.65 U of Bioline Taq polymerase. Reaction conditions consisted of an initial denaturation step at 94 °C for 5 minutes followed by 36 cycles of 45 s at 94 °C, 30 s at 58 °C and 90 s at 72 °C and a final step at 72 °C for 6 minutes. Visualisation of PCR products followed that outlined above.

2.3.4.2.5 *Sequencing of PCR products*

The PCR products were purified using the Invitek MSB® Spin PCRaparel 250 (Germany) kit. Because of the high number of amplified samples from the nested PCR, only a subset of the PCR products obtained was sequenced. DNA sequencing reactions were performed using the Big Dye system (version 3.1 dye terminators, Applied Biosystems, USA). The total reaction volume was 10 µl and contained 5 × Buffer, 0.4 mM of each primer. The conditions consisted of an initial denaturation step at 95 °C for 60 seconds followed by 30 cycles of 10 s at 95 °C, 5 s at 50 °C and 4 minutes at 60 °C and a final step at 60 °C for 30 s. The electrophoresis of the products was performed on an ABI 3130XL Genetic Analyser at the Central Analytical Sequencing Facility at Stellenbosch University. Sequences obtained for both directions were evaluated using the software, Geneious 3.5.6 (Biomatters Ltd., New Zealand) and manually edited using Sequence Alignment Editor v. 2.0a11. Species identification was done by using the megablast function of the NCBI's GenBank nucleotide database (www.ncbi.nlm.nih.gov).

2.3.4.2.6 *Restriction enzyme identification of* Phaeoacremonium *spp. from PCR products*

Sequence analysis of the PCR products that were amplified using the Pm1/Pm2 primer pair showed that DNA from other fungi such as *Diplodia seriata* was also amplified. As a result, restriction maps of the ITS sequences generated with the Pm1/Pm2 primer pair were defined using the NEBcutter V2.0 (New England Biolabs Inc.) to generate discriminate profiles for the *Phaeoacremonium* species. The restriction enzyme Hpy 188I (New England, Biolabs) resulted in unique fragment profiles to identify *Phaeoacremonium* species. The conditions for enzyme digestion were as follows: 1 µl of enzyme, 17 µl of PCR product and 2 µl of enzyme buffer in a final reaction volume of 20 µl and digested for an hour at 37 °C. Restriction fragments were separated on an ethidium bromide-stained 3 % agarose gel using 1× TAE buffer in the electrophoresis tank. An undigested PCR product was used as a control for non-digestion and a 100 bp molecular size marker was used to determine the size of each band. The digestion profile was visualized under UV light as described above.

2.3.4.3 Detection of grapevine trunk pathogens from pruning wounds on which pruning wound traps were placed

The basal internodes of the pruned canes collected before the placement of the pruning wound traps were taken to the laboratory for fungal isolation to verify the presence or absence of trunk disease pathogens. This would indicate pathogens present in the shoots prior to pruning. The pruned pieces were surface sterilized by immersion in 70 % ethanol for 30 seconds, 2 minutes in 3.5 % sodium hypochlorite and again for 30 seconds in 70 % ethanol. Shoots were then split longitudinally and small wood fragments (1 × 2 mm) were cut from either side of the pith and plated onto PDA in two Petri dishes. Dishes were incubated at 23 – 24 °C, exposed to approximately 12 hours day light and 10 hours of darkness and monitored daily for fungal growth for four weeks. For each of the three pruning times, the four-week-old pruning wounds were pruned off and taken to the laboratory for isolation of trunk disease pathogens. The same isolation procedure was followed. To determine whether arthropods could be involved in infection of pruning wounds, arthropods collected from pruning wounds traps were also screened for trunk disease pathogens as described above.

2.3.5 The influence of environmental factors on the presence of trunk disease pathogens on arthropods

Environmental variables measured and tested were total rainfall and average temperature for each week that arthropods were collected. Total rainfall was determined by adding the daily amount of rainfall for the seven days of each week of collection. The average temperature was determined by adding the daily average temperature and dividing the total by seven. The weather data was obtained from the Nietvoorbij and Rawsonville-Blaarfontein weather stations.

The effect of site (the two vineyards) and collection years (2010 and 2011) on both the total abundance of arthropods (inclusive of all taxa collected during the study) and the three most abundant arthropod taxa collected were statistically determined with Chi-squared (χ^2) tests using the Freq Procedure of SAS statistical software (Version 9.2, SAS Institute Inc., USA). We therefore, tested the null hypothesis that abundances of arthropods and these groups of arthropods were equal between sites (Chenin blanc and Pinotage vineyards) and years (2010 and 2011). In addition, the degree of linear relationship between the abundance of the arthropod groups mentioned above and those that carried trunk disease pathogens, and rainfall and average temperature were determined using Pearson's correlations using the Corr Procedure of SAS statistical software (Version 9.2, SAS Institute Inc., USA).

2.4 RESULTS

2.4.1 Arthropod collection

2.4.1.1 Arthropods associated with declining grapevines

The number of arthropod individuals per taxon collected from the Pinotage and Chenin blanc vineyards are shown in Table 1. Only those that were collected more than twice and/or those that tested positive for grapevine trunk disease pathogens at least once are included. The remainder were considered as either rare or not being able to acquire spores of the pathogens of interest.

Arthropods collected from grapevines in the two vineyards belonged to more than 31 families. In total, 10 875 arthropod individuals were collected during the two years of sampling and of these, 6402 were from the Chenin blanc and 4473 were from the Pinotage

vineyard. The trunk and cordon traps yielded the highest number of arthropod individuals (7858), followed by the pruning wound traps (1765 individuals) and the visual searches (1252 individuals). The majority of the arthropod individuals collected belonged to the Diplopoda (millipedes), Araneae (spiders), Coleoptera (beetles), Hymenoptera (ants and wasps), Collembolla (springtails), Orthoptera (crickets), Blattodea (cockroaches), Dermaptera (earwigs), Thysanoptera (thrips) and Hemiptera (bugs).

Ants were represented by cocktail ants, *Crematogaster peringueyi* Emery, and Argentine ants, *Linepithema humile* (Mayr) as well as the common pugnacious ants, *Anoplolepis custodiens* (F. Smith). Most abundant beetle families represented in the collection included the Coccinellidae (ladybird beetles), Elateridae (click beetles), Curculionidae (weevils) and Staphylinidae (rove beetles). The spiders were represented by the Gnaphosidae (ground spiders), Clubionidae (sac spiders), Miturgidae (long-legged sac spiders), Salticidae (jumping spiders), Corinnidae (dark sac spiders), Philodromidae (running crab spiders), Lycosidae (wolf spiders) and Theridiidae (web spiders). The families of Hemiptera included Pentatomidae (stinkbugs), Cicadellidae (leafhoppers), Reduviidae (assassin bugs), Pyrrhocoridae (cotton stainers) and Pseudococcidae (mealybugs). Orthopterans mainly consisted of species in the family Gryllidae (crickets). Only a single species of millipede was collected namely, the Portuguese millipede, *Ommatoiulus moreleti* (Lucas). The abundance of these taxa differed between the two vineyards. For example, cockroaches, click beetles, cocktail ants and pugnacious ants were more abundant in the Chenin blanc vineyard whereas Argentine ants, millipedes and earwigs were abundant in the Pinotage vineyard (Table 1).

2.4.1.2 Arthropods collected from pruning wound traps

Traps placed around freshly made pruning wounds trapped several arthropod morphospecies (Table 2). The main arthropod taxa consistently captured on the pruning wound traps included cocktail ants, beetles, spiders, wasps, springtails, earwigs, thrips and stinkbugs. Millipedes were not commonly captured on the pruning wound traps but they were observed in large numbers on the freshly made pruning wounds during the night in the Pinotage vineyard (Figs. 2A, B). Millipede faeces were observed on many pruning wounds in vineyards during the day. Similarly, cocktail ants were observed in large numbers on the freshly made wounds during the day in the Chenin blanc vineyard (Fig. 2C). In addition, a sac spider (*Cheiracanthium furculatum* Karsch) was observed on a pruning wound at night

in the Pinotage vineyard (Fig. 2D). Rove beetles, springtails, thrips and wasps were only captured using the pruning wound traps and not by any other collection method.

2.4.2 Screening of arthropods for presence of Petri and other grapevine trunk disease pathogens

Millipedes, spiders and cocktail ants yielded the target pathogens fairly consistently using both plating (culturing) and molecular techniques and subsequently, numerous individuals tested positive for pathogens (Tables 1 and 2). The numbers of arthropods that tested positive for grapevine trunk disease pathogens, for some taxa, exceeded 40 %. Overall, a total of 1873 out of 5677 (33 %) samples tested positive for trunk pathogens, of which 37 % (685/1873) was recovered from millipedes, 22 % (420/1873) from cocktail ants, 15 % from spiders whereas various unidentified beetles and cockroaches contributed 4 % each to the total number of positive samples. Rove beetles contributed 3 % while Argentine ants, earwigs and ladybird beetles contributed 2 % of the positive samples, respectively. Springtails, crickets, common pugnacious ants, click beetles, stinkbugs, parasitic wasps and various unidentified arthropods contributed 1 %, respectively. The remaining taxa contributed less than 1 % to samples that tested positive for grapevine trunk disease pathogens using both plating and molecular techniques.

2.4.2.1 Identification of fungal pathogens obtained from water washings using plating techniques

The grapevine trunk disease pathogens identified from the fungal cultures obtained from the water samples included *Pa. chlamydospora*, *Phaeoacremonium* species, species from the Botryosphaeriaceae, Diatrypaceae as well as Diaporthales. The BLAST results of the species identifications are provided in Table 3. Grapevine trunk disease pathogens were isolated from several arthropod species from both vineyards and monitoring seasons. Some trunk pathogens were predominant in one vineyard over another (Table 4). For example, *Phaeoacremonium sicilianum* Essakhi, Mugnai, Surico, and Crous was the most common species of *Phaeoacremonium* associated with arthropods and mainly from those in the Chenin blanc vineyard. The most common species in the Botryosphaeriaceae was *D. seriata* isolated mostly from arthropods collected in the Pinotage vineyard. *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous was second in abundance of the Botryosphaeriaceae isolates and was commonly found on arthropods from

the Chenin blanc vineyard. *Cryptovalsa ampelina* (Nitschke) Fuckel and *Phomopsis viticola* (Sacc.) Sacc. were the most commonly isolated pathogens within the Diatrypaceae and Diaporthales, respectively.

2.4.2.2 Identification of fungal pathogens obtained from water washings using molecular techniques

2.4.2.2.1 *Detection of Petri disease fungi using nested PCR*

Five thousand six hundred and seventy-seven water samples, collected during the two seasons from both vineyards, were tested using species-specific primers for *Pa. chlamydospora* and genus-specific primers for *Pheaoacremonium* species. Examples of amplification results for primer pairs Pch1/Pch2 and Pm1/Pm2 are shown in Figs. 3 and 4, respectively. Seven hundred and twenty-one samples tested positive for the presence of *Pa. chlamydospora*, whereas 1020 samples tested positive for *Phaeoacremonium* species (Table 5). Sequencing of a subset of the PCR products (182 samples) for *Phaeoacremonium* spp. indicated that the most common species associated with the collected arthropods was *Pm. sicilianum* (75 %) and *T. minima* (15 %). However, due to the non-specificity of the PCR, 18 of the samples sequenced were generated from DNA of *D. seriata*, *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton and various unidentified taxa. Seventy-three of the PCR products generated using the primer pair Pch1/Pch2 were sequenced and all were confirmed to be from *Pa. chlamydospora*.

2.4.2.2.2 *Restriction enzyme identification of* Phaeoacremonium *spp. from PCR products*

It was possible to distinguish between *Phaeoacremonium* spp. and other fungal taxa by their restriction fragment length polymorphism patterns. The Hpy188I-digested amplicon for *Phaeoacremonium* species resulted in two prominent bands. One was a smaller band of approximately 145 bp for *T. minima* and 153 bp for *Pm. sicilianum*, and a larger band of approximately 170 bp for both species. The enzyme digestion of *D. seriata* and *S. sapinea* DNA also resulted in two fragments, one of approximately 128 bp and another of approximately 260 bp. Digestion of the other unidentified fungi with the enzyme Hpy 188I resulted in two fragments, one of approximately 225 bp and another of approximately 254 bp. Of the 1062 PCR products that were digested, 856 had the diagnostic banding pattern of *Phaeoacremonium* species.

2.4.2.3 Detection of grapevine trunk pathogens from pruning wounds on which pruning wound traps were placed

Isolations from the initial pruning of shoots showed that 12 shoots (for both years and vineyards) were infected with either *Pa. chlamydospora* (4 shoots), *Pm. aleophilum* (found together with *Pa. chlamydospora* in 1 shoot), *Phomopsis viticola* (3 shoots) or *D. seriata* (5 shoots) prior to the placement of the traps. Thirty-eight wounds became infected with the above mentioned pathogens during the experimental time. Sixteen wounds were infected with *Pa. chlamydospora*, 11 with *D. seriata*, nine became infected with *Phomopsis viticola* and two became infected with *Pm. aleophilum*. Table 6 summarizes the fungi isolated from pruning wounds, which were clean from Petri disease fungi, but were infected during the experimental time, and the arthropod taxa from which either *Pa. chlamydospora* or *Phaeoacremonium* species was detected on the same wound. In the Pinotage vineyard, no arthropod collected from pruning wound traps tested positive for the specific trunk disease fungus that was isolated from the same pruning wound. However, in the Chenin blanc vineyard, five arthropod taxa collected from pruning wound traps tested positive for the same trunk disease fungi that were also isolated from the pruning wounds on which these traps were placed. During 2010, four pruning wounds that became infected with trunk pathogens were associated with arthropods that tested positive for the same fungi. Two of these wounds contained two arthropod morphospecies each, which tested positive for the same pathogen. Only a single wound in 2011 tested positive for a trunk disease pathogen (Table 6).

2.4.3 The influence of environmental factors on the presence of trunk disease pathogens on arthropods

Chi-squared tests revealed that arthropod abundance differed significantly between sites ($\chi^2_{d.f=1} = 342.16$, $P < .0001$) and years ($\chi^2_{d.f=1} = 66.91$, $P < .0001$). Higher numbers of arthropods were collected during 2011 than 2010 at both sites. The Chenin blanc vineyard had a higher number of arthropods collected compared to the Pinotage vineyard. Millipedes were found to be more abundant in the Pinotage vineyard compared to the Chenin blanc vineyard ($\chi^2_{df=1} = 208.27$, $P < .0001$) and a higher number of individuals was collected in 2011 in comparison to 2010 ($\chi^2_{df=1} = 122.01$, $P < .0001$) for both vineyards. Spiders were found to be more abundant in the Chenin blanc vineyard compared to the Pinotage vineyard ($\chi^2_{df=1} = 215.55$, $P < .0001$) and 2010 had a higher number of spiders compared to 2011

($\chi^2_{df=1}$ = 11.42, P = .0007) for both vineyards. Cocktail ants were more abundant in the Chenin blanc vineyard ($\chi^2_{df=1}$ = 2440.52, P < .0001), however, there was no significant difference ($\chi^2_{df=1}$ = .5448, P = .4172) between the number of ants collected in 2010 and 2011 for both vineyards. Arthropods were present throughout the monitoring period in both vineyards with millipedes, spiders and cocktail ants as the most abundant taxa (Figs. 5 and 6). Arthropods confirmed to carry trunk disease pathogens were also collected throughout the monitoring period (Figs. 7 and 8).

Significant correlations found were between the total number of arthropods (Categories: arthropods (all taxa included), millipedes, ants and spiders) and the numbers that tested positive for grapevine trunk diseases (Table 7). All of these correlations were positive indicating that an increase in arthropod numbers was coupled with an increase in numbers of positive arthropods. In terms of environmental variables, average temperature had a significant negative correlation with the total number of ants, number of positive ants, total number of spiders and number of positive spiders as well as a significant positive correlation with number of millipedes and positive millipedes. Rainfall had a significant but weak positive correlation with the total number of arthropods, total numbers of ants and total number of spiders (Table 7). However, no significant correlation was found between an increase in rainfall and the numbers of arthropods that tested positive for grapevine trunk pathogens (Table 7). The proposed guidelines regarding the strength of the correlation coefficient, used in this study, are provided in Table 8.

2.5 DISCUSSION

A large diversity of arthropod morphospecies collected from declining grapevines was found to carry fungi associated with Petri disease and esca. These were often collected from within the cracks and crevices of diseased vines. This indicates that cracks and crevices within diseased grapevines provide a protective habitat for arthropods seeking shelter in a habitat with low structural diversity, which probably also explains why the trunk and cordon traps yielded the highest number of collected arthropods. A lot of different taxa, each with different ecology and dispersal potential, will present the fungi with numerous different ways of being dispersed.

As pruning wounds are the main portals of entry of Petri disease fungi and pathogens associated with esca in vineyards (Eskalen and Gubler, 2001; Rooney-Latham *et al*., 2005;

Rolshausen *et al*., 2010; Van Niekerk *et al*., 2011), any of the arthropods associated with wounds and that have been shown to carry spores of the fungi could potentially vector the fungi to healthy plants. Arthropod taxa that were consistently observed and/or collected from pruning wound traps included millipedes, beetles, spiders, wasps, springtails, earwigs, ants, thrips and stinkbugs. All except for the thrips tested positive for trunk disease pathogens at least once. Most of these arthropods are likely to be attracted to the plant sap exuded from these wounds as it is very rich in sugars (Van der Meer *et al*., 1990). For example, shortly after sunset, large numbers of Portuguese millipedes were observed feeding directly on the plant sap flowing from freshly made wounds. Millipedes are usually detritivores (Crawford, 1990) but often also feed on roots, fruits and germinating seeds of living plants (Baker, 1978; Ebregt *et al*., 2007). Similarly, cocktail ants were observed in large numbers on fresh pruning wounds feeding on plant sap. These are known to prefer sugary and high protein foods (Longino, 2003; Mgocheki and Addison, 2009; Nyamukondiwa and Addison, 2011). Predatory arthropods such as spiders commonly found at wounds are probably also attracted to these since numerous potential prey items are found at these sites. Based on abundance of individuals that tested positive for trunk disease pathogens and the numbers of individuals found in association with pruning wounds, millipedes, ants and possibly spiders seem to be the most likely candidates for arthropod vectored dispersal of the grapevine trunk disease pathogens mentioned in the present study.

The presence of fungal pathogens on arthropods is not uncommon. Fungal pathogens have been isolated from the bodies of different kinds of arthropods, including beetles (Russin *et al*., 1984; Webber, 1990; Lilleskov and Bruns, 2005), mites (Talbot, 1952; Roets *et al*., 2006) and springtails (Talbot, 1952; Lilleskov and Bruns, 2005). However, for grapevine trunk disease pathogens, there is very limited information to suggest that arthropods could acquire spores of the causal organisms. Arthropods reported to carry Petri disease and esca fungi include non-grapevine associated bark beetles (Kubatova *et al*., 2004), rove beetles (Epstein *et al*., 2008) and possibly also mites and collembolans (Edwards *et al*., 2001a). The potential of arthropod taxa such as millipedes, ants, other beetle taxa and spiders to serve as vectors of grapevine trunk disease pathogens is therefore, novel.

Arthropod abundance differed significantly between vineyards and years. The geographical location and differential seasonal conditions of these vineyards probably resulted in differences in the numbers of arthropods collected. The abundance of many

morphospecies is also linked to a specific locality. For example, the cocktail ant species, *Crematogaster peringueyi*, is found abundantly in certain wine growing areas in the Western Cape of South Africa (such as in the Chenin blanc vineyard included in our study), but is less widespread in other areas (Kriegler and Whitehead, 1962). A significant positive linear relationship was found between the total number of arthropods and the number of arthropods that tested positive for grapevine trunk disease pathogens. This therefore, shows that the risk of contamination of pruning wounds with trunk disease pathogens would increase with an increase in spore bearing arthropods, especially with those associated with pruning wounds. If these arthropods aid in infections, any control effort for the spread of grapevine trunk disease pathogens should also include the control of arthropod numbers.

Previous studies have described a link between the occurrence of Petri disease pathogens in vineyards to the occurrence of rainfall (Eskalen and Gubler, 2001; Rooney-latham *et al*., 2005). However, in our study an increase in rainfall had no apparent influence on the abundance of arthropods found to carry pathogens. If fungal sporulation is indeed linked to rainfall, our results may suggest that pathogens are capable of surviving for extended periods of time on the exoskeleton of arthropods and therefore, could be detected on arthropods independent of rainfall events. The results may also suggest that fruiting bodies are able to produce and release spores over longer periods than previously believed. Average temperature had a significant positive association with millipede abundance. This is consistent with their reproduction cycle, where they lay eggs in winter (at low temperatures) and emerge in spring/early summer when their numbers increase until autumn (Baker, 1978; Widmer, 2006). The number of cocktail ants was negatively correlated with average temperature. The activities of ants in time are largely determined by their physiological status, especially their tolerance to ranges of temperatures and humidity. Most diurnal ants have been reported to be more active at dawn and at dusk and the activities declining around midday (Hölldobler and Wilson, 1990). This could explain why the numbers of cocktail ants decreased with the increase in temperature during the sampling periods. A significant negative correlation between the number of spiders and average temperature was also observed. This is consistent with a study by Soomro *et al*. (2010) who observed a decline in the population of spiders with increase in temperature during an investigation of spider activities on sunflower. The same trend was also observed in a study by Martins *et al*. (2004) who found that the numbers of the sun spider, *Mummucia coaraciandu* Martins, Bonato, Machado, Pinto-Da-Rocha & Rocha, are negatively

correlated with mean temperature, but showed no correlation with monthly rainfall. It should be noted, however, that correlation analysis just measures an association between variables and it does not provide a direct explanation of the relationships (Taylor, 1990).

A diverse range of trunk disease pathogens was isolated from a wide variety of arthropod taxa. No pathogen was restricted to a specific arthropod taxon. Also, in some instances, a single arthropod individual was found to carry more than one trunk disease pathogen simultaneously. It is known that different grapevine decline pathogen species can occur in a single esca-diseased vine (Ari, 2000; Armengol *et al*., 2001; Edwards and Pascoe, 2004; White *et al*., 2011) and therefore, arthropods could easily come into contact with spores of numerous taxa if these sporulate at the same time. All of the major fungal taxa associated with grapevine trunk diseases were isolated in this study. *Phaeomoniella chlamydospora* and *Phaeoacremonium* species were found more abundantly than species of the Botryosphaeriaceae, Diatrypaceae and Diaporthales. This is because these fungi were detected using both the traditional plating and molecular techniques. The molecular methods resulted in much higher numbers of samples that were positive for these pathogens because they are sensitive and have the ability to detect both viable and nonviable propagules of pathogens (Rawsthorne *et al*., 2009). It is noteworthy that the most abundant *Phaeoacremonium* species isolated from the surface of arthropods in this study was *Pm. sicilianum* instead of *Pm. aleophilum*, which is the most common species isolated from grapevines world-wide. *Phaeoacremonium sicilianum* was first reported in South Africa by White *et al*. (2011), being the second most common species associated with esca diseased vines after *Pm. aleophilum*. Therefore, its importance seems to be major. Interestingly, some other fungal taxa known to be pathogenic to other crops than grapevines, such as *Aplosporella prunicola* Damm & Crous [isolated from dieback symptoms of *Prunus* L. trees (Damm *et al*., 2007)], *Diplodia medicaginis* Brunaud [isolated from alfalfa, *Medicago sativa* L. (Phillips *et al*., 2008)] and *Diplodia scrobiculata* J. de Wet, Slippers & M.J.Wingf. [pathogen on *Pinus* L. spp. (Bihon *et al*., 2011)] were recovered from arthropods. Their presence on arthropods from vineyards suggests that the arthropods could transport viable propagules from other hosts, such as pine or stone fruit trees, that are commonly planted around vineyards. The pathogenicity of these fungi on grapevines is currently unknown and therefore, it remains unclear if their presence on arthropods occurring on grapevines is of economic importance.

The potential exists for any contaminated arthropod to brush off pathogens onto pruning wounds and cause infection. If any of these arthropods taxa feed on the fungi, there is also a distinct possibility that fungal propagules will be easily transferred to healthy plants during feeding on sap and/or they may acquire propagules from the sap. Circumstantial evidence suggests that arthropods have been able to transfer infective spores to fresh pruning wounds during the study period. Some wounds were found to be infected with trunk disease pathogens after initial isolations from shoots failed to show their presence. A few arthropod taxa including a *Cheiracanthium* C.L. Koch species (spider), *C. peringueyi* (ant), collembolan (springtail) and a coccinellidae species (beetle) were found to carry spores of the same fungus that was isolated from the same wound that the arthropod was collected from. Even though aerial inoculum cannot be ruled out, this suggests that arthropods do have the potential to inoculate healthy grapevines with pathogenic fungi.

The presence of diverse arthropod taxa in high abundance in vineyards and their ability to acquire numerous different pathogens poses numerous disease management problems. Having an arthropod as a possible disease vector means that three organisms (pathogen, host and vector) require attention to effectively control disease spread instead of the standard two (host and pathogen) (Leach, 1940). The formulation of a good control strategy against grapevine trunk diseases is therefore, more complicated because the biology and ecology of all three organisms would need to be reasonably well understood (Purcell and Almeida, 2005). In this study, we have identified the most likely arthropods to vector Petri disease and esca pathogens as Portuguese millipedes and cocktail ants because they were found to be abundant, carried pathogens consistently and were directly linked to pruning wounds. However, data for spider species such as *Cheiracanthium* spp. also agreed with these criteria. Since spiders are considered good biological control agents of pests, this presents additional management problems. The specific role of each of the arthropod taxa identified in this study, to potentially vector trunk disease pathogens should thus, be carefully evaluated in future studies.

Pruning wound protection has been the main focus of efforts to combat the incidence of grapevine trunk diseases in the vineyard (Serra *et al*., 2008; Rolshausen *et al*., 2010). Since arthropods carrying inoculum were found on fresh pruning wounds, and there is a possibility of the inoculum being deposited on the wounds, it is therefore, worthwhile to protect pruning wounds. Elimination of vectors with insecticides is usually the first step that is considered in controlling diseases caused by insect-borne pathogens (Purcell, 2006).

Although they have been effective in reducing the spread of insect-borne diseases (Purcell, 2006), insecticides have diverse toxic effects on beneficial insects (such as predators and parasitoids) (Thomson, 2012). Several methods should be combined to achieve effective control (Purcell, 2006). Sanitation (removal of infected plants and pruning debris) in the vineyard is recommended as a cultural practice to reduce inoculum load (Mugnai *et al*., 1999). Arthropods use cracks and crevices of diseased vines as habitats. Removing dead or diseased vines therefore, will not only reduce inoculum in the vineyard, but also reduce populations of the potential vectors as well as reduce the numbers of the arthropods carrying fungal spores. Alternatively, instead of controlling the numbers of arthropods in the vineyard, efforts can be focused on blocking their movement to pruning wounds. This could be achieved by applying sticky barriers near the wounds or the use of baits at the base of the plants (Purcell, 2006). Ants are routinely controlled in vineyards as part of the mealybug control program (Nyamukondiwa and Addison, 2011). In South African vineyards, ant control methods include insecticidal sprays and chemical stem banding (Addison, 2002). However, the use of chemical stem barriers has been found not to be effective in controlling cocktail ants because they are arboreal (Addison, 2002). The use of toxic baits has been suggested to probably offer effective control of these ants (Mgocheki and Addison, 2009) because food is shared among nest mates (Longino, 2003).

It is important to note that the mere association of arthropods with diseased plants or the presence of inoculum on the arthropod's body cannot be used to conclude that a certain arthropod is a vector of a disease. Any evidence that presents a certain arthropod as a vector of a plant disease should always meet Leach's postulates for proof of arthropod transmission (Leach, 1940). This study has shown that several arthropod species can acquire Petri disease and esca pathogens. However, further experiments are required to assess whether arthropods can vector trunk disease pathogens to healthy pruning wounds. Only once this is established, it can be ascertained whether it is necessary to include arthropod control as part of an integrated management strategy to prevent pruning wound infections by trunk disease pathogens.

2.6 REFERENCES

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Table 1. Arthropods collected using visual surveys, pruning wound traps as well as trunk and cordon traps during surveys of declining Chenin blanc and Pinotage vineyards for 2010 and 2011. Total percentage of arthropod individuals per taxon that tested positive for trunk disease pathogens, using both plating and molecular techniques, are presented in brackets.

Order (common names)	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2011	2010	2011
Diplopoda (Millipedes)	**Julidae**				
	Ommatoiulus moreleti Lucas	425 (50)	1325 (47)	1093 (73)	1620 (46)
Hymenoptera (Ants and wasps)	Parasitic wasps (numerous taxa)	85 (25)	0	114 (4)	0
	Formicidae				
	Crematogaster peringueyi Emery	1187 (47)	1347(53)	10 (0)	8 (25)
	Linepithema humile Mayr	4 (25)	28 (18)	32 (22)	143 (50)
	Anoplolepis custodiens (F. Smith)	33 (0)	17 (47)	0	3 (0)
Coleoptera (Beetles)	**Staphylinidae**	102 (17)	98 (43)	15 (0)	24 (24)
	Coccinellidae	94 (14)	44 (20)	90 (8)	136 (15)
	Elateridae	23 (35)	24 (13)	1 (0)	1 (0)
	Curculionidae	7 (29)	7 (57)	10 (40)	3 (0)
	Various beetles	203 (17)	97 (25)	88 (10)	59 (17)

Table 1. Continued

Order (common names)	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2010	2011	2011
Orthoptera (Crickets)	**Gryllidae**	7 (43)	10 (50)	9 (0)	5 (60)
Unidentified arthropods	**Various taxa**	19 (37)	6 (0)	29 (48)	4 (0)
Blattodea (Cockroaches)		36 (42)	154 (48)	1 (0)	1 (0)
Hemiptera (True bugs)	**Pentatomoidea**	51 (14)	16 (19)	1 (100)	13 (15)
	Pseudococcidae	6 (33)	6 (67)	0	3 (33)
	Cicadellidae	10 (0)	0	4 (0)	4 (0)
	Pyrrhocoridae	0	11 (55)	0	0
	Reduviidae	0	0	0	11 (18)
Collembolla (Springtails)		71 (55)	15 (7)	426 (17)	29 (7)
Thysanoptera (Thrips)		7 (0)	7 (0)	6 (0)	1 (0)
Dermaptera (Earwigs)	**Labiduridae**	1 (0)	11 (18)	75 (41)	41 (32)
Araneae (Spiders)	**Ammoxenidae**				
	Ammoxenus sp. Simon	2 (100)	0	0	0
	Araneidae				
	Neoscona blondeli Simon	0	0	9 (11)	1 (0)
	Neoscona hirta C. L. Koch	0	0	3 (67)	0

Table 1. Continued

Order (common names)	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2010	2011	2011
Araneae (Spiders)	**Araneidae**				
	Neoscona quincasea Roberts	1 (0)	0	2 (0)	0
	Neoscona subfusca C. L. Koch	2 (50)	0	8 (38)	5 (20)
	Clubionidae				
	Clubiona pulchella Canestrini	6 (33)	13 (23)	13 (46)	5 (20)
	Clubiona africana Lessert	0	1 (0)	1 (0)	0
	Clubiona sp. 1 Latreille	3 (33)	4 (25)	18 (28)	3 (33)
	Clubiona sp. 2	3 (0)	1 (100)	0	0
	Corinnidae				
	Afreceto martini	7 (14)	26 (38)	0	3 (33)
	Eresidae				
	Dresserus sp.	1 (0)	1 (100)	0	1 (0)
	Gnaphosidae				
	Asemesthes sp. Simon	0	1 (100)	4 (0)	1 (0)
	Camillina sp. Berland	19 (26)	21 (33)	9 (0)	6 (33)
	Drassodes sp. C. L. Koch	2 (0)	0	2 (50)	1 (0)

Table 1. Continued

Order (common names)	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2010	2011	2011
Araneae (Spiders)	**Gnaphosidae**				
	Pterotricha auris Tucker	0	0	0	7 (57)
	Scotophaeus relegates Purcell	5 (60)	5 (0)	1 (0)	2 (0)
	Upognampa biamenta Tucker	0	0	1 (0)	1 (0)
	Xerophaeus capensis Purcell	5 (20)	8 (75)	4 (0)	7 (14)
	Xerophaeus sp. Purcell	0	2 (50)	0	0
	Zelotes sp. Koch	13 (23)	11 (36)	9 (22)	19 (37)
	Linyphiidae				
	Meioneta sp. Hull	1 (0)	0	1 (0)	1 (0)
	Pelecopsis janus Jocque	2 (0)	43 (30)	0	1 (0)
	Lycosidae				
	Allocosa lawrencei Sundevall	2 (0)	0	0	2 (50)
	Pterartoriola sagae Purcell	3 (67)	8 (25)	0	3 (33)
	Miturgidae				
	Cheiracanthium furculatum Karsch	55 (25)	24 (30)	13 (8)	6 (67)

Table 1. Continued

Order (common names)	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2010	2011	2011
Araneae (Spiders)	**Miturgidae**				
	Cheiracanthium sp. C.L. Koch	19 (32)	17 (47)	7 (14)	4 (25)
	Oxyopidae				
	Oxypes sp. 1	1 (0)	0	0	1 (100)
	Oxypes sp. 2	0	0	0	3 (67)
	Salticidae				
	Cyrba dotata Blackwall	23 (22)	4 (0)	1 (0)	2 (0)
	Evarcha sp. Clerck	1 (0)	3 (33)	0	0
	Heliophanus modicus Blackwall	17 (29)	0	1 (0)	0
	Heliophanus sp. Blackwall	0	4 (50)	0	0
	Thyene sp. Simon	0	2 (50)	0	0
	Sparassidae				
	Olios sp. Walckenaer	0	1 (100)	6(17)	1 (0)
	Philodromidae				
	Philodromus thanatellus Strand	1(0)	2 (50)	6 (17)	3 (67)
	Thanatus vulgaris Simon	4 (0)	1 (0)	2 (100)	1 (0)

Table 1. Continued

Order (common names)	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2010	2011	2011
Araneae (Spiders)	**Philodromidae**				
	Thanatus thanatellus Simon	0	1 (0)	4 (25)	7 (14)
	Tibellus minor Thorell	0	0	0	1 (100)
	Thomisidae				
	Xysticus sp. C. L. Koch	0	1 (0)	1 (0)	1 (100)
	Theridiidae				
	Euryopis episinoides Walckenaer	178 (26)	101 (30)	11 (36)	3 (0)
	Latrodectus geometricus C. L. Koch	0	2 (0)	0	1 (0)
	Steatoda capensis Sundevall	0	0	0	1 (100)
	Theridion sp. 1 Walckenaer	8 (38)	11 (18)	11 (18)	2 (0)
	Theridion sp. 2	0	0	3 (67)	0
	Unidentifiable spiders	22 (20)	11 (43)	12 (17)	6 (60)

*The percentages were calculated as the number of arthropod individuals that carried trunk disease pathogens divided by the total number of arthropod individuals collected.

*Only arthropods that were collected more than twice and/or those that tested positive for grapevine trunk disease pathogens at least once are included.

Table 2. Arthropods collected from pruning wound traps in Chenin blanc and Pinotage vineyards during 2010 and 2011. Total percentage of arthropod individuals per taxon that tested positive for trunk disease pathogens, using both plating and molecular techniques, are presented in brackets.

Order	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2011	2010	2011
Diplopoda	**Julidae**				
	Ommatoiulus moreleti	1 (0)	2 (100)	0	5(0)
Hymenoptera	Parasitic wasps (numerous taxa)	85 (25)	0	114 (4)	0
	Formicidae				
	Crematogaster peringueyi	112 (30)	57(35)	10 (0)	6 (17)
	Linepithema humile	1(0)	8 (0)	17 (6)	19 (42)
	Anoplolepis custodiens	1 (0)	3(0)	0	0
Coleoptera	**Staphylinidae**	102 (17)	98 (43)	15 (0)	24 (24)
	Coccinellidae	9 (33)	5 (40)	10 (0)	0
	Elateridae	0 0	3(33)	0	0
	Curculionidae	0	2 (50)	10 (0)	3 (0)
	Various beetles	144 (11)	50 (28)	68 (13)	42 (14)
Unidentified arthropods	**Various taxa**	11 (55)	5 (0)	23 (17)	3(0)

Table 2. Continued

Order	**Family** *Genus/ Species*	**Chenin blanc** **Number of arthropod individuals (% positive)**		**Pinotage** **Number of arthropod individuals (% positive)**	
		2010	2011	2010	2011
Hemiptera	**Pentatomoidea**	7 (43)	4(25)	0	7 (0)
	Cicadellidae	9 (0)	0	2 (0)	0
Collembolla		69 (57)	14 (7)	395 (17)	29 (7)
Thysanoptera		7 (0)	7 (0)	6 (0)	1 (0)
Dermaptera	**Labiduridae**	1 (0)	1 (0)	1 (0)	0
Araneae	**Araneidae**				
	Neoscona subfusca	1 (0)	0		1 (0)
	Clubionidae				
	Clubiona pulchella	0	0	1 (100)	0
	Clubiona sp. 1	1(100)	1(100)	2(50)	1 (0)
	Gnaphosidae				
	Zelotes sp.	0	0	2 (50)	0
	Linyphiidae				
	Pelecopsis janus	0	41(32)	0	1 (0)
	Miturgidae				
	Cheiracanthium sp.	2 (100)	3 (33)	0	0

Table 2. Continued

Order	Family *Genus/ Species*	Chenin blanc Number of arthropod individuals (% positive)		Pinotage Number of arthropod individuals (% positive)	
		2010	2011	2010	2011
Araneae	**Salticidae**				
	Cyrba dotata	9 (0)	0	0	0
	Heliophanus modicus	2(50)	0	0	0
	Philodromidae				
	Philodromus thanatellus	0	1(100)	1 (0)	1(0)
	Thanatus vulgaris	3 (0)	0	1 (100)	0
	Thanatus thanatellus	0	1 (0)	1 (0)	2 (0)
	Theridiidae				
	Euryopis episinoides	3 (33)	1 (0)	0	0
	Theridion sp. 1	0	3 (0)	0	0
	Unidentifiable spiders	3 (0)	4 (25)	2 (0)	3 (0)

*The percentages were calculated as the number of arthropod individuals that carried trunk disease pathogens divided by the total number of arthropod individuals collected

*Only arthropods that were collected more than twice and/or those that tested positive for grapevine trunk disease pathogens at least once are included

Table 3. BLAST (from Genbank) identification results of the fungal species isolated from the surface of arthropods collected from vineyards.

Fungal taxon	Total number of cultures	Representative culture	Compared to GenBank accession	% Similarity	% Gaps
Phaeoacremonium sicilianum Essakhi, Mugnai, Surico & Crous	225	PMC 220	FJ 872409.1	99	0
Togninia minima (Tul. & C. Tul.) Berl.	90	PMC 397	AY 179939.1	99	1
Phaeoacremonium parasiticum (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf.	8	PMC 240	HQ 605022.1	99	0
Phaeoacremonium alvesii L. Mostert, Summerb. & Crous	2	PMC 206	EU 883990.1	97	3
Diplodia seriata De Not.	65	PMC 161	JQ 659282.1	99	0
Diplodia scrobiculata J. de Wet, Slippers & M.J. Wingf.	2	PMC 198	DQ 458899.1	98	0
Diplodia mutila (Fr.) Mont.	1	PMC 121	JQ 411412.1	99	0
Diplodia medicaginis Brunaud	1	PMC 361	EU 673318.1	99	0
Spencermartinsia viticola (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous	19	PMC 174	AY 905555.1	99	0
Spencermatinsia sp.	4	PMC 224	EU 673323.1	99	0
Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips	2	PMC 160	AY 228097.1	99	0
Neofusicoccum australe (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips	1	PMC 202	EU 375516.1	99	0

Table 3. Continued

Fungal taxon	Total number of cultures	Representative culture	Compared to GenBank accession	% Similarity	% Gaps
Cryptovalsa ampelina (Nitschke) Fuckel	21	PMC 109	AY 920391.1	100	0
Eutypella australiensis Trouillas, Sosnowski & Gubler	1	PMC 207	HQ 692479.1	100	0
Eutypella sp. (Pers.) Sacc.	2	PMC 130	HQ 008913.1	88	4
Eutypa lata (Pers.) Tul. & C. Tul.	1	PMC 123	AY 462541.1	99	0
Phomopsis viticola (Sacc.) Sacc.	14	PMC 173	FJ 790861.1	99	0
Diaporthe helianthi Munt.-Cvetk., Mihaljč. & M. Petrov	2	PMC 128	AJ 312353.1	99	0
Aplosporella prunicola Damm & Crous	2	PMC 339	EF 564376.1	100	0

Table 4. Number of arthropod samples (one sample = 1 - ≤20, depending on size of individual arthropods) that tested positive for grapevine trunk disease pathogens assessed using culturing techniques. Percentages of samples that tested positive for pathigens are given in brackets. Samples were collected from Chenin blanc and Pinotage vineyards during 2010 and 2011 using trunk and cordon traps, pruning wound traps and visual searches.

Pathogen	**Chenin blanc**		**Pinotage**	
	2010 (%)	**2011 (%)**	**2010 (%)**	**2011 (%)**
Phaeomoniella chlamydospora	32 (2)	75 (4)	20 (2)	23 (2)
Phaeoacremonium sicilianum	69 (4)	150 (8)	4	2
Togninia minima	13 (1)	27 (2)	17 (2)	33 (3)
Phaeoacremonium parasiticum	1	0	1	6
Phaeoacremonium alvesii	1	0	1	0
Diplodia seriata	6	5	31 (3)	23 (2)
Diplodia mutila	0	0	1	0
Spencermartinsia viticola	9 (1)	9 (1)	0	1
Spencermartinsia sp.	0	3	1	0
Neofusicoccum parvum	0	0	2	0
Neofusicoccum australe	0	0	1	0
Cryptovalsa ampelina	12 (1)	7	1	1
Eutypella australiensis	1	0	0	0
Eutypella sp.	2	0	0	0
Eutypa lata	0	0	1	0
Phomopsis viticola	1	8	2	3
Diaporthe helianthi	0	0	2	0
Total number of samples tested	**1619**	**1854**	**931**	**1273**

Table 5. Number of arthropod samples (one sample = 1 - ≤20, depending on size of individual arthropods) that tested positive for Petri disease pathogens assessed using nested PCR. Samples were collected from Chenin blanc and Pinotage vineyards during 2010 and 2011 using trunk and cordon traps, pruning wound traps and manual searches.

Pathogen	**Chenin blanc**		**Pinotage**		**Total**
	2010 (%)	**2011 (%)**	**2010 (%)**	**2011 (%)**	
[a]*Phaeomoniella chlamydospora*	202 (12)	209 (11)	142 (15)	168 (13)	721
[b]*Phaeoacremonium* spp.	227 (14)	456 (25)	62 (7)	275 (22)	1020
Total number of samples tested	**1619**	**1854**	**931**	**1273**	

[a]The species-specific primer-pair of Tegli *et al*. (2000) was used to identify *Pa. chlamydospora*.

[b]The genus-specific primer pair of Aroca and Raposo (2007) was used to identify species of *Phaeoacremonium*.

Table 6. Arthropods, collected from pruning wound traps set in the Chenin blanc vineyard, that were associated with newly infected pruning wounds including the Petri disease fungi that were isolated from these, respectively.

Trap no.	Pruning time	Pathogen isolated from		Arthropod taxa	Time of isolation from arthropod after pruning	Pathogen isolated from arthropod
		Freshly pruned shoot	4-week old pruning wound			
1	June 2010	-	*Pa. chlamydospora*	*Cheiracanthium* sp.	1 week	*Pa. chlamydospora*
1	June 2010	-	*Pa. chlamydospora*	Collembolla	4 weeks	*Pa. chlamydospora*
2	June 2010	-	*Pa. chlamydospora*	Coccinellidae	2 weeks	*Pa. chlamydospora*
4	June 2010	-	*Pa. chlamydospora*	Unidentified species	1 week	*Pa. chlamydospora*
4	June 2010	-	*Pa. chlamydospora*	*C. peringueyi*	4 weeks	*Pa. chlamydospora*
8	June 2010	-	*Phaeoacremonium* sp.	*C. peringueyi*	3 week	*Phaeoacremonium* sp.
15	June 2011	-	*Pa. chlamydospora*	*C. peringueyi*	1 week	*Pa. chlamydospora*

Table 7. Pearson correlation coefficients for association between abundance of various arthropod taxa and groups collected and environmental variables.

	Rainfall	Average temp.	Number of arthropods	Positive arthropods	Number of millipedes	Positive millipedes	Number of ants	Positive ants	Number of spiders	Positive spiders
Rainfall	_									
Average Temp	**-0.38**[a]	_								
Number of arthropods	**.22**[b]	.01	_							
Positive arthropods	.11	.11	**.85**[a]	_						
Number of millipedes	.11	**.19**[c]	**.68**[a]	**.84**[a]	_					
Positive millipedes	.04	**.24**[b]	**.67**[a]	**.89**[a]	**.95**[a]	_				
Number of ants	**.23**[b]	**-0.25**[b]	**.48**[a]	**.27**[b]	-0.08	-0.08	_			
Positive ants	.14	**-0.19**[c]	**.44**[a]	**.33**[b]	-0.06	-0.07	**.89**[a]	_		
Number of spiders	**.19**[c]	**-0.27**[b]	**.32**[b]	.11	**-0.22**[b]	-0.17	**.53**[a]	**.48**[a]	_	
Positive spiders	.15	**-0.33**[b]	**.20**[b]	.17	-0.14	-0.09	**.28**[b]	**.30**[b]	**.67**[a]	_

*Values in bold indicate significant associations: a < .001, b < .01, c < .05.

Table 8. Guidelines proposed to describe the strength of the correlation coefficient.

Strength of association/relationship	Correlation coefficient, *r*	
	Positive	Negative
Weak	.1 to .3	-0.1 to -0.3
Moderate	.3 to .5	-0.3 to -0.5
Strong	.5 to 1.0	-0.5 to -1.0

Figure 1. Traps used to collect arthropods from vineyards. **A.** Corrugated fiberboard trunk and cordon traps. **B.** A transparency paper funnel that served as a pruning wound trap.

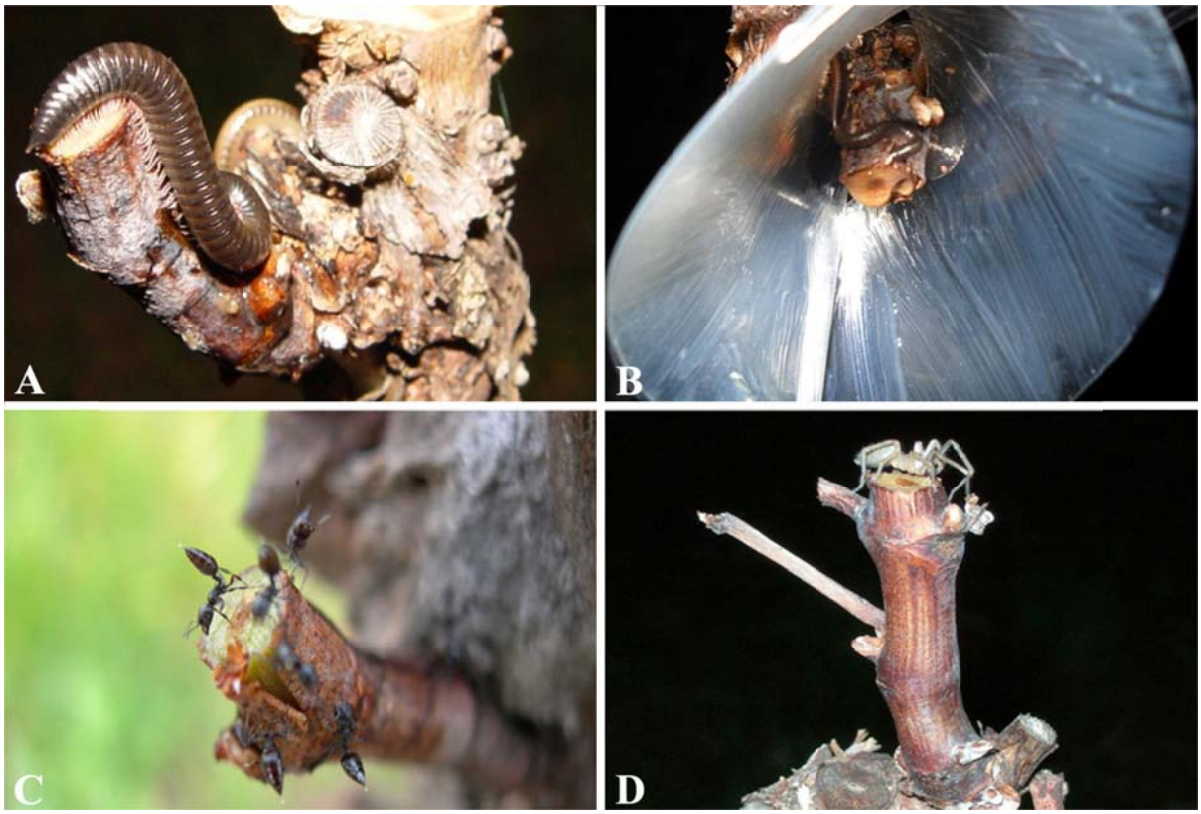

Figure 2. Visual searches revealed direct association of various arthropods with fresh pruning wounds. **A.** Portuguese millipedes (*O. moreleti*) observed at night. **B.** Portuguese millipede observed on a pruning wound with a trap at night. **C.** Cocktail ants (*C. peringueyi*) observed during the day a few minutes after pruning. **D.** A sac spider (*C. furculatum*) seen on a pruning wound at night.

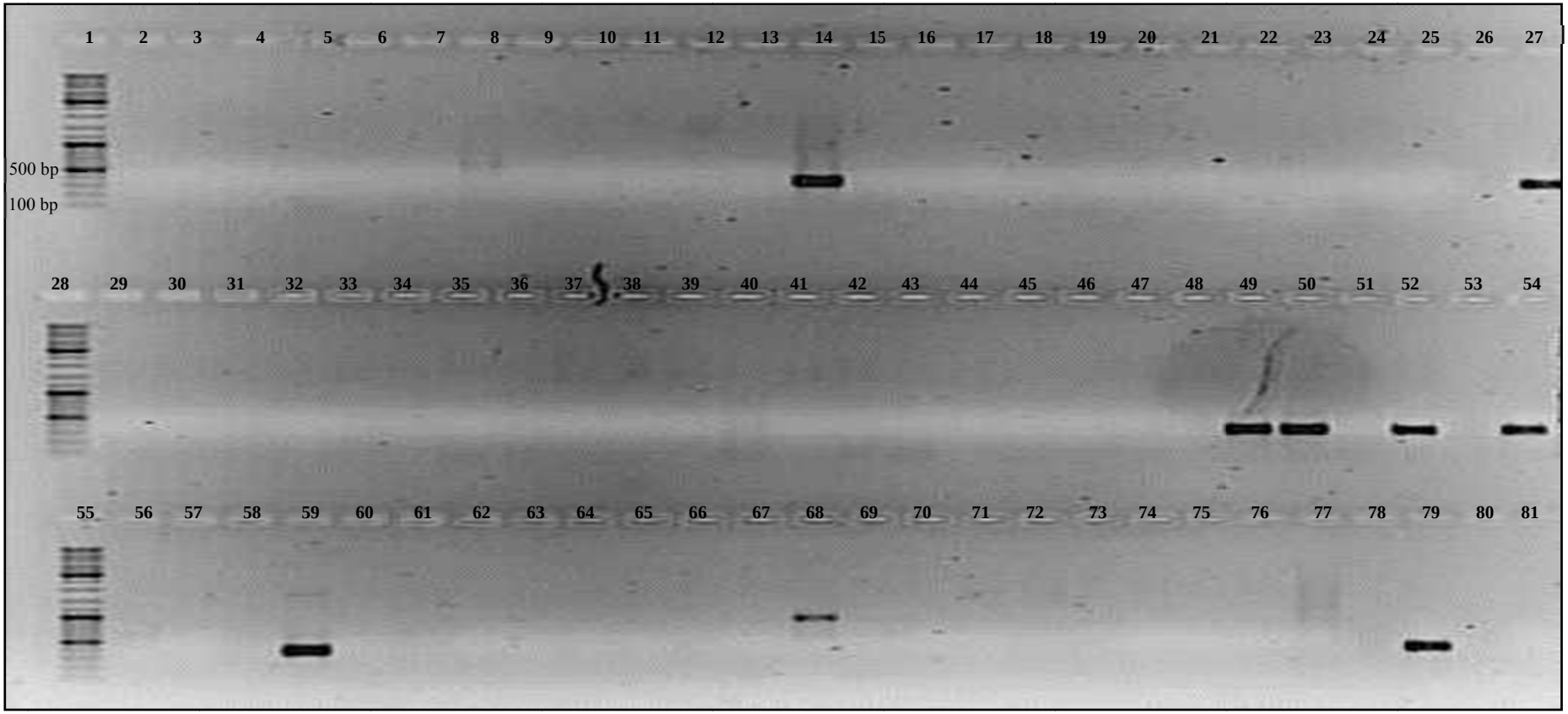

Figure 3. Example of a gel electrophoresis profile of the nested PCR products amplified using primer pair Pch1/Pch2 for the detection of *Pa. chlamydospora*. Lanes 14, 27, 49, 50, 52, 54 and 59 tested positive for *Pa. chlamydospora* with the 360 bp diagnostic fragment. Lane 79 is the positive *Pa. chlamydospora* control and lane 81 is the negative water control. Lanes on the left (1, 28 and 55) contained a 100 bp ladder (Promega).

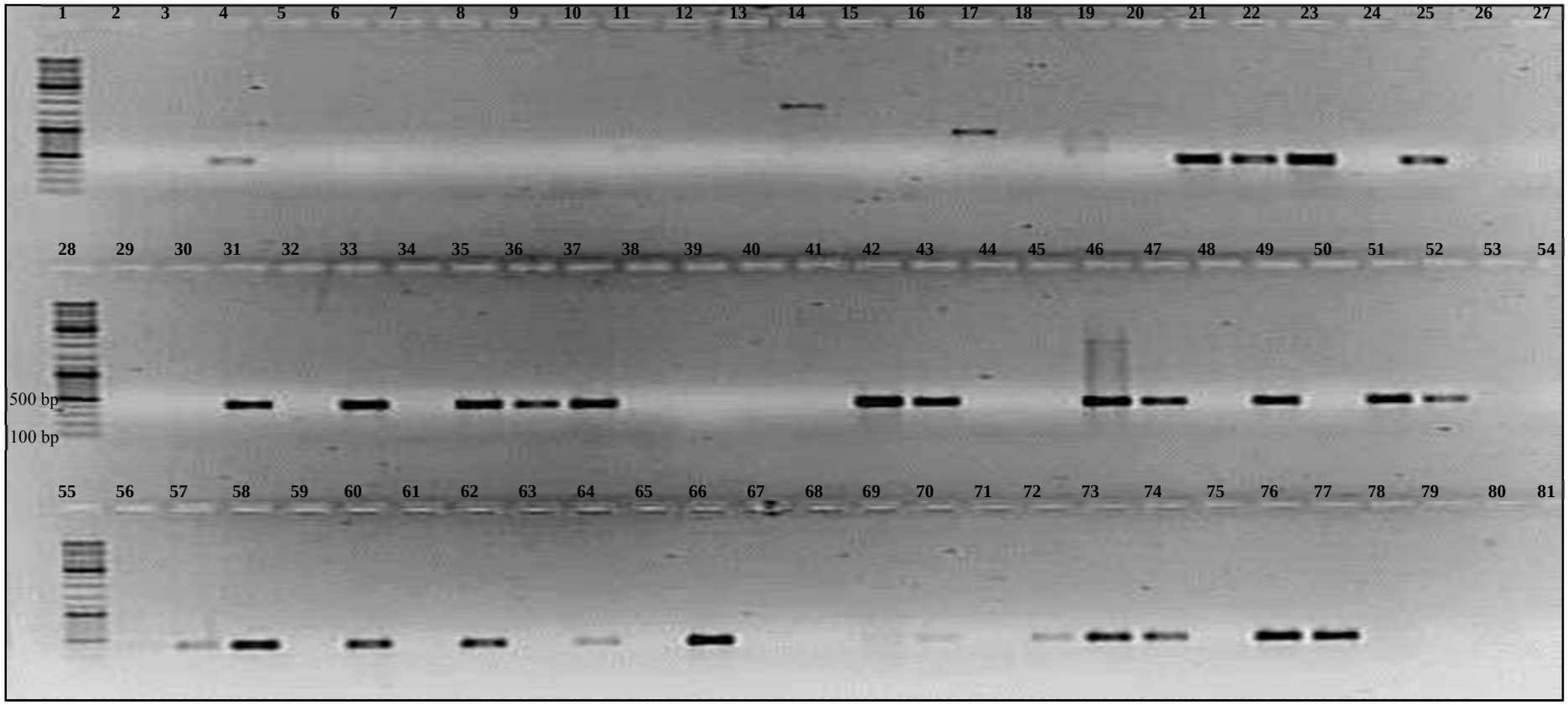

Figure 4. Example of a gel electrophoresis profile of the nested PCR products amplified using primer pair Pm1/Pm2 for the detection of *Phaeoacremonium* species. Lanes with bands tested positive for *Phaeoacremonium* species with the 415 bp diagnostic fragment. Lane 77 is the positive *Phaeoacremonium aleophilum* control and lane 78 is the negative water control. Lanes on the left contained a 100 bp ladder (Promega).

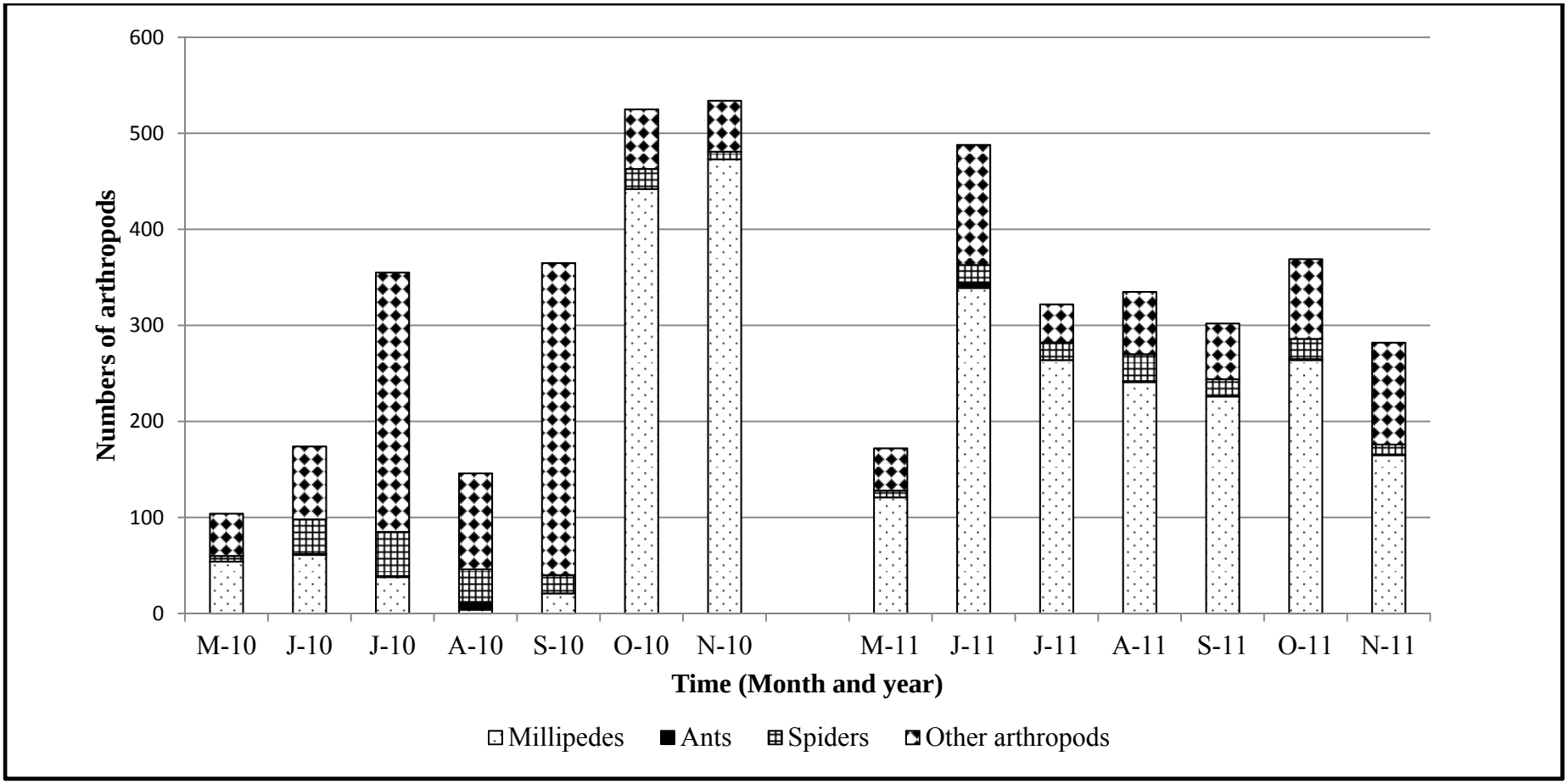

Figure 5. Total number of arthropods collected for each month from the Pinotage vineyard, for the years 2010 (-10) and 2011 (-11). Collection was done from May (M) until November (N).

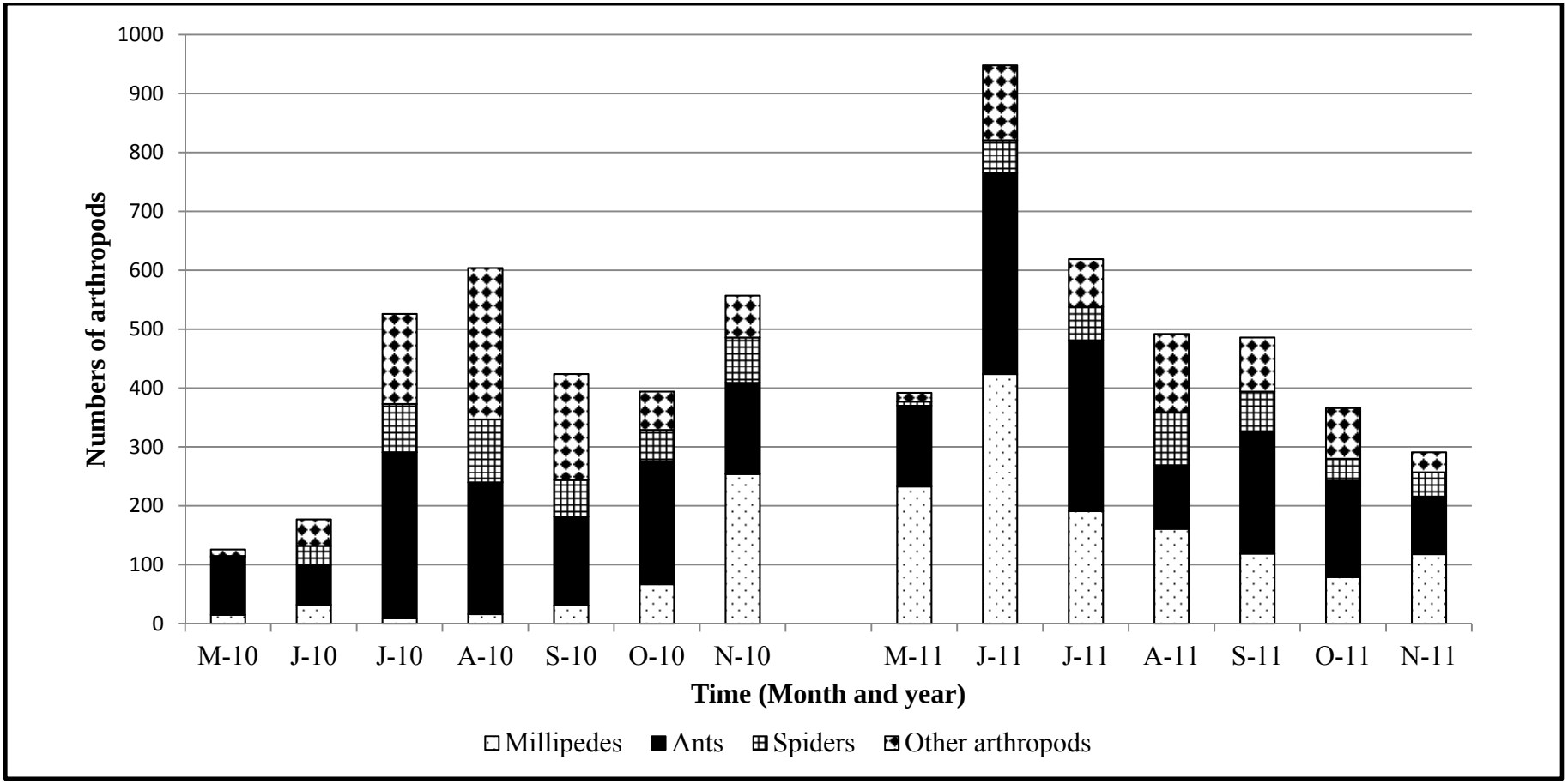

Figure 6. Total number of arthropods collected for each month from the Chenin blanc vineyard, for the years 2010 (-10) and 2011 (-11). Collection was done from May (M) until November (N).

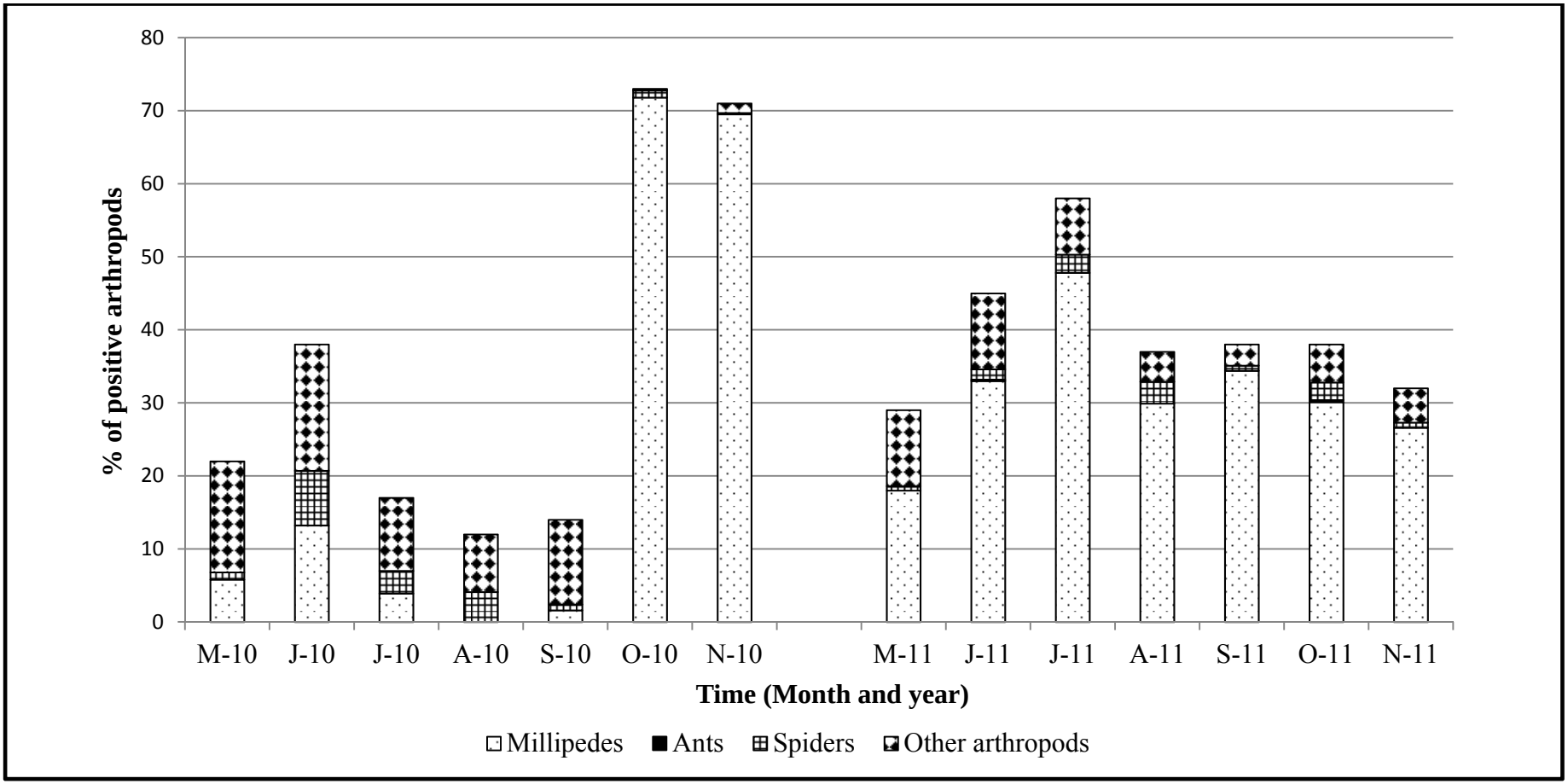

Figure 7. Percentages of arthropods that tested positive for grapevine trunk disease pathogens, calculated for each month from the Pinotage vineyard, for the years 2010 (-10) and 2011 (-11). Collection was done from May (M) until November (N).

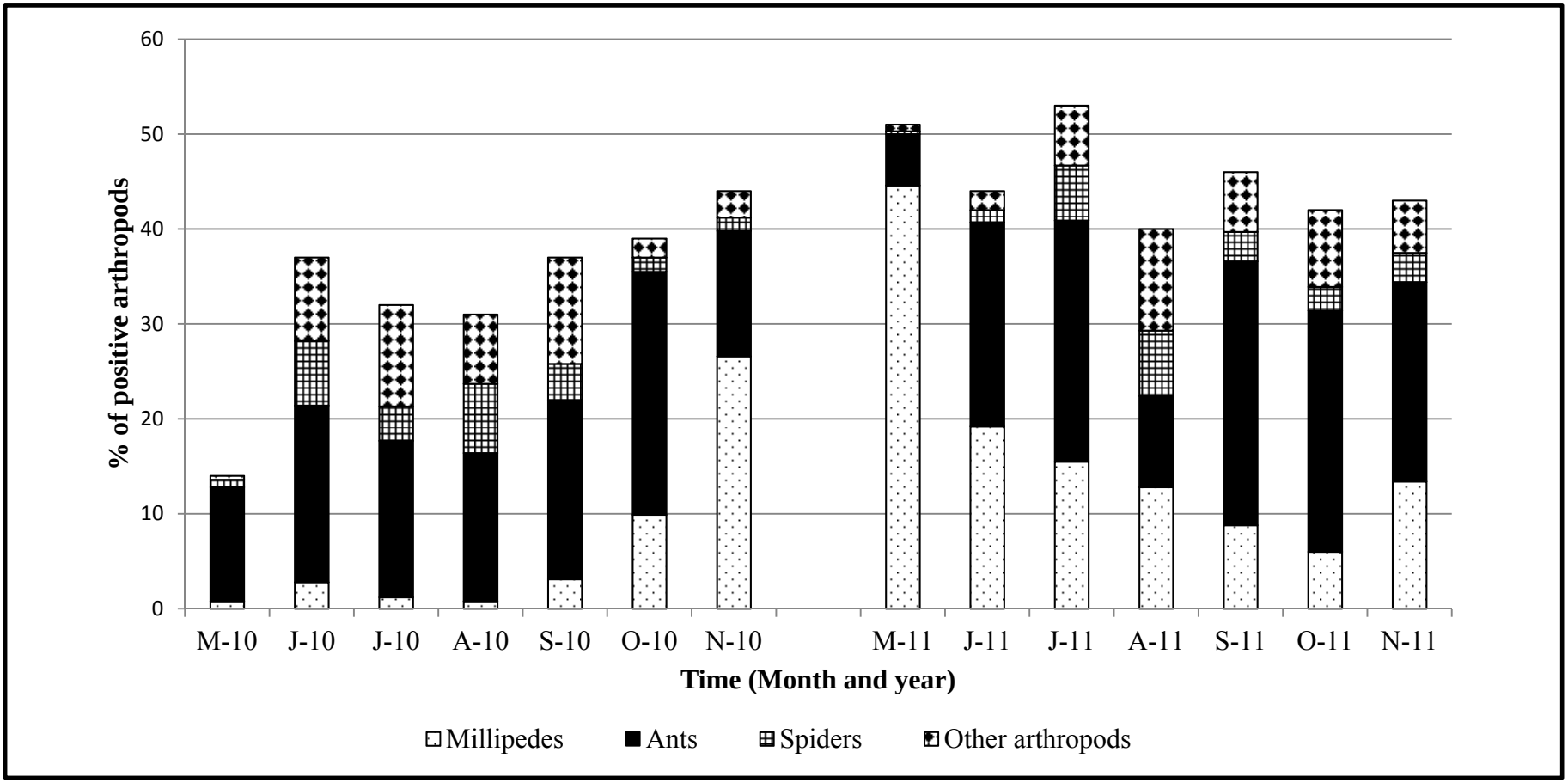

Figure 8. Percentages of arthropods that tested positive for grapevine trunk disease pathogens, calculated for each month from the Chenin blanc vineyard, for the years 2010 (-10) and 2011 (-11). Collection was done from May (M) until November (N).

CHAPTER 3

ANTS AND MILLIPEDES AS VECTORS OF *PHAEOMONIELLA CHLAMYDOSPORA* TO GRAPEVINE PRUNING WOUNDS

3.1 ABSTRACT

Phaeomoniella chlamydospora is one of the causal organisms of Petri disease and esca, which are important trunk diseases on grapevines globally. However, limited information exists on its epidemiology. In this study, we investigated the potential of Portuguese millipedes and cocktail ants to vector this fungus to fresh pruning wounds. We determined whether sap formed after pruning of shoots is a potential food source for these taxa, *in vitro*. Millipedes were offered a choice between grapevine sap and water in Petri dishes and monitored for ingestion of sap. Laboratory-kept colonies of ants were presented with a choice of grapevine sap, water and tuna and monitored to identify feeding preferences. Both arthropod species preferred grapevine sap over the other items presented to them, implying that they would visit fresh pruning wounds for sap. Subsequently, it was determined whether both arthropod taxa can effectively transmit a DsRed transformed *Pa. chlamydospora* isolate to fresh wounds and cause infection *in vitro*. Arthropods were exposed to the fungus for 24 hours and transferred to the base of pruned healthy plants (potted) and pruned healthy shoots (in polystyrene strips floating in water) and were removed after three days. Isolations from pruning wounds, one month later, confirmed that both arthropod species were able to vector the fungus to pruning wounds and cause infection. The fungus was not isolated from control plants. Since millipede feacal matter is often observed on wounds under field conditions, the survival of infective spores through the gut of these was also determined. Millipedes were exposed to *Pa. chlamydospora* for 24 hours, surface-sterilized and allowed to defecate in sterile Petri dishes. Feacal pellets were recovered, macerated in water and plated onto potato dextrose agar. The fungus was recovered from pellets in a viable state. These results provide compelling evidence that arthropods need to be taken into consideration when managing Petri disease and esca pathogens.

3.2 INTRODUCTION

Petri disease and esca are important grapevine trunk diseases that cause economic losses in vineyards and are reported in all viticultural areas around the world (Chicau *et al*., 2000; Armengol *et al*., 2001; Fischer, 2002; Edwards and Pascoe, 2004; Rooney-Latham *et al*., 2005b; Zanzotto *et al*., 2007; White *et al*., 2011). *Phaeomoniella* (*Pa.*) *chlamydospora* (W. Gams, Crous and M.J. Wingf. and L. Mugnai) Crous & W. Gams and *Phaeoacremonium* (*Pm.*) species W. Gams, Crous & M.J. Wingf. are the causal agents of Petri disease (Scheck *et al*., 1998; Mugnai *et al*., 1999; Groenewald *et al*., 2001) while basidiomycetes together with Petri disease fungi cause esca (Surico, 2009).

Previous studies on the dispersal of Petri disease pathogens in established vineyards has indicated a role of aerial spores that can infect vines through fresh pruning wounds (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). However, recent studies failed to detect spores of these fungi in the air. Michelon *et al*. (2007) failed to trap spores of these pathogens, in an Italian vineyard, using glass microscope slides and volumetric spore traps. Similarly, volumetric spore traps used in a vineyard in Stellenbosch, South Africa, showed an absence of spores of these fungi in the air despite their isolation from pruning wounds (Van Niekerk *et al*., 2010). This suggests that these pathogens may not be primarily dispersed by air. One of the potential agents for dispersal of these pathogens includes arthropods (Chapter 2).

Petri disease fungi sporulate on diseased grapevine wood (Edwards and Pascoe, 2001; Eskalen *et al*., 2002; Eskalen *et al*., 2005a, 2005b; Rooney-Latham *et al*., 2005a). *Phaeomoniella chlamydospora* produces pycnidia on old pruning wounds or in cracks and crevices of grapevine wood (Edwards and Pascoe, 2001; Eskalen *et al*., 2002). Perithecia of *Togninia* (Tul. & C. Tul.) Berl, the teleomorph of *Phaeoacremonium*, are found in cracks and crevices on trunks and cordons of diseased vines, and have long necks (Mostert *et al*., 2003; Rooney-Latham *et al*., 2005a) that are oriented towards openings of cracks in the bark, pruning wounds and/or insect tunnels (Rooney-Latham *et al*., 2005a; Eskalen *et al*., 2005a, 2005b). Sticky droplets of ascospores are produced at the tips of these (Rooney-Latham *et al*., 2005a), an adaptation usually associated with arthropod spore dispersal (Leach, 1940; Abbott, 2002). Sporulation of Petri disease fungi has been linked to the occurrence of rainfall (Eskalen and Gubler, 2001; Rooney-Latham *et al*., 2005a; Serra *et al*., 2008) and the cracks

and crevices of diseased vines may provide a protected humid environment favourable for sporulation (Serra *et al*., 2008). These environments are also favourable habitats for numerous arthropods. Sporulation of these pathogens in areas that are often occupied by arthropods indicates a possible role of arthropods in spore dissemination. In Chapter 2, more than 31 families of arthropods collected from grapevines in South Africa were found to be carriers of trunk disease pathogens. However, no direct experimental evidence exists to implicate any arthropod species as a vector of trunk disease pathogens.

Dissemination of fungal spores by arthropods occurs as a result of arthropods being contaminated with the fungi while visiting infected plants and then transporting the spores to healthy plants (Agrios, 2005). Arthropods can only be considered effective vectors if they are able to transfer spores to healthy plants under conditions that are suitable for inoculation and infection (Leach, 1940). Portuguese millipedes, *Ommattoiulus moreleti* (Lucas), and cocktail ants, *Crematogaster peringueyi* Emery are associated with grapevine pruning wounds (Chapter 2), presumably to feed on the sugar-rich grapevine sap, and consistently yielded grapevine trunk disease pathogens from their exoskeletons (Chapter 2). However, it has not been experimentally demonstrated that these arthropods can inoculate pruning wounds of healthy grapevines with spores and cause infection.

Fungal spores may be transported either externally on the exoskeleton of arthropods (El-Hamalawi and Menge, 1996; Kluth *et al*., 2002; Storer *et al*., 2004), or internally through the digestive tract (Leach, 1940; El-Hamalawi and Menge, 1996; Prom and Lopez, 2004; Lilleskov and Bruns, 2005). External transport of fungal spores has been established for numerous pathogens (El-Hamalawi and Menge, 1996; Kluth *et al*., 2002; Storer *et al*., 2004) and also for the grapevine trunk pathogens (Kubatova *et al*., 2004; Chapter 2). An increasing number of studies indicate that faecal material of arthropods may contain viable propagules of pathogens which make faeces a possible important source of inoculum contributing significantly to fungal dispersal (Leach, 1940; Carter, 1973; Prom and Lopez, 2004). For example, *Fusarium vasinfectum* (G.F.Atk) can be disseminated in the faecal pellets of grasshoppers that ingest infected tissues (Leach, 1940). Similarly, spores of *Claviceps paspali* Stev. can pass through the intestinal tract of the green fly *Pyrellia coerulea* (Wied.) in a viable state (Carter, 1973). Prom and Lopez (2004) recovered viable spores of *Claviceps africana* Freder., Mantle & De Milliano from excreta of adult corn earworm moths, *Helicoverpa zea* (Boddie) after being fed on ergot-infected sorghum panicles. Interestingly,

the passage of fungal spores through the digestive tract of arthropods and small mammals may even enhance their germination (Stanghellini and Russel, 1972; Cork and Kenagy, 1989; Castillo-Guevara *et al*., 2011). Faecal matter provide an immediate supply of moisture and nutrients and can be seen as a concentrated 'spore pocket' (Castillo-Guevara *et al*., 2011) when compared to dispersal by agents such as wind. This increases chances for survival of spores, as with dispersal agents such as wind, some spores may land on nutrient poor substrates. Faecal pellets of millipedes were observed on many pruning wounds on grapevines (Chapter 2) and if spores of grapevine trunk disease pathogens can survive the journey through the digestive tract of these, it is likely that millipede faecal pellets act as inoculum sources on pruning wounds.

The aims of this study were therefore, to determine whether Portuguese millipedes and cocktail ants are attracted to and/or can feed on grapevine sap and to determine whether these arthropods could acquire inoculum of a *Pa. chlamydospora* and disseminate it to grapevine pruning wounds to cause infection. This study also investigated whether millipedes can feed on material containing *Pa. chlamydospora* and excrete the propagules in a viable state.

3.3 MATERIALS AND METHODS

3.3.1 Grapevine sap as food source for millipedes and ants

3.3.1.1 Millipedes

Millipedes (*O. moreleti*) were collected by hand from the arms and trunks of grapevines as well as from plant debris on the soil surface in a Cabernet Sauvignon vineyard at Nietvoorbij, Stellenbosch, South Africa, during July 2012. Individuals were placed in 9 cm diameter Petri dishes and starved for 8 hours. Grapevine sap was collected by cutting shoots from Cabernet Sauvignon grapevines and collecting the sap at the surface with a pipette. One drop (200 µl) of water and one drop (200 µl) of sap was placed at opposite sides of the Petri dish ca. 60 mm apart and ca. 15 mm from the side. The positioning of the water or sap samples in the Petri dishes was such that, in half of the plates the vine sap was on the right and in the other half, the sap was on the left. The experiment was replicated with 30 different

millipede individuals serving as replicates. In addition, 20 millipede individuals were presented with two 200 µl drops of water only in the same experimental setup.

The starved millipedes were placed at the edge of the Petri dishes, halfway between the two drops (Fig. 1A). Observations of millipedes were made every 30 minutes (ca. 1 min observation time) for a total observation period of 4 hours. The experiment was conducted at night (main activity period of millipedes) and repeated once. The drop from which the millipedes were presumably feeding (with mouthparts on the drop) was noted and the total number of individuals observed to ingest water, sap or nothing was recorded. The millipedes were indicated to have ingested a substance regardless of how many times they were observed to feed and were indicated to have fed on both items if they were found to feed on both drops during the experiment. The number of millipedes that ingested or did not ingest a substance, during the two nights of experimentation, was combined. A Chi-squared (χ^2) test was conducted using the Freq Procedure of SAS statistical software (Version 9.2; SAS Institute Inc., USA) to test the null hypothesis that the number of millipedes that ingested a substance was independent of the identity of the substance.

3.3.1.2 Cocktail ants

Grapevine wood that contained nests of cocktail ants (*C. peringueyi*) were collected from a declining Chenin blanc vineyard in Rawsonville, South Africa, in July 2012, and kept under laboratory conditions in perspex boxes measuring 600 mm x 450 mm x 300 mm. Nest sizes were not quantified and likely contained variable numbers of foraging worker ants. Food was withheld from the ants for 24 hours prior to experimentation. The experiment consisted of seven replicates and was conducted over a 3 day period. One box (replicate) was used during the first day and three boxes per day in the following days, with a different ant nest used for each replicate. Pieces of wood containing ant nests were placed at the centre of the boxes while food samples were placed at both ends of the boxes. Ants were offered a choice of 0.5 g tuna, 0.5 ml water and 0.5 ml grapevine sap collected from the Cabernet Sauvignon vineyard used for the millipede feeding studies. The different food items were placed in Petri dishes of 65 mm in diameter, with two Petri dishes of each food item in each box. A set of food items was placed on one side of the nest and another set on the opposite side (Fig. 1B). The number of ants observed feeding on (with their mouthparts on the food item) each item was recorded at 15 minute intervals (ca. 1 minute observation time) for a

total duration of 3 hours. The total number of ants found feeding on a particular food item during the entire time of observation was used for statistical analyses for each of the seven replicates. Analyses of food preference data were conducted using a General Linear Model (GLM) analysis of variance (ANOVA) in SAS version 9.2 with the number of ants (counts) found on each item as the dependent variable. This was followed by LSD post hoc t-test (α = .05) to compare means. Counts were Log (x+1) transformed prior to statistical analysis and replicates were entered as block factors.

3.3.2 Transmission of fungal propagules from external surfaces of arthropods to pruning wounds

3.3.2.1 Fungal isolate used

A DsRed-Express transformed *Pa. chlamydospora* isolate (STE-U 6520, McLean *et al*., 2009) from the Stellenbosch University culture collection was used in this study. Use of this isolate made visualisation of successful infection of freshly pruned grapevine shoots easy and also ensured that positive detection of infection was by the experimental isolate only. The fungus was maintained in Petri dishes containing potato dextrose agar (PDA, Biolab, South Africa) for 14 days at 24 °C prior to experimentation.

3.3.2.2 Preparation of grapevine plants

3.3.2.2.1 *Potted plants*

One-year-old rooted dormant canes of the cultivar Chardonnay were obtained from a certified nursery and were planted in individual plastic pots, measuring 13 cm in diameter and 23 cm in height in a glass-house. Thirty vines were used for each of the two treatments (inoculations with spore-carrying cocktail ants or millipedes) and ten served as control for each treatment, respectively. A cylinder, constructed from transparency paper, was placed around each plant and was fastened to the inside of the plastic pots using adhering tape. Cheesecloth was placed over the top of the cylinder and fastened using adhering tape to prevent arthropods from escaping. Soil within pots was covered with filter paper to prevent arthropods from digging into the soil (Fig. 2A). A single shoot per vine was pruned immediately prior to inoculation with arthropods, which were placed on the filter paper. After

inoculation with arthropods, plants were grown for a month before isolations were carried out.

3.3.2.2.2 *Floating canes*

One-year-old dormant grapevine canes of the cultivar Chardonnay were soaked in water for 4 hours after which they were surface sterilized by dipping in Sporekill™ (ICA International Chemicals, South Africa) at 150 ml/100L for 5 minutes and dried at room temperature. The canes were stored for 2 months at 4 °C in air tight bags containing moistened perlite. These were then subjected to hot water treatment (50 °C for 30 minutes) and cut into two-node pieces. Each piece was placed through a 9 mm hole in polystyrene strips floated in water, such that half of the length of each cane was immersed. Two plastic basins (600 mm x 300 mm x 200 mm) were used as experimental arenas, one for millipedes and the other for ants. Each basin contained 26 shoots placed on polystyrene strips. Canes were pruned to one bud immediately before arthropods were placed onto the polystyrene strips. Basins were covered with cheesecloth to prevent arthropods from escaping (Fig. 2B). Water within the basins was changed twice weekly and the vines were allowed to grow for a month prior to isolations.

3.3.2.3 Arthropod collection, treatment and placement on grapevine plants

Millipedes and ants were collected from the above-mentioned vineyards. Millipedes were washed in distilled water for 10 seconds and dried using filter paper. Because of their small size, ants were used unwashed. Collected millipedes (3 per dish) and ants (30 per dish) were allowed to associate with sporulating colonies of the DsRed-Express transformed *Pa. chlamydospora* in Petri dishes for 24 hours. These were removed from the Petri dishes using sterile tweezers and immediately placed on either the filter paper (for potted plants) or on the polystyrene strips (for floating canes) and left for 3 days, after which they were removed. Three millipedes and ten ants were used for each of the potted vine treatments, respectively. Control potted plants received the same numbers of individuals but these were placed onto PDA plates without any fungal colonies prior to inoculation. Each cane inserted into the polystyrene strip received either three millipedes or ten ants (depending on the treatment) that were placed directly onto the strip.

3.3.2.4 Inoculum viability and spore load of *Pa. chlamydospora* on millipedes and ants

The viability of the inoculum was determined by allowing individuals of each arthropod species to walk on PDA plates containing the fungus for 24 hours (as described above). Three individuals of each species were then placed individually in 2-ml tubes containing 1 ml of sterile water. The tubes were vortexed for 60 seconds to loosen spores. Arthropods were removed and the suspension was serially diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) with sterile water, and 100 µl aliquots of each dilution were spread onto Petri dishes containing potato dextrose agar. Three replicate dishes were used for each dilution. Plates were incubated at 24 °C for 3 days and the number of *Pa. chlamydospora* colonies growing on each was determined using a stereo microscope. Number of colonies growing on stock suspension (10^0) plates could not be determined due to the large number of colonies growing on them. For the rest, the number of colonies was counted and the average percentage germination was determined by dividing the number of geminating spores by the total number of spores in the plates.

Average spore loads [number of fungal spores carried on the exoskeleton of arthropods (Jacobi *et al*., 2007)] on arthropods that were allowed to associate with the fungal colonies were determined. Three individuals of each arthropod species were placed separately in sterile 2-ml eppendorf tubes containing 1 ml of sterile water, which were vortexed for 60 seconds and the arthropods removed. The number of spores in the suspensions was estimated using a haemocytometer. The volume of suspension examined in each replicate was 5 µl. The average number of spores per species was calculated and represented it as the approximate spore load.

3.3.2.5 Transmission of inoculum onto pruning wounds

The one-month-old pruning wounds from both the potted and floating vines were pruned and examined for the presence of the DsRed-Express transformed *Pa. chlamydospora*. The pruned pieces were surface sterilized by immersion in 70 % ethanol for 30 seconds, 60 seconds in 3.5 % sodium hypochlorite and again for 30 seconds in 70 % ethanol. Shoots were then split longitudinally and small wood fragments (1 x 2 mm) were cut from either side of the pith and plated onto PDA amended with chloromycetin (250mg/L) in Petri dishes. Dishes were incubated at 23 – 24 °C, exposed to approximately 12 hours day light and 10 hours of darkness and monitored daily for fungal growth for four weeks. The

fluorescence of the *Pa. chlamydospora* colonies growing on the PDA plates was observed in water mounted slides of mycelium using an epifluorescence microscope (Zeiss Axioskop, West Germany).

3.3.3 Millipede faecal pellets as sources of inoculum on grapevine pruning wounds

Millipedes were collected from a Cabernet Sauvingnon vineyard at Nietvoorbij. Forty-five millipedes were placed in Petri dishes (n = 15) containing PDA with *Pa. chlamydospora* colonies for 24 hours, each dish containing three millipedes. Twenty-four millipedes for the control experiment were placed in PDA dishes without any fungal colonies (n = 8). Millipedes were aseptically removed, using sterile tweezers, and surface sterilised; these were placed in 50-ml Falcon tubes containing 10 ml of sterile water and vortexed for 60 seconds. Millipedes were then transferred to fresh tubes containing 2.5 % sodium hypochlorite and vortexed for 30 seconds and thereafter, were transferred to tubes containing 10 ml of 70 % ethanol and vortexed for 10 seconds. The millipedes were then washed by vortexing in 10 ml sterile water for 60 seconds. This final wash-step was repeated. Different sterilisation regimes were tested to ensure effective surface sterilisation and not to kill the millipedes. They were dried on sterile paper towels and placed in sterile empty Petri dishes and left overnight. Faecal pellets of the millipedes were aseptically picked from Petri dishes the next morning and placed in 2-ml tubes containing 1 ml of sterile water. A suspension of the faecal pellets was made by macerating in sterile water. To determine whether the faecal pellets contained viable propagules of the fungus, 100 µl of the 1 ml suspension of faecal matter was plated on PDA. We also tested the efficacy of the surface sterilisation technique by plating possible spores from the final millipede wash step. These water samples were centrifuged for 10 minutes at 10000 rpm in an eppendorf centrifuge 5810R (Eppendorf International, Germany), the supernatant discarded and the pellet was re-suspended in 200 µl of sterile water. One-hundred microliters of this suspension was plated onto PDA and monitored for the growth of *Pa. chlamydospora*. The experiment was repeated.

3.4 RESULTS

3.4.1 Grapevine sap as food source for millipedes and ants

Forty-six of the 60 millipedes presented with a choice of water and sap were observed to feed throughout the duration of the experiment while the remaining 14 were not observed to feed. Forty-two of these ingested vine sap (Fig. 3A) while only four drank water. Of the 42 individuals, 39 chose to initially feed on the grapevine sap while only four chose to initially ingest the water. However, not one of the 39 individuals that started feeding on the grapevine sap first switched to water at a later stage whilst three of the four that chose water first switched to the sap later on. Chi-square tests showed that there was a significant ($\chi^2_{d.f=1}$ = 50.90, P < .0001) tendency for millipede individuals to feed on grapevine sap when faced with a choice between water or sap. When these had no choice (only water offered), only three of the 20 individuals were observed drinking, only once. Similarly, significantly more workers of *C. peringueyi* preferred grapevine sap over the protein and water offered to them (F = 12.60, d.f = 6, P < .05, Fig. 3B and Fig. 4).

3.4.2 Transmission of fungal propagules from external surfaces of arthropods to pruning wounds

The viability of the inoculum was found to be approximately 96 % and the spore load per individual millipede was estimated to be approximately 1.8 x 10^6 spores per ml of water while that of ant individuals was approximately 5.5 x 10^4 spores per ml of water. Some millipede and ant individuals were observed to move to pruning wounds on potted vines immediately after placement on filter paper (Figs. 5A and 5B). Ants were, however, much more active than millipedes and constantly moved around in the experimental arenas. Similar behaviour was observed for arthropods placed on the floating polystyrene strips. *Phaeomoniella chlamydospora* was isolated from 47 % (14 out of 30 plants) and 27 % (8 out of 30 plants) of the pruning wounds on potted vines that were exposed to millipedes and ants, respectively. The fungus was not isolated from any pruning wounds of control plants. In the case of the floating canes, the fungus was isolated from 15 % (4 of the 26 shoots) and 12 % (3 out of 26 shoots) of the canes for millipedes and ants, respectively. All isolates recovered from the pruning wounds were confirmed to be that of the DsRed-Express transformed *Pa.*

chlamydospora initially used for inoculations as these fluoresced red when viewed under the epifluorescence microscope.

3.4.3 Millipede faeces as sources of inoculum on grapevine pruning wounds

Plating the suspensions from the water in which the millipedes were rinsed revealed that the sterilisation technique used was fairly effective as 74 % (34 of the 46 dishes in both experiments) did not contain any fungal or bacterial growth. For the remaining 26 % of the dishes, 5 out of 12 plates produced a few colonies of *Pa. chlamydospora* while the rest were contaminated by bacteria and other fungal taxa (very few colonies when compared to dishes where faecal suspensions were plated).

Seventy-three percent (22 out of 30 dishes for both experiments) of dishes that contained suspensions of faecal pellets produced high numbers of *Pa. chlamydospora* colonies (Fig. 6). *Phaeomoniella chlamydospora* was not observed in the remaining 27 % of these dishes as these were overgrown by contaminants. The pathogen was not recovered from faeces of control millipedes exposed to PDA only.

3.5 DISCUSSION

The fungi that cause Petri disease and esca are vascular pathogens that infect grapevines *via* pruning wounds in the field (Eskalen and Gubler, 2001; Rolshausen *et al*., 2010). It has long been suspected that arthropods may play a role in this process, but this has never been empirically tested. In this study, both millipedes and ants were shown to be attracted to grapevine pruning wounds to feed on the sugar rich (Van der Meer *et al*., 1990) sap. In the process, these were shown to be able to effectively transfer spores of one of the causal agents of Petri disease and esca (*Pa. chlamydospora*) to pruning wounds on healthy plants and cause infection. Other grapevine trunk disease pathogens may be dispersed in a similar manner since they are ecologically fairly similar. These results may explain why Petri disease fungi were isolated from pruning wounds in vineyards where aerial spores of the pathogens could not be detected (Michelon *et al*., 2007; Van Niekerk *et al*., 2010).

Generally, millipedes are known to be detritivores (Crawford, 1992) although some, like the Portuguese millipede, have also been reported to feed on roots, fruits and germinating seeds of different plants (Baker, 1978; Ebregt *et al*., 2007) as well as on grapevine leaves

(Roets, personal comm.). This study, therefore, presents the first evidence that some taxa can also feed on plant sap produced from wounded grapevines. The choice of grapevine sap by the cocktail ants is not surprising as these have a high affinity for sugary food sources, although they are also known to feed on protein (tuna or dead insects) food sources (Longino, 2003; Mgocheki and Addison, 2009; Nyamukondiwa and Addison, 2011). It is noteworthy that these taxa might not necessarily visit pruning wounds for sap alone, but may also be feeding on the gum that forms later. The sap is usually only available for a day or two but these arthropods were observed to continue visiting pruning wounds even after the sap has stopped flowing (data not shown). Pruning wounds have been found to be susceptible for several weeks after pruning (Eskalen *et al*., 2007; Serra *et al*., 2008; Van Niekerk *et al*., 2011) and this may promote infection during feeding on gum exudates by contaminated arthropods.

In the field, inoculum can be acquired from the cracks and crevices on trunks, cordons and spurs of diseased vines (Edwards *et al*., 2001; Rooney-Latham *et al*., 2005a) as well as from sap flowing from fresh pruning wounds on diseased vines (Rooney *et al*., 2001). Although arthropods used in the study were exposed to the pathogen under conditions that would probably not occur naturally, they were able to acquire large quantities of pathogen spores and transmit the inoculum to pruning wounds immediately after exposure to fungal cultures. Under field conditions, numerous spores carried on the exoskeleton will be lost in transit or may die from exposure to less favourable conditions and lead to much lower inoculation success rates.

Numerous arthropod taxa have been shown to be associated with fungal pathogens. De Nooij (1988) demonstrated that weevils, *Ceutorhynchidius troglodytes* Germar, were able to carry the fungus, *Phomopsis subordinaria* (Desm.) Traverso, internally and externally, after exposing the weevils to the sporulating fungus in Petri dishes for 48 hours. The tortoise beetles, *Cassida rubiginosa* Muller, and alate aphids [*Uroleucon cirsii* (Linnaeus)] were able to transport spores of the rust fungus, *Puccinia punctiformis* (F.Strauss) Rohl. between diseased and healthy thistles, *Cirsium arvense* (L.) Scop. These aphids were exposed to the infected leaves for 24 hours in Petri dishes (Kluth *et al*., 2002). Argentine ants, *Iridomyrmex humilis* (Mayr), which were fed on sugary exudate (in Petri dishes) containing *Phytophthora citricola* Sawada were able to acquire and transmit the fungus and cause 73 % infection in wounded stem cuttings of a lauraceous tree, *Persea indica* (L.) Spreng. (El-Hamalawi and

Menge, 1996). Storer *et al*. (2004) showed that twig beetles in the genus *Pityophthorus* Eichhoff were able to vector the pitch canker pathogen, *Fusarium circinatum* Nirenberg & O'Donnell, to Monterey pine branches. To the best of our knowledge, this study is the first to show that millipedes can also vector plant pathogenic fungi.

Inoculum of pathogens can not only be transported on external structures of arthropods, but also internally. This was clearly demonstrated in the current study where *Pa. chlamydospora* was recovered from the faeces of millipedes. Therefore, *Pa. chlamydospora* propagules are resistant to the digestive enzymes of millipedes. However, the identity of the infective unit (whether it is the spores or mycelium) in the faeces is still unknown and it still needs to be determined how long the millipede faeces can remain infective. Several authors have noted the presence of fungal spores in droppings of arthropods implicating their importance as a dispersal agent. For example, the faecal pellets of the millipede, *Harpaphe haydeniana* (Wood), were found to contain the ectomycorrhizal fungus, *Tomentella sublilacina* (Ellis & Holw.) and could serve as inoculation source of seedlings of the Bishop Pine, *Pinus muricata* D. Don. (Lilleskov and Bruns, 2005). El-Hamalawi and Menge (1996) found that faeces of garden snails (*Helix aspera* Muller), when fed avocado plants (*Persea americana* Mill.) infected with *Phytophthora citricola*, contained viable propagules of the fungus. The same study showed that inoculation of wounds of healthy avocado plants with contaminated faeces resulted in 77 % infection success (El-Hamalawi and Menge, 1996). Turner (1967) found that sporangium of *Phytophthora palmivora* Butler survived the passage through the digestive tract of the African snail *Achatina fulica* Bow. and remained infective for 14 weeks.

The release of propagules of Petri disease and esca pathogens occurs mostly in winter and has been closely linked to the occurrence of rainfall (Eskalen and Gubler, 2001; Rooney *et al*., 2005a, but see chapter 2). This is also the time when millipedes start to emerge and become more abundant (Baker, 1978; Bailey and Kovaliski, 1993; Carey and Bull, 1986; Widmer, 2006). This coincides with the time when grapevine pruning is usually carried out and this suggests that millipedes may play a major role in the inoculation of vines with Petri disease and esca pathogens. *Crematogaster* ants are arboreal, usually nesting under the bark or in hollow branches of trees (Longino, 2003). *Crematogaster peringueyi* is more abundant in old vineyards containing a lot of dead wood (Kriegler and Whitehead, 1962). These same niches are also sporulation areas for Petri disease pathogens (Edwards *et al*., 2001; Rooney-

Latham *et al*., 2005a). These ants can therefore, easily become contaminated with pathogens and transfer these to fresh wounds on healthy vines while foraging. Personal observations confirm that the cocktail ants often move from one vine to the next via trellising wires.

Results from this study show that it is necessary to integrate the control of arthropods, particularly cocktail ants and millipedes, into the efforts to manage Petri disease pathogens. These management strategies should include protection of pruning wounds from pathogen inoculation by arthropods and removal of dead and diseased wood to reduce inoculum load. This will also help reduce arthropod populations as dead wood is considered a prime habitat for millipedes and cocktail ants. The movement of arthropods to fresh pruning wounds should be limited by e.g. placing barriers below wounds or the use of baits at the base of the plants (Purcell, 2006). The movement of millipedes can easily be prevented by placing smooth vertical or rounded barriers at the base of trunks (Widmer, 2006). The importance of the control of cocktail ants and other ant species found in vineyards is already well established (Kriegler and Whitehead, 1962; Nyamukondiwa and Addison, 2011). Ants tend the vine mealybug, *Planococcus ficus* (Signoret), which is a serious pest in South African vineyards, by consuming the honeydew it produces and thereby stimulate outbreaks of this arthropod (Mgocheki and Addison, 2009; Nyamukondiwa and Addison, 2011). Several control methods are in place in South Africa (Mgocheki and Addison, 2009; Nyamukondiwa and Addison, 2011). These need to be adhered to in order to help curb the spread of grapevine trunk disease pathogens.

Further studies are needed to determine the relative importance of other arthropod taxa, which were found to carry grapevine trunk disease pathogens (Chapter 2), in the transmission of these pathogens to fresh pruning wounds. Control practices need to be evaluated on a per-taxon basis as some of these spore-carrying arthropods are also beneficial to the plants. Spiders, for example, are good control agents for many pest species, but these have also been identified as carriers of the trunk disease pathogens (Chapter 2). The use of broad spectrum insecticides is thus, not advocated here and a greater focus on sanitation practices will likely negate the role of most arthropods in disease transmission.

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Figure 1. Experimental set-up for food choice experiments. **A.** Petri dish (with lid open) showing the arrangement of water and grapevine sap with a millipede (M). **B.** Perspex box used to test food preferences of cocktail ants. Petri dishes contained grapevine sap, water or tuna were placed at either side of wood pieces (in the centre) containing ant nests.

Figure 2. Experimental set-up for determining vector potential of arthropods. **A.** Young grapevines in plastic pots inoculated with either millipedes or cocktail ants. **B.** Grapevine canes placed through holes in polystyrene strips floating in a plastic container filled with water.

Figure 3. Illustrations of food choice experiments. **A.** A millipede (M) observed feeding on grapevine sap in a Petri dish. **B.** Petri dishes containing tuna, sap and water, respectively which were placed in a perpex box containing ant nests. Several cocktail ants can be seen feeding on the grapevine sap (middle) compared to tuna (left) and water (right).

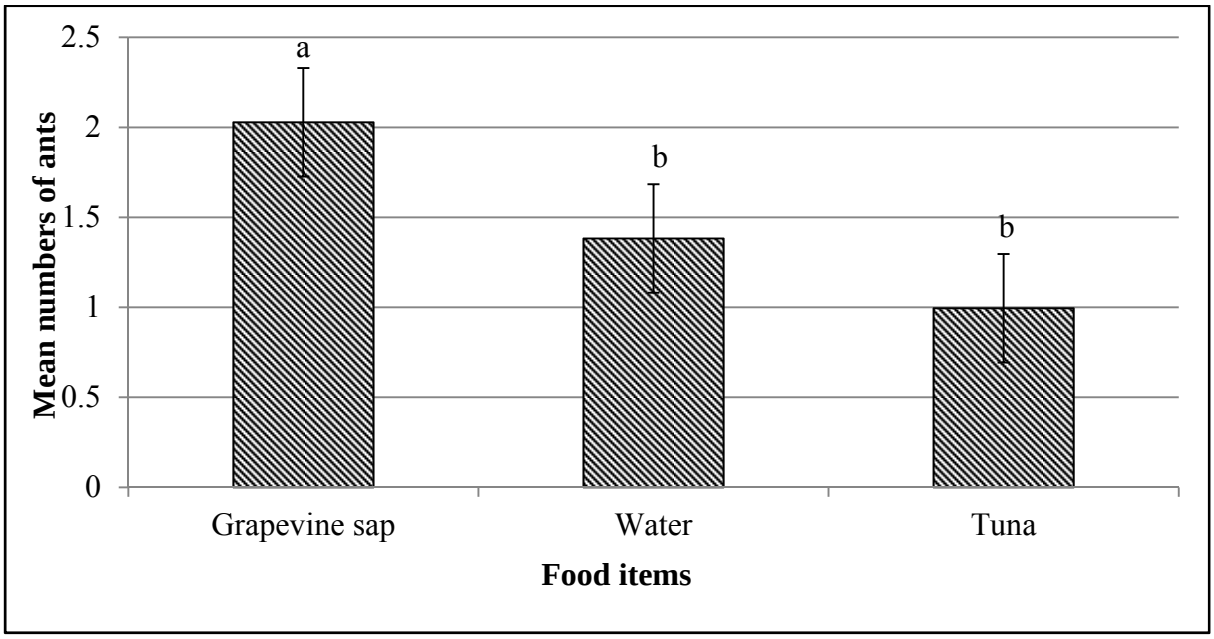

Figure 4. Mean number of ants (Log (x+1) transformed means) that fed on a specific food item throughout the three hour observation period. Different letters above bars indicate significant differences between means at $P < .05$.

Figure 5. Transmission of *Pa. chlamydospora* to freshly pruned potted grapevines. **A.** A millipede (indicated by arrow) on a pruning wound. **B.** An ant (indicated by arrow) is observed on a pruning wound of one of the potted grapevines.

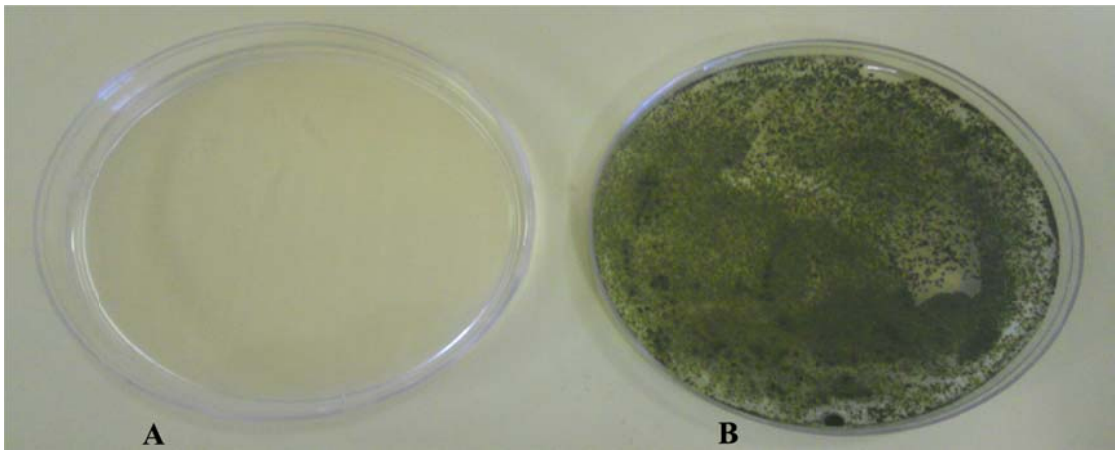

Figure 6. Millipede faecal pellets as sources of *Pa. chlamydospora*. **A.** Potato dextrose agar plate (with lid open) in which the suspension from millipede washings (control for efficacy of surface sterilisation technique) was plated. The dish does not contain any growth of any microorganism 16 days after plating was done. **B.** *Pa. chlamydospora* is seen covering the plate (with lid open) in which the macerated faecal pellets of the same millipedes as in A, was plated.

CHAPTER 4

GENERAL DISCUSSION

Petri disease and esca are important diseases of grapevines and have had a large impact on the grapevine industry in the last two decades. Several sources of inoculum and dispersal mechanisms of the pathogens responsible for these two grapevine trunk diseases have been studied and are discussed in Chapter 1. Although extensive research has been carried out to understand the epidemiology of Petri disease and esca pathogens, several questions remain uncertain with regards to dissemination in vineyards. Arthropods have been suspected to play a role in the dispersal of grapevine trunk disease pathogens in the field (Edwards *et al*., 2001; Rooney-Latham *et al*., 2005). Prior to the present investigation, only one study had shown the presence of *Diplodia seriata* (associated with black dead arm) on rove beetles, caught on pruning wounds in a vineyard (Epstein *et al*., 2008). The overall objectives of this study were to identify arthropods that harbour Petri disease and esca fungi and their potential to transmit the fungi to fresh grapevine pruning wounds. Understanding the sources of inoculum and dispersal mechanisms of any pathogen is important in order to develop effective control measures. Therefore, the knowledge gained with this study could be used to determine whether it is necessary to include the control of certain arthropods in the disease management of grapevine trunk diseases.

4.1 Arthropods as carriers of pathogens associated with Petri disease and esca in vineyards

Arthropods associated with declining grapevines were collected from two vineyards and were tested for the presence of grapevine trunk disease pathogens. The study succeeded in identifying arthropods occurring on declining grapevines and showing that a wide variety of arthropods carry grapevine trunk disease pathogens. These findings are consistent with other studies which have demonstrated that arthropods are capable of carrying and transmitting pathogens to healthy plants (De Nooij, 1988; El-Hamalawi and Menge, 1996; Kluth *et al*., 2002; Storer *et al*., 2004; Lilleskov and Bruns, 2005). The arthropod taxa which were associated with diseased vines and found to carry trunk disease pathogens are listed in Table 1 of Chapter 2. The presence of a variety of arthropod species found carrying trunk

disease pathogens in vineyards present a great number of arthropod species that may serve as vectors of pathogens.

Although the aim of the study was to focus on fungi associated with Petri disease and esca, all the major groups of fungi associated with grapevine trunk diseases were detected (*via* plating on PDA) and reported on. Molecular detection (nested PCR) was more sensitive since it detected a higher number of samples with Petri disease fungi compared to plating on media. However, it is important to note that the high number of samples does not indicate a high number of viable pathogen propagules because PCR has the ability to detect both viable and non-viable propagules of fungi (Rawsthorne *et al*., 2009). The presence of trunk disease fungi on arthropods helps to explain why some studies have not caught spores of Petri disease fungi in the air. Studies by Michelon *et al*. (2007) and Van Niekerk *et al*. (2010) failed to trap spores of these pathogens in vineyards using volumetric spore traps.

4.2 Ants and millipedes as vectors of *Phaeomoniella chlamydospora* to grapevine pruning wounds

The first phase of the work (Chapter 2) showed that high numbers of Portuguese millipedes and cocktail ants carried trunk disease fungi, indicating their potential of vectoring trunk pathogens. These arthropod species were used in subsequent studies to determine their vector status. The first aim of Chapter 3 was to test the hypothesis that these arthropods were associated with grapevine pruning wounds partly because of their attraction to and/or fed on sap flowing from freshly made wounds. The results of this study supported the hypothesis that the arthropods are attracted to and feed on grapevine sap. These findings suggested that further work needed to be conducted to: i) test whether arthropods carrying inoculum on their exoskeleton can successfully deposit it on grapevine pruning wounds during feeding on the sap and cause infection and ii) determine whether *Pa*. c*hlamydospora* propagules consumed by millipedes can withstand the digestive enzymes in the alimentary canal and be passed out in a viable state in the faecal pellets. These studies, as discussed in Chapter 3, were valuable in that they conclusively proved that ants and millipedes do not only carry inoculum, but are capable of depositing it and cause infection on pruning wounds as they explore them for grapevine sap. These results agree with those of El-Hamalawi and Menge (1996) and Lilleskov and Bruns (2005) who reported that Argentine ants and millipedes can carry and transfer inoculum to healthy plants, respectively.

Fungal pathogens have undetermined means of dispersal, thus they could use any means possible for their dispersal. This is demonstrated in the recovery of viable propagules of *Pa. chlamydospora* in the faeces of millipedes. Several studies have also showed that arthropods such as snails (El-Hamalawi and Menge, 1996), grasshoppers (Leach, 1940) and adult corn earworm moths (Prom and Lopez, 2004) ingest fungal propagules and pass out the propagules in a viable state. These findings demonstrate that faecal material of millipedes, which was frequently observed on pruning wounds, should be considered as an important source of inoculum for *Pa. chlamydospora* and probably other fungal trunk pathogens in vineyards.

The results of this study confirm speculations by other studies (Edwards *et al*., 2001; Rooney-Latham *et al*., 2005; Van Niekerk *et al*., 2010; Gramaje and Armengol, 2011) that arthropods may have a role in the dissemination of trunk disease pathogens. These results are important as they contribute immensely in understanding the epidemiology of grapevine trunk disease pathogens and demonstrate that more integrated strategies are needed for the proactive management of these pathogens in vineyards.

4.3 Conclusion and future research

Understanding how plant pathogens are dispersed is an essential part of plant pathology. The knowledge of pathogen dispersal mechanisms is important for effective control of plant diseases because it allows prevention of dispersal of disease as well as breaking the infection chain, by discontinuation of the life cycle of the pathogen (Brown, 1997). Although the study did not quantify the relative importance of arthropods compared to aerial dispersal of *Pa. chlamydospora*, it is evident from observations made in this study that arthropods may play a role in the dispersal of Petri disease and esca pathogens. These observations include the high diversity of arthropod species associated with diseased grapevines and those carrying trunk disease pathogens, the ability of ants and millipedes to transmit *Pa. chlamydospora* to healthy vines and the ability of the fungus to survive inside the gut of millipedes. It seems likely that other grapevine trunk disease pathogens may also be dispersed by arthropods internally.

The management of millipedes and ants needs to form part of the management strategies to combat grapevine trunk diseases in vineyards. Management strategies of grapevine trunk diseases range from chemical and biological protection of pruning wounds to cultural practices such as use of disease free planting material and removal of dead or

diseased plant parts (Gramaje and Armengol, 2011). No current advice is available to producers in South Africa regarding the management of arthropods such as millipedes in reducing grapevine trunk diseases since it was not known that they are vectors of trunk disease pathogens. This study therefore, provides the basis for developing and testing management strategies against such arthropods in future research.

Although only millipedes and ants were used in the transmission studies, it is important that further studies be undertaken to determine the vector status of other arthropods that were found in high numbers, such as the *Cheiracanthium* species (spiders) as well as *Euryopis episinoides* (spiders). Rove beetles and springtails were only captured using pruning wounds traps. This demonstrates that these are associated with pruning wounds and therefore, future work should also aim to determine if they can transmit inoculum to wounds and cause infection. Snails occurred in considerably high numbers in vineyards, but were not included in this study. However, studies have demonstrated that snails vector plant pathogens internally and externally (Turner, 1967; El-Hamalawi and Menge, 1996; Alvarez *et al*., 2009; Borkakati *et al*., 2009). Therefore, future studies should also consider determining their vector status of grapevine trunk pathogens.

The pathogen status of *Diplodia scrobiculata*, *D. medicaginis* and *Aplosporella prunicola*, which are pathogens on *Pinus* spp. (Bihon *et al*., 2011), *Medicago sativa* (Phillips *et al*., 2008) and *Prunus* spp. (Damm *et al*., 2007), respectively, needs to be determined on grapevines. These fungi were present on arthropods in grapevines and their possible role in grapevine trunk diseases is unknown.

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