INSECT-FUNGAL INTERACTIONS IN TREE NUT CROP ORCHARDS IN SOUTH AFRICA, WITH SPECIFIC REFERENCE TO PECANS

By

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DECLARATION

"I declare that the thesis hereby submitted by me for the Master of Science degree in Entomology at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore concede copyright of the dissertation to the University of the Free State."

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Jaco Saaiman

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie (1867 − 1934)

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Chapter 1

Insect-Fungal Associations on Tree Nut Crops in Semi-Arid Regions of South-Africa

1.1 Introduction

Fungal pathogens are the leading cause of disease in most crop production systems (Agrios, 2005). These organisms cause more crop diseases than all other micro-organisms combined. This is due to adaptations which allow fungal pathogens to survive within many diverse biotic and abiotic environments. Successful dispersal mechanisms of fungal propagules contribute largely to the success of fungi (Kendrick, 2000). They are dispersed by wind, water, seeds, other vegetative plant material, their own mechanical forces, soil, insects and other organisms (Agrios, 2005). Although fungi use a variety of methods to spread between their host plants, insects remain one of the most important methods of dissemination. They disseminate fungi through a variety of activities such as pollination, feeding, oviposition and/or by merely moving around on the surface of a plant. Insects can thus contribute to the spread and occurrence of disease within crop production. The association between insects and fungal plant pathogens may have a detrimental effect on the establishment and production of important new crops, such as tree nuts (*e.g.* pistachios, walnuts and pecans) in South Africa.

This literature review is focussed on insect-fungal associations and their affect on the production of tree nut crops in semi-arid regions of South Africa. The first section focuses on the role of insects as vectors in the dissemination of fungi, compared to other modes of dispersal. The next section focuses on the dissemination of fungal plant pathogens by several insect families; Pentatomidae (Hemiptera), Cicadellidae (Hemiptera), Chrysomelidae (Coleoptera) and Curculionidae (Coleoptera). During this masters study, the role of species from these four families in the dissemination of fungi in tree nut crop orchards was investigated. Examining the insect-fungal associations occurring on other crops might show ways in which members of these families disseminate and interact with fungal pathogens. The final section of the review will offer background information on the production of tree nut crops (pistachios, walnuts and pecans) within semi-arid regions of South Africa and documented insect-fungal associations known to occur worldwide on these crops.

1.2 The role of insects in disseminating fungi

Fungi are among the most successful groups of organisms in terms of their ecological and evolutionary diversity (Desprez-Loustau *et al.*, 2007). The fungal kingdom is around 900 million

years old (Blackwell, 2000) and some estimate that there are at least 5.1 million species of which only about 400,000 have been described (Blackwell, 2011). Fungi are essential components of biodiversity, besides the great number of species, but also for their socio-economic, ecological, and evolutionary significance (Desprez-Loustau *et al*., 2007). Economically, fungi are of considerable importance due to their beneficial uses, including as an important food source, being used for industrial enzymes, and antibiotics. They also function as agents of disease in both plants and animals (including humans). The ability of fungi to cause disease on crops is significant since fungal pathogens are the leading cause of disease in most crop production systems. Fungi fulfil an ecological function as decomposers, which drive the carbon cycle and also as symbionts that sustain forests and grasslands *via* mycorrhizal associations (Desprez-Loustau *et al*., 2007).

Two important factors have contributed to the success of fungi *viz*. their ability to colonize new habitats and the ability to produce unique fruiting structures that disperse their spores (Ingold, 1953). Fungi are successful colonists since they are found in almost all aerobic environments. Dissemination of fungal propagules is the most important factor to ensure the survival of a species. Consequently, fungi have evolved ways to exploit several abiotic (hydrophily and anemophily) and biotic (vectored) dispersal mechanisms (Ingold, 1953). The dispersal of plant pathogenic propagules is important in agriculture, resulting in the spread and occurrence of disease.

1.2.1 Hydrophily: The dispersal of fungal propagules by water

Fungal propagules that are disseminated by water tend to be light and are easily carried by currents and water flow (Begon, Harper & Townsend, 1996). Several fungi from the Chytridiomycota, as well as members of the fungal-like phyla Hyphochytriomycota and Oomycota are aquatic and use water to disperse their spores (*e.g*. *Pythium* and *Phytophtora*) (Kendrick, 2000). The spores of these taxa are also commonly equipped with flagella (known as zoospores) that aids in their dispersal through water. Due to the small size of these spores their dispersal is limited by the absence of water or vectors. Several hyphomycetous fungi produce spores that may either be dispersed by floating on the surface of water or by being washed below the surface. These spores have distinctive appendages or shapes that allow them to be easily dispersed by water currents (Kendrick, 2000), however, dispersal by means of water has its

constraints in terms of spreading propagules to distant water bodies (ponds and rivers) that are not connected (Begon *et al*., 1996). Several fungi produce specialized spores that can be dispersed by water, but can also produce spores that are dispersed by wind in order to overcome this problem (Ingold, 1971).

1.2.2 Anemophily: The dispersal of fungal propagules by air

The use of air currents for dispersal is a passive method that relies on chance (Begon *et al*., 1996). In most cases the organism has no control of where the propagules will eventually land. To overcome this, certain organisms forcibly eject their spores towards certain sites while other produces a mass of spores to increase the odds of the propagules to eventually reach a suitable habitat. A variety of organisms use this method of dispersal which mostly include plants, fungi and insects (Begon *et al*., 1996). The most common method fungi use to disperse their spores is by means of air currents (Gregory, 1962). A cubic meter of air can contain as much as 200,000 fungal spores.

Fungal spores are dispersed through air in several ways (Roets, 2006). Some ascomycetes and certain basidiomycetes forcibly eject their spores while most of the hyphomycetes produce chains of spores that are easily dislodged and suspended in air. The spores of fungi that are dispersed *via* air are usually dry and can travel hundreds of kilometres. Fungi that rely on specialized substrata that are dispersed by air face certain limitations. Chances of these spores reaching a suitable uncolonized niche are very low and will likely perish before reaching suitable substrates. In this case the use of focused spore dispersal to reach limited or selective substrata would be more beneficial for the fungus. Species that use focused spore dispersal (such as vectoring) have a competitive advantage over species that rely solely on air dispersal. These species would be able to have the same success rate in their survival, but with less energy used in the production of high numbers of spores and resources may rather be used for other physiological processes (Roets, 2006).

1.2.3 Vectoring: The dispersal of fungal propagules by insects

Vectoring is defined as: "*dispersal by an organism, which consciously or unconsciously aids in the dispersal of another*" (Kendrick, 2000). According to Roets (2006), when taking this

definition into account, all fungal species are subject to dispersal by one or several vectors. Fungi that require specific substrata (*e.g.* a specific host plant) or that do not have access to either water or air, rely on vectors, such as insects, for the dispersal of their propagules. This is an effective strategy since fungi that require specific substrata often occupy the same niches as their vectors. The vector organisms are also well suited (*e.g.* they are very mobile or have specialized olfactory abilities to locate specific substrata) for the dispersal of fungal spores to distanced niches. Species from all fungal groups (ascomycetes, basidiomycetes, hyphomycetes and zygomycetes) have evolved to exploit vectored dispersal (Roets, 2006).

Research has been conducted to some extent on the dispersal of fungal propagules by mammals. Fungi that rely on mammals for dispersal (*e.g. Tuber* spp.) produce strong alluring odours to lure a variety of mammals (Fogel & Trappe, 1978). The animals feed on the fungal fruiting bodies and the fungal propagules are disseminated in their droppings (Claridge & May, 1994). Smaller fungal species, such as members of the hyphomycetes and ascomycetes, require substrates that are not accessible by mammals and, therefore, require smaller vectors such as insects and other arthropods (Roets, 2006).

The most important vectors of fungi are insect related. Insects disseminate plant pathogenic fungi thus contributing to the spread and occurrence of disease in crop production (Agrios, 2005). The role of insects in the dispersal of plant pathogenic fungi has been greatly underestimated in the past, even though they play a significant role in the occurrence of disease. Insects can disseminate fungi in a variety of ways including pollination, feeding, oviposition, and/or by merely moving around on the surface of a plant. Insects can disseminate plant pathogens specifically or non-specifically. Specific dissemination refers to the vectoring of viruses as well as phloem inhabiting fungi and bacteria where the pathogens are internally carried, however, the most common method of fungal dissemination by insects is non-specific, where the fungal propagules are carried on the exoskeleton (Agrios, 2005).

Several fungal species have evolved morphological adaptations to suit this mode of dispersal. The most notable of these are the production of spores in slimy droplets (*e.g. Leptographium* spp., *Ophiostoma* spp., *Verticillium* spp. and *Fusarium* spp.) or layers of slimy spores on the surface of fruiting bodies (*e.g. Phallus* spp.) (Abbott, 2002). Slimy spores attach to the bodies of insects as they move over the spores and are thus dispersed as the insects move on.

Although the dispersal of slimy spores is highly effective, fungi that produce dry spores are also dispersed by insects and not only by air (*e.g. Cephalotrichum* spp.). This is known as the "feather duster effect" and occurs when insects move through masses of synnemata, spores are then released in small clouds, effectively "dusting" the insects (Abbott, 2002).

Due to the intimate relationships between fungi and insects it is not surprising that fungi tend to form specific associations with their insect disseminators. The formation of such associations eventually results in symbiosis between the insect vectors and fungi (Roets, 2006). The most epidemiological and historically important mutualistic associations, in terms of pathogenic fungi being vectored by insects, are between wood boring ambrosia beetles (*e.g. Dendroctonis* spp., *Ips* spp. and *Xyleboris* spp.), and their symbiotic ophiostomatiod fungi (*e.g. Ceratocystis* spp., *Ceratocystiopsis* spp. and *Ophiostoma* spp.) (Paine, Raffa & Harrington, 1997; Harrington, 2005). This association has received special attention due to the pathogenic nature of the interaction and the ability of the fungi to kill off mature trees as a result of their dispersal by insects. The importance of this association is discussed in more detail in the next section.

Dissemination of ophiostomatoid fungi by wood boring ambrosia beetles

Several wood boring ambrosia beetles have specialized structures called mycangia in which both the sexual (ascospores) and asexual (conidia) spores of ophiostomatoid fungi are carried between hosts (Six, 2003). The evolution of structures like mycangia indicate that a very old (60-80 million years) relationship exists between these beetles and their symbionts (Farrell *et al*., 2001). Thus, the fungi associated with the ambrosia beetles are completely dependent on their vectors for dispersal and the survival of their species (Six, 2003).

Among the associations between ambrosia beetles and the ophiostomatoid fungi the most destructive and well documented relationship exists between *Ophiostoma novo-ulmi* and the European bark beetle, *Scolytus multistriatus* (Coleoptera: Curculionidae: Scolytinae) and a bark beetle native to North America, *Hylurgopinus rufipes* (Coleoptera: Curculionidae: Scolytinae) (Negron *et al*, 2005). This is a mutualistic relationship where both the insects and the fungi benefit (Bleiker *et al*. 2009). Beetles rely on the fungus to weaken and ultimately kill the trees, which provide preferable breeding sites for the beetles while the fungus relies on the beetles to be dispersed to new un-colonised hosts (Bleiker *et al*. 2009). The association between the beetles

and this fungus results in the disease known as Dutch elm disease (Connor & Wilkinson, 1998). Dutch elm disease was first noted in France (1919) and the first publicized report of its occurrence came from Holland (1921) (Brasier & Buck, 2002). Since then, the disease has spread throughout Europe, parts of Asia, and through most of the temperate regions of North America. The rapid spread of this disease is due to the interaction between the causative fungus and scolytid beetles, and it is believed that 99% of elm tree infections are a result of beetle transmission. The disease is characterized by the wilting of leaves and defoliation, followed by the die back of branches and eventually the whole tree. *Ophiostoma novo-ulmi* occurs and propagates underneath the bark of the trees on the inside of tunnels (also known as galleries) made by the ambrosia beetles. During the fruiting stages, the ascomata form long necks with ostioles at the tip. Spores ooze out of the ostioles to form slimy droplets at the tip of the neck. When the beetles move around within the tunnels, the spores cling to their bodies and ensure dissemination when the beetles move to a new tree. The mode of dissemination and subsequent transmission is so successful that this pathogen has almost completely decimated the American elm tree populations of Northern America and Europe. This association has led to one of the most important historical epidemics in plant pathology with the loss of trees. This is coupled with the removal of dead trees from urban areas amounting to millions of dollars annually (Brasier & Buck, 2002). The establishment of such associations may lead to great economic losses in other important crop production systems.

1.2.4 Importance of research concerning insect-fungal associations

Insects play a distinctive role in the dissemination of plant pathogenic fungi and much of the research concerning insect-fungal associations focuses on whether certain insect species disseminate plant pathogens. In most cases these insects are also pests (Steffan, Daane & Yokota, 2000; Sanderlin & Melanson, 2010) and research in this field normally aims to gain extensive knowledge on how these interactions function to effectively control the pathogen or the insect, or both. The knowledge gained from this can be used to limit the spread and occurrence of disease, increasing profits made from crop production. An investigation into the diversity of fungi associated with insects can identify potential pathogens, especially in new crops (*e.g.* tree nut crops in South Africa). Research on insect-fungal associations can also be incorporated into a bio-monitoring protocol to identify fungal pathogens (especially those that

have mutualistic relationships with insects such as the ophiostomatiod fungi). Proactive measures can thus be implemented to reduce the spread and occurrence of disease in previously uninfected areas.

An investigation into the diversity of fungi associated with insects also provides mycologists with the opportunity to discover and describe new fungal species. These studies might also reveal new entomopathogenic fungi that can be used to manage insect pests (Cottrell & Shapiro-Ilan, 2009; Down *et al.*, 2009). The use of such fungi to control pests is a method that is preferred since the use of synthetic pesticides is declining (Cottrell & Shapiro-Ilan, 2009; Down *et al*., 2009). Similarly, pathogens of weeds might also be discovered which, if applied, can reduce the use of herbicides (Kluth, Kruess & Tscharntke, 2001). The use of insects to disperse phytopathogens can be of considerable value to agriculture. An example is the control of the creeping thistle, *Cirsium arvense* (Asteraceae) that is a serious problem in wheat production, with *Puccinia punctiformis* (thistle rust) that is dispersed by the gall midge *Mycodiplosis coniophaga* (Diptera) (Kluth *et al*., 2001).

1.3 Dissemination of phytopathogenic fungi by Pentatomidae (Hemiptera), Cicadellidae (Hemiptera), Chrysomelidae (Coleoptera) and Curculionidae (Coleoptera)

Members belonging to the families, Pentatomidae (shield or stink bugs) and Cicadellidae (leafhoppers) from the order Hemiptera, and Chrysomelidae (leaf beetles) and Curculionidae (snout beetles or true weevils) from the order Coleoptera were used in this MSc study and, therefore, are discussed here. The role of these families in the dissemination of plant pathogens is discussed, as this might reveal the interaction between these insects and phytopathogens.

1.3.1 Hemiptera

Hemiptera, or true bugs, is the largest exopterygote insect order that shows considerable diversity within the group (Scholtz & Holm, 1985; Picker, Griffiths & Weaving, 2002; Triplehorn & Johnson, 2005). Species and families from this order vary greatly in their body shape, wings, antennae, life histories and feeding habits (Triplehorn & Johnson, 2005). This order has evolved a great range of structural features and adaptations to exploit a wide range of

different environments (Scholtz & Holm, 1985). Hemiptera was previously classified as two orders, Heteroptera and Homoptera (Picker *et al*., 2002). These orders were grouped together under the order, Hemiptera, due to their unique opistognathous piercing-sucking mouthparts. This is the most mutual characteristic that exists within the group (Scholtz & Holm, 1985; Triplehorn & Johnson, 2005). The structure of the mouthparts enables these insects to successfully extract fluids from both animals and plants, and has remained fairly unaltered, even though a wide range of other structural adaptations has occurred. Several members of this order are of economic importance as pests of crops (Steffan *et al*., 2000; Picker *et al*., 2002). Due to their method of feeding they are also capable of vectoring viruses, bacteria, phytoplasmas and fungi that cause diseases on plants and animals (Steffan *et al*., 2000; Picker *et al*., 2002).

1.3.1.1 Pentatomidae

Pentatomidae is a diverse and well-studied group (Triplehorn & Johnson, 2005). There are about 300 known species of pentatomids in Southern Africa and roughly 200 species in North America (Picker *et al*., 2002; Triplehorn & Johnson, 2005). These insects range from small to medium sized and show considerable variation in shape, colour and habits (Scholtz & Holm, 1985; Picker *et al*., 2002). They are easily distinguishable from other bugs by their round or ovoid body shape and their five-segmented antennae (Triplehorn & Johnson, 2005). Species of this family are also armed with glands that release pungent odours when they are threatened (Picker *et al*., 2002; Triplehorn & Johnson, 2005). Most species of this family are phytophagous, but species from the sub-family Asopinae feed on soft-bodied insects such as beetle larvae (Picker *et al*., 2002). Several species are of economical importance as pests of crops (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005), such as the southern green stink bug, *Nezaria viridula*, that attacks several crops throughout the tropical regions of the world (Triplehorn & Johnson, 2005).

Dissemination of fungal plant pathogens by members of Pentatomidae

Members of the Pentatomomorpha (Pentatomidae and Coreidae) are the most frequent disseminators of fungi within the order Hemiptera (Mitchell, 2004). Their feeding methods may lead to the forming of infection courts, providing entry points for fungi and other pathogens to infect plants, however, this group is more frequently involved in the direct vectoring or primary

facilitation of fungal plant pathogen transmission (Mitchell, 2004). Species within this family have been found to be involved in the dissemination of *Botryosphaeria dothidea* that causes panicle shoot blight and both *Nematospora coryli* and *Aureobasidium pullulans* that cause stigmatomycosis on pistachios. The dissemination of these pathogens by pentatomids is discussed further in the section regarding insect-fungal associations on pistachios. The rice stink bug, *Oebalus pugnax,* is also involved in the transmission of several fungal pathogens, *viz*. *Fusarium oxysporum*, *Cochliobolus miyabinus*, *Curvularia lunata*, *Alternaria alternata* and *A*. *padwickii* that cause pecky rice disease on rice (*Oryza sativa* L.). Stigmatomycosis and pecky rice are two of the most intensively studied fungal diseases transmitted by pentatomids (Mitchell, 2004). Both these diseases involve direct insect feeding damage to the seeds and grains, which consequently leads to fungal infection (Mitchell, 2004).

The rice stink bug (Oebalus pugnax) *and* Fusarium oxysporum*,* Cochliobolus miyabinus*,* Curvularia lunata*,* Alternaria alternata *and* A. padwickii *on rice* (Oryza sativa L.)

Pecky rice is a disease caused by stink bug feeding on rice grains during the dough stage causing subsequent fungal infections that discolour the grains (Mitchell, 2004). Several fungal species are involved with this disease complex, *viz*. *F. oxysporum*, *C. miyabinus*, *C. lunata*, *A. alternata* and *A*. *padwickii* (Mitchell, 2004). Lee *et al.* (1993) investigated the role of the rice stink bug (*O. pugnax*) in the severity of the disease due to the transmission of these fungal pathogens between rice plants (*O. sativa* L.). Trials were conducted in both glass house and field tests with bugs that were captured and not artificially inoculated with pathogens. They found evidence that supported the claims that *O. pugnax* is involved in the vectoring of pathogens that cause pecky rice. This included (1) the caryopsis of the rice grains which only became discoloured when wounded during inoculation; (2) isolations of the pathogens that cause kernel discolouration from the saliva and stylets of the rice stink bug; (3) the substantial kernel discolourations on insect infested panicles in contrast to the absence of kernel discolouration on kernels without insects; and (4) a lower incidence of discoloured kernels in insecticide treated plots in contrast to higher incidence of the symptoms occurring in the fungicide treated and control plots. Lee *et al*. (1993) postulated a “loose vector relationship” between the pathogens and the rice stink bug with infection caused during feeding and feeding damage, providing entry points for pathogens.

1.3.1.2 Cicadellidae

Cicadellidae represents a large and important insect family (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). Members from this family are small and sometimes brightly coloured, but mostly bright green (Picker *et al*., 2002). More than 350 species has been described from Southern Africa and more than 2500 from North America (Picker *et al*., 2002; Triplehorn & Johnson, 2005). Leafhoppers feed on a variety of plants that range from shrubs, grasses, field and garden crops, forest, shade and orchard trees (Scholtz & Holm, 1985). Leafhoppers mainly feed on the leaves of these plants (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). Most members from this family are monophagous, feeding on a single or closely related plant species, while others are polyphagous. There are several economically important species within this family that are pests of crops (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). Leafhoppers cause damage to crops in five ways; (1) the excessive removal of plant sap from the leaves and the destruction of chlorophyll cells causing leaves to be covered in yellow and white spots; (2) the mechanical blocking of the xylem or phloem by salvia that is injected during feeding resulting in the browning of entire leaves; (3) the oviposition of eggs in green twigs can cause the terminal ends of twigs to wilt and die; (4) the inhibition of growth in the undersurface of leaves where the leafhoppers feed resulting in stunted growth and leaf curling; and (5) the vectoring of plant pathogens that can cause disease (Triplehorn & Johnson, 2005).

Dissemination of fungal plant pathogens by members of Cicadellidae

Leafhoppers are well known for their ability to disseminate plant pathogens, especially viruses and phytoplasmas (Mitchell, 2004; Agrios, 2005). Leafhoppers in combination with aphids (Hemiptera: Aphididae) form the basis for the vectoring of most of the important plant viruses that cause yield loss (Mitchell, 2004). However, their role in the dissemination of fungal pathogens in particular is not well documented. Evidence suggests that the potato leafhopper, *Empoasca fabae* (Cicadellidae) and pea aphid, *Acyrthosiphon pisum* (Aphididae) are associated with root rot caused by *Fusarium* spp. on red clover, white clover, and alfalfa plants.

Potato leaf hopper, Empoasca fabae *(Cicadellidae) and the pea aphid,* Acyrthosiphon pisum *(Aphididae) and* Fusarium *spp. on white clover, red clover, and alfalfa.*

Leath and Byers (1977) evaluated the interaction between the pea aphid and the potato leafhopper that occurs on the leaves and stems of red clover, white clover, and alfalfa plants, and *Fusarium* species that cause root rot. Red clover, white clover, and alfalfa are important pasture plants that are used as animal feed. To investigate the interaction, separate groups of plants were exposed to *Fusarium*, aphids and leafhoppers, *Fusarium* and aphids, or *Fusarium* and leafhoppers, in factorial combinations. The plants most severely damaged were those that were inoculated with *Fusarium* spp. and subjected to aphid feeding (Leath and Byers, 1977). This indicates the importance of interactions between insect feeding and disease incidence and that they are not separate, discrete problems. Insect damage may contribute towards disease incidence with insect feeding amplifying the effect of diseases either through dissemination or by causing physical stresses that increases the susceptibility of plants.

1.3.2 Coleoptera

Coleoptera is the most diverse and successful group of insects (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). They represent 40% of all known Hexapoda with an estimated 370 000 species in some 23 000 genera (Scholtz & Holm, 1985; Triplehorn & Johnson, 2005). There are 18 000 species known from southern Africa and more than 30 000 described species from North America (Picker *et al*., 2002; Triplehorn & Johnson, 2005). Members from this order vary greatly in size, shape, colour, habits, and biological requirements (Scholtz & Holm, 1985). The most distinctive characteristic of this order is the structure of the wings. The front wings or elytra of these insects are well-sclerotized, hardened or leathery and brittle (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). The hind wings are membranous and longer than the front wings, and folds away beneath the elytra when not in use. All beetles have biting-chewing mouth parts and utilize a variety of food sources. A large proportion of the species are phytophagous while others are predacious or parasitic (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). Beetles that live on plants may feed directly on the foliage and other plant parts, bore into the stems or fruits of plants, or live as leaf miners (Triplehorn & Johnson, 2005). Coleoptera are of great economic importance since

they feed on stored food stuffs, crops, cloths, and other important organic material (Triplehorn & Johnson, 2005).

1.3.2.1 Chrysomelidae

Members from the family Chrysomelidae, vary greatly in their shape, size and colour (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). They are related to the insect family, Cerambycidae (longhorned beetles), due to their tarsal structure, but some also closely resemble members from the family, Coccinellidae (ladybirds), due to their colour and body shape. All the adults and the larvae of this family are phytophagous, feeding primarily on leaves and flowers. Some species bore into the stems, roots and seeds of plants while some larvae live as leaf miners. Several members of this family are considered economically important pests of cultivated plants (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005).

Dissemination of fungal plant pathogens by members of Chrysomelidae

Chrysomelidae is considered an important family that vectors plant pathogenic viruses (Anderson & Morales, 1994). Members of this family have also been found to be involved in the dissemination of fungal plant pathogens. The bean leaf beetle (*Cerotoma trifurcata*) is a suspected disseminator of *Alternaria tenuissima* on soybean (*Glycine max*) and the flea beetle (*Phyllotreta cruciferae*) that vectors *Alternaria brassicola* on cabbage (*Brassica oleracea* var. *capitata*).

The bean leaf beetle (Cerotoma trifurcata) *and* Alternaria tenuissima *on soybean* (Glycine max)

Interactions between the bean leaf beetle (*C. trifurcata*) and *A. tenuissima*, which both cause damage to soybean plants (*G. max*), were investigated by Shortt *et al*. (1981). They sampled pathogens from pods and seeds to quantify beetle and pathogen damage, isolations from the heads and abdomens of beetles were made, and pathogenicity tests were conducted on relevant fungi. Several pathogens of soybean were isolated that included *A. tenuissima, A. alternata*, *Epicoccum* sp., *Nematospora* sp., *Phoma* sp., *Fusarium equiseti*, *F. graminearum*, *F. tricinctum* and *Gliocladium roseum*. *Alternaria tenuissima* was the predominant pathogen isolated from all the samples. Shortt *et al*. (1981) concluded that beetle damage is not required

for pathogens to infect soybean plants. The fungi isolated from the insects are reflected by the mycoflora in the environment. The associations between *A. tenuissima* and *C. trifurcata* suggest that the transmission of this pathogen by the beetles is possible. Beetle feeding damage causes wounds that may facilitate the entry of pathogens into soybean plants, even though it is not necessarily required. This may increase the dispersion of *A. tenuissima* through feeding, ultimately contributing to the incidence of disease.

Flea beetles (Phyllotreta cruciferae) *and* Alternaria brassicicola *on cabbage* (Brassica oleracea var. capitata)

Dillard, Cobb & Lamboy (1998) investigated the association between flea beetles (*P. cruciferae*) and *A. brassicicola* that causes alternaria leaf spot on cabbage (*B. oleracea var. capitata*). The aim of their study was to determine whether there was a relationship between beetle infestation and disease incidence. Initially, flea beetles were collected and *A. brassicicola* was isolated from the specimens. The incidence of *A. brassicicola* on the beetles also increased as the season progressed. Three trials were conducted on cabbage plants to observe disease incidence. These included non-inoculated plants, inoculated plants and plants subjected to flea beetle transmission. The highest disease incidence was observed on the inoculated plants, but several and substantial lesions caused by *A. brassicicola* were also observed on plants subjected to flea beetle transmission. Further studies included the microscopic examination and isolation of *A. brassicicola* from flea beetle faecal matter. In both cases *A. brassicicola* was observed. Scanning electron microscopy (SEM) was also used to examine flea beetles externally for any propagules and conidia of *A. brassicicola* and this fungus was observed on all body parts of the flea beetles. It was also established that feeding damage to the margins of cabbage leaves, caused by beetles, functioned as entry points for *A. brassicicola*. The combination of saliva and faecal matter with the fluids excreted from injured leaves provided ample moisture for the conidia to germinate. Dillard *et al*. (1998) concluded that flea beetles can increase the incidence of *A. brassicicola* through four possible ways; (1) the passive deposition of *A. brassicicola* on stem and leaf surfaces, (2) the dissemination of *A. brassicicola* conidia externally from the body, (3) feeding sites that serve as entry for passive deposition of conidia, and (4) the deposition of viable conidia *via* the faeces and mouthparts.

1.3.2.2 Curculionidae

Curculionidae is commonly referred to as snouted beetles or true weevils, which includes the associated bark and ambrosia beetles (Triplehorn & Johnson, 2005). Curculionidae is the most diverse group of organisms in the world and contains more than 48 000 described species (Scholtz & Holm, 1985; Picker *et al*., 2002). There are more than 2500 species in southern Africa and 3000 species from 500 genera in North America (Picker *et al*., 2002; Triplehorn & Johnson, 2005). Members range from small to large (1 - 60 mm) and show great diversity in their shape, size and colour (Scholtz & Holm, 1985; Picker *et al*., 2002). All curculionids possess a slender rostrum referred to as a “snout” with mandibles at the end (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). The snout can vary, ranging from short and broad to long and thin, in certain cases three times the length of the body. Both adults and larvae are phytophagous, feeding on all plant parts. The larvae usually feed on the inside of plant tissue while the adults bore into fruits, nuts, and other plant parts (Scholtz & Holm, 1985; Picker *et al*., 2002; Triplehorn & Johnson, 2005). Members of this family are wide spread and several are serious pests of crops and stored products. Most plant species host at least one or several species (Picker *et al*., 2002).

Dissemination of fungal plant pathogens by members of Curculionidae

Several species of Curculionidae are involved in the dissemination of fungi. The most notable of these are species from the sub-family Scolytinae. These insects are commonly known as ambrosia beetles (*e.g. Dendroctonis* spp., *Ips* spp. and *Xyleboris* spp.). They form close mutualistic relationships with their fungal partners (Harrington, 2005; Paine *et al*., 1997). The fungi disseminated by these insects include the ophiostomatoid fungi that mostly belong to the genera *Ceratocystis*, *Ceratocystiopsis*, *Leptographium* and *Ophiostoma* (Paine *et al*., 1997; Harrington, 2005). The fungi associated with the ambrosia beetles are completely dependent on their vectors for dissemination. This relationship received special attention due to the pathogenic nature of their interaction and the ability of the fungi to kill mature trees (Agrios, 2005). Amongst the associations between ambrosia beetles and the ophiostomatoid fungi, the most destructive and well documented relationship exists between *Ophiostoma novo-ulmi*, the European bark beetle, *Scolytus multistriates* (Curculionidae: Scolytinae) and a bark beetle native to North America, *Hylurgopinus rufipes* (Curculionidae: Scolytinae). The interaction between

these beetles and the fungus, results in the disease known as Dutch elm disease that has almost completely decimated the North American elm population (Connor & Wilkinson, 1998). The interaction between these beetles and their associated fungi has been previously discussed. Recently, it has also been found that the walnut twig beetle, *Pityophthorus juglandis* (Curculionidae: Scolytinae), is responsible for the dissemination of the thousand cankers disease caused by *Geosmithia morbida* (Cranshaw & Tisserat, 2010). The association between these organisms has the potential to destroy black walnut populations in its native range in a similar manner previously demonstrated by introduced species such as the pathogens that causes Dutch elm disease (affecting American elm) and chestnut blight (affecting American chestnut) (Cranshaw & Tisserat, 2010; Grant *et al*., 2011). The interaction between ambrosia beetles and phytopathogenic fungi has also been documented within pecan orchards (Alvidrez-Villarreal *et al*., 2012). Research conducted in Mexican pecan orchards provided evidence that the trunk and branch borer, *Euplatypus segnis* (Coleoptera: Curculionidae: Platypodinae) is associated with several fungal species, *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata* and *Lasiodiplodia theobromae*, that are able to cause die back of pecan trees. It was found that the beetles, in combination with fugal invasion, eventually results in the death of trees and that *E. segnis* contributes to the spread of fungi in pecan orchards (Alvidrez-Villarreal *et al*., 2012). The dissemination of *G. morbida* by *P. juglandis* on walnut, and phytopathogenic fungi by *E. segnis* on pecan, is discussed further in the sections regarding insect-fungal associations on walnut and pecan respectively. Other fungal plant pathogens associated with members from the family Curculionidae include the dissemination of *Heterobasidion annosum* by the large pine weevil (*Hylobius abietis*) to conifer species, the transmission of *Fusarium oxysporum* f. sp. *medicaginis* by *Sitona hispidulus* (the clover root curculio) to alfalfa plants (*Medicago sativa*), and the dissemination of a *Fusarium* sp. by a leaf mining beetle, *Phylloplatypus pandani* (Curculionoidae), to *Pandanus boninensis* (Pandanaceae), a plant native to the north-western Pacific Ogasawara (Bonin) islands.

Hylobius abietis *and* Heterobasidion annosum *on conifer species*

Heterobasidion annosum is a cosmopolitan fungal plant pathogen that causes white rot, a disease of various tree species (Kendrick, 2000). This disease causes economic loss in the forestry industry and threatens natural forest in numerous parts of the world (Kendrick, 2000).

Kadlec, Stary & Zumr (1992) investigated the potential of the large pine weevil (*H. abietis*) to vector *H. annosum* between conifers. They analysed adult weevils, and their excreta, for the presence of *H. annosum* and found that these insects vector this pathogen. They concluded that the dispersal of *H. annosum* by *H. abietis* may be significant in comparison to other modes of dispersal and that weevil feeding damage on the bark of conifers might also serves as an entry point for many pathogens, such as *H. annosum* (Kadlec *et al*., 1992).

The clover root curculio (Sitona hispidulus) *and* Fusarium oxysporum f. sp. medicaginis *on alfalfa plants* (Medicago sativa)

Leath & Hower (1993) investigated whether the clover root curculio (*S. hispidulus*) transmitted *F. oxysporum* f. sp. *medicaginis* to alfalfa plants (*M. sativa*) and if larval feeding damage assisted the pathogen in the colonisation of alfalfa plants. *Fusarium oxysporum* f. sp. *medicaginis* is a pathogen that causes fusarium wilt on alfalfa plants. Roots with weevil feeding damage were collected and three isolations were made, centripetal from each feeding site. The three different isolations yielded 75%, 30% and 5% *Fusarium* colonization respectively with the highest infestation occurring in the centre of the feeding site. Two *Fusarium* spp. were isolated from the heads of collected larvae and pathogenicity tests were conducted with both *Fusarium* spp. causing vascular necrosis and wilting in alfalfa plants. They also sterilized *S. hispidulus* eggs and deposited them on the surface of soil treated with *F. oxysporum* f. sp. *medicaginis* and buried them with alfalfa plants during planting. Once the eggs hatched after 77 days, the larvae burrowed beneath the soil surface and fed on the roots of the alfalfa plants. The alfalfa plants suffered from fusarium wilt and *F. oxysporum* f. sp. *medicaginis* was isolated from all the symptomatic plants. Increased feeding damage by the larvae of the clover root curculio increased the occurrence of fusarium wilt on alfalfa plants (Leath & Hower, 1993).

Phylloplatypus pandani *and a* Fusarium *sp. on* Pandanus boninensis

Sugiura & Masuya (2010) investigated the role of adult weevils (*P. pandani*) in the dissemination of phytopathogens amongst *P. boninensis* plants. This is a weevil that mines leaves of *P. boninensis* during its adult stage. Mining of leaves by adult insects is a phenomenon that is rarely reported. Initially, it was observed that the areas surrounding the galleries of these insects showed a reddish-brown discolouration caused by fungal infection. They isolated fungi

from both the insects and their galleries. Seven fungal species were isolated from the insects and nine from their galleries. A single *Fusarium* sp. was isolated from both the insects and galleries. Pathogenicity tests were conducted under field conditions by inoculating the fungus into the healthy leaves of *P. boninensis*. The isolated *Fusarium* sp. caused leaf discolouration and was found to be pathogenic towards the pandanus plant. It was proven that *P. pandani* is a vector of this fungus and contributes to its dispersal. The association between the *Fusarium* sp. and the beetles can be attributed to two scenarios; (1) a mutualistic relationship between insect and fungus where the insects either feed on the fungus or uses the fungus to overcome the pandanus plants anti-herbivore defences, such as the excretion of resin within the galleries and (2) the adult beetles unintentionally transfer the fungus between the leaves of the pandanus plants (Sugiura & Masuya, 2010).

1.4 Production of tree nuts in semi-arid regions of South Africa

The semi-arid regions of South Africa cover most of the central and western higher altitude regions of the country (Milton & Dean, 1998; Woyessa, Hensley & Van Rensburg, 2006). These areas are characterized by low and sporadic rainfall, low humidity, and long hot summers with cold winters (Allemann, 2008; De Villiers & Joubert, 2008; Van Zyl, 2009). Currently, pistachios, walnuts, and pecans are the only tree nut crops that are produced on a commercial scale in these regions. All these tree nut crops require low rainfall and humidity, and long hot summers with cool winters for maximum production (Allemann, 2008; De Villiers & Joubert, 2008; Van Zyl, 2009). The semi-arid regions of South Africa are ideal for the production of these tree nut crops. Production areas in the semi-arid regions include Prieska (A-grade), Hopetown (A-grade), Upington (A-grade), and the Vaalharts-irrigation scheme (B-grade) (De Villiers & Joubert, 2008). A-grade areas are ideal, or almost ideal, ecological conditions while B-grade areas have good prospects for commercial production (De Villiers & Joubert, 2008). All the A-grade production areas are found in the semi-arid regions of South Africa.

Currently, the main tree nut crop cultivated in the semi-arid region of South Africa is pecans (De Villiers & Joubert, 2008). Walnuts and pistachios are relatively young industries in South Africa (orchards have only been established in the last two decades) (Allemann, 2008; Van Zyl, 2009). The pecan industry has much potential, producing nuts of exceptional quality

and providing an excellent market opportunity for export, especially with the growing demand for pecans by countries such as China (Anon, 2011[A]). Additionally, tree nut crop production may also aid in work creation. The production of these crops may have a major effect on downstream employment, which can improve the living standards of several communities in the production areas (Anon, 2011[A]).

Tree nuts are increasingly being grown commercially and in home gardens for nuts or shade in South Africa (Von Broembsen, 2006). With an increase in acreages of these crops, diseases, insects and weeds are becoming a major problem (Von Broembsen, 2006). Together with the fast expansion of the industry, the influence of insect pests and pathogens are not well understood in South Africa. A large proportion of research focuses primarily on the pests or the pathogens while few studies focus on the interaction and associations between the two. The dissemination of disease causing organisms is sometimes associated with the presence of certain insects. Therefore, homeowners and commercial growers alike should be aware of such relationships since it can negatively influence the production, quality and safety of the nuts produced in South Africa (Von Broembsen, 2006). It is important to determine the association between insect pests and fungal pathogens as associations like these may be exploited to obtain better control of both insect pests and pathogens within the agro-ecosystems.

1.4.1 Pistachios

1.4.1.1 History and background

The pistachio (*Pistacia vera* L), which is a deciduous, dioecious, and wind-pollinated tree species, is a member of the family Anacardiaceae (Hormaza & Wünsch, 2007; Allemann, 2008). There are more than a dozen species belonging to the genus *Pistacia,* but only *P. vera* yields commercially acceptable and edible fruits (Janick, 2002; Eskalen *et al.*, 2010). According to Janick (2002), the pistachio tree is native to western Asia, and has been widely cultivated and domesticated in several Mediterranean countries some 2000 years ago. Even before the advent of agriculture, pistachios were used by the Stone Age man and his successors as a highly prized food source (Caruso, 2005; Allemann, 2008). It was during the height of the Roman Empire that seeds were collected and spread throughout the Mediterranean to countries such as Iran, Iraq, Turkey, Greece, Tunisia and Italy were seedlings were successfully propagated and grown

(Kaska, 2005; Allemann, 2008). However, presently pistachios are cultivated all over the world (Janick, 2002). In India and Afghanistan, nuts are still generally obtained from wild trees. Furthermore, the orchard cultivars of Iran, Turkey, and Italy differ little from wild pistachio populations. These cultivars were obtained from selective breeding and, generally, trees were selected due to their large nut size. Pistachio cultivars are currently being produced and improved in Californian orchards. These are mainly bred to produce larger, better flavoured nuts (Janick, 2002).

The production of pistachio's in South Africa started with the first orchards being established in 1990 at Green Valley Nuts (GVN) on the bank of the Orange River near Prieska in the Northern Cape Province (Louw, 2003; Haddad & Louw, 2006). Pistachio trees were introduced into South Africa by the Industrial Development Corporation (IDC) since it is a potentially high yielding crop (Allemann, 2008). Several constraints exist with the commercial production of pistachio nuts in South Africa, including rainfall, soil conditions, and temperature (Kanber *et al*., 2004; Allemann, 2008). To overcome these constraints, regular irrigation and fertilization are needed to ensure optimal yields (Allemann, 2008). Fortunately, pistachio trees prefer areas that have cool winters and long hot summers; which makes the high altitude of the western parts of the country an ideal region to produce this crop (Crane & Iwakiri, 1981).

Pistachios are commercially propagated by grafting. This is done by grafting certain varieties on certain root stocks to increase disease resistance, growth, and yield (Hormaza & Wünsch, 2007; Allemann, 2008). The pistachio plant is a small to medium sized and bushy tree that grows slowly to a height of about nine meters (Hormaza & Wünsch, 2007). Pistachio trees may have a single or several trunks (Crane & Iwakiri, 1981). Maximum yields can be expected at the age of 7 to 10 years and trees may produce for up to forty years (Allemann, 2008). Leaves are hairy when young and glabrous when old, and consist of three to five oval leaflets (Crane & Iwakiri, 1981). Pistachios are dioecious and both the staminate and pistillate inflorescences are panicles formed in the axils of the previous year's growth, consisting of up to several hundred individual flowers (Crane & Iwakiri, 1981). Pistachios are a major export crop for many countries worldwide; especially Iran where desert conditions make it difficult to produce other crops on a large scale (Janick, 2002).

Pistachio nuts are most often eaten whole, either fresh or roasted and salted (Soetaert, 2003). They are also used in ice cream and confections such as baklava. Pistachios are also listed as one of the specific nuts that can reduce the risk of heart disease. By eating 43 gram of most nuts per day, including pistachios, as part of a of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease (Soetaert, 2003).

1.4.1.2 International production and export

The USA, Iraq and Turkey are the three top producers of pistachios (Table 1.1) (Anon, 2011[B]). These three countries produce most of the worlds pistachio supply. Other countries that produce pistachios on a notable scale include; Syria, Greece and China. During the 2010/2011 season the global production of pistachio nuts totalled 625,800 tons (Anon, 2011[B]).

Table 1.1: The top 6 pistachio producing countries (In Shell Basis - Metric Tons) for the 2010/2011 season and the production projections for the top three countries for the 2011/2012 season (Anon, 2011[B]).

Top pistachio producers		2010/2011	2011/2012 (Projected)
1	USA	240,200	181,000
2	Iran	195,000	240,000
3	Turkey	112,000	72,000
4	Syria	50,000	-
5	Greece	5000	-
6	China	5000	-
7	Other	18,600	-
	Total:	**625,800**	**-**

The European Union (EU) is the largest importer of tree nuts, especially tree nuts produced by the United States of America (USA) (Medina, 2011). The EU imports roughly 33% of the world’s tree nuts. Other important markets include; East Asia (28 %), China & Hong Kong (18 %), and the Middle East (14 %). The most important tree nuts imported by the EU are hazelnuts and shelled almonds that represents 60% of the tree nuts imported by the EU. Other important tree nuts include pistachios and walnuts. The US and Iran are the two largest producers and exporters of pistachios, with these two countries accounting for almost 100% of the pistachios imported by the EU (Medina, 2011). Due to the high aflatoxin levels found in the

pistachios produced by Iran, the largest proportion of pistachios imported by the EU originates from the USA that produces better quality pistachios (Zheng, 2011). This has led to a major growth of the pistachio industry in the USA (Zheng, 2011).

The Global Agricultural Information Network (GAIN) reports that South Africa exported 219 tons of pistachio nuts during the 2009/2010 marketing year to the European Union (Medina, 2011). In comparison to the major pistachio nut exporting countries this is an almost negligible amount. However, the pistachio industry is still in its infancy in South-Africa, and even though no significant export has been made thus far, the industry shows promise (Louw, 2003; Haddad & Louw, 2006).

1.4.1.3 Insect-fungal associations

Aflatoxin levels are important to the production and export of pistachio nuts. Aflatoxins are produced by a variety of *Aspergillus* species, but *Aspergillus flavus* is the most notorious producer of this mycotoxin (Thomson & Mehdy, 1978; Smith & Henderson, 1991). Aflatoxin poisoning can pose a severe threat to the health of consumers and this might have a negative effect on global pistachio purchases (Zheng, 2011). In the USA alone, the total loss in sales due to aflatoxin contamination can reach $50 million per year and is much higher in seasons with greater insect damage (Cardwell *et al*., 2001; Zheng, 2011). Insects are however not the main source of aflatoxin contamination in pistachios (Zheng, 2011). Contamination can occur in the field, during the harvest season, in storage, and during the processing procedures whenever the surrounding environment is humid enough for the dry core of pistachios to absorb moisture (Zheng, 2011). Due to the increase of aflatoxin levels during years of greater insect damage, some research has been conducted on insect-fungal associations in pistachio orchards. Several insect species or groups of insects have been noted to be involved in the dissemination of fungal pathogens of pistachios. These include the navel orange worm, *Amyelois transitella* (Lepidoptera: Pyralidae), which is involved in the dissemination of *Aspergillus* species that causes *Aspergillus* blight and produces aflatoxins (Shahidi Bonjar, 2004), as well as several hemipteran species, predominantly stink bugs and leaf footed bugs (Hemiptera: Pentatomidae and Coreidae), that are involved in the dissemination of *Botryosphaeria dothidea* that causes panicle shoot blight (Michailides & Morgan, 1996), and both *Nematospora coryli* and

Aureobasidium pullulans that cause stigmatomycosis on pistachios (Michailides, Morgan & Doster, 1995).

Navel orange worm, Amyelois transitella *(Lepidoptera: Pyralidae) and* Aspergillus *spp. on pistachios*

Aflatoxins were discovered in the late 1960's and first found in pistachios during the early 1970's (Anon, 2009[A]). Since then, research has been conducted on the aflatoxin contamination of pistachios (Anon, 2009[A]). Aflatoxins are important in pistachio production since it poses a major threat to the industry, affecting the safety of the nuts for human consumption and influences the desirability of the commodity. Aflatoxins are produced by both *Aspergillus flavus* and *Aspergillus parasiticus*, but *A. flavus* is the predominant fungus causing aflatoxin contamination in pistachios (Shahidi Bonjar, 2004). Aflatoxin contamination has been found in several other crops other than pistachios (Anon, 2009[A]). This includes other tree nuts, peanuts, figs, corn, cottonseed meal, rice, and spices. The ingestion of aflatoxins by livestock such as poultry and other animals causes acute poisoning resulting in liver disorders and death. In humans, aflatoxin poisoning is rarely acute and they rather act as chronic toxins and are thus associated with liver cancer (Anon, 2009[A]).

Insects and fungal infestation of pistachio nuts occur during nut split (Shahidi Bonjar, 2004). The nut is relatively safe from both insect and fungal infection until natural splitting just before harvest, however, in certain cases early splitting occurs where the nuts remain attached to the shells, causing the shells to split with the nuts and exposing it to invasion by either pathogens or insects (Shahidi Bonjar, 2004). The navel orange worm commonly infects early split of pistachio nuts, causing feeding damage and kernel discolouration (Thomson & Mehdy, 1978; Holtz, 2002; Campbell, Molyneux & Schatzki, 2003). During this process, *A. flavus* is disseminated to the nuts. The damage caused during feeding provides entry points for *A. flavus* spores on the surface of the nuts (Anon, 2009[A]). *Aspergillus flavus* is considered a weak pathogen that has to rely on alternative methods to infect pistachio nuts. Early split of pistachios, in combination with navel orange worm infestation, contributes to 90% of pistachio nuts to be infected with *A. flavus* (Anon, 2009[A]). The navel worm is thus considered the most important insect that contributes to the occurrence of aflatoxin contamination in pistachios (Dowd, 2003).

Hemipteran pests (Hemiptera: Pentatomidae and Coreidae) and Botryosphaeria dothidea *on pistachios*

Botryosphaeria dothidea is a fungal pathogen that causes panicle shoot blight of pistachios (Steffan *et al*., 2000). This is a serious disease that can cause severe losses to pistachio yields (Steffan *et al*., 2000). *Botryosphaeria dothidea* has been recorded in pistachio orchards of several countries (Michailides, 2006). These countries include USA, Greece, Italy and South Africa (Michailides, 2006). The first association between hemipteran pests and *B. dothidea* was noted by Michailides and Morgan (1996). Isolations were made from the fruit with lesions caused by hemipterans, and *B. dothidea* was eventually found to be associated with both the punctures and the lesions. Their objective was to determine the involvement of hemipteran insects in transmitting *B. dothidea*. Michailides and Morgan (1996) used four hemipteran species, *viz. Thyanta pallidovirens* (Hemiptera: Pentatomidae), *Chlorocroa uhleri* (Hemiptera: Pentatomidae), *Leptoglossus clypealis* (Hemiptera: Coreidae), and *Liorhyssus hyalinus* (Hemiptera: Rhopalidae), in trials to determine their role in the dissemination of *B. dothidea.* All the species were found to transmit *B. dothidea.* However, *L. clypealis* and *L. hyalinusi* were more successful disseminators, since they were able to infect more fruit than the other species (Michailides & Morgan, 1996).

Steffan *et al*. (2000) furthered the understanding of the interactions between *B. dothidea* and hemipteran pests of pistachios, and to improve the effective management of both. Over 15,000 hemipteran individuals were collected from pistachio orchards. The insects were cultured on acidified potato-dextrose agar (APDA) and observed for the presence of *B. dothidea*. Few insects (less than 0.12%) naturally carried *B. dothidea* spores, which were only found externally on the insects. This indicated that hemipteran pests can assist in the infection of nuts by *B. dothidea*, providing germination sites for spores that are already present on the nut surface. Hemipteran pests such as stink bugs can thus contribute to the dispersal of *B. dothidea*, but that the fungus is rather spread by other means such as rain, wind, other insects, equipment and birds. According to Steffan *et al*. (2000), efforts to control *B. dothidea*, should focus on removing the major sources within pistachio orchards, however, the initiation of the disease by hemipteran feeding punctures in orchards is of major significance in the epidemiology of the disease.

Hemipteran pests (Hemiptera: Pentatomidae and Coreidae) associated with Nematospora coryli *and* Aureobasidium pullulans *on pistachios*

Stigmatomycosis of pistachios is a disease caused by *Nematospora coryli* and *Aureobasidium pullulans* (Michailides *et al*., 1995; Eskalen *et al*, 2010). The term, stigmatomycosis, describes a disease that occurs on several crops including peanuts, pecan, pomegranates, citrus and pistachios. This disease has been found to occur in almost all the countries that produce pistachios. With pistachios, the disease is characterized by a malformed and brown necrotic kernel that is wet, smelly, rancid, and slimy in appearance. Stigmatomycosis is found in split or closed nuts and incidences of the disease as high as 80% of the nuts have been reported (Michailides *et al*., 1995; Eskalen *et al*, 2010). *Nematospora coryli* and *A. pullulans* is transmitted by large coreid and pentatomid species (Michailides *et al*., 1995). The three most common pentatomid pest species nl. *Thyanta pallidovirens* (Hemiptera: Pentatomidae), *Chlorochroa uhleri* (Hemiptera: Pentatomidae) and *Chlorochroa ligata* (Hemiptera: Pentatomidae), and a leaffooted bug, *Leptoglossus clypealis* (Hemiptera: Coreidae), have been shown to transmit *N. coryli* and *A. pullulans* and cause symptoms of stigmatomycosis in pistachio kernels. Smaller hemipteran pests are unable to transmit this disease since they cannot puncture the epicarp of the nuts (Michailides *et al*., 1995).

1.4.2 Walnuts

1.4.2.1 History and background

According to Mehlenbacher (2003), there are a number of different walnut species (Juglandaceae: *Juglans*). These include the black or wild walnut (*Juglans nigra*), the butternut or white walnut (*Juglans cineria*) of the United States, and the English or Persian walnut (*Juglans regia*). A limited number of black and white walnut types have been domesticated within the USA and these are not cultivated on a notable scale. The English or Persian walnut, which hails from Europe, is extensively cultivated throughout southern Europe, in China, and more recently at the Pacific coast of the United States (Mehlenbacher, 2003).

The black walnut is one of the outstanding timber trees of the eastern deciduous hardwood belts in the United States (Adem *et al.*, 2000; Janick, 2002). Natural stands have been severely decimated for timber and veneer. Nuts are still collected from wild trees for home

consumption and, in certain rural areas, for commercial sale (Janick, 2002). The husk of the black walnut does not easily separate from the inner shell and the husk strongly stains hands and clothing. The black walnut has a hard-to-crack inner shell, which makes it difficult to properly utilize the nut as a food source. The combination of these factors has caused the black walnut industry to fail in making any great commercial strides (Janick, 2002). In contrast, the Persian or English walnut has none of these problematic characteristics. It is commercially produced worldwide for the valuable nuts and is, therefore, the most economically important species in the genus, *Juglans* (Janick, 2002; Van Zyl, 2009).

The Persian walnut is native to the temperate regions in the mountains of Eastern Europe and Central Asia, extending from Turkey, Iran and western China eastward to the Himalayan regions of India and Nepal (Leslie & McGranahan, 1998). Records indicate that the Persian walnut was cultivated by the ancient Greeks and Romans (Sauer, 1993). This nut was spread throughout Europe during the 1500's and is now grown worldwide (Sauer, 1993). According to historical observation, walnuts have been cultivated on a small scale in the Aberdeen and Oudtshoorn areas of South Africa, but the industry has not expanded further due to a lack of suitable technology and cultivars (Van Zyl, 2009). In 2000, prospects by the Industrial Development Corporation of South Africa (IDC) proposed the establishment of a new walnut industry in South Africa (Adem *et al*., 2000). The aim was to establish 600 hectares of walnuts for commercial production and export (Adem *et al*., 2000). The largest proportion of these trees was planted at Rotondo Walnuts on the banks of the Orange River near Aliwal North in the southern area of the Free State Province (Van Zyl, 2009).

Walnuts are commercially propagated by grafting (Van Zyl, 2009). Certain varieties are grafted onto certain root stocks to increase disease resistance, growth and yield (Van Zyl, 2009). After planting, the first notable yields can be expected within 5 years. Maximum yields can only be expected at an age of 10 years and older (Adem *et al*., 2000). All *Juglans* species are monoecious, with catkins being borne laterally on one-year-old shoots, and pistillate flowers borne terminally or laterally on the current season's shoots (Van Zyl, 2009). Although walnuts are genetically self-fruitful they exhibit the phenomenon of dichogamy, being either protandrous or protogynous depending on cultivar. Therefore, walnuts are mostly cross-pollinated by wind (Polito, 1998; Van Zyl, 2009).

Walnuts are used in several commercial practices (Adem *et al*., 2000) *e.g*. they are considered excellent timber trees, the husks and bark of the trees are used as dyes and the nuts are a sought after food commodity (Adem *et al*., 2000). The nuts can be eaten raw or roasted and salted (Adem *et al*., 2000). It is regarded as highly valuable by an increasingly health-conscious world society due to the various health benefits the nuts provide (Savage, McNeil & Dutta, 2001). Walnuts are good sources of the minerals; copper, magnesium and phosphorus (Anon. 2010). They are naturally low in sodium, contain dietary fibre and protein, and contain no cholesterol. Walnuts are also a great source of vitamin E and omega-3 fatty acids and have the ability to lower the level of cholesterol and reduce the risks of heart disease (Savage *et al.* 2001).

1.4.2.2 International production and export

The three top countries of walnut production are China, USA and Turkey (Table 1.2) (Anon, 2011[B]). These countries supply most of the world's walnuts. Other countries that produce walnuts on a notable scale include Chile, France and India. During the 2010/2011 season the global production of walnuts totalled 1,119,000 tons (Anon, 2011[B]).

Table 1.2: The top 6 walnut producing countries (In Shell Basis - Metric Tons) for the 2010/2011 season and the production projections for the top three countries for the 2011/2012 season (Anon, 2011[B]).

Top walnut producers		2010/2011	2011/2012 (Projected)
1	China	490,000	480,000
2	USA	455,000	445,000
3	Turkey	66,000	62,000
4	Chile	32,000	-
5	France	32,000	-
6	India	29,000	-
7	Other	15,000	-
	Total:	**1,119,000**	**-**

Walnuts are considered one of the important tree nut crops imported by the EU, making it the largest importer of walnuts (Medina, 2011). The EU imports roughly a third of the world's walnut produce (Anon, 2011[C]). China and the USA produce roughly 75% of the world's walnuts. Currently, the largest exporter of walnuts is the USA even though it is only the second largest

producer. The reason is that most of the massive walnut production in China is destined for domestic consumption (Anon, 2011[C]).

All the major walnut production countries are situated in the Northern Hemisphere, resulting in an increased demand from the European Union, the major walnut importer, for Southern Hemisphere walnuts over the traditional Christmas period (Van Zyl, 2009). The nuts from the southern hemisphere are fresher during this period. This is an exceptional opportunity for countries in the Southern Hemisphere due to higher exchange rates and a guaranteed market (Van Zyl, 2009).

The cultivation of walnuts (*J. regia*) in South-Africa is currently a young but promising industry (Van Zyl, 2009). Although South Africa is listed amongst the countries in the southern hemisphere where walnut cultivation is rapidly developing, the local industry is minute in comparison to other southern hemisphere producers such as Australia, Chile and Argentina. Although production in these countries is not comparable to the major walnut producing countries, it indicates that their walnut industries are flourishing in comparison to that of South-Africa (Van Zyl, 2009).

1.4.2.3 Insect-fungal associations

Both the navel orange worm, *A. transitella* (Lepidoptera: Pyralidae), and stink bugs (Hemiptera: Pentatomiidae) are pests of walnuts (Campbell *et al*, 2003; Klass, 2011). Similarly to pistachios, both of these insects might be involved in the dissemination of fungal pathogens. However, there is no evidence that suggests this. Aflatoxins produced by *Aspergillus* spp. have been detected in walnuts and it is suspected that the navel orange worm might be involved in the transmission of these *Aspergillus* spp., contributing to the occurrence of aflatoxins in walnuts (Campbell *et al*, 2003). *Botryosphaeria dothidea* is also a fungal pathogen of walnuts and stink bugs are possibly involved in the spread of this pathogen (Michailides, 2006). Evidence suggests that the walnut twig beetle, *Pityophthorus juglandis* (Coleoptera: Curculionidae: Scolytinae) is responsible for the dissemination of the thousand cankers disease caused by *Geosmithia morbida* (Cranshaw & Tisserat, 2010).

Walnut twig beetle, Pityophthorus juglandis *(Coleoptera: Curculionidae: Scolytinae) and* Geosmithia morbida *on walnuts*

Thousand cankers is a disease of black walnuts that is caused by *Geosmithia morbida,* a fungus that was described in 2008 (Cranshaw & Tisserat, 2010; Grant *et al*., 2011). Thousand cankers is a disease complex consisting of the walnut twig beetle, *P. juglandis*, and *G. morbida*. The beetles tunnel into the tree, disseminating the fungus as they move through the tree. As the fungus grows it produces thousands of small cankers which grow together and girdle the branches. The cankers consist of necrotic plant tissue that blocks water and nutrient flow to parts of the tree, causing it to decline and die (Cranshaw & Tisserat, 2010; Grant *et al*., 2011).

Currently, the walnut tree beetle is the only known disseminator of *G. morbida*, although other possible disseminators are being investigated. Since the research on this complex has been initiated, no effective treatments have been found. On-going research to identify treatments for the walnut twig beetle and/or the fungal canker (Cranshaw & Tisserat, 2010; Grant *et al*., 2011). It has been estimated that if the thousand cankers disease had been introduced into Missouri (the largest walnut producing state) in 2010, it would have caused an economic loss of $851 million over the next 20 years. Presently, the thousand cankers disease is having a devastating effect on the black walnuts found throughout the western states of the USA (Cranshaw & Tisserat, 2010; Grant *et al*., 2011). It is feared that the situation can become catastrophic if the walnut twig beetle colonizes the native areas of the black walnut, such as Missouri (Cranshaw & Tisserat, 2010; Cranshaw, 2011). Ultimately, this may have the potential to destroy black walnut population stands in its native range in a manner previously demonstrated by a fungal species responsible for causing the Dutch elm disease (affecting American elm) and chestnut blight (affecting American chestnut) (Cranshaw & Tisserat, 2010; Grant *et al*., 2011). These associations may lead to catastrophic consequences in the production of tree nut crops such as walnuts. The thousand cankers complex thus highlights the importance of research on the associations between insects and fungal pathogens.

1.4.3 Pecans

1.4.3.1 History and background

The Pecan (*Carya illinoenensis*) belongs to the family Juglandaceae (the Hickory family) the same as the Persian walnut (De Villiers & Joubert, 2008). Several species of hickory (the genus *Carya*) yield edible nuts, but *C. illinoensis* is responsible for the pecan cultivars that presently support a sizable orchard industry (Janick, 2002). These thin-shelled ("paper shell") varieties of pecan account for about half of the domestic production of pecan nuts in North America. These varieties are also predominantly produced worldwide in other countries (Janick, 2002).

The pecan was originally indigenous to North America, where it grows wild in the states along the Gulf of Mexico and around the Great Lakes (Oosthuizen, 1991; De Villiers & Joubert, 2008). Pecan nuts were used as a food source by Native Americans long before European and Spanish settlers arrived in the Americas (Andersen & Crocker, 2009). It is believed that Native Americans and fur traders spread the pecan from the Mississippi Valley eastward (Andersen & Crocker, 2009). Once the European and Spanish settlers reached the Americas the pecan was highly regarded as a wild nut tree and considered an important trade item (Janick, 2002; Andersen & Crocker, 2009). The first recorded shipment of pecan nuts to England was documented in 1761 (Andersen & Crocker, 2009). Since then, pecans have been spread and cultivated across the world (Janick, 2002).

The first pecan trees were imported to South Africa during the late 1800's (Oosthuizen, 1991; De Villiers & Joubert, 2008). The first grafted pecan trees were imported in 1912 and initial plantings of these trees were made within the sub-tropical regions around Nelspruit. Currently, the Vaalharts irrigation scheme in the Northern Cape Province of South-Africa is the largest production area in the country. Other important production areas include Tzaneen, Louis Trichardt, Kwazulu-Natal, the middleveld around Pretoria and certain parts along the Orange River (Oosthuizen, 1991; De Villiers & Joubert, 2008). Although original planting of pecan trees were made in the sub-tropical areas of the country, pecans are not truly sub-tropical plants (De Villiers & Joubert, 2008). Due to the high rainfall in sub-tropical regions, diseases are a major problem and only resistant and tolerant pecan cultivars can be planted. Overall, pecans are

successful in any part of the country where there are short cool winters and long hot summers with low rainfall and humidity (De Villiers & Joubert, 2008).

Pecan trees are fast growing and can become very tall unless properly managed (De Villiers & Joubert, 2008; Andersen & Crocker, 2009). They can reach a height of more than 25 meters with a 2 meter trunk diameter (Andersen & Crocker, 2009) and are propagated by means of grafting (De Villiers & Joubert, 2008). This is done by grafting certain varieties on certain root stocks to increase disease resistance, growth and yield (De Villiers & Joubert, 2008). Once established, it takes 5 years for the trees to produce the first notable harvest. Maximum yields can only be expected from an age of 10 years and onward, depending on the cultivar (Andersen & Crocker, 2009).

Pecans are monoecious with catkins (male flowers) being borne laterally on one-year-old shoots, and pistillate (female flowers) flowers are borne terminally or laterally on the current season's shoots (Andersen & Crocker, 2009). Pecans are wind pollinated and cross pollination is needed for maximum productivity. Nuts typically occur in clusters of 2 to 6 nuts and are enclosed by a thick, green husk. The husk supplies photosynthate and protects the developing nut (Andersen & Crocker, 2009). With maturity, the outer husk splits open into four parts, revealing an inner shell that surrounds the 2 kernels (Janick, 2002). The use of mechanical harvesting machinery to shake the nuts from the trees is the most widely used method to harvest pecan nuts commercially (Janick, 2002).

The nuts are dried, or "cured," for a few weeks after harvesting, and they are then ready for eating without roasting or any other treatments (Janick, 2002). Cracking and shelling machines handle most of the commercial crop, which is usually marketed shelled (Janick, 2002). Pecan nuts contain high levels of mono-unsaturated oil which is almost the highest among the various nut types (Anon, 2009[B]). Mono-unsaturated oil is rich in oleic and linoleic acids, which reduces harmful low-density lipoproteins in the blood and therefore lowers cholesterol and reduces the risk of heart disease. Pecan nuts also contain high levels of other minerals such as potassium, iron, phosphorous, calcium and vitamins such as vitamin A, vitamin E, thiamine (B1) riboflavin (B2), and niacin (Anon, 2009[B]).

1.4.3.2 International production and export

The top four pecan nut producing countries in the world for the 2010/2011 season were the USA, Mexico, South Africa and Australia (Table 1.3) (Anon, 2011[B]). Other countries that produce pecans on a smaller scale include Israel and Peru (Hadjigeorgalis, lilywhite & Herrera, 2005). The global production of pecan nuts for the 2010/2011 season totalled 82,979 tons (Anon, 2011[B]).

Table 1.3: The top 4 pecan nut producing countries (Kernel Basis - Metric Tons) for the 2010/2011 season and the production projections for the top three countries for the 2011/2012 season (Anon, 2011[B]).

Top Pecan nut producers		2010/2011	2011/2012 (Projected)
1	USA	52,989	64,125
2	Mexico	24,494	36,743
3	South Africa	3,682	3,675
4	Australia	1,004	-
5	Other	810	-
	Total:	**82,979**	**-**

The largest commercial producer and exporter of pecans is the USA that on average produces 80% of the world's pecan nuts (Gorscak, 2012). During the first 11 months of 2011 the USA exported more than 50,000 metric tons of pecans. The three largest importers of USA pecans are Hong Kong, Mexico and the EU. These markets import almost 70% of the pecan nuts produced by the USA (Gorscak, 2012). The demand of pecans by the Chinese has caused the pecan industry in the USA to grow considerably (Wessel, 2010). Before 2007, China imported almost no pecans from the USA, and since 2009 it has imported 25% of the total USA pecan produce (Wessel, 2010). The total export market during 2010 for USA pecans was worth $143 million for unshelled pecans and $109 million for shelled pecans (Geisler, 2011).

The volume projections for the production of pecan nuts in South Africa show that the country will produce approximately 28,247 tons per annum by the year 2020 (Anon, 2011[B]). This is almost a hundred percent increase over the next decade from what South Africa is currently producing. In contrast to this, Mexico, which is the second largest producer of pecans, does not show much growth in their pecan industry. Trejo & Hernandez (2003) stated that

Mexico produced 34, 000 tons per annum on average. This is close to what Mexico produced during the 2010/2011 season (Anon, 2011[B]). Projections show that the pecan industry in South Africa is thriving and that it has much promise.

1.4.3.3 Insect-fungal associations

Stink bugs (Hemiptera: Pentatomidae) are considered one of the important insect pests of pecans (De Villiers & Joubert, 2008). As with pistachios and walnuts, it is suspected that hemipteran pests such as stink bugs are responsible for the spread and occurrence of *B. dothidea,* a serious pathogen of pecans. On pecans, this pathogen causes water stage nut drop or stem end blight, and its occurrence is associated with insect feeding (Ree, 2003; Anon, 2006; Pijut, 2010). Hemipteran pests are also suspect for the dissemination of *N. coryli* and *A. pullulans* which causes stigmatomycosis in pecans (Michailides *et al*., 1995). The symptoms of this disease and the process of transmission on pecans are similar to that of pistachios (Michailides *et al*., 1995). Recently, research conducted by Alvidrez-Villarreal *et al*. (2012) indicated that the trunk and branch borer, *Euplatypus segnis* (Coleoptera: Curculionidae: Platypodinae) is associated with several fungal species including *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata,* and *Lasiodiplodia theobromae* that are able to cause the die back of pecan trees. Another example illustrating the involvement of insects in the spread of disease in pecan orchards is *Xylella fastidiosa,* a bacterial pathogen that responsible for pecan bacterial leaf scorch that is transmitted by leafhoppers (Hemiptera; Cicadellidae) and spittlebugs (Hemiptera: Cercopidae) (Sanderlin & Melanson, 2010). The dissemination of this pathogen reflects the importance of insect-pathogen interactions in pecan production.

Trunk and branch borer, Euplatypus segnis *(Coleoptera: Curculionidae: Platypodinae) and* Fusarium oxysporum, Fusarium solani, Alternaria alternata *and* Lasiodiplodia theobromae *on pecans*

The ambrosia trunk and branch borer, *Euplatypus segnis*, is an economic pest of pecan in several regions of Mexico where pecans are produced commercially (Alvidrez-Villarreal *et al*., 2012). This pest is found in more than 20% of the pecan orchards in these regions, where its presence is attributed to 4% yield loss annually. Speculations exist that *E. segnis* have symbiotic relations with fungi occurring in the pecan orchards, as with other ambrosia beetle species

occurring on other tree species. Research was conducted by Alvidrez-Villarreal *et al*. (2012) to determine what fungal species are associated with *E. segnis* occurring in pecan orchards and whether these fungal species may be responsible for the die back of pecan branches and ultimately the death of trees. Both insects and diseased wood were investigated for the presence of fungi. Several fungal species were isolated and identified to be associated with the diseased wood and the beetles. These included *Helminthosporium* sp., *Aspergillus* sp., *Penicillium* sp., *Phoma* sp., *Ascochyta* sp., *Paecilomyces* sp., *Umbeliopsis* sp., *Torula* sp., *Fusarium solani*, *Alternaria alternata*, *Fusarium oxysporum*, and *Lasiodiplodia theobromae*. Pathogenicity tests were conducted by inoculating the stems of 3 year old healthy pecan trees with representatives of the above mentioned fungal species. The results indicated that four of the fungal species nl. *Fusarium oxysporum, Fusarium solani, Alternaria alternata* and *Lasiodiplodia theobromae*, isolated from both the insects and the diseased wood, were able to cause the die back of pecan trees after 84 days. Alvidrez-Villarreal *et al*. (2012) concluded that insects in combination with fugal invasion eventually results in the death of trees and that *E. segnis* contributes to the spread of fungi in pecan orchards.

Leafhoppers (Hemiptera: Cicadellidae) and spittlebugs (Hemiptera: Cercopidae) associated with Xylella fastidiosa *on pecans*

Xylella fastidiosa was first described as a pathogen of pecans in 2000 (Sanderlin & Heyderich-Alger, 2000). The pathogen abides in the xylem of the plant and causes bacterial leaf scorch. The symptoms of the scorch are described as necrotic areas that appear at the margins of leaflets and then spread to the midrib of the leaflet. The advancing lesions are defined by black margins that eventually lead to the falling of leaflets from the tree (Sanderlin & Heyderich-Alger, 2000). As a result the trees are defoliated that negatively affects harvests and, in extreme cases, the death of the trees (Sanderlin & Melanson, 2010). *Xylella fastidiosa* is not only limited to pecans, it can cause leaf scorch on several other important horticultural crops. These include grape, peach, plum, almond, sycamore, oak and red maple. The primary method of transmission of *X. fastidiosa* in these crops is by insect vectoring. The main vectors of this pathogen include insects from the families Cicadellidae and Cercopidae. Sanderlin & Melanson (2010) investigated the role of several species of cicadellids and cercopids in the transmission of *X. fastidiosa* between pecan trees. They identified three effective vectors of *X. fastidiosa*, including

the pecan spittle bug, *Lepyronia quadrangularis,* the glassy-winged sharpshooter, *Homalodisca vitripennis* and the Johnson-grass sharpshooter, *Homalodisca insolita.* The pecan spittle bug is able to function as a vector and, because these bugs spend their entire life on pecan trees, they can become significant vectors of this pathogen within pecan orchards. The glassy-winged sharpshooter and the Johnson-grass sharpshooter are major threats in the spread of this pathogen, especially the glassy-winged sharpshooter. The latter is a voracious polyphagous feeder and can contribute to the spread of this pathogen between orchards or from other hosts (Sanderlin & Melanson, 2010). The dissemination of pathogens such as *X. fastidiosa* by insects can seriously injure the pecan industry resulting in great economic losses.

1.5 Conclusion

Fungal plant pathogens are the leading cause of disease within crop production, affecting the quality, quantity and the safety of produce. Diseases caused by phytopathogenic fungi can, therefore, have major economic implications and directly threatens food security. The two most important factors influencing the success of fungal plant pathogens is the ability to colonise hosts and to spread between hosts. Evidently, fungal pathogens are successful in colonising their hosts when considering the degree of disease caused by these organisms in crop production. The spread of phytopathogenic fungal propagules between and within crop production systems directly influences the occurrence of disease. Fungal plant pathogens have evolved to utilise a variety of methods to spread their propagules including anemophily, hydrophily and vectored dispersal. Anemophily and hydrophily are successful modes of dispersal but relies on chance, whereas vectored dispersal is preferred by fungi that require specific substrata *e.g*. specific host plants.

Among the vectors exploited by phytopthogenic fungi, insects are the most important. Insects are well suited to be vectors of fungi since they are mobile and possess specialized morphological (*e.g*. mycangia), and physiological (*e.g*. olfactory systems) adaptations. This allows them to carry fungal propagules and quickly locate host plants. Insects can also assist phytopathogenic fungi in gaining entry into host plants by creating infection courts through feeding damage. Due to the important role that insects play in the dissemination of phytophotogenic fungi they directly contribute to the spread and thus the occurrence of disease. The interaction between insects and phytopthogenic fungi might be detrimental to the establishment of important new crops in South Africa and hinder commercial production.

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Chapter 2

Fungi Associated with Insects Occurring in Pistachio and Walnut Orchards in South Africa

2.1 Introduction

Pistachios (*Pistacia vera* L.) and walnuts (*Juglans regia* L.) are relatively new crops produced commercially in South Africa, with both industries remaining in their infancy (Louw, 2002; Haddad & Louw, 2006[A]; Van Zyl, 2009). Due to the importance of both commodities internationally, and the high potential of these crops, their establishment and production is supported by the Industrial Development Corporation (IDC) of South Africa (Louw, 2002; Allemann, 2008; Van Zyl, 2009). The establishment of both crops forms part of a project to provide labour for South Africans, while generating revenue as export items (Allemann, 2008; Van Zyl, 2009). Since the establishment of these crops, several constraints have been identified that may potentially limit the yield production. These include environmental conditions (rainfall and temperature), the availability of propagative material, and the presence of pests and diseases. Several of these constraints have been managed with agricultural practices and the local production of propagative material (Allemann, 2008; Van Zyl, 2009). However, knowledge is limited regarding the pests and diseases and their interaction on these crops.

Since the establishment of pistachio orchards in South Africa, much research has been conducted regarding potential pests and their control (Louw, 2002; Haddad, 2003; Louw & Fourie, 2004; Haddad, Louw & Dippenaar-Schoeman, 2004[A]; Haddad, Louw & Dippenaar-Schoeman, 2004[B]; Haddad, Dippenaar-Schoeman & Pekár, 2005; Haddad & Dippenaar-Schoeman, 2006; Haddad & Louw, 2006[A]; Haddad & Louw, 2006[B]). New diseases and their effect on this crop have also been identified and reported for the first time (Swart & Botes, 1995; Blodgett & Swart, 1998; Allemann, 2008). The role of pests in the dissemination of fungal pathogens within pistachio orchards, however, is not well documented. Swart (2002) conducted research regarding the role of *Sparrmannia flava* (Coleoptera: Scarabaeidae: Melolonthinae) and *Nysius natalensis* (false chinch bug, Hemiptera: Lygaeidae: Orsillinae) as disseminators of fungal phytopathogens in pistachio orchards. It was concluded that both species may act as potential disseminators of fungal pathogens within *P. vera* orchards and might negatively influence the production of this crop. During research conducted by Haddad & Louw (2006[A]), it was observed that feeding damage to developing nuts caused by *Atelocera raptoria* Germar (Hemiptera: Pentatomidae) might function as entry points for opportunistic fungi. *Atelocera raptoria*, commonly known as the powdery stink bug, has been identified as a species that occurs

in sufficient densities within orchards to pose a major threat to pistachio production. This bug feeds on the petioles of young leaves, causing premature leaf drop, and on young developing nuts, resulting in lesions forming on the kernels (Haddad & Louw, 2006[A]). The dissemination of fungal pathogens by Hemiptera and other insects in pistachio orchards, from various regions around the world, has also been reported. These include the navel orange worm, *Amyelois transitella* (Lepidoptera: Pyralidae), that disseminate *Aspergillus* species responsible for Aspergillus blight and the presence of aflatoxins (Dowd, 2003; Shahidi Bonjar, 2004; Anon, 2009). Several hemipteran species, predominantly stink bugs and leaf footed bugs (Hemiptera: Pentatomidae and Coreidae), have also been noted to be involved in the dissemination of *Botryosphaeria dothidea* that causes panicle shoot blight (Michailides & Morgan, 1996; Steffan, Daane & Yokota, 2000), and both *Nematospora coryli* and *Aureobasidium pullulans* that cause stigmatomycosis on pistachios (Michailides, Morgan & Doster, 1995).

Little research has been conducted in the Free State Province with regards to pests and diseases that adversely affect walnuts, although stink bugs (Hemiptera: Pentatomidae) and leafhoppers (Hemiptera: Cicadellidae) do occur and feed on trees of various ages* (Rotondo Walnuts, personal communication). Recently it has been found that the walnut twig beetle, *Pityophthorus juglandis* (Coleoptera: Curculionidae: Scolytinae) is responsible for the dissemination of the thousand cankers disease caused by *Geosmithia morbida* in walnuts indigenous to North America (Cranshaw & Tisserat, 2010). Presently, this disease has a devastating effect on black walnuts found throughout the western states of the USA (Cranshaw & Tisserat, 2010; Grant *et al*., 2011). The thousand cankers disease complex consists out of the walnut twig beetle, *P. juglandis*, and *G. morbida*. This complex may ultimately have the potential to destroy black walnut population stands in its native range in a manner previously demonstrated by introduced species such as the pathogens that cause Dutch elm disease (affecting American elm) and chestnut blight (affecting American chestnut) (Cranshaw & Tisserat, 2010; Grant *et al*., 2011).

Insects as disseminators of fungal phytopathogens may play a vital role in the spread and occurrence of disease in both pistachio and walnut orchards. This may adversely affect the establishment and production of both crops in South Africa. The primary aim of this chapter was to investigate whether certain insect species occurring in pistachio and walnut orchards are

*Personal communication with the staff at Rotondo Walnuts.

associated with fungal phytopathogens. The second aim was to determine whether a single insect group (*i.e.* stink bugs in pistachio and walnut orchards) harbour the same fungi. The objective here was to determine whether the fungi form a closer association with the insects or the crops involved. This study also investigated whether different insects occurring on the same crop (*i.e.* stink bugs and leafhoppers on walnuts) harbour the same fungi. This was to determine whether the ethology and ecology of the insects, or the environment determine the presence of fungi on the insects.

2.2 Materials and Methods

2.2.1 Study sites and insect specimen collection

The site where insects were collected on pistachio trees was Green valley nuts (GVN), near Prieska in the Northern Cape Province (S 29º 33.717', E 22º 55.007'). *Atelocera raptoria* Germar (Hemiptera: Pentatomidae) was collected on the pistachio trees as this species is most likely to be associated with fungal pathogens and their dissemination (Figs. 2.1). Trees used in this study were between the ages of 17 and 18 years. A total of 100 specimens were collected by actively searching trees and capturing individuals by hand, placing each specimen separately into a sterile Polytop^{TM}. The Polytops were sterilized by soaking them in 76% ethanol for 24 hours and letting them air dry, top down on a sterile cloth in a laminar flow hood. Upon capture, the specimens were placed into a cooler box to prevent fungal colonies from growing on the stink bugs. Specimens were then transported to the laboratories at the University of the Free State where they were placed in a freezer at -20 ˚C for 5 minutes. This killed the stink bugs without affecting the fungal propagules present on the specimens.

Insects were collected on walnut trees at Rotondo Walnuts, west of Aliwal North (S 30° 41.10', E 26° 42.37'). Rotondo Walnuts is currently the largest producer of walnuts in South Africa. *Coenomorpha nervosa* Dallas (Hemiptera: Pentatomidae), commonly known as the grey-brown stink bug, was cited as the likely species to harbour and disseminate fungal pathogens in the walnut orchards (Fig. 2.2). Similarly to GVN, the same protocol was used to collect specimens. The stink bugs were collected during October 2009 on trees between the ages of 8 and 10 years. *Empoasca citrusa* Theron (Hemiptera: Cicadellidae), commonly known as the green citrus leafhopper, was also observed to be abundant on smaller trees between the age of 1

and 3 years (Fig. 2.3). These trees were kept in nurseries covered with 60% shade netting (Fig. 2.4). Leafhopper specimens were collected during February 2012. It was observed that the trees infested with the leafhoppers had severe hopper burn and fungal infections on their leaves (Figs. 2.5 - 2.6). A total of 100 leafhopper specimens were collected by actively searching trees, capturing individuals by hand, and then placing each specimen into a sterile 1.5 mℓ Eppendorf™ tube. The Eppendorf tubes were prepared by autoclaving. Upon capture, the specimens were placed into a cooler box and were transported to the laboratories at the University of the Free State. The leafhopper specimens were killed by freezing as described above. Confirmation of the identification of the various insect species was done by the Biosystematics Division of the Agricultural Research Council (ARC) in South Africa.

2.2.2 Isolation of fungal colonies from insect specimens

Potato dextrose agar (PDA) (Biolab) was used for the isolation of fungi from the insects and was prepared according to manufacturer specifications three days prior to insect collection. The PDA medium is high in nutrients and supports rapid non reproductive growth of fungal colonies that makes it ideal for the isolation of fungi. The agar was autoclaved for 20 min. at 100 kPa steam pressure (121 ˚C). It was then cooled in a warm bath to 55 ˚C. A total of 0.3 mℓ BioStrep™ (Streptomycin Sulphate) was added to each litre of PDA medium. The BioStrep™ inhibits bacterial growth and helps to reduce contamination. The agar medium was poured into 90 mm diam. empty Petri plates (approximately 20 ml PDA per plate). The plates were left for 12 hours in a laminar flow hood in order to solidify and clearing access water on the lids of the plates.

The euthanized insects were individually plated on PDA plates, by using a sterile pincer. The insects were placed in the middle of the plates with their mouthparts and legs facing downwards. The inoculated PDA agar plates were then placed in an incubator at 25 ºC for seven days. This provided ideal conditions for spore germination and colony growth.

The agar plates were removed from the incubator Each PDA plate was examined and each fungal colony was subcultured to a corn meal agar (CMA, Difco) plate. The latter medium is low in nutrients and supports the slow reproductive growth of fungal colonies, making it ideal for the identification of fungi. The agar plates were prepared by suspending 17 g of CMA agar

powder in one litre distilled water. The CMA plates were prepared following the same procedures as with the preparation of the PDA plates. The re-isolation of fungal colonies was done by aseptically cutting an agar disk (0.5x0.5mm) from each fungal colony on the PDA plates and plating it on CMA. Agar pieces were cut on the edges of the fungal colonies to obtain vigorous growing hyphae and to limit contamination. The agar pieces were placed as such so that the re-isolated fungal material faced downwards to make contact with the agar medium. The plates were then placed in the incubator at 25 ºC for seven days. The plates were removed from the incubator, checked for contamination, and placed in a sporulation chamber. Agar plates were subjected to 12-hour UV/dark cycles for seven days. This was done to stimulate fruiting structure development and to enhance sporulation.

2.2.3 Identification of fungal colonies

Fungal colonies were identified based on their morphological characteristics such as fruiting structures, spore shape and size, as well as colony texture and colour. Various literature sources were used as aids in the identification of fungi (see references list). Light and stereo microscopy was used to examine the fungal colonies and fruiting structures, and an attempt was made to identify all fungi to species level. Fungal structures were aseptically removed and mounted on microscope slides. The fungal material was stained with lactophenol cotton blue and covered with a coverslip, after which it was examined by using a light microscope. Fungi that did not sporulate initially were further incubated until fruiting structures were observed. To aid the isolates that had difficulties in producing fruiting structures, sterilised pine needles were added to old and re-isolated colonies. The needles were autoclaved for 40 min., left at room temperature for 24 hours, and autoclaved again for 40 min. This was to eliminate any spore forming organisms that could be resistant to heat. The process of identification and re-isolation was repeated until all the fungal colonies were identified to species level. In certain samples, yeasts were also isolated from the insects. Identification of the yeasts was done by the department of Microbial, Biochemical and Food Biotechnology at the University of the Free State. All the yeast colonies were identified to species level based on molecular sequencing. Seven-day growth studies were performed to distinguish between species of *Penicillium*, whereas sequencing techniques were used to identify *Fusarium* species. All other fungi were identified based on

morphological characteristics. All prominent fungi that were isolated were deposited in the CGM culture collection at the department of Plant Sciences at the University of the Free State.

Identification of Penicillium *spp.*

The basis on which the morphological identification of *Penicillium* spp. is done focuses on a seven-day growth study that compares morphological and physiological characteristics such as the effects of temperature, water relations, pigmentation, exudate formation and colony development on certain growth media (Pitt, 1991). The technique is based on growing cultures for 7 days on three different agar media at 25 ºC [Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N)], and one of these nl. CYA at 5 ºC and 37 ºC. Preparation of the media is discussed in detail in Pitt (1991).

Purified *Penicillium* cultures were inoculated on CYA, MEA and G25N plates by using a sterile dissection needle (Pitt, 1991). Each of the plates was inoculated with a single culture at three points, equidistant from the edge and centre of the plates, and from each other (Fig. 2.7). Care was taken to avoid colonies from stray spores (Pitt, 1991). To prevent this, spores were suspended in a deterrent based water solution. The solution was made up by dissolving 5 mℓ polysorbitan 80 (tween 80) in 100 mℓ sterile distilled water and transferring 1 mℓ of the solution to a sterile Eppendorf tube. Spore suspensions were made by transferring a needle point of spores to the Eppendorf tubes and lightly shaking the tubes for several seconds until the spores were dispersed through the solution. A sterile dissection needle was dipped in the spore suspension and point inoculations were made. The plates were then placed in incubators set to the respective temperatures mentioned above. After seven days the plates were removed, examined with a binocular compound and stereo microscope, and colonies were measured. The various *Penicillium* spp. were identified based on the key system provided by Pitt (1991).

Molecular identification of Fusarium *spp.*

Molecular techniques were used for identification purposes due to the difficulties of identifying *Fusarium* spp. on the basis of morphology alone. Representatives of each morphotypic group of *Fusarium* strains isolated from the insects were selected to be identified. The fungal cultures were grown on water agar (WA) plates that were incubated for 24 hours at 25 ^{o}C. The latter medium is low in nutrients and supports the slow and sparse growth of fungal

colonies. To ensure the genetic uniformity of cultures, individual hyphal tips were subcultured from the WA plates. This was done by microscopically and aseptically removing a piece of agar (0.5x0.5mm.), containing a single hyphal tip, from each *Fusarium* colony with a needle and plating it on PDA. In total, three separate hyphal tips were collected and plated. The WA plates were prepared by suspending 20 g agar bacteriological powder (Biolab) in one litre distilled water. The medium was autoclaved and poured in Petri plates similar to the preparation of other growth media as described previously. The PDA plates were incubated at 25°C for three days, after which the three hyphal tip colonies were compared to the original culture. The healthiest growing, non pinotal colony was selected and subcultured on a PDA plate for DNA extraction purposes. The inoculated PDA plates were placed in the incubator at 25 ºC for 14 days.

The fungal material was removed from the PDA plates by aseptically scraping it off with a scalpel and transferring the material to a 2 mℓ sterile Eppendorf tube. Care was taken not to collect agar during removal and transferring of fungal material since this might adversely affect the DNA extraction process. The Eppendorf tube was sealed with Parafilm^{TM} in which three holes were made with a hot dissection needle. The purpose of the Parafilm^{TM} was to prevent contamination and to stop material from shooting out of the Eppendorf tubes during freeze drying. The tubes were freezed at -80 ºC and then lyophilised for 24 hours in a Virtis-freeze mobile II freeze dryer. Two sterile steel beads were placed in each tube and the samples were tissuelised for one minute (30 seconds per side) in a Qiagen tissue-lizer at 300 rpm to free the DNA from the fungal cells.

A hexadecyltrimethylammonium bromide (CTAB) extraction method was used to extract DNA from the fungal material (Velegraki *et al*., 1999; Jana *et al*., 2003; Brandfass & Karlovsky, 2008). In total, 750 µl CTAB buffer was added to each sample. A 20 mℓ CTAB buffer solution (enough for 24 extractions) was prepared by suspending 2 mℓ. 1 M Tris-Cl (100 mM), 0.8 mℓ 0.5 M EDTA (20 mM) (pH 8.0), 5.6 mℓ 5 M NaCl (1.4 M), 4 mℓ 10% CTAB (2%) and 40 µl β-mercapto-ethanol (0.2%) in 7.56 mℓ double distilled sterilised water. The solution was prepared in a laminar flow hood to prevent contamination and exposure to harmful volatiles. The samples were vortexed for ± 30 seconds to mix the tissualised fungal material with the buffer and incubated in a warm bath at 65 ºC for one hour. The DNA and the fungal material were separated from each other by adding 500 µl chloroform ($ChCl_3$) / isoamylalcohol (IAA) (24:1 v/v) to the

samples. The solution was thoroughly mixed by vortexing and centrifuged at 12 000 *g* for 10 minutes at 4 ºC. The DNA was precipitated from the aqueous phase with 500 µl isopropanol to sterile 1.5 mℓ Eppendorf tubes. The solutions were thoroughly mixed and incubated at room temperature for 20 minutes. The samples were then again centrifuged for 10 min. The supernatant was discarded from the samples and all the liquid was drawn off using a water pump. Care was taken not to suck in the pellets at the bottom of the Eppendof tubes. The pellets were wash precipitated at room temperature by adding 500 µl ice-cold 70% (v/v) ethanol and incubated for 20 minutes at room temperature. The samples were centrifuged and the liquid was drawn off using a water pump and the pellets were air dried for one hour at room temperature. The pellets were then re-suspended in 200 µl TE buffer and left overnight at 4 ºC. In total, 2 µl RNase A (10 mg/ml) was added to the samples and incubated in a warm bath at 37 ºC for two hours to eliminate RNA present in the pellets. The DNA was extracted from the solutions with 20 µl 7.5 M ammonium acetate and 200 µl $ChCl_3$ / IAA (24:1). The solutions were thoroughly mixed and centrifuged as above. The DNA was precipitated from the aqueous phase to 1.5 mℓ Eppendorf tubes with 500 µl ice-cold 100% ethanol and left overnight at -20 ºC. The solutions were centrifuged, where after all the liquid was drawn off using a water pump. The pellets were washed twice with 500 µl ice-cold 70% (v/v) ethanol and centrifuged as mentioned above. All the liquid was drawn off using a water pump and the pellets were air dried for one hour at room temperature. The pellets were dissolved in 50 µl TE buffer by leaving them overnight at 4 ºC. When the pellets were properly dissolved the concentration and purity of the extracted DNA was determined using a nano drop 2000 spectrophotometer (Thermo scientific) and the samples were prepared for the PCR process.

For the PCR process the DNA extracted from the *Fusarium* isolates was diluted to 5 ng/µl templates by adding calculated volumes of the extracted DNA to appropriate volumes of DEPC water. DNA segments from the Elongation Factor-1α region were amplified with the primers EF1 (5´-ATGGGTAAGARGACAAGAC-3´) and EF2 (5´-GGARGTACCAGTSATCATGTT-3´) (Kristensen *et al*., 2005; Alastruey-Izquierdo *et al*., 2008). Primers were obtained from Inqaba Biotechnical Industries (Pty) Ltd and prepared by dissolving the powderised primers in TE buffer as prescribed. A 100 *p*mol/µl primer set was prepared by adding 10 µl of both primers from the stock solutions to 80 µl of DEPC water. To aid in preparing the PCR reaction a mixture was compiled from the primer set, DEPC water and

a Taq-pol master mix (5 X FIREPOL master mix, ready to load (Bolie Biodyne)). The mixture was compiled according to the number of PCR reactions required. In total, 15 µl PCR reaction mixtures were prepared containing 13 µl of the complied mixture and 2 µl of the 5 ng/µl DNA templates. A control PCR reaction mixture was also prepared in the same manner except that the DNA template was substituted with 2 µl of DEPC water. The PCR reaction mixtures were prepared in 60 µl sterilised Eppendorf tubes. The reactions were carried out in a G-Storm PCR (Vacutec). The cycling parameters that were used for the amplification of the EF-1α region included an initial denaturation of 3 min at 94 ºC, followed by 35 cycles of 20 s at 94 ºC, 30 s at 58.2 ºC and 60 s at 72 ºC, and a final elongation step of 5 min at 72 ºC. Once the reactions were completed the integrity of the PCR products was confirmed on a 1% (w/v) electrophoresis agarose gel stained with ethidium bromide. The gel was prepared by suspending 0.6 g of agarose (Conda laboratorios) in 50 mℓ of 0.5 % TAE buffer and heating the solution in the microwave. The solution was cooled under running tap water and 2 µl ethidium bromide was added. The solution was mixed by light swirling and poured in to a mold with a comb to create a square gel containing 14 wells. The gel was left for an hour to solidify where after it was removed from the mold and placed in a flatbed system where it was submerged in 0.5% TAE buffer. To confirm the fragment size of the PCR products, 2.5 µl of a DNA ladder (1 kb Gene ruler (Fermentas) was used. To confirm that no contamination occurred in the PCR product preparations, one well was loaded with 5 µl of the control PCR product. In total, 5 µl of the PCR products were loaded in each well, after which the gel was run at 100 volts for 30 min. The results were visualized under UV illumination (Fig. 2.8).

The samples were prepared for sequencing by cleaning the PCR products using a Favorgen® (Biotech Corp.) gel/PCR purification kit designed to recover and concentrate DNA fragments. This was done in four steps. The first step included the preparation of 50 µl aliquots of the PCR products by adding 40 µl DEPC water to the 10 µl PCR products in 1.5 mℓ Eppendorf tubes. Five volumes (250 µl) of FADF Buffer were added to the one volume (50 µl) of the samples. The samples were then mixed by vortexing. For the second step FADF columns were placed in 2 mℓ collection tubes and the sample mixtures from the previous step were placed in the FADF columns. The FADF Buffer binds the DNA fragments to the glass fiber matrix of the spin column to prevent the loss of DNA during the cleaning process. The samples were then centrifuged at 12 000 *g* for 1 min at 4 ºC. The flow-through was discarded and the FADF

columns were placed back in the collection tubes. The third step was to place 700 µl of Wash Buffer (ethanol added) in the FADF columns. The samples were then centrifuged at 12 000 *g* for 1 min at 4 ºC. Once complete the flow-through was discarded and the columns were centrifuged at 12 000 *g* for 3 min 30 s at 4 ºC to dry the column matrixes. During this step all contaminants were washed out by the Wash Buffer containing the ethanol. The fourth and final step focuses on the recovery of the purified DNA from the columns with a low salt Elution Buffer. The dried columns from the previous step were transferred to new micro-centrifuge tubes and 25 µl of Elution Buffer was added to the center of each column. The columns were then left for 3 min at room temperature to allow the Elution Buffer to be absorbed by the matrix. The columns were then centrifuged at 12 000 *g* for 2 min 30 s at 4 ºC to elute the purified DNA from the columns. Once cleaned the purified DNA was checked by using a 1% (w/v) agarose gel, prepared as mentioned previously. (Fig. 2.9).

To prepare for the sequencing reaction process of the purified DNA a 3.2 *p*mol/µl primer set was prepared for both EF1 and EF2. A total of 3.2 µl of each primer from the stock solutions was added to 96.8 µl DEPC water in two separate 1.5 mℓ Eppendorf tubes and mixed by vortexing. To aid in preparing the sequence reaction, a mixture was compiled from a primer set (ITS 1f or ITS 4), DEPC water, a sequencing ready reaction mixture (BigDye® Terminator V-3.1 Cycle sequencing ready reaction mix – 100 (Applied Biosystems)) and a sequencing buffer (BigDye® Terminator 5 X sequence buffer (Applied Biosystems)). The mixture was compiled according to the number of sequence reaction mixtures required. In total, 10 µl sequence reaction mixtures were prepared containing 8 µl of the complied mixture and 2 µl of the purified DNA templates. The sequence reaction mixtures were prepared in 60 µl sterilised Eppendorf tubes. The reactions were carried out in a G-Storm PCR (Vacutec) programmed for an initial denaturation of 1 min at 96 ºC, followed by 25 cycles of 10 s at 96 ºC, 5 s at 55 ºC, 4 min at 60 ºC, and a final elongation step of 5 min at 60 ºC.

Before sequencing a final clean-up procedure was followed. The sequence reaction products were cleaned with an EDTA precipitation clean-up protocol. The 10 µl sequence products were transferred to new 1.5 mℓ sterile Eppendorf tubes. A total of 10 µl DEPC water, 5 µl 125 MM EDTA solution and 60 µl 100% ethanol were added to the sequence reaction products in the Eppendorf tubes. The solutions were mixed by vortexing and incubated at room

temperature for 15 min. The solutions were then centrifuged for 15 min. The supernatant was removed after which 60 µl 70% ethanol was added. The solutions were then re-centrifuged for 5 min 30. The supernatant was once again removed and the pellets were left overnight to dry in the dark. The sequence data was collected on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems®). The resulting sequence data for the *Fusarium* spp. were analyzed using GeneiousPro V-5.6.5 and searched on MycoBank for corresponding fungal sequences.

2.2.4 Statistical analysis

The percentage of insect specimens associated with each fungal species was calculated using Microsoft Excel. The fungi were grouped by known pathogens of pistachios and walnuts, potential pathogens and non-pathogenic fungal species that supposedly pose no threat to the nut crops on which they occurred. The potential pathogens and contaminants were selected based on the nature of the group and/or their known pathogenicity towards other agricultural crops, or relations with pathogens that can cause disease on either pistachio or walnut. The most abundant fungal species (5% or higher incidence) were graphically represented with a bar chart. Based on the number of pathogens and potential pathogens/contaminants isolated from the insects, it was assessed whether the insects played a role in harbouring and the possible dissemination fungal pathogens. To analyze the similarities of species assemblages between different samples, *Sørenson's quantitative similarity index* was used (Junninen *et al*., 2006; Osono, 2008). The Sørenson's index (Dice Coefficient) is used to measure the similarity between samples and provides a value ranging from 0 (0% similarity) to 1 (100% similarity).This calculated by using the statistical program PAST (Hammer, Harper & Ryan, 2001).

2.3 Results and Discussion

2.3.1 Pistachio (*P. vera*)

In total, 24 species from 14 fungal and yeast genera were isolated from the *A. raptoria* specimens collected on pistachio trees at Green Valley Nuts (Table 2.1). These are represented by 8 species that are pathogenic, 8 that could be considered potentially pathogenic or as contaminants, and 8 that are considered non-pathogenic towards pistachio. Pathogens that were isolated include *Alternaria alternata* and *A. tenuissima* (causing Alternaria late blight),

Aspergillus flavus and *A. niger* (Aspergillus blight), *Cladosporium cladosporioides* (kernel decay), *Epicoccum nigrum* (kernel decay), *Fusarium oxysporum* (hull and kernel decay), and *Rhizopus stolonifer* (kernel decay) (Michailides, 2006; Eskalen *et al*., 2010). Potential pathogens and contaminants that were isolated include; *Aspergillus terreus*, *Cochliobolus spicifer*, *Epicoccum sorghi*, *Fusarium incarnatum*, *F. nygamai*, *Khuskia oryzae*, *Mucor hiemalis* and *Phoma pomorum*. The non-pathogenic fungi that were isolated include two *Acremonium* species, *Chaetomium funicola*, *C. globosum*, *Chaetomium* sp., *Cryptococcus magnus*, *C. roseus* and *Rhodotorula glutinis*. In addition, no microorganisms were isolated from 1% of the specimens. In total, 10 of the fungal species that were isolated can be considered abundant (Fig. 2.10). These include 5 pathogenic species, 2 potential pathogenic/contaminating species and 3 non-pathogenic species.

2.3.1.1 Most abundant fungal species isolated from *A. raptoria*

The second most commonly isolated pathogen was *Alternaria alternata* (Fig. 2.10). This fungus was isolated from 23% of the *A. raptoria* specimens. *Alternaria alternata* is one of the three most important fungal pathogens that cause disease on pistachios (Allemann, 2008). This pathogen causes the disease Alternaria late blight which is responsible for major economic losses in pistachio production (Allemann, 2008). The first record of Alternaria late blight on pistachio was from Egypt in 1974 (Michailides, 2006). The disease has since been reported in California, Italy, Australia, and South Africa. In serious cases it causes shell staining and the early defoliation of trees, which reduce yield and weakens the trees (Michailides, 2006).

Aspergillus flavus was isolated from 13% of the *A. raptoria* specimens (Fig. 2.10). This fungus may cause a disease on pistachios known as Aspergillus blight (Michailides, 2006; Eskalen *et al.*, 2010). Furthermore, it might also be involved in the post-harvest spoilage of the nuts (Eskalen *et al.*, 2010). *Aspergillus flavus* is known to produce mycotoxins called aflatoxins in pistachios (Boutrif, 1998). Aflatoxins produced by *Aspergillus* spp. have been found so often with pistachio nuts that it has become a health concern worldwide (Eskalen *et al*., 2010). Research suggests that pre-harvest contamination with *A. flavus* is responsible for the high aflatoxin levels. The occurrence of *Aspergillus* spp. has been found to be higher on pistachio nuts infested by the navel orange (*Amyelois transitella*) worm than in non-infested nuts. This might

indicate the important role that insects play in the dissemination of pathogenic *Aspergillus* spp. (Eskalen *et al*., 2010).

Cladosporium cladosporioides was isolated from 8% of the *A. raptoria* specimens (Fig. 2.10). This fungus may cause kernel decay on pistachio nuts (Michailides, 2006). Little is known about the infection biology of fungi that cause kernel decay on pistachio. The fungal species that cause this disease are mostly saprophytes, which most likely survive on dead and decaying material within orchards. The spores of this fungus are mostly dispersed by wind and the severity of the disease varies from orchard to orchard. Kernel decay incidence is usually not higher than 1 - 2 % of the nuts affected. It has been observed that the incidence of this disease is at its highest when significant rains occurred early in the season. Kernel decay is also more abundant on early split nuts and on nuts infested with the navel orange worm. Nearly all infection of nuts occurs before harvest, and improper storage conditions that allow moisture to build up can increase infection and yield losses. The disease is characterized by the dark brown discoloration or "staining" of the shell exterior. Most of the shell discoloration, however, is probably caused by the rupturing of the hull and not by fungal infection. In cases where the decay is extensive, the kernel is discoloured beneath the seed coat (Michailides, 2006).

The most commonly isolated pathogen was *Epicoccum nigrum* (Fig. 2.10), which was isolated from 27% of the *A. raptoria* specimens. *Epicoccum nigrum* may cause kernel decay on pistachio nuts (Michailides, 2006). The disease symptoms and characteristics of the kernel decay are the same as for *C. cladosporioides* (Michailides, 2006). *Epicoccum nigrum* has also been found to be involved in the postharvest spoilage of pistachio nuts (Nawar, 2008).

Rhizopus stolonifer was isolated from 5% of the *A. raptoria* specimens (Fig. 2.10) and may be one of the causative agents of kernel decay on pistachio nuts (Michailides, 2006). The disease symptoms and characteristics of the kernel decay caused by *R. stolonifer* on pistachios are also similar to *C. cladosporioides* (Michailides, 2006).

The most commonly isolated potential pathogen and/or contaminant was *Epicoccum sorghi* (Fig. 2.10) that was isolated from 15% of the *A. raptoria* specimens. *Epicoccum sorghi* was previously classified as *Phoma sorghina*, a cosmopolitan endophyte that has been isolated from several important agricultural crops planted across the world (Perelló & Moreno, 2005). It

is suspected that *E. sorghi* is a weak pathogen that can cause disease symptoms when plants are under stress. This fungus has also been found to produce several mycotoxins. These include tenuazonic acid that causes the inhibition of protein synthesis in humans and animals (Perelló & Moreno, 2005), as well as an unknown mycotoxin that has been implicated in onyalai (a blood disorder occurring in southern Africa) It is unclear whether *E. sorghi* can cause disease on pistachio trees, but it may be a potential pathogen.

The second most commonly isolated potential pathogen and/or contaminant was *Fusarium incarnatum* (Fig. 2.10), which was isolated from 9% of the *A. raptoria* specimens. *Fusarium incarnatum* is not classified as a pathogen of pistachio, although a collection of *Fusarium* spp. has been found to cause kernel decay (Michailides, 2006). Furthermore, *Fusarium* spp. also produce clinical important mycotoxins (Schaafsma *et al*. 1998). The production of these mycotoxins may pose a health risk and lower the value of the crop yield (Schaafsma *et al*. 1998).

The most commonly isolated and apparently non-pathogenic fungus was *Acremonium* sp. 1. This fungus was isolated from 17% of the *A. raptoria* specimens and 5% of the stink bug specimens. The genus, *Acremonium*, is described as filamentous, cosmopolitan fungi with most of the species being saprophytic, occurring on dead plant material and in soil (Domsch, Gams & Anderson, 2007). The genus *Acremonium* represents more than a 100 species. The conidia of *Acremonium* are usually one-celled, hyaline or pigmented and mostly aggregated in slimy heads at the apex of each phialide. The slimy heads ensure easy dissemination by insects. This fungal genus is saprophytic and occurs on decaying wood and leaves; it is widely distributed throughout the atmosphere, but has never been classified as a pathogen of pistachios (Domsch *et al*., 2007).

The second most commonly isolated non-pathogenic fungus *Chaetomium globosum* (Fig. 2.10) was isolated from 10% of the *A. raptoria* specimens. *Chaetomium* spp. are dematiaceous filamentous fungi found in the soil, air and plant debris. They are saprophytic and play a vital role in the decomposition of organic material (Domsch *et al*., 2007). The most common species within the genus include *C. atrobrunneum*, *C. funicola*, *C. globosum* and *C. strumarium* (Domsch *et al*., 2007). *Chaetomium* spp. are often associated with insects, since they are regularly isolated from this niche (Kendrick, 2000). *Chaetomium* spp. have hairy appendages on their fruiting bodies that cling to insects as they move over fungal colonies. This allows the

spores to be easily disseminated by insects. *Chaetomium* species are not considered pathogens of pistachio.

Various pathogenic, potential pathogenic, and non-pathogenic fungi were isolated from *A. raptoria* individuals collected on pistachio trees at GVN (Table 2.1). This indicates that *A. raptoria* is involved in harbouring and possibly disseminating fungal pathogens within pistachio orchards. A large proportion of the fungal isolates were pathogenic towards pistachios, and most of them have been cited in literature to be the causative agents of serious diseases in pistachio nuts (Michailides, 2006); these include *A. alternata* and *A. flavus.* Several potential-pathogenic fungi, as well as fungi that might cause postharvest decay, were isolated. Some of these fungal species can also produce potentially harmful mycotoxins. The production of mycotoxins can compromise the safety of nuts for consumption by humans and animals. Additionally, fungal development in the nuts can also affect the palatability and aesthetic appearance. Results also indicate that the insects harboured beneficial microorganisms such as epiphytic yeasts (*Cryptoccocus magnus*, *C. roseus* and *Rhodotorula glutinis*). Although the incidence of these organisms isolated was low, they may still have a positive effect against disease development within the pistachio orchards. Overall the results have proven that *A. raptoria* can harbour, and possibly disseminate, pathogenic fungi in large quantities within pistachio orchards. These stink bugs likely form an important link within the epidemiology of fungal diseases in pistachio production areas.

2.3.2 Walnuts (*J. regia*)

A total of 19 species that represent 15 fungal genera were isolated from *C. nervosa* and a total of 17 species, belonging to 10 fungal genera, were isolated from *E. citricola* at Rotondo Walnuts (Table 2.2). Overall, 25 species from 17 fungal genera were isolated from insects at this site. These were represented by 2 species that are pathogenic, 18 species that can be considered potentially pathogenic or as contaminants, and 5 species that are considered non-pathogenic. Pathogens that were isolated included *Alternaria alternata* and *Fusarium oxysporum* (Fusarium canker) (Mircetich, 2001). Other potential pathogens and contaminants isolated included *Alternaria tenuissima*, *Aspergillus flavus*, *Aspergillus niger*, *Byssochlamys spectabilis*, *Cladosporium cladosporioides*, *Cochliobolus sativus*, *Cunninghamella echinulata*, *Epicoccum nigrum*, *E. sorghi*, *Fusarium incarnatum*, *F. laceratum*, *Khuskia oryzae*, *Mucor plumbeus*,

Penicillium chrysogenum, *P. janthinellum*, *P. veriabile*, *Pestalotiopsis* sp. and *Phoma glomerata*. The non-pathogenic fungi that were isolated included *Acremonium strictum*, *Chaetomium funicola*, *C. globosum*, *Gonatobotrys simplex* and *Sordaria fimicola*. In addition, no microorganisms were isolated from 10% of the *E. citricola* specimens and unknown bacteria were isolated from 22% of the *C. nervosa* specimens and from 3% of the *E. citricola* specimens. In total, 7 of the fungal species isolated from the *C. nervosa* specimens were considered abundant (Fig. 2.11). These include 1 pathogenic, 4 potential pathogenic, and 2 non-pathogenic species. In total, 7 of the fungal species isolated from the *E. citricola* specimens were abundant (Fig. 2.12). These include 1 pathogenic, 5 potential pathogenic, and 1 non-pathogenic species.

2.3.2.1 Most abundant fungal species isolated from *C. nervosa*

The only abundant pathogen isolated from *C. nervosa* was *Alternaria alternata* (Fig. 2.11) that occurred on 17% of the insect specimens. *Alternaria alternata* is considered a miscellaneous pathogen of English walnut and it is known to cause fungal leaf spots (Mircetich, 2001). This fungus causes the premature defoliation of trees in the fall which weakens the trees and results in yield loss in the following season.

The most common potential pathogen and/or contaminant isolated was *Cunninghamella echinulata* (Fig. 2.11). This fungus was isolated from 13% of the *C. nervosa* specimens. It is a filamentous fungus found in soil and plant material, particularly in mediterranean and subtropical zones (Domsch *et al*., 2007). It has also been recovered from animal material, cheese, and Brazil nuts. The genus *Cunninghamella* currently contains seven species with *C. bertholletiae*, *C. elegans* and *C. echinulata* being the most common (Domsch *et al*., 2007). *Cunninghamella echinulata* can be considered as a potential contaminant, but it is not classified as a pathogen of walnuts.

The second most common potential pathogen and/or contaminant isolated was *Epicoccum nigrum* (Fig. 2.11). It was isolated from 8% of the *C. nervosa* specimens. This fungus is a cosmopolitan, dematiaceous fungus that is primarily isolated from plants and soil (Domsch *et al*., 2007). It has a saprophytic or epiphytic life style, occurring on dead or dying plant material. It is often considered as a secondary invader of damaged plant parts. *Epicoccum nigrum*

is also often associated with insects (Domsch *et al*., 2007). The fungus is not considered a pathogen of walnuts but it might be involved in the postharvest decay of stored nuts.

Khuskia oryzae was isolated from 5% of the *C. nervosa* specimens (Fig.2.11). This fungus is a filamentous dematiaceous fungus that is widely distributed occurring in soil, decaying plants and seeds (Domsch *et al*., 2007). *Khuskia oryzae* has a saprophytic lifestyle and is often considered as a contaminant of various substances such as food (Domsch *et al*., 2007). *Khuskia oryzae* is not known as a pathogen of walnuts, but it might be involved in postharvest spoilage.

Byssochlamys spectabilis, also known as *Byssochlamys spectabilis*, was isolated from 7% of the *C. nervosa* specimens (Fig. 2.11). Members of *Byssochlamys* are cosmopolitan filamentous fungi that are commonly isolated from soil, decaying plants and food products (Domsch *et al*., 2007). Certain species of *Byssochlamys* are also isolated from insects. The colonies of *Byssochlamys* spp. are very similar to those of *Penicillium* but vary greatly in colour (Domsch *et al*., 2007). *Byssochlamys spectabilis* is not known as a pathogen of walnuts, although it is known to cause postharvest decay.

The most common non-pathogenic fungus isolated was *Chaetomium globosum* (Fig. 2.11) that was isolated from 30% of the *C. nervosa* specimens. Additionally, *C. funicola* was isolated from 8% of the stink bug specimens. Similar to pistachios, *C. globosum* and *C. funicola* are not considered pathogens of walnuts.

2.3.2.2 Most abundant fungal species isolated from *E. citricola*

The only most abundant pathogen isolated from *E. citricola* was *Fusarium oxysporum* (Fig. 2.12), which was isolated from 17% of the insect specimens. *Fusarium oxysporum* is known to cause fusarium canker on black walnut (Mircetich, 2001). In the case of the English or Persian walnut this disease is caused by *F. solani* (Mircetich, 2001). *Fusarium oxysporum* is known to be a highly opportunistic and destructive plant pathogen (Domsch *et al*., 2007). It is possible that *F. oxysporum* can cause this disease on the English walnut, although the disease is considered minor or miscellaneous, and does not cause considerable economic losses of walnut production (Mircetich, 2001).

Aspergillus flavus was isolated from 10% of the *E. citricola* specimens (Fig. 2.12) and is known to contaminate a number of foodstuffs, including a variety of nuts such as peanuts and several tree nuts (FAO, 2003). *Aspergillus flavus* is known to synthesize mycotoxins including aflatoxins. It is proven that by chronic ingestion these mycotoxins possess a carcinogenic potential in animals. Amongst these mycotoxins, the well-known aflatoxins may induce hepatocellular carcinoma. *Aspergillus flavus* is not classified as a pathogen of walnuts, but it might be implicated in post-harvest spoilage. As a result of this, and the production of mycotoxins, *Aspergillus flavus* may cause economic losses of walnut production.

The most commonly isolated potential pathogen and/or contaminant was *Cladosporium cladosporioides* (Fig. 2.12). This fungus was isolated from 22% of the *E. citricola* specimens. *Cladosporium* spp. are dematiaceous fungi that are widely distributed in air and rotten organic material, and frequently isolated as contaminants of foodstuffs (Domsch *et al*., 2007). It is not considered a pathogen of walnuts but it might be involved in the postharvest spoilage of harvested and stored nuts.

The second most common potential pathogen and/or isolated was *Fusarium incarnatum* (Fig. 2.12), which was isolated from 14% of the *E. citricola* specimens. Additionally, *Fusarium laceratum* was isolated from 9% of the leafhopper specimens. Members of *Fusarium* are filamentous and widely distributed on plants and in the soil (Domsch *et al*., 2007). They form part of the normal mycoflora of commodities such as rice, beans, soybean and other economically important crops. While most species are more common in tropical and subtropical areas, certain species inhabit the soil in areas with a more temperate climate. *Fusarium* spp. are well known as being common contaminants and plant pathogens. Furthermore, *Fusarium* spp. also produce clinically important mycotoxins (Domsch *et al*., 2007). Ingestion of grains and other foodstuffs, contaminated with these toxins, may give rise to allergic reactions and are known to be carcinogenic when consumed over a long period of time (Schaafsma *et al*. 1998). There are no indications that *F. incarnatum* and *F. laceratum* are causative agents of disease on walnuts, although due to their nature they can be considered as potential pathogens and/or contaminants of walnuts.

Penicillium chrysogenum was isolated from 12% of the *E. citricola* specimens (Fig. 2.12). Most of the species in the genus, *Penicillium,* are considered filamentous fungi that are

widespread and their spores are found in soil, decaying vegetation and the air (Domsch *et al*., 2007). The genus, *Penicillium,* has several species and the most common species include *P. chrysogenum*, *P. citrinum*, *P. janthinellum*, *P. marneffei* and *P. purpurogenum* (Domsch *et al*., 2007). Species within the genus, *Penicillium,* are usually associated with food spoilage and they are able to contaminate a wide variety of foodstuffs. Certain *Penicillium* spp. are known to produce as much as 13 different mycotoxins that can range from almost non-toxic to highly toxic (Pitt, 1991). *Penicillium* spp. are not classified as pathogens of walnuts, but they might be involved in postharvest spoilage. As a result of this, and the production of mycotoxins, this genus may cause economic losses in walnut production.

The only prominent non-pathogenic fungus isolated from *E. citricola* was *Acremonium strictum* (Fig. 2.12). This fungus was isolated from 14% of the *E. citricola* specimens. Similar to the *Acremonium* species isolated from the stink bugs on pistachio, *Acremonium strictum* is not considered a pathogen of walnuts.

Various fungal species were isolated from *C. nervosa* and *E. citricolo* collected at Rotondo Walnuts (Table 2.2). These included various pathogens, potential pathogens and contaminants, and non-pathogenic fungi. The only pathogens isolated were *Alternaria alternata* and *Fusarium oxysporum*. This indicates that both insect species have the ability to harbour, and possibly disseminate, fungal pathogens. The largest proportion of the fungi isolated from these insects were, however, saprophytic and of no threat to walnut production. The fungal genus most isolated from *C. nervosa* was *Chaetomium,* which was isolated from more than a third of the stink bug specimens (Fig. 2.12). *Cladosporium cladosporioides* was the most commonly isolated from the *E. citricola* specimens and, even though this fungus may act as a contaminant of walnuts, it is saprophytic in nature, occurring mostly on dead and decaying organic material. Furthermore, only miscellaneous pathogens and potential pathogenic fungi were isolated and none of the serious pathogenic fungal genera, which are known to cause notably economic losses on walnuts, were found. This is contradictory to the samples taken in the pistachio orchard, where the largest proportion of the fungi isolated was pathogenic. This might be an indication that serious pathogenic fungal species are absent from the orchards rather that they are not harboured and disseminated by insects. Regardless of the fact that fungal pathogens were isolated in low proportion, some of the fungi isolated may cause postharvest damage and

produce harmful mycotoxins that pose a risk to human and animal health. The production of mycotoxins can compromise the safety of nuts for consumption. Fungal development within the nuts can also influence the palatability and aesthetic appearance of the nuts.

2.3.3 Sample comparisons and Sørenson's similarity index

The Sørenson's index, comparing the similarity of the species assemblages between the fungi isolated from the *A. raptoria* specimens collected on pistachio and those isolated from the *C. nervosa* specimens collected on walnuts, showed that they had a low level of similarity. The Sørenson's index value was 0.51163. When the Sørenson's index value is below 0.7 it indicates that there is no direct association between the two samples and that the diversity differs from each other (Gardner *et al*., 2011). The results suggest that the fungi associated with the two stink bug species are not specific to a particular insect group, but rather associated with the crop. Therefore, the ability of these insects to harbour and disseminate fungal pathogens is dependent on the presence of a particular pathogen within an orchard or within the environment. The dissemination of pathogens by these two species within pistachio and walnut orchards may be merely accidental and not due to a specific association with a particular fungal group; however, the stink bugs may provide suitable infection portals through feeding, aiding pathogens present on them or the plants, with infection and colonisation of the plants. Other factors that might influence the distribution of fungal species may be the particular stink bug species involved and the difference in location. The Sørenson's index showed that species assemblages of fungi isolated from the *C. nervosa* and *E. citricola* specimens collected on walnuts were different. The Sørenson's index value was 0.63158. The results indicate that the diversity differs from each other. This suggests that the different insects on the same crop harbour a different selection of fungi. This might be due to the ethology and ecology of a particular insect species. Other factors that might influence this distribution of fungal species may be time of collection and the age of the trees.

2.4 Conclusions

A large variety of fungi and yeasts were isolated from the insect specimens collected in both the pistachio and walnut orchards. These included several known pathogens of the crops, potential pathogens and non-pathogenic fungi. It can be concluded that insects occurring in pistachio and walnut orchards play an important role in the dynamics of fungal populations in these orchards. Of consequence is the role that these insects may play in harbouring and disseminating phytopathogens. The largest proportion of fungi isolated from the *Atelocera raptoria* specimens in the pistachio orchards was pathogenic; these include *Alternaria alternata* and *Aspergillus flavus*, two of the most serious pathogens of this crop. This suggests that these insects may play a role in the spread of disease in South African pistachio orchards. Compared to the occurrence of pathogens on the stink bug specimens collected in the pistachio orchard, the presence of pathogens on *Coenomorpha nervosa* and *Empoasca citricola* in the walnut orchards was minimal. Only two pathogens were isolated, which were *Alternaria alternata* and *Fusarium oxysporum*. These pathogens are considered minor or miscellaneous and none of the serious fungal pathogens that threaten this crop was found. This suggests that the serious pathogenic fungal species are absent from the orchards rather than that they are not harboured and disseminated by insects. Fungal species were isolated from all three insect species that may cause postharvest damage and can produce health threatening mycotoxins. Postharvest damage and the production of mycotoxins can cause significant losses in both industries. By comparing the similarity of the different samples it was found that the fungi associated with the insects occurring in the orchard is most likely based on the prevailing mycoflora of the particular orchard rather than on a particular insect group. The ecology and ethology of a particular insect species may also influence which fungal species occur on it. Overall, the study has shown that the insects occurring in pistachio and walnut orchards are associated with fungal pathogens of these crops and that these insects have the capability to harbour and disseminate these pathogens.

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2.6 Tables & Figures

Figure 2.1: *Atelocera raptoria* Germar (Hemiptera: Pentatomidae) on pistachio leaves (Photo by DeV. Fourie).

Figure 2.2: *Coenomorpha nervosa* Dallas (Hemiptera: Pentatomidae) on a walnut (Photo by J. Saaiman).

Figure 2.3: *Empoasca citrusa* Theron (Hemiptera: Cicadellidae) on a walnut leaf (Photo by J. Saaiman).

Figure 2.4: Walnut saplings grown at the Rotondo Walnuts nursery (Photo by J. Saaiman).

Figure 2.5: Damage to a young leaf of a walnut sapling (Photo by J. Saaiman).

Figure 2.6: A damaged leaflet compared to a healthy leaflet (Photo by J. Saaiman).

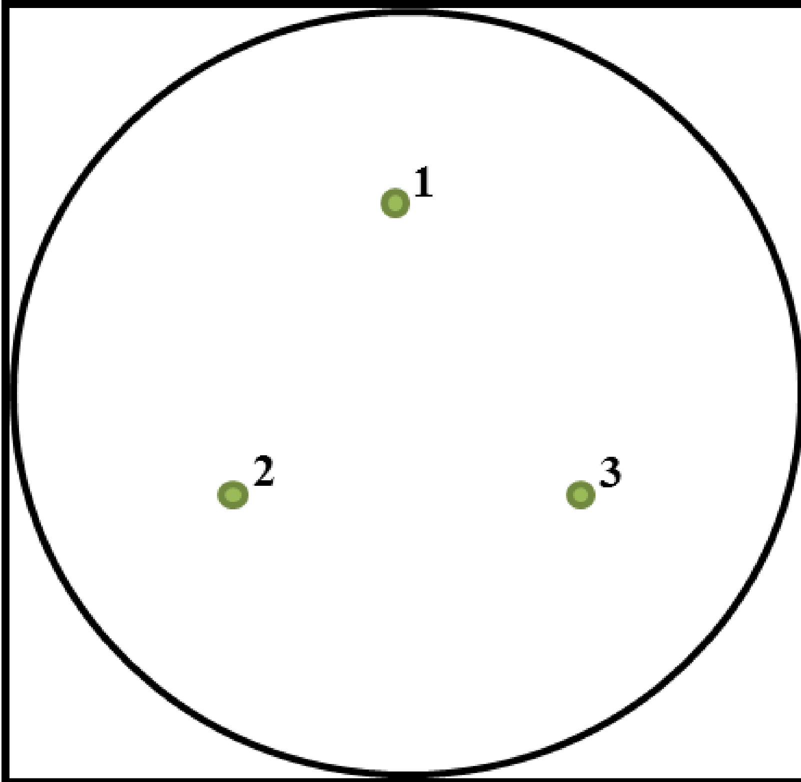

Figure 2.7: The equidistant inoculation of *Penicillium* spp. on agar plates during the seven day growth studies.

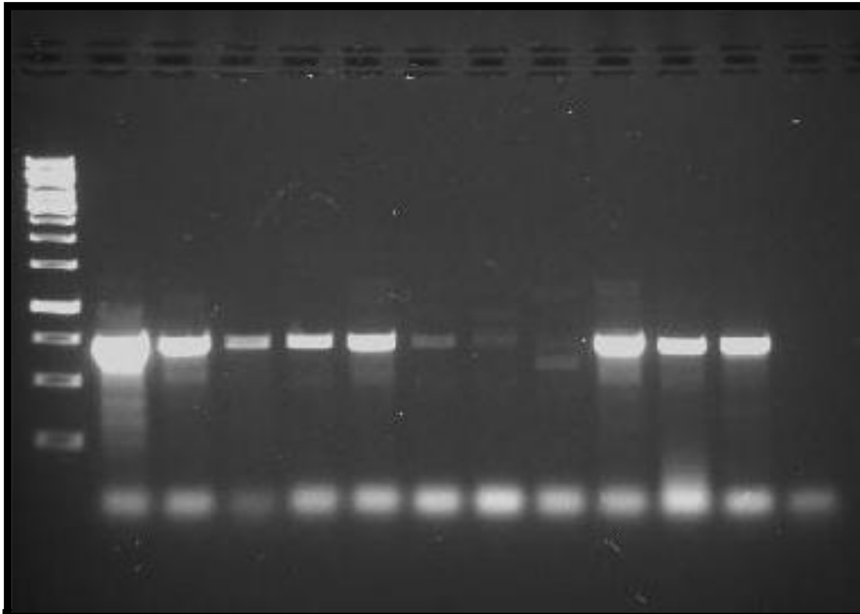

Figure 2.8: The illumination of PCR products from *Fusarium* isolates on an agarose gel showing amplification of the EF-1α gene region (Photo by J. Saaiman).

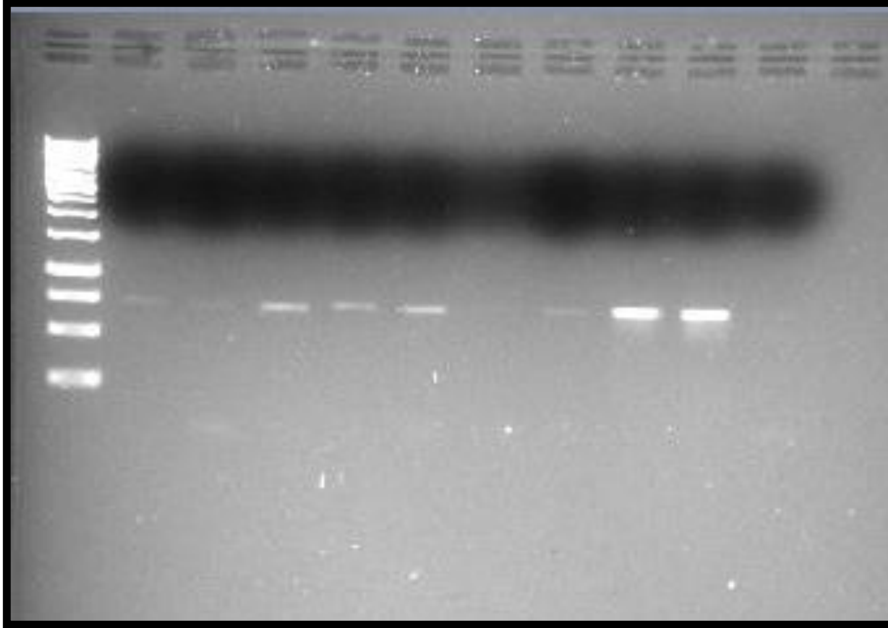

Figure 2.9: The results from the DNA clean-up of the PCR products for the *Fusarium* isolates, visualised on an agarose gel with UV illumination (Photo by J. Saaiman).

Table 2.1: Fungal species isolated from *A. raptoria* collected on pistachio trees at Green valley Nuts near Prieska in the Northern Cape Province during April 2009.

Fungal Species Isolated:	Percentage:	Disease:
Pathogens:		
Alternaria alternata	23	Alternaria late blight
Alternaria tenuissima	3	Alternaria late blight
Aspergillus flavus	13	Aspergillus blight
Aspergillus niger	3	Aspergillus blight
Cladosporium cladosporioides	8	Kernel decay
Epicoccum nigrum	27	Kernel decay
Fusarium oxysporum	3	Hull and kernel decay
Rhizopus stolonifer	5	Kernel decay
Potential Pathogens & Contaminants:		
Aspergillus terreus	2	N/A
Cochliobolus spicifer	2	N/A
Epicoccum sorghi	15	N/A
Fusarium incarnatum	9	N/A
Fusarium nygamai	2	N/A
Khuskia oryzae	1	N/A
Mucor hiemalis	2	N/A
Phoma pomorum	3	N/A
Non-Pathogenic Fungi:		
Acremonium sp.1	17	N/A
Acremonium sp.2	5	N/A
Chaetomium funicola	4	N/A
Chaetomium globosum	10	N/A
Chaetomium sp.	3	N/A
Cryptococcus magnus	2	N/A
Cryptococcus roseus	1	N/A
Rhodotorula glutinis	3	N/A
Other:		
None	1	N/A

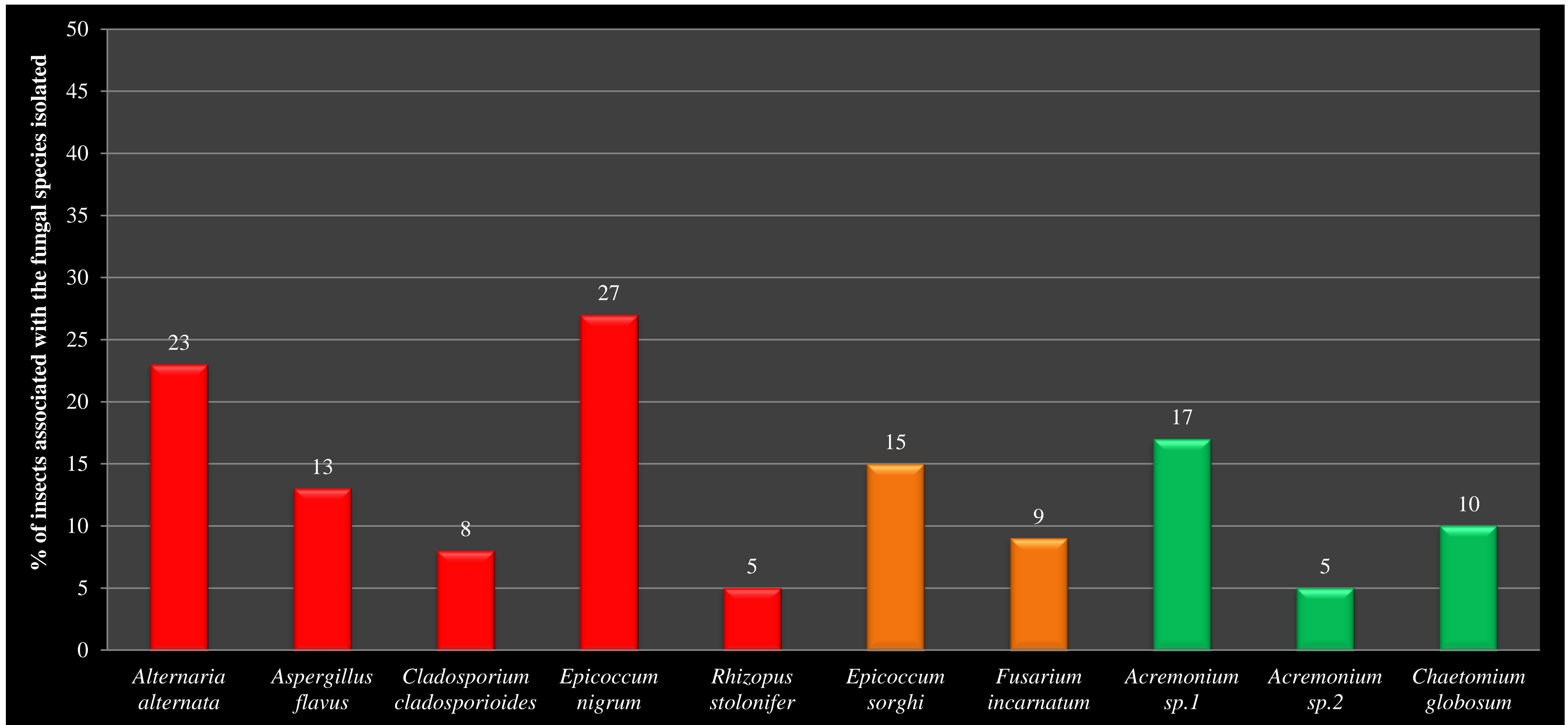

Figure 2.10: The percentage of insects associated with the most abundant fungal species isolated from *A. raptoria* collected on pistachio trees at Green valley Nuts near Prieska in the Northern Cape Province during April 2009 (Red bars - Pathogens, Orange bars - Potential pathogens & Green bars - Non-pathogenic fungi).

Table 2.2: Fungal species isolated from *C. nervosa* and *E. citricola* collected on walnut trees at Rotondo Walnuts near Aliwal North in the Free-State Province during October 2009 and February 2012.

Fungal Species Isolated:	**Percentage:**		**Disease:**
	C. nervosa	***E. citricola***	
Pathogens:			
Alternaria alternata	29	1	Alternaria leaf spot
Fusarium oxysporum	4	17	Fusarium canker
Potential Pathogens & Contaminants:			
Alternaria tenuissima	0	4	N/A
Aspergillus flavus	3	10	N/A
Aspergillus niger	3	2	N/A
Byssochlamys spectabilis	7	0	N/A
Cladosporium cladosporioides	1	22	N/A
Cochliobolus sativus	2	0	N/A
Cunninghamella echinulata	13	0	N/A
Epicoccum nigrum	8	2	N/A
Epicoccum sorghi	1	3	N/A
Fusarium incarnatum	4	14	N/A
Fusarium laceratum	0	9	N/A
Khuskia oryzae	5	0	N/A
Mucor plumbeus	1	4	N/A
Penicillium chrysogenum	0	12	N/A
Penicillium janthinellum	0	3	N/A
Penicillium veriabile	0	4	N/A
Pestalotiopsis sp.	0	2	N/A
Phoma glomerata	2	3	N/A
Non-Pathogenic Fungi:			
Acremonium strictum	1	5	N/A
Chaetomium funicola	8	0	N/A
Chaetomium globosum	30	0	N/A
Gonatobotrys simplex	1	0	N/A
Sordaria fimicola	3	0	N/A
Other:			
Bacteria	22	3	N/A
None	0	10	N/A

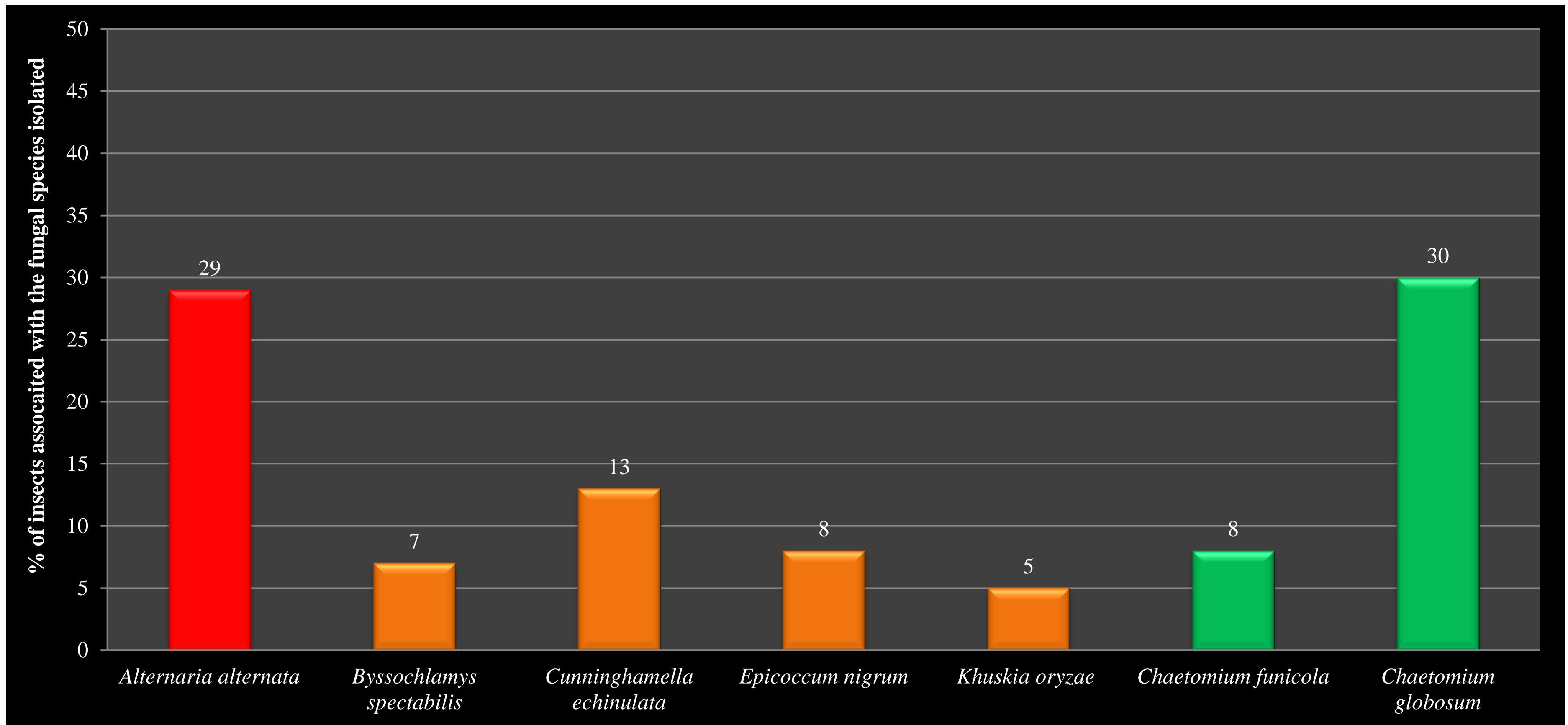

Figure 2.11: The percentage of insects associated with the most abundant fungal species isolated from *C. nervosa* collected on walnut trees at Rotondo Walnuts near Aliwal North in the Free-State Province during October 2009 (Red bars - Pathogens, Orange bars - Potential pathogens & Green bars - Non-pathogenic fungi).

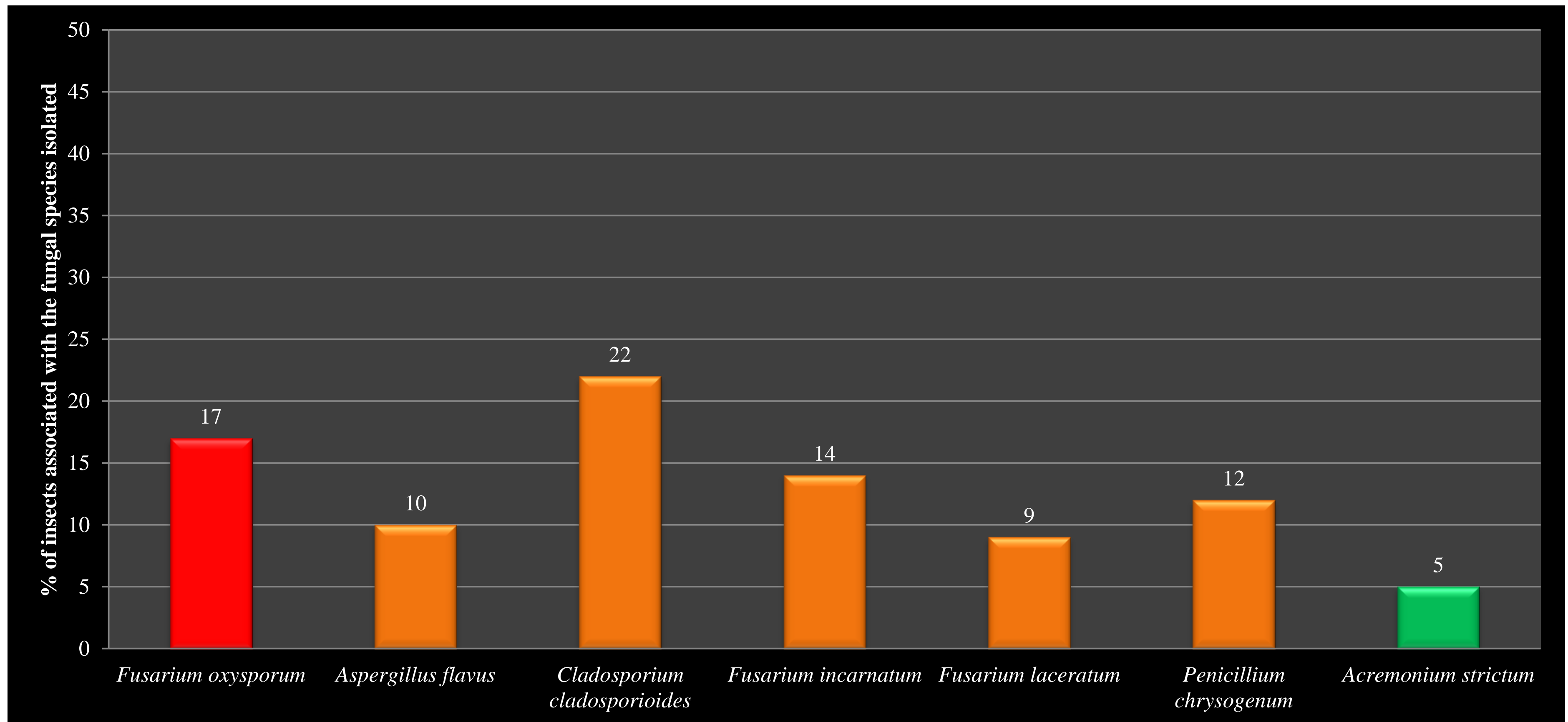

Figure 2.12: The percentage of insects associated with the most abundant fungal species isolated from *E. citricola* collected on walnut trees at Rotondo Walnuts near Aliwal North in the Free-State Province during February 2012 (Red bars - Pathogens, Orange bars - Potential pathogens & Green bars - Non-pathogenic fungi).

Chapter 3

Fungi Associated with Insects Occurring in Pecan Orchards in South Africa

3.1 Introduction

The pecan (*Carya illinoensis*) is a tree nut crop indigenous to North America, where it grows wild in the states along the Gulf of Mexico and around the Great Lakes (Oosthuizen, 1991; De Villiers & Joubert, 2008). Pecans belong to the same plant family as the Persian walnut *viz*. Juglandaceae (Hickory family) (De Villiers & Joubert, 2008). Several species of hickory (the genus *Carya*) yield edible nuts, but *C. illinoensis* is responsible for the pecan cultivars that today support a sizable industry worldwide (Janick, 2002). Pecans were imported to South-Africa during the late 1800's and the first grafted trees were imported in 1912 (Oosthuizen, 1991; De Villiers & Joubert, 2008). Initial plantings were made in sub-tropical regions around Nelspruit (Oosthuizen, 1991; De Villiers & Joubert, 2008). Due to the high rainfall and humidity, diseases are a major problem and only resistant and tolerant pecan cultivars can be planted (De Villiers & Joubert, 2008). Pecan production has since then expanded to other regions in South Africa, mostly the Northern Cape Province that has long hot summers, short cool winters and low rainfall and humidity. Pecan production areas in the Northern Cape Province include Prieska (A-grade), Hopetown (A-grade), Upington (A-grade) and the Vaalharts-irrigation scheme (B-grade). Currently, the Vaalharts irrigation scheme in the Northern Cape Province of South-Africa is the largest production area in the country. Overall, pecans adapt well in any part of the country with short cool winters and long hot summers with low rainfall and humidity (De Villiers & Joubert, 2008).

Since the establishment of pecans in South Africa the production of this crop has greatly increased (De Villiers & Joubert, 2008). Together with the increased cultivation of the crop the effect of pests and diseases on the production of pecans has become a major concern. Insect pests feed on, and damage, the pecan trees and nuts, which adversely affect production. Furthermore, insect pests occurring in pecan orchards can serve as vectors for the spread of fungal phytopathogens that may cause a decrease in potential yields. Although some case studies have focused on insect-fungal interactions, the role of insects in the spread of fungal phytopathogens has generally been neglected in the past; even though they are considered the most important vectors of fungi, and more specifically phytopathogens (Agrios, 2005). Little knowledge exists regarding the role that insects play in the spread and occurrence of disease in pecan orchards. Recently, research conducted by Alvidrez-Villarreal *et al*. (2012) in Mexican pecan orchards

illustrated that the trunk and branch borer, *Euplatypus segnis* (Coleoptera: Curculionidae: Platypodinae), is associated with several fungal species nl. *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata* and *Lasiodiplodia theobromae*, that are able to cause die back of pecan trees. They concluded that feeding of insects, in combination with fungal invasion, eventually results in the death of trees and that *E. segnis* contributes to the spread of fungi in pecan orchards (Alvidrez-Villarreal *et al*., 2012). No research has been conducted in South African pecan orchards regarding the associations between fungal phytopathogens and insects.

The primary aim of the study was to investigate whether insect species occurring in pecan orchards are associated with fungal phytopathogens. The study also aimed to compare the fungi associated with insects from two different environments such as an orchard in the Northern Cape Province (Prieska) (semi-arid region) to the fungi associated with insects occurring in a pecan orchard in the Kwa-Zulu Natal Province (Van Reenens) (subtropical region). The fungi associated with two insect species occurring in the pecan orchard at Van Reenens, at two different time intervals, were also compared. This was done to determine whether fungi associated with insects in the beginning of the season differs from the fungi associated with insects towards the end of the season.

3.2 Materials and Methods

3.2.1 Study sites and insect specimen collection

Two study sites were selected to investigate whether insects occurring in pecan (*Carya illinoensis*) orchards are associated with fungal phytopathogens. The first site was Oranje Landgoed near Prieska in the Northern Cape Province (S 29º 34.72’, E 22º 51.72’) and the second, Excelsior farm near Van Reenen’s Pass in the Kwa-Zulu Natal Province (S 28º 26.750’, E 29º 31.382’). At Oranje Landgoed 100 *Empoasca* sp. (Hemiptera: Cicadellidae) individuals were collected during March 2009 on trees between the ages of 10 and 12 years (Fig. 3.1). Leafhoppers were abundant in the pecan orchards at Oranje Landgoed, and it was observed that feeding damage resulted in the stunted growth of the pecan leaves (Fig. 3.2). This was likely a result of the blocking of the phloem by saliva injected by the leafhoppers during feeding. At Excelsior near Van Reenen’s Pass 100 *Sciobius cf. granosus* Fahraeus (Coleoptera: Curculionidae) and 100 *Panafrolepta dahlmani* (Jacoby) (Coleoptera: Chrysomelidae)

individuals were collected during March 2010 and December 2010, respectively. Specimens of both species were collected in the same orchard on trees between the ages of 28-30 years (Fig. 3.3). During the collections, it was observed that *S. cf. granosus* (citrus snouted weevil) fed on the pecan leaves, which resulted in typical punch-hole damage to the leaves (Figs. 3.4 & 3.5). It was also observed that several of the pecan nuts were heavily infected with fungal phytopathogens (Fig. 3.6). At both sites specimens were collected by actively searching trees and capturing individuals by hand. Each individual was placed in a sterile 1.5 mℓ Eppendorf™ tube to prevent cross contamination between specimens. Upon capture, the specimens were placed in a cooler box to preserve the integrity of the samples. The specimens were then placed in a freezer at -20 ˚C for 5 minutes. The identity of *Panafrolepta dahlmani* was confirmed by the Biosystematics Division of the Agricultural Research Council (ARC), while *Sciobius cf. granosus* and the *Empoasca* sp. was identified by the Department of Zoology and Entomology, University of the Free State.

3.2.2 Isolation of fungal colonies from insect specimens

Potato dextrose agar (PDA, Biolab) was used to isolate the fungi associated with the insects collected in the pecan orchards. The medium was prepared according to the manufacturer's specifications and autoclaved. In total, 0.3 mℓ of BioStrep™ (Streptomycin Sulphate) was added to each litre of medium after cooling to 55 ˚C. The euthanized insects were individually plated, mouthparts and legs facing downwards, on the PDA plates. The plates were then placed in an incubator at 25 ˚C for seven days to allow fungal development.

The plates were microscopically examined and fungal colonies were subcultured from the insects. The fungi were plated on corn meal agar (CMA, Difco) for identification purposes. This medium is low in nutrients and supports the slow reproductive growth of fungal colonies which makes it ideal for the identification of fungi. The CMA plates were prepared according to the manufacturer's specifications by using 17 g CMA per litre distilled water. Re-isolation of fungal colonies was done by aseptically removing a piece of agar (0.5x0.5mm) from each fungal colony on the PDA plates and subculturing it on a CMA plate. The agar pieces were cut from the edges of the fungal colonies to obtain vigorous growing hyphae and to limit contamination. The inoculated CMA plates were then placed in the incubator at 25 ºC for seven days, after which

they were placed under UV lights with 12-hour dark/UV cycles for an additional seven days (See chapter 2).

3.2.3 Identification of fungal colonies

Fungi were identified based on their morphological characteristics such as fruiting structures, spore shape and size, as well as colony texture and colour. Several literature sources were used as aids in the identification of fungal colonies (see references for the literature used during identification). Light and stereo microscopy was used to examine the fungal colonies and fruiting structures, and an attempt was made to identify all the fungi isolated to species level. Relevant information observed was also noted, for example the difference in spore shape, different colony forms or the diversity of colour of colonies in members of the same fungal genus. To aid isolates that had difficulties in producing fruiting structures and spores, sterilised pine needles were added to the colonies. The pine needles were twice autoclaved for 40 min at 100 kPa steam pressure (121 ˚C) at 24 hour intervals. Yeasts that were isolated were identified by the department of Microbiology at the University of the Free State using sequencing techniques. The identification of *Penicillium* and *Fusarium* species were done as described in detail in chapter 2.

3.2.4 Statistical Analysis

The percentage of insect specimens associated with each fungal species isolated from the insects was calculated using Microsoft Excel. The fungi were grouped by known pathogens of pecan, potential pathogens and/or contaminants and non-pathogenic fungal species that apparently pose no threat to pecan production. The potential pathogens and contaminants were selected based on the nature of the group and/or their known pathogenicity towards other agricultural crops, or relations with pathogens that can cause disease on pecan. The occurrence of the most abundant fungal species (5% or higher incidence) was plotted on a bar chart. Based on the number of pathogens and potential pathogens isolated from insects it was assessed whether the insects played a role in harbouring (and the possible dissemination) of fungal pathogens in the pecan orchards. To analyze the similarities of species assemblages between different samples, the *Sørenson's quantitative similarity index* was used (Junninen *et al*., 2006; Osono,

2008). The Sørenson's (Dice Coefficient) index was determined using the statistical program PAST (Hammer, Harper & Ryan, 2001).

3.3 Results and Discussion

A total of 17 species from 12 fungal genera were isolated from the *Empoasca* sp. individuals collected at Oranje Landgoed. A total of 22 species from 17 fungal genera were isolated from the *S. cf. granosus* individuals and a total of 25 species, representing 21 genera, were isolated from *P. dahlmani* collected on pecan trees at Excelsior farm (Table 3.1). In total, 42 species from 27 genera were isolated from all three species of insects. These were represented by 6 species that are pathogenic, 25 species that can be considered potentially pathogenic or as contaminants, and 11 species that are non-pathogenic on pecan. Pathogens isolated included *Alternaria alternata* (causing fungal leaf spot & die-back), *A. tenuissima* (fungal leaf spot), *Aureobasidium pullulans* (stigmatomycosis), *Botriosphaeria ribis* (water stage nut drop & stem end blight), *Fusarium oxysporum* (die-back) and *Trichothecium roseum* (pink rot) (Michailides, Morgan, & Doster, 1995; Anon., 2006; Alvidrez-Villarreal *et al*., 2012). Potential pathogens and contaminants isolated included *Aspergillus flavus*, *A. japonicus*, *A. niger*, *Byssochlamys spectabilis, Cladosporium cladosporioides*, *Cochliobolus sativus*, *C. spicifer*, *Crysonilia sitophila*, *Cunninghamella echinulata*, *Curvularia clavata*, *Epicoccum nigrum*, *E. sorghi*, *Fusarium equiseti*, *F. incarnatum*, *Khuskia oryzae*, *Macrophomopsis* sp., *Mucor circinelloides*, *M. hiemalis*, *M. plumbeus*, *Penicillium brevicompactum*, *P. chrysogenum*, *Pestalotiopsis* sp., *Phoma glomerata*, *Rhizopus oryzae* and *R. stolonifer*. The non-pathogenic fungi isolated included *Acremonium* spp., *Acremonium strictum*, basidiomycetous spp., *Chaetomium globosum*, *C. funicola*, *Cryptococcus magnus*, *C. roseus*, *Isaria farinosa*, *Rhodotorula glutinis*, *Trichoderma koningii* and *T. viride*. In addition, no microorganisms were isolated from 2% of the *Empoasca* sp. specimens and from 4% of *P. dahlmani*. Unknown bacteria were isolated from 7% of the *S. cf. granosus* specimens and from 4% of *P. dahlmani* (Table 3.1). In total, 6 of the fungal species isolated from the *Empoasca* sp. specimens were the most abundant, including 2 pathogenic-, 2 potential pathogenic-, and 2 non-pathogenic species (Fig. 3.7). In total, 9 of the fungal species isolated from the *S. cf. granosus* specimens were the most abundant (Fig. 3.8), including 3 pathogenic- and 6 potential pathogenic species. From the *P. dahlmani* specimens 6

fungal species were the most abundant (Fig. 3.9), that included 2 pathogenic-, 2 potential pathogenic-, and 2 non-pathogenic species.

3.3.1 Most abundant fungal species isolated from *Empoasca* sp.

The most commonly isolated pathogenic fungus was *Alternaria alternata* (Fig. 3.7) that was isolated from 38% of the *Empoasca* specimens. In addition, *A. tenuissima* was isolated from 9% of the leafhoppers. *Alternaria* includes cosmopolitan dematiaceous fungi commonly isolated from plants, soil, food and indoor air environments (Ellis, 1971). *Alternaria alternata* is the most common species within the group and it has been reported to cause disease on many different economically important crops (Agrios, 2006). *Alternaria* spp. are considered miscellaneous pathogens on pecans that can cause fungal leaf scorch (Anon., 2006). The disease causes premature defoliation of trees in the fall. Infected leaves turn to reddish-brown and the infection occurs along the leaf margin or at the tips of leaves, causing the infected area to roll upwards. With age, the reddish-brown areas become a dull brown with small black spots scattered over the lesions (Anon., 2006). Recent research done by Alvidrez-Villarreal *et al*. (2012) showed that *A. alternata* can cause the die-back of pecan branches. They found that the presence of insects in combination with fungal invasion eventually results in the death of trees, and that the ambrosia trunk and branch borer, *Euplatypus segnis* (Coleoptera: Curculionidae: Platypodinae) contributes to the spread of fungi in pecan orchards (Alvidrez-Villarreal *et al*., 2012). Furthermore, *Alternaria* spp. are known to produce toxic secondary metabolites that include over 70 compounds of varying toxicity (Pryor, 2003). Some of these metabolites are harmful mycotoxins with mutagenic and teratogenic properties, and have been linked to certain forms of cancer. Subsequently, the occurrence of secondary metabolites of *Alternaria* spp. in foodstuffs is becoming an increasing environmental concern (Pryor, 2003).

The second most common potential pathogen and/or contaminant isolated was *Aspergillus flavus* (Fig. 3.7) that was isolated from 6% of the *Empoasca* specimens. *Aspergillus flavus* is a filamentous, cosmopolitan and ubiquitous species which is found in most environments (Domsch, Gams & Anderson, 2007). This fungus and other members of *Aspergillus* are commonly isolated from soil, plant debris and indoor air environments (Domsch *et al*., 2007). *Aspergillus flavus* has been implicated in the contamination of peanuts and nuts.

This fungus is not classified as a pathogen of pecan, but it may be involved in postharvest spoilage. Consequently, in addition to the production of mycotoxins, *A. flavus* has the potential to cause economic losses in pecan production.

The most commonly isolated potential pathogen and/or contaminant was *Cladosporium cladosporioides*, which was isolated from 34% of the *Empoasca* sp. specimens (Fig. 3.7). *Cladosporium cladosporioides* is a dematiaceous fungus that is widely distributed in air and rotten organic material and frequently isolated as a contaminant on foodstuffs (Domsch *et al.*, 2007). Due to the abundance of *C. cladosporioides* it has been suspected to be pathogenic on pecans in South Africa, however, no evidence exists supporting these speculations. This fungus is not considered a pathogen of pecan but it might be involved in the postharvest spoilage of stored nuts.

The most common non-pathogenic fungus isolated was *Acremonium strictum* (Fig. 3.7). This species was isolated from 5% of the *Empoasca* specimens. Another unknown *Acremonium* species was isolated from 5% of the leafhopper specimens. The conidia of *Acremonium* are mostly aggregated in slimy heads at the apex of each phialide. This makes the spores especially easy to be disseminated by insects, but has never been classified as a pathogen of pecan (Domsch *et al.*, 2007).

3.3.2 Most abundant fungal species isolated from *S. cf. granosus*

The second most common pathogenic fungus isolated was *Alternaria alternata* (Fig. 3.8). This fungus was isolated from 35% of the *S. cf. granosus* specimens. Additionally, *A. tenuissima* was isolated from 7% of the weevil specimens, and has the capability to cause branch die-back on pecan trees (Alvidrez-Villarreal *et al.*, 2012).

The most common pathogenic fungus isolated was *Trichothecium roseum*, which was isolated from 38% of the *S. cf. granosus* specimens (Fig. 3.8). *Trichothecium roseum* is a filamentous mitosporic fungus with a world-wide distribution and is often isolated from decaying plant substrates, soil, seeds of corn and food-stuffs (Domsch *et al.*, 2007). The fungus is a known pathogen of pecans and is commonly referred to as pink mold (Anon., 2006). This pathogen usually occurs on nuts infected with the scab fungus (*Fusicladium effusum*). The pink mold

fungus apparently enters nuts through scab lesions on shucks and continues to produce masses of pink spores on the shuck surfaces until late fall. The fungus sometimes invades the kernel of thin shelled varieties of pecan, causing "pink rot" which is characterized by an oily appearance of the nut shell and a rancid odour. Pink mold rarely occurs on the shucks of nuts in the absence of scab disease. If scab is controlled, pink mold is usually not a problem on pecans. Despite this, if the pathogen is disseminated by insects while feeding on the nuts, it might gain entry into the husks and cause disease without the presence of pecan scab (Anon., 2006).

Aspergillus flavus was isolated from 6% of the *S. cf. granosus* specimens (Fig. 3.8). As previously mentioned, *A. flavus* is not known as a pathogen of pecan, but could be associated with post-harvest spoilage

Cochliobolus sativus was isolated from 6% of the *S. cf. granosus* specimens (Fig. 3.8), a fungus that is wide spread and occurs on numerous plant species and in soil (Domsch *et al*., 2007). It is saprophytic in nature and can cause disease on various crops. *Cochliobolus sativus* is associated with cereals and not regarded as a pathogen of pecans but may be considered a potential pathogen (Domsch *et al*., 2007).

Curvularia clavata was isolated from 5% of the *S. cf. granosus* specimens (Fig. 3.8). *Curvularia* spp. are described as dematiaceous filamentous fungi (Ellis, 1971). Most species of *Curvularia* are facultative pathogens occurring in soil, plants and cereals in tropical or subtropical areas. *Curvularia* contains several species, including *C. brachyspora*, *C. clavata*, *C. geniculata*, *C. lunata*, *C. pallescens*, *C. senegalensis* and *C. verruculosa*. Members of *Curvularia* are known to cause leaf spots on several tree species (Ellis, 1971), however, it is unknown whether these fungi are pathogenic to pecans.

The second most common potential pathogen and/or contaminant isolated was *Epicoccum nigrum* (Fig. 3.8). This fungus was isolated from 7% of the *S. cf. granosus* specimens. *Epicoccum nigrum* is a cosmopolitan, dematiaceous fungus that is primarily isolated from plants and soil (Domsch *et al*., 2007). *Epicoccum nigrum* is not considered a pathogen of pecan but may invade damaged plant parts and cause postharvest decay of nuts.

The most common potential pathogen and/or contaminant isolated was *Mucor hiemalis* that was isolated from 18% of the *S. cf. granosus* specimens (Fig. 3.8). Additionally, *M.*

circinelloides was isolated from 6% of the weevil specimens. The genus, *Mucor,* contains several species of which *M. amphibiorum*, *M. circinelloides*, *M. hiemalis*, *M. indicus*, *M. racemosus*, and *M. ramosissimus* are most common (Domsch *et al*., 2007). Members of *Mucor* are often considered to be food spoilage fungi that could contribute to postharvest decay of pecan nuts (Domsch *et al*., 2007).

3.3.3 Most abundant fungal species isolated from *P. dahlmani*

The most commonly isolated pathogenic fungus was *Alternaria alternata* that was isolated from 10% of the *P. dahlmani* specimens (Fig. 3.9). Members of *Alternaria* include miscellaneous pathogens that can cause fungal leaf scorch (Anon., 2006). *Alternaria alternata* has the capability to cause branch die-back on pecan trees (Alvidrez-Villarreal *et al*., 2012).

The second most common pathogen isolated was *Fusarium oxysporum* that was isolated from 6% of the *P. dahlmani* specimens (Fig. 3.9). *Fusarium oxysporum* is known to be a highly opportunistic and destructive plant pathogen (Domsch *et al*., 2007). This species causes die-back on pecan trees (Alvidrez-Villarreal *et al*., 2012). It is suspected that the ambrosia trunk and branch borer (*E. segnis)* can spread this fungus and provide suitable entry points on pecan trees. Die back is considered an important disease on pecan (Alvidrez-Villarreal *et al*., 2012).

The most common potential pathogen and/or contaminant isolated was *Fusarium equiseti* (Fig. 3.9). This fungus was isolated from 25% of the *P. dahlmani* specimens, and *F. incarnatum* was isolated from 5% of the leaf beetle specimens. *Fusarium* spp. are filamentous and widely distributed on plants and in the soil (Anon., 2007). *Fusarium* spp. are well known as being either common contaminants or plant pathogens. Furthermore, *Fusarium* spp. can also be mycotoxigenic (Domsch *et al*., 2007). There are no indications that *F. equiseti* and *F. laceratum* are involved in causing disease on pecan, although due to their nature they can be considered as potential pathogens and/or contaminants of pecan.

The second most common non-pathogenic fungus isolated from the *P. dahlmani* specimens was an *Acremonium* sp., which was isolated from 12% of the *P. dahlmani* specimens (Fig. 3.9). Due to their saprophytic nature, *Acremonium* species cannot be considered pathogens of pecan.

Isaria farinosa was the most abundant non-pathogenic fungus isolated from the *P. dahlmani* specimens (Fig. 3.9). This fungus was isolated from 19% of the *P. dahlmani* specimens. It has a world-wide distribution occurring in both temperate and tropical zones. It is commonly isolated from forest soils and wood, but probably originated from insects in these habitats (Domsch *et al.,* 2007). *Isaria farinosa* was previously known as *Paecilomyces farinosus* and recently transferred to the genus *Isaria* (Zimmermann, 2008). The species *I. farinosa*, is a well-known entomopathogenic fungus with a wide host range (Demirci *et al*., 2011). This fungus has been recorded on a wide range of insect hosts, including Lepidoptera, Diptera, Hemiptera, Coleoptera, Hymenoptera, and Arachnida. It successfully controls several agricultural pests. *Isaria farinosa* is not considered to be a pathogen of pecan, but as a beneficial organism that can reduce the numbers of insect pests in pecan orchards (Demirci *et al*., 2011).

The results show that a large variety of fungal species are associated with the insect specimens collected on pecan. These included several pathogens, potential pathogens and contaminants, and non-pathogenic fungi. The most abundant pathogens isolated were *Alternaria alternata*, *Fusarium oxysporum* and *Trichothecium roseum*. This suggests that all three insect species have the capabilities to be involved with harbouring and possibly the dissemination of fungal pathogens within pecan orchards. The largest proportion of the fungi isolated from *Empoasca* sp. were pathogenic, potentially pathogenic or contaminants. The two species of fungi most isolated from the *Empoasca* sp. specimens was *A. alternata* and *C. cladosporioides.* Both species were isolated from more than a third of the leafhopper specimens (Fig. 3.7). All the most abundant fungal species isolated from *S. cf. granosus* is pathogenic on pecan or potentially pathogenic. The two fungal species most frequently isolated were *A. alternata* and *T. roseum*, both are known pathogens of pecan (Fig. 3.8). In contrast to the other two samples the more abundant fungi isolated from *P. dahlmani* were only potentially pathogenic or non-pathogenic. The two fungal species most frequently isolated were *Fusarium equiseti* and *Isaria farinosa* (Fig. 3.9). There is a possibility that the pathogens were not present at the time of collection, since several pathogens were isolated from the *S. cf. granosus* specimens that were collected at a later period of the season. The presence of *T. roseum* on *S. cf. granosus* and the absence thereof on *P. dahlmani* reflects this, since *T. roseum* is a pathogen that attacks pecan nuts late in the season. Apart from the pathogens isolated, several of the other fungal species isolated may cause postharvest decay and produce harmful mycotoxins. The production of mycotoxins can

compromise the safety of the use of nuts for consumption. Fungal development in the nuts can also influence the palatability and aesthetic appearance of the nuts. The results also indicate that the insects are involved in the harbouring of positive microorganisms like the epiphytic yeasts (*Cryptococcus magnus*, *C. roseus* and *Rhodotorula glutinis*) and *I. farinosa* that were isolated (Table 3.1). The presence of these organisms may have a negative effect on disease development and pest populations within the pecan orchards. Overall, results indicate that insects occurring in pecan orchards can harbour and possibly disseminate pathogenic and potential pathogenic fungi. Insects likely form an important link within the epidemiology of fungal diseases within pecan production.

3.3.4 Sample comparisons and Sørenson quantitative similarity index

The Sørenson's index showed that the species assemblages between the fungi isolated from *Empoasca* sp. at Oranje Landgoed are different from those of *S. cf. gronsus* and *P. dahlmani* at Excelsior. The Sørenson's index value for *Empoasca* sp. *vs*. *S. cf. gronsus* was 0.47619 and for the *Empoasca* sp. *vs*. *P. dahlmani* 0.34783. When the Sørenson's index value is below 0.7 it indicates that the diversity differs from each other (Gardner *et al*., 2011). These results indicate that the fungi associated with insects occurring in a semi-arid region differ from the fungi associated with insects occurring in areas with more rain. As a result, insects occurring in different regions on the same crop might be involved in the dissemination of different fungal pathogens.

The Sørenson's index showed that the species assemblages between the fungi isolated from the *S. cf. gronsus* and *P. dahlmani* specimens collected in the same orchard at Excelsior at different periods in the season were different. The Sørenson's index value was 0.44. The results suggest that different fungal species are associated with insects occurring in the same orchard at different periods of the season. Therefore, certain insect species might disseminate certain fungal pathogens at certain periods. The selective control of these insect species at particular intervals might help reduce the spread and occurrence of disease at certain periods of the season.

3.4 Conclusions

A large variety of fungal and yeast species were isolated from the insect specimens collected on pecan. These include several known pathogens of the crop, potential pathogens and non-pathogenic species. In conclusion, insects occurring in pecan orchards play an important role in the dynamics of fungal populations in these orchards. Of importance is the role that these insects may play in the dissemination of pathogens. The most abundant fungal species isolated from *Empoasca* sp. and *S. cf. granosus* were pathogenic. These include *Alternaria alternata, A. tenuissima* and *Trichothecium roseum*. Pathogens were also isolated from *P. dahlmani*, which included *Alternaria alternata* and *Fusarium oxysporum*, however, a large proportion of the fungi isolated from these specimens were non-pathogenic. These include the well known entomopathogenic fungus *Isaria farinosa.* Certain fungal species were isolated from all three insect species that may cause postharvest decay and produce harmful mycotoxins that can cause significant losses. Insects occurring in different regions on the same crop might be involved in the dissemination of different fungal pathogens. Different fungal species are associated with insects occurring in the same orchard at different periods of the season and that certain insect species might disseminate certain fungal pathogens at certain periods. Overall, the study has shown that insects may play a role in the spread and occurrence of disease in South African pecan orchards.

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3.6 Tables & Figures

Figure 3.1: *Empoasca* sp. (Hemiptera: Cicadellidae) collected on pecans at Oranje Landgoed (Photo by V. R. Swart).

Figure 3.2: The stunted growth of pecan leaves, likely caused by the feeding activities of leafhoppers (Photo by V. R. Swart).

Figure 3.3: The Pecan orchard at Van Reenen's Pass in which the *Sciobius cf. granosus* and *Panafrolepta dahlmani* specimens were collected (Photo by V. R. Swart).

Figure 3.4: *Sciobius cf. granosus* (Coleoptera: Curculionidae) collected on pecan at Excelsior farm near Van Reenen's Pass (Photo by V. R. Swart).

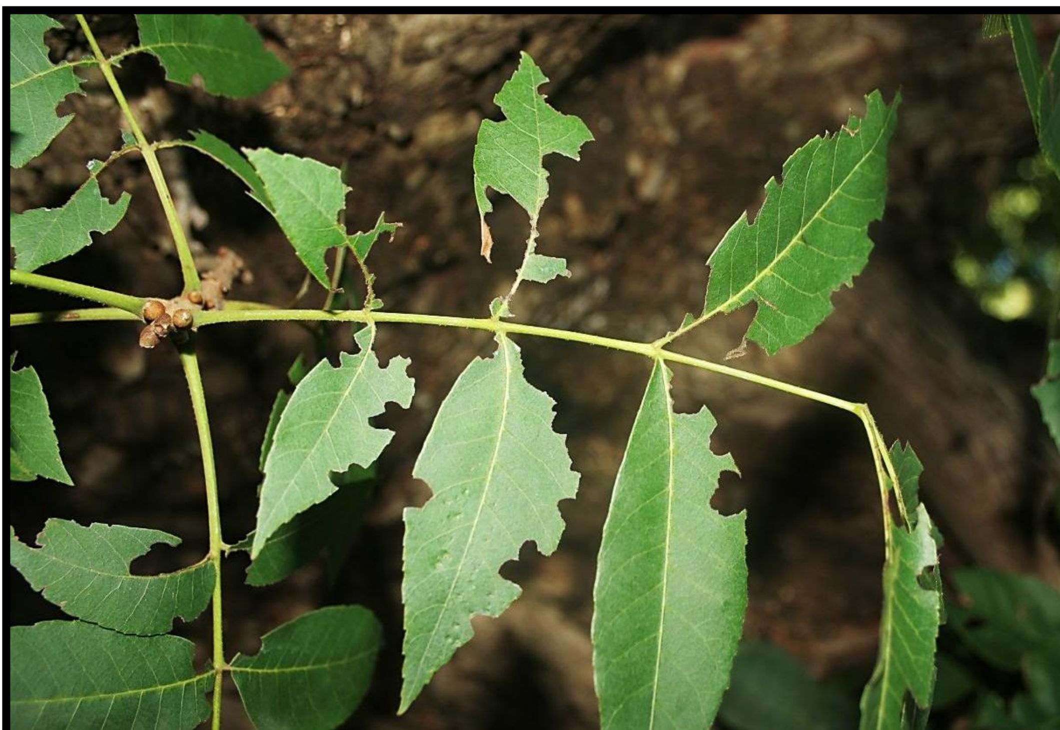

Figure 3.5: Punch-hole damage caused by the feeding activities of *Sciobius cf. granosus* to an individual pecan leaf (Photo by V. R. Swart).

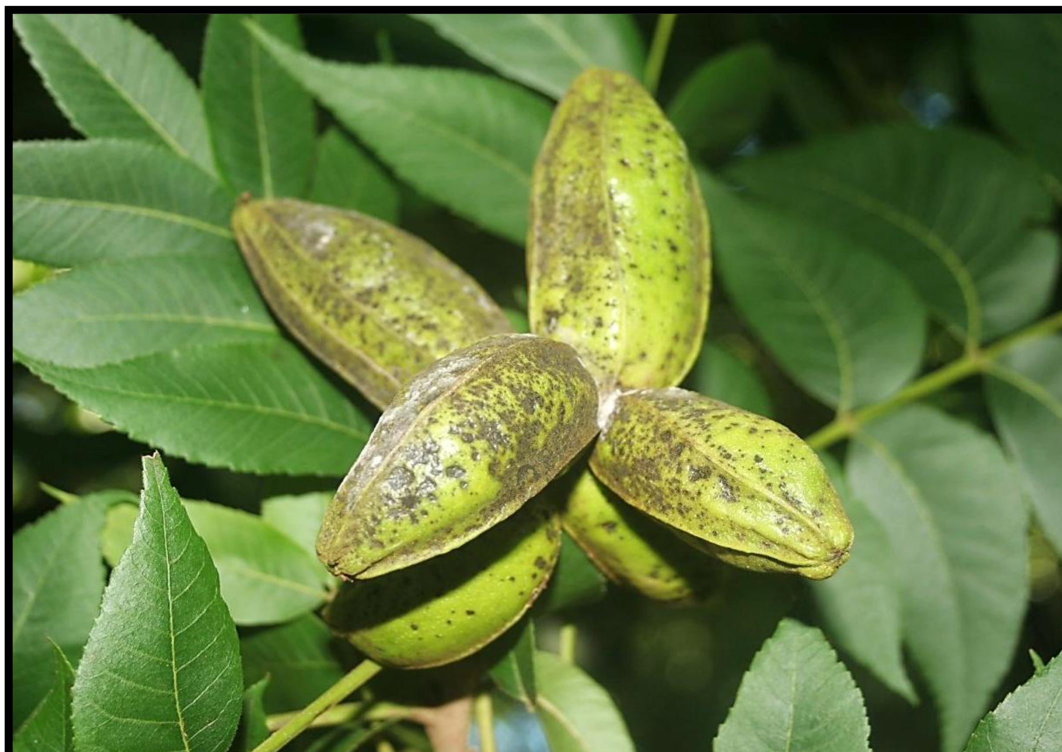

Figure 3.7: Pecan nuts infected with fungal phytopathogens, observed during the March 2010 specimen collection (Photo by V. R. Swart).

Table 3.1 (A): Fungal species isolated from *Empoasca* sp., *S. cf. granosus* and *P. dahlmani* collected on pecan trees.

Fungal Species Isolated:	Percentage			Disease:
	Empoasca sp.	*S. cf. granosus*	*P. dahlmani*	
Pathogens:				
Alternaria alternata	38	35	10	Fungal leaf spot
Alternaria tenuissima	9	7	0	Fungal leaf spot
Aureobasidium pullulans	0	0	2	Stigmatomycosis
Botryosphaeria ribis	0	1	0	Water stage nut drop & Stem end blight
Fusarium oxysporum	0	1	6	Die-back
Trichothecium roseum	0	38	0	Pink rot
Potentail Pathogens & Contaminants:				
Aspergillus flavus	6	6	2	N/A
Aspergillus japonicus	0	0	1	N/A
Aspergillus niger	4	1	0	N/A
Byssochlamys spectabilis	0	0	2	N/A
Cladosporium cladosporioides	34	3	3	N/A
Cochliobolus sativus	1	6	0	N/A
Cochliobolus spicifer	0	0	1	N/A
Crysonilia sitophila	0	2	0	N/A
Cunninghamella echinulata	0	0	1	N/A
Curvularia clavata	1	5	1	N/A
Epicoccum nigrum	1	7	1	N/A
Epicoccum sorghi	3	4	3	N/A
Fusarium equiseti	0	0	25	N/A
Fusarium incarnatum	0	0	5	N/A
Khuskia oryzae	0	1	4	N/A
Macrophomopsis sp.	0	0	2	N/A
Mucor circinelloides	0	6	0	N/A
Mucor hiemalis	1	18	0	N/A
Mucor plumbeus	0	3	0	N/A
Penicillium brevicompactum	0	1	3	N/A
Penicillium chrysogenum	1	0	0	N/A
Pestalotiopsis sp.	0	0	2	N/A
Phoma glomerata	2	0	3	N/A
Rhizopus oryzae	0	0	3	N/A
Rhizopus stolonifer	0	1	0	N/A

Table 3.1 (B): Fungal species isolated from *Empoasca sp., S. cf. granosus* and *P. dahlmani* collected on pecan trees.

Fungal Species Isolated:	**Percentage**			**Disease:**
	Empoasca sp.	***S. cf. granosus***	***P. dahlmani***	
Non-Pathogenic Fungi:				
Acremonium spp.	5	0	12	N/A
Acremonium strictum	5	0	0	N/A
Basidiomycetous spp.	0	1	4	N/A
Chaetomium globosum	0	1	1	N/A
Chaetomium funicola	1	0	0	N/A
Cryptococcus magnus	2	0	0	N/A
Cryptococcus roseus	1	0	0	N/A
Isaria farinosa	0	0	19	N/A
Rhodotorula glutinis	2	0	0	N/A
Trichoderma koningii	0	1	0	N/A
Trichoderma viride	0	0	1	N/A
Other:				
Bacteria	0	7	4	N/A
None	2	0	4	N/A

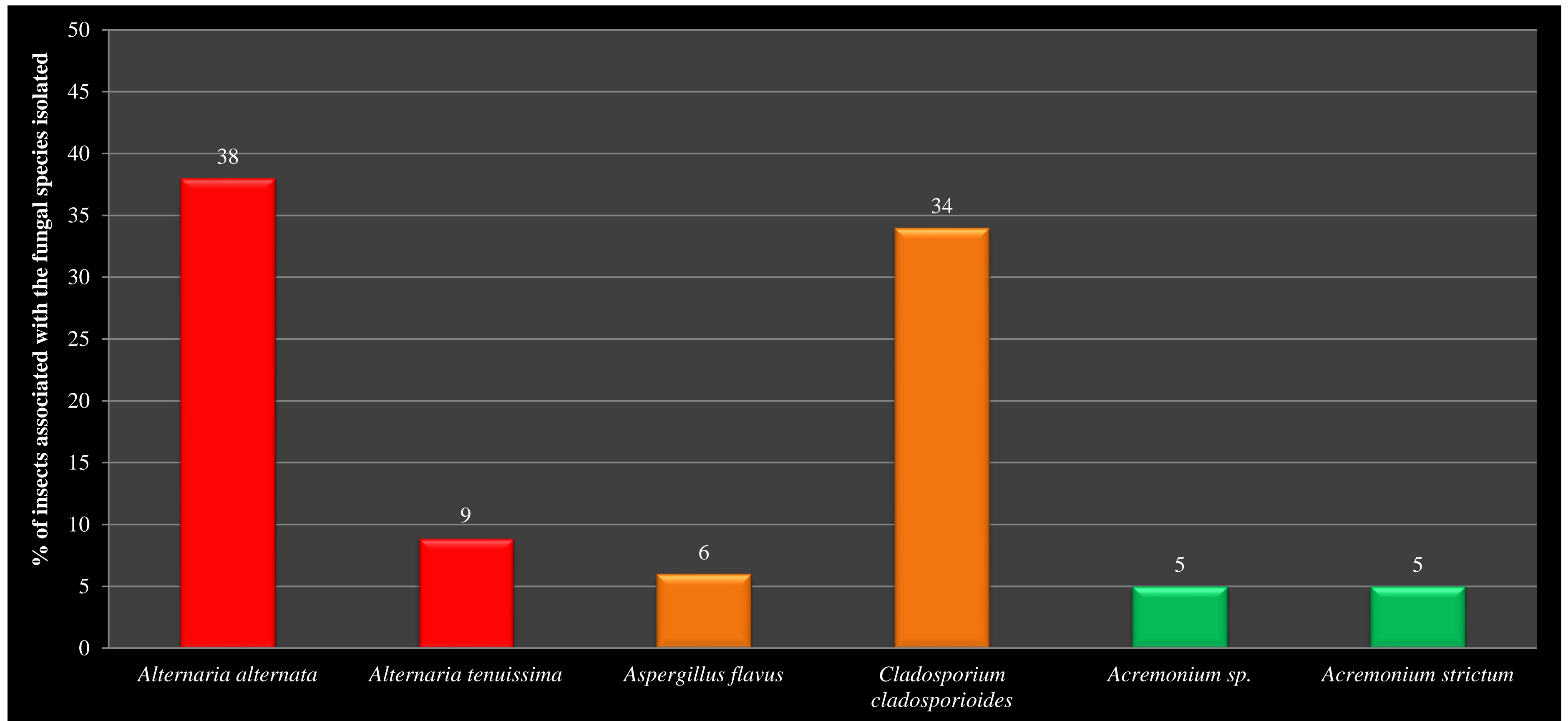

Figure 3.7: The percentage of insects associated with the most abundant fungal species isolated from *Empoasca* sp. collected on pecan trees at Oranje Landgoed near Prieska in the Northern Cape Province during March 2009 (Red bars - Pathogens, Orange bars - Potential pathogens & Green bars - Non-pathogenic fungi).

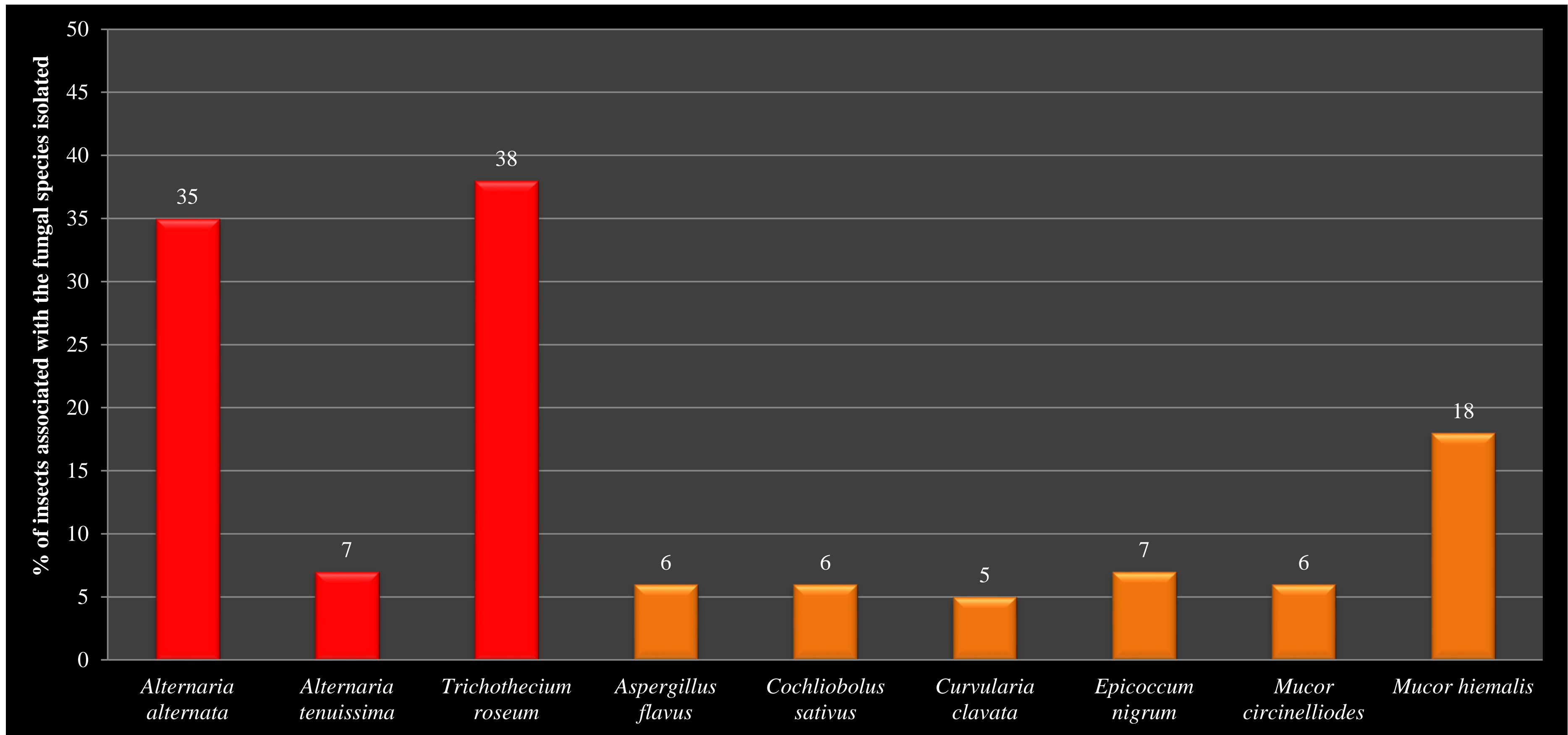

Figure 3.8: The percentage of insects associated with the most abundant fungal species isolated from *S. cf. granosus* collected on pecan trees at Excelsior farm near Van Reenen's Pass in the Kwa-Zulu Natal Province during March 2010 (Red bars - Pathogens, Orange bars - Potential pathogens & Green bars - Non-pathogenic fungi).

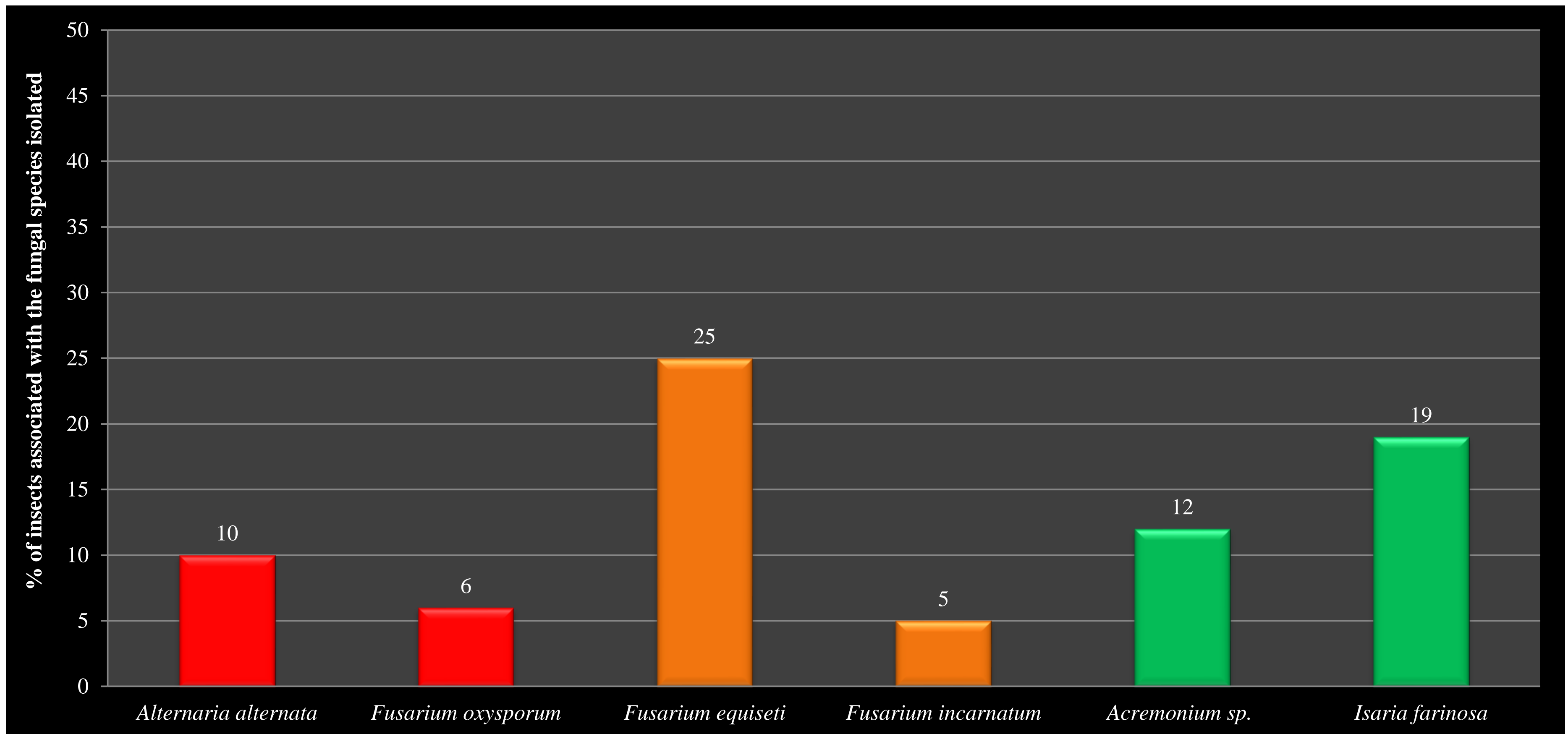

Figure 3.9: The percentage of insects associated with the most abundant fungal species isolated from *P. dahlmani* collected on pecan trees at Excelsior farm near Van Reenen's Pass in the Kwa-Zulu Natal Province during December 2010 (Red bars - Pathogens, Orange bars - Potential pathogens & Green bars - Non-pathogenic fungi).

Chapter 4

The Succession of Fungal Populations Associated with the Grey-Brown Stink Bug (*Coenomorpha nervosa*) in South African Pecan Orchards

4.1 Introduction

Stink bugs (Hemiptera: Pentatomidae) are a large group of insects of which several species are economically important pests of sub-tropical and tropical crops, such as pecans (Scholtz & Holm, 1985; Van den Berg, De Villiers & Joubert, 2001; Picker *et al*., 2002; Triplehorn & Johnson, 2005). Some species feed exclusively on these types of crops while others are polyphagous and feed on other important crops, such as vegetables. Stink bug feeding on small, developing fruits and nuts results in premature drop, while feeding on larger fruits and nuts results in blemishes and kernel necrosis. Stink bugs also feed on the shoots of trees, which may ultimately wither and die. Several stink bug species are recognized pests of pecan in South Africa. These include *Atelocera raptoria* Germar (woolly stink bug), *Bathycoelia natalicola* Distant (two-spotted stink bug), *B. rodhaini* Distant (yellow-spotted stink bug), *Farnya* sp. (variegated stink bug), *Nezara prunasis* Dallas (small green stink bug) and *N. viridula* (L.) (green vegetable stink bug). *Coenomorpha nervosa* Dallas (grey-brown stink bug) is not a recognized pest of pecans, but is an important pest of sub-tropical crops such as macadamia and avocado in South Africa (Van den Berg *et al*., 2001). The grey-brown stink bug has been observed to be the most abundant stink bug species occurring in pecan orchards in the Vaalharts district in North-West Province of South Africa[*]. This insect most likely forms part of a stink bug-complex that is responsible for stink bug damage to pecan nuts in this region).

Apart from their pest status, stink bugs may also be involved in the spread of diseases, which may reduce yields. They have been found to be involved in the dissemination of fungal phytopathogens on other tree nut crops such as pistachio (Michailides & Morgan, 1996, Steffan, Daane & Yokota, 2000). Research conducted by Michailides & Morgan (1996) indicated that an association exits between stink bugs from pistachio orchards and *Botryosphaeria dothidea,* a pathogen of pistachios that causes panicle shoot blight. Steffan *et al*. (2000) indicated that stink bugs assist in *B. dothidea* infection of pistachio nuts, providing germination sites for spores that are present on the nut and shoot surfaces that ultimately increase disease severity. Stink bugs are involved in the dissemination of both *Nematospora coryli* and *Aureobasidium pullulans* that are causative agents of stigmatomycosis in pistachios and other tree nut crops such as pecans (Michailides, Morgan & Doster, 1995). Stink bugs occurring in South African pecan orchards may play a similar role in the spread of fungal phytopathogens.

[*] Personal communication with Mr Vaughn Swart, Department of Zoology and Entomology, UFS.

The main aim of the study was to determine whether fungal phytopathogens of pecans are associated with *Coenomorpha nervosa* individuals from pecan orchards. The aim was also to determine the occurrence and incidence levels of fungal phytopathogens associated with stink bugs at different developmental stages of pecan nuts, and to compare the possible different sources (*e.g.*, soil, air, the phyllosphere, and stink bugs) of fungal phytopathogens.

4.2 Materials and Methods

4.2.1 Study site and sample collection

The succession of fungi associated with the grey-brown stink bug, *C. nervosa* Dallas (Hemiptera: Pentatomidae), was investigated on pecans in a single pecan orchard, farm 7M8 (S 27°38.475' E 24°44.794'), in the Vaalharts region (Northern Cape Province, South Africa) as a study site. The Vaalharts region is currently the largest production area for pecan nuts in South Africa (De Villiers & Joubert, 2008). The orchard contains trees ranging from 25-30 years of age, including cultivars such as Barton, Choctaw, Ukulinga, Wichita and Western Schley (Fig.4.1). Stink bug, air, soil, leaf and nut samples were collected on three dates [15 November 2011 (C1), 14 February 2012 (C2), 16 April 2012 (C3)]. The sampling dates represented the growing season for pecan nuts in South Africa, covering from the flowering stage until the pecan nuts are harvested (Fig. 4.2). Stink bugs were sampled from a single row of Western Schley, as this cultivar exhibited the most significant stink bug damage within the specific orchard. Air, soil, leaf and nut samples were randomly taken at ten selected trees (S1 - S10) that were spread out over the orchard. Trees were marked with a Garmin GPSmap 62s and plastic labels in order to collect material from the same trees on all three sampling occasions (Fig. 4.3).

Stink bug specimen collection: A total of 100 grey-brown stink bug specimens (*C. nervosa* Dallas) were collected during each sampling session by actively searching pecan trees and placing each individual stink bug into a sterile Polytop^{TM} (Figs. 4.4 & 4.5). During the second collection (C2) an additional ten stink bugs were collected in order to investigate the presence of fungal propagules on the exterior of the stink bugs with scanning electron microscopy. Upon capture, the specimens were placed into a cooler box to prevent fungal development and possibly influence the integrity of the samples. The stink bugs were placed in a

freezer at -20 ˚C for 5 minutes. The identity of the collected stink bug species was confirmed by the Biosystematics Division of the Agricultural Research Council (ARC).

Air sampling: Samples were taken with an AES Sampl'air air sampler in close proximity to the ten selected trees respectively within the orchard during all three sampling dates. Sampling commenced by running the air sampler for one and five minutes respectively to represent high and low loads of fungal propagules. Samples were taken with both malt salt agar (MSA) and potato dextrose agar (PDA). The Petri dishes containing MSA and PDA were prepared three days prior to sampling. The PDA medium is rich in nutrients and is ideal for the isolation of most of the fungal propagules occurring in the air, whereas MSA medium is ideal for the isolation of fungi that grow at low water activities. The AES air sampler was set up at each of the trees by elevating the vacuum pumps from the soil to prevent the intake of propagules originating from the soil (Fig. 4.6). The sampled Petri plates were then placed in an incubator at 25 ˚C for three days after which fungal colonies were counted and identified microscopically.

Soil sample collection: Samples were collected at the base of ten selected trees in the orchard during all three sampling dates. Soil was collected at two points around the trees using a small hand shovel. Approximately 100 g of soil was collected at each tree from the top 20 cm and placed in 50 mℓ sterile centrifuge tubes. The sampled tubes were transported in a cooler box to the UFS laboratories for fungal isolation and identification.

Leaf and nut sample collection: Leaf and nut samples were collected by randomly removing ten leaflets and ten nuts from the ten selected trees in the orchard. Leaf samples were collected during all three collection dates, whilst the nut samples were only collected on the second and third collection dates since the trees were flowering during the first collection. The leaflets and nuts were collected by physically removing them from the trees by hand. Collected leaves and nuts from each tree were placed separately in plastic bags that were labelled accordingly. Samples were transported to the UFS in a cooler box where the fungi associated with the leaves and nuts were isolated and identified.

4.2.2 Scanning electron microscopy of stink bug specimens

To confirm that *C. nervosa* harbours fungal propagules the exoskeleton of ten captured specimens were examined using scanning electron microscopy (SEM). The *C. nervosa* specimens were placed in a freezer at -20 ˚C for 5 minutes to kill the individuals. Once euthanized the specimens were fixed onto SEM stubs by means of plastic adhesives. The specimens were fixed so that the ventral side, especially the mouth parts, would be visible during observation, since the ventral parts are most in contact with the plant surfaces. Once fixed, the specimens were placed in a desiccator containing unsaturated Drierite ($CaSO_4$) crystals for 48 hours. The specimens were placed in a desiccator to remove moister that may adversely affect the conduction of electrons during the SEM process. The specimens were sputter coated with gold and examined using an SEM microscope. All body parts were examined for signs of fungal propagules.

4.2.3 Isolation of fungal colonies from samples

Isolation of fungi from stink bug specimens: Once the stink bugs were euthanized they were individually plated onto PDA plates, with their mouthparts and legs facing downwards. The inoculated PDA agar plates were then placed in an incubator at 25 ºC for seven days to allow the fungal propagules on the stink bugs to germinate and grow. The plates were then removed from the incubator and the fungal colonies isolated were identified.

Isolation of fungi from soil samples: In order to isolate the fungi from soil a modification of the standard dilution plate technique was used (Nesmith & Jenkins, 1979; Vargas Gil, Pastor & March, 2009; Gaddeyya *et al*., 2012). Soil samples were diluted in a range from 10^{-1} to 10^{-6}. In total, 10 g of the sample was transferred to 100 mℓ sterile distilled water. The suspension was mixed using a magnetic stirrer (VOSS Instruments Ltd.) at 700 rpm for 5 min. The suspension was diluted four times by transferring 1 mℓ of the original suspension to 9 mℓ of sterile water. The dilution was mixed again by vigorous shaking and 1 mℓ of the dilution was transferred to 9 mℓ of sterile water. The process was repeated until all four dilutions were completed. From the original suspension, aliquots of 1 mℓ and 0.1 mℓ were plated to both PDA and MSA plates, respectively. These represented the 10^{-1} and 10^{-2} dilutions. The Petri dishes were placed in an

incubator at 25 ºC for seven days and fungal colonies were counted and identified based on morphological characteristics.

Isolation of fungi from leaf and nut samples: The isolation of fungi from the leaves and nuts were done according to literature (Okigbo & Osuinde, 2003; Gangadevi & Muthumary, 2008; Osono, 2008; Motlagh, 2010) with some modification to the methods. Leaf and nut samples were initially washed in sterilised distilled water to remove dust and dirt. The samples were surface sterilized with 76% ethanol after which they were twice rinsed in sterile distilled water. The samples were allowed to air dry in a laminar flow hood for one hour. Five disks of approximately 1 x 1 cm were aseptically cut from each leaf and from the husk of each nut. The husks were used since it is known that most fungal pathogens infect the husks, *e.g. Fusicladium effusum* that causes scab (De Villiers & Joubert, 2008). The disks cut from each leaf and nut, were placed equidistantly on Petri plates containing PDA. The plates were placed in an incubator at 25 ºC for seven days to allow fungal development. Colonies were identified and counted microscopically.

4.2.4 Identification of fungal colonies

The fungal colonies isolated from the various samples taken were identified on the original plates based on their morphological characteristics such as fruiting structures, spore shape and size, as well as colony texture and colour. Literature sources were used as guidelines during the identification of fungal colonies (see references). Light and stereo microscopy was used to examine the fungal colonies and fruiting structures, and an attempt was made to identify all the fungi isolated to species level.

Representatives of both the fungal colonies that could not be identified on the original plates and the colonies that were identified were subcultured to plates containing PDA. The plates were then incubated at 25 ˚C for seven days. Those fungal isolates that did not sporulate were kept in the incubator for longer. To aid isolates that had difficulties in producing fruiting structures and spores, sterilized pine needles were added to the colonies. These were kept in the incubator at 25 ˚C and examined weekly until all the fungal colonies were identified. Other groups that provided problems with identification included *Penicillium* spp., coelomycetous fungi and *Fusarium* spp. Seven-day growth studies were used to identify *Penicillium* spp.,

whereas coelomycetous fungi and *Fusarium* spp. were identified to species level using molecular techniques.

Molecular identification of coelomycetous fungi and Fusarium *spp.*

Due to the difficulties of identifying coelomycetous fungi and *Fusarium* spp. morphologically, representative cultures of each morphotypical coelomycetous and *Fusarium* group were selected to be identified by means of sequencing. Single spore isolates, DNA extraction and PCR products were prepared in the same manner as described in detail in chapter 2. Extracted DNA was diluted to 5 ng/µl templates by adding calculated volumes DEPC water. For the coelomycetous fungi the ITS region of the rDNA operon was amplified using the primers ITS 1F (5´ CTT GGT CAT TTA GAG GAA GTAA 3´) and ITS 4 (5´ TCC TCC GCT TAT TGA TAT GC 3´). The ITS region, especially with ITS 1F and ITS 4 primers, has been used successfully in the identification of coelomycetous fungi and several other fungal groups (Barber, *et al*., 2005; Farr *et al.*, 2005; Szentiványi, *et al*., 2005; Dickie & FitzJohn, 2007; Manter & Vivanco, 2007). For the *Fusarium* spp. DNA segments from the Elongation Factor-1α region were amplified with the primers EF1 (5´-ATGGGTAAGARGACAAGAC-3´) and EF2 (5´-GGARGTACCAGTSATCATGTT-3´) (Kristensen *et al*., 2005; Alastruey-Izquierdo *et al*., 2008). The same procedure, as discussed in chapter 2, was followed for the molecular identification of both the coelomycetous fungi and *Fusarium* spp., however, for the coelomycetous fungi the following cycling parameters were used during PCR; initial denaturisation of 1 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 60 s at 72 ºC and a final elongation step of 5 min at 72 ºC. The following cycling parameters were used during sequencing; initial denaturisation of 1 min at 96 ºC, followed by 25 cycles of 10 s at 96 ºC, 5 s at 55 ºC and 4 min at 60 ºC, and a final elongation step of 5 min at 60 ºC.

4.2.5 Statistical Analysis

Once all the fungal colonies were identified to species level the results of the study were statistically analysed. The percentage of stink bug specimens, leaves and nuts associated with each fungal species isolated from the insects was calculated using Microsoft Excel. The number of fungi isolated from the air samples was expressed as colony forming units (CFU) per m^3 of air using the correspondence table (*n*-*N*) in the AES air sampler manual. Fungi isolated from the soil

samples were expressed as CFU per gram soil. This was calculated by multiplying the number of fungi per dilution by corresponding factors of ten for the fungal species that were more than 30 per plate. When a fungal species was isolated less than 30 times from a plate it was multiplied by a single factor of ten in order to note the incidence of these fungal species. The fungi were grouped by known pathogens of pecan, potential pathogens and/or contaminants and non-pathogenic fungal species that pose no threat to pecan production for each sample type. The potential pathogens and contaminants were selected based on the nature of the group and/or their known pathogenicity towards other agricultural crops or relations with pathogens that can cause disease on pecan. The occurrence of the most abundant fungal species was graphically represented using bar graphs. For the air samples species isolated in at least one site visit at 10 CFU's/m^3 air or higher, for the soil samples at 30 CFU's/g soil or higher and for the leaves, nuts and stink bugs at 5% or higher incidence were considered abundant. Based on the number of pathogens and potential pathogens isolated, and the occurrence patterns of the most abundant species isolated, it was assessed whether a sample type could be a source for pathogens and whether stink bugs can act as disseminators of fungi in pecan orchards. To analyze the similarities of species assemblages between different sampling dates and different sample types, Sørenson quantitative similarity index was used (Junninen *et al.*, 2006; Osono, 2008). Refer to Chapter 2 for more detail.

4.3 Results and Discussion

4.3.1 Scanning electron microscopy of stink bug specimens

Fungal material, in the form of hyphae, were observed to occur on the exoskeleton of the stink bugs (Figs. 4.7, 4.8 & 4.9). Most of these were observed to occur on the legs. Hyphae were observed on the pre-tarsal segments, but mostly on the setae of the legs (Figs. 4.7 & 4.8). Fungal hyphae may act as propagules that can give rise to new colonies and may cause disease (Kendrick, 2000). Certain fungal species, *e.g. Chaetomium* spp., have evolved to have complex spores and fruiting structures that have hooks and spines that can attach to the setae of arthropods (Kevan, Chaloner & Savile; 1975; Kendrick, 2000). These augmentations aid in their dispersal by arthropods (Kevan *et al.*, 1975; Kendrick, 2000). Therefore, stink bug specimens have the potential to spread fungi within pecan orchards. Fungal material was also observed in the groove

on the abdomen of the stink bugs in which their piercing-sucking mouthparts rest (Fig. 4.9). The presence of fungal material close to the mouthparts suggests that stink bugs can disseminate fungi as they feed. Feeding courts may provide suitable entry points for fungal phytopathogens to gain entry into developing pecan nuts. Unfortunately, the identification of the fungi is very difficult and most of the times unachievable with SEM. Therefore it is not known if the observed fungal material is related to pathogens of pecan. In this thesis it was important to confirm that fungal propagules were present on the exoskeleton of the stink bugs. These results suggest that stink bugs occurring in pecan orchards have the potential to harbour and disseminate fungal phytopathogens. The presence of fungal propagules on the stink bugs may increase the chances of survival and spread of fungal phytopathogens. Repeated observation of fungal propagules on the insect body may give rise to the assumption that the acquisition and spread of these propagules is indeed possible.

4.3.2 Fungal species isolated

In total, 91 fungal species, representing 42 genera, were isolated. These are represented by various known pathogens of pecan, potential pathogens and contaminants, and non-pathogenic fungi that bare no threat to pecan production. The fungal species isolated were from five different sample types (air, soil, leaf, nut & stink bug samples) collected on three occasions (C 1, C 2, & C 3) throughout the 2011-2012 season. The fungal species isolated from each sample are discussed separately in the following sections.

4.3.2.1 Fungal species isolated from air samples

In total, 38 fungal species, representing 19 genera, were isolated from the air samples collected on the three visits (C 1, C 2 & C 3) (Table 4.1). These were represented by 4 species that are pathogenic, 32 that can be considered potentially pathogenic or as contaminants and 2 species that are non-pathogenic on pecan trees. Pathogens that were isolated include *Alternaria alternata* (causing fungal leaf spot & die-back), *A. tenuissima* (fungal leaf spot), *Aureobasidium pullulans* (stigmatomycosis), and *Fusarium oxysporum* (die-back) (Michailides, Morgan, & Doster, 1995; Anon., 2006; Alvidrez-Villarreal *et al*., 2012). Potential pathogens and contaminants isolated included *Aspergillus awamori, A. candidus, A. flavus*, *A. niger*, *A. niveus*, *A. ochraceus*, *A. terreus*, *A. versicolor*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *C.*

pseudocladosporioides, *Colletotrichum coccodes*, *Coniothyrium fuckelli*, *Epicoccum nigrum*, *E. sorghi*, *Eurotium repens*, *Fusarium equiseti*, *F. incarnatum*, *Microsphaeropsis olivacea*, *Mucor circinelloides*, *M. hiemalis*, *Paecilomyces lilacinus*, *Penicillium aurantiogriseum*, *P. brevicompactum*, *P. canescens*, *P. citrinum*, *P. crustosum*, *P. decumbens*, *P. janthinellum*, *Peyronellaea sancta*, *Rhizopus oryzae* and *Sclerotinia sclerotiorurm*. The non-pathogenic fungi isolated included *Acremonium* sp. and *Trichoderma ghanense*. In total, 8 of the fungal species isolated from the air samples can be considered to be abundant (Fig. 4.11). These include 1 pathogenic-, 6 potential pathogenic-, and 1 non-pathogenic species.

Most abundant fungal species isolated from the air samples

The only abundant pathogen isolated from the air samples was *Alternaria tenuissima* (Fig. 4.11). The results suggest that the incidence of *A. tenuissima* was more abundant at the beginning of the season after which it decreased towards the middle of the season and slightly increasing towards the end of the season. *Alternaria* spp. are cosmopolitan dematiaceous fungi commonly isolated from plants, soil, food and indoor air environments (Ellis, 1971). *Alternaria tenuissima* is one of the more common species within the group and it is known to cause disease on a variety of economically important crops (Kendrick, 2000). *Alternaria* spp. are considered miscellaneous pathogens on pecans that can cause fungal leaf scorch and die-back (Anon., 2006; Alvidrez-Villarreal *et al*., 2012).

The most abundant fungal species, and potential pathogen and contaminant, isolated from the air was *Cladosporium cladosporioides* (Fig. 4.11). Its incidence followed a similar trend when compared to *A. tenuissima* where the incidence of *C. cladosporioides* was most abundant at the beginning of the season after which it decreased towards the middle of the season, and increased again towards the end of the season. *Cladosporium cladosporioides* is a dematiaceous fungus that is widely distributed in air and rotten organic material, and frequently isolated as a contaminant in foodstuffs (Domsch, Gams & Anderson, 2007). This fungus has been suspected to be pathogenic to pecans in South Africa, but no evidence exists to support this. *Cladosporium cladosporioides* is not considered a pathogen of pecan but, because of its association with postharvest problems, could influence the quality of stored nuts

The results showed that the incidence of *Epicoccum nigrum* was more abundant at the beginning of the season, decreasing towards the middle of the season, and increasing to its highest levels towards the end of the season (Fig. 4.11). *Epicoccum nigrum* is a cosmopolitan, dematiaceous fungus that is primarily isolated from plants and soil (Domsch *et al*., 2007) where it grows saprophytically or epiphytically on dead or dying plant material (Domsch *et al*., 2007). *Epicoccum nigrum* is not considered a pathogen of pecan but may invade damaged plant parts and be implicated in postharvest decay of nuts.

The presence of *Epicoccum sorghi* followed a similar trend compared to *E. nigrum* where the incidence of *E. sorghi* was higher at the beginning of the season decreasing towards the middle of the season and increasing to its highest abundance towards the end of the season (Fig. 4.11). It is unclear whether *E. sorghi* can cause disease on pecan trees, but it may be a potential pathogen of the crop.

The second most abundant potential pathogen and contaminant isolated from the air samples was *Penicillium janthinellum* (Fig. 4.11). Other *Penicillium* species that were also isolated included *P.brevicompactum* and *P. citrinum*). The incidence of *Penicillium* spp. was most abundant in the beginning of the season where after their incidence diminished. *Penicillium* spp. are not known to be pathogens of pecan, but they might be involved in postharvest spoilage, and can have an effect on the health of consumers because of their mycotoxigenic nature.

The only abundant non-pathogenic fungal species isolated from the air was *Trichoderma ghanense* (Fig. 4.11). These results follow a similar trend when compared to *Epicoccum nigrum* and *E. sorghi* where the incidence of *T. ghanense* was initially high, decreased towards the middle of the season, and increased to its highest incidence towards the end of the season. *Trichoderma* spp. are filamentous fungi that are widely distributed and occurs in the soil, plant material, decaying vegetation and wood (Domsch *et al.*, 2007). They are not considered to be pathogens of pecans, and could possibly be beneficial to the plants within pecan orchards. Several strains of *Trichoderma* spp. have been developed as biocontrol agents against fungal diseases (Kendrick, 2000). Most of the biocontrol agents are represented by the species *T. harzianum*, *T. viride* and *T. hamatum*. The biocontrol agents grow in their natural habitat, the root surface, and negatively affect root diseases in particular. These agents can also be effective

against foliar diseases. The biocontrol agents are so successful that they have thus far controlled every pathogenic fungus for which control has been sought (Kendrick, 2000).

Several fungal species were isolated from the air samples throughout the 2011-2012 season (Table 4.1). These included several pathogens, potential pathogens and contaminants and non-pathogenic fungi. The most abundant and the second most common fungal pathogen isolated was *Alternaria tenuissima* (Fig. 4.11).The largest proportion of fungi isolated can be considered potential pathogens or contaminants. The effect of postharvest decay on pecan nut can be dramatic where several of the contaminants isolated are also associated with the production of mycotoxins. Mycotoxins present in pecan nuts can reduce the safety of the nuts for human consumption. The most abundant potential pathogen and contaminant isolated was *Cladosporium cladosporioides*. All the other fungal species were isolated at considerable lower incidences. The results also suggest that both these species were the most abundant at the beginning of the season where after the incidence declined, and increased towards the end of the season. This might indicate that both these species grow saprophytically and sporulate on dead and decaying plant material, such as leaves and nuts, which are abundant at these times. Other factors that might influence the dispersion may include climatic conditions such as temperature and humidity. The mid-season in the Northern Cape Province is usually extremely hot and dry which might create unfavourable conditions for fungal species to grow and produce conidia. According to the results it would seem that the incidence of fungi in the air within pecan orchards may be dependent on substrate availability and climatic conditions. The results support the notion that the air might be a source for both pathogens and potential pathogens and contaminants.

4.3.2.2 Fungal species isolated from the soil samples

In total, 44 species from 19 fungal genera were isolated from the soil samples taken on the three visits (C 1, C 2 & C 3) (Table 4.2), which is represented by 5 species that are pathogenic, 34 species that can be considered potentially pathogenic or as contaminants, and 5 species that are non-pathogenic on pecan. Pathogens isolated included *Alternaria alternata*, *A. tenuissima*, *Aureobasidium pullulans*, *Botryosphaeria ribis* (water stage nut drop & stem end blight) and *Fusarium oxysporum* (Anon., 2006). Potential pathogens and contaminants isolated included *Aspergillus aliaceus*, *A. candidus*, *A. carneus*, *A. niger*, *A. ochraceus*, *Cladosporium*

cladosporioides, *Colletotrichum coccodes*, *Coniothyrium insitivum*, *Epicoccum nigrum*, *E. sorghi*, *Eurotium repens*, *Fusarium equiseti*, *F. incarnatum*, *F. laceratum*, *Gliocladium* sp., *Khuskia oryzae*, *Mucor circinelloides*, *M. hiemalis*, *Penicillium aurantiogriseum*, *P. brevicompactum*, *P. citreonigrum*, *P. citrinum*, *P. crustosum*, *P. decumbens*, *P. janczewskii*, *P. janthinellum*, *P. pinophilum*, *P. restrictum*, *P. spinulosum*, *P. variabile*, *P. veruculosum*, *Peyronellaea coffeae-arabicae*, *P. sancta,* and *Rhizopus oryzae*. The non-pathogenic fungi isolated included *Humicola fuscoarta*, *Sporothrix* sp., *Trichoderma ghanense*, *T. reesei,* and *T. viride*. In total, 12 of the fungal species isolated from the soil samples can be considered abundant (Fig. 4.12). These include 1 pathogenic species, 10 potential pathogenic species and 1 non-pathogenic species.

Most abundant fungal species isolated from soil samples

The most abundant pathogen isolated from the soil samples was *Alternaria tenuissima* (Fig. 4.12). These results suggest that the incidence of *A. tenuissima* was at its lowest at the beginning of the season and increased to its highest incidence at the end of the season. As mentioned previously *Alternaria* spp. are considered miscellaneous pathogens that can cause fungal leaf scorch and die-back.

The results show that the incidence of *A. ochraceus* was low at the beginning of the season where after it increased to its highest towards the middle of the season to once again decreasing towards the end of the season (Fig. 4.12). *Aspergillus* spp. are filamentous, cosmopolitan and ubiquitous organisms that can be found in most environments (Domsch *et al*., 2007). *Aspergillus ochraceus* and other members of *Aspergillus* are commonly isolated from soil, plant debris and indoor air environments (Domsch *et al*., 2007). *Aspergillus ochraceus* is not classified as a pathogen of pecan, but it may be involved in postharvest spoilage, in combination with the potential production of mycotoxins such as ochratoxin A, it may cause economic losses in pecan production.

The most abundant fungal species, and potential pathogen and contaminant, from soil was *Cladosporium cladosporioides* (Fig. 4.12). The results suggest that the presence of *C. cladosporioides* was most abundant during the beginning of the season, decreasing towards the middle of the season and slightly increasing towards the end of the season.

Fusarium equiseti followed a similar trend to the incidence of *A. ochraceus* in the soil and it suggests that the presence of *F. equiseti* was low at the beginning of the season, increasing to its highest incidence towards the middle of the season, and decreasing towards the end of the season (Fig. 4.12). There are no indications that *F. equiseti* is involved in causing disease on pecan, but due to the nature of *Fusarium* spp. it can be considered as potential pathogens and/or contaminants of pecan.

Results of the incidence of *Mucor hiemalis* suggest a similar pattern to the incidence of *A. ochraceus* and *F. equiseti* in the soil, it shows that the presence of *M. hiemalis* was low at the beginning of the season increasing to its highest incidence towards the middle of the season, and decreasing towards the end of the season (Fig. 4.12).

The second most abundant potential pathogen and contaminant to be isolated from the soil samples was *Penicillium brevicompactum* (Fig. 4.12). Results indicated that the incidence of this fungus was low at the beginning of the season after which the incidence increased to its maximum during the middle of the season, and then decreased towards the end of the season. In contrast, the incidence of the other *Penicillium* spp. were low during the beginning and middle of the season and highest at the end of the season.

The only abundant non-pathogenic fungal species isolated from the soil samples was *Trichoderma ghanense* (Fig. 4.12). These results followed a similar trend as with the incidence of *A. tenuissima* in the soil where the incidence of *T. ghanense* was at its lowest during the beginning of the season, increased to its highest level at the end of the season.

Several fungal species were isolated from the soil samples throughout the 2011-2012 season (Table 4.12). These included known pathogens of pecan, potential pathogens, contaminants and non-pathogenic fungi. The only abundunt pathogen isolated was *Alternaria tenuissima* (Fig. 4.12). However, *A. tenuissima* was isolated at lower incidences when compared to the potential pathogens and contaminants isolated from the soil. Its incidence was low when compared to other samples taken. Similar to air samples, the largest proportion of fungi isolated from the soil can be considered potential pathogens or contaminants of pecan. Several of these species are also known to produce mycotoxins. The most abundant potential pathogen and contaminant isolated was *Cladosporium cladosporioides* and the second most common was

Penicillium brevicompactum. Both these species are commonly isolated from the soil (Domsch *et al*., 2007). These two species were also the most abundant species isolated from the soil throughout the season. The incidence of *C. cladosporioides* followed a similar trend when compared to the incidence in the air. Its incidence was initially high decreasing towards the middle of the season and increasing towards the end of the season. Similar to air samples, this dispersion pattern may be due to substrate availability and climatic conditions. In contrast, the incidence of *P. brevicompactum* was at its highest during the middle of the season. The level of incidence of most of the other potential pathogens and contaminants were also highest towards the end of the season. These species may tolerate the climatic conditions better than *C. cladosporioides* or their incidence may have increased due to the lower occurrence of *C. cladosporioides* in the soil. The results suggest *C. cladosporioides* is the most abundant species within the orchard's soil and its reduced incidence may open niches that were previously unavailable to other species. As a result the incidence of the other species may have increased due to reduced competition from *C. cladosporioides*. Due to the low occurrence of pathogens it would seem that the soil does not act as a source for pathogens within the orchard; however, the soil can act as a source for potential pathogens and contaminants throughout the season.

4.3.2.3 Fungal species isolated from leaf samples

In total, 36 species from 20 fungal genera were isolated from leaf samples taken on the three visits (C 1, C 2 & C 3) (Table 4.3). These were represented by 3 species that are pathogenic, 28 species that can be considered potentially pathogenic or as contaminants, and 5 species that are non-pathogenic on pecan. Pathogens that were isolated include *Alternaria alternata*, *A. tenuissima* and *Fusarium oxysporum*. Potential pathogens and contaminants isolated include *Aspergillus awamori*, *A. niger*, *Byssochlamys spectabilis*, *Cladosporium cladosporioides*, *Cochliobolus spicifer*, *Colletotrichum coccodes*, *Coniothyrium pyrium*, *Epicoccum nigrum*, *E. sorghi*, *Fusarium equiseti*, *F. incarnatum*, *F. sporotrichioides*, *Khuskia oryzae*, *Mucor circinilloides*, *M. hiemalis*, *Penicillium chrysogenum*, *P. citrinum*, *P. crustosum*, *P. decumbens*, *P. janthinellum*, *Phoma glomerata*, *Peyronellaea americana*, *P. coffeae-arabicae*, *P. eucalyptica*, *P. glomerata*, *P. sancta* and *Rhizopus oryzae*. The non-pathogenic fungi isolated include *Acremonium* sp., *Gonatobotrys simplex*, *Leptodontium* sp., *Trichoderma ghanense*, *Truncatella angustata.* In total, 7 of the fungal species isolated from the soil samples can be

considered to be abundant (Fig. 4.13). These include 1 pathogenic species and 6 potential pathogenic species.

Most abundant fungal species isolated from leaf samples

The only abundant pathogen isolated from the leaf samples was *Alternaria tenuissima* (Fig. 4.13). The results suggest that the incidence of *A. tenuissima* was at its lowest during the beginning of the season and increased to towards the end of the season.

The most abundant potential pathogen and/or contaminant, isolated from the leaf samples was *Cladosporium cladosporioides* (Fig. 4.13). The results followed a similar trend when compared to *A. tenuissima* where the incidence of *C. cladosporioides* was at its lowest during the beginning of the season and increased towards the end of the season.

Epicoccum nigrum followed a similar trend when compared to *A. tenuissima* and *C. cladosporioides* (Fig. 4.13). The incidence was initially low where after it builds up during the course of the season.

The second most abundant potential pathogen and contaminant to be isolated from the leaf samples was *Epicoccum sorghi* (Fig. 4.13). It followed similar trends than most of the other fungi where relatively low incidence was observed in the beginning of the season that increased towards the end of the season.

The incidence of *Fusarium incarnatum* peaked during the middle of the season, which is different to most of the other fungi discussed. This fungus should be considered as a potential pathogen and contaminant in pecans due to the pathogenic nature of *Fusarium* spp. in general.

Results suggest that the incidence of *Khuskia oryzae* differs from most of the other fungi in that its incidence was highest during the beginning of the season, decreased during the middle of the season, and then increased again towards the end of the season (Fig. 4.13). *Khuskia oryzae* is not known to be a pathogen of pecan, although it might be involved in postharvest damage.

Penicillium crustosum was only isolated from the leaf samples during the middle of the season (Fig. 4.13). The reason for only occurring in the middle of the season on surface sterilised leaves is, however, unknown. It could be an indication that the fungus could be endophytic and

that some of the test plants were under stress, allowing a non-pathogenic fungus to colonise the leaves.

Several fungal species were isolated from the leaf samples throughout the 2011-2012 season (Table 4.3). These include known pathogens of pecan, potential pathogens and contaminants and non-pathogenic fungi. The only abundant pathogen isolated was *Alternaria tenuissima* , which was also the second most common fungal species isolated from the leaf samples. Similar to the air and soil samples the largest proportion of fungi isolated from the leaves can be considered potential pathogens or contaminants of pecan. Several of these species are also known to produce mycotoxins. The most abundant potential pathogen and contaminant isolated was *Cladosporium cladosporioides* and the second most common *Epicoccum sorghi*. The incidence of all three previously mentioned species and several of the other less abundant species followed similar trends. The incidence of these species was initially low and increasing towards the end of the season. This might indicate that the infection of the leaves occurs at the beginning of the season after which they grow endophytically, sheltered from extreme conditions, and consequently their incidence can steadily increase towards the end of the season. Infection sources at the beginning of the season might include the air, soil and stink bugs since the species incidence was high within these areas during the start of the season. These species might also occur endophytically in the twigs and branches of trees from which they can migrate into the leaves.

4.3.2.4 Fungal species isolated from nut samples

In total, 28 species from 18 fungal genera were isolated from the nut samples collected during the three visits (C 1, C 2 & C 3) to the site (Table 4.4). These species were represented by 4 species that are pathogenic, 20 species that can be considered potentially pathogenic or as contaminants and 4 species that are non-pathogenic on pecan. Pathogens isolated include *Alternaria alternata* (Causing fungal leaf spot & Die-back), *A. tenuissima* (Fungal leaf spot), *Fusarium oxysporum* (Die-back) and *Neofusicoccum parvum* (Fungal leafspot & Blackening of the husk) (Michailides *et al*., 1995; Anon., 2006; Alvidrez-Villarreal *et al*., 2012). Potential pathogens and contaminants isolated include *Aspergillus niger*, *A. versicolor*, *Byssochlamys spectabilis*, *Cladosporium cladosporioides*, *Colletotrichum coccodes*, *Coniothyrium fuckelli*, *Epicoccum nigrum*, *E. sorghi*, *Fusarium equiseti*, *F. incarnatum*, *F. nygamai*, *Khuskia oryzae*,

Microsphaeropsis olivacea, *Penicillium aurantiogriseum*, *P. brevicompactum*, *P. decumbens*, *P. janthinellum*, *Peyronellaea coffeae-arabicae*, *P. sancta* and *Rhizopus oryzae*. The non-pathogenic fungi isolated include *Acremonium* sp., *Chaetomium globosum*, *Gonatobotrys simplex* and *Leptodontium* sp. In total, 8 of the fungal species isolated from the soil samples can be considered to be most abundant (Fig. 4.14). These include 2 pathogenic-, 3 potential pathogenic-, and 3 non-pathogenic species.

Most abundant fungal species isolated from nut samples

The most abundant pathogen isolated from the nut samples was *Alternaria tenuissima* (Fig. 4.14). The results suggest that the incidence of *A. tenuissima* was at its lowest during the beginning of the season and increased to its highest level of incidence towards the end of the season.

The second most abundant pathogen isolated from the nut samples was *Neofusicoccum parvum* (Fig. 4.14). This fungus is a member of the *Botryosphaeriaceae* (*Botryosphaeriales*, *Dothideomycetes*, *Ascomycota*), which can be parasitic, saprophytic or endophytic on a variety of plant species (Mohammadi *et al.,* 2013). The group has a worldwide distribution and several species are known to cause disease on economically important plant species (Mohammadi *et al*., 2013). *Neofusicoccum parvum* has been found to be pathogenic on several plant species of which its effect on grapevine is probably the most economically important (Baskarathevan *et al*., 2012; Sakalidis *et al*., 2013). This particular species is genetically diverse and has been found to be associated with at least 90 hosts across six continents and in 29 countries (Sakalidis *et al*., 2013). *Neofusicoccum parvum* has not been previously found to be associated with pecan. During the course of this thesis it has been proven that *N. parvum* can cause disease on pecan (See Chapter 5). It has been observed that this species can cause both leaf spots and the blackening of the husk surrounding the pecan nut itself. Therefore, *N. parvum* can be considered a pathogen of pecan, however, the extent of its distribution and effect on pecan production remains unknown.

Cladosporium cladosporioides was isolated at considerable lower incidences from the nuts than in the other samples. This might be due to competition with other fungal species such as *A. tenuissima* and *N. parvum* which are pathogenic on pecan nuts (Chap 5). is not considered a pathogen of pecan but it could play a role in postharvest spoilage of stored nuts (Fig. 4.14).

The second most abundant potential pathogen and contaminant isolated from the nut samples was *Fusarium incarnatum* (Fig. 4.14). It is unknown whether *F. incarnatum* is involved in causing disease on pecan.

The most abundant potential pathogen and contaminant isolated from the nut samples was *Microsphaeropsis olivacea* (Fig. 4.14). Members of *Microsphaeropsis* are closely related to the genus, *Coniothyrium* (Sutton, 1980; Pethybridge *et al*., 2008), of which some members were also isolated in this study. Morphologically the separation of the two groups is based on the production of conidia (Pethybridge *et al*., 2008). *Microsphaeropsis* spp. produce small dark aseptate conidia on phialides where *Coniothyrium* spp. produce theirs annelidically (Pethybridge *et al*., 2008). *Microsphaeropsis* spp. are widely dispersed and commonly isolated from many different habitats (Verkley *et al*., 2004). The group has received attention as potential biological control agents as fungal antagonists (Carisse, El Bassam & Benhamou, 2001; Verkley *et al*., 2004; Pethybridge *et al*., 2008), potential bioremediators (Da Silva *et al*. 2003; Verkley *et al*., 2004), and as producers of antibiotics and other secondary metabolites (Tsuda *et al*. 2003; Verkley *et al*., 2004; Hormazabal *et al*., 2005). The group also contains several plant pathogens that cause necrotic spots and/or lesions (Pethybridge *et al*., 2008). *Microsphaeropsis* spp. are not classified as pathogens of pecan, but due to their nature they can be considered as potential pathogens of this crop.

The most abundant non-pathogenic fungal species isolated from the nut samples was *Acremonium* sp. (Fig. 4.14). This fungal genus is saprophytic and occurs on decaying wood and leaves; it is widely distributed in nature, but has never been implicated as a pathogen of pecan (Domsch *et al*., 2007).

Chaetomium spp. are dematiaceous and mostly saprophytic filamentous fungi found in the soil, the air and plant debris. These fungi are not considered as pathogens of pecans.

Gonatobotrys simplex is a widely distributed parasitic fungal species that attacks and lives on other fungi (Barron, 1968), and is commonly isolated from soils. *Gonatobotrys simplex* has been found to be antagonistic towards several *Alternaria* spp. It is not considered a pathogen of pecan, and may have a negative effect on pathogen occurrence and development within pecan orchards (Barron, 1968).

Several fungal species were isolated from the nut samples throughout the 2011-2012 season (Table 4.4). These included known pathogens of pecan, potential pathogens, contaminants and non-pathogenic fungi. Two of the most abundant pathogens isolated were *Alternaria tenuissima* and *Neofusicoccum parvum* (Fig. 4.14). These two species were also generally the most abundant species isolated from the nut samples. The potential pathogens, contaminants, and the non-pathogenic fungal species were isolated at far lower incidences than these two species. The incidence of all the most abundant species isolated from the nut samples followed the same trend. Their incidence was initially low, increasing to its highest level of incidence towards the end of the season. This trend is similar to the distribution pattern observed with the most abundant species isolated from the leaf samples. It is most likely that the same factors that influence the incidence of the fungal species within the leaves also influence the incidence of the fungal species in the nuts.

4.3.2.5 Fungal species isolated from stink bugs

In total, 57 species from 35 fungal genera were isolated from the stink bug samples collected during the three visits (C 1, C 2 & C 3) at the site (Table 4.5). These species represented 6 species that are pathogenic, 36 that can be considered potentially pathogenic or as contaminants, and 15 that are non-pathogenic on pecan. Pathogens isolated include *Alternaria alternata*, *A. tenuissima*, *Areobasidium pullulans*, *Botryosphaeria ribis*, *Fusarium oxysporum* and *Neofusicoccum parvum* (fungal leafspot & Blackening of the husk). Potential pathogens and contaminants isolated include *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *Botrytis cinerea*, *Byssochlamys spectabilis*, *Cladosporium cladosporioides*, *Cochliobolus lunatus*, *C. sativus*, *C. spicifer*, *Colletotrichum coccodes*, *Curvularia clavata*, *Epicoccum sorghi*, *Fusarium camptoceras*, *F. equiseti*, *F. incarnatum*, *Khuskia oryzae*, *Microdochium nivalae*, *Mucor circinelliodes*, *M. hiemalis*, *Neosatorya fisheri*, *Paecilomyces lilacinus*, *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. citreonigrum*, *P. decumbens*, *P. janthinellum*, *P. spinilosum*, *Pestalotiopsis* sp., *Phoma brasiliensis*, *Peyronellaea eucalyptica*, *P. glomerata*, *P. pomorum*, *P. sancta*, *Rhizopus oryzae*, *Sclerotia . sclerotiorum* and *Ulocladium* sp. The non-pathogenic fungi isolated include *Acremonium* sp., *Arthrobotrys oligospora*, *Chaetomium funicola*, *C. globosum*, *Chaetomium* sp., *Humicola fuscoarta*, *H. grisea*, *Melanospora* sp., *Moniliella acetobutans*, *Monodictys paradoxa*, *Preussia* sp., *Sporothrix* sp., *Trichoderma*

ghanense, *T. reesei* and *Trichoderma viride*. In total, 15 of the fungal species isolated from the soil samples can be considered to be the most abundant (Fig. 4.15). These include 2 pathogenic-, 11 potential pathogenic-,and 2 non-pathogenic species.

Most abundant fungal species isolated from stink bugs

The most abundant pathogen isolated from the stink bug samples was *Alternaria tenuissima* (Fig. 4.15). The results suggest that the incidence of *A. tenuissima* was highest during the beginning of the season decreasing towards the middle of the season, and slightly increasing towards the end of the season. Alvidrez-Villarreal *et al*. (2012) showed that *A. alternata* can cause the die-back of pecan branches and that insects, in combination with fungal invasion, eventually kill the trees. Additionally, the ambrosia trunk and branch borer, *Euplatypus segnis* (Coleoptera: Curculionidae: Platypodinae) contributes to the spread of fungi in pecan orchards (Alvidrez-Villarreal *et al*., 2012).

The second most abundant pathogen isolated from the stink bug samples was *Neofusicoccum parvum* (Fig. 4.15). The results suggest that the incidence of *N. parvum* was high during the beginning of the season increasing to its highest level towards the middle of the season and then absent at the end of the season.

The most abundant potential pathogen and contaminant isolated from the stink bug samples was *Cladosporium cladosporioides* (Fig. 4.15). The incidence of *C. cladosporioides* on the stink bugs followed the same trend as the incidence of *A. tenuissima* where it was at its highest during the beginning of the season, decreasing towards the middle of the season, and slightly increasing again towards the end of the season.

The results suggest that the incidence of *Cochliobolus spicifer* was low at the beginning of the season, increasing to its highest level towards the middle of the season, and decreasing towards the end of the season. *Cochliobolus* spp. are mostly saprophytic in nature, but can cause disease on various crops (Domsch *et al*., 2007). *Cochliobolus* spp. are mostly associated with cereals and not known as a pathogen of pecans but could be considered a potential pathogen.

The results show that the presence of *E. sorghi* was at its highest at the beginning of the season after which it decreased towards the end of the season (Fig. 4.15).

The presence of *Fusarium equiseti* on the stink bugs followed the same pattern as the presence of *E. sorghi* where it is at its highest at the beginning of the season and decreased towards the end of the season. It is unknown whether *F. equiseti* can cause disease on pecan nuts.

The second most prominent potential pathogen and contaminant isolated from the stink bug samples was *Mucor circinelloides* (Fig. 4.15). In addition, *M. hiemalis* was also isolated at high incidences. These results show that the presence of both *Mucor* spp. was low at the beginning of the season, increased to its highest towards the middle of the season, and once again decrease towards the end of the season

Penicillium aurantiogriseum was only isolated from the stink bugs at the middle of the season. The presence of *P. chrysogenum* was at its highest at the beginning of the season after which it decreased. *Penicillium decumbens* was only isolated from the stink bugs at the end of the season.

Peyronellaea eucalyptica was only present on the stink bugs at the end of the season. *Peyronellaea* forms part of the larger *Phoma* group (Boerema *et al.*, 2004). *Peyronellaea* is characterized by the possession of multicellular chlamydosporal structures, which may occur in combination with unicellular chlamydospores. *Peyronellaea* spp. are wide spread and some are known to be opportunistic plant pathogens (Boerema *et al*., 2004). *Peyronellaea* spp. are not known to be pathogens of pecan but can be considered potential pathogens.

Rhizopus oryzae was absent on the stink bugs during the beginning of the season, increasing in incidence to its highest during the middle of the season (Fig. 4.15). *Rhizopus* spp. are cosmopolitan filamentous fungi found in soil, decaying fruit and vegetables, animal faeces and various foodstuffs (Anon., 2007). While *Rhizopus* spp. are common contaminants, certain species are considered plant pathogens. *Rhizopus oryzae* is a saprophytic fungus that is involved with the spoilage of many food products (Domsch *et al*., 2007). but is not classified as a pathogen of pecans, although it might be involved in postharvest spoilage.

The most abundant non-pathogenic fungal species isolated from the stink bug samples was *Acremonium* sp. (Fig. 4.15). The incidence of this fungus followed a similar trend as the incidence of *E. sorghi* and *F. equiseti* where it was highest during the beginning of the season, decreasing towards the end of the season.

The second most abundant non-pathogenic fungal species isolated from the stink bug samples was *Chaetomium globosum* (Fig. 4.15). The incidence of *C. globosum* on the stink bugs was low during the beginning and middle of the season, but slightly increased towards the end of the season.

Generally, the largest variety of fungal species was isolated from the stink bugs in comparison to the other samples taken. This suggests that the isolation of fungi from insects in tree-nut crop orchards, such as pecan orchards, may give a good indication of the mycoflora of the environment. The fungal species isolated from the stink bugs throughout the 2011-2012 season included known pathogens of pecan, potential pathogens and contaminants, and non-pathogenic fungi (Table 4.5). Two abundant pathogens were isolated including *Alternaria tenuissima* and *Neofusicoccum parvum* (Fig. 4.15). *Alternaria tenuissima* was generally the most common fungal species isolated from all samples while *N. parvum* was one of the more abundant on the insects and nuts. As with the air, soil, and leaf samples the largest proportion of fungi isolated from the stink bugs can be considered potential pathogens or contaminants of pecan. Several of these species are also known to produce mycotoxins. The most abundant potential pathogen and contaminant isolated was *Cladosporium cladosporioides*. This fungus was overall the second most common fungal species isolated. The incidence of both *A. tenuissima* and *C. cladosporioides* followed a similar trend to the presence of these species in the air and soil samples. The incidence of these fungal species was initially high after which it declined in the middle of the season and increased towards the end. This was contradictory to the incidence of these species in the leaves and nuts. This might indicate that the incidence of these two species is linked to the incidence of these species in the air and soil and that it might be affected by climatic conditions and substrate availability in the environment. It is most likely that the stink bugs have no specific association with these species and their ability to harbour and disseminate these species will depend on the availability of conidia in their environment. These species can, however, be present on the stink bugs at high levels and the stink bugs may provide suitable entry points into pecan trees and nuts for these fungi through feeding wounds. The incidence of *N. parvum* on the stink bugs did not follow the same pattern as the incidence of this species in the nuts; however, this species was only isolated from the nuts and from the stink bugs. It was completely absent from all the other samples taken throughout the whole season. This might indicate an association between the stink bugs and *N. parvum*, where the stink bugs act as

disseminators spreading this fungus between developing nuts as they feed. The results also indicate that if the stink bugs act as disseminators of fungi in pecan orchards that this dissemination will happen early in the season when the fungi occur most on the stink bugs. This may be one of the modes of action that fungi utilise to gain entry into the leaves and nuts after which they grow endophytically. Generally, the results show that stink bugs occurring in pecan orchards are associated with fungal pathogens and several potential pathogens and contaminants. The results also show that the incidence of *A. tenuissima* and *C. cladosporioides* on the stink bugs is variable and most likely influenced by the environment. Additionally, it has been shown that stink bugs are most likely disseminators of *N. parvum*.

4.3.3 Sample comparisons and Sørenson quantitative similarity index

Tables 4.6 - 4.10 show the Sørenson's quotation values comparing the similarity between the different site visits for each sample type. The Sørenson's index comparing the similarity of the species assemblages between the fungi isolated from the three air samples shows that there is no similarity between the different samples taken, since there are no high pairwise similarity values (Table 4.6). The results suggest that the fungal diversity at the beginning of the season was more similar to the fungal diversity at the end of the season than the diversity at the middle of the season. The diversity at the middle of the season differed the most from the diversity at the end of the season. These results reflect the frequencies at which the fungal species were isolated, where there was a drop in the incidence of the most abundant fungal species during mid-season (Fig 4.11). This drop might be due to substrate availability and climatic conditions. The results suggest that pathogens, potential pathogens and contaminants are most likely dispersed by air during the beginning and the end of the season.

The Sørenson's index comparing the similarity of the species assemblages between the fungi isolated from the three soil samples shows no similarity between the different samples (Table 4.7). The results suggest that the fungal diversity at the beginning of the season was more similar to the fungal diversity at the end of the season then the diversity at the middle of the season. The highest similarity was found between the diversity at the middle and end of the season. The similarity between the beginning and end of the season may be attributed to the low incidence of abundant fungal species such as *Cladosporium cladosporioides* during the middle of the season and their high incidence at the beginning and end of the season (Fig. 4.12). The high

similarity between the middle and end of the season may be due to the absence of the less abundant species at the beginning of the season and their incidence during the middle and end of the season (Fig. 4.11). The results suggest that the distribution and occurrence of fungal species in pecan orchard soil is highly variable. As mentioned previously the incidence of pathogens in the soil was low and that the soil in this case most likely does not function as a source of pathogens.

The Sørenson's index comparing the similarity of the species assemblages between the fungi isolated from the three leaf samples shows that there is no similarity between the different samples (Table 4.8). These results show that the similarity increases as the season progresses as C 2 was more similar to C 3 than C 1 and as C 1 to C 2. These results are reflected in the frequencies at which the fungal species were isolated where the abundance of the species increased from the beginning to the end of the season (Fig. 4.13). This might indicate that the infection of the leaves by the prominent species occurs at the beginning of the season after which they grow endophytically, shelter from extreme conditions and as a result, their presence can steadily increase towards the end of the season.

The Sørenson's index comparing the similarity of the species assemblages between the fungi isolated from the two nut samples shows that there is no similarity between the different samples (Table 4.9). The dissimilarity between the samples might be due to the increase of species towards the end of the season (Fig. 4.14).

The Sørenson's index comparing the similarity of the species assemblages between the fungi isolated from the three stink bug samples shows that there is no similarity between the different samples (Table 4.10). These results show that all three the samples were almost equally similar. The low similarity fungal species may be due to the presence and absence of several of the less prominent species on the stink bugs (Fig. 4.15). This indicates that that as previously mentioned, the presence of fungi on the stink bugs is highly variable. This variability might be due to the availability of conidia in their environment, which can be effected by substrate availability or climatic conditions. Therefore, the ability of the stink bugs to act as disseminators of fungal pathogens might be influenced by the environment.

Tables 4.11 - 4.13 shows the Sørenson quotation values comparing the similarity between the leaf and nut samples, stink bug and leaf samples, and stink bug and nut samples. The Sørenson's index comparing the similarity of the species assemblages between the fungi isolated from the leaf and nut samples shows that there is no similarity. These results show that the similarity between the samples increases as the season progresses, as the highest similarity was found between the leaf and nut samples taken at the end of the season. It is, therefore, possible that the mycoflora of the leaves and nuts are linked to a certain extent. This might indicate that fungal species occurring endophytically in the leaves and other plant parts can spread through the plant system to the nuts when they form, and as the season progresses the mycoflora occurring in the nuts becomes more similar to the mycoflora in other plant parts.

The Sørenson's index comparing the similarity between the fungi isolated from the stink bug and leaf, and the stink bug and nut samples shows that there is no similarity between the different (Table 4.9). The Sørenson's index values are given in table 4.10. Overall, the results show that there is not a high similarity between the fungi occurring on the stink bugs and those found in the leaves and nuts. The presence of the prominent fungal species on the stink bugs follows a similar pattern to the prominent species found in the air and soil samples. The Sørenson's index could not be used to compare these samples and confirm this assumption since they are measured in different units. These results might indicate that the fungi occurring on the stink bugs are obtained from their entire environment and that the presence of fungal species such as *A. tenuissima* and *C. cladosporioides* in the leaves and nuts cannot, in whole, be attributed to vectoring by stink bugs. However, *N. parvum* only occurred on the stink bugs and on the nuts and this might indicate an association between the stink bugs and *N. parvum*, where the stink bugs act as disseminators spreading this fungus between developing nuts as they feed.

4.4 Conclusions

A large variety of fungal species were isolated from the samples taken throughout the 2011-2102 season in the pecan orchard. These included several known pathogens of the crop, potential pathogens and non-pathogenic species. The greatest diversity of fungi was found to be associated with the stink bug specimens. This indicates that stink bugs, most likely insects in general, play an important role in the dynamics of fungal populations within pecan orchards. As a result the isolation of fungi from insects in tree-nut crop orchards, such as pecan orchards, may give a good indication of the environment's mycoflora. Overall, the two most abundant fungal species isolated from the stink bugs and the other samples were *Alternaria tenuissima* and *Cladosporium cladosporioides. Alternaria* spp. are considered miscellaneous pathogens of pecan while *C. cladosporioides* is considered a potential pathogen and contaminant. The incidence of these two species on the stink bug samples followed a similar pattern to the incidence of these two species in the air and soil. However, the incidence of these two species on the stink bugs differed from their incidence in the leaves and nuts. This may indicate that the fungi occurring on the stink bugs is obtained from their entire environment and that the incidence of fungal species such as *A. tenuissima* and *C. cladosporioides* in the leaves and nuts cannot be solely attributed to vectoring by stink bugs. *Neofusicoccum parvum* was only isolated from the nuts and from the stink bugs. It was completely absent from all the other samples taken throughout the whole season. This might indicate an association between the stink bugs and *N. parvum*, where the stink bugs act as disseminators spreading this fungus between developing nuts as they feed. *Neofusicoccum parvum* has not been previously found to be associated with pecan. During the course of this study it has been proven that *N. parvum* can cause disease on pecan (See Chapter 5). It has been found that this species can cause both leaf spots and the blackening of the husk surrounding the pecan nut itself. This study has shown that the grey-brown stink bug occurring in pecan orchards is associated with fungal phytopathogens and that it has the capability of acting as a disseminator of these pathogens.

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4.6 Tables & Figures

Figure 4.1: The pecan orchard in which the progression of fungal populations associated with the grey-brown stink bug was investigated (Photo by J. Saaiman).

Figure 4.3: The layout of the study site indicating the row in which the stink bug specimens were collected and the 10 selected trees (S1-S10) where the other samples (air, soil, leaf & nuts) were taken (earth.google.com).

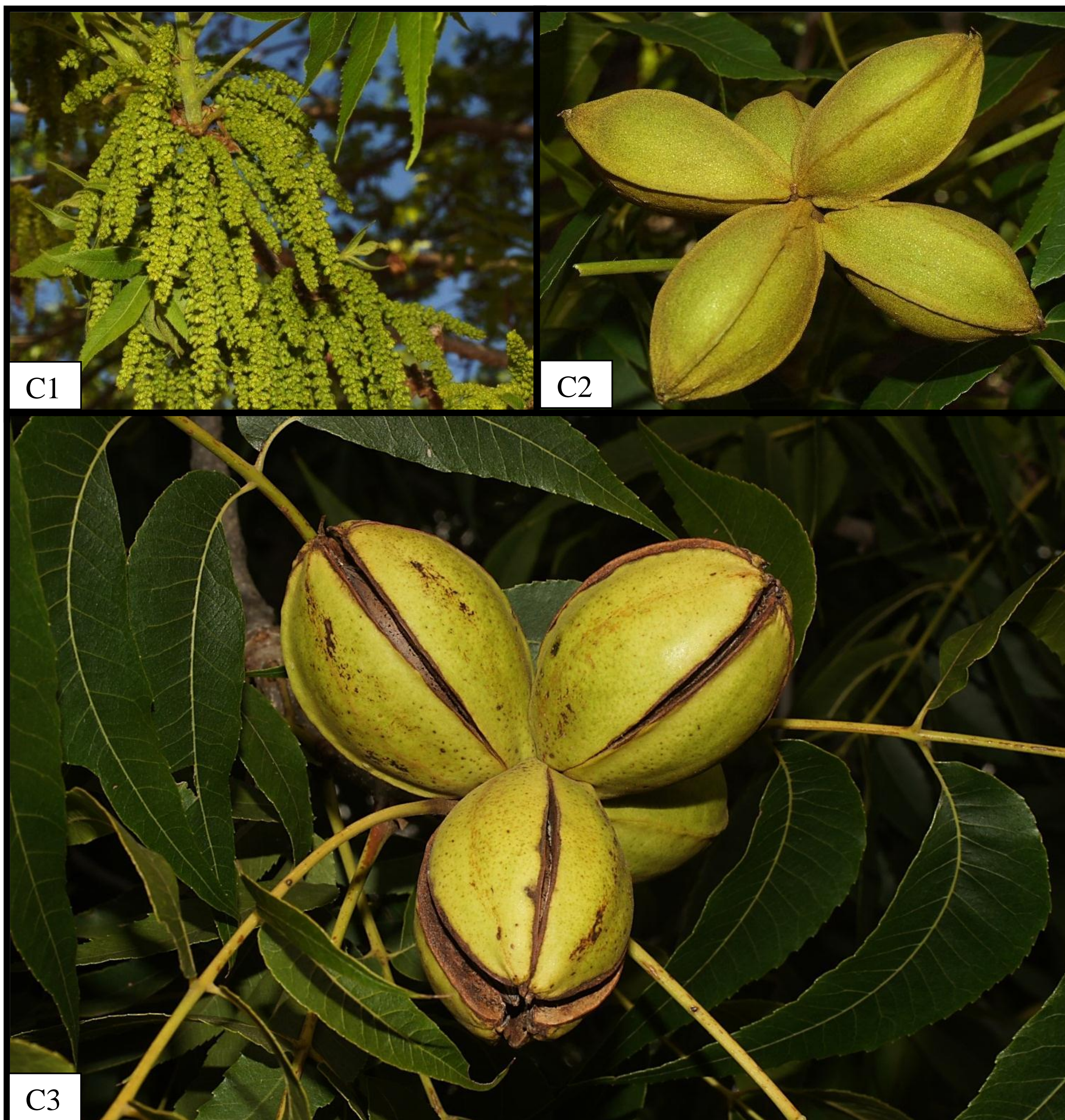

Figure 4.2: The different developmental stages of the pecan nuts on each of the sampling dates (C1, C2 and C3), (Photo by J. Saaiman).

Figure 4.4: A grey-brown stink bug visiting a pecan nut (Photo by J. Saaiman).

Figure 4.5: The ventral side of the grey-brown stinkbug showing its opisthognathous piercing-sucking mouth parts that it uses to feed on pecan nuts (Photo by J. Saaiman).

Figure 4.6: The AES air sampler setup for the collection of fungal propagules from the air at one of the selected trees in the pecan orchard (Photo by J. Saaiman).

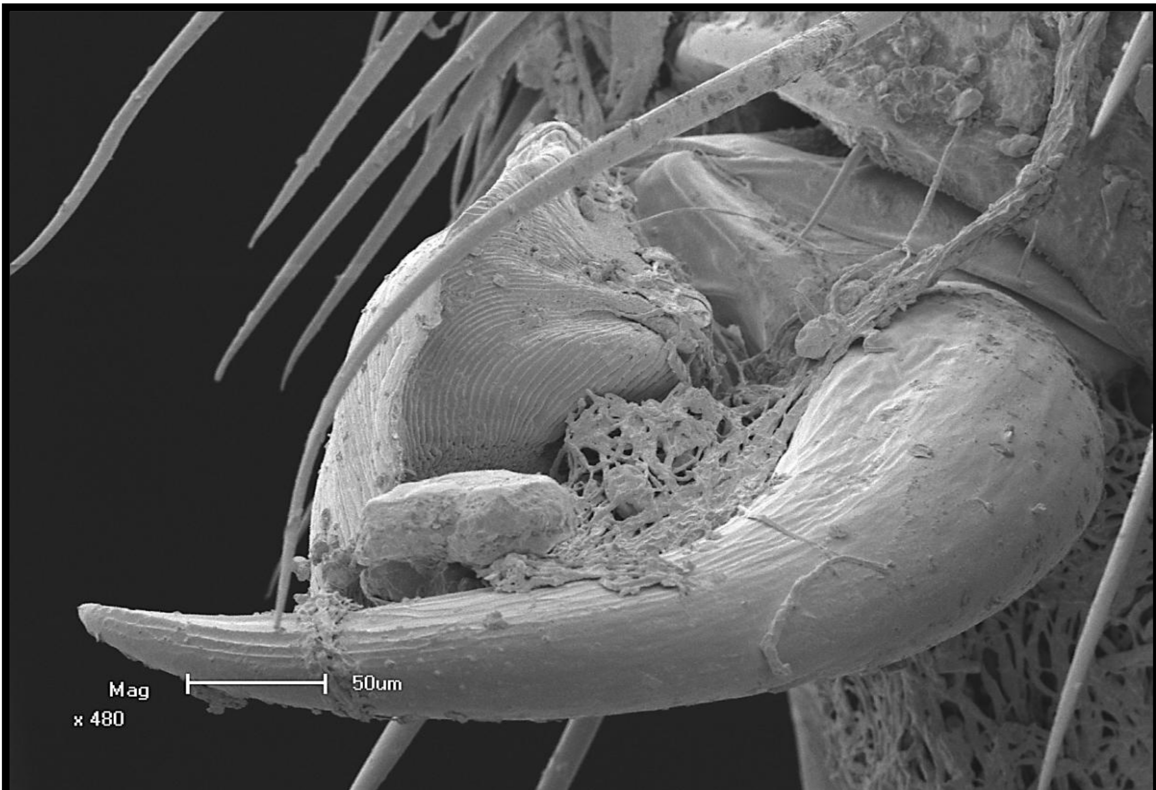

Figure 4.7: The pre-tarsal segment of a grey-brown stinkbug entangled with fungal hyphae (Photo by J. Saaiman).

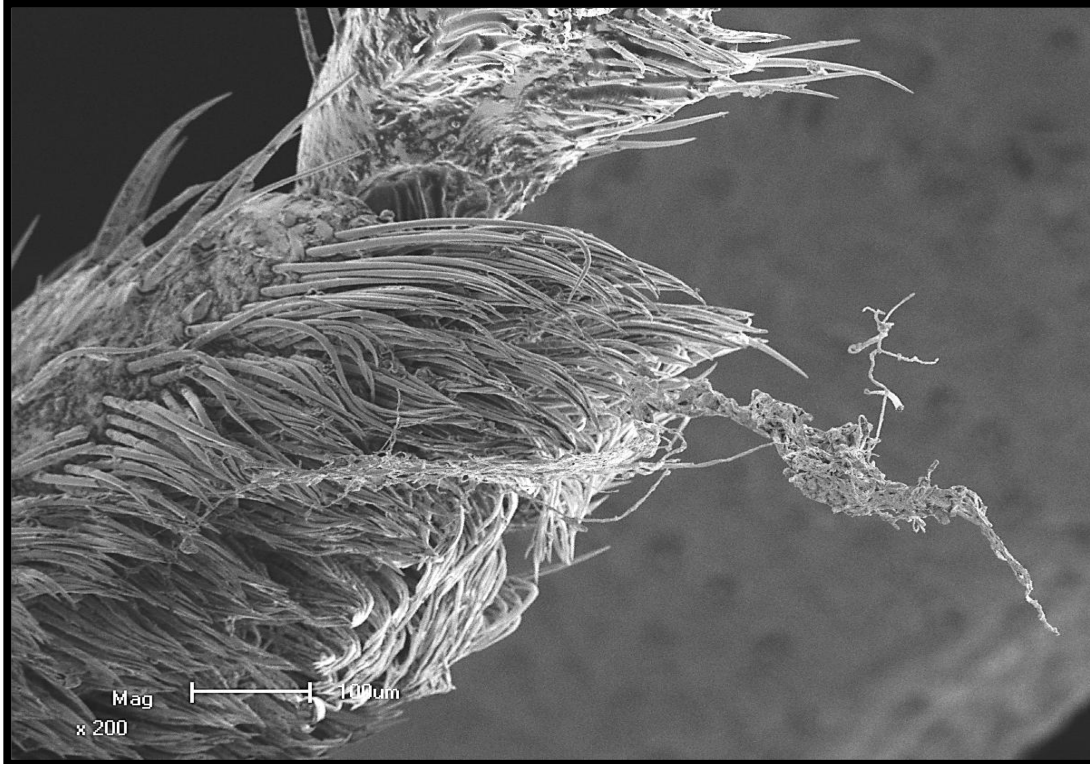

Figure 4.8: The seta on a tarsal segment of the grey-brown stink bug with a lose hanging piece of hyphae (Photo by J. Saaiman).

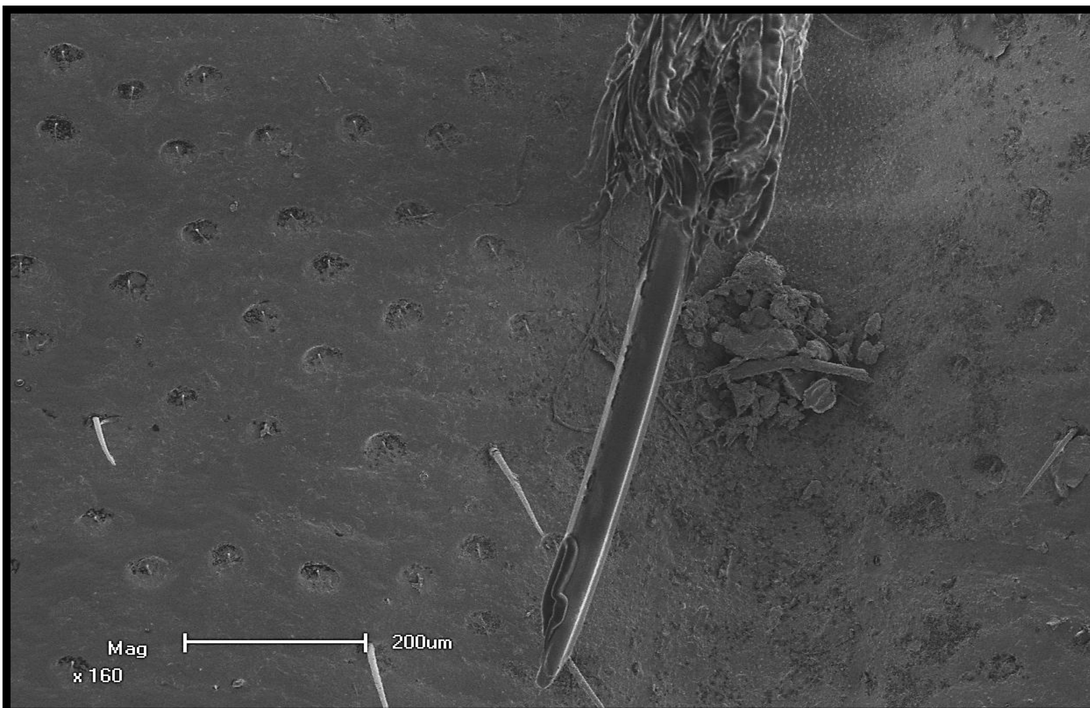

Figure 4.9: The tip of the grey-brown stink bugs piercing-sucking mouthparts that are used to pierce pecan nuts and apparent fungal material in the grove on its abdomen in which the mouthparts rest (Photo by J. Saaiman).

Figure 4.10: Extreme infection and colonisation of pecan nuts by fungal contaminants (Photo by J. Saaiman).

Table 4.1 (A): Fungal species isolated from the air samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Colony Forming Units (CFU's)/ m^3 Air			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Pathogens:				
Alternaria alternata	2	0	7	Fungal leafspot & Die-back
Alternaria tenuissima	280.4	41.2	57.6	Fungal leafspot
Aureobasidium pullulans	0	0	9	Stigmatomycosis
Fusarium oxysporum	6	0	1	Die-back
Potential Pathogens & Contaminants:				
Aspergillus awamori	6.6	0	0	N/A
Aspergillus candidus	2	0	0	N/A
Aspergillus flavus	3.4	0	0	N/A
Aspergillus niger	5	0.2	0	N/A
Aspergillus niveus	0	0	0.2	N/A
Aspergillus ochraceus	1.6	0.6	4.4	N/A
Aspergillus terreus	0	0.2	0	N/A
Aspergillus versicolor	0	0	0	N/A
Botrytis cinerea	3.8	0	0	N/A
Cladosporium cladosporioides	1385	637	870	N/A
Cladosporium pseudocladosporioides	0	4	0	N/A
Colletotrichum coccodes	0	0	1.2	N/A
Coniothyrium fuckelli	0	1	0	N/A
Epicoccum nigrum	4.2	0.4	12.6	N/A
Epicoccum sorghi	11.8	8.2	15.2	N/A
Eurotium repens	0.1	0	1.2	N/A
Fusarium equiseti	0	0	7	N/A
Fusarium incarnatum	7	2.8	13	N/A
Microsphaeropsis olivacea	0	0	1.4	N/A
Mucor circinelloides	0.4	1	2.8	N/A
Mucor hiemalis	4.6	0.2	0	N/A
Paecilomyces lilacinus	0	3.8	0	N/A
Penicillium aurantiogriseum	0	0.4	6.2	N/A
Penicillium brevicompactum	44.4	0	13.2	N/A
Penicillium canescens	2	0	0	N/A
Penicillium citrinum	11.6	0	0	N/A
Penicillium crustosum	0	0.6	0	N/A

Table 4.1 (B): Fungal species isolated from the air samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Colony forming Units (CFU's)/ m^3 Air			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Potential Pathogens & Contaminants (Cont.):				
Penicillium decumbens	0	0	8.6	N/A
Penicillium janthinellum	49.2	12.6	0	N/A
Peyronellaea sancta	7.2	0	1	N/A
Rhizopus oryzae	1.6	0.2	9.8	N/A
Sclerotinia sclerotiorum	6	0	1	N/A
Non-Pathogenic Fungi:				
Acremonium sp.	0	0	0.2	N/A
Trichoderma ghanense	2.6	1.2	10.2	N/A

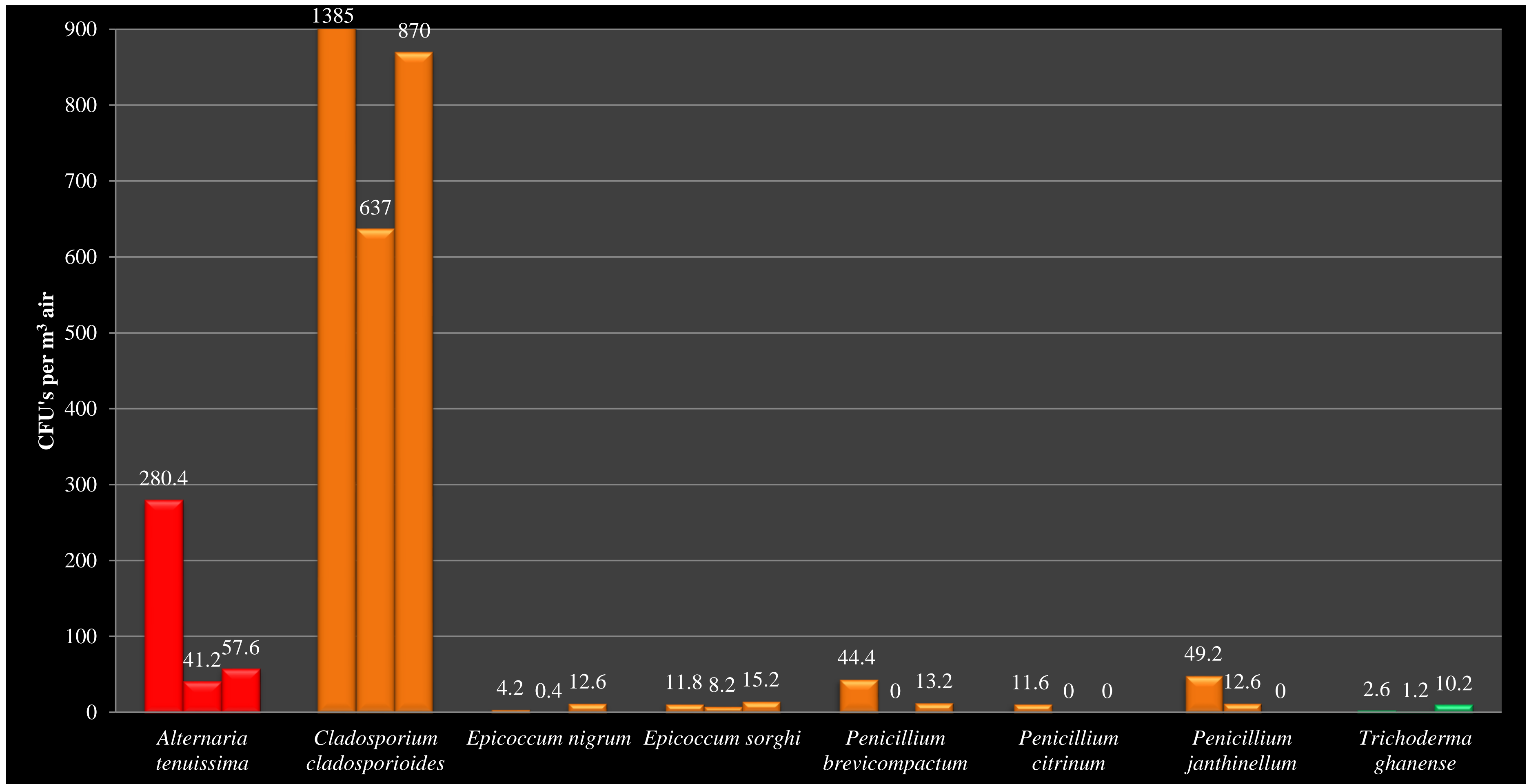

Figure 4.11: The colony forming units (CFU's) per m^3 of air of the most prominent fungal species isolated from the air samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Red bars - Pathogens, Orange bars - Potential pathogens & contaminants & Green bars - Non-pathogenic fungi).

Table 4.2 (A): Fungal species isolated from the soil samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Colonie forming Units (CFU's)/ g Soil			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Pathogens:				
Alternaria alternata	0	0	12	Fungal leafspot & Die-back
Alternaria tenuissima	5	3	31	Fungal leafspot
Aureobasidium pullulans	2	0	12	Stigmatomycosis
Botryosphaeria ribis	1	0	0	Water stage nut drop & Stem end blight
Fusarium oxysporum	4	3	1	Die-back
Potential Pathogens & Contaminants:				
Aspergillus aliaceus	0	32	0	N/A
Aspergillus candidus	0	2	0	N/A
Aspergillus carneus	2	0	0	N/A
Aspergillus niger	3	14	0	N/A
Aspergillus ochraceus	30	79	38	N/A
Cladosporium cladosporioides	5201	103	1112	N/A
Colletotrichum coccodes	2	0	0	N/A
Coniothyrium insitivum	0	0	1	N/A
Epicoccum nigrum	2	0	8	N/A
Epicoccum sorghi	3	27	14	N/A
Eurotium repens	0	1	0	N/A
Fusarium equiseti	12	85	54	N/A
Fusarium incarnatum	0	16	0	N/A
Fusarium laceratum	1	0	0	N/A
Gliocladium sp.	1	0	0	N/A
Khuskia oryzae	2	0	0	N/A
Mucor circinelloides	11	7	8	N/A
Mucor hiemalis	11	93	51	N/A
Penicillium aurantiogriseum	0	10	0	N/A
Penicillium brevicompactum	432	658	40	N/A
Penicillium citreonigrum	1	0	0	N/A
Penicillium citrinum	0	32	30	N/A
Penicillium crustosum	0	1	60	N/A
Penicillium decumbens	1	0	0	N/A

Table 4.2 (B): Fungal species isolated from the soil samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Colonie forming Units (CFU's)/ g Soil			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Potential Pathogens & Contaminants (Cont.):				
Penicillium janczewskii	0	0	40	N/A
Penicillium janthinellum	4	2	54	N/A
Penicillium pinophilum	1	0	0	N/A
Penicillium restrictum	0	1	0	N/A
Penicillium spinulosum	0	0	40	N/A
Penicillium variabile	9	0	0	N/A
Penicillium veruculosum	0	2	0	N/A
Peyronellaea coffeae-arabicae	0	0	2	N/A
Peyronellaea sancta	0	0	2	N/A
Rhizopus oryzae	0	6	6	N/A
Non-Pathogenic Fungi:				
Humicola fuscoarta	0	0	1	N/A
Sporothrix sp.	2	0	0	N/A
Trichoderma ghanense	4	13	58	N/A
Trichoderma reesei	1	0	0	N/A
Trichoderma viride	2	0	0	N/A

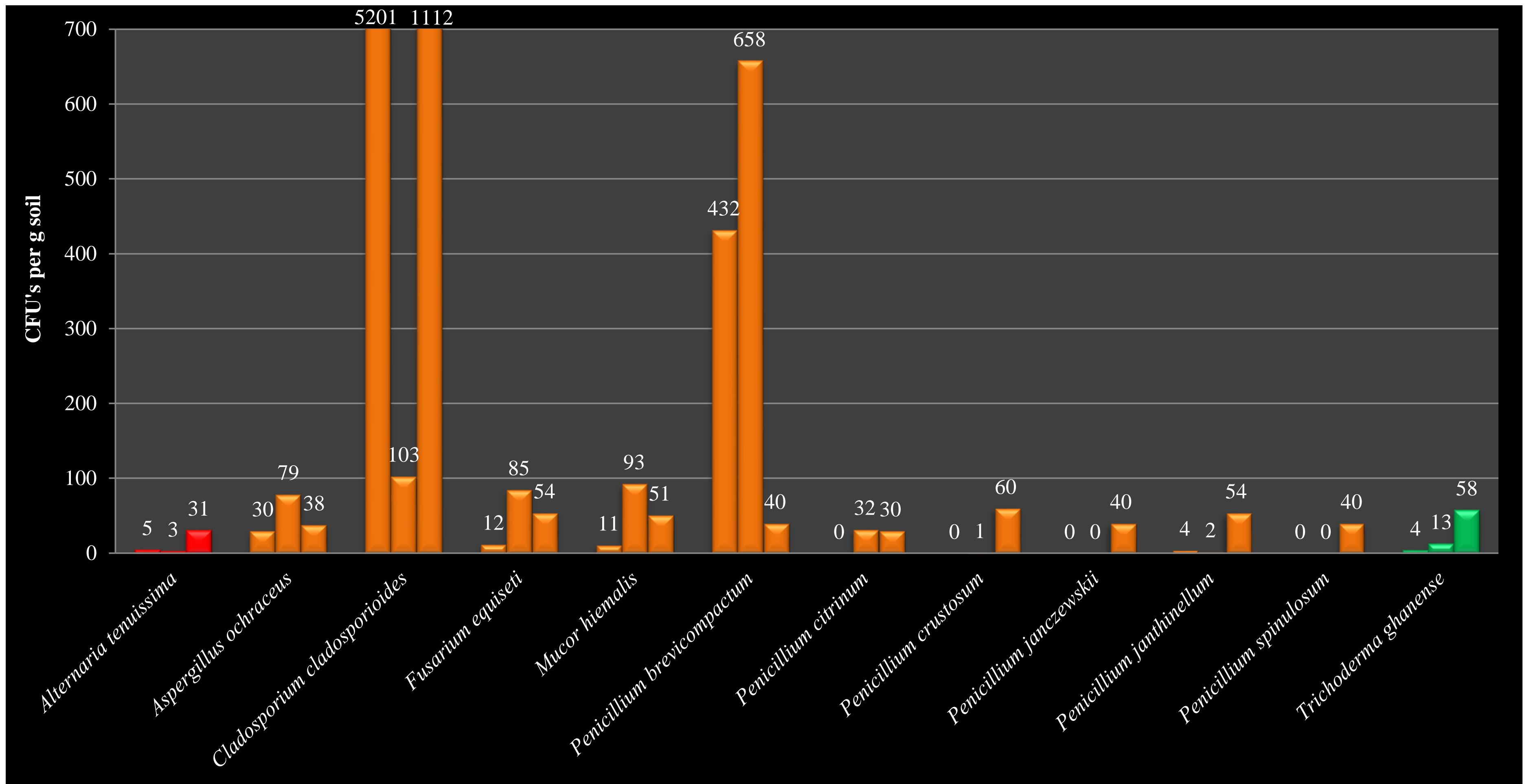

Figure 4.12: The colony forming units (CFU's) per gram soil of the most prominent fungal species isolated from the soil samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Red bars - Pathogens, Orange bars - Potential pathogens & contaminants & Green bars - Non-pathogenic fungi).

Table 4.3 (A): Fungal species isolated from the leaf samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Percentage (%)			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Pathogens:				
Alternaria tenuissima	26.6	88.2	97.4	Fungal leafspot
Aureobasidium pullulans	0.4	0.2	0	Stigmatomycosis
Fusarium oxysporum	0.6	1	1.6	Die-back
Potential Pathogens & Contaminants:				
Aspergillus awamori	0.2	0	0	N/A
Aspergillus niger	0.2	0.6	0.2	N/A
Byssochlamys spectabilis	0	0.2	0	N/A
Cladosporium cladosporioides	82.2	96	97.2	N/A
Cochliobolus spicifer	0	2.4	0	N/A
Colletotrichum coccodes	0	0	0.2	N/A
Coniothyrium pyrium	0.4	0	0	N/A
Epicoccum nigrum	1	5.2	12	N/A
Epicoccum sorghi	3.2	69.4	78	N/A
Fusarium equiseti	0.6	2.4	3.8	N/A
Fusarium incarnatum	0.8	31.8	6.2	N/A
Fusarium sporotrichioides	0.2	0	0	N/A
Khuskia oryzae	6.2	1	3.6	N/A
Mucor circinelloides	0	0.8	0.2	N/A
Mucor hiemalis	0	0	4.4	N/A
Penicillium chrysogenum	2.8	0	0	N/A
Penicillium citrinum	0.4	0	0	N/A
Penicillium crustosum	0	7	0	N/A
Penicillium decumbens	0	0	2.6	N/A
Penicillium janthinellum	0.4	0	0	N/A
Phoma glomerata	0	0	7	N/A
Peyronellaea americana	0	5.2	0	N/A
Peyronellaea coffeae-arabicae	0.2	0	0	N/A
Peyronellaea eucalyptica	0	3.8	0	N/A
Peyronellaea glomerata	0	0.2	0	N/A
Peyronellaea sancta	1.6	9.6	0.6	N/A
Rhizopus oryzae	1.6	2.2	2.2	N/A

Table 4.3 (B): Fungal species isolated from the leaf samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Percentage (%)			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Non-Pathogenic Fungi:				
Acremonium sp.	0.2	0	0.4	N/A
Gonatobotrys simplex	0	0	0.2	N/A
Leptodontium sp.	0	0	0.2	N/A
Trichoderma ghanense	0.4	0.4	1.2	N/A
Truncatella angustata	0	0.2	0	N/A

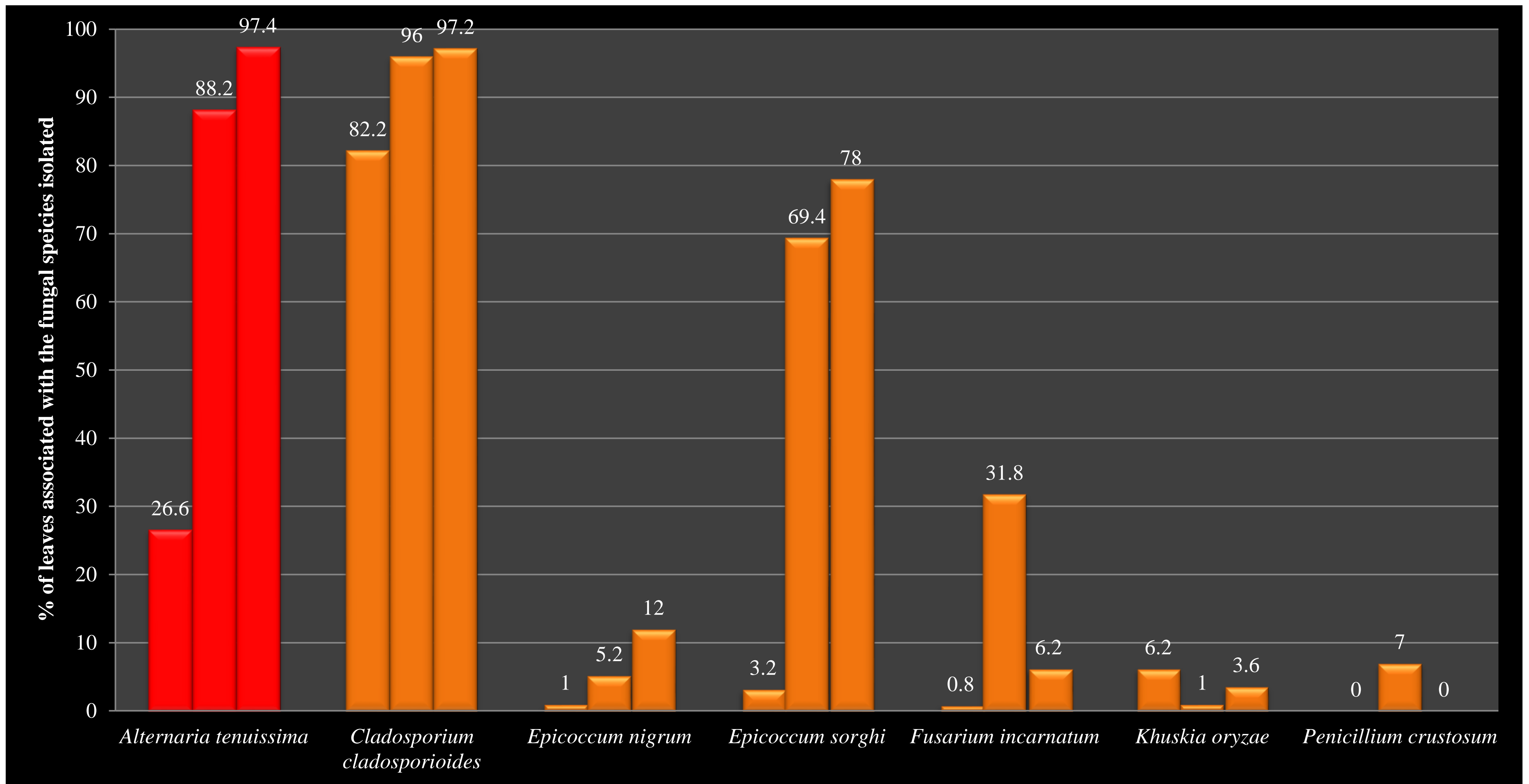

Figure 4.13: The percentage of leaves associated with the most prominent fungal species isolated from the leaf samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Red bars - Pathogens, Orange bars - Potential pathogens & contaminants & Green bars - Non-pathogenic fungi).

Table 4.4: Fungal species isolated from the nut samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Percentage (%)			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Pathogens:				
Alternaria alternata	0	0	2.2	Fungal leafspot & Die-back
Alternaria tenuissima	0	11.2	59.4	Stigmatomycosis
Fusarium oxysporum	0	0	0.2	Die-back
Neofusicoccum parvum	0	25.2	41.8	Fungal leafspot & Blackening of the husk
Potential Pathogens & Contaminants:				
Aspergillus niger	0	0.2	0	N/A
Aspergillus versicolor	0	0.2	0	N/A
Byssochlamys spectabilis	0	0	0.4	N/A
Cladosporium cladosporioides	0	0.2	5	N/A
Colletotrichum coccodes	0	0.2	0	N/A
Coniothyrium fuckelli	0	1	0	N/A
Epicoccum nigrum	0	0	2.6	N/A
Epicoccum sorghi	0	0.8	4	N/A
Fusarium equiseti	0	0	1	N/A
Fusarium incarnatum	0	0	6.2	N/A
Fusarium nygamai	0	0	0.4	N/A
Khuskia oryzae	0	0.2	0.6	N/A
Microsphaeropsis olivacea	0	5.4	10.6	N/A
Penicillium aurantiogriseum	0	0.8	0	N/A
Penicillium brevicompactum	0	0	0.4	N/A
Penicillium decumbens	0	0.6	0	N/A
Penicillium janthinellum	0	2.2	0	N/A
Peyronellaea coffeae-arabicae	0	0.4	0	N/A
Peyronellaea sancta	0	1.8	1.8	N/A
Rhizopus oryzae	0	0	1.4	N/A
Non-Pathogens:				
Acremonium sp.	0	0.2	9	N/A
Chaetomium globosum	0	0	5	N/A
Gonatobotrys simplex	0	**0**	6	N/A
Leptodontium sp.	0	0	0.2	N/A

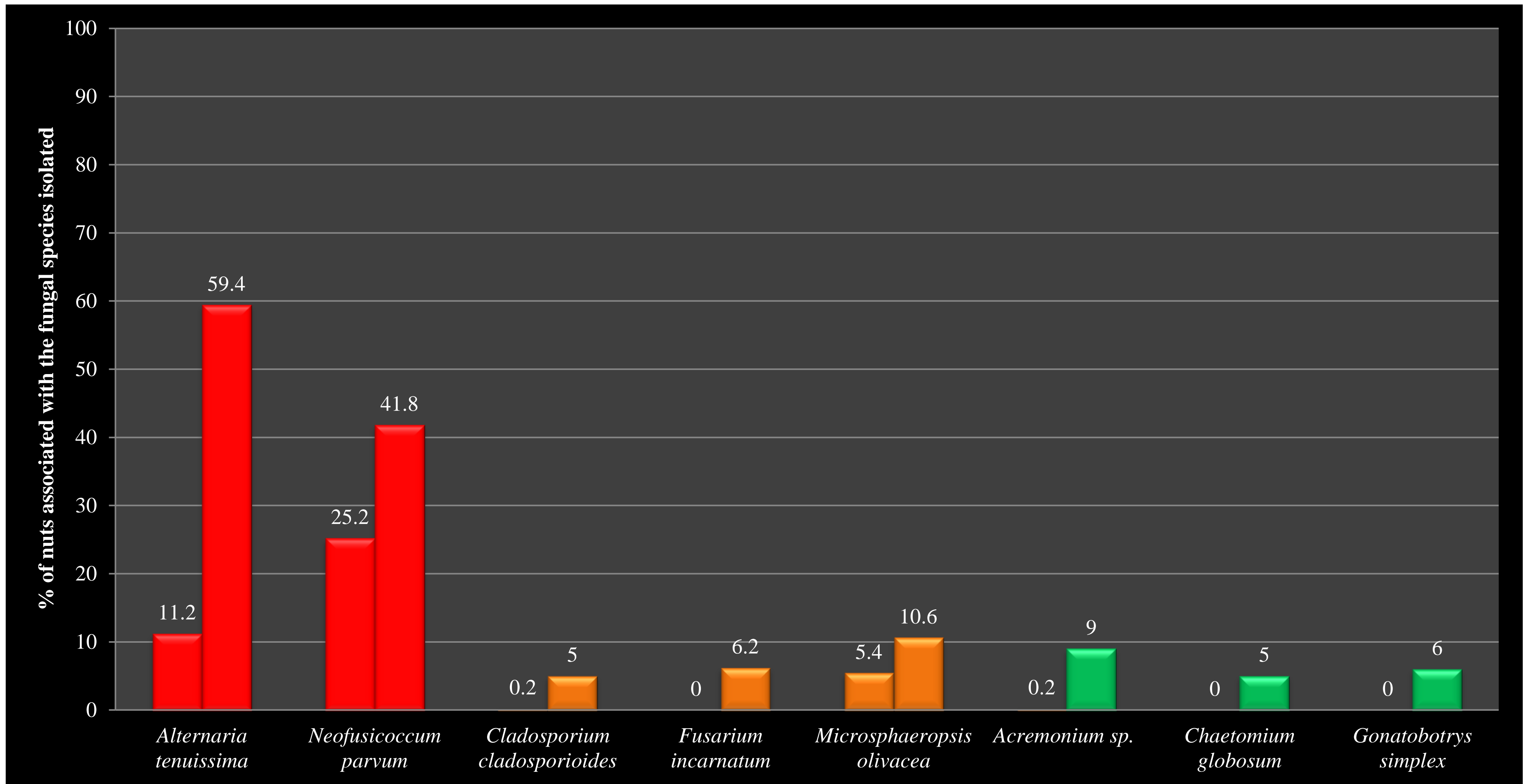

Figure 4.1: The percentage of nuts associated with the most prominent fungal species isolated from the nut samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Red bars - Pathogens, Orange bars - Potential pathogens & contaminants & Green bars - Non-pathogenic fungi).

Table 4.5 (A): Fungal species isolated from the stink bug samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Percentage (%)			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Pathogens:				
Alternaria alternata	0	1	1	Fungal leafspot & Die-back
Alternaria tenuissima	46	20	22	Fungal leafspot
Aureobasidium pullulans	1	0	0	Stigmatomycosis
Botryosphaeria ribis	1	0	0	Water stage nut drop & Stem end blight
Fusarium oxysporum	1	4	6	Die-back
Neofusicoccum parvum	9	12	0	Fungal leafspot & Blackening of the husk
Potential Pathogens & Contaminants:				
Aspergillus flavus	0	2	1	N/A
Aspergillus niger	2	1	1	N/A
Aspergillus ochraceus	2	0	0	N/A
Botrytis cinerea	1	0	0	N/A
Byssochlamys spectabilis	1	0	1	N/A
Cladosporium cladosporioides	35	0	15	N/A
Cochliobolus lunatus	0	1	0	N/A
Cochliobolus sativus	0	1	0	N/A
Cochliobolus spicifer	1	6	0	N/A
Colletotrichum coccodes	0	0	1	N/A
Curvularia clavata	0	1	0	N/A
Epicoccum sorghi	15	4	3	N/A
Fusarium camptoceras	0	1	9	N/A
Fusarium equiseti	13	6	4	N/A
Fusarium incarnatum	0	3	0	N/A
Khuskia oryzae	4	3	1	N/A
Microdochium nivalae	0	0	1	N/A
Mucor circinelloides	4	19	1	N/A
Mucor hiemalis	5	11	0	N/A
Neosartorya fischeri	0	0	2	N/A
Paecilomyces lilacinus	0	0	1	N/A
Penicillium aurantiogriseum	0	14	0	N/A
Penicillium chrysogenum	12	1	3	N/A

Table 4.5 (B): Fungal species isolated from the stink bug samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Northern Cape Province, South Africa).

	Percentage (%)			
Fungal Species Isolated:	C 1	C 2	C 3	**Disease:**
Potential Pathogens & Contaminants (Cont.):				
Penicillium citreonigrum	0	1	0	N/A
Penicillium decumbens	0	0	9	N/A
Penicillium janthinellum	4	0	0	N/A
Penicillium spinilosum	4	0	0	N/A
Pestalotiopsis sp.	0	0	1	N/A
Phoma brasiliensis	1	0	0	N/A
Peyronellaea eucalyptica	0	0	9	N/A
Peyronellaea glomerata	2	0	1	N/A
Peyronellaea pomorum	0	0	2	N/A
Peyronellaea sancta	9	2	0	N/A
Rhizopus oryzae	0	8	7	N/A
Sclerotinia sclerotiorum	1	0	0	N/A
Ulocladium sp.	0	0	2	N/A
Non-Pathogenic Fungi:				
Acremonium sp.	7	2	2	N/A
Arthrobotrys oligospora	0	1	0	N/A
Chaetomium funicola	0	1	0	N/A
Chaetomium globosum	2	1	5	N/A
Chaetomium sp.	0	1	0	N/A
Humicola fuscoarta	1	0	0	N/A
Humicola grisea	0	0	1	N/A
Melanospora sp.	0	0	1	N/A
Moniliella acetobutans	0	0	2	N/A
Monodictys paradoxa	1	0	0	N/A
Preussia sp.	0	0	2	N/A
Sporothrix sp.	1	0	0	N/A
Trichoderma ghanense	0	0	1	N/A
Trichoderma reesei	0	1	0	N/A
Trichoderma viride	4	0	0	N/A
Other				
None	11	21	19	N/A

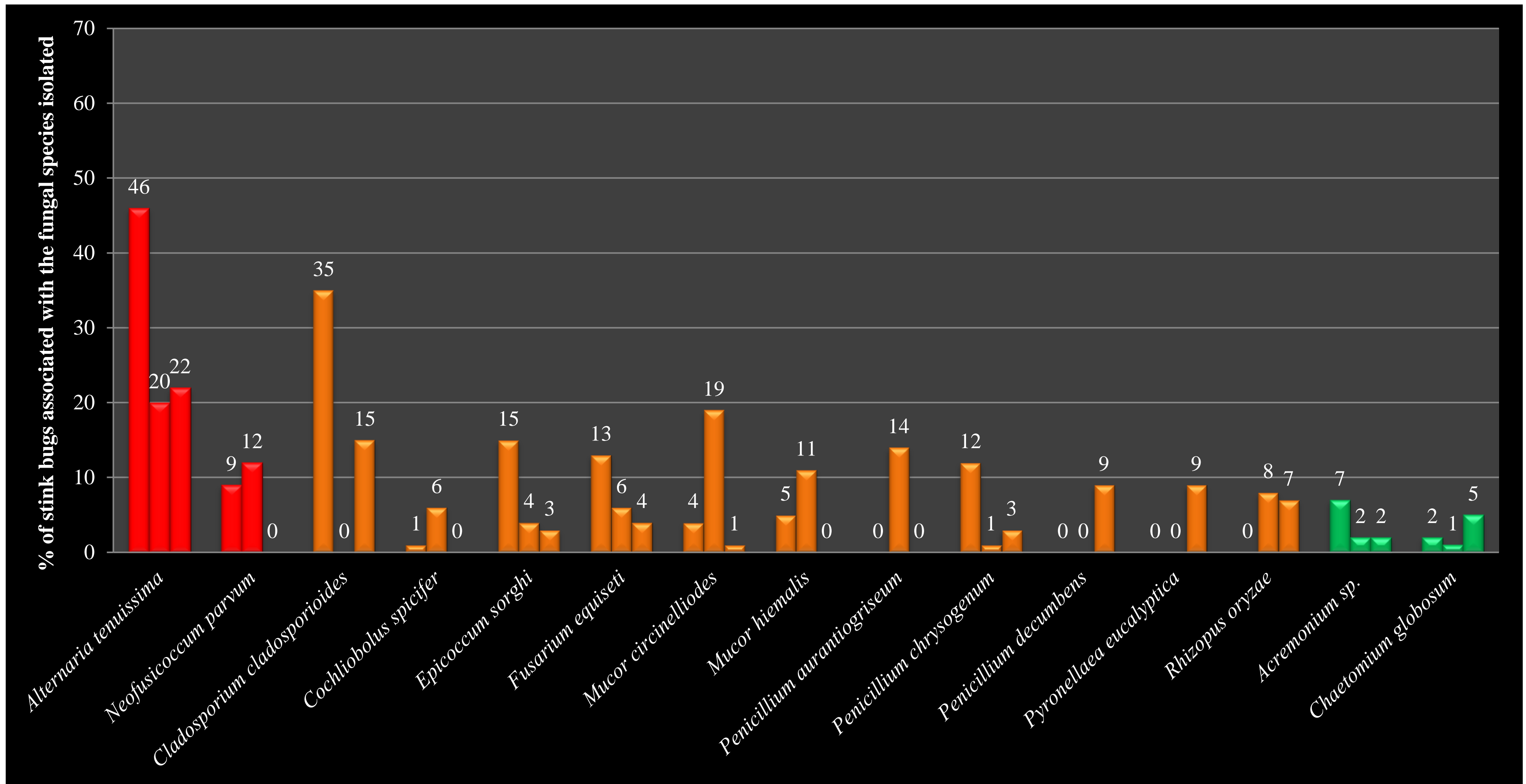

Figure 4.15: The percentage of stink bugs associated with the most prominent fungal species isolated from the stink bug samples taken during three site visits (C 1, C 2 & C 3) throughout the 2011-2012 season in a pecan orchard on the farm 7M8 in the Vaalharts region (Red bars - Pathogens, Orange bars - Potential pathogens & contaminants & Green bars - Non-pathogenic fungi).

Table 4.6: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the air samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Air Sample:	**C 1**	**C 2**	**C 3**
C 1	1	0.57143	0.6383
C 2	0.57143	1	0.4878
C 3	0.6383	0.4878	1

Table 4.7: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the soil samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Soil Sample:	**C 1**	**C 2**	**C 3**
C 1	1	0.4898	0.52
C 2	0.4898	1	0.62222
C 3	0.52	0.62222	1

Table 4.8: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the leaf samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Leaf Sample:	**C 1**	**C 2**	**C 3**
C 1	1	0.57143	0.56
C 2	0.57143	1	0.61224
C 3	0.56	0.61224	1

Table 4.9: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the nut samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Nut Samples:	**C 1**	**C 2**	**C 3**
C 2	**N/A**	1	0.44444
C 3	**N/A**	0.44444	1

Table 4.10: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the stink bug samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Stink bug Samples:	**C 1**	**C 2**	**C 3**
C 1	1	0.49123	0.43333
C 2	0.49123	1	0.47458
C 3	0.43333	0.47458	1

Table 4.11: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the nut & leaf samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Samples:	**C 1 (Leaves)**	**C 2 (Leaves)**	**C 3 (Leaves)**
C 2 (Nuts)	0.48649	0.32432	0.5
C 3 (Nuts)	0.53659	0.53659	0.65

Table 4.12: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the Leaf & stink bug samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Samples:	**C 1 (Stink bugs)**	**C 2 (Stink bugs)**	**C 3 (Stink bugs)**
C 1 (Leaves)	0.48	0.46809	0.42308
C 2 (Leaves)	0.44	0.46809	0.42308
C 3 (Leaves)	0.44898	0.52174	0.5098

Table 4.13: The Sørenson's quotation values obtained from comparing the similarity of the fungal species assemblages associated with the nut & stink bug samples taken during the three different sampling visits (C 1, C 2 & C 3) throughout the 2011-2012 season.

Samples:	**C 1 (Stink bugs)**	**C 2 (Stink bugs)**	**C 3 (Stink bugs)**
C 2 (Nuts)	0.3913	0.35556	0.33333
C 3 (Nuts)	0.4	0.4898	0.38462

Chapter 5

The Pathogenicity of Fungi Associated with the Grey-brown Stink Bug on Pecans

5.1 Introduction

The pecan (*Carya illinoensis*) is subject to attack by several fungal pathogens (De Villiers & Joubert, 2008). Currently, in South Africa the only disease of consequence is scab caused by *Fusicladium effusum* (De Villiers & Joubert, 2008). Other diseases and potential pathogens that have been noted to occur at minor incidences include anthracnose caused by *Colletotrichum gloeosporioides* and leaf spot caused by *Cercospora caryae* (Crous, Phillips & Baxter, 2000; De Villiers & Joubert, 2008). These diseases are mostly a problem in the lowveld, where the high rainfall and humidity favour disease development, especially scab (De Villiers & Joubert, 2008). Conditions in the western high altitude regions (Hartswater and Orange River regions) of South Africa are not conducive to diseases and pathological problems are usually caused in combination with nutrient deficiencies such as zinc and nickel (De Villiers & Joubert, 2008). Disease symptoms of unknown sources have been noted as the pecan industry expanded in this region over the last decade.

During the course of this study it has been noted that a combination of *Alternaria* spp. and *Cladosporium cladosporioides* are the most abundant fungal species to occur on insects in pecan orchards (Chapter 3 & 4). It is suspected that these fungi might function as pathogens of pecans in South Africa. No evidence suggests that *C. cladosporioides* is a pathogen of pecans; however, this fungus can cause disease on other tree nut crops such as macadamia where it is responsible for raceme blight (Van den Berg *et al.*, 2008) and blossom blight on almonds (Crous *et al*., 2000). *Alternaria* spp. are known as miscellaneous pathogens of pecans, being able to cause leaf spots that lead to premature leaf and nut drop (Anon., 2006). This phenomenon has not yet been documented in South Africa. Another potential pathogen that was noted is *Neofusicoccum parvum* (Chapter 4). The research showed that this fungus only occurred on stink bugs and on the pecan nuts. *Neofusicoccum parvum* is a member of the Botryosphaericeae, a group of fungi that contains several serious pathogens of trees (Crous *et al*. 2000; Damm, Crous, & Fourie, 2007). No previous evidence exists that indicates an association between *N. parvum* and pecans. Since *N. parvum* only occurred on the sampled stink bugs and pecan nuts it is likely that an association exists between the insect and the fungus to spread to the nuts. Such associations have been noted to occur between other members of the Botryosphaericeae (*Botryosphaeria dothidea*) and stink bugs occurring in pistachio orchards (Michailides &

Morgan, 1996; Steffan, Daane & Yokota, 2000). The main aim of the study was to confirm the grey-brown stink bug is associated with fungal pathogens of pecan. Secondly, the pathogenicity of *Alternaria tenuissima*, *Cladosporium cladosporioides* and *Neofusicoccum parvum* was determined on pecans. Thirdly, the effect of wounding *vs*. non-wounded on disease development was compared.

5.2 Materials and Methods

5.2.1 Fungal isolates

5.2.1.1 Fungal isolation from the grey-brown stink bug

Isolates were obtained from the collected grey-brown stink bug specimens (Fig. 5.1) from the Vaalharts region as described in detail in Chapter 4. Single hyphal tips were removed from pure colonies of *Alternaria tenuissima*, *Cladosporium cladosporioides* and *Neofusicoccum parvum* as described in detail in Chapter 2. The tips were sub-cultured and used to confirm the identity of the fungi to be used in the pathogenicity trials.

5.2.1.2 Identification of fungal isolates

The fungal colonies were initially identified based on their morphological characteristics such as fruiting structures, spore shape and size, as well as colony texture and colour. Confirmation of the identity of *A. tenuissima*, *C. cladosporioides* and *N. parvum* was done by using molecular techniques. The single hyphal tip cultures were grown on PDA plates for 14 days. The harvesting of fungal material and DNA extraction was done as described in Chapter 2. Once extracted the ITS region of the rDNA operon was amplified using the primers ITS 1F (5´-CTTGGTCATTTAGAGGAAGTAA) and ITS 4 (5´-TCCTCCGCTTATTGATATGC). Additional gene regions, other than the ITS region, were also analysed for each of the three species. Both genomic DNA and pure cultures were sent to Inquaba Biotec for analyses. For *A. tenuissima* the TEF1 gene region was amplified with the primers EF1-728F (5´-CATCGAGAAGTTCGAGAAGG) and EF1- 986R (5´-TACTTGAAG GAACCCTTACC) (Carbone & Kohn, 1999; Woudenberg *et al.*, 2013) and the RPB2 region with RPB2–5F2 (5´-GGGGWGAYCAGAAGAAGGC) and fRPB2–7cR (5´-CCCATRGCTTGYTTRCCCAT) (Liu, Whelen & Hall, 1999; Sung, Sung, Hywel-Jones & Spatafora, 2007; Woudenberg *et al.*, 2013).

For *C. cladosporioides* the Actin gene region was amplified with the primers ACT-512F (5´-ATGTGCAAGGCCGGTTTCGC) and ACT-783R (5´-TACGAGTCCTTCTGGCCCAT) (Carbone & Kohn, 1999). For *N. parvum* the EF gene region was amplified with the primers EF-AF (5'-CATCGAGAAGTTCGAGAAGG) and EF-BR (5´-CRATGGTGATACCRCGCTC) (Pavlic *et al.*, 2009) and the RPB2 region with RPB2bot6F (5´-GGTAGCGACGTCACTCCC) and RPB2bot7R (5´-GGATGGATCTCGCAATGCG) (Pavlic *et al.*, 2009). The resulting sequence data was analyzed using GeneiousPro V-5.6.5 and searched on MycoBank for corresponding fungal sequences.

5.2.1.2 Preparation of the fungal colonies for the pathogenicity trials

Pure colonies of the test fungi, including *Alternaria tenuissima*, *Cladosporium cladosporioides* and *Neofusicoccum parvum* were grown on PDA plates to obtain enough inoculum for the pathogenicity trials.

5.2.2 Pathogenicity trials

Trials were conducted under both laboratory and greenhouse conditions. The effect of the isolates on pecan nuts was tested using a modification of the detached leaf assay protocol proposed by Keith & Zee (2010). The pathogenicity of the isolates on pecan leaves was tested on young pecan trees kept under greenhouse conditions using a similar protocol as with the detached leaf assay. This option was selected due to the difficulties that some isolates had in producing measurable levels of conidia.

5.2.2.1 Detached leaf (nut) assays

Nuts were collected at a pecan farm in the Petrusburg district, Free State province, South Africa. A single pecan cultivar, Wichita, was selected for the pathogenicity trials. This cultivar was selected, since it is most commonly planted in the western regions of South Africa. Nuts were collected during February 2013 from Wichita trees between 10 – 12 years of age. During February, the nuts are almost fully grown but the endosperm remains soft since the nuts are still ripening (Fig. 5.2). Nuts were collected by hand from ± 20 trees. Only healthy nuts, that showed no disease symptoms or physiological abnormalities, were collected. The nuts were transported in a cooler box to the laboratories of the University of the Free State.

The nuts were initially washed in sterilized distilled water to remove dust and dirt. The nuts were then surface sterilized by soaking them in 76% ethanol for one minute, after which they were twice rinsed in distilled and sterile water for one minute. The nuts were air dried in a laminar flow hood for one hour. The nuts were then inoculated with ± 0.3 x 0.3 cm agar plugs, which were cut from the fungal colonies grown on the PDA plates. The plugs were cut using a sterile scalpel and then removed from the plates using a sterile dissection needle. The plugs were placed in the centre of the nuts with the fungal growth against the surface of the husk. The plugs were gently pressed against the nuts with the dissection needle so that it would remain in place (Fig. 5.3). A similar process was followed for the control, except that the nuts were inoculated with PDA plugs devoid of any fungal material. In total, 60 nuts were inoculated with plugs from each of the fungal isolates and with PDA plugs for the control respectively. To investigate the role of wounding 30 of the 60 nuts for each isolate and the control were wounded in a fashion that represents stink bug feeding. This was done by piercing the husks of the nuts with a thin, sterilized dissection needle (Fig. 5.4). The needle was pressed through the husk into the shell of the nut. The plugs were then placed over the wound. To observe the effect of wounding on the husk an additional ten nuts were wounded but not inoculated.

Once the nuts were inoculated, they were placed in 25 ℓ plastic containers (Fig. 5.5 & 5.6). The nuts inoculated with a particular fungal isolate were kept in the same container, but separate from nuts inoculated with the other fungal isolates to prevent cross contamination. The nuts were suspended ± 4 cm from the bottom on diamond mesh (Fig. 5.7). Before the nuts were placed in the containers the bottom was filled with 3 ℓ sterile distilled water. The water prevented the nuts from drying out and also provided ample humidity for disease development. The plastic containers were sterilized with 76% ethanol and the diamond mesh by autoclaving. The lid of the container was sealed with a layer of plastic to prevent contamination and drying of the nuts and leaves. The containers were incubated at 25 ˚C for the duration of the study (Fig. 5.8). The containers were exposed to 12 hour light / dark cycles

The nuts were inspected for disease symptoms at four and five day intervals (*e.g.* day 4, 9, 14, 19 & 23). The husks were used since most fungal pathogens that affect pecan nuts directly infect the husks, *e.g. Fusicladium effusum* that causes scab (De Villiers & Joubert, 2008). Destruction of the husk by pathogens results in growth deficiencies of the nuts or premature nut

drop (De Villiers & Joubert, 2008). The presence or absences of symptoms were noted and when symptoms did appear the lesions formed were measured using an electronic calliper (Helios Digital). This was done to determine the rate at which disease developed. Only lesions larger than 0.5 X 0.5 cm were taken into account. The size of the infected nuts was also measured to determine the percentage of the nut affected by a fungal isolate.

5.2.2.2 Greenhouse trials

For the pathogenicity trials in the greenhouse ten three-year old (Height ± 150 cm) Wichita saplings were purchased at a local pecan nursery. The saplings were placed in a greenhouse on the premises of the University of the Free State (Fig. 5.9). The saplings were kept in the greenhouse for one month before the trials were conducted. This was done to allow them to acclimatize and to observe them for any disease symptoms and physiological disorders that might affect the results of the study. The greenhouse was kept at day temperatures of 28 ˚C and night temperatures of 22 ˚C with an average temperature of 25 ˚C. During the course of the study the trees were watered twice a week and no chemicals (pesticides or fertilizers) were used that might affect the results.

Selected leaves of each tree were inoculated with the three different fungal isolates and a control. On each tree four healthy leaves were selected halfway up the stem of the trees. On each leaf six leaflets were selected, three on either side of the rachis, in the middle of each leaf. The selected leaves and leaflets are also the recommended leaves and leaflets that should be sampled for leaf analysis in pecan orchards (De Villiers & Joubert, 2008). Before each leaf was inoculated an attempt was made to surface sterilize the leaflets selected for inoculation. Initially the dust and dirt was wiped from the leaflets with a sterile cotton cloth dipped in sterile distilled water. The leaflets were then wiped with a cotton cloth soaked in 76% ethanol. The ethanol residues were wiped off with a cotton cloth dipped in sterile distilled water. This was done to minimize contamination with other microorganisms in the vicinity. The leaflets were then inoculated similarly to the nuts used in the detached leaf assay. The leaflets were inoculated with plugs of ± 0.3 X 0.3 cm cut from fungal colonies grown on PDA plates. The plugs were placed to one side of the midrib in the centre of the leaflets using a sterile dissection needle. The plugs were placed with the fungal growth against the surface of the leaflets. The plugs were then gently pressed against the leaflet with the dissection needle so that it would stay in place. The plugs

were covered with small blocks of adhesive tape to ensure that the plugs stay in contact with the leaflets for the duration of the study (Fig. 5.10 & 5.11). A similar process was followed for the control, except that the leaflets were only inoculated with PDA plugs without any fungal material. A single leaf, six leaflets in total, of each tree was inoculated with each of the three fungal isolates and a control. To investigate the effect of wounding, three of the six leaflets to one side of the midrib were wounded before inoculation. This was done for each fungal isolate and the control. The leaflets were wounded by making several puncture wounds in the blade of each leaflet with a sterile dissection needle (Fig. 5.12). The plugs were then placed on the puncture wounds during inoculation. Once all the leaves were inoculated they were covered with a plastic bag tied down at the base of the petiole (Fig. 5.13). The plastic bags were kept on the leaves for the first 24 hours after inoculation to raise the humidity that aids with initial infection.

The leaves were inspected for disease symptoms at four and five day intervals (*e.g.* day 4, 9, 14, 19 & 23). The presence or absences of symptoms were noted and when symptoms did appear the lesions formed were measured using an electronic calliper (Helios Digital). Only lesions larger than 0.5 X 0.5 cm were measured. The size of the affected leaves was also measured to determine the percentage of the surface of the leaves affected by a fungal isolate at the end of the study.

5.2.2.3 Confirmation of pathogenicity with Koch's postulates

Once both the detached leaf assays and greenhouse trials were completed the presence of the disease causing organism was confirmed with Koch's postulates on nuts and leaves that showed disease symptoms. Disks of ± 0.5 X 0.5 cm were cut from the lesions that formed on the nuts and leaves after inoculation. These disks were plated on PDA plates. The inoculated PDA plates were incubated at 25 ºC for 7 days to allow fungal development. The fungal colonies were identified based on morphology.

5.2.3 Statistical analysis

Once the results were gathered it was statistically analysed. The presence or absence of infection was noted. The resulting disease incidence was graphically expressed as the percentage of nuts/leaves that showed symptoms on each day of observation. The rate of disease development was calculated using logistic transformation data of the percentage area of the nut

or leaf affected at the end of the study. The rate of disease development between wounded and non-wounded nuts and leaves was determined using regression statistics and compared with a T-test in Microsoft Excel. The rates for the logistic transformation data of the percentage area of the nut or leaf affected was also illustrated with a scatter plot graph in Excel. To determine the percentage area of the nut affected the area of the lesions formed and the size of the nuts/leaves was calculated using the formula for the area of an ovoid shape (Pi x A x B; where A= ½ of the length and B= ½ of the breadth). This formula was selected since it most closely represented the shape of the lesions formed and that of the nuts/leaves. In addition, the rate of onset (5% of the area colonised) and the rate to 100% area of the nut/leaf colonised was calculated with the region statistics and used to compare the effect of wounding and non-wounding on disease development for each isolate.

Any significant differences between the effect of wounding and non-wounding and between the ability of the various isolates to cause disease, an analysis of variance (ANOVA) report was generated using the NCCS (2007) program. The ANOVA report was generated using the area of the lesion, log transformed data for the area of the lesion, the percentage of the nut/leaf affected, arcsine transformed data for the percentage of the nut/leaf affected and area under the curve data for wounded and non-wounded nuts/leaves affected by each isolate. The area under the curve was also calculated with NCCS for each replicate on each day of observation for both wounded and non-wounded nuts/leaves with the areas of the lesions formed. The resulting data from the ANOVA report was illustrated in tables and the differences between wounding and non-wounding and the various isolates was determined using the corresponding LSD values. Differences were indicated on the tables by alphabetical assignment for the area of the lesion, the percentage of the nut/leaf affected and area under the curve. The alphabetical assignment for the area of the lesion, the percentage of the nut/leaf affected was based on the log transformed data for the area of the lesion and the arcsine transformed data for the percentage of the nut/leaf affected.

5.3 Results and Discussion

5.3.1 Pathogenicity and symptoms caused by *Alternaria tenuissima*, *Neofusicoccum parvum* and *Cladosporium cladosporioides* on pecan nuts and leaves

Disease symptoms were observed on nuts and leaves inoculated with both *Alternaria tenuissima* and *Neofusicoccum parvum* (Table 5.1). Symptoms appeared on both wounded and non-wounded nuts and only on the wounded pecan leaves. In contrast to this, no disease symptoms were observed on both the wounded or non-wounded nuts and leaves inoculated with *Cladosporium cladosporioides* and the control. The symptoms caused by *A. tenuissima* was the formation of dark-brown to black, oval to round and shallow sunken lesions on the surface of the husk around the point of inoculation (Fig. 5.14). These were the typical symptoms observed on both the wounded and non-wounded nuts inoculated with *A. tenuissima*. The symptoms caused by *N. parvum* to both wounded and non-wounded nuts include the systemic blackening and shrivelling of the husk surrounding the pecan nut over time, until the whole husk was blackened and shrivelled (Figs. 5.15 - 5.17). This type of damage may have a detrimental effect on the growth and development of a pecan nut, since the husk supplies the nut with photosynthetic assimilates necessary to reach maturity. The symptoms caused by *A. tenuissima* to wounded pecan leaves were dark, oval to round leaf spots forming around the point of inoculation (Fig. 5.18). Similar symptoms were caused by *N. parvum* to wounded pecan leaves, except that the lesions were irregular in shape varying from angular to round or oval (Fig. 5.19). During heavy infestation with pathogens that cause leaf spots, the photosynthetic capabilities of plants are reduced, influencing the yield and plant health.

The results of the subculturing made from the lesions formed on the nuts and leaves, inoculated with *A. tenuissima* and *N. parvum*, showed that *A. tenuissima* occurred in 100% of the wounded and 97% of the non-wounded nuts (Table 5.2). *Neofusicoccum parvum* occurred in 100% of both the wounded and non-wounded nuts. *Alternaria tenuissima* occurred in 92% of the wounded and 0% of the non-wounded leaves while *N. parvum* occurred in 100% of the wounded and 0% of the non-wounded leaves. These results confirm the presence of both *A. tenuissima* and *N. parvum* in the nuts and leaves that showed symptoms. The non-inoculated wounded control

on both the nuts and leaves did not produce lesions larger than 0.2 x 0.2 cm (Fig. 5.15 - 5.20). Therefore, it can be accepted with certainty *A. tenuissima* and *N. parvum* were the causative agents of symptoms on the wounded nuts and leaves, and not to damage caused during wounding. In addition, *C. cladosporioides* and the control did not have an effect on both nuts and leaves while a notable affect was observed on the nuts and leaves that were inoculated with *A. tenuissima* and *N. parvum* (Figs. 5.21 - 5.26). Based on the results, both *A. tenuissima* and *N. parvum* can be considered pathogens of pecan while *C. cladosporioides* can be considered non-pathogenic.

5.3.2 Incidence of disease symptoms caused by *Alternaria tenuissima* and *Neofusicoccum parvum* on wounded and non-wounded pecan nuts and leaves

Symptoms were only observed at day 14 on both the wounded and non-wounded nuts inoculated with *A. tenuissima* (Fig. 5.27). On day 14, 20% of the wounded nuts and 3% of the non-wounded nuts were affected. On day 19, 37% of the wounded nuts and 17% of the non-wounded nuts were affected while on day 23, 47% of the wounded nuts and 27% of the non-wounded nuts were affected. With *N. parvum*, symptoms were observed on the wounded nuts on day 5 where 23% of the nuts showed symptoms (Fig. 5.28). Symptoms were observed on the non-wounded nuts on day 9 where 13% of these nuts showed symptoms and 87% of the wounded nuts showed symptoms. On day 14, 100% of the wounded nuts and 73% of the non-wounded nuts were affected. Only on day 23 were 100% of the non-wounded nuts affected. These results show that wounding is not a necessity required by *A. tenuissima* and *N. parvum* to be able to infect pecan nuts and that both species can penetrate and colonise the pecan husk without external help; however, wounding can increase the rate of penetration of the husk for both species and as a result allow for quicker infestation and colonisation. With regard to *A. tenuissima*, wounding also increased disease incidence. The results also suggest that *N. parvum* is more virulent than *A. tenuissima* on pecan nuts. *Neofusicoccum parvum* was able to cause disease symptoms on day 5 on the wounded nuts and on day 9 on the non-wounded nuts and *N. parvum* was able to affect 100% of both the wounded (day 14) and non-wounded (day 23) nuts (Figs. 5.27 & 5.28). *Alternaria tenuissima* only caused symptoms on both the wounded and non-wounded nuts on day 14 and *A. tenuissima* could only affect 47% of the wounded and 27% of the non-wounded nuts within the 23 day period. These results indicate that *N. parvum* might be a

serious pathogen of pecans that can severely affect yields. It should be considered that *Alternaria tenuissima* is likely a miscellaneous pathogen of pecan that cause disease symptoms on pecan nuts under stress since the onset of symptoms only appeared on the pecan nuts 2 weeks after harvested from the trees.

On the leaves, symptoms were observed at day 14 after been inoculated with *A. tenuissima* (Fig. 5.29). On day 14, 20% of the wounded leaves showed symptoms and on day 19, 27% were affected. Incidence did not increase and on day 23 only 27% of the wounded leaves showed symptoms. With *N. parvum* symptoms were observed on the wounded leaves on day 5, where 60% of the leaves showed symptoms (Fig. 5.30). On day 9, 87% of the wounded leaves showed symptoms. The incidence did not increase after day 9 and at the end of the study (day 23) only 87% of the wounded leaves were affected. Both *A. tenuissima* and *N. parvum* were unable to infect and colonise the non-wounded leaves and as a result 0% of the non-wounded leaves were affected by both fungi. The results show that wounding is crucial for both *A. tenuissima* and *N. parvum* to be able to infect and colonise pecan leaves. *Neofusicoccum parvum* can also be considered more virulent on pecan leaves than *A. tenuissima* (Figs. 5.29 & 5.30). Both *A. tenuissima* and *N. parvum* are less virulent on pecan leaves than on pecan nuts since the level of incidence of both species were higher on the nuts than on the leaves. Both species were also able to cause disease symptoms on the non-wounded nuts but were unable to cause symptoms on the non-wounded leaves. It would seem that ideal conditions are required by *N. parvum* to infect and colonise pecan leaves. Infection of the leaves by *N. parvum* most likely occurred within the first 24 hours after inoculation when the leaves were covered with the plastic bags. The results reflect this by the high incidence on day 5 and day 9, after which no additional infection and colonisation occurred.

5.3.3 Rate of disease development, days to onset and days to 100% colonisation on wounded and non-wounded pecan nuts and leaves for *Alternaria tenuissima* and *Neofusicoccum parvum*

The results for the T-test, based on the summary output data of the regression statistics for the nuts (Tables 5.3 - 5.6), showed that there was no significant difference between the rate of disease development on wounded and non-wounded nuts infected with *A. tenuissima* and *N.*

parvum (Table 5.11). Therefore, the rate of disease development is the same on wounded and non-wounded nuts once infection has occurred. Wounding does not increase the rate at which nuts are colonised. This occurrence can be observed visually on figures 5.31 & 5.32 where there are no significant differences between the slopes of wounded and non-wounded nuts infected with *A. tenuissima* and *N. parvum*. In contrast, the results for the T-test, based on the summary output data of the regression statistics for the leaves (Table 5.7 - 5.10), showed that there was a significant difference between the rate of disease development on wounded and non-wounded leaves infected with *A. tenuissima* and *N. parvum* (Table 5.11). This occurrence can be observed visually in figures 5.33 & 5.34 where there are significant differences between the slopes of wounded and non-wounded leaves. This difference can be attributed to the fact that there was no colonisation of the non-wounded leaves infected with *A. tenuissima* and *N. parvum*. The wounding of leaves indirectly increases leaf colonisation since it cannot occur without wounding.

The days to onset is of great importance (5% of the area colonised) and days to 100% of the area of the nuts and leaves colonised (Table 5.12). For *N. parvum* the days to onset on the wounded nuts were 7 and on the non-wounded 16. The days to 100% of the area of the nuts colonised were 21 and on the non-wounded 29. These results suggest that even though wounding does not influence the rate of disease development it does reduce the time required by *N. parvum* to infect and colonise nuts. This occurrence can also be observed visually on figure 5.31 where there is a difference between the y-intercepts of wounded and non-wounded nuts infected by *N. parvum*, showing that a larger area on the wounded nuts were colonised than on the non-wounded nuts on the same day after inoculation. Wounding to nuts can consequently shorten the time in which significant damage can be caused and it may also reduce the time available to control this pathogen. For *A. tenuissima* the days to onset on the wounded nuts were 21 and on the non-wounded 22. The days to 100% of the area of the nuts colonised were 42 and on the non-wounded 42. These results confirm that *A. tenuissima* is less virulent than *N. parvum* on nuts and shows that wounding did not provide a benefit to *A. tenuissima* during the colonisation of nuts. This phenomenon can also be observed visually on figure 5.32 where there is no significant difference between the y-intercepts of wounded and non-wounded nuts infected by *A. tenuissima*. As a result the two regression lines are almost superimposed with a small difference on the first days of observation showing that there were no differences between the colonisation

of wounded and non-wounded nuts on each day of observation. For *N. parvum* the days to onset on the wounded leaves were 19 and for *A. tenuissima* 34. The days it took to reach 100% colonisation of the total area of wounded leaves for *N. parvum* were 55 and for *A. tenuissima* 68. These occurrences can be observed visually on figures 5.33 & 5.34 where there were significant differences between the y-intercepts of wounded and non-wounded leaves infected by *A. tenuissima* and *N. parvum*. These results show that both *A. tenuissima* and *N. parvum* are more virulent on nuts than on leaves and that *A. tenuissima* is less virulent than *N. parvum* on leaves. The results also show that wounding is a necessity for both *A. tenuissima* and *N. parvum* to be able to infect and colonise leaves.

5.3.4 Comparisons between the effects of wounding and non-wounding and the ability of *Alternaria tenuissima*, *Neofusicoccum parvum* and *Cladosporium cladosporioides* to cause disease on pecan nuts and leaves.

The results of the ANOVA reports comparing the average area of the lesions formed, the average percentage of the area of the nut affected, the area under the disease development curve for the three fungal isolates and the control for wounded and non-wounded nuts (Tables 5.13 - 5.15), showed that there is a significant difference between wounding and non-wounding. The reports also suggest that the effect that *N. parvum* has on pecan nuts is significantly different than that of *A. tenuissima, C. cladosporioides* and the control. The effect of *A. tenuissima* is significantly different than that of *C. cladosporioides* and the control in terms of the average area of the lesions formed and the average percentage of the area of the nuts affected, but not in terms of the area under the disease development curve. There were no significant differences between *C. cladosporioides* and the control. These results confirm that both *A. tenuissima* and *N. parvum* are pathogenic on pecan nuts while *C. cladosporioides* is not. *Neofusicoccum parvum* is more virulent than *A. tenuissima*. Although *A. tenuissima* can be considered a pathogen on pecan nuts it is most likely an endophyte that will cause disease symptoms on pecan nuts if the plants are under stress, since there is no significant difference between its effect compared to the effect of *C. cladosporioides* and the control in terms of the area under the disease development curve. The results also confirm that wounding provides a benefit to pathogens resulting in larger lesions, a larger percentage of the nut affected, and quicker disease development over time. This can be

attributed to an increased rate of onset allowing the pathogens to colonise and cause symptoms faster on wounded nuts.

The ANOVA reports comparing the average area of the lesions formed on the leaves, the average percentage of the area of the leaves affected and the area under the disease development curve for the three fungal isolates and the control for wounded and non-wounded leaves (Tables 5.16 - 5.18), showed that there is a significant difference between wounding and non-wounding. The reports also suggests that the effect that *N. parvum* has on pecan leaves is significantly different than that of *A. tenuissima, C. cladosporioides* and the control. The effect of *A. tenuissima* is significantly different than that of *C. cladosporioides* and the control in terms of the average area of the lesions formed and the average percentage of the area of the leaves affected, but not in terms of the area under the disease development curve. There were no significant differences between *C. cladosporioides* and the control. These results confirm that both *A. tenuissima* and *N. parvum* are pathogenic on pecan leaves while *C. cladosporioides* is not. *Neofusicoccum parvum* is more virulent on pecan leaves than *A. tenuissima*. Although *A. tenuissima* can be considered a pathogen on pecan leaves it is most likely a miscellaneous pathogen that will only cause symptoms when plants are under stress. The results also confirm that wounding provides a benefit to pathogens resulting in larger lesions, a larger percentage of the leaf affected and quicker disease development over time. This can be attributed to the fact that wounding is required for the tested pathogens to infect and colonise pecan leaves.

5.4 Conclusions

The identification of new fungal phytopathogens is of great importance in crop production. The identification of new pathogens is crucial for the rapidly expanding South African pecan industry, since very little information is available on pathogens that attack this crop in the country. Both *Alternaria tenuissima* and *Neofusicoccum parvum,* which is associated with the grey-brown stink bug, are pathogenic on pecan nuts and pecan leaves while *Cladosporium cladosporioides* cannot be considered a pathogen of this crop. However, *C cladosporioides* is a species complex and the isolate that we used might not be pathogenic, but others in the complex may be pathogenic. Both *A. tenuissima* and *N. parvum* are more virulent on pecan nuts than on pecan leaves. *Alternaria tenuissima* is less virulent than *N. parvum* and can be considered a miscellaneous pathogen of pecan that will cause disease if plants that are under stress. Wounding of pecan nuts increases disease incidence on nuts inoculated with *A. tenuissima* and is a necessity for both *A. tenuissima* and *N. parvum* to be able to infect, colonise and cause symptoms on pecan leaves. Wounding does not increase the rate of disease development on wounded nuts infected with *A. tenuissima* and *N. parvum*. Wounding, however, reduces the time required by *N. parvum* to infect and colonise pecan nuts. Due to this, wounding to nuts can shorten the time in which significant damage can be caused to the nuts and a may also reduce the time available to control this pathogen. In contrast, wounding does not provide any benefit to *A. tenuissima* during the initial colonisation of pecan nuts. This study confirms that stink bugs occurring in pecan orchards are associated with fungal phytopathogens. These insects feed on pecan nuts providing wounds that can assist the pathogens in causing disease on pecan nuts. The grey-brown stinkbug is likely a disseminator of *N. parvum* spreading this fungus to developing nuts and, as it feeds, providing suitable entry points through which this pathogen can infect and cause disease on the nuts.

5.5 References

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5.6 Tables & Figures

Figure 5.1: The grey-brown stinkbug (*Coenomorpha nervosa* Dallas) visiting a pecan nut (Photo by J. Saaiman).

Figure 5.2: The growth stage at which the pecan nuts were collected for the detached nut assays (Photo by J. Saaiman).

Figure 5.3: Pecan nuts inoculated with the control agar plugs (Photo by J. Saaiman).

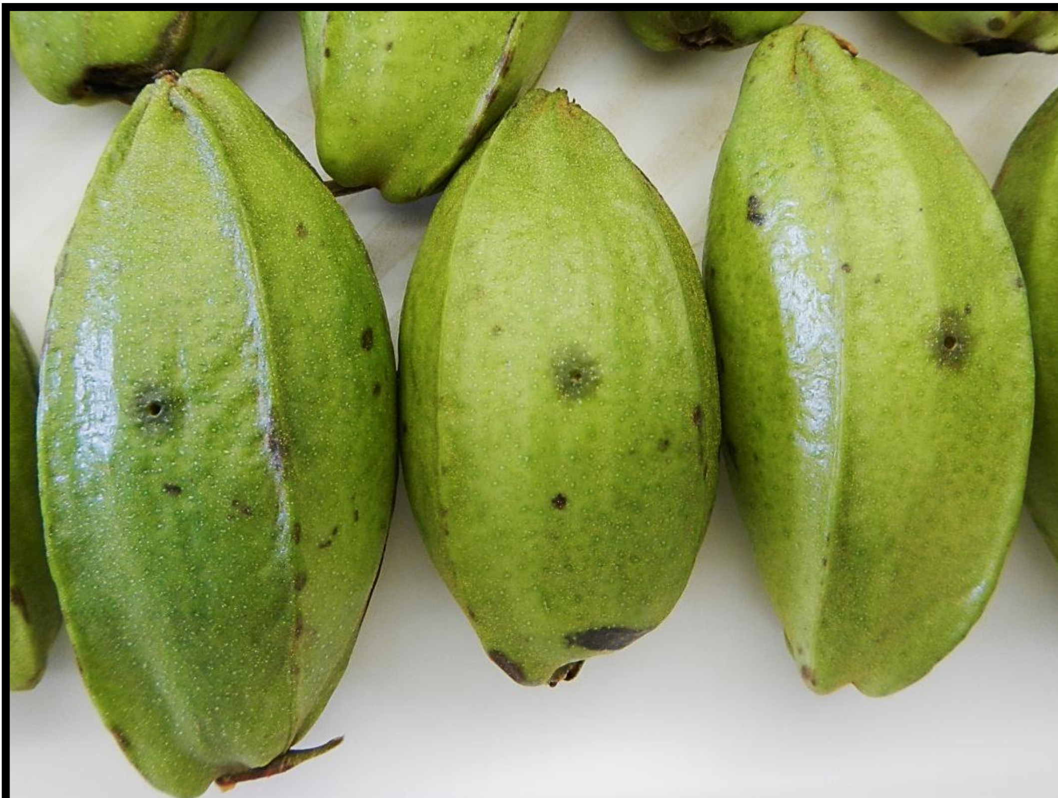

Figure 5.4: The wounds created on the pecan nuts to simulate stink bug feeding damage (Photo by J. Saaiman).

Figure 5.5: Plastic containers set up for use in the detached leaf assay trial (Photo by J. Saaiman).

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Figure 5.8: The plastic containers placed in an incubator (Photo by J. Saaiman).

Figure 5.9: Three year old pecan saplings in the greenhouse at the University of the Free State (Photo by J. Saaiman).

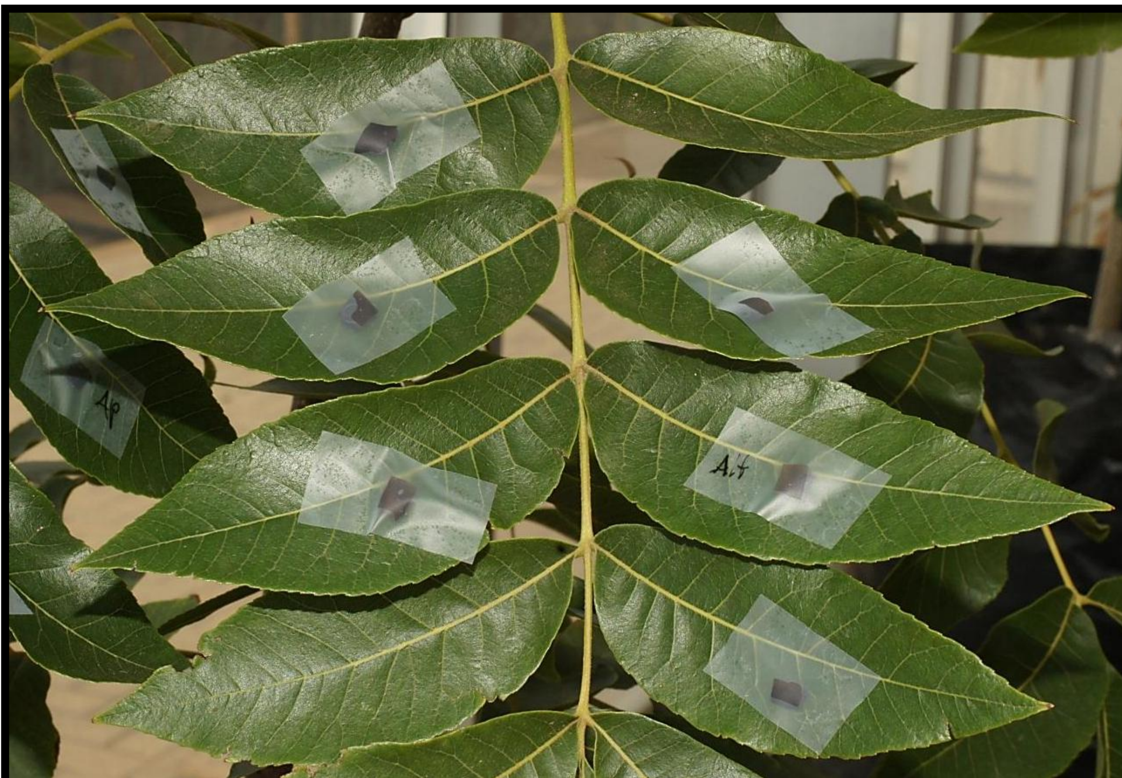

Figure 5.10: Pecan leaflets inoculated with *Alternaria tenuissima* plugs, three wounded on one side (left) and three non-wounded on the other (right) (Photo by J. Saaiman).

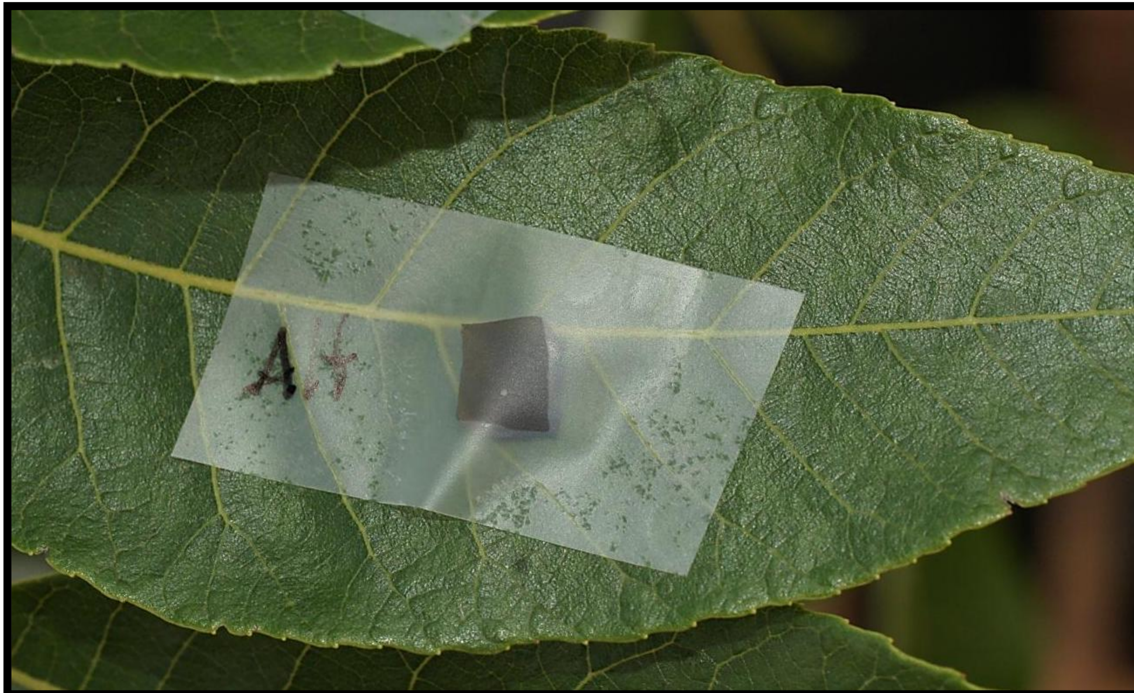

Figure 5.11: A pecan leaflet inoculated with an *A. tenuissima* plug, kept in place with adhesive tape (Photo by J. Saaiman).

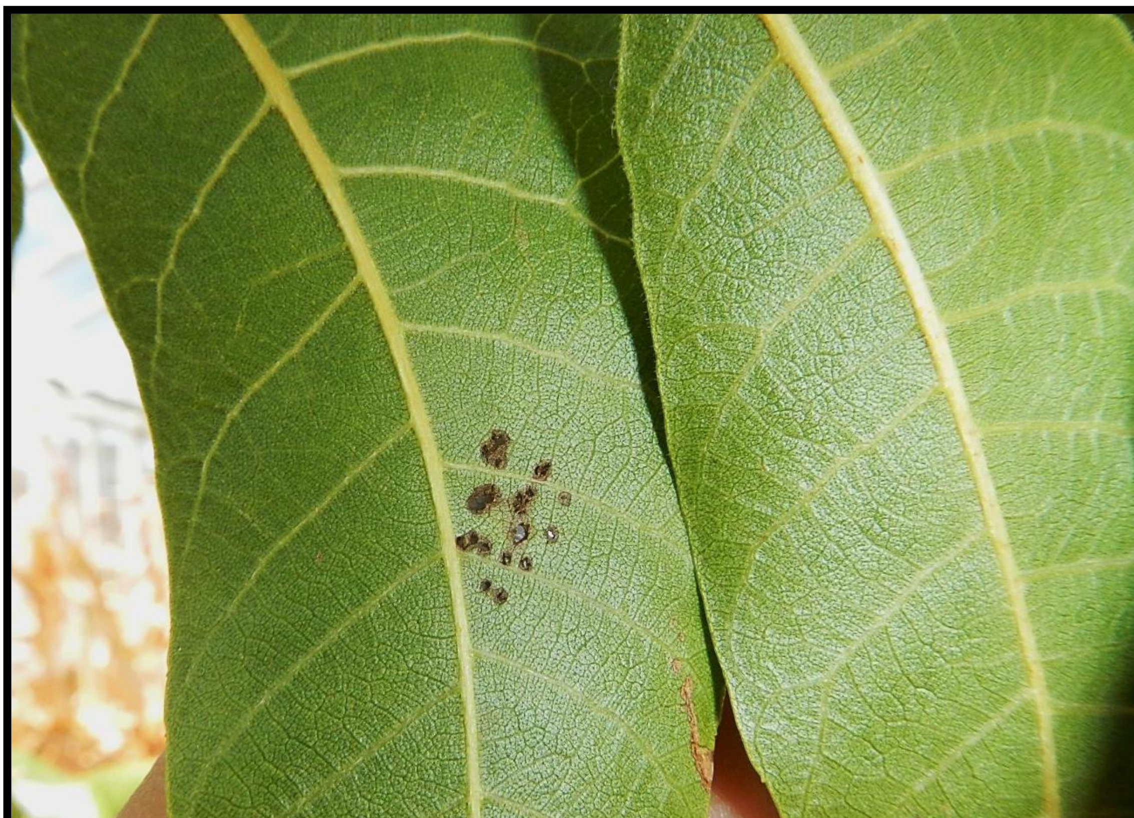

Figure 5.12: The puncture wounds made on the pecan leaflets to investigate the effect of wounding on disease development (Photo by J. Saaiman).

Figure 5.13: The inoculated leaves of the pecan saplings placed in bags (Photo by J. Saaiman).

Figure 5.14: Symptoms caused on wounded pecan nuts by *A. tenuissima* (Day 14) (Photo by J. Saaiman).

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Figure 5.16: Symptoms caused on wounded pecan nuts by *N. parvum* (Day 9) (Photo by J. Saaiman).

Figure 5.17: Symptoms caused on wounded pecan nuts by *N. parvum* (Day 14) (Photo by J. Saaiman).

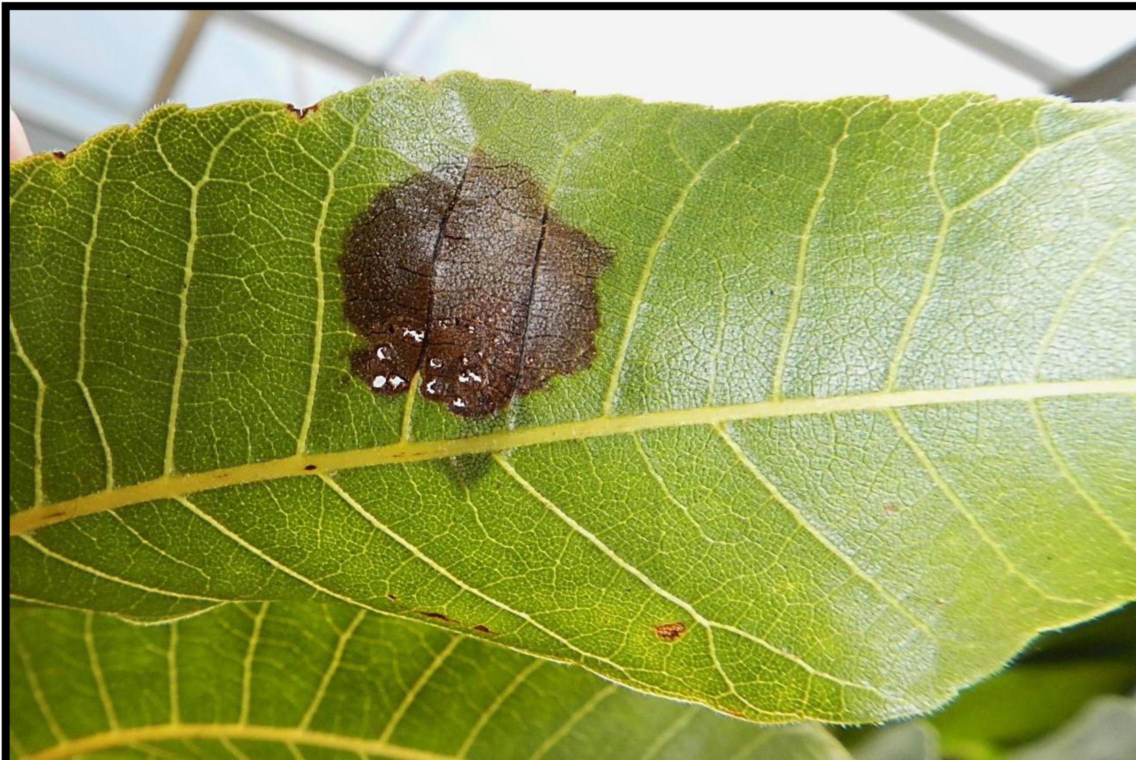

Figure 5.18: Symptoms caused on a wounded pecan leaf by *A. tenuissima* (Day 9) (Photo by J. Saaiman).

Figure 5.19: Symptoms caused on wounded pecan leaves by *N. parvum* (Day 9) (Photo by J. Saaiman).

Figure 5.20: Non-inoculated wounded pecan leaves (Day 9) (Photo by J. Saaiman).

Figure 5.21: The effect of the control on wounded (top) and non-wounded (bottom) pecan nuts (Day 14) (Photo by J. Saaiman).

Figure 5.22: The effect of *C. cladosporioides* on wounded (bottom) and non-wounded (top) pecan nuts (Day 14) (Photo by J. Saaiman).

Figure 5.23: The effect of *A. tenuissima* on wounded (top) and non-wounded (bottom) pecan nuts (Day 14) (Photo by J. Saaiman).

Figure 5.24: The effect of *N. parvum* on wounded (top) and non-wounded (bottom) pecan nuts compared to the non-inoculated wounded control (middle) (Day 9) (Photo by J. Saaiman).

Figure 5.25: The effect of *N. parvum* on wounded (top) and non-wounded (bottom) pecan nuts compared to the non-inoculated wounded control (middle) (Day 14) (Photo by J. Saaiman).

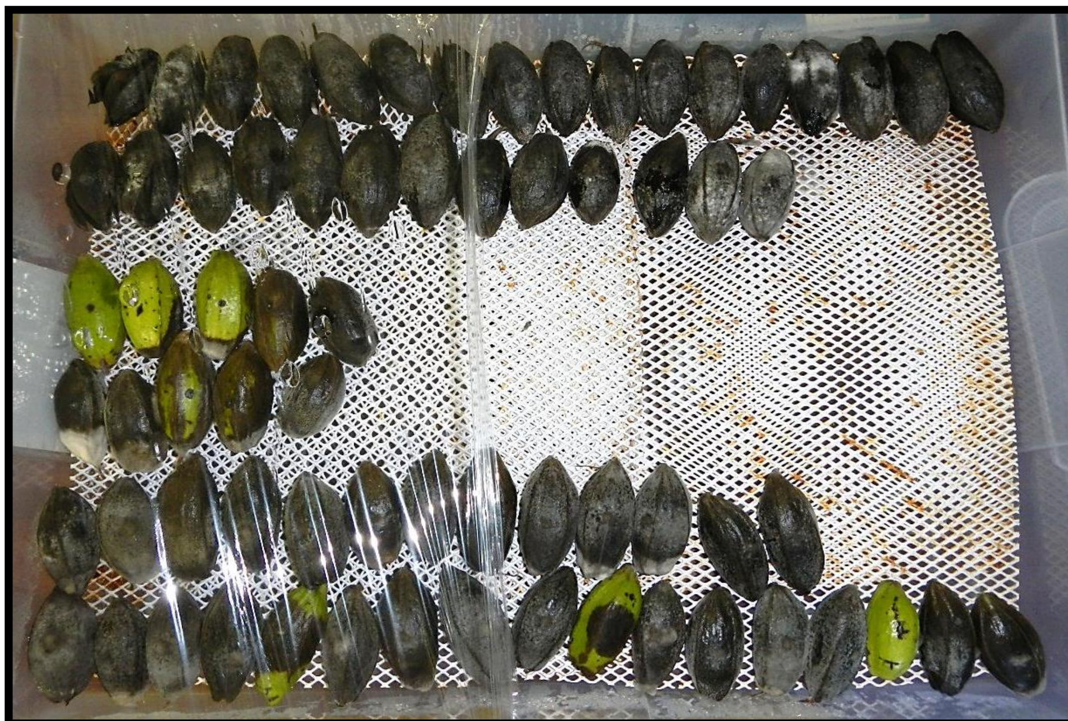

Figure 5.26: The effect of *N. parvum* on wounded (top) and non-wounded (bottom) pecan nuts compared to the non-inoculated wounded control (middle) (Day 23) (Photo by J. Saaiman).

Table 5.1: The percentage of pecan nuts and leaves affected over time by *Alternaria tenuissima*, *Cladosporium cladosporioides*, *Neofusicoccum parvum* and a control.

Nuts	**W *vs.* N-W**	**Day 5**	**Day 9**	**Day 14**	**Day 19**	**Day 23**
Alternaria tenuissima	Wounded	0	0	20	37	**47**
	Non-wounded	0	0	3	17	**27**
Cladosporium cladosporioides	Wounded	0	0	0	0	**0**
	Non-wounded	0	0	0	0	**0**
Neofusicoccum parvum	Wounded	23	87	**100**	**100**	**100**
	Non-wounded	0	13	73	93	**100**
Control	Wounded	0	0	0	0	**0**
	Non-wounded	0	0	0	0	**0**
Leaves	**W *vs.* N-W**	**Day 5**	**Day 9**	**Day 14**	**Day 19**	**Day 23**
Alternaria tenuissima	Wounded	0	0	20	27	**27**
	Non-wounded	0	0	0	0	**0**
Cladosporium cladosporioides	Wounded	0	0	0	0	**0**
	Non-wounded	0	0	0	0	**0**
Neofusicoccum parvum	Wounded	60	87	87	87	**87**
	Non-wounded	0	0	0	0	**0**
Control	Wounded	0	0	0	0	**0**
	Non-wounded	0	0	0	0	**0**

Table 5.2: The recovery percentage of *Alternaria tenuissima* and *Neofusicoccum parvum* from affected nuts and leaves to confirm their pathogenicity with Koch's postulates.

Nuts	**W *vs.* N-W**	**Recovery Percentage**
Alternaria tenuissima	Wounded	100
	Non-wounded	97
Neofusicoccum parvum	Wounded	100
	Non-wounded	100
Leaves	**W *vs.* N-W**	**Recovery Percentage**
Alternaria tenuissima	Wounded	92
	Non-wounded	0
Neofusicoccum parvum	Wounded	100
	Non-wounded	0

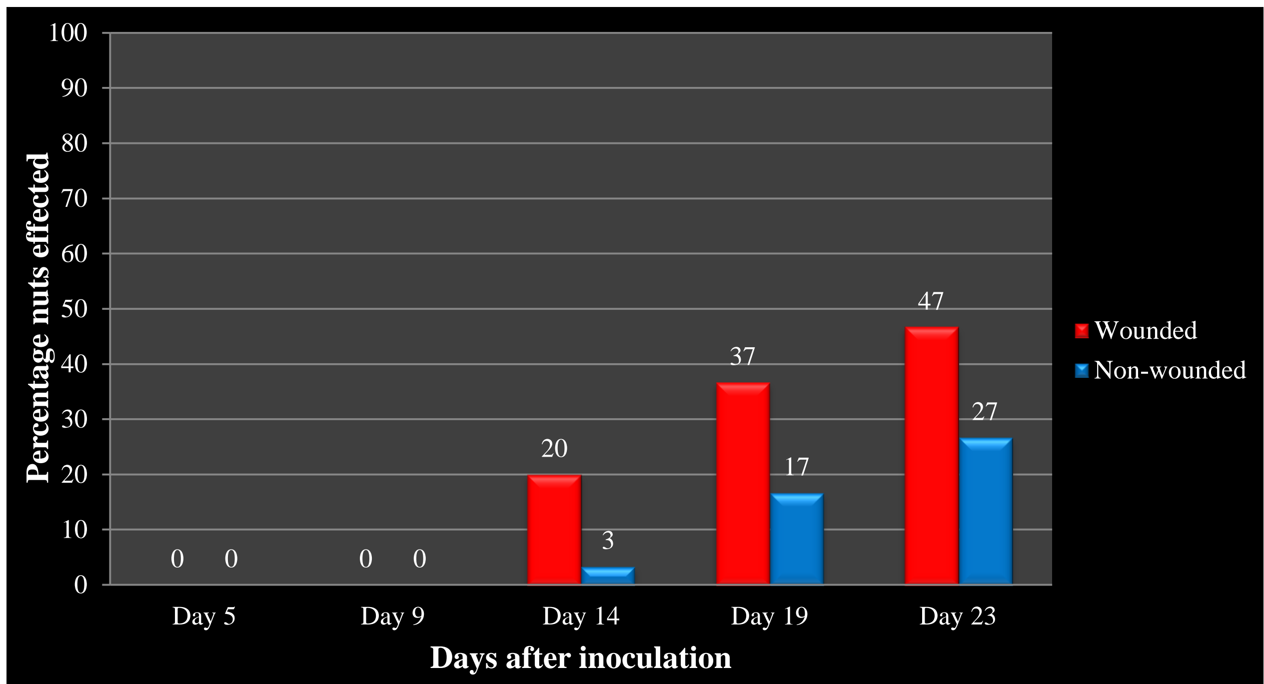

Figure 5.27: The percentage of wounded and non-wounded nuts affected by *Alternaria tenuissima* over time.

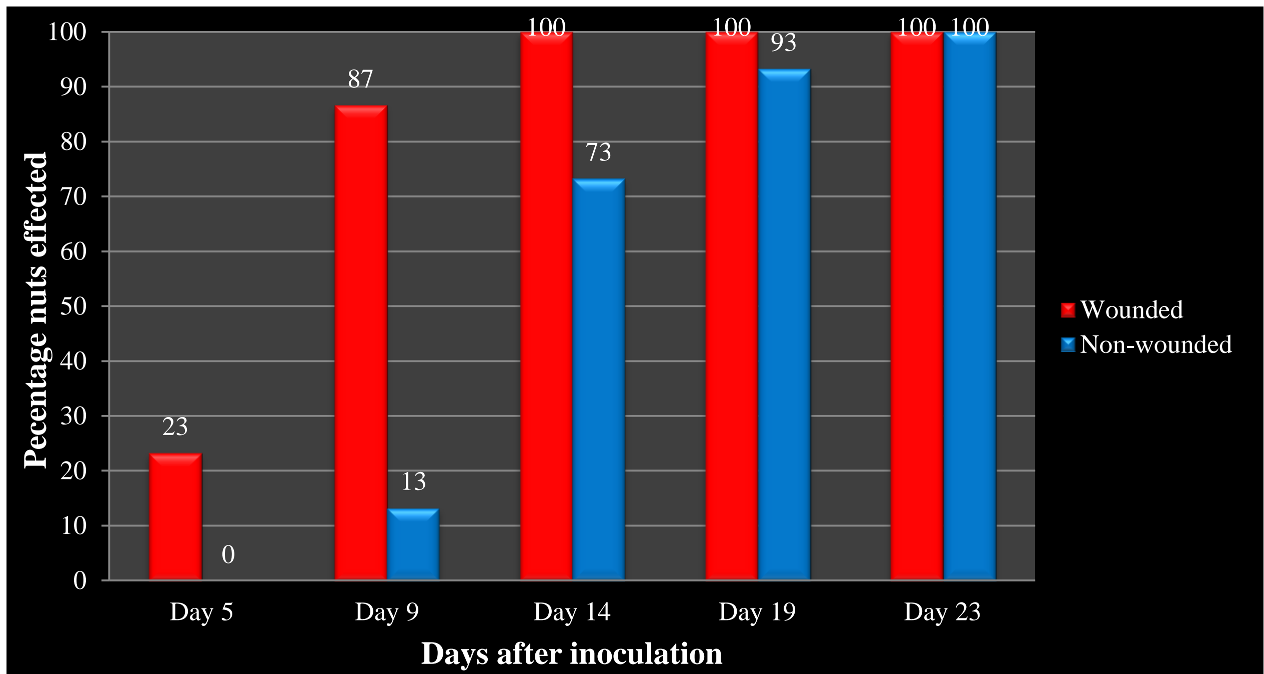

Figure 5.28: The percentage of wounded and non-wounded nuts affected by *Neofusicoccum parvum* over time.

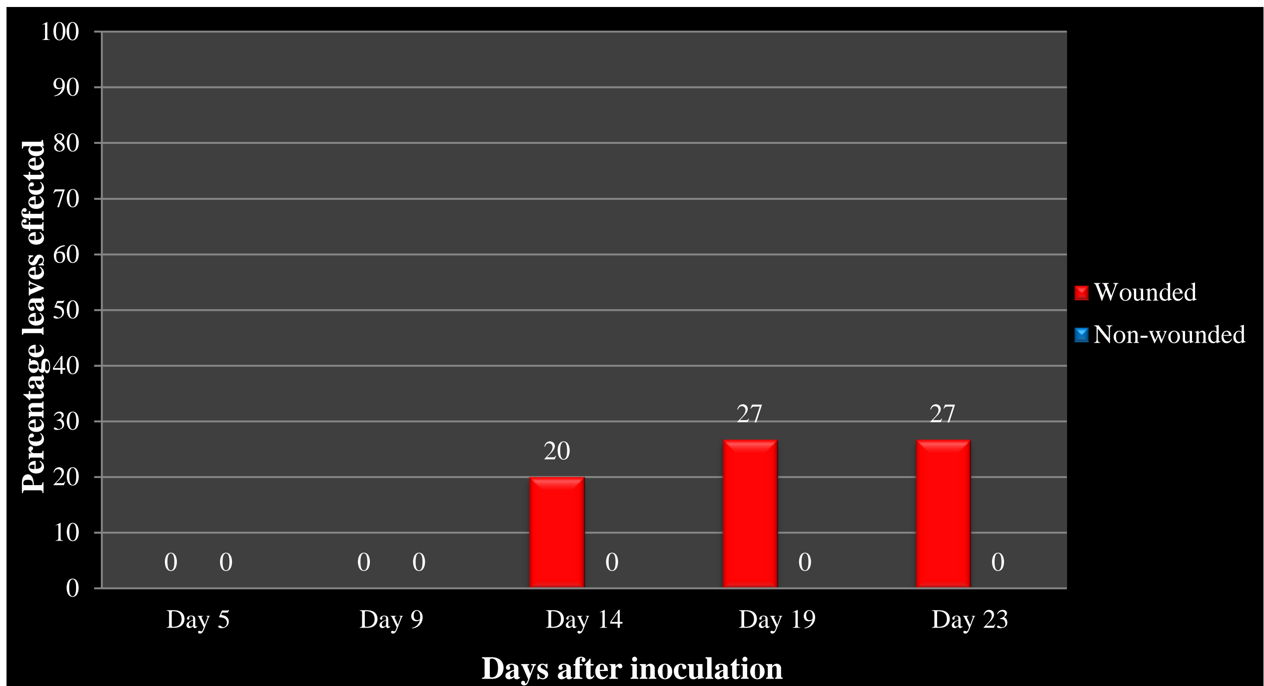

Figure 5.29: The percentage of wounded and non-wounded leaves affected by *Alternaria tenuissima* over time.

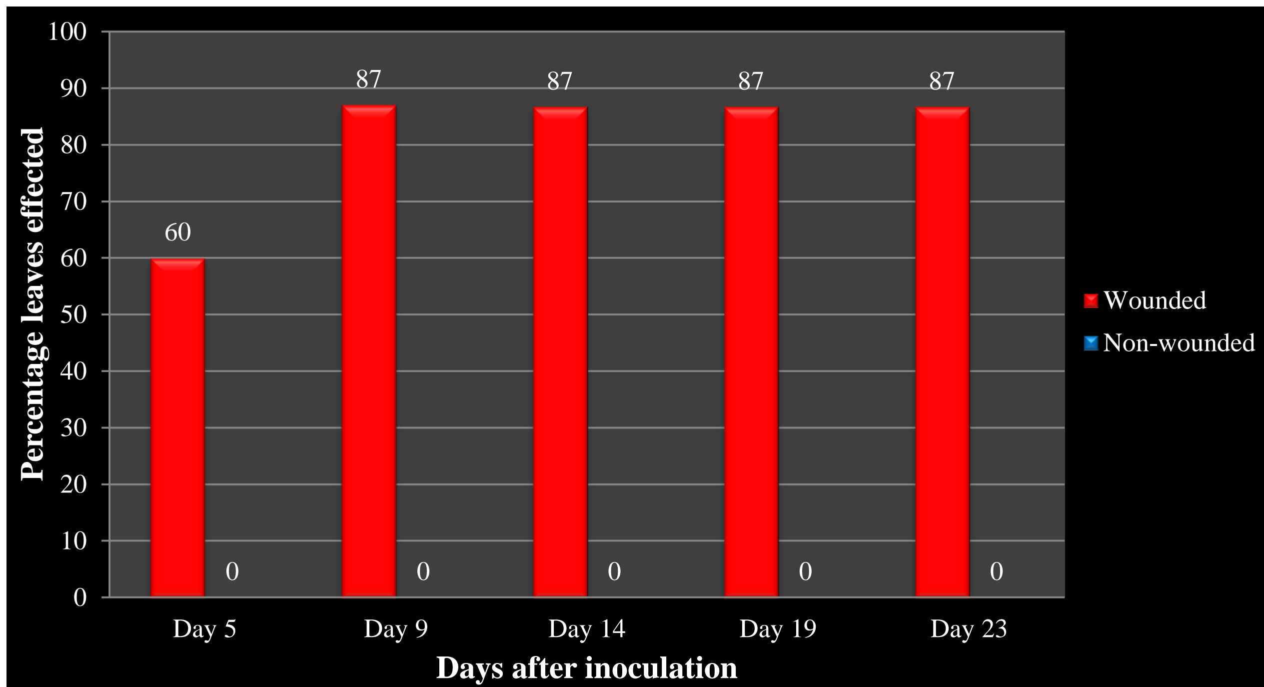

Figure 5.30: The percentage of wounded and non-wounded leaves affected by *Neofusicoccum parvum* over time.

Table 5.3: A summary output for the regression statistics based on the log-transformed data for the percentage of the area of wounded nuts affected by *Neofusicoccum parvum* over time.

Regression Statistics	
Multiple R	0.974919342
R Square	0.950467723
Adjusted R Square	0.933956964
Standard Error	1.72861687
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	172.0155986	172.0155986	57.56656775	0.004750088
Residual	3	8.964348854	2.988116285		
Total	4	180.9799474			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-9.684090801	1.83309065	-5.282930662	0.013227638	-15.51780337	-3.850378234	-15.51780337	-3.850378234
X Variable 1	0.900774296	0.118721894	7.587263522	0.004750088	0.522948243	1.278600349	0.522948243	1.278600349

Table 5.4: A summaery output for the regression statistics based on the log-transformed data for the percentage of the area of non-wounded nuts affected by *Neofusicoccum parvum* over time.

Regression Statistics	
Multiple R	0.965336573
R Square	0.931874699
Adjusted R Square	0.909166265
Standard Error	2.002262502
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	164.5176005	164.5176005	41.03650243	0.007706728
Residual	3	12.02716538	4.009055128		
Total	4	176.5447659			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-16.32357515	2.123274819	-7.687923867	0.004573028	-23.08078326	-9.566367053	-23.08078326	-9.566367053
X Variable 1	0.880923616	0.137515953	6.405973964	0.007706728	0.443286479	1.318560753	0.443286479	1.318560753

Table 5.5: A summary output for the regression statistics based on the log-transformed data for the percentage of the area of wounded nuts affected by *Alternaria tenuissima* over time.

Regression Statistics	
Multiple R	0.910709929
R Square	0.829392575
Adjusted R Square	0.772523433
Standard Error	2.685238811
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	105.1596907	105.1596907	14.58422879	0.031596192
Residual	3	21.63152241	7.21050747		
Total	4	126.7912131			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-17.52968706	2.847528704	-6.156105482	0.008625072	-26.59179427	-8.467579861	-26.59179427	-8.467579861
X Variable 1	0.704298429	0.184422959	3.81893032	0.031596192	0.117382265	1.291214592	0.117382265	1.291214592

Table 5.6: A summaery output for the regression statisticts based on the log-transformed data for the percentage of the area of non-wounded nuts affected by *Alternaria tenuissima* over time.

Regression Statistics	
Multiple R	0.926882377
R Square	0.859110941
Adjusted R Square	0.823888676
Standard Error	2.34882416
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	134.5652683	134.5652683	24.39113282	0.00782383
Residual	4	22.06789973	5.516974932		
Total	5	156.633168			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-15.76042135	1.708851956	-9.222812598	0.000768075	-20.504955	-11.0158877	-20.504955	-11.0158877
X Variable 1	0.598767091	0.121238886	4.938737978	0.00782383	0.262153979	0.935380203	0.262153979	0.935380203

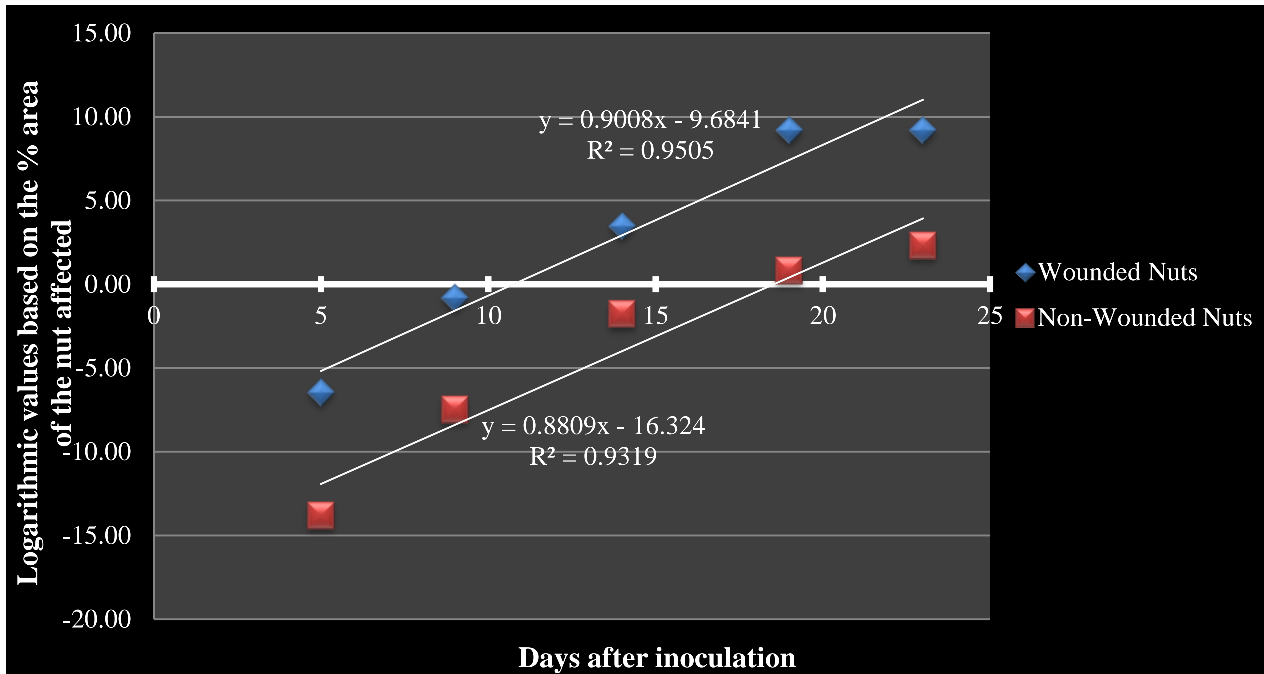

Figure 5.31: The comparison between the the log-transformed data for the percentage of the area of wounded and non-wounded nuts affected by *Neofusicoccum parvum* over time.

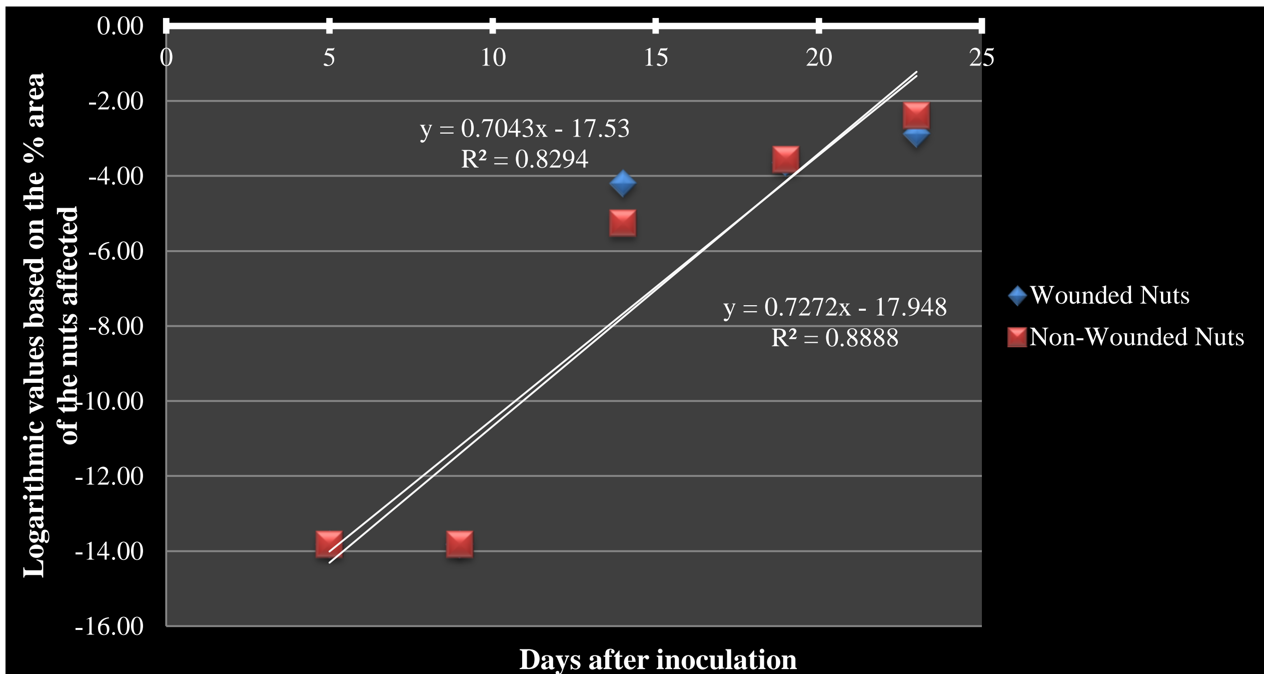

Figure 5.32: The comparison between the log-transformed data for the percentage of the area of wounded and non-wounded nuts affected by *Alternaria tenuissima* over time.

Table 5.7: A summary output for the regression statistics based on the log-transformed data for the percentage of the area of wounded leaves affected by *Neofusicoccum parvum* over time.

Regression Statistics	
Multiple R	0.807917631
R Square	0.652730898
Adjusted R Square	0.536974531
Standard Error	0.328365849
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.608002336	0.608002336	5.638833646	0.09809312
Residual	3	0.323472392	0.107824131		
Total	4	0.931474728			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-4.581435049	0.348211554	-13.15704488	0.000948499	-5.689599624	-3.473270474	-5.689599624	-3.473270474
X Variable 1	0.05355311	0.022552259	2.374622843	0.09809312	-0.018218244	0.125324463	-0.018218244	0.125324463

Table 5.8: A summary output for the regression statistics based on the log-transformed data for the percentage of the area of non-wounded leaves affected by *Neofusicoccum parvum* over time.

Regression Statistics	
Multiple R	1
R Square	1
Adjusted R Square	1
Standard Error	0
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0	0	N/A	N/A
Residual	3	0	0		
Total	4	0			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-13.81550956	0	65535	N/A	-13.81550956	-13.81550956	-13.81550956	-13.81550956
X Variable 1	0	0	65535	N/A	0	0	0	0

Table 5.9: A summary output for the regression statistics based on the log-transformed data for the percentage of the area of wounded leaves affected by *Alternaria tenuissima* over time.

Regression Statistics	
Multiple R	0.918099161
R Square	0.842906069
Adjusted R Square	0.790541426
Standard Error	1.553772317
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	38.86116255	38.86116255	16.09685492	0.027788035
Residual	3	7.242625236	2.414208412		
Total	4	46.10378779			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-16.1151932	1.647678878	-9.780542444	0.002271301	-21.35884276	-10.87154365	-21.35884276	-10.87154365
X Variable 1	0.4281441	0.106713521	4.012088598	0.027788035	0.08853405	0.76775415	0.08853405	0.76775415

Table.5. 10: A summary output for the regression statistics based on the log-transformed data for the percentage of the area of non-wounded leaves affected by *Alternaria tenuissima* over time.

Regression Statistics	
Multiple R	1
R Square	1
Adjusted R Square	1
Standard Error	0
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0	0	N/A	N/A
Residual	3	0	0		
Total	4	0			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-13.81550956	0	65535	N/A	-13.81550956	-13.81550956	-13.81550956	-13.81550956
X Variable 1	0	0	65535	N/A	0	0	0	0

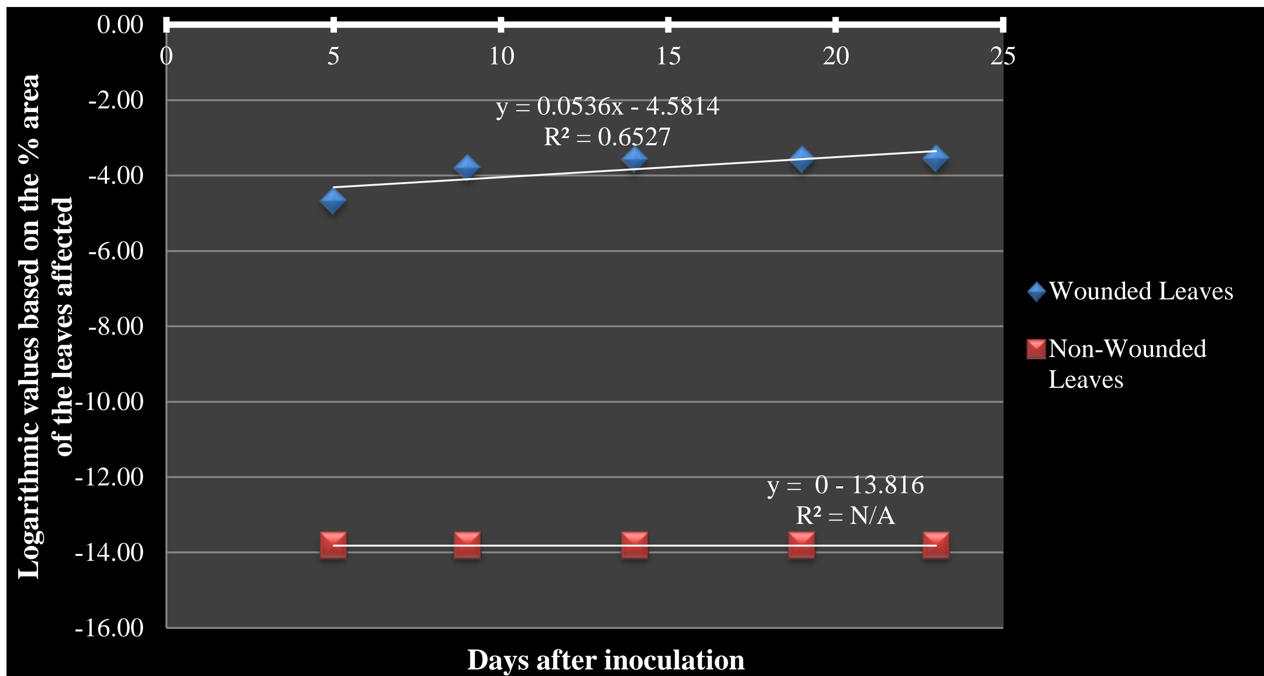

Figure 5.33: The comparison between the the log-transformed data for the percentage of the area of wounded and non-wounded leaves affected by *Neofusicoccum parvum* over time.

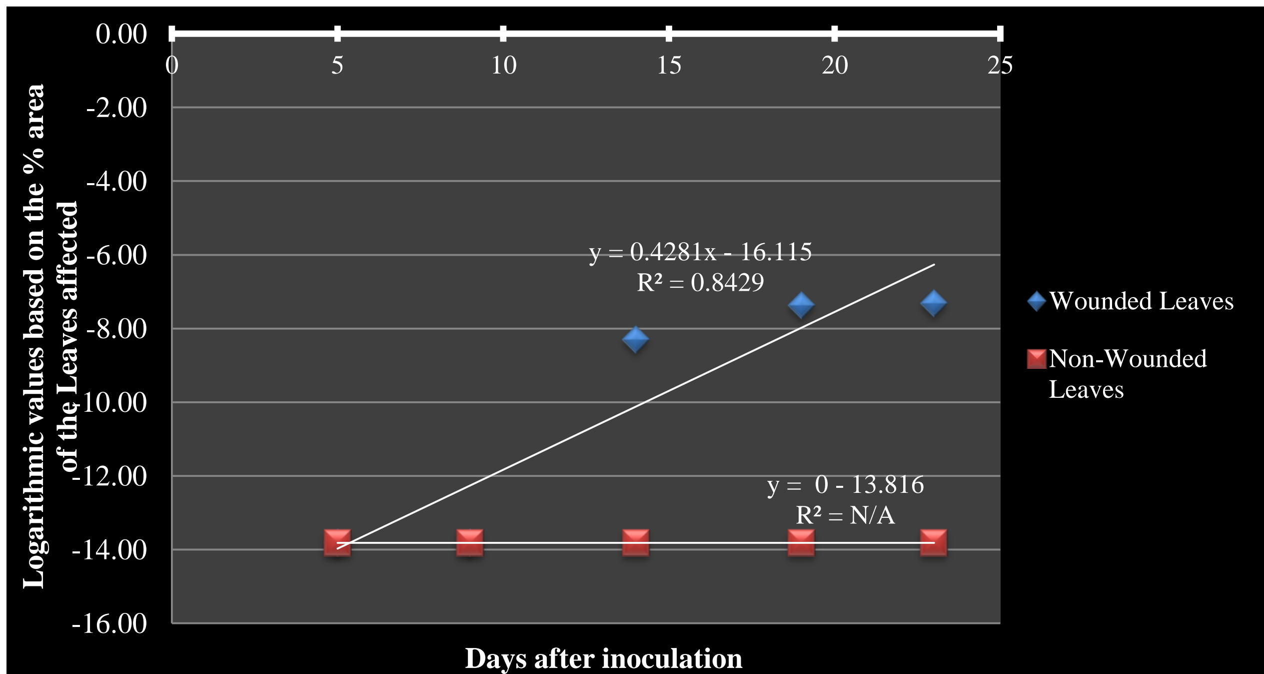

Figure 5.34: The comparison between the log-transformed data for the percentage of the area of wounded and non-wounded leaves affected by *Alternaria tenuissima* over time.

Table 5.11: Comparisons between the rate of disease development on wounded and non-wounded nuts and leaves for both *Neofusicoccum parvum* and *Alternaria tenuissima*.

	Wounded vs. Non-Wounded	**Significance**
Nuts:		
N. parvum	-0.109265251	Non-Significant
A. tenuissima	-0.478155636	Non-Significant
Leaves:	**Wounded vs. Non-Wounded**	**Significance**
N. parvum	-2.374622843	Significant
A. tenuissima	-4.012088598	Significant

Table 5.12: The days to onset and days to 100% colonisation of wounded and non-wounded nuts and leaves by *Neofusicoccum parvum* and *Alternaria tenuissima*.

	N. parvum		***A. tenuissima***	
Nuts:	**Wounded**	**Non-Wounded**	**Wounded**	**Non-Wounded**
Days to onset (5%)	7	16	21	22
Days to 100% colonisation	21	29	42	42
Leaves:	**Wounded**	**Non-Wounded**	**Wounded**	**Non-Wounded**
Days to onset (5%)	19	N/A	34	N/A
Days to 100% colonisation	55	N/A	68	N/A

Table 5.13: The analysis of variance (ANOVA) table comparing the average area of the lesions formed by the three fungal isolates and the control on wounded and non-wounded nuts at day 23.

Isolate	Wounded	Non-Wounded	Mean	
1. *Neofusicoccum parvum*	4683.04	4152.47	4417.75	A
2. *Alternaria tenuissima*	198.44	60.91	129.68	B
3. *Cladosporium cladosporioides*	0.00	0.00	0.00	C
4. Control	0.00	0.00	0.00	C
Mean	1220.37	1053.34	1136.86	
	A	B		

Table 5.14: The analysis of variance (ANOVA) table comparing the average percentage of the area of the nut affected by the three fungal isolates and the control on wounded and non-wounded nuts at day 23.

Isolate	Wounded	Non-Wounded	Mean	
1. *Neofusicoccum parvum*	100.00	93.91	96.96	A
2. *Alternaria tenuissima*	4.69	1.49	3.09	B
3. *Cladosporium cladosporioides*	0.00	0.00	0.00	C
4. Control	0.00	0.00	0.00	C
Mean	26.17	23.85	25.01	
	A	B		

Table 5.15: The analysis of variance (ANOVA) table comparing the area under the disease development curve for the three fungal isolates and the control on wounded and non-wounded nuts over time.

Isolate	Wounded	Non-Wounded	Mean	
1. *Neofusicoccum parvum*	88286.92	29932.95	59109.94	A
2. *Alternaria tenuissima*	947.52	214.74	581.13	B
3. *Cladosporium cladosporioides*	0.00	0.00	0.00	B
4. Control	0.00	0.00	0.00	B
Mean	22308.61	7536.92	14922.77	
	A	B		

Table 5.16: The analysis of variance (ANOVA) table comparing the average area of the lesions formed by the three fungal isolates and the control on wounded and non-wounded leaves at day 23.

Isolate	Wounded	Non-Wounded	Mean	
1. *Neofusicoccum parvum*	128.04	0.00	64.02	A
2. *Alternaria tenuissima*	12.09	0.00	6.04	B
3. *Cladosporium cladosporioides*	0.00	0.00	0.00	C
4. Control	0.00	0.00	0.00	C
Mean	35.03	0.00	17.52	
	A	B		

Table 5.17: The analysis of variance (ANOVA) table comparing the average percentage of the area of the nut affected by the three fungal isolates and the control on wounded and non-wounded leaves at day 23.

Isolate	Wounded	Non-Wounded	Mean	
1. *Neofusicoccum parvum*	3.37	0.00	1.68	A
2. *Alternaria tenuissima*	0.32	0.00	0.16	B
3. *Cladosporium cladosporioides*	0.00	0.00	0.00	C
4. Control	0.00	0.00	0.00	C
Mean	0.92	0.00	0.46	
	A	B		

Table 5.18: The analysis of variance (ANOVA) table comparing the area under the disease development curve for the three fungal isolates and the control on wounded and non-wounded leaves over time.

Isolate	Wounded	Non-Wounded	Mean	
1. *Neofusicoccum parvum*	2019.24	0.00	1009.62	A
2. *Alternaria tenuissima*	114.34	0.00	57.17	B
3. *Cladosporium cladosporioides*	0.00	0.00	0.00	B
4. Control	0.00	0.00	0.00	B
Mean	533.39	0.00	266.70	
	A	B		

Chapter 6

Final Conclusions and Recommendations

- Tree-nuts are increasingly being grown in South Africa on a commercial basis, but also in home gardens for nuts or shade. Together with increased acreages of these crops diseases, insects and weeds are becoming a major problem.

- A large proportion of research on these pests and pathogens focuses primarily on one or the other, and very few studies focus on the interaction and associations between the two. It is important to determine the association between insect pests and fungal pathogens as such associations may be exploited to obtain better control against insect pests and pathogens within the crop production system.

- Phytopathogens rely on several methods of dispersal, of which insects are one of the more important vectors. As disseminators of fungal phytopathogens insects may contribute to the spread and occurrence of disease in tree-nut crop production which will result in economic losses.

- This study has shown that insects occurring in tree-nut crop orchards are associated with a large variety of fungal species, which include known pathogens of the crops, fungal species that can be considered as potential pathogens and contaminants, and fungi that apparently pose no threat to tree-nut crop production.

- By studying the fungi associated with insects occurring in pistachio and walnut orchards it was found that the largest proportion of fungi isolated from the *Atelocera raptoria* specimens in the pistachio orchards was pathogenic. Fungal species include *Alternaria alternata* and *Aspergillus flavus*, two of the most serious pathogens of this crop. This indicates that these insects may play a role in the spread and occurrence of disease in South African pistachio orchards.

- In contrast to the occurrence of pathogens on the stink bug specimens collected in the pistachio orchard, the incidence of pathogens on the *Coenomorpha nervosa* and *Empoasca citricola* specimens, collected in the walnut orchards, was minimal. Only two pathogens were isolated which were *Alternaria alternata* and *Fusarium oxysporum*. This

might be an indication that the serious pathogenic fungal species are absent from the orchards rather than that they are not harboured and disseminated by insects.

- When comparing the similarity of the samples collected on pistachio and walnut, it was found that the fungi associated with the insects were most likely based on the prevailing mycoflora of the particular orchard rather than on a particular insect group. In addition, it was found that the ecology and ethology of a particular insect species may also influence the fungal species occurring on it.

- By studying the fungi associated with insects occurring in pecan orchards it was found that the most abundant fungal species isolated from the *Empoasca* sp. (Prieska) and the *Sciobius cf. granosus* (Van Reenen's Pass) specimens were pathogenic. These include *Alternaria alternata, A. tenuissima* and *Trichothecium roseum*.

- Pathogens were also isolated from the *Panafrolepta dahlmani* specimens collected on pecan at Van Reenen's Pass, which include *Alternaria alternata* and *Fusarium oxysporum*. However, a large proportion of the fungi isolated from these specimens were non-pathogenic. These included the well known entomopathogenic fungus *Isaria farinosa.*

- From these results it can be concluded that insects occurring in pecan orchard have the potential to harbour and disseminate fungal phytopathogens.

- By comparing the similarity of the samples collected on pecan it was found that insects occurring in different regions on the same crop might be involved in the dissemination of different fungal pathogens. It was also found that different fungal species are associated with insects occurring in the same orchard at different periods of the season, and that specific insect species might disseminate specific fungal pathogens at certain periods.

- By studying the succession of fungal species associated with *Coenomorpha nervosa* (grey-brown stink bug) through a pecan nut production season, and comparing it to other

samples (air, soil, leaf & nut samples) it was found that the highest diversity of fungi is on stink bug specimens.

- This indicates that stink bugs, most likely insects in general, play an important role in the dynamics of fungal populations within pecan orchards. As a result, the isolation of fungi from insects in tree-nut crop orchards, such as pecan orchards, may give a good indication of the mycoflora in the environment.

- Overall the two most abundant fungal species isolated from stink bugs and the other samples were *Alternaria tenuissima* and *Cladosporium cladosporioides*. *Alternaria* spp. are considered miscellaneous pathogens of pecan while *C. cladosporioides* is considered a potential pathogen and contaminant.

- The presence of these two species on stink bugs followed a similar pattern to the presence of these two species in the air and soil. However, the incidence of these two species on the stink bugs differed from their incidence in the leaves and nuts. This may indicate that the fungi occurring on the stink bugs is obtained from their entire environment and that the incidence of fungal species such as *A. tenuissima* and *C. cladosporioides* in the leaves and nuts cannot generally be attributed to vectoring by stink bugs.

- *Neofusicoccum parvum* was only isolated from the nuts and from the stink bugs. It was completely absent from all the other samples taken throughout the whole season. This indicates an association between the stink bugs and *N. parvum*, where the stink bugs act as disseminators spreading this fungus between developing nuts as they feed. *Neofusicoccum parvum* has not been previously found to be associated with pecan.

- This study has shown that the grey-brown stink bug occurring in pecan orchards is associated with fungal phytopathogens and that it has the capability of acting as a disseminator of these pathogens.

- By studying the pathogenicity of fungal species associated with the grey-brown stink bug occurring in pecan orchards, it was found that both *Alternaria tenuissima* and

Neofusicoccum parvum are pathogenic on pecan nuts and leaves, while *Cladosporium cladosporioides* cannot be considered a pathogen of this crop.

- Both *A. tenuissima* and *N. parvum* are more virulent on pecan nuts than on pecan leaves. *Alternaria tenuissima* is less virulent than *N. parvum* and can be considered a miscellaneous pathogen of pecan which will most likely only cause disease when plants are under stress.

- Wounding of pecan nuts increases disease incidence on nuts inoculated with *A. tenuissima*, and is a necessity for both *A. tenuissima* and *N. parvum* to be able to infect, colonise and cause symptoms on pecan leaves.

- Wounding does not increase the rate of disease development on nuts infected with *A. tenuissima* and *N. parvum*. However, it reduces the time required by *N. parvum* to infect and colonise pecan nuts. Due to such wounding, time is shortened in which significant damage is caused and also may reduce the time available to control this pathogen. In contrast, wounding does not provide any benefit to *A. tenuissima* during the initial colonisation of pecan nuts.

- This study confirmed that stink bugs in pecan orchards are associated with fungal phytopathogens. The stink bugs feed on pecan nuts, making wounds that can assist the pathogens in causing disease on pecan nuts. The grey-brown stink bug is likely a disseminator of *N. parvum*, spreading this fungus to developing nuts through wounding and feeding.

- Overall, the study has shown that insects occurring in tree-nut orchards have the capability to harbour and disseminate fungal phytopathogens. These insects feed on various parts of the trees causing damage and, in addition, provide suitable entry points into the trees through which pathogens gain entry to infect, colonise and cause disease.

SUMMARY

This study investigates insect-fungal-plant interactions on tree-nut crops (pistachio, walnut and pecan) in South Africa. The main aim was to determine whether insects occurring in tree-nut crop orchards are assocaited with fungal phytopathogens and whether they have the capability to harbour and dissiminate them. Isolations from *Atelocera raptoria* (powdery stink bug) on pistachio showed that the largest proportion of fungi associated with these insects are pathogenic, some serious pathogens of the crop. Isolations from *Coenomorpha nervosa* (grey-brown stink bug) and *Empoasca citricola* (green citrus leafhopper) on walnut showed that the insects were associated with fungi but the presence of pathogens was minimal. The isolation of fungi associated with insects occurring in pecan orchards showed that the most abundant fungal species from *Empoasca* sp. (leafhoppers) and the *Sciobius cf. granosus* (citrus snouted weevil) were pathogenic. Pathogens were also isolated from *Panafrolepta dahlmani*, however, a large proportion of the fungi isolated from these specimens were non-pathogenic. Fungi from the grey-brown stink bug, collected through a pecan nut production season and comparing it to other ways of dispersal (air, soil, leaf & nut samples), showed that the greatest diversity of fungi was found to be associated with the stink bug specimens. This indicates that stink bugs, most likely insects in general, play an important role in the dynamics of fungal populations within pecan orchards. As a result the isolation of fungi from insects in tree-nut crop orchards may give an indication of the mycoflora in the environment. Overall, the two most abundant fungal species isolated from stink bugs and other niches were *Alternaria tenuissima* and *Cladosporium cladosporioides.* The incidence of these two species on stink bugs followed a similar trend to the incidence of these two species in the air and soil. However, the incidence of these two species on the stink bugs differed from their incidence in the leaves and nuts. This indicates that the fungi occurring on the stink bugs are from their environment and that the presence of fungal species such as *A. tenuissima* and *C. cladosporioides* in the leaves and nuts cannot necessarily be attributed to vectoring by stink bugs. *Neofusicoccum parvum* was only isolated from the nuts and from the stink bugs. It was completely absent from all the other samples taken throughout the whole season. This suggests an association between the stink bugs and *N. parvum*, where the stink bugs act as disseminators spreading this fungus between developing nuts as they feed. Pathogenicity trials indicated that *A. tenuissima* and *N. parvum* are pathogenic on pecan nuts and leaves, while *C. cladosporioides* cannot be considered a pathogen of this crop. It was also found that

wounding of pecan nuts increases disease incidence on nuts inoculated with *A. tenuissima* and is a necessity for both *A. tenuissima* and *N. parvum* to be able to infect, colonise and cause symptoms on pecan leaves. Wounding does not increase the rate of disease development, but it reduces the time required by *N. parvum* to infect and colonise pecan nuts. In contrast, wounding does not provide the same benefit to *A. tenuissima*. Overall, the study has shown that insects occurring in tree-nut orchards have the capability to harbour and disseminate fungal phytopathogens. These insects feed on various parts of the trees causing damage and, in addition, provide suitable entry points into the trees through which pathogens gain entry to infect, colonise and cause disease.

Key terms: Insect-fungal associations, Tree-nut crops, Pecan, Stink bugs, *Coenomorpha nervosa*, Dissemination, Phytopathogens, *Alternaria tenuissima*, *Cladosporium cladosporioides*, *Neofusicoccum parvum*.

OPSOMMING

Hierdie studie ondersoek insek-swam-plant interaksies op neut gewasse (pistachio, okkerneut en pekanneute) in Suid-Afrika. Die hoofdoel was om te bepaal of insekte wat in neutboorde voorkom geassosieer is met swam fitopatogene en of hulle oor die vermoë beskik om vir hierdie 'n hawe te bied en hulle te versprei. Isolasie vanaf *Atelocera raptoria* (wollerige stinkbesie) op pistachio het getoon dat die grootste gedeelte van swamme wat op hierdie insekte voorkom patogene is, insluitend ernstige patogene van die gewas. Isolasie vanaf *Coenomorpha nervosa* (grys-bruin stinkbesie) en *Empoasca citricola* (groen sitrus bladspringer) individue wat versamel is op okkerneute het getoon dat die insekte geassosieer is met swamme, maar die teenwoordigheid van patogene was minimaal. Die isolasie van swamme vanaf insekte wat in pekanneutboorde versamel is het getoon dat die mees prominente swamspesies wat voorkom op *Empoasca* sp. (bladspringers) en *Sciobius cf. granosus* (sitrus kalander) patogenies is. Patogene is ook geïsoleer vanaf die *Panafrolepta dahlmani* individue. 'n Groot gedeelte van hierdie swamme was egter nie-patogenies nie. Die isolasie van swamme vanaf die grys-bruin stinkbesie wat versamel is deur die loop van 'n pekanneut produksie seisoen, en wat vergelyk is met ander maniere van verspreiding (lug, grond , blaar en neut monsters), toon dat die grootste verskeidenheid van swamme geassosieer word met die stinkbesies. Dit dui daarop dat stinkbesies, waarskynlik insekte in die algemeen, 'n belangrike rol speel in die dinamika van swampopulasies binne pekanneutboorde. Die dui ook aan dat die isolasie van swamme vanaf insekte, wat in neutboorde voorkom, moontlik gebruik kan word om 'n goeie aanduiding van die omgewing se mikoflora te kry. Die twee mees prominente swam spesies wat algeheel die meeste geïsoleer is vanaf stinkbesies en vanaf ander nisse was *Alternaria tenuissima* en *Cladosporium cladosporioides*. Die teenwoordigheid van hierdie twee spesies op die stinkbesies het 'n soortgelyke patroon gevolg as die teenwoordigheid in die lug en grond. In teenstelling het die teenwoordigheid van hierdie twee spesies op die stinkbesies verskil van hul teenwoordigheid in die blare en neute. Dit kan daarop dui dat die swamme wat op die stinkbesies voorkom verkry word uit die omgewing en dat die teenwoordigheid van swam spesies soos *A. tenuissima* en *C. cladospioroides* in die blare en neute nie, in geheel, toegeskryf kan word aan verspreiding deur stinkbesies nie. *Neofusicocum parvum* is net geïsoleer vanaf die neute en die stinkbesies. Dit was afwesig in al die ander monsters wat geneem was tydens die hele seisoen. Dit dui 'n assosiasie aan tussen die stinkbesies en *N. parvum*, waar die stinkbesies optree as verspreiders wat hierdie

swam versprei tussen ontwikkelende neute tydens voeding. Patogenisiteitsproewe het gevind dat *A. tenuissima* en *N. parvum* patogenies is op pekanneute en pekanneut blare, terwyl *C. cladosporioides* nie as 'n patogeen van hierdie gewas beskou kan word nie. Dit is ook bevind dat wonding van pekanneute die voorkoms van siektes verhoog op neute wat geïnokuleer is met *A. tenuissima*, en dat dit 'n noodsaaklikheid is vir beide *A. tenuissima* en *N. parvum* om in staat te wees om pekanneutblare te infekteer, te koloniseer en simptome te kan veroorsaak. Wonding versnel nie die tempo van siekte ontwikkeling nie, maar dit verkort die tyd wat benodig word deur *N. parvum* om pekanneute te infekteer en te koloniseer. In teenstelling verskaf wonding nie dieselfde voordeel aan *A. tenuissima* nie. In die geheel het die studie bewys dat insekte wat in neutboorde voorkom die vermoë het om 'n hawe te bied vir fitopatogene en hulle te kan versprei. Hierdie insekte voed op verskillende dele van die bome, wat skade veroorsaak en daarbenewens geskikte toegangs punte verskaf waardeur patogene toegang verkry om bome te infekteer, te koloniseer en siekte te veroorsaak.