Declaration.

I declare that this dissertation is my own work. It is being submitted for the Degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has not been submitted to any other university for any other purpose or examination.

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Musa Donald Marimani

_____ day of_____2010

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Abstract.

The aims of this project were twofold: (a) to compare the accumulation patterns of antifungal diene (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene) and triene (*Z,Z,E*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene) compounds in harvested and unharvested "Fuerte' avocado fruits in response to inoculation with *Colletotrichum gloeosporioides* and relate these patterns to observations of the infection cycle, and (b) to use rDNA ITS nucleotide sequence analysis to confirm the identities of some important fungal pathogens of avocado. One of these pathogens was an apparent complex of *C. gloeosporioides* and *Pseudocercospora* sp. (CgP complex). Inoculation of harvested and unharvested fruits with *C. gloeosporioides* at approximately 240 d after fruit set caused an increase in diene and triene levels as measured by HPLC and HPLC-MS compared with controls. In both experiments, these levels reached a maximum during the first 2 d, and subsequently decreased during the 7 d monitoring period. However, there were some notable differences in fluxes of these compounds between experiments. In harvested fruits during the active period, diene levels were higher in the flesh than the peel, whereas triene levels were higher in the peel than the flesh and triene levels decreased to below those of controls in the later stages whereas diene levels did not. Similar patterns emerged for unharvested fruits but diene levels in unharvested fruits were relatively higher than in harvested fruits whereas triene levels were similar in harvested an unharvested fruits. By employing SEM ellipsoid spores characteristic of *C. gloeosporioides* were first observed on the harvested inoculated fruit surfaces at 2 d, followed by surface hyphae at 3 d. However, the infection process of *C. gloeosporioides* in unharvested fruits was delayed, relative to harvested fruits, probably due to high antifungal diene and triene contents of unharvested

fruits. In contrast to SEM observations, ellipsoid spores of *C. gloeosporioides* were observed on the inoculated fruit surfaces at 7 d by using CLSM, and no surface hyphae were noted. The ITS universal primers were employed to confirm the identities of the fungal pathogens; Primer pair of ITS5 and ITS4 was used to amplify genomic rDNA from the CgP complex, *Pseudocercospora* sp. and *Phomopsis* sp., while ITS1 and ITS4 primers were used to amplify *Colletotrichum* sp. genomic DNA. Expected PCR product sizes of ~550 base pairs were obtained for all amplifications. Phylogenetic analysis revealed that the CgP sequences were closest to a *Phomopsis* sp., and confirmed the identity of *Pseudocercospora purpurea*, *Colletotrichum gloeosporioides* and *Phomopsis perseae*.

CHAPTER 1: General introduction.

1.1. The avocado host: history, distribution and cultivars.

Persea genus is one of 50 genera in the family *Lauraceae*, which also includes camphor, cinnamon and different significant wood species (Zentmyer, 1994). The avocado (*Persea americana* Miller) originated in South America, where its fruit formed an essential part of the local diet for thousands of years (Snowdon, 1990). In the 17th century, avocados were established in the Caribbean islands but spread beyond Central America in the 19th century (Whiley, 1991). These fruits were introduced into South Africa by Dutch settlers, by bringing seedlings from the West Indies and other Dutch colonies (Durand, 1990). Three botanical varieties may be identified within *P. americana* and they include viz. Mexican (*P. americana* Miller var. drymifolia Schlecht. & Cham. Blake), Guatemalan (*P. nubigena* L.O. Williams var. guatemalensis L.O. Williams) and West Indian (*P. americana* Miller var. *americana*) (Whiley, 1991). Mexican varieties consist of cultivars such as Zutano, Bacon and Duke, and originated in the cool subtropical highland forests of Mexico. The fruit from these cultivars is of little economic significance in South Africa, however, Zutano and especially Duke are essential as rootstock cultivars. Guatemalan varieties are originally from the tropical highlands of Guatemala and consist of cultivars such as Edranol and Hass. Additionally, West Indian cultivars are originally from Central America, but are of little economic significance in South Africa (Durand, 1990). 'Fuerte', which is the most popularly grown cultivar in South Africa, is a natural hybrid of the Mexican and Guatemalan varieties (Durand, 1990).

1.2. Biology of avocado.

Trees of avocado are evergreen, can grow to a height of 8-20 metres and vary somewhat in shape (Jackson, 1986). New growth occurs in flushes, but the period between flushes differs with cultivar and locality (Nakasone and Paull, 1998a). Avocado trees are mostly propagated by grafting on seedling or clonal rootstocks with the required characteristics (Zentmyer, 1994). Trees grown from seed can start to produce within four to eight years, but asexually propagated trees can start within three years (Fintrac Inc., www.marketasia.org 1998, last accessed on the 30 November 2009). Small, pale-green flowers are borne on multi-branched panicles and possess a unique flowering behaviour because flowers open twice, the first time as a functional female (pistil receptive) and the second time, as a functional male (pollen shedding) (Whiley, 1991). Avocados are grouped into two types with respect to their flowering patterns viz. A (e.g. Hass; Guatemala) and B (e.g. Bacon; 'Fuerte') (Nakasone and Paull, 1998a).

Based on Botany, avocado fruit are considered to be berries (Zentmyer, 1994). The single large seed is composed of two cotyledons enclosing the embryo and is surrounded by a mesocarp which is thick (Nakasone and Paull, 1998a). Avocado fruits differ greatly with respect to variety. Oil content may range from 3-30 %, and the West Indian varieties have the lowest oil content (Fintrac_Inc., www.marketasia.org 1998, last accessed on the 30 November 2009). Fruit shape is usually pyriform to ovoid (Nakasone and Paull, 1998a). Development periods of fruit are different depending on variety, with the Guatemalan varieties having the longest period viz. 10-15 months from fruit set to maturity. Harvest maturity is dependent on variety and production area, and is determined using the oil

content, percentage dry matter and colour analysis (Fintrac Inc., www.marketasia.org 1998, last accessed on the 30 November 2009).

1.3. Economic importance.

Avocados are grown in approximately 50 countries (Zentmyer, 1994), and are the sixth-most essential subtropical crop in the world (Ploetz, 1994a). World production of these fruits is mostly dominated by countries such as Mexico, Brazil, USA, Spain and Israel (Van Zyl and Ferreira, 1995). However, Mexico and Brazil do not export in large quantities, although they are large producers, because 97 % of all fruit produced in Mexico is for local use, while cultivars produced in Brazil are not acceptable for export because of poor fruit quality (Van Zyl and Ferreira, 1995). Hass is the main avocado variety produced world-wide, but internationally, varieties such as 'Fuerte', Crillo, San Miguel and Taylor are also popular (Graef, 1995). Countries that compete with South Africa on the European market for avocado fruit export are Israel, Spain, Mexico and Kenya (Ernst, 1997).

Approximately 12 400 hectares are currently utilized for avocado production in South Africa. Total production ranges between 85 000 and 100 000 tons per annum. Export production ranges between 35 000 and 43 000 tons per annum, with the remainder being consumed by local markets. The season for exporting is between March and October (http://www.westfalia.co.za, last accessed on the 20 July 2010). Seventy percent of avocados cultivated in South Africa are exported, 95 % of them to Europe (South African Avocado Growers' Association). Consequently, as the South African subtropical fruit industries are very export-oriented, a high premium is put on ensuring fruit quality.

However, one of the most significant threats to the maintenance of such standards is post-harvest diseases such as anthracnose on avocados caused by *Colletotrichum gloeosporioides* (Jeffries *et al*., 1990) and *Cercospora* spot on avocados caused by *Pseudocercospora purpurea*. Therefore, this study was essential in terms of investigating aspects of the biology of anthracnose infection caused by *C. gloeosporioides* and to understand the accumulation patterns of natural antifungal compounds with a view to manipulating them as a possible form of biocontrol. In addition, the study investigated the phylogenetic associations amongst common fungal pathogens of avocado.

1.4. The main aims of this study were:

- To monitor the accumulation patterns of the antifungal diene and triene compounds in harvested and unharvested avocado fruits (cv. 'Fuerte') in response to infection by *C. gloeosporioides* and relate these to stages of the infection cycle.
- To identify *C. gloeosporioides* and associated fungal pathogens using molecular techniques aimed at targeting and amplifying the internal transcribed spacer (ITS) region of the ribosomal DNA.

CHAPTER 2: Literature review.

2.1. Biology of *Colletotrichum* and *Pseudocercospora* pathogens of avocado.

Section 2.1 introduces the biology, infection strategies, symptoms and control of anthracnose and *Cercospora* spot caused by *C. gloeosporioides* and *P. purpurea*, respectively. Moreover, it also aims to evaluate the role of antifungal compounds during quiescent infections of *C. gloeosporioides*. It provides background to the first major aim of the study.

2.1.1. Anthracnose and *Colletotrichum* species.

Anthracnose is a disease characterized by sunken necrotic tissue where orange conidial masses are produced by *Colletotrichum* species (Bailey *et al*., 1992). *Colletotrichum* species cause anthracnose on a variety of temperate, subtropical and tropical fruits worldwide, and are especially a severe problem in tropical areas (Bailey and Jeger, 1992). Although many cultivated fruit crops are infected by *Colletotrichum* species, the most significant economic losses are observed when fruits are infected after harvest (Freeman *et al*., 1998). In general, conidia germinate on the fruit surface and produce appressoria and quiescent infections which only develop after the fruits begin to ripen. The fungal pathogen *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. has been associated with quiescent infections and post-harvest diseases on a wide variety of fruits. These include avocado, mango, papaya, guava, passion fruit, citrus, apple and grapes (Simmonds, 1965; Hartill, 1992; Alahakoon *et al*., 1994; Timmer *et al*., 1998). *Colletotrichum acutatum* has been reported on many temperate and subtropical fruits including apple (Bernstein *et al*., 1995; Shi *et al*., 1996), grapes (Kummuang *et al*., 1996), kiwi (Ushiyama *et al*., 1996), peach

(Bernstein *et al*., 1995; Adaskaveg and Hartin, 1997), almond (Adaskaveg and Hartin, 1997) and pecan (Bernstein *et al*., 1995). Except for causing anthracnose, *C. gloeosporioides* also causes dieback, root rot, leaf spot, blossom rot and seedling blight on a variety of crops including avocado, almond, peach (Freeman *et al*., 1998), peppers (Manandhar *et al*., 1995), papaya (Dickman, 1994), mango (Ploetz, 1994b), *Stylosanthes* species (Chakraborty and Jones, 1993), citrus (Timmer *et al*., 1994), rubber trees (Brown and Soepena, 1994), passion fruit (Jeffries *et al*., 1990) and strawberry (Denoyes and Baudry, 1995). Cross-inoculation studies with *C. gloeosporioides* on a wide variety of hosts have been described (Maas and Howard, 1985; Alahakoon *et al*., 1992; Freeman *et al*., 1996). Differential virulence of *C. gloeosporioides* isolated from many different hosts was shown when inoculated into other hosts (Quimio and Quimio, 1975; Freeman and Shabi, 1996). In addition, the pathogen was shown to genetically adapt to the new host, ultimately inducing the same level of disease as isolates from the host in question (Alahakoon *et al*., 1992). Several hosts susceptible to *C. gloeosporioides* are grown world-wide and losses where multiple hosts such as mango, avocado, coffee, papaya and citrus are grown in close proximity could be significant (Freeman *et al*., 1998). This is true in South Africa, where the climatic zones suited for cultivation of subtropical crops are small, and avocados and mangoes are often grown in adjacent blocks or orchards (Sanders and Korsten, 2003).

Based on morphology, South African isolates of *C. gloeosporioides* from avocado and mango are as phenotypically different as isolates from other countries over the world, with colony colours ranging from pale salmon pink to dark gray green. Isolates from mango are

often less different than those from avocado. Many isolates produce appressoria terminally on germ tubes and only minute differences can be observed between appressoria of different isolates, their mean length being 14.6 µm (Sanders and Korsten, 2003). Four different types of conidial shapes have been described, and both avocado and mango isolates mainly produce cylindrical conidia with a tapered base and obtuse apex. Conidial length differs considerably, but width remains relatively constant. But, no correlation is observed between the length/width ratio of conidia and the virulence of isolates when inoculated into avocado and mango host fruits (Sanders and Korsten, 2003).

2.1.2. Disease cycle, epidemiology and symptoms.

C. gloeosporioides conidia are produced in large numbers on dead leaves and twigs entangled in the canopy of trees (Fitzell, 1987). Infected fruit still hanging on the tree can be an essential source of inoculum in cultivars such as 'Fuerte'. In rainy weather, conidia are washed down through the canopy of the tree. Infection often occurs during extended periods of warm rainy weather, and fruit are susceptible at all stages from fruit set to harvest (Peterson, 1978; Coates *et al*., 1993a). In the presence of free water, many conidia deposited on the fruit surface germinate within 7 hours (Parbery, 1981). Each germinated conidium produces a germ tube that has a length of 10-20 µm. Approximately 5-6 hours after germ tube production, development of a terminal appressorium proceeds. During initial stages, the apex of the germ tube becomes swollen, enlarging back towards the conidium (Parbery, 1981).

An infection peg is produced from the germ-pore and invades the outer wax layer and cuticle of the fruit skin. Growth of the infection peg is restricted in the cuticular region, where it remains dormant until fruit ripening (Prusky *et al*., 1990; Coates *et al*., 1993b). It is thought that the fungus is not able to colonize unripe tissue because of the presence of antifungal compounds in the avocado fruit peel (Prusky *et al*., 1983). During ripening, levels of antifungal compounds decrease in the peel, enabling fungal growth to proceed. Cells underlying the peel and flesh are colonized, causing symptom development. In extensive stages of lesion development, acervuli are produced beneath the fruit surface. Consequently, the cuticle and epidermal cell walls are ruptured, and conidia are produced in a mucilaginous matrix followed by water dispersal (Whiley, 2002).

In Type 1 lesions which occur prior to harvest in cultivars such as 'Fuerte', it has been hypothesized that insect- or mechanically-induced wounds result in localized ripening, causing a decline in the levels of antifungal compounds in affected areas (Fitzell, 1987). Presumably, such a decline enables the fungus to colonize tissue that would normally be resistant. Type 2 lesions appear to be capable of developing without any skin damage (Fitzell, 1987). Coates (1991) was able to reproduce Type 2 lesions at specified times of the year by inoculating developing avocados in the orchard with 10^6 conidia ml^{-1} of *C. gloeosporioides*. Interestingly, Type 2 symptoms were not produced when fruit were inoculated with 10^4 conidia ml^{-1}, thus, showing that high inoculum concentrations in the field may be a critical factor in the development of pre-harvest anthracnose symptoms (Whiley, 2002). A diagram illustrating the life cycle of *Colletotrichum gloeosporioides* is shown in Figure 2.1.

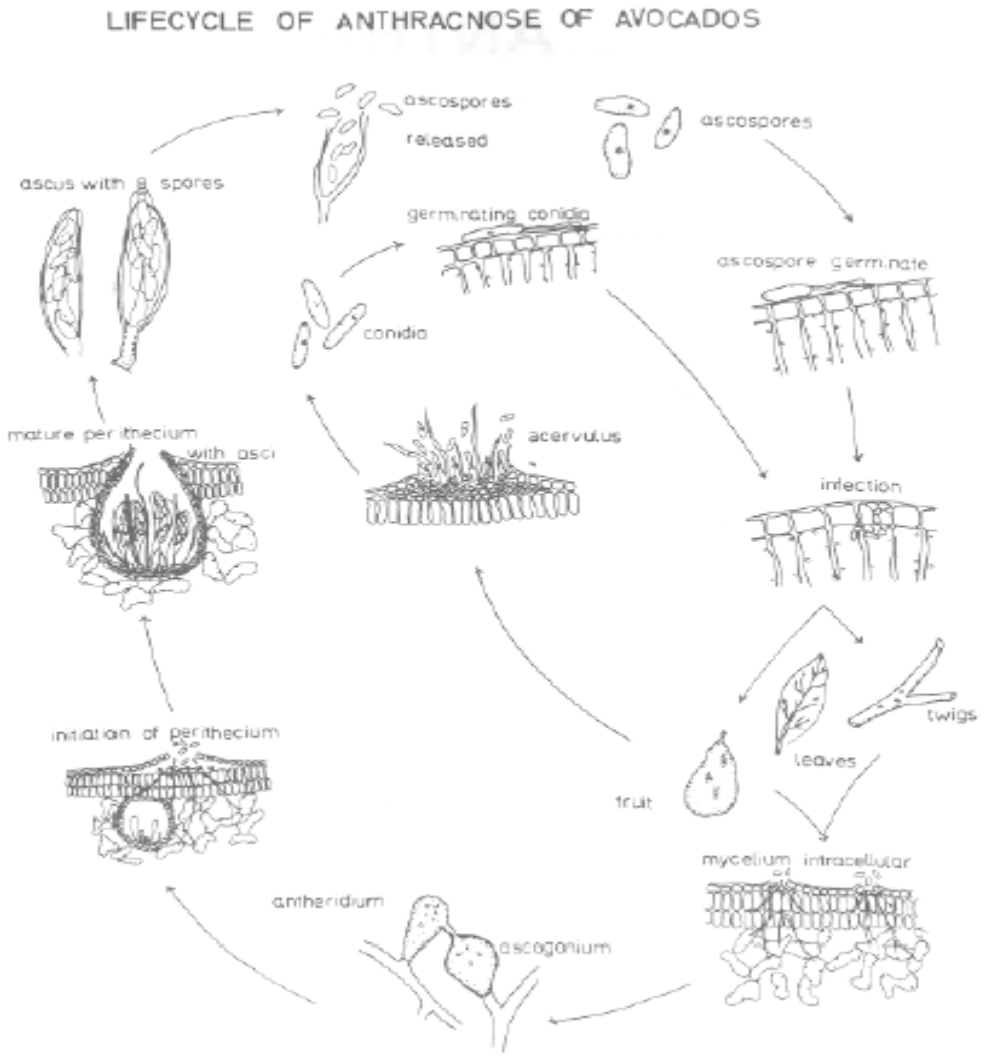


Figure 2.1. Diagrammatic illustration of the life cycle of *Colletotrichum gloeosporioides* on avocado fruits (Kotzè, 1978).

Anthracnose symptoms can manifest on flowers, fruit, leaves or twigs. Infected fruit is the most important concern, but most fruit damage does not manifest until after harvest. In groves, unhealthy or dead leaves are the most obvious symptoms. Spots develop on leaves, starting as yellow, then brown discolourations that coalesce into large dead areas. Necrosis manifests across or between leaf veins, on leaf margins and generally at leaf tips. If the disease is severe, trees drop many leaves at an early stage. Brown or purplish lesions develop on new shoots and shoots may die back (http://www.ipm.ucdavis.edu/, last accessed on the 21 April 2008).

Prior to harvest, brown to black lesions less than 5 mm in diameter manifest around lenticels on infected fruit. These small discolourations may be ignored while fruit are still on the tree and lesions often do not enlarge until fruit ripens after harvest. Sometimes, large lesions manifest on avocados on the tree, often after infected fruit is injured by insects or mechanical wind rubbing. Lesions become blacker, larger and increasingly sunken after harvest. Ultimately, lesions spread over the entire fruit surface and throughout the pulp. When the fruit is cut in half through one of the lesions, rot advancing into the flesh generally exhibits a hemispherical pattern. Initially, decayed pulp is firm, but becomes soft and putrid as decay advances. Pink spore masses may be produced on the fruit surface and under wet conditions; a slimy mass of pink spores erupts through the fruit skin (http://www.ipm.ucdavis.edu/, last accessed on the 21 April 2008). An infected avocado fruit exhibiting typical anthracnose symptoms is shown in Figure 2.2.

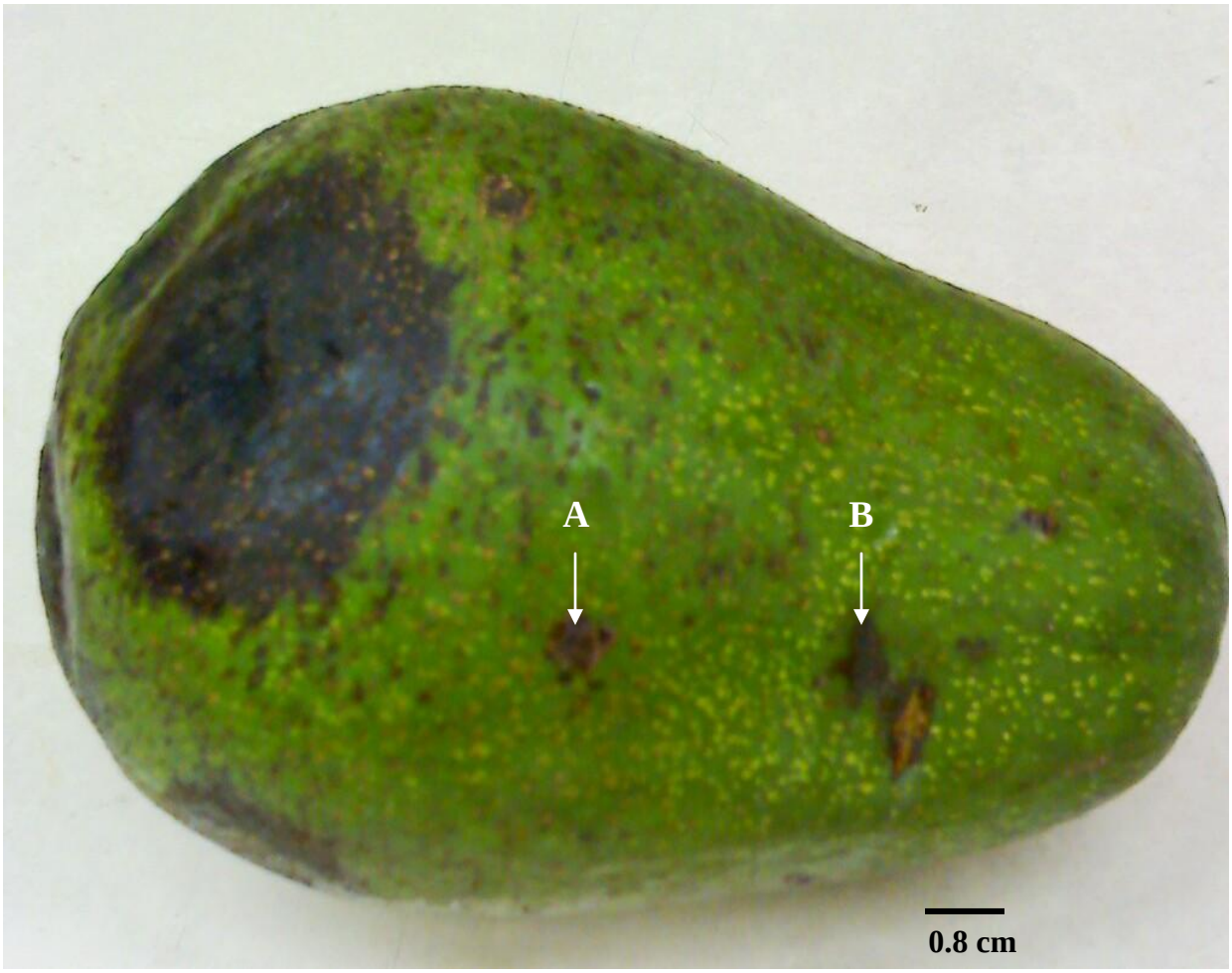


Figure 2.2. Anthracnose symptoms in avocado showing 'Fuerte' avocado fruit with black specks (A) and anthracnose lesions (B) caused by *Colletotrichum gloeosporioides*.

2.1.3. Pathogenesis and infection process of *Colletotrichum* species.

The pathogenesis of *Colletotrichum* diseases is different, stemming from the fundamental nutritional and ecological diversities within the genus. Some species (e.g. *C. circinans* and *C. capsici*) are necrotrophs (Pring *et al*., 1995). After penetrating the host surface, these necrotrophs employ a subcuticular, intramural infection strategy, involving an intercellular mode of growth with large production of cell wall degrading enzymes. A few *Colletotrichum* species (mostly *C. gloeosporioides* and *C. acutatum*) are either mutualistic endophytes or quiescent endophytes which switch to necrotrophy after a latency spell (Prusky and Plumbley, 1992; Latunde-Dada *et al*., 1999). In most cases, endophytic species directly penetrate host leaves or may penetrate through stomatal openings and then ramify the mesophyll intercellularly without symptom development (Akinwunmi and Latunde-Dada, 2001).

Quiescent fungal species such as *C. gloeosporioides* have a prolonged phase of pre-penetrative growth restricted in synchrony with the physiological state of the infected organ. Other *Colletotrichum* species, such as *C. orbiculare* species aggregate (consisting of *C. lagenarium, C. malvarum, C. trifolii* and *C. lindemuthianum*), *C. graminicola, C. sublineolum, C. destructivum, C. truncatum* and *C. linicola* are hemibiotrophs (Bailey *et al*., 1992, 1996, 1997; Skipp *et al*., 1995; Latunde-Dada *et al*., 1996). The strategy of hemibiotrophic species initiates from a transient post-penetrative asymptomatic biotrophy that is rapidly accompanied by a phase of destructive necrotrophy resulting in the appearance of disease symptoms and production of conidiomata by the pathogen. In all cases, apart from the *C. orbiculare* species aggregate where spore septation does not

manifest, conidial germination requires the formation of an equatorial septum along the breadth of the spore. In addition to the need for conidial and appressorial adhesion, there are two major pre-requisites for direct host penetration by forcible means. These include melanin (the permeability barrier) and osmolytes (such as glycerol, for production of osmoticum from which the high turgor pressure and invasive forces derive). The magnitude of the internal pressures and forces formed within melanized appressoria for host penetration needs a secure anchoring of these structures to host surfaces. Such is the tenacity of this adhesion that strong mechanical treatments such as ultrasonication do not remove the basal walls of melanized appressoria from their substratum (Mendgen and Deising, 1993; Deising *et al*., 2000).

But, no direct measurements of the adhesive forces have been reported, but as noted by Talbot (1999) 'if the adhesion force did not exceed the penetration force, the cell (i.e. the appressorium) will simply lift away from the leaf' (Akinwunmi and Latunde-Dada, 2001). The significance of appressoria in the infection process has been indicated in many studies with melanin deficient mutants and inhibitors of melanin biosynthesis (Kubo and Furusawa, 1991; Mendgen and Deising, 1993). Moreover, many detailed light and electron microscopy studies have been based on the infection process of *Colletotrichum* and reviewed in detail by Bailey *et al*. (1992) and O`Connel *et al*. (2000). Although pre-penetration steps are often the same in all *Colletotrichum* species, main differences become evident after penetration, when two types of infection strategies can be identified: intracellular hemibiotrophy and subcuticular, intramural necrotrophy (Bailey *et al*., 1992; Skipp *et al*., 1995). Several *Colletotrichum* species initially establish infection by utilizing

a brief biotrophic phase, which is associated with large intracellular primary hyphae. Later, they resort to a destructive, necrotrophic phase, associated with narrower secondary hyphae, which ramify throughout the host tissue. In species which employ this strategy, the starting biotrophic phase can differ in duration from less than 24 hours to over 72 hours (O`Connel *et al*., 1985; Latunde-Dada *et al*., 1996; Wharton and Julian, 1996). Primary hyphae produced by such species can also differ greatly in morphology (Wharton and Diegue`z-Uribeondo, 2004).

Unlike ripening fruits, unripe fruits have extremely high concentrations of pre-formed antimicrobial compounds. These compounds may accumulate in the immature pericarp at concentrations of up to 1 mg g^{-1} fresh weight, and include 5-substituted resorcinols such as 5-(12-cisheptadecenyl)-resorcinol in mango, dienes such as (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene in avocado, and saponins like a-tomatine in tomato (Prusky and Plumbley, 1992; Prusky, 1996). Because of such a chemically adverse environment, the fungus has to bide its time. However, how the fungus times the infection process to match with fruit ripening is not understood (Flaishman and Kolattukudy, 1994). The ability to attain a foothold in the host without encountering the arsenal of passive chemical resistance or inducing those of active resistance is inherent in a number of fruit-infecting *Colletotrichum* species such as *C. gloeosporioides, C. acutatum* and *C. musae*. Conidia, melanized appressoria and penetration pegs of these quiescent species have developed mechanisms for physiological inactivity, being quiescent until the ripening process starts, thus, allowing the avoidance of host defences within unripe, pre-climacteric fruits. These species, especially *C. gloeosporioides*, are field-to-store pathogens whose conidia are

invariably regarded as surface contaminants with pre-harvest fruits. Physiological inactivity of quiescence also allows avoidance of detection by humans. The surface wax of avocado, and not that of any other plant species, selectively induces germination and appressorium formation only by the conidia of *C. gloeosporioides* (Podila *et al*., 1993). Moreover, conidia of quiescent *Colletotrichum* species need contact with a hard surface in order to recognize the host signals that trigger spore germination (Liu and Kolattukudy, 1998).

2.1.4. Reproduction and genetics of *Colletotrichum* species.

The life cycle of *Colletotrichum* species has a sexual and an asexual stage. Generally, the sexual stage is responsible for the genetic variability, while the asexual stage is responsible for fungal dispersal. Sexual recombination in many *Colletotrichum* species is rare in nature and to date only 11 out of about 20 *Colletotrichum* species have *Glomerella* teleomorphs. Furthermore, sexual reproduction in *Glomerella* is more complex than is usual for most ascomycete fungi. Fungal species that reproduce sexually can often be classified as either self-fertile (homothallic), or self-sterile (heterothallic). But, *Glomerella* is not usual because within a single species some strains are both self-fertile and cross-fertile, while others are cross-fertile but self-sterile (Chilton and Wheeler, 1949; Wheeler, 1954). Based on extensive studies on the genetics of mating in *Glomerella cingulata*, it was concluded that heterothallism is obtained from homothallism through mutations in genes controlling steps in the morphogenetic pathway necessary for self-fertility (Wheeler, 1954).

2.1.5. *Cercospora* spot and *Pseudocercospora* species.

Cercospora spot or blotch of avocados was first described by Cooke (1878). Later it was found to be mostly scattered along the East Coast of the USA on seedling avocado plantings in Florida (Stevens, 1920) and was referred to as the most important disease of avocados there (Zentmyer, 1953). In a list of the main industry problems, *Cercospora* spot was mentioned from French West Indies, Martinique and Cameroon (Gustafson, 1976). It was the second most common avocado disease in Mexico (Turu, 1969).

Avocado black spot (*Cercospora* spot) is caused by the fungus *Pseudocercospora purpurea* (Cooke) Deighton (teleomorph, *Mycosphaerella*) and is still the most important pre-harvest avocado disease in South Africa. The disease is noted by raised shiny black spots (1-6 mm in diameter in the early stage), with spots becoming sunken in later stages (Darvas, 1982). According to Brodrick *et al*. (1974), this disease was first noted in South Africa in the Tzaneen area (Limpopo province of South Africa). It has since spread throughout the Limpopo Province and now also occurs in the Nelspruit (Mpumalanga Province of South Africa).

The economic significance of the disease was illustrated by Brodrick *et al*. (1974) who reported that untreated orchards only produced 20 % exportable fruit, while 85-90 % of the fruit was exportable from trees regularly sprayed with fungicides. There are two different *Pseudocercospora* species namely *P*. *perseae* and *P*. *purpurea* which are capable of infecting *Persea* (avocado) trees. From preliminary studies of the fungi participating in fruit and leaf spot, it appears that *P*. *purpurea* is predominant. This theory was consolidated

by several experiments in which typical symptoms were induced by inoculating fruit and leaves with spore suspension of the organism accompanied by successful re-isolation (Darvas, 1977). *P. purpurea* possesses relatively short conidiophores in compact to spreading fascicles. Conidia are long, 1-9 septate, straight to curved, ranging 2-4, 5 X 20-100 μ (Chupp, 1953). In pure cultures, particularly on rich media, conidia are much longer, often with 11 or more septa. Cultures sporulate abundantly under near ultraviolet treatment (Darvas, 1977).

2.1.6. Disease cycle, epidemiology and symptoms.

Although an ascomycete teleomorph may exist, *Cercospora* spot infection often manifests through conidia, which are present all year, particularly when relative humidity is high. Most of the inoculum probably comes from leaves that are infected (Pohronezny *et al*., 1994). Sporulation is advanced during warm rainy weather. Conidia are spread easily by wind, insects, splashing rain and irrigation to start new infections (Pegg and Coates, 1993; Pohronezny *et al*., 1994). Fungal penetration can be direct or through wounds, after which the fungus remains latent for about 3 months. Infections manifesting early in the season causes the highest disease incidence and severity at harvest. Late season fruit are less susceptible, while fruit less than 40 mm in diameter and those reaching maturity are immune to infection (Pohronezny *et al*., 1994). Fruit are more susceptible when the size range is between 25-75 % of their final size. In South Africa, the fungus remains latent for up to three months after infection before spots are observed. It has been observed that the severity of *Cercospora* spot is correlated with the severity of root rot caused by *Phytophthora cinnamomi* in South Africa (Pohronezny *et al*., 1998; Manicom, 2001).

On the fruit lesions appear as small, scattered, brown, slightly sunken spots which have a definite outline but irregular shape. Brodrick *et al*. (1974) described disease symptoms on the fruit as minute, raised, black, shiny spots of not more than 3 mm in diameter and are generally associated with a cracking and corking at the lenticels. Grayish spore-bearing structures of the fungus are observed on the spots (3 to 6 mm in diameter), which later develop cracks, enabling entry of other fungi which cause fruit decay. *P*. *purpurea* also causes small angular spots on leaves (Zentmyer, 1953). The final phase is probably the easiest to note and this is where other fungi have excess to cause rapid rot of fruit. The typical brownish cracking and surrounding tissues often turn black, hence the misleading name "black spot" used for *Cercospora* spot in Tzaneen and other regions (Darvas, 1977). An infected avocado fruit exhibiting typical *Cercospora* spot symptoms is shown in Figure 2.3.

Figure 2.3. *Cercospora* spot symptoms in avocado showing 'Fuerte' avocado fruit with characteristic minute, raised, black and shiny spots caused by *Pseudocercospora purpurea*.

2.1.7. The role of antifungal compounds during quiescent infections in unripe fruits.

Different reasons have been provided to explain the manifestation of quiescent infections by a pathogen in unripe fruits. These include insufficient enzyme production, nutritional requirements and the presence of antifungal compounds which restricts pathogen development in unripe fruits, but not in ripening fruits (Verhoeff, 1974; Swinburne, 1983). The action of antifungal compounds in quiescent infections of unripe fruits was suggested to be the cause of either their triggering or presence as preformed compounds (Prusky *et al*., 1989). Accumulation of capsicannol phytoalexins, 1-deoxycapsidiol and the endesmadienol (Watson and Brooks, 1984), has been associated with the manifestation of quiescent infections in *Capsicum annuum* fruits infected with *C. gloeosporioides* (Adikaram *et al*., 1982). In contrast to this, Karni *et al*. (1989) reported a notable decline in the diene compound [(*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene] concentration in freshly harvested fruits, and postulated that it is possible that this manifests due to harvesting stress. Although the diene declined to subfungitoxic levels, the period of low concentration is short and fungal development does not manifest until fruit ripening 7-10 days later. Wounding, inoculation, or exposure to CO_2 or ethylene can trigger the preformed antifungal diene compound. However, the biosynthetic pathway of the diene compound has never been reported. The presence of a multiple double bond in the antifungal compound molecules of avocado fruit peel of (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene and the triene compound [(*Z,Z,E*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene] (Prusky and Keen, 1995; Domergue *et al*., 2000) may imply that desaturation is a critical step in the biosynthesis of these compounds (Madi *et al*., 2003).

Among the primary fatty acid synthesis products of the diene compound are palmitoyl-acyl-carrier (16:0-ACP) and Δ^9 stearoyl-ACP (18:0-ACP). The soluble plastid enzyme called stearoyl-ACP desaturase (EC1.14.99.6) introduces the first double bond into stearoyl-ACP, between carbons 9 and 10, to form oleoyl-ACP (18:1 Δ^9-ACP). More double bonds are introduced into oleic acid or palmitoleic acid by Δ^{12} desaturases after incorporation of the fatty acids into lipids. Δ^9-stearoyl-ACP desaturase activity is a critical determinant of the net level of fatty acid desaturation (Thompson *et al*., 1991; Shanklin and Somerville, 1992).

It appears as though fungal quiescence or fruit resistance depends on the period of, or modulation of the time of decline in diene concentration (Prusky *et al*., 1991). Lipoxygenase activity regulates a decrease in the concentration of the antifungal diene which, in turn, is regulated by a decrease in the concentration of the endogenous antioxidant, epicatechin (Prusky *et al*., 1988; Karni *et al*., 1989).

The antifungal diene compound has many other biological activities and includes acting as a growth inhibitor of the silkworm larvae *Bombyx mori* L. (Chang *et al*., 1975), an inducer of lactating mammary necrosis of mice (Rodriguez-Saona *et al*., 1997) as well as a feeding deterrent to larvae of *Spodoptera exigua* (Kashman *et al*., 1969). Additionally, the antifungal compound (*Z,Z,E*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene has also been isolated and identified from idioblast oil cells. This compound has antifungal activity, as it inhibits *C. gloeosporioides* spore germination and germ tube elongation similarly to the diene (Domergue *et al*., 2000). Kobiler *et al*. (1993) also showed antifungal

activity of these two compounds (diene and triene) against the fungus *Colletotrichum gloeosporioides*. Chemical structures of the antifungal triene and diene are shown in Figure 2.4.

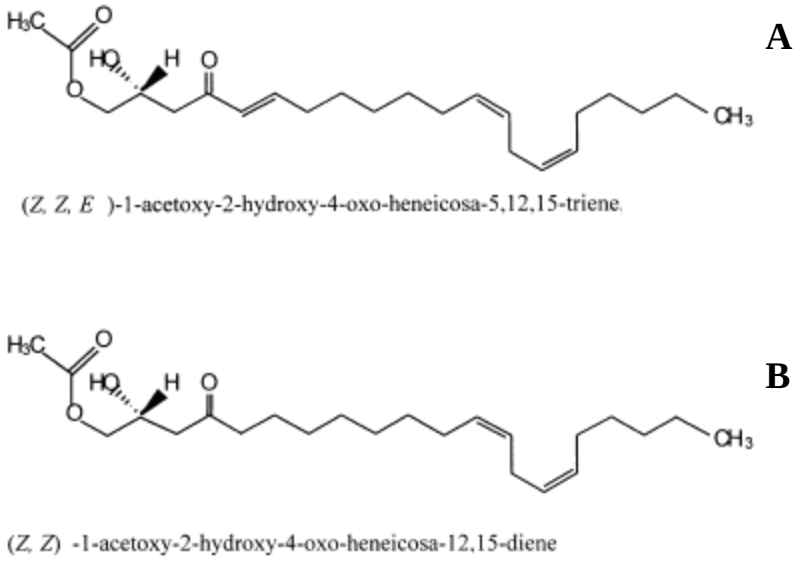


Figure 2.4. Chemical structures of the triene (A) and diene (B) compounds as determined by Domergue *et al*. (2000).

2.1.8. Control of anthracnose and *Cercospora* spot.

Effective control of *C. gloeosporioides* diseases often requires the utilization of one or combination of the following: (a) resistant cultivars, (b) cultural control, (c) chemical control, and (d) biological control using antagonistic organisms.

(a) Resistant cultivars.

Resistance to disease may be more essential for the control of disease in agricultural crops. However, it has been utilized to a lesser degree in fruit crops, particularly due to long duration needed for breeding and selecting for resistance, relative to shorter-term advantages of chemical control. Resistant cultivars in fruit are complicated by the ability of many *Colletotrichum* pathogens to form quiescent infections. In most host-pathogen interactions, resistance needs the induction of host defence responses that inhibit or restrict pathogen development and may be conditioned by a single gene pair, a host resistant gene and a pathogen avirulence gene (Flor, 1971). Generally, such gene-for-gene interactions are usually not involved in fruit resistance to post-harvest diseases caused by *Colletotrichum* species (Prusky *et al*., 2000). Instead, resistance is often the result of many genes interacting in a way that is not well understood. Fruit resistance to post-harvest pathogens has been reported as a "dynamic incompatibility" (Prusky *et al*., 2000). The response of the host resistance genes to products of a pathogen's avirulence genes inhibits or restricts pathogen development under specific host physiological conditions (Wharton and Diegue´z-Uribeondo, 2004).

Physiological changes during ripening result in softening of tissues and manifestation of decay symptoms (Prusky *et al*., 1984). Resistance of unripe avocado fruits to post-harvest pathogens has been suggested to be more associated with the presence in the peel of the antifungal compound (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene (Prusky *et al*., 1982, 1983). The diene compound has an ED_{50} for inhibition of germ tube elongation of 420 μg ml^{-1} (Prusky *et al*., 1982). Levels of this compound generally decrease during fruit ripening from concentrations of 1000 μg g^{-1} fresh weight to subfungitoxic concentrations below 350 μg g^{-1} fresh weight and this decrease coincides with the development of decay in fruit infected by *C. gloeosporioides* (Prusky *et al*., 1982).

(b) Cultural control.

Cultural control normally involves the range of methods used to control diseases, mainly employing tactics aimed at disease avoidance through phytosanitation, manipulation of crop patterns or by improving resistance and avoiding predisposition. In fruit crops, it requires the utilization of proper sanitation methods during the processing of harvested fruit, transportation, packaging and storage, to avoid contact of the fruit to the pathogen. The ubiquitous nature of inoculum sources of *Colletotrichum* diseases and their general rapid epidemic development under desirable conditions decreases the effectiveness of many pre-harvest general phytosanitory methods. However, orchard hygiene is critical in disease control, as removal of inoculum sources such as diseased leaves and fruit can improve the efficiency of chemical control (Waller 1972, 1988).

(c) Chemical control.

In most cases, chemical control methods are used on fruit crops, partly because the value of the produce gained normally offsets the relatively expensive inputs in terms of materials and labour, machinery, transportation and storage. In general, *Colletotrichum* diseases can be controlled by many chemicals which include copper compounds, dithiocarbamates, benzimidazole and triazole compounds. Other fungicides include chlorothalonil, imazalil and prochloraz (Waller, 1992).

Control of *C. gloeosporioides* and other post-harvest avocado and mango pathogens is based mainly on inoculum decrease and inhibition of latent infections. As such, it is essential to have an appropriate pre-harvest spray programme in place for this purpose. In South Africa, previously registered fungicides included monthly pre-harvest applications of benomyl, accompanied by cupric hydroxide or copper oxychloride for avocados, and copper oxychloride or with alternate mancozeb or benomyl sprays for mangoes. Apart from benomyl, the above compounds are all contact fungicides and timing of application should correlate with periods of high rainfall when inoculum is dispersed (Sanders *et al*., 2000).

Introduction of benzimidazole fungicides such as benomyl, carbendazim and thiophanates in the early 1960`s improved disease control. However, extensive utilization of these compounds caused selection for resistant pathogen genotypes, which remained dominant for many years after discontinued use. The first cases of resistance were reported in fungi with short life cycles, e.g. *Botrytis cinerea* in vineyards (Sanders *et al*., 2000). Resistance has further been described for fungi isolated from different of sources, e.g. *Venturia*

inaequalis from apple, *Penicillium digitatum* and *P. italicum* from citrus and *C. gloeosporioides* from rambutan and mango. Currently, prochloraz and thiabendazole are only registered for post-harvest treatment of avocados, the former also for post-harvest use on mangoes (Leroux and Clerjeau, 1985; Lonsdale, 1992a).

(d) Biological control using antagonistic organisms.

Biological control methods aimed at controlling *Colletotrichum* diseases have not received much attention, although the potential of biological control through the effect of phyllosphere antagonists has been observed (Lenn`e and Parbery, 1976). Effects of surface microflora on the incidence of anthracnose diseases such as coffee berry disease, avocado anthracnose and mango anthracnose are currently being clarified with the aim of improving naturally occurring biocontrol mechanisms (Masaba and Waller, 1992; Korsten and Jeffries, 2000). Although most of the technology is still at the research level, current progress has resulted in a number of new commercial products, e.g. Aspire™, BioSave™, Trichodex™, AQ10™ and Avogreen ™ (Wilson, 1997; Janisiewicz, 1998; Korsten *et al*., 1998).

Cercospora spot is generally controlled by two to five applications of copper fungicides during the rainy season, with benomyl normally being included once per season. The currently increasing need to decrease the amount of copper applied to orchards, thereby decreasing copper build-up in soils, is driven by export markets and future sustainability of farming operations. Trials performed during the 2000/01 season in a high disease pressure orchard demonstrated that certain products should be evaluated further. Application of

copper oxychloride followed by Lime Sulphur, Ortiva, Solanacure or Avogreen resulted in similar control. Avogreen and Solanacure are antagonists, and because Avogreen is already a registered commercial product, neither was further assayed. Of the strobilurins assayed, Ortiva (azoxystrobin) gave the best control of *Cercospora* spot and demonstrated potential for anthracnose control and was further evaluated in the 2001/02 season (Duvenhage, 2002).

2.2. Morphological and molecular identification of the fungal pathogens.

Section 2.2 discusses the morphological and molecular identification of *C. gloeosporioides* and *P. purpurea*, and also introduces the *Colletotrichum gloeosporioides-Pseudocercospora* (CgP) complex. This provides background to the second major aim of the study.

2.2.1. Morphological identification of *Colletotrichum* species.

Corda established the genus *Colletotrichum* in 1831 for fungal species with hyaline, curved fusiform conidia and setose acervuli (Jeffries *et al*., 1990). But, there has been uncertainty and confusion since the first description, as to which fungi could be in this genus. Genera such as *Vermicularia* Tode were considered as part of the genus *Colletotrichum* (Baxter *et al*., 1985) and it was difficult to distinguish *Gleosporium* Desm. & Mont. from *Colletotrichum* (Jeffries *et al*., 1990). Von Schrenk and Spaulding introduced the generic name *Glomerella* in 1905 with five original species, including *G. cingulata* (Sutton, 1992). Monographs by von Arx (1957) and (1970) decreased confusion in the *Colletotrichum* genus by employing cultural characteristics and host-specificity as criteria. This reduced

the number of described taxa significantly. As such, many species of *Gleosporium* and *Vermicularia* were included and the revised genus was broader, but taxonomically simpler (Jeffries *et al*., 1990) with 39 so-called accepted species (Sutton, 1992). *Glomerella* is recently recognized to have about 10 species and *Colletotrichum* over 20 (Skipp *et al*., 1995).

Although limits for genus definition have been well reported, the species still remain poorly understood (Skipp *et al*., 1995). Evolution of different systems for identification of different species is mainly caused by changing concepts regarding the significance of morphology in correlation with advancing knowledge about host-pathogen relationships and host ranges (Sutton, 1992). System development for the identification of *Colletotrichum* species varies little from those employed to describe other groups of mitosporic fungi (Sutton, 1992). *Colletotrichum* species readily grow in culture and cultural characteristics may be placed to criteria available for species differentiation. The minimal number of morphological characteristics employed for this purpose, in addition to inherent morphological plasticity and phenotypic overlap, has lead to much controversy concerning authoritative identifications (Sreenivasaprasad *et al*., 1996). This controversy caused broad, unreliable concepts about species based on classical criteria such as conidial shape and size, presence, absence and morphology of setae, presence of sclerotia and appressoria and symptom manifestation on hosts (Sutton, 1992).

Consequently, morphology alone does not allow strain identification and when breeding programmes, control measures and quarantine methods need this degree of accuracy,

identification of an isolate as belonging to a species complex is of little value (Sutton, 1992). *Colletotrichum* species may be divided into two main groups, one with straight conidia and the other with falcate conidia (Skipp *et al*., 1995). Conidial shape may provide a reliable way of identifying certain species and has been used to determine the identity of *Colletotrichum* species pathogenicity to strawberry (Denoyes and Baudry, 1995). However, in some cases, identification may be complicated because of overlapping ranges of conidial morphology and differences in colony characteristics (Adaskaveg and Hartin, 1997). Moreover, characteristics such as benomyl sensitivity and temperature response can be employed to show similarity between isolates, but cannot be utilized as distinguishing features of a species (Adaskaveg and Hartin, 1997).

Host-specificity is shown at a number of levels, with some species having a wide host range (*C. acutatum* JH. Simmonds, *C. crassipes* (Speg.) v. Arx, *C. dematium* and *C. gloeosporioides*). In contrast, some species are restricted to a specific host or family (*C. circinans* (Berk.) Voglino, *C. coccodes* (Wallr.), S. Hughes and *C. musae* (Berk. and M.A. Curtis.) von Arx). Within a host-specific species or form, physiological races specific for a specific host genotype have been identified, and it is possible that gene-for-gene systems are being used (Skipp *et al*., 1995). In other cases, specificity is defined within a species, such as *C. gloeosporioides* on *Stylosanthes* in Australia, where two different biotypes have been reported. These biotypes are host-specific and have been indicated to differ in morphology, symptomlogy, double stranded RNA, restriction fragment length polymorphisms (RFLPs) and molecular karyotypes (Manners *et al*., 1992).

2.2.2. Molecular identification of *Colletotrichum* species.

Implementation of different systems for classification and elucidation of evolutionary pathways has been a subject of debate for many years. Evolutionary histories of organisms are currently elucidated using phylogenetic relationships together with phenetic classification. This phylogenetic character mapping requires the use of molecular and morphological data to assist in defining the rise of adaptations and subsequent evolutionary development. Molecularly-based trees must be constructed, so as to enable correct identification of an organism using molecular characteristics (Mitchell *et al*., 1995). However, for characters to be useful in elucidating evolutionary relationships, they must be diverse, but must also be conserved to some extent (Michelmore and Hulbert, 1987). Sequences from genes that have the evolution rate, are present only once in the genome, or act like a single copy region are the most useful for this purpose. Genes that fulfill these criteria are the ribosomal RNA (rRNA) and mitochondrial RNA (mtRNA) genes from the nuclear and mitochondrial genomes, respectively (Mitchell *et al*., 1995). Most fungal phylogenetic studies utilize sequences from the ribosomal gene cluster, as they are present in large numbers as tandem repeats and evolve as a single unit. As a consequence, the rDNA repeat unit has been widely utilized for restriction enzyme studies and affords an ideal priming target for amplification (Mitchell *et al*., 1995).

Almost all sequence studies in fungi have been based on these genes because of the universally conserved regions that behave as ideal primer sites (Bruns *et al*., 1991). The mtDNA is a desirable source of genetic markers, because it is easy to purify and is found in high copy numbers. Additionally, the genome is small enabling simple banding patterns

when digested with restriction enzymes. Isolates of certain fungal species also have mitochondrial plasmids, and natural variation manifests either if they are present, absent or there are differences in plasmid size or homology (Michelmore and Hulbert, 1987). However, they are of minimal usage due to lack of conservation and consequent transmission. Mitochondrial DNA is not widely employed for comparative purposes as rDNA because length mutations manifest at a high frequency, thereby, complicating analyses such as restriction fragment length polymorphisms (RFLPs). However, mtDNA is widely employed for evolutionary studies in fungi (Bruns *et al*., 1991).

The ribosomal gene cluster is present as a series of head to tail tandem repeats with 60-200 copies per haploid genome (Henson and French, 1993). This cluster is composed of three structural regions coding for the 5.8S, 18S and 28S rRNA genes. In between these genes are the transcribed spacer regions. Genes that lie between the 18S and 5.8S, 5.8S and 28S genes are called the internally transcribed spacer (ITS) regions. The region that separates this cluster along the chromosome is called the non-transcribed spacer (NTS) region. Prior to the 18S gene is another small spacer region called the externally transcribed spacer (ETS) (Mitchell *et al*., 1995). The ETS and NTS regions together form the intergenic spacer (IGS) region (Mitchell *et al*., 1995). Despite conservation of rRNA genes, there is enough variation to utilize them as targets for specific amplification (Henson and French, 1993). More variation is present in the ITS regions, followed by NTS region, while the most variation is found in the IGS region (Henson and French, 1993). These regions, structural genes, transcribed spacers and non-transcribed spacers evolve at different rates and sequences can be used to distinguish between taxa at different levels (Sherriff *et al*.,

1994). Structural genes are highly conserved and can be employed to differentiate to order level (Mitchell *et al*., 1995). Due to their high variability, the ITS and IGS regions can be utilized to distinguish taxa from class to species (Sherriff *et al*., 1994).

The recent taxonomic status of *Colletotrichum* species is unclear (Sreenivasaprasad and Talhinhas, 2005). The wide host range of many species has resulted in problems for plant pathologists who need to identify specific plant pathogens to control disease. Molecular techniques have provided additional data to assist the naming of fungi, and have been used to taxonomically difficult genera including *Fusarium* (O′Donnell *et al*., 1998), *Pestalotiopsis* (Jeewon *et al*., 2002; Lui *et al*., 2007), *Mycosphaerella* and its anamorphs (Crous *et al*., 2000; Mancini *et al*., 2006), but to a lesser degree to *Colletotrichum* (Sreenivasaprasad *et al*., 1996; Moriwaki *et al*., 2002; Du *et al*., 2005; Photita *et al*., 2005).

For example, *Colletotrichum gloeosporioides* is broadly accepted as the etiological agent of yam anthracnose (Singh *et al*., 1966; Winch *et al*., 1984). But, identification and differentiation of *Colletotrichum* species utilizing morphological characteristics have normally been inadequate (Brown *et al*., 1996), as demonstrated in a study of yam anthracnose fungi, in which some fungal isolates appeared similar to both *C. gloeosporioides* and *C. acutatum* (Abang *et al*., 2001). Isolates identified as *C. gloeosporioides* varied greatly in morphology and virulence (Abang *et al*., 2001), as well as in randomly amplified polymorphic DNA (RAPD) banding pattern (Thottappilly *et al*., 1999). For this reason, molecular techniques such as arbitrarily primed polymerase chain reaction (AP-PCR) (Freeman and Rodriguez, 1995), polymerase chain reaction (PCR)

using species-specific primers (Adaskaveg and Hartin, 1997), ITS-RFLP and ITS sequence analysis (Sreenivasaprasad *et al*., 1994, 1996; Martín and García-Figueres, 1999) have been utilized to resolve systematic challenges in *Colletotrichum* species.

Additionally, utilization of molecular marker techniques has enhanced the accuracy and speed of identification as well as classification of phytopathogenic fungi. Among these molecular techniques, DNA fragment analysis (e.g. RAPD and AP-PCR) has been broadly utilized to investigate relationships between isolates of many fungal genera including *Colletotrichum* species. In the same way, nucleotide sequence information from the 5.8S gene and the ITS region of rDNA has been utilized to design *Colletotrichum* species-specific primers for diagnostic purposes and phylogenetic analysis (Tapia-Tussell *et al*., 2008).

Fungal pathogens *C. gloeosporioides* and *C. acutatum* are especially difficult to identify using traditional taxonomic methods because of their morphological variability, the ample range of their hosting crops, and the broad variety of their cultured isolates. Therefore, traditional taxonomic methods need to be complemented with molecular techniques (Andrade *et al*., 2007; Whitelaw-Weckert *et al*., 2007). Consequently, *C. gloeosporioides* and *C. acutatum* specific oligonucleotides have been broadly employed in differentiating these two fungal pathogens by using PCR (Freeman *et al*., 1998, 2001; Peres *et al*., 2002b; Afanador *et al*., 2003; Sanabria, 2007). The clear identification of the pathogen obtained using this technique has sometimes led to discarding crossed infection hypotheses (i.e. those stating that just one fungal species is responsible for infecting different fruit crops)

(Freeman and Katan, 1997). Furthermore, AP-PCR and polymorphisms in nuclear DNA, rDNA, mtDNA, and AT-rich DNA have been utilized to distinguish between populations of *C. acutatum, C. coccodes*, *C. fragariae*, *C. gloeosporioides*, *C. kahawae*, *C. magna* and *C. orbiculare* (Correll *et al*., 1993; Freeman *et al*., 1993; Hodson *et al*., 1993; Sreenivasaprasad *et al*., 1993; Sherriff *et al*., 1994; Brown *et al*., 1996; Johnston and Jones, 1997). Due to lower conservation of nucleotide sequences in the NTS and ITS regions between the small and large nuclear rDNA subunits relative to the coding regions, these have been utilized to recognize current evolutionary divergence within *Colletotrichum* species (Freeman *et al*., 2000; Ford *et al*., 2004).

2.2.3. Morphological identification of *Cercospora* species.

Cercospora Fres. is a heterogeneous genus with more than 2,000 particular epithets placed to it (Chupp, 1954; Ellis, 1971; International Mycological Institute, 1971–1998). Traditionally, the identification of *Cercospora* species is mainly focused on conidial characters and host association (Chupp, 1954; Ellis, 1971, 1976). But, it is becoming increasingly apparent that some species in this genus are conspecific with a broad host range, while others should be placed in other genera (Johnson and Valleau, 1949; Jones, 1959; Deighton, 1974, 1976, 1979; Ellis, 1976; Siboe and Braggins, 1994). *Cercospora apii*-group is a group of *Cercospora* species with morphological and cultural similarity to *C. apii* (the lectotype species), and *Pseudocercospora purpurea* has been classified as part of the *C. apii*-group (Siboe *et al*., 2000).

In general, these *Cercospora* species form well-defined brown or pale brown to tan necrotic spots on their host plants. The necrotic lesions possess characteristic raised and brown, dark brown, or reddish purple borders. Conidia are hyaline or subhyaline, acicular, obclavate, truncate at the base, with a hilum thicker than the wall of the conidium, and tapering toward the apex (Chupp, 1954; Siboe, 1989). Evidence from biochemical and pathogenicity data suggests that the *C. apii*-group fungi are all similar to *C. apii* with no discernible host specialization (Johnson and Valleau, 1949; Jones, 1959; Ellis, 1976; Siboe, 1989). However, mycologists and plant pathologists have not been convinced enough to efficiently reduce these species of *Cercospora* to synonymy with *C. apii*, but proceed to report as new any *Cercospora* found on a host for the first time, although they appreciate the major limitation of the host association criterion in the *C. apii*-group species delimitation. Fifteen to 20 new species names on average have been published every year since 1971 (International Mycological Institute, 1971–1998).

Although *Cercospora* species have usually been dismissed as weak parasites (Chupp, 1954; Ellis, 1971), attention has been focused on the increasing virulence and epidemic outbreaks of previously unimportant species. For example, high yield losses due to gray leaf spot disease caused by *Cercospora zeae-maydis* Tehon & E.X. Daniels are recently being recorded worldwide on maize (*Zea mays* L.) (Ward *et al*., 1997). For this reason, as the economic importance of *Cercospora* diseases of crop plants increases, there is a need for the unequivocal taxonomy of taxa within the genus for accurate identification (Siboe *et al*., 2000).

Accurate identification of the causative agent of a disease is an epidemiological need for efficient disease management and control. Information on the host range of a pathogen is critical in the establishment of sources of inocula and virulent pathotypes for plant crops. To be able to understand the relationship between the *C. apii*-group species, genetic information on members of the group are essential. Diagnosis of *Cercospora* spot is only possible from sporulating necrotic lesions or cultures. The setbacks associated with this method are that *Cercospora* species are time-consuming to isolate into axenic culture and challenging to sporulate *in vitro*. Moreover, diagnosis at the first sign of disease is usually too late for economical and safe control of the disease. Because of the setbacks of microscopy in disease diagnosis, there is a need for a rapid method for detecting infection in the host plants at the latent stage (Siboe *et al*., 2000).

2.2.4. Molecular identification of *Cercospora* species.

The integration of morphological and molecular data sets in *Mycosphaerella* is beset with a number of problems. *Pseudocercospora purpurea* is a teleomorphic species of the *Mycosphaerella* genus and many hypotheses have been suggested to divide the genus into separate parts, groups, sections or genera. Recently, the molecular work has mainly supported one major clade of *Mycosphaerella*, and two minor clades, typified by *Dissoconium* and *Cladosporium* (Crous *et al*., 2000, 2001b). The view that anamorph concepts have not always correlated with different groups in *Mycosphaerella*, but have been demonstrated to have evolved more than once within the genus, has created confusion. However, in some cases, integration has simplified or decreased the genera. For example, species of *Mycovellosiella* (superficial mycelium, conidia in chains),

Phaeoramularia (no superficial mycelium, conidia in chains) and *Passalora* (no superficial mycelium, conidia solitary) mostly cluster together, resulting in synonymy of the genera under the older name *Passalora* (Crous *et al*., 2001b).

Pseudocercospora is another confusing example. Published work by Crous *et al*. (2001b) demonstrates that many other genera are also clustered with *Pseudocercospora*, and these include *Pseudophaeoramularia* U. Braun, and *Stigmina*, while the position of *Stenella* was not clearly resolved. As more taxa have been placed for analyses, it has become evident that *Stenella* and *Stigmina* are in fact good genera, whereas *Pseudophaeoramularia* clusters with *Pseudocercospora*. These observations are still in agreement with those of Crous *et al*. (2001b), namely that conidial catenulation is not an ideal feature at a generic level, and that the extent of thickening of spore scars is critical, rather than just presence or absence (Crous *et al*., 2000). Molecular data also influences the way a teleomorph is defined. Preliminary data propose that ascospore septation, and the presence of pseudoparenchymatal stromatic tissue around the ostiole is of lesser taxonomic importance at the generic level. This will cause many older names having to be re-evaluated, and may ultimately need the conservation of *Mycosphaerella*, to safely preserve the name for future use over older, but lesser known names (Crous *et al*., 2000).

2.2.5. The *Colletotrichum gloeosporioides-Pseudocercospora* (CgP) complex.

The *Colletotrichum gloeosporioides-Pseudocercospora* complex is an apparent fungal complex comprising of the fungal pathogens *Colletotrichum gloeosporioides* (teleomorph,

Glomerella cingulata) and a *Pseudocercospora* sp. Originally a culture of '*Glomerella cingulata*' (PPRI 6008) that had been isolated from an infected avocado was obtained from the Plant Protection Research Institute (PPRI). However, after a spore morphological verification it was hypothesized that this species was in fact not *Glomerella cingulata*, but, rather a fungal complex comprising *Colletotrichum gloeosporioides* and a *Pseudocercospora* sp. Despite numerous attempts, the two fungi of the complex could not be separated and as such the complex is referred to as the *Colletotrichum gloeosporioides-Pseudocercospora* (CgP) complex (Giovanelli, 2008).

Because of apparent similarities in spore morphology between the CgP complex and the genus *Phomopsis*, another common fungal pathogen of avocado, *Phomopsis perseae*, was brought into the phylogenetic investigation.

2.3. The specific objectives of this study were:

- To inoculate pre- and post-harvested avocado fruits with spores of *C. gloeosporioides*.
- To observe the infection process of *C. gloeosporioides* by scanning electron microscopy and confocal laser scanning microscopy.
- To determine the levels of the antifungal diene and triene compounds in harvested and unharvested avocado fruits at different times using high performance liquid chromatography and relate these to stages of the infection process.
- To identify the fungal pathogens *Colletotrichum gloeosporioides*, *Pseudocercospora purpurea*, *Phomopsis perseae* and the *Colletotrichum gloeosporioides-Pseudocercospora* complex using molecular techniques.

CHAPTER 3: Materials and methods.

3.1. Source and maintenance of isolates.

Fungal strains of *Glomerella cingulata* (Penz.) Penz. & Sacc. (PPRI 6008), the redesignated fungal complex comprising *Colletotrichum gloeosporioides* and a *Pseudocercospora* sp. (Giovanelli, 2008) and *Phomopsis perseae* (PPRI 6006) the causative agent of stem-end rot and trunk canker in avocado fruits were obtained from the Plant Protection Research Institute (PPRI) culture collection.

Additionally, *Pseudocercospora* sp. and *Colletotrichum* sp. were isolated from infected avocado fruits (Roodewal farm, Nelspruit) exhibiting *Cercospora* spot and anthracnose symptoms, respectively using the tissue transplanting technique described by Ratanacherdchai *et al*. (2007), with some modifications (Figure 3.1). Infected fruits were wiped with 70 % (v/v) ethanol (Merck). The disease plant parts were cut at advanced margin of lesions into small pieces (about 5 mm X 5 mm) and surface disinfected with 1 % (v/v) sodium hypochlorite (JIK) for 1 min. Lesions were rinsed in sterile deionised water, transferred onto Malt Extract Agar (MEA, 50 g l^{-1}, Biolab) and Potato Dextrose Agar (PDA, 39 g l^{-1}, Biolab), and incubated at 25 °C (Ratanacherdchai *et al*., 2007). All fungal isolates were maintained by either inoculation of avocado fruits once every 3 months or sub-culturing onto (MEA) and (PDA) at 25 °C every 3 weeks. Preliminary identifications of *Pseudocercospora* sp. and *Colletotrichum* sp. to genus level were made using the key of Barnett (1965) and tentatively classified as *P. purpurea* and *C. gloeosporioides* based on host source and subject to molecular verification.

Figure 3.1. 'Fuerte' avocado fruits exhibiting symptoms of *Cercospora* spot (A) and anthracnose (B) from which the fungal pathogens *Pseudocercospora* sp. and *Colletotrichum* sp. were isolated.

3.2. Experimental site.

The experimental site was at Roodewal farm in Nelspruit, South Africa (Figure 3.2) on Orchard 7, which contained 'Fuerte' avocado trees. Orchard 7 was 2.5 hectares in area and the trees were young and small (2-3 metres in both height and width) with 2-3 metres between each tree. The size of the orchard was 247 m X 199 m X 339 m.

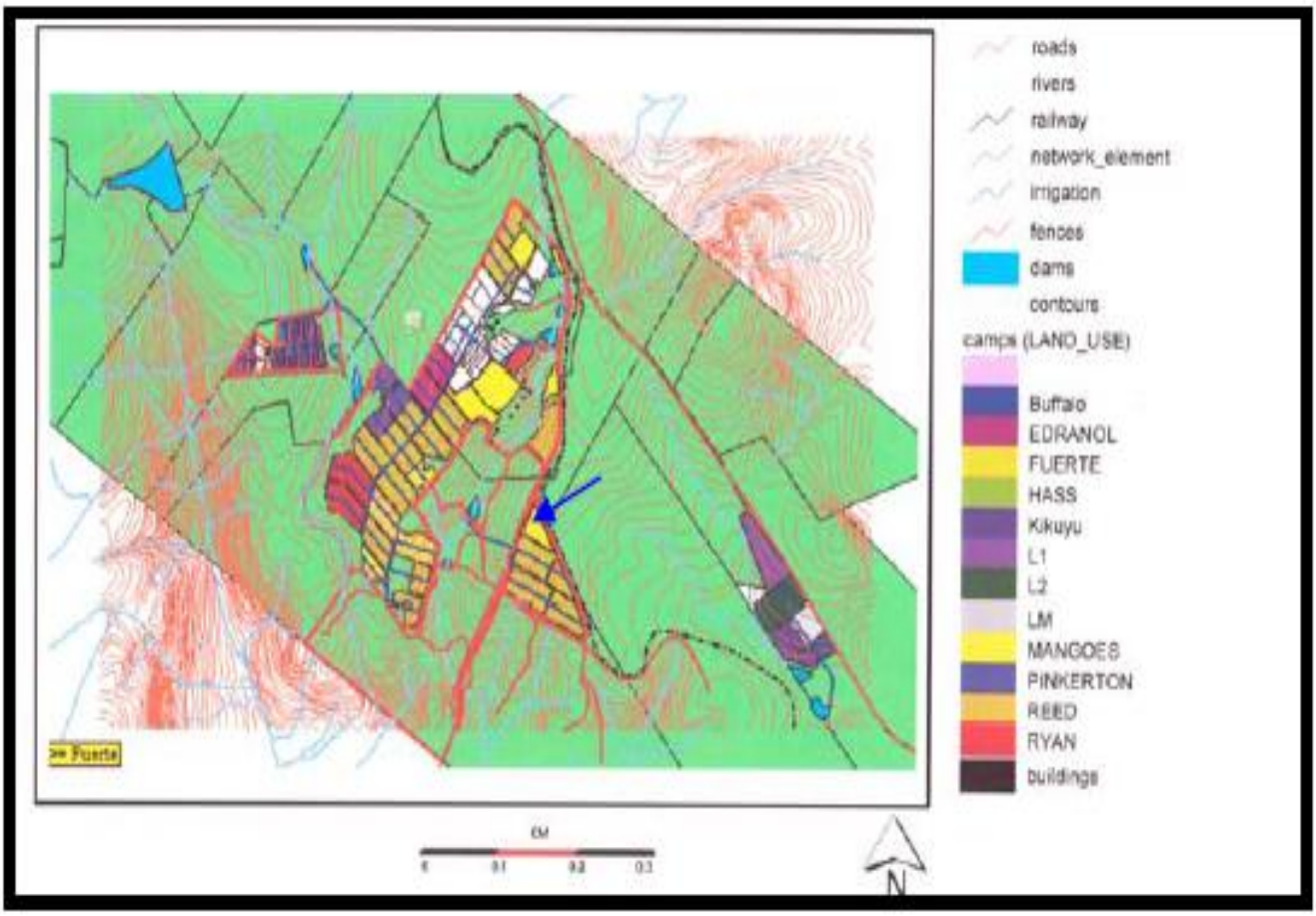


Figure 3.2. Geographic map of Roodewal farm, Nelspruit, South Africa generated on ESRI ArchExplorer 1.1. Orchard 7 is indicated by an arrow.

3.3. Experiment to monitor the accumulation of (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene and (*Z,Z,E*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene in harvested and unharvested fruits.

Figure 3.3 shows the experimental design of the diene and triene experiment.

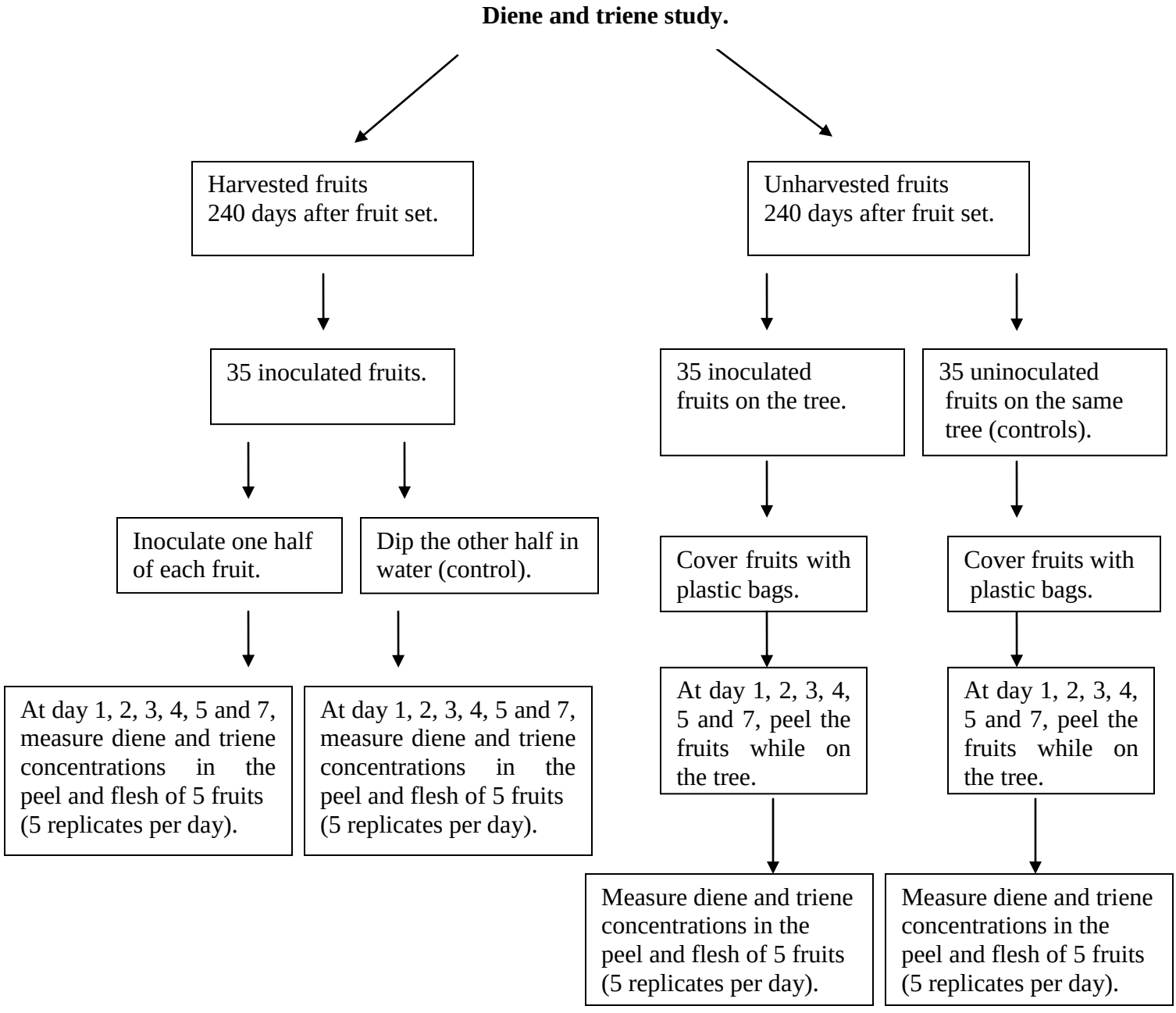


Figure 3.3. Experimental design of experiment to monitor accumulation patterns of the antifungal diene and triene.

3.3.1. Inoculation of avocado fruits with *Colletotrichum gloeosporioides* to monitor antifungal diene and triene accumulation.

Fresh fungal cultures of *C. gloeosporioides* were grown on MEA (50 g l^{-1}, Biolab) and PDA (39 g l^{-1}, Biolab) at 25 °C until sporulation. Spore suspensions (conidia) of the fungus were prepared by adding 10 ml of 0.05 % Tween-80 (Sigma-Aldrich) to the fungal cultures. Spores were harvested by using a glass spreader and filtered through four layers of sterile cheesecloth to remove the fungal mycelial debris, centrifuged at 10 000 *g* (Spectrafuge 24D, Labnet) for 1 min and adjusted to 1 x 10^7 spores per ml^{-1} using a haemocytometer (Marienfeld, Germany) (Prusky *et al*., 1990).

Prior to inoculation, all fruits were surface sterilized by immersion in 1 % (v/v) sodium hypochlorite (JIK) for 10 min, rinsed three times in sterile deionised water and allowed to air-dry. Fruits were wiped with 70 % (v/v) ethanol (Merck) and allowed to air-dry (Giovanelli, 2008). One hour after harvest, fruits were inoculated by immersing the longitudinal half of the fruit in a continuously stirred spore suspension (1 x 10^7 spores per ml^{-1}) containing 0.05 % Tween-80 (Sigma-Aldrich) for 1 min. The stem end was prevented from touching the inoculum. The other longitudinal halves of the fruits were dipped in sterile deionised water (controls) (Prusky *et al*., 1990). Fruits were incubated at 25 °C inside aluminium trays covered with aluminium foil and humidity was maintained by placing a petridish (90 mm, Biolab) filled with sterile deionised water at the centre of each tray. Symptom development on inoculated halves was monitored daily for up to 7 d after inoculation.

Whole unharvested fruits were inoculated by painting them with a spore suspension using a cotton swab and covered with plastic bags. Uninoculated fruits from the same tree were used as controls. Experiments were performed at approximately 240 d after fruit set. This is because inoculation of freshly harvested fruit at approximately 240 d after fruit set causes an increase in the diene concentration in the fruit flesh and peel (Prusky *et al*., 1990). Figure 3.3 shows the experimental design of the diene and triene experiment.

3.3.2. Theoretical background to HPLC-MS technique.

In order to measure the characteristics of individual molecules, a mass spectrometer converts them to ions to enable them to be moved about and manipulated by external electric and magnetic fields. The three important functions of a mass spectrometer, and the associated components, are: (a) the ion source, in which a small sample of compound is ionized, usually to cations by losing an electron, (b) the mass analyzer, in which the ions are sorted and separated according to their mass and charge, and (c) the detector, which detects and tails separated ions (Downard, 2007).

Each of the three tasks may be achieved in different ways. Generally, ionization is effected by a high energy beam of electrons, and ion separation is done by accelerating and focusing the ions in a beam, which is then bent by an external magnetic field. The ions are then detected electronically and the resulting information is stored and analyzed in a computer using appropriate software. The most important component of the spectrometer is the ion source, in which the molecules of the sample are bombarded by electrons arising from a heated filament. This is called an EI (electron-impact) source. Gases and volatile liquid

samples (e.g. triene and diene compounds) are allowed to leak into the ion source from a reservoir, but non-volatile solids and liquids may be introduced directly. Cations formed by the electron bombardment are pushed away by a charged repellor plate (anions are attracted to it), and accelerated toward other electrodes, having slits through which the ions pass as a beam. Some of these ions fragment into smaller cations and neutral fragments. When the ion beam experiences a strong magnetic field perpendicular to its direction of motion, the ions are deflected in an arc whose radius is inversely proportional to the mass of the ion. Lighter ions are deflected more than heavier ions. A mass spectrum is often presented as a vertical bar graph, in which each bar represents an ion having a specific mass-to-charge (*m/z*) ratio and the length of the bar shows the relative abundance of the ion. The most intense ion is assigned an abundance of 100, and it is called the base peak. Most of the ions formed in a mass spectrometer have a single charge, so the (*m/z*) value is equal to the mass itself. The highest-mass ion in a spectrum is normally considered to be the molecular ion, and lower-mass ions are fragments from the molecular ion, assuming the sample is a single pure compound (Downard, 2007).

In this study, the triene and diene compounds in the crude extracts were identified based on their respective (*m/z*) ratios in relation to elution profiles obtained in a previous study performed by Domergue *et al*. (2000). Since standards of the compounds were unobtainable, comparative relative abundances are expressed as peak heights in millimetres (mm).

3.3.3. Extraction, High Performance Liquid Chromatography (HPLC) and Mass Spectroscopy (MS) analysis of diene and triene compounds.

The antifungal compounds in avocado peel and flesh were extracted according to the method described by Prusky *et al*. (1982), with some modifications. Harvested fruits were picked and peeled, while unharvested fruits were peeled while on the tree. Ten grams of fruit peel and flesh (adjacent to the inoculated sites) were weighed and placed in 95 % (v/v) ethanol (Merck) for transport to the laboratory and homogenized (ULTRA-TURRAX T25, IKA-Labortechnik) in 50 ml of 95 % (v/v) ethanol (Merck) for 3 min. The homogenate was filtered using a 212 µm sieve (ENDECOTTS LTD. London, England). After filtration, the filtrate was concentrated to 5 ml in a Rotary evaporator (Roteva, EQUITRON) at 40 °C under vacuum and partitioned twice with 10 ml of 99.5 % (v/v) dichloromethane (Merck). The organic layers were pooled, dried with anhydrous magnesium sulphate (Merck), filtered using miracloth and dried in a Rotary evaporator at 40 °C under vacuum. The active compound was collected by adding 5 ml of 100 % (v/v) ethanol (Merck) (Prusky *et al*., 1990), and this provided a crude extract.

Determination of the presence of the diene and triene compounds was done by HPLC analysis (SRI 210 D LIQUID CHROMATOGRAPHY) of the active fraction in 100 % (v/v) ethanol (Merck). For both experiments (harvested and unharvested), 60 µl of the sample was pumped into a reversed-phase column (C18, 25 cm, Phenomenex). Compounds were eluted with 80 % (v/v) ethanol (HPLC Grade, Sigma-Aldrich) diluted with 20 % (v/v) deionized water at a flow rate of 1.5 ml min^{-1} and monitored with UV detection at a

wavelength of 205 nm (Domergue *et al*., 2000). Data analysis was achieved using PeakSimple 3.29 software.

MS analysis of the crude extracts was achieved using a method described by Domergue *et al*. (2000), with some modifications. MS data were obtained using an LXQ Finnigan mass spectrometer (Thermo Electron Corporation), positive atmospheric pressure chemical ionization (+APCI) and the following parameters: injection volume 1 µl, discharge current 5 µA, capillary voltage 8 V, capillary temperature 180 ºC, APCI vaporizer temperature 300 ºC, a delay time of 4 min between HPLC-MS separations, sheath gas flow and auxiliary gas flow at 40 and 5 l min^{-1}, respectively. The MS was coupled to a Surveyor HPLC system (Thermo Electron Corporation) equipped with a photodiode array (PDA) detector. Compounds were eluted using a gradient of 95 % (v/v) and 100 % (v/v) methanol (HPLC Grade, Merck) at a flow rate of 1 ml min^{-1}. Data analysis was achieved using the Xcalibur 2.0, LXQ 1.0 software.

3.3.4. Purification of diene.

The antifungal diene was isolated from the crude extract by flash chromatography using a method described by Prusky *et al*. (1982), with some modifications. The silica gel 60 H (55 µm, Merck) was poured into a 25 ml column (Omnifit) to a depth of 5 cm, packed and pre-equilibrated with 50 ml of 98 % (v/v) hexane (Merck). Five millilitres of the crude extract was added into the column and allowed to settle. Considerable inactive material was eluted with 100 ml of 99.5 % (v/v) dichloromethane (Merck), while the majority of the active compound was eluted with 50 % (v/v) ethyl acetate (Merck) in dichloromethane (Merck)

and dried in a Rotary evaporator (Roteva, EQUITRON) at 40 °C under vacuum. The active compound was collected by adding 5 ml of 100 % (v/v) ethanol (Merck) (Prusky *et al*., 1982).

3.3.5. Bioassay for antifungal activity.

The bioassay for antifungal activity was performed using a method described by Prusky *et al*. (1982), with some modifications. A spore suspension of *C. gloeosporioides* (1 x 10^7 spores per ml^{-1}) was used for all experiments. The isolate was sub-cultured on MEA (50 g l^{-1}, Biolab) and PDA (39 g l^{-1}, Biolab) at 25 °C and its pathogenicity was tested by inoculating avocado fruits once every 3 months. Purified diene compounds (from 2 d unharvested flesh), crude extracts (from 2 d unharvested flesh) or methyl linoleate [(*Z*,*Z*)-9,12-octadecadieneoic acid] (Merck) were dissolved in 100 % (v/v) ethanol (Merck). Extracts from unharvested flesh at 2 d were employed due to high antifungal diene levels. Methyl linoleate was tested for antifungal activity due to its structural similarity with the diene compound. Both purified diene compounds and crude extracts were concentrated to 2 ml by evaporating the ethanol, and the following range of dilutions (3 replicates) was prepared as follows: 1/20, 1/40, 1/60, 1/80, and 1/100 for methyl linoleate, the purified diene and crude extract.

Diluted samples as well as control samples (lacking the pure diene compounds, crude extracts or methyl linoleate) were spotted onto 25 mm diameter filters (0.65 μm pore size, Millipore) that were placed in the wells of glass depression slides. After removal of the ethanol by drying, 40 μl of the *C. gloeosporioides* spore suspension in the assay solution

[(0.05 % (v/v) Tween-80 (Sigma-Aldrich) and 0.0015 % (v/v) dimethyl sulfoxide (Merck) in 5 % ethanol] were placed on the disk and the slides were incubated in a moist chamber at 25 °C and observed after 16 h. The activity of each compound was determined by measuring the percent of spore germination and germ tube elongation (considered inhibited when length was less than twice the length of the spore) (Prusky *et al*., 1982).

3.3.6. Statistical analysis.

Results of diene (Appendix 13) and triene (Appendix 14) studies, as well as bioassay experiments (Appendix 15) were expressed as mean ± standard error of mean (SEM) and analyzed by One-Way Analysis of Variance (ANOVA) followed by Tukey`s Honestly-Significant-Difference Test as post test using SYSTAT 12 software. *p* values less than 0.05 were considered as significantly different.

3.4. Observation of infection process of *C. gloeosporioides* on avocado fruits by Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM).

Experimental design of this study is shown in Figure 3.4.

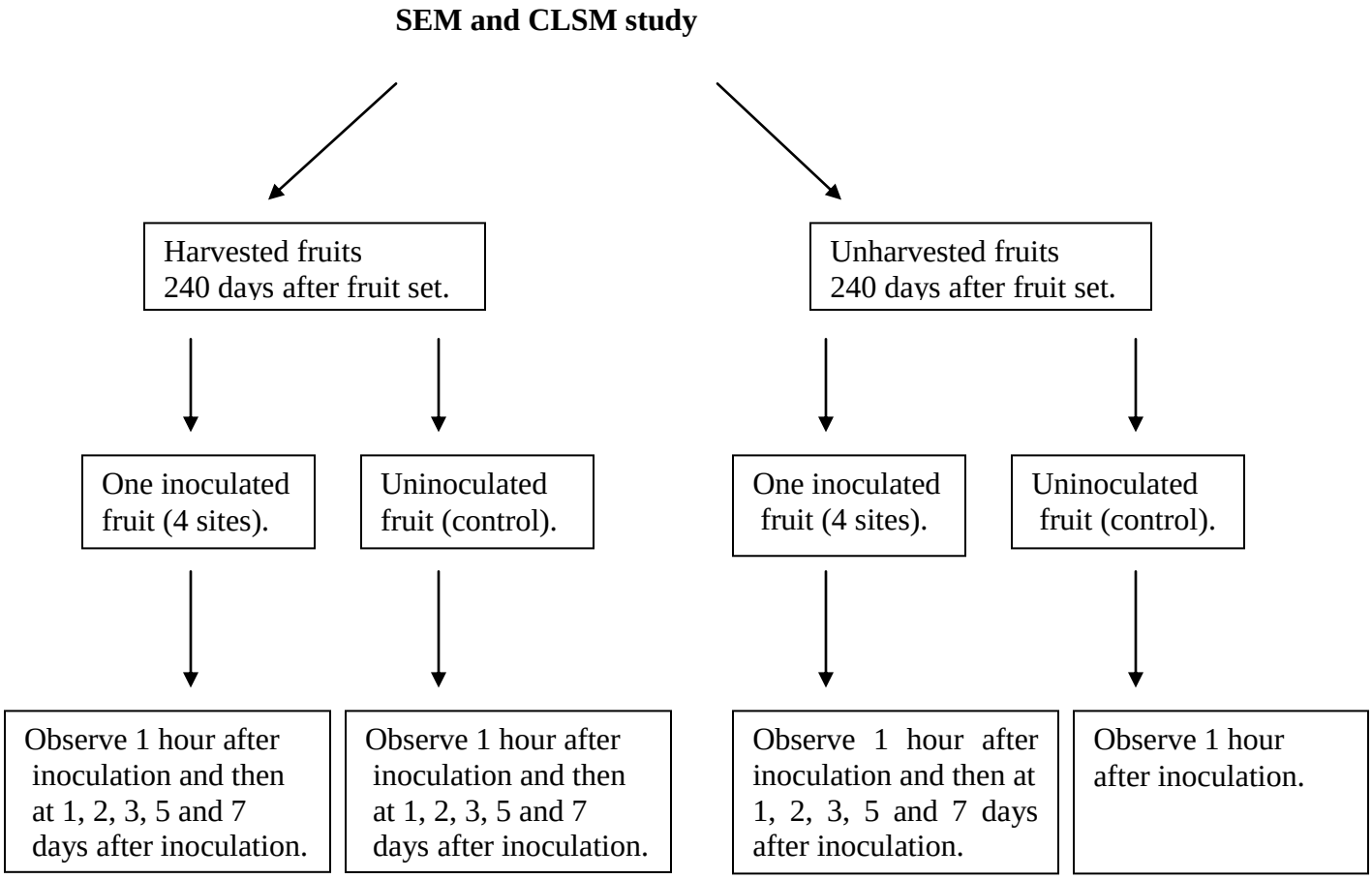


Figure 3.4. Experimental design of infection process in fruit infected with *Colletotrichum gloeosporioides*.

3.4.1. Inoculation of avocado fruits with *Colletotrichum gloeosporioides* to study the infection process.

Each harvested fruit was wounded on 4 sites using a sterile needle (2 mm length and 1 mm thick) and inoculated with 100 µl of *C. gloeosporioides* spore suspension (1 x 10^7 spores per ml^{-1}). One uninoculated fruit was used as a control for both harvested and unharvested experiments. Harvested fruits were placed inside an aluminium tray, covered with aluminium foil and incubated at 25 °C. Humidity was maintained by placing a petridish filled with sterile deionised water at the centre of each tray. The control fruit was placed in a separate tray to prevent the spread of infection. Symptom development was monitored daily for up to 7 d after inoculation.

In contrast to this, the unharvested fruit was wounded and inoculated with the *C. gloeosporioides* spore suspension while on the tree. Both the inoculated and uninoculated fruits were covered with plastic bags and symptom development was monitored daily for up to 7 d after inoculation. The experimental design of infection process in fruit infected with *C. gloeosporioides* employing SEM and CLSM is shown in Figure 3.4.

3.4.2. Scanning Electron Microscopy (SEM) of harvested and unharvested avocado fruits.

SEM was performed using a method described by Palhano *et al*. (2004), with some modifications. Pieces of fruit peel were excised using a sterile scalpel and fixed with 2 % (v/v) glutaraldehyde (Merck) in 0.1 M potassium phosphate buffer (pH 7.0) for 3 h. After fixing, tissues were briefly rinsed in 0.1 M potassium phosphate buffer (pH 7.0) and

sequentially dehydrated in a graded ethanol series (10, 30, 50, 70 and 90 % for 15 min with a double change) and in 100 % (v/v) ethanol (Merck) for 1 h. The ethanol was removed from the tissues by critical point drying (HITACHI, HCP-2 Critical point Dryer). Tissues were mounted on the stubs using graphite and allowed to air-dry, covered with carbon and coated with gold, and observations were made with a JEOL, JSM-840 instrument operated at 15 kV (Palhano *et al*., 2004).

3.4.3. Confocal Laser Scanning Microscopy (CLSM) of harvested avocado fruits.

CLSM was performed using a method described by Brundrett *et al*. (1994), with some modifications. Pieces of fruit peel were excised using a sterile scalpel and cleared in 2.5 % (v/v) potassium hydroxide (Merck) for 10 min at 90 °C in a water bath (Merck). Tissues were rinsed three times in tap water and acidified by soaking them in 20-50 volume of 1 % (v/v) hydrochloric acid (Merck) for 24 h (Brundrett *et al*., 1994).

Acidified tissues were stained with 0.05 % (v/v) Trypan blue in acidic glycerol for 15 min at 90 °C. Staining quality was substantially improved by destaining tissues in 50 % glycerol (v/v) (Merck) and 1 % (v/v) hydrochloric acid for 4 d prior to observation. Tissues were mounted onto glass slides (76 X 26 mm, B&C, Germany) and covered with cover slips (22 X 50 mm, Deckglaser), and observations were made with a Zeiss LSM-410 instrument (Brundrett *et al*., 1994).

3.5. Molecular identification of fungal pathogens.

3.5.1. DNA extraction.

Genomic DNA was extracted from fungal mycelia of the CgP complex, *Pseudocercospora* sp., *Colletotrichum* sp. and *Phomopsis* sp. using a method described by Raeder and Broda (1985), with some modifications. Fungal mycelia were harvested by carefully scrapping off the MEA plate using a sterile scalpel and ground using a sterile pestle and mortar. The ground material (300-600 mg) was transferred into a sterile 2 ml graduated microcentrifuge tube (Whitehead Scientific), kept in a freezer (Glacier, ultra low temperature freezer) at -70 °C overnight and freeze-dried (lyophilized) for 4 h. For lyophilization, holes were drilled into the lid of the tube to enhance evaporation. The lyophilized material was ground to fine powder using a sterile pestle and mortar, and 30-60 mg was transferred into a sterile tube (Raeder and Broda, 1985).

Five hundred microlitres of the extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) was added to the tube, resuspended by briefly vortexing and mixed using a pipette tip. Three hundred and fifty microlitres of saturated phenol (v/v) (Molecular Grade, Sigma-Aldrich) was added and mixed by inverting the tube. One hundred and fifty microlitres of 99.8 % chloroform (v/v) (HPLC Grade, Sigma-Aldrich) was added, mixed and centrifuged at 13 000 rpm (Spectrafuge 24D, Labnet) for 1 h. The upper aqueous phase was transferred into a microcentrifuge tube with 25 µl of RNAse A solution (Fermentas Life Sciences, South Africa) and incubated in a water bath (Merck) at 37 °C for 10 min. One hundred microlitres of chloroform was added, briefly mixed and

centrifuged at 13 000 rpm for 10 min. The upper aqueous phase was immediately transferred into another microcentrifuge tube (Raeder and Broda, 1985).

DNA was precipitated by adding 250 µl of 99.5 % (v/v) isopropanol (Molecular Grade, Fluka) and quickly spun for 10 s. The supernatant was removed using a pipette and quickly spun for another 10 s. The pellet was rinsed with 70 % (v/v) ethanol (Molecular Grade, Sigma-Aldrich), and the alcohol removed using a pipette. The tube was inverted on a paper towel (Kimwipes, Kimberly-Clark) for 30 min to evaporate excess ethanol. The pellet was resuspended in 100 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and kept at -20 °C until use (Raeder and Broda, 1985).

3.5.2. Polymerase chain reaction (PCR).

PCR was performed in 4 separate PCR tubes (Biorad) in a final volume of 25 µl. The first tube was composed of: 12.5 µl of 2 X PyroStart Fast PCR Master Mix (Hot start *Taq* DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs), 0.5 µl (10 µM) of each primer ITS5 (forward, 5`-GGAAGTAAAAGTCGTAACAAGG-3`) and ITS4 (reverse, 5`-TCCTCCGC TTATTGATATGC-3`), 1.25 µl of CgP complex genomic DNA and 10.25 µl of nuclease free water.

The second tube was composed of: 12.5 µl of 2 X PyroStart Fast PCR Master Mix (Hot start *Taq* DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs), 0.5 µl (10 µM) of each primer ITS5 (forward, 5`-GGAAGTAAAAGTCGTAACAAGG-3`) and ITS4 (reverse, 5`-TCCTCCGC TTATTGATATGC-3`), 1.25 µl of *Pseudocercospora* sp. genomic DNA and

10.25 µl of nuclease free water. The ITS5 and ITS4 primers described by Siboe *et al*. (2000) were used to target and amplify the ITS region of both the CgP complex and *Pseudocercospora* sp.

The third tube was composed of: 12.5 µl of 2 X PyroStart Fast PCR Master Mix (Hot start *Taq* DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs), 0.5 µl (10 µM) of each primer ITS1 (forward, 5`-TCCGTAGGTGAACCTGCGG-3`) and ITS4 (reverse, 5`–TCCTCCGC TTATTGATATGC-3`), 1.25 µl of *Colletotrichum* sp. genomic DNA and 10.25 µl of nuclease free water. The ITS1 and ITS4 primers described by Lubbe *et al*. (2004) and, Kumar and Shukla (2005) were used to target and amplify the ITS region of *C. gloeosporioides* and other *Colletotrichum* species.

The fourth tube was composed of: 3.2 µl of Dream *Taq* polymerase (5 units μl^{-1}), 3.2 µl of 10 X Dream *Taq* buffer, 3.2 µl of $MgCl_2$ (25mM), 3.2 µl dNTP mix (10mM each), 0.5 µl (10 µM) of each primer ITS5 (forward, 5`-GGAAGTAAAAGTCGTAACAAGG-3`) and ITS4 (reverse, 5`-TCCTCCGC TTATTGATATGC-3`), 1.25 µl of *Phomopsis* sp. genomic DNA and 9.95 µl of nuclease free water. The ITS5 and ITS4 primers described by Uddin *et al*. (1997) were used to target and amplify the ITS region of *Phomopsis* sp.

All the PCR reagents employed were from Fermentas (Fermentas Life Sciences, South Africa), apart from the primers and dNTP mix which were synthesized by Inqaba (Inqaba Biotech Industries, South Africa) and Biorad (Biorad Laboratories, South Africa), respectively.

For all four PCR reactions, a negative control sample containing all reaction components except genomic DNA was included to test that no self amplification or DNA contamination occurred. For control samples, nuclease free water was added in place of genomic DNA. All PCR tubes were briefly centrifuged at 13 000 rpm (Spectrafuge 24D, Labnet) for 10 s to settle all the PCR reaction components. All the PCR amplification reactions were performed using a PCR thermocycler (MyCycler, Biorad) and the following cycling conditions were utilized for all amplifications: One cycle of 94 °C for 3 min (initial denaturation), 35 cycles of 94 °C for 30 s (denaturation), 52.9 °C for 1 min (annealing) and 72 °C for 2 min (elongation). Final elongation was achieved at 72 °C for 10 min.

3.5.3. Agarose gel electrophoresis.

A 1 % (w/v) agarose gel was prepared by dissolving 0.5 g of Molecular Grade agarose (low EEO, Whitehead Scientific) into 50 ml of 1 X Tris-Borate EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). The solution was mixed by gently shaking in a 100 ml bottle, dissolved in a microwave oven for about 45 s and allowed to cool to about 45 °C. Two microlitres of ethidium bromide (10 mg ml^{-1}, Biorad) was added and mixed gently by shaking. The mixture was poured gently into a gel tray and allowed to solidify for about 20 min. A comb was inserted into the gel tray to form wells (lanes) into which the PCR products can be loaded. After solidifying, a comb was removed and the gel tray transferred into an electrophoretic tank (Biorad). A 1 X TBE buffer was added into the tank, such that it covered the gel. Two microlitres of 6 X (v/v) orange loading dye (Fermentas Life Sciences, South Africa) was placed on a sheet of parafilm and mixed thoroughly with 5 µl of 1 kb DNA ladder (0.1 µg $µl^{-1}$) (O`GeneRuler, Fermentas Life

Sciences, South Africa) and/or 5 µl of the PCR products. The molecular weight marker was loaded into the first lane and PCR products were loaded into the following lanes. The PCR products were electrophoresed by applying a constant voltage of 100 for about 1.5 h.

Amplified PCR products were viewed under UV transillumination using the Gel Doc System (BioDoc-it System, Biorad). The molecular weight marker was used to determine the size of the amplified PCR products. A PCR product size of ~550 base pairs was expected for the amplification of *Pseudocercospora* sp. (Siboe *et al*., 2000) and *Phomopsis* sp. (Uddin *et al*., 1997) genomic DNA using the ITS5 and ITS4 universal primers. Similarly, a PCR product size of ~550 base pairs was expected for the amplification of *Colletotrichum* sp. genomic DNA using the universal primers ITS1 and ITS4 (Lubbe *et al*., 2004; Kumar and Shukla, 2005).

3.5.4. DNA sequencing, subtyping and phylogenetic analyses.

Once the specificity of the amplifications were confirmed, PCR products were used as template DNA for sequencing and the products were sequenced using both the forward and reverse primers used in the PCR reaction. Subsequent PCR amplifications were performed so that three PCR products each for the CgP complex and *Phomopsis* sp, and one each for *Pseudocercospora* sp. and *Colletotrichum* sp. were sent for sequencing. Sequencing was performed by Stellenbosch University Sequencing Unit (South Africa) using an ABI3130xl Genetic analyzer. DNA sequences were edited using FinchTV 1.4.0 software and manual adjustments were made where necessary.

For subtyping, edited DNA sequences were compared with those already deposited in the GenBank (NCBI) using the Basic Local Alignment Search Tool (BLAST). DNA sequences and GenBank reference sequences were multiply aligned using the multiple sequence alignment tool (DNAMAN Version 4.0), and unrooted phylogenetic trees were constructed using the neighbour-joining method (DNAMAN Version 4.0) with a bootstrapping stringency of 1000, so as to reveal the phylogenetic relationship and degree of relatedness between DNA sequences in question and GenBank reference sequences (Sreenivasaprasad *et al*., 1996).

CHAPTER 4:

RESULTS: Accumulation patterns of diene and triene antifungal compounds in harvested and unharvested fruits in relation to infection cycle of *C. gloeosporioides*.

4.1. Macroscopic symptoms of inoculated fruit.

No symptoms were observed in any harvested inoculated fruit halves for up to 4 d (Figure 4.1 A). However, as from 5 d, minor anthracnose symptoms, in the form of black fruit rot, were observed in the flesh of inoculated halves, but absent in the fruit peel (Figure 4.1 B). Moreover, severe anthracnose symptoms characterized by advanced black fruit rot and greenish discolourations were observed in harvested inoculated fruit halves at 7 d (Figure 4.1 C). Consequently, these inoculated fruit halves became softer at 7 d relative to 5 d. However, anthracnose symptoms were not observed in harvested control fruit halves which were dipped in sterile deionised water.

Conversely, anthracnose symptoms were not observed in either unharvested inoculated fruits or in unharvested control fruits for up to 7 d.

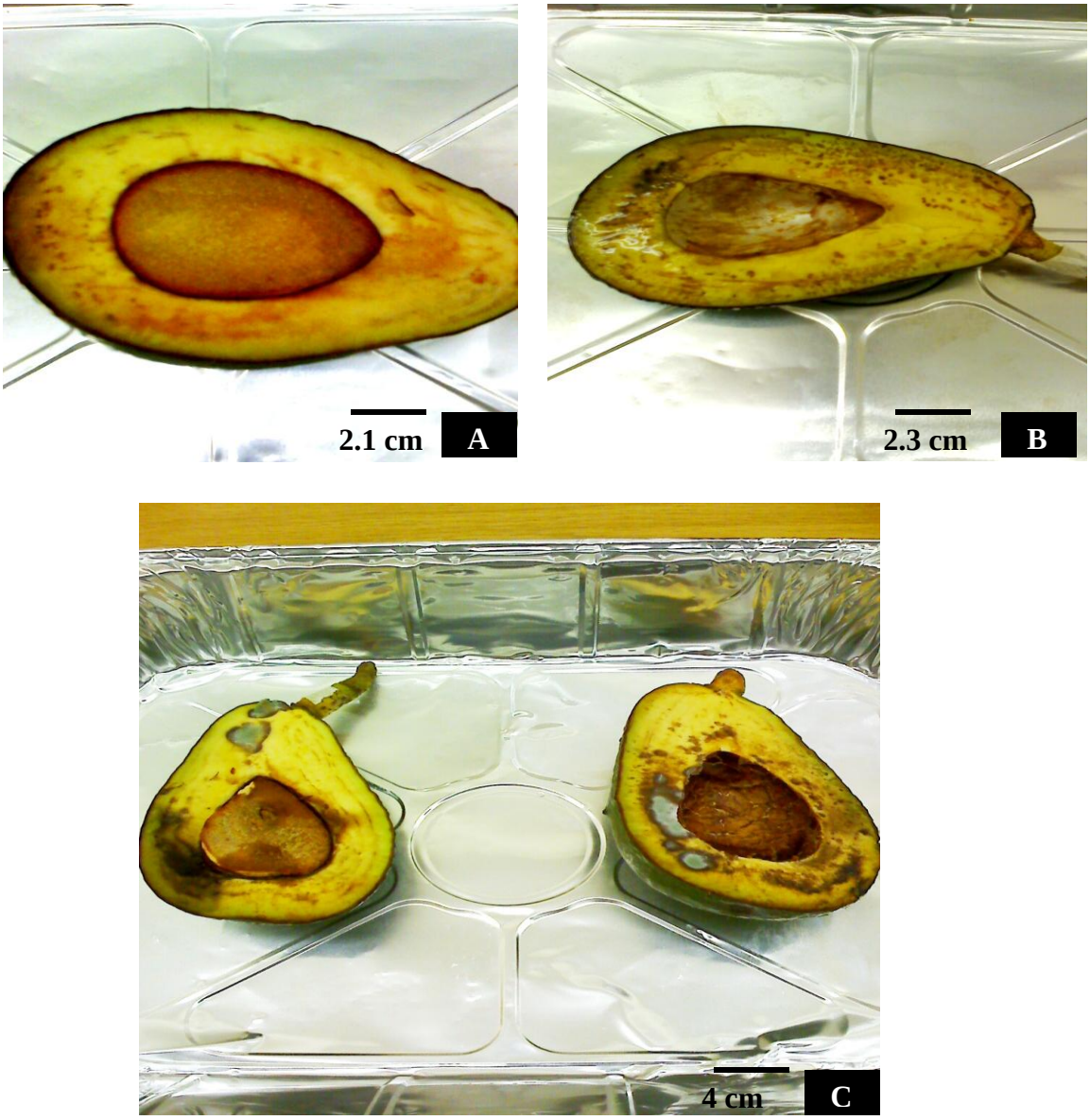


Figure 4.1. Anthracnose symptom development in harvested avocado fruit halves after inoculation with conidial spore suspension of *C. gloeosporioides*. A, 4 d after inoculation; B, 5 d after inoculation; C, 7 d after inoculation.

4.2. Antifungal diene and triene accumulation patterns in harvested and unharvested fruits.

Figure 4.2 shows the HPLC separation scan of the inoculated harvested flesh crude extract sample from 2 d and MS fragmentation scans which identified the triene and diene compounds. The average elution time of the antifungal triene and diene from the crude extracts (i.e. both the flesh and peel crude extracts) was at approximately 6.58 and 7.27 min, respectively, and remained relatively constant during subsequent HPLC-MS separations. Further HPLC-MS separations of harvested crude extracts are shown in Appendices 5, 6, 9 and 10.

In general, inoculation of freshly harvested fruits with the fungal pathogen *C. gloeosporioides* at approximately 240 d after fruit set caused an increase in the diene (Figure 4.3) and triene (Figure 4.4) levels of both the fruit peel and flesh from 1 to 2 d, followed by declines until 7 d. However, there were differences in the patterns of accumulation and decline between diene and triene compounds. Diene levels in inoculated and uninoculated control fruits were similar at 0 d, but levels in both the peel and flesh of inoculated fruits started to increase reaching a maximum at 2 d with the flesh producing higher levels than the peel. From 2 d, diene levels decreased to those of the control with the rate and level of decrease between 4 d and 7 d being similar in peel and flesh extracts (Figure 4.3). Diene levels in both peel and flesh of control fruits remained similar and relatively constant during the 7 d period.

In contrast, triene levels in inoculated and uninoculated control fruits, in both peel and flesh were similar between 0 d and 1 d, whereafter levels in inoculated fruits increased significantly, reaching a peak at 2 d with the levels in the peel being higher than those in the flesh, and control levels being similar to each other (Figure 4.4). From 2 d, levels declined with the rate and level of decline in peel and flesh extracts being similar between 4 d and 7 d (Figure 4.4). Triene levels in both peel and flesh of control fruits remained similar and relatively constant during the 7 d period, but significantly higher than inoculated fruits between 4 d and 7 d.

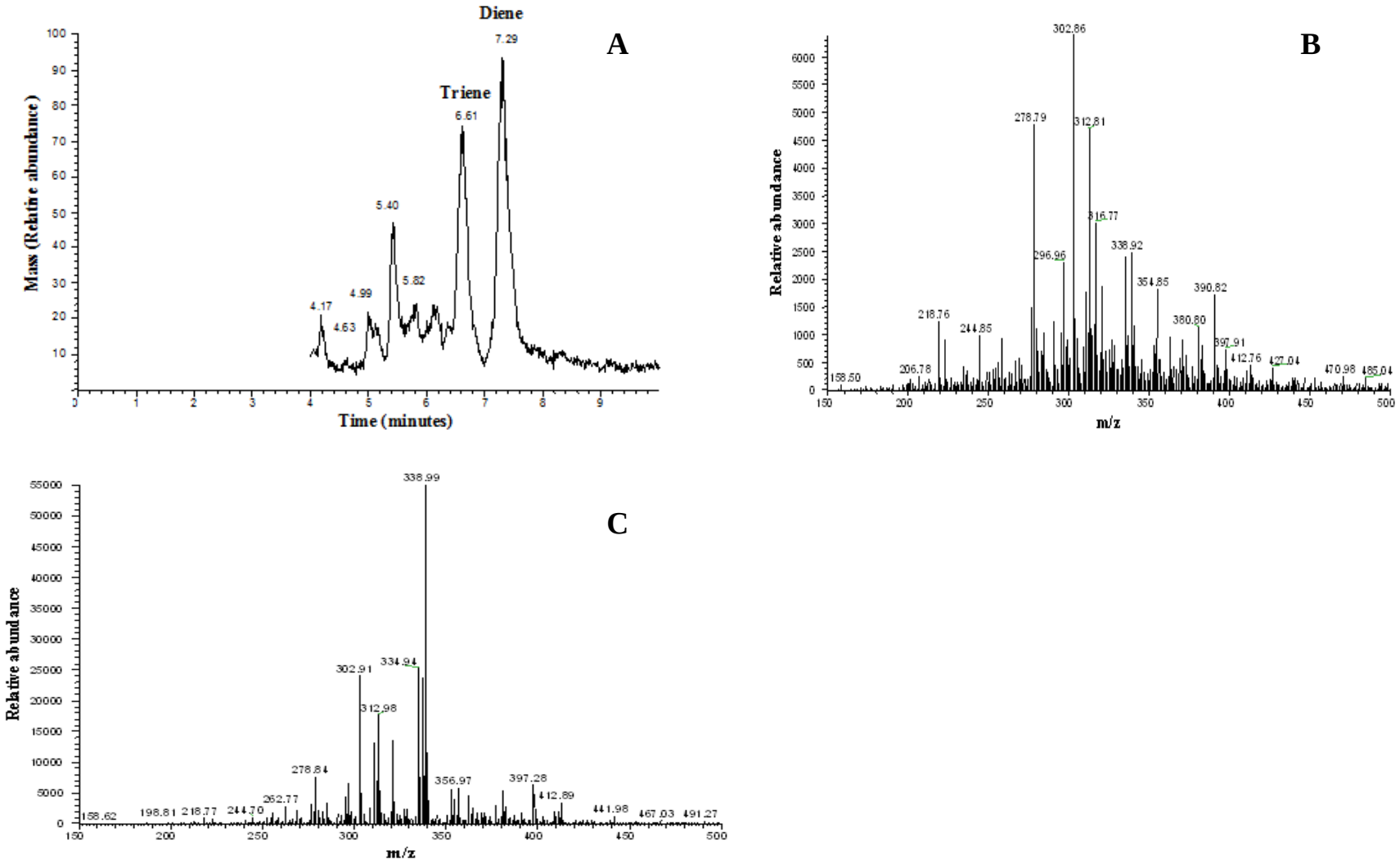


Figure 4.2. HPLC separation and fragmentation of harvested flesh crude extract sample from 2 d.

HPLC separation (**A**) and MS fragmentation of compounds identified as triene (**B**) and diene (**C**) based on analysis of Domergue *et al*. (2000). Harvested flesh crude extract was separated using a reversed-phase column (C18, 25 cm, Phenomenex) and eluted using a gradient of 95 % (v/v) and 100 % (v/v) methanol at a flow rate of 1 ml^{-1}min. The HPLC eluant was monitored with (+APCI) mass spectrometric detection.

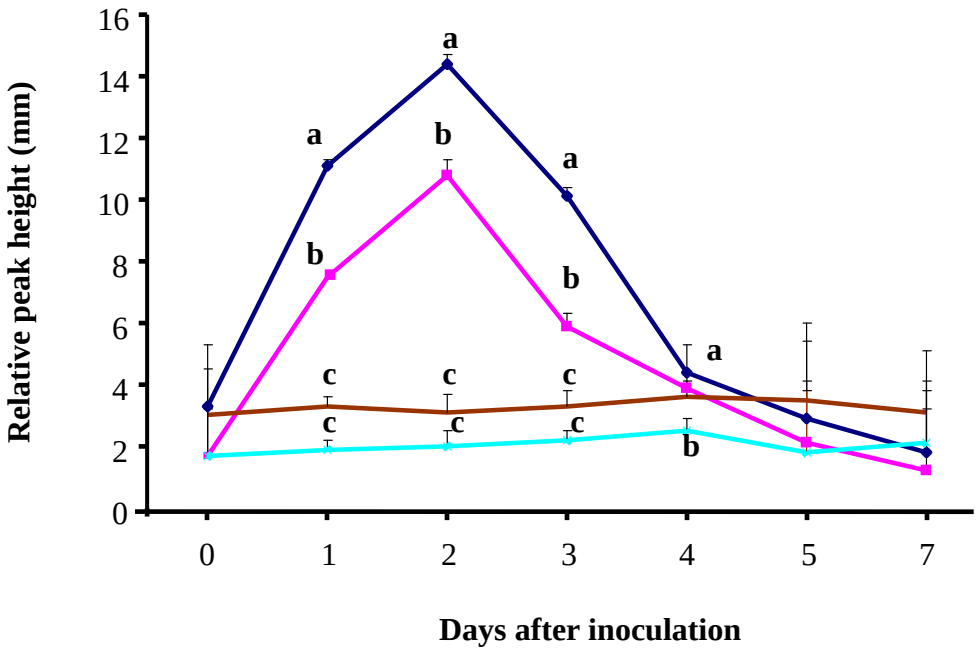


Figure 4.3. Diene levels of harvested avocado fruits.

Changes in diene levels in the peel and flesh of harvested 'Fuerte' avocado fruits inoculated with *C. gloeosporioides* at approximately 240 d after fruit set. Values represent the means ± SEM of 5 replicate extracts. ♦, flesh inoculated; ■, peel inoculated; —, flesh control; —, peel control. Different letters indicate significant differences between means ($p < 0.05$) at each day.

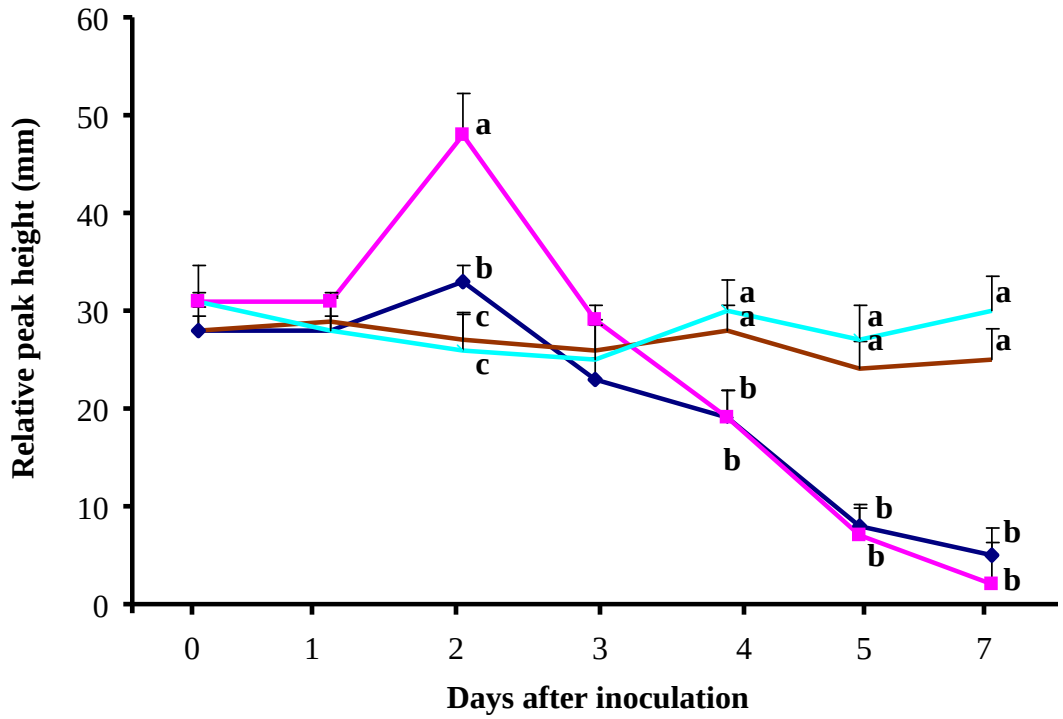


Figure 4.4. Triene levels of harvested avocado fruits.

Changes in triene levels in the peel and flesh of harvested 'Fuerte' avocado fruits inoculated with *C. gloeosporioides* at approximately 240 d after fruit set. Values represent the means ± SEM of 5 replicate extracts. ♦, flesh inoculated; ■ , peel inoculated; —, flesh control; —, peel control. Different letters indicate significant differences between means ($p < 0.05$) at each day.

Figure 4.5 shows the HPLC separation scan of the unharvested peel crude extract sample from 2 d and MS fragmentation scans which identified the triene and diene compounds. The average triene and diene elution time from crude extracts (i.e. both the flesh and peel crude extracts) was at approximately 6.57 and 7.27 min, respectively, and remained relatively constant during subsequent HPLC-MS separations. Further HPLC-MS separations of unharvested crude extracts are displayed in Appendices 7, 8, 11 and 12. The diene levels were relatively higher in unharvested than harvested fruits, with the triene levels being relatively similar in both harvested and unharvested fruits.

Similarly to harvested fruits, inoculation of unharvested fruits with *C. gloeosporioides* at approximately 240 d after fruit set resulted in an increase in the antifungal diene levels from 0 d until 2 d after inoculation (Figure 4.6) and triene levels from 1 d after inoculation until 2 d (Figure 4.7). Once again, patterns of accumulation and decline in levels were different between diene and triene compounds. Diene levels of peel and flesh extracts of inoculated and uninoculated control fruits were similar at 0 d, whereafter levels in inoculated fruits increased to reach a peak at 2 d, with levels in the flesh being higher than those in the peel (Figure 4.6). From 2 d, levels in both components declined becoming similar to each other and those of the controls by 5 d. Levels of peel and flesh controls were similar and relatively constant over the 7 d period (Figure 4.6).

Fluctuations in triene levels in unharvested fruits were less clear cut. Between 0 d and 1 d, inoculated peel and flesh extracts were similar to their respective controls (Figure 4.7). However, by 2 d the triene levels in inoculated peel and flesh extracts had peaked at the same

level and from 4 d to 7 d were higher in the peel than the flesh. By 4 d inoculated peel values had dropped to those of the flesh control. Inoculated peel and flesh values persisted in their decline so that by 7 d the peel control showed the highest value followed by the flesh control value, the inoculated flesh value, and then the inoculated peel with the lowest value (Figure 4.7). Throughout the 7 d period, peel control values were higher than flesh control values, significantly so between 2 d and 7 d.

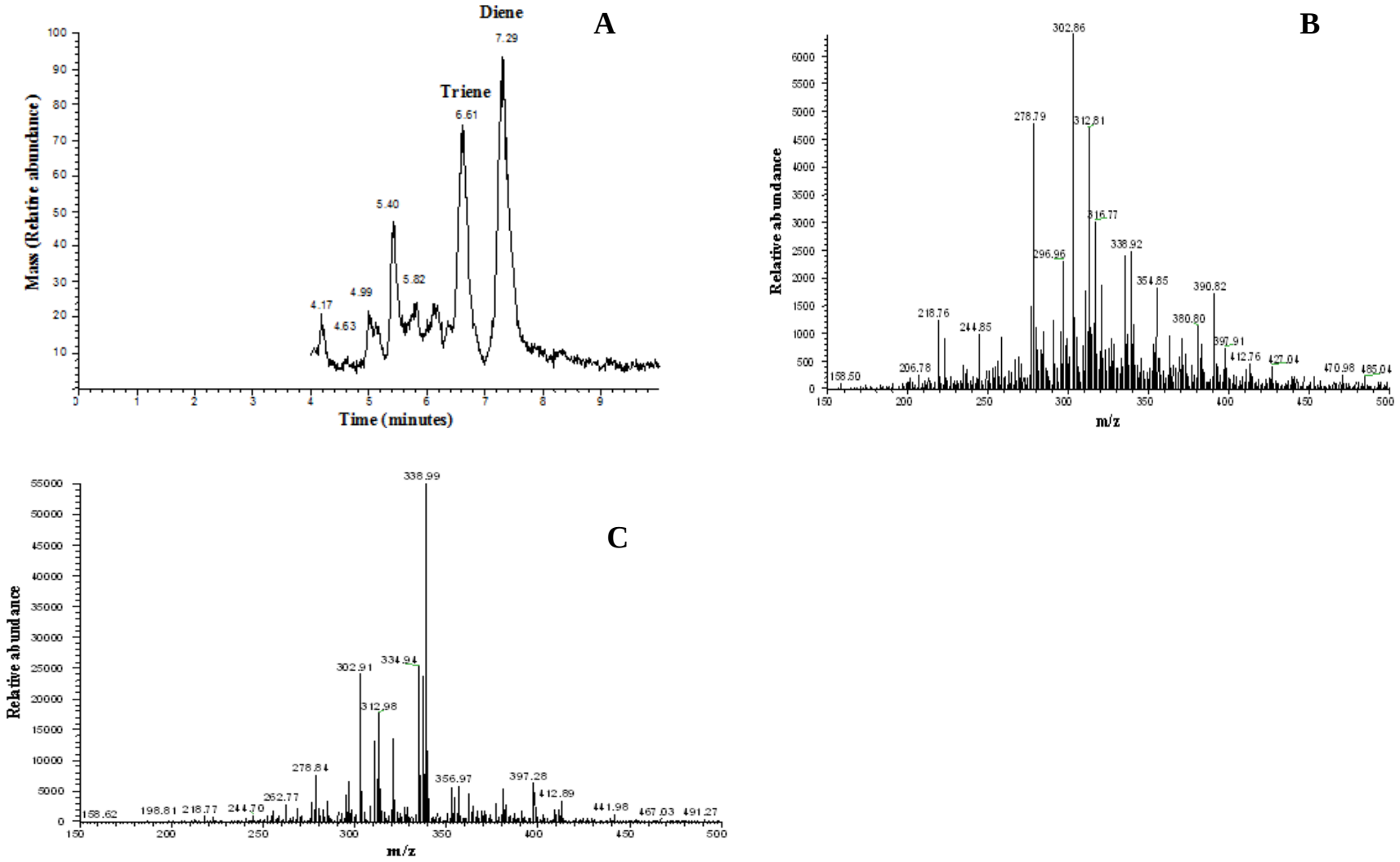


Figure 4.5. HPLC separation and fragmentation of unharvested peel crude extract sample from 2 d.

HPLC separation (**A**) and MS fragmentation of compounds identified as triene (**B**) and diene (**C**) based on analysis of Domergue *et al*. (2000). Unharvested peel crude extract was separated using a reversed-phase column (C18, 25 cm, Phenomenex) and eluted using a gradient of 95 % (v/v) and 100 % (v/v) methanol at a flow rate of 1 ml^{-1}min. The HPLC eluant was monitored with (+APCI) mass spectrometric detection.

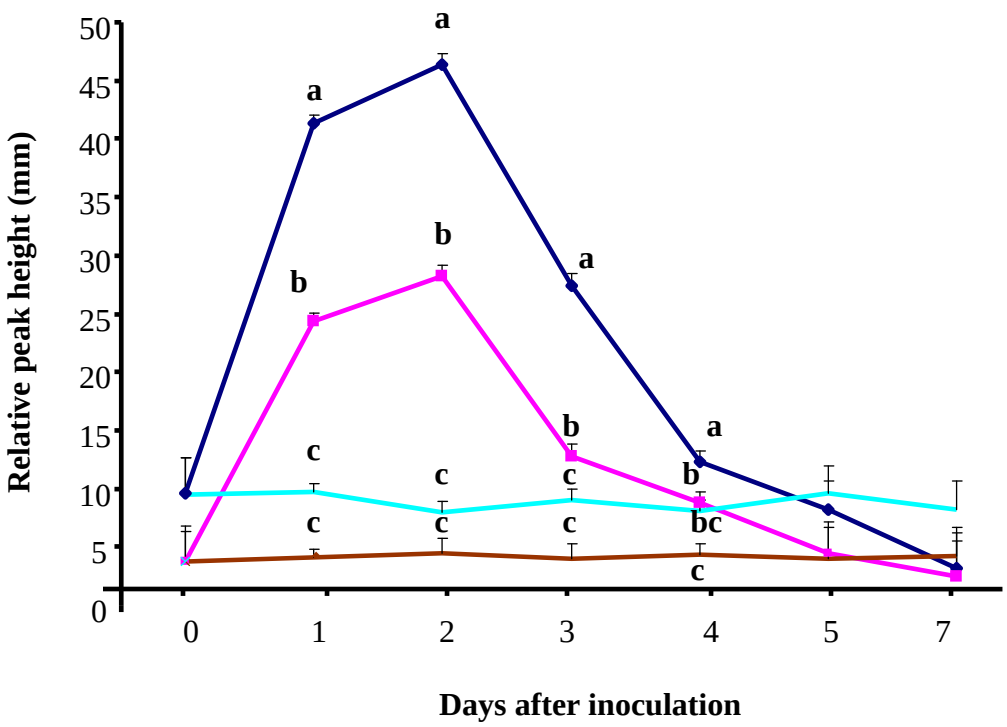


Figure 4.6. Diene levels of unharvested avocado fruits.

Changes in diene levels in the peel and flesh of unharvested 'Fuerte' avocado fruits inoculated with *C. gloeosporioides* at approximately 240 d after fruit set. Values represent the means ± SEM of 5 replicate extracts. ♦, flesh inoculated; ■ , peel inoculated; —, flesh control; —, peel control. Different letters indicate significant differences between means ($p < 0.05$) at each day.

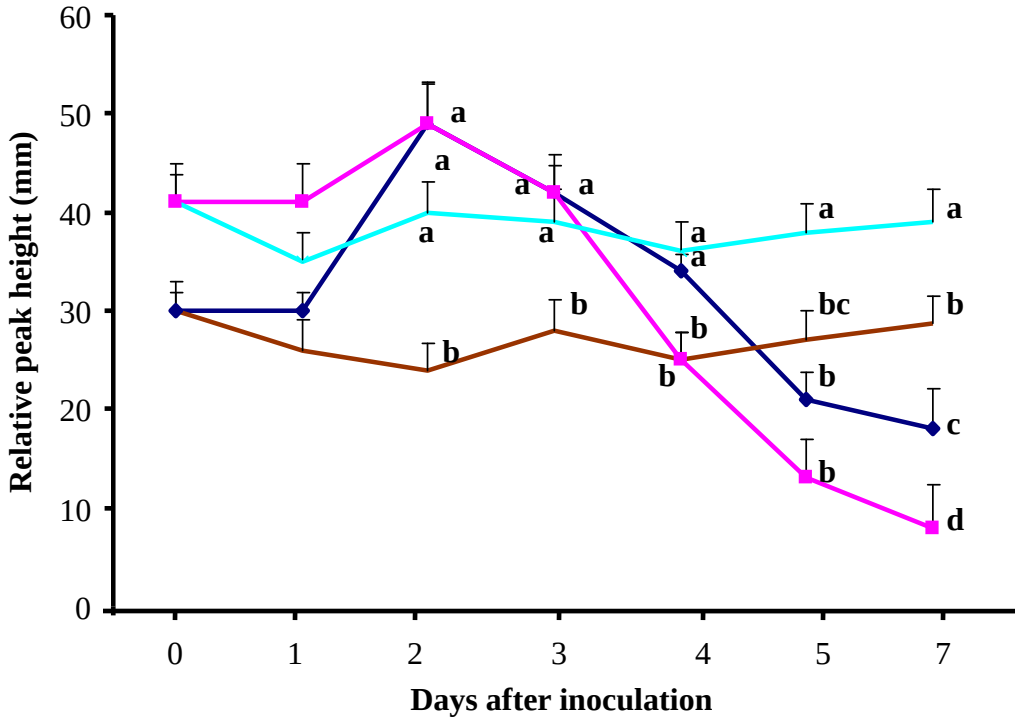


Figure 4.7. Triene levels of unharvested avocado fruits.

Changes in triene levels in the peel and flesh of unharvested 'Fuerte' avocado fruits inoculated with *C. gloeosporioides* at approximately 240 d after fruit set. Values represent the means ± SEM of 5 replicate extracts. ♦, flesh inoculated; ■, peel inoculated; —, flesh control; —, peel control. Different letters indicate significant differences between means ($p < 0.05$) at each day.

4.3. Bioassay for antifungal activity.

As methyl linoleate and the antifungal diene compound are very similar in structure, and the latter diene has been shown to inhibit spore germination and germ tube elongation of *C. gloeosporioides* (Prusky *et al*., 1991), both compounds were compared for antifungal activity together with the flesh crude extracts from 2 d. Structural characterization of methyl linoleate is composed of four conjugated diene hydroperoxides, 13-hydroperoxy-9-*cis*, 11-*trans*, 13-hydroperoxy-9-*trans*, 11-*trans*, 9-hydroperoxy-10-*trans*, 12-*cis*, 9-hydroperoxy-l0-*trans*, 12-*trans*-octadecadienoic acid methyl esters, while that of the diene compound is (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene.

Figure 4.8 indicates that all three compounds had inhibitory effects on fungal growth. All undiluted extracts showed similar strong antifungal activity causing an almost complete inhibition (~95 %) in spore germination and germ tube elongation. Thereafter, up to the 60 X dilution the purified diene and crude extract (which would have contained both the triene and diene compounds, as well as other possibly inhibitory compounds) showed similarly higher activity than the methyl lineolate, and at higher dilutions the crude extract had the highest inhibitory effect followed by the purified diene (Figure 4.8). It was also observed that the crude extract still displayed inhibitory activity at 500 X dilution whereas the other compounds were no longer effective at this concentration (data not shown). Between 5 x (data not shown) and 60 X dilutions (Figure 4.8), the purified diene and crude extract showed similarly higher antifungal activities than methyl linoleate, and at higher dilutions the crude extract displayed the highest inhibitory effect followed by the purified diene and methyl linoleate, respectively.

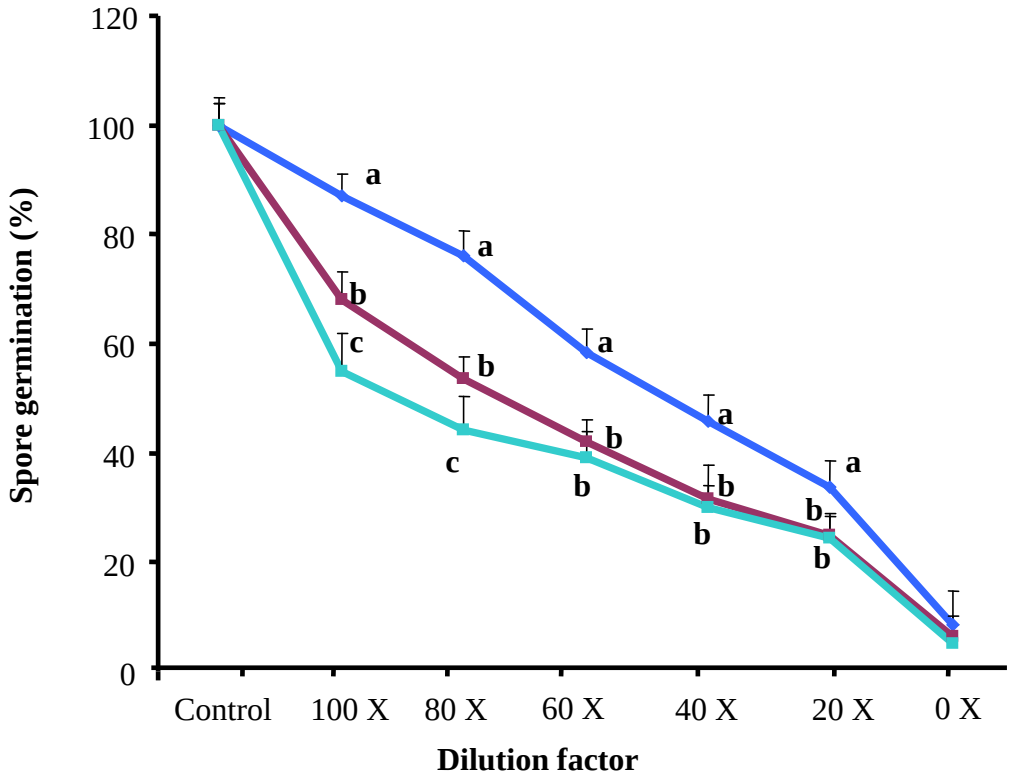


Figure 4.8. Antifungal activity of compounds towards *C. gloeosporioides*.

Biological activity of methyl linoleate, purified diene compound and crude extract towards *C. gloeosporioides*. The activity of each compound was determined by measuring the percent of spore germination and germ tube elongation (considered inhibited when length was less than twice the length of the spore). Values represent the means ± SEM of 3 replicate samples.♦, methyl linoleate;■, purified diene compound;■, crude extract. Different letters indicate significant differences between means ($p < 0.05$) at each dilution factor.

4.4. Observation by SEM of the infection process of *C. gloeosporioides* in harvested and unharvested fruits.

No fungal structures were observed in any of the four inoculated fruit sites (harvested and unharvested) from 1 h to 1 d (Figure 4.9 A). However, as from 2 d, a few dispersed ellipsoid spores characteristic of *C. gloeosporioides* were observed on the inoculated fruit surfaces of harvested fruits (Figure 4.9 C), but, at this stage no fungal hyphae were noted. No fungal presence was detected on control fruits at this time (Figure 4.9 B). Conversely, as from 3 d, fungal hyphae were observed on inoculated fruit surfaces of harvested fruits, some of which were attached to the fruit surface with or without spores, while some were interacting together to form a mycelium (Figure 4.9 D). In harvested fruit, severe tissue damage and destruction were observed at 5 d and 7 d with some hyphae protruding out of the lenticels, resulting in cell wall and cuticle destruction (Figure 4.10 A and 4.11 A). In most inoculated fruits, by 7 d hyphae had successfully colonized the fruit surface, resulting in an increased number of spores being observed on the fruit surfaces in both harvested and unharvested fruits (Figure 4.11 B and D). Fungal structures were not observed in any uninoculated fruits (controls) for up to 7 d, indicating that there was no fungal infection and/or contamination.

Contrary to harvested inoculated fruits, in which ellipsoid spores of *C. gloeosporioides* were observed on fruit surfaces at 2 d and hyphae at 3 d, respectively, spores and surface hyphae were observed simultaneously at 3 d in unharvested fruits. Although an increased number of spores were observed at 5 d, only a few hyphae were observed to have penetrated the fruit surface (Figure 4.10 B). As a consequence, hyphal interaction to form a

mycelium and extensive colonization was not observed. However, in some inoculated sites, cell wall and cuticle destruction occurred, thereby destructing the exocarp and exposing the mesocarp layer, but no fungal structures were observed at 5 d (Figure 4.10 C) and 7 d (Figure 4.11 C). An increase in the number of spores was also observed on the fruit surface at 5 d (Figure 4.10 D) relative to 3 d (data not shown). In some inoculated fruits, cell wall and cuticle destruction was increased, as more exocarp destruction occurred, thereby, increasing the exposure of the mesocarp layer and the number of spores on the fruit surface was increased (Figure 4.11 B and D). In general, only minor symptoms of infection were expressed in unharvested fruits relative to harvested fruits. However, fungal structures were not present in any uninoculated unharvested fruits (controls), indicating that there was no fungal infection and/or contamination.

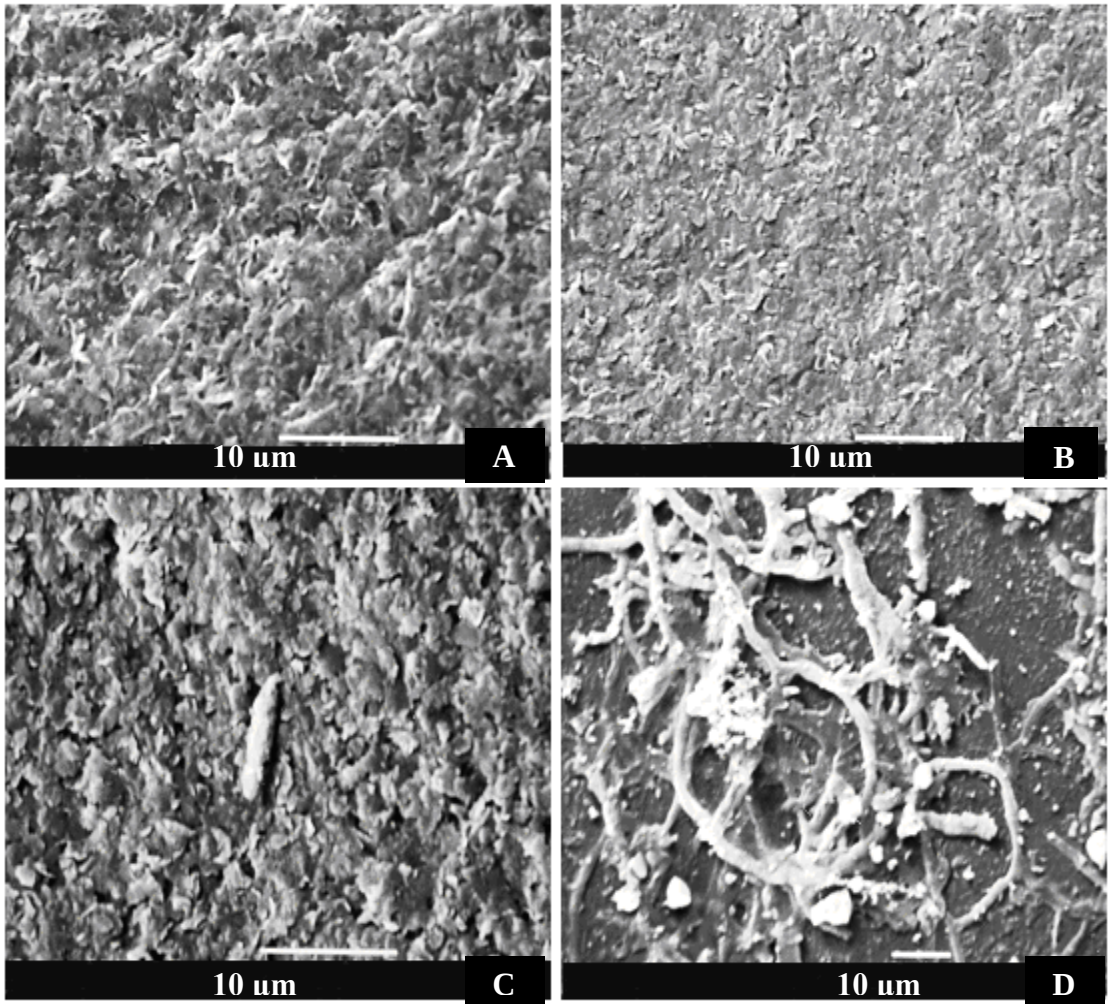


Figure 4.9. Scanning electron micrographs of avocado fruit from 1 h to 3 d after inoculation with *C. gloeosporioides*. Unharvested inoculated fruit site at 1 h (**A**). Uninoculated fruit (control) at 2 d (**B**). Ellipsoid spore of *C. gloeosporioides* on the inoculated fruit surface of harvested fruit at 2 d (**C**). Surface hyphae interacting with each other to form a mycelium at 3 d on harvested fruit (**D**).

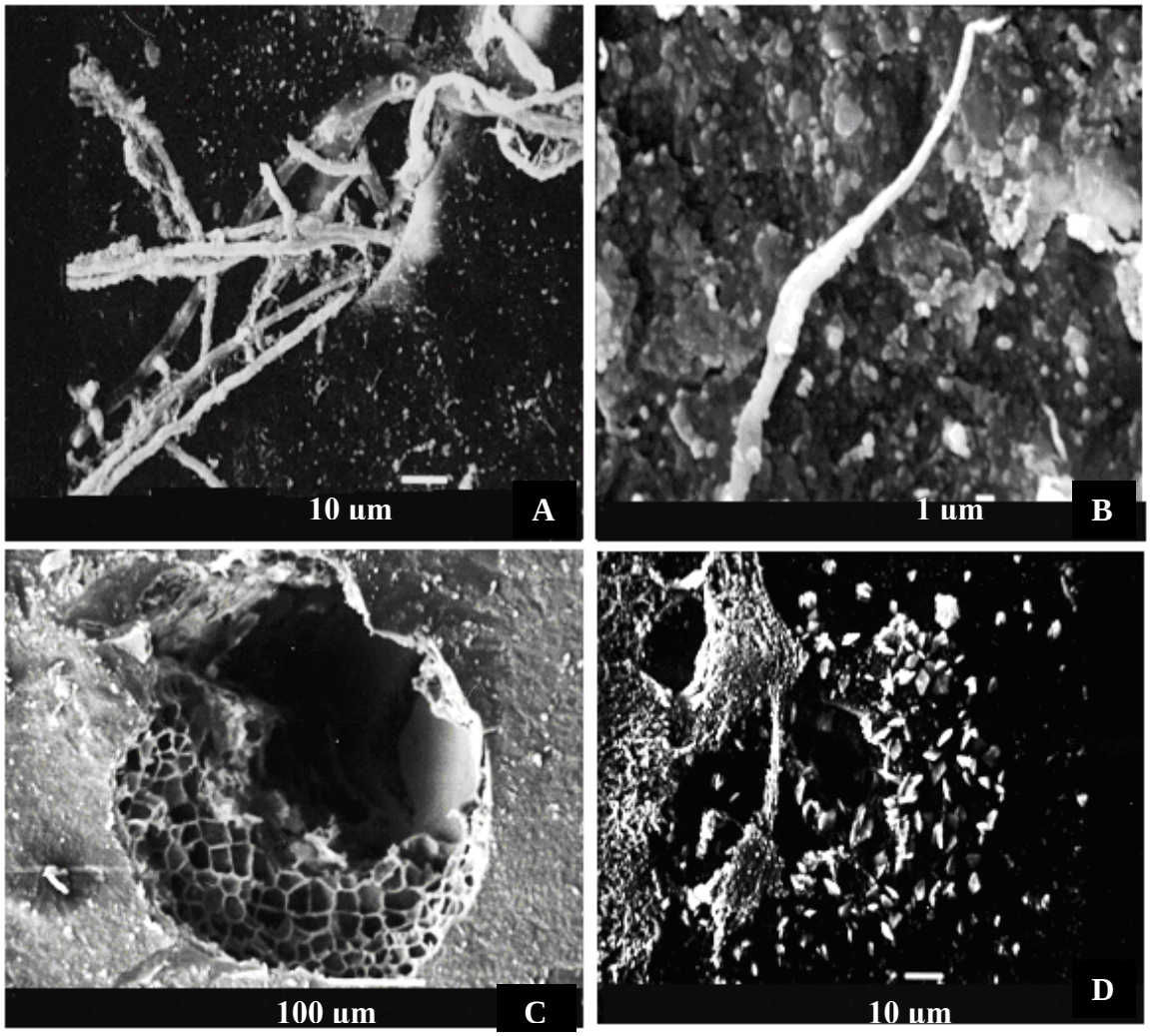


Figure 4.10. Scanning electron micrographs of avocado fruit at 5 d after inoculation with *C. gloeosporioides*. Hyphae protruding out of lenticel in harvested inoculated fruit (**A**). Surface hyphae protruding out of the unharvested inoculated fruit surface (**B**). Destruction of the exocarp, leading to subsequent exposure of the mesocarp layer in unharvested inoculated fruit (**C**). An increased number of *C. gloeosporioides* spores on the surface of unharvested inoculated fruit (**D**).

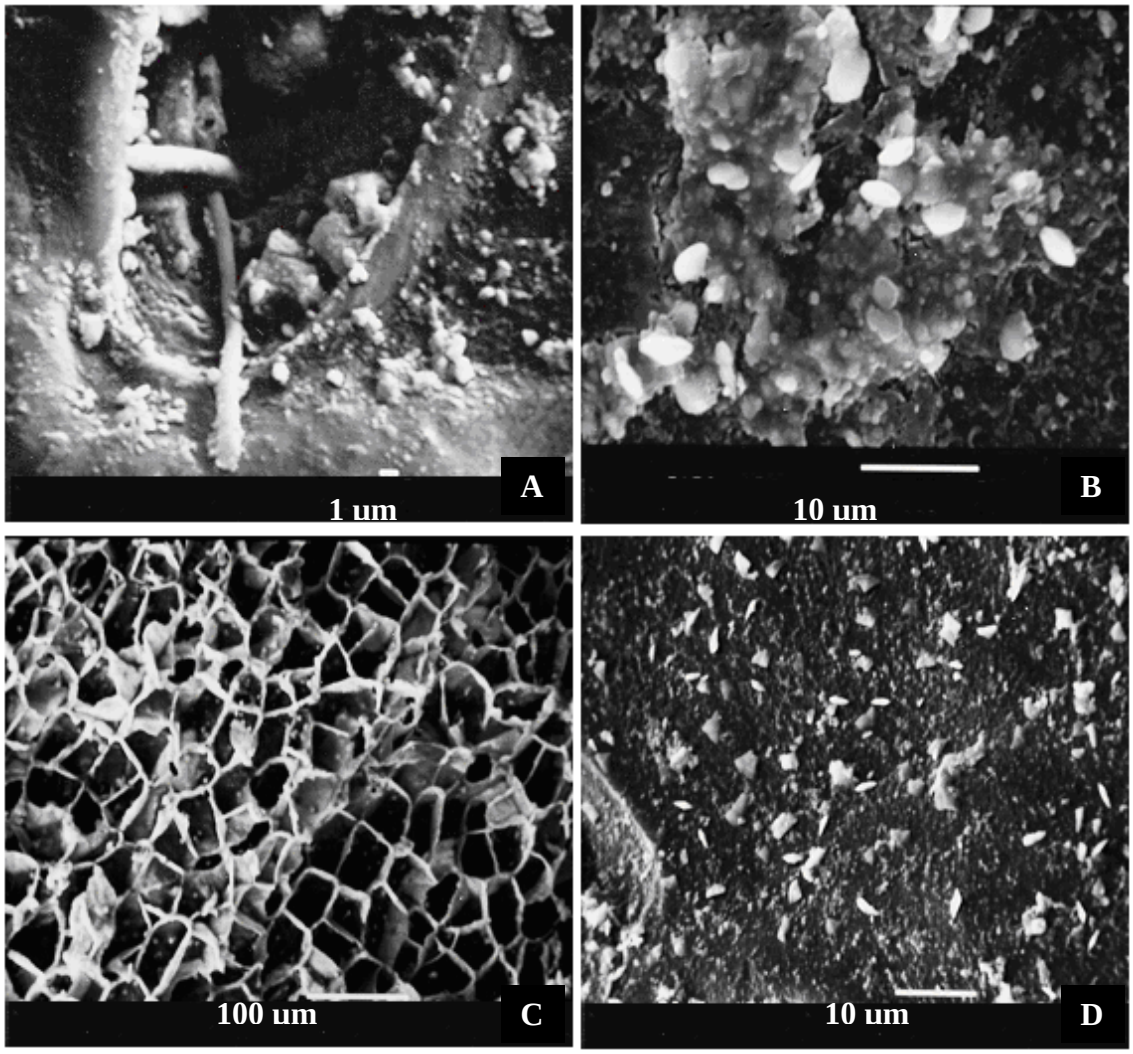


Figure 4.11. Scanning electron micrographs of avocado fruit at 7 d after inoculation with *C. gloeosporioides*. Hyphae protruding out of lenticel in harvested inoculated fruit (**A**). An increased number of ellipsoid spores of *C. gloeosporioides* on the harvested inoculated fruit surface (**B**). Increased exposure of the mesocarp layer, due to an increase in the destruction of the fruit exocarp layer in unharvested inoculated fruit (**C**). An increased number of ellipsoid *C. gloeosporioides* spores on the surface of unharvested inoculated fruit (**D**).

4.5. Observation by CLSM of the infection process of *C. gloeosporioides* in harvested fruits.

No fungal structures were observed in any of the four harvested inoculated fruit sites from 1 h to 6 d (Figure 4.12 A and B). However, at 7 d, ellipsoid spores characteristic of *C. gloeosporioides* were observed on the inoculated fruit surfaces (Figure 4.12 C and D), but, no fungal hyphae were noted.

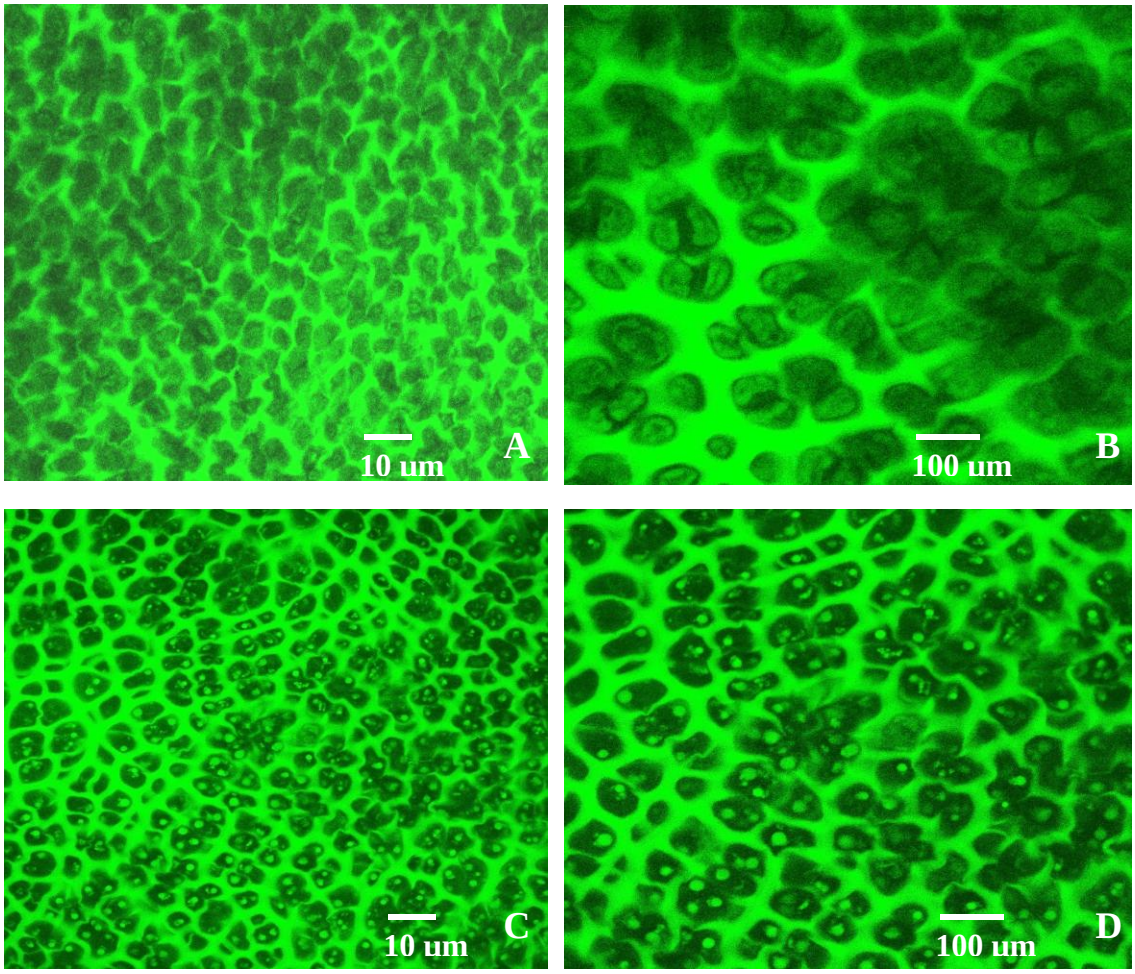


Figure 4.12. Confocal laser scanning micrographs of avocado fruit from 1 d to 7 d after inoculation with *C. gloeosporioides*. Uninoculated fruit (control) (**A**). Harvested inoculated fruit surface at 5 d (**B**). *C. gloeosporioides* spores on the surface of inoculated harvested fruit at 7 d (**C** and **D**).

4.6. Discussion and conclusion.

Although several cultivated fruit crops are infected by *Colletotrichum* species, the most significant economic losses manifest when fruits are infected after harvest (Freeman *et al*., 1998). The fungal pathogen *Colletotrichum gloeosporioides* has been associated with quiescent infections and post-harvest diseases on many fruits, and they include avocado, mango, papaya, guava, passion fruit, citrus, apple and grapes (Simmonds, 1965; Hartill, 1992; Alahakoon *et al*., 1994; Timmer *et al*., 1998).

This study was aimed at studying the accumulation patterns of the antifungal diene and triene compounds measured by HPLC and HPLC-MS and relate these to the infection process as observed by SEM and CLSM of *C. gloeosporioides* in both harvested and unharvested avocado fruits. HPLC-MS confirmed the presence of the triene and diene compounds, in which mass units of 302.91 (at about 300 *m/z*) and 397.28 (at about 400 *m/z*) were observed for the triene compound (Figure 4.2 and 4.5). Moreover, mass units of 302.86 (at about 300 *m/z*) and 397.91 (at about 400 *m/z*) were observed for the diene compound (Figure 4.2 and 4.5). These observations were in close agreement with those obtained by Domergue *et al*. (2000) in which mass units of 302 (at about 300 *m/z*) and 395 (at about 400 *m/z*), as well as 304 (at about 300 *m/z*) and 398 (at about 400 *m/z*) were observed for the triene and diene compounds, respectively.

Prior to inoculation of both harvested and unharvested fruits, the peel of uninoculated (control) fruits possessed similar diene levels to the flesh (Figure 4.3 and 4.6). Following inoculation, the diene levels were always higher in the flesh than peel of inoculated fruits,

and apparently higher in unharvested than harvested fruits based on comparative peak heights. Consequently, the inoculated flesh of unharvested fruits possessed the highest diene levels.

In inoculated harvested fruits, in both the flesh and peel, the following diene accumulation patterns were observed (Figure 4.3). There was a sharp increase in levels from 0 d to 1 d followed by a steeper increase to 2 d, and a subsequent decrease in levels from 3 d to 7 d. Diene levels of control fruits remained relatively constant during the 7 d period, with no significant differences. Prusky *et al*. (1990) observed similar patterns but with small variations in time scales. In their study, flesh diene levels increased dramatically from 0 d to peak at 3 d followed by a rapid decline to control levels at 7 d. They also observed an increase in flesh control levels at 5 d. In the peel, there was a dip in diene levels at 2 d, followed by a peak at 4 d and then a subsequent decline to 10 d. As in the flesh, control levels of the peel also showed an increase at 5 d. Prusky *et al*. (1990) also observed the flesh to have higher levels than the peel during the 'active' period of 0 d to 5 d. The diene accumulation patterns in inoculated unharvested fruits (Figure 4.6) were similar to those in harvested fruits (Figure 4.3). In contrast, in unharvested fruits Prusky *et al*. (1990) observed a sharp increase in levels in the flesh between 0 d and 1 d followed by a rapid decline reaching control levels at 4 d. Lower peel levels followed a similar, but more muted pattern with the controls remaining similarly low throughout the incubation period. Despite the differences in diene flux patterns between this study and that of Prusky *et al*. (1990), which might be due to differences in experimental conditions, the overall patterns are similar. Harvesting causes a fall in diene levels from ambient levels, in both the flesh and the peel

(possibly due to harvesting stress – Prusky *et al*. (1990) but this is followed by a spike following inoculation up to 2 d or 3 d. From then, levels start to decline to pre-existing levels which in the case of harvested fruits may not be sufficient to retard infection. High diene levels in unharvested relative to harvested fruits may be attributed to very high concentrations of pre-formed antimicrobial compounds present in unharvested fruits and this arsenal of constitutive resistance may accumulate in the immature pericarp at concentrations up to 1 mg g^{-1} fruit fresh weight (Prusky and Plumbley, 1992; Prusky, 1996).

Although the triene compound from avocado has been identified and characterized by HPLC-MS and its antifungal activity confirmed (Domergue *et al*., 2000) no monitoring of its accumulation pattern over time in response to inoculation of avocado fruit by *C. gloeosporioides* has been done, therefore, triene accumulation patterns were observed as follows. Triene levels in harvested inoculated and uninoculated control fruits, in both peel and flesh remained relatively constant between 0 d and 1 d, this may be due to a lack of symptom development in inoculated fruits as the fungal pathogen *C. gloeosporioides* has been reported as being capable of producing quiescent infections (Prusky, 1996). However, levels in inoculated fruits increased significantly, reaching a maximum at 2 d with the levels in the peel being higher than those in the flesh, and control levels being similar (Figure 4.4), and this may suggest that the triene compound is more expressed and/or synthesized in the peel relative to the flesh of inoculated fruits. Interestingly, from 4 d to 7 d in the case of harvested fruit and 5 d to 7 d in the case of unharvested fruit the triene levels of both the inoculated peel and flesh were lower than those of their respective

controls (Figure 4.4 and 4.7). This may suggest that the triene compound is more unstable relative to the diene compound with respect to its antifungal activity resulting in possible triene degradation, or the fungal pathogen *C. gloeosporioides* is capable of detoxifying the triene compound or there is a repression of the biosynthetic pathway leading to triene synthesis resulting in lower levels in inoculated fruits from 4 d or 5 d to 7 d. This may be due to the (*Z*,*Z*,*E*) configuration of the triene compound which may cause the chain to bend, thereby, restricting the conformational freedom of the fatty acid (Bernard *et al*., 2002). In general, triene accumulation patterns in inoculated unharvested fruits (Figure 4.7) were relatively similar to those in harvested fruits (Figure 4.4).

The following broad scenario might be suggested. Both dienes and trienes are active anti-fungal compounds in unharvested fruits but trienes act as a first line of defence with higher levels in the peel than the flesh. If fruits are infected on the tree there is an immediate stimulation of diene and triene production with levels returning to ambient levels after a few days, sufficiently high in the case of dienes to contain the pathogen and maintain it as a quiescent infection. In the case of trienes, after the initial stimulation, exposure to the pathogen may cause a decline in triene biosynthesis to levels no longer effective (although for how long would require further experiments). Upon harvesting, diene levels decline allowing any quiescent infections to develop but triene levels do not decline. If infection occurs after harvesting, there is a rapid stimulation of both diene and triene synthesis but levels decline after a few days, to non-effective concentrations in the case of dienes, and to degradation/complete inhibition of synthesis in the case of trienes.

Although the antifungal activity of diene (Prusky *et al*., 1982; Domergue *et al*., 2000) and triene (Domergue *et al*., 2000) against *C. gloeosporioides* has been tested before it was important to confirm that the extracts from this study showed similar inhibitory activity against the fungal isolate used. Since methyl linoleate has a similar chemical structure to these compounds it was included in the bioassay for comparative purposes (Bull and Carman, 1994). Methyl linoleate, diene and the crude extract all had inhibitory effects on the growth of the fungal pathogen *C. gloeosporioides* (Figure 4.8). The pure diene compound exhibited a higher antifungal activity relative to methyl linoleate, thereby inhibiting more spore germination and germ tube elongation. Interestingly, at most dilutions the inhibition patterns of both compounds were relatively similar which may be attributed to a high structural similarity between them. In contrast to these, the crude extract possessed a higher antifungal activity, thereby inhibiting more spore germination and germ tube elongation relative to the diene and methyl linoleate. This might have been an expected outcome since the crude extract would possess both the triene and diene antifungal compounds, and other possibly inhibitory compounds. In general, the inhibitory patterns of the purified diene compound were in close agreement with those observed by Domergue *et al*. (2000) (Figure 4.8) although in their study absolute concentrations of dienes and trienes were determined.

Macroscopic anthracnose symptoms were not observed in the peel of inoculated sites of either harvested or unharvested fruits employed for the SEM study for up to 7 d (Figure 4.9 A). This may have been due to the ability of the fungal pathogen *Colletotrichum gloeosporioides* to produce quiescent infections and post-harvest diseases on many fruits,

including avocado (Simmonds, 1965; Hartill, 1992; Alahakoon *et al*., 1994; Timmer *et al*., 1998). No fungal structures were observed in inoculated fruits from 1 h to 1 d (supposedly due to high diene and triene contents during that period), but ellipsoid spores of *C. gloeosporioides* were observed on the fruit surface from 2 d (Figure 4.9 C). This was in agreement with the description of Akinwunmi and Latunde-Dada (2001), that after inoculation, the ungerminated aseptate conidium of *Colletotrichum* attaches to the cuticle and germinates, with or without septation, to form a germ-tube that differentiates into a melanized appressorium. Moreover, conidia of quiescent *Colletotrichum* species need to be in contact with a hard surface in order to recognize the host signals that trigger spore germination (Liu and Kolattukudy, 1998). By 3 d fungal hyphae emerged, most of which successfully invaded and colonized the fruit surface by interacting with each other to form a mycelium (Figure 4.9 D). During this period of fungal penetration and invasion, an increased number of spores were observed on the fruit surfaces (data not shown). Previous studies by O`Connel *et al*. (1985), Latunde-Dada *et al*. (1996), as well as Wharton and Julian (1996) have indicated that many *Colletotrichum* species initially establish infection by using a brief biotrophic phase, associated with large intracellular primary hyphae.

Extensive fruit damage and destruction were observed at 5 d (Figure 4.10 A-D) and 7 d (Figure 4.11 A-D), as an increased number of hyphae invaded the fruit surface. Consequently, some hyphae caused fruit damage by protruding out of the lenticels (Figure 4.10 A), resulting in cell wall and cuticle destruction, as well as exocarp destruction leading to mesocarp exposure. Previous studies have shown that after *Colletotrichum* species have established infection by using a brief biotrophic phase (associated with large intracellular

primary hyphae), they later resort to a destructive, necrotrophic phase (associated with narrower secondary hyphae) which ramify throughout the host tissue (O`Connel *et al*., 1985; Latunde-Dada *et al*., 1996; Wharton and Julian, 1996). Furthermore, in most inoculated fruits, an increased number of surface hyphae successfully colonized, penetrated and invaded the fruit surface by interacting with each other to form a mycelium (data not shown).

In contrast to harvested inoculated fruits, in which ellipsoid spores characteristic of *C. gloeosporioides* were observed on inoculated fruit surfaces as from 2 d and hyphae at 3 d, respectively, fungal hyphae and spores were observed simultaneously at 5 d in unharvested fruits (Figure 4.10 B and D). A delay in fungal hyphae and spore emergence in inoculated unharvested relative to inoculated harvested fruits may be attributed to very high concentrations of pre-formed antimicrobial compounds found in unharvested fruits (Prusky and Plumbley, 1992; Prusky, 1996). Although an increased number of spores were observed at this stage (Figure 4.10 D), only a few hyphae penetrated the fruit surface (Figure 4.10 B). As a consequence, hyphal interaction to form a mycelium and extensive colonization were absent. Moreover, previous studies postulated that inhibition of fungi during quiescent infections of *C. gloeosporioides* on unripe avocado fruits was proposed to manifest because of the presence of the preformed antifungal compound (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene (Prusky *et al*., 1982).

Unlike ripening fruits, unripe fruits contain very high concentrations of pre-formed antimicrobial compounds. This constitutive arsenal of resistance may accumulate in the

immature pericarp at concentrations up to 1 mg g^{-1} fruit fresh weight, and include 5-substituted resorcinols such as 5-(12-cisheptadecenyl)-resorcinol in mango, dienes such as (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene in avocado, and saponins like a-tomatine in tomato (Prusky and Plumbley, 1992; Prusky, 1996). However, in some inoculated fruits, cell wall and cuticle destruction occurred, thereby exposing the mesocarp (Figure 4.10 C), but, at this stage fungal structures were not observed. In general, only minor symptoms of infection were expressed in unharvested fruits relative to harvested fruits, supposedly due to extremely high concentrations of antifungal compounds (triene and diene) present in unharvested fruits. However, no fungal structures were observed in any uninoculated fruits (controls) for up to 7 d, suggesting that there was no fungal infection and/or contamination.

In contrast to the SEM study in which macroscopic anthracnose symptoms were not observed in any of the fruits (both harvested and unharvested) for up to 7 d, anthracnose symptoms were observed at 6 d in all harvested inoculated fruits used for the CLSM study. No fungal structures were observed in inoculated fruits from 1 h to 6 d (Figure 4.12 A and B). However, ellipsoid spores of *C. gloeosporioides* were observed on the fruit surfaces at 7 d (Figure 4.12 C and D). Once again, this may have resulted due to the ability of the fungal pathogen *C. gloeosporioides* to produce quiescent infections and post-harvest diseases on many fruits, including avocado (Simmonds, 1965; Hartill, 1992; Alahakoon *et al*., 1994; Timmer *et al*., 1998). In contrast to the SEM in which fungal hyphae were observed at 3 d, no fungal hyphae were observed on inoculated fruit surfaces of avocados used for the CLSM study. This may have resulted from the fact that the SEM technique is

more sensitive and possesses higher magnifications relative to the CLSM technique. This is because SEM images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern (Suzuki, 2002), while CLSM images the sample surface by scanning it with a laser beam (Prasad *et al*., 2007), thus, allowing SEM to be more sensitive relative to CLSM.

Ultimately, the infection process of *C. gloeosporioides* was observed to be more rapid, efficient, successful and prominent in harvested than unharvested fruits. For this reason, the correlation between the antifungal diene levels (using HPLC and HPLC-MS) to the infection process of *C. gloeosporioides* (using SEM and CLSM) in avocado fruits is that: during the period of high diene (i.e. 1-2 d) and triene (i.e. 2 d) levels, no macroscopic anthracnose symptoms were expressed and no fungal infection was observed in both harvested and unharvested fruits. However, during the period of subsequent diene (i.e. 3-7 d) and triene (i.e. 3-7 d) decline, anthracnose infection was subsequently expressed, particularly in harvested fruits with low concentrations of antimicrobial compounds relative to unharvested fruits (Prusky and Plumbley, 1992; Prusky, 1996).

Although the presence of the fungus could be observed under SEM, no anthracnose symptoms were noted for up to 7 d in unharvested fruits. During this period the diene and triene levels were higher than those in harvested fruits, particularly at 2 d, thereby, probably inhibiting pathogen growth in unripe fruits (Verhoeff, 1974; Swinburne, 1983). This may have led the fungal pathogen *C. gloeosporioides* to produce quiescent infections in unharvested fruits (Alahakoon *et al*., 1994). Additionally, this observation was in

agreement with previous studies which postulated that fungal inhibition during quiescent infections of *C. gloeosporioides* on unripe avocado fruits was proposed to manifest because of the presence of the preformed antifungal compounds (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene (Prusky *et al*., 1982) and (*Z,Z,E*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene (Prusky and Keen, 1995; Domergue *et al*., 2000).

In harvested fruits, macroscopic symptoms of anthracnose were not observed for up to 4 d (Figure 4.1 A) and during this period, the diene and triene levels were higher in inoculated fruit halves than controls, particularly at 2 d. Symptoms were observed in inoculated fruit halves as from 5 d (Figure 4.1 B), when minor anthracnose symptoms in the form of black fruit rot were observed in the flesh of inoculated halves, but absent in the fruit peel. Moreover, severe anthracnose symptoms characterized by advanced black fruit rot and greenish discolourations were observed in the flesh of inoculated fruit halves, but absent in the peel at 7 d (Figure 4.1 C). The absence of anthracnose symptoms in the fruit peel may have resulted due to the ability of the fungal pathogen *C. gloeosporioides* to form quiescent infections and post-harvest diseases on several fruits, including avocado (Simmonds, 1965; Hartill, 1992; Alahakoon *et al*., 1994; Timmer *et al*., 1998). During this period of symptom expression (i.e. 5-7 d), there was in general, a significant decrease in antifungal diene and triene levels. Karni *et al*. (1989) reported a significant decrease in the diene concentration in freshly harvested fruits, and postulated that it is possible that this manifests as a result of harvesting stress. In the latter case, although the diene decreased to subfungitoxic levels, the period of low concentration is short and fungal growth does not manifest until fruit ripening 7-10 d later. It appears that fungal quiescence or fruit resistance depends on the

duration of, or modulation of the period of decline in diene concentration (Prusky *et al*., 1991).

The biosynthesis of antifungal compounds in avocados may result from a general activation of fatty acid biosynthetic pathways that initiate from acetate and/or from intermediate precursor molecules, e.g. oleic acid or linoleic acid (Fehling and Mukherjee, 1991; Leikin-Frenkel and Prusky, 1998; Madi *et al*., 2003). In a previous study, Madi *et al*. (2003) proposed that the improved accumulation of oleic acid in the course of Δ^9 desaturation was an important step in antifungal diene biosynthesis. Similarly, Wang *et al*. (2004) showed that activation of Δ^{12} fatty acid desaturase may also control the accumulation of the antifungal diene. Once the putative precursor, linoleic acid, is produced, the elongation step may become the rate limiting step in the biosynthesis of the diene compound beyond the threshold level that confers resistance. Very-long-chain fatty acids (VLCFA) are synthesized in a microsomal fatty acid elongation system by respective additions of C2 moieties from malonyl-CoA to pre-existing C18 fatty acids produced from the *de novo* fatty acid biosynthesis pathway in the plastid (Fehling *et al*., 1992; Millar and Kunst, 1997).

In avocado infected with *C. gloeosporioides*, reactive oxygen species (ROS) are formed by the pathogen during the biotrophic stage (Legendre *et al*., 1993; de Jong *et al*., 2002; Wang *et al*., 2004). Reactive oxygen species are chemically-reactive molecules that contain oxygen (e.g. oxygen ions and peroxides). In summary, the following model can be

suggested: the quiescence of *C. gloeosporioides* in unripe avocado is triggered by the formation of ROS by the pathogen and the host (Beno-Moualem and Prusky, 2000; Wang *et al*., 2004). During this process ROS would directly trigger fatty acid precursors of antifungal diene, higher diene levels and the inhibition of fungal growth. This model is supported by the fact that ethylene, cold stress and fungal inoculation, which trigger diene production in avocado fruits (Prusky and Keen, 1993; Rivero *et al*., 2002; Madi *et al*., 2003; Wang *et al*., 2004), were demonstrated to induce production of hydrogen peroxide (H_2O_2) in other systems (de Jong *et al*., 2002; Rivero *et al*., 2002). As such, it can be proposed that H_2O_2 behaves as a signal molecule with a central role in the stimulation of resistance in avocado fruits. At the last stage of fruit ripening, genes responsible for diene synthesis are shut down and diene is degraded slowly (Prusky *et al*., 1983; Madi *et al*., 2003; Wang *et al*., 2004). Once below the inhibitory threshold of the diene, the quiescent fungus is released from the quiescent biotrophic phase (Prusky, 1996) to become an active necrotroph displaying the typical disease symptoms (Wang *et al*., 2004).

In conclusion, inoculation of harvested and unharvested 'Fuerte' avocado fruits with *C. gloeosporioides* at approximately 240 d after fruit set causes an increase in the antifungal diene and triene levels, particularly at 2 d. Unharvested fruits have higher diene contents relative to harvested fruits, thus, enabling them to be more resistant to infection by *C. gloeosporioides* ultimately leading to no anthracnose or low anthracnose symptom expression. Consequently, the infection process of *C. gloeosporioides* was more efficient and prominent in harvested fruits with low antifungal diene contents, than in unharvested fruits with higher diene contents. The crude extract possessing both the triene and diene

antifungal compounds, and other possibly inhibitory compounds has the highest inhibitory effect towards *C. gloeosporioides*, thus, inhibiting more spore germination and germ tube elongation, followed by the purified diene and methyl linoleate, respectively.

CHAPTER 5:

RESULTS: Characterization and identification of fungal pathogens.

5.1. Morphology of fungal pathogens.

Spores from a sporulating culture of *C. gloeosporioides* were ellipsoid in morphology with an average size of 11.9 X 4.7 µm (*n*=60) (Figure 5.1). The *C. gloeosporioides* spore size range was observed to be from 6–23 X 4–6 µm. The fungus possessed simple conidiophores that were elongate, while conidia were hyaline, unicellular, ovoid or oblong. The sporulating culture of the *Colletotrichum gloeosporioides-Pseudocercospora* (CgP) complex employed by Giovanelli (2008) produced two morphologically distinct types of spores (Figure 5.2): filiform and ellipsoid spores. Filiform spores had an average size of 23 X 1.5 µm (*n*=60), while ellipsoid spores had an average size of 7 X 2.5 µm (*n*=60). The size range of CgP filiform spores was observed to be from 2-4.5 X 17-90 µm, while that of ellipsoid spores was observed to be from 5-22 X 3-5 µm.

The *Pseudocercospora* sp. isolated failed to sporulate and was, thus, only identified using molecular techniques. However, spores from a sporulating culture of *Pseudocercospora* sp. employed by Giovanelli (2008) were filiform in morphology with an average size of 20 X 3.5 µm (*n*=60) (Figure 5.3). The spore size range of the *Pseudocercospora* sp. was observed to be from 2-4.9 X 19-99 µm. The fungus possessed conidia that were hyaline or dark, filiform and multicellular. Similarly to the CgP complex, *Phomopsis perseae* (PPRI 6006) produced two morphologically distinct types of spores (Figure 5.4): alpha conidia

that were colourless, fusiform, non-septate and with an average size of 7.5 X 2.5 µm (*n*=60), as well as beta conidia that were filiform, curved or bent, hyaline, unicellular and with an average size of 13.2 X 1.3 µm (*n*=60). The size range of *Phomopsis perseae* alpha conidia was observed to be from 1-3 X 11-45 µm, while that of beta conidia was observed to be from 2-4 X 15-70 µm.

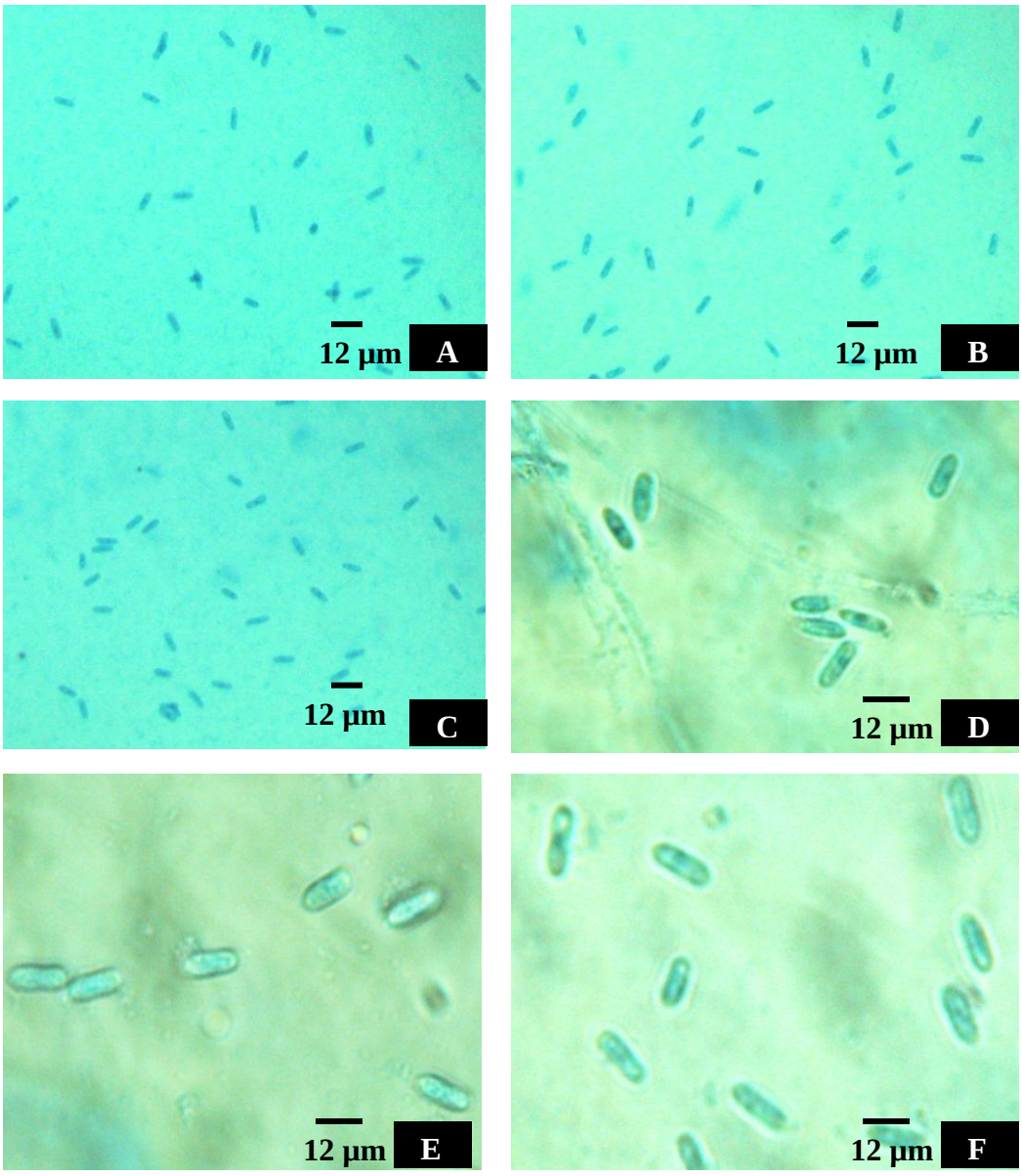


Figure 5.1. Morphological identification of *Colletotrichum gloeosporioides* from ellipsoid spores examined under the light microscope using low power (A-C) and high power (D-F).

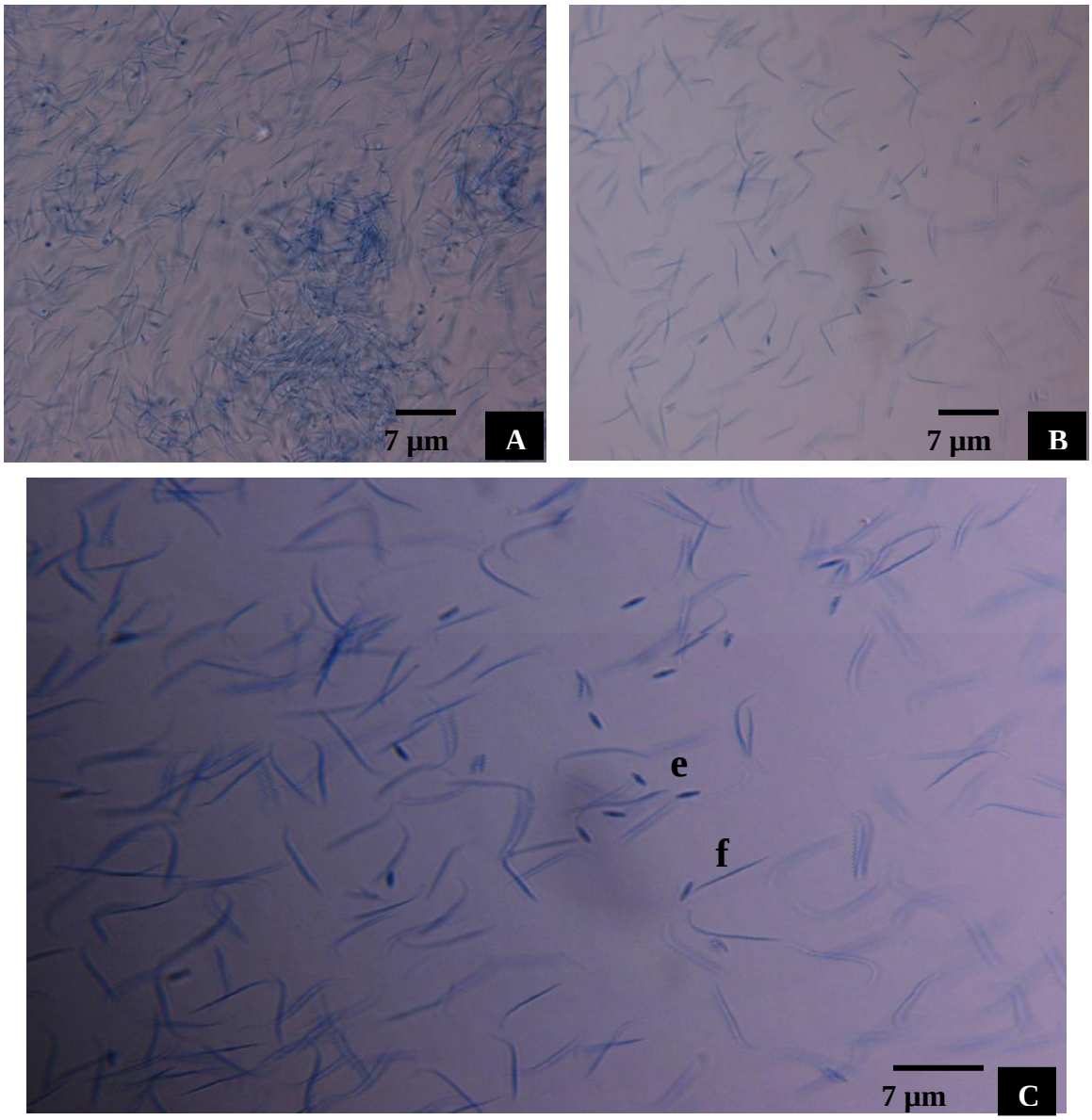


Figure 5.2. Morphological identification of the *Colletotrichum gloeosporioides-Pseudocercospora* complex (CgP) complex from spores examined under the light microscope using low power (A and B) and high power (C). e = ellipsoid spores; f = filiform spores.

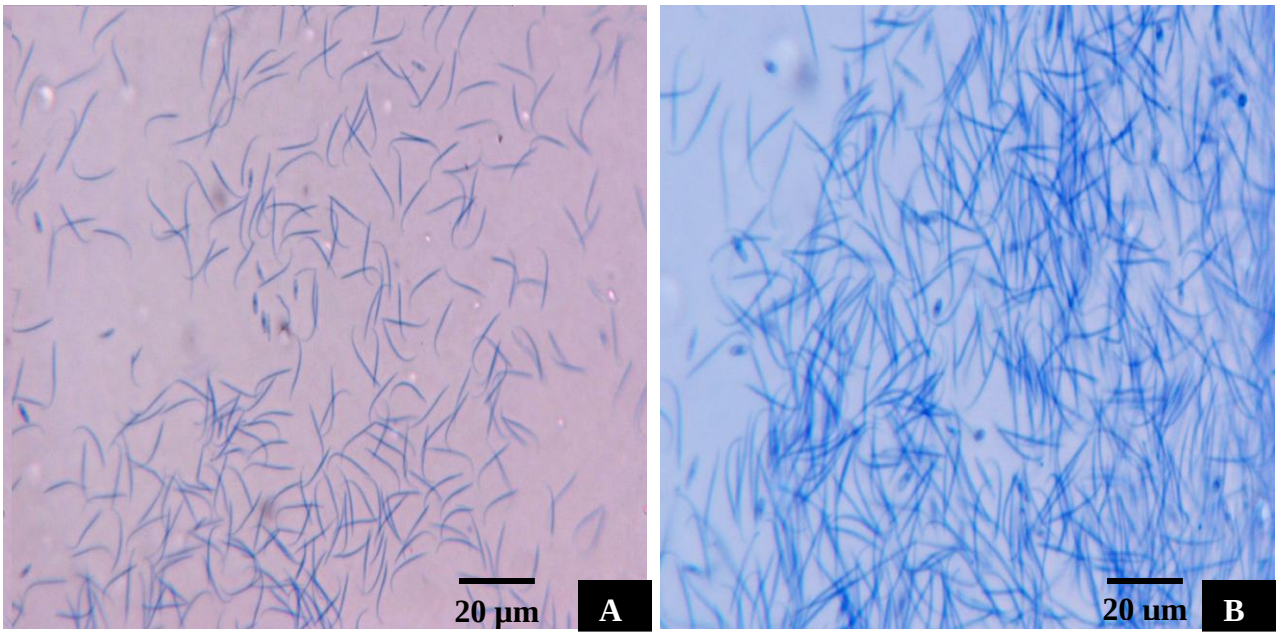


Figure 5.3. Morphological identification of *Pseudocercospora* sp. from filiform spores examined under the light microscope using low power (A) and high power (B).

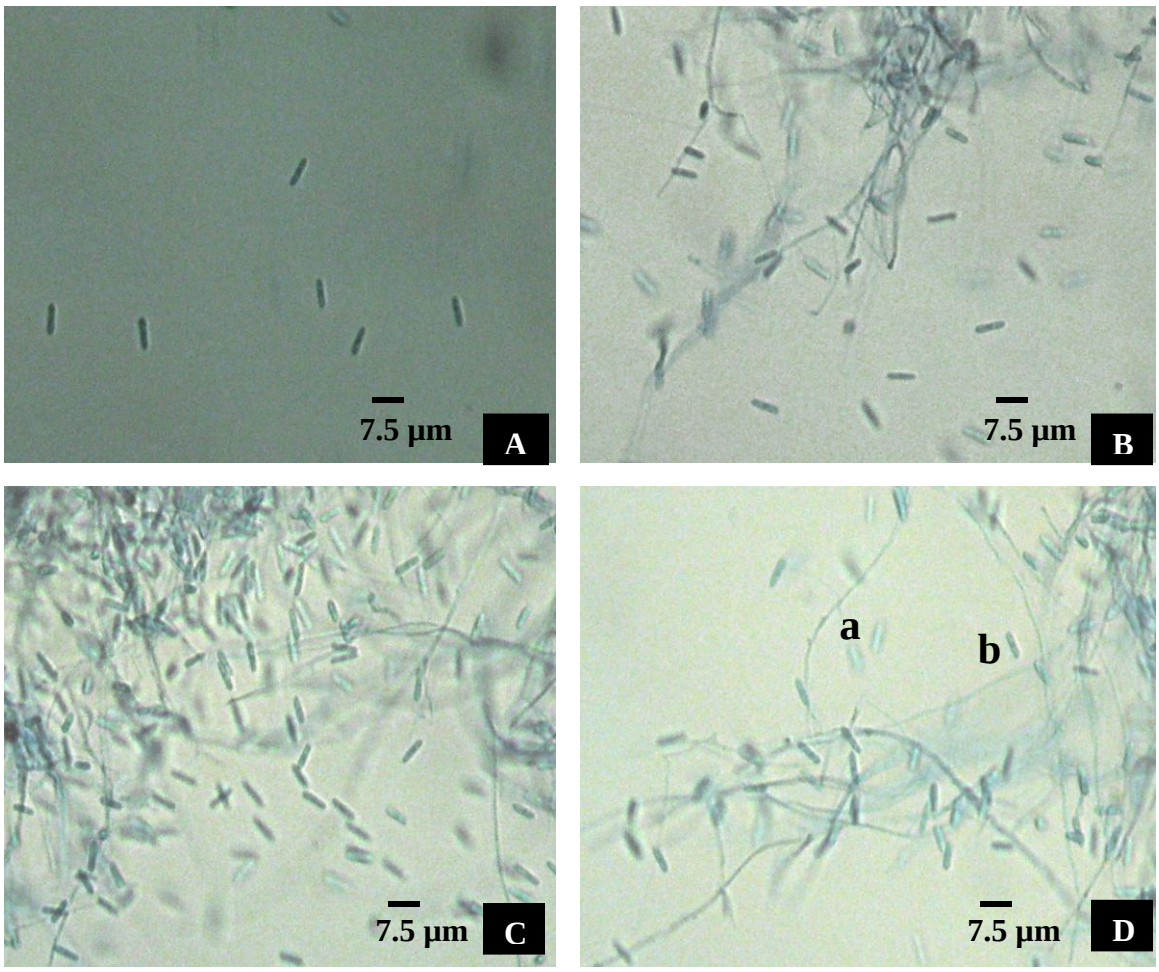


Figure 5.4. Morphological identification of *Phomopsis perseae* from alpha and beta conidia examined under the light microscope using low power (A-D). a = alpha conidia; b = beta conidia.

5.2. Cultural characteristics of *Pseudocercospora* sp. and *Colletotrichum* sp. after isolation from infected avocado fruits.

Pseudocercospora sp. and *Colletotrichum* sp. were successfully isolated from two avocado fruits exhibiting symptoms of *Cercospora* spot and anthracnose, respectively, using the tissue transplanting technique. The *Pseudocercospora* sp. isolate (Figure 5.5 A) was relatively slow-growing compared to *Colletotrichum* sp., and was dark gray to white in colony colour. In contrast to this, the *Colletotrichum* sp. isolate (Figure 5.5 B) was fast growing relative to *Pseudocercospora* sp., and was light gray to white in colony colour.

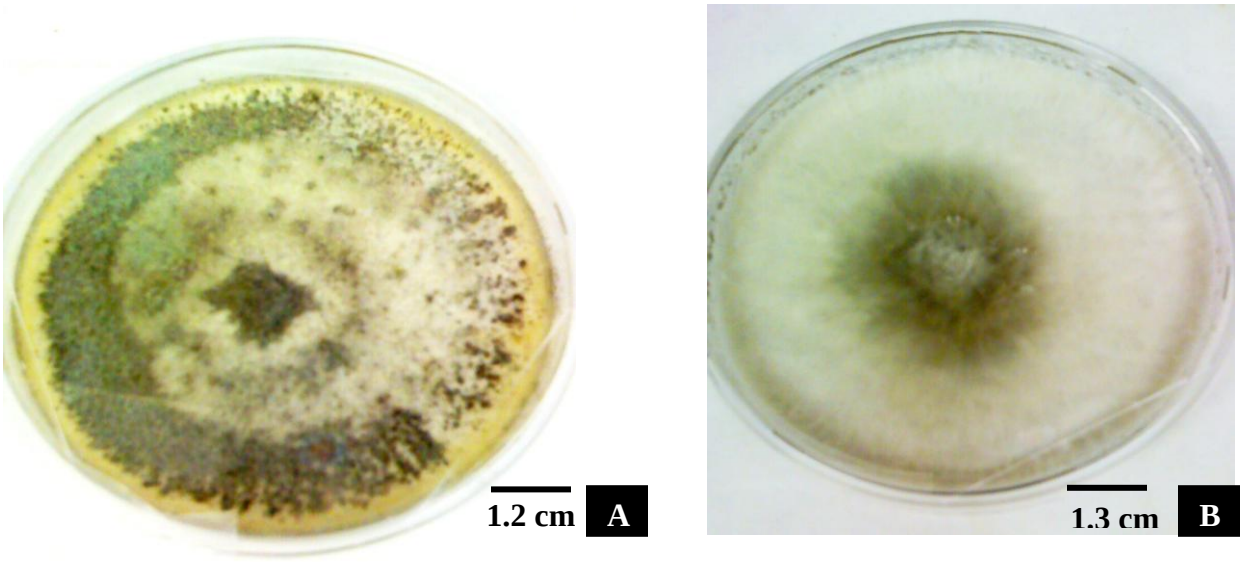


Figure 5.5. Cultures of *Pseudocercospora* sp. (A) and *Colletotrichum* sp. (B) isolated from infected avocado fruits expressing symptoms of *Cercospora* spot and anthracnose, respectively.

5.3. Amplification of fungal genomic DNA using ITS universal primers.

Fungal genomic DNA of all fungal pathogens was amplified using the ITS universal primers. The CgP complex genomic DNA was successfully amplified using the universal primers ITS5 and ITS4, generating a PCR product size of ~550 base pairs (Figure 5.6). Similarly, genomic DNA from *Pseudocercospora* sp. and *Phomopsis* sp. was also amplified using the universal primers ITS5 and ITS4, generating an expected PCR product size of ~550 base pairs (Figure 5.6).

In contrast to these, *Colletotrichum* sp. genomic DNA was amplified using the universal primers ITS1 and ITS4, generating an expected PCR product size of ~550 base pairs (Figure 5.6). No amplification was observed in lane 6, 7, 8 and 9 where the CgP complex, *Pseudocercospora* sp., *Colletotrichum* sp. and *Phomopsis* sp. negative control samples were loaded, respectively, indicating that there was no self-amplification or contamination (Figure 5.6).

5.4. DNA sequencing, subtyping and phylogenetic analyses.

5.4.1. Sequence characteristics.

The multiple sequence alignments of the 550 base pair nucleotide sequences of the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the CgP complex, *Pseudocercospora* sp., *Colletotrichum* sp. and *Phomopsis* sp. investigated in this study with GenBank reference sequences of the same gene regions are illustrated in Appendices 1, 2, 3 and 4, respectively.

5.4.2. Phylogenetic analyses.

Neighbour joining analysis was employed as it is one of the most accurate phylogenetic methods when attempting to determine which sequences are most closely related by using a tree as a visual analysis. Therefore, this method was ideal to use when identifying unknown CgP sequences to generic and/or species level, as well as confirming the identity of *Pseudocercospora* sp., *Colletotrichum* sp. and *Phomopsis* sp. sequences to species level. A neighbour joining tree was constructed using the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the CgP complex, *Pseudocercospora* sp., *Colletotrichum* sp., *Phomopsis* sp, as well as closely related GenBank reference sequences of the same gene regions, so as to establish accurate phylogenetic relationships and deduce sequence homology between the fungal pathogens.

Fungal DNA sequences were reliably edited with the FinchTV 1.4.0 software. Due to subsequent PCR amplifications, three PCR products each for the CgP complex and *Phomopsis* sp. were sent for sequencing (resulting in CgP sp. PCR product, CgP sp. PCR product 2 and CgP sp. PCR product 3, as well as *Phomopsis* sp. PCR product, *Phomopsis* sp. PCR product 2 and *Phomopsis* sp. PCR product 3, respectively). However, only one PCR product each for *Pseudocercospora* sp. and *Colletotrichum* sp. were sent for sequencing (resulting in *Pseudocercospora* sp. PCR product and *Colletotrichum* sp. PCR product, respectively). For phylogenetic analysis, the three CgP sequences did not intermingle with GenBank reference sequences, but formed their own unique cluster and were closely related to *Phomopsis* reference sequences (Figure 5.7). This is particularly true for CgP sp. PCR product, which revealed amongst others, a 99 % (**AY485742**), 97 %

(**EU002917** and **FJ441631**), 96 % (**AY745085**, **EU977307**, **EU977308**, **EU002918**, **EU002925**, **EU977226** and **FJ884148**) and 95 % (**AF001014**, **AY620999**, **DQ286286**, **EU814480** and **FJ440701**) sequence similarity with *Phomopsis* reference sequences, respectively. This sequence identity was mainly based on the 18S rRNA (partial sequence), ITS1, 5.8S rRNA, ITS2 (complete sequence) and 28S rRNA (partial sequence) gene regions.

In contrast to the CgP sequences, the *Pseudocercospora* sp. sequence was closely related to and intermingled with *Pseudocercospora* GenBank reference sequences. Thus, revealing amongst others, a 100 % (**DQ073923** and **EU726632**), 99 % (**DQ303083**, **DQ676519**, **EU726632**, **EF394858** and **EF394859**)**,** 98 % (**AF362055** and **AF362057**) and 97 % (**AF362056**) sequence similarity with *Pseudocercospora* reference sequences, respectively (Figure 5.8). Similarly to the CgP sequences, sequence similarity was also mainly based on the 18S rRNA (partial sequence), ITS1, 5.8S rRNA, ITS2 (complete sequence) and 28S rRNA (partial sequence) gene regions.

The *Colletotrichum* sp. sequence was also closely related to and intermingled with *Colletotrichum* GenBank reference sequences, thus, revealing amongst others, a 100 % (**AY266377**, **EU734584**, **EU734585**, **EU734586**, **EU520087** and **FJ459928**), 99 % (**EU054411**, **FJ904824** and **FJ904831**) and 98 % (**AB051406**, **EU847425**, **FJ449913** and **FJ449914**) sequence similarity, respectively (Figure 5.9). Sequence similarity was also mainly based on the 18S rRNA (partial sequence), ITS1, 5.8S rRNA, ITS2 (complete sequence) and 28S rRNA (partial sequence) gene regions.

Similarly to CgP sequences, the three *Phomopsis* sp. sequences were highly similar to *Phomopsis* GenBank reference sequences, although they did not intermingle with reference sequences but formed their own unique cluster, thus, revealing a 100 % (**AF230762**, **AY620999**, **EU814480**, **FJ440701**, **GQ250197**, **GQ250196**, **GQ250195**, **GQ250194**, **GQ250193**, **GQ250198**, **GQ250192** and **GU967697**) and 99 % (**AF001014**, **AF230750**, **DQ286286**, **GQ281808**, **GQ281807**, **GQ281796**, **GQ281809** and **GQ281798**) sequence similarity, respectively (Figure 5.10). Once again, sequence similarity was also mainly based on the 18S rRNA (partial sequence), ITS1, 5.8S rRNA, ITS2 (complete sequence) and 28S rRNA (partial sequence) gene regions.

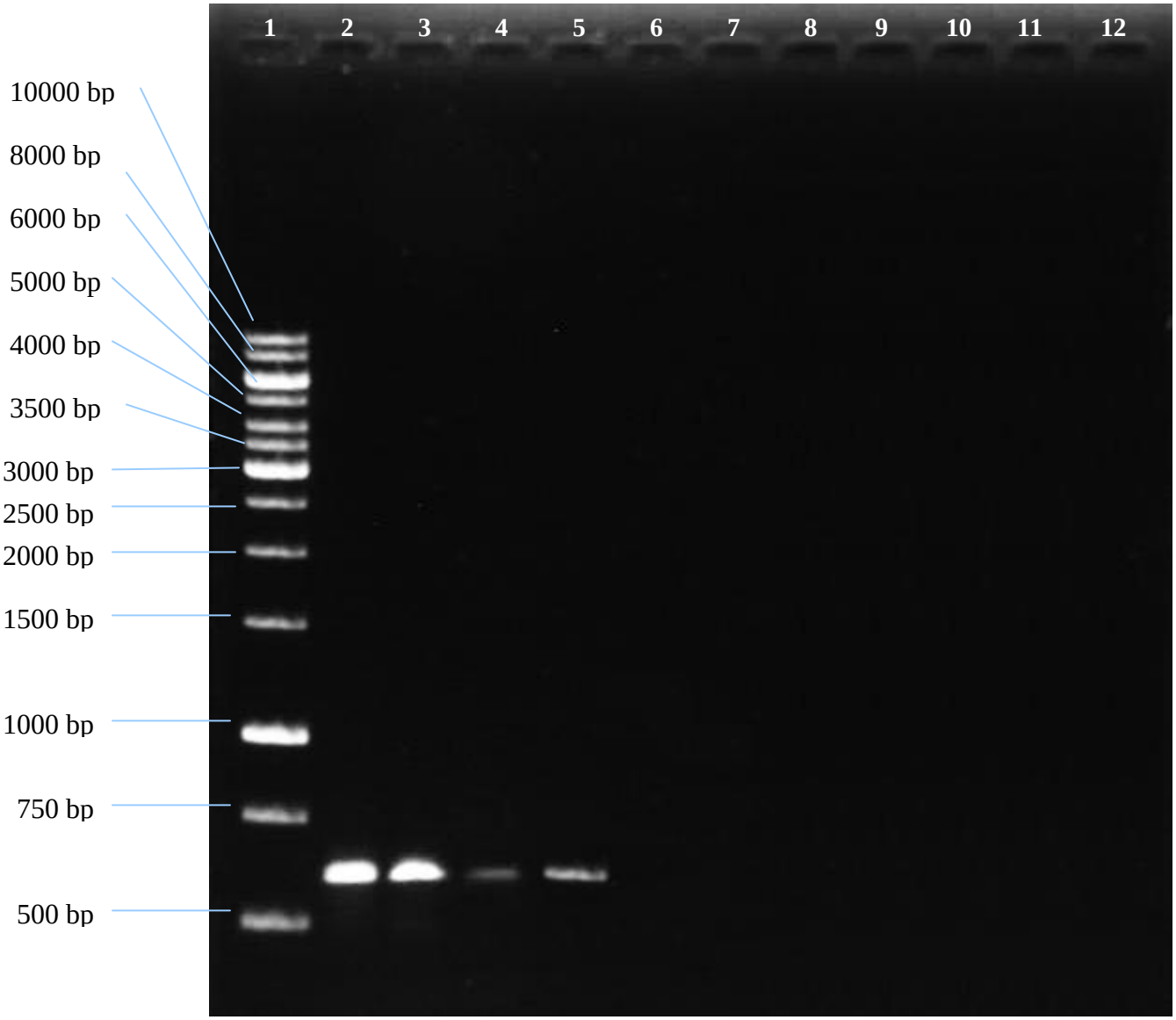


Figure 5.6. One percent agarose gel profile of fungal genomic DNA amplification.

Lane 1: 1 kb DNA ladder. Lane 2: CgP complex PCR product. Lane 3: *Pseudocercospora* sp. PCR product. Lane 4: *Colletotrichum* sp. PCR product. Lane 5: *Phomopsis* sp. PCR product. Lane 6: CgP complex negative control. Lane 7: *Pseudocercospora* sp. negative control. Lane 8: *Colletotrichum* sp. negative control. Lane 9: *Phomopsis* sp. negative control.

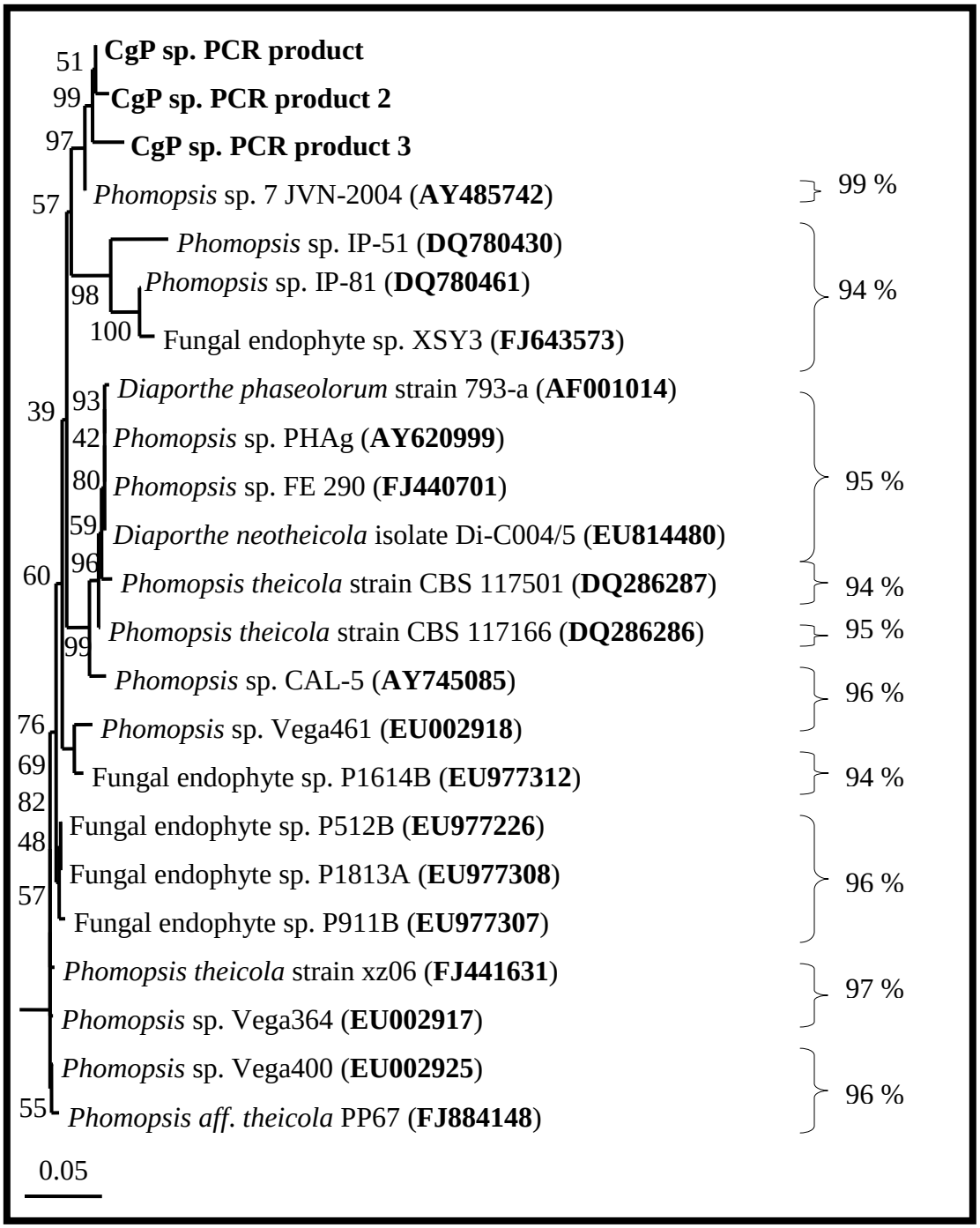


Figure 5.7. Phylogenetic analysis of the *Colletotrichum gloeosporioides-Pseudocercospora* (CgP) complex nucleotide sequences.

Unrooted neighbour-joining tree of sequences of the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of three CgP sequences. Fungal sequences used in the experiment are shown in bold (without accession numbers). Accession numbers of GenBank reference sequences are displayed in bold (in brackets). The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1000 replications) are indicated at the internodes.

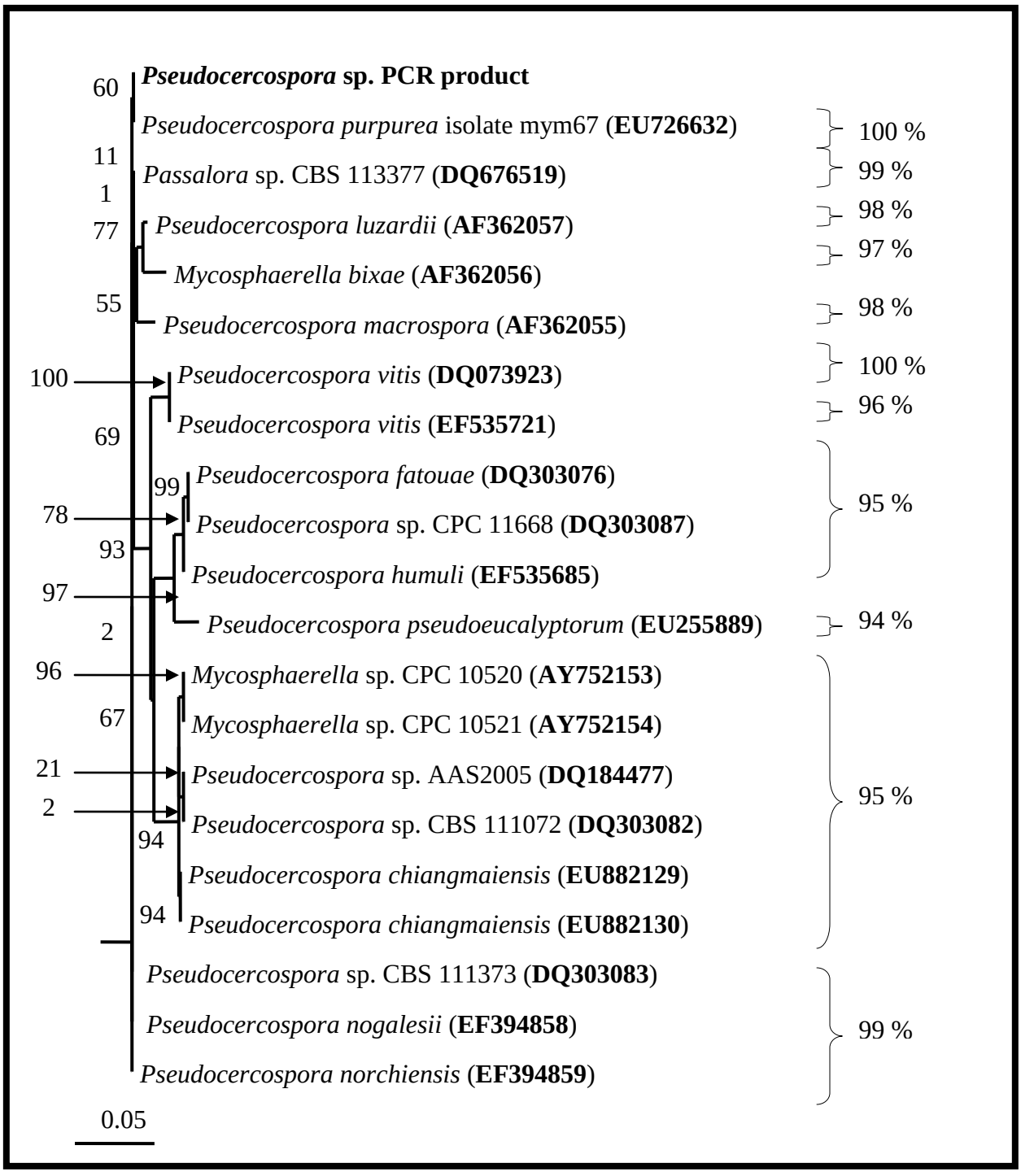


Figure 5.8. Phylogenetic analysis of *Pseudocercospora* nucleotide sequences.

Unrooted neighbour-joining tree of sequences of the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of *Pseudocercospora*. The fungal sequence used in the experiment is shown in bold (without an accession number). Accession numbers of GenBank reference sequences are displayed in bold (in brackets). The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1000 replications) are indicated at the internodes.

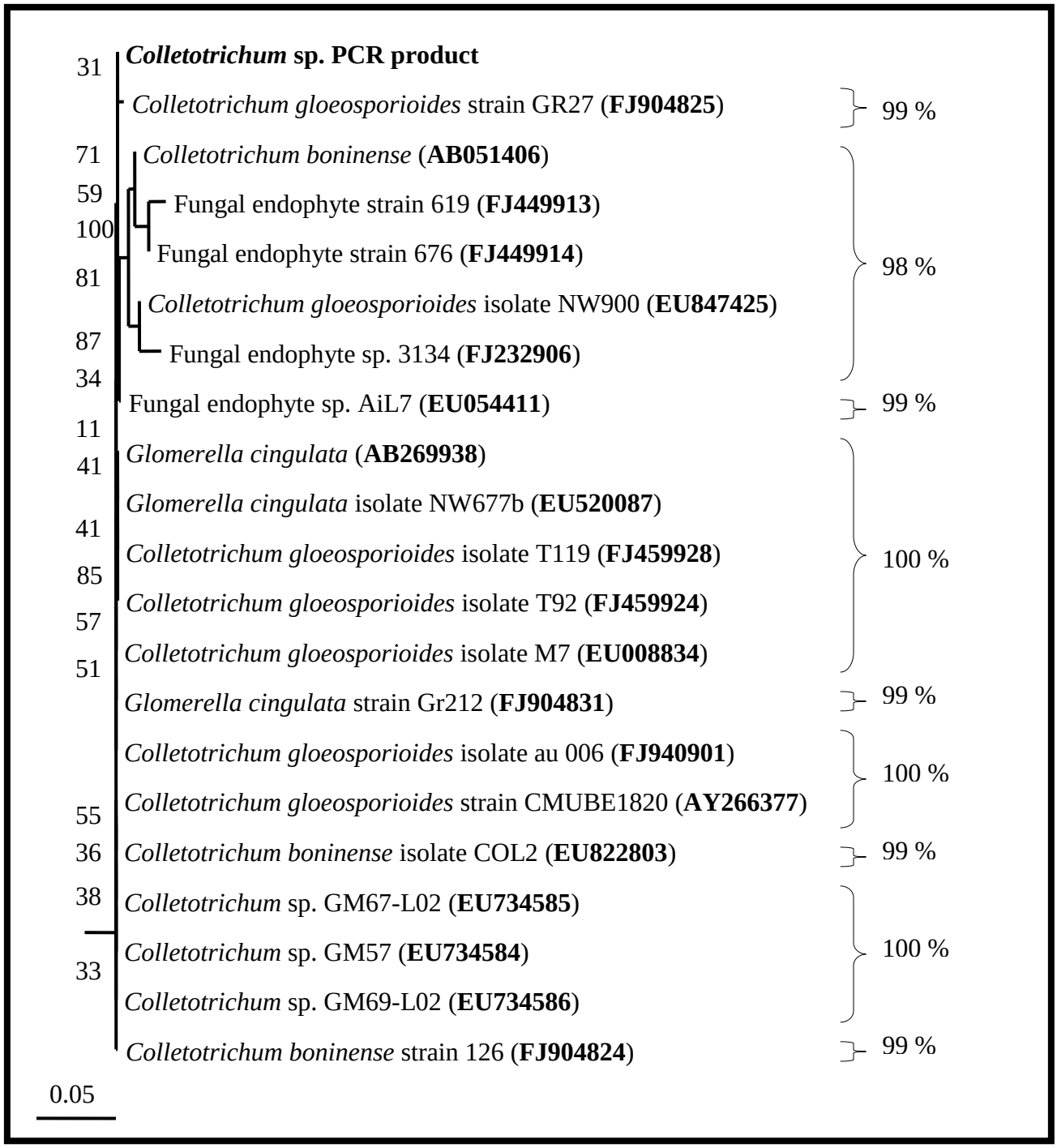


Figure 5.9. Phylogenetic analysis of *Colletotrichum* nucleotide sequences.

Unrooted neighbour-joining tree of sequences of the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of *Colletotrichum*. The fungal sequence used in the experiment is shown in bold (without an accession number). Accession numbers of GenBank reference sequences are displayed in bold (in brackets). The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1000 replications) are indicated at the internodes.

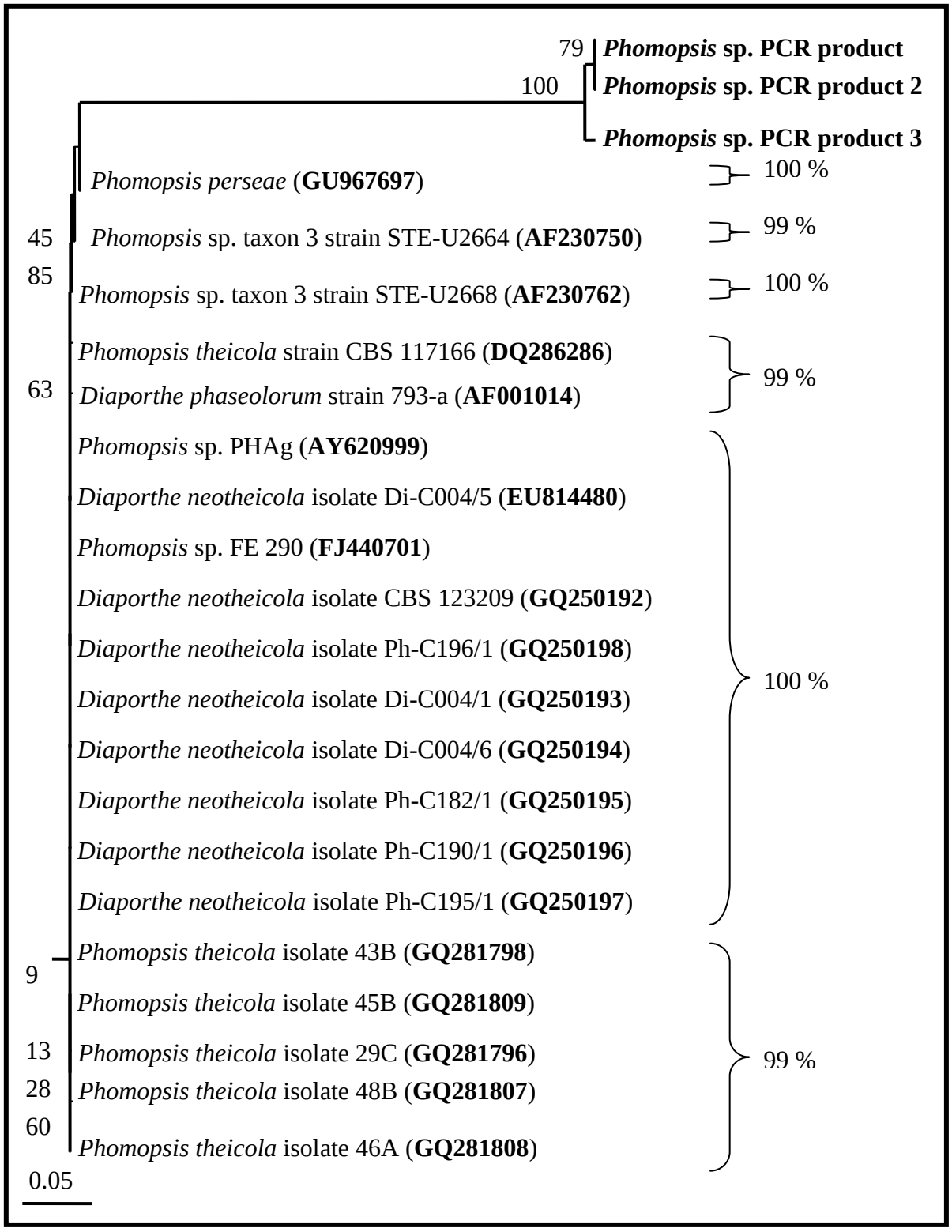


Figure 5.10. Phylogenetic analysis of *Phomopsis* nucleotide sequences.

Unrooted neighbour-joining tree of sequences of the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of three *Phomopsis* sequences. The fungal sequences used in the experiment are shown in bold (without accession numbers). Accession numbers of GenBank reference sequences are displayed in bold (in brackets). The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1000 replications) are indicated at the internodes.

Additionally, the edited CgP, *Pseudocercospora* sp., *Colletotrichum* sp. and *Phomopsis* sp. nucleotide sequences were compared using the BLAST tool (NCBI), so as to reveal the degree of relatedness between the four fungal species. All four fungal nucleotide sequences were multiply aligned using the multiple sequence alignment tool (DNAMAN Version 4.0), and an unrooted phylogenetic tree was constructed using the neighbour-joining method (DNAMAN Version 4.0) with a bootstrapping stringency of 1000 (Figure 5.11). Interestingly, the CgP and *Pseudocercospora* sp. sequences revealed a 95 % sequence similarity. Similarly, the CgP and *Phomopsis* sp. sequences showed a 95 % sequence similarity, while a sequence similarity of 92 % was obtained when comparing the CgP and *Colletotrichum* sp. sequences (Figure 5.11).

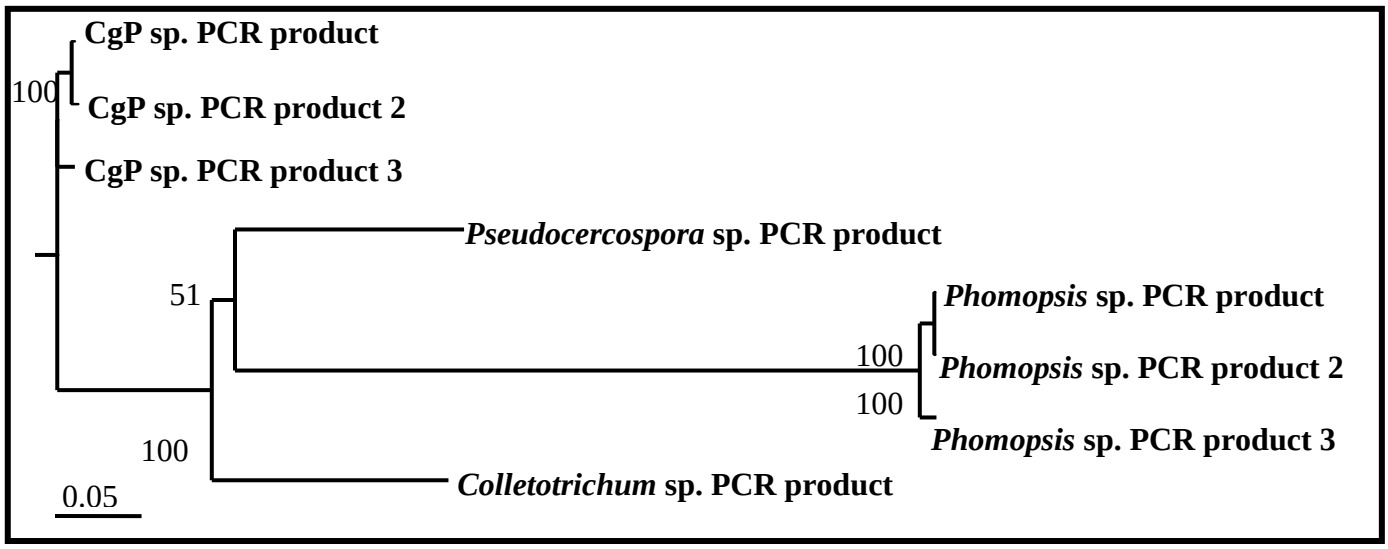


Figure 5.11. Phylogenetic analysis of *Colletotrichum gloeosporioides*-*Pseudocercospora* (CgP) complex, *Pseudocercospora* sp., *Colletotrichum* sp. and *Phomopsis* sp. nucleotide sequences.

Unrooted neighbour-joining tree of sequences of the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the CgP complex, *Pseudocercospora* sp., *C. gloeosporioides* and *Phomopsis* sp. Fungal sequence comparison was achieved using the BLAST tool (NCBI). Fungal sequences used in the experiment are shown in bold. The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1000 replications) are indicated at the internodes.

5.5. Discussion and conclusion.

Anthracnose is a disease characterized by sunken necrotic tissue where orange conidial masses are formed by *Colletotrichum* species (Bailey *et al*., 1992). The fungal pathogen *Colletotrichum gloeosporioides* has been associated with quiescent infections and post-harvest diseases on many fruits, including avocado (Simmonds, 1965; Hartill, 1992; Alahakoon *et al*., 1994; Timmer *et al*., 1998). Avocado black spot (*Cercospora* spot) is caused by the fungus *Pseudocercospora purpurea* and is still the most important pre-harvest avocado disease in South Africa.

Conidial shape may supply a reliable way of distinguishing between certain species and has been utilized to determine the identity of *Colletotrichum* species pathogenic on strawberry (Denoyes and Baudry, 1995). However, identification may be complicated because of overlapping ranges of conidial morphology and differences in colony characteristics in some cases (Adaskaveg and Hartin, 1997). Similarly, the identification of *Cercospora* species is mainly focused on conidial characteristics and host association (Chupp, 1954; Ellis, 1971, 1976). However, it is becoming more apparent that some species in this genus are conspecific with a broad host range, while others should be placed in other genera (Johnson and Valleau, 1949; Jones, 1959; Deighton, 1974, 1976, 1979; Ellis, 1976; Siboe and Braggins, 1994).

The overall morphological identification of the *Colletotrichum* isolate was in agreement with that described by the key of Barnett (1965), which describes *Colletotrichum* spp. as possessing simple conidiophores that are elongate, hyaline conidia that are unicellular,

ovoid or oblong, and are parasitic in imperfect stages of *Glomerella*. The isolate used in this study also produced ellipsoid spores of 11.9 X 4.7 µm (Figure 5.1; section 5.1). This spore size range and morphology were in close agreement with the results obtained by Cano *et al*. (2004), in which straight, cylindrical, apex obtuse and base truncate conidia of *C. gloeosporioides* of 6–25 X 4–6 µm were observed. On the basis of the morphological similarities between the isolate understudy and the description of Cano *et al*. (2004) and the host source, the *Colletotrichum* isolate was regarded as *Colletotrichum gloeosporioides* and was subject to molecular confirmation.

The sporulating culture of the CgP complex employed by Giovanelli (2008) produced spores that were filiform (resembling *Pseudocercospora* sp), while some were ellipsoid (resembling *Colletotrichum gloeosporioides*) in morphology. Filiform spores had an average size of 23 X 1.5 µm, while ellipsoid spores had an average size of 7 X 2.5 µm (Figure 5.2; section 5.1). *Pseudocercospora* sp. isolated by Giovanelli (2008) produced spores that were filiform in morphology with an average size of 20 X 3.5 µm (Figure 5.3; section 5.1). The overall morphological identification was in agreement with that described by the key of Barnett (1965), which describes *Pseudocercospora* spp. as having dark, simple conidiophores arising in clusters, and bursting out of leaf tissue, and bearing conidia successively on new growing tips, possessing conidia that are hyaline or dark, filiform, multicellular and parasitic on higher plants. The spore size range and morphology were in close agreement with the results obtained by Palmateer *et al*. (2006) in which *P. purpurea* produced filiform spores of 20-100 X 2-4.5 µm.

The fungal culture of *Phomopsis perseae* obtained from PPRI produced two morphologically distinct types of spores: alpha conidia that were colourless, fusiform, non-septate and had an average size of 7.5 X 2.5 µm, as well as beta conidia that were filiform, curved or bent, hyaline, unicellular and had an average size of 13.2 X 1.3 µm (Figure 5.4; section 5.1). The overall morphological identification was in agreement with that described by the key of Barnett (1965), which describes *Phomopsis* spp. as having dark pycnidia, which are ostiolate, immersed, erumpent and nearly globose, simple conidiophores, hyaline conidia that are unicellular, ovoid to fusoid (alpha conidia) and filiform, or bent stylospores (beta conidia). The spore size range and morphology were in close agreement with the results obtained by Ploetz (2003), in which *Phomopsis* sp. produced alpha conidia of 7-10 X 2-3 µm, and Swarup *et al*. (1966), in which another *Phomopsis* species (*Phomopsis manihotis*) produced beta conidia of 12.0-20.0 X 1-1.5 µm.

The overlapping spore morphologies between *Colletotrichum*, *Pseudocercospora* and *Phomopsis* therefore created doubt about the designation of the CgP complex as a mixture of *Colletotrichum gloeosporioides* and *Pseudocercospora* sp.

Therefore, the ultimate aim of this study was to attempt to unravel the identity of the CgP complex using molecular techniques aimed at targeting and amplifying the ITS region (encompassing the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions) of the fungal pathogen. Genomic DNA from the CgP complex, the isolated *Pseudocercospora* sp. and *Phomopsis* sp. was successfully amplified using the universal primers ITS5 and ITS4, generating an expected PCR product size of ~550 base pairs (Figure 5.6). These results

were in agreement with those obtained by Siboe *et al*. (2000) and Uddin *et al*. (1997), in which genomic DNA from various *Pseudocercospora* species and *Phomopsis* sp. were amplified using the ITS5 and ITS4 universal primers, generating PCR product sizes of ~550 base pairs, respectively.

In contrast to these, *Colletotrichum* sp. genomic DNA was amplified using the universal primers ITS1 and ITS4, generating an expected PCR product size of ~550 base pairs (Figure 5.6) This was in agreement with the results obtained by Lubbe *et al*. (2004), in which genomic DNA from various *Colletotrichum* species was amplified using the ITS1 and ITS4 universal primers, generating PCR product sizes of ~550 base pairs. Kumar and Shukla (2005) also used the ITS1 and ITS4 universal primers to amplify genomic DNA from *Aspergillus fumigatus, Aspergillus flavus, Candida albicans, Candida krusei, Candida parapsilosis*, *Cryptococcus neoformans*, *Fusarium* spp., *Sporothrix schenckii* and *Trichophyton mentagrophytes*, generating PCR product sizes of ~550 base pairs.

Phylogenetic analysis revealed that the three CgP sequences showed 94-99 % sequence similarity with *Phomopsis* spp., *Diaporthe*, the teleomorph of *Phomopsis*, or unknown fungal endophytes (Figure 5.7). However, they did not intermingle with *Phomopsis* GenBank reference sequences, but formed their own unique cluster. This was an unexpected outcome, since the CgP complex was thought of comprising *Colletotrichum gloeosporioides* and a *Pseudocercospora* sp., based on morphological identification using conidial characteristics. The CgP complex morphological misidentification may have resulted because *C. gloeosporioides* spores are ellipsoid in morphology, while those of

Pseudocercospora sp. and *Phomopsis* sp. are filiform and fusiform, respectively. Filiform and fusiform spore morphologies are at times difficult to differentiate as they may resemble each other. Additionally, some *Phomopsis* species such as *Phomopsis manihotis* (Swarup, 1966), *Phomopsis* sp. (Ploetz, 2003) as well as *Phomopsis juniperovora* (http://www.na.fs.fed.us/spfo/pubs/fidls/phomopsis/phomopsis.htm, last accessed on the 20 July 2010) contain both alpha conidia (which resemble ellipsoid spores of *C. gloeosporioides*) and beta conidia (which resemble filiform spores of *Pseudocercospora purpurea*), which may have led to the misidentification of the *Phomopsis* sp. as the '*Colletotrichum gloeosporioides-Pseudocercospora* (CgP) complex'. Consequently, molecular identification revealed that all three CgP sequences are those of a *Phomopsis* species and not a fungal complex comprising *Colletotrichum gloeosporioides* and a *Pseudocercospora* sp., as suggested by morphological identification. The results corroborated the view of Adaskaveg and Hartin (1997), that in some cases, identification may be complicated because of overlapping ranges of conidial morphology and differences in colony characteristics.

The *Pseudocercospora* sp. sequence showed 94-100 % sequence similarity to various *Pseudocercospora* spp. or *Mycosphaerella*, the anamorph of *Pseudocercospora* or *Passalora* sp. with 100 % similarity to *Pseudocercospora purpurea* and *Pseudocercospora vitis* (Figure 5.8). *Passalora* is closely related to *Pseudocercospora* and *Stenella* at a genus level (Crous *et al*., 2001b). Although it seems that all three anamorph genera have evolved more than once within *Mycosphaerella*, and that these genera are retained best as separate entities, examples have been found of *Passalora* morpho-types clustering within

Pseudocercospora (Crous *et al*., 2001b). *Pseudocercospora vitis*, the anamorph of *Mycosphaerella personata*, is the causative agent of leaf spot in the plant family *Vitaceae*. For, this reason, it is very likely that the *Pseudocercospora* sp. isolated from an infected avocado fruit is *Pseudocercospora purpurea*, and not *Pseudocercospora vitis*.

The *Colletotrichum* sp. sequence showed 98-100 % sequence similarity to various *Colletotrichum* spp. or unknown fungal endophytes or *Colletotrichum boninense* (causative agent of leaf anthracnose) or *Colletotrichum gloeosporioides* and *Colletotrichum* sp. (Figure 5.9). *Colletotrichum boninense* previously fell within the broad species concept of *Colletotrichum gloeosporioides* but is differentiated from *C. gloeosporioides* by colony shape, conidial morphology, and molecular phylogenetic analysis of ITS sequences (Moriwaki *et al*., 2003; Lu *et al*., 2004). For, this reason, it is very likely that the *Colletotrichum* sp. isolated from an infected avocado fruit is *Colletotrichum gloeosporioides*, and not *Colletotrichum boninense*.

Similarly to the three CgP sequences, phylogenetic analysis also revealed that all three *Phomopsis* sp. sequences were representing *Phomopsis* species, showing 99-100 % sequence similarity with *Phomopsis* spp., *Diaporthe*, the teleomorph of *Phomopsis*, or *Phomopsis perseae* (Figure 5.10). However, they did not intermingle with *Phomopsis* GenBank reference sequences, but formed their own unique cluster. This inevitably confirmed that the *Phomopsis* (PPRI 6006) obtained from the Plant Protection Research Institute (PPRI) culture collection is *Phomopsis perseae*.

Furthermore, a 95 % sequence similarity was revealed after comparing the CgP and *Pseudocercospora* sp. nucleotide sequences (Figure 5.11). Similarly, the CgP and *Phomopsis* sp. sequences showed a 95 % sequence similarity, while a sequence similarity of 92 % was obtained when comparing the CgP and *Colletotrichum* sp. nucleotide sequences. Consequently, this suggested that the 'CgP complex' (*Phomopsis* sp.) is more closely related phylogenetically to *Pseudocercospora* sp. and *Phomopsis* sp. than *Colletotrichum* sp.

In conclusion, morphological identification alone may not allow accurate strain identification of fungal pathogens. For this reason, morphological identification needs to be complemented with molecular identification for reliable strain identification to a genus and/or species level, especially with a host like avocado where a susceptible cultivar such as 'Fuerte' can be infected simultaneously by a number of fungal pathogens on different organs, causing both, or either, pre- and post-harvest diseases. A combination of identifications on the basis of host source, cultural characteristics, conidial morphology and analysis based on rDNA ITS nucleotide sequences confirmed the isolates used in this study to be *Colletotrichum gloeosporioides*, *Pseudocercospora purpurea*, *Phomopsis perseae* and the so-called CgP complex likely to be a species of *Phomopsis*.

CHAPTER 6:

Conclusion, recommendations and future studies.

Based on the antifungal diene and triene accumulation patterns in harvested and unharvested fruits measured using HPLC and HPLC-MS, these compounds proved to be capable of controlling anthracnose infection as they were able to neutralize the fungal pathogen *Colletotrichum gloeosporioides* (particularly in unharvested fruits), leading to absence of anthracnose symptoms in the early stages of SEM study. Furthermore, this was consolidated by an impressive and convincing antifungal activity of the diene compound after being assayed for antifungal activity, in which its activity was directly proportional to its concentration (i.e. diene activity increased with an increase in concentration).

Although the triene compound was not purified and assayed for antifungal activity, the close structural similarity between diene and triene compounds suggests that it may possess similar antifungal activity towards *C. gloeosporioides* as the diene compound. This view is supported and consolidated by a close similarity in inhibitory patterns between the diene compound and another closely related compound methyl linoleate after being assayed for antifungal activity towards the anthracnose pathogen *C. gloeosporioides*, although the former displayed a higher antifungal activity relative to the latter. Therefore, as a recommendation, there is a need to synthesize these compounds (diene and triene) and evaluate their potential as biocontrol agents against anthracnose infection, particularly at an early stage so as to prevent pathogen entry and penetration. Furthermore, there is also a need to evaluate the potential of methyl linoleate as a biocontrol agent against anthracnose infection as it is already commercially available. Since methyl linoleate is already produced

commercially, the synthesis of the diene and triene compounds as biocontrol agents should be feasible, due to the close structural similarities between these compounds, and as such a relatively similar synthetic pathway may be employed for the synthesis of the diene and triene compounds.

Despite the production of a wide variety of synthetic organic fungicides, copper fungicides still dominate the field of fungicidal plant disease control. Copper fungicides have been employed for the control of many vegetable, fruit and flowering plant diseases. Copper oxychloride is probably the most widely used copper fungicide. It is a low soluble copper in different formulation as wettable powder, colloidal liquid or ready to use dust (Gharieb *et al*., 2004). However, the use of copper compounds (e.g. copper oxychloride) is beset with a number of hazardous health problems including irritation of the skin, eyes and respiratory tract, metallic taste and nausea. In more severe cases, there may be blood in vomit, and jaundice and enlarged liver may develop. This is because soluble salts are corrosive to mucous membranes and the cornea, and organic copper compounds are more absorbable and display greater systemic toxicity than inorganic compounds (http://www.pesticideinfo.org, last accessed on the 29 November 2010).

Contrary to copper compounds, high diene and triene concentrations in avocados have not been reported as a human health concern, as humans ingest fresh and processed avocados and avocado oil is mostly used in cosmetics. One possible explanation for this lack of reported toxicity may be due to the nature of the idioblast cells. These cells have a tough cell wall that is probably not degraded by human digestion, so that the cells may pass

through the digestive system intact. But, substantial information will be required on field rates, mammalian toxicity, photostability and phytotoxicity before the compound can be considered for commercialization. Moreover, it may also be possible to develop more photostable, more selective, or more potent analogs (Bull and Carman, 1994).

As far as cost analysis is concerned, the technology used to produce copper fungicides is relatively simple and inexpensive. The simplicity of copper-chemical production and the availability of cheap copper-bearing secondaries have resulted in the field possessing a large number of small producers, especially in less-developed countries (Piret, 1997). In contrast to this, the technology used to produce methyl linoleate involves the use of soybean or canola oil and methanol, thus, making it less economically feasible relative to that of copper oxychloride. The most common procedure employed for the synthesis of methyl linoleate uses methanol (converted to sodium methoxide) to produce methyl esters (commonly referred to as fatty acid methyl ester) as it is the cheapest alcohol available, though ethanol can be used to produce an ethyl ester (commonly referred to as fatty acid ethyl ester) (Strahan, 2008). Consequently, the use of copper oxychloride as a fungicide is estimated to cost about R 2000 per hectare in one season (D. Taylor, Roodewal Farm, pers. comm,), while that of methyl linoleate is estimated to cost about R 3020 per hectare in one season (Sigma-Aldrich Handbook, 2008-2009).

Should these compounds prove to be successful biocontrol agents towards anthracnose infection, further studies should therefore focus on the antifungal activities of these compounds towards other fungal pathogens of avocado, including *Pseudocercospora*

purpurea (the causative agent of *Cercospora* spot) and *Phomopsis perseae* (the causative agent of dieback and trunk canker). Ultimately, further studies should also evaluate the activity of these compounds towards other microorganisms which infect the avocado fruit, including bacteria (e.g. *Erwinia carotovora*, and *Xanthomonas campestris*, the causative agents of bacterial soft rot and bacterial canker, respectively), as well as insects, including those that cause mechanical fruit damage, thereby exposing the fruit to a wide spectrum of infectious microorganisms. At a fundamental level, we need to investigate the biosynthetic pathways leading to diene and triene production in the fruit as part of an integrated biochemical study investigating diene and triene production and activity in relation to other general or hypersensitive resistance responses.

In this present study, it was further consolidated that morphological identification alone may not allow accurate strain identification of fungal pathogens. As such, morphological identification needs to be complemented with molecular identification (of which PCR was employed in this study followed by DNA Sequencing) for reliable strain identification to a genus and/or species level, especially with a host like avocado where a susceptible cultivar such as 'Fuerte' can be infected simultaneously by a number of fungal pathogens on different organs, causing both, or either, pre- and post-harvest diseases. For this reason, before attempting to evaluate the potential of the diene, triene and methyl linoleate as possible biocontrol agents towards fungal pathogens, bacteria, other microorganisms or insects, it will be of critical importance to validate the identity of these infectious agents using molecular techniques.

CHAPTER 7:

References.

Abang MM, Green KR, Wanyera NW, Iloba C (2001). Characterization of *Colletotrichum gloeosporioides* Penz. from yam (*Dioscorea* spp.) in Nigeria. *In*: *Symposium of the International Society for Tropical Root Crops–Africa Branch, Cotonou, Bénin*. Root crops in the 21st century. *Proceedings of the 7th Triennial*: 613-615.

Adaskaveg JE, Hartin RJ (1997). Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology* **87**: 979-987.

Adikaram NKB, Brown AE, Swinburne TR (1982). Phytoalexin involvement in the latent infection of *Capsicum annuum* L. fruit by *Glomerella cingulata* (Stonem). *Physiological Plant Pathology* **21**: 161-170.

Afanador L, Minz D, Maymon M, Freeman S (2003). Characterization of *Colletotrichum* isolates from tamarillo, pasiflora and mango in Colombia and identification of a unique species from genus. *Phytopathology* **93** (5): 579-587.

Akinwunmi O, Latunde-Dada AO (2001). *Colletotrichum*: tales of forcible entry, stealth, transient confinement and breakout. *Molecular Plant Pathology* **2** (4): 187–198.

Alahakoon PW, Sreenivasaprasad S, Brown AE (1992). Selection of a genetic variant within *Colletotrichum gloeosporioides* isolates pathogenic on mango by passaging through wounded tomato fruits. *Physiological and Molecular Plant Pathology* **41**: 227–240.

Alahakoon PW, Brown AE, Sreenivasaprasad S (1994). Cross-infection potential of genetic groups of *Colletotrichum gloeosporioides* on tropical fruits. *Physiological and Molecular Plant Pathology* **44**: 93-130.

Andrade EM, Uesugi CH, Ueno B, Ferreira M (2007). Morphocultural and molecular characterization of *Colletotrichum gloeosporioides* isolates pathogenic to papaya. *Fitopatologia Brasileira* **32** (1): 21-31.

Bailey JA, O`Connell RJ, Pring RJ, Nash C (1992). Infection strategies of *Colletotrichum* species. *Commonwealth Agricultural Bureaux*: 88-120.

Bailey JA, Jeger MJ (1992). *Colletotrichum*: Biology, Pathology and Control. *Commonwealth Agricultural Bureaux*: 88-120.

Bailey JA, Nash C, Morgan LW, O`Connell RJ, TeBeest DO (1996). Molecular taxonomy of *Colletotrichum* species causing anthracnose on the *Malvaceae*. *Phytopathology* **86**: 1076–1083.

Bailey JA (1997). rDNA sequencing: an essential guide to the taxonomy and diagnosis of *Colletotrichum*. *In*: *Diagnosis and Identification of Plant Pathogens* **11**. Developments in Plant Pathology. *Academic Publishers*, Dordrecht, Netherlands, Kluwer: 47–51.

Barnett HL (1965). Illustrated genera of imperfect fungi. West Virginia University, *Burgess publishing company*, Morgantown, West Virginia. **2**: 242-443.

Baxter AP, Westhuizen GCAV, Eicker A (1985). A review of literature on the taxonomy, morphology and biology of the fungal genus *Colletotrichum*. *Phytophylactica* **17**: 15–18.

Beno-Moualem D, Prusky D (2000). Early events during quiescent infection development by *Colletotrichum gloeosporioides* in unripe avocado fruits. *Biochemistry and Cell Biology* **90** (5): 553-559.

Bernard CG, Hammond SM, Hampson SE, Eves A, Crowder MJ (2002). Influence of supplementary vitamins, minerals and essential fatty acids on the antisocial behavior of young adult prisoners. *The British Journal of Psychiatry* **181**: 22–28.

Bernstein BE, Zehr EI, Dean RA, Shabi E (1995). Characteristics of *Colletotrichum* from peach, apple, pecan and other hosts. *Plant Disease* **79**: 478-482.

Brodrick HT, Pretorius WJ, Frean RT (1974). Avocado diseases. *Farming in South Africa*. Avocado (H1): 1-8.

Brown AE, Soepena H (1994). Pathogenicity of *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* on leaves of *Hevea* spp. *Mycological Research* **98**: 264–266.

Brown AE, Sreenivasaprasad S, Timmer LW (1996). Molecular characterization of slow-growing orange and key lime anthracnose strains of *Colletotrichum* from *Citrus* as *C. acutatum*. *Phytopathology* **86**: 523-527.

Brundrett MC, Melville L, Peterson L (1994). Practical methods in mycorrhizal research. *Mycologue Publications*, Ontario, Canada: 161.

Bruns TD, White TJ, Taylor JW (1991). Fungal molecular systematics. *Annual Review of Ecological Systems* **22**: 525-564.

Bull SD, Carman RM (1994). Synthesis of the avocado antifungal, (*Z,Z)*-2-hydroxy-4-oxohenicosa-12,159 dien-1-yl acetate. *Australian Journal of Chemistry* **47**: 1661-672.

Cano J, Guarro J, Gene´ J (2004). Molecular and morphological identification of *Colletotrichum* species of clinical interest. *Journal of Clinical Microbiology* **42** (6): 2450-2454.

Chakraborty S, Jones PN (1993). A rapid bioassay for the assessment of pathogenic variation in *Colletotrichum gloeosporioides* infecting *Stylosanthes scabra*. *Plant Disease* **77**: 1016–1020.

Chang CF, Isogai A, Kamikado T, Murakoshi S, Sakurai A, Tamura S (1975). Isolation and structure elucidation of growth inhibitors for silkworm larvae from avocado leaves. *Agricultural and Biological Chemistry* **39** (5): 1167.

Chilton SJP, Wheeler HE (1949). Genetics of *Glomerella*. VII. Mutation and segregation in plus cultures. *American Journal of Botany* **36** (10): 717-721.

Chupp C (1953). A monograph of the genus *Cercospora*. *Ithaca*, New York: 667.

Chupp C (1954). A Monograph of the Fungus Genus *Cercospora*. *Ithaca*, New York: 667.

Coates LJ (1991). A collaborative theory of inversion: Irony in dialogue. Masters thesis, University of Victoria, Victoria, BC.

Coates LM, Irwin JAG, Muirhead IF (1993a). The use of a benomyl resistant mutant to demonstrate latency of *Colletotrichum gloeosporioides* in avocado fruit. *Australian Journal of Agricultural Research* **44**: 763-772.

Coates LM, Muirhead IF, Irwin JAG, Gowanlock DH (1993b). Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit. *Mycological Research* **97**: 1363-1370.

Cooke MC (1878). North American Fungi. *Hedwigia* **17**: 37-40.

Correll JC, Rohoads DD, Guerber JC (1993). Examination of mitochondrial DNA restriction fragment lengh polymorphisms, DNA fingerprints, and randomly amplified polymorphic DNA of *Colletotrichum orbiculare*. *Phytopathology* **83**: 1199-1204.

Crous PW, Aptroot A, Kang JC, Braun U, Wingfield MJ (2000). The genus *Mycosphaerella* and its anamorphs. *Studies in Mycology* **45**: 107-121.

Crous PW, Kang JC, Braun U (2001b). A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequence and morphology. *Mycologia* **93**: 1081-1101.

Darvas JM (1977). *Cercospora* spot. *South African Avocado Growers' Association Proceedings of the Technical Committee* **1**: 3-6.

Darvas JM (1982). Etiology and control of some fruit diseases of avocado (*Persea americana* Mill) at Westfalia Estate, D.Sc. (Agriculture) thesis, University of Pretoria, Pretoria.

de Jong AJ, Yakimova ET, Kapchina VM, Woltering EJ (2002). A critical role for ethylene in hydrogen peroxide release during programmed cell death in tomato suspension cells. *Planta* **214**: 537-545.

Deighton FC (1974). Studies on *Cercospora* and allied genera.V. *Mycovellosiella* Rangel and a new species of *Ramulariopsis*. *Mycological Papers* **137**: 1–75.

Deighton FC (1976). Studies on *Cercospora* and allied genera. *Mycological Papers* **140**: 1–168.

Deighton FC (1979). Studies on *Cercospora* and allied genera. VII. New species and redisposition. *Mycological Papers* **144**: 1–56.

Deising HB, Werner S, Wernitz M (2000). The role of fungal appressoria in plant infection. *Microbes and Infection* **2**: 1631–1641.

Denoyes B, Baudry A (1995). Species identification and pathogenicity study of French *Colletotrichum* strains isolated from strawberry using morphological and cultural characteristics. *Journal of Phytopathology* **85**: 53–57.

Dickman KB (1994). Part V. Papaya: Anthracnose. *In*: *Compendium of tropical fruit diseases*. *APS Press*. St Paul, Minnesota: 58–59.

Domergue F, Helms GL, Prusky D, Browse J (2000). Antifungal compounds from idioblast cells isolated from avocado fruits. *Phytochemistry* **54**: 183-189.

Downard KM (2007). William Aston - the man behind the mass spectrograph. *European Journal of Mass Spectrometry* **13** (3): 177–190.

Du M, Schardl CL, Vaillancourt LJ (2005). Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. *Mycologia* **97**: 641-658.

Durand BJ (1990). Introduction to avocado growing in South Africa. A1 *In*: *Farming in South Africa*. *Directorate of Agricultural Information*. Pretoria: 1.

Duvenhage JM (2002). Evaluation of new generation fungicides for control of *Cercospora* spot on avocado fruit. *South African Avocado Growers` Association Yearbook* **25**: 11-14.

Ellis MB (1971). Dematiaceous Hyphomycetes. *Commonwealth Mycological Institute*, England.

Ellis MB (1976). More Dematiaceous Hyphomycetes. *Commonwealth Mycological Institute*, England.

Ernst AA (1997). Annual Chairman's Report. *South African Avocado Growers` Association Yearbook* **20**: vii-ix.

Fehling E, Mukherjee KD (1991). Acyl-CoA elongase from a higher plant (*Lunaria annua*): metabolic intermediates of very-long-chain acyl-CoA products and substrate specificity. *Biochimica et Biophysica Acta* **1082**: 239-246.

Fehling E, Lessire R, Cassagne C, Mukherjee KD (1992). Solubilization and partial purification of constituents of acyl-CoA elongase from *Lunaria annua*. *Biochimica et Biophysica Acta* **1126**: 88-94.

Fitzell RD (1987). Epidemiology of anthracnose disease of avocados. *South African Avocado Growers` Association Yearbook* **10**: 113-116.

Flaishman A, Kolattukudy PE (1994). Timing of fungal invasion using host's ripening hormone as a signal. *Proceedings of National Academy of Sciences* **91**: 6579–6583.

Flor HH (1971). The current status of the gene-for-gene concept. *Annual Review of Phytopathology* **9**: 275-296.

Ford R, Banniza S, Photita W, Taylor PWJ (2004). Morphological and molecular discrimination of *Colletotrichum truncatum* causing anthracnose on lentil in Canana. *Australasian Plant Pathology* **33** (4): 559-569.

Freeman S, Pham M, Rodriguez RJ (1993). Molecular genotyping of *Colletotrichum* species based on arbitrarily primed PCR, A-T-rich DNA, and nuclear DNA analyses. *Experimental Mycology* **17**: 309-322.

Freeman S, Rodriguez RJ (1995). Differentiation of *Colletotrichum* species responsible for anthracnose of strawberry by arbitrarily primed PCR. *Mycological Research* **99**: 501-504.

Freeman S, Katan T, Shabi E (1996). Characterization of *Colletotrichum gloeosporioides* isolates from avocado and almond fruits with molecular and pathogenicity tests. *Applied and Environmental Microbiology* **62**: 1014–1020.

Freeman S, Shabi E (1996). Cross-infection of subtropical and temperate fruits by *Colletotrichum* species from various hosts. *Physiological and Molecular Plant Pathology* **49**: 395–404.

Freeman S, Katan T (1997). Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. *Phytopathology* **87**: 516-521.

Freeman S, Katan T, Shabi E (1998). Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease* **82**: 596-605.

Freeman S, Minz D, Jurkevitch E, Maimon M, Shabi E (2000). Molecular analyses of *Colletotrichum* species from almond and other fruits. *Phytopathology* **90**: 608-614.

Freeman S, Minz D, Maymon M, Zveibil A (2001). Genetic diversity within *Colletotrichum acutatum sensu* Simmonds. *The American Phytopathological Society* **91** (6): 586-592.

Gharieb MM, Ali MI, El-Shoura AA (2004). Transformation of copper oxychloride fungicide into copper oxalate by tolerant fungi and the effect of nitrogen source on tolerance. *Biodegradation* **15:** 49–57.

Giovanelli LC (2008). Evaluation of an ethanolic extract of propolis as a potential pre- and post-harvest fungicide for 'Fuerte' avocado (*Persea americana* Mill.) fruits and orchards. MSc-Dissertation, University of the Witwatersrand, Johannesburg.

Graef J (1995). World market for avocado. *Regional Agribusiness Project Market Information Bulletin* **10**:1-8.

Gustafson CD (1976). World avocado production. *California Avocado Society Yearbook*: 74-90.

Hartill WFT (1992). Post-harvest rots of avocado in New Zealand and their control. *Brighton Crop Protection Conference*: 1157-1162.

Henson JM, French R (1993). The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology* **31**: 81-109.

Hodson A, Mill PR, Brown AE (1993). Ribosomal and mitochondrial DNA polymorphisms in *Colletotrichum fragariae* in strawberry crowns. *Phytopathology* **58**: 540-541.

Jackson D (1986). Temperate and subtropical fruit production. *Butterworths Horticultural Books*, New Zealand.

Janisiewicz WJ (1998). Biocontrol of post-harvest diseases of temperate fruits. *Plant Microbe Interactions and Biological Control*. *Marcel Dekker Incorporation*, New York: 171-198.

Jeewon R, Liew ECY, Hyde KD (2002). Phylogenetic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA sequences and morphological characters. *Molecular Phylogenetics and Evolution* **25**: 378-392.

Jeffries P, Dodd JC, Jeger MJ, Plumbley RA (1990). The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathology* **39**: 343–366.

Johnson EM, Valleau WD (1949). Synonymy in some species of *Cercospora*. *Phytopathology* **39**: 763–770.

Johnston PR, Jones D (1997). Relationship among *Colletotrichum* isolates from fruit rots assessed using rDNA sequences. *Mycologia* **89**: 420-430.

Jones PJ (1959). Purple stain of soybean seeds incited by several *Cercospora* species. *Phytopathology* **49**: 430–432.

Karni L, Prusky D, Kobiler I, Bar-Shira E, Kobiler D (1989). Involvement of epicatechin in the regulation of lipoxygenase activity during quiescent *Colletotrichum gloeosporioides* infections of ripening avocado fruits. *Physiological and Molecular Plant Pathology* **35**: 367-374.

Kashman Y, N`eeman I, Lifshitx A (1969). New compounds from avocado pear. *Tetrahedron* **25**: 4617-4631.

Kobiler L, Prusky D, Midland S, Sims JJ, Keen NT (1993). Compartmentation of antifungal compounds in oil cells of avocado fruit mesocarp and its effect on susceptibility to *Colletotriohum gloeosporioides*. *Physiological and Molecular Plant Pathology* **43**: 319-328.

Korsten L, Towsen E, Claasens V (1998). Evaluation of Avogreen as a post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. *South African Avocado Growers` Association Yearbook* **21**: 83-87.

Korsten L, Jeffries P (2000). Potential of biological control of diseases caused by *Colletotrichum*. *Commonwealth Agricultural Bureaux*: 266-291.

Kotzè JM (1978). Anthracnose of avocados. *South African Avocado Growers` Association Yearbook*. **2**: 45-47.

Kubo Y, Furusawa I (1991). Melanin biosynthesis: prerequisite for successful invasion of the plant by appressoria of *Colletotrichum* and *Pyricularia*. *In*: *The fungal spore and disease initiation in plants and animals*. *Plenum Publishing*, New York: 205-217.

Kumar M, Shukla PK (2005). Use of PCR targeting of internal transcribed spacer regions and single-stranded conformation polymorphism analysis of sequence variation in different regions of rRNA genes in fungi for rapid diagnosis of mycotic keratitis. *Journal of Clinical Microbiology* **43** (2): 662-668.

Kummuang N, Smith BJ, Diehl SV, Graves CH Jr (1996). Muscadine grape berry rot diseases in Mississippi: disease identification and incidence. *Plant Disease* **80**: 238-243.

Latunde-Dada AO, O`Connell RJ, Nash C, Pring RJ, Lucas JA, Bailey JA (1996). Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum*) from cowpea (*Vigna unguiculata*). *Mycological Research* **100**: 1133–1141.

Latunde-Dada AO, O`Connell RJ, Nash C, Lucas JA (1999). Stomatal penetration of cowpea (*Vigna unguiculata*) leaves by a *Colletotrichum* species causing latent anthracnose. *Plant Pathology* **48**: 777–785.

Legendre L, Rueter S, Heinstrin PF, Low PS (1993). Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glyceine max*) cells. *Plant Physiology* **102**: 233-240.

Leikin-Frenkel A, Prusky D (1998). Ethylene enhances the antifungal lipid content in idioblasts from avocado mesocarp. *Phytochemistry* **49**: 2291-2298.

Lenn`e JM, Parbery DG (1976). Phyllosphere antagonists and appressoria formation in *Colletotrichum gloeosporioides*. *Transactions of the British Mycology Society* **66**: 334-336.

Leroux P, Clerjeau M (1985). Resistance of *Botrytis cinerea* Pers. and *Plasmopara viticola* (Berk. & Curt.) Berl. and de Toni to fungicides in French vineyards. *Crop Protection* **4**: 137-160.

Liu ZM, Kolattukudy PE (1998). Identification of a gene product induced by hard-surface contact of *Colletotrichum gloeosporioides* conidia as a ubiquitin-conjugating enzyme by yeast complementation. *The Journal of Bacteriology* **180**: 3592–3597.

Lonsdale JH (1992a). Evaluation of systemic fungicides as pre-harvest treatments of avocados. *South African Avocado Growers' Association Yearbook* **15**: 35–38.

Lu G, Cannon PF, Reid A, Simmons CM (2004). Diversity and molecular relationships of endophytic *Colletotrichum* isolates from the Iwokrama Forest Reserve, Guyana. *Mycological Research* **32**: 108:53-63.

Lubbe CM, Denman S, Cannon PF, Groenewald JZ, Lamprecht SC, Crous PW (2004). Characterization of *Colletotrichum* species associated with diseases of *Proteaceae*. *Mycologia* **96** (6): 1268-1279.

Lui AR, Xu T, Guo LD (2007). Molecular and morphological description of *Pestalotiopsis hainanensis* sp. nov., a new endophyte from a tropical region of China. *Fungal diversity* **24**: 23-26.

Maas JL, Howard CM (1985). Variation of several anthracnose fungi in virulence to strawberry and apple. *Plant Disease* **69**: 164–166.

Madi L, Wang X, Kobiler I, Lichter A, Prusky D (2003). Stress on avocado fruits regulates Δ^9-stearoyl ACP desaturase expression, fatty acid composition, antifungal diene level and resistance to *Colletotrichum gloeosporioides* attack. *Physiological and Molecular Plant Pathology* **62**: 277–283.

Manandhar JB, Hartman GL, Wang TC (1995). Anthracnose development on pepper fruits inoculated with *Colletotrichum gloeosporioides*. *Plant Disease* **79**: 380–383.

Mancini N, Perotti M, Ossi CM, Cavallero A, Matuka S, Paganoni G, Burioni R, Rama P, Clementi M (2006). Rapid molecular identification of fungal pathogens in corneal samples from suscepted keratomycosis cases. *Journal of Medical Microbiology* **55**: 1505-1509.

Manicom BQ (2001). Diseases. *In*: *The cultivation of avocado*. *Institute of Tropical and Subtropical Crops,* Nelspruit, South Africa: 214-230.

Manners JM, Masel AM, Braithwaite KS, Irwin JAG (1992). Molecular analysis of *Colletotrichum gloeosporioides* pathogenic on the tropical pasture legume *Stylosanthes* spp. *In*: *Colletotrichum, Biology, Pathology and Control*. *CAB International*, Wallingford, UK: 250–268.

Martín MP, García-Figueres F (1999). *Colletotrichum acutatum* and *C. gloeosporioides* cause anthracnose on olives. *European Journal of Plant Pathology* **105**: 733–741.

Masaba D, Waller JM (1992). Coffee Berry Disease: The current status. *CAB International*, Wallingford, UK: 237–249.

Mendgen K, Deising H (1993). Infection structures of fungal plant pathogens-a cytological and physiological evaluation. *New Phytologist* **124**: 193–213.

Michelmore RW, Hulbert SH (1987). Molecular markers for the genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology* **25**: 383-404.

Millar AA, Kunst L (1997). Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal* **12**: 121-131.

Mitchell JI, Roberts PJ, Moss ST (1995). Sequence or structure? A short review on the application of nucleic acid sequence information to fungal taxonomy. *Mycologist* **9**: 67-75.

Moriwaki J, Tsukiboshi T, Sato T (2002). Grouping of *Colletotrichum* species in Japan based on r DNA Sequences. *Journal of General Plant Pathology* **68**: 307-320.

Moriwaki J, Sato T, Tsukiboshi T (2003). Morphological and molecular characterization of *Colletotrichum boninense* sp. nov. from Japan. *Mycoscience* **44**: 47-53.

Nakasone HY, Paull RE (1998a). Avocado. *In*: *Tropical fruits*. *CAB International*, Wellington: 76-102.

O`Connell RJ, Bailey JA, Richmond DV (1985). Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. *Physiological Plant Pathology* **27**: 75–98.

O`Connell RJ, Perfect S, Hughes B, Carzaniga R, Bailey J, Green J (2000). Dissecting the cell biology of *Colletotrichum* infection processes. *In*: *Host Specificity, Pathology and Host-Pathogen Interactions*. *APS Press*, St. Paul, Minnesota, USA: 57–77.

O`Donnell KO, Cigelnik E, Nirenberg HI (1998). Molecular systematics and phyleogeography of the *Gibberella fujikoroi* species complex. *Mycologia* **90**: 465-493.

Palhano FL, Vilches TTB, Santos RB, Orlando MTD, Ventura JA, Fernandes PMB (2004). Inactivation of *Colletotrichum gloeosporioides* spores by high hydrostatic pressure combined with citral or lemongrass essential oil. *International Journal of Food Microbiology* **95**: 61– 66.

Palmateer AJ, Ploetz RC, Harmon PF (2006). Florida Plant Disease Management Guide: Avocado (*Persea americana*). University of Florida, IFAS Extension: 1-10.

Parbery DG (1981). Biology of anthracnose of leaf surfaces. *In*: *Microbial ecology of the Phylloplane*. *Academic Press*, London, UK: 135-154.

Pegg KG, Coates LM (1993). *In*: *Diseases of Fruit Crops*. Department of Primary Industries, Queensland: 20-24.

Peres N, Kuramae E, Dias M, Souza N (2002b). Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil. *Journal of Phytopathology*. **150**: 128-134.

Peterson RA (1978). Susceptibility of 'Fuerte' avocado fruit at various stages of growth to infection by anthracnose and stem end rot fungi. *Australian Journal of Experimental Agriculture and Animal Husbandry* **18**: 158-160.

Photita W, Taylor PWJ, Ford R, Lumyong P, McKenzie HC, Hyde KD (2005). Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Diversity* **18**: 117-133.

Piret NL (1997). Economic and environmental aspects of producing copper chemicals. *Journal of the Minerals, Metals and Materials Society* **49** (10): 33.

Ploetz RC (1994a). Introduction. *In*: *Compendium of tropical fruit diseases*. *APS Press*, St Paul, Minnesota: 1.

Ploetz RC (1994b). Part III. Mango: Anthracnose. *In*: *Compendium of tropical fruit diseases*. *APS Press*, St Paul, Minnesota: 35–36.

Ploetz RC (2003). Diseases of Tropical Fruit Crops. *CAB International*. Wallingford: 1-71.

Podila GK, Rogers LM, Kolattukudy PE (1993). Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. *Plant Physiology* **103**: 267-272.

Pohronezny KI, Simone GW, Kotze JM (1994). *Pseudocircospora* spot (blotch). *In*: *Compendium of tropical fruit diseases*. *The American Phytophathological Society*, St. Paul, Minnesota: 81.

Pohronezny KL, Simone GW, Kotzè JM (1998). *Pseudocercospora* spot (Blotch). *In*: *Compendium of tropical fruit diseases*. *APS Press*, St. Paul, Minnesota, USA: 79-80.

Prasad V, Semwogerere D, Weeks ER (2007). Confocal microscopy of colloids. *Journal of Physics*: *Condensed Matter* **19** (11): 1-25.

Pring RJ, Nash C, Zakaria M, Bailey JA (1995). Infection process and host range of *Colletotrichum capsici*. *Physiological and Molecular Plant Pathology* **46**: 137–152.

Prusky D, Keen NT, Sims JJ, Midland SL (1982). Possible involvement of an antifungal compound in latency of *Colletotrichum gloeosporioides* after harvest of unripe avocado fruits. *Phytopathology* **72**: 1578-1582.

Prusky D, Keen NT, Eaks I (1983). Further evidence for the involvement of a preformed antifungal compound in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Physiological and Molecular Plant Pathology* **22** (2): 189-198.

Prusky D, Ascarelli A, Jacoby B (1984). Lack of involvement of nutrients in the latency of *Colletotrichum gloeosporioides* in unripe avocado fruits. *Phytopathologische Zeitschrift* **110**: 106-109.

Prusky D, Kobiler I, Jacoby B (1988). Involvement of epicatechin in cultivar susceptibility of avocado fruits to *Colletotrichum gloeosporioides* after harvest. *Phytopathologische Zeitschrift* **123**: 140-146.

Prusky D, Gold S, Keen NT (1989). Purification of an endopolygalacturonase produced by *Colletotrichum gloeosporioides*. *Physiological and Molecular Plant Pathology* **35**: 367-374.

Prusky D, Karni L, Kobiler I, Plumbley RA (1990). Induction of the antifungal diene in unripe avocado fruits: Effect of inoculation with *Colletotrichum gloeosporioides*. *Physiological and Molecular Plant Pathology* **37**: 425-435.

Prusky D, Plumbley RA, Kobiler I (1991). The relationship between antifungal diene levels and fungal inhibition during quiescent infection of *Colletotrichum gloeosporioides* in unripe avocado fruits. *Plant Pathology* **40**: 45-52.

Prusky D, Plumbley RA (1992). Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. *In*: *Colletotrichum*: *Biology, Pathology and Control*. *CAB International*, Wallingford, UK: 289–307.

Prusky D, Keen NT, Eaks I (1993). Further evidence for the involvement of preformed antifungal compounds in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Physiological Plant Pathology* **22**: 189198.

Prusky D, Keen NT (1993). Involvement of preformed antifungal compounds in the resistance of subtropical fruits to fungal decay. *Plant Disease* **77**: 115-119.

Prusky D, Keen NT (1995). Inducible preformed compounds and their involvement in the resistance of plants to pathogens. *In*: *Novel Approaches to Integrated Pest Management*. Boca Raton, Lewis: 139–52.

Prusky D (1996). Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology* **34**: 413–434.

Prusky D, Ardi R, Koblier I, Beno-Moalem D, Leikin A (1998). Mechanism of resistance of avocado fruits to *Colletotrichum gloeosporioides* attack. *Australian Centre for International Agricultural Research Proceedings* **80**: 63–71.

Prusky D, Kobiler I, Ardi R, Beno-Moalem D, Yakoby N, Keen NT (2000). Resistance mechanisms of subtropical fruits to *Colletotrichum gloeosporioides*. *In*: *Colletotrichum*: *Host Specificity*, *Pathology and Host-Pathogen Interaction*. *The American Phytopathological Society*, St. Paul, Minnesota: 232-244.

Quimio TH, Quimio AJ (1975). Notes of Philippine grape and guava anthracnose. *Plant Disease Reporter* **59**: 221–224.

Raeder U, Broda P (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17-20.

Ratanacherdchai K, Wang HK, Lin FC, Soytong K (2007). RAPD analysis of *Colletotrichum* species causing chilli anthracnose disease in Thailand. *Journal of Agricultural Technology* **3** (2): 211-219.

Rivero RM, Ruiz JM, Garcia PC, Lopez-Lefebre LR, Sanches E, Romero L (2002). Response of oxidative metabolism in watermelon plants subjected to cold stress. *Functional Plant Biology* **29**: 643-648.

Rodriguez-Saona C, Millar JG, Trumble JT (1997). Growth inhibitory, insecticidal, and feeding deterrent effects of (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,-15-diene, a compound from avocado fruit, to *Spodoptera exigua*. *Journal of Chemical Ecology* **23** (7): 1819.

Sanabria A (2007). Identification of *Colletotrichum* species causing anthracnose on Tahiti lime, tree tomato and mango. MSc-thesis.Institute of Biotechnology, Faculty of Sciences, Universidad Nacional de Colombia, Bogota.

Sanders GM, Korsten L, Wehner FC (2000). Survey of fungicide sensitivity in *Colletotrichum gloeosporioides* from different avocado and mango production areas in South Africa. *European Journal of Plant Pathology* **106**: 745–752.

Sanders GM, Korsten L (2003). Comparison of cross inoculation potential of South African avocado and mango isolates of *Colletotrichum gloeosporioides*. *Microbiological Research* **158**: 143-150.

Sanders GM, Korsten L (2003). A comparative morphological study of South African avocado and mango isolates of *Colletotrichum gloeosporioides*. *Canadian Journal of Botany* **81** (8): 877–885.

Shanklin J, Somerville C (1992). Stearoyl-acyl-carrier desaturase from higher plants is structurally unrelated to the animal and fungal homologs. *Proceedings of the National Academy of Sciences USA* **88**: 2410–54.

Sherriff C, Whelan MJ, Arnold GM, Lafay JF, Bry-goo Y, Bailey JA (1994). Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. *Experimental Mycology* **18**: 121-138.

Shi Y, Correll JC, Guerber JC, Rom CR (1996). Frequency of *Colletotrichum* species causing bitter rot of apple in Southeastern United States. *Plant Disease* **80**: 692-696.

Siboe GM (1989). Morphological and biochemical inter-relationships among isolates of *Cercospora* from Kenya. PhD-thesis, University of Reading, UK.

Siboe GM, Braggins JE (1994). A proposed species concept in the genus *Cercospora* Fres. with reference to Kenyan isolates. *In*: *Proceedings of the Thirteenth Plenary Meeting of AETFAT* **1**. *National Herbarium and Botanic Gardens of Malawi*, Malawi: 627–634.

Siboe GM, Murray J, Kirk PM (2000). Genetic similarity among *Cercospora apii*-group species and their detection in host plant tissue by PCR/RFLP analyses of the rDNA internal transcribed spacer (ITS). *Journal of General and Applied Microbiology* **46**: 69–78.

Simmonds JH (1965). A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. *Queensland Journal of Agricultural and Animal Sciences* **22**: 457-459.

Singh RD, Prasad N, Mathur RL (1966). On the taxonomy of the fungus causing anthracnose of *Dioscorea alata* L. *Indian*. *Phytopathology* **19**: 65-71.

Skipp RA, Beever RE, Sharrock KR, Rikkerink EHA, Templeton MD (1995). *Colletotrichum In*: *Pathogenesis and Host-parasite Specificity in Plant Diseases*: *Histological*, *Biochemical*, *Genetic and Molecular Basis* **2**. *Elsevier Science Limited* (*Pergamon Press*), Oxford, UK: 119–143.

Snowdon AL (1990). Miscellaneous tropical and subtropical fruits. *In*: *A colour atlas of post-harvest diseases and disorders of fruits and vegetables* **1**. *General Introduction and fruits*. *Wolfe Scientific*, London: 92-93.

Sreenivasaprasad S, Brown AE, Mills PR (1993). Coffee brown disease pathogen in Africa: genetic structure and relationship to the group species *Colletotrichum gloeosporioides*. *Mycological Research* **97**: 995-1000.

Sreenivasaprasad S, Mills PR, Brown AE (1994). Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. *Mycological Research* **98** (2): 186–188.

Sreenivasaprasad S, Mills PR, Meehan BM, Brown AE (1996). Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. *Genome* **39**: 499-512.

Sreenivasaprasad S, Talhinhas P (2005). Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Molecular Plant Pathology* **6** (4): 361–378.

Stevens HE (1920). Some diseases of the avocado and other subtropical fruits. *Florida State Horticultural Society Proceedings* **33**:77-91.

Strahan D (2008). Green fuel for the airline industry. *New Scientist* **119** (2669): 34–37.

Sutton BC (1992). The genus *Glomerella* and its anamorph *Colletotrichum*. *In*: *Colletotrichum*: *Biology, Pathology and Control*. *CAB International*, Wallingford: 1–26.

Suzuki E (2002). High-resolution scanning electron microscopy of immunogold-labelled cells by the use of thin plasma coating of osmium. *Journal of Microscopy* **208** (3): 153–157.

Swarup J, Chauhan LS, Tripathi RC (1966). Two new *Phomopsis* spp. from India. *Mycopathologia* **28** (4): 1-3.

Swinburne TR (1983). Quiescent infections in post-harvest diseases. Postharvest pathology of fruits and vegetables. *Academic Press*, London: 1-21.

Talbot NJ (1999). Forcible entry. *Science* **285**: 1860–1861.

Tapia-Tussell R, Quijano-Ramayo A, Cortes-Velazquez A, Lappe P, Larque-Saavedra A, Perez-Brito D (2008). PCR-based detection and characterization of the fungal pathogens *Colletotrichum gloeosporioides* and *Colletotrichum capsici* causing anthracnose in papaya (*Carica papaya* L.) in the Yucatan Peninsula. *Molecular Biotechnology* **40**: 293–298.

Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kird JC, Knauf VC (1991). Primary structures of precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. *Proceedings of the National Academy of Sciences USA* **88**: 2578–82.

Thottappilly G, Mignouna HD, Onasanya A, Abang MM, Oyelakin O, Singh NK (1999). Identification and differentiation of isolates of *Colletotrichum gloeosporioides* from yam by random amplified polymorphic DNA markers. *African Crop Science Journal* **7**: 195-205.

Timmer LW, Agostini JP, Zitko SE, Zulfiquar M (1994). Postbloom fruit drop, an increasingly prevalent disease of citrus in the Americas. *Plant Disease* **78**: 329–334.

Timmer L, Brown GE, Zitko SE (1998). The role of *Colletotrichum* spp. in post-harvest anthracnose of citrus and survival of *C. acutatum* on fruit. *Plant Disease* **82**: 415-418.

Turu T (1969). Avocados south of the border. *California Avocado Society Yearbook* **70**: 31-37.

Uddin W, Stevenson KL, Pardo-Schultheiss RA (1997). Pathogenicity of a species of *Phomopsis* causing a shoot blight on peach in Georgia and evaluation of possible infection courts. *Plant Disease* **81**:983-989.

Ushiyama K, Aono N, Kita N, Ogawa J (1996). First report of pestalotia disease, anthracnose and angular leaf spot of kiwi fruit and their pathogens in Japan. *Annals of the Phytopathological Society of Japan* **62**: 61-68.

Van Zyl JA, Ferreira SG (1995). An overview of the avocado industry in South Africa. *South African Avocado Growers` Association Yearbook* **18**: 23-30.

Verhoeff K (1974). Latent infections by fungi. *Annual Review of Phytopathology* **12**: 99-110.

von Arx JA (1957). The species of the genus *Colletotrichum* Cda. *Phytopathology* **29**: 413-468.

von Arx JA (1970). A revision of the fungi classified as *Gloeosporium*. *Bibliography* of *Systematic Mycology* **24**: 1-203.

Waller JM (1972). Water-borne spore dispersal in coffee berry disease and its relation to control. *Annals of Applied Biology* **71**: 1-18.

Waller JM (1988). New developments in integrated disease management of tropical plantation crops. *Proceedings of the Brighton Crop Protection Conference - Pests and Diseases*: 297-305.

Waller JM (1992). *Colletotrichum* diseases of perennial and other cash crops. *The American Phytopathological Society*: 167-185.

Wang X, Lichter A, Kobiler I, Leikin-Frenkel A, Prusky D (2004). Expression of Δ^{12} fatty acid desaturase during the induced accumulation of the antifungal diene involved in resistance to *C. gloeosporioides* in avocado fruits. *Molecular Plant Pathology* **5**: 575-585.

Ward JMJ, Laing MD, Rijkenberg FHJ (1997). Frequency and timing of fungicide applications for the control of gray leaf spot in maize. *Plant Disease* **81**: 41-48.

Watson DG, Brooks CJW (1984). Formation of capsidiol in *Capsicum annuum* fruits in response to non-specific elicitor. *Physiological Plant Pathology* **24**: 331-337.

Wharton PS, Julian AM (1996). A cytological study of compatible and incompatible interactions between *Sorghum bicolor* and *Colletotrichum sublineolum*. *New Phytologist* **134**: 25–34.

Wharton PS, Diegue`z-Uribeondo J (2004). The biology of *Colletotrichum acutatum*. *Anales del Jardin Botanico de Madrid* **61**: 3-22.

Wheeler HE (1954). Genetics and evolution of heterothallism in *Glomerella*. *Journal of Phytopathology* **44**: 342.

Whiley AW (1991). *Persea americana* Miller. *In*: *Edible fruits and nuts*. *Plant Resources of South-East Asia* **2**. *PROSEA, Pudoc*, Wageningen, Netherlands: 249-254.

Whiley AW (2002). Crop management. *In*: *Avocado*: *Botany, Production and Uses*. *CABI Publishing*, Oxon, UK: 231-258.

Whitelaw-Weckert MA, Curtin SJ, Huang R, Steel CC, Blanchard CL, Roffey PE (2007). Phylogenetic relationships and pathogenicity of *Colletotrichum acutatum* isolates from grape in subtropical Australia. *Plant Pathology* **56**: 448–463.

Wilson CJ (1997). Biocontrol of aerial plant diseases in agriculture and horticulture: current approaches and future prospects. *Journal of Industrial Microbiology and Biotechnology* **19**: 188-191.

Winch JE, Newhook FJ, Jackson GVH, Cole JS (1984). Studies of *Colletotrichum gloeosporioides* disease on yam, *Dioscorea alata* in Solomon Islands. *Plant Pathology* **33**: 467-477.

Zentmyer GA (1953). Diseases of the avocado. *Yearbook of Agriculture*: 875-881.

Zentmyer GA (1994). Part IV. Avocado. *In*: *Compendium of tropical fruit diseases*. *APS Press*, St Paul, Minnesota: 71-84.

Internet sources:

Fintrac Inc., www.marketasia.org 1998 (last accessed on the 30 November 2009).

http://www.ipm.ucdavis.edu/ (last accessed on the 21 April 2008).

http://www.na.fs.fed.us/spfo/pubs/fidls/phomopsis/phomopsis.htm (last accessed on the 20 July 2010).

http://www.pesticideinfo.org (last accessed on the 29 November 2010).

http://www.westfalia.co.za (last accessed on the 20 July 2010).

Appendix 1: Sequence alignment of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the three CgP strains. Hyphens (-) indicate similar bases. Species names represented as accession numbers indicate that the sequences were obtained from the GenBank.

```
CgP sp. PCR product     ....................................AGG    3
CgP sp. PCR product 2..............................acggagtAGG      10
CgP sp. PCR product 3 ......................................      0
AF001014              aaagtcgtaacaaggtctccgttggtgaaccagcggagGG     47
AY485742             ....................................agGG      4
AY620999              aaagtcgtaacaaggtctccgttggtgaaccagcggagGG     47
AY745085              ................................ggagGG      6
DQ286286              ..................................GG      2
DQ286287              ..................................GG      2
DQ780430              ........................ttttaaaccgggAGG     15
DQ780461              aaagtcgtaacaaggtctccgttggtgaaccagcggagGG     47
EU002917              ..................cgttggtgaaccagcggagGG     21
EU002918              ....tcgtaacaaggtctccgttggtgaaccagcggagGG     36
EU002925              ..................cgttggtgaaccagcggagGG     21
EU814480              ..................................agGG      4
EU977226              ..................................gagGG      5
EU977307              ..............................tgcggagGG      9
EU977308              ..................................gGG      3
EU977312              ............................acctgcggagGG     12
FJ440701              ..................................agGG      4
FJ441631              ......gtaacaaggtctccgttggtgaaccagcggagGG     34
FJ643573              aaagtcgtaacaaggtctccgttggtgaaccagcggagGG    240
FJ884148              ............................ccagcggagGG     11
Consensus
```

Appendix 2: Sequence alignment of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the *Pseudocercospora* strain. Hyphens (-) indicate similar bases. Species names represented as accession numbers indicate that the sequences were obtained from the GenBank.

```
Pseudocercospora sp. PCR product .....................................0
AF362055                         .....................................0
AF362056                         .....................................0
AF362057                         .....................................0
AY752153                         .....................................0
AY752154                         .....................................0
DQ073923                       gagtacaatttaaatcccttaacgaggaacaattggaggg480
DQ184477                         .....................................0
DQ303076                         .....................................0
DQ303082                         .....................................0
DQ303083                         .....................................0
DQ303087                         .....................................0
DQ676519                         .....................................0
EF394858                         .....................................0
EF394859                         .....................................0
EF535685                         .....................................0
EF535721                         .....................................0
EU255889                         .....................................0
EU726632                         .....................................0
EU882129                         .....................................0
EU882130                         .....................................0
Consensus
```

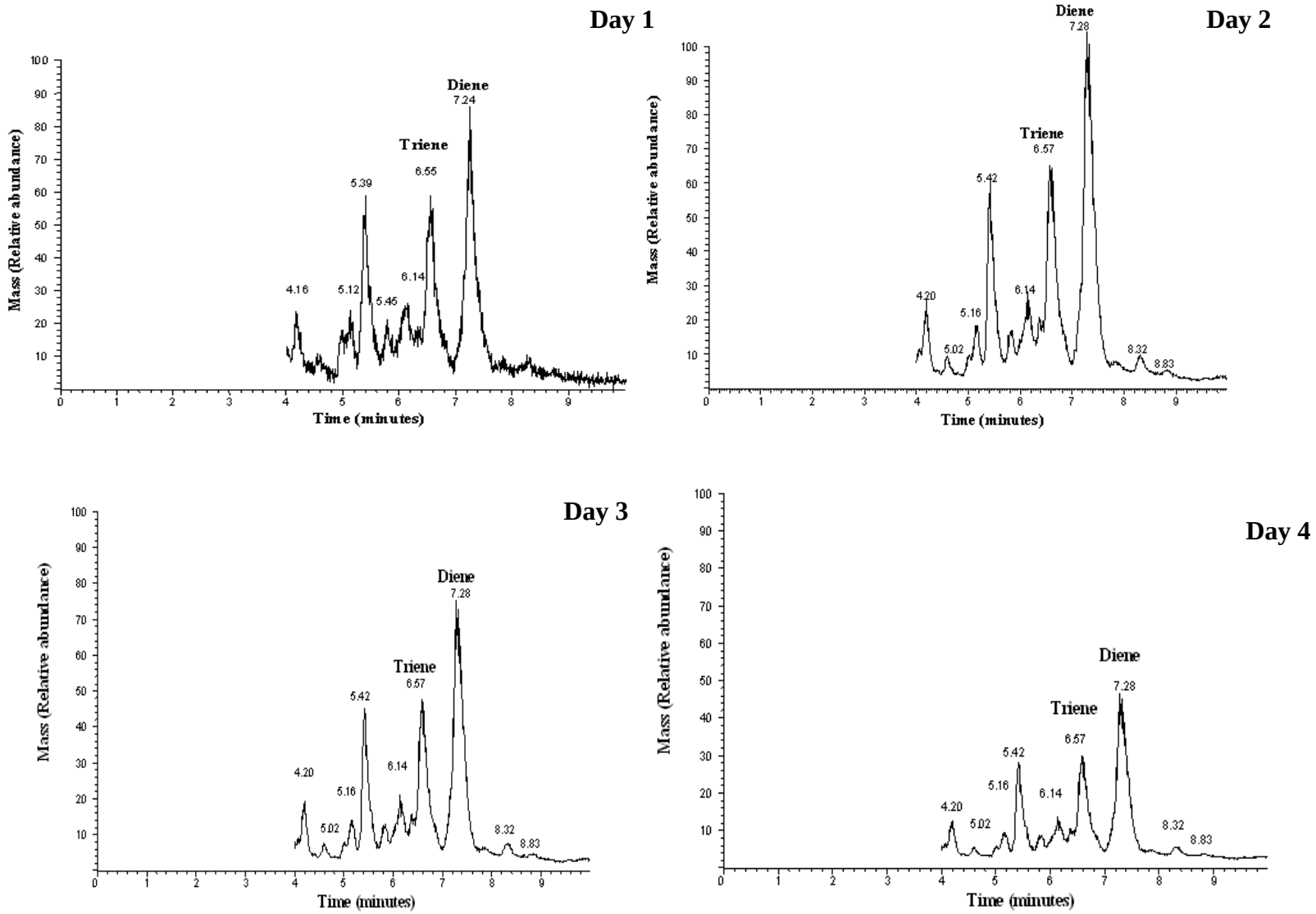
Appendix 3: Sequence alignment of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the *Colletotrichum* strain. Hyphens (-) indicate similar bases. Species names represented as accession numbers indicate that the sequences were obtained from the GenBank.

```
Colletotrichum sp. PCR product.....................................  0
AB051406                          .....................................  0
AB269938                          .....................................  0
AY266377                          .....................................  0
EU008834                          .....................................  0
EU054411                          .....................................  0
EU520087                          .....................................  0
EU734584                          .....................................  0
EU734585                          .....................................  0
EU734586                          .....................................  0
EU822803                          taccgattgaatggctcagtgaggctttcggattgactca 80
EU847425                          .....................................  0
FJ232906                          .....................................  0
FJ449913                          .....................................  0
FJ449914                          .....................................  0
FJ459924                          .....................................  0
FJ459928                          .....................................  0
FJ904824                          .....................................  0
FJ904825                          .....................................  0
FJ904831                          .....................................  0
FJ940901                          .....................................  0
Consensus
```

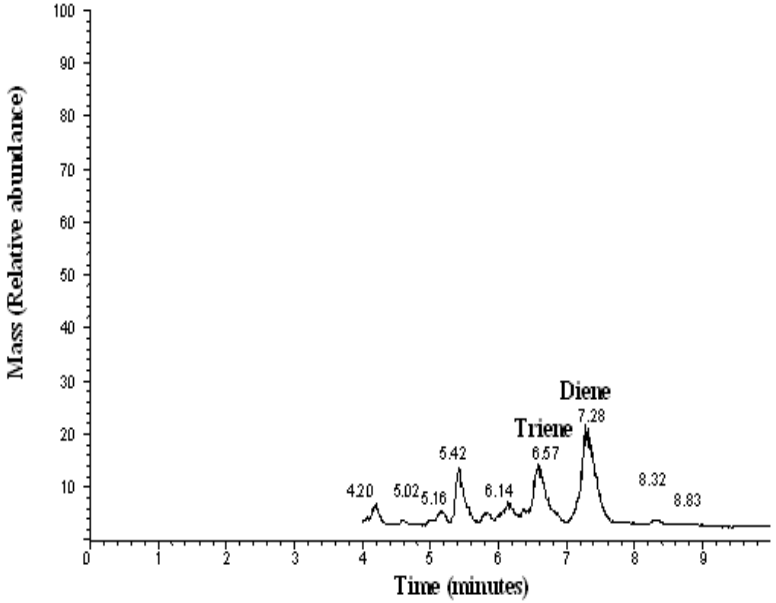
Appendix 4: Sequence alignment of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions of the three *Phomopsis* strains. Hyphens (-) indicate similar bases. Species names represented as accession numbers indicate that the sequences were obtained from the GenBank.

```
Phomopsis sp. PCR product      ..................................... 0
Phomopsis sp. PCR product 2    ..................................... 0
Phomopsis sp. PCR product 3    ..................................... 0
AF001014                    ggaagtaaaagtcgtaacaaggtctccgttggtgaaccag   40
GU967697                    ......aaaagtcgtaacaaggtctccgttggtgaaccag   34
AF230750                    .....................................      0
AF230762                    .....................................      0
AY620999                    ggaagtaaaagtcgtaacaaggtctccgttggtgaaccag   40
EU814480                    .....................................      0
FJ440701                    .....................................      0
GQ250192                    .....................................      0
GQ250193                    .....................................      0
GQ250194                    .....................................      0
GQ250195                    .....................................      0
GQ250196                    .....................................      0
GQ250197                    .....................................      0
GQ250198                    .....................................      0
GQ281798                    ......................tccgtaggtgaacctg   16
GQ281808                    ......................tccgtaggtgaacctg   16
GQ281809                    ......................tccgtaggtgaacctg   16
GQ281796                    ......................tccgtaggtgaacctg   16
DQ286286                    .....................................      0
GQ281807                    ......................tccgtaggtgaacctg   16
Consensus
```

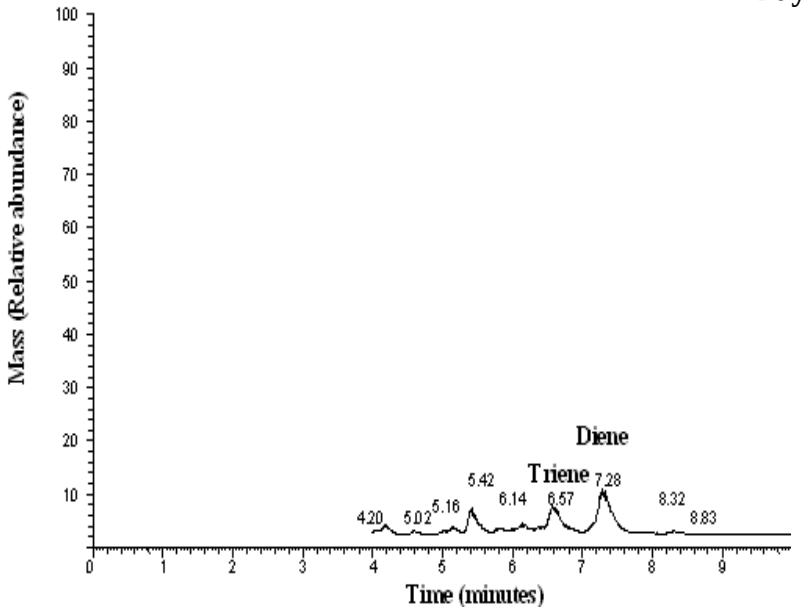
Appendix 5: HPLC separation of harvested flesh crude extracts from 1 to 7 d.



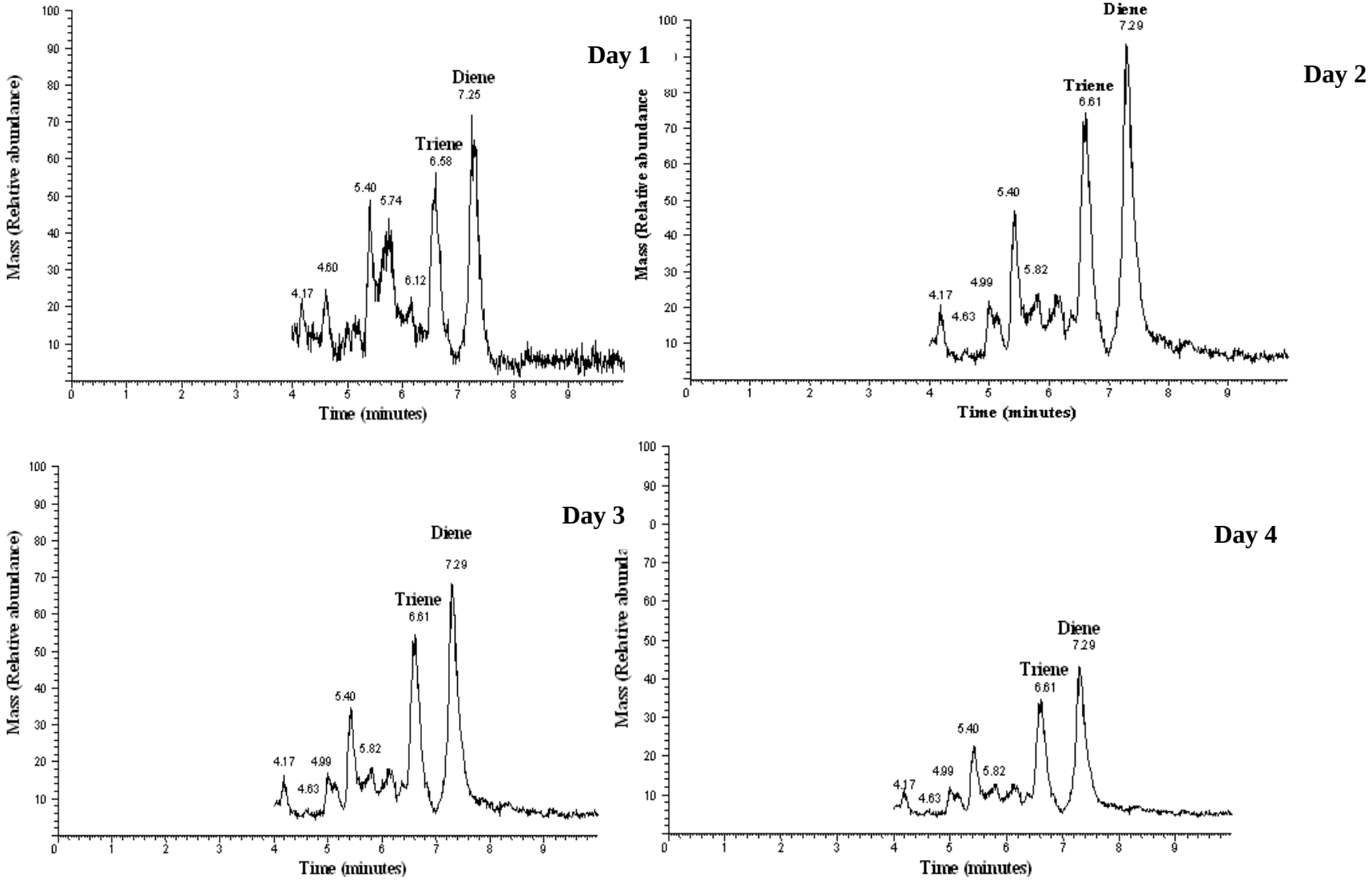
Day 5

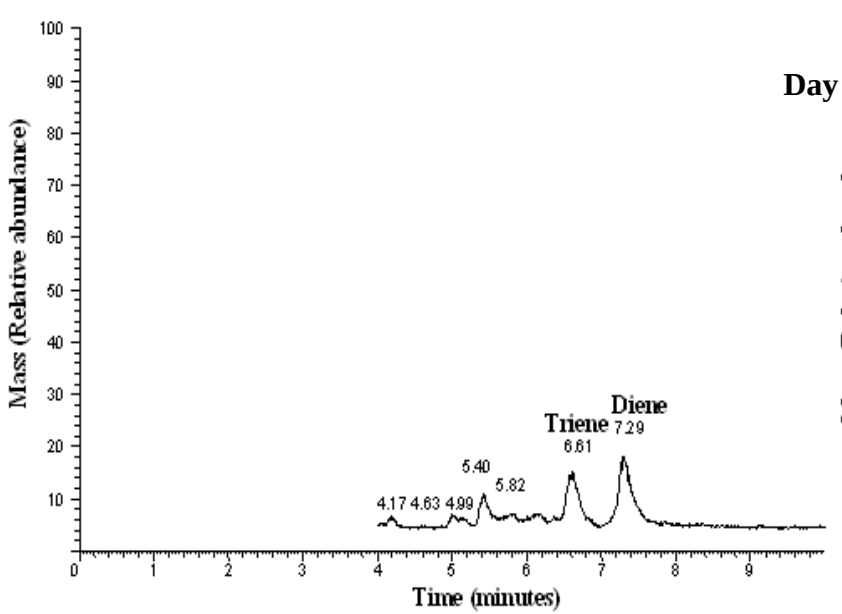

Mass (Relative abundance)
Time (minutes)
Diene
Triene
7.28
6.57
5.42
6.14
5.16
5.02
4.20
8.32
8.83

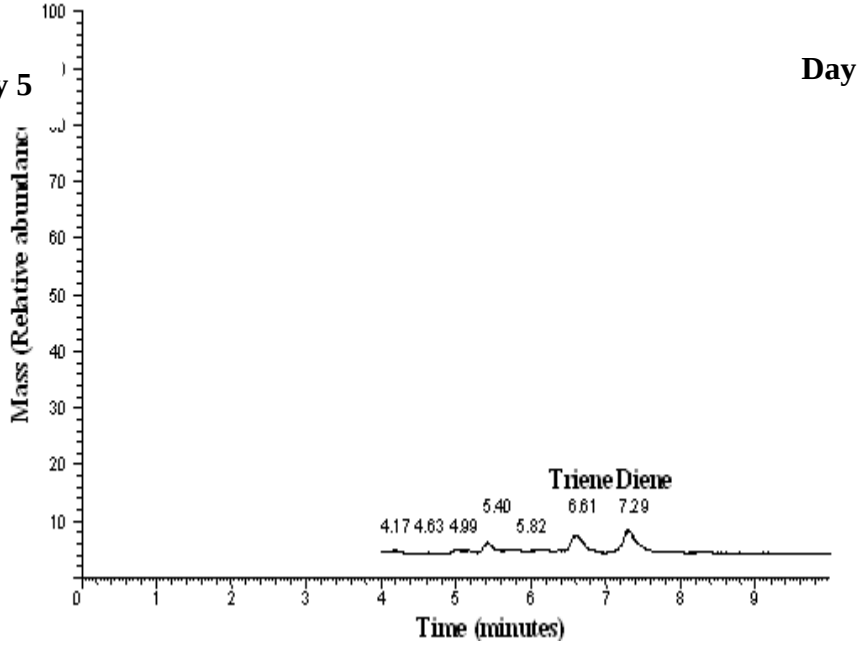
Day 7


Mass (Relative abundance)
Time (minutes)
Diene
Triene
7.28
6.57
5.42
6.14
5.16
5.02
4.20
8.32
8.83

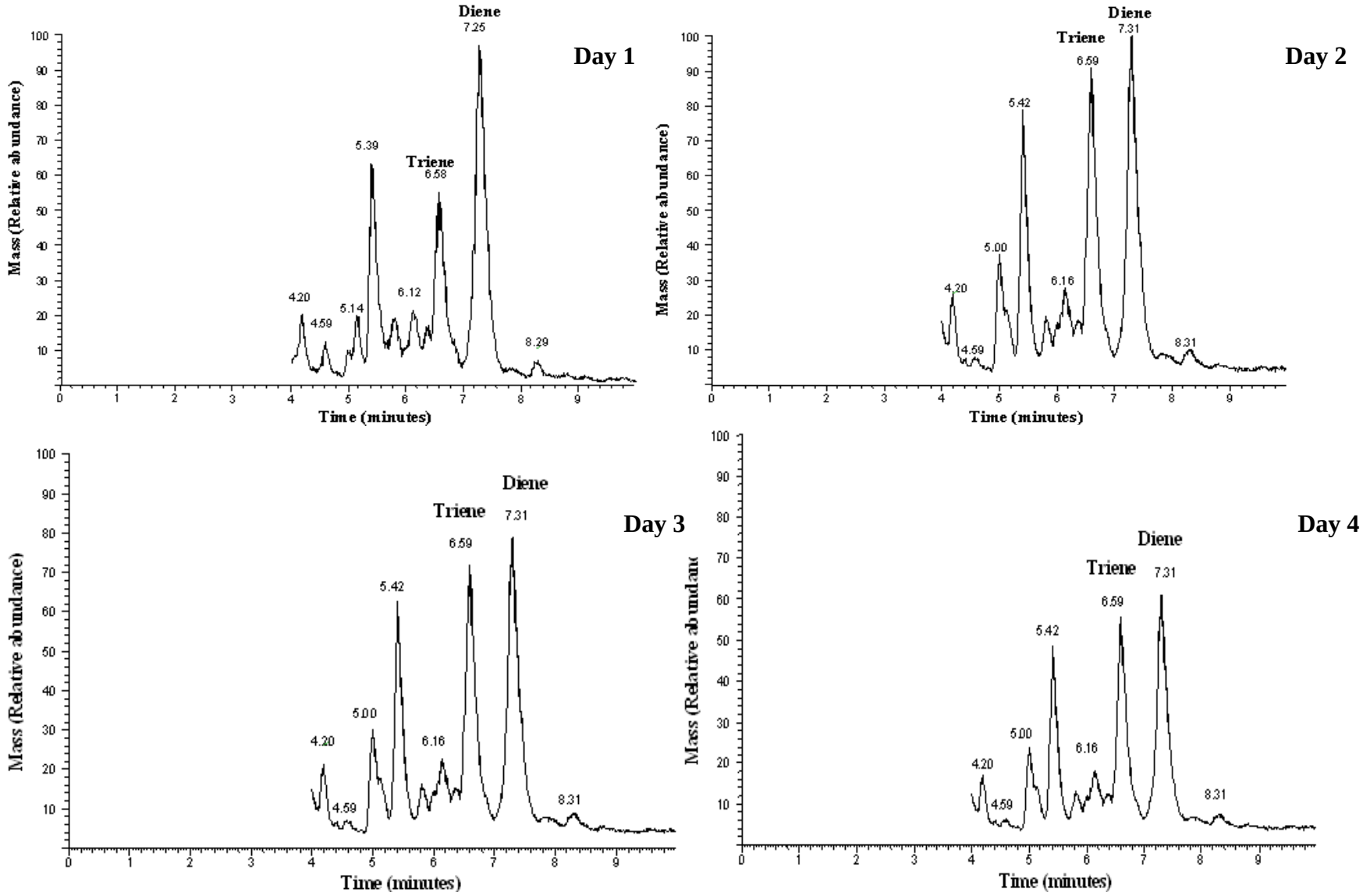
Appendix 6: HPLC separation of harvested peel crude extracts from 1 to 7 d.

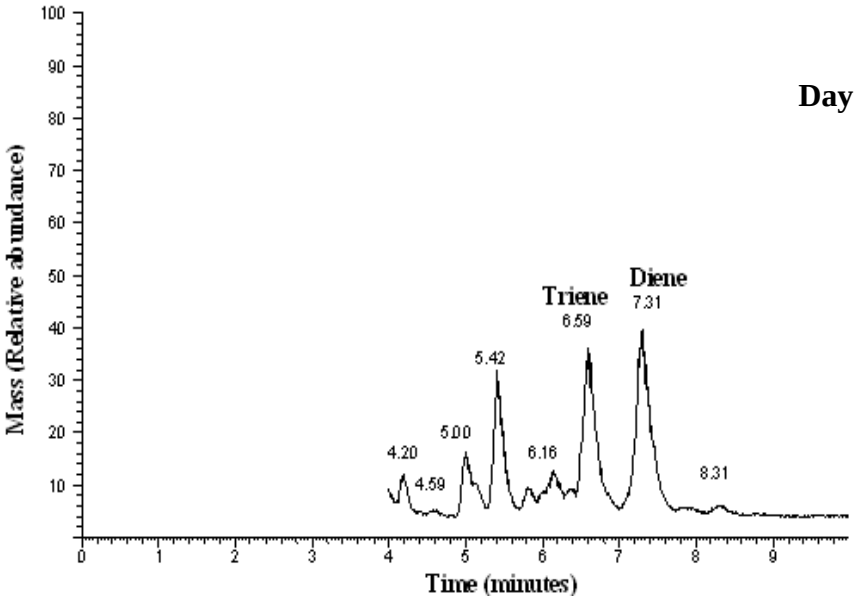


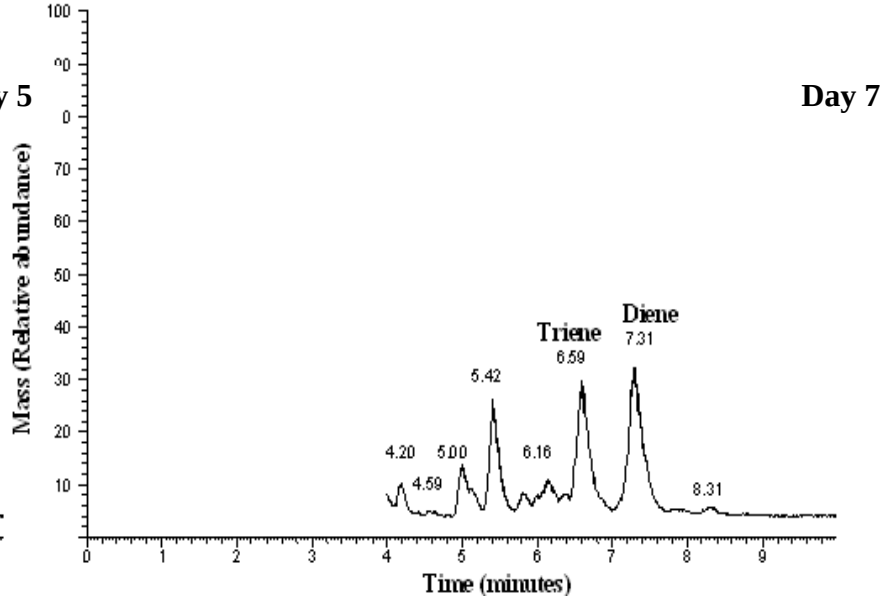

Day 5
Mass (Relative abundance)
Time (minutes)
Diene
Triene
7.29
6.61
5.40
5.82
4.17 4.63 4.99


Day 7
Mass (Relative abundance)
Time (minutes)
Triene Diene
5.40
6.61
7.29
5.82
4.17 4.63 4.99

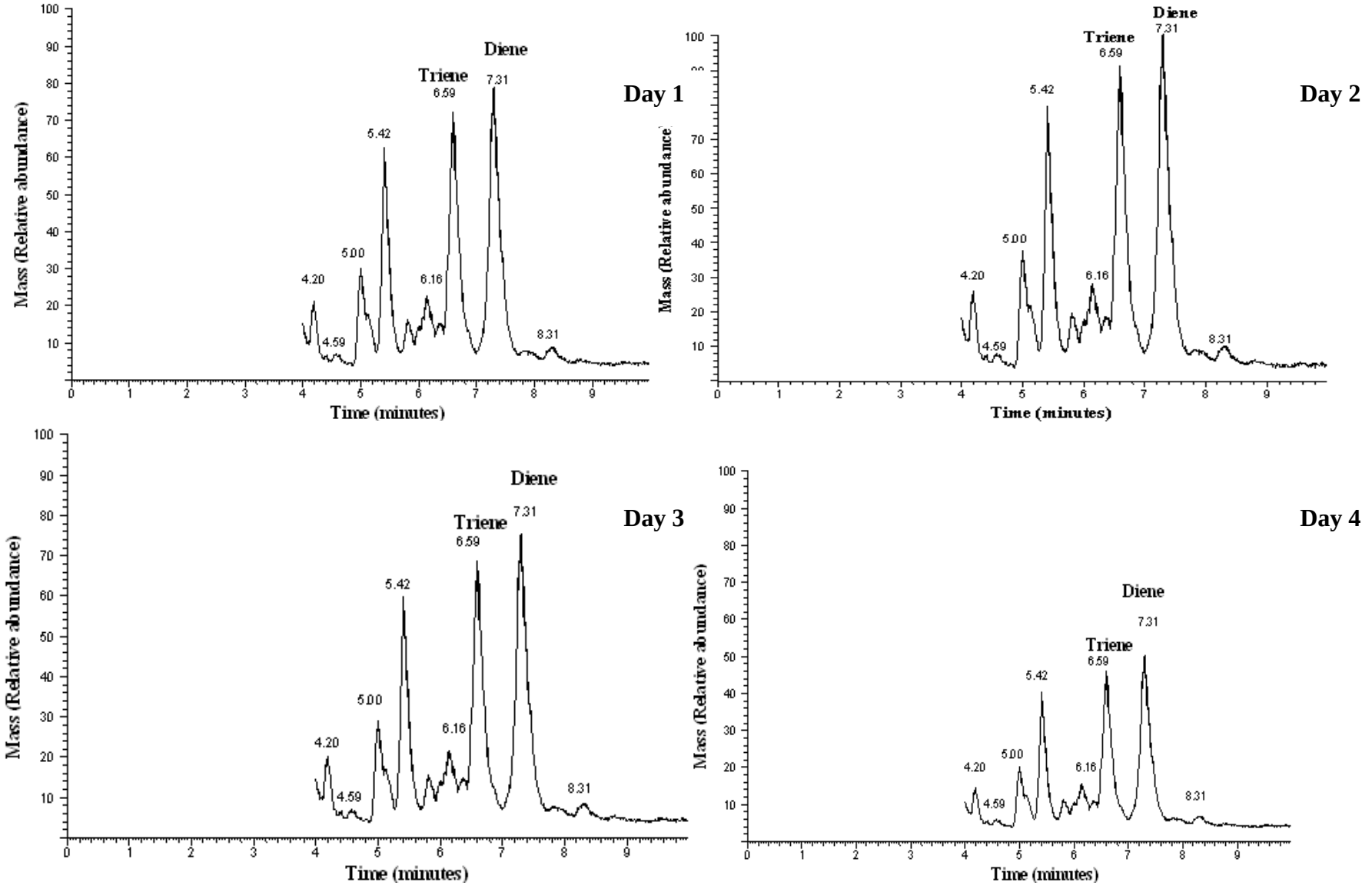
Appendix 7: HPLC separation of unharvested flesh crude extracts from 1 to 7 d.

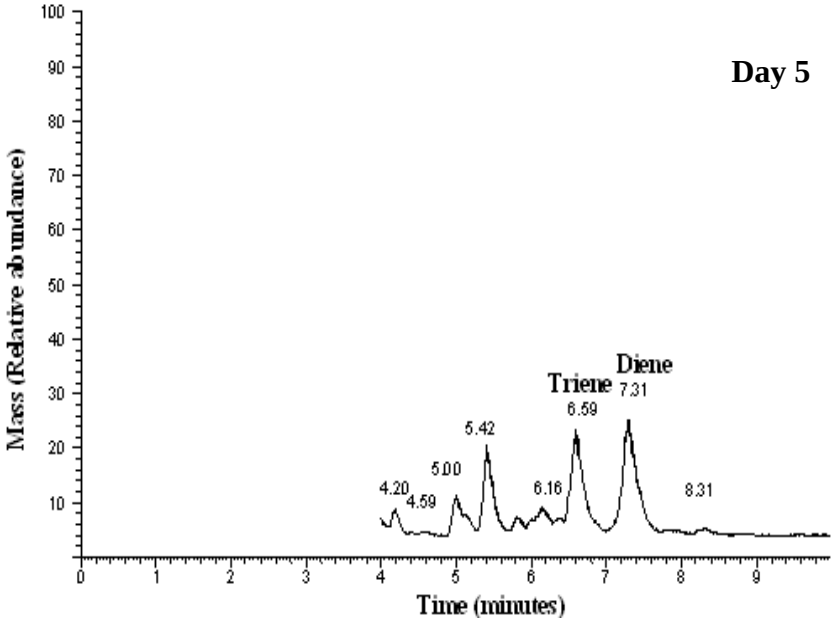


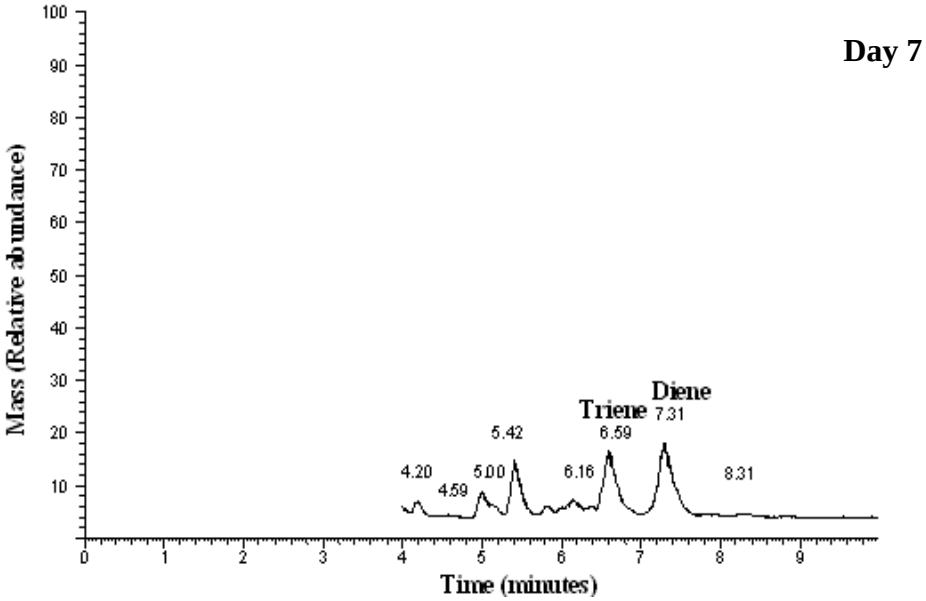

Day 5
Mass (Relative abundance)
Time (minutes)
Triene
6.59
Diene
7.31
5.42
5.00
4.20
4.59
6.16
8.31


Day 7
Mass (Relative abundance)
Time (minutes)
Triene
6.59
Diene
7.31
5.42
4.20
5.00
4.59
6.16
8.31

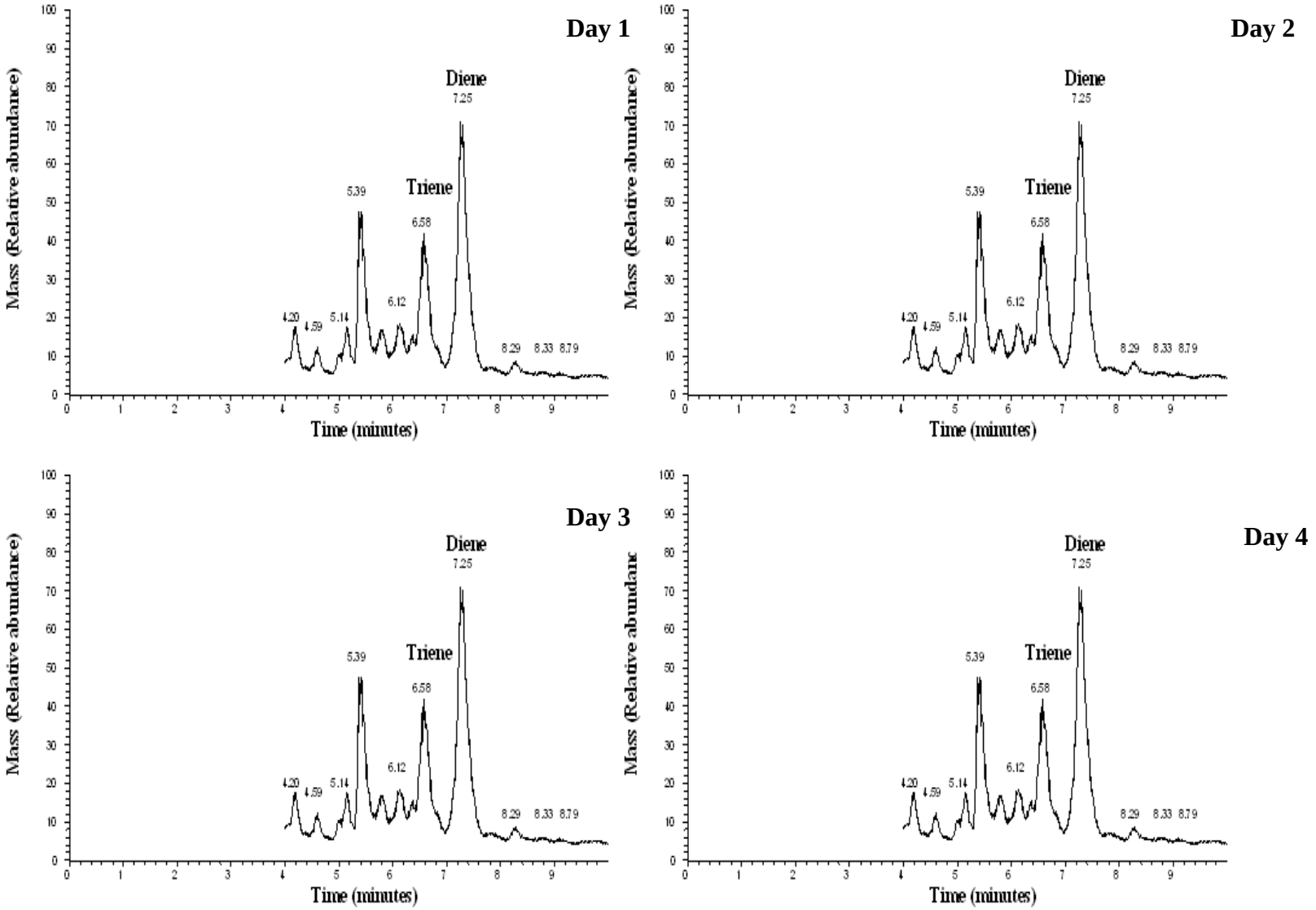
Appendix 8: HPLC separation of unharvested peel crude extracts from 1 to 7 d.



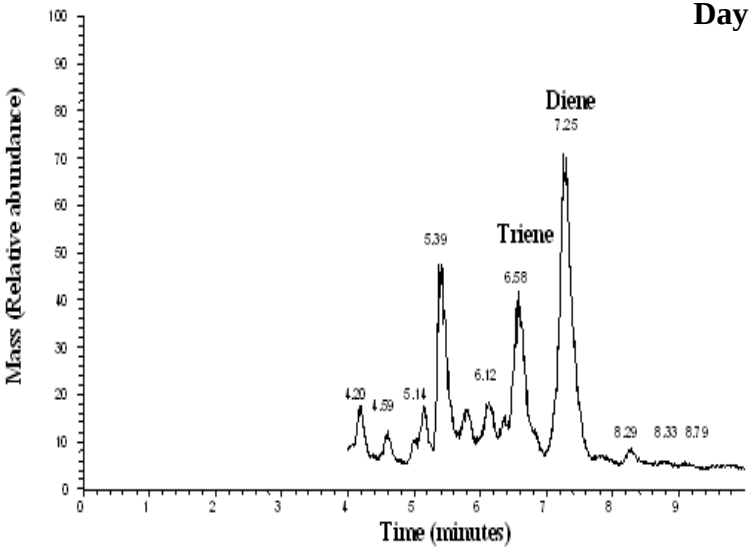

Day 5
Mass (Relative abundance)
Time (minutes)
Diene
Triene
7.31
6.59
5.42
5.00
4.20
4.59
6.16
8.31


Day 7
Mass (Relative abundance)
Time (minutes)
Diene
Triene
7.31
6.59
5.42
4.20
4.59
5.00
6.16
8.31

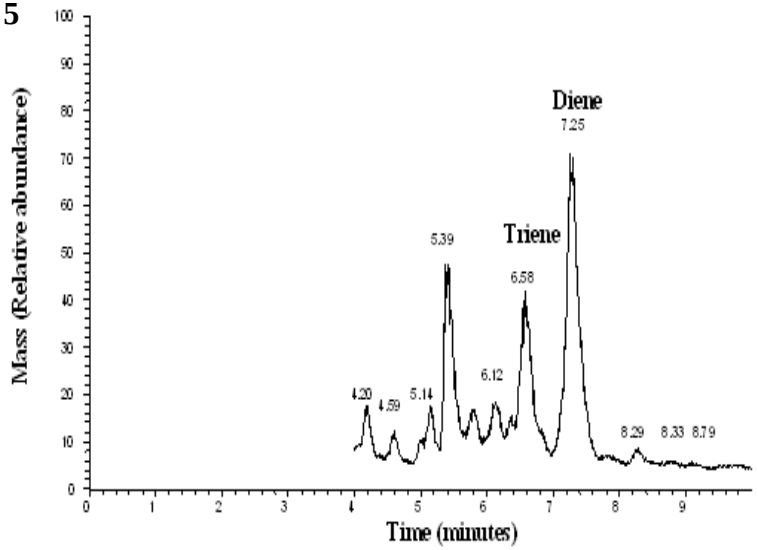
Appendix 9: HPLC separation of uninoculated harvested flesh crude extracts from 1 to 7 d.



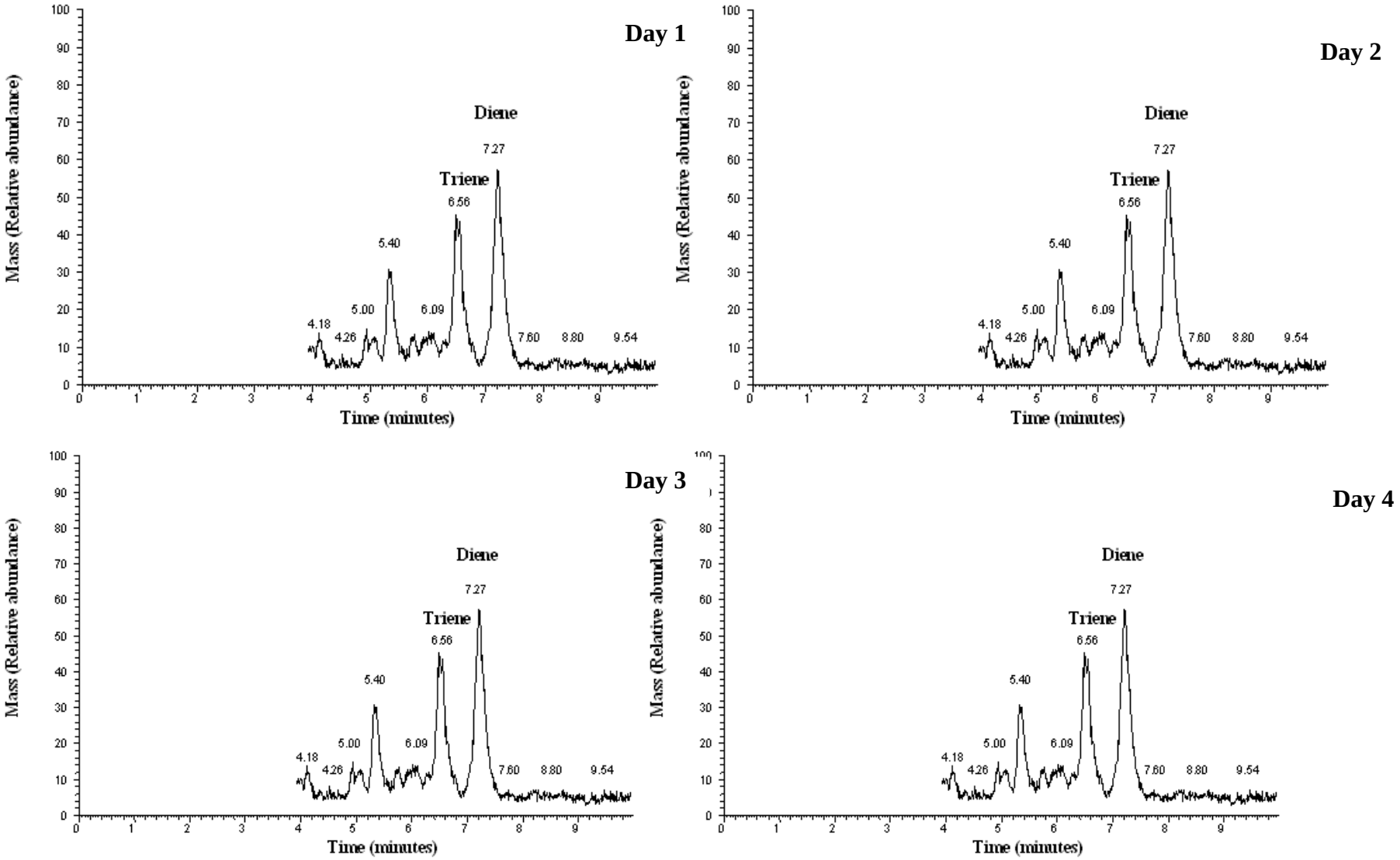
Day 5

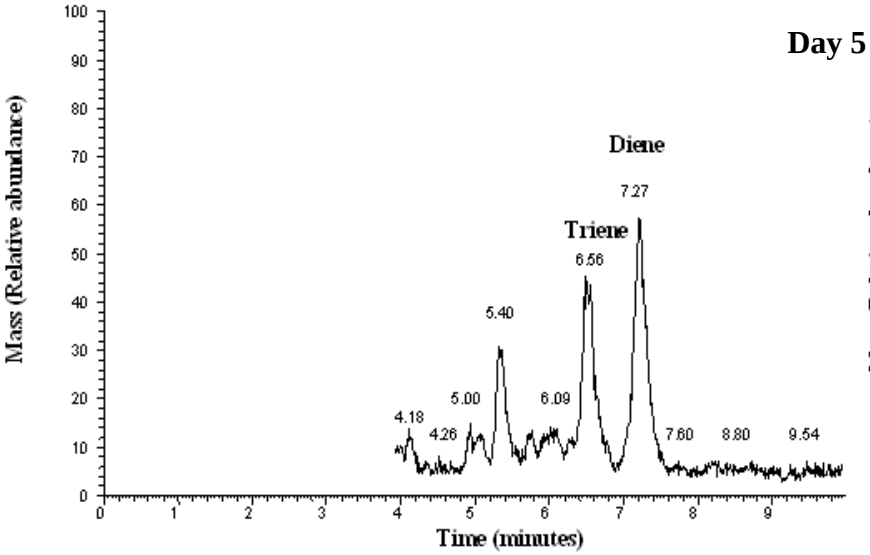

Mass (Relative abundance)
Time (minutes)
Diene
7.25
Triene
6.58
5.39
6.12
5.14
4.59
4.20
8.29
8.33
8.79

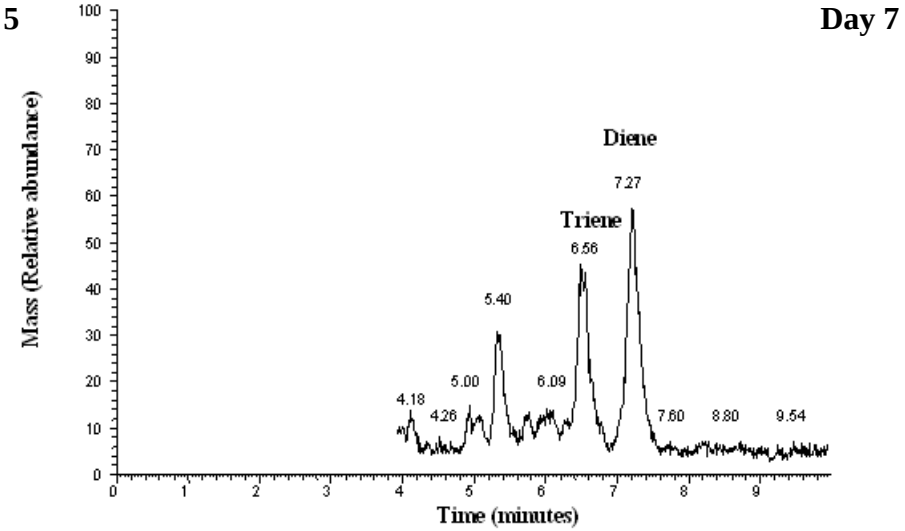
Day 7


Mass (Relative abundance)
Time (minutes)
Diene
7.25
Triene
6.58
5.39
6.12
5.14
4.59
4.20
8.29
8.33
8.79

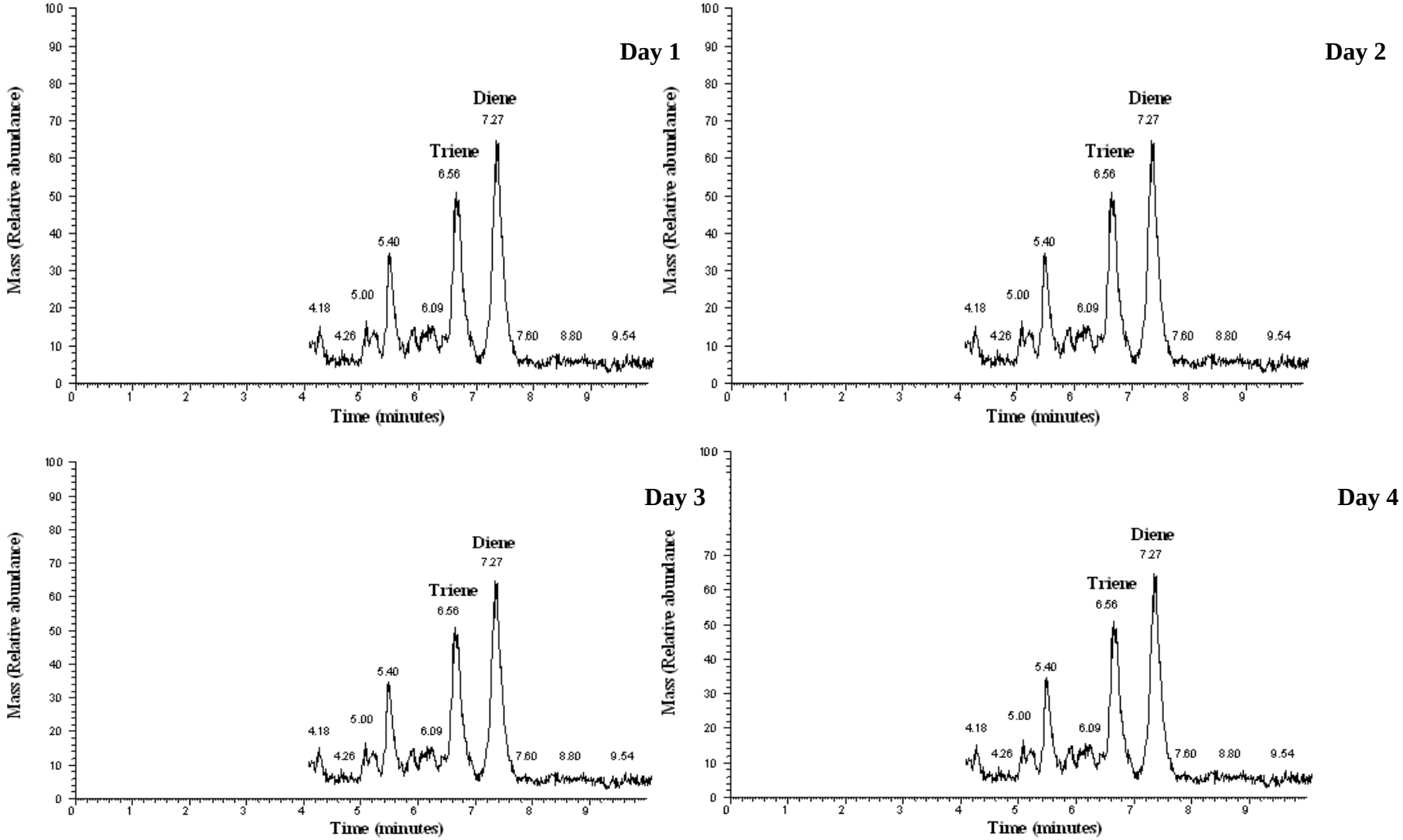
Appendix 10: HPLC separation of uninoculated harvested peel crude extracts from 1 to 7 d.

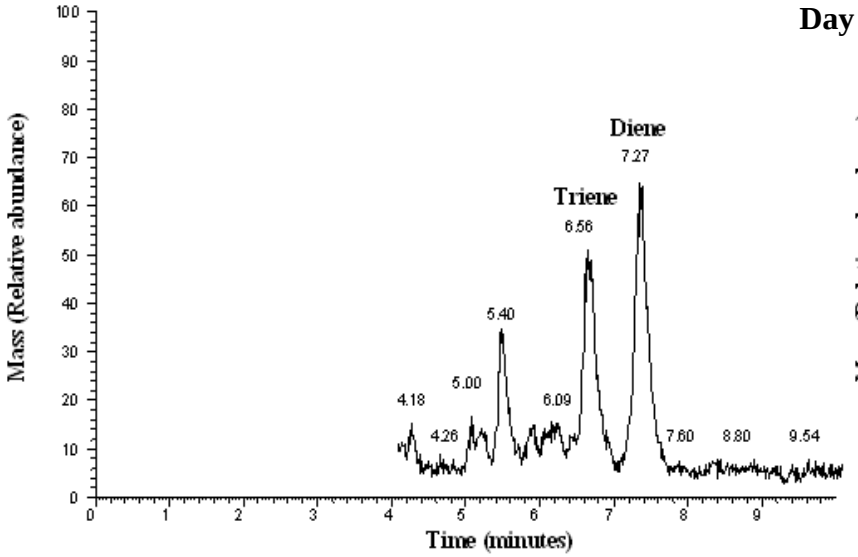


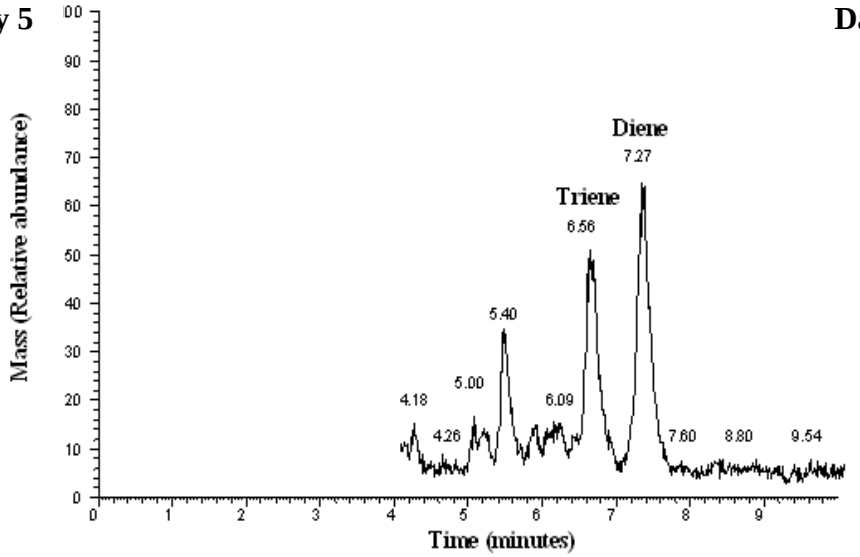

Day 5
Mass (Relative abundance)
Time (minutes)
Diene
Triene
4.18
4.26
5.00
5.40
6.09
6.56
7.27
7.60
8.80
9.54


Day 7
Mass (Relative abundance)
Time (minutes)
Diene
Triene
4.18
4.26
5.00
5.40
6.09
6.56
7.27
7.60
8.80
9.54

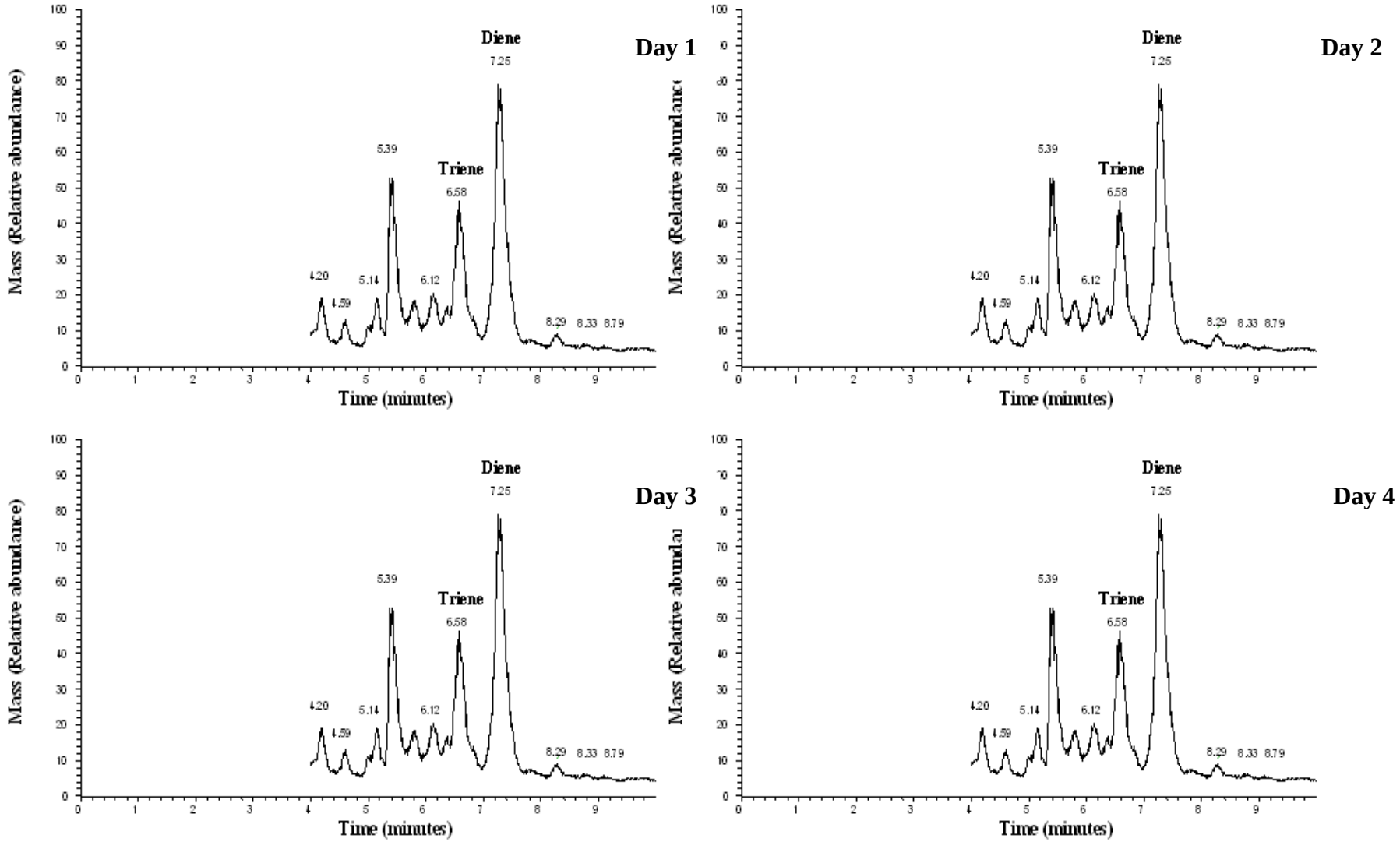
Appendix 11: HPLC separation of uninoculated unharvested flesh crude extracts from 1 to 7 d.

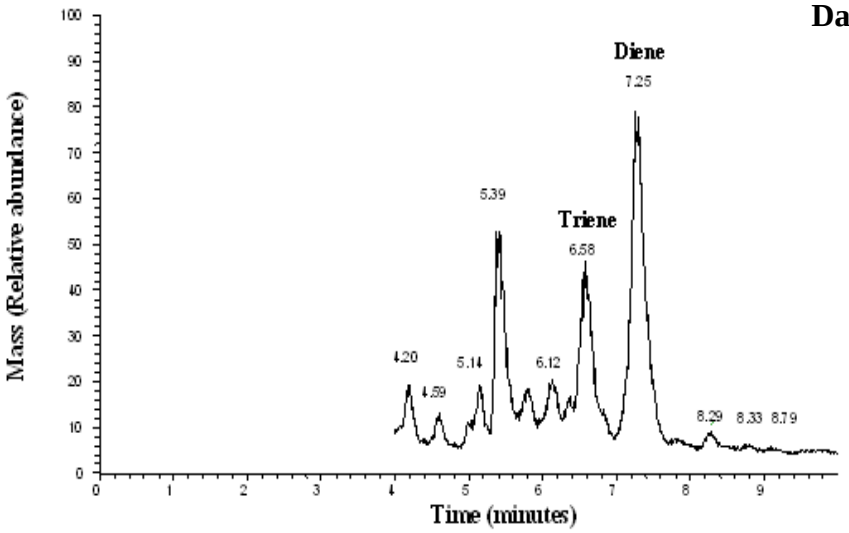


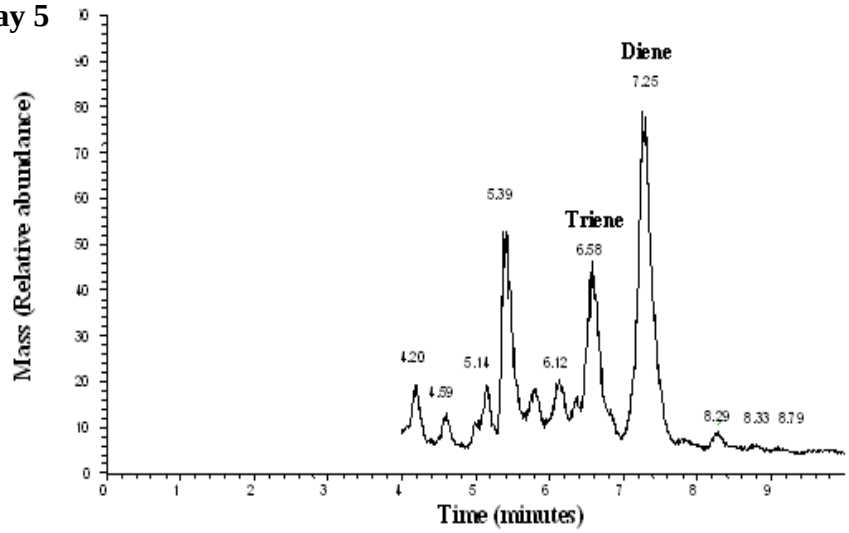

Day 5
Mass (Relative abundance)
Time (minutes)
Diene
7.27
Triene
6.56
5.40
5.00
4.18
4.26
6.09
7.60
8.80
9.54


Day 7
Mass (Relative abundance)
Time (minutes)
Diene
7.27
Triene
6.56
5.40
5.00
4.18
4.26
6.09
7.60
8.80
9.54

Appendix 12: HPLC separation of uninoculated unharvested peel crude extracts from 1 to 7 d.




Day 5
Diene
7.25
Triene
6.58
5.39
4.20
4.59
5.14
6.12
8.29
8.33
8.79
Mass (Relative abundance)
Time (minutes)


Day 7
Diene
7.25
Triene
6.58
5.39
4.20
4.59
5.14
6.12
8.29
8.33
8.79
Mass (Relative abundance)
Time (minutes)

Diene study:

Appendix 13. Statistical significance between the experimental and control samples of harvested and unharvested crude extracts (relative peak heights in mm), using One-Way and Post ANOVA Tests. Values were expressed as mean ± standard error of mean (SEM) using SYSTAT 12 software. *p* values less than 0.05 were considered as significantly different.

Harvested fruits:

Day 1:

Comparison	**Mean difference**	***q***	***p* value**
Flesh vs peel	3.640	9.298	*** $p<0.001$
Flesh vs flesh control	7.820	19.976	*** $p<0.001$
Flesh vs peel control	9.240	23.603	*** $p<0.001$
Peel vs flesh control	4.180	10.678	*** $p<0.001$
Peel vs peel control	5.600	14.305	*** $p<0.001$
Flesh control vs peel control	1.420	3.627	ns $p>0.05$

Day 2

Comparison	**Mean difference**	***q***	***p* value**
Flesh vs peel	3.600	8.272	*** $p<0.001$
Flesh vs flesh control	11.310	25.988	*** $p<0.001$
Flesh vs peel control	12.400	28.493	*** $p<0.001$
Peel vs flesh control	7.710	17.716	*** $p<0.001$
Peel vs peel control	8.800	20.221	*** $p<0.001$
Flesh control vs peel control	1.090	2.505	ns $p>0.05$

Day 3

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	3.910	6.348	*** $p<0.001$
Flesh vs flesh control	9.065	14.717	*** $p<0.001$
Flesh vs peel control	10.160	16.494	*** $p<0.001$
Peel vs flesh control	5.155	8.369	*** $p<0.001$
Peel vs peel control	6.250	10.147	*** $p<0.001$
Flesh control vs peel control	1.095	1.778	ns $p>0.05$

Day 4

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	2.076	1.958	ns $p>0.05$
Flesh vs flesh control	6.055	5.768	ns $p>0.05$
Flesh vs peel control	7.140	16.494	*** $p<0.001$
Peel vs flesh control	5.196	6.772	ns $p>0.05$
Peel vs peel control	3.984	3.785	ns $p>0.05$
Flesh control vs peel control	5.085	1.037	ns $p>0.05$

Unharvested fruits:

Day 1

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	10.240	3.931	* $p<0.05$
Flesh vs flesh control	24.275	9.318	*** $p<0.001$
Flesh vs peel control	21.980	8.437	*** $p<0.001$
Peel vs flesh control	14.035	5.388	** $p<0.01$
Peel vs peel control	11.740	4.507	* $p<0.05$
Flesh control vs peel control	-2.295	0.881	ns $p>0.05$

Day 2

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	10.877	3.585	*** $p<0.001$
Flesh vs flesh control	26.584	8.622	*** $p<0.001$
Flesh vs peel control	25.359	8.224	*** $p<0.001$
Peel vs flesh control	15.745	5.106	** $p<0.01$
Peel vs peel control	14.520	4.709	* $p<0.05$
Flesh control vs peel control	-1.225	0.397	ns $p>0.05$

Day 3

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	9.065	7.187	*** $p<0.001$
Flesh vs flesh control	17.320	13.732	*** $p<0.001$
Flesh vs peel control	15.375	12.190	*** $p<0.001$
Peel vs flesh control	8.225	6.545	*** $p<0.001$
Peel vs peel control	6.310	5.003	** $p<0.01$
Flesh control vs peel control	-1.945	1.542	ns $p>0.05$

Day 4

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	3.529	5.723	** $p<0.01$
Flesh vs flesh control	9.364	15.620	*** $p<0.001$
Flesh vs peel control	8.299	13.46	*** $p<0.001$
Peel vs flesh control	8.225	9.901	*** $p<0.001$
Peel vs peel control	6.111	7.722	ns $p>0.05$
Flesh control vs peel control	-1.335	2.165	ns $p>0.05$

p values < 0.01 were considered to be significantly different, *p* values < 0.0001 were considered to be extremely different, *q* values > 3.813 indicated that $p<0.05$.

Triene study:

Appendix 14. Statistical significance between the experimental and control samples of harvested and unharvested crude extracts (relative peak heights in mm), using One-Way and Post ANOVA Tests. Values were expressed as mean ± standard error of mean (SEM) using SYSTAT 12 software. *p* values less than 0.05 were considered as significantly different.

Harvested fruits:

Day 1

Comparison	**Mean difference**	***q***	***p* value**
Flesh vs peel	-2.945	2.542	ns $p>0.05$
Flesh vs flesh control	3.095	2.778	ns $p>0.05$
Flesh vs peel control	3.076	2.958	ns $p>0.05$
Peel vs flesh control	5.055	4.768	ns $p>0.05$
Peel vs peel control	4.196	5.772	ns $p>0.05$
Flesh control vs peel control	2.984	2.785	ns $p>0.05$

Day 2

Comparison	**Mean difference**	***q***	***p* value**
Flesh vs peel	4.640	10.298	***$p<0.001$
Flesh vs flesh control	13.035	4.388	**$p>0.01$
Flesh vs peel control	14.745	4.106	**$p>0.01$
Peel vs flesh control	10.877	3.585	***$p<0.001$
Peel vs peel control	24.359	9.224	***$p<0.001$
Flesh control vs peel control	-1.235	2.055	ns $p>0.05$

Day 3

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	-4.945	4.542	ns $p>0.05$
Flesh vs flesh control	5.100	6.711	ns $p>0.05$
Flesh vs peel control	-2.335	3.165	ns $p>0.05$
Peel vs flesh control	-1.226	0.398	ns $p>0.05$
Peel vs peel control	-2.294	0.880	ns $p>0.05$
Flesh control vs peel control	2.074	1.956	ns $p>0.05$

Day 4

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	4.640	10.298	ns $p>0.05$
Flesh vs flesh control	5.820	17.976	***$p<0.001$
Flesh vs peel control	7.240	21.603	***$p<0.001$
Peel vs flesh control	5.180	11.678	***$p<0.001$
Peel vs peel control	6.600	15.305	***$p<0.001$
Flesh control vs peel control	3.420	5.627	ns $p>0.05$

Day 5

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	1.000	0.339	ns $p>0.05$
Flesh vs flesh control	-16.000	5.425	**$p>0.01$
Flesh vs peel control	-19.000	6.442	**$p>0.01$
Peel vs flesh control	-17.000	5.764	**$p>0.01$
Peel vs peel control	-20.000	6.781	**$p>0.01$
Flesh control vs peel control	-3.000	1.017	ns $p>0.05$

Day 7

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	3.000	1.125	ns $p>0.05$
Flesh vs flesh control	-20.000	7.519	*** $p<0.001$
Flesh vs peel control	-25.000	9.339	*** $p<0.001$
Peel vs flesh control	-23.000	8.647	*** $p<0.001$
Peel vs peel control	-28.000	10.527	*** $p<0.001$
Flesh control vs peel control	-5.000	1.880	ns $p>0.05$

Unharvested fruits:

Day 1

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	0.420	2.627	ns $p>0.05$
Flesh vs flesh control	0.090	1.505	ns $p>0.05$
Flesh vs peel control	3.095	3.778	ns $p>0.05$
Peel vs flesh control	4.076	3.958	ns $p>0.05$
Peel vs peel control	7.196	8.772	ns $p>0.05$
Flesh control vs peel control	6.085	2.037	ns $p>0.05$

Day 2

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	0.000	0.000	ns $p>0.05$
Flesh vs flesh control	25.000	7.996	*** $p<0.001$
Flesh vs peel control	9.000	2.879	ns $p>0.05$
Peel vs flesh control	25.000	7.996	*** $p<0.001$
Peel vs peel control	9.000	2.879	ns $p>0.05$
Flesh control vs peel control	-16.000	5.181	* $p<0.05$

Day 3

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	-1.295	1.881	ns $p>0.05$
Flesh vs flesh control	9.877	4.585	*** $p<0.001$
Flesh vs peel control	24.584	10.622	ns $p>0.05$
Peel vs flesh control	22.359	5.224	*** $p<0.001$
Peel vs peel control	-1.355	2.175	ns $p>0.05$
Flesh control vs peel control	6.225	7.901	*** $p<0.001$

Day 4

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	14.320	10.732	*** $p<0.001$
Flesh vs flesh control	7.065	5.187	*** $p<0.001$
Flesh vs peel control	-2.000	0.669	ns $p>0.05$
Peel vs flesh control	0.000	0.000	ns $p>0.05$
Peel vs peel control	9.877	2.585	*** $p<0.001$
Flesh control vs peel control	-11.000	3.682	*** $p<0.001$

Day 5

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	8.000	3.096	ns $p>0.05$
Flesh vs flesh control	-6.000	2.322	ns $p>0.05$
Flesh vs peel control	-17.000	6.580	** $p<0.01$
Peel vs flesh control	-14.000	5.419	** $p<0.01$
Peel vs peel control	-25.000	9.676	*** $p<0.001$
Flesh control vs peel control	-11.000	4.258	* $p<0.05$

Day 7

Comparison	Mean difference	*q*	*p* value
Flesh vs peel	10.000	5.106	* $p<0.05$
Flesh vs flesh control	-10.800	5.515	** $p<0.01$
Flesh vs peel control	-21.000	10.724	*** $p<0.001$
Peel vs flesh control	-20.800	10.621	*** $p<0.001$
Peel vs peel control	-31.000	15.830	*** $p<0.001$
Flesh control vs peel control	-10.200	5.209	** $p<0.01$

p values < 0.01 were considered to be significantly different, *p* values < 0.0001 were considered to be extremely different, *q* values > 3.813 indicated that $p<0.05$.

Appendix 15. Statistical significance between methyl linoleate, crude extract and purified diene compound using One-Way and Post ANOVA Tests. Values were expressed as mean ± standard error of mean (SEM) using SYSTAT 12 software. *p* values less than 0.05 were considered as significantly different.

100 X:

Comparison	**Mean difference**	***q***	***p* value**
Methyl linoleate vs crude extract	32.000	17.827	****p*<0.001
Methyl linoleate vs purified diene	19.000	10.585	****p*<0.001
Crude extract vs purified diene	-13.000	7.242	***p*<0.01

80 X:

Comparison	**Mean difference**	***q***	***p* value**
Methyl linoleate vs crude extract	31.833	11.119	****p*<0.001
Methyl linoleate vs purified diene	20.667	7.219	****p*<0.001
Crude extract vs purified diene	-11.167	3.900	**p*<0.05

60 X:

Comparison	**Mean difference**	***q***	***p* value**
Methyl linoleate vs crude extract	19.333	23.047	****p*<0.001
Methyl linoleate vs purified diene	16.333	19.471	****p*<0.001
Crude extract vs purified diene	-3.000	3.576	ns *p*>0.05

40 X:

Comparison	Mean difference	*q*	*p* value
Methyl linoleate vs crude extract	15.667	16.974	***$p<0.001$
Methyl linoleate vs purified diene	14.000	15.169	***$p<0.001$
Crude extract vs purified diene	-1.667	1.806	ns $p>0.05$

20 X:

Comparison	Mean difference	*q*	*p* value
Methyl linoleate vs crude extract	9.333	6.662	**$p<0.01$
Methyl linoleate vs purified diene	8.667	6.186	*$p<0.05$
Crude extract vs purified diene	-0.667	0.476	ns $p>0.05$

10 X:

Comparison	Mean difference	*q*	*p* value
Methyl linoleate vs crude extract	6.667	5.410	*$p<0.05$
Methyl linoleate vs purified diene	6.000	4.869	*$p<0.05$
Crude extract vs purified diene	-0.667	0.541	ns $p>0.05$

5 X:

Comparison	Mean difference	*q*	*p* value
Methyl linoleate vs crude extract	6.333	6.582	**$p<0.01$
Methyl linoleate vs purified diene	6.000	6.235	*$p<0.05$
Crude extract vs purified diene	-0.333	0.346	ns $p>0.05$

0 X:

Comparison	Mean difference	*q*	*p* value
Methyl linoleate vs crude extract	3.667	6.736	***p*<0.01
Methyl linoleate vs purified diene	2.333	4.287	ns *p*>0.05
Crude extract vs purified diene	-1.333	2.449	ns *p*>0.05

p values < 0.01 were considered to be significantly different, *p* values < 0.0001 were considered to be extremely different, *q* values > 3.813 indicated that *p*<0.05.