Comparative studies of *Dothiorella* on avocado

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SUMMARY

COMPARATIVE STUDIES OF *DOTHIORELLA* ON AVOCADO

PROMOTER: PROF. L. KORSTEN
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A market survey was conducted to determine the incidence of stem-end rot (SE) on avocado fruit obtained from the Pretoria Fresh Produce Market representing the Tzaneen production area. *Dothiorella aromatica* isolates collected from this survey were compared in terms of physiological characteristics i.e. growth and temperature, carbon and nitrogen utilization and pH response as well as genetic relatedness using random amplified polymorphic DNA (RAPD's). The incidence of SE was found to be as high as 31 % and anthracnose 18 %. Symptom development was more apparent when fruit was evaluated at the overripe than eating ripe stage. Of the 12 identified fungi isolated from SE lesions, *D. aromatica* was by far the most frequently isolated fungus. All *D. aromatica* isolates tested were found to be pathogenic using the fruit inoculation technique. Based on lesion size, isolates were separated into two groups of virulent and less virulent. Most isolates grouped within the one cluster, with only one isolate falling in the second group being less virulent. Although similar groupings were found between physiological tests, a lack of consistency as to which isolate belonged to which group was found. The optimum temperature for growth was 25 °C and an initial pH of 6. The mean colony growth rate was 5 mm day^{-1}. Isolates grew at a minimum of eight to a maximum of 27 mm within 24 hours. Isolates grew best on pectin and poorly on sorbitol when used as a carbon source. Urea supported growth best and poor growth was found on casein-amended sources. At a molecular level, the RAPD technique could be used successfully to seperate isolates into three groups based on cluster analyses. OPC02 was the most discrimatory primer and was therefore used in this study. Isolates produced DNA fragments ranging from 1500 bp to 450 bp. The results obtained from RAPDs could not be correlated with the pathogenicity and physiological tests. Future studies should focus on comparing isolates from different avocado production areas and testing different primers for the ability to distinguish between isolates of *D. aromatica*.

CHAPTER 1

GENERAL INTRODUCTION

The avocado (*Persea americana* Mill.) also known as alligator pear (Jackson, 1986), or aguacate (Nakasone and Paull, 1998) belongs to the family Lauraceae and is one of the few commercially significant members of the genus *Persea* (Zentmyer, 1994). The avocado fruit is native to Mexico and Latin America (Samson, 1986), and from there it was introduced to the rest of the world. Today, avocado is produced in nearly 50 countries around the world (Zentmyer, 1994). Of these, Mexico is by far the biggest producer, accounting for approximately 36 % of world production (Van Zyl and Ferreira, 1995). Other major producers are Brazil, USA, Spain, Israel, South Africa and Chile (Zentmyer, 1994).

There are three major recognized races of avocado: Mexican, Guatemalan and West Indian (Jackson, 1986; Nogalingam, 1993). In addition to these races, there are hybrids such as the 'Fuerte' variety, which is a natural cross between the Mexican and Guatemalan races (Durand, 1990). The two major cultivars in South Africa are 'Fuerte' and 'Hass', which accounts for about 42 % and 33 % of production area planted to avocados respectively (South African Avocado Growers' Association, 1998). Other cultivars grown in South Africa include 'Ryan' and 'Pinkerton' accounting for 11 and 8.5 % respectively. According to the 1998 tree census done by the South African Avocado Growers Association, it was estimated that a total area of 12 500 ha has been planted with these cultivars.

The South African avocado industry exports the bulk of its fruit to European countries (Toerien, 1992; Van Zyl and Ferreira, 1995). The total production for the 1999 season was approximately 55 793 metric tonnes of which 38 % were exported to mainly European countries amounting to $15 199 000 earnings in foreign exchange (FAOstat Database, www.fao.org 2001). The majority of avocados are exported by sea in refrigerated containers (South African Avocado Growers' Association, 1998). From on farm packing to reaching its final destination at the retail end in European countries it can take about 25 day, strict control of all links in cold chain is vital in order to maintain high standards of fruit quality. These extended cold storage periods during sea transport increases the risk of post-harvest problems, caused by physiological disorders and post-harvest decay. Due to the latent nature

of these post-harvest diseases, symptoms only develop on fruits once they start to ripen (Darvas, 1982). Decay is therefore only detected on fruit once it is on the overseas markets, resulting in major losses at the retail end, which represent the accumulative cost of packing, shipping and marketing. These factors severely hamper future growth and impact negatively on the South African avocado industry.

Anthracnose, stem-end rot (SE) and *Dothiorella/Colletotrichum* fruit rot complex (DCC) are the most important post-harvest diseases of avocado (Darvas, 1992). Post-harvest losses due to SE can be as high as 13 % on certain export consignments (Korsten *et al.*, 1991), whilst on locally marketable fruit SE has been implicated to cause losses as high as 25 % (Sanders and Korsten, 1997). To date, limited control of SE has been achieved by means of pre-harvest fungicide sprays using copper oxychloride or post-harvest application of prochloraz (Darvas *et al.*, 1987; Darvas and Kotzé; 1987b; Lonsdale and Kotzé, 1989). Although prochloraz is currently registered for control of SE (Nel *et al.*, 1999), tolerance levels specified by most of the importing countries including France are very low. As a result most growers do not use this fungicide (Derek Donkin, personal communication).

According to Darvas (1982), SE was first described in California and has subsequently been reported in several other avocado producing countries such as Australia (Peterson, 1978; Muirhead *et al.*, 1982); South Africa (Darvas and Kotzé, 1987a); the USA (Spalding and Reeder, 1976) and Israel (Prusky & Keen, 1994). The disease occasionally causes severe losses, particularly when anthracnose is well controlled and storage conditions are suboptimal (Johnson and Kotzé, 1994). In general, the disease is more common in stored fruit with symptoms developing at the retail end (Fitzell and Coates, 1995). Jacobs (1974), first reported SE in South Africa, and described it as a problem associated with irradiated fruit. The causal agent was later described as *Botryodiplodia theobromae* Pat. Gorter, (1977).

Since then, several fungal pathogens have been implicated as causal agents of SE and the status of each differed between countries and regions. In South Africa, Darvas (1987a) described *Thyronectria pseudotrichia* (Schw.) Seeler, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph *Glomerella cingulata* (Stonem.) Spauld. & Schrenk), *Phomopsis perseae* Zevora and *Dothiorella aromatica* (Sacc.) Petrak and Sydow as the most important while *Fusarium*

sambucinum Fuckel, *Fusarium decemcellulare* Brick, *Fusarium solani* (Mart.) Appel & Wr. Emend. Synd. Hans., *Drechslera setariae* (Sewada) Subram. & Jain, *Pestalotiopsis versicolor* (Speg.) Steyart and *Rhizopus stolonifer* (Ehrenb. Ex Fr.) Vuill were the least important. Of these, *D. aromatica* has been implicated in most countries as one of the most important pathogens of SE (Peterson, 1978; Darvas, 1987a).

However, despite the economic importance of SE and the prevelance of *D. aromatica,* little is still known about the etiology and taxonomic status of this causal agent. Currently, there is conflicting evidence in the literature about the correct identity of *Dothiorella* spp. Sutton (1980), first noted that Petrak (1922) made a link between *Botryosphaeria (B. berengeriana* De Not.) and *Fusicoccum*, by which time Petrak referred to *Fusicoccum* as *Dothiorella*. This probably marks the beginning of an extended period of confusion regarding the application of the name *Dothiorella* to specimens that have hyaline spores (Sutton, 1980). Recently, Crous and Palm (1999) re-examined *Dothiorella* and its teleomorph *Botryosphaeria* and suggested that these taxa need extensive revision to confirm their correct taxonomic status. Denman *et al.* (2000) re-evaluated the anarmorphs of *Botryosphaeria* on proteaceae and concluded that anarmophs with hyaline conidia should be accommodated in the genus *Fusicoccum.* Since this fungus is commonly known as *Dothiorella* in the South African avocado industry, the name *Dothiorella* has been retained for the purpose of this study.

In order to better understand the epidemiology of this pathogen and ultimately develop more effective control strategies, it is important to know as much as possible about the pathogen itself. To date, fungal pathogens have been identified using standard morphological characteristics. Isolates have been compared using a number of techniques including inoculation studies (Korsten *et al.*, 1994, Swart, 1999), physiological tests (Swart, 1999), protein profiles (Lattore *et al.*, 1995), isozyme analyses (Bonde *et al.*, 1991), DNA hybridization (Bruns *et al.*, 1991), DNA mapping (Bruns *et al.*, 1991), restriction fragment length polymorphisms (RFLP's) (Bruns *et al.*, 1991), random amplified polymorphic DNA (RAPD's) (Foster *et al.*, 1993) and amplified fragment length polymorphisms (AFLPs) (Majer *et al.*, 1996).

In view of the above, a study was undertaken to elucidate the incidence and diversity of *D. aromatica* in the Tzaneen area. The investigation entailed the following:

- A market survey to determine:
  - The incidence of SE in commercially available fruit from the Tzaneen area
  - The role and importance of *D. aromatica* in comparison to other SE pathogens

- To compare isolates based on:
  Pathogenicity
  Physiology
  RAPD analysis

Once established, this study will lay a foundation for further work, where the isolates from different cultivars, production areas and countries can be compared to eventually clarify the taxonomic status and global importance of this pathogen.

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CHAPTER 2

LITERATURE REVIEW

THE HOST

History, distribution and cultivars

The avocado (*Persea americana* Mill.) is native to the highlands of Mexico and Latin America, where it has been consumed for more than 10,000 years (Samson, 1986). Avocados were cultivated in a variety of habitats, including the highlands of Guatemala, the rainforest, lowland tropics, various Montane habitats, and subtropical areas in Latin America (Zentmyer, 1994). Today, the avocado is cultivated in the subtropical and tropical regions of the world as both a commercial and subsistence crop.

The avocado has been known in South Africa since the early arrival of Dutch settlers from the West Indies and other Dutch colonies (Durand, 1990). All planting materials were initially obtained from the West Indian Islands and at a later stage improved planting materials were imported from Mexico, Guatemala and California (Van Zyl and Ferreira, 1995). Different cultivars, amongst others 'Fuerte', were imported during 1924 and 1927, which marked the beginning of a sophisticated commercial industry (Durand, 1990). The first commercial orchard was planted in KwaZulu-Natal and subsequently in other subtropical regions of South Africa. Since then, the avocado industry expanded rapidly to become one of most important subtropical export industries in South Africa.

The area planted to avocados in South Africa has expanded steadily over the past 30 years, from approximately 2000 ha in 1970 to 12 500 ha (South African Avocado Growers' Association, 1998). Since South Africa export mainly to the European markets, the industry had to change the type of cultivar planted for export, to one more generally preferred by the European market. This trend is reflected where 'Fuerte' accounts for only 13 % and 'Hass' for 33 % of all newer plantings as from 1995 (South African Avocado Growers' Association, 1998). Other commercially important local cultivars include 'Ryan' and 'Pinkerton', accounting for 12 and 41 % respectively of all newer plantings (South African Avocado Growers' Association, 1998).

Biology

The avocado is shallow-rooted and varies in canopy shape, from tall, upright to widely spreading with multiple branches (Nakasone and Paull, 1998). Although avocado is classified as an evergreen subtropical tree (Jackson, 1986), some cultivars shed leaves during flowering whilst others shed their leaves gradually (Nakasone and Paull, 1998). Two types of flowers are recognized viz. category A (e.g. 'Hass') and B (e.g. 'Fuerte'), which restricts self-pollination and encourages cross-pollination between complementary groups. The avocado fruit is a one seeded berry (Nakasone and Paull, 1998), and the fruits are mainly pear shaped (Nogalingam, 1993). The skin colour of the ripe fruit varies from green to purple. On ripening, the pulp changes from a firm to a smooth, buttery texture (Nogalingam, 1993). The fruit is high in fat, proteins and minerals but low in carbohydrates (Kadam and Salunkhe, 1995).

Marketing and international trade

The avocado is currently produced in nearly 50 countries around the world both as a commercial and or subsistence crop (Zentmyer, 1994). Some of the important avocado production areas and their world production are shown in (Table 1) (FAOstat Database, www.fao.org 2001). Mexico has been the largest producer of avocado over the past three years, with total production of 940 000 metric tonnes in 2001. Other important world producers are USA, Indonesia, Chile, Brazil, South Africa and Spain.

The majority of exported fruits are traded in Europe, with the largest percentage going to France (64 %). Some of the important avocado exporting countries in world trade are shown in (Table 2) (Faostat Database, www.fao.org 2001). Mexico has been one of the major exporters of fresh avocados between 1997 to 1999, with Spain being the second largest followed by Chile, South Africa and Israel (Table 2). Other countries such as Canada, Saudi Arabia and Singapore represent potential future markets that can be developed (Van Zyl and Ferreira, 1995). Approximately 50 % of the total South African exports are destined for France while 25 % goes to the UK and 20 % to other European countries such as Scandinavia (Van Zyl and Ferreira, 1995). The main competitors for South African fruit on the European markets are Israel, Spain, Mexico, Chile and Kenya.

Post-harvest diseases

The three most important post-harvest diseases of avocado are anthracnose, stem-end rot (SE) and *Dothiorella/Colletotrichum* fruit rot complex (DCC) (Darvas, 1992; Korsten, 1993). *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph *Glomerella cingulata* (Stonem.) Spauld. & Schrenk) causes anthracnose, DCC and SE, whilst *Dothiorella aromatica* (Sacc.) Petrak and Sydow causes DCC and SE. Darvas and Kotzé (1987b), described twelve pathogens involved in SE which includes the above-mentioned two and *Thyronectria pseudotrichia* (Schw.) Seeler, *Phomopsis perseae* Zerova, and to a lesser extent, *Fusarium decemcellulare* Brick, *Fusarium sambucinum* Fuckel, *Fusarium solani* (Mart.) Appel & Wr. emend. Snyd. & Hans., *Pestalotiopsis versicolor* (Speg.) Steyart, *Lasidioplodia theobromae* (Pat.) Griffon & Maubl., *Drechslera setariae* (Sawada) Subram. & Jain, and *Rhizopus stolonifer* (Ehrenb. Ex Fr.) Vuill.. Hartill (1991) also reported *Sphaceloma perseae* Jenkin in New Zealand, which has never been reported in South Africa.

THE DISEASE

Stem-end rot

The spectrum of pathogens that cause SE is predetermined by environmental conditions (Johnson and Kotzé, 1994). Hot conditions promote infection by *L. theobromae*, whereas wet conditions promote infection by *C. gloeosporioides* and *T. pseudotrichia*. Water stress promotes endophytic infection and subsequent storage conditions determine which pathogens predominate. Cool storage promotes *C. gloeosporioides* and *P. perseae* over *T. pseudotrichia*, whereas *L. theobromae* (if present) will predominate over *Dothiorella* spp. at 30°C. Peterson (1978) reported *D. aromatica* and *C. gloeosporioides* as the main causal agents of SE in Australia while Darvas and Kotzé (1987b) and Darvas *et al.* (1987) described *T. pseudotrichia* as the most important pathogen of SE under South African conditions when fruit were ripened both at ambient temperature and after cold storage. In contrast, Korsten *et al.* (1994a) described *C. gloeosporioides* and *D. aromatica* as the most frequently isolated pathogens in locally marketed avocados ripened at ambient room temperature. Compared to other cultivars, 'Fuerte' is highly susceptible to SE (Kotzé, 1978).

According to Darvas (1982), SE was first described in California. Since then, the disease has been reported in several other avocado producing countries including Australia (Peterson, 1978); South Africa (Darvas and Kotzé, 1979); USA (Spalding and Reeder, 1976), and Israel (Prusky and Keen, 1994). In South Africa, SE was first reported by Jacobs (1974). He believed that it was mainly a problem on irradiated fruit. The causal agent was later described as *Botryodiplodia theobromae* (Pat.) Griffon & Maubl. syn *L. theobromae* (Gorter, 1977). The incidence of SE in South Africa is lower than that of anthracnose but the former still results in significant losses which can be as high as 25 % on locally marketable fruit (Sanders and Korsten 1997). This is particularly true when anthracnose is well controlled and storage conditions are suboptimal (Johnson and Kotzé, 1994). In general, SE is more common in stored fruit and symptoms only develop at the retail end when fruits ripen (Fitzell and Coates, 1995). Considering these losses, SE is regarded as an important factor limiting storage and export of avocado (Muirhead *et al.*, 1982).

Symptoms

Initial symptoms of SE start as a firm, dark-brown to black rot of fruit beginning at the pedicel end proceeding towards the blossom end (Pegg, 1991; Pegg and Coates, 1993) (Fig. 1.1). The rest of the fruit remains firm while the affected pedicel end softens (Fitzell and Coates, 1995). Symptoms also develop internally at the stem-end as the fruit ripens, appearing within four to seven days when stored at 25 °C (Johnson and Kotzé, 1994). When infected fruits are cut in half, a dark brown rot can be seen extending down into the body of the fruit (Fitzell and Coates, 1995) (Fig. 1.2). The internal quality of the fruit deteriorates rapidly once the fruit softens (Snowdon, 1990). If fruits are placed in warm, humid conditions, a grey fungal mat will appear over the neck of the fruit (Fitzell and Coates, 1995).

The SE pathogens also affect the vascular bundles, causing a brown discoloration extending in advance of the decaying flesh (Johnson and Kotzé, 1994). According to Johnson and Kotzé (1994), SE can be detected externally in advance of the internal flesh decay. Once the button is removed, the presence of mycelium can be seen on the abscission scar. The disease has also been reported to be associated with an unpleasant odour particularly when severely infected (Darvas and Kotzé, 1987b).

Disease cycle and epidemiology

The infection process leading to SE in avocado is not completely understood, but several different mechanisms may be involved (Fitzell and Coates, 1995). *Dothiorella* spp., *L. theobromae, P. perseae, P. versicolor*, and *C. gloeosporioides* occur as endophytes in avocado stems (Johnson and Kotzé, 1994). Infection may occur when the fungus moves into the flower and stem-end tissue of fruit before harvest.

Spores of *L. theobromae*, *P. perseae*, *C. gloeosporioides* and *T. pseudotrichia* are produced in tree litter, dead leaves, twigs and branches where fruiting bodies may occur particularly after long periods of continual wet weather (Johnson and Kotzé, 1994). Spores from these fruiting bodies can also germinate and initiate SE infections through wounds before harvest (Fitzell and Coates, 1995), or for some fungi, by direct penetration of the surface (Darvas, 1982). With direct penetration, the newly deposited spore will germinate on the moist fruit and produce a germ tube, which will grow into the lenticel and penetrate the skin (Darvas, 1982). The fungus grows considerably in the lenticel, but is unable to attack and destroy the living skin until the fruit softens. Latent infections through lenticels do not take place until the fruit diameter reaches 37 mm. Regardless of how infection occurs, symptoms do not generally appear until fruit ripening (Fitzell and Coates, 1995).

Fruit infection may also occur at harvest (Johnson and Kotzé, 1994), through the cut surface of the stem (Fitzell and Coates, 1995). Ring-necrosis can also develop prior to the development of SE, and both are promoted by water stress (Johnson and Kotzé, 1994). According to Johnson & Kotzé (1994), symptoms of SE occur in decreasing frequency in fruit harvested from east, north, and the remaining sides of the tree. The disease is more common in fruit picked in rain, or in fruit with stems broken during harvesting (Fitzell and Coates, 1995). Fruit with broken stems are particularly likely to develop SE if washed or dipped in dirty water.

THE PATHOGEN

Morphology of *Dothiorella aromatica*

According to Darvas (1982), Petrak and Sydow (1927) described *D. aromatica* as follows: Pycnidia are produced sub-epidermally in groups or scattered, often covering large areas of the leaves. The lower halves of the pycnidia are embedded in the mesophyll and the upper halves are at first covered by the epidermis, but erumpent later. They are usually round or irregular in shape and measure 20-300 µm in diameter with a flat, indistinct ostiole, which opens through an irregular round pore. Pycnidial walls usually measure between 15-25 µm, but in some cases are up to 30 µm thick consisting of several layers of indistinct and irregular shaped dark cells of 5-8 µm in size. Conidia are oblong or spindle shaped at both ends being more pointed on the side of the attachment. They are straight or slightly curved, one celled, hyaline with indistinct fine-grained plasma and measuring 1-23 x 5-7 µm. Conidiophores are simple, pointed at the end with an average of 6-16 µm, seldom up to 20 µm in length and 2 µm wide at the base.

Control

Limited control of avocado post-harvest diseases can be achieved by means of pre-harvest fungicide sprays using copper oxychloride or post-harvest applications of prochloraz (Muirhead *et al.*, 1982; Darvas *et al.*, 1987; Lonsdale and Kotzé, 1989). However, copper oxychloride leaves unsightly residues, which must be removed manually in the packhouse (Korsten *et al.*, 1994b). Although prochloraz is currently registered for control of SE (Nel *et al.*, 1999), tolerance levels specified by most of the importing countries including France are very low as a result most growers do not use this fungicides (Derek Donkin, personal communication). However, in view of public concern about environmental pollution, health risks and the possibility of pathogens developing resistance to fungicides, it has become important to explore alternative control measures (Droby *et al.*, 1991).

Biocontrol research on fruit crops has largely been successful in the laboratory but was often a failure in the field (Wisniewski *et al.*, 1991; Wisniewski and Wilson, 1992). Nevertheless, a few notable exceptions have progressed to commercial field or packhouse applications. For avocado, successful biocontrol of post-harvest diseases has been achieved with both pre- and post-harvest applications of

Bacillus subtilis sprayed onto trees at monthly intervals during fruit development, or when applied as a post-harvest dip or wax treatment (Korsten *et al.*, 1991; Korsten *et al.*, 1995). Pre-harvest biocontrol with *B. subtilis* was reported to be more effective in reducing SE than standard copper oxychloride sprays (Korsten *et al.*, 1989; Korsten *et al.*, 1994), whilst post-harvest application of the same organism controlled SE on 'Fuerte' avocado fruit as effectively, and even better than prochloraz. The biocontrol agent *B. subtilis* has subsequently been commercialized and registered as Avogreen for pre-harvest applications to control avocado fruit diseases (Korsten *et al.*, 1998).

Recently detergents and/or sanitizers that are used on routine basis in the food processing and dairy industries to reduce inoculum of spoilage organisms have been tested for use on avocado to control post-harvest diseases (Boshoff *et al.*, 1995). When these disinfectants were applied as post-harvest dip treatments, a higher than commercially recommended concentration of SU319 (Quarternary ammonium compound), a commercially recommended concentration of Stericlen (Quarternary ammonium compound) and Iodet (Iodine) could effectively control anthracnose and SE. However, Agrisan (Quarternary ammonium compound), Stericlen and 76 % ethanol controlled anthracnose better than SE (Boshoff *et al.*, 1995; Van Dyk *et al.*, 1997). Of the disinfectants tested, the 76 % ethanol resulted in drastic lenticel damage (Boshoff *et al.*, 1995). Kremer-Köhne (1996) has also shown a decrease in the incidence of anthracnose and SE when fruits were treated with chlorine or wax either alone or in combination.

MOLECULAR TECHNIQUES USED FOR COMPARISON OF ISOLATES

Fingerprinting techniques

DNA fingerprinting involves the separation of DNA fragments from a specific DNA sample (Vos *et al.*, 1995). A variety of DNA fingerprinting techniques are presently available, most of which use PCR for detection of fragments i.e. restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) and amplified fragment length polymorphisms (AFLPs). Molecular markers are being increasingly used to characterize fungal plant pathogen populations (Majer *et al.*, 1996). Markers can be used to evaluate levels of genetic diversity and phylogenetic relationships within and between species, and to identify particular races and pathotypes.

Restriction fragment length polymorphisms

Restriction fragment length polymorphisms (RFLPs) is a powerful method for the detection of specific fragments of DNA (Muthukumaran and Darrell, 1996). It can therefore be used to assess the genetic variability and taxonomic relationships of plant pathogens (Kennedy, 1996). In this method, two approaches may be followed, viz. PCR/RFLP and RFLP. In a PCR based method, a known polymorphic restriction site in a DNA sequence is specifically amplified, usually the internal transcribed spacer (ITS) and the intergenic (IGS) spacers using a pair of synthetic oligonucleotide primers complementary to alternate strands of the target genomic DNA sequence and DNA polymerase (Buscot *et al.*, 1996). The second approach entails restriction of the entire genome with restriction enzymes followed by southern blotting using selected probes (Adachi and Tsuge, 1994). RFLP analyses is a reliable technique however, it is time consuming, expensive and requires personnel trained in molecular genetics (Buscot *et al.*, 1996).

Random amplified polymorphic DNA

Another method of obtaining markers for evaluation of genetic information of organisms is random amplified polymorphic DNA (RAPDs) (Muthukumaran and Darrell, 1996). This technique provides a PCR fingerprint for related organisms based on the genome rather than individual genes (Mitchell *et al.*, 1995). Important strains of fungi and the origins of populations can be identified, especially the groupings that are heterogenous like the Zygomycetes and the Deutoromycetes where life history is patchy (Mitchell *et al.*, 1995). A PCR reaction is conducted with a single primer, usually nine or ten nucleotides in length (Foster *et al.*, 1993). Because the sequence is short and arbitrary, it will bind to many sites in genomic DNA amplifying a variety of differently sized fragments. These fragments can be separated by electrophoresis to give a specific banding pattern (Mitchell *et al.*, 1995).

The results of RAPDs are quick, and the whole analysis, from the initial reaction to gel observations can be completed in one working day (Foster *et al.*, 1993). In addition, there is no need for a specific gene library or any form of a clone required as a probe (Theodore *et al.*, 1995). RAPD analysis requires less DNA and no radioisotopes, which can result in cost reduction and enhanced safety.

Despite the advantages, several shortcoming of these techniques have been identified including the difficulty in standardisation due to the nature of the primers, lack of ability to distinguish homo- and heterozygotes, and differences in band intensities which make scoring patterns ambiguous (Bruns *et al.*, 1991).

Amplified fragment length polymorphisms

Amplified fragment length polymorphisms (AFLP) is a relatively new technique that is based on the detection of genomic restriction fragments by PCR amplification (Vos *et al.*, 1995). DNA of any origin or complexity can be used in this technique. This method allows differentiation of microorganisms without prior knowledge of specific DNA information using a limited set of generic primers (Vos *et al.*, 1995). The number of fragments detected in a single reaction can be modified by selection of specific primer sets. Prior to amplification, adapters with known sequences are ligated to digested genomic DNA, and for PCR, primers which perfectly match the adapter sequences are used (Van der lee *et al.*, 1997). These stringent reaction conditions used for primer annealing renders AFLP to be more reliable and more reproducible than RAPDs (Vos *et al.*, 1995).

Variability using this method is assessed at a large number of independent loci, since the markers are 'neutral' (Majer *et al.*, 1996). Furthermore variation is revealed in any part of the genome. A recent report has been published where AFLPs have been successfully used to characterize isolates of *Colletotrichum* pathogens of alfalfa (O'Neill *et al.*, 1997).

Other molecular techniques for comparison of isolates

Protein electrophoresis

Gel electrophoresis of total soluble proteins has been employed as a tool in fungal taxonomy at the intrageneric level (Michelmore and Hulbert, 1987). Most genera studied thus far, interspecific differences in banding patterns were greater than intraspecific differences. However, small differences in total protein band patterns between biotypes or formae speciales may be inconsistent (Sherrif *et al.*, 1995). Despite this, a report has been published where protein profiles of isolates of

Phytophthora cryptogea Pethbr. & Lafferty resulted in groupings consistent with host, geographic area or enzyme phenotype (Latorre *et al.*, 1995).

Isozymes

Isozyme analysis is a biochemical technique that has numerous applications in plant pathology (Bonde and Micales, 1993). It is now used routinely to settle taxonomic disputes, identify cultures, fingerprint patentable fungal lines, analyze genetic variability, trace pathogen spread, follow the segregation of genetic loci, and identify ploidy levels of fungi and other plant pathogens (Bonde and Micales, 1993). Isozymes are molecular forms of an enzyme. The forms usually have similar, if not identical, enzymatic properties but slightly different amino acid sequences. Only those isozymes with amino acid compositions with variable net charge, or those that result in major differences in the shape of an enzyme, can be separated by electrophoresis (Bonde and Micales, 1993). Although isozyme analysis is not as sensitive as molecular techniques such as RFLPs or RAPDs, it remains a simple, efficient, and inexpensive technique that can be used to solve many taxonomic and phytopathological problems.

MOLECULAR DIFFERENTIATION VERSUS MORPHOLOGICAL TRAITS

Academic debates on the value of molecular information versus morphological traits in classification have been waged over the last thirty years (Mitchell *et al.*, 1995). Genetic studies require accurate, readily determinable characters or markers (Michelmore and Hulbert, 1987). Classical fungal taxonomy relies heavily on the size and shape of fruiting structures, spore morphologies and release mechanisms, coloration and habitat to define taxa (Mitchell *et al.*, 1995). Although classical fungal taxonomy adequately describes the grouping, it does not always help explain its origins. According to Mitchell *et al.* (1995) a new approach, i.e. 'Phylogenetic character mapping', has been adopted which involves plotting morphological, or other, characters defining taxa along a molecular lineage, or vice versa, and merging both molecular and morphological data to define the rise of adaptation (Mitchell *et al.*, 1995). To correctly identify an organism using molecular characteristics, molecular based trees must be constructed. Sequences with useful phylogenetic information are obtained from genes that have the same function in all taxa, evolve at approximately the same rate and are present

only once in a genome or behave like a single copy region (Mitchell, *et al.*, 1995). The genes that fulfil these criteria are the ribosomal RNA genes from the nuclear and mitochondrial genome.

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Table 1 World production of avocados according to country (Metric tonnes)

*Country	1999	2000	2001
Mexico	879,083	933,337	940,000
USA	164,000	164,500	164,000
Indonesia	126,480	128,000	130,000
Chile	82,000	98,000	120,000
Brazil	85,000	85,000	85,000
South Africa	55,793	80,546	81,000
Israel	55,900	73,300	68,000
Spain	58,000	47,000	47,000

Source: (FAOstats Database www.fao.org 2001) updated: 7 November 2001

*Table based on selected avocado production countries

Table 2 Avocado imports to Europe according to country of origin (Metric tonnes)

*Country	1997	1998	1999
Mexico	49,824	71,226	55,402
Spain	44,116	56,268	37,301
Chile	16,702	48,163	34,788
South Africa	22,604	52,080	34,483
Israel	39,943	29,024	31,938

Source: (FAOstats Database www.fao.org, 2000) updated: 22 December 2000

*Table based on selected avocado production countries

Fig. 2.1 Early external symptoms of stem-end rot on 'Fuerte' avocado fruit.

Fig. 2.2 Internal symptoms of stem-end rot on 'Fuerte' avocado fruit.

CHAPTER 3

MARKET SURVEY OF STEM-END ROT ON 'FUERTE' AVOCADOS

ABSTRACT

A market survey of commercially available fruit from the Tzaneen area was conducted on a weekly basis during the 1999 season to determine the incidence of post-harvest diseases. The incidence of stem-end rot (SE) was higher than that of anthracnose. Variations in the levels and severity of SE and anthracnose occurrence were observed. Symptom development of SE was higher when fruit were evaluated at the overripe stage. *Dothiorella aromatica* (31 %) was by far the most frequently isolated fungus, followed by *Phomopsis perseae* (14 %) and *Lasidioplodia theobromae* (8.5 %). *Acremomium charticola* and *Gliocladium roseum* were found to be associated with the SE disease complex but are not known pathogens of SE. *Pestaloptiopsis versicolor*, *Fusarium solani*, *Fusarium* spp, *Colletotrichum gloeosporioides, Drechslera setariae*, and *Alternaria alternata* were also isolated but at lower frequencies. Pathogenicity of *D. aromatica* isolates was confirmed and variation in virulence was observed. Cluster analysis revealed three groups of which most isolates grouped into the highly virulent category.

INTRODUCTION

The avocado originated from the highlands of Mexico and Latin America and is currently produced in most of the tropical and subtropical regions of the world as both a subsistence and commercial crop (Samson, 1986; Zentmyer, 1994). In South Africa, avocados are the second most important subtropical crop cultivated mainly in the warm subtropical regions of the Northern and Mpumalanga provinces and to a lesser extent KwaZulu Natal (Van Zyl and Ferreira, 1995). Production of 81 000 metric tons in the 2001 season has been recorded (FAOstats Database www.fao.org, 2001), with the bulk of this fruit being exported to mainly European countries. The quality of South African fruit on these export markets has been excellent despite the extended periods of cool storage during shipping, which can be as long as 25 days from picking to marketing (South African Avocado Growers' Association, 1998).

Anthracnose and stem-end rot (SE) are generally regarded as the most important post-harvest diseases of avocado in South Africa (Darvas and Kotzé 1987a; Darvas, 1992). The most important pathogens associated with SE include: *Thyronectria pseudotrichia* (Schw.) Seeler, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph *Glomerella cingulata* (Stonem.) Spauld & Schrenk), *Phomopsis perseae* Zevora, *Dothiorella aromatica* (Sacc.) Petrak and Sydow, *Lasidioplodia theobromae* syn *Botryodiplodia theobromae* (Pat.) Griffon & Maubl. (Darvas and Kotzé 1987a; 1987b). Lesser important SE pathogens include *Drechslera setariae* (Sewada) Subram. & Jain, *Fusarium decemcellulare* Brick, *F. sambucinum* Fuckel, *F. solani* (Mart.) Appel & Wr. Emend. Synd. Hans., *Pestaloptiosis versicolor* (Speg.) Steyart and *Rhizopus stolonifer* (Ehrenb. Ex Fr.) Vuill.

In a study conducted by Darvas and Kotzé (1987a), the most frequently isolated fungus was *T. pseudotrichia* followed by *C. gloeosporioides*. Later reports in South Africa suggested that *C. gloeosporioides* followed by *D. aromatica* are the most important SE pathogens (Korsten *et al.*, 1994). These results were in agreement with Peterson (1978) who reported that *D. aromatica* and *C. gloeosporioides* were the most important pathogens in the SE disease syndrome respectively. *Dothiorella* spp. occur as endophytes in avocado stems and symptoms of endophytic infections are not apparent in the stem-end tissue until fruit development is well advanced (Johnson and Kotzé, 1994). This is particularly important due to extended transit periods prior to marketing, which can result in latent symptom development as fruit begins to ripen at the consumers' end of the fruit chain.

Losses due to SE have been reported on the overseas market and can be as high as 15 % (Korsten *et al.*, 1991), while up to 25 % of locally marketed fruit developed SE (Sanders and Korsten, 1997). Although *D. aromatica* has been reported to play an important role in causing SE, information of the etiology of this fungus is still lacking. The purpose of this study was therefore to conduct a market survey in the Tzaneen area to determine the role and importance of *D. aromatica* on Fuerte avocado fruit, to confirm pathogenicity and to compare virulence of representative isolates.

MATERIALS AND METHODS

Isolation of post-harvest pathogens

'Fuerte' fruit were collected from the Pretoria Fresh Produce Market at weekly intervals for eight weeks from 18 March to 13 May 1999. Five trays were collected as representative of the Tzaneen area with fruit ranging in size from 8 to 19 per tray. A total of 505 fruit was evaluated in this survey. Fruit were left to ripen at ambient temperature and were rated for post-harvest diseases at three different stages of ripeness viz. eating ripe, slightly overripe and overripe. This was done to mainly allow complete symptom expression, since SE only develops when fruit starts to soften. At each ripeness stage, the number of fruit per tray with SE symptoms was noted.

Fruit were surface sterilized with ethanol and three isolations were made from the edge of SE lesion of the fruit by aseptically cutting a 0.5 mm section from the pulp of the fruit. Sample segments were plated onto potato dextrose agar (PDA) (Biolab) and plates were incubated at 25 °C for up to five days before subcultures were made. Pure cultures were subsequently made onto oatmeal agar (20 g oatmeal, 20 g agar (Biolab), 1 L distilled water) and incubated at 25 °C. Once visible growth was observed, the cultures were incubated at ambient temperature under mixed irradiation from near ultraviolet and daylight type fluorescent tubes (Phillips TL 40 W/08RS, F40 B43 and TL 40W/ 33RS respectively) until spore formation was observed. Isolates were preserved in sterile water and by plating onto PDA slants.

Selected *D. aromatica* isolates (Appendix 1) were taken up in the culture collection of the Department of Microbiology and Plant Pathology. The Biosystematic division of the Agricultural Research Council, Pretoria, confirmed identity of the isolates. For further studies isolates of *D. aromatica* were randomly selected from the culture collection obtained in this investigation. Data were statistically analyzed with the SAS system using analysis of variance and Duncan's multiple range tests to separate means.

Evaluation of pathogenicity

Twenty-seven *D. aromatica* isolates (Appendix 2) were selected and grown on PDA plates, incubated at 25 °C for 14 days. 'Fuerte' fruit collected from the Johannesburg Fresh Produce Market were used for pathogenicity tests using the plug inoculation technique of Sanders *et al.* (1996). Prior to inoculation, all fruit were swabbed with 70 % ethanol and left to air dry. Ten-millimeter deep plugs were aseptically cut from fruit using a four-millimeter diameter sterile stainless steel cork borer. Plugs for inoculum were aseptically cut from the fungal edge of an actively growing culture on PDA. Three replicates from each culture were placed separately into holes made into the side of three different fruit. The holes were made on the sides of the fruit to prevent fungal lesions from growing over each other and becoming difficult to measure. Fruit plugs were replaced and covered with parafilm. For the control, fruit were inoculated with plugs cut from uninoculated PDA. Fruit were incubated upright at ambient room temperature and the parafilm was removed as soon as visible symptoms appeared. After seven days, the results were evaluated by measuring the length and breadth of the lesions. Data was statistically analyzed using the SAS system as described. Data was also subjected to Ward's agglomerative hierarchical clustering method based on the sum-of squares criteria. The resulting hierarchies were plotted as a dendogram.

RESULTS

The general quality of fruit was considered good, except for those collected during the sixth week, which showed a lot of mechanical damage. In this study it was found that the incidence of SE was much higher (31 %) than that of anthracnose (18 %). Significant differences were not found between the incidence of SE and anthracnose at weekly intervals (Fig. 3.1). Levels of SE per fruit ripeness stage were not significant (Fig. 3. 2), except for the fifth week, during which levels of SE was highest at overripe stage, followed by slightly overripe and eating ripe which had almost no SE symptoms.

Significant differences were found with the incidence of SE when fruit were evaluated at different ripeness stages over the entire observation period (P=0.0001) (Fig. 3.3). As expected, disease incidence was higher when fruits were evaluated at the overripe stage. However, no significant

difference was found in terms of SE incidence when fruit were slightly overripe or eating ripe. Prior to softening of the fruit, no symptom development of SE was visible.

All 12 fungal species described before to be associated with SE were isolated from SE in this study. The most frequently isolated fungus was *D. aromatica* (31 %), followed by *P. perseae* (14 %), *L. theobromae* (8.5 %), and *Acremomium charticola* W.Gams (7.8 %). Other fungi were isolated at frequencies of less than 5 % of fruit affected (Table 1).

All twenty-seven isolates of *D. aromatica* tested in this study were pathogenic. Highly significant differences (P=0.0001) were found in virulence between isolates (Table 2). Isolates produced lesion sizes on fruit that ranged from 56.2 to 13.8 mm. A dendogram constructed from the mean lesion size produced on the fruit, separated isolates into two groups at the Euclidean distance of 21 (Fig. 3.4). The majority of isolates were within the second group whilst the first group contained only one isolate (96b).

DISCUSSION

The results obtained in this study confirmed previous reports that SE remains an important post-harvest disease of 'Fuerte' avocado fruit, causing significant losses at the consumer end. The incidence of SE (31 %) was higher than that of anthracnose (18 %) in this study. This is in contrast with similar work previously done with 'Fuerte' fruit from the Tzaneen area where the incidence of anthracnose (48 %) was higher than that of SE (15 %) (Sanders and Korsten, 1997). It is interesting to note that in their study they referred to higher rainfall (127 mm) recorded during the 1996 season and correlated it with increased levels of anthracnose. This compared to the lower (25 mm) rainfall figures of the previous season (1995) where the incidence of SE was 25%. Most fungi causing SE occur endophytically in stems of avocado and according to Johnson and Kotzé (1994), drier conditions promote endophytic infection. This may explain the prevalence of SE in this study, which was done during a drier 1998 season (21 mm) (ARC-Institute for soil, climate and water). Overall the incidence of SE tended to increase whilst anthracnose decreased (Fig. 3.1). The incidence of SE and anthracnose is known to vary from year to year and from area to area depending on amongst others, climatic conditions (Sanders and Korsten, 1997; Swart, 1999).

Disease incidence of SE was more prominent when fruit was evaluated at an overripe stage. This finding is in agreement with numerous other reports (Darvas, 1982; Darvas and Kotzé 1987a; Johnson and Kotzé, 1994) which suggest that symptom development occur after harvest as the fruit ripens. This study also showed that it is important to monitor symptom development at different stages of fruit ripeness to enable slower developing lesions to appear. Evaluating fruit for SE incidence too early might result in undetected infections. With the study of Korsten *et al.* (1994) fruit were evaluated only at a single ripeness stage, this way symptom expression might have not occurred and the results may be misleading.

This study has shown that a number of fungi could be isolated from SE similar to the ones previously described as major and minor pathogens. The most frequently isolated fungus in this study was *D. aromatica.* This is in agreement with the investigation by Korsten *et al.* (1994), where *C. gloeosporioides* and *D. aromatica* were the most frequently isolated pathogens. In their study, fruit were also stored under ambient conditions prior to evaluation. In other reports the frequency of isolation of *D. aromatica* remained similar irrespective of the storage conditions prior to ripening (Darvas and Kotzé, 1987a). In a study done by Darvas and Kotzé (1987b), *D. aromatica* was isolated infrequently from both cold or ambient stored fruit, whilst *T. pseudotrichia* followed by *C. gloeosporioides* were the most commonly isolated fungi. In addition, Darvas and Kotzé (1987a) reported *T. pseudotrichia* to be the most frequently isolated fungus from SE symptoms. This was particular noticeable in fruit that was ripened at ambient temperature. Subsequent studies by Darvas *et al.* (1987a), also confirmed that *T. pseudotrichia* is the most commonly occurring SE pathogen.

The association of *P. versicolor*, *D. setariae, F. solani* and other *Fusarium* spp with SE symptoms was in accordance with previous investigations (Darvas and Kotzé, 1979; Darvas and Kotzé, 1987a; Darvas and Kotzé 1987b; Korsten *et al.*, 1994). Contrary to previous reports (Darvas and Kotzé 1987a; Darvas and Kotzé 1987b; Korsten *et al.*, 1994), *L. theobromae* was found to be important in this study. Korsten *et al.* (1994) reported no incidence of this pathogen on fruits collected from the Tzaneen area, whilst Darvas and Kotzé, (1987a), reported that this fungus occured only at low frequencies. Of interest was the relative high frequency of isolating *A. charticola* during this study, which has not previously been reported as a SE pathogen of avocado. This fungus is however a typical saprophyte, which may explain its presence in the SE lesions of particularly older fruit.

Trichothecium roseum (Persoon) Link was isolated at a low frequency in this study. Although this pathogen was also previously isolated from SE symptoms, it was not regarded as an important pathogen (Darvas and Kotzé, 1987a). Korsten *et al.* (1994) also isolated this fungus at a low frequency and confirmed its pathogenicity. It was interesting to note that they reported that *T. roseum* was highly virulent which was in contrast with studies of Hartill (1991) who also occasionally isolated this pathogen from SE but described it as not being highly virulent. Of importance to the avocado industry is that *T. roseum* produces mycotoxins called trichothecin (Patterson, 1973; Shishiyama *et al.*, 1993; Reddy *et al.*, 1997). These mycotoxins have a cytotoxic effect on eukaryotic cells damaging actively dividing cells, such as bone marrow thereby resulting in extensive haemorrhages. (Patterson, 1973; Ishii *et al.*, 1986; Shishiyama *et al.*, 1993; Reddy *et al.*, 1997). Toxicity on the liver has also been reported on eukaryotic cells (Patterson, 1973). The fact that this pathogen has been isolated sporadically over the years from the Tzaneen avocado production area should raise some concern for exporters who need to conform to Hazard Analysis Critical Control Point, Food Safety Standards.

In this study, the pathogenicity of *D. aromatica* and its importance as a fruit spoilage organism was confirmed. Darvas *et al.* (1987b) evaluated isolates of *D. aromatica* and other SE fungi for pathogenicity and found that although these isolates were less virulent than *C. gloeosporioides*, they played an important role in the SE disease syndrome. Based on lesion size two groups were found within the population of *D. aromatica* isolates i.e. virulent and less virulent. From this study, it can be concluded that a number of fungi are involved in the disease syndrome of SE and that *D. aromatica* plays an important role. In addition, variation in virulence within *D aromatica* isolates exists. Further studies will focus on other complimentary techniques such as physiological and molecular tests to confirm the groupings.

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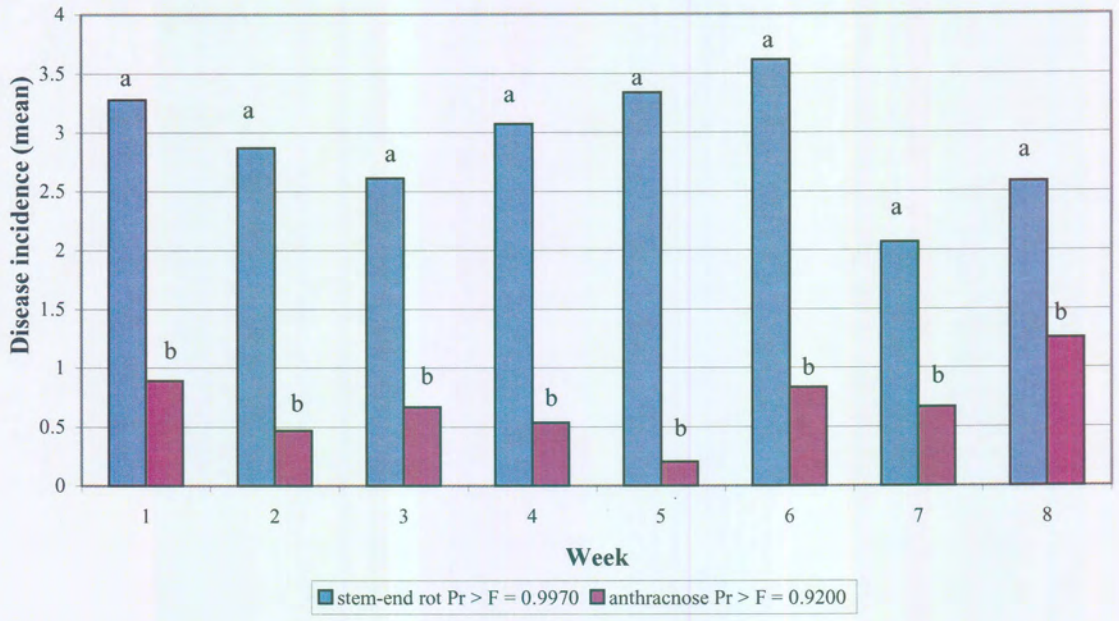
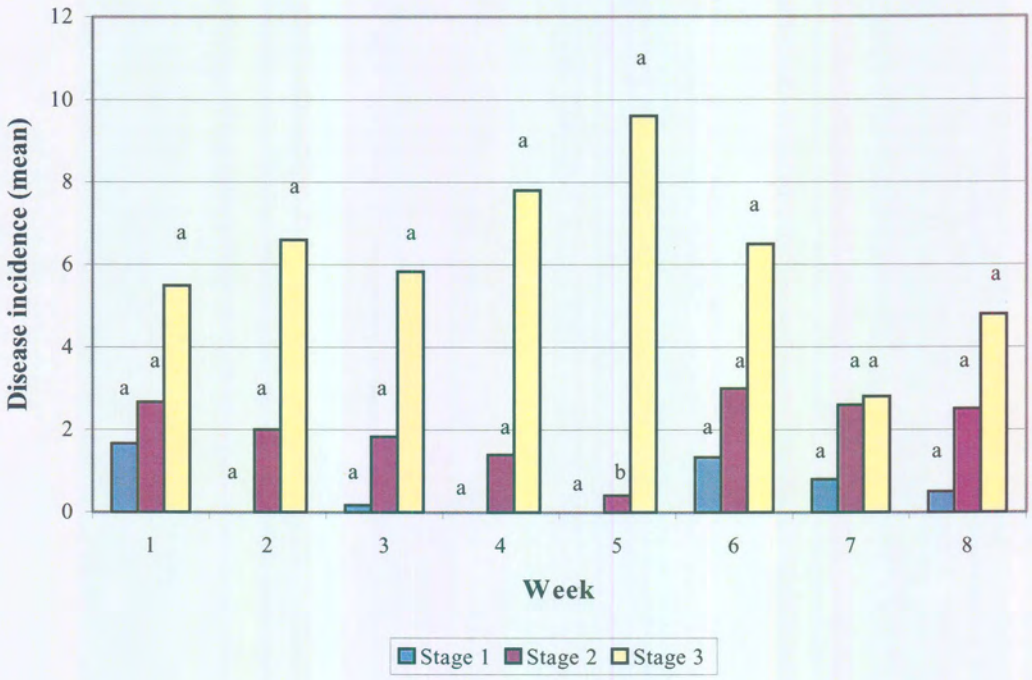


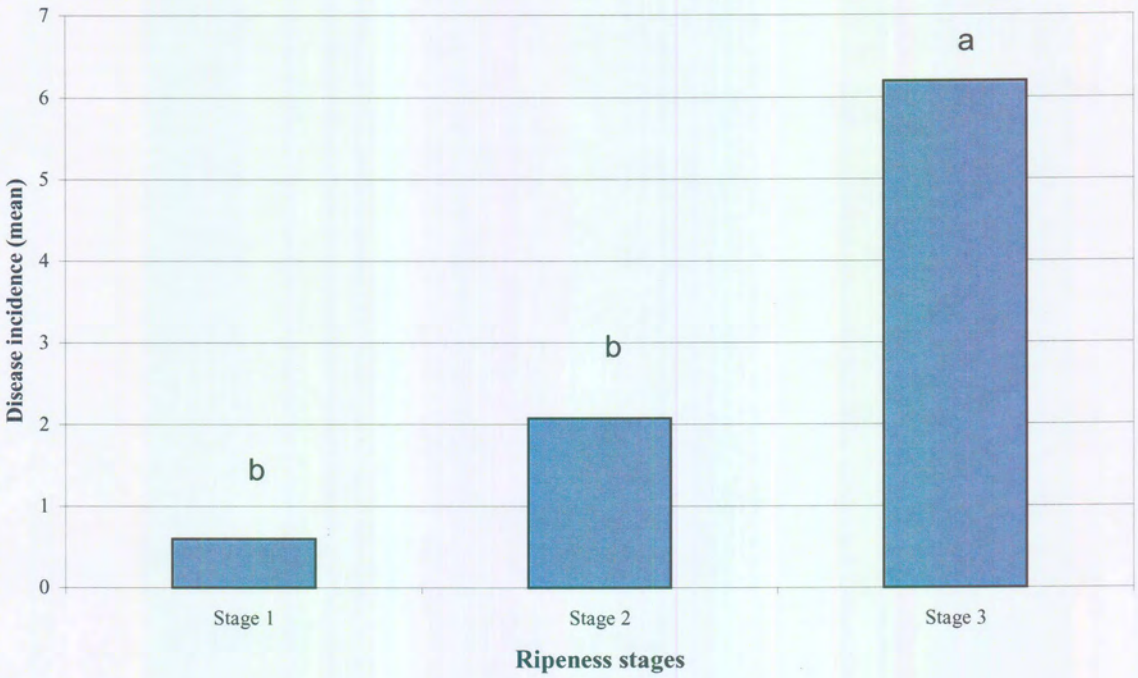
Bars with the same letter are not significantly different according to Duncan's multiple range test

Fig. 3.1 Comparison of the incidence of anthracnose and stem-end rot over an eight-week observation period.



Bars with the same letter are not significantly different according to Duncan's multiple range test (Week1: P=0.4761; Week2: P=0.2935; Week3: P=0.2837; Week4: P=0.0919; Week5: P=0.0237; Week6: 0.2927; Week7: P=0.7213; Week8: 0.4872).

Fig.3.2 Comparison of the incidence of stem-end rot evaluated at the three different ripeness stages over an eight-week period.



Bars with the same letter are not significantly different according to Duncan's multiple range test (P=0.0001).

Fig. 3.3 Incidence of stem-end rot at different stages of ripeness over the entire observation period.

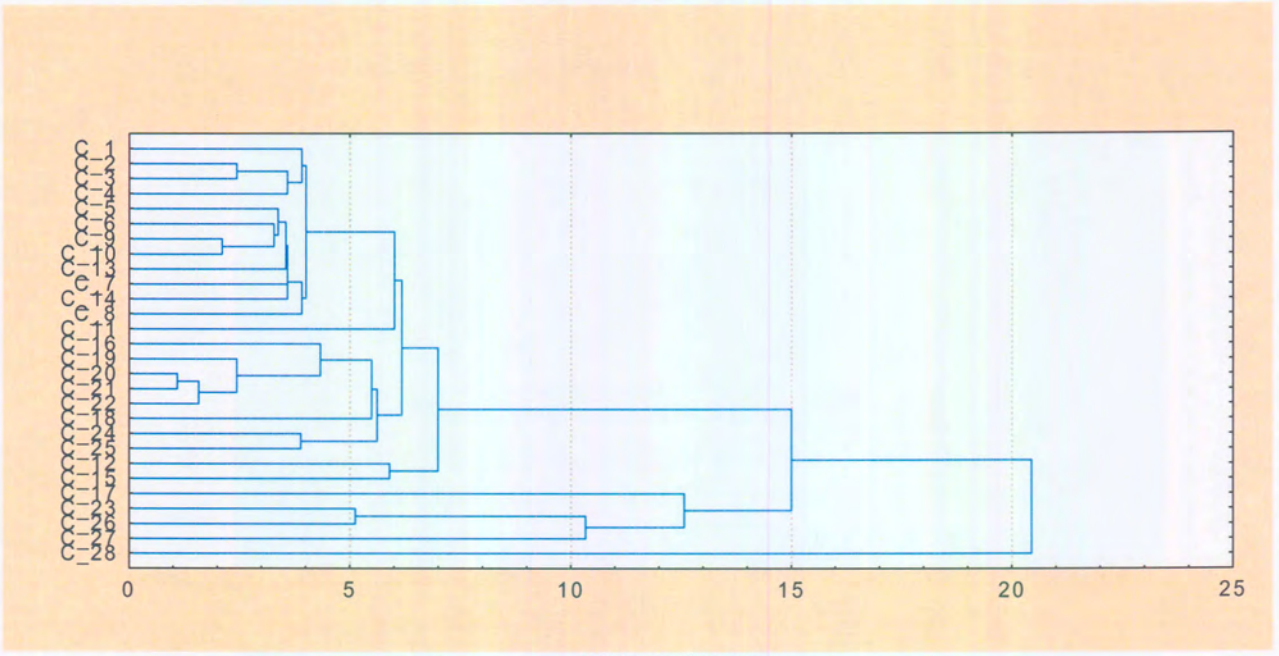
Table 1 Frequency of isolation of stem-end rot pathogens from 'Fuerte' avocado fruit

Pathogen name	Frequency of isolation (%)
Dothiorella aromatica	31
Phomopsis perseae	14
Lasidioplodia theobromae	8.5
Acremomium charticola	7.8
Thyronectria pseudotrichia	5.4
Gliocladium roseum	4.7
Pestaloptiopsis versicolor	3.9
Fusarium solani	3.1
Fusarium spp	2.3
Colletotrichum gloeosporioides	2.3
Drechslera setariae	1.6
Alternaria spp.	1
Sterile cultures	14.7

Table 2 Evaluation of pathogenicity of *Dothiorella aromatica* isolates by inoculation on 'Fuerte' fruit

Isolate number	Mean lesion size (mm)[a]
12	56.2a
23	50.8ab
34	50.7ab
14	50.6ab
5	49.9abc
1	49.8abc
11	48.7abcd
9	48.0bcd
32	47.3bcde
15	47.1bcdef
6	46.7bcdef
2	46.0bcdef
21	45.7bcdef
27	44.8bcdef
25	44.0bcdef
3	43.8bcdef
26	43.5bcdef
7	43.2bcdef
24	41.8cdefg
4	40.3defg
8	39.4efg
17	38.9fg
13	34.6g
39	24.8h
22	22.6h
36	14.9I
31	13.8I
Control	0.0j

[a] Means with the same letter do not differ significantly according to Duncan's multiple range test (P=0.0001).



C-values are isolate case number designations assigned by Statistica

Fig. 3.4 A dendogram of *Dothiorella aromatica* isolates showing relatedness amongst isolates with regards to lesion size produced on ''Fuerte'' avocado fruit.

CHAPTER 4

PHYSIOLOGICAL COMPARISON OF *DOTHIORELLA* ISOLATES OBTAINED FROM AVOCADO

ABSTRACT

Dothiorella aromatica isolates from avocado were compared using physiological characteristics. Isolates grew significantly better at 25 than 15 and 37 °C. However no significant differences were found between isolates when compared at 25 °C. Pectin as a carbon source supported growth most effectively and isolates could be segregated into two clusters. Isolates grew significantly better on urea as a nitrogen source. Isolates were also separated into two groups on urea. Like many fungi, the best growth was observed at pH 6 and least at pH 8. At pH 6, isolates were separated into two clusters with the one having only one isolate. Similar but inconsistent groupings were found with all physiological tests, and these results were consistent with that described in the previous chapter using pathogenicity studies.

INTRODUCTION

Dothiorella aromatica (Sacc.) Petrak and Sydows is one of the most frequently isolated fungi in stem-end rot (SE) lesions of avocado (Fitzell and Coates, 1995). To date this fungus was mostly identified using morphological criteria (Darvas, 1982). However, fungal diagnosis based on only one such method is insufficient for systematic and population studies (Mitchell *et al.*, 1995). Furthermore, it may result in different fungi being assigned to the same genera and species. Traditional taxonomy has largely been based on morphological criteria, supported by biochemical or physiological markers (Maclean *et al.*, 1993). However, knowledge of physiological characteristics of *D. aromatica* is still lacking.

In addition to the above, limited control of SE can be achieved with the use of preharvest fungicide sprays such as copper oxychloride or post-harvest prochloraz applications (Darvas and Kotzé, 1987; Lonsdale & Kotzé, 1989). Although prochloraz is currently registered for control of SE (Nel *et al.*, 1999), tolerance levels specified by most of the importing countries including France are very low as

a result most growers do not use this fungicides (Derek Donkin, personal communication). Furthermore, copper oxychloride often leaves visible residues, which must be removed manually in the packhouse (Korsten *et al.*, 1994). Due to these constraints SE has been difficult to contain and it therefore requires appropriate alternative control measures. One such alternative method that has been developed for avocado fruit diseases is biological control. Korsten *et al.* (1989) showed the potential of using *Bacillus subtilis* in controlling amongst others SE. However, inconsistent control has forced researchers to adopt a more ecological approach of better understanding the nutrient requirements of both the pathogen and the antagonist. By providing this information better product formulation can be developed that favour antagonist growth.

In order to design a more effective control strategy and to better understand the etiology of the fungus, it is important to know as much as possible about the organism. This study was conducted to provide basic information on the physiology of *D. aromatica* by comparing isolates using growth rate, temperature, pH response and carbon and nitrogen requirements as criteria.

MATERIALS AND METHODS

Growth rate and temperature response

Twenty-five starter cultures of D. *aromatica* (Appendix 3) were plated onto potato dextrose agar (PDA) (Biolab) and incubated at 25 °C for 10 days. Details of these isolates and the source of their origin are shown in Appendix 1. Growth rate was determined based on the method of Swart (1999). Briefly four-millimeter plugs were cut from the colony edge and plated centrally onto three replicate Czapek-Dox (Merck) plates. Plates were incubated at 15, 25 and 37 °C for the duration of the experiment. Four-colony diameters were measured twice a day for seven days, and the mean daily growth rates were subsequently calculated. Data was statistically analyzed with SAS using analysis of variance as described in Chapter Three.

Carbon and nitrogen utilization

Twenty-seven isolates of *D. aromatica* (Appendix 4) were prepared as described. Details of these isolates are shown in Appendix 1. To determine the influence of different carbon and nitrogen sources on the growth of *D. aromatica* isolates, the method of Paterson and Bridge (1994) was used, where carbon 1 % (w/v) and nitrogen 0.2 % (w/v) was added to the basal media. The only exception was sodium nitrite, which was added to a final concentration of 20 mM. The final pH of all media was 5.5 except for sodium nitrite, where the pH was adjusted to 7 to reduce potential toxicity. The following carbon sources were evaluated: DL-mannitol (Saarchem), sorbitol (BDH), pectin (BDH), and cellulose (carboxymethyl cellulose) (BDH). Nitrogen sources that were evaluated include ammonium dihydrogen phosphate $NH_4H_2PO_4$ (Saarchem), sodium nitrate ($NaNO_3$) (Saarchem), sodium nitrite ($NaNO_2$) (Saarchem), casein hydrolysate (Biolab) and urea (Merck). All sources were added to the basal medium prior to autoclaving except for mannitol, sorbitol and urea, which were filter-sterilised through a 0.22 µm filter and added aseptically to autoclaved medium.

Plugs were cut from 10-day old starter cultures of *Dothiorella* and placed centrally on three plates containing either a carbon or nitrogen source. For the control, plugs were also placed on three replicates on basal media without a carbon or nitrogen source. Plates were incubated at 25 °C in darkness and four colony diameters were measured after seven days and the mean calculated. Statistical analysis of data was done as described.

pH response

Twenty-five isolates of *D. aromatica* (Appendix 5) were prepared as described above. Essentially the method of Swart (1999) was followed to determine the optimum pH range of *Dothiorella* isolates. Briefly, citrate-phosphate buffer was used for determining the response of *Dothiorella* isolates at pH 5. Phosphate and boric acid-borax buffer was used for pH 6 or 7, and pH 8 respectively. Basal medium was then added to each litre of buffer (Swart, 1999). Twelve grams per litre of Bacto Agar (Difco) was added to each buffer. Four-millimeter plugs punched from the edge of the cultures were plated onto three replicate plates per pH level tested, viz., 5, 6, 7, and 8. Plates were incubated at 25

°C in darkness and four colony diameters were measured after seven days. The mean for the four measurements was calculated. Statistical analysis of data was conducted as described previously.

RESULTS

Growth rate and temperature

Highly significant differences in the growth rate of *D. aromatica* isolates were observed at three different temperature responses tested i.e. 15, 25 and 37 °C (P=0.0001). Isolates grew profusely at 25 °C followed by 37 and 15 °C respectively (Fig. 4.1). Isolates were compared at 25 °C for differences in growth rate since this temperature was found to be the optimum. At this temperature, no significant differences were found between isolates according to Duncan's multiple range test (P=0.9944) and the data was therefore not included. Overall, isolates grew a mean growth rate of 5 mm day^{-1}. Isolates grew a minimum of 8 to a maximum of 27 mm, with an average growth of 14.8 mm within 24 hours. After 72 hours, some isolates were already growing at the maximum of 80 mm.

Carbon and nitrogen source utilization

Dothiorella isolates showed highly significant differences in growth on all carbon sources tested (P=0.0001). Isolates grew best on pectin resulting in a mean colony diameter of 74 mm, followed by mannitol (67 mm), starch (63 mm) and cellulose (55 mm) (Fig. 4.2). Sorbitol amended-media was the least utilized carbon source with a mean colony diameter of 38 mm after seven days. Since pectin supported growth most effectively, isolates were compared or growth on this source. Hierarchial comparison of isolates on pectin revealed two groups at a linkage distance of 37.9 (Fig. 4.3). Most isolates could be grouped within the first cluster whereas the other one comprised only one isolate (114a). The first group could be further classified into two groups at a linkage distance of 23 with one isolate (164c) on its own in one group.

Significant differences were found between isolates when grown on different nitrogen sources (P=0.0001) (Fig. 4.4). Isolates grew best on urea, followed by sodium nitrite, ammonium phosphate, sodium nitrate and casein. However, no significant differences were found between isolates when

grown on sodium nitrite, ammonium phosphate and sodium nitrate. Isolates were compared for growth on urea since this source supported growth the best. Hierarchial comparison of isolates based on their ability to utilise urea revealed two groups at a linkage distance of 26.5 (Fig. 4.5). Most of the isolates grouped within the first cluster, with the second consisting of only one isolate (114a). The first cluster was further subdivided into two groups at a linkage distance of 10.8, with a second subgroup consisting of only one isolate (95c). The differences in the linkage distance between pectin and urea source utilization clearly show that isolates are more closely related in terms of urea utilization compared to pectin utilization (Fig 4.5 and Fig 4.3).

pH response

Highly significant differences in growth of *Dothiorella* isolates were observed at four pH levels investigated i.e. 5, 6, 7 and 8 (P=0.0001) (Fig. 4.6). Isolates grew best at pH 6 and least on medium with an initial pH of 8. However, hierarchial comparison of isolates based on pH 6 revealed two groups at a linkage distance of 12.1 (Fig. 4.7). Most isolates grouped within the first cluster whilst the second comprised only one isolate (164c). The groupings were similar but not consistent with the results of carbon and nitrogen utilization.

DISCUSSION

The effects that carbon and nitrogen as well as, temperature and pH had on the growth rate of *D. aromatica*, was investigated in this study. Isolates could be segregated into two groups based on carbon and nitrogen utilization. Of these the one group consisted of the majority of isolates, whilst the other had only one isolate (114a). The temperature optima for *D. aromatica* isolates of 25 °C, correlates with that of other fungi (Lilly and Barnett, 1951; Wastie, 1972; Swart, 1999). Temperature growth response has been used to distinguish between *C. gloeosporioides* of avocado and almond (Freeman *et al.*, 1998; Swart, 1999). In this study, isolates could not be separated at 25 °C. This not only shows that isolates from the Tzaneen area cannot be separated according to temperature but may indicate that adaptability to a specific region may occur. Swart (1999), reported isolates of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. from cooler regions of KwaZulu Natal growing significantly better at 15 °C and also found that they were adapted to cold conditions.

Better growth at 37 °C compared to 15 °C could indicate that isolates are better adapted to warm than cooler conditions. It is perhaps important to note that isolates were representative of the Tzaneen area with an annual summer temperature of 25 to 30 °C. According to Griffith (1994), fungi are characteristically tolerant of low pH and most have optimum pH ranges for growth of between 5.0 and 7.0. The observed pH optimum of 6 is in accordance with other similar fungi (Cochrane, 1958; John and Berry, 1975; Swart, 1999). The acid value of avocado oil ranges from the minimum of 1 to maximum of 7 (Biale and Young, 1971; Human, 1987), which may explain poor fungal growth on pH 8, which is much higher than the pH of the host. This was also evident in a study by Swart (1999) done with *C. gloeosporioides* isolates from avocado.

From this study, it was found that *D. aromatica* isolates are capable of utilizing a wide range of carbon and nitrogen sources. Carbohydrates are the most common carbon sources for fungal growth (Hawker, 1950). Pectin was the best-utilized carbon source. According to Biale and Young (1971), the middle lamella of avocado fruit starts to disintegrate during ripening which is coupled with an increase in water-soluble pectin, which may be available for use by fungi.

Utilization of starch, which is an insoluble polysaccharide, suggests that *D. aromatica* isolates may produce the necessary enzymes such as amylase, which hydrolyses starch. In addition, starch has not been reported as a common carbohydrate in avocado (Nogalingam, 1993; Kadam and Salunkhe, 1995). Isolates were also capable of utilizing cellulose as a carbon source. Avocado is regarded as the most representative of those crops in which cellulases are of primary importance (Biale and Young, 1971). This may suggest that enzymes from the host itself, hydrolyses cellulose to simple compounds that can be utilized by the fungus.

Significantly more growth was found on mannitol- than sorbitol-amended media. Mannitol has been reported to occur naturally in fruits such as cherry (Desai and Salunkhe, 1995) and olive (Raina, 1995; Olías and García, 1997), whilst sorbitol is found in prunes, pears and apples (Lindsay, 1985), peach and nectarines (Joshi and Buthani, 1995), and cherry (Desai and Salunkhe, 1995). The ability of isolates to utilize mannitol more effectively may suggest that in the absence of the host, the fungi may survive on other hosts. The teleomorph of *Dothiorella/Fusicoccum* (*Botryosphaeria ribis*) has been reported to occur on various woody crops such as kiwi, mango, citrus and apple (Von Arx,

1987; Punithalingam, 1980; Sutton, 1980; Pennycook & Samuels, 1985; Johnson, 1992). *Dothiorella* isolates were capable of utilizing nitrites, which are considered to be toxic (Griffith, 1994). Isolates utilized nitrites, nitrates and ammonium phosphate similarly as nitrogen sources.

Though many findings of this study remain unexplained, it can be concluded that *D. aromatica* isolates can be segregated into two groups according to their physiological characteristics, however differences found in this study will need to be confirmed at a molecular level.

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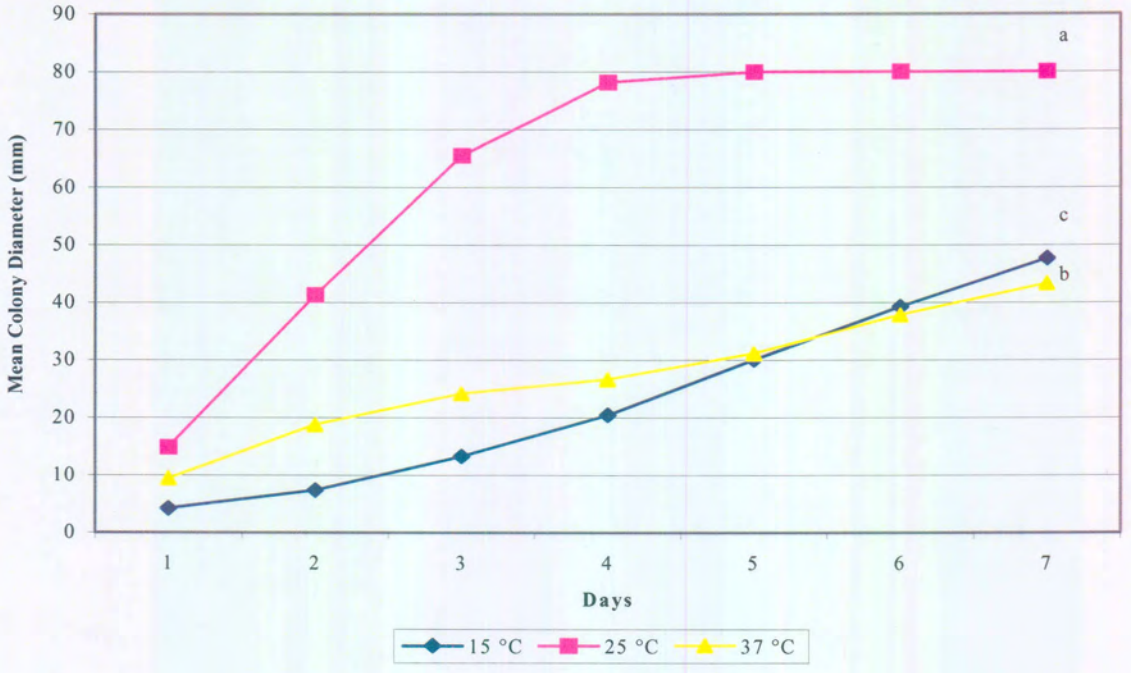
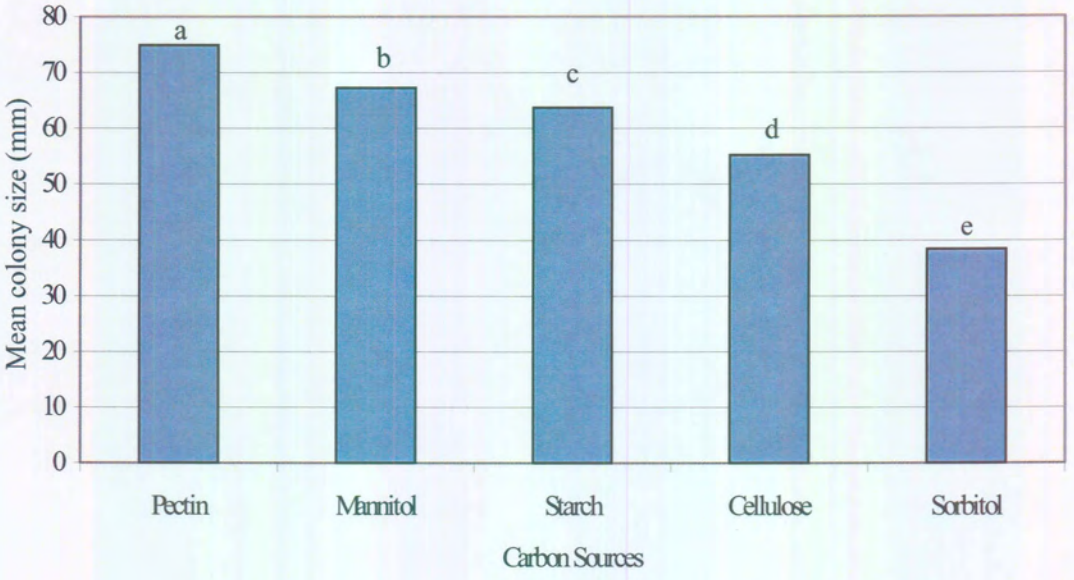


Colony size indicates the average colony size for all isolates at a specific temperature for each day. Line graphs with the same letters do not differ significantly according to Duncan's multiple range test (P=0.0001).

Fig.4.1 Comparison of *Dothiorella aromatica* isolates using growth responses at three different temperature ranges.

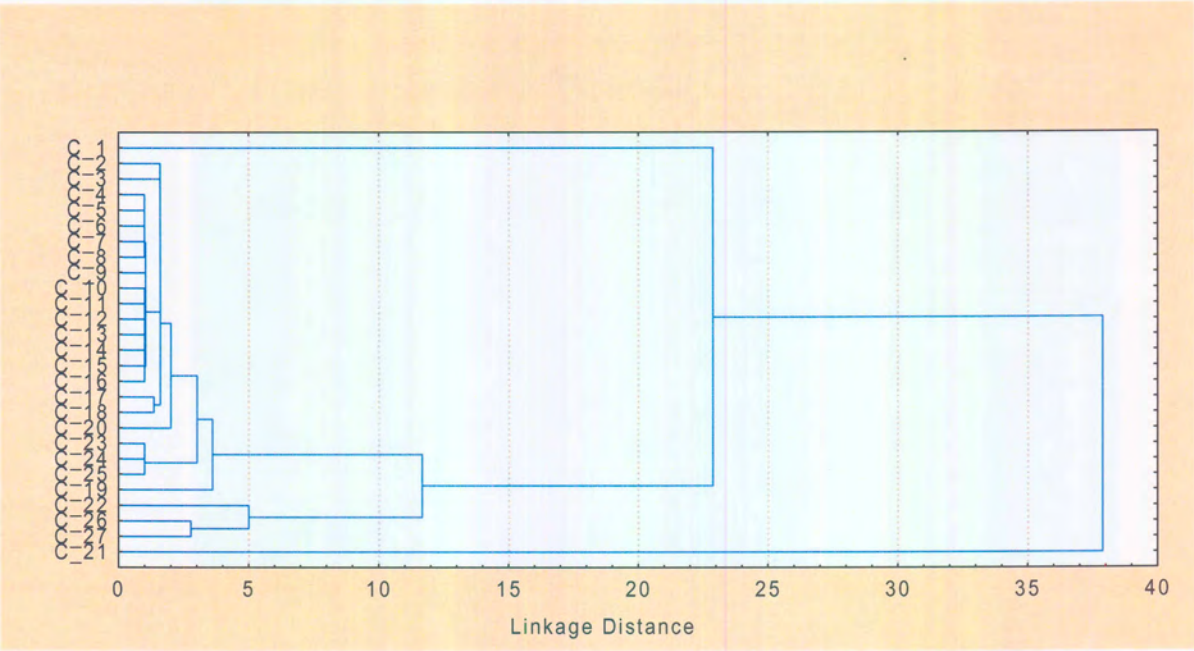


Bars with the same letter are not significantly different according to Duncan's multiple range test (P = 0.0001).

Fig. 4.2 Comparison of *Dothiorella aromatica* growth responses on different carbon source amended media.

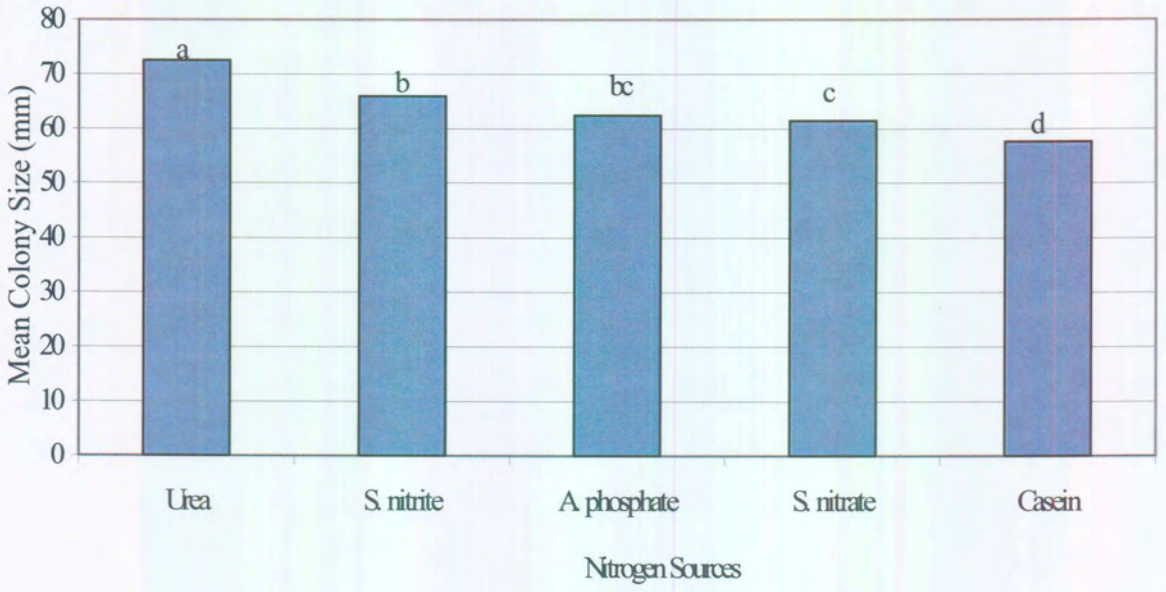
Ward's method

Euclidean distances



C-values are isolate case number designations assigned by Statistica

Fig. 4.3 Hierarchical comparison of growth responses of *Dothiorella aromatica* isolates on pectin using Ward's method of clustering according to Euclidean distances.

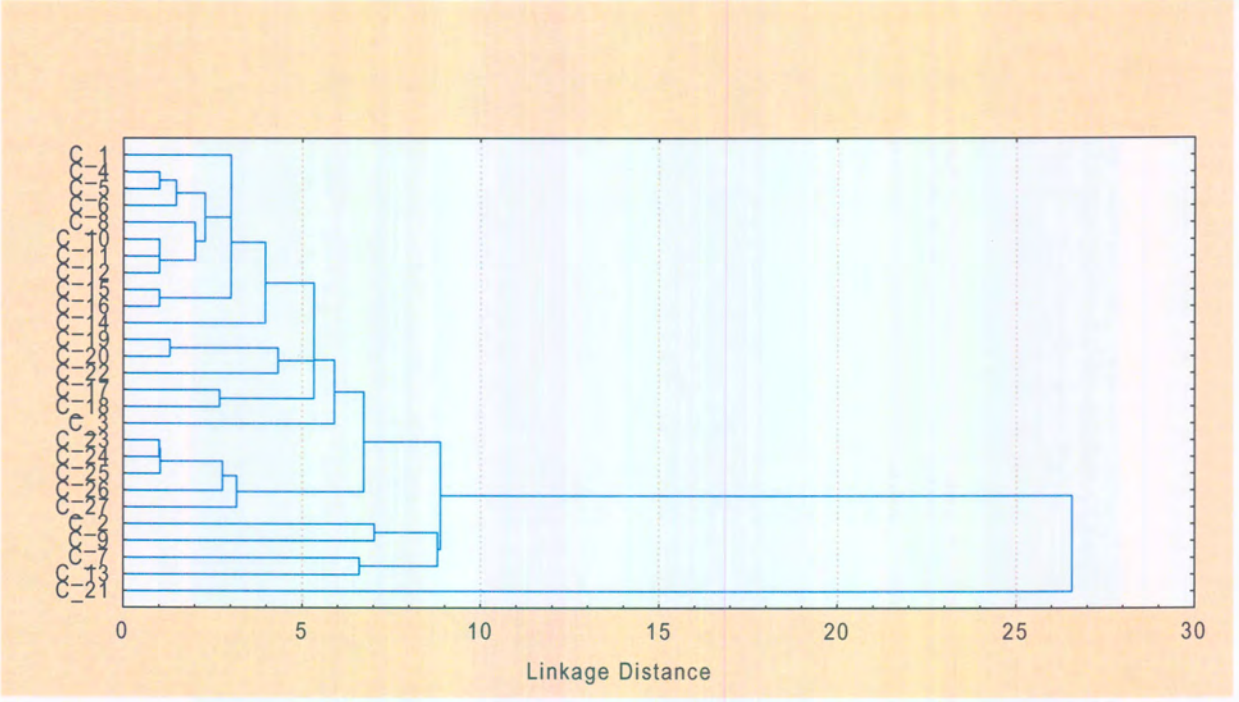


Bars with the same letter are not significantly different according to Duncan's multiple range test (P=0.0001).
*S=sodium, A=ammonium

Fig. 4.4 Comparison of *Dothiorella aromatica* growth responses on different nitrogen source amended media.

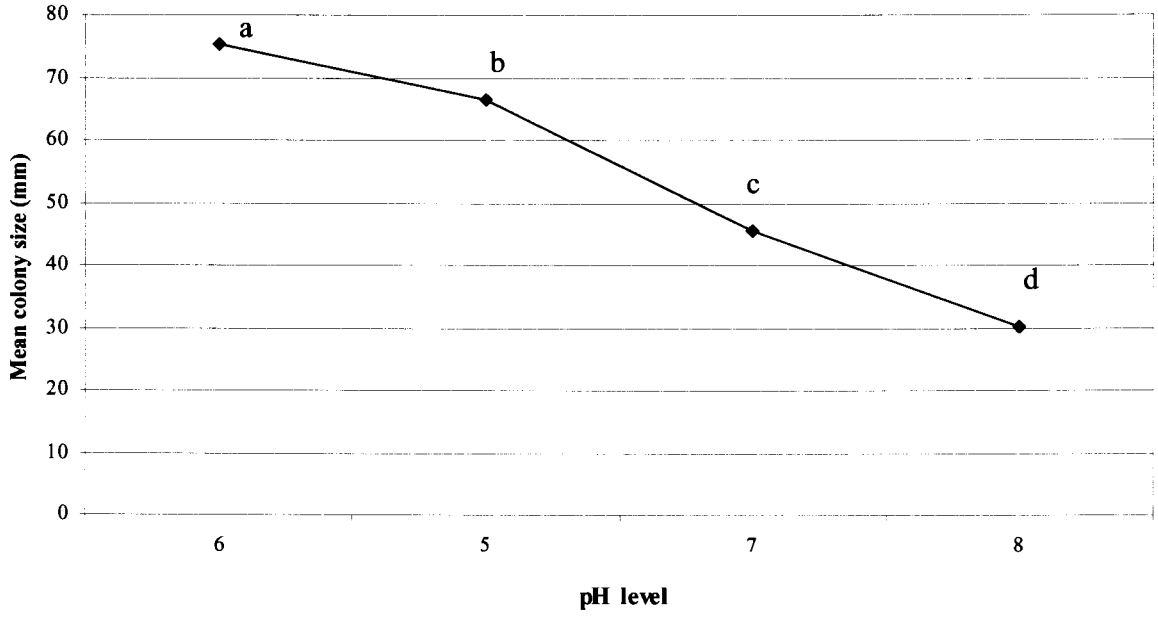
Ward's method

Euclidean distances



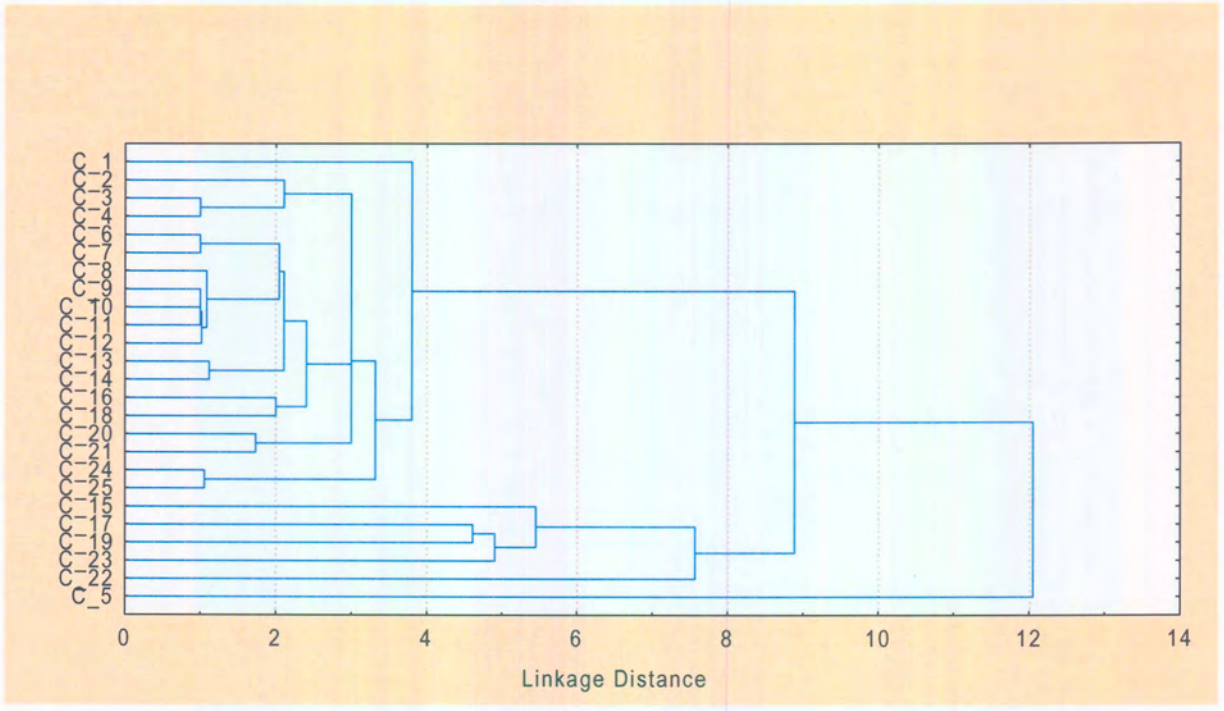
C-values are isolate case number designations assigned by Statistica

Fig. 4.5 Hierarchical comparison of growth responses of *Dothiorella aromatica* isolates on urea using Ward's method of clustering according to Euclidean distances.



Points with the same letter do not differ significantly according to Duncan's multiple range test (P=0.0001).

Fig 4.6 Growth response of *Dothiorella aromatica* isolates at different pH levels.



C-values are isolate case number designations assigned by Statistica

Fig. 4.7 Hierarchical comparison of growth responses of *Dothiorella aromatica* isolates on pH 6 using Ward's method of clustering according to Euclidean distances.

CHAPTER 5

RAPD COMPARISON OF *DOTHIORELLA AROMATICA* ISOLATES FROM AVOCADO

ABSTRACT

Genetic comparison among thirty-six isolates of *Dothiorella aromatica* was carried out using random amplified polymorphic DNA (RAPDs). Three primers (OPC02, OPE14 and OPA02), were screened for their ability to distinguish between isolates. The primer OPC02 produced polymorphic patterns that were reproducible and could discriminate between *D. aromatica* isolates, *Colletotrichum gloeosporioides* and *Lasidioplodia theobromae*. Further comparisons were subsequently carried out using OPC02. Isolate profiles produced three to eight bands with DNA fragments ranging from 1500bp to 450bp. A dendogram constructed from the number of bands showed that isolates could be separated into three groups (I–III). The results of this study were not consistent with previous results from pathogenicity and physiological tests.

INTRODUCTION

Dothiorella aromatica (Sacc.) Petrak and Sydow is regarded as one of the important causal agents of stem-end rot of avocados (Darvas and Kotzé, 1987). However, detailed characterisation of the genetic variability among isolates of *D. aromatica* has not been reported despite the prevalence and economic importance of the fungus. Such characterisation would be useful for pathogenicity studies and to trace the spread of the disease amongst geographic regions. This has been the case with bacterial blackspot studies on mango (Pruvost *et al.*, 1998). Molecular studies with other pathogens suggest that many fungi may spread clonally so that populations consist of morphologically identical groups that may differ genetically (Mitchell *et al.*, 1995). This is of agricultural and taxonomic importance since it provides information on how a pathogen spreads in a population and whether it exchange genes which may have implications in control strategies (Mitchell *et al.*, 1995).

Isolates of *D. aromatica* have been identified almost exclusively based on morphological and cultural characteristics (Darvas, 1982). Characters such as conidial morphology and the shape of fruiting structures have been used to differentiate fungal species (Maclean *et al.*, 1993). While these phenotypic characteristics can adequately describe the grouping it does not always help explain its origins. Pathogenicity and physiological tests are often time consuming, too costly, insensitive for use in routine diagnosis or ineffective in distinguishing closely related isolates (Schneider *et al.*, 1997). This was confirmed in the previous chapter where results from different tests were not always consistent. Other methods such as immunoblotting and western blotting (Vigrow *et al.*, 1991) and protein banding patterns using SDS (PAGE) (Swart, 1999) have been investigated in order to differentiate between isolates. However these methods rely on culture of isolates and gene expression rather than whole genomic DNA and are still relatively slow (Theodore *et al.*, 1995).

Several methods based on analysis of genomic DNA for the identification of fungal pathogens have been developed (Silva *et al.*, 1998). These techniques include restriction fragment length polymorphisms (RFLPs) (Buscott *et al.*, 1996), random amplified polymorphic DNA (RAPD) (Manzanares-Daulex, 2001) and amplified fragment length polymorphic DNA (Steele *et al.*, 2001).

RAPD analysis is a simple and rapid technique for comparing fungal isolates (Williams *et al.*, 1990). This technique has been used successfully to differentiate and identify isolates of many fungi, amongst others *Colletotrichum* spp. causing anthracnose of various fruits, sorghum and *Stylosanthes* spp. (Freeman *et al.*, 1998; Guthrie *et al.*, 1992; Munaut *et al.*, 1998), *Podosphaeria leucotricha* (Ell. et Ev.) Salm. causing powdery mildew in apples (Urbanietz and Dunemann, 2000), *Eutypa lata* (Pers:Fr.) Tul & C. Tul. causing dieback of grapevines (Peros *et al.*, 1997), and *Elsinoe* spp. causing scab of citrus (Tan *et al.*, 1996). The purpose of this study was to compare isolates using RAPD's and to confirm the results of pathogenicity tests (Chapter Three) and physiological tests (Chapter Four).

MATERIALS AND METHODS

Culture collection

Thirty-six monoconidial isolates of *D. aromatica* used in this study were randomly selected from the culture collection obtained in Chapter Three (Appendix 6). Details of their source of origin are shown in Appendix 1. Two isolates of *Lasidioplodia theobromae* (Pat.) Griffon & Maubl. and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph *Glomerella cingulata* (Stonem.) Spauld. & Schrenk) each were included for comparative purposes. Isolates were cultured and maintained as described in Chapter Three. The *L. theobromae* isolate was obtained from the culture collection described in Chapter Three. The *C. gloeosporioides* isolate was obtained from Dr Gina Swart in the Department of Microbiology and Plant Pathology.

Fungal mycelium preparation

To produce mycelium for DNA extraction, all isolates were grown for five days under conditions described in Chapter Three. For fungal mycelium preparation, the method of Sreenivasaprasad *et al.* (1993) was used. Briefly, the inoculum of eight 4 mm diameter discs were cut from the edge of actively growing cultures and placed in a 50 ml volume of liquid medium (10 g glucose; 1 g NH_4H_2PO4; 0.2 g KCl; 0.2 g $MgSO_4.7H2O$; 5 g yeast extract (Difco); 1 ml of 0.5 % w/v $CuSO_4.5H_2O$; 1 ml of 1 % w/v $ZnSO_4.7H_2O$ in 1L of sterile distilled water). Mycelium was harvested by filtration through Whatman No. 3 filter paper, immediately frozen in liquid N_2, pulverized, lyophilized, ground to a fine powder and stored at –70 °C until required.

DNA extractions

Template DNA extractions were done using a modified method of Raeder and Broda (1985) as follows: 1000 µl of extraction buffer (200 mM Tris-HCl; 250 mM NaCl; 25 mM EDTA; 0.5 % w/v SDS) was added to 50 mg of mycelium in an eppendorf tube. The mixture was vortexed and centrifuged at high speed for two minutes. The upper aqueous phase was transferred to another tube and phenol-chloroform-isoamyl alcohol (25:24:1) was added, mixed well and centrifuged as

described above. This step was repeated until the supernatant was clear. The aqueous phase was transferred into another eppendorf tube and 2.5 µl RNAse A (Sigma) was added and incubated for 30 minutes at 37 °C to remove ribonucleic acid (RNA). The volume was adjusted to 500 µl with sterile double distilled water and DNA precipitated by adding 50 µl 3M ammonium acetate and 1000 µl of 97 % ice-cold ethanol. Tubes were then placed at –20 °C for 1-2 hours. The mixture was centrifuged at high speed for 15 minutes, and the pellets rinsed with 70 % ethanol to remove salts. The pellets were dried under vacuum for five minutes and the DNA was dissolved in 100 µl sterile distilled water and stored at -20 °C until use. The presence of DNA and hence its purity and concentration was confirmed by running a 10 µl sample on a 0.8 % agarose gel (Promega).

PCR amplification with random primers

Three random 10-mer primers (Operon Technologies Inc.) (OPC02: 5'-GTGAGGCGTC-3', OPE14: 5'-TGCGGCTGAG-3'and OPA02: 5'-TGCCGAGCTG-3') were screened for their ability to distinguish between *D. aromatica* isolates. Reactions were performed in 50 µl volumes with 0.5 µl template DNA, 0.6 µl of each respective 10-mer primer, 5 µl recommended 10 x buffer (supplied with *Taq* polymerase), 2 mM $MgCl_2$, 100 µM each of dCTP, dGTP, dATP and dTTP (Promega) and 0.5 U *Taq* polymerase (Promega). PCR was performed using the following conditions: one initial denaturation cycle at 94 °C for two minutes, followed by 35 cycles of denaturation at 94 °C, annealing at 35 °C and extension at 72 °C for 90 seconds, followed by a single final extension cycle of 72 °C for 10 minutes. Analysis was carried out by adding 3 µl loading buffer (Maniatis *et al.*, 1982) to 10 µl PCR product which was loaded onto a 2 % horizontal agarose gel using a Tris-borate buffer (TBE) (Maniatis *et al.*, 1982) system. Electrophoresis was performed at 75 V for two hours. Gels were stained for 30 minutes with 0.5 µg ml^{-1} ethidium bromide (Merck) and viewed under UV light. Sizes of products were confirmed by comparison with a molecular mass marker (100 bp, Promega), which was loaded onto each gel. The gels were digitised and analysed with the GelCompar 4.0 computer programme (Applied Maths Kortrijk, Belgium). The Dice-coefficient was used to construct a distance matrix. A tolerance of 4.0 % in band positions was allowed. The unweighted pair group method of arithmetic averages (UPGMA) was used to cluster the isolates.

RESULTS

DNA was successfully extracted from the 37 isolates studied (Fig. 5.1). All three primers tested gave banding patterns for all isolates tested, however primer OPC02 consistently produced discriminatory banding patterns. The size of the DNA fragments ranged from 1500 bp to 450 bp. From the screening results, OPC02 was selected for use in further studies because of the number of polymorphisms generated by this primer. DNA extractions and subsequent RAPD analyses were done in duplicate and were found to be reproducible.

A level of genetic variation was observed in the *D. aromatica* population tested in this study (Fig. 5.2). A dendogram constructed from this data showed three distinct groups based on the number of bands (Fig. 5.3). The first group consisted of only three isolates with two isolates containing RAPD profiles of five bands. The other isolate had seven bands. Most isolates grouped together into the second cluster with profiles containing from five to eight bands, the last group had the least number of bands. The second group could be further subdivided into two isolates of almost equal number of isolates with RAPD profiles of four to eight and 5-7 bands. Group II and III were closely related and group I was the most dissimilar of the three.

Two isolates of *L. theobromae* and *C. gloeosporioides* that were included for reference were clearly different from *D. aromatica* isolates and from each other. Isolates of *L. theobromae* were more related to *Dothiorella* than *C. gloeosporioides*. The *C. gloeosporioides* isolate had only one band whilst *L. theobromae* contained five bands. None of the *D. aromatica* isolates contained RAPD profiles with only one band.

DISCUSSION

Analysis of RAPD profiles has proven to be an effective, efficient and rapid technique for comparing isolates of *D. aromatica*. This analysis has shown that there are differences between *D. aromatica* isolates. In this study, the OPC02 primer gave more distinct bands than OPE14 and OPA02. The reason that OPC02 worked better may be due to the fact that this simple repeat sequences occurs more often in isolates tested. Based on the data obtained in this study, it could be speculated that

three different populations within *D. aromatica* may occur in the Tzaneen area. However, these results were not consistent with those found using physiological tests in Chapter 4. Isolates that belong to these populations can therefore be identified by their unique RAPD patterns using OPC02.

Taxonomy of *D. aromatica* is faced with a number of shortcomings. Firstly, the taxonomy of this fungus is based exclusively on classical descriptive criteria such as shape of the conidia, structure of the fruiting body (Darvas, 1982) and the properties of the colony (Johnson and Kotzé, 1994). These characters cannot always distinguish between species, and may result in the same species name assigned to different species or vice versa. Hering and Nirenberg (1995) compared isolates of *Fusarium* spp. using RAPD and found that some of the isolates expected to be *F. sambucinum* based on their morphology could not be placed under this the same group based on the band patterns. For conclusive identification of microorganisms both molecular and phenotypic characters should be applied.

Coupled to this problem, is the fact that *D. aromatica* is a slow sporulating fungus and fruiting bodies are rarely seen (Darvas *et al.*; 1987) even when cultures are placed under UV light. There is therefore a need for a quick diagnostic tool. In addition, members of Deuteromycetes have no true sexual stage and the morphological variation between isolates can be taxonomically confusing (Mitchell *et al.*, 1995). RAPD allows differentiation between isolates within a few days of obtaining a sample (Theodore *et al.*, 1995). The speed at which results can be produced would aid in the confirmation of the identity of the fungus as the main cause of SE, assist in tracing the origin and consequently enable development of control strategies. In addition, RAPD can be a useful tool as it allows differentiation of microorganisms without knowledge of specific DNA sequence information (Welsh and McClelland, 1990). To date, none of the previous studies have characterized isolates of *D. aromatica* at the molecular level.

In this study, *D. aromatica* isolates were successfully compared at a molecular level. This data could serve as a first step in the identification of this fungus South Africa. Sequencing of the subset of randomly chosen isolates included in this study suggests that the isolates from avocado mostly group within the species *Botryosphaeria ribis* (anamorph *Fusicoccum* sp.) (Jacobs *et al.*, unpublished). This data corresponds with published data since this species is commonly isolated throughout the

world as the dominant *Botryosphaeria* spp. from various woody crops such as kiwifruit, mango, citrus and apple (Von Arx, 1976; Punithalingam, 1980; Sutton, 1980; Pennycook & Samuels, 1985; Johnson, 1992).

Future studies should focus on comparing isolates from different avocado production areas and testing different primers for the ability to distinguish between isolates of *D. aromatica*. Although the RAPD technique proved effective in distinguishing isolates, techniques such as RFLP or AFLP may be more reliable and repeatable for future use. These techniques have been used extensively in fungal species identification for many *Botryosphaeria* spp (Johnson, 1992; Jacobs *et al.*, unpublished). Based on the sequence data, an identification system can be developed with these techniques, which may facilitate the future identification of *Dothiorella* spp. from avocado in South Africa. These results should be used in conjunction with morphological traits, as these are by far the easiest to use when identifying a fungus since it is usually cheap and quick. Inclusion of molecular information will assist in systematic and population studies and hence clarify the taxonomy of the fungus.

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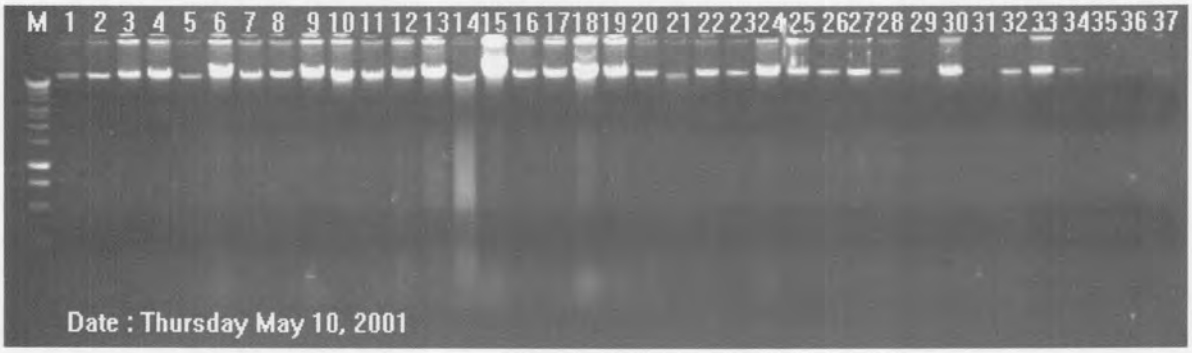
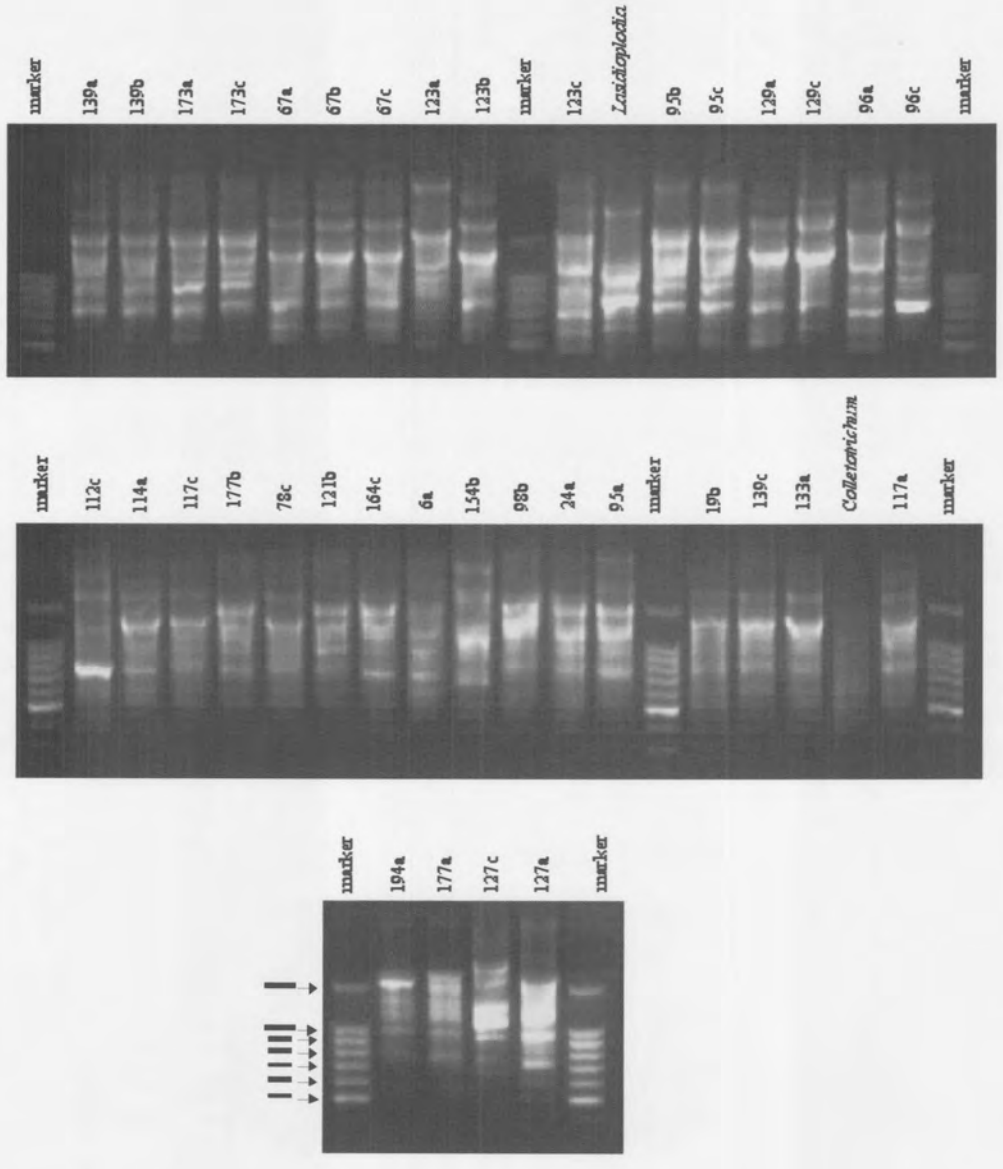


Electrophoresis was carried out on 0.8 % agarose gel run at 75V for two hours

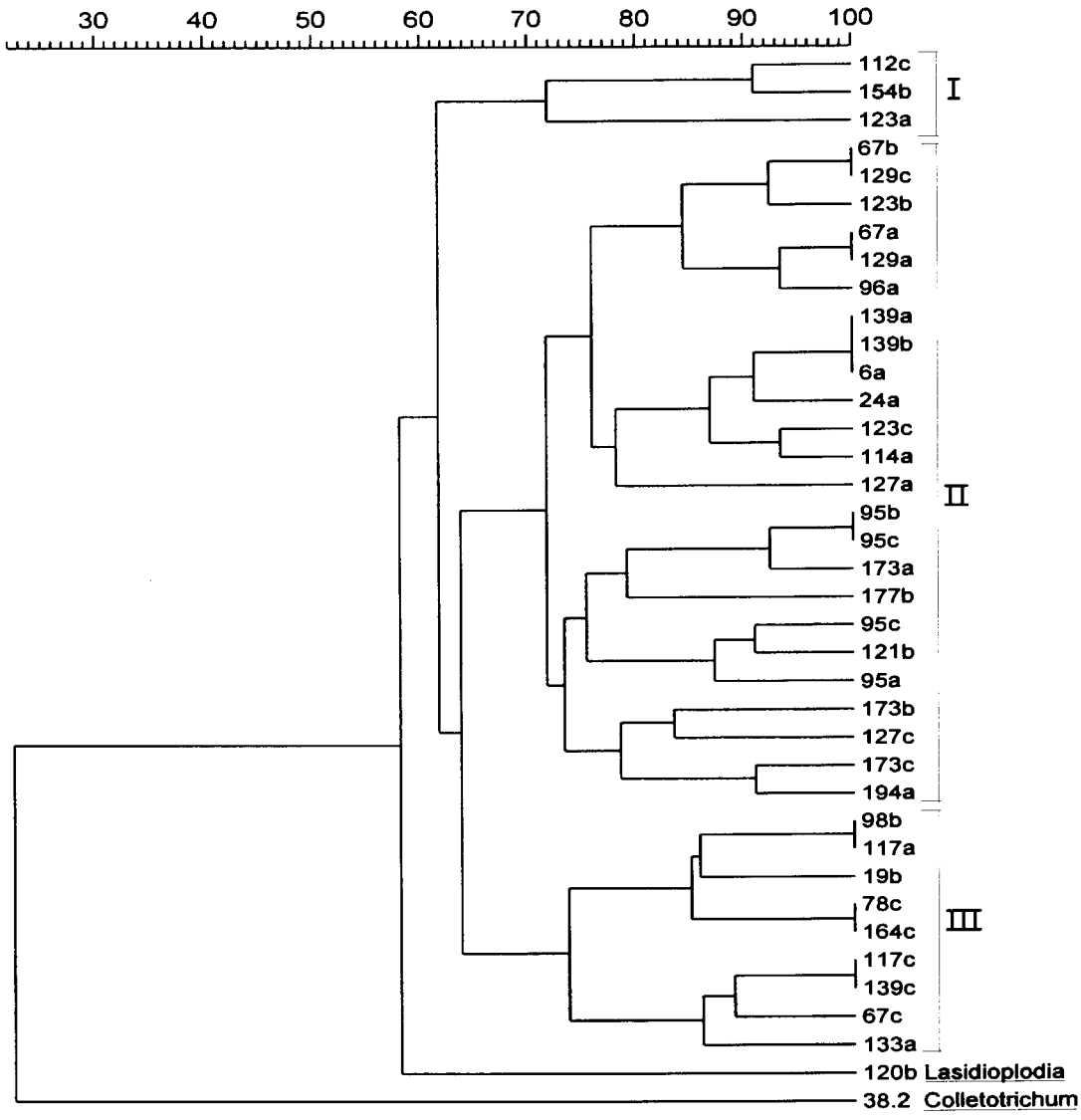
Lane 1:139a, Lane 2:19b, Lane 3:173a, Lane 4:173c, Lane 5:67a, Lane 6:67b, Lane 7:67c, Lane 8:123a, Lane 9:123b, Lane 10:123c, Lane 11:120b, Lane 12:95b, Lane 13:95c, Lane 14:129a, Lane 15:129c, Lane 16:96a, Lane 17:96c, Lane 18:112c, Lane 19:114a, Lane 20:117c, Lane 21:177b, Lane 22:78c, Lane 23:121b, Lane 24:164c, Lane 25:6a, Lane 26:154b, Lane 27: 98b, Lane 28:24a, Lane 29: 95a, Lane 30:Mag5, Lane 31:139c, Lane 32: 133a, Lane 33: 19a, Lane 34: 117a, Lane 35: 194a, Lane 36: 173c, Lane 37: 127c.

Fig. 5.1 Agarose gel electrophoresis showing DNA extraction results of 37 isolates of *Dothiorella aromatica.*



Electrophoresis was carried out on 2 % agarose gel run at 75V for two hours
Lane 1,11,20,21,34,40,41,and 46 are the 100bp ladders.

Fig. 5.2 Agarose gel electrophoresis of RAPD fragments from DNA of *Dothiorella aromatica* generated by primer OPC02.



The unweighted pair group of arithmetic averages method (UPGMA) of clustering was applied to cluster isolates.

Fig. 5.3 Dendogram showing the grouping of representative RAPD band profiles of *Dothiorella aromatica* isolates.

CHAPTER 6

GENERAL DISCUSSION

Avocados (*Persea americana* Mill.) are one of the most important subtropical crops in South Africa, with an annual production of 71 524 tonnes and export volumes amounting to 37 598 tonnes during the 2000/2001 (Abstract of Agricultural Statistics, 2001). Avocado production in South Africa is an export-orientated industry, aimed primarily at the European markets, where there is a great demand for high quality fruit (South African Avocado Growers' Association, 1998). In South Africa, losses due to post-harvest diseases represent a substantial portion of export revevue, since the majority of the fruit (97 %) is exported by sea thus being exposed to long periods of cold storage during shipping (Kohne, 1999). During extended cold storage conditions, fruit quality can be severely affected by post-harvest diseases and physiological disorders, which mostly only develop post-harvestly.

Stem-end rot (SE) is one of the three most important post-harvest diseases of avocado (Snowdon, 1990; Darvas, 1992). Losses due to SE as high as 25 % have been reported on certain export consignments (Sanders and Korsten, 1997). Data for fruit obtained from the local market quantifying the incidence of post-harvest diseases, is scarce. Thus far, only one study could be traced and that was by Sanders and Korsten (1997). In addition, a number of pathogens are associated with the SE syndrome and limited control of the disease is primarily achieved by means of fungicides (Lonsdale and Kotzé, 1989). Furthermore, investigations into alternative control measures for SE such as biological control has been hampered by a lack of knowledge on the relationship between host, pathogen and associated microflora, as well as the characteristics of the plant surface and environment (Korsten, 1995). In addition, knowledge on the life cycle of the fungi associated with SE is also insufficient as well as their nutrient requirements and specific growth requirements.

In the view of the above, this study was undertaken in order to quantify the incidence of SE and investigate the importance of *Dothiorella aromatica* (Sacc.) Petrak and Sydow in relation to other SE pathogens. Isolates of *D. aromatica* obtained from the market survey were compared on the basis of physiological characteristics i.e. growth rate and temperature response, carbon and nitrogen utilization as well as pH response and genetic relatedness using RAPDs.

Results obtained from this study shows that SE plays an important role in post-harvest losses in the South African avocado industry. The incidence of SE was found to be 31 % compared to 18 % for anthracnose. The higher incidence of SE compared to findings of Sanders and Korsten (1997), may be ascribed to drier conditions experienced during the 1998/99 season. Dry conditions promote endophytic infection (Johnson and Kotzé, 1994) and most fungi associated with SE are endophytes. High incidences of SE were found particularly when fruit was evaluated at the overripe than eating ripe stage, which signifies the importance of evaluating fruit at the correct ripeness stage. This confirms that fruit mostly develop symptoms only when it is at the consumer end on the overseas market, thereby damaging the reputation of the South African industry (Johnson and Kotzé, 1994).

A number of fungi were isolated from the SE lesions of 'Fuerte' avocado fruit. Of these, *D. aromatica* was the most frequently isolated fungus, which is in contrast to findings of Darvas and Kotzé (1987), where *Thyronectria pseudotrichia* (Schw.) Seeler was the most frequently isolated pathogen. In the case of *D. aromatica*, it is known to infect uninjured fruit whilst *T. pseudotrichia* is unable to infect through an uninjured rind of fruit (Darvas *et al.*, 1987). This observation may be attributed to the increased awareness of growers when handling fruit from the orchard to the market. This finding also emphasizes the importance of *D. aromatica* in the SE syndrome. The lower isolation frequency of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph *Glomerella cingulata* (Stonem.) Spauld & Schrenk) may be attributed to drier conditions during the 1998/99 season. This pathogen has a minimum moisture requirement for successful infection and colonization (Jeffries *et al.*, 1990), and it can be concluded that conditions could have been unfavorable. This corroborates the lower incidence of anthracnose compared to SE found in this study.

Of interest was the isolation of *Trichothecium roseum* (Persoon) Link which is known to produce the trichotecene mycotoxin, which has a negative effect on eukaryotic cells (Patterson, 1973). These effects include damage to the liver and bone marrow causing haemorrhages (Patterson, 1973). Sporadic isolation of this fungus from the Tzaneen area should raise concerns for exporters who need to conform to Hazard Analysis Critical Control Point (HACCP), Food Safety Standards.

In this study, pathogenicity of *D. aromatica* was confirmed. All 'Fuerte' fruit inoculated with *D. aromatica* isolates developed lesions. Although differences were found between isolates, caution should be taken when interpreting these results. Changes in pathogenicity have been reported to occur in different isolates at different times (Satyaprasad *et al.*, 2000), rendering the pathogenicity test technique to be an unreliable means of making direct comparison on its own. Variation in virulence was found suggesting that isolates from the Tzaneen area are different. In order to explain the cause of these differences, isolates from different cultivars and geographic regions should be studied. Differences in virulence amongst isolates could have implications in the effectiveness of disease control strategies especially chemical control (Agrios, 1997). This includes development of resistant strains, rendering the control agent to be ineffective by only selecting for susceptible strains.

Data obtained from the physiological studies clearly indicates that there are differences between isolates of *D. aromatica*. For all physiological parameters evaluated in this study, two groups were found between isolates. The majority of isolates were highly virulent with only one isolate being less virulent. Isolates grew better at 25 °C than 15 and 37 °C respectively. The Tzaneen area has an average summer temperature range of 25 to 30 °C and it can be concluded that Tzaneen is a potentially high risk area for SE and therefore the control measures should be adapted accordingly. Nevertheless, isolates from cooler regions such as KwaZulu Natal should be studied to rule out the possibility of adaptability by isolates to a specific environment. It is also concluded that in order to obtain optimum growth, isolates should be grown on media with a pH of 6; this is in agreement with findings on many fungi (John and Berry, 1975; Swart, 1999).

This study has also shown that isolates of *D. aromatica* are capable of utilizing a wide range of compounds as carbon and nitrogen sources. The ability of isolates to grow on pectin shows that *D. aromatica* has the necessary enzymes to degrade complex carbohydrates. The ability of isolates of *D. aromatica* to utilize nitrates and nitrites indiscriminately suggested that isolates are capable to metabolise nitrites, which are generally toxic to fungi (Griffith, 1994). Though many of these findings remain unexplained this information provides a broad idea about compounds that can be utilised by *D. aromatica*.

Molecular analysis using RAPD's has confirmed that there is genetic heterogeneity amongst isolates of *D. aromatica* and clearly separated this fungus from other fungi tested. RAPD's have been used to characterise fungal strains or even individuals within species (Guthrie *et al.*, 1992; Tan *et al.*, 1996; Peros *et al.*, 1997; Freeman *et al.*, 1998; Munaut *et al.*, 1998; Urbanietz and Dunemann, 2000). In this study, isolates were segregated into three groups with profiles having three to eight bands. One hundred percent similarities found between some isolates suggest a possibility of true clonality which imply interchange within a population (Satyaprasad *et al.*, 2000). This may be partially explained by the fact that the sexual stage of this fungus is rarely seen (Darvas, 1982). Although these results were not consistent with that of pathogenicity and physiological tests, this data could serve as a first step in the characterisation of this fungus in South Africa. In conclusion, this study has shown that differences exist between isolates of *D. aromatica*. However it is important to use a combination of techniques to confirm these differences. Future studies should focus on obtaining a library of *D. aromatica* isolates from different cultivars, geographic regions and countries and compare isolates using a variety of methods.

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Appendix 1 Details of *Dothiorella aromatica* isolates used in the comparative studies

Isolate code[a]	Isolation week [b]	Stage of ripeness [c]
6A	W1	S3
14a	W1	S2
19A	W1	S2
19B	W1	S2
24A	W1	S2
53A	W2	S2
67A	W1	S3
67B	W1	S3
67C	W1	S3
78C	W1	S3
83B	W3	S1
95A	W2	S3
95B	W2	S3
95C	W2	S3
96A	W3	S3
96B	W3	S3
96C	W3	S3
98B	W2	S2
112C	W3	S2
114A	W2	S2
117B	W2	S3
117C	W2	S3
120B	W2	S3
121B	W2	S3
123A	W2	S3
123B	W2	S3
123C	W2	S3
127A	W2	S3

127B	W2	S3
127C	W2	S3
129A	W3	S2
129B	W3	S2
129C	W3	S2
133A	W3	S3
139A	W3	S3
139B	W3	S3
139C	W3	S3
154A	W4	S3
154B	W4	S3
154C	W4	S3
164A	W3	S3
164C	W3	S3
173A	W4	S3
173C	W4	S3
194A	W4	S3
Mag5	W4	S3

[a] denotes a number designated to a particular isolate when isolations were made from fruit. Isolates with the same number are from the same lesion but different areas.

[b] denotes a week at which isolations were made. W1=Week 1 (23/03/99); W2=Week 2 (6/04/99); W3=Week3 (14/04/99); W4=Week 4 (21/04/99).

[c] denotes stage of fruit ripeness when isolations were made. S1=Stage one: Eating ripe; S2=Stage two: Slightly overripe; S3=Stage three: Overripe.

Appendix 2 *Dothiorella aromatica* isolates used in hierarchical comparison of pathogenicity test

Number[a]	Isolate code[b]
C.1	164c
C.2	6a
C.3	139c
C.4	123a
C.5	123c
C.6	112c
C.7	24a
C.8	67a
C.9	173c
C.11	98b
C.12	120b
C.13	129c
C.14	139a
C.15	96a
C.17	129a
C.21	95a
C.22	19a
C.23	139b
C.24	154c
C.25	Mag5
C.26	95c
C.27	95a
C.31	129b
C.32	127c
C.34	67c
C.36	53a
C.39	96b

[a] denotes a number designating a particular isolate code in the Wards's method.

[b] denotes a number designated to a particular isolate when isolations were made from fruit. Isolates with the same number are from the same lesion but different areas.

Appendix 3 *Dothiorella aromatica* isolates used in growth rate and temperature response test

Isolate number[a]	Isolate code[b]
1	173c
2	96a
3	117b
4	78c
5	164c
6	95a
7	95c
8	133a
9	24a
10	6a
11	194a
12	120b
13	112c
14	Mag5
15	129c
16	139c
17	123a
18	67a
19	139a
20	129a
21	154c
22	123c
23	96b
24	14a
25	127a

[a] denotes a number designating a particular isolate code in the Wards's method.

[b] denotes a number designated to a particular isolate when isolations were made from fruit. Isolates with the same number are from the same lesion but different areas.

Appendix 4 *Dothiorella aromatica* isolates used in hierachical comparison of carbon and nitrogen utilization test

Isolate number[a]	Isolate code[b]
C.1	164C
C.2	6A
C.3	139C
C.4	123A
C.5	123C
C.6	112C
C.7	24A
C.8	67A
C.9	173C
C.10	121B
C.11	98B
C.12	120B
C.13	129C
C.14	139A
C.15	96A
C.16	194A
C.17	129A
C.18	133A
C.19	78C
C.20	117B
C.21	114A
C.22	19A
C.23	139B
C.24	154C
C.25	Mag5
C.26	95C
C.27	95A

[a] denotes a number designating a particular isolate code in the Wards's method

[b] denotes a number designated to a particular isolate when isolations were made from fruit. Isolates with the same number are from the same lesion but different areas.

Appendix 5 *Dothiorella aromatica* isolates used in hierachical comparison of pH response test

Isolate number[a]	Isolate code[b]
C.1	164C
C.2	6A
C.4	123A
C.5	123C
C.7	24A
C.8	112C
C.9	67A
C.11	173C
C.12	120B
C.13	129C
C.14	139A
C.15	96A
C.17	129A
C.18	133A
C.19	78C
C.20	117B
C.21	114A
C.22	19A
C.24	154C
C.25	Mag5
C.26	95C
C.27	95A
C.28	83B
C.29	127A
C.30	127C

[a] denotes a number designating a particular isolate code in the Wards's method

[b] denotes a number designated to a particular isolate when isolations were made from fruit. Isolates with the same number are from the same lesion but different areas.

Appendix 6 *Dothiorella aromatica* isolates used in RAPDs

Isolate number[a]	Isolate code[b]
1	139a
2	19b
3	173a
4	173c
5	67a
6	67b
7	67c
8	123a
9	123b
10	123c
11	120b
12	95b
13	95c
14	129a
15	129c
16	96a
17	96c
18	112c
19	114a
20	117c
21	177b
22	78c
23	121b
24	164c
25	6a
26	154b
27	98b
28	24a
29	95a
30	Mag5
31	139c
32	133a
33	19a
34	117a
35	194a
36	173c
37	127c

[a] denotes a number designating a particular isolate code in the Wards's method.

[b] denotes a number designated to a particular isolate when isolations were made from fruit. Isolates with the same number are from the same lesion but different areas.