GENETIC VARIATION AMONG *Verticillium dahliae* ISOLATES USING PATHOGENICITY AND AFLP ANALYSIS

by

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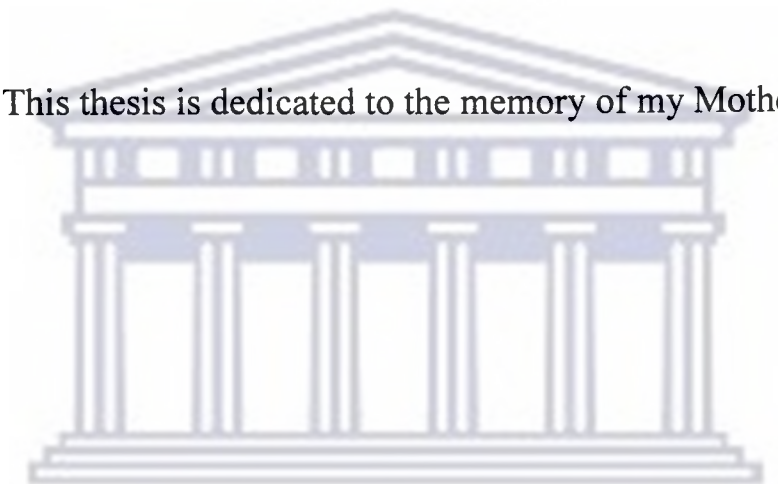
This thesis is dedicated to the memory of my Mother



DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Ms. Marinda Visser Date

PREFACE

This thesis is presented as a compilation of three chapters. Each chapter is introduced separately and is written according to the format of Plant Disease.

Chapter 1 Introduction and Literature review
Biology and molecular characterisation of the plant pathogen *Verticillium dahliae*

Chapter 2 Research Results
Pathogenicity and virulence of *Verticillium dahliae* isolates from cotton, potato, tomato, and avocado on tomato seedlings

Chapter 3 Research Results
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Summary

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CHAPTER 1

Literature Review

Biology and Molecular Characterisation of the Plant Pathogen *Verticillium dahliae*

Chapter 1

Biology and Molecular Characterisation of the Plant Pathogen *Verticillium dahliae*

1. INTRODUTION

Soilborne plant pathogenic fungi have had an important economic and social impact on humans since the evolution of agricultural societies (Stakman, 1959). In South Africa, the soilborne pathogen *Verticillium dahliae* Kleb, causes wilt and early dying, which results in extensive losses in the production of potatoes and tomatoes (Uys *et al.*, 1995). Control of soilborne diseases however remains elusive. Since roots are hidden from view, disease development and subsequent yield loss are not recognised until harvest or when symptom expression, in the form of plant death, occurs, by which time it is too late to react with any control measure. Management of soil-borne diseases is a continual challenge to growers. The structural, physical, and biological complexity of the soil environment, in which pathogens interact with plant roots, inherently limits the options available for disease control. The oldest and most fundamental approach employed by man to control plant diseases is the modification of cultural practices. Most cultural practices used to control plant diseases are preventative in nature. Some of the most proven control measures remain resistance and, in some situations, fungicides. Despite efforts to effectively combine these options with other control strategies, soil-borne diseases continue to constrain crop production (Ligoxigakis & Vakalounakis,

1992). Therefore, the study of soilborne diseases and their control poses a continuing challenge for plant pathologist.

2. HISTORY AND BACKGROUND OF THE GENUS *VERTICILLIUM*

The genus *Verticillium* (subdivision - *Deuteromycotina*, form-class *Hyphomycetes*) was established by Nees von Esenbeck in 1816 (Isaac, 1967). Based on conidiophore morphology, 40 species were described (Rossmann *et al.*, 1987). Verticillium wilt was first reported in 1879 from Germany as causing early maturity disease of potato. This disease was confused with Fusarium wilt as part of the 'sleepy disease' of tomato in Britain in 1896 and 1910, and was detected as a distinct disease in 1922 (Ashworth *et al.*, 1979). In South Africa, *Verticillium* was first described as a pathogen of tomatoes in 1953 (Gorter, 1977).

2.1 Host range of *Verticillium*. The genus *Verticillium* has a wide host range, and the genus includes species which infect mushrooms, insects and nematodes (Gams, 1988). The genus *Verticillium* contains vascular and nonvascular pathogens of higher plants, the former being of greater economic importance, particularly in temperate and tropical regions of the world. These include Israel, Greece, New Zealand, Canada, USA and Europe (Pegg, 1974). The two most important species causing Verticillium wilt are *V. albo-atrum* Reinke and *V. dahliae*. *V. dahliae* is more widely involved as a pathogen, is the most widespread and most destructive species. *V. dahliae* is known to be a

pathogen of more than 160 plant species (Schnathorst, 1981). The host range includes all dicotylodonous plants, herbaceous as well as woody plants (Pegg, 1974). The host species of *V. dahliae*, for example, consist of a wide array of cultivated plants. The hosts include: peanut (*Arachis hypogaea L.*), (Melouk *et al.*, 1983); cotton (*Gossypium hirsutum L.*), (Ashworth, 1983); potato (*Solanum tuberosum L.*), (Nachmias & Krikun, 1984; Strausbaugh, 1993); tomato (*Lycopersicon esculentum Miller*), (Schaible *et al.*, 1951); hops (*Humulus lupulus L.*), (Horner, 1954); alfalfa (*Medicago sativa L.*), (Graham *et al.*, 1977); eggplant (*Solanum melongena L.*), (Elmer & Ferrandino, 1994); olive (*Olea europea L.*), (Boyle, 1963); avocado (*Persea americana L.*), strawberry (*Fragaria species*) (Visser & Kotze, 1975); Cucumber (*Cucumis sativus L.*), (Gubler *et al.*, 1978) and woody ornamentals (Chen, 1994) among many others (Melouk *et al.*, 1983).

3. VERTICILLIUM WILT TAXONOMY

Most species of the genus *Verticillium* produce abundant conidia when grown at 20-23°C for 7 to 10 days on any standard agar medium such as potato-dextrose agar (PDA), malt-extract agar (MEA), or cornmeal agar (CMA). The prostrate hyphae, which are first produced, are hyaline. In *V. dahliae* the mycelium is initially white when incubated under near UV illumination. After a week, the colony usually turns black due to the production of black microsclerotia (MS) within the medium. Hyaline sectors often arise in the white colonies (Hawksworth & Talboys 1970b). More or less erect hyaline, verticillately branched conidiophores are produced abundantly, with 3-4 phialides (16-35

x 1-2.5 μm) arising from each node (Hawksworth, 1970). These phialides are sometimes secondarily branched. At the end of each phialide, ellipsoidal, hyaline, unseptate, but occasionally 1-septate conidia (2.5-8 x 1.4 - 3.2 μm) develop (Isaac, 1949). Dark brown to black, microsclerotia (15-50 μm diameter) develop in the centre of the culture, consisting of swollen globular cells. These microsclerotia arise from single conidia by repeated budding (Hawksworth, 1970). Dark brown to black resting mycelia are only formed in association with microsclerotia (Hawksworth & Talboys, 1970b). *V. dahliae* forms melanized microsclerotia as resting structures. Resting structures germinate in the presence of adequate moisture at 22.5°C with no evidence to indicate that any rest period is necessary, however, germination declines with increasing age of the resting bodies (Isaac & MacGarvie, 1966).

V. albo-atrum can be distinguish from *V. dahliae* by the absence of microsclerotia and the production of resting mycelia. *V. dahliae* produces microsclerotia (Hawksworth & Talboys 1970a). The conidiophores are erect, septate, and branched, with the short branches forming whorls. Terminal branches of the conidiophores usually are flask-shaped and pointed at the tips (Hawksworth, 1970). Conidia are borne terminally, either singly or in small clusters. Conidia are unicellular, ovoid to ellipsoid or spherical, and hyaline to orange-brown (Hawksworth & Talboys, 1970a).

V. tricorpus Isaac is distinguished from all other *Verticillium* species by the orange-yellow colour of the first formed prostrate hyphae, the simultaneous production of chlamydospores, microsclerotia, and resting mycelia (Hawksworth & Talboys, 1970a). It also has a typical yeast-like growth at temperatures above 30°C. *V. dahliae* grows profusely at temperatures of 30°C whereas the growth of *V. tricorpus* and *V. albo-atrum* is limited at high temperatures. A low pH (5.3-7.2) favours *V. dahliae*, while *V. tricopus* prefers a pH of 7.2-8.0 and *V. albo-atrum* a pH of 8.0-8.6 (Hawksworth, 1970). *V. albo-atrum* favours glycerine as a carbon source while other *Verticillium* species thrive on carbohydrates such as sucrose and dextrose (Hawksworth,1970; Hawksworth & Talboys, 1970a).

4. VERTICILLIUM WILT OF POTATO, TOMATO AND COTTON

4.1 Verticillium wilt of potatoes. Diseases are one of the most important causes of yield and tuber quality losses in potato production world-wide (Hooker, 1981). Agronomic factors which influence the production cycle of disease include rotation, planting material, (Huisman & Ashworth, 1976), cultivar selection, (Hooker, 1981), soil management and tillage, (Heale & Isaac, 1965), irrigation, pesticide application, haulm destruction and harvesting, (Howard, 1996), crop residues (Heale, 1988), and storage (Heale & Isaac, 1965). All have a profound influence on the incidence and severity of Verticillium wilt. There are also strong interactions with other pathogens in causing damage and loss. The potato early dying disease is the result of a synergistic interaction

between *V. dahliae* and the root-lesion nematode *Pratylenchus penetrans* (Joaquim & Rowe, 1991; Platt, 1986; Stevenson *et al.*, 1976). Although there is evidence that various species of *Pratylenchus* interact differently with *V. dahliae* in potato early dying, little is known about the effects of virulence differences among various isolates of either disease-causing agent (Joaquim & Rowe, 1991). *V. dahliae* is the major component in the potato early dying syndrome (Powelson, 1970; Krikun & Orion, 1979). Various *Verticillium* species are associated with potato early dying (Busch & Edgington, 1967; Pegg, 1974). The resultant wilt disorder severely reduces yield and quality. Producers suffer further losses in seed sales and usage as infected plants produce tubers which are infected or infested with these pathogens (Jeger *et al.*, 1996). Colonisation of the tubers also causes an increase in soil-borne levels of inoculum, and hence, an ever-increasing disease potential in the fields used for potato production (Wheeler *et al.*, 1994). Verticillium wilt results from the planting of infested seed or planting seed in contaminated soil (Easton *et al.*, 1972). The role of surface- and internally borne inoculum of seed-potato tubers in disseminating the pathogen has been pointed out by Krikun *et al.* (1983); MacGarvie & Hide, (1966) and Thanassoulopoulos & Hooker, (1968). Susceptible weed species, the presence of symptomless carriers and soil-borne population levels maintained by non-hosts are additional important contributors (Pegg, 1974).

Soilborne fungal pathogens including *V. dahliae* may pose particular problems in potato production because of their long-term persistence, the difficulties of controlling

inoculum and the lack of good sources of resistance. Current management procedures for potato early dying are often based on estimates of soil populations of *V. dahliae* prior to planting. Existing quantitative soil assay procedures for *V. dahliae* rely on the assumption that all propagules that grow on selective agar media are equally pathogenic and, therefore, that enumeration of colonies is a direct measure of inoculum potential (Rouse, 1985). Among several factors that could affect the relationship between inoculum potential and inoculum density in field soils are the occurrence of mixtures of either pathogenic and non-pathogenic strains of varying virulence capabilities (Schnathorst, 1981).

In potato, symptoms of *V. dahliae* are difficult to distinguish from normal senescence and may initially involve only reduced growth (Street & Cooper, 1984). Early foliar symptoms may appear as unilateral chlorosis of lower leaves on a few plants. Later some wilting of whole leaflets or leaves may occur, but the unilateral death of lower leaves is more typical (Isaac & Harrison, 1968). Inoculated potato plants produce no symptoms until tuberisation commences (Busch & Edgington, 1967), suggesting that before symptom expression of Verticillium wilt becomes evident, the host must be in an advanced stage of development (Busch *et al.*, 1978). Kotcon *et al.* (1984) associated disease incidence of *V. dahliae* with reduced root growth, foliar weight, and tuber yield. Infected plants exhibited lower specific leaf areas (area produced/dry weight of leaf tissue), and under dry conditions, lower relative growth rates and lower leaf growth rates

(Rowe, 1985). A lesser root length of potato plants due to *V. dahliae* may decrease the water supply and cause the development of foliar symptoms (Kotcon *et al.*, 1984).

4.2 Verticillium wilt of tomatoes. Verticillium wilt first described as "Sleepy disease" in tomato is responsible for considerable financial losses in tomato production throughout the world. In most cases the disease is stress related and can only be controlled by preventative treatment. Verticillium wilt caused by *V. dahliae* is a prominent disease of tomato throughout all the tomato growing regions of South Africa (Visser & Hatting, 1981). Wilt symptoms on tomatoes caused by *Verticillium* species, are similar to those caused by *Fusarium* species and are characterised by a browning of the vascular tissue (Dobinson & Lazarovitz, 1994). Plants infected by *Verticillium* are usually stunted, while the internodes especially the younger, are badly developed (Baergen *et al.*, 1993). Susceptible cultivars like Rooi Kakie, Bonny Best and Roodeplaat Albesto show severe symptoms on the foliage during the seedling stage (Uys *et al.*, 1995; Visser 1982). These infected plants grow fairly well when transplanted, but yield is markedly reduced (Uys *et al.*,1995; Visser, 1982). When the temperature and light are favourable to the fungus, the disease symptoms appear suddenly and the plants wilt while still green (Bewley, 1922). During the night, the plants may recover their turgidity, only to wilt again as the morning advances. The leaves wither from the base of the plant upwards, adventitious roots emerge from the stem and the plant dies. Death is much slower when conditions are less favourable to the fungus: yellow blotches appear on

individual leaflets on the lower leaves and these leaflets whither. The fungus moves up one side of the plants first and produced a wilt on this side only. After a time the fungus works round the stem and induces wilting on all sides of the plants (Bewley, 1922). The pathological symptoms in the anatomy of plants suffering from wilt disease are limited to a brown discoloration in the vascular tissue and the presence of fungal hyphae within them (Bewley, 1922). Stunting is a symptom of Verticillium wilt of tomato especially when plants are infected in an early growth stage (Thanassoulopoulos & Kitsos, 1974).

Two races of *V. dahliae* are recognised on the basis of pathogenecity to tomato cultivars carrying the dominant Ve allele (Schnathorst & Mathre, 1966). Cultivars carrying the Ve gene are resistant to race 1 isolates. The race 2 isolates produce characteristic symptoms of early dying, including severe chlorosis and wilting (Dobinson *et al.*, 1996). Both races have been recorded in South Africa (Ferreira *et al.*, 1990), based on virulence on the differential tomato cultivars Flora Dade (V1 resistant) and Rooi Kakie (susceptible).

Factors such as soil pH, soil and air temperature, photoperiod, host cultivar and virulence of the pathogen have a marked influence on the development of Verticillium wilt of tomatoes (Dobinson & Lazarovitz, 1994). Susceptibility to Verticillium wilt appears to be a complex response depending on host cultivar, fungal strain, and inoculum concentration (Visser, 1977). Much of the controversy regarding the severity of

Verticillium wilt may be due to differences in the virulence of the pathogen (Visser, 1977).

4.3 Verticillium wilt of cotton. Verticillium wilt of cotton is now recognised as one of the major problems of irrigated cotton in most cotton-growing areas of the world (Bell, 1992). The causal agent, *V. dahliae* is capable of infecting plants throughout the growing seasons (Paplomatas *et al.*, 1992; Willie & Devay, 1970).

Disease symptoms in cotton plants infected by *V. dahliae* are variable and often influenced by the strains of the pathogen (Bejarano-Alcazar *et al.*, 1995). Among strains, which infect cotton, two major groups have been recognised in pathogenecity tests, those, which defoliate and those, which do not (De Vay *et al.*, 1997). Leaf symptoms in cotton plants with Verticillium wilt are typified by interveinal chlorotic areas which later become necrotic (Ashworth, 1983). The chlorosis is caused by the occlusion of leaf veins in limited areas of leaves. The first appearance of these leaf symptoms, usually on lower leaves, is associated with cessation of both plant growth and fruit development. Less recognised symptoms mimic potassium deficiency on infected plants; these symptoms do not stop growth although they may cause reductions in growth and lint yields (De Vay *et al.*, 1997). Potassium deficiency symptoms associated with infections of cotton plants by *V. dahliae* first appear in younger leaves and begin with bronzing at leaf margins. The

leaves eventually develop a metallic sheen and become thick and brittle, often resulting in extreme distortion of leaf tissues (De Vay *et al.*, 1997).

In the field, plants sometimes exhibit general chlorosis with slight to extensive vascular discoloration in stems and epinasty that is followed by sudden defoliation (Schnathorst & Mathre, 1966). Disease incidence is usually assessed as percent foliar symptoms or vascular discoloration. The incidence of foliar symptoms of Verticillium wilt is directly correlated to lint and seed losses (Paplomatas *et al.*, 1992). Vascular discoloration is a good indicator of infection by *V. dahliae*, but it appears to have little influence on lint yield. Losses of lint yield depend mainly on the development stage of plants when foliar symptoms appear. Development of foliar symptoms in infected plants is influenced by several factors, of which the most important are the susceptibility of cotton cultivars, the environmental conditions, and the pathotypes of *V. dahliae* (Bejarano-Alcázar *et al.*, 1996). Pathotypes of *V. dahliae* infecting cotton are differentiated mainly by whether or not they defoliate the plant. The defoliating pathotype has a more pronounced effect than the non-defoliating pathotype on growth parameters of the affected plants and reduction of lint yield (Bejarano-Alcázar *et al.*, 1996).

The disease is managed by the use of wilt-tolerant cultivars, crop rotation, and cultural practices. Highly virulent strains or high inoculum densities of the pathogen

(Bejarano-Alcázar *et al.*, 1996) often overcome the effectiveness of tolerant cultivars. Because Verticillium wilt is a single cycle disease, inoculum levels of *V. dahliae* in the soil at planting time play a critical role in disease development and are directly reflected in lint yield losses (Paplomatas *et al.*, 1992).

5. LIFE CYCLE AND ECOLOGY OF *VERTICILLIUM DAHLIAE*

Verticillium dahliae is classified as a soil invading or root inhabiting fungus (Powelson, 1970). These fungi are characterised by a parasitic phase on the living host plant and by a saprophytic phase after the death of the host (Powelson, 1970). The fungus first infects the roots but does not cause a rot. It then grows within the vascular system that feeds a section of the plant towards the main stem and sporulates within the vascular system. Dispersal of the pathogen occurs within the vascular system and eventually, the vessels become clogged and the flow of water from the roots to the rest of the plant is reduced (Mol & Termorshuizen, 1995). At the end of the disease cycle microsclerotia are formed on above and below ground parts of the senescing plant, from where the microsclerotia are released into the soil again (Powelson, 1970). Microsclerotia levels are maintained in the soil by susceptible weed species that are symptomless carriers as well as the roots of non hosts crops (Martinson & Horner, 1962; Pegg, 1974).

5.1 Survival Rate of *V. dahliae*. The fungus overwinters in plant debris as poppy-seed sized balls of hyphae called microsclerotia. The microsclerotia, in contrast to mycelium and conidia, can survive for long periods in the soil, and still remain infectious or dormant for up to four to eight years (Ashworth *et al.*, 1974; Mol & Termorshuizen, 1995). In this state, *V. dahliae* can survive in the soil for as long as 8 years and *V. albo-atrum* can survive for 3 years, even in the absence of susceptible hosts plants (Ferrandino, 1995). Ben-Yephet & Szmulewich, (1985) reported that microsclerotia of *V. dahliae* survive longer in the field than in the laboratory. Wilhelm (1955) found that *V. dahliae* persisted for 14 years in field soil with no hosts present. The long persistence of the fungus in the field is probably due to its ability to colonise and produce new microsclerotia on the root systems of nearly all plant species including monocotyledons (Martinson & Horner, 1962). Microsclerotia have the ability to germinate more than once. Farley *et al.* (1971), showed that microsclerotia germinated and sporulated every time after re-moistening soil with a sucrose solution or water up to nine times. The high survival potential of microsclerotia and the wide host range make *V. dahliae* endemic to many agricultural soils (Powelson, 1970). Persistence for up to 8 years, and the ability to withstand drying and antagonistic soil factors account for the apparent ability of the fungus to survive for long periods in soil without host plants (Schnathorst, 1981).

6. PATHOGENIC VARIATION AND HOST SPECIALISATION WITHIN *V. DAHLIAE.*

Plant pathogenic species, such as *V. dahliae*, are not homogenous populations and frequently exhibit extensive genetic variation. Variation may be evident in such phenotypic traits as growth rates, pigmentation, fecundity, and production of important toxins or extracellular enzymes (Woudt et al., 1991). This variation may result in discrete variation in aggressiveness and virulence. Variation in host range is also present within many pathogenic species (McDermot & McDonald, 1989). Pathogenic and virulence variation that impacts efforts to control the disease incited by the pathogen is the main concern (McDonald & McDermot, 1993). Variation in sensitivity to fungicides and bactericides, or variation in host range and virulence also impact on efforts to control plant diseases (Burdon & Silk, 1997).

Until recently, only observing the pathogenic behaviour could identify pathogenic variation and virulence of isolates of the pathogen, hence the original use of the term physiological race. Physiological race is used most frequently to denote variation in virulence among fungal pathogens, characterised by pathogenesis on a host differential series where genotypes of the same host species differ in genes for resistance (McDonald & McDermot, 1993).

An interesting case of variation in aggressiveness is with *V. dahliae* on cotton (Verticillium wilt). On cotton, *V. dahliae* is divided into two groups according to its ability to cause defoliation and non defoliation (Daayf *et al.*, 1995; De Vay *et al.*, 1997). It is however uncertain what the correlation between cotton isolates and tomato isolates is, and it appears that sub-populations of *V. dahliae* are not completely isolated genetically (Daayf *et al.*, 1995). There is, however, considerable variation in morphology and pathogenecity of isolates of this pathogen (Puhalla, 1979).

Intraspecific variation in *V. dahliae* has been considered the exception rather than the rule, in contrast to the situation in *F. oxysporum*, where many special forms and races are distinguished. Adaptation of isolates of *V. dahliae* to hosts is, in comparison to *F. oxysporum*, a rare phenomenon which has been reported only from cotton, the cruciferae, peppermint (*Mentha piperata*), brussel sprouts (*Brassica oleracea var. botrytis*) and horseradish (*Armoracia rusticana*) (Subbarao *et al.*, 1995). Host adaptation of *V. dahliae* appears not to be a rigid character because, for example, several peppermint isolates which were initially non-pathogenic to tomato became pathogenic after one or more passages through tomato plants (Fordyce & Green, 1963). The general lack of specificity in *V. dahliae* has been confirmed by showing that single isolates of *V. dahliae* can infect many hosts (Subbarao *et al.*, 1995), including the roots of monocotyledonous plants such as barley, onion, tulip and wheat (Malik & Milton, 1980).

Identification of subspecific groupings (i.e., formae speciales, races, or pathotypes) within *V. dahliae* has been difficult. With a few exceptions, host specificity is rare among isolates of *V. dahliae*; host reactions, when employed, have not generally been useful to differentiate among strians (Bender & Shoemaker, 1984). Physiologic races of *V. dahliae* have only been defined in tomato with the Ve gene (Alexander, 1962). Similar to *F.oxysporum* f. sp. *lycopersici*, *V. dahliae* has different physiological races in tomato (Bender & Shoemaker, 1984; Ligoxigakis & Vakalounakis, 1992). Race 2 of the pathogen was first recorded in South Africa in 1988 (Ferreira *et al.*, 1990), and it readily infects cultivars carrying the Ve gene. Isolates of the pathogen are therefore usually differentiated into two physiological races according to their virulence on tomato cultivars carrying the Ve gene, which determines resistance to race 1 (Bender & Shoemaker, 1984; Okie & Gardner, 1982). Both physiological races have been reported from South Africa (Ferreira *et al.*, 1990). The virulence of these isolates, however, varies considerably throughout the country. This could largely be attributed to environmental influences and the occurrence of disease complexes (Ferreira *et al.*, 1990).

Because variation in virulence has a great impact on management of disease by host resistance, considerable research has been devoted to this subject. Whereas variation in aggressiveness also can affect disease management, this is more frequently related to degree of disease management and not complete success or failure. Variation in virulence (or fungicide resistance) may result in a complete failure of the management

system if that system is dependant on host resistance that is no longer effective due to a change in pathogen virulence traits (Woudt *et al.*, 1991)

7. DISEASE CONTROL

Control by chemical soil fumigation at the commercial level is a temporary and costly practice which, with the current concern for hazards due to the chemical contamination of groundwater, might best be avoided. The choice of cultivar, crop rotation, and cultural practices will not be sufficient to keep the crop from infection, but they will still be key factors in controlling the severity of the disease (Mol & Termorshuizen, 1995). In Canada the incidence and severity of disease caused by *V. dahliae* are increasing because of (1) the growing proportion of susceptible crops (potato, sugar beet) in present rotation systems and (2) the restriction of chemical soil disinfecting for environmental reasons (Lazarovitz, 1987). The pathogen forms microsclerotia that remain viable in soil for many years. Because of its polyphagous nature, the pathogen causes problems not only in crops grown in short rotations but also in other host plants. Examples are wilt disease in lane trees and roses on fields previously grown with potatoes (Platt & Bollem, 1995). In many crops, Verticillium wilt is managed with a combination of chemical and cultural methods. The wide host ranges of the organism compromises the effectiveness of crop rotation as a sole method of control. The effectiveness of certain cultural practices in controlling *V. albo-atrum* and the ineffectiveness of the same

practices in controlling *V. dahliae;* the resting structures of *V. dahliae* are likely capable of much longer survival in soil (Schnathorst, 1981).

Conventional chemical control procedures have been relatively ineffective for reducing soilborne diseases, primarily because when added to soils, chemicals lose efficacy due to dilution, leaching or inactivation through interactions with soil or soil microbes (Green, 1981; Heale, 1988). In cases where production is severely limited by disease, chemical sterilants or fumigants are often utilised as a last resort. Disease control is therefore restricted to fumigation procedures. However, fumigants are expensive, environmentally dangerous and may be removed from the market place.

Constant and rapid monitoring of pathogen levels is very important in regions where *Verticillium* wilt is a major disease. Climate and cultivar changes can lead to critical shifts in the occurrence and predominance of *V.dahliae* and *V. albo-atrum* (Celetti & Platt, 1987; Wheeler *et al.*, 1994). Such observations emphasise the need for an accurate method of species identification and quantification which is sufficiently rapid for a producer or inspector to make appropriate recommendations since control options differ depending on pathogen type (Heale, 1988). At present, the main method of detection is field inspection. However, Verticillium wilt can have the same symptoms as drought stress, and mature plant senescence (Dobinson *et al.*, 1996; Lazarovitz, 1987).

To determine the actual causal agent and frequency of occurrence, plants and soils must be taken to laboratories for assessment. Unfortunately, the methods available for pathogen assay are time consuming (up to 7 weeks), labour intensive and often of limited accuracy. Results are at best semi-quantitative and most current testing techniques fail to distinguish *V. albo-atrum* from *V. dahliae* and other similar species (Huisman & Ashworth, 1976). It can thus be concluded that the long survival times and wide hosts range of *V. dahliae* compromise the effectiveness of crop rotation as a sole method of control.

8. IMPORTANCE OF GENETIC ANALYSIS OF PLANT PATHOGENS

The study of pathogen variation and the forces contributing to this variation usually involves the study of population genetics. A population is a community or individuals at a given locality sharing a common gene pool (Boeger & McDonald,1991). Population genetics needs genetic markers that are potentially unrelated to strongly selected characters e.g. virulence and pesticide resistance as genetic markers, they are subject to strong selection pressures. Genetic analysis of plant pathogens has been used to a great extent to assist plant pathologists in their study of pathogens. The importance and uses of genetic analysis in plant pathology will be discussed briefly.

The full range of options for disease prevention and management will be unknown until more details are known of the molecular events controlling compatibility, and their

distribution among diverse host-pathogen systems (Maclean *et al.*,1993). However, the application of techniques of molecular biology should help resolve this deficiency. Fungal populations with high levels of genetic variation are likely to adapt more rapidly to fungicides or resistant hosts than populations with little genetic variation (McDonald & McDermot, 1993). Information on the distribution of genetic variation may be used to decide how best to deploy resistance genes or fungicides to achieve control.

Many pathogen populations contain a large reservoir of genetic variation for DNA markers and pathogenecity. This finding suggest that many or most pathogenic fungi possess sufficient genetic variation to quickly adapt to the genetically uniform monocultures that dominate modern agriculture (Horward, 1996). The genetic analysis of populations for DNA variation is thus important in view of the impact it can have on current control measures.

8.1 Control measures. Genetic structure refers to the amount and distribution of genetic variation within and between populations (McDermot *et al.*, 1989). Knowledge of the genetic structure of pathogen populations has direct applications to agricultural ecosystems. For instance the amount of genetic variation being maintained within a population indicates how rapidly a pathogen can evolve, and this information may eventually be used to predict how long a control measure is likely to be effective (McDonald & Martinez, 1991).

8.2 Taxonomy. Fungal classification is based primarily on the morphology of sexual organs and associated reproductive structures, and asexual resting structures, spores and sporophores However, many fungi lack suitable morphological markers, and thus present a problem to the systematic taxonomist (McDermot *et al.*, 989). The prime examples are the *Deuteromycotina*, some of which fail to produce any reproductive structures other than mycelia and / or sclerotia (Bachman, 1994; Nei & Li, 1979). Other fungi exhibit pleomorphism either in the field or in culture, and may not produce the required diagnostic structures 'on cue' for the taxonomist (Maclean *et al.*,1993). DNA-based techniques will complement the position or confirmation of such fungi in the overall scheme of fungal phylogeny (McDonald & McDermot, 1993).

Because the morphology of all organisms is programmed by the information stored in their DNA, classification based on genome structure and sequence homology, would at first sight appear to present the ultimate taxonomic system (Bursdall,1990). However, two qualifications are immediately apparent. First, the interpretation of genome and sequence homologies may be controversial (Li *et al.*, 1994; Smith, 1989). Second, even if taxonomy based solely on DNA comparisons were possible, it would be of little practical use. Taken in isolation, DNA comparisons represent the ultimate in taxonomic reductionism, and give no overview of how whole organisms or their component parts work within themselves or their environment (Brown, 1996; Bursdall, 1990; Evams *et al.*, 1997; Thompson & Burdon, 1992; Williams *et al.*, 1991). The future

of taxonomy lies somewhere in between, and DNA analysis should be a necessary primary character, along with morphological and biochemical / physiological characters, in aligning organisms into useful taxonomic groups (Systma, 1990). Today powerful genetic tools have provided other means of identifying organisms into useful taxonomic groups but should not be seen in isolation. Morphological, along with pathological characterisation will still be important in future taxonomy of plant pathogens.

8.3 Detection and Identification of plant pathogens. Population genetic analyses are also used for detection and identification of plant pathogenic fungi, since this is becoming increasingly important as the demand for pathogen-free propagation and planting material increases (Randles *et al.*, 1996). This is mainly the result of the need to reduce the number and amount of crop protection chemicals (Nachmias *et al.*, 1982). Until a decade ago, the development of detection and identification methods was limited to classical methods requiring hard-won skills and taxonomic knowledge. DNA fingerprinting technology tools have become available to develop tests based on unequivocal parameters such as species-specific molecules or DNA sequences (Randles *et al.*, 1996). They do not assay pathogenicity, so biological methods are still needed to test these. Molecular techniques can be used to tag individual pathotypes whose dispersal and persistence can be monitored directly in the environment (McDermot *et al.*, 1989).

Intracellular plant pathogens are widely distributed in all crops and the diagnosis of known pathogens and the identification of new pathogens is essential to minimise their impact on plant health and productivity (Brown, 1996). There is wide scope for the use of technologies based on molecular biological methods for diagnosis. They offer rapid, sensitive, reliable and efficient means of identifying pathogens in plants (Jefferies *et al.*, 1988). They can be used to set up specific tests for known pathogens, or they may be used to identify a new pathogen where a disease of unknown etiology occurs (Evams *et al.*, 1997).

9. POPULATION CHARACTERISATION OF *V. DAHLIAE*:

The population characterisation of plant pathogens requires the identification of genetic markers that are unambiguous and informative (polymorphic) (Henson & French, 1993). Population analysis of plant pathogenic fungi is important because pathogens evolve. Pathogen populations must constantly adapt to changes in their environment to survive. In agricultural ecosystems, environmental changes may include resistant varieties, applications of fungicides and fertilisers (Keit, 1959), irrigation, and crop rotation (Hu *et al.*, 1993). Control strategies must target a population instead of an individual if they are to be effective.

9.1 Morphological markers

9.1.1 Vegetative compatibility groups (VCGs) VCGs have been very useful for identifying clones of fungi that are largely asexual, although it has been shown that isolates with the same VCG are not always members of the same clone, as was the case with several *Verticillium* species (Bachman, 1994; Carder & Barbara, 1991; Chen, 1994; Daayf *et al.*, 1995; Joaquim & Rowe, 1991; Pulhalla & Hummel, 1983; Strausbaugh, 1993). The primary advantage of VCG analysis is that it is relatively easy and inexpensive to implement. A significant disadvantage is that VCGs are phenotypes instead of genotypes and, thus, are not useful for measuring population similarity and differentiation (Joaquim & Rowe, 1990). Another disadvantage is that some isolates will not form nitrogen nonutilizing (nit) mutants (McDonald & Martinez, 1991).

9.1.2 Virulence and fungicide-resistance markers. Bachman (1994), used fungicide-resistance markers and virulence markers that describe genetic variation in plant pathogen populations. However these characters have attributes that limit their usefulness for fully understanding population genetics (Drenth *et al.*, 1993). Because virulence and fungicide sensitivity are under strong selection in agricultural systems, they are likely to provide biased estimates of the potential for genetic change in pathogen populations (McDonald & Martinez, 1991). Virulence and resistance markers may represent only a small portion of the total genetic variation present in the population (Drenth *et al.*, 1993). Furthermore, races of *V. dahliae* have only been reported for

tomatoes, thus limiting the use of virulence markers in characterisation of *V. dahliae* populations. Conclusions drawn exclusively on race phenotypes of plant pathogenic fungi which were derived by this gene-for-gene concept (Flor, 1971), may not give sufficient information on the level of other genetic differences either between races, or between independent collections of a race (McDermot *et al.*, 1989; McDonald & McDermot 1993).

9.2 Genetic DNA markers. The choice of genetic markers can have a substantial impact on the analysis and interpretation of data. When working with fungi that reproduce mainly asexually, a population structure is produced that is composed largely of clonal lineages. In these cases, a neutral marker such as a DNA fingerprint may be used to address questions about selection because of the complete correspondence between genotype (e.g., DNA fingerprint) and phenotype (selected marker, e.g., pathotype) (Randles *et al.*, 1996). It is best to use genetic markers that are selectively neutral, highly informative, reproducible, and relatively easy to assay (Randles *et al.*, 1996).

The development of a molecular probe which could be used for rapid (hours to days) identification and quantification of *Verticillium* species would clearly have a major impact on various sectors of the potato industry as well as a commercial value. Recent studies in the laboratories of Nazar *et al.* (1991) and Hu *et al.* (1993) have indicated the

feasibility of developing molecular probes based on genomic DNA sequencing, recombinant DNA techniques and newly developed PCR technology for *Verticillium* species (Mullis *et al.*, 1986).

9.2.1 DNA fingerprints. A fingerprint is a unique pattern for the identification of an individual; it is desirable, but not essential, those fingerprints show familial relationships among parents and siblings (Jefferies *et al.*, 1988). It offers an especially powerful tool for population studies of asexually reproducing organisms, such as many fungi, because they can be used to distinguish among different clonal lineages in a population with a high degree of certainty (McDonald & Martinez, 1991). DNA fingerprints associated with particular biotypes, races and strains of plant pathogens, will be of considerable practical value to plant pathologists monitoring pathogen populations. Many DNA fingerprinting techniques have been developed in the past few years and are generally based on one of two strategies: (1) Classical, hybridisation-based fingerprinting, such as restriction fragment length polymorphisms (RFLPs) (Carder & Barbara, 1991), (2) polymerase chain reaction (PCR) techniques (Mullis et al., 1986), such as random amplified polymorphic DNA (RAPD), (Levy *et al.*, 1991; Williams *et al.*, 1991).

9.2.2 DNA restricted fragment length polymorphisms (RFLP). RFLPs detect variation in DNA sequences among homologous sections of chromosomes. RFLPs are

based on the hybridisation of DNA probes to fragments of DNA that have been digested with specific restriction endonucleases and size fractionated on agarose gels (Beckmann & Soller, 1983). Because RFLPs can detect variation in both coding and noncoding regions of DNA, they are much more variable than isozymes, and they have the advantage of being on the average selectively neutral and codominant (Carder & Barbara, 1991). RFLPs can distinguish between heterozygotes and homozygotes. Fingerprint RFLPs are from moderately repetitive regions and give more complex patterns with more useful data than single band patterns (Okoli *et al.*, 1994). The main drawbacks of RFLPs is that they require relatively large amounts (5 to 10 μg) of DNA from each individual so the fungus must be grown in pure culture prior to DNA extraction, more specialised equipment are required and they are more expensive to assay than isozymes (McDonald & McDermot, 1993).

Restriction fragment length polymorphism (RFLP) of *Verticillium* species have recently led to the recognition of two major subspecific groups within each species, with little variation between isolates within these groups (Carder & Barbara, 1991; Okoli *et al.*, 1993). Differentiation of specific pathotypes from *V. dahliae* and *V. albo-atrum* using RFLPs has been reported (Okoli *et al.*, 1994). Griffen *et al.* (1997) used RFLPs to assess genetic variation in isolates of *V. albo-atrum* pathogenic to hop, lucerne and other plants. While restriction fragment length polymorphism (RFLP) techniques will

continue to be used widely in research settings, they have little future in routine diagnostic applications.

9.2.3 Random amplified polymorphic DNA (RAPD). The detection of genetic variation using the polymerase chain reaction (PCR) has rapidly gained favour because of its conceptual simplicity and potential to quickly screen a large number of individual with a minimal amount of sample DNA (Robb *et al.*, 1994). Variation usually is detected as the presence or absence of amplified DNA sequences. The genetic variation detected appears to be a random sampling of the genome (Nazar *et al.*, 1991). The number of genetic markers that can be generated is functionally unlimited. RAPDs have some drawbacks. They are generally dominant, meaning that heterozygotes and homozygotes cannot be differentiated; results can be sensitive to experimental conditions and can also have problems with reproducibility (McDonald & McDermot 1993). Technical limitations associated with RAPDs make them difficult to reproduce between laboratories and sometime within laboratories. There are also analytical problems associated with RAPDs. RAPDs have only two alleles (amplification or non-amplification) for each amplicon locus. Although this is ideal for genetic mapping, it is a drawback for measures of genetic diversity affected by the number of alleles at a locus (McDonald & Martinez, 1991).

RAPDs utilise short single primers of arbitrary or random base sequence, with over 50% G + C content to amplify genomic DNA at low stringency conditions (Li *et al.*, 1994). Only the sequences that have proximal priming sites in the correct orientation will be amplified and due to the low stringency, some mismatch annealing may occur giving rise to further products (Liew *et al.*, 1991).

The development of random amplification of polymorphic DNA (RAPD) has allowed the generation of reliable, reproducible DNA fragments or fingerprints in several fungi (Crowhurst *et al.*, 1991; Goodwin & Annis, 1991; Williams *et al.*, 1991). Messner *et al.* (1996) have investigated the molecular heterogeneity and phylogenetic position of the plant pathogen *V. dahliae* using RAPD-PCR and sequencing of the 18SrDNA, and found no correlation between geographic location of the isolates and the RAPD-pattern. Barasubiye *et al.* (1995) used RAPDs to discriminate between lucerne and potato isolates of *V. albo-atrum*. RAPD analysis has been found useful as a molecular tool in identification and phylogenetic studies of pathogenicity groups in *Verticillium* species (Koike *et al.*, 1995; 1996).

9.2.4 PCR and sequencing. The polymerase chain reaction (PCR) is starting to make a contribution to indexing for pro- and eukaryotic pathogens. The strategy of

amplifying specific genes and subsequently analysing them for sequence differences by direct sequencing can be expected to have a major impact on plant pathology because it will allow the relationship between biological properties and sequence to be established (Randles *et al.*, 1996).

PCR-based DNA assays have been developed for the identification, quantification and to differentiate *Verticillium* species. Sequence variation was utilised in the internal transcribed spacer regions (ITS 1 and ITS 2) of rDNA to design specific primers for the polymerase chain reaction (PCR) capable of differentiating between species of *Verticillium* (Nazar *et al.*, 1991). There have been many attempts to separate *Verticillium albo-atrum* and *V. dahliae* and other *Verticillium* species at the species level recently (Carder *et al.*, 1991; 1994; Li *et al.*, 1994; Moukhamedov *et al.*, 1994; Nazar *et al.*, 1991). Robb *et al.* (1994) have monitored the spread of *V. dahliae* and *V. albo-atrum* in individual, infected host plants by PCR quantitation of fungal biomass. Although the traditional plating method for detecting this fungus generally correlates with PCR detection, PCR is more sensitive. Target sequences were detected by PCR immediately after host inoculation in top segments of host plants, but the pathogen was undetected by the traditional assay until 24 hr after inoculation (Okoli *et al.*, 1993). In addition, fungal quantitation by plating methods requires 3-4 days, with up to six weeks for positive fungal identification, whereas PCR detection requires only one day (Nazar *et al.*, 1991).

On the basis of the nucleotide sequences of an amplified mitochondrial small rRNA gene region, Li *et al.* (1994), developed specific primers that amplified a 140-bp region of *V. dahliae* DNA. The *V. dahliae*-specific PCR primer may aid in more rapid and specific detection of the pathogen directly in plant and soil samples. Morton *et al.* (1995) used sub-repeat sequences in the RNA intergenic regions and sequences of the internal transcribed spacers (ITS) and reported differences between haploid and diploid isolates of *V. dahliae*

9.3 Amplified fragment length polymorphism (AFLP). A very recent approach put forward by KEYGENE, is called "selective restriction fragment amplification", resulting in "amplified fragment length polymorphisms" (AFLPs) (Vos *et al.*, 1995). This novel, DNA typing method, is potentially suitable for molecular typing of plant pathogens. This technique represents a combination of RFLP analysis and PCR, resulting in highly informative fingerprints (Matthes *et al.*, 1998). The technique resembles RFLPs in that genomic restriction fragments are detected, but with the difference that instead of Southern hybridisation which involves several laborious steps, PCR amplification is used for detection of restriction fragments. The resemblance with the RFLP technique was the basis to choose the name AFLP (Matthes *et al.*, 1998). In contrast to the RFLP technique, AFLPs will display presence or absence of restriction fragments rather than length polymorphisms. AFLP analyses combine the reliability of the RFLP technique

with the power of the PCR technique (Matthes *et al.*, 1998). The disadvantages of AFLPs are that they require more technical expertise than RAPDs (ligations, restriction enzyme digestions, and polyacrylamide gels), and they suffer the same analytical limitations as RAPDs (McDonald & McDermot, 1993). AFLP markers combine the best characteristics of other DNA markers (RAPDs, RFLPs) while avoiding their disadvantages. Unlike RFLPs, AFLP technology is PCR-based, requires only minimal amounts of starting DNA, and is readily automatable (Keim *et al.*, 1997). AFLP analysis requires no prior sequence knowledge of the target genome, and therefore has no upfront characterisation costs (Jones *et al.*, 1997). AFLPs are more reliable than RAPDs, since more stringent reaction conditions are used for primer annealing, than in RAPDs (Matthes *et al.*, 1998). AFLPs have a further advantage over RAPDs because more loci are screened in each reaction and the longer primers make it more likely that an AFLP will be reproducible (Powell *et al.*, 1996).

The technique will generate fingerprints of any DNA regardless of the origin or complexity. Scientists presented AFLP fingerprints of DNA's differing in genome size as much as 100 000 fold (Jones *et al.*, 1997). It was also stated that the number of restriction fragments that may be detected by the AFLP technique in complex genomes is virtually unlimited depending on the frequency of the restriction enzyme sites (Vrieling *et al.*, 1997). A single enzyme combination will already permit the amplification of 100 000s of unique AFLP fragments, of which 50 - 100 are selected for each AFLP reaction.

This is depended on the genome size and the base composition (Vos *et al.*, 1995). Fingerprints are produced without prior sequence knowledge using a limited set of generic primers. The number of fragments detected in a single reaction can be "tuned" by selection of specific primer sets (Vrieling *et al.*, 1997).

In most organisms AFLPs will prove to be the most effective way to construct genetic DNA marker maps compared to other existing marker technologies (O' Neill *et al.*, 1997). AFLP technology offers the fastest, most reproducible and most cost-effective way to generate high-density genetic maps for marker-assisted selection of desirable traits. It is also the ideal tool for determining varietal identity and assessing trueness to type (Jones *et al.*, 1997).

The technology is based on the selective amplification of a subset of genomic restriction fragments using PCR. DNA is digested with restriction endonucleases, and double stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotides extending into the restriction fragments are added to the 3' ends of the PCR primers such that only a subset of the restriction fragments are recognised. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The subset of

amplified fragments is then analysed by denaturing polyacrylamide gel electrophoresis to generate the fingerprint (Vos *et al.*, 1995).

To prepare an AFLP template, genomic DNA is first isolated and digested with two restriction endonucleases simultaneously. This step generates the required substrate for ligation and subsequent amplification. The restriction fragments for amplification are generated by two restriction endonucleases: *Eco*RI and *Mse*I. *Eco*RI is a 6bp cutter and *Mse*I is a 4bp cutter that generates small DNA fragments that will amplify well and are in the optimal size range for separation on denaturing polyacrylamide gels (Vos *et al.*, 1995).

The number of fragments to be amplified is reduced by using *Eco*RI. These fragments are preferentially amplified as a result of primer design and amplification strategy. The success of the AFLP technique is dependent upon complete restriction digestion; therefore, much care should be taken to isolate genomic DNA of the highest quality, intact without contaminating nucleases or inhibitors. A major factor affecting the quality of an AFLP fingerprint is the quality of the genomic DNA (Vos *et al.*, 1995).

Typically, 50 to 100 restriction fragments are co-amplified and detected in each AFLP reaction following denaturing gel electrophoresis. This technique therefore, is extremely powerful for the identification of DNA polymorphisms. The AFLP analysis

system has been optimised for use with genome sizes of general crop plants including tomato, corn, soybean, cucumber, lettuce, barley and brassica (Maughan *et al.*, 1996).

When the AFLP fingerprints of closely related plants or fungi are compared, fingerprints are generally very similar. When differences are observed in an otherwise identical fingerprint, such differences are referred to as DNA polymorphisms (Vos *et al.*, 1995). These polymorphisms are detected in DNA fingerprints obtained by restriction sites. The DNA polymorphisms identified using AFLP are typically inherited in Mendelian fashion and may therefore be used for typing, identification of molecular markers, and mapping of genetic loci (Vos *et al.*, 1995).

These fingerprints may be used as a diagnostic tool for determining the identity of a specific DNA sample or to assess the relatedness between individual samples. Fingerprints are also used as a source for genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and / or genetic loci (Hill *et al.*, 1996).

9.3.1 AFLPs in Plant Pathology. *Fusarium oxysporum* f.sp. *ciceris* was distinguished from other formae speciales and from other chickpea pathogens such as, *Fusarium eumartii* and *Fusarium solani*, using AFLPs (Kelly *et al.*, 1995). An analysis, which can clearly distinguish between fungi likely to be present in the same host or

environment, is obviously an advantage. The above findings suggest that AFLP analysis provides a rapid method of differentiating between isolates of *F. oxysporum* f.sp. *ciceris* inducing chickpea yellowing or wilt. Current work involves further AFLP analysis using other primers and the characterisation of the syndrome-specific bands, to develop race group specific primers or probes for the in planta or in soil detection and quantification of *F. oxysporum* f.sp. *ciceris* (Kelly *et al.*, 1995).

The AFLP technique was used to assess the levels of genomic variations among species and isolates of the genus *Colletotrichum* (O'Neill *et al.*, 1997). Their objective was to characterise at the molecular level two lucerne pathogens, which were unusually aggressive to anthracnose-resistant lucerne cultivars, and the taxonomic position of, which was uncertain, based on morphological criteria. The diversity detected with AFLP within *Colletotrichum* species from lucerne suggested that this technique will be useful for identification of individual isolates within this complex genera because of its ability to generate large numbers of polymorphisms and the consistency of PCR amplification (O' Neill *et al.*, 1997).

Keim *et al.* (1997) found that the AFLP marker system appears to be a useful approach for generating high-density genetic maps in soybean. AFLP markers were also evaluated for determining the phylogenetic relationships in *Lactuca* species It was found

that AFLPs represent reliable PCR-based markers for studies of genetic relationships at a variety of taxonomic levels (Hill *et al.*, 1996).

The AFLP technique was also used to assess the extent of variation in cultivated and wild soybean, and to determine genetic relationships among soybean accessions (Maughan *et al.*, 1996). AFLP markers tightly linked to the locus conferring resistance to the leaf rust *Melampsora larici-populina* in *Populus* has also been identified (Cervera *et al.*, 1996). These markers can be useful in current breeding programs and are the basis for future cloning of the resistance gene. No AFLP studies have been performed on the genus *Verticillium* up until now.

10. FUTURE CONTIBUTIONS OF GENETIC ANALYSES TO THE SCIENCE OF PLANT PATHOLOGY

With its emphasis on identification of particular pathogens and genetic analysis of populations, the discipline of plant pathology will require simple molecular methods to identify individuals and biotypes. One would envisage two tiers of technique application. The first tier is in the research laboratory, which will place pathogens of interest in the scheme of systematic taxonomy by sequence-related techniques, and find genetic loci that discriminate biotypes and individuals of the pathogen (Brown, 1996; Burdon & Silk 1997; Henson & French, 1993). The second tier will be to develop user-friendly techniques to identify the putative causal agents of disease in the field, ultimately to the

level of pathogenic race. PCR-based techniques would seem to have the greatest potential, as levels of specificity can be attained that can simplify sample preparation protocols (Maclean *et al.*, 1993).

Research to date has just begun to develop specific applications, and it is likely that the implementation of PCR-based diagnostic tests will grow rapidly in the near future. Central questions in plant pathology can be addressed at a level of precision that was impossible just a few years ago. As PCR methods for detection of pathogens become available, more research will focus on using these tools to study pathogen populations, biology, ecology, variability, and host-pathogen interactions (Henson & French, 1993).

Eventually, experiments using molecular markers should reveal more about the process of coevolution between host and pathogen in agricultural ecosystems (McDermot *et al*,1989). As advanced PCR-based markers become available, it may become possible to amplify specific DNA sequences from soil or root samples and make a direct assessment of the genetic structure of populations of soilborne fungi without first making pure cultures (McDonald & McDermot, 1993; Williams *et al.*, 1991). Selective PCR-amplification can also be used to quantify pathogens directly from soil or plant material in a relatively short period.

11. CONCLUSION

This review highlights the need for integrating a basic understanding of the taxonomy, biology, ecology, and genetics of *V. dahliae* and species of important plant-pathogenic fungi into population genetic studies. More knowledge about the genetic structure of populations of plant pathogens is needed to implement effective control strategies, thus the need to implement a potential PCR-based technique.

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CHAPTER 2

RESEARCH RESULTS

Pathogenicity and Virulence of *Verticillium dahliae* isolates from cotton, potato, avocado and tomato on tomato seedlings.

Chapter 2

Pathogenicity and Virulence of *Verticillium dahliae* Isolates from Cotton, Potato, Tomato, and Avocado on Tomato Seedlings.

ABSTRACT

Verticillium dahliae isolates obtained from cotton, potato, tomato, and avocado were evaluated for pathogenicity and virulence on two tomato cultivars (Rooi Kakie and Flora Dade) in the greenhouse, using a root-dip inoculation technique. Disease evaluations were based on percentage leaf yellowing, shoot length and shoot dry mass of plants. Isolations were made from vascular tissue of plants to confirm infection. *V. dahliae* isolates from cotton, potato, and avocado all caused typical Verticillium wilt symptoms on both tomato cultivars and were successfully re-isolated from the seedlings in two experiments conducted. The cotton, potato, and avocado isolates were thus pathogenic on tomato. Isolates from tomato showed few foliar symptoms and could not be re-isolated from plants in the second trial. The pathogenic isolates differed in degree of virulence. Variation in virulence was also observed among isolates obtained from diseased plants belonging to the same species. None of the isolates tested were host specific, because cotton, potato and avocado isolates induced foliar symptoms on both tomato cultivars. Leaf yellowing was found to be a better parameter for disease assessment than shoot dry mass since a higher correlation was found between isolation of the pathogen and leaf yellowing than between pathogen isolation and shoot dry mass.

INTRODUCTION

Verticillium dahliae Kleb. has been isolated from many plant species world-wide and has a wide host range which includes various tree species, shrubs, wild plants, vegetables, ornamentals and weeds (Pegg, 1974). In South Africa, *V. dahliae* was first found in diseased cotton plants at Barberton during the season of 1938-1939 (Doidge, 1950). *V. dahliae* was responsible for a wilt disease in cotton in the vicinity of Kakamas, Upington as well as in eggplant in the vicinity of Tulbagh, and in potatoes in the Cape Province (Doidge, 1950). It was later also identified as the casual organism of a wilt disease in strawberries (Visser & Kotze, 1975). Today cotton, potatoes and tomatoes are still three of the economically important crops that are affected annually, due to losses in production following *Verticillium* infections (Heale, 1988).

V. dahliae is a root infecting fungus and infection leads to blockage of the vascular tissue of the host plant (Pegg, 1974). Verticillium wilt of potato causes the discolouration and deterioration of the root following colonisation by the pathogen (Nachmias & Krikun, 1984). Root infection may, or may not result in the expression of foliar symptoms (Mol & Termorshuizen, 1995). Often symptoms do not develop until plant flowering (Busch & Schooley, 1970). When such symptoms develop, they start from the lower leaves and progress upward. Affected leaves exhibit chlorosis followed by necrosis (Pegg, 1984). Symptoms often start near the leaf margins and progress inward along the veins, resulting in a characteristic V-shaped lesion (Nachmias & Krikun,

1984). Symptoms commonly develop on one-half of a leaf and the water conducting tissues of infected plants are discoloured.

Severe economic losses are caused by *V. dahliae* in field-grown tomatoes in many production areas, and the pathogen limited yields to a great extent before the discovery of genetic resistance. Tomato is the only host where formae speciales or races are described for *V. dahliae.* This is because of a lack in host specificity of the pathogen (Subbarao *et al.*, 1995). Two races of *V. dahliae* are recognised on the basis of pathogenicity to tomato cultivars, carrying the dominant Ve allele. Isolates are designated as race 2 when they have the ability to overcome resistance conferred by the Ve gene, and as race 1 if they do not have the ability (Bender & Shoemaker, 1984). In tomato, *V. dahliae* infects susceptible plants through the roots and invades and blocks the water conducting tissue. As a result, chlorosis, stunting and decrease in yield are normally observed (Pegg, 1974).

Since the original discovery of Verticillium wilt in cultivated cotton the disease has been found in all major cotton-growing regions of the world (Doidge, 1950; Schnathorst & Mathre, 1966). Two groups of *V. dahliae* which infect cotton, namely 'defoliating' (aggressive), and "non-defoliating' (non-aggressive) were described (Daayf *et al.*, 1995). Several additional characters were used to distinguish these pathotypes, including optimal growth, temperature, germination rate of conidia, production rate of microsclerotia and physiological factors induced in host plants (Willie & Devay, 1970). Isolates of *V. dahliae* from cotton range in virulence from those causing defoliation and

death, to those, which incite only mild chlorosis in one or more leaves of the host plant. In addition to these dramatic foliar symptoms, the host plant is also stunted, and its overall vegetative growth is reduced. The size of mature leaves is reduced and the stem between successive leaves is shortened. Differences in pathogenic specialisation may also occur between *V. dahliae* isolates from the same host (Willie & Devay, 1970).

V. dahliae generally lacks host specificity with isolates having a wide host range (Subbarao *et al.*, 1995). Isolates from cauliflower caused wilt symptoms on potato, tomato, strawberry and watermelon, while all isolates from crucifer crops caused wilt on cauliflower (Subbarao *et al.*, 1995). However, it has been found that there can be a certain degree of host specialisation in that isolates are more pathogenic on the host from which they were originally isolated than on other hosts (Krikun & Bernier, 1987). This holds an important consequence for cultural control methods such as crop rotation and disease management in general.

Thus, from an ecological, disease management, and taxonomic perspective, the potential pathogenic specialisation, or lack thereof, within *V. dahliae* is an important consideration when implementing disease control strategies. The objective of the present study was to determine the pathogenicity (the capability of a pathogen to cause disease), (Agrios, 1978) and the virulence (the degree of pathogenicity of a given pathogen) (Agrios, 1978), of *V. dahliae* isolates from cotton, potato, tomato and avocado on two tomato cultivars.

MATERIALS AND METHODS

Isolation of *Verticillium* isolates. *Verticillium* isolates from potato: *Verticillium* was isolated from diseased potato plants from the Sandveld area in the Western Cape province. Stems were removed from plants and surface sterilised for 60 s in 95% ethanol. Vascular tissues were plated onto corn meal agar (CMA) and water agar (WA). The plates were incubated at 21 ± 2°C under intermittent light for one week. Growth was examined with a light microscope (10x). Single spore isolations were made from all positively identified isolates according to Hawksworth & Talboys, (1970b).

Verticillium isolates from cotton, tomato and avocado: The isolates from cotton were obtained from the *Verticillium* culture collection maintained at the Tobacco & Cotton Research Institute of the Agricultural Research Council (ARC). The tomato and avocado isolates were from the culture collection maintained at ARC-Roodeplaat. *Verticillium* species were identified by I. Rong (ARC-Plant Protection Research Institute (PPRI) and C. Millard (ARC-Roodeplaat) according to Hawksworth & Talboys (1970b). The host, geographic origin, and race of the isolates that form part of this study are listed in Table 1.

Inoculum preparation and inoculation procedure. All isolates were grown on CMA at room temperature for 7-14 days. Millet seed (40 g in 60 ml of water) was

sterilised over two consecutive days (Duvenhage *et al.*, 1993). Each flask was inoculated with *V. dahliae* colonised agar plugs and left to grow for two weeks. Inoculum was prepared by adding 200 ml of sterile distilled water to colonised millet seed in Erlen Meyer flasks. The resulting suspension was sieved through sterile muslin cloth and adjusted to a concentration of $1x10^{6}$ conidia per millimetre using a heamacytometer.

Tomato seedling production and inoculation. The two tomato cultivars Rooi Kakie (susceptible), and Flora Dade (resistant, Ve gene) were grown in Hygromix (Hygrotech) in seedling trays with 200 compartments at 25 ± 5°C in the greenhouse under controlled environmental conditions. Seedlings were irrigated daily with water at pH 6.5± 0.5 and fertilised every second week with Chemicult until the first true leaf stage, ±5 weeks. A day prior to inoculation the plants were subjected to water stress, by omission of watering. The seedlings were removed from the trays and inoculated by dipping the roots in the spore suspension ($1x10^{6}$), for 5-min (Okie & Gardner, 1982; Venter, 1990). Inoculated seedlings were then transplanted in individual cups (300 ml) with two plants per cup, six cups (replications) per treatment. The sequence in which seedlings were inoculated and transplanted in the cups was based on a completely randomised design. After inoculation, plants were grown in the greenhouse for 6 weeks at 25 - 30°C under natural light. Plants were watered daily for the first four weeks, and every second day for the last two weeks. Plants were subjected to these water stress periods to assist symptom development. Control plants were subjected to the same conditions. The plants were fertilised twice after inoculation, at two and four weeks. The

entire experiment was conducted twice, once in September (experiment 1) and again in June (experiment 2).

Disease evaluation. Experiment 1: Disease symptoms were assessed at 6 weeks after inoculation. Plants were evaluated for leaf yellowing, shoot dry mass, shoot length and vascular browning. Recovery of the pathogen after infection (re-isolation), was also one of the parameters used. The total number of yellow leaves was expressed as a percentage of the total number of leaves on the plants. A mean percentage was determined for the six replicates used, with two plants being a replicate. The dry shoot mass of each of three replicate plants, (one plant per replicate), per cultivar/isolate combination were determined by cutting the stems at soil level and drying the aerial portion to constant weight at 70° C. Shoot length was determined by measuring the stem segment from the soil level to the end of the stem segment. To confirm pathogenicity, vascular colonisation of each isolate was determined by removing vascular stem sections from the remaining 6 replicate plants per cultivar/isolate combination, and transferring it to Petri dishes containing CMA. These were incubated at room temperature for seven days and then examined for the presence of the pathogen. The fungus was identified microscopically by the formation of typical verticillately branched conidiophores. The total number of isolates tested was 30.

Experiment 2: The disease evaluation for experiment 2, was the same as experiment 1, except that shoot length was not determined, and that foliar symptoms were also assessed at 3 weeks. A total of 28 isolates were tested.

Statistical analyses. Experiment 1: Standard one-way analysis of variance was performed on the total yellow leaves, shoot length, and shoot dry mass using SAS V 6.12 Statistical Software (SAS, 1990). Shapiro-Wilk test was performed to test for non-nomality (Shapiro & Wilk, 1965). Student's t-Least Significant Difference was calculated at the 5% level to compare the treatment means.

Experiment 2: Standard one-way analysis of variance was performed on the total yellow leaves and shoot dry mass using Stat graphics (V 4.1), followed by subsequent Tukey pairwise tests ($P \leq 0.05$) (Zar, 1984). The mean values of parameters of the group of isolates collected from cotton, potatoes, tomatoes and avocado were also submitted to one way analysis of variance followed by subsequent Tukey pairwise tests ($P \leq 0.05$). Correlation between percentage isolation from plants, leaf yellowing and dry mass was investigated using Spearman's correlation coefficient (Zar, 1984).

RESULTS

Symptoms of inoculated plants resembled those described in the literature for Verticillium wilt of tomato. Foliar symptoms were evident in both cultivars infected with *V. dahliae* isolates. Typical leaf yellowing and browning were present **(Fig. 1a, b)**. A

few plants took several weeks to develop foliar symptoms and some did not develop symptoms at all.

Experiment 1: All isolates from the four hosts plants caused foliar symptoms in the form of leaf yellowing on the two cultivars, Rooi Kakie (no Ve gene) and Flora Dade (Ve gene present). Evaluations were conducted at six weeks only. The percentage leaf yellowing did not differ significantly for both Rooi Kakie ($P = 0.3605$) and for Flora Dade ($P = 0.5100$) (Table 2). No significant differences for shoot length and shoot dry mass for both Rooi Kakie ($P = 0.1457$ and $P = 0.1446$) respectively, and Flora Dade ($P = 0.6841$ and $P = 0.1290$) respectively, could be found.

The means for percentage leaf yellowing among isolates from different hosts did not differ significantly ($P \leq 0.05$) on Rooi Kakie with mean disease incidences of 34.6 % (cotton), 34.7 % (potato), 33.6 % (avocado) and 33.6% (tomato) (Table 3). The same results were obtained for the resistant cultivar Flora Dade with overall disease incidence of 28.9 % (cotton), 31.4 % (potato), 26.3 % (avocado) and 32.1 % (tomato). Means for shoot lengths as well as shoot dry mass showed no significant difference ($P \leq 0.05$). In experiment 1 all isolates were found to be pathogenic and were re-isolated from all symptomatic inoculated plants. During experiment 1 the conditions in the glasshouse was not optimal. The temperature could not be controlled, and plants were subjected to heat stress. This heat could have contributed to the expression of more severe disease symptoms. Isolations from asymptomatic control plants were negative.

Experiment 2: Isolates had a significantly different ability to cause leaf yellowing on Rooi Kakie ($P<0.0001$) and Flora Dade ($P< 0.0001$) at 3 weeks and 6 weeks (Table 2). All cotton, potato and avocado isolates had the ability to induce foliar symptoms in the form of leaf yellowing on the two cultivars, Rooi Kakie and Flora Dade. The analysis of variance revealed significant differences ($P<0.0001$) between shoot dry mass of Rooi Kakie seedlings inoculated with the different isolates. There was also a significant difference for shoot dry mass of Flora Dade seedlings ($P< 0.0001$). There was a significant difference between the mean values of all isolates collected from different hosts, for leaf yellowing and shoot dry mass on both Rooi Kakie ($P< 0.0001$) and Flora Dade ($P< 0.0001$) at 3 and 6 weeks.

The expression of disease symptoms (yellow leaves) was fairly pronounced at 3 weeks, for the first assessment (Table 4). At 6 weeks, the percentage leaf yellowing means for the isolates from different host, ranged from 37.8 % (cotton), 37.7 % (potato), 51.3 % (avocado), and 24.7 % (tomato) at 6 weeks for Rooi Kakie. On Flora Dade percentage leaf yellowing means ranged from 41.5 %, 43.8 %, 53.5 %, and 14.1% for isolates from cotton, potato, avocado, and tomato respectively, at 6 weeks. The cotton and potato isolates, according to the Tukey pairwise test ($P \leq 0.05$), did not differ significantly in the degree of pathogenicity, whereas the avocado isolates did differ significantly for leaf yellowing at 6 weeks on Rooi Kakie. The highest percentage leaf yellowing symptoms was caused by the avocado isolates, and the least disease symptoms

developed from tomato isolates at six weeks. Shoot dry mass means were least for the avocado isolates and highest for the tomato isolates. All potato and cotton isolates were pathogenic on both tomato cultivars but varied in virulence with some isolates being more virulent than others.

The avocado isolates, VA 3-1 and 31fd, was found to cause the highest percentage of diseased leaves on both cultivars, 48.0% and 54.6% on Rooi Kakie, respectively, and 49.0% and 58.0% on Flora Dade, respectively (Table 4). These avocado isolates scored the highest mean value for inducing foliar symptoms (leaf yellowing) on both tomato cultivars. Isolate VA 3-1 had the overall lowest shoot dry mass mean of 6.72 g and 8.82 g on Rooi Kakie and Flora Dade, respectively. Isolate VA 3-1 caused a significantly lower mass ($P \leq 0.05$) on Rooi Kakie, than on Flora Dade. The group of isolates (avocado) with the highest disease symptoms (leaf yellowing) also had the lowest drymass values (Table 4).

Symptom expression of the tomato isolates (66, CCU 91, CCU 90, VT2-4, 67fd, S1, 90m, 90d) on the two differential hosts were minimal for both host cultivars. These tomato isolates scored the lowest mean values for leaf yellowing (6 weeks), ranging from 24.7% on Rooi Kakie to 14.1% on Flora Dade at ($P \leq 0.05$). All the tomato isolates showed higher means for shoot dry mass than isolates from potato and cotton in the case of Flora Dade (Table 4). Although all the tomato isolates were pathogenic during the first experiment, a loss in pathogenicity was observed for tomato isolates after experiment

two. There was no prior indication that these isolates lost their pathogenicity, until after the data for experiment two was analysed.

The highest pathogen recovery rate were found among the avocado (100% for Rooi Kakie and Flora Dade) isolates, followed by the potato (84.6% for Rooi Kakie and 61% for Flora Dade) and cotton (72% for Rooi Kakie and Flora Dade) isolates (Table 4).

V. dahliae could be re-isolated from each isolate x cultivar combination except the tomato isolates during experiment 2. All control plants remained symptomless during the trial and attempts to isolate the pathogen from control plants were negative.

Vascular discolouration in the lower stems was found **(Fig. 2)**, but not sufficiently pronounced in both experiments to be used for disease evaluations. No vascular browning from plants infected with the tomato isolates was detected in experiment 2.

A significant negative correlation was found between shoot dry mass and re-isolation of the pathogen at 6 weeks ($R= -0.43$, $P \leq 0.05$) on Rooi Kakie (Table 5). A higher significant correlation was found for leaf yellowing and the recovery of the pathogen when compared at both 3 weeks ($R = 0.83$, $P \leq 0.05$) and 6 weeks ($R= 0.79$, $P \leq 0.05$), respectively. The correlation coefficients between leaf yellowing and drymass were not significant at both 3 weeks ($R = -0.35$, $P \leq 0.05$) and 6 weeks ($R= -0.38$, $P \leq 0.05$) of leaf yellowing evaluation for Rooi Kakie.

On Flora Dade, a significant negative correlation between shoot dry mass and re-isolation of the pathogen was present ($R= -0.44$, $P \leq 0.05$) (Table 6). Leaf yellowing and the recovery of the pathogen had a higher significant correlation at both 3 weeks ($R= 0.61$, $P \leq 0.05$) and 6 weeks ($R= 0.82$, $P \leq 0.05$). The correlation between leaf yellowing and shoot dry mass at 6 weeks was not significant ($R= -0.26$, $P \leq 0.05$).

DISCUSSION

The parameters used for the assessment of pathogenicity and virulence of *V. dahliae* isolates in the study were leaf yellowing, shoot length (only experiment 1) and shoot dry mass of tomato seedlings. Leaf yellowing was found to be a better parameter for disease assessment than shoot dry mass, since a significantly higher correlation was found between isolation of the pathogen and leaf yellowing (at 3 and 6 weeks on both cultivars), than between pathogen isolation and shoot dry mass. An assessment of leaf yellowing at 3 weeks seems to be a good indicator of the disease development and the expected results at 6 weeks. No significant correlation was found between reduction in shoot dry mass and leaf yellowing as also found by Baergen *et al.* (1993). Ratings based on foliar symptoms have also been found previously to be more reliable than ratings based on reduction in plant height, degree of vascular discolouration, or presence of the pathogen within the stem of tomato seedlings (Grogan *et al.*, 1979; Thanassoulopous & Kitsos, 1974). A reduction in shoot length (stunting), and the consequent reduction in dry weight have been found to be unreliable indications of pathogenicity (Bender &

Shoemaker, 1984; O' Garro & Clarkson, 1988). Thus, leaf yellowing is considered to be a more accurate parameter for assessment of pathogenicity and virulence of *V. dahliae* isolates on tomato seedlings than dry mass, and should be used for future pathogenicity and virulence analysis.

Symptom expression developed quicker under the heat stress conditions of experiment 1 than the cool controlled conditions of experiment 2. Suboptimal temperatures can cause an increase in the wilt development by *V. dahliae* (Termorshuizen & Mol, 1995). Ashworth *et al.* (1976) found that climatic conditions and irrigation influenced disease symptoms significantly. Kehr *et al.* (1962) also found that the pathogenicity of *Fusarium oxysporum* f. sp *cepae* isolates on onion lines was markedly greater at 38°C than at 34°C, and the differences in pathogenicity were less at 38°C than at 34°C. It is possible that some of the plant injury that appeared to be due to the action of the pathogen may be attributed to the adverse effect of high temperature on seed germination and plant growth (Kehr *et al.*, 1962). The analysis of variance for disease evaluations revealed no significant differences for experiment 1 while significant differences were observed for experiment 2. The increase in temperature could have reduced the chances of differences between isolate effects in experiment 1. A temperature study done by Ben-Yephet, (1979) and Joaquim & Rowe, (1991) found that with an increase in temperature, the chances for significant differences in disease development were drastically reduced. Temperature of 26°C and higher induced severe

pathogenic effects and tended to minimise differences between the experiments and among isolates of *F. oxysporum* f. sp *cepae* (Kehr, *et al.*, 1962).

The tomato isolates lost their pathogenicity and their virulence during the second trial and could thus not be compared with cotton, potato and avocado isolates. Disease symptoms were initially present, but did not increase significantly as with isolates from cotton, potato and avocado. It was demonstrated that *V. dahliae* isolates vary in pathogenicity to different hosts (Jeger *et al.*, 1996; Minogochi & Clarkson, 1994; Tjamos, 1981). Researchers dealing with *V. dahliae* share the experience that results of experiments are often inconsistent, apparently as a consequence of the fact that the behaviour of this pathogen is insufficiently understood (Termorshuizen & Mol, 1995). It is known that great variability is possible in *Verticillium* species (Mol & Termorshuizen, 1995; Vigoroux, 1971). Researchers found that the biotic and abiotic environment have a profound effect on disease progress and yield loss (Ashworth *et al.*, 1976).

The cotton, potato and avocado isolates were pathogenic on both tomato cultivars, and no non-pathogenic isolates were found amongst these isolates. Basu (1961) and Schnathorst & Mathre (1966) also found that isolates from various other hosts (melon, cucumber, potato, strawberries) were pathogenic to tomato. However, Schnathorst & Mathre (1966) found that highly virulent cotton isolates were essentially non-pathogenic to tomato. In this study all cotton isolates were found to be pathogenic, with different levels of virulence on both tomato cultivars. Cotton isolates used in this study were

classified as defoliating. Two different pathotypes are found within the defoliating strains in South Africa, which differ in virulence but not in pathogenicity (Personal communication, A. Swanepoel).

Krikun & Bernier (1987) found that isolates from pea were more pathogenic and virulent on pea than the potato isolates, thus indicating a certain degree of specialisation among *V. dahliae* isolates. In this study no indications were found for a certain degree of host specialisation, since isolates from cotton were equally pathogenic on the solanaceous host (tomato) than isolates isolated from a solanaceous host (potato). Results of this study rather agrees with that of Visser (1977), who also found a lack of specialisation between virulence and host of isolation, when a rose isolate was more virulent on a tomato host than isolates from tomato (Visser, 1977). Similar results have also been found, indicating that quantitatively higher pathogenicity rather than specific adaptation to a host is important (Grogan *et al.*, 1979; Minogochi & Clarkson, 1994). *V. dahliae* isolates are characterised by a high pathogenic flexibility or levels of variation, and virulence of soilborne *V. dahliae* isolates occurs as a continuum from weakly virulent to highly virulent isolates (Ashworth, 1983; Daayf *et al.*, 1995;). Potato and cotton isolates were equally virulent in both trials, and 31fd, isolated from avocado, was found to be the most virulent. Results of the study agree with the findings of other authors in that differences in virulence rather than specific adaptation to a host is important (Ashworth, 1983; Daayf *et al.*, 1995; Grogan *et al.*, 1979; Minogochi & Clarkson, 1994; Visser, 1977).

The results of this study suggest that host specificity was not common in *V. dahliae* isolates, although given isolates may have exhibited differential virulence against the hosts. A continuum of virulence levels on isolates ranging from slightly to highly virulent was observed in this study. This variability in virulence among *V. dahliae* isolates raises a question about the usefulness of predicting the severity of Verticillium wilt based on soil inoculum density. It can thus be concluded that in agricultural cropping systems in South Africa the virulence and pathogenicity of *V. dahliae* isolates will not be dependent on the previous crop planted, since no correlation was found between host of isolation and virulence. Results from the study indicate that inherent virulence of an isolate seem to be more important than host of isolation. This is very important in the evaluation of host resistance, since genotypes that seems resistant in one study may perform poorly in another study due to the difference in virulence of isolates (Baergen *et al.*, 1993). Host plant resistance is relative to the virulence of the isolates used to test for that resistance (Strausbaugh, 1993). In breeding programs it is crucial that prospective *Verticillium* resistant lines be exposed to the most virulent pathotype for proper evaluation (Joaquim & Rowe, 1991). The choice of cultivar, crop rotation, and cultural practises will not be sufficient to keep the crop from infection but they will be key factors in controlling the severity of the disease (Tjamos, 1981). Thus, for the screening of cultivar resistance the most virulent isolates must be used.

Table 1. Geographical origin, host and race description of *Verticillium dahliae* isolates used in pathogenicity studies.

Isolate number	Host	Geographical Origin	Race	Source [a]
VD94/1	Cotton	Springbokvlakte	Defoliating	1
VD94/3	Cotton	Springbokvlakte	Defoliating	1
VD94/4	Cotton	Springbokvlakte	Defoliating	1
VD94/5	Cotton	Springbokvlakte	Defoliating	1
VD94/6	Cotton	Springbokvlakte	Defoliating	1
VD94/8	Cotton	Springbokvlakte	Defoliating	1
G10	Potato	Sandveld	-[b]	4
G12	Potato	Clanwilliam	-	4
G15	Potato	Sandveld	-	4
G18	Potato	Sandberg	-	2
G19	Potato	Clanwilliam	-	2
G20	Potato	Clanwilliam	-	2
G21	Potato	Sandberg	-	2
G22	Potato	Lambertsbaai	-	2
G23	Potato	Sandveld	-	3
G24	Potato	Lambertsbaai	-	3
G25	Potato	Ceres	-	3
G26	Potato	Vanrhynsdorp	-	3
G27	Potato	Worcester	-	3
G28	Potato	Worcester	-	3
G29	Potato	Eastern Cape	-	3
VA 3-1	Avocado	Hartbeeshoek	2	4
31fd	Avocado	Roodeplaat	2	4
CCU 90	Tomato	Mooketsi	2	4
67fd	Tomato	Roodeplaat	1	4
66	Tomato	Mooketsi	1	4
VT 2-4	Tomato	Mpumalanga	2	4
90m	Tomato	Mooketsi	2	4
90d	Tomato	Mooketsi	2	4
CCU 91	Tomato	Mooketsi	1	4
225	Tomato	Roodeplaat	1	4
S1	Tomato	Roodeplaat	1	4
72vd	Tomato	Roodeplaat	1	4
27d	Tomato	Mooketsi	1	4
WS	Tomato	Elsenburg	1	3

[a] Isolates supplied and identified by 1 = A. Swanepoel,(ARC-Tobacco & Cotton Research Institute), 2 = M Visser, 3 = A. Mc Leod, (ARC-Roodeplaat), 4 = MDR Uys (ARC- Roodeplaat) and S. Visser (ARC Roodeplaat)

[b] no known race groups on potato

Table 2. Analysis of variance for percent leaf yellowing (LY), shoot length (SL) and shoot dry mass (SDM) following inoculation of tomato cultivars by isolates of *Verticillium dahliae.*

Cultivar	Degrees of freedom	Mean square	F value	P
Experiment 1				
Rooi Kakie				
LY	29	21.38	1.11	0.3605
SL	29	15.69	1.39	0.1457
SDM	29	0.41	1.39	0.1446
Flora Dade				
LY	29	40.12	0.98	0.5100
SL	29	7.83	0.84	0.6841
SDM	29	0.84	1.43	0.1290
Experiment 2 at 3 weeks				
Rooi Kakie				
LY	27	82.01	9.58	< 0.0001
LY [b] c x p x a x t x C	4	250.75	16.87	< 0.0001
Flora Dade				
LY	27	31.87	4.01	< 0.0001
LY c x p x a x t x C	4	94.14	9.58	< 0.0001
Experiment 2 at 6 weeks				
Rooi Kakie				
LY	27	82.50	9.64	< 0.0001
SDM	27	5.10	4.59	< 0.0001
L Y c x p x a x t x C	4	250.50	16.76	< 0.0001
SDM c x p x a x t x C	4	14.56	8.08	< 0.0001
Flora Dade				
LY	27	31.33	3.78	< 0.0001
SDM	27	2.38	2.84	< 0.0001
LY c x p x a x t x C	4	84.73	8.26	< 0.0001
SDM c x p x a x t x C	4	3.52	2.84	0.029

[b] Comparisons among cotton (c), potato (p), avocado (a) and tomato (t) isolates and controls (C)

Table 3. Leaf yellowing, shoot length, and shoot dry mass of tomato (cv. Rooi Kakie and Flora Dade) inoculated with *Verticillium dahliae* isolates in experiment 1.

	Rooi kakie			Flora dade		
V.dahliae isolate	Leaf yellowing (%)	Shoot length (cn)	Shoot dry mass (g)	Leaf yellowing (%)	Shoot length (cm)	Shoot dry mass (g)
Cotton						
VD 94/1	35.33 a-e	3.267 d	2.04 a-f	25.66 b-d	6.73 a-c	1.55 cd
VD 94/3	33.33 b-e	9.13 a-c	2.24 a-f	31.00 a-d	8.31 ab	3.31 a
VD 94/4	33.33 b-e	6.35 a-d	1.83 b-f	26.66 b-d	7.08 a-c	2.37 a-d
VD 94/5	38.33 a-c	6.80 a-d	1.47 f	36.00 ab	6.25 a-c	2.93 ab
VD 94/6	33.00 b-e	6.23 a-d	2.39 a-e	23.33 d	6.13 a-c	3.07 ab
VD 94/8	34.33 a-e	11.63 a	2.36 a-f	31.00 a-d	9.48 a	2.29 a-d
mean	34.61a[y]	7.23	2.05	28.94a	7.33	2.59
Potato						
G10	33.66 a-e	7.20 a-d	2.15 a-f	28.33 a-d	6.36 a-c	3.13 ab
G12	33.00 b-e	10.20 ab	1.70 d-f	30.66 a-d	5.60 a-c	2.24 a-d
G15	39.00 ab	11.66 a	2.46 a-d	33.00 a-d	5.96 a-c	2.57 a-d
G18	36.33 a-d	8.97 a-c	2.09 a-f	32.33 a-d	7.95 ab	2.49 a-d
G19	32.33 b-e	6.46 a-d	1.56 ef	27.66 a-d	7.13 a-c	2.12 a-d
G20	35 66 a-e	9.11 a-c	2.18 a-f	31.00 a-d	8.35 ab	2.13 a-d
G21	36.66 a-d	7.46 a-d	2.68 ab	31.00 a-d	8.16 ab	3.31 a
G22	33.00 b-e	10.20 ab	2.16 a-f	31.00 a-d	9.96 a	2.27 a-d
G23	33.00 b-e	10.40 ab	2.71 ab	37.33 a	9.50 a	3.00 ab
mean	34.73a	9.07	2.19	31.36a	7.66	2.58
Avocado						
VA 3-1	33.67 a-e	4.38 cd	1.76 c-f	26.33 b-d	6.95 a-c	2.49 a-d
mean	33.67a	4.38 cd	1.76	26.33ab	6.95 a-c	2.49
Tomato						
225	33.00 b-e	7.91 a-d	1.62 d-f	28.66 a-d	6.81 a-c	2.95 ab
69	33.66 a-e	8.75 a-d	1.86 b-f	33.66 a-d	5.71 a-c	3.11 ab
67	31.00 de	7.83 a-d	2.13 a-f	25.00 dc	8.60 ab	2.45 a-d
66	31.00 de	10.01 ab	2.23 a-f	25.33 dc	5.18 a-c	2.71 a-c
VT 2-4	36.00 a-e	8.73 a-d	1.87 b-f	37.33 a	7.60 a-c	2.02 b-d
90m	37.66 a-d	6.33 a-d	2.36 a-f	30.00 a-d	2.76 c	1.33 d
90d	35.33 a-e	6.78 a-d	2.61 a-c	29.66 a-d	9.45 a	2.08 a-d
CCU 91	34.66 a-e	5.13 b-d	2.06 a-f	28.00 a-d	6.53 a-c	2.73 a-c
S1	40.66 a	8.271 a-d	1.97 a-f	35.33 a-c	8.15 ab	2.35 a-d
72 vd	31.33 c-e	3.93 dc	2.82 a	25.66 b-d	7.38 a-c	1.50 cd
27d	31.00 de	5.58 b-d	2.13 a-f	28.33 a-d	4.10 bc	2.89 ab
WS	29.00 e	7.85 a-d	2.16 a-f	28.00 a-d	8.48 ab	2.20 a-d
mean	33.69 a	7.03	2.12	32.11 a	6.74	2.37

[y] Means within a column followed by the same letter are not significantly different according to the LSD test

Table 4: Leaf yellowing, shoot dry mass, and pathogen recovery of tomato (cv. Rooi Kakie and Flora Dade) inoculated with *Verticillium dahliae* isolates in experiment 2 at 3 and 6 wks.

	Rooi kakie				Flora Dade			
V.dahliae isolate	Leaf Yellowing at 3wks (%)[x]	Leaf Yellowing at 6wks (%)[x]	Shoot Dry mass (g)	Pathogen recovery (%)[xx]	Leaf yellowing at 3wks (%)[x]	Leaf Yellowing at 6wks (%)[x]	Shoot dry mass (g)	Pathogen recovery (%)[xx]
Cotton								
VD 94/1	13.27 b-e	25.15 a-c	10.28b-d	33	6.86 a-c	37.83 d-j	10.32 abc	33
VD 94/3	14.13 c-e	53.40 c-f	9.85a-d	100	9.19 a-c	38.83 d-j	9.58 abc	100
VD 94/4	12.82 b-e	35.00 a-f	10.60 cd	33	5.45 ab	40.00 e-j	11.12 bc	33
VD 94/5	14.935 de	36.21 a-f	11.08 d	100	9.14 a-c	39.50 d-j	8.59 abc	100
VD 94/6	14.33 c-e	47.17 c-f	8.59a-d	100	10.16 a-c	55.00 i-j	10.10 abc	100
VD 94/8	12.80 b-e	28.68 a-f	8.20a-d	66	5.30 a	37.66 d-j	10.39 abc	66
mean	13.73 c[y]	37.87 b	9.76 c	72	7.68 ac	41.47 b	10.10 b	72
Potato								
G18	14.32 c-e	37.56 a-f	7.06 ab	100	7.75 a-c	44.33 g-j	9.62 abc	100
G19	14.18 c-e	30.11 a-f	7.83a-d	33	5.99 ab	58.00 j	10.40 abc	33
G20	12.47 c-f	37.58 a-f	9.82a-d	66	5.69 ab	35.33 c-i	9.24 abc	66
G21	12.41 c-f	27.11 a-e	8.57a-d	66	9.18 a-c	31.16 b-h	10.51 abc	0
G22	13.92 c-e	37.66 a-f	9.45a-d	100	5.90 ab	39.83 d-j	8.04 a	100
G24	12.38 c-f	44.50 b-f	8.90a-d	100	5.08 a	45.00 g-j	10.08 abc	100
G25	13.16 b-e	37.43 a-f	8.77a-d	100	9.21 a-c	44.83 g-j	8.86 abc	100
G26	9.29 b-e	51.00 c-f	7.78a-d	100	12.66 c	55.00 ij	9.11 abc	100
G27	9.59 b-f	42.96 b-f	7.25 a-c	100	8.07 a-c	41.25 f-j	10.78 abc	100
G28	6.99 a-c	36.08 a-f	7.40 a-c	66	6.37 a-c	44.00 g-j	10.42 abc	66
G29	8.22 b-d	33.05 a-f	9.57a-d	100	7.54 a-c	43.16 g-j	9.49 abc	100
mean	11.54 c	37.73 b	8.335 ab	84.6	7.63 a-c	43.80 bc	9.70 ab	61
Avocado								
VA 3-1	9.86 b-f	48.01 c-f	6.72 a	100	8.45 a-c	49.05 h-j	8.82 abc	100
31fd	14.03 c-e	54.60 f	7.17 ab	100	11.61 bc	58.00 ij	8.42 ab	100
mean	11.95 bc	51.30 c	6.95 a	100	10.03 c	53.52 c	8.62 a	100

Table 4: continued

	Rooi kakie				Flora Dade			
V.dahliae isolate	Leaf yellowing at 3wks (%)[x]	Leaf yellowing at 6wks (%)[x]	Shoot dry mass (g)	Pathogen recovery (%)[xx]	Leaf yellowing at 3wks (%)[x]	Leaf yellowing at 6wks (%)[x]	Shoot dry mass (g)	Pathogen recovery (%)[xx]
Tomato								
CCU 90	5.18 ab	15.50 a	9.48 a-d	0	4.14 a	14.33 a-c	10.97 a-c	0
67fd	14.10 c-e	36.39 a-f	7.95 a-d	0	7.68 a-c	19.50 a-f	11.51 c	0
66	4.71 ab	30.65 a-f	10.83 d	0	5.74 ab	6.33 a	10.77 a-c	0
VT 2-4	10.91 b-f	19.58 ab	7.75 a-d	0	4.07 a	22.33 a-f	8.66 a-c	0
90m	11.00 b-f	25.36 a-d	9.48 a-d	0	5.45 ab	9.66 ab	9.75 a-c	0
90d	5.41 ab	30.03 a-f	10.90 d	0	4.41 a	17.13 a-d	9.43 a-c	0
CCU 91	15.95 e	20.38 ab	9.46 a-d	0	6.21 ab	11.00 ab	9.25 a-c	0
S1	8.50 b-d	20.00 ab	10.80 d	0	5.22 a	12.83 a-c	9.45 a-c	0
mean	9.44 b[y]	24.73a	9.58 c	0	5.37 ab	14.13 a	9.96 ab	0
Control	1.02 a	13.00 a	9.61a-d	0	3.87 a	17.83 a	10.85 a-c	0

[x] Leaf yellowing based on percentage of plants displaying yellowing symptoms

[xx] Percentage recovery of pathogen after re-isolation from infected plants

[y] Means within a column followed by the same letter are not significantly different according to the Tukey test

Table 5. Correlation coefficients for three disease parameters in tomato cultivar Rooi Kakie.

Variable	Drymass	Leaf yellowing [y]	Pathogen recovery
Rooi Kakie (3 weeks) [y]			
Drymass	1.00	-0.35	-0.41*
Leaf yellowing (3 weeks) [y]	-0.35	1.00	0.83*
Rooi Kakie (6 weeks) [y]			
Drymass	1.00	-0.38	-0.43*
Leaf yellowing (6 weeks) [y]	-0.38	1.00	0.79*

[y] The analysis was conducted at week 3 and week 6 of evaluation for leaf yellowing.
Numbers followed by * were significantly different ($P \leq 0.005$)

Table 6. Correlation coefficients for three disease parameters in tomato cultivar Flora Dade.

Variable	Drymass	Leaf yellowing [y]	Pathogen recovery
Flora Dade (3 weeks) [y]			
Drymass	1.00	-0.30	-0.44*
Leaf yellowing (3 weeks) [y]	-0.30	1.00	0.61*
Flora Dade (6 weeks) [y]			
Drymass	1.00	-0.26	-0.44*
Leaf yellowing (6 weeks) [y]	-0.26	1.00	0.82*

[y] The analysis was conducted at week 3 and week 6 of evaluation for leaf yellowing.
Numbers followed by * were significantly different ($P \leq 0.05$)

Fig. 1a Symptoms of Verticillium wilt of tomato, chlorosis of lower leaves on the tolerant cultivar Flora Dade

Fig. 1b Symptoms of Verticillium wilt of tomato, chlorosis of lower leaves on susceptible cultivar, Rooi Kakie

Fig. 2 Vertical cut on stem showing vascular discoloration

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CHAPTER 3

RESEARCH RESULTS

Molecular characterisation of *Verticillium dahliae* using AFLPs

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Molecular Characterisation of *Verticillium dahliae* Using AFLPs

ABSTRACT

A PCR-based fingerprinting technique based on amplified fragment length polymorphism (AFLPs) was used to screen fourteen isolates of *Verticillium dahliae* from potato, cotton, avocado and tomato for genetic variation. Results from pathogenicity tests showed that isolates from potato, cotton, avocado and tomato to be pathogenic to two tomato cultivars. After the second trial, however, it was discovered that the tomato isolates lost their pathogenicity. Each of the five primer combinations generated fingerprint patterns markedly distinct from the other primers. The fingerprint patterns obtained were complex, but enabled the distinction among isolates from the four host plants. DNA polymorphisms and similarities (common band sharing) were found among the *V. dahliae* isolates obtained from the same host. Repeated amplification of the same DNA extracted at different times revealed no artefactual variation, confirming the reproducibility of the technique. The AFLP technique will be a valuable tool for the identification of individual isolates of *V. dahliae* genera because of its ability to generate large numbers of polymorphisms, and the consistency of the polymerase chain reaction amplification.

INTRODUCTION

The hyphomycete, *Verticillium dahliae* Kleb. is a root- infecting fungus of world-wide distribution, has a wide host range on many plants of economic value in which it

causes Verticillium wilt (Pegg, 1974). In the genus *Verticillium* there are several plant pathogenic species of which *V. dahliae* is more widely involved as a pathogen, and is the most widespread and most destructive species (Pegg, 1974). Host specific isolates and races of *V. dahliae* are indistinguishable on the basis of morphological characters (Hu *et al.*, 1993; Pegg, 1984).

The Sandveld constitutes one of the largest producing areas of seed and table potatoes in the Western Cape. A major question concerning *Verticillium* diseases is the cause of the recent dramatic increase in the number and severity of epidemics in the Sandveld area. Despite losses in yield and quality of the commercial crop, producers suffer further losses in seed sales, since infected potato plants produce tubers that are also infected. Diagnostic tests are at best semi-quantitative, and in the case of seedborne infection, isolation of the pathogen proved to be difficult and time consuming. The continuing spread of this disease in many crops suggests that current diagnostic methods are relatively ineffective thus limiting disease management options.

The development of the polymerase chain reaction (PCR) technology has resulted in new opportunities in plant pathogen diagnostics (Henson & French, 1993). One potential alternative approach to detection of *Verticillium* species in infected material is the use of a PCR-based tool. PCR-based DNA assays have been developed for the identification, quantification and to differentiate *Verticillium* species recently (Carder & Barbara, 1991; Griffen *et al.*, 1997; Li *et al.*, 1994; Nazar *et al.*, 1991; Robs *et al.*, 1994; Williams *et al.*,

1991). *V. dahliae* specific PCR primers may aid in more rapid and specific detection of the pathogen directly in plant and / or soil samples. PCR-based tools offer several advantages compared to traditional methods of plant disease diagnosis. These include convenience, sensitivity that could be used for the monitoring of wilt diseases, testing of farm soils, and certification of seed tubers. The application of these techniques will be useful for identification of *V. dahliae* in infected potatoes and tomatoes, detection of *V. dahliae* in infected plants that lack visible morphological structures or symptoms. In view of the economic importance of *Verticillium*-induced wilt diseases, molecular techniques allowing differentiation between strains, genotypes, or individuals have become indispensable tools in population genetic surveys and disease control (Maclean *et al.*, 1993).

DNA fingerprinting is rapidly becoming a tool for the interpretation of family and evolutionary relationships and is also applicable to questions of systematics, taxonomy and population genetics. The AFLP technology, a new DNA fingerprinting technique, is a powerful tool for the detection and evaluation of genetic variation (Vos *et al.*, 1995). Specific sequence information of the organism under investigation is not required and amplification of genomic DNA is initiated at target sites, which are distributed throughout the genome (Vos *et al.*, 1995). The system is based on the use of highly stringent PCR conditions, facilitated by adding double-stranded adapters on the ends of restriction fragments which serve as primer sites during amplification (Vos *et al.*, 1995). Selective amplification is achieved by adding one or more bases on to the PCR primers, which will only then be successfully extended if the complementary sequence is present in the

fragment flanking the restriction site (Donini *et al.*, 1997). The technique is robust and reliable because stringent reaction conditions are used for primer annealing (Vrieling *et al.*, 1997). In comparison to RFLPs, AFLPs will display presence or absence of restriction fragments rather than length differences (Vrieling *et al.*, 1997). Polymorphic fragments are the result of variation in the number of appropriate primer-matching sites of different DNAs (Donini *et al.*, 1997). It is visualised as the presence or absence of a particular band in the fingerprint.

The aim of the study was to optimise AFLP analysis as a possible tool for evaluating genetic variation of *V. dahliae* isolates.

MATERIALS AND METHODS

Fungal Cultures. The substrate, origin and pathogenicity of *V. dahliae* isolates are given in **(Table 1)**. Pathogenicity experiments were described in Chapter 2. Fungal cultures were grown from a single spore and maintained on corn meal agar. Potato dextrose broth (Difco) was used as a growth medium. Fungal material was grown in liquid rather than solidified media prior to DNA isolation. Each flask was inoculated with a spore suspension. Efficient aeration was needed for large-scale growth. The medium constitutes no more than one fifth of the total volume of the flask and was incubated at 22°C for 7-14 days on a rotary shaker at 220 rpm. Work was done in a biological safety hood.

Large-scale accumulation of fungal material for DNA isolation. The mycelia were harvested from the culture medium by vacuum filtration using a Buchner funnel or by centrifugation (15 min, 2500 x g, and room temperature). The mycelia were subsequently washed twice with sterile water, and was either used immediately for genomic DNA isolation or frozen in liquid nitrogen and stored at -20°C.

Genomic DNA Isolation. Several versions of the DNA isolation procedure described by Lee *et al.* (1990) and Raeder & Broda (1985) were investigated. None of these methods yielded good quality DNA. A combination of the two protocols, with minor modifications was used. The protocol is a sodium dodecyl sulfate (SDS) based method, making use of strong detergents to lyse fungal cell walls (Lee *et al.*, 1990; Raeder & Broda, 1985).

Freshly harvested mycelia were ground using a mortar and pestle in the presence of liquid nitrogen and sterile carborandum. Ground mycelium were then resuspended in 10 ml extraction buffer (200 mmol Tris HCl (pH 8.5), 250 mmol NaCl, 25 mmol EDTA (pH 8.0), 0.5% SDS). The isolation buffer was prewarmed to 65°C before addition to the ground mycelia. The suspension was incubated at 65°C for 15-30 min with gentle mixing every 5 min. This was followed with the addition of 7 ml phenol (Merck), mixed gently by inversion, then added 3 ml chloroform / isoamyl alcohol (24:1) and subjected to centrifugation at 16,000 rpm (24,000 x g) for 60 min at 4°C. The supernatant (top layer) was carefully transferred to a fresh tube, avoiding the material at the interface. If any

interface material was carried over, the chloroform / isoamyl alcohol wash was repeated. The DNA was precipitated by the addition of 0.54 volumes of ice cold 100% isopropanol. DNA was allowed to precipitate overnight at room temperature. This was spin at 5,000 rpm for 5 min at 4°C and gently pouring off the supernatant, keeping the pellet. The pellet was washed with 70% ice-cold ethanol, then incubated at 5 min at room temperature then spun at 5,000 rpm at 4°C for 5 min. The supernatant was decanted and the tubes inverted to dry the DNA pellet. Pellets were resuspended in double distilled water. If the pellet was not fully resuspended, after 1 hr, it was incubated at 65°C for 5-10 min, swirling gently if required. DNA was transferred to sterile Eppendorf tubes and stored at -20°C. If an excessive amount of polysaccharides was present in the pellet, it was resuspended in 1x TE containing 2 M NaCl and reprecipitated with 2 volumes of ethanol. Duplicate DNA isolations were performed on a number of samples to verify the reproducibility of the analysis.

Assessing DNA concentration. (i) Fluorometrically: DNA concentrations of the samples were determined following the instructions supplied with the Dyna Quant 200 (Hoefer) flourometer. (ii) Agarose gel electrophoresis: DNA samples were subjected to agarose gel electrophoresis and compared to λ DNA (50 ng - 300 ng) of a known concentration, after visualisation by ethidium bromide staining. λ DNA was applied to the same gel.

AFLP Reactions. The AFLP reactions were carried out following the instructions supplied with the GIBCO Life Technology AFLP ™ kit.

Restriction-Ligation Reactions. Fungal genomic DNA (200 ng) was digested using *Eco*RI and *Mse*I. Restriction digestions were performed at 37°C for 3 hr or overnight. The restriction enzymes were then heat inactivated at 70°C for 10 min. The DNA fragments were ligated to *Eco*RI and *Mse*I adapters at 20°C overnight. Duplicate samples for a number of isolates were prepared.

Preamplification Reactions. The restriction-ligation reaction mixture was diluted 10-fold with sterile 1x TE and used as template for the preselective amplification reactions. The sequences of the adaptors and restriction sites serve as primer binding sites for the preselective amplification. The preselective primers each having 1 "selective" nucleotide, which will recognise the subset of restriction fragments having the matching nucleotide downstream from the restriction site, were used. Preselective amplification was performed in a MJ Research PTC-200 thermal cycler, using a temperature profile of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min for 30 cycles. Ten microliters (10µl) of the preamplification products were applied to a 1.5% agarose gel to confirm that amplification has taken place before continuing with the selective amplification reactions.

Selective Amplification Reactions. For selective amplification, the preamplified

fragments were diluted 10-fold and used as template for selective amplification. *Eco*RI +3 and *Mse*I +3 primers, were used for amplification. For visualisation of PCR product by autoradiography, the *Eco*RI +3 primer was end-labelled with γ-^{33}P-ATP. Selective amplification was performed at 94°C for 30 s, 65°C for 30 s, (-0.7°C/cycle) and 72°C for 1 min for 13 cycles, followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min for 23 cycles.

Duplicated digested DNA samples were subjected to the pre-amplification and selective amplification reactions. Pre-amplified lemon control DNA, obtained from the laboratories at Infruitec was used as controls in the selective amplification.

Polyacrylamide gel electrophoresis. Amplification products were mixed 1:1 with 80% formamide, containing 10 mmol EDTA, 1mg/ml each of xylene cyanol and bromophenol blue. Samples were either used immediately, or stored at -20°C. Prior to gel electrophoresis, samples were denatured for 3-4 min at 94°C and placed on ice immediately. The amplification products were separated by electrophoresis, using a 5% denaturing polyacrylamide (19:1) sequencing like gel (GIBCO BRL Life Technologies Inc.). Gels were run at 45°C at 80W for 90 min. Gels were dried directly onto Whatman 3M paper on a slab gel dryer for 75 min. Gels were exposed to BioMax-MR film for 24-48 hr.

Visualisation of PCR products. Non-radioactive silver staining was performed

according to the Promega DNA Silver Staining System. The Kodak C/RA-1 duplicating film was used for recording these results on film. Amplification products of selective PCR using γ-^{33}P-ATP, were visualised by autoradiography. The autoradiograph was then developed using Cronex developer and fixer.

RESULTS

All the isolates used in this study were pathogenic except for the two tomato isolates **(Table 1)**. Although the two tomato isolates were pathogenic during the first trial, a loss in pathogenicity was observed for these isolates after the second trial. There was no prior indication that these isolates lost their pathogenicity, until after the data for the second trial was analysed and subjected to AFLP analysis.

The isolation of DNA from the filamentous fungi, *V. dahliae*, proofs to be the most difficult procedure of the analysis. Most time was spent on optimising a DNA extraction method. Fungal cultures grown in liquid media resulted in higher yields of mycelium for genomic DNA isolation (**Fig. 1**). Degraded and intact genomic DNA was obtained after initial DNA extractions (**Fig. 2a, b, c**). DNA with low yields was obtained. The quality and yield of fungal DNA preparations were influenced by the condition of the starting material. It was found that fresh material resulted in higher yields and more intact DNA. Restriction digestion of some DNA samples were complete within three hours as described in the AFLP protocol, other samples were only partially digested following this period.

Completely digested DNA resulted in amplification product in the range of 100-500 bp, **(Fig. 3a, b, c)**, partially digested DNA resulted in amplification products in the range of 500-1000 bp **(Fig. 3d, e)**.

Five primer combinations were used for selective amplification. On average 40 fragments were visualised per analysis. Polymorphisms were revealed with each primer combination used. Polymorphism refers to the presence and absence of a particular band in a fingerprint.

With a limited number of primer combinations, an array of polymorphisms were found among the isolates from the different hosts. Polymorphisms were found among potato, tomato, cotton and avocado isolates (**Fig. 4, 5, 6, 7, 8**). Polymorphisms between isolates of the same host were also detected. Within cotton isolates, VD 94/5 and VD 94/8 were found to be polymorphic (**Fig. 5**). Potato isolates, G18 and G19 showed a polymorphism (**Fig. 6**), and the fingerprints of avocado isolates 31 and 31fd were polymorphic (**Fig. 9**).

The AFLP analysis also reflected the similarities between the tomato, avocado, cotton, and potato isolates. All isolates shared an intense low molecular weight banding pattern (**Fig. 4, 5, 6, 7**).

The technique proved to be highly reproducible. Repeated amplification of the same DNA revealed no artefactual variation and resulted in identical fingerprints **(Fig. 5, 6, 7, 8, 9)**. Silverstaining and radiolabelling were both effective for the visualisation of selective amplification products. The bands visualised with silverstaining were faint compared to those obtained with radiolabelling **(Fig. 8)**. Bands appeared fuzzier in the silverstaining than the radiolabelling method. No bands specific to geographical origin were observed, this could clearly be seen among the cotton isolates **(Fig. 5)**.

DISCUSSION

Restriction fragment length polymorphism (RFLP) studies have recently led to the recognition of two major subspecific groups within *V. dahliae* and *V. albo-atrum*, with little variation between isolates within these groups (Carder & Barbara, 1991; Michelmore & Hulbert, 1987; Okoli *et al.*, 1993, 1994). However, the AFLP analysis using only five primer combinations, produced sufficient polymorphisms to assess genetic variation among the *V. dahliae* isolates in this study. Polymorphisms were common among isolates from the four different host plants potato, cotton, avocado and tomato. Barasubiye *et al.* (1995) used random amplified polymorphic DNA (RAPD) to discriminate between lucerne and potato isolates of *V. albo-atrum*. In this study polymorphisms within the isolates from the same host were also present. The polymorphisms found between the two potatoes, cotton, and avocado isolates in this study shows that they are genetically distinct. Thus sufficient genetic variation was present among all isolates. The degree to which intraspecific

variation is expressed in a particular group of isolates varies with each primer. However, similarities amongst the isolates from the different hosts were also found using the five primer combinations. All *V. dahliae* isolates shared commonalities to a certain extent, but the polymorphisms showed that genetic variation was present. Genetic variation could be correlated with the pathogenicity groupings of the isolates, which could be used for differentiation (Morton *et al.*, 1995; Nazar *et al.*, (1991); Robb *et al.*, (1994)). Despite the common ability of *V. dahliae* isolates from potato, cotton and avocado and tomato to cause disease on tomato cultivars, pathogenic variation was evident. However, the relationship between genotypic and pathogenic variation is not well understood in this study. Pathogenic variation has been demonstrated within host-adapted subpopulations of *V. dahliae* including those pathogenic on cotton, potato, and tomato (Daayf *et al.*, 1995; Powelson, 1970; Puhalla & Hummel, 1983). Dobinson *et al.* (1998) also found that pathogenic variation was quite common but the relationship between pathogenic and genotypic variation is not well understood.

The polymorphisms found within the *V. dahliae* isolates confirms that the pathogen possess sufficient genetic variation to quickly adapt to the genetically uniform monocultures that dominate modern agriculture (Howard, 1996). Characterisation of isolates that differ genetically may reveal specific physiological differences with respect to virulence, host range, cultivar specificity and interactions with other pathogens, information that would be directly relevant to the management of Verticillium wilt (Dobinson *et al.*, 1998). It is conceivable however, that *V. dahliae* isolates with highly similar genetic backgrounds could

differ at only one or a few genetic loci that determine cultivar specificity or host range. Isolates of different genetic backgrounds could have in common the genetic information that allows them to cause disease on different tomato cultivars (Dobinson *et al.*, 1998). The results of the study are consistent with both possibilities.

No clear geographic pattern was found for the isolates when studying the AFLP fingerprints. Messner *et al.* (1996) investigated the molecular heterogeneity and phylogenetic position of the plant pathogen *V. dahliae* using RAPD-PCR and sequencing of the 18SrDNA and also found no correlation between geographic location of the isolates and the RAPD-pattern . It has been suggested that isolates from geographical separate regions and even within one locale, can be genetically distinct (Baergen *et al.*, 1993; Minogochi & Clarkson, 1994; O' Garro & Clarkson, 1988; Tjamos, 1981). This phenomenon has been found in this study.

The most common problems encountered in the isolation of pure, high molecular weight genomic DNA from fungal mycelia were the high production of potential contaminants such as polysaccharides and phenols, inhibitory to subsequent analysis (Kremer *et al.*, 1998; Weising *et al.*, 1995). Since AFLPs are based upon restriction digestion of DNA, the quality of the extracted DNA and the method of extraction could affect the profiles obtained (Jones *et al.*,1997). The combination of the DNA isolation methods used gave higher yields and good quality DNA compared to previous methods tested (method not shown). Optimisation strategies most importantly concerned the

ingredients and also the pH of the isolation buffer. Other critical parameters were the condition of the starting material, the pH of the buffered phenol, and the concentration of the SDS used in the extraction buffer. Liquid nitrogen facilitates the grinding process considerably. Over-enthusiastic grinding during DNA extractions should be avoided as this can damage the DNA.

Initial DNA samples obtained could not be digested due to low quality and impurities in the DNA. This resulted in partial digestions of DNA. Optimisation of the extraction method resulted in intact DNA samples of high quality. The most important source of variability in this study was any deterioration in DNA quality. The optimisation of the DNA extraction method for *V. dahliae* still needs further attention. A prerequisite for AFLP fingerprinting is high quality DNA (Vos *et al.*, 1995).

Restriction site variation was detected after silverstaining and autoradiography. Both silver staining and autoradiography were effective. Radiolabelling was preferred for visualisation of selective amplification products because of a higher sensitivity, one or two orders of magnitude over silver staining. The resolution was also much better with radiolabelling then silver staining as the background was much clearer.

An important aspect of fingerprinting is the reproducibility of results (Vos *et al.*, 1995). The stringent reaction conditions used in the current study ensured reproducibility and produced a dense but reliable banding pattern each time. With a widespread pathogen

like *V. dahliae*, it will be necessary to establish that this banding pattern is constant or retains constant discriminatory features at a species level. Repeated amplification of the same DNA revealed no artefactual variation. In all applications of fingerprinting involving species not previously studied, it is important, where possible, to establish that the bands being detected in the fingerprint occur independently of each other (Kremer *et al.*, 1998).

More primer combinations should be tested to develop race group specific primers or probes for the detection in soil and quantification of *V. dahliae*. Using such primers with a larger number of isolates from a range of sources, banding patterns can be generated, scored and submitted to an appropriate cluster analysis to investigate the pathogenic and genotypic relationship between isolates. Such DNA profiles could be used to supplement other phenotypic data on isolates obtained from standard pathogenicity tests.

A more sophisticated approach would be to excise a band or bands to use as probes for detection and identification. DNA probes can be obtained by excising bands from the gel that are species specific (Robb *et al.*, 1994). Specific primers, obtained by sequencing such a band, could be used to develop a PCR-based test. On the basis of the nucleotide sequences of an amplified mitochondrial small rRNA gene region, Li *et al.* (1994), developed specific primers that amplified a 140-bp region of *V. dahliae* DNA. This could provide the means for both accurate identification and sensitive, rapid detection. The technique is ideal for assessing genetic analysis at the DNA level, but as a routine diagnostic test it will not be cost-effective. The technique can be used to complement the

more 'traditional' methods of identification, facilitating the task of interpretation. The fingerprints can then be used as a possible diagnostic method for differentiating isolates of different race types of *V. dahliae*. The technique has been successfully used for differentiating between isolates of *Fusarium oxysporum* f.sp.*ciceris* causing chickpea yellowing and wilt (Kelly *et al.*, 1994).

The above findings suggest that AFLP analysis is an excellent new tool for differentiating and investigating genetic variation between isolates of *V. dahliae* inducing Verticillium wilt. The AFLP approach is objective, repeatable, and unaffected by environmental factors, and it provides a direct assessment of the genetic relationships of the fungus at the DNA level. Further work is needed to discriminate between isolates of the pathogen belonging to different races, associated with host specificity, race specificity, and virulence and avirulence groups. The information obtained in this study can be used for further studies of the genetic variation among isolates of *V. dahliae*. The use of resistant genes in selection of cultivars and fungicides might pose problems in disease control, in view of the apparent genetic variation present in *V. dahliae* populations in South Africa. Fungal populations with high levels of genetic variation are likely to adapt more rapidly to resistant hosts or fungicides than populations with little genetic variation (McDonald & McDermot, 1993). The amount of genetic variation being maintained within a population indicates how rapidly a pathogen can evolve and this has direct applications to agricultural ecosystems. The genetic analysis of populations for DNA variation is thus important in view of the impact it can have on current control measures.

Table 1. Origins of *Verticillium dahliae* isolates used in this investigation

Fungi	Isolate	Substrate and origin	Pathogenicity [a]		Source [b]
			Exp.1	Exp.2	
V. dahliae	VD94/1	Cotton, Springbokvlakte	+	+	1
V. dahliae	VD94/4	Cotton, Springbokvlakte	+	+	1
V. dahliae	VD94/5	Cotton, Springbokvlakte	+	+	1
V. dahliae	VD94/6	Cotton, Springbokvlakte	+	+	1
V. dahliae	VD94/8	Cotton, Springbokvlakte	+	+	1
V. dahliae	G18	Potato, Sandberg	+	+	2
V. dahliae	G19	Potato, Clanwilliam	+	+	2
V. dahliae	G20	Potato, Clanwilliam	+	+	2
V. dahliae	G22	Potato, Lambertsbaai	+	+	2
V. dahliae	G24	Potato, Lambertsbaai	+	+	3
V. dahliae	VA 3-1	Avocado, Hartbeeshoek	+	+	4
V. dahliae	31fd	Avocado, Mooketsi	+	+	4
V. dahliae	VT 2-4	Tomato, Mpumalanga	+	-	4
V. dahliae	S1	Tomato, Roodeplaat	+	-	4

[a] Results after the pathogenicity experiments, + = pathogenic, and - = non-pathogenic,
[b] 1 = A. Swanepoel, 2 = M Visser, 3 = A. Mc Leod, 4 = MDR Uys and S. Visser

Table 2. Selective primer combinations used in AFLP analysis

***Eco* RI +3**	***Mse* I +3**
E-ACG	M-CTT
E-ACG	M-CAA
E-ACG	M-CTG
E-ACC	M-CTG
E-ACT	M-CTG

Fig. 1 Fungal isolate grown in liquid culture, potato dextrose broth, resulted in higher yields.

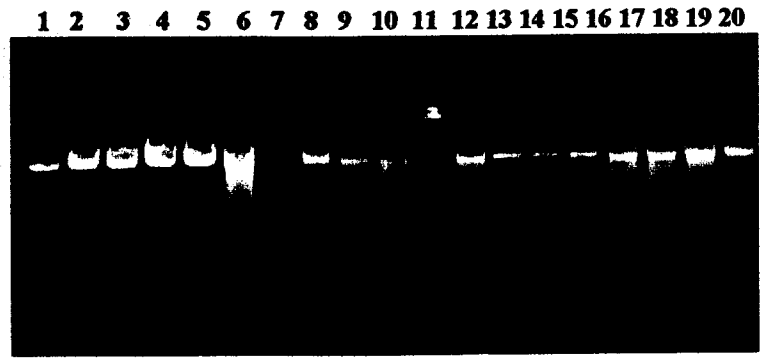

Fig. 2a DNA isolations after optimisation. Lanes 1-5= λ DNA, (50-250ng/µl) 6= 31fd, 7= degraded, 8= G20, 9= G24, 10= Vt2-4, 11= S1, 12= degraded, 13= 94/5, 14= 94/8, 15= G18, 16= G19, 17= VA3-1, 18= 94/1, 19= G22, 20= G24

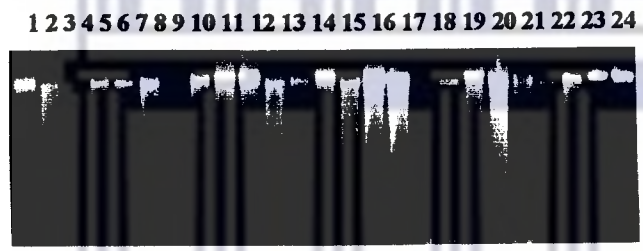

Fig. 2b DNA isolations after optimisation. Lanes 1= 94/4, 2= 67, 3= degraded, 4= 90m, 5= G20, 6= 94/5, 7= VA3-1, 8= VT2-4, 10= G18, 11= G22, 12= S1, 13= 94/1, 14=94/6, 15= 31fd, 16= 91, 17-18=degraded, 19= 94/4, 20= 94/5, 21= 90d, 22= 94/5, 23= 94/8, 24= G21

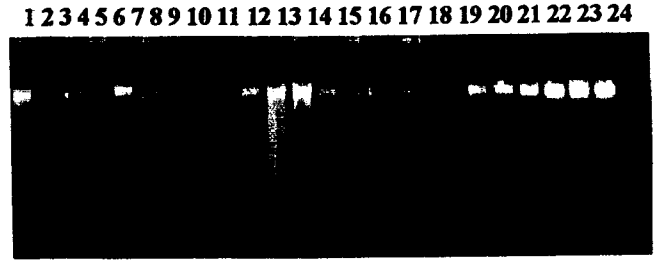

Fig. 2c DNA isolations after initial optimisation. 1= 31fd, 2=degraded, 3= G23, 4-5=degraded, 6= Va3-1, 7-11=degraded, 12= VT2-4, 13= S1, 14= G18, 15= G19, 16= G20, 17=G22, 18= degraded,

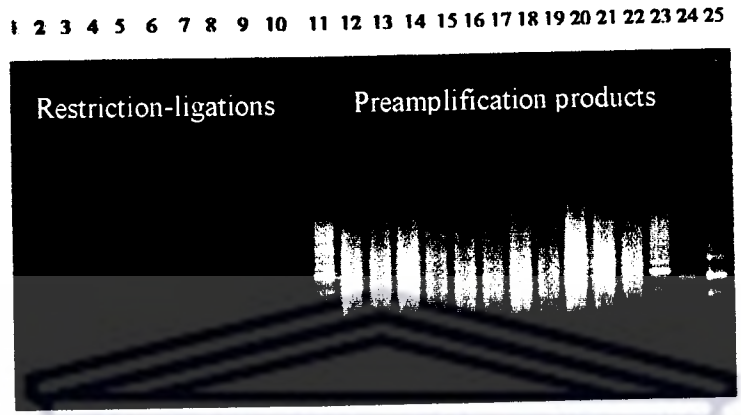

Fig. 3a Completely digested DNA resulted in good preamplification product. Lanes 1= 94/1, 2= 94/4, 3= 94/5, 4=94/8, 5= G18, 6= G19, 7=G20, 8= G22, 9= VA 3-1, 10= VT2-41, 11= marker, 12= 94/1, 13= 94/4, 14= 94/5, 15= 94/8, 16= G18, 17= G19, 18= G20, 19= G22, 20= VA 3-1, 21= VT2-4, 22= marker, 23= tomato control, 24= *Arabidopsis* control

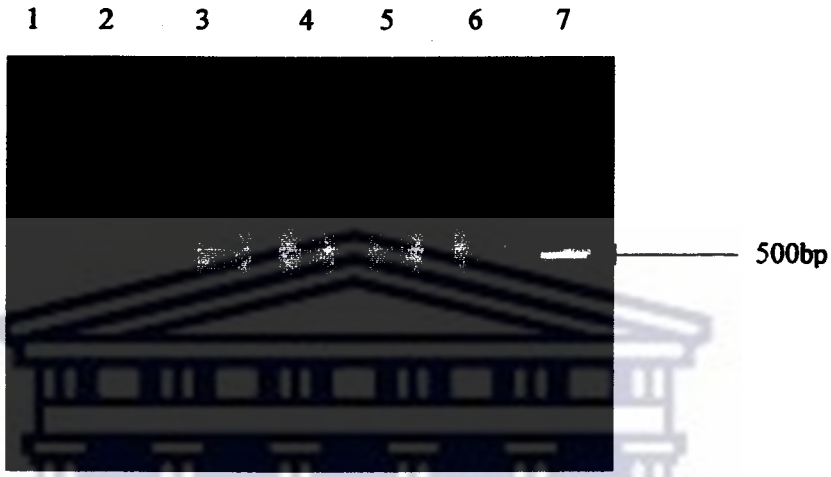

Fig. 3b Preamplification products of duplicate samples. Lanes 1-2= G19(P*), 3-4= 94/4 (C*), 5-6= G18 (P), 7= marker
* P= potato, C= cotton isolates

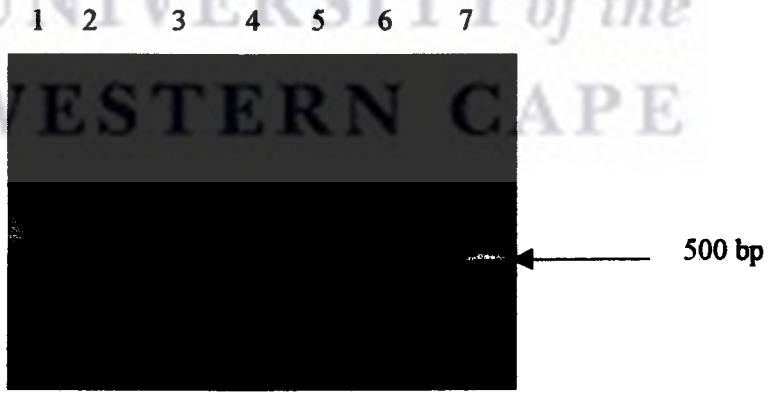

Fig. 3c Preamplification products Lanes 1= G22 (P*), 2=not loaded, 3= S1(T*), 4= 94/6 (C*), 5= not loaded, 6= 31fd(A), 7= marker
* P= potato, T= tomato, C= cotton

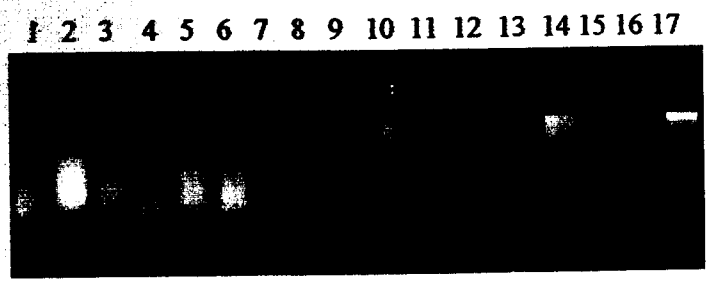

Fig. 3d Partially digested DNA samples resulted in low yield of preamplification products. Lanes 1=94/5 (C*), 2= G23 (P*), 3= 94/6 (C), 4= S1(T*), 5= 94/1(C), 6= 94/4(C), 7= 31fd (A), 8= VT2-4(T), 9= not loaded, 10=94/5(C), 11= G23(P), 12= 94/6(C), 13= S1(T), 14= 94/1(C), 15= 94/4 (A), 16= 31fd (A), 17=VT2-4 (T)
*** C= cotton, P= potato, T= tomato, A= avocado**

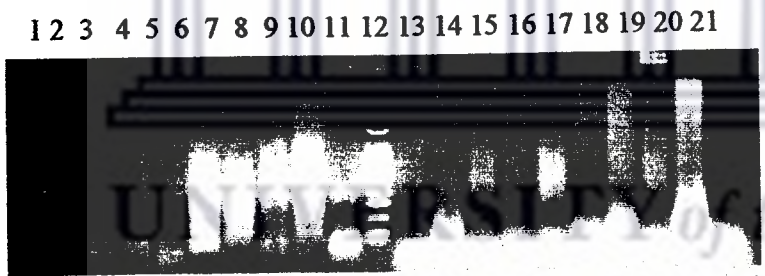

Fig. 3e Partially digested DNA samples resulted in low yield of preamplification products. Lanes 1=S1(T*), 2= G22(P*), 3-4= G18(P), 4-5= 94/6(C*), 6-7= VA3-1(A), 8= 94/5(C), 9= 31fd(A), 10= G19(P), 11= marker, 12= S1(T), 13= G22(P), 14-15= G18(P), 16-17= 94/6(C), 18-19= VA3-1(A), 20= 94/5(C), 21=31fd(A)
*** T= tomato, P= potato, C= cotton, A= avocado**

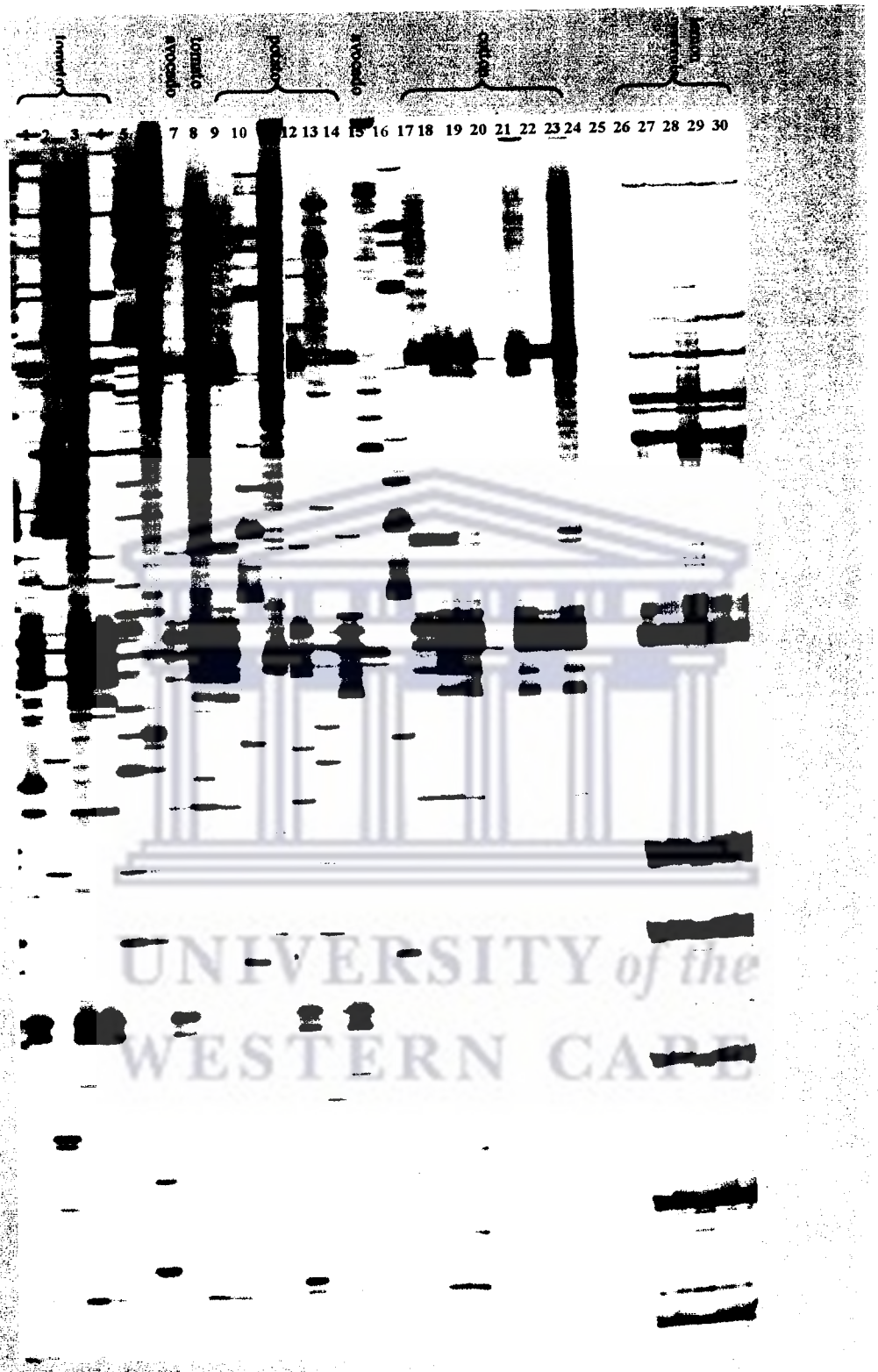

Fig. 4 AFLP fingerprints generated using primer combination E-ACG & M-CTT. Lanes 2, 5, 6, 10, 11 and 16 tomato and Arabidopsis controls, 26-30=lemon controls
Lanes 1= 90m, 3=VT2-4, 4=VT2-4, 7 = 31fd, 8=S1, 9= G20, 12= G22, 13= G24, 14= G 18, 15= VA 3-1, 17= 94/1, 18-19= 94/4, 20-21= 94/5, 22= 95/6, 23= 94/8. Area in brackets indicate the low molecular weight banding pattern shared by all isolates.

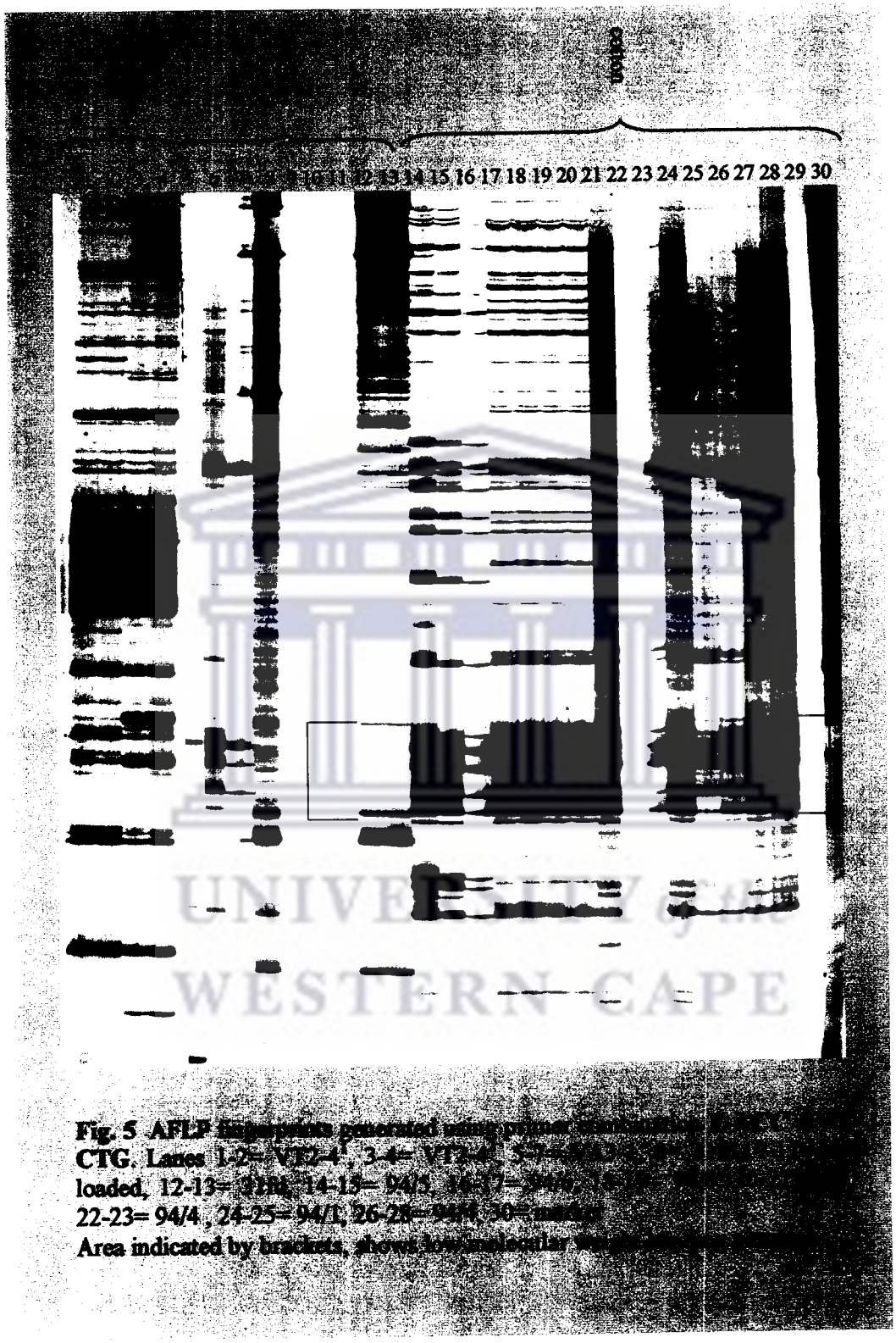

Fig. 5 AFLP fingerprints generated using primer combination
CTG. Lanes 1-2= VT2-4 , 3-4= VT2-4 ,
loaded, 12-13= 91B, 14-15= 94/5, 16-17= 94/6,
22-23= 94/4 , 24-25= 94/1, 26-28= 94/9, 30= marker
Area indicated by brackets, shows low molecular

Fig. 6 AFLP fingerprints generated using primer combination E-ACG & M-CTG. Arrow indicates polymorphism between two potato isolates, G18 and G19. Lanes 1-3 = 94/1, 4-5 = 94/3, 6-7 = 94/5, 8= VA 3-1, 10-11 = G18, 12-13 = G19, 14-15 = 94/8, 16-17 = 94/6, 18-19 = 94/41, 20-23 = 94/6, 24-26 = 94/8, 27 = tomato control, 28 = molecular weight marker

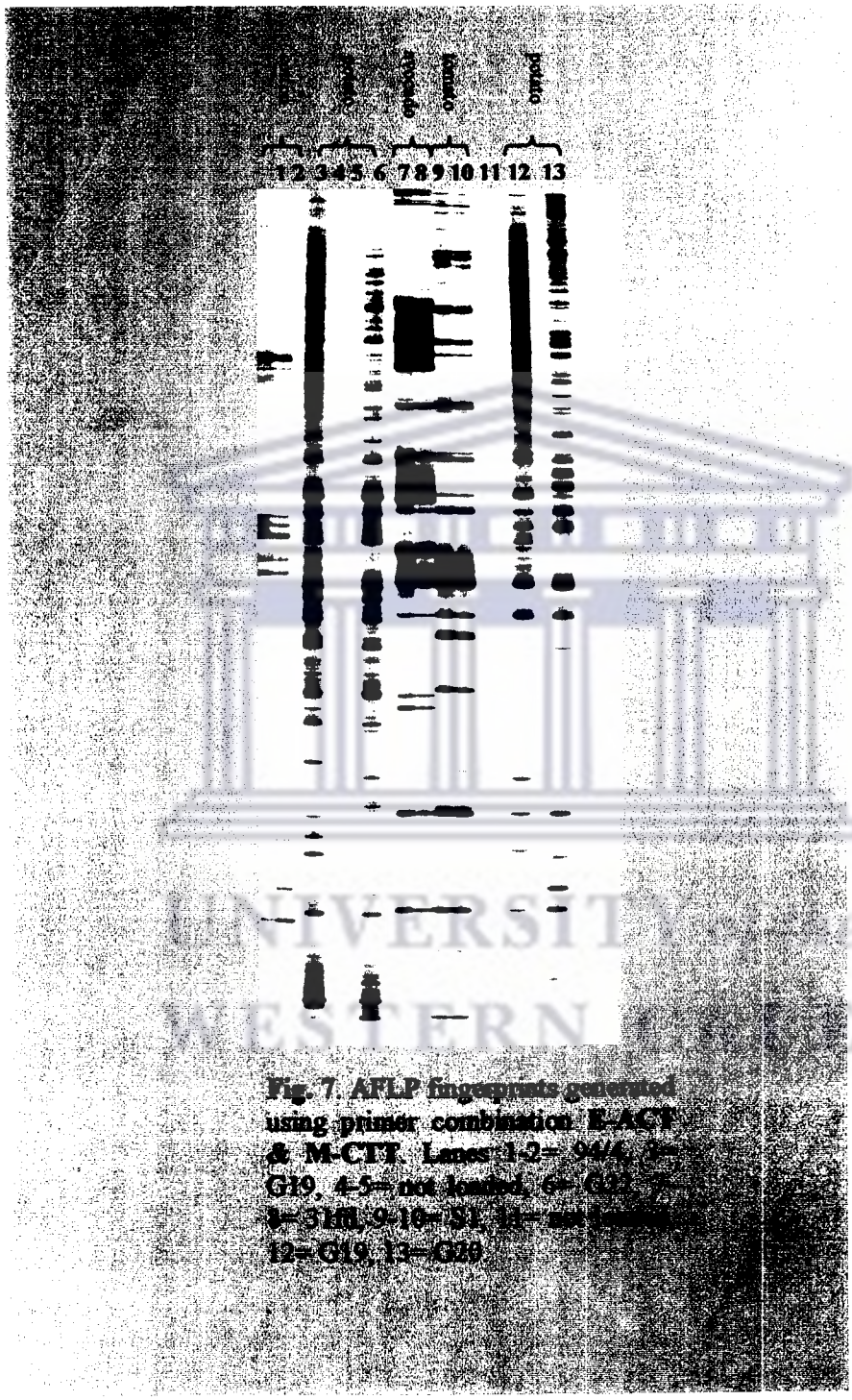

Fig. 7. AFLP fingerprints generated using primer combination E-ACT & M-CTT. Lanes: 1-2= 94/4, 3= G19, 4-5= not loaded, 6= G2[illegible], 7= [illegible], 8= 31M, 9-10= S1, 11= not loaded, 12= G19, 13= G20

Fig. 8 AFLP fingerprints after silver staining using primer combination E-ACG & M-CTG. Lanes 1= marker, 2-3= not loaded, 4-5= 94/1, 6-7= 94/5, 8-9= 94/8, 10-11= 33/1

Fig. 9 AFLP fingerprints generated using primer combination E-ACT / M-CTG. Lanes 1= marker, 2-3= V 1-2, 4-5 S1, 6-7= VA3-1, 8-9= 3/93, 10= marker, 11-12= 94/8, 13= G24, 14-15= 94/5.

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SUMMARY

SUMMARY

In South Africa under specific climatic regimes, wilt and early dying caused by *Verticillium dahliae* Kleb, results in extensive losses in the production of potatoes, tomatoes and a range of other hosts. The aim of the study was to use pathogenicity and AFLP analysis for differentiating *V. dahliae* isolates from different host plants with respect to virulence and DNA polymorphisms.

Verticillium dahliae isolates obtained from cotton, potato, tomato, and avocado were evaluated for pathogenicity and virulence on two tomato cultivars (Rooi Kakie and Flora Dade) in the greenhouse, using a root-dip inoculation technique. Disease evaluations were based on percentage leaf yellowing, shoot length and shoot dry mass of plants. Isolations were made from vascular tissue of plants to confirm infection. *V. dahliae* isolates from cotton, potato, and avocado all caused typical Verticillium wilt symptoms on both tomato cultivars and were successfully re-isolated from the seedlings in two experiments conducted. The cotton, potato, and avocado isolates were thus pathogenic on tomato. Isolates from tomato showed few foliar symptoms and could not be re-isolated from plants in the second trial. The pathogenic isolates differed in degree of virulence. Variation in virulence was also observed among isolates obtained from diseased plants belonging to the same species. None of the isolates tested were host specific, because cotton, potato and avocado isolates induced foliar symptoms on both tomato cultivars. Leaf yellowing was found to be a better parameter for disease assessment than shoot dry mass since a higher correlation was found between isolation of the pathogen and leaf yellowing than between pathogen isolation and shoot dry mass.

A PCR-based fingerprinting technique based on amplified fragment length polymorphism (AFLPs) was used to screen fourteen isolates of *Verticillium dahliae* from potato, cotton, avocado and tomato for genetic variation. Results from pathogenicity tests showed that isolates from potato, cotton, avocado and tomato to be pathogenic to two tomato cultivars. After the second trial, however, it was discovered that the tomato isolates lost their pathogenicity. Each of the five primer combinations generated fingerprint patterns markedly distinct from the other primers. The fingerprint patterns obtained were complex, but enabled the distinction among isolates from the four host plants. DNA polymorphisms and similarities (common band sharing) were found among the *V. dahliae* isolates obtained from the same host. Repeated amplification of the same DNA extracted at different times revealed no artefactual variation, confirming the reproducibility of the technique. The AFLP technique will be a valuable tool for the identification of individual isolates of *V. dahliae* genera because of its ability to generate large numbers of polymorphisms, and the consistency of the polymerase chain reaction amplification.