

**Occurrence, identification
and a potential management
strategy of *Fusarium*
species causing wilt of
potatoes in South Africa**

by

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DECLARATION

I hereby declare that this dissertation submitted to the University of Pretoria for the degree of MSc Microbiology has not been previously submitted by me in respect of a degree at any other University

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OCCURRENCE, IDENTIFICATION AND A POTENTIAL MANAGEMENT STRATEGY OF *FUSARIUM* SPECIES CAUSING WILT OF POTATOES IN SOUTH AFRICA

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ABSTRACT

Fusarium is a soilborne fungus which can live in soil for long periods of time. It is known to cause wilt, root rot and crown rot diseases in a diverse group of crop plants. Of all the diseases caused by *Fusarium* the most important are the vascular wilts. Pathogens that cause wilting usually enter their host plant through young roots and then grow into and up the water-conducting vessels of the root and stem. The vessels become blocked and water supply to the leaves is limited. This results in the potato plant being weak resulting in yellowing of leaves, browning of stems and production of smaller tubers. *Fusarium* is diverse and widely distributed and can be isolated from agricultural soils and plant material. The study was done to determine the occurrence of this pathogen in the South African potato industry. Samples of plant material showing wilt symptoms were collected from nine potato production regions. Fungal isolations were made from tubers using a *Fusarium* selective medium, i.e Peptone PCNB Agar. The isolates were examined morphologically and those resembling *Fusarium* were further identified using molecular techniques. DNA sequence analysis of the translation elongation factor 1- α gene was done on the isolates. DNA-based techniques have increasingly become the tool of choice for understanding the genetic diversity and phylogeny of *Fusarium* species. The pathogenicity of the isolates from all the regions was also investigated on potato cultivar Caren. The DNA results confirmed *Fusarium* as the pathogen causing Fusarium wilt on potatoes. Two species of *Fusarium* were identified; namely *F. oxysporum* and *F. solani*. *F. oxysporum* was more prevalent and occurred in all regions compared to *F. solani*. *F. oxysporum* is best known for the plant pathogenic strain, which cause wilt, root rot and crown rot diseases on a wide variety of crops, often limiting crop production. It is also known to be phylogenetically diverse. In the

pathogenicity test, the isolates were found to be virulent and one was highly virulent therefore confirming their ability to cause wilting of potatoes. The effect of silicon on Fusarium wilt of potatoes was investigated in this study to assess its effectiveness in the control of Fusarium wilt. An *in vitro* study using potassium silicate was done to determine if silicon can inhibit the growth of *Fusarium* at different concentrations. The results showed that at low concentrations of potassium silicate the growth of *Fusarium* was not inhibited, while at a high concentration, there was inhibition. Greenhouse pot trials were conducted to determine the effect of silicon soil amendments on Fusarium wilt of potatoes, tuber yield and the production of phenolics in the cell wall of potato peels. The levels of chlorogenic, caffeic and ferulic acids were also investigated. The following treatments were used: control, silicon ash (~99% Si), slag (30% Si), fly ash (50% Si) and lime (calcium carbonate) as a pH control. Treatments were divided into those inoculated with *Fusarium* and those without *Fusarium*. Results showed that for silicon treatments not inoculated with *Fusarium*, slag had the highest tuber yield, followed by lime, fly ash and silicon ash when compared to the control. Silicon treatments inoculated with *Fusarium* did not improve the yield as the control had the highest yield and the occurrence Fusarium wilt was not reduced in silicon treatments. In this regard silicon did not have an effect on Fusarium wilt because symptoms were visible in the silicon amended treatments. The results for phenolic acid content showed that ferulic acid levels were too low for analysis; for chlorogenic acid, concentrations were generally lower in the silicon treatments than in the treatments without silicon; and caffeic acid levels were higher in silicon treatments than treatments without silicon, as a result of increased production of as defence mechanism against invading pathogens. However, this is the first study on the effect of silicon on Fusarium wilt of potatoes and its influence on the production of phenolics. Further research is required to understand the role of silicon in potato pathosystems.

Keywords: *Fusarium*, *Solanum tuberosum*, wilt, silicon

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND INFORMATION

Potatoes are an important source of food for humans and animals (Oerke *et al.*, 1994). The potato plants develop by forming tubers, and increase their yield until the foliage dies down (Oerke *et al.*, 1994). This is not always the case because certain diseases can affect the growth of potatoes. There are numerous soilborne diseases that are persistent and reoccur in potato production areas and these result in reduced plant growth, lower tuber quality and reduced marketable yield. For certain potato diseases chemical fumigants and seed treatments provide some control (Larkin and Griffin, 2007). However, these are not always effective as diseases can occur at every stage of development when plants are susceptible to attack by pathogens (Oerke *et al.*, 1994). Losses occur due to reduction in tuber yield and reduction of quality during the growing season. This is due to storage rots and further quality changes attributable to diseases (Hide and Horrocks, 1994).

Fusarium is the most widely recognized and understood fungal genus. The species of this genus are the most abundant and widespread in soil communities (Summerell *et al.*, 2002). *F. oxysporum* is well represented among the communities of soilborne fungi (Fravel *et al.*, 2003). *Fusarium* causes a number of plant diseases, one of them being wilt. It infects a diverse group of crop plants (Marshall *et al.*, 1981) and a number of its species are associated with potato wilts and stem rots (Nelson *et al.*, 1981). In South Africa, *Fusarium oxysporum* Schlecht. emend. Snyder & Hans. (section *Elegans*) and *Fusarium solani* (Mart.) Sacc. emend Snyder & Hans. (Snyder and Hansen, 1940) are the most important causal agents of wilting on potatoes (Visser, 1999).

Fusarium wilt is a disease of global importance that can result in huge agricultural losses if not controlled (Nelson *et al.*, 1981). *Fusarium* wilt of potatoes is referred to in this way to distinguish it from other wilt diseases (Rich, 1983). *Fusarium* wilt pathogens show a high level of host specificity based on the plant species and cultivar they infect (Fravel *et al.*, 2003).

The management of Fusarium wilt has primarily been by means of the use of resistant cultivars and fumigating the soil with methyl bromide (Larena *et al.*, 2003). Recent research has focused on methods to stimulate plant defence mechanisms by using non-pathogenic strains of *F. oxysporum* (Panina *et al.*, 2007) and amendment of the soil with mineral elements including silicon (Fawe *et al.*, 1998). Silicon fertilizers have been applied to rice and sugarcane to promote high and sustainable crop yields (Ma and Yamaji, 2006). The benefits of silicon amendments include enhanced resistance against pathogens and pests (Epstein, 1994; Ma and Yamaji, 2006; Richmond and Sussman, 2003). Silicon can also act as a physical barrier by strengthening the cell wall through impregnation beneath the cuticle layer (Ma and Yamaji, 2006; Richmond and Sussman, 2003). Silicon plays an indirect role in stimulating host defence responses by promoting the production of phenolics and phytoalexins in response to pathogen infection and enhancing the activity of defence-associated enzymes (Hammerschmidt, 2005; Ma and Yamaji, 2006).

In this study, the occurrence of *Fusarium* species on potatoes in South Africa (SA) and their identification using DNA sequence information was investigated. The use of silicon soil amendments as a potential management strategy against this pathogen in order to minimize the occurrence of wilting of potatoes was also investigated.

1.2 FUNDAMENTAL OBJECTIVE

- To study Fusarium wilt in SA and investigate which *Fusarium* species are responsible for causing wilt of potatoes; to investigate the effect of potassium silicate on the growth of *F. oxysporum in vitro* and to study the effect of silicon soil amendments on Fusarium wilt of potatoes on the production of certain phenols within the potato plant.

1.3 SPECIFIC OBJECTIVES

- To identify which species of *Fusarium* cause Fusarium wilt on potatoes in SA.; which species are dominant and the distribution of the respective species.
- To determine the virulence of the isolates on potato cultivar Caren grown in SA.
- To test the *in vitro* effect of potassium silicate on the growth of *F. oxysporum*.

- To test the effect of silicon of Fusarium wilt of potatoes when applied to soil artificially inoculated with *F. oxysporum* and the effect on the production of certain phenols within the potato plant

All these objectives will contribute to a better understanding of the disease and the formulation of effective methods that can be used in improving the management of Fusarium wilt.

1.4 CHAPTER OUTLINE

Chapter 2 The literature review focuses on the causal agent *Fusarium* and Fusarium wilt disease.

Chapter 3 The aim of this study was to identify the species of *Fusarium* isolates, responsible for Fusarium wilt on potatoes in SA and to determine the pathogenicity of *Fusarium* from different potato growing regions using a SA cultivar, Caren

Chapter 4 To determine the *in vitro* effect of silicon on the growth of *Fusarium oxysporum* on PDA media supplemented with potassium silicate. To investigate the effects of silicon soil amendments on Fusarium wilt of potatoes and on the production of phenolic acids in potato tubers.

Chapter 5 General discussion

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Fusarium is an economically important plant pathogen (Booth, 1971) and the genus itself is widely recognized and well understood. Its name is inspired by the distinctive banana-shape macroconidia which it produces (Summerell *et al.*, 2002). Its species represent the most abundant and widespread genus (Nelson *et al.*, 1981) and this diverse and adaptable group of fungi can be found in numerous types of soils (Marshall *et al.*, 1981; Summerell *et al.*, 2002).

Fusarium spp. cause cortical rots, blights, leaf spots, root rots, cankers and vascular wilt diseases (Marshall *et al.*, 1981). Of all the diseases caused by *Fusarium*, vascular wilt is the most important because these causal agents attack a diverse group of crop plants including tomato, banana, pea, cotton, potato and sweet potato (Marshall *et al.*, 1981). A number of *Fusarium spp.* are associated with potato wilts and stem rots (Nelson *et al.*, 1981).

Fusarium oxysporum is the most common species that causes vascular wilt diseases in a wide variety of economically important crops (Roncero *et al.*, 2003). In SA, *F. oxysporum* and *F. solani* are the most important causal agents of *Fusarium* potato wilt (Visser, 1999). A study conducted in SA by Venter and co-workers found that isolates of *F. oxysporum* f. sp. *tuberosi* caused different disease symptoms such as stem-end rot, dry rot and wilt, and could often be placed into distinct vegetative compatibility groups (VCGs) consistent with the type of symptom (Venter *et al.*, 1992).

2.1.1 *Fusarium oxysporum*

F. oxysporum is an asexual fungus (Edel *et al.*, 2001), common, widespread and found in soil (Kistler, 1997). However, very little is known about the amount, distribution and genetic variation of these populations in soils (Bao *et al.*, 2002; Edel, 1997; Edel, 2001; and Lori *et al.*, 2004). *F. oxysporum* has the ability to exist as a saprophyte in soils and degrade lignin and complex carbohydrates. It is also a pervasive plant endophyte that can colonize plant roots and is pathogenic to many agriculturally important plants (Nelson *et al.*, 1981).

Pathogenic *Fusarium oxysporum* strains

F. oxysporum is an anamorphic species that includes non-, human -, animal- and plant pathogenic strains. The pathogenic forms may have evolved from non-pathogenic strains (Bao *et al.*, 2002; Roncero, 2003). The pathogenic strains of *F. oxysporum* have a very broad host range, ranging from animals, insects and humans to both gymnosperm and angiosperm plants (Summerell *et al.*, 2002).

Non-pathogenic *Fusarium oxysporum* strains

F. oxysporum strains have been isolated from asymptomatic roots of crop plants and they have been shown to be non-pathogenic on the plant species from which they were recovered (Gordon *et al.*, 1989; Hancock, 1985). Although these strains are aggressive colonizers of the root cortex, (Schneider, 1984) they are unable to cause wilt diseases, due either to their inability to enter the vascular tissue or due to a rapid response of the host, which localizes the infection. Thus, *F. oxysporum* can persist in hosts without causing disease (Gordon, 1997).

Non-pathogenic strains of *F. oxysporum* can induce resistance to Fusarium wilts in various plants (Steinberg *et al.*, 1997). Available limited studies on non-pathogenic strains occurring in individual fields show a high level of diversity (Correll *et al.*, 1986). These non-pathogenic strains differ in ecological characteristics (Edel, 2001). The relationship between the pathogenic and non-pathogenic forms of *F. oxysporum* might shed light on the evolution of the pathogenic strains of *F. oxysporum* (Alves-Santos *et al.*, 1999; Summerell *et al.*, 2002). Most research is focused on pathogenic strains and studies on non-pathogenic strains have been neglected. There has been some interest in the use of non-pathogenic *F. oxysporum* strains as biological control agents (Geiser *et al.*, 1994).

Formae speciales

Formae speciales are used to describe the physiological capabilities of fungi. This concept has been useful to plant pathologists in identifying isolates. *Formae speciales* are also used to characterize intra-specific relationships (Gordon, 1997). Pathogenic strains of *F. oxysporum* often show high levels of host specificity and, therefore, are divided into *formae speciales* based on the specific host they infect (Marshall *et al.*,

1981). *Formae speciales* differ in symptomology, epidemiology and cultivar susceptibility (Roncero, 2003).

2.1.2 *Fusarium solani*

Fusarium solani is widely distributed in soil and is also an important plant pathogen in agriculture. It infects crops such as soybean, bean and potato, causing root rot and wilting of the upper plant parts. *F. solani* is sub-classified into *formae speciales* based on host specificity. *F. solani* isolates compose a highly genetically variable species that can be related to its wide host range (Brasileiro *et al.*, 2004).

2.1.3 Molecular markers used to identify *Fusarium* species

For the purposes of direct sequence identification and analysis, various genomic regions have been evaluated as taxonomic markers (O'Donnell and Cigelnik, 1997; O'Donnell *et al.*, 1998, 2000; Schweigkofler *et al.*, 2004). The ribosomal RNA (rRNA) internal transcribed spacer (ITS) gene is widely used for other fungi (Bruns *et al.*, 1991) but this region has been proven not to be effective for classifying *Fusarium spp.* This is due to the presence of two divergent and non-orthologous copies of the ITS2 region in most *Fusarium spp.* examined (O'Donnell and Cigelnik, 1997; O'Donnell *et al.*, 1998). Nuclear ribosomal DNA intergenic spacer (IGS) regions and the mitochondrial ribosomal DNA small subunit (SSU) gene (; Baayen *et al.*, 2000; O'Donnell *et al.*, 1998; O'Donnell *et al.*, 2004) have also been utilized in *Fusarium* identification. The IGS region will differentiate most fungi to species level and below but it is often highly variable even at the intraspecific level (Bruns *et al.*, 1991). The mtDNA SSU region is the least informative and as it codes for a functional product, it therefore does not contain as many variable sites (O'Donnell *et al.*, 2004). Instead, the gene encoding the TEF-1 α has become the marker of choice as it is a single-copy gene that is highly informative among closely related species (Geiser *et al.*, 2004).

The commonly targeted regions for identification of *Fusarium spp.* are the nuclear translation elongation factor 1-alpha (TEF-1 α) gene introns. The TEF-1 α sequence information is widely used and taxonomically informative (Geiser *et al.*, 2004). TEF-1 α is a highly conserved ubiquitous protein involved in translation (Cho *et al.*, 1995) and has been used to study intra- and inter-specific variation and phylogeny for a wide variety of fungi including *Fusarium* (Knutsen *et al.*, 2004). Non-orthologous copies of the gene

have not been detected in the genus and universal primers have been designed that work across the phylogenetic breadth of the genus (Geiser *et al.*, 2004). In this regard, the commonly targeted regions are the nuclear translation elongation factor 1-alpha (TEF-1 α) gene introns.

2.1.4 *Fusarium* morphological characteristics

Fusarium spp. may produce three types of spores called macroconidia, macroconidia, and chlamydospores (Fig 2.1) (Agrios, 2005; Nelson, 1983); however, some species do not produce all three. Morphology of the macroconidia is the key characteristic for characterization of the genus *Fusarium*, as well as species differentiation. The macroconidia are produced by sporodochia, structures where a spore mass is supported by a mass of short monophialides or polyphialides bearing the macroconidia. Macroconidia of *Fusarium spp.* are of various shapes and sizes but the shape of the macroconidia formed in sporodochia by a species is a relatively consistent feature when the fungus is grown on natural substrates under standard conditions (Burgess, 1988; Nelson, 1983). Dimensions of the macroconidia may show considerable variation within individual species (Nelson *et al.*, 1994).

Microconidia are produced in the aerial mycelium; these can be produced in false heads only or in chains on either monophialides or polyphialides (Nelson *et al.*, 1994). The presence or absence of microconidia is a primary character in *Fusarium* taxonomy. If microconidia are present, the features considered are the shape, size and the mode of formation, which is best observed on carnation leaf agar (Fisher *et al.*, 1982). Chlamydospores (Fig 2.2) are thick-walled asexual spores produced by hyphae or conidia. They can be formed on dead host plant tissue in the final stages of the wilt disease (Marshall *et al.*, 1981). Chlamydospores may either be present or absent; if they are present, they may be formed singly, in pairs, in clumps, or in chains, with either rough or smooth walls (Nelson *et al.*, 1994). All these spores can survive in the soil for long periods of time without a suitable host plant (Marshall *et al.*, 1981).

2.2 FUSARIUM WILT OF POTATOES

2.2.1 Introduction

Fusarium wilt is a disease of global importance that can result in large agricultural losses if not controlled (Nelson *et al.*, 1981). Fusarium wilt occurs in many potato production areas and is difficult to determine its distribution in each of the continents because it is often confused with other wilt diseases (Rich, 1983). The literature on this disease is not that extensive, although this fungus had been identified by several authors in a number of potato producing countries. Fusarium wilt of potatoes should, however, not be considered as a negligible disease (Thanassouloupoulos and Kitsos, 1985a, b).

Fusarium wilt disease on potatoes is referred to in this way in order to distinguish it from Verticillium wilt and other wilts caused by bacteria. Isolation of the causal pathogen is necessary for a positive diagnosis (Zitter and Loria, 1986). Fusarium wilt is a warm weather disease in contrast to Verticillium wilt, which is a cool weather disease. Fusarium wilt development is favoured by hot weather and irrigation, while wet soil or high soil moisture can suppress wilt symptoms (Rich, 1983). Sometimes *Verticillium* and *Fusarium* affect host plants in the same field and even the same plant; therefore, the two diseases can easily be confused (Mace *et al.*, 1981).

2.2.2 Causal agents

Fusarium wilt is caused by soilborne *Fusarium* spp, which can survive in soil for long periods of time, without their host plants, as free-living saprophytes (Garrett, 1970). It is now accepted that three species of *Fusarium* cause wilt of potato plants in the field, namely, *F. solani* f. sp. *eumartii* and *F. avenaceum* (Nelson *et al.*, 1981). *Fusarium* spp. causing wilt, vary from area to area and can all be isolated from lower stem pieces, tubers and discoloured vascular tissue (Hooker, 2001).

2.2.3 Disease cycle

The characteristic and defining feature of vascular wilt diseases is the containment of the pathogen within the vascular system of the host (Fig 2.3) throughout the period of pathogenesis. There are two conditions which must be satisfied before a wilt disease can develop: the fungus must first gain entry to the vascular system of the host and it must have the ability to continue to colonize the vascular system (Talboys, 1972).

Fusarium spp. cannot infect intact tubers or lenticels. Certain potato production practices result in wounding of the potato, which provides an opportunity for infection (Hooker, 2001). When a susceptible host plant is present, fungal spores can come into contact with young roots. Spore germination is stimulated by the diffusion of nutrients from the roots through the soil. Spores then penetrate through the cortex of the root apical region. They pass through to the endodermis which offers incomplete resistance of passage to the xylem vessels, where the spores migrate upwards into the aerial shoots and the main leaf vessels (Garrett, 1970).

The movement of the fungus in the vascular bundles is due to the rapid growth of the mycelium upwards in the xylem vessel, where it is able to differentiate and produce macroconidia, which can be found in the xylem sap. These conidia are transported to the rest of the vascular tissue, where they germinate. The mycelium grows from one vessel to another through pits. When the macroconidia germinate, chlamydospores are formed on mycelium and these chlamydospores are responsible for the long-term survival of the pathogens in soil free of host plants (Garrett, 1970).

2.2.4 Symptoms

Fusarium wilt results in a variety of symptoms on tubers ranging from surface decay to vascular discolouration. Several species of *Fusarium* can infect potatoes and cause wilt symptoms on plants, and different symptoms on tubers. The pathogen causes a sunken brown necrotic area at the stem attachment, firm brown circular lesions on the tuber surface and brown discolouration of the vascular tissues. A shallow cut through the stem end reveals the streaky vascular discolouration referred to as stem-end browning. This disease can also cause light brown discolouration a short distance on each side of the vascular system in the tuber. This tissue is firm and does not produce cheesy exudates like bacterial ring rot caused by *Corynebacterium sepedonicum* (Zitter and Loria, 1986).

The symptoms of the fungal infection occur in the middle of the growing season when the infected plants become lighter in colour (Garrett, 1970). The lower leaves wilt, turn yellow, and then brown and eventually drop from the plant; the vascular system also turns brown (Garrett, 1970; Rich, 1983). Wilt diseases result in chlorosis, loss of turgidity and death. Vascular dysfunction in wilt diseases is due to high resistance to the flow of water through infected plants. The leaves receive less water and as a consequence they

wilt (Mehrotra, 1980). The internal symptom of Fusarium wilt is the discolouration of the vascular tissue (Fig 2.4) (Forsyth *et al.*, 2006). After harvest the remaining infected potato stems have a characteristic pink colour due to *Fusarium* spore masses (Fig 2.5).

2.2.5 Nutritional requirements

Fusarium spp. have been shown to utilize various carbon sources in their metabolism. These carbon compounds include fatty acids, long carbon chain acids, the alcohols and polysaccharides, including starches, hemicelluloses, and true celluloses. This renders *Fusarium spp.* as destructive enemies of roots in crops (Mace *et al.*, 1981). *F. oxysporum* has a rapid, superficial and spreading habit of growth; this may be associated with a greater oxygen requirement and may account for the frequent colonization of xylem tissue by this rapid growth (Nelson *et al.*, 1981).

The virulence of wilt fungi depends on their growth rate, which is influenced by nutrients, oxygen, water, pH and temperature (Marshall *et al.*, 1981). Differences in these factors have an effect on disease severity in the greenhouse and in the field (Ben-Yephet and Shtienberg, 1997). In nature the wilt fungi receive their nutrients from root and xylem sap and are able to utilize various sugars as a carbon source. *Fusarium* is able to grow under low oxygen levels but this can reduce sporulation and pigmentation. Its optimum growth temperature range is 24°-32°C. *Fusarium* does not require vitamins but certain ions such as zinc can stimulate growth (Marshall *et al.*, 1981).

2.3 Control

Fusarium wilt is difficult to control, but the use of resistant cultivars and crop rotation can reduce losses. Potato fields must not be over-irrigated (Rich, 1983). Chemical control of *Fusarium* in banana fields has been unsuccessful due to the production of thick-walled, long living chlamydospores which are resistant to chemical fumigation (Bailey and Lazarovits, 2003; Jeger *et al.*, 1996). In suppressive soils, despite the presence of a virulent pathogen and a susceptible host, the disease either does not develop or the severity and spread of the disease is restricted. Suppressive soils are, however, only active in limited geographical areas and are crop specific (Forsyth *et al.*, 2006). Fusarium wilt cannot be controlled by means of chemical fungicides. Cultural practices are usually implemented to manage the disease. The use of a three year crop rotation system with crops such as maize and wheat does not eliminate the pathogen but

reduces the levels in the soil (Denner *et al.*, 2003). Avoid the planting seed tubers that are infected with *Fusarium* (Hooker, 2001; Oerke *et al.*, 1994).

Silicon

Silicon is taken up by the roots in the form of silicic acid [Si (OH)₄] (Ma and Takahashi, 2002; Ma and Yamaji, 2006). It is translocated to the shoot via the transpiration stream and is polymerized and accumulates on the cell wall (Ma and Takahashi, 2002). Silicon affects the absorption and translocation of several macronutrient and micronutrient elements. It has also been shown to positively affect the growth and development of many plants, mostly by contributing to the mechanical strength of cell walls and their function of keeping plants erect and their leaves well positioned for light interception (Epstein, 1994). The impregnation of cell walls with silicon contributes to the resistance of plants to attacks by fungi, parasitic higher plants, and herbivores, including phytophagous insects (Epstein, 1994).

Silicon fertilization in natural soils with low levels of silicon, offers promising results in terms of disease control and yields. In rice it reduces susceptibility to fungal diseases (Datnoff *et al.*, 1997). Silicon fertilizers have been applied to rice and sugarcane to enhance high and sustainable crop yields (Ma and Yamaji, 2006). Bekker *et al.* (2006) demonstrated that silicon has the ability to enhance plant defence mechanisms against infection by *Phytophthora cinnamomi* and that potassium silicate in amended potato dextrose agar media had the ability to suppress mycelial growth of plant pathogens, including *F. oxysporum* and *F. solani*. This could be a potential management strategy for *Fusarium* wilt of potatoes, but that very little work has been done on it.

2.3 CONCLUSION

Fusarium oxysporum was found to be the main causal agent of *Fusarium* wilt of potatoes. Studying the interactions between *Fusarium* spp., potatoes and the environment is important since *Fusarium* has the ability to grow under diverse conditions. Its ability to survive in soil for long periods of time and colonize roots of plants is a major stumbling block in trying to control this disease. It is, therefore, able to continually re-infect newly planted potatoes. *Fusarium* wilt results in losses due to the reduction in tuber yield and quality at the time of harvest. Thus, finding ways to control this and other soilborne diseases will continue to be important in future strategies for

improving potato production. It has become clear that it is unlikely that any single measure will provide adequate control against this disease. The study on the effect of silicon on the disease might provide the industry with additional management options to the already existing ones. Integrated management measures will continue to be necessary to control this disease.

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<http://vegetablemdonline.ppath.cornell.edu/>. Accessed 3/7/2008, 11:37am

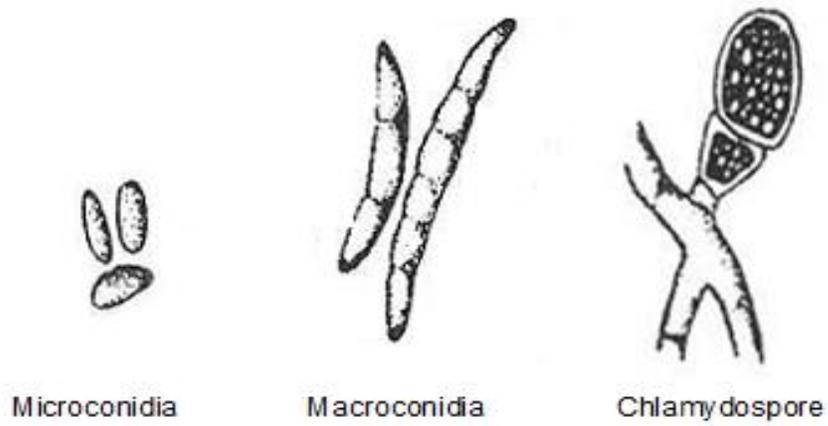


Fig. 2.1 Typical *Fusarium* spores (Agrios, 2005).

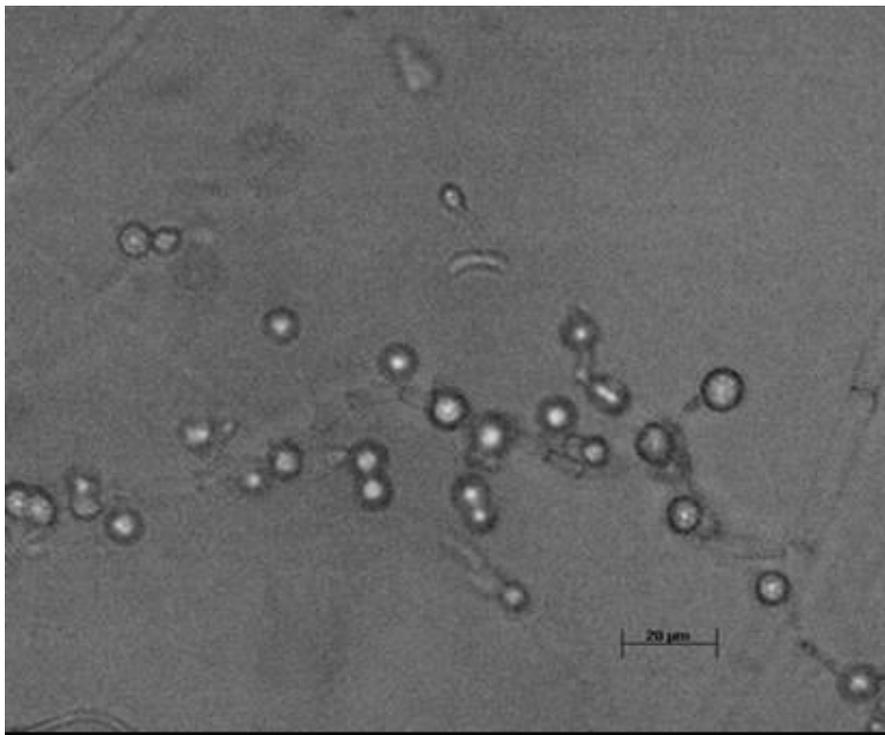


Fig. 2.2 *Fusarium* single and paired chlamydospores (Nelson, 1981).

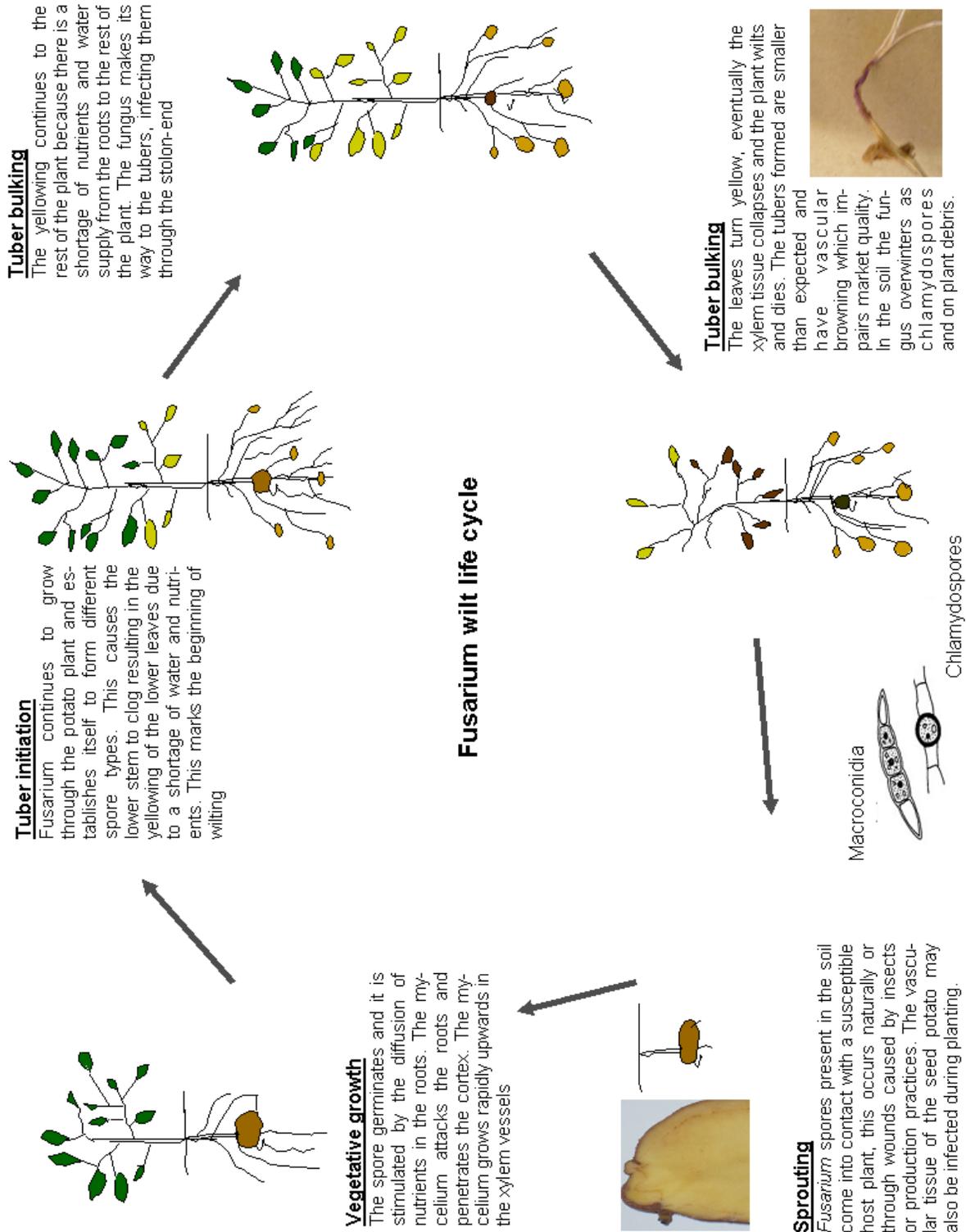


Fig. 2.3 Disease cycle of Fusarium wilt on potatoes, redrawn from Agrios (2005).



Fig. 2.4 Potato tuber showing vascular ring discolouration caused by *Fusarium* species (Forsyth *et al.*, 2006).



Fig. 2.5 Characteristic browning of wilted potato stem; the pink colour is due to *Fusarium* spore masses.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF *FUSARIUM* SPECIES CAUSING FUSARIUM WILT OF POTATOES IN SOUTH AFRICA

3.1 ABSTRACT

Fusarium wilt is a disease of global importance that causes considerable agricultural losses. In South Africa (SA), *Fusarium* is a major cause of a vascular wilt disease in potato plants. The objectives of this study were to identify the *Fusarium* species isolated from potato plants showing these symptoms in different growing regions in SA and to determine their pathogenicity. Isolates were examined morphologically and those resembling *Fusarium spp.* were subjected to DNA sequence analysis of the translation elongation factor 1- α gene. A maximum likelihood phylogenetic tree was drawn using these sequences. The isolates were identified as *Fusarium oxysporum* and *F. solani*, with *F. oxysporum* being the dominant species identified in the study. The phylogenetic tree showed that the isolates from the same location did not group together. Results from the pathogenicity tests showed that all isolates of both *Fusarium spp.* were pathogenic on potato plants. Isolate K4KZN, was found to be the most aggressive of the isolates used in the study. This study thus confirmed that Fusarium wilt is widespread in SA and poses a serious threat to the potato industry. Identification of the *Fusarium spp.* that cause wilting of potatoes will help in improving control methods used against this disease in SA.

3.2 INTRODUCTION

The genus *Fusarium* contains economically important pathogens (Booth, 1971) and is the most widely recognized and understood fungal genus. Its species represent the most abundant and widespread microbes of the global soil microflora (Summerell *et al.*, 2002). *Fusarium* causes vascular wilt diseases on a diverse range of crops including tomato, banana, pea, cotton, tobacco and sweet potato (Marshall *et al.*, 1981). A number of *Fusarium spp.* are associated with potato wilts and stem rots (Nelson *et al.*, 1981). *F. oxysporum* Schlecht. emend. Snyd. & Hans. (section *Elegans*) and *F. solani* (Mart.) Sacc. emend Snyd. & Hans. (Snyder and Hansen, 1940) are the most common species that cause these symptoms (Roncero *et al.*, 2003). *F. solani* is widely distributed in soil and is an important plant pathogen in agriculture. It infects crops such as soybean, bean

and potato causing root rot and wilting of the upper plant parts (Brasileiro *et al.*, 2004). In SA *F. oxysporum* and *F. solani* appear to be the most important causal agents of potato wilt (Visser, 1999). *F. oxysporum* is well represented among the communities of soilborne fungi (Fravel *et al.*, 2003). *F. oxysporum* f. sp. *tuberosi* has been identified as causing a true potato wilt (Thanassouloupoulos and Kitsos 1985).

Potato (*Solanum tuberosum*) is a crop of significant economic importance in many countries (Ayed *et al.*, 2006). The full economic value of this crop is not known because of the impact of several soilborne diseases that are persistent and reoccur in potato production areas. One of these diseases is Fusarium wilt. Fusarium wilt results in reduced plant growth, lower tuber quality and reduced marketable yield (Larkin and Griffin, 2007). The symptoms of the fungal infection occur in the middle of the growing season when the infected plants become lighter in colour (Garrett, 1970). The lower leaves wilt, turn yellow in colour and eventually brown, after which they drop from the plant (Garrett, 1970; Rich, 1983). The internal symptom of Fusarium wilt is the discolouration of the vascular tissue, which is especially visible on tubers (Forsyth *et al.*, 2006).

DNA-based techniques have increasingly become the tool of choice for identification and understanding the genetic diversity and phylogeny of *Fusarium spp.* (O'Donnell *et al.*, 1998). The translation elongation factor 1- α (TEF-1 α) was used in this study. TEF-1 α is a highly conserved ubiquitous protein involved in translation and has been used to study intra- and site-specific variation and phylogeny for a wide variety of fungi including *Fusarium spp.* (Chow *et al.*, 1995; Knutson *et al.*, 2004).

The aim of this study was to identify *Fusarium* isolates causing Fusarium wilt on potatoes in SA and to determine the relative pathogenicity of these isolates from different potato growing regions.

3.3 MATERIALS AND METHODS

3.3.1 Isolation of *Fusarium*

Isolations were made from the following potato production regions (Fig.3.1); KwaZulu-Natal (KZN), Mpumalanga (MP), Eastern Free State (EFS), Sandveld (SV), Northern Cape (NC), Western Free State (WFS), North West (NW), Ceres (C) and Eastern Cape (EC).

Fusarium isolates were obtained from potato plants showing signs of wilt. Potato tubers were washed with running tap water to remove excess soil and then surface sterilized by soaking them in NaOCl (0.5% v/v) for five minutes. The tubers were rinsed three times in sterile distilled water to remove the NaOCl and left on paper towels for 30 min to dry completely. The tubers were sliced from the stolon end and then four pieces (5 x 5mm in size) of the potato were cut from the diseased vascular ring or tissue. The potato pieces were placed on *Fusarium* selective medium which consisted of 15g peptone, 1g potassium hydrophosphate, 0.5g magnesium phosphate, 20g agar, 0.8g pentachloronitrobenzene (PCNB) and 1mg of streptomycin per litre agar. The inoculated plates were incubated at 25°C for 5 - 7 days. The resulting fungal colonies were purified and single spored. These were grown on half strength potato dextrose agar (PDA) plates and incubated at 25°C for 2 weeks for identification of *Fusarium* (Nelson, 1981).

3.3.2 Morphological Identification

For morphological identification, single spore isolates were grown for 10-15 days on Potato Dextrose Agar (PDA) medium (Nelson *et al.*, 1983), and on Carnation Leaf Agar (CLA) medium prepared following a modification of the method described by Fisher *et al.* (1982). Young leaves from carnations (*Dianthus carophyllus* L) were cut into small pieces of approximately 5mm², placed in glass Petri dishes and autoclaved for 20 min. CLA was prepared by aseptically placing two or three leaf pieces onto Petri dishes and floating them on sterile water agar (WA). As prescribed by Nelson *et al.* (1983), gross cultural characteristics of each isolate were determined from 10-15 day old PDA cultures, whereas microscopic features of microconidia, macroconidia, conidiophores and chlamydospores were determined based on 10-15 day old CLA cultures.

3.3.3 DNA extraction and PCR

DNA extraction of *Fusarium* isolates was done using a commercial DNA extraction kit, Dneasy Plant kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. For identification of isolates the translation elongation factor 1 α was used in this study (TEF-1 α). The TEF-1 α gene was amplified using the primers, EF1 5'-ATGGGTAAGGA(A/G)GACAAGAC-3' and EF2 5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3' under conditions as described by O'Donnell *et al* (1998).

PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the respective PCR primers. For sequencing, the BigDye terminator sequencing kit (Version 3.1, Applied Biosystems) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used.

3.3.4 Sequence analyses and identification

Sequences were edited on Chromas Lite version 2.1 (http://www.technelysium.com.au/chromas_lite.html) and BioEdit version 7.0.0. Copyright ©1997-2004 Tom Hall and aligned using MAFFT online alignment (<http://align.bmr.kyushu-u.ac.jp/mafft/online/server/>). Individual isolates were aligned with sequences from the *Fusarium* identification database (<http://fusarium.cbio.psu.edu>; Geiser *et al.*, 2004) for TEF-1. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). The outgroup species, *Fusarium spp.* (NRRL 25184), was selected for rooting the tree and represented a putative sister group to the *F. oxysporum* complex (O'Donnell and Cigelnik, 1997; O'Donnell *et al.*, 1998).

3.3.5 Pathogenicity testing

For the pathogenicity study, 27 *Fusarium* isolates representing all growing regions were used. Isolates were grown on half strength PDA and incubated at 25°C for seven to ten days. These were used to inoculate 50g of sterile red millet seed from Smith Seeds, 586 Moreleta Street, Silverton, Pretoria, SA. Each bag containing millet seed was inoculated with fifteen 5x5mm fungal plugs and incubated for two to three weeks at 25°C. The inoculated millet was mixed with 2kg virgin sandy loam soil. Potato cultivar Caren mini-tubers were used to test pathogenicity of isolates, as it has a medium length growing period and is moderately susceptible to *Fusarium* dry rot (Denner *et al.*, 2003). Sprouted

tubers were planted in the soil and grown at $\pm 28^{\circ}\text{C}$. Plants were irrigated twice a week and fertilized once every two weeks with 100ml of 0.5g/l of Multifeed solution. Plants were monitored for the development of symptoms. They were examined fortnightly from 9 weeks until 13 weeks after emergence for the presence of wilting. Symptoms were assessed by visually dividing stems into three equal sections and assigning a class value to each plant according to a 5-point scale used by Isaac and Harrison (1968):

1= no wilting or yellowing

2= wilting and yellowing in one third of the stem

3= wilting and yellowing in two thirds of the stem

4= total wilting and yellowing

5=whole plant dead.

Fifteen weeks after planting, lower stem isolations were made from the plants on *Fusarium* selective Peptone pentachloronitrobenzene (PCNB) medium. Each isolate was classified into a wilt reaction category based on the modified index of Corsini *et al.* (1988) and Millard (2003),:

$\{(\text{Presence of wilt symptoms, 0 or 1}) \times (\text{wilt severity, 1-5})\} + (\text{re-isolation of pathogen, 0 or 1})$

Based on the index, isolates were rated as:

≤ 2.2 = not pathogenic

2.3-4.0 = virulent

≥ 4.1 = highly virulent

3.3.6 Data analysis

Data were analyzed using the statistical program GenStat® (Payne *et al.*, 2007). The experiment was a completely randomized design (CRD). Analysis of variance (ANOVA) was applied to test for differences in pathogenicity between three *Fusarium* isolates from each of the nine potato production areas. The experiment was repeated and the results were combined. The data was acceptably normal, with homogenous treatment variances. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level of significance (Snedecor and Cochran, 1980).

3.4 RESULTS

3.4.1 *Fusarium* identification

Morphology

Morphological identification of *Fusarium* spp. involves the examination of cultural characteristics and microscopic features such as the presence or absence of microconidia, macroconidia and chlamydospores; and the shape of the conidiogenous cells. The presence of microconidia, macroconidia and chlamydospores was observed for all the isolates. Some were observed to contain short microconidia-bearing monophialides which are used to distinguish *F. oxysporum*, from *F. solani*. No isolates could be positively identified as *F. solani* from the observations.

Molecular characterization

The sequenced isolates were compared to *Fusarium* (Table 3.1) using the NCBI database (National Centre for Biotechnology Information; www.ncbi.nih.gov) using the BLASTN search tool. Some were identified as *formae speciales* of *F. oxysporum* which cause vascular wilt in a wide variety of crops, while some individual pathogenic strains within the species have limited host ranges. Most of the isolates identified were *F. oxysporum* and only two were identified as *F. solani*. The *F. oxysporum* f. sp. Identified were: *F. oxysporum* f. sp. *gladioli*, *F. oxysporum* f. sp. *lupine*, *F. oxysporum* f. sp. *asparagi*, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *vasifectum*, *F. oxysporum* f. sp. *lisi*, *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *dianthi* and *F. oxysporum* f. sp. *spinaciae*. These occurred randomly throughout the different potato growing regions, but not all f. sp. occurred in all regions. None of the isolates identified were positively identified as *F. oxysporum* f. sp. *tuberosi*, which is a f. sp. of *F. oxysporum* that has been found to have the ability to cause Fusarium wilt on potatoes (Thanassoulopoulos and Kitsos, 1985).

3.4.2 Phylogenetic analyses

The evolutionary history was inferred using the maximum likelihood method (Sneath and Sokal, 1973). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood

method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Condon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset.

The phylogenetic tree was drawn using TEF-1 α sequence data which separated the isolates into five distinct groups (Fig. 3.2). Isolates belonging to different regions grouped randomly throughout the tree and this indicating that the isolates are randomly distributed throughout the potato growing regions. Isolate K4 in group 1 from KwaZulu Natal (KZN) did not group with any other isolate and perhaps represents a new form of *Fusarium* isolates from the other regions which appeared in both groups. Groups 2, 3 and 5 isolates, belonging to different regions, grouped randomly throughout. Group 4 consisting of authentic *F. solani* did not group with any of *F. solani* isolates identified in this study.

3.4.3 Pathogenicity test

The results in Table 3.2 show that all the *Fusarium* isolates obtained from the different potato production areas are pathogenic to potato cultivar Caren according to the severity index. All 27 of the isolates tested were virulent, while one isolate, from K4KZN, was highly virulent. This indicates that these isolates are capable of causing Fusarium wilt on potato plants.

The mean results indicate that the *Fusarium* strains isolated are capable of causing wilt on potato plants. The first symptoms of yellowing appeared at around 9 weeks after emergence and the progress of wilting was monitored up to 13 weeks, at which time almost the entire plant was yellow. After 13 weeks the plants were brown in colour and some died earlier than others. The control was still healthy and green at this stage. At harvest, only a few small tubers were formed by some of the plants. The results of tuber yield were not included in the study because most of the plants did not form tubers. When the *Fusarium* isolates from the potato production areas are compared, they all had ratings between 4 and 5 which show their ability to infect and cause wilting (Fig 3.3). These isolates were not significantly different to each other in terms of causing wilting symptoms.

Koch's postulates were confirmed for all the *Fusarium* isolates tested, by re-isolating from the diseased plants and plating on *Fusarium* selective media to confirm the presence of *Fusarium*.

3.5. DISCUSSION

In this study *Fusarium* isolates were obtained from diseased potato plant material from different potato growing regions in SA. Morphological identification of the isolates was conducted and DNA sequence identifications were done to correctly identify the isolates. The isolates identified represent a small portion of the *Fusarium spp.* that exists in SA. From the identified isolates, *Fusarium* was confirmed to be the causal agent of Fusarium wilt of potatoes in SA as previously reported by Visser (1999). *F. oxysporum* was the most prevalent species and some of the *F. oxysporum* isolates were further identified to subspecies level but none were positively identified as *F.oxysporum f. sp. tuberosi*. The results indicate that *Fusarium* readily occurs throughout the potato growing regions in SA and is not restricted to one region. This can be explained by the fact that *Fusarium spp.* commonly exists as a soilborne pathogen (Summerell *et al.*, 2003).

The identification of *Fusarium spp.* causing diseases on potatoes and other solanaceous crops has proved to be difficult (Romberg and Davis, 2007). The concept of f. sp. was developed to distinguish morphologically similar isolates of *F. oxysporum* with the ability to cause disease on different plants (Kistler, 1997). Normally the host range of f. sp. is restricted to a few plant species (Katan, 1997). However; some f. sp. have a broader host range (Menzies *et al.*, 1990). In this study, isolates of different f. sp. were able to cause Fusarium wilt on potatoes. Perhaps the sampling regions could have been previously used to plant other plant species such as spinach, melons, carnations and bean before they were used as potato fields.

The occurrence of plant diseases is governed by the host, pathogen and the environment. For wilting, the reaction of the host plant to the pathogen following inoculation is an important factor which influences wilt development, and then the severity of the wilt is determined by the pathogen and the environment (Ben-Yephet and Shtienberg, 1997). The difference in climate and local variation in weather can limit the number of *Fusarium spp.* observed (Summerell *et al.*, 2003). A similar observation from

this study could have been the case as there was little variation observed among the *Fusarium* isolates. It is therefore important to study *Fusarium* strains within local populations, as this may provide an indication of whether pathogenic forms of *Fusarium* have evolved within the population or been introduced through infested seeds or by long distance dispersal of soil particles and fungal spores (Skovgaard *et al.*, 2002).

Pathogenicity assessment is important to determine aggressiveness of isolates in different potato production regions (Wu *et al.*, 2005). Our findings indicated similarities in aggressiveness among *Fusarium* isolates from all potato growing regions. This would suggest that wilt-causing *Fusarium spp.* are dispersed among most potato production regions in SA. This could pose a potential threat to the potato industry in future if the environmental conditions for disease to occur become optimal due to climate change.

Fusarium can cause serious losses in the potato industry because of its ability to penetrate roots, tubers and young sprouts (Thanassoulopoulos and Kitsos, 1985). *Fusarium spp.* overwinter in soil or as colonizers of living plant matter or debris (Nelson *et al.*, 1981), thus resulting in high levels of soilborne inoculum pathogenic to potatoes. Disease occurrence will result in losses due to reduction in tuber yield and quality at the time of harvest (Hide and Horrocks, 1994). The amount of damage caused by this disease is often underestimated (Thanassoulopoulos and Kitsos, 1985). Planting cultivars that are less susceptible to infection by *Fusarium*, in addition to other control measures, could play an important role in the integrated management of Fusarium wilt. The findings from this study could be used as part of a disease management strategy for Fusarium wilt of potatoes in SA.

The knowledge of genetic structures of pathogen populations can offer insight into the future potential of that particular population to evolve in terms of dispersal and virulence, as well as how it will react to control methods such as the application of fungicides (McDonald and Linde, 2002). Molecular characterization of isolates is important in helping to develop new control methods, as it will ensure that all representatives of *Fusarium* are considered when testing new fungicides or host cultivars. In this way, the management of the disease will focus on control strategies to target a population and not individual strains (McDonald *et al.*, 1989). It is important to know the identity of *Fusarium*

species that occur in each region because this will enable growers to determine the risk of Fusarium wilt.

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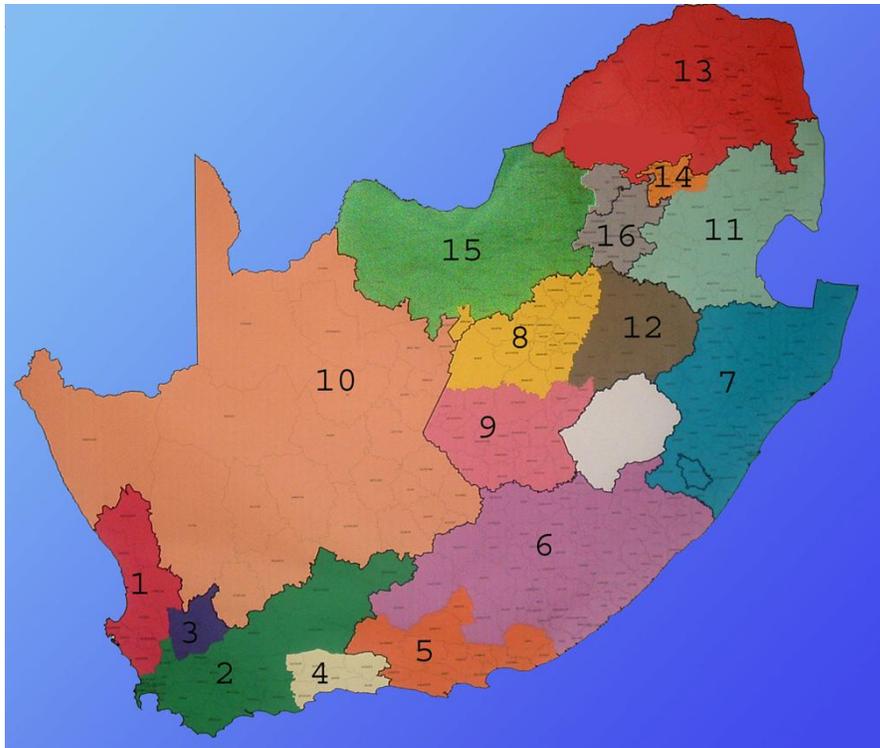


Fig. 3.1 Map of SA showing potato production regions (www.potatoes.co.za)

1. Sandveld (SV),
2. South Western Cape (SWC),
3. Ceres (C),
4. Southern Cape (SC),
5. Eastern Cape (EC),
6. North Eastern Cape (NEC),
7. KwaZulu-Natal (KZN),
8. Western Free State (WFS),
9. South Western Free State (SWFS),
10. Northern Cape (NC),
11. Mpumalanga (MP),
12. Eastern Free State (EFS),
13. Limpopo (L),
14. Marble Hall (MH),
15. North West (NW),
16. Gauteng (G)

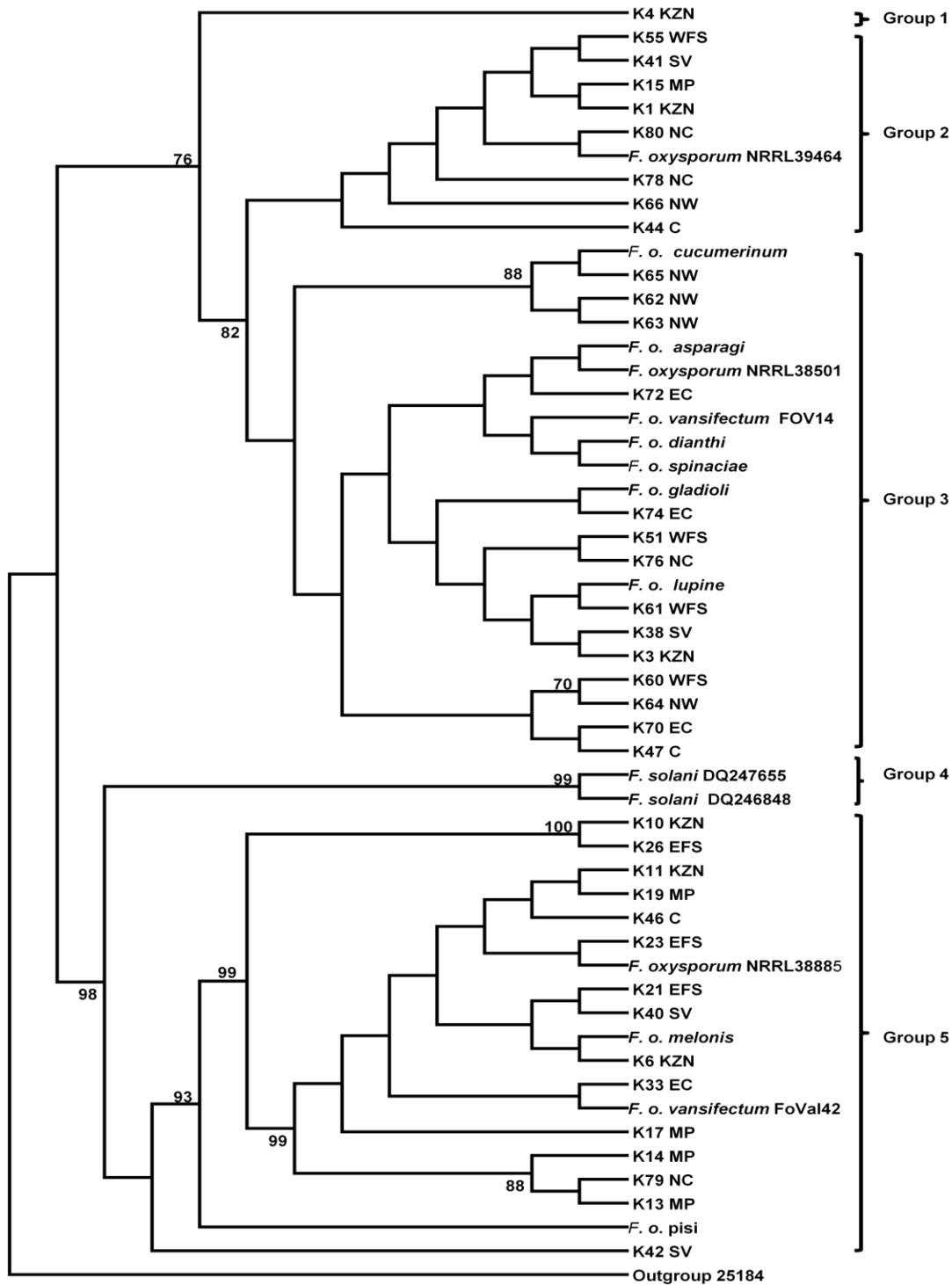
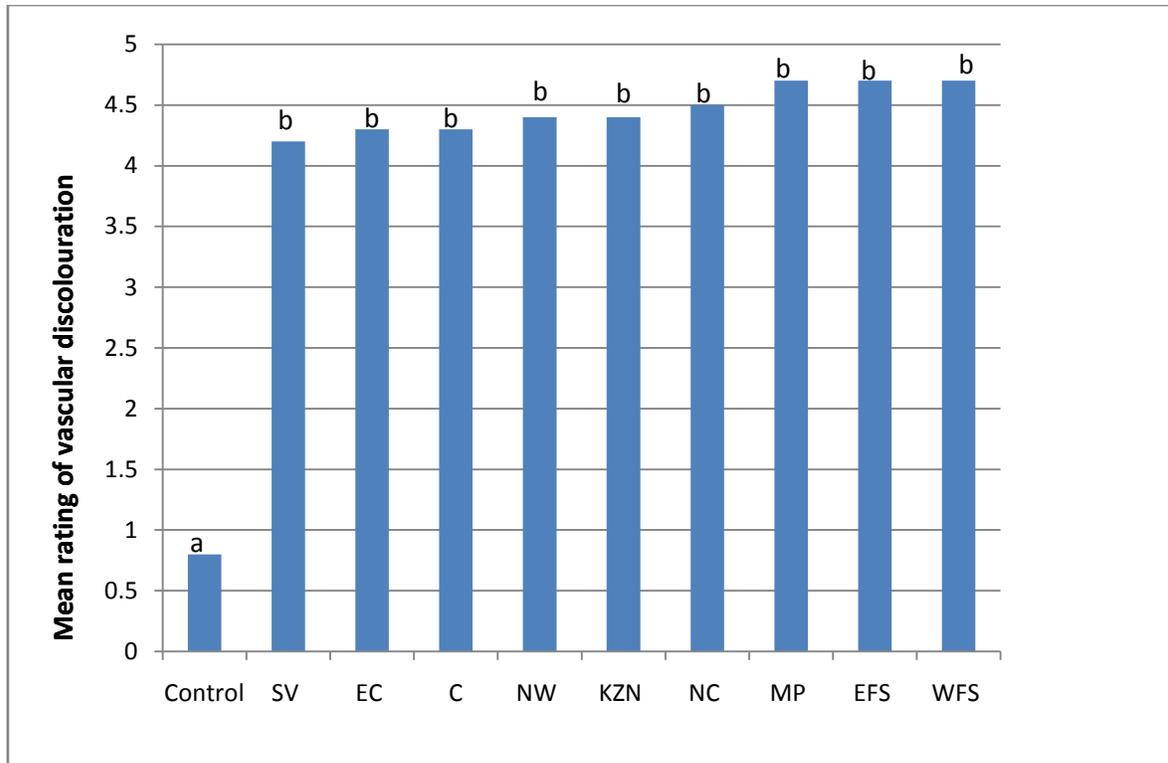


Fig. 3.2 Phylogenetic tree of the *Fusarium* isolates based on the elongation factor 1 α gene. Bootstrap support values of 70% and higher are shown above nodes.



Represents the regions namely SV - Sandveld, EC - Eastern Cape, C - Ceres, NW - North West, KZN - KwaZulu Natal, NC - Northern Cape, MP - Mpumalanga, EFS - Eastern Free State and WFS - Western Free State

Fig. 3.3 Comparison between uninoculated and potato plants inoculated with *Fusarium* isolates from different potato production areas. P value = 5%, LSD: 0.37

Table 3.1 *Fusarium* isolates identified using the TEF-1 α

Isolate	^a Geographic	<i>Fusarium</i> species	^b Name	Accession
K1	KZN	<i>F. o. gladioli</i>	<u>FD_01261</u>	AF246847
K3	KZN	<i>F. o. lupine</i>	<u>FD_00117</u>	FJ985285
K4	KZN	<i>F. o. asparagi</i>	<u>FD_01243</u>	AF246865
K6	KZN	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K10	KZN	<i>F. solani</i>	<u>FD_01317</u>	DQ247655
K11	KZN	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K13	MP	<i>F. oxysporum</i> NRRL 39464	<u>FD_00802</u>	FJ985419
K14	MP	<i>F. oxysporum</i> NRRL 38501	<u>FD_00700</u>	FJ985403
K15	MP	<i>F. oxysporum</i> NRRL 39464	<u>FD_00802</u>	FJ985419
K16	KZN	<i>F. o. asparagi</i>	<u>FD_01243</u>	AF246865
K17	MP	<i>F. o. melonis</i>	<u>FD_00785</u>	DQ837696
K18	MP	<i>F. o. vasifectum</i> FoVal42	<u>FD_00809</u>	EU313535
K19	MP	<i>F. o. pisi</i>	FD_00617	EU313540
K20	MP	<i>F. o. melonis</i>	<u>FD_00785</u>	DQ837696
K21	EFS	<i>F. o. cucumerinum</i>	<u>FD_00785</u>	FJ985379
K22	KZN	<i>F. o. melonis</i>	<u>FD_00785</u>	DQ837696
K23	EFS	<i>F. o. dianthi</i>	<u>FD_00793</u>	DQ452429
K24	EFS	<i>F. o. dianthi</i>	FD_00793	DQ452429
K25	EFS	<i>F. o. dianthi</i>	FD_00793	DQ452429
K26	EFS	<i>F. solani</i>	FD_01047	DQ246848
K29	EFS	<i>F. o. dianthi</i>	FD_00793	DQ452429
K28	EFS	<i>F. o. dianthi</i>	FD_00793	DQ452429
K30	KZN	<i>F. o. vasifectum</i> FOV14	<u>FD_01376</u>	DQ877695
K32	EFS	<i>F. o. melonis</i>	<u>FD_00785</u>	DQ837696
K33	EC	<i>F. oxysporum</i> NRRL 39464	<u>FD_00802</u>	FJ985419
K35	EC	<i>F. o. pisi</i>	<u>FD_00617</u>	EU313540

Isolate	^a Geographic	<i>Fusarium</i> species	^b Name	Accession
K36	KZN	<i>F. o. dianthi</i>	FD_00793	DQ452429
K38	SV	<i>F. oxysporum</i> NRRL 39464	<u>FD_00802</u>	FJ985419
K40	SV	<i>F. o. spinaciae</i>	<u>FD_00211</u>	DQ837687
K41	SV	<i>F. o. gladioli</i>	<u>FD_01261</u>	AF246847
K42	SV	<i>F. o. vasifectum</i> FOV14	<u>FD_01376</u>	DQ877695
K44	C	<i>F. o. lupine</i>	<u>FD_00117</u>	FJ985285
K46	C	<i>F. o. spinaciae</i>	<u>FD_00211</u>	DQ837687
K47	C	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K 51	WFS	<i>F. o. cucumerinum</i>	<u>FD_00785</u>	FJ985379
K55	WFS	<i>F. o. gladioli</i>	<u>FD_01261</u>	AF246847
K60	WFS	<i>F.oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K61	WFS	<i>F. o. lupine</i>	<u>FD_00117</u>	FJ985285
K62	NW	<i>F. o. dianthi</i>	<u>FD_01267</u>	AF246846
K63	NW	<i>F. o. dianthi</i>	<u>FD_01267</u>	AF246846
K64	NW	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K65	NW	<i>F. o. cucumerinum</i>	<u>FD_00785</u>	FJ985379
K66	NW	<i>F. o. gladioli</i>	<u>FD_01261</u>	AF246847
K69	NW	<i>F. o. dianthi</i>	<u>FD_00793</u>	DQ452429
K70	EC	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K72	EC	<i>F. o. asparagi</i>	<u>FD_01243</u>	AF246865
K74	EC	<i>F. o. gladioli</i>	<u>FD_01262</u>	AF246846
K76	NC	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K78	NC	<i>F. oxysporum</i> NRRL 38885	<u>FD_00801</u>	FJ985418
K79	NC	<i>F. o. gladioli</i>	<u>FD_01261</u>	AF246847
K80	NC	<i>F. oxysporum</i> NRRL 39464	<u>FD_00802</u>	FJ985419

^aRepresents the regions namely KZN - KwaZulu Natal, MP - Mpumalanga, SV - Sandveld, C - Ceres, WFS - Western Free State, EFS - Eastern Free State, NW - North West, EC - Eastern Cape and NC - Northern Cape

^b*Fusarium* identification database name

Table 3.2 Relative virulence of *Fusarium* isolates on potato cultivar Caren

Production area	Isolate no.	Severity index ^b	Rating
KZN	K 1	3.8 abc	Virulent
	K 4	4.4 f	Highly virulent
	K 11	3.9 bcd	Virulent
MP	K 13	3.3 a	Virulent
	K 14	4.0 bcde	Virulent
	K15	3.9 bcd	Virulent
EFS	K 21	3.9 bcd	Virulent
	K 23	3.7 abc	Virulent
	K 26	3.9 bcd	Virulent
SV	K 40	3.7 abc	Virulent
	K 41	3.7 abc	Virulent
	K 42	3.8 abc	Virulent
NC	K 76	3.9 bcd	Virulent
	K 78	4.0 bcde	Virulent
	K 79	3.7 abc	Virulent
WFS	K 60	3.8 abc	Virulent
	K 55	3.8 abc	Virulent
	K 51	4.0 bcde	Virulent
NW	K 63	3.7 abc	Virulent
	K 64	3.5 a	Virulent
	K 65	3.7 abc	Virulent
C	K 46	3.8 abc	Virulent
	K 47	3.9bcd	Virulent
	K 44	3.7abc	Virulent
EC	K 70	3.7abc	Virulent
	K 72	3.7abc	Virulent
	K 74	3.8abc	Virulent

^aRepresents the regions namely KZN - KwaZulu Natal, MP - Mpumalanga, SV - Sandveld, C -Ceres, WFS - Western Free State, EFS - Eastern Free State, NW - North West, EC - Eastern Cape and NC - Northern Cape

^bMean of 3 replicates; values followed by the same letter do not differ significantly according to Fishers' protected t-test least significant difference at P value = 5%, LSD: 0.3018

CHAPTER 4

THE *IN VITRO* EFFECT OF SILICON ON THE GROWTH OF *FUSARIUM OXYSPORUM* AND THE EFFECT OF DIFFERENT SILICON SOIL AMENDMENTS ON *FUSARIUM* WILT DISEASE AND POTATO TUBER YIELD

4.1 ABSTRACT

Fusarium wilt is a disease of global importance resulting in large agricultural losses of numerous crops if not managed successfully. In SA, *Fusarium oxysporum* is the most important causal agent of potato wilt and there are no reliable control measures available. One option is the use of silicon (Si) to improve host resistance. Si is essential for normal growth and development of plants, and has been shown to improve host plant resistance to pathogen attack. Its effect on controlling *Fusarium* wilt on potatoes has not previously been investigated. In this study, the *in vitro* effect of potassium silicate (KSi) (20.7% silicon dioxide) on the growth of *F. oxysporum* was investigated. The effect of different Si soil amendments, namely Si ash (~99% Si), slag (30% Si), fly ash (50% Si) and lime on tuber yield and on the production of phenol acids was also investigated. Potato dextrose agar (PDA) was amended with different concentrations of KSi, namely 0, 5, 10, 20, 40 and 80ml KSi per litre agar. Following the addition of KSi (pH 12.7), the pH of PDA solutions increased and thus pH controls were included. Plates were inoculated with *F. oxysporum* and percentage inhibition was calculated seven days post inoculation. At 80ml KSi.l⁻¹PDA the growth of *F. oxysporum* was inhibited by 92% while 40ml KSi.l⁻¹ of PDA showed only 5% inhibition. Interestingly, at low concentrations, 5ml.l⁻¹ 10ml.l⁻¹ and 20ml.l⁻¹ KSi.l⁻¹PDA, *F. oxysporum* growth was enhanced. Since there was no growth inhibition in the pH controls, Si was shown to be responsible for the inhibition. In the pot trials, a lime (calcium carbonate) treatment was included as the pH control. Results showed a definite increase in yield when compared to the uninoculated soil control, thus Si had a significant effect on yield. The results for the phenolic acids studies were variable, perhaps because the amounts of Si absorbed by the plant were too low to stimulate increased production of phenolic compounds. Further research is necessary to confirm the efficacy of Si soil amendments in management of *Fusarium* wilt of potatoes.

4.2 INTRODUCTION

Fusarium wilt is a disease of global importance that can result in significant agricultural losses to numerous plant species if not controlled (Nelson *et al.*, 1981). It is widely distributed throughout the world (Rich, 1983) and a number of *Fusarium* spp. are associated with potato wilt (Nelson *et al.*, 1981), with the most prevalent species being *Fusarium oxysporum*. This *Fusarium* spp. causes vascular wilt disease in a wide variety of economically important crops (Roncero *et al.*, 2003).

In SA *F. oxysporum* and *F. solani* are the most important causal agents of potato wilt (Visser, 1999). *F. oxysporum* is well represented among the communities of soilborne fungi (Fravel *et al.*, 2003). Once this fungus has infested the soil, it becomes difficult to eradicate because it produces chlamydospores that have prolonged persistence in the soil (Larena *et al.*, 2003; Smith *et al.*, 2005). This complicates management of the disease in the field and there is no effective fungicide treatment for its control (Borrero *et al.*, 2004). The management of *Fusarium* wilt has been strongly focused on using resistant cultivars and fumigating the soil with methyl bromide (Larena *et al.*, 2003). Due to the environmentally unfriendly properties of methyl bromide and the appearance of more races of the pathogen that can overcome plant resistance, there is a need to find alternative management practices (Larena *et al.*, 2003).

Recent research has focused on methods to stimulate plant defence mechanisms. These include the use of microorganisms such as non-pathogenic strains of *F. oxysporum* (Panina *et al.*, 2007) and amendments of soil with mineral elements including Si (Fawe *et al.*, 1998). Si is the second most abundant element both on the earth's crust and in the soil (Epstein, 1999). It is accumulated in plants at levels equivalent to that of macronutrient elements such as calcium, magnesium and phosphorus (Epstein, 1999). Si is taken up by the roots in the form of silicic acid [$\text{Si}(\text{OH})_4$] (Ma and Takahashi, 2002; Ma and Yamaji, 2006). The benefits of Si amendments include enhanced resistance against pathogens and pests, drought and heavy metal tolerance and increased quality and yield of agricultural crops. These effects are primarily associated with substantial deposition in cell walls, which lead to mechanical strengthening and rigidity (Epstein, 1994; Richmond and Sussman, 2003; Ma and Yamaji, 2006). Si can play a direct role in stimulating host defence responses. Soluble Si can promote the production of phenolics

and phytoalexins in response to pathogen infection and enhance the activity of defence-associated enzymes (Hammerschmidt, 2005; Ma and Yamaji, 2006).

Various sources of Si are available for use in agriculture, including slag and fly-ash. Slag contains mainly calcium silicate and is produced as a by-product of the steel and iron industries. Slag has been used as a Si fertilizer in Japan (Ma and Takahashi, 2002). Fly-ash, produced from coal power plants, is also used as a Si source in agriculture mixed with potassium carbonate or potassium hydroxide and magnesium hydroxide (Ma and Takahashi, 2002).

Si fertilization of soils with low levels of Si offers promising results in terms of disease control and yields. In rice it reduces susceptibility to fungal diseases (Ma and Yamaji, 2006). Si fertilizers have been applied to rice and sugarcane to increase crop yields on a sustainable basis (Ma and Yamaji, 2006). In SA, Bekker *et al.* (2006) demonstrated that Si has the ability to enhance plant defence mechanisms against infection by *Phytophthora cinnamomi* and that potassium silicate in amended potato dextrose agar media can suppress mycelial growth of plant pathogens, including *F. oxysporum* and *F. solani*. It has also been shown to control *Pythium* species in cucumber plants (Chérif *et al.*, 1992).

There are no reports available regarding the effect of Si on improving the resistance of potato plants to diseases. In a study by Bekker *et al.* (2007) they found that Si application on avocado resulted in increased resistance against *P. cinnamomi* infection due to the increase of phenolic levels in the roots. Nara *et al.* (2006) found that free (chlorogenic and caffeic acid) and bound-form (ferulic acids) phenolic acids in potato peels are an effective source of antioxidative activity. Ghanekar *et al.* (1984) found that the three main phenolic compounds in potato tubers, namely, chlorogenic, caffeic and ferulic acids, possess antibacterial activity against soft rot bacteria and were more effective in combination than individually, even at low combination concentrations. The oxidation of these phenolic acids can, therefore, also be involved in reducing soft rot development (Hammerschmidt, 2005). Ferulic acid may play an important role in cell wall extensibility and growth rate (Nara *et al.*, 2006).

A few studies have been conducted to evaluate the effect of Si on the incidence of diseases caused by soilborne fungi. The aim of this study was to assess the *in vitro* effect of soluble potassium silicate on the growth of *F. oxysporum* and to determine the effect of different Si sources in soil on Fusarium wilt caused by *F. oxysporum*. Pot trials were conducted to investigate the effect of Si on the potential yield of potatoes and the effect it may have on production of phenolic acids.

4.3 MATERIALS AND METHODS

4.3.1 Isolation and identification of *Fusarium oxysporum*

An isolate of *Fusarium* was obtained from potato plants showing wilting symptoms. Potato tubers from infected plants were obtained from Kwa-Zulu Natal, washed with running tap water to remove excess soil and then surface sterilized by soaking them in NaOCl (0.5% v/v) for five minutes. The tubers were rinsed three times in sterile distilled water to remove the NaOCl and left on paper towels for 30 minutes to dry completely. The tubers were sliced longitudinally from the stolon end and then four pieces, 5 x 5mm in size, of the potato were cut from the diseased vascular ring or tissue. The potato pieces were placed on *Fusarium* selective media, Peptone pentachloronitrobenzene (PCNB) agar (Nelson *et al.*, 1983). The plates were incubated at 25°C for 5 - 7 days. Single fungal colonies were cut out and transferred onto half strength potato dextrose agar (PDA) plates and incubated at 25°C for 7 – 10 days. The identity of the isolates was confirmed using morphology by plating isolates on Carnation Leaf Agar (CLA), grown for 10 days and viewed using a stereomicroscope to observe the presence of macroconidia, microconidia, chlamydospores and the conidiophore structures (Nelson, 1981).

Identity was also confirmed by DNA extraction of the *Fusarium* isolate using a commercial DNA extraction kit, Dneasy Plant kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. For identification the TEF-1 α was used and the gene was amplified using the primers, EF1 and EF2 under conditions as described by O'Donnell *et al.* (1998). PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the respective PCR primers. For sequencing, the BigDye terminator sequencing kit (Version 3.1, Applied Biosystems) and an ABI PRISMTM 3100 DNA sequencer (Applied Biosystems) were used. The sequence was edited on Chromas Lite version 2.1 (http://www.technelysium.com.au/chromas_lite.html) and BioEdit version 7.0.0. Copyright

©1997-2004 Tom Hall. The isolate was aligned with sequences from the *Fusarium* Identification database (<http://fusarium.cbio.psu.edu>; Geiser *et al.*, 2004) and was confirmed to be *F. oxysporum*.

***In-vitro* trial**

4.3.2 Agar preparation

Potato dextrose agar (PDA) was prepared and autoclaved. AgriSil K50 (Plant Health Products, SA) potassium silicate liquid containing 33g/kg potassium and 96g/kg silica was filtered using a 0.45µm Millipore filter and added to the autoclaved PDA, at concentrations of 5, 10, 20, 40 and 80ml per litre of PDA, respectively. The negative control was unamended PDA. Potassium silicate was mixed with PDA using magnetic stirrers to ensure even distribution. The pH values for all the different concentrations were measured before decanting the media into Petri dishes. Plates were incubated for two days before use to ensure that no contamination took place during amendment with Si.

4.3.3 Antifungal activity assay

One *F. oxysporum* isolate was randomly selected for this study and it was first tested for pathogenicity on healthy potato tubers which were inoculated at the stolon end. The tubers were placed into brown paper bags and incubated at 25°C for ten days. Ten replicates were used per treatment and 5mm discs of actively growing *F. oxysporum* were placed in the centre of potassium silicate amended and control agar plates. All treatments were incubated at 25°C. Colony growth of all *F. oxysporum* isolates was recorded after seven days for each treatment. Percentage inhibition was calculated according to the following formula (Biggs *et al.*, 1997):

$$\text{Percentage inhibition} = \frac{(C-T) \times 100}{C}$$

Where: C = colony diameter (mm) of the control

T = colony diameter (mm) of the treatment

4.3.4 pH determination

Potassium silicate at 5, 10, 20, 40 and 80ml.l⁻¹ of autoclaved PDA increased the pH of the PDA to 8.8, 9.6, 10.2, 10.6, and 10.8, respectively; while unamended PDA had a pH

of 5.2. A high pH is known to suppress growth of *F. oxysporum* (Bekker *et al*; 2006), therefore the effect of high pH in the absence of Si had to be investigated. A 1Mol stock solution of potassium hydroxide with a pH of 12.8 was prepared to adjust the pH of the PDA media to 8.8, 9.6, 10.0, 10.4, and 10.6, respectively without the addition of Si. These plates served as the pH controls.

4.3.5 *In-vitro* data analysis

The experiment was designed as a completely randomized design (CRD). Analysis of variance (ANOVA) was applied to test for differences between the six treatments, as well as the treatment by inoculation interaction. Treatment means were separated using Fishers' protected t-test least significant difference (LSD) at the 1% level of significance (Snedecor and Cochran, 1980). GenStat® statistical program was used to analyse the results (Payne, 2007).

Pot trials

4.3.6 Preparation of inoculum and soil

The same *F. oxysporum* isolate used for the *in vitro* study was used to inoculate 150g of sterile red millet seed (Smith's Seed, 586 Moreleta Street, Pretoria, South Africa) in plastic bags. Each bag was inoculated with ten 5x5mm fungal plugs and incubated for three weeks at 25°C. The inoculated millet was mixed with 2kg soil. Virgin sandy loam soil was used and the soil analysis performed by the Department of Plant Production and Soil Science, University of Pretoria. The results were as follows: 123.9mg/kg calcium, 46.3 mg/kg magnesium, 39.6 mg/kg potassium, 11.7 mg/kg sodium and pH (water) of 5.4. Potato mini-tubers, cultivar Caren, were used for this trial. Caren is known to be susceptible to Fusarium wilt.

4.3.7 Preliminary Pot trial I

The treatments used included Si ash (~99% Si), slag (30% Si) and fly ash (50% Si). Lime (calcium carbonate) was used as a pH control in the soil, as it contains no Si. Each treatment was split into pots containing soil inoculated with *F. oxysporum* and pots containing uninoculated soil to quantify the effect of the pathogen on the production of phenolic acids. Slag, fly-ash and calcium carbonate were applied at a rate 2t/ha i.e. 0.9g/2kg soil; while Si ash was applied at 0.5t/ha (i.e. 0.225g/2kg soil. Lime was used as

a pH control. Distilled water was added to pots to bring the soil to field capacity after applying the treatments. Each treatment consisted of five replicates and all the treatments were kept at $\pm 28^{\circ}\text{C}$ in greenhouse compartments for a period of 18 weeks. Multifeed-P, which does not contain Si, was applied fortnightly at a rate of 100ml of 0.5g of Multifeed diluted in one litre of water until the potato plants were harvested. The pot plants were all irrigated with 200ml of distilled water every third day.

4.3.8 Pot trials II and III

The treatments used included Si ash (~99% Si), slag (30% Si) and fly ash (50% Si). Lime (calcium carbonate) was used as a pH control in the soil. The treatments were divided into artificially inoculated soil and uninoculated soil. Plastic bags each containing 150g of sterile red millet seed (Smith Seeds, 586 Moreleta Street, Silverton, Pretoria, SA) were inoculated with fifteen 5x5mm fungal plugs and incubated for three weeks at 25°C . The inoculated red millet was used to inoculate the soil. Slag, fly-ash and calcium carbonate were applied at a rate of 2t/ha, which was equivalent to 1.8g/4kg soil. Si ash was applied at 0.5t/ha i.e. 0.45g was added to 4kg soil. For each soil amendment treatment there was an inoculated and an uninoculated treatment. Lime was used as a pH control. Distilled water was added to pots to bring them to field capacity after applying the treatments. Each treatment consisted of five replicates and all treatments were kept under greenhouse conditions at $28 \pm 2^{\circ}\text{C}$. Multifeed-P water soluble fertilizer was used as described above.

4.3.9 Disease Assessment

For both pot trials, plants were visually observed for incidence and severity of wilting symptoms twelve weeks after planting. Stems were divided into three equal sections and a class value was assigned to each plant according to a 5-point scale used by Isaac and Harrison (1968).

- 1= no wilting or yellowing
- 2= wilting and yellowing in one third of the stem
- 3= wilting and yellowing in two thirds of the stem
- 4= total wilting and yellowing
- 5=whole plant dead

Plants were harvested fifteen weeks after planting and isolations were made from each treatment to determine if the causal agent was present. The average wet mass of tuber yields for the different treatments was determined at harvest.

4.3.10 Identification and quantification of phenolic compounds

Identification and quantification of chlorogenic acid, caffeic acid and ferulic acid from tubers was done by HPLC. Tuber skins are known to contain high concentrations of phenolic acids, while much lower concentrations are found in the tuber flesh (Lewis *et al.*, 1998). Hence, in this study, tuber peels were used for extraction of the phenolic acids for quantification. Generally potato tubers contain low levels of phenolic acids (Ramamurthy *et al.*, 1992).

4.3.11 Extraction of phenolic compounds

Tuber peels were freeze-dried for five days and then ground using a mortar and pestle to a fine powder. For each sample, 200mg of the powder was put through a 1mm sieve and placed in a 1.5ml Eppendorf tube for extractions. Aliquots of 1ml of a cold mixture of methanol: acetone: ultra-pure water (7:7:1, v: v: v) were added, vortexed and ultrasonified for 5min. After sonification, samples were shaken for 20min at 160rpm while on ice. Samples were centrifuged for 5min and the supernatant of each sample was transferred to a 20ml centrifuge tube. This process was repeated three times and the supernatant evaporated in a laminar flow cabinet at room temperature. The residue was dissolved in 1ml sterile, ultra-pure water. Finally, samples were filtered through 0.45µm, 25mm, Ascrodise, GHP, syringe filters (Separations, SA). Samples were stored at 4°C until analysed using reverse phase – high performance liquid chromatography (RP-HPLC).

4.3.12 Reverse Phase – High Performance Liquid Chromatography (RP-HPLC)

For identification and quantitative analysis of samples, 10µl of purified extract per sample was analysed using RP-HPLC (Hewlett Packard Agilent 1100 series) with a UV diode array detector at 325 and 340nm. Separation was achieved using a Gemini 3µ, C18, 110A (Phenomenex®) reverse phase column (250mm length, 5µm particle size, 4.6mm inner diameter). A gradient elution was performed with water (pH 2.6 adjusted

with H_3PO_4) and acetonitrile (ACN) and consisted of 0min, 7% ACN; 0 – 20min, 20% ACN; 20 – 28min, 23% ACN; 28 – 40min, 27% ACN; 40 – 45min, 29% ACN; 45 – 47min, 33% ACN; 47 – 50min, 80% ACN. Solvent flow rate was $0.7\text{ml}\cdot\text{min}^{-1}$. Identification of the phenolic compounds was done by comparing their retention times and UV apex spectrum to those of standards for chlorogenic acid, caffeic acid and ferulic acid. The column was re-equilibrated with initial conditions for 10min, after each run. Peaks were detected at 280nm, although this wavelength is not optimal for ferulic acid (Zhou *et al.*, 2004).

4.3.13 Data analysis

The experiment was designed as a completely randomized design (CRD). Analysis of variance (ANOVA) was applied to test for differences between five treatments, two inoculations as well as the treatment by inoculation interaction. The data was acceptably normal, with homogeneous treatment variances. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level of significance (Snedecor and Cochran, 1980).

4.4 RESULTS

***In-vitro* trial**

4.4.1 Percentage inhibition

At $80\text{ml KSi}\cdot\text{l}^{-1}$ PDA soluble Si suppressed the growth of *F. oxysporum* by 92% and at $40\text{ml KSi}\cdot\text{l}^{-1}$ PDA soluble Si showed some degree of growth inhibition (5%). At concentrations of $5\text{ml KSi}\cdot\text{l}^{-1}$ PDA, $10\text{ml KSi}\cdot\text{l}^{-1}$ PDA and $20\text{ml KSi}\cdot\text{l}^{-1}$ PDA colony growth was enhanced, thereby resulting in a negative percentage inhibition of -44.5, -44.5% and -30.9% respectively (Fig 4.1).

4.4.2 pH determination

A negative percentage inhibition indicates that the colony growth was enhanced. Colony growth was enhanced at the lowest and highest pH values when compared to the control (Table 4.1). pH does not play a role in the inhibition of *F. oxysporum*.

Pot trials

4.4.3 Preliminary pot trial I

In the first pot trial, initial wilting symptoms of the lower leaves were observed on the inoculated and Si amended potato plants (Fig 4.2). All the potato plants had an average disease rating of 4 for the slag, Si-ash and fly-ash and lime. All the plants were wilted and showed symptoms of yellowing with dried brown leaves and dry stems. The control treatment also had an average disease rating of between 4 and 5 which was expected. At harvest potatoes were rated as 5 because the plants were dead. It is likely that this was because the inoculum level used was too high. The millet seed also caused the soil to clump together so the potato plants were not able to grow normally. The uninoculated treatments grew normally to maturity. Progeny tubers were only produced by the uninoculated plants for all the treatments.

The tuber yield for Si amended and uninoculated plants was significantly higher than for the control (Fig 4.3). There were no significant differences in yield between the lime, slag, Si-ash and fly-ash treatments although slag had the highest yield. These results do not give an indication of how Si soil amendments affect the intensity of *Fusarium* wilt, but they do show that addition of Si to the soil in the absence of *F. oxysporum* can influence the growth of potatoes probably as a result of a pH effect and a Si effect.

4.4.4 Pot trials II and III

The potato plants for the Si amended and inoculated treatments showed symptoms for total wilting and yellowing at harvest, after twelve weeks. The slag and lime treatments had an average disease rating of 3, while Si-ash and fly-ash and the control had a disease rating of 4. For the slag and lime, wilting was only observed for two thirds of the whole stem.

For the yield results from pot trials II and III, there were no significant differences between Si amended and uninoculated treatments of lime, Si-ash and fly-ash and the control, while slag was significantly different from the other treatments (Fig 4.4). For Si amended and inoculated treatments yield results, the control was significantly higher than the Si and lime treatments. When comparing the yield of the Si treatments, slag had a slightly higher yield than the other treatments and this could be due to the fact that slag also contains calcium. The unexpectedly high yield of the control treatment could be due

to the difference in the amount of millet used in the preliminary pot trial and pot trials II and III, which were 150g of millet in 2kg soil and 100g of millet in 4kg soil, respectively. Hence, there was a low level of *F. oxysporum* inoculum in the pot and thus did not appear to affect plant growth. When comparing the uninoculated and inoculated treatments the yield for progeny tubers was slightly higher for the slag treatment at 27.5g and 22g, respectively, and for lime, Si-ash and fly-ash treatments there were no significant differences.

4.4.5 Phenol quantification

For both pot trials II and III the concentrations of ferulic acid were too low for analysis and were thus excluded from the study. The chlorogenic acid levels for the uninoculated treatments were significantly higher than the inoculated amended treatments for lime, slag and Si-ash, but not for the fly-ash treatment. Lime had the highest level, followed by the control and slag treatments. In the inoculated treatments the control and Si-ash were significantly different from lime, slag and fly-ash. For the uninoculated treatments lime had the highest concentration of chlorogenic acid at 19 μ g/DW, followed by slag at 15.3 μ g/DW, fly-ash at 13 μ g/DW and 14 μ g/DW respectively and slag had higher levels than the control, while the Si-ash treatment had the lowest level of chlorogenic acid (Fig 4.5).

The caffeic acid levels in tubers were generally higher than those of chlorogenic acid. The levels of caffeic acid in the uninoculated treatments were significantly lower than in the inoculated treatments, except for the Si-ash treatment. Lime, slag and fly-ash inoculated treatments had high levels of caffeic acid at 32, 28 and 30 μ g/DW respectively, which were significantly different to the control and Si-ash (Fig 4.6).

4.5 DISCUSSION

Diseases are one of the most important causes of yield and tuber quality losses in potato production worldwide (Hooker, 2001; Oerke *et al.*, 1994). Plant pathology is dedicated to the development and application of management practices that reduce the adverse effect of disease on food production. To be successful, these practices must be economically feasible and environmentally acceptable. Understanding the factors that trigger the development of plant disease epidemics is essential if we are to create and implement effective strategies for disease management (De Wolf and Isard, 2007). Si is

not essential for growth of plants, but can be beneficial and can directly or indirectly affect growth (Allison, 1968).

Bekker *et al.* (2006) reported the *in vitro* amendment of potassium silicate at 20, 40 and 80ml.l⁻¹ PDA to have an inhibitory effect on colony growth of *F. oxysporum*. In our study, inhibition was visible only at high concentrations of 80ml KSi.l⁻¹ PDA. This could be due to the high Si levels efficiently inhibiting the growth of *F. oxysporum*. At 40ml.l⁻¹ PDA the inhibitory effect was very low and at 5, 10 and 20ml.l⁻¹ PDA there was no growth inhibition.

The high pH in the medium in the absence of Si also enhanced the growth of *F. oxysporum*. This indicated that Si was responsible for the inhibition effect and not the increased pH. Low Si concentrations had no effect on the growth of *F. oxysporum* while a high concentration suppressed the colony growth. *In vitro* results have revealed that Si has the ability to suppress growth of *F. oxysporum* but it is not clearly understood which mechanisms are involved in suppression of colony growth. A clear understanding of such results can help prevent development of resistance of plant pathogens to Si.

The application of different Si sources to soil has been reported to alleviate both abiotic and biotic diseases of a variety of plants and improve crop yields (Ma and Takahashi, 2002). In the first pot trial, the application of lime, Si slag, fly ash and Si ash increased the yield of cultivar Caren when compared to the control. In 1955 slag was used as a liming agent in Europe and recognized as a Si fertilizer in Japan and the world for the first time (Ma and Takahashi, 2002).

For the pot trial investigation different sources of Si were used which were in a powder form. These were mixed with the soil for planting potatoes. The potassium silicate could not be used for the pot trial assessments because when studying soil-borne diseases, it was difficult to use liquid Si for spraying or drenching.

In the first pot trial, the soil was not inoculated with *Fusarium* and the presence of Si in soil improved the yield of potatoes. In the second and third trials however, the Si amendments inoculated with *F. oxysporum* did not improve the yield of the potatoes and the control yielded the highest average. The Si amendments without *F. oxysporum*

slightly improved plant growth and yield of tubers when compared to the control. The slag treatment had the highest tuber yield. In this regard Si did not have an effect on Fusarium wilt development because wilting symptoms were visible on the Si amendment treatments. The results showed that slag gave a better yield compared to other treatments. The presence of calcium in the slag could have played a role in the increased yield of this treatment because calcium is a structural component of cell walls and other plant membranes (Gunter, 2002). Ayres (1966) reasoned that the increased yield from calcium silicate treatment in their studies was probably due to the combination of calcium and Si.

Nutrient analysis of the soil before and after planting would be important to establish if the combination of increased Si and calcium concentrations in the soil plays a role in plant growth and development. It is important to separate the effects of Si from those of calcium (Savant *et al.*, 1999). As the optimum Si application rate is not yet known, Si sources could cause nutrient imbalances in the soil, which will result in differences in how the specific Si sources affect Fusarium wilt on potatoes.

It has been suggested that Si may activate a form of defence response, leading to phenol production and release at infection sites (Koga *et al.*, 1988). As early as 1935 Walker and Link (1935) suggested that the presence of phenolic compounds in host plants does not indicate that they play a role in the resistance of the host to a given pathogen, in this case *Fusarium*. Also, phenols may be present in plants but at such low concentrations that their inhibitory effect on the pathogen is negligible (Dixon and Paiva, 1995). In this study the levels of Si taken up by the potatoes in the presence of *F. oxysporum* might have been too low to increase production of phenols.

Generally the levels of chlorogenic acid were lower in the inoculated Si treatments compared to uninoculated Si treatments although we expected them to be slightly higher because this compound is produced by the plant as defence against invading pathogens. Perhaps the presence of *F. oxysporum* was, as mentioned earlier, too low to stimulate production of higher levels of chlorogenic acid. The fact that the concentration of caffeic acid was higher in tuber peels than that of chlorogenic acid was surprising. This could be explained by the fact that chlorogenic acid is the storage form of caffeic acid, which can be converted during stress conditions to caffeic acid (Ghanekar *et al.*,

1984). For lime, slag, fly ash and the control the levels of caffeic acid were higher for the inoculated treatment when compared to uninoculated. For the Si treatments perhaps it is an indication that the potato plant had increased production of caffeic acid in the presence of Si as a defense mechanism against *Fusarium*. The invasion by *Fusarium* resulted in higher levels of caffeic acid being produced as a result of stress.

Future research should focus on the effect of Si application on microbial flora in the soil and how this influences plant health. The tendency of potassium silicate applications *in vitro* to inhibit fungal growth with time highlights the importance of determining the intervals at which Si should be applied to the soil and growth media to suppress the growth of pathogenic *F. oxysporum*. Further studies could closely examine the relationships between available Si content in soil, Si within the plant, deposition of (insoluble) Si, enhancement of structural and biochemical defence responses, and the subsequent suppression of diseases (Dann and Muir, 2002). This is because Si did not have a direct detrimental effect on the germination and growth (Lee *et al.*, 2004) of *Fusarium* as this pathogen was re-isolated from the potato plants in Si amended soil. Artificial inoculation of potted plants may not be reliable enough in predicting the wilt response that would occur under natural conditions (Smith *et al.*, 2001). This study would also need to be repeated under natural field conditions to fully investigate the effect of Si on disease development and growth of the plant. Determining the amount of Si that potatoes can accumulate would be important for use in industry.

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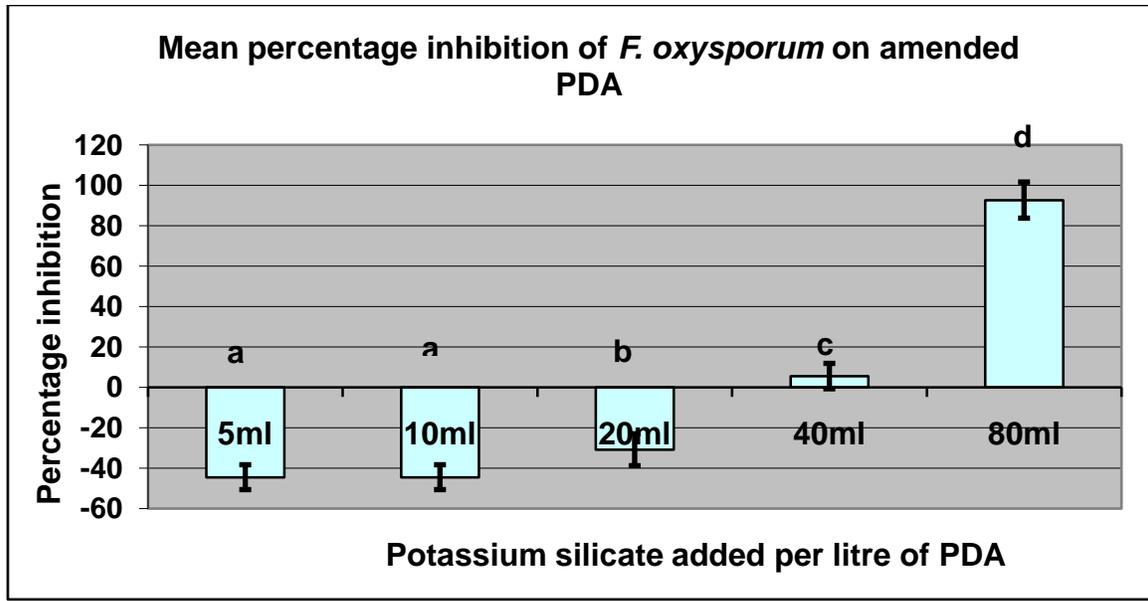


Fig. 4.1 Average percentage growth inhibition of different potassium silicate concentrations amended to PDA. Treatments with the same letters are not significantly different to each other. P value = 1%, LSD: 5.953

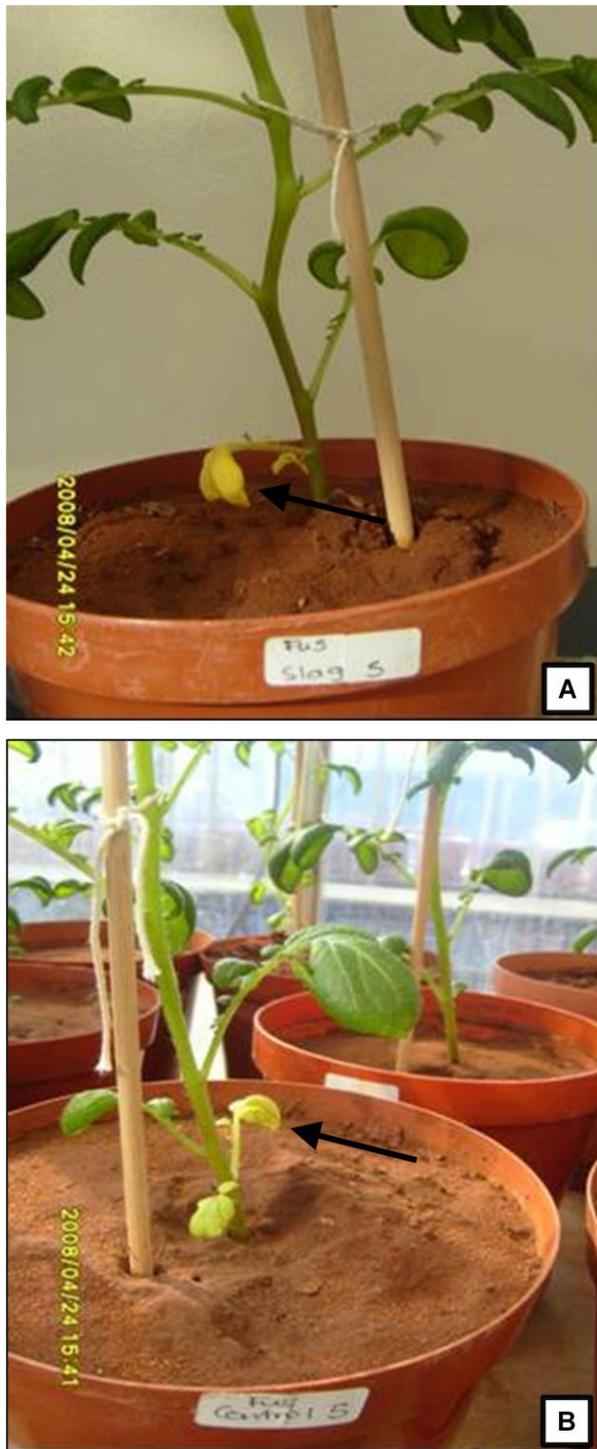


Fig. 4.2 Fusarium wilt symptoms on potato plants. (A) Soil inoculated with *Fusarium oxysporum* and amended with slag; (B) Unamended soil inoculated with *F. oxysporum*.

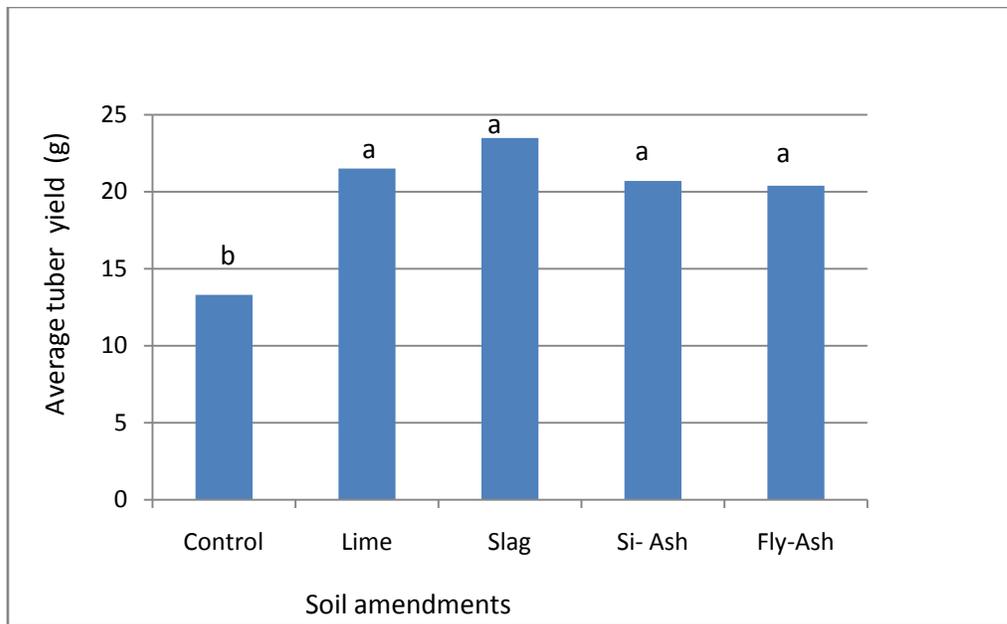


Fig. 4.3 The effect of silicon soil amendments on potato yield obtained in the preliminary pot trial. P value = 1%, LSD: 4.26

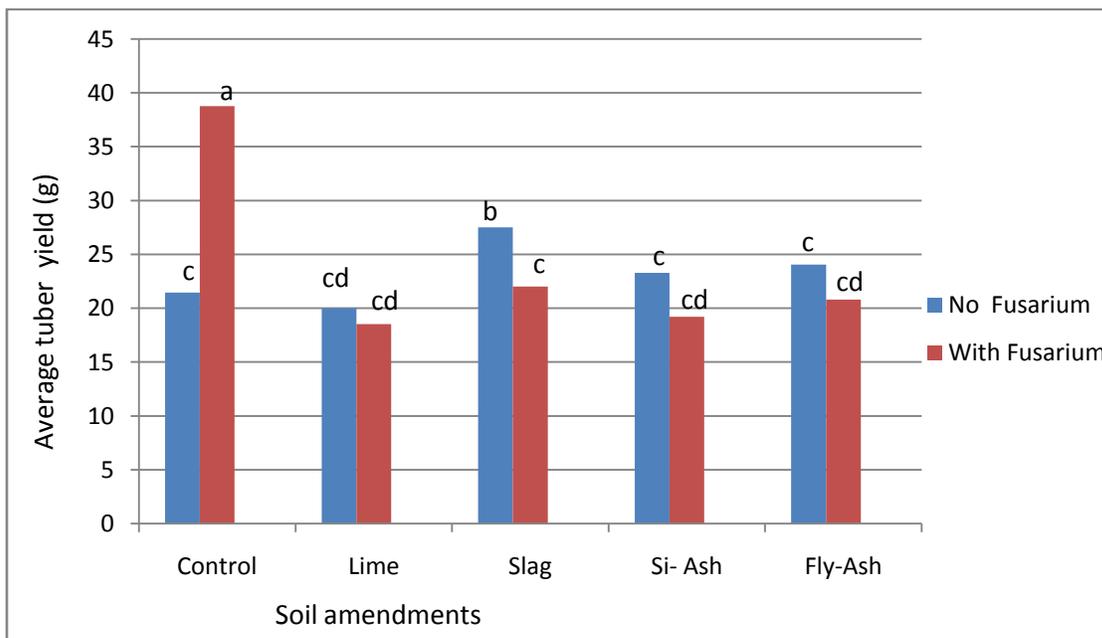


Fig. 4.4 Effect of silicon soil amendments on potato yield in pot trials II and III. P value = 5%, LSD: 4.18

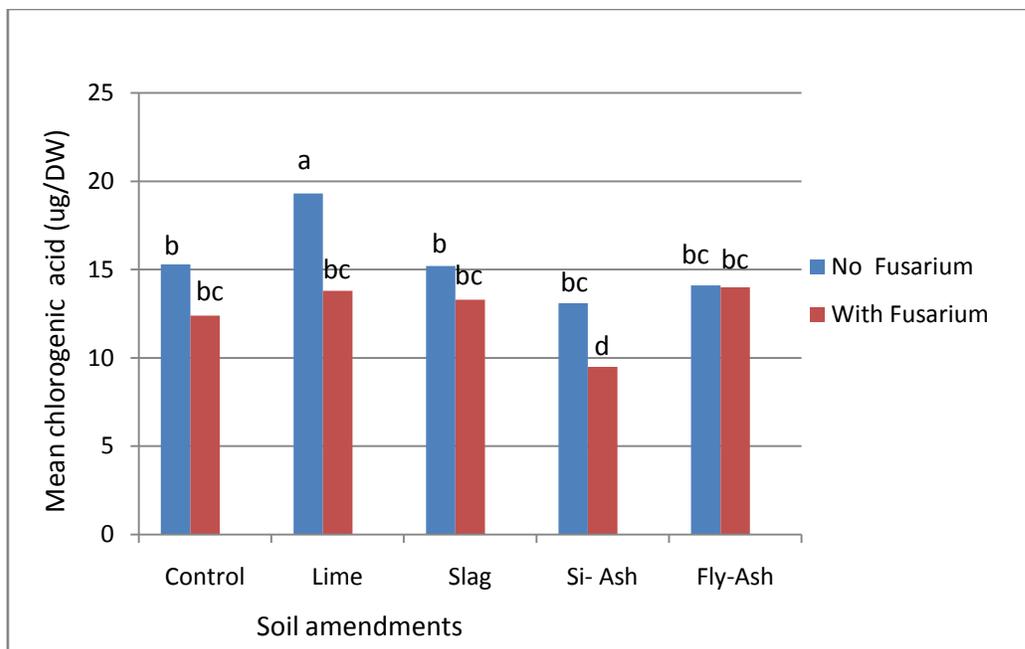


Fig. 4.5 Effect of silicon amended soil on the concentration of chlorogenic acid in tuber peel. P value = 1%, LSD: 4.10

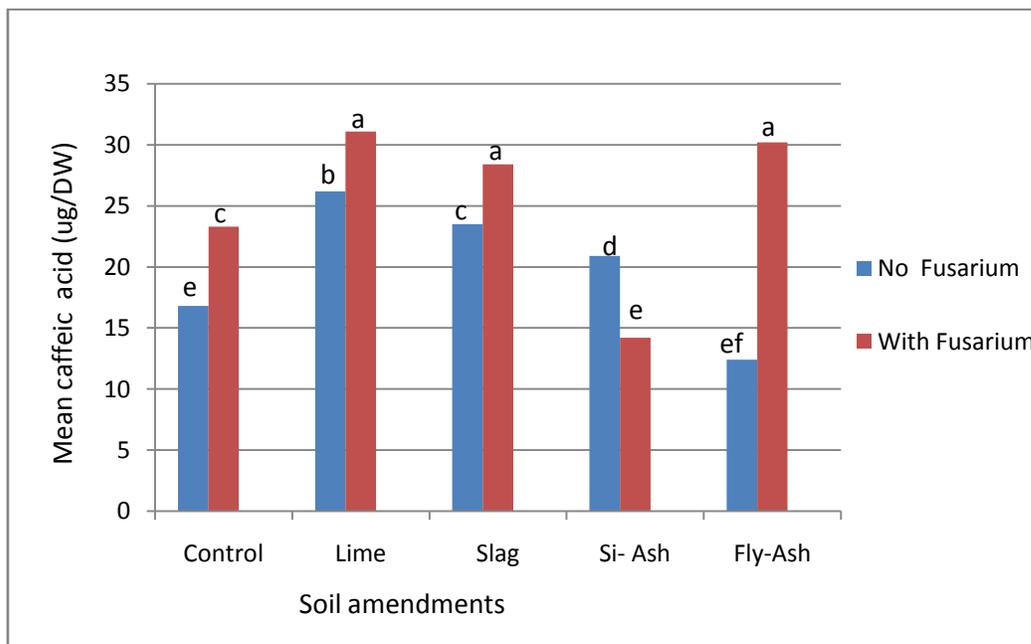


Fig. 4.6 Effect of silicon amended soil on the concentration of caffeic acid in tuber peels. P value = 1%, LSD: 2.76

Table 4.1 Mean colony diameters and percentage inhibition of *Fusarium oxysporum* on PDA at different pH values

	Control	pH 8.8	pH 9.6	pH 10.0	pH 10.4	pH 10.6	F Pr.
Colony diameter (mm)	64.95	77.35	78.95	78	77.5	75.55	<0.001
% Inhibition	1.1.1	-18.95	-19.01	20.68	-21.45	-30.64	

CHAPTER 5

GENERAL DISCUSSION

In this study the occurrence of *Fusarium* and its ability to cause Fusarium wilt on potatoes in SA was investigated. *Fusarium* is a diverse and widely distributed fungal genus and it can be isolated from agricultural soils and plant material. For the study, *Fusarium* was isolated from potato plants showing wilting symptoms. Two species of *Fusarium* were identified as causing Fusarium wilt on potatoes, namely *F. oxysporum* and *F. solani*. *F. oxysporum* was more prevalent and occurred in all the regions from where samples were collected.

Fusarium is a soilborne fungus and is difficult to eradicate because it produces chlamyospores that have prolonged persistence in the soil (Larena *et al.*, 2003); this results in difficulties in the management the disease. Fusarium wilt cannot be controlled by means of chemical fungicides (Borrero *et al.*, 2004) so cultural practices are often implemented. The use of a three year crop rotation system with non-host crops like maize and wheat will reduce disease levels in the soil, although it will not eliminate the pathogen. Avoiding the use of susceptible cultivars in areas where Fusarium wilt is a problem and the use of certified seed tubers (Denner *et al.*, 2003) helps to reduce disease incidence in the field. In the past, the management of the disease has focused on using resistant cultivars and fumigating the soil with methyl bromide (Larena *et al.*, 2003). Recently research has focused on methods to stimulate plant defence mechanisms and these include the use of microorganisms such as non-pathogenic strains of *F. oxysporum* (Panina *et al.*, 2007) and amendments of soil with elements such as Si (Fawe *et al.*, 1998).

The effect of Si on Fusarium wilt of potatoes was investigated in this study to assess its effectiveness in the control of Fusarium wilt. The *in vitro* results showed that Si can inhibit the growth of *Fusarium* only at high concentrations when using potassium silicate. Thus for this study, slag, fly ash and silicon ash were used as silicon sources. It is thus likely that the amount of Si accumulated by potatoes measured would be undetectable or very low because the Si was only applied once to the soil throughout the season. Hence, even if potatoes are able to accumulate Si, additional applications would be

needed for it to be detectable in plant tissues. This type of study may be difficult to replicate in the field.

A higher concentration of Si may be required for direct soil application where soil-borne pathogens such as *Fusarium* are found. Perhaps more silicon would be more available for the potato plant to take up and might also have a direct effect on the growth or germination of the *Fusarium* in the soil, which could result in a decrease in disease incidence. Once the optimum application rate of silicon needed by potatoes has been established, more potato cultivars would have to be tested to compare results, especially between tolerant, resistant and susceptible cultivars to Fusarium wilt. The uptake and accumulation of Si between the cultivars could also differ.

For the successful acceptance of Si as part of a Fusarium wilt management strategy on potatoes, further detailed studies are required in the following areas:

- Quantification of the amount of Si which is absorbed by the potato plant through the roots;
- Deposition of silicon in the potato plant when it is absorbed and whether it acts as a physical barrier and/or plays a role in being able to stimulate the natural defence mechanisms in potatoes;
- Information on which source of silicon gives the best results and how to apply it. The use of foliar applications versus slow release formulations in controlling soilborne diseases and
- Rate of applications that will be practical in the field because we are not well informed on how well Si is absorbed by potato plants in the field.

It is clear that *Fusarium* comprises a wide range of species, which can be sub-classified further into *formae speciales*. To date, in SA only *F. oxysporum* and *F. solani* have been identified as the major causes of Fusarium wilt on potatoes. *F. oxysporum*, which is more complex than *F. solani*, appears to be more prevalent in many areas. The genetic characterization of the isolated strains of *Fusarium* will give an indication of where certain strains occur with respect to the different potato growing regions in SA. These findings will elucidate whether certain areas have specific strains and whether this is related to environmental conditions, which play a role in disease development. The genetic relatedness or differences between the isolates will also give an indication of

whether the same strains that cause Fusarium wilt occur throughout the country or are confined only to certain regions.

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APPENDIX

Peptone PCNB Agar

Peptone	15g
KH ₂ PO ₄	1.0g
MgSO ₄	0.5g
PCNB (Pentachloronitrobenzene)	750mg
Agar	20g
H ₂ O	to 1L

Streptomycin is added as a stock solution of 5g of in 100ml distilled water and is used at the rate of 20ml/L of medium.