EFFECT OF INOCULUM SOURCE, ALTERNATIVE HOST AND CULTIVAR ON DEVELOPMENT OF BROWN SPOT AND BLACK PIT 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 (Agric) Plant Pathology has not previously been submitted by me in respect of a degree at any other University.

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Effect of inoculum source, alternative host and cultivar on development of brown spot and black pit of potatoes in South Africa

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Abstract

In recent years two new diseases, brown spot and black pit, have been observed on potatoes in South Africa. Brown spot symptoms appear on the foliage as small brown lesions, whereas black pit symptoms appear on the tubers as small dark sunken lesions.

In this study the causal organism of brown spot and black pit of potatoes in South Africa was determined. During initial isolation, one fungus and two bacteria were isolated, which were included in the trial. Only the treatments where the fungal isolate was used in inoculation resulted in the development of brown spot lesions. The causal organism of brown spot and black pit were identified as *Alternaria alternata* which is consistent with other research.

To better understand the spread of *Alternaria alternata* between the plants and/or tubers a pot trial was conducted. It was observed that when planting an inoculated seed tuber brown spot may develop on foliage. But the daughter tubers harvested from plants infected with *A. alternata* will not necessarily develop black pit. Daughter tubers are most likely infected by *A. alternata* during harvesting and black pit lesions develop in high humidity in storage.

Cultivar resistance is one of the most important measures in controlling plant diseases. Cultivar susceptibility of thirteen South African potato cultivars (Avalanche, Buffelspoort, BP1, Fabula, Fianna, Frodo, Hertha, Labadia, Lanorma, Mondial, Pentland Dell, Up-To-Date and Van Der Plank) was evaluated. Pot trials showed that all the evaluated cultivars are susceptible to infection by *Alternaria alternata.*

Various crops (tomatoes, cabbage, mustard, wheat, oats, tobacco and maize) were assessed to determine the host range of *Alternaria alternata* (potato pathotype) in rotation crops in South Africa potato growing regions. Of the crops evaluated, the pathogen was able to infect only tomato crops. Only wheat, maize and oats can safely be used in the crop rotation in South Africa, as various potato pathogens attack cabbage, mustard and tobacco plants.

This study will lead to a better understanding of brown spot and black pit diseases of potatoes in South Africa and globally. The study emphasise the need for further research which will help to reduce brown spot and black pit diseases of potato.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 BACKGROUND AND MOTIVATION OF THE STUDY

Potatoes (*Solanum tuberosum* L.) are one of the major food crops worldwide with more than 322 million tons of potatoes produced in 2012. In South Africa 1 322 774 tons of potatoes were produced during 2012 (Potatoes South Africa, 2012). There are sixteen regions in South Africa where potatoes are produced namely Ceres, Eastern Free State, Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Loskop Valley, Mpumalanga, North-Eastern Cape, Northern Cape, North-West, Sandveld, Southern Cape, South-Western Cape, South-Western Free State and Western Free State (Potatoes South Africa, 2012).

Potatoes are susceptible to a wide variety of diseases caused by fungal, bacterial and viral pathogens (Stevenson *et al.,* 2001). Two potato diseases that were previously of little interest in potato production in South Africa are brown spot and black pit of potatoes. In recent years these diseases caused a problem in potato production in South Africa (Van der Waals *et al.,* 2011). The causal agent of brown spot and black pit is *Alternaria alternata* (Fr.) Keissler. *A. alternata* infects both the foliage (brown spot) and tubers (black pit) of the potatoes (Stevenson *et al.,* 2001). The diseases were first described in 1984 by Droby *et al*. (1984a; 1984b). Since then little research has been done on these diseases.

For this reason a study was conducted to better understand these diseases of potatoes.

1.2 FUNDAMENTAL OBJECTIVES

The aim of this study was to investigate if *A. alternata* causes brown spot and black pit diseases in South Africa, and to determine cultivar susceptibility to these diseases, to determine if other crops may be susceptible to the causal agent of brown spot and black pit of potatoes and to determine how the pathogen spreads between plants.

1.3 SPECIFIC OBJECTIVES

The objective of the study was to determine:

- The causal organism of brown spot and black pit in South Africa.
- The susceptibility of potato cultivars to brown spot and black pit diseases.

- The spread of the brown spot and black pit pathogen between plants.
- If any other crops (tomatoes, cabbage, mustard, wheat, oats, tobacco and maize) are susceptible to the causal agent of brown spot and black pit of potatoes.

1.4 CHAPTER OUTLINE

Chapter 2 The literature review focuses on the causal organisms, symptoms, disease occurrence and control of brown spot and black pit of potatoes.

Chapter 3 The causal organism of brown spot and black pit was determined according to Koch's Postulates. The causal organism was identified by sequencing.

Chapter 4 It was determined how brown spot and black pit pathogen may possibly spread between potato plants, tubers and vice versa. The treatments of the pot trial where the spread of the pathogen between plants was determined were: (i) inoculated tubers, sterilised soil, uninoculated plants; (ii) uninoculated tubers, inoculated soil covered, uninoculated plants; (iii) uninoculated tubers, inoculated soil uncovered, uninoculated plants; (iv) uninoculated tubers, sterilised soil, inoculated plants; (v) uninoculated tubers, sterilised soil, uninoculated plants. The treatments of the *in vitro* trial where the spread of the pathogen between tubers was determined were: (i) inoculated tubers, incubated; (ii) uninoculated tubers, incubated with inoculum; (iii) uninoculated tubers, incubated.

Chapter 5 The susceptibility of potato cultivars to brown spot and black pit of potatoes were determined. The cultivars were: Avalanche, Buffelspoort, BP1, Fabula, Fiana, Frodo, Hertha, Labadia, Lanorma, Mondial, Pentland Dell, Up-To-Date and Van Der Plank.

Chapter 6 It was determined if any crops used in crop rotation practices with potatoes are an alternative hosts for the causal organism of brown spot and black pit. The crops inoculated were: tomatoes, cabbage, mustard, wheat, oats, tobacco and maize.

Chapter 7 General discussion and final conclusion which includes suggestions for future research.

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CHAPTER 2
LITERATURE REVIEW OF BROWN SPOT AND BLACK PIT OF POTATO

2.1 INTRODUCTION

The potato (*Solanum tuberosum* L) crop is ranked fourth in the global production of food crops (Luck *et al.,* 2011) and in 2011 the total global potato production was 374 million tons, with South Africa contributing 2.1 million tons (FAOSTAT, 2011).

In South Africa potatoes are produced in sixteen different growing regions namely Ceres, Eastern Free State, Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Loskop Valley, Mpumalanga, North-Eastern Cape, Northern Cape, North-West, Sandveld, Southern Cape, South-Western Cape, South-Western Free State, Western Free State (Potatoes South Africa, 2012).

There are a number of diseases affecting potato crops worldwide. The causal agents of these diseases include fungi, bacteria, viruses and nematodes. Not only do pathogens produce disease on potato plants, but so too can environmental factors play an important role in the health of the plants (Stevenson *et al.,* 2001).

Two of these potato diseases caused by fungi are black pit and brown spot of potatoes. These diseases are caused by the same pathogen, *Alternaria alternata* (Fr.) Keissler (Rotem, 1994). Although relatively little is known about these diseases, they are known to occur in Brazil, China, Israel, South Africa and USA (Droby *et al.,* 1984a; Boiteux & Reifschneider, 1994; Kirk *et al.*, 2007; van der Waals *et al.*, 2011, Zheng & Wu, 2013). Zheng & Wu (2013) however reported that the disease complex is caused by *Alternaria tenuissima* (Kunze:Fr.) Wiltshire in China. Potato brown spot is a foliar disease of potato while black pit lesions mostly develop in storage on potato tubers (Stevenson *et al.,* 2001).

2.2 CAUSAL ORGANISM

The causal organism of brown spot and black pit of potatoes was first described in Israel in 1984, as *A. alternata* (Droby *et al.,* 1984a). The genus *Alternaria* was established in 1817 with *A. alternata* (*Alternaria tenuis* Nees) as the type strain. Due to the lack of a sexual stage of most isolates the genus was classified into the phylum Fungi Imperfecti (Agrios, 1997; Tomma, 2003). Today the following classification for *Alternaria* spp. is used:

Pleosporaceae, Pleosporales, Pleosporomycetidae, Dothideomycetes, Pezizomycotina, Ascomycota, Fungi; Deuteromycota, Hyphomycetes, Porosporae (Ellis, 1971; Rotem, 1994).

The conidia of *A. alternata* are catenate, ovoid or obclavate and sometimes rostrate, multi-celled and brown in colour (Ellis, 1971). The majority of the conidia have 1-7 transverse septa, 1-2 longitudinal septa and are 26.7-28.0 x 10.8µm in size (Droby *et al.,* 1984a).

Culture characteristics such as conidial size and septation of *A. alternata* are highly variable (Simmons & Roberts, 1993). In culture the aerial mycelium of *A. alternata* appears fluffy and is lighter in colour than *Alternaria solani* Sorauer (Ellis). *A. alternata* appears to have a vast amount of different mycelium colours that are dependent on the specific isolate (Petrunak & Christ, 1992).

In *Alternaria alternata* f. sp. *spenicleae* cultures, Masangkay *et al.* (2000) found that the sporulation of the cultures was influenced by nutrition, temperature, light and moisture and that the greatest number and most virulent conidia were produced on half strength Potato Dextrose Agar (PDA) at 28°C. Culture media can also influence the conidial morphology of *Alternaria* spp.. Misaghi *et al*. (1978) noted that conidia isolated from stem cancers on tomato (*Lycopersicon esculentum* L.) plants were larger and more uniform in size and morphology than conidia produced from isolates grown on culture media.

Von Ramm and Lucas (1963) reported that *A. alternata* that cause disease on tobacco (*Nicotiana tabacum* L.) plants, can easily become avirulent and develop non-sporulation sectors under certain conditions *in vitro*. Pathogenicity of the cultures furthermore declines with repeated sub-culturing onto PDA (Lloyd, 1972).

2.3 SYMPTOMS AND SIGNS

Alternaria spp. is the single most widespread plant pathogenic fungal genus in the world. There are more than 4000 Alternaria-host associations recorded in the USDA Fungal Host Index, with some *Alternaria* spp. causing disease on a wider host range than others (Rotem, 1994; Lawrence *et al.,* 2008).

Symptoms caused by *Alternaria* spp. on plants include leaf spots, blotches, and blights (Yu, 1992; Thomma, 2003). *A. solani* and *A. alternata* cause lesions on the leaves and tubers of potatoes. *A. solani* is associated with early blight of potatoes, while *A. alternata* causes

brown spot and black pit. Brown spot refers to the symptoms on the leaves, and black pit the symptoms on the tubers (Droby *et al.,* 1984a; 1984b; Stevenson *et al.,* 2001).

Brown spot lesions (Figure 2.1) are small, brown lesions on leaves and stems and when occurring on the leaves, initially develop on the abaxial side of the leaf. The lesions may coalesce to form larger lesions, and severely infected leaves can die and drop off prematurely. Black pit symptoms on the tubers appear as sunken dark lesions (Figure 2.2) with defined borders, the underlying tissue is leathery to corky in texture. The symptoms usually fully develop in storage (Droby *et al.,* 1984b; Stevenson *et al.,* 2001, Kirk & Wharton, 2012).

Figure 2.1 Brown spot lesions on a potato leaf.

Figure 2.2 Black pit lesions on a potato tuber.

2.4 DAMAGE AND ECONOMIC IMPACT

Alternaria spp. have a large host range and thus cause a large combined economical loss throughout the world (Munuera *et al.*, 2001). Pathogens that cause disease on leaves influence the photosynthetic ability of the plants, which results in yield loss. Younger leaves play a greater role in photosynthesis than older leaves (Rotem, 1994). In the field *A. alternata* can cause disease on the potato leaves as early as 58 days after emergence; however the disease incidence at this stage is still low. At 65 days the disease incidence will have more than doubled compared to that at 58 days, and leaves may drop prematurely (Droby *et al.,* 1984a; Stevenson *et al.,* 2001). As *A. alternata* causes symptoms on young leaves, it has the ability to cause major yield loss in potato fields.

Brown spot of potatoes reduces the photosynthetic leaf area resulting in an imbalance between nutrient demand in the tubers and the nutrients supplied by the leaves, ultimately leading in a reduced yield (Simmons, 2000).

2.5 DISEASE OCCURRENCE

A. alternata causes similar infection patterns in different hosts. In order for a plant pathogen to infect a host plant, the correct environmental conditions need to be present (Agrios, 1997). *A. alternata*, like most *Alternaria* spp. are highly resistant to adverse weather conditions and disease can develop in a wide range of temperatures, with the general optimal, minimum and maximum temperatures being 22.5˚C, 10˚C and 35˚C respectively (Rotem, 1994).

2.5.1 Disease cycle

Temperature, relative humidity (RH) and moisture play the biggest role in host infection by *Alternaria* spp. (Rotem, 1994). Brown spot of potatoes depends mostly on moisture (fog, dew, rain, irrigation), and leaf nutrition (Stevenson *et al*., 2001). Infection of tomato plants by *A. solani* is affected by inoculum concentration, leaf wetness duration, plant age and host susceptibility (Vloutoglou & Kalogerakis, 2000).

Conidiophores of *A. alternata* develop in a wide range of temperatures with the maximum and minimum temperatures being 33˚C and 5˚C respectively, and the optimal temperature being 27˚C (Rotem, 1994). Temperature and humidity also influence the production of conidia (Rotem, 1994; Timmer *et al.*, 1998). On potato plants and debris *A. alternata* spores are produced between 4°C to 30°C (Kirk & Wharton, 2012). There are multiple *A. alternata* spore production cycles in a single growing season, resulting in secondary spread in the brown spot disease cycle (Kirk & Wharton, 2012).

Wind transfer is the most important dissemination method of *A. solani, Alternaria brassicicola* (Schwein.), Wiltshire, and *A. alternata* (Rands, 1917; Rotem, 1994; Chen *et al.,* 2003). Due to their large bodies, the spores float in the wind and get trapped in high atmosphere, travelling great distances (Rotem, 1994). *Alternaria* spp. spores are passively removed from the lesions, as they do not actively release their spores into the atmosphere (Rotem, 1994). Wind and humidity are the main agents in releasing the spores from the infected leaves (Aylor, 1978). According to Leach (1975) the spores of *A. alternata* are actively propelled into the air by drying, followed by a rapid increase in humidity and exposure to infrared light.

Alternaria spp. conidia that land on host plants where the optimal environmental conditions are present will germinate and develop germ tubes within a few hours. The germ tube will penetrate the host plant via wounds, cuticles, stomata and directly through cells (Rotem,

1994). In potato and citrus (*Citrus* spp. L.), *A. solani* and *Alternaria* spp. spores penetrate the leaves directly or through the stomata to cause disease (Solel & Kimchi, 1998).

Temperature and relative humidity influence germination of *Alternaria* spp. conidia (Rotem, 1994). The infection temperatures in most species range between 10˚C and 40˚C. The optimal temperature for infection of potatoes by *A. solani* is 25°C in wet conditions - either rain or dew (Zachmann, 1982). *A. alternata* spores require a temperature between 20°C and 30°C to germinate and infect the plant tissue. A minimum wetting period and relative humidity are needed for the pathogen to successfully establish in the host plant. Different *Alternaria* spp. will require unique relative humidity percentages and wetting periods (Rotem, 1994; Solel & Kimchi, 1998).

Brown spot lesions develop firstly on the leaves near the soil surface (Soleimani & Kirk, 2012). Early blight lesions develop 2½ to 3 days after potato plants have been inoculated with *A. solani* (Spletzer & Enyedi, 1999; Schaefer *et al.,* 2005). In citrus Alternaria brown spot, caused by *A. alternata*, black necrotic lesions appear on the leaves 2 to 3 days after infection (Timmer *et al.,* 2003). Brown spot lesions on potato foliage develop 2 to 3 days after initial infection (Kirk & Wharton, 2012).

A. solani can penetrate the potato tuber through wounds. Early experiments even suggested that *A. solani* can penetrate the potato skin directly or through the lenticels (Folsom & Bonde, 1925; Gratz & Bonde, 1927). *A. alternata* penetrates the tubers through the lenticels and injured skin (Stevenson *et al.,* 2001). As with *A. solani*, the tubers also become contaminated with *A. alternata* during harvesting (Kirk & Wharton, 2012).

2.5.2 Survival of *Alternaria* species

Species survival depends on the ability of the pathogens to survive in unfavourable environmental conditions; this ability is inherited and also forms with mutations (Rotem, 1994).

Alternaria spp. have no known specially developed overwintering stage (Rotem, 1994). *Alternaria dauci* (Kühn) overwinters in plant debris, volunteer hosts and infected seeds whereas *Alternaria brassicae* (Berk.) Sacc overwinters in plant debris, infected seeds and in soil (Ansari *et al.,* 1989; Pryor *et al.,* 2002). The brown spot pathogen, *A. alternata*, overwinters in plant debris (Kirk & Wharton, 2012).

A. alternata conidia are exposed to UV radiation that can affect their survival (Rotem *et al.,* 1985; Rotem, 1994). The conidia are thus adapted to withstand the effects of radiation. Adapting methods to tolerate radiation include melanin pigments and multi-celled structures (Rotem, 1994).

2.5.3 Other factors involved in disease occurrence

Susceptibility of plants to *Alternaria* spp. is generally increased when the plant becomes aged, or when the plant is experiencing stress (Rotem, 1994; Agrios, 1997). Wounding of plants also increases the infection by *Alternaria* spp.. Weaker *Alternaria* spp., which include *A. alternata*, require wounds or natural openings in order to infect the host plant (Rotem, 1994). In onions (*Allium cepa* L.) pre-wounding is essential for infection by *A. alternata* and *A. tenuissima* (Skiles, 1953). In potato tubers wounding is necessary for *A. alternata* and *A. solani* to cause disease (Droby *et al.,* 1984b; Stevenson *et al.,* 2001).

2.6 DISEASE CONTROL

The main aspect in controlling potato diseases is the reduction of inoculum in the field. This includes a crop rotation system of 3 to 5 years with non-host plants, removing of any contaminated plant debris and planting disease-free tubers (Shuman, 1995; Agrios, 1997; Westcott, 2001).

In order to control fungal diseases sufficiently, chemical control is needed (De Waard *et al.*, 1993). To control brown spot effectively the application of foliar fungicides is essential (Kirk & Wharton, 2012). Maneb, mancozeb, chlorothalonil, triphenyltin hydroxide, fluopyram and a mixture of fluopyram and pyrimethanil are effective against both early blight and brown spot. Fungicides: famoxadone, pyrimethanil, fenamidone and boscalid seem to be effective against brown spot (Kirk & Wharton, 2012). The potato plants should be sprayed with the fungicides as soon as the first signs of brown spot are observed (Kirk & Wharton, 2012; Fairchild *et al.,* 2013).

A combination of fungicide-resistant plant pathogens and public concern about fungicide residues in the environment has led to the investigation of other methods to control plant diseases (Ishii, 2006). Methanol extracts from eucalyptus, peppermint and lavender inhibited the mycelial growth and spore germination of *A. alternata* (isolated form potato) *in vitro* (Zaker & Mosallanejad, 2012). Soleimani and Kirk (2012) found that by applying commercially available resistance inducers, ASM and Chitosan as foliar sprays, brown spot disease on potatoes can be reduced. Foliar fertilizers: Basfoliar 12-4-6, ADOB Mn and

Solubor DF, have shown to significantly inhibit the growth of *A. alternata* (isolated from potato) *in vitro* (Cwalina-Ambroziak, 2012).

Alternara alternata infects the tubers mainly at harvesting (Soleimani & Kirk, 2012). *A. alternata,* like *A. solani* needs a wound or lenticel as entry point in order to cause disease on potato tubers (Droby *et al.,* 1984b; Stevenson *et al.,* 2001). By allowing the tubers to mature before harvesting and minimizing mechanical damage at harvesting, the infection of potato tubers can be limited (Shuman, 1995). A method of controlling black pit post-harvest is necessary to effectively control the disease. Mills *et al.* (2004) evaluated the effect of copper sulphate, mancozeb, organic – and inorganic salt compounds on potato postharvest pathogens, including *A. alternata*. They showed that sodium metabisulfite and propyl-paraben inhibit the mycelium growth and sporulation *in vitro* and aluminum acetate, alum, copper sulphate and mancozeb inhibits spore germination *in vitro*.

Research on postharvest disease control of other potato diseases may be effective against black pit of potatoes (Droby *et al.,* 1984b). Postharvest control of potato diseases like silver scurf (*Helminthosporium solani* Durieu & Mont.), dry rot (*Fusarium* spp.) and soft rot ((*Pectobacterium spp.*) (van Hall) Dye) has been achieved by altering the controlled atmosphere in storage with chlorine and treating potato tubers before storage with inorganic and organic salts (Yaganza *et al*., 2001; Hervieux *et al*., 2002; Mecteau *et al*., 2002; Tweddell *et al.,* 2003). It was also found that by dipping tubers into sodium benzoate and potassium sorbate, soft rot and silver scurf were reduced during storage (Yaganza *et al*., 2001; Hervieux *et al*., 2002). However Yaganza *et al.* (2003) stated that aluminium- and sulphite-containing compounds may have certain health concerns for humans when used to treat potato tubers and that more research needs to be done regarding the effect of aluminium-and sulphite-containing compound on human health. Postharvest control of mango fruit (*Mangifera indica* L.) infected by *A. alternata* includes the dipping of the mango fruit in chlorine, which reduces the disease incidence of Alternaria rot of mango in storage. Black spot of persimmons (*Diospyros kaki* L.) is reduced by altering CO_2 levels in storage (Prusky *et al.,* 1997; 2002).

Resistant cultivars are an important aspect in controlling plant diseases (Agrios, 1997). Droby *et al*. (1984a) determined that potato cultivars Blanka, Desiree and Spunta are most susceptible to brown spot, where cultivar Cardinal had medium susceptibility, and Up-To-Date least susceptibility to brown spot. Brown spot disease severity seems to be similar between early, medium and late maturing cultivars, where black pit disease seems to be

higher in medium to late maturing cultivars (Kapsa & Osowski, 2011). In Table 2.1, potato cultivar susceptibility to brown spot and black pit can be seen (Kapsa & Osowski 2011).

Table 2.1 Potato cultivar susceptibility to brown spot and black pit (Kapsa & Osowski 2011).

Very Early & Early Cultivars			**Medium Early Cultivars**			**Medium Late & Late Cultivars**		
Cultivar	***Alternaria alternata***		**Cultivar**	***Alternaria alternata***		**Cultivar**	***Alternaria alternata***	
	Brown spot	**Black pit**		**Brown spot**	**Black pit**		**Brown spot**	**Black pit**
Augusta	+++	+++	Albatros	+++	++	Danusia	+	+++
Bard	+	+++	Andromeda	+	++	Fiana	+	+++
Delikat	++	+++	Asterix	+	+++	Neptun	+++	++
Dorota	+++	++	Clarissa	+++	+++	Pasja	+++	+++
Felka	+	+++	Cycloon	++	+++	Rudawa	+++	+++
Gabi	+++	+++	Monsen	+++	+++	Saturna	+	+++
Gracja	+++	+++	Pasat	++	+++	Skawa	+	+
Innowator	+	+++	Romula	+	+++	Sonda	++	+++
Korona	+	+++	Sarina	++	+++	Syrena	+++	+++
Lady Clare	+	+++	Victoria	++	+++	Ślęza	++	++
Lord	++	+++	Zebra	+++	+++	Umiak	++	+++
Molli	+	+++						
Rosalind	+	+++						
Vitara	++	+++						

	Key	Brown spot	Black pit
Resistant	+++	< 11% disease severity	< 8% disease severity
Medium sensitive	++	11 – 13% disease severity	8 – 12% disease severity
Sensitive	+	> 13% disease severity	> 12% disease severity

Insects are able to spread pathogens between plants. Insects can carry plant pathogens internally or externally and transfer the pathogens to the host plants (Agrios, 1997). Leafminers (*Lyriomyza trifolii* Burgess) that causes injury to potato crops increased the number of entry points for *A. alternata*, thus increasing brown spot severity on potato crops (Deadman *et al.,* 2002). Nine point fifty three percent of the white grubs (*Brahmina coriacea* Hope) found in potato fields in North West Indian hills were infected with *A. alternata*. White grubs cause injury on potato crops and may thus be able to transfer *A. alternata* to the potato plants (Sharma *et al.,* 2012). Controlling insect damage to the cops may reduce brown spot disease severity (Deadman *et al.,* 2002).

A range of information is available regarding the control of *A. alternata* affecting other crops. Brown spot severity of tobacco plants was reduced by 65% by applying non-pathogenic

Alternaria spp. to tobacco leaves a few days before infection with pathogenic *A. alternata* (Harvey & Spurr, 1977). Various studies have been done on controlling postharvest pathogens of tomato plants with essential oils. The studies showed that cassia (*Cassia fistula* L.), thyme (*Thymus* spp. L.), dill (*Anethum graveolens* L.) and ajowan (*Trachyspermum ammi* Sprague) extracts had antifungal properties against *A. alternata* isolated from tomato plants (Feng & Zheng, 2007; Abdolahi *et al.,* 2010; Tian *et al.,* 2011). In a study done by Carvalho *et al.* (2011), plant extracts from *Anadenanthera colubrine* (Vell.) Brenan reduced the development of *A. alternata* (causal agent of brown spot of tangerines (*Citrus tangerine* Tanakah)) to levels similar to those achieved by commercial fungicides. In combination with essential oils, salts such as KCl and NaCl can be used to improve the antifungal activity of essential oils, and thus lowering the amount of essential oils needed to control post-harvest diseases (Feng & Zheng, 2006).

Biological control refers to the use of microbial antagonists to suppress pathogens (Heydari & Pessarakli, 2010). Studies have been done on controlling different *Alternaria* spp. with biological agents. In a study done by Pastor *et al.* (2012) *Pseudomonas fluorescens* (Flügge) Migula strain PC12 not only showed potential in inhibiting *Alternaria alternata* f. sp. *lycopersici in vitro*, but also enhanced the root growth of the tomato plants. A few strains of *Bacillus amyloliquefaciens* (ex Fukumoto) Priest have been tested against *Alternaria* spp. isolates as possible biological control agents. The effect of *B. amyloliquefaciens* on *Alternaria* spp. includes the reduction of the infection rate and reduction in disease severity (Chen & Wu, 1999; Wu *et al.,* 2007).

2.7 SUMMARY

A. alternata causes brown spot and black pit diseases of potatoes in Brazil, USA, Israel, China and South Africa. *A. alternata* has the potential to cause major yield losses as it infects young leaves and tubers. Limited research has been done on these diseases. It is necessary to conduct more research on brown spot and black pit of potatoes to better the understanding and control of these diseases.

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CHAPTER 3

IDENTIFICATION OF THE CAUSAL AGENT OF BROWN SPOT AND BLACK PIT ON POTATOES IN SOUTH AFRICA

ABSTRACT

In recent years two new diseases, brown spot and black pit, have been observed on potatoes in South Africa. Brown spot symptoms appear on the foliage as small brown lesions, whereas black pit symptoms appear on the tubers as small dark sunken lesions. Trials were conducted to determine the causal organism of brown spot and black pit of potatoes in South Africa. *Alternaria alternata* was identified as the causal organism of both diseases.

3.1 INTRODUCTION

Fungi, bacteria and viruses cause diseases on potatoes. Some of the major potato (*Solanum tuberosum* L.) diseases include: soft rot, early blight and late blight (Tek *et al.,* 2004). *Alternaria* spp. cause the diseases early blight, brown spot and black pit of potatoes. Early blight is caused by *Alternaria solani* Sorauer (Ellis), while *Alternaria alternata* (Fr.) Keissler is the causal organism of brown spot and black pit (Stevenson *et al.,* 2001).

In the first quarter of 2013 new diseases on plants caused by *A. alternata* were reported, which included *A. alternata* causing disease on Japanese pepper (*Zanthoxylum piperitum* L.) in China (Yang *et al.,* 2013), leaf spot of bananas (*Musa* spp. L.) in the USA (Parkunan *et al.,* 2013), postharvest black spot of date palm (*Phoenix dactylifera* L.) fruit in Spain (Palou *et al.,* 2013) and leaf spot in *Withania coagulans* Dunal in India (Sharma *et al.,* 2013).

Brown spot caused by *A. alternata* has been reported in Israel, Brazil, USA and South Africa* (Droby *et al.,* 1984a; Boiteux & Reifschneider, 1994; Kirk, 2007; van der Waals *et al.*, 2011, respectively). In 2013 *Alternaria* leaf blight of potatoes caused by *Alternaria tenuissima* (Kunze:Fr.) Wiltshire was reported in China (Zheng & Wu, 2013). Black pit on tubers caused by *A. alternata* has only been reported in Israel (Droby *et al.,* 1984b).

Brown spot lesions are small, brown lesions on the leaves and stems of the potato plant. The lesions on the leaves initially develop on the abaxial side of the leaf, as the lesions mature they may coalesce. In severe brown spot infections the leaves die and drop prematurely. Black pit symptoms on the tubers typically develop in storage as sunken dark lesions with defined borders (Droby *et al.,* 1984a; 1984b; Stevenson *et al.,* 2001).

* Note: Some of the findings in this study were included in the article: Van der Waals, J.E., Pitsi, B.E., Marais, C., Wairuri, C.K., 2011. First report of *Alternaria alternata* causing leaf blight of potatoes in South Africa. *Plant Disease* 95(3):363

In the past few years brown spot and black pit potato lesions have been observed on potatoes in South Africa. The aim of this study was to determine if the symptoms observed were those of brown spot and black pit disease, if the causal organism of these diseases is *A. alternata,* and if both symptoms are caused by the same strain of *A. alternata*.

3.2 MATERIALS AND METHODS

3.2.1 Fungal isolation

Potato leaves with brown spot symptoms and potato tubers with black pit symptoms were received from across South Africa. The lesions were surface sterilized with 2% NaOCl solution for one minute and rinsed twice in sterile distilled water. The lesions were excised from the leaf and placed on half strength Potato Dextrose Agar (PDA) and Nutrient Agar (NA) and incubated at 25˚C for 7 days in the dark. After fungal growth occurred pure fungal cultures was re-isolated onto half strength PDA and labelled (Table 3.1). Yellow and white bacteria were also isolated from the first brown spot lesions (origin Mpumalanga) in the same manner as described above.

Table 3.1 *Alternaria alternata* brown spot and black pit cultures isolated from potatoes in South Africa.

Isolate number	Province	Year isolated	Host	Isolated from
AA1	Free State	2010	Potato	Tubers
AA2	Western Cape	2012	Potato	Tubers
AA3	Mpumalanga	2012	Potato	Tubers
AA4	Mpumalanga	2010	Potato	Tubers
AA5	Limpopo	2010	Potato	Tubers
AA6	Eastern Cape	2011	Potato	Tubers
AA7	Gauteng	2011	Beans	Leaves
AA8	KwaZulu-Natal	2012	Potato	Leaves
AA9	Mpumalanga	2012	Potato	Leaves
AA11	Free State	2012	Potato	Leaves
AA12	Free State	2012	Potato	Leaves
AA13	Western Cape	2012	Potato	Tubers
AA15	KwaZulu-Natal	2011	Potato	Tubers
AA16	Mpumalanga	2010	Potato	Leaves
AA17	Free State	2012	Potato	Leaves
AA18	KwaZulu-Natal	2012	Potato	Leaves
AA20	Northern Cape	2012	Potato	Tubers

3.2.2 Identification

The first *Alternaria* sp. cultures isolated from diseased potato leaves and tubers were sent to the Biosystematic Division Plant Protection Research Institute, South Africa for identification and were identified as *Alternaria alternata*.

Identification of the fungi by sequencing

All isolates causing brown spot and black pit obtained from samples across South Africa were identified by sequencing. The fungal DNA was extracted from pure fungal cultures using the ZR Soil Microbe DNA MiniPrep Kit (Zymo Research). A 1µL aliquot of this DNA solution was used for PCR, which was performed in 25µL reactions. The ITS region of all the isolates listed in Table 3.1 was amplified by PCR using primers ITS 5 (5′ to 3′ GGAAGTAAAAGTAACAAGG) and ITS 3 (5′ to 3′TCCTCCGCTTATTGATATGC) (White *et al.,* 1990). The amplification was performed in 25µL reactions, containing 0.1µL Taq polymerase, 10µL Taq buffer, 1µL of each primer, 1µL10-100ng DNA. The PCR was performed in a Bio Rad MJ Mini Personal Thermal Cycler, Model PTC 1148. The thermocycler conditions were 94°C for 10min, followed by 40 cycles of 60°C for 60s, 72°C for 60s, and a final extension step of 72°C for 10min. 5µL of amplified product was subjected to electrophoresis through a 1% agarose gel in 1 x TBE Buffer at 100V. The remainder of the amplified product was cleaned with the DNA Clean & Concentrator™ -5 kit from Zymo Research.

The cleaned amplified product was amplified in preparation for sequencing. The amplification was performed in 10µL reactions, containing 2µL Terminal Big Dye, 1µL Buffer, 1µL of only one primer, 1µL amplified product. The PCR was performed in a Bio Rad MJ Mini Personal Thermal Cycler, Model PTC 1148. The thermocycler conditions were the same as described above. The amplified product was cleaned by adding ice-cold 10µL Sabax sH_2O, 2µL 3M Sodium acetate and 50µL 100% ethanol. The solution was vortexed and incubated on ice for 10min where after the solution was centrifuged at 13000rpm for 30min at 4°C. The supernatant was discarded and 250µL of 70% ethanol was added to the pellet and centrifuged again for 5min at 13000rpm at 4°C. The supernatant was discarded and left overnight to dry. The products were sent to the DNA Sequencing Facility, University of Pretoria, where the products were precipitated and sequenced on an ABI 3100 PRISM TM Genetic Analyzer. The DNA sequences obtained were visually edited in BioEdit version 7.1.11 (Hall, 2013) and compared with the NCBI database using the BLASTN procedure (Altschul *et al.,* 1990). Additional comparative analysis of DNA sequences were done using online data from international reference fungal strains retrieved from MYCOBANK (Robert *et al.,* 2005).

Microbiological test on bacteria

Microbiological tests were conducted on the bacteria in order to aid in identification. The microbiological tests conducted were: Gram staining, Oxidative-Fermentative Test (O/F test), growth on NA, Murashige and Skoog medium supplemented with 2 mg/l 2,4-D and 3% sucrose solidified with 0.6% agarose (MSP), Crystal violet polypectate medium (CVP), Yeast extract-dextrose-$CaCO_3$ medium, (YDC) and Tween medium, Nitrate reduction reaction, Voges Proskauer test, Indole test, Methyl red test, and growth on various sugars as described in Schaad *et al.* (2001).

3.2.3 Inoculation trials

Brown spot

To fulfil Koch's postulates a pot trial was conducted in order to determine the causal organism(s) of brown spot. Twenty disease-free BP1 G0 seed tubers (origin Mpumalanga province) were planted separately in pots containing 4kg steam sterilised red top soil. The physical characterisitcs of the soil include a pH of 4.2, 74.8% coarse sand, 17.5% clay and 7.5% silt. For more information see Appendix C and D. High humidity was obtained by placing the pots in polyethylene bags for the duration of the trial. The plants were watered every second day. To induce stress conditions no fertilizer was added. The greenhouse temperature was kept under 30°C. The plants were inoculated 65 days after emergence, as Droby *et al.* (1984a) found that the disease susceptibility was highest at 65 days. The trial consisted of 8 treatments with 5 replications of each. A replicate consists of one pot containg one plant. The treatments were: (i) plants inoculated with *Alternaria* sp., (ii) plants inoculated with yellow bacteria, (iii) plants inoculated with white bacteria and (iv) uninoculated control.

Potato leaves from Mpumalanga province were obtained. A detailed description of the morphology of the potato brown spot lesions was made before isolation. Isolations were made from the lesions as described in 3.2.1. One fungal isolate was chosen and labelled labbelled AA16. Two bacteria (yellow and white) were also isolated from the brown spot lesions in the same manner as described in 3.2.1. The fungal and bacterial cultures isolated were included in the brown spot Koch's postulates trials.

The isolate fungal (AA16) was plated on V8-juice medium and incubated at 25˚C in the dark for 3 weeks to encourage sporulation. A spore suspension was made by adding 1ml sdH_2O to the cultures aseptically and harvesting the spores aseptically. The spore suspension was added to a sterile flask. The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 1.5 x 10^4 spores per ml. The bacterial cultures were plated onto NA and incubated at 25˚C for 48 hours. Bacterial suspensions were made by adding

1ml sdH_2O to the cultures hand harvesting the bacteria aseptically. The bacterial suspension was transfered to a sterile flask. The concentration of the suspension was determined in a Petroff-Hauser bacterial counter and adjusting to 1 x 10^6 bacteria per ml.

The plants were inoculated with the bacterial and fungal cultures at 65 days after emergence according to the treatments mentioned. The plants were first damaged by lightly tapping the leaves with a sterile stick. The leaves were inoculated by spraying with 30ml of the *Alternaria* sp. spore suspension (1.5 x 10^4 conidia per ml) and/or bacterial suspensions (1 x 10^6 bacteria per ml). After appearance of lesions descriptions of the brown spot lesions were made and pathogens were isolated from the lesions as described above. The treatments where the bacteria alone were inoculated were also isolated on agar plates although the leaves did not have any lesions.

Black pit

A trial was conducted to determine the causal organism of black pit of potatoes. Black pit lesions were observed on Frodo G0 seed tubers (unknown origin). The lesions were described and isolations made from the lesions as described in 3.2.1. One fungal isolate was chosen and labbelled AA4.

The *A. alternata* cultures were placed on V8-juice medium and incubated at 25˚C for 3 weeks to encourage sporulation. A spore suspension was made as described in 3.2.1. A total of 30 disease-free G0 seed tubers were inoculated with *Alternaria* sp. spore suspension (1.5 x 10^4 conidia per ml) or sdH_2O as control. The disease-free seed tubers originate form Ceres, Mpumalanga, Northern Cape and North West. The treatments were: (i) Frodo tubers dipped in spore suspension, (ii) BP1 tubers dipped in spore suspension, (iii) Up-To-Date tubers dipped in spore suspension, (iv) Frodo tubers uninoculated control, (v) BP1 tubers uninoculated control, (vi) Up-To-Date tubers uninoculated control.

The tubers were dipped for one minute in either the *A. alternata* spore suspension (1.5 x 10^4 conidia per ml) or sdH_2O, according to the treatments mentioned. The tubers were placed in a plastic bag in order to create highly humid conditions and incubated for 2 weeks where after disease incidence was measured. After appearance of lesions, description of lesions was made and isolations were made from the lesions onto half strength PDA as described in 3.2.1. The experiment was conducted twice.

Single *Alternaria alternata* strain causing both brown spot and black pit disease

A trial was conducted to determine if the *A. alternata* isolated from potato black pit lesions can cause potato brown spot disease, and if the *A. alternata* isolated from potato brown spot lesions can cause potato black pit disease. *A. alternata* was isolated from brown spot (isolate AA16) and black pit (isolate AA4) lesions separately and spore suspensions were made of the isolated *A. alternata* cultures, as described in 3.2.1. The treatments were: (i) potato foliage inoculated with *A. alternata* (AA4) spore suspension, (ii) positive control: potato foliage inoculated with *A. alternata* (AA16) spore suspension, (iii) potato foliage uninoculated control, (iv) potato seed tubers dipped in *A. alternata* spore suspension (AA16), (v) positive control: potato seed tubers dipped in *A. alternata* spore suspension (AA4), (vi) potato seed tubers uninoculated control. Each treatment was replicated five times in this trial. Each replicate consisited of either one pot containing one plant or one tuber inoculated with *A. alternata*.

Before inoculation the leaves were wounded by tapping them with a sterile stick. The leaves were then inoculated by spraying them until runoff with either one of the *Alternaria* sp. spore suspensions (1.5 x 10^4 conidia per ml) of AA16, AA4, or sdH_2O. The disease incidence of brown spot was measured 2 weeks after inoculation. The BP1 G0 seed tubers (origin Mpumalanga province) were dipped for one minute in either one of the *Alternaria* sp. spore suspensions (1.5 x 10^4 conidia per ml) AA16, AA4, or sdH_2O, without wounding the tubers beforehand. The tubers were placed in a humidity chamber for 1 week, where after disease incidence was measured. Lesion description was made of the black pit and brown spot lesions before isolation. Lesion isolation was done as described in 3.2.1. The experiment was conducted twice.

Confirmation of causative organism for both black pit and brown spot lesions

Pure fungal cultures isolated from the treatments as described in 3.2.1, 3.2.2 and 3.2.3 were re-isolated onto half strength PDA. The cultures were morphologically examined to confirm identity. One isolate from each treatment was identified by means of PCR. The isolates were not sequenced as the isolates (AA16 and AA4) used in inoculation were already sequenced and identified as *A. alternata*. The PCR was done only to confirm the identification of te isolated obtained in the Koch's Postulates trials as *A. alternata*. The fungal DNA was extracted from pure fungal cultures using the ZR Soil Microbe DNA MiniPrep Kit (Zymo Research). A 1µL aliquot of this DNA solution was used for PCR, which was performed in 25µL reactions. *Alternaria* sp.-specific primers (1µL of each primer), A. alt-F3 (5′ to 3′ TCTAGCTTTGCTGGAGACTC) and A. alt.-R1.1 (5′ to 3′ AGACCTTTGCTGATAGAGAGT) (Schuhegger *et al.,* 2006), were used to generate short amplicons of 95bp. The PCR was performed and the thermocycler conditions were 94°C for 10min, followed by 40 cycles of

95°C for 15s, 60°C for 60s, 72°C for 60s, and a final extension step of 72°C for 10min. The amplified products were subjected to electrophoresis through a 1% agarose gel in 1 x TBE Buffer at 100V.

3.3 RESULTS

3.3.1 Inoculation trials

Brown spot

The lesions found on the potato plants initially submitted from Mpumalanga province were typically those of brown spot and were small brown lesions that appeared on the abaxial side of the leaves. As the lesions matured they became visible on the adaxial side of the leaves and developed concentric rings. Mature lesions were smaller than those of early blight (caused by *A. solani*).

During initial isolation of lesions submitted, both fungal and bacterial cultures were isolated. The spores of the fungal cultures morphologically resembled an *Alternaria* species, but not those of *A. solani*. The fungal isolate was identified as *A. alternata* via sequencing.

During the early isolations of the lesions, two bacteria were isolated as well. These bacteria were only isolated from the leaves and not the potato tubers. The bacterial isolates were included in the Koch's postulates trial. The bacterial cultures were not identified; however the results of biochemical tests that were conducted are presented in Table 3.2.

In the Koch's postulates trial, two weeks after inoculation brown spot lesions developed on the treatments inoculated with *A. alternata*. No lesions developed on the potato plants inoculated with only the bacteria. After isolation *A. alternata* was isolated from all the plants inoculated with *A. alternata*. *A. alternata* was isolated from the treatments inoculated with *A. alternata* and bacteria as well. Thus *A. alternata* had to be used in inoculation to cause disease on the plants. The bacteria were also isolated from the potato leaves inoculated with the bacteria.

Table 3.2 Microbiological test results conducted on the bacteria isolated from brown spot of potatoes.

Biochemical test	**Yellow bacterial isolate results**	**White bacterial isolate results**
Gram staining	Gram negative	Gram negative
O/F test	+/+	+/+
Colour on NA	Yellow	White
Tween Medium	Does not hydrolyze tween	Hydrolyze tween
Growth on MSP	Yellow mucoid	Yellow mucoid
Growth on CVP	No pits	No pits
Growth on YDC	Yellow	White
Nitrate reaction	Negative	Negative
Voges Proskauer test	Negative	Negative
Indole test	Positive	Negative
Methyl red	Negative	Negative
Growth on sugars: Sucrose	Negative	Negative
Inositol	Negative	Negative
Sorbitol	Yellow growth	Yellow growth
Mannitol	Negative	Yellow growth
Erythritol	Negative	Negative

The brown spot lesions observed in the trial were small dark lesions (Figure 3.1) on the abaxial side of the leaves, which developed concentric rings (Figure 3.2) and became visible on the adaxial side of the leaves as the lesions matured. The infection was severe, and some of the infected leaves dropped prematurely. Lesions developed on the stem of the plants as well. The lesions were dark brown with no specific form but with a distinct border. Lesions from the plants inoculated with *A. alternata* and *A. alternata* in combination with the bacteria were identical. *Alternaria* sp. isolated from the different treatments were confirmed by PCR.

Figure 3.1 Brown spot of potatoes.

Figure 3.2 Severe symptoms of brown spot on potatoes.

Black pit

The lesions found on the potato tubers submitted were dark and sunken with a defined border. The lesions typically developed in the lenticels of the tubers. Only a fungus was isolated from the tubers. The fungus was morphologically similar to those isolated from brown spot of potatoes, a small spored *Alternaria* sp.. The fungus was sequenced to identify the isolates as *A. alternata*. The results obtained in the Koch's postulates experiment for black pit, resulted in similar symptoms appearing in the lenticels of the tubers for all the treatments inoculated with *A. alternata* after one week of incubation. The symptoms were identical to those observed in the potato tubers from which the initial isolations were made. The black pit lesions observed in the trial were small sunken dark lesions with a distinct border, forming in the lenticels of the tubers (Figure 3.3). The control treatments developed no symptoms and no organisms were isolated from them. The isolates isolated from the trials were confirmed as *A. alternata* with PCR.

Figure 3.3 Black pit symptoms on a potato tuber.

Single *Alternaria alternata* strain causing both brown spot and black pit disease

It was determined that a single isolate, from either brown spot or black pit lesions, can cause both brown spot and black pit disease of potatoes. The potato leaves inoculated with *A. alternata* (AA4) isolated from black pit lesions, resulted in brown spot lesions similar to those observed in the Koch's postulates brown spot trial. The positive control treatment, where the potato plants were inoculated with *A. alternata* (AA16) resulted in similar lesions on the plant leaves. The lesions of both treatments mentioned were small, brown necrotic areas that appeared on the abaxial side of the leaves. As the lesions matured they developed concentric rings and became visible on the upper side of the leaves as well. Isolation from lesions resulted in the isolation of *A. alternata*, confirmed by PCR.

The potato tuber inoculated with *A. alternata* isolate AA16 resulted in black pit lesions on the tubers. The lesions on the tubers were similar to those observed in the Koch's postulates black pit trial. The positive control treatment inoculated with *A. alternata* isolate AA4 resulted in similar lesions. The lesions of both treatments mentioned developed in the lenticels of the tubers. The lesions were dark sunken with defined borders. The isolation from the lesions resulted in the isolation of *A. alternata* that was confirmed by PCR.

The plants and tubers in the negative control treatments inoculated with sdH_2O developed no symptoms and no *A. alternata* were isolated from them. A single *A. alternata* isolate, isolated from either brown spot or black pit lesions is able to cause both brown spot and black pit lesions.

3.3.2 Identification

Identification of the fungi by sequencing

The cultures were identified as *A. alternata* by comparing the sequences with the NCBI database using the BLASTN procedure (Altschul *et al.,* 1990) and by using BioloMICS online software from MYCOBANK (Robert *et al.,* 2005). The cultures had between 90-100% similarities with other *A. alternata* cultures on these databases. In Figure 3.4 the phylogenetic analysis of isolate sequences of brown spot and black pit of potatoes can be seen.

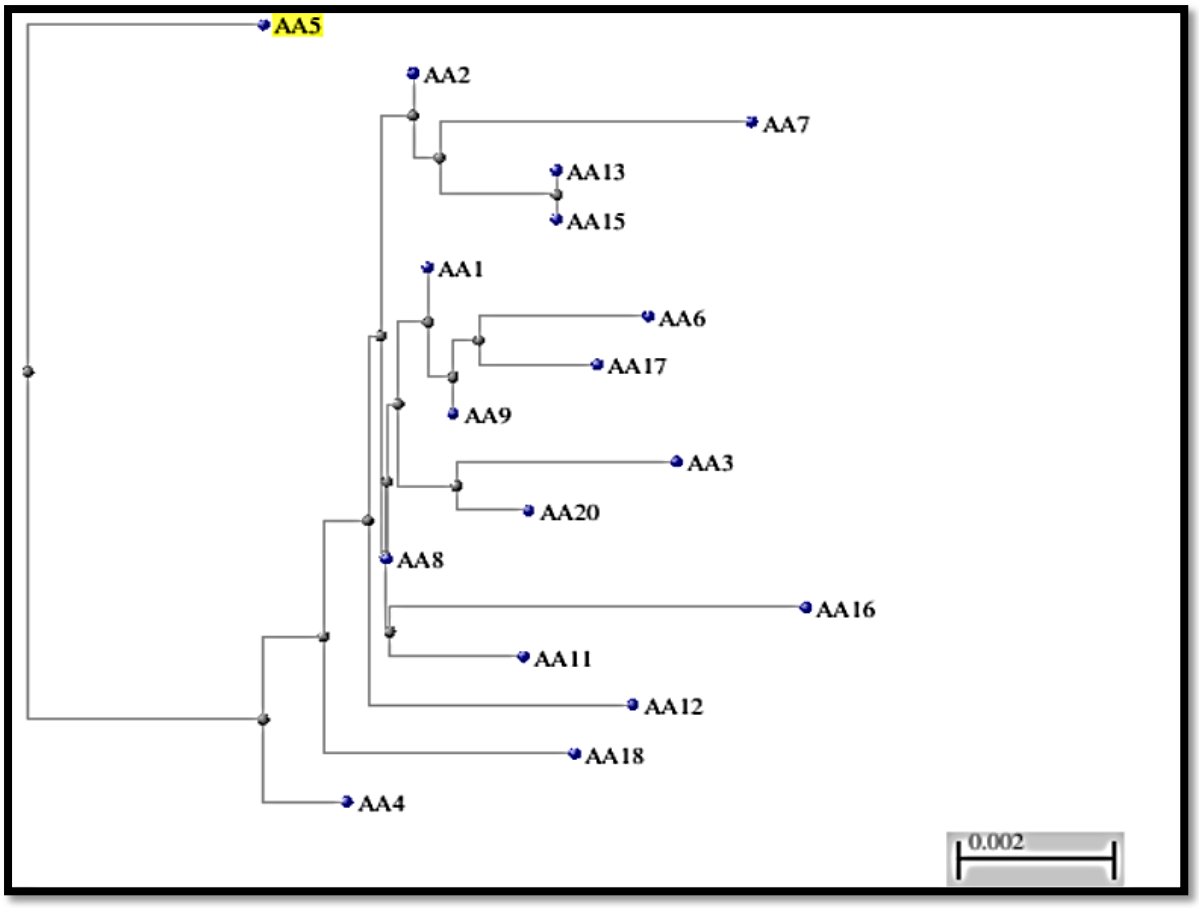

Figure 3.4 Phylogenetic analysis of isolate sequences of brown spot and black pit of potatoes.

Confirmation of causative organism

Results obtained using Alternaria specific primers can be seen in Figure 3.5 -3.7. In the brown spot Koch's postulates first and second trials 95bp PCR band were obtained for the treatments where the leaves inoculated with *A. alternata*, where the leaves inoculated with *A. alternata* and yellow bacteria, where the leaves inoculated with *A. alternata* and white bacteria and where the leaves were inoculated with *A. alternata* and both bacteria, which confirms the precense/identification of *Alternaria* spp. in those treatments.

In the black pit Kochs postulate first and second the treatments where Frodo, BP1 and UTD tubers were inoculated with *A. alternata* 95bp bands were obtained, which confirms the presence/identification of *Alternaria* spp. in those treatments.

In the trials where potato leaves were incoluated with spore suspension isolated form black pit, and in the trials where tubers were inoculated with spore suspension isolated form brown spot the treaments where the leaves were inoculated with black pit spore suspension, the

leaves inoculated with brown spot spore suspension, the tubers inoculated with black pit spore suspension, and the tubers inoculated with brown spot spore suspension 95bp PCR bands were obtained, which confirms the presence/identification of *Alternaria* spp. in those treatments.

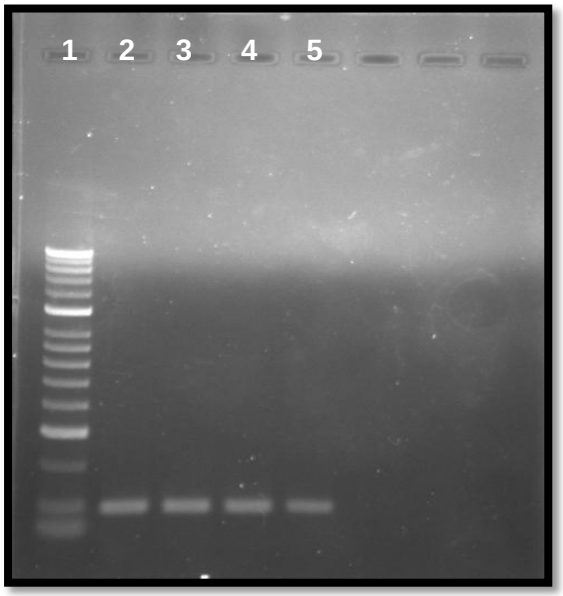

Figure 3.5 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* in the Koch's postulates first trial for brown spot. Description of lanes: 1, Hyper ladder V; 2, leaves inoculated with *A. alternata*; 3, leaves inoculated with *A. alternata* & yellow bacteria; 4, leaves inoculated with *A. alternata* & white bacteria; 5 leaves inoculated with *A. alternata* & both bacteria.

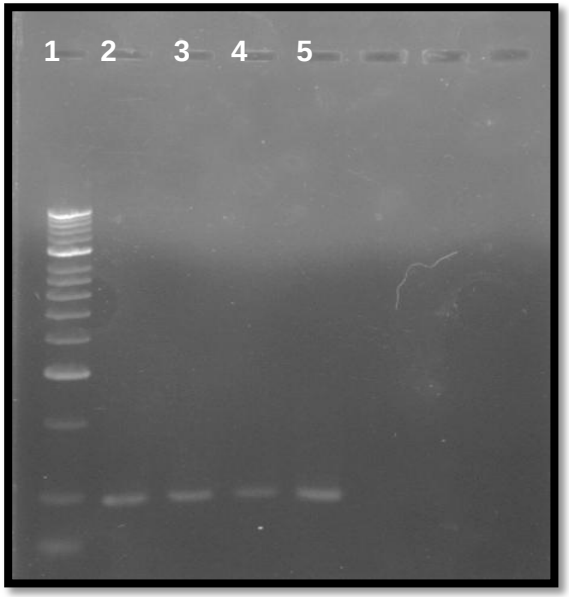

Figure 3.6 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* in the Koch's postulates second trial for brown spot. Description of lanes: 1, Hyper ladder V; 2, leaves inoculated with *A. alternata*; 3, leaves inoculated with *A. alternata* & yellow bacteria; 4, leaves inoculated with *A. alternata* & white bacteria; 5 leaves inoculated with *A. alternata* & both bacteria.

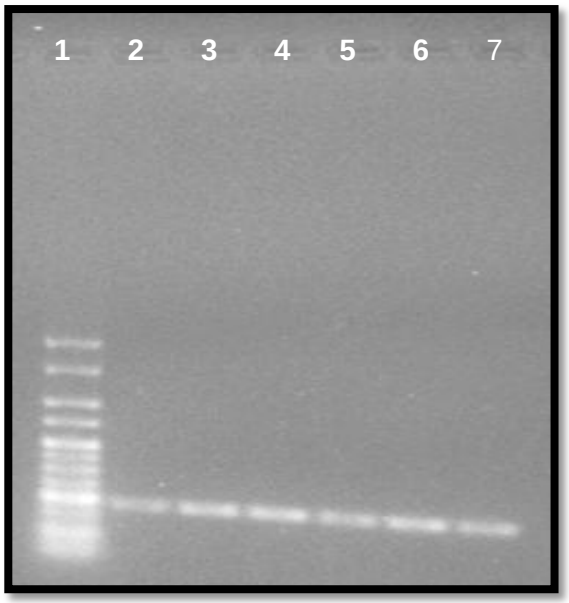

Figure 3.7 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* in the Koch's postulates first and second trial for black pit. Description of lanes: 1, Hyper ladder V; 2, Frodo tubers inoculated with *A. alternata* trial I; 3, BP1 tubers inoculated with *A. alternata* trial I; 4, UTD tubers inoculated with *A. alternata* trial I; 5 Frodo tubers inoculated with *A. alternata* trial II; 6, BP1 tubers inoculated with *A. alternata* trial II; 7, UTD tubers inoculated with *A. alternata* trial II.

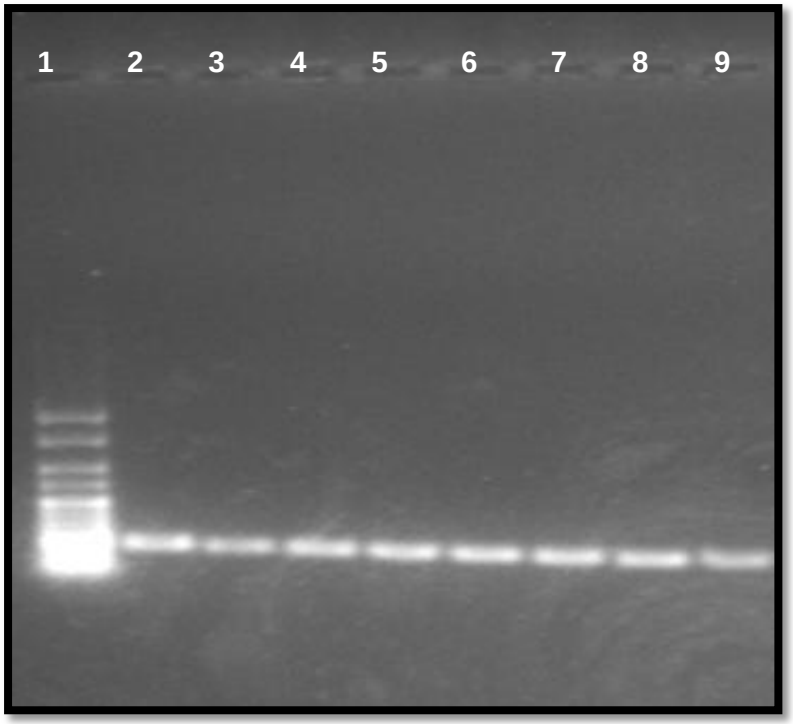

Figure 3.8 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* in the trials where potato leaves were inoculated with spore suspension isolated from black pit, and in the trials where tubers were inoculated with spores suspension isolated from brown spot. Description of lanes: 1, Hyper Ladder V; 2, leaves inoculated with black pit spore suspension (I); 3, leaves inoculated with brown spot spore suspension (I); 4 leaves inoculated with black pit spore suspension (II); 5, leaves inoculated with brown spot spore suspension (II); 6, tubers inoculated with brown spot spore suspension (I); 7, tubers inoculated with black pit spore suspension (I); 8, tubers inoculated with brown spot spore suspension (II); 9, tubers inoculated with black pit spore suspension (II).

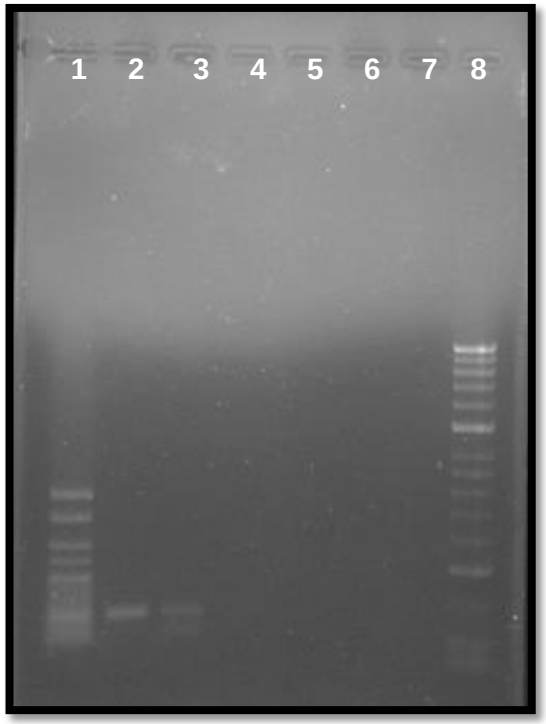

Figure 3.9 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* and *Alternaria solani* with PCR primers A. alt-F3 A. alt.-R1.1. Descriptions of Lanes: 1, Hyper Ladder V; 2, *Alternaria alternata*; 3, *Alternaria solani*; 8, Hyper Ladder II.

3.4 DISCUSSION

During initial isolation from brown spot samples from Mpumalanga that were submitted to the Potato Pathology Programme @ UP Diagnostic Clinic, were identified as *A. alternata* and two bacterial cultures being isolated (a white and yellow bacterium). All three isolates were used in the Koch's postulates trial. All three isolates were re-isolated from the inoculated plants two weeks after inoculation. However only the treatments where the *A. alternata* isolate was used in inoculation resulted in the development of brown spot lesions on the potato foliage. The bacteria may thus have only been epiphytic bacteria.

If the bacteria used in this study were plant pathogens it could be expected that lesions would have formed. Some plant pathogenic bacteria are able to survive and multiply on the surface of the plants and some are able to survive and multiply on both the surface and the interior of the plants. Mutant strains of plant pathogens that are not able to cause disease are however still able to establish both interior and exterior populations but no lesions are formed on the plants (Allington & Chamberlain, 1949; Smitley & McCarter 1982; Lindemann *et al.,* 1984 Rouse *et al.,* 1985; Hirano & Upper, 1990; Kamoun & Kado, 1990; Huang *et al.,* 1991; Yang *et al.,* 1996). As no lesions formed in the treatments where only the bacteria were used in inoculation, they cannot be considered as plant pathogens. The bacteria were also not always isolated from brown spot lesions later in the study and they were not isolated from black pit lesions. Thus the bacteria used in the trials are probably only epiphytes able to survive on the potato leaves (Leben, 1965; Kinkel *et al.,* 1995; Beattie & Lindow, 1999).

A. alternata was determined as the causal organism of brown spot of potato in Brazil, Israel and USA (Droby *et al.,* 1984a; Boiteux & Reifschneider, 1994; Kirk, 2007). The lesions observed on the foliage are small dark lesions that become visible on the abaxial side of the leaves. As the lesions mature they become visible on the adaxial side of the leaves. The symptoms observed during the leaf inoculation with *A. alternata* spore suspension correspond with the descriptions made by Droby *et al.* (1984a), Boiteux & Reifschneider (1994) and Kirk (2007).

A. alternata was the causal organism of black pit of potato tubers; this is consistent with the results from Droby *et al.* (1984b) who concluded that the causal organism of black pit of potatoes in Israel was *A. alternata*. The black pit lesions were small sunken dark lesions with distinct boarders, forming in the lenticels of the tubers. The symptoms observed during the tuber inoculation with *A. alternata* spore suspension are the same as observed by Droby *et al.* (1984b). The last report or mention of black pit of potatoes was almost 30 years ago.

It is not known if the *A. alternata* isolates found in South Africa are unique in its ability to cause black pit of potatoes. It may be that the incidence of black pit is lower in other countries and that the disease goes unnoticed or that the symptoms are confused with those caused by *Fusarium* spp. or *Pectobacterium* spp. (van Hall) Dye, as these pathogens cause similar disease symptoms on the tubers, infecting the lenticels of the tubers (Stevenson *et al.*, 2001).

In recent years brown spot and black pit of potatoes have been observed in South Africa. Brown spot symptoms appear on the abaxial side of the potato leaves as small dark lesions that become visible on the adaxial side of the leaves as the lesions mature. In severe infections the leaves drop prematurely. Black pit symptoms appear as dark sunken lesions in the lenticels of tubers. The resulst of the current study confirm the causal organism of brown spot and black pit of potatoes as *A. alternata*.

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CHAPTER 4

EFFECT OF DIFFERENT INOCULUM SOURCES OF *ALTERNARIA ALTERNATA* ON SYMPTOM EXPRESSION ON POTATOES

ABSTRACT

Alternaria alternata is the causal agent of brown spot and black pit of potatoes. To determine the primary source of inoculum of brown spot and black pit disease a pot and *in vitro* trials was conducted. The treatments of the pot trial was: (i) inoculated tubers, sterilised soil, uninoculated plants; (ii) uninoculated tubers, covered inoculated soil, uninoculated plants; (iii) uninoculated tubers, uncovered inoculated soil, uninoculated plants; (iv) uninoculated tubers, sterilised soil, inoculated plants; (v) uninoculated tubers, sterilised soil, uninoculated plants. The treatments of the *in vitro* trial were: (i) inoculated tubers, incubated; (ii) uninoculated tubers, incubated with inoculum; (iii) uninoculated tubers, incubated. It was observed that when planting an inoculated seed tuber brown spot may develop on foliage; however when brown spot disease is present on the leaves, black pit will not necessarily develop on the tubers. To limit brown spot and black pit diseases it is important to plant disease free tubers and harvest in dry weather.

4.1 INTRODUCTION

Alternaria alternata (Fr.) Keissler causes brown spot and black pit of potatoes (*Solanum tuberosum* L.). The foliar disease is known as brown spot. The lesions are small brown lesions on leaves and stems, which initially develop on the abaxial side of the leaf (Stevenson *et al.,* 2001). The disease of the tubers is known as black pit. Black pit symptoms on the tubers appear as sunken dark lesions with defined borders that can develop in storage (Droby *et al.,* 1984b; Stevenson *et al.,* 2001).

Pathogens can be disseminated through the air, soil, water and infected plant material in the environment, on equipment and in association with vectors (Agrios, 1997). The primary dispersal method of *A. alternata* is by wind (Rotem, 1994). *A. alternata* spreads through the air and can travel by air currents for a remarkable distance. *A. alternata* spores are adapted to float in wind due to their large bodies (Rotem, 1994).

Plant pathogens require a certain range of temperatures to cause disease on a host plant (Agrios, 1997). Moisture is critical for the germination of the fungi and its ability to penetrate the plants (Agrios, 1997).

A. alternata is known to be a foliar pathogen rather than a root and tuber pathogen (Thomma, 2003). On potatoes, both *A. alternata* and *Alternaria solani* Sorauer (Ellis) cause disease of tubers. Wounded tubers are infected with *A. solani* at harvesting and the disease subsequently develops in storage. *A. solani* can cause early blight of the tubers through the lenticels of the tubers (Venette & Harrison, 1973; Stevenson *et al.,* 2001).

No studies have yet been conducted on the translocation of *A. alternata* from foliage to tubers or vice versa. As black pit is thought to be a storage disease, it is unknown at what stage in the production the tubers are infected with *A. alternata* causing black pit, and if brown spot will develop if *A. alternata*-infected tubers are planted (Droby *et al.,* 1984b). The aim of this experiment was to determine the sources of inoculum of *A. alternata* of potatoes.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of pathogen and preparation of spore suspension

Potato leaves were obtained from Mpumalange province. Brown spot lesions on potato leaves were surface sterilized with 2% NaOCl solution for one minute and rinsed twice in sterile distilled water. The lesions were excised and placed on half strength Potato Dextrose Agar (PDA) and Nutrient Agar (NA) and incubated at 25˚C for 7 days in the dark. Pure fungal cultures were sub-cultured onto half strength PDA and labeled AA16. The isolate (AA16) that was used as inoculum was previously confirmed as *A. alternata* by sequencing of the ITS region as described in chapter 3.

The *A. alternata* isolate (AA16) was plated on V8-juice medium and incubated at 25˚C for 3 weeks in the dark to encourage sporulation. A spore suspension was made by adding 1ml sdH_2O to the cultures aseptically and harvesting the spores aseptically. The spore suspension was transferred to a sterile flask. The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 1.5×10^4 spores per ml.

4.2.2 Trials

A pot trial was conducted to determine the source of inoculum of *A. alternata* that cause brown spot symptoms. To determine the source of inoculum causing the black pit symptoms, a second trial in a high humidity chamber was conducted. The experiments were repeated once.

Source of inoculum for brown spot symptom development

The treatments of the pot trial were: (i) inoculated tubers, sterilised soil, uninoculated plants; (ii) uninoculated tubers, covered inoculated soil, uninoculated plants; (iii) uninoculated tubers, uncovered inoculated soil, uninoculated plants; (iv) uninoculated tubers, sterilised soil, inoculated plants; (v) uninoculated tubers, sterilised soil, uninoculated plants.

The experiments with treatments that included inoculated tubers, sterilised soil, uninoculated plants (i) was conducted as follows. Six unwounded disease-free BP1 G0 seed tubers (from Mpumalanga province), were dipped into the *A. alternata* spore suspension (refer to 4.2.1) for one minute. The tubers were placed in a humidity chamber for 2 weeks at 25°C to allow the development of black pit symptoms on the seed tubers. Tubers showing symptoms after 14 days were then planted in 4kg steam-sterilised red top soil. The physical characterisitcs of the soil include a pH of 4.2, 74.8% coarse sand, 17.5% clay and 7.5% silt. For more information see Appendix C and D. In the treatment (ii) uninoculated tubers, covered inoculated soil, uninoculated plants, disease free BP1 G0 seed tubers were planted individually into 6 pots containing steam-sterilised red top soil that was inoculated by drenching the soil with 30ml *A. alternata* spore suspension (1.5×10^4 conidia per ml) per 4kg pot. The soil was covered with black plastic as soon as the potato plant emerged. In the treatment (iii) uninoculated tubers, inoculated soil uncovered, uninoculated plants, the same method was followed as in treatment ii, except the soil was not covered with black plastic. This was done to prevent *A. alternata* spores present in the soil from splashing onto the leaves. In the treatment (iv) uninoculated tubers, sterilised soil, inoculated plants, six disease free BP1 G0 seed tubers were planted individually into pots filled with 4kg steam-sterilised red top soil. At 50 days after emergence the plants were inoculated by spraying the leaves with *A. alternata* spore suspension (1.5×10^4 conidia per ml) until runoff. For the uninoculated control treatment (v) six disease free BP1 G0 seed tubers were planted individually into pots filled with 4kg steam-sterilised red top soil.

High relative humidity was obtained by placing the pots in polyethylene bags that stayed on for the remainder of the growing season. The plants were watered every second day until field capacity. To induce stress conditions no fertilizer was added. Disease severity and incidence of brown spot were visually evaluated once a week for 12 weeks, starting at 4 leaf stage, except in the case of the treatment where the plant leaves were inoculated at 50 days, in which case the disease incidence was measured once a week for 4 weeks starting one week after inoculation. Brown spot severity was measured using the scale for percentage leaf area infected (Appendix A; James, 1971), (refer to 4.2.3). The experiments were repeated once.

At harvest the foliage was removed first by cutting off the stem at the base of the soil. Daughter tubers were removed from the soil, washed and placed in a plastic bag in order to create highly humid conditions for 3 weeks at 25°C to promote black pit disease development.

Source of inoculum for black pit symptom development

The treatments of the *in vitro* trial were: (i) inoculated tubers, incubated; (ii) uninoculated tubers, incubated with inoculum; (iii) uninoculated tubers, incubated. In the treatment inoculated tubers, incubated (i), six disease free BP1 G0 seed tubers were dipped into the *A. alternata* spore suspension (1.5 x 10^4 conidia per ml) for one minute. In the treatments (ii) uninoculated tubers, incubated with inoculum, six disease free BP1 G0 seed tubers (origin Mpumalanga provinde) were placed in a plastic bag with brown spot infected leaves (BP1, inoculated with *A. alternata* spore suspension as described above, with 20% brown spot disease severity) in order to create highly humid conditions. The tubers and leaves were inoculated for 3 weeks in the dark at 25°C. For the uninoculated control (iii) six disease free BP1 G0 seed tubers were dipped into the sdH_2O for one minute. The BP G0 seed tubers were obtained from Mpumalanga province.

The tubers were placed in a high humidity chamber in the dark for 3 weeks at 25°C. Disease severity and incidence of black pit were visually evaluated once a week for 3 weeks, starting one week after tuber inoculation. Severity of black pit on tubers was measured by using the percentage tuber area infected scale (Appendix B; James, 1971). The experiments were repeated once.

4.2.3 Disease assessment

Both brown spot and black pit disease severity were visually evaluated using different percentage scales (0-100%). Severity of brown spot on the plants was measured by using the percentage leaf area infected scale (Appendix A; James, 1971), and severity of black pit on tubers was measured by using the percentage tuber area infected scale (Appendix B; James, 1971). Area Under Disease Progress Curve (AUDPC) was calculated for both of the trials, using the following formula (Shaner and Finney, 1977):

$$AUDPC = \sum_{i=1}^{n} \left[\frac{(y_i + y_{i+1})}{2}\right](t_{i+1} - t_i)$$

Where, *yi* is the % diseased area on the *ith* date, *ti* is the date on which the disease was scored *(ith* day), n is the number of dates on which disease was scored (Shaner and Finney,

1977). The results were also statistical analysed using GenStat (GenStat for Windows, 2012).

4.2.4 Confirmation of causative organism for both black pit and brown spot lesions

The symptomatic leaves or tubers obtained from the trials were surface sterilized with 2% NaOCl solution for one minute and rinsed twice in sdH_2O. The lesions were excised and placed on half strength PDA and NA and incubated at 25˚C for 1 week in the dark. Pure fungal cultures were sub-cultured onto half strength PDA. The culture's spores were microscopically examined to confirm identity. One isolate from each treatment was identified with PCR. The fungal DNA was extracted from pure cultures using the ZR Soil Microbe DNA MiniPrep Kit (Zymo Research). A 1µL aliquot of this DNA solution was used for PCR, which was performed in 25µL reactions. *Alternaria* sp. -specific primers (1µL of each primer), A. alt-F3 (5′ to 3′ TCTAGCTTTGCTGGAGACTC) (Schuhegger *et al.,* 2006), and A. alt.-R1.1 (5′ to 3′ AGACCTTTGCTGATAGAGAGT) (Schuhegger *et al.,* 2006), were used to generate short amplicons of 95bp. The PCR was performed under thermocycler conditions of 94°C for 10min, followed by 40 cycles of 95°C for 15s, 60°C for 60s, 72°C for 60s, and a final extension step of 72°C for 10min. The amplified products were subjected to gel-electrophoresis in a 1% agarose gel in 1 x TBE Buffer at 100V.

4.3 RESULTS

When inoculated seed tubers were planted, brown spot symptoms developed on the lower leaves of the emerged plants within two weeks (Figures 4.1, 4.2, 4.7 and 4.9). When referring to the AUDPC (Figures 4.3 and 4.4), this inoculum source resulted in significantly (p value < 0.001) more brown spot disease than the other treatments. The daughter tubers that were placed in a humidity chamber for 3 weeks did not develop any black pit disease.

No brown spot symptoms developed in the treatment where the soil was inoculated before planting and covered. Brown spot did develop when the inoculated soil was left uncovered. The lower leaves near the ground developed brown spot symptoms two weeks after emergence. In the watering process, soil splashed onto the lower leaves, possibly splashing *A. alternata* inoculum onto the leaves that ultimately caused brown spot disease. As indicated by the AUDPC (Figures 4.3 and 4.4), the uncovered soil treatments resulted in significantly (p value < 0.001) more brown spot disease than the control treatment, but significantly (p value < 0.001) less brown spot disease than when the inoculated seed tubers were planted. Again the daughter tubers did not develop black pit disease.

Brown spot developed on the plants inoculated with spore suspension at 50 days post emergence. In this treatment all the replicates became diseased with brown spot. No secondary infection occurred. The polyethylene that covered the plants for the duration of the growing season limited the aerial dissemination of the conidia. As indicated by the AUDPC (Figures 4.3 and 4.4), this treatment resulted in significantly (p value < 0.001) more brown spot disease than the control treatment. Again the daughter tubers did not develop black pit disease.

In all the treatments where brown spot disease developed, the diseased leaves wilted and dropped prematurely within a few weeks. Although the disease severity was low in both experiments it was high enough for the leaves to drop prematurely.

The inoculated tubers developed severe black pit disease two weeks post inoculation. The AUDPC values (Figures 4.5 and 4.6) indicate this source of inoculum resulted in the development of significantly (p value < 0.001) more black pit disease than the other treatments. When tubers were placed nect to brown spot infected leaves as inoculum source, black pit disease developed, but significantly (p value < 0.001) less than in the treatments where the tubers were inoculated with spore suspension and incubated in a high humidity chamber. No black pit disease developed on the control tubers, indicating that the seed tubers used were disease free.

Results obtained using *Alternaria* specific primers can be seen in Figure 4.8 and 4.9. A 95bp PCR band was obtained which confirmed the indentities of *Alternaria* spp. infection in trial.

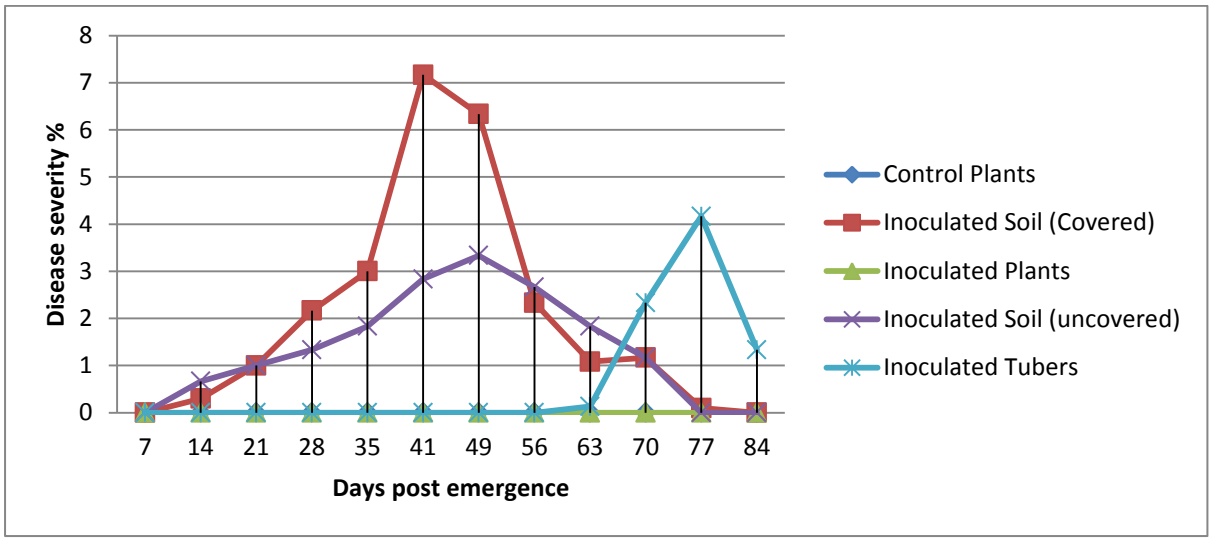

Figure 4.1 Disease progress curve for different inoculum sources of *Alternaria alternata* for brown spot trial I.

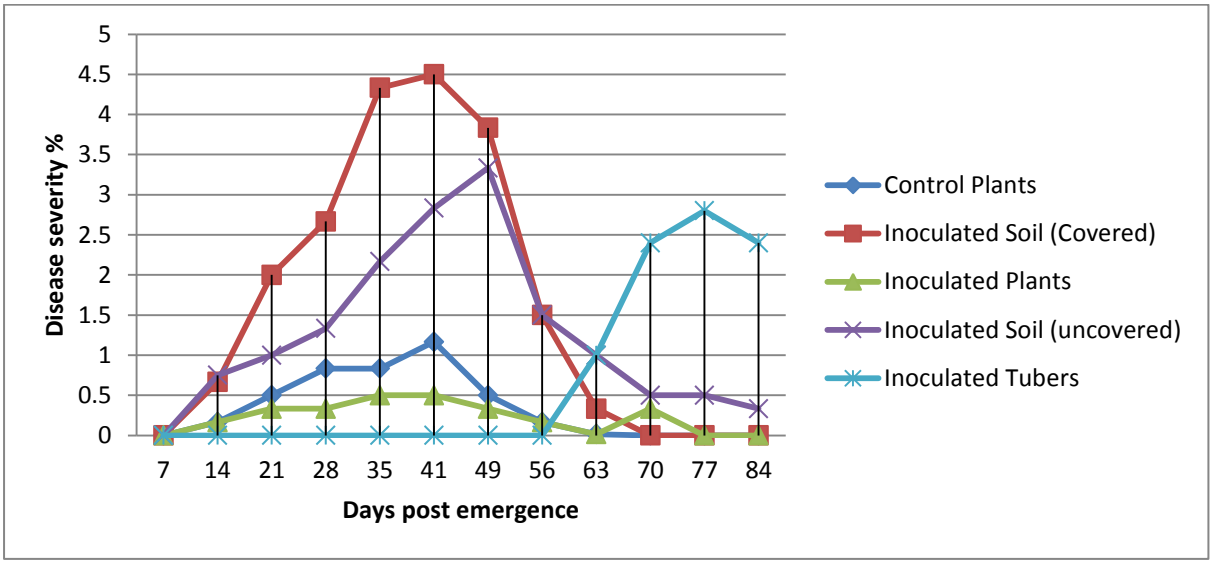

Figure 4.2 Disease progress curve for different inoculum sources of *Alternaria alternata* for brown spot trial II.

AUDPC reflects the total amount of disease over the growing period. Treatments with the lowest values have less disease and thus these sources of inoculum are less important for disease development.

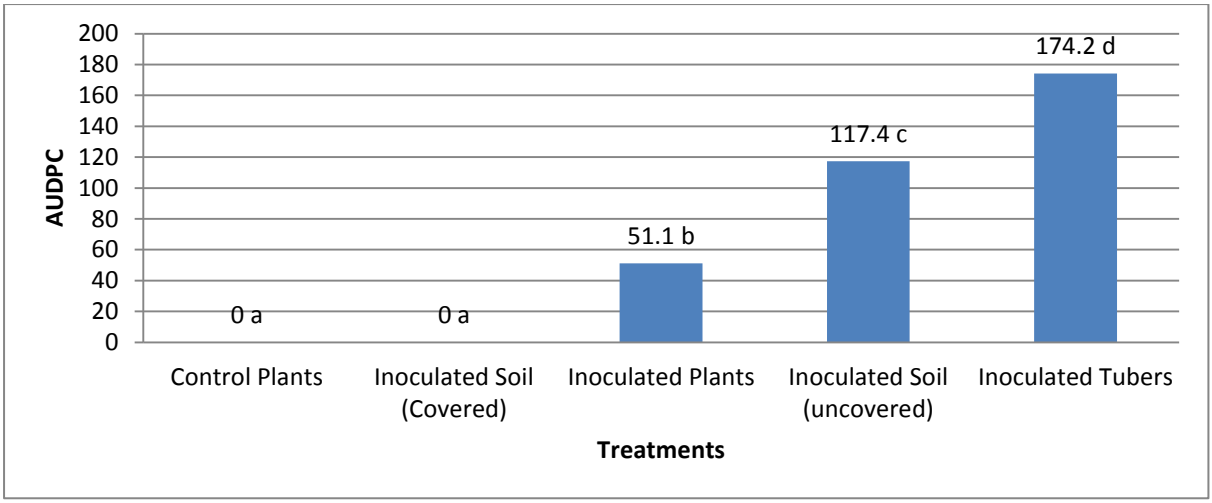

Figure 4.3 Area under disease progress curve (AUDPC) for different inoculum sources of *Alternaria alternata* for brown spot trial I.
$p<0.001$, CV = 11.4%
Values on bars followed by the same letter are not significantly different at the 5% level of significance.

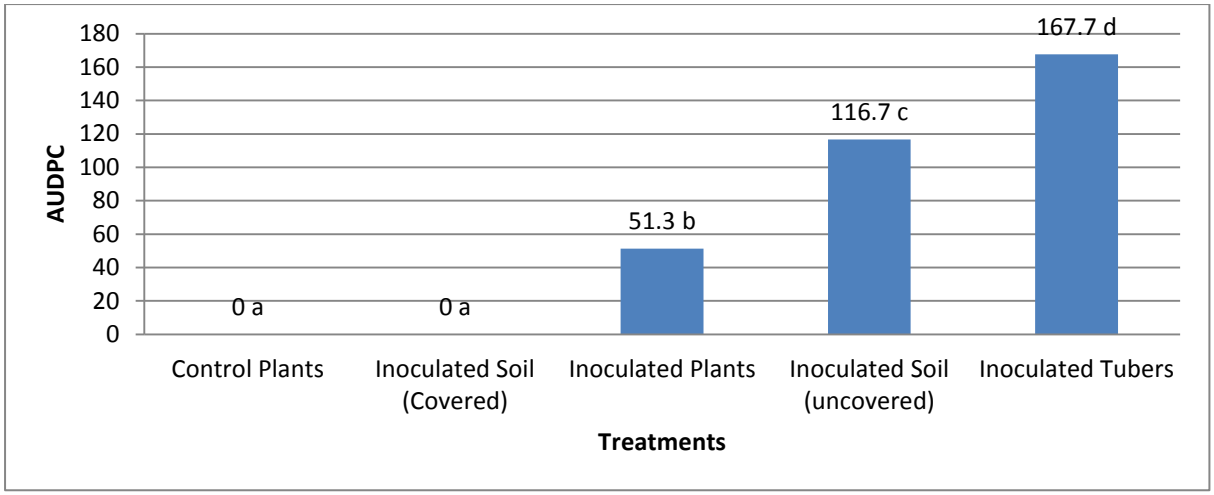

Figure 4.4 Area under disease progress curve (AUDPC) for different inoculum sources of *Alternaria alternata* for brown spot trial II.
p<0.001, CV = 5.6%
Values on bars followed by the same letter are not significantly different at the 5% level of significance.

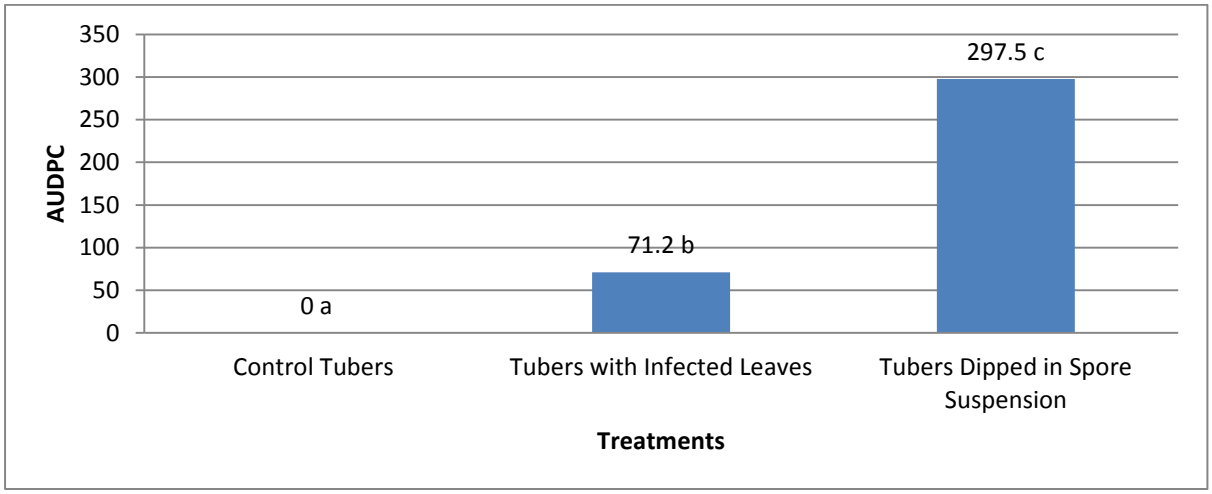

Figure 4.5 Area under disease progress curve (AUDPC) for different inoculum sources of *Alternaria alternata* for black pit trial I.
p<0.001, CV = 9.3%
Values on bars followed by the same letter are not significantly different at the 5% level of significance.

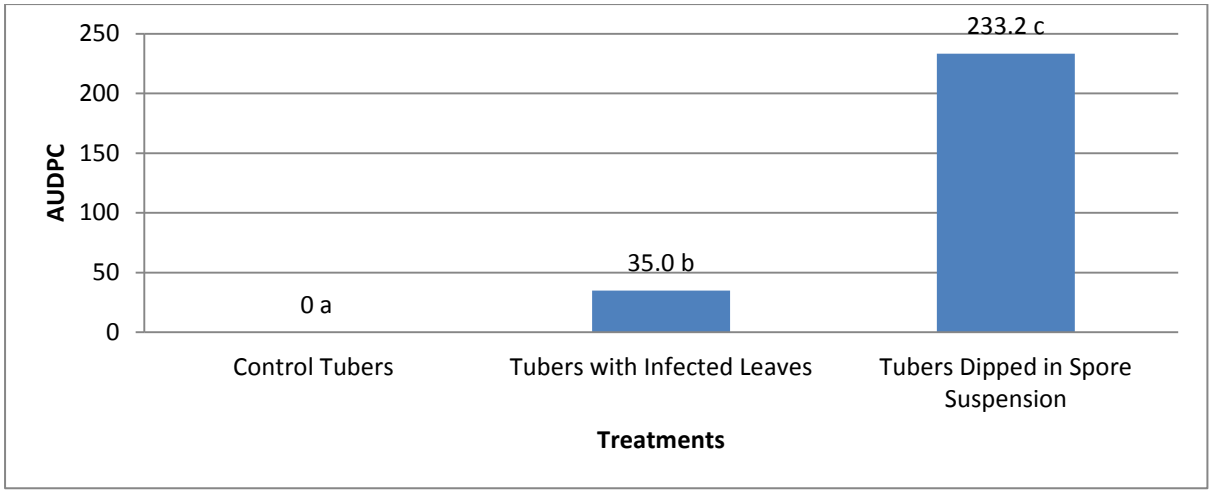

Figure 4.6 Area under disease progress curve (AUDPC) for different inoculum sources of *Alternaria alternata* for black pit trial II.

$p<0.001$, CV = 23%

Values on bars followed by the same letter are not significantly different at the 5% level of significance.

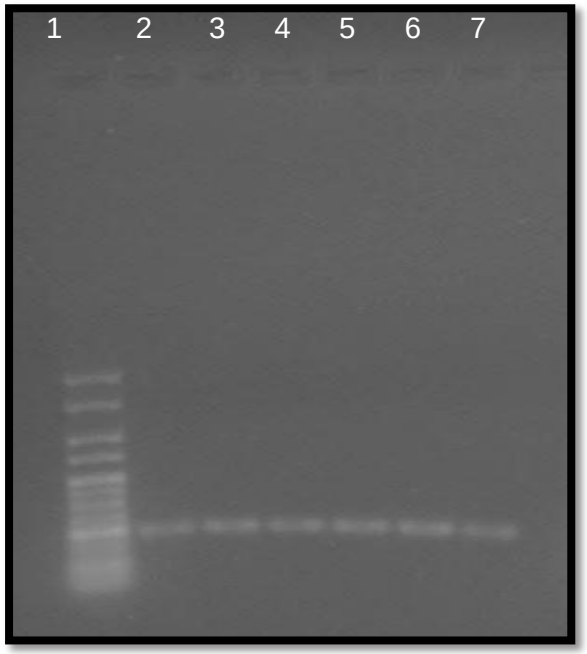

Figure 4.7 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* in inoculum sources of brown spot trial I & II. Description of lanes: 1, Hyper Ladder V; 2, Inoculated Tubers trial I; 3, Inoculated Soil (uncovered) trial I; 4, Inoculated Plants trial I; 5, Inoculated Tubers trial II; 6, Inoculated Soil (uncovered) trial II; 7, Inoculated Plants trial II.

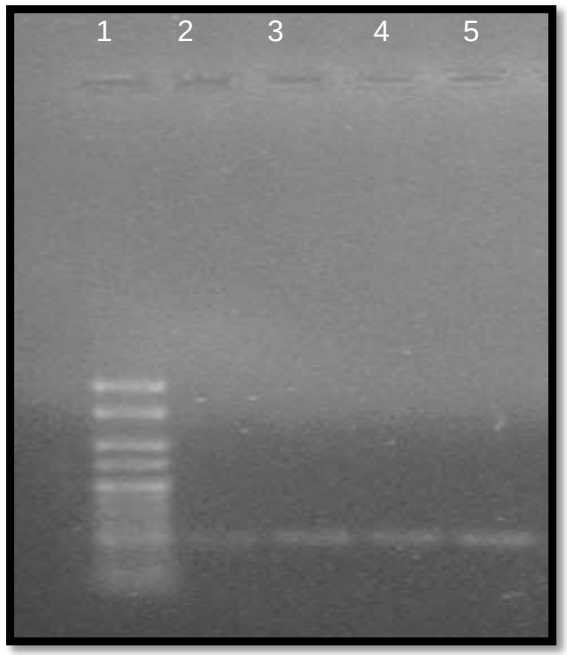

Figure 4.8 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* in inoculum sources of black pit trial I & II. Description of lanes: 1, Hyper Ladder V; 2, Dipped Tubers trial I; 3, Tubers with Leaves trial I; 4, Dipped Tubers trial II; 5, Tubers with Leaves trial II.

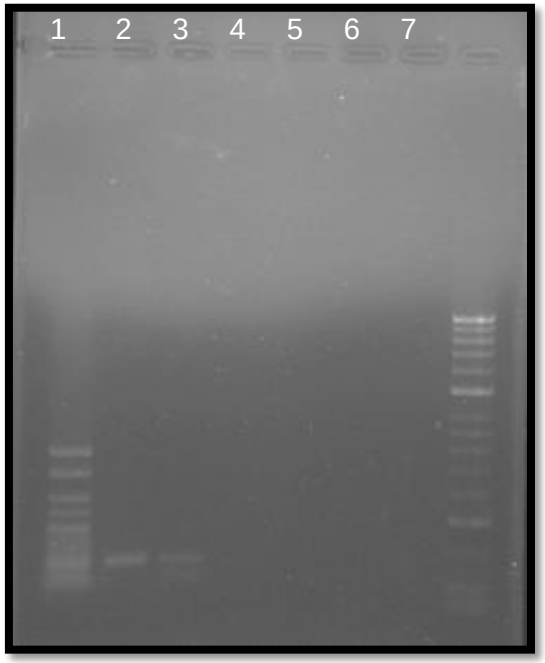

Figure 4.9 Agarose gel electrophoresis amplified DNA product to test for the presence of *Alternaria alternata* and *Alternaria solani* with PCR primers A. alt-F3 A. alt.-R1.1. Descriptions of Lanes: 1, Hyper Ladder V; 2, *Alternaria alternata*; 3, *Alternaria solani*; 8, Hyper Ladder II.

4.4 DISCUSSION

Preliminary trials indicated that when *A. alternata* inoculated tubers were planted, the plants developed brown spot symptoms. When potato plant foliage was inoculated with *A. alternata* the tubers did not develop black pit symptoms. It was also observed that when the soil was inoculated with *A. alternata* spores the lower leaves developed more brown spot symptoms than the younger leaves.

The planting of *A. alternata* inoculated tubers resulted in significantly higher disease severity than the other treatments, as shown by the AUDPC. Planting of inoculated tubers plays an important role, as the tubers are a source of inoculum that may increase black pit disease. Planting disease free tubers may limit the amount of initial inoculum, thus reducing brown spot disease. None of the daughter tubers of any of the treatments developed black pit disease. Thus, when planting an inoculated tuber, the foliage may become diseased with brown spot; however the daughter tubers may not develop black pit disease. Apparently brown spot developed on the lower leaves of the plants near the soil surface first. The leaves came into direct contract with the soil, and soil splashed onto the leaves during watering. Various *Alternaria* spp. have been shown to survive in the soil (Maude *et al.,* 1972; Ansari *et al.,* 1989; Pryor *et al.,* 2002). It is thus possible that *A. alternata* that causes brown spot and black pit may be able to survive in the soil, at least for a short period. In neither of the trials the daughter tubers that were in direct contact with the inoculated soil developed black pit.

By removing the foliage before harvesting the amount of inoculum that came into contact with the daughter tubers were limited. None of the daughter tubers developed black pit disease, and no disease developed when the daughter tubers were placed in conditions favouring the development of black pit disease. It can be concluded that the daughter tubers did not become infected with *A. alternata* spores in the soil. The optimal inoculum concentration and soil environment needed for the development of black pit on tubers is unknown. Under different conditions (e.g. concentration of inoculum) the tubers may develop black pit.

The pot trial was conducted in a greenhouse that limited air currents, and as *Alternaria* spp. are dispersed mainly by wind (Rotem, 1994), no secondary spread of *A. alternata* occurred in the pot trials. The plants were also covered with polyethylene bags that limited the secondary spread between the plants.

According to Stevenson *et al*. (2001) premature dropping of leaves only occurs with severe infection. Premature dropping of leaves occurred in the following treatments: (i) inoculated tubers, sterilised soil, uninoculated plants; (iii) uninoculated tubers, inoculated soil uncovered, uninoculated plants; (iv) uninoculated tubers, sterilised soil, inoculated plants. The disease severity on the remaining leaves was less than 1%. If the plants were grown in commercial fields and secondary spread of inoculum occurred, the disease severity throughout the growing season may have been higher.

In the black pit symptom development trial, the treatment where the tubers were dipped in an *A. alternata* spore suspension resulted in a significantly higher black pit disease severity than the tubers that were only placed next to brown spot infected leaves. When the tubers were dipped in the *A. alternata* spore suspension the spores came into direct contact with the entire tuber surface and the favourable environmental conditions resulted in a higher disease severity.

At harvesting the ideal would be to harvest the daughter tubers with sufficient care in order to prevent contamination with *A. alternata* spores that can cause black pit disease. In commercial fields this is however not possible. As *A. alternata* infected tubers develop black pit disease under conditions of high humidity, it is advisable that the relative humidity in storage facilities should be low to prevent the development of black pit disease.

In conclusion, by planting tubers showing symptoms of black pit, brown spot may develop on foliage as the infected tubers act as a source of inoculum for *A. alternata*. When brown spot disease is present on the foliage, black pit will not necessarily develop on the tubers in storage. To prevent brown spot and black pit symptom development, it is important to plant disease free tubers and harvest with care in dry weather especially when brown spot has been observed on the foliage. More research is still needed on the control of brown spot and black pit in commercial potato fields. As black pit is mosly a post-harvest disease it is important to conduct research on the contamination of A. alternata in the packhouses and to determine what effect storage conditions have on symptom development.

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CHAPTER 5
SUSCEPTIBILITY OF VARIOUS POTATO CULTIVARS TO ALTERNARIA ALTERNATA

ABSTRACT

Alternaria alternata causes brown spot and black pit of potatoes. Potato cultivars have significant different levels of susceptibility to diseases. Trials were conducted to determine the disease susceptibility of selected cultivars to brown spot and black pit of potatoes. Potato cultivars included in the investigation differed in their susceptibility to brown spot and black pit. Cultivar Lanorma showed the lowest disease severity to both brown spot (trial I: 4.6%, trial II: 4%). Cultivars Buffelspoort and Avalance showed the lowest disease severity to black pit in trial I (1.4%) and trial II (3.8%) respectfully. Cultivars Avalanch and Frodo showed the highest disease severity for brown spot (trial I: 14.6%, trial II: 7.1%) and black pit (trial I: 19%, trial II 13.2%) respectfully.

5.1 INTRODUCTION

Alternaria alternata (Fr.) Keissler causes brown spot and black pit of potatoes (*Solanum tuberosum* L.). The foliar disease is known as brown spot. The lesions are small and brown on the leaves and stems and initially develop on the abaxial side of the leaf (Droby *et al.,* 1984a; Stevenson *et al.,* 2001). The disease of the tubers is known as black pit. Black pit symptoms appear as sunken dark lesions with defined borders and develop mainly in storage (Droby *et al.,* 1984b; Stevenson *et al.,* 2001).

Different potato cultivars display different levels of disease susceptibility, such as differences in susceptibility to early blight, caused by *Alternaria solani* Sorauer (Ellis). The potato cultivars Mondial, Fianna and BP1 are moderately susceptible to early blight, whereas cv. Hertha is moderately resistant (Visser, 1999).

Early maturing cultivars are more susceptible to *A. solani* than late maturing cultivars because the late maturing cultivars keep on forming leaves and new tubers until the environment changes to such an extent that disease development is favoured (Johanson & Thurtson, 1990; Buckskin &Katahdin, 1991). This may be the same in the case of brown spot and black pit of potatoes caused by *A. alternata* (Stevenson *et al.,* 2001). Droby *et al.* (1984b) inoculated potato tuber cv. Blank, Cardinal, Desiree, Spunta and Up-To-Date with an *A. alternata* spore suspension to determine if different cultivars may have different susceptibility to black pit. He found that cv. Up-To-Date showed a lower black pit incidence

than the other cultivars, and it seems that similar to early blight mechanical injury plays a major role in the infection of *A. alternata* of potato tubers. It is of interest to remember that the foliage and tubers may have different susceptibility levels to brown spot and black pit of potatoes. Potato cultivar Avalanche has a medium susceptibility to foliar late blight (caused by *Phytophthora infestans*), and low tuber susceptibility (British Potato Council, 2000). Similar differences in susceptibility between foliage and tubers can be seen in cv. Pentland Dell and Shepody (British Potato Council, 2000). Brown spot disease of potatoes, caused by *A. alternata*, seems to have similar disease severity between early, medium and late maturing cultivars, where as black pit disease severity seems to be higher in medium to late maturing cultivars (Kapsa & Osowski, 2011). Potato cultivars tested by Kapsa & Osowski (2011), that are less susceptible to brown spot are: Augusta, Dorota, Gabi, Gracja, Albatros, Clarissa, Monsen, Zebra, Neptun, Rudawa, and Syrena.

There is no true resistance to *A. solani* in cultivated potato cultivars. Jansky *et al.* (2008) conducted an experiment to determine if wild relatives of cultivated potatoes contain any tolerance to early blight. They found that tolerance is evident in some species but the tolerance was not species-specific and that diploid species had higher levels of tolerance to early blight than the tetraploid European cultivated potato varieties.

Planting resistant cultivars is one of the most important measures in controlling a disease (Agrios, 1997). It is thus of interest to determine which potato cultivars are least susceptible to brown spot. The aim of this study was to determine if there is a difference in resistance to potato black pit and brown spot between selected cultivars.

5.2 MATERIALS AND METHODS

5.2.1 Isolation of pathogen and preparation of spore suspension

Leaves with brown spot lesions (obtained from Mpumalanga province) were surface sterilized with 2% NaOCl solution for one minute and rinsed twice in sterile distilled water. The lesions exised from the leaves were placed on half strength Potato Dextrose Agar (PDA) and Nutrient Agar (NA) and incubated at 25˚C for 7 days. Fungal growth form the plated tissue was subcultured onto half strength PDA and labeled AA16. The isolate (AA16) that was used as inoculum was confirmed as *A. alternata* by sequencing the ITS region as described in chapter 3.

The *A. alternata* isolate (AA16) was plated on V8-juice medium and incubated at 25˚C for 3 weeks in the dark to encourage sporulation. A spore suspension was made by adding 1ml

sdH_2O to the cultures aseptically and harvesting the spores aseptically. The spore suspension was transferred to a sterile flask. The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 1.5 x 10^4 spores per ml.

5.2.2 Trials

A trial was conducted to determine the susceptibility of different cultivars to brown spot and black pit of potatoes caused by *A. alternata*. The experiments were repeated once. The cultivars were chosen based on availability at the time.

Susceptibility of potato cultivars to brown spot

The following cultivars were used in the pot trial experiments: Avalanche, BP1, Buffelspoort, Fabula, Fianna, Frodo, Labadia, Lanorma, Mondial and Up-To-Date. Ten disease free generation zero seed tubers (from form Ceres, Mpumalanga, Northern Cape and North West) of each cultivar were planted individually into pots filled with 4kg steam-sterilised red top soil. The physical characterisitcs of the soil include a pH of 4.2, 74.8% coarse sand, 17.5% clay and 7.5% silt. For more information see Appendix C and D. Fourteen days after emergence of the potato plants the leaves were inoculated by firstly tapping the leaves lightly with a sterile stick, and then spraying the leaves with *A. alternata* spore suspension (1.5 x 10^4 conidia per ml sdH_2O) until runoff. Uninoculated control plants were sprayed with sdH_2O in the same manner as described above. High relative humidity was obtained by placing the pots in polyethylene bags that stayed on for the remainder of the growing season. The plants were watered every second day until field capacity. In order to stress the plants no fertilizer was added. Greenhouse temperature was kept under 30°C. Disease severity and incidence of brown spot were visually evaluated once a week, starting 1 week after inoculation. The disease severity for the early maturing potato cultivars (Avalanche, Buffelspoort and Labadia) were measured for 11 weeks until 91 days post emergence. The disease severity of the medium maturing potato cultivars (BP1, Fabula, Fianna, Lanorma and Up-To-Date,) was measured until 105 days post emergence. The disease severity of the late maturing potato cultivars (Frodo and Mondial) was measured for 15 weeks, until 119 days after emergence. Brown spot severity was measured using the percentage leaf area infected scale (Appendix A; James, 1971), (refer to 5.2.3).

Susceptibility of potato cultivars to black pit

The following potato G0 tuber cultivars were used in the experiments: Avalanche, BP1, Buffelspoort, Fianna, Frodo, Hertha, Mondial, Pentland Dell, Up-To-Date and Van Der Plank.

Ten disease free G0 seed tubers (from Ceres, Mpumalanga, Northern Cape and North West) of each cultivar were dipped into the *A. alternata* spore suspension (1.5 x 10^4 conidia per ml) for one minute. Uninoculated control disease free tubers were dipped into sdH2O for one minute. The tubers were placed in a plastic bag in order to create highly humid conditions and incubated in the dark for 3 weeks at 25°C. Disease severity and incidence of black pit were visually evaluated once a week for 3 weeks, starting one week after tuber inoculation. Severity of black pit on tubers was measured by using the percentage tuber area infected scale (Appendix B; James, 1971). The experiment was repeated once.

5.2.3 Disease assessment

Severity of brown spot on the plants was measured by using the percentage leaf area infected scale (Appendix A; James, 1971), and severity of black pit on tubers was measured by using the percentage tuber area infected scale (Appendix B; James, 1971). Area Under Disease Progress Curve (AUDPC) was calculated for both of the trials, using the following formula (Shaner & Finney, 1977):

$$AUDPC = \sum_{i=1}^{n} \left[\frac{(y_i + y_{i+1})}{2}\right](t_{i+1} - t_i)$$

Where, *yi* is the percent of diseased area on the *ith* date, *ti* is the date on which the disease was scored *(ith* day), n is the number of dates on which disease was scored. The results were also statistical analysed using GenStat (GenStat for Windows, 2012).

The average brown spot disease development (AUDPC) and average black pit disease severity (in %) of early, medium and late maturing cultivars were also calculated.

5.3 RESULTS

Results from the first and second brown spot pot trials showed varying levels of brown spot throughout the growing season in all ten cultivars (Figure 5.1 and 5.2). The greenhouse used was not able to control the temperature effectively in the summer and the temperature rised to 35°C in the day during the second trial, resulting in a higher temperature in trial II. In the first trial cv. Avalanche and Fianna developed the significantly highest level of brown spot disease, based on the Area Under Disease Progress Curve (AUDPC) (Figure 5.3). The AUDPC reflects the total amount of disease over the growing period. Cultivars with the highest AUDPC values are more susceptible to the disease. Cultivars Buffelspoort and Up-To-Date developed significantly less disease than the other cultivars. The medium maturing cultivars generally developed less brown spot disease, followed by early maturing cultivars, and the late maturing cultivars developed the highest average brown spot disease (Table

5.1). No disease development was observed on control plants in this trial. The highest and lowest mean disease severity measured at any time in the growing season for the potato cultivars were Avalanche with 14.6% disease severity and Lanorma with 4.6% disease severity.

In the second trial, cv. Avalanche, Frodo and Mondial developed significantly more brown spot disease (Figure 5.3) and cv. Buffelspoort, Lanorma and Up-To-Date developed significantly less disease compared to the other cultivars. No disease development was observed on plants from the control pots in this trial. In general the late maturing cultivars developed less brown spot disease. Early maturing cultivars developed on average the most disease, followed by the medium maturing cultivars (Table 5.1). The highest and lowest mean disease severity measured at any time in the growing season were once again recorded on Avalanche with 7.1 % disease severity and Lanorma with 4% disease severity.

Results from the first and second black pit trial showed varying levels of black pit disease between the ten cultivars (Figure 5.4 and 5.5). In the first trial cv. Van Der Plank, Up-To-Date and Frodo developed significantly more disease compared to the other cultivars. Cultivars Avalanche and Buffelspoort (first trial only) developed significantly less disease than the other cultivars, with no significant difference to the control plants. No disease development was observed on tubers from the control treatments in this trial. Late maturing cultivars had on average the highest disease severity, followed by medium maturing cultivars and early maturing cultivars.

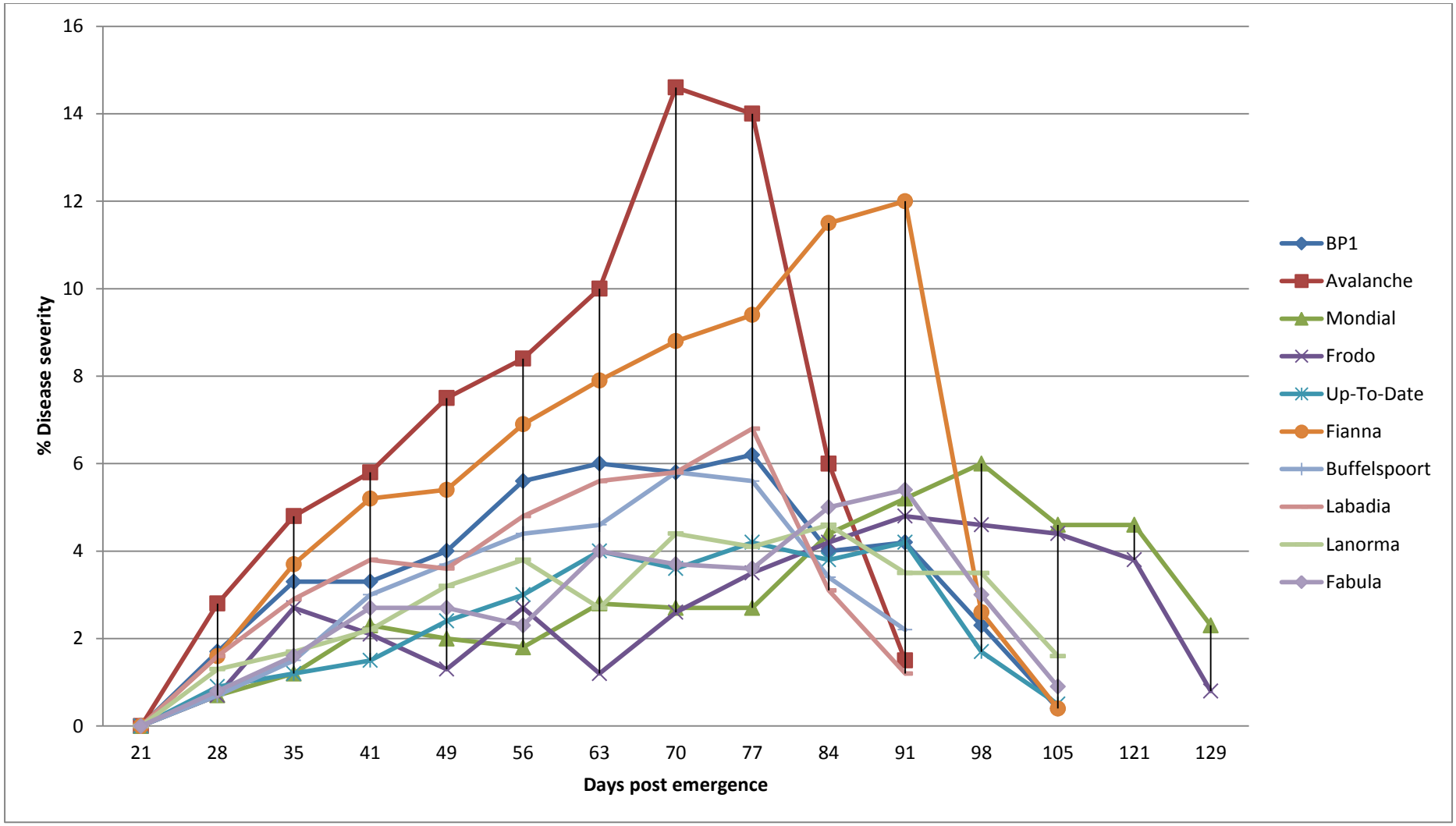

Figure 5.1 Disease progress curves for cultivar susceptibility evaluation against brown spot potato disease caused by *Alternaria alternata*, greenhouse trial I.

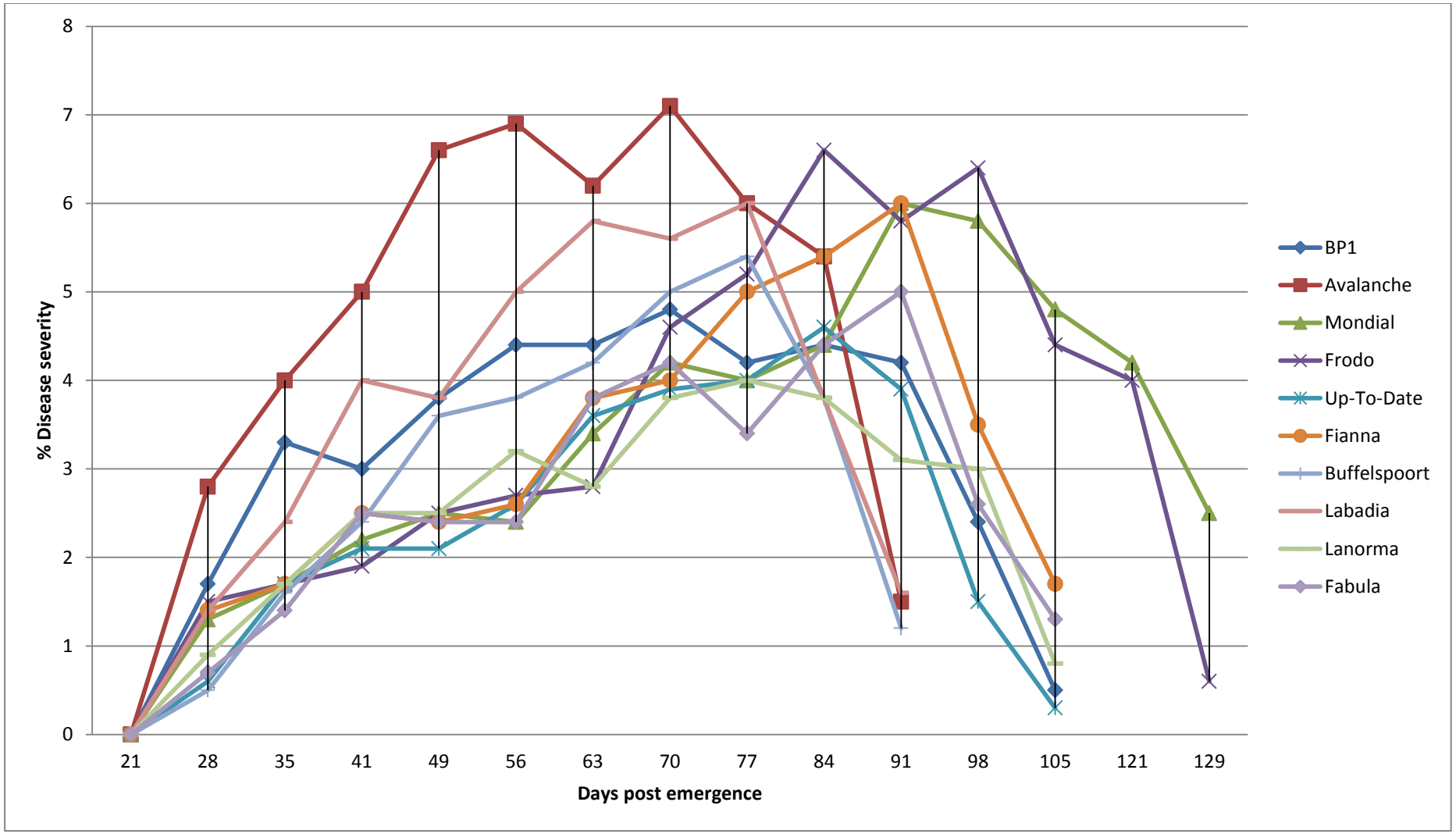

Figure 5.2 Disease progress curves for cultivar susceptibility evaluation against brown spot potato disease caused by *Alternaria alternata*, greenhouse trial II.

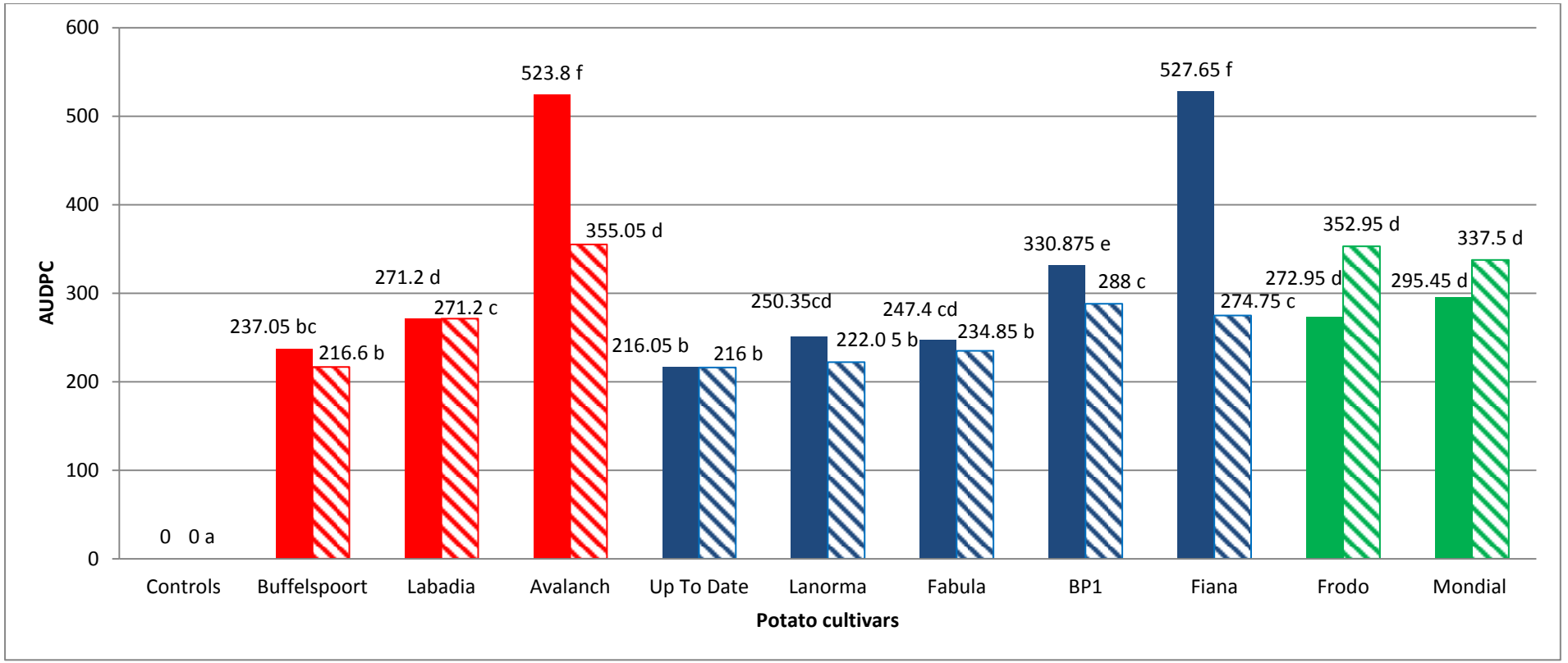

Figure 5.3 Area under disease progress curve (AUDPC) values indicating susceptibility of potato cultivars to brown spot disease caused by *Alternaria alternata* trial I (solid bars) and trial II (stripe bars).

Trial I: $p<0.001$, CV = 3.8%

Trial Ii: $p<0.001$, CV = 5.4%

Values on bars followed by the same letter are not significantly different at the 5% level of significance. Red bars indicate early maturing cultivars, blue bars indicate medium maturing cultivars and green bars indicate late maturing cultivars.

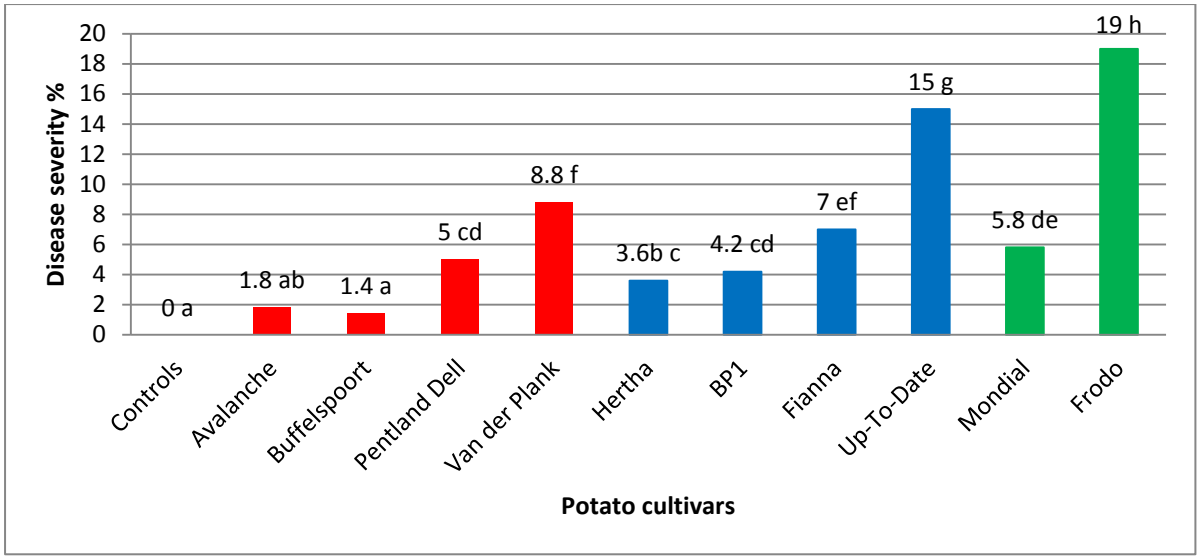

Figure 5.4 Mean disease susceptibility of potato cultivars to black pit disease caused by *Alternaria alternata* in greenhouse trial I.

p<0.001, CV = 12%

Values on bars followed by the same letter are not significantly different at the 5% level of significance. Red bars indicate early maturing cultivars, blue bars indicate medium maturing cultivars and green bars indicate late maturing cultivars.

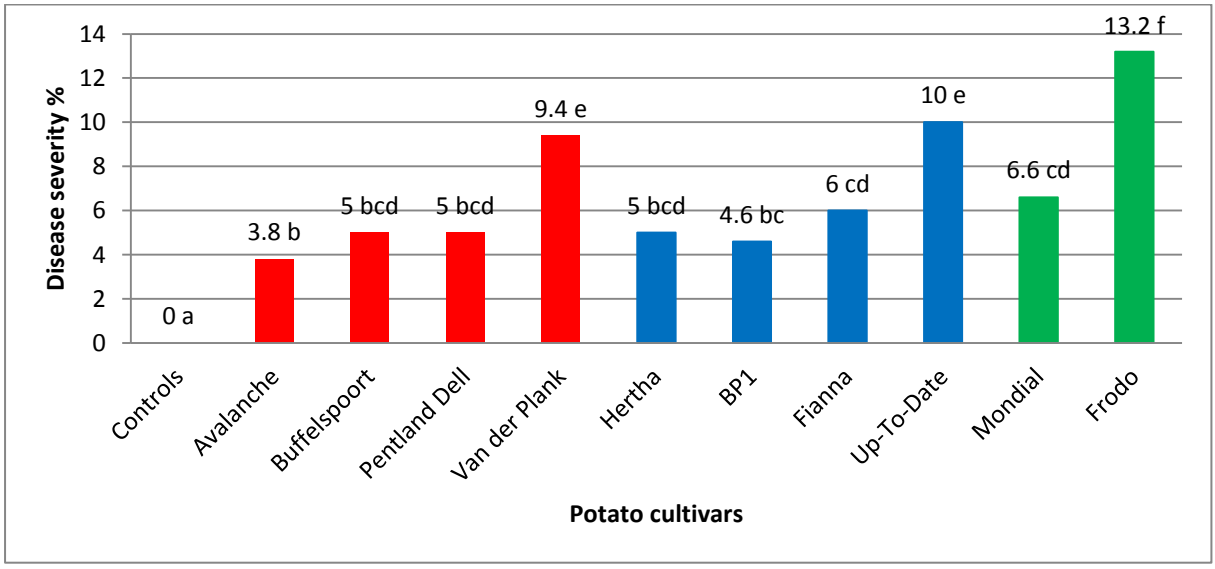

Figure 5.5 Mean disease susceptibility of potato cultivars to black pit disease caused by *Alternaria alternata* during greenhouse trial II.

p<0.001, CV = 11.7%

Values on bars followed by the same letter are not significantly different at the 5% level of significance. Red bars indicate early maturing cultivars, blue bars indicate medium maturing cultivars and green bars indicate late maturing cultivars.

Table 5.1 Average brown spot and black pit disease development of potato cultivars grouped into early, medium and late maturing cultivars.

	Early maturing cultivars	**Medium maturing cultivars**	**Late maturing cultivars**
Brown spot first trial (AUDPC)	281	247	345
Brown spot second trial (AUDPC)	344	314	284
Black pit first trial (% disease severity)	4.3%	7.5%	12.4%
Black pit second trial (% disease severity)	5.8%	6.4%	9.9%

5.4 DISCUSSION

Planting resistant cultivars is one of the most important control measures in controlling plant diseases (Agrios, 1997). In both trials Up-To-Date had the lowest disease susceptibility to brown spot, correlating with the results obtained by Droby 1984b, however Up-To-Date was one of the cultivars that was the most susceptible to black pit disease, indicating that there may not always be correlation between brown spot and black pit disease susceptibility. In this study cv Avalanche, Fianna and Mondial also had different susceptibility levels to brown spot and black pit of potatoes. Kapsa & Osowski (2011) obtained similar results.

The disease severity in trial I was double of that in trial II. It may be because the environmental conditions in the greenhouse were more favourable for the disease. The greenhouse used was not able to always control the temperature effectively, resulting in temperatures rising to 35°C in summer. Trial I was planted in March and trial II was planted in November, resulting in higher temperatures in trial II. *A. alternata* disease can develop in a wide range of temperatures, with the general optimal, minimum and maximum temperatures being 22.5˚C, 10˚C and 35˚C respectively (Rotem, 1994). Thus 35˚C in the greenhouse would have caused a less desirable temperature for *A. alternata*, resulting in less disease than in trial I.

Early, medium and late maturing cultivars have different susceptibility levels to early blight, with late maturing cultivars being more resistant (Wharton & Kirk, 2007). The early maturing cultivars used in these experiments were: Avalanche, Buffelspoort, Labadia (brown spot trials), Pentland Dell (black pit trials) and Van Der Plank (black pit trials). The medium maturing cultivars were: BP1, Fabula (brown spot trials), Fianna, Hertha (black pit trials)

Lanorma (brown spot trials) and Up-To-Date. The late maturing cultivars were Frodo and Mondial. The late maturing cultivars seem to be more susceptible to black pit, and early maturing cultivars less susceptible. Similar results were obtained by Kapsa & Osowski (2011); they determined that on average late maturing cultivars seem to have higher susceptibility to black pit than early and medium maturing cultivars. In the two brown spot trials there were no differences between the susceptibility of early, medium and late maturing cultivars. Kapsa & Osowski (2011) determined that the average brown spot disease severity between early, medium and late maturing cultivars are similar. The experiment of Kapsa and Ososki (2011) was however conducted on detached leaves, and disease susceptibility to the potato cultivars may differ in pot trial, as spray-inoculation of intact plants represents a more realistic means of determining the pathogenicity of *Alternaria* spp. than detached leaf technique (Peever *et al.,* 1999).

No cultivars were 100% resistant against brown spot or black pit diseases of potatoes. Based on the results obtained in these experiments, cv. Up-To-Date is the least susceptible to brown spot and cv. Avalanche and Buffelspoort are the least susceptible to black pit disease of potatoes.

Potato tubers seem to have different resistance levels against black pit of potatoes. Potato cultivar choice may be an important measure in controlling the spread and incidence of brown spot and black pit of potatoes. Up-To-Date and Buffelspoort are the least susceptible to brown spot, and cv. Avalanche is the least susceptible to black pit. To help growers make the best decision based on their specific needs, it will be of interest to screen more South African potato cultivars for susceptibility or tolerance against brown spot and black pit.

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CHAPTER 6

ALTERNATIVE HOSTS FOR *ALTERNARIA ALTERNATA*

ABSTRACT

Agricultural spoilage due to *Alternaria alternata* is estimated at 20% worldwide. *A. alternata* causes brown spot on the foliage and black pit on the tubers of potatoes. To determine if *A. alternata* isolated from potatoes can cause disease on other crops, a pot trial was conducted. The crops inoculated were tomatoes, cabbage, mustard, wheat, oats, tobacco and maize. Only the tomato plants became diseased when inoculated with *A. alternata.*

6.1 INTRODUCTION

Alternaria spp. are able to cause disease on many different plants with various different symptoms, both in the field and as post-harvest decay (Rotem, 1994; Thomma, 2003). An estimated 20% of all agricultural spoilage is caused by *Alternaria* spp. (Potkar & Jahav, 2012).

On potatoes (*Solanum tuberosum* L.), *Alternaria* spp. cause early blight, brown spot and black pit. *Alternaria alternata* (Fr.) Keissler causes the latter two diseases. The foliar disease is known as brown spot. Brown spot lesions are small and brown, and occur on the leaves and stem of the plants. On the leaves the lesions initially develop on the abaxial side (Stevenson *et al.,* 2001). The disease of the tubers is known as black pit. Black pit symptoms on the tubers appear as sunken, brown to black lesions with defined borders and can develop in storage (Droby *et al.,* 1984b; Stevenson *et al.,* 2001). *Alternaria solani* Sorauer (Ellis) causes early blight of potatoes. The symptoms are dark concentric lesions typically found on the older leaves (Stevenson *et al.,* 2001). *A. solani* can also cause disease on damaged tubers.

The *A. alternata* tobacco pathotype causes brown spot of tobacco (*Nicotiana tabacum* L.). Symptoms on the leaves appear as brown circular lesions with a yellow halo, about 2 to 8 days after inoculation (Slavov *et al.,* 2004).

Alternaria arborescens Simmons, also known as *Alternaria alternata* f. sp. *lycopersici*, causes black mould and leaf blight of tomato plants, *Solanum esculentum* (L.) H. Karst. (Pearson & Hall, 1975; Akhtar *et al.,* 1994). The pathogen causes a black sunken lesion on the tomato fruit surface (Pearson & Hall, 1975), whereas tomato leaf blight symptoms develop from the tip of the leaf as a brown concentric lesion and then yellowing of the leaves. As the disease progresses, the lesions coalesce and spread along the margin of the

leaf petiole. The symptoms typically appear on the lower leaves and spread to the upper leaves of the plant, usually under high humidity. Under severe infection, defoliation can occur (Akhtar *et al.,* 1994). In a study by Mesbah in 2000, 196 Solanaceae species were tested against the AAL toxin produced by *A. arborescens.* Out of these 25 plant species 14 of the Solanum genera tested sensitive for the toxin (Mesbah *et al.,* 2000).

Weir *et al.* (1998) using RAPD-PCR-analysis found no significant genetic difference between *A. alternata* isolates from potato and tomato plants. However a large and significant difference was found between isolates of *A. solani* isolated from potato and tomato plants. It is still unclear if the *A alternata* strains that causes disease on potatoes can cause disease on any plants other than tomato. It is important to determine if *A. alternata* isolated from potatoes, can cause disease on crops used in crop rotation with potatoes.

The aim of this study was to determine if any alternative hosts exist for this specific *A. alternata* strains used in this study, and if this specific *A. alternata* strains can cause disease on plants used in rotation with potatoes in South Africa.

6.2 MATERIALS AND METHODS

A pot trial was conducted to determine which of the following crops could be a host for the brown spot pathogen, *A. alternata*. The crops used in the preliminary trial were: mustard (*Brassica juncea* L.), wheat, barley (*Hordeum vulgare* L.), maize (*Zea mays* subsp. *mays* L.), cabbage (*Brassica oleracea* L.) and tomatoes. The crops used in the first and second trials are presented in Table 6.1.

Table 6.1 Crops used in the *Alternaria alternata* alternative hosts trials I and II.

Crops	Cultivar I	Cultivar II	Cultivar III
Tomato	Oxheart	Floradade	Rodade
Cabbage	Drumhead	Cape Spitz	Zoe
Mustard	Florida Broadleaf	Green Giant	Joek
Wheat	CRN 826	SST 835	SST 822
Oats	Witteberg	Maluti	Overberg
Tobacco	BS3	LK8	
Maize	DKC 78-15B	Pannar 62-308bt	Pannar 62 36 B

Tomatoes were included as a positive control (Weir *et al.,* 1998). Untreated tomato, wheat, oats (*Avena sativa* L.) and maize seeds were used. The cabbage, mustard and tobacco seeds were pelleted seed (coated with kaolin), which makes planting easier. The cultivars

were chosen based on availability at the time. The following seed were obtained from Starke ayres: tomato seeds (cv. Oxheart, Floradade, Rodade) cabbage seeds (cv Drumheas and Cape Spitz) mustard seeds (cv, Florida Broadleaf, Green Giant, Joek). Cabbage cultivar Zoe was obtained form Premier seeds. The wheat (cv. CRN 826, SST 835, SST 822) and oats (cv. Witteberg, Maluti and Overberg) were obtained from Griekwaland-Wes Korporatief. The tobacco (cv. BS3 and LK8) were obtained from LARSS. The maize (cv. Pannar 62-308bt and Pannar 62 36 B) were obtained from Pannar and the maize cv. DKC 78-15B was obtained from Monsanto.

6.2.1 Isolation of the pathogen

Potato leaves with brown spot lesions were surface sterilized with 2% NaOCl solution for one minute and rinsed twice in sdH_2O. The lesions were excised from the leaves and placed on half strength Potato Dextrose Agar (PDA) and Nutrient Agar (NA) and incubated at 25˚C for 7 days in the dark. Pure fungal cultures were sub-cultured onto half strength PDA and the isolate labelled AA16. The isolate (AA16) that was used as inoculum was confirmed as *A. alternata* by sequencing the ITS region as described in chapter 3.

The *A. alternata* isolate (AA16) was plated on V8-juice medium and incubated at 25˚C for 3 weeks in the dark to encourage sporulation A spore suspension was made by adding 1ml sdH_2O to the cultures aseptically and harvesting the spores aseptically. The spore suspension was transferred to a sterile flask. The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 1.5×10^4 spores per ml.

The different crops were inoculated with the fungus at 3 different stages in their life cycle: as seedlings, before flowering and at fruiting.

6.2.2 Trial at seedling stage

Twenty seeds of each of the crops and cultivars were germinated in a polystyrene tray in the greenhouse and filled with steam sterilised vermiculite. Before planting, the tray was dipped twice in 2% NaOCl solution for one minute, and rinsed twice in sdH_2O. The first six seedlings that emerged within a day of each other, for each crop and cultivar, were removed with the root system and vermiculite intact and replanted into a second polystyrene tray that was cleaned as described previously. This was done to ensure that all the plants were the same age at inoculation. Fourteen days after planting, the seedlings were inoculated by spraying the leaves with *A. alternata* spore suspension (1.5×10^4 conidia per ml) until runoff. High humidity was obtained by placing the trays in polyethylene bags that stayed on for the remainder of the trial. The seedlings were watered every second day until field capacity.

The greenhouse temperature was kept under 30°C. In order to stress the plants no fertilization was added. Disease severity of brown spot was measured once a week for 3 weeks, starting 7 days after inoculation. Brown spot severity was measured using the percentage leaf area covered scale (Appendix A; James, 1971), (refer to 6.2.5).

6.2.3 Trial at mature plant stage before flowering

Twenty seeds of each of the crops and cultivars were germinated in a polystyrene tray in the greenshouse and filled with steam sterilised vermiculite. Before planting, the tray was dipped twice in 2% NaOCl solution for one minute, and rinsed twice in sdH2O. The first ten seedlings that emerged within a day of each other, for each crop and cultivar, were removed with the root system and vermiculite intact and planted into 10 pots filled with steam sterilised red top soil. The physical characterisitcs of the soil include a pH of 4.2, 74.8% coarse sand, 17.5% clay and 7.5% silt. For more information see Appendix C and D. Six seedlings for each crop and cultivar were chosen that were morphologically at the same height and growth stage. These plants were inoculated 3 to 4 weeks after sowing, by spraying the leaves with *A. alternata* spore suspension (1.5 x 10^4 conidia per ml) until runoff. All the crops were inoculated 4 weeks post planting, except cabbage and mustard, which were inoculated at 3 weeks due to their shorter growing season. High humidity was obtained by placing the trays in polyethylene bags that stayed on for the remainder of the trial. The plants were watered every second day until field capacity. The greenhouse temperature was kept constant at 25°C. In order to stress the plants no fertilization was added. Disease severity of brown spot was measured once a week for 3 weeks, starting 7 days after inoculation. Brown spot severity was measured using the percentage leaf area covered scale (Appendix A; James, 1971), (refer to 6.2.5).

6.2.4 Trial at fruiting stage

Twenty seeds of each of the crops and cultivars were germinated in a polystyrene tray in the greenshouse and filled with steam sterilised vermiculite. Before planting, the tray was dipped twice in 2% NaOCl solution for one minute, and rinsed twice in sdH2O. The first ten seedlings that emerged within a day of each other, for each crop and cultivar, were removed with the root system and vermiculite intact and replanted into 10 pots filled with steam sterilised red top soil. The physical characterisitcs of the soil include a pH of 4.2, 74.8% coarse sand, 17.5% clay and 7.5% silt. For more information see Appendix C and D. Six seedlings for each crop and cultivar were chosen that were morphologically at the same height and growth stage. These plants were inoculated as the fruits started to develop, by spraying the fruit with *A. alternata* spore suspension (1.5 x 10^4 conidia per ml) until runoff. High humidity was obtained by placing the trays in polyethylene bags that stayed on for the

remainder of the trial. The plants were watered every second day until field capacity. The greenhouse temperature was kept under 30°C. In order to stress the plants no fertilization was added. Disease severity of brown spot was measured once a week for 3 weeks, starting 7 days after inoculation. Brown spot severity was measured using the percentage leaf area covered scale (Appendix A; James, 1971), (refer to 6.2.5).

6.2.5 Disease assessment

Brown spot disease was visually evaluated using the percentage leaf area covered scale (0-100%), presented in Annex A (James, 1971). Area Under Disease Progress Curve (AUDPC) was calculated for both of the trials, using the following formula (Shaner & Finney, 1977):

$$AUDPC = \sum_{i=1}^{n} \left[\frac{(y_i + y_{i+1})}{2}\right](t_{i+1} - t_i)$$

Where, *yi* is the percent of diseased area on the *ith* date, *ti* is the date on which the disease was scored *(ith* day), n is the number of dates on which disease was scored. The results were also statistical analysed using GenStat (GenStat for Windows, 2012).

6.2.6 Confirmation of causative organism from lesions on crops

Leaves with lesions obtained from the trials were surface sterilized with 2% NaOCl solution for one minute and rinsed twice in sdH_2O. The lesions were excised from the leaves and placed on half strength PDA and NA and incubated at 25˚C for 1 week. Pure fungal cultures were sub-cultured onto half strength PDA. For preliminary identification the isolates were morphologically compared to the original cultures. Isolates were further identified by means of PCR.

One isolate from each treatment was identified by means of PCR. The fungal DNA was extracted from pure cultures using the ZR Soil Microbe DNA MiniPrep Kit (Zymo Research). A 1µL aliquot of this DNA solution was used for PCR, which was performed in a total of 25µL reactions. *Alternaria* sp.-specific primers (1µL of each primer), A. alt-F3 (5′ to 3′ TCTAGCTTTGCTGGAGACTC) (Schuhegger *et al.,* 2006), and A. alt.-R1.1 (5′ to 3′ AGACCTTTGCTGATAGAGAGT) (Schuhegger *et al.,* 2006), were used to generate short amplicons of 95bp. The PCR was performed under thermocycler conditions of 94°C for 10min, followed by 40 cycles of 95°C for 15s, 60°C for 60s, 72°C for 60s, and a final extension step of 72°C for 10min. The amplified products were subjected to gel-electrophoresis in a 1% agarose gel in 1 x TBE Buffer at 100V.

6.3 RESULTS

In the preliminary trial, tomato and mustard plants developed lesions after inoculation with *A. alternata*. Isolations from the tomato lesions yielded fungi morphologically similar to *A. alternata* isolate AA16, used in the inoculation. Isolations from the mustard lesions yielded fungi morphologically similar to *Alternaria* spp. but not *A. alternata* isolate AA16. The cultures were not identified as *A. alternata* by means of PCR as this was only the preliminary trial. Crop susceptibility to a pathogen may differ throughout its life cycle (Agrios, 1997). It was decided to test the susceptibility of the different crops at three points in the life cycle.

6.3.1 Trial I and II

In all the different life stages evaluated on the different crops only the tomato plants became diseased. As shown in Figures 6.1 to 6.3, none of the other crops were susceptible to *A. alternata*. *A. alternata* caused brown spot disease on all three tomato cultivars (Oxheart, Floradade and Rodade) under evaluation.

At seedling stage tomato cultivar Oxheart was statistically more susceptible to *A. alternata* than the other cultivars (Figure 6.4). Before flowering tomato cultivar Rodade seedlings were statistically most susceptible to *A. alternata* (Figure 6.5). At fruting stage of the tomato plants the AUDPC values (Figure 6.6) indicate that there is no statistically difference between the susceptibility of the three tomato cultivars to *A. alternata*. As only the tomato plants became diseased, only the tomato plants results are shown on all of the figures.

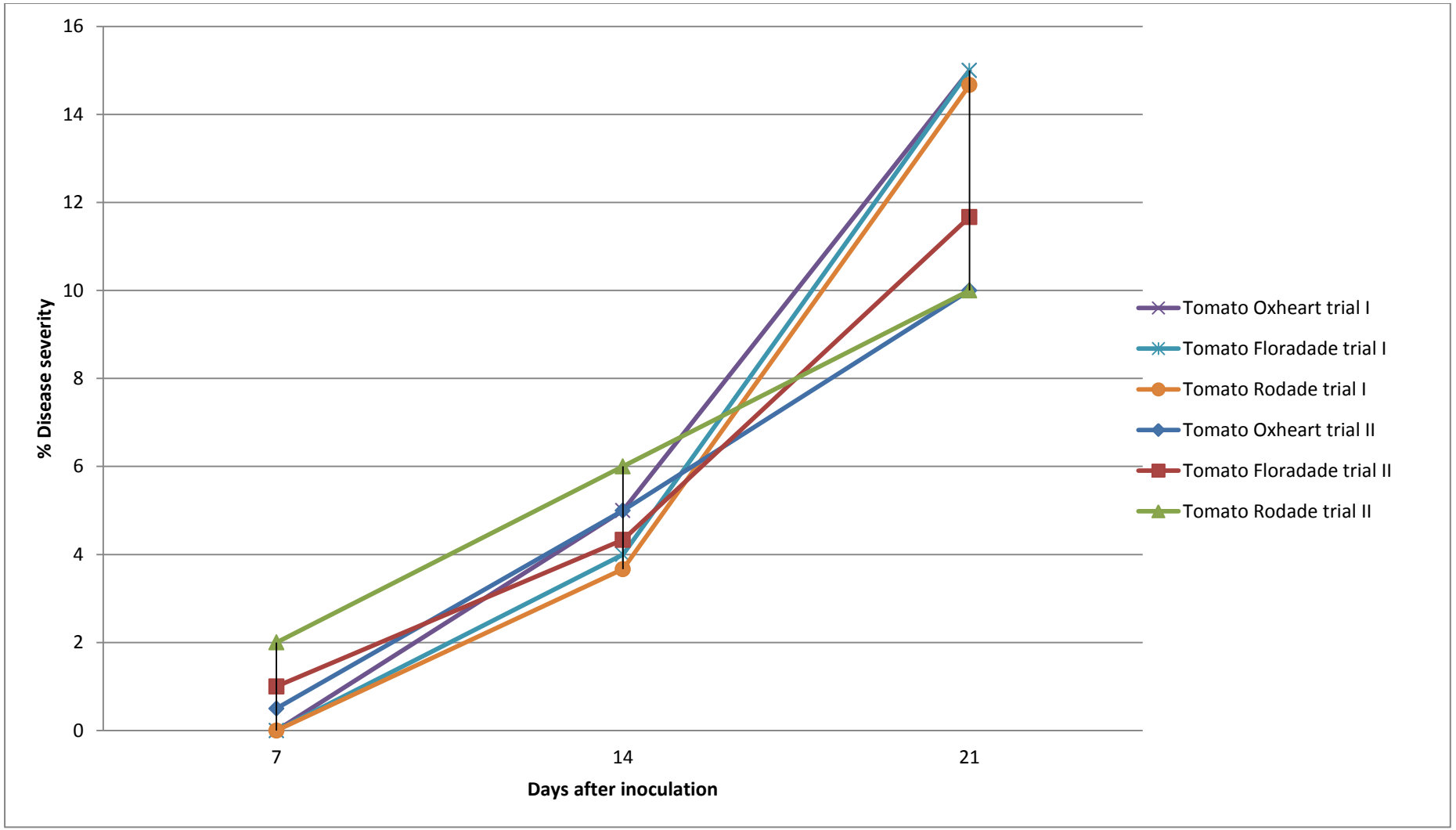

Figure 6.1: Susceptibility of tomato cultivars to *Alternaria* alternata (potato strain) at seedling stage as indicted by menas of disease progress curve (trial I and II).

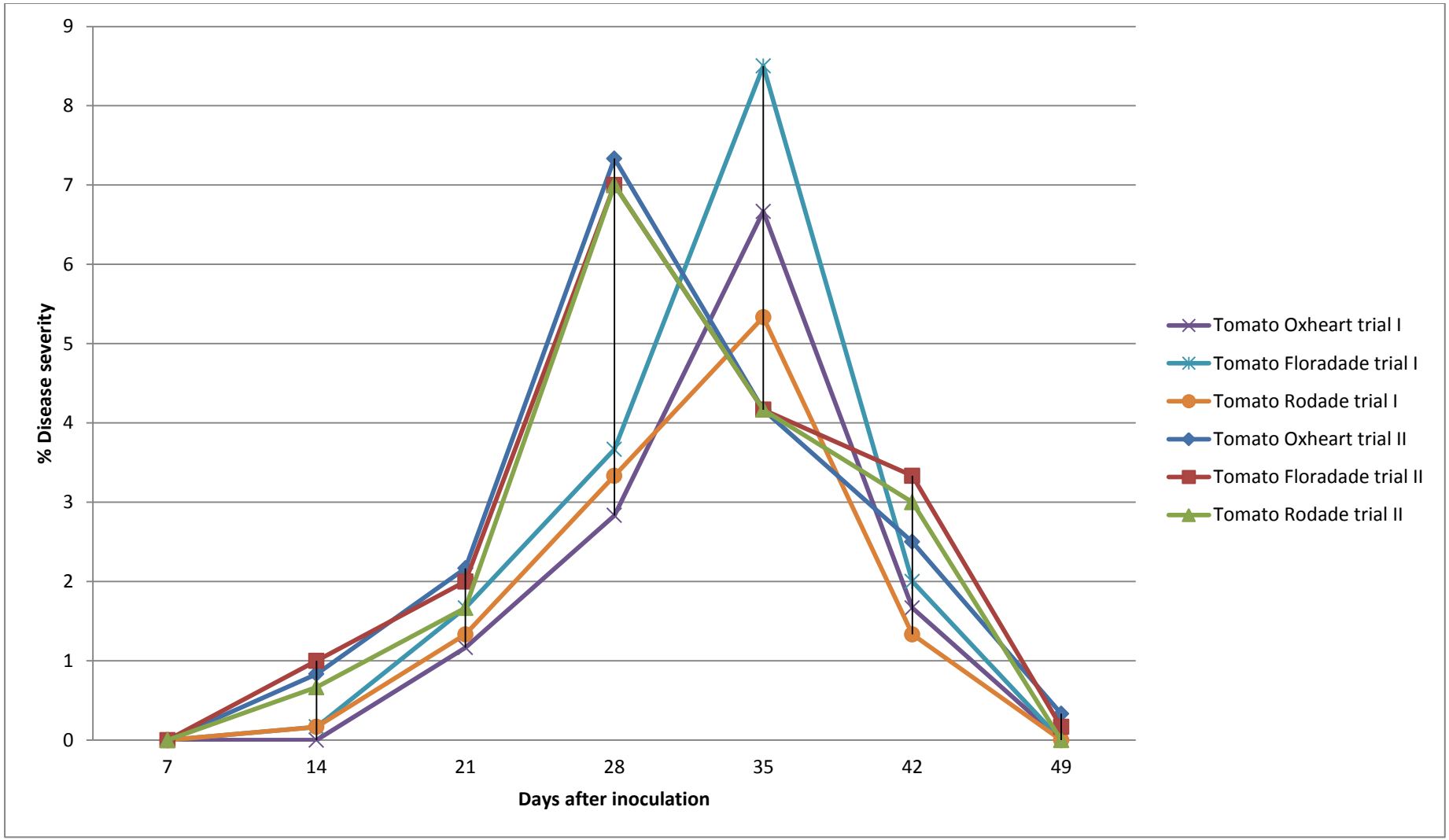

Figure 6.2: Susceptibility of tomato cultivars to *Alternaria* alternata (potato strain) before flowering as indicted by menas of disease progress curve (trial I and II).

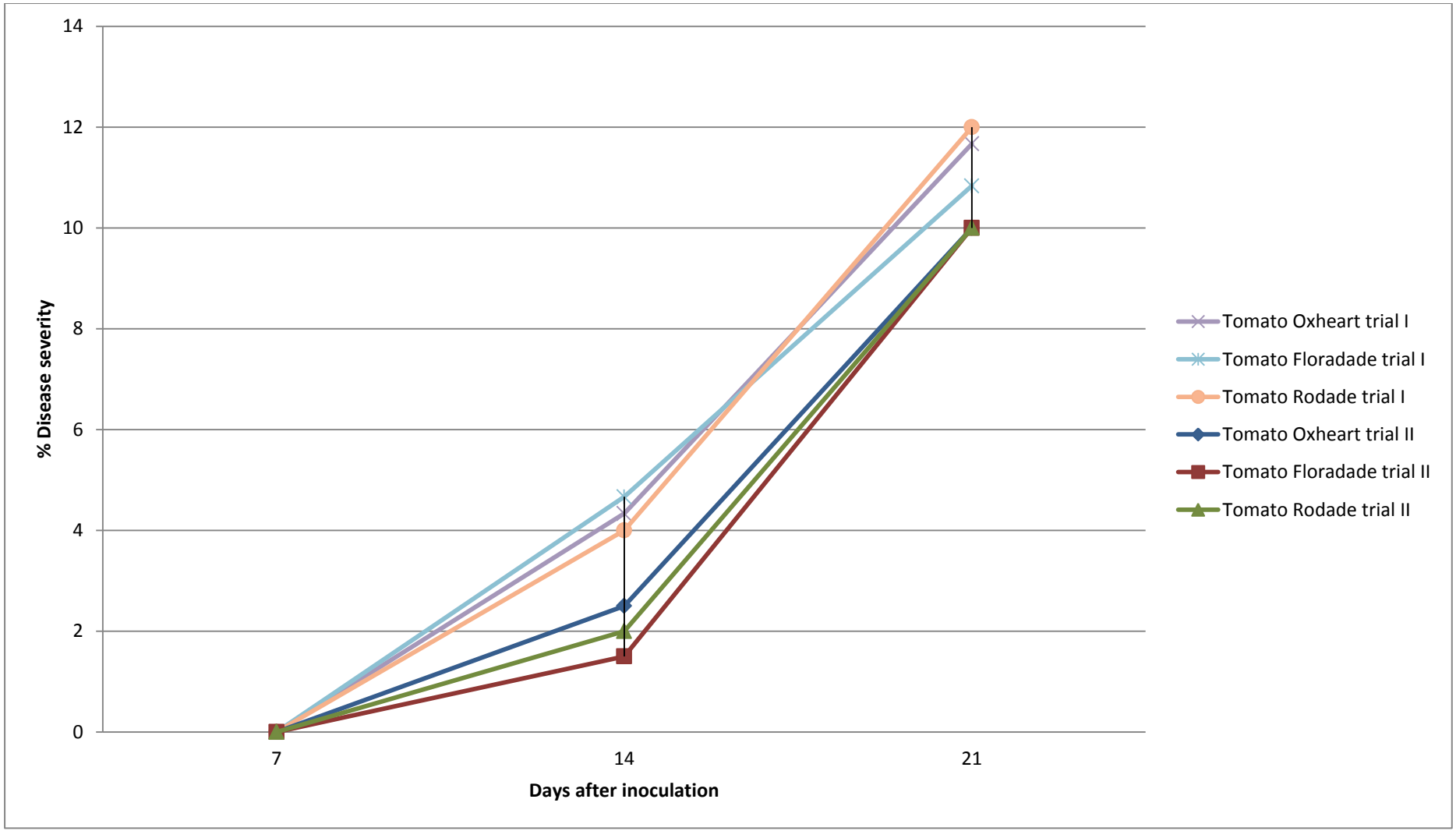

Figure 6.3: Susceptibility of tomato cultivars to *Alternaria* alternata (potato strain) at fruiting stage as indicted by menas of disease progress curve (trial I and II).

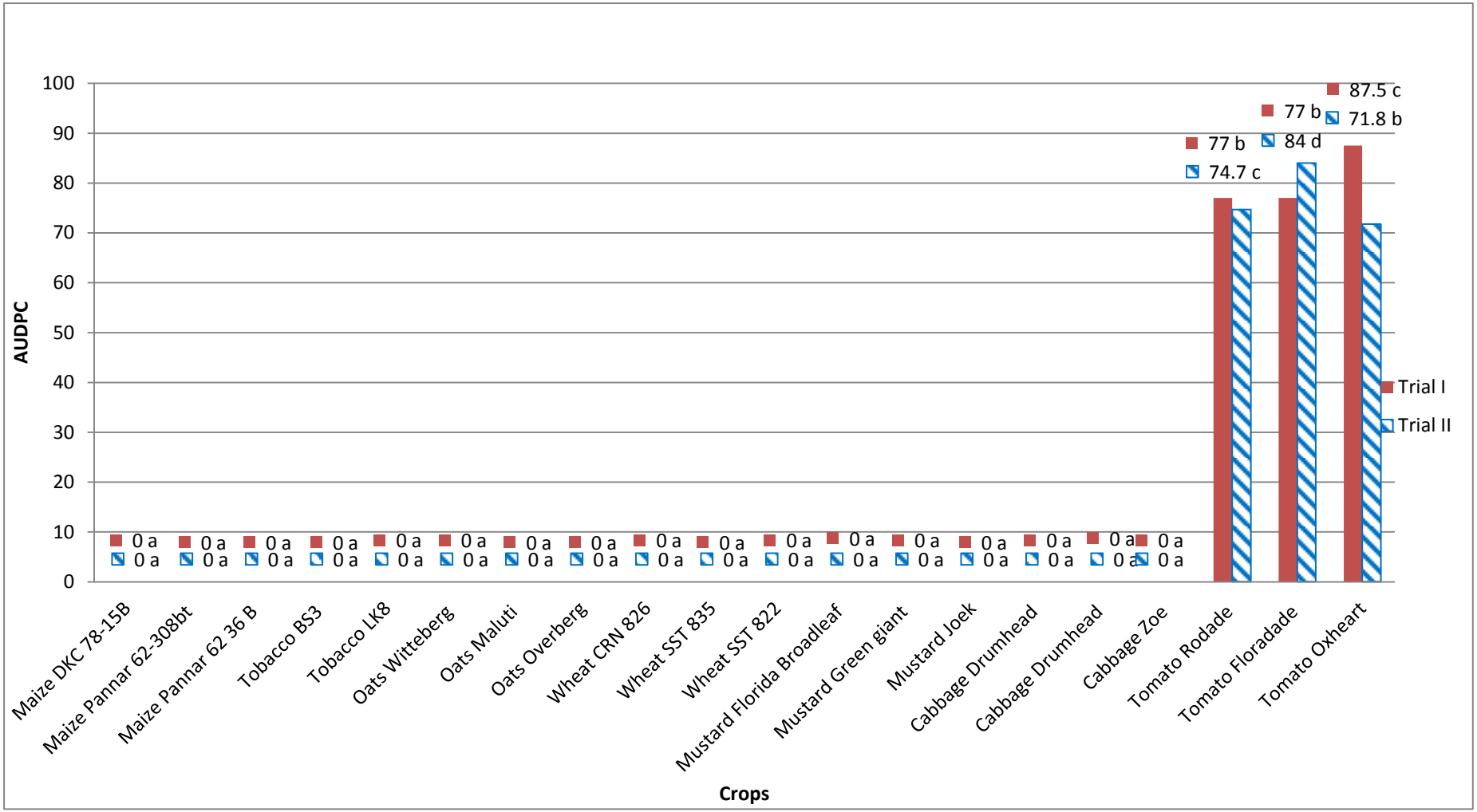

Figure 6.4: Susceptibility of different crops at seedling stage to *Alternaria alternata* (potato strain) expressed as area under disease progress curve (AUDPC) (trial I and II).

Trial I: $p<0.001$, CV = 10%

Trial II: $p<0.001$, CV = 12.8%

Bars with the same letters above them are not statistically different at the 5% level of significance per trial.

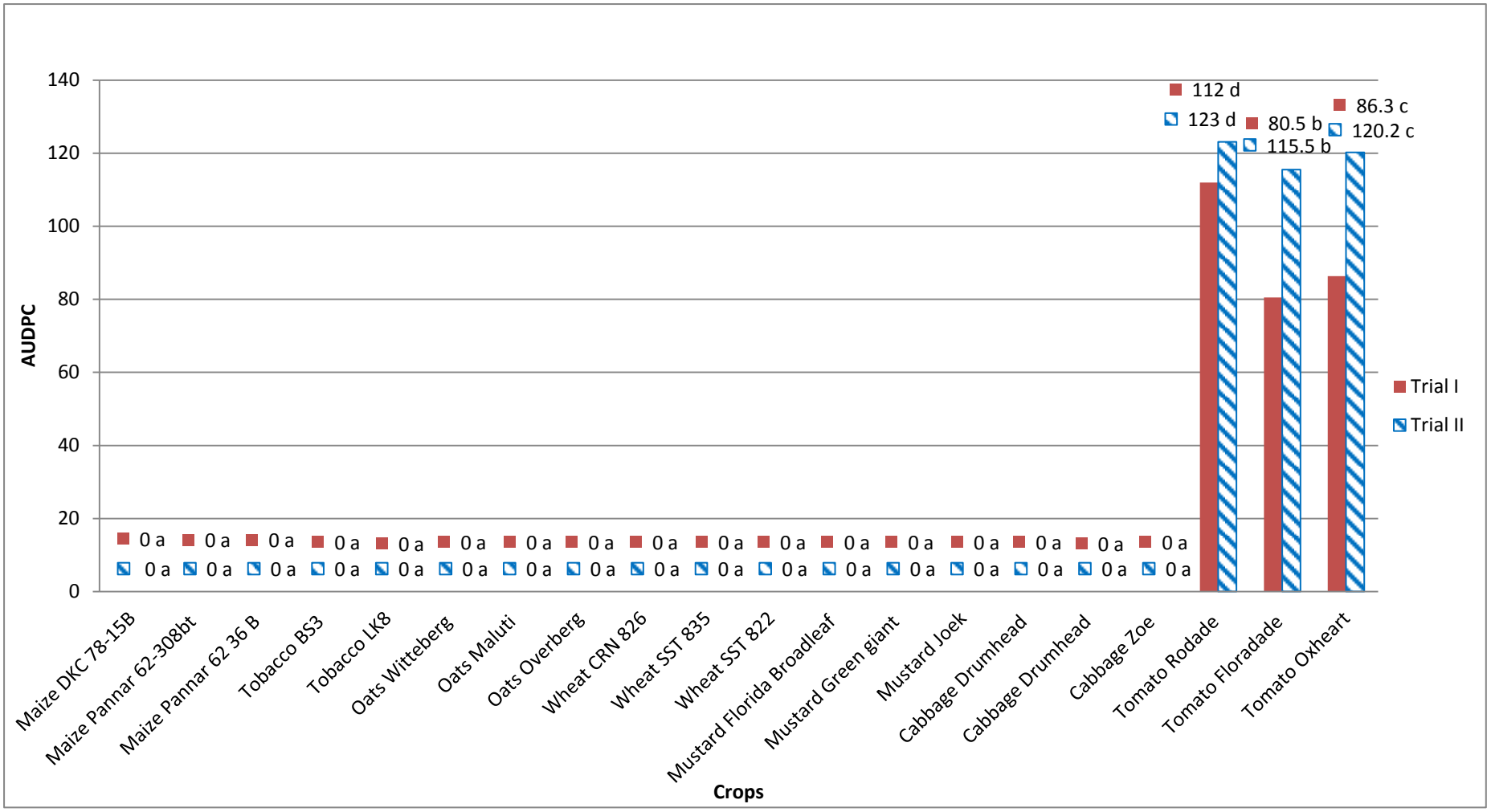

Figure 6.5: Susceptibility of different crops before flowering to *Alternaria alternata* (potato strain) expressed as area under disease progress curve (AUDPC) (trial I and II).
Trial I: $p<0.001$, CV = 8.4%
Trial II: $p<0.001$, CV = 6.6%
Bars with the same letters above them are not statistically different at the 5% level of significance per trial.

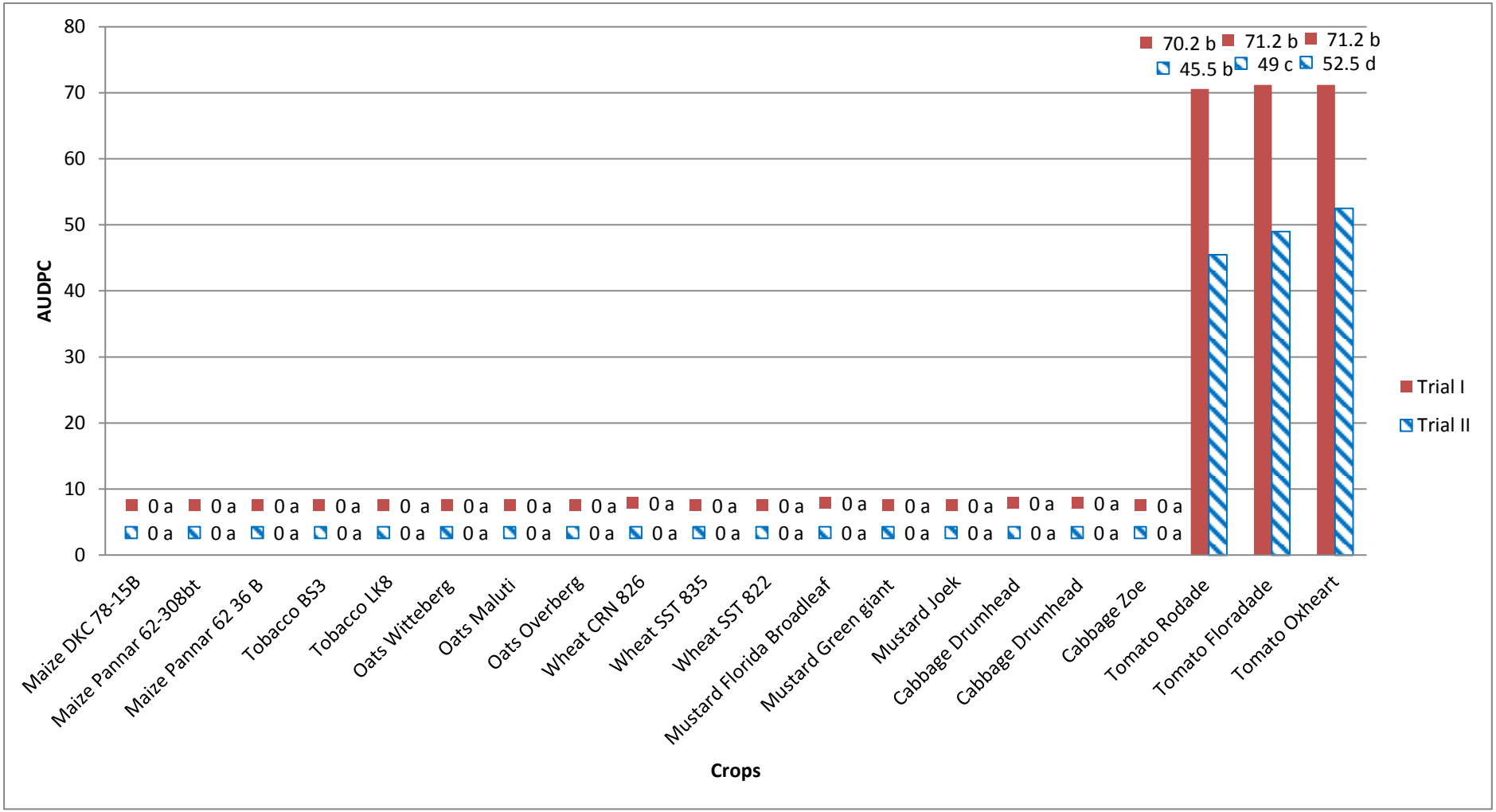

Figure 6.6: Susceptibility of different crops at fruiting stage to *Alternaria alternata* (potato strain) expressed as area under disease progress curve (AUDPC) (trial I and II).
Trial I: $p<0.001$, CV = 6.2%
Trial II: $p<0.001$, CV = 16.5%
Bars with the same letters above them are not statistically different at the 5% level of significance per trial.

6.3.2 Confirmation of causative organism for lesions on crops

Various types of lesions were observed on some of the crops after inoculation. The number of lesions on each crop and cultivar is indicated in Table 6.2. A total of 216 isolations were made from lesions on half strength PDA. Spores were microscopically identified based on morphology as being those of *Alternaria* spp. or not.

The foliar lesions on the tomato plants were dark concentric rings that developed a yellow halo as the lesions matured (Figure 6.7). The lesions on the fruit were small sunken dark lesions. The lesions were similar to those described in the literature when referring to tomato *A. alternata* f. sp. *lycopersici*, which causes black mould and leaf blight of tomato plants (Pearson & Hall, 1975; Akhtar *et al.,* 1994). One isolate from each tomato cultivar in each trial was confirmed as *A. alternata* by means of PCR.

Cabbage and mustard plants developed a few lesions in both trials at different inoculation stages, these lesions were however not caused by *Alternaria* spp., as the spores did not resemble those of *Alternaria* spp. The tobacco, maize, wheat and oats plants developed no lesions in the trials.

Figure 6.7 Lesions on a tomato plant at seedling stage, caused by *A. alternata* after artificial inoculation in the greenhouse.

Table 6.2 Fungi isolated form lesion and confirmation as *Alternaria alternata*, for the trials: alternative host for *Alternaria alternata* potato strain, trials I and II.

Trial	Stage of inoculation	Crop	Cultivar	Number of lesions isolated from	*Alternaria alternata* isolates based on spore morphology on ½ PDA	PCR confirmation
Trial I	Inoculation at seedling	Tomato	Oxheart	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at seedling	Tomato	Floradade	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at seedling	Tomato	Rodade	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at 4 weeks	Tomato	Oxheart	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at 4 weeks	Tomato	Floradade	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at 4 weeks	Tomato	Rodade	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at fruiting	Tomato	Oxheart	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at fruiting	Tomato	Floradade	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at fruiting	Tomato	Rodade	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at seedling	Tomato	Oxheart	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at seedling	Tomato	Floradade	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at seedling	Tomato	Rodade	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at 4 weeks	Tomato	Oxheart	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at 4 weeks	Tomato	Floradade	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at 4 weeks	Tomato	Rodade	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at fruiting	Tomato	Oxheart	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at fruiting	Tomato	Floradade	12	Small spore *Alternaria* spp.	Yes
Trial II	Inoculation at fruiting	Tomato	Rodade	12	Small spore *Alternaria* spp.	Yes
Trial I	Inoculation at seedling	Cabbage	Drumhead	3	No, not *Alternaria* spp. spores	N/A
Trial I	Inoculation at seedling	Cabbage	Cape Spitz	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Cabbage	Zoe	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Cabbage	Drumhead	1	No, not *Alternaria* spp. spores	N/A

Trial I	Inoculation at 4 weeks	Cabbage	Cape Spitz	5	No, not *Alternaria* spp. spores	N/A
Trial I	Inoculation at 4 weeks	Cabbage	Zoe	2	No, not *Alternaria* spp. spores	N/A
Trial I	Inoculation at fruiting	Cabbage	Drumhead	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Cabbage	Cape Spitz	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Cabbage	Zoe	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Cabbage	Drumhead	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Cabbage	Cape Spitz	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Cabbage	Zoe	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Cabbage	Drumhead	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Cabbage	Cape Spitz	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Cabbage	Zoe	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Cabbage	Drumhead	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Cabbage	Cape Spitz	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Cabbage	Zoe	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Mustard	Floridade Broadleaf	1	No, not *Alternaria* spp. spores	No
Trial I	Inoculation at seedling	Mustard	Green Giant	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Mustard	Joek	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Mustard	Floridade Broadleaf	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Mustard	Green Giant	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Mustard	Joek	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Mustard	Floridade Broadleaf	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Mustard	Green Giant	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Mustard	Joek	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Mustard	Floridade Broadleaf	2	No, not *Alternaria* spp. spores	N/A

Trial II	Inoculation at seedling	Mustard	Green Giant	3	No, not *Alternaria* spp. spores	N/A
Trial II	Inoculation at seedling	Mustard	Joek	1	No, not *Alternaria* spp. spores	N/A
Trial II	Inoculation at 4 weeks	Mustard	Floridade Broadleaf	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Mustard	Green Giant	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Mustard	Joek	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Mustard	Floridade Broadleaf	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Mustard	Green Giant	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Mustard	Joek	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Tobacco	BS3	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Tobacco	LK8	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Tobacco	BS3	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Tobacco	LK8	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Tobacco	BS3	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Tobacco	LK8	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Tobacco	BS3	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Tobacco	LK8	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Tobacco	BS3	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Tobacco	LK8	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Tobacco	BS3	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Tobacco	LK8	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Wheat	CRN 826	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Wheat	SST 835	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Wheat	SST 822	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Wheat	CRN 826	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Wheat	SST 835	No lesions	N/A	N/A

Trial I	Inoculation at 4 weeks	Wheat	SST 822	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Wheat	CRN 826	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Wheat	SST 835	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Wheat	SST 822	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Wheat	CRN 826	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Wheat	SST 835	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Wheat	SST 822	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Wheat	CRN 826	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Wheat	SST 835	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Wheat	SST 822	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Wheat	CRN 826	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Wheat	SST 835	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Wheat	SST 822	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Oats	Witteberg	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Oats	Maluti	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Oats	Overberg	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Oats	Witteberg	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Oats	Maluti	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Oats	Overberg	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Oats	Witteberg	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Oats	Maluti	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Oats	Overberg	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Oats	Witteberg	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Oats	Maluti	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Oats	Overberg	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Oats	Witteberg	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Oats	Maluti	No lesions	N/A	N/A

Trial II	Inoculation at 4 weeks	Oats	Overberg	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Oats	Witteberg	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Oats	Maluti	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Oats	Overberg	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Maize	DKC 78-15B	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Maize	Pannar 62- 308bt	No lesions	N/A	N/A
Trial I	Inoculation at seedling	Maize	Pannar 62- 36 B	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Maize	DKC 78-15B	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Maize	Pannar 62- 308bt	No lesions	N/A	N/A
Trial I	Inoculation at 4 weeks	Maize	Pannar 62- 36 B	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Maize	DKC 78-15B	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Maize	Pannar 62- 308bt	No lesions	N/A	N/A
Trial I	Inoculation at fruiting	Maize	Pannar 62- 36 B	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Maize	DKC 78-15B	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Maize	Pannar 62- 308bt	No lesions	N/A	N/A
Trial II	Inoculation at seedling	Maize	Pannar 62- 36 B	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Maize	DKC 78-15B	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Maize	Pannar 62- 308bt	No lesions	N/A	N/A
Trial II	Inoculation at 4 weeks	Maize	Pannar 62- 36 B	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Maize	DKC 78-15B	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Maize	Pannar 62- 308bt	No lesions	N/A	N/A
Trial II	Inoculation at fruiting	Maize	Pannar 62- 36 B	No lesions	N/A	N/A

6.4 DISCUSSION

Knowing the host range of *A. alternata* (potato strain) is of important as it has an impact on disease management. If the same pathogen attacks more than one crop, disease management will be made more difficult (Oyarzun *et al.,* 1998). This study determined that tomato is a host of *A. alternata* potato strain, as all three tomato cultivars (Rodade, Floradade, Oxheart) became diseased when inoculated with this pathogen. Only in the preliminary trial did the mustard plants become infected with *A. alternata*. Mustard may still be susceptible to *A. alternata* potato strain. It was also determined that cabbage, wheat, oats, tobacco and maize are not hosts of *A. alteranta* potato strain.

All the tomato cultivars tested in this study were susceptible to *A. alternata* (potato strain) infection throughout their life cycle. *A. alternata* f. sp. *lycopersici* however only causes disease on mature tomato plants. Only 25% of the tomato cultivars Grogan *et al.* (1975) tested against *A. alternata* f. sp. *lycopersici* were susceptible (Grogan *et al.,* 1975). Weir *et al.* (1998) using RAPD-PCR-analysis however found that there is no genetic variation between *A. alternata* isolated from tomato and potato plants. It is unsure how closely *A. alternata* potato strain and *A. alternata* f. sp. *lycopersici* may be related; further investigation into this matter is needed.

Planting of tomato crops near potato crops should be avoided as alternative hosts may act as natural sources of inoculum. It may be possible that *A. alternata* potato strain can also cause disease on other plants from the Solanaceae family. It would also be of interest to determine whether a toxin is produced by *A. alternata* potato strain as the sensitivity of a host to a toxin often correlates to the host specificity of the fungal isolate producing the toxin. Toxin production is required by *Alternaria* spp. to cause disease (Kohmoto *et al.,* 1991; Otani, *et al.,* 1995).

It would also be important to determine if any weeds typically found in South African potato fields are susceptible to *A. alternata* as weeds have shown to be hosts of other potato disease (Raid & Pennypacker, 1987; Souza-Dias *et al.,* 1993; Leyva-López *et al.,* 2002). Fifteen weed species from the families: Amaranthaceae, Chenopodiaceae, Compositae, Convolvulaceae, Cruciferae, Gramineae, Malvaceae, Oxalidaceae, Polygonaceae, Solanaceae act as alternative hosts for *Colletotrichum coccodes* (Wallr.) Hughes in the USA (Raid & Pennypacker, 1987). Potato leaf roll virus (PLRV) causes disease on 5 Solanum spp. weeds: *Solanum aculeatissimum*, *S. lycocarpum*, *S. paniculatum*, *S. variabile* and *S.*

erianthum (Souza-Dias *et al.,* 1993), and the causal organisms of potato purple top and potato hair sprouts were detected in weeds surrounding potato fields in Mexico (Leyva-López *et al.,* 2002).

It is unknown whether *A. alternata* potato strain can survive in the soil, as some *Alternaria* spp. are known to survive in soil/fields for long periods of time (Maude *et al.,* 1972). Crop rotation can be an effective mechanism of reducing potato soil disease in a field (Honeycutt *et al.,* 1996). Wheat, maize, oats, tobacco, and cabbage may be used as crop rotation crops as *A. alternata* potato strain is unable to cause disease on these plants. Wheat, maize and oats are known not to serve as hosts of any pathogen infecting potato crops (Denner *et al*., 2003; Aegerter *et al.,* 2008). However cabbage and tobacco are attacked by various fungal, bacterial, viral pathogens and nematodes which also attack potatoes and could therefore sustain populations of these pests and pathogens in the absence of a potato crop (Shew & Lucas, 1991; Rimmer *et al.,* 2007).

Brassica spp. plants are susceptible to the following pathogens that also attack potato plants: *Botrytis cinerea* (De Bary) Whetzel*, Pythium debaryanum* (R. Hesse) Nieuwl., *Phytophthora megasperma* Drechsler*, Rhizoctonia solani* J.G. Kühn*, Verticillium dahliae* Kleb*, Sclerotinia sclerotiorum* (Lib.) de Bary*,* Cucumber mosaic virus, and *Meloidogyne* spp. Göldi. (Rimmer *et al.,* 2007). Tobacco plants are susceptible to the following potato pathogens: *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen*, R. solani, Sclerotium rolfsii* Sacc.*, Verticillium albo-atrum* Reinke & Berthold, *V. dahliae, Ralstonia solanacearum* Smith) Yabuuchi et al.*, Pectobacterium* spp. (van Hall) Dye and the following viruses: Tobacco mosaic virus, Tobacco necrosis virus, Tobacco rattle virus, Tobacco streak virus, Alfalfa mosaic virus, Cucumber mosaic virus. The nematodes that attack both tobacco and potatoes are *Meloidogyne hapla*, *Meloidogyne incognita* and *Meloidogyne javanica*. (Shew & Main, 1990; Shew & Lucas, 1991).

The results of the current study are useful for the selection of rotation crops for the management of brown spot of potatoes. Tomato plants are susceptible to *A. alternata* potato strain. In the management it is important to use non-host crops such as maize, wheat and oats as crop rotation crops.

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CHAPTER 7
GENERAL DISCUSSION

Potato (*Solanum tuberosum* L.) is one of the most important food crops throughout the world (Luck *et al.,* 2011). Brown spot and black pit of potatoes was first reported in Israel in 1984 (Droby *et al.,* 1984a; 1984b) and have been a problem in South African potato production in recent years (van der Waals *et al.,* 2011). Brown spot lesions can be found on the foliage of the potato plant and black pit lesions on the tubers. The causal organism of brown spot and black pit was determined by Droby *et al.* (1984a; 1984b) as *Alternaria alternata* (Fr.) Keissler. The research presented in this thesis will in part contribute to the knowledge and control of brown spot and black pit diseases of potatoes.

The main objectives of this research were to identify the causal organism of brown spot and black pit of potatoes in South Africa (Chapter 3), assess the spread of the causal agent between plants (Chapter 4), determine the susceptibility of potato cultivars to brown spot and black pit diseases (Chapter 5), and to test whether other crops may be susceptible to the brown spot and black pit causal agent (Chapter 6).

In order to determine the causal organism of brown spot and black pit of potatoes in South Africa Koch's postulates were tested. During initial isolation, one fungus and two bacteria were isolated, which were included in the trial. The treatments where the fungal isolate was used in inoculation resulted in the development of brown spot lesions. As no lesions formed in the treatments where only the bacteria were used in inoculation, the bacteria cannot be considered plant pathogens (Allington & Chamberlain, 1949; Agrios, 1997). The bacteria used in the trials were probably only epiphytes able to survive on the potato leaves (Leben, 1965; Kinkel *et al.,* 1995; Beattie & Lindow, 1999). The causal organism of black pit of potatoes was also determined in another Koch's postulates trial. The causal organism of brown spot and black pit were identified as *A. alternata* which corroborates the findings of other research (Droby *et al.,* 1984a; Boiteux & Reifschneider, 1994; Kirk *et al.*, 2007).

Brown spot of potatoes has been reported in a number of countries (Droby *et al.,* 1984a; Boiteux & Reifschneider, 1994; Kirk *et al.*, 2007; van der Waals *et al.*, 2011, Zheng & Wu, 2013). As the last report of black pit was almost 30 years ago it is not known if the *A. alternata* isolates found in South Africa are unique in their ability to cause black pit of potatoes or if the black pit symptoms in other countries are being confused with those caused by *Fusarium* spp. or *Pectobacterium* spp. (van Hall) Dye (Stevenson *et al.*, 2001).

By identifying the causal agent of brown spot and black pit of potatoes in South Africa, control strategies against these diseases can effectively be developed in the future.

To better understand the spread of *A. alternata* between the plants and/or tubers a pot trial and *in vitro* trial was conducted (Chapter 4). In the trials it was observed that when planting an inoculated potato seed tuber brown spot may develop on foliage. But the daughter tubers harvested from plants infected with *A. alternata* will not necessarily develop black pit. Daughter tubers are most likely infected by *A. alternata* during harvesting and black pit lesions develop in high humidity in storage. It is thus advisable that the relative humidity in storage facilities should be low to prevent the development of black pit disease and that care should be taken during harvesting to limit the amount of inoculum the tubers may come in contact with.

In Chapter 5 potato cultivar susceptibility to brown spot and black pit was tested. Planting resistant cultivars is one of the most important measures in controlling plant diseases (Agrios, 1997). There are no commercial potato cultivars that are completely resistant to brown spot and black pit of potatoes (Kapsa & Osowski, 2011). During the current study of South African potato cultivars Avalanche, Buffelspoort, BP1, Fabula, Fianna, Frodo, Hertha, Labadia, Lanorma, Mondial, Pentland Dell, Up-To-Date and Van Der Plank were all susceptible to both brown spot and black pit disease. There is also no connection between the susceptibility to brown spot and black pit diseases in a single cultivar as cv.Up-To-Date had the lowest disease susceptibility to brown spot but a medium disease susceptibility to black pit. Based on the results obtained in the current study, cv. Avalanche and Buffelspoort were the least susceptible to black pit disease of potatoes and cv. Up-To-Date the least susceptible to brown spot. It is of importance to screen more South African cultivars to help growers make the best decision based on their specific need.

In Chapter 6 crop susceptibility to *A. alternata* potato strain was assessed. Crop rotation can be an effective mechanism of reducing potato disease in a field (Honeycutt *et al.,* 1996). Of the tested crops cabbage, wheat, oats, tobacco and maize were not susceptible to *A. alternata* potato strain. All three tomato cultivars (Rodade, Floradade, Oxheart) were however susceptible to *A. alternata* infection throughout their life cycle, with seedlings being most susceptible. Planting of tomato crops near potato crops should be avoided as alternative hosts may act as natural sources of inoculum (Agrios, 1997).

Wheat, maize and oats may be used in rotation with potatoes, however cabbage and tobacco are attacked by various pathogens which also attack potatoes and could therefore

sustain populations of these pests and pathogens in the absence of potato crops (Shew & Main, 1990; Shew & Lucas, 1991; Rimmer *et al.,* 2007). The results obtained in this study are useful for the selection of rotation crops for the management of brown spot of potatoes. It would be important to determine if any weeds typically found in South African potato fields are susceptible to *A. alternata* as these may pose a risk as alternative hosts.

The results of the studies presented in this thesis will contribute to the knowledge of brown spot and black pit potato diseases not only in South Africa but internationally. This will allow growers to make informed decisions regarding problems they face in the field during the production season. Brown spot and black pit of potatoes are diseases that need to be studied further, especially regarding control strategies to reduce the impact that they have on potato production around the world.

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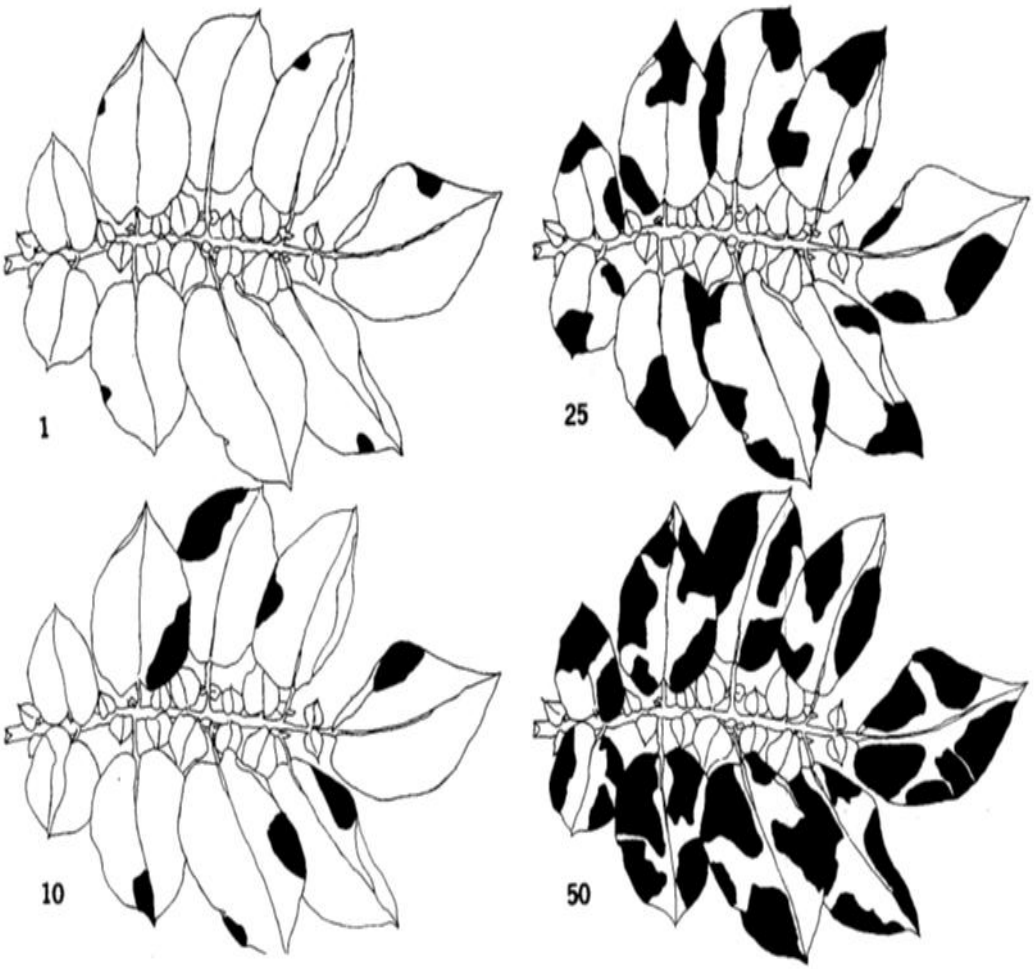
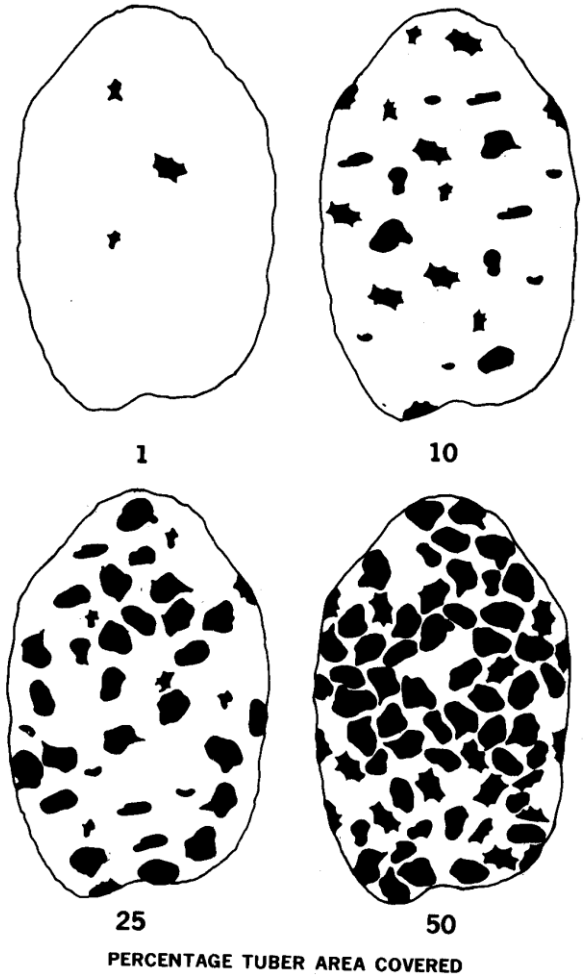
APPENDIX A: Disease assessment key indicating percentage leaf area affected (James, 1971).



APENDIX B: Disease assessment key indicating percentage tuber area affected (James, 1971).



APPENDIX C: Soil analys of the red top soil used in the study: effect of inoculum source, alternative host and cultivar on development of brown spot and black pit of potatoes in South Africa, analysed by the Department of Plant Production and Soil Science, University of Pretoria.

GROND ONTLEDING:

SOIL ANALYSIS:

18 May 2009

Aan/To:

onbekend

Fax No:

Datum Ontvang: 2009/05/13

Taak /Task No: 78

University of Pretoria
Universiteit van Pretoria
Department of Plant Production and Soil Sience
Departement Plantproduksie en Grondkunde
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						Ammonium Acetaat Oplosbare : / Ammonium Acetate Extractable :									
Jaar / Year	LabNo	VeldNo / FieldNo	pH H2O	Weerstand / Resistance / ohm	P / Bray I / mg/kg	Ca / mg/kg	K / mg/kg	Mg / mg/kg	Na / mg/kg	C / %	Totale N / Total N / %N	NH4 / mg/kg	NO3 / mg/kg	%	P / %
2009	1329	BE1	4.2		10.6	41	33	11	29		0.00	3.08	6.78	0.00	0.00

APPENDIC D: Particle analysis of the red top soil used in the study: effect of inoculum source, alternative host and cultivar on development of brown spot and black pit of potatoes in South Africa, analysed by the Department of Plant Production and Soil Science, University of Pretoria.

DEELTJIE GROOTTE ONTLEDING:

PARTICLE SIZE ANALYSIS:

18 May 2009

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Datum Ontvang: 2009/05/13

Taak/Task No: 78

Jaar / Year	LabNo	VeldNo / FieldNo	Growwe Sand / Coarse Sand %	Medium Sand / Medium Sand %	Fyn Sand / Fine Sand %	Baie Fyn Sand / Very Fine Sand %	Slik / Silt %	Klei / Clay %	Totaal / Total %
2009	1329	BE1	74.8				7.5	17.5	99.8