

Causal agent and control of brown spot of potatoes in South Africa

By

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Submitted in partial fulfillment of the requirement for the degree of

MInst(Agrar): Plant Protection

In the Faculty of Natural and Agricultural Sciences

Department of Microbiology and Plant Pathology



UNIVERSITEIT VAN PRETORIA
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July 2013

Declaration

I, the undersigned, declare that this thesis, which I hereby submit for the degree Master of Agricultural Management in Plant Protection at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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July, 2013

Acknowledgements

I would like to pass my special thanks to:

- My supervisor Dr Jacquie Van der Waals, Mrs. Rudi Horak, Miss Puleng Tsie and the rest of the Potato Pathology Program at UP for their guidance, support, patience and friendship.
- Mr Charles Wairuri, for assistance with the molecular work done in this study.
- Potatoes South Africa, University of Pretoria and THRIP for their financial assistance.
- Syngenta, Bayer, Plaaskem, BASF, Du Pont and Villa for donation of the fungicides
- My family for their patience, guidance and support.
- The all mighty God our heavenly father for everything

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Chapter 1: General introduction

The potato (*Solanum tuberosum* L.) is one the most important crops in the world. Potatoes are the fourth most cultivated crop with more than 315 million tons produced worldwide (FAO, 2012). In sub Saharan Africa, South Africa is the largest potato grower and third in Africa after Egypt and Algeria (FAO, 2012).

South Africa is not ideally suited for potato production due to water scarcity; moreover, potatoes have a weak root system that causes the plant to be susceptible to water deficiency. South Africa however encompasses a range of diversified microclimates and soil characteristics around the country that allows a year-round production of the crop (Steyn, 2003). The country is divided into 16 potato production regions namely Limpopo, Loskop Valley, Northwest, Mpumalanga, Gauteng, eastern and western Free State, south western Free State, KwaZulu Natal, Sandveld, Ceres, South western Cape, Southern Cape, Northern Cape and north eastern Cape (Potatoes South Africa, 2012). The most cultivated varieties in South Africa are Mondial, BP1, UTD, Buffelspoort, Sifra, Valor, Avalanche, Fianna and Van der Plank (VDP) (Potatoes South Africa, 2012).

In the past decade drastic changes in climatic conditions have been noted to influence crop production practices (Rosenzweig *et al.*, 2005) These crop production practices can be either positive or negative and are largely influenced by the adaptability of pests and pathogens (Ghini *et al.*, 2008; Newton *et al.*, 2010). Pests, diseases and water scarcity remain among the most

constraining factors affecting potato production in South Africa with commercial farmers having to rely heavily on irrigation, improved varieties and chemicals to increase yield and suppress pests and diseases (Steyn, 2003).

In recent years brown spot lesions have been noticed to occur on potato plants grown in South Africa. *Alternaria alternata* (Fries) Keissler has been continuously isolated from these lesions. According to Soleimani & Kirk (2012) potato brown spot is a common and destructive disease in areas of high moisture. This disease reduces the photosynthetic leaf area and as a result causes yield loss due to a reduced supply of carbohydrates from the leaves to the developing tubers (Droby *et al.*, 1984).

Yield losses due to this disease are estimated to be around 30% in South Africa (Van der Waals *et al.*, 2011). Soleimani & Kirk (2012) have reported yield losses to reach up to 80% in North America if the disease is left uncontrolled. Increased yield losses are noticed when the brown spot occurs in conjunction with other diseases such as blackleg, early blight and Verticillium wilt (Jansky *et al.*, 2008). For a number of years there was uncertainty surrounding the causal agent of this disease in South Africa. It was not until 2011 that the causal agent was confirmed to be *A. alternata* (Van der Waals *et al.*, 2011) following the results from this study. The occurrence of brown spot, even under multiple applications of various fungicides registered for controlling early blight (caused by *Alternaria solani*) suggests that these fungicides may be ineffective against *A. alternata*, the causal agent of brown spot.

Reuveni & Sheglov (2002) reported that *in vitro* mycelial growth of *A. alternata* isolates causing moldy-core of apples, were less sensitive to azoxystrobin and trifloxystrobin and more sensitive

to difenoconazole. Pasche *et al.* (2005) reported isolates of *Alternaria solani* to have acquired resistance towards fungicides affecting mitochondrial respiration and that this kind of resistance is due to a F129L mutation which is also detectable in *Alternaria alternata* isolates. According to Van der Waals *et al.* (2005) fungicides from this group are amongst some of the most frequently applied active ingredients in South Africa to control early blight on potatoes. *In vitro* tests were conducted (Chapter 4 of this study) to evaluate the efficacy of different fungicides to mycelial growth inhibition of *A. alternata*. Fungicides such as AC crop oil (A tank mixture of Acanto[®], Capitan[®] and H&R Crop oil), Nativo[®], Bellis[®] and No-Blite[®] inhibited over 87% mycelial growth. Most of the effective fungicides contain multiple active ingredients, but resistance of *A. alternata* towards Pristine[®], containing the same two active ingredients as Bellis[®] has been reported (Avenot *et al.*, 2008). Rotation of fungicides during field applications may be necessary to attain effective disease control and to delay resistance towards active ingredients (Staub, 1991; Brent, 1995).

Motivation of the study

South Africa experienced a foliar disease outbreak on potatoes in recent years. According to Van der Waals *et al.* (2011) this disease has raised concerns among farmers in South Africa. Symptoms resemble those of early blight, but instead of isolating *Alternaria solani* which is known to cause early blight, *Alternaria alternata* was continuously isolated from these lesions. This disease occurred regardless of multiple applications of various fungicides aimed at controlling early blight. It was therefore hypothesized that the fungicides registered for controlling early blight may be unable to control the brown spot disease. This study aimed at

identifying the causal organism of brown spot on potatoes, by conducting Koch's postulates with isolates obtained from symptomatic potato leaves and to test the activity of different fungicides which are registered for controlling *Alternaria* species causing diseases on various crops in South Africa, for their *in vitro* mycelial growth inhibition efficacy against *A. alternata*.

Chapter outline

Chapter 2: Literature review

Little is known about *A. alternata* on potatoes in South Africa, therefore this review aims at giving an overview of *A. alternata* as a pathogen, the disease cycle, the symptoms, culture characteristics and to highlight the current trend in taxonomy of the organism, identification and control of the fungus.

Chapter 3: Application of Koch's postulates to determine the cause of brown spot on potato in South Africa.

This chapter will focus on determining the causal organism of brown spot on potatoes and a scanning electron microscope is used as a tool to verify colonization and infection by the organism.

Chapter 4: *In vitro* chemical growth inhibition of *Alternaria alternata* isolates associated with brown spot of potato in South Africa.

A. alternata isolates are subjected to various fungicides registered to control *Alternaria* species on numerous crops. The purpose of this chapter is to conduct a preliminary study to evaluate

which fungicide combinations are most effective in inhibiting the organism *in vitro*, to lay a foundation for future field trials and fungicide selection for the industry.

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Chapter 2: Literature review

Abstract

Trading networks around the world influence introduction of pathogens to new areas where they were not previously present. The ability of such pathogens to flourish is however dependent on climatic conditions of the area and the availability of a susceptible host. Emphasis has recently focused on the importance of climate change in causing shifts in crop production practices, emergence and reemergence of plant pests and pathogens around the world. *Alternaria alternata* is one of the most widely distributed fungi in the world and is found to occur in a wide range of climates either as a saprophyte, primary or secondary pathogen, depending on the host. Although *A. alternata* has been recognized as a weak opportunistic fungus, it has been constantly isolated from brown spot symptoms recently noted on potato plants in South Africa. The disease causes considerable yield losses. *A. alternata* species morphology and cultural properties show a discrepancy even within the same isolate. This has lead to misidentification of the fungus in the past, however molecular techniques are available today to accurately identify the organism. This should be used in combination with conventional methods for accurate identification of the causal organism. This literature review discusses physiological, molecular and morphological characteristics of *A. alternate*, as well as the symptoms, geographic distribution, alternative hosts, disease cycle and control of the pathogen.

1. Introduction

The ability of plant pathogens to infect and flourish on new plant hosts causes a major threat to global food safety and security (Chakraborty & Newton, 2011). Agricultural trade networks and tourism, within and between countries paces the spread and occurrence of new and novel plant disease outbreaks. This is influenced by the introduction and adaptability of pathogenic species and races in to a new niche, or with mutation or resistance to existing control options (Newton *et al.*, 2010).

The role of climate change on plant and pathogen interactions cannot be overlooked. Recent reports stress the effects of climate change to have a positive or negative influence on global crop production and integrated disease and pest management systems (Coakley *et al.*, 1999). With the aid of simulation models Hijmans (2003) estimated a potato yield reduction of 18-38% and Rosenzweig *et al.* (1995) reported a global shift in planting time, with a shift in production of various crops to other areas.

The distribution of pests and diseases may also be affected; plant diseases and pests which were previously known to be of less economic importance may become more aggressive and as a result cause more severe damage to crops than before, correspondingly more damaging pests may become of less economic importance (Ghini *et al.*, 2008; Newton *et al.*, 2010).

Disease outbreaks caused by *Alternaria* species have been reported to occur on various plant hosts including potatoes (Giha, 1973). *A. alternata* can adapt to diverse environments and has been reported to cause disease on over 100 plant species (Rotem, 1994). This fungus is known to cause disease due to host weaknesses caused by biotic or abiotic factors. For example *A. alternata*

has been known to cause secondary infections with other plant pests and pathogens such as *Alternaria macrospora* Zimmerman on cotton (Bashan *et al.*, 1991), *Lyriomyza trifolii* Burgess (Deadman *et al.*, 2002) and *A. solani* on potatoes. Furthermore Sharma & Kolte (1994) reported that potassium deficiency is a prerequisite to *Alternaria* leaf spot on oilseed rape.

Droby *et al.* (1984) however reported *A. alternata* to be the principal pathogen causing *Alternaria* blight on potatoes in Egypt. In recent years brown spot lesions have been observed to occur on potato plants grown in South Africa. The disease symptoms appear as necrotic brown spots occurring on the foliage of potatoes. These symptoms are similar to those caused by *A. solani* (Giha, 1973) except for the absence of concentric rings within the necrotic spots (Neergaard, 1945).

2. The pathogen

2.1. Taxonomy and classification

Alternaria alternata (Fries.) Keissler belongs to the Eukaryomycota, Kingdom Fungi, class Deuteromycota, order Moniliales, family Dematiaceae; this class is well recognized as the Fungi Imperfecti due to the unknown sexual stage (Rao, 1971; Simmons, 2002). Correct classification of microorganisms cannot be overlooked. This is critical for attaching unique characteristics to specific genera so that the species' distinct behaviour can be accurately predicted (Roberts *et al.*, 2000). The genus *Alternaria* was first recognized in 1817 with *A. alternata* previously known as *A. tenuis* (Neergaard, 1945; Rao, 1971; Bart & Thomma, 2003).

The species occurring within the genus *Alternaria* have overlapping morphological characteristics. This makes it difficult for morphological identification of *Alternaria* species, due to misrepresentation of criteria used for identification and therefore most report papers rely exclusively on spore measurements for identification (Roberts *et al.*, 2000). This has lead to descriptions of *Alternaria* species that have not been verified by others (Bart & Thomma, 2003)

Species of *Alternaria* have been divided into subgeneric groups, due to their large diversity. The subgeneric groups of *Alternaria* species are differentiated based on chain formation of conidia (Neergaard, 1945; Simmons & Roberts, 1993; Roberts *et al.*, 2000). *A. alternata* isolates have a wide host range, possess morphologically similar characteristics and different isolates have the ability to cause distinct symptoms (Stuart *et al.*, 2009).

Intra-subspecific classification is used to classify the species based on pathological differences. For example, isolates causing *Alternaria* brown spot on the citrus fruit peel are referred to as *A. alternata* tangerine pathotype and those attacking citrus leaves are referred to as *A. alternata* rough lemon pathotype. These isolates occur on the same host but produce different host selective toxins (Stuart *et al.*, 2009). In the case of host specificity *formae specialis* is adopted (Rotem, 1994).

Thin Layer Chromatography and Liquid Chromatography Mass Spectroscopy have been used to perform secondary metabolite profiling of fungal isolates based on metabolite production patterns as a tool for taxonomic relatedness. Bhagobaty & Joshi (2011) reported that a biochemical metabolite must not be used as a biochemical marker to ascertain taxonomic identity because one

fungal isolate may produce a mixture of numerous metabolites given a set of conditions and this may cause misunderstanding.

2.2. Identification of *A. alternata*

According to Rotem (1994), *A. alternata* produces small spores that are gray-green to pale yellowish brown. The conidia are produced in single or branched chains (Stuart, 2009). The conidia contain melanin that is concentrated on the outer region, which arises from the primary cell wall (Bart & Thomma, 2003).

The conidia are multiseptate and muriform with dimensions ranging from 5-14 by 10-43µm. (Ellis, 1971). Rotem (1994) reported the conidia of *A. alternata* to have 3-7 transverse septa with a short conical or cylindrical beak. *Alternaria* species are primarily differentiated by conidium characteristics.

Classification of *Alternaria* based on morphological characteristics is further complicated due to the presence of fungal genera such as *Ulocladium* and *Stemphylium*, which produce morphologically similar conidia to that of *Alternaria* species. According to Pryor & Gilbertson (2000), *Ulocladium* and *Alternaria* are differentiated by the basal end of immature conidia, of which *Ulocladium* conidia are ovoid and non-beaked (Simmons, 1969). *Stemphylium* is distinguished from *Ulocladium* and *Alternaria* based on the conidiophore proliferation (Simmons, 1969).

In a study using ITS sequences Pryor & Gilbertson (2000) reported that a significant molecular distinction was evident between *Ulocladium*, *Stemphylium* and *Alternaria* isolates. According to

Ferrer *et al.* (2001) universal primers have been developed from multicopy gene targets to detect fungi. Ferrer *et al.* (2001) reported that internal transcriber spacer (ITS) regions of fungal ribosomal DNA are very different and characteristic; therefore reliable for identification of fungi. The ITS1 and ITS2 transcriber regions are located between 18S and 5.8S and between 5.8S and 28S of the ribosomal RNA (Ferrer *et al.*, 2001).

Weir *et al.* (1998) used ITS1 and ITS2 primers with Random Amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR) to differentiate between *A. alternata* and *A. solani* isolates from different hosts. Stuart *et al.* (2009) developed specific primers to amplify specific gene loci for *A. alternata* on lilac and further reported that this allowed PCR detection without morphological identification of spores.

The accuracy of this molecular approach may however become limited if previously undescribed nucleotides from unique isolates are encountered, because molecular techniques such as screening for rRNA regions are narrowed by the fact that new sequences have to be compared with known sequences (Bhagobaty & Joshi, 2011). Therefore two or more techniques must be used for precise identification of fungal isolates (Frisvad *et al.*, 2007).

2.3. Toxin production

Alternaria species are known to produce low molecular weight phytotoxic metabolites (Agrios, 2011; Mamgain *et al.*, 2013). These are divided into two categories with regard to host specificity namely, host specific and non host specific toxins (Dehpour *et al.*, 2010). According to Markham & Hille (2001) *A. alternata* is the most widespread fungus that has a pathotypic variant producing toxins of this nature.

The presence of phytotoxins is evident when necrotic spots appear on the leaves prior to hyphal penetration (Dehpour *et al.*, 2007). The manifestation of a chlorotic halo surrounding the point of infection is a characteristic of necrotrophic plant pathogens. This zonation is caused by diffusion of fungal metabolite-like toxins into the plant tissue (Bart & Thomma, 2003).

These toxins have distinct structures and primary target sites on host plants (Slavov *et al.*, 2004). Non host-selective toxins, followed by their mechanism of action include: brefeldin A, that disassembles the golgi complex; zinnol that affects membrane permeability of cells; tenuazonic acid which inhibits protein synthesis; while curvularin and tentoxin inhibit photophosphorylation and cell division (Bart & Thomma, 2003). Numerous workers have also reported the production of host selective toxins (HST) by certain *A. alternata* pathotypes. Such toxins include AT, AC, AM, AK, AF and AL toxins. These HSTs are responsible for symptom development and their involvement in pathogenesis is limited to a specific host (Rotem, 1994; Dehpour *et al.*, 2010).

Siler & Gilchrist (1982) isolated and purified a host selective toxin derived from *A. alternata* f.sp. *lycopersici* and reported that this toxin alone can produce symptoms on susceptible tomato plants similar to those caused by the fungus. It was evident that tenuazonic acid has a role in pathogenesis after causing wilting of seedlings, necrosis on leaves, inhibition of shoot and root growth of germinating seedlings of groundnuts (Devi *et al.*, 2010).

These toxins are not responsible for reproduction or growth of the fungus but have been reported to play a role in symptom development (Stuart *et al.*, 2009). Bart & Thomma (2003) reported that these toxins are not absolutely required for establishing disease; though act as virulence factors

for pathogenesis. However extracellular proteins and enzymes are also required for overall pathogenicity (Markham & Hille, 2001).

2.4. Sporulation in culture

Light, pH, temperature, nutrition, moisture and carbon source have an effect on sporulation and virulence of *A. alternata* (Masangkay *et al.*, 2000). According to Carvalho *et al.* (2008) sporulation and growth conditions of the fungus are closely related to virulence.

Masangkay *et al.* (2000) reported that conidial production of *A. alternata* increased under constant UV light on V8 juice agar (VJA) at 28°C but decreased on half strength PDA; however continuous darkness reduced conidia production on VJA and caused an increase on half strength PDA. Addition of 20g/l of calcium carbonate in all the media and 2ml of sterile distilled water on cultured mycelium optimized conidia production (Masangkay *et al.*, 2000).

Culturing the fungus on half strength PDA containing 20g/l of calcium carbonate, incubated at 18°C produced the most virulent conidia (Masangkay *et al.*, 2000). Monosaccharides enhance vegetative growth biomass by up to 75.6% compared to sucrose whereas sucrose decreases vegetative growth and enhances sporulation of *A. alternata* (Gupta *et al.*, 1979). According to Carvalho *et al.* (2008) mycelium stress techniques and incubation under white light or UV light are useful in testing for pathogenicity because they induce sporulation of *A. alternata*.

2.5. Variation in culture

Isolates of *A. alternata* are genetically different and bear heterocaryotic mycelia. Conidia arising from such mycelia may also be genetically different (Slavov *et al.*, 2004). Three isolates of *A.*

alternata isolated from three potato varieties were noted to differ in cultural, morphological and conidial measurements; variation was also evident within the same isolate (Giha, 1973). Pusz (2009) reported that isolates of *A. alternata* which are pathogenic to *Amaranthus* species have different linear growth and RAPD-PCR amplification of this fungus revealed heterogeneity to occur within the same species. According to Barksdale (1969) disease screening requires various culture selections for an accurate representation of wild type isolates since single spore cultures may produce a genetically distinct isolate. Re-culturing of *A. alternata* leads to a drop in pathogenicity (Ramm & Lucas, 1963). Non-sporulating sectors often occur in culture, even under optimum conditions for sporulation and this leads to loss of virulence (Slavov *et al.*, 2004). Lloyd (1969) reported that this is caused by prolonged vegetative growth of mycelia which induces loss of sporulative ability that pilots total saprophytism.

3. The disease

3.1. Geographic distribution

Alternaria alternata is a widespread fungus and has been found in many areas of the world on various different crops (Rotem, 1994). Brown spot of potato however, is prevalent in arid and semi-arid climatic conditions whereas early blight occurs in more humid regions of the world (Giha, 1973). *Alternaria* brown spot disease on potatoes has been reported to occur in at least nine countries namely, Sudan (Giha, 1973), Israel (Droby *et al.*, 1984), Italy (Pellegrini *et al.*, 1990), Brazil (Boiteux & Reifshneider, 1994), Yugoslavia (Cakarevic & Bosokovic, 1997), United Kingdom (Deadman *et al.*, 2002), United States of America (Dillard & Cobb, 2008), Germany (Leiminger *et al.*, 2010). and South Africa (Van der Waals *et al.*, 2011) The disease is most

problematic where potatoes are usually grown under irrigation, or in areas of high rainfall and humidity (Rotem, 1994) and can either occur alone or in association with other organisms (Giha, 1973). *Alternaria alternata* requires slightly less humidity to grow compared to *A. solani* (Giha, 1973).

3.2. Host range

According to Rotem (1994) *A. alternata* has been reported to occur on at least 115 plants from 43 families. Agnihotri (1963) reported that *A. alternata* is not specialized and it has a wide host range. Subspecies of *A. alternata* are reported on a wide range of crop plants and weeds, some of which include cotton, sunflower, onions, pistachio, citrus, carrot, sesame, cannas, *Zinnia elegans*, tobacco, sugarcane, groundnuts, eggplant (Rotem, 1994), nightshades, *Chrysanthemum* species, pear, European wild apple, *Doracaena* species, *Tagetes* species, tomato, *Pandonus tectorius*, (Agnihotri, 1963), peas, castello, castor bean, *Rhynocosia memnonoa*, *Datura metel* (Giha, 1973) and a variety of species from the families cucurbitaceae and brassicaceae (Rotem, 1994). It should however be note that some *Alternaria* isolates may have been previously misidentified due to the high diversity and variability of the genus *Alternaria* (Xia & Tiang-Yu, 2008) and therefore modern molecular techniques may be necessary to confirm *A. alternata* as a pathogen on some of these hosts.

3.3. Economic importance

Alternaria brown spot has been reported to cause reductions in yield due to loss of green foliage (Droby *et al.*, 1984). This is due to increased respiration rates and reduced photosynthesis which leads to a decrease in tuber bulking. Droby *et al.* (1984) reported that a 28% foliar infection is

followed by 18% yield reduction. According to Van der Waals *et al.* (2011) *Alternaria* brown spot can cause yield reduction of up to 30% under favorable conditions in South Africa. In North America yield losses ranging from 20 to 80% under uncontrolled conditions have been reported (Soleimani & Kirk, 2012). The occurrence of brown spot with other disease such as early blight, Verticillium wilt and blackleg has been reported to cause severe yield losses (Soleimani & Kirk, 2012). The control of brown spot with fungicides has significantly reduced disease severity and thus resulted in increased yield (Droby *et al.*, 1984).

3.4. Symptoms

The symptoms start by chlorosis surrounding the necrotic spots on the lower leaves progressing to the upper leaves into the emerging leaflets (Droby *et al.*, 1984; Akhtar *et al.*, 2004). Necrotic spots appear early in the season. These are small sunken lesions that are circular or oval in shape which occur as interveinal necrotic spots with raised margins with an indistinguishable zonation and may increase in size to coalesce and cause blight of the leaves (Neeraj & Verma, 2010, Hubballi *et al.*, 2010). Giha (1973) observed chlorosis to begin from the edge of the leaves and extend inwards.

Disease progress and severe infections lead to drying out of leaflets which finally fall off (Tafforeau & Lactorse, 2010). Chlorotic spots may also appear on the stems (Van der Waals *et al.*, 2011). Droby *et al.* (1984) reported symptoms caused by *A. alternata* on potato to appear under the leaves, whereas Cakarevic & Boskovic (1997) reported necrosis to appear on the upper side of potato leaves.

A. alternata can cause quiescent infection and remain dormant without any visual symptoms until the environment or inherent host properties are conducive for symptom development (Bart & Thomma, 2003). Slavov *et al.* (2004) reported that symptoms on tobacco may be visible within two to eight days after artificial inoculation in a favourable environment with a maximum incubation period of 35 days before visible symptoms appear and as little as 24 hours on citrus (Dehpour *et al.*, 2007).

Alternaria brown spot symptoms can be confused with those of early blight (Giha, 1973; Droby *et al.*, 1984; Leiminger *et al.*, 2010), tomato spotted wilt virus (TSWV) (Abad *et al.*, 2005) and environmental factors such as magnesium deficiency and manganese toxicity (Tafforeau & Lactose, 2010). Characteristic early blight symptoms consist of rings within the necrotic lesions (Neergaard, 1945, Stevenson *et al.*, 2001) and TSWV symptoms exhibit ring patterns without necrosis (Abad *et al.*, 2005). Turkensteen & Spoelder (2011) recorded lesions indistinguishable from typical early blight symptoms and neither *A. solani* nor *A. alternata* were isolated from these lesions, as a result creating confusion with mineral toxicity and deficiencies on potato plants. According to Leiminger *et al.* (2010) visual diagnosis of symptoms is not reliable; therefore identification by PCR screening of the causal organism by using species specific primers is a reliable tool to distinguish between different organisms and symptoms.

4. The disease cycle of *Alternaria alternata* on potatoes

4.1. Overwintering and dispersal

Brown spot is a polycyclic disease and primary inoculum can survive as mycelium or spores arising from conidiophores in decaying plant material on the soil for a considerable time (Bart

&Thomma, 2003). According to Agnihotri (1963) spores of *A. alternata* can survive for up to 6 months in the soil. The inoculum is produced in spring when the conidia break off from conidiophores and dispersed by water splash or wind to the lower leaves (Rotem, 1994, Bart & Thomma, 2003) and adjacent plants (Dehpour *et al.*, 2007). The numbers of *A. alternata* spores in the air are increased by the presence of diseased plants in the field but are constantly present in the atmosphere (Bashan *et al.*, 1991).

4.2. Infection

Once the *Alternaria* spore is in contact with the plant surface it attaches by producing extracellular material which assists with adherence (van den Berg *et al.*, 2003). Multiple germ tubes protrude from conidia, branching in random directions (Slavov *et al.*, 2004; Dehpour *et al.*, 2010). Extracellular material is also produced by the germ tube for grip on the plant surfaces exerting pressure to aid with penetration (van den Berg *et al.*, 2003).

Penetration of the plant may be through wounds, stomata and direct penetration of the cell walls by the germ tube (Droby *et al.*, 1984). According to Slavov *et al.* (2004) virulent species of *A. alternata* may penetrate directly through the cell wall while less virulent species penetrate through wounds and/or stomata. Penetration of the stomata is mostly by chance (Dehpour *et al.*, 2007) and the germ tube can penetrate the plant with or without producing appressoria (Bart & Thomma, 2003; van den Berg *et al.*, 2003).

The cuticle of the plant forms the first line of defense for direct penetrating fungi, but plant pathogenic fungi are able to produce enzymes that break down these barriers (Agrios, 2011). Enzymes such as cutinases that destroy the cuticle are produced by *A. brassisicola* on cabbage.

Lipase, cellulose, pectinase, pectin methyl esterase and galacturonidase have been reported to be produced by *Alternaria* species (Bart & Thomma, 2003). For example, *A. citri* has been reported to depend heavily on galacturonidase production for breaking down the cell wall and consequently establishing an infection (Bart & Thomma, 2003).

4.3. Colonization and secondary sporulation

After penetration the germ tubes produce endophytic mycelia that colonize the plant organs by invading intra- and intercellular spaces and advancing to healthy cells (Droby *et al.*, 1984). Conidiophores may protrude through wounds and stomata on the surface of the infected leaves (van den Berg *et al.*, 2003; Dehpour *et al.*, 2007) which may lead to secondary infection. High humidity was shown to increase sporulation of *A. alternata* on citrus. Sporulation was reported to start ten days after symptom development and the spores remained abundant in the atmosphere for 20 to 40 days (Reis *et al.*, 2006).

4.4. Epidemics

Water and temperature are important for growth of fungi and affect the number of spores produced and released in the air (Lyon *et al.*, 1984). Michailides & Morgan (1993) reported that *Alternaria* blight of pistachio is noticeable when sprinkler irrigation or flooding is practiced. A positive correlation has been illustrated between disease severity and increased irrigation rates or flooding (Droby *et al.*, 1984). Neeraj and Verma (2010) reported that the highest disease intensity was observed when temperatures were between 25°C and 28°C with an average relative humidity of 80%. This is due to the prolonged periods of leaf wetness and moisture around the plants (Droby *et al.*, 1984). Bashan *et al.* (1991) further reported that high humidity contributes to

disease severity by inducing production of conidiophores from necrotic areas of the infected leaves. According to Droby *et al.* (1984), brown spot is closely related to early blight since both diseases become more severe later in the season due to the slow build-up of inoculum. The release of conidia is stimulated by rain drops and a drastic drop in humidity or when dew dries in the morning and conidia are dispersed by wind during the day (Dehpour *et al.*, 2007).

4.5. Host nutrition

Infection of plants by *Alternaria* species is largely influenced by external and inherent host factors (Scholze & Ding, 2005). Mineral nutrients are not only important for growth and development of plants and microorganisms but also affect plant disease interactions (Spann *et al.*, 2010). Fertilizer applications such as nitrogen, phosphorus and potassium have been reported to have an effect on disease severity (Sharma & Kolte, 1994). For example, black spot disease caused by *Alternaria brassicae* (Sacc.) Berk., on oil seed rape becomes more virulent when the soil is treated with NP (N90 kg/ha and P40 kg/ha) applied as urea and superphosphate than on plants from unfertilized control (Sharma & Kolte, 1994).

Primary disease resistance is influenced by production of antioxidants, phytoalexins and flavonoids which rely on availability and assimilation of nutrient elements (Spann *et al.*, 2010). Resistance towards *Alternaria* diseases was induced when potassium was applied together with nitrogen; where smaller lesions were observed compared to plants that received either of the elements alone. Fertilization with high nitrogen and phosphorus has also been reported to increase disease severity (Sharma & Kolte, 1994).

The differences in host susceptibility towards *Alternaria* leaf spot of cotton was linked to potassium content in the host plant (Sharma & Kolte, 1994). On the other hand Van der Waals *et al.* (2001) noted that low phosphorus, high nitrogen and medium to low potassium reduce susceptibility towards early blight and that this is due to extended vegetative growth, reduced fruiting and reduction in tuber formation. Increased rates of nitrogen fertilization cause losses in tuber quality and yield but have a positive effect by reducing infection rates and disease severity of early blight (Mackenzie, 1981).

According to Sharma & Kolte (1994), potassium fertilized plants produce a high content of phenolic compounds which are responsible for suppressing disease. Scholze & Ding (2005) noted that black spot disease of cabbage caused by *A. brassicola* increased with a decrease in phenolic content in plants. Spann *et al.* (2010) further stated that potassium is required for synthesis of cellulose that builds up the cell wall and that potassium deficiency causes the cell walls to leak thus creating an opportunity for infection. Each nutrient may however affect plant disease either positively or negatively, depending on the disease complex (Spann *et al.*, 2010).

4.6. Host susceptibility

The middle leaves were the most heavily affected part of the plant on both young and older potato plants (Droby *et al.*, 1984). The most severe disease was observed on potato plants at ages of 58 to 74 days. According to observations made by Droby *et al.* (1984) physiological age has a significant effect on the disease since older plants were more susceptible to disease than younger plants; the fungus can however infect plants at any stage of growth.

Droby *et al.* (1984) reported that older leaves of the potato plant are more susceptible than the middle leaves, with younger leaves displaying smaller lesions compared to older leaves. According to Rotem (1994), tomato plants established an increase in infection as they grew older. This type of resistance is however age conditioned and temporary and should therefore be differentiated from permanent resistance which persists throughout the plant's life cycle (Van der Waals *et al.*, 2001).

A varied level of genetic resistance is found in different cultivars. In an *in vitro* study of host defense mechanisms of potatoes against *A. alternata* in Italy, Pellegrini *et al.* (1990) reported cultivar Chiquita to have resistance against the fungus and Superior is regarded to be susceptible. This kind of resistance observed in cultivar Chiquita may be due to increased production of phenolic compounds (Pellegrini *et al.*, 1990).

Droby *et al.* (1984) tested five potato cultivars grown in Israel against *Alternaria* brown spot. Cultivar Up-to-date and Cardinal were more resistant to disease followed by Spunta with intermediate resistance, whereas Blanka and Desiree were considered to be susceptible. Complete resistance was never recorded and symptoms were detected on all cultivars tested and therefore differences in cultivar resistance levels were more evident towards the end of the season (Droby *et al.*, 1984).

5. Control

Different options are available for the control of diseases caused by fungi (Agrios, 2011). These methods are dependent on the nature of the disease cycle, some of which include cultural control practices that are employed to lessen infection caused by the initial inoculum (Madden *et al.*,

1978). A 3-5 year crop rotation of potatoes with non host crops such as grains and forage crops reduces the accumulation of inoculum and as a result reduces disease severity and incidence (Van der Waals *et al.*, 2001).

Field sanitation, providing proper plant nutrition, selection of resistant cultivars, avoiding water stress and planting disease free seeds, are methods available to suppress disease. Field sanitation engages the removal of decaying vines, weeds such as *Solanum nigrum* (black nightshade) and volunteer plants which may carry inoculum; which is done before planting a new crop (Ferreira, 1998). Tillage practices can be used to bury vines deep enough to limit exposure of spores to the germinating crops (Agrios, 2011). These methods may not eradicate the disease, but reduce disease severity by lowering initial inoculum (Madden *et al.*, 1978).

Under favourable climatic conditions the pathogen becomes more aggressive and can multiply and infect freely which renders cultural control options ineffective (Surviliene & Dambrauskiene 2006). It is in these cases that chemical applications cannot be neglected. Fungicides have been reported to provide satisfactory results and are considered to be the most effective method of controlling the disease (Madden *et al.*, 1978; Van der Waals *et al.*, 2001). Proper timing and fungicide coverage of the crops is important to reduce disease and yield loss. Early application of fungicides during crop growth until the vines are dead has proven to be effective.

Droby *et al.* (1984) found promising results after field testing of fungicides such as maneb, imazalil and iprodione. It is however economically unsuitable to continuously apply fungicides (Kashyap & Dhiman, 2010). Injudicious fungicide applications also cause accumulation of residues and development of resistance (Kashyap & Dhiman, 2010). It is for this reason that

computerized early warning systems which forecast disease development by employing environmental parameters and cultivar type are used. Decision support systems such as FAST (forecast system for *Alternaria solani* on tomato) and PLANT-Plus (forecast system for *Alternaria solani* on potato) have been implemented for efficient timing of spray programs and as a result fungicide applications were minimized without any effect on yield compared to the conventional calendar-based spray program (Madden *et al.*, 1978; Van der Waals *et al.*, 2003).

These models however, have not been designed to control *Alternaria* brown spot. According to Van der Waals *et al.* (2001) South African farmers heavily rely on chemicals to control blight diseases of potatoes, although this might be the only effective and most readily available method under high disease pressure, development of resistance turned out to be a setback (Ferrar *et al.*, 2004; Pasche *et al.*, 2005; Kirk *et al.*, 2009).

In the potato growing regions of the USA a near 100% resistance level of *A. solani* towards application of strobilurin fungicides has been reached (Kirk *et al.*, 2009) as a result of the F129L mutation (Pasche *et al.*, 2005). Complete disease control cannot be obtained by a single control measure (Surviliene & Dambrauskiene, 2006). Therefore implementation of integrated disease and pest management programs is important for efficient disease control (Surviliene & Dambrauskiene, 2006). Various microorganisms and plant extracts have been reported to have an inhibitory effect on *A. alternata* (Begum *et al.*, 2010; Neeraj & Verma, 2010). Fifty eight percent mycelial inhibition was achieved by methanol extracts from stems and leaves of *Myoporum bontioides* (Neeraj & Verma, 2010).

6. Conclusion

Little attention has been given to the management of *Alternaria* brown spot on potatoes. There is sufficient evidence that supports *A. alternata* to have the potential to cause considerable reduction of yield (Van der Waals *et al.*, 2011) especially in arid and semi arid regions under relatively low humidity (Giha, 1973). Characteristic *Alternaria* brown spot symptoms have been observed on potato fields in South Africa and it appears even under spray programs designed to control early blight (Van der Waals *et al.*, 2011). This has lead to the hypothesis that current fungicides registered to control *A. solani* may have little or no inhibitory effect on *A. alternata*, hence the emergence of brown spot in potato fields. Numerous workers describe *A. alternata* as a weak opportunistic pathogen that causes disease due to host weaknesses (Rotem, 1994; Deadman *et al.*, 2002; Leiminger *et al.*, 2010) but there is evidence that supports the ability of the fungus to cause disease on healthy plants (Giha, 1973; Droby *et al.*, 1984), however little damage is caused in such occasions (Giha, 1973).

According to Giha (1973) the ability of *A. alternata* to cause disease is more dependent on the environment than it is on inherent properties of the fungus. According to Van der Waals *et al.* (2011) brown spot can cause serious damage under favourable conditions and if the disease is left untreated. Recent reports place emphasis on the ability of climate change to render pathogens previously known to be weak to become more serious depending on locality (Newton *et al.*, 2010).

Giha (1973) reported *A. alternata* isolated from different hosts to have great morphological differences even within the same isolates. This causes great confusion in identification of the

fungus (Pryor & Gilbertson, 2000). Molecular techniques such as DNA fingerprinting are available for identification of fungi; however these methods should never be used alone (Frisvad *et al.*, 2007).

Various cultural and chemical control options are available to suppress the development of *A. alternata* (Neeraj & Verma, 2010). It is in respect of the uncertainties behind the causal agent of brown spot on potato and unavailability of intrinsic control options thereof that this research has focused on determining the causal agent of brown spot disease on potato and *in vitro* evaluation of different registered fungicides to control *A. alternata*.

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Chapter 3: *Alternaria alternata*, causal agent of potato brown spot in South Africa

Abstract

Alternaria alternata has frequently been isolated from brown spot symptoms on potato in various production regions of South Africa. This disease was never reported before in South Africa. Koch's postulates conducted in a greenhouse under controlled conditions confirmed that *A. alternata* is the causal agent of brown spot disease on potato plants. Both detached leaves and potcultured plants which were inoculated, developed brown spot while all the uninoculated treatments remained disease free. Isolations were done from both inoculated and uninoculated treatments but *A. alternata* was only recovered from inoculated treatments, and identity was confirmed by means of a PCR with species-specific primers. Scanning electron micrographs of the leaf discs revealed that *A. alternata* had colonized and infected the potato leaves of both pot cultured plants and detached leaves. Nothing was observed on uninoculated treatments which acted as control.

3.1. Introduction

In recent years *Alternaria alternata* (Fr.) Keissler has frequently been isolated from brown spot symptoms observed on potato foliage (*Solanum tuberosum*.L) in various production regions of South Africa (Van der Waals *et al.*, 2011). The leaves of potato first appear yellow, after which circular brown sunken lesions appear on both the abaxial and adaxial sides of the affected leaves. Under high disease pressure lesions coalesce and stems may also become blighted. Additionally Akhtar *et al.* (2004) described symptoms caused by *A. alternata* f.sp. *lycopersici* on tomato as starting with yellowing and browning of the lower leaves, progressing to the upper leaves under

high humidity, which leads to formation of necrotic lesions (Anderson *et al.*, 2005) causing defoliation. Similar symptoms were first reported in Sudan (Giha, 1973), followed by Israel, where necrosis occurred on both tubers and foliage of potato plants (Droby *et al.*, 1984). Cakarevic & Boskovic (1997) reported necrosis caused by *A. alternata* to appear on the abaxial side of potato leaves. Furthermore, Droby *et al.* (1984) described relative similarities between symptoms caused *A. alternata* and *Alternaria solani* Sorauer on potato foliage; however early blight symptoms consist of concentric rings contained by the necrotic leaf area (Neergaard, 1945, Stevenson *et al.*, 2001).

A. alternata has previously been reported to cause foliar blight on numerous plant families (Rotem, 1994) and it is recognized as an opportunistic cosmopolitan pathogen (Survaliane & Dambrauskiene, 2006). According to Cakarevic & Boskovic (1997) *A. alternata* is a common pathogen of the family Solanaceae. Droby *et al.* (1984); Boiteux & Reifschneider (1994) and Cakarevic & Boskovic (1997) reported *A. alternata* as the predominant pathogen causing brown spot on potatoes. Hudec & Rohacik (2002) reported *A. alternata* to be widely spread by wind and that some plants such as wild beet can be a source of inoculum to other important crops though disease symptoms may differ depending on the host.

A. alternata is a facultative parasite and it attacks due to predisposition of the host to biotic or abiotic stresses, such as primary disease infections, mineral deficiencies or drought (Boiteux & Reifschneider, 1994). For example, *A. alternata* was isolated together with *A. solani* from potatoes showing symptoms of early blight (Boiteux & Reifschneider, 1994). A succession of infections of the potato plant by *A. solani* which weakens the host is often followed by *A.*

alternata infection, as a result causing severe necrosis and blight on foliar parts of potatoes. This disease is well known as the *Alternaria* complex (Kirk *et al.*, 2007). A similar relationship was observed when leafminer (*Lyriomyza tryfolii*) and *A. alternata* infected potato plants to cause severe brown spot symptoms on leaves (Deadman *et al.*, 2002).

A. alternata is known to produce conidia that colonize and infect foliar parts of the potato (Droby *et al.*, 1984; Agrios, 2005), however, little is known about the infection processes of *A. alternata* on potatoes. Bart & Thomma (2003) reported that spores produced by *Alternaria* species germinate, producing germ tubes which can penetrate through wounds, stomata or by direct penetration through the plant cuticle. *A. alternata* penetrates cotton leaves through stomata and this process is affected by moisture availability (Bashan *et al.*, 1991).

Nishimura *et al.* (1978) reported *A. alternata* isolates to produce host specific toxins (HST) that incite disease symptoms on a specific crop. According to Nishimura & Komoto (1983) HSTs produced by *A. alternata* assist the pathogen during tissue attachment, infection and symptom development (Quayyum *et al.*, 2003; Agrios, 2011). The HST secreted by the pathogen causes yellowing surrounding the point of infection, progressing in to the healthy tissue of the host (Quayyum *et al.*, 2003; Agrios, 2011).

The disease is more prevalent in arid and semi-arid regions where irrigation is practiced (Giha, 1973). Michailides & Morgan (1993) reported that *Alternaria* blight of pistachio is noticeable when sprinkler irrigation or flooding is practised. Bashan *et al.* (1991) further reported that high humidity contributes to disease severity by inducing production of conidiophores from necrotic

areas of the infected leaves. Optimal growth of *A. alternata* is reported to be between 25 and 30°C (Cakarevic & Bosokovic, 1997).

Although *A. alternata* is reported to cause leaf blight on a wide range of plants (Agrios, 2005; Feng & Zheng, 2007) which includes the Solanaceae crops (Cakarevic & Bosokovic, 1997; Bart & Thomma, 2003), until recently there was still uncertainty whether *A. alternata* was the causal agent of the foliar brown spot observed on potatoes in South Africa. The aim of this study was to investigate the pathogenicity of *A. alternata* on potatoes in South Africa.

3.2. Materials and methods

3.2.1. Isolation of the pathogen from host

Potato plant samples showing brown spot symptoms were collected from various potato growing regions in South Africa. Leaves were surface disinfected with 1% sodium hypochlorite and cultured on V8 juice agar (V8; Simmons, 1992) amended with 250mg/L of chloramphenicol. The V8 agar plates were incubated for eight days under alternating 12 hour UV light/ 12 dark cycle at 25°C.

3.2.2. Identification of the pathogen

To identify the pathogen cultures were grown on V8 agar media for eight days. The plates were incubated for eight days under 12 hour UV light/ 12 dark cycle at 25°C to induce sporulation. According to Carvalho *et al.* (2008) mycelium stress techniques and incubation under white light or UV light are useful for pathogenicity studies because they induce sporulation of *A. alternata*.

The resulting mycelia and spores were morphologically identified according to Pryor & Michalides (2002) by means of a light microscope.

To confirm morphological identification of the fungus, a polymerase chain reaction (PCR) was used to amplify internal transcribed spacer (ITS) region. Fungal mycelium and conidia from 12 isolates grown on PDA for 2 weeks at 25°C were scraped into eppendorf tubes and DNA extractions were done using a Zymo DNA extraction kit (Zymo Research, California). Amplifications were carried out with a forward and reverse primer AAF2 (5'-TGCAATCAGCGTCAGTAACAAAT-3') and AAR3 (5'-ATGGATGCTAGACCTTTGCTGAT-3') at an expected fragment size of ~ 340bp (Konstantinova *et al.*, 2002). This primer pair was used for the PCR amplification of rDNA containing the ITS 1, the 5.8S gene and ITS2 (Konstantinova *et al.*, 2002). A 25 µl reaction volume containing a reaction mixture of 18.25 µl of sterile double-deionised water, 5U My Taq buffer, 0.25U Taq DNA polymerase, 0.25 µl of the respective primer sets (200 nM) and 1 µl template DNA (15 ng/µl) was used. MJ Mini: Personal Thermal Recycler (Bio-Rad) was used for PCR amplifications of the ITS primers. The process ran for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, and elongation at 72°C for 1 min. A final extension was performed at 72°C for 10 min. The PCR products were analyzed in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. An hpII ladder was used as a molecular weight marker.

3.2.3. Preparation of 10% vegetable oil emulsion spore suspension

A lightly warmed 9 ml aliquot of pure vegetable oil (Sunfoil, Pietermaritzburg, South Africa) was mixed with 0.1ml of Tween 80 (Sigma-Aldrich). The oil-surfactant mixture was stirred using a

magnetic stirrer while 90ml of distilled water was added (Babu *et al.*, 2003). The solution was emulsified by stirring for 5 minutes in a laboratory mixer. Two milliliters of the vegetable oil emulsion was poured on to two-week old V8 juice agar plates bearing *A. alternata* conidia. The conidia were loosened from the mycelium by gently scraping the cultures using a glass rod. The final oil emulsion spore suspension was sieved through cheese cloth to remove excess mycelia. The spore concentration was adjusted to 5×10^4 conidia/ml with the aid of a haemocytometer.

3.2.4. *In vitro* detached leaves and *in vivo* pot plant inoculations

Twenty leaves were randomly detached from 8 week old BP1 potato plants and disinfected with 1% sodium chloride solution for 5 min, rinsed in sterile distilled water for a minute and inoculated by painting ten leaves to run-off with a 10% oil emulsion containing *A. alternata* spores adjusted to 5×10^4 spores/ml, while the remaining ten leaves were painted with a sterile 10% oil emulsion to act as a control. The leaves were placed in 90mm Petri dishes containing a sterilized blotter paper and 5ml of sterile distilled water to maintain a high humidity and the plates were kept at natural day and night conditions in a glasshouse at 30°C ($\pm 1^\circ\text{C}$) for a week. Symptoms were recorded daily.

Twenty BP1 potatoes were planted in 20kg pots. Ten plants were inoculated by painting the leaves with the 10% oil emulsion spore suspension (5×10^4 conidia/ml) of *A. alternata* to run-off as soon as the first 3-5 leaves emerged. The remaining plants were painted with a sterile 10% oil suspension. The plants were kept at 30°C ($\pm 1^\circ\text{C}$) in a fogging system which went on for 2hrs twice a week and re-inoculation was repeated once a week until symptoms appeared.

3.2.5. Scanning electron microscopy

To confirm infection of the potato plant by *A. alternata*, 10 randomly selected leaves from 5 inoculated and un-inoculated plants were cut into 5 mm² pieces three days post inoculation. At this stage small irregular brown lesions were visible with a magnifying lens on the inoculated leaves and none were observed on uninoculated leaves. The leaf pieces were fixed in 2.5% (v/v) glutaraldehyde in 0.075 M phosphate buffer (pH 7.4). The material was rinsed in the same buffer, post-fixed in 0.25% (w/v) aqueous osmium tetroxide for two to four hours and then rinsed three times in distilled water. The material was subjected to an ascending ethanol series (30, 50, 70, 80, 95 and 100%), dried in a critical point dryer and mounted on stubs. The specimens were coated with gold in a Polaron sputter coater and examined with a Jeol JSM 840 scanning electron microscope at 5 kV.

3.2.6. Confirmation of the pathogen

At the end of each experiment, all of the detached leaves and pot plants of both the control and treatment were screened for the presence of *Alternaria alternata*. The pathogen was re-isolated and morphologically identified using a light microscope. The identity of the causal agent was confirmed by means of a PCR as it was described during initial identification.

3.3. Results and discussion

3.3.1. *In vitro* detached leaf assay and *in vivo* pot plant inoculation

Small irregular brown spot lesions were observed on all detached leaves inoculated with *A. alternata* three days post inoculation and no symptoms developed on the control treatment (Fig.1,

2 & 3). Brown sunken irregular lesions appeared on both the abaxial and adaxial side of the detached leaves (Fig. 1 & 2). The leaflets became chlorotic then necrotic. This started at the leaf margins and progressed interveinally to the leaf interior (Fig. 1). Mycelial growth was observed on the necrotic areas of the adaxial side of the infected leaves (Fig. 1. arrows).

Symptoms in the pot trials were only visible eight days after inoculation. All inoculated plants were infected (Fig. 4) while no symptoms developed on the control plants. Infected pot plants exhibited the same symptoms as those on the detached leaves. Yellowing of the older leaflets progressed from the leaf blades towards the interior of the leaves followed by brown irregular lesions that occurred between the leaf veins (Fig. 4). The symptoms were also visible on both sides of the leaves.

According to Giha (1973) *A. alternata* causes chlorosis that begins on the leaf edges and extends inwards. Yellowing of the of the older leaves progressing to young emerging potato leaves is characteristic of symptoms caused by *A. alternata* (Droby *et al.*, 1984; Akhtar *et al.*, 2004). Agrios, (2011) and Quayyum *et al.* (2003) reported that yellowing around the point of infection is the result of host selective toxins (HST), characteristic of *Alternaria* species. HSTs assist species such as *A. alternata* with host infection by assisting the pathogen to progress to healthy tissues (Akhtar *et al.*, 2004).

The incubation period in this study is comparable to that described by Slavov *et al.* (2004) which occur between two to eight days. According to Bart & Thomma (2003) *A. alternata* can cause quiescent infection and remain dormant without any visual symptoms until the environment or inherent host properties are conducive for symptom development. Neeraj & Verma (2010)

reported *A. alternata* to cause a sunken brown necrotic area with indistinguishable margins. These brown necrotic areas occurred between the leaf veins. These observations are comparable with the findings in this study.

3.3.2. Scanning electron microscopy

The conidia were not dislodged during SEM preparation and this led to the conclusion that they strongly adhered to the leaf surface. The conidia produced extracellular amorphous material and multiple germ tubes which randomly grew across the leaf surface (Fig.5A). Previous studies of various *Alternaria* species reported similar observations (van den Berg *et al.*, 2003; Slavov *et al.*, 2004; Dehpour *et al.*, 2010). An extracellular amorphous material was also produced by the mycelium (Fig.3B). Extracellular amorphous material has been linked with conidia and germ-tubes of *A. cassiae* and *A. solani* on cowpea and potatoes respectively (van den Berg *et al.*, 2003; Dita *et al.*, 2007) and has been reported to have an adhesive function (van den Berg *et al.*, 2003).

The germ-tubes established infection by directly penetrating the leaf epidermis (Fig.5A and B) and closed stomata (Fig.5C). Secondary sporulation was evident when conidiophores emerged through stomata (Fig.5B). According to van den Berg *et al.* (2003) conidiophores of *A. cassiae* emerged through the stomata and leaf epidermis four days after inoculation, however Reis *et al.* (2006) reported that sporulation of *A. alternata* started ten days after symptom development on citrus.

Results from this study showed that secondary conidiophores were produced on plant tissue three days post inoculation. This interval is relatively similar to that of *A. cassiae* on cowpea as reported by van den Berg *et al.* (2003). Giha (1973) and Rotem (1994) reported that mycelial

growth and sporulation may be variable in the genus *Alternaria* because some *Alternaria* species and isolates are more aggressive than others. This disparity was reported to occur within the same species and amongst isolates (Giha, 1973). This genetic difference in isolates is attributed to variation caused by heterokaryotic mycelia which bear genetically different conidia (Slavov *et al.*, 2004).

Production of appressoria was not witnessed in this study which may have been influenced by the time elapsed before SEM was conducted, however, van den Berg *et al.* (2003) and Dehpour *et al.* (2007; 2010) reported that production of appressoria is not necessary to initiate infection through direct penetration, wounds or open and closed stomata. The germ-tubes showed no specific penetration sites (Dehpour *et al.*, 2010); however colonization in this study was observed through closed stomata (Fig. 5C) and directly through the epidermis as indicated on Figure 5A (di) and Figure 5B (di). According to Rotem (1994) and Dehpour *et al.* (2010) the infection cohort in less pathogenic *Alternaria* species may be limited to wounds and stomata. Slavov *et al.* (2004) reported that virulent species of *A. alternata* may penetrate directly through the cell wall.

It should be noted that SEM in this study was only used as a tool to verify colonization and an extensive SEM and light microscope studies are necessary to determine the infection behavior and specific penetration sites.

3.3.3. Identification of the pathogen

Morphological identification of the fungus by means of a light microscope revealed light brown multiseptate conidia with a diameter of 20- 25.33 μm . The conidia were borne on long chains with 3-7 transverse septa and short conical beaks, similar to that described by Weir *et al.* (1998)

and Akhtar *et al.* (2004). Molecular identification by means of PCR confirmed the identity of the fungus to be *A. alternata*, as it was identified morphologically. In the infection studies *A. alternata* was only recovered from diseased plants and not from healthy plants which acted as controls. There were no differences found between the pre-inoculation identification of *A. alternata* and the re-identification which was done post symptom development.

3.4. Conclusion

The SEM conducted in this study serves as a basis for infection studies of *A. alternata* on potatoes, as it would appear that this is the first such study in this pathosystem. It should be noted that the SEM in this study was only used as a tool to verify the establishment of infection in the inoculated treatments. Conidia attached to and infected the inoculated leaves. Furthermore inoculated treatments had already developed microscopic symptoms three days post inoculation, prior to fixation for SEM.

The development of symptoms on inoculated plants and leaves served as a confirmation that *A. alternata* conidia attached and infected potato leaves as was observed with SEM. The initial stages of disease development are evident when colonization and infection occur, when conidia attach and germinate to penetrate the host (Van Den Berg *et al.*, 2003; Slavov *et al.*, 2004; Dehpour *et al.*, 2010).

Both detached leaf and pot plant experiments produced symptoms identical to those which were observed in the field. Re-isolations were done from both inoculated and uninoculated pot plants and leaves. However visual diagnosis of symptoms is misleading, therefore identification of the causal organism by PCR with species specific primers is a reliable tool (Leiminger *et al.*, 2010).

A. alternata in this study was recovered only from inoculated plants and its identity was confirmed by both conventional and molecular techniques after re-isolations.

Since no *A. alternata* or other fungi were recovered from uninoculated plants, this was conclusive that no quiescent infection occurred and confirmed *A. alternata* to be the causal agent of brown spot on potatoes in South Africa.



Figure 1: *Alternaria alternata* inoculated adaxial side of a detached BP1 leaf with irregular brown spot lesions, chlorosis and mycelial growth (arrows).



Figure 2: *Alternaria alternata* inoculated abaxial side of a detached BP1 leaf with irregular brown spot lesions and chlorosis.



Figure 3: An un-inoculated symptom free detached BP1 leaf, which acted as a control



Figure 4: Chlorosis followed by irregular brown spot lesions developing from the leaf edges of the pot plant and proceeding towards the leaf interior, eight days after inoculation.

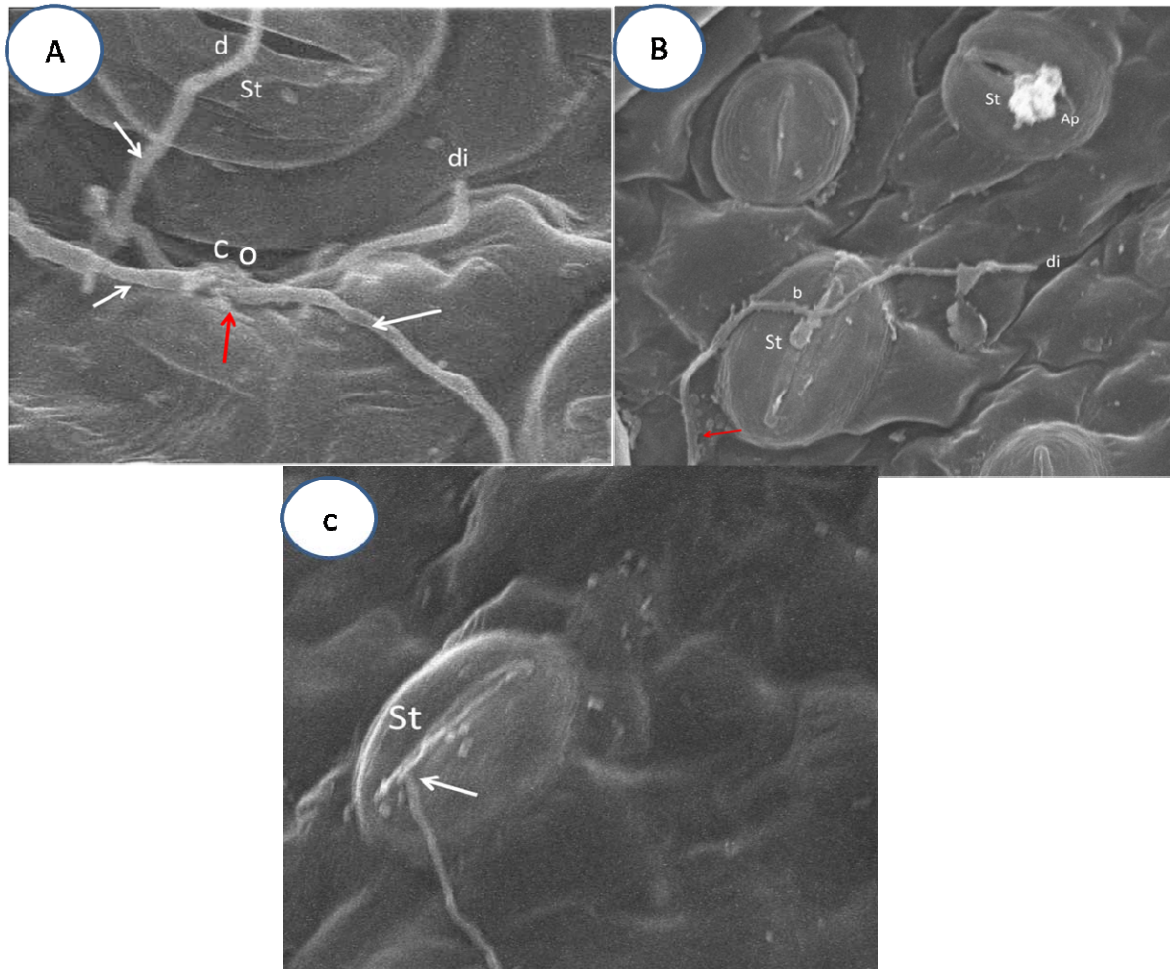


Figure 5: Scanning electron micrographs of *A. alternata* on potato leaves. Photo (A): A conidium germinates (co; red arrow), and produces multiple germ-tubes (white arrows) that randomly grow across the leaf surface. Extracellular amorphous material is produced by the conidium (red arrow). A germ-tube grows over open stoma (St) without penetration (d). A germ-tube directly infects (di) the epidermis on lower surface of the leaf. Photo (B): mycelium of *A. alternata* produces extracellular amorphous material (red arrow). The mycelium grows over (b) a closed the stoma (St) and directly penetrates the epidermis without production of appressoria; conidiophores (Ap) emerge through stoma Photo (C) mycelium penetrates a closed stoma.

3.5. References

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Chapter 4: *In-vitro* chemical control of *Alternaria alternata* associated with brown spot of potato in South Africa

Abstract

Alternaria brown spot disease on potatoes caused by *Alternaria alternata* has reached epidemic proportions in South Africa. It would appear that fungicides that are registered for control of early blight (*Alternaria solani*) have little effect on *Alternaria* brown spot. Different fungicides registered for control of *Alternaria* species on various crops in South Africa including potatoes were tested for the inhibition of *A. alternata in vitro*. All the treatments were fungistatic and mycelial growth inhibition ranged between 64 and 90%. Fungicides with active ingredient(s) that have never been used on potatoes before generally had an increased inhibitory effect when compared to those which have been previously used. Fungicides with a triazole as an active ingredient generally performed better than other fungicides. Azoxystrobin showed the least mycelial growth inhibition, however incorporation of azoxystrobin by mixture with other active ingredients from alternative fungicide groups produced better results than azoxystrobin used alone.

4.1. Introduction

In recent years *Alternaria alternata* (Fr.) Keissler has frequently been isolated from brown spot symptoms observed in potato production regions of South Africa (van der Waals *et al.*, 2011). First symptoms of the disease on potatoes (*Solanum tuberosum* L.) are circular brown sunken lesions on the underside of leaves. Later these lesions appear on the upper sides of leaves, after

which leaves may become chlorotic. Under high disease pressure severe coalesced lesions can completely blight stems and leaves.

Similar symptoms were first reported in Sudan (Giha, 1973). Giha (1973), Droby *et al.* (1984) and Leiminger *et al.* (2010) described similarities between symptoms caused *A. alternata* and *A. solani* on potatoes, except that early blight symptoms consist of concentric rings around the necrotic leaf area (Neergaard, 1945).

A. alternata has previously been reported to cause foliar blight on diseased vegetables and is recognized as an opportunistic cosmopolitan pathogen (Surviliene & Dambrauskiene, 2006). Boiteux & Reifschneider (1994) reported that *A. alternata* is a weak pathogen, usually attacking stressed or wounded plants. According to Cakarevic & Boskovic (1997) *A. alternata* is a common pathogen of the family Solanaceae. Furthermore Giha (1973), Droby *et al.* (1984), Pellegrini *et al.* (1990), Boiteux & Reifschneider (1994); Cakarevic & Boskovic (1997), Deadman *et al.* (2002), Dillard & Cobb (2008), Leiminger *et al.* (2010) and Van der Waals *et al.* (2011) reported *A. alternata* as a pathogen causing brown spot of potatoes in Sudan, Israel, Italy, Brazil, Yugoslavia, United Kingdom, United States of America, Germany and South Africa respectively.

Considerable yield losses have been encountered to warrant the control of brown spot on potatoes. However attempts to control *Alternaria* blight by using chemicals have been reported to be unsuccessful or rather complicated on many crops (Bashan *et al.*, 1991). Application of fungicides such as benomyl, iprodione and mancozeb to control mouldy-core on apples produced ineffective results (Reuveni & Sheglov, 2002).

Resistance and unsatisfactory control of *A. alternate* has previously been reported due to application of numerous fungicides such as Aliette® (contains iprodione and fosetyl-Al as active ingredients) to control brown spot on citrus (Timmer & Zitko, 1997), as well as of iprodione and tebuconazole for control of brown spot of *Minneola tangelo* in South Africa (Swart *et al.*, 1998). Management of *Alternaria* diseases cannot be attained by a single control measure especially under high inoculum pressure where multiple applications of fungicides on crops are required (Surviliene & Dambrauskiene, 2006).

Regardless of the trend to restrict fungicide applications on crops to a minimum, chemical control remains important for effective disease control (Gullino *et al.*, 2000). In the recent development of fungicides, some compounds have been reported to act effectively against numerous species of *Alternaria*, including *A. alternata* on apples (Reuveni & Prusky, 2007). Pryor *et al.* (2002) reported the use of fungicides to suppress *Alternaria* leaf blight caused by *Alternaria dauci*. Fungicides such as Signum® (contains boscalid and pyraclostrobin as active ingredients) and trifloxystrobin were reported to be relatively effective against several *Alternaria* species *in vitro* (Surviliene & Dambrauskiene, 2006).

Growers have experienced epidemic proportions of brown spot on potatoes and it would appear that the current fungicides registered for control of early blight on potatoes are not entirely successful in controlling brown spot. In addition, most of these fungicides have not been tested for control of *A. alternata* which causes brown spot of potatoes in South Africa. The aim of this study was to conduct preliminary screening for the mycelial inhibitory effect of different

fungicides *in vitro* against *A. alternata* isolated from brown spot diseased potato plants in Mpumalanga, South Africa.

4.2. Materials and methods

4.2.1. Agar preparation

Thirty nine grams of Potato dextrose agar (PDA) (Sigma-Aldrich) was poured in 1L of distilled water, autoclaved and cooled to 50°C. Different quantities of each fungicide were added to the cooled PDA and adjusted accordingly to the required concentrations as listed in Table 1, excluding the control which had no fungicide added to the PDA.

4.2.2. Antifungal activity assay

The extent to which each fungicide inhibits the radial growth of *A. alternata* was determined. Fungicide amended agar plates were inoculated with a 5mm diameter plug from a one week old actively growing culture of *A. alternata*. The plug was grown on unamended PDA to act as a control. All the treatments were incubated at 25°C for seven days and the fungal growth readings were taken by measuring colony diameter on the eighth day and percentage inhibition determined as described by Kaiser *et al.* (2006). The experiment consisted of 10 replicates per treatment and was repeated twice.

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

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

T = colony diameter (mm) of the test plate.

4.2.3. Mode of fungicide activity

To determine the fungistatic or fungicidal potential of each fungicide mycelium plugs of *A. alternata* were removed from the antifungal assay treatments on the eighth day after exposure to each chemical treatment including the control. Each plug was then transferred to fresh PDA and incubated at 25°C. Further mycelial growth from the plugs was evaluated after eight days following the transfers on to fresh PDA. The colony diameter of each treatment was recorded and compared to the control plugs using the formula described by Kaiser *et al.* (2006) as given above. The data was recorded as percentage growth relative to the control and the experiment was repeated twice.

4.2.4. Statistical analysis

Analysis of variance was determined at the 95 % confidence interval using the SAS program. The Fisher's t-distribution was used for multiple comparison of each fungicide in terms of mycelial growth inhibition and percentage germination.

4.3. Results and discussion

The mycelial growth inhibition in this study ranged from 64% to 90%. The most effective treatments which showed most mycelial inhibition were AC crop oil (Acanto[®] + Capitan[®] + H&R Crop oil) and Nativo[®] with an average mycelia inhibition of 88.7% and 88.2% respectively. The second most effective treatments were Bellis[®] and No-Blite[®] which inhibited growth by 87.2% and 87% respectively (Fig 5). There were no significant differences in percentage inhibition between Nativo[®] and AC Crop oil and the second most effective fungicides, Bellis[®] and No-Blite[®].

There were however significant differences found between AC crop oil and the second most effective fungicides Bellis[®] and No-Blite[®]. Amistar Top[®] and Score[®] were the third most effective in inhibiting mycelial growth by 85.1% and 84.9% with no significant difference found between the two treatments. Significant differences were found between Proxan[®], Barrier[®], TU Crop oil (Tanos[®] + Unizeb[®] + H&R crop oil), Amistar Opti[®] and Twist[®] which caused 82.8%, 80.4%, 76.7%, 68.8%, and 65.6% growth inhibition respectively (Fig 5). All mycelial plug transfers to fresh PDA exhibited 100% growth compared to the control and it was thus concluded that all treatments have a fungistatic mode of action.

Our results were consistent with that of Allemann (2007) as it was observed that boscalid-pyraclostrobin, picoxystrobin-flusilazole, tebuconazole and difenoconazole inhibited over 84% of mycelial growth. Fungicides containing a strobilurin active ingredient such as azoxystrobin and trifloxystrobin used alone or in combination with a contact fungicide such as in the case of azoxystrobin-chlorothalonil combination showed the least mycelial growth inhibition. These results are consistent with the findings of Ma *et al.* (2003) who reported resistance to azoxystrobin by *A. alternata* isolates occurring on pistachio; moreover Whiteside (1970) reported chlorothalonil to produce unsatisfactory results against *A. alternata*.

According to Issiakhem & Bouznad (2010), difenoconazole, which has been reported by Van der Waals *et al.* (2005) to have been used less often than chlorothalonil in controlling early blight on potatoes; was more efficient in controlling *A. solani* and *A. alternata in vitro*, a similar effect was observed in this study when fungicides containing difenoconazole as the active ingredient inhibited the highest percentage of mycelia than chlorothalonil, with azoxystrobin being one of

the active ingredients in both difenoconazole and chlorothalonil as in the case of Amistar Opti (chlorothalonil + azoxystrobin) and Amistar Top (difenoconazole + azoxystrobin).

While Amistar Top[®], Bellis and No-Blite have two active ingredients, of which all consist of a strobilurin and a second active ingredient, Twist[®] consists of trifloxystrobin as the only active ingredient. Trifloxystrobin is also a strobilurin and fungicides in this group inhibit spore germination (Pasche *et al.*, 2004); therefore, mycelial inhibition by Twist[®] may be expected to be poor. Fungal resistance of *Alternaria* species to strobilurin fungicides has previously been reported (Farrar *et al.*, 2004). The efficacy of trifloxystrobin was enhanced when tebuconazole is added as an alternative active ingredient in one of the best performing fungicides, Nativo[®].

According to Miles *et al.* (2005), fungicides with multiple active ingredients that have different mechanisms of action are likely to be most effective in disease suppression and resistance management. For example the efficiency of strobilurin fungicides was not compromised when they were integrated into an anti-resistance strategy (Miles *et al.*, 2005).

Field application of trifloxystrobin or azoxystrobin incorporated with mancozeb and copper to control *A. alternata* on citrus produced significantly better results than either fungicide used alone (Miles *et al.*, 2005). The mycelial inhibitory effects of Score[®] and Amistar Top[®] were insignificant. This was unexpected since Amistar Top[®] contains two active ingredients namely, difenoconazole and azoxystrobin, while Score[®] only contains difenoconazole. It should thus be noted that azoxystrobin is a fungicide from the strobilurin group, against which resistance has previously been reported in *A. alternata* (Farrar *et al.* 2004).

According to Reuveni & Prusky (2007) active ingredients are not only combined to enhance mycelial inhibition but for numerous reasons. This includes widening the spectrum of antifungal activity, to delay resistance, to exploit the synergistic interactions between compounds, to increase activity or to reduce the amount of fungicide used without compromising the loss of activity. The latter two reasons may explain why Score[®] and Amistar Top[®] mycelial inhibition were insignificant. According to Ben-Noon *et al.* (2001) the combination of two or more active ingredients from different groups may be weak and produce no significant difference in comparison to either of the active ingredients used alone, but this may extend the life span of the fungicide by combating resistance development (Miles *et al.*, 2005).

The active ingredients found in TU Crop oil (Tanos[®] (cymoxanil+famoxadone) + Unizeb[®] (copper hydroxide) + H&R crop oil (mineral oil), Proxan[®] (copper hydroxide) and Barrier[®] (procymidone + zinc oxide), excluding mineral oil have previously been registered and extensively used to control early blight of potatoes (Van der Waals *et al.*, 2005). As hypothesised by Van der Waals *et al.* (2011) it appears that fungicides which were previously used to control early blight have little effect on *Alternaria* brown spot. This statement is supported by the observation that fungicides which performed fairly well against *in vitro* mycelial inhibition of *A. alternata* in this study have either been recently registered for use on potatoes, or the combination of the active ingredients is being for the first time tested to control *A. alternata in vitro*, for example, AC crop oil.

Despite Amistar Opti[®] and Amistar Top[®] both having azoxystrobin as one of the two active ingredient, while Amistar Top[®] and Score[®] both contain difenoconazole. It is noted that although

Score[®] contains difenoconazole as the only active ingredient, Amistar Top[®] and Score[®] showed greater inhibition of mycelial growth than Amistar Opti[®], which was indicative that difenoconazole inhibited more mycelial growth than chlorothalonil. Amistar Opti[®] had however higher percentage of mycelial inhibition than Twist[®], probably due to Twist[®] having trifloxystrobin as the only active ingredient which has been reported to inhibit less mycelial growth than azoxystrobin (Miles *et al.*, 2005).

Significant differences may be expected amongst fungicides of the same group which have the same mode/mechanism of action because the physiochemical properties of the active ingredient have an influence on the biological activity (Ben-Noon *et al.*, 2001). The better performance of Proxan[®] over Amistar Opti[®] may be attributed to the increased mycelial growth inhibition by dichlorophen when compared to chlorothalonil which are active ingredients of Proxan[®] and Amistar Opti[®] respectively.

Bellis and No-Blite contain boscalid and fenamidone respectively as one of their active ingredients, which have been reported to be as effective as tebuconazole in inhibiting mycelia growth of *A. alternata in vitro* (Surviliene & Dambrauskiene, 2006). Avenot *et al.* (2008) however, reported multiple resistance of *A. alternata* against Pristine[®] (Pyraclostrobin + boscalid) which contains the same active ingredients as Bellis[®]. This suggests a spray program with a rotation of fungicides containing multiple active ingredients that are not from the same group, applied to effectively control brown spot in the field. This may be done during field applications so that effective control and reduced resistance can be attained (Staub, 1991; Brent, 1995; Avenot *et al.*, 2008).

4.4. Conclusion

Constant application of fungicides with a single or multiple active ingredients with the same mechanism of action that cause a 100% mycelial inhibition *in vitro* is not ideal. Not only will this practice result in ineffective control, but will also exert a high selection pressure which increases the chance for development of resistance.

The Fungicide Resistance Action Committee (FRAC) recommends that an anti-resistance strategy must be used in fungicide applications. This includes limiting the strobilurin application to one third of the total number of the fungicides applied without compromising efficacy (Miles *et al.*, 2005).

Effective suppression of spore germination and mycelial growth is important for control to suppress gradual increase of inoculum during the season. According to Surviliene & Dambrauskiene (2006) effective control of diseases caused by *Alternaria* species requires multiple applications of fungicides.

A combination of different active ingredients with at least one active ingredient without a previous history of extensive use or resistance has proven to give good mycelial growth inhibition. This anti-resistance development strategy has proven to enhance the inhibitory effect of a strobilurin in a previous study (Miles *et al.*, 2005). A similar effect was evident in our study when the activity of trifloxystrobin (Twist[®]), one of the worst performing active ingredients, was enhanced by combination with tebuconazole, an active ingredient from another group with an alternative mechanism of action, as in the case of Nativo[®].

The combination strength of multiple active ingredients has proven to be important in inhibition of *A. alternata* mycelial growth. Combinations of different active ingredients which differ in their mechanisms of action such as that of AC crop oil and Nativo[®] respectively inhibited the most mycelial growth in this study.

Van der Waals *et al.* (2011) hypothesized that the severe occurrence of *Alternaria* brown spot even when fungicides previously used to control early blight on potatoes are under field application may be due to little or no effect on *A. alternata*. Extensive application of fungicides from the same group with similar a mechanism of action may reduce the sensitivity of the active ingredient towards the targeted organism (Brent, 1995).

Results from our study as supported by Whiteside (1970) have proven chlorothalonil to be unsatisfactory by inhibiting the least mycelial growth. Moreover findings from an *in vitro* study of mycelial inhibition by Issiakhem & Bouznad (2010) confirmed that *A. alternata* is more tolerant to both difenoconazole and chlorothalonil than *A. solani*, which is very sensitive to both fungicides. This suggested that there may be a build-up of difenoconazole, chlorothalonil and azoxystrobin resistance in *A. alternata* isolates in South Africa. A near 100% resistance has been reached in *A. alternata* due to application of strobilurin fungicides in the potato growing regions of the USA (Kirk *et al.*, 2009) as a result of F129L mutation (Pasche *et al.*, 2005). This statement is inconclusive in a South African context and a follow up study to screen for resistance of *A. alternata* isolates to different fungicides is underway. Furthermore field trials to test for fungicide activity are to be carried out to confirm results from this study.

Table 1: Fungicides tested for the efficacy to inhibit mycelia growth of *A. alternata* *in vitro*.

Fungicide	1. Amistar Opti	2. Amistar Top	3. Score	4. Bellis	5. No-Ethra	6. Twist	7. Native	8. Tanos – Unizeb – H&R Crop Oil (TU Crop oil)	9. Acanto – Capitan – H&R Crop Oil (AC Crop oil)	10. Proxan	11. Bantler
Company	Syngenta	Syngenta	Syngenta	BASF	Bayer	Bayer	Bayer	DuPont and Villa	DuPont	Flaachem	Flaachem
Active ingredients	Azoxystrobin (strobilurin) – chlorothalonil (pyrithalimide)	Azoxystrobin (strobilurin) – difenoconazole (triazole)	difenoconazole (triazole)	Boscalid (carbamate) – pyraclostrobin (strobilurin)	Fenamidone (imidazolinone) – mancozeb (dithiocarbamate)	Trifloxystrobin (strobilurin)	Trifloxystrobin (strobilurin) – tebuconazole (triazole)	Cymoxanil – Fenoxadone – Mancozeb (copper) – Mineral Oil	Picoxystrobin (strobilurin) – Fusilarole (triazole) – Mineral Oil	Copper hydroxide – Dichlorofen	Maneb (dithiocarbamate) – Proxynidone (dicarb oxamide) – Zinc oxide
Mode of action	Systemic contact	Systemic contact	Systemic	Systemic contact	Systemic contact	Contact	Systemic contact	Contact	Systemic	Contact	Contact locally systemic
Active ingredient concentration	45.8 % W W of chlorothalonil and 4.6 % W W of azoxystrobin	11.2 % W W of azoxystrobin and 11.4 % W W of difenoconazole	25.2 % W W of difenoconazole	252 g kg of boscalid and 128 g kg of pyraclostrobin	50% water dispersible granules (WG) of mancozeb and 10% WG of fenamidone	45.7% W W of trifloxystrobin	25.0% W W of trifloxystrobin and 50.0% W W of tebuconazole.	250 g kg of cymoxanil and 250 g kg fenoxadone and 800g kg of mancozeb	25g l of picoxystrobin and 23.6% w w of fusilarole	125g l of copper hydroxide and 62g l of dichlorophen	525g l of maneb, 125g l of proxynidone and 4.7g l of zinc oxide
Registered dose for treatment of Alternaria as amended in FDA	12.5 ppm of Amistar Opti	3.75 ppm of Amistar Top	1.75 ppm of Score	3 ppm of Bellis	10 ppm of No-Ethra	1.5 ppm of Twist	3 ppm of Native	A tank mixture of 5 ppm of Tanos, 20 ppm of Unizeb and 5 ppm of H&R crop oil	3 ppm of Acanto, 4 ppm of Capitan and 5 ppm of H&R crop oil	6 ppm of Proxan	6 ppm of Bantler

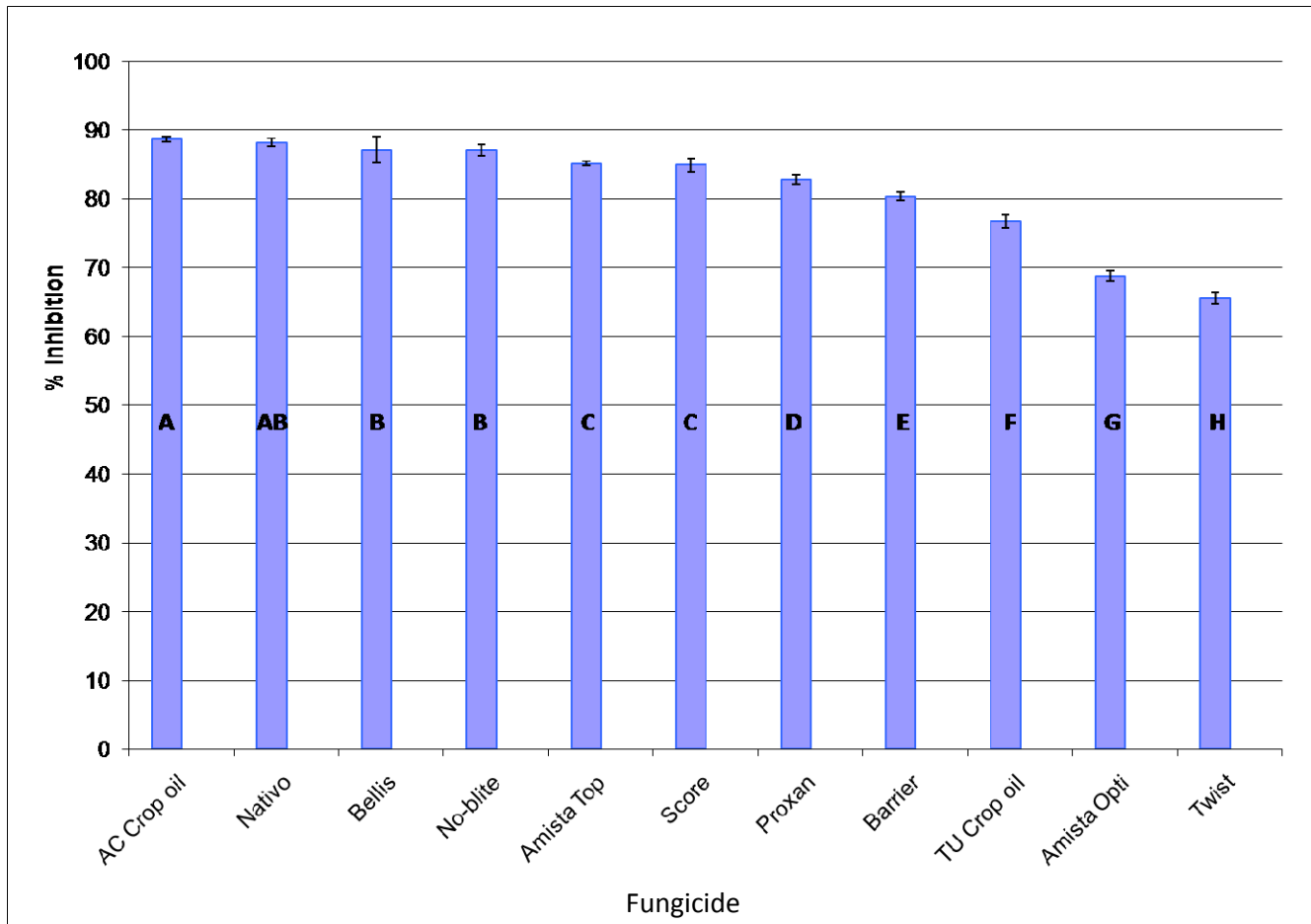


Figure 6: *In vitro* effects of various fungicides on percentage mycelial inhibition of *A. alternata* causing brown spot on potatoes in South Africa. Minimum significant difference of the mean is 1.3596. Means with the same letter are not significantly different as determined by a least significant difference test ($P \leq 0.05$).

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Chapter 5: General discussion

A plant pathogen requires a set of conditions which influences its interaction with its hosts to cause disease. Some plant pathogens are not specialized, as a result they thrive in diverse environments and are pathogenic to numerous plant species (Maryann & Williams, 2012). This plant pathogen-host interaction determines the initial barrier or door to a disease outbreak (Maryann & Williams, 2012; Agrios, 2011). The impact of climate change on plant disease outbreaks, re-emergence and disappearance of diseases in various regions of the world is inevitable (Rosenzweig *et al.*, 2005). In recent years *A. alternata* has been reported to cause disease on plant hosts on which it has never previously occurred or been reported. Some of these diseases and hosts include leaf blight of noni in Hawaii (Hubballi *et al.*, 2010), brown spot on potato (Van der Waals *et al.*, 2011) and leaf blight of sunflower (Kgatlé, 2013) in South Africa. According to Rosenzweig *et al.* (2005) these diseases may cause changes in production practices to combat depreciating yield and quality of various crops.

The objective of chapter 3 of this study was to determine the causal agent of brown spot of potatoes in South Africa. Results from this chapter proved *A. alternata* to cause brown sunken irregular symptoms on potato leaves through Koch's postulates. These symptoms took three to eight days to appear; this incubation period agreed with that of Slavov *et al.* (2004). Although these symptoms were visible on both the adaxial and abaxial side of the potato leaves they were more apparent on the adaxial side which is consistent with the results previously reported by Cakarevic & Boskovic (1997) and Neeraj & Verma (2010), with raised margins and an indistinguishable border surrounding the sunken necrotic area. Furthermore, these symptoms were comparable to those described by Giha (1973) with chlorosis beginning from the leaf edges followed by necrosis which extended to the leaf interior to cause leaf blight. An electron microscope scan revealed that *A. alternata* spores had produced germ-tubes, conidiophores and extracellular material. This provides substantial evidence that *A. alternata* had attached and colonised the potato leaves, because according to

Slavov *et al.* (2004) extracellular material is associated with attachment of spores to host surfaces. Furthermore Dehpour *et al.* (2007) reported that fungi produce germ-tubes which penetrate host surfaces and as a result infect and colonize the host for disease to occur and symptoms to appear. further infection studies need to be conducted to identify infection processes and sites, since SEM in this study was only conducted to detect the presence of *A. alternata* and its colonization on potato leaves.

The causal agent was confirmed by re-isolation of the pathogen from inoculated and detached leaves then subjected to identification by morphological characteristics and PCR. Results from this study confirmed that *A. alternata* is the causal agent of brown spot on potatoes since the pathogen was only recovered from inoculated diseased leaves. Morphological characteristics of the fungi in the present study were similar to *A. alternata* as previously described by Rotem (1994), Weir *et al.* (1998) and Akhtar *et al.* (2004), producing small multiseptate conidia with 3-7 transverse septa and a short cylindrical beak.

Due to the presence of genera such as *Ulocladium* and *Stemphylium* which produce morphologically similar conidia as *Alternaria* species (Pryor & Gilbertson, 2000), classification based solely on morphological characteristic may produce inconclusive results. According to Frisvad *et al.* (2007) two or more techniques are required to accurately identify the fungal isolates. To avoid misidentification a PCR method was used as a second complementary tool to confirm the identity of *A. alternata*. This PCR technique was applied as described by Konstantinova *et al.* (2002) in which forward and reverse species specific primers that detect the presence *A. alternata* were used. These primers amplified a ~ 340bp PCR amplicon of the ITS region which gave conclusive evidence that *A. alternata* is the causal organism of brown spot of potatoes in South Africa.

Various methods are available to manage *Alternaria* brown spot on various crops. These methods include physical and cultural control options. These methods may however, not eradicate, or prevent disease

development but reduce the buildup of initial inoculum (Agrios, 2011; Kemmitt, 2002; Stevensons, 1993). Though criticized due to environmental pollution and resistance development (Gullino *et al.*, 2000), chemical control is an option available to offer disease prevention and therapeutic treatment of already infected plants. Neither chemical control nor physical and cultural control options used alone can control the *Alternaria* disease especially under high disease pressure (Survaliene & Dambrauskiene, 2006). *Alternaria* diseases require an integrated disease management strategy which will reduce the damage caused to tolerable levels (Survaliene & Dambrauskiene, 2006). The aim of chapter 4 of this study was to investigate the efficacy of different registered fungicides in inhibition of *A. alternata* mycelial growth *in vitro*. Although various authors have reported numerous active ingredients to be ineffective against *Alternaria* diseases (Timmer & Zitko, 1997; Swart *et al.*, 1998), most of these fungicides were never tested on *A. alternata* that causes brown spot of potatoes in South Africa and it is evident that fungicides which are registered to control early blight on potatoes in South Africa are not entirely successful in controlling brown spot.

After a test of various fungicides to inhibit mycelial growth *in vitro* it was observed that numerous fungicides are capable of inhibiting *A. alternata*. Complete inhibition of mycelial growth was not achieved, although it is not ideal to attain 100% control because this will cause a high selection pressure which induces resistance development. It was evident that combinations of multiple active ingredients with different mechanisms of action without a history of extensive use provide good mycelial inhibition. Reuveni & Prusky (2007) reported that a tank mixture of bromeiconazole or difenoconazole with captan effectively treated moldy core on Red Delicious apple than either of the fungicides used alone. This did not only effectively reduce disease development but also reduced the chances of resistance development (Reuveni & Prusky, 2007). According to Avenot *et al.* (2008) a continuous field application of a single fungicide or fungicides with the same mechanism of action may cause resistance build-up and as a result poor disease control. A further field and

resistance study is necessary to confirm the results obtained *in vitro*. According to Timmer & Zitko (1997) proper timing and application rates of fungicides are necessary to effectively control disease.

The potato industry of South Africa is faced with various challenges, some of which include pest and diseases that reduce yield and quality of the harvest. Amongst other diseases, brown spot of potatoes posed a threat to the potato industry in recent years due to an unidentified causal organism and as a result this caused uncertainties as to which control options are available to combat the disease. Results from this study paved a path to control brown spot of potatoes by identifying the causal organism and further giving guidance of already registered fungicides that are likely to control the disease.

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