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Characterization of *Alternaria alternata* isolates causing brown spot of potatoes in South Africa

By

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Submitted in partial fulfilment of the requirements for the degree of Master in Science (Agriculture) Plant Pathology

In the faculty of Natural and Agricultural Sciences Department of Microbiology and Plant Pathology

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DECLARATION

I, Joel Prince Dube, declare that the thesis, which I hereby submit for the degree Master of Science (Agriculture) Plant Pathology at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

Signed: ______________________________

Date: _______________________________

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Abstract

Alternaria alternata (Fr.) Keissler is now widely recognized as the causal agent of brown spot and black pit of potatoes. Brown spot is a foliar disease with potential to cause 30% yield loss and postharvest losses of up to 10%. Losses are mainly due to premature defoliation.

In this study, morphological and epidemiological characteristics of *A. alternata* were studied in order to understand the extent to which different light regimes influence radial growth, sporulation and pathogenicity of this pathogen. The role of low temperatures on initial sporulation was also investigated. Exposure of isolates to low temperatures (4°C) in the dark increased number of spores produced by isolates under all light conditions. Light did not have any effect on pathogenicity and isolate genetic capability had no influence on radial

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growth of isolates. The combined isolate and light effect (gene-environment interaction), had significant influence on both radial growth and disease severity.

The taxonomy of the genus *Alternaria* has been highly debateable over the years, especially in small spored *Alternaria* species where identification is based entirely on morphological characteristics. This is mainly due to presence of closely related taxa such as *Ulocladium, Macrosporium* and *Stemphylium* that complicate correct identification of this genus.

An investigation was carried out to determine the phylogenetic relationship as well to determine the relationship between molecular characterization and morphological identification. All isolates were identified as *A. alternata* based on morphology. The identity was further confirmed by molecular phylogeny using the GAPDH, EF1α and a combined phylogeny of these gene regions. All isolates formed one section with *A. alternata*. The isolates also grouped together with *A. arborescens*, *A. tenuissima*, *A. longipes* and *A. gaisen*, which were all recently characterized into the *Alternata* section. Comparison of RFLP digests of the ITS1 and ITS4 region revealed no genetic variability. The GAPDH and EF gene regions can therefore be used to delineate among *Alternaria* isolates and was in congruence with morphological identification. PCR-RFLP can be a useful tool in detecting genetic variability among isolates.

Control of brown spot has mainly been through the use of strobilurins; however, recent disease epidemics on potatoes in South Africa led to an investigation into the failure of strobilurins to control brown spot. Samples were collected during the 2012-2013 growing season and eight *Alternaria* isolates were recovered from five growing regions. *In vitro* sensitivity tests showed that six of the eight isolates had reduced sensitivity to azoxystrobin. Sequence analysis of the cytochrome b gene revealed a mutation that led to an amino acid substitution which consequently led to reduced sensitivity.

This study will lead to a better understanding of this new disease of potatoes that has proven to be of economic importance. Correct identification is paramount in disease management and this study has shown some reliable molecular technics that can be used to identify species in this genus correctly. This study was also able to link failure to control

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brown spot to fungicide resistance, and alternative control strategies can now be recommended to control this pathogen.

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Chapter 1

General introduction

"For of all gainful professions, nothing is better, nothing more pleasing, nothing more delightful, nothing better becomes a well-bred man than agriculture" (Marcus Tullius Cicero). Agriculture plays an important role in the economies of many countries especially developing countries where it contributes about 30% of the Gross Domestic Profit (GDP) (FAO, 2012). In South Africa agriculture contributes 3% towards GDP (World Bank, 2012).

Potato (*Solanum tuberosum*. *L*.) is ranked the fifth major food crop in the world, exceeded only by wheat, rice, maize, and barley (Potatoes South Africa, 2010) and is considered the most important dicotyledonous source of human food (Stevenson et al. 2001). In South Africa alone annual production is approximately 2.2 million tonnes (Potatoes South Africa, 2012) and production is spread over 16 different geographic areas with a wide range of soils and climatic conditions (Potatoes South Africa, 2012).

South Africa ranks 28th in the world in terms of total potato production (tons per country) and contributes about 0.3 % to the global potato production (Du Preez, 2011). In terms of the African continent, area under production is only 3.5 % of the total area, but South Africa contributes 11 % of the total potato production (Potatoes South Africa, 2010).

It is characteristically a crop of the cool, temperate regions or of elevation of approximately 2,000 m or more in the tropics (Stevenson et al. 2001). It requires cool nights and well-drained soil with adequate moisture and does not produce well in low altitude, warm, tropical environments (Alptekin, 2011). Commercial production of most potatoes is primarily through vegetative propagation by means of lateral buds formed on the tuber, a modified stem (Hooker, 1981).

There are over 31 diseases caused by 30 fungal pathogens in South Africa (Truter, 2005) and recently a new foliar disease known as brown spot, caused by *Alternaria alternata* (Fr.) Keissl., was identified in South Africa (Van der Waals et al. 2011). Kirk et al. (2007) reported on an "*Alternaria* complex" formed by *A. alternata* and a related fungus, *Alternaria solani*

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Sorauer, when they occur together on a potato plant. The tuber stage of brown spot has been reported under long term storage as black pit of tubers (Nolte, 2008).

Brown spot is colloquially known as "the other early blight" (Nolte, 2008) and is often confused with early blight which is caused by a member of the same genus, *A. solani*, as it forms similar lesions to that of early blight. Identification of *Alternaria* species is based primarily on the morphology and development of conidia and conidiophores, and to a lesser degree on host plant association and colony morphology (Konstantinova et al. 2002). The genus *Alternaria* has a complex taxonomy (Pryor and Michailides, 2002) and poses a great challenge in identification (Roberts et al. 2000), especially among small spored *Alternaria* taxa mainly due to morphological plasticity and presence of morphologically similar genera such as *Stemphylium, Ulocladium*, and *Macrosporium*. Molecular tools can be used to correctly identify members of the genus *Alternaria* and molecular phylogeny has been valuable over the years to delineate among fungal taxa.

Disease management is a key building block in effective and safe crop management Programme (Stevenson et al. 2001). The early and accurate diagnosis of plant disease is the most crucial component of any crop management system because plant diseases can be managed most effectively if control measures are introduced at an early stage of disease development (Miller and Martin, 1988). Brown spot has the potential to cause yield reduction of up to 30% and if left uncontrolled, the disease can be very destructive (Kirk and Wharton, 2012). Yield potential is reduced mainly due to premature defoliation (Agrios, 2005). Postharvest losses due to *A. alternata* as black pit can be as high as 10% (Boyd, 1972; Delleman et al 2005) and most of the affected potatoes are not fit for processing.

The most common and effective control method for brown leaf spot is application of foliar fungicides, one of the most commonly used being strobilurins. The huge impact of the strobilurins on agriculture is well reflected by the current status of azoxystrobin, a member of the strobilurins, which is now registered for use on 84 different crops in 72 countries, representing over 400 crop/disease systems (Bartlett et al. 2002). However, over the years failure to control brown spot with strobilurins has been reported in many potato growing regions. Continuous and accurate monitoring of sensitivity is necessary to provide information regarding the sensitivity of field populations in certain key crop-pathogen

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combinations and to track the evolution of resistance genes (Ma et al. 2003). Use of molecular based methods can be used for fast and accurate detection of resistant populations amongst plant pathogenic fungi allowing the development of resistance monitoring programmes that will enable potato growers to respond quickly based on test results.

Research in the present study aims at providing information on *A. alternata*, the causal agent of brown spot of potatoes and is presented in the following chapters.

Chapter 2: A review of brown spot on potatoes is given. The review is divided into two main categories; 1. The host: where information on anatomy, origin and distribution, optimum agronomic conditions and major disease affecting potato was given. 2. The pathogen: in this category the taxonomy, the brown spot disease, morphological and molecular characteristics, host range, geographical distribution, epidemiology, disease cycle, management and resistance to conventional fungicides were reviewed.

Chapter 3: Three *A. alternata* isolates, all isolated and purified from potato leaves showing typical brown spot lesions were randomly selected from the Potato Pathology @ UP culture collection. Variability and epidemiological aspects of *A. alternata* were investigated. The objectives of this trial were to investigate effects of different wavelengths of light and temperature on radial growth and sporulation of *A. alternata* as well as to investigate the role of low temperatures on initial sporulation of *A. alternata* isolates.

Chapter 4: Aims to characterize small-spored *Alternaria* species isolated from infected potato leaves using molecular and morphological tools.

Chapter 5: Continuous and accurate monitoring is necessary to provide information regarding the sensitivity of field populations in certain key crop-pathogen combinations and to track the evolution of resistance genes (Ma et al. 2003). The objectives of this study were to:

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1. Screen *A. alternata* isolates collected from various potato growing regions in South Africa for sensitivity to a QoI fungicide azoxystrobin

2. Investigate possible mechanisms of azoxystrobin resistance, if any, in these *A. alternata* isolates by comparing the cytochrome b (cyt b) gene from sensitive and resistant isolates.

Chapter 6: The final chapter is the general discussion which concludes the investigations and studies done on *A. alternata*, including recommendations and implications for industry and further research.

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Chapter 2

A Review of Brown Spot on Potatoes

2.1 The Host: *Solanum tuberosum*

The cultivated potato (*Solanum tuberosum* L.), is the most consumed vegetable in the world (Soleimani and Mohajer, 2011). It is also the most economically important vegetable and is considered the most important dicotyledonous source of human food (Hooker, 2001). The potato is best known for its carbohydrate content (approximately 26 grams in a medium potato) and it is either eaten directly by humans with the rest being fed to animals or used to produce starch (FAO, 2008). Globally, approximately 18.5 million hectares is under potato production and annual potato yield is estimated to be approximately 322 million tonnes (FAO, 2010).

2.1.1 Origin and Distribution

The potato originated in the Andes Mountains of South America where it served as the staple food for the native people for millennia (Stevenson et al. 2001). The Inca Indians in Peru were the first to cultivate potatoes around 8 000 BC to 5 000 BC and Chile is considered to be a sub-centre of origin for the cultivated potato (Spooner et al. 2005). The Spanish invasion of the Inca Empire in 1532, led to the introduction of the potato to the rest of Europe and it has since spread around the world and become a staple crop in many countries (Francis, 2005). Many researchers believe that the potato's arrival in northern Europe brought an end to famine there since when compared with grains, tubers are inherently more productive (Mann, 2011).

There is no certainty about how and when this crop reached South Africa but it is generally believed that the Dutch East India Company brought potatoes to South Africa from the Netherlands (NAMCCT, 2011). Potatoes are rated as one of South Africa's most important staple foods (Syngenta, 2013) and production is spread over 16 different geographic areas (Figure 2.1) with a wide range of soils and climatic conditions (Potatoes South Africa, 2010). Potatoes are mainly produced under full irrigation, although successful dry land production still occurs in some regions, such as the Eastern Free State. The main producing regions are

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found in Limpopo, Free State, Western Cape, Mpumalanga, KwaZulu Natal and Eastern Cape (Potatoes South Africa, 2010).

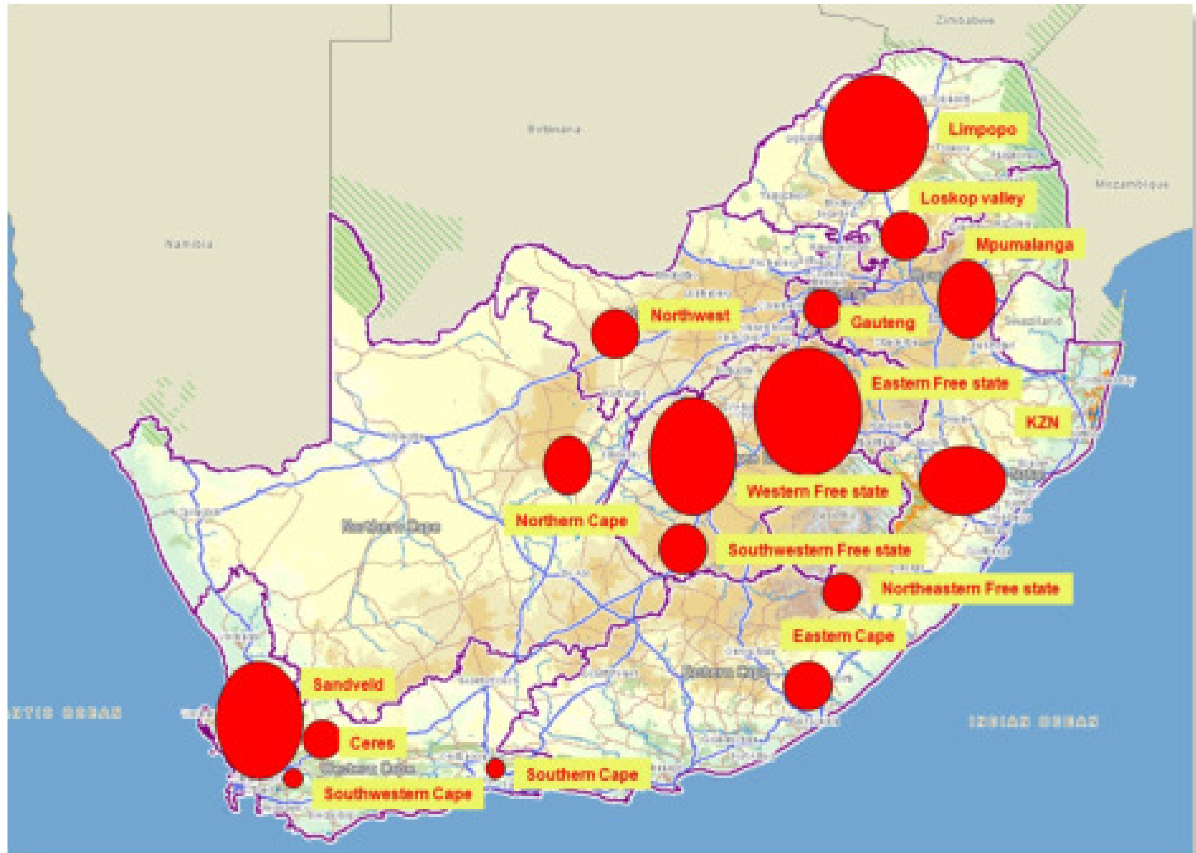

Figure 2.1. Potato producing regions in South Africa (adopted from http://nbsystems.co.za/potato/index_3.htm)

2.1.2 Agronomic Conditions

Two cultivar groups have been developed Andigenum, which is adapted to short day conditions and is mainly grown in the Andes, and Chilotanum, which is cultivated around the world (Rodriguez et al. 2010). Potato is grown in more than 100 countries, under temperate, subtropical and tropical conditions (Black, 2008). It is essentially a cool weather crop, with temperature being the main limiting factor to production. Tuber growth is greatly inhibited in temperatures below 10°C and above 30°C, while optimum yields are obtained where mean daily temperatures are in the 18 to 20°C range (Niederwieser, 2003). Vegetative growth is best at temperatures of 24°C and tuber development at 20°C (Black, 2008).

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Optimum planting dates differ from region to region (Visser, 2011). In South Africa, potatoes are planted from August to January in frosty areas and in frost free areas they can be planted from February to early June (Potatoes South Africa, 2012). Deep, well drained and aerated soils with a pH of 5.2-6.4 are considered ideal (Hollebrandse and Steyn, 2004). A planting depth of 13cm is recommended and then cover with soil up to 25-30cm deep in furrows (Le Roux et al. 2003).

There are about 83 potato varieties grown in South Africa (Potatoes South Africa, 2012) and these can be characterized into 3 groups, short, medium and long growers, according to the length of their growing periods. Short growing season cultivars usually take less than 90 days from planting to harvesting, medium-growing season cultivars take 90 -110 days from planting to harvesting and they form the bulk of potatoes grown in South Africa. Longer growing season cultivars take longer than 110 – 150 days from planting to harvesting (Black, 2008).

Potatoes are harvested when the above ground parts are completely dead and the time frame is dependent on variety (Jauron, 2000). Yellowing of the leaves and easy separation of the tubers from the stolons indicate that the crop has reached maturity (Stark and Love, 2003). To facilitate harvesting, the potato vines should be removed two weeks before the potatoes are dug up as they interrupt the harvesting process. Potatoes are harvested using a spading fork, a plough or commercial potato harvesters that unearth the plant and shake or blow the soil from the tubers (Willemse, 2013). If the potatoes are to be stored rather than consumed immediately, they should be left in the soil to allow their skins to thicken (Jauron, 2000). Thick skins prevent storage diseases and shrinkage due to water loss (Agriculture, Fisheries and Aquaculture, 2004).

2.1.3 Potato Diseases

Potatoes, like any other crop, are constantly under threat from viruses, viroids, bacteria, fungi, nematodes and phytoplasma (Stevenson et al. 2001). Nine significant potato viruses are found in South Africa (Black, 2008). The most serious viruses in terms of crop losses in South Africa, which are monitored by the potato certification scheme, are potato leaf roll virus, potato virus Y and tomato spotted wilt virus (Thompson and Strydom, 2003). Aphids

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are the main source of dissemination of viruses (Thomas, 1983), although some mechanical transmission may also occur, hence constant monitoring of aphid populations is required (Bradshaw, 2006). Aphids spread viruses to seed and table stock potatoes, which can reduce yields and quality. High populations of aphids can cause foliage to degenerate and fall off (Jacobsen and Zidack, 2012).

Several bacterial pathogens also affect potatoes causing wilts and rots of above ground plants parts such as stems and leaves, as well as tubers in the field or in storage (Christ, 1998). Bacterial diseases of importance on potatoes include the destructive bacterial wilt also known as brown rot caused by *Ralstonia solanacearum* (Wairuri et al. 2012), common scab caused by *Streptomyces scabiei* (Lambert and Loria, 1989; Wanner, 2006), as well as soft rot and black leg caused by *Pectobacterium* spp (Czajkowski et al. 2011). All of these bacterial diseases infect tuber or stem tissue (Stevenson et al. 2001).

Fungi are generally the most important group of plant pathogens (Agrios, 2005) and cause the greatest damage on potatoes as many different genera attack potato (Stevenson et al. 2001). At least 13 fungal diseases have been identified on potatoes (International Potato Centre, 1996; Stevenson et al. 2001). In South Africa fungal pathogens of economic importance include, powdery scab caused by *Spongospora subterranea* f.sp. *subterranea* J.A. Toml. (Wright, 2012), black dot caused by *Colletotricum coccoides* (Wallr.) S. Hughes (Lees and Hilton, 2003), late blight caused by *Phytophthora infestans* (Wallr.) S. Hughes (Stevenson, 2009), early blight caused by *A. solani* (van der Waals et al. 2004), stem canker and black scurf caused by *Rhizoctonia solani* (Kühn) (Wilson et al. 2008) *Fusarium* dry rot and wilt caused by *Fusarium* spp. (Stevenson et al. 2001) and of late, brown spot and black pit caused by *Alternaria alternata* (Fr.) Keissl. (van der Waals et al. 2011).

Prevalence of potato diseases including brown spot and black pit can result in great economic loses given the various ways that potato can be used for both domestic and industrial purposes. Once harvested, potatoes are used for a variety of purposes, and not only as a vegetable for cooking at home (Stark and Love, 2003). Other uses include retained as seed potato, Stock feed for cattle, pigs and chicken (Maynard and Hochmuth, 2006), processed for starch in the brewing industry where the fermentable sugars are used in the

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distillation of alcoholic beverages such as vodka and akvavit (Bayer, 2008). Potato starch is also widely used by the pharmaceutical, textile, wood and paper industries as an adhesive, binder, texture agent and filler, and by oil drilling firms to wash boreholes (IPC, 2013). Potato starch is a 100% biodegradable substitute for polystyrene and other plastics and used, for example, in disposable plates, dishes and knives (Gopal and Khurana, 2006). Therefore it is quite important that integrated disease management programmes be implemented to minimize losses due to diseases.

2.2 The Pathogen: *Alternaria alternata*

2.2.1 The Genus *Alternaria*

Species in the genus *Alternaria* (Pleosporaceae, Pleosporales, Dothideomycetes, Ascomycota) are very common, abundant and occur worldwide in a variety of habitats (Tweedy and Powell, 1963; Rotem, 1994). Over 1200 Alternaria species have been published since 1796; however, Simmons (2007) only accepted about 300. Recently, Woudenberg et al. (2013) redefined the genus and proposed 32 new combinations and 10 new names based on morphology and molecular characterization.

The genus *Alternaria* includes saprophytic and pathogenic species, as well as borderline species that become one or the other depending on environmental conditions (Agrios, 2005). The saprophytic species are involved in degradation of plant debris and other products (Rotem, 1994). Several taxa have also been associated with a lot of post-harvest diseases as well as common allergens among immune compromised people (Battilani et al. 2009).

Alternaria is a genus of ascomycete fungi with major plant pathogens (Lawrence et al. 2012). They are ubiquitous in the environment and they are a natural part of fungal flora almost everywhere (Pscheidt and Stevenson, 1986). The assumed absence of *Alternaria* spp. in any environment has been attributed to lack of a suitable host, unfavourable environment or no desire in tracking its presence (Rotem, 1994). The spores are airborne and found in the soil and water, as well as indoors and on surfaces (Simmons 1992). The production of dark-coloured conidia with longitudinal and transverse septa has remained a

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key taxonomic characteristic of the genus (Pryor and Gilbertson, 2000). Rotem (1994) describes four main ecological and physiological similarities among pathogenic *Alternaria* and these include:

1. All pathogenic *Alternaria* species are highly resistant to adverse weather
2. They grow in a wide range of temperatures and use the locally available source of moisture
3. They sporulate best on necrotic and dead tissue and produce a relatively small number of spores, mainly at the end of the season; and,
4. They are decisively affected by the age-conditioned susceptibility of host plants.

2.2.2 Brown Spot of Potato Caused by *Alternaria alternata*

Alternaria alternata causes brown spot and black pit of potatoes (Nolte, 2008; Kirk and Wharton, 2012) and is one of the most prevalent plant pathogens. It causes leaf spots on different hosts in South Africa as well as other parts of the world (Thomma, 2003). Brown spot of potatoes, colloquially known as “malroes”, a new or emerging disease of potatoes in South Africa (van der Waals et al, 2011), has resulted in serious yield losses in many production regions of the country over the past 5 to 8 years (Potatoes South Africa, 2012). It is a foliar disease that occurs throughout the growing season causing premature defoliation and symptoms are often confused with those of early blight caused by *Alternaria solani* (Nolte, 2008).

In South Africa, brown spot of potatoes was reported in 2011 (van der Waals et al. 2011). Brown spot symptoms can be seen any time from 50 days post emergence (or earlier), often before early blight (Nolte, 2008) and brown spot lesions are seen on the middle canopy leaves (Kirk and Wharton, 2012), while the first early blight symptoms appear on the lower senescing leaves (Nolte 2008). Early symptoms of brown spot on potatoes are seen as small, round, dark brown necrotic lesions on leaves which are usually 10mm in diameter, first visible on the abaxial side of leaves (van der Waals et al. 2011). These may coalesce to form large necrotic lesions with dark brown margins and concentric rings (Stevenson et al. 2001; Kirk and Wharton, 2012). Severely affected leaves dry up and result in premature defoliation

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(Stevenson et al. 2001). Spots may also be found on stems and petioles which when coalesced form elongated, superficial brown or black patches of dead tissue (Kirk and Wharton, 2012).

This pathogen has also been reported as a postharvest disease on potatoes as black pit (Nolte, 2008). Black pit symptoms occur under severe infection and favourable conditions and usually form black sunken areas of corky tissue with definite margins on the tuber. Lesions are usually 10mm in diameter and may be sunken (Droby et al. 1984a). These are similar in appearance to pits caused by common scab but are usually deeper, narrower and darker (Kirk and Wharton, 2012). Bruised and stressed tubers are most severely affected and these symptoms develop during storage (Droby et al. 1984b).

Black pit is a postharvest tuber disease mostly affecting those tubers damaged during harvest. Losses of up to 10% have been recorded in stored potatoes (Boyd, 1972; Delleman et al 2005) and most of the affected potatoes are not fit for processing. Extent of damage depends greatly upon environmental conditions, cultivar, pathogen strain, and postharvest handling (Droby et al. 1984a). In their study, Droby et al (1984b) observed that disease incidence increased six-fold in mechanically harvested tubers compared to manually harvested tubers.

2.2.3 Morphology and Cultural Characteristics

To date, classification and identification of *Alternaria* species have been based primarily on conidium characteristics and patterns of conidium catenation (Simmons and Roberts 1993; Xia and Tian-Yu, 2008). *Alternaria alternata* grows in long chains, with dark brown conidiophores and conidia (Ellis, 1971). Conidia of *A. alternata* have short beaks, compared to *A. solani*, and may be smooth or finely warty with both transverse and longitudinal septa. Conidiophores have one to four septa, straight to geniculate and have prominent conidial scars (Simmons, 1999). Conidia range from 20-60 by 9-18 µm in dimensions. Conidia are greenish brown, obclavate and may be straight to geniculate (Stevenson et al. 2001). Larger conidia are in contact with the conidiophores whilst smaller conidia are found at the distal end of the chain (Rands, 1917).

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2.2.4 Molecular Characterization

Traditionally, *Alternaria* species have been characterized and identified on the basis of conidial shape, ornamentation, and size, as well as by their association with particular crop plants (Ellis, 1971; Brun et al. 2013). However, small-spored *Alternaria* species are a taxonomically challenging group of fungi with few morphological or molecular characters that allow unambiguous discrimination among taxa (Andrew et al. 2009, Woudenberg et al. 2013). Identification of some *Alternaria* species still offers considerable difficulties owing to their high variability and the polymorphism occurring even in pure cultures (Simmons, 1992; Yu 1992). The existence of morphologically related pathogens such as *Ulocladium* and *Stemphylium*, which produce morphologically similar conidia to *Alternaria,* contributes to inaccurate identification of this genus (Lawrence et al. 2013).

Molecular methods have been increasingly employed in phylogenetic and taxonomic studies of fungi (Bruns et al. 1991). Subsequent molecular studies have confirmed 14 morphological groups as monophyletic lineages as previously proposed by Simmons (Simmons, 1992; Andrew et al. 2009). However; molecular studies also revealed multiple non-monophyletic genera within the *Alternaria* complex and *Alternaria* species clades, which do not always correlate to species-groups based on morphological characteristics (Woudenberg et al*.* 2013). In their studies, Woudenberg et al (2013) showed that the 18S nrDNA (SSU), 28S nrDNA (LSU), the internal transcribed spacer regions 1 and 2 and intervening 5.8S nrDNA (ITS) phylogenies display a low resolution, which was reflected by poor to no support of the sections; however the combined phylogeny based on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNA polymerase second largest subunit (RPB2) and translation elongation factor 1-alpha (TEF1) gene regions showed better resolution as these genes had the best resolution to delineate *Alternaria* spp.

2.2.5 Host Range

Alternaria alternata has been recorded to cause leaf spots and other diseases on over 380 host species and it is the most frequently reported *Alternaria* species in the literature (Rotem, 1994). It is an opportunistic pathogen on numerous hosts causing leaf spots, rots

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and blights on many plant parts (Guo-yin et al. 2013). It has also been known to cause rot in storage of tomatoes and potato tubers (Clouse and Gilchrist, 1987). The pathogen may also cause quiescent infections in which the hyphae remains dormant until the organ ripens and its susceptibility increases, hence making it a major post-harvest pathogen (Prusky et al. 1981).

The host range of *A. alternata* has been on the increase over the years. In South Africa it was reported on potatoes in 2011 (Van der Waals, 2011) and has been reported to cause black spot of pomegranate in Israel (Ezra et al. 2010), *Alternaria* leaf spot of almond in California (Teviotdale et al. 2001), leaf spot of kiwi in Turkey (Karakaya and Celic, 2012), leaf spot on aloe vera in Louisiana (da Silva and Singh, 2012), leaf blight of tomato in Pakistan (Akhtar et al. 2004), *Alternaria* blight on ginseng in USA (Putnam and du Toit, 2003), *Alternaria* brown spot of citrus in Spain (Vicent et al. 2000), *Alternaria* leaf spot of banana in USA (Parkunan, 2013) and leaf blight on pepper in China (Liu et al. 2013). The host range of *A. alternata* can be extended to its use as a biological control agent of weeds such as water hyacinth (*Eichhornia crassipes*) in India (Babu et al. 2003), *Lantana camara* in Egypt (Sanodiya et al. 2010) and *Amaranthus* spp. in the UK (Ghorbani and Leifert, 2000).

2.2.6 Geographic Distribution

Diseases caused by *Alternaria* spp are common and worldwide in their occurrence (Laemmlen, 2002). *A. alternata* is a common saprobe found on many plants and other substrata worldwide (Guo et al. 2004). With global increase in temperatures due to climate change, distribution of *A. alternata* is expected to increase as shown by occurrence of the pathogen on new hosts in countries such as such as Turkey (Karakaya and Celic, 2012), India (Maiti et al. 2007), Spain (Vicent et al. 2000), South Africa (Van der Waals et al. 2011), USA (da Silva and Singh, 2012) and in Pakistan (Akhtar, 2004). Brown spot of potatoes is now widely reported in many other parts of the world other than South Africa such as United States (Woudeberg et al. 2013), Israel (Droby et al. 1984a), Yugoslavia (Cacarevik and Boskovik, 1997) and Iran (Ardestani et al. 2010).

2.2.7 Toxins

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Most phytotoxins employed by a fungal invader are chemically diverse secondary metabolites, low molecular weight components that are not required for normal growth or reproduction (Thomma, 2003). Two major features of *Alternaria* spp. are the production of melanin, especially in the spores, and the production of host specific toxins in the case of pathogenic species (Tanabe et al. 1995). Though most of them live a saprophytic lifestyle some have developed plant-fungus interactions that give them the ability to overcome plant defence mechanisms by producing host specific and non-host specific toxins (Markham and Hille, 2001). Production of these toxins contributes greatly to ability of plant pathogens to cause disease on susceptible hosts (Thomma, 2003). *A. alternata* was found to be amongst seven of the *Alternaria* pathogenic variants that are known to produce host specific toxins (Mckay et al. 1999). In *Alternaria* non host specific toxins have been identified such as tenuazonic acid, tentoxin and zenion which exert phytotoxic activity through different modes of action (Fujiwara et al. 1988). *Alternaria alternata* is known to produce a host specific toxin, AAL-toxin, which closely resembles the mycotoxin Fumonisin B1, that was identified as a *Fusarium moniliforme* toxin (Chen et al. 1992).

2.2.8 Epidemiology and Disease Cycle

Alternaria alternata lives saprophytically in almost any kind of plant remains and infection occurs when windblown spores land on potato foliage at any growth stage of the plant. Penetration of tubers is predominantly through wounding and of leaves may be via stomata (Stevenson et al. 2001). Long dew periods and temperatures over 18ºC are favourable for spore germination and penetration; whilst infection occurs over a temperature range of 10-35ºC (Misaghi, 1978). The mycelia of the pathogen survive between growing seasons in infested plant debris and soil, in infected potato tubers, and in overwintering debris of susceptible crops and weeds (Kirk and Wharton, 2012).

It is primarily a weak pathogen, attacking already stressed plants, but may also be pathogenic on healthy plants (van der Waals et al. 2011). In order to infect a potato tuber, *A. alternata* requires that the tuber is first wounded (Droby et al. 1984a). Wounding may occur during harvest and lead to post-harvest and storage infections of tubers, or by insects and nematodes (Maynard and Hochmuth, 2006). Insects not only make infection possible,

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but they also stress the entire host-plant system and thereby reduce overall resistance and resilience (Rotem, 1994). Furthermore, insects may act as vectors by dispersing inoculum throughout the crop. Infection pathways also include nutrient, water, or microclimate-originated stresses (Westcott, 2001). Figure 2.2 illustrates the disease cycle of *A. alternata* causing brown spot and black pit of potatoes.

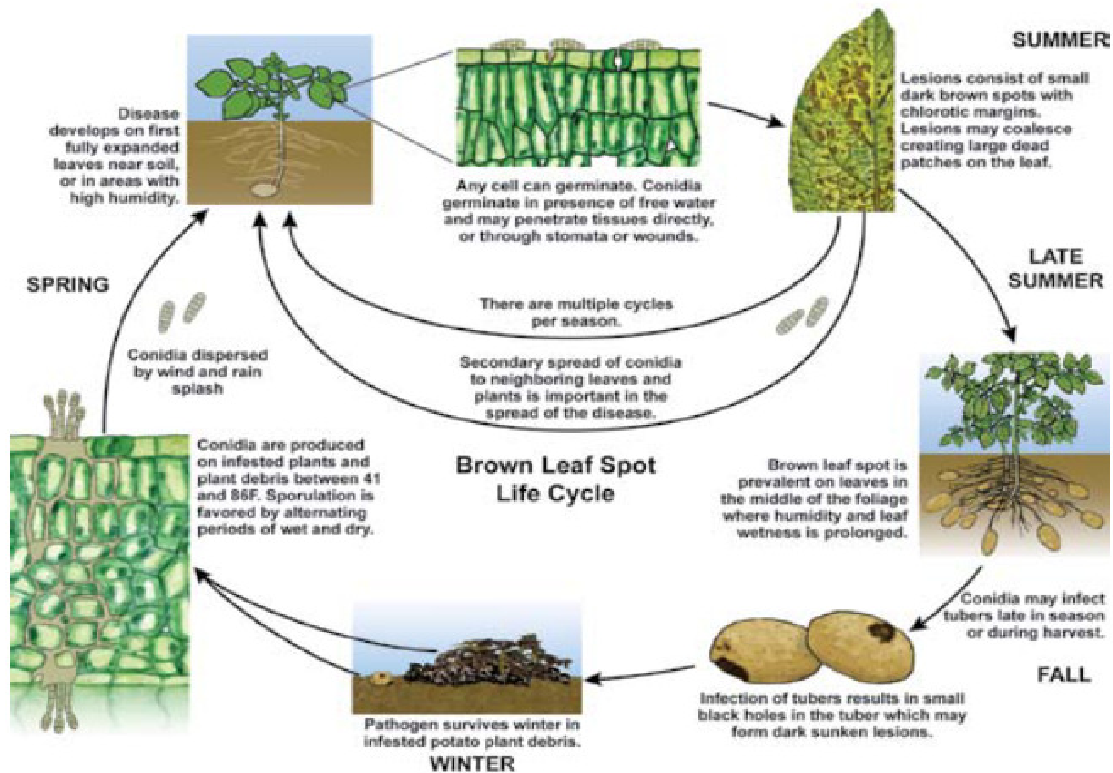

Figure 2.2. The disease cycle of brown spot pathogen *Alternaria alternata* on potatoes (Kirk and Wharton, 2012).

2.3 Management

Disease management is a key building block in an effective and safe crop management program (Agrios, 2005; Stevenson et al. 2001). The disease is controlled primarily through the use of cultural practices and foliar fungicides (Kirk and Wharton, 2012). A number of strategies have been used and made available for control of brown spot of potatoes. Westcott (2001) recommends planting a 3-year rotation to control *A. solani* and *A. alternata* on potato using non-host plants such as wheat, canola, rye, millet, carrots, stubble and clover to reduce inoculum levels during the overwintering stage. Removing and burning of infected debris and eradication of weed hosts further reduces inoculum (Agrios, 2005).

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Using drip irrigation rather than overhead irrigation is recommended as it has been shown to reduce the time of disease onset and the rate of disease progression (Ben-Noon et al. 2003). Use of resistant cultivars is therefore one of the key components of disease management, and development of disease resistant cultivars is an important goal of potato breeding programs (Stevenson et al. 2001). However, screening for brown spot resistant cultivars has not been done in South Africa and therefore provides a niche for research.

Keeping plants healthy by optimal fertilization and irrigation is recommended for growers (Mikkelsen, 2006), and to minimize tuber infection after harvest, tubers should be stored under conditions that promote rapid suberization because *A. alternata* is unable to infect through intact periderm (Kirk and Wharton, 2012). This will also assist in reduced disease carry-over to next season when the stored potatoes are retained as seed.

Use of fungicides remains one of the most important tools for managing diseases in many crops (Damicone and Smith, 2009). Use of registered fungicides at recommended rates is imperative for successful control of brown spot (BASF, 2012). Unlike insecticides and some herbicides which kill established insects or weeds, fungicides are most commonly applied to protect healthy plants from infection by fungal plant pathogens (Beckerman, 2008). Fungicides have been used for over 200 years to protect plants against disease attack by fungi (Krämer and Schirmer, 2007).

To be effective, fungicides must be applied before infections become established and in a sufficient spray volume to achieve thorough coverage of the plant or treated area (FRAC, 2011). Protection with fungicides is temporary because they are subject to weathering and breakdown over time.

2.3.1 Resistance

Poor disease control with fungicides can result from several causes including insufficient application rate, inherently low effectiveness of the fungicide on the target pathogen, improper timing or application method, and excessive rainfall (Lyr, 1995). Resistance, which is defined as lack of sensitivity, to fungicides in fungal pathogens is another major cause of poor disease control (Hewitt, 1998). Resistance development has been documented to

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varying degrees in virtually every chemical mode of action as described by the Fungicide Resistance Action Committee (FRAC) (Pasche and Gudmestad, 2008).The development of fungicide resistance is influenced by complex interactions of factors such as the mode of action of the fungicide, that is, how the active ingredient inhibits the fungus; the biology of the pathogen; fungicide use pattern; and the cropping system. Understanding the biology of fungicide resistance, how it develops, and how it can be managed is crucial for ensuring sustainable disease control with fungicides (Beckerman, 2008).

The problem of fungicide resistance became apparent following the registration and widespread use of the systemic fungicide benomyl (Benlate) in the early 1970s (Lyr, 1987). Many of the fungicides developed and registered since the introduction of benomyl are also systemic, have a site-specific mode of action, and are at increased risk for resistance problems (Damicone and Smith, 2009).

2.3.2 Quinone outside Inhibitors (QoIs)

Azoxystrobin belongs to a group of fungicides called Quinone outside Inhibitors (QoIs) (Brent and Hollomon, 1998; Rosenzweig et al. 2008). These are registered as Code 11 fungicides under the FRAC classification code (FRAC, 2012). QoI fungicides include three fungicide families, strobilurins and two newer families, represented by fenamidone and famoxadone (Bartlett et al. 2002).

QoI fungicides, also known as strobilurins, act at the quinol outer binding site of the cytochrome bc1 complex (complex III of fungal respiration) (Chapman et al. 2011). Thus, these fungicides act by inhibiting fungal mitochondrial respiration that stops energy production in the fungus and results in its death (Grasso et al. 2006). This group of fungicides should be applied preventively or as early as possible in the disease cycle (Ma and Michailides, 2005). They are effective against spore germination and early fungal growth (Ma et al. 2003). Once the fungus is growing inside the leaf tissue, QoI fungicides have little or no effect (FRAC, 2011). Use of strobilurins has become an integral part of disease management programs all over the world and this can be attributed to one of the many advantages they have over other fungicides (Surviliene and Dambrauskiene, 2006). These

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properties include broad spectrum action, good retention on leaves, growth enhancement, having less phytotoxicity, control of fungi that are resistant to other fungicides with other modes of action, low use rates and excellent yield and quality benefits (Brent and Hollomon, 1998; Bartlett et al. 2002). These fungicides have been shown to demonstrate high levels of preventative as well as curative activity (Bartlett et al. 2002), mainly because of their potent inhibitory effect on spore germination and zoospore motility (Stevenson and James, 1999).

QoI fungicides are active only at one specific site, therefore the risk of fungicide resistance is high (Mueller and Bradley, 2008). QoI resistance among fungal pathogens was first discovered in cereals when QoI resistant isolates of *Erysiphe graminis* f. sp. *tritici* were identified in Northern Germany in 1998 (Bartlett et al. 2002). To date, there are at least 20 different plant pathogens that have some level of resistance to QoI fungicides (Hewitt, 1998; FRAC, 2012).

Table 2.1. List of pathogens with resistance to QoI fungicides. Adopted from FRAC 2012 available at: www.frac.info.

Species name	Host	Geographical distribution	Mutation	References
Alternaria alternata, Alternaria mali, Alternaria arborescens	Pistachio	USA	G143A	Ma et al. 2003; 2004.
Alternaria mali	Apple	USA	G143A	Lu et al. 2003
Alternaria solani	Potato	USA	F129L	Pasche et al. 2004
Blumeria graminis f. sp. *tritici* and *hordei*	Wheat and barley	France, Sweden, Denmark	G143A	Sierotzki et al. 2000a
Colletotrichum graminicola	Turf grass	USA	G143A	Avila-Adame et al. 2003

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Table 1.1. List of pathogens with resistance to QoI fungicides. Adopted from FRAC 2012 available at: www.frac.info continued.

Species name	Host	Geographical distribution	Mutation	Reference
Corynespora cassiicola	Cucumber	Japan	G143A	Ishii, 2004
Didymella bryoniae	Cucurbit	USA	G143A	Langston, 2002
Glomerella cingulata (*Colletotrichum gloeosporioides*)	Strawberries	Japan	G143A	Ishii, 2004
Mycosphaerella fijiensis	Banana	Central and South America, Cameroon, Philippines	G143A	Sierotzki et al. 2000b.
Mycosphaerella graminicola	Wheat	EU	G143A	Fraaije et al. 2005; Sierotzki et al. 2005
Mycovellosiella nattrassii	Eggplant	Japan	G143A	Ishii, 2004.
Plasmopara viticola	Grape	EU	G143A; F129L	Heaney et al. 2000.
Pseudoperonospora cubensis	Cucurbits	EU, Asia	G143A	Heaney et al. 2000; Ishii et al. 2001.
Pyrenophora teres and *P. tritici-repentis*	Barley and wheat	EU	G143A	FRAC. 2010.

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Table 1.1. List of pathogens with resistance to QoI fungicides. Adopted from FRAC 2012 available at: www.frac.info continued.

Species name	Host	Geographical distribution	Mutation	Reference
Pyricularia grisea	Turf grass	USA	G143A; F129L	Vincelli and Dixon, 2002; Kim et al. 2003.
Pythium aphanidermatum	Turf grass	USA	F129L	Gisi et al. 2002
Spheárotheca fuliginea	Cucurbits	US	G143A	Heaney et al. 2000, Ishii et al. 2001.
Venturia inaequalis	Apple	EU	G143A	Steinfeld et al. 2002

QoI resistance has been shown to be conferred by point mutations within the cyt b gene (Lesniak et al. 2011). The molecular mode of resistance to QoI fungicides is understood to an advanced level and at least 15 different point mutations have been described in the cyt b gene leading to resistance (Brasseur et al. 1996). In field isolates of different pathogen species, the major mechanism of resistance is the amino acid substitution of glycine with alanine at position 143 (G143A) of the cytochrome b protein (Gisi et al. 2002). The amino acid substitution confers resistance in mutant isolates (Russel, 2004). Other mutations such as the F129L mutation have also been reported to be associated with moderate levels of resistance, and populations of these fungi are still controlled with QoI fungicides used at manufacturer recommended rates (Bartlett et al. 2002). The G143A mutation has been reported in at least 18 pathogens including *A. alternata* (Table 1).

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Chapter 3

Morphological and Epidemiological Characteristics of *Alternaria alternata* Affecting Potato Plants in South Africa

Abstract

Three *Alternaria alternata* isolates from potatoes were selected from 11 *Alternaria* isolates obtained from 6 potato growing regions in South Africa. The role of different light intensities (continuous near-UV, continuous white fluorescent, continuous dark and alternate white fluorescence and dark 16/8hrs) on cultural variability, radial growth rate and pathogenicity of isolates was investigated. The investigation was done under standardized conditions of 25°C and 85% relative humidity. The role of low temperatures on initial sporulation was also assessed and both experiments were analysed as a factorial analysis. Light had an effect on colony morphology as four different pigmentations were observed for the four different light regimes in all isolates, ranging from olive grey (normal pigmentation), and dark greyish brown to dark brown with yellow aerial mycelium. However; isolate SPD had 2 different pigmentations for isolates under conditions of continuous near-UV. The interaction of each isolate and light had a significant effect on radial growth rate (P=0.0163) on artificial media and on pathogenicity (P=0.0002). Exposure of isolates to low temperatures of 4°C in dark for 48h resulted in an increase in number of spores/ml compared to when isolates were incubated only at 25°C. All isolates recorded an increase in number of spores/ml under all conditions except for isolate FWN under continuous dark conditions. Isolate 3Vp was more virulent to potato under all conditions. The interaction of light (environment) and isolate (genetic capability) had a significant effect on both growth rate and pathogenicity of *A. alternata*. It is evident that variations do exist among *A. alternata* isolates from potatoes.

Key words: cultural variability, genetic capability, interaction, pathogenicity, radial growth rate.

3.1 Introduction

Alternaria alternata (Fr.) Keissl. is now widely recognized as the causal agent of brown spot in potato and other crops, causing small brown spots that start on the underside of the

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infected leaves. It has also been reported to cause black pit on stored potato tubers where the fungus was consistently isolated from the black sunken lesions on the tuber (Droby et al. 1984; Nolte, 2008). In South Africa brown spot was first reported in 2011 (Van der Waals et al. 2011) and has been widely observed in many potato production regions across South Africa.

Genetic variability is known to exist among *Alternaria* species and Slavov et al. (2004) reported that any given mycelium may become heterokaryotic because of the nature of the pathogen and this consequently leads to variability. A number of parameters such as light, humidity, temperature, pH of the growth media, and age of isolate determine the variations in characteristics of an isolate with respect to conidial size, shape and segmentation (Vakalounakis and Christias, 1985).

Pathogenicity of *A. alternata* is also known to be variable (Slavov et al. 2004) and these variations in isolates of the same pathogen may account for this instability. Hubballi et al. (2010) established that even isolates of the same pathogenic species exhibit differences in environmental requirements for optimal growth, sporulation and infection. Von Ramn and Lucas (1963) reported the occurrence of non-sporulating sectors in cultures under optimum conditions after weekly subculturing on potato dextrose agar (PDA) which resulted in a drop in pathogenicity. Slavov et al. (2004) stated that brown spot lesions on tobacco can be seen 2-8 days post inoculation, but in experiments done by Norse (1971), an incubation period of up to 35 days was observed in some isolates showing how variable *Alternaria* isolates can be. Spur and Main (1974) made 13 serial transfers on V-8 agar and PDA, and found that sub-culturing did not alter the virulence of isolates. The current investigation into some aspects of *A. alternata* from potato was initiated after numerous futile pathogenicity tests were carried out with different isolates in the greenhouse. The objectives of this trial were to: i. investigate effects of different wavelengths of light on radial growth and sporulation of *A. alternata*. ii. investigate the role of low temperatures on initial sporulation of *A. alternata* isolates iii. and to assess virulence of the isolates under different wavelengths of light.

3.2 Materials and Methods

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3.2.1 Pathogen: Samples of infected plant material were collected from six potato producing regions in South Africa. Isolations were made by cutting small pieces (5 mm x 5 mm) from the margin of brown spot lesions, surface disinfesting them in 1% sodium hypochlorite for 5 minutes and rinsing twice in sterile distilled water. The pieces were left to dry and then placed on V8 agar medium (200 ml V8 juice, 3 g $CaCO_3$, 20 g agar) and the plates were incubated at 25°C in darkness for seven days. Conidia from resulting colonies were picked up with a sterile inoculation loop and suspended in 500 µl sterile distilled water from which a 20 µl suspension was plated to water agar (WA) plates (20g Agar bacteriological, Biolab, Merck), which were then incubated at 25°C in darkness for 16 h. Using a sterile needle under a light microscope a single germinating spore was picked up and transferred to fresh Potato Dextrose Agar (PDA) plates (Biolab, Merck) and incubated at 25°C in darkness. Isolates were identified based on morphological characteristics.

3.2.2 Culture variability: Rapid growth is an important characteristic for fungal invasion and colonization (Solheim et al*.* 2001), therefore linear growth rate of each isolate was determined. Two-week old cultures on PDA incubated at 25ºC in the dark were used as inoculum. Round agar plugs from the unsporulated mycelial mat were cut using an 8mm-diameter cork borer, as adopted from Dimbi et al. (2004). Each agar plug was then transferred singly onto the centre of a fresh PDA agar plate and sealed with parafilm. Each isolate was exposed to four different light conditions (continuous near-UV, continuous white fluorescence, continuous dark and alternate white fluorescence and dark 16/8hrs) at 25ºC. Radial growth was recorded for each isolate on the seventh day using two cardinal diameters, through two orthogonal axes previously drawn on the bottom of each petri dish to serve as reference. The experiment was carried out in a completely randomized design with three replicates for each isolate in all treatments. The experiment was repeated to confirm reproducibility. Effect of isolate (genetic capability) and effect of different light regimes (environment) was analysed as a factorial analysis in JMP stats to see the effect of each individual factor and the interaction between isolate and light (gene-environment interaction).

3.2.3 Conidial production: The abundant production of conidia is an important requirement in the infection process of this pathogen and it is well established that sporulation is

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dependent upon light and temperature (Vakalounakis and Christias, 1985). The sporulation abilities of each isolate were investigated under different light conditions. Fresh PDA plates were inoculated as described for mycelium growth. Inoculated plates were exposed to four light regimes (continuous near-UV, continuous white fluorescent light, continuous dark and alternate white fluorescence and dark 16/8hrs) for seven days at 25°C. The experiment was repeated, but this time the isolates were kept at 4°C in darkness for 48hrs before the light treatments to assess the role of low temperatures and darkness for initial sporulation. Spores were brushed from petri dishes and suspended in 10ml distilled water and number of spores per ml was determined using a haemocytometer. The experiment had three replicate plates for each isolate in all treatments.

3.2.4 Pathogenic variability: Pathogenic variability was carried out using a detached leaf assay (Sofi et al. 2013) on a susceptible cultivar BP1. Healthy potato leaves of the same size and all from the middle canopy of the plant were selected and surface sterilized. Leaves were pin pricked (Lakshmanan et al. 1990) and inoculated with a $4x10^5$ conidia/ml spore suspension of each isolate respectively. Control plants were inoculated with distilled water. Each isolate was incubated at four different light intensities, (continuous near-UV, continuous white fluorescent light, continuous dark and alternate white fluorescence and dark 16/8hrs), a mean temperature of 25°C and relative humidity of 85%. Three replications were maintained for each isolate and as well as the control plates. Symptoms were observed three days post inoculation. Disease severity (the area affected by the pathogen) was evaluated using a disease severity scale from 0-5 (0= no disease; 1= 0-20% infection, 2= 26-40% infection; 3= 41-60% infection, 4= 61-80% infection and 5= 81-100% infection)

3.2.5 Statistical analysis: A one way analysis of variance was generated using JMP stats (version 10.0) to analyse culture variability and effect of temperature and light on conidial production. Effect of light and the genetic capability of each isolate on growth rate and sporulation were analysed. Effect of light and genetic capability of isolate on growth were analysed individually and in combination to understand their effect.

Pathogenic variability was also analysed in a factorial design where the effect of different isolates, different wavelengths of light and the combined effect of isolate and light on pathogenicity was evaluated in a one way analysis of variance.

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3.3 Results

3.3.1 Pathogen: Eleven isolates were obtained from six potato production regions (Table 3.1).

Table 3.1: *Alternaria alternata* isolates isolated from potato leaves.

Isolate	Location	Variety
SPD	Mpumalanga	Shepody
FWN	Mpumalanga	Fianna
DRS	Mpumalanga	Darius
3Vp	Limpopo	Mondial
4Vp	Limpopo	Mondial
5Vp	Limpopo	Mondial
T3	Northern Cape	Fabula
T5	Cape town	Fianna
T8	KwaZulu Natal	Mondial
P1	Northern cape	Darius
P2	Northern Cape	Darius

Three *A. alternata* isolates, SPD, FWN and 3Vp were randomly selected from the collected isolates and used in downstream experiments (Table 3.1).

3.3.2 Culture variability: When grown on PDA under 4 different standardized light conditions (continuous near-UV, continuous white fluorescent, continuous dark and alternate white fluorescence and dark 16/8hrs), isolates showed colony morphology

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variations. Colony pigmentation varied from olive grey (normal pigmentation), dark greyish brown to dark brown with yellow aerial mycelium. These variations were recorded for all isolates under different light conditions (Figure 3.1). However; on isolate SPD, colony variation was observed between replicate plates incubated under continuous near-UV light (Figure 3.2).

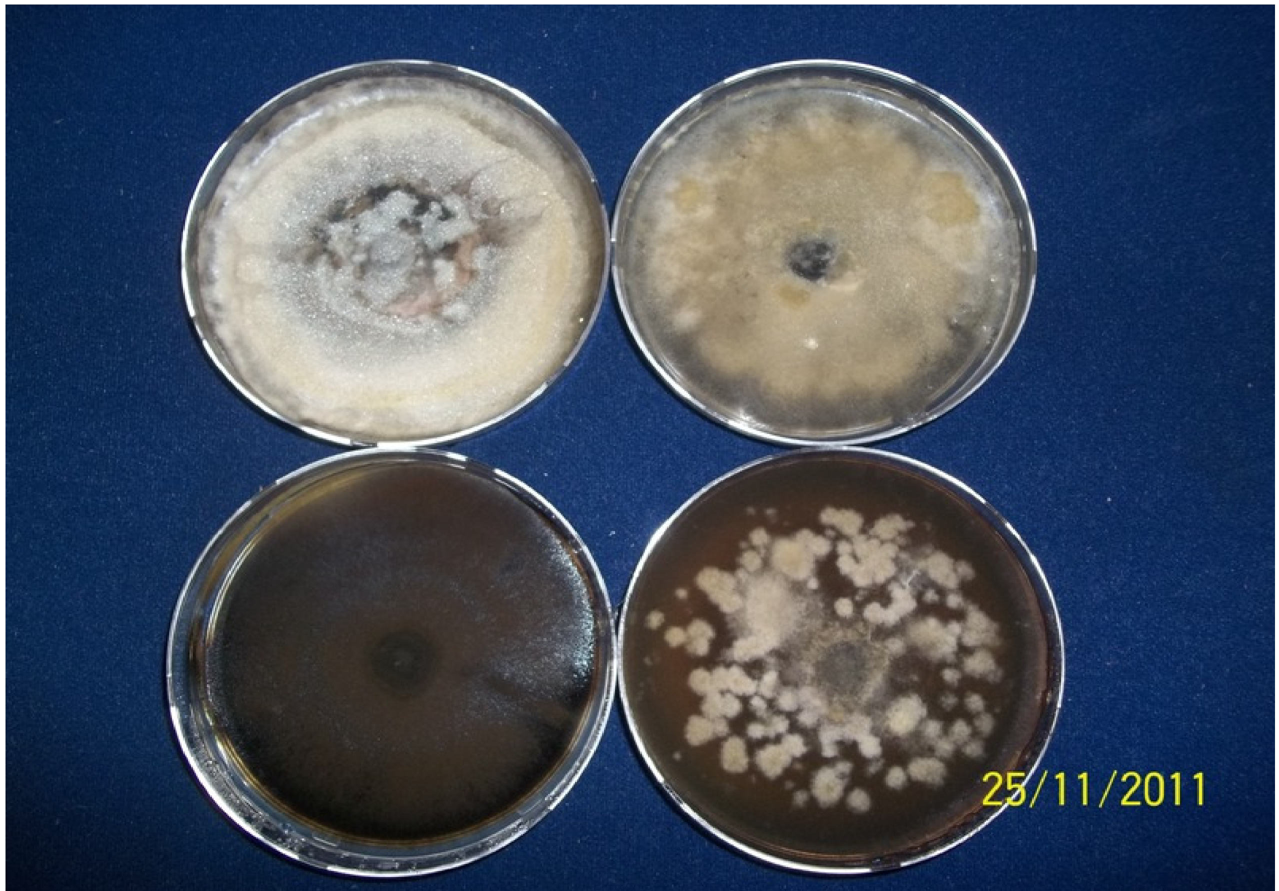

Figure 3.1: Variations in isolate 3Vp under four different light conditions. (Top left- continuous white fluorescent light, top right- continuous near-UV, bottom left- continuous dark and bottom right- alternate white fluorescence and dark 16/8hrs).

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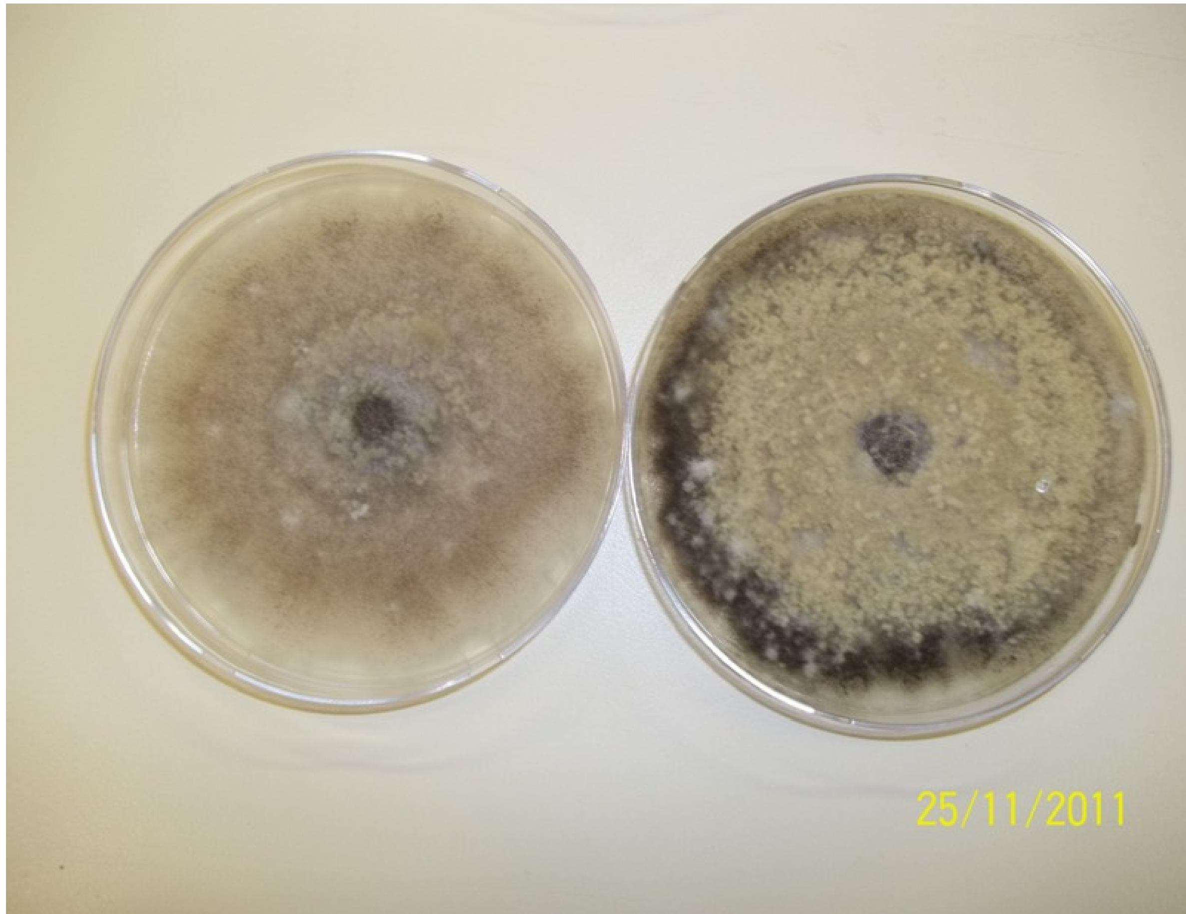

Figure 3.2: Variation in isolate SPD of *A. alternata* under continuous near-UV light.

Different patterns in radial colony growth were observed among isolates. The mycelia had either a uniform growth pattern or irregular growth where mycelial growth was characterized by wavy margins. Isolate 3Vp and SPD showed a general circular growth pattern under all light conditions, whilst isolate FWN had irregular growth under alternate light and dark 16/8hrs and continuous darkness, but all isolates under continuous near-UV light and under continuous light had circular growth.

From the experiment it was observed that different light regimes had a significant influence on growth rate (P=0.0163). Isolate 3Vp had filled all the plates by day 7 under all light conditions. Isolate SPD and FWN showed significant difference among treatments with conditions of near-UV promoting the greatest mycelial growth followed by continuous dark conditions in both isolates. In isolate FWN conditions of alternate white fluorescence and dark (16/8) had the least mycelial growth, whilst isolate SPD had the least mycelial growth under conditions of continuous fluorescent light (Figure 3.3).

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Although isolate 3Vp grew significantly faster under all light treatments than SPD and FWN, whilst Isolate SPD showed least mycelial growth under all light conditions, the isolates genetic capability had no significant influence on growth rate of isolates (P=0.3667). However; there was a highly significant interaction from light and isolates (P<0.0001). All means were analysed at 95% confidence interval and all means were separated using all pairs Tukey-Kramer test.

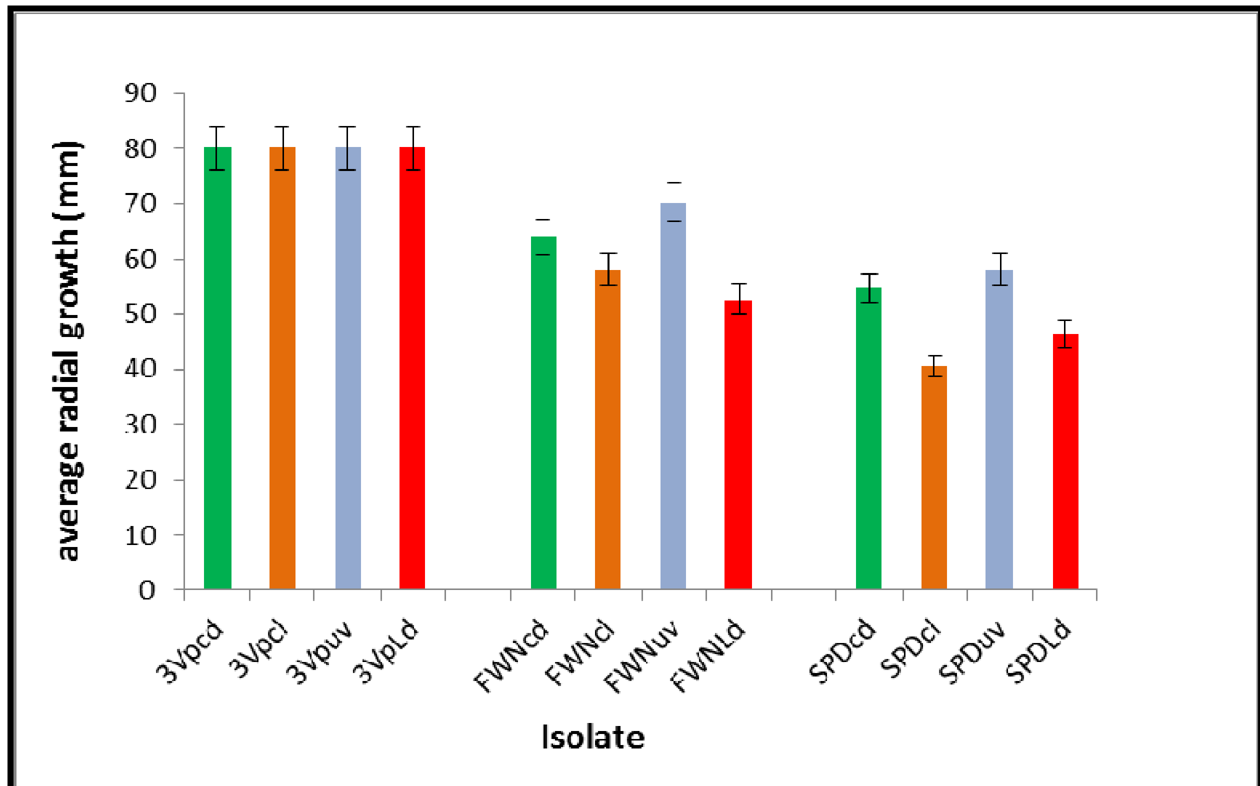

Figure 3.3: Average radial growth of *A. alternata* on PDA after 7 days at four different light conditions CD= continuous dark, CL=continuous fluorescent light, UV= continuous near-UV light, LD= alternate fluorescent light and dark (16/8hrs). LSD= 3.60563; DF= 35.

3.3.3 Conidial production: Spores were produced under all light conditions except for isolate FWN that did not produce spore under continuous dark condition. The greatest mean spores produced was under conditions of continuous near-UV light in all isolates while the lowest number was recorded under continuous dark for isolate FWN and continuous light for isolate SPD. Except for continuous near-UV light, the effect of the other treatments on sporulation differs between isolates. The percentage increase after exposure to low

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temperatures in dark for 48h was calculated and it was observed that isolate 3Vp under alternating fluorescent light and dark (16/8hrs) had the greatest increase (0.16%) whist isolate FWN under conditions of continuous dark had the least percentage increase (0%) in spore production as it recorded no spores before and after exposure to low temperature for 48h in darkness (Figure 3.5). It was, however, observed that there was no significant difference in pre-treating isolates to low temperatures in the dark.

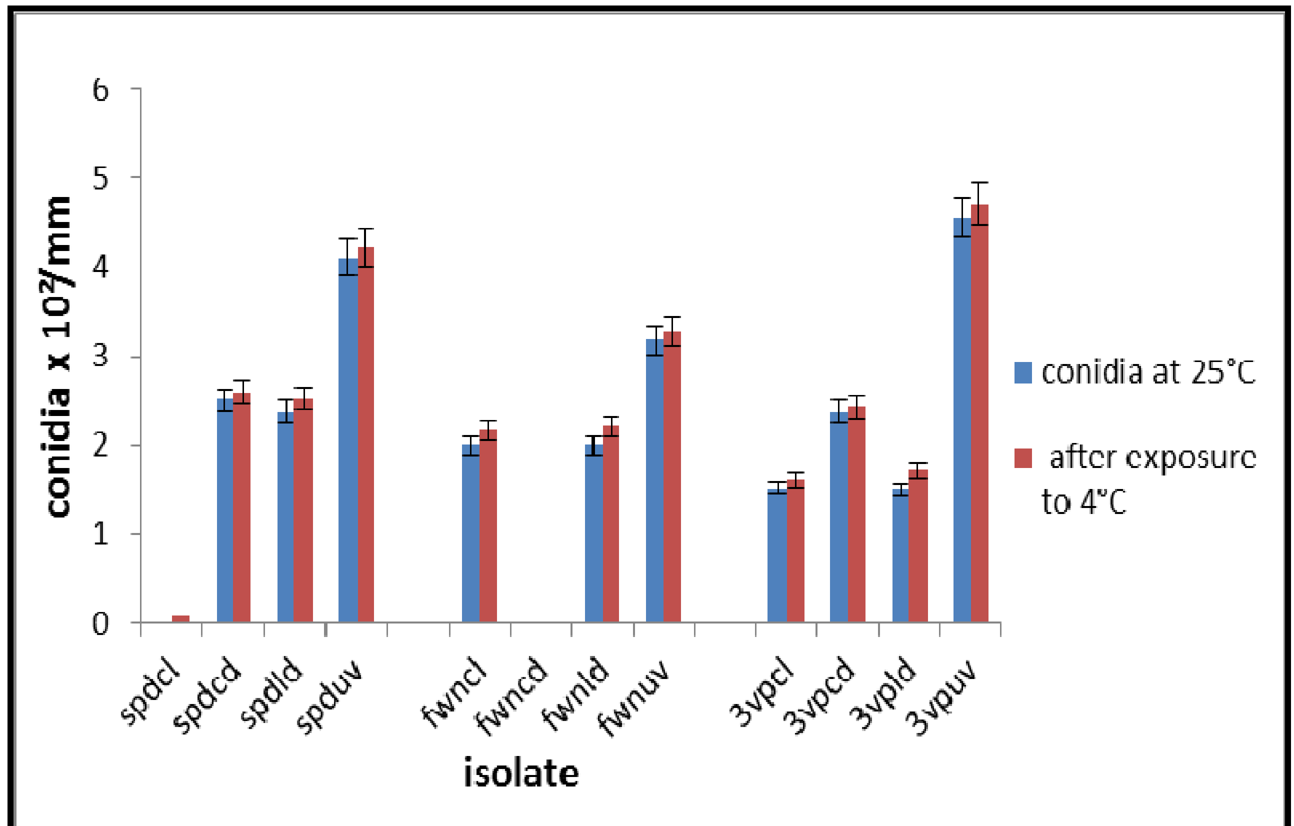

Figure 3.4: Response to pre-treating isolates with low temperature (4°C) and darkness for 48hrs, followed by incubation at 25°C at various light conditions. CL= continuous fluorescent light, CD= continuous dark, LD= alternate fluorescent light and dark (16/8hrs), UV= continuous near-UV light.

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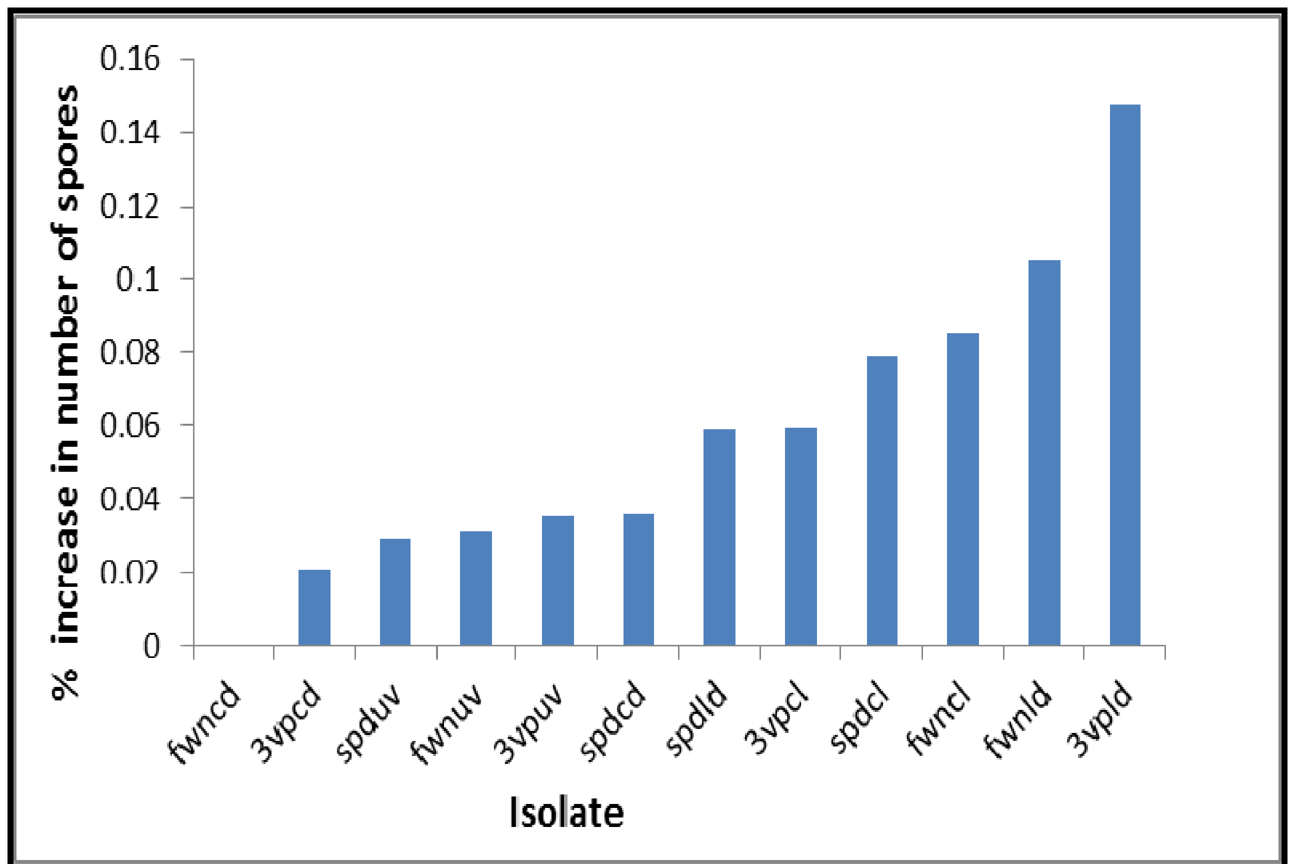

Figure 3.5: Percentage increase in number of spores after exposure to 4°C for 48h. CD= continuous dark, CL=continuous fluorescent light, LD= alternate fluorescent light and dark (16/8hrs), UV= continuous near-UV light.

3.3.4 Pathogenic variability: Disease incidence was observed on the second day for all isolates under all conditions tested except for isolate SPD under continuous light conditions. Disease incidence was evaluated as the presence of brown lesions on the potato leaves. Disease severity was recorded on the third day post inoculation (Figure 3.6). Differences in severity were observed among the different isolates and for each isolate under different light conditions. The greatest severity was recorded for isolate 3Vp under all conditions when compared to the other isolates.

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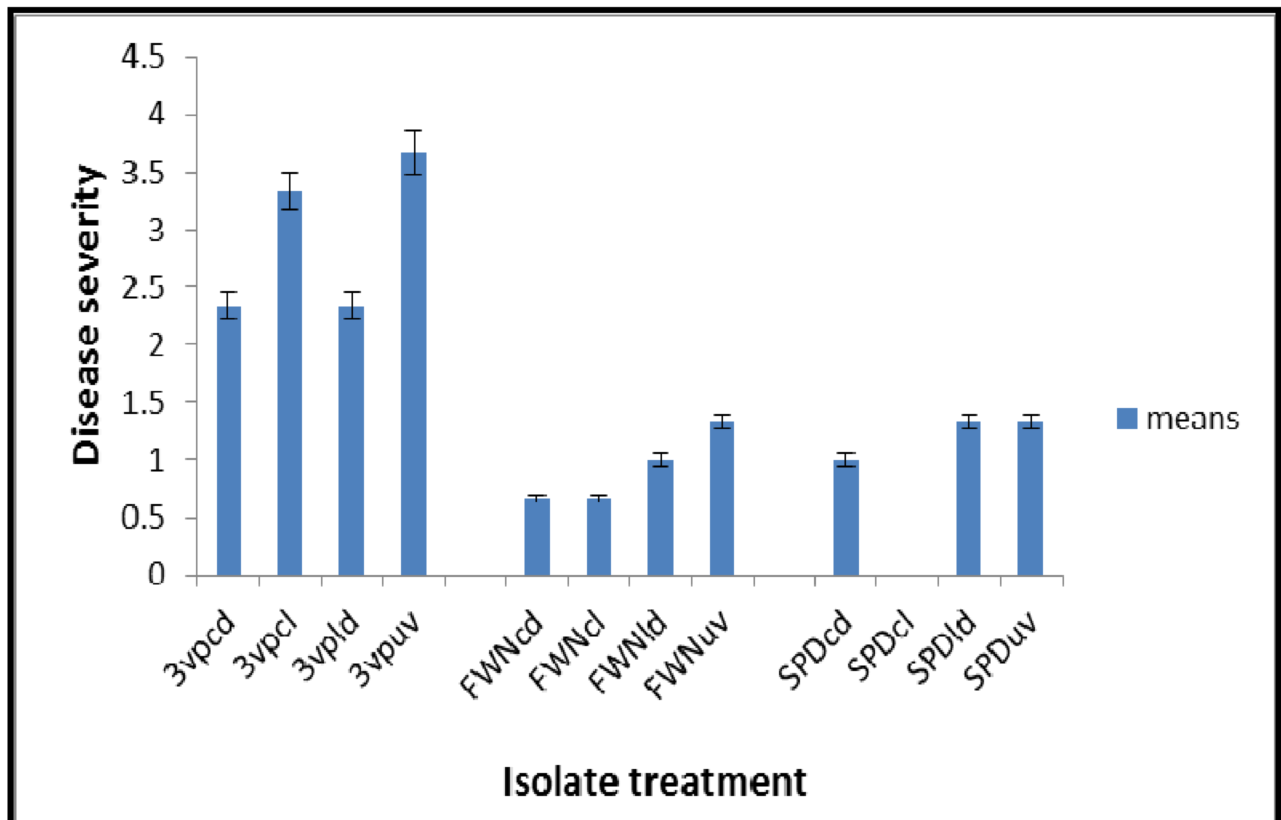

Figure 3.6: Disease severity on detached BP1 leaves 3 days post inoculation. CD= continuous dark, CL=continuous fluorescent light, LD= alternate fluorescent light and dark (16/8hrs), UV= continuous near-UV light. LSD= 1.215; DF=22.

Although all isolates had the highest disease severity under continuous near-UV light, statistical analysis revealed that the different light regimes had no significant influence on severity (P=0.1267). It was however; shown that the isolate genetic capability had a highly significant influence on severity (P<0.0001). The combined effect of light conditions and genetic capability of isolates had a significant influence on severity (P=0.0002) at 95% confidence interval and all means were analysed using all pairs Tukey-Kramer test.

3.4 Discussion and Conclusion

It is well established that illumination can alter the morphology of some fungi (Vakalounakis and Christias, 1985). In their study, Vakalounakis and Christias (1985) were able to prove that light intensity affects conidial morphology in *Alternaria cichorii* Nattrass, and also pointed out that light intensity and light quality are two aspects of light that are involved in sporulation. Johnson and Haplin (1954) proved that spores of *Alternaria solani* Sorauer and

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Pyricularia oryzae Cavara tended to be longer under light than in the dark. In their study, as in ours, the greatest number of spores was recorded in isolates exposed to continuous near-UV light and this may be due to the fact that continuous near-UV light, as explained by Vakalounakis and Christias (1986), is known to be involved in the first two categories of fungal photoresponses. These photoresponses include spore stimulation (phototropism and carotenoid synthesis) and photoresponses associated with reproduction.

In our findings, variations in colony pigmentation were evident among isolates under different light intensities as shown in Figure 3.1 and this can be explained by the findings of Honda (1969) and Kumagai and Oda (1969) who all showed that a photoreceptor system called a "mycochrome" exists and is involved in a blue and near-UV reversible photoreaction. This photoreaction is involved in photo-morphogenesis in achlorophyllous organisms resulting in photo-reversible photochromic pigments which have been reported in marine fungi (Wagner and Wagner, 1995).

Light has a profound effect on mycelial growth of *A. alternata* (Hubballi et al. 2010) and this was statistically proven in our experiments as different light intensities had significant influence on radial growth of mycelia. In their experiments Hubballi et al*.* (2010) also observed a significant difference in radial growth of isolates when exposed to different light regimes. They were able to observe that conditions of continuous dark resulted in slower radial growth, however in our experiments the least radial growth recorded varied between continuous light and alternating light and dark 16/8h in isolate SPD and FWN respectively. This shows how variable the *A. alternata* isolates from potato tested in the present study were in response to different light intensities.

In most studies conducted to assess the sporulating abilities of *Alternaria* isolates, highest sporulation was observed under near-UV (Slavov et al. 2004) and under diurnal light (Barksdale, 1969). Although all our isolates had greatest number of spores recorded under near-UV light, spores were recorded under continuous dark conditions in our experiments and this would correspond to the findings of Shahin and Shepard (1979), who devised that profuse sporulation of *Alternaria* spp can be achieved by growing the pathogen on a slightly alkaline medium in the dark at 18°C. However not all of their isolates showed this. Just as in

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the case of Shahin and Shepard (1979), isolates SPD and 3Vp corresponded with their findings as they had more spores under continuous dark conditions when compared to alternating light and dark (16/8h), but isolate FWN recorded no spores under continuous dark conditions which further confirms that there is a lot of variability among *Alternaria* isolates.

In experiments done by Honda and Aragaki (1978) using different *Alternaria* spp it was observed that *Alternaria tomato* (Cke.) Webber sporulated under dark and under continuous light at 18°C, they also observed that sporulation of *Alternaria passiflorae* Simmons, *Alternaria solani* Sorauer, and *Alternaria porri* Ellis was inhibited by continuous fluorescent light at 27°C. This further explains variations that occur among members of the genus *Alternaria*.

Temperature is also known to affect spore morphology (Vakalounakis and Christias, 1985; Rotem 1994). Spores of *Alternaria longipes* Elvis and Everhart (Tisdale and Watkins, 1931) and *Alternaria alternata* f.sp *lycopersici* (Misaghi et al. 1978) were longer at lower temperatures than high temperatures. The role of low temperatures and darkness in the initiation of sporulation was assessed and it was observed that there was an increase in spore concentration in almost all isolates under all light conditions after exposure to 4°C under dark conditions for 48 hours. This confirmed that low temperatures and a dark period play an important role in initiation of sporulation. However some isolates had no spores even after exposure to a low temperature and this can be explained by the variability in *Alternaria* spp which includes sporulation ability of the isolates (Slavov et al. 2004).

Since it has been established that genetic variations do occur among *A. alternata* isolates, even among isolates derived from single spore isolates due to the nature of the mycelium to become heterocaryotic under optimum conditions (Slavov et al. 2004), this may lead to differences in growth rates and sporulation ability consequently affecting pathogenicity and virulence of the pathogen (Rotem, 1994). The genetic capability of any pathogen maybe reached if conditions are favourable and genetic variability in isolates may enable virulent isolates to overcome unfavourable conditions.

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In conclusion isolate 3Vp was more virulent under all conditions when compared to other isolates. Despite light having no significant influence on pathogenicity, it was observed that all isolates were more virulent under conditions of near-UV light. Isolate FWN was observed to be the least pathogenic isolate among the three isolates. From this study it is concluded that variations do exist among *A. alternata* isolates from potatoes. Light has no significant influence on pathogenicity but on growth rate; while genetic capability of isolates had no significant influence on growth rate, it had a highly significant influence on pathogenicity. Never the less a combination of light (environment) and isolate (genetic capability) had a significant effect on both growth rate and pathogenicity of *A. alternata*.

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Chapter 4

Morphological and Molecular Characterization of *Alternaria alternata* Isolates Associated with Brown Leaf Spot of Potatoes in South Africa.

Abstract

Potato leaves showing symptoms of brown spot were collected from eight growing regions in South Africa. Thirteen isolates were obtained from six potato cultivars. Isolates were identified morphologically based on conidia size, shape, septation, surface ornamentation, apical extension and proliferation. The isolates were also characterized based on partial β-tubulin (BT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and translation-elongation factor 1-α (TEF1) gene regions. Phylogenetic analysis of the isolates together with reference sequences from GenBank was based on parsimony and Maximum likelihood using 1000 bootstrap replications. PCR-RFLP of the internal transcribed spacer (ITS) region was performed to detect if any genetic variation existed among isolates. Conidia size ranged from 8-15 μm x 15-44 μm. Conidia were produced in short chains of 2-6 conidia with a number of secondary and tertiary branches. Conidia had 1-3 longitudinal and 2-6 transverse septa, typical of *Alternaria alternata*. Analysis based on GADPH, TEF1 and concatenated GAPDH and TEF1 showed most parsimonious and maximum likelihood bootstrap support values of between 98% and 100% for potato isolates belonging to the *Alternata* section. Sequence data from BT region provided no resolution between the species and was therefore not included in the concatenated data set. PCR-RFLP showed no variations in the digestion profile between isolates. Morphological characteristics used in combination with multilocus phylogenetic analyses can be used in identification and characterization of *A. alternata* isolates from potatoes.

Key words: β-tubulin, glyceraldehyde-3-phosphate, PCR-RFLP, phylogenetic trees, translation-elongation factor 1-α.

4.1 Introduction

It is well established that *Alternaria alternata* (Fr:) Keissl. is the causal agent of brown leaf spot and black pit of potatoes (Droby et al. 1984; van der Waals et al. 2011; Kirk and

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Wharton, 2012). The disease is characterized by small necrotic lesions that start on the abaxial side of the leaf (van der Waals et al. 2011), and coalesce with time to form large necrotic lesions (Stevenson et al. 2001). Yield losses of 30% or more may be experienced if left uncontrolled and postharvest losses of up to 10% have been recorded in stored potatoes (Boyd, 1972; Delleman et al. 2005).

Small-spored *Alternaria* species are cosmopolitan saprophytes, plant pathogens, allergens and mycotoxin producers that provide a huge challenge in identification in a broad range of disciplines; hence the need for techniques to accurately identify isolates (Roberts et al. 2000). The genus *Alternaria* has a complex taxonomy (Pryor and Michailides, 2002) that is based primarily on the morphology and development of conidia and conidiophores, and to a lesser degree on host plant association and colony morphology (Konstantinova et al. 2002). However; there are several small-spored catenulate *Alternaria* spp. with morphological characteristics that overlap each other especially in the case of *A. alternata*; the most commonly known are *Alternaria tenuissima* (Kunze) Wiltshire and *Alternaria infectoria* (E. G. Simmons) (Pryor and Michailides, 2002).

Roberts et al. (2000) described the three-fold problems associated with these overlapping morphological characteristics. First, is the inherent phenotypic plasticity observed in *Alternaria* cultures grown on non-standardized media and under uncontrolled growth conditions. Secondly, the divergent views on sub-generic classification and the resulting inconsistent application of names in the modern literature. Thirdly, the widespread practice of applying the name *A. alternata* to small-spored taxa without critical examination of living material under defined conditions. Most *Alternaria* spp., including *A. alternata*, exhibit considerable phenotypic plasticity that is dependent upon cultural conditions such as substrate, temperature, light, and humidity (Misaghi et al. 1978; Simmons, 2007). Pryor and Michailides (2002) stated that even within a particular *Alternaria* isolate, there is a considerable range of variation in conidium morphology with regard to size, shape, septation, colour, and ornamentation.

In addition to morphology, variation exists among small-spored *Alternaria* spp. with respect to host range. Overlapping morphological characteristics within these taxa resulted in the proposal that host-specific taxa should be referred to as pathotypes or as *formae speciales*

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of *A. alternata* (Nishimura et al. 1978; Nishimura and Kohmato, 1983; Rotem, 1994). Examples of host-specific small-spored *Alternaria* include *Alternaria mali* Roberts on apple (Johnson et al. 2000), *Alternaria gaisen* Nagana on pear (Xia and Tian-Yu, 2008), *Alternaria longipes* (Ellis and Everh.) E.W. Mason on tobacco (Tisdale and Wadkins, 1931), *A. alternata* pathotype strawberry on strawberry (Kusaba and Tsuge 1995), *Alternaria citri* Ellis and N. Pierce (pathotypes tangerine and rough lemon) on citrus (Peever et al. 2004), and *A. alternata f.* sp. *lycopersici* Keissl. on tomato (Misaghi et al. 1978; Pryor and Michailides, 2002). Existence of closely related genera such as *Stemphylium, Ulocladium*, and *Macrosporium* that also possess phaeodictyosporic and morphologically similar conidia to *Alternaria* spp. further complicates the classification of certain species, as well as the practical identification of some of the plant pathogenic members of these genera (Simmons, 1967).

The genus *Alternaria* was recently monographed by Simmons (2007), who accepted 276 species distinguished by three-dimensional sporulation patterns in addition to conidial morphology. However morphological identification is not always reliable in cases where isolates may have been transferred to unsuitable media and grown under inappropriate conditions where spore production is hampered, or strains may have become sterile after years in culture (Simmon, 2007; Brun et al. 2013). There is also a mounting body of evidence that the *A. alternata*-pathotype classification is inconsistent due to the morphological, pathological, and some physiological differences among these fungi (Roberts et al. 2000).

Other identification methods such as chemotaxonomy (Brun et al. 2013) as well as advanced molecular techniques can be useful in delineating among these fungi. Phylogenetic studies have demonstrated a clear distinction between large and small-spored *Alternaria* species (Peever et al. 2004; Woudenberg et al. 2013). However; it is among the small spored *Alternaria* spp. that molecular phylogenies of this group have revealed little to no variation in the genetic loci commonly employed in fungal systematics (Andrew et al. 2009). The objectives of the current study were to:

i. Delineate *Alternaria* isolates isolated from potato in South Africa based on partial sequence data of translation elongation factor 1-α (TEF1), β-tubulin (BT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene regions.

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ii. Determine the relationships among morphology and phylogeny that can be useful in characterizing *Alternaria alternata* isolates from potato in South Africa.

4.2 Materials and Methods

4.2.1 Pathogen: Samples of infected plant material were collected from different potato producing regions in South Africa. Small pieces of approximately 25 mm^2 were cut from symptomatic leaves with a sterile scalpel blade. Infected material was surface sterilized by immersing it in 1% sodium hypochlorite for 5 minutes and then rinsing twice in sterile distilled water for one minute. The plant material was plated onto V8 media and incubated at 25°C under UV-light with a 12 h photoperiod for 7 days. Single spore isolations were performed by plating 0.1 µl spore suspension on water agar and incubated overnight. Single germinating spores were picked up using a sterile needle while viewing under a stereo microscope and transferred to potato dextrose agar (PDA) in order to obtain pure cultures. PDA plates were incubated at 25°C under UV-light with a 12 h photoperiod for 7 days.

4.2.2 Morphological identification: Resultant single spore isolates were plated onto potato carrot agar (PCA) (Simmons, 1992) and incubated at 25°C under UV-light with a 12 h photoperiod for 6 days. Conidial characteristics were examined using the scotch-tape method using lactophenol as mounting fluid. Photographs of characteristic structures were made with a Zeiss microscope at 40X magnification. This method was used mainly to preserve attachment of conidia to conidiophores (Navi et al. 1999). Identification was based on conidia size, shape, septation, surface ornamentation apical extension and proliferation.

4.2.3 PCR and sequencing: The DNA from each isolate was extracted using the Zymo fungal DNA extraction kit (Inqaba Biotech) according to manufacturer's protocol and stored at -20°C. The BT region was amplified with the primers Bt1a (5'-CAGCTCGAGCGTATGAACGTCT-3') and Bt1b (5'-TGTACCAATGCAAGAAAGCCTT-3') (Quayyum et al. 2005); the ITS region with primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al. 1990); the TEF1 gene with primer pair EF1-728F (5'-CATCGAGAAGTTCGAGAAG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTAC-3') (O'Donnel et al. 1998) and GAPDH region with primer pair gpd1 (5'-CAACGGCTTCGGTCGCATTG-3') and gpd2 (5'-GCCAAGCAGTTGGTTGTGC-3') (Berbee et al. 1999). These gene regions were

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selected based on their high support values and high resolution in separating clades within the genus *Alternaria* especially when they are all used in combination.

Thermo cycler conditions for GAPDH region included an initial denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 52ºC for 45 sec, extension at 72ºC for 1 min, and a final extension at 72ºC for 5 minutes. Conditions for ITS, BT and TEF1 were the same, except for annealing at 56°C, 54°C and 53°C, respectively. The 25 µl reaction mix contained 19.5 µl Sabax water, 2.5 µl reaction buffer, 1.25 µl $MgCl_2$, 0.5 µl dNTPs, 0.25 µl of each primer, 0.25 µl of Taq polymerase (Bioline) and 0.5 µl of the DNA template. PCR products and Hyperladder II (Bioline) were run in 1% agarose gel at 120 volts for 45 minutes and viewed under UV light.

PCR products were cleaned using Sephadex G50 (Sigma Aldrich) and sequenced in both directions using the PCR primers for BT, GAPDH and TEF1. The sequencing PCR was conducted in a reaction mix containing 4 µl Sabax water, 2 µl Big Dye, 1 µl sequencing buffer, 1 µl each primer and 2 µl of the cleaned PCR product. Thermo-cycling conditions for sequencing PCR included 25 cycles of denaturation at 96ºC for 10 sec, annealing at 52ºC for 1 min and extension at 60ºC for 4 minutes. Sequencing products were cleaned using Sephadex G50 (Sigma Aldrich). Each sample was sequenced using the ABI 3500xl capillary array genetic analyser and edited using Mega 5. Forward and reverse sequences were combined to obtain consensus sequences using CLC Main workbench 6.7 (CLC Bio).

4.2.4 Phylogenetic analysis: Multiple sequence alignments were generated with MAFFT version 7 (Katoh and Standley, 2013) separately for each region together with reference sequences acquired from GenBank mostly based on the work of Woudenberg et al. (2013) but also based on nucleotide BLAST searchers against the nucleotide database in GenBank (Table 4.1). The Q-INS-i algorithm was chosen for all the alignments. Subsequently, the aligned sequences were concatenated for phylogenetic analysis. No manual ‘‘improvements” or ‘‘corrections” were done on the alignment, except edges consisting of incomplete sequences. Gaps were treated as missing data in the subsequent analysis. Phylogenetic analysis was based on parsimony and Maximum likelihood analysis.

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Parsimony analysis was performed using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods version 4) (Swofford, 2002). Heuristic searches were done with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPAR effective and MaxTrees set to auto-increase. The combinability of the datasets was determined by the partition homogenicity test (Farris et al. 1994). The consistency (CI) and retention (RI) indices were determined for the data set. Bootstrap analyses with 1000 replications were performed to determine branching point confidence intervals for the most parsimonious trees generated for the respective data sets.

Maximum likelihood analysis with 1000 bootstrap replicates was performed using PhyML version 3.0 (Guindon and Gascuel, 2009). Models, with gamma-distributed substitution rates, for each region as well as the combined dataset were selected using jModelTest 2.1.4 (Darriba et al. 2012). The phylogenetic tree was rooted with *Embellisia annulata* as monophyletic sister outgroup to the rest of the taxa.

4.2.5 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP): PCR amplification followed by restriction enzymes is a common technique used in detecting genetic variability among isolates. PCR products of the ITS region were digested using two restriction enzymes Hind III and Alu I (Life Technologies). These two enzymes are some of the most commonly used enzymes in molecular biology and have been widely used as a potent tool in delineating the genus *Alternaria*.

Digestion with restriction enzymes was carried out in a reaction mix containing 7.5 µl Sabax water, 2 µl reaction buffer, 10 µl PCR product and 0.5 µl restriction enzyme. The reaction mix was incubated at 37°C for 3hrs and left overnight at room temperature. Digests were examined on a 2% agarose gel at 100 volts for an hour and viewed under UV light. PCR-RFLP analysis was performed twice to confirm reproducibility and consistency in digestion of products.

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Table 4.1: GenBank accession numbers for *Alternaria* reference isolates and out-group isolates used for phylogenetic analyses.

Species name	GenBank accession number		
	BT	GAPDH	TEF1
A. alternata	JQ811937	AY278808	KC584634
A. arborescens	JQ811950	AY278810	KC584636
A. caricis	JQ671961	AY278826	KC584726
A. conjuncta	JQ671992	AY562401	KC584649
A. ethzedia	JQ671971	AY278795	KC584657
A. gaisen	AY293874	KC584116	KC584658
A. infectoria	JQ671970	AY278793	KC584662
A. limoniasperae	JQ672041	AY562411	KC584666
A. longipes	JQ672026	AY278811	KC584667
A. macrospora	JQ672066	KC584124	KC584668
A. porri	JF331581	KC584132	KC584679
A. scirpicola	JQ671962	KC584163	KC584728
A. solani	JF417707	KC584139	KC584688
A. tenuissima	JQ811947	AY278809	KC584693
Embellisia annulata	JQ671942	JN383467	KC584709

*BT = β-tubulin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; TEF1 = translation elongation factor 1-α.

4.3 Results

4.3.1 Pathogen: Thirteen *Alternata* isolates were obtained from diseased material (Table 4.2).

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Table 4.2: List of *Alternaria* isolates obtained from infected potato leaves.

Isolate number	Province	Potato Variety
2G	KwaZulu Natal	Fianna
7Vp	Northwest Cape	Darius
AA15	Northern Cape	Mondial
Den1	Limpopo	Darius
DRS	Mpumalanga	Darius
FWN	Mpumalanga	Fianna
Clsf4	Western Cape	Sifra
CS6	Gauteng	Fabula
SPD	Mpumalanga	Shepody
Z21a	Limpopo	Mondial
Z21d	Limpopo	Mondial
Z21h	Limpopo	Mondial
9	Northern Cape	Mondial

4.3.2 Morphological identification: Isolates showed a typical dark brown to grey-black colour on PCA. Colony growth was circular with alternating dark and light concentric sporulation rings due to alternating UV light and dark regimes. Dark brown septate mycelia were sparsely produced on PCA. The conidiophores were mostly single rather than in clusters and were septate. The conidiophores produced brown pigmented conidia in acropetal succession of predominantly simple chains with a few branched chains (Figure 4.1). Chains of conidia were produced at the beak of a spore and the chains were made up of 2-6 conidia with a number of secondary and tertiary branches. Conidia were obclavate and muriform with 1-3 longitudinal and 2-6 transverse septa (Figure 4.2). Conidia size ranged from 8-15 µm x 15-44 µm.

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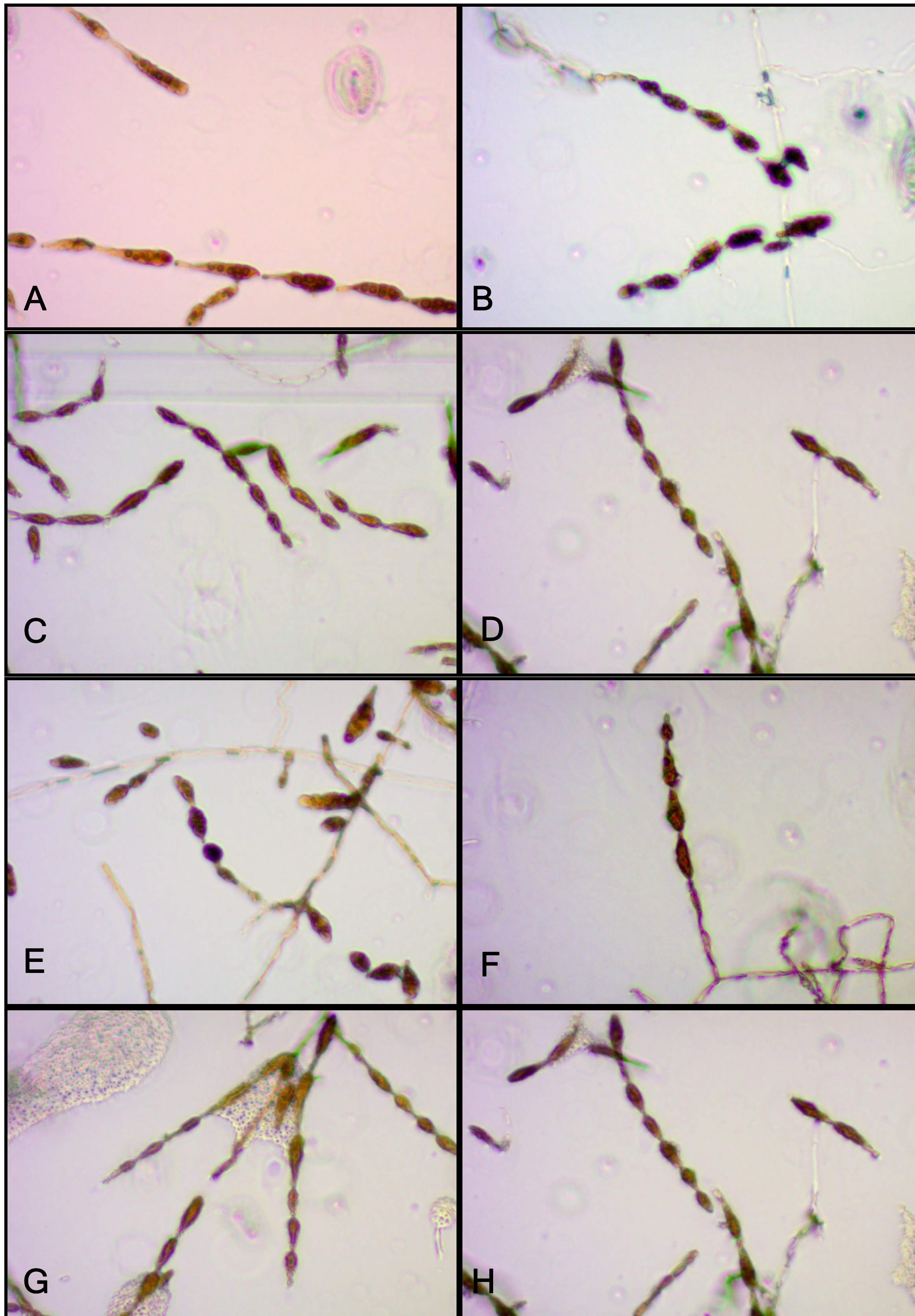

Figure 4.1: Variation in branching of selected *Alternaria* isolates from foliar lesions of potato (*Solanum tuberosum* L.). A= 2G; B= Den1; C=SPD; D=FWN; E=Z21a; F= Z21d; G=Z21h; H=DRS.

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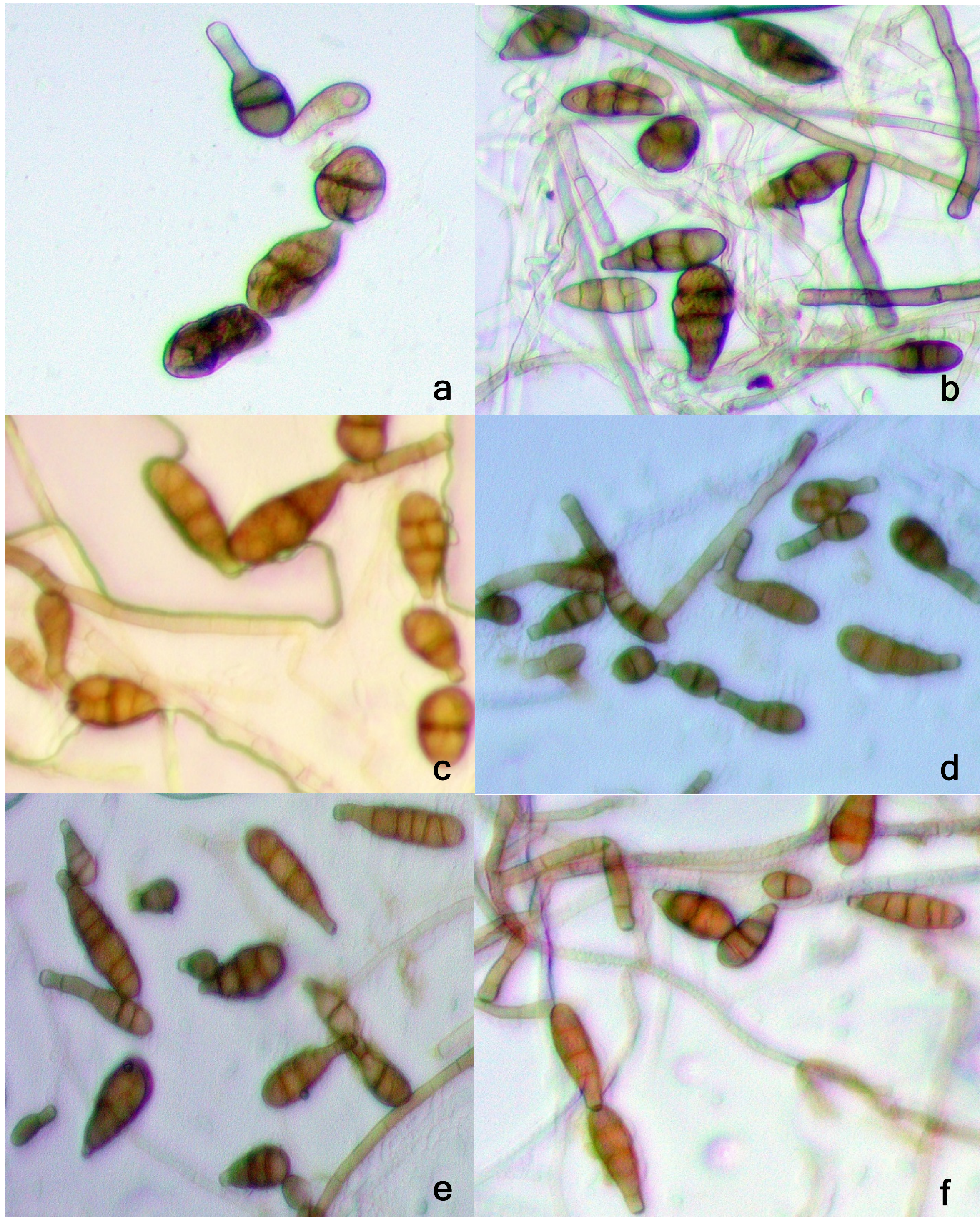

Figure 4.2: Conidia of selected isolates showing longitudinal and transverse septa. a= Z21a; b= Z21h; c= 2G; d= Z21d; e= CS6; f= Clsf 4.

4.3.3 Phylogenetic analyses: PCR of the BT, GAPDH, ITS and TEF1 regions resulted in the amplification of fragments of about 480, 550, 600 and 300 bp in length. Alignment by inserting gaps resulted in a total of 359, 582, 344 and 1285 characters used in the BT,

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GAPDH, TEF1 and combined data set for the GAPDH and TEF1 gene sequences, respectively. All parsimony-uninformative and constant characters were excluded, resulting in 203, 106, 72 and 381 parsimony-informative characters for the BT, GAPDH, TEF1 and combined data set, respectively. Heuristic searches on the data set generated 100 most parsimonious trees and partition homogeneity tests indicated that GAPDH and TEF1 data sets could be combined. The BT data set was excluded from the combined data set because the BT data set did not resolve the relationships amongst the species (data not shown).

Based on analyses of the TEF1 gene, four different sections were observed (Figure 4.3). All 13 *Alternaria* isolates from potato grouped together with *A. alternata*, *Alternaria arborescens, A. gaisen*, *A. limoniasperae* and *Alternaria longipes* to form the *Alternata* section with a bootstrap support value of 100% for both MP and ML. Isolates were also compared to other related genera and these include *Alternaria macrospora* (Zimm.) Nishikado and Oshima, *Alternaria solani* (Sorauer), *Alternaria porri* (Ellis) which formed the *Porri* section; *Nimbya scirpicola* (Fuckel) Sivan, *Nimbya caricis* (E.G. Simmons) Woudenberg and Crous formed the *Nimbya* section; and *Alternaria ethzedia* (E.G. Simmons), *Alternaria conjuncta* (E.G. Simmons) and *Lewia infectoria* (Fuckel) M.E. Barr and E.G. Simmons formed the *Infectoria* section.

Based on the analyses of the GAPDH gene (Figure 4.4); all 13 *Alternaria* isolates from potato grouped together in the *Alternata* section with bootstrap support values of 100% for MP and 98% for ML. Phylogenetic analysis of the BT gene revealed no resolution between the potato and the reference isolates (data not shown). Seven of the *Alternaria* isolates from potato clustered together with *A. solani* and *A. porii*. The other five isolates clustered together and to none of the reference sequences, suggesting that they were a different species. The isolates grouped with none of the small spored reference isolates. Therefore, the BT dataset was excluded from the combined data set. The analyses of the combined GAPDH and TEF1 datasets (Figure 4.5) showed a similar result compared to GAPDH and TEF1 with all the potato isolates grouping together in the *Alternata* section with bootstrap support values of 100% for both MP and ML.

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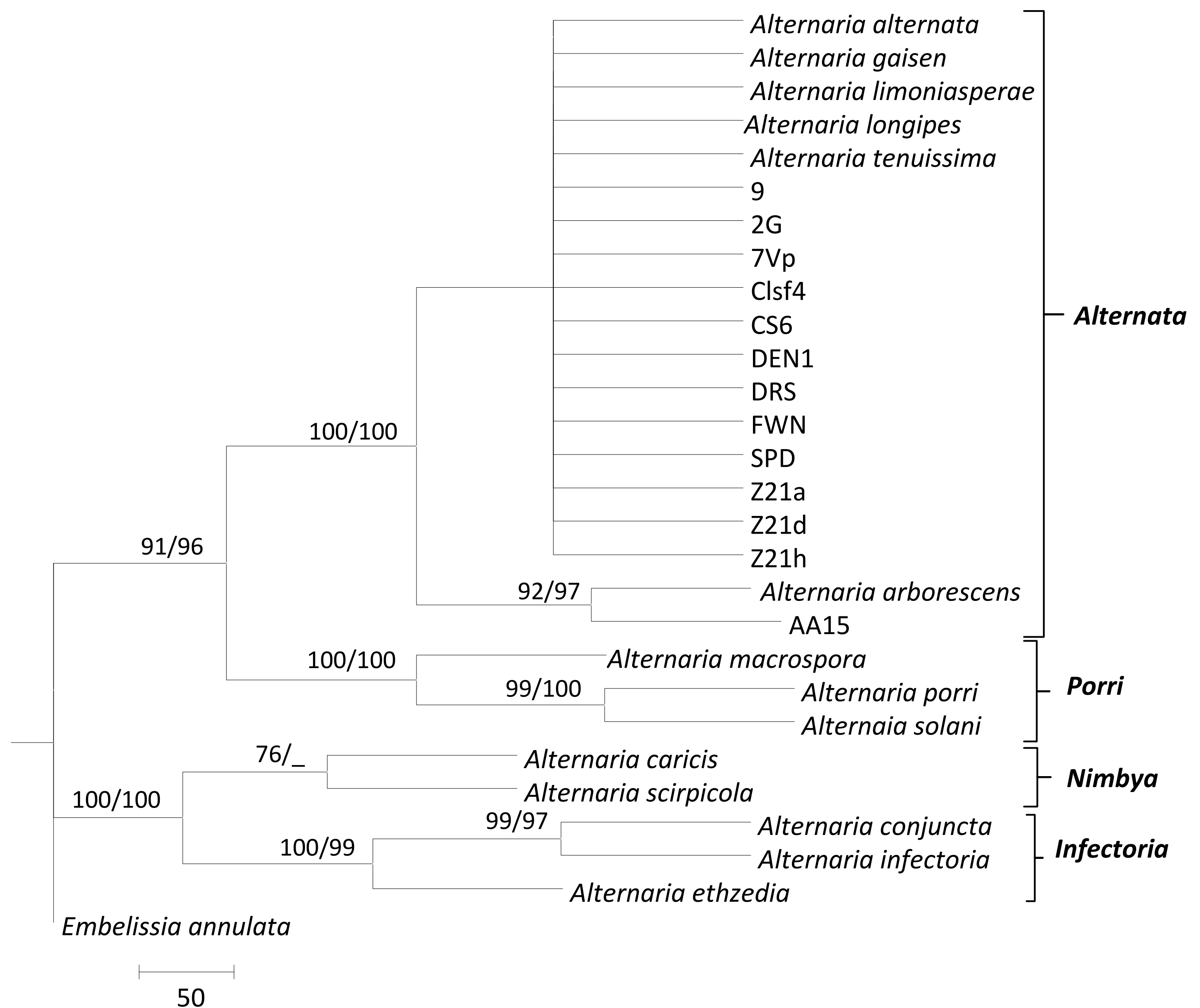

Figure 4.3: Most parsimonious tree based on the TEF1 sequences of 27 *Alternaria* isolates. The most parsimonious (MP) bootstrap support values and maximum likelihood (ML) bootstrap support values are given at the nodes (MP/ML) and values less than 75% are not shown. The tree was rooted to *Embellisia annulata* (CBS 302.84).

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Figure 4.4: Most parsimonious tree based on the GAPDH sequences of 27 *Alternaria* isolates. The most parsimonious (MP) bootstrap support values and maximum likelihood (ML) bootstrap support values are given at the nodes (MP/ML) and values less than 75% are not shown. The tree was rooted to *Embellisia annulata* (CBS 302.84).

The combined parsimonious analysis of both GAPDH and TEF datasets (Figure 4.5) showed all isolates grouping together with *A. alternata*, *A. arborescens, A. gaisen*, *A. limoniasperae* and *A. longipes*, forming one section with a bootstrap value of 100% for both ML and MP. Three other sections; *Nimbya*, *Porri* and *Infectoria*, were formed with reference cultures.

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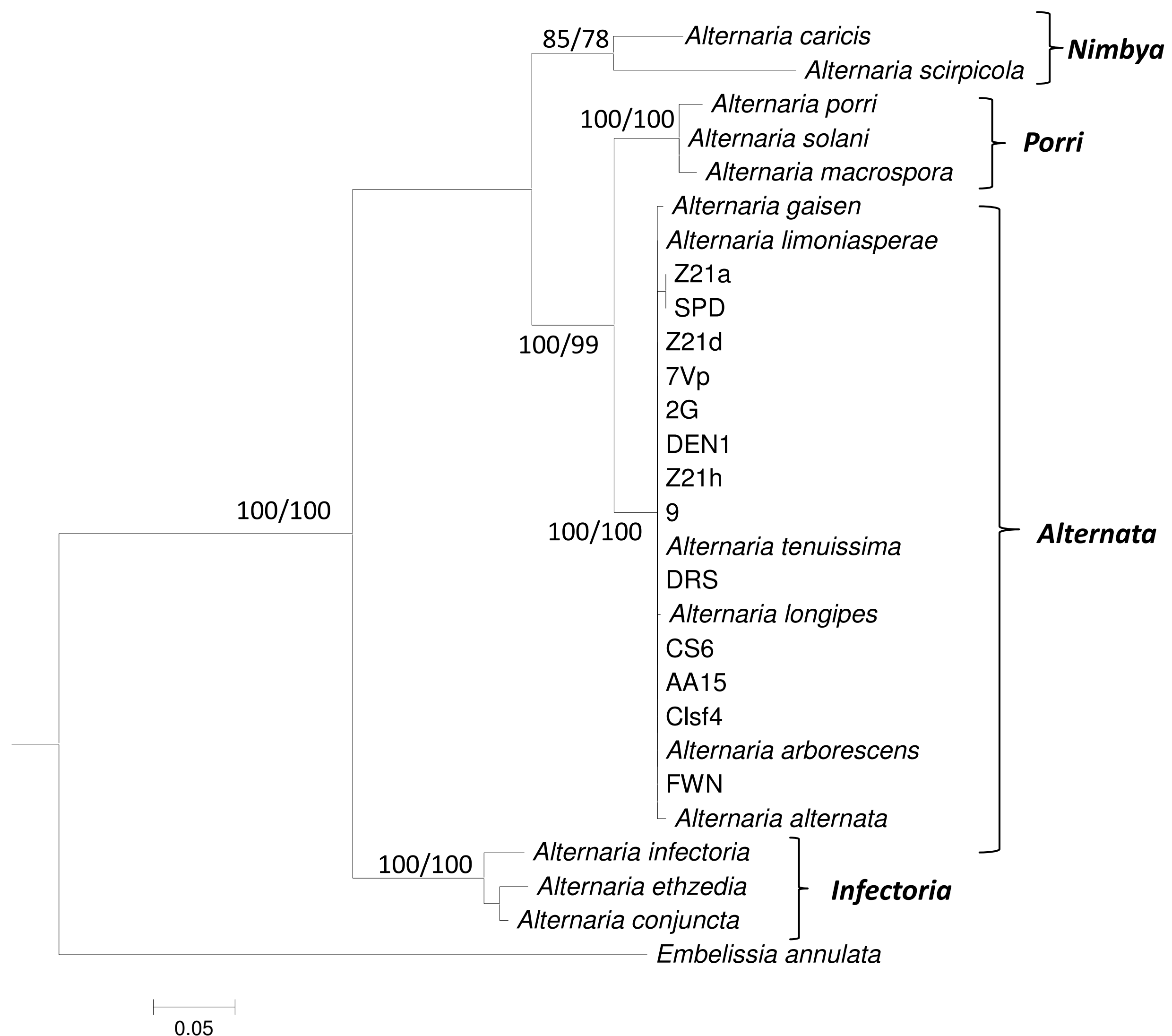

Figure 4.5: The most parsimonious consensus tree based on the combined GAPDH and TEF1 datasets of 27 *Alternaria* isolates. The most parsimonious (MP) bootstrap support values and maximum likelihood (ML) bootstrap support values are given at the nodes (MP/ML) and values less than 75% are not shown. The tree was rooted to *Embellisia annulata* (CBS 302.84).

4.3.4 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP): RFLP based on digestion with enzyme Alu I (Figure 4.6) and Hind III (Figure 4.7) showed no difference in the profiles between the isolates. The same band size and number of digests were observed after gel electrophoresis. Isolates were run against a molecular marker (Hyper-ladder II) to compare size of digests. A negative control was included to check for false positives and

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contamination. A positive control of undigested PCR product was included to compare digested and undigested products and indicate residual PCR products after digestion, if any were present.

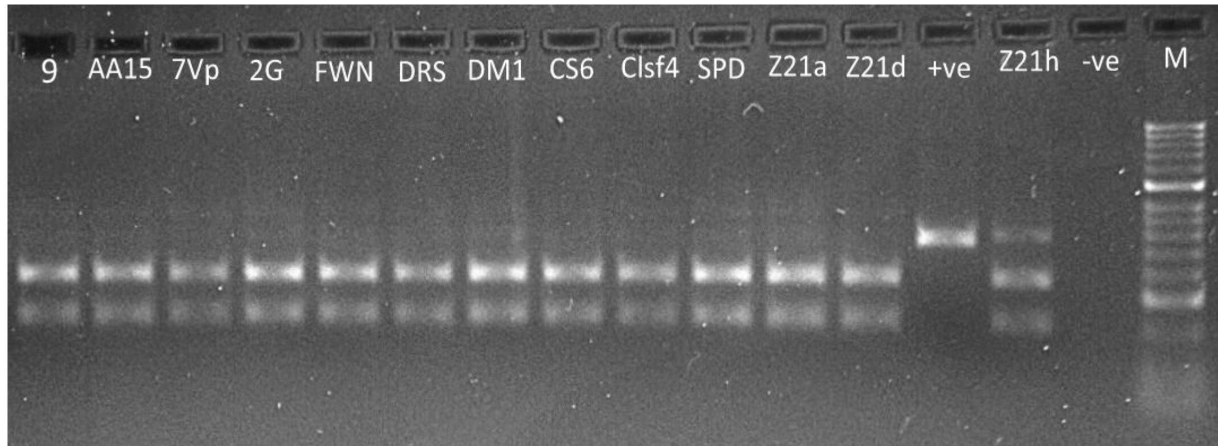

Figure 4.6: PCR-RFLP profile from digestion of the ITS region using restriction enzyme Alu I.

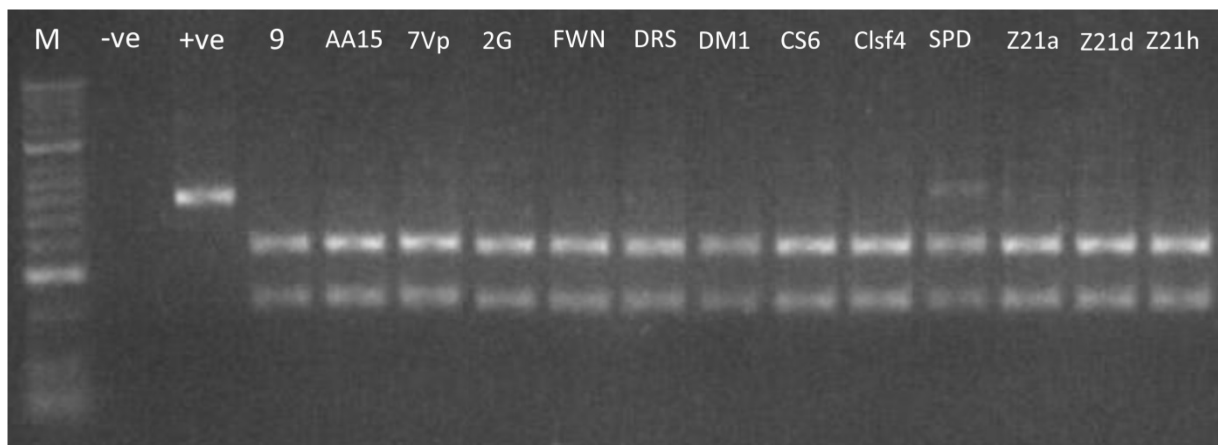

Figure 4.7: PCR-RFLP profile from digestion of the ITS region using restriction enzyme Hind III.

4.4 Discussion and Conclusion

Brown spot and black pit of potato is an emerging disease complex caused by *A. alternata*, a pathogen on over 380 hosts including humans. The success of this pathogen has been attributed to extensive ecological and physiological plasticity (Rotem, 1994). The genus *Alternaria* is of great economic and medical importance; hence correct identification of pathogenic species is of great value to researchers.

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Correct identification of members of the genus *Alternata* has been a great challenge especially among the small spored *Alternaria* (Andrew et al. 2009) and this has been attributed to the great morphological variability among these species (Slavov et al. 2004). This confusion is compounded by the presence of other morphologically similar and closely related pathogens such as *Ulocladium* and *Stemphylium*, which produce morphologically similar conidia (Lawrence et al. 2012).

Inaccurate identification of this genus was further complicated by inability of common house-keeping genes such as the ITS (Guo-yin et al. 2013) and the mitochondrial small subunit (mtSSU) ribosomal DNA to delineate among small spored *Alternaria* as there is a lack of correlation between morphology and molecular phylogeny (Chou and Wu 2002). It has been reported in Basidiomycetes that DNA sequence data from the ITS region are insufficient to distinguish all species, as some taxa could evolve more quickly than mutations could accumulate in their ITS regions (Gardes et al. 1991, Anderson and Stasovski, 1992). Furthermore, the same has also been found in some Ascomycete genera, where sequence data of the BT and histone regions were required to distinguish species in the *Cylindrocladium spathiphylli* and *C. floridanum* species complexes that otherwise appeared similar based on their ITS data sets (Kang et al. 2002). No resolution was obtained with the mitochondrial large subunit (mtLSU) ribosomal DNA, BT, actin, calmodulin, chitin synthase, TEF1 or 1.3.8-trihydroxynaphthalene (THN) reductase gene regions either (Peever et al. 1999; 2004).

One of the aims of this study was to infer phylogenetic relationships among *Alternaria* isolates isolated from potato in South Africa. The GAPDH and TEF1 regions obtained in this study separated all 13 isolates into the *Alternaria* section. This section includes *A. alternata*, *A. arborescens, A. gaisen*, *A. limoniasperae* and *A. longipes* all which were recently classified as belonging to the *A. alternata* section by Woudenberg et al. (2013). Andrew et al. (2009) also supported this grouping when they proposed that *A. alternata, A. arborescens* and *A. tenuissima* be classified as *Alternata* based on a multilocus phylogeny using endopolygalacturonase (endoPG) gene and the anonymous noncoding region, OPA10-2.

This *A. alternata* grouping was supported in both individual and phylogenetic analysis with both GAPDH and TEF1 gene regions. These two gene regions were selected as they were

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proven by Woudenberg et al. (2013) to have strong support to delineate the *Alternaria* spp and other related genera. In their study, Woudenberg et al. (2013) showed that the GAPDH and TEF1 gene regions resulted in Bayesian posterior probability (PP) support of 0.90-0.94 and 0.95-0.99, respectively, when used singly and PP of 1.00 when used in combination..

However; the BT gene was not able to delineate *Alternaria* isolates from potato as it could not distinguish the small spored from the large spored *Alternaria* species. The BT gene was also in contradiction to the findings of Woudenberg et al. (2013), as it delineated small spored *Alternaria* spp. into three different groupings. It also grouped *A. macrospora* with *A. arborescens* and *A. tenuissima*. It is therefore concluded that the β-tubulin gene is not able segregate among members of the genus *Alternaria*.

Morphological analysis of isolates concluded that all isolates belonged to *A. alternata* based on conidial characteristics. The conidia had short, beaks and 2-6 transverse septa and 1-3 longitudinal septa were observed in most of the conidia examined. Conidia were obclavate and muriform (Akhtar et al. 2004; Simmons, 2007) which is typical of *A. alternata*. Molecular characterization delineated all isolates into the *Alternata* section hence they were consistent with morphological characteristics. It is therefore concluded that the GAPDH and TEF1 gene regions used in combination with morphology can be used to delineate and identify *A. alternata* isolates from potato in South Africa as they showed good resolution and support for *A. alternata*.

PCR-RFLP is one of the most common molecular techniques that have been adopted in an approach to establish a consensus between morphological and molecular methods to delineate among small spored *Alternarias* (Pryor and Michailides, 2002). In their studies, Pryor and Michailides (2002) resolved *A. alternata* and *A. tenuissima* groups as a single section with no segregation of morphological types and these were also indicated by Woudenberg et al. (2013) through phylogenetic analysis with three high resolution gene regions. It therefore renders this method reliable to characterize small spored *Alternaria*. In this study a PCR-RFLP digestion of the ITS region with restriction enzymes Alu I and Hind III gave a reproducible digestion with a consistent number of digests per isolate. The RFLP profiles for all isolates were identical and this therefore indicates that the isolates were similar with respect to the ITS region. From this study it was observed that only one species

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of *Alternaria* was recovered from brown spot lesions. However, use of more restriction enzymes should be employed to determine if any variations occur among *Alternaria* isolates, since different enzymes have different restriction sites.

The objectives of this study were to determine the phylogeny of the *Alternaria* isolates isolated from potato in South Africa using sequence data of the BT, TEF1 and GAPDH gene regions of the genome as well as to determine the relationships among morphology and phylogeny that can be useful in characterizing *A. alternata* isolates. From this study it was observed that multilocus phylogeny using GAPDH and TEF1 gene regions was able to identify *Alternaria* isolates from potato as *A. alternata*. Multilocus phylogeny, together with PCR-RFLP was in congruence with morphological characterization; hence these molecular tools can be useful in identification and characterization of *Alternaria* isolates from potato.

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Chapter 5

Reduced sensitivity to QoI fungicides in *Alternaria alternata* isolates from potato in South Africa

Abstract

Alternaria alternata is now world widely known to be the causal agent of Brown spot and black pit. Brown spot is a foliar disease with potential to cause up to 30% yield reduction and black pit is a post-harvest disease with a potential 10% yield loss if left uncontrolled. Control of the brown spot pathogen has mainly been through the use of strobilurins fungicides; however recent disease epidemics of brown spot on potatoes in South Africa led to an investigation of the possible development of reduced sensitivity to strobilurins in the *A. alternata* population. Sensitivity of *A. alternata* isolates was determined *in vitro* for the QoI fungicide, azoxystrobin. Thirteen isolates from brown spot in five potato production regions in South Africa were obtained for sensitivity tests. Eight isolates were confirmed to be *A. alternata* by polymerase chain reaction using species specific primers AAF2 and AAR3. The effective concentration to reduce growth by 50% (EC_{50}) was determined for each isolate. Isolate PPRI 13611 and 13614 had low EC_{50} values of 0.11 and 0.23µg/ml respectively, with a mean of 0.17µg/ml showing their relative sensitivity to azoxystrobin. Isolates PPRI 13610, 13613, 13612, 13609, 13607 and 13608 had EC_{50} values of 51.88, 53.11, 54.57, 58.60, 96.49 and 114.92 µg/ml respectively, with a mean of 71.60 µg/ml, showing their reduced sensitivity to azoxystrobin. Sequence analysis of the partial Cytochrome b gene showed strong correlation of reduced sensitivity in isolates PPRI 13607, 13608, 13609, 13610, 13612 and 13613 to a base substitution from glycine to alanine resulting in an amino acid change at position 143 (G143A). Although reduced sensitivity has been reported on other crops where azoxystrobin has been used to control different pathogens, this is the first report of resistance to azoxystrobin in field isolates of *A. alternata* on potatoes in South Africa. Identification of resistance will help to explain failure to control this disease using strobilurins and further yield losses can be reduced through implementation of anti-resistance strategies.

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Key words: *Alternaria alternata*, azoxystrobin, EC_{50}, QoI, reduced sensitivity, resistance.

5.1 Introduction

Brown spot caused by *Alternaria alternata* (Fr.) Keissl. has become one of the most important fungal foliar diseases on potato worldwide (Nolte, 2008). It causes small circular brown necrotic spots that start on the abaxial side of leaves and are later observed on the adaxial side as the disease progresses (Van der Waals et al. 2011). Under severe conditions, lesions coalesce to form large necrotic areas resulting in premature defoliation (Leiminger and Hausladen, 2008). Elongated, superficial brown or black lesions may also form on stems and petioles (Kirk and Wharton, 2012). Yield reduction is mainly due to premature defoliation and although yield losses are rarely above 20%, if left uncontrolled, the disease can be very destructive (Kirk and Wharton, 2012). Brown spot lesions usually develop on the middle leaves late in the season, however early season brown spot has been observed in stressed and poorly nourished plants (Nolte, 2008). This disease can also occur over a wide range of climatic conditions depending on foliage wetting from rainfall, fog, dew, or irrigation; the nutritional status of foliage and on cultivar susceptibility (Stevenson et al. 2001).

The use of fungicides was one of the most innovative introductions during the green revolution as it allowed for the maintenance of healthy crops and yields of high quality (Brent and Hollomon, 1998). Treatment of crops with fungicides is part of an integrated approach in control of plant diseases (Ma and Michailides, 2005) where they are most commonly applied as protectants from fungal plant pathogens; however, the development of resistance has become a major limiting factor in their efficacy. Resistance is the genetic adjustment by a fungus that results in reduced sensitivity (Damicone and Smith, 2009) and this adjustment has mainly been attributed to the mode of action of the active ingredient. The development of resistance is an inherent problem in crop protection (Benting et al. 2004).

Strobilurins are a broad spectrum class of chemicals important in the management of plant pathogenic fungi (Ma et al. 2003). They belong to the group of chemicals classified as Quinone outside inhibitor (QoI) fungicides (Fungicide Resistance Action Committee [FRAC]

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group 11) (Gisi et al. 2002; Pasche et al. 2005; Grasso et al. 2006). Strobilurins act on a wide range of fungal processes including spore germination, fungal growth, and reproduction (sporulation) (Damicone and Smith, 2009). They act by inhibiting electron transport in the Cytochrome b (cyt b) complex of mitochondria of fungi (Bartlett et al. 2002). QoI fungicides target the cyt b gene located within the mitochondrial bc1 complex III and inhibit the transfer of electrons required for cellular respiration and subsequent ATP production (Anke et al. 1977). Trials with multiple sprays of strobilurins, first registered as azoxystrobin in California showed excellent control of *A. alternata, A. tenuissima* (Kunze) Wiltshire and *A. arborescens* E.G. Simmons; however two to three seasons after its registration there were reports of its failure to control *Alternaria* spp. on almond and pistachbotaio (Luo et al. 2007). There is strong evidence that the exclusive use of strobilurin fungicides contributed to the spread of early blight by shifting *Alternaria solani* Sorauer populations from the wild type to the resistant type (Pasche et al. 2005). Understanding the biology of fungicide resistance, how it develops, and how it can be managed is crucial for ensuring sustainable disease control with fungicides (Brent and Hollomon, 1998). Based on their novel mode of action, strobilurins have a higher risk of resistance development hence raising the need to elucidate mechanisms of resistance.

Field resistance of strobilurins has been reported on different pathogens from different countries that have also used this fungicide (Sierotzki et al. 2000). Jiang et al. (2008) reported resistance to azoxystrobin in *Botrytis cinerea* Pers:Fr. and resistance from field isolates was also reported in *Blumeria graminis* f. sp. *tritici* (DC.) Speer, *Blumeria. graminis* f. sp. h*ordei* (Bartlett et al. 2002), *Didymella bryoniae* (Fuckel) Rehm (Ma and Michelledes, 2005), and *Plasmopara viticola* Berk. and M.A. Curtis (Sierotzki et al. 2000).

In previous studies, QoI resistance was shown to be conferred by point mutations within the cyt b gene (Lesniak et al. 2011). A point mutation at position 143 of the cyt b gene caused by a base substitution and resulting in an amino acid change from guanine to alanine is reported to confer resistance to QoI fungicides (Grasso et al. 2006). In 2012, FRAC published a revised list of pathogens with resistance towards QoI fungicides. Fifteen of the 18 different pathogens were reported to have the G143A mutation in field isolates (FRAC, 2012). Other mutations including F129L and G137R have also been reported and associated with reduced

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sensitivity; however, fungi with these mutations are still controlled with QoI fungicides used at recommended rates (Bartlett et al. 2002). The G143A mutation is currently the most common mutation discovered to date in QoI-resistant pathogens recovered from field surveys (Lesniak et al. 2011).

Continuous and accurate monitoring is necessary to provide information regarding the sensitivity of field populations in certain key crop-pathogen combinations and to track the evolution of resistance genes (Ma et al. 2003). Conventional monitoring methods are based on *in vitro* sensitivity tests (Luo et al. 2007). Bioassays provide essential information on sensitivity to fungicides. However, these methods can be slow and costly, especially for obligate pathogens that cannot be grown on artificial media. DNA-based methods targeted at specific resistance genes offer rapid, cost-effective alternatives (Grasso, 2005). Molecular methods can provide accurate and powerful tools to detect the early appearance of resistant isolates or to follow populations where resistance already exists. The objectives of this study were to screen *A. alternata* isolates collected from different potato production regions of South Africa for sensitivity to a QoI fungicide, azoxystrobin, and to investigate possible mechanisms of azoxystrobin resistance, if any, in these *A. alternata* isolates by comparing the cyt b gene sequences from sensitive and resistant isolates. We hypothesized that there are resistant *A. alternata* isolates in South African potato fields in which azoxystrobin has been used to control early blight and brown spot, and due to the recent increase in brown spot epidemics in many of the potato growing regions in the country (J. E van der Waals, *personal observation*)

5.2 Materials and Methods

5.2.1 Collection, identification and purification of isolates: Samples of infected plant material were collected from five potato producing regions in South Africa. Isolations were made by cutting small pieces (5 mm x 5 mm) from the margin of brown spot lesions, surface disinfesting them in 1% sodium hypochlorite for 5 minutes and rinsing twice in sterile distilled water. The pieces were left to dry and then placed on V8 agar medium (200 ml V8 juice, 3 g $CaCO_3$, 20 g agar) and the plates were incubated at 25°C in darkness for seven days. Conidia from resulting colonies were picked up with a sterile inoculation loop and

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suspended in 500 µl sterile distilled water from which a 20 µl suspension was plated to water agar (WA) plates (20g Agar bacteriological, Biolab, Merck), which were then incubated at 25°C in darkness for 16 h. Using a sterile needle under a light microscope a single germinating spore was picked up and transferred to fresh Potato Dextrose Agar (PDA) plates (Biolab, Merck) and incubated at 25°C in darkness.

Isolates were identified using polymerase chain reaction (PCR) with species specific primers. DNA extraction was performed using Zymo fungal bacterial DNA extraction kit according to manufacturer protocol. Primer pair AAF2 (5'-TGCAATCAGCGTCAGTAACAAAT-3') and AAR3 (5'-ATGGATGCTAGACCTTTGCTGAT-3') (Konstantinova et al. 2002) was used for identification and a PCR was run in a reaction mix containing 19.5 µl Sabax water (Sigma Aldrich), 2.5 µl buffer, 1.25 µl $MgCl_2$, 0.5 µl dNTPs, 0.25 µl of each primer, 0.25 µl MyTaq polymerase (Bioline) and 0.5 µl of template DNA. The reaction was run under the following conditions: initial denaturation at 94°C for 2 min, annealing at 57°C for 30 s, extension at 72°C for 1 min for 35 cycles and a final extension at 72°C for 10 min, followed by holding temperature at 4°C until recovery of the samples. A 5 µl aliqot of the amplification product was separated in a 1% agarose gel by electrophoresis in 1% TAE buffer (40 mM Tris–HCl and 2 mM EDTA pH 8.0) alongside a Hyper-Ladder IV 100bp (Bioline) molecular weight marker and visualized under UV light.

5.2.2 *In vitro* determination of isolate sensitivity to strobilurin fungicides: Sensitivity of *A. alternata* isolates to azoxystrobin was determined using an *in vitro* assay and evaluating relative conidial germination on fungicide amended media using a protocol adopted from Ma et al. (2003). Technical grade azoxystrobin was dissolved in acetone at a concentration of 100 µg/ml. Salicylhydroxamic acid (SHAM) was also dissolved in methanol at a concentration of 100 µg/ml. Water agar was prepared and allowed to cool to 55°C before amending with 0, 1.0, 2.5, 5.0, 10, 25, 50, 75, 85 and 100 µg of azoxystrobin per ml of media. The media was also amended with 100 µg/ml SHAM to inhibit alternative oxidase pathways. A 0.1 ml aliquot of $1x10^4$ conidia/ml of each isolate was transferred to strobilurin amended or control plates by spreading the spores on the plates with a sterile hockey stick. The control plates were amended with SHAM alone. Two plates for each fungicide concentration were used for each isolate. Plates were incubated at 25°C in a completely

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randomised design under darkness for 16 h. One hundred conidia per plate were examined and percentage conidia germinated was recorded. The EC_{50} for each isolate was determined. The experiments were repeated to validate findings and eliminate possible errors.

5.2.3 Statistical analysis: The dose effect of the fungicide on germination and the EC_{50} (the dose that kills 50% of the pathogen) of each isolate was computed using the Probit analysis. The Probit analysis is used for regression modelling of binary response variables (Finney, 1952). This analysis was adopted as it takes into account natural motility and total number of observations and was run at 95% confidence interval. Maximization of the likelihood function was analysed using the Newton-Raphson algorithm. The analysis was computed using XLSTAT (Version 2013.4.02).

5.2.4 Sequence analysis of the partial cyt b gene of *Alternaria* isolates: The cyt b gene was partially sequenced using primers CBF1 (5'-TAT TAT GAG AGA TGT AAA TAA TGG-3') and CBR2 (5'-AAC AAT ATC TTG TCC AAT TCA TGG-3') (Ma et al. 2003) to amplify a 287 bp fragment. Total genomic and mitochondrial DNA was extracted by growing each isolate on PDA at 24°C for 7 days. Mycelia was extracted by scraping it off using a sterile surgical blade and subjected to DNA extraction using fungal/bacterial DNA MiniPrep kit (Inqaba, Zymo Research). A PCR was performed in a 25 µl volume with 50 ng of DNA, 0.5 µl of each primer, 7.5 µl MyTaq buffer and 0.5 µl MyTaq polymerase (Bioline). The PCR program was as follows: Initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 90°C for 40 sec, annealing at 50°C for 40 sec, extension at 72°C for 1.5 min, and for the A-tailing reaction, the elongation time of the last cycle was prolonged to 20 minutes at 72°C. PCR products were run on 2% agarose gel and TAE buffer alongside a Hyper-Ladder IV 100bp (Bioline) molecular weight marker and purified using Sephadex (G50, Sigma Aldrich).

In order to get full length sequences a protocol from Sirchia et al. (2001) was adopted in which PCR products from each isolate were ligated into the pGEM-T Easy vector (Promega) in a reaction containing 3 µl of vector DNA (100 ng), 1 µl insert DNA (17 ng), 1µl Ligase 10X Buffer, 0.5 µl T4 DNA Ligase (Weiss units) and 4.5 µl of nuclease-free water to a final volume of 10 µl. The ligation reaction was set up using pGEM®-T Easy vector and LigaFast™ Rapid

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DNA Ligation System using a PCR product: vector molar ratio of 6:1. The reaction mix was incubated over night at 4°C, centrifuged at 10000 rpm for one minute after which 2µl of the ligation mix was added to 50 µl of *Escherichia coli* (strain DH5α) competent cells and transformed using the electroporation method. Transformation efficiency was tested on nutrient agar plates (Biolab) amended with 0.5 µg/ml of ampicillin, 40 µl of IPTG (isopropyl thiogalactoside) (SIGMA) and 40 µl of Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (SIGMA) that were spread on top of the plates with a hockey stick spreader and allowed to dry for 30 min before plating the transformed cells. White colonies containing the relevant insert were selected for downstream experiments.

Plasmid extraction was performed using a Quiagen plasmid mini kit according to manufacturer's protocol. PCR of the recombinant plasmids confirmed that the PCR products from *A. alternata* isolates had been cloned into the recombinant plasmids. The primers CBF1 and CBR2 were used in PCR analysis. Each sequencing sample included 4 µl of water, 2.5 µl of sequencing dye, 0.5 µl of Big Dye, 1 µl of each primer in separate reactions and 4 µl of the pre-cleaned PCR product. A sequencing PCR was run for 30 cycles under the following conditions: Denaturation at 96°C for 10 s, annealing at 53°C for 5s and extension at 60°C for 4 min. Both strands of the cloned fragments were sequenced using primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') (Youssef et a*l*. 2001). Sequencing was conducted using an ABI 3500xl capillary array genetic analyser.

Results

5.3.1 Collection, identification and purification of isolates: A total of 13 isolates were collected from five potato growing regions. Eight of the 13 were morphologically identified as *A. alternata* and further confirmed using species-specific primers AAF2 and AAR3. The other 5 isolates were morphologically identified as *A. solani* and tested negative when amplified with *A. alternata* specific primers AAF2 and AAR3. A 340bp amplicon was amplified in *A. alternata* isolates (Konstantinova et al. 2002). Isolate 12Vp identified as *A. alternata* in previous studies was used as a positive control in the PCR. The eight *A. alternata* isolates were deposited in the National Collections of Fungi (PPRI collection), Plant

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Protection Research Institute, Agricultural Research Council, Pretoria, South Africa (Table 5.1).

Table 5.1: *Alternaria alternata* isolates used for screening for resistance

PPRI No[1]	**Isolate No**	**Region**	**Variety**
13607	Clsf2	Western Cape, Clanwilliam	Sifra
13608	Clsf4	Western Cape, Clanwilliam	Sifra
13609	CS4	Western Free State, Christiana	Fabula
13610	P2.0	Northern Cape, Vryburg	Darius
13611	P3.1	Loskop valley, Marble Hall	Shepody
13612	P3.2	Loskop valley, Marble Hall	Darius
13613	P3.3	Loskop valley, Marble Hall	Fianna
13614	P4.0	Limpopo, Dendron	Mondial

[1]PPRI: National Collections of Fungi, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa

5.3.2 *In vitro* determination of isolate sensitivity to strobilurin fungicides: Isolates PPRI 13611 and 13614 had great sensitivity to azoxystrobin as maximum inhibition to spore germination was reached at 5 µg/ml and 10 µg/ml respectively. The rest of the isolates had reduced sensitivity to fungicide activity as a highly significant number of spores ($P<0.0001$) germinated under all fungicide concentrations (Figure 5.1).

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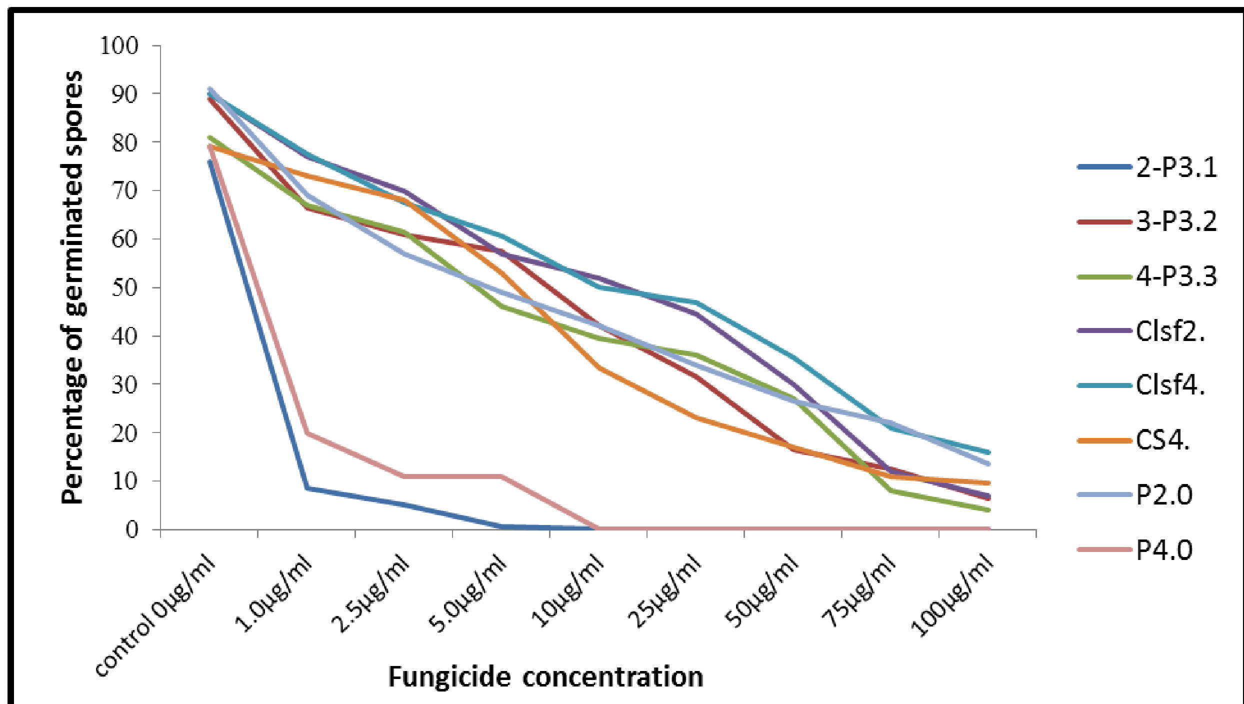

Figure 5.1: Average percentage spore germination of each *A. alternata* isolate at different azoxystrobin concentrations.

The EC_{50} of each isolate was determined by Probit analysis. Isolates PPRI 13611 and 13614 had an EC_{50} of 0.11 and 0.23 µg/ml respectively with a mean of 0.17 µg/ml, showing their relative sensitivity to azoxystrobin. Isolates PPRI 13610, 13613, 13612, 13609, 13607 and 13608 had EC_{50} values of 54.57, 53.11, 96.49, 114.92, 58.60 and 51.88 µg/ml respectively, with a mean of 71.60 µg/ml, showing their reduced sensitivity to azoxystrobin. The Log(Dose) variable and the mortality for all isolates were significantly greater ($P<0.0001$) when compared to the control.

5.3.3 Sequence analysis of the partial cyt b gene from *Alternaria* isolates: A 287 bp fragment was amplified using the primer pair CBF1 and CBR2 in all eight isolates that were tested. Sequences were edited using CLC Main workbench (version 6.7) and aligned in Mega 5.05. Sequences were blasted in GenBank at the National Centre for Biotechnology Information (NCBI). Sequences of isolates PPRI 13607, 13608, 13609, 13610, 13612 and 13613, all with reduced sensitivity to azoxystrobin, showed 100% homology to isolates of *A. arborescens* 37E3 (AY263414.1), *A. tenuissima* 37C3 (AY263412.1), *A. alternata* 37D5 (AY263410.1) and *A. alternata* 37B12 (AY263409.1) all of which were reported to be

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resistant to strobilurins (Ma et al. 2003). Isolates PPRI 13611 and 13614 showed a 100% homology with *A. alternata* (A039), *A. tenuissima* (A068), and *A. arborescens* A067 (AY263413.1) which were reported as sensitive to strobilurins (Ma et al. 2003).

The multisequence alignment of all isolates indicated that isolates PPRI 13607, 13608, 13609, 13610, 13612 and 13613 had a point mutation from wild type GGT (sensitive) to mutant GCT (resistant) at base 227 (Figure 5.2). This point mutation caused an amino acid change from glycine to alanine (Figure 5.3) and this has been confirmed in related studies as the cause of resistance to QoIs in fungal isolates (Ma et al. 2003).

Figure 5.2: Partial sequence of the cytochrome b gene showing the GGT-GCT mutation at 227 bp position.

Figure 5.3: Protein translation of the partial cytochrome b gene showing the G143A mutation.

5.4 Discussion and conclusion

QoI fungicides are known to act mainly on spore germination (Pasche et al. 2004) and therefore an *in vitro* spore germination study was carried out to assess the baseline sensitivity of *A. alternata* isolates. This assessment helped to achieve one of the objectives

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of this study, which was to screen *A. alternata* isolates causing brown spot on potatoes in South Africa for sensitivity to the QoI fungicide azoxystrobin. In this study six out of eight isolates showed reduced sensitivity to azoxystrobin. From the results it could be clearly seen that isolates PPRI 13612 and 1361 are highly sensitive to azoxystrobin, compared to the other isolates. Based on the high EC_{50} values of isolates PPRI 13607, 13608, 13309, 13610, 13612 and 13613, it was established that resistant isolates of *A. alternata* do exist among field isolates collected from potatoes in South Africa. This is the first report of strobilurin resistant field isolates of *A. alternata* from potatoes in South Africa.

Strobilurins are known to have a single site mode of action (Brent and Hollomon, 1996; Pasche et al. 2004, 2005) and in order to determine mechanism of azoxystrobin resistance a comparison was made of the cyt b gene from sensitive and resistant isolates. These results revealed a point mutation in the resistant isolates which was absent in the sensitive isolates. This shift in sensitivity among *A. alternata* isolates therefore explains the significant decrease in control of brown spot across South African potato fields observed during field trips conducted prior to this study.

The introduction of strobilurins in the late 1990s was revolutionary mainly because they are inspired by a group of fungicidally active natural products (Bartlett et al. 2002). Azoxystrobin was the first strobilurin on the market in 1996 and so far seven other strobilurins have been made available on the international market; these include kresoxym-methyl, metominostrobin, trifloxystrobin, picoxystrobin, pyraclostrobin, famoxadone and fenamidone (Von Stackelberg, 2012). In South Africa the first strobilurin, azoxystrobin, was registered for broad spectrum control of plant pathogenic fungi on maize and potatoes in 2001 (Syngenta, 2012). Resistance to strobilurins was reported as soon as 2 years after registration for control of *A. alternata* causing late blight on Pistachio in California (Avenot and Michailides, 2007). In this study on potato, resistance of *A. alternata* to strobilurins is reported 12 years after registration of strobilurins on potatoes in South Africa.

Pathogen biology plays an important role in development of resistance. *A. alternata* is known to produce a large number of spores under favourable conditions (Rotem, 1994) and it has been well established that fungal pathogens that have a high rate of reproduction are

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more prone to developing resistance (Guest and Brown, 1996). Fungi with high reproduction capacity produce many individuals which when exposed to selection pressure have a greater probability of surviving and overcoming fungicidal effects (Damicone and Smith, 2009). *A. alternata* is known to be cosmopolitan, ubiquitous and polycyclic (Rotem, 1994), and these characteristics enable resistant individuals to reproduce rapidly under selection pressure of a fungicide.

The appearance of resistant isolates of *A. alternata* in potato fields brings about the need to put in place resistance management strategies. Strategies such as use of tank mixes and alternating azoxystrobin with fungicides utilizing different modes of action can be implemented (Avenot and Michailides, 2007). An integrated approach to disease management that incorporates both chemical and cultural methods should be put in place. Disease management practices such as crop rotation, use of certified disease-free seed, good nutrient management, suitable planting dates, removing and burning infected plant debris, eradicating weed hosts to help reduce the inoculum levels in subsequent plantings, avoiding irrigation in cool, cloudy weather and timing irrigation to allow plants time to dry before night fall should be adopted (Kirk and Wharton, 2012). With more strobilurins available on the market these anti-resistance strategies need to be implemented when azoxystrobin and other strobilurins are used extensively for the control of *A. alternata* brown spot of potatoes.

Despite the fact that brown spot of potatoes has been observed to cause significant yield losses, the economic importance of this disease has continuously been ignored and *A. alternata* is still viewed as a weak pathogen. In recent disease forecasting studies based on global climatic change, it was shown that the cumulative relative development rate (cRDR) of brown spot is likely to increase in some potato growing regions in South Africa by 2050 (Van der Waals et al. 2013)**.** In our study the number of resistant isolates was more than that of sensitive isolates, therefore continued solitary use of strobilurin fungicides can cause these populations to proliferate further, posing a higher risk to potato production and other crops that this fungicide is registered on.

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Results of this study have shown that azoxystrobin-resistant *A. alternata* isolates are present in the Western Cape and Northern Cape provinces, and Loskop valley (parts of Gauteng, Mpumalanga and Limpopo provinces). However, screening of *Alternaria* populations in all the other potato production regions should be carried out to determine the distribution of resistance in South Africa. The frequency of resistance to QoI in *Alternaria* populations in South Africa is likely to increase as long as strong strobilurin selection pressure is present. The probable mechanism of spatial and geographical spread of resistance of *Alternaria alternata* isolates between different potato production regions in South Africa should be investigated further.

In South Africa azoxystrobin plays a pivotal role in potato production as it is one of the most recommended products, together with other strobilurins, for control of brown spot, early blight and late blight (Syngenta, 2012). However, successive spraying of this fungicide over the years might have increased selection pressure on *Alternaria* populations which has consequently led to resistance. It is therefore imperative that additional management practices be employed to avoid the selection and spread of these resistant isolates.

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Chapter 6

General Conclusion

Potato (*Solanum tuberosum* L.) is one of the most important food crops in the world and is the most consumed vegetable in the world (Soleimani and Mohajer, 2011). In South Africa production is spread over sixteen growing regions and approximately two million tonnes are harvested from approximately 52 000 hectares under potato production (Du Plessis and Van Zyl, 2012).

Brown spot of potatoes is an emerging foliar disease of potatoes in South Africa (Van der Waals et al. 2011) with a potential yield loss of 30% if uncontrolled and postharvest losses of up to 10% due to black pit on tubers (Kirk and Wharton, 2012). Brown spot and black pit of potatoes are caused by *Alternaria alternata* (Fr.) Keissl. (Nolte, 2008; van der Waals et al. 2011) which is also known to cause leaf spots on over 380 other hosts species (Rotem, 1994). Brown spot is becoming a major problem as it is frequently observed in potato fields across the country and failure to control this disease with current fungicides has allowed further spread of this disease.

Alternaria alternata belongs to the small spored species of the genus *Alternaria*, a taxonomically challenging group of fungi with great morphological plasticity in undefined conditions (Pryor and Michailides, 2002) and few molecular characteristics that allow unambiguous discrimination among taxa (Andrew et al. 2009). This, together with presence of morphologically similar genera such as *Stemphylium, Ulocladium*, and *Macrosporium*, complicates the practical identification of members of these genera (Simmons, 1967).

The main objectives of this study were: (i) to investigate effects of different wavelengths of light on radial growth and sporulation of *A. alternata*; (ii) to investigate the role of initial low temperatures on initial sporulation of *A. alternata* causing brown spot of potatoes; (iii) to assess the virulence of the isolates under different wavelengths of light (Chapter 3); (iv)to delineate *Alternaria* isolates isolated from potato in South Africa using sequence data from translation elongation factor 1-alpha (TEF1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene regions; (v) to determine the relationships among morphology and phylogeny that can be useful in characterizing *A. alternata* isolates from

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potato in South Africa (Chapter 4); (vi) to screen *A. alternata* isolates collected from different potato production regions of South Africa for sensitivity to a QoI fungicide, and (vii) to investigate possible mechanisms of resistance to QoI fungicides, if any, in these *A. alternata* isolates by comparing the cytochrome b (cyt *b*) gene sequences from sensitive and resistant isolates (Chapter 5).

After successive fruitless pathogenicity trials done in the summer of 2011 a study was conducted to investigate the morphological and epidemiological characteristics of *A. alternata* causing brown spot of potatoes in South Africa. Variations in colony pigmentation were observed for each isolate when exposed to four different standardized light conditions. Colony pigmentation varied from olive grey (normal pigmentation), dark greyish brown to dark brown with yellow aerial mycelium. These variations may be explained by a photoreceptor system called a mycochrome (Honda, 1969; Kumagai and Oda, 1969) that is involved in photo-morphogenesis. This mycochrome system results in formation of photochromic pigments in fungi (Wagner and Wagner, 1995).

Illumination can alter the morphology of fungi (Vakalounakis and Christias, 1985). Near-UV light plays a role in phototropism, carotenoid synthesis as well as photoresponses associated with reproduction (Vakalounakis and Christias, 1985; 1986), hence isolates grew faster and sporulated more under conditions of near UV light. Light has a profound effect on mycelial growth (Hubbali et al. 2010) and it was observed to have a significant influence in radial growth of some isolates when exposed to different light regimes.

Sporulation is required for dispersal, preservation and genetic variation and it has been directly linked to disease epidemics (Xu and Ridout, 1998). Environmental factors influence the rate at which epidemics arise (Campbell and Madden, 1990). Low temperatures induce conidial initiation (Slavov et al. 2004) and near-UV is involved in photosporogenesis (Vakalounakis and Christias, 1985). Exposure to low temperatures in the dark for 48hrs increased number of spores/mm produced in *Alternaria* for each isolate when compared to those without cold pre-treatment. Conditions of near-UV light promoted greater sporulation and disease severity was greatest in conditions of near-UV in all isolates. However; variations in sporulation were observed among isolates under different conditions.

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Due to their economic and medical impact, correct identification of *Alternaria* spp. would be of great value to researchers and medical mycologists (Woudenberg et al. 2013). Identification is based primarily on morphology (Konstantinova et al. 2002), however; advances in molecular techniques provide an accurate and consistent method of identifying and delineating members of this genus. All thirteen *Alternaria* isolates obtained from infected potato material and used in this study were characterized as *A. alternata* based on morphology. The isolates had dark grey septate mycelia, with mostly single conidiophores. Conidiophores had short chains of conidia ranging from 2-6 conidia with secondary and tertiary branching. Conidia had 1-3 longitudinal and 2-6 transverse septa. Conidia size ranged from 15-44 µm in length and 8-15 µm wide.

Multi-locus phylogeny using the TEF1 and GAPDH gene regions grouped isolates together into the *A. alternata* clade. The gene regions were consistent in characterizing the isolates as they grouped all isolates into the *A. alternata* clade when used in separate and in combined analysis. Combined phylogeny of the GAPDH and TEF1 gene regions gave a bootstrap support value of 100% with *Alternaria gaisen* (Nagana), *Alternaria arborescens* E.G. Simmons and *Alternaria longipes* Ellis and Everh and E.W. Mason which all belongs to the section *Alternata* and is in agreement with results from (Woudenberg *et al*. 2013). PCR-RFLP digestion of internal transcribed spacer regions 1 and 2 and intervening 5.8S nrDNA (ITS) with restriction enzymes AluI and HindIII produced the same number and same sized digestion products showing no genetic variability among isolates with regards to the ITS region.

Molecular identification was in congruence with morphological identification and both techniques showed that all the thirteen isolates from potatoes belong to the *A. alternata* species. The multi-locus phylogenetic analysis with the GAPDH and the TEF1 genes were able to resolve or delineate *Alternaria* isolates recovered from infected potato leaves. PCR-RFLP indicated that no genetic variation existed among isolates with respect to the ITS region. These molecular techniques were useful to group the small spored *Alternaria* isolates.

Disease management forms a key building block in an effective and safe crop management program (Agrios, 1997; Stevenson et al. 2001). Control of brown spot has been mainly

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through use of a strobilurin fungicide, azoxystrobin. Strobilurins have a single site mode of action and therefore fungi are prone to resistance over time (Mueller and Bradley, 2008). Therefore, constant monitoring of fungal populations for reduced sensitivity is of paramount importance (Ma et al. 2003).

Isolates were characterized as sensitive or as having reduced sensitivity based on *in-vitro* tests. *In vitro* sensitivity test on water agar amended with azoxystrobin showed that isolates PPRI 13610, 13613, 13612, 13609, 13607 and 13608 had reduced sensitivity based on high EC_{50} values of 51.88, 53.11, 54.57, 58.60, 96.49 and 114.92 µg of active ingredient/ml of agar respectively. Isolates PPRI 13611 and 13614 were sensitive to azoxystrobin and had low EC_{50} values of 0.11 and 0.23µg/ml respectively. Sequence analysis of the cyt *b* gene revealed a point mutation at position 227bp. Protein translation of the partial cyt *b* gene revealed an amino acid substitution from guanine to cytosine at position 143. The G143A mutation is currently the most common mutation discovered to date in QoI-resistant pathogens recovered from field surveys (Lesniak et al. 2011).

Pathogen biology plays a very important role in development of resistance (Rotem, 1994) and it has been well established that fungal pathogens that have a high rate of reproduction are more prone to developing resistance (Guest and Brown, 1996). *A. alternata* is known to be a cosmopolitan, ubiquitous and polycyclic fungus (Rotem, 1994), and these characteristics enable resistant individuals to reproduce rapidly under selection pressure of a fungicide.

In conclusion, morphological and epidemiological characteristics were investigated in this study and it was observed that variations do occur among *A. alternata* isolates causing brown spot of potatoes. Low temperatures under dark conditions promote greater sporulation among isolates; however, there was no significant difference in sporulation recorded after cold pre-treatment. Conditions of near-UV light promote greater radial growth and sporulation in some *A. alternata* isolates. Pathogenicity greatly differed between isolate as some isolates performed better than others. The combined effect of environment and isolate influenced both radial growth and pathogenicity of *A. alternata* isolates.

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A. alternata was the only pathogen isolated from brown spot lesions from potato. Molecular identification was in congruence with traditional morphological characterization. Multi-locus phylogeny using GAPDH and TEF1 gene regions was able to delineate and accurately identify *Alternaria* isolates from potato. PCR-RFLP is a useful tool to identify genetic variability among *Alternaria* isolates.

Screening of isolates for resistance to azoxystrobin revealed reduced sensitivity among field isolates of *A. alternata*. The G143A mutation is responsible for reduced sensitivity of *A. alternata* to azoxystrobin on potatoes. The emergence and subsequent increase of brown spot of potatoes can be attributed to the presence of resistant isolates that cannot be controlled with azoxystrobin. Future research should focus on the presence of cross resistance of azoxystrobin to other fungicides as well as finding alternative methods to control brown spot of potatoes.

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