Seed-borne fungi of herbs cultivated in South Africa and evaluation of non-chemical seed treatments to control *Alternaria* sp. on coriander

by

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DECLARATION

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

SIGNATURE: ……..

DATE: ……...

SUMMARY

Seed-borne mycoflora associated with eight herb seed species, viz. basil (*Ocimum basilicum* L.), chives (*Allium schoenoprasum* L.), coriander (*Coriandrum sativum* L.), dill (*Anethum graveolens* L.), parsley [*Petroselium crispum* (Mill.) Fuss], sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) and wild rocket [*Diplotaxis tenuifolia* (L.) DC.], and their effects on seed germination were studied. Studies on the pathogenicity of isolated seed-borne *Alternaria tenuissima* (Kunze) Wiltshire were also performed; whereof evaluations of non-chemical methods were conducted to control the aforementioned pathogen.

Seed health tests detected ten genera of fungi associated with herb seed lots, which included *Alternaria*, *Aspergillus, Cladosporium, Curvularia, Epicoccum, Fusarium*, *Penicillium, Rhizoctonia*, *Rhizopus* and *Trichoderma*. It was observed that *Alternaria, Aspergillus, Fusarium* and *Penicillium* were the predominant fungi. This study represents the first record of seed-borne fungi associated with herbs of the Apiaceae and Lamiaceae plant families in South Africa. Findings of seed germination tests showed that all herb seed lots were above their minimum acceptable levels, except for seed lots of dill and wild rocket. In addition, correlation analysis showed that incidence of seed-borne fungi was positively correlated with the number of diseased seedlings raised from the aforementioned seed lots ($r= 0.239$, $p<0.01$). Pathogenicity tests showed that the seed-borne fungus, *A. tenuissima*, was both seed-transmitted and pathogenic on coriander.

In -vitro screening tests to investigate the antifungal effects of plant extracts of *Allium sativum* L, *Carica papaya* L, *Datura stramonium* L, *Lantana camara, Tagetes minuta* and *Zingiber officinale* Roscoe were conducted against pathogenic *A. tenuissima*. Based on the agar infusion method, it was observed that most acetone and ethyl acetate extracts effectively inhibited growth of *A. tenuissima* when applied at low concentrations, which had minimum inhibitory concentration (MIC) values of ≤5 mg/ml. However, most water extracts demonstrated poor antifungal activities; thus, recorded high MIC values (>10 mg/ml). From the antifungal tests using the disc diffusion method, the ethyl acetate extract of *Allium*, acetone extracts of *Datura* and *Zingiber*, and the water extract of *Lantana* were selected for further evaluations in the greenhouse as they demonstrated good antifungal activities against *A. tenuissima*.

Furthermore, a preliminary *in vitro* study was conducted to examine the optimum hot water treatment temperature-time combination that effectively controls *A. tenuissima* associated with coriander seeds. Findings of this study showed that seeds soaked in hot water at 54ºC for 15 mins resulted in a considerable reduction of the incidence of *A. tenuissima* with minimal effect on seed germination. However, soaking coriander seeds at temperatures above 54ºC significantly lowered seed germination.

Naturally infected coriander (cultivar American long) seeds treated with plant extracts of *Allium*, *Datura*, *Lantana* and *Zingiber*; hot water at 54ºC for 15 mins and biological control agents of *Bacillus subtilis* (Ehrenberg) Cohn and *Trichoderma harzianum* Rifai were evaluated for their effects on seed germination, seedling emergence and incidence of *Alternaria* leaf spot disease incited by *A. tenuissima*. This study showed that all seed treatments effectively lowered the incidence of *A. tenuissima* on coriander, which resulted in improvement of percentage seed germination. However, *Datura* extracts negatively affected seed germination as it resulted in higher numbers of abnormal seedlings. Greenhouse experiments showed that sowing treated seeds significantly increased seedling emergence and seedling growth. Thus, seedlings raised from treated seeds were significantly longer and had broader leaf surface area, which contributed to higher seedling fresh and dry mass compared to untreated seeds. The incidence and severity of *Alternaria* leaf spot disease was less pronounced on coriander seedlings raised from coriander seeds treated with extracts of *Allium*, *Zingiber* and the biological control agent, *Bacillus* sp.

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Table of contents

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

a.i= active ingredient

cm= centimetre

CV= Coefficient of Variation

DNA= Deoxyribonucleic acid

EC_{50}= half maximal effective concentration

ISTA= International Seed Testing Association

ISR= Induced systemic resistance

ITS= Internal transcribed spacer

l= litre

LSD= Least Significant Difference

m= metre

mg= milligram

min= minute

mm = millimetre

mM= millimolar

^{o}C = degree Celsius

PCA= Potato Carrot Agar

PCR= Polymerase chain reaction

PDA= Potato dextrose agar

ppm= Parts per million

r = coefficient of correlation

RAPD= Random Amplified Polymorphic DNA

rDNA= ribosomal deoxyribonucleic acid

RFLP= Restriction fragment length polymorphism

rpm= revolutions per minute

rRNA= ribosomal ribonucleic acid

SAS= Statistical Analysis Software

™= Trademark

WP= Wettable powder

α= alpha

β= beta

µg= microgram

µm= micrometre

®= Registered

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

General introduction

1.1 Motivation of study

Apiaceae and Lamiaceae are two important plant families encompassing aromatic and medicinal herbs with a cosmopolitan distribution (Plunkett and Downie, 1999; Harley et al. 2004). Basil (*Ocimum basilicum* L.), dill (*Anethum graveolens* L.), chives (*Allium schoenoprasum* L.), coriander (*Coriandrum sativum* L.), and parsley (*Petroselium crispum* Mill.), which are representative crops within these families, are popularly used in the culinary industry (Hinneburg et al., 2006; Daly et al. 2010). Fresh vegetative parts of different herbs are used in mixed salads, to decorate dishes and are major components of traditional Asian, Mexican, Mediterranean, and Thai dishes (Diederichsen, 1996).

In South Africa, it is estimated that a total of 26.6 million people consume medicinal herbs as they are perceived as cheaper alternatives to inorganic medicine (Mander et al. 2007). Many people are showing preference in consuming herbs as alternatives to inorganic medicine for their healing and health promotion effects (Gilbert and Khokhar, 2008). For this reason, there is a huge demand in the supply of medicinal herbs throughout the world (Uniyal et al., 2002). Even though statistical data of international trade of herbs tracks few plants used in the culinary industry(Purohit and Vyas, 2004), their global market value was conservatively valued at R662.16 billion in the year 2006 and is projected to be R53.4 trillion by 2050 (Sher, 2013). The South African herb industry was valued at a net value of R2.9 billion (Mander et al. 2007).

Despite the increase of economic importance of aromatic and medicinal herbs, global productivity is still minimal to satisfy elevated demands (Singh and Jha, 2008). Therefore, cultivation of herbs has shifted from conservative harvesting from the wild to more intensive commercial scale farming. Although considerable production outputs were reported under such monoculture production systems in Asia, northern America, central Europe and Australia (Mathias, 1994; Ravi et al. 2007); commercial cultivation of herbs has often been associated with outbreaks of previously unidentified diseases. For example, a seedling mortality of approximately 50% on established basil seedlings has been reported due to *Fusarium* wilts (Truemen and Wick, 1996). In another study,

seed damage caused by *Phoma multirostrata* (P. N. Mathur, S. K. Menon & Thirum.) Dorenb. & Boerema reduced 84% of an established coriander crop stand as a result of root decay and discolourations (Hashmi and Ghaffar, 1991). In some of the diseases, infected plants rarely die but the presence of blighted and spotted lesions reduces their commercial value (Garibaldi et al. 2011). Since the vegetative foliar parts represent the greater proportion of economic yield of culinary herbs, any practical means to guarantee yields of cultivated herbs must be highly prioritised (Garibaldi et al. 1997; Gamliel and Yarden, 1998).

Disease management in most herb production regions is done by application of chemical pesticides. Although little research has been done on herbs, farmers depend on unregistered pesticides that have been tested and evaluated on crops other than herbs. Everett and Neilson (1996) showed consistency in the control of leaf spot disease by foliar application of dichlofluanid and propineb. Field trials conducted by Xu et al. (2013) recorded highest reduction of leaf spot disease by applying pyrimethanil (91.52%) followed by mancozeb (82.66%), cyprodinil (77.1%), difenoconazole azole (75.43%), and flusilazole prochloraz (71.03%). Although use of pesticides proved to be reliable in controlling foliar diseases in most of these examples; continued usage of synthetic chemicals is currently being discouraged globally due to problems relating to chemical toxicity coupled with their hazardous effects on the environment (Harris et al. 2001). As more strict policies and laws are being enforced to curb further use of synthetic chemicals, alternative non-chemical methods have been proposed (Stark, 2008; Jacometti et al. 2010).

Planting of resistant cultivars is an alternative conservative non-chemical approach in disease management. However, compared to major crops there are few crop breeding companies that focus their research on improving herb cultivars (Wyenandt et al. 2010). Since breeding programs for the development of pathogen resistance within herbs is limited, disease management can be done by use of plant extracts. Various plant species have been identified as reservoirs of chemotherapeutants (Hostettmann and Wolfender, 1997; Mdee et al. 2009). Organic constituents of various plant metabolites show varying levels of fungistatic or fungicidal effects and are also easily biodegradable (Al-Samarrai et al. 2011; Ownagh et al. 2012; Znini et al. 2013). Research has shown that active components of most plant extracts are non-phytotoxic and exhibit high

antifungal properties against a wide range of leaf spot pathogens such *Alternaria* spp., *Ascochyta* spp. and *Septoria* spp. (Agbenin and Marley, 2006; Guleria and Kumar, 2006; Tegegne et al. 2008; Mishra et al. 2009).

Alternatively, the use of beneficial microorganisms for managing plant diseases has presented many of opportunities. Different microbes have been reported to show varying fungicidal and fungistatic effects against pathogenic fungi by invading and directly parasitizing or mycolysing plant pathogens (Agrios, 2005; Jabaji-Hare and Neate, 2005). Certain beneficial microorganisms are also known to enhance plant growth and development of the host (Howell, 2003; van Wees et al. 2008). Many studies have shown various *Trichoderma* spp. to be highly effective against *Alternaria* spp. (Aghighi et al. 2004; Nallathambi et al. 2009; Gveroska and Ziberoski, 2011; Ambuse et al. 2012). Commercial products consisting of *Trichoderma* spp. as their active ingredients are increasingly being registered under different trade names such as; TrichoFlow WP™ (New Zealand), and Trichoplus (South Africa) (Altintas and Bal, 2008; Gwynn and Maniania, 2010). Likewise, bacterial formulations such as *Bacillus* sp. are now available commercially and registered as Kodiak®, which is effective against *Rhizoctonia solani* J.G. Kühn and *Fusarium* spp. (Brannen and Kenney, 1997) and Serenade used for controlling *Cercospora* spp., *Botrytis cinerea* Pers. and powdery mildew of lettuce (*Lactuca sativa* L.) (Jacobsen et al. 2004; Ongena and Jacques, 2008).

The use of physical control measures as novel approaches for disease management is gaining popularity. Although little work has been reported on herbs, years of research on other vegetable and cereal crops have reported numerous milestone advances in use of hot water treatments in controlling *Alternaria* leaf spot (Fallik, 1996; Hermansen et al. 1999; Nega et al. 2003).

Despite an increase in reports of diseases hampering successful cultivation of herbs (Blodgett and Swart, 2002; McLeod et al. 2006; Sharma et al. 2012; Lee et al. 2013), little research has been done to establish the primary sources and causes of infection of herbs particularly under South African conditions. Herbs are perceived as minor crops and little priority is therefore given for research on improving productivity of cultivated herbs (Gilardi et al. 2013). In this regard, management of most herb diseases is complicated as there are few pesticides registered in South Africa and also due to the risk of presence of residues at harvesting (Leadbeater and Gisi, 2010; Croplife, 2014).

1.2 Aim of the study

The purpose of this study is to examine and identify seed-borne mycoflora associated with commercial herb seeds and to determine whether any of these fungi result in seed transmitted diseases. This research aims to promote local cultivation of herbs, particularly under organic farming and provide non-chemical control of identified seed-borne fungi to reduce the risk of pesticide residues associated with disease control in herbs.

1.3 Hypothesis tested

- There are no seed-borne pathogens associated with commercial herb seeds including basil, chives, coriander, dill, parsley, sage, thyme and wild rocket.

- Use of bio-control agents, botanical extracts, and physical treatments in seed sanitation will not effectively control seed-borne pathogens associated with herb seeds.

1.4 Specific objectives

The project was targeted to achieve the following objectives:

- To examine the mycoflora associated with commercial herb seeds i.e. basil, chives, coriander, dill, parsley, sage, thyme and wild rocket.
- To determine the effect of seed-borne mycoflora on seed germination of commercial herb seeds i.e. basil, chives, coriander, dill, parsley, sage, thyme and wild rocket.
- To investigate pathogenicity of seed-borne *Alternaria tenuissima* associated with seeds of coriander.
- To evaluate the efficacy of alternative, non-chemical, seed treatments in controlling pathogenic *A. tenuissima*. This includes use of bio-control agents, hot water treatments and plant extracts.

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CHAPTER 2

Literature review

According to the Herb Society of America's New Encyclopedia, the term "herb" is a noun describing any small seed bearing plant with fleshy rather than woody parts from which the term "herbaceous" was derived (Deni, 2001). However, based on the definition by Craig (1999), a herb is any plant valued for its flavour, fragrance and medicinal qualities, and pesticidal properties with a wide range of industrial uses.

2.1 Significance of seed-borne microorganisms associated with herbs

A microorganism is described as seed-borne when it occurs on the surface of seed due to contamination or progeny infection (Agarwal and Sinclair, 1997).

The practice of sowing clean, disease-free seed is recommended in order to avoid or evade different microbes from affecting normal seed development and later infections (Agrios, 2005). Several seed-borne microorganisms have been shown to weaken and predispose seeds to future attack as they are more vulnerable to a variety of soil-borne pathogens (Mustafa, 2009). Alternatively, infected seeds often show compromised performance due to varying levels of abnormalities ranging from seed discolouration, necrosis, seed abortion or seed rotting (Coles and Wicks, 2003). Utobo et al. (2011) reported that some seed-borne pathogens may cause seed abortion, seed rot or seed necrosis, which often result in complete failure of seeds to germinate and reduced seedling emergence.

Garibaldi et al. (2004b) mentioned that seed-borne diseases impose a significant constraint on overall yield of cultivated herbs. Previous seed health tests conducted by different workers led to detection of various microorganisms associated with herb seeds. These microorganisms include *Alternaria* spp., *Fusarium* spp., and *Pythium* spp., which negatively affect seed physiological processes and lower or reduce yields (Chiocchetti et al. 1999; Bralewski et al. 2005). A total of 84% coriander (*Coriandrum sativum* L.) plants showed discolouration and decay of roots due to seed damage by *Phoma multirostrata* (P.N. Mathur, S.K. Menon & Thirum.) Dorenb. & Boerema (Hashmi and Ghaffar, 1991). Trueman and Wick (1996) reported losses of close to 50% of basil seedlings due to sowing of *Fusarium* sp. infested seeds.

Besides loss in yields, seed-borne microorganisms have been associated with huge economic losses in international trade. Interception of *Septoria petroselini* Desm associated with parsley seed consignments accounted for devastating economic consequences to several farmers in Harpenden, England (Moore, 1946; 1947). Advances in mycology have facilitated timely detections of high risk microorganisms residing in seeds with the possibility of return, treating, or destruction, or rejection of the whole consignment at the expense of the farmer.

Research has reported dangers of pathogenic seed-borne microorganisms being introduced into new non-diseased regions by use of infected seeds (Gilardi et al. 2013a). In that way, infected seed act as primary inoculum in transmission of diseases to disease-free areas (Agrios, 2005). Basil seeds infected by pathogenic *Fusarium oxysporum* f. sp. *basilici* Tamietti & Matta incited *Fusarium* wilt disease which caused rapid death of seedlings (Chiocchetti et al. 1999). The disease was first recorded in southern European Russia in 1956. The disease was then reported in Italy in 1975, France in 1982, and in the United States of America in 1992 (Vannacci et al. 1999). The widespread occurrence of the disease and its rapid spreading led to etiology investigations by Gamliel et al. (1996) who proved the disease to be seed-borne and also spread by planting infested seeds. Another severe disease, downy mildew of basil, incited by *Peronospora belbahrii* Thines was observed to be transmitted by infected seeds, first recorded in Uganda in 1937 by Hansford, later reported in Switzerland and Italy in 2003 (Belbahouri et al. 2005), then spread and recorded in France (Garibaldi et al. 2004a) as well as South Africa (McLeod et al. 2006).
Herb seeds like any other seeds can be infested by various fungi, bacteria, nematodes, and viruses. In view of that, a summary of records of previous work of seed health tests showing association of various microorganisms on herb seeds is presented hereafter.

2.1.1 Basil (*Ocimum basilicum* L.)

Ocimum basilicum L. (Figure 2.1) belongs to the Labiatae family. Basil leaves and stems are used as a food spice, for flavourings, fragrances and for medicinal purposes (Lee et al. 2005). Information on seed-borne mycoflora associated with basil seed continues to increase with the publication of records of their occurrence (Table 2.1). Gilardi et al. (2013b) assayed 18 basil seed samples from farms affected by the disease in Piedmont during the fall of 2010 for the presence of *Alternaria* spp. Isolations done from basil

experimental lines recorded 1.18% from non-disinfected seeds and 0.43% from disinfected seeds. It was observed that commercial seeds were infested with *Alternaria* spp. isolated at 7.29% and 2.62%, respectively, for non-disinfected and disinfected seed samples. Kuzmanovic and Popovic (2012) showed that basil seed lots were infected with the following fungi *Alternaria* spp*. Aspergillus flavus* Link*, Aspergillus niger* Tiegh*, Cladosporium* spp*., Fusarium oxysporum* Schltdl*, Fusarium proliferatum* (Matsush.) Nirenberg*, Fusarium semitectum* Berk. & Ravenel, *Fusarium solani* (Mart.) Sacc.*, Fusarium* spp*., Fusarium verticillioides (*Sacc.) Nirenberg*, Penicillium* spp., and *Pleospora herbarum* (Pers.) Rabenh*.*

Figure 2.1: Foliage of an *Ocimum basilicum* plant.

(http://www.midsummernightmeadows.com/herbs/the-basil-plant/). Accessed on 29 August 2014.

2.1.2 Coriander (*Coriandrum sativum* L.)

Coriandrum sativum L. (Figure 2.5) is an important herb of the Apiaceae (Umbelliferae) plant family mainly cultivated for production of spices and vegetables (Aćimović et al. 2011). Seed-borne mycoflora associated with *C. sativum* have been investigated by many researchers as shown in Table 2.1. Although investigations conducted by Nagy (1971) showed that *Pseudomonas* sp., the cause of bacterial umbel blight and seed decay was not seed-borne, further research by Taylor and Dudley (1980) proved the pathogen to be both seed-borne and seed-transmitted. In another study, *Pseudomonas syringae* pv*. coriandricola* (*Psc*)*,* was detected on coriander seeds in Germany by Toben and Rudolph (1996). Seed health tests conducted by Dwivedi et al. (2006) recorded a total of 88 seed-borne fungi on coriander seed lots collected from Rajasthan, India, of which *Alternaria alternata* (Fr.) Keissl., *A. flavus*, *Curvularia lunata* (Wakker) Boedijn, *C. pallescens* Boedijn, *Drechslera tetramera* (McKinney) Subram. & B.L. Jain, *Fusarium moniliforme* J. Sheld [now known as *Fusarium verticillioides* (Sacc.) Nirenberg] and *F. oxysporum* were important. Pant (2011) found a total of 23 fungi associated with 30 coriander seed samples making them unfit for human consumption. Amongst the fungi, *Protomyces macrosporus* Unger was isolated in more than 50% seed samples and *A. niger* had the highest incidence. In another study, following the direct plate method recommended by the International Seed Testing Association (ISTA) to isolate fungi, Jayshree (2011) recorded a total of seven *Aspergillus* species associated with coriander seeds which include; *A. niger, A. flavus, Aspergillus fumigatus* Fresen., *Aspergillus ochraceus* Wilh*, Aspergillus terreus* Thom*, Aspergillus sydowi* (Bainier & Sartory) Thom & Church *,* and *Aspergillus versicolor* (Vuill.) Tirab.

2.1.3 Dill (*Anethum graveolens* L.)

Zehtab-salmasi et al. (2006) reported *Anethum graveolens* L. (Figure 2.2) as a herb of the Apiaceae family with leaves and seeds used for flavouring and seasoning. Research work on mycoflora associated with seeds of *A. graveolens* is summarised in Table 2.1. Maude (1996) and Bralewski et al. (2005) found that dill seed samples were predominantly infected by *A. alternata*. In a study to determine influence of seed stalk architecture on fungal mycoflora associated with the dill cultivar Amat, Szopińska and Bralewski (2006) isolated *A. alternata*, *Cladosporium* spp., *Epicoccum purpurascens* Ehrenb*.*, *Gonatobotrys simplex* Corda and *Trichothecium roseum* (Pers.) Link with the

highest frequency. Seed health tests conducted by Szopińska et al. (2008) using the deep-freeze blotter method recorded new incidences of seed-borne fungi; viz. *Alternaria dauci* (J.G. Kühn) J.W. Groves & Skolko, *Alternaria tenuissima* (Kunze) Wiltshire, *Botrytis cinerea* Pers., *Drechslera* spp., *Fusarium* spp., *T. roseum* and *Ulocladium* sp. Significant reduction of seed germination of dill seed lots by various seed-borne fungi were recorded; viz. *A. alternata* (65.5%), *Alternaria radicina* Meier, Drechsler & E.D. Eddy (6.8%), *Fusarium equiseti* (Corda) Sacc. (2.3%), *Fusarium avenaceum* (Fr.) Sacc. (1.8%), *Phoma* sp. (0.8%), and *Stemphylium botryosum* Sacc. (2.0%) (Janas, 2013).

Figure 2.2: Inflorescence of an *Anethum graveolens* plant. (http://upload.wikimedia.org/wikipedia/commons/b/bf/Anethumgraveolens.jpg.) Accessed on 29 August 2014.

2.1.4 Parsley (*Petroselium crispum* Mill.)

Petroselium crispum Mill. (Figure 2.3) is a member of the Apiaceae family widely used as a fresh vegetable. A summary of studies conducted on seed-borne mycoflora of parsley are shown on Table 2.1. Research has shown that *Septoria petroselini* and

Stemphylium spp. are the most common fungi associated with parsley seed lots (Tahvonen, 1978). Koike et al. (2013) isolated pathogenic *Stemphylium vesicarium* from parley seeds; it caused leaf spot disease on inoculated parsley seedlings. Seed health studies by other researchers recorded many other fungal species associated with parsley seeds, these include; *A. alternata, A. radicina*, *F. avenaceum*, *F. oxysporum, Fusarium culmorum* (Wm.G. Sm.) Sacc. and *Phoma anethi* (Pers.) Sacc. (Nawrocki, 2004; Koike et al. 2013).

Figure 2.3: *Petroselium crispum* plant. (http://awaytogarden.com/growing-and-storing-a-year-of-parsley/). Accessed on 29 August 2014.

2.1.5 Sage (*Salvia officinalis* L.)

Salvia officinalis L. (Figure 2.4) is a perennial shrub of the Lamiaceae plant family with greyish leaves bearing blue to purplish flowers (Santos-Gomes and Fernandes-Ferreira, 2001). Slobodan et al. (2006) conducted seed health tests of sage seed samples harvested from commercial fields during the period 2001 to 2005. The results of the

seed health tests performed using the blotter and agar plate methods indicated that a total of thirteen fungal species were isolated from sage seeds, these included; *A. alternata*, *F. oxysporum*, *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas, *Fusarium equiseti*, *A. flavus*, *A. niger*, *Epicoccum purpusescens*, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Chaetomium* spp., *Doratomyces* spp., *Verticillium* spp., *Penicillium* spp. and *Rhizopus* spp.

Figure 2.4: *Salvia officinalis* plant (http://natureforcities.snre.umich.edu/streetside-gardens/plant-library/perennials/east-friesland-sage/). Accessed on 29 August 2014.

Table 2.1: Seed-borne fungi associated with selected herbs

Crop	Seed-borne fungi	Disease	Losses	Selected references
Basil	*Fusarium oxysporum* f. sp. *Basicili*	*Fusarium* wilts and Crown rots	14%	Elmer et al. (1994); Keinath, (1994); Trueman and Wick (1995); Gamliel et al. (1996); Vannacci et al. (1999)
	Fusarium proliferatum, F. solani (*Haematonectria haematococca*), *F. verticillioides* (*Gibberella fujikuroi*), *F. semitectum* (*Fusarium incarnatum*)			Kuzmanovic and Popovic (2012)
	Peronospora lamii	Downy mildew	50%	Farahani-Kofoet et al. (2012)
	Peronospora belbahrii			Garibaldi et al. (2004a)
	Alternaria spp.			Bulajić et al. (2009); Kuzmanovic and Popovic (2012)
	Pleospora herbarum			Kuzmanovic and Popovic (2012)
	Cladosporium spp.			Kuzmanovic and Popovic (2012)
	Aspergillus spp., *A. niger, A. flavus*			Kuzmanovic and Popovic (2012)
	Penicillium spp.			
Coriander	*Protomyces macrosporus*	Stem gall	15%	Gupta (1954); Pant (2011)
	Fusarium oxysporum, F. verticillioides (Gibberella fujikuroi), *F. solani*	Vascular wilts	56%	Trueman et al. (1996); Dwivedi et al. (2006); Pant (2011)
	Pseudomonas syringae pv. *Coriandricola*	Umbel blight		Toben and Rudolph (1996)
	Phoma multirostrata			Hashmi and Ghaffar (1991); Shrivastava and Jain (1992)
	Alternaria alternata, A. dauci	Lowered germination		Iovaishene and Strukchinskas (1992); Dwivedi et al. (2006); Jain and Jain (1995); Shrivastava and Jain (1992); Pant (2011); Jayshree et al. (2011)
	Aspergillus spp.			Priya Rani et al. (1995); Dwivedi et al. (2006); Jayshree et al. (2011)
	Curvularia lunata, C. pallescens (*Pseudocochliobolus pallescens*)			Dwivedi et al. (2006); Pant (2011); Shrivastava and Jain (1992); Jayshree et al. (2011)
	Rhizopus spp.			Pant (2011)
	Verticillium dahnia			
Dill	*Alternaria alternata*	Lowered germination		Maude (1996); Bralewski et al. (2005), Szopińska and Bralewski (2006); Janas (2013)
	Epicoccum purpusescens (*Epicoccum nigrum*)			Szopińska and Bralewski (2006)
	Gonatobotrys simplex (*Melanospora damnosa*)			Szopińska and Bralewski (2006)

	Alternaria dauci and A. tenuissima, A. radicina			Szopińska et al. (2008); Janas (2013)
	Verticillium spp.			Szopińska and Bralewski (2006)
	Fusarium spp.	Lowered germination		Szopińska and Bralewski (2006); Szopińska et al. (2008); Janas (2013)
	Penicillium spp.			Szopińska and Bralewski (2006)
	Rhizopus spp.			Szopińska and Bralewski (2006)
	Stemphylium consortiale (Ulocladium consortiale)*; S. botryosum* (*Pleospora tarda*)	Lowered germination		Szopińska and Bralewski (2006); Szopińska et al. (2008); Janas (2013)
	Botrytis cinerea			Szopińska et al. (2008)
	Drechslera spp.			Szopińska et al. (2008)
	Trichothecium roseum			Szopińska et al. (2008)
	Ulocladium sp.			Szopińska et al. (2008)
	Phoma multirostrata	lowered germination		Szopińska et al. (2008); Janas (2013)
Parsley	*Septoria petroselini*		31%	Moore (1947)
	Stemphylium spp.			Koike et al. (2013)
	Alternaria spp.			Nawrocki (2004); Koike et al. (2013)
	Fusarium spp.			Nawrocki (2004)
	Ulocladium sp.			Koike et al.(2013)

2.2 The Host: *Coriandrum sativum* L.

Based on seed health tests performed by the Seed Science research group (University of Pretoria, South Africa) to investigate the range of seed-borne mycoflora associated with a variety of herb seeds, the highest incidence of microorganisms was detected on coriander seeds. Of these microorganisms, *Alternaria tenuissima* (Kunze) Wiltshire was found to be pathogenic in greenhouse pathogenicity tests. Hence *C. sativum* was selected as the main host plant for this study.

Coriander is a leafy green annual herbaceous salad crop (Ishikawa et al. 2003). Coriander leaves and fruits (seed) have been used from ancient times in traditional medicine and culinary purposes (Diederichsen, 1996). Consumption and use of coriander has increased over the years due to the essential and fatty oil content of the seeds, which make them suitable as raw materials for various industrial applications (Sahib et al. 2013). In the year 2010, the Centre for the Promotion of Imports from developing countries (CBI) reported a large increase in the distribution and marketing of coriander due to their unique aroma and flavour. Present day cultivation of coriander

is widespread; however, commercial production is mainly done in India, central Europe, Morocco and the former Soviet Union (Ravi et al. 2007). A report on the increase of coriander imports in South Africa during the 2008 to 2012 period (Phahlane, 2013) clearly indicated that local production of herbs is still under capacity and therefore unable to meet the increased demand.

2.2.1 History and nomenclature

The name coriander is an ancient French word: *Coriandre* derived from Latin: Coriandrum originating from ancient Greek: *Korianno* (Attokaran, 2011). Coriander is a noun describing cultivation of a buggy smelling plant (Diederichsen, 1996). Botanical classification of the plant is summarized as follows;

Family: Apiaceae (*Umbelliferae)*

Tribe: Coriandreae

Order: Apiales

Genus: *Coriandrum*

Species*:* *Coriandrum sativum* Linn

Synonyms: *Coriandrum majus* Gouan

Coriandrum diversifolium Gilib.

Coriandrum testiculatum Lour.

Coriandrum globosum Salisb.

Coriandrum melphitense Ten. et Guss.

Selinum coriandrum E. H. L. Krause in Sturm, Deutschl.

Common names: Chinese-yuan sui, hu sui

Dutch-koriander

English-coriander, collender, chinese parsley

French-coriandre, persil arabe

German-koriander, wanzendill, schwindelkorn

Greek-koriannon, korion

Italian-coriandolo

Polish-kolendra

Russian-koriandr, koljandra, kinec, kinza, vonju eezel'e, klopovnik

Spanish-coriandro, cilantro, cilandrio

(Diederichsen, 1996)

Historical evidence has shown that coriander is one of the oldest cultivated herbs dating from around 5000 BC (Zohary and Hopf, 1993). Diederichsen (1996) reported coriander to be native to the Near East region, viz. Western Asia, Eastern Mediterranean region and Egypt, from which it was distributed and naturalized in India, China, North America and the rest of the world (Maroufi et al. 2010; Kothari et al. 1989).

Archaeological deposits of coriander seeds left in Egyptian Pharaohic tombs during the 21st Dynasty, between 1091 and 961 BC are enough evidence to support its cultivation as it does not grow as a wild plant in Egypt. In the early 19th century Reinhardt mentioned that primitive trade led to importation of better yielding coriander cultivars from Asia to Egypt (Diederichsen, 1996). Early domestication of the crop was documented in an ancient library of Ashurbanipal, King of Assyria, in 700 BC (Diederichsen, 1996). Ancient religious literature reported on the use of coriander as one of the bitter herbs consumed during the Jewish Passover ritual referred to in the old testament around 1500 BC, where manna was described as being white like a coriander seed (Exodus 16:31).

Today commercial cultivation of coriander is done in Bulgaria, Central Europe, India, Morocco and Russia (Sriti et al. 2009). In general, coriander can be grown as a drought resistant crop under diverse climatic conditions (Maroufi et al. 2010). The crop thrives in sandy silt soils (Carrubba et al. 2001). Cool climatic environments are recommended in the plant's early stages, whilst high temperatures and sunny weather optimizes flowering (Angelini et al. 1997).The minimum temperatures necessary for emergence are 4-6°C, but only at temperatures of 15-17°C will emergence be as early as two weeks (Luk'janov and Reznikov, 1976). Diederichsen (1996) reported that coriander plants grown in greenhouses which received more illumination from artificial light and higher temperatures than plants grown outside had maximum fruiting capacity. This shows that amounts of light as well as high temperatures are paramount variables affecting fruit production.

2.2.2 Botanical description

Coriander is an annual herb that grows in an upright fashion reaching a height of 25 to 50cm at maturity (Figure 2.5a). The plant has a soft hairless and light green stem with several side branches which sub-divide to end with compound umbels (Sahib et al. 2013). At the basal node of each compound umbel is an involucral bract where five smaller umbellates attach (Figure 2.5c). At least three flowers are borne in each

umbellate. The flowers are borne in small umbels, white or very pale pink, asymmetrical, with the petals pointing away from the centre of the umbel longer (5–6 mm) than those pointing towards it (only 1–3 mm long). Upon successful pollination, empty pollen sacs fall off whereas the remaining reproductive structure develops into coriander seed (Diederichsen, 1996).

Seeds are globular shaped and normally pale white to brown in colour with a diameter ranging between 3 to 5 mm and 1.5 to 3 mm depending on the specific variety (Figure 2.5b), viz. *C. sativum var. vulgare* and *C. sativum var. microcarpum,* respectively (Coşkuner and Karababa, 2007). The weight of one thousand seeds range between 8.72 to 9.71 g (Coşkuner and Karababa, 2006). In general, fruit split into two distinct mericarps both with sclerotified pericarp on the outer convex and a well-defined hollow centre filled with a tiny carpophore (an elongated axis raising the stem of the pistil above stamens). On the surface of each mericarp are six longitudinal ridges, straight side ribs on the convex outside, which alternate with five waved main ribs (Sahib et al. 2013). The interior convex is aligned with two longitudinal vittae which contain the essential oil of the ripe fruit (Coşkuner and Karababa, 2007).

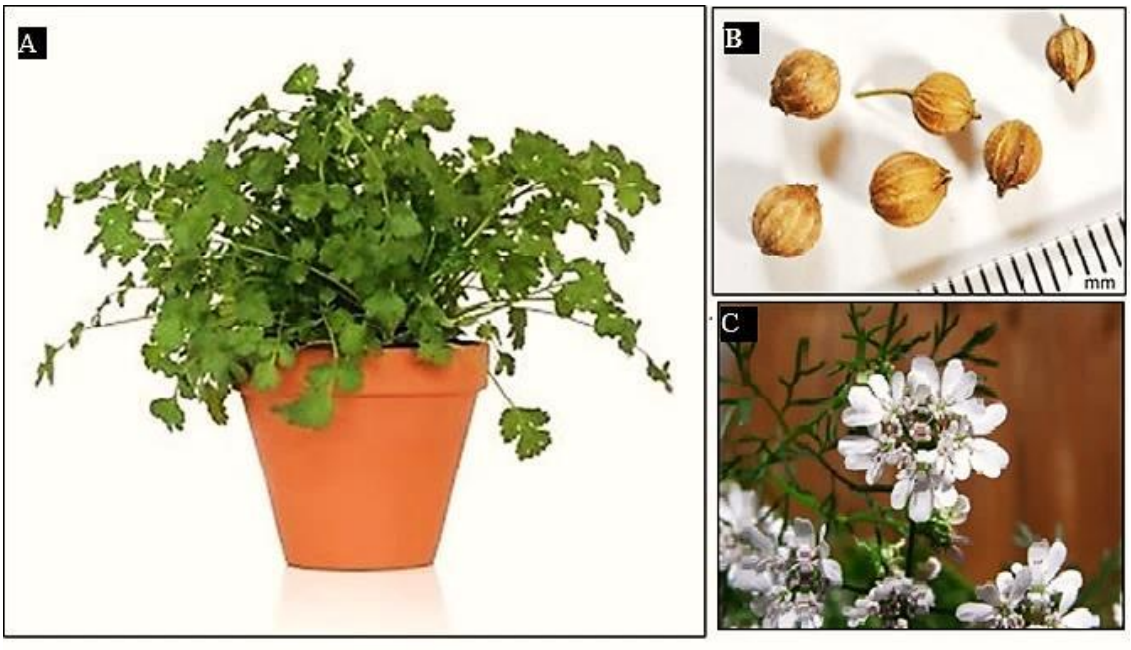

Figure 2.5: *Coriandrum sativum*; (A) Plant (B) Seeds (C) Inflorescence (http://www.guide-to-houseplants.com/growing-cilantro.html; http://commons.wikimedia.org/wiki/File:Coriander_seeds.jpg; http://stephens-views.blogspot.com/herb.html). Accessed on 21 March 2014.

Coriander leaf morphology is highly variable with a unique pinnate characteristic and is green or pale green in colour with a shiny waxy underside. The leaves change to a red

or violet colour in the course of flowering and normally wither before the first fruits are ripe starting from the bottom branches. Leaves on the upper nodes are highly pinnatifid, deeply incised with a clear division, and arranged in a rosette fashion whereas bottom leaves are stalked and more tripinnatifid (Diederichsen, 1996).

2.2.3 Composition and importance of Coriander

Today coriander has a wide variety of applications based on different edible plant parts, viz. green herb and seed. Matasyoh et al. (2009) has shown that the aroma produced by the green plant is due to the aldehydes of the essential oil. A study conducted by Prakash (1990) has shown coriander leaves contain high levels of vitamin C (ascorbic acid, up to 160 mg/100 g), vitamin A (carotin, up to 12 mg/100 g) and vitamin B2 (up to 60 mg/100 g). In Asia, Iran, Iraq and America consumption of fresh green leaves have gained popularity as it has become an ingredient of ethnic cookery of Mexican and Thai dishes, and is used in a variety of soups, salads and chutneys (Norman, 1990; Kamat et al. 2003). Extensive research by numerous workers indicate traditional pharmacological importance of coriander leaves for its anti-diabetic, antidiuretic, antifungal, antioxidant and anthelmintic properties (Bakkali et al. 2005; Shyamala et al. 2005; Jabeen et al. 2009).

Mature coriander seed is the most widely used component of the plant. The rich aroma and flavour of coriander seeds is derived from the high concentration of essential oil deposits in the mericarp (Sahib et al. 2013). Coriander seed contains high levels of petroselinic acid, making it ideal for a wide range of industrial applications (Bhuiyan et al. 2009). In this regard, coriander seeds are important raw materials in the manufacturing of softeners, emulsifiers, detergents, and soaps (Reiter et al. 1998). Oil from coriander seed has been used for flavouring pastry, baking, tobacco products and in the perfumery industry (Bandoni et al. 1998).

Today there is an increasing demand in local and international markets for value added products obtained from coriander (Raju, 1990; Aburjai and Natsheh, 2003). Coriander seeds constitute a major component (25-40%) in curry powder (Wangensteen et al. 2004) and are extensively used as condiments in preparation of pickling spices, sausages and seasonings. Coriander has been used in manufacturing of alcoholic beverages as its addition in beer increases its inebriating effects. In addition, coriander

seeds are primarily used in modern medicine as a flavouring agent to reduce the irritating effects of other medicines (Ravi et al. 2007).

2.3 The pathogen: *Alternaria tenuissima*

Alternaria tenuissima (Kunze) Wiltshire is a cosmopolitan hyphomycete that belongs to the genus *Alternaria* Nees (Reddy et al. 2002). The small-spored fungus is ubiquitous in nature surviving as a secondary pathogen or saprophyte on several host plants (Andersen et al. 1996; Pyror and Michailides, 2002; Honda et al. 2001). Depending on the nature of host association, phytopathogenic *A. tenuissima* penetrates and incites an array of abnormalities on leaves, stems, buds and pods, which may include blights, spots, premature leaf defoliation, and/or staining of fruits (Shortt et al. 1982; Rahman et al. 2002).

Due to a scarcity of concise identification of *Alternaria* in the literature, there has been vast confusion over the years in distinguishing most small-spored species of the *Alternaria* genus (Pryor and Gilbertson, 2002). As such, attempts in systematics and taxonomy often lacked clarity resulting in inaccurate naming and identification of some *Alternaria* species (Simmons and Roberts, 1993).

In the last decade, advancement and standardisation of morphological and molecular methods has helped to solve the confusion in systematics and taxonomy of *Alternaria* spp. (Pryor and Michailides, 2002). At present, reports that are correctly identifying *A. tenuissima* associated with different hosts have increased (Serdani et al. 2002; Uzabakiriho et al. 2013). Despite an increase of reports of *A. tenuissima* on various hosts, no studies have shown its association with cultivated herbs.

2.3.1 History and nomenclature

Kingdom:	Fungi
Subkingdom:	Eumycotera
Phylum:	Ascomycota
Class:	Dothideomycetes
Subclass:	Pleosporomycetidae
Order:	Pleosporales
Family:	Pleosporaceae

Genus:	*Alternaria*
Species*:*	*A. tenuissima* (Fries) Wiltshire (1933)

In 1816, Nees originally described the *Alternaria* genus consisting of *A. alternata* as the only species (Rotem, 1994). Further studies led to the conclusion that *A. alternata* is a representative species complex that consists of several heterogeneous small-spored and catenulate species-groups of *A. alternata*, *A. tenuissima* and *A. arborescens* E.G. Simmons (Pryor and Michailides, 2002). Extensive convergent characteristics of conidia of various *Alternaria* spp. confused many mycologists working on its systematics as they found Nees' morphological identification notes to be ambiguous (Simmons, 1999). Therefore, small-spored *Alternaria* spp. are often misidentified (Ray et al. 2005). Work conducted by Fries in 1882 synonymized both *A. alternata* and *Torula alternata* Fr. giving rise to the genus *Macrosporium*. Description of phaeodictyosporic hyphomycetes by Wallroth in 1883 further intensified the confusion in the classification of the genus as two genera were added, *Stemphylium* and *Ulocladium,* respectively (Woundenber et al. 2013). A combination of work conducted by Rotem (1994) and Simmons (1994) led to introduction of the catenation method in classification of the genus *Alternaria,* which subdivided the genus into three groups, viz. longicatenatae, brevicatenatae and noncatenatae (Chuo and Wu, 2002). According to Rotem (1994), *A. tenuissima* is categorised under the brevicatenatae.

Simmons (1997) introduced a standardised protocol that is now used by many mycologists for distinguishing the small-spored species of the *Alternaria* genus. The basis of proper identification is on a given set of specific growing conditions, viz. a particular growth medium exposed at a specified light intensity, temperature and a defined time period which all have an overall effect on three dimensional sporulation patterns of cultured fungi. By adopting these specific parameters, work of several mycologists have successfully sub-divided the *Alternaria* genus into 14 sub-generic groups (Roberts et al. 2000; Andersen et al. 2001, 2002). In this method, the species group name is derived from a single representative species, for instance, the *Brassicicola* group, the *Infectoria*, the *Porri* group etc. (Pryor and Gilbertson, 2000; Simmons, 1992). Although this method has not resolved the confusion at the species level, it has been successful in organizing the various *Alternaria* spp. at the sub-generic level.

2.3.2 Cultural and morphological characteristics of *A. tenuissima*

It was observed that small-spored species of the genus *Alternaria*, when cultured under a defined, specified, set of growth conditions will yield a unique three dimensional sporulation pattern (Simmons, 1999). Andersen et al. (2002) identified very close similarities in sporulation pattern for *Alternaria arborescens*, *A. infectoria* E.G. Simmons and *A. tenuissima* when cultured on potato carrot agar (PCA) and examined at a magnification of X50 (Figure. 2.6). *Alternaria infectoria* cultures produce long, intercalary secondary conidiophores, often with several conidiogenous loci, resulting in a complex branching pattern (Figure 2.6A). Primary conidiophores of *A. infectoria* isolates are usually short and produced in clumps on PCA giving the cultures a granular look to the naked eye. *Alternaria arborescens* isolates are characterised by long primary conidiophores, which produce conidia in branched chains (Figure. 2.6B) (Andersen et al. 2002). Pryor and Michailides (2002) showed that when *A. tenuissima* is cultured on PCA at 22^{o}C under a 10/14 hour dark and cool-white fluorescent light it has an olive grey cottony appearance with a very faint thin (1-2 mm) white margin as shown in Figure 2.7A. When the cultures are exposed to light at a close proximity, sporulation occurs in rings of solitary chains of up to 12 conidia on branching hyphae.

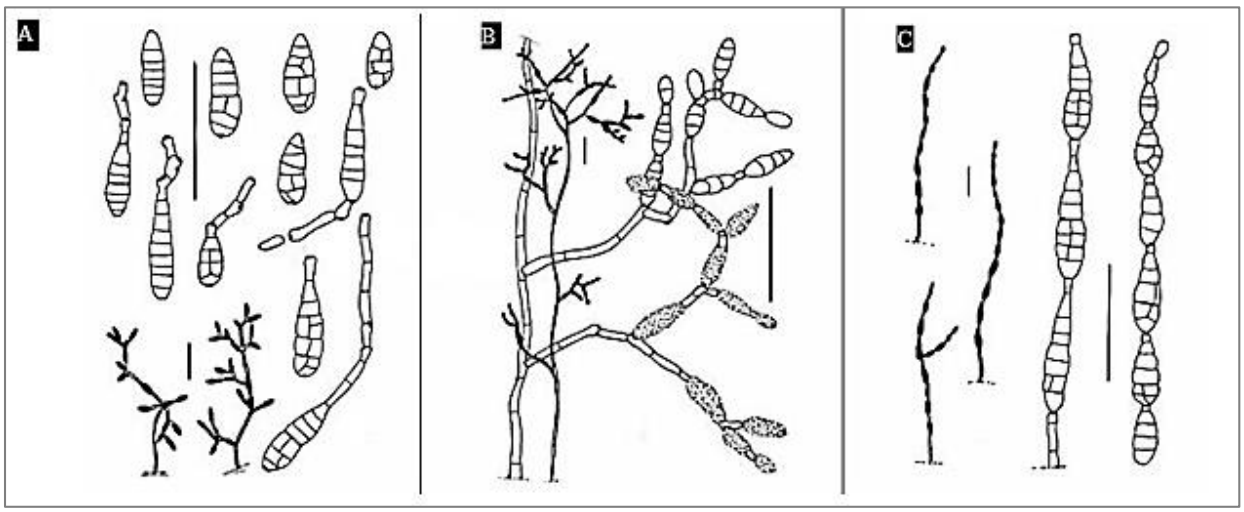

Figure 2.6: Sporulation patterns of enlarged representative conidia; (A) *Alternaria infectoria*, (B) *A. arborescens* and (C) *A. tenuissima.* Bars=50 µm (Andersen et al. 2002).

However, when grown in the dark, colonies are more branched with tufted development of hyphae bearing simple branching conidia chains of moderate length (Simmons and

Roberts, 1993). Cultures grown for more than 7 days under fluorescent light will show longer chains and an unfamiliar branching whereas light-deprived cultures display an aerial development of long, sub-erect hyphae with lateral branches, each of which produces a short chain of a few conidia. The conidia are medium golden brown in colour (Simmons, 1994).

Alternaria tenuissima cultures grown on V-8 agar show a close resemblance with those grown on PCA; however, have densely crowded spores when exposed to light (Figure 2.7B). After 5-7 days of incubation both cultures, growing on PCA and V8, measure 5 cm diameter and when observed under 50X magnification, 2-3 pairs of poorly defined concentric rings of growth and sporulation can be seen.

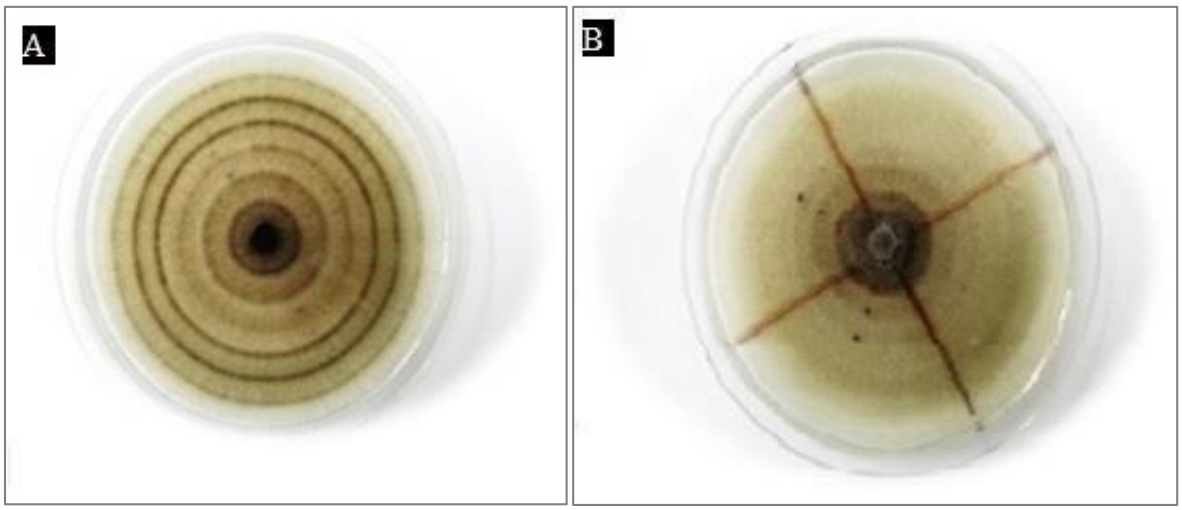

Figure 2.7: *A. tenuissima* when cultured on; (A) PCA (B) V8 agar. (Source: Mangwende, E. 2014).

Additional differences in sporulation are normally observed when *A. tenuissima* is cultured on V-8 agar (Simmons, 2007). After 24 hours, the first 2-3 conidia chains of *A. tenuissima* will show a distinct narrowly tapered upper half with transverse septa as the only dominant feature and spores seldom exceed 50 x 8 µm (Figure 2.8). Extension of the incubation period increases distal addition of secondary conidia resulting in long tapered beaked spore bodies. In most cases, the long-taper aspect decreases, and most new conidia in a chain terminate abruptly in a short 1-2-celled secondary conidiophore. In general, light-exposed cultures are characterized by rings of colonies dominated, at 4 days after incubation, by unbranched chains of 6-10 conidia (Figure 2.6C). Branching is never from the apex as in *A. arborescens*; however, if it occurs the short simple secondary conidiophores would usually have originated from the conidial

body. Terminal conidia of a fully developed chain usually have transverse septa; only one or two mature conidia in a chain have the helpfully diagnostic median, sub-constricting transverse septum that is such a striking feature of field conidia. These median-constricted conidia may be ovoid with a short apical secondary conidiophore or obclavate and broadly tapered into an apical conidiophore. Conspicuous constriction at the median transeptum is present in *ca* 10% of conidia (Ellis and Ellis, 1997).

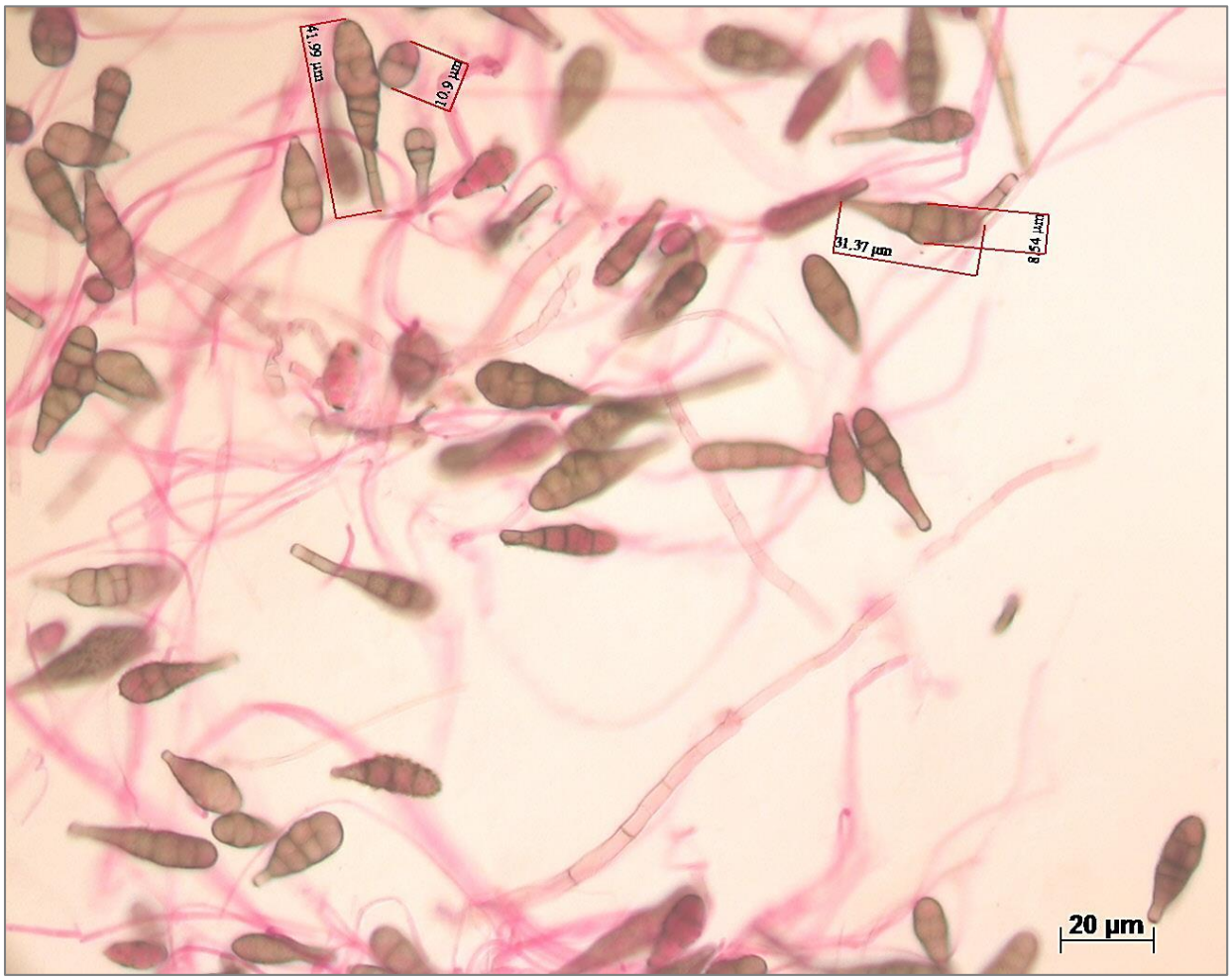

Figure 2.8: Conidia morphology of *Alternaria tenuissima*: image captured at X40 magnification. (Source: Mangwende, E. 2014).

2.3.3 Molecular characterization of *A. tenuissima*

Due to the strong resemblance of different *Alternaria* spp. making it difficult to distinguish using morphological methods, a number of mycologists are now resorting to the use of advanced cytogenetic and molecular markers for identification. Progression of technology in the last decade has facilitated perfection in identification of *A. tenuissima*

with more clarity from many other small-spored species of the genus *Alternaria* (Ray et al. 2005).

Results from RAPD fragment analysis combined with cluster analyses show high species or species-group specificity (Cooke et al. 1998; Peever et al. 1999; Roberts et al. 2000). *Alternaria tenuissima* was classified under the sporulation Group 5 among six taxonomically distinct clades of *Alternaria* pathotypes represented on the dendogram when analysed by random amplified polymorphic DNA (RAPD) (Roberts et al. 2000). DNA sequence analysis of internal transcribed spacer region (ITS) 1 and 2 of the nuclear-encoded rRNA gene (rDNA), Serdani et al. (2002) identified *A. tenuissima* as the major pathogen that caused core rot disease of apples in South Africa. Use of ITS 1 and 4 by Bensassi et al. (2009) successfully distinguished three closely related *Alternaria* species; *A. alternata, A. tenuissima* and *Alternaria japonica* Yoshii.

However, cluster analysis of the RAPD and PCR-RFLP fragment patterns did not show clear distinctions between *A. tenuissima* and *A. alternata* (Pryor and Michaliades, 2002). In addition, sequencing or parsimony analysis of nuclear rDNA, ITS region and β-tubulin genes do not seem to resolve morphologically similar or closely related species or genera (Kusaba and Tsuge, 1995; Pryor and Gilbertson 2000). Pryor and Michaliades (2002) showed that the ITS region failed to bring clarity in the identification of a mixture of small-spored *Alternaria* species as it resulted in one complex monophyletic clade that consisted of three different species groups.

Work conducted by Andrew et al. (2009) has shown potential use of the endopolygalacturonase (endoPG) gene and *Alternaria* allergen a1 (Alta1) in delineating closely related species within the *A. alternata*- complex. Harteveld et al. (2013) rejected the hypothesis that *A. alternata* was the only pathogen that incited leaf blotch and fruit spot diseases of apple with isolation of *A. tenuissima* and other small-spored *Alternaria* species.

2.3.4 Metabolic profiling in systematics of *A. tenuissima*

As a complimentary method, classification and identification of small-spored *Alternaria* species can be done by profiling secondary exudates. Studies by Andersen (1995) and Andersen and Thourane (1996) showed that metabolites secreted by different *Alternaria* spp. cultured on standardised media can be profiled into distinct morphological species-

groups identified as *A*. *infectoria* species-group, the *A*. *arborescens* species-group and the *A. tenuissima* species-group as shown in Table 2.2. Both *A. tenuissima* and *A. arborescens* produced tenuazonic acid. Although *A*. *infectoria* species produced - unknown metabolites that were not found in *A*. *alternata*, *A*. *arborescens* species group or *A*. *tenuissima* species-group isolates; *A*. *infectoria* species did not produce alternariol, alternariol monomethyl ether, or any of the other known metabolites (altenuene, altertoxin I, tentoxin or tenuazonic acid). All *Alternaria* spp. except for *A*. *arborescens* species-group isolates (91-149 and ST11- 57b) and two *A*. *tenuissima* species-group isolates (91-77 and ST11-47a) consistently produced alternariol and alternariol monomethyl ether (Andersen and Thourane, 1996). Altenuene and altertoxin I were produced consistently by all *A*. *alternata isolates*, whereas 53 out of 56 *A*. *tenuissima* species-group isolates produced altertoxin I. Altenuene was produced by 16 *A*. *arborescens* species and by 12 *A*. *tenuissima* species and these isolates are located either in the bottom *A*. *arborescens* cluster or in the two uppermost clusters of the dendogram (not shown). Production of tenuazonic acid is common in both *A*. *arborescens* and *A*. *tenuissima*. Fourteen isolates in the bottom sub-cluster in the *A*. *arborescens* cluster did not produced tenuazonic acid and neither did the two *A*. *arborescens* species-group isolates in the top cluster.

Table 2.2: Secondary metabolites produced by *Alternaria* spp. (Andersen et al. 2002)

	Species groups (number of isolates)			
Metabolite* (RI value)	*A. infectoria* (35)	*A. arborescens* (59)	*A. tenuissima* (56)	*A. alternata* (3)
Altenuene (RI: 809)	0	16	12	3
AOH (RI: 868)	0	57	55	3
AME (RI: 1014)	0	58	54	3
Altertoxin I (RI: 856)	0	16	53	3
Tentoxin (RI: 894)	0	2	15	0
TeA (RI: 808)	0	42	49	0
Unknown A (RI: 652)a	0	11	5	3
Unknown B (RI: 714)a	0	0	4	0
Unknown 2 (RI: 839)b	33	0	0	0
Unknown X (RI: 907)	28	0	0	0
Unknown Y (RI: 941)	0	2	53	0
Unknown C (RI: 1425)a	0	55	35	0

* AOH, alternariol; AME, alternariol monomethyl ether; TeA, tenuazonic acid.
a Unknown metabolites used in Andersen et al. (2001).
b Unknown metabolite used in Andersen and Thrane (1996).

2.3.5 Epidemiology and host range of *A. tenuissima*

Alternaria tenuissima has been reported on different hosts in many geographical regions of the world (Gannibal et al. 2007). Many researchers have detected occurrence of *A. tenuissima* on different plant hosts including: apple fruits (*Malus domestica* Borkh.) in South Africa (Serdani et al. 2002), broad bean leaves (*Vicia faba*) in Japan (Honda et al. 2001), cereal grains in North Europe (Andersen et al. 1996; Kosiak et al. 2004), English walnut (*Juglans regia* L.) and hazelnut (*Corylus avellana* L.) (Belisario et al. 2004), pistachio leaves (*Pistacia vera* L.) in the USA (Pryor and Michailides, 2002) and strawberry fruit (*Fragaria ananassa* Duch.) in Korea (Lee et al. 2001). *Alternaria tenuissima* is a common pathogen of thistle leaves (*Silybum marianum* L.) in Russia (Gannibal et al. 2007), blueberry (*Vaccinium cyanococcus* L.) and pepper (*Capsicum annuum* L.) in China (Luan et al. 2007; Li et al. 2011), respectively. More recently, *A. tenuissima* was isolated from morama beans [*Tylosema esculentum* (Burch) L. Schreib.] grown in the Southern Kalahari region (Uzabakiriho et al. 2013) and pigeon pea [*Cajanus cajan* (L.) Millsp.] in India (Balai and Singh, 2013).

Pathogenic *A. tenuissima* may only infect and cause disease symptoms provided that the host is susceptible and the prevailing environmental conditions are favourable. As

shown in Figure 2.9, *Alternaria* spp. are mainly transmitted through infected seeds or dead and dying plant parts from which overwintering mycelia or spores are dispersed by the wind or human activity (Agrios, 2005). Gannibal et al. (2007) reported consistent detection of hundreds of conidia from *Alternaria* spp. in 1 m^3 of air sampled 15 m above ground in Europe, USA and Australia.

When the conidium has landed on a susceptible plant surface, it starts germinating and directly penetrates the host tissues. Following a successful penetration, fungal mycelia will infect susceptible cells and tissues of the host procuring nutrients from them (Agrios, 2005). It has been reported that stressed and/or old plant tissues are more susceptible to infection particularly at a temperature range of 15 to 25ºC following a 12 hour dew period (Reis et al. 2006). Mycelia of pathogenic fungi may continue to grow between the cells until most of the plant tissues are colonised to produce visible lesions or necrosis of the whole affected organ (Figure 2.9). The rate of tissue invasion by mycelia was reported to be reduced under refrigerated storage conditions (Oprea et al. 1985). Once the mycelium has colonised the whole tissue, new conidia are produced for further dispersal. When humidity is high, above 72%, preferably with poor circulation of air, *Alternaria* spp. normally takes three days to start producing new conidia which are further dispersed by rain splash or wind (Rotem, 1994).

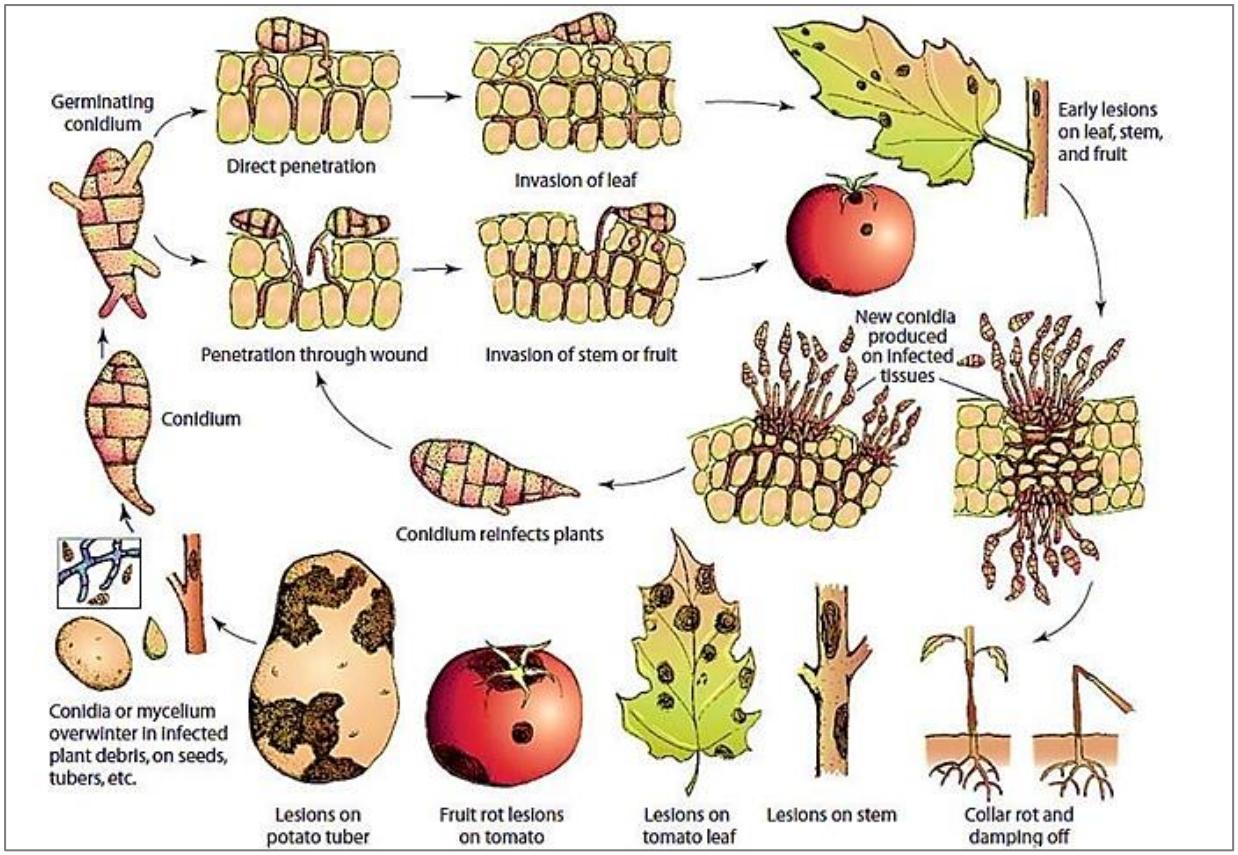

Figure 2.9: The disease cycle of *Alternaria* spp. (Agrios, 2005)

2.3.6 Diseases and symptoms caused by *A. tenuissima*

There are many reports of pathogenic *A. tenuissima* associated with different plants (cereals, vegetables, and fruits) causing severe damage and substantial crop loss. In the event of a successful infection from germinating conidia, disease symptoms may appear after 3 to 5 days of pathogen incubation (Rotam, 1994). Seed-borne *Alternaria* spp. may attack the seedling, usually after emergence, and cause damping-off or stem lesions and collar rot (Agrios, 2005).

Disease caused by *A. tenuissima* on above ground vegetative parts may appear as leaf or fruit spots and blights (Sharma et al. 2012). Small brown necrotic spots, 11 mm in diameter, were observed on stems, buds, and pods of pigeon peas irrespective of the age of the host. The spots coalesced rapidly to form large concentric rings. As the disease progressed, infected plant parts eventually died (Sharma et al. 2012). In addition to aforementioned symptoms, Luan et al. (2007) observed necrotic spots incited by *A. tenuissima* on plant leaves as brown to grey circular reddish leaf spots with brownish red borders and an insignificant canker on stems. *A. tenuissima* was reported

to incite leaf spot on several hosts. *A. tenuissima* caused development of brown to black, circular to oval, necrotic spots on the leaves of smooth amaranth (*Amaranthus hybridus* L.) (Blodgett and Swart, 2002). More than 95% of leopard plant leaves [*Farfugium japonicum* (L.) Kitam.] were infected by *A. tenuissima* and developed very small light brownish spots which gradually increased in colour and size as the disease progressed (Lee et al. 2013). During the late stage of disease, leaf spots became hollow and completely dehydrated. Li et al. (2011) reported 70-80% leaf spot disease on a pepper field. Initially very tiny, circular, brown necrotic spots were observed on the leaves. As disease developed with time, the spots gradually enlarged becoming irregular in shape whilst few others remained circular with concentric rings or zones. In the later stages of infection, these spots coalesced, resulting in withering, extensive drying and shedding of leaves.

Disease caused by *A. tenuissima* can cause a tremendous reduction in economic yield of some cultivated plants. Yield reductions of up to 44.19% were recorded on a pigeon pea field (Balai and Singh, 2013). Field surveys by Rahman et al. (2002) showed that more than 90% of broad bean plants were infected with leaf spots incited by *A. tenuissima*

There are also reports of *A. tenuissima* causing deterioration of harvested agricultural commodities. Studies on stored cereal degradation showed that *A. tenuissima* decreased fat, fibre and protein content of Pearl millet [*Pennisetum glaucum* (L.) R. Br.], sorghum [*Sorghum bicolor* (L.) Moench], and rice (*Oryza sativa* L.) (Fapohunda and Olajuyigbe, 2006). *Alternaria tenuissima* has been reported to reduce the economic value of harvested cereal grains because of contamination by mycotoxins (Andersen et al. 1996). High levels of *A. tenuissima* toxins; alternariol, alternariol monomethyl ether, tenuazonic acid, altertoxin I and other metabolites have been detected in high concentrations in affected wheat grains (Gannibal et al. 2007). *A. tenuissima* was isolated with a relative incidence of 16.1% of the pathogens that caused postharvest fruit rot of apple cultivars, Red Delicious, Golden Delicious, Granny Smith, and Fuji (Konstantinou et al. 2011). Dark sunken lesions were observed on the surface of ripening blueberries (Wright et al. 2004). Swart and Kriel (2002) observed small chlorotic spots on the cuticle of cactus pears caused by *A. tenuissima*. Later the spots coalesced

forming raised grey scabs. Uzabakiriho et al. (2013) observed that *A. tenuissima* reduced the economic value of morama bean by inciting necrotic spots on seed pods.

2.3.7 Control of *Alternaria tenuissima*

This section discusses some successful control strategies of *A. tenuissima* reported on different crops through use of resistant varieties, chemicals and application of bio-control agents. Advances in the field of genetics and crop breeding has presented vast potential in the field of crop protection by enabling the transfer of disease resistant molecular markers into plants to control or reduce infection caused by pathogens (Agrios, 2005). Resistance against leaf spot caused by *A. tenuissima* was reported in some apricot hybrids (*Prunus armeniaca* L.) and varieties: Callatis, Saturn, Olimp, Condor, Neptun, Cannette, Cafona, Wenatchee, Early Orange, Mekteb, Skaha, Early Bee, M 22/4, M 55/6, M 37/26, and B 33/13 (Oprea et al. 1985).

Currently, breeding programs of herbs are still in their early stages; hence, disease management in production of herbs is mainly done using chemicals. Tests to evaluate efficacy of 98 fungicides against *Alternaria* leaf spot of schisandra (*Schisandra chinensis*) were conducted by Xu et al. (2013). The antifungal activity was measured using the mycelia growth rate method. The ten best performing fungicides were then evaluated in a field trial and it was found that cyprodinil and procymidone had a stronger toxicity, with EC_{50} values lower than 0.1 mg/L compared to flusilazole which was only effective at EC_{50} values ranging from 0.1 to 1.0 mg/L (Xu et al. 2013). In another study, field tests showed that pyrimethanil had the greatest effect against *Alternaria* leaf spot diseases with a control effect of 91.52%, followed by mancozeb, cyprodinil, difenoconazole, flusilazole, prochloraz, which reduced the disease by 82.66%, 82.05%, 77.19%, 75.43%, 81.03%, respectively. The application of Thiram 75 WP (0.3%) on the first appearance of spots on apricot leaves has also been shown to control *A. tenuissima* (Oprea et al. 1985). Field trials conducted by Balai and Singh (2013) showed a decline of losses caused by *Alternaria* blight disease in the grain yield of pigeon pea by spraying Mancozeb 75 WP (0.25%). In this trial, plots that were sprayed with one, two, three and four sprays of Mancozeb recorded mean percentage disease control of 29.88%, 53.45%, 68.88% and 73.09%, respectively. Everett and Neilson (1996) observed that leaf spot disorder of New Zealand Gold Splash was effectively controlled by a wide range of curative fungicides. Efficacy of bitertanol, chlorothalonil, iprodione, prochloraz,

and vinclozolin declined with time whereas captafol, dichlofluanid and propineb constantly inhibited *A. tenuissima* using the leaf disc technique (Everett and Neilson, 1996).

Due to an increase in reports of pesticide toxicity towards the environment, alternative non chemical control methods have been proposed in protecting plants from pathogen damage. These include the use of biological agents, plant extracts and physical methods to reduce or destroy inoculum sources and prevent onset or further development of disease. *In vitro* studies conducted by Khare et al. (2003) showed that the bio-control agents *Aspergillus flavus, Aspergillus niger* and *Chaetomium globosum* Kunze inhibited the growth of *A. tenuissima*. Preliminary studies conducted by Shanguang (2007) showed potential use of three strains of actinomycetes, RA-1, RA-2 and RA-3 as they recorded high antimicrobial activity against *A. tenuissima*. When determining the efficacy of these actinomycetes against black patch of Dongzao (*Ziziphus jujuba* Mill.) it was observed that actinomycete RA-2 secreted more metabolites that effectively reduced 52% of the disease as compared to RA-1, RA-3 and the standard fungicide Kangji, which had no significant effect on disease development.

There is great potential in the application of *Pseudomonas* isolates in controlling phytopathogenic fungi. Jošić et al. (2012) investigated the antifungal activity of different *Pseudomonas* isolates (Q16, B25 and PS2) against *A. tenuissima* associated with purple coneflower [*Echinacea purpurea* (L.) Moench]. It was observed that the *Pseudomonas* isolates reduced conidial germination of *A. tenuissima* by 54.65 – 85.22%. Antagonistic effects of different *Trichoderma* species were investigated using the dual plate technique (Ambuse et al. 2012). Results showed high antagonistic activity of all *Trichoderma* species, *T. viride* Pers., *T. harzianum* Rifai*, T. virens* J.H. Mill., Giddens & A.A. Foster) Arx*, T. koningii* Oudem. and *T. pseudokoningii* Rifai, against *A. tenuissima*. It was also observed that *T. viride, T. koningii* and *T. pseudokoningii* recorded antagonistic activity of more than 80% in the case of the sensitive isolate of the test fungus.

Studies were conducted to control *A. tenuissima* on blackberry (*Morus nigra* L.) stem cuttings using different plant extracts (Ramulu et al. 2010). *In vitro* evaluations of plant extracts were done using the amended agar technique. Plant extracts of mesquite [*Prosopis juliflora* (Sw.) DC.] showed the maximum inhibition on mycelial growth (81.2%)

which were followed by *L. camara* (66.7%). *In vivo* trials showed that the plant extract *P. juliflora* reduced *A. tenuissima* by 55%, which was comparable to the chemical fungicide Mancozeb (Ramulu et al. 2010).

The ability of salicylic acid (SA) and the newly prepared bioactive matter "Agrileen" (AG) to suppress the leaf spot disease of tomato was evaluated by Agamy et al. (2013). Application of both SA (0.5 and 1.0 mM) and AG (2.5 and 5%) enhanced the growth and plant yield of tomato in addition to the reduction of infection of *A. tenuissima*. Conclusions were made that applications of SA or AG could protect tomato plants against *A. tenuissima* infection either through direct strength of the defence system or by reduction of the severity of the pathogen (Agamy et al. 2013).

2.4 Non-chemical seed treatment in controlling *Alternaria* spp.

Non-chemical seed treatment is a term referring to addition of a non-chemical antimicrobial or fungicidal coat applied to the seed surface prior to planting (Koch et al. 2010). This normally involves use or application of organic compounds, which reduce or eliminate various microorganisms on or in the surface of seeds (Burges, 1998; Singh and Mathur, 2010). This description of non-chemical seed treatments qualifies all biogenic compounds lacking synthetic chemicals, which include botanical extracts, mechanical or physical processes such as heat, magnetism, electricity or radiation (Yao et al. 2005; Schmitt et al. 2009).

Alternaria tenuissima is a cosmopolitan fungus affecting numerous seeds of different hosts, cereals, vegetables and fruits (Gannibal et al. 2007). In South Africa, Rang et al. (2002) first reported *A. tenuissima* causing dry core rot of apple and *Alternaria* black rot of citrus however; there is no previous report of the pathogen on herbs of the Lamiaceae and Umbelliferae family. Under favourable conditions, the pathogen germinates and infects the host causing a wide array of symptoms ranging from seed discolouration, seed decay, and seed abortion. These early signs and symptoms of disease normally go unnoticed and appear at later growth stages of the host as different infections ranging from seedling damping-off and leaf blights to leaf spots causing severe damage and substantial crop loss. Belisario et al. (2004) reported a 30% yield reduction caused by *Alternaria* black spot disease caused by *A. tenuissima*. *Alternaria* infected fruits are typically rejected at the markets due to skin blemishes, which render them unmarketable, resulting in important economic losses (Vicent et al. 2000).

In an attempt to control these devastating effects, there is a large dependence on use of conventional fungicides applied as a seed coat, or foliar spray for reducing or controlling pathogens and or disease development during sowing and later in the field (Mamgain et al. 2013). Although synthetic chemicals proved to be effective and efficient in controlling or eradicating diseases, their continued use is greatly discouraged in organic production systems as the European Union has passed strict regulations restraining their continued use due to pesticide toxicity and environmental concerns (Harris et al. 2001). In this regard, the search for alternative methods for controlling, reducing and eliminating pathogen and disease development in organic farming is still underway. Alternative approaches in crop protection that is safe and eco-friendly are being explored. One important option is to make use of plant based products as plant metabolites and plant based pesticides have been proven to be equally effective with minimal environmental impact (Varma and Dubey, 1999). In the past, hot water treatments were reported to be highly effective in eradicating seed-borne microorganisms associated with cereal and vegetable crops (Nega et al. 2003). It is essentially important to determine specific treatment temperature/time parameters for each and every crop for its applicability on different seeds of various host species (Forsberg, 2004).

This section provides details of some possible non-chemical control methods with emphasis on their mechanism and mode of action on different *Alternaria* spp.

2.4.1 Hot water seed treatment

The history of use of hot water in seed sanitation can be traced back to the late 19th century (Forsberg, 2004). In this era, the method increasingly became a standard technique in seed sanitation of cereal seeds as it proved to be effective in controlling many phytopathogenic fungi (Gray, 1962; Sharma, 1981; Pryor et al. 1994). As years went by, chemical seed treatments were introduced resulting in temporal abandonment of use of hot water treatments since chemicals give better control of seed-borne pathogens. However; continued use of chemicals in controlling phytopathogenic organisms is increasingly restricted due their harmful effects on human health and the environment (Harris et al. 2001). In addition, present intensification of organic farming systems has led to a huge disparity and a need for alternatives to chemical methods to guarantee expected crop yield from pathogen damage. This has greatly justified the use

of hot water seed sanitation. There are reports showing success of usage of hot water in seed treatment of various crops in controlling different *Alternaria* spp. without affecting their seed physiological processes (Hermansen et al. 1999; Vilchez et al. 2000). A study conducted by Nega et al. (2003) showed a 95% reduction in incidence of pathogenic *Alternaria* spp. associated with different vegetable crops such as, carrot, cabbage, celery and parsley, without significant losses of germination due to hot water treatments at 50^{o}C (20 to 30 mins) up to 53^{o}C (10 to 30 mins).

The principle and mechanism of hot water seed sanitation is based on disruption of pathogen ultrastructure due to thermal coagulation of the complex structures of proteins and lipids. For hot water seed treatment to be effective, precision on gauging differential sensitivity between seeds and seed-borne microorganisms is important. Microorganisms to be controlled must have a lower tolerance to high temperatures than seeds being treated (Forsberg, 2004). Pryor et al. (1994) showed that higher treatment temperature-time combinations above 55^{o}C (5, 10 and 20 mins) resulted in significant reduction of seed germination capacity with moderate control of *Alternaria* spp. Therefore, it is particularly important to determine optimum temperature-time combinations for each and every crop to establish pathogen-free seeds while reducing plant injury as shown in Figure 2.10 (Forsberg, 2001). It was also observed that shorter time intervals must be used at higher temperatures to avoid reduced germination of sensitive crops (Nega et al. 2003).

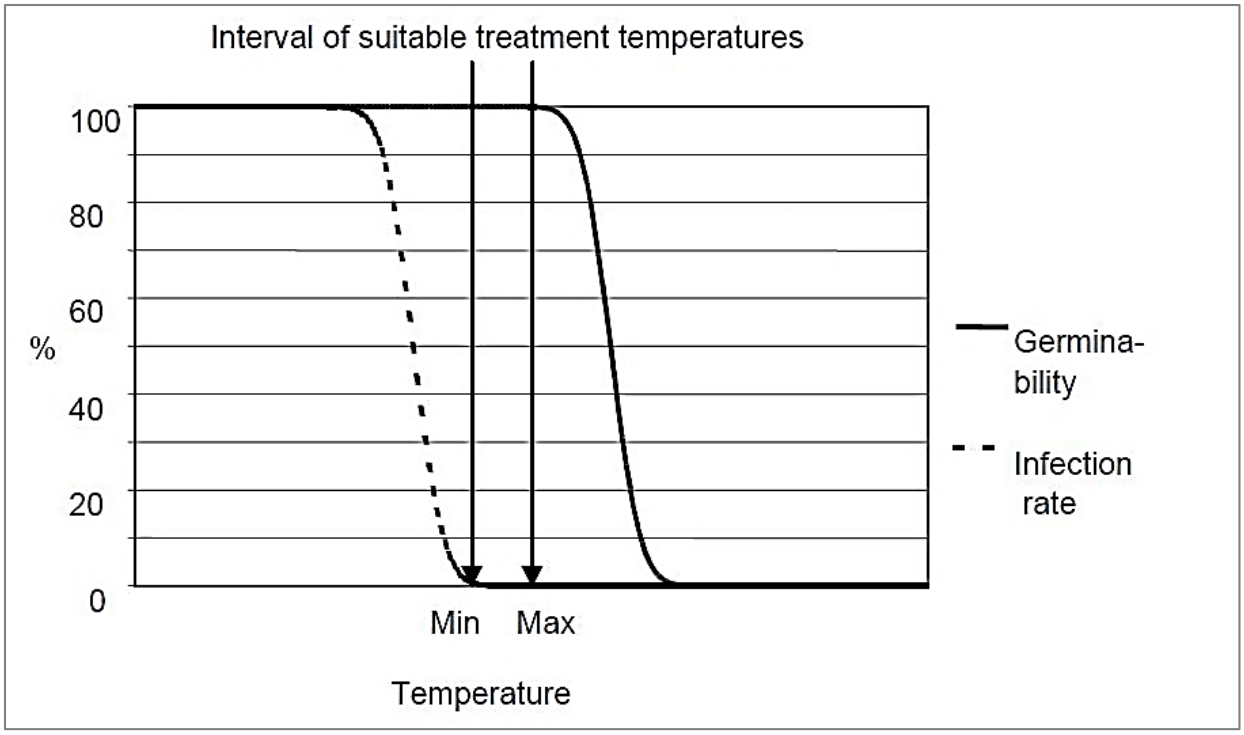

Figure 2.10: Schematic diagram showing mechanism of thermal seed treatment. Treatment temperatures within the interval [Min; Max], treated seeds have full germination capacity developing into disease-free plants (Forsberg, 2001).

Alternatively, research has shown the possibility of increasing the efficacy of hot water seed treatment by combining with other non-chemical control agents such as plant extracts, organic compounds and certain microorganisms. Efficacy evaluations of hot water treatments and applications of biological control agents as seed treatments for controlling *A. dauci* and *A. radicina* were conducted by Koch et al. (2010). Although, best results were obtained with seeds treated with *Pseudomonas* sp. strain MF416 and *Clonostachys rosea* strain IK726, good and consistent control was also recorded with seeds treated with hot water. The efficacy of hot water seed treatment was significantly improved when combined with a *Pseudomonas* sp. MF 416 or *C. rosea* IK726 treatment (Koch et al. 2010).

2.4.2 Bio-control agents in controlling *Alternaria* spp.

The demand of natural production by consumers has increased use of non-chemical methods in disease management particularly under organic farming systems (De Ceuster and Hoitink, 1999). Thus, there is a shift from dependence on use of convention chemicals to more environmentally sustainable production systems of which application of microbes on seeds is promising due to its great precision and minimized impact on

the environment compared to chemicals (Stiling and Cornelissen, 2005; Chandler et al. 2011). In the case of seed production, many steps have been taken with success in exploiting beneficial microorganisms (Mastouri et al. 2010; Johnson et al. 2011; Maldonado-González et al. 2012; Martins et al. 2014). Beneficial microbes are being applied on seeds to promote nitrogen fixation, phosphate solubilisation, plant growth-promotion and biological control of plant pathogens or pests (Junaid et al. 2013; Bashan et al. 2014). To control pathogenic microbes associated with seeds, knowledge of beneficial and pathogenic microbes is required. It is also important to consider issues regarding stability of beneficial microbes during and after the application process (Elliott et al. 2001). Sufficient numbers of the inoculant must survive the application process and be able to grow in the environment of germinating seed. More importantly, selected bio-control agents must be able to withstand shelf life conditions since treated seed will be stored for some time before being sown (Stiling and Cornelissen, 2005). In respect of the above variables, this section discusses applicability of selected bio-control agents in the control of *Alternaria* spp.

Bacillus [*Bacillus subtilis* (Ehrenberg) Cohn] is a rod shaped gram-positive bacterium classified as an obligate aerobe (Joshi and McSpadden, 2006). Shanmugam et al. (2011) mentioned that *Bacillus* sp. by virtue of its ability to form heat and desiccation resistant spores can be formulated readily into stable products, which when applied as seed treatments may control disease and improve seed germination (Kloepper et al. 2004). Generally, application of *Bacillus* sp. has been widely investigated because the genetic and biochemical analysis of bacteria and its mass production are straight forward compared to those of fungi (Leifert et al. 1995; Mateescu et al. 2004; Souto et al. 2004). Seeds treated with a strain of *B. subtilis* were reported to show significant reduction of incidence of *A. radicina* with improved seed health and germination (Hentschel, 1991). *Bacillus subtilis* has been commercially registered as Kodiak (Gufstafson Biologicals, Plano, TX), Subtilex (Becker Underwood, Ames, IA) and Serenade (Agraquest Inc., Davis, CA) (Joshi and McSpadden, 2006). Formulations containing *B. subtilis* are being used in disease management of various plant diseases including early blight, fire blight, downy mildew, and a variety of leaf spots on crops such as cotton, soybean and vegetables. A low disease incidence of 28.7% was recorded in plots treated with formulations of *B. subtilis* as compared to that of non-treated pathogenic *A. solani* control of 56.4%. *In vitro* assays conducted by Chung et al. (2008)

observed clear zones of inhibition when *B. subtilis* was used to control *Alternaria brassicicola, Alternaria porri* (Ellis) Cif., *Alternaria mali* Roberts, *Alternaria solani,* and *Alternaria rosifolii* E.G. Simmons & C.F. Hil.

Research has shown the principle mechanism of *B. subtilis* to be based on production of antibiotics and volatiles which exert a fungistatic and fungicidal effect (Linderman and Gilbert, 1975). *In vitro* production of siderophore, chitinase, and β-1, 3-glucanase was identified from dual culture assays with *B. subtilis* and *Alternaria* spp. (Shanmugam et al. 2011). Culture filtrate analysis of *B. subtilis* isolate ME488 extracts has shown production of bacilysin as confirmed by GC–MS and iturin by *B. subtilis* ME488 as confirmed by thin-layer chromatography (TLC) of butanol extracts (Chung et al. 2008). Sharma and Sharma (2008) observed that *B. subtilis* strain UK-9 produced antifungal metabolites, which caused morphological alterations in cell membranes which resulted in reduced spore germination on leaves and disease incidence of the pathogen. *Bacillus subtilis* had a plant growth-promoting effect on the treated plots (Sharma and Sharma, 2008).

Chowdappa et al. (2013) observed that tomato seedlings recorded enhanced systemic resistance when seeds were treated with *B. subtilis* OTPB1 and OTPB3 to control early blight and late blight, incited by *A. solani* and *Phytophthora infestans* (Mont.) de Bary, respectively. When compared in terms of growth hormones, the levels of indole-3-acetic acid (IAA) and gibberellic acid (GA3) were increased significantly in roots of seedlings treated by OTPB1 or OTPB3 by 29.12% and 45.82% or 54.34% and 67.59%, respectively, as compared to non-inoculated controls (Shanmugam et al. 2011). Treatment with OTPB1 or OTPB3 enhanced the levels of defence–related enzymes including peroxidase, polyphenol oxidase and superoxide dismutase in tomato plants. This study also showed that in addition to plant growth and antibiosis, OTPB1 and OTPB3 enhanced systemic resistance in tomato seedlings through induction of growth hormones and defence enzymes (Chowdappa et al. 2013).

***Trichoderma* spp**. are classified as imperfect fungi largely distributed all over the world as free living, present in soil and root ecosystems, opportunistic avirulent plant symbionts that also parasitise other fungi (Harman et al. 2004). Ozbay and Newman (2004) reported first use of *Trichoderma* species as bio-control agents in the early 1930s. Years of continued research cumulatively identified and listed a wide range of

diseases which can be controlled by *Trichoderma* spp. (Howell, 2003). Some registered commercial products containing *Trichoderma* as the principle ingredient are being marketed as TrichoFlow WP™ (New Zealand), ArborGuard™, Binab (USA, Sweden), BioTrek 22G (Europe) GreenMax and Trichoplus (South Africa) (Altintas and Bal, 2008).

Trichoderma spp. has been reported to be effective in controlling several *Alternaria* spp. Vannacci and Harman (1987) conducted a screening assay to determine efficacy of 42 microorganisms in controlling *Alternaria raphani* J.W. Groves & Skolko and *A. brassicicola*. Results of germination tests of infected seeds showed that *Trichoderma harzianum* Rifai was among the five beneficial microorganisms that effectively reduced pod infection by *Alternaria* spp. In another study, *Alternaria*, which caused 3.3% post-emergence damping off and 12.5% root rot infection, was effectively reduced and resulted in significant increase of sugar-beet root weight when seeds were treated with *Trichoderma* sp. (Abada, 1994). Ambuse et al. (2012) showed that *Trichoderma viride* Schumach., *Trichoderma koningii* Oudem and *Trichoderma pseudokoningii* Rifai reduced growth of *A. tenuissima* by more than 80%.

Past research has suggested different mechanisms for the bio-control activity of *Trichoderma* spp., which are involved in attacking other fungi and enhancing plant and root growth. Such mechanisms may include, competition for space and nutrients, secretion of chitinolytic enzymes that affect the structure and function of fungal cell walls, mycoparasitism, production of inhibitory compounds, inactivation of the pathogen's enzymes and induced resistance (Haram et al. 1996; Ozbay and Newman 2004). *Alternaria alternata* is a pathogenic fungus which infects the host by secreting endogalacturonase (endo-PG0) and pectate lyase (PL) enzymes hydrolysing the cell walls. *In vitro* antifungal investigations of pathogenic *A. alternata* using *T. harzianum* showed that presence of *T. harzianum* decreased endo-PGase secretion of *A. alternata* by about 50% irrespective of the presence of plant growth regulators (Roco and Pérez, 2001).

2.4.3 Plant extracts as seed treatment in controlling *Alternaria* spp.

The search for plants with anti-microbial activity was recorded in the 1940s (Russell, 2005). As the years passed, there has been an increase in publications of information on application of different plant extracts to inhibit or control microorganisms on or in the surface of seeds resulting in an improvement of germination and emergence. The

mechanism and principles of action of seed dressings using plant extracts is dependent on the composition of plant extract, or their secondary metabolites, to bring about inhibition or reduction of biological activity of microorganisms (Rios and Recio, 2005; Park et al. 2008).

Garlic (*Allium sativum* L.) is a bulbous plant in the genus *Allium*. When the garlic bulbs are crushed, the highly permeable compound, allicin is dislodged and made available (Pai and Platt, 1995). Muhsin et al. (2001) mentioned that inhibitory action of garlic extracts against fungi is based on the presence of allicin as the major antifungal component. After a fungus has absorbed it, allicin undergoes thio-disulphide exchange reactions with free thiol-groups in proteins and inhibits enzyme production by fungi (Slusarenko et al. 2008). Decomposition of allicin produces various products, like ajoene, which possess antifungal properties (Curtis et al. 2004). Absorption of allicin inactivates the ability of fungal pathogens to digest plant cell wall elements thereby preventing pathogens from penetrating the host tissues thereby translating to loss of pathogenicity (Miron et al. 2000). Although allicin is reported to be effective against certain fungal pathogens, it was shown that *A. sonchi* Davis and *A. solani* maintained a constant enzymatic activity after exposure to garlic extracts (Muhsin et al. 2001). Aqueous extracts of garlic, ginger (*Zingiber officinale* Roscoe), neem (*Azadirachta indica* A. Juss.) and onion (*Allium cepa* L.) were evaluated for their efficacy in controlling seed-borne fungi associated with mustard seeds (Latif et al. 2006). Seed treated with garlic extracts effectively reduced seed-borne mycoflora and promoted seed germination capacity. Singh et al. (1990) reported that ajoene, an unsaturated disulfide compound produced from garlic, was very effective in inhibiting spore germination at a concentration of 25 µg/ml and in some cases spore germination was inhibited by 100% at 100 µg/ml. Results of *Alternaria* infested carrot seeds treated with garlic extracts were comparable with the industrial seed dressing Aatiram® (Thiram), which resulted in improvements of germination rates and uniformity of germination (Koch et al. 2010).

Ginger (*Zingiber officinale* Roscoe) is an erect, herbaceous perennial plant in the family Zingiberaceae grown for its edible rhizome (Ravindran and Babu, 2004). Studies conducted by Ficker et al. (2003) identified the crushed extracts to be composed of 6, 8 and 10-gingerdiol as the main antifungal compounds. However, Sharma and Tiwari (2013) found 1,8-cineole as the major component. Secondary metabolites of ginger

extracts are absorbed by the fungi and interfere with various metabolic activities of the fungi resulting in varying levels of fungistatic or fungicidal activity (Srivastava, 1994; Singh et al. 1999). Although seed health tests conducted by Latif et al. (2006) showed lower antifungal activity of ginger extracts compared to neem -extracts in reducing seed-borne *Alternaria* spp., Mahapatra and Das (2013) showed that four different concentrations of aqueous extracts of *Zingiber* sp. rhizome namely, 5, 10, 15, 20%, adequately managed *Alternaria* leaf blight of mustard under field conditions compared to untreated controls. The study also showed that ginger rhizome extract significantly reduced the severity and leaf infection percentage incited by *Alternaria brassicae* (Berk.) Sacc. and *A. brassicicola* (Mahapatra and Das, 2013). Fawzi et al. (2009) performed *in vitro* antifungal evaluations from which cold water extracts of *Zingiber* sp. effectively reduced growth of *A. alternata* and their hydrolytic enzymes, β-glycosidase, pectin lyase and protease. Sharma and Tiwari (2013) recorded minimum inhibitory concentration of 500 ppm of essential oil extracted from ginger for control of *A. alternata.* Ginger and garlic extracts at 5, 10, and 15% effectively outcompeted other botanical extracts of eucalyptus, polyalthia, communist weed, neem, clerodendron and bougainvilla against growth of *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout on tomato plants (Ngoc et al. 2013).

Jimson weed (*Datura stramonium* L.) is an annual herb that belongs to the family Solanaceae (Berkov et al. 2006). The plant can grow into a bush that is up to 1.5m tall with long (8-20 cm), smooth toothed irregular undulate leaves (Weaver and Warwick, 1984). Discovery of several bioactive properties of the plant has opened a wide range of applications in the field of mycology. Its extracts comprise of 5,6-Dihydro-6-pentyl-2H- pyran-2-1, diphenylamine and tetratetracontane which render them with their high inhibitory activity against a wide range of phytopathogenic fungi. Although limited literature is available for the use of *Datura* sp. as seed treatments, a lot of work has been done to illustrate the plant's general activity against *Alternaria* spp. Zhen-guo et al. (2012) showed that high concentrations, up to 1200 mg/L, of *Datura* extracts translated to an increase in inhibition rates against *A. alternata* and other phytopathogenic fungi. Studies conducted by Shivpuri et al. (1997) showed that ethanol leaf extracts of *D. stramonium,* and other plants, were highly effective in controlling *A. brassicicola* at concentrations of 1000 µg/ml. *In vitro* efficacy evaluations showed that ethanol extracts (1:1) of *D. stramonium,* and other plant extracts, effectively inhibited

spore germination and mycelia growth of *Alternaria macrospora* (Sacc.) Mussat (Bambawale et al. 1995). However *in vivo* evaluations gave inconclusive results as treated cotton plants did not develop appreciable amount of disease even in the control. On the contrary, well defined disease development in untreated control plots led to the conclusion that *D. stramonium* effectively controlled *A. solani* at a concentration of 5% as fruit yield increased by 76.2% (Nashwa and Abo-Elyousr, 2012).

Kakiebos (*Tagetes minuta* L.) is a marigold plant that is native to the northern and southern parts of America (Tankeu et al. 2013). The plant grows on a straight stem extending to a height of 1 to 2 m tall with compound leaves bearing small creamy yellow flowers (Hulina, 2008). *Tagetes minuta* contains various secondary metabolites such as acyclic, monocyclic and bicyclic monoterpenes, sesquiterpenes, flavonoids and thiophenes (Soule, 1996). Zygadlo et al. (1994) reported that *A. solani* was inhibited at concentrations of 5000 ppm and 3000 ppm by two volatile constituents of *Tagetes* sp. (ocimene-rich and ocimenone-rich). In another study, *T. minuta* crude extracts showed no antifungal activity against *Alternaria passiflorae* J.H. Simmonds by the diffusion method (Rugutt et al. 2006).

Pawpaw (*Carica papaya* L.) is a large tree-like plant belonging to the family Caricaceae which originated from the tropics of America (Ali et al. 2010). It grows with a single stem extending to a height of 5 to 10 m tall, with spirally arranged leaves confined to the top of the trunk (Ali et al. 2010). Extracts of the plant parts have been reported to be bioactive (Chávez-Quintal et al. 2011). Cowan (1999) and Chávez-Quintal et al. (2005) reported that *C. papaya* derived its bioactivity from its leaves and seeds which contain proteolytic enzymes (papain, chymopapain), alkaloids (carpain, carpasemine), sulfurous compounds (benzyl isothiocyanate), flavonoids, triterpenes, organic acids and oils. *In vitro* antifungal tests conducted by Suleiman (2010) showed that methanol extracts of pawpaw and neem effectively inhibited *A. solani* by the disk infusion assay. An increase of inhibition action of the extracts was observed with an increase in concentration.

Tickberry *(Lantana camara* L.) is a species of flowering plant in the verbena family, Verbenaceae. Plant extracts of *L. camara* exhibit high antifungal activity due to the presence of sabinene, *β*-caryophyllene, 1,8-cineole, bicyclogermacrene and *α*-humulene (Venkatachalam et al. 2011). Biochemical properties of these components

explain their relative permeability through the cell membrane at the same time disrupting the lipid bilayer (Kurade et al. 2010). *In vitro* evaluations were done using the cup plate method to investigate efficacy evaluations of 18 plants against five seed-borne fungi (Pawar, 2011). *Lantana camara* aqueous extracts ranked third in their activity against *A. alternata* with a zone of inhibition of 20.3 mm. Antifungal evaluations conducted by Boughalleb et al. (2005) against *A. solani* showed that *Lantana* sp. flower extracts had the highest antifungal activity (38%) followed by leaf extracts (27.1%) and stems (26.6%). Studies conducted by Manzoor et al. (2013) indicated that both nature or type of extraction solvent and preparation method employed to obtain the extracts have an overall effect on the performance of the antioxidant and antimicrobial effects of the extracts. Methanolic *L. camara* extracts produced by ultrasound-assisted stirring, recorded the highest antimicrobial activity against *A. alternata* followed by ultrasonic-assisted stirring and last by manual stirring.

2.5 Summary of literature review

Over the years, herbs were neglected crops and were generally regarded as minor crops. This has contributed to less priority in productivity and advances in research of its crop improvement. However, the change in diet and lifestyle in the last decades has led to an increase in the demand for herb products and has prompted investigations to improve productivity of herbs. Hence, information relating to possible diseases affecting production is increasingly becoming available with publication of new diseases. Considerable studies have reported some diseases on herbs. Since vegetative aerial parts are the major component of the economic yield of cultivated herbs, a growing concern has prompted search for alternative non-chemical strategies in management of diseases limiting cultivation of herbs. Consumers show more preference for organic or natural food products as they are free from chemical residues. In addition, continued use of synthetic chemicals in plant disease management is largely disregarded due to their detrimental effects towards the environment. Therefore, the literature discussed aspects of non-chemical seed treatment in managing *Alternaria* foliar disease of coriander which include bio-control agents, plant extracts, and physical control methods.

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CHAPTER 3

Seed-borne fungi associated with herbs and their effect on seed germination

Abstract

South Africa, with its warm climatic conditions, has the potential for expansion of cultivation of aromatic and medicinal herbs. Although considerable yield increments were reported on some herb cultivars, cultivation of herbs has often been associated with outbreaks of previously unreported diseases. Investigations to determine local sources of diseases have not been conducted, and general information relating to management of herb disease is scarce. Hence, this study was conducted to determine whether seeds are the primary source of diseases. The agar plate method was used to evaluate seed health status of 13 herb seed lots. Surface sterilized seeds were plated in 10 replicates of 20 seeds and incubated at 25^{o}C for 7 days, from which a total of 11 fungal species were identified. A new fungus was isolated from coriander and basil seed samples i.e. *Alternaria tenuissima* and occurred with the highest incidence on coriander seed lot 34184 (73%). Pathogenicity tests proved *Alternaria tenuissima* to be pathogenic as it incited lesions on coriander cultivar American long. Furthermore, infected seed produced seedlings with lesions from which *A. tenuissima* was isolated and Koch's postulates proved that the pathogen was seed transmitted. *Penicillium* sp. was the most common fungi isolated from basil seed lot M043803 CAC (3.5%), chives seed lot 31744 (2%), all coriander seed lots (3.5, 9, 5%, respectively), sage seed lot H071408JJH (4%), thyme seed lot L095502FAB (5.5%) and wild rocket seed lot 33013 (3.5%). Germination tests were performed following ISTA rules (2013). The study showed that seed-borne mycoflora had a weak correlation with poor germination of herb seeds ($r= -0.129$, $p<0.01$). However, seed-borne mycoflora associated with herb seeds had a positive correlation with the percentage of diseased seedlings ($r= 0.239$, $p<0.01$). Results therefore indicated that further studies to prove seed transmission and pathogenicity of the other seed-borne fungi other than *Alternaria* sp. are warranted.

3.1 Introduction

Apiaceae and Lamiaceae plant families embody more than 360 genera with close to 9 000 plant species distributed all over the world (Hortus, 1976; Bulajić et al. 2009). The two plant families consist of economically important plants popularly known as aromatic and medicinal herbs (Plunkett et al. 1996; Harley et al. 2004; Scheen and Albert, 2009). Discovery of their medicinal and healing properties has resulted in a global increase in demand of herb products (Hoareau and Da Silva, 1999; Gilbert and Khokhar, 2008). Commercial cultivation of herbs is mainly done in France, India, Israel, Italy, Morocco, Russia, United States of America and South Africa (Garibaldi et al. 2003; Ravi et al. 2007; Homa et al. 2014).

The global change in life style, particularly diet, in the last decade has contributed to increased consumption of herbs (Kelly et al. 2005; Gilbert and Khokhar, 2008), which emphasises the need to increase production of herbs (Diederichsen, 1996; Gilardi et al. 2013). Although yield increases were recorded in a number of herb farms scattered across the globe, this has often been associated with outbreaks of previously unreported diseases, among which seed-borne and seed-transmitted diseases are of great importance (Chiocchetti et al. 2001; Garibaldi et al. 2004; Bulajić et al. 2009; Gilardi et al. 2014).

The continual occurrence of new, unfamiliar diseases is the biggest challenge limiting further expansion of herbs (Gamliel et al. 1996). Some of the major causes of diseases reported in South Africa include *Peronospora belbahrii* Thines and *Fusarium oxysporum* f. sp. *basilici* Tamietti & Matta (Swart and Van Niekerk, 2003; McLeod et al. 2006). The establishment and distribution of such pathogens is known to occur in a short period of time by means of infected seeds (Gamliel and Yarden, 1998; Gilardi et al. 2013). Losses due to infection by seed-borne and seed-transmitted mycoflora may include reduced germination and/or seedling damping-off that normally account for mortality of established crop stands (Coles and Wicks, 2003; Tylkowska et al. 2003). Early symptoms of seed-borne infection normally go unnoticed, leading to catastrophic disease incidences when they manifest at a later stage of plant growth (Mathur et al. 1972; Hashmi and Ghaffar, 1991; Pawar, 2011). There are several reports of spread of herb diseases into non-diseased areas by means of infected seeds (Gamliel and Yarden, 1998; Garibaldi et al. 2004; Moya et al. 2004; Farahani-Kofoet et al. 2012). The

spread of leaf spot disease of wild rocket [*Diplotaxis tenuifolia* (L.) DC.] caused by *Plectosphaerella cucumerina* (Lindf.) W. Gams is an example of a recent outbreak (Gilardi et al. 2013).

Since reports of herb diseases are increasing, it is important to determine the possible sources and causes of disease for effective disease management. Although some studies have been conducted abroad, little is known on the health status of herb seeds produced under South African weather conditions. In this regard, investigations were conducted to determine seed-borne fungi associated with commercial herb seed lots. This study also evaluated the effects of seed-borne fungi mycoflora on seed germination.

3.2 Materials and Methods

3.2.1 Source of seed

Untreated herb seed samples were obtained from South African seed companies as shown in Table 3.1.

Table 3.1: List of herb seed lots used in the study

Herb common name	Botanical name	Lot/Ref No.
Basil	*Ocimum basilicum* L.	33590
Basil	*Ocimum basilicum* L.	M043803CAC
Chives	*Allium schoenoprasum* L.	31744
Coriander	*Coriandrum sativum* L.	34184
Coriander	*Coriandrum sativum* L.	M074163HAC
Coriander	*Coriandrum sativum* L.	N056404HAD
Dill	*Anethum graveolens* L.	33442
Parsley	*Petroselium crispum* (Mill.) Fuss	33614
Parsley	*Petroselium crispum* (Mill.) Fuss	M051102CAC
Sage	*Salvia officinalis* L.	H071408JJH
Thyme	*Thymus vulgaris* L.	33591
Thyme	*Thymus vulgaris* L.	L095502FAB
Wild Rocket	*Diplotaxis tenuifolia* (L.) DC.	33013

3.2.2 Seed health tests

A modification of the ISTA standard agar plate method was used to detect seed-borne fungi associated with herb seeds (ISTA, 2013). Two hundred seeds of each herb seed lot were randomly selected, surface sterilised in 1% sodium hypochlorite solution for five minutes and rinsed three times in sterile distilled water. Sterilised seeds were dried on paper towels spread in a laminar flow. Ten seeds were plated in 9 cm diameter plastic Petri dishes containing sterile potato dextrose agar (PDA, Biolabs) amended with 25 mg/L streptomycin sulphate (Biolabs). Petri dishes were wrapped with Parafilm® before incubation at 25°C for 7 days under alternating cycles of 12 h ultra violet (UV) light and darkness. The experiment was arranged in four replicates of fifty seeds and set up in a completely randomized design. The experiment was repeated twice.

3.2.2.1. Morphological identification of fungi

After 7 days of incubation, fungal cultures growing on the seeds were isolated and purified using the single spore technique before morphological examination under a Zeiss light microscope. Morphological characteristics recorded for each fungus included the shape of spores, colour, arrangement of conidiophores, catenulation of conidia, and description of mycelium. Those fungal cultures identified as *Alternaria* spp. were cultured on potato carrot agar (PCA) by doing single spore isolations, whereas, spores of cultures identified as *Fusarium* were transferred onto carnation leaf agar (Simmons, 1992; Burgess et al. 1994). Inoculated plates were incubated at 23°C and 10/14 h cool fluorescent-light/darkness cycles for 7 days (Pryor and Michailides, 2002). All other fungal isolates were sub-cultured on PDA and incubated at 25°C for 7 days under alternating cycles of near UV light and darkness. Reference manuals of Mathur and Kongsdal (2003) and Ellis and Ellis (1997), were used to identify and distinguish fungi to the species level. Identification of *Fusarium* spp. was based on the morphology of macroconidia and microconidia as described by Leslie and Summerell (2006), and Nelson et al. (1981; 1983).

Names of identified isolates were verified by an expert mycologist (Dr M. Truter, Mycology Unit, Biosystematics, Agricultural Research Council-Roodeplaat, South Africa). Fungal isolates were stored on half strength PDA at 4°C and as mycelia blocks in double sterile distilled water in McCartney bottles for further experiments. The

frequencies of each fungal species isolated were calculated using the formula derived by Marasas et al. (1988);

$$\text{Frequency of isolated fungi (\%)} = \frac{\text{No. of samples of occurrence of a fungal species}}{\text{Total no. of samples}} \times 100$$

3.2.3 Standard seed germination test

Seed germination tests were performed according to the rules of the International Seed Testing Association (ISTA, 2013), however, only two replications of 200 seeds divided into four sub-replicates of 25 seeds. Germination tests of seed samples of basil, dill, parsley, thyme, wild rocket and sage were performed using the top of paper method. Seeds were placed on the surface of three moistened filter papers (Whatman No. 1) aligned inside glass Petri dishes. Before the germination test on basil and sage was conducted, physiological dormancy of seed samples was broken by pre-chilling the plated seeds by exposing them to 5-10^{o}C for up to 7 days, on filter papers moistened with 0.2% KNO_3 solution instead of distilled water, respectively.

Germination tests of chives and coriander were performed following a modified between paper method (ISTA, 2013). For each herb species, four replicates of fifty seeds were placed on top of three layers of moistened germination paper before covering them with a fourth layer of moistened germination paper. The layers of germination paper were rolled, sealed in a polythene bag and incubated at 25^{o}C under an alternating 12 hours darkness and normal light regime. Seedlings were evaluated according to ISTA rules, where percentages of normal or abnormal (deformed and diseased) seedlings was determined after 21 days.

Data collected from seed health and germination tests was arcsine transformed and analysed for variance using SAS Version 9.3 (SAS Institute, 2010). Statistical means were separated using the Fisher's LSD test.

3.2.4 Pathogenicity test

Pathogenicity tests were done in growth chambers at the Plant Sciences Complex, Faculty of Natural and Agricultural Sciences, University of Pretoria (South Africa). Since seed health tests revealed that *Alternaria tenuissima* was isolated with the highest incidence from coriander seeds, the pathogenicity and seed transmission of only this fungus was determined. Coriander cultivar American long seeds, naturally infected with

A. tenuissima, were sown in 5 cm diameter pots (one seed/pot) filled with pasteurised loamy soil and grown at 25^{o}C /17^{o}C day-night temperatures, respectively. The photoperiod was maintained at 16 hours with an average humidity of 80-85%. Pots were watered with sterile distilled water once every day for 8 weeks before inoculation with *A. tenuissima*.

Pathogenicity tests were conducted by modifying the protocol designed by Blodgett and Swart (2002). Fungal inoculum was prepared from a 14 day old *Alternaria* sp. culture grown on PCA. Mycelia were harvested by scraping the surface of the culture and flooding the fungal plates with sterile distilled water amended with two drops of Tween 20 (Merck). Thereafter, the suspension was filtered through double cheesecloth and the concentration of the filtrate determined and adjusted to 5x10^{5} spores/ml using a haemocytometer.

For the pathogenicity trial, half of the 8 week old coriander seedlings were wounded with a sterile pin in the centre of the leaves with a diameter of 0.5 µm and the other half left unwounded. The wounded and unwounded plants were inoculated with the *A. tenuissima* inoculum sprayed with an automatic aerosol sprayer until runoff. Positive and negative controls comprised wounded leaves sprayed with sterile distilled water and non-wounded leaves sprayed with sterile distilled water, respectively. A high humidity (>95%) was maintained by covering inoculated plants with polythene bags for 72 h. Experimental units were arranged in a completely randomised design and assessed for disease development two weeks after inoculation. The experiment was repeated twice. Evaluation of severity of disease was based on the intensity of leaf spot disease determined by the rating scale developed for this study as shown in Table 3.2.

Table 3.2: *Alternaria* leaf spot disease severity scale

Index value	Description of disease severity
0	No infection
1	Leaf lesions (diameter<5 mm) on surface of leaf ≤2; (1–20%)
2	3≤ leaf lesions (diameter<5 mm) on surface of leaf <5; (21-40%)
3	5≤ leaf lesions (diameter>5 mm) on surface of leaf≤7; (41-60%)
4	8≤ leaf lesions (diameter>5 mm) with coalescing of lesions to form one necrotic spot; (61-80%)
5	>9 leaf lesions with severe to complete damage of leaf; (81-100%)

3.3 Results

3.3.1 Seed health test

The single spore isolates of seed-borne fungi associated with herb seeds used for identification of fungal isolates to species level are shown in Figure 3.1. The fungal culture shown in Figure 3.1A was identified as *Aspergillus flavus*. When grown on PDA, *A. flavus* appeared as yellowish to green colony (Figure 3.1A) bearing rough surfaced conidia with globose to subglobose shapes measuring 3-5 µm in diameter [Figure 3.1B] (Mathur and Kongsdal, 2003). Thyme seed lot 33591 recorded the highest incidence of *A. flavus* [5.5%] (Table 3.3). There was no statistical difference between thyme seed lot L095502FAB (3.5%) and wild rocket seed lot 33013 (3%). However, incidence of *A. flavus* was significantly higher on both seed lots compared with coriander seed lot M074163HAC (1.5, 2.0%, respectively) and coriander seed lot 34184 (1.0; 2.5%, respectively).

Fungal cultures of *Aspergillus niger* produced brown to black globose heads on long hyaline conidiophores (Figure 3.1C). Brown globose conidia had a diameter of 4.5 µm (Figure 3.1D). *Aspergillus niger* was detected with the highest incidence on sage seed lot H071408JJH (4.5%) and coriander seed lot N056404HAD [4%] (Table 3.3). The occurrence of *A. niger* on coriander seed lots 34184 and M074163HAC and wild rocket seed lot 33013 was significantly lower compared to that of sage seed lot H071408JJH and coriander seed lot N056404HAD.

Long straight chains of spores were observed when viewed under a microscope (Figure 3.1E) and were similar to the description for *Alternaria tenuissima* mentioned by Pryor and Michailides (2002) and Mirkova and Konstantinova (2003). *Alternaria tenuissima,* when cultured on PCA media, appeared as a uniform circular fluffy growth with an olive-brown upper surface. The colony consisted of long unbranched conidial chains composed of 15 to 22 spores (Figure 3.1F). Conidia were short, varying from 16 x 7 µm to 45 x 13 µm, oval and bean shaped with 1-6 transverse septa and 0-2 longitudinal septa. The highest incidence of *A. tenuissima* was recorded on coriander seed lot 34184 (73%) followed by coriander seed lot N056404HAD (45%) which was significantly higher by 16.5% when compared with coriander seed lot M074163HAC. Parsley seed lot 33614 recorded the lowest incidence of *Alternaria* sp. (4.5%).

A cream coloured fluffy mycelial growth shown in Figure 3.1G was identified as *Curvularia pallescens* as it produced smooth-walled straight conidia with 3-4 septa

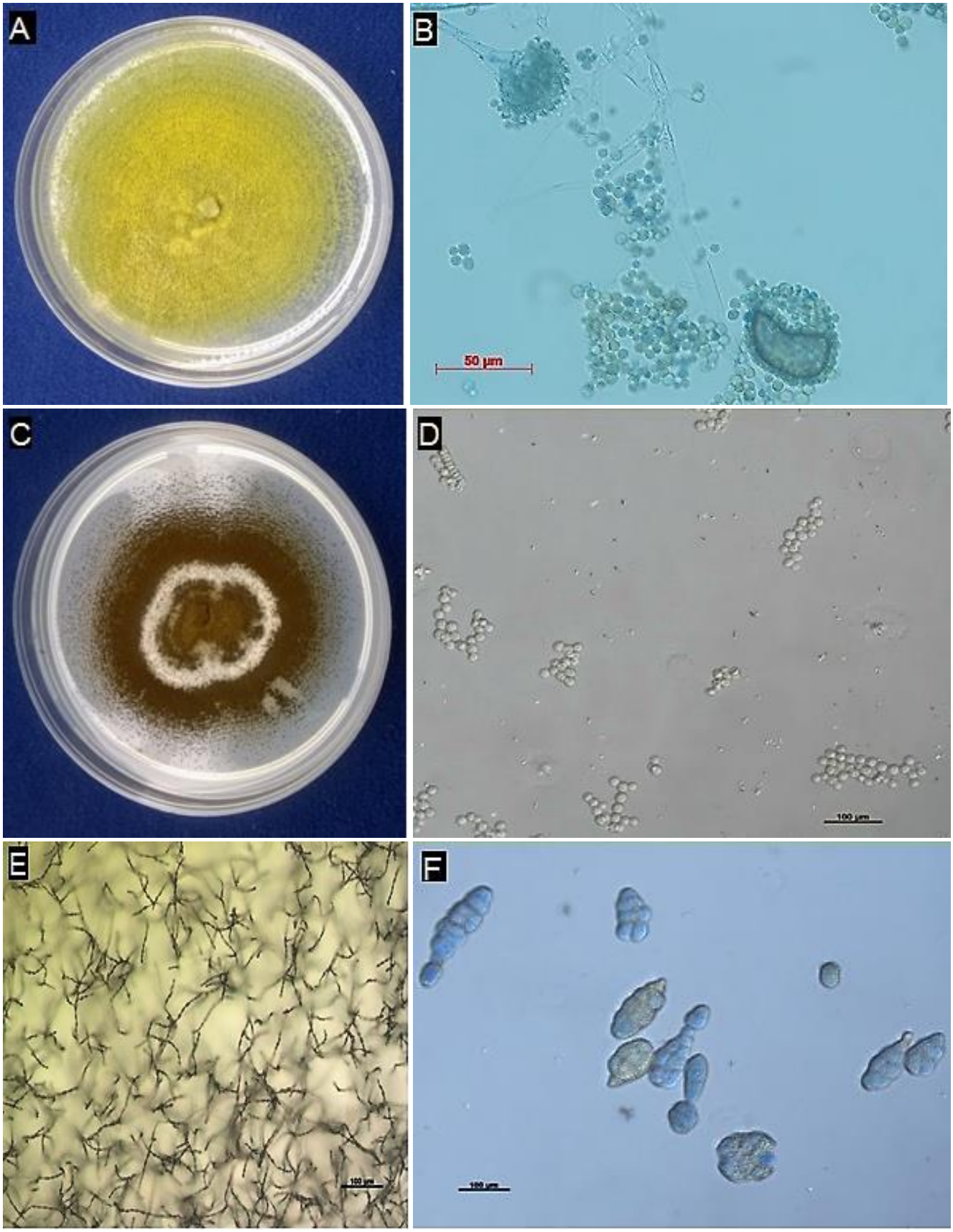

Figure 3.1: Morphological characteristics of seed-borne fungi associated with herb seeds. (A) Culture of *Aspergillus flavus*, (B) Subglobose conidia and conidial heads of *A. flavus*, (C) Culture of *Aspergillus niger*, (D) Brown globose conidia of *A. niger*, (E) Straight chain sporulation pattern of *Alternaria tenuissima*, (F) Conidia of *A. tenuissima*. [All microscopic images were captured at X40 magnification, except (E) that was captured at X20 magnification].

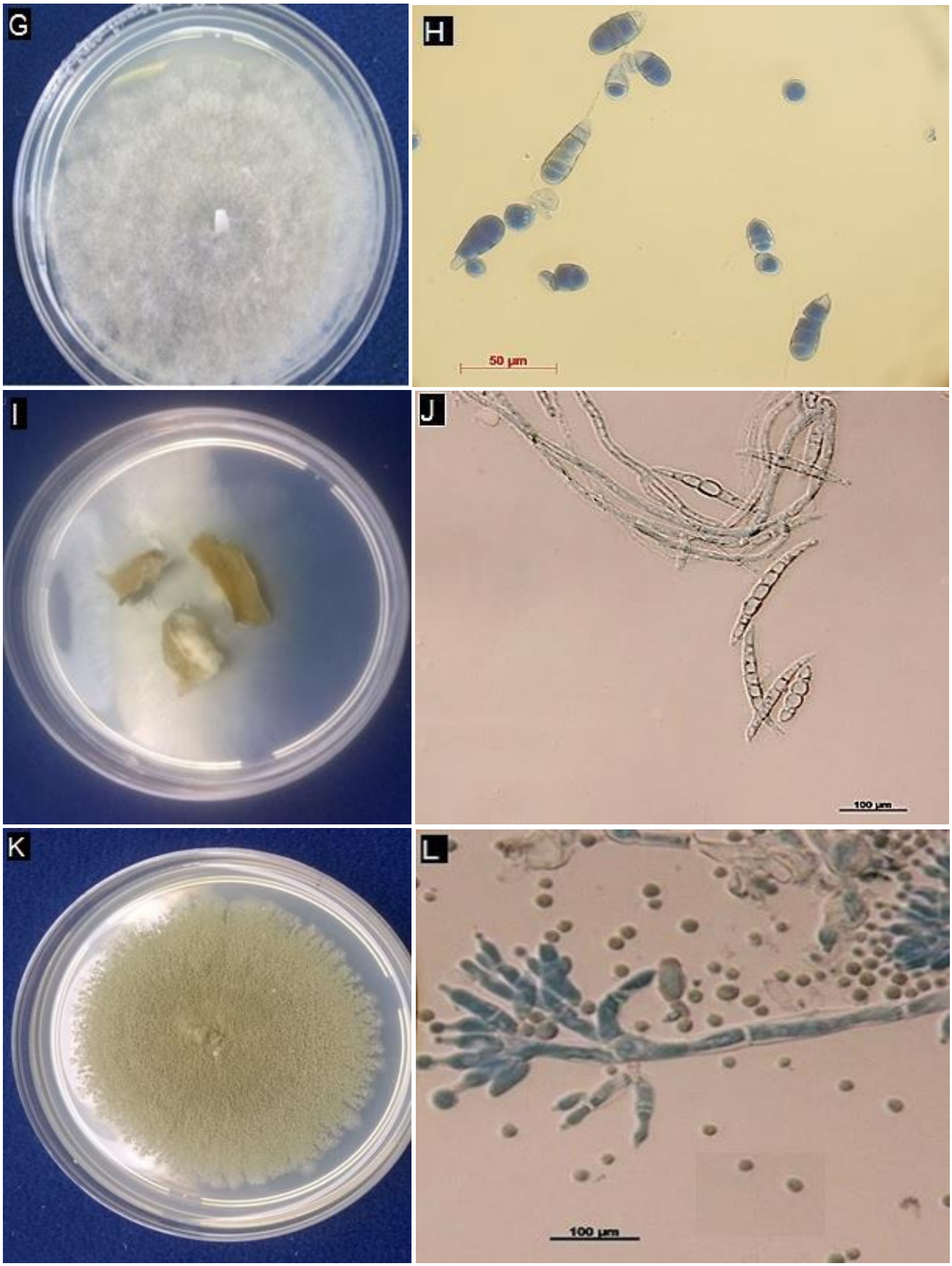

Figure 3.1: Morphological characteristics of seed-borne fungi associated with herb seeds. (G) Culture of *Curvularia pallescens*, (H) Conidia of *C. pallescens*, (I) *Fusarium oxysporum* cultured on carnation leaf agar, (J) Macroconidia of *F. oxysporum*, (K) Culture of *Penicillium expansum*, (L) Hyaline conidiophore of *P. expansum.* [All microscopic images were captured at X40 magnification].

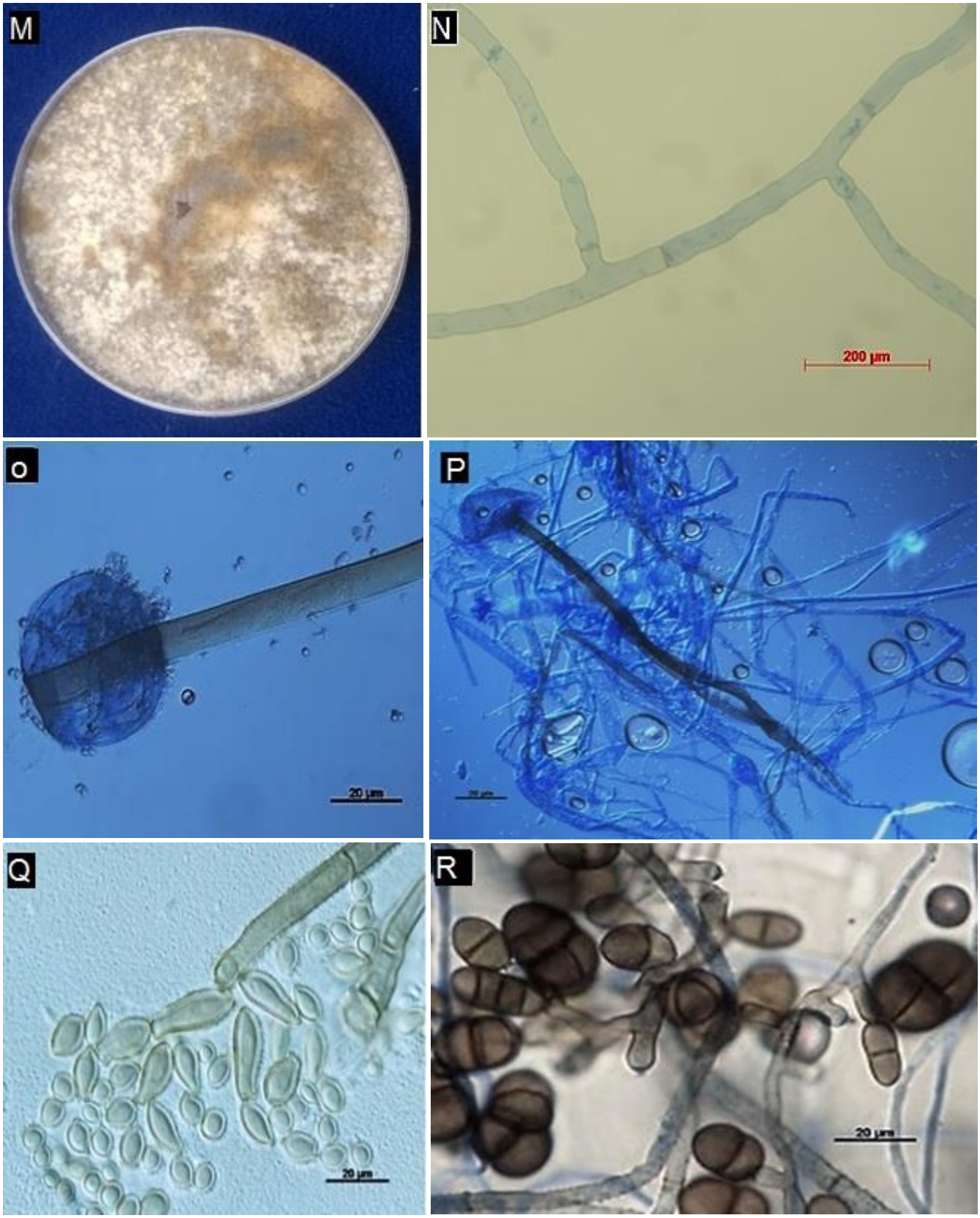

Figure 3.1: Morphological characteristics of seed-borne fungi associated with herb seeds. (M) Culture of *Rhizoctonia solani,* (N) Hyphae of *R. solani* branching at right angles, (O) Sporangia of *Rhizopus* sp., (P) Rhizoids at the base of sporangia of *Rhizopus* sp., (Q) Spores of *Cladosporium* sp., (R) Conidia of *Epicoccum purpurascens*. [All microscopic images were captured at X40 magnification, except for image P captured at X20].

(Figure 3.1H). Average size of conidia measured 19.5 x 8 µm. *Curvularia* sp. was only detected on two seed lots of herb cultivars; sage H071408JJH (3.5%) which was significantly higher than thyme seed lot 33591 (Table 3.3).

Fungi cultured on leaf carnation agar produced white or cream coloured mycelia (Figure 3.1I), and produced numerous thin walled 3-5 septate macroconidia measuring 45 x 4 µm in size, matching the description of *Fusarium oxysporum* given by Leslie and Summerell (2006) [Figure 3.1J]. *Fusarium oxysporum* was most prominent on chives 31744 seed lot (7.5%) compared to the other herb seed cultivars. Incidences of *F. oxysporum* detected on basil seed lot M043803CAC, coriander seed lots M074163HAC and coriander N056404HAD were all 5% lower when compared to chives 31744 seed lot. The lowest incidence of *F. oxysporum* was recorded on sage seed lot H071408JJH.

The fungal culture shown in Figure 3.1K produced bluish green powdery colonies with hyaline conidiophores measuring 500 x 3 µm. Penicillates are known to branch at the apex, with branches terminating in groups of 5 to 9 phialides which are typical features described for *Penicillium expansum* [Figure 3.1L] (Ellis and Ellis, 1997). *Penicillium expansum* was the most common fungus, which was detected on chives 31744 seed lot (2%); basil seed lot M043803CAC (3.5%); all coriander seed lots (3.5; 9; 5%, respectively), wild rocket seed lot 33013 (3.5%), sage seed lot H071408JJH (4%) and thyme seed lot L095502FAB (5.5%).

A brown mycelium growing in a radiating manner from single spore cultures isolated from parsley was observed (Figure 3.1M). Further observations under a light microscope showed distinct 90^{o} branching characteristic of *Rhizoctonia solani* [Figure 3.1N] (Mathur and Kongsdal, 2003).

Sporangiophores of a fungus shown in Figure 3.1O have an average length of 1500 µm and 18 µm in width, are aseptate and highly branched and grow from stolons opposite rhizoids (Figure 3.1P). This description matched that of *Rhizopus oryzae* (Ellis and Ellis, 1997).

Single spore isolate of the fungus shown on Figure 3.1Q produced smooth aseptate conidia measuring 4-9 µm x 3-5 µm. Based on the description of Ellis and Ellis (1997),

Table 3.3: Presence and frequency of seed-borne fungi associated with herb seeds of the Apiaceae and Lamiaceae family

Herb seed	Lot/Ref No.	Incidence (%) of seed-borne fungi[a]											
		A.t*	***A.n****	***A.f****	***C.h****	***C.p****	***E.p****	***F.o****	***P.e****	***R.s****	***R.sp****	***T.v****	**Total**
Basil	33590	-	-	-	-	-	-	-	-	-	-	-	0
Basil	M043803CAC	17.5 d	-	-	-	-	-	2.5 b	3.5 bc	-	-	-	23.5
Chives	31744	-	-	-	-	-	-	7.5 a	2 cd	-	-	-	9.5
Coriander	34184	73 a	2.5 b	0.5 c	-	-	-	-	3.5 bc	-	-	-	79.5
Coriander	M074163HAC	28.5 c	1.5 b	2b c	-	-	-	2.5 b	9 a	-	-	-	43.5
Coriander	N056404HAD	45 b	4 a	-	-	-	-	2.5 b	5 b	-	3 a	-	59.5
Dill	33442	28.5 c	-	-	-	-	1.5 c	-	-	-	-	-	30.0
Parsley	33614	4.5 e	-	-	-	-	-	-	2 cd	1.0 a	-	-	7.5
Parsley	M051102CAC	-	-	-	3.5 a	-	2.5 b	-	-	-	-	2 a	8.0
Sage	H071408JJH	-	4.5 a	-	-	3.5 a	-	0.5 c	4 bc	-	3 a	-	15.5
Thyme	33591	5 e	-	5.5 a	1.5 b	1.5 b	7 a	-	-	-	-	-	19.0
Thyme	L095502FAB	-	-	3.5 b	-	-	-	-	5.5 b	-	-	-	9
Wild Rock	33013	-	1.5 b	3 b	-	-	-	2.5 b	3.5 bc	-	-	-	10.5
LSD		0.136	0.114	0.111	0.061	0.132	0.064	0.061	0.107	0	0.022	0	
CV%		23.55	68.57	67.76	94.69	266.56	54.53	31.04	29.36	0	51	0	

[a]Means in the same column followed by the same letter are not significantly different according to Fisher's LSD test at $p<0.05$.
**A.t= Alternaria tenuissima*; *A.n= Aspergillus niger*; *A.f= Aspergillus flavus*; *C.h= Cladosporium herbarum*; *C.p= Curvularia pallescens*; *E.p= Epicoccum purpurascens*; *F.o= Fusarium oxysporum*; *P= Penicillium expansum*; *R.s= Rhizoctonia solani*; *R= *Rhizopus* sp.; *T.v= Trichoderma viride*.

the isolate was identified as *Cladosporium herbarum*, which was detected on parsley seed lot M051102CAC (3.5%) and was significantly higher than thyme seed lot 33591 (Table 3.3).

Cultures identified as *Epicoccum purpurascens* produced brown granular sporodochia containing numerous spherical black conidia with a diameter range of 15-25 µm (Figure 3.1R). Thyme seed lot 33591 recorded the highest incidence of *Epicoccum* sp. (7.0%). Dill seed lot 33442 had the lowest incidence of *Epicoccum* sp. (1.5%).

3.3.2 Seed germination tests

Results of the seed germination tests are shown in Table 3.4. The highest number of normal seedlings was recorded on basil seed lot 33590 (86.8%); however, not significantly different than basil seed lot M043803CAC (82.5%) and parsley seed lot 33614 (83.2%). Percentage normal seedlings of chives seed lot 31744 and all coriander seed lots was significantly lower (5.8-9.8%) compared to basil seed lot 33590. The lowest percentage normal seedlings was recorded for sage (68.5%).

Seed germination tests yielded abnormal seedlings which included both deformed and diseased seedlings. The average percentage of deformed seedlings ranged from 5.5-7% for wild rocket seed lot 33013, basil seed lot M043803CAC, coriander seed lot M074163HAC, dill seed lot 33442, parsley seed lot 33614, sage seed lot H071408JJH and all thyme seed lots. The remaining seed lots had a significantly lower percentage of deformed seedlings, which ranged from 3.5-4.5% with the lowest percentage recorded on chives seed lot 31744.

Generally, diseased seedlings accounted for the larger percentage of abnormalities compared to deformed seedlings. Parsley seed lot M051102CAC recorded the highest percentage of diseased seedlings (8%) and there was no statistical difference when compared with coriander seed lot N056404HAD and basil seed lot M043803CAC seed lots (7.5%, both). In addition, the percentage of diseased seedlings that germinated from chives seed lot 31744, coriander seed lot M074163HAC and dill seed lot 33442 seed lots (6.5%; 7%; 6.5%, respectively) showed no statistical difference when compared with those of coriander seed lot N056404HAD and basil seed lot 33590. Coriander seed lot M074163HAC and wild rocket seed lot 33013 seed lots yielded the lowest percentage of diseased seedlings; however, no statistical differences were observed when

compared with basil seed lot 33590 and sage seed lot H071408JJH (3.5%; 3.8%, respectively).

Table 3.4: Standard seed germination tests of herb seeds of Apiaceae and Lamiaceae plant families.

			Abnormal seedlings (%)*	
Herb seed	**Lot/Ref No.**	**Normal seedlings (%)***	**Deformed**	**Diseased**
Basil	33590	86.8 a	4.5 bc	3.5 ef
Basil	M043803CAC	82.5 abc	6.3 a	7.5 a
Chives	31744	79.8 bc	3.5 c	6.5 abc
Coriander	N056404HAD	77.5 c	4.0 c	7.5 a
Coriander	M074163HAC	78.8 c	4.0 c	2.8 f
Coriander	34184	72.8 c	5.5 ab	7.0 ab
Dill	33442	74.0c	5.8 ab	6.5 abcd
Parsley	33614	83.3 ab	4.5 bc	4.5 de
Parsley	M051102CAC	79.5 bc	5.5 ab	8 a
Sage	H071408JJH	68.5 d	6.3 a	3.8 ef
Thyme	33591	78.8 c	5.8 ab	5.0 bcde
Thyme	L095502FAB	81.0 bc	6.0 a	5.0 cde
Wild Rocket	33013	71.3 d	7.0 a	2.8 f
LSD		0.1001	0.0803	0.0846
CV%		3.213	15.789	12.857

*Means in the same column followed by the same letter are not significantly different according to Fisher's LSD test (at $p<0.05$) using the GLM procedure. Values are means from two hundred seeds in four sub-replicates.

Correlation analysis showed that amount of seed-borne fungi had a weak correlation with germination of herb seeds ($r= -0.129$, $p<0.01$). The percentage of diseased plants recorded were positively correlated with the incidence of seed-borne fungi detected on herb seeds ($r= 0.239$, $p<0.01$). The study also indicated no relationship between amount of seed-borne fungi and the percentage of deformed seedlings yielded from germinating herb seeds ($r = -0.369$, $p<0.01$).

3.3.3 Pathogenicity test

The pathogenicity test showed that seed-borne *Alternaria tenuissima* was pathogenic to coriander cv. American long (Figure 3.2). Inoculated coriander plants displayed symptoms after 9 days. The symptoms appeared as small, dark brown to black circular lesions (diameter <5 mm) [Figure 3.2A]. As time progressed, lesions enlarged and

coalesced to form dark brown blotches (Figure 3.2B). These symptoms were observed on both wounded and non-wounded plants (Figure 3.3). *Alternaria* disease was most severe on wounded coriander leaves that were inoculated with the pathogen (64%) compared to non-inoculated wounded and non-wounded leaves (Figure 3.3).

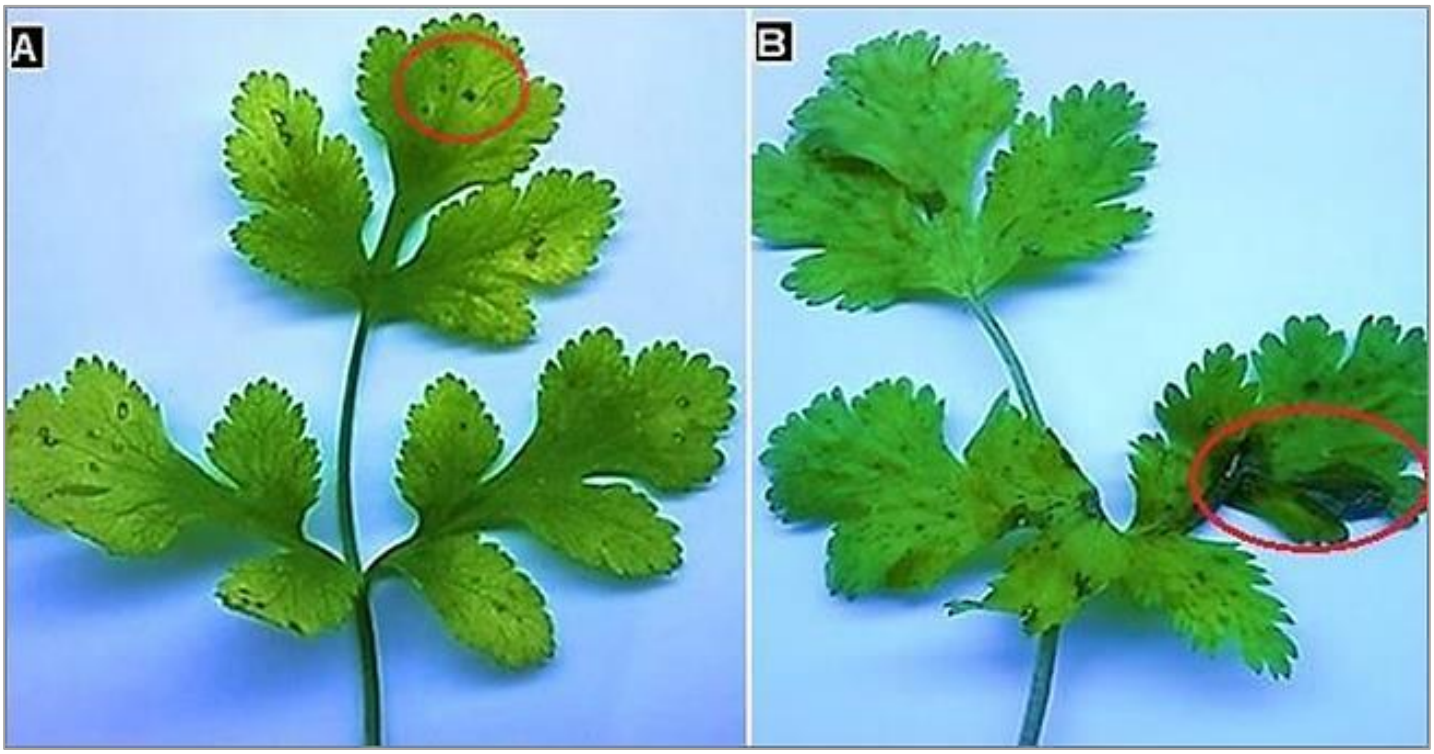

Figure 3.2: Symptoms of leaf spot disease caused by inoculating wounded coriander leaves with *A. tenuissima*; (A) small leaf lesions encircled in red [diameter <5 mm] (B) coalescing of leaf lesions [diameter >5 mm] forming a necrotic spot as highlighted in red.

The pathogenicity assay showed that seed-borne *A. tenuissima* is pathogenic and symptoms of *Alternaria* leaf spot disease also appeared on wounded and unwounded control plants (Figure 3.3). Wounded and inoculated plants had the highest severity of disease (64%). Pathogenicity of *A. tenuissima* on coriander was confirmed and it was re-isolated from diseased leaves of both inoculated and uninoculated plants thereby fulfilling Koch's postulates and also proving seed transmission of the pathogen.

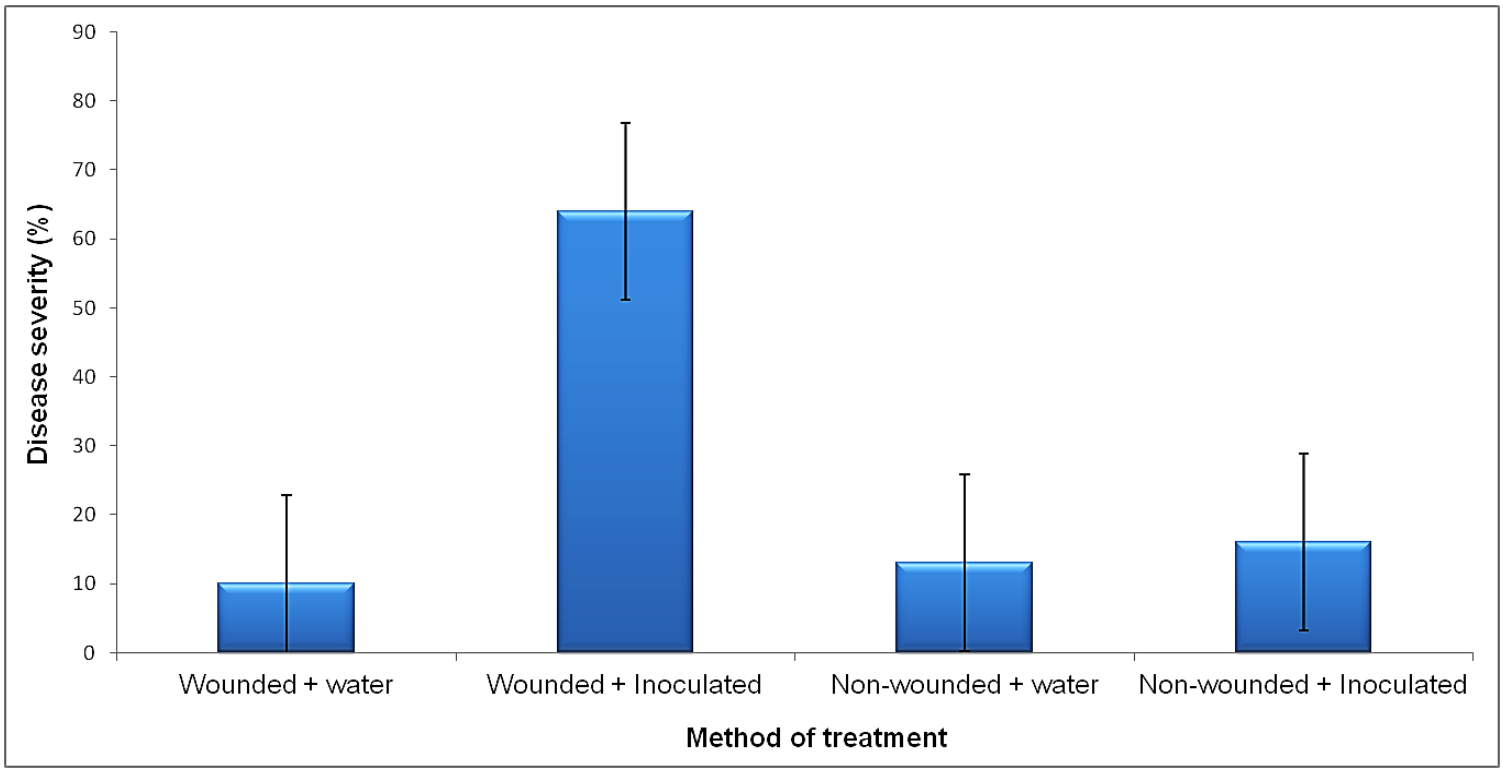

Figure 3.3: Severity of *Alternaria* leaf spot disease on coriander recorded after 14 days of inoculation. Results presented are mean values of two trials with five replicates per trial.

3.4 Discussion

It is important to test seeds for the presence or absence of disease causing organisms (ISTA, 2013). Apart from introducing new sources of inoculum into the field, infected seeds may also spread diseases into non-diseased areas (Agrios, 2005). Reports of incidences of seed-borne and seed-transmitted diseases of herbs continue to increase (Moya et al. 2004; Farahani-Kofoet et al. 2012). In South Africa, only a few diseases have been reported on local herb farms and little has been done to establish their possible causes and sources (Swart and van Niekerk, 2003; McLeod et al. 2006). It is possible that commercial Apiaceae and Lamiaceae seed produced in South Africa are potential sources of diseases reported on local herb farms.

There are no set standards for certification of herb seed in South Africa (Plant improvement Act, 1976), except for parsley, for which a minimum germination of 50% has been stipulated. This study showed that parsley seed lots 33616 and M051102CAC were above this value by 33.25% and 29.5%, respectively. With reference to Vermont Seed Standard Regulations (2014), 75% and 80% are the minimum percentage germination percentages required for seed germination tests of dill and wild rocket,

respectively. However, this study showed that both seed lots were below the minimum acceptance level by 1% and 8.75% for dill and wild rocket, respectively. It was also found that coriander seed lots N056404HAD, M074163HAC and 34184 were above the minimum germination level (70%) by 7.5; 8.75; 2.75%, respectively, which is acceptable according to the International Seed Federation (2013). Likewise, sage was above the acceptable germination percentage of 60% by 8.5%.

A total of 10 genera of fungi were associated with herb seed samples. The diversity of seed-borne mycoflora detected in this study was low compared to previous studies in which between 13 to 17 genera of fungi were detected (Szopińska and Bralewski, 2006*;* Pavlović et al. 2008; Szopińska et al. 2008; Jayshree et al. 2011). It is possible that the technique used was not sensitive enough to detect all of the seed-borne mycoflora. For instance, Chiocchetti et al. (2001) showed that detection thresholds of *P. cucumerina* were improved by using molecular techniques (PCR and RAPD) as opposed to the use of conventional techniques. The difference observed in the diversity of seed-borne mycoflora recorded for locally produced seeds in South Africa compared to other countries might have been attributed to differences in weather conditions in the respective localities. Prevailing weather patterns and availability of nutrients during plant development, particularly during the reproductive stage affect the distribution and concentration of inoculum as well as the susceptibility of the host to infection by the pathogen. Thus, if the conditions are unfavourable for the plant, chances of disease development are high and could result in infected seeds (Angulo-Romero et al. 1999; Mitakakis et al. 2001; van der Waals et al. 2003; Gofroń and Rapiejko, 2009).

Infection of Apiaceae and Lamiaceae seeds with *Alternaria* spp. has been reported by other workers (Pryor et al. 1994; Kwasna, 1992; Bulajić et al. 2009; Pant, 2011). The seed health test results of coriander and basil seed lots support these findings; however, A*. tenuissima* was not previously recorded on these herb seeds. In previous studies *A. alternata* was reported as the most commonly isolated seed-borne fungi associated with seeds of Apiaceae and Lamiaceae (Slobodan et al. 2006; Szopińska and Bralewski, 2006; Szopińska et al. 2008; Bulajić et al. 2009; Pant, 2011; Janas, 2013). It is possible that with the confusion in the systematics of the genus *Alternaria*, several small-spored *Alternaria* spp. may have been previously misidentified resulting in inaccurate deductions being made from statistics on the occurrence and epidemiology of *Alternaria*

spp. (Simmons and Roberts 1993; Ray et al. 2005). Since a variation in growth conditions affect the three dimensional sporulation patterns of *Alternaria* spp., Simmons and Roberts (1993) and Simmons (1997) highlighted the importance of the use of a standardised set of growth conditions to minimise differences observed during morphological and molecular identification of *Alternaria* spp. Subsequently, studies are increasingly being reported with better clarity and success in identifying various *Alternaria* spp. (Garrido et al. 2013; Oviedo et al. 2013; Zheng and Wu, 2013).

The current study of pathogenicity of seed-borne *A. tenuissima* showed development of leaf spots on inoculated coriander cv. American long seedlings. The appearance of disease symptoms on inoculated wounded coriander leaves and uninoculated controls proves that *A. tenuissima* is pathogenic and both seed-borne and seed-transmitted as was also shown by previous studies on other crops (Shortt et al. 1982; Nasir, 2003; Sultana and Ghaffar, 2009). There are several reports of *A. tenuissima* causing diseases on different hosts i.e. broad bean (*Vicia faba* L.) leaves (Honda et al. 2001), cereal grains (Kosiak et al. 2004), cotton (*Gossypium herbaceum* L.) (Davis et al. 1977), strawberry (*Fragaria ananassa* Duchesne) fruits (Lee et al. 2001) and many others. In spite of reports of *A. tenuissima* on apples (*Malus domestica* Borkh.), cactus pear (*Opuntia littoralis* Mill.) and amaranth (Amaranthus hybridus L.) in South Africa (Serdani et al. 2002; Swart and Kriel, 2002; Blodgett and Swart, 2002), its association with seeds of coriander and other herbs has not been previously recorded probably because little research has been conducted on herbs in South Africa.

A low incidence of seed-borne *Fusarium* sp., and *Rhizoctonia* sp. was detected on herb seed samples. Previous studies also showed similar results in which infection by *Fusarium* spp. was lower than 5% on herb seeds (Slobodan et al. 2006; Sigillo et al. 2013). Further studies are required to determine if this *Fusarium* sp. is a pathogen and is seed-transmitted.

Since most herb seeds are mainly used in the production of spices (Blank and Grosch, 1991), it is important that seed health tests be done regularly to monitor the microbiological profile of spices. Although other workers have reported contamination of spices by toxigenic moulds (Jayshree et al. 2011), little data is available on the occurrence of toxigenic fungi of herbs and spices produced in South Africa (Baxter and Holzapfel, 1982). Apart from reducing normal plant development, some of the seed-

borne fungi isolated in this study, such as *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* are important toxigenic fungi that should not be consumed by humans (Pant, 2011). Future toxigenic studies should take the presence of these fungi on herb seeds into account.

The amount of seed-borne fungi had a weak correlation with germination of herb seeds and this may be the reason why different amounts of fungi detected on seeds of the same herb species had no significant effect on seed germination percentage. For instance, no significant differences were observed in germination capacities of all coriander seed lots irrespective of the amount of seed-borne mycoflora associated with them. Despite higher concentrations of seed-borne mycoflora associated with parsley seed lot M051102CAC compared with parsley seed lot 33614, there was no significant difference in the number of parsley seed that germinated. The presence of *Trichoderma* sp. on seed lot M051102CAC might explain this observation. Several *Trichoderma* spp. are well-known for their ability to parasitize other fungi (Howell, 2003; Harman, 2006). Several studies have also shown that *Trichoderma* sp. can out-compete many pathogenic fungi by competing for nutrients; however, mycoparasitism has been shown to be the principle mechanism of biological control (Howell, 2002; John et al. 2010). The low germination capacity of sage may be contributed to the fact that seed supplied was harvested in the 2011/2012 agricultural season which is two years before this study was conducted. A study conducted by Slobodan et al. (2006) supported this observation as it showed rapid decrease of germination capacity of sage seeds with an increase in storage time.

In conclusion, the current study showed that commercial herb seeds were infected with various seed-borne fungi, which may be possible sources of inoculum in local fields (Swart and van Niekerk, 2003). In addition, detection of seed-borne fungi has indicated risk of spread of seed-borne fungi into new unaffected areas as previously reported by Elmer et al. (1994), Keinath (1994), Chiocchetti et al. (1999), Garibaldi et al. (2004) and Moya et al. (2004). Since the study showed a positive correlation between amount of seed-borne mycoflora and percentage of diseased seedlings, use of healthy certified seed is recommended for farming and export purposes. In addition, this study showed the presence of fungi of possible toxigenic nature which emphasises the importance of conducting microbiological contamination tests if seeds are intended for consumption.

Further investigations to assess the pathogenicity of isolated seed-borne fungi would be helpful to determine whether they are a potential threat to the herb industry. There is also a need for continued research to improve and develop fast and more sensitive detection techniques for a timely decision in trade or seed movement for phytosanitary certification.

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CHAPTER 4

In vitro evaluation of plant extracts and hot water seed treatment for the control of *Alternaria tenuissima* associated with seeds of coriander (*Coriandrum sativum* L.)

Abstract

The current study was conducted to screen acetone, ethyl acetate and water plant extracts of *Allium*, *Carica*, *Datura*, *Lantana*, *Tagetes* and *Zingiber* for their antifungal activities against *Alternaria tenuissima*. In addition, effects of hot water treatments of coriander seeds on incidence of *A. tenuissima* and seed germination were studied. Minimum inhibitory concentrations (MIC) of plant extracts were determined using an agar infusion assay. The MICs of acetone plant extracts of *Allium*, *Carica*, *Datura*, *Lantana* and *Zingiber* were 2.5, 15, 10, 15 and 2.5 mg/ml, respectively; whereas, MICs of ethyl acetate extracts were 5, 15, 5, 10 and 5 mg/ml, respectively. MICs for water extracts were only recorded for *Carica*, *Lantana*, and *Zingiber* (15, 15 and 10 mg/ml, respectively). Antifungal activities of plant extracts were evaluated using the disc diffusion assay. Discs impregnated with acetone extracts of *Allium*, *Datura* and *Zingiber* at a concentration of 15 mg/ml completely inhibited growth of *A. tenuissima* (100%) and discs impregnated with extracts of *Tagetes* had the lowest antifungal activity (33.2%). Ethyl acetate plant extracts at 15 mg/ml showed an antifungal activity which was comparable to that of the synthetic fungicide fludioxonil and mefenoxam (the commercial product Celest® XL), except for *Carica* and *Tagetes* which were significantly lower by 19.35 and 82.66%, respectively. Discs impregnated with water extracts of *Lantana* at 15 mg/ml showed the highest inhibition (93.21%). However, discs impregnated with *Tagetes* at a concentration of 5 mg/ml yielded the lowest antifungal activity (1.42%) against *A. tenuissima*. Naturally infected coriander seeds were hot water treated at temperatures ranging from 39 to 57ºC at intervals of 3ºC for periods of 1 to 60 mins. Soaking coriander seeds in a 54ºC water

bath for 15 mins resulted in the highest seed germination percentage (85%). Although hot water treatments at higher temperature-time parameters resulted in the lowest incidence of *A. tenuissima*, germination capacity was significantly reduced. Based on the results of this study, it can be suggested to substitute chemicals with organic extracts of *Allium*, *Datura* and *Zingiber* (15 mg/ml) and hot water treatment at 54ºC for 15 mins to eradicate *A. tenuissima* associated with coriander seeds. However, there is a need for further evaluations to determine the efficacy of the seed treatments under greenhouse and field conditions.

4.1 Introduction

Alternaria is a cosmopolitan genus that embraces more than 300 species, which occur as saprophytic, endophytic and pathogenic fungi (Kirk et al. 2008). Taxonomy of the genus *Alternaria* has been under continuous review due to confusion associated with the overlapping morphological characteristics of the closely related fungal species (Simmons 1992; Andrew et al. 2009). Of particular interest is *A. alternata* (Fr.) Keissl., *A. arborescens* E.G. Simmons, *A. infectoria* E.G. Simmons and *A. tenuissima* (Kunze) Wiltshire, which are often confused with each other as they share strong morphological similarities. However, Simmons and Robert (1993) introduced a more concise and comprehensive classification criterion that categorised most *Alternaria* spp. into 14 distinctive species-groups, which consequently improved the detection of small-spored *Alternaria* spp. In this regard, there has been an increase in reports concerning the isolation of *A. tenuissima* from different hosts including morama beans [*Tylosema esculentum* (Burch.) Schreiber] (Uzabakiriho et al. 2013), pigeon pea [*Cajanus cajan* (L.) Millsp.] (Balai and Singh, 2013), blueberry (*Vaccinium corymbosum* L.) (You et al. 2014) and sunflower (*Helianthus annuus* L.) (Wang et al. 2014). More recently, the Seed Science Research Group (University of Pretoria, South Africa) detected high incidences of pathogenic *A. tenuissima* (73%) on coriander (*Coriandrum sativum* L.) seeds (unpublished).

Pathogenic *A. tenuissima* can cause seedling damping-off, leaf blight, stems collar rot and fruit lesions on a wide range of host plants (Gannibal et al. 2007; Sharma et al. 2012). Although actual losses have not been documented for herbs, yield declines of more than 40% have been

recorded on other crops (Balai and Singh, 2013; Harteveld et al. 2013). Currently, disease management of *Alternaria* leaf spot is done using synthetic chemicals. Several synthetic fungicides have been reported to be effective in controlling *A. tenuissima*, e.g. cyprodinil, Dithane M-45, mancozeb, pyrimethanil etc. (Kushwaha et al. 2010; Xu et al. 2013). However, the continued excessive use of pesticides in plant disease management programs is being strongly discouraged due to the toxic effects associated with use of these chemicals on humans and the environment (Harris et al. 2001).

Therefore, due to the above mentioned challenges, the search for nature-friendly and safer non-chemical methods in the management of plant diseases has intensified (Nashwa and Abo-Elyousr, 2012). Research has shown potential for the use of hot water seed treatments and plant extracts as substitutes to synthetic chemicals (Khare et al. 2003; Shanguang, 2007; Jošić et al. 2012). Apart from the fact that these control methods are cheap, easy to prepare and biodegradable; various plant extracts from many plant families such as Zingiberaceae, Amaryllidaceae and Verbenaceae have been reported to possess antifungal effects on various pathogenic *Alternaria* spp. (Huie, 2002; Nashwa and Elyousr, 2012; Zaker, 2013; Taware et al. 2014). Although studies have shown use of hot water treatments to be effective in cereals and seeds of other vegetables, few studies have been done to evaluate control of *Alternaria* spp. associated with coriander seeds (Nega et al. 2003; Koch et al. 2010).

On the basis of the above, the objectives of this study were: (i) to determine the minimum concentration of organic (acetone and ethyl acetate) and water extracts of *Allium*, *Carica*, *Datura*, *Lantana*, *Tagetes* and *Zingiber* required for inhibiting growth of *A. tenuissima*, (ii) to screen organic and water extracts of the above listed plants for their antifungal activities against *A. tenuissima*, and (iii) to determine the optimal hot water temperature-time treatment combinations that will effectively eradicate *A. tenuissima,* without reducing seed germination.

4.2 Materials and Methods

4.2.1 Source of seed and pathogen isolate

From previous seed health tests (Chapter 3), pathogenic *Alternaria tenuissima* was isolated from a naturally infected coriander cv. American long seed lot provided by a commercial seed company. Pure fungal cultures were maintained on half strength PDA at 4^{o}C until further use.

4.2.2 Plant collection

Selection of plants was based on local availability and previous reports of efficacy against several *Alternaria* spp. on other crops (van der Wolf et al. 2008; Seetha Ramulu et al. 2010; Mahapatra and Das, 2010; Nashwa and Abo-Elyousr, 2012). *Datura* leaves (*Datura stramonium* L.), Spanish flag leaves *(Lantana camara* L.) and khakhibos *(Tagetes minuta* L.) were collected at the Hatfield Experimental farm (University of Pretoria). Pawpaw (*Carica papaya* L.) leaves were sourced from Limpopo province. Fresh garlic cloves (*Allium sativum* L.) and ginger rhizomes (*Zingiber officinale* Roscoe) were purchased from a fresh vegetable supermarket (Food Lover's Market, Brooklyn, Pretoria, South Africa). Sample specimens of each plant material were identified at the H.G.W.J Schweickerdt Herbarium, Department of Plant Science at the University of Pretoria, where voucher numbers were assigned to each (Table 4.1).

4.2.3 Preparation of crude plant extracts

Plant parts were separately dried in the shade at room temperature and ground to a fine powder using a Macsalab mill (Model 200 LAB, Eriez®, USA). Powdered plant material (1 kg for each sample) was submitted for extraction by dissolving in 2 l of each organic solvent (acetone or ethyl acetate) and water [90% sterile distilled water plus 10% dimethyl sulphoxide to increase polarity and increase penetration (DMSO, Merck)]. Thereafter, each mixture was thoroughly shaken at 1500 rpm with a hand held homogeniser (PRO 250, Monroe, CT USA) for 5 min. Solvent powder mixtures were left on a rotary shaker at 110 rpm for 48 h after which filtration was done using a 0.45 µl filter funnel connected to a vacuum pump (Masangwa et al. 2013). For organic filtrates, organic solvents were vaporised in a Buchi-Rotavapor (Model R-200, Switzerland). Water filtrates were concentrated to powder using a freeze drier (Edwards

High vacuum International, Sussex, England) at -80ºC. Final powdered plant extracts were put in glass vials, weighed and stored at -20ºC until required for further tests.

4.2.4 Determination of MIC of plant extracts

The agar infusion method described by Kritzinger et al. (2005) was used to determine the minimal inhibitory concentration (MIC) of plant extracts. Final plant preparations were done by dissolving the extracts in separate solvents, viz. water, acetone and ethyl acetate, to form stock solutions, which were sterilized using filters with a pore size of 0.45 µm (Millex-HA, Merck, Pretoria, South Africa). Sterile stock solutions were prepared to obtain different concentrations (0.5, 1.0, 2.5, 5.0, 10.0 and 15 mg/ml) and incorporated aseptically to sterile molten potato dextrose agar (PDA, Merck). The mixing temperature was not allowed to exceed 40ºC to prevent inactivation of plant extracts. The amended PDA media was gently shaken to homogenise distribution of the plant extracts before pouring into 50 mm diameter Petri dishes. Similarly, concentrations of 0.5, 1.0, 2.5, 5.0, 10 and 15 mg/ml Celest® XL (25 ai/l fludioxonil and 10 g ai/l mefenoxam) were incorporated as standard treatments (positive controls), whereas, separate solvents used to prepare plant extracts (water, acetone or ethyl acetate) were incorporated in molten PDA as negative controls. A 5 mm diameter mycelial plug of *A. tenuissima*, taken from the edges of a seven day old culture, was placed in the centre of solidified agar plates (Figure 4.1). Inoculated plates (replicated thrice) were randomly arranged in a 25ºC incubator and left to grow for 5 days after which MICs of plant extracts were determined by measuring the radial growth of *A. tenuissima*. The assay was repeated twice and the MIC values were determined by visual observation of the first concentration at which growth of *A. tenuissima* was completely inhibited.

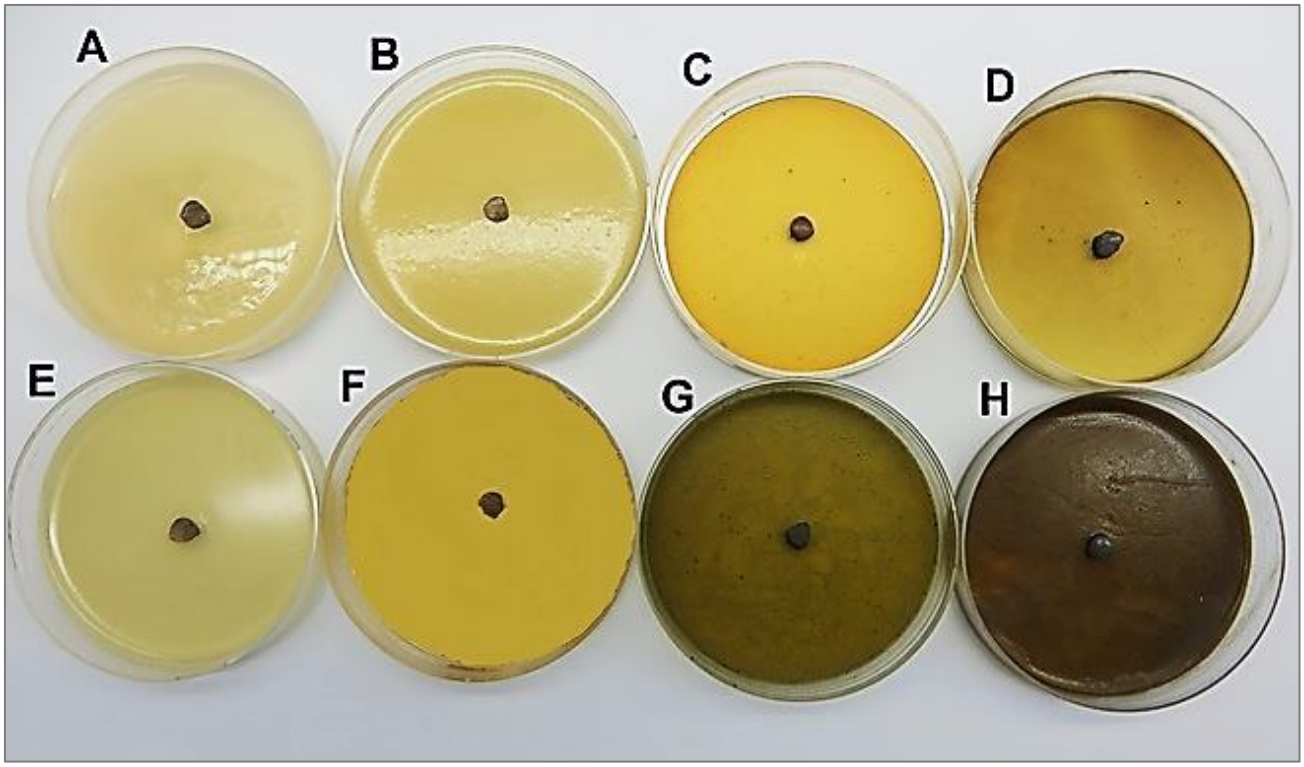

Figure 4.1: A generalised outline of experimental units, different treatments used to amend PDA agar, in the agar infusion assay; A: Celest® XL, B: *Allium*, C: *Zingiber*, D: *Tagetes*, E: media amended with one of acetone, ethyl acetate or water, F: *Carica*, G: *Datura*, H: *Lantana*.

4.2.5 Preparation of pathogen inoculum

Inoculum was prepared by scraping the surface of a 14 day old *A. tenuissima* culture and flooding the plates with sterile distilled water amended with Tween 20 and rubbing the culture with a sterile glass rod to dislodge the mycelia. The mycelial suspension was filtered through three layers of cheesecloth before adjusting the concentration to 10^5 spore/ml using a haemocytometer.

4.2.6 Antifungal activity of plant extracts against *A. tenuissima*

Powdered plant extracts (prepared as described in section 4.2.2) were dissolved in the same solvent used to prepare them, viz. 10% DMSO, ethyl acetate or acetone, to yield stock solutions of 0.5, 1, 2.5, 5.0, 10.0 and 15.0 mg/ml. Thereafter, stock solutions were sterilised by passing through a 0.45 µm syringe filters and their antifungal activity examined using a modified disk diffusion method used by Murray et al. (1995). In this assay, 100 µl of *A.*

tenuissima inoculum concentrated to 10^5 spore/ml (prepared as described in section 4.2.4) was spread on 90 mm Petri dishes containing PDA. Sterile filter paper disks (6 mm in diameter) were impregnated with 10 µl of sterile stock solution of plant extracts, at a concentration of 0.5, 1, 2.5, 5.0, 10.0 and 15.0 mg/ml, and aligned on the surface of an inoculated Petri dish (Figure 4.2). Sterile disks impregnated with the same solvents used to dissolve the plant extracts, viz. 10% DMSO, acetone or ethyl acetate, served as negative controls. The experimental units were all compared against the positive treatments, which consisted of discs impregnated with 0.5, 1, 2.5, 5, 10 and 15 mg/ml of Celest® XL (25 ai/l fludioxonil and 10 g ai/l mefenoxam). Treatments were replicated three times and randomly arranged inside a 25ºC incubator.

The antifungal activity of plant extracts was determined by measuring the inhibition zones of *A. tenuissima* produced around the disks after three and six days of incubation. The *in vitro* assay was repeated twice. The percentage inhibition was calculated using the following formula (Soumya and Nair, 2012):

% inhibition of growth of fungi = $\frac{Dt}{Dc}$x100

Where, D_c = zone of inhibition around disc impregnated with Celest® XL.

D_t = zone of inhibition around disc impregnated with plant extract or solvent

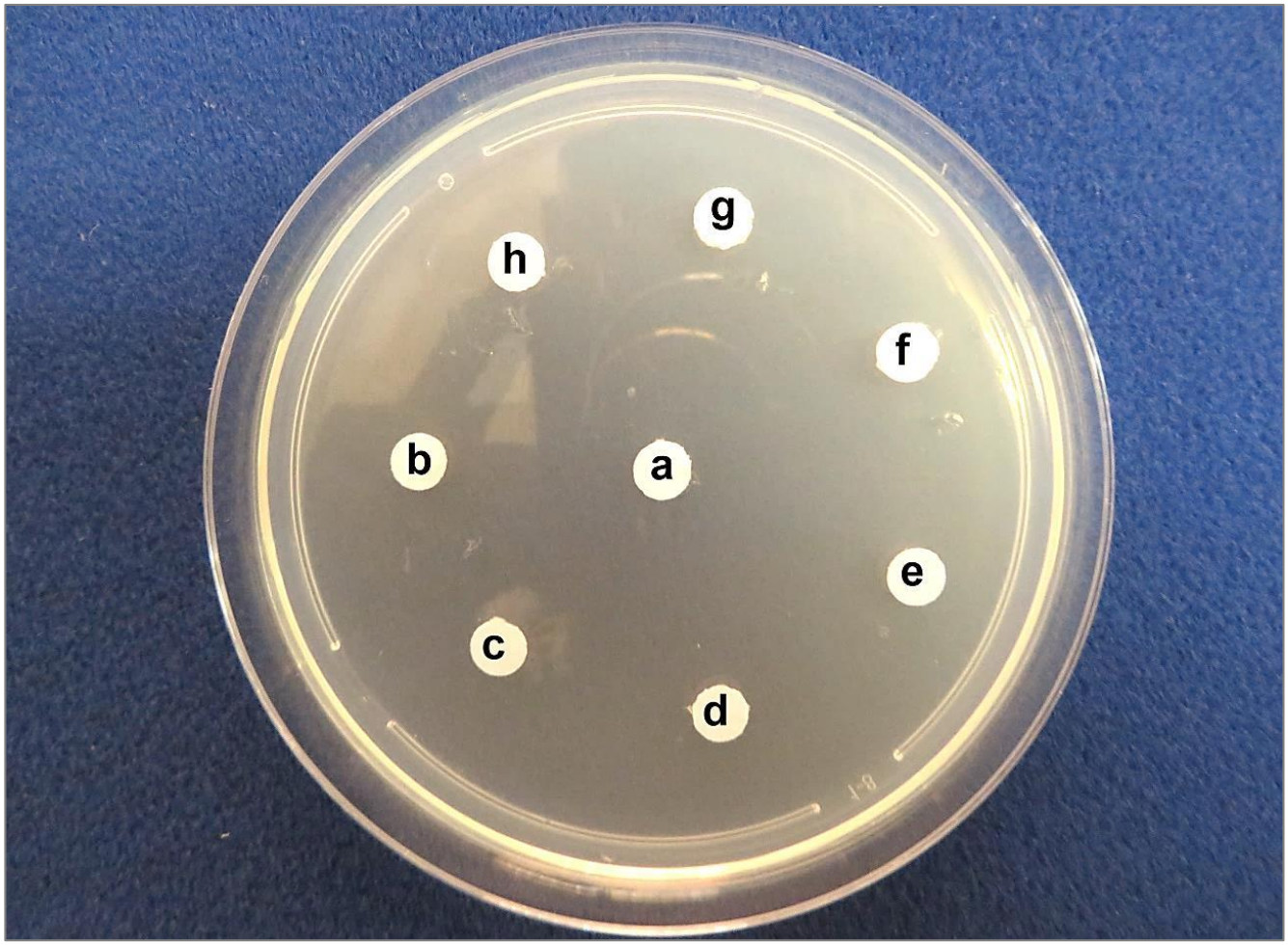

Figure 4.2: A generalised illustration of arrangement of experimental units consisting of different treatments impregnated on filter discs numbered; a: negative control (acetone, water, or ethyl acetate), b: positive control (Celest® XL), c: *Allium*, d: *Carica*, e: *Datura*, f: *Lantana*, g: *Tagetes*, h: *Zingiber*.

The percentage inhibition data was arcsine transformed and statistically analysed for variance at 5% level of significance and mean separation was done using the Fisher's Least Significant Difference test (LSD). Values are presented as untransformed data.

4.2.7 Control of *A. tenuissima* using hot water seed treatments

Hot water seed treatments were performed on naturally infected coriander seed according to the protocol used by Masum et al. (2009). A sample size of one hundred seeds was randomly counted and loosely tied in cheesecloth to form an aliquot. For each specific treatment, eight

aliquots were soaked in sterile distilled water at room temperature for 4 h before immersion in a hot water bath (Labotec, Model: 132A).

Glass beakers (600 ml) were filled with 300 ml sterile distilled water and preheated at 2ºC higher than the target temperature prior to introduction of aliquots of seeds. When the temperature reading reached the initial temperature of 39ºC, the aliquots of seeds were soaked for different time periods of 1, 15, 30, 45 and 60 mins. Thereafter, temperature of the hot water bath was gradually raised with 3ºC until a maximum temperature of 57ºC. Aliquots of seed soaked at the above mentioned temperature-time combinations were opened, seeds spread on sterile paper towels and left overnight to air dry on a laminar flow bench. Seeds soaked in sterile distilled water and left for 60 mins at standard room temperature and air dried as above served as controls.

Seed health and seed germination tests were then performed on seeds that were subjected to the different hot water treatments, using modified methods of the International Seed Testing Association (ISTA, 2014; described in 3.2.3). For seed health tests, each replicate comprised of 50 seeds plated on the surface of PDA in a 90 mm Petri dish (10 seeds/ plate). Thus, four replicates of 50 seeds were evaluated for the incidence of *A. tenuissima* after seven days of incubation in a 25ºC incubator. Seed health tests were repeated twice. For seed germination tests, a total of 400 seeds per treatment were sub-divided into eight replicates of 50 seeds. In each sub-replicate, 50 seeds were placed on three layers of moistened paper dolls and covered with the fourth moistened layer then rolled, sealed and incubated at 25ºC under an alternating 12 h darkness and normal light regime. Seedlings were evaluated according to ISTA rules, where percentages of normal or abnormal (deformed and diseased) seedlings were determined after 21 days.

Percentage data of results taken from seed health and germination tests were arcsine transformed to normalize data before statistical analysis of variance using SAS Version 9.3 (SAS Institute, 2010). Statistical means were separated using the Fisher's LSD test (at $p<0.05$).

4.3 Results

4.3.1 Yield of plant extracts

Plant parts of the selected species were extracted using acetone, ethyl acetate and water yielded different quantities of powder extracts as shown in Table 4.1. In general, the highest quantities of plant extracts were harvested using water whereas the lowest quantities were harvested using ethyl acetate.

Table 4.1: Quantities of plant extracts harvested from organic solvents and water.

		Harvested plant extract (g)		
Plant name	**Voucher number***	**Acetone**	**Ethyl acetate**	**Water**
Allium sativum	PRU119639	20.5	13	318
Carica papaya	PRU119726	37	13	96
Datura stramonium	PRU119637	35	17	142
Lantana camara	PRU119636	42	18.25	112
Tagetes minuta	PRU119725	24	21	220
Zingiber officinale	PRU119638	18	12	263

*Voucher numbers were assigned by the University of Pretoria H.G.W.J. Schweickerdt Herbarium.

4.3.2 Antifungal activity of plant extracts

Antifungal tests were performed using the agar infusion method from which MIC values of plant extracts were determined (Table 4.2). Organic extracts of *Allium*, *Zingiber* and the ethyl acetate extract of *Datura* performed well at low concentrations (≤5 mg/ml), where they completely inhibited growth of *A. tenuissima*. Water extracts of *Carica*, *Lantana* and *Zingiber* recorded the highest MIC values of 15, 15 and 10 mg/ml, respectively. At all concentrations evaluated, water extracts of *Allium*, *Datura* and *Tagetes* failed to inhibit growth of *A. tenuissima*; hence, no MIC values were assigned to them. The same trend was observed for organic extracts of *Tagetes*. Celest® XL consistently inhibited *A. tenuissima* at its lowest concentration (0.5 mg/ml) for all extraction solvents (Table 4.2).

Table 4.2: Antifungal activity [expressed as MIC (mg/ml)] of organic and water plant extracts against *A. tenuissima* using the agar infusion method.

	MIC values (mg/ml)		
Plant extract	**Acetone solvent**	**Ethyl acetate solvent**	**Water solvent**
Allium sativum	2.5	5	-*
Carica papaya	15	15	15
Datura stramonium	10	5	-*
Lantana camara	15	10	15
Tagetes minuta	-*	-*	-*
Zingiber officinale	2.5	5	10
Celest® XL	0.5	0.5	0.5

*No inhibition at the maximum concentration (15 mg/ml).

There were no significant differences between repeats of the disc diffusion assays hence, results were pooled and statistical analysis was done. Means of percentage inhibition are shown in Table 4.3. Results presented are for concentrations and plant extracts that only exhibited significant antifungal activities, thus ≥5 mg/ml. In general, the antifungal activities of plant extracts increased over time, from which maximum zones of inhibition were recorded after six days of incubation (Table 4.3). In this regard, description of observations was based on antifungal performance of plant extracts against *A. tenuissima* in plates incubated for six days. At all concentrations tested, Celest® XL effectively inhibited growth of *A. tenuissima*. Discs impregnated with plant extracts recorded a significantly higher antifungal activity against *A. tenuissima* compared to those impregnated with extraction solvents (controls), except for performance of water extract of *Tagetes* at 5 mg/ml (1.4%) (Table 4.3).

Generally, acetone plant extracts showed an increase in antifungal activities with an increase in concentration against growth of *A. tenuissima*. Discs impregnated with acetone extracts of *Allium*, *Carica*, *Datura* and *Zingiber* at 15 mg/ml showed higher antifungal activities (0.4, 1.4, 1.04 and 2.1%, respectively) compared to discs impregnated with Celest® XL; although the difference was statistically insignificant (Table 4.3). Discs impregnated with acetone extracts of *Lantana* and *Tagetes* at 15 mg/ml had a significantly lower antifungal activity against *A. tenuissima* (20.6, 66.8%, respectively) compared to discs impregnated with Celest® XL. At a concentration of 10 mg/ml, only discs impregnated with *Zingiber* and *Datura* extracts were

comparable with Celest® XL. Zones of inhibition of *A. tenuissima* around discs impregnated with other plant extracts, viz. *Allium*, *Carica* and *Lantana*, at a concentration of 10 mg/ml were significantly smaller by 0.4, 2.9 and 28.1%, respectively, when compared to Celest® XL (Table 4.3). Compared to other plant extracts extracted with acetone, discs impregnated with *Tagetes* extracts at 5, 10 and 15 mg/ml had the lowest inhibition zones against growth of *A. tenuissima* (1.9, 4.6 and 6.2 mm) (Table 4.3).

Discs impregnated with ethyl acetate extracts showed an increase in antifungal activity with an increase in concentration, except for a slight decrease of 1.6 and 3.0% recorded when the concentration was elevated from 10 to 15 mg/ml on discs impregnated with *Tagetes* and *Zingiber* extracts, respectively. In general, discs impregnated with ethyl acetate extracts had greater zones of inhibition compared to discs impregnated with acetone and water extracts, except for discs impregnated with *Allium*, *Datura*, *Tagetes*, *Zingiber* at 15 mg/ml and *Carica* extracts at all concentrations as well as discs impregnated with acetone extract of *Tagetes* at 10 mg/ml (Table 4.3). Discs impregnated with ethyl acetate extracts of *Allium* and *Datura* at a concentration of 10 mg/ml showed greater zones of inhibition (0.3 and 0.01%, respectively) compared to discs impregnated with Celest® XL against *A. tenuissima*, although the differences were statistically insignificant. At the highest concentration (15 mg/ml), performance of discs impregnated with ethyl acetate extracts was statistically similar to that of Celest® XL, except for discs impregnated with *Carica* and *Tagetes* in which antifungal effects were significantly lower by 19.4 and 82.7%, respectively (Table 4.3).

Discs impregnated with water extracts showed the smallest zones of inhibition against *A. tenuissima* compared to those measured around discs impregnated with acetone and ethyl acetate plant extracts (Table 4.3). In general, there was an increase of antifungal activity with discs impregnated with water extracts with an increase in concentration, except for a significant decrease of 1.3% observed when the concentration of *Zingiber* was elevated from 10 to15 mg/ml (Table 4.3). Compared to other water extracts, discs impregnated with extracts of *Lantana* at 15 mg/ml recorded the greatest zones of inhibition against *A. tenuissima* (16.5 mm), However, discs impregnated with *Tagetes* at a concentration of 5 mg/ml yielded the lowest antifungal activity (0.3 mm) compared with other plant extracts against *A. tenuissima* (Table 4.3).

Table 4.3: *In vitro* antifungal activity of plant extracts tested against mycelial growth of *A. tenuissima* using the disk diffusion method (six days of incubation).

Plant	Concentration (mg/ml)	Inhibition of *A. tenuissima* [(%)[a], mm*] Acetone solvent	Ethyl acetate solvent	Water solvent
Allium	5	(97.5) 17.8 c	(98.1) 18.1 ab	(18.4) 3.2 j
	10	(99.6) 18.4 b	(100.2) 18.7 ab	(32.5) 5.7 i
	15	(100.4) 18.6 ab	(99.8) 18.7 ab	(37.9) 6.7 h
Carica	5	(79.7) 14.5 e	(64.6) 11.9 h	(5.6) 1.0 o
	10	(97.07) 17.9 c	(78.8) 14.7 g	(12.9) 2.3 kl
	15	(101.4) 18.8 ab	(80.7) 15.1 fg	(17.9) 3.2 j
Datura	5	(77.5) 14.2 fg	(98.5) 18.2ab	(53.4) 9.3 f
	10	(100.02) 18.4 ab	(100.01) 18.7 ab	(65. 6) 11.6 e
	15	(101.04) 18.8 ab	(99.1) 18.6 ab	(71.1) 12.6 d
Lantana	5	(60.6) 11.1 i	(83.03) 15.3 ef	(44.0) 7.7 g
	10	(71.9) 13.3 h	(94.97) 17.8 c	(81.6) 14.4 c
	15	(79.4) 14.8 ef	(98.8) 18.6 ab	(93.2) 16.5 b
Tagetes	5	(10.3) 1.9 l	(19.8) 3.7 i	(1.4) 0.3 p
	10	(24.7) 4.6 k	(18.95) 3.5 i	(9.0) 1.6 m
	15	(33.2) 6.2 j	(17.3) 3.3 i	(18.7) 3.3 j
Zingiber	5	(83.5) 15.3 d	(88.7) 16.4 d	(7.5) 1.3 mn
	10	(100.9) 18.6 ab	(100.4) 18.8 a	(13.8) 2.4 k
	15	(102.1) 19.0 a	(98.4) 18.4 ab	(12.6) 2.2 l
Celest® XL	5	(100.0) 18.3 ab	(100.0) 18.4 ab	(100.0) 17.5 a
Celest® XL	10	(100.0) 18.4 ab	(100.0) 18.7 ab	(100.0) 17.6 a
Celest® XL	15	(100.0) 18.6 ab	(100. 0) 18.7 ab	(100.0) 17.7 a
Control	5	(1.3) 0.2 m	(1.1) 0.2 j	(0.0) 0.0 p
Control	10	(0.8) 0.2 m	(1.2) 0.2 j	(0.0) 0.0 p
Control	15	(1.2) 0.2 m	(1.01) 1.2 j	(0.0) 0.0 p
LSD		0.027	0.020	0.013
CV%		3.14	3.78	5.021

*Values bearing some letters are mean zones of inhibition measured around discs impregnated with different treatments; where in each column, values followed by the same letter do not differ significantly according to Fisher's LSD test (at $p<0.05$).
[a]Values in brackets are percentage inhibition of treatments.

4.3.3 Hot water seed treatments

The effects of different hot water treatments on the incidence of *A. tenuissima* and germination percentage of coriander seed are shown in Figure 4.3. The highest percentage of normal seedlings was recorded for seeds soaked in a 54ºC water bath for 15 mins (85%). Further

increase in temperature above 54ºC resulted in a significant decrease in germination percentage so that coriander seeds soaked at temperature-time combinations beyond 57ºC for 1 min resulted in complete failure of seeds to germinate. In general, the percentage of normal seedlings yielded by soaking seeds at 39ºC were not significantly different compared to the control, except when seeds were soaked at 39ºC for 1 h. Soaking seeds at 42ºC for 15 mins resulted in a significantly higher percentage of normal seedlings (1.5%, both) compared to the control. However, when seeds were soaked at 42ºC for 1 min it resulted in a similar germination percentage.

Soaking coriander seeds in a water bath at 45ºC for 1 minute resulted in the highest number of diseased seedlings, which was comparable to the percentage of diseased seedlings recorded at 39ºC for 45 mins and the control. Treating coriander seeds at a temperature of 39ºC for 1 min resulted in significantly lower (2.5%) number of diseased seedlings compared to the control. Except for aforementioned hot water temperature-time treatments, the remaining treatments (>39ºC) significantly reduced the percentage diseased seedlings compared to the control. In general, there is a decreasing trend on the percentage of diseased seedlings with an increase in exposure time at all temperatures.

The highest incidence of *A. tenuissima* was recorded on seeds treated for 1 minute at 48ºC. In general, hot water seed treatment at 39 and 42ºC maintained a constant incidence of *A. tenuissima*, which ranged from 65 to 70%. Further increases in temperature resulted in a fluctuation of the incidence of *A. tenuissima,* with the highest being recorded at shorter exposure times. Soaking coriander seeds in a 57ºC water bath resulted in the lowest incidence of *A. tenuissima* and there was no sign of the fungus at 30, 45 and 60 mins of soaking.

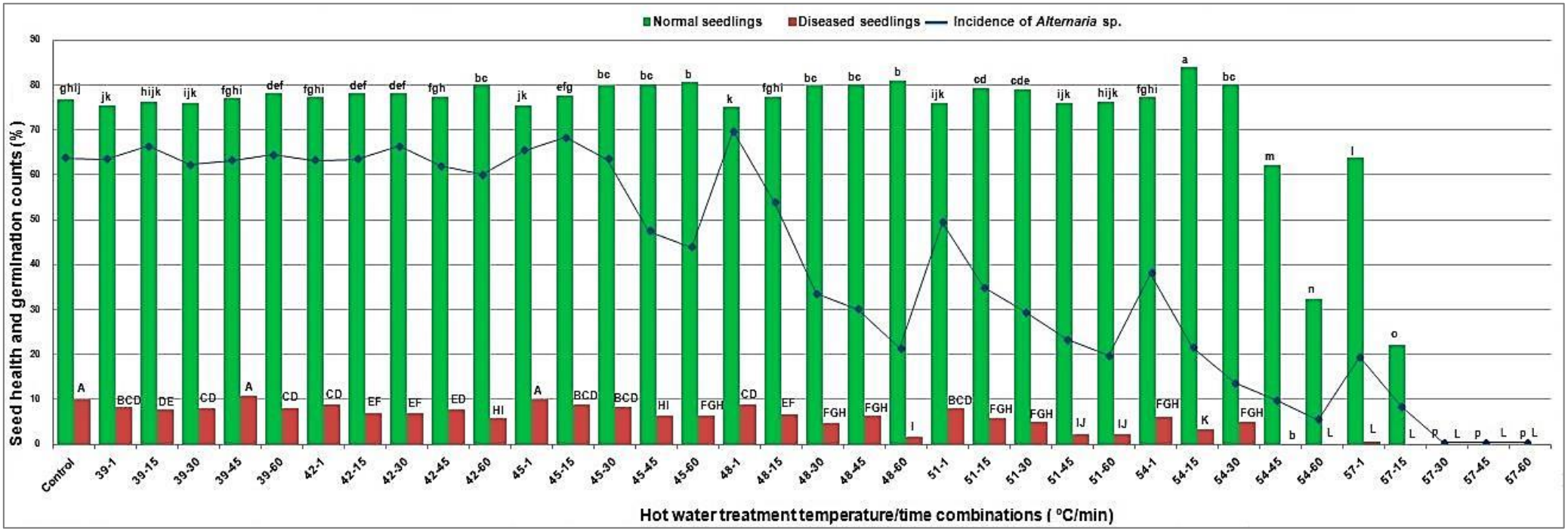

Figure 4.3: Effect of different hot water temperature-time combinations on the incidence of seed-borne *A. tenuissima* and coriander seed germination. Means with different letters indicate significant differences according to Fisher's LSD test (P<0.05). (Normal seedlings: LSD= 1.32; CV= 3.71%. Diseased seedlings: LSD= 0.877; CV= 11.50%. Incidence of *A. tenuissima*: LSD= 2.179; CV= 3.71%).

4.4 Discussion

The future use of synthetic chemicals for the management of plant diseases is being discouraged globally due to their hazardous effects on the environment (Harris et al. 2001). Hence, there is a continuous search for equally effective non-chemical methods for managing plant diseases. In this regard, this study was initiated to screen organic and water plant extracts of *Allium*, *Carica*, *Datura*, *Lantana*, *Tagetes* and *Zingiber* as substitutes for synthetic fungicides in controlling *A. tenuissima*. In addition, evaluations were performed on the use of hot water seed treatments as another alternative non-chemical method for controlling seed-borne *A. tenuissima* associated with seeds of coriander.

The highest quantities of plant extracts were harvested using water solvents compared to those yielded from organic solvents. Similarly, Masangwa et al. (2013) reported that the highest quantities of plant extracts were extracted using water. Lapornik et al. (2005) reported that the quantity of plant extract harvested by different solvents is dependent on the physiochemical properties of antifungal compounds to be extracted and the polarity of the extraction solvent. Since water is a polar solvent, this property may have contributed to increased tissue and cellular permeability during extraction, which may have a significant cumulative effect on the bulkiness of water extracts due to exhaustive extraction of various cellular components (Reichardt, 1994). Indeed, lower quantities of plant extracts were harvested in the current study using non-polar aprotic solvents of acetone and ethyl acetate as they have been reported to show low permeability in plant tissues (Gurjar et al. 2012).

All plant extracts exhibited different levels of antifungal activity against *A. tenuissima*. Similar results were reported by many authors working on various *Alternaria* spp. (Chethana et al. 2013; Ravikumar et al. 2013; Zaker, 2013; Singh et al. 2014). At six days after incubation, ethyl acetate extracts applied at 15 mg/ml showed antifungal activities in descending order of *Allium*> *Datura*> *Lantana*> *Zingiber*> *Carica* and lastly *Tagetes*; whereas, for acetone extracts the order was *Zingiber*> *Carica*> *Allium*> *Datura*> *Lantana*> *Tagetes.* The difference in degree of the antifungal activity exhibited by the plant extracts may be due to the composition of different bioactive compounds present in the respective plant species. For instance, Zhen-guo et al. (2012) reported an abundance of 5,6-dihydro-6-pentyl-2H-pentyl-2-H-pyran-2-one in *Datura* extracts, which was identified as the principle antifungal component. On the other hand, allicin and other thiosulfate compounds were reported as bioactive

antifungal constituencies of *Allium* extracts (Lanzotti et al. 2013). Generally, organic plant extracts recorded higher antifungal activities against *A. tenuissima* compared to water extracts. Ultee et al. (2002) reported the presence of hydrophobic bioactive components, which are highly permeable through cell membranes, by plant extracts harvested from organic compounds. Since harvesting of plant extracts using organic solvents allowed exhaustive extraction of non-polar bioactive plant constituencies (Curtis et al. 2004), this may explain the higher antifungal activities recorded for organic extracts compared to water extracts. In addition, it is possible that micro molecules of extraction solvents interacted at the molecular or cellular level resulting in varying antifungal levels due to synergism and or incompatibility of organic and water extracts against *A. tenuissima* (Hassanein et al. 2008; Ramulu and Ramanjaneyulu, 2010).

In general, an increase in antifungal activities of plant extracts was observed with an increase in concentration of plant extracts. Similar results were reported for the antifungal activities of plant extracts against different *Alternaria* spp. (Tedeschi et al. 2007; Fawzi et al. 2009; Afifi and Zayan, 2010; Zaker and Mosallanejad, 2010). At a low concentration, biologically active compounds may be present in insufficient amounts to show antifungal activity; however, as the concentration increases the amount of biological active compounds increase (Vadlapudi and Kaladhar, 2013). In the case of water extracts, in which plant extracts of *Carica*, *Lantana* and *Zingiber* were tested, no antifungal activities against *A. tenuissima* were observed when applied at ≥10 mg/ml, which is a possible indication of a lack of activity or presence of other unknown compounds counteracting their antifungal activities.

The highest improvement of percentage normal seedlings was recorded when naturally infected coriander seeds were soaked in hot water at 54ºC for 15 mins, which was 8% higher than the control. In addition, the incidence of *A. tenuissima* was reduced by 52% at this temperature time combination. A similar study done by Nega et al. (2003) showed that lower temperature-time combinations of 50ºC for 30mins and 53ºC for 10 mins effectively reduced incidences of *Alternaria dauci* (J.G. Kühn) J.W. Groves & Skolko and *Alternaria radicina* Meier by 85 to 98%, respectively, coupled with high improvements in percentage normal carrot seedlings. Furthermore, soaking cabbage (*Brassica oleracea* L.) seeds in a 53ºC hot water bath for 10 mins effectively reduced the incidence of *Alternaria brassicicola* (Schwein.) Wiltshire by 92 to 99% (Nega et al. 2003). The differences in efficacy of hot water seed treatments observed for different pathosystems mentioned in the above studies may be

explained by differences in chemical composition of physiological structures of the respective host-pathogen combinations. Thermal treatment may have affected chemical processes such as protein hydration and synthesis of macromolecules to various degrees (Baker, 1962). Forsberg (2004) mentioned that seeds of different plant species have different tolerances to high temperatures. However, in the case of the same seeds of coriander used in this study, there were also some differences in heat tolerance within the same species. In this way, there was differential improvement in seed health and seed germination and may be supported by a known fact that seeds of the same plant species, even if they have been harvested during the same year and stored under similar conditions, may show different tolerance to high temperatures (Forsberg, 2004).

Hot water treatment above 54ºC resulted in a significant decrease in incidence of *A. tenuissima*. However, this caused a complete failure in seed germination. It is possible that soaking coriander seeds at the highest temperatures might have caused thermal inactivation of subcellular structures (Simak et al. 1957), and organelles such as mitochondria (Abu-Shakra and Ching, 1967); thus, reducing viability of both the pathogen and seeds. However, soaking seeds at the lowest temperature showed opposite results; there was the highest number of diseased seedlings which was comparable to the control for seeds soaked at 39ºC for 45 mins and 45ºC for 1 min. Nega et al. (2003) also mentioned that soaking vegetable seeds at temperatures below 50ºC had no significant effect on the incidence of *Alternaria* spp.

Since there are limited options for the management of herb diseases in South Africa, the use of plant extracts and hot water treatments for the control *A. tenuissima* has much potential as illustrated by findings of this study. However, additional *in vivo* evaluation on the performance of acetone extracts of *Datura* and *Zingiber*; ethyl acetate extracts of *Allium* and water extract of *Lantana* as seed treatments should be conducted to determine the potential of the plant extracts for disease control. This study also showed good performance hot water seed treatment at 54ºC for 15 mins; therefore, it can be recommended as an alternative seed treatment method for coriander crops, both in organic and conventional farming conditions.

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CHAPTER 5

Management of *Alternaria* leaf spot of coriander (*Coriandrum sativum* L.) using bio-control agents, plant extracts and hot water seed treatments

Abstract

The present study was conducted to evaluate the effects of non-chemical seed treatments, viz. plant extracts, hot water treatments and bio-control agents, on the incidence and severity of *Alternaria* leaf spot disease, caused by seed-borne *Alternaria tenuissima*. Seed health tests showed that *Bacillus subtilis* and *Trichoderma harzianum* effectively lowered the incidence of *A. tenuissima* and compared well with the synthetic fungicide mefenoxam + fludioxonil (Celest® XL) seed treatment. From seed germination tests, all seed treatments showed a significant improvement of germination, from which the highest percentage of normal seedlings was recorded on seeds treated with *Bacillus* sp. (86.8%), which was 1.8% higher than normal seedlings from seeds treated with Celest® XL. In comparison with the other seed treatments, coriander seeds treated with *Lantana* extracts yielded the most diseased seedlings (5%); whereas, significantly higher percentages of abnormal seedlings were obtained from seeds treated with *Datura* extracts and hot water at 48ºC for 60 mins (7.8 and 7.3%, respectively). The greenhouse trial showed that seed treatments significantly improved percentage seedling emergence, except for seeds treated with *Lantana* and hot water at 48ºC for 60 mins, when compared to untreated seeds. The highest seedling emergence was recorded on seeds treated with *Bacillus* sp. (83.3%). There was a significant difference in growth parameters between seedlings sown in September and October. For seeds sown in October, the longest shoot lengths was from seeds treated with *Trichoderma* sp. (119.8 mm), which were significantly higher (17.7 mm) than seedlings raised from seeds treated with Celest® XL. There was no incidence of *Alternaria* leaf spot disease on seedlings from seeds treated with *Bacillus* sp. and an extract of *Allium,* which compared well with seeds treated with Celest® XL. Since there are limited chemicals registered for management of diseases affecting herb production, the results of this study have shown that seed treatment with both bio-control agents (*Trichoderma* and *Bacillus*) and extracts of *Allium* and *Zingiber* are potential replacements of the synthetic fungicide (Celest® XL) in controlling *Alternaria* leaf spot disease on coriander plants.

5.1 Introduction

Coriander (*Coriandrum sativum* L.) is a member of the Apiaceae family and is native to the Near East region, viz. Western Asia, Eastern Mediterranean region and Egypt (Diederichsen, 1996; Ishikawa et al. 2003). Today coriander is distributed and naturalised world over; however, commercial cultivation is mainly done in Bulgaria, Central Europe, India, Morocco and Russia (Sriti et al. 2009). Cultivation of coriander is relatively new in South Africa (SADC Trade, 2014).

The market for herbs is increasing with the improvement of living standards worldwide (Sher, 2013). The Centre of Promotion of Imports from developing countries (CBI, 2014) reported that imports of spices and herbs into the European Union member countries amounted to 520 000 tons with a value of R24.786 billion between the period of 2009 and 2013. Phahlane (2013) reported a net increase of 6% on imports of coriander and saffron (*Crocus sativus* L.) into South Africa between the periods 2008 to 2012. All parts of the coriander plant are edible, but dried fruits are the most valued products (Pathak et al. 2011). The fruits are a major component of pickling spices (Ramadan and Mörsel, 2002). Essential oil extracted from fruits is extensively used in perfumery, aromatherapy and production of soap, creams and lotions (Cooksley, 2003; Aburjai and Natsheh, 2003; Coşkuner and Karababa, 2007). The fresh green leaves are most popularly used for culinary purposes and are shown to be rich sources of various nutrients such as vitamins A, B_1, B_2, C, calcium, carotene, phosphorus and oxalic acid (Sarimeseli, 2011; Divya et al. 2014; Santos et al. 2014).

Despite the economic importance of coriander, productivity has shown a wide gap between its genetic potential and yield realized at the farmer's field due to several biotic and abiotic stresses (Meena and Malhotra, 2006). Among biotic factors are some diseases caused by different fungi. Manoranjitham et al. (2003) has reported a 60% loss of coriander yield due to wilting disease caused by *Fusarium oxysporum* f. sp. *coriandrii* (Fusacr). Although plants infected with *Alternaria* spp. and *Phoma multirostrata* (P.N. Mathur, S.K. Menon & Thirum) Dorenb & Boerema seldom die, presence of lesions and other foliar blemishes may reduce their market value (Raghunath, 1963; Hashmi and Ghaffar, 1991; Boedo et al. 2012). Although previous losses were not documented on coriander crops, Balai and Singh (2013) reported more than 40% yield reduction of a pigeon pea [*Cajanas cajan* (L.) Millsp.] crop due to blights caused by pathogenic *Alternaria tenuissima* (Kunze) Wiltshire.

Since the vegetative parts represent the greater proportion of economic yield, cultivation of herbs is frequently accompanied by application of synthetic fungicides throughout the growing season for production to be economically viable. In South Africa, management of diseases is complicated as there are limited chemicals registered for herbs (Croplife, 2014). Although application of various unregistered chemical groups result in considerable suppression of diseases (Gilardi et al. 2013), some compounds of synthetic chemicals may react causing degradation of constituents of different plant parts responsible for fragrance of aromatic herbs such as linalool (McFrederick et al. 2008). For this reason, there is an increased emphasis on prioritizing maintenance of natural proportions of the components of essential oils from production in the field to final extraction as they confer the characteristics for which aromatic plants are used in pharmaceutical, food and fragrance industries (Anitescu et al. 1997). In addition, application of synthetic pesticides may pose danger of pesticide toxicity due to the remains of chemical residues in/on plant parts at harvesting (Gahukar, 2012). Accordingly there is a need for safer and eco-friendly alternatives to synthetic chemicals for management of plant diseases (Masangwa et al. 2013; Singh et al. 2014).

This study was conducted to evaluate the efficacy of non-chemical seed treatments in controlling *Alternaria* leaf spot disease occurring on coriander in the greenhouse. Since the disease is seed-borne, investigations were also conducted to determine the effects of non-chemical seed treatments on the incidence of *Alternaria* sp. with the aim of improving seed germination percentage of coriander seeds. The treatments used in this study included hot water seed treatments at temperature-time combinations of 48^{o}C for 60 mins and 54^{o}C for 15 mins, and the application of plant extracts of *Allium*, *Datura* and *Lantana*. Two commercial biological formulations of *Bacillus subtilis* (Ehrenberg) Cohn and *Trichoderma harzianum* Rifai were also included. *In vivo* treatments were compared to the performance of a standard seed treatment chemical mefenoxam + fludioxonil (Celest® XL).

5.2 Materials and Methods

5.2.1 Source of seed

Experiments were conducted using untreated, naturally infected coriander (cultivar American long) seeds obtained from a commercial seed company in South Africa.

5.2.2 Preparation of plant extracts

Plant extracts used in this study were selected based on *in vitro* screening tests described in Chapter 4. The *in vivo* tests were performed using acetone extracts of *Datura stramonium* L. and *Zingiber officinale* Roscoeas, ethyl acetate extract *of Allium sativum* L. and water extract of *Lantana camara* L. since they showed good antifungal activity against *A. tenuissima*. Acetone and ethyl acetate plant extracts listed above were prepared following the same protocol described in section 4.3.3 and dissolved in 1% DMSO solution to yield final concentrations of 15 mg/ml. Water extracts were prepared using sterile distilled water.

5.2.3 Seed treatments

5.2.3.1 Seed treatments with bio-control agents

Two commercial bio-control agents, viz. a powder formulation of *Trichoderma harzianum* (EcoT™) concentrated at $2x10^9$ spores/g, and a $2x10^{11}$ spores/ml liquid suspension of 5% *Bacillus subtilis* strain MBI 600, were supplied by Plant Health Products (Pvt.) Ltd (Kwazulu Natal, South Africa) and Becker Underwood (Pvt) Ltd (Kwazulu Natal, South Africa), respectively. An aliquot of 900 coriander seeds was soaked in a liquid suspension containing *Bacillus* sp. applied at the recommended dosage of 147.87 ml/45 kg seed, whereas dried *Trichoderma* sp. granules were applied at a rate of 0.1 g/g of seed and vigorously shaken by hand to ensure homogenous application. Coriander seeds were soaked in the liquid suspension for 4 h to ensure uniformity of coating of *Bacillus* sp. and then air dried overnight on sterile paper towels in a laminar flow bench.

5.2.3.2 Chemical seed treatment

Syngenta South Africa (Pvt.) Ltd. supplied the systemic fungicide Celest®XL (25 ai/l fludioxonil and 10 g ai/l mefenoxam), which was used as a standard or reference seed treatment chemical in this study. Chemical seed treatment was applied by soaking seeds in a diluted mixture of fungicide and sterile distilled water, which were calibrated to cover the recommended rate of 100 ml/100 kg seed. After a soak period of 4 h, seeds were left to dry on sterile paper towels in a laminar flow bench.

5.2.3.3 Hot water seed treatment

Two hot water treatment temperature-time combinations (48^{o}C for 60 mins and 54^{o}C for 15 mins) were selected for *in vivo* studies as they showed significant reduction of incidences of *A. tenuissima* without lowering coriander seed germination in a preliminary study (Chapter 4). Hot water seed treatments were performed according to the protocol of Masum et al. (2009) as described in section 4.4.3. Thereafter, seeds were drained of excess water and air dried on sterile paper towels in the laminar flow bench.

5.2.3.4 Seed treatment with plant extracts

Coriander seeds were soaked in 250 ml Erlenmeyer flasks filled with different crude plant extracts and thoroughly shaken for 10 min. Thereafter, the flasks were incubated for 12 h at 25^{o}C under dark conditions. Finally, excess water was drained and wet seeds were air dried by spreading them on sterile paper towels aligned in the laminar flow bench.

5.2.4 Effect of seed treatments on the occurrence of *A. tenuissima*

Seeds treated with chemical and non-chemical seed treatments (listed above) were evaluated for the incidence of *A. tenuissima* using a modified standard agar plate method (ISTA, 2014). In this test, ten seeds from each treatment were plated on sterile potato dextrose agar plates (PDA, Biolabs) and incubated at 25^{o}C for 7 days under alternating cycles of 12 h ultra violet light followed by 12 h of darkness. Each treatment consisted of 200 seeds arranged in four replicates of 50 seeds. Coriander seeds soaked in sterile distilled water and 1% DMSO, respectively, served as positive controls; whereas, untreated seeds were included as the negative control of the experiment. The entire experiment was repeated twice. Morphological characteristics of fungal colonies growing around seeds were observed under a stereomicroscope, and relative incidence of *A. tenuissima* was determined using the following formula (Marasas et al. 1988; Simmons, 1992; Ellis and Ellis, 1997):

$$\text{Incidence of } A.\ tenuissima\ (\%) = \frac{\text{No. of samples associated with } A.\ tenuissima}{\text{Total no. of samples}} \times 100$$

5.2.5 Effect of seed treatments on seed germination

The effect of chemical and non-chemical seed treatments on the germination of coriander seeds was tested using a modified between paper method (ISTA, 2014). In this test,

untreated coriander seeds were incorporated as the negative control and seeds soaked in sterile distilled water and 1% DMSO represented positive controls. For each seed treatment method, a sample size of 200 treated seeds was placed between layers of moistened blotters. Thus, four replicates of 50 seeds were placed on top of three layers of moistened blotters before covering with the forth layer of moistened germination paper. Thereafter, germination papers containing seeds were rolled, sealed in polythene bags and incubated at 25ºC under alternating cycles of 12 h darkness followed by 12 h normal light regime. The experiment was repeated twice, and evaluations were done after 21 days in which experimental units were scored as normal or abnormal (deformed and diseased) seedlings.

Data collected from the seed health and germination tests was arcsine transformed and analysed for variance (ANOVA) using SAS Version 9.3 (SAS Institute, 2010), however data is presented in percentages. Statistical means were separated using the Fisher's LSD tests ($p<0.05$).

5.2.6 Greenhouse trial

The experiments were conducted during the summer season (sowing date: 04 September and 18 October 2014) in the Plant Pathology Greenhouse located at the Experimental farm (University of Pretoria, South Africa, latitude: 25° 45' 6.94'' S, longitude: 28°15' 34.69'' E, and at an elevation of 1 380 m above sea level). Coriander seeds treated with one of the seed treatments listed above (section 5.3.2) were sown in a 6 cavity plastic seedling trays filled with pasteurized loam soil, where each cavity measured 55 mm (l) x 50 mm (w) x 65 mm (h). In this study each seedling tray represented a replicate with one seed sown per cavity. The experimental units were replicated four times and arranged on greenhouse benches in a complete randomized block design. Seedling trays with seeds treated with Celest® XL served as standard positive control seed treatments; whereas seedling trays with untreated seeds served as the negative control. The temperature of the glasshouse was set at 25±2ºC and seedling trays were watered daily. Plants were left to grow until 21 days and thereafter number of emerged seedlings was recorded. Thereafter, plants were left to grow for three more weeks and assessed for the presence and severity of *Alternaria* leaf spot disease. The severity of *Alternaria* leaf spot disease was determined based on the disease severity scale described in section 3.2.4. Thereafter, plants were harvested (three plants/replicate) and fresh mass of aerial vegetative parts (stem and leaves) determined. Measurements of shoot length were taken and the surface area of coriander leaves was determined by the standard

graph paper method (five leaves/plant) (Tagliipour and Salehi, 2008). Furthermore, plants were oven dried at 85^{o}C for 48 h until constant weight and the dry mass recorded (Marichali et al. 2014).

The entire experiment was repeated twice and data was statistically analysed for variation using SAS 9.3 (SAS institute, 2010), in which differences between means were compared using the Fisher's LSD test ($p<0.05$).

5.3 Results

5.3.1 Effect of seed treatments on the incidence of *A. tenuissima* and seed germination of coriander

The study showed that application of seed treatment agents significantly improved the health status of coriander seeds in comparison to untreated seeds (Table 5.1). *Alternaria tenuissima* associated with coriander seeds was completely reduced by application of *Trichoderma* and was similar to results of the seed treatment with the synthetic fungicide Celest® XL (0%). Seed treatments with *Bacillus* sp. and a hot water at 48^{o}C for 60 mins reduced the incidence of *A. tenuissima* by 56.8 and 52.3%, respectively, compared to the untreated seeds; however, soaking seeds in sterile distilled water and 1% DMSO promoted the incidences of *A. tenuissima* (4.8 and 2.5%, respectively).

There was a significant improvement in percentage normal seedlings yielded from seeds treated with different seed treatments compared to untreated seeds (Table 5.1). Seed treated with *Allium*, *Zingiber*, *Datura*, *Lantana*, biological agent *Trichoderma*, and hot water seed treatments (48^{o}C-60 min and 54^{o}C-15 min) yielded significantly higher percentages of normal seedlings, which ranged between 6.5 to 21.5% (Table 5.1). In fact, coriander seeds treated with *Bacillus* sp. recorded 1.8% more normal seedlings compared to the standard chemical treatment Celest® XL, although the difference was not significant.

Furthermore, seedlings raised from treated seeds had significantly lower percentage diseased seedlings when compared to the untreated control, except for seeds treated with *Lantana* extract (Table 5.1). Among the non-chemical seed treatments tested; coriander seeds treated with *Allium* extract, *Bacillus*, *Datura* and *Trichoderma* yielded percentage diseased seedlings that compared well with Celest® XL treatment (Table 5.1).

Table 5.1: Effect of seed treatments on the incidence of *A. tenuissima* and seed germination of coriander.

Seed treatment	Incidence of *A. tenuissima* (%)*	Germinated seedlings*		
		Normal	Diseased	Deformed
Allium ethyl acetate	5.0 f	82.5 b	2.0 def	3.8 cd
Datura acetone	13.3 d	71.8 fg	1.3 f	7.8 a
Lantana water	29.5 c	78.0 e	5.0 ab	3.5 de
Zingiber acetone	7.8 e	80.3 cd	2.8 cde	4.3 c
Bacillus subtilis	0.3 h	86.8 a	1.8 def	2.0 ef
Trichoderma harzianum	0.0 h	84.0 a	1.5 ef	1.8 f
Hot water 48^{o}C-60min	4.7 f	78.5 de	2.3 cde	7.3 ab
Hot water 54^{o}C-15min	2.5 g	82.0 bc	2.5 cde	3.0 e
Water	61.8 a	70.3 g	4.5 ab	2.0 ef
Celest®XL	0.0 h	85.0 a	1.3 f	2.8 de
Control	57.0 b	65.3 h	5.3 a	6.5 b
1% DMSO	59.5 ab	72.5 f	3.8 bcd	3.3 c
LSD	2.727	2.113	1.325	1.636
CV%	14.87	1.826	33.06	26.97

*The means not sharing a common letter in a column differ significantly. Statistical analysis was performed using one way ANOVA and means separated using the Fisher's LSD test at $p<0.05$.

In comparison to all seed treatments, seeds treated with *Datura* extract had the highest percentage of deformed seedlings (7.8%). Soaking seeds in a hot water bath at a temperature of 48^{o}C for 60 min also yielded significantly high percentage deformed seedlings (7.3%), but not significantly different to seed treatment with *Datura* extract and the control (untreated coriander seeds) (Table 5.1). The percentage of deformed seedlings recorded for all the remaining seed treatments were significantly lower than the control.

5.3.2 Effect of seed treatments on seedling growth

All seed treatments significantly improved percentage seedling emergence, except for seeds treated with *Lantana* and hot water at 48^{o}C for 60 mins, which were not significantly different when compared to untreated seeds (Table 5.2). Sowing seeds treated with *Bacillus* resulted in a higher (4.2%) seedling emergence compared to seeds treated with Celest® XL, although the difference was not significant. However, seedling emergence of seeds treated with *Trichoderma* and hot water at 54^{o}C for 15 mins were lower by 6.3 and 4.2%, respectively, compared to seedling emergence recorded for seeds treated with Celest® XL.

In general, there was a significant difference between length, fresh and dried mass of seedlings sown in October compared with seedlings sown in September ($p<0.05$). In this regard, *in vivo* evaluations of efficacy of seed treatments were based on results taken from the October crop. The crop had a significantly better growth as shown by the length of shoot, surface area of leaves and mass of seedlings (both fresh and dried), except for (i) lower length of seedlings raised from seeds treated with an extract of *Zingiber* and hot water treatment at 48ºC for 60 mins, (ii) smaller surface area of leaves from seedlings from seeds treated with an extract of *Datura* and (iii) lower fresh and dry mass of seedlings from seeds soaked in hot water and Celest® XL (Table 5.3).

Seeds treated with *Trichoderma* sown in October yielded the longest seedlings and were significantly higher (17.7 mm) compared to seedlings grown from seeds treated with Celest® XL. In addition, seedlings raised from seeds treated with *Lantana*, *Bacillus* and sterile distilled water yielded seedlings that were significantly longer (15.7, 12.5 and 7.4 mm) than seedlings sown from seeds treated with Celest® XL.

There was a significant enhancement of mean leaf area of seedlings from treated seeds compared to untreated seeds, except for significantly lower mean leaf areas of seedlings raised from seeds treated with *Datura* and *Zingiber* (2.63 and 3.09 mm^2, respectively). The highest leaf area was recorded for seedlings raised from seeds treated with an extract of *Lantana* (4.90 mm^2). Seedlings treated with *Bacillus*, *Trichoderma*, and hot water bath (both, 48ºC for 60 mins and 54ºC for 15 mins) had significantly broader leaves (4.82, 4.62, 4.53 and 4.47 mm^2, respectively) compared to mean leaf area of seedlings raised from seeds treated with Celest® XL (4.16 mm^2).

Seedling fresh mass of coriander was significantly improved by most non-chemical seed treatments except for hot water treated seeds (at 48ºC for 60 mins) (Table 5.2). Seedlings from seeds treated with *Bacillus* had the highest fresh mass (1.64 g), which was significantly higher than seedlings from Celest® XL treated seeds. Fresh mass of seedlings grown from seeds treated with *Trichoderma* and *Zingiber* (1.45 and 1.60 g, respectively) were comparable to fresh mass of seedlings grown from seeds treated with Celest® XL.
In contrast to the untreated seeds, all seed treatments significantly increased the seedling dry mass, except for seedlings from seeds soaked in a hot water bath at 48ºC for 60 mins. The highest seedling dry mass was obtained from seeds treated with *Bacillus* (0.113 g). Dry mass of seedlings from seeds treated with *Trichoderma* and *Zingiber* (0.100 and 0.106 g,

Table 5.2: Effect of seed treatments on emergence, shoot length, leaf area, fresh and dry mass of coriander seedlings.

Treatment	Seedling emergence (%)*	Shoot length (mm)*		Leaf area (mm^2)*		Fresh mass (g)*		Dry mass (g)*	
		Sept	Oct	Sept	Oct	Sept	Oct	Sept	Oct
Allium ethyl acetate	66.5 cd	85.4 b	93.6 e	3.36 de	3.83 f	1.25 c	1.34 e	0.080 de	0.083 ef
Datura acetone	66.7 bcd	76.9 cd	94.9 e	3.12 fg	2.63 i	0.90 e	1.04 f	0.067 fgh	0.076 g
Lantana water	54.2 cde	94.4 a	117.8 ab	3.62 bc	4.90 a	1.22 c	1.41 de	0.081 de	0.093 d
Zingiber acetone	70.8 b	76.2 cd	73.3 f	3.54 cd	3.09 h	1.52 b	1.60 ab	0.101 ab	0.106 b
Bacillus subtilis	83.3 a	98.2 a	114. 7 b	4.16 a	4.82 ab	1.50 b	1.64 a	0.098 bc	0.113 a
Trichoderma harzianum	72.8 ab	79.3 c	119.8 a	3.74 b	4.62 bc	1.44 b	1.45 cd	0.089 cd	0.100 cd
Hot water 48°C-60min	50.0 de	68. 7 e	64.1 g	2.88 hi	4.47 cd	0.75 f	0.57 g	0.060 h	0.050 j
Hot water 54°C-15 min	75.0 ab	87.1 b	92.0 e	3.28 def	4.53 bc	1.05 d	1.00 f	0.074 efg	0.079 fg
Water	62.5 cd	74.0 d	109.6 c	2.97 gh	4.21 de	0.77 f	0.99 f	0.065 gh	0.069 h
Celest®XL	79.2 ab	96.2 a	102.2 d	3.98 a	4.16 e	1.67 a	1.52 bc	0.119 a	0.102 bc
Control	45.8 e	57.0 f	52.2 h	2.74 i	3.41 g	0.66 g	0.65 g	0.057 h	0.063 i
1% DMSO	62.5 cd	68.9 e	73.6 f	3.16 efg	4.14 ef	0.76 f	1.37 de	0.077 ef	0.086 e
LSD	2.83	4.51	4.58	0.22	0.31	0.08	0.1	0.01	0.01
CV%	17.847	6.973	6.146	8.202	9.281	9.053	10.315	15.485	8.665

*Means within each column followed by the same letters are not significantly different according to Fisher's LSD test ($p<0.05$).

respectively) were not significantly different to those from seeds treated with Celest® XL (0.102 g).

5.3.3 Effect of seed treatments on incidence and severity of *Alternaria* leaf spot

The highest incidence of *Alternaria* leaf spot was recorded on untreated seeds (46%), which did not differ significantly when compared to seedlings raised from seeds soaked in sterile distilled water and 1% DMSO (37.5 and 29%, respectively). All seed treatments significantly reduced the incidence of *Alternaria* leaf spot when compared with the untreated seeds. Celest® XL, *Zingiber Bacillus* and *Allium* extract treatments were most effective at reducing disease incidence, with the latter two having zero disease incidence (Figure 5.1).

Coriander seedlings from untreated seeds had the highest severity of leaf spot disease (13.8%), which did not differ significantly from those of seeds soaked in 1% DMSO, *Lantana* extract and sterile distilled water (12.1, 10 and 8.8%, respectively) (Figure 5.1). Seeds treated with Celest® XL, *Trichoderma*, *Zingiber* and hot water soak at 54^{o}C for 15 mins recorded the lowest severity of *Alternaria* leaf spot, but did not differ from seeds treated with *Bacillus* and *Allium*, which yielded disease-free seedlings (Figure 5.1).

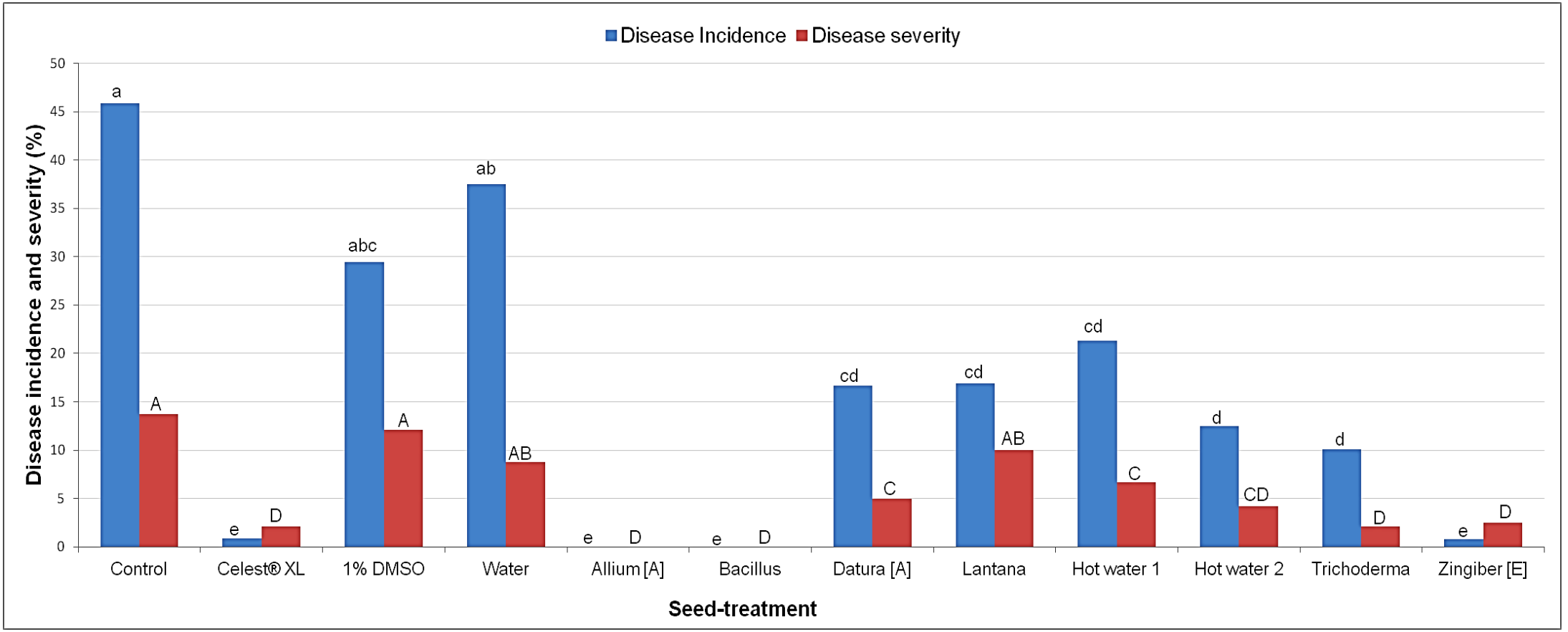

Figure 5.1: Effect of different seed treatments on the incidence and severity of *Alternaria* leaf spot on coriander seedlings. Means with different letters indicate significant differences according to Fisher's LSD test ($p<0.05$). (Disease Incidence: LSD= 0.972; CV= 13.51%. Disease severity: LSD= 0.648; CV= 10.07%).

Keynotes: [A]= acetone extract, [E]= ethyl acetate extract, Hot water 1= hot water seed treatment at 48^{o}C for 60 mins and Hot water 2= hot water seed treatment at 54^{o}C for 15 mins.

5.4 Discussion

The use of healthy, disease-free seed is important in order to minimise infections by seed-borne and seed-transmitted pathogens (Mancini and Romanazzi, 2014). Since pathogen infected seed is normally characterised by reduced germination and poor seedling emergence, the practice of treating seed is of great significance for enhanced seedling survival (Strandberg, 1983; Pryor, 2002). Over the years, seeds were treated using synthetic chemicals. However, restrictions in registration and use of chemicals particularly for cultivation of crops under organic farming have intensified the demand for alternative non-chemical methods in the control of diseases (Du Toit, 2004; Groot et al. 2006). In this study, different non-chemical seed treatments, viz. plant extracts, biological and hot water, effectively improved seedling emergence and growth of coriander seeds infected with *A. tenuissima*.

Seed health tests indicated high incidences of *A. tenuissima* on non-treated coriander seeds, which may have contributed to occurrence of diseased seedlings, observed during the germination test and accounted for reduced seedling emergence in the greenhouse. Since there are no set standards for certification of coriander seeds in South Africa (Plant improvement Act, 1976), results of this study were compared to those set by the International Seed Federation (2013). This showed germination of untreated coriander seed to be 4.8% below the accepted tolerance value of 70%. The roughness of the surface of coriander seeds and absence of any antifungal agent may have provided a niche for *A. tenuissima* as shown by other studies for carrot [*Daucus carota* (Hoffm.) Schübl. & G. Martens] and tomato (*Solanum lycopersicum* L.) seeds infected by other *Alternaria* spp. (Kirkpatrick and Bazzaz, 1979; Mancini and Romanazzi, 2014). *Alternaria tenuissima* may interfere with the cellular and metabolic processes of seed germination and seedling development through production of toxic metabolites such as alternariol, tenuazonic acid, altertoxin I etc. (Davis et al. 1977; Andersen et al. 2002). For instance, tenuazonic acid has been shown to inhibit incorporation of amino acids into protein, which affects proper seed development as newly formed proteins are immobilised inside ribosomes (Shiqeura and Gordon, 1963). Tylkowska et al. (2003) showed that exposure of carrot seeds to extracts containing alternariol, altenuene and tenuazonic acid reduced percentage normal seedlings and also promoted an increase of percentage diseased seedlings.

In the current study, it was observed that sowing variously treated seeds resulted in an improvement of seedling growth parameters compared with untreated seeds. In fact, coriander seeds treated with *Trichoderma* recorded the longest seedlings and a considerably broader leaf surface area. Similarly Kleifeld and Chet (1992) showed that *Trichoderma* spp. caused an increase in emergence of seeds, plant height, leaf area and dry weight of bean (*Phaseolus vulgaris* L. 'Brittle Wax'), radish (*Raphanus sativus* L.), tomato [*Lycopersicon lycopersicum* (L.) H. Karst.], pepper (*Capsicum annum* L.) and cucumber (*Cucumis sativus* L.). Although the current study showed that *Trichoderma* sp. effectively reduced the incidence of seed-borne *A. tenuissima* associated with coriander, a study conducted by Windham et al. (1986) proved that the mechanism by which *Trichoderma* spp. increased the rate of seed germination and dry weight of tobacco (*Nicotiana tabacum* L.) and tomato shoots involved a growth regulating factor as opposed to direct mycosis and competition for nutrients against plant pathogens. In addition, the growth-promotion effect of *T. harzianum* was observed after inoculation on the cucumber root system (Yedidia et al. 2001). The surface area of leaves and length of shoots resulting from seeds treated with *Lantana* were considerably greater than those from seeds treated with Celest® XL.

This study showed that different seed treatments were effective in reducing incidence of seed-borne *A. tenuissima* associated with coriander seeds, which may have contributed towards reduced percentage of diseased seedlings and improved seedling emergence for the respective treatments. In comparison to all seed treatments, seeds treated with biological agents (*Bacillus* and *Trichoderma*) recorded the lowest incidence of *A. tenuissima* and diseased seedlings, which were both comparable to results of seeds treated with Celest® XL. Similar studies showing effectiveness of *Trichoderma* spp. as seed treatments against some seed-borne and soil-borne pathogens including *Pythium, Fusarium* etc. resulted in improved seedling emergence and plant growth (Ahmad and Baker, 1987; Kleifeld and Chet, 1992; Ha, 2010). Sivapalan (1993) showed that seed treatment with *T. harzianum* increased seedling emergence of broccoli (*Brassica oleracea*) seeds infected with *Alternaria brassicicola* (Schwein.) Wiltshire. The biological action of *Bacillus* sp. has been shown to involve production of antibiotics or biosurfactants (Dow et al. 2000; Lugtenberg and Kamilova, 2009), which may have inhibited growth of *A. tenuissima* on seeds in the current study resulting in complete reduction of incidence and severity of *Alternaria* leaf spot. The significant increase in

growth of seedlings raised from seeds treated with *Bacillus* sp. is in agreement with studies which reported *Bacillus* spp. as growth promoting rhizobacteria (Raupach and Kloepper, 1998; Ramamoorthy et al. 2001; Mena-Violante and Olalde-Portugal, 2007). In addition, it is possible that *Bacillus* may have stimulated phytohormones or improved solubilisation and mobilisation of phosphate or induced systemic resistance (Gutiérrez-Mañero et al. 2001; Çakmakçi et al. 2006; Richardson et al. 2009; Wang et al. 2009) that may have enhanced seedling growth as reflected by increases in fresh and dry mass of coriander seedlings. In addition the increase in shoot length and fresh and dry mass of coriander seedlings from seeds treated with *Bacillus* may occur by elicitation of ISR by *Bacillus* spp., which has been reported to involve some ultrastructural and cytochemical alteration that help cushion the host from pathogen attack resulting in more energy being allocated for cellular metabolic processes contributing to growth and development (Kloepper et al. 2004).

Seed treatments with plant extracts also reduced the incidence of *A. tenuissima.* This was in agreement with previous studies which showed that plants extracts contain natural antimicrobial compounds, such as sulphur containing compounds in the case of *Allium*, which may either act on the pathogen directly (Amadioha, 2000) or induce systemic resistance in host plants to cause a reduction on the incidence of the disease (Kagale et al. 2004; Nashwa and Abo-Elyousr, 2012). This might have contributed to the increase in seed germination and improved percentage emergence of coriander observed in this study. This study also showed that *Zingiber* extracts effectively reduced the incidence of *Alternaria* leaf spot disease of coriander. This may have been attributed to its high antifungal activity as other studies showed *Datura* extracts to possess high levels of secondary metabolites such as 6, 8 and 10-gingerdiol (Singh et al. 1999; Sharma and Tiwari, 2013). However, this contradicted with a study conducted by Mahapatra and Das (2013), which showed higher incidence of *Alternaria* blight disease on mustard [*Brassica juncea* (L.) Vassiliĭ Matveievitch Czernajew] despite seed treatment and foliar applications of an extract of *Zingiber*.

Although significant improvements of percentage normal seedlings were recorded on seeds treated with plant extracts, they were lower than those of seeds treated with bio-control agents (*Bacillus* and *Trichoderma*) and the synthetic fungicide (Celest® XL). In comparison to the other plant extracts, extracts of *Datura* yielded a significantly lower

percentage of normal seedlings. In addition, seeds treated with *Datura* extracts recorded the highest percentage of deformed seedlings. Similar observations were reported when *Alternaria* spp. infected seeds of sunflower (*Helianthus annuus* L.), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) were treated with *Datura* extracts (Lovett and Potts, 1987). This may have been caused by toxic allelopathic compounds contained in *Datura* extracts, which caused a reduction in seed germination. Lovett et al. (1981) showed the presence of tropane alkaloids, scopolamine and hyoscyamine as allelopathic chemicals in seeds and leaves of *Datura*.

Soaking coriander seeds in a hot water bath at 54^{o}C for 15 mins resulted in significant reduction in the incidence of *A. tenuissima* and yielded disease-free seedlings, which compared well with seeds treated with *Bacillus* and *Allium.* Similar studies showed that hot water treatment at a temperature of 53^{o}C for 30 mins resulted in more than 95% control of *Alternaria dauci* (J.G. Kühn) J.W. Groves & Skolko and *Alternaria radicina* Meier, Drechsler & E.D. Eddy for the reduction of disease symptoms in carrot (Nega et al. 2003). The differences in specificity of hot water seed treatment parameters in the aforementioned studies support previous reports which indicated that the efficiency of different temperature-time combinations vary between different plant species (Forsberg, 2004; Mancini and Romanazzi, 2014). Although there was significant improvement of seedling emergence for hot water treatment (54^{o}C for 15 mins) of coriander seeds, fresh and dry mass of the seedlings was significantly lower than that of Celest® XL treated seed.

In conclusion, this study has shown that the incidence of seed-borne *A. tenuissima* may lower the potential seed germination and seedling emergence of coriander by 21.5% and 37.5%, respectively. On the basis of findings of this study, seed treatments with biological control agents (*Bacillus* and *Trichoderma*) can be recommended to replace chemical seed treatment (Celest® XL) as they were equally effective in improving seed health status and percentage germination of coriander seeds infected with *A. tenuissima*. Apart from the bio-control agents, seed treatments with plant extracts of *Allium* and *Zingiber* may also be recommended for the management of *Alternaria* leaf spot on coriander. Therefore, this justifies further biochemical profiling to elucidate bioactive compounds responsible for antifungal properties of *Allium* and *Zingiber* extracts. The aforementioned seed treatments also showed a general trend towards an

increase in fresh and dry mass of coriander seedlings, which compared well with seedlings grown from seeds treated with Celest® XL. In view of the toxicity of *Datura* extracts demonstrated by higher percentage of deformed seedlings and poor improvement on seedling emergence, it should not be used as a seed treatment for coriander.

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CHAPTER 6

General discussion

There are vast opportunities in the generation of employment to alleviate poverty in South Africa through cultivation, processing, packaging and transportation of herbs (Mander et al. 2007). Although there are attempts to increase local productivity of herbs, this has often been associated with outbreak of previously unreported diseases (Swart and van Niekerk, 2003; McLeod et al. 2006). Furthermore, sustainable cultivation is limited by the lack of registered pesticides for the management of herbs in South Africa (Croplife, 2014). Although some reports mention the spread of diseases by infected seeds, limited research has been done to determine the health status of locally produced seeds to determine whether they are sources of inoculum of these diseases. In this regard, this study was initiated to examine and identify seed-borne mycoflora associated with commercial herb seeds and to determine whether any of these fungi result in seed transmitted diseases. Since this study was targeted to promote organic farming of coriander, emphasis was on evaluation of non-chemical methods for the management of *Alternaria* leaf spot disease.

In Chapter 3, seed health tests were performed on herb seed lots, viz. basil (*Ocimum basilicum* L.), chives (*Allium schoenoprasum* L.), coriander (*Coriandrum sativum* L.), dill (*Anethum graveolens* L.), parsley [*Petroselium crispum* (Mill.) Fuss], sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) and wild rocket [*Diplotaxis tenuifolia* (L.) DC.], from which a total of ten genera of fungi were detected. Most of the seed lots were infected with *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*. Some of the other fungi detected on herb seed lots included *Cladosporium*, *Curvularia*, *Epicoccum*, *Rhizoctonia and Rhizopus*. However, this study showed low mycoflora diversity when compared to some previous studies, which detected more than thirteen fungal genera (Szopińska and Bralewski, 2006*;* Pavlović et al. 2008; Szopińska et al. 2008; Jayshree et al. 2011). This variation may be attributed to differences in localities from where seeds were produced as these are often characterised by a heterogeneous diversity of mycoflora due to the prevailing weather conditions at the respective locations (Mitakakis et al. 2001; van der Waals et al. 2003; Gofroń and Rapiejko, 2009). From seed germination tests, it was observed that all the herb seed lots examined were above their minimum tolerance accepted for seed certification of germination as stipulated by the Vermont

Seed Standard Regulations, except for dill and wild rocket that recorded lower (1 and 8.75%, respectively) than their accepted germination levels of 75 and 80%, respectively (Vermont Seed Standard Regulations, 2014). Correlation analysis of results of seed tests indicated that seed-borne mycoflora associated with herb seed lots were positively correlated with the number of diseased seedlings. However, due to time constraints pathogenicity tests were only done using seed-borne *Alternaria tenuissima* (Kunze) Wiltshire isolated from coriander seeds, which proved to be pathogenic in causing *Alternaria* leaf spots on coriander seedlings. Further studies to prove seed transmission and pathogenicity of the other seed-borne fungi other than *Alternaria* sp. are warranted.

Since seed-borne *A. tenuissima* was shown to cause disease on coriander plants, it was important to investigate effective non-chemical methods that are equally effective as synthetic chemicals in the management of the disease. *In vitro* studies performed in the current study (Chapter 4) compared the efficacy of acetone, ethyl acetate and water extracts of *Allium sativum* L., *Carica papaya* L., *Datura stramonium* L., *Lantana camara* L., *Tagetes minuta* L. and *Zingiber officinale* Roscoe in controlling *A. tenuissima*. Results of the antifungal agar infusion assay showed that minimum concentrations of 5 mg/ml of most acetone and ethyl extracts effectively inhibited growth of *A. tenuissima*. Water extracts of *Carica*, *Lantana*, and *Zingiber* were effective at higher concentrations (10 mg/ml). However, antifungal evaluations using the disc diffusion method showed no zones of inhibition around discs impregnated with the respective extracts, which may indicate that this method is not suitable for use with these extracts. However, using the disc diffusion method, application of acetone and ethyl acetate extracts of *Allium*, *Datura* and *Zingiber* and water extract of *Lantana* water at 15 mg/ml effectively inhibited growth of *A. tenuissima*. The extracts used for further evaluation in the greenhouse were selected from these two assays. As another non-chemical control method, hot water seed treatments were investigated *in vitro*. After soaking naturally infected coriander seeds at different treatment temperature-time conditions, it was observed that hot water seed treatments at 54ºC for 15 mins was the optimal hot water treatment temperature-time combination, which significantly lowered the incidence of *A. tenuissima* with a resulting considerable improvement of seed germination. Although hot water treatments at higher temperature-time combinations (>54ºC) resulted in the lowest incidence of *A. tenuissima*, seed germination was significantly reduced. The temperature-time

combinations used in the present study were higher compared to similar studies on seeds of vegetables and cereals against various *Alternaria* spp. (Nega et al. 2003; Amein et al. 2011; Saeideh and Mohammad, 2012). The difference compared to the pathosystems mentioned above may be due to the fact that seeds of different plant species have distinctive tolerance to thermal treatments; hence, it is important to determine specific optimal hot water seed treatment temperature-time combinations that effectively control the incidence of pathogens without affecting survival of different plant species (Forsberg, 2001; Mancini and Romanazzi, 2014).

In chapter 5 the effects of non-chemical seed treatments on the seedling emergence, plant growth and management of *Alternaria* leaf spot disease was evaluated. Seed health tests performed on seeds treated with non-chemical agents showed significant reduction of incidence of *A. tenuissima* compared to the untreated seed lot. Coriander seeds treated with the biological control agents *Bacillus subtilis* (Ehrenberg) Cohn and *Trichoderma harzianum* Rifai had the lowest incidence of *A. tenuissima* and also the highest improvement in seed germination. In fact, higher percentage of normal seedlings was obtained from seeds treated with *Bacillus* sp. compared to seedlings grown from seeds treated with the synthetic fungicide Celest® XL (25 ai/l fludioxonil and 10 g ai/l mefenoxam). In addition, findings of this study have showed that seed treatment with plant extracts of *Allium* and *Bacillus* sp. can be used to replace seed treatment with synthetic chemicals for the management of *Alternaria* leaf spot disease of coriander as they were equally effective as Celest® XL. Seedlings from seeds treated with plant extracts of *Allium* and *Zingiber* and the bio-control agents *Bacillus* and *Trichoderma* showed significant increases in growth parameters, viz. longer shoots and a broader leaf surface area, which might have contributed to an increase in fresh and dry mass of coriander seedlings.

In conclusion, this study has revealed that most commercial herb seeds produced in South Africa are infected with several seed-borne fungi, which positively correlated with percentage of diseased seedlings. Seed-borne *A. tenuissima* was shown to be pathogenic on coriander. In addition, this study also showed that plant extracts of *Allium*, *Datura*, *Lantana*, and *Zingiber* effectively inhibited growth of the fungus *in vitro*; whereas, hot water seed treatment at 54ºC for 15 mins effectively reduced the incidence

of *A. tenuissima* associated with coriander seeds. Plant extracts of *Allium* and *Zingiber*, bio-control agents *Bacillus* and *Trichoderma* and a hot water treatment at 54ºC for 15 mins may be recommended for further field evaluations against *Alternaria* leaf spot of coriander before they are commercialised.

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