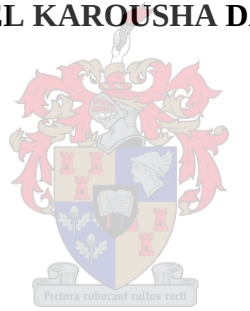
THE EFFECT OF GARLIC EXTRACTS ON THE CONTROL OF POSTHARVEST PATHOGENS AND POSTHARVEST DECAY OF APPLES

By

CHANEL KAROUSHA DANIEL



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at Stellenbosch University

Supervisor: Dr Cheryl Lennox

Co-supervisor: Dr Filicity Vries

April 2014

Stellenbosch University http://scholar.sun.ac.za

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any other qualification.

_____ _____

Chanel Karousha Daniel Date

Copyright © 2014 Stellenbosch University
All rights reserved

Stellenbosch University http://scholar.sun.ac.za

SUMMARY

Apples are an important export commodity for the South African market, and postharvest losses that occur as a result of decay due to infection with pathogenic fungi such as *Botrytis cinerea* Pers., *Penicillium expansum* (Link) Thom. and *Neofabraea alba* (E.J. Guthrie) are of major concern for all parties concerned with fruit production and distribution.

Decay control of these fungi is primarily managed through the use of synthetic fungicides; however, pathogen development of resistance to these fungicides and recent worldwide concern over healthier living and a greener environment has called for the discriminate use of synthetic chemicals. This has opened up an avenue for the development of safer and more environmentally friendly alternatives to control postharvest decays. The use of plant extracts and essential oils are favoured as natural sources of antimicrobials whilst still being safe for human consumption and having no negative impact on the environment.

Allium sativum (garlic) is one such plant species that is well documented for its value in improving human health and is readily available for consumption not just as a flavour component of food but also to be taken as a daily herbal diet supplement. Given the antimicrobial effectiveness of garlic against human pathogens and ailments, its value as an antifungal agent against postharvest pathogens causing grey mould, blue mould and bull's eye rot of apples was investigated *in vitro* and *in vivo* within this study. Furthermore, an attempt was made to elucidate the chemical components of garlic extracts by gas chromatography-mass spectrometry (GC-MS).

All experiments in this study were carried out with garlic extracts prepared from fresh garlic bulbs. For the *in vitro* experiments, two extract preparations of garlic, one containing ethanol (Extract 1) and one where ethanol had been removed by evaporation (Extract 2), was tested for antifungal action within an amended media experimental design. Both extract preparations were each subjected to two dilution series (0-80% garlic extract) with water and ethanol as diluents. Both extract preparations were successful at retarding pathogen mycelial growth and spore germination; however, concentrations of Extract 2 (ethanol evaporated) and diluted with distilled water provided markedly better inhibition of *B. cinerea* and *P. expansum* than the ethanolic dilutions of extract 2. Both extract preparations yielded similar inhibitory results when tested against *N. alba*. Due to the results achieved in the amended media experiments, the use of a crude garlic extract without ethanol and diluted in

Stellenbosch University http://scholar.sun.ac.za

water was considered to be the best option for further tests throughout the remainder of the study. *In vitro* volatile effects of crude garlic extracts at concentrations between 0 and 40% garlic extract were subsequently tested. Garlic volatiles were effective in inhibiting pathogen mycelial growth and spore germination of all three pathogens, at lower concentrations compared to the amended media experiments. *In vitro* volatile exposure with garlic extracts was more effective at inhibiting *N. alba* than direct application of the extracts.

Curative and protective application of garlic extracts and clove oil for increased fungal inhibition through synergism was tested by direct and volatile exposure to the pathogens *in vivo* on three economically important apple cultivars; 'Granny Smith', 'Golden Delicious', and 'Pink Lady'. Direct exposure of artificially wounded and inoculated fruit to the garlic extract and clove oil revealed that garlic extracts applied curatively but not protectively effectively controlled decay caused by *B. cinerea* and *P. expansum* on all apple cultivars. Both curative and protective applications were ineffective in controlling *N. alba*. *In vivo* volatile exposure to the garlic extracts and clove oil did not inhibit decay on any of the cultivars and was not effective against any of the three pathogens investigated.

A full chemical profile analysis was done by GC-MS analysis of garlic extract samples. The compounds diallyl disulphide, allyl methyl trisulphide, allyl methyl disulphide and dimethyl trisulphide were detected in relatively high amounts. This result suggests that the abundance of sulphur and sulphur related compounds detected may be responsible for the antifungal action noted in the experimental studies.

In conclusion, garlic was shown to have antifungal activity against *B. cinerea*, *P. expansum* and *N. alba*. The pathogens used in this study were not compared with each other, but undoubtedly each pathogens reacts differently to exposure to the garlic extracts. It would therefore be advisable to investigate the effects of the extracts on each of the pathogens in a more in-depth study. More investigations into the application of the garlic extracts is required before it may be recommended for use; however, results for the use of garlic extracts against these postharvest pathogens and the postharvest decay they cause are promising.

Stellenbosch University http://scholar.sun.ac.za

OPSOMMING

Appels is ‘n belangrike uitvoerproduk vir die Suid-Afrikaanse vrugtebedryf, maar noemenswaardige na-oes verliese word weens bederf deur patogeniese swamme soos *Botrytis cinerea* Pers., *Penicillium expansum* (Link) Thom. en *Neofabraea alba* (E.J. Guthrie) ervaar. Dit raak alle partye betrokke met die produksie en verspreiding van hierdie vrugsoort.

Hierdie swamme word hoofsaaklik met behulp van kunsmatige swamdoders beheer, alhoewel weerstand-ontwikkeling en wêreldwye bewusmaking van ‘n gesonder leefstyl en omgewing die gebruik van kunsmatige middels streng aanspreek en die ontwikkeling van veiliger en meer omgewingsvriendelike alternatiewe middels verlang. Plant-ekstrakte en essensiële olies kan dien as sulke middels en is natuurlike bronne van anti-mikrobiese aktiwiteit, is veilig vir menslike verbruik en het ook geen negatiewe invloed op die omgewing nie.

Allium sativum (knoffel) is so ‘n plantspesie wat as alternatiewe middel gebruik kan word. Dit is bekend vir sy waarde in die verbetering van menslike gesondheid, is maklik bekombaar en word nie net as ‘n geurmiddel vir voedsel gebruik nie, maar ook as ‘n daaglikse krui-aanvulling. Gegewe die anti-mikrobiese doeltreffendheid van knoffel teenoor menslike patogene en kwale, is die werking (*in vitro* en *in vivo*) teen na-oes patogene wat grys skimmel, blou skimmel en teikenvrot in appels veroorsaak, in hierdie studie ondersoek. Bepaling van die chemiese samestelling van die knoffel-ekstrak is ook met behulp van gas-chromatografie massa spektrometrie (GK-MS) onderneem.Vars knoffelbolle is vir elke eksperiment in hierdie studie gebruik met die voorbereiding van die knoffel-ekstrak. Vir die *in vitro* eksperiment is twee knoffel-ekstrakte voorberei, naamlik: ‘n ekstrak wat etanol bevat (Ekstrak 1) en een waarvan die etanol verwyder is met verdamping (Ekstrak 2). Die ekstrakte is getoets vir werking teen fungi in kultuur-medium.. Albei ekstrakte is verdun tot twee konsentrasie reekse (0-80%) met water en etanol as verdunningsmiddels. Albei ekstrakte het suksesvolle werking getoon teenoor die patogene ten opsigte van vertraging van miselium-groei en spoor-ontkieming, alhoewel konsentrasies van Ekstrak 2, verdun met gesuiwerede water, patogene *B. cinerea* en *P. expansum* beter onderdruk het as Ekstrak 2 verdunnings met etanol.. Beide ekstrakte en hul afsonderlike verdunnings met etanol en water het soortgelyke resultate gelewer met onderdrukking van *N. alba*.

Volgens resultate wat verkry is van die kultuur-medium eksperimente, is Ekstrak 2 verdun met gesuiwerde water beskou as die geskikste vir verdere toetse in hierdie studie Die

Stellenbosch University http://scholar.sun.ac.za

vlugtige effek van Ekstrak 2 is *in vitro* getoets by konsentrasies tussen 0 tot 40%. Die vlugtige stowwe van knoffel het al drie patogene se groei en spoor-ontkieming effektief onderdrukby laer konsentrasies as wat gebruik is in die kultuur-medium eksperiment. Dus is *in vitro* blootstelling van *N. alba* aan die vlugtige stowwe meer effektief as direkte toediening van die ekstrakte.

Die voorkomende en beskermende effek van die knoffel-ekstrak, asook naeltjie-olie, is *in vivo* ondersoek om te bepaal of die stowwe saam sterker onderdrukking van die patogene kon bewerkstellig. Direkte en vlugtige blootstelling is op drie ekonomies-belangrike appel-kultivars getoets, naamlik: 'Granny Smith', 'Golden Delicious' en 'Pink Lady'. Direkte blootstelling met die knoffel-ekstrak en naeltjie-olie aan gewonde en ge-inokuleerde vrugte het aangedui dat *B. cinerea*- en *P. Expansum*-bederf net beheer kon word indien knoffel voorkomend toegedien is vir al die ondersoekte appel-variëteite. Voorkomende en beskermende toediening was onsuksesvolle om *N. alba* te beheer. *In vivo* blootstelling van die drie patogene aan die knoffel-ekstrak en naeltjie-olie se vlugtige stowwe kon nie enige van die patogene effektief onderdruk nie en was onsuksesvol in bederf-beheer.

'n Volledige chemiese profiel is saamgestel deur GK-MS ontleding van die knoffel-ekstrakte. Hoë vlakke van verbindings dialliel disulfied, alliel-metiel-tri-sulfied, alliel-metiel-disulfied en dimetiel-trisulfied is bespeur. Die aantal vrye sulfied en sulfied-verwante verbindings in die ekstrak kan moontlik 'n verduideliking bied vir die anti-swam werking waargeneem gedurende hierdie studie.

Ten slotte: knoffel toon 'n anti-swam werking teenoor *B. cinerea*, *P. expansum* en *N. alba*. Die patogene in hierdie studie is nie met mekaar vergelyk nie, omdat elkeen uniek en uiteenlopend op knoffel reageer het. Alhoewel die huidige studie alreeds belowende resultate gelewer het, moet die ekstrak se effek op elke patogeen onderskeidelik nog in diepte ondersoek word, asook die wyse van die toediening in die na-oes praktyk voordat hierdie middel aanbeveel kan word vir gebruik.

Stellenbosch University http://scholar.sun.ac.za

ACKNOWLEDGEMENTS

"For I know the plans I have for you, declares the Lord, plans to prosper you and not harm you, plans to give you hope and a future". – Jeremiah 29:11.

I humbly dedicate my work to the Almighty God, for it is through His spirit and divine will upon my life that I found the wisdom, the knowledge, the understanding, and the strength to complete this task.

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- My supervisors Drs Cheryl Lennox and Filicity Vries for expert advice and guidance in the planning and execution of experiments, input added to the writing of this thesis, and for being a sounding board on general matters.
- The technical staff at the Agricultural Research Council (ARC) Infruitec-Nietvoorbij (Postharvest Pathology) for assisting in the execution of experiments.
- The Department of Plant Pathology at Stellenbosch University for making it a fun, fruitful and intellectually stimulating environment to work in.
- The ARC, Technology and Human Resources for Industry Programme (THRIP) and Stellenbosch University for financial support.
- Jessica Rochefort and Sonja Coertze for assistance with the language components.
- Mardé Booyse at the ARC for assistance with the statistical analysis.
- Lucky Mokwena at the Central Analytical Facility (CAF- Stellenbosch University) and Dr Oluwafemi Caleb for their assistance with the GC-MS work.
- My family – Dad, Mum, Sister, Brother for their loving support. To my parents especially for their continued guidance and prayers.
- Craig Swartland – your words of encouragement through the last stretch and prayers said on my behalf in my hours of distress were greatly appreciated.
- Friends and colleagues at the Department of Plant Pathology, and especially the "A-Team" for your their and assistance.

Stellenbosch University http://scholar.sun.ac.za

CONTENTS

Stellenbosch University http://scholar.sun.ac.za

Stellenbosch University http://scholar.sun.ac.za

Stellenbosch University http://scholar.sun.ac.za

Stellenbosch University http://scholar.sun.ac.za

1.
THE POTENTIAL OF USING GARLIC EXTRACTS IN THE MANAGEMENT OF POSTHARVEST DECAY OF APPLES

1.1. INTRODUCTION

Fruit are among the most important foods of humans as they are nutritive and valuable for the maintenance of human health (Shahi *et al*., 2003). South Africa`s climate and soil conditions provide ideal conditions for many varieties of fruit to be grown. Citrus, deciduous fruit (grapes, pome and stone fruits) and subtropical fruit are all grown in various regions throughout the country (Polderdijk *et al*., 2006).

Pome fruits are the most important temperate zone fruits (Snowdon, 1990). Apples (*Malus domestica* Borkh.) have been recorded as the second most consumed fruit following oranges, and in the United States it remains the third most valuable fruit crop (Geisler, 2011). Global apple production for 2011/2012 period was estimated to have reached a record 65.23 million tons (Negro and Lojo, 2011).

In South Africa, apples constitute the bulk of deciduous fruit produced. Apples are an important export commodity for the South African market (Snowdon, 1990), with roughly half of the apples produced being exported (Mogala, 2012). Although South Africa is a comparatively small apple grower in terms of global hectares, the country exports large volumes globally (Morokolo, 2011). For the 2011/2012 season, approximately 20 million cartons of apples were exported (PPECB, 2013). Apples harvested in South Africa are sold to various buyers. In the 2010/2011 season, approximately 43% of total apple production was exported, 30% sold locally, 28% was processed and the remaining 0.2% was used in dried fruit production (Mogala, 2012).

During cold storage several fungal decays occur that cause economic losses (Calvo *et al*., 2007). Postharvest decay infection of fruit often starts in the field and may occur along the postharvest handling chain, with symptoms only manifesting after storage, and is often only detected at distribution points overseas. Low temperatures are used for long-term storage and when exporting apples to slow the development of storage diseases and to sustain fruit quality (Tian and Bertolini, 1995). The major postharvest pathogens associated with various apple cultivars are *Botrytis cinerea* Pers., the cause of grey mould, *Penicillium*

Stellenbosch University http://scholar.sun.ac.za

expansum (Link) Thom., the cause of blue mould and *Neofabraea* spp., which causes lenticel rot or bull's eye rot. In a survey conducted in Washington State on the postharvest diseases of apples, grey and blue mould accounted for approximately 28 and 32% of decayed fruit (Kim and Xiao, 2008). With half of the South African apple production being distributed internationally, postharvest decays are of major concern. A myriad of factors such as handling, storage, transportation, and packaging play a role in the quality of the end product (Mogala, 2012) and postharvest decays may arise at any point during the export process. International regulations that govern the export of pome fruit allow only 2% decay within bins, in some cases. If this level is exceeded, the shipment may have to be repacked, often at the expense to the packhouse and producer. Decay on apples may also give rise to rejection claims. Therefore, postharvest decay of apples puts economic strain on all parties involved in the South African apple export chain.

1.2. POSTHARVEST PATHOGENS OF APPLES

1.2.1. *Botrytis cinerea*

Fifty species of *Botrytis* exist that contribute in part to a wide array of plant infections. The pathogenic fungus, *B. cinerea,* is the cause of grey mould infections. The name *Botrytis* is derived from the Greek word for grape, since the fungus produces spores like bunches of grapes. *Botryotinia fuckeliana* (De Bary) Whetzel (Mirzaei *et al*., 2007) is the teleomorphic stage of the pathogen, with *B. cinerea* being its anamorph. All *Botrytis* species are necrotrophic, since plant cells are actively killed during pathogenesis (Laluk and Mengiste, 2010).

1.2.1.1. Disease Cycle

Botrytis can be found overwintering as mycelium or sclerotia in decomposing plant debris and soil. The pathogen favours a cool, moist climate for optimal growth, spore formation and release, germination and establishment of infections (Shtienberg and Elad, 1997; Williamson *et al*., 2007). The pathogen is active at low temperatures and causes considerable losses on crops kept for long periods in storage, even if the temperatures are between 0 and 10°C (Elad *et al*., 1996). Germinating spores penetrate tissues through wounds and produce mycelium on aged flower petals, dried foliage, dead bulbs, and other plant

Stellenbosch University http://scholar.sun.ac.za

debris. Sclerotia usually germinate by producing mycelial threads that can infect directly, but during the sexual cycle sclerotia become spermatized thus producing apothecia and ascospores. In temperate regions, the beginning of spring triggers the production of conidiophores and conidia which serve as the primary inoculum source within a crop. The conidia follow a precise sequence of initiation, production and dissemination that is regulated by temperature and humidity fluctuations (Williamson *et al*., 2007). Conidia become airborne and are carried off by air currents to settle and cause infection elsewhere. They may also be dispersed by water droplets (Coertze and Holz, 2002).

Diseases caused by *Botrytis* are varied and with a wide host range. Although it will normally appear as blossom blights or fruit rots, it can also present as damping-off, stem cankers and rots, leaf spots, tuber and bulb rots (Williamson *et al*., 2007). On fruit and under humid conditions, the fungus produces a typical rot with a noticeable grey-mould layer on the affected tissues that is characteristic of *Botrytis* diseases (Elad *et al*., 1996).

1.2.1.2. Symptoms

Although this pathogen is synonymous with grape infections, *B. cinerea* infects approximately 200 crop species worldwide. The pathogen has shown to be most active on mature or senescent tissue, but this is apparently as a result of an infection that has occurred early in crop development and which remains dormant until the environmental and host conditions become favourable. These latent infections are the cause of severe damage that is expressed after the harvesting of apparently healthy crops and subsequently transporting of these crops to distant markets at which stage the losses become evident (Williamson *et al*., 2007).

Botrytis can be observed wherever host plants are grown, from subtropical areas to temperate zones. Plants may be infected at any stage, but new succulent growth, newly injured tissues and ageing or dead foliage are ideal for this disease. *Botrytis* typically manifests as lesions on leaves and stems that rapidly produce a grey/brown furry spore mass which may resemble a pile of ash, thus the name ‘grey mould’. With progression of the disease, the lesions continue to grow and encircle stems and leaf petioles and will ultimately cause plant collapse. Spores of this fungus can also develop on flower petals, particularly under growing conditions that favour moisture and humidity. Once flower petals have been infected, disease development in young fruit will occur rapidly, with the fruit tissue swiftly disintegrating into a water-soaked mass. On pome and stone fruit *Botrytis* is known to cause a

Stellenbosch University http://scholar.sun.ac.za

soft, spongy rot that has a sweet, cider-like odour. With progression of the rot the fungus produces masses of grey spores on the surface of infected fruits. These infections have the ability to spread from one fruit to the next during storage, producing “nests” or “pockets” of decayed fruit. Small black resting bodies called sclerotia may ultimately form on decayed fruit (Xiao, 2006).

On pome fruit and specifically apples, *Botrytis* may be present in the calyx end throughout the growing season without manifesting any symptoms of infection (Bryk, 1986); leading to the conclusion that infection may be of a latent nature with infection occurring in the orchard followed by disease development in storage. Bryk (1986) identified two important points of infection: preharvest during blooming (calyx end infection); and at the time of, or just after, picking (postharvest puncture infection).

1.2.1.3. Management of *Botrytis*

The management of *Botrytis* spp is aimed at reducing the inoculum load and relies heavily on an integrated management system that incorporates cultural and chemical strategies. Fungicidal control is practiced worldwide and several fungicide classes are available for use. Up until recently, dicarboximide fungicides such as iprodione were used extensively for the control of *Botrytis* in grapes and many other fruit and vegetable production systems (Russell, 2005). Fungicidal control starts in the orchards with fungicides such as iprodione and procymidone applied as two full cover sprays preharvest for the control of calyx end decay on pome fruit (Van Zyl, 2011). Iprodione is also used on apples for the control of postharvest decays caused by *Botrytis* and is applied as a dip or drench (Van Zyl, 2011). However, numerous cases of resistance to these fungicides have resulted in the need to decrease the use of dicarboximides, and other classes of fungicides have had to be used since. Resistance management has become easier with the introduction of three highly effective fungicide classes that include anilinopyrimidines (pyrimethanil), phenylpyrrols, and the hydroxyanilides. Recently, a carboximide fungicide showing activity towards *Botrytis* has also been introduced. The simultaneous use of more than one fungicide class in a season has greatly improved the overall control of *Botrytis* (http://www.compendium.bayercropscience.com).

Stellenbosch University http://scholar.sun.ac.za

1.2.2. *Penicillium expansum*

The genus *Penicillium* is an ascomycete fungus that is of major importance in the natural environment as well as to the food and drug industry (Chutia and Ahmed, 2012). There are approximately 150 species of *Penicillium* and only a small percentage of these species effect agriculturally significant crops (Oliveri *et al*., 2007). Species of *Penicillium* are common soil fungi that prefer cool and temperate climates and are ubiquitous wherever organic material is available. Commonly known as moulds, species of *Penicillium* are among the chief causes of food spoilage (Chutia and Ahmed, 2012).

1.2.2.1. Disease Cycle

Blue mould rot by *P. expansum* is the disease that is most frequently reported on. However, there are a number of other lesser known pathogenic species that are usually also less destructive. All species of *Penicillium* that cause blue mould are primarily wound pathogens that usually gain access to fruit via fresh mechanical injuries. The pathogen produces hardy spores that survive between seasons on infected objects, on which *Penicillium* has the ability to develop and produce spores in abundance. Contamination can also come from an array of other sources which include soil carried on bins brought in from the orchard, decaying fruit, the air, drenching solutions, and contaminated water used in the packhouses (Gardner *et al*., 1986).

1.2.2.2. Symptoms

Penicillium rots (blue and green mould rots and core rots) are the most common and usually the most destructive of all postharvest diseases, which account for up to 90% of decay in transit, storage and in the market. *Penicillium expansum* has been identified as being the main cause of these infections in the postharvest context (van der Walt *et al*., 2010). The pathogen gains entry to tissues through wounds. However, infection can spread from one fruit to another through uninjured skin, the stem end, the open calyx tube and lenticels (Neri *et al*., 2006). Typically, rots first appear as soft, watery, slightly discoloured spots on the surface of the fruit. These spots start off shallow but deepen quickly, and at room temperature the infected fruit decays rapidly within a few days. Once decay has set in, a white mould develops on the surface of the fruit and subsequently produces spores. The sporulating area has different variations of blue to green coloring that is encircled by a white mycelium and a

Stellenbosch University http://scholar.sun.ac.za

ring of water-soaked tissue (Rathod, 2010). Fruit that has been decaying give off a musty odour and under dry conditions may shrink and become mummified. Under moist conditions and at temperatures between 20-25°C, the soft, watery lesions rapidly enlarge, and there is a distinct differentiation between rotted tissues versus the firm healthy tissue. When conditions are humid, spores that are bluish green in colour will form on lesion surfaces (Snowdon, 1990). Even though warm conditions are more favourable, infection can occur at low temperatures with slower onset of decay. However, once the fruit is moved to warmer storage conditions, rapid decay development takes place.

Penicillium expansum is amongst the species of *Penicillium* that is known to produce a mycotoxin, called patulin, which has been reported to be mutagenic and can have a neurotoxic, immunotoxic and gastrointestinal effect on animals (Welke *et al*., 2011). This mycotoxin can be found mainly in low quality fruit, which is usually used in processed apple products, such as juices and baby food. It is therefore important to monitor and attempt to curb the accumulation of such a toxin. In South Africa, and in most countries, the maximum allowed dosage of patulin is 50µg/kg (Kubo, 2012).

1.2.2.3. Management of *Penicillium*

The control of *Penicillium* rots may be achieved by orchard fungicide sprays as well as calcium sprays to increase resistance to fungal infections. However, the most important method to control infection involves careful handling and sanitation in both the orchard and packhouse. Postharvest fungicide dips and drenches may also be effective, but the application time is critical as delays of just a few hours can result in large increases in decay (Snowdon, 1990). Currently, postharvest decay by *Penicillium* is treated with fungicide dips, drenches and atomizer sprays such as chlorine dioxide, iprodione, pyrimethanil, and dimethyl didecyl ammonium chloride, which is registered solely for use against *P. expansum*, for fruit to be treated for 10 minutes shortly after harvest or storage at regular atmosphere (van Zyl, 2011). The possibility of the emergence of fungicide resistant strains makes the concept of using a range of fungicides that differ with regards to mode of action an advisable one.

1.2.3. *Neofabraea alba*

The fungal species responsible for pome fruit diseases such as bull's-eye rot (*Neofabraea alba*), anthracnose (*N. malicoriticis*) and perennial cankers (*N. perennans*) were previously considered to be part of the genus *Pezicula*; however, these organisms have now

Stellenbosch University http://scholar.sun.ac.za

been reclassified as *Neofabraea* species (de Jong *et al*., 2001). Bull's-eye rot of apple and pear is an important postharvest disease for pome fruit producing countries (Gariepy *et al*., 2005; Spotts *et al*., 2009). Amongst the four species of *Neofabraea* causing fruit decay, *Neofabraea alba* is considered to be the main causal agent of bull's-eye rot in apples (Henriquez and Spotts, 2004) in specific regions in the USA and Europe. Gariepy *et al.* (2005) noted that in previous incidence reports of postharvest pathogens in packhouses in British Columbia, bull's-eye rot decay occurred on 40% of 'Golden Delicious' and 9% of 'McIntosh' apples.

1.2.3.1. Disease Cycle

The disease cycle of *N. alba* is not clearly understood. The fungus lives saprophytically on dead bark and the leaves of pome fruit. Conidia are released from acervuli by water throughout the year, and infection of unripe fruit occurs through the lenticels. The pathogen remains latent until the fruit reaches optimal maturity and infects ripened tissue (Neri *et al*., 2009).

Cankers on trees in the orchard are a possible source of inoculum for bull's eye rot of fruit in storage (Gariepy *et al*., 2005); however, canker development has not been linked directly to *N. alba* infections on apples.

1.2.3.2. Symptoms

Fruit infection by *N. alba* occurs in the orchard with disease symptoms appearing after long term cold storage, manifesting as lesions on the fruit (Spotts *et al*., 2009). The lesions are generally flat and slightly sunken, with a brown colour, usually with a lighter brown centre. Decayed tissue remains firm, with acervuli prevalent on older lesions (Snowdon, 1990; Spotts *et al*., 2009).

1.2.3.3. Management of *Neofabraea*

In South Africa, there are no fungicides currently registered specifically for the treatment of *Neofabraea.* Preharvest spray programmes implemented for apple scab are adopted to control this fungus. In recent years, hot water treatments and biofumigation have gained interest as a means of controlling postharvest decay naturally. Plant volatile compounds are being investigated for their antifungal activity and safety at low concentrations. *In vitro* studies of plant volatiles against *N. alba* have shown promising results for decay control

Stellenbosch University http://scholar.sun.ac.za

(Neri *et al*., 2009). The fungicides pyrimethanil, thiophanate-methyl, thiabendazole and pyroclostrobin plus boscalid has shown effectiveness for the control of bull's eye rot caused by *N. alba* on pear fruit overseas (Spotts *et al*., 2009). The fungicide pyrimethanil, under the tradename "Penbotec 400 SC", has been effective for postharvest use against anthracnose and perennial cankers caused by *N. perrenans* and *N. malicorticis* (Janssen, 2008) and this provides the basis for testing this fungicide against *N. alba* on apples in the South Africa.

1.3. POSTHARVEST DISEASE CONTROL STRATEGIES

Crop losses may result from physiological disorders such as superficial scald, pathological decays to fungi and mechanical injury to fruit that occurs during transport and handling. During export, postharvest pathogens such as *B. cinerea, P. expansum* and *Neofabraea* spp. cause major economic losses due to postharvest latent infections that only manifest later on in the export chain (Calvo *et al*., 2007).

The control of postharvest pathogens is of great importance for the apple industry since the development of resistance to currently available commercial fungicides is becoming an important factor in determining the end point quality of fruit. However, it is important to note that postharvest diseases may begin in the field (preharvest) and thus management strategies should be applied to all phases of production and distribution.

Over the years, an assortment of disease management strategies has been employed to reduce spoilage caused by pathogenic microorganisms. Standard methods for managing postharvest diseases include cultural and physical methods, temperature manipulation and controlled atmosphere storage, plant breeding and chemical and biological control strategies.

1.3.1. Cultural and Physical Strategies

Cultural and physical activities represent non-chemical strategies that entail manipulation of the environment to reduce disease pressure. For example, it is important to reduce the length of leaf wetness periods in the orchard as this is the time which is essential for spore germination and penetration. This can be done by increasing plant distance, trimming of the canopy, ventilation, and control of temperature and relative humidity (Elad *et al*., 1996). Some of the other strategies that are employed for the management of postharvest diseases of fruit include sanitation, handling and storage, heat treatments and irradiation (Schirra *et al*., 2011). Successful postharvest handling of fruit requires careful coordination

Stellenbosch University http://scholar.sun.ac.za

and integration of the many steps leading from harvest operations to consumer level in order to maintain the initial fruit quality.

1.3.1.1. Sanitation

Sanitation is commonly practised for the reduction of inoculum sources. This can be achieved by starting with clean material and keeping pruned plant material away from the crop. The removal of infected plant parts (leaves, branches, fruit, etc), as well as any other plant debris that could harbour the pathogen, aids in reducing the inoculum load as well as the likelihood of the pathogen infecting healthy tissue that still exists. In relation to this, the washing and disinfecting of picking boxes, packhouses, drench water tanks (with hypochlorite) and equipment such as knives, pruning shears and other such tools contributes greatly to limiting the amount of disease that may develop later (Llyas, 2010). Along with practising good sanitation, handling with care should be practised to limit mechanical injuries incurred by the fruit during picking, packing, in transit and in storage. Reducing injuries to the crop will help to reduce re-contamination or spread of the disease as well as prevent moisture loss which will keep the crop at optimum vitality. Separation of healthy fruits from decayed fruit in storage reduces possible sources of inoculum and helps to prevent contamination (Ritenour *et al*., 2011). Whilst a great deal of importance is placed on adopting good sanitation and handling practices, it is important to note that the incorrect application of these practises could yield opposite effects. Sargent *et al*. (1995) reported that many postharvest decay problems that occurred within packhouses were as a result of the incorrect use of hypochlorite for sanitising the dump tanks and hydro coolers. While many packers regularly added sodium or calcium hypochlorite to their water handling systems, the effectiveness of this treatment was decreased because the recommended guidelines for packinghouse water sanitation was incorrectly followed.

1.3.1.2. Storage Temperature and Atmosphere

The use of low temperature levels during storage and transit of fresh produce is considered to be the most important method of postharvest disease management. The idea is to cool the fruit after harvesting and then to maintain the low storage temperature for the duration of the postharvest process up until the time that it gets to the consumer. The produce is maintained at storage temperatures between -2 to 14ºC, depending on the type of crop.

Stellenbosch University http://scholar.sun.ac.za

Storage at these low temperatures delays pathogen growth and disease development and also prolongs the physiological postharvest lifespan of the crop (Wu, 2010).

The maintenance of a controlled atmosphere (CA) during storage and transport has been used to suppress respiration of both the host and the pathogen, thereby suppressing the development of postharvest rots. Low oxygen (2-5%) and high carbon dioxide (5-20%) levels are widely used to reduce the respiration of crop and promote the postharvest lifespan and has been shown to extend the edible shelf life of certain produce from 14 to 21 days (Zagory, 1999).

In South Africa, it is recommended that 'Golden Delicious' apples and red cultivars such as 'Starking' be cooled to a core temperature of -0.5°C within the first 48 hours of harvest and held for the duration of storage at 90-95% relative humidity. However, 'Granny Smith' apples should be cooled to 0°C and then raised to 0.5°C and held there for the duration of storage. Under a CA regime, a gas ratio of 3% oxygen and 1.5% carbon dioxide should be attained within 48 hours of storage. It is recommended that fruit destined for the export market should be treated in the same manner, whilst adhering to specific fungicide dips and drench application requirements (van der Merwe *et al*., 2012).

1.3.1.3. Heat Treatments

Heat treatments are reportedly a beneficial means of controlling postharvest diseases (Mirshekari *et al*., 2012), especially with regard to controlling insect pests, preventing fungal rots and to affect the ripening of fruit (Lurie, 1998). The three methods of heat treatment that are commonly used are: (1) hot water which is used for fungal control and insect disinfestations, (2) vapour heat, used particularly for insect control and (3) hot air which has been used for the control of fungi and insects, as well as to monitor the response of the crop to elevated temperatures (Lurie, 1998). Heat treatments (40°C for 5 and 10 minutes) elicited defence responses in fruit which inhibited the growth of *Monilinia fructicola* and reduced overall decay in peach fruit without impairing the quality of the fruit itself (Liu *et al*., 2012). Also, hot-air curing of corn and tobacco leaves helps to remove most of the moisture, thus protecting them from attack by fungal and bacterial saprophytes. Hot water dipping at a temperature of approximately 50°C for 3 minutes was found to significantly reduce storage rots caused by *Neonectria gallingena*, *B. cinerea* and *P. expansum* on apples (Maxin *et al*., 2012).

Stellenbosch University http://scholar.sun.ac.za

Overall, the use of heat treatments is widely viewed to be a non-damaging physical treatment. Suggested application of hot water dipping of apples would be at harvest, following short term cold storage and CA to maintain fruit quality during storage (Maxin *et al*., 2012).

1.3.1.4. Irradiation

The use of irradiation is a technology which was thought to have the potential to control postharvest diseases. Various types of electromagnetic radiation (UV light, X-rays, and gamma rays) have been studied for their ability to control postharvest diseases by killing the disease-causing pathogens of various fruit and vegetable commodities. However, some important microorganisms are not killed at the maximum allowed dosage of radiation treatment. In addition, factors such as temperature, atmospheric composition and physiological state of the produce at the time of treatment all play a role in the final outcome of the treatment (Zagory, 1999).

When tested for its application on apple and pear cultivars, low dose irradiation reduced decay on apples caused by *P. expansum* by up to 80% but failed to have any impact on disease incidence caused by *B. cinerea*. Furthermore, the firmness of the apple was lost to a small extent and while the outer colour was not affected, there was noticeable colour change to the inside flesh of the apples due to irradiation exposure (Drake *et al*., 1998).

1.3.2. Chemical Strategies

At present, the application of chemical agents remains the primary method of choice for the management of postharvest diseases. The strategies adopted here, usually take the form of pre-and/or postharvest sprays, dip or drench treatments, and fumigation. Quite often postharvest pathogens infect produce before harvest. In such cases it is necessary to apply the fungicides in the field. For example, in the control of mango anthracnose, trees are routinely sprayed with a protectant fungicide such as mancozeb during flowering and fruit development (Coates and Johnson, 1997). In general, preharvest sprays control the surface inoculum, and provide preventative control of contamination and infection during harvest and postharvest. Fungicides applied during the postharvest process need to control latent infections and protect against infections which may occur along the postharvest handling chain, including during storage (Coates and Johnson, 1997).

Stellenbosch University http://scholar.sun.ac.za

The application of postharvest fungicides is accomplished through the use of dips, sprays, fumigants, treated wraps and box liners or in waxes and coatings. Dips and sprays are commonly used and depending on the compound, these can take the form of aqueous solutions, suspensions or emulsions. The fungicides that are commonly applied as dips or sprays include the benzimidazoles (e.g. benomyl and thiabendazole) and the demethylation inhibitor fungicides (e.g. prochloraz and imazalil). Other fumigants used include carbon dioxide, ozone and ammonia (Coates and Johnson, 1997). Fruit wraps or box liners impregnated with the fungicide biphenyl have been used in some countries for the control of postharvest decays of citrus fruit (Erasmus *et al*., 2011). An integration of waxes and fungicides is another popular disease control method which also adds to the aesthetic appeal of the fruit, for example, in the use of liquid wax plus imazalil treatment of citrus against *Penicillium* spp. (Erasmus *et al*., 2011).

Fungicides that are principally used for controlling postharvest diseases have been well scrutinized as posing oncogenic and other major health related risks. Furthermore, pathogen resistance to commonly used fungicides has become a major issue. As a result of the perceived negative effects that chemical fungicides pose and the problem of fungicide resistance, there is an international demand for the discovery of safer alternatives that can control postharvest diseases adequately (Tripathi *et al*., 2008).

1.3.3. Alternative Control Strategies

The need to reduce the use of fungicides on export fruit has opened the door for innovative alternative measures ("green" alternatives) to control postharvest diseases. The successful development of alternative measures for decay control would provide a more environmentally friendly and "consumer-acceptable" substitute for the current synthetic fungicides and would provide a competitive advantage to the South African pome fruit producers and exporters in international markets.

Biological control is one of the most promising alternatives to the chemical treatment of postharvest diseases. Over the last two decades, a number of biological control agents have been studied for their use on a multitude of pathogens and fruit crops (Spadaro and Gullino, 2004). Biologically controlling postharvest diseases may be achieved by the use of antagonistic microorganisms, the application of naturally derived compounds, or by enhancing the innate resistance of a commodity (Narayanasamy, 2006).

Stellenbosch University http://scholar.sun.ac.za

1.3.3.1. Antagonistic Microorganisms

The use of antagonistic microorganisms has proved to be the most promising alternative for chemical control, and can be used either alone or as part of an integrated pest management strategy. An ideal antagonist has been described as being an organism which is genetically stable, can be effective at low concentrations and acts against a broad range of pathogens on various fruit commodities. The antagonist should have simple nutritional requirements, have the ability to survive in unfavourable environmental conditions and should be able to grow on cheap substrates in fermenters. In addition, an ideal antagonist should be one that lacks pathogenicity for the host plant and does not produce metabolites that are toxic to plants and humans. It should also be resistant to the most frequently used pesticides and should be compatible with other chemical and physical treatments (Spadaro and Gullino, 2004). An effective antagonistic microorganism that possesses the above traits will work against pathogenic organisms by either the production of antibiotics, competing for nutrients and space, parasitism or direct interaction with the pathogen, or by inducing resistance within the host tissue (Mari and Guizzardi, 1998).

A review conducted on twenty years of biological control research by Droby *et al*. (2009) reported that at the time that the review was done there existed only two commercially available products for postharvest use, namely: "Biosave" (*Pseudomonas syringae* Van Hall) registered in the USA and used for the control of sweet potato and potato diseases and "Shemer" (*Metschnikowia fructicola*) which is registered in Israel and used commercially for the control of sweet potato and carrot diseases. Two yeast-based products on the market at the time of the review included Aspire ™ (Ecogen, US) and YieldPlus™ (Anchor Yeast, South Africa), which is still currently registered for use in South Africa for postharvest application on apples and pears. In addition, a commercial formulation of *Candida sake* was developed and registered for use on pome fruit in Spain (Droby *et al*., 2009). Antagonistic microorganisms such as *Cryptococcus albidus*, *Agrobacterium radiobactor* and *Bacillus subtilis* have been registered for use on various fruit commodities in South Africa (van Zyl, 2011).

Whilst there are many advocates for the use of biological control agents; however, use of such a method does come with a few drawbacks. In a study conducted to control *Northern jointvetch* weed disease on rice, the authors pointed out some of the disadvantages of biological control include a) high costs for initial and subsequent treatments, b) specific to that study was that the control was only on one weed species infecting rice and c) biological

Stellenbosch University http://scholar.sun.ac.za

control agents are often more sensitive to the environment than herbicides or fungicides (Daniel *et al*., 1973).

Over the past years there has been increased research focused on identifying microorganisms to be used in the control of postharvest diseases. The expectation is that at least a small portion of diseases that are of economic importance can be effectively managed with the use of biocontrol agents; however, the successful application of these products depend largely on their ability to control postharvest diseases in a reliable, cost effective and user friendly manner (Narayanasamy, 2006).

1.3.3.2. Secondary Compounds in Plants

In recent years, plant bioactive substances have been studied as a new approach to postharvest disease management (Mari *et al*., 2010). Plants produce an array of secondary metabolites, which in many cases have been found to be biologically active, and a rich source of antimicrobial, allelopathic, antioxidant and bioregulatory properties (Tripathi *et al*., 2008). The suggestion that plant extracts may be good alternatives to the traditionally used fungicides to control phytopathogenic fungi are attributed to the presence of bioactive chemicals such as flavonoids, phenols, tannins, alkaloids, quinons, saponins and sterols (Burt, 2004). Naturally occurring biologically active compounds from plants are believed to be more adaptable, acceptable and less harmful than artificial compounds, and therefore represent a wealthy source of prospective disease-control agents (Tripathi *et al*., 2008; Amini *et al.,* 2012). Some extracts and essential oils of "medicinal" plants have been found to be effective against fungal and bacterial pathogens (Amini *et al.,* 2012). Furthermore, these plant products are biodegradable compounds which could be used in an integrated pest management program (Soylu *et al*., 2006), and many have shown low mammalian toxicity (Tzortzakis and Economakis, 2007).

Essential oil production by plants is considered to be a mechanism of plants to defend themselves against pathogens and pests (Hadizadeh *et al*., 2009). Certain aromatic components produced by fruits during ripening showed antifungal activity (Mari and Guizzardi, 1998). The fungicidal activity of essential oils from citrus, eucalyptus and thymus has already been demonstrated in a number of studies. For example, *in vitro* studies have shown that the oil of eucalyptus inhibits mycelial growth of important soilborne and postharvest disease pathogens such as *Pythium spp, Rhizoctonia solani* (Katooli *et al*., 2011; Huy *et al*., 2000) and *Collectotrichum gloeosporioides* (Huy *et al*., 2000). A study done by

Stellenbosch University http://scholar.sun.ac.za

Nosrati *et al.* (2011) proposed that spearmint essential oil could be used in the control and management of *Fusarium oxysporum f. sp. radicis-cucumerinum* which is the causal organism of stem and crown rot of greenhouse cucumber. Pawar and Thaker (2007) examined the effect of essential oils obtained from several different plants on *Alternaria porri* and *Fusarium oxysporum f. sp. cicer*, and found that the most active essential oils were those of lemongrass, clove, cinnamon bark, cinnamon leaf, cassia, fennel, basil and evening primrose.

The essential oil of clove is extracted from the leaves, twigs and flower buds of the clove plant *Eugenia aromatica*. The plant has been extensively studied and certainly adds to the arsenal of plant extracts that are effective against microbial pathogens. Bacterial plant pathogens *Agrobacterium tumefaciens*, *Erwinia carotovora* pv. *carotovora*, *Pseudomonas syringae* pv. *syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *pelargonii*, *Rhodococcus fascians* and *Streptomyces* spp. have all shown sensitivity to clove oil extracts (Huang and Lakshman, 2010). Clove essential oil has also been recommended to control postharvest decay fungi such as *B. cinerea* (Siripornvisal *et al*., 2009).

The antibacterial and antifungal activities of plant extracts and essential oils have been demonstrated extensively *in vitro*; however, positive effects of plant extracts and essential oils on postharvest diseases have only been demonstrated on a few fresh commodities (Plotto *et al*., 2003). In constrast, Plotto *et al.* (2003) showed that essential oil vapours of thyme, oregano, lemongrass and cilantro were unsuccessful in halting disease development in artificially inoculated tomatoes. Some oil vapours appeared to provoke a phytotoxic effect on treated fruit under long periods of exposure; however, their study showed that oil emulsions of thyme and oregano did in fact reduce disease development in tomatoes inoculated with *B. cinerea* and *Alternaria aborescens* when applied as dip treatments. An investigation into reducing the postharvest fungal rot caused by *Alternaria alternata* showed that nettle oil reduced decay by approximately 46% and treatments with this oil also did not result in any visible disorders or off-odours to the fruits (Hadizadeh *et al*., 2009).

Plant extracts and essential oils are made up of many different compounds and the composition of the oils often varies between species of plant. It is therefore rather difficult to associate antifungal activity to single compounds or the chemical classes they fall into (Mishra and Dubey, 1994). It may very well be that the inhibitory effects displayed may be due to synergistic interactions between the different compounds (Bagamboula *et al*., 2004)

Stellenbosch University http://scholar.sun.ac.za

suggesting that resistance of plant pathogenic fungi to the essential oils is unlikely to develop. This is yet another reason that plant extracts and essential oils are promising compounds in the development of natural fungicides.

1.4. *ALLIUM SATIVUM*

1.4.1. Description

Allium sativum L., commonly known as garlic, is native to central Asia, and has long been a staple in the Mediterranean region as well as Asia, Africa, and Europe. It was known to ancient Egyptians, and has been used for both culinary and medicinal purposes since their time (Harris *et al*., 2001).

A garlic plant may grow to be 30-90 cm tall. The bulb below ground is the main part of the plant and is divided into segments called cloves, with each bulb containing between 6-12 cloves. A vertical stem grows from the garlic bulb to form an umbrella shaped arrangement of flowers in a cluster, with linear leaves growing from the base of the stem. The flower cluster varies in colour from purplish white to pale pink or a reddish white, according to the variety, soil and chemical influences. Garlic's recognizable smell is derived from its' sulphur-containing constituents, which are also considered to be the source of its medicinal properties (Ankri and Mirelman, 1999; Davies, 2012).

1.4.2. Distribution and Habitat

There are approximately 300 varieties of garlic, which is cultivated worldwide, particularly in hot, dry regions. De La Cruz Medina and Garcia (2007) reported that garlic is one of the twenty most important vegetables in the world, with an annual production of roughly three million metric tons. Major garlic growing countries include the USA, China, Egypt, Korea, Russia and India and South Africa (De La Cruz Medina and Garcia, 2007).

1.4.3. Cultural Practices

Garlic has been used by many cultures throughout history for both culinary and medicinal purposes. In ancient Egypt, the workers building the great pyramids were fed garlic on daily basis. The Bible makes reference to the Hebrews having enjoyed their food with garlic (Numbers 11:5, KJV). In the First World War, garlic was extensively used as an antiseptic to prevent gangrene (De La Cruz Medina and Garcia, 2007). *In vitro* studies on

Stellenbosch University http://scholar.sun.ac.za

garlic has established the antiprotozoal, antiviral, antibacterial and antifungal properties of this plant (Harris *et al*., 2001).

1.4.4. Medicinal Uses

The effectiveness of garlic against cardiovascular diseases *in vitro* has prompted many clinical trials focusing on disease conditions such as atherosclerosis, hyperlipidemia, thrombosis, hypertension and diabetes. These clinical trials have yielded some positive results (Banerjee and Maulik, 2002). Studies have shown that an allicin containing supplement could prevent against attack by the common cold (Josling, 2001). This has the backing of long tradition in herbal medicine and Cherokee culture, which has used garlic for hoarseness and coughs (Pandya *et al*., 2011).

Garlic extracts also show *in vitro* activity against influenza A and B, cytomegalovirus, rhinovirus, and HIV. The active inhibitors of such infections seem to be mainly allicin, diallyl trisulphide and ajoene (Harris *et al*., 2001; Cardelle-Cobas *et al*., 2010). Josling (2001) conducted a study in which volunteers were randomly selected to receive either a placebo or allicin-containing garlic supplement on a daily basis over a period of 12 weeks. The study revealed that the active-treatment group had significantly fewer colds than the placebo group, which recorded more days with viral symptoms, with a significantly longer duration of symptoms. It was therefore concluded that intake of an allicin containing supplement can protect against attack by the common cold (Josling, 2001). Harris *et al*. (2001), reported on an article which proposed that in the case of HIV, ajoene may act by inhibiting the integrin-dependant processes. Allyl alcohol and diallyl disulfide have also proven to be effective against HIV-infected cells (Shoji *et al*., 1993). No activity has been observed with alliin or S-allyl cysteine suggesting that only allicin and allicin-derived substances have any inhibitory activity against viral pathogens (Harris *et al*., 2001).

Under certain conditions, allicin degrades to diallyl trisulphide, which is a more stable chemical than the extremely volatile allicin, and is easily synthesised (Amagase, 2006; Cardelle-Cobas *et al*., 2010). In China, a commercially available preparation of this called "Dasuansu" has been prescribed for the control of *Entamoeba histolytica* and *Trichomonas vaginalis* infections (Lun *et al*., 1994). Garlic may also be antigiardial, removing the symptoms from patients within 24 hours and completely removing any indication of giardiasis from the stool of patients within 72 hours when a dosage of 1 mg ml^{-1} twice daily

Stellenbosch University http://scholar.sun.ac.za

aqueous extract or 0.6 mg ml^{-1} commercially prepared garlic capsules was administered (Harris *et al*., 2001).

1.4.5. Chemical Compounds

Garlic's main active constituent is alliin (Figure 1) (Benkeblia and Lanzotti, 2007). Alliin, when crushed, converts to allicin which is an antibiotic. Garlic also contains other sulphur-containing compounds such as ajoene, diallylsulfide, dithiin, S-allylcysteine, and enzymes, B vitamins, proteins, minerals, saponins, flavonoids, and Maillard reaction products, which are non sulphur-containing compounds. Furthermore, a phytoalexin (allixin) has been found (Pandya *et al.*, 2011). This is a non-sulphur compound with a γ-pyrone skeleton structure that has antioxidant effects, antimicrobial effects, antitumor promoting effects, inhibits aflatoxin B2 DNA binding, and neurotrophic effects (Yamasaki *et al*., 1991).

Non-volatile sulphur containing compounds such as g-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin) are both abundant in intact garlic. These sulfoxides are then converted into thiosulphinate (such as allicin) through enzymatic reactions (Amagase, 2006). Other thiosulphinate and oil-soluble components such as ajoenes, vinyldithiins and sulphides such as diallyl sulphide (DAS), diallyl disulphide (DADS), and diallyl trisulphide (DATS), also contribute to garlic's characteristic flavour and odour and biological properties (Cardelle-Cobas *et al*., 2010). Like allicin, these compounds are all volatile and are possibly quite unstable (Amagase, 2006). The antibacterial, antifungal, antiviral and antiprotozoal effects of garlic has been ascribed to the abovementioned constituents of the plant.

1.4.6. Antimicrobial Properties

Garlic has been used for centuries to combat various diseases. In India, it has been used to prevent wound infection and food spoilage (Arora and Kaur, 2007). More recently garlic has proven to be effective against a host of gram-positive, gram-negative and acid-fast bacteria, including *Pseudomonas*, *Proteus*, *Staphylococcus aureus, Escherichia coli*, *Salmonella*, *Klebsiella*, *Micrococcus*, *Bacillus subtilis*, *Clostridium*, *Mycobacterium* and *Helicobacter* (Delaha and Garagusi, 1985; Harris *et al*., 2001). The antibacterial activity of garlic is widely attributed to allicin. This is supported by the observation that when stored at room temperature the antibacterial capacity of garlic extract is greatly reduced when compared to extracts that have been stored at 0–4°C, suggesting thermal instability of the

Stellenbosch University http://scholar.sun.ac.za

active components (Harris *et al*., 2001). The intracellular effects of allicin are not well understood; however, it is known that allicin has sulfhydryl modifying activity and is therefore capable of inhibiting sulfhydryl enzymes. Cysteine and glutathione neutralize the thiolation activity of allicin, and on addition to the reaction mixture, the antibacterial activity is reduced (Harris *et al*., 2001).

Garlic extracts have also been shown to decrease oxygen uptake of microbes, reduce the growth of pathogenic organisms, and to inhibit the synthesis of lipids, proteins and nucleic acids and damage to membranes of microorganisms (Harris *et al*., 2001). Once again, studies in this area have shown that it is the allicin and allicin-derived constituents that contribute to the antifungal properties of garlic. In a study done by Hughes and Lawson (1991), a sample of pure allicin was shown to be antifungal but the removal of allicin from the reaction by solvent extraction decreased the antifungal activity (Harris *et al*., 2001).

While the literature shows that garlic has long been used as a source of antibiotic for human pathogens, investigations on its value as a deterrent to plant pathogens are recent. Extracts of garlic have been shown to have a strong inhibitory effect on the mycelial development of plant pathogenic fungi such as *Fusarium solani*, *Rhizoctonia solani*, *Pythium ultimum* and *Colletotrichum lindemuthianum* (Bianchi et *al*., 1997). Garlic has shown promising results for the control of powdery mildew on cucumbers (Seo *et al*., 2006). Methanolic garlic extracts have been shown to elicit a complete inhibitory effect on the fungal growth of *Penicillium digitatum* (Kanan and Al-Najar, 2008).

Garlic has also been investigated for its usage as a green insecticide. For example, Koul *et al.* (2008) found that garlic oil was highly toxic to the eggs of the diamond black moth (*Putella xylostella*).

A garlic pesticide is available for commercial use under the trade name "Mole Repellent" (Efekto™) (Pesticides 2010: www.croplife.co.za). Recent products added to the list with garlic extracts as an active ingredient include "Kannar Garlic Repellent 930", Kannar KangroShield100" and "Kanguard 940", all of which have been registered for use on cherries and no minimum residue level (MRL) specification exists for the garlic component of these products (HORTGRO Science, 2013).

Stellenbosch University http://scholar.sun.ac.za

1.5. AIMS AND OBJECTIVES

The aim of this study was to evaluate the antifungal efficacy of crude garlic extracts against mycelial growth and spore germination of the postharvest pathogens *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*, both *in vitro* and *in vivo.*

Objective 1: *In vitro* assays

(a) The first objective was to investigate the antifungal activity of crude garlic extract preparations (containing ethanol or no ethanol), diluted to different concentrations using two diluents (ethanol and water, respectively) on the mycelial growth and spore germination of the target fungal pathogens, and subsequently, to determine whether the effect was fungistatic or fungicidal in nature.

(b) The ability of the crude garlic extracts to elicit antifungal activity via the vapour phase was investigated in combination with storage temperatures and effective concentrations highlighted by objective 1a above.

Objective 2: *In vivo* assays

(a) To determine the curative or protective effect of crude garlic extracts on postharvest pathogen decay on different apple cultivars alone, and in combination with the essential oil of clove bud.

(b) To determine the curative or protective effects that the volatile vapours of garlic extracts and clove bud essential oil would have on the control of postharvest pathogen decay on different apple cultivars

Objective 3: Full chemical profile analysis of crude garlic extracts

(a) Garlic extracts were subjected to a full profile chemical analysis using gas chromatography-mass spectrometry (GC-MS) in order to identify possible compounds responsible for the observed antifungal activity.

Stellenbosch University http://scholar.sun.ac.za

1.6. LITERATURE CITED

Amagase, H. 2006. Significance of garlic and its constituents in cancer and cardiovascular disease: Clarifying the real bioactive constituents of garlic. The Journal of Nutrition 2: 716-725. Online: www.jn.nutrition.org Retrieved 09-01-2013.

Amini, M., Safaie, N., Salmani, M.J. and Shams-Bakhsh, M. 2012. Antifungal activity of three medicinal plant essential oils against some phytopathogenic fungi. Trakia Journal of Sciences 10: 1-8.

Ankri, S. and Mirelman, D. 1999. Antimicrobial properties of allicin from garlic. Microbes and Infection 2: 125-129.

Arora, S.D. and Kaur, G.J. 2007. Antibacterial activity of some Indian medicinal plants. Journal of Natural Medicine 61: 313-317.

Bagamboula, C.F., Uyttendaele, M. and Debevere, J. 2004. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. Food Microbiology 21: 33-42.

Banerjee, S.K. and Maulik, S.K. 2002. Effect of garlic on cardiovascular disorders: A review. Nutrition Journal 1: 4.

Bayer CropScience crop compendium: *Botrytis cinerea*. Online: http://compendium.bayercropscience.com. Retrieved 20-06-2012.

Benkeblia, N. and Lanzotti, V. 2007. *Allium* thiosulfinates: Chemistry, biological properties and their potential utilization in food preservation. Food 1: 193-201.

Bianchi, A., Zambonelli, A., Zechini D'Aulerio, A. and Bellesia, F. 1997. Ultrastructural studies of the effects of *Allium sativum* on phytopathogenic fungi *in vitro*. Plant Disease 81: 1241-1246.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods – A review. International Journal of Food Microbiology 94: 223-253.

Bryk, H. 1986. The time of infection of apples by *Botrytis cinerea* Pers. Acta Agrobotanica 39: 385-395.

Calvo, J., Calvente, V., de Orellano, M.E., Benuzzi, D. and Sanz de Tosetti, M.I. 2007. Biological control of postharvest spoilage caused by *Penicillium expansum* and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*. International Journal of Food Microbiology 113: 251-257.

Stellenbosch University http://scholar.sun.ac.za

Cardelle-Cobas, A., Soria, A.C., Corzo-Martinez, M. and Villamiel, M. 2010. A comprehensive survey of garlic functionality. In: Garlic Consumption and Health. Nova Science Publishers, Inc.

Chutia, M. and Ahmed, G.U. 2012. Diversity, antimicrobial activities and associated microbiota of soil *Penicillia* from virgin forest floor. Journal of Microbiology, Biotechnology and Food Sciences 1: 1279-1294.

Coates, L.M. and Johnson, G. 1997. Postharvest diseases of fruit and vegetables. In: Plant Pathogens and Plant Diseases. Rockvale Publications, Armidale, Australia 533-548.

Coertze, S. and Holz, G. 2002. Epidemiology of *Botrytis cinerea* on grape: wound infection by dry, airborne conidia. South African Journal of Enology and Viticulture 23: 72-77.

Daniel, J.T., Templeton, G.E., Smith, R.J. and Fox, W.T. 1973. Biological control of Northern Jointvetch in rice with an endemic fungal disease. Weed Science 21: 303-307.

Davies, J.R. Herbs hands healing: Traditional western herbal products. Online: Extracts from: In a nutshell 'Garlic' http://www.herbs-hands-healing.co.uk/singleherbs/garlic.html. Retrieved: 08-03-2012.

de Jong, S.N., Levesque, C.A., Verkley, G.J.M., Abeln, E.C.A., Rahe, J.E. and Braun, P.G. 2001. Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the *b*-tuberlin gene. Mycological Research 105: 658-669.

De La Cruz Medina, J. and Garcia, H.S. 2007. Garlic: Postharvest Operations. Instituto Tecnologico de Veracruz.

Delaha, E.C. and Garagusi, V.F. 1985. Inhibition of mycobacteria by garlic extract (*Allium sativum*). Antimicrobial Agents and Chemotherapy 27: 485–486.

Drake, S.R., Sanderson, P.G. and Neven, L.G. 1998. Quality of apples and pears after exposure to irradiation as a quarantine treatment. USDA, ARS-TFRL. Online: http://mbao.org/1998airc/079drake.pdf Retrieved: 18-11-2013.

Droby, S., Wisniewski, M., Macarisin, D. and Wilson, C. 2009. Review: Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biology and Technology 52: 137-145.

Elad, Y., Malathrakis, N.E. and Dik, A.J. 1996. Biological control of *Botrytis*-incited diseases and powdery mildews in greenhouse crops. Crop Protection 15: 229-240.

Stellenbosch University http://scholar.sun.ac.za

Erasmus, A., Lennox, C.L., Jordaan, H., Smilanick, J.L., Lesar, K. and Fourie, P.H. 2011. Imazalil residue loading and green mould control in citrus packhouses. Postharvest Biology and Technology 62: 193-203.

Gardner, P.D., Eckert, J.W., Baritelle, J.L. and Bancroft, M.N. 1986. Management strategies for control of *Penicillium* decay in lemon packinghouses: economic benefits. Crop Protection 5: 26-32.

Gariepy, T.D., Rahe, J.E., Levesque, C.A., Spotts, R.A., Sugar, D.L. and Henriquez, J.L. 2005. *Neofabraea* species associated with bull's-eye rot and cankers of apple and pear in the Pacific Northwest. Canadian Journal of Plant Pathology 27: 118-124.

Geisler, M. 2011. Commodity apple profile. Agricultural Marketing Resource Centre. Online: http://www.agmrc.org/. Retrieved 10-04-2012.

Hadizadeh, I., Peivastegan, B. and Hamzehzarghani, H. 2009. Antifungal activity of essential oils from some medicinal plants of Iran against *Alternaria alternata*. American Journal of Applied Sciences 6: 857-861.

Harris, J.C., Cottrell, S. and Lloyd, D. 2001. Antimicrobial properties of *Allium sativum* (garlic). Applied Microbiology and Biotechnology 57: 282-286.

Henriquez, J.L. and Spotts, R.A. 2004. Etiology of bull's eye rot of pear caused by *Neofabraea* spp. in Oregon, Washington, and California. Plant Disease 88: 1134-1138.

HORTGRO Science Freshnotes Technical Update. 2013. Industry guideline information on restrictions on the use of plant protection products on deciduous fruit for 2013/14, version 2. Online: http://www.hortgro.co.za/production-techical-information/mrls/documents. Retrieved: 17-10-2013.

Huang, Q. and Lakshman, D.K. 2010. Effect of clove oil on plant pathogenic bacteria and bacterial wilt of tomato and geranium. Journal of Plant Pathology 92: 701-707.

Hughes, B.G. and Lawson, L.D. 1991. Antimicrobial effect of *Allium sativum* L. (garlic) *Allium ampeloprasum* (elephant garlic), and *Allium cepa* L. (onion), garlic compounds and commercial garlic supplement products. Journal of Phytological Research 5: 154-158.

Huy, J.S., Ahn, S.Y. and Koh, Y.J. 2000. Antimicrobial properties of cold-tolerant *Eucalyptus* species against phytopathogenic fungi and food-borne bacterial Pathogens. Plant Pathology Journal 16: 286-289.

Stellenbosch University http://scholar.sun.ac.za

Janssen Preservation and Material Protection (PMP). 2008. PENBOTEC™ labelled for the control of bull's-eye rot on pome fruit (*Neofabraea perennens* and *N. malicorticis*). Janssen PMP Research Bulletin and Label Update for Deliveries to the US.

Josling, P. 2001. Preventing the common cold with a garlic supplement: A double-blind, placebo controlled survey. Advances in Natural Therapy 18: 4.

Kanan, G.J. and Al-Najar, R.A. 2008. *In vitro* antifungal activities of various plant crude extracts and fractions against citrus postharvest disease agent *Penicillium digitatum*. Journal of Biological Sciences 1: 89-99.

Katooli, N., Maghsodlo, R. and Razavi, S.E. 2011. Evaluation of *eucalyptus* essential oil against some plant pathogenic fungi. Journal of Plant Breeding and Crop Science 3: 41-43.

Kim, Y.K. and Xiao, C.L. 2008. Distribution and incidence of *Sphaeropsis* rot in apple in Washington State. Plant Disease 92: 940-946.

Koul, O., Walia, S. and Dhaliwal, G.S. 2008. Essential oils as green pesticides: Potential and constraints. Biopesticides International 4: 63-84.

Kubo, M. 2012. Mycotoxins legislation worldwide. Leatherhead Food Research. Online: http://services.leatherheadfood.com/eman/FactSheet. Retrieved: 07-11-2013.

Laluk, K. and Mengiste, T. 2010. Necrotroph attacks on plant: wanton destruction or covert extortion. Pages 1-34 in: The Arabidopsis Book. American Society of Plant Biologists.

Liu, J., Sui, Y., Wisniewski, M., Droby, S., Tian, S., Norelli, J. and Hershkovitz, V. 2012. Effect of heat treatment on inhibition of *Monilinia fructicola* and induction of disease resistance in peach fruit. Postharvest Biology and Technology 65: 61-68.

Llyas, S.M. 2010. Best practices for the efficient postharvest management of fruits and vegetables for higher value addition and profitability. Pages 48-71 in: 2010 AARDO Workshop on Technology on Reducing Post-Harvest Losses and Maintaining Quality of Fruits and Vegetables.

Lun, Z.R., Burri, C., Menzinger, M. and Karminsky, R. 1994. Antiparasitic activity of diallyl trisulfide (Dasuansu) on human and animal pathogenic protozoa (*Trypanosoma* sp., *Entamoeba histolyica* and *Giardia lamblia*) *in vitro*. Annales de la Societe Belge de Medecine Tropicale 74:51–59.

Lurie, S. 1998. Review: Postharvest heat treatments. Postharvest Biology and Technology 14: 257-269.

Stellenbosch University http://scholar.sun.ac.za

Mari, M. and Guizzardi, M. 1998. The postharvest phase: Emerging technologies for the control of fungal diseases. Phytoparasitica 26: 59-66.

Mari, M., Neri, F. and Bertolini, P. 2010. New approaches for postharvest disease control in Europe. In: Prusky, D., and Gullino, M.L. Plant Pathology in the 21st Century: Contributions to the 9th International Congress of Postharvest Pathology. Springer Dordrecht.

Maxin, P., Weber, R.W.S., Linhard Pederson, H. and Williams, M. 2012. Hot-water dipping of apples to control *Penicillium expansum*, *Neonectria gallingena* and *Botrytis cinerea*: effects of temperature on spore germination and fruit rots. European Journal of Horticultural Science 77: 1-9.

Mirshekari, A., Ding, P., Kadir, J. and Ghazali, H.M. 2012. Effect of hot water dip treatment on postharvest anthracnose of banana var. *Berangan*. African Journal of Agricultural Research 7: 6-10.

Mirzaei, S., Goltapeh, E.M. and Shams-bakhsh, M. 2007. Taxonomical studies on the genus *Botrytis* in Iran. Journal of Agricultural Technology 3: 65-76.

Mishra, A.K. and Dubey, N.K. 1994. Evaluation of some essential oils for the toxicity against fungi causing deterioration of stored food commodities. Applied and Environmental Microbiology 60: 1101-1105.

Mogala, M. 2012. A profile of the South African apple market value chain. Department of Agriculture, Forestry and Fisheries, Arcadia, Pretoria, South Africa. Online: www.daff.gov.za Retrieved 20-09-2013.

Morokolo, B. 2011. A profile of the South African apple market value chain. Department of Agriculture, Forestry and Fisheries, Arcadia, Pretoria, South Africa. Online: www.daff.gov.za Retrieved 03-03-2012.

Narayanasamy, P. 2006. Postharvest pathogens and disease management. Pages 357-433 in: Chapter 9: Biocontrol agents for disease management. Wiley-Interscience, New Jersey, USA.

Negro, D.R. and Lojo, J. 2011. World apple production reached a new record. Online: http://www.freshplaza.com/. Retrieved 10-04-2012.

Neri, F., Mari, M., Brigati, S. and Bertolini, P. 2009. Control of *Neofabraea alba* by plant volatile compounds and hot water. Postharvest Biology and Technology 51: 425-430.

Neri, F., Mari, M. and Brigati, S. 2006. Control of *Penicillium expansum* by plant volatile compounds. Plant Pathology 55: 100-105.

Stellenbosch University http://scholar.sun.ac.za

Nosrati, S., EsmaeilzadehHosseini, S.A., Sarpeleh, A., SoflaeiShahrbabak, M. and SoflaeiShahrbabak, Y. 2011. Antifungal activity of (*Mentha spicata L.*) essential oil on *Fusarium oxysporum f. sp. radicis-cucumerinum* the causal agent of stem and crown rot of greenhouse cucumber in Yazd, Iran. 2011 International Conference on Environmental and Agriculture Engineering Volume 15.

Oliveri, C., Campisano, A., Catara, A. and Cirvilleri, G. 2007. Characterisation and fAFLP genotyping of *Penicillium* strains from postharvest samples and packinghouses. Plant Pathology 89: 29-40.

Pandya, K., Solanki, B., Maniar, K., Gurav, N. and Bhatt, S. 2011. Natural herbal supplements – A study on the nutritional value and their phytochemical constituents. International Journal of Pharmaceutical Science and Research 2: 1480-1494.

Pawar, C. and Thaker, V.S. 2007. Evaluation of the anti *Fusarium oxysporum f. sp. cicer* and *Alternaria porri* effect of some essential oils. World Journal of Microbiology and Biotechnology 23: 1099-1106.

Perishable Products Export Control Board (PPECB). 2013. PPECB Export Directory 2013. South Africa, 11th edition.

Pesticides 2010. Online: http://www.croplife.co.za/. Retrieved 31-08-2012.

Plotto, A., Roberts, R.G. and Roberts, D.D. 2003. Evaluation of plant essential oils as a natural postharvest disease control of tomato (*Lycopersicon esculentum*). Acta Horticulture 628: 737-745.

Polderdijk, J.J., Van Dyk, F.E., Ferrerira, D., Gius, G.W. and Keller, S. 2006. Fruitful: Integrated supply-chain information system for fruit produce between South Africa and The Netherlands. Agro-food Chains and Networks for Development: 129-140.

Rathod, G.M. 2010. Survey of post-harvest fungal diseases of some fruits from Marathwada regions of Maharashtra, India. Journal of Ecobiotechnology 2: 7-10.

Ritenour, M.A., Zhang, J. and Dewdney, M. 2011. Postharvest decay control recommendations for Florida citrus fruit. Institute of Food and Agricultural Sciences, University of Florida.

Russel, P.E. 2005. Centenary Review: A century of fungicide evolution. Journal of Agricultural Science 143: 11-25.

Sargent, S.A., Ritenour, M.A. and Brecht, J.K. 1995. Handling, cooling and sanitation techniques for maintaining postharvest quality. Institute of Food and Agricultural Sciences, University of Florida; HS719.

Stellenbosch University http://scholar.sun.ac.za

Schirra, M., D'Aquino, S., Cabras, P. and Angioni, A. 2011. Control of postharvest diseases of fruit by heat and fungicides: efficacy, residue levels, and residue persistence. A review. Journal of Agricultural and Food Chemistry 59: 8531-8542.

Schmidt, R.J. 2013. *Alliaceae – 1 Agapanthus – Tulbaghia* (onion family). Online: http://www.botanical-dermatology-database.info/BotDermFolder/ALLI-1.html Retrieved: 27-08-2013.

Seo, S., Lee, J., Park, J., Han, K. and Jang, H. 2006. Control of powdery mildew by garlic oil in cucumber and tomato. Research in Plant Disease 12: 51-54.

Shahi, S.K., Patra, M., Shukla, A.C. and Dikshit, A. 2003. Use of essential oil as botanical-pesticide against post harvest spoilage in *Malus pumilo* fruits. BioControl 48: 223-232.

Shoji, S., Furuishi, K., Yanase, R., Miyazaka, T. and Masayasu, K. 1993. Allyl compounds selectively killed human deficiency virus-type 1-infected cells. Biochemical and Biophysical Research Communications 194: 610–621.

Shtienberg, D. and Elad, Y. 1997. Incorporation of weather forecasting in integrated, biological-chemical management of *Botrytis cinerea*. Phytopathology 87: 332-340.

Siripornvisal, S., Rungprom, W. and Sawatdikarn, S. 2009. Antifungal activity of essential oils derived from medicinal plants against grey mould (*Botrytis cinerea*). Asian Journal of Food and Agro-Industry (Special Issue): 229-223.

Snowdon, A.L. 1990. A Colour Atlas of Postharvest Diseases and Disorders of Fruits and Vegetables. Wolfe Scientific, Spain.

Soylu, E.M., Soylu, S. and Kurt S. 2006. Antimicrobial activities of the essential oils of various plants against tomato late blight disease agent *Phytophthora infestans*. Mycopathologia 161: 119-128.

Spadaro, D. and Gullino, M.L. 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. Journal of Food Microbiology 91: 185-194.

Spotts, R.A., Seifert, K.A., Wallis, K.M., Sugar, D., Xiao, C.L., Serdani, M. and Henriquez, J.L. 2009. Description of *Cryptosporiopsis kienhilzii* and species profiles of *Neofabraea* in major pome fruit growing districts in the Pacific Northwest USA. Mycological Research 113: 1301-1311.

The Holy Bible. New King James Version. 1982. Numbers 11:5, pp. 190. Thomas Nelson Bibles, Nashville, Tennessee, USA.

Stellenbosch University http://scholar.sun.ac.za

Tian, S. P. and Bertolini, P. 1995. Effects of low temperature on mycelial growth and spore germination of *Botrytis allii* in culture and on its pathogenicity to stored garlic bulbs. Plant Pathology 44: 1008-1015.

Tripathi, P., Dubey, N.K. and Shukla, A.K. 2008. Use of some essential oils as postharvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. World Journal of Microbiology and Biotechnology 24: 39-46.

Tzortzakis, N.G. and Economakis, C.D. 2007. Antifungal activity of lemongrass (*Cymbopogon citrates* L.) essential oil against key postharvest pathogens. Innovative Food Science and Emerging Technologies 8: 253-258.

Van der Merwe, J.A., da Silva, N. and van Bodegom, P. 2012. Chapter 6: Recommended temperature and gas regimes for cold storage of South African commercial apple and pear cultivars. In: South African apple and pear producers' association CA operator's manual. Deciduous Fruit Producers Trust, Paarl, South Africa.

van der Walt, L., Spotts, R.A., Visagie, C.M., Jacobs, K., Smit, F.J. and McLeod, A. 2010. *Penicillium* species associated with preharvest wet core rot in South Africa and their pathogenicity on apple. Plant Disease 94: 666-675.

van Zyl, K. 2011. The Control of Fungal, Viral and Bacterial Diseases in Plants. A Croplife South Africa compendium, 1st ed. AVCASA, South Africa.

Welke, J.E., Hoeltz, M., Dottori, H.A. and Noll, I.B. 2011. Patulin accumulation in apples during storage by *Penicillium expansum* and *Penicillium griseofulvum* strains. Brazilian Journal of Microbiology 42: 172-180.

Williamson, B., Tudzynski, B., Tudzynski, P. and Van Kan, J.A.L. 2007. Pathogen profile *Botrytis cinerea:* the cause of grey mould disease. Molecular Plant Pathology 8: 561-580.

Wu, C. 2010. An overview of postharvest biology and technology of fruits and vegetables. 2010 AARDO Workshop on Technology on Reducing Post-harvest Losses and Maintaining Quality of Fruits and Vegetables. 2-11.

Xiao, C.L. 2006. Postharvest fruit rots in d'Anjou pears caused by *Botrytis cinerea*, *Potebniamyces pyri*, and *Sphaeropsis pyriputrescens*. Online: Plant Health Progress http://www.plantmanagementnetwork.org/pub/php/diagnosticguide/2006/pears/ Retrieved: 16-09-2013.

Stellenbosch University http://scholar.sun.ac.za

Yamasaki, T., Teel, R.W. and Lau, B.H. 1991. Effect of allixin, a phytoalexin produced by garlic, on mutagenesis, DNA-binding and metabolism of alfatoxin B1. Cancer Letters 59: 89-94.

Zagory, D. 1999. Effects of post-processing handling and packaging on microbial populations. Postharvest Biology and Technology 15: 313-321

Stellenbosch University http://scholar.sun.ac.za

1.7. FIGURES

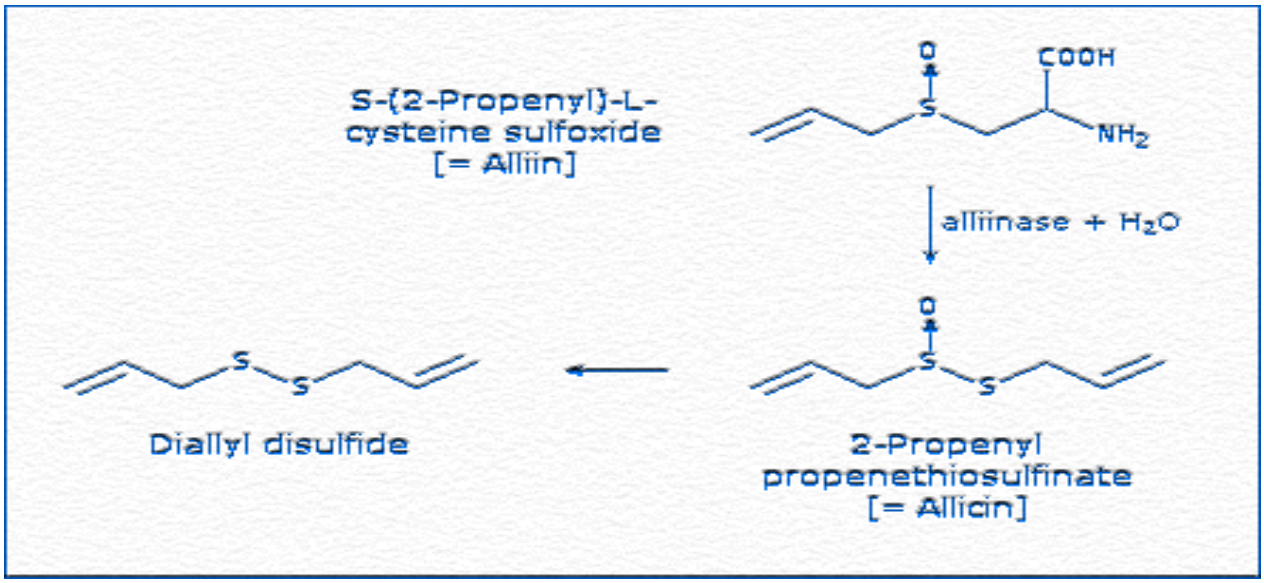


Figure 1: The Main Chemical Constituents of *Allium sativum* (garlic) (after Schmidt, 2013)

Stellenbosch University http://scholar.sun.ac.za

2.

IN VITRO EFFECTS OF GARLIC EXTRACTS ON THE PATHOGENIC FUNGI *BOTRYTIS CINEREA, PENICILLIUM EXPANSUM* AND *NEOFABRAEA ALBA*

ABSTRACT

This study aimed to determine the *in vitro* antifungal activity of garlic extract preparations and the volatile activity of these extracts at different concentrations on the postharvest pathogens *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*. Extracts subjected to rotary evaporation to remove ethanol and extracts where ethanol was not removed, were tested by using aqueous and ethanol solvents to make up the required concentrations. Mycelial growth of *B. cinerea* and *P. expansum*, on garlic extract amended PDA media, was inhibited by aqueous and ethanol dilutions of both extracts with or without removal of ethanol, prior to preparing dilutions. Generally, it was indicated that extracts where ethanol was removed, yielded a more potent extract in comparison to the extract still containing ethanol at time of dilution preparations. Furthermore, aqueous dilutions of the extract where ethanol was removed, performed better than where ethanol was used for dilution purposes. EC_{50} values pertaining to the extract excluding ethanol indicated that 13.36% and 8.09% aqueous dilutions of the garlic extract could be used to limit the growth of *B. cinerea* and *P. expansum*, respectively. *Neofabraea alba* growth was similar when treated with the aqueous and ethanolic dilutions of the garlic extracts from evaporated and non-evaporated extracts (with or without ethanol). EC_{50} values generated for *N. alba* either bordered on or exceeded the sample concentration range. It was concluded that extract preparation method and the type of solvent used play important roles in the antifungal activity of garlic extracts. Based on the outcome of the amended media experiments, the use of the evaporated extracts (without ethanol) was chosen for all subsequent experimentation within the overall study. Garlic volatiles are able to inhibit mycelial growth and spore germination at concentrations as low as 20% garlic extract. Individual pathogens reacted differently to volatiles of garlic extracts, with *N. alba* being most sensitive to garlic extract volatiles, followed by *P. expansum* and lastly *B. cinerea*. Overall, the antifungal activity of garlic extracts for the control of *B. cinerea* and *P. expansum* was confirmed. No solid conclusions with regard to the antifungal effect of garlic extracts could be reached for *N. alba,* although

Stellenbosch University http://scholar.sun.ac.za

volatiles of garlic seem to be more effective against this pathogen than direct application of the extracts. This is the first report of antifungal activity of garlic extracts against *N. alba*.

Stellenbosch University http://scholar.sun.ac.za

2.1. INTRODUCTION

Several pathogenic fungi such as *Botrytis cinerea* Pers., *Penicillium expansum* (Link) Thom. and *Neofabraea alba* (E.J. Guthrie) are major infectious agents of apples especially in the postharvest stage. Postharvest fungal attack of fruit often leads to problems relating to diminished fruit quality, nutritional value, deterioration of organoleptic characteristics and reduced shelf life (Serrano *et al*., 2008). Pathogenic fungi are controlled primarily through the use of synthetic fungicides; however, restrictions are being placed on the use of chemicals due to the perceived negative effects that pesticides may have on human health and the environment. The increasing regulations on the use of synthetic fungicides, and the emergence of pathogen resistance to the most valuable fungicides being used, validate the search for novel control strategies (Dellavalle *et al*., 2011).

In recent years, a number of plant extracts, their essential oils and their volatile components have been reported to have strong antifungal activity (Siripornvisal *et al*., 2009). In general, antimicrobial activity of plant extracts have been well documented and in more recent times, studies on the effects of plant extracts and essential oils on plant pathogens have also received attention (Tripathi *et al.*, 2008). The antimicrobial properties of plant extracts have been reported with increasing frequency from different parts of the world. For example, a large proportion of the South American population use extracts obtained from medicinal plants as remedies for many infectious diseases (Dellavalle *et al*., 2011). In the agricultural sector, plant extracts, essential oils and their components are gaining increasing interest due to their volatility, reasonably safe status, their eco-friendly and biodegradable properties and wide consumer acceptance (Tzortzakis and Economakis, 2007). Application of essential oils in the vapour phase is preferred to liquid phase application due to their volatility, which reportedly leads to more activity, allows for the use of lower concentrations, has no to little effect on the sensory properties of foodstuffs (Laird and Phillips, 2011) and in some instances, have been reported to have low or no residue detection levels (Sholberg, 2009).

Garlic *(Allium sativum L.*) has long been used against human pathogens. There are; however, few references on the use of garlic extracts to control plant pathogens. Examples include the work of Russel and Mussa (1977) controlling *Fusarium oxysporum* f.sp *phaseoli*; Garcia and Garcia (1990) controlling *Aspergillus* spp., and Obagwu *et al*. (1997) controlling *Collectotrichum capsici*. Inhibitory effects of garlic against *Penicillium digitatum* have also been reported (Obagwu and Korsten, 2003; Kanan and Al-Najar, 2008). The antifungal

Stellenbosch University http://scholar.sun.ac.za

activity of garlic is widely attributed to the presence of allicin or allicin-derived compounds, which is considered to be the main active ingredient in garlic. Whilst many studies have highlighted the antimicrobial action of garlic on pathogens, little research has been done relating to postharvest plant pathogens (Obagwu and Korsten, 2003) and specifically the postharvest pathogens of apples.

The objectives of this study was to (1) evaluate the antifungal efficacy of garlic extracts, of which ethanol was either removed by evaporation or not, prior to preparation of aqueous and ethanolic dilutions for efficacy testing in amended culture media and (2) to evaluate the volatile activity of garlic extracts at room temperature (RT), and in combination with low temperature storage (-0.5°C) on the *in vitro* mycelial growth and spore germination of apple postharvest pathogens *B. cinerea*, *P. expansum* and *N. alba*.

2.2. MATERIALS AND METHODS

2.2.1. Pathogen isolates

Three pathogens, *Botrytis cinerea* (B62-SUN) isolated from pears and *Penicillium expansum* (P1110-SUN) and *Neofabraea alba* (DOK7-SUN) isolated from apples were selected. All three pathogens were obtained from the fungal collection of Stellenbosch University Plant Pathology Department. All the isolates were tested for pathogenicity on fruit and pure isolations were prepared. The fungi were identified on the basis of morphological characteristics, viz. formation on media, mycelial and types of conidial characteristics. Both *B. cinerea* and *P. expansum* were cultured on potato dextrose agar (PDA, pH 5.6 Merck) at 25°C for three days for mycelial plugs and seven days for the production of conidia. *Neofabraea alba* was cultured on acidified potato dextrose agar (aPDA, Merck) for one month at 25°C. Conidia were harvested by flooding the media surface with sterile distilled water together with Tween 80 and gently agitating the plate to dislodge spores. A conidial suspension was prepared in Tween 80 solution (0.05% w/v) and the final inoculum concentration was adjusted to 1 x 10^4 conidia ml^{-1}, respectively, for each pathogen.

2.2.2. Preparation of garlic extracts

Fresh garlic (*Allium sativum* L.) cloves were purchased from a retail store (Woolworths, Stellenbosch). The garlic cloves were peeled and surface sterilized using

Stellenbosch University http://scholar.sun.ac.za

ethanol (99.9%). The garlic cloves were allowed to dry and 800g of garlic was weighed out and crushed in a blender. One litre of ethanol was added to the crushed garlic, to obtain an 80% garlic extract mixture which was then placed into a glass container and extracted overnight at room temperature. The extract was then filtered using a Büchner funnel using Whatman qualitative filter paper (No. 4).

With the extracts prepared initially ethanol was not evaporated (Extract 1); however, the extracts prepared thereafter was subjected to a rotary evaporator (at 60-80°C) to remove the ethanol, thus leaving only a semisolid extract solution (Extract 2). This extract was considered as the 100% concentrate of the extract. All extracts were stored in a refrigerator at 4°C until subsequent use.

2.2.3. Effect of garlic extracts on mycelial growth

A range-finding protocol was used to test concentrations ranging from 80 – 2.5% (wt/v) garlic extract to determine at which concentrations the extract would render complete inhibition of pathogen growth. This was determined following the poisoned food technique of Shahi *et al*. (2003) with slight modification. The respective concentration of the garlic extract was prepared by adding the requisite amount of the extract to ethanol or sterile distilled water up to a volume of 2.8 mL, which was then added to 140 mL of PDA medium (pH 5.6), and 20 mL aliquots of the amended PDA was poured into 90 mm Petri plates. Control sets consisted of non amended PDA. A preliminary trial was carried out that incorporated 2.8 mL of 70% and 100% ethanol into media. No significant difference between the non-amended media, and the sterile distilled water and ethanol amended media was noted (results not shown).

Mycelial discs of 3mm diameter cut out from the periphery of three day old cultures (B62 and P1110) and seven day old culture (DOK7) of the test pathogens were aseptically transferred mycelium side down, onto the surface of the agar. Petri plates were incubated at 25 °C for three days for both *B cinerea* and *P. expansum* and 7-21 days for *N. alba*. Twenty one day incubation of *N. alba* was done as it was observed that pathogen growth could not be adequately measured at seven days for this fungus. Radial mycelial growth was measured using digital calipers. Percentage of mycelial growth inhibition (MGI) was calculated as follows: MGI (%) = (dc-dt) x 100/dc where, dc = mycelial growth diameter in control sets, dt = mycelial growth diameter in treatment sets.

Stellenbosch University http://scholar.sun.ac.za

The nature of antifungal activity [fungistatic (temporary inhibition)/fungicidal (permanent inhibition)] of the garlic extract was determined by the transfer of the inhibited fungal discs from the abovementioned method onto unamended PDA in a Petri plate and growth was recorded. Three replicates were done for each of the three pathogens and for each concentration tested and the experiment was repeated once.

2.2.4. Effect of garlic extracts on conidial germination of *B. cinerea* and *P. expansum*

One-hundred micro liters of fungal conidia suspensions (1 x 10^4 conidia mL^{-1}) were pipetted onto the center of garlic-amended PDA plates prepared as per description in Section 2.2.3. Inoculated plates were incubated at 25°C for three days. The control plates consisted of the pathogen on unamended PDA. Three replicates were done per concentration. Plates were evaluated for germinated (+) and non-germinated (-) conidia. The experimented was repeated once.

2.2.5. Effect of garlic volatiles on mycelial growth and spore germination

A phytatray chamber assay was used to determine the effect of the volatile vapour of garlic extracts on the pathogens *in vitro*. A glass Petri dish containing 5 mL of garlic extract 2, diluted with water to concentrations of 0%, 20%, 30% or 40% was fixed to the base of a disposable phytatray (Zibo, Brackenfell). Sterilised distilled water was used as the control. Four 65 mm PDA Petri plates inoculated with the respective fungi were fixed to the sides of the phytatray. Each chamber contained two plates inoculated with 3 mm mycelial plugs cut from the leading edge of an actively growing culture and placed mycelial side down onto the PDA, as well as two plates inoculated with 100µl of a 1 x 10^4 conidia mL^{-1} conidial suspension by means of a spread plate method. The lid was closed and the chamber was then incubated at 20°C (RT) and -0.5°C for a total of three days for the fungi *B. cinerea* and *P. expansum,* and seven days for the fungus *N. alba,* before evaluation. Plates incubated at -0.5°C was further incubated at 20°C for three and seven days, respectively, to simulate fruit shelf life. A total of three replicates with five phytatray chambers were done for each concentration. Plates inoculated with mycelial plugs were evaluated by measuring mycelial growth of the fungi using digital callipers. Fungal spore plates were evaluated for germination (+) and non-germination (-) and subsequently converted to a percentage for statistical analysis. The experiment was repeated with a modification to the makeup of the

Stellenbosch University http://scholar.sun.ac.za

garlic extracts. In Experiment 1, the extracts was evaporated down to a volume of 150 mL and in Experiment 2 (repeated experiment), the extracts was evaporated down to 300 mL.

2.2.6. Statistical analysis

For the garlic amended media study, the experimental design was completely randomised (CRD) with 8 x 2 factorial and three replications. The factors was the eight concentrations (0%, 2.5%, 5%, 10%, 20%, 40%, 60% and 80% garlic extract) and two extract preparations (ethanol and water as solvents) for the dilution range. The data was analysed separately for crude extracts of which ethanol was evaporated (Extract 2) or not (Extract 1), prior to preparing dilutions, using water or ethanol as solvents, for subsequent efficacy testing. For the garlic volatile study**,** the experiment was CRD with 3 factors being temperature, day (three or seven days incubation) and concentrations (0%, 20%, 40% and 60% garlic extract) and three replications. Conidial germination data was binary (present or absent) summed over the Petri dishes expressed as percentage. Mycelial growth was measured in diameter (mm) with both data types being converted to percentage inhibition which was analysed by an appropriate analysis of variance (ANOVA). The treatment means were compared by a Student's t- test with least significant difference (LSD) at 5% ($P = 0.05$) (Ott, 1993). A logarithmic growth curve was fitted to the concentration range to calculate EC_{50} values. The percentage inhibition and EC_{50} values were submitted to an appropriate ANOVA to compare treatments. Analysis was performed using SAS version 9.2 statistical software (SAS, 2012). For experiments carried out, all three pathogens were analysed separately and no comparison was made between them.

2.3. RESULTS

2.3.1. Effect of garlic extracts on mycelial growth

Results generated for garlic Extract 1 (ethanol not evaporated) shows that aqueous dilutions of the extracts significantly inhibited the mycelial growth for all pathogens tested when compared to the control, but to a varied degree. The highest mycelial inhibition was recorded for *P. expansum* with a concentration of 80% garlic aqueous dilution rendering a 74.35% inhibition of the pathogen (Table 1). Ethanol diluted extracts were significantly inhibitory to all three pathogens at all the respective concentrations, compared to their

Stellenbosch University http://scholar.sun.ac.za

controls. Both the 60% and 80% crude extracts of which ethanol was not evaporated prior to dilutions with ethanol, showed complete inhibition of *P. expansum,* with the effect being fungistatic at 60% and fungicidal at the 80% concentrations. Overall, the rate of growth was influenced by concentration of the extracts, as indicated by the decrease in colony diameter with increasing extract concentration (Table 1).

The aqueous and ethanol dilutions of Extract 2 (ethanol evaporated), showed complete inhibition (100%) of *B. cinerea* at the higher concentration ranges (80% and 60%), with the effect at both concentrations being fungicidal (Table 2). Aqueous dilutions of Extract 2 at 40% showed 92.08% inhibition of *B. cinerea*. At a concentration of 80%, the aqueous and ethanol dilutions of the extracts inhibited *P. expansum* by 96.21% and 99.21% respectively. Ethanol dilutions seemed to be more effective against *N. alba* with the 80% extract showing 79.63% inhibition. Non statistical comparison between the extracts indicated that, in comparison to garlic Extract 1, both the aqueous and ethanol dilutions of garlic Extract 2 (ethanol evaporated), had an overall greater inhibitory effect on the pathogens tested, with the aqueous dilutions of the extracts providing significantly better results than the ethanol diluted extracts (Table 2).

The effective concentrations at which 50% pathogen inhibition (EC_{50}) resulted from the use of garlic extracts was calculated. When using garlic Extract 1, where ethanol was not removed from the crude extract prior to testing, 25.53%, 11.66% and 11.52% of the ethanol diluted extract was needed to control *B. cinerea, P. expansum* and *N. alba* respectively. Alternatively, an aqueous diluted extract at concentrations of 72.48%, 30.25% and 58.92% would render the same results for each of the respective pathogens (Table 3). Garlic Extract 2, where ethanol was removed from the crude extract prior to testing, showed that *B. cinerea* could be controlled using 20.59% of an aqueous dilution or 13.36% of an ethanol diluted extract. For *P. expansum*, a 19.95% ethanol diluted extract or 8.09% aqueous extract could be used to gain control, while results indicate that 50% control of *N. alba* would not occur unless either extract is at a concentration of no less than 79.51% (Table 3). In conclusion garlic Extract 2, which had been subjected to the rotary evaporator to remove ethanol, had better antifungal activity compared to garlic Extract 1, requiring lower dilution concentrations of the crude extract to inhibit *B. cinerea* and *P. expansum* fungal growth to the EC_{50} value, using water as solvents for the dilutions of the extract. The effective concentration required to reduce the growth of *N. alba* could be reduced drastically by using garlic Extract 1 (ethanol not removed) and by using ethanol for dilution of the extract.

Stellenbosch University http://scholar.sun.ac.za

2.3.2. Effect of garlic extracts on conidial germination of *B. cinerea* and *P. expansum*

Aqueous and ethanolic diluted extracts of garlic, subjected to evaporation of ethanol from the crude extract, were tested for their antifungal activity against conidial viability of the pathogens *B. cinerea* and *P. expansum* at concentration ranges between 0-80%. After a three day incubation period, Petri plates were examined for germination (+) or non-germination (-). *Botrytis cinerea* and *P. expansum* still grew when exposed to concentration ranges of 0-10% aqueous diluted garlic extract (Table 4). Germination of both pathogens was completely inhibited at the higher concentration ranges of 20-80% aqueous diluted garlic extract. For both pathogens exposed to the ethanol diluted garlic extracts, conidial germination was completely inhibited at all concentrations with the exception of the lowest concentration of 2.5% garlic extract (Table 4).

2.3.3. Effect of garlic volatiles on mycelial growth and spore germination *in vitro*

The volatile vapour of garlic were strongly active against mycelial growth of all fungi tested especially at concentrations of 30% and 40% in Experiment 1, where ethanol was evaporated to a volume of 150 mL (Table 5). However, the volatile vapours in Experiment 2 (repeated experiment where ethanol was evaporated to a volume of 300 mL) showed weaker activity against *B. cinerea*. Overall, volatile vapour of garlic was strongly active against *P. expansum* and *N. alba* for both experiments conducted. Furthermore, for all pathogens tested, percentage mycelial inhibition increased with an increase in garlic concentration (Table 5).

Conidial germination of all fungi tested was almost completely inhibited by volatile vapour of garlic extracts in Experiment 1(Table 6), irrespective of the concentration of the extract. Volatile vapours were more effective against *P. expansum* and *N. alba* in preventing conidial germination. A higher concentration of garlic crude extract was required if the extract was evaporated to 300 mL, to effectively reduce germination of *B. cinerea*.

Mycelial growth for plates incubated at -0.5°C showed complete inhibition against all pathogens tested, irrespective of the garlic extract concentration or the evaporated volume of the crude extract (Table 7). This was most likely due to the low incubation temperature (Table 7). When phytatrays were incubated further at 20°C, 30% and 40% concentrations were strongly active against mycelial growth of all fungi tested, for extracts evaporated to 150 mL. The same results were generated for *P. expansum* and *N. alba* in the repeated experiment (Experiment 2), however, a significant drop in mycelial inhibition was recorded

Stellenbosch University http://scholar.sun.ac.za

for *B. cinerea* across the concentration range. The combination of garlic volatiles with low temperature (-0.5°C) resulted in a stronger antifungal activity especially against *P. expansum* and *N. alba* compared to phytatrays only incubated at 20°C as is suggested by the difference between control and treatment sets.

Similar results as for the mycelial growth were observed for conidial germination trays incubated at -0.5°C (data not presented). The data showed that conidial germination was completely inhibited for all three fungi. Phytatrays taken from -0.5°C storage and incubated further at 20°C showed persistent complete inhibition (100%) of conidia exposed to extracts at concentrations ranging 20-40%. A combination of garlic volatile with low temperature showed stronger inhibition of conidia compared to phytatrays incubated at 20°C.

2.4. DISCUSSION

Garlic extracts had a significant effect on the growth of the pathogens tested. This finding agrees with earlier reports on the antifungal properties of garlic (Obagwu *et al*., 1997). The effect of garlic extracts on postharvest pathogens was determined by direct exposure of pathogens to garlic extracts as well as through the volatile action of garlic vapours.

Extracts not subjected to rotary evaporation (Garlic Extract 1) and extracts subjected to rotary evaporation (Garlic Extract 2) to remove ethanol after the extraction process were tested on *B. cinerea*, *P. expansum* and *N. alba* to determine which extract would be more effective in inhibiting mycelial growth of each pathogen. The method by which a plant extract is prepared will influence the type of activity (antifungal or other) it has (Arora and Kaur, 2007; Raghavendra *et al*., 2009). Different extraction and dilution solvents will affect the extraction of different chemical compounds and physiological properties within a plant, and therefore different extracts may contain different compounds, or the same compounds in varying quantities (Mendonca-Filho, 2006; Senhaji *et al*., 2013).

With focus on Extract 1 (extracts not subjected to a rotary evaporator to remove ethanol), it was observed that ethanolic dilutions of the extracts gave better activity than the aqueous dilutions of the same extract for all pathogens tested. In the case of *B. cinerea*, an 80% ethanol dilution of the garlic extract gave stronger activity than the aqueous preparation (Table 1). In contrast to garlic Extract 1, aqueous dilutions of garlic Extract 2 (ethanol

Stellenbosch University http://scholar.sun.ac.za

evaporated) gave better activity than the ethanol dilutions of the same extract. Previous studies both support (Gull *et al*., 2012) and contradict (Saravanan *et al*., 2010) this finding; however, all studies do support the fact that aqueous garlic extract preparations do result in antimicrobial activity. Overall, the extracts subjected to a rotary evaporator to remove ethanol were much more effective at inhibiting mycelial growth of *B. cinerea* (Table 2). When comparing aqueous preparations of extracts to preparations by other organic solvents and tested against *B. cinerea*, a parallel may be drawn between results from the present study and those achieved by Senhaji *et al*., (2013); where the present study revealed that complete (100%) mycelial inhibition could be achieved by using a 60% aqueous dilution of crude garlic Extract 2 (Table 2), whereas the same concentration of 60% aqueous dilution of crude garlic Extract 1 only elicited a 50% mycelial inhibition (Table 1) of *B. cinerea*. The aqueous and ethanol dilutions of the garlic extract without ethanol (Extract 2) did perform better overall when compared to the garlic Extract 1. Timothy *et al*., (2012) noticed a dose dependant antifungal activity of leaf extracts of *Cassia alata* when tested against clinical isolates of pathogenic fungi, including a species of *Penicillium*. The same trend can be noted with this study with *B. cinerea* and *P. expansum* each eliciting a decrease in colony diameter with increasing concentration of garlic extract.

For the pathogen *N. alba*, percentage inhibition of mycelial growth was significantly different across the concentration range for both the garlic extracts tested. However, for each of the extracts tested, results indicate that the ethanol extracts were more effective at reducing mycelial growth of the pathogen. Due to the nature of the results, no firm conclusions can be made regarding the effect of garlic extracts on *N. alba* when incorporated into growth media. Tentative results indicate that when applying extract 2, in order to reduce the growth of this pathogen by 50%, a concentration of 79.51% ethanol dilution or 81.39% aqueous dilution of the garlic extract preparation (Table 3) would be needed.

Plant extracts of *Allium* and *Capsicum* have been shown to completely inhibit spore germination of *B. cinerea* (Wilson *et al*., 1997). The effect that garlic extracts would have on conidial viability of *B. cinerea* and *P. expansum* was tested and in this instance, across the chosen concentration range, both pathogens behaved in an identical manner with regards to exposure to the aqueous and ethanol extracts. Exposure to the extracts diluted with ethanol yielded a greater inhibitory effect on pathogen conidial viability. This would confirm the use of ethanol at concentrations of 70% and above as a surface sterilant, however, ethanol used as a control for pilot experiments (results not shown) did not result in any antifungal activity.

Stellenbosch University http://scholar.sun.ac.za

In recent years, a number of studies have been carried out concerning the application of essential oils as microbial agents (Barratta *et al*., 1998). Most of the information published on this subject has reported on the antifungal activity of essential oils and plant extracts exposed directly to fungus (as per method described above using amended media). However, only few studies concerning the antifungal activity of volatiles of plant essential oils and extracts have been performed (Chee and Lee, 2007).

Garlic contains of more than 200 compounds, some of which are volatile substances such as allicin, ajoene and alliin (Goncagul and Ayaz, 2010). An investigation into the effectiveness of garlic extracts for the control of pathogenic bacteria and fungi has demonstrated that allicin (the putative active ingredient of garlic) supplied via the vapour phase was effective in reducing *Phytophthora infestans in vitro* (Curtis *et al*., 2004). With that said it should also be stated that investigations into the volatile effects of garlic on fungi is quite scarce; however, literature on the topic does exist for a few of the more common plant essential oils and extracts.

In the present *in vitro* study, the volatiles released from garlic crude extracts were effective in limiting mycelial growth and condial germination of all the pathogens tested. The individual pathogens responded differently to the garlic extracts, with *N. alba* being the most sensitive to the volatile vapours of the garlic extracts.

At 20°C, conidial germination and mycelial growth of all three pathogens, *B. cinerea, P. expansum* and *N. alba,* were effectively inhibited by garlic volatiles across the concentration range, with pathogen inhibition being increased as concentration of garlic extract volatile vapour increased (Table 5 and 6). Volatile substances released from essential oils derived from *Ocimum sanctum, Prunus persica* and *Zingiber officinale* were reported to have a similar effect on the control of *B. cinerea* on grapes (Tripathi *et al*., 2008). A recent study showed that the vapours of thyme, peppermint and citronella oils with increasing concentrations causeded a gradual inhibition on the growth of *P. expansum* and other postharvest pathogens (Sellamuthu *et al*., 2013).

When incubated at -0.5°C, the low temperature affected growth of all pathogens, as was indicated in the reduced growth of the control sets of *B. cinerea* and *P. expansum*. However, for both these pathogens, complete inhibition (100%) was noted on all plates exposed to the garlic treatments (Table 7). Furthermore, the shelf life study indicated that mycelial growth was inhibited, while conidial germination remained 100% inhibited (data not presented) for all three pathogens even when the temperature was raised to 20°C. This

Stellenbosch University http://scholar.sun.ac.za

suggests a synergistic relationship between the low temperature and the garlic extracts. Where under standard conditions incubation at -0.5°C is supposed to suppress growth of pathogens in storage, the garlic extracts enhanced pathogen inhibition and allowed for added control of pathogens as can be seen when comparing the control sets to the treated sets (Table 7). In an *in vivo* experiment done, it was highlighted that thyme oil in the volatile phase in combination with modified atmosphere packaging (MAP) at a concentration of 8% carbon dioxide and 2% oxygen showed potential to maintain fruit quality for up to 18 days at 10°C cold storage and a further 5-10 days at market shelf (Sellamuthu *et al*., 2013). Similar findings have been reported with banana and peach commodities (Anthony *et al*., 2003; Arrebola *et al*., 2010) where essential oils and MAP have been shown to have good synergistic effect for the control of postharvest diseases, and for overall fruit quality.

No comparison was made between the pathogens in this study and results indicate that while all three pathogens were sensitive to the garlic extracts, each individual pathogen reacted differently to the extracts. When exposed to garlic in the vapour phase, *N. alba* was most sensitive to the extracts at both temperatures tested, followed by *P. expansum* and *B. cinerea*. When the experiments were repeated, a slight reduction in the activity of the garlic extracts on the mycelial growth of *N. alba* and *P. expansum* was noted. Although a significant difference ($P < 0.05$) was noted for each experiment of extracts and each concentration, overall inhibitory activity of the garlic extracts for mycelial growth of these pathogens still remained high. No difference between the experiments was noted for conidial germination (data not presented). However, and with the sole exception of incubation at -0.5°C, the significant difference between experiments of garlic tested on *B. cinerea* is quite evident. At 20°C, conidia of *B. cinerea* were completely inhibited when exposed to 20% garlic at first; however, no control of this pathogen was noted at this concentration when the experiment was repeated, suggesting a possible concentration effect due to the modification made to the preparation of the extracts in the repeated experiment. The resistant behaviour of *B. cinerea* to garlic extracts in the second trial of this study could also be attributed to the repeated subculturing of the pathogen over the period of time. It was noted with *Venturia inequalis*, that isolates became more sensitive to the fungicide flusilazole after repeated subculturing (Köller *et al*., 1991). While the opposite behaviour was noted in this study, the reasoning is completely plausible when coupled with the fact that isolates of different pathogens behave differently in culture at different times of the year. This study was undertaken in the colder months of the year (April – June) where *B. cinerea* tends to start

Stellenbosch University http://scholar.sun.ac.za

producing sclerotia in culture. In their study, Curtis *et al*. (2004) reported that some fungi, e.g. *Alternaria brassisicola*, were unable to recolonise the inhibition zone after prolonged cultivation, whereas *B. cinerea* was able to do so relatively quickly. This observed difference between pathogens' behaviour to garlic extracts (the present study included) is significant to the potential of using garlic extract preparations to reduce inoculum load. Therefore it was suggested that each pathosystem would have to be investigated individually to assess the feasibility of using garlic extracts to treat diseases (Curtis *et al*., 2004).

Although small, a drop in inhibitory activity of garlic extracts for mycelial growth at 20°C in the repeated experiment was also noted for *P. expansum* and *N. alba*. Besides the fact that the pathogens' individual sensitivity to the garlic extracts was obviously different, the data seems to indicate a concentration effect due to the fact that extracts in the first experiment were evaporated to half the volume (150 mL) of extracts used for the second experiment (300 mL). Results concluded from the garlic amended media experiments (Section 2.3.1.) would speak directly to this finding as it was shown that an aqueous dilution of the extract not subjected to a rotary evaporator to remove ethanol (Extract 1) had a reduced inhibitory effect on all three test pathogens as opposed to aqueous dilutions of extracts subjected to a rotary evaporator to remove ethanol (Extract 2).

In conclusion, the present study showed that garlic extracts can have a significant effect on preventing the growth of *B. cinerea* and *P. expansum*. However, growth of *N. alba* was not significantly suppressed by either of the garlic extract preparations. The extraction method (ethanol evaporated or not evaporated), as well as the solvent used for dilution concentrations (water or ethanol) had an influence on the antifungal activity of the garlic extract. Crude garlic extract where ethanol was evaporated prior to dilutions, was significantly better at inhibiting fungal growth of all pathogens than a crude garlic extract where ethanol was not evaporated. This might be due to the observed fact that the longer the extracts were exposed to the ethanol the more the antifungal activity was reduced. Furthermore, this study showed that depending on the chosen extract preparation method, either water or ethanol could be used as an appropriate solvent. For the purpose of the overall study, crude aqueous diluted garlic extracts were favored for further experimentation.

When tested in the vapour phase, garlic extracts were able to control growth of *B. cinerea, P. expansum* and *N. alba*. These findings confirm those of fellow researchers who stated that application in the vapour phase is preferred due to increased volatile activity and the ability to use lower concentrations (Laird and Phillips, 2011). In the present study,

Stellenbosch University http://scholar.sun.ac.za

concentrations used in the volatile experiment were at a lower garlic concentration than the amended media experiments. Furthermore, increased antifungal activity was noted. This is a significant finding as it gives a possible lead into using a garlic preparation as a fumigant to control pathogens that may be present in the air and on regular surfaces in a packhouse or containers. Also, where garlic extracts is combined with effective storage conditions, this application may be able to be adopted into a closed packaging system.

In conclusion, volatile vapour of garlic extracts showed potent antifungal activities against conidial germination compared to mycelial growth of the test fungi. Volatile vapours of garlic extracts were more effective than agar amended medium as efficacy in volatile assays was at concentrations 20-40% compared to amended agar at 60-80%.

2.5. LITERATURE CITED

Anthony, S., Abeywickrama, K. and Wijeratnam, S.W. 2003. The effect of spraying essential oils of *Cymopogon nardus, Cymbopogon flexuosus* and *Ocimum basilicum* on postharvest diseases and storage life of Embul banana. Journal of Horticultural Science and Biotechnology 78: 780-785.

Arora, S.D. and Kaur, G.J. 2007. Antibacterial activity of some Indian medicinal plants. Journal of Natural Medicine 61:313-317.

Arrebola, E., Sivakumar, D., Bacigalupo, R. and Korsten, L. 2010. Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases. Crop Protection 29: 369-377.

Barratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, C., Barroso, J.G. and Ruberto, G. 1998. Antimicrobial and antioxidant properties of some commercial essential oils. Flavour and Fragrance Journal 13: 235-244.

Chee, H.Y. and Lee, M.H. 2007. Antifungal activity of clove essential oil and its volatile vapour against dermatophytic fungi. Mycobiology 35: 241-243.

Curtis, H., Noll, U., Stormann, J. and Slusarenko, A.J. 2004. Broad-spectrum activity of the volatile phytoantocipin allicin in extracts of garlic (*Allium sativum* L.) against plant pathogenic bacteria, fungi and oomycetes. Physiological and Molecular Plant Pathology 65: 79-89.

Stellenbosch University http://scholar.sun.ac.za

Dellavalle, P.D., Cabrera, A., Alem, D., Larrañaga, P., Ferreira, F. and Rizza, M.D. 2011. Antifungal activity of medicinal plant extracts against phytopathogenic fungus *Alternaria* spp. Chilean Journal of Agricultural Research 71: 231-239.

Garcia, R.P. and Garcia, M.I. 1990. Laboratory evaluation of plant extracts for the control of *Aspergillus* growth and alfatoxin production. Proceeding of the Japanese Association of Mycotoxicology. Horticultural Abstracts 60:6543.

Goncagul, G and Ayaz, E. 2010. Antimicrobial effect of garlic (*Allium sativum*) and traditional medicine. Journal of Animal and Veterinary Advances 9: 1-4.

Gull, I., Saeed, M., Sahukat, H., Aslam, S.M., Samra, Z.Q. and Athar, A.M. 2012. Inhibitory effect of *Allium sativum* and *Zingiber officinale* extracts on clinically important drug resistant pathogenic bacteria. Annals of Clinical Microbiology and Antimicrobials 11:8.

Kanan, G.J. and Al-Najar, R.A. 2008. *In vitro* antifungal activities of various plant crude extracts and fractions against citrus postharvest disease agent *Penicillium digitatum*. Jordan Journal of Biological Sciences 1: 89-99.

Köller, W., Smith, F.D. and Reynolds, K.L. 1991. Phenotypic instability of flusilazole sensitivity in *Venturia inequalis*. Plant Pathology 40: 608-611.

Laird, K. and Phillips, C. 2011. Vapour phase: a potential future use for essential oils as antimicrobials? Letters in Applied Microbiology 54: 169-174.

Mendonca-Filho, R.R. 2006. Chapter 1: Bioactive Phytocompounds: New Approaches in the Phytosciences. In: Modern Phytomedicine. Turning Medicinal Plants into Drugs. Wiley-VCH Verlag GmbH & Co. Weinheim, Germany.

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effects of extracts of garlic (*Allium sativum*) bulb and neem (*Azadirachta indica*) seed on the mycelial growth and sporulation of *Collectotrichum capsici.* Journal of Agricultural Technology 5: 51-55.

Obagwu, J. and Korsten, L. 2003. Control of citrus green and blue moulds with garlic extracts. European Journal of Plant Pathology 109: 221-225.

Ott, R.L. 1993. An Introduction to Statistical Methods and Data Analysis. Duxbury Press: Belmont, California.

Raghavendra, M.P., Satish, S. and Raveesha, K.A. 2009. Alkaloid extracts of *Prosopis juliflora* (Sw.) DC. (Mimosaceae) against *Alternaria alternata*. Journal of Biopesticides 2: 56-59.

Stellenbosch University http://scholar.sun.ac.za

Russel, P.E. and Mussa, A.E.A. 1977. The use of garlic (*Allium sativum*) extracts to control foot rot of *Phaseolus vulgaris* caused by *Fusarium solani* f.sp. *phaseoli*. Annals of Applied Biology 86: 369–372.

Saravanan, P., Ramya, V., Sridhar, H., Balamurugan, V. and Umamaheswari, S. 2010. Antibacterial activity of *Allium sativum* L. on pathogenic bacterial strains. Global Veterinaria 4: 519-522.

SAS Institute, Inc. 2012. SAS Version 9.2 64 bit. SAS Institute Inc, SAS Campus Drive, Cary, North Carolina.

Sellamuthu, P.S., Sivakumar, D. and Soundy, P. 2013. Antifungal activity and chemical composition of thyme, peppermint and citronella oils in vapour phase against avocado and peach postharvest pathogens. Journal of Food Safety 33: 86-93.

Senhaji, B., Ben Hmamou, D., Salghi, R. 2013. *Asteriscus imbricatus* extract: antifungal activity and anticorrosion inhibition. International Journal of Electrochemical Science 8: 6033-6046.

Serrano, M., Martinez-Romero, D., Giullen, F., Valverde, J.M., Zapata, P.J., Castillo, S. and Valero, D. 2008. The addition of essential oils to MAP as a tool to maintain the overall quality of fruits. Trends in Food Science and Technology 19: 464-471.

Shahi, S.K., Patra, M., Shukla, A.C. and Dikshit, A. 2003. Use of essential oil as botanical-pesticide against post harvest spoilage in *Malus pumilo* fruits. BioControl 48: 223-232.

Sholberg, P. 2009. Control of postharvest decay by fumigation with acetic acid or plant volatile compounds. Fresh Produce 3 (special issue 1): 80-86. Global Science Books.

Siripornvisal, S., Rungprom, W. and Sawatdikarn, S. 2009. Antifungal activity of essential oils derived from medicinal plants against grey mould (*Botrytis cinerea*). Asian Journal of Food and Agro-Industry (Special Issue): 229-223.

Timothy, S.Y., Wazis, C.H., Adati, R.G. and Maspalma, I.D. 2012. Antifungal activity of aqueous and ethanolic leaf extracts of *Cassia alata* Linn. Journal of Applied Pharmaceutical Science 2: 182-185.

Tripathi, P., Dubey, N.K. and Shukla, A.K. 2008. Use of some essential oils as postharvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. World Journal of Microbiology and Biotechnology 24: 39-46.

Tzortzakis, N.G. and Economakis, C.D. 2007. Antifungal activity of lemongrass (*Cymbopogon citrates* L.) essential oil against key postharvest pathogens. Innovative Food Science and Emerging Technologies 8: 253-258.

Stellenbosch University http://scholar.sun.ac.za

Wilson, C.L., Solar, J.M., El Ghaouth, A. and Wisniewski, M.E. 1997. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. Plant Disease 81: 204-210.

Stellenbosch University http://scholar.sun.ac.za

2.6. TABLES AND FIGURES

Table 1: Garlic Extract 1 (ethanol not removed by rotary evaporation) - Inhibitory effect of aqueous and ethanol dilutions of garlic extracts on mycelial growth of *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*

	Inhibition (%)		
Concentration of garlic extracts (%)	***B. cinerea***	***P. expansum***	***N. alba***
Aqueous diluted extract			
0	0.00 k	0.00 k	0.00 h
2.5	0.21 k	5.83 j	26.79 fg
5	1.33 k	8.58 j	18.38 g
10	6.00 i	22.38 h	32.70 g
20	23.78 g	34.01 g	36.8 d-f
40	42.06 e	55.95 e	47.55 c-e
60	50.47 d	67.89 d	52.41 bc
80	51.53 d	74.35 c	48.97 cd
Ethanol diluted extract			
0	0.00 k	0.00 k	0.00 h
2.5	3.91 i	15.80 i	35.98 ef
5	9.12 h	25.06 h	27.47 fg
10	23.03 g	42.09 f	64.18 ab
20	38.13 f	60.07 e	52.56 bc
40	57.89 c	79.27 b	67.67 a
60	75.90 b	100.0 a[1]	68.08 a
80	80.86 a	100.0 a[2]	75.93 a

Mean values down the column with the same letter (s) represent data that is not significantly different ($P < 0.0001$). [1] Indicates fungistatic effect (temporary inhibition). [2] Indicates fungicidal effect (permanent inhibition).

Stellenbosch University http://scholar.sun.ac.za

Table 2: Garlic extract 2 (ethanol removed by rotary evaporation) - Inhibitory effect of aqueous and ethanol dilutions of garlic extracts on mycelial growth of *Botrytis cinerea, Penicillium expansum* and *Neofabraea alba*

	Inhibition (%)		
Concentration of garlic extracts (%)	***B. cinerea***	***P. expansum***	***N. alba***
Aqueous diluted extract			
0	0.00 g	0.00 h	0.00 e
2.5	11.50 e	28.82 f	5.95 de
5	12.91 e	29.48 f	6.03 de
10	36.36 d	60.03 d	30.08 bc
20	52.05 c	70.60 c	6.43 de
40	92.08 a	83.82 b	34.29 bc
60	100.0 a[2]	95.68 a	40.15 b
80	100.0 a[2]	96.21 a	25.05 bc
Ethanol diluted extract			
0	0.00 g	0.00 h	0.00 e
2.5	0.86 g	5.10 g	21.53 cd
5	0.44 g	11.59 g	1.94 e
10	1.62 g	10.92 g	4.31 e
20	42.17 c	41.17 e	6.68 de
40	69.20 b	72.88 c	7.26 de
60	100.0 a[2]	85.40 b	39.26 b
80	100.0 a[2]	99.21 a	79.63 a

Mean values down the column with the same letter (s) represent data that is not significantly different ($P < 0.0001$). [1] Indicates fungistatic effect (temporary inhibition). [2] Indicates fungicidal effect (permanent inhibition).

Stellenbosch University http://scholar.sun.ac.za

Table 3: Calculated concentrations of garlic extracts that will effectively inhibit fungal growth by 50% (EC_{50})

Extract preparation		Garlic extract concentration, for each dilution solvent (%)	
	Pathogen*	**Aqueous**	**Ethanol**
Garlic Extract 1 (ethanol not removed)	*Botrytis cinerea*	72.48 a	25.53 b
	Penicillium expansum	30.25 a	11.66 b
	Neofabraea alba	58.92 a	11.52 b
Garlic Extract 2 (ethanol removed)	*Botrytis cinerea*	13.36 a	20.59 b
	Penicillium expansum	8.09 a	19.95 b
	Neofabraea alba	81.39 b	79.51 a

* Analysis conducted between water and ethanol as a dilution solvent, for each pathogen, respectively. Mean values followed by the same letter (s) represent data that are not significantly different ($P < 0.05$).

Table 4: Effect of Garlic Extract 2 on conidial germination and viability

	Pathogens			
	Botrytis cinerea		*Penicillium expansum*	
Garlic extract concentration (%)	Aqueous dilution	Ethanolic dilution	Aqueous dilution	Ethanolic dilution
0	+	+	+	+
2.5	+	+	+	+
5	+	-	+	-
10	+	-	+	-
20	-	-	-	-
40	-	-	-	-
60	-	-	-	-
80	-	-	-	-

(+) Germinated; (-) Non-germinated

Stellenbosch University http://scholar.sun.ac.za

Table 5: Volatile action of garlic crude extracts against pathogen mycelial growth after incubation of phytatrays at 20°C

	Inhibition (%)					
	Botrytis cinerea[*]		*Penicillium expansum*[*]		*Neofabraea alba*[*]	
Garlic extract concentration (%)	Exp[**] 1	Exp[***] 2	Exp 1	Exp 2	Exp 1	Exp 2
0	0.00 e	0.00 e	0.00 f	0.00 f	0.00 d	0.00 d
20	61.55 b	1.06 e	75.41 d	66.72 e	100.0 a	72.03 c
30	97.14 a	18.61 d	89.60 b	82.35 c	100.0 a	88.74 b
40	99.35 a	41.88 c	100.0 a	90.85 b	100.0 a	98.02 a

[*]Pathogens not compared statistically. [**]Experiment 1 – ethanol was evaporated until garlic extracts reached a volume of 150 mL. [***]Experiment 2 (repeat of experiment 1 with modification) – ethanol was evaporated until garlic extracts reached a volume of 300 mL.

Table 6: Volatile action of garlic crude extracts against pathogen spore germination after incubation of phytatrays at 20°C

	Inhibition (%)					
	Botrytis cinerea[*]		*Penicillium expansum*[*]		*Neofabraea alba*[*]	
Garlic extract concentration (%)	Exp[**] 1	Exp[***] 2	Exp 1	Exp 2	Exp 1	Exp 2
0	0.00c	0.00c	0.00b	0.00b	0.00b	0.00b
20	100.0a	0.00c	96.67a	100.0a	100.0a	100.0a
30	100.0a	60.00b	100.0a	100.0a	100.0a	100.0a
40	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a

[*]Pathogens not compared statistically. [**]Experiment 1 – ethanol was evaporated until garlic extracts reached a volume of 150 mL. [***]Experiment 2 (repeat of experiment 1 with modification) – ethanol was evaporated until garlic extracts reached a volume of 300 mL.

Stellenbosch University http://scholar.sun.ac.za

Table 7: Volatile effects of garlic crude extracts against pathogen mycelial growth at -0.5°C and shelf life storage (20°C)

Inhibition (%)						
Temperature (-0.5°C)	*Botrytis cinerea*[*]		*Penicillium expansum*[*]		*Neofabraea alba*[*]	
Garlic extract concentration (%)	Exp[**] 1	Exp[***] 2	Exp 1	Exp 2	Exp 1	Exp 2
0	0.00e	0.00e	0.00e	100.0a	100.0a	100.0a
20	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a
30	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a
40	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a
20°C[a]						
0	0.00e	0.00e	0.00e	0.00e	0.00c	0.00c
20	69.41b	11.98d	97.76bc	90.89d	100.0a	87.17b
30	97.62a	32.47c	99.14ab	96.21c	100.0a	95.45a
40	97.12a	60.93b	100.0a	100.0a	100.0a	100.0a

[*]Pathogens not compared statistically. [**]Experiment 1 – ethanol was evaporated off until garlic extracts reached a volume of 150 mL. [***]Experiment 2 (repeat of experiment 1 with modification) – ethanol was evaporated off until garlic extracts reached a volume of 300 mL. [a]Trays initially incubated at -0.5°C (3-7days), were incubated further for the same amount of time at 20°C as per pathogen requirement.

Stellenbosch University http://scholar.sun.ac.za

3.

IN VIVO APPLICATION OF GARLIC EXTRACTS AND CLOVE OIL TO PREVENT POSTHARVEST DECAY CAUSED BY *BOTRYTIS CINEREA, PENICILLIUM EXPANSUM* AND *NEOFABRAEA ALBA* ON APPLES

ABSTRACT

Curative and protective application of garlic extracts and clove oil directly or through volatile exposure, were tested *in vivo* for potential to inhibit decay caused by postharvest pathogens *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba* on three apple cultivars, 'Granny Smith', 'Golden Delicious' and 'Pink Lady'. Curative application of the extracts by direct exposure treatment proved to be more effective than a protective application for decay management of *B. cinerea* and *P. expansum* on all three cultivars. The efficacy of garlic extracts and clove oil, as well as a combination treatment, did not differ significantly from each other; however, all treatments significantly reduced decay when compared to the control treatments. Direct exposure of fruit artificially inoculated with *N. alba* to the extracts, did not show any decay inhibition when compared to that of the control treatments. Curative or protective exposure of inoculated fruit to the volatile components of the extracts did not inhibit postharvest decay on any of the apple cultivars; and, in some cases, application of the extracts resulted in the observation of increased pathogen lesion diameters. This study highlighted curative application of garlic extracts as having the potential to reduce postharvest decay caused by *B. cinerea* and *P. expansum,* when applied directly to the fruit.

Stellenbosch University http://scholar.sun.ac.za

3.1. INTRODUCTION

Postharvest losses of fruit are significant and can reach levels of up to 50% depending on the commodity (Janisiewicz and Korsten, 2002; Singh *et al*., 2011). Apples are among the fruit commodities that are prone to postharvest losses due to infection and subsequent decay caused by postharvest pathogens such as *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba.* Government regulations regarding minimum residue levels (MRL's), coupled with public concern about the possible harmful effects of fungicides (Singh *et al*., 2012), as well as the concern over pathogens acquiring resistance to commonly used fungicides have promoted the search for new and novel alternatives to agrochemicals (Combrink *et al*., 2011). Plant extracts and essential oils are a rich source of natural compounds that have antimicrobial potential (Okigbo *et al*., 2009; Shinde *et al*., 2011) and have thus come to the forefront in the search towards safer agrochemical production.

Extracts and essential oils derived from thyme, cinnamon, peppermint, sweet basil, ginger, chilly, clove and garlic (Ziedan and Farrag, 2008; Okigbo *et al*., 2009; Combrink *et al*., 2011; Singh *et al*., 2012) are among some of the plant extracts that have been investigated for their antimicrobial effects against plant pathogens. Investigations into garlic (*Allium sativum* L), has highlighted a wide degree of antimicrobial applications (Ankri and Mirelman, 1999) for this plant. In relation to antimicrobial action against plant pathogens, Nashwa and Abo-Elyousr (2012) reported on the ability of garlic to reduce early blight disease on tomato, Okigbo *et al*. (2009) showed that garlic was effective at inhibiting mycelial growth of root rot pathogens affecting cassava and garlic was also found to be effective in controlling species of *Penicillium* (Obagwu and Korsten, 2003).

The use of plant extracts and essential oils targets a niche market such as organic farming; however, any available products are registered primarily for use preharvest (Dayan *et al.*, 2009), leaving an open market in the postharvest sector. Furthermore, the majority of the work revolving around the use of plant extracts postharvest has focused on *in vitro* suppression of microbial pathogens and little work has been done on the *in vivo* effects of plant extracts and essential oils on fruits and vegetables, postharvest (Combrink *et al*., 2011; Singh *et al*., 2011). Therefore the objectives of this chapter was to test ability of garlic extracts and clove oil to inhibit decay caused by the postharvest pathogens *B. cinerea*, *P. expansum* and *N. alba* on apples. Investigations into the curative or

Stellenbosch University http://scholar.sun.ac.za

protective mode of action of the compounds in the extract by the direct application of the extracts onto fruit, as well as the ability of the extracts to prevent decay on apples by volatile release of substances was investigated.

3.2. MATERIALS AND METHODS

3.2.1. Fruit

The three apple cultivars 'Granny Smith', 'Golden Delicious' and 'Pink Lady' were harvested from the Grabouw region in the Western Cape, South Africa and were sourced from "Fruit Ways" Packhouse. Fruit were removed from cold storage and left overnight at room temperature prior to any execution of the trials.

3.2.2. Pathogenic isolates

Three pathogens, *B. cinerea* (B62-SUN) isolated from pears, and *P. expansum* (Pen 1-SUN) and *N. alba* (DOK7-SUN) isolated from apples, were selected. All three pathogens were obtained from the fungal collection of Stellenbosch University Plant Pathology Department. All the isolates were tested for pathogenicity on fruit and pure isolations were prepared. The fungi were identified on the basis of morphological characteristics, viz. formation on media, mycelial characters and types of conidial characters. Both *B. cinerea* and *P. expansum* were cultured on potato dextrose agar (PDA, pH 5.6, Merck) at 25°C for seven days. *Neofabraea* was maintained on acidified potato dextrose agar (aPDA, Merck) or V8 agar for one month at 25°C. In the case of *B. cinerea* and *P. expansum*, conidia were harvested by flooding the media surface with sterile distilled water together with Tween 80 and gently agitating the plate to dislodge spores. A conidial suspension was prepared in Tween 80 (0.05% w/v) and the final inoculum concentration adjusted with a haemocytometer to a concentration of 1 x 10^4 spores mL^{-1} respectively for each pathogen. Mycelial plugs (3 mm) taken from the periphery of actively growing cultures of *N. alba* were used as the inoculum source for this pathogen.

3.2.3. Preparation of garlic and clove oil extracts

Fresh garlic (*Allium sativum* L.) cloves were purchased from a retail store (Woolworths, Stellenbosch). The garlic cloves were peeled and cleaned cloves were surface

Stellenbosch University http://scholar.sun.ac.za

sterilized using ethanol. One thousand grams of garlic was weighed out and crushed in a blender together with 1250 mL of ethanol. The mixture was then placed into a glass container and extracted overnight at room temperature. The extract was then filtered through a Büchner funnel and Whatman qualitative filter paper (No. 4). After filtration, the extract was subjected to a rotary evaporator (at 60-80°C and 30 rpm) to remove the ethanol, thus leaving only a semisolid extract solution (crude extract). This extract was considered as the 100% concentrate of the extract. The extracts were stored in a refrigerator at 4°C until subsequent use.

Clove bud oil (100% organic) manufactured by SOIL essential oils™ was bought from a local pharmacy. The oil was diluted with 10% ethanol to prepare the required concentrations.

3.2.4. Curative and protective action of garlic extracts and clove oil, by direct application, on disease control

The curative and protective actions of garlic and clove oil were evaluated. Five treatments were applied to the three apple cultivars, consisting of garlic extracts alone at concentrations of 50% and 40%, respectively, 1% clove oil, a combination treatment of 1% clove oil plus 40% garlic extract and sterile distilled water as the control.

To test for curative action of treatments, fruit were surface sterilized in 70% ethanol and allowed to dry. The fruit were then wounded with a sterile wounding apparatus (3x3mm), with two wounds inflicted on each fruit. Fruit were then inoculated by delivering with the aid of a sterile pipette, a volume of 20µl of the respective spore suspensions (1x 10^4 cfu mL^{-1}) for *B. cinerea* and *P. expansum* into the wound on the fruit while inoculation of *N. alba* was done by aseptically inserting a 3 mm mycelial plug into the wound. Pathogen germination was allowed for 24 hours in a humidity chamber (90-95% RH, 20°C). After 24 hours incubation, fruit were removed from the humidity chamber and treated with 20µl of the respective treatments, aseptically delivered using sterile pipettes. Fruit were then further incubated until seven days at 20°C (90-95% RH).

Protective action of the treatments was tested in a similar manner as above. However, wounded fruit were first treated with the respective treatments and allowed 2 hours for the treatment to settle into the wounds. Afterwards, the fruit were inoculated with the respective pathogens as mentioned above and incubated at 20°C and 90-95% RH for seven days.

Stellenbosch University http://scholar.sun.ac.za

Following the incubation period, fruit were assessed by measuring lesion diameter in millimeters (mm) using digital vernier calipers.

3.2.5. Curative and protective action of garlic and clove oil on disease control by the release of extracts volatile vapours

The ability of garlic and clove oil to elicit an antifungal effect on *B. cinerea* and *P. expansum* via the vapour phase was tested *in vivo*. Fruit boxes were lined with green polyliner bags (37.5 microns) with pulp trays placed inside. A total of ten apples were positioned around a Petri dish fixed to the centre of the pulp tray. Curative and protective action of the extracts were tested in a similar way as per method described previously; however, exposure to the extracts was through the volatile phase instead of through direct wound inoculation as described above. Inoculated fruit were exposed to the volatile vapours by placing 5 mL of the respective treatment into the Petri dishes inside the boxes. The polyliner bags were immediately sealed once the treatments were placed into the Petri dishes. All fruit was incubated for seven days at 20°C and 90-95% RH. Fruit were assessed by measuring lesion diameter (mm) using digital vernier calipers. *Neofabraea alba* was exposed to volatiles of 40% garlic and a control treatment only and this was done only on the apple cultivar 'Pink Lady'.

3.2.6. Statistical Analysis

For the first experiments described, the experimental design was completely randomised (CRD) with comparison between treatment and cultivar for each method of application (curative or protective) for *B. cinerea* and *P. expansum*. Cultivar, treatment and method were compared when analysing data for *N. alba*. Three random replications of each treatment were conducted, using 10 fruit per treatment. Lesion diameter (mm) was measured and analysed by an appropriate analysis of variance (ANOVA). Levene's test for homogeneity of variance (Levene, 1960) and a weighted analysis (John and Quenouille, 1977) of data was applied for *B. cinerea* and *P. expansum*. A combined analysis of the curative and protective methods was carried out for pathogens in the volatile fruit trial. The treatment means for all pathogens were compared by a Student's t-test with least significant difference (LSD) at 5% ($P = 0.05$) (Ott, 1993). Analysis was performed using SAS version 9.2 statistical software (SAS, 2012).

Stellenbosch University http://scholar.sun.ac.za

3.3. RESULTS

3.3.1. Curative and protective action of garlic extracts and clove oil, by direct application, on disease control

For each of the three apple cultivars ('Granny Smith', 'Golden Delicious' and 'Pink Lady') a similar trend between cultivar and treatment was noted. Significant differences occurred between treatments and controls. No significant difference between the extract treatments was observed. All treatments were effective at controlling the growth of the *B. cinerea* curatively on all cultivars as is evident by a reduction in lesion diameter in the treatments when compared to the control (Tables 1 and 2). Even though data showed no significant difference between the garlic extracts and clove oil treatments, the garlic treatments at concentrations of 40% and 50% garlic extract elicited a slightly smaller lesion diameter compared to the garlic extract + clove oil treatment, indicating that the garlic treatments alone performed better at reducing pathogen development. When garlic and clove oil treatments were applied protectively to the apples, a treatment and cultivar interaction was observed (Table 3 and 4). The cultivars responded differently to each of the treatments applied, with the combination treatment of 1% clove and 40% garlic giving significantly better control of *B. cinerea*. With this treatment there was no significant difference observed for the respective cultivars tested. A significant reduction in lesion diameter relative to the control is noted on all three cultivars (Table 4).

Curative application of garlic extracts and clove oil by direct exposure of the pathogen to the extract shows a significant treatment and cultivar interaction ($P \leq 0.05$) for the control of *P. expansum* (Table 5). All treatments significantly reduced the lesion diameter on all three cultivars compared to the control (Table 6). Although not significantly different to other treatments of garlic and clove individually, the combination treatment worked best to inhibit pathogen growth on 'Granny Smith' and 'Golden Delicious' apples. When applied protectively for the control of *P. expansum*, no significant cultivar treatment interaction was noted ($P = 0.56$) (Table 7). For each of the cultivars respectively, no significant reduction in lesion diameter was observed from any of the treatments applied (Table 8). In the case of 1% clove oil, protective application seemed to enhance pathogen growth on all three cultivars, giving a larger lesion diameter in comparison to the control set. The application of the 50% garlic extract was the only treatment to significantly reduce lesion diameter on 'Granny Smith' apples when compared to the control.

Stellenbosch University http://scholar.sun.ac.za

A combined ANOVA was carried out for the direct application of garlic extracts and clove oil effect on *N. alba* (Table 9). The ANOVA revealed no significant interaction when comparing cultivar with method (P = 0.83), cultivar with treatment (P = 0.83) or when comparing cultivar, method and treatment (P = 0.80). A significant interaction (P = 0.001) was observed when comparing method with treatment. This is evident in the comparison of 50% garlic treatment for the curative versus protective methods (Table 10) where lesion diameter for the curative application on 'Pink Lady' cultivar was significantly different to the lesion diameter for the protective application of that treatment on 'Pink Lady'. In the case of treatments applied curatively on all three cultivars, application of 40% garlic alone as well as in combination with 1% clove oil enhanced pathogen growth as was evident by lesion diameters larger than the control sets (Table 10). The lesion diameters assessed from the 1% clove oil treatment was not significantly different to the control for each of the cultivars. On each of the cultivars, protective application, resulted in lesion diameters that were larger than the respective control (Table 10), indicating an antagonistic response of the pathogen to the treatments. A 1% clove oil application had a smaller lesion diameter compared to the control; however, it was not significant. Results for all treatments relative to the control indicate no pathogen control of *N. alba* by the extracts on any of the apple cultivars.

3.3.2. Curative and protective action of garlic and clove oil on disease control by the release of extracts volatile vapours

ANOVA results (Table 11 and 12) indicated no significant interaction ($P > 0.05$) between cultivar, method (curative or protective) and treatments for either *B. cinerea* or *P. expansum*. With the exception of 1% clove oil, applied protectively to 'Granny Smith' and 'Pink Lady' apples for *B. cinerea*, all treatments were not significantly different to the respective controls or other treatments (Table 13). Similarly, for *P. expansum*, most of the treatments did not differ significantly from the control and those that did were marginal in value (Table 14). Volatile vapours of garlic extracts and clove oil failed to induce any pathogen inhibition. Only a concentration of 40% garlic was tested on *N. alba* and only on the 'Pink Lady' cultivar. Results indicated no significant difference between treatment and method of application. No decay control was achieved with either curative or protective application of 40% garlic extract via the vapour phase (Table 15).

Stellenbosch University http://scholar.sun.ac.za

3.4. DISCUSSION

Garlic extracts and clove oil have been investigated by many researchers for their antimicrobial properties. The antifungal activity of crude garlic extracts against the postharvest pathogens *B. cinerea*, *P. expansum* and *N. alba* was demonstrated *in vitro* (Chapter Two). Clove oil's antimicrobial capacity has been demonstrated by authors that have looked at the effect on bacterial pathogens (Huang and Lakshman, 2010) and fungal pathogens, including *B. cinerea* (El-Zemity and Ahmed, 2005; Combrink *et al*., 2011). The aforementioned pathogens have been termed throughout this study as being postharvest pathogens; however, in the case of all three, infections by these pathogens may begin preharvest. Therefore, the ability of garlic and clove oil extracts to exhibit the same antifungal action seen *in vitro* was investigated *in vivo* with curative and protective applications on apple cultivars.

In the effort to test the value of using plant extracts and essential oils as alternatives to fungicides, methods have been used such as direct wound exposure, amendments with fruit coatings, volatile applications, and dipping. In this study, directly treating pathogen inoculated wounds with the garlic extracts and clove oil showed that for *B. cinerea* and *P. expansum*, all treatments successfully inhibited decay on apples caused by these pathogens. Furthermore, curative application of extracts was highlighted as giving the best control on all apple cultivars. When applied curatively, all the treatments did not differ in the inhibitory effect seen on the apples. The curative and protective application of essential oils supplemented into fruit waxes and applied on citrus gave a slightly different response than the one seen in the present study as 100% decay inhibition of *P. digitatum* was noted when they were applied protectively, whilst the curative treatment reduced decay by 63% (du Plooy *et al*., 2009).

The possible synergistic interaction between compounds may lead to a combination of essential oils that has increased antimicrobial action in contrast to the two products acting individually (van Vuuren, 2008). This was the opinion of the researchers in the planning stages of this experiment and was confirmed in the outcomes of the study when 1% clove oil and 40% garlic extract were combined. However, if focusing on the curative application, the results are revealed as not being statistically different to the two treatments on their own. Allicin and eugenol are the respective active compounds of garlic and clove oil. Both these compounds constitute approximately 70-90% of the samples (Ankri and Mirelman, 1999;

Stellenbosch University http://scholar.sun.ac.za

Alma *et al*., 2007; Combrink *et al*., 2011). Even, though they fall under different classes of chemical compounds with clove being a phenylpropene and allicin being a sulphur compound, the similarity between them is the presence of an allyl chain. Since no work could be found on the mechanism of action of allyl chain derivatives, it could be possible that the similar antifungal action shared by these two extracts may have to do with the possession of the allyl chain in their respective biochemistries and could be cause for future work.

Extracts of *Anadenanthera colubrina* successfully suppressed lesions caused by *Alternaria alternata* on Murcott tangor fruits. Other plant extracts in that study showed no effects on disease management *in vivo,* even though *in vitro* application proved to be promising (Carvalho *et al*., 2011). In line with this, Plaza *et al*. (2004) reported on the inability of essential oils to control species of *Penicillium* when applied directly to inoculated fruit. In contrast to du Plooy *et al*. (2009), a different study reported on the failure of essential oils to exhibit any antifungal action even when employed in citrus wax or packaging. On the topic of citrus wax amendments with essential oils, the study reported "severe rind damage" on citrus (Plaza *et al*., 2004).

Natural plant volatile compounds and volatiles released from plant extracts and essential oils have been studied *in vitro* for thier ability to reduce pathogen decay. Studies have reported on the potential of using volatile compounds for storage fumigation, modified atmosphere storage and packaging and active packaging on a range of fruit and vegetable commodities (Combrink *et al*., 2011; Wood *et al*., 2013). Plaza *et al.* (2004) was also of the opinion that application of extracts in the volatile phase may have some merit, even though the essential oils used in that particular study failed to exhibit an antifungal response when applied directly to fruit and with amendments to fruit coatings. Vapour phase application of volatile compounds and essential oils have had some reported success on fruit, for example, there has been reported control of *Monilinia laxa* and *Rhizopus stolonifer* on stone fruit (Neri *et al*., 2007; Ziedan and Farrag, 2008). Dipping and spray application of essential oil of Shiraz thyme showed antifungal activity against *P. digitatum* only in a dip treatment (Solaimani *et al*., 2009), favouring direct application over volatile application. This is in line with the present study, where exposure of the pathogens to the volatile vapours of garlic extracts and clove oil did not result in any decay inhibition on any of the apple cultivars, regardless of curative or protective application, despite promising results from *in vitro* experiments (Chapter Two). Testing volatile compounds *in vitro* and *in vivo*, the results obtained in this study with regards to *N. alba* are validated up by those of Neri *et al*. (2009),

Stellenbosch University http://scholar.sun.ac.za

where fumigation of plant volatiles *in vitro* consistently inhibited *N. alba*. However, the *in vivo* data showed only a slight inhibition of *N. alba* on inoculated fruit from only one of the nine plant volatile compounds that showed promised *in vitro* (Neri *et al*., 2009).

The garlic extracts and clove oil treatments used in this study showed some promise for direct curative application on 'Granny Smith', 'Golden Delicious' and 'Pink Lady' apple cultivars for the management of decays caused by *B. cinerea* and *P. expansum*. No *in vivo* success has been reached for the use of garlic or clove oil on *N. alba*. The ability of plant extracts and essential oils to exhibit antimicrobial action via the vapour phase may have promise; however, this was not achieved in the current study and a proven, reproducible, cost effective method of application still needs to be found.

3.5. LITERATURE CITED

Alma, M.H., Ertas, M., Nitz, S. and Kollmannsberger, H. 2007. Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum* L.). BioResources 2: 265-269.

Ankri, S. and Mirelman, D. 1999. Antimicrobial properties of allicin from garlic. Microbes and Infection 2: 125-129.

Carvalho, D.D.C., Alves, E., Camargos, R.B., Oliveira, D.F., Scolforo, J.R.S., de Carvalho, D.A. and Batista, T.R.S. 2011. Plant extracts to control *Alternaria alternata* in Murcott tangor fruits. Revista Iberoamericana de Micologia 28: 173-178.

Combrink, S., Regnier, T. and Kamatou, G.P.P. 2011. *In vitro* activity of eighteen essential oils and some major components against common postharvest fungal pathogens of fruit. Industrial Crops and Products 33: 344-349.

Dayan, F.E., Cantrell, C.L. and Duke, S.O. 2009. Natural products in crop protection. BioOrganic and Medicinal Chemistry 17: 4022-4034.

du Plooy, W., Regnier, T. and Combrink, S. 2009. Essential oil amended coatings as alternatives to synthetic fungicides in citrus postharvest management. Postharvest Biology and Technology 53: 117-122.

El-Zemity, S.R. and Ahmed, S.M. 2005. Antifungal activity of some essential oils and their major chemical constituents against some phytopathogenic fungi. Journal of Pest Control and Environmental Science 13: 61-72.

Stellenbosch University http://scholar.sun.ac.za

Huang, Q. and Lakshman, D.K. 2010. Effect of clove oil on plant pathogenic bacteria and bacterial wilt of tomato and geranium. Journal of Plant Pathology 92: 701-707.

Janisiewicz, W.J. and Korsten, L. 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40: 411-41.

John, J.A. and Quenouille, M.H. 1977. Experiments: design and analysis (Combined experiments and weighted ANOVA). Charles Griffin and Company LTD, London and High Wycombe.

Levene, H. 1960. "Robust test in the equality of variance". In: Contributions to probability and statistics. Iolkin Palo Alto, CA: Stanford. University Press pp 278-292.

Nashwa, S.M.A. and Abo-Elyousr, K.A.M. 2012. Evaluation of various plant extracts against the early blight disease of tomato plants under greenhouse and field conditions. Plant Protection Science 48: 74-79.

Neri, F., Mari, M., Brigati, S. and Bertolini, P. 2007. Fungicidal activity of plant volatile compounds for controlling *Monilinia laxa* in stone fruit. Plant Disease 91: 30-35.

Neri, F., Mari, M., Brigati, S. and Bertolini, P. 2009. Control of *Neofabraea alba* by plant volatile compounds and hot water. Postharvest Biology and Technology 51: 425-430.

Obagwu, J. and Korsten, L. 2003. Control of citrus green and blue moulds with garlic extracts. European Journal of Plant Pathology 109: 221-225.

Okigbo, R.N., Okorie, R.E. and Putheti, R.R. 2009. *In vitro* effects of garlic (*Allium sativum* L.) and African basil (*Ocimum gratissimum* L.) on pathogens isolated from rotted cassava roots. Interciencia 34: 742-747.

Ott, R.L. 1993. An introduction to statistical methods and data analysis. Belmont, California: Duxbury Press: pp 807-837.

Plaza, P., Torre, R., Usall, J., Larmaca, N. and Vinas, I. 2004. Evaluation of the potential of the commercial postharvest application of essential oils to control citrus decay. Journal of Horticultural Science and Biotechnology 79: 935-940.

SAS Institute, Inc. 2012. SAS Version 9.2 64 bit. SAS Institute Inc, SAS Campus Drive, Cary, North Carolina.

Shinde, S.L., Junne, S.B. and Wadje, S.S. 2011. Utilization of medicinal plants to control seed borne pathogens of selected seeds. Asian Journal of Pharmaceutical and Health Sciences 1: 79-82.

Stellenbosch University http://scholar.sun.ac.za

Singh, H., Al-samarai, G. and Syarhabil, M. 2012. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of citrus. International Journal of Scientific and Research Publications 2: 1-4.

Singh, H., Fairs, G. and Syarhabil, M. 2011. Anti-fungal activity of *Capsicum frutescence* and *Zingiber officinale* against key post-harvest pathogens in citrus. International Conference on Biomedical Engineering and Technology. IPCBEE 11: IACSIT Press, Singapore.

Solaimani, B., Ramezani, S., Rahemi, M. and Saharkhiz, M.J. 2009. Biological control of postharvest disease caused by *P. digitatum* and *P. italicum* on stored citrus fruits by Shiraz Thyme essential oil. Advances in Environmental Biology 3: 249-254.

van Vuuren, S.F. 2008. Antimicrobial activity of South African medicinal plants. Journal of Ethnopharmacology 119: 462-472.

Wood, E.M., Miles, T.D. and Wharton, P.S. 2013. The use of natural plant volatile compounds for the control of potato postharvest diseases, black dot, silver scurf and soft rot. Biological Control 64: 152-159.

Ziedan, E.H.E. and Farrag, E.S.H. 2008. Fumigation of peach fruits with essential oils to control postharvest decay. Research Journal of Agriculture and Biological Sciences 4: 512-519.

Stellenbosch University http://scholar.sun.ac.za

3.6. TABLES AND FIGURES

Table 1: Analysis of variance (ANOVA) to compare cultivar and treatment for curative application of garlic and clove oil extracts, applied directly into artificially inflicted wounds, on apples cultivars inoculated with *B. cinerea*

Source	[c] DF	Mean Square	Pr > F
[a] Cultivar	2	94.093297	0.0025
[b] Treatment	4	1194.510119	<.0001
Cultivar x Treatment	8	21.279786	0.1491
Model	14	202.345211	<.0001
Error	30	1.885447	
Corrected total	44		

[a] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [b] Five treatments incorporating garlic and clove oil extracts; [c] DF = Degrees of freedom.

Table 2: Effects of curative application of garlic extracts and clove oil, applied directly into artificially inflicted wounds, for the control of *Botrytis cinerea* on three apple cultivars 'Granny Smith' (GS), 'Golden Delicious' (GD), and 'Pink Lady' (PL) measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

	Mean lesion diameter (mm)		
Treatment	GS	GD	PL
Control (water)	33.81 a	34.17 a	22.31 b
50% Garlic	3.72 cde	2.46 e	2.79 de
40 % Garlic	4.08 cde	2.66 de	1.32 e
1% Clove	6.44 cde	6.80 cde	4.70 cde
1% Clove + 40% Garlic	9.39 c	8.54 cd	3.36 de

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Stellenbosch University http://scholar.sun.ac.za

Table 3: Analysis of variance (ANOVA) to compare cultivar and treatment for protective application of garlic and clove oil extracts, applied directly into artificially inflicted wounds, on apples cultivars inoculated with *B. cinerea*

Source	[c] DF	Mean Square	Pr > F
[a] Cultivar	2	23.264491	0.039
[b] Treatment	4	535.887959	<.0001
Cultivar x Treatment	8	67.800085	<.0001
Model	14	202.345211	<.0001
Error	30	1.885447	
Corrected total	44		

[a] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [b] Five treatments incorporating garlic and clove oil extracts; [c] DF = Degrees of freedom.

Table 4: Effects of protective application of garlic extracts and clove oil, applied directly into artificially inflicted wounds, for the control of *Botrytis cinerea* on three apple cultivars 'Granny Smith' (GS), 'Golden Delicious' (GD), and 'Pink Lady' (PL) measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

	Mean lesion diameter (mm)		
Treatment	GS	GD	PL
Control (water)	33.57ab	22.64e	26.09de
50% Garlic	28.84cd	36.95a	32.07bc
40 % Garlic	24.83de	33.10ab	31.04bc
1% Clove	34.34ab	28.18cd	23.72e
1% Clove + 40% Garlic	12.47f	14.90f	11.31f

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Stellenbosch University http://scholar.sun.ac.za

Table 5: Analysis of variance (ANOVA) to compare cultivar and treatment for curative application of garlic and clove oil extracts, applied directly into artificially inflicted wounds, on apples cultivars inoculated with *P. expansum*

Source	[c] DF	Mean Square	Pr > F
[a] Cultivar	2	2.348183	0.3023
[b] Treatment	4	690.399073	<.0001
Cultivar x Treatment	8	8.317538	0.0013
Model	14	202.345211	<.0001
Error	30	1.885447	
Corrected total	44		

[a] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [b] Five treatments incorporating garlic and clove oil extracts; [c] DF = Degrees of freedom.

Table 6: Effects of curative application of garlic extracts and clove oil, applied directly into artificially inflicted wounds, for the control of *Penicillium expansum* on three apple cultivars 'Granny Smith' (GS), 'Golden Delicious' (GD), and 'Pink Lady' (PL) measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

Treatment	Mean lesion diameter (mm)		
	GS	GD	PL
Control (water)	23.78 a	23.61 a	19.63 b
40 % Garlic	2.36 def	2.77 c-f	4.12 cd
50% Garlic	2.67 c-f	2.07 def	3.57 cde
1% Clove	1.91 def	2.87 c-f	3.62 cde
1% Clove + 40% Garlic	1.53 ef	0.94 f	4.74 c

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Stellenbosch University http://scholar.sun.ac.za

Table 7: Analysis of variance (ANOVA) to compare cultivar and treatment for protective application of garlic and clove oil extracts, applied directly into artificially inflicted wounds, on apples cultivars inoculated with *P. expansum*

Source	[c] DF	Mean Square	Pr > F
[a] Cultivar	2	21.1438452	0.0123
[b] Treatment	4	69.2943992	<.0001
Cultivar x Treatment	8	3.5493323	0.5617
Model	14	24.847139	<.0001
Error	30	4.1392515	
Corrected total	44		

[a] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [b] Five treatments incorporating garlic and clove oil extracts; [c] DF = Degrees of freedom.

Table 8: Effects of protective application of garlic extracts and clove oil, applied directly into artificially inflicted wounds, for the control of *Penicillium expansum* on three apple cultivars 'Granny Smith' (GS), 'Golden Delicious' (GD), and 'Pink Lady' (PL) measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

Treatment	Mean lesion diameter (mm)		
	GS	GD	PL
Control (water)	20.40 bc	19.66 bcd	15.93 ef
40 % Garlic	18.35 b-e	20.21 bc	18.47 b-e
50% Garlic	16.03 ef	17.31 c-f	16.68 def
1% Clove	24.49 a	24.13 a	21.50 ab
1% Clove + 40% Garlic	17.05 c-f	17.50 c-f	14.93 f

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Stellenbosch University http://scholar.sun.ac.za

Table 9: Analysis of variance (ANOVA) for the curative and protective application of garlic extracts and clove oil, applied directly into artificially inflicted wounds, for the control of *Neofabraea alba* on apples.

Source	DF[d]	Mean Square	Pr > F
[a] Method	1	23.1279143	<.0001
[b] Cultivar	2	50.416375	<.0001
Cultivar x Method	2	0.2417522	0.8325
[c] Treatment	4	23.2715202	<.0001
Method x treatment	4	6.8684642	0.0011
Cultivar x Treatment	8	0.6870824	0.8349
Cultivar x Method x Treatment	8	0.7392381	0.8044
Error	60	1.3148998	
Corrected Total	89		

[a] Two methods for analysis: curative and protective application of extracts; [b] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [c] Five treatments incorporating garlic extracts and clove oil; [d] DF = degrees of freedom.

Table 10: Effects of curative and protective application of garlic extracts and clove oil, applied directly into artificially inflicted wounds, for the control of *Neofabraea alba* on three apple cultivars 'Granny Smith', 'Golden Delicious', and 'Pink Lady' measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

	Mean lesion diameter (mm)					
	'Granny Smith'		'Golden Delicious'		'Pink Lady'	
Treatment	Curative	Protective	Curative	Protective	Curative	Protective
Control (water)	6.85 lm	8.31 h-l	8.29 h-l	7.97 j-m	9.99 c-i	8.80 g-k
50% Garlic	9.51 e-j	9.92 c-i	10.92 a-f	11.61 abc	11.87 b	12.32 a
40 % Garlic	6.25 m	9.48 e-j	8.44 h-l	11.28 a-e	9.11 f-j	12.37 a
1% Clove	7.21 klm	8.13 i-l	8.63 g-l	10.01 b-h	10.44 b-g	11.40 a-d
1% Clove + 40% Garlic	9.50 e-j	9.55 d-j	11.02 a-e	11.26 a-e	11.72 abc	12.54 a

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Stellenbosch University http://scholar.sun.ac.za

Table 11: Analysis of variance (ANOVA) for the volatile action of garlic extracts and clove oil against *Botrytis cinerea*.

Source	[d]DF	Mean Square	Pr > F
[a] Method	1	0.0741813	0.8904
[b] Cultivar	2	141.3024506	<.0001
Cultivar x Method	2	12.0631125	0.0517
[c] Treatment	4	5.9123423	0.206
Method x Treatment	4	1.956718	0.7321
Cultivar x Treatment	8	5.9910385	0.1606
Cultivar x Method x Treatment	8	4.5446207	0.3302
Model	29	14.5712697	<.0001
Error	60	3.8738087	
Corrected Total	89		

[a] Two methods for analysis: curative and protective application of extracts; [b] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [c] Five treatments incorporating garlic and clove oil extracts; [d]DF = Degrees of freedom.

Stellenbosch University http://scholar.sun.ac.za

Table 12: Analysis of variance (ANOVA) for the volatile action of garlic and clove oil extracts against *Penicillium expansum*

Source	[d]DF	Mean Square	Pr > F
[a] Method	1	0.0380278	0.8841
[b] Cultivar	2	135.1067139	<.0001
Cultivar x Method	2	3.4599885	0.151
[c] Treatment	4	12.3048576	0.0001
Method x Treatment	4	4.5007283	0.0491
Cultivar x Treatment	8	1.6209503	0.5112
Cultivar x Method x Treatment	8	0.6865182	0.9233
Model	29	12.5121905	<.0001
Error	60	1.7731689	
Corrected Total	89		

[a] Two methods for analysis: curative and protective application of extracts; [b] Three apple cultivars: 'Granny Smith', 'Golden Delicious' and 'Pink Lady'; [c] Five treatments incorporating garlic and clove oil extracts; [d]DF = Degrees of freedom.

Table 13: Volatile effects of the curative and protective application of garlic extracts and clove oil for the control of *Botrytis cinerea* on three apple cultivars 'Granny Smith', 'Golden Delicious', and 'Pink Lady' measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

	Mean lesion diameter (mm)					
	'Granny Smith'		'Golden Delicious'		'Pink Lady'	
Treatment	Curative	Protective	Curative	Protective	Curative	Protective
Control	5.28 g-m	5.92 e-m	8.73 a-f	6.00 e-l	10.25 abc	11.10 a
40 % Garlic	3.60 lm	4.75 i-m	7.94 a-i	7.64b-j	10.04 a-d	8.78 a-f
50% Garlic	5.27 g-m	4.90 h-m	6.98 d-k	5.95e-m	9.69 a-d	8.83 a-e
1% Clove	5.24 g-m	7.51 b-i	8.08 a-h	4.02 klm	8.18 a-g	7.20 c-k
1% Clove + 40% Garlic	4.56 j-m	2.78 m	5.58 f-m	6.27 e-l	10.64 ab	8.23 a-g

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Stellenbosch University http://scholar.sun.ac.za

Table 14: Volatile effects of the curative and protective application of garlic extracts and clove oil for the control of *Penicillium expansum* on three apple cultivars 'Granny Smith', 'Golden Delicious', and 'Pink Lady' measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

	Mean lesion diameter (mm)					
	'Granny Smith'		'Golden Delicious'		'Pink Lady'	
Treatment	Curative	Protective	Curative	Protective	Curative	Protective
Control	25.02 a-d	25.90 ab	21.08 hij	22.40 e-i	26.38 a	25.07 a-d
40 % Garlic	24.98 a-d	25.78 ab	21.11 hij	20.40 ijk	24.57 a-e	24.14 b-f
50% Garlic	24.23 a-f	23.78 b-g	21.01 hij	19.34 jk	22.24 f-i	23.09 d-h
1% Clove	24.90 a-d	25.43 abc	21.67 ghi	21.01 hij	24.15 b-f	22.26 f-i
1% Clove + 40% Garlic	24.89 a-d	23.29 c-g	20.82 ijk	18.78 k	23.53 c-g	23.31 c-g

Values followed by the same lettering indicate no significant difference between treatments and cultivar, respectively at $P \leq 0.05$. Letters in descending order indicates largest lesion diameter (no pathogen control) to smallest lesion diameter (pathogen control).

Table 15: Volatile effects of the curative and protective application of garlic extracts for the control of *Neofabraea alba* on 'Pink Lady' apple cultivar measured by mean lesion diameter (millimetres) after seven days incubation at 20°C.

	Mean lesion diameter (mm)	
Treatment	Curative	Protective
Control	9.31 a	10.89 a
40 % Garlic	9.16 a	9.47 a

Values followed by the same lettering indicate no significant difference between treatments at $P \leq 0.05$.

Stellenbosch University http://scholar.sun.ac.za

4.

FULL CHEMICAL PROFILE ANALYSIS OF *ALLIUM SATIVUM* CRUDE EXTRACT BY GAS CHROMOTOGRAPHY-MASS SPECTROMETRY

ABSTRACT

Members of the *Allium* genus are rich sources of sulphur compounds that have antimicrobial potential. *Allium sativum* (garlic) is one such plant that is well documented for its ethnopharmacological, culinary and medicinal applications. This study aimed to characterise the compounds present in garlic. Crude garlic extracts were prepared from fresh garlic bulbs and Gas chromatography-Mass Spectrometry was used to generate a full profile chemical analysis of crude garlic extracts where the compounds were identified according to the chemical library standards and quantified. The analysis identified 43 compounds present in the crude garlic extract and revealed that sulphur compounds and derivatives comprised approximately 85.95% of the garlic sample. Allicin, the putative active compound in garlic, could not be identified but compounds such as allyl methyl sulphide, allyl methyl disulphide, allyl methyl trisulphide, allyl propyl disulphide, diallyl disulphide; diallyl trisulphide, dimethyl disulphide and dimethyl trisulphide were found to be abundantly present in the extracts.

Stellenbosch University http://scholar.sun.ac.za

4.1. INTRODUCTION

Garlic (*Allium sativum* L.) has been used for centuries as a food supplement and has been well documented for its medicinal properties in traditional and conventional medicine (Khadri *et al*., 2010; Clemente *et al*., 2011). The wide range of antifungal, antibacterial and antifungal activity of garlic has been widely attributed to the high concentrations of sulphur-containing compounds it possesses (Ankri and Mirelman, 1999; Khadri *et al*., 2010).

Cavallito and Bailey (1944) discovered an oxygenated sulphur compound named allicin (diallyl thiosulphinate) which they considered to be responsible for the aroma and flavour of garlic. Many researchers have since attributed the antimicrobial action of garlic to allicin being present as the main active component (Cavallito and Bailey, 1944; Ankri and Mirelman, 1999; Bocchini *et al*., 2001; Josling, 2001). Allicin was found to be a highly volatile compound which decomposes rapidly into other compounds such as diallyl disulphide (Davis, 2005; Amagase, 2006).

The volatility and rapid decomposition rate of allicin has sparked some debate as to whether or not it is in fact responsible for the antimicrobial activity of garlic. The mystery that revolved around a difference in smell detected in whole garlic cloves versus crushed garlic cloves (Verma *et al*., 2008) has also added to this; however, the smell has been explained to be due to the conversion of an amino acid (alliin) to allicin via the action of the enzyme allinase (Ankri and Mirelman, 1999). The conversion of alliin to allicin is an event that occurs rapidly when intact garlic bulbs are crushed (Ankri and Mirelman, 1999; Clemente *et al*., 2011). The formation of allicin is followed by its rapid decomposition into the other sulphur derived compounds such as diallyl disulphide (DADS), diallyl sulphide (DAS), diallyl trisulphide, sulphur dioxide, allyl propyl disulphide, diallyl tetrasulphide (Amagase, 2006; Verma *et al*., 2008) and it is for this reason that it has also been proposed that antimicrobial activity may actually be due to a complex of sulphur and sulphur related compounds rather than just one compound (Harris *et al*., 2001; Josling, 2001).

Gas chromatography-mass spectrometry (GC-MS) is an effective analytical tool for the chemical profiling of a mixture of compounds. The analysis separates the components and then provides a representative spectral output. A full profile chemical analysis of garlic from South Africa has not been published previously. Therefore, the aim of this study was to employ GC-MS analysis to determine the chemical constituents present in a garlic extract from samples sourced from South African, and subsequently, to use this information to

Stellenbosch University http://scholar.sun.ac.za

highlight the possible active compound or complexes thereof that contributes to the antifungal effects provided by garlic crude extracts, as reported in Chapter Two and Three of this study.

4.2. MATERIALS AND METHODS

4.2.1. Preparation of garlic extracts

Fresh garlic (*Allium sativum* L.) cloves were purchased from a retail store (Woolworths, Stellenbosch). The garlic cloves were peeled and surface sterilized using ethanol (100%). The garlic cloves were allowed to dry and 800g of garlic was weighed out and crushed in a blender. A liter of ethanol was added to the crushed garlic, and the 80% garlic extract mixture was then placed into a glass container and extracted overnight at room temperature. The extract was then filtered using a Büchner funnel lined with Whatman qualitative filter paper (No. 4). The filtrate was subjected to a rotary evaporator (at 60-80°C at 30rpm) to remove the ethanol, leaving only a semisolid crude extract solution. This product was considered to be a 100% concentrate of the extract. All extracts were stored in a refrigerator at 4°C until use.

4.2.2. Determination of compounds present in crude garlic extracts through GC-MS analysis

Approximately 1 mL of garlic crude extract was transferred to 20 mL solid phase microextraction (SPME) vials for analysis. The crude extract present within the vials were allowed to equilibrate for two minutes in the heating chamber of the CTC autosampler maintained at 30°C. The volatile compounds were extracted by exposure of a 50/30m divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre (Supelco^{TM}) on the headspace of the samples. Following extraction, desorption of the volatile compounds from the fibre coating was carried out for 10 minutes in the injection port of the gas chromatography-mass spectrometry (GC–MS) operated in splitless mode. The temperature of the injection port was maintained at 240°C. Separation of the volatile compounds was performed on an Agilent 6890 N (Agilent, Palo Alto, CA) gas chromatograph coupled with an Agilent 5975 MS (Agilent, Palo Alto, CA) mass selective detector. Chromatographic separation was performed on a DB-FFAP (60 m length; 250 μm inner diameter; and 0.5 μm film thickness) capillary

Stellenbosch University http://scholar.sun.ac.za

column from Agilent Technologies. Analyses were carried out using helium as a carrier gas with a flow rate of 1.9 mL min^{-1} operated in constant flow mode. The injector temperature was maintained at 240°C.

The oven temperature was as follows: 70°C for one minute; and then ramped up to 225°C at 5°C min^{-1} and held for three minutes. The MSD was operated in full scan mode and the ion source and quadropole were maintained at 230°C and 150°C, respectively. The transfer line temperature was maintained at 280°C and total run time was approximately 46 minutes. Authentic standards were unavailable so compounds were tentatively identified by comparison with mass spectral libraries (NIST05 and Wiley 275.L). For quantification, the automatically calculated relative abundances were used and are expressed as a percentage. The sample was run twice with three repetitions respectively.

4.3. RESULTS

Based on the GC-MS analysis of crude garlic HS-SPME extract, a total of 43 volatile compounds were identified (Table 1). From this total, 25 compounds were identified as sulphur or sulphur-derived compounds, two were alcohols and one ester was identified.

Sulphur and sulphur-derived compounds made up approximately 85.95% of the entire sample concentration. The relative abundances (%) of all sulphur groups identified in this study are presented in Table 2. Allyl methyl sulphide (7.93%), allyl methyl disulphide (7.86%), allyl methyl trisulphide (13.85%), diallyl disulphide (24.10%) and dimethyl trisulphide (11.36%) were present with the highest percentages within the sample (Table 2). The chromatograph (Figure 1) highlights compound abundance relative to the retention time, with compounds generating the highest peaks in the sample being highlighted. The percentage relative abundances (%) presented in this study was calculated automatically using the peaks obtained from the chromatograph. Similar results were obtained for the second sample run with the exception of the detection of three additional compounds, namely, 3-vinyl-1,2-dithiacyclohex-4-ene, 3-vinyl-1,2-dithiacyclohex-5-ene and 1-Oxa-4,6-diazacyclooctane-5-thione.

Stellenbosch University http://scholar.sun.ac.za

4.4. DISCUSSION

Over the years different analytical techniques such as steam distillation, high performance liquid chromatography (HPLC) (Bocchini *et al*., 2001), principle component analysis (PCA) (Clemente *et al*., 2011) and GC-MS have been employed to profile chemical compounds in garlic and other plant essential oils. With regard to garlic extracts, available literature reveal common compounds cited across the various studies but also, uncommon compounds have been found in some studies, suggesting that even though these methods are all quite sensitive to their purpose, variation in compound detection can occur between sample sets. This is possibly owing to a variety of reasons relating to the type of analysis carried out, the analytical techniques applied, the conditions surrounding the study but also due to the garlic sample itself – with sample preparation and cultivar type also playing a role in the compounds that are detected (Clemente *et al*., 2011).

Over 35 different compounds have been identified in garlic (Clemente *et al*., 2011) with the sulphur-containing compounds being the main focus of research studies conducted on garlic and related species. The full profile analysis in this study rendered a total of 43 compounds highlighted within the garlic sample and further investigation found that the total amount of sulphur-containing compounds made up approximately 85.95% of the sample tested. Allicin (diallyl thiosulphinate) could not be directly detected in this study; however, it has been reported that allicin decomposes to diallyl disulphide, diallyl trisulphide and sulphur dioxide (Amagase, 2006) and these compounds together with other volatiles typically present in crushed garlic were found in relatively high amounts in this study. Furthermore, exposure time of extract preparation until it was analysed could be integral to detecting allicin due to its rapid decomposition rate.

The major sulphides that have been identified in garlic include diallyl sulphide, allylmethyl, and dimethyl, mono- tohexasulphides together with small amounts of allyl 1-propyl and methyl 1-propyl, di-, tri-, and tetrasulphides (Amagase, 2006), although different studies (Khadri *et al*., 2010; Clemente *et al*., 2011; Borrego *et al*., 2012), including the present study, report different amounts of these compounds present. A study by Khadri *et al*. (2010) reported the two major compounds present in the garlic sample tested to be methyl allyl trisulphide (34.61%) and diallyl disulphide (31.65%). Both these compounds were found in the sample tested in this study, but at lower concentrations of 13.85% and 24.10% respectively. According to the authors, no other reports of allyl methyl trisulphide had been made previously and they concluded that the cultivar used represented a new chemotype

Stellenbosch University http://scholar.sun.ac.za

typical of eastern Algeria (Khadri *et al*., 2010); however, this cannot be the case since the garlic used in the present study was not sourced from that geographical region. The compounds 3-vinyl-1,2-dithiacyclohex-4-ene and 3-vinyl-1,2-dithiacyclohex-5-ene have been reported as the compounds responsible for allinase activity (Chen *et al*., 2007). These compounds were detected in the second sample analysis that was carried out (results not shown) but not in the first analysis. A possible reason for this could be owed to the amount of time allowed to lapse between extraction of garlic and analysis of the extract. The first batch of extracts was analysed about a month after the extracts were prepared; however, the second analysis was carried out within seven days of extract preparation. Another compound detected in the second analysis but not the first but which is worth mentioning is 1-Oxa-4,6-diazacyclooctane-5-thione which was found by researchers as a compound present in "rosy garlic" (*Allium roseum* L.) and was also listed in that study as not being found in any other literature on the topic (Zouari *et al*., 2013).

Due to the various sulphur-linked compounds highlighted in different studies on components of garlic, Amagase (2006) speculated that while garlic is recognised for the abundance of sulphur compounds present, compounds other than allicin could also contribute to the various antimicrobial activities. The present study supports this hypothesis, as allicin was not found in the sample tested; however, other sulphur and sulphur derivative compounds were found in large amounts. It is plausable that a complex of compounds, rather than one individual compound, is responsible for the antifungal activity noted for garlic samples in this study (Chapters 2 and 3). It is recommended that if individual compounds can be sourced then each should be subjected to antimicrobial screening to determine the contribution to the antimicrobial action of garlic extracts.

4.5. LITERATURE CITED

Amagase, H. 2006. Significance of garlic and its constituents in cancer and cardiovascular disease: Clarifying the real bioactive constituents of garlic. The Journal of Nutrition 2: 716-725. Accessed online: www.jn.nutrition.org Retrieved 09-01-2013.

Ankri, S. and Mirelman, D. 1999. Antimicrobial properties of allicin from garlic. Microbes and Infection 2: 125-129.

Stellenbosch University http://scholar.sun.ac.za

Bocchini, P., Andalo, C., Pozzi, R., Galletti, G.C. and Antonelli, A. 2001. Determination of diallyl thiosulfinate (allicin) in garlic (*Allium sativum* L.) by high-performance liquid chromatography with a post-column photochemical reactor. Analytica Chemica Acta 441: 37-43.

Borrego, S., Valdes, O., Vivar, I., Guiamet, P., Battistoni, P., Gomez de Saravia, S. and Borges, P. 2012. Essential oils of plants as biopesticides against microorganisms isolated from Cuban and Argentine documentary heritage. International Scholarly Research Network Microbiology 2012: 1-7.

Cavallito, C. and Bailey, J.H. 1944. Allicin, the antibacterial principle of *Allium sativum*. Isolation, physical properties and antibacterial action. Journal of the American Chemical Society 66: 1950-1952.

Chen, Z., Zhang, H., Liu, B., Yang, G., Aboul-Enein, H.Y., Wang, W., Ding, R., Du, H. and Li, H. 2007. Determination of herbicide residues in garlic by GC-MS. Chromatographia 66: 887-891.

Clemente, J.G., Williams, J.D., Cross, M. and Chambers, C.C. 2011. Analysis of garlic cultivars using head space solid phase microextraction/gas chromatography/mass spectroscopy. The Open Food Science Journal 6: 1-4.

Davis, S.R. 2005. An overview of the antifungal properties of allicin and its breakdown products – the possibility of a safe and effective antifungal prophylactic. Mycoses 48: 95-100.

Harris, J.C., Cottrell, S. and Lloyd, D. 2001. Antimicrobial properties of *Allium sativum* (garlic). Applied Microbiology and Biotechnology 57: 282-286.

Josling, P. 2001. Preventing the common cold with a garlic supplement: A double-blind, placebo controlled survey. Advances in Natural Therapy 18:4.

Khadri, S., Boutefnouchet, N. and Dekhil, M. 2010. Antibacterial activity evaluation of *Allium sativum* essential oil compared to different *Pseudomonas aeruginosa* strains in Eastern Algeria. Scientific Study and Research Chemistry and Chemical Engineering, Biotechnology, Food Industry 11: 421-428.

Verma, S.K., Jain, V. and Verma, D. 2008. Garlic – "The spice of life": Composition, cooking chemistry and preparations. Journal of Herbal Medicine and Toxicology 2: 21-28.

Stellenbosch University http://scholar.sun.ac.za

Zouari, S., Ketata, M., Boudhrioua, N. and Ammar, E. 2013. *Allium roseum* L. volatile compounds profile and antioxidant activity for chemotype discrimination – Case study for the wild plant of Sfax (Tunisia). Industrial Crops and Products 41: 172-178.

Stellenbosch University http://scholar.sun.ac.za

4.6. TABLES AND FIGURES

Table 1: Full profile analysis of garlic extracts

Compounds detected	RT (min)	Percentage	±SD
1-Propene	3.06	1.74	0.14
Methanethiol	3.36	0.96	0.06
Sulphur dioxide	4.00	0.31	0.03
2-Propen-1-thiol	4.41	0.75	0.17
Ethanol	4.71	2.17	0.14
Dimethyl disulphide	7.20	3.16	0.14
2-Propen-1-ol	7.75	0.81	0.15
Allyl sulphide	9.05	7.93	0.21
Di(1-propenyl)sulphide	9.95	0.16	0.02
cis,cis-Di-1-propenyl sulphide	10.01	0.06	0.03
Methyl propyl disulphide	11.60	0.03	0.00
3,4-Dimethylthiophene	12.38	0.04	0.01
Methyl allyl disulphide	13.44	11.23	0.19
Ethyl methanesulphinate	14.83	0.02	0.00
Tetradecane	16.97	0.02	-
Dimethyl trisulphide	17.45	11.36	0.22
Allyl propyl disulphide	18.82	0.06	0.00
1H-Imidazole, 2-ethyl-4,5-dihydro-4-methyl-	19.96	0.03	0.00
Diallyl disulphide	21.14	24.1	0.29
1,3-Butadiene, 1-(ethylthio)-3-methyl-	21.49	0.03	0.00
Silanol, trimethyl-, nitrate	21.62	0.02	0.00
4-Methyl-5-vinylthiazole	23.51	0.04	0.02
Methyl allyl trisulphide	25.63	13.85	0.94
Benzaldehyde, 4-methyl-	27.55	0.04	0.00
2-Methyl-1,3-butadiene	27.83	0.09	0.00
Octane, 3-methoxy-	29.99	0.04	0.02
3,4-Dihydro-3-vinyl-1,2-dithiin	30.49	4.66	0.22
Trimethylene trisulphide	30.85	0.34	0.04
2-Cyclohexylidene-1,3-dithiolane	30.96	0.27	0.03
Diallyl trisulphide	31.87	5.07	0.21
1-Chlorbenzo[c]tricyclo[4.1.0.0(2,7)]hept-3-en	33.05	0.06	0.01
Bicyclo[6.3.0]undeca-1,3,5,7-tetraene	33.17	0.07	0.02
Thiophene, 2-propyl-	35.12	0.02	0.00
Trimethylenebisethylsulphide	36.91	0.01	-
Thiophene, 2-ethyl-	37.16	0.02	0.00
Spiro[bicyclo[2.2.1]hept-5-ene-2,2'-[1,3]dithiolane]	37.35	0.03	0.00
2-(aminomethylbutanoic acid	38.43	0.20	0.03
Quinazoline, 4-ethoxy-	39.17	0.04	0.02
2-(2-thia-4-pentenyl)-1-thia-cyclohex-5-ene	39.98	0.02	0.00
3-(2-thia-4-pentenyl)-1-thia-cyclohex-5-ene	40.95	0.03	0.01
Hexadecanoic acid, ethyl ester	41.63	0.03	0.00
(S)-(-)-(4-Chlorophenyl)methyl Sulfoxide	42.84	0.03	0.00

RT – retention time; SD – standard deviation; percentages reflected is an average of three sample runs

-compound present in only one replicate, therefore SD could not be calculated

Stellenbosch University http://scholar.sun.ac.za

Table 2: Levels of sulphur and sulphur-derived compounds that may contribute to the antimicrobial action of garlic

Compound[1]	Percentage[2] (%)	Chemical formula
Allyl Methyl Sulphide	7.93	C_4H_8S
Allyl Methyl Disulphide	11.23	$C_4H_8S_2$
Allyl Methyl Trisulphide	13.85	$C_4H_8S_3$
Allyl Propyl Disulphide	0.06	$C_6H_{12}S_2$
Diallyl Disulphide (DADS)	24.10	$C_6H_{10}S_2$
Diallyl Trisulphide (DATS)	5.07	$C_6H_{10}S_3$
Dimethyl Disulphide	3.16	$C_2H_6S_2$
Dimethyl Trisulphide	11.36	$C_2H_6S_3$
Methanethiol	0.96	CH_4S
3.4-Dihydro-3-vinyl-1,2-dithiin	4.66	$C_6H_8S_2$

[1]Compound identification based on mass spectrum and retention index matching reference samples from the mass spectral libraries (NIST05 and Wiley 275.L). [2]Percentage reflected is the mean of triplicate analysis expressed as the relative percentage of compound in the garlic sample.

Stellenbosch University http://scholar.sun.ac.za

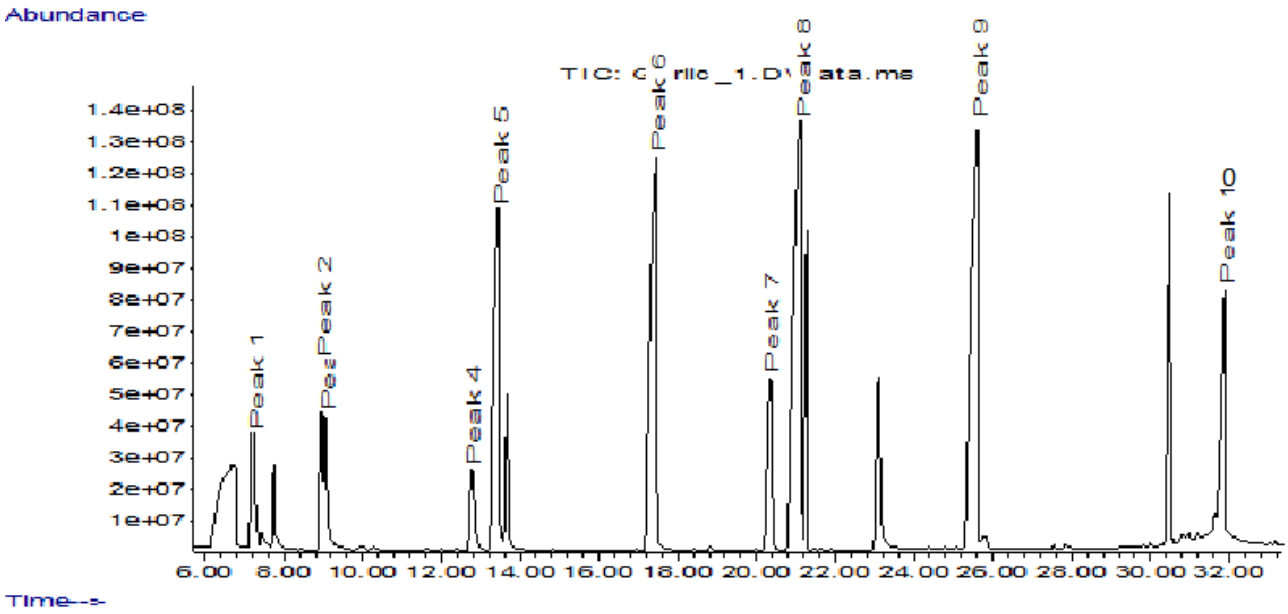


Figure 1: GC-MS (Full scan) chromatogram showing some of the peak areas corresponding to compounds present in the garlic extract in relation to retention time (RT). Peaks 1-10 correspond to the compounds: Dimethyl disulphide, Diallyl sulphide, Allyl methyl sulphide, Allyl methyl disulphide, Dimethyl trisulphide, 1-Oxa-4,6-diazacyclooctane-5-thione, Diallyl disulphide, Allyl methyl trisulphide, Diallyl trisulphide, respectively, with Allyl methyl sulphide corresponding to Peaks 3 and 4 on the chromatograph.