Antimicrobial effects of chitosan and essential oils on postharvest diseases of pomegranate fruit

By

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Declaration

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Summary

Pomegranate (*Punica granatum* L.) fruit diseases often caused by a range of fungi and bacteria, pose significant financial, nutritional and postharvest losses along the value chain. This study aimed at identifying pomegranate postharvest diseases in South Africa and improving the shelf life of whole and minimally processed pomegranates using chitosan and essential oils (EOs). To identify pomegranate postharvest pathogens and their origin in the value chain, samples of leaves and fruit at different development stages were collected from commercial orchards of cultivars 'Herskawitz' (mid harvest) and 'Wonderful' (late harvest) located in the Western Cape region of South Africa. Fungi were isolated from healthy and intact pomegranate flowers (open, closed, diheased), immature fruitlets buds, immature green fruit and ripe pomegranate fruit as well as leaves. Isolated fungal pathogens were identified using phylogenetic analysis of the internal transcribed spacer (ITS: ITS1 and ITS2) of the nuclear ribosomal DNA and the 5.8S ribosomal RNA genes.

Aspergillus niger Tiegh., *Cytospora* spp., *Clonostachys* spp., *Embellisia eureka* E.G. Simmons, *Pestalotiopsis* spp., *Nigrospora oryzae* and *Rhizopus stolonifer* Ehrenb., were associated with non-disinfected plant materials. The major pomegranate spoilage pathogens were isolated from surface sterilised samples and comprised of *Alternaria* spp., *Aureobasidium pullulans*, *Botrytis* spp., *Penicillium* spp., and *Pilidiella granati* Sacc. (syn. *Coniella granati* [Sacc.] Petr. & Syd.). The open flower stage had the highest incidence of spoilage pathogens and the same pathogens were isolated from fruit at postharvest. Pathogenicity tests were carried out on the major postharvest spoilage pathogens isolated namely *Botrytis* sp., *Penicillium* sp. and *P. granati*. This is the first report of *P. granati* on pomegranate fruit in South Africa. A restriction fragment length polymorphism (RFLP) tool was developed for the detection of pomegranate postharvest pathogens. This tool will be important in the monitoring of pomegranate pathogens in orchards and packhouses.

The antifungal activity of crab shell chitosan (0-10 g/L) and fludioxonil (0-1.0 g/L) was tested against *Botrytis* sp., *Penicillium* sp. and *P. granati* previously isolated from pomegranate. Pathogen sensitivity to crab shell chitosan based on the EC_{50} values (concentration causing a 50% reduction in mycelial growth) showed that *P. granati* (EC_{50}-0.47 g/L) was the most sensitive followed by *Botrytis* sp. (EC_{50}-1.19 g/L) and *Penicillium* sp. (EC_{50} 2.21 g/L). For fludioxonil *Penicillium* sp. (EC_{50} 0.02 mg/L) was the most sensitive followed by *P. granati* (EC_{50} 0.48 mg/L) and *Botrytis* sp. (EC_{50} 0.09 mg/L). Pre-treating wounded fruit (preventive treatment) with chitosan prior to pathogen inoculation gave better disease control (30-66% decay reduction) compared to introducing the chitosan after pathogen inoculation as a curative strategy (18-38%). Applying chitosan (0, 2.5, 7.5 and 15 g/L) as an edible coating on minimally processed pomegranate arils prior to cold storage significantly

($P<0.05$) lowered counts for mesophilic aerobic bacteria, yeast and moulds. In addition, the chitosan treatment also maintained the physico-chemical attributes of the arils (total soluble solids (TSS), titratable acidity (TA), moisture, colour, firmness, total phenolics, anthocyanins and ascorbic acid). The findings demonstrate that crab shell chitosan can be considered as a potential green fungicide for postharvest disease management of both whole and minimally processed pomegranate fruit.

A follow up study to enhance the antimicrobial properties of chitosan was conducted using chitosan as a polymeric carrier of volatile EOs (cinnamon, lemongrass and oregano). The EOs were assayed for antifungal activity against *Botrytis* sp., *Penicillium* sp., and *P. granati*. Lemon grass was the least effective EO as it failed to provide complete inhibition of any of the fungal pathogens while oregano EO was the most potent as it gave complete inhibition of *P. granati* by both vapour and direct contact methods. *In vivo* application of chitosan-oregano as an edible coating effectively controlled fungal growth by 59-100% but induced negative effects on the fruit skin. When applied as active film, the chitosan-oregano film still significantly ($P < 0.05$) reduced fruit decay by 34-100% without elucidating cosmetic damage to the fruit rind. The findings revealed the potential application of chitosan-EO based films in developing antimicrobial based active food packaging systems.

To further improve use of EOs in antimicrobial packaging, cinnamon and oregano EOs were encapsulated in β-cyclodextrin (β-CD) and a nanofibrous matrix based on chitosan and polyvinyl alcohol (PVA) to reduce the thermal instability of the EOs and achieve prolonged release. A GC-MS analysis revealed that the β-CD was significantly ($P < 0.05$) more efficient in encapsulation of cinnamon EO (4.86%), compared to oregano EO (1.75%). However, similar EO concentrations were obtained when the essential oils were incorporated into electrospun chitosan based nanofibres. The EO based β-CD microcapsules and nanofibres both had antimicrobial effect on *Botrytis* sp. and thus could be used in antimicrobial packaging.

This study identified the major postharvest spoilage pathogens of pomegranates in the Western Cape Region of South Africa and further determined that the orchard was the major source of these pathogens. Crab shell chitosan independently and in combination with EOs was shown to effectively control pomegranate postharvest pathogens on whole and minimally processed fruit. While exposing the pomegranate fruit to direct contact with EO emulsions reduced postharvest spoilage it also resulted in a negative cosmetic appearance of the fruit rind. However, exposing fruit to in-direct contact with encapsulated EOs controlled postharvest spoilage without affecting the fruit cosmetic appearance.

Opsomming

Vrugtesiektes in granate (*Punica granatum* L.) word dikwels deur ’n wye reeks swamme en bakterieë veroorsaak en lei tot groot finansiële en na-oes verliese in die waardeketting, asook verliese aan voedsaamheid. Die doel met hierdie studie was om na-oes siektes by granate in Suid-Afrika te identifiseer en om die raklewe van vars en minimaal geprosesseerde granate met die hulp van chitosan en essensiële olies (EOs) te verbeter. Om die identiteit van patogene op na-oes granate asook hulle bronne in die waardeketting vas te stel is monsters van blare en vrugte op verskillende stadiums van hulle ontwikkeling vanaf kommersiële vrugteboorde in die Weskaap, Suid-Afrika versamel. Die monsters is geneem van die kultivars ‘Herskawitz’ (mid-oes) en ‘Wonderful’ (laatoes). Swamme is vanaf gesonde en heel granaatblomme (oop en toe), onvolwasse blomme, onvolwasse groen vrugte en ryp granate, asook blare geneem. Die geïsoleerde swam patogene is deur die filogetiese ontleding van die interne transkribeerde spasieerder (ITS: ITS1 and ITS2) van die atoom ribosomale DNA en die 5.8S ribosomals RNA gene identifiseer.

Aspergillus niger Tiegh., *Cytospora* spp., *Clonostachys* spp., *Embellisia eureka* E.G. Simmons, *Pestalotiopsis* spp., *Nigrospora oryzae* en *Rhizopus stolonifer* Ehrenb. is met nie-besmette plantmatriaal assosiëer. Die belangrikste bederfpatogene is vanaf oppervlakte gesteriliseerde monsters geneem en hulle is *Alternaria* spp., *Aureobasidium pullulans, Botrytis* spp., *Penicillium* spp., en *Pilidiella granati* Sacc. (syn. *Coniella granati* [Sacc.] Petr. & Syd.). Die oopblom stadium het die hoogste voorkoms van bederfspatogene en dieselfde patogene is op no-oes vrugte gevind. Toetse is gedoen op die hoof na-oes bederfpatogene naamlik *Botrytis* sp., *Penicillium* sp. en *P. granati*. Dit is die eerste keer wat *P. granati* op granate in Suid Afrika rapporteer is. ’n Instument (RFLP) is ontwikkel vir die opsporing van granaat na-oes patogene. Hierdie instrument kan help met die monitering van granaatpatogene in vrugteboorde en pakhuise,

Die antiswam aktiwiteit van kropdop chitosan (0-10 g/L) and fludioxoniel (0-1.0 g/L) is op *Botrytis* sp., *Penicillium* sp. en *P. granati* wat vooraf van granate verkry is, getoets. Hoe sensitief die patogene is vir krapdop chitosan baseer op EC_{50} waardes (konsentrasies wat ’n 50% vermindering in swamvlok groei veroorsaak) toon dat *P. granati* (EC_{50} 0.47 g/L) die mees senstief is, gevolg deur *Botrytis* sp. (EC_{50} 1.19 g/L) en *Penicillium* sp. (EC_{50} 2.21 g/L). Wat betref fludioxoniel was *Penicillium* sp. (EC_{50} 0.02 mg/L) die sensitiefste gevolg deur *P. granati* (EC_{50} 0.48 mg/L) en *Botrytis* sp. (EC_{50} 0.09 mg/L). Die voorafbehandeling van vrugte (voorkomende behandeling) met chitosan lei tot beter siektebeheer (30-66% vermindering in bederf) invergeleke met die gebruik van chitosan na die inspuiting van patogene as ’n genesende strategie (18-38%). Die aanwending van chitosan (0, 2.5, 7.5 and 15 g/L) as n eetbare bedekking op minimaal geprosesseerde granate voor koelberging

verminder die telling ($P < 0.05$) van mesofiliese bakterieë, swamme en skimmels. Verder hou die chitosan behandeling ook die fisiko-chemiese eienskappe van die granate (totale oplosbare vastestowwe (TSS), titreerbaarheid, klamheid, kleur, fermheid en askorbiensuur) in stand. Die bevindinge toon dat krapdop chitosan beskou kan word as ’n potensiële swamdoder vir die na-oes siektebeheer van beide vars en minimal geproseseerde granate

’n Opvolgstudie is gedoen om die antimikrobiale eienskappe van chitosan te verbeter. Chitosan is gebruik as ’n polimeriese draer van vlugtige essensiële olies EOs (kaneel, lemongrass and oregano). Die EOs is getoets vir antiswam aktiwiteite teen *Botrytis* sp., *Penicillium* sp., en *P. granati*. Lemon grass was die minste effektief en het nie die swam patogene heeltemal inhibeer nie, terwyl die oregano EO die sterkste was en *P. granati* deur waterdamp en direkte kontak heeltemal inhibeer. Die *in vivo* aanwending van chitosan-oregano as ’n eetbare bedekking het swamgroei effektief beheer (59-100%) maar het ’n negatiew effek op die skil gehad. As dit as flies gebruik is, het die chitosan-oregano flies nog steeds ($P < 0.05$) die bederf van die granate met 34-100% verminder sonder om die skil te benadeel. Die bevindinge bewys dus dat die aanwending van chitosan-EO gebaseerde fliese potensiaal het in die verpakking van vrugte.

Om die gebruik van EOs in antimikrobiale verpakking verder te verbeter is kaneel en oregano EOs toegemaak in capsules met β-cyclodextrin (β-CD) en ’n nanovessel matrieks gebaseer op chitosan and poliviniel alkohol (PVA) om die termiese onstabiliteit van die EOs te verminder en om langer vrylating te bewerk. ’n GC-MS analise toon dat die β-CD heelwat ($P < 0.05$) meer doeltreffend is in die kapsule form van kaneel (4.86%), invergeleke oregano EO (1.75%). Dieselfde EO konsentrasies is egter verkry toe die EO inkorporeer is in chitosan-baseerde nanovesels. Die EO-baseerde β-CD mikrokapsules en die nanovesels het albei ’n antimikrobale effek op *Botrytis* sp. en kan dus in antimikrobale verpakking gebruik word.

Tydens hierdie studie is die hoof na-oes bederfpatogene van granate in die Weskaap Streek van Suid-Afrika identifiseer en is daar verder vasgestel dat die vrugteboord die hoofbron van hierdie patogene is. Daar is bewys dat krapdop chitosan op sy eie of in kombinasie met EOs granaat na-oes patogene op vars en minimal geprosesseerde granate kan beheer. Die blootstelling van granate aan EO emulsies verminder die bederf maar het ook ’n negatiewe effek op die voorkoms van die skil. Maar die blootstelling van die vrug aan indirekte kontak met EOs in kapsules beheer die bederf sonder om die voorkoms van die skil te affekteer

List of published/submitted articles

1. **Munhuweyi, K**., Lennox, C.L., Meitz-Hopkins, J.C., Caleb, O.J. & Opara, U.L. (2016). Major diseases of pomegranate (*Punica granatum* L.), their causes and management-A review. *Scientia Horticulturae*, **211**, 126–139**.**
2. **Munhuweyi, K.,** Lennox, C.L., Meitz-Hopkins, J.C., Caleb, O.J., Sigge, G.O. & Opara, U.L. Fungal genera associated with preharvest and postharvest rot of pomegranate fruit. Submitted to *Crop Protection*.
3. **Munhuweyi, K.,** Lennox, C.L., Meitz-Hopkins, J.C., Caleb, O.J., Sigge, Gunnar O. & Opara, U.L. (2016). *In vitro* effects of crab shell chitosan against mycelial growth of *Botrytis* sp., Penicillium sp. and Pilidiella granati. *Acta Horticulturae*, **1144**, 403–408.
4. **Munhuweyi, K.,** Lennox, C.L., Meitz-Hopkins, J.C., Caleb, O.J. & Opara, U.L (2017). Investigating the effects of crab shell chitosan on fungal mycelial growth and postharvest quality attributes of pomegranate fruit. *Scientia Horticulture* **220,** 78-79.
5. **Munhuweyi, K.,** Caleb, O.J., Lennox, C.L., Reenen, A.J. van & Opara, U.L. (2017). *In vitro* and *in vivo* antifungal activity of chitosan-essential oils against pomegranate fruit pathogens. *Postharvest Biology and Technology*, **129**, 9–22.
6. **Munhuweyi, K.,** van Reenen A. J., Caleb, O.J. & Opara, U.L Physical and antifungal properties of active cinnamon and oregano essential oil based β-cyclodextrin microcapsules and electrospun nanofibrous films. Prepared for submission to *LWT - Food Science and Technology*.

List of conference presentations

1. **Munhuweyi, K.,** Cheryl L. Lennox, J. C. Meitz-Hopkins, Oluwafemi J. Caleb, Gunnar O. Sigge & Opara, U.L. (2014). The role of latent preharvest infection on postharvest diseases of pomegranate in South Africa. 26th of November 2014 at the 10th International IOBC-WPRS Workshop on Pome Diseases.

2. **Munhuweyi, K.,** Cheryl L. Lennox, J. C. Meitz-Hopkins, Oluwafemi J. Caleb, Gunnar O. Sigge & Opara, U.L. (2015). PCR detection and RFLP differentiation of fungal species associated with postharvest decay of pomegranates. 49th Congress of The Southern African Society for Plant Pathology (SASPP), 18-21 January 2015.

3. **Munhuweyi, K.,** Cheryl L. Lennox, J. C. Meitz-Hopkins, Oluwafemi J. Caleb, Gunnar O. Sigge & Opara, U.L. (2015). In vitro effect of crab shell chitosan against mycelial growth of *Botrytis sp.*, *Penicillium* sp. and *Pilidiella granati* from pomegranate. III International Symposium on Postharvest Pathology-Using science to increase food availability. Bari (Italy), 7 - 11 June 2015 Villa Romanazzi Carducci.

4. **Munhuweyi, K,** Lennox, C.L., Meitz-Hopkins, J. C., Caleb O. J., Sigge G O, & Umezuruike Linus Opara (2015). A forensic approach to detecting and reducing pathological causes of fruit spoilage along the value chain – A case study of pomegranates. 5TH African Higher Education week and RUFORUM Biennial Conference. Century City Conference Centre, Cape Town, South Africa, 17-21 October 2016.

5. **Munhuweyi, K,** Lennox, C.L. & Opara, U. L. (2016). Antifungal activity by vapour and direct contact of essentials oils added to chitosan against orchard diseases of pomegranates. II South African Postharvest Innovation Symposium: Competitiveness through innovation. Stellenbosch (South Africa), 21 - 22 November 2016.

Note

This dissertation presents a compilation of manuscripts where every chapter is an individual entity and some duplication between chapters, therefore, has been unavoidable.

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Chapter 1

General introduction

The pomegranate (*Punica granatum* L.) is regarded as a 'super fruit' due to its rich content of polyphenols that is comparable to that of red wine and green tea (Gil *et al.*, 2000). This ancient and precious fruit crop of the Mediterranean region and Near East cultures is savoured as a delicacy and essential dietary component, celebrated in symbolism and greatly appreciated for its medicinal properties (Stover & Mercure, 2007). Currently, a diversity of pomegranate cultivars is grown across the globe, to meet the growing demand for the pomegranate's nutritional and medicinal benefits (Holland *et al.*, 2009). Clinical studies show that pomegranate fruits contain polyphenol antioxidants (primarily ellargic acid, and punicagalgin) that may lower risk of heart disease and retard cancer progression (Opara *et al.*, 2009; Bhowmik *et al.*, 2013)

South Africa is an important producer of pomegranate in the Southern Hemisphere, competing alongside Argentina, Australia, Chile, and Peru (Fawole & Opara, 2013). There is growing potential for pomegranate producers located in the Southern Hemisphere region as they enjoy the benefits of exporting their produce during the counter-season window in spring and early summer months of the Northern Hemisphere when there is limited market competition (Brodie, 2009). Although the South African pomegranate production volumes have increased tremendously over a short period, the gains are negated by the resurgence of devastating diseases like bacterial *blight and wilt* (Petersen *et al.*, 2010). The invasion of fruit by postharvest diseases is inevitable during postharvest storage. The severity of the problem is exacerbated when there is a high incidence of latent fungal infections and or large proportion of superficially damaged fruit at the time of harvest (Holland *et al.*, 2009).

Fungal disease infections were reported to cause pomegranate fruit losses of up to 50% in Greece (Tziros & Tzavella-Klonari, 2008). In Spain, pomegranate infetions of approximately 65-70% were caused by *Botrytis* latent infections (Palou *et al.*, 2013). The causal spoilage pathogens invade the fruit and cause considerable damage to the tissues, thus rendering the fruit unmarketable. Postharvest diseases of pomegranate depend on local preharvest (cultivar, climate, growing conditions, etc.) factors (Elyatem & Kader, 1984; Palou & del Rio, 2009). Therefore, the importance of determining the etiology and potential postharvest diseases for each growing area should be highly emphasised

To maintain an all year-round supply of pomegranate fruit, appropriate disease management strategies must be practised and this requires identifying the causal pathogens first. Both the wound pathogens (postharvest infections) and latent pathogens (field infections) must be identified. The conventional management of fruit diseases heavily relies on the application of chemical fungicides

which are often applied pre- and postharvest as chemical dips. The advent of consumer awareness regarding the benefits of eating chemical free foods has driven research initiatives into finding alternative greener preservative techniques. At present, there is limited information on the characterisation and identification of postharvest pathogens of pomegranate in South Africa. There are also limited registered synthetic fungicides currently permitted for postharvest treatment of pomegranates in South Africa. This negates on the country's potential to tap into the global pomegranate industry. Attention should therefore be devoted to the evaluation of alternative antifungal treatments in the context of an integrated disease management.

Therefore, the overall aims of this research were to determine the postharvest diseases of pomegranate fruit grown in South Africa using science based tools and to control their proliferation using crab shell chitosan and essential oils. The specific objectives were to:

a. Investigate the pre- and postharvest diseases of pomegranate cultivars 'Herskawitz' and 'Wonderful' using molecular techniques
b. Test the antimicrobial effects of chitosan and essentials oils (cinnamon, lemongrass and oregano) against fungal pathogens isolated from pomegranate fruit
c. Determine the antimicrobial and physical properties of encapsulated essentials oils using β-cyclodextrin and nanofibrous films for their controlled release

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Chapter 2

Major diseases of pomegranate (*Punica granatum* L.), their causes and management - A review

Abstract

Pomegranate (*Punica granatum L.*) diseases often caused by a range of fungi and bacteria, pose direct significant financial, nutritional and postharvest losses along the value chain. Common postharvest pathogens of pomegranate fruit include *Botrytis cinerea, Alternaria alternata, Penicillium implicatum, Coniella granati* and *Aspergillus niger*. A major bacterial disease of pomegranate is bacterial blight caused by *Xanthomonas axanopodis pv. punicae*. Field application of fungicides is necessary to control pathogens, which infect the fruit during developmental stages. Additionally, postharvest fungicides could be used to control localised infections in the fruit tissue or to prevent new infections during postharvest handling processes and storage. However, global trends towards reducing application of chemical fungicides are growing, with the demand for 'green' and cost effective strategies. This review discusses the details of various pomegranate postharvest diseases, symptoms and their causative microorganism. The importance and types of disease control and management strategies for pomegranate fruit and fruit tree are critically evaluated. This review will provide a critical guide to all the role players along the pomegranate value chain.

Keywords: Diseases, fruit quality, biopesticides, postharvest management, *Punica garanatum*

1. Introduction

Long term storage of pomegranate fruit is compromised by pathogens that cause postharvest rots and decay. Pomegranates are predisposed to attack from various pathogens at pre- and/or postharvest stage, which has a significant impact on fruit quality and storage life. These pathogens often cause damage to the tissues, thereby making the fruit unsaleable. A significant portion of pomegranate pre- and postharvest losses is attributed to diseases associated with various bacterial and fungal species (Tziros & Tzavella-Klonari, 2008; Mondal *et al*., 2012). Additionally, commercial marketability of pomegranate fruit is further limited by physiological disorders like rind cracking, chilling injury, husk scald, and excessive weight loss (Caleb *et al*., 2012).

Poor or inadequate postharvest handling practices also have detrimental influence on the overall quality and shelf life of pomegranate fruit. In India, inadequate packaging and storage facilities cause pomegranate losses of about 25 – 38% annually (Murthy *et al*., 2009; Sudharshan & Nadu, 2013). The unavailability of fruit due to postharvest losses affects both the producers and consumers. Postharvest fruit losses impede on the producer's potential to maintain and grow their

market share; i.e. to retain a competitive edge this often leads to financial losses and price hikes (Hess-pierce & Kader, 2003; Defilippi *et al*., 2006; Petersen *et al*., 2010). For the consumers' losses cause reduced availability and higher prices due to increased cost of production of fruit that never reaches the consumer, as well as higher unit costs of fruit marketing and transportation. The losses also limit the retention and extension of the nutritional quality of the produce (Kerch, 2015). Fungal infections in pomegranate can spread across nested fruit during cold storage (Northover & Zhou, 2002; Palou & del Rio, 2009). Therefore, evaluating the incidence and impact of diseases is a crucial factor towards developing effective and sustainable management strategies.

Fruit disease refers to a combination of several symptoms that result from the infection of plant material and manifest in various external and internal symptoms. These diseases are of three general types: fruit surface rots, internal fruit infections and stem end rots (Ammar & El-Naggar, 2014). Fruit infections occur in all producing areas and can cause serious yield losses. Postharvest losses of 10-25% from disease infections are common (Palou *et al*., 2013; Thomidis, 2014). Severe disease outbreaks can wipe out an entire orchard or fruit consignment in a short space of time (Petersen *et al*., 2010; Day & Wilkins, 2011).

There are different pathways through which pathogens infect pomegranates and these include flowers (via the petals, stigmas, styles, or stamens); on fruit through stigmata, pedicels, wounds (due to insect exit holes, bird pecks, thorn punctures, and natural cracking); or directly through the cuticle (Thomidis, 2014). The disease infections can spread by cross contamination between infected and healthy fruit packed together (Badawy & Rabea, 2009). Diseases typically associated with pomegranate postharvest storage losses, are often overlooked due to the slow development and late symptom expression by quiescent pathogens (Michailides *et al*., 2010). The pathogens invade the fruit during flowering stage. As fruit development progresses the incidence declines as some of the infected material probably dies off from the diseases, however some may remain latent until postharvest. In the absence of proper sanitation and hygiene, the diseases can rapidly spread, causing extensive fruit breakdown, and even spoiling an entire consignment (Day & Wilkins, 2011).

2. **Important pathogens of pomegranate fruit**

Pathogens such as *Botrytis cinerea* Pers.: Fr. (grey mould rot), *Alternaria alternata* (Fr.) Keissl. (heart rot), *Aspergillus niger* (Tiegh.) Speg., (heart rot), blue/green mould rot (*Penicillium* spp.: *P. implicatum* Biourge, and *P. expansum* Link), *Coniella granati* (Sacc.) Petr. & Syd., *Colletotrichum gloeosporioides* and *Pestalotiopsis versicolor* are all important pomegranate fungal diseases. While, bacterial blight caused by *Xanthomonas axanopodis pv punicae* (Hingorani and Singh, 1959), has been implicated to cause severe preharvest infections of pomegranate; alongside *B.*

cinerea (Mirzaei *et al*., 2008), *Penicillium* spp. (Palou *et al*., 2013), *Al. alternata* (Gat *et al*., 2012), *As. niger*, *Aureobasidium pullulans* (de Bary and Lowenthal) G. Arnaud (Thomidis, 2014), *C. granat*i (Mirabolfathy *et al*., 2012), and *Cytospora punicae* Sacc., which causes wood canker and branch dieback (Hand *et al*., 2014; Palavouzis *et al*., 2015b). Severe branch dieback and wood canker of pomegranate was reported in Tunisia, with symptoms including leaf yellowing, canker formation and wood lesion (Triki *et al*., 2015). Preharvest disease infections of pomegranate can remain quiescent only to become symptomatic during postharvest storage (Thomidis, 2014). These pathogens lead to fruit spoilage and facilitate fruit rind and arils decay during storage (Yehia, 2013). Table 1 summarises the major pre- and postharvest diseases of pomegranate reported across the world.

2.1. Common pomegranate disease symptoms

Infected pomegranate fruit trees display symptoms of wilting, stunted growth, reduced vigour, dieback of branches and general tree decline (Somasekhara, 1999). Leaf symptoms (such as discolouration, spots and lesions) can at times be followed by wilting of the entire plant leading to death (Jadhav & Sharma, 2011; Xu *et al*., 2011; Celiker *et al*., 2012). Pomegranate fruit disease symptoms can be visualised in the form of surface rots, shrivelling, discolouration and the display of undesirable cosmetic attributes such as spots or lesions. Moulding can appear on the affected areas depending and on the type of pathogen the colour of the moulds can develop as either fluffy grey or powdery blue/green mould (Pala *et al*., 2009; Ezra *et al*., 2010; Yehia, 2013). All fruit disorders eventually lead to rind break down, aril browning and decay (Zhang & McCarthy, 2012; Khokar *et al*., 2013).

Some pathogens only attack the fruit from the inside, while the external surface of the fruit remains asymptomatic (Zhang & McCarthy, 2012). Diseased fruit display poor shelf life and flavour quality attributes (Kader, 2008). Changes include losses in sugars, acids, characteristic aroma and development of off flavours due to fermentative metabolism and transfer of undesirable odours, such as sulphurous compounds mainly from fungi (Kader, 2008). Close examination of affected fruits can at times reveal distinct symptoms of crown rot, and internal damage of the calyx area (Pala *et al*., 2009). Therefore, identifying and quick characterisation of these disease symptoms is paramount to their effective control and management. Fig. 1 illustrates various disease symptoms of pomegranate fruit (Kader, 2008; Gat *et al*., 2012).

2.2. Sources of causal agents

Disease inoculum can originate from previously infected plant debris in soil, stem infections, weeds, old fruit left on the tree or on orchard floor (Coates & Johnson, 1997: Jadhav & Sharma,

2011). This was evident by recovery of pathogenic fungi from pomegranate orchard soil samples (Jamadar *et al*., 2011). Surface wounds created by mechanical injury or insect damage, even if microscopic in size; provide main ports of entry for pathogens (Coates & Johnson, 1997; Holland *et al*., 2009; Karaca *et al*., 2014). Abiotic factors such as sunburn, hail damage and fruit cracking due to uneven rain and /or irrigation as well as day/night temperature fluctuations can contribute to sites for pathogen infection (Pala *et al*., 2009)

Pathogenic fungi can spread as air borne inoculum as confirmed from spore trapping studies by Ma *et al*. (2003). The spore traps were used to detect and quantify airborne ascospores of different fruit disease pathogens (Ma *et al*., 2003; Meitz-Hopkins *et al*., 2014). Disease causing pathogens can spread through wind borne or rain splash, which facilitates the release of aerial inoculum in fruit orchards (Pala *et al*., 2009). Spores landing on senescent flower tissue such as stamens (anther pollen sacks and filaments), germinate and cause infection when there is free water on the plant surface from rain, dew, fog, or irrigation. Michailides *et al*. (2010) identified the petal fall stage in pomegranate fruit development as the most susceptible phase to infection. However, pomegranate infections can still occur at any time during fruit development until harvest and during postharvest.

Fungal diseases of pomegranate fruit can be classified based on how the infection was initiated or how the disease developed (Table 1). Initially, 'quiescent' or 'latent' fungi invade fruit during flower bloom and early fruit set, but remain inactive or dormant until favourable physiological changes in host tissue, which allows for further infection to occur (Coates & Johnson, 1997; Forster & Adaskaveg, 2000; Sanzani *et al*., 2012). The dramatic changes during fruit ripening stimulate quiescent infections inside the host tissue, which results in the production of toxins and enzymes that degrade the cuticle and cell wall by the pathogen (Zhang & Mccarthy, 2012). In response to this attack, the host release antimicrobial compounds such as phytoalexins and reactive oxygen species to strengthen its physical barriers, or initiates localised cell death to stop the disease (Zhang & Mccarthy, 2012). Pathogen identification can be achieved by microscopic examination of the morphological characteristics, mode of sporulation, conidia and the use of DNA based molecular techniques (Petersen *et al*., 2010; Lawrence *et al*., 2013).

3. **Major pomegranate diseases**

3.1. Heart rot

One of the major diseases impacting pomegranate production is 'black heart' or 'heart rot' caused by *Al. alternata* (Zhang & McCarthy, 2012). In addition to *A. alternata*, *Aspergillus niger* has also been implicated in causing internal fruit decay characteristic of heart rot (Yehia, 2013; Thomidis,

2014). Heart rot disease has been reported in Greece (Tziros *et al*., 2008), California (Michailides *et al*., 2008), and India (Pala *et al*., 2009). Pomegranate heart rot cannot be managed by postharvest treatments. Field sanitation and fungicide application during pre-harvest are recommended to controlling spread of the disease (Reddy *et al*., 1998).

3.1.1. Symptoms

Alternaria 'heart rot' is hard to detect externally and can contaminate packhouse during fruit processing into ready-to-eat products (Zhang & Mccarthy, 2012). The hard-leathery rind of infected fruit appears healthy and remains firm while the inner core of the affected fruit is partially or completely decayed starting from its calyx end (Yehia, 2013). Affected fruit can reveal a slightly abnormal skin colour in the peel, and are of lighter weight from the internal decay (Zhang & Mccarthy, 2012). Heart rot infected fruit generally float in water and can at times be distinguished for the heathy fruit.

3.1.2. Disease cycle and epidemiology

'Heart rot' originates from the orchard during flowering and early fruit development often after rainfall (Gat *et al*., 2012). Pomegranate infection by *Alternaria* spp. causing black heart is mostly during the anther dehiscence stage (open flower) as according to Michailides *et al*. (2008) whose study determined the optimal infection period using serial inoculations, starting from bloom until harvest period. This finding is further corroborated by Ezra *et al*. (2015a, b); the authors observed that *Al. alternata* spores sprout on stigmata of open flowers to invade the style. Fungal mycelium can also reach the lower loculus and become latent for about three to four months until fruit ripening (Ezra *et al*., 2015b). Initial disease development on the arils is marked by brown soft rot, and as the fungus grows this becomes black and dry. The pathogen spreads internally with fruit development eventually causing aril decay (Zhang & Mccarthy, 2012). The disease was reported in Central Greece (Tziros *et al*. 2008), with significant fruit losses of approximately 40-50% being reported in India (Pala *et al*., 2009).

3.2. Alternaria black spot

Pomegranate black spot caused by *Al. alternata* (Fr.) Keissl was reported in Israel (Ezra *et al*., 2010; Gat *et al*., 2012) and Spain (Berbegal *et al*., 2014). There is variation between the *Al. alternata* that cause black spot of pomegranate and that which causes 'heart rot' (Gat *et al*., 2012). This difference or variation could be attributed to the mode of infection and symptoms expressed in the fruit (Ezra *et al*., 2010). Damage caused by black spot fruit is confined to the rind surface, and the edible tissue is intact. Heart rot on the other hand is restricted to the internal area, while the rind

surface shows no sign of the disease (Gat *et al*., 2012). Black spot of pomegranate is caused by specific isolates of *Al. alternata*, which are different from isolates associated with heart rot infected fruit based on polymerase chain reaction (PCR specific primers, apPCR-based) characterization (Ezra *et al*., 2010). A molecular marker based on approximately 1200-bp sequence was even developed to accurately discriminate the highly virulent pomegranate black spot causing *Al. alternata* isolates from those isolates that did not cause this disorder (Gat *et al*., 2012).

3.2.1. Symptoms

Alternaria black spot (*Alternaria alternata*) is characterised by small reddish brown/black circular spots on pomegranate fruit surfaces and leaves (Ezra *et al*., 2010; Berbegal *et al*., 2014). The spots coverage of the fruit surface can range from 1% to more than 50% (Gat *et al*., 2012), and it consists of a necrotic lesion surrounded by green-yellow halo (Ezra *et al*., 2010). Diseased leaves become chlorotic and abscised. Fruit damage is initially limited to the surface of peel, while edible tissue is unaffected (Gat *et al*., 2012).

3.3. Aspergillus fruit rot

Aspergillus niger is a common decay pathogen reported on most fruits and is often described as a wound pathogen. In pomegranate, the pathogen was associated with general fruit rot and heart rot in California (Day & Wilkins, 2011), Greece and Cyprus (Kanetis *et al*., 2015), India (Jamadar *et al*., 2011; Khokhar & Tetarwal, 2012), Italy (D'Aquino *et al*., 2009), Spain (Palou & del Río, 2009), and Saudi Arabia (Yehia, 2013).

3.3.1. Symptoms

Infected fruit generally are slightly off-colour, pale red, with some yellow to brownish red discoloration. The disease is associated with rind fissures or cracks, which often enables secondary infections on fruit stored at higher temperature. Hence, the fruit rot can be followed by bacteria (*Erwinia sp*.) and yeast (*Saccharomyces* sp.) infection (Yehia, 2013).

3.3.2. Disease cycle and epidemiology

Aspergillus (As) rot infections originate from the orchard during flowering and early fruit development after rainfall. The fungus continues to develop within the fruit without any external symptom just like *Alternaria* fruit rot. Fruit colonisation by *As. niger* is often associated with insect infestations, or fruit injuries, or cracking probably caused by sunburn, bird pecking, and russeting (Yehia, 2013).

3.4. Grey mould rot

Grey mould caused by *B. cinerea* is by far the most economically important pomegranate storage disease, and causes up to 30% postharvest losses (Holland *et al*., 2009; Day & Wilkins, 2011). This necrotrophic fungus affects the pomegranate calyx creating a reservoir of infection that extends into the fruit causing in rot development (Thomidis, 2014). The impact of the decay is most severe following mechanical injury.

3.4.1. Symptoms

Grey fungal matter can be spotted on the crown area, with development of light brown or tan lesions, spreading gradually downwards from the neck of the fruit (Michailides *et al*., 2010). The lesions become darker with time, most probably due to bleeding of the red juice from the arils as the fungus advances. Cracks become noticeable, as the tissue and skin expands. This is followed by the appearance of grey mycelial growth at advanced stages of fruit decay. 'Nests' can occur between adjacent fruits even in cold storage because the pathogen can grow (though slowly), at temperatures as low as -0.5 °C (Badawy & Rabea, 2009).

3.4.2. Disease cycle and epidemiology

Botrytis cinerea infections occur both in the orchard as well as during storage from latent and wound infections. During tree flowering, airborne spores are spread to the open flowers or the crown of young fruits. The pathogen germinates on the flower parts and sporulates into a typical grey coating (Palou & del Río, 2009). Conidia and mycelia contaminating the fruit surface can infect through skin injuries and cracks. Propagules of *B. cinerea* (e.g. sclerotia, mycelium and conidia) can be found in the soil, plant litter and in the air (Lennox *et al*., 2003). Sclerotia often germinate during early spring to produce conidia that can be disseminated as either wet or dry, thereby, providing inoculum for blossom or fruit infection (Lennox *et al*., 2003).

'Grey' mould originating from latent infections established before harvest are more critical, as these infections are easily activated by free water during postharvest sanitation washes and use of elevated relative humidity conditions (Palou *et al*., 2007; Day & Wilkins, 2011). Postharvest fungicide treatment can penetrate the crown of the fruit preventing further spread of infection. However, orchard sanitation remains crucial as the most important preventive strategy. The incidence of grey mould can be controlled by fungicides such as fenhexamid and fludioxonil (Holland *et al*., 2009). A combination of postharvest chemical treatments with chlorine, hypochlorite, and fungicide dips are recommended prior to storage, to avoid development of fungicide resistance. Carbon dioxide enriched atmospheres are fungistatic and combined with optimum cold temperature storage can inhibit growth of *B. cinerea* (Day & Wilkins, 2011)

3.5. Blue/Green mould

Penicillium infections via wounds inflicted on rind surface at harvest and subsequent postharvest handling practices are responsible for blue/green mould development in affected tissues. Different species of *Penicillium* spp. have been implicated in pomegranate spoilage and decay (Table 1). These include *P. implicatum* in Spain (Labuda *et al*., 2004) and Pakistan (Hammerschmidt *et al*., 2012; Khokar *et al*., 2013), *P. glabrum* (an endophyte) in Uzbekistan (Hammerschmidt *et al*., 2012), *P. crustosum*, *P. expansum*, *P. minioluteum* Diercks and *P. solitum* in the Slovak Republic (Labuda *et al*., 2004). Unidentified *Penicillium* spp. have been cited in postharvest pomegranate losses from different regions including Greece (Thomidis, 2014), Spain (Palou & del Río, 2009; Palou *et al*., 2013), Turkey (Pala *et al*., 2009; Ozguven *et al*., 2012).

3.5.1. Symptoms

This fungus is characterised by powdery blue/green mould symptoms, developing from emergent circular lesions of soft, brown tissues. Initial symptoms include water-soaked lesions on the outer fruit surface (Khokhar *et al*., 2013). Later, a green to blue green powdery mould may appear on the surface of fruit lesions. Infected areas are tan or grey coloured when cut. At advanced stages, mycelium grows inside the fruit through connective tissues and infected arils may disintegrate into watery rot. The moulds can expand rapidly and are often covered with globose yellowish pycnidia that turn to dark greenish-brown over time (Palou *et al*., 2010).

3.5.2. Disease cycle and epidemiology

Penicillium is principally a wound pathogen and cannot invade the healthy intact tissues of calyx in comparison to *B. cinerea* and *C. granati* (Munoz *et al*., 2011). Invasion of pomegranate fruit can occur through wounds or bruises but colonisation usually occurs on the surface of senescent plant tissue. A common saprophyte found on plant debris and senescent plant tissue, the pathogen survives continually in the orchard primarily as conidia or conidiospores. Airborne spores initiate the infection through the peel by following mechanical injury. Contamination spreads when spores are dispersed from infected fruit during postharvest handling and processing. Inoculum sources include surfaces of infected fruit, open air, packing areas, storage rooms, transport containers and/or market places.

Spore growth and development heavily relies upon moisture development. Nutritionally, the pathogen is necrotrophic, and requiring nutrients only sufficient for it to germinate around the wound spot (Janisiewicz *et al*., 2000). A minor injury to the fruit skin during harvest and transportation promotes infection. Green mould develops most rapidly at ambient conditions but slower at temperatures below 10 °C and above 30 °C. Decay is almost totally controlled at freezing temperature

(0-1 °C) (Plaza *et al*., 2004). Blue mould can sometimes be found on fruit in the field, but symptoms generally appear during storage (Palou & Guardado, 2010).

3.6. Wilt

Wilt is an important disease of pomegranate, which adversely affects crop cultivation (Somasekhara, 1999; Benagi *et al*., 2011). *Ceratocystis fimbriata* Ellis and Halsted is regarded as the primary cause of pomegranate wilt. Also, fungal pathogens *Fusarium oxysporum* Schltdl. and *Verticillium dahliae* Kleb. 1913, have been identified as causal agents of pomegranate wilt (Jadhav & Sharma, 2011). Pomegranate wilt disease has been reported in China (Xu *et al*., 2011), Greece and India (Jadhav & Sharma, 2011; Sataraddi *et al*., 2011).

3.6.1. Symptoms

Affected branches show yellow leaves, followed by wilting and drooping of foliage of one or more branches of the plant. The entire tree eventually dies off within few weeks to display complete wilting (Somasekhara, 1999; Jadhav & Sharma, 2011). Wilt symptoms can at times spontaneously appear causing immediate senescing of the entire plants' foliage at once (Sharma *et al*., 2010). Infected plants reveal dried foliage and fruits attached to the branches for many months (Sharma *et al*., 2010). The xylem area becomes dark reddish-brown to purple deep-brown or black staining. Cross and vertical sections of infected plant parts generally reveal dark greyish-brown streaks in vascular and adjoining cortex tissues (Somasekhara, 1999). The fungus can spread between adjacent trees and at times randomly across different locations in the orchard. The disease can be managed efficiently by integrated management practices involving sanitation, cultural methods, chemical control and use of resistant cultivars (Sharma *et al*., 2010).

3.6.2. Disease cycle and epidemiology

Primary source of inoculum is soil and secondary are conidia and water. The disease is more prevalent in heavy soil with high moisture content (Sharma *et al*., 2010). *Ceratocystis fimbriata* and *F. oxysporum*, are soil borne and survive in soil through their thick-walled conidia, alleurioconidia and chlamydospores, respectively. The fungus is easily dispersed as mycelium, conidia, aleurioconidia or ascopores. These can spread via infected seedlings, irrigation and rain water, root contact, insects, implements, pruning and budding tools (Sharma *et al*., 2010). 'Wilt' disease attacks both wounded and intact healthy roots. After entering the host, the disease progresses through the xylem in water-conducting cells causing rapid wilting of the plant and broad dark discolouration of the vasicular system.

3.7. Bacterial blight rot

Bacterial blight is an important disease of pomegranate leading to severe crop losses (Petersen *et al*., 2010). This disease is caused by *Xanthomonas axonopodis* pv. *punicae.* Outbreaks of the disease in pomegranate orchards have been reported in South Africa (Petersen *et al*., 2010) India (Jadhav & Sharma, 2011; Yenjerappa *et al*., 2013) and China (Xu *et al*., 2011). Moderate to severe pomegranate crop damage and even entire field losses were observed in the affected areas (Petersen *et al*., 2010; Jadhav & Sharma, 2011).

3.7.1. Symptoms

The disease affects all parts of the plant and manifests as minute, dark brownish, regular to irregular water soaked lesions on leaves and fruit. Under severe cases soaked lesions can result in premature leaf defoliation (Jadhav & Sharma, 2011). Blight lesions on fruits are characterised by formation of small fissures even at the initial stages. Coalescing of these lesions can cause fruit splitting and the development of secondary infections such as blue mould rot and decay (Jadhav & Sharma, 2011). Infected stems and twigs may undergo girdling which leads to drying and breaking off branches. In progressed stages of infection canker formation can take place on the main stem and branches (Jadhav & Sharma, 2011).

3.7.2. Disease cycle and epidemiology

The bacterium is a polar flagelate gram negative, aerobic, and forms shiny non-spore yellowish mucoid colonies on nutrient agar medium (Jadhav & Sharma, 2011; Mondal *et al*., 2012). Infected cuttings and wind splashed rains provide the primary and secondary sources of inoculum, respectively. In advanced stages of infection canker formation takes place on main stem end (Jadhav & Sharma, 2011). Bacterial blight can survive at temperature range of 9-43 °C and lower humidity, but infection becomes severe under high humid conditions (>80%) and moderate temperatures (25-35 °C) during the rainy season (Jadhav & Sharma, 2011).

3.8. Coniella granati (Synonym: Pilidiella granati) fruit rot

Coniella granati was found to cause postharvest pomegranate fruit rots in California (Michailides *et al*., 2010), Greece (Thomidis, 2015), Spain (Palou *et al*., 2010), Iran (Mirabolfathy *et al*., 2012) and Turkey (Celiker *et al*., 2012). Affected fruit completely rot during storage resulting in yield loss of up to 50%. Celiker *et al*. (2012) identified *C. granati* as the causal pathogen of crown rot and shoot blights in pomegranate trees.

3.8.1. Symptoms

Coniella granati, also commonly referred to as pomegranate leaf botch, causes stem and crown cankers, resulting in decline and eventual death of young pomegranate shoots (CABI/EPPO, 2016). Early symptoms appear first as small circular spots, which further develop and increase in size into expanded brown lesions (Tziros & Tzavella-Klonari, 2008). Affected fruit show cream-coloured colonies of velvety appearance and abundant dark brown to black spherical pycnidia (Palou *et al*., 2010). The rind of rotted fruit is often covered with pycnidia. Infection turns the arils brown, soft and juicy. Fruit membranes and the rind also turn brown. Black fungal pycnidia with characteristic large, elliptical, colourless, one celled spores can develop on the surface of the arils, membranes, and surface of rind (fruit skin). *Coniella granati* rot differs from *Alternaria* fruit rot; *Aspergillus* fruit rot, and grey mould, in that it causes breaking of the peel, unevenly distributed black dots on fruit peel, and a black circle around the black dots (Pala *et al*., 2009).

3.8.2. Disease cycle and epidemiology

Coniella granati is isolated frequently from pomegranates, but the disease in only sporadically found in orchards. Pycnidia can be found in the bark of trunks, dead shoots, rotten fruit (mummies), stem cankers, and thorns, as well as on the surface of leaves (Thomidis, 2015). The optimum temperature for the pathogen's growth ranges from 25-30 °C, while the fungus grows slowly at 15 °C, but completely inhibited at 35 °C.

3.9. Anthracnose

Anthracnose disease of pomegranate is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. All stages of pomegranate fruit development are susceptible to the disease. Latent infection may occur in visibly healthy plants causing extensive fruit losses due to anthracnose disease related symptoms (Munoz *et al*., 2009). Chemical control of postharvest diseases of fruit and vegetables are limited therefore control anthracnose and other related latent diseases is crucial during preharvest to reduce potential carryover of the disease from the field into postharvest.

3.9.1. Symptoms

Infected leaves turn yellow and drop off. Diseased portions initially reveal minute circular spots with yellowish halos, later becoming irregular, and dark brown that eventually cover the fruit partly or wholly with sunken centres (Benagi *et al*., 2011; Sataraddi *et al*., 2011; Thomidis, 2014). The disease is severe when there is high humidity and temperatures of 20-27 °C. Symptoms of the disease have been reported in Greece (Thomidis, 2014) and India (Sataraddi *et al*., 2011).

3.9.2. Disease cycle and epidemiology

Anthracnose conidia can be derived from the lower leaves of dying infected petioles (Ali *et al*., 2010). Primary and secondary source of inoculum are from infected leaves and windborne conidia, respectively. The fungus can penetrate the fruit directly by producing an extracellular cutinolytic enzyme, enabling the pathogen to enter unwounded fruit. The symptoms only become apparent when the fruit ripens. The disease is favoured by wet, humid, warm conditions and can spread by infected seeds, rain splash and moist winds.

3.10. Other common diseases of pomegranate

Rhizopus stolonifer (Her. Ex Fr.) Lind is a common disease pathogen of pomegranate, which at times is the most destructive of the postharvest pathogens (Jamadar *et al*., 2011). The fungus invades pomegranate fruit through the wounds and rapidly rots the entire internal tissues, leaving intact only the enclosing cuticle. Infected fruit is covered by coarse grey mycelium with black macroscopic sporangia. In contrast to other pathogens, *R. stolonifer* soft rot is capable of spreading quickly to other fruit in container, and an entire carton of fruit may be rotted within a few days (Palou *et al*., 2013). These result in fruit cracking and rotting of inner pulp. Nevertheless, with careful sanitation and avoidance of wounds, the disease may be kept under control (Holland *et al*., 2009; Jamadar *et al*., 2011; Palou *et al*., 2013)

Neofusicoccum parvum (Pennycook & Samuels) caused pomegranate shoot blight in Northern Greece (Palavouzis *et al*., 2015a). It was observed that, the shoots of pomegranate cv. 'Wonderful' wilted and was blighted, taking a distinct dark colour, with black pycnidia formed on the surface of blighted shoots. The disease is also pathogenic to peaches and pistachio (Palavouzis *et al*., 2015a). Turkolmez *et al*. (2016) reported on crown and root rot of pomegranate in Turkey caused by *Phytophthora palmivora*. The stems of affected pomegranate plants showed signs of bark cracks above ground level, and below the soil line brown lesions on root collars are visible, with no capillary root formation. Additionally, a soft decaying main stem with a characteristic sour smell was noted. The disease was found to decline in all inoculated plants within a month after planting (Türkölmez *et al*., 2016).

4. Disease management

Eradication of fruit diseases is difficult to control, once they have colonised the orchard. Disease management options are therefore, mostly preventative and limited to controlling infection risk (Palou *et al*., 2007). Hence, early detection of fungal infection/ diseased fruit at harvest and during storage is crucial for developing disease prediction models, improving timing and efficiency

of control applications. Most postharvest disease begins in the field, therefore control measures must also begin in the field. The various disease management practices for pomegranate are discussed further in this sub-section.

4.1. Preharvest practices

4.1.1. Chemical protection

Chemical treatments have been by far the most effective approach to against plant diseases. Fungicides such as thiophanate methyl and tebuconazole are important in controlling shoot blight and fruit rot in pomegranate trees (Thomidis, 2015). Field evaluations of five fungicides viz., carbendazim (0.05%), mancozeb (0.25%), companion (0.25%), copper oxychloride (0.3%) and captan (0.3%) against fruit spot and rot diseases of pomegranate were conducted in India (Khosla *et al*., 2008). The results indicated that captan, companion and copper oxychloride reduced the diseases significantly irrespective of interval period. Copper oxychloride was however, observed to leave scars of dried spray materials on the fruit which reduced the market acceptability (Khosla *et al*., 2008). The drawback of chemical usage is that they can leave unwanted trace residues on fresh produce surface that are of concern to human health. Fungicide resistant strains may also develop, which could lead to reduced efficacy of synthetic chemicals (Palou *et al*., 2007).

4.1.2. Cultural control practices

Cultural practices are non-chemical approaches, which involve management of the environment to reduce disease inoculum pressure (Jadhav & Sharma, 2011). In pomegranate on-field management systems and improvement of soil drainage by means of ridging is important as these facilitate air movement and drainage (Wohlfarter *et al*., 2010). Stress conditions such as improper soil preparation, fertilisation and irrigation in fruit orchards can predispose plants to poor growth condtions that favour disease infections (Wohlfarter *et al*., 2010). Water stress should be avoided as this may cause fruit cracking. Field requirements require removal of old branches and fruit, training pickers, graders and others in identification of disease symptoms and protecting pruning wounds from getting infected. Protective pastes can be applied to protect cut ends of pruned branches (Wohlfarter *et al*., 2010; Jadhav & Sharma, 2011). Fruit can be bagged whilst on the tree as a protective barrier from insect and bird damage; however, this may not be economically feasible (Jadhav & Sharma, 2011). Healthy-appearing but infected fruit can be removed prior to harvesting by gently shaking the tree and allowing them to drop off to the ground. Preventing insect damage to avoid fruit wounds is important.

4.1.3. Selective cultivar breeding

Host plant resistance is a valuable complementary disease control measure where fungicides must be applied. This entails the selection of appropriate varieties that show disease and or insect resistance (Verma *et al*., 2014; Thomidis, 2015). Insect resistance studies are primarily focused on on transgenic plants. One of the most serious pests of pomegranates is the fruit borer (*Virachola isocrates*) and studies inducing insect resistance against this pathogen have been explored (Verma *et al*., 2014). Limited studies have been conducted to assess the level of susceptibility of pomegranate cultivars to different diseases. One study screened 19 pomegranate genotypes under *in vitro* conditions using detached leaf technique for anthracnose disease resistance caused by *C. gloeosporoides* (Jayalakshmi *et al*., 2013). None of the tested cultivars showed resistance to the disease with the most susceptible being Araktha, Ganesh and Keasr (Jayalakshmi *et al*., 2013). In another study Kumari & Ram (2015) screened 63 pomegranate cultivars under natural field conditions for resistance to dry fruit rot and leaf spot caused by *Coniella granati*. The majority of the cultivars tested were moderately resistant to the disease while a select few showed moderate susceptibility (Kumari & Ram, 2015). More studies still need to be done to identify the most disease resistant pomegranate cultivars to control fruit losses. The limited knowledge on the mechanisms and sources of disease resistance in pomegranate necessitates the need for further extensive molecular and genomic studies to come up with superior plants (Chauhan & Kanwar, 2012).

4.1.4. Genetic transfer technology

Genetic manipulations could assist increasing the disease resistance, insect/pest resistance, herbicide tolerance, salt tolerance, shelf life, cold tolerance, and fruit quality of pomegranates (Gomez-Lim & Litz 2004; Terakani *et al*., 2007). Most pathogens require wounds for infection; therefore preventing insect damage limits ports of entry for pathogens and the spreading of disease inoculum Gomez-Lim & Litz, (2004). Verma *et al*. (2014) successfully transformed pomegranate genetic plants by inducing insect resistance using *Agrobacterium*-mediated Cry1A (b) gene transfer into pomegranate cv. Kandhari Kabuli.

Previously agronomic traits and morphological characteristics were the sole means of selecting superior genotypes and desirable fruit traits such as weight, size, quality and taste (Chauhan & Kanwar, 2012). However, morphological markers can vary significantly depending on environmental and cultivation conditions. Application of molecular markers in conventional breeding offers a tool for shortening the breeding process, while gene transfer technology offers a more powerful strategy for the insertion of useful traits directly into elite genotypes (Chauhan & Kanwar, 2012). This would entail traditional breeding for disease resistance and fruit quality and genetic

enhancement of host species with specific genes (Chauhan & Kanwar, 2012). So far the *Agrobacterium*-mediated genetic transformation is deemed to be the most effective technique to transfer desirable genes into fruit crops including pomegranate (Chauhan & Kanwar, 2012). Detailed screening of pomegranate germplasm is required to ascertain resistant genotypes using biotechnological tools. (Gomez-Lim Miguel & Litz Richard, 2004)

4.1.5. Biocontrol and biopesticides

Biocontrol research is based on the application of microorganisms to manage soil borne, leaf and fruit diseases. This is highly recommended as a sustainable agricultural practice (Chavan *et al*. 2016). Successful studies have been conducted from which integrated nutrient management practices using biofertilisers to increase plant growth, nutrition, fruit yield, metabolism and *rhizosphere* enzyme activities of pomegranate in harsh field conditions of the Indian Thar Desert were obtained (Aseri *et al*., 2008). The effect of nitrogen fixing and arbuscular mycorrhizal fungi was found to be most effective growth treatment (Aseri *et al*., 2008).

Various species of microbes have been proven to retard fungal decay of pome fruits such as *Bacillus subtilis* subsp spizizenii (Avogreen) and pome fruit *Cryptococcus albidus* (Saito) C.E. Skinner (YieldPlus) in South Africa (Janisiewicz & Korsten, 2002). Chavan *et al*. (2016) assessed the biocontrol potential of 40 *actinomycete* strains isolated from natural sources against *Xanthomonas axonopodis* pv. punicae, responsible for oily spot disease of pomegranate. One of the ascomycete strains (A5) effectively prevented the growth of 36 *Xanthomonas* strains inoculated on pomegranate fruits, and showed its potential as a biocontrol agent against the disease (Chavan *et al*. 2016). Other biocontrol agents including *Pseudomonas fluorescens, Ps. Aeruginosa, Bacillus subtilis,* and *Lactobacillus* spp. have also been tested on the disease but were not as successful (Chavan *et al*. 2016).

Trichoderma formulations have been found to be suitable agents in protection of bacterial blight and anthracnose of pomegranate (Sataraddi *et al*., 2011). The mechanisms used by *Trichoderma* include production of antibiotics, mycoparasitism, competition for nutrients and space with pathogenic fungi as well as stimulation of host resistance (Mutawila *et al*., 2015; Sataraddi *et al*., 2011). The mode(s) of action for effective biocontrol agents is paramount both for enhancing their performance and the development of product formulations. This enhances expression of valuable traits and provides a criterion for evaluating potential antagonists.

Biopesticides present an emergent generation of crop protectants sourced from microbial communities naturally occurring on plant surfaces and their extracts (Yenjerappa *et al*., 2013).

Microbial pesticides are selected antagonistic micro-organisms, which are developed for their anti-pathogenic activity. Biopesticides disease control mechanisms involve various modes of actions: production of antibiotics, induction hot resistance, synthesis of phytoalexins and/or the accumulation of an extra cellular matrix, competition for nutrients and space, siderophores production and direct interaction with the pathogen and/or volatile production are involved (Yenjerappa *et al*., 2013). Moawad & Al-Barty, (2011) evaluated *in vitro* effect of medicinal and ornamental plant extracts toward pomegranate aphid, *Aphis punicae* (Passerini). 'Ruta chalepensis' showed the best results with the highest repellence (75%) and mortality (80%) effect at 0.015% concentration (Moawad & Al-Barty, 2011). Aphids are among the most serious and widespread pests in pomegranate orchards, responsible for transmittance of viral diseases and secret honey dew on which fungi grow (Moawad & Al-Barty, 2011). They have a high reproductive capacity and extensive usage of insecticides can eventually lead to disease resistance (Moawad & Al-Barty, 2011).

Biological control technologies are however limited as they often present variable efficacies (Wisniewski *et al*., 2001; James *et al*., 2010). Therefore, to reach comparable levels of efficacy as that presented by conventional chemicals, the use of microbial antagonists integrated with commercial chemicals (Mutawila *et al*., 2015), hot water (Yuan *et al*., 2013), chloride salts (Siddiqui *et al*., 2011), carbonate salts (Sivakumar *et al*., 2002) and/or with natural plant extracts (Sukorini *et al*., 2013) and other physical treatments is required (Artes *et al*., 2000). Postharvest hot water dipping of muskmelon at 53 ºC for 3 min effectively ($p < 0.01$) reduced the decay incidence, 18.9% lower than that in control fruit after 18 days of storage (Yuan *et al*., 2013).

4.2. Postharvest practices

4.2.1. Fruit sorting

Maximum care is required after harvesting to prevent mechanical punctures, bruising, and abrasions on fruit skin. Systematic sorting and grading of pomegranates for any discrepancies including discoloration and cracking should be done to prevent the packing of diseased fruit (Opara, *et al*., 2015). Packing house operators and shippers conduct regular separation of sound fruits from decayed ones to minimise secondary spread of diseases such as *B. cinerea* during storage and marketing of the fruit (Elmer & Michailides, 2000). Inspections of at least 2% percent of each consignment before shipment for diseased fruit symptoms can be conducted. Minimising physical damage of the calyx (crown) and fruit during postharvest handling of pomegranates is very important.

4.2.2. Chemical treatments

Water (chlorinated) rinsing of fresh produce is a common practice prior to storage to remove surface dirt and spoilage pathogens. However, pre-storage water treatment alone can actually facilitate microbial growth compared to the 'dry-treatment' (arils not subjected to any pre-wash treatments) (O'Grady *et al*., 2015). Water treated pomegranate arils showed 3.30 and 4.74 log CFU/g after 6 and 8 days of storage while <1 log CFU/g was detected for dry-treated arils after the 8 days of storage (O'Grady *et al*., 2015). Chlorine washes help lessen the risk of grey mould and *Penicillium* spp. decays. These washes are often accompanied by dips in postharvest fungicides like fludioxonil and fenhexamid (Forster & Adaskaveg, 2000). Fenhexamid and fludioxonil treatments can effectively reduce the natural incidence of grey mould caused by *B. cinerea* in pomegranates, especially in the crown region (Forster & Adaskaveg, 2000; D'Aquino *et al*., 2009; Holland *et al*., 2009). Most fungicides can be applied as dips, sprays, fumigants, treated wraps and box liners. Waxes and coatings can also be applied to enhance fruit quality/appearance. Fungicides can be incorporated with fruit waxes to control postharvest disease and extend pomegranate shelf life in ambient and cold storage conditions (Ghatge *et al*., 2005).

4.2.3. Storage management

Several physiological and biochemical processes occur, affecting changes in colour, taste, texture and ultimate decline in nutritional quality during postharvest (Arendse *et al*., 2015). Application of combined physical and chemical treatments often referred to as hurdle technology, results in better quality (Opara *et al.*, 2015). Integrated pre-treatments can involve using combinations of potassium sorbate, calcium propionate accompanied by Ultra Violet C or heat. Gamma irradiation has been demonstrated as a feasible approach especially in controlling food contamination. The additive properties of both treatments enhance quality attributes better than when treatments are used individually due to a broad spectrum effect. In addition to postharvest treatments, good crop management strategies should be emphasised if the full potential of the fruit is to be realised (Opara *et al*., 2015).

Storing pomegranates properly can help avoid further decay. Pomegranate fruit are susceptible to dehydration at low relative humidity after harvest and prone to peel break down, a physiological injury which can predispose fruit to decay. Hence, temperature and humidity management in the postharvest arena is crucial to avoid deterioration of produce and the initiation of infection (Arendse *et al*., 2015). The optimum postharvest storage temperature for most pomegranate fruits is 5 °C for up to two months and 7 °C for longer than two months at 90 to 95% relative humidity (Caleb *et al*., 2012). Pomegranates can be cold stored from 5 – 10 °C, 80 – 90% relative humidity for

two to three months (Pareek *et al*., 2015). Pomegranate is sensitive to low O_2 (<5 Pka) atmospheres, chilling injury, weight loss and decay (Arendse *et al*., 2015). Storage of pomegranate is best at 7 ºC and 90-95% relative humidity, because the fruit is least susceptible to chilling injury.

Postharvest fruit loss can vary depending on the level of latent microbial infection at the time of harvest. Temperature conditions must always be kept in check to provide an environment that does not favour pathogen growth and that does not also cause fruit stress at the same time. Pathogen mould growth is accelerated with temperature elevation >5°C, while at lower temperatures than this the pomegranate becomes susceptible to husk scald and chilling injury followed by decay (Arendse *et al*., 2015). Investigating the suitability of different pomegranate cultivars to postharvest handling and storage may be an important primary factor to address the control of avoidable postharvest fruit loss. Relative humidity affects postharvest transpiration rate of pomegranate. Additionally, modified atmosphere packaging (MAP) helps prolong shelf life of both whole and minimally processed fruit (Caleb *et al*., 2012).

4.2.4. Modified packaging atmosphere

Modified atmosphere packaging (MAP) in combination with optimum low storage temperature has been successfully used to prolong fruit and vegetable shelf life. This is achieved by hermetically sealing fresh respiring produce into polymeric film and allowing the atmosphere within the package to be modified passively by interplay of produce respiration rate and film permeability properties (Banda *et al*., 2015a). This process slows down physiological and biochemical processes and retards senescence. Suitable equilibrium atmosphere is achieved by proper matching of fresh produce respiration rate and film permeability characteristics (Caleb *et al*., 2012, Banda *et al*., 2015a). Active MAP achieved by flushing of desired gas mixtures into packages accelerates faster establishment of equilibrium atmospheres than passive MAP (Banda *et al*., 2015a). The recommended levels for desired equilibrium composition for packaging fresh produce are low oxygen (2-5 kPa O_2 and 10-20 kPa CO_2) (Banda *et al*., 2015a). Additionally, perforation-mediated MAP (micro-perforation) technique can be applied towards improving gas permeability of high barrier films. Perforation-mediated MAP offers the benefit of avoiding in package anaerobiosis, extending the shelf life and maintaining minimally processed fresh produce (Hussein *et al*., 2015).

Palou *et al*. (2007) reported synergistic effects between antifungal treatments and controlled atmosphere (CA) of 5 kPa O_2 and 15 kPa CO_2 in 'Wonderful' pomegranates artificially inoculated with *B. cinerea*. Porat *et al*. (2009) demonstrated the potential of MAP systems to prolong pomegranate quality using special bags (Xtend®) which have small holes (micro perforations). The bags provided a 5% CO_2 and 12-14% O_2 environment, reducing scald and weight loss significantly.

The extend bags effectively reduced pomegranate crown decay over 16 weeks of storage at 6 °C (Holland *et al*., 2009).

Postharvest stress predisposes fresh produce to loss in plant defence mechanisms and response. A decline in relative humidity from 96 to 76% can increase transpiration rate of minimally processed pomegranate arils by six-fold (Caleb *et al*., 2013). To achieve desirable shelf life, the humidity and temperature conditions must be monitored and maintained. Reducing storage temperature from 22 to 5 C can retard pomegranate respiration rate by 75% (Aindongo *et al*., 2014). Furthermore, active and intelligent packaging can help maintain a sterile environment by releasing antimicrobials into the packaging that can serve to control growth and spread of disease pathogens.

Active packaging refers to an interactive packaging system that can release or absorb gases and antimicrobial compounds to maintain product shelfife. On the other hand, intelligent packaging monitors the condition of packaged food and/or the environment that gives real time feedback about different factors during transportation and storage (Pereira de Abreu *et al*., 2012). Active packaging systems extend the shelf life of processed foods by means of adsorbing/scavenging (for example of oxygen, ethylene, liquid and moisture, and odour) or releasing systems for antimicrobials. Cherry tomatoes inoculated with *B. cinerea* showed significantly less decay symptoms compared to control fruit after 5 and 10 days of storage (Rodriguez-Lafuente *et al*., 2010). In a different study Montero-Prado *et al*. (2011) applied an active label with cinnamon to extend shelf life of peach fruit for 12 days at room temperature. The control fruit showed 86% decay, while the active label packaged fruit experienced 13% decay (Montero-Prado *et al*., 2011). Active packaging was also observed to preserve the quality attributes of wild strawberry better than the control (Almenar *et al*., 2007). Fruit wraps or box liners impregnated with fungicides such as biphenyl have been successfully used to control *Penicillium* spp. in citrus (Coates & Johnson, 1997). Appropriate time-temperature combinations may reduce chilling injury, and control decay, without impairing ripening, or physico-chemical quality (Escribano & Mitcham, 2014).

4.2.5. Heat treatment and storage

Different physical and chemical postharvest treatments can be applied to enhance the quality, storage and shelf life of pomegranate fruit (Opara *et al*., 2015) as shown in Table 2. Physical treatments include curing and heat treatment at 50- 60 ºC as either wet or dry to control postharvest diseases and fruit quality (Wisniewski *et al*., 2001; Mirdehghan *et al*., 2006). Heat treatments serve as part of an integrated, pesticide-free alternative for control of insects, decay and chilling injury (Mirdehghan *et al*., 2007; Li *et al*., 2013). Thermal hot water pre-treatment of pomegranates has been reported to reduce chilling injury, electrolyte and potassium leakage (Artes *et al*., 2000). Postharvest

heat treatments induce increases in free putrescene and spermidine during storage, which may be responsible in reduced fruit softening and reduction of chilling injury severity (Mirdehghan *et al*., 2007). Higher polyamine levels and maintenance of fatty acids ratio during storage may play a role in the maintenance of high membrane integrity and fluidity (Mirdehghan *et al*., 2007). The heat treatment could induce a mechanism of tolerance to cold temperature through the stimulation of polyamine biosynthesis.

Pomegranate precondition or curing at moderate temperature (30- 40 ºC) and high RH (90-95%) for short periods (1-4 days), can be applied before conventional refrigerated storage (Pareek *et al*., 2015). Heat treated pomegranate arils exhibit higher total antioxidant activity (total phenolics, ascorbic acid and anthocyanin content), sugars (glucose and fructose) as well as organic acids (malic, citric and oxalic acids) compared to controls (Table 2). A comparison study was conducted to assess the effect of intermittent warming (2 h at 20 ºC every 9 days), hot water treatment (3 min, at 52 ºC) and heat conditioning (24 h at 5 ºC) on quality pomegranate cv. Jbali (Ben Abda *et al*., 2010). The fruits were stored for two months at 2 and 6 ºC at 90-95% RH followed by a simulated marketing period of 7 days at 20 ºC. Chilling injury, electrolyte leakage and fruit were significantly reduced by the hot water treatment and less efficiently by the heat conditioning treatment respectively. Intermittent warming was found inefficient at controlling the same disorders. Treatments did not significantly affect acidity, total soluble solids and sensorial quality. The lightness intensity L^* values for hot water and heat conditioning treated fruits were however slightly affected (Ben Abda *et al*., 2010).

Various heat treatments have been applied to pomegranate. In one study pomegranate cv. Mollar de Elche was stored at 2 or 5 °C and 95% RH for 90 days. Heat curing at 33 °C was applied in cycles of 1 day at 33 °C every 6 days, followed by commercialisation periods of 6 days at 15 °C and 70% RH (Artes *et al*., 2000). Intermittent cooling at 2 °C considerably reduced chilling injury symptoms and maintained pomegranate fruit quality in terms of retention of anthocyanins and organic acids (Artes *et al*., 2000). The same pomegranate cv. Molar de Elche was also heat treated by hot water dipping at 45 °C for 4 min and then storing at 2 °C and 90% RH for 90 days (Mirdehghan *et al*., 2007). Chilling injury symptoms (skin browning, electrolyte leakage) could be correlated to tissue softening and loss of fatty acids was significantly reduced in heat-treated pomegranates.

Combination of heat treatments with other postharvest technologies (such as ethylene inhibitors, plant growth regulators, edible coatings, biological control agents and adequate packaging) could not only maintain, but also improve the sensory quality of the commodity (Escribano & Mitcham, 2014). Gutierrez-Martinez *et al*. (2012) combined hot recirculating water with two yeast

antagonists, *Candida guilliermondii* and *Pichia membranaefactiens*, to control the natural infection of *Botrytis cinerea* in tomato. The heat treatment enhanced the efficacy of the two antagonistic yeasts. Different defense related enzymes were induced in the fruit (Gutierrez-Martinez *et al*., 2012)

Liu *et al*. (2012) studied both the direct inhibition of *Monilia fruticola* and the elicitation of defense responses by a hot water treatment applied to reduce peach brown rot. The application triggered the accumulation of reactive oxygen species, collapsed mitochondrial membrane potential and decreased the ATP levels in the fungus while inducing the activity of defense-related enzymes in the fruit. Yuan *et al*. (2013) looked into the effect of hot water dipping on natural decay incidence and mechanisms involved in muskmelon fruit. The treatment melted epicuticular waxes, enhanced the activities of defense-related enzymes and resulted in accumulation of antifungal compounds, while reducing the activities of cell wall degrading enzymes and softening.

Although heat treatments help maintain the physico-chemical and nutritional quality of fresh produces; special attention must be given to potential adverse effects on sensory quality (Escribano & Mitcham, 2014). Heat treatments were reported to enhance the accumulation of phenolic compounds, flavonoid, lignin and hydroxyproline-rich glycoproteins (HRGPs) of muskmelons during postharvest storage (Yuang *et al.,* 2013). Exposing pomegranate juice on the other hand to thermal heat treatment at 70-90 °C for 90 min caused loss in antioxidant activity of approximately 31 and 10% of ascorbic and total phenolic content compared to control (Paul & Ghosh, 2012). Intermitent waming and cooling causes a physiological stress and abrupt changes in respiaion which may be detrimental to the crop therefore the coenzyme Q10 must be considered as it gives a better idea on how the crop responds to temperature variation. There is little effect when inoculation occurs after heating. Drawback of curing and intermittent warming is that the fruit becomes more susceptible to water loss (Wisniewski *et al*., 2001). It does not appear that high temperature treatments alone can control postharvest disease; hence an integrated approach of applying heat treatment with antagonists (biological antimicrobials) and chloride based products would be commercially more acceptable (Wisniewski *et al*., 2001). Further research is needed to successfully integrate heat treatments into the commercial postharvest chains (Escribano & Mitcham, 2014).

4.2.6. Organic acid and salt treatment of arils

Organic acids have been applied to slow down both enzymatic and non-enzymatic browning, texture loss and microbial growth on fresh produce, as well as reduce the respiration of the spoilage microorganisms. Treatment of pomegranate arils dipped in citric acid (10 g/L) for 60 s prior to storage at 5, 10,15 and 20 ± 2 ºC over 5 days maintained safety and respiration rate (Banda *et al*., 2015b). Low temperature combined with citric acid treatment effectively retarded respiration rate and was

lowest at 5 ºC. Pre-treatment of pomegranate arils with antibrowning agents (4-hexylresorcinol (0.001 M), potassium sorbate (0.005 M) and ascorbic acid (0.5 M) were observed to improve microbial quality of pomegranate stored at 5 ºC for 15 days (Caleb *et al*., 2015).

Salt solutions of sodium and potassium bicarbonate provide fungicidal activity against foliar pathogens. Common food additives (sodium bicarbonate, potassium sorbate) were compared to fludioxonil fungicide on pomegranates artificially inoculated *Botrytis cinerea* stored at 7.2 ºC in either air or controlled atmosphere (CA, 5 kPa O2 + 15 kPa CO2) conditions (Palou *et al*., 2007). The food additives did not improve efficacy alone without fludioxonil and so could be used as complementary treatments for reduced fungicide usage (Palou *et al*., 2007).

4.2.7. Essentia oils

Essential oils (EOs) represent a rich potential source of an alternative environmentally safe antimicrobial compounds. The bioactivity of EOs in the vapour phase makes them attaractive as possible fumigants for staored product protection. The volatile oils are obtained for plant materials such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Burt, 2004). Essential oils also find uses as food flavouring, perfumery fragrances and for pharmaceutical functional properties. Plant extracts are generally recognised as safe (GRAS) (FDA, 2014), and therefore considered as ‘reduced risk’ pesticides. Cinnamon oil incorporated into chitosan coatings effectively controlled decay to below 5% in sweet peppers (Xing *et al*., 2010). The antimicrobial activity of EOs can be attributed to their diret effect on the mycelial growth of the pathoegns and spore germination by affecting cellular metabolism of the pathoegens (Sivakumar & Bautista Banos, 2014). The hydrophobicity of EOs and their components allows the oil to partition in the lipid layers results in disruption of membrane structures eventually leaing to cell death (Burt, 2004). Deformation and dysfunction of the membrane could lead to the interference in the generation of ATP within the fungal cell, inhibiting enzymes and substrate usage of ATP (Sivakumar & Bautista-banos, 2014). Cinnamon, oregano, lemograss EOs are amont the most researched antimicrobials for fresh produces.

4.2.8. Packaging combined with edible coatings

The use of natural compounds provides safer measures of disease control without posing consumer health risks. Chitosan, a natural compound derived from the cell wall of many fungi is a good example of readily biodegradable antimicrobial (Bautista-Banos *et al*., 2006). Application of chitosan coating (0.5 and 1%) inhibited bacterial and fungal growth on the surface of pomegranate arils stored at 4 ºC for 12 days (Ghasemnezhad *et al*., 2013). It was even able to enhance and maintain physicochemical attributes by stimulating a number of biochemical processes including production of chitinase, accumulation of phytoalexins and increase lignific (Rabea *et al*., 2009; Varasteh *et al*.,

2012; Ghasemnezhad *et al*., 2013; Kou *et al*., 2014). Chitosan can extend shelf life due to its ability to form a semi-permeable coating minimising the rate of respiration and reducing water loss (Bautista-Banos *et al*., 2006).

Aloe vera is an important medicinal plant has also been used to control pomegranate fruit decay and maintain pomegranate arils during storage (Martinez-Romero *et al*., 2013; Nabigol & Asghari, 2013). The efficacy of *Aloe vera* gel was assessed on *Penicillium digitatum* and *Aspergillus niger*. To gain similar inhibition as for *P. digitatum,* the necessary *A. vera* gel concentration was 2-fold higher for *A. niger*. Complete inhibition for *P. digitatum* was achieved at 500 ml/L compared with 64% for *A. niger*. Differences exist in leaf properties and chemical composition of the gels of several Aloe spp. (Zapata *et al*., 2013). *Aloe vera gel* alone or in combination with organic acids (ascorbic and citric) leads to lower counts for mesophilic aerobics, yeasts and moulds (Martinez-Romero *et al*., 2013). The antifungal activity of *Aloe* gel has been correlated with the content of aloin, one of the major phenolic compounds of *Aloe* leaves (Zapata *et al*., 2013).

Many plant-derived extracts and essential oils demonstrate fungicidal or insecticidal properties (Burt, 2004; Zapata *et al*., 2013). It is expected that as more of the different plant compounds are investigated, further compounds with potential use as postharvest treatments to control decay will be identified. In particular, interesting results could be identified to provide synergistic activity. Development of alternative postharvest disease control options using natural plant extracts (Sanchez-Gonzalez *et al*., 2011; Severino *et al*., 2015) have become more important as successful commercial applications. Together with proper sanitation these disease control measures have the potential of being good replacement for current chemical/fungicide treatments.

4.2.9. Gamma irradiation

Microorganisms, especially acid tolerant bacteria and fungi such as yeasts and moulds, can easily spoil minimally processed fresh produces. Gamma irradiation can be applied to produce fruit juices with improved shelf life. Irradiation at doses greater than 2kGy completely inactivated microbial growth during storage however higher decline in anthocyanin content was observed above 2 kGy. Alighourchi *et al*., (2008) successfully diminished the total bacteria and fungi count and retarded microbial growth during storage of pomegranate juice. This was achieved exposing the juice to gamma irradiation at 2.0 kGy. Dosage above 2 kGy had an adverse effect on total anthocyanin content (Alighourchi *et al*., 2008).

5. Conclusions and future prospects

Pomegranates are susceptible to various decay causing pathogens. To prolong pomegranate storage periods and achieve an all year round fruit supply, the physiological disorders and diseases of pomegranate must be understood. This requires the assessment of wound pathogens (postharvest infections) and latent pathogens (field infections). This review provided a comprehensive overview on diseases associated with pomegranate and emphasized on the need for further research towards better understanding of the disease epidemiology. Strict sanitation practices during pre- and postharvest handling can significantly impede incidence of fruit decay. Pomegranate disease control traditionally relies on the use of chemical pesticides and fungicide which. Future research interests favour nonchemical postharvest treatments of pomegranates and other fresh produces. Effective nonchemical methods such as heat treatment, biocontrol, organic acids and edible coatings, have revealed additional benefits on fresh produce such as extending shelf life, improving chilling tolerance, fruit decay and in some cases nutritional quality. However, recent studies have shown that non-chemical strategies alone can present variable efficacies and require supplementation with chemical fungicides to reach comparable efficacies.

Thus, concerted research effort towards the development of alternative disease control and integrated management strategies, with novel approach such as biopesticides and innovative packaging technology systems is critical. Additionally, the use of natural antimicrobials will address consumer health concerns. Viruses, bacteria, fungi and other micro-fauna have been shown to possess some level of disease control ability. Hence, more research is needed to utilize the vast natural antimicrobial potentials. Genetic engineering of microbial antagonists to enhance plant protection could be useful in the development of desirable disease resistant cultivars. Successful adaptation of these alternatives may require a more integrated and holistic understanding of postharvest disease management.

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Table 1: Pomegranate diseases reported in Literature

Pathogen	disease	Symptoms	Area	Source
Al. alternata	*Alternaria* black spot	Small reddish brown circular spots on pomegranate fruit surfaces and leaves	United States and Israel	(Ezra *et al.*, 2010; Gat *et al.*, 2012)
Al. alternata (Al. alternata, Al. tenuissima and Al. arborescens)	*Alternaria* internal black rot)	Brown aril decay and black sporulation; rind decay in advanced stage, puffy appearance	Greece and United Sates	(Tziros *et al.*, 2008; Zhang & McCarthy, 2012; Kanetis *et al.*, 2015b)
Aspergillus niger	*Aspergillus* heart rot	Decay of fruit rind; brown aril decay and black sporulation inside fruit; pale skin colour	Saudi Arabia	(Yehia, 2013;
Emericella variecolor	Fruit rot	Softening of rind and underlying pulp. Browning of affected rind turning black at advanced stage of infection.	India	(Sharma *et al.*, 1982)
Alternaria spp., Fusarium spp. and Aspergillus niger	Fruit rot	Heart rot, fruit spot, fruit rot and styler end rot symptoms	Egypt	(Ammar & El-Naggar, 2014)
B. cinerea Botrytis spp.	Grey mould rot	Decay of fruit rind and arils; greyish mycelium	Iran, Greece and Spain	(Mirzaei *et al.*, 2008; Bardas *et al.*, 2009; Palou & del Río, 2009)
Ceratocystis spp.	Wilt	Yellowing and wilting of leaves leading death within several weeks	India	(Somasekhara, 1999)
Colletotrichum gloeosporioides	Anthracnose	Circular to irregular, dark brown spots with sunken centers resulting in fruit decay	India	(Sataraddi *et al.*, 2011)
Cytospora punicae		wood canker and branch die back	United states	(Hand *et al.*, 2014)
Cytospora punicae	Fruit rot	Apoplexy collar rot	Greece	(Palavouzis *et al.*, 2015)
P. glabrum	Endophyte	Not pathogenic	Uzbekistan	(Hammerschmidt *et al.*, 2012)
Penicillium implicatum	Blue mould fruit rot	Water-soaked areas on fruit surface, later, a green to blue green powdery mould develops. Infected areas are tan or grey when cut.	Pakistan	(Khokhar *et al.*, 2013)
Penicillium spp., *Botrytis cinerea, C. granati,*	Fruit rot	Fruit decay	Spain	(Palou *et al.*, 2013)
Penicillium spp., B. cinerea Pers.: Fr, Aspergillus niger van Tiegh	Fruit rot	Fruit decay	Spain	(Palou & del Río, 2009)
P. implicatum	Blue mould fruit rot		Spain	(Labuda *et al.*, 2004)
C. granati report	Crown rot	Decay of fruit rind and arils, pycnidia on trunk, shoots, thorns, leaves, and fruit	Greece, United States,	(Tziros & Tzavella-Klonari, 2008; Michailides *et*

				al., 2010; Thomidis & Exadaktylou, 2011)
C. granati report	Die back and fruit rot, dry rot	Death of aerial tree parts, fruit decay and formation of pycnidia around the fruit	India and Iran	(Sharma & Tegta, 2011; Mirabolfathy *et al.*, 2012)
Al. alternata, As. niger, Bacterial blight, Botrytis spp., C. cladosporioides, C. granati, Phytophtora spp., Aspergillus spp., Au. Pullulans and Penicillium spp.	Fruit rot	Fruit decay	Greece, Turkey	(Pala *et al.*, 2009; Özgüven *et al.*, 2012; Thomidis, 2014)
Xanthomonas axanopodis pv punicae	Bacterial blight:	Dark brownish, regular to irregular water soaked lesions on leaves and fruits resulting in premature defoliation under severe cases.	South Africa and India	(Petersen *et al.*, 2010; Jadhav & Sharma, 2011; Mondal *et al.*, 2012; Yenjerappa *et al.*, 2013)

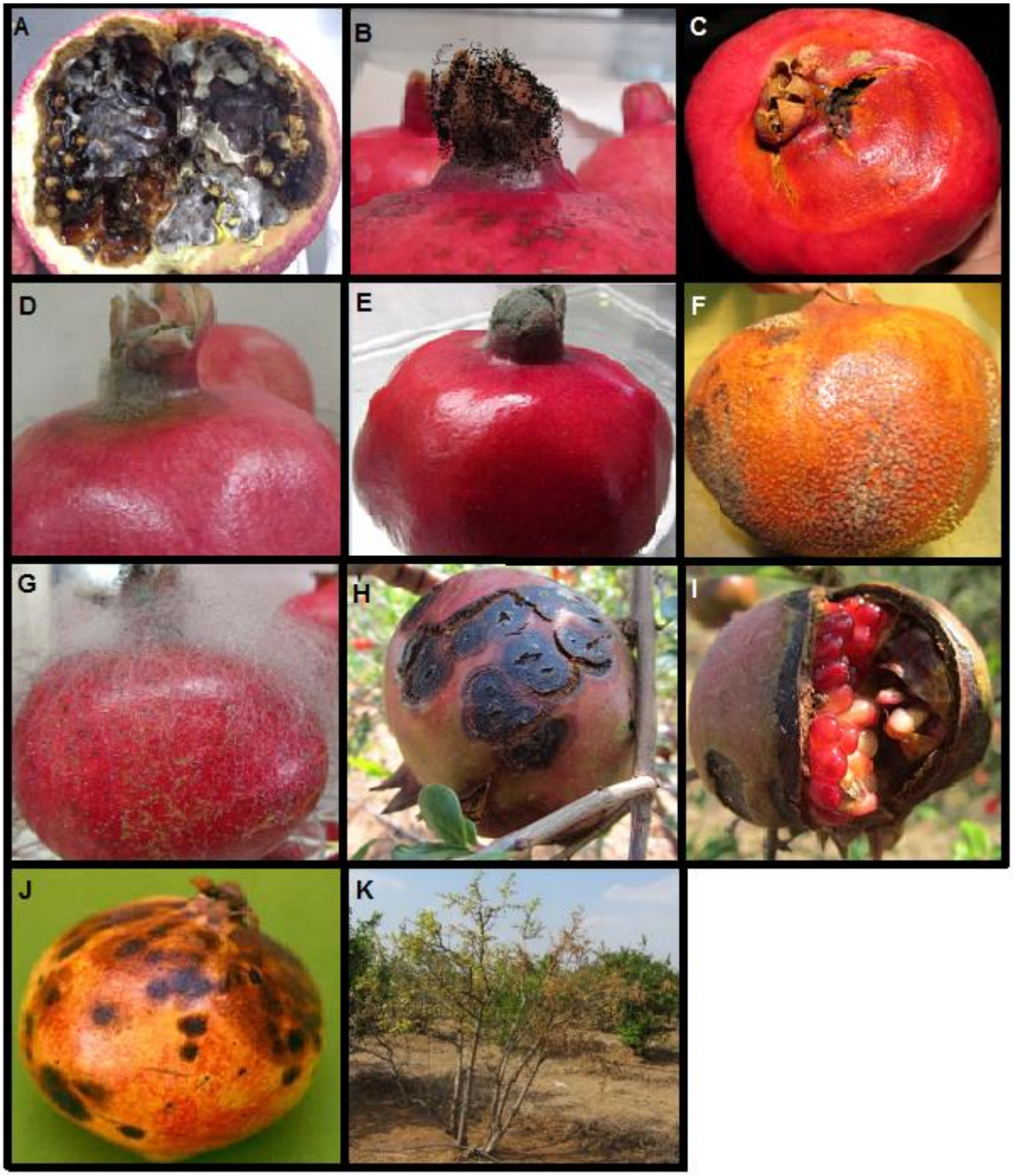

Figure 1. Pomegranate disease symptoms: Pictures **A** (*Alternaria alternata* 'heart rot'), **B** (*Aspergillus niger* fruit rot), **C** (*Aspergillus niger* 'heart rot'), **D** (*Botrytis cinerea* 'grey mould'), **E** (*Penicillium spp.* 'blue mould'), and **F** (*Coniella granati*), courtesy of Stellenbosch University Plant pathology Lab; **G** (*Rhizopus stolonifer* 'fruit rot'), **H** (*Xanthomonas axanopodis pv punicae* 'Bacterial blight rot'), **I** (*Xanthomonas axanopodis pv punicae* 'Bacterial blight rot'), **J** (*Colletotrichum gloeosporioides* 'Anthracnose') adopted from Anonymous, (2012), and **K** (*Ceratocystis* spp. ('Wilt'))

Table 2 Postharvest treatments of pomegranate

Scope of study	Cultivar	Fruit fraction	Source
Modelling effects of storage temperature	'Bhagwa',	Aric-sac	(Aindongo *et al.*, 2014)
Gamma irradiation preservation technique	'Gorche Shahvar Yazdi', 'Malase Yazdi', 'Vahshe Kane Tehran', 'Mesri Torshe Kazeron', 'Jangali Pust Germeze Rodbare Torsh' and 'Torshe Mamoli Lasjer'	juices	(Alighourchi *et al.*, 2008)
Active-modified atmosphere of pomegranate arils	'Wonderful'	arils	(Banda *et al.*, 2015a)
Effect of citric acid treatment	'Wonderful'	arils	(Banda *et al.*, 2015b)
Pre-treatments and modified atmosphere packaging	'Bhagwa'	arils	(Caleb *et al.*, 2015)
Chitosan aril coating	Iran pomegranate	arils	(Ghasemnezhad *et al.*, 2013)
Perforation packaging	'Acco'	arils	(Hussein *et al.*, 2015)
Aloe vera coating	'Mollar de Elche'	arils	(Martínez-Romero *et al.*, 2013; Nabigol & Asghari, 2013)
Postharvest water dipping pre-storage treatment	'Bhagwa'	Whole fruit and arils	(O'Grady *et al.*, 2015)
Antifungal and controlled atmosphere disease control	'Wonderful'	Whole fruit	(Palou *et al.*, 2007)
Anthocyanin changes in chitosan-coated	'Rabbab-e-Neyriz'	juice	(Varasteh *et al.*, 2012)
Non chemical disease control	United States		(Wisniewski *et al.*, 2001)
Aloe vera gels from different species	Spain		(Zapata *et al.*, 2013)

Chapter 3

Advancement in active packaging of fruits and vegetables - A review

Abstract

Food packaging systems have been extensively shown to offer measurable benefits to the quality and safety of fresh fruit and vegetables. The dynamics along the supply chain, coupled with produce physiological response, limitations of the packaging material, and continuous microbial growth pose a considerable challenge. This often limits the application of conventional packaging for fruit and vegetables. The use of active food packaging systems that is responsive to these dynamics and offers the possibilities of overcoming some of the pitfalls of conventional passive packaging systems. This chapter presents a comprehensive review of different categories of active packaging currently investigated for fruit and vegetable preservation. The concept and different approaches employed in developing active packaging systems are discussed in detail. The technical benefits of applying this technology on the physicochemical quality attributes, physiological response and microbial safety of fresh produces is discussed. Future prospects towards research and development into active and intelligent packaging technologies are also highlighted.

Key words: active packaging, fruits and vegetables, microbial safety

1. Introduction

Fruit and vegetable consumption is essential to maintain a healthy lifestyle and therefore packaging is very important to presereve their quality. The variation in nutritional composition of fresh produce has serious implications on public health, and this requires consumption of a variety of fruits and vegetables. For instance, the respective lycopene and total carotenoid content of water melon is 30-36 and 6-21 times higher than in cantaloupe, orange, pineapple and red grapes, while the lycopene content of carrot is 3-4 times higher than cucumber and 4-6 times higher than celery (Opara & Al-Ani, 2010). The drive towards achieving a healthy lifestyle has created an ever-growing demand for a variety of fresh and ready-to-eat foods. This increased demand has in turn greatly influenced the evolution of conventional packaging systems towards extending the shelf life of food products (Wen *et al.*, 2016).

Packaging materials provide a vehicle for marketing and transporting food products to meet the needs and expectations of consumers anywhere, while overcoming seasonal barriers and at an affordable cost (Pereira de Abreu, *et al.*, 2012). Packaging that performs some desired role in food

preservation aside from providing an inert barrier to external conditions automatically becomes "active" (Conte *et al.*, 2006). Active packaging is, therefore, defined as a system that plays an interactive role in creating a specific environment contributing to slowing down the deterioration process and extending the shelf-life of the produce (Gonzalez-Aguilar *et al.*, 2009). Targeted preservation techniques are employed with active packaging. Food oxidation can be controlled by incorporating scavengers or antioxidants into the packaging system (Aday & Caner, 2013). Moisture absorbers may be introduced in cases of quality deterioration from moisture or condensation build-up (Gonzalez-Aguilar *et al.* 2009; Rux *et al.*, 2016). In other cases, antimicrobial agents may be used to control microbial growth, and manage disease prevalence during postharvest storage (de Paiva *et al.*, 2017). Table 1 summarises targeted preservation techniques commonly applied in active packaging to address common spoilage problems associated with postharvest fruit and vegetable products.

The most important drivers for innovation in packaging technology include, changes in consumer lifestyle, cost saving and logistics, efficiency, sustainability trends, convenience and continual improvement (Rundh, 2009). The emergence of an ageing population and increase in single person households has also driven innovation both in food choice and packaging, ease of product handling and disposal, impacting food portions and pack sizes (Rundh, 2009). A study conducted to assess consumer values in fresh fruit quality, revealed that flavour, sweetness, firmness and colour were the main drivers of consumer satisfaction (Opara *et al.*, 2007). Interestingly, a large majority of the consumers (38%) were even willing to pay 25% or more on unit price for guaranteed good quality produce (Opara *et al.*, 2007). This feedback informs the food industry that consumers expect nothing else but the best of quality produce and value for their money. Packaging that can extend the flavour and nutritional shelf life of food is therefore of added benefit. Therefore, if innovating the current packaging systems can pro-long product quality then it is an important avenue worth exploring as consumer are prepared to pay more for guaranteed quality fruit and vegetables.

The advent of active packaging technologies has presented an effective and feasibly affordable technique for food processors to extend the shelf life of high value commodities such as whole and minimally processed fruit and vegetables (Pereira de Abreu *et al.*, 2012). Active packaging systems offer the most relevant innovative idea in delivering nutritional and safe quality horticultural commodities for consumer satisfaction. Thus, advancement into active packaging related researches will enable the development of new and even more improved active systems. This review therefore focused on the application of active packaging technologies of fruits and

vegetables. The prospects of developing active packaging systems from biodegradable and nano-active materials is also discussed providing a guide for all the role players in fresh produce value chain and researchers on future prospect of active packaging systems.

2. Concepts of active packaging systems

Active packaging systems make use of scrubbers or scavengers, absorbers, emitters and controlled release systems to extend the shelf life of food products. These systems can be derived from the concept that polymers can provide a wide range of permeability for different applications, justifying studies aimed towards enhancing packaging functionality. Important package performance properties include mechanical, thermal, optical and mass transport properties, since polymeric matrices are permeable to water vapour, oxygen (O_2), carbon dioxide (CO_2), nitrogen and low molecular compounds because critically low O_2 triggers anaerobic microoganisms. (Del Nobile *et al.*, 2009). Polymeric films are advantageous over other materials such as paper-based materials that are often perceived as too permeable while glass and metal packaging are not permeable to low molecular weight compounds and cannot provide a platform for further optimisation of barrier properties for various applications. The initial food quality, processing operations, distribution method, package design and disposal also play a significant role in selecting appropriate packaging materials (Del Nobile *et al.*, 2009).

Fruits and vegetables vary in their specificity in quality attributes, storage conditions, expected shelf life and packaging tools applications; therefore, proper selection of headspace conditions is critical. Modified atmosphere packaging (MAP) is a widely used strategy to protect products against deteriorative effects which may include discolouration, off-flavour and off-odour development, nutrient loss, texture changes, pathogenicity, enzymatic and microbial degradations. Under MAP, headspace environment may change during storage but there is no manipulation of the internal environment (Caleb *et al.*, 2012). In essence, MAP involves replacement of air in a pack with a fixed single gas or mixtures of gas proportions (Belay *et al.*, 2017). The prime goal of MAP applications is to generate sufficiently low oxygen (O_2) conditions to influence the metabolic process and reduce respiration rate, oxidative stress, tissue senescence and ethylene synthesis (Belay *et al.*, 2017).

Although MAP addresses the increasing demand for fresh, natural and chemical free products, there are drawbacks to this technology which can be addressed and/or complemented with active packaging. Some of the drawbacks of MAP packaging applications are related to costs of equipment and appropriate packaging materials and the limitation on retails for the increased

pack volume of bags (Rodriguez-Aguilera & Oliveira, 2009). Active packaging, on the other hand, offers extended shelf life preservation through the continued release and/or absorption using active agents to maintain the desired packaging environment. For example, Montero-Prado *et al.* (2011) applied a self-adhesive active label consisting of cinnamon essential oil (EO) attached to a macro perforated polyethylene (PET) tray to extend the shelf life of peach fruit at room temperature for 12 days. The current strategies involved in generating active packaging systems for fruits and vegetables are discussed below.

3. **Active packaging applications of fruit and vegetables**

3.1. Oxygen regulation

Oxygen generally supports fresh produce respiratory process and stimulates growth of aerobic bacteria; hence, its deprivation is important in the control and/or suppression of the growth of spoilage microorganisms. In addition, scavenging or reducing O_2 concentration helps to control spoilage caused by aerobic microoganisms and the respiration rate of fresh produce, thereby slowing down metabolic processes and extending shelf life. The scavengers are usually included in packaging system to remove O_2 by means of a chemical reaction (Aday & Caner, 2013). Different studies showed that the use of O_2 scavengers lead to faster reduction and to lower concentrations of residual O_2, as compared to nitrogen flushing (Charles *et al.*, 2003; Kartal *et al.*, 2012). The most common substances used are iron powders and ascorbic acid. Table 2 provides a brief overview of studies where O_2 scavengers were applied in the preservation of fruit and vegetables.

Charles *et al.* (2003) studied the effects of a commercial iron-based O_2 scavenger (ATCO® LH100) in sachet-form. The sachets were placed in low-density polyethylene pouches to carry tomatoes. The scavenging device effectively reduced the transient period to reach O_2 steady state by 50% compared to the control, and suppressed CO_2 peak owing to rapid oxygen depletion (Charles *et al.* 2003). The O_2 scavengers effectively controlled respiration rate thereby extended the shelf life of the tomatoes kept at 20 ºC for 13 d. In a follow up study, Charles *et al.* (2008) successfully employed oxygen scavengers based on reduced iron this time in combination with MAP to extend the shelf life of endives kept in polypropylene pouches at 20 ℃ for 8 days (Table 2). The combination of MAP and the oxygen scavengers maintained O_2 and CO_2 partial pressure, reduced transient period by 52%, and delayed greening and browning of the endives (Charles *et al.*, 2008).

Oxygen scavengers effectively extended the shelf life strawberries from 28 d to 35d (Kartal *et al.*, 2012). This was achieved by applying a commercial O_2 scavenger (ATCO-210) on strawberries kept at 4 ºC for 35 d in PVC/PE sealed in biaxially oriented and micro-perforated polypropylene (BOPP) (Kartal *et al.*, 2012). The active packaged strawberries maintained the best quality attributes; (total soluble solids) TSS, pH and firmness (Kartal *et al.*, 2012). In a similar study, Aday & Caner (2013) applied commercial O_2 scavengers: ATCO-100 and ATCO-240 on strawberries stored at 4 ºC for 28 d in polylactic acid (PLA) trays. The authors found that scavengers successfully lowered respiration rate, maintained electrical leakage, colour, firmness and mould reduction of the strawberries. In addition, the total decay in treated trays was comparably lower (15-20%) than that of the control (72%).

Oxygen scavengers can be applied alone or in combination with other active scavengers to provide a dual or multifold active packaging system, which is capable of absorbing or generating desirable compounds or removing other undesirables, aside from regulating oxygen. In this regard, Lu *et al.* (2015) evaluated the combination of a chlorine dioxide (ClO_2) generator, CO_2 emitter, O_2 scavenger on iceberg lettuce kept at 4, 10 or 22 ºC for 21 d using Nylon/EVOH/polyethylene oxygen barrier bags (Table 2). The active agents effectively inhibited microbial growth of *Escherichia coli* and total aerobic bacteria; however, the use of an allyliothiocyanate generator was ineffective at controlling microbial growth (Lu *et al.*, 2015). The ClO_2 generator was the most effective (no *E. coli* detected) while the O_2 scavenger presented *E. coli* log counts of approximately 0.44 and 1.85 log CFU/g at 10 and 22 ºC, respectively (Lu *et al.*, 2015). In contrast to the active treatments, the control fruit experienced higher loads of *E. coli* of 0.83, 3.51 and 5.99 log CFU/g at 4, 10 and 22 ºC, respectively, after 21 d (Lu *et al.*, 2015).

Antioxidants may also be incorporated into packaging systems to control oxidative degradation of fruit and vegetables during storage. Antioxidant-active food packaging can be produced by incorporating ascorbic acid, ferulic acid, quercetin, and green tea extract into an ethylene vinyl alcohol copolymer matrix (Lopez-de-Dicastillo *et al.*, 2012). Pathirana *et al.* (2013) monitored changes in lipid oxidation stability and antioxidant properties of avocado using 1 µL/L of 1-methylcycloprone (1-MCP) for 24 h at 20 ºC coupled with a low O_2 active atmosphere environment (3.5% O_2 and 96.5% N_2 at 5 ºC for 21 d) and then stored at 20 ºC for 14 d to simulate shelf storage conditions. The treatment lowered peroxide levels, and increased iodine levels, which effectively controlled lipid oxidation and ripening (Pathirana *et al.* 2013). A high antioxidant activity and no off flavours were detected throughout the storage trial for the treated fruit, however, control fruit developed off flavour at the over-ripe stage (Pathirana *et al.* 2013).

The successes of O_2 scavengers in active packaging of fruit and vegetable offer a host of benefits to all stakeholders across the production chain right up to the consumer. These include retention of physico-chemical quality attributes while assuring consumer satisfaction, thereby, reducing postharvest losses. However, opposed to the currently available chemical based O_2 scavengers, additional research into O_2 scavenging systems derived from natural and biological components could have added advantages towards consumer perception and environmental sustainability.

3.2. Carbon dioxide regulation

Desirable CO_2 concentrations must be maintained otherwise an elevated evolution of CO_2 may induce detrimental anaerobic fermentation reactions leading to product decay. Thus, the regulation of CO_2 concentration inside packaged fresh or minimally processed produce is important. For instance, Aday *et al.* (2011) compared two commercial CO_2 absorbers: EMCO A (46% sodium carbonate peroxyhydrate, 24% sodium carbonate (Na_2CO_3), 16% bentonite clay) and EMCO B (50% sodium carbonate peroxyhydrate, 20% Na_2CO_3, 14% sodium chloride, 16% bentonite clay) in combination with an O_2 scavenger (ATCO 210) on strawberry quality stored at 4 ºC for 28 d. The authors showed that applying the gas scavengers effectively maintained soluble solid content (TSS), electrical conductivity, firmness and sensory attributes of the strawberries kept in polylactic acid (PLA) trays compared to the non-treated control. Similarly, Veasna *et al.* (2012) assessed the effect of a CO_2 absorbent (Lipmen, Korea) and 1-MCP on eggplants stored at 1, 4 and 8 ºC for 15 d, and 3 d at room temperature. The eggplants were packed in antifogging oriented polypropylene (OPP) and PE films. Veasna *et al.* (2012) reported that the CO_2 scavenger and 1-MCP combination delayed chilling injury deterioration of the strawberries, across all treatments. Similarly, the reduction of CO_2 and ethylene production was reported to have positive effects on the quality of pears (Nugraha *et al.* 2015). Nugraha *et al.* (2015) evaluated the quality of pears over 7 months at 1 ºC in the presence of a CO_2 adsorbent (Ageless, Japan) and an ethylene (C_2H_4) absorber (Sensitech, United Technologies, USA). The study demonstrated that the removal of CO_2 and C_2H_4 could control internal browning of pears especially during the first 3 months of storage at 1 ºC compared to the control (Nugraha *et al.*, 2015).

Carbon dioxide regulation is a commonly used strategy to control microbial growth. Carbon dioxide possesses the ability to suppress bacterial growth and can remove offensive odours in fresh products due to its bacteriostatic and fungistatic properties (Aday *et al.*, 2011; Lu *et al.*, 2015). Carbon dioxide inhibits anaerobic bacterial growth. However, certain pathogens such as

Clostridium perfrigens and *C. botulinum* are not affected by the presence of CO_2 and their growth is encouraged by anaerobic conditions. The antimicrobial activity of CO_2 is associated with alteration of cell membrane function including nutrient uptake and absorption; direct inhibition of enzyme system reactions; changes of intracellular pH; and direct changes to physico-chemical properties of proteins (Amanatidou *et al.*, 1999; Kader & Ben-Yehoshua, 2000). Lu *et al.* (2015) successfully applied active nylon/EVOH/polyethylene 3-phase active-polymer films generating either CO_2, chlorine dioxide (ClO_2) or allyl isothiocyanate (AIT), and scavenging O_2 to control growth of *E. coli* O157:H7 and total aerobic on iceberg lettuce. Table 4 presents a summary of various literatures on successful application of CO_2 scavenger.

3.3. Ethylene regulation

Ethylene (C_2H_4) is responsible for a wide variety of undesirable effects if left unmonitored in storage or packaging systems. It accelerates the respiration rate of fruit and vegetables, as well as softening and ripening processes leading to quick senescence, and it is responsible for a number of other specific post-harvest disorders (Meyer & Terry, 2010). Removal of ethylene from packages of fruit and vegetables is, therefore, of the utmost importance. Most substances designed to remove ethylene from packages are applied as sachets or directly integrated into the packaging material. The most commonly used are based on potassium permanganate ($KMnO_4$), activated carbon and activated earth (Meyer & Terry, 2010). For example, Meyer & Terry (2010) investigated the effect of 1-MCP and a newly developed palladium (Pd)-promoted ethylene scavenger (e + ®Ethylene Remover) on changes in firmness, colour, fatty acids and sugar content of early and late season avocadoes (*Persea americana* Mill.), cv. 'Hass', during storage at 5 °C and subsequent ripening at 20 °C. The results showed that the ®Ethylene Remover definably delayed ripening of avocadoes retaining fruit firmness of 47.5 N compared to control (5.5 N). Although, the 1-MCP treatments was more effective to the ethylene scavenger, in delaying fruit ripening, however it impaired subsequent ripening of the avocados by lowering the ethylene by almost four folds (Meyer & Terry, 2010).

The effect of ethylene scavengers on quality of different fresh produces has been demonstrated with successful results on various other fresh produces including kimchi (fermented cabbage) (Shin *et al.*, 2002), tomatoes (Bailen *et al.*, 2006; Martinez-romero *et al.*, 2009; Garcia-Garcia *et al.*, 2011), strawberries (Aday & Caner, 2011), plums and peaches (Rudra *et al.*, 2013) (Table 4). Ethylene gas regulation is important in order to control produce respiration rate. The maintenance of ethylene gas production using a CO_2 absorbent consisting of $Ca(OH)_2$ sachets and

zeolite powder in combination with sodium carbonate reduced pressure build up and volume expansion of kimchi packages stored at 15 ºC for 14 d in 10 µm thick high density polyethylene (HDPE), 20 µm thick polyolefin (PD941) packages (Shin *et al.*, 2002). The CO_2 partial pressure was maintained at 0.8 bar after 8 d, while the control showed double the CO_2 build-up at 1.98 bar (Shin *et al.*, 2002).

An effective way to extend shelf life of tomatoes can be achieved by means of prolonging the ripening phase of the tomatoes. Granular activated carbon sachets: GAC and Palladium acetate in combination with MAP: 4 and 10 kPa O_2 add CO_2 respectively, effectively lowered ethylene accumulation, fruit softening, weight loss and decay, retained better odour and flavour quality of tomatoes stored at 8 ºC for 28 d (Bailen *et al.*, 2006). Likewise, Terry *et al.* (2007) inhibited fruit ripening using a 2.5% palladium-zeolite based ethylene scavenger on avocadoes stored at 12 ºC, 10 d, bananas (16 ºC, 9 d) and strawberries (5 ºC, 13 d) (Table 4). The scavenger supressed ethylene levels to < 0.1 µL/L for the three fruits, while control samples expressed 78.41, 76.97 and 67.99 µL/L for avocado, banana and strawberry, respectively (Terry *et al.*, 2007).

Delaying fruit ripening through ethylene suppression promotes shelf life extension by reducing decay susceptibility of the packaged product for extended periods. For instance, fruit decay after 28 days was 39% compared to 100% for the control tomatoes kept at 8 ºC for 35 d (Bailen *et al.*, 2006). In a similar study, Martínez-romero *et al.* (2009) stored tomatoes at 8 ºC for 28 d in the presence of 70 g activated carbon-1% palladium alone or with a 3 h cyclic heater (175 ºC) in OPP bags. The treatments lowered ethylene accumulation, fruit softening, weight loss and decay (Martínez-romero *et al.*, 2009). In addition, the active packaged tomatoes retained better odour and flavour quality with ethylene levels of 0.16-0.9 $\times 10^{-4}$ kPa, which was way less that of the controls at 3×10^{-4} kPa (Martínez-romero *et al.*, 2009). Another related study on ethylene regulation of tomatoes was conducted by Garcia-Garcia *et al.* (2011). Controlled respiration rate of transmission for cherry tomatoes was achieved at 20 ºC for 30 d using a polylactic acid (PLA; 3% w/v) coating on cardboard wrapped with a low-density polyethylene (LDPE) (Garcia-Garcia *et al.*, 2011). The application of ethylene scavengers has been extensively demonstrated in literature as an effective tool for controlling ethylene production and/or accumulation (Table 4)

3.4. Moisture regulation

The benefits of placing moisture absorbers are listed in Table 5. Moisture control is very important in regulating shelf life of fruit and vegetables. To demonstrate this, Garcia-Garcia *et al*. (2013) extended tomato shelf life by controlling humidity and respiration rate transmission using

polylactic acid (PLA; 3% w/v) coating on cardboard tray (Table 5). In a separate study, a multifunctional agar-based biofilm with Na_2CO_3 and/or sodium glycinate as CO_2 absorbent was used to successfully suppress losses in physico-chemical quality of mushrooms stored at 10 ºC for 5 d (Wang *et al.*, 2015). Rux *et al.* (2015) also worked on extending the shelf life of mushrooms through moisture regulation. The authors showed that a humidity regulating tray consisting of 18% NaCl embedded in polypropylene/EVOH/polyethylene packages maintained better quality of mushroom. However, the trays could not prevent occurrence of condensation after day 6 of storage at 4, 12 and 20 ºC, due to higher transpiration rate of the mushroom. In a different study, humidity regulating trays (polyethylene (outside)/foamed hygroscopic ionomer (active layer) and hygroscopic ionomer (sealing layer)) containing 12% NaCl were found to be effective in regulating in-package relative humidity (RH) and maintain overall quality of strawberries and tomatoes stored at 13 ºC for 7 d (Rux *et al.*, 2016).

Furthermore, An (2016) applied a combination of active agents; CO_2 absorbent ($Ca(OH)_2$) absorbent polymer, moisture absorbent (sodium polyacrylate, crosslinked) and monitored changes in mushroom quality along the entire supply chain. The authors showed that active agents improved mushroom preservation by effectively reducing yeast/mould growth and decay through moitsture control (An, 2016). It is noteworthy to state that beside the benefits of moisture regulation, the application of the various approaches can induce a higher weight loss compared to the controls. The moisture absorbers created a less humid environment, thereby, rendering the produce inside active packaging to be more prone to transpiration weight losses. For example, Wang *et al.* (2015) observed a 9-12.6% weight loss for active packaged mushrooms compared to 2.1% for control mushrooms. Similarly, Rux *et al.* (2016) recorded a 0.3-0.6% weight loss for control strawberries and tomatoes stored at 13 ºC for 7 d which was lower than 1% weight loss for tomatoes and 2-3% for strawberries kept in regulated humidity environment under the same temperature for the same duration.

3.5. Microbial control

Control of postharvest pathogens falls amongst the greatest challenges within the fruit and vegetable industry (de Paiva *et al.*, 2017). In fact, the most investigated active packaging systems are centred into fabrication of packaging with antimicrobial properties as illustrated in Table 6. Colonisation of fruits and vegetables by naturally occurring bacteria and fungi may induce infections, causing extensive postharvest spoilage and decay (Holland *et al.*, 2009; Caleb *et al.*, 2012). Some microbes can potentially cause food borne illnesses or lead to food toxicity

(Muhammad *et al.*, 2004). Common spoilage and pathogenic microorganisms of fresh produces include *E. coli* O157, *Listeria monocytogenes*, *Bacillus ceres*, *Staphylococcus aureus*, *Salmonella*, *Campylobacter*, *Clostridium perfringens*, *Aspergillus niger*, *Botrytis cinerea*, *Fusarium*, *Penicillium*, and *Saccharomyces cerevisiae* (Table 6). The growth of these pathogens is affected by a variety of intrinsic factors, such, as pH, presence of O_2 and extrinsic factors associated with storage conditions, including temperature, relative humidity and the duration (Caleb *et al.* 2012).

An important biodegradable antimicrobial compound that has been greatly researched for preservative role of fresh products is chitosan. The fungicidal effect of chitosan alone or in combination with other preservative compounds has been illustrated on various commodities as summarised in Table 6. Strawberry fruit previously inoculated with *B. cinerea* and *Rhizopus Stolonifer* were subjected to crab shell chitosan (10 and 15 mg/mL) coating and held at 13℃, 14 d (El-Ghaouth *et al.*, 1992). A 50% reduction in decay was observed for the coated fruit compared to control and the authors speculated that defence enzymes were activated on freshly cut fruit and not whole fruit (El-Ghaouth *et al.*, 1992). In another study, Chitosan 1.5% effectively controlled fruit decay, and maintained fruit firmness and did not influence TSS and weight loss of papaya inoculated with *C. gloeosporioides* and stored at ambient temperature (25-28℃) for 5 d.

Bautista-Banos *et al.* (2004) compared the antimicrobial effects of chitosan with *Capsicum frutescens*, hinokitiol pepper cayenne, and yam bean extracts against *P. expansum* inoculated apples and found that the chitosan application gave the best decay control results. The molecular weight (Mw) of chitosan is important. Badawy & Rabea (2009) observed that chitosan was the most effective at 5.7 x 10^4 Mw and that increasing the Mw any further had no added beneficial effects. Chitosan can also be integrated into food packaging as a polymeric carrier of volatile antimicrobials such as essential oils (EOs); carvacrol, mandarin, bergamot and lemon (Severino *et al.*, 2015).

Apart from chitosan several other compounds have been shown to possess antimicrobial properties applicable for fruit and vegetable active packaging. For example, the encapsulation of garlic EO using β-cyclodextrin effectively controlled growth of mesophilic bacteria, yeast and moulds on fresh-cut tomato stored at 8 ºC for 3 d (Ayala-Zavala *et al.*, 2008). Other researches have explored the use of paraffin (Rodriguez-Lafuente *et al.* 2010), gum Arabic (Maqbool *et al.*, 2010), pectin (Melgarejo-Flores *et al.*, 2013), *Aloe vera* gel (Nabigol & Asghari, 2013), sodium alginate (Guerreiro *et al.*, 2017) and whey protein (Marquez *et al.*, 2017) for developing edible antimicrobial films for fruit and vegetable storage (Table 6).

Biological control using microbial antagonists has also emerged as a promising technique to control postharvest pathogens and decrease synthetic fungicide use (de Paiva *et al.*, 2017). Yeasts present desirable antagonistic properties suitable for incorporating into antimicrobial packaging systems. For instance, yeast possess the ability to grow rapidly and colonise surface wounds and subsequently out-compete pathogens for nutrients and can persist throughout storage space as demonstrated by (de Paiva *et al.*, 2017). Accordingly, antagonistic yeast strains, *Hanseniaspora. opuntiae* L479 and *Metschikowia pulcherrima* L672 effectively controlled the development of *Penicillium expansum* in wounded cherries stored at 1 ºC for 35 d (de Paiva *et al.* 2017). An important drawback concerning the industrial application of biocontrol antagonists is related to the lack of consistent efficiency (de Paiva *et al.* 2017). In this regard, more research has been dedicated into combining the effectiveness of biocontrol agents with other alternative treatments to give results comparable to synthetic fungicides.

However, despite the abundance of existing, numerous simulated studies focused on developing antimicrobial systems, real applications are limited by technical, aesthetic and regulatory barriers (de Paiva *et al.* 2017). To this regard, a few recent examples can be cited. Liu *et al.* (2016) investigated antimicrobial and antioxidant bioactive films based on poly(lactic acid)/poly(trimenthylene carbonate) incorporated with oregano EO solvent cast films. Tapia *et al.* (2008) applied alginate and gellan based coatings to improve barrier, texture and nutritional properties of fresh-cut papaya. Mantilla *et al.* (2013) assessed the effect of a multi-layered edible coating with a microencapsulated antimicrobial complex (β-cyclodextrin and trans-cinnamaldehyde) in enhancing the quality and shelf life of fresh-cut pineapple.

4. Future prospects of active packaging technologies

Active packaging systems are continuously evolving in response to growing challenges from a modern society. Nanotechnology presents a new route for further development for innovation in food packaging systems. Advancement in nanotechnology and improvement of nanomaterials will further facilitate the development of optimized active and intelligent packaging systems (Pereira De Abreu *et al.*, 2012), to meet the needs of the consumers and the industry. Nanospheres, nanofibers, and encapsulated microfibers can retain or entrap bioactive molecules, such as volatile EOs for their controlled and steady release during storage (Pereira De Abreu *et al.*, 2012).

More research focused towards developing value added active packaging systems are required towards shifting or switching the current conventional food packaging systems to a bio-

based packaging. The use of biodegradable polymers for packaging in the development of novel, biodegradable polymeric materials from natural sources, offers as an alternative to reduce amount of waste and environmental impacts. Biopolymers "green polymers" represent a huge platform towards reducing environmental pollution (Wen *et al.*, 2016). Continued interest in the use of natural substances requires the need for research into the search for healthy, environmentally friendly, and economically attainable formulations. Among the biopolymers, cellulose may be used to produce films bearing high flexibility and improved smoothness and water solubility (De Moura *et al.*, 2011). In this view De Moura *et al.*, (2011) generated highly stable edible films by incorporating chitosan nanoparticles into carboxymethylcellulose films. Similarly, Azizi *et al.* (2014) dispersed cellulose nanocrystals and zinc oxide (CCs/ZnO) nano composites as bio-functional fillers of poly(vinyl alcohol) (PVA) and chitosan to generate antimicrobial active films. The bio-composite film showed antibacterial activity against *Salmonella choleraesuis* and *Staphylococcus aureus* (Azizi *et al.*, 2014).

Apart from providing food preservation, active packaging technologies can be used as a vehicle of delivering functional foods including vitamins, thereby tackling against some of the existing drawbacks associated with the fabrication of functional foods (Pereira De Abreu *et al.*, 2012). Active packaging materials capable of releasing antimicrobial compounds can inhibit or delay microbial growth of food products during storage. In this bid, chitosan nanoparticles prepared by ionic crosslinking with tripolyphosphate (TPP) were generated for the encapsulation and release of proteins (Jarudilokkul *et al.*, 2011). Use of polymers such as β-cyclodextrin (β-CD) provides a feasible alternative for the encapsulation and controlled release of natural antimicrobials. The success of any emerging packaging is governed by its integration into existing or future packaging as an affordable, easy to use, accurate, reliable, simple, reproducible (i.e. range of operation), and environmentally friendly material.

5. Conclusions

Active packaging can significantly improve postharvest quality of fruits and vegetables by slowing down produce respiration through monitoring O_2, CO_2, C_2H_4, moisture and microbial growth levels in packaging systems. Combining different individual active agents provides multiple active packaging systems, which often most, if not all cases, guarantee food safety and preservation. The success of any new packaging will depend on making its cost, applicability, effect on the environment. Successful smart packaging must be economically mass-produced and remain at all times food contact safe. First preference is currently directed towards antimicrobials

tagged as natural, efficient and non-toxic a due to the safety and ecological concerns. Incorporation of nanofibers into packaging systems could improve antimicrobial delivery and preservation of fruit and vegetable attributes

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Table1. Summary of the fundamental concepts of active packaging technology of fruits and vegetables

Problem*	Active packaging solutions	Mechanism
High oxygen (O_2)	• Scavengers, iron powders, ascorbic acid, photosensitive dyes • UV light activated films with O_2 scavenger extruded into multilayer films (polyolefin: polyethylene and polypropylene)	• Slows down respiration rate of produce • Restricts microbial growth • Prevents oxidative damage of plant tissue pigments • Eliminates formation of oxidative by-products
Carbon dioxide (CO_2) regulation	• Scavengers and emitters • Calcium, Sodium, Potassium hydroxide, Calcium oxide, Silica gel, Palladium, zeolite etc.	• Combined with low O_2 helps to slow down respiration rate of produce • Supress surface microbial growth
Ethanol build up	• Ethylene scavengers	• Delays ripening, respiration rate, colour changes, and fruit softening by supressing ethylene production
Moisture condensation	• Drip absorbers, dehumidifiers, • Super absorbent polymers • Silica gel (sachet), Clays (sachet)	• Lowers water activity to supress microbial growth • Enhances product appearance and freshness
Microbial spoilage	• Slow release of preservatives • Fixed preservatives e.g. edible coatings • Surface modified package materials • Organic acids and their salts (sorbic acid) • Miscellaneous (Triclosan), bacteriocins (nisin), Spice and herb extracts, volatile fragrances e.g. essential volatiles	• Control microbial population • Target specific microorganisms to provide higher safety and quality products • Significant impact on shelf-life extension • Volatiles possess aromatic compounds with antimicrobial properties

*Sources: Martinez-Romero *et al.*, 2009; Sandhya 2010; Pereira de Abreu *et al*, 2012; Rocha *et al*. 2013; Rux *et al*., 2015

Table 2. Selected studies on the use and implications of O_2 scavengers in active packaging systems of fresh produce.

Product and storage conditions	Active compounds	Packaging materials	Outcome	Shelf life attributes		Source
				Control	Active packaging	
Tomatoes (20 ° C for 13 d)	Commercial iron-based O_2 scavenger (ATCO® LH100) sachets	LDPE pouch	Controlled RR	O_2 reached EMA after 100 h, and CO_2 accumulated after 20 h	O_2 (3kPa) steady state took 50 h, and suppressed CO_2 accumulation	Charles *et al*. (2003)
Endives (20 ºC for 7 d)	O_2 scavengers (reduced iron); MAP 3 and 4.5 kPa of O_2 and CO_2 respectively	Macro-perforated OPP pouches (30µm), and LDPE (50µm)	Rapid EMA attained, delayed greening and browning	4 days for O_2 to reach steady state, CO_2 build up after 2 d	2 days to reach O_2 steady state CO_2 peak disappeared	Charles *et al*. (2008)
Strawberries (4 ºC for 35 d)	Commercial O_2 scavenger: ATCO-210	PVC/PE sealed with micro-perforated BOPP	Maintained the best TSS, pH and firmness	Limited shelf life of 28 d	Extended shelf life up to 35 d	Kartal *et al*. (2012)
Strawberries (4 ºC for 4 wks.)	Commercial O_2 scavengers: ATCO-100 and ATCO-240	PLA trays	Lowered RR, maintained colour, firmness and reduced mould	72% decay after 28 d	15-20% decay after 28 d	Aday & Caner (2013)
Iceberg lettuce (4, 10, and 22 ºC for 21 d)	Chlorine dioxide (ClO_2) generator, CO_2 emitter, O_2 scavenger	Nylon/EVOH/polyethylene bag	Effectively inhibited microbial growth, allyliothiocyanate generator was ineffective	*E. coli* O157:H7 load of 0.8, 3.5 and 5.9 log CFU/g at 4, 10 and 22 ºC, respectively, after 21 d	O_2 scavenger: *E. coli* 0.44 and 1.85 CFU/g at 10 and 22 ºC, respectively, after 21 d	Lu *et al*. (2015)

LDPE: Low-density polyethylene OPP: Oriented polypropylene BOPP: biaxially-oriented polypropylene; PLA: Polylactic acid; EMA: Equilibrium modified atmosphere; ND: not detectable; RR: respiration rate

Table 3. Regulation of CO_2 by means of active packaging systems for fresh horticultural commodities.

Product and storage conditions	**Active components**	**Packaging materials**	**Outcome**	**Shelf life attributes**		**Source**
				Control	**Active packaging**	
Strawberries (4 ºC for 28 d)	Commercial CO_2 absorbers: EMCO A and B (46-50% Na_2CO_3	PLA trays Sachets were paper coated with perforated polypropylene	Maintained TSS, electrical conductivity, firmness, colour, and sensory attributes	11% decay	Trace decay observed	Aday *et al*. (2011)
Eggplant (1, 4 and 8 ºC for 15 d and 3 d at room temp.)	CO_2 absorbent and 1-MCP	Antifogging OPP and PE films	Suppressed chilling injury deterioration at 4 and 8 ºC; severe chilling injury at 1 ºC for both treated and untreated fruit	Severe chilling injury (80%) at 4 and 8 ºC	Slight (< 10%) chilling injury at 4 and 8 ºC	Veasna *et al*. (2012)
Iceberg Lettuce 4, 10, or 22 ºC for 21 d	Films generating either CO_2, ClO_2 or allyl isothiocyanate (AIT), and scavenging O_2	Nylon/EVOH/PE 3-phase active-polymer films (CSP Technologies)	Effectively controlled growth of *E. coli* O157:H7 and total aerobic bacteria under all storage conditions	*E. coli* O157:H7 load of 3.5 and 5.9 log CFU/g at 10 and 22 ºC, respectively	*E. coli* O157:H7 load of 0.44 and 1.85 CFU/g at 10 and 22 ºC, respectively	Lu *et al*. (2015)
Pears (1 ºC for 7 months)	CO_2 adsorbent and C_2H_4 adsorbent	control film (permeability: 100 mL/day/m^2) active film (permeability: 2000 mL/day/m^2)	Reduced CO_2 and C_2H_4 levels	Severe internal browning at 5 months	Severe internal at 7 months	Nugraha *et al*. (2015)

TSS: soluble solids content; PE: polyethylene; 1-MCP: 1-methylcyclopropene

Table 4. Summary of selected studies on the use and implications of using ethylene scavengers in active packaging systems of fresh produce.

Product and storage condition	**Active compounds**	**Packaging materials**	**Outcome**	**Shelf life attributes**		**Source**
				Control	**Active packaging**	
Kimchi (15 ºC for 14 d)	CO_2 absorbent: $CA(OH)_2$ sachets Zeolite powder combined with sodium carbonate	10 µm thick HDPE, 20 µm thick polyolefin (PD941)	Reduced CO_2 partial; pressure build up volume expansion of kimchi packages	1.98 bar CO_2 partial pressure after 8 d	0.84 bar CO_2 partial pressure after 8 d	(Shin *et al*. 2002)
Tomatoes (8 ºC for 35 d)	Granular activated carbon sachets: GAC (Sigma-Aldrich, Madrid, Spain) and Palladium acetate MAP: 4 and 10 kPa O_2 add CO_2	20 µm non-perforated OPP bags	Reduced ethylene accumulation, prevented fruit softening, weight loss, and decay.	100% decay after 28 d	39% decay after 28 d	(Bailen *et al*. 2006)
Avocado (stored at12 ºC, 10 d), banana (stored at16 ºC, 9 d) strawberry (5 ºC, 13 d)	2.5% Palladium-zeolite	Open trays or vented plastic punnets	Inhibited ethylene-induced ripening	Ethylene levels of 78.41, 76.97 and 67.99 µL/L for avocado, banana and strawberry	Ethylene levels of <0.1 µL/L for avocado, banana and strawberry	(Terry *et al*. 2007)
Tomato (8 °C for 28 d)	70 g activated carbon-1% palladium alone or with a 3h cyclic heater (175 °C)	Metal device	Low concentrations of ethylene and CO_2 maintained.	3×10^{-4} kPa ethylene accumulation	0.16-0.9 $\times10^{-4}$ kPa ethylene accumulation	(Martínez-romero *et al*. 2009)
Avocado (5 ºC for 26 d)	Palladium-ethylene scavenger (e + ® Ethylene remover) or 1-methylcyclopropene (1-MCP,1.5 µL/L)	Polypropylene boxes	delayed ripening, 1-MCP although more effective it impaired subsequent normal ripening,	Ethylene levels 0.07 µL/L 5.5 N firmness	Ethylene levels 0.02 µL/L 47.5 N firmness	(Meyer & Terry, 2010)

Strawberries (4°C for 21 d)	ethylene-moisture sachets		Maintained global appearance and acidity	11% decay	trace decay visibility	(Aday & Caner, 2011)
Cherry tomatoes (20 °C for 30 d)	polylactic acid (PLA; 3% w/v) coating on cardboard tray	Coated cardboard wrapped with a low-density polyethylene (LDPE)	controlled respiration rate of transmission for cherry tomatoes	~3.3 log CFU/g bacterial count ~3.3 log CFU/g yeast and mould count	~2.3 log CFU/g bacterial count ~2.4 log CFU/g bacterial count	(Garcia-Garcia *et al*. 2011)
Plum and peach (32 °C for 6 d)	Sodium permanganate, antimicrobials spray coating (potassium sorbate, potassium metabisulphite)	Wrapping paper coatings	growth inhibition of *Candida pelliculosa* (58%), but Potassium metabisulphite was not effective	0% growth inhibition	23-58% *Candida* growth inhibition Retained fruit freshness	(Rudra *et al*. 2013)

CFU: colony forming unit; LDPE: low density polyethylene; HDPE: high density polyethylene; MAP: modified atmosphere packaging; MCP: methylcyclopropene; OPP: oriented polypropene; PLA: polylactic acid

Table 5. Regulated moisture control by means of active packaging systems for fresh horticultural commodities.

Products and storage conditions	Active compounds	Packaging materials	Outcome	Shelf life		Source
				Control	Active packaging	
Cherry tomatoes (20°C for 30 d)	(PLA; 3% w/v) coating on cardboard tray	Coated cardboard wrapped with a low-density polyethylene (LDPE)	controlled humidity and respiration rate transmission	20% decay	5% decay	(Garcia-Garcia *et al*., 2013)
Mushroom (10 ºC for 5 d)	Multifunctional agar-based biofilm with sodium carbonate and or sodium glycinate as CO_2 absorbent	Perforated OPP with a HDPE tray	generated desired atmosphere, giving best quality attributes	2.73 log CFU/g bacterial count 2.1% weight loss	1.82 log CFU/g bacterial count 9-12.6% weight loss	(Wang *et al*. 2015)
Mushrooms (4.12 and 20 ºC for 6 d)	Sodium chloride (18% NaCl) embedded humidity regulating tray	Polypropylene/EVOH/ polyethylene	maintained quality of mushroom but could not prevent condensation	100% RH in headspace	Maintained 93% RH Adsorbed 4.1 g (~ 68%) of the water vapour	(Rux *et al*. 2015)
Mushroom (15-20 ºC, for 128 h, ~ 5 d.)	CO_2 absorbent Ca $(OH)_2$ absorbent polymer, Moisture absorbent Sodium polyacrylate	perforated (8mm) 30 µm thick OPP placed inside a 40 µm low density polyethylene bag	effectively reduced yeast/mould growth a decay	CO_2 0.1% and O_2 20.5% gas levels 4.3 log CFU/g Aerobic bacteria 4.0 log CFU/g yeast ad mould count	CO_2 3.3-4.0% and O_2 8.8-10.3% gas levels 44-4.5 log CFU/g Aerobic bacteria 3.2-3.4 log CFU/g yeast ad mould count	(An, 2016)
Strawberries and tomatoes (13 ºC for 7 d)	humidity regulating trays (12% NaCl)	polyethylene (outside)/ hygroscopic ionomer (active layer) polypropylene (control film)	regulated in package relative humidity (RH), and maintained produce quality	0.3-0.6% weight loss	Weight loss of 1% for tomatoes and 2-3% for strawberry	(Rux *et al*. 2016)

CFU: colony forming unit; EVOH: ethylene vinyl alcohol (EVOH); HDPE: high density polyethylene; LDPE: low density polyethylene; PLA: polylactic acid; RH: relative humidity

Table 6. Applications of antimicrobial active packaging on a range of fruits and vegetables

Product and storage condition	Active compounds	Target pathogen	Outcome	Source
Strawberry fruit (13℃, 14 d)	Crab shell chitosan (10 and 15 mg/mL) coating	*Botrytis cinerea* Pers *Rhizopus Stolonifer*	50% reduction in decay compared to control. Defense enzymes activated	(El-Ghaouth *et al*. 1992)
Papaya (25-28 °C, 5 d)	Chitosan (0.5 and 1.5%), extract of custard apple	*C. gloeosporioides*	Chitosan 1.5% control fruit decay, treatment maintained fruit firmness	(Bautista-Banos *et al*. 2003)
Apples (18-20 °C,15 d and 4 °C,12 weeks)	Chitosan (High MW) and extracts of: *Capsicum frutescens*, hinokitiol pepper cayenne, and yam bean extracts	*P. expansum* Link	Chitosan 1% was the best treatment to reduce apple rot. The extracts were not effective	(Bautista-Banos *et al*. 2004)
fresh-cut tomato (8 ºC, 3 d)	Garlic EO encapsulated with β-cyclodextrin	Mesophilic bacteria, yeast and moulds	Maintained antimicrobial quality	(Ayala-Zavala *et al*. 2008)
Tomato (25 °C, 3 d and 2 °C, 21 d)	Chitosan with different molecular weights (0.5×10^4 - 2.9×10^5)	*B. cinerea*	Chitosan treatments elicited biochemical defense responses	(Badawy & Rabea, 2009)
Tomato, grape (4 and 24 °C, 10 d)	Chitosan (1.0 and 2.5% w/v)	*Colleotrichum* sp.	Better lesion control by chitosan treatment only seen at 24˚C. No treatment differences at 4℃.	(Munoz *et al*. 2009)
Cherry tomatoes (25°C, 10 d)	Active paraffin coating with bark cinnamon, oregano, clove essential oils	*Alternaria alternata*	Bark cinnamon and oregano gave bets results.	(Rodriguez-Lafuente *et al*. 2010)
Bananas (13°C, 28 d)	Gum Arabic (10%), Lemmon grass (0.05%), and cinnamon (0.4%) coatings	*C. musae* and *C. gloesporiodes*	Gum arabic/cinnamon gave best results (300% less decay to control fruit and improved phytochemical attributes)	(Maqbool *et al*. 2010)
Table grapes (1-2 ºC, 22 d)	Hydroxypropylmethylcellulose, chitosan and bergamont EO	Mesophilic bacteria, yeast and moulds	Suppressed microbial growth over 19 d by at least 50%	(Sanchez-Gonzalez *et al*. 2011)
Bananas (22 °C, 12 days)	Oligochitosan (5 to 20 g/L)	*C. musae*	50% reduction in radial growth and the activation of defense related enzymes	(Xiangchun *et al*. 2012)
Banana, papaya and dragon fruit (13 °C, 80 RH, 28 d)	Nano emulsions of 1% Low MW crab shell chitosan (200 - 1000nm)	*C. musae*, and *C. gloeosporioides*	Best results obtained using 200nm (banana) and 600nm (papaya and dragon fruit) droplet sizes	(Zahid *et al*. 2012)

Pomegranate arils (4 ºC,12 d)	Chitosan coating	Mesophilic bacteria, yeast and moulds	40-50% reduction in microbial growth	(Ghasemnezhad *et al*. 2013)
Table grapes (10 °C, 15 d)	Cinnamon leaf oil water emulsions (0-5 g/L), vapours (0 – 0.588 g/ L) or as chemical in pectin coatings (0 and 36.1 g/L).	*B. cinerea*	100% inhibition by pectin/cinnamon composite coating, the cinnamon Emulsion odour was not acceptable.	(Melgarejo-Flores *et al*. 2013)
Strawberry (5 °C, 7d)	Nanosized silver, chitosan, irradiated chitosan	*B. cinerea*	Composite treatment gave best results (10% decay) compared to control (90%).	(Moussa *et al*. 2013)
Pomegranate arils (5 °C, 21 d)	*Aloe vera* gel (0-250 mL/L)	*Penicillium digitatum* and *Aspergillus niger*	*Aloe vera* inhibition of *A. niger* was 2-fold higher than, *P. digitatum.* Decay and weight loss were.	(Nabigol & Asghari, 2013)
Strawberry (20°C, 4 d and 0°C, 7 d plus 3 d shelf life)	Crab shell chitosan (1% w/v) in 1% (v/v) acetic, hydrochloric, glutamic or, mic acid respectively and a commercial chitosan based, Chito-Plant formulation (1% w/v)	*B. cinerea*, *rhizopus* rot and blue mould. (storage symptoms)	Commercial chitosan gave best results.	(Romanazzi *et al*. 2013)
Pomegranate arils (3 ºC, 12 d)	*Aloe-vera* coating	Yeast and moulds, total aerobic count	100% inhibition of microbial growth.	(Martinez-Romero *et al*. 2013)
Strawberries (4 and 25 ºC, 15 d)	thymol 8% or carvacrol 8%	Fungal growth	Extended produce shelf life.	(Ramos *et al*. 2013)
Pineapples (4 ºC, 15 d)	Multilayer edible coating: with a microencapsulated (β-cyclodextrin and cinnamaldehyde)	Yeast and moulds, total aerobic count	Inhibited microbial growth, preserved fruit colour, texture and pH.	(Mantilla *et al*. 2013)
Green beans (4°C, 13 d)	Chitosan-essential oil (carvacrol, mandarin, bergamont and lemon) nanoemulsion, modified atmosphere and gamma irradiation	*Escherichia coli* O157:H7 and *Salmonella Typhimurium*	Antimicrobial coating (carvacrol nano emulsion), gamma irradiation and MAP gave undetectable levels of microbial populations.	(Severino *et al*. 2015)
Strawberries (21 ºC, 6 d)	Polyvinyl alcohol/cinnamon EO/β-cyclodextrin	*Staphylococcus aureus* and *Escherichia coli*	*E. coli* and *S. aureus* inhibition zones of 28.9 ± 0.3 mm and 30.5 ± 0.4 mm, respectively.	(Wen *et al*. 2016)

Sweet cherries (1 ºC, 35 d)	yeast strains, *H. opuntiae* and *M. pulcherrima*	*Penicillium expansum*	Antagonistic yeasts, particularly *M. pulcherrima delayed P. expansum* development, incidence and severity.	(de Paiva *et al*. 2017)
Apples (fresh-cut) (4 ºC, 8 d)	Edible coatings (sodium alginate (2%)/eugenol (0.1%)/citral (0.15%)/ pectin (0.15%). Anti-browning agents (ascorbic acid (1%)/citric acid (1%)/sodium chlorite (0.05%)	Reduced microbial spoilage without significantly affecting sensory and nutritional qualities.	Sodium alginate better performed pectin in reducing browning index, loss in firmness, weight loss, and microbial spoilage. Ascorbic acid was the best anti-browning agent.	(Guerreiro *et al*. 2017)
Fresh cut apples, potatoes and carrots (4-6 ºC, 10 d)	whey protein/pectin/trans-glutaminase coating	Weight loss reductions: Soy protein (20%), whey protein (40%), whey, protein/pectin/transglutaminase (80-100%), 100% weight loss reduction.	Marked inhibitory effect on mesophilic colonies growth. Preserved carrot phenolic and carotenoid content.	(Marquez *et al*. 2017)

Chapter 4

Fungal genera associated with preharvest and postharvest rot of pomegranate fruit

Abstract

Spoilage of pomegranate (*Punica granatum* L.) initiated by fungal pathogens during storage is a major cause of postharvest and economic loss. Fungal infections often occur in orchards from where they could be carried over into storage and its rot is initiated under conducive conditions. Thus, this study investigated fungal pathogens associated with the leaves, flowers closed, open and petal fall, fruitlet buds, immature (green) and commercially ripe pomegranate fruit (cvs. 'Herskawitz' and 'Wonderful') from the orchard and packhouse. Fungal pathogens were isolated and identified morphologically and confirmed by sequence analysis of the internal transcribed spacer regions of the nuclear ribosomal RNA genes (ITS: ITS1 and ITS2). Pathogens namely *Penicillium* spp., *Botrytis* spp., small-spored *Alternaria* spp., and *Pilidiella granati* Sacc. (syn. *Coniella granati* [Sacc.] Petr. & Syd.) were isolated from flowers, fruitlet buds, immature green and commercially ripe fruit. At commercial maturity *Penicillium* spp. and *Botrytis* spp. were the most prevalent spoilage pathogens in cv. Wonderful and Herskawitz, respectively. The incidence of *Botrytis* spp. (17 – 50%) and *Penicillium* spp. (24 – 63%) was lower in fruit obtained from the packhouse. Based on the analysis of ITS sequence data, a rapid molecular polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP detection assay was designed. This inexpensive molecular tool can be used for rapid and reliable monitoring of pomegranate disease-causing fungi without the need for advanced instrumentation.

***Keywords*:** Latent pathogens; pomegranate diseases; postharvest

1. Introduction

Long term storage of pomegranate fruit is compromised by pathogens that cause postharvest rots (Holland *et al.*, 2009). Fruits containing high levels of sugars and nutrients provide a desirable substrate for fungal growth and pomegranates are no exception. The impact of fungal infection is generally recognised as a postharvest quality problem;

however, the infection may originate from the orchard (Zhang & McCarthy, 2012). The challenge of postharvest spoilage on the commercial marketability of pomegranate is further exacerbated by various physiological disorders such as chilling injury, weight loss, husk scald and cracking, leading to decay (Caleb *et al.*, 2012). In addition, storage of pomegranates at suboptimal conditions also enhances fungal growth and mycotoxin production by *Alternaria* sp. for example (Ammar & El-Naggar, 2014).

The most common pathogens associated with pomegranate fruit rot include *Botrytis cinerea Pers.: Fr.* (grey mould), *Alternaria alternata* Fr. Keissl (heart rot), *Aspergillus niger* Tiegh. (heart rot), *Pilidiella granati* Sacc. (wet rot) and several species of the genus *Penicillium.* (Palou *et al.*, 2010; Thomidis, 2014). *Penicillium* green/blue mould rot of pomegranate is caused by *P. implicatum* Biourge, *P. expansum* Link, and *P. sclerotiorum* J.F.H. Beyma (Palou *et al.*, 2010, Khokhar *et al.*, 2013), while *Penicillium glabrum* (Wehmer) Westling is a reported endophyte occurring on pomegranate (Hammerschmidt *et al.*, 2012). Other fungal species that have previously described on pomegranate include *Colletotrichum gloeosporioides* Penz, *Pestalotiopsis versicolor* Speg. (Palou & del Río, 2009) and *Cytospora punicae* Sacc., which causes pomegranate collar rot of trees and wood cankers (Palavouzis *et al.*, 2015; Hand *et al.*, 2014).

The onset of disease infection of pomegranates must be investigated in order to assist in managing the problem of fruit losses where they are most critical. Rapid detection and confirmation of plant-borne pathogens is necessary for successful disease management, especially when introducing foreign plant materials (McCartney *et al.*, 2003). Fungal pathogens impede on the fruit quality of pomegranate especially on the export market where huge turnaround profits can be made or lost. The control of these diseases is particularly complicated by legislation on the postharvest use of synthetic chemicals. Control strategies for these pathogens is greatly compromised by the ability of the causal agents to remain latent in the host tissue until ripening, when the susceptibility rises due to an increase in sugars and the decrease in the content of defensive secondary metabolites (Michailides *et al.*, 2005). Therefore, an early and accurate diagnosis of the pathogen in symptomless pomegranates might be extremely useful for a proper management of the disease, or alternatively, to choose the lots of fruit that can be stored safely for long periods (Sanzani *et al.*, 2012). It will prevent unnecessary potential delays and monetary losses as appropriate

control measures can be implemented prior to further disease spread and introduction (McCartney *et al.*, 2003). This is essentially important given that most plant-based products have a limited shelf life (Alwakeel, 2013).

Pathogen identification can be achieved using cultural methods and molecular techniques. Cultural methods require skilled taxonomists to reliably identify the pathogens based on morphological characters, nutrient requirements and *in vitro* growth studies (McCartney *et al.*, 2003). This technique is however, time-consuming and any slight differences in medium composition can impair effective comparison of mycelial characters even by well-seasoned experts (Walker *et al.*, 2011). The application of molecular techniques to detect a variety of fungi provides a better alternative with respect to precision, accuracy and time efficiency (Lecellier *et al.*, 2014). Polymerase chain reaction (PCR)-based species identification with primers in the internal transcribed spacers (ITS1 and 2) regions has been widely used (White *et al.*, 1990). The ITS region can be readily sequenced and amplified with universal primers (Sanzani *et al.*, 2012). Depending on the ITS sequencing results, further identification to species level might require sequence analysis of other genetic loci to distinguish some closely related fungal species, such as small-spored *Alternaria* spp. (Woudenberg *et al.*, 2013). However, despite these advances, cultural diagnoses still persist, mainly due to the technical skills and costs associated with the molecular studies (Sanzani *et al.*, 2012).

Thus, the aim of this study was to investigate the incidence of pathogens causing pomegranate postharvest decay from the farm to packhouse and the application of a rapid identification method using molecular techniques that do not require sequencing. The objectives of this study were; a) to identify and characterize pathogens associated with pre- and postharvest fruit rots of selected pomegranate cultivars ('Herskawitz' and 'Wonderful'), and, b) to design and apply a molecular identification tool based on polymerase chain-restriction fragment length polymorphism (PCR-RFLP) for identification of pomegranate fruit pathogens.

2. **Materials and Methods**

2.1. Sample collection

Leaves, flowers (closed, open and petal fall), fruitlet buds, immature green fruit and

commercially ripe pomegranate fruit cv. 'Herskawitz' (mid harvest) and 'Wonderful' (late harvest) were aseptically collected from three commercial orchards located in the Wellington region (Western Cape, South Africa, GPS S33° 39.276 E18° 59.399). In each orchard, ten trees were tagged from which the samples were collected at all sampling times (different phenological stages). A total of 10 units of closed flowers (S1), open flowers (S2), petal fall (S3), fruitlet buds (S4), immature green fruit (S5), commercially ripe fruit (S6), packhouse processed fruit (S7) and leaves (S8), were collected per tree (Fig. 1). This study focused on the local production of pomegranate fruit and fungal pathogen sampling from the same geographical area. The selected region accounts for over 65% of the country's pomegranate production hence insight on the diseases present in this region was of great significance to the South African pomegranate industry. A similar assessment of disease during cold storage of commercially-handled pomegranate at 7 ºC and 90% relative humidity (RH) for about 7 months was conducted (Palou *et al.*, 2013).

2.2. Sample preparation

The orchard samples obtained were divided into two equal batches, the first batch was surface sterilised prior to analysis, while the second batch was not sterilised to serve as the control. Non-fruit material (S1, S2, S3 and S8) was surface sterilised by dipping in 70% (v/v) ethanol for 30 s, 0.5% (v/v) sodium hypochlorite (NaOCl) for 2 min, and 70% (v/v) ethanol for 30 s (Fourie *et al.*, 2002). After surface sterilisation, the plant material was cut into quarters and placed in a petri dish containing sterile potato dextrose agar (PDA), with the anthers and stamens touching the medium (direct plating method). Only leaf portions with lesions were plated onto PDA. The lidded Petri dishes were not parafilmed so as to induce fungal sporulation for all the samples and these were left for approximately two weeks at 25 °C, after which they were checked for fungal growth.

Fruit material (S4, S5, S6 and S7) were surface sterilised by dipping in 70% (v/v) ethanol for 30 s, followed by 0.5% (v/v) NaOCl for 2 min and 70% (v/v) ethanol for 30 s, after which they were rinsed in distilled water for 60 s. After sterilisation the fruits were then immersed in 0.3% (v/v) paraquat, (200 g/L bipyridyl), (WPK Agricultural, Cape Town, South Africa) for 60 s (Sanzani *et al.*, 2012) and then rinsed in distilled water for 60 s. Paraquat induces latent infections and the establishment of wound pathogens to emerge in a shorter time by hastening fruit breakdown (Biggs, 1995; Michailides *et al.*, 2005).

Fruits were then left to air dry before incubation in moisture chambers at 25 °C under diurnal light conditions. The humidity chambers contained paper towels wetted with sterile distilled water. The chambers were opened once a week to ensure fresh air and growth stimulation. The fruit was assessed daily for rind breakdown and appearance of fungi.

2.3. Processing and postharvest storage

Total of 150 pomegranates (representing 50 fruit per orchard) of each cv. 'Herskawitz' and 'Wonderful') originating from the same three orchards were sampled after standard commercial packhouse processing. The packhouse processing involved the following steps; fruit were dipped in 0.5% Sodium hypochlorite for 2 min, and thereafter in fludioxonil (Syngenta Crop Protection Inc., Greensboro, NC, USA) for 30 s at ambient conditions (21 °C) at the recommended dose of 600 mg/L. After the fungicide treatment, fruit was sorted into different count sizes and culling of external defects. The fruit was then packed in 'Xtend' poly liner bags and placed into 50 x 30 x 10 cm^3 unlidded cardboard boxes lined with a cardboard cavity tray to prevent fruit contact. Fruit from the packhouse was also incubated in moisture chambers at the Department of Plant Pathology, Stellenbosch University without any further form of surface sterilisation for at least two weeks at 25 °C. Each fruit was individually placed in a labelled Petri dish lid and any fruit with rot symptom were immediately isolated and removed from the chambers.

For long-term cold storage, only pomegranates (cv. Herskawitz) from the packhouse were investigated, packed boxes were brought into the lab and stored at 7 °C, 90% RH for 7 months. The choice of cv. Herskawitz for long-term storage trial was based on a preliminary study conducted that showed the cultivar was more susceptible to *Botrytis* spp. than 'Wonderful'. A total of 56 fruits (7 boxes with 8 fruits each) were assessed for the incidence of postharvest decay during long-term storage. Each commercial box was treated as a replicate. Fungi were isolated from all visible decay symptoms (e.g. crown rot, aril decay, skin lesion, and moulding).

2.4. Morphological identification of isolated fungi

Mono-conidial cultures of isolates were established, plated onto PDA, and checked for morphological characteristics; conidia shape or branching, hypha segmentation and colour of colony on media (Bardas *et al.*, 2009). Genera were identified using microscopy with

the aid of keys from the 'Illustrated genera of imperfect fungi' (Barnett & Hunter, 1998). A total of 145 samples were isolated and identified from pre- and postharvest sampling. These were then stored as PDA blocks in 9 mL sterile distilled water McCartney bottles at 4 °C at the Department of Plant Pathology, Stellenbosch University. All isolates that could not easily be distinguished to species level (i.e. the principal natural taxonomic unit, ranking below a genus) were reported on the basis of their fungal genera. The cryptic small-spored *Alternaria* spp. such as *Al. alternata* and *A. tenuissima* were collectively referred to as *Alternaria* spp., *B. cinerea,* and other cryptic species were referred to as *Botrytis* spp.

2.4.1. Pathogenicity test

Fungal pathogens that prevailed during cold storage were tested for pathogenicity on healthy fruit. Virulence and pathogenicity of predominant postharvest fungal isolates was verified by fulfilling the Koch's postulates. This was achieved by inoculating mature healthy pomegranate (cvs. Herskawitz and Wonderful) with fungal pathogens (*Botrytis* sp., *P. granati* and *Penicillium* sp.) isolated from decayed fruit during cold storage. Cultures were grown on Potato Dextrose Agar (Merck Pty. Ltd., Modderfontein, South Africa) for 7-14 days at 25 °C before each trial. Healthy whole fruit samples (6 replicates of 5 fruits per replicate) were wounded using a sterile cork-borer (with 5 mm deep by 5 mm wide) to make two wounds per fruit, one on either opposite side of the fruit. Wounded fruits were artificially inoculated with the mycelia from fungal pathogens *Botrytis* sp., *Penicillium* sp. and *P. granati*, respectively and incubated at 25 °C for 14 days. Control treatment consisted of fruits (30 per cultivar) wounded and inoculated with plain PDA plugs and stored at 25 °C for 14 days. Control fruit did not show any symptom of the disease. For the purpose of culture collection and recording keeping the test fungal pathogens *Botrytis* sp., *P. granati* and *Penicillium* sp. were stored in the Stellenbosch University, Department of Plant Pathology (STE-U) culture collection under the given accession numbers STE-U 7866, STE-U7864, and STE-U 7865, respectively. The assigned Genbank accession numbers of these isolates are KT279813, KT279817 and KT279814 respectively.

2.5. Molecular identification

2.5.1. DNA isolation, amplification and sequencing

Confirmation and validation of species identification was done using molecular

methods on isolates obtained from decayed pomegranate fruit samples. Mycelia (60 mg of each sample) was used to extract DNA from monospore cultures on PDA using a standard Phenol-Chloroform extraction protocol, as described by Meitz-Hopkins *et al.* (2014). The extraction buffer contained 0.1 M Tris pH 8.0, 0.05 M EDTA, 0.5 M NaCl, 0.7% β-mercaptoethanol and 0.25% sodium dodecyl sulphate. An RNAseA (Sigma cat. no. R4875, 0.6 U per sample) and a proteinase K (Bioline cat. no. BIO-37037, final concentration at 50 mg/L digest step of 30 min each at 37 °C was included before Phenol-Chloroform purification. DNA was resuspended in 20 µL $TE_{0.1}$ (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0), enabling 260/280 optical density measurement of the positive control in a neutral pH buffer. The concentration of each DNA sample was determined using NanoDrop® ND-Spectrophotometer (NanoDrop 1000 Spectrophotometer v 3.3.1, Thermo Scientific, USA) and samples were diluted to a final concentration of 25 mg/L.

The internal transcribed spacer (ITS) regions, including the 5.8S ribosomal RNA (ribonucleic acid) gene, were amplified and sequenced using universal primers (ITS1, 5'-TCC GTA GGT GAA CCT GCG G - 3' and ITS4, 5 '– TCC GCT TAT TGA TAT GC – 3') (White *et al.*, 1990). Polymerase chain reactions (PCR) were carried out using a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems, Foster City, CA). Amplification consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing temperature of 54 °C for 1 min and a final extension step at 72 °C for 1 min. PCR reagents were used at the following concentrations: 1× Bioline reaction buffer (Bioline Ltd., London, UK), deoxynucleoside triphosphates (dNTPs) each 300 µM (Bioline Ltd., cat. no. BIO-39025), ITS 1 and ITS4 primers (each 0.50 µM), 1.50 mM $MgCl_2$, 1.00 g/L BSA, and 0.125 U Biotaq DNA polymerase (Bioline Ltd., cat. no. BIO- 21040). The ABI 3130XL Genetic Analyser at the Central Analytical Facility, Stellenbosch University, was used for sequencing. Sequencing reactions were conducted using the BigDye system (version 3.1 dye terminators, Applied Biosystems) after the PCR products had been purified using the MSB®Spin PCRapace Clean-up System (Invitek GmbH, Berlin). Samples were amplified in an Applied Biosystems 2720 Thermal cycler and sequenced with the same primers as used for PCR amplification, using 1 µL PCR product in a 10 µL reaction. The sequencing program followed an initial denaturation of 1 min at 94 °C, followed by 30 cycles of 10 s at 95 °C, 5 s at 50 °C, and 4 min at 60 °C and a final step at 60 °C for 30 s. The generated sequencing

data obtained from 5'and 3' directions were used to generate a consensus sequence using software Geneious 3.5.6 (Biomatters Ltd., New Zealand, (available from http://www.geneious.com/) and manually edited using Sequence Alignment Editor V.2.0a11.

Species identification was done using the megablast function of the NCBI's GenBank nucleotide database (www.ncbi.nlm.nih.gov) by finding homology in the fungal nucleotide sequence using Nucleotide Basic Local Alignment Search Tool (BlastN) analysis and alignment of sequences with reference sequences from the Centraalbureau voor Schimmelcultures (CBS; www.cbs.knaw.nl) using a multiple sequence alignment program (MAFFT; Katoh & Toh, 2008).

2.5.2. PCR-RFLP analysis

A fast-molecular identification method was designed to confirm and validate the fungal pathogens identified on decayed pomegranate fruit. Inter and intraspecific variations between fungal species were compared in an alignment of sequences using Geneious 3.5.6 to find a restriction enzyme for use in Restriction Fragment Length Polymorphism (RFLP) sequence analyses. Thus, PCR products of the ITS regions 1 and 2 were digested with 10 U restriction enzyme Tas1 (Tsp509I, Thermo Scientific F01354). This restriction enzyme recognizes ^AATT sites and cuts best at 65 °C. The digest was performed overnight at 65 °C. Fragments were separated by electrophoresis on a 3% agarose gel and the size of each fragment was estimated by comparison with 100 bp DNA ladder (Gene Ruler Bioline, Thermo Scientific SM0331).

2.6. Statistical analysis

Analysis of disease incidence at the different sampling stages for each cultivar was conducted using a general linear model (GLM) analysis of variance (ANOVA) in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) (Palou *et al.*, 2013). This was followed by least significant difference (LSD) post hoc t-test ($p < 0.05$) to compare means.

3. Results and discussion

3.1. Fungal genera associated with pomegranate fruit

A total of 11 fungi genera were isolated from pomegranate cvs. 'Herskawitz' and

'Wonderful' fruit. *Rhizopus stolonifer* Ehrenb., *A. niger*, *Cytospora* spp., *Clonostachys* spp., *Embellisia eureka* E.G. Simmons, *Pestalotiopsis* spp. and *Nigrospora oryzae* were predominantly isolated from non-disinfected plant materials. While, *Aureobasidium pullulans* (de Bary & Lowenthal) Arnaud, *Alternaria* spp., *Botrytis* spp., *Penicillium* spp., and *P. granati* pathogens were isolated from flowers, fruitlet buds, immature green fruit and commercially ripe fruit while they also caused rots in stored fruits (7 °C and 90% RH for 7 months) for both cultivars (Table 1). However, *A. pullulans* was not detected on any of the fruit samples (S5-7), while *P. granati* was also not found on the closed flowers (S1). The fungal genera isolated from non-disinfected plant materials were considered/described as sporadic fungi as they were not found residing on the fruit surface material after surface sterilisation.

The fungal pathogens identified in this study are consistent with several fungi that have been associated with pomegranate fruit rot across the globe. These include, *A. alternata*, *B. cinerea* (Egypt), *A. niger*, *N. oryzae*, *P. versicolor*, *P. granati* (Turkey and Greece), *C. gloeosporioides* (Venezuela), and *C. punicae* (China) (Jadhav & Sharma, 2011; Pala *et al.*, 2009; Thomidis, 2014; Tziros *et al.*, 2008). Most of the diseases affect all plant parts and depending on the pathogen, and infection mode. These can be manifested as the minute, dark brownish, regular to irregular water-soaked lesions on leaves and fruits (Jadhav & Sharma, 2011). The ones not identified in this study could probably be due to variances in agro-climatic conditions, and cultivar susceptibility (Palou *et al*., 2013). These pathogens are often site-specific and vary from region to region depending on the local factors; preharvest (cultivar, climate, cultivation practices etc.), harvest (fruit maturity, frequency of wound infections), and postharvest (e.g. packing house handling, available storage) conditions (Palou *et al*., 2013).

3.2. Effect of fruit surface disinfection

Surface disinfection of pomegranate fruit prior to incubation had a significant influence on the number isolates. Sporadic fungal species were isolated from the non-disinfected fruit samples, these included *R. stolonifer*, *A. niger*, *Cytospora* sp., *Clonostachys* sp., *E. eureka*, *Pestalotiopsis* sp., and *N. oryzae*. Additionally, the incidence of latent fungal pathogens was significantly higher for non-disinfected samples in comparison to disinfected fruit ($p < 0.05$) as shown in Table 2. Tracing the pomegranate

fruit development stages revealed that the prevalence of predominant fungal pathogen was 2- to 3-fold higher in the non-disinfected than disinfected fruit. This result showed that the removal of surface flora by washing and disinfection of fresh produce prior to investigation is of paramount importance for the reduction of microbial load and infestation of fruit during long term storage (O'Grady *et al.*, 2015).

Chloride-based washes alone are however less effective compared to fungicide treatments as the application of fludioxonil at the packhouse significantly reduced pathogen incidence for both cultivars even further (Ghatge *et al.*, 2005). The major drawback of fungicide application is the possibility of finding trace chemical residues on fruit and development of fungicide resistant strains, which may result in reduced efficacy of synthetic chemicals (Palou *et al.*, 2007). The low incidence of *A. pullulans* and *Al. alternata* in the postharvest phase compared to preharvest could probably be as a result of effective preharvest fungicide spray applied by the orchards investigated. In other studies, *A. alternata/tenuissima* was consistently isolated from heart rot infections of pomegranate cv. Kapmaditika in Central Greece, causing significant yield losses of 40-50% (Tziros *et al.*, 2008) and 1-9% loss of ripe 'Wonderful' pomegranates in Italy (Ezra *et al.*, 2010). *Alternaria alternata* was also reported to be the causal agent of pomegranate black spot disease in Israel (Gat *et al.*, 2012).

3.3. Phenological stages

3.3.1. Pomegranate flower materials, immature fruit and leaves

Fungal infections were detected mostly on sample materials S2 (open flower) and S3 (petal fall). The closed flowers (S1) were the least affected by all the prevailing pathogens except in the case of *A. pullulans* where the highest score of 23-25% was recorded. *Aureobasidium pullulans* was the most frequently isolated pathogen from 'Herskawitz' (11-25%) and 'Wonderful' (10-23%). This was followed by *Botrytis* spp. and *Alternaria* spp. infections ranging between 4-29% and 1-14% for both pomegranate cultivars, respectively. *Penicillium* spp. was detected in 4-5% and 2-5% of 'Herskawitz' and 'Wonderful' flower materials, respectively while *P. granati* was only detected on 1-3% of the same pomegranate cultivars.

As the flowers developed into fruitlets (S4) and then into immature green fruits (S5)

a high incidence of *Botrytis* spp. was recorded for both 'Herskawitz' (15-29%) and 'Wonderful' (14-28%) followed by *Alternaria* spp. (10-13%), *A. pullulans* (5-14%), *Penicillium* spp. (5-8%) and *P. granati* (1-7%). 'Wonderful' pomegranate cultivar was the most susceptible to *Penicillium* while 'Herskawitz' showed a higher infestation of all the other fungal pathogens (Table 2). On the leaf samples (S8) *Alternaria* spp. had the highest occurrence (45%) followed by *Botrytis spp.* (18%), *A. pullulans* (12-15%), *Penicillium* spp. (3%) and *P. granati* (< 2%). The most susceptible phenological stage for the flowering phase was found to be the open flower (dehiscence) and no-petal (petal fall) stage. Similar findings were reported by Michailides *et al.* (2008) who observed a high frequency of black heart development at the anther dehiscence stage (open flower) of 'Wonderful' pomegranates. They concluded that most of the infections by *Alternaria* spp. occur at bloom time.

3.3.2. Mature pomegranate fruit

Infections were characterised by browning of the skin, which gradually extended and could be clearly distinguished with time, by the evident development of pathogen sporulation. *Penicillium* spp. and *Botrytis* spp. were mainly isolated from the calyx area (Fig. 2). *P. granati* was observed to infect all parts of the fruit while *Alternaria* spp. was only visualised upon cutting open the fruit (Fig. 2). A high predominance of *Penicillium* spp. was detected in the crown area of 'Herskawitz' (31-85%) and 'Wonderful' (68-96%) but, despite this high incidence, green/blue mould fruit decay was only present in cracked and wounded fruit regions. Several studies have reported different *Penicillium* spp. (*P. crustosum* Thom, *P. expansum* Link, *P. implicatum*, *P. sclerotiorum*, and *P. minioluteum* Diercks) associated with pomegranate fruit rot in Spain (Palou *et al.*, 2010), the Slovak Republic (Labuda *et al.*, 2004) and Pakistan (Khokhar *et al.*, 2013). It is important to note that not all *Penicillium* spp. isolated from decaying pomegranates are pathogenic, some are endophytes (Palou *et al.*, 2010). In this study, *Alternaria* spp., *Botrytis* spp. and *P. granati* were regarded as latent pathogens causing fruit rots on healthy fruit postharvest, while *A. pullulans* was considered as an endophyte. Fruit rots caused by *Penicillium* spp. in this study, were majorly localised around wounded fruit regions, and was therefore considered as a potential latent wound pathogen.

Pomegranate cv. 'Herskawitz' (S6) was significantly ($p < 0.05$) more susceptible to

Botrytis spp. (21-41%) compared to 'Wonderful' (7-8%). Variation observed in cultivar susceptibility to fungal pathogens is consistent with other reports in literature. For instance, variations in the frequency of latent pathogen infection were observed in three different table grape varieties ('Victoria', 'Italia', and 'Red Globe') grown in Spain (Sanzani *et al.*, 2012). The 'Red globe' variety was regarded to be the most suitable cultivar for long term storage, and this was attributed to its strong skin, firmness, favourable sugar/acid ratio and resistance to grey mould (Sanzani *et al.*, 2012). Factors that could contribute to variation in pomegranate pathogenic load include cultivar type, fertilisation, orchard practices, application of pre-harvest fungicides and dynamic weather patterns (Holland *et al.*, 2009, Munhuweyi *et al.*, 2016). Jayalakshmi *et al.* (2013) screened 19 pomegranate genotypes for anthracnose disease resistance caused by *C. gloeosporioides* while Kumari & Ram (2015) screened 63 pomegranate cultivars for resistance to leaf spot and dry fruit rot caused by *P. granati*. Both studies confirmed differences in pomegranate cultivar susceptibility to the diseases. Some of the cultivars tested were moderately resistant to the diseases while a select few showed moderate susceptibility (Kumari & Ram, 2015). In the present study, 'Wonderful' was more suitable for long term storage compared to 'Herskawitz', due to its resistance to postharvest grey mould in particular.

3.3.3. Postharvest handling

The frequency of fungal infections at commercial ripening (S6) declined after pack house processing and handling (S7) (Table 2). The incidence of *P. granati* ranged from 1-3% for both cultivars. *Alternaria* spp. was detected on 5% of S6 for both 'cultivars and the incidence declined to < 2% after pack house processing (S7). The low incidence of *Alternaria* spp. especially after packhouse processing in this study could be from the culling of suspected defect fruit. Heart rot infected fruit generally float in water as they are lighter than healthy fruit (Zhang & McCarthy, 2012) and can, therefore, be easily distinguished from healthy fruit. Fludioxonil application in the packhouse significantly ($p < 0.05$) reduced the frequency and occurrence of *Botrytis* spp. and *Penicillium* spp. by 13-51% and 23-64%, respectively. Fungicide treatment continued to reduce the development of stem and calyx-end decay in the long-term cold storage of the pomegranates for at least 3 months at 7 °C and 90% RH. However, after 7 months of storage, 71% of total fruit had decayed and the most abundant pathogens associated with the decay were *Botrytis* spp. (58%), *Penicillium*

spp. (30%) and *P. granati* (12%).

Grey mould caused by *B. cinerea* has been reported as the primary limiting factor for long-term storage of pomegranates (Holland *et al.*, 2009; Day & Wilkins, 2011). Propagules of *B. cinerea* (e.g. sclerotia, mycelium and conidia) can be found in the soil, plant litter and in the air (Lennox *et al.*, 2003). After blossom infection, infection inevitably spreads from the mesocarp tissue and adjoining sepals in a circular manner to reach the vascular bundles (Michailides *et al.*, 2005). To control grey mould, attention should be devoted to the implementation of field treatments to reduce the incidence of latent infections, by using synthetic fungicides such as fenhexamid and fludioxonil treatments (Holland *et al.*, 2009). The use of non-polluting, postharvest antifungal treatments during cold storage and commercial handling of pomegranates are recommended for controlling blue mould (Palou *et al.*, 2013). This can be achieved by using natural biodegradable coatings such as chitosan and putrescine (Shiri *et al.*, 2013).

Application of 0.5 and 1% chitosan coating was shown to inhibit bacterial and fungal growth on the surface of pomegranate arils stored at 4 °C for 12 d (Ghasemnezhad *et al.*, 2013). *Aloe vera* gel coating was used to control pomegranate fruit decay and maintain pomegranate arils during storage (Martínez-Romero *et al.*, 2013; Nabigol & Asghari *et al.*, 2013). Other non-chemical methods such as heat treatment, biocontrol, organic acids and modified atmosphere packaging have revealed additional benefits such as extending shelf life, improving chilling tolerance, decreasing fruit decay and in some cases maintaining nutritional quality (Opara *et al.*, 2015). These postharvest tools should be combined with regular inspection and grading of pomegranates for any disorders including discoloration and cracking, to prevent the packing of diseased fruit and disease spreading (Opara *et al.*, 2015).

3.4. Molecular identification of fungal pathogens

PCR amplification (ITS1/ITS2) of the pomegranate sampled fungal isolates gave amplification products ranging between 500-614 bp. ITS sequences of the fungal isolates were aligned with GenBank ITS reference sequences from pomegranate and other hosts (Table 3). Based on the ITS sequencing data, some of the isolates were identified accurately (100%) to species level as *A. pullulans*, *E. eureka*, *P. crustosum* (synonym *P. solitum*), *P. glabrum* and *P. granati* (Table 3). The remaining isolates could not be differentiated further

to species level using ITS primers and were grouped at genus level as *Alternaria spp.* (*A. alternata, A. tenuissima, A. arborescens*), *Botrytis* spp. *(B. fabae, B. perlagonii, B. cinerea, B. pseudocinerea), Clonostachys* sp. *and Pestalotiopsis* sp. (Table 3). The results obtained from this study are in agreement with the suggestion that although ITS sequences can be used for species identification. Some of the fungal genera have identical or near identical ITS sequence data and require further molecular characterisation (Sanzani *et al.*, 2012). This in turn, necessitates the use of specific primers that can accurately reveal the identity of culprit pathogens prior to their spreading. Therefore, future studies should address clarifying the taxonomical status of the unresolved individual species from the present study. A possible molecular technique to identify small-spored *Alternaria* species is by using the anonymous OPA1-3 (Optic Athropy) region (Visagie *et al.*, 2013). *Penicillium* and *Botrytis* spp. species can be identified through partial beta-tubulin PCR and special primers can also be designed (Visagie *et al.*, 2013; Berbegal *et al.*, 2014).

Restriction digests (PCR-RFLP) of the amplified ITS region of fungal pathogens isolated from pomegranate sample materials were performed with Tas1 enzyme. The fungal isolates yielded different band fragments (> 70 bp) ranging from one to four depending on the species level (Table 4). Based on the Tas1 restriction digest highest (4) and least (1) number of band fragments was observed for one of the *Alternaria* and *P. glabrum* isolates, respectively. The 17 latent fungal isolates were separated into six fungal genera namely; *Botrytis* spp., *Alternaria* spp., *E. eureka*, *A. pullulans*, *Penicillium* spp. and *P. granati* using a 100 bp gene ruler DNA ladder mix (Fermentas, South Africa) (Fig. 3). *Aureobasidium pullulans, E. eureka, Penicillium* spp. and *P. granati* were identified to species level, while *Botrytis* spp. and *Alternaria* spp. could only be identified up to genus level using the Tas1 enzyme (Fig. 3). Similarly, Sanzani *et al.* (2012) developed a real-time quantitative PCR detection method based on a probe designed *on B. cinerea* intergenic spacer (IGS) regions. The system proved to be highly specific and sensitive, enabling quantification of as little as 10 fg of *B. cinerea* DNA and detection of single conidia in artificially inoculated grape berries. In this current study, PC-RFLP assay was successful in validating and confirming the identification of pomegranate fruit decay pathogens.

4. Conclusions

This study successfully identified sporadic, endophytic and latent fungal

pathogens associated with selected pomegranate cultivars. Sporadic fungi consist of species i*solated directly from the surface of non-disinfected fruits, which included R. stolonifer*, *Cytospora* sp., *N. oryzae* and *Pestalotia* sp. While *Penicillium* spp., (although predominant during postharvest storage of pomegranate) was characterised as wound pathogens, because it was majorly localised around wounded fruit regions. In the present study *Penicillium* spp. were identified as causing pomegranate decay at genus level. *Botrytis,* spp., and *P. granati,* both originated from the orchards and persistent during postharvest storage of pomegranate were characterised as latent fungal pathogens and also verified via pathogenicity test. This showed that samples obtained from open flowers (S2) or petal fall samples (S3) can be reliably assessed for early detection of latent fungal pathogens of pomegranate.

Based on the morphological characters and ITS gene region sequence the following fungal genera were characterised up to species level; *A. pullulans*, *P. crustosum*, *P. glabrum*, *P. granati*, and *E. eureka*, while *Botrytis* spp., *Alternaria* spp. were characterised up to genus level. The PCR-RFLP as a molecular tool adequately confirmed and characterised fungal pathogens isolated from decayed pomegranate fruit. Thus, this can be used in the industry for rapid detection and identification of disease-causing microorganisms affecting pomegranate fruit from farm to packhouse. However, further study would be useful to test the specificity of PCR-RFLP against other sporadic fungal pathogens such as *Cytospora* sp. and *A. niger* associated with pomegranate fruit decay. Additionally, *Botrytis* spp. in this study was morphologically identified as *Botrytis cinerea*, while ITS gene was inconclusive. Hence, further analysis of other gene regions such as the beta tubulin gene region would help confirm that none of the isolates belong to the closely related species *B. pseudocinerea*.

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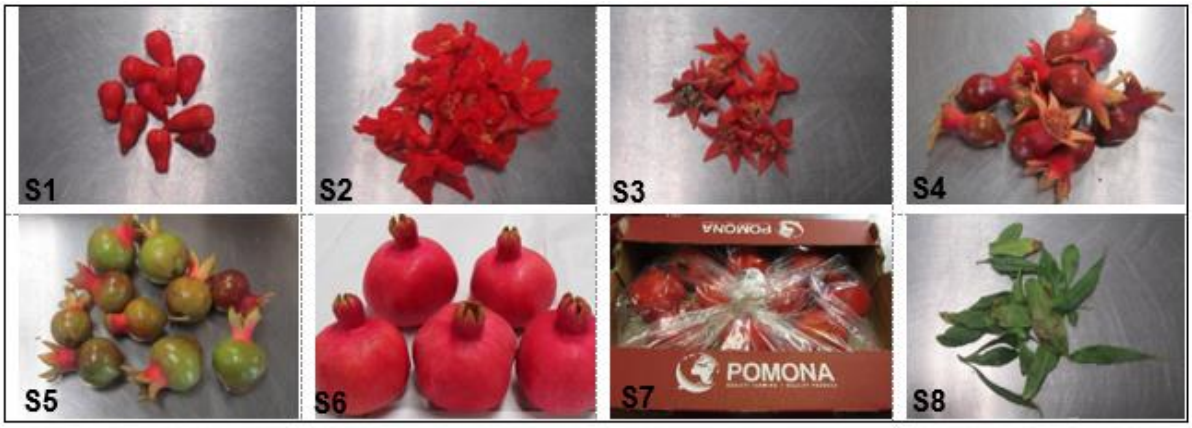

Figure 1. Types of pomegranate samples collected from three orchards; closed flower (S1), open flower (S2), petal fall (S3), fruitlet (S4), immature fruit (S5), ripe fruit (S6), packhouse fruit (S7) and leaves (S8).

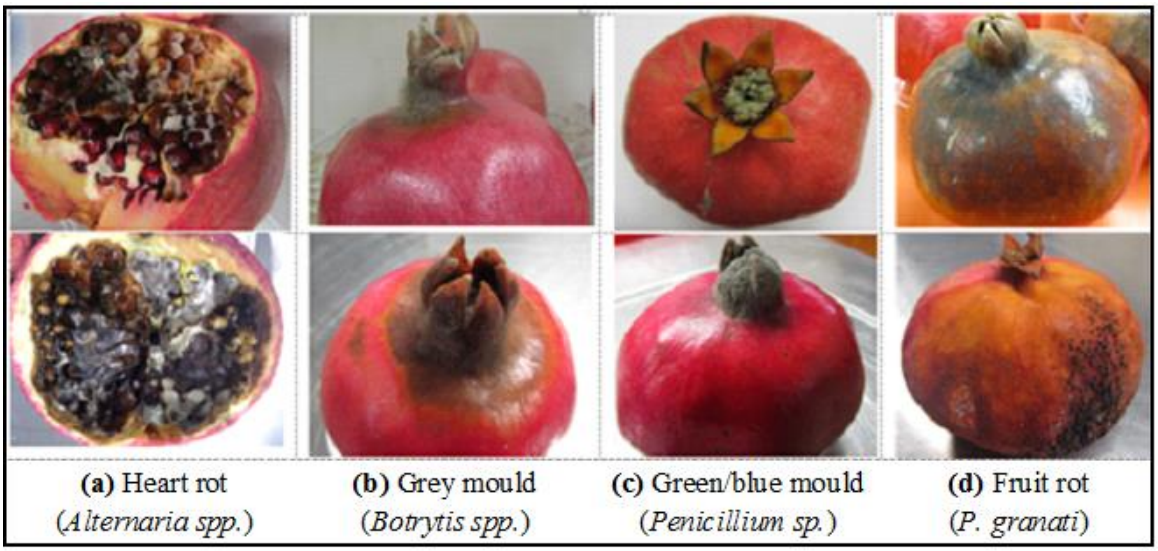

Figure 2 Pictorial presentation of pomegranate fruit affected with (a) *Alternaria* spp., (b) *Botrytis* spp., (c) *Penicillium* spp. and (d) *Pilidiella granati* on cv. 'Herskawitz' and 'Wonderful' pomegranate fruit.

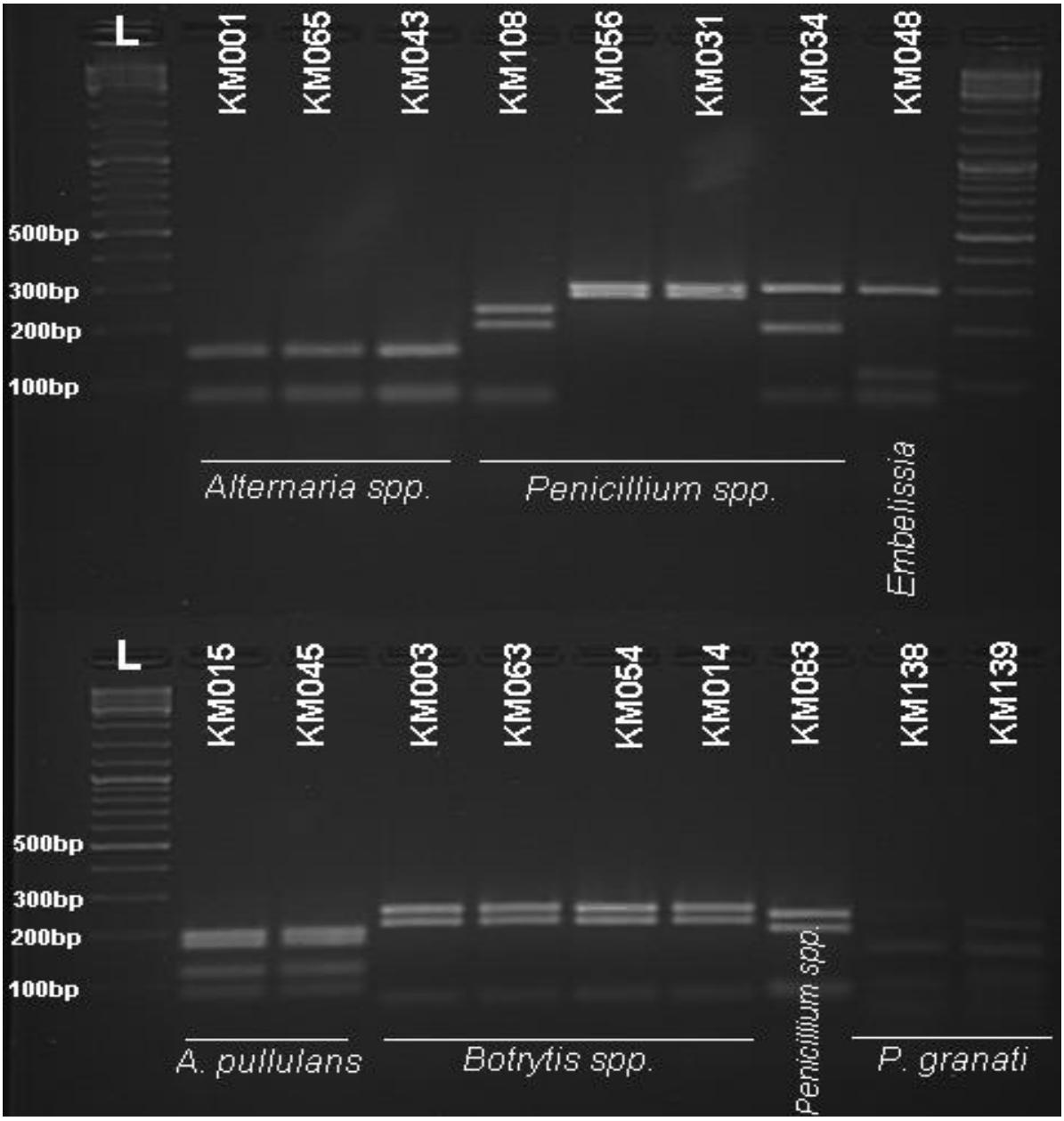

Figure 3 Tas1 restriction digest of internal transcribed spacer (ITS) polymerase chain reaction (PCR) amplification product. L: 100 bp ladder; *Alternaria* spp. (KM001, KM043, KM065); *Penicillium crustosum* (KM083, KM108); *Penicillium glabrum* (KM031, KM056); *Penicillium chermesinum* (KM034); *Embelissia eureka* (KM048); *Aureobasidium pullulans* (KM015, KM045); *Botrytis* spp. (KM003, KM014, KM054, KM063); *Pilidiella granati* (KM138, KM139).

Table 1. Fungal species identified and characterised from different part of pomegranate plant material.

Sample type	Description	Fungal species identified[a]								
		Alternaria spp.	*Aureobasidium pullulans*	*Botrytis* spp.	*Penicillium* spp.	*Pilidiella granati*	*Rhizopus stolonifer*	*Cytospora* sp.	*Nigrospora oryzae*	*Pestalotiopsis* sp.
S1	Closed flower	✓	✓	✓	✓	✓	✓	✓	✓	✓
S2	Open flower (petals)	✓	✓	✓	✓	✓	✓	✓	✓	✓
S3	Petal fall (no petals)	✓	✓	✓	✓	✓	✓	✓	✓	✓
S4	Fruitlet bud	✓	✓	✓	✓	✓	x	✓	✓	✓
S5	Immature fruit	✓	✓	✓	✓	✓	✓	✓	✓	✓
S6	Ripe fruit	✓	x	✓	✓	✓	✓	✓	✓	x
S7	Packhouse fruit	**	**	**	**	**	**	**	**	**
S8	Leaves	✓	✓	✓	✓	✓	✓	x	x	x
		Fungal species identified[b]								
		Alternaria spp.	*Aureobasidium pullulans*	*Botrytis* spp.	*Penicillium* spp.	*Pilidiella granati*	*Rhizopus stolonifer*	*Cytospora* sp.	*Nigrospora oryzae*	*Pestalotiopsis* sp.
S1	Closed flower	✓	✓	✓	✓	x	x	x	x	x
S2	Open flower (petals)	✓	✓	✓	✓	✓	✓	x	x	x
S3	Petal fall (no petals)	✓	✓	✓	✓	✓	✓	x	✓	✓
S4	Fruitlet bud	✓	✓	✓	✓	✓	x	x	x	✓
S5	Immature fruit	✓	x	✓	✓	✓	x	x	x	x
S6	Ripe fruit	✓	x	✓	✓	✓	x	x	x	x
S7	Packhouse fruit	✓	x	✓	✓	✓	x	x	x	x
S8	Leaves	✓	✓	✓	✓	✓	✓	x	x	x

[a] Fungal genera present on non-disinfected pomegranate plant material.
[b] Fungal genera present on disinfected pomegranate plant material.
Present (✓) and absent (x), ** Packhouse provided only surface disinfectant fruit

Table 2. The mean[a] (*n* = 10) percentage incidence of fungal pathogens on sterile and non-sterile pomegranate samples 'Herskawitz' and 'Wonderful' sourced from three orchards in Wellington area.

Pathogen	[*] Treatment	Cultivar	[°] Sample type(s) (S1)	(S2)	(S3)	(S4)	(S5)	(S6)	(S7)	(S8)
Alternaria spp.	S	Herskawitz	0.67^{f}	14.00^{bc}	12.67^{bcd}	10.00^{cde}	18.67^{b}	5.33^{def}	1.33^{f}	45.00^{a}
		Wonderful	0.67^{f}	14.00^{bc}	12.67^{bcd}	10.00^{cde}	18.67^{b}	5.33^{def}	1.33^{f}	45.00^{a}
	NS	Herskawitz	9.33^{efg}	37.33^{c}	34.67^{c}	18.67^{de}	14.67^{ef}	0^{g}	**	70.00^{a}
		Wonderful	11.33^{ef}	34.67^{c}	28.00^{cd}	12.67^{ef}	14.67^{ef}	7.33^{g}	**	56.67^{b}
Auriobasidium pullulans	S	Herskawitz	25.33^{a}	10.67^{cb}	13.33^{b}	4.67^{cd}	0^{d}	0^{d}	0^{d}	15.00^{b}
		Wonderful	23.33^{a}	10.00^{cb}	14.00^{b}	0^{d}	0^{d}	0^{d}	0^{d}	11.67^{cb}
	NS	Herskawitz	79.33^{a}	25.33^{cd}	16.67^{d}	0.67^{d}	0^{e}	0^{e}	**	68.33^{b}
		Wonderful	80.00^{a}	29.33^{c}	19.33^{cd}	0^{e}	1.33e	0^{e}	**	70.00^{ab}
Botrytis cinerea	S	Herskawitz	4.00^{g}	26.67^{bc}	29.33^{b}	15.33^{def}	22.67^{bcd}	41.33^{a}	20.67^{bcd}	18.33^{cde}
		Wonderful	4.00^{g}	21.33^{bcd}	28.00^{bc}	14.00^{defg}	20.00^{bcd}	8.07^{efg}	6.67^{fg}	18.33^{cde}
	NS	Herskawitz	14.0^{e}	42.00^{ab}	45.33^{a}	33.33^{bc}	29.33^{cd}	45.56^{a}	**	15.00^{e}
		Wonderful	13.33^{e}	33.33^{bc}	40.00^{abc}	28.67^{cd}	29.33^{cd}	20.00^{de}	**	$18.33d^{e}$
Penicillium spp.	S	Herskawitz	4.00^{de}	5.33^{de}	4.67^{de}	0.67^{e}	0.67^{e}	84.67^{a}	31.33^{c}	3.33^{de}
		Wonderful	2.67^{de}	2.00^{de}	4.67^{de}	8.00^{d}	3.33^{de}	90.00^{a}	68.67^{b}	3.33^{de}
	NS	Herskawitz	9.33^{bcde}	10.00^{bcde}	16.00^{b}	3.33^{efg}	2.67fg	96.00^{a}	**	3.33^{efg}
		Wonderful	10.67^{bcd}	8.00^{cdefg}	14.00^{bc}	4.67^{defg}	1.33g	93.17^{a}	**	8.33^{cdef}
Pilidiella granati	S	Herskawitz	0^{c}	2.67^{c}	1.33^{c}	7.33^{b}	24.00^{a}	3.33^{c}	2.67^{c}	1.67^{c}
		Wonderful	0^{c}	0.67^{c}	1.33^{c}	2.00^{c}	10.00^{b}	2.67^{c}	1.33^{c}	0.00^{c}
	NS	Herskawitz	0.67^{cd}	4.67^{bcd}	4.00^{bcd}	9.33^{b}	16.00^{a}	4.6^{7bcd}	**	5.00^{bcd}
		Wonderful	5.33^{d}	9.33^{b}	6.67^{b}	5.33^{bcd}	6.00^{bc}	5.33^{bcd}	**	0^{d}

[a]Means indicated by different letters in each row differed at the 95% level according to Fischer's least significant difference (LSD) test.
[*]Surface disinfected (S) vs. Non-surface disinfected (NS) pomegranate plant materials
[°]Description of sample type(s) is presented in Figure 1.
**Packhouse provided only surface disinfectant fruit

Table 3. Isolates of fungal genera, and their associated GenBank references

Genus	*STE-U ID	Genbank ID	Genbank Ref	Score (%)	Source
Alternaria sp.	7868	KT279816	K465761	100	Lecellier *et al.,* 2014
Alternaria sp.	7872	KT279818	JN383490	100	Lawrence *et al.,* 2012
Botrytis sp.	7866	KT279813	AJ716303	100	Staats *et al.,* 2005
A. Pullulans	7869	KT279811	FJ150875	100	Zalar *et al.,* 2008
Penicillium sp.	7865	KT279817	JN942857	100	Schoch *et al.,* 2012
Penicillium sp.	7871	KT279815	FJ491802	100	Van der Walt, 2010
Penicillium sp.	7870	KT279812	FJ491804	100	Van der Walt, 2010
P. granati	7864	KT279814	JN815313	99.7	Mirabolfathy *et al.,* 2012

*Isolates submitted to the culture collection at Stellenbosch University (STE-U), Department of Plant Pathology.

Table 4. Amplicon size and band sizes (bp) derived from the internal transcribed spacer (ITS1 and ITS2) of the nuclear ribosomal DNA and the 5.8S ribosomal RNA gene after restriction digest of the amplicon using Tas1 restriction enzyme.

Species	Assertion Number	PCR Amplicon	Tas1* Band sizes
Alternaria spp.	KM001	567	147-81-78-75
	KM043	548	147-81-78-75
	KM065	568	159-147-75
Penicillium glabrum	KM031	528	272
Penicillium spp.	KM034	569	287-191-75
	KM056	570	272
Penicillium crustosum	KM083	536	232-196-79
	KM108	500	232-196-79
Embellisia eureka	KM048	569	294-143-107-77-71
Aureobasidium pullulans	KM015	581	193-173-86
	KM045	576	193-173-86
Botrytis spp.	KM003	543	246-214
	KM063	539	246-214
	KM014	533	254-214
	KM054	541	246-214
Pilidiella spp.	KM138	614	305-95-92
	KM139	614	305-95-92

*Represents Tas1 enzyme restriction fragments >70 bp

Chapter 5

Investigating the effects of crab shell chitosan on fungal mycelial growth and postharvest quality attributes of pomegranate fruit

Abstract

Postharvest disease management of pomegranate fruit remains a critical challenge and the need for effective treatments is essential in-order to minimise crop losses. This study investigated the *in-vitro* and *in-vivo* antifungal activities of crab shell chitosan and fludioxonil (a registered postharvest fungicide) as a control against *Botrytis* sp., *Penicillium* sp. and *Pilidiella granati* isolated from pomegranate fruit. Mycelial growth inhibition was evaluated using potato dextrose agar amended with varying concentrations of 0 to 1 g/L for fludioxonil, and 0 to 10 g/L of chitosan. Complete mycelial growth inhibition was observed at 0.10 g/L and 10 g/L for fludioxonil and chitosan, respectively. Chitosan concentrations causing a 50% reduction in mycelial growth (EC_{50}) were 0.47, 1.19, and 2.21 g/L for *P. granati*, *Botrytis* sp., and *Penicillium* sp., respectively. While, for fludioxonil the EC_{50} concentrations were 0.02, 0.48, and 0.09 mg/L for *Penicillium* sp., *P. granati*, and *Botrytis* sp., respectively. For *in-vivo* investigation, pomegranate fruits were artificially inoculated with mycelial plugs of *Botrytis* sp., *Penicillium* sp. and *P. granati* and kept at 25 °C for 21 days. Chitosan effectively reduced rot incidence by 18-66% and was most efficient when applied as a preventative treatment regardless of cultivar ('Herskawitz' and 'Wonderful'). Additionally, chitosan treatments (0, 2.5, 7.5 and 15 g/L) were applied on minimally processed pomegranate arils as edible coating prior to packaging and storage at 4 °C for 14 days. The chitosan treated arils best maintained physico-chemical quality attributes and significantly lower microbial counts for mesophilic aerobic bacteria, yeast and moulds. This study showed that crab shell chitosan has a potential as a green fungicide for postharvest disease management of pomegranate.

***Keywords*:** antifungal, chitosan, diseases, pomegranate (*Punica granatum* L.)

1. Introduction

Pomegranate (*Punica granatum* L.) has become one of the most lucrative super fruit globally, due to the health benefiting properties such as vitamins, minerals, dietary fibre

and polyphenols (tannins, anthocyanins and ellargic acid) (Gil *et al.*, 2000; Bhowmik *et al.*, 2013; Mditshwa *et al.*, 2013). The antioxidant activities of pomegranates can neutralise nearly twice as many free radicals as red wine and seven times as many as green tea (Gil *et al.*, 2000; Seeram *et al.*, 2008). Clinical studies have shown that polyphenols from pomegranate can lower risk of heart disease and retard cancer progression (Stover & Mercure, 2007; Aviram & Rosenblat, 2012; Bhowmik *et al.*, 2013).

Pomegranates are colonised by various naturally occurring and/or pathogenic fungi and bacteria (Petersen *et al.*, 2010; Munhuweyi *et al.*, 2016). The colonisation of the fruit can be superficial, or may cause extensive internal postharvest spoilage and decay (Palou *et al.*, 2009; Munhuweyi *et al.*, 2016). Pomegranate disease management is predominantly achieved by the application of synthetic fungicides pre- and postharvest. One of the commonly applied fungicides during commercial handling of pomegranates and other pome fruit is fludioxonil (Palou *et al.*, 2009; Karaoglanidis *et al.*, 2011). However, intensive fungicide usage is limited due to public health concerns, and the emergence of fungicide-resistant strains of the various fungal genera. This has necessitated the need to explore alternatives for synthetic fungicides (Liu *et al.*, 2007). The alternative fungicides are aimed to either substitute or provide a compliment for synthetic fungicides.

Chitosan, a non-toxic biodegradable compound derived from the outer shell of crustaceans has shown potential as a new class of plant protectant against pre- and postharvest diseases of horticultural commodities (Bautista-Banos *et al.*, 2006). For instance, chitosan (1.5 and 2.0%) was found to effectively control postharvest anthracnose (*Colletotrichum gloeosporioides* Penz.) of 'Eksotika II' papaya (Ali *et al.*, 2010). According to Ali *et al.* (2010) the *in-vitro* mycelial growth inhibition of chitosan ranged between 90-100% and *in-vivo* disease symptoms were delayed by 3-4 weeks during 5 weeks' storage of papaya at 12 ± 1 °C. Interestingly, the authors found that chitosan coating was less effective at 2.0% compared to the 1.5% for fruit kept at ambient temperature (28 ± 2 °C). Furthermore, Xu *et al.* (2007) reported a synergistic effect between chitosan (1%) and grapefruit seed extract (0.1%) against postharvest fungal rot caused by *Botrytis cinerea* on 'Red globe' grapes incubated for 7 days at 25 °C. Similarly, Liu *et al.* (2007) investigated the inhibitory effects of chitosan on *B. cinerea* and *Penicillium expansum* rot of tomatoes stored at 2 and 25 °C for 21 and 3 days, respectively. The authors found that chitosan

strongly inhibited spore germination, germ tube elongation, *in vitro* mycelial growth, and disrupted the plasma membranes of spores for both pathogens.

In another separate study, Sayyari *et al.* (2016) investigated the effects of salicyclic acid, chitosan and salicylolyl chitosan treatments on chilling injury and nutritional quality of pomegranate 'Mallas Saveh' stored at 2°C for 5 months. Salicyloyl chitosan treatment alleviated chilling injury better than salicylic acid and chitosan alone. In addition, salicyloyl chitosan treatment gave fruit with higher hydrophilic and lipophilic antioxidant activity and this was associated with a high unsaturated to saturated free fatty acids ratio of ~9.6 mg/100g FW compared to 6 mg/100 g FW in control samples, which demonstrated higher membrane integrity as the treatment effectively delayed external and internal browning and electrolyte leakage (Sayyari *et al.*, 2016). All these studies showed that the application of chitosan has the potential to control fruit decay, delay the ripening process and maintain quality attributes of a fresh food product. However, there is limited information in the literature on the effective concentrations of crab shell chitosan, which could inhibit mycelial growth on pomegranate fruit (cvs. 'Herskawitz' and 'Wonderful'). Also, the role of chitosan as a preventative or curative alternative to synthetic fungicide for pomegranates has not been investigated.

Thus, the aims of this study were; (i) to investigate the antifungal efficacy of crab shell chitosan on fungal pathogens isolated from pomegranate cultivars (cvs.) 'Herskawitz' and 'Wonderful' fruit, and (ii) to elucidate on the role of crab shell chitosan as an effective edible coating for minimally processed pomegranate arils. To achieve these aims, three objectives were investigated; a) the *in-vitro* sensitivity of mycelial growth of *Botryti*s sp., *Penicillium* sp., and *P. granati* isolated from pomegranate fruit to varying concentrations of chitosan and fludioxonil (positive control); b) antifungal effect of crab shell chitosan as an edible coating on whole pomegranate fruit before infection (as preventative) and post infection (as curative); and c) the effects of chitosan as an edible coating on the physico-chemical and microbial quality of pomegranate arils during cold storage at 4°C for 14 days.

2. **Materials and methods**

2.1. Plant material

Pomegranate fruit cvs. 'Herskawitz' (an early season cultivar) and 'Wonderful' (a late

season cultivar) were aseptically harvested at commercial maturity from an orchard located in Wellington, Western Cape region, South Africa (GPS S33° 39.276 E18° 59.399). Fruit of uniform size, shape, and free of any external defects were selected and washed with tap water (1 min). Thereafter, samples were washed with 70% (v/v) ethanol for 30 s, 0.35% (v/v) sodium hypochlorite (NaOCl) for 2 min, and 70% (v/v) ethanol for 30 s as described by Fourie *et al.* (2002), then dried overnight under laminar airflow. This was done to avoid dirt and any contamination from unwanted surface flora. A separate batch of fruit (100 per cultivar) was set aside for aril processing.

2.1.1. Aril preparation and packaging

Processing and packaging of arils was performed at a commercial packhouse facility at 12 ºC, in accordance with good manufacturing practice guidelines recommended for food product. Pomegranate fruit husks were manually processed for aril extraction by trained personnel Crab shell chitosan (Sigma-Aldrich, Steinheim, Germany) was purified as described by Laflamme *et al.* (1999) and dissolved in 1% glacial acetic acid and pH adjusted to 5.6 to make a 15 g/L stock solution. Arils were uniformly mixed and divided into four equal lots for the following coating treatments: (a) control (untreated with chitosan), but arils were washed in 1% acetic acid v/v, (pH 5.6) in order to expose the samples to similar acetic acid concentration used in chitosan preparation; (b) 2.5 g/L chitosan; (c) 7.5 g/L chitosan; and (d) 15 g/L chitosan. Treatments were performed by dipping each batch of arils in respective solution for 60 s, thereafter drained with a colander, and collected on a tray before air drying. Aril portions of 125 g were weighed into 15.5 x 11.5 x 3.5 cm^3 polypropylene trays (Blue Dot Packaging, Cape Town, South Africa) that had been previously sterilised with ethylene oxide. The trays were heat sealed using a semi-automated machine (Food Processing Equipment, South, Africa) with a polymeric film POLYID® 107 polyethylene (thickness 55 µm; carbon dioxide permeability rate, 600-700 mL/m^2.day at 38 °C, 90% relative humidity and 1 Bar) provided by Barkai Ployon Ltd., (Kibbutz Barkai, Israel). A label of 7.0 x 3.8 cm^2 area was placed onto each package film to simulate the labels found in the retails market packages.

The packaged products were immediately cooled down to 2 °C whilst at the packhouse, before transportation in ice-packed cooler boxes fitted with data loggers (Gemini Data Loggers, United Kingdom) to the plant pathology research laboratory. The temperature

inside the cooler boxes ranged between 3.5-4.5 °C, on arrival. Packaged samples were stored at 4 °C and 95% for 14 d. Microbial and physico-chemical properties of pomegranate fruit samples were taken on fresh arils prior to packaging and storage as a baseline on day 0. Four packages from each experimental condition were taken for analyses on each sampling day 3, 7, 10 and 14.

2.2. Pathogen sampling

Fungal pathogens, *Botrytis* sp. (STE-U 7866), *Pilidiella granati* (STE-U7864) and *Penicillium* sp. (STE-U 7865) were previously isolated and characterised from naturally infected pomegranates (cv. Herskawitz) harvested from a commercial orchard located in the Wellington area, Western Cape, South Africa (GPS S33° 39.276 E18° 59.399). Virulence and pathogenicity of the isolates was verified by fulfilling the Koch's postulates. The pathogen isolates are stored in the Stellenbosch University, Department of Plant Pathology (STE-U) culture collection under the given accession numbers. Cultures were grown on Potato Dextrose Agar (PDA) (Merck Pty. Ltd., Modderfontein, South Africa) for 7-14 days at 25 °C before each trial.

2.3. Media preparation

Fludioxonil (Sigma-Aldrich, Steinheim, Germany) was dissolved in acetone to make a 10 g/L stock solution. Pomegranate packhouses make use of chlorinated water to sanitise the fruit and fludioxonil dips to cotrol postharvest spoilage of fruit. Crab shell chitosan (Sigma-Aldrich, Steinheim, Germany) was purified as described by Laflamme *et al.* (1999) and dissolved in 1% glacial acetic acid and pH adjusted to 5.6 to make a 10 g/L stock solution. The purification was achieved by extensive grinding of chitosan to a powder, washing repeatedly in distilled water, pelleting at low-speed centrifugation (Eppendorf AG, Hamburg, Germany), and then air drying. The chitosan sheets were subsequently solubilized by stirring in 0.25 M HCl, centrifuged at 13 000 g for 10 min at 4 °C to remove insoluble material, and precipitated by neutralisation with 2.5 M NaOH. The chitosan pellets, recovered by centrifugation at 25 000 g for 15 min, were thoroughly washed with deionised water to remove salts and lyophilised. Mycelial growth inhibition was tested on PDA amended with 0 (control), 0.05, 0.1, 0.5, 0.75 and 1.0 g/L fludioxonil, or with 0.15, 0.3, 0.45, 0.6, 0.75, 1.5, 3.0, 4.5, 6.0 and 10 g/L chitosan. In all cases, the final

concentration of acetone for all fludioxonil amended media was 0.1%, including control plates. All chitosan solutions were adjusted to pH 5.6 prior to amending the PDA, including the control.

2.4. In vitro assay

Mycelial plugs (5 mm diameter) taken from margins of an actively growing colony were placed in the centre of amended PDA plates with the mycelium facing down as described by Abd-alla & Haggag (2010). Plates were incubated at 25 °C in the dark and the radial colony growth was measured every 24 h for *Botrytis* sp. and *P. granati*, while *Penicillium* sp. a slow-growing pathogen was measured every 48 h. The diameter of each colony was measured twice perpendicularly per plate. Percentage inhibition relative to the control was calculated from the day 3 colony diameters for *Botrytis* sp. isolates, day 5 for *P. granati* isolates and day 8 for *Penicillium* sp. A total of 6 PDA plates per isolate were used for the *in-vitro* experiments consisting of two repetitions with 3 PDA plates per isolate.

2.5. In-vivo antifungal assay on whole fruit

Pomegranate fruit were artificially inoculated with the mycelia from fungal pathogens *Botrytis* sp., *Penicillium* sp. and *P. granati.* Healthy whole fruit samples were wounded using a sterile cork-borer (with 5 mm deep by 5 mm wide) to make two wounds per fruit, one on either side of the fruit. The wounding method applied was adopted from Palou *et al.* (2010) and was most suitable for pomegranate thick husk. For the preventative investigation (T1), wounded fruit were dipped in 15 g/L crab shell chitosan for 60 s, air dried (for at least 6 h). After air drying the fruit samples were inoculated with mycelial plugs (5 mm diameter) taken from margins of actively growing colonies of each respective fungal pathogen with mycelium facing down onto the wounded area. To evaluate the curative effect (T2), wounded fruit were first inoculated with the fungal pathogens and after 6 h of incubation period, the fruit samples were treated with the chitosan. Identical experiments were conducted using pomegranate cvs. 'Herskawitz' and 'Wonderful' treated with 1% v/v acetic acid adjusted to pH 5.6 using 1 M (NaOH) as controls. Fludioxonil was not applied as an edible coating in this experiment.

In all trials the fruit were kept at 25 °C in sterile plastic chambers containing sterile wet paper to ensure high relative humidity for 21 days. The percentage reduction in decay was

189 determined by expressing the difference in lesion diameter for treated fruit over the control
190 (disease inoculated fruit not exposed to chitosan), using day 4 lesion diameters for *P.*
191 *granati* (fast growing) and day 16 for *Botrytis* sp. and *Penicillium* sp., respectively. *In vivo*
192 experiments were made using 3 replicates (5 fruits per replicate) of artificially inoculated
193 fruits. The entire experiment was independently repeated once to validate the results. Fruit
194 were considered decayed when infected by any of the pathogens. Positive control fruit
195 inoculated with *P. granati* had the fastest growing lesion diameters of around 50-60 mm
196 after 4 days, which was 4-5 times fold larger than those observed for *Botrytis* sp. and
197 *Penicillium* sp. isolates. To get comparable lesion growth the lesion diameters for *Botrytis*
198 and *Penicillium* were evaluated at day 16.

199 ### *2.6. Physico-chemical analysis*

200 #### *2.6.1. Texture and mass loss*

201 Firmness of arils was measured using texture analyser (TA-XT Plus, Stable Micro
202 Systems, Surrey, England), with a 35 mm diameter cylindrical probe. Firmness was
203 expressed as maximum compression force (N). A test speed of 1.0 mm/s and distance of
204 9.5 mm were used. An average of 20 arils was measured individually for each experimental
205 condition. Mass of pomegranate arils was recorded on days 0, 3, 7, 10 and 14. Cumulative
206 losses in mass were calculated as the percentage of initial mass lost as described by Fawole
207 & Opara (2013).

208 #### *2.6.2. Total soluble solids and titratable acidity*

209 Arils (125 g) for each pack were juiced separately using a LiquaFresh juice extractor
210 (Mellerware, Cape Town, South Africa), and the juice was directly used for total soluble
211 solid (TSS) measurement using a digital refractometer expressed as% Brix (Atago, Tokyo,
212 Japan). Titratable acidity (TA) expressed as citric acid equivalent g/L was measured by
213 titration at an end point of pH 8.2 using a Metrohm 862 compact titrosampler (Metrohm
214 AG, Herisau, Switzerland). All values obtained for TSS and TA were presented as mean (n
215 = 4) ± standard error (S.E).

216 #### *2.6.3. Colour*

217 Aril colour was measured using a Minolta Chroma Meter CR-400 (Minolta Corp, Osaka,
218 Japan). Before each measurement, the apparatus was calibrated against a white tile

background (Illuminants C: Y = 93.6, x = 0.3133, y= 0.3195). Approximately 20 g of arils were placed into a Petri dish and the measurements were taken from 5 different points of the dish. Hunter colour parameters; *L** (lightness), *a** (redness and greenness) and *b** (yellowness and blueness) were measured. Chroma (*C**) values, which indicate the quantitative attribute of colour intensity and hue angle (*h*°) which is the qualitative of colour of sample was calculated using equations (1) and (2), respectively, (Pathare *et al.*, 2013). Results were presented as mean ± S.E. of 10 replicates.

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$h^\circ = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (2)$$

2.6.4. Ascorbic acid

Ascorbic acid was determined using a colourimetric method as described by Barros *et al.* (2007) with some modifications. Pomegranate juice (1 mL) was diluted with 1% metaphosphoric acid (MPA), the mixture was vortexed, then sonicated (Ultrasonic cleaner DC400H, United Scientific, Holon, Israel) for 5 min in cold water and centrifuged at 10,000 g for 5 min at 4 °C. The samples were diluted with 0.0025% 2,6-dichlorophenolindophenol dye and incubated in a dark environment for 10 min. Ascorbic acid concentration was measured spectrophotometrically using a UV-vis spectrophotometer (Thermo Scientific technologies, Madison, USA) at 510 nm wavelength. The concentration of ascorbic acid in pomegranate juice was quantified using a standard curve of known concentration (0.01-0.10 mg/L) of L-ascorbic acid (Sigma-Aldrich, Steinheim, Germany) and final results were expressed as mean (n = 4) ± S.E of ascorbic acid in crude juice (g/L).

2.6.5. Total anthocyanin content

Total anthocyanin content was determined by pH-differential method using 2 buffer systems comprising of potassium chloride (pH 1, 0.025 M) and sodium acetate (pH 4.5, 0.4 M). Pomegranate juice sample (1 mL) was mixed with 9 mL of pH 1.0 and pH 4.5 buffers, separately. Absorbance was measured at 520 and 700 nm and the total anthocyanin content was expressed as cyanidin-3-glucoside equivalents using the following equations:

$$A = (A_{520} - A_{700})_{pH1.00} - (A_{520} - A_{700})_{pH4.5} \quad (3)$$

$$\text{Total monomeric anthocyanin} = \left[\frac{A \times MW \times DF \times 100}{\varepsilon \times L}\right] \quad (4)$$

where A = Absorbance, MW = anthocyanin molecular weight (449.2) g/mol for cyanidin-3-glucoside; DF = dilution factor; L = path length (1 cm); ε = Cyd-3-glucoside molar extinction coefficient (26,900). All analyses were done as four replicates for each treatment. Final results are expressed as mean (n = 4) ± S.E. for cyanadin-3-glucoside equivalents (C_3gE) per litre of pomegranate juice (g/L).

2.6.6. Total phenolics content

Total phenolic concentration was determined in triplicate by the Folin-Ciocalteu (Folin-C) colourimetric method (Makkar *et al.*, 2007). This was carried out spectrophotometrically at 750 nm by adding 500 µL of 1 M Folin C and 2.5 mL of 2% sodium carbonate to 50 µL fruit juice extract. The mixture was incubated in the dark for 40 min. Results were expressed as the mean (n = 4) ± SE of gallic acid equivalent of crude juice (g/L).

2.6.7. Antioxidant activity

The antioxidant capacity of fruit juice was measured by the DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay. A methanolic extract of pomegranate juice sample (15 µL) was diluted with methanol (735 µL) in test tubes followed by the addition of methanolic DPPH solution (750 µL, 0.1 mM). The mixtures were incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm and compared with the standard curve (R^2 = 0.95) of ascorbic acid (0-2000 µM). The free-radical capacity of pomegranate juice was expressed as mean value (n = 4) ± S.E. of ascorbic acid (g) equivalents (AAE) per litre of juice (g/L). Ascorbic acid was used a standard of reference (despite being susceptible to oxidation to dehydro-ascorbic acid) because it allowed for the quantification of the rate of change among treatments. Similarly, measuring the total phenolic content would help track the the changes in phenolic content.

2.7. Microbial quality

Microbiological stability was screened by total plate count. Plate count agar (PCA) was used for aerobic mesophilic bacteria and for the yeast and mould counts potato dextrose agar (PDA) acidified with 10% tartaric acid. For indicator microorganisms *Escherichia coli*

were analysed using tryptone bile x-glucuronide (TBX) agar. Packages were opened under sterile conditions, and 10 g of each sample was obtained aseptically and homogenized with 90 mL of sterile physiological solution (PS). Further 3-fold dilutions were prepared using 1.0 mL of diluent into 9.0 mL of PS. In order to enumerate microbial load, 1.0 mL of each dilution was pour-plated in triplicate onto appropriate media, PCA for aerobic mesophilic bacteria and PDA for yeast and moulds. Plates for aerobic mesophilic bacteria were incubated at 37 °C for 2 days, *E. coli* were incubated for 2 days at 35 ºC and at 25 °C for 5 days for yeast and moulds. The microbial colonies were counted between 30 and 300, and results were presented as mean value (n = 6) ± S.E. expressed as log CFU/g.

2.8. Statistical analysis

The effective concentration inhibiting mycelial growth by 50% *in-vitro* (EC_{50}) was calculated by regression analysis. Calculations were performed using SAS (SAS Institute Inc., Cary, North Carolina, USA). All other experimental data were treated for analysis of variance (ANOVA) at 95% confidence interval to evaluate the effect of crab shell chitosan and storage duration on the quality attributes of pomegranate.

3. Results and discussion

3.1. In-vitro mycelial inhibition by chitosan and fludioxonil

Chitosan and fludioxonil had significant effects on the in vitro inhibition of fungal mycelial growth ($P < 0.05$) as shown in Table 1. Mycelial growth was observed to be markedly reduced by the increase in concentrations of fludioxonil and chitosan (Fig. 1). All the pathogens were completely inhibited at 1 g/L and 10 g/L of fludioxonil and chitosan, respectively. The effective concentration to reduce mycelial growth by 50% (EC_{50}) varied for fludioxonil and chitosan among the different pathogens ranging from 0.1 to 0.5 mg/L and 0.5 to 2.2 g/L for fludioxonil and chitosan, respectively (Table 1). For example, *P. granati* was the most sensitive pathogen to chitosan *in-vitro*, with an EC_{50} of 0.48 ± 0.01 mg/L, while, Penicillium sp. was the most resistant to chitosan with an EC_{50} of 2.21 ± 0.05 g/L. However, when exposed to fludioxonil, *Penicillium* sp. was the most sensitive (0.02 ± 0.01 mg/L) followed by *Botrytis* sp. (0.09 ± 0.0001 mg/L) and *P. granati* (0.4 7± 0.02 mg/L).

The findings from this study on the sensitivity of fungal pathogens to fludioxonil and chitosan compounds are in agreement with pervious results reported in literature (Li & Xiao, 2008; Badawy & Rabea, 2009; Karaoglanidis *et al*., 2011). For example, Karaoglanidis *et al*. (2011) reported EC_{50} sensitivity values to fludioxonil in the range of 0.13-0.47 mg/L for *P. expansum* isolated from various apple packing houses in Greece. Li & Xiao (2008) also reported lower EC_{50} values of 0.01-0.07 mg/L for fludioxonil against *P. expansum* isolated from apple fruit in the United States. Badawy & Rabea (2009) also reported comparable EC50 values of 1.4-2.4 g/L for chitosan of different molecular weights against *B. cinerea* isolated from tomato in Egypt. The *in-vitro* antimicrobial activity of chitosan has been demonstrated on a host of pathogens including *Colletotrichum gloeosporioides*, *B. cinerea*, *P. expansum*, and *Alternaria* sp. (Bautista-Baños *et al*., 2006, Xiangchun *et al*., 2012; Romanazzi *et al*., 2013).

Postharvest application of chitosan offers several advantages. As a polycationic biocide, chitosan can interfere with spore germination and mycelial growth of fungi by halting growth development (Yu *et al*., 2007; Romanazzi *et al*., 2013). To substantiate this, marked morphological contraction of mycelium and the development of granular inclusions in the cytoplasm were observed for *B. cinerea* treated with chitosan-based formulations in a study carried out by Reglinski *et al*. (2010). Additionally, chitosan can act as a biological elicitor, inducing host defence responses such as accumulation of β-1,3-glucanases and phenolic compounds, and through the induction of host tissue maceration enzymes thus boosting host resistance (Bautista-Baños *et al*., 2006). All control fruit were adjusted to pH 5.6 prior to the study was to match the pH of the chitosan solutions. This was to demonstrate that biochemical aspects related to infection, such as Ph, did not affect the infection process of the pathogen.

3.2. In-vivo antifungal assay for whole fruit

Antifungal sensitivity of *Botrytis* sp., *P. granati* and *Penicillium* sp. to crab shell chitosan *in-vivo* varied significantly ($P < 0.05$) depending on the pathogen type (Fig. 2). Chitosan treatment effectively halted fruit decay by the three pathogens when expressed as a percentage of the decay observed for the positive controls (inoculated fruit not exposed to chitosan). Crab shell chitosan as a preventative treatment provided 60-66%, 61-63% and 30-34% inhibition of *Botrytis* sp., *P. granati* and *Penicillium* sp., respectively (Fig. 2). The

preventative treatment against the fungal pathogens showed similar percentage of inhibition on both pomegranate fruit cultivars, except for the *Botrytis* sp. infected fruit where decay reduction was 66% for 'Herskawitz' and 60% 'Wonderful'. Application of chitosan as a curative treatment to pomegranate fruit that were pre-inoculated with fungal pathogens showed 19-24%, 32-39% and 21-22% inhibition of *Botrytis* sp., *P. granati* and *Penicillium* sp., respectively (Fig. 2).

Effective control of fungal diseases requires treatments that simultaneously provide both curative and preventative activities. The present study demonstrates that chitosan was most effective as a preventative treatment compared to the curative strategy. Protective fungicides have the advantage of being present on or in the plant before the pathogen arrival or begin to develop and prevent infection from occurring by acting as a protective barrier (Yu *et al*., 2007). the inhibitory effect of chitosan is considerably lower when applied after the pathogen is already established and actively growing within the infection site in the fruit peel (Wisniewski *et al*., 2001; Usall *et al*., 2008). Accordingly, Yu *et al*. (2007) observed that chitosan offers a preventative action in delaying the onset of disease rather than offering an enduring resistance or a curative effectiveness. The current study demonstrates that chitosan can prevent fungal growth development even on fruit with established fungal infections. This implies that chitosan can be applied as a plant protectant for pomegranate fruit prior to postharvest storage. Furthermore, in this study decay reduction by chitosan was least effective against *Penicillium* sp. in comparison to *Botrytis* sp. and *P. granati*. Differences in sensitivity to chitosan were also observed by Ramos-García *et al*. (2010) who reported that *Fusarium oxysporum* was most sensitive to chitosan at 0.5%, while *Penicillium digitatum* and *R. stolonifer* were least affected at 1.5% concentration.

3.3. Quality of coated minimally processed pomegranate arils

3.3.1. Texture and mass loss

At harvest the aril hardness for 'Herskawitz' 161.44 ± 2.98 N and 194.28 ± 2.48 N for 'Wonderful', are comparably higher than 79.8 N and 118.4 N reported by Fawole & Opara (2012) for the same cultivars respectively (Table 2). Aril hardness continuously declined during the 14 d storage at 4 °C. The interactions between chitosan treatment and

storage duration were significant ($P < 0.01$) for both pomegranate cultivars 'Herskawitz' and 'Wonderful'. Aril hardness values were notably higher for samples treated with chitosan, especially at the highest chitosan concentration of 15 g/L during cold storage. While control samples (untreated arils) recorded the highest textural losses of 7-19% on day 3 of cold storage and these losses increased to 49-51% after day 14 of storage. At the end of storage day 14, arils coated with 7.5 and 15 g/L chitosan showed the best texture retention (57%) compared to control samples (48-51%). These changes can be attributed to tissue senescence and, cell membrane deterioration as well as sample water loss (Fawole & Opara, 2013). According to Vargas *et al*. (2006), aril firmness retention is facilitated by chitosan's ability to reduce metabolic effects; thereby facilitating the retention of water loss and turgor pressure by the arils. Other, edible coatings applied on pomegranate arils, such as *Aloe vera* gel and starch, also delayed softening during storage and resulted in lower respiration rates and metabolic activity (Martínez-Romero *et al*., 2013; Oz & Ulukanli, 2012). Martínez-Romero *et al*. (2013) confirmed the retardation of respiration rate by *Aloe vera* gel by noting significant decreases in O_2 as carbon dioxide (CO_2) increased.

Mass loss of arils for both cultivars increased continuously during storage (Table 2). Chitosan treatment as well as storage duration had significant influence on the mass loss of arils ($P < 0.05$) as shown in Table 2. Chitosan treatment and storage duration had significant influence on the mass loss of arils ($P < 0.05$) as shown in Table 2. However, there was no significant interaction between chitosan treatment and storage duration for both cultivars; 'Herskawitz' ($P = 0.0738$) and 'Wonderful' ($P = 0.2212$). At the end of storage period, arils coated with 15 g/L chitosan had better moisture retention than the control arils. The chitosan effect on mass loss was similar for both cultivars, however, cv. 'Wonderful' presented slightly higher values (0.3-0.7%) compared to 'Herskawitz' (0.2-0.3%) at end of storage day 14 at 4°C. Ghasemnezhad *et al*. (2013) suggests that the chitosan film formed on the surface of the fruit delays the migration of moisture from the fruit into the environment, thus facilitating better moisture retention during storage. Edible coating of pomegranate arils can tremendously alleviate mass loss during storage. Oz & Ulukanli, 2012 showed that coating arils of pomegranate 'Silifke asısı (33 N 16)' with a starch based solution (containing *Nigella sativa* cold pressed oil) stored at 4°C for 12 days effectively reduced mass loss by 6-fold.

3.3.2. Total soluble solids and Titratable acidity

The initialtotal soluble solid (TSS) values recorded at harvest were 15.75 ± 0.05% and 16.50 ± 0.04% for cvs. 'Herskawitz' and 'Wonderful', respectively (Table 2). These values are comparable to those reported by Fawole & Opara (2012) for the same cultivars; 'Herskawitz' (15.5%) and 'Wonderful' (16.3%). A slight increase in TSS was observed until day 7, ranging between 16.30-16.53% and 16.40- 16.95% for cvs. 'Herskawitz' and 'Wonderful', respectively, but, thereafter declined. The increase in TSS was higher for the control samples in comparison to arils coated with chitosan. At the end of storage untreated arils (control) had the lowest TSS value of 14.30 ± 0.20% and 15.98 ± 0.07% for cvs. 'Herskawitz' and 'Wonderful', respectively, while chitosan (15 g/L) coated arils had 15.45 ± 0.26% and 16.30 ± 0.49%, respectively. The interaction between chitosan treatment and storage duration significantly ($P < 0.01$) influenced the changes in TSS for both cultivars. Similar patterns in TSS fluctuations were observed for both cultivars during the cold storage (4°C).

The decline in TSS after day 10 of cold storage is in line with the results presented by Zahran *et al*. (2015) who also reported a decline in TSS in fresh-cut 'Wonderful' coated with irradiated chitosan and stored at 5 °C for 15 d. The change in juice TSS can be attributed to the active hydrolysis of starch to sugars in fruit during storage (Fawole & Opara, 2013). Furthermore, Zahran *et al*. (2015) attributed the decrease in TSS to increased metabolic activities of pomegranate arils during storage, which included the conversion of soluble sugars into other organic acids such as citric, malic, oxalic and succinic. The authors suggested that the process is accelerated by packaging environments surrounded by a rich oxygen (O_2) concentration (Zahran *et al*., 2015).

Titratable acidity (TA) was notably higher for cv. 'Herskawitz' (18. 2 ± 0.10 g/L) compared to that for cv. 'Wonderful' (14.20 ± 1.2 g/L) at harvest. Considerable variation was observed in TA during storage for both cultivars and these changes were mainly driven by storage duration ($P < 0.01$) and chitosan treatment ($P < 0.05$). However, the interaction between these factors was only significant for 'Wonderful' ($P = 0.0188$) and not for 'Herskawitz' ($P = 0.7905$). An initial increase in TA for all the arils was observed during the first 10 d of storage, and thereafter declined significantly regardless of treatment. The increase in TA was generally slower for arils treated with 7.5 and 15 g/L chitosan compared

to 2.5 g/L chitosan and control arils. After 14 d of cold storage at 4°C, the TA levels had increased by 8-17% for control arils and remained stable (0-3% changes) for arils coated with chitosan (15 g/L). An increase in acidity can be considered as a positive characteristic from an acceptance point of view for low acidic cultivars such as 'Mollar de Elche' can be (Martínez-Romero *et al*., 2013). In corroboration with the findings from this study, Ghasemnezhad *et al*. (2013) reported an increase in TA over time with the least increments being observed for the highest chitosan concentration of 0.5 and 1% in pomegranate 'Tarom' after 12 days at 4 °C.

At harvest, the TSS/TA ratio for 'Wonderful' was 11.9 and fluctuated from 10.02-13.0 during storage. Other studies recorded slightly higher TSS/TA ratio values of 14.3-15.8 for cv. 'Wonderful' at postharvest (Fawole & Opara, 2013; Zahran *et al*. 2015). The TSS/TA ratio of cv. 'Herskawitz' was 8.66 ± 0.05 and this was comparably lower than that for 'Wonderful' of 11.87 ± 1.01 at harvest (Table 2). Average TSS/TA values remained constant for both cultivars during the first 7 days of cold storage at 4˚C. Changes in TSS and TA during postharvest storage resulted in increases in TSS/TA during the first 7 days and a gradual decline thereafter. A gradual decline in TSS/TA was observed form day 10 of storage and this was most pronounced for 'Herskawitz' control arils. Both the chitosan and storage duration had significant effects on the TSS/TA ratio regardless of cultivar ($P < 0.05$). The chitosan-treatments maintained high aril juice TSS/TA ratio compared to control. Other studies recorded slightly higher TSS/TA ratio values of 14.3-15.8 for cv. 'Wonderful' at postharvest (Fawole & Opara, 2013; Zahran *et al*. (2015). This may be attributed to the variation in TA values observed in that study compared to the current study. The ratio of TSS/TA is considered to be a good indicator of fruit maturity (as sour, sweet-sour or sweet), this is largely dependent on cultivar and agro-climatic regions (Mditshwa *et al*., 2013). The TSS/TA ratio for the arils was found to be consistent during storage for arils treated with 7.5 and 15 g/L chitosan.

Suppression in changes in overall TSS/TA ratio by chitosan is attributed to the inhibitory effect of chitosan on respiration and other bioactivities occurring in arils that consume sugars (Zahran *et al*., 2015). This is achieved by formation of an oxygen barrier on fruit surface leading to reduced metabolic rates and consequently, less acidity variation in chitosan-treated fruits (Ghasemnezhad *et al*., 2013). In addition, Meighani *et al*., (2015)

reported that postharvest application of carnauba wax on pomegranates for120 days storage at 4.5 °C and 3 additional days at 20 °C could significantly lower respiration rate and weight loss better than chitosan. The combining of carnauba wax with chitosan can considerably improve pomegranate postharvest and the respiration will be controlled while chitosan enhances the antimicrobial safety of the fruit due to its antimicrobial properties.

3.3.3. Colour

The colour attributes of the pomegranate arils treated with chitosan and their controls are shown in Table 3. At harvest, 'Wonderful' had comparably lower values of C^* (20.02 ± 0.93), a^* (17.85 ± 0.83), and L^* (18.27 ± 1.33) when compared to 'Herskawitz' of C^* (24.76 ± 1.48), a^* (23.09 ± 1.26), and L (24.37 ± 2.57). In contrast, 'Herskawitz' had lower $h°$ (20.25 ± 1.12) and b^* (8.57 ± 0.92) values to that for 'Wonderful' at $h°$ (27.02 ± 1.43) and b^* (9.15 ± 0.65). Aril redness (a^*) and colour intensity (C^*) were 23% and 20% higher for 'Herskawitz' than for 'Wonderful' at harvest.

Colour parameters C^*, $h°$, L, and b^* varied significantly ($P < 0.05$) among the chitosan treatments (Table 3). The storage duration was the main driving factor ($P < 0.05$) for changes in aril chroma (C^*), lightness (L^*), redness (a^*), and yellowness (b^*), indices for both cultivars. All the colour indices increased for both cultivars especially during the first 10 d of storage. Chitosan application had a significant ($P < 0.05$) effect on $h°$ ($P = 0.0032$) and b^* ($P = 0.0072$) of cv. 'Herskawitz'. Furthermore, the interaction between chitosan treatment and storage duration was also significant for $h°$ ($P = 0.0022$) and a^* ($P = 0.0180$) of the same cv. 'Herskawitz'. The changes/fluctuations in colour attribute observed during storage were not always significant ($P > 0.05$) among the treatments. As result, despite the variations in colour stability over the 14 days of storage at 4 ° C, the changes in colour were not statistically lower from the harvest data across all treatments for both cultivars.

Aril colour measured as CIE C^*, $h°$ L^*, a^*, and b^* during cold storage at 4 °C for 14 days did not change significantly ($P > 0.05$) among the chitosan treatments for both cultivars. These findings are also in agreement with data reported for other minimally processed pomegranate aril cultivars (Caleb *et al*., 2013a; Banda *et al*., 2015; Ayhan & Eştürk, 2009). The colour of arils can be positively correlated with the increase in anthocyanin content (Martínez-Romero *et al*., 2013). Therefore, the change in colour may

be correlated with either decrease or accumulation of these plant pigments (Miguel *et al*., 2004). The findings from this study indicate that dipping of pomegranate arils in chitosan better-maintained aril colour attributes especially at 7.5 and 15 g/L.

Colour attributes of fresh produce influences consumer's choice and preferences and is determined by the chemical, biochemical, microbial and physical changes which occur in produce over time (Pathare *et al*., 2013). Cultivar differences have also been implicated to play a role in the way colour attributes change over time. In the current study the changes in colour parameters did not differ significantly between the two cultivars 'Herskawitz' and 'Wonderful'. Similar findings were reported by Fawole & Opara (2013) who observed negligible changes in aril colour for 'Ruby' and 'Bhagwa' during postharvest storage irrespective of storage condition. However, significant changes in colour intensity (C^*) were observed over time for 'Ruby' and not in 'Bhagwa' (Fawole & Opara 2013) as well as in 'Acco' and 'Herskawitz' (Caleb *et al*., 2013b) under similar storage conditions.

3.3.4. Ascorbic acid

The ascorbic acid activity of coated and uncoated arils is presented in Fig 3 A-B. 'Herskawitz' recorded a high ascorbic acid content of 1.78 ± 0.01 g/L when compared to 'Wonderful' (1.70 ± 0.03 g/L) on day 0. Ascorbic acid activity decreased significantly during storage. A 3-6% decline in ascorbic acid was observed on day 3 for cv. 'Herskawitz' (1.60-1.79 g/L) and cv. 'Wonderful' (1.60-1.68 g/L). On day 10 of storage, the losses in ascorbic acid ranged between 4-12% ('Wonderful') and 16-27% ('Herskawitz'). A further decline was observed at the end of storage and was highest in control arils than those treated with chitosan. After 14 days of cold storage, the concentration of ascorbic acid had declined by about 11-24% from 1.70 ± 0.03 g/L (at harvest) to range between 1.29-1.51 g/L for 'Wonderful' and by 16-27% from 1.78 ± 0.01 g/L (at harvest) g/L to range between 1.30-1.50 g/L for cv. 'Herskawitz'. Chitosan treatments and storage duration had a significant effect ($P < 0.01$) on the ascorbic acid content of the arils. On day 14 the control arils had lost 24-31% while those treated with of 15 g/L had recorded losses of only 2-5% in ascorbic acid. The losses in ascorbic acid were slightly higher for 'Herskawitz' compared to 'Wonderful'. Treating the arils held back ascorbic acid reduction across the treatments with statistical significance ($P < 0.05$) being observed especially for the 15 g/L dosage.

The ascorbic acid concentration at harvest for 'Wonderful' (1.70 g/L) and 'Herskawitz' (1.78 g/L) were comparable to those reported in other studies of pomegranates. For instance, Zahran *et al*. (2015) reported ascorbic acid content of 1.25 g/L for 'Wonderful', and Mirdehghan *et al*. (2006) reported 1.06 g/L for 'Mollar de Elche'. Chitosan treated arils maintained higher concentration of ascorbic acid throughout this experiment. Similarly, high ascorbic acid concentration in the chitosan treatments pomegranates was reported by Zahran *et al*. (2015). This observation was attributed to limited O_2 supply caused by the barrier effectiveness imposed by chitosan coating, thereby suppressing the oxidation of ascorbic acid (Zahran *et al*., 2015).

3.3.5. Total anthocyanin content

The total anthocyanin content reported here as cyanidin-3-glucoside a major anthocyanin pigment in pomegranate as reported by Varasteh *et al*. (2012) dropped with storage duration for all treatments (Fig. 3 C-D). At harvest, total anthocyanin content was 1.65 ± 0.02 g/L and 1.31 ± 0.04 g/L of pomegranate juice for 'Herskawitz' and 'Wonderful', respectively. These findings corroborated the report by Ghasemnezhad *et al*. (2013) who recorded 10-19% decline in anthocyanin content of 'Tarom' pomegranate during cold storage. The concentration of chitosan coating and storage duration had significant ($P < 0.01$) effects on the retention of total anthocyanins by the arils. Increasing the chitosan concentration treatment resulted in a lower reduction in anthocyanin content over time. An approximate 2-12% loss in anthocyanins was observed for both cultivars after three days of cold storage to give 1.05-1.18 g/L and 145-1.63 g/L for 'Wonderful' and 'Herskawitz', respectively. On day 10 control arils of either cultivar had lost between 18-34% total anthocyanin content while the losses for arils treated with 15 g/L ranged between 9-14%. After 14 days of storage, the arils treated with chitosan retained about 80-85% anthocyanin content and this was significantly ($P < 0.05$) higher than control samples (64-66%). The final anthocyanin contents ranged between 0.86-1.04 g/L and 1.06-1.41 g/L for cvs. 'Wonderful' and 'Herskawitz', respectively.

Pomegranate arils pre-treated with chitosan retained higher anthocyanin contents when compared to the untreated controls. A similar observation was reported by Ghasemnezhad *et al*. (2013) who found higher anthocyanin content (0.72 g/L) for arils

553 treated with Chitosan (1%) in comparison to control arils (0.65 g/L). In contrast to the
554 findings from this study, minor increases in anthocyanin content were reported for chitosan-
555 coated 'Rabbab-e-Neyriz' pomegranate after the first 45 days of refrigerated storage with
556 higher values recorded in control fruit (Varasteh *et al*., 2012). Anthocyanin synthesis
557 continues in harvested whole fruit even at low storage temperatures (Fawole & Opara,
558 2013). However, according to Varasteh *et al*. (2012) postharvest treatments of fresh-cuts
559 may affect anthocyanin biosynthesis and/or degradation. Postharvest procedures that
560 enhance anthocyanin content preservation are of great benefit as they determine colour
561 while exhibiting a wide range of biological, pharmacological, anti-inflammatory, ant
562 oxidative, and chemo-protective properties (Fawole & Opara, 2013). The chitosan barrier
563 effect is presumed to modify the fruit's endogenous CO_2 and O_2 levels, which could result
564 in reduced O_2 supply required for the enzymatic oxidation degradation of anthocyanin
565 (Bautista-Baños *et al*., 2003; Ghasemnezhad *et al*., 2013). Reduction in anthocyanin
566 content could not be directly correlated with changes in colour measurements probably
567 because the degradation of the anthocyanins was not significant enough to affect overall
568 aril colour attributes.

569 *3.3.6. Total phenolics*

570 There was a significant interaction effect of chitosan treatment and storage duration
571 on total phenolics for both cultivars ($P < 0.05$). A high content of total phenols was recorded
572 in pomegranate juice of cv. 'Herskawitz' (3.74 ± 0.15 g/L) than 'Wonderful (2.40 ± 0.07
573 g/L) at harvest (Fig. 3 E-F). Furthermore, cv. 'Herskawitz' recorded the highest total
574 phenolic content of 2.09-3.74 g/L during the 14 days of cold storage at 4 °C when compared
575 to 'Wonderful' (1.25-2.40 g/L). The total phenolic concentrations for the two cultivars
576 investigated in this study were within the range of 0.1-5.3 g/L observed for eight
577 pomegranate cultivars grown in different regions of South Africa by Fawole & Opara
578 (2012). An even wider variation in phenolic content of 4.2-9.3 g/L was reported for eight
579 pomegranate cultivars grown in Iran by Mousavinejad *et al*. (2009).

580 Total phenolic content decreased with increase in storage duration for both
581 cultivars. Chitosan treatment, storage duration and their interaction had significant ($P <$
582 0.05) effect on total phenolic content for both cultivars. Higher concentration of total
583 phenolics was found in pomegranate arils treated with higher concentration of chitosan of

7.5 and 15 g/L. Losses in total phenolics ranged between 20-21% for untreated control arils, while arils treated with 15 g/L chitosan had 5-7% loss after 3 d of storage. At the end of storage day 14, the total phenolic content had declined significantly for both cultivars by at approximately 44-48% in the controls and by 36% for chitosan coated arils (15 g/L). Total phenolic content for control arils was 2.09 ± 0.10 g/L for 'Herskawitz' and 1.25 ± 0.02 g/L for 'Wonderful' on day 14. Chitosan coating effectively suppressed the decline in aril phenolic content during storage for both pomegranate cultivars. This was evidenced by a high content in total phenols that could be correlated with an increase in chitosan concentration.

Total phenolic concentration was more stable for arils treated with 7.5 and 15 g/L chitosan, but declined with increase in storage duration (Fig. 3 C-D). Similarly, Ghasemnezhad *et al*. (2013) also observed significant retention of total phenolics by as much as 74, 67, and 65% in arils pre-treated 0.25, 0.5 and 1.0% chitosan, respectively, when compared to 63% retained by the control arils. The decline in phenolics is associated with loss in astringency during fruit maturation (Fawole & Opara, 2013). Liu *et al*. (2007) ascribed the decrease in phenolic compounds at the end of storage to the cell structural breakdown, as part of senescence process during storage. In contrast to these findings Zahran *et al*. (2015) reported an increase from 0.9 to 1.2 g/L for total phenols in fresh-cut pomegranate 'Wonderful' during 15 d of storage at 5 °C, regardless of whether they had been pre-treated with irradiated chitosan or not. Ghasemnezhad *et al*. (2013) suggested that chitosan probably induces phenolic contents in plants while also delaying changes in contents of anthocyanin and polyphenol peroxidase activity in fresh-cut 'Tarom' during cold store

3.3.7. Antioxidant activity

The initial antioxidant activity (DPPH radical scavenging capacity) was 1.22 ± 0.01 and 0.97 ± 0.02 g/L of crude juice for 'Herskawitz' and 'Wonderful', respectively (Fig. 3 G-H). Similarly, Çam *et al*. (2009) reported that cultivar variation may affect the antioxidant activity of pomegranate juice resulting in varying levels of sweet and sourness Antioxidant activity was significantly affected by both chitosan and storage duration ($P < 0.05$). However, the interaction between these two factors was only significant for

'Wonderful' (P = 0.0159) and not for 'Herskawitz' (P = 0.2171). The total antioxidant activity gradually decreased over time. Losses in antioxidant activity were quite negligible (< 2%) for arils coated with chitosan (15 g/L), after 3 days of storage, while control arils had lost 13-27%. At the end of day 10, approximately 8-27% losses were observed for chitosan (15 g/L) coated arils while control arils recorded 17-32% losses in antioxidant activity. Loss in total antioxidant activity was about 16-27% for 15 g/L, 18-32% for 7.5 g/L, and 25-32% for 2.5 g/L chitosan coated arils, while for the controls was 28-33% at the end of storage day 14. Antioxidant activity values ranged between 0.88-1.03 g/L for 'Herskawitz' and 0.58-0.70 g/L for 'Wonderful' after 14 days of cold storage. In comparison to fruit at harvest, the antioxidant activity declined by approximately 16-30% and 27-33% after 14 days of storage at 4 °C for 'Herskawitz' and 'Wonderful', respectively.

Previous studies observed that there was a positive correlation between antioxidant activity and total phenolic content (Mirdehghan *et al*., 2006; Ghasemnezhad *et al*., 2013). Mirdehghan *et al*. (2006) correlated total antioxidant activity to high levels of total phenolics and to a lesser extent to ascorbic acid and anthocyanin content of heat treated 'Mollar de Elche' pomegranate. Therefore, the change in total antioxidant capacity in the current study could probably be attributed to the decline in total anthocyanin and phenolic content. In contrast to this study, Zahran *et al*. (2015) recorded an increase of around 50% from 0.3-0.45 g/L ascorbic acid equivalent in antioxidant activity for minimally processed 'Wonderful' pomegranate arils treated with irradiated chitosan and stored at 5 °C for 15 d. The authors also observed an increase in the total phenolic content but a decline in the total anthocyanin content. Çam *et al*. (2009) suggest that antioxidants may react in different ways depending on the antioxidant assay resulting in no direct correlation existing between the total phenolic content and antioxidant activity

3.3.8. Microbial quality

Chitosan treatment and storage duration as well as their interaction had significant influence (P < 0.05) on microbial growth for both cultivars. At harvest, initial aerobic mesophilic bacteria and yeast and mould counts on fresh arils were below 1 log CFU/g. The yeast and mould count remained below detection limit (<1.00 log CFU/g) for arils coated with 7.5 and 15 g/L chitosan for both cultivars until day 3 of storage for 'Herskawitz' and until day 7 for 'Won- derful' (Fig. 4). Increase in total aerobic mesophilic bacterial

count followed a similar trend to that observed for fungal growth for both cultivars. After 14 days of cold storage only arils treated with 7.5 and 15 g/L chitosan recorded aerobic mesophilic bacterial count below 7 log CFU/g, which is the maximum limit for fresh/fresh-cut fruit (FCDA, Act 54, 1979). Also, by day 7 of storage the yeast and mould counts for untreated arils 'Herskawitz' (5.4 log CFU/g) exceeded the legislated maximum limit of 5 log CFU/g for fresh/fresh-cut fruit (FCDA, Act 54, 1979).

Treatment with 15 g/L chitosan significantly reduced microbial growth compared with other chitosan concentrations and the control. Similarly, Ghasemnezhad *et al*. (2013) who reported lower bacterial counts of approximately 40–50 CFU/mL in arils treated with 0.25–1.0% chitosan compared to 70 CFU/mL in con- trol samples. The antimicrobial activity of chitosan in reducing microbial populations and fruit decay has been shown in previ- ous reports (Bautista-Banos *et al*., 2003; Badawy & Rabea, 2009; Ghasemnezhad *et al*., 2013). The antimicrobial activity of chitosan is largely attributed to amine group that alters the cell structure of microbial membranes leading to their disruption. Furthermore, it was observed in this study that arils treated with chitosan did not present symptoms of gas build up in the package headspace com- pared to untreated control samples at the end of storage (Fig. 5).

Headspace gas composition was in the range of 10–20% CO_2 and 4–12% O_2 for arils treated with chitosan, and ≈26% CO_2 and <0.05% O_2 for untreated control samples at the end of day 14. Accumulation of gas in package headspace has been suggested to be a combination of produce respiration and microbial spoilage effects (Caleb *et al*., 2013a, b). There was no *E. coli* detected in all the arils for both control samples and those coated with chitosan. The absence of *E. coli* colonies on any of the arils throughout the duration of stor- age in this study highlights the importance of good-agricultural and manufacturing practices in the processing and packaging of fresh arils.

4. Conclusion

This study has demonstrated the *in-vitro* anti-fungal activity of crab shell chitosan in inhibiting the growth of *Botrytis* sp., *Penicillium* sp. and *P. granati.* The species-specific sensitivity to chitosan was demonstrated in this study and *P. granati* was identified as the most sensitive to chitosan at 0.47 g/L. Application of crab shell chitosan coating (15 g/L) as an edible coating for whole pomegranate fruit was effective in controlling fungal growth

both as a preventive and curative treatment. The results from this study indicate that crab shell chitosan might be effective in controlling postharvest fungi and fruit quality in pomegranate. Both *in-vitro* and *in-vivo* experiments obtained significant evidence to recommend further studies on upscaling the chitosan application for commercial purposes as an integrated postharvest treatment method.

Chitosan, being a natural, biodegradable compound offers the possibility for the development of safe fungicide and/or food preservatives. Chitosan application to minimally processed pomegranate arils provided beneficial attributes in maintaining quality of the arils (firmness, colour and bioactive compounds) compared to untreated samples. In addition, the growth of spoilage microorganisms was significantly reduced with the application of chitosan compared with control. Chitosan coatings extended the shelf life base for fresh-cut pomegranate from less than 7 days to 10 days. Future studies on investigating the mechanism of pathogen inhibition by chitosan. Further research is also required to understand the physiological response of fruit to chitosan treatment.

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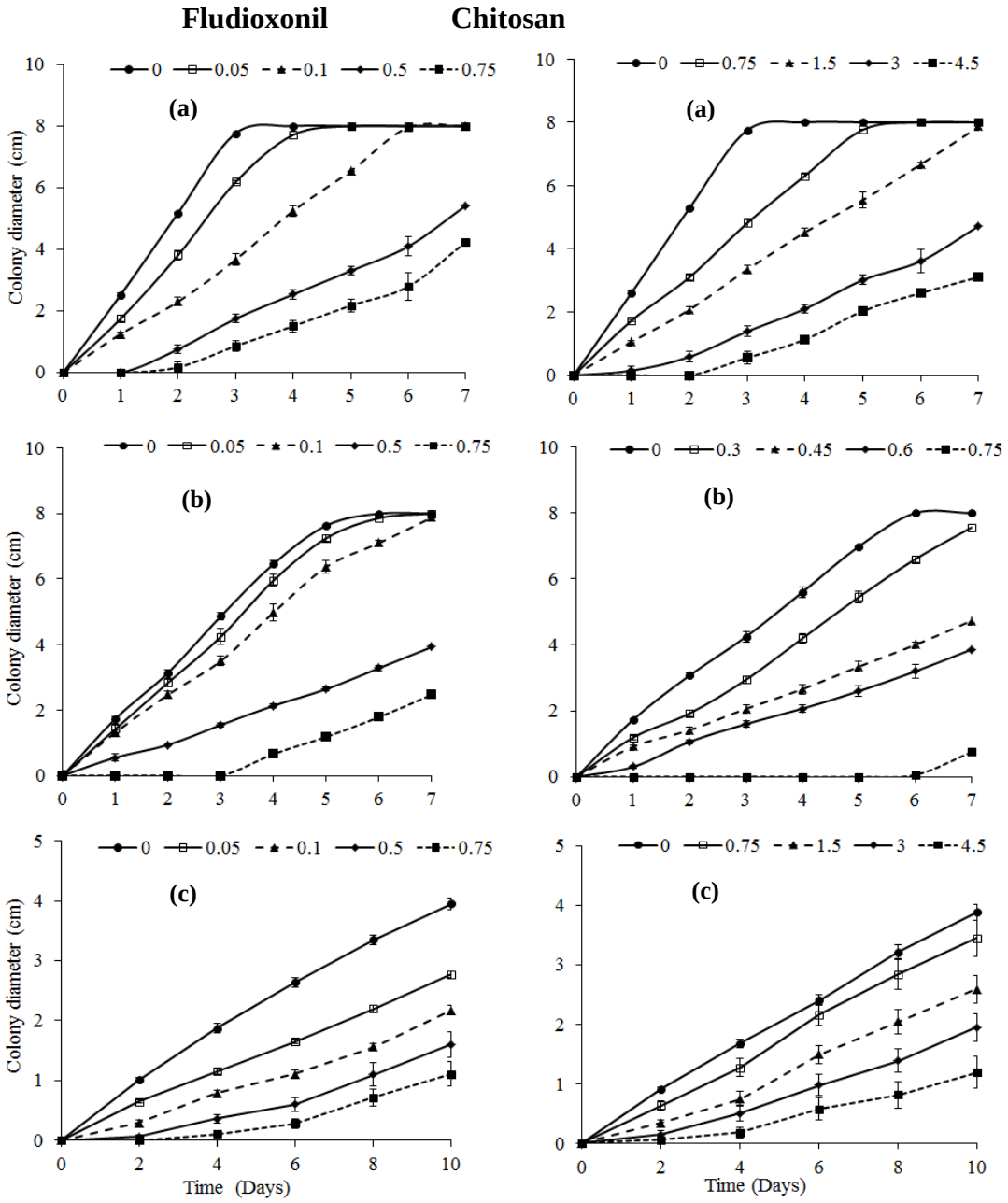

Figure 1 Mycelial growth inhibition of pathogens *Botrytis* sp. **(a)**, *P. granati* **(b)** *and Penicillium* sp. **(c)** isolated from pomegranate fruit by fludioxonil (mg/L) and chitosan (g/L) at varying concentrations.

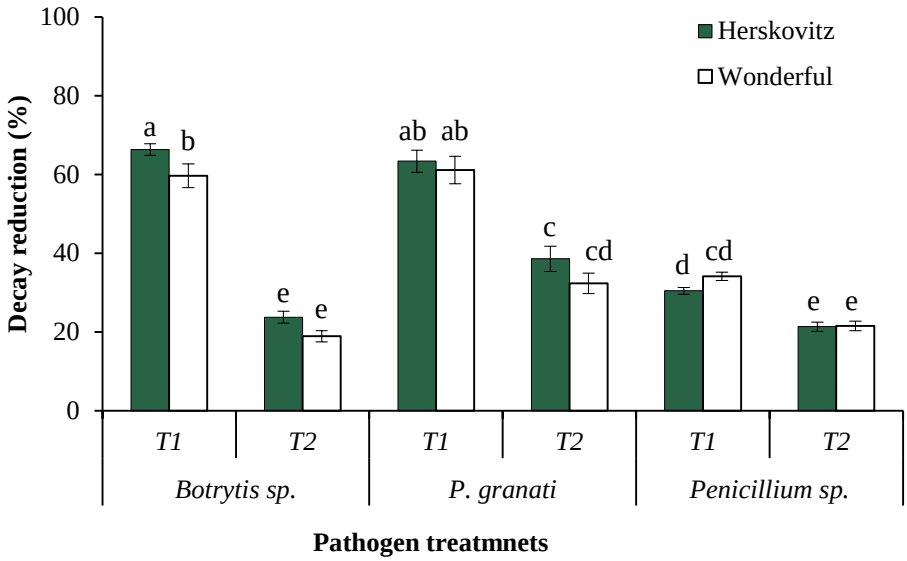

Figure 2 Decay reduction of pomegranate postharvest rots *Botrytis* sp., *P. granati* and *Penicillium* sp. treated with chitosan (15 g/L) as a preventative (T1) and curative (T2) antifungal agent at 25 °C and evaluated on day 4 for *P. granati* and for *Botrytis* sp. and *Penicillium* sp. on day16. Bars represent mean (n = 30) ± standard error, and bars with same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$)

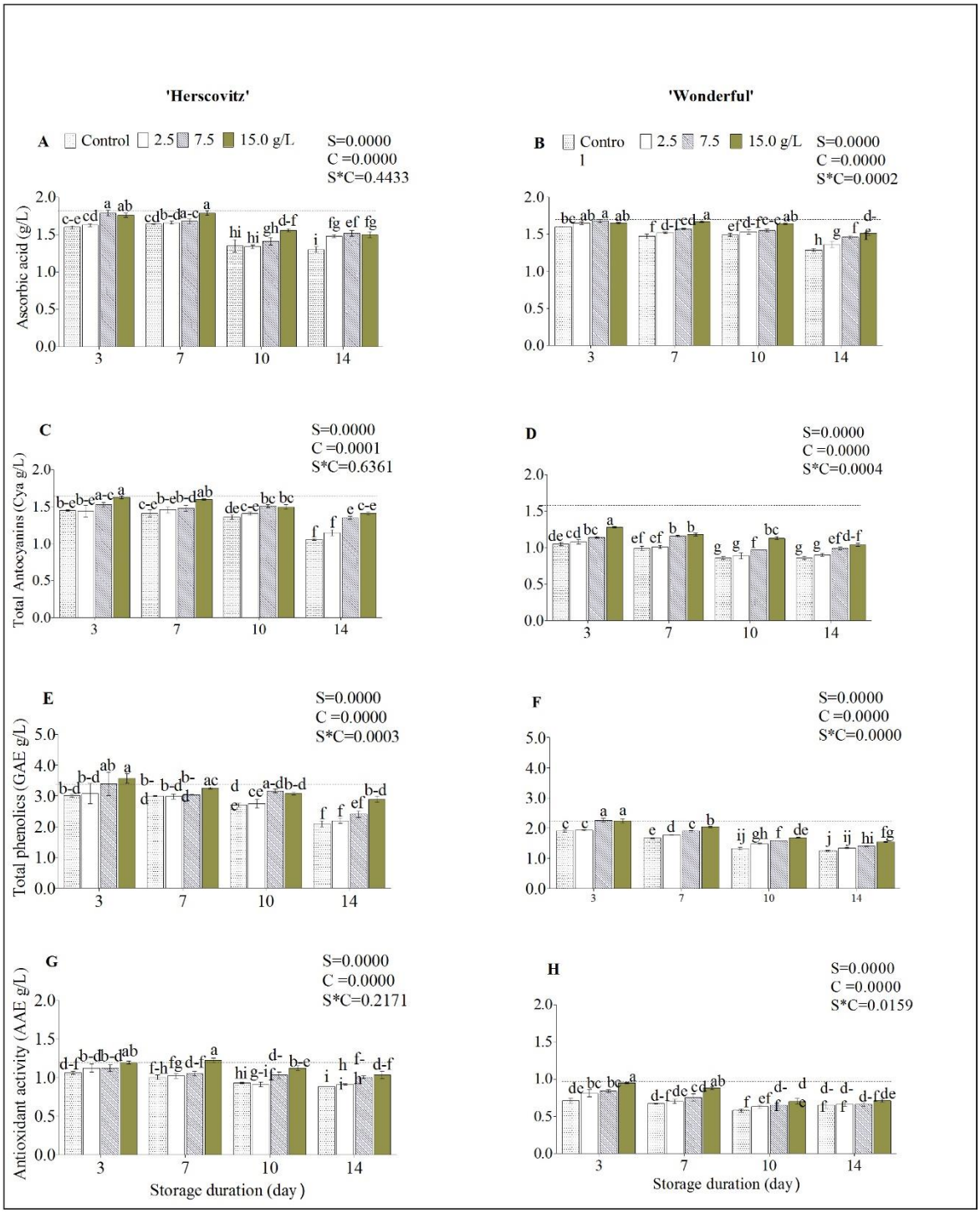

Figure 3 Changes in ascorbic acid (A-B), total anthocyanins (C-D), total phenolic content (E-F) and antioxidant activity (G-H) of pomegranate cvs. 'Herskawitz' and 'Wonderful' arils treated with crab shell chitosan stored at 4 ± 0.5 °C for 14. Each column represents mean value (n = 4) ± S.E and values with different letters are statistically different according to Duncan's test ($P < 0.05$). Continuous line represents mean value at harvest (day 0); experimental factors: Chitosan treatment (C) and Storage duration (S).

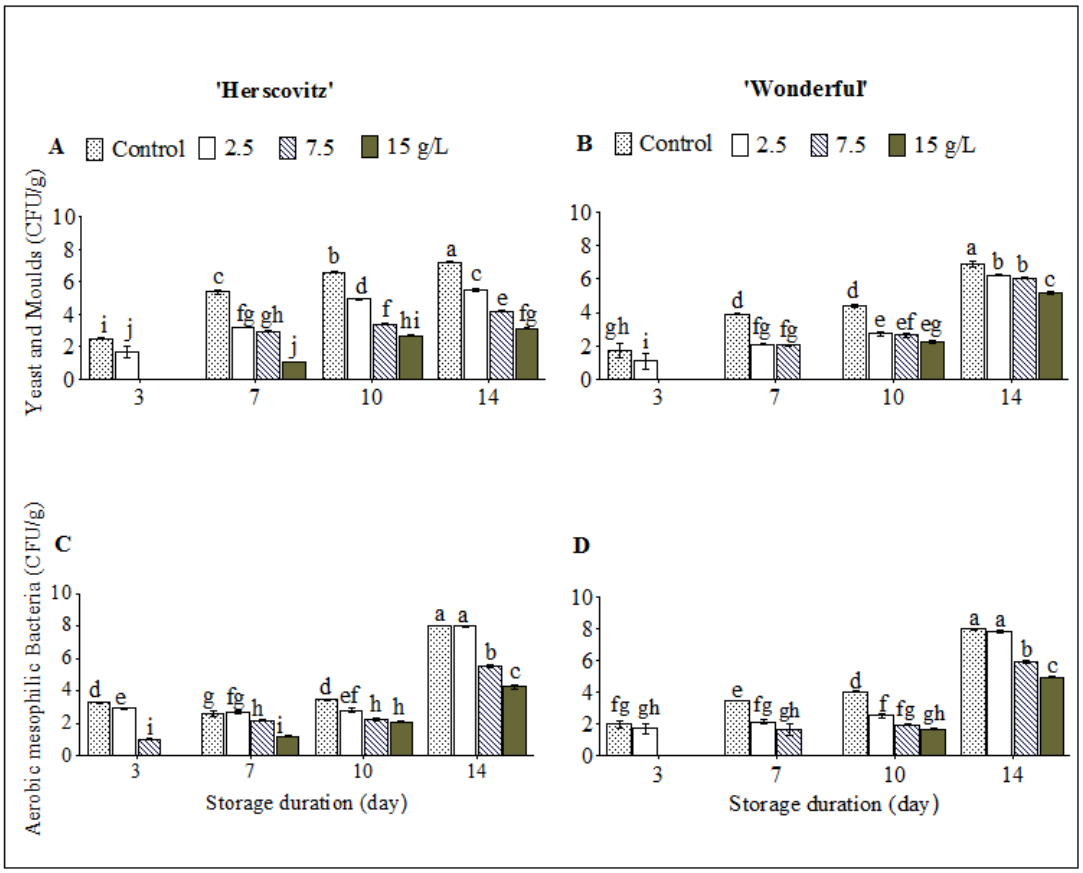

Figure 4 Effect of crab shell chitosan on growth of yeast and moulds (A-B) and aerobic mesophilic bacteria (C-D) and in pomegranate arils 'Herskawitz' and 'Wonderful' stored 4 °C for 14 days. Columns represent mean (n = 6) ± S.E for control and crab shell chitosan treatments at 2.5, 7.5 and 15 g/L respectively. Values with different letters are statistically different according to Duncan's test ($P < 0.05$).

Fig. 5. Visual observation and comparison of bulged package for untreated (control) 'Wonderful' pomegranate arils (a) and packaged arils treated with 15 g/L chitosan (b), after 14 days of storage at 4 ºC.

Table 1. Sensitivity (EC_{50}) of *Botrytis* sp., *P. granati and Penicillium* sp. to fludioxonil and chitosan based on *in-vitro* inhibition of mycelial growth.

Pathogen	Mean *EC_{50} values	
	Fludioxonil (mg/L)	Chitosan (g/L)
Botrytis sp.	0. 09 ± 0.01^{b}	1.19 ± 0.09^{b}
Pilidiella granati	0. 48 ± 0.01^{a}	0.47 ± 0.02^{c}
Penicillium sp.	0. 02 ± 0.01^{c}	2.21 ± 0.05^{a}

Means (n = 4) ± standard error in the same column bearing a common letter/no letters are insignificantly different at $P < 0.05$
*EC50 is the effective concentration of fludioxonil and chitosan that inhibited radial mycelial growth by 50%.

Table 2 Effect of chitosan on pomegranate aril hardness, mass loss, total soluble solids (TSS), titratable acidity (TA) and ratio at 4 °C for 14 days.

Day	Cultivar Herskawitz						Cultivar Wonderful				
	Chitosan (g/L)	Hardness (N)	Mass loss (%)	TSS (%)	TA (g/L)	TSS:TA	Hardness (N)	Mass loss (%)	TSS (%)	TA (g/L)	TSS:TA
0	Harvest	161.97 ± 0.44^{a}	0.00^{h}	15.75 ± 0.05^{cd}	18.20 ± 0.10^{g}	8.66 ± 0.05^{ab}	194.28 ± 2.48^{a}	0.00^{h}	16.50 ± 0.04^{abc}	14.20 ± 1.20^{bcd}	11.87 ± 1.01^{abc}
3	0	149.69 ± 1.29^{f}	0.04 ± 0.00^{fgh}	16.25 ± 0.03^{ab}	19.40 ± 0.10^{abcd}	8.39 ± 0.05^{bc}	157.13 ± 1.06^{g}	0.12 ± 0.00^{efgh}	16.95 ± 0.03^{a}	14.40 ± 0.40^{abcd}	11.8 ± 0.36^{abc}
	2.50	152.68 ± 0.63^{cde}	0.04 ± 0.01^{fgh}	16.20 ± 0.06^{b}	18.80 ± 0.30^{defg}	8.65 ± 0.12^{ab}	159.44 ± 1.12^{fg}	0.08 ± 0.04^{fgh}	16.70 ± 0.00^{ab}	16.40 ± 1.50^{ab}	10.43 ± 0.92^{bcd}
	7.50	152.68 ± 0.41^{cde}	0.03 ± 0.00^{gh}	15.75 ± 0.03^{cd}	18.60 ± 0.30^{efg}	8.51 ± 0.12^{ab}	165.45 ± 0.87^{de}	0.06 ± 0.02^{fgh}	17.00 ± 0.04^{a}	15.00 ± 0.50^{abcd}	11.37 ± 0.34^{abcd}
	15.00	158.99 ± 1.24^{b}	0.01 ± 0.00^{h}	15.75 ± 0.09^{cd}	18.40 ± 0.20^{fg}	8.60 ± 0.12^{ab}	171.03 ± 1.10^{b}	0.04 ± 0.00^{gh}	15.33 ± 0.19^{d}	13.80 ± 0.30^{cd}	11.17 ± 0.32^{abcd}
7	0	146.51 ± 0.54^{g}	0.11 ± 0.00^{cd}	16.53 ± 0.13^{ab}	19.60 ± 0.20^{abc}	8.42 ± 0.01^{abc}	157.22 ± 1.08^{g}	0.20 ± 0.00^{defg}	16.80 ± 0.92^{ab}	15.70 ± 0.60^{abc}	10.80 ± 0.81^{bcd}
	2.50	150.19 ± 1.24^{ef}	0.09 ± 0.02^{cdf}	16.50 ± 0.04^{ab}	19.00 ± 0.30^{cdef}	8.68 ± 0.13^{ab}	162.87 ± 1.36^{ef}	0.16 ± 0.00^{defgh}	16.78 ± 0.05^{ab}	13.00 ± 0.50^{d}	12.97 ± 0.54^{a}
	7.50	152.30 ± 1.00^{cde}	0.08 ± 0.01^{dfg}	16.55 ± 0.09^{a}	19.10 ± 0.30^{bcde}	8.66 ± 0.15^{ab}	166.07 ±1.94^{cde}	0.08 ± 0.00^{fgh}	16.70 ± 0.09^{ab}	13.60 ± 0.20^{cd}	12.29 ± 0.24^{ab}
	15.00	154.67 ± 0.68^{c}	0.05 ± 0.01^{fgh}	16.30 ± 0.06^{ab}	18.70 ± 0.10^{efg}	8.75 ± 0.08^{a}	170.08 ± 1.22^{bc}	0.12 ± 0.00^{efgh}	16.40 ± 0.12^{abc}	13.40 ± 0.02^{cd}	12.24 ± 0.15^{ab}
10	0	143.17 ± 0.35^{h}	0.23 ± 0.04^{b}	14.93 ± 0.10^{f}	19.80 ± 0.20^{a}	7.54 ± 0.06^{ef}	148.52 ± 1.81^{h}	0.48 ± 0.12^{b}	16.55 ± 0.09^{abc}	15.60 ± 0.50^{abc}	10.66 ± 0.39^{bcd}
	2.50	149.82 ± 1.23^{f}	0.14 ± 0.01^{c}	15.35 ± 0.09^{e}	19.60 ± 0.10^{abc}	7.85 ± 0.09^{de}	156.59 ± 0.77^{g}	0.22 ± 0.02^{cdefg}	16.53 ± 0.13^{abc}	16.50 ± 0.50^{a}	10.02 ± 0.34^{cd}
	7.50	150.32 ± 1.12^{def}	0.10 ± 0.02^{cd}	16.43 ± 0.03^{ab}	19.00 ± 0.20^{cdef}	8.70 ± 0.08^{ab}	168.05 ± 1.13^{bcd}	0.20 ± 0.04^{defg}	16.40 ± 0.00^{abc}	15.30 ± 0.60^{abcd}	10.76 ± 0.45^{bcd}
	15.00	152.14 ± 0.34^{def}	0.12 ± 0.02^{cd}	16.48 ± 0.05^{ab}	18.80 ± 0.20^{defg}	8.76 ± 0.08^{a}	170.40 ± 1.84^{bc}	0.24 ± 0.04^{cdef}	15.78 ± 0.13^{cd}	14.80 ± 0.90^{abcd}	10.74 ± 0.55^{bcd}
14	0	82.57 ±0.27^{k}	0.32 ± 0.04^{a}	14.30 ± 0.20^{g}	20.10 ± 0.20^{a}	7.14 ± 0.15^{g}	95.03 ± 1.70^{j}	0.68 ± 0.16^{a}	15.98 ± 0.07^{bcd}	16.60 ± 0.60^{a}	9.65 ± 0.30^{d}
	2.50	89.64 ± 0.52^{j}	0.20 ± 0.02^{b}	14.65 ± 0.03^{f}	19.80 ± 0.20^{ab}	7.42 ± 0.08^{fg}	93.30 ± 1.80^{j}	0.34 ± 0.02^{bcd}	16.03 ± 0.03^{bcd}	15.10 ± 0.80^{abcd}	10.70 ± 0.66^{bcd}
	7.50	92.93 ± 0.41^{i}	0.19 ± 0.01^{b}	14.90 ± 0.12^{f}	18.80 ± 0.00^{defg}	7.94 ± 0.06^{d}	104.70 ± 1.58^{i}	0.40 ± 0.08^{bc}	15.70 ± 0.00^{cd}	13.90 ± 0.50^{cd}	11.36 ± 0.43^{abcd}
	15.00	92.74 ± 0.37^{i}	0.14 ± 0.01^{c}	15.45 ± 0.26^{cde}	19.10 ± 0.20^{cde}	8.11 ± 0.22^{cd}	108.57 ± 1.00^{i}	0.30 ± 0.02^{cde}	16.30 ± 0.49^{abc}	13.40 ± 0.50^{cd}	12.26 ± 0.76^{ab}
	P-value										
	Chitosan (C)	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0424	0.1511	0.0054
	Storage (S)	<0.0001	0.0028	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	C x S	0.0006	0.0738	<0.0001	0.7905	<0.0001	<0.0001	0.2212	0.0002	0.0188	0.0005

Means (n = 4) ± standard error in the same column bearing a common letter/no letters are insignificantly different at P < 0.05 according one-way ANOVA using Duncan's multiple range test. Data for each cultivar was analysed separately.

Table 3 Effect of chitosan on CIELa*b* colour indices of pomegranate arils stored at 4°C for 14 days

Day	Chitosan (g/L)	Cultivar Herskawitz					Cultivar Wonderful				
		*C**	*h°*	*L*	*a**	*b**	*C**	*h°*	*L*	*a**	*b**
0	Harvest	24.76 ± 1.48^{abc}	20.25 ± 1.12^{ef}	24.37 ± 2.57^{a}	23.09 ± 1.26^{abcd}	8.57 ± 0.92^{c}	20.02 ± 0.93^{b}	27.02 ± 1.43^{bc}	18.27 ± 1.33^{abc}	17.85 ± 0.83^{b}	9.15 ± 0.65^{bcd}
3	0	25.16 ± 1.48^{abc}	20.65 ± 1.12^{def}	24.77 ± 2.57^{a}	23.49 ± 1.26^{abcd}	8.97 ± 0.92^{c}	19.82 ± 0.93^{b}	26.82 ± 1.43^{bc}	18.07 ± 1.33^{abc}	17.65 ± 0.83^{b}	8.95 ± 0.65^{cd}
	0.25	22.90 ± 0.91^{b}	27.81 ± 1.16^{bc}	25.66 ± 1.39^{a}	20.24 ± 0.88^{cd}	10.65 ± 0.51^{bc}	18.78 ± 2.20^{b}	28.17 ± 2.21^{bc}	14.49 ± 1.47^{c}	16.57 ± 2.05^{b}	8.70 ± 1.04^{d}
	0.75	22.43 ± 1.83^{c}	27.74 ± 1.10^{bc}	25.64 ± 1.45^{a}	19.87 ± 1.76^{cd}	10.35 ± 0.67^{bc}	25.70 ± 1.77^{ab}	27.92 ± 1.05^{bc}	20.63 ± 2.25^{abc}	22.64 ± 1.44^{ab}	12.12 ± 1.12^{abcd}
	1.50	23.11 ± 1.34^{c}	27.86 ± 1.55^{bc}	20.41 ± 1.87^{a}	20.47 ± 1.45^{cd}	10.64 ± 0.37^{bc}	23.73 ± 2.13^{ab}	26.87 ± 2.01^{bc}	17.24 ± 2.89^{bc}	21.06 ± 1.89^{ab}	10.80 ± 1.26^{abcd}
7	0	28.03 ± 2.95^{abc}	25.73 ± 0.98^{bcdef}	18.42 ± 2.64^{a}	25.15 ± 2.50^{abc}	12.35 ± 1.63^{ab}	23.48 ± 1.7^{ab}	31.97 ± 3.55^{abc}	24.47 ± 4.86^{ab}	19.82 ± 1.89^{ab}	12.17 ± 1.16^{abcd}
	0.25	22.26 ± 2.28^{c}	35.34 ± 4.32^{a}	23.05 ± 3.56^{a}	18.32 ± 2.53^{d}	12.18 ± 1.04^{ab}	24.89 ± 2.90^{ab}	32.84 ± 2.87^{ab}	21.40 ± 3.03^{abc}	20.98 ± 2.72^{ab}	13.14 ± 1.55^{ab}
	0.75	26.43 ± 2.22^{abc}	27.91 ± 2.5^{bc}	19.29 ± 2.48^{a}	23.29 ± 2.09^{abcd}	12.25 ± 1.33^{ab}	24.58 ± 2.06^{ab}	31.88 ± 2.89^{abc}	21.74 ± 3.76^{abc}	20.76 ± 1.94^{ab}	12.90 ± 1.35^{abc}
	1.50	30.30 ± 1.72^{a}	29.12 ± 0.44^{b}	21.98 ± 2.11^{a}	26.45 ± 1.45^{ab}	14.77 ± 0.95^{a}	24.25 ± 2.75^{ab}	35.43 ± 3.18^{a}	19.04 ± 3.02^{abc}	19.75 ± 2.58^{ab}	13.80 ± 1.57^{a}
10	0	23.91 ± 1.39^{c}	26.08 ± 2.8^{bcde}	21.98 ± 2.59^{a}	21.33 ± 1.48^{bcd}	10.34 ± 1.03^{bc}	28.65 ± 3.19^{a}	28.62 ± 1.21^{abc}	27.50 ± 2.87^{a}	25.19 ± 2.94^{a}	13.58 ± 1.38^{a}
	0.25	24.11 ± 1.39^{c}	26.28 ± 2.8^{bcde}	22.18 ± 2.59^{a}	21.53 ± 1.48^{abcd}	10.54 ± 1.03^{bc}	24.96 ± 1.69^{ab}	28.73 ± 2.51^{abc}	23.37 ± 4.91^{abc}	21.62 ± 1.04^{ab}	12.25 ± 1.71^{abcd}
	0.75	24.13 ± 1.45^{c}	21.85 ± 1.14b^{cdef}	21.33 ± 2.16^{a}	22.38 ± 1.36^{abcd}	8.98 ± 0.69^{c}	23.87 ± 2.38^{ab}	25.43 ± 0.74^{c}	18.01 ± 2.27^{abc}	21.51 ± 2.08^{ab}	10.33 ± 1.21^{abcd}
	1.50	30.20 ± 1.82^{ab}	26.39 ± 1.79^{bcd}	22.75 ± 2.43^{a}	27.07 ± 1.87^{a}	13.25 ± 0.75^{ab}	23.17 ± 2.56^{ab}	30.69 ± 2.69^{abc}	26.36 ± 3.11^{ab}	19.88 ± 2.31^{ab}	11.72 ± 1.45^{bcd}
14	0	22.90 ± 0.91^{c}	27.81 ± 1.16^{bc}	25.66 ± 1.39^{a}	20.24 ± 0.88^{cd}	10.65 ± 0.51^{bc}	20.02 ± 0.93^{b}	27.02 ± 1.43^{bc}	18.27 ± 1.33^{abc}	17.85 ± 0.83^{b}	9.15 ± 0.65^{bcd}
	0.25	22.43 ± 1.83^{c}	27.74 ± 1.10^{bc}	25.64 ± 1.45^{a}	19.87 ± 1.76^{cd}	10.35 ± 0.67^{bc}	19.82 ± 0.93^{b}	26.82 ± 1.43^{bc}	18.07 ± 1.33^{abc}	17.65 ± 0.83^{b}	8.95 ± 0.65^{cd}
	0.75	24.76 ± 1.48^{abc}	20.25 ± 1.12^{ef}	24.37 ± 2.57^{a}	23.09 ± 1.26^{abcd}	8.57 ± 0.92^{c}	18.78 ± 2.20^{b}	28.17 ± 2.21^{bc}	14.49 ± 1.47^{c}	16.57 ± 2.05^{b}	8.70 ± 1.04^{d}
	1.50	25.16 ± 1.48^{ab}	20.65 ± 1.12^{def}	24.77 ± 2.57^{a}	23.49 ± 1.26^{abcd}	8.97 ± 0.92^{c}	25.70 ± 1.77^{ab}	27.92 ± 1.05^{bc}	20.63 ± 2.25^{abc}	22.64 ± 1.44^{ab}	12.12 ± 1.12^{abcd}
P-value											
Chitosan (C)		0.0712	0.0032	0.8068	0.0703	0.0072	0.5752	0.6330	0.3500	0.6778	0.4004
Storage (S)		0.0251	0.0008	0.0364	0.0157	<0.0001	0.0253	0.001	0.0062	0.0871	0.0006
C x S		0.0641	0.0022	0.7699	0.0180	0.3806	0.0872	0.9359	0.3860	0.1002	0.2334

Means (n = 10) ± standard error in the same column bearing a common letter/no letters are insignificantly different at P < 0.05 according one-way ANOVA using Duncan's multiple range test. Data for each cultivar was analysed separately.

Chapter 6

In vitro and i*n vivo* antifungal activity of chitosan-essential oil combinations against pomegranate fruit pathogens

Abstract

This study investigated the effect of chitosan combined with essential oils (cinnamon, lemon, and oregano) as edible films against the growth of three important disease-causing pathogens of pomegranate (*Botrytis* sp., *Penicillium* sp., and *Pilidiella granati*). Essential oils (EOs) were characterised by gas chromatography analysis and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). *In vitro* antifungal activity of the EOs against the pathogens was conducted on agar media inoculated with fungal spores using the inverted lid and direct contact with agar techniques. Cast films were prepared from chitosan of different (low, medium, and high) molecular weights (Mw), and assessed for use as biodegradable packaging material. Thereafter, chitosan films (medium Mw) was used in combination with 0.0, 1.0, 5.0 or 10% (w/w) concentrations of the EOs for *in vitro* investigation. The use of ATR-FTIR effectively discriminated the EOs based on the energy levels of the atomic bond vibrations present, as indicated by their characteristic absorption peaks. Based on GC-MS analysis of the selected EOs, 35 volatile compounds belonging to eight different chemical classes were tentatively identified. In addition, differences in chitosan Mw were found to have significant influence on film functional properties such as colour, thickness, contact angle, and tensile strength. Based on *in vitro* investigation, chitosan-oregano or chitosan-cinnamon EOs films at 1.0% (w/w) concentration exhibited complete inhibitory effect against all three pathogens. Maximum inhibitory effects of chitosan-lemongrass films were dependent on EO concentration, with *P. granati*, *Botrytis* sp., and *Penicillium* sp. inhibited completely at 10, 50, and 50 g L^{-1}, respectively. Antifungal *in vivo* study on pomegranate fruit artificially inoculated with *Botrytis* sp. and treated with chitosan-oregano EO (emulsions and films) showed that both treatment methods inhibited *Botrytis* sp. growth. However, the inhibitory effect was higher for fruit directly dipped into the chitosan-EO emulsions as opposed to samples exposed to vapour contact via the films. This study showed that chitosan-EOs applied as edible coatings or casted films can be used to control postharvest fungal pathogens associated with pomegranate fruit.

Key words: *Punica granatum* L.; antimicrobials; volatiles; edible coatings.

1. Introduction

Postharvest storage duration of pomegranate fruit could be critically restricted due to fungal decay. Diseased pomegranate fruit may appear as blemishes such as black spot caused by *Alternaria alternata* or *Colletotrichum gloeosporiodes*. In other instances, the fruit may present symptoms of moulding due to either *Botrytis cinerea* or *Penicillium* sp. infections, while other disease such as heart rot caused by either *Al. alternata* or *Aspergillus niger* may only be detected when the fruit is cut open (Berbegal *et al.*, 2014; Thomidis, 2014; Thomidis & Exadaktylou, 2011). Regardless of the symptoms, postharvest diseases cause economic losses and the diseased fruit poses a potential health risk. Pathogenic fungi contribute to foodborne outbreaks and epidemics causing illnesses from stomach cramp to fatality (Martinovic *et al.*, 2016). Filamentous fungi, in particular, can invade and degrade food products by means of extracellular enzymes (Al-Hindi *et al.*, 2011). Major fungi genera present along the pomegranate value chain include *Botrytis*, and *Penicillium* (Thomidis, 2014; Munhuweyi *et al.*, 2016), which can be identified based on conidia structure and their ability to grow even in cold storage (Munhuweyi *et al.*, 2016).

Postharvest fungal infections are predominantly controlled using fungicides. This contributes to the development of resistant pathogen populations (Rahman *et al.*, 2014), which in turn results in the use of higher fungicide dosages and the consequent increase in toxic residues on fresh produce. Furthermore, consumers' demand for safe and nutritional food, as well as the negative perception towards the use of synthetic preservatives in food (Hyldgaard *et al.*, 2012); has heightened the research need for safer alternative antimicrobial agents across the globe. Conventional food preservation techniques, based on thermal treatments such as pasteurisation and sterilisation ensure microbiological safety at the cost of partial loss in nutritional and sensory qualities (Lado & Yousef, 2002). An alternative non-thermal preservation approach involves the use of natural antimicrobials to improve the lethal effects of the non-thermal treatments while maintaining the nutritional and sensory attributes of the produce (Severino *et al.*, 2014). Natural antimicrobials can be applied to fruit and vegetables, by means of using edible coatings enriched with active compounds.

Essential oils (EOs) can be utilised as a natural alternative in food preservation and their usage complies with consumers' expectations for natural food with marginal chemical treatments (Arrebola *et al.*, 2010; Hromis *et al.*, 2016). Application of EOs is considered a safe treatment for the control of postharvest decay of fresh produce, and therefore, should not have any regulatory issues (Sivakumar & Bautista-Banos, 2014). However, when a single EO is

applied on fresh produce, it is often not effective enough to prevent or delay decay. In addition, it could cause negative organoleptic effects especially at higher doses that can provide an antimicrobial effect (Hyldgaard *et al.*, 2012). To address this drawback, various studies have investigated the synergistic effects of combining different EOs with other antimicrobial compounds. For instance, Severino *et al.* (2014) demonstrated a strong antimicrobial synergism between a chitosan coating containing 0.05% mandarin essential oil and γ-irradiation treatment against *Listeria innocua* in green beans kept at 4 °C for 14 days. Similarly, Oz & Ulukanli (2012) applied 300 and 600 ppm of *Nigella sativa* (black cumin) EO into a starch-based edible coating onto "Silifke as¸ısı (33 N 16)" pomegranate arils kept at 4 °C for 12 days. The combination of a starch-based coating plus *N. sativa* oil significantly reduced weight loss, browning, decay rates and retained the highest overall aril quality compared with the control. Bioactive substances such as EOs can be incorporated into film-forming materials in order to create controlled-release systems (Gemili *et al.*, 2009). However, significant losses of the volatile compounds occur during the drying stage of the film. Therefore, micro- and nano-encapsulation of EOs could improve the efficiency of the films further (Sanchez-Gonzalez *et al.*, 2011).

Emission of bioactive vapour/volatile compounds inside packaged food product offers the advantage of preventing surface growth of microbes without having to come into direct contact with the food product. Melgarejo-Flores *et al.* (2013) investigated the effects of emulsions, vapours and coatings of cinnamon leaf oil on table grape stored at 10 °C for 15 days. Application of the cinnamon oils as a vapour phase was found to be most effective in reducing fungal decay and maintain antioxidant activity of the grapes, while the emulsion treatment negatively influenced odour acceptability of the grapes (Melgarejo-Flores *et al.*, 2013). The use of polymers as carriers of antimicrobials not only permits the controlled release of these antimicrobials but also prevents dramatic reductions in their antimicrobial activities through inactivation by components in foods by means of dilution below active concentration due to migration into the bulk food matrix (De Azeredo, 2013). These films also reduce the amount of active ingredient required for direct incorporation into the food, satisfying consumer demand for fewer additives. Therefore, the set objectives of this study were to; (i) elucidate on the impacts of chitosan Mw on the functional properties of the casted films; and, (ii) investigate the *in vitro* and *in vivo* inhibitory effects of chitosan-EOs (of varying concentrations lemongrass, cinnamon, and oregano oils) via vapour emission and direct coating against *Botrytis* sp., *Penicillium* sp., and *Pilidiella granati* pathogens of pomegranate fruit.

2. Materials and methods

2.1. Plant material

Pomegranate fruit cv. 'Wonderful' were harvested manually under aseptic conditions at commercial maturity from an orchard located in the Wellington region (GPS S33° 39.276 E18° 59.399), Western Cape, South Africa. Selected fruit of uniform size, shape, and free of any external defects were washed with tap water (1 min), dipped in 70% (v/v) ethanol for 30 s, 0.35% (v/v) sodium hypochlorite (NaOCl) for 2 min, and 70% (v/v) ethanol for 30 s (Fourie *et al.*, 2002). Thereafter, the pomegranate fruit samples were air-dried overnight under laminar airflow prior to the *in vivo* study.

2.2. Fungal cultures

A preliminary study was conducted before this study to identify fungal pathogens associated with pomegranate cv. 'Wonderful' postharvest. *Botrytis* sp., *Pilidiella granati* and *Penicillium* sp. were isolated from diseased pomegranate fruit during cold storage. Pathogenicity and virulence of these fungal isolates were investigated and confirmed according to the Koch's postulates. The isolated fungal pathogens *Botrytis* sp., *Pilidiella granati* and *Penicillium* sp were thereafter stored at the Stellenbosch University, Department of Plant Pathology (STE-U) culture collection under the given accession numbers STE-U 7866, STE-U7864, and STE-U 7865, respectively. These isolated fungal pathogens were grown in Potato Dextrose Agar (PDA) (Biolab, Modderfontein, South Africa) incubated at 25 °C for 7-14 days before each trial in order to obtain spores. A solution of distilled water amended with Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.01 mL/L was added to the PDA plates of each fungal colony. This solution was then filtered through sterile cheesecloth with two layers to obtain spore concentrations. Spores were counted using haemocytometer (Neubauer, Marienfeld-Superior, Lauda-Konigshofen, Germany) and optical microscope (Leica Wild M8 Transmitted Light Stereo Microscope, Wild Heerbrugg, Switzerland). Final adjusted spore concentration of 1 x 10^6 spores/mL was used for each fungal culture.

2.3. Characterisation of essential oils (EOs)

Pure extracts of cinnamon (*Cinnamon verum*) leaf and lemongrass (*Cymbopogon citratus*) oils were obtained commercially from Soil (Durban, South Africa), while Oregano (*Origanum vulgare*) leaf extract was obtained from Clive Teubes Africa (Randburg, South Africa). The EOs were kept refrigerated at 4°C prior to further analysis. Attenuated total

Reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was used to determine the chemical and structural characteristics of the EOs. Measurements were conducted using a Nicolet™ iS™ 10 Fourier transform infrared spectrophotometer fitted with a smart diamond ATR accessory (Thermo Fischer Scientific, Waltham, USA). Sixty-four scans were recorded for each sample with a wavenumber resolution of 4 cm^{-1} using the Attenuated Total Reflectance (ATR) mode.

Volatile organic compound (VOC) composition of these EOs were determined via gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890N GC (Agilent, Palo Alto, CA) gas chromatograph coupled with an Agilent 5975B MS (Agilent, Palo Alto, CA) mass selective detector. The ZB-FFAP column (30 m x 0.25 mm id x 0.25 µm) part number 7HG-G009-11 was used for the separation of components. Helium was used as a carrier gas at a constant flow rate of 1.3 mL/min. Samples of each EO were individually diluted in hexane (1:10000), and 1 µL of the mixture was thereafter injected into the column with a split ratio of 10:1. Oven temperature was maintained as follows: 50 °C for 3 min, then ramped up to 80 °C at the rate of 4 °C/min for 2.5 min; and, finally ramped up to 250 °C at a rate of 10 °C/min, and held for 3 min. Mass spectra were analysed in the scan mode over the range of 35 to 600 m/z. Under the previously mentioned GC-MS conditions, the main components of essential oils were analysed and the total ion currents were obtained. Compounds were tentatively identified by their retention times (RT) and Kovats retention index (KI) values. Mass spectra obtained for each sample was compared with the National Institute of Standards and Technology library (NIST; version 2.0). The mean (n = 3) percentage relative abundances of VOCs based on the integrated peaks were used (Babushok *et al.*, 2011; Caleb *et al.*, 2013). Kovats retention index was calculated using Eq. 1: $I = 100n+100(N\text{-}n)\left[\frac{\log RtA - \log Rtn}{\mathrm{Log}RtN - \log Rtn}\right]$

(1)

where n is the number of carbon atoms in the smaller alkane; N is number of carbon atoms in the larger alkane, and Rt is the retention time.

2.4. In vitro antimicrobial Screening of EOs

The inhibitory effects of the oils were tested using a modified disc diffusion method described by Wen *et al.* (2016). Two methods of inhibition were investigated this included a vapour contact and direct contact. Potato dextrose agar (PDA) was inoculated with 1 x 10^6 CFU/mL spores of *Botrytis* sp. *Penicillium* sp. and *P. granati*, respectively. In the direct contact method, filter paper disks (6 mm diameter (Ø)) were cut using a hole-puncher. Both sides of

filter disks were sterilised under UV irradiation for 2 h (each side for 1 h), followed by the addition of EO (5 µL). Paper disks containing EOs were placed directly onto the surface of the inoculated media. For the vapour contact method, the filter disks (Ø = 6 mm) containing 5 µL of EO were placed on the inside surface of the medium-free top lid cover of each petri dish, with no direct contact between the cover and the fungi. The Petri dishes were sealed using parafilm to prevent leakage of EO vapour, followed by incubating at 25 ± 2 °C and 55 ± 2% relative humidity for 72 h. The inhibition radius around the oil disc (colony-free perimeter) was measured using a digital Vernier calliper (Mitutoyo, Kawasaki, Japan). All analyses were conducted in triplicate. Fungistatic index was calculated using Eq. 2, and expressed as percentage (%) fungistatic inhibition:

$$\text{Fungistatic inhibition (\%)} = 1 - \left(\frac{Ri}{Rc}\right) \times 100 \quad (2)$$

where *Rc* represents the mean value of the colony radius of control media and *Ri* is the zone of inhibition radius of the paper disk-EO media.

2.5. Edible film preparation

All chemical reagents used in film preparation were purchased from Sigma-Aldrich (Steinheim, Germany). Crab shell chitosan of high Mw (H-Mw), medium Mw (M-Mw), and low Mw (L-Mw) with 800-2000 cp, 200-800 cp, and 20-200 cp viscosity, respectively, were prepared separately by dissolving the chitosans (2 w/w%) in acetic acid solution (1 v/v%). The mixtures were then stirred continuously for 24 h at room temperature. Thereafter, glycerol (30 w/w% of chitosan), and Tween 20 (0.2% v/v) were added to the mixture. The resultant film forming solutions (FFS) were filtered through a sintered glass filter to remove any undissolved particles.

Chitosan films enriched with EOs of different concentrations (0.0, 1.0. 5.0 and 10.0% (w/v) were prepared by adding the EO to the FFS mixture and the final product was homogenised using Ultra Turrax (Ultra Turrax IKA T25, IKA, Staufen, Germany) at 20, 000 g for 1 min at room temperature. Cast films were prepared by casting 25 g of the final FFS per Petri dish, and dried for 72 h in a fume hood that was fitted with a conventional 30 cm desk fan (SCE, Cape Town, South Africa). Films were kept in Petri dishes covered with aluminium foil and placed in a desiccator at ambient condition to ensure complete removal of water. Prior to every analysis, the films were carefully peeled off from Petri dish and conditioned at 25 °C and 55% RH for 48 h in a controlled environment chamber (MLR-32, Panasonic, Osaka, Japan)

before testing.

2.5.1. *Film thickness and tensile properties*

Film thickness (µm) was measured to the nearest 0.001 mm with a hand-held digital micrometer (Mitutoyo, Tokyo, Japan). Measurements were taken at ten randomly selected points on each film to calculate average value. The mechanical properties of the films were determined as described by Wang *et al.* (2011) using a TA-XT2i texture analyser (LRX, Lloyd Instruments Ltd, Royston, UK). In order to determine tensile strength (TS, MPa) and elongation at break (EB,%), films were cut into strips (6 mm wide) and mounted between the tensile grips (Ta 96). Initial grips separation was 50 mm and crosshead speed was 0.8 mm/s. For each sample, five replications were performed. Tensile strength (TS, MPa) was calculated as:

$$\mathrm{TS} = F_t / \left(\frac{L}{W}\right) \quad (3)$$

where F_t is the maximum stretching strength (N), L is the thickness of the films (mm), W is the width film samples (6 mm).

2.5.2. *Film colour*

Colour values were measured using a portable Minolta Chroma Meter CR-400 (Minolta Corp, Osaka, Japan) as described by Leceta *et al.* (2013). Film disks were placed on a white plate, and CIELAB colour coordinates L^* = 0 to 100 (describing black to white), -a^* to +a^* values (which describes greenness to redness) and –b* to +b^* values (which describes blueness to yellowness) recorded. Standard values for white calibration plate were L^* =97.39, a^*= 0.03 and b^* =1.77. Changes in colour parameters L^*, a^*, and b^* were evaluated by comparing total colour differences between films, and, chroma (C^*), hue angle (h^*) and yellowness indices (YI) were calculated from Eq. 4, 5 and 6, respectively:

$$C^* = \sqrt{(a^*)^2 + (b)^2} \quad (4)$$

$$h^\circ = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (5)$$

$$YI = \frac{142.86^*}{L^*} \quad (6)$$

All values were expressed as the means ± SE of ten measurements from different areas of each film.

2.5.3. Film contact angle of water

The static contact angle was determined from sessile droplet dimensions. Magnification was achieved by using a Zeiss microscope unit. A drop of about 1 µL of distilled water was placed onto the sample mat and immediately the magnified image was captured using a Nikon SMZ-2T (Tokyo, Japan). Image analysis software (Carl AxioVision LE) was used to determine the contact angle. Ten replicates were made per formulation.

2.6. Antifungal activity of chitosan-EO active films

For the *in vitro* investigation, antifungal activity was evaluated based on a method described by Du *et al.* (2009). Spores from 7 day old cultures were suspended in sterile distilled water and 0.02% Tween 80 and counted in a haemocytometer and then diluted to 0.1 mL of 10^6 spores of each fungal isolate and then plated onto 65 mm PDA plates. The inoculum was spread evenly throughout each plate and then left to dry for 5 min in a biosafety hood. Medium Mw chitosan films (2 wt.%) with different concentrations (0.0, 1.0. 5.0 and 10.0%, w/v) of either cinnamon, lemongrass or oregano EO were aseptically cut into 50 mm diameter discs and then tapped onto the lids of the PDA plates, that had been previously spread with spore suspensions. Parafilm was used to seal the edge of each PDA plate. The inhibition radius (absence of fungal growth) on each PDA plate was checked after 72 h of incubation at 25 °C.

An *in vivo* investigation was carried out using fruits previously inoculated with 5 mm diameter mycelial plugs of *Botrytis* sp. (a principal fungal pathogen of pomegranate). Briefly, pomegranate fruit (cv. 'Wonderful') were surface sterilised with 5% sodium hypochlorite and then wounded (5 mm deep by 5 mm wide) with a sterile cork borer to make two wounds per fruit, one on either side of the fruit. Mycelial plugs (5 mm diameter) taken from margins of actively growing colonies of Botrytis sp. were then placed onto the wounds. Inoculated fruits were subsequently dipped into chitosan FFS containing; (a) 0.25%, (b) 0.5%, (c) 1.0% oregano EO, and (d) the control solution (1% acetic acid v/v, pH 5.6) had no EO. The concentrations of oregano EO used for the *in vivo* study were based on findings from preliminary experiments. A separate batch of inoculated pomegranate fruit samples was placed into cleansed 4-Side Lock 9.0 L (27 x 21 x 16 cm^3) polypropylene containers (ADDIS, Cape Town, South Africa) previously functionalised by coating with the chitosan-EO (oregano) film solutions. To prepare these, approximately 225 g of medium Mw chitosan (2wt%) enriched with oregano EO at concentrations of 0, 0.5, 1.0 or 1.0% (w/v) were separately poured into polypropylene containers and dried overnight in a fume hood fitted with a conventional 30 cm desk fan (SCE,

Cape Town, South Africa). A similar amount of FFS (225 g) was poured to dry onto the adjoining Silicon Seal lids of the containers. The bottom of the plastic containers was lined with a gridded in lay to prevent the fruit from being in direct contact with the films. A glass Petri dish containing 20 mL of sterile distilled water was placed at the centre of the gridded inlay to ensure high relative humidity. Fig. 1 shows the set up used for the containers. The percentage reduction in decay was determined by expressing the difference in lesion diameter for treated fruit over the control. Lesion diameters of *Botrytis* sp. on artificially inoculated fruits on day 10 were considered (with 3 replicates of 4 fruits each). The entire experiment was independently conducted again to ensure repeatability.

2.7. Statistical analysis

Analysis of variance (ANOVA) was performed on all data using SAS-Statistical software (SAS Institute Inc., Cary, North Carolina, USA). Data was subjected to analysis of variance (ANOVA) and means were separated by least significant difference (LSD; $P < 0.05$) according to Duncan's multiple range test. All samples are reported as mean ± standard error

3. Results

3.1. Characterisation of essential oils

Based on GC-MS analysis of the selected EOs, 35 volatile compounds belonging to eight different chemical classes were tentatively identified (Table 1). The most abundant chemical groups of VOCs were monoterpenes (29%), followed by the alcohols (26%), aldehydes (11%) and esters (11%), sesquiterpenes (9%) and ketones (9%), epoxides (3%) and ethers (3%). Cinnamon EO chemical composition presented eugenol (56.9%), benzyl benzoate (4.8%), β-caryophyllene (4.8%), safrole (4.7%), cinnamaldehyde (3.4%) and eugenyl acetate (2.6%) as major constituents. On the other hand, lemongrass consisted of geranial (41.4%), neral (32.5%), myrcene (7.8%), linalool L (0.9%), 4-caranone (3.4%), and geraniol (3.2%). While, oregano EO had unique compounds such as carvacrol (51.5%), car-2-en-4-one (11.7%), *p*-cymene (10.0%), thymol (3.6%), caryophyllene oxide (2.4%) and β-caryophelene (2.1%), with trace amount of 1,8-ciole, pinene, myrcene, α-terpineol, α-humelne, camphor, and γ-Terpinene.

Fourier transform infrared (FTIR) spectroscopy effectively discriminated the EOs based on the energy levels of the atomic bond vibrations present. The characteristic differences in the absorption peaks of the three EOs indicated the variation in their chemical constituents

(Fig. 3). The broad band at 3600 cm^{-1} and 3200 cm^{-1} in cinnamon and oregano EO spectrum was attributed to the O-H stretching. This band was absent in the lemongrass spectrum. The strong bands at 2931cm^{-1} (cinnamon EO), 2959 cm^{-1} (oregano EO), and 2966 cm^{-1} (lemongrass EO) indicate C-H stretching for alkane. The other important bands of cinnamon were at 1603 cm^{-1} and 1511 cm^{-1} and these revealed presence of carbonyl group C=O bond for aldehyde. The band at 1270 cm^{-1} and 1029 cm^{-1} correspond to C-O vibration (Fig. 3). In lemongrass spectrum, peak 1451 cm^{-1} represents a strong methyl band and the band at 797 cm^{-1} (methyl rocking vibration) is indicative of a long-chain linear aliphatic structure (Fig. 3).

3.2. In vitro antifungal screening of EOs

In vitro screening of the EOs showed that they all possessed inhibitory effects against *Botrytis* sp., *Penicillium* sp. and *P. granati*, in the following order of efficacy: oregano > cinnamon > lemongrass (Fig. 2). Observed inhibitory effects of the EOs against fungal pathogens investigated were found to be influenced by the type of contact method employed. Direct contact technique provided better fungal growth inhibitory effect compared to the vapour technique. For instance, vapour inhibition ranged from 26-34% for *Botryti*s sp., 32-100% for *P. granati*, and 16-43% for *Penicillium* sp., while, fungal inhibition was higher by direct contact method ranging from 46-51% for *Botrytis* sp., 51-100% for *P. granati*, and 28-69% for *Penicillium* sp., as shown in Figure 2. Although, cinnamon EO showed an inhibitory effect comparable to that demonstrated by oregano, however, it did not provide complete inhibition for *P. granati* using the vapour contact method as was observed for oregano EO. While lemongrass EO was the least effective and failed to achieve complete inhibition for all three fungal pathogens.

3.3. Edible film properties

3.3.1. Film thickness

There were no significant ($P < 0.05$) differences in film thickness (0.07-0.08 mm) for the three different types of chitosan before incorporation of glycerol and EO additives (Table 2). Chitosan film thickness increased to range between 0.08-0.12 mm upon the introduction of glycerol. The variation in thickness increased further (0.12-0.14 mm) when EO was added to the FFS. Films prepared with High-Mw chitosan were the most affected by the incorporation of glycerol (0.12 mm) and cinnamon EO (0.14 mm) with the highest film thickness compared to the other two types of chitosan.

3.3.2. Film tensile properties

Tensile strength (TS) increased with increase in molecular weight of chitosan as shown in Table 2. Low Mw chitosan scored the lowest TS of 49.41 ± 2.37 Mpa followed by medium Mw chitosan with 55.63 ± 1.32 Mpa and high Mw chitosan with 62.99 ± 1.64 Mpa (Table 2). The Elasticity at break point (EB) differed for chitosan of low Mw (7.83 ± 0.48%), medium Mw (6.23 ± 0.50%), and high Mw (4.97 ± 0.29). A 32-56% decrease in TS was observed when glycerol was added to the film formulation. In contrast, the EB value increased by 5-7 folds when glycerol was introduced to the chitosan film solutions. Incorporation of cinnamon-EOs further reduced the mechanical strength by an additional 27-46%, while increasing the EB further by 18-28%. The higher Mw chitosan retained the highest TS (23.24 ± 0.93%) compared to medium Mw (17.37 ± 0.91%) and low Mw (15.81 ± 1.51%) after adding cinnamon-EO. The EB values remained high for the lower Mw chitosan compared to high Mw chitosan (Table 2).

3.3.3. Film contact angle of water

The contact angle for chitosan with high Mw (97.20 ± 1.82°) was significantly ($P < 0.05$) greater than that observed for medium Mw (90.85 ± 2.21°) and low Mw (87.54 ± 1.31°) chitosan films (Table 2). A significant drop in contact angle was observed for all the films following the addition of glycerol. The contact angle further declined upon the incorporation of cinnamon EO. The alterations of the film surface parameters were highest in the case of films made from low Mw chitosan. Introduction of EO further lowered the contact angle values by 41-50% to give values of 44.21 ± 1.96°, 52.08 ± 2.53°, and 56.94 ± 2.39° for low, medium, and high Mw chitosan, respectively (Table 2).

3.3.4. Film colour

All neat chitosan films formed transparent films with a slight hint of yellow tinting. The yellow tint (*YI*) was more prominent for low (14.67 ± 0.25) and medium (14.50 ± 0.38) Mw chitosan, and least for the high Mw chitosan (6.09 ± 0.06) as shown in Table 3. The *a** values for the plain films were close to 0 indicating that the films were more neutral in terms of red or greenness. Differences in C* were evident between the high Mw (3.93 ± 0.03) and medium Mw (9.33 ± 0.24) as well as low Mw (9.60 ± 0.21) chitosan films. The lightness intensity values (*L**) did not vary with chitosan Mw, while *a** and *b** values were significantly least for the high Mw chitosan films compared to the other two types of chitosan. Addition of glycerol significantly increased the *C**, *b** and *YI* values for all three chitosans, while *a** values declined. The changes were further intensified by addition of cinnamon EO to the FFS (Table

3).

3.4. FTIR of chitosan (M-Mw) films enriched with cinnamon, lemongrass and oregano EO

Figure 4 summarises the FTIR spectra of chitosan plasticised with glycerol, and chitosan enriched with either cinnamon lemongrass, or oregano EOs. The presence of cinnamon EO in the chitosan FFS can be associated with the characteristic peaks at 1511 cm^{-1}, 1270 cm^{-1} (Fig. 4b). Lemongrass and oregano EOs were detected between the 3000 and 2800 cm^{-1} region (Fig. 4b, c). The FTIR spectrum of plasticised chitosan revealed that N–H and O–H stretching vibration at 3500 cm^{-1} and 3200 cm^{-1} remained unchanged. On the other hand, the $–CH_3$ symmetric stretch at 2927 cm^{-1} shifts to 2915cm^{-1} for the lemongrass active film, and the C=O stretching vibration at 1639 cm^{-1} shifts to 1643 cm^{-1} and 1647 cm^{-1} for lemongrass and oregano EO active films, respectively. All three oils distorted the N–H stretching vibration at 1553 cm^{-1}. In addition, based on comparison to spectra for EOs only (Fig. 3), the C–OH stretching vibration at 1026 cm^{-1} shifted to 1029 cm^{-1}, while, the CH_3 bending vibration at 1412 cm^{-1} shifted to 1371 cm^{-1} and 1423 cm^{-1} for lemongrass and oregano EO films, respectively (Fig. 4).

3.5. Antifungal activity of chitosan-EO films in vitro

Antifungal activity of the active films was determined by visual inspection for the presence or absence of fungal growth. Chitosan films enriched with either oregano or cinnamon exhibited complete inhibition against *Botrytis* sp. *Penicillium* sp. and *P. granati* sp. at 1%. In addition, *P. granati* was susceptible to all active films with 1% (w/v) EO concertation. Lemongrass active films exhibited the least antifungal inhibitory effect against the other two pathogens at 1% (w/v) as shown in Figure 5. Complete inhibition of *Botrytis* sp. and *Penicillium* sp. by lemongrass active films was achieved only at 10.0% (w/v) concentration. In contrast, cinnamon EO active films achieved complete inhibition for *Botrytis* sp. and *P. granati* at 1%, and *Penicillium* sp. at 5% (w/v) concentration, while, oregano EO active films achieved complete inhibition of all three fungal pathogens at concentration of 1%. Therefore, for further antifungal investigation *in vivo* oregano EO was selected based on its efficacy.

3.6. Fungistatic activity of chitosan-EO films in vivo

Chitosan film forming suspensions (FFS) enriched with; 0.25%, 0.5%, or 1% (w/v) concentration of oregano EO was used for the *in vivo* study. Fruit dipped in plain chitosan

revealed a 43.11 ± 5.99% reduction in pomegranate fruit decay compared to untreated samples. Further reduction in fruit decay was observed for samples coated in chitosan-EO emulsion or active film (Fig. 6). Reduction in incidence of decay was most effective for fruit samples coated with the chitosan-EO emulsions. In addition, increase in EO concentration within the FFS significantly enhanced inhibitory effects as shown in Figure 6. Pomegranate fruit samples coated with chitosan-oregano EO (1.0%, w/v) emulsion had 100% decay reduction. Similarly, vapour contact via active chitosan-EO films of varying concentrations (0.25, 0.5 and 1.0%, w/v) resulted in 33.69 ± 8.15%, 46.64 ± 7.21% and 100% inhibition of *Botrytis* sp. respectively.

However, exposing the fruit inoculated with *Botrytis* sp. to the chitosan-EO by both vapour and direct contact method had a significant effect on the peel colour of the fruit as presented in Figure 7. The visual appearance of the fruits dipped in the chitosan-EO experienced the highest change in peel colour compared to those exposed to vapour contact. Dipping fruit with chitosan alone gave the best visual appearance compared to the other different treatments.

4. Discussion

4.1. Composition of essential oils

The major constituents for the three EOs assessed in this study corroborate with those reported in the literature by Avila-sosa *et al.* (2012). For instance, lemongrass EO was associated with geranial and geranial acetate, while carvacrol and thymol were predominant in oregano EO (Avila-sosa *et al.*, 2012). Similarly, cinnamon EO was found to be rich in eugenol and cinnamaldehyde among the major constituents (Avila-sosa *et al.*, 2012). The discrepant variation in relative percentage composition of VOCs in EOs may be attributed to differences in geographical location (agro-climatic regions), the plant part extracted and method of extraction (Burt, 2004). For example, the bark of the cinnamon plant is rich in cinnamaldehyde (62-90%), while the leaves are associated with eugenol (up to 80%), and the flowers are predominantly rich in (E)-cinnamyl acetate and caryophyllene (Nabavi *et al*., 2015). Also, to illustrate the importance of the extraction method Kasim *et al*. (2014) recovered 3.71-5.22% EO from the Cinnamon cassia bark using different solvents by soxhlet extraction and only 1.82% of the EO when using the hydro-distillation method.

The distinctive fragrances, tastes and antimicrobial activity of EOs are presumably derived from the different consortium of compounds present and the possible synergistic

interactions between them (De Oliveira *et al*., 2013; Adinew, 2014; Sivakumar & Bautista-Banos, 2014). These compounds may include aldehydes, hydrocarbons, esters, ketones, phenols, and terpene alcohols (Hyldgaard *et al*., 2012). In addition to providing inhibitory effects, EO volatile components such as thymol or eugenol are presumed to enhance the increment in antioxidant levels (polyphenols, flavonoids, and anthocyanins) of plants thereby, improving the oxygen absorbance and scavenging capacity of the fruit tissue (Wang *et al*., 2008). This in turn could improve the resistance of plant tissue to pathogens and reduce physiological deterioration (Wang *et al*., 2008).

4.2. Characterisation of EOs using ATR-FTIR

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy differentiated cinnamon, lemongrass and oregano EOs on basis of their spectrums. The spectral information obtained allows attribution of the signal to the main macromolecular constituents, in a qualitative and quantitative manner for lipids, polysaccharides, nucleic acids and proteins, etc. (Madivoli *et al.*, 2012; Lecellier *et al.*, 2014). The spectrum provides a global 'molecular fingerprint', which can be used for characterisation, differentiation and identification of chemical compounds (Lecellier *et al.*, 2014). The main absorption peaks between 3500 and 3200 cm^{-1} in the spectra of cinnamon and oregano EOs are indicative of the O–H vibration; these groups are able to form hydrogen bonds by means of electrostatic interaction in the presence of cationic groups (Abugoch *et al.*, 2011). The strong methylene/methyl band (1424 cm^{-1}) and weak methyl band around 1365 cm^{-1} in the lemongrass spectra indicate the presence of a long-chain linear aliphatic structure (Madivoli *et al.*, 2012). Carbonyl compounds are often the strongest band in the spectrum and will lie between 1875 and 1575 cm^{-1}, and its exact position being dependent upon its immediate substituent (Madivoli *et al.*, 2012). The FTIR spectra for lemongrass EO presented in the current study is comparable to that presented by Madivoli *et al.* (2012). For instance, the characteristic absorption peak at 1669 cm^{-1} (Fig. 3c) is similar to that observed at 1670 and 1671 cm^{-1} by Madivoli *et al.* (2012) and Vazquez-Briones *et al.* (2015), respectively. The FTIR spectra for oregano EO presented by Ferrandiz *et al.* (2015) is very much identical to the one presented in the current study.

4.3. Film thickness

The Mw of chitosan had no significant bearing on the film thickness of the plain chitosan films. This observation was in agreement with findings reported by Leceta *et al.* (2015). Also, the film thickness of the plain chitosan films (0.07-0.08 mm) was comparable to

that (0.06) mm reported by Leceta *et al*. (2015). In a different study, Leceta *et al*. (2013) reported an increase in film thickness when glycerol (15 and 30 wt.% of chitosan) was added to the FFS. Likewise, the incorporation of 10 wt.% EOs to chitosan FFS was shown to significantly increase the film thickness by (Wang *et al*., 2011). Film thickness was shown to increase from 0.10 mm by 3-fold for the chitosan-cinnamon and by 4-fold for the chitosan-clove films (Wang *et al*., 2011). According to Martínez-Camacho *et al.* (2010), the increase in film thickness may be due to compacting differences of the chitosan chains. The authors suggested that the mixture possibly gels during the process of film formation, thereby affecting the alignment, sorting, and compacting of the interaction between the chitosan, oil, and plasticiser molecules. The use of compounds, plasticisers or antimicrobial agents, with a molecular volume greater than anion, in this case, acetic acid, allows for the attainment of softer films that can be used to produce multilayer or covered films. Therefore, the presence of EOs into the chitosan film matrix probably loosens the microstructure of the film matrix thereby altering the overall thickness of the film (Wang *et al.*, 2011).

4.4. Film mechanical properties

Food packaging films are required to maintain film integrity in order to withstand the stress that occurs during shipping, handling and storage (Kanatt *et al*., 2012). Hence, the mechanical properties of any given film are of critical important. The tensile strength of the films was significantly ($P < 0.05$) affected by the addition of glycerol and cinnamon EO, especially films in which the low molecular weight chitosan was used. Films made from high Mw chitosan had the highest TS of 62.99 ± 1.64 Mpa, but the TS declined to 23.24 ± 0.93 Mpa after the introduction of cinnamon EO. Film mechanical properties could be related to the film network microstructure and the intermolecular force (Peng & Li, 2014). The mechanical properties obtained for crab shell chitosan are consistent with those reported by Leceta *et al.* (2015). It was observed that the addition of 30 wt.% glycerol increased the percentage elongation from 4.59 to 30.51%, and from 4.58 to 37.67%, giving more flexible films compared to those without glycerol and EO. The use of plasticiser and emulsification causes significant changes in film mechanical properties (Peng & Li, 2014), and these changes are probably favoured by the occurrence of the Maillard reaction (Leceta *et al.*, 2015). The higher elasticity values of the chitosan-EO comparing with chitosan-glycerol and control can be explained in terms of molecular weight of chitosan, which can influence the type and number of polymer-solvent, polymer-plasticiser, and polymer-plasticiser-EO interactions (Peng & Li, 2014; Leceta *et al.*, 2015).

4.5. Film contact angle of water

The water contact angle indicates the degree of hydrophilicity of films, being low when the hydrophilicity of the films is high (Leceta *et al.*, 2015). In practice, large ($\theta > 65°$) and small ($\theta < 65°$) contact angles represent the quantitative definition of a hydrophobic and hydrophilic surface, respectively (Vogler, 1998; Kurek *et al.*, 2014). The contact angle values provided in this study (87-91°) are comparably lower than those (106-116°) recorded by Leceta *et al.* (2015). In the current study, 2 wt.% chitosan was used in the formulation while a 1 wt.% formulation was used in the one by Leceta *et al.* (2015). This difference alone could have a huge effect on the overall film properties, as the amount of chitosan used is double that from the literature study. As observed in the present study, a higher contact angle was recorded for H-Mw chitosan (116°) compared L-Mw chitosan (106°) (Leceta *et al.*, 2015). Adding of glycerol to the chitosan FFS slightly decreases the water contact angle. This can be attributed to the hydrophilic character of the plasticiser (Leceta *et al.*, 2015). Chitosan-EO films presented lower contact angles than for chitosan-glycerol and control films, indicating less water resistance. This phenomenon may be due to the reorientation in molecular structure by the EOs. The oils might also increase the hydrophobic interaction within the chitosan system, only to project the polar groups toward the film surface (Salarbashi *et al*., 2014).

The contact angle is dependent on the relative magnitude of cohesive and adhesive molecular forces that exist respectively within the liquid and between the solid. The swelling (hydrophilicity) of material is desirable from an application point of view for targeted controlled release of active compounds (Kurek *et al.*, 2014). The surface phenomenon plays an important role in the mechanism of permeation. Films with higher moisture contents have lower contact angles, indicating hydrophilicity to absorb more water (Leceta *et al.*, 2013). Regardless of the temperature, as soon as the film was exposed to high humidity (foodstuff), the active compound will be released and will provide an immediate antimicrobial efficiency (Kurek *et al.*, 2014).

4.6. Film colour

Film colour governs the appearance, marketability and suitability of packaging materials for various applications. Chitosan films with similar discrepancies in film colouration were reported by Leceta *et al.* (2015) who observed higher lightness (L^*) intensity of 96.19 ± 0.38 for H-Mw chitosan compared to 95.65 ± 0.4 for L-Mw chitosan. In the current study, the plain chitosan films were more transparent with less yellow-tinting with an increase in Mw

(Table 3). Similarly, Leceta *et al.* (2015) observed higher *b** 5.11-8.55 for L-Mw films compared to H-Mw (2.59-3.68) after 90 days of storage 5.11-8.55 for low Mw films. Similarly, Leceta *et al.* (2013) also observed, a remarkable increase in (*C**) and *h*°, value for both low and high Mw chitosan films with addition of glycerol (15 and 30 wt.% of chitosan), while the *a** values declined. The yellowness intensified with the incorporation of EOs. Peng & Li (2014) observed that the colour change of the edible films is governed by the type of oil, with some oils having a stronger effect on opacity than others do. The changes in film colour indicate the presence of Maillard reaction taking place (Leceta *et al.*, 2015). The maillard reaction is facilitated by chemical modification of the free amino groups present in the FFS. The maillard reaction is preceded by the degradation and formation of varying different Mw compounds (Leceta *et al.*, 2015). Film permeability studies could provide more detail on the physical properties of the films; however, the focus of this study was on the characterisation of anti-microbial effects of these films *in-vitro* and *in-vivo*.

4.7. FTIR analysis of chitosan films

Changes were observed in the spectrum of plasticised chitosan and with the introduction of EOs by comparing the spectral differences in the 500- 4000 cm^{-1} FTIR spectra region. The shift relative intensity of the band at 1412 cm^{-1} decreased with the incorporation of EOs. The effect was most pronounced for cinnamon and lemongrass EO active films. The decrease in the 1412 cm^{-1} and the increase in the 1207 cm^{-1} to 1371 cm^{-1} could be attributed to chitosan deacetylation, allowing higher mobility in the polymer and favouring maillard reaction (Leceta *et al.*, 2015). Aside for the spectra for lemongrass EO, the chitosan peak at 1024 cm^{-1}, which is related to C–O remained unchanged. The presence of the characteristic absorption spectra of the individual EOs spectra indicates that the oils were efficiently incorporated by the chitosan FFS. Additionally, the presence of changes in peak position and strength in the FTIR spectrum demonstrates the existence of interaction among the chemical constituents of the FFS (Wen *et al.*, 2016). Similarly, Bahram *et al.* (2014) associated the peak between 3500 and 3000 cm^{-1} to the after effect of active whey-protein film combined with cinnamon leaf oil.

4.8. Antifungal activity of EOs in vitro

All three EOs (cinnamon, lemongrass and oregano) possessed antimicrobial properties. The major components of cinnamon and oregano EOs were eugenol (57%) and carvacrol (52%) which are phenolic compounds well known for their antifungal properties. Phenols interact

with membrane proteins leading to deformations in the membrane structure and functions. Therefore, EOs such as oregano and cinnamon containing volatiles like carvacrol, eugenol and thymol, are anticipated to exhibit strong antimicrobial properties against microbial pathogens (Lambert *et al*., 2001; Hyldgaard *et al*., 2012). The lack of phenolic compounds in the lemongrass EO could explain the relatively lower antifungal activity of lemongrass EO compared to those of cinnamon and oregano. However, the terpenoids, neral (32%) and geraniol (42%) in the lemongrass EO also possess antifungal activity whose mode of action also involves penetration and disruption of the membrane structure of microorganisms (Sivakumar & Bautista-Banos, 2014). Citral, a major volatile component of lemongrass EO, is a mixture of geranial and neral, which induces toxic effects on the structure and function of cellular membrane of pathogens (de Oliveira *et al*., 2013). In addition, Hyldgaard *et al*. (2012) highlighted that the antimicrobial activity of EOs is not entirely dependent on the presence of the major compounds, but is rather a collective synergism of both the major and minor compounds.

4.9. Antifungal activity of active chitosan-EO films

The study demonstrated the potential of EOs added to edible films as antifungal agents by vapour contact. Significant differences ($P < 0.05$) in fungistatic activity were observed for the various chitosan-EOs films. In addition, higher EO concentration increased the fungicide efficacy. Chitosan alone is only antimicrobial in the dissolved state (Wang *et al.*, 2011). To achieve antimicrobial activity, the positively charged amino groups of the chitosan monomer units, which can react with the anionic groups of the microbial cell surface must be freely available (Wang *et al*., 2011). In this regard, pure chitosan films presumably cannot display any antimicrobial activity in the solid state, solely because the chitosan molecules are fixed within the film matrix, and cannot diffuse to induce antimicrobial activity (Wang *et al.*, 2011). Hence, combining chitosan with EOs enhances the overall antimicrobial efficiencies of both compounds. The EOs being volatile have a short window period of providing antimicrobial activity. Using chitosan as a polymeric carrier of the EOs facilitates a sustained release of antimicrobials for longer periods. Changes in the water contact angle showed that the incorporation of the EOs increases the hydrophilic nature of the chitosan film surface. This, in turn, facilitates the ability of the of the chitosan films to release their antimicrobial activity the moment they get into contact with moist substances (Salarbashi *et al.*, 2014).

Similarly, Avila-sosa *et al.* (2012) observed that a higher EO concentration was

required as vapour contact to reach similar inhibition as that achieved with direct. They also observed similar findings that oregano and cinnamon EOs performed better than lemongrass. In similar study, Melgarejo-Flores *et al.* (2013) effectively controlled *Botrytis* decay of table grapes kept at 10 ºC for 15d using emulsions (0,0.5,2.5, and 5 g/L), vapours (0, 0.196, 0.392, and 0.588 g/L), and as an active agent incorporated into pectin coatings (0 and 36.1 g/L). The cinnamon emulsions were most effective at the highest concentration (5 g/L). The emulsions were, however, less antimicrobial compared with the cinnamon vapour which even enhanced the flavonoid and antioxidant activity of the berries. To achieve this, the authors placed table grapes in polypropylene containers, and exposed them to varying concentrations of cinnamon EO vapours (0-0.6 g/L). The highest berry antioxidant activity was observed when the cinnamon was incorporated into the pectin and there was no fungal decay observed. The findings from that study revealed that cinnamon as vapours or coatings can be used to control decay and increase the antioxidant health benefits of grapes (Melgarejo-Flores *et al*., 2013).

5. Conclusion

This study confirmed the variation in chemical profile of the selected EOs by GC-MS and FTIR spectroscopy. Furthermore, this study showed that the functional properties of chitosan films vary depending on the molecular weight and the type of chitosan used. The findings suggest that careful consideration should be made in the selection of chitosan for the production of desired edible films. Addition of glycerol and EOs compromised the optical and tensile strength of these chitosan films, leading to the reduction in opacity and mechanical strength, while the film thickness and elasticity increased. Incorporation of EOs enhanced the fungistatic activity of the chitosan films as shown in this study. However, the type of EO added into the chitosan emulsion significantly influenced the films antifungal efficacy. Chitosan film incorporated with oregano EO had the highest antifungal activity, and followed by cinnamon and lemongrass EO. The major volatile compositions differed for each EO, although similar minor compounds were detected across some of the oils. Detailed studies on the antifungal effect of chitosan-EO films on important fungi causing food spoilage provided insights into finding alternative safe packaging materials for fresh fruit and vegetables. Chitosan-EO films showed great application potential as bioactive compound matrices. These matrices can provide activity to food packaging films, by humidity and temperature induced mechanisms. Thus, there is need for further research in the field of encapsulation of bioactive plant extracts into active films to advance the potential benefits of active packaging.

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Figure 1 Functionalised polypropylene containers coated with chitosan-oregano active film layer used for the storage of inoculated (with mycelial plugs of *Botrytis* sp.) pomegranate fruit.

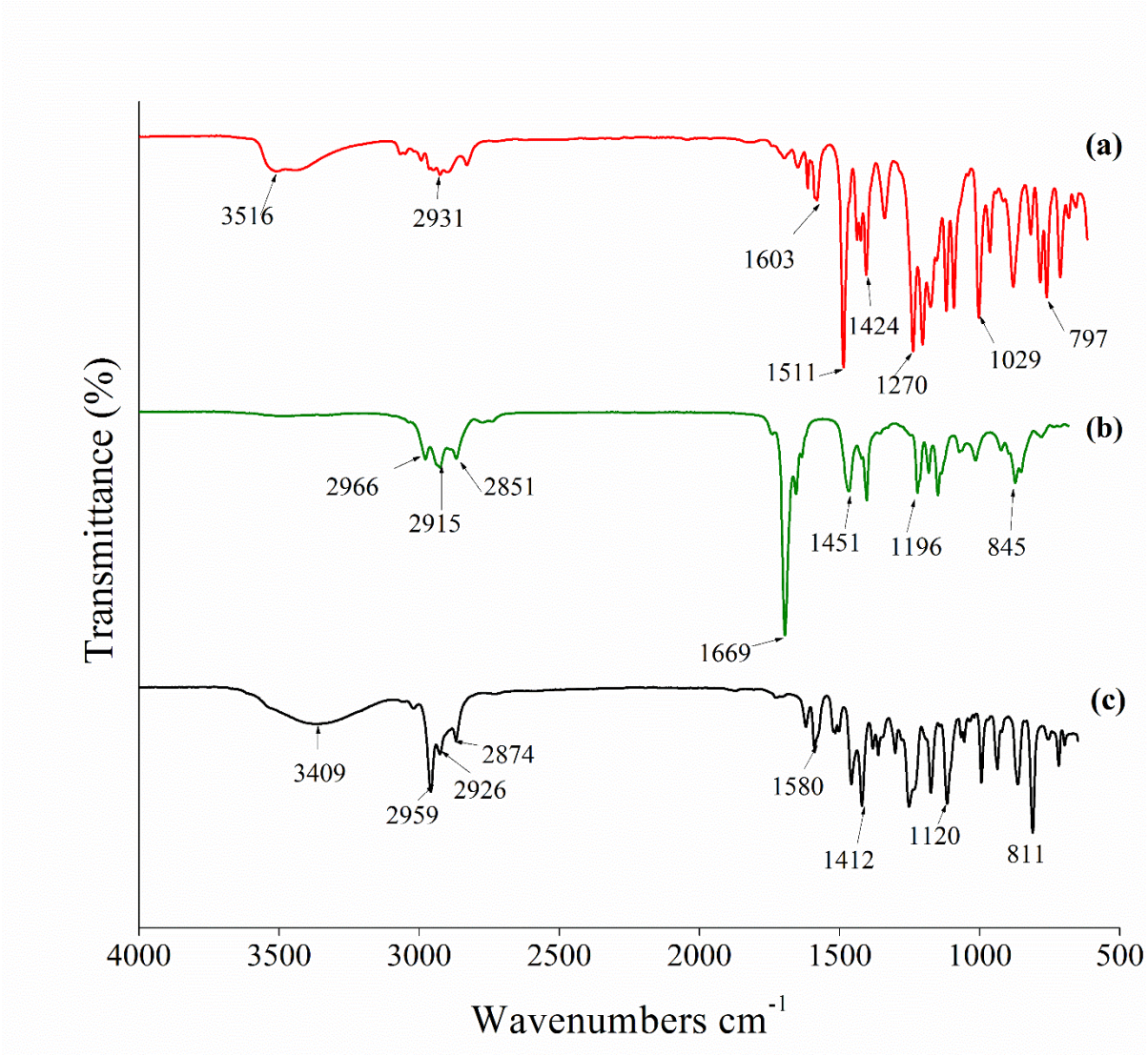

Figure 2 Fourier transform infrared spectroscopy (FTIR) spectra of the various essential oils used in this study; (a) cinnamon; (b) lemongrass and (c) oregano essential oil.

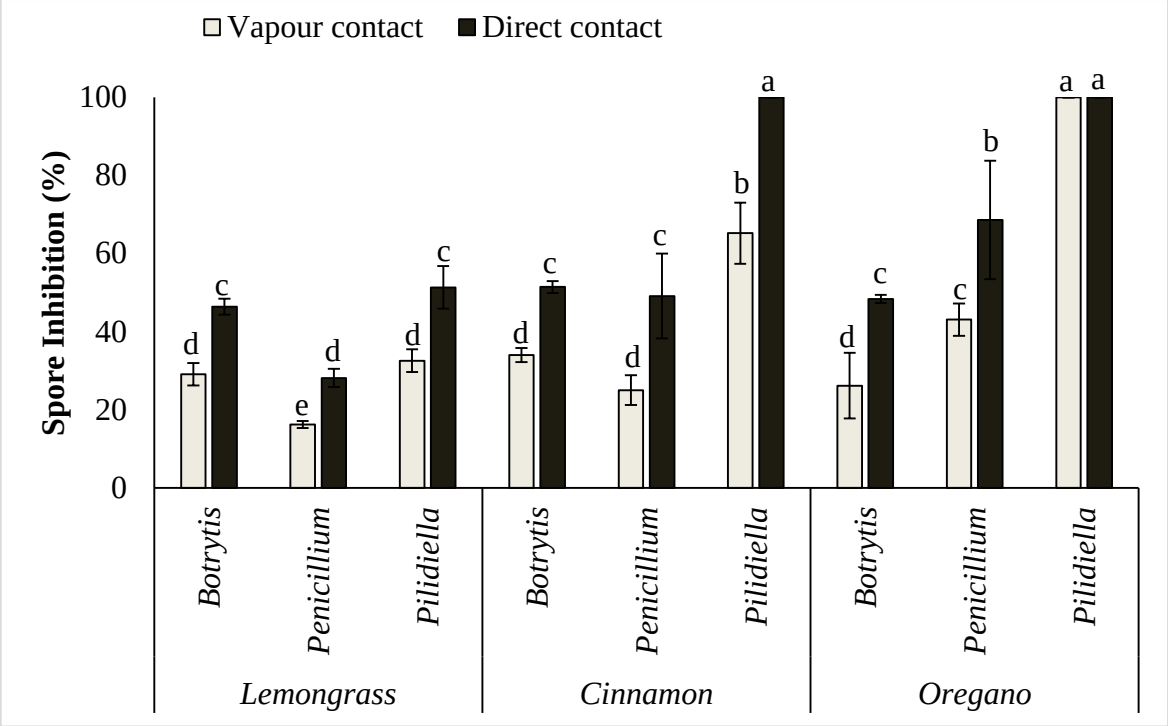

Figure 3 Percentage spore inhibition of lemongrass, cinnamon and oregano essential oils against *Botrytis* sp., *Penicillium* sp. and *P. granati* by exposure to vapour and direct contact tests. Error bars represent standard error (SE) of mean values (n = 6) at 95% confident interval.

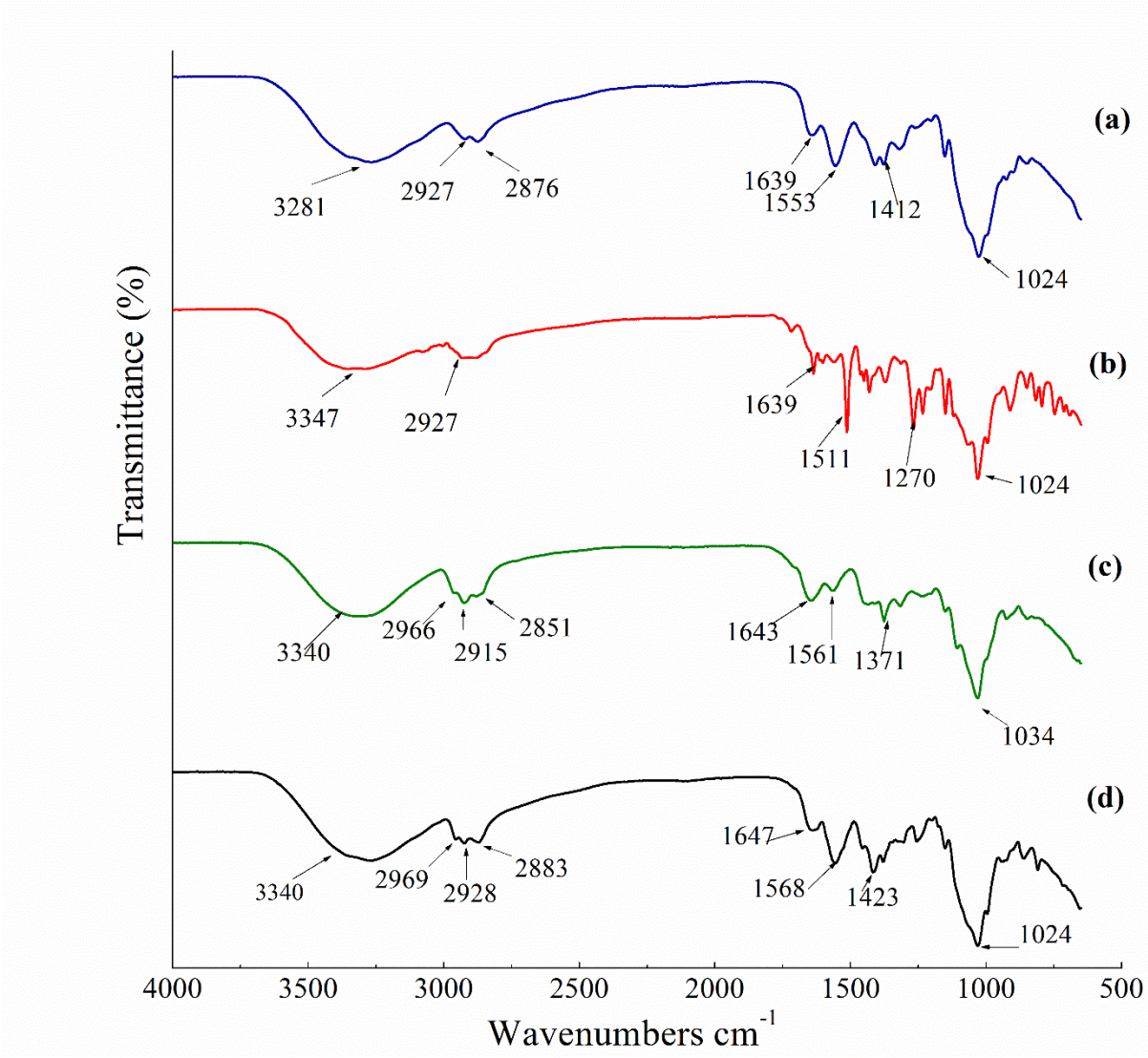

Figure 4 Fourier transform infrared spectroscopy spectra of (a) chitosan film made with 2 wt.% chitosan medium Mw + glycerol 30 wt.% of chitosan (b) chitosan film enriched with cinnamon EO; (c) chitosan film enriched with lemongrass EO and (d) chitosan film enriched with oregano essential oils.

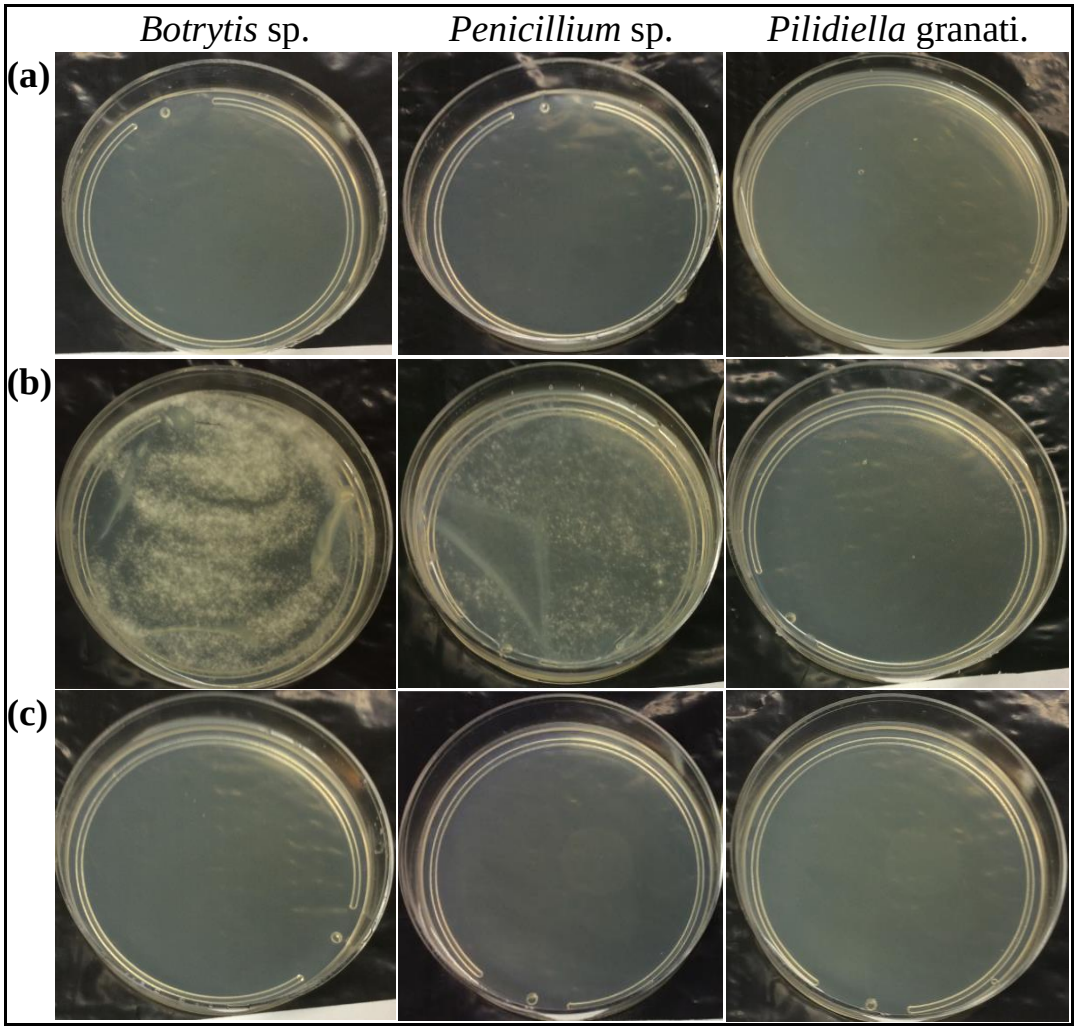

Figure 5 Inhibition of *Botrytis* sp., *Penicillium* sp. and *Pilidiella granati* by active chitosan films enriched with 1% essential oil of (a) cinnamon, (b) lemongrass, and (c) oregano.

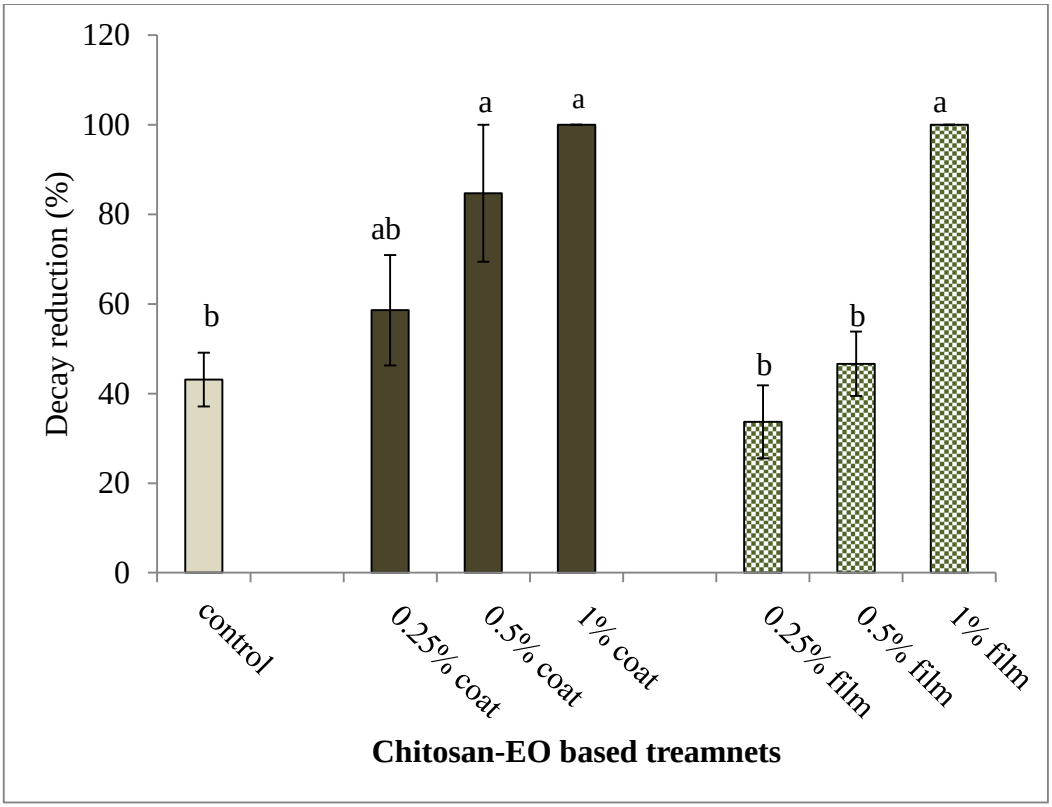

Figure 6 Percentage reduction in incidence of decay for pomegranate fruit against *Botrytis* sp. investigated *in vivo* via direct contact (coating) or by exposure to vapour released from active chitosan–oregano EO film treatments. Error bars represent standard error (SE) of mean values (n = 24) at 95% confident interval.

Figure 7 Pomegranates artificially inoculated with *Botrytis* sp. and treated with; (a) represent untreated fruit samples (control), (b) samples dipped into chitosan alone, (c) fruit dipped in chitosan-EO emulsion, and (d) fruit exposed to chitosan-EO active prior to storage at 25 °C for 10 days.

Table 1. Composition and relative abundance (%) of volatile compounds of cinnamon, lemongrass and oregano essential oils via GC-MS analysis.

Compound (***classes***)	KI (DB-5) *		Relative abundance (%)		
	Exp.	Lit.**	Cinnamon	Lemongrass	Oregano
Monoterpenes					
α-Pipene	949	944[a]	1.05 ± 0.09[a]	ND	1.03 ± 0.07[a]
Camphene	968	964[a]	0.40 ± 0.07	ND	ND
β-Pinene	996	990[a]	0.34 ± 0.06[b]	ND	0.90 ± 0.04[a]
Myrcene	1007	999[e]	ND	7.76 ± 0.07[a]	0.88 ± 0.04[b]
α-Phellandrene	1029	1025[e]	1.31 ± 0.19	ND	ND
p-Cymene	1056	1042[e]	1.91 ± 0.26[b]	ND	10.01 ± 0.40[a]
γ-Terpinene	1083	1073[e]	ND	ND	0.34 ± 0.01
4-Caranone	1221	1197[d]	ND	3.45 ± 0.04	ND
β-Selinene	1491	1492[b]	0.88 ± 0.08	ND	ND
β-Bisabolene	1680	1525[a]	ND	0.88 ± 0.02	ND
Ether					
1,8-Cineole	1061	1054[e]	ND	ND	1.60 ± 0.01
Ketones					
6-Methyl-5-hepten-2-one	1020	994[d]	ND	0.71 ± 0.02	ND
Camphor	1191	1171[h]	ND	ND	0.51 ± 0.08
Car-2-en-4-one	1370	1215[f]	ND	ND	11.68 ± 1.79
Alcohols					
Linalool L	1135	1109[a]	1.74 ± 0.04[b]	0.90 ± 0.02[c]	3.28 ± 0.0 [a]
l-Borneol	1213	1177[a]	ND	ND	1.79 ± 0.03
Terpinen-4-ol	1223	1197[c]	ND	ND	0.95 ± 0.04
α-Terpineol	1241	1208[h]	ND	ND	0.85 ± 0.06
Citronellol	1271	1240[a]	ND	0.82 ± 0.01	ND
Geraniol	1302	1265[a]	ND	3.22 ± 0.04	ND
Thymol	1343	1289[h]	ND	ND	3.58 ± 0.09
Carvacrol	1370	1308[d]	ND	ND	51.53 ± 8.09
Eugenol	1432	1390[c]	56.92 ± 2.22	ND	ND
Aldehydes					
Neral	1295	1256[a]	ND	32.46 ± 0.21	ND
Geranial	1333	1285[a]	ND	41.37 ± 0.19	ND
Cinnamaldehyde	1351	1267[h]	3.40 ± 0.57	ND	ND
Safrole	1347	1314[c]	4.74 ± 0.14	ND	ND
Sesquiterpenes					
α-Copaene	1398	1401[h]	0.56 ± 0.04	ND	ND
β-Caryophyllene	1452	1431[a]	4.82 ± 0.04[a]	ND	2.05 ± 0.09[b]
α-Humulene	1490	1469[b]	ND	ND	0.73 ± 0.02
Esters					
Geranyl acetate	1414	1387[a]	ND	0.64 ± 0.01	ND
Cinnamyl acetate	1514	1444[h]	3.05 ± 0.10	ND	ND
Eugenyl acetate	1583	1524[h]	2.57 ± 0.12	ND	ND
Benzyl benzoate	1754	1764[i]	4.77 ± 0.25	ND	ND
Epoxide					
Caryophyllene oxide	1621	1658[e]	1.05 ± 0.04[b]	ND	2.44 ± 0.11[a]

The relative abundance the average of three analyses (n = 3) ± standard error (SE). Values in the same row with different superscript lower case letters are significantly different ($P < 0.05$), based on Duncan's multiple range test.

*DB-5 was the column used for GC-MS analysis.

**Literature Sources: [a](Baranauskiene *et al.*, 2003); [b](Boukhris *et al.*, 2012); [c](Cardeal *et al.*, 2006); [d](Kallio *et al.*, 2006); [e](Figiel *et al.*, 2010); [f](Mastelic *et al.*, 2006); [e](Miyazaki *et al.*, 2011); [h](Trajano *et al.*, 2012); ND refers to the compounds that were not present/below detection limit of GC-MS.

Table 2. Tensile strength (TS), elongation at break (EB), thickness and water contact angle of chitosan-based films of different molecular weight.

Chitosan	Treatment	TS (MPa)	EB (%)	Thickness (mm)	Contact angle (°)
Low Mw	Plain	49.41 ± 2.37^{c}	7.83 ± 0.48^{e}	0.08 ± 0.00^{d}	87.54 ± 1.31^{b}
	Glycerol	21.63 ± 0.51^{f}	43.22 ± 1.55^{c}	0.08 ± 0.00^{d}	87.44 ± 0.87^{b}
	Cinnamon (5%)	15.81 ± 1.15^{g}	55.01 ± 1.10^{a}	0.12 ± 0.00^{c}	44.21 ± 1.96^{e}
Medium Mw	Plain	55.63 ± 1.32^{b}	6.23 ± 0.50^{e}	0.08 ± 0.00^{d}	90.85 ± 2.21^{b}
	Glycerol	25.95 ± 1.39^{e}	41.85 ± 2.67^{c}	0.11 ± 0.00^{c}	91.70 ± 1.25^{ab}
	Cinnamon (5%)	17.37 ± 0.91^{g}	49.32 ± 0.88^{b}	0.13 ± 0.01^{b}	52.08 ± 2.53^{c}
High Mw	Plain	62.99 ± 1.64^{a}	4.97 ± 0.29^{e}	0.07 ± 0.00^{d}	97.20 ± 1.82^{a}
	Glycerol	42.83 ± 0.97^{d}	33.81 ± 1.26^{d}	0.12 ± 0.00^{c}	87.50 ± 2.96^{b}
	Cinnamon (5%)	23.24 ± 0.93^{ef}	43.18 ± 1.83^{c}	0.14 ± 0.01^{a}	56.94 ± 2.39^{c}

Values in the same column bearing a common letter are not significantly different at ($P < 0.05$) based on Duncan's multiple range test.

Table 3. Colour mean values (*C**, *L**, *a**, *b** and yellowness index (*YI*)) of chitosan-based films

Chitosan	Treatment	*C**	*L**	*a**	*b**	*YI*
Low Mw	Plain	9.60 ± 0.21^{f}	91.89 ± 0.65^{a}	-1.76 ± 0.40^{c}	9.44 ± 0.43^{f}	14.67 ± 0.25^{e}
	Glycerol	15.81 ± 0.78^{d}	88.87 ± 0.69^{c}	-3.53 ± 0.29^{f}	15.41 ± 1.74^{d}	24.80 ± 1.38^{d}
	Cinnamon (5%)	51.49 ± 0.55^{a}	74.63 ± 1.05^{e}	5.38 ± 1.27^{a}	51.2 ± 1.19^{a}	98.09 ± 1.86^{a}
Medium Mw	Plain	8.72 ± 0.25^{f}	90.1 ± 0.20^{abc}	-1.86 ± 0.13^{c}	9.14 ± 0.53f	14.50 ± 0.38^{e}
	Glycerol	9.33 ± 0.24^{f}	90.16 ± 0.34^{abc}	-2.04 ± 0.16^{dc}	8.48 ± 0.53^{f}	19.99 ± 0.32^{d}
	Cinnamon (5%)	44.45 ± 2.21^{b}	82.44 ± 1.13^{d}	-0.72 ± 0.70^{b}	44.44 ± 4.97^{b}	77.23 ± 4.68^{b}
High Mw	Plain	3.93 ± 0.03^{g}	91.26 ± 0.12^{ab}	-0.53 ± 0.02^{b}	3.89 ± 0.07^{g}	6.09 ± 0.06^{f}
	Glycerol	12.90 ± 0.13^{e}	90.18 ± 0.62^{abc}	-2.70 ± 0.07^{de}	12.61 ± 0.28^{e}	13.43 ± 0.37^{e}
	Cinnamon (5%)	28.55 ± 0.71^{c}	89.22 ± 0.41^{bc}	-2.81 ± 0.13^{e}	28.41 ± 1.61^{c}	45.52 ± 1.31^{c}

Mean values (n = 20) ± standard error in the same column with same lowercase letter are not significantly different at ($P < 0.05$) based on Duncan's multiple range test

Chapter 7

Physical and antifugal properties of active essential oil-based β-cyclodextrin microcapsules and electrospun nanofibrous films for antimicrobial packaging

Abstract

Microcapsules of β-cyclodextrin (β-CD) can serve as a carrier vehicle for functional compounds such as bioactives, flavours and antimicrobials. This study developed active microcapsules and nanofibers derived from reacting β-CD with cinnamon (CIN) and oregano (OREG) essential oils (EOs) and determined the *in vitro* antifungal activity against postharvest pathogen *Botrytis* sp. which is an important disease of pomegranates. Solutions of β-CD and either cinnamon or oregano (OREG) EOs were subjected to co-precipitation to induce micro-encapsulation and the properties of the complexed microcapsules characterised. Solid state nuclear magnetic resonance (NMR) showed the emergence of new carbon peaks in the β-CD spectra which were absent in the plain β-CD spectra confirming encapsulation the EOs. Encapsulation of EOs was further confirmed by thermogravimetric analysis (TGA) which indicated that the thermal evaporation/degradation of the β-CD-EO complex occurred over a lower temperature (270 ºC) compared to the plain β-CD (300 ºC). Gas chromatography-mass spectrometry (GC-MS) showed that the amount of volatile EOs released was lowest for oregano EO than cinnamon EO suggesting that the strength of interaction between the EOs β-CD and cinnamon EO was stronger than that of β-CD and oregano EO. Nanofibers (~100-300 nm) were successfully fabricated from chitosan/polyvinyl alcohol/β-cyclodextrin (CH/PVA/β-CD) solution that served as carriers of cinnamon and oregano EOs. The release of EOs volatiles from the nanofibers was confirmed via GC-MS and the nanofibers had antifungal activity against *Botrytis* sp. This study demonstrates the potential application of micro-encapsulation in the design of active nanofiber matrices applicable for active packaging systems.

***Keywords*:** Antimicrobial packaging, *Botrytis* sp., Food safety, Volatiles,

1. Introduction

The shelf life of fresh and minimally processed fruit and vegetables is often limited by the growth of spoilage microorganisms. The use of antimicrobial packaging materials offers the potential to extend shelf life by retarding the growth rate of the microorganisms' (Espitia

et al., 2014). Several antimicrobial agents have been used in the making of antimicrobial packaging systems. These include inorganic, organic and biologically active compounds such as plant extracts and several polymers (Jianglian & Shaoying, 2013). Among these compounds those regarded as non-toxic, biodegradable, natural agents and generally recognised as safe (GRAS) by the U.S. Food and Drug Administration (FDA), are the most preferred (Wen *et al*., 2016).

Essential oils are considered 'green' alternatives in the nutritional, pharmaceutical, and agricultural fields due to the reported antimicrobial, nematicidal, insecticidal, and antioxidant properties (Turek & Stintzing, 2013; Sivakumar & Bautista-Banos, 2014). For example, Ali *et al*. (2015) demonstrated the retention of quality attributes and antifungal activity of chitosan enriched with lemongrass against anthracnose for bell pepper kept at room temperature for 21 days. In another study, Arrebola *et al*. (2010) combined an antagonist *Bacillus amyloquefacians* with thyme and lemongrass EO in a pad delivery system that successfully controlled *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* on peach fruit. Application of *B. amyloquefacians* with lemongrass as a biodegradable modified atmosphere packaging (MAP) system provided complete disease control and maintained overall acceptance of peaches after cold storage at 4 ºC for 14 days and at market shelf conditions (20 ºC for 2 d) (Arrebola *et al*., 2010).

The volatility of EOs complicates their application as food preservatives as they are thermally unstable. Encapsulation of EOs can improve their solubility and stability, while masking their undesirable flavour (Ayala-Zavala *et al*., 2008). For instance, Woranuch & Yoksan, (2013) reported that encapsulating eugenol loaded-chitosan nanoparticles using an emulsion-ionic gelation crosslinking method, enhanced its stability against light oxidation (Woranuch & Yoksan, 2013). Microcapsules of β-cyclodextrin (β-CD) can serve as a carrier vehicle for functional compounds. Cyclodextrins (CD) are cyclic oligosaccharides that exhibit a truncated hollow cone, made up of 7 D-glucose monomers linked by α-(1.4) bonds (Kayaci *et al*., 2014). The exterior of the β-CD molecule is hydrophilic, while the inner cavity is hydrophobic (Kayaci *et al*., 2014). Cyclodextrins are capable of forming non-covalent host-guest inclusion complexes with several molecules including EOs, fragrances/flavours and antioxidants (Kayaci *et al*., 2014). The encapsulation of garlic oil in β-CD by Ayala-Zavala and González-Aguilar (2010), effectively provided a release system of antimicrobial volatiles that was able to inhibit microbial growth and preserve the shelf life of fresh-cut tomatoes without impairing the sensory quality of the tomatoes at 5 ºC for 7 days.

Furthermore, the application of nano-scale antimicrobial materials provides improved delivery of active antimicrobial agents and enhances inhibitory activities compared to traditional materials (Ignatova *et al*., 2013; Rieger *et al*., 2015). Amna *et al*. (2015) developed an antimicrobial hybrid-packaging material composed of biodegradable polyurethane and supplemented with virgin olive oil and zinc oxide by electrospinning and effectively inhibited the growth of *Staphylococcus aureus* and *S. typhimurium*. Currently, there is need for further development of active nanofibers for food packaging application purpose and investigation of other bioactive antimicrobial agents. Electrospinning of environmentally friendly, biocompatible and non-toxic polymers such as chitosan and polyvinyl alcohol (PVA) is most preferred compared to using traditional petroleum-based polymers such as polyethylene. In this regard Wen *et al*., (2016) developed nanofilms via electrospininning of encapsulated cinnamon EO in PVA/β-CD for preservation of strawberries. The nanofilms extended the shelf life of the strawberries kept at 4 ºC for 6 days compared to 4 days by the control. So far limited work is available on control of storage diseases of pomegranate such as *Botrytis* spp. using this technology.

Therefore, the aim of this study was to encapsulate cinnamon and oregano EOs in β-CD, and to fabricate chitosan/PVA/β-CD/EO nanofibrous films by means of electrospinning. The study also investigated the antifungal activity of the developed microcapsules and nanofibrous films against Botrytis sp. using the agar diffusion technique.

2. **Materials and methods**

2.1. Fungal culture

Botrytis sp. (STE-U 7866), was obtained from pomegranates (cv. Herskawitz) located in the Wellington area, Western Cape, South Africa (GPS S33° 39.276 E18° 59.399). The pathogen isolate was grown on Potato Dextrose Agar (PDA; Biolab, Modderfontein, South Africa) for 7-14 days at 25 °C before each trial. Spore concentrations were freshly prepared by filtering each culture grown in PDA through two layers of cheesecloth with distilled water amended with Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.01 mL/L. Spores were counted with a haemocytometer (Neubauer, Marienfeld-Superior, Lauda-Konigshofen, Germany) and an optical microscope (Leica Wild M8 Transmitted Light Stereo Microscope, Wild Heerbrugg, Switzerland), and final concentration was adjusted to 1 x 10^6 spores/mL.

2.2. Essential oils (EOs)

Cinnamon leaf EO (*Cinnamon verum*) was obtained commercially from Soil (Durban, South Africa) and oregano EO (*Origanum vulgare* leaf extract) was obtained commercially from Clive Teubes Africa (Randburg, South Africa). The oils were previously extracted from fresh or partly dried leaves by means of steam distillation based on the supply data sheet provided. Samples were kept refrigerated at 4°C until the start of experiment.

2.3. Microencapsulation process

Microcapsules of cinnamon:β-CD (CIN/β-CD) and oregano:β-CD (OREG/β-CD) were prepared separately as described by Ayala-Zavala *et al.* (2008). A portion of β-CD (50 ± 0.01 g) was dissolved in ethanol (10 w/v%) at 55 ± 2 ºC and then EO was slowly added to the warm β-CD solution to obtain a weight ratio of 12:88 (EO:β-CD). During the addition of the oil solution, the β-CD solution was continuously stirred at a constant temperature 55 ± 2 ºC. Afterwards, the heater was turned off and the resultant mixture was covered and stirred for 4 h. The final solution was maintained overnight at 4 ºC. The precipitated microcapsules were recovered by filtration and freeze-dried for 48 h. The microcapsule complexes were thereafter removed from the freeze-drier and allowed to air-dry at 25 ºC in a desiccator for additional 24 h. The obtained EO:β-CD microcapsules were weighed at equilibrium and the amount of recovered active-microcapsules (on dry weight basis) was calculated in percentage. The initial weight prior to the addition of active agents was compared to the recovered active-microcapsules. Finally, the active-microcapsules were stored at 25 ºC in an airtight bottle. Each sample was prepared and analysed in triplicate.

2.4. Edible film preparation

Solution preparation was based on the method described by Wen *et al.* (2016) with a few modifications. For instance, Wen *et al.* (2016) did not use chitosan in their formulation mix, which was included in this current study. The advantage of using chitosan is that its possesses many useful properties such as biocompatibility, antimicrobial activity and can be electrospun into nanofibers (Wen *et al.*, 2016). Briefly, chitosan (3% w/w) was prepared by dissolving 3 g of low molecular chitosan (L-Mw) in 100 g of 70% acetic acid under constant stirring using a magnetic stirrer (RY5, IKA, Germany) at room temperature for 4 h. Polyvinyl alcohol (7% w/w) was dissolved in 100 g distilled water at 80 ºC for 3 h under constant stirring. The concentration of acetic acid (70%) used was derived from preliminary studies during which

chitosan nanofiber formation was observed. The two solutions were subsequently combined based on 30:70 ratio of chitosan to PVA.

Thereafter, 2 g of β-cyclodextrin (β-CD) was dissolved into the chitosan-PVA solution by constant stirring at 55 ºC for 1 h. Then, cinnamon and oregano essential oil (≈3 g) were respectively added to the chitosan-PVA-β-CD solution and mixed for 3 h. The final mixture obtained in the end was a turbid white solution. For adequate comparison and to ensure that the EOs were successfully incorporated, the solutions of chitosan-PVA and chitosan-PVA- β-CD were also prepared separately. The viscosity of the solution (20 g) was measured at controlled temperature of 22 ± 1ºC using a DV II+Pro viscometer (Brookfield, Middleboro, USA) with S63 and S64 spindle at 20 rpm. In addition, the conductivity of each solution was measured using a conductivity meter (CRISON Instruments, Barcelona, Spain). All tests were carried out in triplicate and the data presented as mean value (n = 3).

2.5. Electrospinning process

Each prepared solution was loaded into a 1 mL syringe fitted with a 21-gauge steel needle. This was driven by a syringe pump (Genie Plus, Kent Scientific, Torrington, USA) to give the solution flow rate of 0.2-0.6 mL/h. Electrospinning was conducted by applying a voltage varying from 13 to 15 kV with a power supply. A grounded collecting plate covered by a piece of aluminium foil was used as the collector for the fibre deposition. The distance between needle tip and collector was approximately 20 cm. Room temperature was maintained at 25 ± 0.5 ºC by air conditioner and the relative humidity (RH) was controlled at 56 ± 2% by dehumidifier. The nanofibrous films were thereafter conditioned at 25 ºC and 56% RH in a controlled environmental chamber (MLR-352H, SANYO, Osaka, Japan) for 12 h.

2.6. Gas chromatography-mass spectrometry (GC-MS)

Headspace gas chromatography-mass spectrometry (GC-MS) analyses were performed on a TRACE 1300 Series GC, equipped with Thermo TSQ 8000 triple quadrupole mass detector and a ZB-Multi residue capillary column (30 m-0.25 mm i.d., and 0.25 µm film thickness). Representative EO:β-CD and active nanofiber samples were transferred into 20 mL headspace vial, and 10 mL of 12% ethanol solution and 2.5 mL (20% sodium chloride) were added to facilitate evolution of volatiles into headspace. In addition, each vial was spiked with 100 µL of anisole as an internal standard. The SPME vials were equilibrated for 5 min at 50 °C in the auto-sampler incubator at 250 rpm. Volatile compounds were adsorbed and extracted

from the vial headspace using headspace solid-phase micro-extraction (HS-SPME). SMPE-fibre (65 µm) coated in divinylbenzene/polydimethylsiloxane (DVB/PDMS) was exposed to the sample headspace for 10 min at 50 °C. After extraction, desorption of the volatile compounds from the fibre coating was carried out in the injection port of the GC–MS with a split ratio of 20:1 for 1 min. The fibre was then conditioned for 15 min between samples for cleaning to prevent cross-contamination. The following temperature cycle was used: an initial 50 ºC was ramped up to 200 ºC at the rate of 6 ºC/min, and held 2 min, then finally from 200 to 250 ºC at a rate of 15 ºC/min, and was held at this final temperature for 3 min. Helium was used as the carrier gas, at a flow rate of 1 mL/min. Identification of compounds was based on the comparison of their mass spectra with those from NIST library data (Version, 2.0) of the GC-MS system. To determine whether the microencapsulation process had any effect on the relative abundance of the volatile organic compounds of the EOs, one major volatile and two minor compounds were selected for analysis of each EO. Analyses were performed for the cinnamon and oregano oil samples before and after microencapsulation with β-CD.

2.7. Characterisation and measurements

The infrared spectra of the electrospun nanofibers were recorded from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans by using a Nicolet™ iS™ 10 Fourier transform infrared spectrophotometer fitted with a smart diamond ATR accessory (Thermo Fischer Scientific, Waltham, USA). Thirty-two scans were recorded for each sample with a wavenumber resolution of 4 cm^{-1} using the Attenuated Total Reflectance (ATR) mode. X-ray diffraction (XRD) data of the nanofibers were collected by using D2 Phaser diffractomer (Bruker, Karlsruhe, Germany) with Cu Kα radiation in the rage of 2θ = 5-30º. Solid ^{13}Carbon state NMR was measured using a VNMRS 500 MHz spectrometer (Varian Inc., Palo Alto, California) with a 15 N to 31P frequency range to confirm the presence of chitosan, PVA, β-CD and EO within the β-CD microencapsulation and nanofiber films. Approximately 50 mg of nanofiber mats, as well as 100 g of powder samples were analysed. The thermal properties of the samples were investigated by using TA Q500 thermogravimetric analyser (TA Instruments, New Castle, USA). In TGA measurements, the nanofibers were heated from room temperature to 600 ºC at a constant heating rate of 20 ºC/min under nitrogen atmosphere. The nitrogen flow was maintained at 60 mL/min. A Merlin, electron microscope (ZEISS, Oberkochen, Germany) was used to investigate the morphology of β-CD microencapsulated samples and nanofilms. The samples were carbon coated prior to SEM analysis. Around 100 fibre diameters of each sample were measured from the SEM images to determine the average

fibre diameter of the nanofiber samples.

2.8. Antifungal activity of EO β-CD microencapsulation and active-nanofiber films

Antifungal activity of EO:β-CD microencapsultions and active-nanofiber films was evaluated *in vitro* against *Botrytis* sp. based on a method described by Du *et al.* (2009). Antifungal activity of plain β-CD (5 g/L) and pure EO (5 g/L) were also investigated as positive and negative controls, respectively. Approximately 0.1 mL of 10^6 spores of each fungal isolate was plated onto 90 mm PDA plates. The EO encapsulates (CIN/β-CD and OREG/β-CD) were diluted into potato dextrose agar (PDA) at different concentrations of 2.5, 12.5, 25 and 50 g/L. Spores of *Botrytis sp.* (1x 10^6/mL) were inoculated by spreading the entire petri dish containing the above-mentioned treatments. After inoculation the plates were incubated for 72 h at 25 °C, and then colony diameter was measured. Four measurements were taken per colony due to the asymmetrical colony diameter, and averaged mean value of six plates (n = 6).

The inhibitory effects of the active-nanofiber films were tested using a diffusion method described by Wen *et al.* (2016). Potato dextrose agar (PDA) was inoculated with 1 x 10^6 CFU/mL spores of *Botrytis* sp., and the active-nanofiber films were cut into 6 mm diameter (Ø) disks using a sterile glass rod and scalpel. Both sides of the nanofiber film (Ø = 6 mm) were sterilised under UV irradiation for 2 h (each side for 1 h), thereafter they were placed onto the surface of the inoculated media. The Petri dishes were sealed using parafilm to prevent leakage of EO vapour, and incubated at 25 ± 2 °C and 55 ± 2% relative humidity for 72 h. The inhibition radius around the nanofiber film disc (colony-free perimeter) was measured using a digital Vernier calliper (Mitutoyo, Kawasaki, Japan) in triplicate. The fungistatic index was calculated using Eqn. 1:

$$\text{Fungistatic inhibition (\%)} = 1 - \left(\frac{\text{Ri}}{\text{Rc}}\right) \times 100 \qquad (2)$$

where *Rc* represents the mean value of the colony radius of control media and *Ri* is the zone of inhibition radius of the essential oil-amended media.

2.9. Statistical analysis

Data analysis was performed using SAS-Statistical software (SAS Institute Inc., Cary, North Carolina, USA). Data was subjected to analysis of variance (ANOVA), and means were separated by Duncan's multiple range tests at 95% confident interval. All values were reported

as mean ± standard error.

3. Results and discussion

3.1. Gas chromatography (GC-MS) analysis

The relative abundance of selected volatile compounds of cinnamon and oregano EOs before and after microencapsulation process are presented in Table 1. The GC-MS spectra revealed eugenol (56.92 ± 2.22%) as the major volatile of pure cinnamon EO with Safrole (4.82%) and β-caryophyllene (4.7%) among the minor constituents. These results were in agreement with reports from other studies on the volatile composition of cinnamon EO (Ayala-Zavala *et al*., 2008; Nabavi *et al*., 2015). On the other hand, carvacrol (51.53 ± 8.09%) was identified as the main compound of oregano EO, while p-cymene (10.01%) and linalool (3.28%) were considered as minor compounds. Similar findings for oregano EO was reported by (Avila-sosa *et al*., 2012) and (Hyldgaard *et al*., 2012). The volatile compounds for both cinnamon and oregano were also observed to possess antimicrobial activity (Hyldgaard *et al*., 2012; Nabavi *et al*., 2015).

The relative abundance of the selected major and minor volatiles was used as a pattern to evaluate the efficiency of the β-CD microencapsulation process. It was observed that the relative abundance of the major constituent of each oil decreased significantly after β-CD-microencapsulation ($p < 0.05$) as shown in Table 1. For instance, the percentage relative contribution of eugenol had declined in the CIN/β-CD microcapsules when compared to that of pure oil by almost 24%. Similarly, the relative abundance of carvacrol volatile had decreased by 27% in the OREG/β-CD complex, although, it was retained as the major constituent (24.07%). In contrast, the relative abundance of β-caryophyllene and safrole was higher after microencapsulation than in free cinnamon EO. The increase in the relative abundance of safrole and β-caryophyllene in the CIN-β-CD complex can be attributed to their stoichiometric formation of stable complexes with β-CD (1:1) and the establishment of a competitive equilibrium between β-CD and the volatiles (Ayala-Zavala *et al.*, 2008).

3.2. Major volatile recovery after microencapsulation

Table 2 presents the summary of recovered cinnamon- and oregano-microcapsules. The weight of active-microcapsules; CIN/β-CD (52.12 g) and OREG/β-CD (49.06 g) recovered were less than the total amount EO/β-CD (59.54 g) originally used. This decrease could probably be due to the amount of free β-CD available for occupation by the EOs before

saturation. The starting ratio of 16:84 of β-CD to EOs applied in this study was based on the findings made by Ayala-Zavala *et al.* (2008). The authors suggested that the maximum inclusion of β-CD with cinnamon and garlic EO during microencapsulation was achieved at approximately 16:84 and 12:88, respectively, and higher starting ratios greater than 16:84 (EO:β-CD) did not significantly affect the EO recovery.

Relative EO-microcapsule recovery based on the initial EO/β-CD was 87.54 ± 0.14% and 82.40 ± 0.56% for CIN/β-CD and OREG/β-CD, respectively. This calculation was obtained by expressing the recovered mass of EO/β-CD after precipitation as a fraction of the original mass of EO and β-CD used. The eugenol volatile content for CIN/β-CD microcapsules was 48.63 ± 0.83 mg/g, while carvacrol content was 17.50 ± 0.24 mg/g for OREG/β-CD (Table 2). The eugenol content load for CIN/β-CD microcapsules of 4.86% was comparable to the 5% reported by Ayala-Zavala *et al.* (2008) the same 16:84 ratios. The recovery of the EO-microcapsule complex may have affected by the amount of EO remaining in the solution after forming microcapsules. Furthermore, losses in volatile content may occur due to evaporation during the microencapsulation process (Kayaci & Uyar, 2011). In addition, significant amount of EO on the surface expected to be lost by evaporation during the drying step, due to the volatility of essential oils, and it is likely that a significant amount of the EOs vapour will occupy the surrounding air space (Bhandari *et al.*, 1998).

3.3. ATR-FTIR characterisation of EO/β-CD microcapsules

The presence of the guest molecule (EO) in the β-CD inclusion complexes was confirmed by the FTIR studies (Fig. 1). The FTIR spectrums of pure cinnamon and oregano EOs, and β-CD (Fig. 1a, b, c), were provided for comparison with CIN/BCD and OREG/BCD FTIR spectrums. Pure cinnamon EO exhibited characteristic peaks at 1510 and 1605 cm^{-1} corresponding to stretching absorption of benzene ring stretching of C=O of the aldehyde group (Fig. 1a). While, oregano EO showed the major IR absorptions at 3367 cm^{-1} (OH stretch), 2826-2959 cm^{-1} (C-H stretch), 1591 cm^{-1} (alkene C=C), and 1400 cm^{-1} (aromatic C=C). The characteristic absorption bands of pure β-CD were observed at around 939, 1022, and 1153 cm^{-1} (Fig. 1c), which corresponded to the coupled C-C/C-O stretching vibrations and the asymmetric stretching vibration of the C-O-C glyosidic present in β-CD (Kayaci & Uyar, (2011).

The IR spectral analysis showed differences in specific band positions of the microcapsule with respect to the inclusion of either cinnamon or oregano EO into the β-CD

matrix. These differences in the IR spectra are typical of β-CD solid-state complexes, due to loss of vibrating and bending of the guest molecule during complex formation (Ayala-Zavala *et al.*, 2010). For the IR spectra of CIN/β-CD and OREG/β-CD microcapsules, both FT-IR bands were almost completely obscured by very intense and broad β-CD bands, which were hardly influenced by molecular complex formation. The absorption bands for OH groups at 3514 and 3367 cm^{-1} experienced a dramatic broadening of the spectra for the prepared EO-microcapsules, and the peaks were shifted toward the lower frequency of 3300 cm^{-1} found in pure β-CD, respectively. This change may be related to the formation of intramolecular hydrogen bonds between the guest and host molecules (Kayaci & Uyar, 2011). The shifts in FTIR after microencapsulation indicated that an interaction had occurred between the EO and β-CD, via hydrogen bonding and that the complexes are not just physical mixtures (Kayaci & Uyar, 2011).

3.4. X-ray diffraction (XRD)

The XRD studies were performed to investigate crystalline structure of CIN/β-CD and OREG/β-CD inclusion complexes (Fig. 2). The XRD patterns of CIN/β-CD and OREG/ β-CD (Fig. 2a, b) and that of plain β-CD (Fig. 2c), have characteristic diffraction peaks in the range of 2θ = 5-30 º. The XRD patterns of EO/β-CD ICs were different from those of plain β-CD (Fig. 2). The XRD pattern of both CIN/β-CD and OREG/β-CD microcapsules show three major peaks centred at 2θ= 6.78, 12.06 and 17.56º, and 2θ= 6.84, 11.86, and 17.71º, respectively. In addition, the change in peak spectrum for plain β-CD when compared against the EO/β-CD suggests that complexation of cinnamon and oregano EOs with β-CD was successful. Kayaci and Uyar (2011), also reported changes in diffraction peak formation in the same range of 2θ = 5-30 º for three types of cyclodextrins α-CD, β-CD and γ-CD) after microencapsulation of vanillin. The XRD patterns of the vanillin/β-CD complexes had revealed the emergence of a distinct peak at 2θ = 11.8 º and at 2θ = 17.8 º, which indicated that the β-CD adopted a channel-type packaging, and confirmed that complexion of the vanillin was successful.

3.5. Thermogravimetric analysis curves

To determine the thermal stability and evaporation of the EO/β-CD encapsulations in comparison to the plain β-CD powder (Fig. 3), TGA study was conducted. Initial weight loss for plain β-CD was below 100 ºC and the major weight loss was at 300 ºC (Fig. 3a). A closely related study by Abbehausen *et al.* (2010) evaluated the thermal behaviour of a hybrid polymer derived from siloxane and β-CD after reacting β-CD with γ- -isocyanatopropyltriethoxysilane

(ITPS). The authors observed weight loss in the β-CD thermal curve centred around 100 ºC, which corresponded to residual water loss. In the study by Abbehausen *et al.* (2010), β-CD showed a fast thermal degradation centred at 340 ºC and started residue degradation above 400 ºC up to 900 ºC. Similarly, Kayaci & Uyar (2011) reported that the TGA curve of β-CD had two main weight losses; an initial weight loss below 100 °C, due to water loss, and the major weight loss at about 300 °C. The authors suggested that the major weight loss at 300 °C corresponded to the degradation of β-CD.

In this study, it was observed that the thermal stability of β-CD was lowered by introduction of the EOs. The degradation temperature of β-CD in the EO/β-CD microencapsulation complex shifted from around 300 ºC to 270 ºC. This could be attributed to hydrogen bonding interaction between hydroxyl groups of β-CD and EOs. The noticeable difference of the TGA curves between plain β-CD and EOs/β-CD complex demonstrates the presence of chemical interaction among the substances. Inclusion of a guest molecule induces a different thermal behaviour of β-CD (Abbehausen *et al.*, 2010). Volatiles in particular have thermal degradation temperatures mostly < 200 ºC and these are associated to the loss of water residues trapped in the polymer (2% m/m) (Abbehausen *et al.*, 2010; Kayaci & Uyar, 2011). The hybrid polymerisation of volatiles with β-CD induces thermal stability of volatile compounds. To demonstrate this, Kayaci & Uyar, (2011) revealed that the weight loss and thermal degradation of vanillin shifted to higher temperature from 80-200 ºC to 150-300 ºC after undergoing cyclodextrin microencapsulation.

3.6. NMR and SEM imaging of β-CD microencapsulation

To ensure that attached and unattached EOs were present in the β-CD microencapsulations, solid-state ^{13}C NMR was conducted. The spectra obtained suggested that due to the presence of cinnamon and oregano EO, new peaks emerged in the 20-40 ppm as well as 140-160 ppm regions for the EO-β-CD complex, which were absent in the plain β-CD spectra (Fig. 4). Solid-state ^{13}C NMR informs that the majority of EOs in the β-CD microencapsulation was physically incorporated and therefore swelling was controlled, while unreacted EOs is likely to be released by the swelling of the encapsulations (Kayaci & Uyar, 2011).

The morphology of the EO-β-CD complex as characterised by SEM imagining is presented in Figure 5. Plain β-CD was characterised as large homogenous plate particles with a compact and regular continuous matrix (Fig. 5a). Sauceau *et al.* (2008) also observed large

pale particles for plain β-CD that were compact, with a smoot surface without pores or cracks. Complexed samples showed a different morphological distortion of the surface structure of the β-CD (Fig. 5b, c). It showed that the homogenous cubic structures had disintegrated into clusters after complex formation. Incorporation of cinnamon and oregano EOs into the β-CD decreased the compact nature to reveal an open amorphous structure. When cinnamon or oregano EO were incorporated into the β-CD matrix, the course surface structure is disrupted and a coarse microstructure can be observed.

3.7. Antifungal activity of β-CD microencapsulations

Figure 6 showed that plain β-CD itself increased the growth of *Botrytis* sp. This can probably be possible because β-CD could act as a carbon source for the fungus (del Toro-Sanchez *et al.*, 2010). However, both free EOs exhibited complete inhibition (100%) of *Botrytis* sp. growth. The CIN/β-CD microcapsules had the lowest fungal growth inhibition at 2.5 g/L (Table 3). Generally, the inhibitory effects of the EO/ β-CD microcapsule increased with the increased in concertation of EO in the complexes. In contrast, OREG/β-CD showed the least inhibition at the 50 g/L (Table 3). Increasing the concentration of OREG/β-CD microcapsule did not result in an increase in antifungal activity of the complex as expected, rather the opposite response was observed. Antifungal activity OREG/β-CD was limited due to the available EO in the OREG/β-CD complex (Fig. 5b). This assumption was corroborated by the GC-MS results, which showed higher encapsulation efficiency for cinnamon EO (4.86%, eugenol) compared to oregano (1.75%, carvacrol). At 2.5 g/L the OREG/β-CD demonstrated comparable fungal growth inhibition comparable to that for CIN/β-CD at the same dosage. However, the more concentrated the complex became the more β-CD became available to promote fungal growth at the expense of the OREG-EO which became more and more limited. Ayala-Zavala *et al.* (2008) compared β-CD microencapsulation of cinnamon and garlic EO, and reported differences in the volatile load with 12.76% recovery for cinnamon EO compared to 20.75% for garlic EO. The authors had achieved complete complexation of garlic EO/β-CD at 12:88, and cinnamon EO/β-CD at 16:84 ratios (Ayala-Zavala *et al.*, 2008).

The current study proved that the antimicrobial property of CIN/β-CD was preserved after microencapsulation process. The microencapsulated volatile constituents were stable enough to preserve its antifungal efficacy against *Botrytis* sp. growth. In contrast, the concentration of preserved volatiles in the OREG/β-CD was limited. Although the OREG/β-CD microcapsule displayed some antimicrobial activity due to the presence of the encapsulated

oregano EO, the overall antimicrobial activity potential of the OREG/β-CD microcapsule was overpowered by the nutritive effects of β-CD present and the limited amount of EO present. Hence an increase in OREG/β-CD microcapsule concentration only provided the substrate with more nutrition to grow. The CIN/β-CD complex inhibited the growth of *Botrytis* more effectively than OREG/β-CD, at all concentrations studied. Hence, the microencapsulated CIN/β-CD could be used as a substitute for synthetic preservatives in packaged fresh food products.

The findings from this study corroborate with those of del Toro-Sanchez *et al.* (2010) who demonstrated the controlled release of thyme EO using β-CD capsules fabricated from a similar precipitation protocol as the one described in this study. In addition, the thymol/β-CD (8:2 ratio) microcapsules effectively controlled *Alternaria alternata* fungal growth by both agar dilution and headspace method (Del Toro-Sanchez *et al.*, 2010). Arana-Sancehz *et al.* (2010) investigated how the antimicrobial and antioxidant activities of *Lippia graveolens* EO are affected by β-CD encapsulation using three types of Mexican oregano EOs. The microencapsulated EOs was shown to preserve its antibacterial activity against *E. coli*, *Pseudomonas aeruginosa* and *S. aureus*, while the antioxidant activity was increased from four to eightfold (Arana-Sancehz *et al.*, 2010). These findings consolidate the result from this study, that microencapsulation of EOs offer an alternative and safe antimicrobial agent against food-borne pathogens and spoilage microorganisms.

3.8. Characterisation of nanofiber films

3.8.1. Viscosity, conductivity and average fibre diameter

In this part of the study, cinnamon and oregano EOs were incorporated into chitosan/poly (vinyl alcohol)/β-cyclodextrin (CH/PVA/β-CD) via electrospinning in order to produce functional nano-fibrous material. Incorporation of cinnamon and oregano EO had a similar effect on the changes in solution viscosity, conductivity, and average nanofiber diameter. Therefore, only results for oregano EO is discussed as shown in Table 4. Initially the viscosity CH/PVA solution was 823.80 cP, and this was not significantly affected by the introduction of β-CD (2%). However, the addition of oregano EO resulted in a significant ($P < 0.05$) four-fold increase in solution viscosity (3230 cP) and at 3% EO inclusion the viscosity had increased by almost 30 fold.

Solution conductivity was 1689 μ/cm for the CH/PVA solution, but adding β-CD and EOs led to a decrease in solution conductivity. The increase in conductivity indicated a lack of

water molecules to dissociate the CH/PVA/β-CD/EO mixture completely (Pakravan *et al.*, 2011). At 3% EO incorporation the conductivity had declined by 10%. In contrast to this finding, Wen *et al.* (2016) recorded a 10% increase in solution conductivity for PVA/CIN/β-CD at 3% inclusion of EO. The differences could be attributed variation in complex formulation, as their solution did not include chitosan.

The nanofiber diameter range was also affected by the incorporation of β-CD and oregano EO with an increase in average fibre diameter from 92.85 to 181.75 nm (Table 4). Similarly, Wen *et al.* (2016) reported an increase in nanofiber diameter from 270 nm to range between 360-401 nm after β-CD and EO was incorporated into the PVA solution. The variation in fibre diameter in the current study with that of Wen *et al.* (2016) could probably be attributed to the incorporation of chitosan in the current study which was absent in the later study. Although chitosan could not form neat fibres alone in the present study it did produce a hint of fibre formation in the range of 50-100 nm, which could have affected the final dimeter of the fibres produced (Fig. 7).

3.8.2. SEM images of electrospun nanofibers

Chitosan being a natural antimicrobial that is biodegradable, and non-toxic, serves as an ideal candidate for the fabrication of nanofibres for application in food processing and preservation (Wen *et al.*, 2016). The major drawback of using chitosan is that is cannot form continuous nanofibers as demonstrated in Figure 7a. The polycationic nature of chitosan, rigid chemical structure, specific inter and intramolecular interactions, makes it is difficult to obtain chitosan-based nano-fibres (Pakravan *et al.*, 2011; Ignatova *et al.*, 2013). To address this drawback water-soluble non-ionogenic polymers such as poly (ethylene oxide) and PVA can be introduced to facilitate fibre formation (Ignatova *et al.*, 2013). Although there is evidence of slight chitosan fibre formation in the range of 50-100 nm, the final product is not suitable for use. Hence, introducing a copolymer such as PVA is necessary to assist in fabricating chitosan-based nanofibers and this gave contiguous nanofibers in the diameter range of 100-200 nm in the present study (Fig. 7).

In the previous section it was evident that β-CD has the ability to encapsulate essential oils for their controlled release. In this regard, plain β-CD (2%) was added to the CH/PVA mix, and although continuous fibres were obtained, here was some evidence of beading (Fig. 7b). Effects of adding β-CD (2% w/w) and cinnamon EO (3 w/w%) separately into CH/PVA as opposed to adding pre-encapsulated EO was illustrated in Figure 7 c, d. Incorporation of β-CD

and cinnamon EO increased the fibre diameter range 200-300 nm (Fig. 7 c). Presence of β-CD in the matrix was evident as shown by the course crystalline structures. These crystals like structures were similar to previously shown SEM images for CIN/β-CD (Fig. 5c, d). Adding the previously fabricated CIN/β-CD microcapsules at 5% into the CH/PVA mix intensified the presence of the β-CD crystal like structures into the fibre matrix and widened the nanofiber diameter range from 150-300 nm (Fig. 7d). Kayaci *et al.* (2014) obtained uniform and bead-free nanofibers from PVA and PVA/geraniol, whereas the aggregates of geraniol/γ-CD crystals in PVA/ geraniol/γ-CD were similar in SEM images to the present study.

3.8.3. FTIR and GC-MS characterisation of nano films

FTIR spectra showed that interactions occurred in the CH/PVA/β-CD/CIN nanofiber films (Fig. 8). The broad band ta 3426 cm^{-1} in the CH/PVA/β-CD nanofiber was attributed to the O-H stretching formed by PVA hydroxyl groups and water, while band at 2927 cm^{-1} resulted from the stretching of C-H. Other important bands include 1421 cm^{-1} and 1029 cm^{-1}, which corresponds to the bending and stretching of C-H chain and the stretching of C-O in the C-O-H bond, respectively. In contrast, the spectrum obtained for CH/PVA/β-CD/CIN showed noticeable variation to CH/PVA/β-CD. The position and strength of O-H stretching peak had an obvious shift in the oil containing fibres from 3326 cm^{-1} to 3309 cm^{-1}. This shift may be attributed to more hydrogen bonds emerging between the reagents resulting in the increase of bond length and decrease of force constant (Kayaci & Uyar, 2016). As shown in Figure 8, the characteristic absorption peaks of cinnamon EO in the mixture of CH/PVA/β-CD/CIN were consistent with those presented earlier in Figure 1a, and were not observed in plain film. This indicated that the cinnamon EO was efficiently included into the cavity of β-CD. In addition, the changes in peak position and strength in the spectrum also demonstrated that there was interaction among CH, PVA, β-CD and cinnamon EO.

GC-MS analysis of the CH/PVA/β-CD/EOs nanofiber films showed that respective fibres contained 1.75 ± 0.12 mg/g of cinnamon EO and 1.38 ± 0.00 mg/g of oregano EO. The analysis revealed that there was better retention of oregano EO in the nanofibrous films compared to the β-CD microcapsules. The presence of chitosan in the mixture might be responsible for the improved oil recovery. This was evident in the antimicrobial activity investigations, in that the antifungal efficacy of oregano EO-nanofiber films was almost comparable to films with cinnamon EO (Table 5). Thus, suggesting that electrospinning is a facile method for preparing nano films with improved antimicrobial activity. Rieger & Schiffman (2014) applied electrospinning technique for solutions containing polyethylene

oxide and cinnamaldehyde without adding chitosan. They reported phase separation. This indicated that a homogenous solution appropriate for electrospinning could only be obtained after the incorporation of chitosan. Chitosan is assumed to act as a stabiliser that physically incorporates the cinnamon EO into the precursor solution.

3.9. Antifungal activity of nanofiber films

The fabricated active nanofilms with cinnamon and oregano EOs showed adequate inhibitory efficacy against *Botrytis* sp. *in vitro*. Our results are in agreement with other related studies on the antimicrobial activity of cinnamon and oregano EOs. Zodrow *et al.* (2012) reported that films fabricated from poly (lactic-*co*-glycolic acid) (PLGA) and 0.1% cinnamaldehyde (from cinnamon) or 0.1% carvacrol (from oregano) had antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus.* Perdones *et al.* (2014) showed that chitosan-cinnamon leaf oil films exhibited antioxidant and antifungal properties against *Aspergillus niger*, *Botrytis cinerea* and *Rhizopus stolonifer* and extended the shelf life of strawberries for 14 d at 10 ºC and 70% RH, or for 4 d at 20 ºC and 80% RH.

Furthermore, the antifungal activity of nanofiber films (CH/PVA/β-CD) with varying concentrations (0, 0.5 and 3%) of cinnamon and oregano EO against *Botrytis* sp. is summarized in Table 5. For the disk diffusion test, the inhibitory effects of nanofilms incorporated with and without β-CD against *Botrytis* sp., were not significantly different for each EO. However, films carrying the β-CD with similar EO concentration showed slightly higher efficacy than the films without β-CD. The stronger antimicrobial activity of the CH/PVA/β-CD/EO nanofilms could be attributed to the presence of higher EOs within its complexes. In addition, the inhibition zones/diameters of the films carrying cinnamon EO were wider than that of films containing oregano EO. This might be attributed to better efficient entrapment of cinnamon EO in the cavity of β-CD compared to oregano EO. This improves the solubility of cinnamon EO leading to the efficient release of antimicrobial volatiles into the agar medium (Ayala-Zavala *et al.*, 2008).

Wen *et al.* (2016) reported a similar phenomenon for PVA/β-CD/CIN films investigated against *Botrytis* sp. compared to microencapsulated-EO. The antimicrobial activity of the same mass of electrospun film increased by 7.2-8.8% against *Botrytis* sp. compared with microencapsulation. The increase may be attributed to the electrospinning method, which allows for better incorporation of active compounds into the polymer and the sub-micron structure of film is favourable for sustained release of antimicrobial agent. The

presence of chitosan in the nanofiber mats offers positively charged free amine groups, which damages bacterial membrane's ability to function as a barrier (Ignatova *et al.*, 2013). The active nanofibers offer an additional mechanism to inactivate microorganisms by direct contact with EO vapours. While nanofibers containing attached EO offer less free amines to interact with microbes, the statistical equivalence of antimicrobial efficacy achieved by all forms of the nanofiber systems confirms that by using a combination of chitosan and EO killing mechanisms, growth inhibition of *Botrytis* fungal pathogen can be achieved. The lipophilic character of the EOs could suggest that they interact with the cellular membrane of the microorganisms. Hydrophobicity of these molecules enables them to partition the lipids of the pathogen's cell membrane, thus disturbing it structure and rendering it more permeable.

4. Conclusions

This study showed that both oregano and cinnamon EOs were successfully microencapsulated using β-CD, and active nanofiber films with smooth and uniform nanofibers were obtained under optimal conditions. The microcapsules of cinnamon-EO/β-CD had higher antimicrobial efficacy compared to oregano-EO/β-CD. The facile process of electrospinning was found to be favourable for maintaining the volatile antimicrobial agents in the nanofibers than microencapsulation. Although the complex CH/PVA/β-CD/EO nanofibers had excellent antimicrobial activity against *Botrytis sp.* (an important postharvest disease causing pathogen), further studies on the long-term controlled-release of encapsulated essential oils are needed to elucidate on the active shelf life for a given product.

Furthermore, the findings from this study highlight on the potential application of microencapsulation and design of nanofibers in the development active food packaging systems. Future research is needed to focus on application of active nanofiber films for fresh products during storage. This would include investigations to establish optimum storage conditions suitable for the maximum performance of nanofibers.

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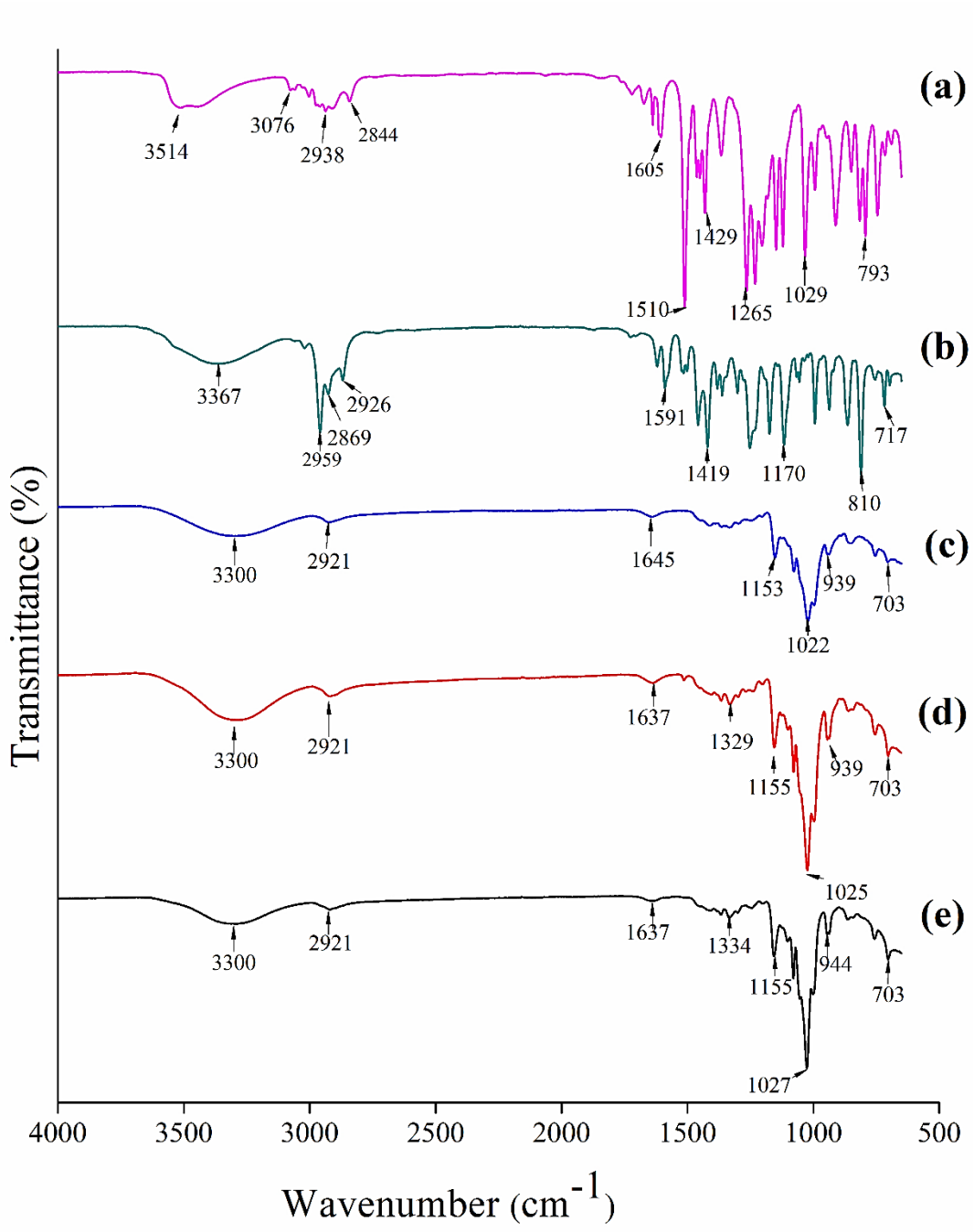

Figure 1. Fourier transform infrared (FTIR) spectra for cinnamon EO **(a),** oregano EO **(b)**, beta-cyclodextrin (β-CD) **(c)**, microencapsulated cinnamon EO (CIN/β-CD) (d), and oregano EO (OREG/ β-CD) (e), respectively.

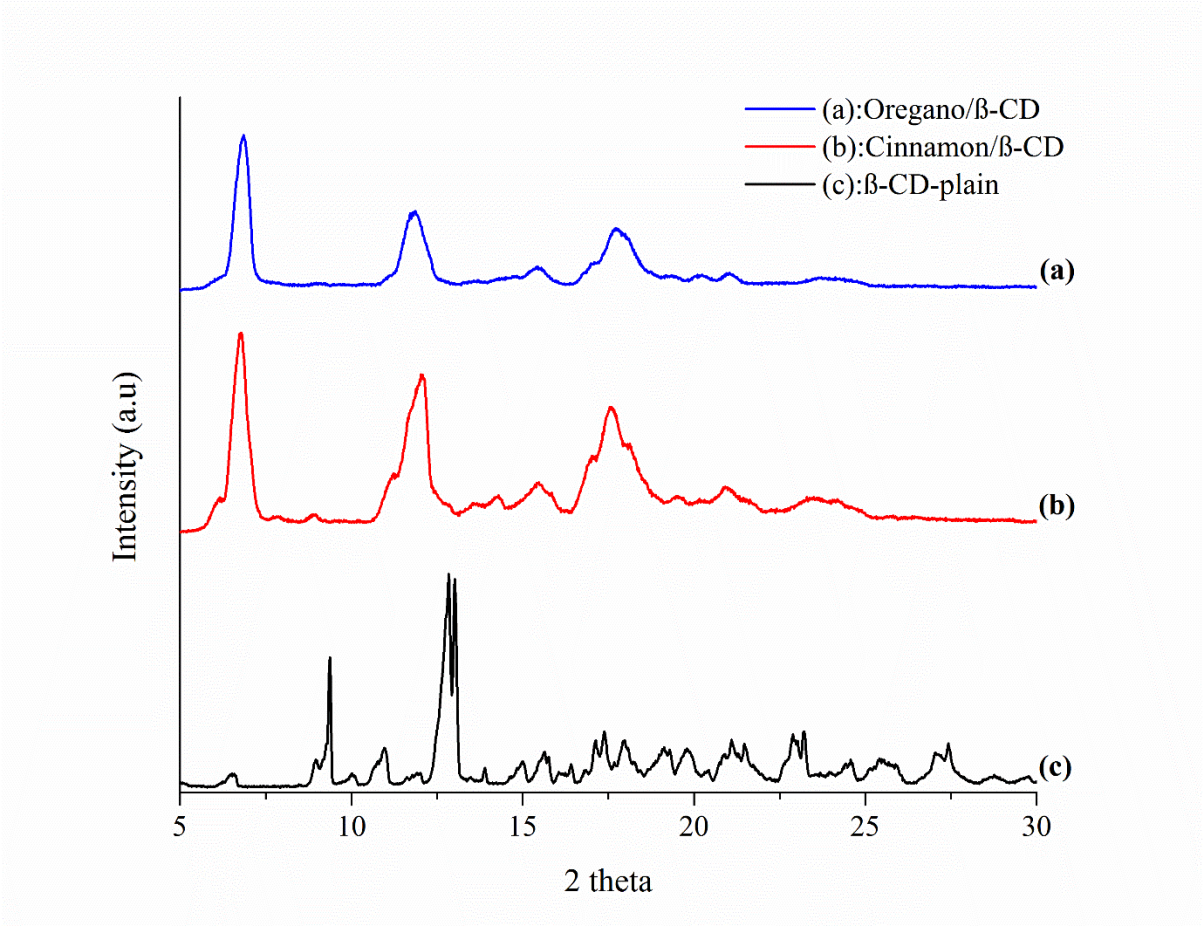

Figure 2. Intensity of the X-ray diffraction (XRD) patterns obtained for microencapsulated cinnamon EO (CIN/β-CD) (a), oregano EO (OREG/β-CD) (b), and plain β-CD (c).

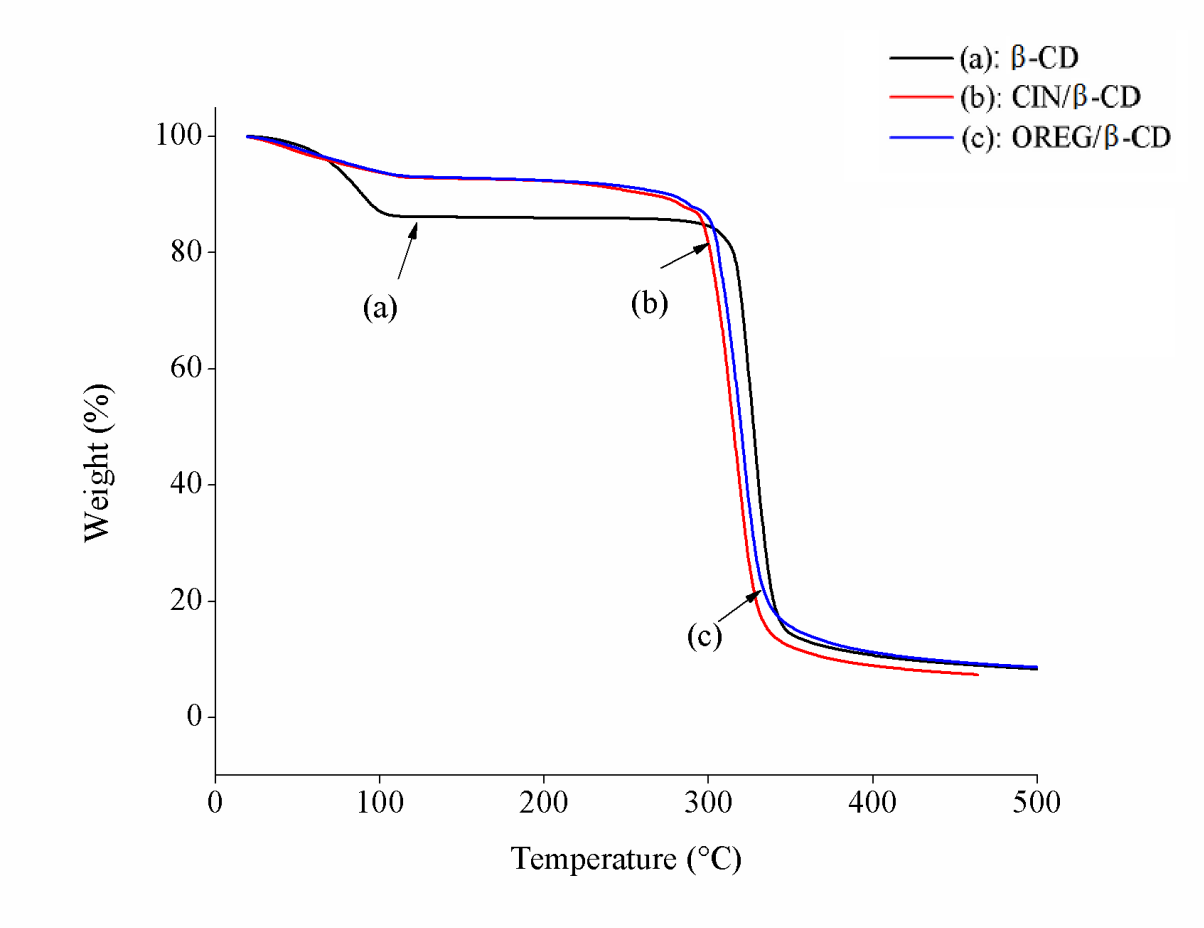

Figure 3. Thermogravimetric analysis (TGA) patterns of plain β-cyclodextrin (β-CD) (a), and microencapsulated cinnamon EO (CIN/β-CD) (b), and oregano EO (OREG/β-CD) (c).

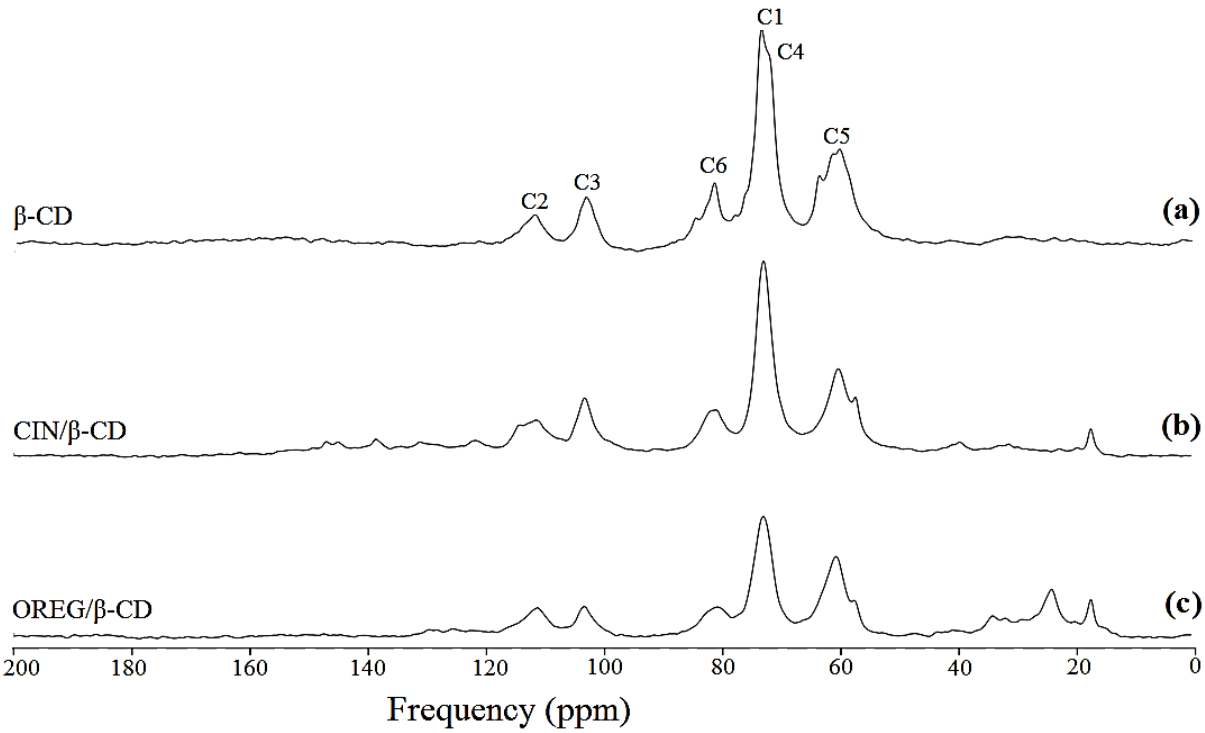

Figure 4. Solid nuclear magnetic resonance (NMR) spectra of (a) plain β-cyclodextrin (β-CD), and microencapsulated (b) cinnamon EO (CIN/β-CD), and (c) oregano EO (OREG/β-CD).

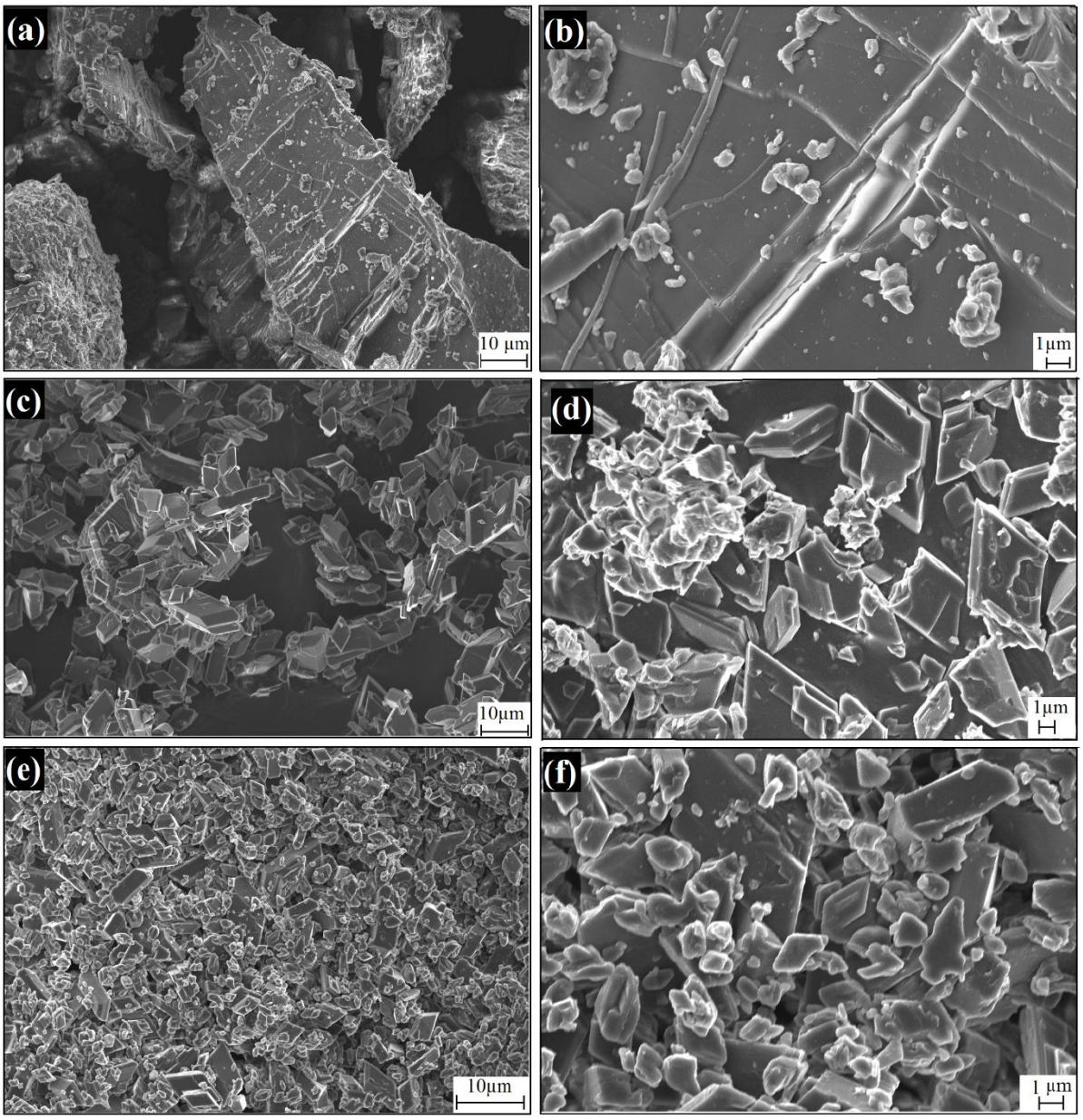

Figure 5. Scanning electron microscopy (SEM) images of plain β-CD (a -b), cinnamon EO/β-CD (c-d), and oregano EO-β-CD (e-f) at 10 and 1 µm magnification, respectively.

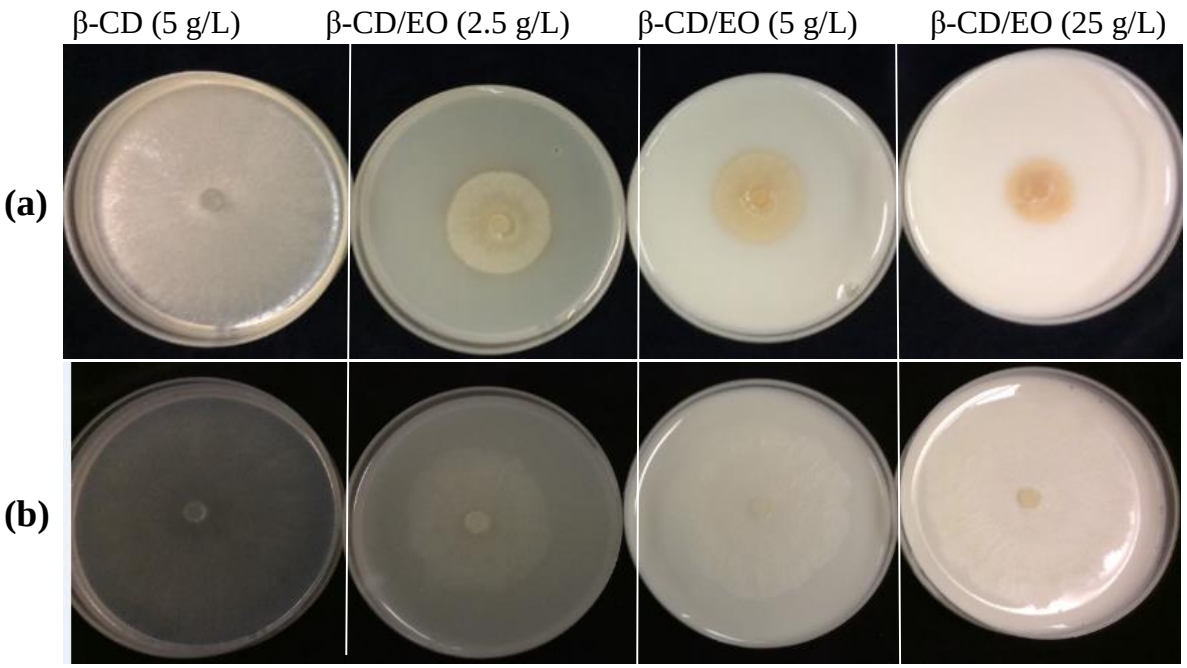

Figure 6. Radial mycelial growth inhibition of *Botrytis* sp. by varying EO concentrations of cinnamon EO/β-CD **(a)**, and oregano EO/β-CD **(b).**

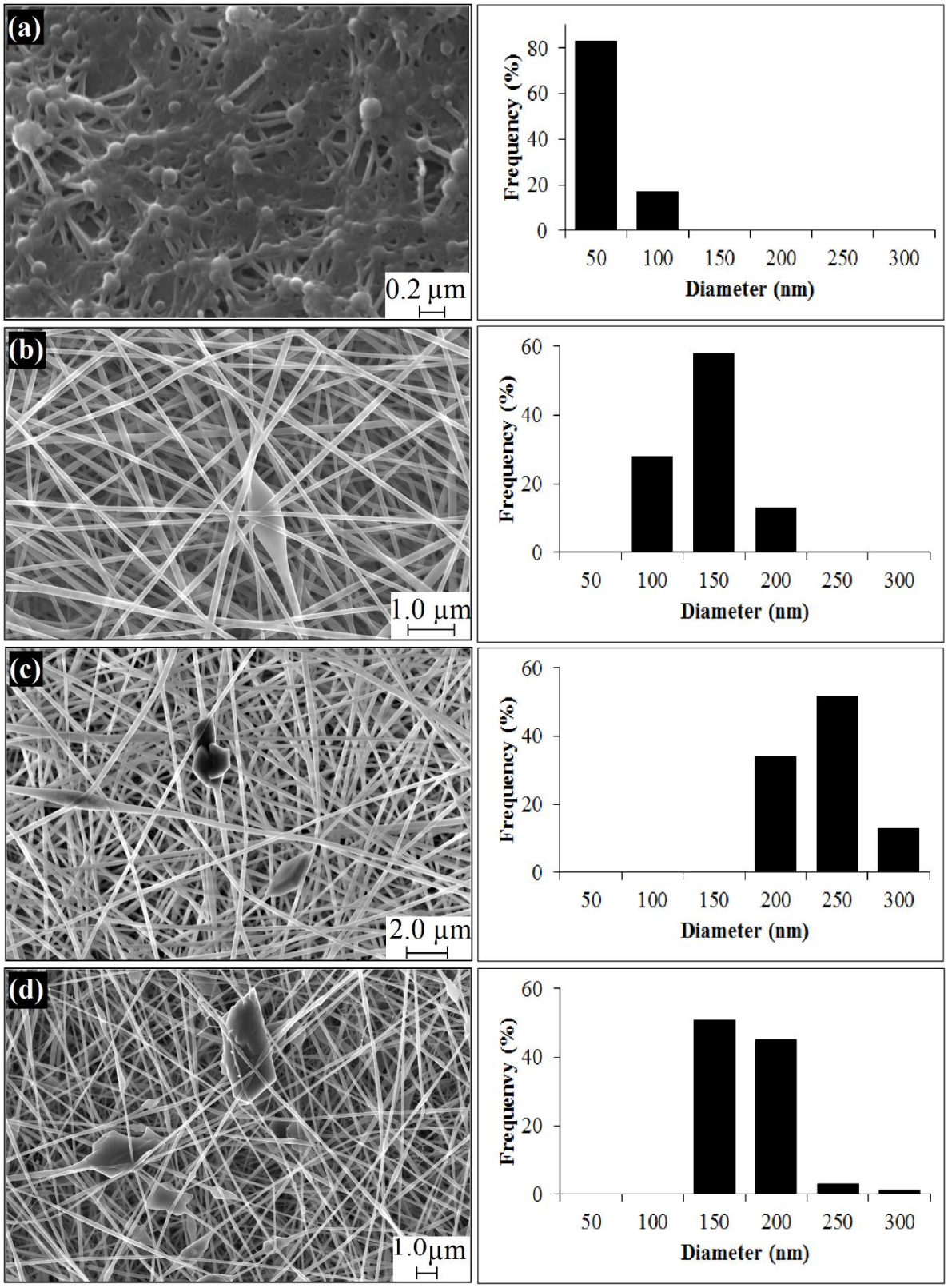

Figure 7. Scanning electron microscopy (SEM) of nanofiber images obtained from 3% chitosan (CH) in 70% v/v acetic acid **(a)**, chitosan/polyvinyl alcohol (PVA) (3:7) **(b)**, CH/PVA/β-CD (2%)/Cinnamon EO (3%) **(c)**, and CH/PVA/(CIN/β-CD-5%) **(d)**, respectively.

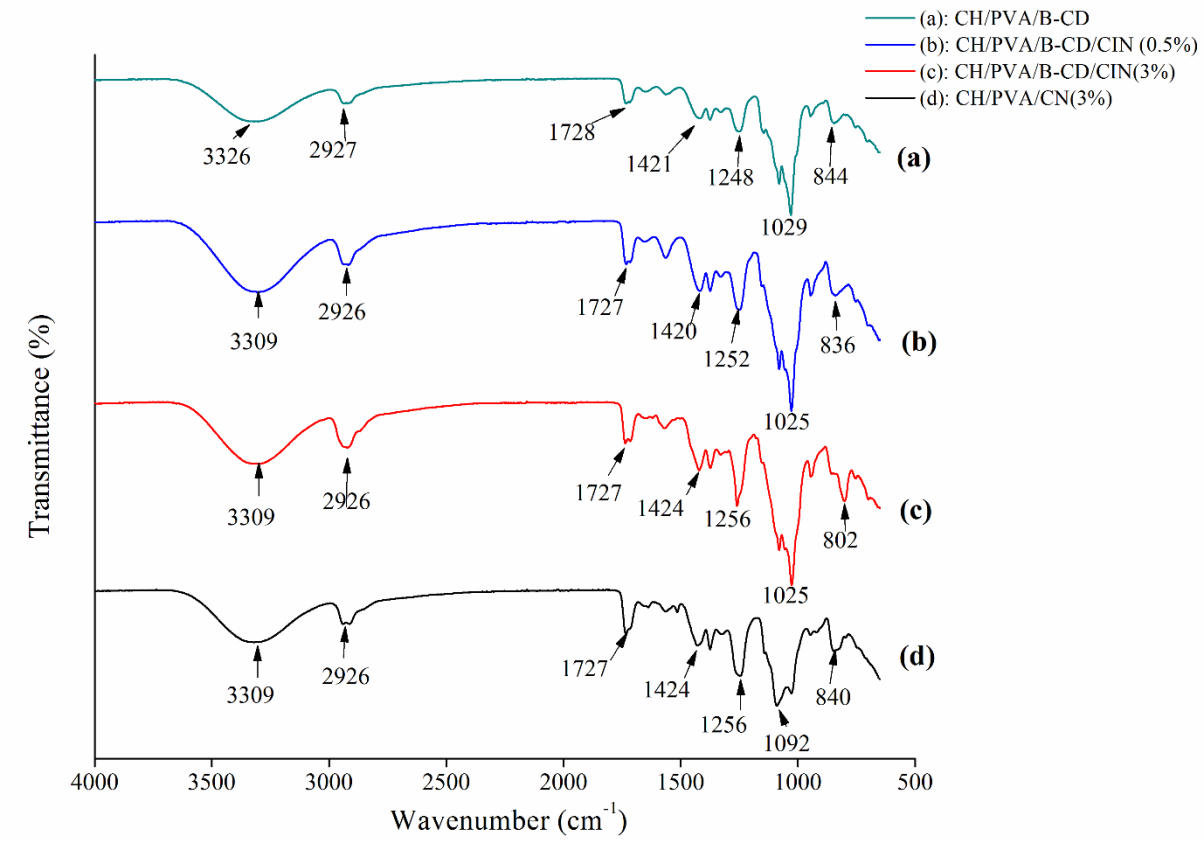

Figure 8. Fourier transform infrared (FTIR) spectra for CH/PVA/β-CD (3:7:2) **(a),** CH/PVA/β-CD/CIN(0.5%) **(b)**, CH/PVA/β-CD/CIN(3%) **(c)** and CH/PVA/CIN(3%) **(d)** films, respectively.

Table 1. Relative abundance composition of major and minor volatile constituents of cinnamon and oregano essentials and their β-cyclodextrin (β-CD) microcapsules

Sample	Volatile compounds	Relative abundance composition (%)	
		Pure EOs	EO/β-CD
CIN/β-CD	Eugenol*	56.92 ± 2.22a	32.73 ± 3.00b
	β-caryophyllene**	4.82 ± 0.04b	11.5 ± 1.04a
	Safrole**	4.74 ± 0.14b	7.69 ± 0.27a
OREG/β-CD	Carvacrol*	51.53 ± 8.09a	24.07 ± 1.31b
	p-Cymene**	10.01 ± 0.40b	19.80 ± 0.61a
	Linalool L**	3.28 ± 0.01c	8.10 ± 0.53c

Values in the same column with similar lower case letter are not significantly different at ($P < 0.05$) based on Duncan's multiple range test.
***** Major VOC
** Minor VOCs

Table 2. Recovery of cinnamon and oregano microcapsules at 16:84 ratio of essential oil versus β-cyclodextrin (β-CD).

Complex	Final EO:β-CD (g. DW)	Microcapsule yield (%)	Major volatile load (%)
CIN/β-CD	52.12 ± 0.08	87.54 ± 0.14	4.86 ± 0.08 (Eugenol)
Oregano/β-CD	49.06 ± 0.34	82.40 ± 0.56	1.75 ± 0.08 (Carvacrol)

*Represents sample ratio of 16:84 (EO:β-CD) whose initial weight was 59.54 g.
Values in the same column with similar lower case letter are not significantly different at ($P < 0.05$) based on Duncan's multiple range test.

Table 3. Antifungal activity of essential oils (EOs), β-cyclodextrin (β-CD), and EO/β-CD complex against *Botrytis* sp. *in vitro*.

Sample	Growth diameter (mm)	Inhibition (%)
CIN EO (5 g/L)	0^{j}	100a
OREG EO (5 g/L)	0^{j}	100^{a}
β-CD (5 g/L)	79.1 ± 0.20^{a}	0^{j}
CIN/β-CD (2.5 g/L)	33.0 ± 0.46^{f}	58.3 ± 0.58^{e}
CIN/β-CD 12.5 g/L)	29.4 ± 0.25^{g}	62.9 ± 0.32^{d}
CIN/β-CD (25 g/L)	23.2 ± 0.32^{h}	70.7 ± 0.40^{c}
CIN/β-CD (50 g/L)	15.4 ± 0.80^{i}	80.5 ± 1.01^{b}
OREG/β-CD (2.5 g/L)	37.0 ± 0.70^{e}	53.3 ± 2.17^{f}
OREG/β-CD (12.5 g/L)	41.0 ± 0.34^{d}	48.2 ± 0.43^{g}
OREG/β-CD (25 g/L)	45.9 ± 0.33^{c}	42.0 ± 0.42^{h}
OREG/β-CD (50 g/L)	48.3 ± 0.29^{b}	38.9 ± 0.15^{i}

Sample ratio of 16:84 used for CIN/β-CD and OREG/β-CD
Values in the same column with similar lower case letter are not significantly different at ($P < 0.05$) based on Duncan's multiple range test.

Table 4. Properties of electrospin solutions and the diameter distributions of the nanofibers generated.

Samples	CH	PVA	β-CD	EO	Viscosity (cP)	Conductivity (μS/cm)	Diameter range (nm)
	(%, w/w)						
CH/PVA	3	7	-	-	823.8 ± 26.00^{d}	1689.0 ± 3.51^{a}	92.9 ± 1.33^{e}
CH/PVA/β-CD	3	7	2	-	890.8 ± 8.66^{d}	1601.3 ± 0.67^{b}	114.6 ± 2.42^{d}
CH/PVA/β-CD/EO 1%	3	7	2	1	3230.0 ± 32.91^{c}	1587.0 ± 0.58^{c}	121.9 ± 1.93^{c}
CH/PVA/β-CD/EO 2%	3	7	2	2	15357.0 ± 69.28^{b}	1553.7 ± 1.20^{d}	143.8 ± 2.35^{b}
CH/PVA/β-CD/EO 3%	3	7	2	3	26334.0 ± 69.28^{a}	1514.3 ± 2.33^{e}	181.8 ± 4.16^{a}

Values in the same column with similar lower case letter are not significantly different at ($P < 0.05$) based on Duncan's multiple range test. CH: chitosan; PVA: polyvinyl alcohol; β-CD: beta-cyclodextrin; EO: oregano essential oil

Table 5. Antifungal activity of the nanofiber films with different active components chitosan, PVA, β-cyclodextrin (β-CD), and essential oils (EOs) complex against *Botrytis* sp. *in vitro*.

Sample	CH	PVA	β-CD	EO	Inhibition (%)
	(%, w/w)				
CH/PVA/CIN-3%	3	7	-	3	51.30 ± 2.48^{a}
CH/PVA/β-CD/CIN-3%	3	7	2	3	53.66 ± 3.19^{a}
CH/PVA/OREG-3%	3	7	-	3	45.77 ± 1.56^{c}
CH/PVA/β-CD/OREG-3%	3	7	2	3	47.34 ± 0.77^{b}

Values in the same column bearing a common letter are insignificantly different at ($P < 0.05$) based on Duncan's multiple range test. CH: chitosan; PVA: polyvinyl alcohol; β-CD: beta-cyclodextrin; EO: oregano essential oil

Chapter 8

Concluding remarks and future perspectives

1. Introduction

The general aim of this study was to improve the postharvest quality of pomegranates by first providing a better understanding of the relevant diseases associated with pomegranate fruit at local level. The review on pomegranate diseases in Chapter 2 provides a detailed overview on the various microbial pathogens associated with pomegranate spoilage from around the world. Chapter 3 reviews the application of active packaging of fruits and vegetables in detail and highlights some of the advances made so far. Antimicrobial active packaging is by far the most researched area regarded as critical for reducing postharvest losses (Chapter 3). The variation in the incidence of the prevailing diseases from one location to the other highlights the validity for this study to identify which pathogens are predominant to South African grown pomegranates. For the South African pomegranate industry to increase pomegranate production and reap more economic revenue when the Northern hemisphere producers are off season, it is important that postharvest losses are kept at minimum. Otherwise increasing production volumes will only translate into more produce losses. Crab shell chitosan was introduced as a green preventative treatment to extend the shelf life of whole and minimally processed pomegranates (Chapter 5). The potential benefits of incorporating essential oils (EOs) into chitosan films was explored to enhance the control of postharvest microbial spoilage of whole and minimally processed pomegranate in Chapter 6. Lastly the potential route of encapsulating EOs using β-cyclodextrin (β-CD) and electrospun nanofibrous films was investigated for their controlled release in Chapter 7.

2. Important postharvest diseases of pomegranate

This study has identified the critical control points for controlling postharvest pathogens of pomegranates currently being grown in the Western Cape region of South Africa. Pomegranate disease control must be done before flowering to reduce pathogen pressure in the orchards. Orchard sanitation and hygiene therefore, remains a crucial preventative strategy in reducing postharvest fruit decay (Munhuweyi *et al*., 2016). The fungal genera *Botrytis*, *Penicillium* and *Pilidiella* often originating from the orchard, were identified to be the major postharvest diseases of pomegranate. This is the first report on *P. granati* in South Africa. This is a significant finding because it shows the importance of studying the etiology of postharvest

diseases in the area of production. In this case fludioxonil was effective against it, but it's possible that other fungicides might not be effective against it.

Botrytis sp. infection is associated with development of brown lesions in affected areas (especially around the crown and neck area) (Thomidis, 2014). The presence of *Penicillium* infection was identified on the basis of presence of a characteristic green/blue mould. A high incidence of *Penicillium* sp. recorded in crown area on the majority of the fruits. *Pilidiella granati* affected all the fruit parts covering the fruit with brown lesions that later developed round brown-back pycnidia around the affected areas. The development of a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay allows for rapid detection of postharvest pathogens occurring anywhere along the pomegranate value chain. This facilitates the prescribing of appropriate control measures for those pathogens. The RFLP tool can also be used to assess the effectiveness of the disease control measures that have been put in place through rapid identification of the pathogens isolated.

3. Chitosan application

The study has shown that chitosan is effective and can be used alone or in combination with fungicides. Often the continuous use of fungicides results in the development of fungicide resistance in pathogen populations and the integrating of the two will reduce resistance development. Chitosan has been demonstrated to play an important role in fruit preservation in other studies as well (Bautisa-Banos *et al*., 2006; Badawy & Rabea, 2009). *P. granati* was the most sensitive pathogen to crab shell chitosan *in vitro* followed by *Botrytis* sp. and *Penicillium* sp. Although, higher concentrations of chitosan were required to achieve microbial inhibition compared to the chemical fungicide fludioxonil, the chitosan application is still regarded as a safer alternative as it is non-toxic for human consumption even at elevated doses and is not harmful to the environment. Fresh cut arils coated with crab shell chitosan had an extended shelf life through suppression of mesophilic bacteria as well as yeast and mould development. Moreover, the physico-chemical attributes of the arils such as anthocyanins, phenolics, colour and textural properties were all better preserved by the chitosan treatment showing the added advantage of applying chitosan as an antimicrobial agent and in the retention of nutritional and sensory attributes of the fruit.

Some pathogens, for example *Penicillium*, only infect through wounds created at harvest and by insects in the field etc. *Penicillium* is a recognised wound pathogen and only causes fruit decay on fruit that is already predisposed to mechanical injury (Thomidis, 2014).

Proactively protecting the wounds with chitosan can prevent fruit decay due to wound pathogens. In the present study, the protective treatment gave significantly ($P < 0.05$) better decay reduction compared to curative control. Protecting fruit from decay is advantageous in that it prevents the spread of the disease and therefore potential fruit losses. However, it should be noted that chitosan works in its hydrated form and if dry it does not work (John *et al.*, 2011) which necessitates the inclusion of other control agents. Once dry, the positively charged amine group, responsible for chitosan' antimicrobial loses bio-activity (John *et al.*, 2011).

The polymeric nature of chitosan allows for it to be used as a carrier of other volatile antimicrobial compounds such as EOs to give an enhanced antimicrobial product The physical properties of chitosan are important when considering the choice of chitosan for application (Badawy & Rabea, 2009). High Mw gave the best optical and mechanical strength properties in the present study, however its viscous nature was a major drawback to work with and thus chitosan of medium Mw was chosen in the current study as the best matrice for incorporation of EOs.

4. Encapsulation of EOs for active biocontrol packaging systems

The variation in chemical composition of the EOs plays a huge role in their overall antimicrobial properties (Sivakumar, & Bautista-Banos, 2014). Oregano EO had the highest antifungal activity followed by cinnamon and lemongrass, respectively. The presence of phenols in oregano and cinnamon probably contributed better antimicrobial activities to that of lemongrass in which monoterpenes were predominant (Chapter 6). Encapsulating EOs using beta-cyclodextrins (β-CDs) and nano-fibrous materials has been demonstrated to extend antimicrobial release of EOs while at the same time preserve the volatiles from rapid degradation through oxidation (Ayala-Zavala & Gonzalez-Aguilar, 2010; Wen *et al.*, 2016). Studies have been conducted that demonstrated the inhibitory presence of EOs in electrospun nanofibers (Rieger *et al.*, 2014, Wen *et al.*, 2016).

The present study adopted the encapsulation method demonstrated by Wen *et al.* (2016) using polyvinyl alcohol (PVA) and β-CD as polymeric carriers and added chitosan into polymer mix similar to that by Rieger *et al.* (2014). The successful encapsulation of the EOs into either the β-CD cavity alone or the chitosan/PVA/β-CD nanofibrous based film could be confirmed using GC-MS and through the demonstration of antimicrobial activity against *Botrytis* sp. previously isolated from pomegranate fruit. The encapsulating efficiency of EOs was also demonstrated by the observed changes in X-ray diffraction patterns (X-RD), nuclear

magnetic resonance (NMR), Fourier transform infrared spectrophotoscopy (FTIR), thermogravimetric analysis (TGA) and gas chromatograms of plain β-CD). Cinnamon EO complexed better with the β-CD moiety when compared to oregano EO, which later demonstrated poor antimicrobial effects after the microencapsulation process.

The results presented demonstrate that EOs are effective antimicrobial agents but they can cause phytoxicity, are prone to volatile loss and for that reason must be encapsulated. Applying chitosan/EO as direct emulsion coating *in vivo* demonstrated higher inhibitory control to vapour contact with films however, the emulsions had a negative effect on the cosmetic appearance of the fruit compared to the active films. Vapour contact of pomegranate with chitosan/EO films provided antimicrobial protection *in vivo* without inducing severe and undesirable cosmetic changes in pomegranate fruit peel when compared with direct emulsion dipping method.

5. Coclusions and future prospects

Postharvest diseases of pomegranate originate from the orchard and make their way into postharvest and can persist as latent (symptomless) infections that only appear during fruit senescence. *Botrytis* and *P. granati* are the most detrimental postharvest spoilage pathogens of pomegranate fruit while *Penicillium* is an important wound pathogen. Chitosan and essential oils impart beneficial preservative attributes on pomegranate whole fruit or arils when combined together. Applied separately, the chitosan is only antimicrobial when in aqueous state, while the EOs vaporise too quickly and unable to generate a sustained release of their antimicrobial properties. Further research is necessary to fully understand encapsulating properties of β-CD and nanofiber technologies for sustained release of volatile antimicrobials. Research into intelligent monitoring of these compounds so as to profile their shelf life is also necessary.

The efficiency of applying biocontrol agents such as chitosan presents a viable route to developing chemical free and environmentally safe packaging materials for the food industry. The challenge that remains is to provide biocontrol matrices that present precise and consistent efficacies. To achieve this, more natural based compounds must be tested for their potential role as food preservatives as well as complimentary substances that they can be combined with to give enhanced disease control. The development of smart/intelligent systems offers the advantage of monitoring real-time changes within any packaged environment. These can be can be introduced to monitor for any changes in active compounds present

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