Murdoch
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Control of *Botrytis cinerea* on post-harvested blueberry (*Vaccinium corymbosum* L.) fruit

This thesis is presented for the degree of

Doctor of Philosophy of Murdoch University

by

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January 2021

Declaration

I declare that this thesis is my account of my research and contains its main content work, which has not previously been submitted for a degree at any tertiary education institution.

Signature: A. AUDA
Date:10/01/2021

Acknowledgment

"In the name of Allah, the Most Gracious and the Merciful."

I gratefully acknowledge the Iraqi Government, represented by the Ministry of Higher Education and Scientific Research, for providing me with a fantastic opportunity to complete my doctoral study in Australia.

I also extend my sincere gratitude to my supervisors, Professor Giles Hardy, Professor Yonglin Ren, and Dr Manjree Agarwal**;** you have been a fabulous supporter and taught me how to think like a scientist. Your advice and guidance regarding my PhD study have been appreciated. I am grateful for the help of Dianne White and Bob Du. Also, I would like to thank my colleague's Farhan Al-Bahadili, Thamer Alshowili, and Dr Mohammed Yaseen.

I dedicate this success to the soul of my father, who believed in me and dreamed of this day. My beloved mom, there are no words to describe my appreciation for everything you have done. My special thanks and gratitude to my family support and love started with my dear wife, my daughters Lamiya and Liyana, my brothers, sisters, and uncle.

Finally, I would like to thank my friends Haider Abd Alrasol, Mohanad Hassan, Azam Ahmed, Hyder Hassan, Adel Hassan, Salih Jalal, Hussain Nasser, and Rian Rashid, your support and help were more appreciated than you could ever imagine.

Abstract

Blueberries (*Vaccinium corymbosum* L.) are cultivated worldwide due to their important commercial value and the health benefits of the fruit. *Botrytis cinerea* is a highly destructive horticulture pathogen with a host range of 1400 plant species, including blueberry. Worldwide its management costs billions of dollars annually. Biosecurity controls require that international shipments of products be pest-free, which is increasingly difficult due to the ban of key pesticides. Instead, biological control strategies can be used to manage postharvest fruit pathogens. Surveys on postharvest blueberry in Western Australia were conducted to assess the fungal species associated with fruit decay and their pathogenicity. The fungi isolated were *Botrytis cinerea, Alternaria alternata, Cladosporium cladosporioides, Penicillium brevicompactum, Epicoccum nigrum, Aureobasidium pullulans, Diaporthe australafricana,* a *Verticillium* sp., and a *Ceriporia* sp. *Botrytis cinerea, A. alternata, C. cladosporioides A. pullulans,* and *D. australafricana* were pathogens. The other four were not. Two methods were used to evaluate the eight fungi as putative antagonists against *B. cinerea*. Firstly, each antagonist was screened for direct antagonism against *B. cinerea* on half-strength potato dextrose agar (PDA). Secondly, the involvement of secondary metabolites was evaluated by growing each antagonist on a dialysis membrane placed on the surface of half PDA for four days before removing the membrane and placing *B. cinerea* in the centre of the PDA plates and monitoring its growth. *E. nigrum* was found to be the most effective antagonist, followed by *Verticillium* sp., the *Ceriporia* sp. and *A. pullulans* using both methods. The eight putative antagonists were then screened for their ability to produce non-volatile organic compounds (NVOCs) in the presence and absence of *B. cinerea* using direct immersion solid-phase microextraction (DI-SPME) coupled with gas chromatography-mass spectrometry (GC-MS). The metabolic analysis by GC-MS confirmed that there were unique compounds identified from all eight fungi, such as *E. nigrum* producing more of these than the others. Future studies are required to determine if any of these compounds can be used to control *B. cinerea* as a postharvest pathogen of blueberry. This study has improved our understanding of postharvest blueberry pathogens in Australia. The results will help refine current *B. cinerea* postharvest management and develop more efficient and environmentally friendly control strategies.

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Chapter one

Literature review

1.1. Introduction

This literature review critically evaluates the diagnosis and control of *Botrytis cinerea* on post-harvest blueberry (*Vaccinium corymbosum*) fruit and its pathology. It then explores new technologies that may be used alone or in combination with existing treatments to control *B. cinerea*. It also discusses current post-harvest blueberry fruit disease practices, including chemical control, physical control, biological control, and new diagnosis and treatment technologies.

Fruit and vegetables are necessary components of a healthy diet. If consumed daily in sufficient amounts, they help prevent major diseases, including chronic diseases such as heart disease, diabetes, cancer, and obesity, and alleviate several micronutrient deficiencies, especially in developed countries (FAO/WHO, 2018). Advances in this direction complement and reinforce other valid reports based on the well-known health benefits of consuming fruit and vegetables as dietary sources of fibre, vegetable proteins, and other protective micronutrients (FAO, 2017). A minimum intake of 400g of fresh plant products per day (excluding potatoes and other starchy tubers) is recommended (McCarthy, 2020).

Blueberries are native to North America and produce edible dark-purple berries. The genus *Vaccinium* includes about 450 species of evergreen and deciduous shrubs. Commercially, blueberry is grown in approximately 27 countries worldwide, most of them in temperate zones (Michalska and Lysiak, 2015). In 2012, the global blueberry industry produced 1,336 billion lbs (605,999 tonnes), consisting of processed and fresh fruit. These were produced globally on some 214,970 acres (Brazelton, 2013). In 2017, global production was 596,813 tonnes (FAO, 2017). The largest blueberry-producing country is the United States of America, which produces over half of the worldwide production (Michalska and Lysiak, 2015). In Australia, blueberry production increased three-fold between 2014 (5,500 tonnes) and 2018 (16,850 tonnes), with a value of AUD 394.4 million (Innovation, 2018). The Australian domestic market consumes most blueberries grown in Australia (Innovation, 2018). Furthermore, the Australian market imports about 1,235 tonnes annually and exports about 356 tonnes (Innovation, 2018).

1.2. *Botrytis*

The genus *Botrytis* belongs to Kingdom: Fungi, Division: Ascomycota, Class: Leotiomycetes, Order: Helotiales, Family: Sclerotiniaceae, Genus: *Botrytis* (Micheli, 1729). Many *Botrytis* species cause diseases in numerous crops, both pre-and post-harvest. Most *Botrytis* species are necrotrophic pathogens (Elmer & Reglinski, 2006) but can also be saprophytic on crops, herbaceous, annual, and perennial plants. *Botrytis* is present anywhere plants are grown. It can grow on many different sources of nutrients, survives well in greenhouses, in the field, and indoors and can attack many different types of plants. The disease caused by *Botrytis* is commonly called *Botrytis* blight or grey mould (Elmer & Reglinski, 2006). Several species in the genus *Botrytis* can cause blights; the most common is *Botrytis cinerea*. *B. cinerea* is the most important *Botrytis* species and is considered the main threat to many plants and plant products in the field and during storage. *B. cinerea* is a well-known pathogen and causes heavy losses in table and wine grapes worldwide (Elad and Stewart, 2007). *Botrytis* infections are favoured by cold, rainy spring, and summer weather, typically around 15°C. Grey mould can be damaging when rainy weather continues over several days (AbuQamar et al., 2006). *Botrytis* is an anamorphic fungus that belongs to the Sclerotiniaceae, and the teleomorph or sexual stage is *Botryotinia fuckeliana. B. cinerea* is responsible for a wide range of symptoms, and these cannot simply be generalized across plant tissues and organs. Mushy rots, followed by collapse and water soaking of parenchyma tissues, accompanied by a rapid appearance of grey conidia masses, are possibly the most common symptoms on leaves and soft fruits. In thick-skinned fruits, such as kiwi fruits, the dark water-soaking symptom is evident after cutting (Williamson et al., 2007). On many fruits and vegetables, the infection begins on attached senescent flowers. Then as soft rot, it develops to affect the adjacent growing fruit (blossom-end rot), as in cucumbers, courgettes (zucchini), strawberries, French beans, and apples. On flower petals, symptoms range from minute to full-scale soft rots depending on the environmental conditions. In greenhouse-grown tomatoes, significant damage occurs on stems in pruning wounds where the fungus can rot through the stem. Soft rot of mature tomato fruits occurs mainly post-harvest; a rare 'ghost spot' symptom in unripe tomato is linked to successful host defence, but in red raspberry (*Rubus idaeus*), the symptom renders fruits unmarketable and away from the devastating grey mould on fruits, the pathogen attacks mature leaves forming a wedge-shaped chestnut brown lesion with a yellow margin that spreads to the node on vegetative stems (primocanes), that gives rise to an apparent pale brown fast-spreading lesion in the primary cortex of the stem (Williamson et al., 2007). Because of periderm layers, the infection does not

enter the axillary buds. However, it postpones the development of the buds at the infected nodes failing to make fertile lateral shoots in the following season. After winter dormancy, the stem lesions in raspberry become white and show black sclerotia that produce grey conidia in spring. While in blackcurrant, symptomless infection of flowers detected by fluorescence microscopy leads to early abscission of developing fruits related to ethylene generation in a form called 'run-off' (Williamson et al., 2007). Over 50 hosts have been reported with seed-borne infection, including flax, lettuce and sunflower (Maude, 1980). In Australia, seed transmission occurs in chickpeas, which can cause complete crop failure (Burgess et al., 1997). In this crop, grey mould often begins by rotting the herbaceous stems at the soil level and leaves and pods with other soft-rot lesions. The widespread internal infection in polyantha plants grown from infected trade seeds with symptoms of the disease only issued three months later at flowering (Williamson et al., 2007).

1.2. Taxonomy

Phylogenetic analysis of 22 species in the genus *Botrytis* determined that *B. cinerea* makes up a small clade, all of which are specialized pathogens (Staats et al., 2005). The conidia (macroconidia) are multinucleate, and the microconidia (male spermatia) are uninucleate (Beever and Weeds, 2007). Also, there are 16 chromosomes during the mitotic metaphase and 16 chromosomes in the developing asci (Shirane et al., 1988). The presence of apothecia is rare in the field and more common in other *Botrytis* spp. Strains are heterothallic, carrying an allele of the mating-type locus MAT1-1 or MAT1-2 (Faretra et al., 1988), but there are field isolates with dual mating phenotypes (Van der Vlugt Bergmans et al., 1993). Sexual crossing in *vitro* includes developing sclerotia of the female (sclerotial) parent for the long term at zero degrees (preconditioning) before fertilizing them with microconidia (spermatia) obtained by irrigation of the culture (Faretra et al., 1988). Genetic differences in *B. cinerea* populations has been studied using a range of molecular techniques such as restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified (Giraud et al., 1997), PCR disclosure of transposable elements (Diolez et al., 1995), RAPD fingerprinting (Van der Vlugt-Bergmans et al., 1993; Kerssies et al., 1997), amplified fragment length polymorphism (AFLP) analysis (Moyano et al., 2003), and DNA sequencing of many gene regions (Albertini and Leroux, 2004).

1.4. Morphology and growth

Botrytis colonies are fast-growing, white, low, flaky, becoming brownish grey to grey with age. *Botrytis* forms two kinds of resting structures (Figure 1.1), firstly sclerotia which are very dark brown or black multi-celled structures, lenticular to irregular in shape, 0.2-0.5mm diameter, comprised of a thickly pressed medulla and a pseudoparenchymatous dull brown to a dark cortical layer of cells, 5-10 µm in diameter (Paul et, al. 1997). Secondly, single-celled, thick dark walled chlamydospores, which are derived from mycelium or sclerotia, and generally framed in tufts, 750 µm to more than 2 mm long, smooth-walled, dark chestnut to brown, 18-23 µm broad in the lower portion, hyaline to pale cocoa in colour. *Botrytis* also forms two types of conidia, macroconidia and microconidia. Macroconidia are globose, ellipsoidal, or egg-moulded, smooth, hyaline to pale brown, for the most part with a protuberant hilum, 8-14 x 6-9 µm. In comparison, microconidia are about three µm in diameter and uninucleate (Pollastro, 1996).

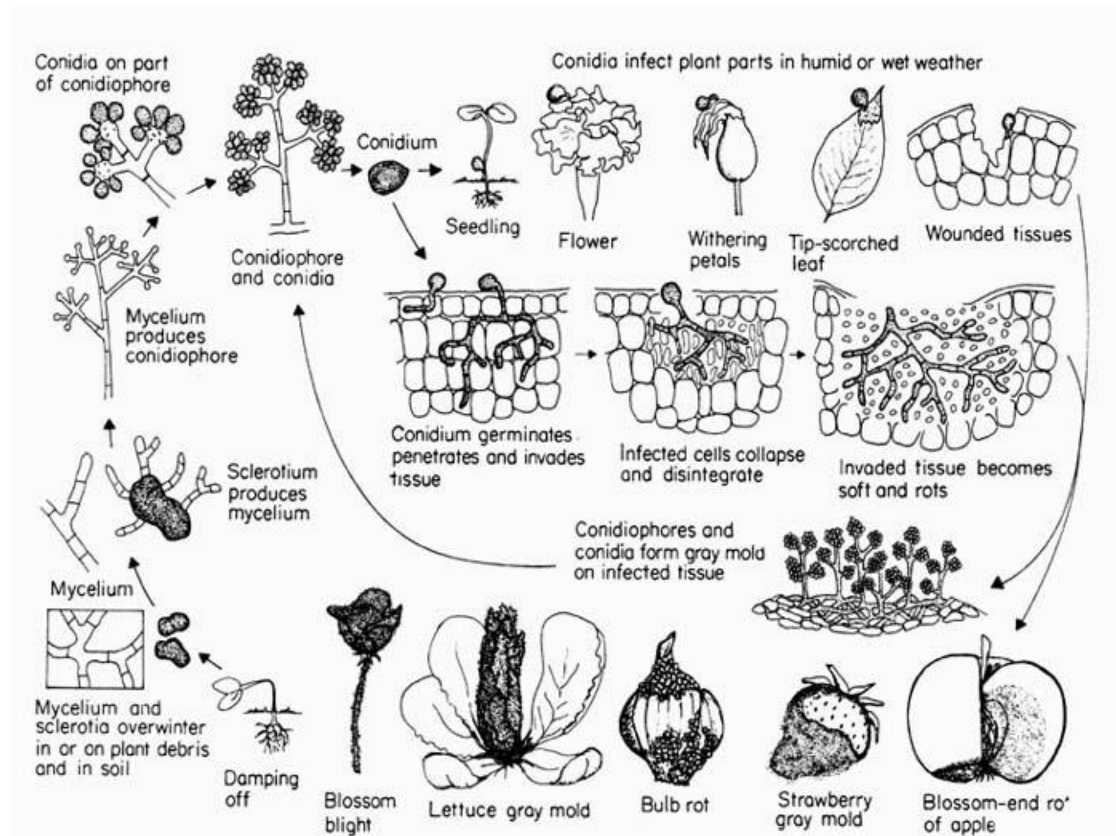

Figure 1. 1. *Botrytis* life cycle (Agrios 2005).

1.5. *Botrytis cinerea*

B. cinerea attacks more than 1400 plant species, including agronomically important crops and harvested commodities (Fillinger et al., 2016). Among fruit and vegetables, *B. cinerea* can infect beans, asparagus, beet, carrot, celery, chicory, eggplant, endive, grape, onion, pepper, lettuce, potato, raspberry, rhubarb, rutabaga, shallot, strawberry, tomato, turnip, and others. *B. cinerea* can infect several ornamental plants like an anemone, begonia, calendula, hawthorn, heather, hydrangea, chrysanthemum, dahlia, rose, snapdragon, sunflower, dogwood, fuchsia, geranium, marigold, pansy, periwinkle, petunia, sweet pea, violet, and zinnia. Droby and Lichter (2004) give a comprehensive list of post-harvest rots caused by *B. cinerea*; these range from grey mould on different plant parts, including fruits, flowers, leaves, roots, and potato tubers. Vegetables (cabbage, beans, lettuce) and fruit crops (strawberry, grape, raspberry, and blackberry) are most severely affected. With increasing international trade in cold-stored products, this fungus has gained significant prominence as a post-harvest pathogen because it can grow effectively at temperatures just above freezing in products like kiwifruit, pears, and apple. Also, the cut-flower trade is adversely influenced by this fungus; gerbera and rose flowers are especially prone to damage. Crops cultivated out of season in both unheated and heated greenhouses or under plastic tunnels increase the chance of infection, mostly in cucumber, tomato, and sweet pepper (Bakr et al., 2002). Many economically important field crops sustain substantial losses due to grey mould. These include chickpeas and protein-rich legumes, which support millions of rural families in Bangladesh, Nepal, and India (Pollastro, 1996). French beans can suffer almost complete losses (Gossen and Platford, 1999). To some degree, most legumes get infected by B. cinerea; however, field bean (*Vicia faba*) is damaged by chocolate spots caused by *B. cinerea*. Blossom blight in alfalfa in Canada causes many seed problems produced in irrigated areas (Gossen and Platford, 1999). Sunflower, which is a significant oil crop, can be infected. In tree plantations, conifer seedlings are vulnerable to grey mould. Industrial hemp (*Cannabis sativa*) grown for fibre products is sensitive to head blight (Williamson et al., 2007).

1.5.1. *B. cinerea* life cycle

Botrytis cinerea overwinters in the soil as mycelium on plants and black sclerotia in soil and plant debris mixed with grain. *B. cinerea* is spread by anything that moves plant or soil debris or carries sclerotia (Figure 1.1). The fungus required free moisture (wet surfaces) for germination and temperatures between 15-25°C (Rivera et al., 2013). The fungus is also active

at low temperatures and can cause infection on vegetables stored for days or months at temperatures ranging from 0 to 10°C. Infection rarely occurs at temperatures above 25°C. Once an infection occurs, the fungus grows over a range of temperatures between 0 to 35^{0}C. *B. cinerea* survives saprophytically through the winter on a diverse host species (Peng et al., 1991). In vineyards, many sources of overwintering inoculum have been shown**;** these include sclerotia, grapevine prunings, and other necrotic grape tissues on the vine and in the ground (Michailides, 2005). The release of new conidia from those sources in the spring gives an abundance of inoculum for infection of floral tissues and tender leaves and growing shoots in the grapevine. Apothecia of the sexual state (*Botryotinia fuckeliana*) have been seen, but their appearance is sporadic (Beever & Weeds, 2007). Senescing floral tissues are susceptible to *B. cinerea*, and significant sporulation is observed in these tissues when conditions favour the pathogen's growth in the spring. During active growth, *B. cinerea* produces a range of hydrolytic enzymes and metabolites to help penetrate and colonise host tissues. Physical and chemical host defences limit the diffusion of early-season infections, and the pathogen becomes quiescent or latent in the host tissue. In berries, *B. cinerea* remains latent until the post-harvest period and then becomes pathogenic as the host defences begin to decline. Latent infection can occur in berry pedicels, and to a lesser extent, in grape bunch rachis (Michailides, 2005). Late season berry infections can also occur in wounded or cracked berries, from immediate infection by airborne conidia or mycelia growing saprophytically on aborted flowers and aborted fruitlets (Seyb, 2004). Periods of prolonged wet conditions late in the season can support pathogen development and leads to the reproduction of *B. cinerea* on necrotic grape leaves and in ripening grapes, resulting in heavy crop losses before harvest. Additional detailed research on the relative significance of early-, mid-, and late-season berry infections has been published (Latorre, 2016).

1.5.2. *B. cinerea* infection

Botrytis cinerea can attack many plant parts, including buds, flowers, leaves, stems, and fruit. However, *B. cinerea* can be mixed with other diseases that cause similar damage. There are many *Botrytis* species and more than one strain of each. *Botrytis* can infect the foliage of nursery stock stored through winter in damp conditions. There are no known resistant plants (Rupp, 2017). *Botrytis* have nutrients or a food source before it infests the plant. Nutrients leaking from wounded plant parts or dying tissues like old flower petals give the required nutrients. The fungus becomes more aggressive from this food base and invades healthy tissue.

Dark to light brown rot forms in the infected tissues. High humidity supports the growth of this fungus. As stated previously, the pathogen is a necrotroph and kills plant cells before using them as a food source. There are reviews on the mechanisms of infection of *B. cinerea*, with an emphasis on microscopic and biochemical research or molecular genetics (Aguirre, 2005). The use of molecular-genetic tools like transformation, differential gene expression analysis, and gene cloning has led to novel insights into the structure of *B. cinerea* genes and their role in the infection process. The mechanisms of how *B. cinerea* infects plants include wounded tissue like large stubs left after taking cuttings, cuttings taken from plants with infestations of *Botrytis*, leaves on which infected flowers have fallen, injured leaves or broken stems, leaves damaged by herbicide damage, over-fertilization, or mechanical injury and seedlings grown under moist, cool conditions. Other losses are estimated at 20-25% of the strawberry plants and 20% of cut flowers in Spain and Holland, respectively, depending on climatic conditions (Leroux, 2004). The cost of fungicidal treatments against *B. cinerea* was about 540 million euros in 2001, representing 10% of the world fungicide market (Lamichhane et al., 2016).

1.5.3. *B. cinerea* management

Due to the economic importance of *B. cinerea,* considerable attention to researching its management has been made. The preharvest methods include chemical, physical, biological, and agricultural methods, while the standard post-harvest methods include fumigation, cold treatment, and biological agents (Huang et al., 2020). Overall, the management of *Botrytis* can be a challenge because many factors contribute to infection and disease development. One of the first steps in managing *B. cinerea* is to control the environment. This involves keeping plants healthy and avoiding any stresses like drought or waterlogging, mechanical or chemical injury and too much or too little fertilizer. Improving air circulation and sunlight is also very effective in reducing disease incidence and severity. For example, heating and ventilating greenhouses to prevent high humidity can be very effective. This may require additional venting early in the day before sunlight has warmed the air and when moisture has condensed. Even lowering the humidity slightly can have a significant effect on *Botrytis* (Albertini, 2004). Sanitation is also an important management tool. Removing dying or dead tissue before planting crops is essential. However, sanitation alone is not enough to control this fungus, as it can produce 60,000 or more spores on a fingernail size of plant tissue (Gleason et al., 2009). Even a single spore can infect under suitable climatic conditions and cause disease. Outdoor planting must be planned to supply good air circulation patterns, which is an important means of inhibiting *Botrytis* growth. The added protection is obtainable for many crops by using

biological control agents or fungicides. However, *Botrytis* is resistant to certain chemicals, and this resistance becomes a problem when fungicides are applied exclusively and extensively over long periods. It is necessary not to depend entirely on a group of chemicals that have similar modes of action. It is best to mix chemicals that act differently (Albertini, 2002).

1.5.3.1. Post-harvest chemical control

A fungicide is a pesticide that controls fungal disease by inhibiting or killing the fungus causing the disease (McGrath, 2004). Synthetic/chemical fungicides play a major role in post-harvest disease control as they can directly inhibit pathogenic fungi and bacteria (Eckert and Ogawa, 1988). These chemicals can protect the fruit from a wide range of infections, from quiescent infections (infection occurring during pre-harvest growth but remaining dormant) to those that arise after harvest, especially during handling, storage, shipment, and marketing (Adaskaveg et al., 2014). Currently, fungicide or chemical treatments are considered the main method for the control of post-harvest fruit losses (Mari et al., 2014). In conventional agriculture, several commercially available fungicides are used for post-harvest disease control (Feliziani and Romanazzi, 2013). Historically sulphur is considered the first chemical that was used for post-harvest disease control (Hahn, 2014). Sulphur was followed by other chemicals, including phenol sodium ortho-phenylphenate, inorganic salts borax, sodium carbonate, and sodium bicarbonate. The next group of fungicides includes methyl benzimidazole, carbamate, thiabendazole, and the demethylation inhibitor imazalil. More recently, the concept of reduced risk new-generation post-harvest fungicides belonging to five chemical classes (FRAC - Fungicide Resistance Action Committee groups), the demethylation inhibitors (Tebuconazole, Propiconazole, and Difenoconazole), the phenylpyrrole Fludioxonil, the hydroxyanilide Fenhexamid, the anilinopyrimidine and Pyrimethanil, and the quinone outside inhibitor Azoxystrobin (Adaskaveg et al., 2014). Some important post-harvest fungicides with their modes of action and active ingredients are presented in Table 1.1.

Post-harvest fungicides can be applied as sprays, dips, fumigants, within wraps and box liners, or with waxes and coatings (Nabigol and Morshed 2011). Sprays and dips are very commonly used and, depending on the compound, can be applied in aqueous suspensions, solutions, or emulsions (Brown and Ogle, 1997). Different approaches such as application in combination with hot water treatment or a sanitizing agent can be used to maximize the effectiveness of post-harvest chemical treatments (Kanetis et al., 2008).

Table 1. 1. Commonly used chemical fungicides (active ingredient) against the different post-harvest diseases of fruit

Fungicide mode of action group	Active ingredient	Pathogen	Diseases	Fruits	Fungicide (available form in Australia)
Methyl Benzimidazole Carbamates	Thiabendazole	*P. digitatum*, *P. italicum*, *P.expansum*,*B. cinerea* , *Colletotrichum* spp.	Green mould, Blue mould, Grey mould, Anthracnose	Citrus, Mango Pome fruit	Thiabendazole 200 plus Thiram 360 (+ thiram), Thiabendazole 500
	Carbendazim	*M. laxa, M. fructigena*	Brown rot	Apple	Carbendazim 500SC, Bavistin FL
DMI	Imazalil	*P. digitatum*, *P. italicum* *Alternaria* spp. *Colletotrichum* spp. *F. roseum, Diaporthe melonis*	Green mould, Blue mould, Alternaria rot Anthracnose, Stem-end rot, Fusarium rot, Phomopsis rot	Citrus, Mango, Musk melon	Imazalil 750 WG, Imazacure 750SG
	Prochloraz	*Alternaria alternata,* *C. gloeosporioides*	Anthracnose, Alternaria rot	Mango	Prochloraz WP, Octave WP, Sportak
	Difenoconazole	*P. expansum*, *B. cinerea*	Blue mould, Grey mould	Pome fruit	Score, Divino 250 EC
	Propiconazole	*P. italicum*, *Geotrichum citri-aurantii, Phomopsis citri* *R. stolonifer, Geotrichum candidum, Monilinia* spp.	Blue mould, Sour rot, Rhizopus rot, Brown rot	Citrus, Stone fruit	Propiconazole 500, Propiconazole 250 EC, Propimax
Hydroxyanilide	Fenhexamide	*Monilinia* spp. *B. cinerea*	Brown rot, Grey mould	Stone fruit, Kiwi fruit, Pomegranate	Teldor 500 SC
Anilinopyrimidine	Pyrimethanil	*P. digitatum*, *P. italicum*, *P. expansum, B. cinerea*, *Monilinia* spp.	Green mould, Blue mould, Grey mould, Brown rot	Citrus, Pome fruit, Pomegranate, Stone fruit,	Scala 400SC; Predict (600g/l SC), Pyrus SC (400g/l ai)
	Cyprodinil	*B. cinerea*	Grey mould	Pomegranate	Switch (+fludoxonil), Solaris
Quinone outside inhibitors (QoIs)	Azoxystrobin	*P. digitatum, Colletotrichum acutatum, Phomopsis citri* *P. italicum, F. moniliforme,* *P. funiculosum, Ceratocystis paradoxa*	Green mould, Anthracnose, Stem end rot, Blue mould, Fruitlet core rot, Black rot	Citrus, Pome fruit, Pineapple	Azoxystrobin 500 WDG, Genfarm Azoxystrobin 250 SC, Amistar 250 SC, Dynasty
Phenylpyrroles	Fludioxonil	*P. digitatum*, *Monilinia* spp. *B. cinerea , Rhizopus stolonifer, F. moniliforme,* *P. funiculosum, C. paradoxa*	Green mould, Brown rot, Blue mould, Alternaria decay, Anthracnose, Grey mould, Rhizopus rot, Fruit let core rot, Black rot	Citrus, Stone fruit, Pome fruit, Kiwi fruit, Pineapple	Maxim 100 FS, Scholar

Adaskaveg et al., 2014; Förster et al., 2007; and Rosenberger 2009.

Application of fungicides as aqueous warm solution/suspension (dipping/drenching/spraying) is the most effective method as water temperature, treatment duration, and fungicide concentration in solution increases the fungicide efficacy positively (Schirra et al. 2011; Altieri et al., 2013). On the other hand, the application of fungicides combined with wax sprayed on fruit is common in Italian packing houses and includes fungicides such as Imazalil applied to citrus (Altieri et al., 2013). It is regarded as an inexpensive, straightforward method with no drainage issues associated with waste fungicide solutions. It can inhibit infection (Kanetis et al., 2007), reduce sporulation from infection sites, reduce water loss or desiccation, and improve the fruit's appearance before marketing (Kanetis et al., 2008; McKay et al., 2012). However, problems with the application of fungicides can reduce effectiveness. These include blocked nozzles and the accumulation of the fungicide on the brushes that often result in waste problems (Altieri et al., 2013). Application of chemical fungicides such as volatile fumigants is sometimes a very effective control method of post-harvest disease of fruits such as strawberry, grape, apple, and pear (Mehrotra and Aggarwal, 2003).

The modes of action of synthetic fungicides on post-harvest pathogens include inhibition of fungal spore germination, germ tube elongation, mycelial growth, and inhibition of enzyme secretion of fungi (involved in plant cell wall degradation). Fungicides also reduce the uptake of glucose and amino acids and decrease the synthesis of various cell components (Allen and Gottlieb 1970; Schirra et al. 2011). Synthetic fungicides are effective, reliable, and convenient to apply when needed. Indeed, fungicidal treatment is often the only means of extending a perishable crop's post-harvest life, particularly when controlled storage (e.g., refrigeration) is unavailable (Eckert and Ogawa 1988). Post-harvest disease management's effectiveness depends on the mixtures and rotations of fungicides with different modes of action and proper application procedures that result in adequate fungicide residues on the fruit (Förster et al., 2007). Combining at least two compounds with different modes of action often helps to minimize pathogen resistance against chemicals (McKay et al., 2012; Feliziani and Romanazzi, 2013). Delayed application or treatment of fruits with post-harvest fungicides may reduce their efficacy for control of disease due to the amount of the fungus on the host tissue or the lack of apoplastic and symplastic mobility (systemic activity) of the fungicide in the host tissues (Mekbib 2007 cited in McKay et al., 2012).

Fungicides are the essential way of controlling post-harvest diseases (Bekele et al., 2007). Around 23 million kg of fungicides are applied to fruit and vegetables each year, and it is mostly accepted that the production and marketing of these perishable products would not be possible without their use. However, as harvested fruit and vegetables are usually processed with fungicides to slow down post-harvest diseases, there is a significant probability of direct human damage due to their use. Moreover, synthetic chemicals to control post-harvest damage have been restricted due to their carcinogenicity, high and acute residual toxicity, long degradation period, environmental pollution, and other side effects on humans (Tripathi and Dubey 2004). The development of resistance to generally used fungicides by post-harvest pathogens has also become a significant problem. For example, many synthetic fungicides are currently used to control the blue mould rot of citrus fruit. However, resistance by *Penicillium italicum* and *Penicillium digitatum* to these fungicides has become a problem in recent years (Askarne et al., 2012).

1.5.3.2. Post-harvest physical control

1.5.3.2.1. Irradiation control

A functional nonchemical approach, irradiation has been used as an alternative treatment to fungicides to control fungal plant pathogens. Ionizing radiation is an effective non-chemical treatment and a viable alternative for controlling post-harvest diseases (Hallman, 2011). Ionizing radiation is radiation with appropriate energy that causes molecules and atoms to become excited or ionized during an interaction with an atom. Ionizing radiation makes free radicals that break chemical bonds and destroy molecules involved in the cell process without leaving any sediment. It gives rise to metabolic and chemical or physiological changes in fungal pathogens. There are only three ionizing radiation sources, i.e., gamma-rays are driven by the isotopes cobalt-60 or electron-beams (e-beams) and cesium-137**;** X-rays generated by a machine source are commercially available for the control of pests. Although e-beams, gamma-rays, or X-rays are produced from different sources, they have the same mode of action. The irradiation dose unit is the grey (Gy) energy absorbed in J kg^{-1} of material. Gamma irradiation has been shown to inactivate fungi from different materials, like stored seeds, paper, soil, and wood (Jeong et al., 2015).

1.5.3.2.2. Modified atmosphere treatments

Modified atmosphere is a method of adjusting the condition of the work environment's internal atmosphere (such as in *vitro*, container, package, etcetera) to improve shelf life (Giuggioli et al., 2017). Due to the short shelf life of food products such as fruit, vegetables, meat, and dairy in the presence of oxygen, this technology presents as a solution. In fresh produce, oxygen presence helps maintain high respiration rates, contributing to shortened shelf life (Boskou and Elmadfa, 2016). Microbiologically, oxygen promotes the growth of aerobic microorganisms (Parry, 2012). Therefore, in modified atmosphere treatments, the oxygen is replaced with nitrogen (N_2) or carbon dioxide (CO_2), as both N_2 and CO_2 prevent the production of energy within the cells and lower the pH of the medium (Goldman et al., 2017). Fungistatic atmospheres are utilized to control *B. cinerea* infections on commodities during storage and shipment (Maude, 1980). Fungistatic atmospheres do not leave unsightly residues associated with most fungicide dip treatments. Carbon dioxide-enriched atmospheres are used to minimize *Botrytis* rot of strawberry fruit during truck shipment (Tshwenyane et al., 2010). A major cause of post-harvest losses due to *B. cinerea* is a fruit being stored for long periods in a modified atmosphere (Valero and Serrano, 2010). Post-harvest treatment using modified atmosphere with low O_2 and/or high CO_2 concentrations reduces the respiration rate of produce, inhibits ethylene production that retards the decrease in titratable acidity (TA) values and induces senescence of fruits, maintains fruit flesh firmness, solid soluble content (SSC), vitamin C and delays fruit deterioration through minimising fruit injury rates and hence extends the storage period of many fruits. For example, modified atmosphere storage consists of 5% O_2 + 10% CO_2 and can effectively control the growth of moulds after two weeks of storage of sweet cherry fruits (Serradilla et al., 2013).

1.5.3.2.3. Heat treatment

Post-harvest heat treatment has been used for pest management in horticultural crops (both fungi and insects) commercially since the 19th century (Schirra et al., 2000). Recently, post-harvest heat treatment has received renewed interest as an attractive physical method to maintain post-harvest quality and manage diseases as an alternative to chemicals (Bokhary et al., 2020; Klein and Lurie 1992; Lurie 1998; Schirra et al., 2000). Heat treatments include hot water (dipping, rinsing, or brushing), vapour heat, and dry, hot air. Hot water and hot air treatments can be used for disease and insect control, whereas; vapour heat is more common for insect control (Palou, 2013; Fallik, 2004). The hot water dipping method (only for a few

minutes) is very effective for fungal disease control (Lurie 1998; Mahajan et al., 2014), but if the exposure time exceeds the recommended time (up to 60 minutes), it can damage the fruit tissue (Klein and Lurie, 1992). The commodity is moved using brush rollers through a pressurized hot water spray in hot water brushing. It is used in commercial sorting lines to clean and reduce the pathogen spores on the perishable surface (Fallik, 2004). In vapour heat, air saturated with water vapour (temperatures of 40–50°C) is used for treating material. In contrast, hot air can be applied by placing fruits or vegetables in a heated chamber with a ventilating fan or applying precisely controlled forced hot air. Both vapour heat and hot air are used in quarantine treatment before fresh market shipments (Lu et al., 2007).

A major benefit of post-harvest heat treatments is that they can be combined with other physical treatments such as ultraviolet type C (UV-C) illumination or ionizing radiation treatments, chemical treatments, and/or biological control treatments (Palou, 2013). Combined treatments can have additive and/or synergistic effects of increasing the effectiveness and/or the persistence of individual treatments (Palou, 2013).

As a physical treatment, heat affects both the plant pathogen and the commodity (Schirra et al., 2000; Pavoncello et al., 2001; Lurie and Pedreschi, 2014). In the host, heat may affect the stability of various proteins, membranes, cytoskeleton structures and alter the efficiency of enzymatic responses in the cell, causing a metabolic imbalance that is important for maintaining quality and shelf life. Heat may also cause the accumulation of reactive oxygen species and sometimes different flavonoids such as quercetin, hesperetin, nringenin, and rutin (Lurie and Pedreschi, 2014), all of which have specific antimicrobial activity. Heat also possesses a direct inhibitory effect on post-harvest pathogens (Zhang et al., 2008; Jemric et al., 2011).

Heat can reduce the post-harvest pathogen by removing the pathogen spores from the fruit surface (during washing), inhibiting fungal germination and growth, and inducing resistance in the host against pathogen infection (Barkai-Golan and Phillips 1991; Klein and Lurie 1992; Lu et al. 2007; Palou, 2013; Mahajan et al., 2014). Heat may also change the pattern of epicuticular wax, in turn covering the micro-cracks on the fruit surface, which may reduce the pathogen invasion (Jing et al., 2010). According to Aguayo et al. (2015), in host-pathogen interactions, heat may increase the host's calcium uptake, increasing cell wall rigidity, thereby increasing resistance to infection. The modes of antifungal action of heat are presented in Table 1. 2. The times and temperatures used in post-harvest heat treatments occur over a wide range (35-39 ˚C

for a day to 63 ˚C for less than a minute) for the control of insects and fungi and depend on the type of produce or horticultural crop (Lurie and Pedreschi, 2014). The physiological tolerance of different commodities varies greatly with heat treatments (Brown and Ogle, 1997). However, the response of fruit to post-harvest heat treatment depends on the cultivar, fruit condition before treatment, temperature and duration of treatment, method of heat application, growing season, and location (due to differences in climate, soil type, season, production practices), maturity at harvest, and fruit size (Fallik, 2004; Fallik et al., 2007). Post-harvest heat treatments are used for disinfection and disinfestation of an increasing variety of crops, including flowers, fruits, and vegetables (Oladele et al., 2020). Some examples of post-harvest heat treatment for post-harvest disease management are presented in Table 1.3.

Table 1. 2. Modes of action of post-harvest heat treatments on post-harvest disease-causing fungi of fruits.

Mode of action	Pathogen	Disease	Host (Scientific name)	References
Direct effect on pathogen				
Inhibition of pathogen growth and germination	*Monilinia fructicola*, *P. digitatum*, *P. expansum*, *Monilinia* sp.	Brown rot, Green mould, Decay	Stone fruit, Citrus (*C. reticulata*, *C. sinensis*), Grapefruit (*C. paradisi*)	Porat et a1., 2000; Spadoni et al., 2013; Liu et al., 2012; Nafussi et al., 2001; Zhang et al., 2008
The killing of pathogen spores, both germinated and ungerminated	*Monilinia laxa*	Brown rot	Peach	(Spadoni et al.., 2014)
Induced resistance in the host	*Monilinia* spp., *P. expansum*, *M. fructicola*, *P. digitatum* *Phlyctema vagabunda*, *C gloeosporioides*	Brown rot, Blue mould, Green mould and decay, Lenticel rot, Anthracnose	Peach; Apple; Grape, Mango, Lemon.	(Nafussi et al., 2001; Pavoncello et al., 2001; Benitez et al., 2006; Liu et al., 2012b; Spadoni et al., 2012; Spadoni et al., 2014)
Indirect effect				
Reducing the population density by removal from the fruit surface	*Penicillium digitatum* *Geotrichum citri-aurantii*	Green mould, Sour rot, Decay	Citrus (*Citrus limon*, *C. sinensis*, *C. reticulata*), Grapefruit (*C. paradisi*), Strawberry (*Fragaria ananassa*).	(Porat et al., 2000; Smilanick et al., 2003; Jing et al., 2010)
Change the pattern of epicuticular wax and then may reduce the pathogen invasion	Various pathogens	Decay	Strawberry (*Fragaria ananassa*), Apples (*Malus domestica*)	(Roy et al., 1994; Jing et al., 2010)
Heat and Ca: Ca uptake increase, and rigidity of cell wall increase and resistance to microbial growth			Apple (fresh cut)	(Aguayo *iet al.,* 2015)

Table 1. 3. Efficacy of post-harvest heat treatment (PHT) for control of post-harvest disease of fruits.

PHT (single or in combination with other treatment)	Pathogen	Disease	Host	Effect on pathogen/disease reduction	References
HWD	Brown rot	*Monilinia laxa,*	Peach, Nectarin	Reduce conidia germination (9%) comparing the control (93%)	(Jemric et al., 2011)
HWD	Brown rot	*Monilinia* spp.	Peach	85.7 to 100% disease incidence reduction	(Spadoni et al., 2014)
HWD	Brown rot,	*Monilinia* sp.,	Peach	More than 78% decay inhibition	(Spadoni et al., 2013)
HWD	Brown rot	*Monilinia fructicola*	Peach	Lower disease incidence (75%) then control (100%)	(Liu et al., 2012b)
HWD	Lenticel rot,	*Phlyctema vagabunda*,	Apples (pink lady)	90% disease incidence reduction	(Spadoni et al., 2012)
HWD+ *Rhodotorula glutinis*	Blue mould,	*Penicillium expansum*,	Pear fruit (*Pyrus pyrifolia* Nakai.)	66.7% decay reduction	(Zhang et al., 2008)
HWD	Green mould,	*Penicillium digitatum*	Lemon fruit	Reduce disease incidence (8-12%) compare to the control (100%)	(Nafussi et al., 2001)
HWRB	Post-harvest decay	Various pathogen	*Strawberry (Fragaria ananassa)*	Reduce Disease incidence (0-22%) then control, but fruit damage become commercially unacceptable	(Jing et al., 2010)
HW	Anthracnose	*Colletotrichum musae*	Banana (*Musa spp.*) var. Berangan	Complete inhibition (100%) of condia germination	(Mirshekari et al., 2012)
HWT+ sodium carbonate	Green mould, Sour rot	*Penicillium digitatum, Geotrichum citri-aurantii*	\`Eureka' lemons (*Citrus limon*) and \`Valencia' oranges (*Citrus sinensis*)	8.9% green mould reduction and 36.7%. sour rot reduction	(Smilanick et al., 2003)
HW + fungicides	Mould	*Penicillium spp.*	Valencia orange	100% control	(Fatemi et al., 2011)
HW + fungicides	Post-harvest decay	-	'Satsuma' mandarin (*Citrus unshiu*).	Complete inhibition of decay	(Sen et al., 2010)
HW +Fungiside	Anthracnose, Stem end rot	*C. gloeosporioides* and *Diploidia natalensis*	Mango (*Mangifera indica*)	Disease severity below 5%	(Waskar and Gaikwad 2005; Yilma et al., 2013)
HW+ Fungicide	Stem end rot		Mango	Reduce disease incidence (1 -20%) then control (70%)	(Akem et al., 2013)
Hot water brushing (HWB)	Green mould	*Penicillium digitatum*	Tangerines, Oranges, and Grape fruit	45 to 55% mould reduction	(Porat et al., 2000)
Microwave (MW)-assisted hot water treatments	Decay		Strawberries	Significant reduction	(Villa-Rojas et al., 2011)
Vapor heat	Anthracnose	*C. gloeosporioides*	Mango	45% reduction of disease incidence	(Sangchote 2013)
HA	Brown rot	*Monilinia* spp	Peach and nectarin *(Prunus persica)*	94 to 100% control	(Casals et al., 2010)
HA+ nano-packing (NP)	Green mould and decay	*Penicillium citrinum*	Chinese bayberry (*Mycira rubra*)	34.6% decay incidence reduction	(Wang et al., 2010)
Steam (ST) + hot air (HA)	Green mould	*Penicillium digitatum*	Sweet orange *(Citrus sinensis)*	Significant reduction	(Aborisade 2014)

HW= Hot water, HWT= Hot water treatment, HWD=Hot water dipping, HWB= Hot water bruising, HA= Hot air, HWRB= Hot water rinsing, and bruising.

Toxicity of heat treatments to human health has not been observed in any report in this review; whereas risks of adverse effects on fruit quality have been reported (Palou, 2013). These adverse effects can be overcome by reducing the time of exposure to heat or altering the methods of application. For example, the adverse effect (weight loss, desiccation, rind staining such as pitting or browning) of curing in citrus (temperatures more than 30 ˚C, relative humidity more than 90% and treatment time 2/3 days) can be successfully avoided by treating the fruits with higher temperatures for reduced periods (18 h at 40°C) or by using intermittent curing (two 18-h cycles at 38°C) (Pérez et al., 2005; Nunes et al., 2007). The main problem of post-harvest heat treatment for disease management is a lack of preventive activity and low persistence on the commodity. Post-harvest heat treatment is simple, fast, inexpensive, compatible, and complementary with other post-harvest disease control methods. Heat can be easily included in integrated disease management programs. Often it increases the efficacy of fungicides, and the produce is generally recognized as safe when fungicides and heat are applied together (Palou 2013; Lurie and Pedreschi, 2014). In organic agriculture, post-harvest heat treatment can mitigate consumer concerns about fungicide residues (Palou 2013; Mahajan et al., 2014).

1.5.4. Post-harvest biological control

Biological control of diseases, weeds, and pests is essential for a successful Integrated Pest Management plan. Biological control is pest management through the use of their natural enemies. Biopesticides are pest limiting agents of biological origin, which include microbial living systems (bacteria, fungi, viruses), entomopathogenic nematodes, insect predators and natural parasites, plant-derived products (botanicals), and insect pheromones (natural and semi chemicals) (Copping and Menn, 2000). Products containing bacteria, fungi, or viruses have recently received much attention from researchers, industry, and authorities. A general classification of biopesticides is based on living organisms and natural products. According to several authors, the term biopesticide should be reserved only for living organisms. However, this definition would not include products derived from biological organisms' metabolism, such as pheromones, allelochemical molecules, or plant extracts. Therefore, in the context of current crop protection, a broader definition of biopesticides, encompassing all molecules of biological origin, seems to be more appropriate (Villaverde et al., 2014). Microbial antagonists, including yeasts, filamentous fungi, and bacteria, are used as control agents of post-harvest diseases, mainly against *Botrytis* and *Penicillium* in fruits and vegetables (Kiewnick, 2001).

Biocontrol products aimed at foliar plant pathogens can serve as alternatives to some chemical

fungicides, especially in cases of fungicide failure. Only a few microorganisms have been commercialised for the control of foliar plant pathogens (Copping and Menn, 2000). One of the studied trade biocontrol agents and the first biocontrol agent to be commercialized and registered for use in greenhouse crops and vineyards against *B. cinerea* is isolate T39 of *Trichoderma harzianum* (Trichodex20sp, Makhteshim Chemical Works). Yeasts have been studied for more than a decade as biological control agents against post-harvest diseases of fruit. Wounded fruit needs protection because wounds are primary sites of infection by several post-harvest fungal pathogens, including *B. cinerea*. *Cryptococcus laurentii* (originally identified as *Debaryomyces hansenii*), *Kloeckera apiculata*, *Sporobolomyces roseus* and *Candida oleophila* have shown biocontrol effectiveness against the grey mould of apple. Typically, biocontrol agents for use on fruit in postharvest storage are isolated from fruit or fruit trees (Filonow et al., 1996).

1.5.5. Diagnostics for *B. cinerea*

There are some traditional isolation techniques that can be used to isolate and identify fungal pathogens. A range of different agar media can be used to isolate fungi into pure culture. For example, potato dextrose agar (PDA) is commonly used for the isolation of plant pathogens. It can be acidified or amended with different antibiotics to prevent bacterial growth. For example, the addition of sterile tartaric acid (10%) is used to reduce the pH of this medium to 3.0 +/- 0.1, to inhibit bacterial growth and facilitate the recovery of pure fungal cultures (Aryal, 2015). The molecular methods, such as phylogenetic analysis and DNA sequencing, to study fungi have greatly improved the resolution and robustness for making estimates of genetic diversity within different taxonomic groups. Molecular studies in the first decade of the 21st century have assisted in the reclassification of the Kingdom Fungi, which is classified into one subkingdom, seven phyla, and ten subphyla (Hibbett et al., 2007).

1.6. Volatile and non-volatile organic compounds

Volatile organic compounds (VOCs) are carbon compounds that evaporate at room temperature (Bennett & Inamdar, 2015). In organisms, they are released as products of metabolic activities or tissue damage. The profile of VOCs may provide very valuable evidence about a variety of relationships and biological status, such as the relationship between insects and their hostage plants (Du, 2001; Jhumur et al., 2007; Verheggen et al., 2008). Also, the inhibitory effect of VOCs on other organisms such as bacteria and fungi (Arroyo et al., 2007**;** Sekine et al., 2007),

the attractive effect on the other sex (Landolt & Phillips, 1997), and as aggregation or defensive secretions against other species (Arnaud et al., 2002; Villaverde et al., 2007). So, because VOCs are affected by biological status, they have been used as indicators for studying metabolic changes (Goh et al., 2016). Hence, VOCs were reported to be robust tools for diagnosing specific diseases that infect humans through the investigation of the differences in the VOCs emitted by patients and healthy people (Belda-Iniesta et al., 2007; Miekisch et al., 2004; Song et al., 2010). Examples of diseases diagnosed using VOCs include respiratory issues, inflammatory diseases, cholesterol biosynthesis and cancer diseases in the human body (Miekisch et al., 2004). For instance, a study about the effect of smoking on human respiration showed that non-smokers produced more saturated hydrocarbons through their respiration, whereas hydrocarbons were predominant in smokers' breath along with other compounds including furan, acetonitrile and benzene (Buszewski et al., 2009). A study of children with asthma and healthy children showed that eight VOCs in exhaled breath could be used as a discriminating tool to differentiate the children with asthma from healthy children (Dallinga et al., 2010).

Fungi produce a diverse blend of compounds that are released into the atmosphere and soils because of their metabolism. To date, 250 fungal compounds have been identified during the primary and secondary metabolism of a range of fungi (Morath et al., 2012). According to Shannon (2012), fungal compounds might contribute to "sick building syndrome" and also can contribute to biological control by some *Trichoderma* species. Gas chromatography-mass spectrometry (GC MS) is the main method for detecting fungal compounds because of its separation power and highly sensitive detection ability. Compounds can be identified through a library or database of mass spectra or by comparing spectra and retention times with known standards (Shannon et al., 2012). In addition, studies on the potential use of VOCs as biomarkers to investigate the infestation of some plant pathogens such as Phytophthora spp. was also proved in many studies (Crespo et al., 2008; Laothawornkitkul et al., 2010; Qiu et al., 2014b). Qiu et al. (2014) used VOCs as a tool for the identification of *Phytophthora cinnamomi* in different growing environments, using specific VOCs like 2-methoxy-4-vinyl phenol to determine the infestation.

The first step to identifying the biological VOCs is applying a noble sampling technique that can deal with the complicated chemical structures and polarities (Zhang & Li, 2010). Solid phase microextraction (DI-SPME) fibres were developed and proposed for the first time by (Arthur & Pawliszyn, 1990; Pawliszyn, 1995). Applying this technique by only inserting (DI-

SPME) fibre into the sample matrix made this method easy to use (Camarasu, 2000). By reducing the extraction steps and increasing efficiency, SPME was considered the most valuable invention in the field 12 of sample preparation (Balasubramanian & Panigrahi, 2011; Zhang & Li, 2010). Three modes to perform SPME were described, including direct extraction, membrane protection extraction and headspace extraction (Balasubramanian & Panigrahi, 2011). Headspace extraction can be implemented by exposing the fibre coating to the headspace of the extraction chamber that contains the biological sample (Laopongsit et al., 2014; Laothawornkitkul et al., 2010; Niu et al., 2015; Qiu et al., 2014b). In biology, the headspace solid-phase microextraction (HS-SPME) of VOCs was commonly applied for its suitability for sampling various types of biological samples, such as animals, plants, and microbial samples (Zhang & Li, 2010). Using HS-SPME coupled with gas chromatography–direct deposition infrared spectrometry (GC–DD-IR) led to a rigorous absence of water and could be applied to trap unknown VOCs directly from within living organisms (Auger et al., 1998). The HS-SPME has been used to extract and sample VOCs from a variety of biological samples (Belliardo et al., 2006; Cai et al., 2007; Crespo et al., 2008; Nakamura & Daishima, 2005). A study that reviewed the significance of using HS-SPME in the analysis of the volatile fraction of aromatic and medicinal plants showed that 108 articles published during 2000–2005 proved the importance of this method (Belliardo et al., 2006).

The HS-SPME techniques coupled with gas chromatography (GC) to analyse fungi-derived VOCs was previously suggested as a method to detect infestations of stored grain (Farag et al., 2006; Nilsson et al., 1996; Pecoraino et al., 2008). However, this method requires further optimisation to enhance the efficacy of VOCs extraction (Laopongsit et al., 2014; Niu et al., 2012). The ability of SPME to absorb chemical compounds is affected by many factors, such as extraction time, temperature, fibre coating and compound types (Balasubramanian & Panigrahi, 2011). A range of commercial coatings of SPME has been used to extract VOCs from the headspace (Balasubramanian & Panigrahi, 2011). For example, 50/30 μm DVB/CARB/PDMS fibre coating can extract many VOCs because it is covered by a mid-polarity coating (Balasubramanian & Panigrahi, 2011; Kataoka et al., 2000). In addition to the effect of the fibre type, the extraction time is also considered a critical factor in collecting more VOCs (Kataoka et al., 2000; Niu et al., 2012; Qiu et al., 2012). Appropriate extraction time is required to maximise the extraction ability of the fibre coating (Kataoka et al., 2000; Niu et al., 2012; Qiu et al., 2012). A short extraction time may not be appropriate to release most of the VOCs, specifically the low volatile compounds, whereas a more extended extraction period

may result in degradation of some of the VOCs, such as the high volatile compounds (Laopongsit et al., 2014). Using a suitable extraction temperature is a significant factor for abundant extraction. High extraction temperatures cause an increase in the extraction rate, but it also reduces the distribution constant (Kataoka et al., 2000). All the above parameters should be taken into consideration to produce reliable and sufficient VOC data.

In current years, non-volatile organic compounds have been studied by many methods of ionization such as field desorption which include laser desorption, secondary ion mass spectrometry, fast atom bombardment, atmospheric pressure ionization, plasma desorption, and direct exposure techniques such as direct and etcetera. There are, still, several compounds whose mass spectra cannot be captured suitably by applying any of these techniques. The impact of coexisting compounds has to be examined not only for getting a good mass spectrum of a compound but for using the rapidly developing technique called mass spectrometry/mass s spectrometry (MS/MS) to non-volatile compounds.

1.6.1. Headspace Solid Phase Microextraction (HS-SPME)

Arthur and Pawliszyn developed solid phase microextraction in 1989 to analyse rapid sample preparation for laboratory conditions and on-site arrangements and provide an efficient method for integrating sample preparation with detection systems separation (Arthur and Pawliszyn, 1990). Following the principle of solid-phase micro extraction (SPME), the technique employs a small volume of polymeric extracting phase coated on the outside of a metal alloy solid support or fused silica (Pawliszyn, 1997). The essential part of SPME is solid fibre support coated with a thin layer of a polymeric stationary phase that is used to extract the analytes by concentrating them from the sample. The fibre is located inside a needle, which protects the fibre coating from damage by injecting it into vial/injector septa penetrations (Pawliszyn, 1997). In general, SPME has been used routinely in combination with GC-FID and GC-MS. However, to analyse non-volatile chemical and thermally labile compounds not amenable to GC-MS, significant improvements were made in the direct coupling of SPME with HPLC and LC-MS. Recently, SPME has been widely applicable in targeted and non-targeted quantitative and qualitative analyses of organic compounds from various gaseous, liquid, solid environmental, food, and biological matrices. The SPME principle relies on placing a thin polymeric coating coated outside of a fused silica fibre directly towards the sample matrix.

Although chemical compounds produced by fungi have been isolated by extraction from mycelia or from whole fungal colonies (Sawoszczuk et al., 2017), some compounds are synthesized and released immediately from the fungi rather than being stored in the colony. In contrast to this, chemicals using the headspace technique from the environment (air, host, and artificial media) surrounding the fungi can be collected. This allows for gains to be made not only on the qualitative structure but also in the relative concentration of the compounds. The values of multicomponent chemicals are usually decipherable for biological activity. The headspace analysis has been used widely in collecting compounds, non-compounds, and pheromones from organisms and substrates (Loulier et al., 2020).

The main strengths of GC–MS for metabolomic analyses include the capacity to detect a large number of intermediates in the central carbon metabolism (organic acids, sugar phosphates, lipids, and amino acids), high quantitative accuracy and reproducibility, precise metabolite identification based on highly reproducible GC retention time and Electron Ionization generated mass spectra and availability of sophisticated signal processing and bioinformatics software packages (Halket et al., 2005). However, GC–MS analyses generally involve more complex sample processing protocols, involving the need for derivatization. GC–MS has been applied successfully to metabolomics analysis of fungi.

Martin et al. (1993) found that postharvest decay fungi produced 15 compounds released from red raspberries and strawberries during ripening. Five of these volatile compounds completely inhibited all fungi directly on fruit at 0.4 µl/ml. Benzaldehyde at 0.04 µL/mL completely inhibited cultures of *Alternaria alternata* and *B. cinerea*. Shimizu et al. (1982) investigated the compounds in grape released in the presence of *B. cinerea*. They found that linalool or terpinen compounds were reduced when *B. cinerea* was grown in these terpenes for 15 days, but only geranial was identified in terpinen supplemented *B. cinerea*. Boch et al., (1986).

1.6.2. Direct immersion Solid Phase Microextraction (DI-SPME)

Solid phase microextraction has been used since 1989 to promote rapid sample preparation and to provide an efficient method to detect chemicals with detection and separation systems (Arthur and Pawliszyn, 1990). The extraction samples can be performed using two methods. The first method is headspace SPME or (HS-SPME), where the fibre is exposed in the vapor phase above a liquid, gas, and gaseous sample. The second method is direct immersion (DI-SPME), where the fibre is directly immersed in the liquid samples (Aulakh et al., 2005).

Samples are extracted by exposing the SPME directly to the headspace or exposing a small sample extraction volume to the SPME (Bojko et al., 2014). After the sample matrix on the SPME coating reaches the distribution equilibrium, the SPME is inserted into a gas chromatograph-mass spectrometer (GC-MS) inlet for thermal desorption or desorption solvent for coupling with LCMS (Pawliszyn, 1997). In addition to studies on the determination of environmental pollutants, SPME has been used to analyse biological samples to detect active compounds in animals, plants, emissions, and microorganisms (Zhang et al., 2012). In environmental samples, SPME has been introduced to extract non-volatile organic compounds. This method has gained a lot of interest in broadly analysing, including in the biological, pharmaceutical, and food sample fields. Also, DI-SPME is used as quantitative analysis in biological samples including plant tissues (Risticevic et al., 2015), pesticides (Aulakh et al., 2005), milk (González-Rodríguez et al., 2005), drugs (Snow, 2000), and water (López-Darias et al., 2010).

1.7. Research gaps and aims

Developing the blueberry industry needs further studies such as pathogens survey. To reduce economic losses in the blueberry industry, farmers routinely use chemical control. This method has many issues, such as chemical pollution, which impacts on the quality of fruits and vegetables and negatively affects the ecosystem; therefore, biological control can be an alternative method. There were three primary aims of this thesis. The first aim was to determine if *B. cinerea* is the major post-harvest pathogen of blueberry in Western Australia. The second aim was to isolate and diagnose potential blueberry fruit antagonists and screen these against *B. cinerea* in *vitro*. Thirdly, to detect the chemical compounds produced by the antagonists using direct immersion solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS). Specifically, each chapter covers the following:

Chapter 2. Variation in pathogen community associated with post-harvest blueberry fruit in Western Australia.

Aim. This study aimed to (a) isolate and identify post-harvest blueberry pathogens in Western Australia and (b) confirm the pathogenicity of the fungal isolates obtained.

Chapter 3. In *vitro* screening of potential antagonists to *B. cinerea*.

Aim. In *vitro* develop potential biological control agents to control *B. cinerea* in post-harvest blueberry.

Chapter 4. Non-volatile chemical compounds produced by antagonists using direct immersion solid-phase microextraction (DI-SPME) coupled with gas chromatography-mass spectrometry (GC-MS).

Aim. Identify the chemical compounds released from *B. cinerea*, its antagonists and their interactions.

Chapter 2.

Variation in pathogen community associated with post-harvest blueberry fruit in Western Australia

2.1. Abstract

Blueberries are cultivated worldwide due to their commercial value and health benefits. In this study, we evaluate and characterize the fungal diversity associated with blueberry grown in Western Australia. One hundred ninety blueberry fruits were collected from four cultivars from two farms and screened for pathogens using culturing techniques. In total, nine fungi were identified. Of these, five fungal species (*Botrytis cinerea, Alternaria alternata, Cladosporium cladosporioides, Aureobasidium pullulans* and *Diaporthe australafricana*) were able to cause significant infection on blueberry. This is the first record of *A. alternata* described from blueberry in Western Australia, and the first to record *D. australafricana* on blueberry in Australia, and both of them were pathogens. Another fungal species found on blueberry fruit for the first time was *Ceriporia* sp. This study shows that the pathogen community associated with blueberry is diverse. The study provides knowledge for better management of the diseases related to the blueberry.

2.2. Introduction

Blueberry (*Vaccinium corymbosum* L.) is a native to North America and produce edible dark-purple berries. In 2012, the global blueberry industry produced a record production of 1,336 million lbs. (605,999 tonnes). In 2017, global production was 596,813 tonnes (Innovation, 2018). The largest blueberry-producing country is the United States of America, which produces over half of the worldwide production (Michalska and Lysiak 2015). Blueberry production increased three-fold between 2014 (5,500 tonnes) and 2018 (16,850 tonnes) in Australia. The Australian domestic market consumes the majority of blueberries grown in Australia (Innovation 2018). Furthermore, Australia imports about 1,235 tonnes annually and exports about 356 tonnes (Innovation 2018). Post-harvest decay, incited by various fungal pathogens, is a significant concern in most blueberry production areas worldwide. Fungal pathogens attack all blueberry parts, including roots, stems, leaves, blossoms, and fruit. Post-harvest decay can happen for many reasons, including berry flesh type, harvest methods, transport, storage, and more (Mehra 2010). Major pathogens of blueberry worldwide include anthracnose caused by *Colletotrichum* species, *Thekospora minima* the cause of blueberry rust, grey mould caused by *Botrytis* species, root rot caused by *Phytophthora* species, and stem blight caused by *Botryosphaeria dothidea, Neofusicoccum australe, N. parvum, N. macroclavatum, N. oculatum, Lasiodiplodia theobromae*, and *L. pseudotheobromae* (Abbey et al., 2019**;** D.P.I. 2019). This study aimed to (a) isolate and identified post-harvest blueberry pathogens in Western Australia and (b) determine their pathogenicity. Finally, the study provides a comprehensive list of post-harvest blueberry pathogens as a reference for future research in Western Australia and elsewhere.

2.3. Materials and Methods

2.3.1 Blueberry sample collection

Blueberry fruit from four cultivars (C99-42, C00-09, Eureka 1403 and EB8-46) from two farms in Western Australia was used (Table 2.1). Once collected, they were kept at 4°C for a maximum of 24 hours before being processed for fungal isolations.

Table 2. 1. Blueberry (*Vaccinum corymbosum*) variety, species, farm address and GPS.

Variety	Harvest seasons	Farm Address	GPS of harvest location
C99-42	August 2016 August 2017	Lot 4 Chitna Rd, Neergabby WA 6503	31o20'44" S 115o37'56" E
C00-09	August 2016 August 2017	Lot 4 Chitna Rd, Neergabby WA 6503	31o20'44" S 115o37'56" E
Eureka 1403	August 2016 August 2017	384 Orange Springs Rd, Regans Ford WA 6507	31o00'15" S 115o40'36" E
EB 8-46	August 2016 August 2017	384 Orange Springs Rd, Regans Ford WA 6507	31o00'15" S 115o40'36" E

2.3.2. Fungal isolation

Each cultivar was divided into two groups, sterilized and non-sterilized. The first group was sterilized by immersing in 70% ethanol for five minutes (Junk et al., 2017) and then left to dry on sterile filter paper for one day in a laminar flow cabinet. The other group was left non-sterilized. A single intact blueberry fruit was then placed onto half-strength potato dextrose agar (1/2 PDA)(9.75 g of Difco potato dextrose agar and 3.75 g of BBL agar (Becton, Dickenson and Company, Sparks, MD, U.S.A. and 500 ml of distilled water) in a Petri dish and kept in the light at room temperature (25°C ±1). There were six replicates for each sample for each blueberry cultivar across the two groups. After five days, fungal colonies growing on the ½ PDA plates were sub-cultured onto fresh 1/2 PDA Petri plates. Fungal samples were subcultured on ½ PDA at least five times to ensure they were not contaminated.

2.3.3. Identification of isolates by DNA extraction

Isolates were grouped visually based on similar morphological and cultural characteristics, and a selection of isolates representing each group were prepared for molecular analysis. These isolates were lodged with the Department of Primary Industries and Regional Development

(DPIRD). As described (Andjic et al., 2007), mycelium from individual isolates was frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted using a hexadecyl trimethyl ammonium bromide (CTAB) protocol but modified by the addition of 100g/ml Proteinase K and 100g/ml RNAs A to the extraction buffer (Andjic et al., 2007). A part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified using the primers ITS-1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR products were cleaned up, as Sakalidis et al. (2011) described. Sequences were matched using BLAST to known sequences in GenBank. The GenBank accessions were obtained from the National Center for Biotechnology Information (United States of America) (Table 2.2.).

2.3.4. Pathogenicity tests

To determine the pathogenicity of each fungal isolate, healthy fruit from the four cultivars were collected and sterilized by immersing in 70% alcohol for 5 minutes and then left to dry on sterile filter paper in a laminar flow for one day at room temperature. Spore suspensions of each fungal isolate were prepared using a cork borer (0.5 cm) to take mycelium and spores from a 4-7-day-old colony grown on ½ PDA and placed into 10 ml sterile distilled water and shaken for 2 mins. Spores were counted using a hemocytometer and then diluted in sterile distilled water to make spore suspensions of 1 x 10^4 spores/mL. The berries were then sprayed to run-off using a sprayer (Canyon model CHS-3AN) and then incubated for seven days in a sealed plastic sterilized rectangular container (Pakplast, A700FG – 700ml, Size:175 x 120 x 55mm) at (25°C ±1) in the dark. Fungal spores growing from the fruit were isolated onto ½ PDA as described above in the section 2.3.2. These were then compared to colonies of the original isolates to confirm Koch’s postulates.

2.3.5. Statistical analysis

Experiments were analyzed using one-way ANOVA to compare the percentage recovery of the pathogens from sterilized and non-sterilized samples and between blueberry cultivars. In all the statistical analyses, the probability level was ≤ 0.05, and SPSS software (SPSS, IBM version 24 Armonk, New York, U.S.A.) was used. A 95% confidence interval was reported.

2.4. Results

A total of 190 isolates from nine fungal were isolated from sterilized and non-sterilized fruit from four post-harvest blueberry cultivars grown commercially in Western Australia (Table 2.2). DNA analysis of the nine fungi confirmed that they were *Botrytis cinerea*, *Alternaria alternata, Cladosporium cladosporioides, Penicillium brevicompactum, Epicoccum nigrum, Aureobasidium pullulans, Diaporthe australafricana,* a *Verticillium* sp., and a *Ceriporia* sp. The results of DNA analysis (Table 2.3) confirmed the gene sequences of these pathogens.

Table 2. 2. DNA analysis and Genbank accession of the fungi isolated from post-harvest blueberry in Western Australia.

Species	Contributor/ Collector	GenBank accession
Cladosporium cladosporioides	SUB8657514 /DTW2116_A05	MW308534
Ceriporia sp.	SUB8657514/ DTW2117_A06	MW308535
Verticillium sp.	SUB8657514/ DTW2118_A07	MW308536
Epicoccum nigrum	SUB8657514/ DTW2119_A08	MW308537
Botrytis cinerea	SUB8657514/DTW2120_A09	MW308538
Penicillium brevicompactum	SUB8657514/DTW2121_A10	MW308539
Diaporthe australafricana	SUB8657514/DTW2122_A11	MW308540
Aureobasidium pullulans	SUB8657514/DTW2123_A12	MW308541
Alternaria alternata	SUB8657514/DTW2127_C04	MW308545

Table 2. 3. Fungal species isolated from sterilized and non-sterilized fruit from four blueberry cultivars post-harvest.

Cultivar	Treatment	Nº of fungal species	Nº of isolates	Pathogens
C99-42	Sterilized	3	6	*B. cinerea*
			8	*Alternaria alternata*
			6	*E. nigrum*
C99-42	Non-sterilized	5	4	*P. brevicompactum*
			8	*E. nigrum*
			8	*C. cladosporioides*
			8	*B. cinerea*
			8	*Alternaria alternata*
Eureka 1403	Sterilized	1	6	*B. cinerea*
Eureka 1403	Non-sterilised	4	8	*B. cinerea*
			8	*P. brevicompactum*
			6	*C. cladosporioides*
			4	*A. pullulans*
EB8-46	Sterilized	4	8	*B. cinerea*
			2	*Alternaria alternata*
			8	*Ceriporia* sp.
			8	*D. australafricana*
EB8-46	Non-sterilised	5	8	*B. cinerea*
			6	*C. cladosporioides*
			6	*Alternaria alternata*
			8	*Verticillium* sp.
			8	*P. brevicompactum*
C00-09	Sterilized	2	8	*B. cinerea*
			8	*Alternaria alternata*
C00-09	Non-sterilized	4	8	*B. cinerea*
			4	*Alternaria alternata*
			4	*P. brevicompactum*
			8	*E. nigrum*

The collector is the label of the samples, and the Contributor is labelled of National Centre for Biotechnology Information.

There were significant differences between sterilized and non-sterilized fruit of the different blueberry cultivars (Figure 2.1). From the non-sterilized blueberries, five fungal species were obtained from cultivars C99-42 and C00-09 and four from cultivars Eureka-1403 EB8-46. In contrast, fewer fungi were isolated from the surface-sterilized berries, with three fungal species each from the cultivar C00-09 and one from cultivar Eureka-1403, and four and two fungal species from the cultivars C99-42 and EB8-46, respectively (Table 2.2).

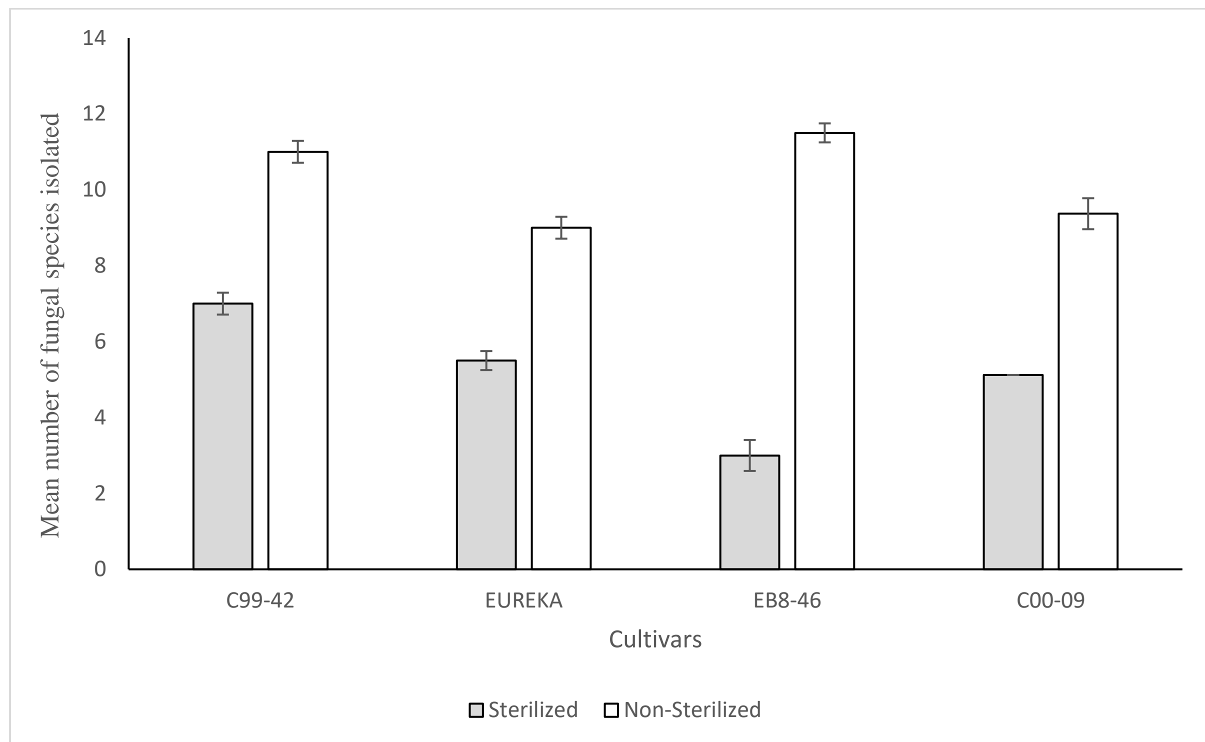

Figure 2. 1. Mean numbers of post-harvest fungi isolated from four cultivars of blueberry fruit that had been surface-sterilized or non-sterilized. Bars represent the standard error.

Botrytis cinerea was the most frequently isolated fungal species from both sterilized and non-sterilized fruit at 87.5 % and 100 %, respectively (Table 2.4). Whereas the *Verticillium* sp. was never isol0ated from surface-sterilized blueberries, and the *Ceriporia* sp. was the least frequently (12.5%) isolated species from non-sterilized blueberries. With respect to cultivar and surface-sterilization, the most fungal species were isolated from cultivar C99-42 and the least from C00-09. Whilst from non-sterilized blueberries, the most and least numbers of fungal species were isolated from EB8-46 and Eureka1403, respectively.

Table 2. 2. Percentages of fungi isolated from surface-sterilized and non-sterilized blueberry fruit.

PATHOGEN	Total		Sterilized		Non- sterilized	
	No of isolations	%	No of isolations	%	No of isolations	%
Botrytis cinerea	60	93.75	28	87.5	32	100
Alternaria alternata	36	56.25	18	56.25	18	56.25
Cladosporium cladosporioides	22	34.37	0	0	22	68.75
Penicillium brevicompactum,	22	34.37	0	0	22	68.75
Epicoccum nigrum	22	34.37	6	18.75	16	50
Aureobasidium pullulans	4	6.25	0	0	4	12.5
Diaporthe australafricana	8	12.5	4	12.5	4	12.5
Verticillium sp.	8	12.5	0	0	8	25
Ceriporia sp.	8	12.5	4	12.5	4	12.5

The pathogenicity testing showed that *B. cinerea*, *A. alternata, C. cladosporioides, A. pullulans* and *D. australafricana* were pathogens to blueberry fruit. While *E. nigrum*, *P. brevicompactum*, *Verticillium* sp. and *Ceriporia* sp. were not pathogens of blueberry fruit.

2.5. Discussion

This study identified nine fungal genera associated with post-harvest blueberries from four cultivars in Western Australia. ITS sequence analysis showed these fungal genera were *Botrytis cinerea, Alternaria alternata, Cladosporium cladosporioides, Penicillium brevicompactum, Epicoccum nigrum, Aureobasidium pullulans, Diaporthe australafricana,* a *Verticillium* sp., and a *Ceriporia* sp.

Botrytis cinerea was the most frequently isolated fungus from sterilized and non-sterilized blueberry across all cultivars. Its frequent isolation from surface-sterilized fruit indicates it was present inside the fruit prior to treatment. It is an important necrotrophic fungus that can infect over 1400 host species and cause grey mould on vegetables, fruits and flowers post-

harvest (Fillinger et al. 2016). This study confirms that *B. cinerea* is a major post-harvest pathogen of blueberry in Western Australia and agrees with other studies where it is generally considered a significant internal pathogen of post-harvest blueberry (Smith et al. 1996**;** Schilder et al. 2002**;** Rivera et al. 2013).

This investigation also showed that sterilizing post-harvest blueberry with 70% alcohol makes it possible to reduce post-harvest disease, especially for *Cladosporium cladosporioides*, *Penicillium brevicompactum*, *Aureobasidium pullulans* and the *Verticillium* sp. These fungi were infrequently isolated from surface-sterilized fruit, which indicates that they were primarily present as surface contaminants. Therefore, a further study focusing on surface sterilization techniques to reduce post-harvest disease in blueberry is suggested.

Alternaria alternata is one of the most common species of *Alternaria* that causes Alternaria fruit rot of post-harvest blueberry in the USA (Zhu 2015) and Argentina (Greco 2012), but also produces mycotoxins in blueberry (Greco 2012). In the present study, *A. alternata* was the second most frequently isolated species from blueberry. Interestingly, this species appeared to be the second most tolerant to surface-sterilization with 70% alcohol. This indicates that it was likely present inside blueberry fruit and consequently not impacted by sterilization. It has been shown as a post-harvest pathogen of blueberry in Chile, Poland, the United States of America, and Argentina (Elfar et al. 2018, Nadziakiewicz et al. 2018, Zhu et al. 2015). You et al. (2014) reported that *Alternaria tenuissima* is a pathogen of blueberry fruit in Western Australia. This is the first record of *A. alternata* from blueberry in Western Australia. It has been recorded in other parts of Australia, such as Tasmania (Yuan et al., 2009).

Four of the species isolated in the present study, *C. cladosporioides*, *P. brevicompactum*, *E. nigrum* and A. *pullulans*, have been recorded previously as opportunistic pathogens on post-harvest fruit (Tournas and Katsoudas 2005**;** Barrau et al., 2006**;** Mehra et al., 2013). Yingying et al. (2017) confirmed that *C. cladosporioides* and *E. nigrum* were pathogens of blueberry in East China. While *P. brevicompactum, C. cladosporioides* and *A. pullulans* have been identified as pathogens in blueberry, strawberry, blackberry and raspberry in the USA (Tournas and Katsoudas 2005).

The blueberry variety EB8-46 was recorded as a novel variety in the United States of America 2015 (Mazzardis 2019). This variety has good quality, large size and good flavour (Mazzardis 2019). In the present study, EB8-46 was the most susceptible variety to fungi, including *D. australafricana* and *Ceriporia* sp. that were recorded for the first time associated with blueberry in Australia. *D. australafricana* is related to blueberry stem canker in Chile (Elfar et al. 2013). The present study is the first isolation study to record *D. australafricana* on

blueberry in Australia, present in both non-sterilized and sterilized fruit. For the first time, another fungal species found on blueberry fruit was *Ceriporia* sp. that is known as a white-rot fungus (Wang et al., 2017). The results confirmed that *B. cinerea*, *A. alternata*, *C. cladosporioides, A. pullulans*, and *D. australafricana* were pathogens to blueberry fruit. These results agreed with Mehra et al. (2013) and Elfar et al. (2018). Whilst the isolates of *E. nigrum, P. brevicompactum, Verticillium* sp. and *Ceriporia* sp. was not pathogenic to blueberry fruit, as also shown by Kerry et al. (2001)**;** Rahimlou et al. (2015) and Bagy et al. (2019).

2.6. Conclusion

The blueberry industry has expanded, and production for the fresh market is increasing. Producers view blueberry disease management as an essential issue. As the industry has matured, disease problems have become more noteworthy. This survey suggests that the Western Australia industry suffers from post-harvest pathogens,and that nine fungal species are present as post-harvest blueberry microorganisms. Five of these nine fungi are pathogens. An essential result of this study were the first reports of two fungi (*Diaporthe australafricana* and a *Ceriporia* sp.) associated with post-harvest blueberry fruit. In future, more attention should focus on monitoring blueberry post-harvest pathogens to manage and improve the shelf life of blueberry and, in return, provide economic benefits to the industry through reduced spoilage.

Chapter 3

In *vitro* screening of potential antagonists for the control of *Botrytis cinerea*

3.1. Abstract

Botrytis cinerea is a severe threat to a wide range of fruit and vegetables, especially continuing to affect post-harvest during storage. Biological control of *B. cinerea* represents a suitable alternative control method to chemicals. Two approaches to evaluate *Alternaria alternata*, *Cladosporium cladosporioides*, *Penicillium brevicompactum*, *Epicoccum nigrum*, *Aureobasidium pullulans*, *Diaporthe australafricana*, *Verticillium* sp., and a *Ceriporia* sp. as potential antagonists of *B. cinerea* were used. The first method directly incubated each potential antagonist with B. cinerea on half-strength potato dextrose agar (1/2 PDA). In the second method, each putative antagonist was inoculated onto a dialysis membrane placed on the surface of ½ PDA for seven days, after which the dialysis membrane was removed, and *B. cinerea* was placed in the centre of the plate to determine if its colony growth was adversely affected. *E. nigrum* most effectively inhibited *B. cinerea*. Whilst *Alternaria alternata*, *Cladosporium cladosporioides*, *Penicillium brevicompactum*, and *Diaporthe australafricana* did not. This study will help develop biological control agents to manage *B. cinerea,* which is important to reduce chemical residues in plant products, environmental pollution, and economic losses.

3.2. Introduction

B. cinerea is a major post-harvest pathogen, and the diseases it causes can reduce the shelf life of many fruits, including blueberries (Gabler et al., 2003, Hancock et al., 2008). *B. cinerea* has a devastating economic impact on different fresh fruit and vegetables (Dean et al., 2012). It can infect all plant parts. In the pre-harvest period, *B. cinerea* may cause apparent disease symptoms or remain inactive until the post-harvest period (Fillinger and Elad, 2016). *B. cinerea* causes between $10 -100 billion losses to pre-and post-harvest crops worldwide (Hua et al., 2018), making it a critical plant pathogen (Dean et al., 2012). *B. cinerea* is challenging to control because it has several attack modes, a broad host range, and sexual and asexual stages to survive in suitable or unsuitable conditions (Fillinger and Elad, 2016). Conidia are the asexual spores of *B. cinerea*, spread by water or wind. Sclerotia are asexual survival structures of *B. cinerea*, allowing the fungus to survive unfavourable conditions (Brandhoff et al., 2017). Synthetic fungicides are the standard way to control grey mould rot caused by *B. cinerea*, and worldwide the annual costs of control exceed $1 billion (Abbey et al., 2019). The effectiveness of fungicides in controlling *B. cinerea* is questionable, as it can develop resistance to fungicides rapidly (Walker, 2013). Also, fungicides are not safe for the environment or humans (Droby et al., 2009). Therefore, biological control is an alternative method to control *B. cinerea* which is considered environmentally friendly, and genetic resistance cannot develop against it. Antagonists are the most important biocontrol agents and bioresource for potential biocontrol applications (Potshangbam et al., 2017). In general, fungi such as *Trichoderma* spp., *Aspergillus* spp., *Petriella* spp., non-pathogenic *Fusarium* spp., and *Gliocladium* spp., have been used as biocontrol agents against a range of fungal plant pathogens (Estrella et al., 2013). In the present study, eight fungi isolated from blueberry were screened for their potential as antagonists to *B. cinerea* previously isolated from blueberry fruit.

3.3 Material and methods

3.3.1. Putative antagonist isolates

A total of 218 isolates belonging to nine fungal genera were isolated from blueberry fruits were isolated from different blueberry cultivars from Lot 4 Chitna Rd, Neergabby WA 6503, and 384 Orange Springs Rd, Regans Ford WA 6507 (Chapter 2). The fungi were *Alternaria alternata*, *Cladosporium cladosporioides*, *Penicillium brevicompactum*, *Epicoccum nigrum*, *Aureobasidium pullulans*, *Diaporthe australafricana*, a *Verticillium* sp., and a *Ceriporia* sp.

These eight fungi and an isolate of *B. cinerea* isolated from post-harvest blueberry were maintained on half-strength potato dextrose agar (1/2 PDA)(9.75 g of Difco potato dextrose agar and 3.75 g of BBL agar (Becton, Dickenson and Company, Sparks, MD, USA, and 500 ml of distilled water) in a Petri dish and kept in the dark at room temperature (25 ±1°C). These isolates were lodged with the Department of Primary Industries and Regional Development (DPIRD). Sequences were matched using BLAST to known sequences in GenBank. The GenBank accessions were obtained from the National Center for Biotechnology Information (United States of America). The isolates were stored in sterile deionized water in McCartney bottles (28 ml) (Arekemase et al., 2011).

3.3.2. Screening for antagonism

The eight putative antagonists were used to study their ability to inhibit the growth of *B. cinerea in vitro* using two methods.

3.3.3. Antagonism using a dual culture method

The antagonistic potential of the putative antagonists to *B. cinerea* was estimated on 1/2 PDA Petri plates by the dual culture method (Fokkema 1978). Mycelial plugs (5 mm diameter) were excised from the five-seven days old cultures of *B. cinerea* using a sterile cork borer and placed on one side of the 1/2 PDA plates (2 cm away from the edge of the plate). In the same plates, perpendicular to the *B. cinerea* inoculum, a 5 mm mycelial disc of a putative antagonist was placed on the opposite side of the plates. Control plates were maintained for each fungus. The samples were incubated for four days in the dark at 25±1°C. After 4-7 days, the inhibition of *B. cinerea* development was assessed in two ways. Firstly, the width of the zone of inhibition was measured at the smallest distance between the *B. cinerea* colony and the colony of the antagonist (Figure 3.1). Secondly, the radial growth of the *B. cinerea* colony was measured by using the equation (Fokkema, 1973).

$$Colony\ radial\ growth\ \% = (r1 - r2)/r1 * 100\% ------------(1)$$

The colony diameter was measured twice at right angles to each other, and the average of the two two distances used. There were three replicate plates for each treatment, and the experiment was repeated three times.

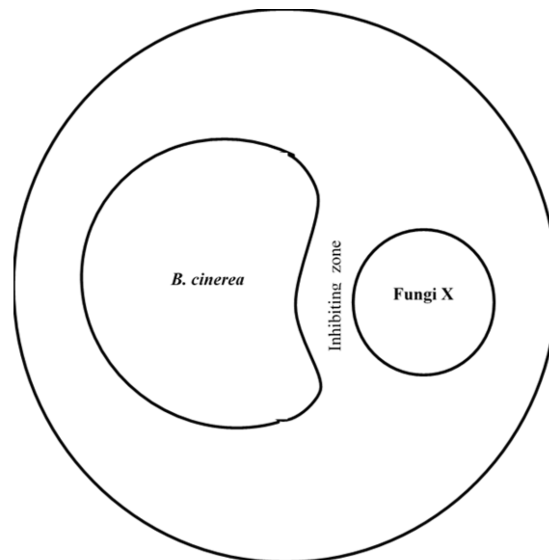

Figure 3. 1. Diagram of the mode of inoculation of the Petri dishes with *B. cinerea* and the putative antagonist. Parameters for measuring antagonism were (a) the length of the inhibition zone between *B. cinerea* and the antagonist and (b) the colony radial growth [100 x (r_1-r_2)/ r_1]. Where r_1 = Radial growth of control (*B. cinerea*); r_2= Radial growth of the antagonist.

3.3.4. Antagonism by dialysis membrane diffusion method

To determine if secondary metabolites were involved in inhibiting the growth of *B. cinerea,* a dialysis membrane method was used (Reino et al., 2008). Firstly, dialysis membrane tubing (Visking code DTV12000.09.30, size 9 Inf Dia 36/32"- 57.2mm: 30m) was cut into 90 mm diameter discs and then boiled for 2 hours. The water is replaced every 30 minutes. The discs were then autoclaved twice for 90 minutes each time, and then an individual disc was placed on top of a ½ PDA in a 90 mm Petri plate. Then a 5 mm diameter core of each antagonist isolate grown for 4- 7 days at 25±1°C on ½ PDA was placed on the surface of the dialysis membrane in the centre of the plate and incubated in a sterilized incubator (model HWS, LET code 0574-88000432, Tianjin- China) at 25±1°C in the dark. The plates were monitored daily until each isolate's colony growth was within 1 cm of the Petri plate's edge. The dialysis membrane containing the antagonist colony was then carefully removed to ensure the agar was not contaminated. The time of dialysis membrane removal varied between 4-7 days, depending on the growth rate of each antagonist. Once the dialysis membrane had been removed, a 5 mm diameter colony of a 4-day-old colony of *B. cinerea* was placed onto the center of each plate and incubated as described above with daily monitoring. A digital vernier (0-150mm,

Electronic Metric Digital Caliper DIN 862 – Linear Code: 49-923-155) was used to measure the colony size of the *B. cinerea* colony every two days until the colony had reached the edge of the Petri-dish (80-85mm). The results were compared to the control group, *B. cinerea* grown on ½ PDA for five days. The procedure of antagonist secretion was applied to the control group. Three replicates were used for each antagonist, and the experiment was repeated once.

3.3.5. Statistical analysis

Data were analyzed using one-way ANOVA on both methods (antagonism using a dual culture method and the dialysis membrane diffusion method). In all the statistical analyses, the probability level was ≤ 0.05, and SPSS software (SPSS, IBM version 24 Armonk, New York, U.S.A.).

3.4. Results

3.4.1. Antagonism on agar plates

There were significant differences among the eight putative antagonists against *B. cinerea* for the area of the inhibition zone and the percentage colony radial growth. *Epicoccum nigrum* was the most antagonistic fungus, with a mean inhibition zone of 11.8 mm (Figure 3.2) and a percentage colony growth of 38% compared to the control (Figures 3.3 and Figure 3.4). The *Verticillium* sp. followed this with an inhibition zone of 5.6 mm and a percentage colony growth of 57% (Figures 3.2, 3.3 and 3.5). With the *Ceriporia* sp. producing a 3.6 mm inhibition zone and a 39% growth in colony diameter, and *A. pullulans* at 2.3 mm and 69% (Figure 3.4).

There were no significant differences between *Penicillium brevicompactum, Alternaria alternata, Cladosporium cladosporioides,* and *Diaporthe australafricana*, where their inhibition zones were 0, 0, 0, and 11 mm (Figure 3.2). While regarding percentages of colony radial growth of *Penicillium brevicompactum, Alternaria alternata, Cladosporium cladosporioides,* and *Diaporthe australafricana* were 42%, 76%, 52% and 40% (Figure 3.3), respectively.

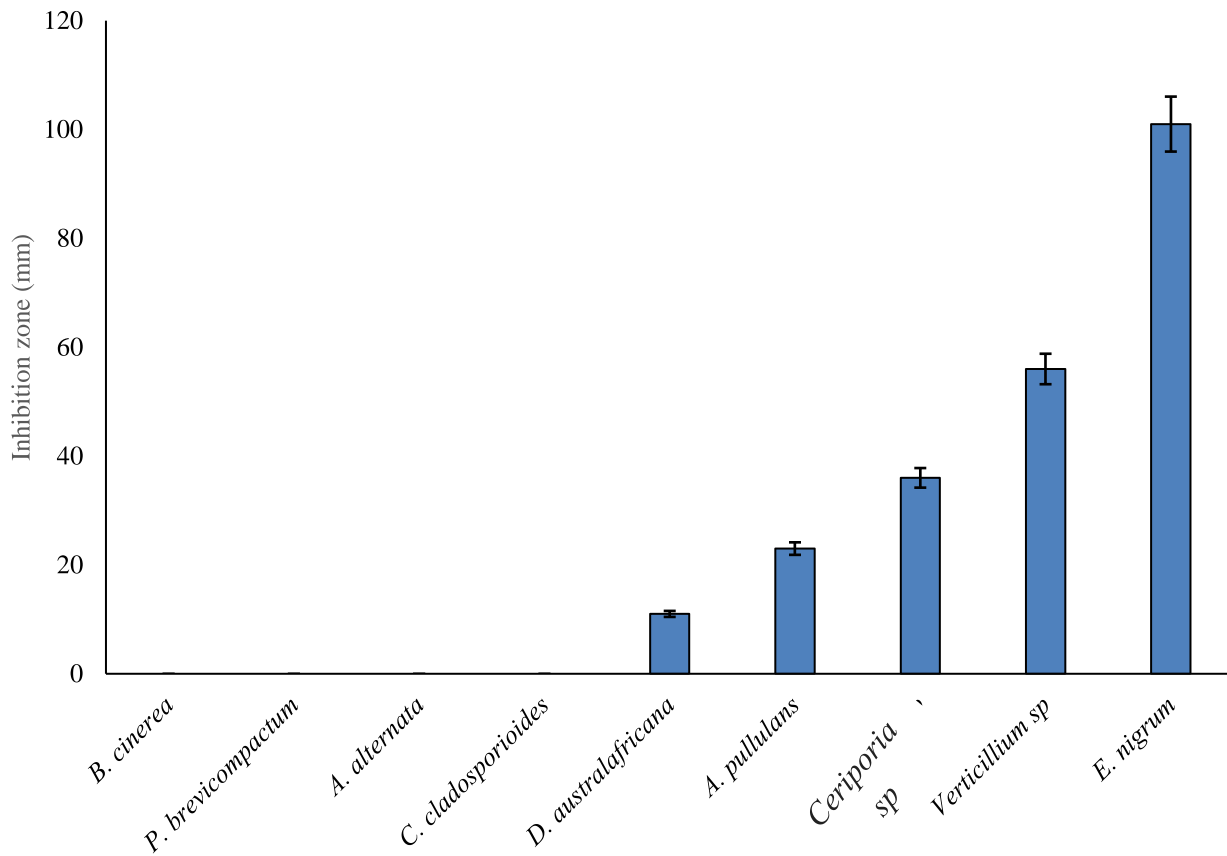

Figure 3. 2. Mean zone of inhibition (mm) between *B. cinerea* and the eight putative antagonists. Bars represent the standard error of the mean.

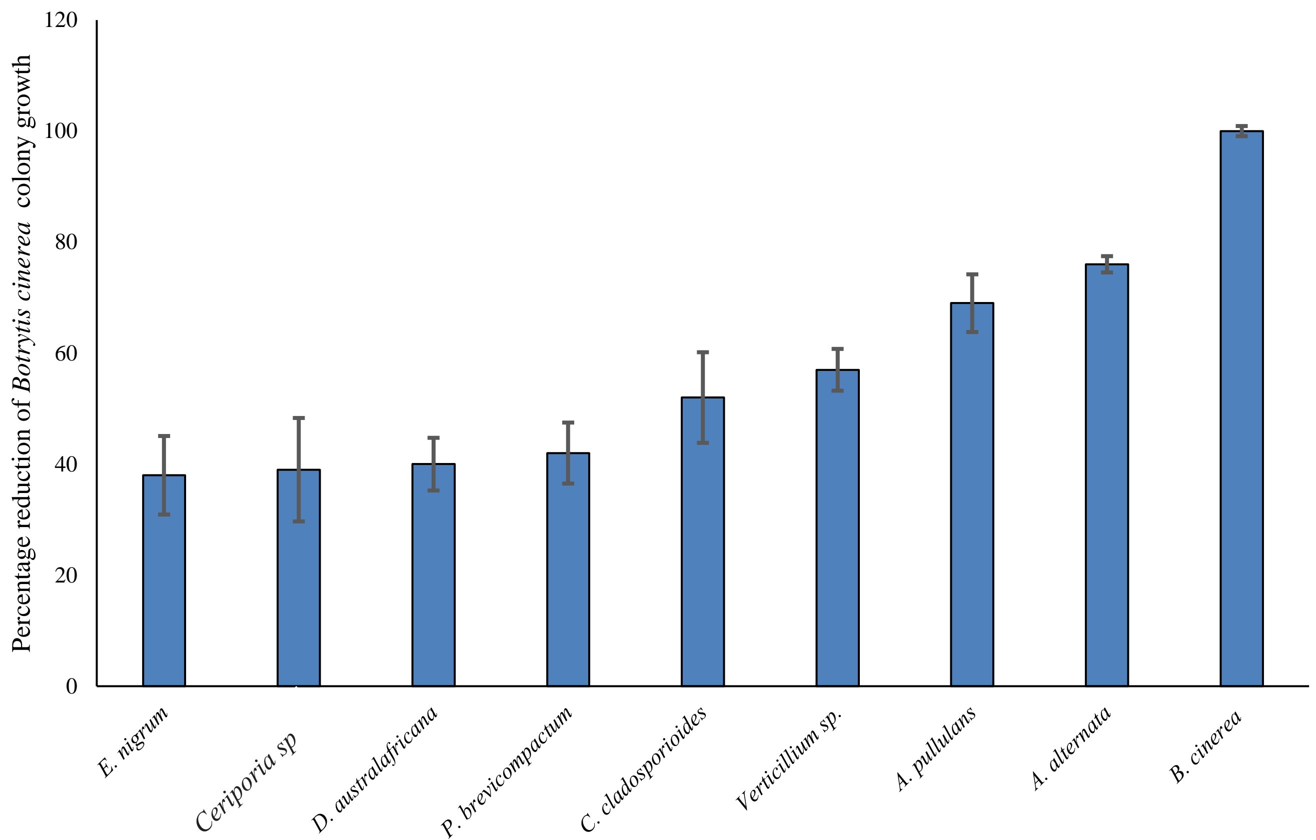

Figure 3. 3. Percentage of colony radial growth reduction of *B. cinerea* in the presence of the eight putative antagonists. Bars represent the standard error of the mean.

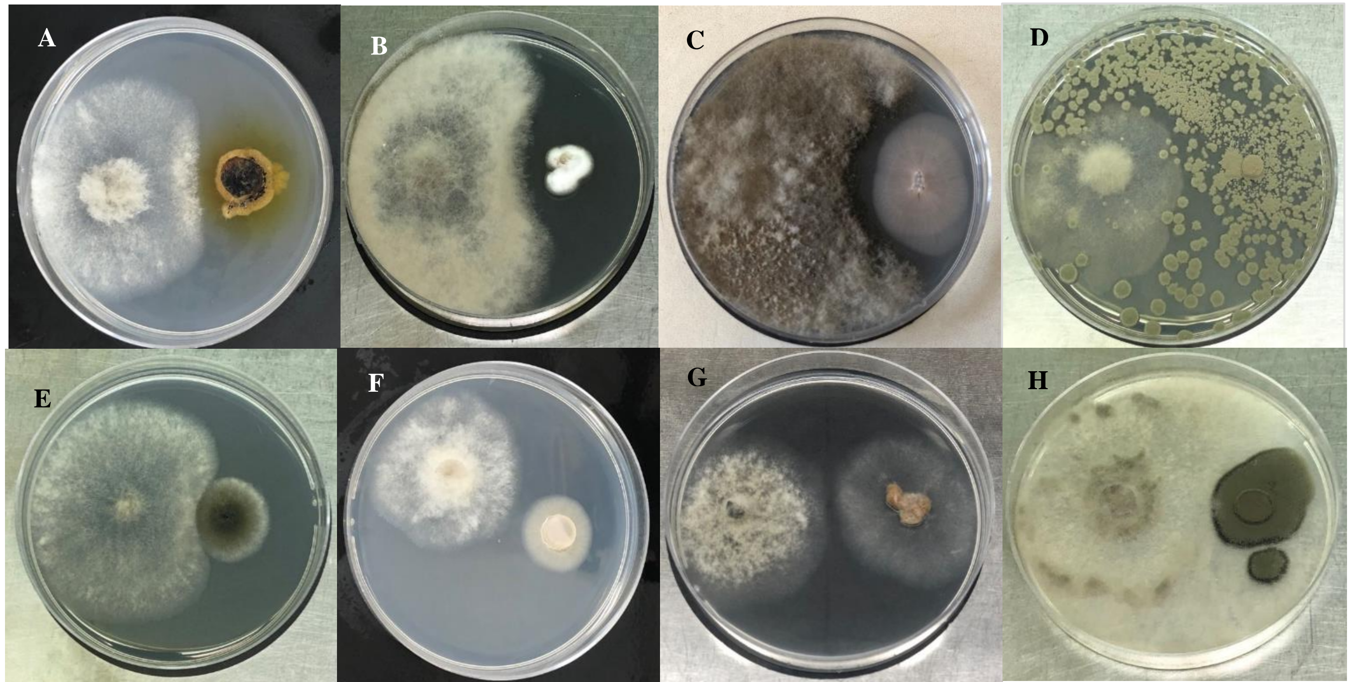

Figure 3. 4. Inhibition zones between A= *B. cinerea* and *E. nigrum*, B= *B. cinerea* and *Verticillium* sp., C= *B. cinerea* and *A. pullulans*, D = *B. cinerea* and *P. brevicompactum*, E = *B. cinerea* and *C. cladosporioides*, F = *B. cinerea* and *Ceriporia* sp., G = *B. cinerea* and *D. australafricana*, H = *B. cinerea* and *A. alternata*.

3.4.2. Antagonism by the dialysis membrane diffusion method

There were significant differences in the colony size of *B. cinerea* (df=7, f= 931.25, p= 0.00) when it was grown on ½ PDA after removing the dialysis membrane and antagonist compared to its growth under the control conditions. The percentage of *B. cinerea* colony growth in the control group was considered 100%. *E. nigrum, Verticillium* sp., *Ceriporia* sp. and *A. pullulans* all had a significant effect on *B. cinerea* colony size (Figures 3.5 and 3.6) with *B. cinerea* colony growth reductions of 21.67, 36.68, 44.44, and 48.55%, respectively. On the other hand, *P. brevicompactum, A. alternata, C. cladosporioides,* and *D. australafricana* had no significant effects on *B. cinerea* colony growth (Figures 3.5 and 3.6), which were 83.63, 85.30, 75.00 and 74.80%, respectively.

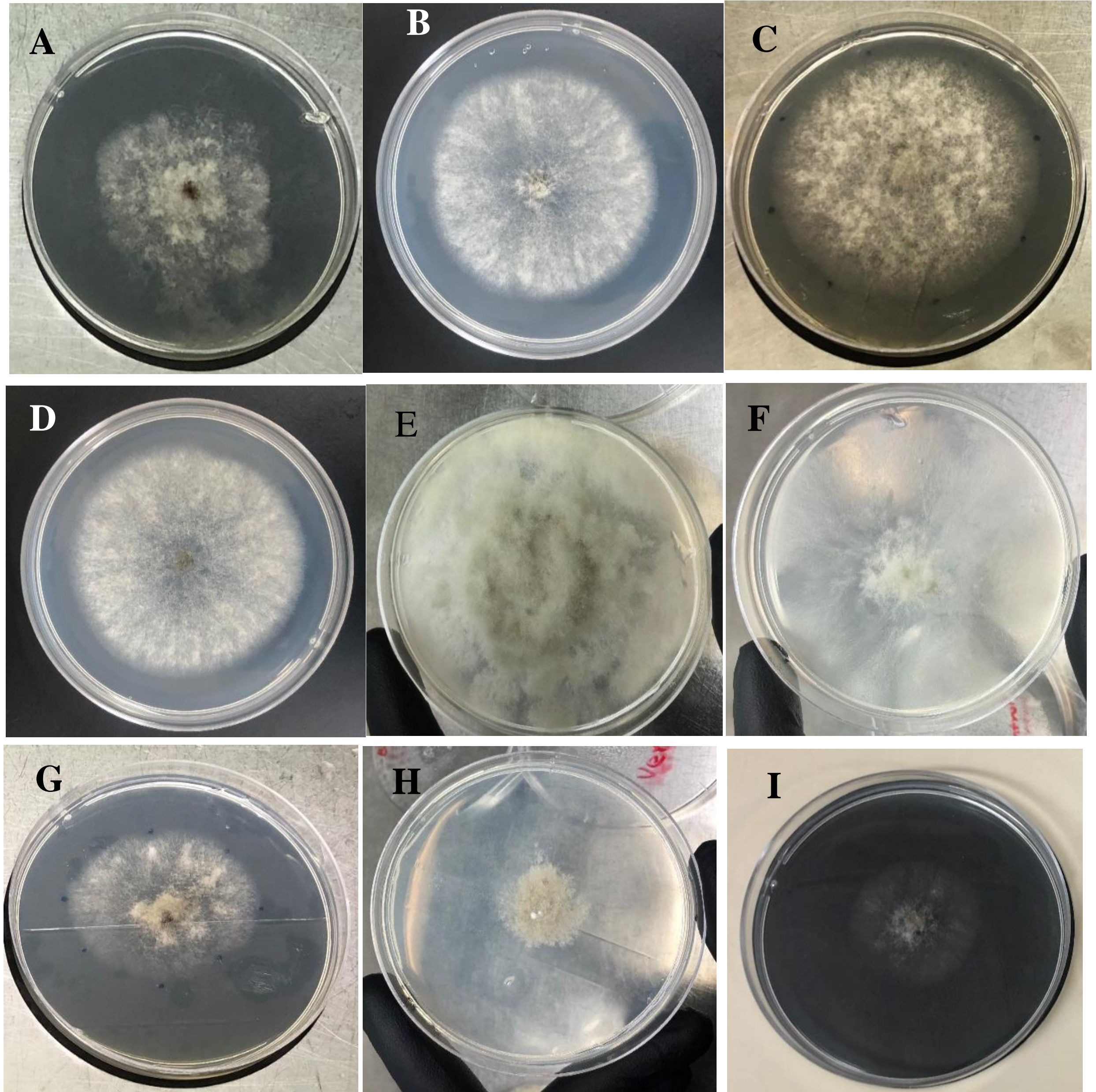

Figure 3. 5. ***Botrytis cinerea*** **colony growth using the dialysis membrane method compared to the control group. A=** ***B. cinerea*** **and** ***A. pullulans*****; B=*****B. cinerea*** **and** ***C. cladosporioides*****; C=*****B. cinerea*** **and** ***P. brevicompactum*****; D =*****B. cinerea*** **and** ***D. australafricana*****; E =*****B. cinerea*** **control; F =*****B. cinerea*** **and** ***A. alternata*****; G =*****B. cinerea*** **and** ***Ceriporia*** **sp.; H =** ***B. cinerea*** **and** ***Verticillium*** **sp.; and I=** ***B. cinerea*** **and** ***E. nigrum*****.**

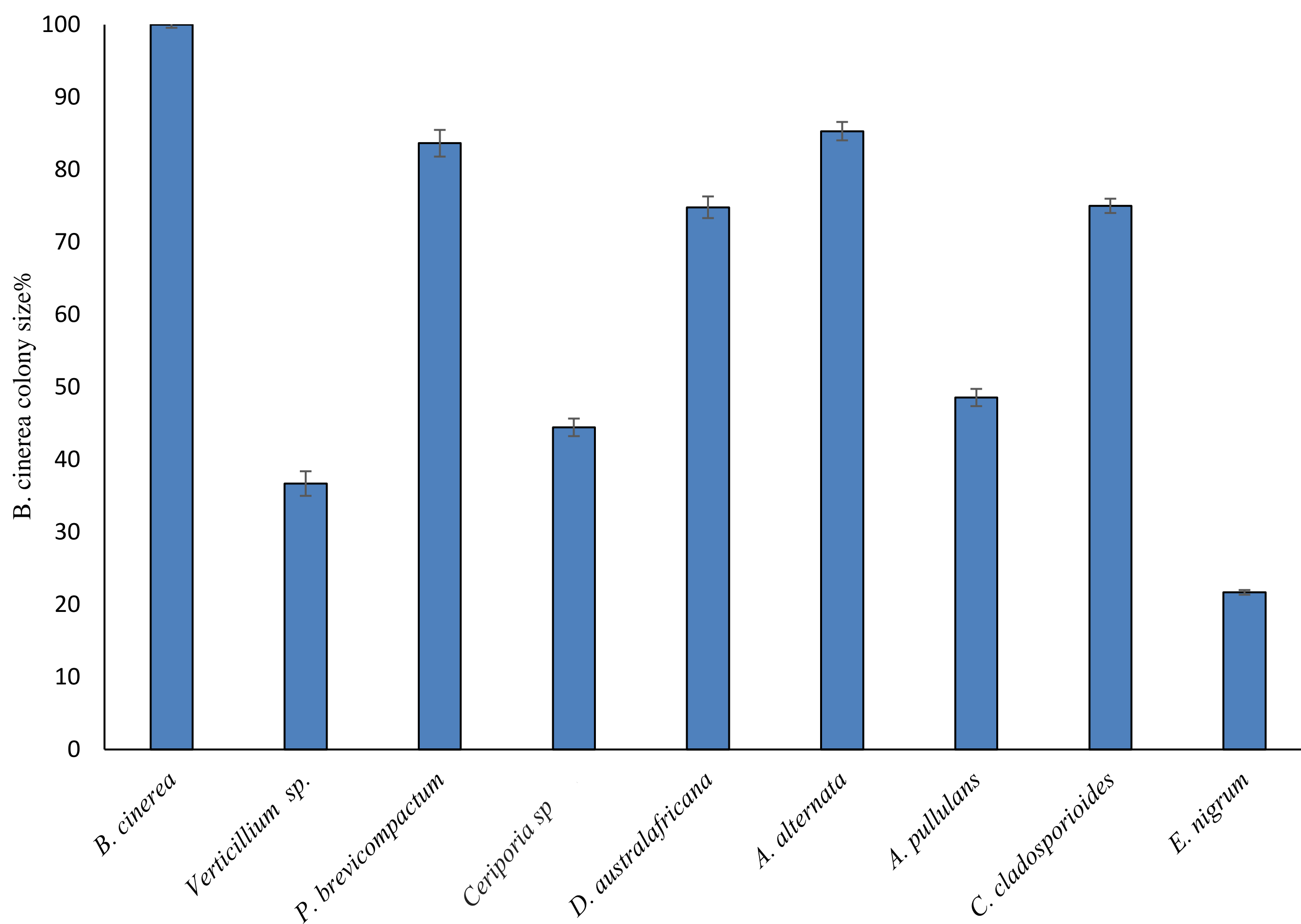

Figure 3. 6. Mean percentage colony growth of *B. cinerea* after exposure to secondary metabolites of eight putative antagonists using the dialysis membrane technique compared to *B. cinerea* colony growth in the absence of secondary metabolites. Bars represent the standard error of the mean.

3.5. Discussion

The inhibition in radial growth of two interacting organisms in dual culture can be attributed to inhibitory substances released by one or both organisms through competition, mechanical obstruction, and hyper parasitism (Dennis and Webster 1971b; Tapwal et al. 2015).

This study shows the *Epicoccum nigrum* isolate as the most promising antagonist to *B. cinerea*, with colony size 38% of the control. These findings are consistent with numerous previous studies showing the ability of *Epicoccum nigrum* to control plant diseases (Kortekamp, 1997; Larena and Melgarejo, 2004; Mari et al., 2007; Bagy et al., 2019). Larena et al. (2005) reported that *Epicoccum nigrum* effectively controlled brown rot on peach fruits in Spain, Italy, and France in 2001 and 2002. This study confirmed that *E. nigrum* inhibits colony growth of *Botrytis cinerea*. Alcock et al. (2015) showed that inhibitory isolates of *E. nigrum* produced epirodin, a polyene antibiotic, and in a diffusible metabolite assay of *E. nigrum*, the *B. cinerea* conidia were germination by up to 94%. In another study, *E. nigrum* strains were evaluated in *vitro* as potential biological agents to control the growth of *B. cinerea*, *Fusarium graminearum, F. avenaceum*, and *F. oxysporum*. The study also showed that *E. nigrum* limited the growth of all isolates of *Fusarium* spp., but not of *B. cinerea* (Ogórek and Plaskowska, 2011). These studies indicate that different isolates of *E. nigrum* do not necessarily control all pathogens they encounter.

The other noticeable result of this study was the zone of inhibition induced by the *Verticillium* sp. against *B. cinerea*, which followed *E. nigrum* ineffectiveness. Other studies have discussed the use of *Verticillium* spp. to control plant diseases. For example, *V. chlamydosporium* has been shown to control nematodes in tomato plants (Dennehy et al., 1993), indicating its intrusiveness, potential to combat, and inhibit the organisms (Dennehy et al., 1993; Kerry, 2011). While many other studies proved that *Verticillium* spp. are pathogens to many plants such as *V. albo-atrum* and *V. dahlia*, which cause vascular wilt diseases in over 200 economically important crops, including dicotyledonous species (Fradin et al., 2009).

This study also showed *Aureobasidium pullulans* inhibited *B. cinerea* growth which agrees with Mari et al. (2012), who found *A. pullulans* to control *B. cinerea* and prevent apple post-harvest decay successfully. The mechanisms of action of *A. pullulans* in a dual culture dish assay could be attributed to the production of chemical compounds generated by the antagonist (Mari et al., 2012).

There are few reports on *Ceriporia* species as plant pathogens or antagonists. Yin and Huang (2018) explored the potential of *Ceriporia lacerata* as a biological control against *Phytophthora capsici* on eggplant. They found that *C. lacerata* inhibited hyphal growth of *P. capsici*, deforming and causing the hyphae to undergo plasmolysis, reducing the incidence of eggplant Phytophthora blight. However, since the *Ceripora* sp. in the present study was inhibitory, it requires further work on its potential as an antagonist, and it needs to be identified to species level.

The present study found that the isolates of *Penicillium brevicompactum* had no antagonistic activity against *B. cinerea.* In *vitro* study showed *Penicillium brevicompactum* was isolated from rot in ginger with an 85% infection rate on the isolated samples (Overy and Frisvad 2005). Also, *Diaporthe australafricana* in the present study showed no antagonistic activity to *B. cinerea*. This is not surprising as Elfar et al., 2013 showed that *Diaporthe australafricana* causes stem canker of blueberry in Chile. Also, *Diaporthe* spp. are plant pathogens causing dieback, blight, wood cankers and fruit rot in a wide range of hosts (Díaz et al., 2017). The present study's results disagree with Köhl et al. (2015), who found that *Cladosporium cladosporioides* can be a biological control agent against plant pathogens, especially against apple scab (*Venturia inaequalis*). However, it is likely that there are differences between isolates, and the isolate used in the present study clearly had no antagonistic ability. Also, *Alternaria alternata* was not inhibitory to *B. cinerea* in the present study. This is not too surprising since *Alternaria alternata* causes many plant diseases such as cherry, *Aloe vera,* and tomato (Wang et al., 2008**;** Ghosh et al., 2016). However, it was included in this study, as it was possible it could interact with *B. cinerea* and hence reduce the impact of the latter as a post-harvest pathogen.

The colony size of *B. cinerea* in the dialysis membrane trial compared to the *B. cinerea* colony size in the control group was adopted to study antagonism. This method helps ascertain if diffusible secondary metabolites are involved in antagonism. *E. nigrum* was the most effective antagonist tested as it limited the growth of *B. cinerea* significantly more than the other seven potential antagonists screened. These results agree with Li et al. (2013), who used the dialysis method to evaluate the potential of *E. nigrum* against *Phytophthora infestans.* The *Ceriporia* sp. was the third most effective fungus in reducing the mycelial growth of *B. cinerea*. However, there are few references about *Ceriporia* in the literature**;** therefore, it is recommended more research should be conducted on the biological control potential of *Ceriporia* sp. This study

also found that *P. brevicompactum, D. australafricana, C. cladosporioides,* and *A. alternata* had no antagonistic ability against *B. cinerea* growth.

3.6. Conclusion

Epicoccum nigrum, the *Verticillium* sp., and the *Ceriporia* sp. inhibited the radial colony growth of *B. cinerea* in *vitro*. The study results reinforced the hypothesis of potential inhibition of *B. cinerea* by potential antagonists, but further studies on more isolates are required to find more effective strains together with *in vivo* studies. The residues of synthetic products such as fungicides on fruit has been and will continue to be one of the main worries of regulatory agencies and consumers. More research is now required concerning the mode of action of the antagonists better to understand the phytopathogen–antagonist–host interactions. In Chapter 4, the putative antagonists' production of nonvolatile organic compounds is examined as a potential mode of action.

Chapter 4

Non-volatile compounds produced by fungal antagonists and *Botrytis cinerea* using direct immersion solid phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS)

4.1. Abstract

This study was performed on *B. cinerea* and eight putative antagonists to diagnose non-volatile chemical compounds as metabolites. Two solvents (ethanol and acetonitrile) with different concentrations and extraction times were used to determine the optimum parameters for the extraction of these compounds. The eight putative antagonists were screened for their ability to produce non-volatile organic compounds in the presence or absence of *B. cinerea* using direct immersion solid-phase microextraction (DI-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) technology. The metabolic analysis by GC-MS confirmed that there were compounds such as Azetidine, 1-nitroso**;** Hexadecanoic acid, Z 11**;** N-Hexadecanoic acid**;** Decanal**;** 2,4-Di-tert-butyl phenol and Hexanedioic acid, bis (2- Ethylhexyl) ester identified from all eight fungi. *E. nigrum, the Verticillium sp. and the Ceriporia sp.* produced more compounds than the other antagonists. Hexanedioic acid, bis (2- Ethylhexyl), N-Hexadecanoic acid and Benzoic acid were produced most abundantly by these fungi. The findings of this study will contribute to understanding the behaviour of antagonists and the control of plant pathogens.

4.2. Introduction

Fungal growth in food and animal feed can lead to considerable spoilage, manifested through nutritional losses, development of mycotoxins and potentially allergenic spores, and unpleasant odours (Filtenborg et al. 1996). Microbial volatile and non-volatile organic compounds appear as middle and end products of several metabolic passageways and belong to many structure types such as ketones, lactones, alcohols, mono- and sesquiterpenes, esters or C8 compounds (Morath et al., 2012; Schnürer et al., 1999). These metabolic volatile and non-volatile organic compounds were shown to be engaged in a different biological procedure like biocontrol or contact between microorganisms and their living environment. Metabolic volatile and non-volatile organic compounds can appear because of the interference between the plant defences and parasites, predators, and diseases and likely produce species competitions (Sivasithamparam and Ghisalberti, 1998).

Biologically, volatile and non-volatile are compounds resulting from the metabolism of organisms such as fungi, bacteria, and insects. The study of these compounds has application in various domains such as in the diagnostics, biology, physiology, and behaviour of different organisms (Chandra, 2019; Wedler, 2017; Riddick, 2020). As a tool, solid phase44 microextraction (SPME) holds promise for the extraction of volatile compounds. It has been demonstrated that SPME has applications in the quantification of different compounds from both solid and liquid matrices in concentrations ranging from low ppt to ppm (Pawliszyn 1997; Jeleń and Wąsowicz, 2000). Locating volatile and non-volatile fungal metabolites usually is done by gas chromatographic (GC) techniques and has been specified for different fungi such as *Fusarium, Phytophthora, Penicillium, Aspergillus* and *Trichoderma*. Following cultivation of the fungi on solid growth medium or in liquid (Pinches and Apps, 2007), non-volatile compounds can be extracted in many different ways, such as with solid-phase extraction or silica gel columns, organic solvents (Keszler et al., 2000; Reithner et al., 2005), or multiple headspace techniques like dynamic headspace, closed-loop stripping examination and solid-phase microextraction. The last technique can be used for the direct and non-invasive extraction of compounds above cultures of fungi. It hence gives the potential of monitoring fungal volatile and non-volatile compound profiles over various growth conditions (Deetae et al., 2007; Meruva et al., 2004; Fiedler et al., 2001; Demyttenaere et al., 2004; Van Lancker et al., 2008).

Direct immersion-solid phase microextraction (DI-SPME) has been employed, then followed by analyses of gas chromatographic-mass spectrometry (GC/MS) for the separation, collection

and identification of compounds (Al-Khshemawee et al. 2018). SPME was used for fast sample preparation and gave an efficient method to detect chemicals in separation and detection systems (Arthur and Pawliszyn, 1990). SPME was used to monitor the production of volatile fungal metabolite by toxigenic strains of *Penicillium roqueforti* and *Fusarium* spp. (Demyttenaere et al., 2002, 2003 and 2004). The development of an analytical technology with powerful quantitative and qualitative capabilities and high specificity is required to study the metabolic of samples. This study investigated the feasibility of using DI-SPME coupled with GC-MS as a high-resolution technology for profiling the metabolism of *B. cinerea* and its antagonists. Specifically, it aimed to identify the non-volatile chemical compounds generated by the antagonists and their interactions on *B. cinerea*.

4.3. Materials and methods

4.3.1. Preparation of antagonist isolates

A total of 190 isolates belonging to nine fungal genera were isolated from different blueberry cultivars from Lot 4 Chitna Rd, Neergabby WA 6503, and 384 Orange Springs Rd, Regans Ford WA 6507 (Chapter 2). The fungi were *Alternaria alternata, Cladosporium cladosporioides, Penicillium brevicompactum, Epicoccum nigrum, Aureobasidium pullulans, Diaporthe australafricana,* a *Verticillium* sp., and a *Ceriporia* sp. These eight fungi and an isolate of *B. cinerea* isolated from post-harvest blueberry were maintained on half-strength potato dextrose agar (1/2 PDA)(9.75 g of Difco PDA and 3.75 g of BBL agar (Becton, Dickenson and Company, Sparks, MD, USA, and 500 ml of distilled water) in a Petri dish and kept in the dark at room temperature (25 ±1°C). These isolates were lodged with the Department of Primary Industries and Regional Development (DPIRD). Sequences were matched using BLAST to known sequences in GenBank. The GenBank accessions were obtained from the NCBI. For long-term storage, the isolates were stored in McCartney bottles (28 ml) (Arekemase et al., 2011).

4.3.2. Apparatus and equipment

GC-MS was used to identify specific compounds produced by *B. cinerea* and the eight antagonists that included *A. alternata*, *C. cladosporioides, P. brevicompactum, E. nigrum, A. pullulans, D. australafricana,* the *Verticillium* sp. and the *Ceriporia* sp. A gas chromatography GC-MS 7890B equipped with a 5977B MSD mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), with an Agilent HP-5MS column (30 m, 0.25 mm, 0.25 μm film thickness) were

used in the experiments. The carrier gas used was helium 99.99% (BOC, Sydney, Australia). The conditions for the operation of the GC-MS were as follows: injector port temperature of 250°C; initial oven temperature of 60°C, which was increased to 250°C (5°C/min); MS Quad 150°C; MS source 230°C; pressure 9.5504 psi. The flow rate was 1.1 mL/min; the splitless model was used. The total running time was set for 48 minutes.

4.3.3. The extraction and analytical procedures

The extraction of chemicals was done with 0.4g of *B. cinerea* and the eight other antagonists as listed above. Mycelial plugs (5 mm diameter) were excised from the five-seven days old cultures of *B. cinerea* using a sterile cork borer and placed on one side of the 1/2 PDA plates (2 cm away from the edge of the plate). In the same plates, perpendicular to the *B. cinerea* inoculum, a 5 mm mycelial disc of an antagonist was placed on the opposite side of the plates. Control plates were maintained for each fungus. The samples were incubated for four days in the dark at 25±1°C. After four days, agar samples were taken from the middle of the interaction area between *B. cinerea* and the antagonist, ensuring no hyphae from either fungus was present. The samples were transferred into 2 mL microtubes containing 1.6 mL of HPLC grade acetonitrile, and then the microtube was sealed with a screw cap. The microtubes were shaken gently by hand for 3 minutes. The samples were homogenised using a Bedbug homogeniser in a 2 mL BeadBug™ microtube containing 1.6 mL HPLC grade acetonitrile for 1 min at 400 rpm and then centrifuged at 8150 x *g* for 3 min using the Dynamic mini centrifuge. The supernatant (1.5 mL) was transferred into a 2 mL amber GC vial with septa.

A solid phase microextraction (SPME) fibre Divinyl benzene/carboxin/polydimethylsiloxane DVB/CAR/PDMS, 50/30 µm (Sigma-Aldrich Australia, catalogue number 57299-U), was used in this study to collect non-volatile organic compounds (Qiu, R et al., 2014). The SPME fibre was inserted into the extract for two h at 25±2°C, immediately after completing the extraction, and the fibre was withdrawn and injected directly into the GC-MS injector for separation of chemicals. Three replicates were used, and the experiment was repeated to confirm the results. Acetonitrile was used as a blank.

4.3.4. Optimisation and validation studies

This study was conducted to optimise the parameters used for the extraction of non-VOCs from *B. cinerea* and the eight antagonists. The SPME was activated manually and calibrated. The optimisation involved testing two different solvents, which were ethanol and acetonitrile at

three concentrations (25%, 50% and 100% v/v), together with four extraction times (one, two, four and eight hours). The optimized parameters were then used for the remainder of the study.

4.3.4. Statistical analysis

The GC–MS signals were collected by the Mass Hunter Acquisition software (Agilent Technologies, Santa Clara, CA, USA). The National Institute of Standards and Technology (NIST) mass spectra library was used to identify chemical compounds. The retention index was used to assist identification. The experiment was repeated three times to confirm the chemicals produced. The area, which represents each peak in the chromatogram, was extracted using Mass Hunter Acquisition software (quantitative analysis) B.06.00 (Agilent Technology, USA). After selecting the compounds, the peak area of each compound was transferred to Microsoft Excel 2016, which was also used for data arrangement and sorting. Differences in chemical compounds between fungi were analysed using ANOVA, followed by t-tests. The asterix (*) in the result tables represents the significant peak area of a compound.

4.4. Results

Three factors were optimised and investigated, including solvent, solvent concentrations and the extraction time. A combination of 50% acetonitrile with a 50% concentration for two hours was optimal, as the numbers and peak area of the extraction compounds were the highest compared to the other combinations used in this study (Figure 4.1 A, B and C).

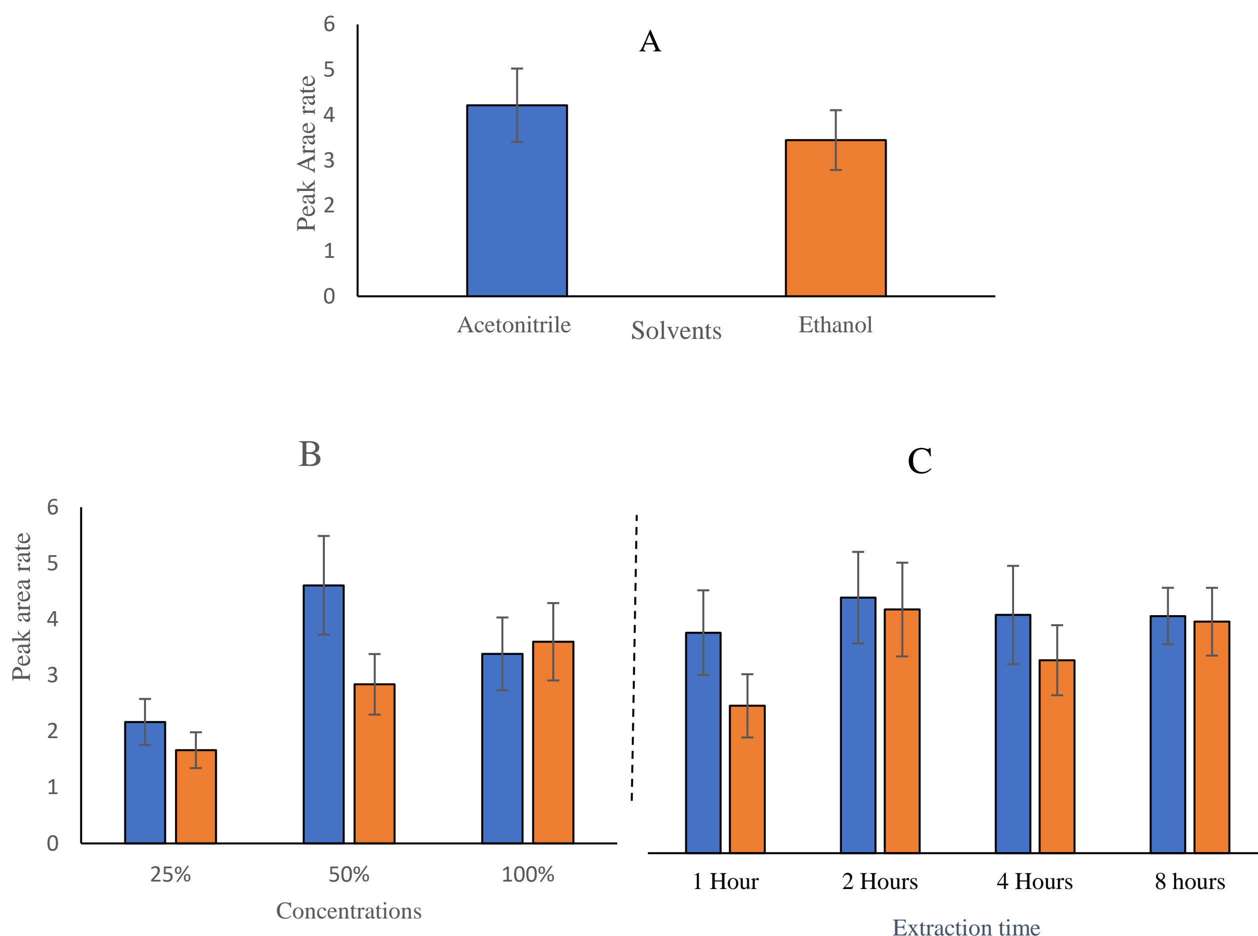

Figure 4. 1. Extraction optimised factors where (A) is between solvents, (B) solvent concentrations, and (C) extraction time. Bars are the standard error, P-value = 0.00.

The peak areas and compounds were used to identify interactions between *B. cinerea* and the antagonists and were used as criteria to compare the results of the metabolic analyses. Generally, the metabolic analyses from *B. cinerea*, the antagonist, and their interactions showed significant differences in the peak areas.

Sixteen compounds were identified in the interaction between *B. cinerea* and *A. alternata.* Eleven of these chemical compounds varied significantly in their peak areas; Hexadecanoic acid, Z 11 had the largest peak area. At the same time, the smallest peak area was for 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (Table 4.1).

For *B. cinerea, A. pullulans* and their interactions, there were ten compounds identified. The interaction between *B. cinerea and A. pullulans* produced six compounds with significant peak areas. (Azetidine, 1-nitroso) gave the most significant peak area, while Cyclopentanemethanol, α-(1-methyl ethyl)-2-nitro had the smallest peak area (Table 4.2).

The interaction between *B. cinerea* and the *Ceriporia* sp. found 15 compounds with significant peak areas from 26 compounds produced. The most prominent peak area was for N-Hexadecanoic acid, and the smallest was for Nonadecane (Table 4.3).

The results of *B. cinerea, C. cladosporioides* and their interactions identified ten compounds, seven of which had significant peak areas (Table 4.4). Decanal had the largest peak area, while Benzofuran-2-one, 4-amino-2,3-dihydro had the smallest chemical compound peak area.

Thirty chemical compounds were identified from *B. cinerea D. australafricana* and their interactions (Table 4.5). The interaction of *B. cinerea* with *D. australafricana* produced 19 compounds with significant peak areas. N-Hexadecanoic acid, Z-11, had the most significant peak area, while 1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl- had the smallest peak area.

Forty chemical compounds were identified from the *B. cinerea* and *E. nigrum* interactions. Nineteen of the forty compounds had significant peak areas (Table 4.6), with Hexanedioic acid, bis (2- Ethylhexyl) ester having the largest peak area, and the smallest peak area was for Pyrano [4,3-b] benzopyran-1,9-dione, 5a-methoxy-9a-methyl-3-(1-propenyl) perhydro (Table 4.6).

The results of the interactions between *B. cinerea* and *P. brevicompactum* were sixteen compounds, of which 12 had significant peak areas (Table 4.7). 2,4-Di-tert-butylphenol had the largest peak area, and Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester had the smallest chemical compound peak area.

Out of the interaction between *B. cinerea* and the *Verticillium* sp., there were ten out of 20 compounds with significant peak areas. The most prominent peak area was for Hexanedioic acid, bis (2- Ethylhexyl) ester, and the smallest was for 1H-Thioxanthene-4-carboxaldehyde, 2,3,5,6,7,8-hexahydro (Table 4.8).

Table 4. 1. Extracted and identified compounds from *Botrytis cinerea*, *Alternaria alternata* and their interactions.

Compound name	RT	RI	*B. cinerea* mean peak area ±SD	*A. alternata* mean peak area ±SD	*B. cinerea & A. alternata*. mean peak area ±SD	Interaction Sig.
2-Furancarboxylic acid, 2-propenyl ester	15.20	1098.00	n. d	n. d	6.51 ± 0.93	*
Decanal	18.81	1204.00	7.14 ± 1.29	n. d	8.17 ± 1.99	
Cyclohexane, isothiocyanato-	19.78	1231.00	15.58 ± 2.83	n. d	19.82 ± 0.14	
Dodecanal	24.48	1409.00	10.89 ± 4.2	n. d	21.92 ± 0.25	*
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	25.61	1474.00	9.9 ± 1.51	n. d	14.59 ± 4.66	
O-Cyanobenzoic acid	26.52	1428.00	n. d	n. d	8.41 ± 0.79	*
2,4-Di-tert-butylphenol	27.12	1519.00	58.38 ± 9.01	140.44 ± 11.98	69.68 ± 8.05	
Benzoic acid, 3-hydroxy-, 2-methylpropyl ester	27.72	1673.00	n. d	7.97 ± 0.52	15.03 ± 0.53	*
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	28.28	1605.00	n. d	n. d	4.21 ± 0.63	*
4-Trifluoromethylbenzoic acid, octyl ester	29.29	1703.00	n. d	12.44 ± 2.84	0	
4-Oxovaleric acid semicarbazone	30.30	1775.00	5.8 ± 0.82	n. d	n. d	
1-(3-(Cyclohexylamino)propyl) guanidine	30.99	1678.00	3.52 ± 0.75	n. d	n. d	
Benzene, 1,3,5-tri-tert-butyl	31.29	1661.00	n. d	8.93 ± 1.57	7.45 ± 1.56	*
Methyl 3,5-tetradecadiynoate	31.75	1714.00	2.78 ± 1.18	n. d	n. d	
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	32.16	1702.00	8.92 ± 1.89	8.18 ± 0.97	n. d	
Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl]-, 4-(1,1,3,3-tetramethylbutyl) phenyl ester	32.36	1781.00	n. d	12.39 ± 3.16	n. d	
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.57	1707.00	2.86 ± 1.21	n. d	n. d	
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	32.74	1710.00	5.870 ± 0.61	n. d	n. d	
Tetradecanoic acid	32.88	1769.00	13.84 ± 2.99	113.30 ± 9.74	10.25 ± 0.27	*
2,4,6-Trimethyl-1,4-dihydropyridine-3,5-dicarbonitrile	33.11	1739.00	n. d	15.41 ± 1.94	n. d	
1-Bromo-11-iodoundecane	33.67	1825.00	5.66 ± 0.78	n. d	n. d	
Pentadecanoic acid	33.83	1848.00	n. d	36.39 ± 1.77	9.12 ± 0.62	*
2-Propenoic acid, tetradecyl ester	34.00	1876.00	n. d	10.68 ± 2.23	n. d	
I-Propyl 13-methyltetradecanoate	34.71	1850.00	5.51 ± 0.50	n. d	n. d	

Hexanedioic acid, mono(2-ethylhexyl) ester	34.98	1872.00	3.30 ± 1.14	n. d	n. d	
9-octadecenoic acid, 2,2,2-trifluoroethyl ester	35.25	2019.00	n. d	133.95 ± 6.85	n. d	
Carbonochloridic acid, 9H-fluoren-9-ylmethyl ester	35.67	1998.00	n. d	n. d	12.84 ± 0.5	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	35.98	1965.00	3.90 ± 0.65	n. d	n. d	
Hexadecenoic acid, Z-11-	36.26	1976.00	8.80 ± 3 .14	n. d	59.7 ± 4.5	*
Isopropyl palmitate	36.43	2013.00	n. d	229.58 ± 9.56	n. d	
Palmitoleic acid	36.76	1936.00	41.03 ± 1.73	n. d	n. d	
N-Hexadecanoic acid	36.88	1968.00	n. d	n. d	15.7 ± 1.5	*
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.08	1924.00	6.37 ± 0.83	n. d	n. d	
Benzaldehyde, 3-[4-(1,1-dimethylethyl) phenoxy]	37.39	2072.00	n. d	17.54 ± 4.03	n. d	
Trimethylsilyl 2-(3a,5-dimethyl-6,8-dioxooctahydro-1H-1,4-methanoinden-1yl) propanoate	37.63	2064.00	n. d	28.65 ± 10.75	n. d	
2-Bromotetradecanoic acid	37.82	2001.00	4.99 ± 0.85	n. d	n. d	
Anisole, 2-sec-butyl-4,6-dinitro	38.08	2008.00	3.44 ± 1.03	n. d	n. d	
Heptadecanoic acid	38.54	2067.00	n. d	11.57 ± 4.21	n. d	
1-Phenyl-3,6-diazahomoadamantan-9-spiro-2-oxirane	38.88	2018.00	n. d	10.27 ± 2.19	n. d	
4-Methoxycarbonylmethylundec-3-enedioic acid, dimethyl ester	39.11	2099.00	n. d	7.93 ± 2.29	n. d	
Linoelaidic acid	40.15	2183.00	n. d	41.27 ± 0.27	n. d	
Oleic Acid	40.27	2134.00	13.86 ± 5.95	n. d	n. d	
2(3H)-Furanone, dihydro-5-tetradecyl	40.44	2178.00	n. d	65.53 ± 6.74	n. d	
Cyclopropaneoctanoic acid, 2-octyl-, methyl ester, trans	40.64	2140.00	n. d	15.33 ± 3.76	n. d	
Hexadecanoic acid, 2-bromo	40.77	2200.00	7.266 ± 2.25	n. d	n. d	
Octadecanoic acid	41.21	2153.00	5.24 ± 1.55	21.19 ± 3.79	n. d	
Glycine, N-(N-glycyl-L-leucyl)	41.81	2287.00	n. d	8.93 ± 2.15	n. d	
Adipic acid, cyclopentylmethyl octyl ester	43.25	2387.00	n. d	n. d	18.51 ± 7.97	*
Adipic acid, 2-ethylhexyl heptyl ester	44.45	2379.00	6.22 ± 2.85	23.32 ± 6.43	n. d	
2-Heptenoic acid, pentadecyl ester	44.96	2382.00	n. d	9.53 ± 1.50	n. d	
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.61	2455.00	1.80 ± 0.79	n. d	n. d	

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 2. Extracted and identified compounds from *Botrytis cinerea*, *Aureobasidium pullulans* and their interactions.

Compound name	RT	RI	*B. cinerea* mean peak area ±SD	*A. pullulans* mean peak area ±SD	*B. cinerea & A. pullulans* mean peak area ±SD	Interaction Sig.
Acetamide	10.29	742	n. d	125.44 ± 0.98	n. d	
1-Pentanol, 2-amino-4-methyl	12.81	975	n. d	n. d	61.75 ± 9.85	*
Azetidine, 1-nitroso-	13.18	927	n. d	n. d	133.17 ± 3.74	*
Methyl isocyanide	14.13	1006	n. d	30.81 ± 2.90	n. d	
1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl	16.50	1164	n. d	9.90 ± 5.03	n. d	
Decanal	18.80	1204	7.14 ± 1.29	12.57 ± 2.26	n. d	
Cyclohexane, isothiocyanato	19.77	1231	15.58 ± 2.83	26.27 ± 5.312	n. d	
Dodecanal	23.48	1409	10.89 ± 4.22	n. d	2.80 ± 0.43	
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	24.21	1474	9.93 ± 1.51	n. d	n. d	
1,8-Naphthyridine, 2,7-dimethyl	25.23	1445	n. d	18.18 ± 13.36	n. d	
O-Cyanobenzoic acid	25.92	1428	n. d	36.34 ± 9.78	n. d	
Cyclopentanemethanol, α-(1-methylethyl)-2-nitro	26.42	1400	n. d	n. d	4.70 ± 0.47	*
2,4-Di-tert-butylphenol	27.11	1519	58.38 ± 9.01	78.20 ± 2.52	21.31 ± 2.87	*
4-Oxovaleric acid semicarbazone	28.29	1775	5.84 ± 0.82	n. d	n. d	
4-Trifluoromethylbenzoic acid, 2-ethylhexyl ester	30.30	1639	n. d	32.92 ± 3.05	3.88 ± 1.03	
1-(3-(Cyclohexylamino)propyl) guanidine	30.98	1678	3.52 ± 0.75	n. d	n. d	
Benzene	31.18	1661	n. d	12.91 ± 3.45	n. d	
Methyl 3,5-tetradecadiynoate	31.54	1714	2.78 ± 1.18	n. d	n. d	
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	32.16	1702	8.92 ± 1.89	n. d	n. d	
1-hydroxy-ethanone	32.27	1707	2.86 ± 1.21	n. d	n. d	
Octasiloxane, 1,1,3,3,5,5,15,15-hexadecamethyl	32.57	1710	5.87 ± 0.61	n. d	n. d	
Tetradecanoic acid	32.81	1769	13.84 ± 2.99	30.98 ± 6.91	10.46 ± 4.49	
1-Bromo-11-iodoundecane	33.27	1825	5.66 ± 0.78	n. d	n. d	
3-(Prop-2-enoyloxy) tetradecane	33.71	1804	n. d	10.42 ± 71.91	n. d	
Pentadecanoic acid	34.32	1869	n. d	n. d	6.19 ± 2.29	*
I-Propyl 13-methyltetradecanoate	34.71	1850	5.51 ± 0.50	n. d	n. d	

Hexanedioic acid, mono(2-ethylhexyl) ester	34.98	1872	3.30 ± 71.14	n. d	n. d	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	35.38	1965	3.90 ± 0.65	n. d	n. d	
Hexadecenoic acid, Z-11	35.85	1976	8.80 ± 3.14	19.46 ± 0.61	17.59 ± 5.64	
Palmitoleic acid	36.46	1936	41.031 ± 1.73	n. d	n. d	
N-Hexadecanoic acid	36.86	1954	n. d	66.69 ± 11.03	n. d	
8a-Hydroxy-1-(2-hydroxyethy tetramethyldecalin(1R,2R,4as,8as)	37.08	1924	6.37 ± 0.83	n. d	55.88 ± 2.55	*
2-B*romotetradecanoic acid	37.62	2001	4.99 ± 0.85	n. d	n. d	
Anisole, 2-sec-butyl-4,6-dinitro	37.98	2008	3.44 ± 1.03	n. d	n. d	
Oleic Acid	40.26	2134	13.86 ± 5.95	n. d	n. d	
2(3H)-Furanone, 5-dodecyldihydro	40.70	2120	n. d	19.79 ± 2.42	n. d	
Hexadecanoic acid, 2-bromo	41.50	2200	7.26 ± 2.25	n. d	n. d	
Octadecanoic acid	42.70	2153	5.24 ± 1.55	n. d	n. d	
Hexanedioic acid, bis(2-ethylhexyl) ester	43.95	2382	n. d	37.83 ± 5.80	n. d	
Adipic acid, 2-Ethylhexyl heptyl ester	44.95	2379	6.22 ± 2.85	n. d	n. d	
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.60	2455	1.80 ± 0.79	n. d	n. d	

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 3. Extracted and identified compounds from *Botrytis cinerea*, *Ceriporia* sp. and their interaction.

Compound name	RT	RI	*B. cinerea* mean peak area ±SD	*Ceriporia.* sp mean peak area ±SD	*B. cinerea* & *Ceriporia* sp. mean peak area ±SD	Interaction Sig.
4-(Methylamino)butyric acid	13.02	1073.00	n. d	11.41 ± 0.67	5.02 ± 2.90	*
2-Furancarboxylic acid, 2-propenyl ester	14.25	1098.00	n. d	n. d	5.37 ± 3.10	*
Nonanal	15.64	1104.00	n. d	n. d	4.96 ± 2.86	*
1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl	16.41	1164.00	n. d	16.39 ± 0.97	n. d	
2-Propyl-tetrahydropyran-3-ol	17.08	1156.00	n. d	n. d	12.13 ±7.00	*
Benzoic acid	17.53	1150.00	n. d	n. d	50.18 ± 2.99	*
Undecanal	18.40	1286.00	n. d	12.88 ± 4.16	n. d	
Decane	18.70	1000.00	n. d	n. d	4.57 ± 2.64	*
Decanal	18.91	1204.00	7.1 ± 1.29	n. d	n. d	
1-Tridecene	19.44	1289.00	n. d	99.83 ± 43.15	n. d	
Cyclohexane, isothiocyanato	20.78	1231.00	15.58 ± 2.83	21.43 ± 2.16	3.41 ± 1.97	
Dodecane	21.50	1214.00	n. d	n. d	5.25 ± 3.03	
N-Decanoic acid	22.18	1372.00	n. d	n. d	5.40 ± 3.11	*
Tridecane	23.20	1313.00	n. d	n. d	7.02 ± 4.05	
Dodecanal	24.48	1409.00	10.89 ± 4.22	14.43 ± 2.85	13.99 ±8.07	
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	25.31	1474.00	9.93 ± 1.51	19.40 ± 2.22	n. d	
Phthalimide	25.93	1450.00	n. d	4.49 ± 1.079	n. d	
Tetradecane	26.70	1413.00	n. d	n. d	15.44 ± 8.91	
2,4-Di-tert-butylphenol	27.12	1519.00	58.38 ± 9.01	54.11 ± 4.80	27.28 ± 5.75	
Dodecanoic acid	28.18	1570.00	n. d	n. d	9.68 ± 5.59	*
Pentadecane	28.86	1512.00	n. d	n. d	13.02 ± 7.51	
4-Oxovaleric acid semicarbazone	29.20	1775.00	5.84 ± 0.82	n. d	n. d	
Isothiazolo[4,5-d] isothiazol-3-one, 4-methoxy-4,5-dihydro	30.31	1629.00	n. d	n. d	8.02 ± 4.63	*
1-(3-(Cyclohexylamino)propyl) guanidine	30.89	1678.00	3.52 ± 0.75	5.20 ± 0.57	n. d	
Benzene, 1,3,5-tri-tert-butyl	31.19	1661.00	n. d	6.54 ± 2.33	n. d	
Methyl 3,5-tetradecadiynoate	31.55	1714.00	2.78 ± 1.18	n. d	n. d	

Compound	RT	RI				Sig
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	32.16	1702.00	8.92 ± 1.89	n. d	n. d	
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.37	1707.00	2.86 ± 1.21	n. d	n. d	
1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyloctasiloxane	32.58	1710.00	5.87 ± 0.61	17.36 ± 1.95	18.30 ± 0.57	*
Tetradecanoic acid	32.72	1769.00	13.84 ± 2.99	7.72 ± 0.90	n. d	
1-Bromo-11-iodoundecane	33.67	1825.00	5.66 ± 0.78	n. d	n. d	
Pentadecanoic acid	33.83	1848.00	n. d	3.17 ± 0.95	n. d	
2-Propenoic acid, tetradecyl ester	34.03	1868.00	n. d	4.17 ± 1.25	n. d	
Cyclododecanecarboxylic acid	34.25	1856.00	n. d	8.08 ± 1.64	n. d	
Octadecane, 6-methyl	34.59	1846.00	n. d	n. d	7.83 ± 4.52	*
Pentadecanoic acid	34.72	1869.00	n. d	n. d	6.42 ± 3.70	*
I-Propyl 13-methyltetradecanoate	34.91	1850.00	5.51 ± 0.50	n. d	0	
Hexanedioic acid, mono(2-ethylhexyl) ester	35.18	1872.00	3.30 ± 1.14	26.22 ± 6.88	4.37 ± 2.52	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	35.48	1965.00	3.90 ± 0.65	n. d	n. d	
4H-1-Benzopyran-4-one, 5-hydroxy-2-methyl-7-(2-propenyloxy)	35.70	1997.00	n. d	n. d	6.00 ± 3.46	*
Hexadecenoic acid, Z-11	36.12	1976.00	8.80 ± 3.14	58.10 ± 8.28	n. d	
Palmitoleic acid	36.46	1936.00	41.03 ± 11.73	n. d	n. d	
I- Propyl 14-methyl-pentadecanoate	36.68	1949.00	n. d	9.38 ± 4.05	12.74 ± 7.35	
N-Hexadecanoic acid	36.88	1968.00	n. d	n. d	73.07 ± 2.19	*
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.08	1924.00	6.37 ± 0.83	n. d	n. d	
Nonadecane	37.52	1910.00	n. d	n. d	2.82 ± 1.63	*
2-Bromotetradecanoic acid	37.72	2001.00	4.99 ± 0.85	n. d	n. d	
Anisole, 2-sec-butyl-4,6-dinitro	37.98	2008.00	3.44 ± 1.03	n. d	n. d	
Oleic Acid	40.27	2134.00	13.86 ± 5.95	13.60 ± 5.94	n. d	
Hexadecanoic acid, 2-bromo	40.50	2200.00	7.26 ± 2.25	n. d	n. d	
Octadecanoic acid	40.90	2167.00	n. d	68.12 ± 55.42	8.93 ± 5.15	
2- Octadecanoic acid	41.61	2153.00	5.24 ± 1.55	12.96 ± 5.00	n. d	
Adipic acid, 2-ethylhexyl heptyl ester	44.95	2379.00	6.22 ± 2.85	n. d	n. d	
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.61	2455.00	1.80 ± 0.79	n. d	n. d	
Tetracosane	47.18	2407.00	n. d	n. d	4.32 ± 2.49	*

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 4. Extracted and identified compounds from *Botrytis cinerea*, *Cladosporium cladosporioides* and their interactions.

Compounds name	RT	RI	*B. cinerea* mean peak area ±SD	*C. cladosporioides* mean peak area ±SD	*B. cinerea & C. cladosporioides* mean peak area ±SD	Interaction Sig.
1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl	16.43	1164	n. d	n. d	6.21 ± 0.73	*
Decanal	18.80	1204	7.14 ± 1.29	n. d	12.47 ± 2.53	*
Cyclohexane, isothiocyanato	19.77	1231	15.58 ± 2.83	n. d	n. d	
Dodecanal	24.48	1409	10.89 ± 4.22	n. d	n. d	
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	25.61	1474	9.93 ± 1.51	n. d	3.80 ± 0.68	*
2,4-Di-tert-butylphenol	27.11	1519	58.38 ± 9.01	59.47 ± 1.94	19.99 ± 2.74	
Benzofuran-2-one, 4-amino-2,3-dihydro	27.51	1584	n. d	n. d	2.83 ± 0.34	*
4-Oxovaleric acid semicarbazone	29.29	1775	5.84 ± 0.82	n. d	n. d	
Isothiazolo[4,5-d] isothiazol-3-one, 4-methoxy-4,5-dihydro	30.30	1629	n. d	n. d	8.04 ± 1.08	*
1-(3-(Cyclohexylamino)propyl) guanidine	30.98	1678	3.52 ± 0.75	n. d	n. d	
Benzene, 1,3,5-tri-tert-butyl	31.18	1661	n. d	n. d	3.35 ± 1.03	*
3-Trifluoroacetoxypentadecane	31.53	1648	n. d	n. d	4.25 ± 1.59	*
Methyl 3,5-tetradecadiynoate	31.84	1714	2.78 ± 1.18	n. d	n. d	
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	32.16	1702	8.92 ± 1.89	n. d	n. d	
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.47	1707	2.86 ± 1.21	n. d	n. d	
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	32.63	1710	5.87 ± 0.61	n. d	n. d	
Tetradecanoic acid	32.71	1769	13.84 ± 2.99	71.57 ± 1.52	9.58 ± 1.94	
1-Bromo-11-iodoundecane	33.37	1825	5.66 ± 0.78	n. d	n. d	
Pentadecanoic acid	33.63	1848	n. d	43.75 ± 5.58	n. d	
I-Propyl 13-methyltetradecanoate	34.81	1850	5.51 ± 0.50	n. d	n. d	
Hexanedioic acid, mono(2-ethylhexyl) ester	35.68	1872	3.30 ± 1.14	n. d	n. d	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	36.28	1965	3.90 ± 0.65	n. d	n. d	
Hexadecenoic acid, Z-11	36.45	1976	8.80 ± 3.14	76.85 ± 5.79	7.80 ± 2.22	
Palmitoleic acid	36.86	1936	41.03 ± 11.73	n. d	n. d	
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.08	1924	6.37 ± 0.83	n. d	n. d	
N-Hexadecanoic acid	37.39	1968	n. d	16.72 ± 0.34	n. d	

2-Bromotetradecanoic acid	37.72	2001	4.99 ± 0.85	n. d	n. d
Hexadecanoic acid, 1,1-dimethylethyl ester	37.95	2092	n. d	13.32 ± 0.36	n. d
Anisole, 2-sec-butyl-4,6-dinitro-	38.55	2008	3.44 ± 1.03	n. d	n. d
Isopropyl palmitate	38.89	2013	n. d	118.51 ± 4.76	n. d
Oleic Acid	40.26	2134	13.86 ± 5.95	n. d	n. d
Hexadecanoic acid, 2-bromo	41.50	2200	7.26 ± 2.25	n. d	n. d
1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	42.73	2203	n. d	60.25 ± 9.09	n. d
Cis-13-Eicosenoic acid	43.22	2374	n. d	43.82 ± 3.94	n. d
Adipic acid, 2- ethylhexyl heptyl ester	44.29	2379	6.22 ± 2.85	n. d	n. d
Hexanedioic acid, bis(2-ethylhexyl) ester	44.99	2414	n. d	13.81 ± 1.11	n. d
2-[5-(2,2-Dimethyl-6-methylene-cyclohexyl)-3-methyl-pent-2-enyl] benzoquinone	45.26	2459	n. d	30.28 ± 5.95	n. d
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.60	2455	1.80 ± 0.79	n. d	n. d

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 5. Extracted and identified compounds from *B. cinerea*, *Diaporthe australafricana* and their interactions.

Compound name	RT	RI	*B. cinerea* mean peak area ±SD	*D. australafricana* mean peak area ±SD	*B. cinerea* & *D. australafricana* mean peak area ±SD	Interaction Sig.
4-(Methylamino)butyric acid	13.89	1073	n. d	13.19 ± 3.22	10.83 ± 1.01	*
Nonanal	15.65	1104	n. d	n. d	7.83 ± 0.45	*
1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl	16.48	1164	n. d	n. d	3.89 ± 0.28	*
Decanal	18.80	1204	7.14 ± 1.29	8.86 ± 1.66	12.13 ± 2.66	
Cyclohexane, isothiocyanato-	19.77	1231	15.58 ± 2.83	8.51 ± 2.29	21.95 ± 1.89	*
Dodecanal	23.40	1409	10.89 ± 4.22	6.26 ± 1.5	n. d	
4H-Pyrrolo 3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	24.61	1474	9.93 ± 1.510	17.54 ± 3.27	23.03 ± 5.39	
O-Cyanobenzoic acid	25.93	1428	n. d	6.46 ± 0.68	6.36 ± 1.45	*
2,4-Di-tert-butylphenol	27.11	1519	58.38 ± 9.01	38.33 ± 3.62	61.17 ± 6.96	
Benzoic acid, 3-hydroxy-, 2-methylpropyl ester	27.81	1515	n. d	10.68 ± 1.98	7.48 ± 1.60	*
Benzophenone	28.15	1603	n. d	n. d	9.15 ± 3.89	
4-Oxovaleric acid semicarbazone	28.89	1775	5.84 ± 0.82	n. d	n. d	
4-Trifluoromethylbenzoic acid, heptyl ester	30.20	1604	n. d	n. d	21.99 ± 3.45	*
2-Benzothiazolamine, 4,6-dimethyl	30.61	1667	n. d	n. d	6.79 ± 1.66	*
1-(3-(Cyclohexylamino) propyl guanidine	30.98	1678	3.52 ± 0.75	6.73 ± 1.25	n. d	
Benzene, 1,3,5-tri-tert-butyl	31.18	1661	n. d	8.68 ± 1.42	12.20 ± 2.76	*
Methyl 3,5-tetradecadiynoate	31.54	1714	2.78 ± 1.18	n. d	n. d	
1,3-Benzenedimethanol, 2-hydroxy-5-methyl	31.75	1726	n. d	n. d	7.46 ± 1.99	*
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2one	31.96	1702	8.92 ± 1.89	14.42 ± 2.71	5.57 ± 1.56	
3,7-Cyclodecadien-1-one, 3,7-dimethyl-10-(1methylethylidene)	32.18	1774	n. d	6.78 ± 1.48	n. d	
1,2-Longidione	32.26	1794	n. d	5.67 ± 0.82	n. d	
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.37	1707	2.86 ± 1.21	n. d	n. d	
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	32.47	1710	5.87 ± 0.61	n. d	n. d	
Tetradecanoic acid	32.61	1769	13.84 ± 2.99	28.85 ± 4.81	43.19 ± 9.48	
Carbamic acid, N-[1-bis(trifluoromethyl)]-, 4-(3-tetramethylbutyl) phenyl ester	32.79	1781	n. d	n. d	8.49 ± 1.82	*
3,4-Tetramethylene-5,5-pentamethyle-2-nepyrazoline	32.93	1773	n. d	n. d	16.35 ± 3.20	*

Cedran-diol, (8S,14)	33.16	1786	n. d	15.57 ± 1.29	n. d	
1-Bromo-11-iodoundecane	33.67	1825	5.66 ± 0.78	n. d	n. d	
Pentadecanoic acid	33.83	1848	n. d	14.30 ± 4.62	23.81 ± 7.86	
Dodecanoic acid, 12-mercapto	34.14	1874	n. d	n. d	7.64 ± 2.13	*
7,11-Hexadecadienal	34.44	1816	n. d	n. d	7.04 ± 2.02	*
I-Propyl 13-methyltetradecanoate	34.61	1850	5.51 ± 0.50	n. d	16.71 ± 5.70	
Hexanedioic acid, mono(2-ethylhexyl) ester	34.98	1872	3.30 ± 1.14	n. d	n. d	
1H- Pyrrolo-3-propanoic acid, 5(4H)-oxo-6,7-dihydro-, methyl ester	35.19	1867	n. d	14.58 ± 3.22	n. d	
2-Oxazolidinone, 3-amino-5-(4-morpholinylmethyl)	35.44	1844	n. d	n. d	20.16 ± 9.33	
4-Phenanthrenol, 1,2,3,4-tetrahydro-4-methyl	36.07	1929	n. d	11.96 ± 1.41	n. d	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	36.28	1965	3.90 ± 0.65	n. d	n. d	
Hexadecenoic acid, Z-11	36.45	1976	8.80 ± 3.14	n. d	56.74 ± 8.04	*
Palmitoleic acid	36.76	1936	41.03 ± 11.73	n. d	n. d	
N- Hexadecanoic acid	36.95	1968	n. d	n. d	24.76 ± 7.60	*
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	37.18	1908	n. d	24.16 ± 2.78	20.11 ± 2.91	*
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.44	1924	6.37 ± 0.83	n. d	n. d	
2-Bromotetradecanoic acid	37.62	2001	4.99 ± 0.85	13.79 ± 2.15	23.58 ± 10.04	
Anisole, 2-sec-butyl-4,6-dinitro-	37.80	2008	3.44 ± 1.03	n. d	n. d	
4-Methoxycarbonylmethylundec-3-enedioic acid, dimethyl ester	37.99	2099	n. d	8.46 ± 0.53	n. d	
Diethylene glycol monolaurate	39.12	2098	n. d	7.61 ± 1.17	n. d	
Oleic Acid	39.76	2134	13.86 ± 5.95	n. d	n. d	
2(3H)-Furanone, dihydro-5-tetradecyl	40.44	2178	n. d	51.94 ± 9.79	n. d	
Linoelaidic acid	40.84	2183	n. d	n. d	85.37 ± 35.84	
Hexadecanoic acid, 2-bromo	41.04	2200	7.26 ± 2.25	n. d	n. d	
Octadecanoic acid	41.60	2153	5.24 ± 1.5	33.16 ± 7.24	26.01 ± 1.34	*
l-Gala-l-ido-octose	41.82	2221	n. d	7.97 ± 0.91	n. d	
N-Chloroacetyl-3,6,9,12-tetraoxapentadec-14-yn-1-amine	42.35	2240	n. d	9.27 ± 1.17	n. d	
Adipic acid, 2-ethylhexyl heptyl ester	43.95	2379	6.22 ± 2.8	n. d	n. d	
2-Heptenoic acid, pentadecyl ester	44.25	2383	n. d	49.98 ± 9.68	26.28 ± 6.94	*
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.60	2455	1.80 ± 0.79	15.90 ± 3.13	n. d	

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 6. Extracted and identified compounds from *Botrytis cinerea*, *Epicoccum nigrum* and their interaction.

Compounds name	RT	RI	*B. cinerea* mean peak area ±SD	*E. nigrum* mean peak area ±SD	*B. cinerea* & *E. nigrum* mean peak area ±SD	Interaction Sig.
Acetic acid, cyano	10.87	921	n. d	713.3 ± 4.60	1.80 ± 0.03	
2-Furancarboxylic acid, 2-propenyl ester	15.19	1098	n. d	986.06 ± 6.65	7.81 ± 5.20	
2-Nonen-1-ol	16.61	1167	n. d	30.64 ± 5.62	8.71 ± 2.30	
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	17.03	1173	n. d	n. d	2.11 ± 0.01	
Benzoic acid	17.66	1150	n. d	160.78 ± 5.70	40.74 ± 1.53	*
Decanal	18.80	1204	7.14 ± 1.29	n. d	5.37 ± 1.29	*
Cyclohexane, isothiocyanato	19.77	1231	15.58 ± 2.83	38.40 ± 10.50	10.54 ± 2.05	
Dodecanal	22.48	1409	10.89 ± 4.22	25.75 ± 6.83	4.04 ± 1.30	
2-Dodecanol, 2-methyl	23.01	1405	n. d	n. d	9.45 ± 7.38	
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	24.61	1474	9.93 ± 1.51	n. d	11.78 ± 3.72	*
O-Cyanobenzoic acid	25.93	1428	n. d	21.05 ± 1.73	n. d	
Phthalic anhydride	26.21	1443	n. d	18.05 ± 43.99	n. d	
2,4-Di-tert-butylphenol	27.11	1519	58.38 ± 9.01	106.46 ± 5.98	48.11 ± 8.48	
Benzoic acid, 3-hydroxy-, 2-methylpropyl ester	28.32	1515	n. d	22.73 ± 6.89	n. d	
Tetradecanal	28.79	1601	n. d	16.53 ± 3.46	n. d	
2-Methyltetradecan-2-ol	29.29	1604	n. d	n. d	3.51 ± 71.76	
4-Trifluoromethylbenzoic acid, 4-octyl ester	29.98	1639	n. d	14.23 ± 2.22	6.78 ± 4.91	*
4-Oxovaleric acid semicarbazone	30.29	1775	5.84 ± 0.82	n. d	n. d	
2-Benzothiazolamine, 4,6-dimethyl	30.60	1667	n. d	14.03 ± 2.21	5.91 ± 1.81	*
1-(3-(Cyclohexylamino)propyl) guanidine	30.88	1678	3.52 ± 0.75	n. d	n. d	
2H-Indeno[1,2-b] furan-2-one, 3,3a,4,5,6,7,8,8b-octahydro-8,8-dimethyl	31.01	1642	n. d	n. d	2.91 ± 0.03	*
Benzene, 1,3,5-tri-tert-butyl	31.47	1661	n. d	13.38 ± 1.49	5.24 ± 1.49	*
Methyl 3,5-tetradecadiynoate	31.85	1714	2.78 ± 1.18	n. d	n. d	
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	32.16	1702	8.92 ± 1.89	n. d	1.53 ± 0.01	
Ppropiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)	32.25	1760	n. d	17.32 ± 3.00	n. d	
Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl]-, 4-(1,1,3,3-tetramethylbutyl) phenyl ester	32.46	1781	n. d	n. d	3.36 ± 1.66	
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.67	1707	2.86 ± 1.21	n. d	n. d	

Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	32.87	1710	5.87 ± 0.61	n. d	n. d	
Tetradecanoic acid	32.91	1769	13.84 ± 2.99	45.11 ± 9.37	23.79 ± 6.92	*
Indantrione, 2-oxime	33.09	1722	n. d	n. d	4.34 ± 1.36	*
Tert-Butyl-2,6-diisopropylphenol	33.23	1724	n. d	n. d	3.38 ± 1.43	*
3,4-Methylenedioxycinnamic acid	33.40	1701	n. d	n. d	3.30 ± 1.55	*
1-Bromo-11-iodoundecane	33.67	1825	5.66 ± 0.78	n. d	n. d	
Pentadecanoic acid	33.83	1848	n. d	18.76 ± 5.15	n. d	
3-Heptadecanol	34.13	1873	n. d	n. d	15.13 ± 3.32	*
5-Hydroxymethyl-1,1,4a-trimethyl-6-methylenedecahydronaphthalen-2-ol	34.44	1868	n. d	n. d	7.61 ± 4.26	
Z-8-Methyl-9-tetradecenoic acid	34.68	1813	n. d	13.98 ± 2.89	n. d	
7,11-Hexadecadienal	34.84	1816	n. d	n. d	8.54 ± 3.77	*
I-Propyl 13-methyltetradecanoate	34.97	1850	5.516 ± 0.50	34.14 ± 11.32	3.66 ± 0.31	
Hexanedioic acid, mono(2-ethylhexyl) ester	35.18	1872	3.30 ± 1.14	n. d	n. d	
Methoprene	35.70	1933	n. d	n. d	17.10 ± 5.81	*
Estra-1,3,5(10)-trien-17β-ol	36.08	1949	n. d	n. d	135.87 ± 8.02	*
Carbonochloridic acid, 9H-fluoren-9-ylmethyl ester	36.27	1998	n. d	20.33 ± 4.91	n. d	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	36.64	1965	3.90 ± 0.65	15.58 ± 3.26	n. d	
Hexadecenoic acid, Z-11	36.79	1976	8.80 ± 3.14	86.51 ± 3.50	n. d	
Palmitoleic acid	36.86	1936	41.03 ± 11.73	n. d	n. d	
N-Hexadecanoic acid	36.98	1969	n. d	155.66 ± 11.31	112.25 ± 4.02	*
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	37.19	1972	n. d	29.65 ± 6.10	n. d	
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.55	1924	6.37 ± 0.83	n. d	n. d	
2-Bromotetradecanoic acid	37.72	2001	4.99 ± 0.85	30.25 ± 9.24	4.54 ± 2.25	
Anisole, 2-sec-butyl-4,6-dinitro	37.98	2008	3.44 ± 1.03	n. d	n. d	
14-Hydroxy-14- methylpentadecanoic acid	38.12	2060	n. d	20.17 ± 4.42	n. d	
N, N-Diethyl-5H-chromeno[4,3-b] pyridin-3-amine	39.11	2057	n. d	22.38 ± 5.47	n. d	
Isopropyl palmitate	39.44	2013	n. d	n. d	3.83 ± 2.26	
Androst-5,7-dien-3-ol-17-one	39.82	2150	n. d	20.15 ± 3.72	n. d	
Oleic Acid	40.26	2134	13.86 ± 5.95	107.17 ± 11.71	41.77 ± 16.45	
Cyclopropaneoctanoic acid, 2-octyl-, methyl ester, trans	40.44	2140	n. d	n. d	9.09 ± 7.63	
Hexadecanoic acid, 2-bromo	40.50	2200	7.26 ± 2.25	n. d	n. d	
Octadecanoic acid	40.70	2153	5.24 ± 1.55	n. d	9.88 ± 3.10	

15-Hexadecenoic acid, 14-hydroxy-15-methyl	41.01	2197	n. d	n. d	3.20 ± 1.96	
2-Nonadecanone, O- methyloxime	42.35	2278	n. d	n. d	5.22 ± 2.41	*
2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl] cyclohexan-1-perhydrol	42.86	2127	n. d	21.55 ± 4.83	n. d	
5-Butyl-5- ethylheptadecane	43.03	2223	n. d	n. d	2.20 ± 0.68	
Pyrano[4,3-b] benzopyran-1,9-dione, 5a-methoxy-9a-methyl-3-(1-propenyl)	43.31	2360	n. d	n. d	1.81 ± 0.04	*
Hexanedioic acid, bis (2- ethylhexyl) ester	44.28	2414	n. d	n. d	200.60 ± 3.75	*
Adipic acid, 2-ethylhexyl heptyl ester	44.95	2379	6.22 ± 2.85	42.376 ± 9.24	n. d	
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.60	2455	1.80 ± 0.79	n. d	n. d	
Isoquinolin-6,7-diol-1-carboxylic acid, N-acetyl-1-methyl	45.93	2447	n. d	n. d	12.35 ± 8.58	

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 7. Extracted and identified compounds from *Botrytis cinerea*, *Penicillium brevicompactum* and their interactions.

Compound name	RT	RI	*B. cinerea* mean peak area ±SD	*Verticillium* sp. mean peak area ±SD	*B. cinerea* & *Verticillium* sp. mean peak area ±SD	Interaction Sig.
2-Nitro-1-propanol	15.65	880.00	n. d	n. d	39.65 ± 10.07	*
1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl	16.41	1164.00	n. d	n. d	5.96 ± 1.56	*
Benzoic acid	17.67	1150.00	n. d	17.12 ± 3.84	0.00	
Decanal	18.81	1204.00	7.14 ± 1.05	10.21 ± 1.68	8.60 ± 1.14	
Cyclohexane, isothiocyanato	19.78	1231.00	15.58 ± 2.31	31.08 ± 2.71	6.11 ± 0.42	*
Dodecanal	23.48	1409.00	10.89 ± 3.44	n. d	n. d	
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	24.61	1474.00	9.93 ± 1.23	n. d	n. d	
2,4-Di-tert-butylphenol	25.12	1519.00	58.38 ± 7.36	9.65 ± 5.09	42.29 ± 7.56	*
Benzoic acid, 3-hydroxy-, 2-methylpropyl ester	26.53	1515.00	n. d	n. d	12.12 ± 0.23	
Pentanoic acid, 2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	27.27	1605.00	n. d	122.50 ± 36.70	3.529 ± 0.06	*
4-Oxovaleric acid semicarbazone	29.30	1775.00	5.84 ± 0.677	n. d	n. d	
1-(3-(Cyclohexylamino)propyl) guanidine	30.79	1678.00	3.52 ± 0.61	n. d	n. d	
Benzene, 1,3,5-tri-tert-butyl	31.29	1661.00	n. d	10.29 ± 0.39	n. d	
Methyl 3,5-tetradecadiynoate	31.65	1714.00	2.78 ± 0.96	n. d	n. d	
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	31.96	1702.00	8.92 ± 1.55	16.99 ± 2.52	n. d	
Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl]-, 4-(1,1,3,3-tetramethylbutyl) phenyl ester	32.28	1781.00	n. d	8.39 ± 1.14	n. d	
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.37	1707.00	2.86 ± 0.99	n. d	n. d	
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	32.58	1710.00	5.87 ± 0.50	n. d	n. d	
Tetradecanoic acid	32.66	1769.00	13.84 ± 2.44	17.02 ± 0.49	n. d	
1-Adamantanecarboxylic acid, 3,5-difluorophenyl ester	32.75	1744.00	n. d	47.00 ± 5.97	n. d	
3-isopropylidene-7,7-ethylenedioxy-decalin	32.92	1734.00	n. d	n. d	7.35 ± 0.81	*
Phenol, 2,6-bis(1,1-dimethylethyl)-4-ethyl	33.15	1767.00	n. d	n. d	6.40 ± 0.65	*
1-Bromo-11-iodoundecane	33.67	1825.00	5.66 ± 0.63	n. d	n. d	
Pentadecanoic acid	33.83	1848.00	n. d	20.29 ± 10.51	6.25 ± 1.16	
I-Propyl 13-methyltetradecanoate	34.61	1850.00	5.516 ± 0.40	n. d	n. d	

Hexanedioic acid, mono(2-ethylhexyl) ester	34.98	1872.00	3.30 ± 0.93	n. d	n. d	
1H-Pyrrolo[2,3-c] pyridine-3-propanoic acid, 5(4H)-oxo-6,7-dihydro-, methyl ester	35.20	1867.00	n. d	n. d	11.08 ± 1.52	*
Methoprene	35.70	1933.00	n. d	n. d	6.5 ± 0.98	*
Estra-1,3,5(10)-trien-17β-ol	35.97	1949.00	n. d	8.08 ± 0.69	n. d	
Carbonochloridic acid, 9H-fluoren-9-ylmethyl ester	36.17	1998.00	n. d	n. d	10.71 ± 3.20	*
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	36.33	1965.00	3.90 ± 0.53	n. d	n. d	
Hexadecenoic acid, Z-11	36.46	1976.00	8.80 ± 2.57	24.67 ± 8.62	51.57 ± 3.40	
N-Hexadecanoic acid	36.75	1968.00	n. d	172.07 ± 9.53	n. d	
Palmitoleic acid	36.91	1936.00	41.03 ± 9.58	n. d	n. d	
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	37.07	1972.00	n. d	n. d	14.85 ± 1.79	*
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.48	1924.00	6.37 ± 0.67	n. d	n. d	
2-Bromotetradecanoic acid	37.62	2001.00	4.99 ± 0.69	n. d	n. d	
1-Methyl-3-phenylsulfanyl-1H-indole	37.78	2056.00	n. d	19.54 ± 8.70	n. d	
Anisole, 2-sec-butyl-4,6-dinitro	37.98	2008.00	3.44 ± 0.84	n. d	n. d	
Oleic Acid	40.27	2134.00	13.86 ± 4.85	n. d	n. d	
Hexadecanoic acid, 2-bromo	40.50	2200.00	7.26 ± 1.83	n. d	n. d	
Octadecanoic acid	42.61	2153.00	5.24 ± 1.26	25.49 ± 5.79	n. d	
Adipic acid, 2-ethylhexyl heptyl ester	44.95	2379.00	6.22 ± 2.33	10.85 ± 2.51	n. d	
Diisooctyl adipate	45.37	2414.00	n. d	20.1 ± 2.65	12.00 ± 2.96	*
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.71	2455.00	1.80 ± 0.64	n. d	n. d	

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

Table 4. 8. Extracted and identified compounds from *Botrytis cinerea*, *Verticillium* sp. and their interactions.

Compound name	RT	RI	*B. cinerea* mean peak area ±SD	*Verticillium* sp. mean peak area ±SD	*B. cinerea* & *Verticillium* sp. mean peak area ±SD	Interaction Sig.
Benzoic acid	17.54	1150	n. d	n. d	6.34 ± 2.37	*
Decanal	18.80	1204	7.14 ± 1.29	5.73 ± 1.48	4.90 ± 1.55	
Cyclohexane, isothiocyanato	19.77	1231	15.58 ± 2.83	4.55 ± 1.24	5.51 ± 1.75	
Dodecanal	24.48	1409	10.89 ± 4.22	n. d	n. d	
2-Dodecanol, 2-methyl	25.00	1405	n. d	n. d	6.53 ± 0.96	*
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	25.61	1474	9.93 ± 1.51	14.29 ± 2.28	n. d	
Oxirane, [[4-(1,1-dimethylethyl) phenoxy] methyl]	27.13	1486	n. d	30.38 ± 3.03	n. d	
2,4-Di-tert-butylphenol	27.11	1519	58.38 ± 9.01	n. d	33.03 ± 6.74	*
4-Oxovaleric acid semicarbazone	30.29	1775	5.84 ± 0.82	n. d	n. d	
4-Trifluoromethylbenzoic acid, 4-octyl ester	30.61	1639	n. d	6.08 ± 1.57	8.07 ± 4.57	
1-(3-(Cyclohexylamino)propyl) guanidine	30.98	1678	3.52 ± 0.75	n. d	n. d	
Benzene, 1,3,5-tri-tert-butyl-	31.18	1661	n. d	1.93 ± 0.31	3.49 ± 0.62	
Methyl 3,5-tetradecadiynoate	31.54	1714	2.78 ± 1.18	n. d	n. d	
4a-Dichloromethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	32.16	1702	8.92 ± 1.89	7.57 ± 1.61	3.10 ± 0.71	
Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl], 4 phenyl ester	32.38	1781	n. d	n. d	3.60 ± 0.73	*
1-[3,3-Dimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclopentyl]-2-hydroxy-ethanone	32.47	1707	2.86 ± 1.21	n. d	n. d	
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	32.57	1710	5.87 ± 0.61	n. d	n. d	
Tetradecanoic acid	32.71	1769	13.84 ± 2.99	22.72 ± 3.64	9.60 ± 1.15	
Trifluoroacetic acid,4-(3-aminobicyclohept-2-yl) phenyl ester	32.92	1795	n. d	8.27 ± 0.97	n. d	
1-(2,6-Dimethyl-4-propoxy-phenyl)-2-methyl-propan-1-one	33.32	1778	n. d	4.41 ± 0.44	n. d	
1-Bromo-11-iodoundecane	33.67	1825	5.66 ± 0.78	n. d	n. d	
Pentadecanoic acid	33.83	1848	n. d	12.24 ± 2.01	3.03 ± 0.60	
I-Propyl 13-methyltetradecanoate	34.41	1850	5.51 ± 0.50	n. d	n. d	
Hexanedioic acid, mono(2-ethylhexyl) ester	34.98	1872	3.30 ± 1.14	n. d	n. d	
Cyclohexanamine, N-(2-methyl-8-oxo-oct-2-en-1-ylidene)	36.28	1965	3.90 ± 0.65	n. d	n. d	

Hexadecenoic acid, Z-11	36.40	1976	8.80 ± 3.14	16.91 ± 3.0	4.45 ± 0.97	
Estra-1,3,5(10)-trien-17β-ol	36.60	1949	n. d	117.91 ± 4.20	n. d	
Palmitoleic acid	36.76	1936	41.03 ± 11.73	n. d	n. d	
N-Hexadecanoic acid	36.99	1968	n. d	n. d	49.33 ± 3.66	*
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	37.16	1972	n. d	n. d	7.40 ± 0.54	*
8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin(1R,2R,4as,8as)	37.38	1924	6.37 ± 0.83	n. d	n. d	
1H-Thioxanthene-4-carboxaldehyde, 2,3,5,6,7,8-hexahydro	37.52	1962	n. d	n. d	1.60 ± 0.25	*
2-Bromotetradecanoic acid	37.72	2001	4.99 ± 0.85	7.57 ± 0.99	n. d	
Anisole, 2-sec-butyl-4,6-dinitro	37.98	2008	3.44 ± 1.03	4.46 ± 0.86	n. d	
Oleic acid	40.22	2134	13.86 ± 5.95	n. d	27.80 ± 3.43	*
Cyclopropaneoctanoic acid, 2-octyl-, methyl ester, trans	40.49	2140	n. d	34.97 ± 2.43	n. d	
Hexadecanoic acid, 2-bromo	40.70	2200	7.26 ± 2.25	n. d	n. d	
Octadecanoic acid	40.90	2153	5.24 ± 1.55	45.21 ± 8.88	8.20 ± 1.97	
2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl] cyclohexan-1-perhydrol	41.04	2127	n. d	10.63 ± 1.95	n. d	
1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	42.34	2203	n. d	11.17 ± 2.04	n. d	
2-Heptenoic acid, pentadecyl ester	43.72	2383	n. d	37.01 ± 3.12	n. d	
Adipic acid, 2-ethylhexyl heptyl ester	44.95	2379	6.22 ± 2.85	16.84 ± 4.38	2.26 ± 0.57	
Hexanedioic acid, bis (2- Ethylhexyl) ester	45.26	2414	n. d	n. d	192.74 ± 3.80	*
1-Propanone, 1-(2,6-dimethyl-4-propoxyphenyl)-3-(1-piperidyl)	45.70	2455	1.80 ± 0.79	n. d	n. d	
1-Phenanthrenecarboxylic acid,7-ethenyl-decahydro-3-hydroxy-4a,7-dimethyl-2-oxo-, methyl ester	46.00	2402	n. d	n. d	4.37 ± 0.80	*

SD is Standard error, and RT is retention time, RI is retention index, * is a significant difference, and n.d is not detected, Sig is significant.

4.5. Discussion

Acetonitrile at 50% concentration for two hours was the best extraction condition. These results agree with many other studies. For example, acetonitrile at 50% concentration for two hours was more economical, shortened the extraction time and had low toxicity compared to ethanol (Sarvin et al., 2018).

The results showed that there were differences between the antagonists in the quality and quantity of the chemical compounds released and their peak area sizes. As there were numerous compounds produced, only those with the largest significant peak areas for each *B. cinerea*-antagonist combination will be discussed. In this study, the headspace-SPME-GC-MS analysis revealed sixteen chemical compounds produced by the interaction between *B. cinerea* and *A. alternata,* and 11 of them had significant peak areas. These included 2-Furancarboxylic acid, 2-propenyl ester**;** Dodecanal**;** O- Cyanobenzoic acid**;** Benzoic acid, 3-hydroxy-, 2-methylpropyl ester**;** Benzene, 1,3,5-tri-tert-butyl**;** Tetradecanoic acid**;** Pentadecanoic acid**;** Hexadecenoic acid, Z-11**;** N-Hexadecanoic acid**;** Adipic acid and cyclopentylmethyl octyl ester. The most significant of the 11 compounds were N-Hexadecanoic, Z-11. Yadav et al. (2020) identified Hexadecanoic acid, Z-11 during a study on the antimicrobial potential of *Alternaria* (GFAV15), which was isolated from the unripe green fruit of *Tinospora cordifolia*, a well-known medicinal plant of the semi-arid region of Rajasthan, India, against two bacterial pathogens, *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative).

There were ten chemical compounds resulting from the interaction between *B. cinerea* and *A. pullulans.* Of these, six had significant peak areas: 1-Pentanol, 2-amino-4-methyl**;** Azetidine nitroso**;** Cycloentanemethanol, α-(1-methylethyl)-2-nitro**;** 2,4-Di-tert-butylphenol**;** 0020 acid and 8a-Hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyldecalin. Cho et al. (2015) identified some of these chemical compounds, such as decanal, Dodecanal and Benzene, when they studied the interaction between *A. pullulans* with *Pichia jadini.* Also, Azetidine nitroso which had the largest peak area in the *B. cinerea* and *A. pullulans* interaction was found to be one of seven unique compounds produced by *Penicillium oxalicum* during the study of Jyoti et al. (2016) on two species of *Penicillium* (*P. oxalicum* and *P. citrinum*).

The interaction between *B. cinerea* and *C. cladosporioides* resulted in ten compounds, seven of which had significant peak areas. These were 1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl**;** decanal**;** 4H-Pyrrolo[3,2,1-ij]quinoline, 1,2,5,6-tetrahydro-4-methyl**;** Benzofuran-2-one, 4-

amino-2,3-dihydro; Isothiazolo[4,5-d]isothiazol-3-one, 4-methoxy-4,5-dihydro; Benzene, 1,3,5-tri-tert-butyl and 3-Trifluoroacetoxypentadecane. Decanal was the most significant of these, and this compound has been isolated from *Trichoderma pseudokoningii* and *Trichoderma viride* by Wheatley et al. (1997), and it is shown as an important determinant involved in the inhibition of the wood decay fungi *Neolentinus lepideus, Postia placenta,* and *Gloeophyllum trabeum*.

Of the 30 chemical compounds produced in the *B. cinerea* and *D. australafricana* interaction, 19 had significant peak areas and included 4-(Methylamino) butyric acid; Nonanal; 1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl; Cyclohexane, isothiocyanato; O-Cyanobenzoic acid; Benzoic acid, 3-hydroxy-, 2-methylpropyl ester; 2-Benzothiazolamine, 4,6-dimethyl; 1-3-Cyclohexylamino propyl guanidine; Benzene, 1,3,5-tri-tert-butyl; 1,3-Benzenedimethanol, 2-hydroxy-5-methyl; Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl], 4-(1,1,3,3-tetramethylbutyl)phenyl ester; 3,4-Tetramethylene-5,5-pentamethyle-2-nepyrazoline; Dodecanoic acid, 12-mercapto; 7,11-Hexadecadienal; Hexadecenoic acid, Z-11; N-Hexadecanoic acid; 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester; Octadecanoic acid and 2-Heptenoic acid, pentadecyl ester. Ferreira et al. (2014) isolated N-Hexadecanoic acid from *Diaporthe* when they characterized the bioactive compounds produced by endophytic fungi of different species belonging to the genera *Penicillium, Diaporthe* and *Hypocrea* associated with *Vellozia gigantea,* an endemic Brazilian plant.

Regarding the interaction between *B. cinerea* and *Ceriporia* sp., a total of 26 chemical compounds were identified. Sixteen of which were significant. These included 4-(Methylamino) butyric acid; 2-Furancarboxylic acid, 2-propenyl ester; Nonanal; 2-Propyl-tetrahydropyran-3-ol; Decane; N-Decanoic acid; Dodecanoic acid; Isothiazolo[4,5-d] isothiazol-3-one, 4-methoxy-4,5-dihydro; 1H,15H-Hexadecamethyloctasiloxane; Octadecane, 6-methyl; Pentadecanoic acid; 4H-1-Benzopyran-4-one, 5-hydroxy-2-methyl-7-(2-propenyloxy); N-Hexadecanoic acid; Nonadecane; Tetracosane and Benzoic acid.

In the interaction between *B. cinerea* and the *Verticillium* sp., a total of 30 compounds were identified, 10 of which had significant peak areas. These included Benzoic acid; 2-Dodecanol, 2-methyl; 2,4-Di-tert-butylphenol; Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl], 4-(1,1,3,3-tetramethylbutyl) phenyl ester; N-Hexadecanoic acid; 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester; 1H-Thioxanthene-4-carboxaldehyde, 2,3,5,6,7,8-hexahydro; Oleic

acid; Hexanedioic acid, bis (2- Ethylhexyl) ester and 1-Phenanthrenecarboxylic acid,7-ethenyl-decahydro-3-hydroxy-4a,7-dimethyl-2-oxo-, methyl ester.

The interaction between *B. cinerea* and *E. nigrum* identified 40 compounds, 19 of which had significant peak areas. These were Benzoic acid; Decanal; 4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl; 4-Trifluoromethylbenzoic acid, 4-octyl ester; 2-Benzothiazolamine, 4,6-dimethyl; 2H-Indeno[1,2-b] furan-2-one, 3,3a,4,5,6,7,8,8b-octahydro-8,8-dimethyl; Benzene, 1,3,5-tri-tert-butyl; Tetradecanoic acid; Indantrione, 2-oxime; Tert-Butyl-2,6-diisopropylphenol; 3,4-Methylenedioxycinnamic acid; 3-Heptadecanol; 7,11-Hexadecadienal; Methoprene; Estra-1,3,5(10)-trien-17β-ol; 2-Nonadecanone, O-methyloxime; N-Hexadecanoic acid; Pyrano[4,3-b] benzopyran-1,9-dione, 5a-methoxy-9a-methyl-3-(1-propenyl) perhydro and Hexanedioic acid, bis(2-ethylhexyl) ester.

Hexanedioic acid, bis(2-ethylhexyl) ester was the most abundant chemical produced by *E. nigrum,* the *Verticillium* sp. and the *Ceriporia* sp. and this result is similar to that of Perveen et al. (2017), who found *E. nigrum* is an important source of bioactive compounds (anticancer and antimicrobial) which included Hexanedioic acid, bis(2-ethylhexyl) ester. Also, the results showed that N-Hexadecanoic acid and Benzoic acid were among the highest peak area compounds that were common in *E. nigrum, Ceriporia* sp. and *Verticillium* sp. (Table 4.9). The results showed that *E. nigrum*, *Verticillium* sp. And *Ceriporia* sp. can be antagonists of *B. cinerea* as they shared some chemical compounds that showed antagonist by other studies such as in *vitro* studies have shown benzoic acid to produce antifungal activities against *Cladosporium cucumerinum* and *Candida albicans* (Lima et al., 2018; Terreaux et al., 1998; López et al., 2002). Several studies confirmed that N-Hexadecanoic acid was found as a metabolite produced by many different fungi such as *Trichoderma harzianum*, *Beauveria bassiana* and *Verticillium* (Soni and Prakash, 2012; Abdullah, 2019). Those studies showed N-Hexadecanoic acid as bioactive components which were potential antifungal to *Phaeosariopsis personata* (Francis et al., 2021; Srivastava and Singh, 2015) and as a crude extract against insect pests such as *Spodoptera littoralis* and *Culex quinquefasciatus* mosquitoes (Soni and Prakash, 2012; Abdullah, 2019).

Sixteen compounds were identified from the interaction between *B. cinerea* and *P. brevicompactum*. Twelve of these had significant peak areas, and included 2-Nitro-1-propanol; 1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl; Cyclohexane, isothiocyanato; 2,4-Di-tert-butylphenol; Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester; 3-

isopropylidene-7,7-ethylenedioxy-decalin**;** Phenol, 2,6-bis(1,1-dimethylethyl)-4-ethyl**;** 1H-Pyrrolo [2,3- c] pyridine-3-propanoic acid, 5(4H)-oxo-6,7-dihydro-, methyl ester**;** Methoprene**;** Carbonochloridic acid, 9H-fluoren-9-ylmethyl ester**;** 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester and Diisooctyl adipate. The present study showed that 2,4-Di-tert-butylphenol (2,4-DTBP) had the largest peak area. 2,4-Di-tert-butylphenol is a typical toxic secondary metabolite produced by many groups of organisms and found in 169 different species, including 11 species belonging to eight families of fungi (Zhao et al., 2020). It is overwhelmingly a major component of essential oils, and it exhibits potent toxicity against almost all organisms tested. 2,4-DTBP is produced by some species of *Penicillium*, *Aspergillus* and *Fusarium*, but experiments showed that it could inhibit the growth of the same fungi (Varsha et al., 2015; Gong et al., 2015). 2,4-Di-tert-butylphenol was also identified from *Penicillium flavigenum* (Zhao et al., 2020). It was found to be effective against the major root-rot fungus *Fusarium oxysporum* by inhibiting hyphal growth and spore germination (Dharni et al., 2014). 2,4-Di-tert-butylphenol also distinctly reduced the mycelial growth of *Phytophthora capsici* when it was tested on seeds of pepper without radicle growth inhibition (Sang and Kim, 2012).

The study focuses on chemical compounds that produced significant peak areas, as this can help in our understanding of fungi with potential antagonistic abilities. The compounds with significant peak areas found in the interactions between *B. cinerea* and the antagonists are shown in Table 4. 9.

Table 4. 9. Presence of significant compounds among interactions of *Botrytis cinerea* and each antagonist.

Compound	*A. alternata*	*Ceriporia* sp.	*E. nigrum*	*C. cladosporioides*	*P. brevicompactum*	*Verticillium* sp.	*D. australafricana*	*A. pullulans*
4-(Methylamino)butyric acid	-	+*	-	-	-	-	+*	-
2-Furancarboxylic acid, 2-propenyl ester	+*	+*	+	-	-	-	-	-
Nonanal	-	+*	-	-	-	-	+*	-
2-Propyl-tetrahydropyran-3-ol	-	+*	-	-	-	-	-	-
Decane	-	+*	-	-	-	-	-	-
N-Decanoic acid	-	+*	-	-	-	-	-	-
Dodecanoic acid	-	+*	-	-	-	-	-	-
Isothiazolo[4,5-d] isothiazol-3-one, 4-methoxy-4,5-dihydro	-	+*	-	+	-	-	-	-
1H,15H-Hexadecamethyloctasiloxane	-	+*	-	-	-	-	-	-
Octadecane, 6-methyl	-	+*	-	-	-	-	-	-
Pentadecanoic acid	+*	+*	+*	-	+	+	+	+*

4H-1-Benzopyran-4-one, 5-hydroxy-2-methyl-7-(2-propenyloxy)	-	+*	-	-	-	-	-	-
N-Hexadecanoic acid	+*	+*	+*	-	-	+*	-	-
Nonadecane	-	+*	-	-	-	-	-	-
Tetracosane	-	+*	-	-	-	-	-	-
Benzoic acid	-	+*	+*	-	+	+*	-	-
Decanal	+	-	+*	+*	+	+	+	-
4H-Pyrrolo[3,2,1-ij] quinoline, 1,2,5,6-tetrahydro-4-methyl	+	-	+*	+	-	-	-	-
4-Trifluoromethylbenzoic acid, 4-octyl ester	-	-	+*	-	-	+	-	-
2-Benzothiazolamine, 4,6-dimethyl	-	-	+*	-	-	-	+*	-
2H-Indeno[1,2-b] furan-2-one, 3,3a,4,5,6,7,8,8b-octahydro-8,8-dimethyl	-	-	+*	-	-	-	-	-
Benzene, 1,3,5-tri-tert-butyl	+*	-	+*	+*	-	+	+*	-
Tetradecanoic acid	+	-	+*	+	-	+	+*	+
Indantrione, 2-oxime	-	-	+*	-	-	-	-	-
Tert-Butyl-2,6-diisopropylphenol	-	-	+*	-	-	-	-	-
3,4-Methylenedioxyci-amic acid	-	-	+*	-	-	-	-	-
3-Heptadecanol	-	-	+*	-	-	-	-	-
7,11-Hexadecadienal	-	-	+*	-	-	-	+*	-
Methoprene	-	-	+*	-	+*	-	-	-
Estra-1,3,5(10)-trien-17β-ol	-	-	+*	-	-	-	-	-
2-Nonadecanone, O- methyloxime	-	-	+*	-	-	-	-	-
Pyrano[4,3-b] benzopyran-1,9-dione, 5a-methoxy-9a-methyl-3-(1-propenyl)	-	-	+*	-	-	-	-	-
Hexanedioic acid, bis(2-ethylhexyl) ester	-	-	+*	-	-	+*	-	-
2-Dodecanol, 2-methyl	-	-	+*	-	-	+*	-	-
2,4-Di-tert-butylphenol	+	+	-	+	+*	+*	+	+*
Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl], 4 phenyl ester	-	-	-	-	-	+*	-	-
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	-	-	-	-	+*	+*	-	-
1H-Thioxanthene-4-carboxaldehyde, 2,3,5,6,7,8-hexahydro	-	-	-	-	-	+*	-	-
Oleic acid	-	-	+	-	-	+*	-	-
1-Phenanthrenecarboxylic acid,7-ethenyl-decahydro-3-hydroxy-4a,7-dimethyl-2-oxo-, methyl ester	-	-	-	-	-	+*	-	-
Dodecanal	+*	+	+	+	-	-	-	-
O-Cyanobenzoic acid	+*	-	-	-	-	-	+*	-
Benzoic acid, 3-hydroxy-, 2-methylpropyl ester	+*	-	-	-	+	-	+*	-
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	+*	-	-	-	-	-	-	-
Hexadecenoic acid, Z-11	+*	-	-	+	+	+	+*	+
Adipic acid, cyclopentylmethyl octyl ester	+*	-	-	-	-	-	-	-
1-Pentanol, 2-amino-4-methyl	-	-	-	-	-	-	-	+*
Azetidine, 1-nitroso	-	-	-	-	-	-	-	+*
Cyclopentanemethanol, α-(1-methylethyl)-2-nitro	-	-	-	-	-	-	-	+*
8a-Hydroxy-1-(2-hydroxyethy tetramethyldecalin(1R,2R,4as,8as)	-	-	-	-	-	-	-	+*
1,3-Cyclohexadiene, 1,2,3,4,5,6-hexamethyl	-	-	-	+*	+*	-	+*	-
Benzofuran-2-one, 4-amino-2,3-dihydro	-	-	-	+*	-	-	+*	-
3-Trifluoroacetoxypentadecane	-	-	-	+*	-	-	-	-
Cyclohexane, isothiocyanato	+	+	+	-	+*	+	+*	-
4-Trifluoromethylbenzoic acid, heptyl ester	-	-	-	-	-	-	+*	-
1,3-Benzenedimethanol, 2-hydroxy-5-methyl	-	-	-	-	-	-	+*	-
Carbamic acid, N-[1-bis(trifluoromethyl)]-, 4-(3-tetramethylbutyl) phenyl ester	-	-	-	-	-	-	+*	-
3,4-Tetramethylene-5,5-pentamethyle-2-nepyrazoline	-	-	-	-	-	-	+*	-
Dodecanoic acid, 12-mercapto	-	-	-	-	-	-	+*	-
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	-	-	-	-	-	-	+*	-
Octadecanoic acid	-	+	+	-	-	+	+*	-
2-Heptenoic acid, pentadecyl ester	-	-	-	-	-	-	+*	-
2-Nitro-1-propanol	-	-	-	-	+*	-	-	-
Pentanoic acid, 2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	-	-	-	-	+*	-	-	-
3-isopropylidene-7,7-ethylenedioxy-decalin	-	-	-	-	+*	-	-	-
Phenol, 2,6-bis(1,1-dimethylethyl)-4-ethyl	-	-	-	-	+*	-	-	-
1H-Pyrrolo[2,3-c] pyridine-3-propanoic acid, 5(4H)-oxo-6,7-dihydro-, methyl ester	-	-	-	-	+*	-	-	-
Carbonochloridic acid, 9H-fluoren-9-ylmethyl ester	+*	-	-	-	+*	-	-	-
Diisooctyl adipate	-	-	-	-	+*	-	-	-

(+*) is significant, (+) is detected, (-) is not detected

4.6. Conclusion

The present study confirmed differences in the in *vitro* non-volatile compounds produced by *A. alternata*, *C. cladosporioides, P. brevicompactum, E. nigrum, A. pullulans, D. australafricana, Verticillium* sp., and *Ceriporia* sp. when grown alongside *B. cinerea*. The results showed that *E. nigrum*, *Verticillium* sp. and the *Ceriporia* sp. could be helpful microorganisms for future research as antagonists of *B. cinerea* as they share some chemical compounds. The different compounds' importance could also be considered as diagnostic or identification tools for potential fungal biocontrol agents; hence, reducing screening times for antagonists. Some of the non-volatile compounds identified in this study have been identified in previous studies with antimicrobial effects for postharvest diseases. Therefore, future research should consider purifying these compounds and examining their effects on different fungal pathogens and the postharvest diseases they cause. The key compounds of interest were Hexanedioic acid, bis(2-ethylhexyl) ester, Benzoic acid, N-Hexadecanoic acid. They were produced by *E. nigrum*, *Verticillium* sp., and *Ceriporia* sp.

5. General Discussion

This study found nine fungal species to be routinely isolated from post-harvest blueberries in Western Australia. These were isolated from C99-42, C00-09, Eureka 1403 and EB 8-46 cultivars from Lot 4 Chitna and 384 Orange Springs farms over two seasons (August 2016 and August 2017). Five of the nine species, *Botrytis cinerea, Alternaria alternata, Cladosporium cladosporioides, Aureobasidium pullulans* and *Diaporthe australafricana,* were able to cause post-harvest disease in blueberry fruit. With *B. cinerea* being the most significant of these, with a 93.75 % infection rate for all blueberry cultivars. The remaining fungi *Alternaria alternata, Cladosporium cladosporioides, Aureobasidium pullulans* and *Diaporthe australafricana* were ordered descending. Of these three, *Epicoccum nigrum*, *Verticillium* sp., and *Ceriporia* sp. had strong potential as biological control agents of *B. cinerea.*

Among plant pathogens, *Botrytis cinerea* is a model organism because of its degrading potential and the enormous economic losses caused by the diseases it causes (Innovation, 2018), especially post-harvest products. Consequently, understanding the biological antagonists of *B. cinerea* led to testing the isolated fungi from blueberry fruit as potential antagonists of this pathogen. Two methods (antagonism on agar plates, antagonism by dialysis membrane diffusion method) were used to evaluate *Alternaria alternata*, *Cladosporium cladosporioides*, *Penicillium brevicompactum*, *Epicoccum nigrum*, *Aureobasidium pullulans*, *Diaporthe australafricana*, *Verticillium* sp., and *Ceriporia* sp. as potential antagonists of *B. cinerea*. Both methods confirmed that *E. nigrum* and the *Ceriporia* sp. were the most promising antagonists of *B. cinerea*. In both methods, the growth of *B. cinerea* was inhibited by *E. nigrum,* at 38% and 21.67%, while for the *Ceriporia* sp. at 39% and 44.44%, for antagonism on agar plates and antagonism by diffusible metabolites (dialysis membrane method), respectively.

The current study focused on analyzing the metabolites produced by the antagonists using the GC-MS technique to understand the mechanism(s) that inhibit the growth of *B. cinerea*. In the current study, the direct immersion solid-phase microextraction (DI-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) was used because it accurately detects more compounds and with less contamination compared with using similar methods to detect volatile organic compounds (Al-Khshemawee et al., 2018). The current study showed that there were non-volatile compounds identified from the antagonists against *B. cinerea*. Hexanedioic acid, bis (2- Ethylhexyl)**;** N-Hexadecanoic acid and Benzoic acid produced the highest peak areas in the interactions between *B. cinerea* and *E. nigrum, Ceriporia* sp. and the *Verticillium* sp. The

results showed that *E. nigrum*, *Ceriporia* sp. and *Verticillium* sp. were potential antagonists to *B. cinerea*, and this could be due to these three compounds inhibiting its growth (Ferreira et al., 2014). Several studies have confirmed the antifungal activities for these three compounds (Lima et al., 2018; Terreaux et al., 1998; López et al., 2002; Soni and Prakash, 2012; Abdullah, 2019; Francis et al., 2021; Srivastava and Singh, 2015).

The future success of plant products management will be driven by the need to deliver high-quality fresh products consistently and cost-effectively to the consumer. Further investigation into the potential of *E. nigrum*, *Ceriporia* sp. and *Verticillium* sp. to be used as biological control agents of *B. cinerea* under controlled in vivo and then commercial conditions is now required. It is also necessary to conduct molecular work to determine the species for the *Ceriporia* and *Verticillium* isolates; if new to science, they will need to be described.

Future research could include:

- More surveys across different regions, seasons, and cultivars to confirm the findings of the current study. Such surveys will also provide the opportunity to find additional fungi for screening as antagonists.
- Elucidate the different volatiles compounds and the mechanisms/modes of action.
- Inoculating the chemical compounds of each antagonist, particularly from *E. nigrum*, *Ceriporia* sp. and *Verticillium* sp., onto blueberry fruit together with *B. cinerea* to determine if they do control the pathogen.
- Test the potential antagonists against more isolates of *B. cinerea* from blueberry and other hosts.
- If they do control the pathogen, then analyse the volatile and non-volatile compounds produced to confirm or not the role of the non-volatile compounds observed in *vitro* with the interactions of the antagonist and *B. cinerea* on agar.
- Confirm whether the antagonists have any detrimental impact on the post-harvest blueberry fruit. For example, on fruit quality, hardiness, acidity, sugar, and shelf life.
- Determine if they can be used whilst blueberries are still on the plants to reduce post-harvest losses.
- Test the non-volatile chemicals on human health.

- Exploring Hexanedioic acid, bis (2- Ethylhexyl)**;** N-Hexadecanoic acid, and Benzoic acid more as chemical compounds and test them individually, in combinations, and at different concentrations against *B. cinerea*.
- This study's procedures and its results can be applied to other post-harvest pathogens.

The study confirmed that *B. cinerea* is the most dominate blueberry pathogen post-harvest in Western Australia. In addition, another eight fungal genera were isolated consisting of post-harvest pathogens and possible endophytes and/or antagonists to *B. cinerea.* The fungi isolated did vary across the four blueberry cultivars. An important result of this study was the potential of *E. nigrum*, *Ceriporia* sp. and *Verticillium* sp. as antagonists of *B. cinerea*. Further in vivo work is now required to demonstrate their potential to reduce the impact of *B. cinerea* on blueberries post-harvest. If effective, they could be developed into beneficial biological agents against *B. cinerea* on blueberry and possibly other crops, reducing the problems associated with chemical residues and environmental pollution caused by fungicides.

Providing data on the chemical profile of the fungi studies was novel and offered a good attempt to understand the modes of action of the potential antagonists against *B. cinerea*. The study provides knowledge for better management of diseases associated with blueberry.

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