Epidemiology and community analysis of stem end rot-associated fungal pathogens and their impact on postharvest disease of commercial avocado (*Persea americana*)

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

Consumer demand for high quality avocados is increasing, owing to the fruit's versatility and high nutritional value. However, avocados suffer from a variety of fungal diseases responsible for decay and loss of yield. A necrotising condition known as Stem End Rot (SER) infects the fruit before ripening and spreads from the stem attachment point down into the mesocarp tissues following exogenous ethylene exposure or during endogenous developmental ripening signals. The fungal pathogens can be partially mitigated with modern fungicides, but the high proportion of fruit that are still failing to meet postharvest quality standards indicates that preharvest fungicide application is an insufficient strategy to prevent crop loss and waste.

Much of the current research is focused on fungal communities in the field, and little is known about pathogen species after the fruit enter the supply chain downstream of the orchard. To investigate the impact of SER associated pathogens at the end of the supply chain, fungal pathogens were cultured from the stem-end of avocados collected from the Greencell distribution centre in Spalding, UK. Fruit physiological characteristics, disease symptoms, and orchard location data were paired with fungal pathogens cultured from each avocado. Factors such as rainfall, temperature, and wind are known to affect which pathogen species thrive in the orchard environment. Koch analysis principles coupled with ITS sequencing were used to establish causation of SER by specific fungal species. A key question was whether the fungal species present from fruit obtained from a specific orchard varied between location and climate type. Climactic data was paired with historical records of disease incidence for selected orchards managed by the Greencell distribution centre. By correlating the identity of the isolated SER causal species with the known abiotic factors and orchard data, this research contributes to a better understanding of pathogen ecology. Whole genome

sequencing of identical morphotypes obtained from different countries of origin provided an insight into the genetic variation present within SER-causing species and illustrated that the country of origin for avocados can harbour multiple strains of the same fungal species. These data lay the foundation for further study of SER causal species ecology and the development of targeted mitigation strategies.

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Abbreviations

B - Bruised avocado mesocarp (also refers to any non-fungal internal browning)

BR - Body Rot

CCA - Canonical Correspondence Analysis

FB - Fungal Browning

GPD - glyceraldehyde-3-phosphate dehydrogenase

ITS - Internal Transcribed Spacer (region of ribosomal RNA)

LRA - Linear Regression Analysis

M - Mycelial cavity

MEA - Malt Extract Agar

MBD - MuddyBoots Dataset

PCA - Principal Component Analysis

PDA - Potato Dextrose Agar

SCD - Supply Chain Dataset

SER - Stem End Rot

VD - Vascular Discolouration

CHAPTER 1: INTRODUCTION

1.1 Avocado Botany and Cultivation

Avocados (*Persea americana*) have increased in popularity in many European countries over the past few decades (Bhuyan et al., 2019). The fruit, once an ancient New World novelty, has now become a staple component of the modern diet. Its reputation as one of the world's most nutritious fruits is also partly responsible to the rise in demand. This is due to its high concentration of healthy oils, protein, and vitamins (Schaffer et al., 2013). Although they are a lucrative crop, avocados suffer from a wide range of fungal disorders in the field and postharvest. This thesis investigated the fungal communities and fruit physiological factors responsible for the postharvest rot disorder Stem End Rot (SER). To understand the context of this disorder, the literature review will first explore the botany and physiology of the avocado tree and avocado breeding. Next, the review presents previously established fungal pathogens, pathogen infection biology, historical measures taken to control infection, and the fruit physiology factors which affect susceptibility. Lastly, the fungal disease ecology and disease risk along the supply chain will be explored. As this research project focused on the fungal communities at the end of the supply chain, the unique opportunities and relevant research questions pertaining to this area of inquiry are discussed in the final introduction section.

To understand why avocados are particularly susceptible to postharvest rots, one must consider their evolutionary history. Gaillard & Godefroy (1995) note that avocados are native to the subtropical climate of Guatemala and southern Mexico, the avocado tree stands at about 10 to 15 meters tall and, whilst yield varies depending on species and area, it can produce up to 120 kg of fruit per tree per growing season (Gaillard & Godefroy, 1995). The avocado tree belongs to an ancient lineage known as

basal angiosperms. Most present-day flowering plants differentiated from the basal angiosperms sometime around the early Cretaceous period. They retain many primitive characteristics in their flower morphology. The sepals and petals are similar in appearance, and the floral parts generally remain unfused. Within this ancient group, avocados are a member of the family Lauraceae, characterised by their thick leathery evergreen leaves (Bergh, 1992; Feild & Arens, 2007).

Classification of *Persea* species varies between taxonomic treatment. Most treatments recognize three horticultural landraces of avocado, based on geographically distinct domestication events. These three races are the Mexican, the Guatemalan, and the West Indian avocado. Each race is adapted to a different climate and exhibits unique fruit characteristics. Mexican avocados thrive in a Mediterranean climate and exhibit good cold tolerance, Guatemalan avocados are fairly cold-tolerant, and Indian avocados prefer humid tropical growing conditions. The commercial avocados most prominently cultivated are of the Guatemalan or Mexican landraces, from which breeders have developed hundreds of cultivars. (Bergh, 1992; Schaffer et al., 2013).

Commercial avocado trees are always propagated by grafting a branch from an established variety onto a disease-resistant rootstock. Grafting ensures that the final product retains the characteristics of the commercial variety, as avocados grown from seed are highly variable in the quality of fruit produced. The rootstock is often obtained from seed, despite this genetic variability, with most countries simply screening specimens susceptible to fungal infection out of the process. The selection of good rootstock in avocado propagation is critical in achieving a uniformity in orchard behaviour and productivity (Castro et al., 2003). Once established, grafted trees are planted in orchards and pruned in accordance with the grower's preferred management strategy. Trees can be pruned shorter to promote airflow through the canopy, and aid in

fruit harvest, but this may yield fewer fruit overall. Trees left to grow tall and sprawling experience higher disease incidence due to the higher humidity levels retained in the canopy, but they also yield more fruit in total (Castro et al., 2003; Schaffer et al., 2013)

In their 2013 foundation text, Schaffer et al. tell us that the avocado tree can reach hights up to 30 meters in its original rainforest environment, but a grafted orchard tree does not usually achieve this level of growth and is often pruned much shorter. The authors go on to explain that in the wild, avocado trees progress through juvenile and adult life phases. The juvenile phase is strictly vegetative and can last up to 15 years depending on the species of avocado. Once the tree has developed to the point where it can respond to flowering cues, it begins the reproductive phase of its life cycle. Schaffer et al. emphasise that the exact nature of these cues remains a mystery, but temperature and sunlight exposure may play a role.

Avocado flowers have developed a unique pollination strategy. Flowers are perfect, with male and female parts fully functional in a single blossom. To prevent cross pollination, each tree is a member of either the Type A or Type B mating group. These are distinguished by the time of day in which the reproductive parts are exposed. Type A trees expose the stigma for fertilisation in the morning one day and close it by noon. On the afternoon of the second day, they expose the stamens and release pollen. Type B trees expose their female parts in the afternoon of the first day and the male parts on the morning of the second day (Figure 1.1). This ensures that reproductive parts between trees of opposite mating types are coordinated to each other in the wild, but a single domesticated cultivar of avocado produces only one type of flower. It was once believed that Type A plants could only interbreed with Type B plants, but subsequent research proved the ability of avocado trees to self-pollinate. However,

another study suggested that self-pollinated fruit often drop from the tree at an early stage of development, resulting in a reduced yield. The timing of flower opening can also vary in response to temperature, shifting the overlap of Type A and Type B trees. Pollination is mainly insect-mediated, and bees are a common avocado pollinator species (Sedgley & Annells, 1981).

Figure 1.1: Anatomy of the avocado flower. Type A trees expose the female reproductive structures (1) in the morning on one day and the male reproductive structures (2) on the following afternoon, whilst type B trees display the opposite pattern. Image: https://lsuagcenter.com/profiles/cdunaway/articles/page1529338129218

Avocado trees produce millions of flowers but only a small percentage of these develop into fruit. The fact that most flowers will drop makes controlled crossing by hand a nonviable breeding strategy. Both flower development and pollination are sensitive to temperature, with pollen tube growth inhibited at temperatures above 33 °C during the day and 28 °C at night (Sedgley & Annells, 1981). Avocados also experience a high percentage of fruit drop in the early stages of fruit set, usually within one week of fruit development. This is thought to be a naturally occurring process of a tree which has set more fruit than it can support.

Even in well-managed orchards, less than 0.1% of avocado flowers and developing fruit progress to the harvest stage (Papademetriou, 1975). Dropping large numbers of developing fruit to better support the remaining yield is a common strategy for both forest and fruit crop trees, however, the volume of avocado fruit drop is above average (Bangerth, 2000). Also, an above average yield one year may impact the tree's ability to set an equally high yield the next year, which is one proposed explanation for the alternate bearing patterns observed in avocado orchards. The most common factors impacting avocado yield variability are climatic events. Often if fruit trees are exposed to freezing temperatures, excessive heat, or low rainfall during flowering or fruit set, the subsequent harvest will be negatively impacted (Jonkers, 1979).

The first six months of fruit development is distinguished by rapid cell division. The rate of cell division varies between cultivars and is responsible for the diversity in shapes and sizes between varieties. This cell division continues throughout avocado development, but the rapid growth at the early stage has been attributed to seed development (Piper & Gardner, 1943). In the next stage of avocado growth, the developing mesocarp tissue collects carbohydrates and proteins. Nutrient transport is critical for both fruit and seed growth, with calcium being particularly important during

development due to its function in cell wall structure (Schaffer et al., 2013).

One of the key characteristics of avocado maturation is the shift in percentage of water and oil content. As the fruit develops, its water content decreases and oil content increases. Many avocado varieties produce a slow growing crop, and the Hass cultivar can be left to 'hang' on the tree for up to 19 months in some cooler growing climates. Avocados do not fully ripen until they are detached or girdled from the tree. The transport of seven carbon sugars and growth regulatory hormones from branch to fruit inhibit avocado development until it is severed from the tree (Cowan, 2004; Robinson et al., 2002; Tingwa & Young, 1975).

1.2 Breeding, Production, and Distribution

Global avocado production has been on the rise since the 1990s, and total global production for the year 2020 was estimated to be around 8.06 million metric tonnes (Shahbandeh, 2022). The world's leading avocado grower is Mexico. This country has been the most dominant avocado growing region worldwide, and in 2020, Mexico produced approximately 4.5 million tonnes of avocado. The other five most prominent avocado producers are the Dominican Republic, Peru, Indonesia, Colombia, and Brazil (Sommaruga & Eldridge, 2021). European production, dominated by Spain, France, and Greece, is expanding, but as the time of this study, it is not able to meet demand. To compensate, Europe imports fruit mainly from Peru, South Africa, Israel, Chile, and Brazil (Grote & Sartorius, 1994; Jacques, 2017).

South Africa is a major avocado production region in terms of global export, generating a five-year average of over 125,600 tons of fruit for commercial distribution, mainly to Europe (Donkin, 2018). The avocado industry therefore contributes a substantial portion toward South Africa's overall export revenue, comprising 21% of its total gross value for subtropical fruits ($0.05 billion) in 2010-2011 (Invest Lebanon,

2016). More recent South African production ranges between 80,000 and 120,000 metric tonnes, and it exported 58,000 tonnes during the 2019 growing season (Louw, 2022). Israel, another major avocado producer, is one of the leading exporters to Europe. As of 2020, Israel produced 187,430 tonnes of fruit, and often up to 70% of this is allocated for export. Israel's key market is the UK, France, and Germany, but it is also expanding into Russia (Dor, 2022; Shahbandeh, 2022).

Once cleaned and sorted in the packing house, fresh unripe avocados are cooled to 8 – 12 ℃. Although they are still firm, the avocados are sensitive to injury and must be packed in specialised crates. Avocados require a controlled atmosphere, with 90% humidity and ventilation within the range of 60 – 80 circulations per hour. Good ventilation prevents the build-up of CO_2 and ethylene, which can cause premature ripening or spoilage. Unripe avocados have a shelf life of approximately 28 days, but most exports to Europe are transported in under seven days via airfreight (Kupfer, 2022). Travel by air is the preferred method to deliver fruit in the freshest condition, but avocados are increasingly being shipped by sea in an effort to reduce the product's carbon footprint (Shorter, 2015).

The Hass and Fuerte cultivars are the two most dominant avocado varieties grown worldwide, with Hass overtaking Fuerte as the industry standard during the 1980s. Discovered by happy accident, the original Hass avocado tree grew form a seed purchased by Rudolph Hass onto which he intended to graft the then popular Fuerte. When the grafts all failed, his family discovered that the dark, pebbly-skinned fruit from this tree possessed a distinctive flavour. Rudolph patented the variety in 1935 and its popularity grew steadily throughout the twentieth century. All Hass avocados originated from this one unusual tree.

Although the Hass variety dominates the current avocado market, the older

classic Fuerte is still widely distributed. With its nutty flavour profile and high oil content, Hass is favoured in savoury dishes in the West, whereas the sweeter, creamier consistency of Fuerte is preferred for Asian dessert dishes. Alongside the two most prominent varieties of *P. americana*, ongoing development of speciality cultivars improve upon the most favoured traits of already established varieties. One such gourmet avocado is the Gem variety, a cultivar patented by the California Avocado Breeding Program. Developed from the Gwen variety, Gem avocados improve upon the unpredictable alternate bearing patterns observed with Hass trees whilst also featuring the rich, nutty flavour prized in Hass (Alder, 2021). In accordance with public demand for rich, savoury avocados the Lamb Hass, a Hass x Gwen hybrid, was bred to produce larger fruit with a flavour and texture almost identical to Hass (Beesman, 2022). Alongside these high-oil cultivars, green-skinned avocados are bred as exotic specialty varieties. Most green-skinned fruit originate from the West-Indian or Guatemalan landraces and feature diverse eating characteristics. Gwen avocados can be sweet and nutty, while Pinkerton are creamy and easy to peel ("Speciality Produce", 1996).

The UK avocado market is dominated by Hass and the green-skinned Fuerte. A world-wide supply chain ensures that these two varieties are available all year, although originating from different countries of origin depending on season (Jacques, 2017). The Greencell distribution centre in Spalding, UK may also circulate a third variety, depending on seasonality and availability; often this is either Gem or Lamb Hass. All avocados used in this doctoral study are of the Hass variety.

1.3 The Main Issue: Avocado Fungal Pathogens

Once seen as a 'high-risk' crop, avocados are still susceptible to a number of fungal pathogens. With improvements in fungicide technology and increased study of

plant disease, the fruits have not only become more feasible, but have provided a sustainable livelihood for those who grow them (Schaffer et al., 2013). The majority of plant infections are caused by fungal pathogens, rather than other biotic entities such as viruses and bacteria (Schumann & D'Arcy, 2010). These pathogens are responsible for devastating yield loss and subsequent food waste, the latter being a significant contribution to rising greenhouse gas levels in the atmosphere.

The exact loss varies between season and growing region, but the two major postharvest disorders Anthracnose and Stem End Rot combined have been shown to cause 10 – 25% yield loss for Hass avocados. Ramirez-Gil et al. (2017) tell us that phytophthora root rot, the most destructive preharvest disorder affecting the avocado industry, can cause up to 50% tree mortality. Other postharvest disorders include improper ripening, skin defect, chilling injury, bruising, and dehydration, but their incidence in Hass usually falls under 7% of total harvest (Ramírez-Gil et al., 2021). The decay of organic waste in municipal landfills releases biogas, which contains an estimated 50% methane. This greenhouse gas is a major component in Anthropocene climate change (Ayalon et al., 2001). A reduction in the amount of fruit wasted in the orchard, declined for import, or rejected by the consumer would therefore support current climate change mitigation strategies. This type of fruit waste is a major issue which the avocado industry is working to alleviate. It is hypothesised that achieving this goal necessitates an understanding of the pathogens.

Considering the fungal diseases plaguing avocados, *Phytophthora cinnamomi* root rot is the most devastating disease for growers overall, but fungal diseases of the fruit have the greatest influence on international trade, where fruit quality assessment effects import rates (Schaffer et al., 2013; Zentmyer, 1984). Two of these, Anthracnose body rot and Stem End Rot (SER), are responsible for the majority of losses due to

postharvest fungal disease, with the latter causing more loss for European avocado distribution. These diseases are a challenge to mitigate, as fungicides applied after harvest are not able to target beneath the avocado skin without leaving a residue. These fungicides are applied via an antimicrobial bath called a 'fungal dip'. The fruit are washed in the water and evenly coated with fungicide, but the chemicals leave a white residue once dry (Gaillard & Godefroy, 1995).

The term 'SER' (SER) was first used by Zentmyer in a 1953 paper exploring other fungal diseases of avocados, but its causal agents had already been recognised and described as early as 1934 (Darvas, 1982). As its name implies, the symptoms of SER begin at the stem end and gradually necrotise mesocarpal tissues as the fungal front moves downward toward the blossom end, with a clearly defined margin. Darkened vascular tissue is a common symptom of severe infections, and the vascular tissue, specifically the xylem elements (Hartill & Everett, 2002), is frequently attacked before the fungal margin reaches farther down the pulp. This is likely due to the ease of movement the long thin fugal hyphae would encounter through the hollow xylem vessels. Cottony-white hyphae are often present within mesocarpal cavities in advanced infections (White et al., 2009). The final stage of disease progression sees the entire fruit consumed by mycelial growth. In an Italian study designed to probe the diversity in disease virulence of certain SER causing pathogens, Guarnaccia et al. (2016) found that pathogen species vary in disease severity. For example, isolates of *Colletotrichum fructicola* and *Diaporthe foeniculacea* caused more pronounced symptoms in Hass avocados, compared to other fruit.

SER is an umbrella term used to describe a collection of symptoms associated with a range of fungal pathogens. It is caused by a wide range of host-specific, generalist, and opportunist fungi species (and genera), all causing similar symptoms.

The condition is referred to as a single disease. It is characterised by a fungal-induced browning of the mesocarp tissue which begins at the stem-end and continues downward until the entire fruit is necrotised. Fungal digestion of the mesocarp can manifest as a hollow, mycelial-filled cavity, even browning of the mesocarp tissue with a clear margin, or a browning of the vascular tissue (Figure 1.2). The most frequently cited causal genera are *Colletotrichum*, *Botryosphaeria*, and *Fusarium* (Darvas & Kotze, 1987; Pérez-Jiménez, 2008). However, the causal species for SER can vary throughout the world depending on orchard conditions, location, and fungal ecology.

Figure 1.2: Typical symptoms of SER include hollow, mycelial-filled cavities near the stem-end (red arrow), fungal-induced browning of the mesocarp tissue beginning at the stem-end (blue arrow), or fungal-mediated discolouration of the vascular tissue (black arrow). Black bar indicates 1 cm.

Studies aimed at identifying the pathogens present at any given time in any given avocado species or orchard often vary, but specific causal species have been confidently identified in avocado orchards around the world. A report by Montealegre, Ramirez, and Riquelme in (2016) identified *Neofusicoccum australe* as the causal pathogen of fruit rots in Chile. Although this was the first time *N. australe* was identified in Chile, the fungus was already known to cause SER in California. Also supporting this finding is another survey of SER causal pathogens in Italy, which investigated disease severity in the Hass and Bacon varieties, as well as disease prevalence. It determined that *Neofusicoccum* genus was the most prevalent in the orchard, and specifically *N. parvum* was found to be most virulent (Guarnaccia et al., 2016).

Pestalotiopsis clavispora and *Pestalotiopsis* spp. were likewise found to cause postharvest SER symptoms in avocados destined for export from Chile. This discovery stemmed from an avocado harvest in 2009 that appeared healthy, but 10% - 14% of this crop went on to develop SER symptoms (Valencia et al., 2011). SER pathogens in California were identified as *Neofusicoccum luteum, Colletotrichum gloeosporioides,* and *Phomopsis* sp., the first of which is associated with branch cankers. Many other opportunists and generalists were associated with SER: *Botryosphaeria lutea, Botryosphaeria dothidea, B. parva, B. ribis, B. rhodina, Glomerella cingulata, Colletotrichum gloeosporioides*, *Glomerella acutata*, *Nectria pseudotrichia*, and species of *Albonectria*, *Alternaria*, *Gibberella*, *Pestalotiopsis*, *Phomopsis*, and *Rhizopus* (Twizeyimana et al., 2013). In this particular survey, *N. luteum* was found to be the most prevalent stem end pathogen in the California orchard, but other papers claim *Fusaria* is also a major causal genus (Guarnaccia et al., 2016; Peres et al., 2002). Overall, there are vast numbers of species identified in literature, thus further

supporting the perception that SER is an opportunistic disease of the stem wound, caused by a fungal species that is present in the orchard at the time.

N. australe was also confirmed as the causal agent of SER on avocados grown in Chile by Montealegre et al. (2016) and by Akgül et al. (2016) in Turkey. By isolating the infected tissue at the stem end, the researchers were able to culture cottony white hyphae, which eventually turned black during later stages of development. The species was confirmed both morphologically and genetically to be *N. australe*, and controlled inoculation of avocados produced identical disease symptoms, thereby fulfilling Koch's postulate for microbial disease relationship. These and many other SER causal species are a member of *Botryosphaeria* (Montealegre et al., 2016).

A less established SER pathogen for avocado is *Colletotrichum gloeosporioides*, which is more commonly associated with a body rot disorder known as Anthracnose. Although Anthracnose contributes significantly to yield loss as a postharvest fruit rot, the *C. gloeosporioides* contribution to SER is also commercially troublesome. Infections from this pathogen are widespread among tropical and subtropical fruits, mainly causing Anthracnose lesions on the skin, but members of the *Colletotrichum* genus were found in 16% of fungal samples isolated from avocados displaying SER symptoms (Guarnaccia et al., 2016; Peres et al., 2002).

The fungal species responsible for SER in South Africa were generally the same as those isolated in other countries. The most frequently cited causal pathogens are *Colletotrichum*, *Botryosphaeria*, and *Fusarium* (Darvas & Kotze, 1987; Pérez-Jiménez, 2008). However, these studies did not specify exactly which piece of tissue was sampled from the avocado. Decayed tissue was taken from SER-positive fruit, but the cultured fungal species could be either a secondary opportunist pathogen or responsible for an unrelated rot disorder. The authors also concluded that only certain species

caused vascular discolouration, but this may be dependent on the pathogens entering from the stem button. Nevertheless, the orchard surveys and isolation studies of fungal pathogens associated with SER have established that symptoms are likely caused by a wide range of fungal species present in the orchard environment. We may find that efforts to mitigate symptoms would be more effective if they focus on the conditions most conducive to disease spread and development, instead of combating a single causal pathogen.

1.4 SER Biology and Senescence

Typically considered a "wound" disorder, SER begins at the cut end of the fruit and works its way to the blossom end. Therefore, researchers have speculated that the disease begins at the opportunistic wound made by cutting the stem of unripe fruit at harvest (Schaffer et al., 2013). The conditions within the fruit during harvest are not favourable to fungal germination, therefore the immature fungus remains dormant on the surface of the skin during a period called quiescence, a term first proposed by Swinburne in (1978).

Infection by a pathogen is a complicated process that requires the fungus to overcome the host plant's biochemical defences, and to activate pathogenicity factors to macerate the plant tissues for its consumption (Prusky, 1996). For example, the alkalization of host tissue by *Colletotrichum gloeosporioides* has been shown to induce the expression of the gene encoding avocado pectate lyase, leading to further tissue degradation (Miyara et al., 2008). However, fungal inoculation of unripe, resistant avocado fruit is also correlated with an increase in reactive oxygen species, such as hydrogen peroxide, causing an induced defensive mechanism (Beno-Moualem & Prusky, 2000).

In general, disease development and progression involve many biochemical

processes, which take place over extended periods of time. Latent infections were experimentally identified by the presence of appressoria on unripe fruit, and rot symptoms developed up to three months after inoculation of symptomless fruit (Binyamini & Schiffmann-Nadel, 1972). The fungus accomplishes this by developing a modified appressoria structure known as an infection peg.

The infection peg ultrastructure of *Colletotrichum sublineolum* isolated from sorghum was carefully studied using both low temperature scanning electron microscopy and transmission electron microscopy. The peg structure was found to be associated with increased melanin and annular wall thickening. A change in the staining of the host cell wall suggested that the infection peg invades the host tissue via both mechanical force and enzymatic digestion (Wharton et al., 2001). Although many SER pathogens are generalist, the complex biochemical interactions involved in infection still require some level of host compatibility. The surface wax of avocado, specifically its fatty alcohol content, was found to induce the germination of *C. gloeosporioides* when other fruit waxes did not, suggesting that at least this particular pathogen can be host specific (Podila et al., 1993). Also, infection pegs that developed after harvest were more efficient in piercing the avocado cuticular wax (Coates et al., 1993).

Once the infection peg has established itself within the avocado skin, its quiescent stage is broken only when exposed to the ripening hormone, ethylene. This phenomenon was first established via electron microscopy analysis, where the SER pathogen *C. gloeosporioides* was observed infecting avocados before they were ripe, and then remaining quiescent in the tissue until exposed to ethylene during the ripening process (Coates et al., 1993).

Fruit ripening is defined as the maturing and softening of cells coupled with a

shift in biochemical composition. This process is regulated by ethylene, the only plant hormone that exists in gaseous form. Like all hormones, it coordinates metabolic processes via signal transduction within the cell. Ethylene is a two-carbon alkene responsible for increasing respiration rates and cellular maturation processes within the fruit tissue. These processes increase with the climacteric rise of ethylene within the fruit. Ethylene is biochemically synthesised from S-adenosylmethione during ripening (Johnson & Ecker, 1998; Oetiker & Yang, 1995). In addition to its vital role in fruit physiology, ethylene is also involved in pathogen infection and defence processes (Johnson & Ecker, 1998).

Ethylene is involved in multiple defensive biochemical responses to pathogen attack, which can be constructive or destructive depending on the specific host-pathogen interaction. Its signal transduction pathway is known to induce other plant defences, such as jasmonic acid production, but sometimes ethylene is also produced by the pathogens themselves (Broekaert et al., 2006).

The release of ethylene results in a softening of the cell-wall and a decline in pre-existing antifungal compounds in the fruit, both of which facilitate pathogen growth. Although ethylene is known to be involved in the biosynthesis of many anti-fungal compounds, the overall effect it has on ripening and softening the fruit consummates a trade-off between pathogen defence and pathogen acquiescence (Prusky et al., 2013). Similar ethylene-mediated disadvantages can also be observed in postharvest SER disorders of other fruit, such as oranges (Porat et al., 1999). One example of the adverse effects of ethylene is the breakdown of (Z,Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene. This compound exists as a constitutive defence molecule in avocado, particularly against *Colletotrichum gloeosporioides*. It is broken down by lipoxygenase in one of the many pathways affected by ethylene, and thus its

breakdown can therefore be countered by the application of the ethylene inhibitor 1-methylcyclopropene (Wang et al., 2006).

Contrary to this, ethylene participates in many defensive pathways, such as induced salicylic acid and jasmonic acid pathogen defence, which are regulated by the Ethylene-Response-Factor-1 gene. Expression of this gene was correlated with increased resistance to necrotrophic pathogens in *Arabidopsis* (Berrocal-Lobo et al., 2002).

For many stone fruits, such as cherry and peach, continuous ethylene exposure was not found to impact commercial quality during cold storage (Palou et al., 2003). However, the cellular microstructure of avocado mesocarp was shown to harbour increased endogenous lipids and cell wall components after ripening with ethylene for 30 – 40 days in cold storage (Ortiz-Viedma et al., 2018). Therefore, avocados should be protected from ethylene until they are ready for the consumer.

1.5 Fungal Control

Strategies to combat fungal infection in the field are varied, and include taking measures to reduce inoculum, eradicate the spread of disease, or prevent disease development. Fungicides are a common approach toward reducing the infection at the molecular level. They have multiple modes of action and target different sites, which range from inhibiting cell division, interfering with sporulation, or destroying fugal hyphae. Attacking spore development would reduce inoculum below an established threshold, thus preventing disease without necessarily eradicating the pathogen completely (Eckert & Ogawa, 1985).

Effective fungicides for both SER and other postharvest disorders include Thiabendazole, a chemical that impedes fungal metabolism by inhibiting the citric acid cycle, and the sterol inhibitor Prochloraz. Both Thiabendazole and Prochloraz were

found to be especially effective against *Colletotrichum gloeosporioides* (Anderson, 1986; Gisi et al., 1985; Muirhead et al., 1982; Sanders et al., 2000). Fungicide synergy occurs when the modes of action for each constituent have a compounding effect greater than the summation of the effects of the individual fungicides. This increases the productivity of the fungicides Thiabendazole, in addition to deterring fungal resistance to any of the individual chemicals. For example, the combination of oxadixyl and mancozeb was found by Gisi et al. (1985) to have strong synergistic effects for both grape and tomato, but the researchers could only speculate as to why.

Despite the aforementioned successes in the development of commercial fungicides, consumer demand is driving research toward alternative methods of fungal control. The reasons for this push include the impact of fungicides on human health, the presence of unwanted fungicide residues on the edible portions of fruit (Papadopoulou-Mourkidou, 1991), sustainability concerns of modern agricultural practises, and the effect fungicides have on the environment.

The avocado industry has historically depended on copper-based fungicides, which have shown deleterious effects on native orchard fauna (Coates et al., 2002). Controlled experiments demonstrated that earthworms avoid soils contaminated by copper-based fungicides; given the important ecological niche of these animals, their absence may impact soil structure and nutrient cycling (Van Zwieten et al., 2004). Overall, fungicide use has been shown to significantly reduce the numbers of non-target microorganisms, such as filamentous fungi and yeasts, thereby impacting other aspects of orchard micro-ecology. This was demonstrated in the same study, which showed that unsprayed control avocados exhibited fewer incidences of *C. gloeosporioides* Anthracnose, suggesting that the non-pathogenic microorganisms may have been competitive species (Stirling et al., 1999).

Yeast antagonists and other biocontrol microorganisms have proven to be effective against fungal pathogens. Ideally, the organism must be applied prior to the pathogenic infiltration, thereby developing a sufficient population size to then overwhelm the pathogen. The exact mode of action could be competition for space or nutrients, fungal cell wall degradation, direct parasitism, and/or oxidative stress (Sharma et al., 2009; Spadaro & Droby, 2016).

In practice, the biocontrol organism *Bacillus subtilis* B246, commercially known as Avogreen, has been proven to successfully mitigate disease symptoms. Demoz et al. (2006) demonstrated that it could effectively colonise avocado flowers. This and similar treatments are most potent when care is taken to ensure that the biocontrol agent is healthy and evenly mixed prior to application (Demoz & Korsten, 2006). Because of its difficult application method, Avogreen has recently fallen out of favour. Although the bacteria were shown to be effective when applied correctly, ensuring that they remained alive and active proved difficult (Demoz & Korsten, 2006).

Alternatively, treatment with the antagonist yeast-like saprophytic species *Aureobasidium pullulans* reduced disease progression but did not prevent disease entirely. However, this research also demonstrated that an intact stem button, and longer stem remnant, could delay SER symptoms (Madhupani & Adikaram, 2017).

Edible coatings making use of different combinations of additives are currently in testing. These can reduce water loss and oxidation, generally creating uninhabitable conditions for fungal growth. Carboxymethyl cellulose has demonstrated effectiveness and can be combined with *Moringa oleifera* Lam. leaf and seed extracts. This then showed increased antimicrobial activity and suppression of metabolic activity, thus increasing avocado shelf life (Tesfay et al., 2017). However, wax coatings also interfere with ethylene absorption and ripening, thereby creating a trade-off between

disease reduction and overall fruit quality.

Another approach to mitigating fungal decay is the induction of internal plant defences by employing well-known plant signalling compounds, such as jasmonates and salicylates, or plant volatiles such as citral (Glowacz et al., 2017; Obianom & Sivakumar, 2017). Applications of gaseous methyl jasmonate were shown to be especially potent at concentrations of 100 µmol/L. The mechanism behind this action was thought to be a combination of increased defensive enzyme activity induced by the hormone, and its interaction with total phenolic compounds already present within the fruit (Glowacz et al., 2017).

Thyme oil effectively inhibited radial mycelial growth of five avocado pathogens in vitro, when used at a concentration of 5 µL/plate, and thyme oil vapor was shown to be successful in vivo. Although the exact mechanism is not completely understood, it was hypothesised to involve the action of the phenolic compounds, with the thyme-derived thymol being the most abundant. Phenolic rings and hydroxyl groups could be responsible for this antifungal activity, as they function by dissolving pathogen cell membranes (Sellamuthu et al., 2013).

Many of the initial trials with plant-derived anti-fungal compounds and induced plant defences have shown promise for commercial application. Consumer demand and increased environmental awareness is challenging the food industry as a whole to reduce pesticide and fungicide use in commercial farming as much as possible. Improvement in technologies with a reduced environmental impact are helping to reach this goal, but even the most effective fungicide can be inadequate when used improperly. Accurate application timing is a crucial counterpart to an effective fungicide, and it must be informed by an understanding of the pathogen life cycle.

1.6 Physiological Factors Affecting Fruit Quality and Chemical Composition

The concentrations of some avocado nutrients, especially the oils and phenols, can influence disease progression and fruit phenolic defence. Therefore, large datasets of measurable traits are required to fully understand avocado fruit physiology. The changes in fruit physiology that occur during ripening drastically alter the texture of the mesocarp. The key to successful preservation during cold storage is in maintaining the proper components, such as the seven carbon sugars mannoheptulose and perseitol, which are energy sources responsible for characteristic ripening behaviour . Sugar content was shown to be an important factor in preserving avocado fruit quality (Blakey et al., 2010; Miles et al., 2012).

The mineral content of the fruit can vary between cultivars but remains fairly consistent within cultivars grafted to a standard rootstock. In a study examining yield and fruit quality of cultivars grown on different rootstocks, Hass was found to have higher potassium and lower calcium levels as compared to Fuerte (Kremer-Köhne & Köhne, 1992). However, the authors admit that their report conflicted with earlier literature. It is possible that the variation in fruit mineral content could be dependent on multiple factors, such as soil quality, fertilisation, tree stress, and moisture levels. Calcium is essential for plant cell wall structure and stability, as well as providing a barrier for pathogen defence both by strengthening the cell wall and functioning as a signal ion during pathogen attack (Thor, 2019).

Nevertheless, this mineral composition is integral to fruit quality and mesocarp structure. In the case of Pinkerton avocados, mesocarp discolouration is a common postharvest issue. The cause was determined to be excess nitrogen, copper, manganese, and boron. This suggested that mineral ratios and combinations are more important factors than the individual minerals themselves (Van Rooyen & Bower, 2005).

The effects of mineral concentrations for avocado and other tree fruits have been well studied, both in terms of disease incidence and fruit texture or quality. High concentrations of calcium and magnesium in relation to potassium content, hastened the ripening process, and high calcium was correlated with reduced vascular browning (Hofman et al., 2002). It was found that fruit that had ripened quickly had a reduced calcium concentration, as well as fruit at later stages of maturity (Cutting et al., 1992). Indeed, the intentional reduction of vegetative growth was thought to increase fruit set of Fuerte avocados by increasing the available calcium levels (Kremer-Kohne, 1987).

One of the more important mineral ratios is the proportion of nitrogen to calcium, and the location of calcium within the tree, as this mineral is involved in cell wall structure and membrane integrity. Applying nitrogen, in the form of a urea spray to the foliage, was shown to increase fruit yield, but at the slight expense of fruit oil content (Aziz et al., 1975). Witney et al. found that calcium was distributed the most in leaves, bark, and roots, and it was found least in immature fruits (Witney et al., 1990). Increased calcium in the fruit is associated with reduced fruit quality and adverse effects on ripening, therefore some disorders which affect fruit quality do so by increasing calcium uptake. For example, vegetative dieback increases the amount of calcium available to the fruit, and *Phytophthora* root rot causes root branching and increased passive calcium uptake (Witney et al., 1990). However, another study by Hartil et al. found no relationship between the mineral content of the fruit and SER incidence. They speculated that if avocados are infected with SER during harvest, the integrity of the cell wall would be irrelevant if the pathogen is able to infect through the broken tissue created by a stem wound (Everett et al., 2007).

Fruit firmness is a standard measurement taken to assess fruit quality, and it is one of the parameters used by the consumer to determine the internal quality of many

types of fruit (Van Woensel et al., 1987). For avocados, intercellular spaces decrease during ripening, thereby reducing the density of the flesh. Researchers also noted a slight decrease in the water content of the fruit as well, but the relationship between water content and ultrasonic velocity (the velocity through which a fixed frequency ultrasonic wave passes through tissue, yielding a measure of fruit ripeness) remains unknown (Self et al., 1994). Nevertheless, the development of non-destructive methods to measure fruit firmness has been successful in identifying fruit with high-quality flesh characteristics, as well as removing fruits which are too ripe for transport. Fruit sensors, such as those which use vibration energy to measure firmness, have been shown to have a 90% accuracy rate in correlating firmness with quality (Peleg et al., 1990).

Tree health also has a marked impact on fruit quality, with stressed trees producing compromised fruit. Water stress has been shown to increase postharvest fruit browning, especially when combined with decreased ventilation. This was due in part by increased polyphenol oxidase activity (Bower & Cutting, 1987). On the whole, flesh browning is a complicated phenomenon, and it can be due in part by the necrotisation of tissues by plant pathogens, or the result of biochemical reactions already in the fruit. Polyphenol oxidase activity is just one example, as it works to oxidise phenols, but the phenol content itself could also be emphasised (Bower & Cutting, 1987).

Plant phenols are a naturally occurring group of molecules which contain at least one aromatic ring and hydroxyl group. They are divided into either flavonoids or non-flavonoids and, in conjugation with sugars, contribute to the secondary metabolite composition of complex foods such as wine and chocolate (Crozier et al., 2006). Environmental pressure can induce phenolic production in plants, which then act as anti-fungal or insecticidal compounds. In a study evaluating vegetable crops, organic

produce was found to have higher phenol content compared to conventionally grown plants which are protected from stress (Davis et al., 2004).

A combination of total phenolic content and polyphenol oxidase activity was found to significantly contribute to the browning of Fuerte avocados. However, the study also notes that some phenols are antagonists of polyphenol oxidase, making the phenomenon of overall browning much more complex (Golan et al., 1977). *In vitro* enzymatic analysis by (Kahn, 1975) confirmed that elevated levels of polyphenol oxidase activity correlated with rapid browning, and both soluble and bound forms of the enzyme contributed to the browning reaction (Kahn, 1977). These experiments initiated the enzyme activity by injury, but the results can also be applied to exposure of plant cells to oxygen during fungal digestion.

Polyphenols are also known for their antioxidant and preservative properties due to their redox activity (Rice-evans et al., 1995). They can both cause and mitigate browning symptoms by counteracting the effects of an oxidation reaction or participating in the browning reaction. A high phenol content correlates with a strong antioxidant capacity for pineapple, banana, and guava in human nutrition, but the species of phenol is more important when considering fruit browning (Alothman et al., 2009). Indeed, polyphenols and flavonoids have been shown to reduce oxidative free radicals in the human body, implying that foods rich in these compounds may contribute to reduced oxidative stress, but the avocado fruits tested in this study were found to have low 'antioxidant power', due in part to a lack of one specific type of phenol, flavonols (García-Alonso et al., 2004).

Whilst the influence of total phenolic compounds on anti-oxidising activity is strong for the aforementioned tropical fruits, a conflicting study found no such correlation when studying 92 edible and non-edible plant products. By measuring

extracted antioxidant activity on methyl linoleate, Kähkönen et al. (1999) found that berries and apples had strong activity but could not find a significant correlation between antioxidant activity and total phenolic content for any of the plant materials studied. Likewise, a review by Frankel and Meyer (2000), criticised the excessively focused methods used to analyse biological components. In the paper, the authors state that antioxidant activity depends on a variety of factors, such as the substrate properties, reaction stages, and localisation of different components to name but a few; these are often not replicated in a laboratory analysis. Consequently, the *in vitro* methyl lineate method stated above appears too simplistic.

Despite the apparent controversy, improvements have been made toward manipulating the browning reaction in avocados by using antioxidant properties of other vegetative materials. Extracts of *Allium* and *Brassica* have been shown to effectively prevent the browning of macerated avocado pulp at 4°C by inhibiting the activity of polyphenol oxidase (Bustos et al., 2015). Research into the use of plant extracts for preserving avocado fruit quality, including defences that already exist in the fruit, is a promising trend in agriculture.

Natural plant defences against pathogen attack are either constitutive or induced. An example of a constitutive defence is the presence of preformed antifungal compounds, whilst induced defences involve the release of new compounds to combat infection. These include the release of reactive oxygen species during the early stages of infection, or circulating substances that antagonise fungal enzymes, such as the modulation of fungal pectolytic enzymes by mango fruit (Prusky, 2009). However, fungi are becoming increasingly resistant to the natural defences acquired by plants as the pathogens evolve and adapt to their host (Morrissey & Osbourn, 1999).

1.7 Fungal Disease Ecology

The first stage in a fungal life cycle is the reproductive spore, which, when produced in host tissues, is called an inoculum. For SER causal species, fungal inocula can originate from living and dead tree branches. Specifically, the spore-producing hyphae are known to infiltrate the extra-cambial tissues of branches, and the inner tissues of leaf pedicels. Hartill and Everett (2002) speculated in an experimental paper that a SER pathogen could be located within the tree, and thereby grow into the fruit through its pedicel. This is a valid theory, since mango SER pathogens have been proven to grow endophytically through stem tissue (Johnson et al., 1992). The density of the fungal pathogen detected within the mango pedicel is so strongly correlated with subsequent SER incidence in the fruit that it can be used in disease screening (Johnson et al., 1991). However, the ripening processes of mango and avocado are so different that comparisons should only be made with caution.

In the conclusion of their report, Hartill and Everett (2002) state, "the evidence supporting endophytic growth through the tree as the source of infection [is not] strong". They go on to say that more robust evidence suggested infection occurred during harvest, with the sterilisation of clippers being a major factor in SER incidence. The same study also suggested that although *Colletotrichum*, *Botryosphaeria*, and *Phomopsis* species were found in the living pedicels, they resided mainly in the extra-cambial tissues and not xylem elements, the tissue more frequently attacked by SER. The substantial reduction in SER incidence demonstrated by sterilising clippers is the strongest evidence for infection occurring during harvest, however other factors may further increase fruit susceptibility.

In contrast, a short study by Köhne and Kremer-Köhne found no increase in SER when picking Hass avocados without a pedicel (where fruit is snapped from the tree). The authors made sure to note that the quicker harvesting times ensured that fruit

would reach the pack house faster, thereby reducing water loss (Bill et al., 2014; Köhne & Kremer-Köhne, 1995). The relationship between water content and SER incidence has not been extensively explored, but water content does affect fruit quality. The issue is further complicated by a 2001 California study by Smilanick et al. which found that snapping avocados from the tree or clipping them could both increase SER incidence, depending on the causal species. Their theory was that the more aggressive endophytic fungi remain in the stem tissue and infect the fruit after being distributed by water. The stem remnant from clipped fruit therefore serves as an innocula. Smilanick et al. (2001) proved that snapped fruit are freed from these pathogens but are then more vulnerable to airborne opportunists which colonize the fresh abscission zone wound. In short, wet conditions result in more SER when fruit are clipped, and dry conditions result in more rot when fruit are snapped.

Another factor that can influence disease susceptibility is the age of the fruit. Fuerte avocados were found to be susceptible to anthracnose infection from very early fruit set until harvest, but they were only susceptible to SER. This was demonstrated by the fact that fruit harvested earlier had less SER incidence when compared to older fruit. However, the authors mentioned a correlation with rainfall and 'timing of harvest', suggesting that environmental factors may have more impact (Peterson, 1978). However, another study by Everett et al. (2007) found no correlation between SER and rain.

The key points in understanding SER infection are to determine exactly when, how, and where the fruit is infected. These questions represent a large knowledge gap in the understanding of the disease ecology of SER causal pathogens. Investigating when the infection occurs could be done via artificial inoculation field studies. In one such study, Binyamini et al. found that the pathogen latent stage can be as long as three

months (Binyamini & Schiffmann-Nadel, 1972). This was not an SER study, and the artificial inoculation methods used are more applicable to body rots. It is also important to note that the nature of artificial inoculation ignores crucial details of the pathogen ecology which may have more impact on disease incidence than just considering when the fruit can be infected. These could be environmental factors surrounding, for example, the fungal development, sporulation, or distribution.

Fungal pathogen distribution follows many of the same patterns studied in other wind-distributed biota, such as pollen or seeds. Factors such as atmospheric currents, airflow through an orchard, presence of suitable host material, fungal lifestyle and pathogenic species present in the surrounding region all contribute to the causal species one might find on the farm (Brown et al., 2002; Gage et al., 1999). Although great strides have been made toward understanding these phenomena, the sheer number of variables involved must always be weighed against the robustness of the conclusions. For example, the genetic composition of an organism can change quite rapidly in the wild given strong selection, and long-distance travel of some fungal pathogens often results in a founder-effect. The genotype of pathogens could most closely resemble that of the surrounding microflora, and would vary by region (Slippers et al., 2007). Pathogen distribution is more often a result of range expansion, with the fungus continually changing and adapting to new environments (Brown & Hovmøller, 2002); a completely different morphotype could alter the way a pathogen interacts with its host.

Sequencing analysis of isolated pathogens should reveal genetic deviation, due to their exposure to new selection pressures, although DNA alone is not enough to determine if these changes affect pathogen behaviour in the field. The literature seems to imply that large groups of opportunists are responsible for SER, instead of specialist pathogens. Multi-locus phylogenetic analysis coupled with morphological examination

have identified numerous species of the *Colletotrichum* genus and the Botrespheriaceae family in spore traps, with the spore count for Botrysphariaceous fungi being highest after rain (Eskalen et al., 2013; Sharma et al., 2017; Slippers et al., 2007). With such a breadth of responsible agents, individual infection strategies become obscured and pathological studies shift focus to the main phenomenon influencing the majority of fungal species, such as sporulation factors.

The most important aspects of infection ecology are the spores and innocula, specifically their location and survival. In general, fungal innocula can be found anywhere, depending on the species. Some fungi thrive in dead plant matter, whilst others colonize soils, and the population dynamics within these micro-ecosystems dictate presiding fungal species at any given time, with a healthy biodiversity often out competing harmful invaders (Altieri, 1999). Of the 15 species found in soils, for example, Darvas (1979) determined the root rot pathogen *Phytophthora cinnamomi* to be the most destructive to avocado.

Native bacterial and fungal microbial populations in mulch were found to suppress the pathogen, but this experiment was carried out using an artificial *in vitro* design. You et al. (1995) incubated *Phytophthora cinnamomi* - inoculated Mira cloth in petri dishes covered with mulch and tested the virulence of the pathogen after a three-week incubation period. It is doubtful whether this design would accurately represent what is occurring in the field. The pathogen could potentially inoculate the host at any point before it fatally succumbs to the other soil microbes. In contrast, the heat generated by decaying mulch was found to be most effective in killing fungal pathogens, as well as competitive microbes and chemical degradation, according to a study by Downer et al. (2008), which determined that fresh plant waste produces temperatures lethal enough to eliminate the pathogen. Proper turning of mulch and

changing old plant matter with new has been shown to be highly effective in eliminating soil pathogens (Downer et al., 2008).

Yet, whilst fungi in the soil and mulch were most affected by temperature and moisture, those found in the canopy and dead leaves are more highly influenced by rainfall and humidity. This is because the innocula for fruit rots and postharvest disease are located on aerial dead plant matter. Spore trap analysis confirmed that the dead leaves and fruit material that had accumulated in the canopy were the most responsible for Anthracnose body rots in avocado. *Botryodiplodia theobromae, Colletotrichum gloeosporioides*, *Dothiorella aromatica*, *Fusarium decemcellulare*, *Pestalotiopsis versicolor*, *Phomopsis perseae* and *Thyronectria pseudotrichia* all seemed to be particularly adapted to bark, twig, leaves, and fruit matter (Darvas et al., 1987). The highly aerated nature of these locations would provide an environment with less fatal heat build-up, and therefore the pathogen would not be eradicated in the same way as compacted soil waste. These types of pathogens are more effectively controlled with fungicide applications.

An extensive three-year study of eight orchards found that the presence of dead branches and high canopy density was highly correlated with body rots, but had no effect on SER, nor did rainfall. To the contrary, factors such as fungicide application, depth of mulch, and fruit shelf-life were all found to have more impact on SER incidence (Everett et al., 2007). Targeted fungicide application would arrest pathogen development and spread directly, whilst proper mulching would suppress fungal inocula found in soil or dead plant matter, and shortened shelf-life would ensure the product reaches the consumer before postharvest rot develops.

1.8 Disease Risk along the Avocado Supply Chain: Points of Increased Risk

Although much of the literature strongly suggests that SER infection occurs at harvest, there is still uncertainty as to exactly why some avocados are more vulnerable to infection than others, and which fruit will be most likely to subsequently develop rot symptoms. Since infection through the stem wound seems the most plausible entry point for SER pathogens, it is the first point of disease risk. In addition to the consequences of snapping fruit from the tree or clipping, injuries suffered by the fruit during harvest must also be considered.

Mechanical damage from dropping fruit from a height less than a meter can cause bruising or opportunistic wounds which increase postharvest fungal disorders. It was thought that bruised tissue and mishandling towards the stem end could both aggravate the opportunistic stem wound and spread fungal spores (Mandemaker et al., 2006). However, Zauberman et al. found that wounding decreased the ripening time for Fuerte avocados and had little effect on ethylene production when compared to unwounded fruit. Both longer ripening times and increased ethylene production have been correlated with increased SER incidence in past experiments (Blakey et al., 2010; Fuchs & Zauberman, 1981; Hopkirk et al., 1994; Wang et al., 2006). A report by Darvas et al. (1990), which explored the effects of targeted fungicide and waxing on SER incidence, added more evidence that SER infection occurs through the stem wound, as sealing and treating this wound reduced the rot. This report also advocated for a reduced ripening time to prevent rot.

The ripening process is another critical stage affecting disease vulnerability. As mentioned previously, the production of ethylene during the ripening process breaks fungal quiescence and initiates rot symptom development. Ethylene is unavoidable in the ripening process, and most literature concerned with storage and ripening is only

postponing the inevitable rot for fruit infected in the field. Indeed, factors such as optimising temperature, whilst also preventing chilling injury, or controlled atmosphere packaging, can prolong avocado shelf life up to 9 weeks (Meir et al., 1997; Palou et al., 2003). This is an accomplishment, but it is diminished by the fact that any infected fruit will go on to develop rot once exposed to normal temperature and atmosphere.

When considering all the studies carried out on avocado postharvest diseases, two main strategies emerge: either inhibit the rot from developing after infection or to prevent infection in the first place. However, the biochemical processes of fungal development are so intricately entwined with fruit maturation that it would be difficult to ripen an infected fruit successfully. Only proper handling and orchard management can prevent infection (Hartill & Everett, 2002).

Optimising postharvest conditions can only maintain the fruit quality, not improve upon it - and fruit quality can always decrease. So, whilst a high-quality fruit can be rendered unsellable after bruising or mistreatment along the supply chain, an SER infected fruit cannot be cured of the disease (Bill et al., 2014). However, Coates et al. (2002) note that with proper storage, such as keeping fruit at a low temperature when it is nearly ripe and controlling temperature and in particular ethylene during the ripening process, most SER and anthracnose rot development can be alleviated. The 2001 Proceedings of California Avocado Research Symposium stated that returning Hass avocados to 8 °C, following long-term cold storage (5 °C) prior to ripening at 18 °C in the presence of ethylene, substantially reduced SER incidence (Smilanick et al., 2001). However, much of this disease reduction would also involve proper field management and attending to the nutrient requirements of the tree (Coates et al., 2002).

1.9 Investigating SER toward the End of the Supply Chain

Whilst there have been numerous field studies exploring the aforementioned

orchard factors, fruit quality dynamics, and fungal populations in the orchard and their relation to SER, fungal disease communities found at avocado distribution centres have not been scrutinised to the same extent. That is to say, Westfalia farms in South Africa conduct extensive research within their own orchards, but they are not as informed on how the avocados fare after shipping. A comparison of orchard dynamics with the corresponding avocados processed at the main avocado distribution centre for Great Britain (Greencell) could therefore provide insight as to how shipping and processing impacts pathogenic fungal populations, and thus potentially identifying weaknesses along the supply chain.

Determining when pathogens infect fruit was already an area of interest but defining where the pathogen infects the fruit will dictate where core samples will be taken. Fungal species will be identified using morphological techniques, such as hyphae morphology and colour, as well as DNA analysis. Sequences used to classify many SER causal species have already been described in the literature, including the internal transcribed spacers ITS1 and ITS2, and the gene encoding the 18S ribosomal RNA region (Montealegre et al., 2016; Twizeyimana et al., 2013). For each avocado, the disease symptoms and any fungal pathogens cultured will be paired with fruit physiological data, which include fresh weight, presence of a stem button, firmness, and dry mass.

The fungal disease data collected can then be combined with other related datasets concerning orchard location or historical records of disease incidence. For the latter, Greencell's quality control database ('MuddyBoots') documents all quality control tests implemented at the distribution centre. Measurements are taken on characteristics such as fruit quality, SER symptoms, firmness, ripening, and colour. These data can be traced back to the orchard in which the avocados were grown.

MuddyBoots data will be used to compare SER disease incidence data to country of origin, orchard, and climate data to investigate how growing conditions impact SER symptoms.

Overall, this project will focus on two main databases: a selection of commercial data from Greencell's 'MuddyBoots' dataset, which will provide a model of weather, climate, country, and seasonal factors that influence SER incidence, and the Supply Chain Dataset (SCD), which records the physical properties and SER symptoms of each avocado, coupled with pathogen species isolated from the fruit. Molecular analysis of the different fungal species discovered from these data will be used for fungal community analysis at the end of the supply chain. Climate data and fruit physical properties will be used to build a predictive model of the most likely SER causal factors. The model will then be tested against fruit processed in the laboratory to make any further adjustments.

Identification of the species, abundance, and distribution of SER causal fungi after distribution would inform suppliers, such as Westfalia, of pathogen diversity in an area of the supply chain from which they are farther removed. The overall aim is to develop a library of SER pathogen diversity for avocados imported into the UK. This database will include other factors thought to have an impact on disease, such as fruit phenolic content, dry mass, orchard location, and climate data.

Patterns in disease incidence could potentially help to identify weaknesses along the supply chain, indicating when avocados are most susceptible to SER development, and if the fungal communities isolated in the UK significantly differ from those found in the orchards of Mexico (October – January), South Africa (May – September), and Israel (January - April). This is a possibility, as in theory, the shipping and handling microenvironment could favour a new microflora that requires a new management

strategy. Even small changes to prevent SER-related loss would have a large impact at an industrial level. Determining how and why certain SER pathogens survive could potentially lead to improvements in processing these much-valued fruits and better assist the farmers who grow them.

1.10 Hypotheses and aims for the thesis

Hypotheses

1. Stem End Rot is caused by a range of fungal pathogens
2. Factors such as preharvest environment, fruit maturity and the country of origin determine the community of fungal pathogens present in the fruit
3. Postharvest factors, such as storage, determine the community of fungal pathogens that develop into Stem End Rot symptoms
4. A greater diversity of pathogens will be present in fruit from countries where avocado has been cultivated for a longer time than from countries where the crop has more recently been domesticated

Aims

1. To establish whether particular environmental conditions in the orchard, spanning the period between flowering and harvest, are correlated with increased postharvest incidence of Stem End Rot [Chapter 3]
2. To identify genera, species and, where possible, the strain of fungi associated with Stem End Rot cultured from avocado fruit at the same point as a consumer would normally receive them at the end of the postharvest supply chain. [Chapter 4]
3. To determine the genetic diversity of the most prevalent morphotypes between

different countries where avocado is commonly cultivated [Chapter 4]

4. To establish the causative relationship between different fungal pathogens cultured from avocado and the development of Stem End Rot symptoms [Chapter 5]
5. To establish which physiological attributes in fruit, assessed at the point at which the fruit enters the retail environment, are associated with the development of Stem End Rot symptoms [Chapter 6]
6. To confirm whether the suspected SER causal species isolated from avocado samples assessed at the point at which the fruit enters the supply chain have the ability to cause Stem End Rot symptoms in unaffected fruit [Chapter 7]

Specific objectives are listed at the end of the introduction for each relevant chapter.

CHAPTER 2: MATERIALS AND METHODS

2.1 Supply Chain Dataset Aggregation

Stem End Rot (SER) was identified by Greencell as the most significant postharvest issue affecting their avocado supply. A large-scale fungal sampling procedure was undertaken to isolate the causal pathogens from avocados which had been grown in different orchard environments and then transferred through the commercial postharvest supply chain to the UK Greencell base. These data inform as to the scale of SER infection, the types of fungi responsible for it, and the identification of problematic orchards and regions. Physiological factors such as fresh weight, dry mass, mesocarp firmness, and browning symptoms were recorded for each fruit to assess factors which may increase susceptibility to rot development. A stem-end core sample was plated on Malt Extract Agar (MEA) to culture any fungal pathogen present, and thereby match a potential fungal causal species to the browning symptoms already recorded for the fruit. No antibiotic was added to the agar to suppress bacterial infection.

2.1.1 Sample Selection

Fully ripened Hass avocado fruits were sourced from the Greencell distribution centre located in Spalding, UK and transported to the laboratory at the University of Reading, Whiteknights, UK under chilled (4 °C) conditions at the same time as similarly treated fruit were transferred to retail stores for the commercial market. At the distribution centre, fruit were exposed to ethylene in specialised ripening chambers until fruit firmness reached customer specifications (2 – 8 Kg). This is measured using a firmometer, or with gentle hand squeezing of the fruit. Only fruit originating from Mexico, South Africa, and Israel were used. Mexico is the site of evolutionary origin for avocados and was therefore expected to have the longest evolutionary history with

the pathogen and highest pathogen diversity. Israel was the largest exporter of avocados to the UK during the five-year period selected for historical commercial data analysis, and the project collaborator in South Africa provided the most accurate weather data since this was the only site to have a weather station within the orchard site.

2.1.2 Avocado Processing and Physiology Measurements

On the day of arrival at the laboratory (day 0), fresh avocados were separated into two groups of 25. The group processed on day 0 was designated as 'Intake' avocados, and the other group was labelled 'Stored' avocados. Stored avocados were stored at 4 °C for 24 hours, and then moved to a climate-controlled environment designed to replicate supermarket shelf temperature and lighting conditions for a period of 72 hours. This sample was processed at the end of 72 hours using the exact same procedure applied to the Intake group. The additional storage procedure replicates what fruit would experience in the commercial retail environment and allows for an examination of shelf-life effects on rot development.

The first 25 avocados were labelled #1-25 using a paint pen on the base of the fruit (the stored avocados were labelled #26-50). Each avocado shipment was designated a shorthand notation (Shipment AA, AB, AC…) alongside the individual fruit number, which corresponded with a master-list linking the fruit label to each Greencell shipment. For example, the 10th avocado processed from shipment AF would be AF10, and all paired data and fungal cultures can be matched to that sample.

To process the avocados: total fresh weight was measured in grams using a standard laboratory balance (Sartorius™ Timberline Toploading Balance, Goettingen, Germany). The presence or absence of a stem button, or remnant petiole, was recorded as either 1 (present) or 0 (absent). Two flesh firmness measurements of the mesocarp

were obtained using a hand-held penetrometer ('Fruit Pressure Tester' FT 011 [0-11 Lbs.]; 11 mm probe; Facchini srl., Alfonsine, Italy). To extract a core sample from the stem end, fruit were first surface sterilised with a 70% ethanol spray. Then, under sterile conditions near a Bunsen burner, the stem button (if present) was removed, and core was excised from the abscission zone to the stone using a 4 mm flame-sterilised fruit coring tool. The extracted core was plated on autoclaved malt extract agar (MAE), with no antibiotic added, prepared according to manufacturer's instructions (Oxoid CM0059). Plates were stored for 72 - 96 hours at 25 °C.

Following the coring procedure, avocados were halved using a knife, the stone was removed, and an image was taken to record browning symptoms. SER symptoms were classified into three major types. Mesocarp browning with a distinct margin was called Fungal Browning (FB), if only the vascular tissue was decayed, with little or no mesocarp browning, this was called Vascular Discolouration (VD), and if the SER pathogen formed cavities in the mesocarp, this was termed Mycelial cavity (M). Examples of these are shown in Figure 2.1. Other non-SER rot symptoms were also recorded but were excluded in the statistical analysis of SER incidence. These were defined as Bruising (B) and Body Rot (BR). Bruising includes any internal discolouration caused by physical injury or temperature abuse of the fruit, such as internal chilling injury. It often has a clear margin but does not usually reach the peel. Body rots are caused by fungal pathogens entering through the skin of the avocado. They often have the same symptoms as SER, such as clear fungal browning margin and hollow cavities in the avocado flesh, but they originate in an area other than the stem end (Figure 2.1)

Photos were taken using a Casio Ex-Z150 Exilim 8.1 Megapixel camera against a blue background. All fungal phenotypes as they appeared after plate incubation were

assigned a morphological description. The fresh weight of one half of each fruit was recorded, and the same material was then dried at 60 °C for seven days until completely desiccated. The fully dried fruit half was weighed again, and the difference in weight was recorded as a percentage of dry mass.

Figure 2.1: Top row avocados display three different manifestations of SER. From left to right, image A is an example of Fungal Browning (FB), showing the characteristic clear margin and dark discolouration beginning from the stem end. Image B is an example of Vascular Discolouration (VD), in which the pathogen first attacks the vascular tissue at the stem end and moves rapidly down the fruit. Image C displays hollow Mycelial (M) cavities more prominently than other rot symptoms. The lower two avocados show non-SER related browning. Image D shows Bruising (B) as darkened mesocarp not originated from the skin or stem end. Image E shows a Body Rot (BR) which is similar to Fungal Browning but originates from the skin. Black bar represents 1 cm.

2.1.3 Plate Culture and Long-Term Storage of Fungal Hyphae

To create a repository of fungal replicates for DNA sequencing, representative fungal samples of the most abundant phenotypes cultured from the avocado cores were preserved for long-term storage. After a 72-hour incubation at 25 °C, samples were sub-plated by excising hyphae from the edge of the culture using a flame-sterilised scalpel and placing it on a new MEA plate. The new plate was then incubated for an additional 72 hours at 25 °C. This sub-plating procedure was repeated at least twice for each sample to ensure that cultures contained a single fungal genotype.

Hyphae from the sub-plated samples was gently scraped from the MEA plate using a flame-sterilised scalpel, carefully separating it from the agar. Hyphae were frozen in a 1.5 mL microcentrifuge tube, containing 1 mL of autoclaved 80% glycerol, at -80 °C according to an established fungal preservation protocol (Humber, 1997).

2.1.4 Fungal Isolation, Propagation, and Morphotyping

After the 72-hour incubation step, the plated avocado core samples were imaged using the same Casio Ex-Z150 camera as described in section 2.1.2. Prior to sequencing, each fungal culture was assigned a two-word morphological description characterised by its colour and texture. Morphotypic fungal phenotype description was recorded based on colour (ex. White, grey, yellow…), and texture (Cotton, Slime, Scalloped, Dense-fur, Striated, and Layered). For example, a white fungus with fine 'fluffy' hyphae was designated White-Cotton. If the fungus produced two colours, both were included in the colour label, eg. WhiteGreyCotton.

2.1.5 Internal Fruit Discolouration Analysis

Rot severity was assessed by measuring the total length of the avocado and dividing this value into thirds. For example, considering a 9 cm avocado, if the fungal browning or vascular discolouration was within 3 cm from the top, it was assigned a 1,

if the rot extended 3 to 6 cm from the top it was assigned 2, and beyond that, it is assigned 3 (Figure 2.2). Absence of rot was designated as 0. To convert it to numerical values, a score of 1 indicates 1-33 % rot, a score of 2 indicates 34-66 % rot, and a score of 3 indicates 67-100 % rot. This assessment is a categorical value, but it is worth noting that rot progresses more rapidly as the fruit ages and cellular integrity degrades.

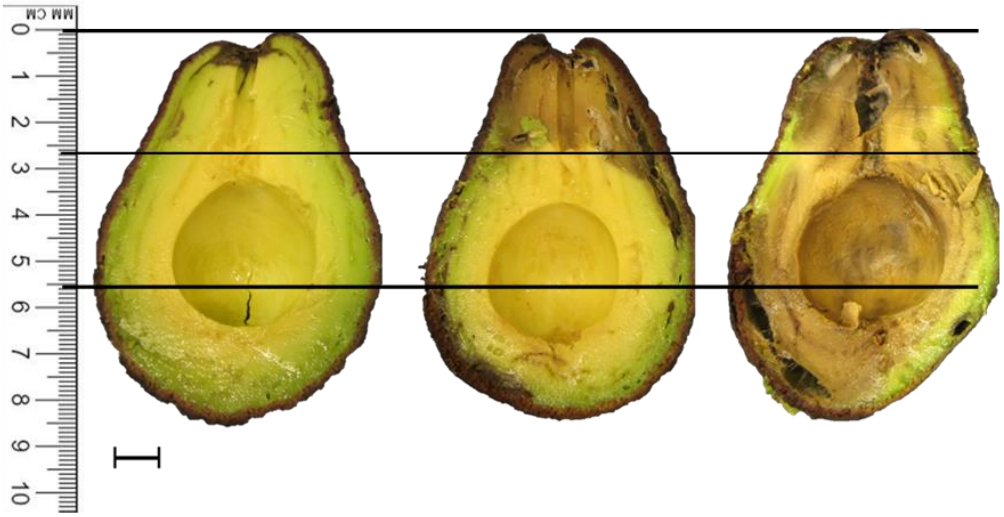

Figure 2.2: Rot severity was assessed by dividing the length of the avocado into thirds and recording whether the fungal browning margin or discoloured vascular tissue fell within the upper third (left), middle third (centre), or lower third (right) of the avocado. Avocados were rated on a scale of 1 – 3, where 1 indicates 1-33 % rot (left), a score of 2 indicates 34-66% rot (centre), and a score of 3 indicates 67-100 % rot (right). Black bar represents 1 cm.

2.2 Statistical Analysis

A 'trial' or 'replicate' was defined as a single box of avocados, sourced from a single grower. Within each replicate, half of the avocados were processed immediately, and the other half were stored under supermarket conditions. Therefore, the replicate should be seen as two sets of data (Intake vs. Stored), and only analysed with their corresponding replicates (i.e., intake trials were compared with other intake trials; stored trials were compared with other stored trials).

For categorical data, Chi-Square tests and Principal Component Analysis (PCA) were most often used. Although a PCA only illustrates statistical correlation of variables, a Chi-Square test was then used to demonstrate the significance of the correlation. For numerical data, a Linear Regression Analysis was used to model the relationship of SER incidence and the explanatory variables. All statistical calculations, as well as associated tables and figures generated, were performed using Statgraphics Centurion 19 (Statgraphics Technologies Inc.).

2.3 Fungal Identification

2.3.1 DNA Extraction and Quality Evaluation

A section of hyphae from the glycerol stock was placed on a new MEA plate and incubated 5 - 7 days at 25 °C. Fungal hyphae were scraped from the plate under sterile conditions using a flame-sterilised scalpel and placed in a 1.5 mL microcentrifuge tube. The DNA was extracted according to the protocol described by Park et al. (2014). Approximately 0.1 g of sand and 300 µL of the KCl buffer [100 mM Tris · HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1M KCl] was added to each tube. Samples were ground thoroughly with a plastic 1.5 mL pestle (Eppendorf, Stevenage, UK).

Once samples were homogenised, 300 µL Chloroform was added to each, and

tubes were inverted 20 times until the solution turned milky. Samples were centrifuged at 12,000 rpm for 1 minute, and the upper aqueous layers were transferred to new 1.5 mL microcentrifuge tubes. Working on ice, 180 µL of cold isopropanol was added to each tube. Samples were inverted by hand 20 times and centrifuged at 12,000 rpm for 1 minute. The supernatant was discarded and 300 µL of 70% ethanol was added to each tube. Samples were centrifuged at 12,000 rpm for 1 minute to pellet the DNA. The pellets were dried at room temperature until all ethanol was evaporated.

DNA pellets were re-suspended in 50 µL filter-sterilised DNA/RNAase free water, warmed to 37 °C. The nucleotide concentration was verified using a nanodrop spectrophotometer (NanoDrop ND-1000) (Table 2.1), and the quality of each extraction was assessed via a 1% agarose gel electrophoresis against a 1 Kbp ladder (Thermofisher 1 Kbp Plus) (Figure 2.3).

Table 2.1: DNA purity as a ratio of A260 and A280 for first set of fungal DNA extractions

Sample ID	ng/ul	A260	A280	260/280	260/230
L8	382.3	7.646	4.157	1.84	1.05
L25	85.42	1.708	0.973	1.76	0.87
M15	185	3.7	1.847	2	1.22
M39	21.61	0.432	0.324	1.33	0.11
M46	1785	35.7	19.036	1.88	1.2
P10	132.69	2.654	1.325	2	1.6
Q13	1089.61	21.792	11.43	1.91	1.23
Q33	394.65	7.893	3.833	2.06	1.93
Q38	382.52	7.65	4.166	1.84	1.03
R1	156.71	3.134	1.669	1.88	1.03
R17	77.64	1.553	0.775	2	1.79
S30	215.21	4.304	2.107	2.04	1.7
S37	107.15	2.143	1.075	1.99	1.59
S47	123.36	2.467	1.21	2.04	1.5

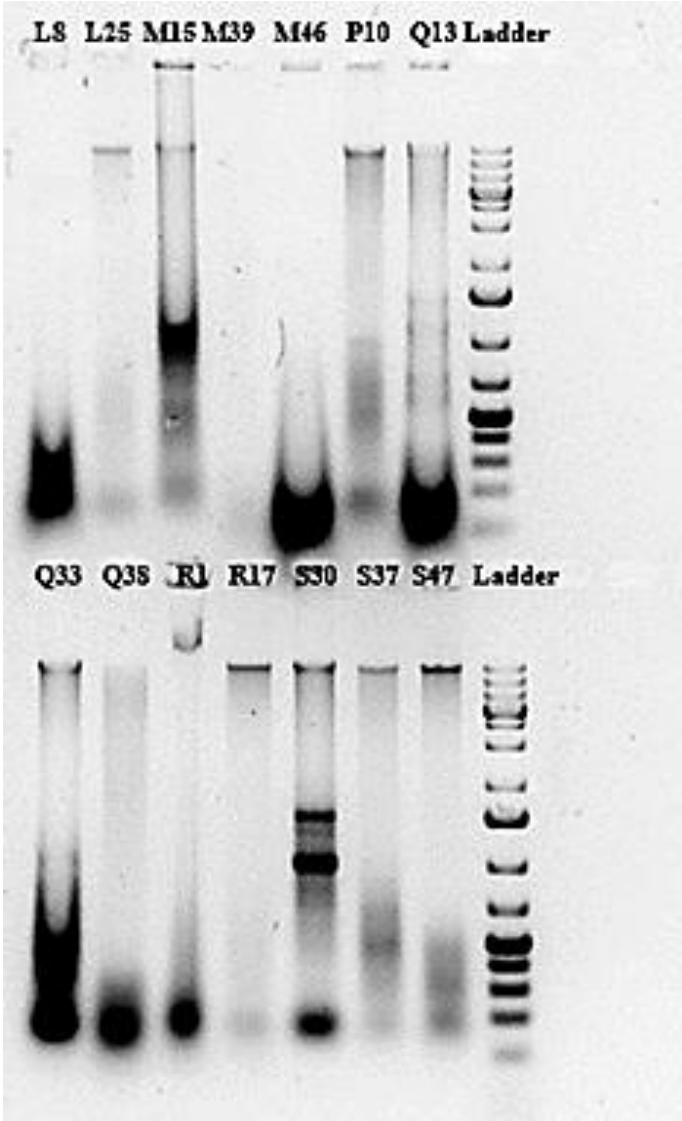

Figure 2.3: A 10 µL sample of each fungal DNA extraction from the first extraction set, verified on a 1% agarose gel visualised with ethidium bromide

2.3.2 Preliminary sequencing

Ideally, fungal DNA isolates would have been identified using both ITSII and β-Tubulin to establish a robust identification. The region of non-coding DNA located between the small and large ribosomal subunit genes, known as the second Internal Transcribed Spacer (ITSII), as well as β-Tubulin, are both highly variable and well-defined regions used in fungal identification (Bazzicalupo et al., 2013; Einax & Voigt, 2003). Unfortunately, the β-Tubulin region failed to amplify the target sequence when using both established protocols and a commercial sequencing service. During the preliminary sequencing phase, many attempts were made to optimise DNA extraction and PCR amplification before switching to a new method and DNA barcode region altogether (Section 2.3.3).

Quality control testing (Section 2.3.1) revealed that the extraction protocol was not ideal, and this was most likely due to the lysis buffer not being optimised to the specific fungal species isolated in this project. Additional lysis formulations were tested, with one of the most successful being the fungal lysis buffer solution (CLS-Y) from a commercial DNA extraction kit (MP Biomedicals). Two separate extractions were performed with this buffer, according to the protocol described in section 2.3.1, one in which the chloroform was added immediately, and another in which the ground fungal sample was incubated in the lysis buffer for five minutes (Figure 2.5, left).

One of the successful extracts shown in Figure 2.5, fungal extraction sample #2, was used as a template to amplify the β-tubulin region using primers established by Einax & Voigt (2003) (Table 2.2). The annealing temperature of these primers was optimised via a gradient PCR using *Penicillium* as a template (Figure 2.4). The PCR protocol, for both gradient PCR test and fungal extract template, was as follows: 1 µL of each primer, 10 µL of GoTaq green polymerase (Promega), 6 µL water, and 2 µL of the template DNA were combined to a final reaction mixture volume of 20 µL. Once

the annealing temperature was established by gradient PCR (Figure 2.4), samples were cycled at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, 52 °C for 1 minute, 72 °C for 1 minute 30 seconds, and a final elongation phase at 72 °C for 10 minutes (Figure 2.5, right). This was carried out using a gradient PCR programme supplied by the thermocycler machine.

As the β-Tubulin protocols were being optimised, whole genomic DNA extractions of fungal isolates, suspended in water at a concentration greater than 20 ng/µL, were submitted on dry ice to Novogene Europe (HK, China) for ITSII sequencing. This was carried out using Paired End 250 (PE250) sequencing with the ITSII primers summarised in Table 2.3.

The whole genomic DNA extraction, suspended in water at a concentration greater than 20.

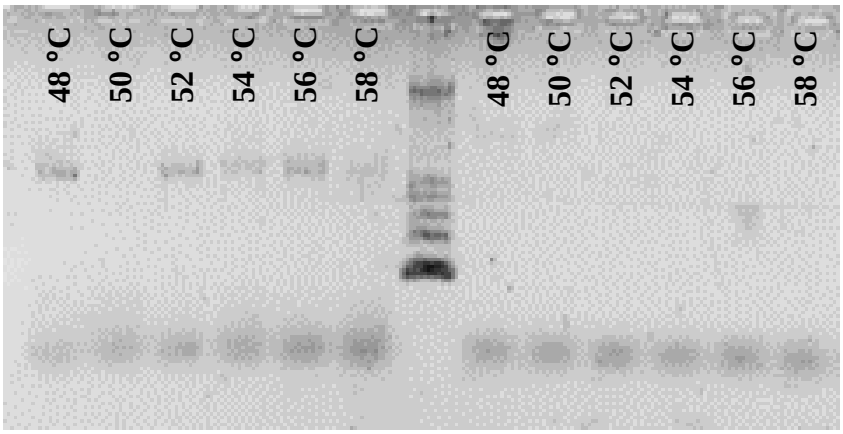

Figure 2.4: Gradient PCR designed to optimise the annealing temperature of the β-tubulin primers described in Table 2.2. Penicillium was used as a template for the six samples to the left of the ladder (GeneRuler 1 kb Plus Ladder, Thermo Scientific), and water was used as a negative control for the six samples to the right of the ladder. The positive control produced a band was produced for 48 °C, 52 °C, 54 °C, 56 °C, and 58 °C, with the strongest band produced at 52 °C

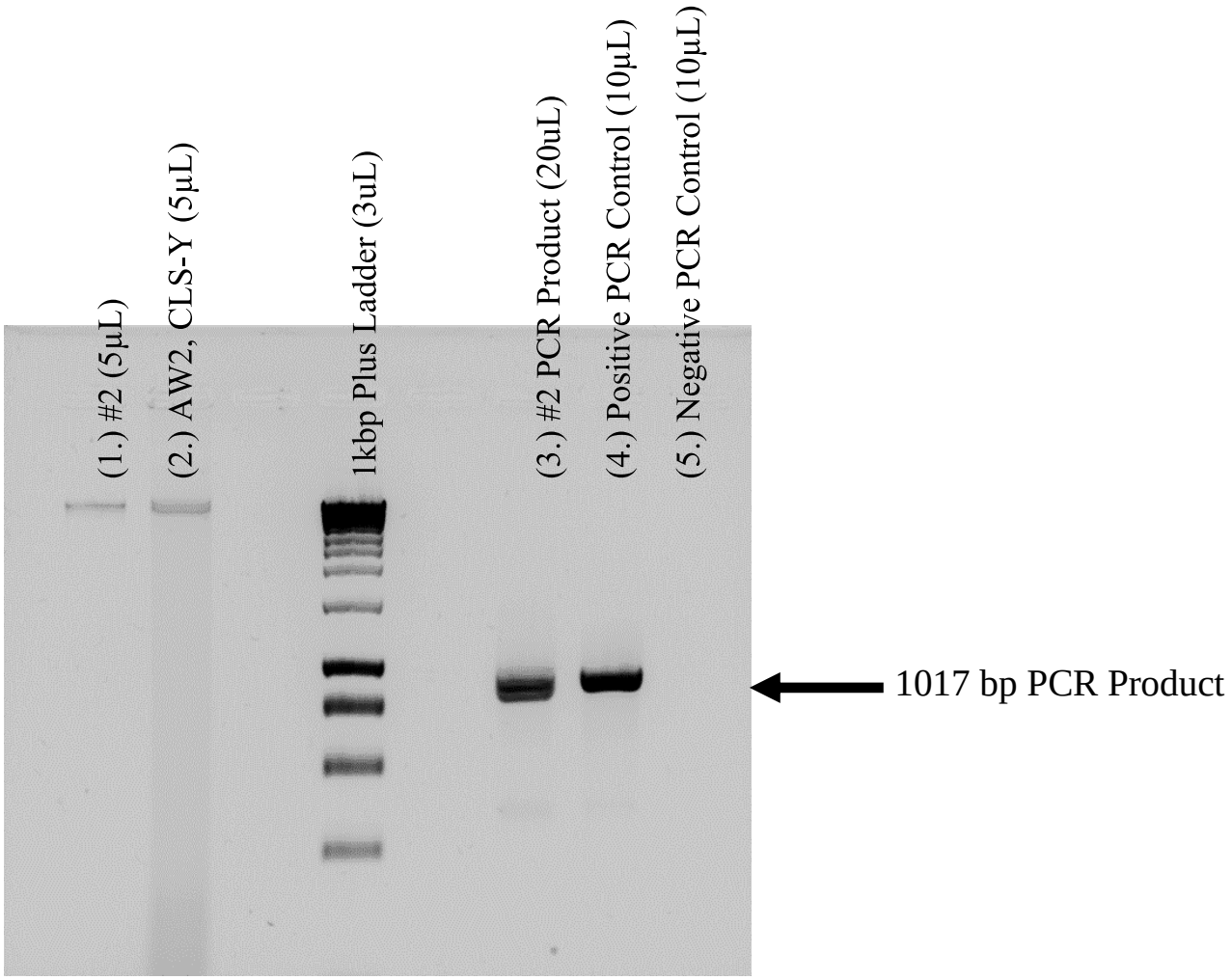

Figure 2.5: Fungal DNA extraction and PCR trials to optimise methods. 1. DNA extraction using commercial (MP Biomedicals) fungal lysis buffer (CLS-Y), 2. DNA extraction using same lysis buffer and five-minute incubation, 3. PCR amplification of extraction #2 which produced two bands, 4. Positive PCR control (penicillin genomic DNA), 5. Negative PCR control (water). The arrow indicates a PCR amplification of the β-tubulin region at the expected size for the positive control, but a double band was produced for the fungal test sample

Table 2.2: β-Tubulin primers designed by Einax & Voigt (2003)

Region	Primer Sequence	Amplicon Length
F-ßtub3 (Forward Primer)	5' TGGGCYAAGGGT Y*AYTAYAC 3'	1017 bp
F-ßtub4r (Reverse Primer)	5' GCCTCAGTRAA*YTCCATYTCRTC CAT 3'	

Table 2.3: Novogene (HK) in house primers used for amplification and sequencing of ITSII

Region	Primer Sequence	Amplicon Length
ITSII (Forward Primer)	5' GCATCGATGAAGAACGCAGC 3'	380 bp
ITSII (Reverse Primer)	5' TCCTCCGCTTATTGATATGC 3'	

2.3.3 Fungal Hyphae Preparation for Macrogen ITS Sequencing

The remaining DNA barcode sequencing of the ITS region was carried out by Macrogen using capillary electrophoresis (Sanger) sequencing. After sub-plating twice to ensure a pure fungal culture, a 5 mm piece of hyphae grown on MEA was harvested from each fungal sample. The hyphae were ground in 300 µL filter-sterilised DNA/RNAase free water with a sterile microcentrifuge pestle. Each suspension was mixed with 900 µL ethanol and centrifuged at 12,000 g for 5 minutes until a pellet formed. Samples were decanted and the centrifugation step was repeated. The samples were left to dry at room temperature until all ethanol evaporated. Samples were sent to Macrogen for ITS and 18s rRNA sequencing using the primers summarised in Table 2.4. As the amplicon length varied between species, Table 2.4 provides an estimate.

Table 2.4: Macrogen Primers

Region	Amplicon Length (Varies by species)	Primer Sequence
ITS1	550 bp	5' TCC GTA GGT GAA CCT GCG G 3'
ITS4		5' TCC TCC GCT TAT TGA TAT GC 3'
NS1	1500 bp	5' GTA GTC ATA TGC TTG TCT C 3'
NS8		5' TCC GCA GGT TCA CCT ACG GA 3'

2.3.4 Fungal Identification and Bioinformatics

All bioinformatics analysis was performed using Geneious Prime software (Geneious Prime 2021.1.1). Trees were constructed after aligning all sequences using MUSCLE alignment, and then applying the Geneious Prime tree builder. All species identity information was obtained through the National Center for Biotechnology Information (NCBI) and the BLAST service.

2.4 Analysis of Orchard Factors Influencing Disease Development and Progression

2.4.1 MuddyBoots Database Preparation

The avocado distribution centre, Greencell, formed in the year 2000 and merged with Westfalia in 2008. The commercial quality control data is recorded on a system called MuddyBoots. Records begin in 2012, however, early data is more sporadic and less complete, with country and location information rarely included in the reports, and grower codes are sometimes missing. For this project, historical data for Hass avocados from 2013 - 2017 were downloaded from MuddyBoots software and compiled into one large Excel spreadsheet.

There are three report detail spreadsheets which have information on SER: Intake Check (tested within 24 hours of receiving fruit), Ripening Trial Check (1-7 days after receiving fruit), and Stored Test (assessing the end of fruit life). Since SER is an age-related disease, the Ripening Trial Check was found to be the most useful. A ripening trial check was performed by selecting a set of avocados at intake, usually 16 - 20, and storing them under retail conditions until the fruit ripened. On each day of the trial, beginning with the first day, the number of avocados which had fully ripened was recorded. Each fruit was cut in half on the day it ripened, and any disease symptoms were recorded. The results from all Ripening Trial Checks from 2013 - 2017 were summarised by calculating a SER incidence for each check. SER incidence per

growing period is defined as the total average of each average Ripening Trial Check outcome. For example, if 20 avocados were ripened during a trial, and three were found to be SER positive, the average SER incidence for this check would be 0.15. All averages from every ripening trial check performed during the country's harvest period were then aggregated and a final average calculated to ascertain the SER incidence. Avocados were only cut open and assessed once they reached eating ripeness. The relationship between the degree of ripeness and disease progression through the mesocarp was not determined.

The MuddyBoots database proved to be so vast it was necessary to focus on a selection of growers which experienced high SER incidence during one harvest period, and which also had comparable periods of no SER during another harvest period. To qualify, the control harvest period had to occur during the same year, or during the same month of a different year to that of the experimental harvest period. Table 2.5 summarises growers chosen for analysis, with control and experimental harvest period.

2.4.2 Statistical Modelling and Weather Analysis

Historical weather data from the global historical weather database Visual Crossing (https://www.visualcrossing.com/weather/weather-data-services#/login) was obtained for the weather station closest to the GPS coordinates associated with each grower. Orchard locations for each country included in the MuddyBoots analysis are summarised on topographical maps in Figures 6 – 11. Hourly weather data nine months before each harvest date was obtained for each site, covering the entire period of avocado development from pollination to harvest. Factors such as temperature (°C), precipitation (cm), and wind speed (m/s) were statistically correlated with SER incidence for control and experimental groups using Statgraphics Centurion 19 (Statgraphics Technologies Inc.).

Table 2.5: Selected commercial grower locations which experienced harvest periods with high SER incidence and corresponding comparable harvest periods with zero rot incidence

	CONTROL GROUP			EXPERIMENTAL GROUP		
	Grower	**Harvest Date**	**SER Incidence**	**Grower**	**Harvest Date**	**SER Incidence**
Columbia	1	Feb-15	0	1	Mar-15	9.09
Columbia	10	Mar-16	0	10	Mar-17	100.00
Columbia	15	Aug-17	0	15	Mar-17	20.00
Chile	41	Nov-16	0	41	Dec-16	4.60
Chile	164	Nov-14	0	164	Nov-13	11.52
Chile	180	Nov-16	0	180	Nov-17	2.32
Chile	184	Nov-15	0	184	Nov-13	10.39
Chile	190	Oct-16	0	190	Dec-16	10.15
Chile	1012	Dec-14	0	1012	Dec-17	1.52
Chile	4113	Dec-17	0	4113	Dec-13	6.10
Chile	5574	Nov-15	0	5574	Nov-16	1.20
Chile	5652	Oct-13	0	5652	Nov-13	2.27
Chile	40004	Nov-15	0	40004	Dec-15	20.83
Chile	40072	Nov-14	0	40072	Nov-15	1.73
Mexico	398383	Jan-17	0	398383	Feb-17	4.13
Mexico	398888	Jan-16	0	398888	Sep-16	3.33
Spain	10*	Mar-17	0	10*	Apr-17	0.93
SouthAfrica	Wat	Apr-15	0	Wat	Jun-15	1.04
Zimbabwe	Z002	Jul-15	0	Z002	Jul-16	37.11
					19 Growers Total	

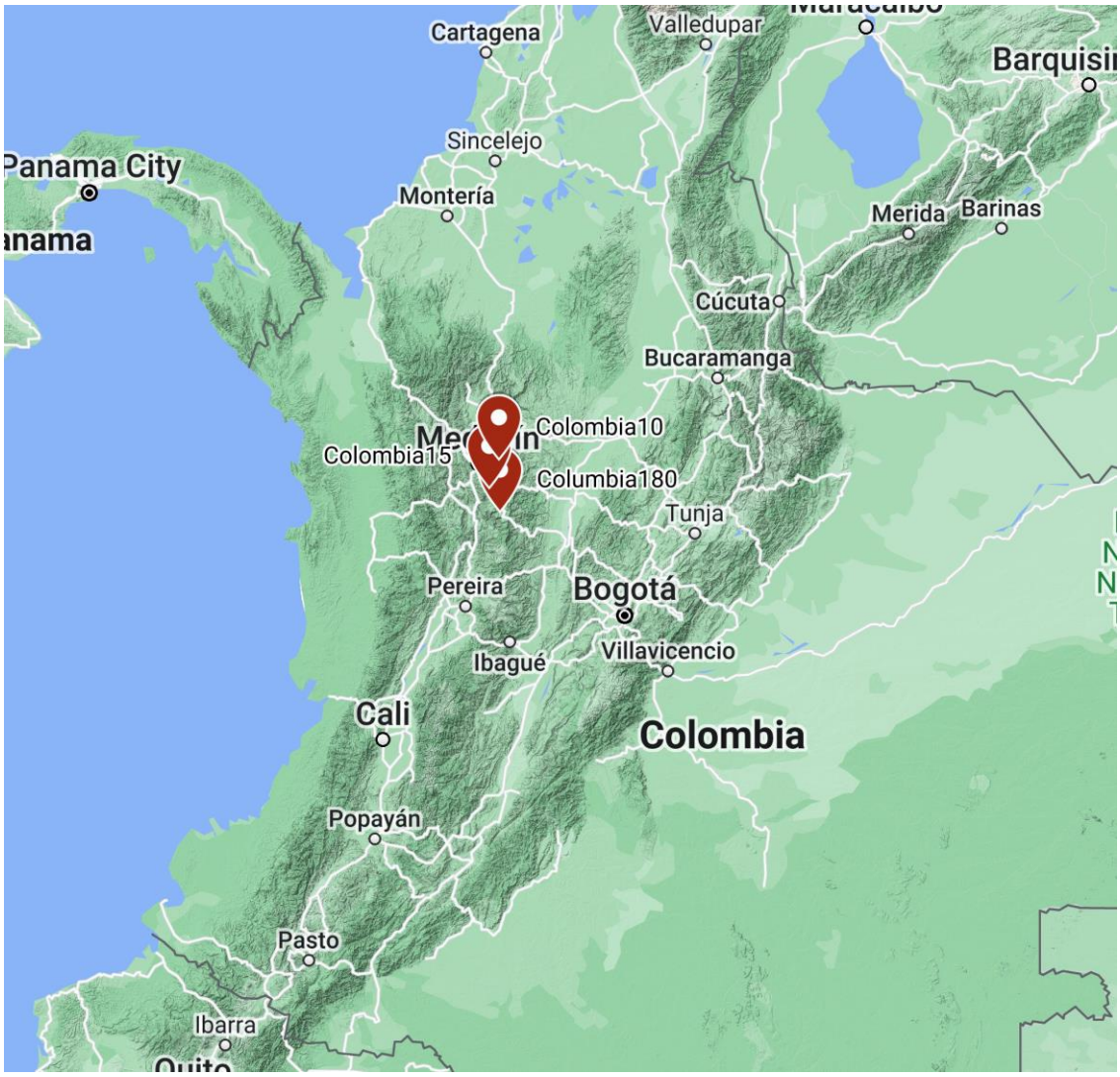

Figure 2.6: Grower locations within Colombia for grower locations Columbia1, Columbia10, Columbia15, and Columbia 180. Grower's Columbia 1 and Columbia 10 shared the same location

Figure 2.7: Grower locations within Mexico for grower locations Mexico398383 and Mexico398888. Both growers shared the same location

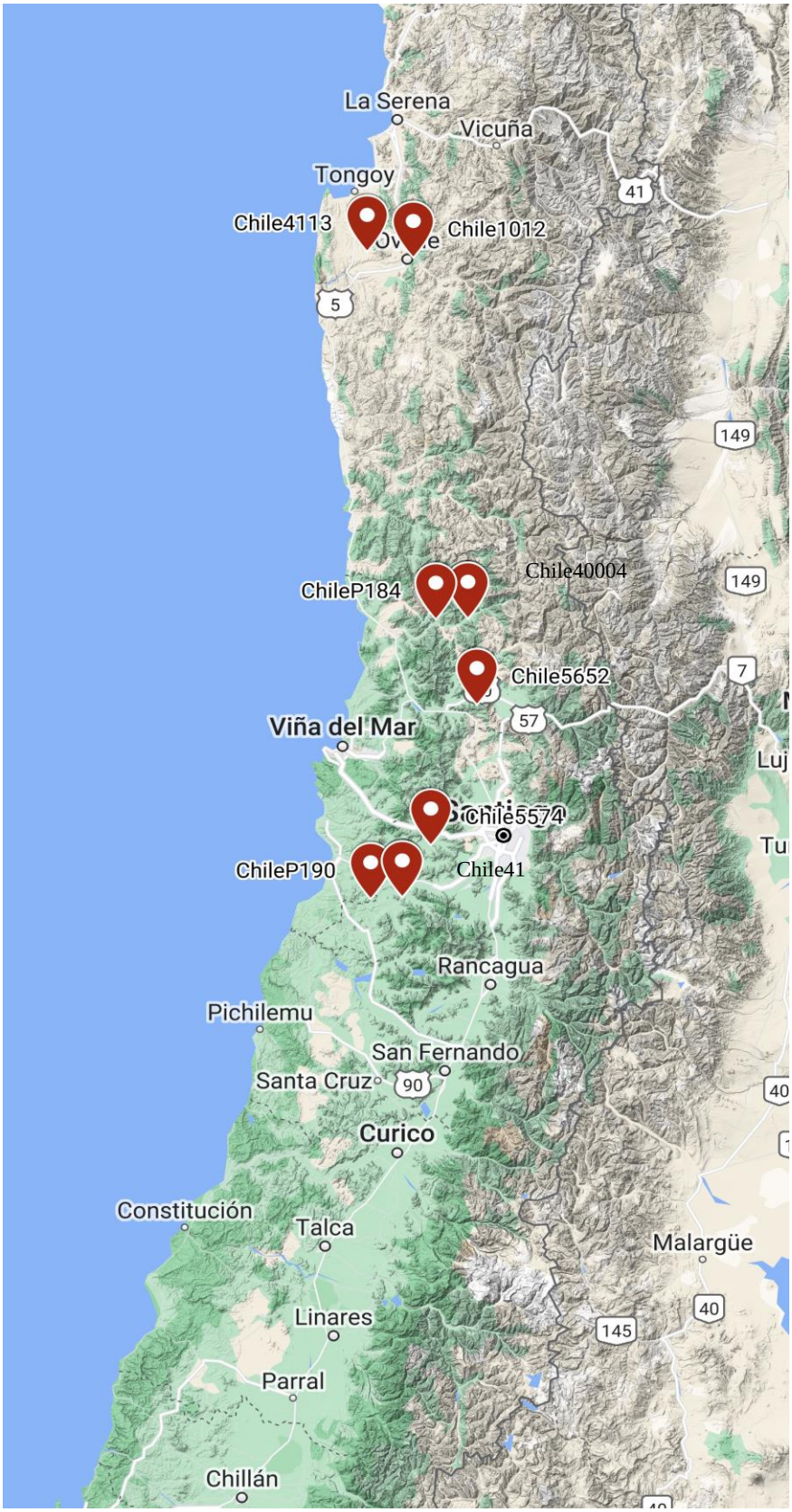

Figure 2.8: Grower locations within Chile for grower locations Chile41, Chile164, ChileP184, ChileP190, Chile1012, Chile4113, Chile5574, Chile5652, Chile40004, and Chile40072. Growers Chile41 and Chile 164 share the same location, and Chile 40004 and Chile40072 share the same location

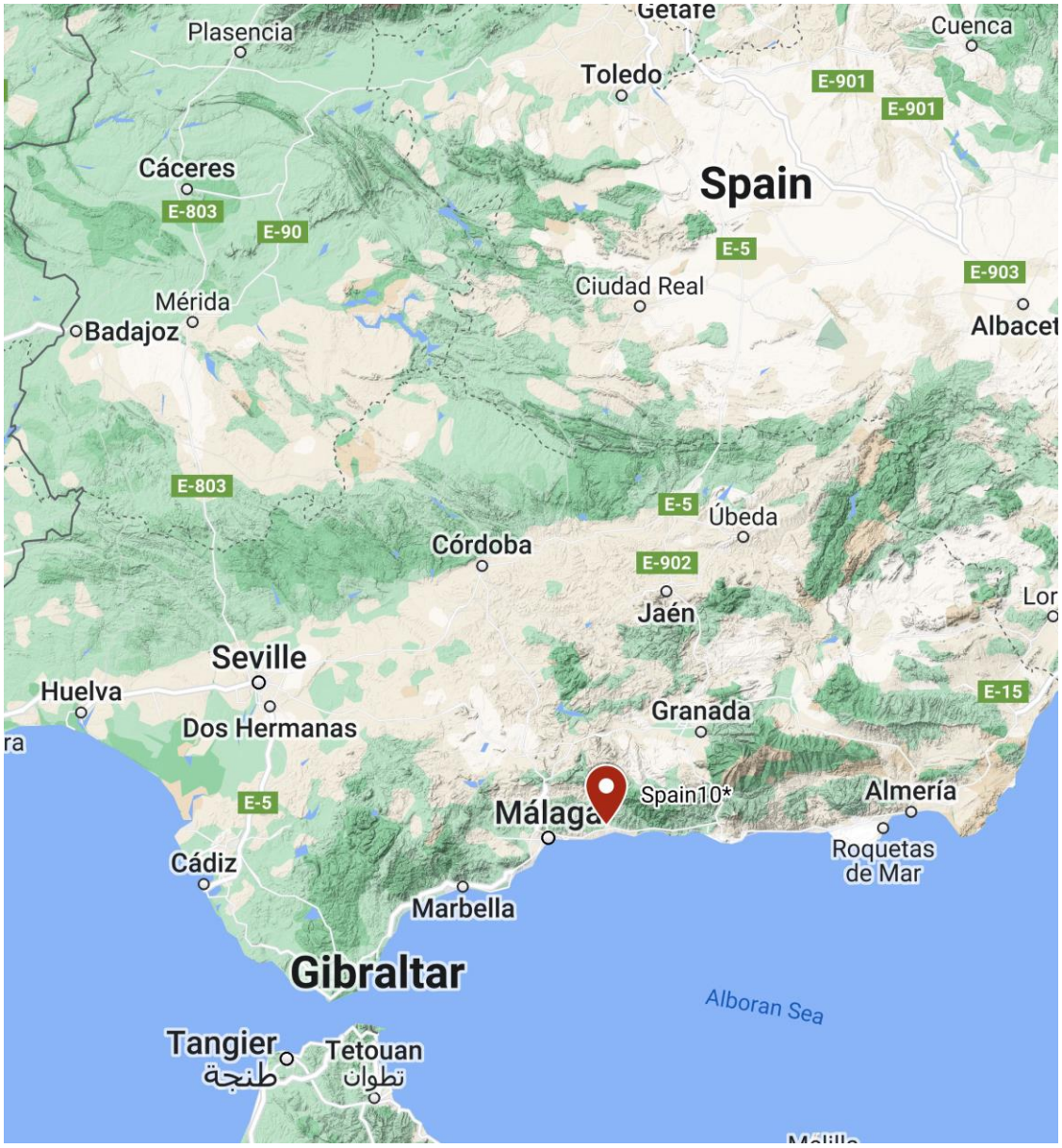

Figure 2.9: Grower location within Spain for one grower, Spain10*

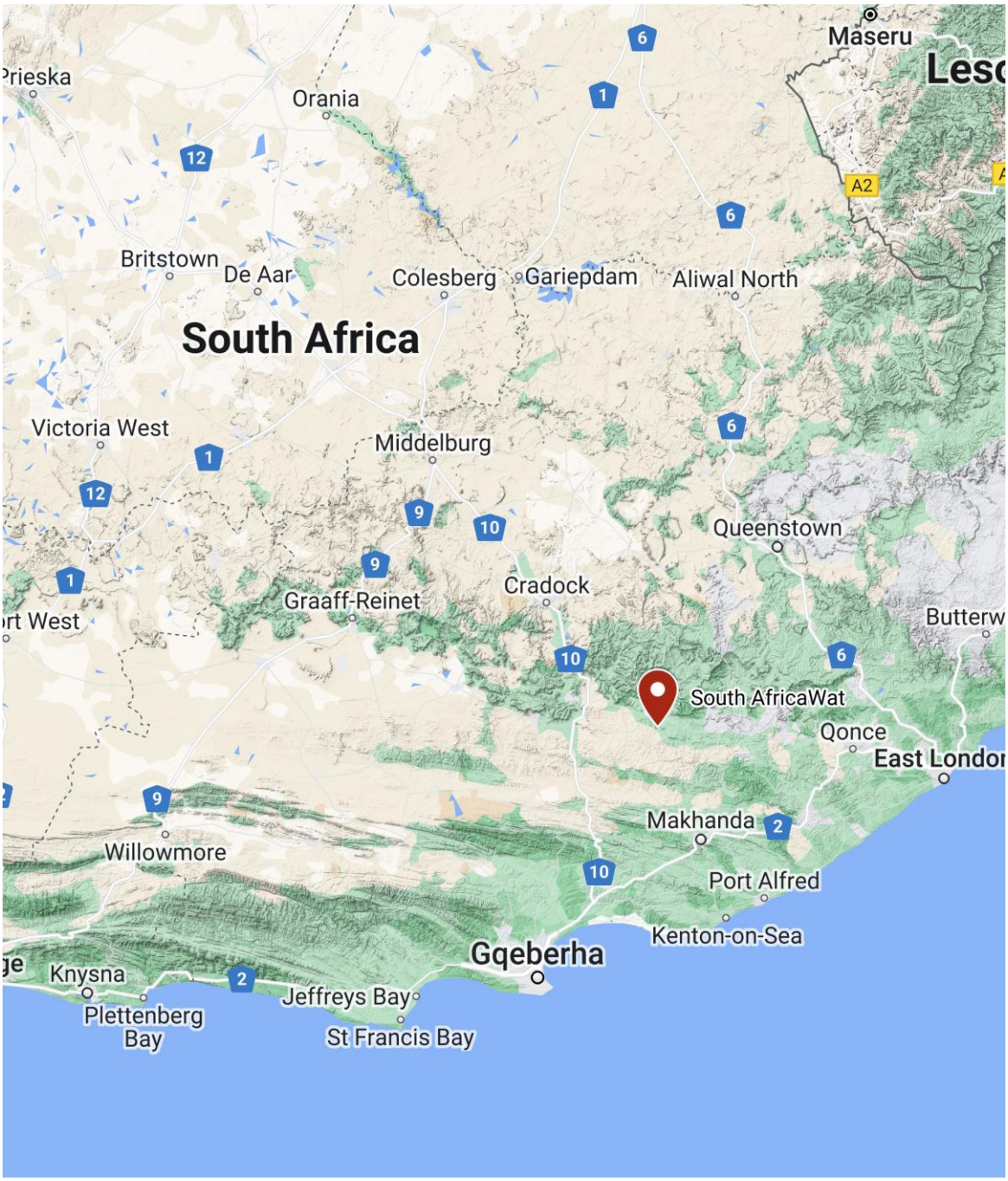

Figure 2.10: Grower location within South Africa for one grower, South AfricaWat

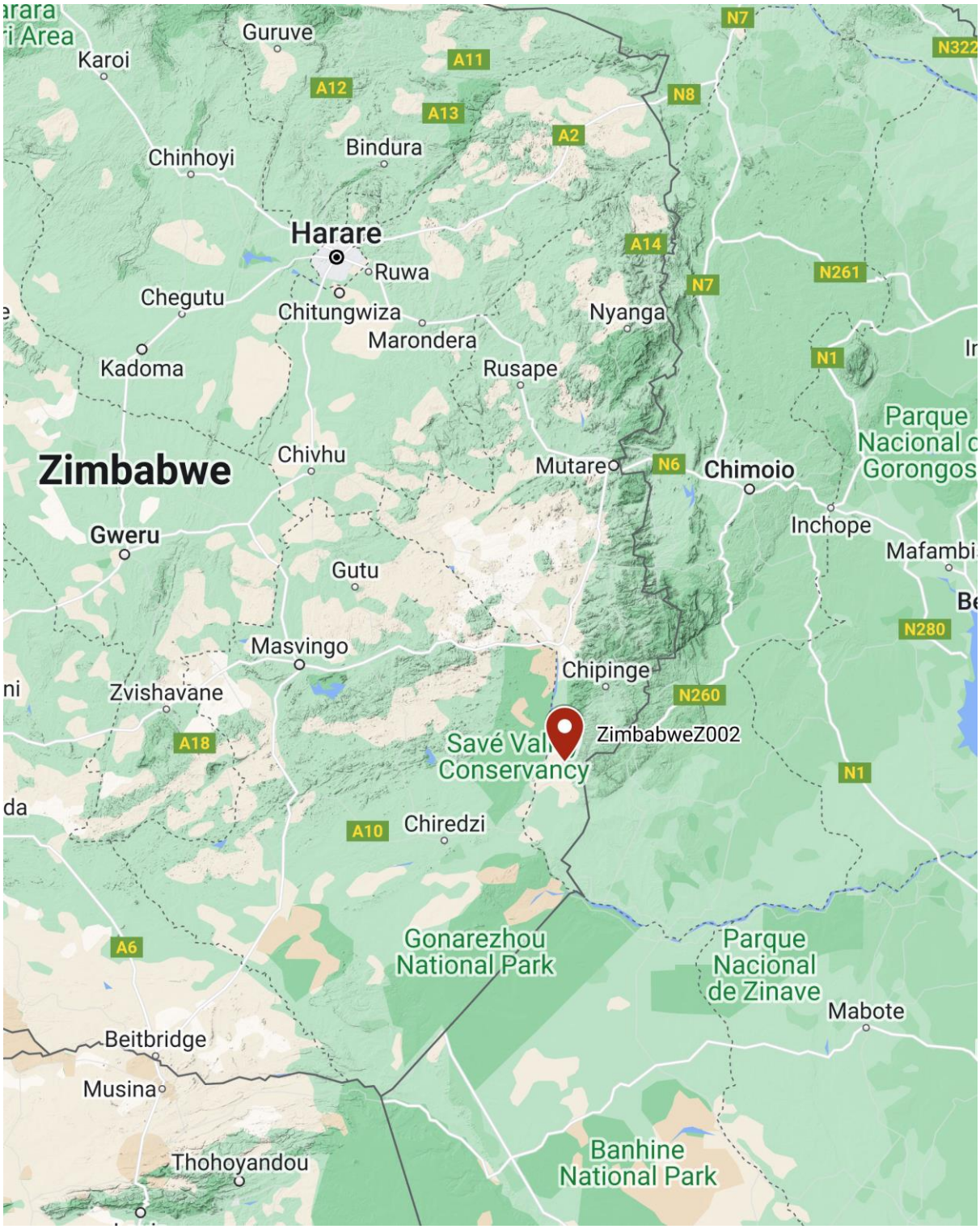

Figure 2.11: Grower location within Zimbabwe for one grower, ZimbabweZ002

2.5 Koch Inoculation Procedure

Filter paper cut into five 1 cm by 1 cm squares were placed on MEA plates next to a fungal hyphae plug and incubated for 72 hours at 25 °C to serve as a reproducible inoculum. Clean avocados, originating from Peru, with no visible rots were washed in a solution of 5% bleach. Once dry, avocados were surface sterilised with a 70% ethanol solution. Under sterile conditions, the top of the avocado was cut off crosswise with a flame sterilised scalpel, approximately 3 cm from the stem button to eliminate any potential quiescent infection in the abscission zone. One piece of filter paper, infused with fungal hyphae, was placed on the exposed avocado mesocarp. An autoclaved piece of un-inoculated 1 cm x 1 cm filter paper was placed on a separate set of fruit to serve as a control. The entire cut ends of the avocados were then sealed with paraffin wax, melted over a candle. The Koch inoculation experiments were carried out in sets of five avocados. The five avocados within a set were inoculated with the same pathogen, therefore serving as five technical replicates of one biological replicate. Multiple sets of avocados were inoculated at once, with only one set of controls serving as the control for the group. There were three control sets in total, for 14 fungal pathogen biological replicates with five technical replicates each.

Avocados were incubated for 96 hours at 25 °C, and then cut in half lengthwise to record disease symptoms. A section of infected tissue was taken from the leading browning margin of the mesocarp and plated on MEA plates with no selection. These samples were incubated at 25 °C for 72 hours to compare the re-isolated morphotype to the originally isolated fungus. Images were recorded using a Casio Ex-Z150 Exilim 8.1 Megapixel camera.

CHAPTER 3: MUDDYBOOTS DATA INFORMATICS

3.1 Introduction

SER disease incidence has been highly correlated with rainfall and humidity, both because these conditions facilitate fungal germination, and because fruit watered in excess are more susceptible to infection (Bower & Cutting, 1987; Twizeyimana et al., 2013). Wind is also a factor in spore dispersal for some species (Brown & Hovmoller, 2002). Proper tree management and breeding may not be enough to overcome a perfect storm of abiotic factors favourable for maximum pathogen growth.

The most effective defence against the postharvest fungal disease of any fruit is the application of appropriate fungicides, most commonly a copper fungicide. Although copper fungicide is an efficient broad-spectrum inhibitor, active against a wide range of fungal genera regardless of strain, the method and timing of application are critical to ensure that the toxin reaches its intended target (Everett et al., 2007). Investigating exactly how SER incidence correlates with rainfall, humidity, and wind speed, or at which temperature fruit are most susceptible to infection, will help inform growers of the ideal time to apply appropriate fungicides.

SER incidence is expected to increase for avocados grown under conditions of increased humidity and rainfall. Disease incidence is also expected to increase with increasing mean temperature up to a point, and then decrease after it exceeds the upper threshold of ideal germination temperature. Spore germination is highly correlated with temperature, and the new spores often require rainfall to disperse, but microflora differ between countries. For example, SER causal species are often related to those found in the environment surrounding the orchard (Schaffer et al., 2013). The temperatures most conducive to SER infection would clarify one aspect of how the environment favours different pathogen species.

The specific objectives of this study were:

1. To compare the patterns of temperature, wind, humidity, and rainfall for orchards that exhibited particularly high SER incidence during one growing season to another growing season in which they were SER free
2. To determine if SER incidence increases when environmental conditions in the orchard match the ideal sporulation conditions established for common SER pathogens in the laboratory
3. To explore fluctuations in the timing and fluctuation of temperature, rainfall, humidity, and wind speed patterns during the flowering and harvest period of the avocado growing season and their potential effects on SER incidence

3.2 Methods and Data Accumulation

An investigation of Greencell's quality control database, MuddyBoots, provided insight into potential variation of SER incidence at the end of the supply chain. The database was extensive. Greencell formed in 2000, it merged with Westfalia in 2008, and the quality control data goes back as far as 2012.

When avocados arrive at the Greencell distribution centre, they are examined for cosmetic and quality control issues, such as skin injury or blemishes that would influence consumer purchase. These data are recorded at intake to alert the grower of potential issues with their shipment. Rot is not evident at this stage, because the avocados must ripen first for symptoms to develop. There are only three reports which record information on SER: the Intake Check (avocados are tested within 24 hours of receiving fruit), Ripening Trial Check (testing 1-7 days after receiving the fruit), and Shelf Life Test (examining the end of fruit life). As SER is an age-related disease, the Ripening Trial Check was found to be the most useful. SER incidence was measured as

the total average of the mean values of SER positive avocados found for all ripening trial checks of a single harvest period. For example, one harvest could have two ripening trial checks associated with it. If one ripening trial check found two avocados positive for SER and another ripening trial check found three SER positive avocados, and the total number of avocados tested were 20 for each, the average for each of these would be 0.10 and 0.15, respectively. The total SER incidence for the growing period would be 0.13.

When all Ripening Trial Check's from 2012 – 2017 were compiled, twenty farmers were identified as having the highest variation in SER incidence. These are listed in Table 2.1. The farms were selected from the original MuddyBoots data if they experienced SER incidence > 0.5 indices for that harvest month. The growers must also have experienced at least one harvest month with no SER incidence, thereby providing a control which affects the fewest possible variables.

Historical weather data from Visual Crossing (https://www.visualcrossing.com/) was obtained for the weather station closest to the GPS coordinates associated with each grower. Hourly weather data nine months before each harvest date covered the entire period of avocado development from pollination to harvest. Factors such as temperature (°C), humidity (%), precipitation (mm), and wind speed (m/s) were statistically correlated with SER incidence for control and experimental groups using a two sample Student's t-test assuming equal variance. The most common and accepted plot for communicating the results of an independent samples t-test is a plot of means with standard error bars. This best represents the theory of a t-test, which mathematically compares two means using their standard errors to assess whether two groups differ more between each other than within themselves.

3.3 Weather Analysis Results

Student's t-test results comparing weather records during a period of high rot with that of zero rot for each of the 19 growers are summarised in Tables 3.1 – 3.3. This was the most appropriate test to visualise the total range for each weather variable for one recorded SER incidence measurement. Other statistical strategies, such as multiple linear regression, would have required multiple comparable SER incidence measurements which were not available in this dataset. The calculations in Table 3.1 compare weather factors throughout the total nine-month growing period. Table 3.2 summarises the t-test calculations for the flowering period, defined as the first 30 days of the growing period, and Table 3.3 summarises the t-test calculations for the harvest period, defined as the last 30 days of the growing season. Figures 3.1 – 3.12 offer a visual representation of the Student's t-test results. Average temperatures for the whole growing period, flowering period, and harvest period for the experimental and control group of each grower, with 95% confidence intervals, are shown in Figrues 3.1 – 3.3. Humidity averages are summarised in Figures 3.4 – 3.6, wind speed in Figures 3.7 – 3.9, and precipitation in Figures 3.10 – 3.12. Overall, higher precipitation and humidity during harvest often resulted in more rot, and increased wind speeds during flowering decreased rot incidence. There were no weather factors which consistently increased SER incidence no matter the location. The Student's t-test was performed on the total hourly weather dataset for each weather variable, which comprised all hourly measurements for nine months.

Table 3.1: Two sample Student's t-test results, assuming equal variance, comparing hourly temperature, humidity, wind, and precipitation levels recorded at a weather station nearest to the orchard during a period of high rot with those recorded during a zero-rot harvest for each of the 19 growers for the entire 9-month avocado growing season

TOTAL GROWING PERIOD					
		df	**t Statistic**	**p value (one-tail)**	**p value (two-tail)**
Columbia01	Temperature	14459	-0.04	0.48	0.97
	Humidity	14459	-0.34	0.37	0.73
	Wind	14441	0.31	0.38	0.76
	Precipitation	14590	0.44	0.33	0.66
Columbia10	Temperature	14603	-13.87	0.00	0.00
	Humidity	14602	10.53	0.00	0.00
	Wind	14600	2.71	0.00	0.01
	Precipitation	14614	2.52	0.01	0.01
Columbia15	Temperature	14557	-0.21	0.42	0.84
	Humidity	14555	-3.96	0.00	0.00
	Wind	14555	1.80	0.04	0.07
	Precipitation	14590	-0.88	0.19	0.38
Columbia180	Temperature	14549	-8.65	0.00	0.00
	Humidity	14548	6.60	0.00	0.00
	Wind	14549	-2.54	0.01	0.01
	Precipitation	14566	0.47	0.32	0.64
Chile41	Temperature	14613	-0.76	0.22	0.45
	Humidity	14613	1.24	0.11	0.22
	Wind	14613	0.75	0.23	0.45
	Precipitation	14639	-0.07	0.47	0.95
Chile164	Temperature	13804	-7.14	0.00	0.00
	Humidity	13804	1.40	0.08	0.16
	Wind	13803	-4.47	0.00	0.00
	Precipitation	13871	-1.13	0.13	0.26
Chile184	Temperature	14394	-13.70	0.00	0.00
	Humidity	14394	13.89	0.00	0.00
	Wind	14393	-2.24	0.01	0.03
	Precipitation	14543	-0.95	0.17	0.34
Chile190	Temperature	14639	-3.25	0.00	0.00
	Humidity	14636	-0.03	0.49	0.98
	Wind	14636	0.93	0.18	0.35
	Precipitation	14662	-0.92	0.18	0.36
Chile1012	Temperature	14392	15.83	0.00	0.00
	Humidity	14392	3.18	0.00	0.00
	Wind	14391	4.21	0.00	0.00
	Precipitation	14659	1.34	0.09	0.18
Chile4113	Temperature	14351	-13.39	0.00	0.00
	Humidity	14351	-0.11	0.46	0.92
	Wind	14348	-3.47	0.00	0.00
	Precipitation	14660	-2.94	0.00	0.00
Chile5574	Temperature	14468	-0.78	0.22	0.44
	Humidity	14468	12.49	0.00	0.00
	Wind	14468	-1.11	0.13	0.27
	Precipitation	14568	-0.44	0.33	0.66
Chile5652	Temperature	14565	-3.31	0.00	0.00
	Humidity	14565	-3.46	0.00	0.00
	Wind	14565	-0.08	0.47	0.94
	Precipitation	14567	-0.07	0.47	0.95
Chile40004	Temperature	14498	-9.14	0.00	0.00
	Humidity	14498	8.26	0.00	0.00
	Wind	14498	-4.68	0.00	0.00
	Precipitation	14639	0.06	0.48	0.95
Chile40072	Temperature	14448	12.64	0.00	0.00
	Humidity	14448	-9.37	0.00	0.00
	Wind	14448	3.19	0.00	0.00
	Precipitation	14544	0.75	0.23	0.45
Mexico398383	Temperature	8348	-2.62	0.00	0.01
	Humidity	8346	-3.96	0.00	0.00
	Wind	7342	1.38	0.08	0.17
	Precipitation	12431	-1.04	0.15	0.30
Mexico398888	Temperature	9525	-2.38	0.01	0.02
	Humidity	9525	-5.42	0.00	0.00
	Wind	8290	2.08	0.02	0.04
	Precipitation	14662	-1.92	0.03	0.05
Spain10	Temperature	14590	-6.69	0.00	0.00
	Humidity	14590	2.14	0.02	0.03
	Wind	14590	-1.30	0.10	0.19
	Precipitation	14590	0.19	0.43	0.85
SouthAfricaWat	Temperature	12810	10.42	0.00	0.00
	Humidity	12810	7.84	0.00	0.00
	Wind	12810	1.67	0.05	0.09
	Precipitation	13102	0.89	0.19	0.37
ZimbabweZ002	Temperature	9373	11.41	0.00	0.00
	Humidity	9367	2.48	0.01	0.01
	Wind	7713	13.15	0.00	0.00
	Precipitation	14615	-1.50	0.07	0.13

Table 3.2: Two sample Student's t-test results, assuming equal variance, comparing hourly temperature, humidity, wind, and precipitation levels recorded at a weather station nearest to the orchard during a period of high rot with those recorded during a zero-rot harvest for each of the 19 growers for the first 30 days of the avocado growing season, defined as the flowering period

FLOWERING PERIOD					
		df	**t Statistic**	**p value (one-tail)**	**p value (two-tail)**
Columbia01	Temperature	1074	8.15	0.00	0.00
	Humidity	1073	-1.41	0.08	0.16
	Wind	912	1.52	0.06	0.13
	Precipitation	1486	-1.09	0.14	0.27
Columbia10	Temperature	1486	-0.85	0.20	0.40
	Humidity	1486	3.37	0.00	0.00
	Wind	1485	-0.34	0.37	0.74
	Precipitation	1486	-0.54	0.29	0.59
Columbia15	Temperature	1482	6.84	0.00	0.00
	Humidity	1482	-9.21	0.00	0.00
	Wind	1482	2.99	0.00	0.00
	Precipitation	1486	-1.30	0.10	0.20
Columbia180	Temperature	1485	-8.91	0.00	0.00
	Humidity	1485	1.52	0.06	0.13
	Wind	1485	-1.21	0.11	0.23
	Precipitation	1486	1.33	0.09	0.18
Chile41	Temperature	1486	-9.24	0.00	0.00
	Humidity	1486	9.29	0.00	0.00
	Wind	1486	-5.91	0.00	0.00
	Precipitation	1486	-0.58	0.28	0.56
Chile164	Temperature	1485	-7.22	0.00	0.00
	Humidity	1485	3.10	0.00	0.00
	Wind	1485	-7.88	0.00	0.00
	Precipitation	1485	3.10	0.00	0.00
Chile184	Temperature	1478	-4.74	0.00	0.00
	Humidity	1478	12.25	0.00	0.00
	Wind	1478	-6.44	0.00	0.00
	Precipitation	1486	0.73	0.23	0.47
Chile190	Temperature	1486	-12.02	0.00	0.00
	Humidity	1486	5.36	0.00	0.00
	Wind	1486	-6.77	0.00	0.00
	Precipitation	1486	-1.01	0.16	0.31
Chile1012	Temperature	1466	18.11	0.00	0.00
	Humidity	1466	-6.59	0.00	0.00
	Wind	1466	2.49	0.01	0.01
	Precipitation	1486	-1.90	0.03	0.06
Chile4113	Temperature	1467	-13.67	0.00	0.00
	Humidity	1467	7.48	0.00	0.00
	Wind	1467	-2.69	0.00	0.01
	Precipitation	1486	0.00	0.00	0.00
Chile5574	Temperature	1479	0.84	0.20	0.40
	Humidity	1479	1.49	0.07	0.14
	Wind	1479	-0.09	0.46	0.92
	Precipitation	1486	1.32	0.09	0.19
Chile5652	Temperature	1486	1.34	0.09	0.18
	Humidity	1486	-10.70	0.00	0.00
	Wind	1486	-2.44	0.01	0.01
	Precipitation	1486	0.71	0.24	0.48
Chile40004	Temperature	1485	-3.27	0.00	0.00
	Humidity	1485	1.42	0.08	0.16
	Wind	1485	-7.92	0.00	0.00
	Precipitation	1486	1.27	0.10	0.20
Chile40072	Temperature	1478	7.60	0.00	0.00
	Humidity	1478	-6.55	0.00	0.00
	Wind	1478	3.74	0.00	0.00
	Precipitation	1486	1.00	0.16	0.32
Mexico398383	Temperature	992	0.00	0.50	1.00
	Humidity	992	0.00	0.50	1.00
	Wind	868	0.00	0.50	1.00
	Precipitation	1486	0.00	0.50	1.00
Mexico398888	Temperature	942	-19.32	0.00	0.00
	Humidity	942	12.91	0.00	0.00
	Wind	817	-2.06	0.02	0.04
	Precipitation	1486	-1.59	0.06	0.11
Spain10	Temperature	1486	15.53	0.00	0.00
	Humidity	1486	1.47	0.07	0.14
	Wind	1486	-2.06	0.02	0.04
	Precipitation	1486	-2.53	0.01	0.01
SouthAfricaWat	Temperature	1465	8.23	0.00	0.00
	Humidity	1465	13.10	0.00	0.00
	Wind	1465	-0.47	0.32	0.64
	Precipitation	1486	3.37	0.00	0.00
ZimbabweZ002	Temperature	1074	8.15	0.00	0.00
	Humidity	1073	-1.41	0.08	0.16
	Wind	912	1.52	0.06	0.13
	Precipitation	1486	-1.09	0.14	0.27

Table 3.3: Two sample Student's t-test results, assuming equal variance, comparing hourly temperature, humidity, wind, and precipitation levels recorded at a weather station nearest to the orchard during a period of high rot with those recorded during a zero-rot harvest for each of the 19 growers for the final 30 days of the avocado growing season, defined as the harvest period

HARVEST PERIOD					
		df	**t Statistic**	**p value (one-tail)**	**p value (two-tail)**
Columbia01	Temperature	1195	-1.94	0.03	0.05
	Humidity	1195	3.31	0.00	0.00
	Wind	823	8.35	0.00	0.00
	Precipitation	1486	2.13	0.02	0.03
Columbia10	Temperature	1485	-13.58	0.00	0.00
	Humidity	1487	7.15	0.00	0.00
	Wind	1486	-2.78	0.00	0.01
	Precipitation	1486	3.37	0.00	0.00
Columbia15	Temperature	1485	-7.05	0.00	0.00
	Humidity	1485	7.75	0.00	0.00
	Wind	1485	-6.99	0.00	0.00
	Precipitation	1486	1.41	0.08	0.16
Columbia180	Temperature	1484	2.59	0.00	0.01
	Humidity	1484	1.10	0.14	0.27
	Wind	1484	-1.26	0.10	0.21
	Precipitation	1486	-1.36	0.09	0.18
Chile41	Temperature	1486	4.00	0.00	0.00
	Humidity	1486	3.57	0.00	0.00
	Wind	1486	1.33	0.09	0.18
	Precipitation	1486	-1.00	0.16	0.32
Chile164	Temperature	1478	2.64	0.00	0.01
	Humidity	1478	-2.34	0.01	0.02
	Wind	1478	0.39	0.35	0.70
	Precipitation	1486	-1.45	0.07	0.15
Chile184	Temperature	1449	0.76	0.22	0.45
	Humidity	1449	-4.34	0.00	0.00
	Wind	1449	3.21	0.00	0.00
	Precipitation	1486	-1.40	0.08	0.16
Chile190	Temperature	1485	15.97	0.00	0.00
	Humidity	1485	-2.82	0.00	0.00
	Wind	1485	4.97	0.00	0.00
	Precipitation	1486	-1.37	0.09	0.17
Chile1012	Temperature	1476	7.49	0.00	0.00
	Humidity	1476	1.38	0.08	0.17
	Wind	1476	-0.59	0.28	0.56
	Precipitation	1486	-0.45	0.33	0.65
Chile4113	Temperature	1474	2.47	0.01	0.01
	Humidity	1474	-2.86	0.00	0.00
	Wind	1474	0.20	0.42	0.84
	Precipitation	1486	-1.00	0.16	0.32
Chile5574	Temperature	1456	7.77	0.00	0.00
	Humidity	1456	-5.78	0.00	0.00
	Wind	1456	0.78	0.22	0.44
	Precipitation	1486	-1.30	0.10	0.19
Chile5652	Temperature	1486	8.38	0.00	0.00
	Humidity	1486	-4.46	0.00	0.00
	Wind	1486	4.91	0.00	0.00
	Precipitation	1486	1.68	0.05	0.09
Chile40004	Temperature	1459	19.12	0.00	0.00
	Humidity	1459	-10.34	0.00	0.00
	Wind	1459	4.79	0.00	0.00
	Precipitation	1486	-2.24	0.01	0.02
Chile40072	Temperature	1455	-1.24	0.11	0.22
	Humidity	1455	7.17	0.00	0.00
	Wind	1455	-0.93	0.18	0.35
	Precipitation	1486	-0.50	0.31	0.62
Mexico398383	Temperature	990	4.89	0.00	0.00
	Humidity	990	-10.34	0.00	0.00
	Wind	884	4.77	0.00	0.00
	Precipitation	1486	-3.71	0.00	0.00
Mexico398888	Temperature	986	27.84	0.00	0.00
	Humidity	986	0.84	0.20	0.40
	Wind	851	-3.05	0.00	0.00
	Precipitation	1486	4.41	0.00	0.00
Spain10	Temperature	1486	6.21	0.00	0.00
	Humidity	1486	-1.77	0.04	0.08
	Wind	1486	-5.41	0.00	0.00
	Precipitation	1486	-1.53	0.06	0.13
SouthAfricaWat	Temperature	1368	0.00	0.50	1.00
	Humidity	1368	0.00	0.50	1.00
	Wind	1368	0.00	0.50	1.00
	Precipitation	1486	0.00	0.50	1.00
ZimbabweZ002	Temperature	1195	-1.94	0.03	0.05
	Humidity	1195	3.31	0.00	0.00
	Wind	823	8.35	0.00	0.00
	Precipitation	1486	2.13	0.02	0.03

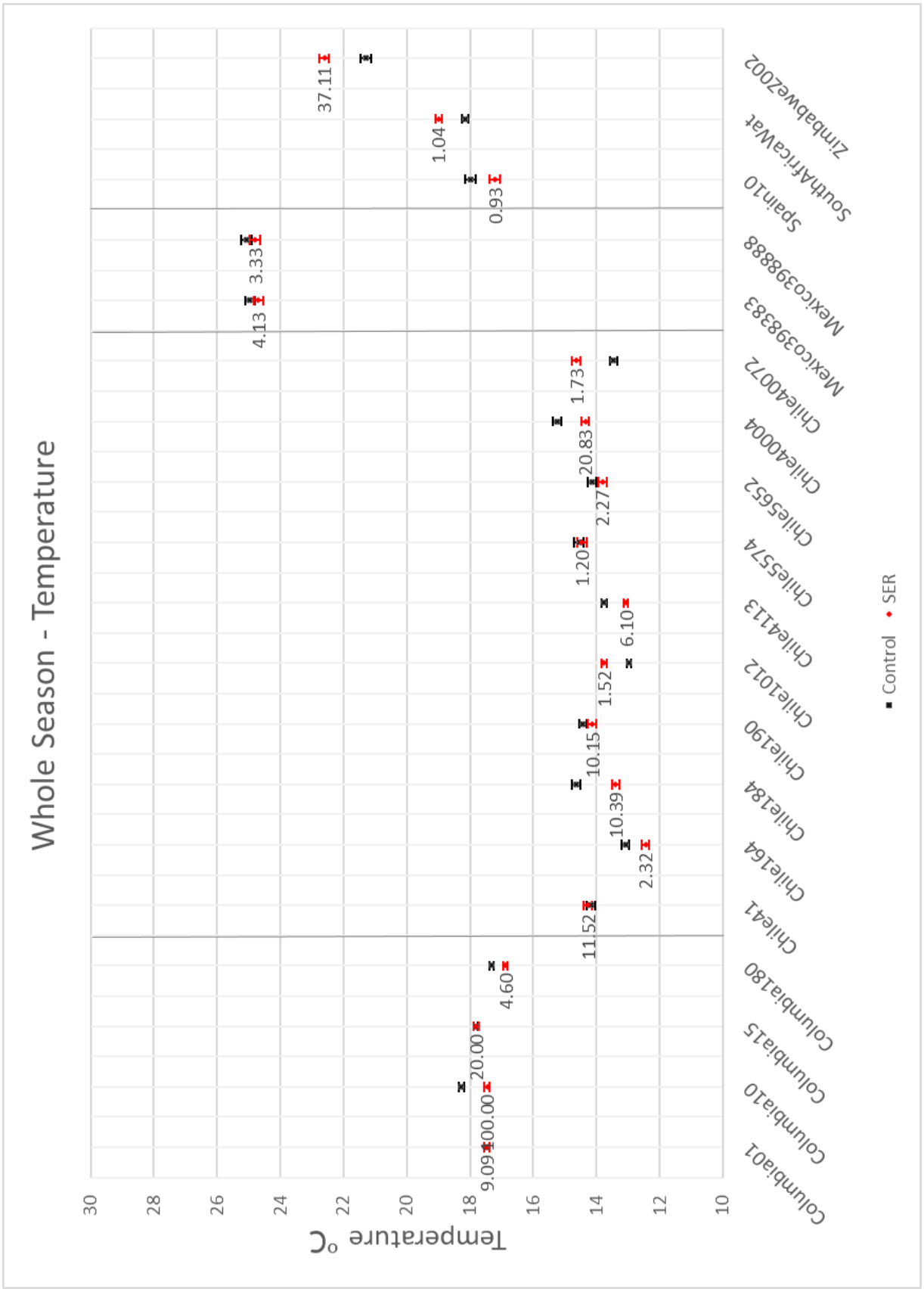

Figure 3.1: Average hourly temperatures recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the entire avocado growing season. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

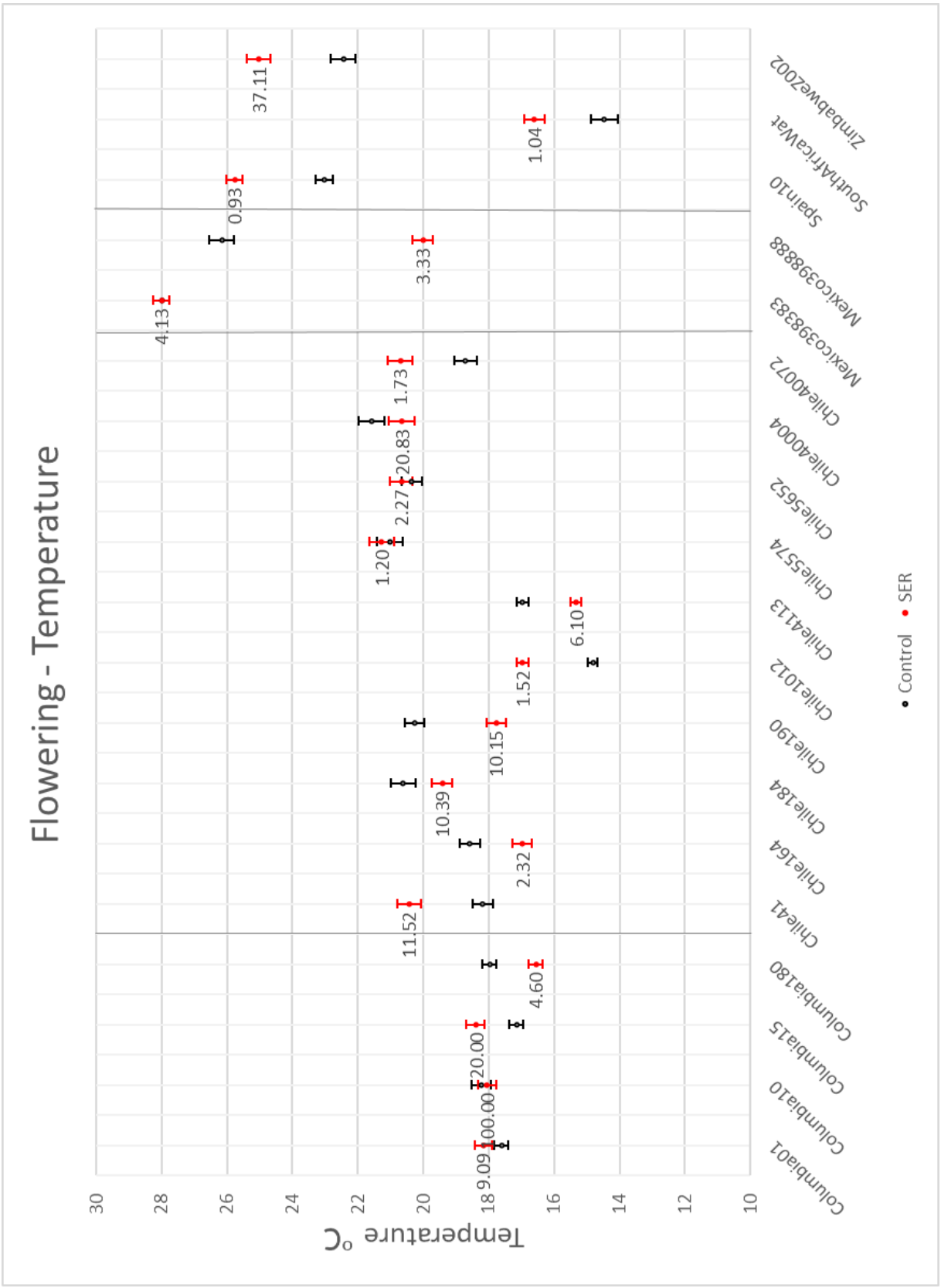

Figure 3.2: Average hourly temperatures recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the first 30 days of the growing season, defined as the flowering period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

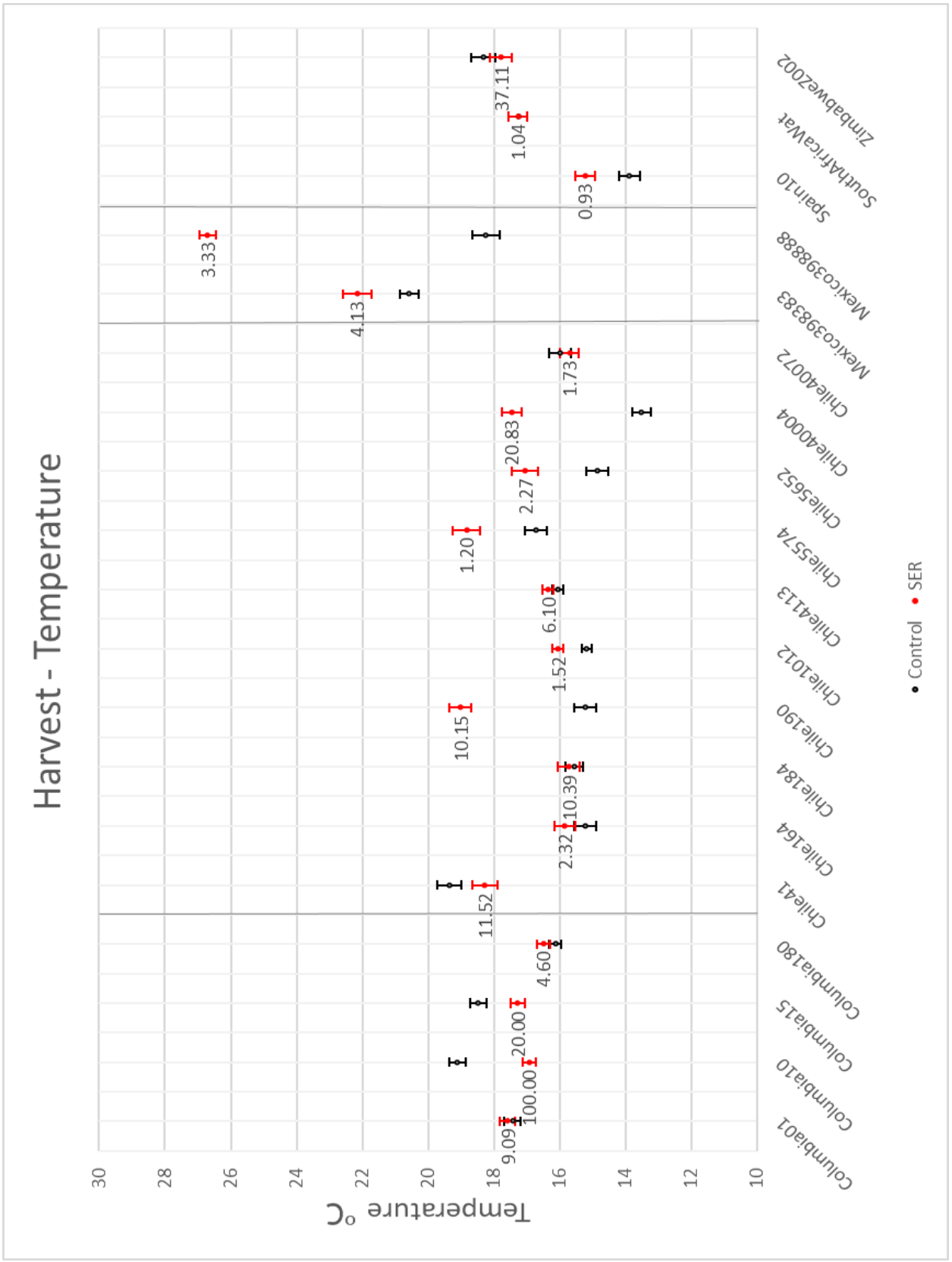

Figure 3.3: Average hourly temperatures recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the final 30 days of the growing season, defined as the harvest period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

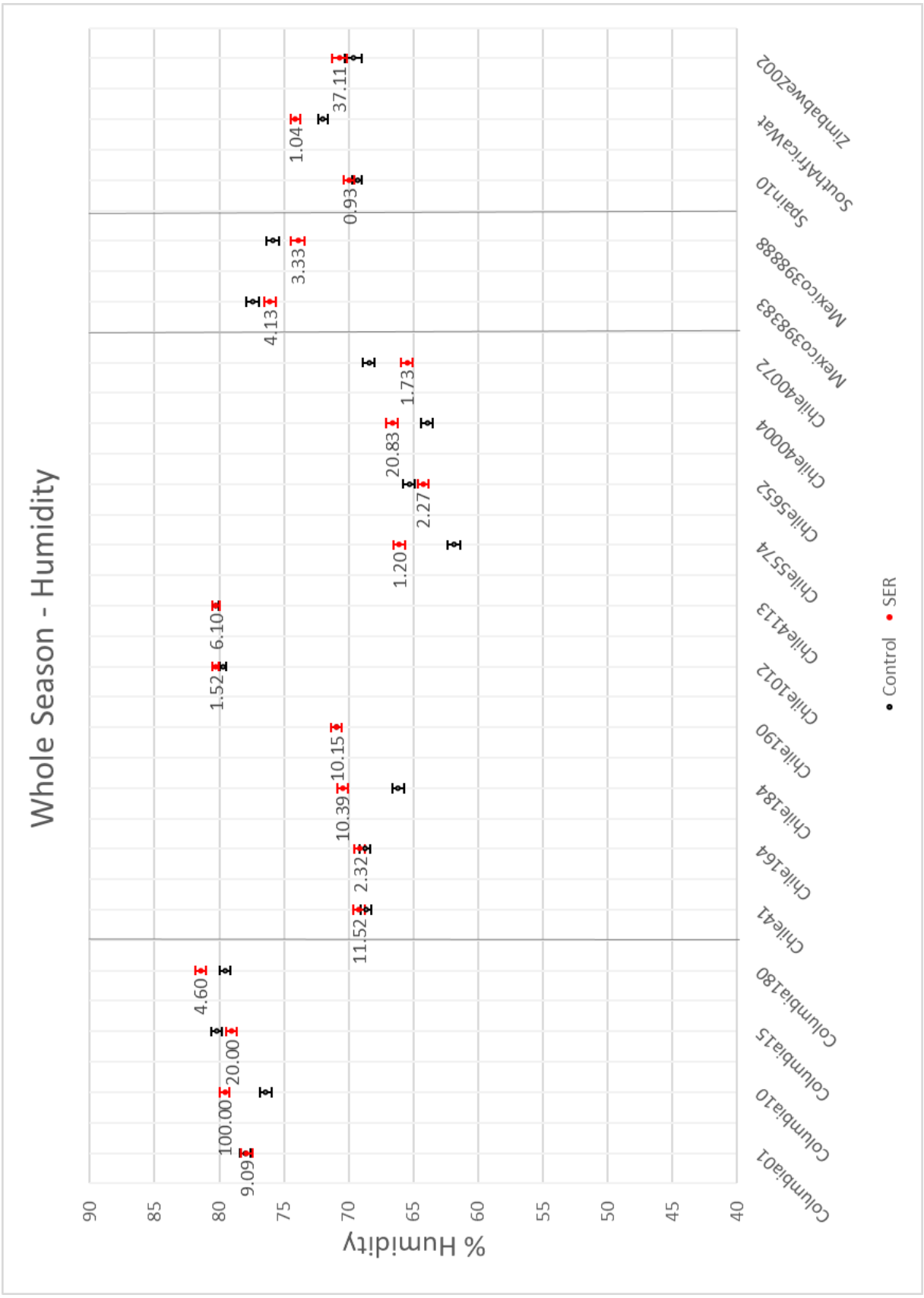

Figure 3.4: Average hourly percent humidity recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the entire avocado growing season. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

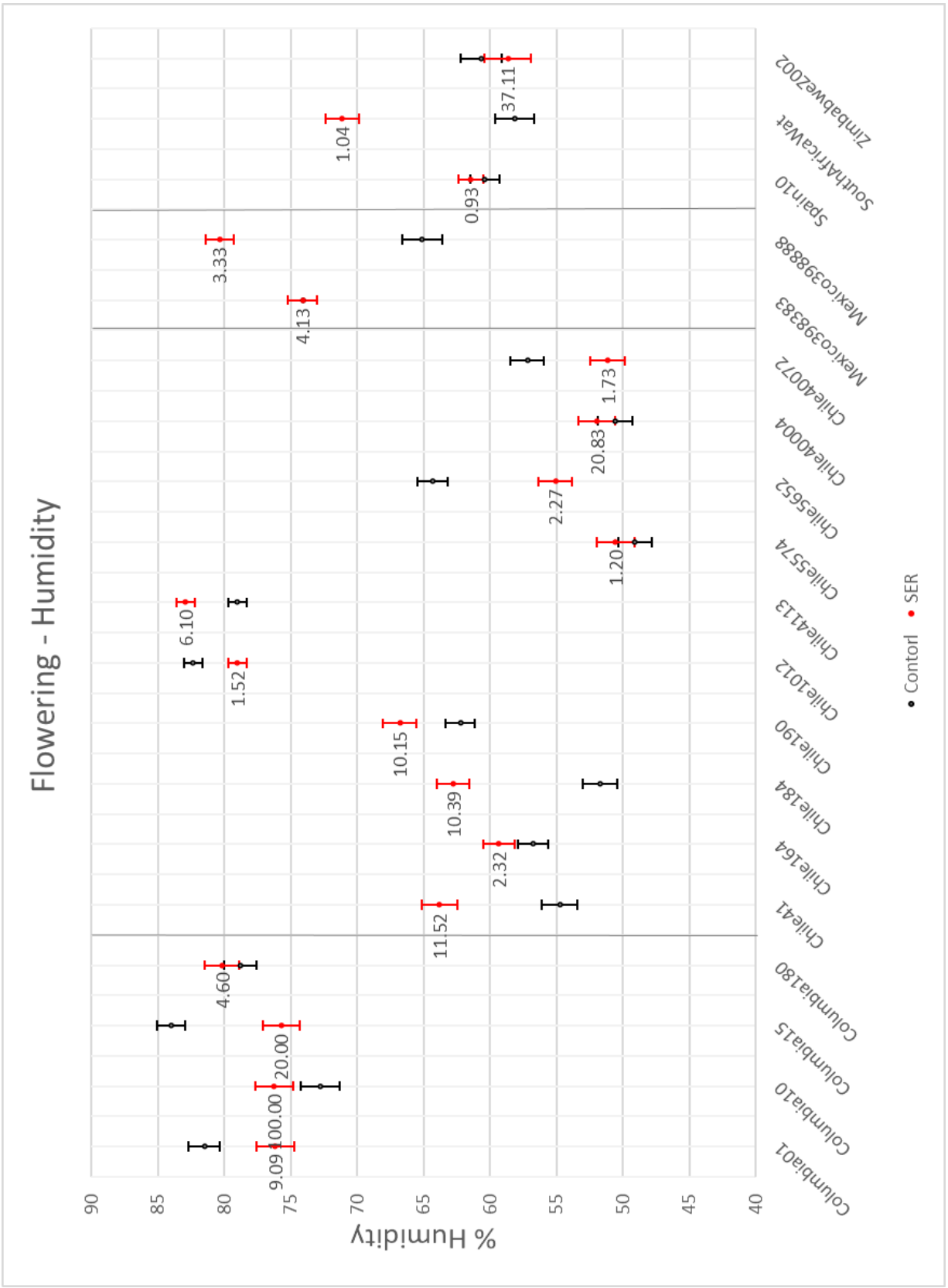

Figure 3.5: Average hourly percent humidity recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the first 30 days of the growing season, defined as the flowering period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

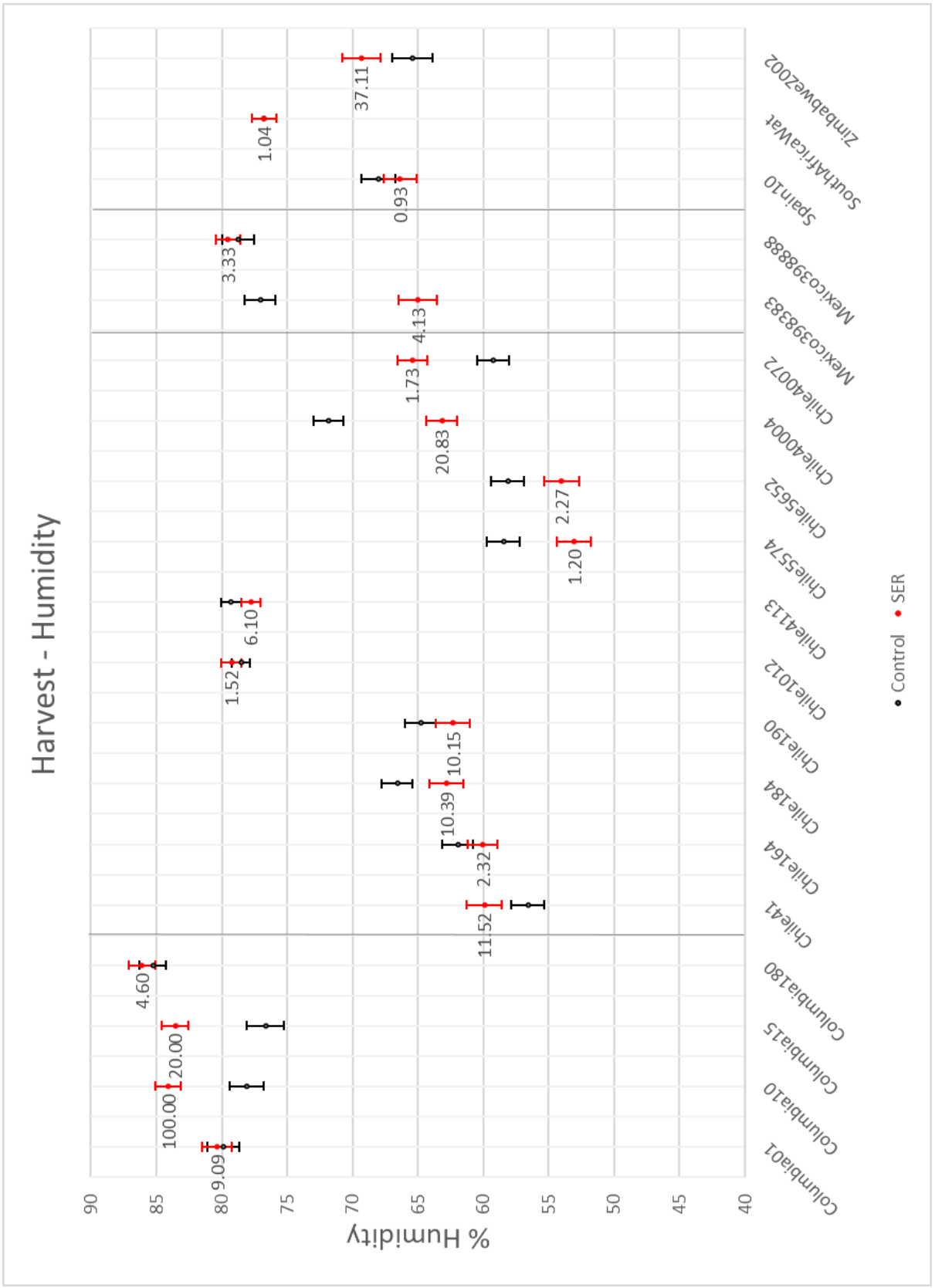

Figure 3.6: Average hourly percentage humidity recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the final 30 days of the growing season, defined as the harvest period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

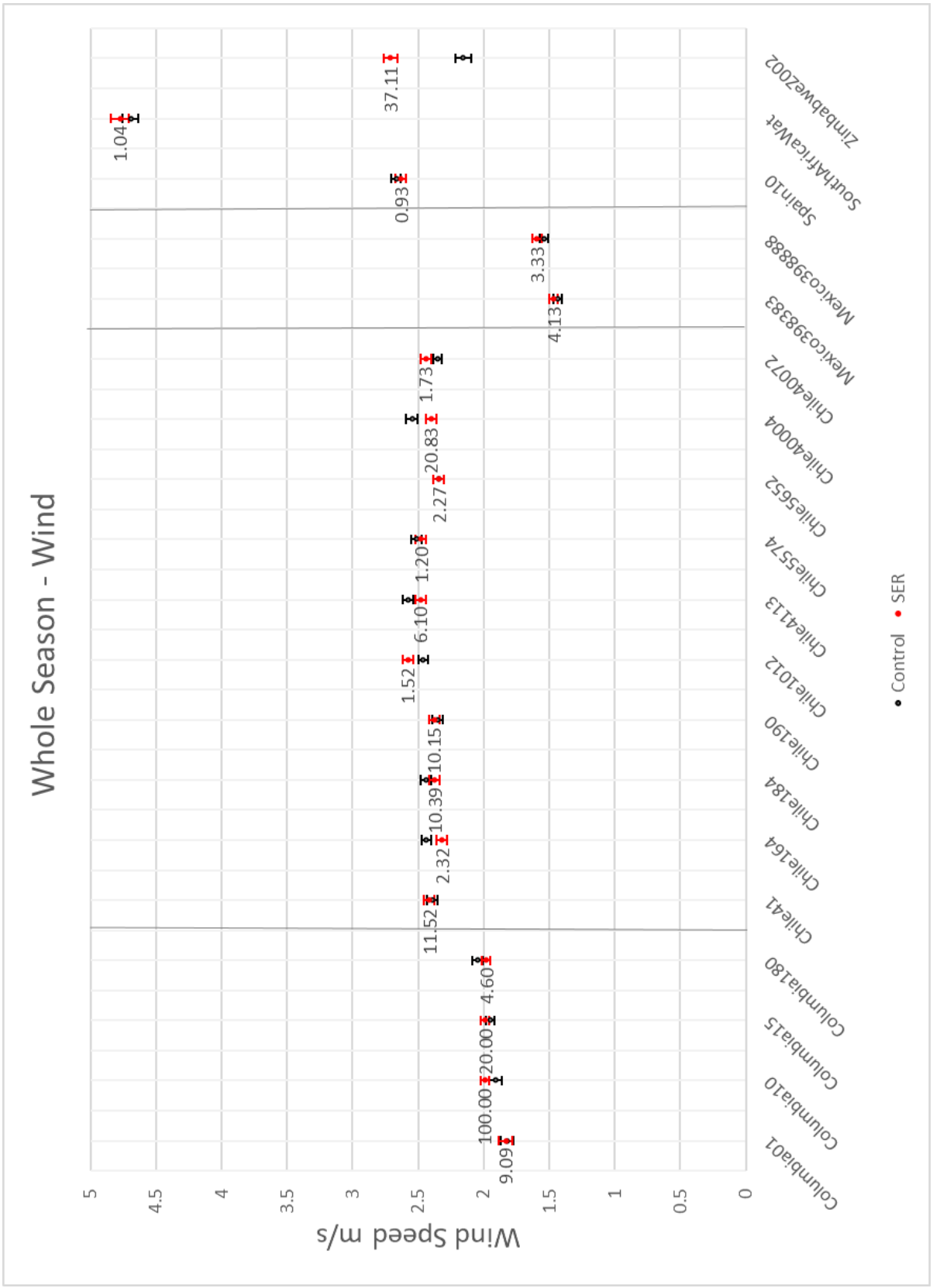

Figure 3.7: Average hourly wind speed recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the entire avocado growing season. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

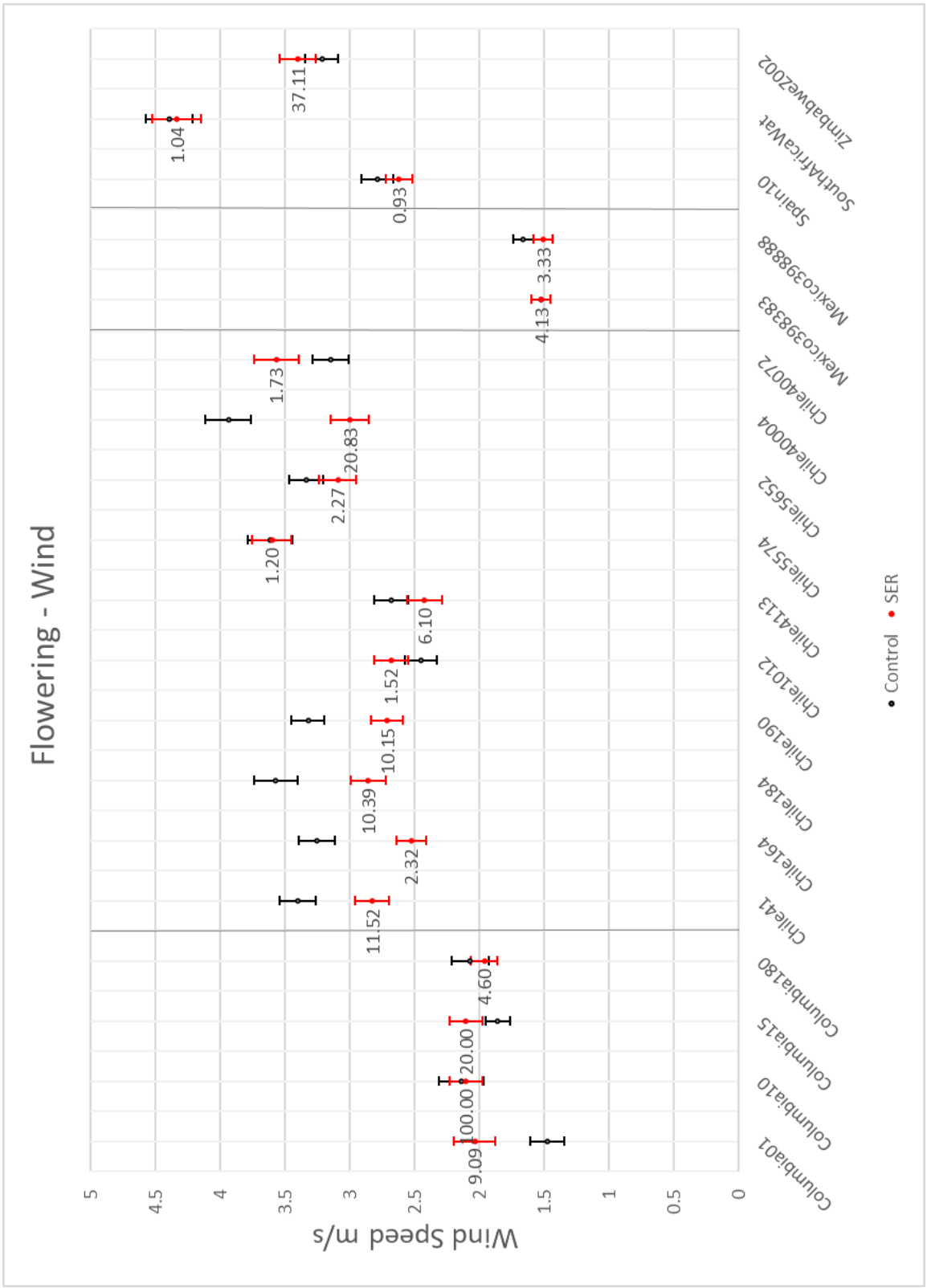

Figure 3.8: Average hourly wind speed recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the first 30 days of the growing season, defined as the flowering period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

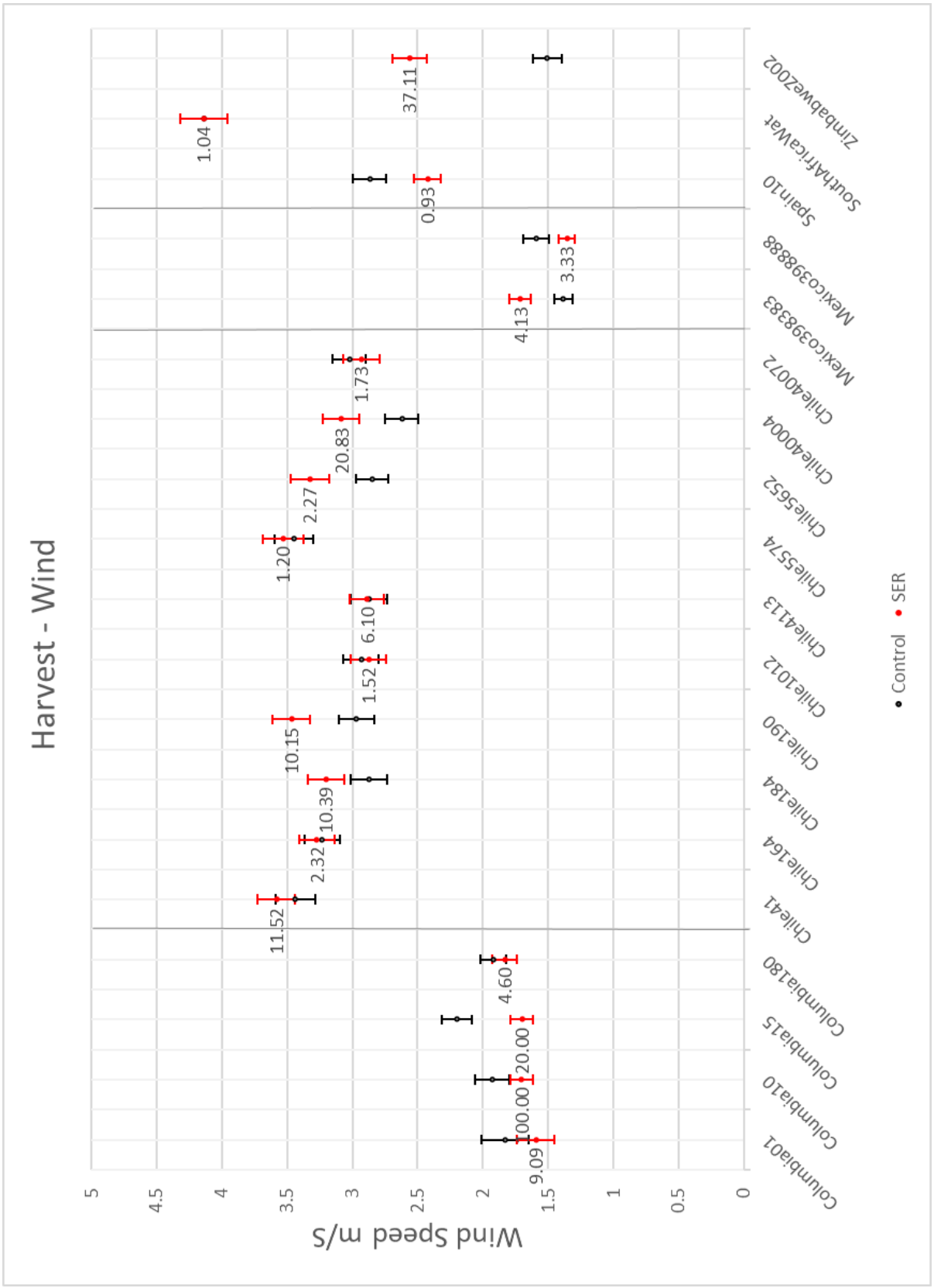

Figure 3.9: Average hourly wind speed recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the final 30 days of the growing season, defined as the harvest period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

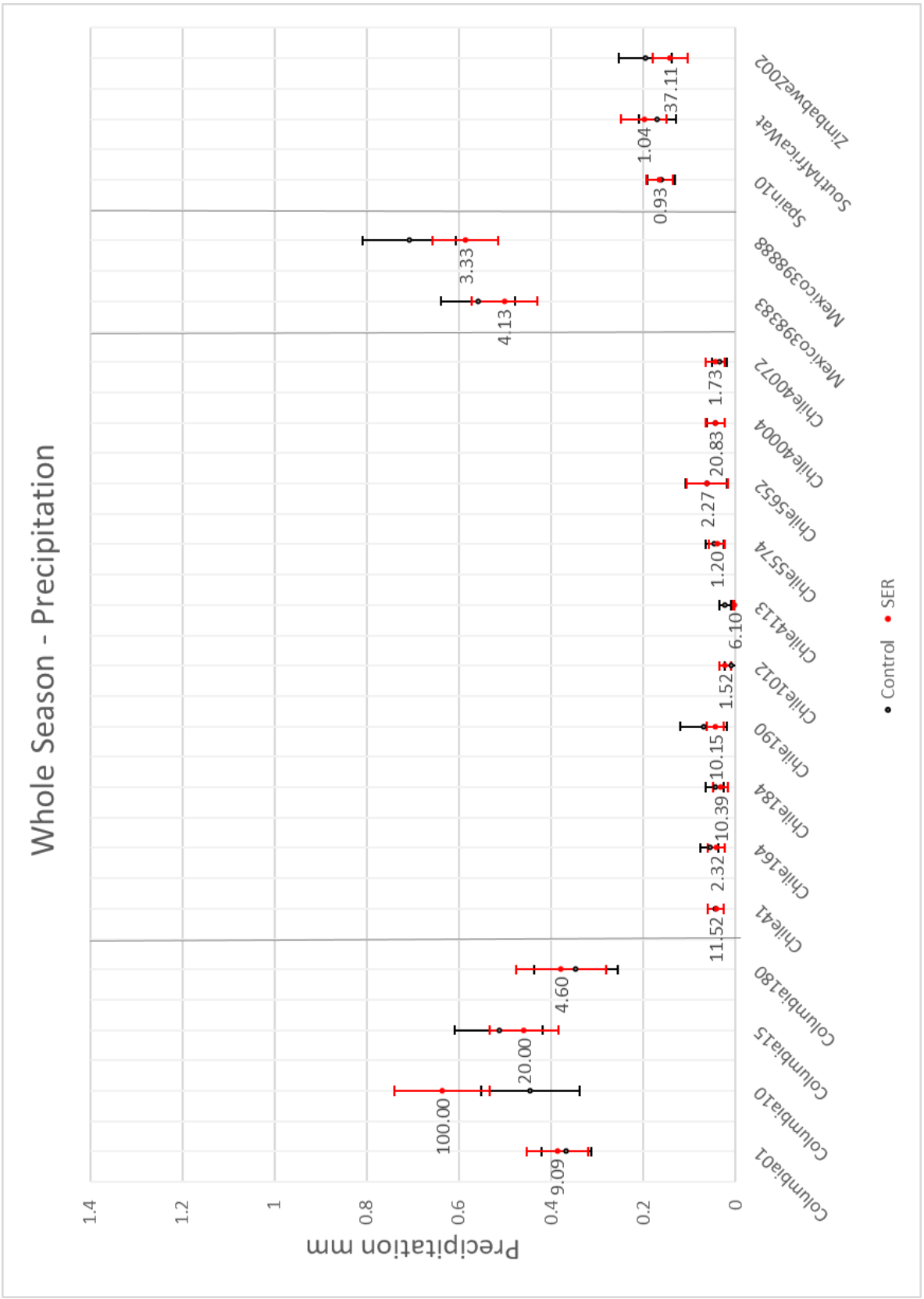

Figure 3.10: Average hourly precipitation recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the entire avocado growing season. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

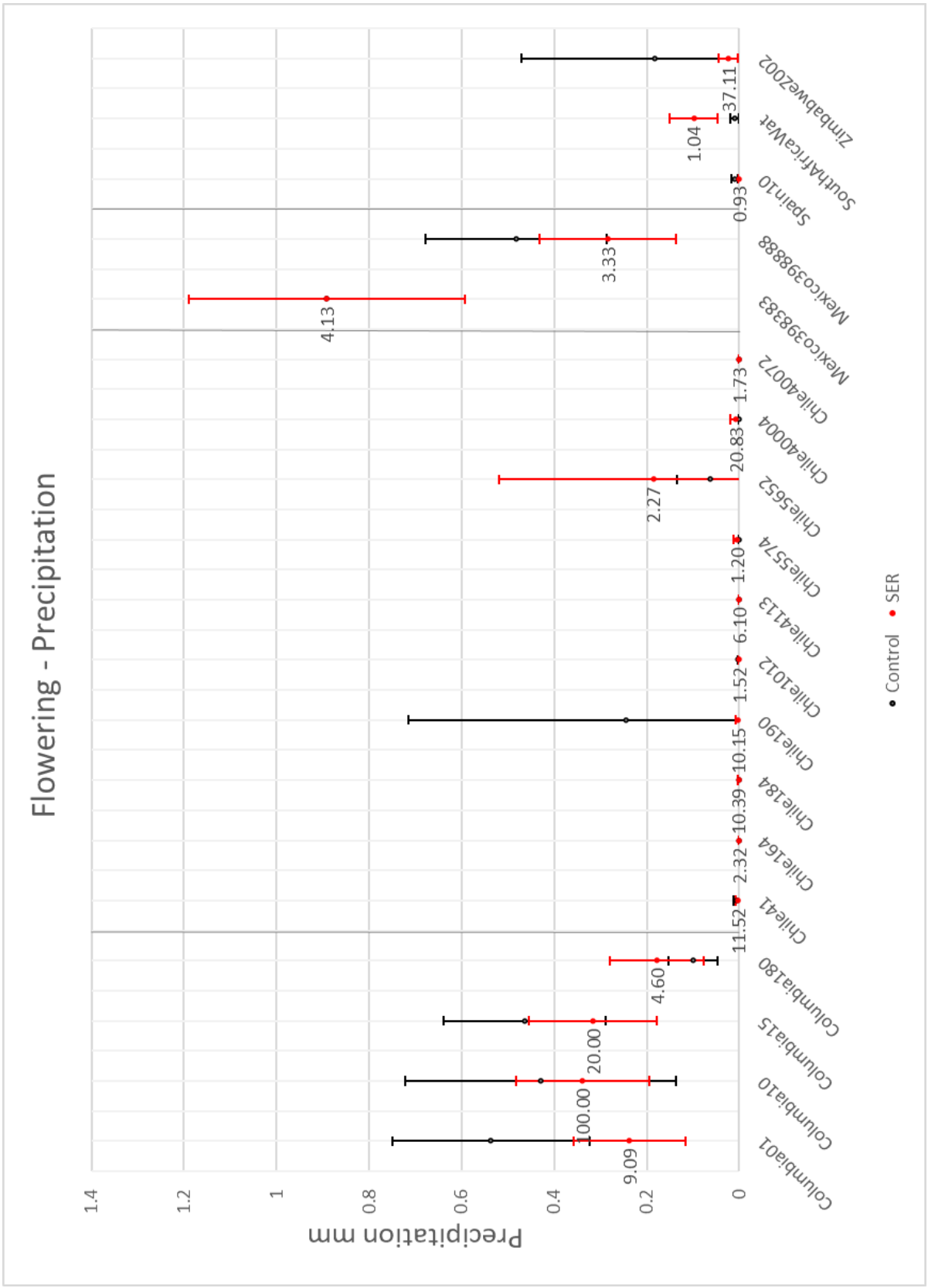

Figure 3.11: Average hourly precipitation recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the first 30 days of the growing season, defined as the flowering period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

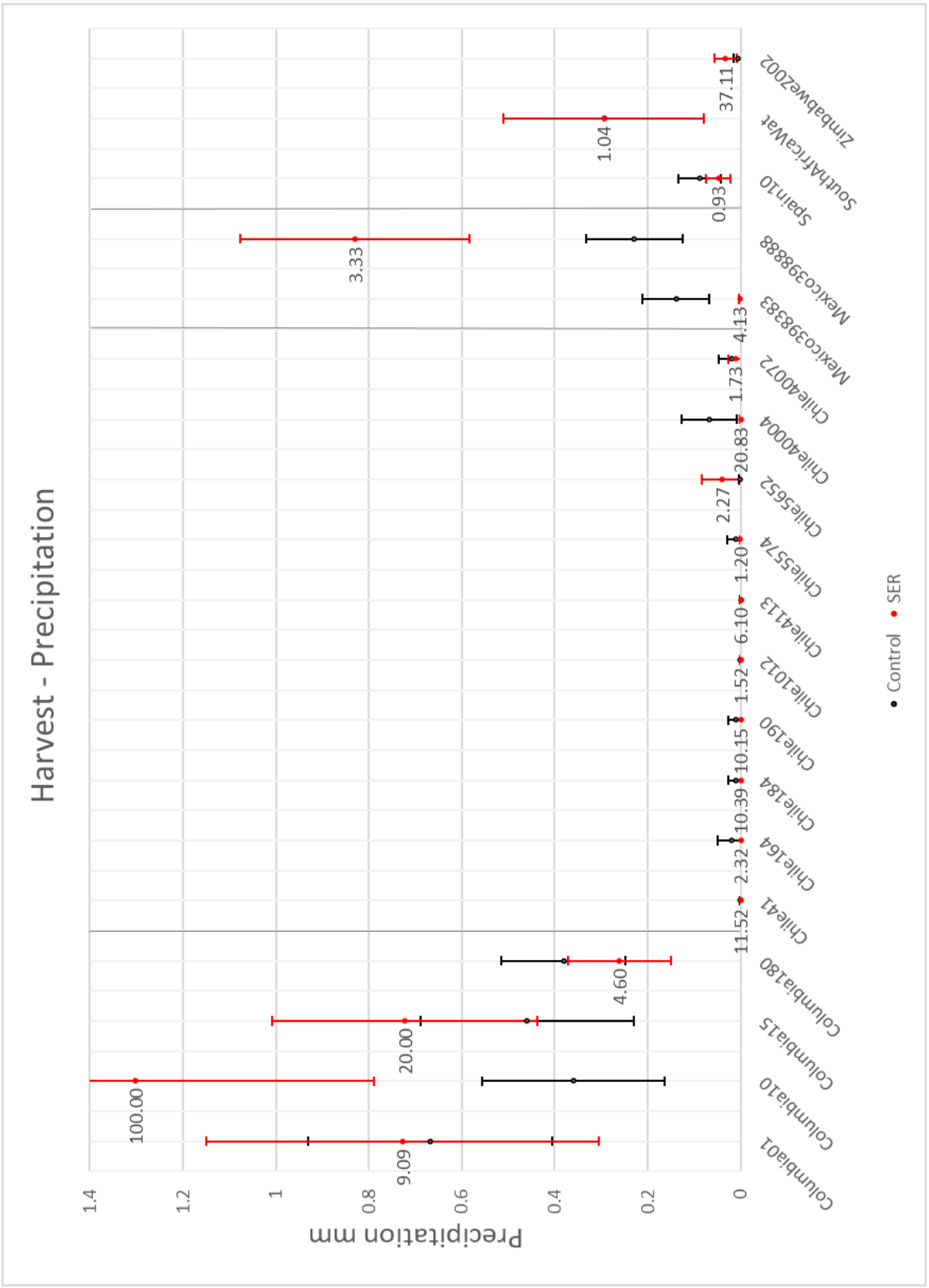

Figure 3.12: Average hourly precipitation recorded at a weather station nearest to the orchard during a period of high rot (red) with those recorded during a zero-rot harvest (black) for each of the 19 growers for the final 30 days of the growing season, defined as the harvest period. Error bars represent a 95% confidence interval and data labels indicate SER incidence as defined in Section 3.2.

3.4 Discussion

Since temperature, wind, and rainfall are considered to be some of the most influential factors affecting SER incidence, it was useful to explore them (Schaffer et al., 2013). Humidity also impacts moisture availability, and it has been observed as an important factor in sporulation (Table 4.2). Examination of the MuddyBoots database revealed an increase in SER incidence during cooler, wetter conditions for some growers. Specifically, growers in Columbia experienced more than twice as much SER when precipitation was more than 0.7 mm and humidity exceeded 80% during the harvest period. Most SER pathogens benefited from increased rain and wind speed, which facilitates spore development and dispersal. The most likely reason for the discrepancy in temperature is that the different locations harboured different fungal pathogen communities. Some pathogens, such as *Diaporthe* prefer cooler and wetter environments compared to the other abundant pathogens isolated during this project (Anco et al., 2012). On the contrary, *Lasiodiplodia* thrives under warm and sunny weather conditions (Saha et al., 2008). High SER incidence most likely depends on the likelihood that a SER pathogen species is prevalent in the orchard during weather conditions most favourable to its germination and spread. The mitigation of SER incidence must therefore focus on a combination of pathogen identification and considering the effects of specific weather events.

The whole season temperature (Figure 3.1) varied too much between all growers to make any distinct conclusion, but increased temperatures during flowering appeared to increase rot incidence for Spain, South Africa, and Zimbabwe and in Mexico during the harvest period. Increased humidity was associated with rot incidence for most of the growers during the flowering period (Figure 3.5), but not the harvest period (Figure 3.6). Wind speed manifested more consistent results per country for harvest and

flowering (Figures 3.8 & 3.9), although the average wind speed for the whole growing period differed little between the high rot and control groups (Figure 3.7). It appears that higher wind speed during flowering reduced rot incidence for most growers, but higher wind speeds during harvest increased rot. This was especially true for Chilean growers, but other countries did not exhibit a clear pattern.

The precipitation data displayed the most variation (Figures 3.10, 3.11, 3.12). This was likely due to the fact that most days would have zero precipitation, with rainfall being a sporadic event throughout the growing season. A dataset populated with mostly zeros and the occasional positive value, especially if these values were instances of high rainfall, would have a higher variance. This variance in precipitation was such that the mean values and confidence intervals often overlapped on all three precipitation analyses. However, the most predictable factor which increased rot incidence for the majority of growers (x %) was an increase in precipitation during harvest.

SER is a complex disorder involving the interplay of factors associated with host susceptibility, pathogen virulence, orchard management, microclimate, and environmental influence. Without any data pertaining to all other variables, an investigation of climate factor impact on SER incidence is too narrow to conclusively define the cause of postharvest rot. However, this research revealed some patterns in the data for certain growers, as well as insight into the interaction between the observed factors. A pattern emerged most clearly from the Columbian growers. Although temperature during flowering or harvest did not appear to affect SER incidence for these sites, high humidity and precipitation during harvest, combined with a lower wind speed, increased rot incidence compared to the control groups.

The Columbian grower with the highest SER incidence (100) had the highest precipitation during harvest, but not flowering (Figures 3.11 & 3.12). It is also interesting that precipitation during flowering correlated well with relative rot incidence for Columbia only. This increase in rot during the harvest period confirms previous findings about the impact of rain during harvest and this may support the theory that infection occurs at harvest rather than flowering (Hartill & Everett, 2002). The fact that temperature did not appear to have a consistent effect on rot incidence for all growers may imply that the rot is caused by a diverse community of fungal pathogens whose sporulation temperatures encompasses a wide range. Average temperatures for both flowering and harvest periods reached as high as 28 ºC and as low as 14 ºC, with no clear pattern on rot incidence.

It is also possible that the weather stations selected for this study were not close enough to the orchards to be accurate (Bourke, 1970). Ideally, weather stations should be located within the orchard to provide the most precise readings. Even microclimate within individual trees can vary, and this effect on overall SER incidence has not yet been thoroughly explored (Everett et al., 2007). If this study were to be repeated, it is imperative to establish an orchard weather station, and further research into tree microclimate could be useful.

Another area of further study is the effects of extreme weather fluctuations on disease incidence. The raw data revealed that some growers exhibited extreme temperature fluctuations, sometimes below freezing, whereas others were more regular. If better data can be obtained, the range of temperature fluctuation throughout the growing season could indicate whether wide temperature fluctuations eliminate potential pathogens, or stress fruit to the point where they are significantly more susceptible to infection.

CHAPTER 4: FUNGAL MORPHOTYPE IDENTIFICATION

4.1 Introduction

Morphological identification of plate cultures is a useful option for researchers in the field who do not have access to a laboratory. Hyphae or spores can be easily cultured out of infected samples, producing consistent genus-specific features as long as the same growth media is used between the sample in question and the morphotype model. Previous studies have demonstrated the hyphal growth characteristics of typical avocado pathogens at the cellular level, and their primary goal was more often to identify SER causal pathogens or study the phylogeny of a specific pathogen (Guarnaccia et al., 2016; Wanjiku et al., 2020). When plate morphology is examined, Potato Dextrose Agar (PDA) is often the media of choice.

Although the growth media used to culture hyphae is arbitrary, as long as it is optimised for fungus, several previous morphological studies of common SER pathogens demonstrate the difference in growth patterns between media. *Lasiodiplodia* (WhiteCotton) hyphae was similar in appearance when grown on PDA and MEA, but it can become indistinguishable from *Neofusicoccum* more often when overgrown on MEA (Wang et al., 2021). Yan et al. (2013) demonstrated the difference between a seven-day old culture and fourteen-day old culture on PDA, showing that the characteristic light grey centre is only apparent during the early growth stage.

Morphological examination of *Neopestalotiopsis* members revealed that the layered growth pattern achieved with MEA is lost on PDA (Biju, 2018). However, many species of WhiteStriated (*Diaporthe*) maintained its form consistently regardless of growth media choice (Thompson et al., 2011).

The specific objectives of this study were:

1. To establish a species identity for each of the seven most abundant

morphotypes isolated during aggregation of the Supply Chain Dataset (SCD)

2. To identify morphological features which were consistently associated with one genus

3. To explore phylogenetic relationships between SER causal species within each morphotype category and in relation to each other

4. To create a morphological plate identification framework that can be used by researchers in the field who do not have access to sequencing technology

4.2 Materials and Methods

All avocado core samples were placed on MEA and incubated at 25 °C for 3 - 4 days as described in Section 2.1.2. Morphotype samples intended for spore analysis were incubated at 25 °C and 100% humidity for 7 days. Hyphal tissue was excised and submerged in a 50% Glycerol solution and arraigned on a slide prior to microscope examination. Gram staining procedure was carried out according to manufacturer's instructions (TCS Biosciences, 2022).

Samples were prepared for sequencing as described in Section 2.1.2. DNA sequencing of the ITS barcode region was performed by Macrogen, using in-house primers (2.3.2) targeting the ITS1 region of the small RNA subunit. All bioinformatics analysis was performed using Geneious Prime software (Geneious Prime 2021.1.1). Neighbor-joining trees were constructed after aligning all sequences using MUSCLE alignment, and then Geneious Prime tree building software. The Maximum Likelihood tree was calculated using PAUP. All species identity information was obtained through the National Center for Biotechnology Information (NCBI) and the BLAST service.

During SCD aggregation, a pattern of common morphotypes emerged. Seven fungal morphotypes in particular were cultured most frequently, with a high drop in occurrence from the seventh to eighth most abundant morphotype. These seven most

abundant morphotypes became the focus of all subsequent research.

Ten biological replicates of each of the seven most abundant morphotypes were chosen for ITS sequencing, however, some of the samples failed to sequence. Many of the failed samples were slime morphotypes. As a result, some morphotypes have fewer than ten sequenced replicates, and WhiteSlime failed completely. Replicates per country are summarised in Table 4.1.

4.3 Results

4.3.1 Morphological Characterisation and Sequencing

Classifying these patterns into clear groups and confirming the species identification laid the groundwork for a reliable field identification system of common SER pathogens. Each fugal morphotype isolated in the SCD was given a descriptive name according to its colour and growth pattern, as described in Section 2.1.4. Examples of the colour, texture combination unique to the seven most abundant phenotypes are shown in Figure 4.1. Some morphotypes were found to grow faster than others, obscuring key morphological differences. WhiteDensefur, WhiteCotton, and WhiteLayered were particularly difficult to separate. When WhiteLayered is overgrown on the plate and the layering feature disappears, it can look exactly like WhiteDensefur. WhiteCotton can also look ‘dense’ when overgrown, and WhiteDensefur turns black under stressful conditions, due to sporulation. Figure 4.2 illustrates the morphological difference between WhiteCotton and WhiteDensefur, even though they were often indistinguishable.

Figure 4.1: The seven most abundant morphotypes isolated from avocado core samples during the SCD collection. Core section was taken from absission zone to fruit stone to capture entire fungal community within the mesocarp. Clockwise from the top left: WhiteDensefur, WhiteLayered, WhiteStriated, WhiteCotton, YellowSlime, WhiteGreyCotton, and WhiteSlime

Figure 4.2: WhiteCotton (upper) and WhiteDensefur (lower) growing together on the same plate illustrates the key difference in these two similar morphotypes

Ideally, ten biological replicates of each fungal morphotype would have been sequenced with both ITS and β-Tubulin. However, the β-Tubulin region failed to sequence for all morphotypes, and variations in yearly fungal pathogen communities prevented adequate sample replication of some morphotypes. Sequencing a partial region of the ribosomal ITS 1 region was sufficient enough to identify fungal morphotypes to species level. Samples chosen for analysis, as well as the BLAST species identification results, are summarised in Table 4.1. A published literature investigation of the ideal sporulation conditions associated with each of these identified genera is summarised in Table 4.2.

Table 4.1: BLAST search results for ITS regions of fungal morphotype replicates. The % Identical Sites refers to the top BLAST hit with the greatest percentage identity.

Sample Code	Country	Fungal Morphotype	Species Identification	% Identical Sites	E Value
CC18	Israel	WhiteCotton	Lasiodiplodia pseudothobromae	100	2.32E-118
AY11	South Africa	WhiteCotton	Lasiodiplodia pseudothobromae	98.9	7.58E-117
AX37	South Africa	WhiteCotton	Lasiodiplodia parva	99.4	1.41E-116
AV3	Peru	WhiteCotton	Lasiodiplodia parva	100	8.15E-118
AQ21	Peru	WhiteCotton	Lasiodiplodia pseudothobromae	100	2.30E-118
AQ8	Peru	WhiteCotton	Lasiodiplodia brasiliensis	100	4.12E-112
BJ21	Israel	WhiteCotton	Lasiodiplodia parva	99.4	4.10E-117
CE48	Israel	WhiteDensefur	Colletotrichum arecicola	99.5	5.91E-117
CK6	Israel	WhiteDensefur	Fusarium perseae	100	1.37E-106
AX15	South Africa	WhiteDensefur	Pestalotiopsis grevilleae	99	4.64E-133
BQ49	Mexico	WhiteDensefur	Pestalotiopsis grevilleae	98.5	2.26E-132
CA27	Israel	WhiteDensefur	Pestalotiopsis papuana	98	3.32E-129
AS35	South Africa	WhiteDensefur	Fusarium pernambucanum	100	2.34E-109
CK10	Israel	WhiteGreyCotton	Neofusicoccum ningerense	100	4.25E-108
CJ40	Israel	WhiteGreyCotton	Neofusicoccum ningerense	100	4.25E-108
CJ30	Israel	WhiteGreyCotton	Neofusicoccum italicum	99.4	5.54E-116
CJ25	Israel	WhiteGreyCotton	Neofusicoccum ningerense	100	4.22E-108
CJ8	Israel	WhiteGreyCotton	Neofusicoccum ningerense	100	6.40E-109
CJ1	Israel	WhiteGreyCotton	Neofusicoccum ningerense	100	4.22E-108
BV14	Mexico	WhiteGreyCotton	Neofusicoccum ningerense	100	2.81E-107
BS4	Mexico	WhiteGreyCotton	Neofusicoccum ningerense	100	1.36E-106
BS2	Mexico	WhiteGreyCotton	Neofusicoccum ningerense	100	2.85E-107
BR50	Mexico	WhiteGreyCotton	Neofusicoccum ningerense	100	9.01E-109
BY29	Mexico	WhiteLayered	Neopestalotiopsis eucalypticola	100	9.83E-111
BX36	Mexico	WhiteLayered	Neopestalotiopsis piceana	100	1.70E-110
BX32	Mexico	WhiteLayered	Neopestalotiopsis eucalypticola	100	9.83E-111
BV48	Mexico	WhiteLayered	Neopestalotiopsis aotearoa	100	7.55E-108
BU36	Mexico	WhiteLayered	Neopestalotiopsis aotearoa	100	7.61E-108
BT30	Mexico	WhiteLayered	Neopestalotiopsis eucalypticola	100	5.40E-115
BS14	Mexico	WhiteLayered	Neopestalotiopsis aotearoa	100	2.63E-114
BQ45	Mexico	WhiteLayered	Neopestalotiopsis aotearoa	100	7.55E-108
BP39	Mexico	WhiteLayered	Neopestalotiopsis eucalypticola	100	7.11EE-111
AX6	South Africa	WhiteLayered	Neopestalotiopsis eucalypticola	100	1.00E-110
CK2	Israel	WhiteStriated	Diaporthe velutina	100	1.60E-111
CK4	Israel	WhiteStriated	Diaporthe macadamiae	100	3.40E-119
CK25	Israel	WhiteStriated	Diaporthe macadamiae	100	9.76E-120
CJ32	Israel	WhiteStriated	Diaporthe velutina	100	5.06E-113
CJ2	Israel	WhiteStriated	Diaporthe macadamiae	100	9.44E-113
BR5	Mexico	WhiteStriated	Diaporthe humulicola	100	7.43E-102
BT40	Mexico	WhiteStriated	Diaporthe caatingaensis	100	5.52E-111
BR36	Mexico	WhiteStriated	Diaporthe cucurbitae	100	4.04E-111
BL38	Mexico	WhiteStriated	Diaporthe humulicola	100	1.14E-102
BU15	Mexico	WhiteStriated	Diaporthe humulicola	100	2.86E-102
P45	South Africa	Slime	Diaporthe macadamiae	100	3.81E+114
YellowSlime1	Israel	Slime	Diaporthe terebinthifolii	100	1.07E-107
YellowSlime2	Israel	Slime	Diaporthe terebinthifolii	100	5.14E-110

Table 4.2: Ideal sporulation conditions for genera assigned to the seven most abundant morphotypes

Morphotype	Genus Identity	Sporulation Temperature	Sporulation Conditions	Source
WhiteStriated	*Diaporthe/ Phomopsis*	21 °C	Increases with longer wetness duration, up to 71 hours	Anco et al., 2012
YellowSlime/ WhiteSlime	[*Phomopsis(?)*]	——	——	——
WhiteGreyCotton	*Neofusicoccum*	28 °C	UVA light	Chan et al., 2020
WhiteLayered	*Neopestalotiopsis*	25 °C	Long periods of wetness ≥72 hours Sporulated with 12-hour photoperiod	Belisario et al., 2020
WhiteCotton	*Lasiodiplodia*	28 °C	Enhanced by light	Saha et al., 2008
WhiteDensefur	*1. Pestalotiopsis* *2.Colletotrichum* *3. Fusarium*	25 °C (±2)	1. 70% Relative Humidity 2. 12h alternating light treatment 3. Substrate pH 6	1. Ranjana et al., 2010 2. Wong et al., 1983 3. Cruz et al., 2019

4.3.2 WHITECOTTON [*Lasiodiplodia*]

WhiteCotton and WhiteDensefur were difficult to distinguish from plate morphology alone. The wispy, cotton-like hyphae were also similar to WhiteGreyCotton, with the only distinction being the even dispersal of grey coloured hyphae throughout the plate for the latter. The seven biological replicates shown in Figure 4.3 depicts a consistent replication of morphotypes designated WhiteCotton which were confirmed by ITS sequencing to be of the genus *Lasiodiplodia*. All sequences aligned well, with only a few point mutations. Interestingly, some of these mutations did not coincide with the species identification predicted in Table 4.1, indicating they may be strain differences. This is reflected in the alignment and phylogeny in Figures 4.4 and 4.5, in which the species identification did not always correspond with membership to a clade. The spore morphology shown in Figure 4.6A and septate hyphae (B) is consistent with what would be expected from *Lasiodiplodia* (Alves et al., 2008), but they are also similar to those of *Fusarium* and *Colletotrichum* (Figure 4.10). It is also important to note that the morphology of this genus can vary between isolates depending on the stage of growth. Figure 4.3 BJ21 and CC18 represent the early stages of fungal growth for *Lasiodiplodia*, before it crowds the plate.

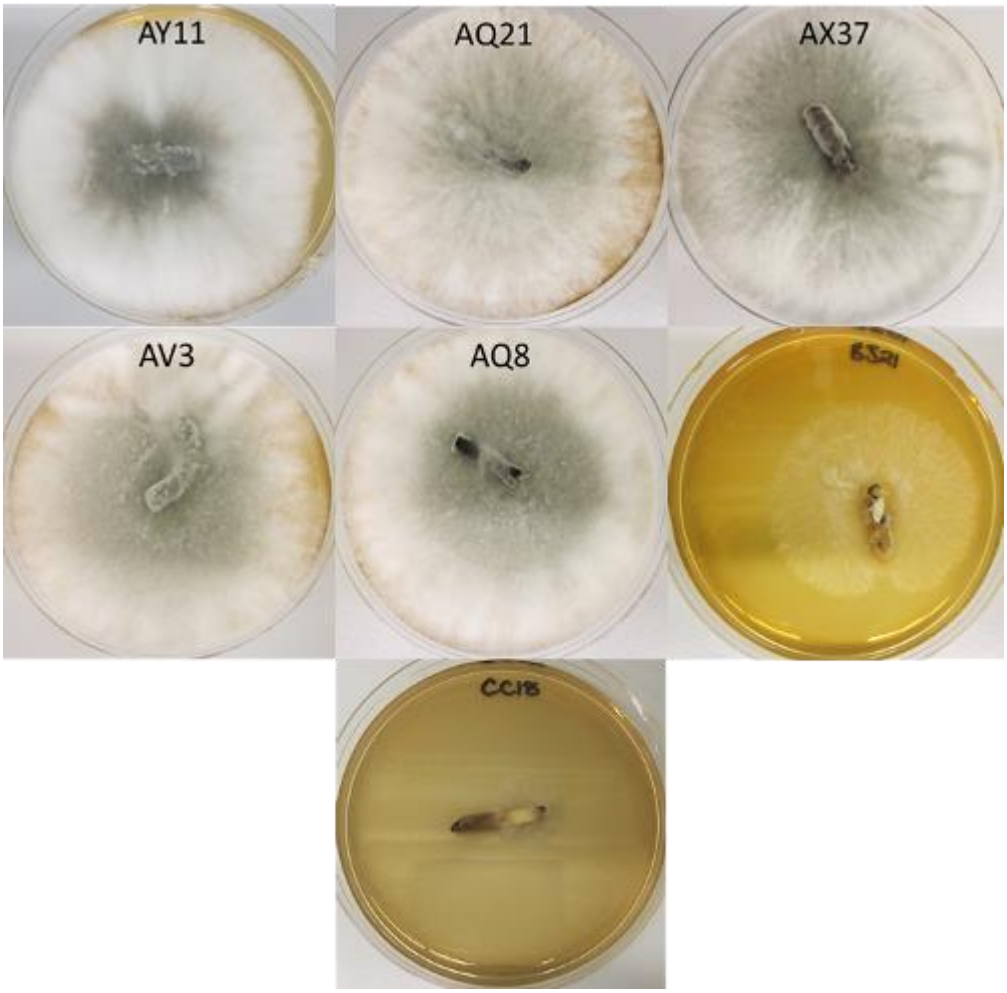

Figure 4.3: Representative WhiteCotton (*Lasiodiplodia*) morphotypes chosen for ITS sequencing. Isolates were cultured from avocados originating from different countries.

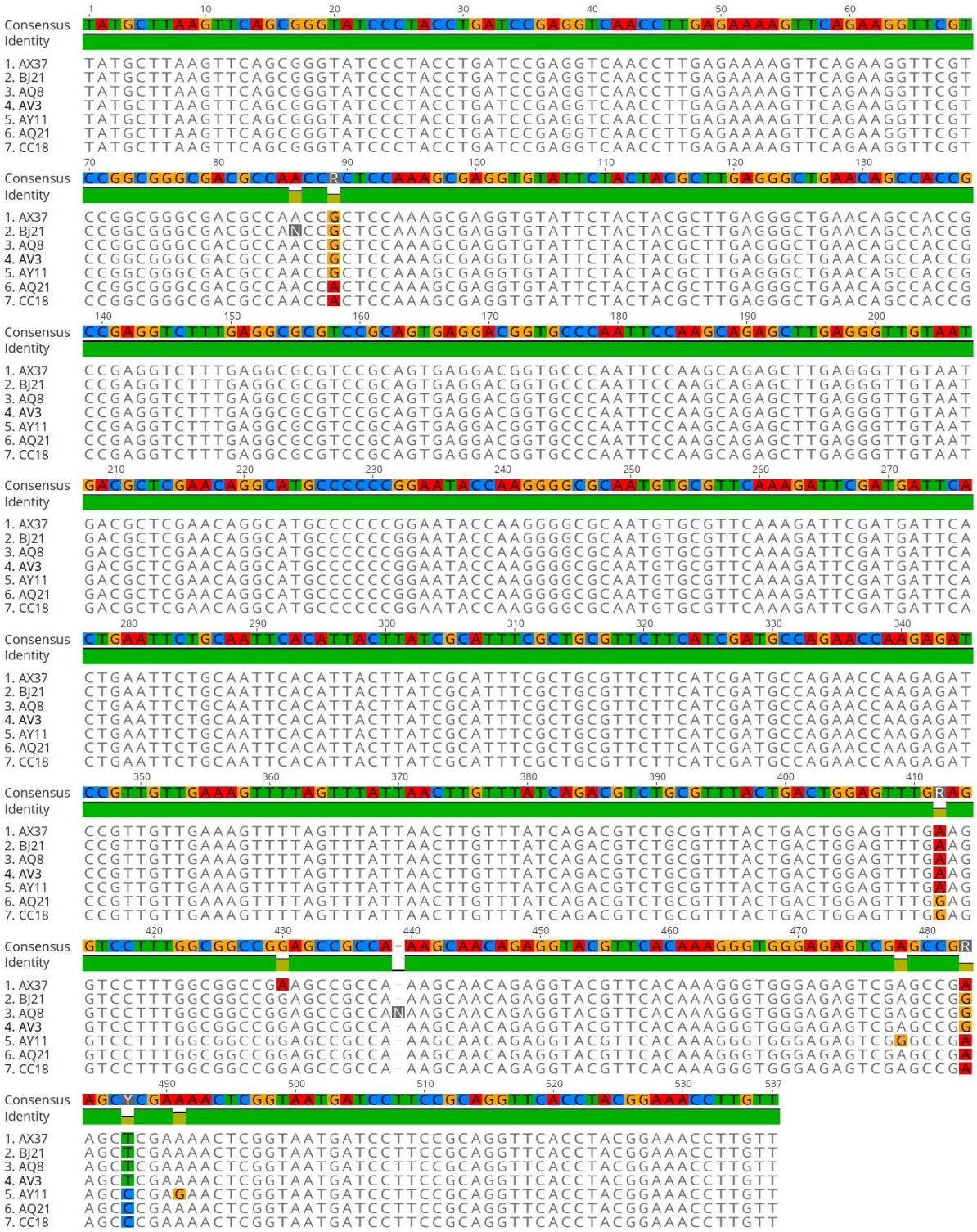

Figure 4.4: ITS sequence alignment of all WhiteCotton morphotypes

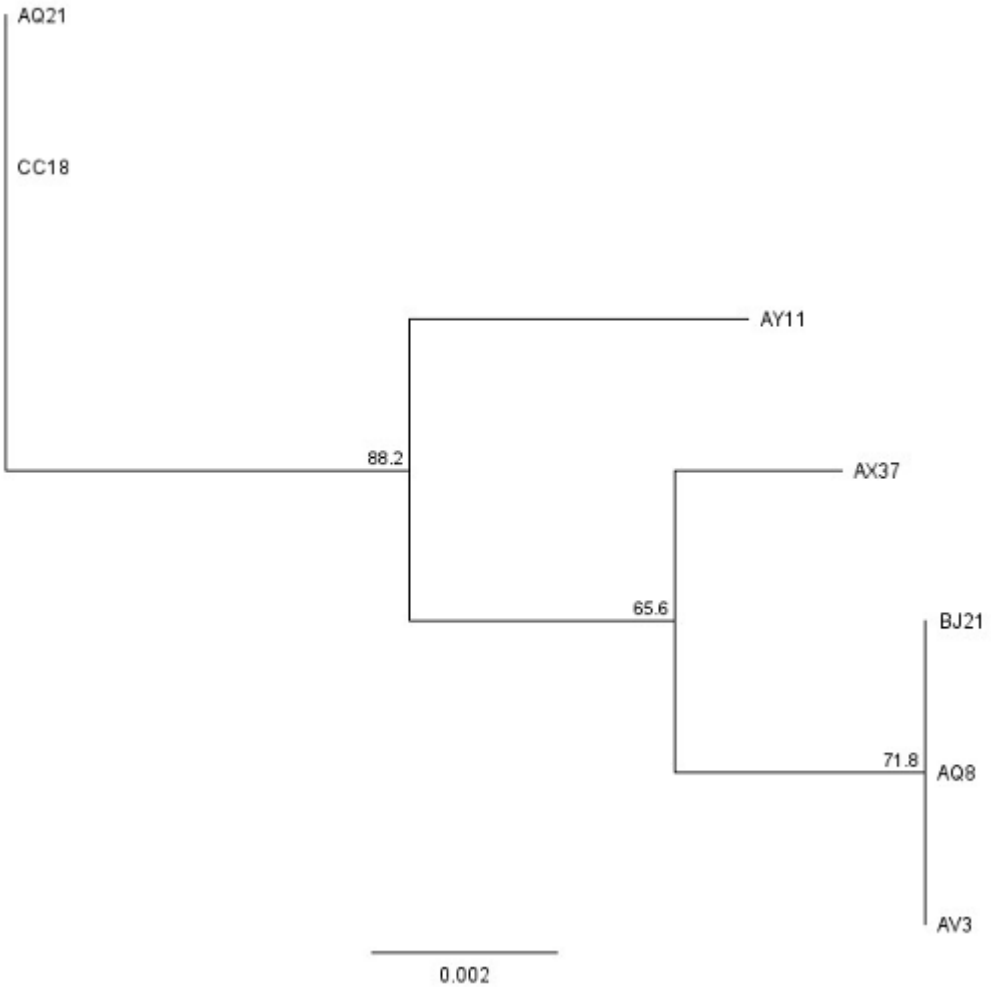

Figure 4.5: Single locus (ITS) neighbour-joining consensus phylogeny of WhiteCotton (*Lasiodiplodia*) samples isolated from avocado mesocarp placed on MEA, incubated at 25 ºC, and sub-cultured twice before DNA extraction and sequencing. Countries of origin for the isolates are as follows: CC18 – Israel, AY11 – South Africa, AX37 – South Africa, AV3 – Peru, AQ21 – Peru, AQ8 – Peru, BJ21 – Israel. Bootstrap support of 500 replicates shown above nodes identify three clades

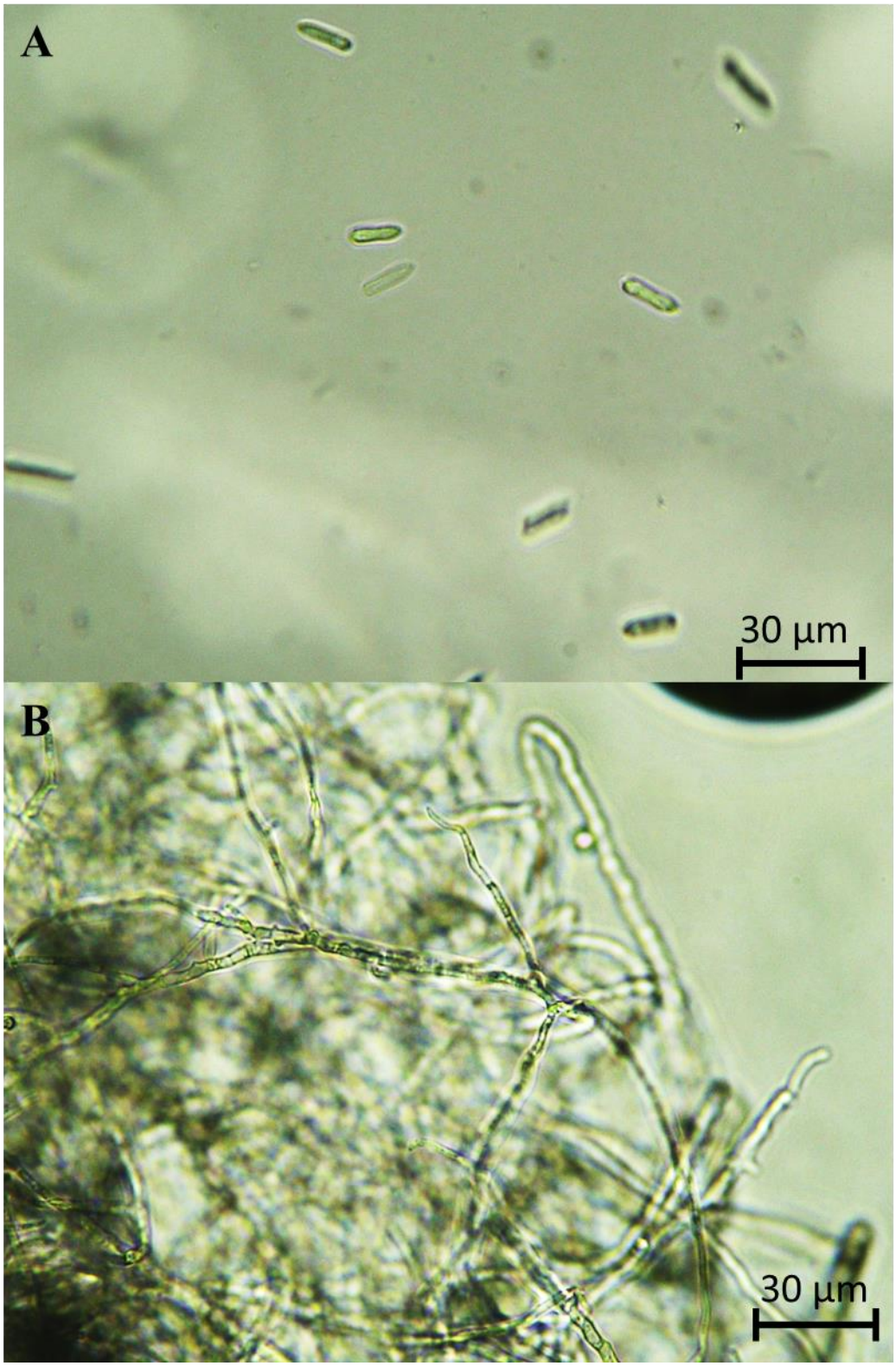

Figure 4.6: Spores collected from the WhiteCotton (*Lasiodiplodia*) morphotype cultured on MEA at 25 °C for five days (A), and fungal mycelia from the same sample (B)

4.3.3 WHITEDENSEFUR [*Pestalotiopsis/Colletotrichum/Fusarium*]

The most difficult morphotype to distinguish was WhiteDensefur. ITS sequencing revealed that plate morphotyping was insufficient to distinguish several genera with a similar growth pattern. For the purpose of fungal community analysis for this project, WhiteDensefur represents a *Pestalotiopsis*, *Colletotrichum*, and *Fusarium* complex. Figure 4.7 shows examples of *Pestalotiopsis* in the upper two plates, *Colletotrichum* are the middle three plates, and one *Fusarium* on the bottommost plate. Naturally, the ITS sequences of three distinct genera did not align well at all with each other in Figure 4.8. However, the phylogeny shows that the members of each genus are more closely related to each other than they are to the other genera, as expected (Figure 4.9). The spore morphology (Figure 4.10) was a similar rod shape for both *Fusarium* and *Colletotrichum*, but *Pestalotiopsis* spores have a morphology resembling those of *Neopestalotiopsis*.

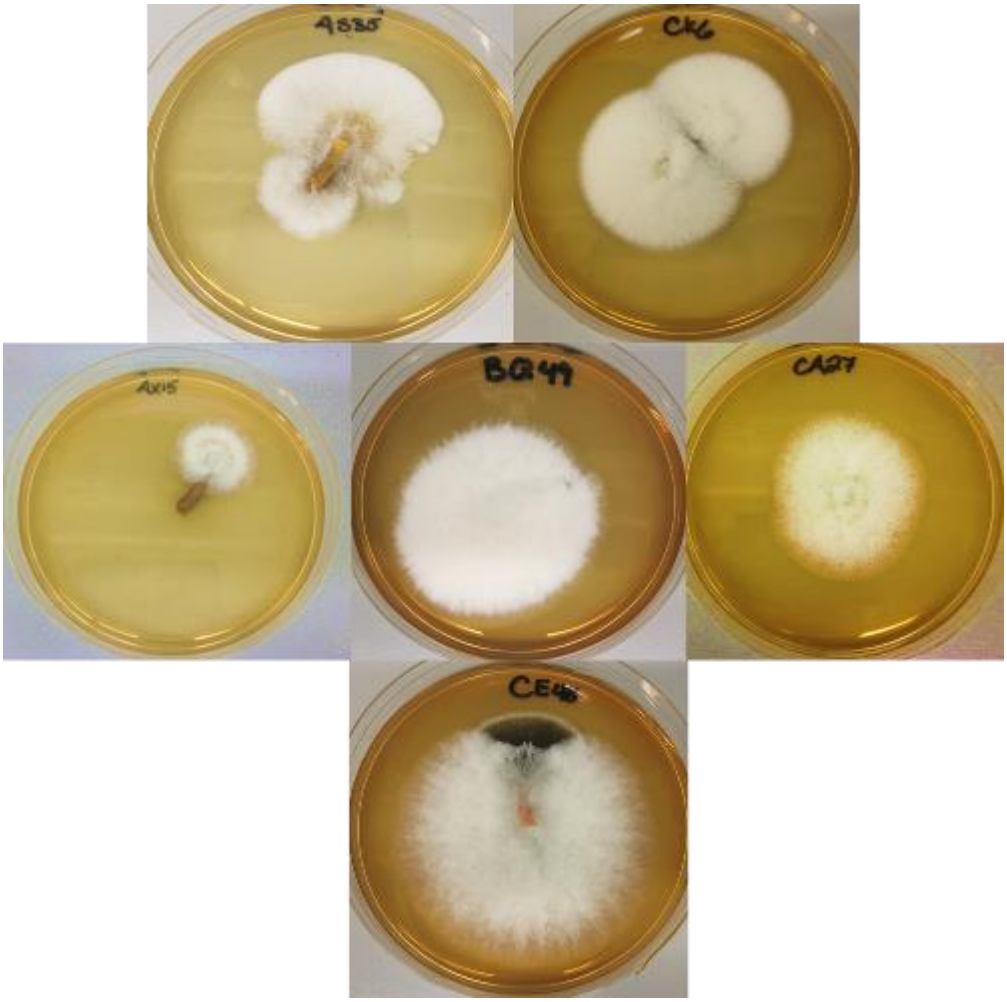

Figure 4.7: Representative WhiteDensefur (Pestalotiopsis/Colletotrichum/Fusarium) morphotypes chosen for ITS sequencing. Isolates were cultured from avocados originating from different countries.

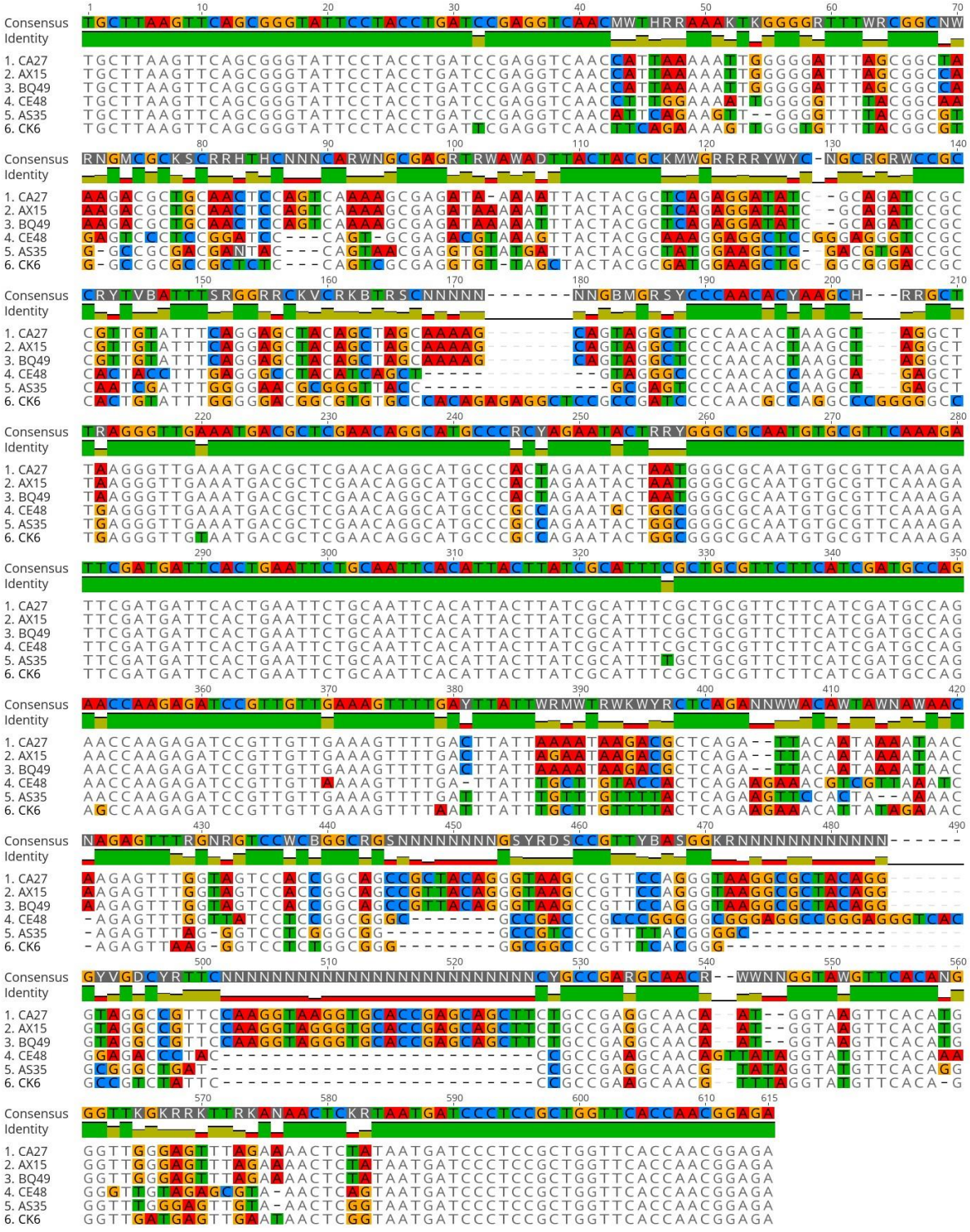

Figure 4.8: ITS sequence alignment of all WhiteDensefur morphotypes

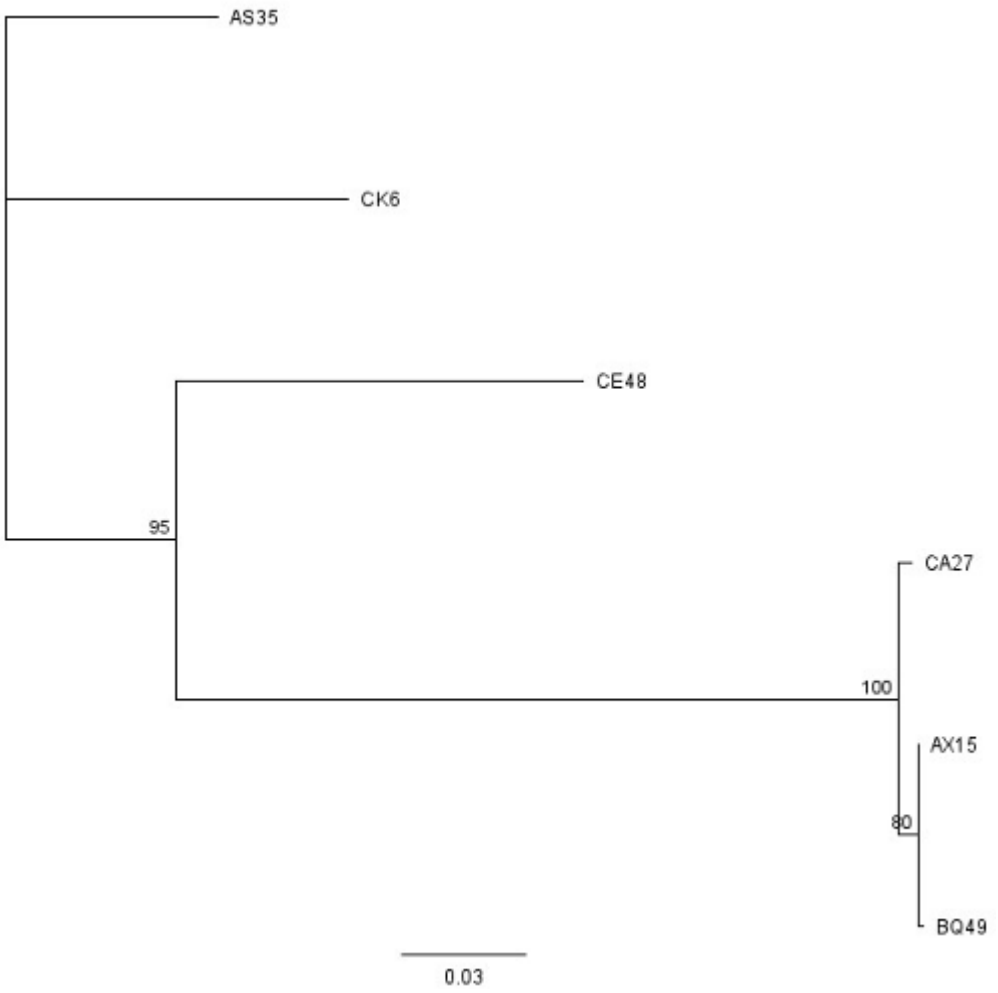

Figure 4.9: Single locus (ITS) neighbour-joining consensus phylogeny of WhiteDensefur (*Pestalotiopsis*/*Colletotrichum*/*Fusarium*) samples isolated from avocado mesocarp placed on MEA, incubated at 25 ºC, and sub-cultured twice before DNA extraction and sequencing. Countries of origin for the isolates are as follows: CE48 – Israel, CK6 – Israel, AX15 – South Africa, BQ49 – Mexico, CA27 – Israel, AS35 – South Africa. Bootstrap support of 500 replicates shown above nodes identify three clades

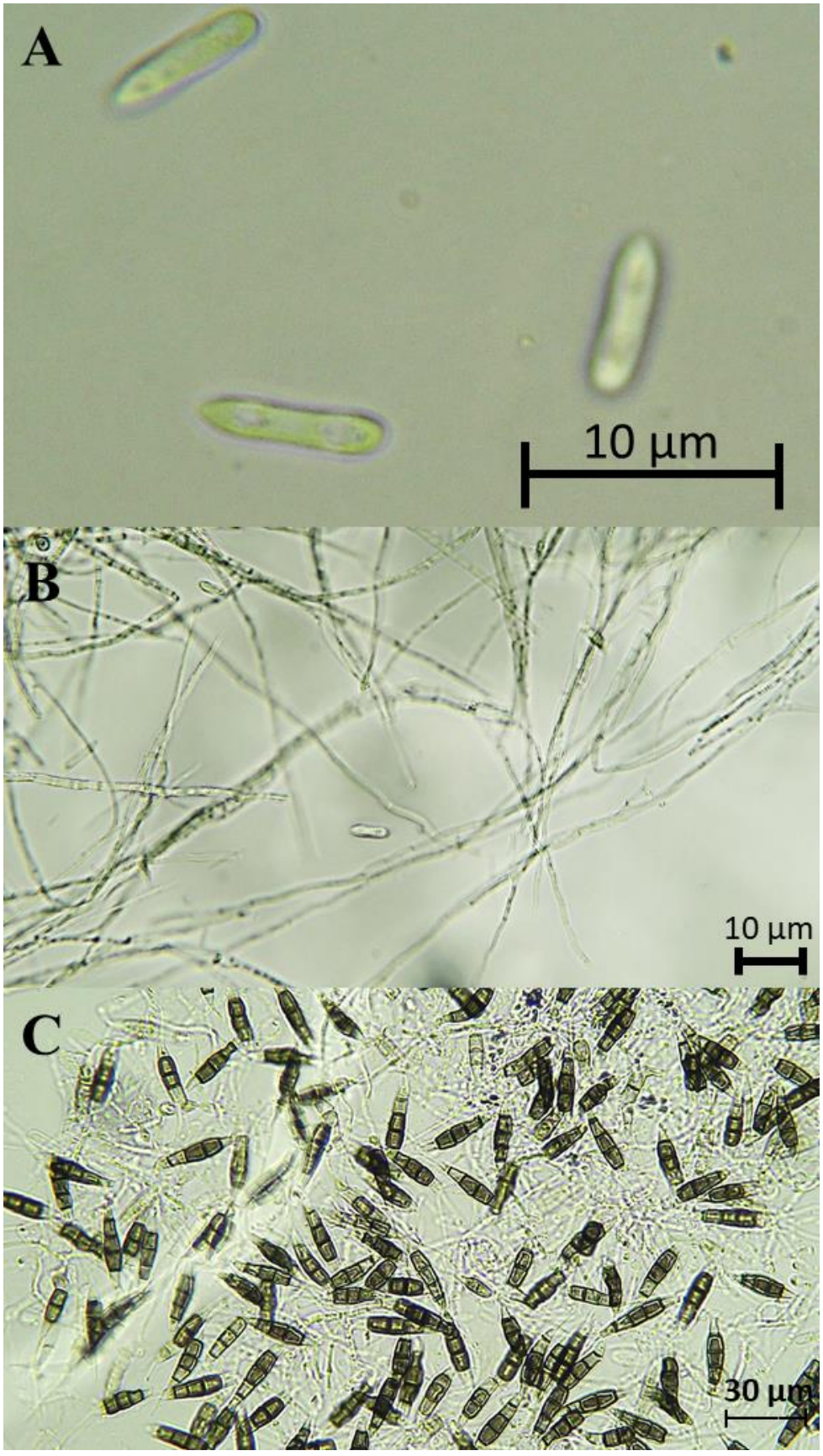

Figure 4.10: Spores collected from samples of the WhiteDensefur morphotype complex cultured on MEA at 25 ºC for five days. These could be (A) *Fusarium*, (B) *Colletotrichum*, and (C) *Pestalotiopsis*

4.3.4 WHITEGREYCOTTON [*Neofusicoccum*]

The WhiteGreyCotton morphotypes shown in Figure 4.11 were consistently identified as species of *Neofusicoccum*. Just like WhiteCotton, the ITS region between samples showed little variation (Figure 4.12) but was still sensitive enough to distinguish two species in Table 1. However, this distinction was not found in the phylogeny (Figure 4.13), which included *N. italicum* within the same clade as some of the other *N. ningerense*. It is possible that the short ITS region revealed variation between strains more so than species. A longer read sequence may also provide a better separation of sequences. The spore morphology was distanced from all other morphotypes, but *Neofusicoccum* still exhibited the same septate hyphae characteristic of all Ascomycetes (Figure 4.14).

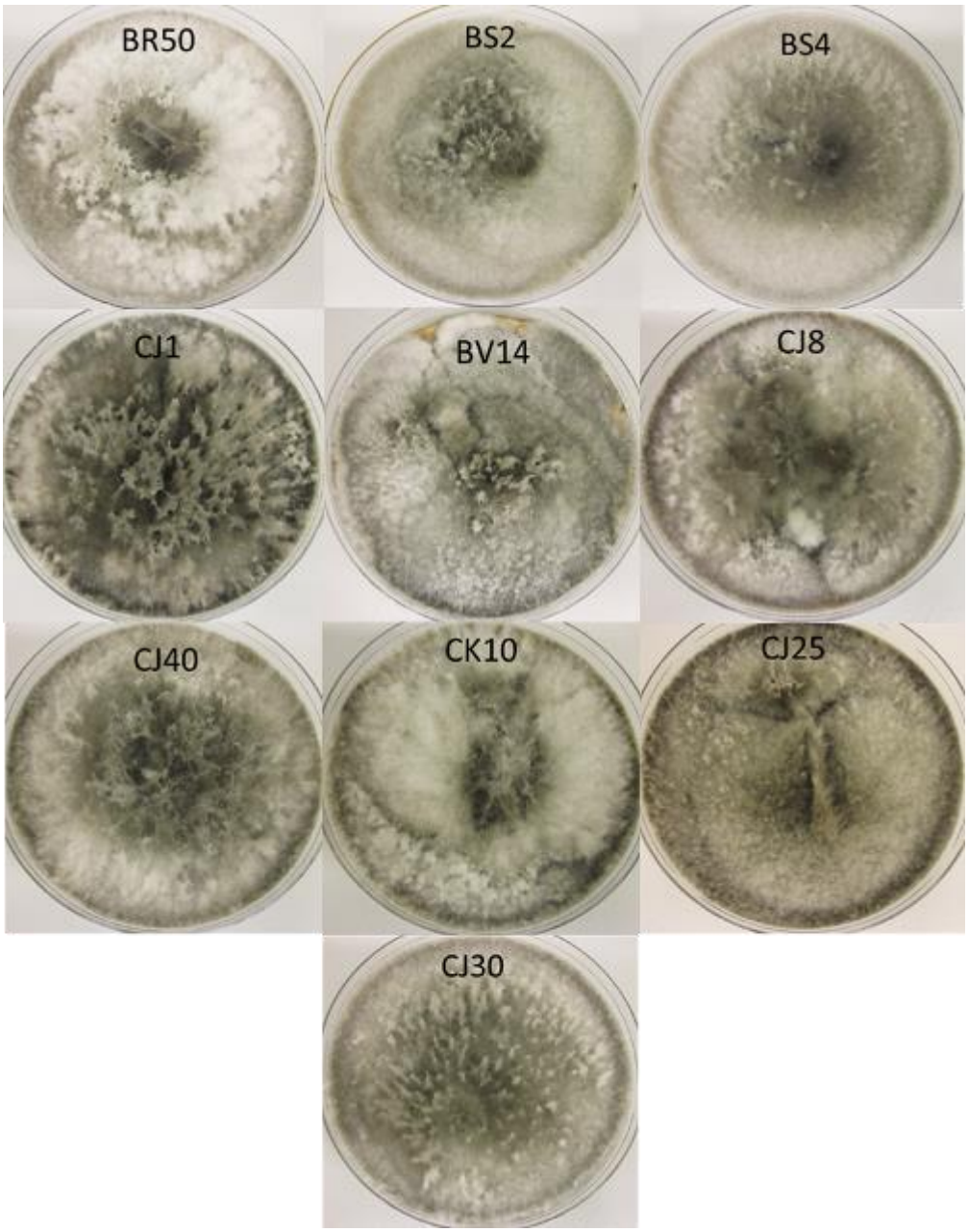

Figure 4.11: Representative WhiteGreyCotton (*Neofusicoccum*) morphotypes chosen for ITS sequencing. Isolates were cultured from avocados originating from different countries.

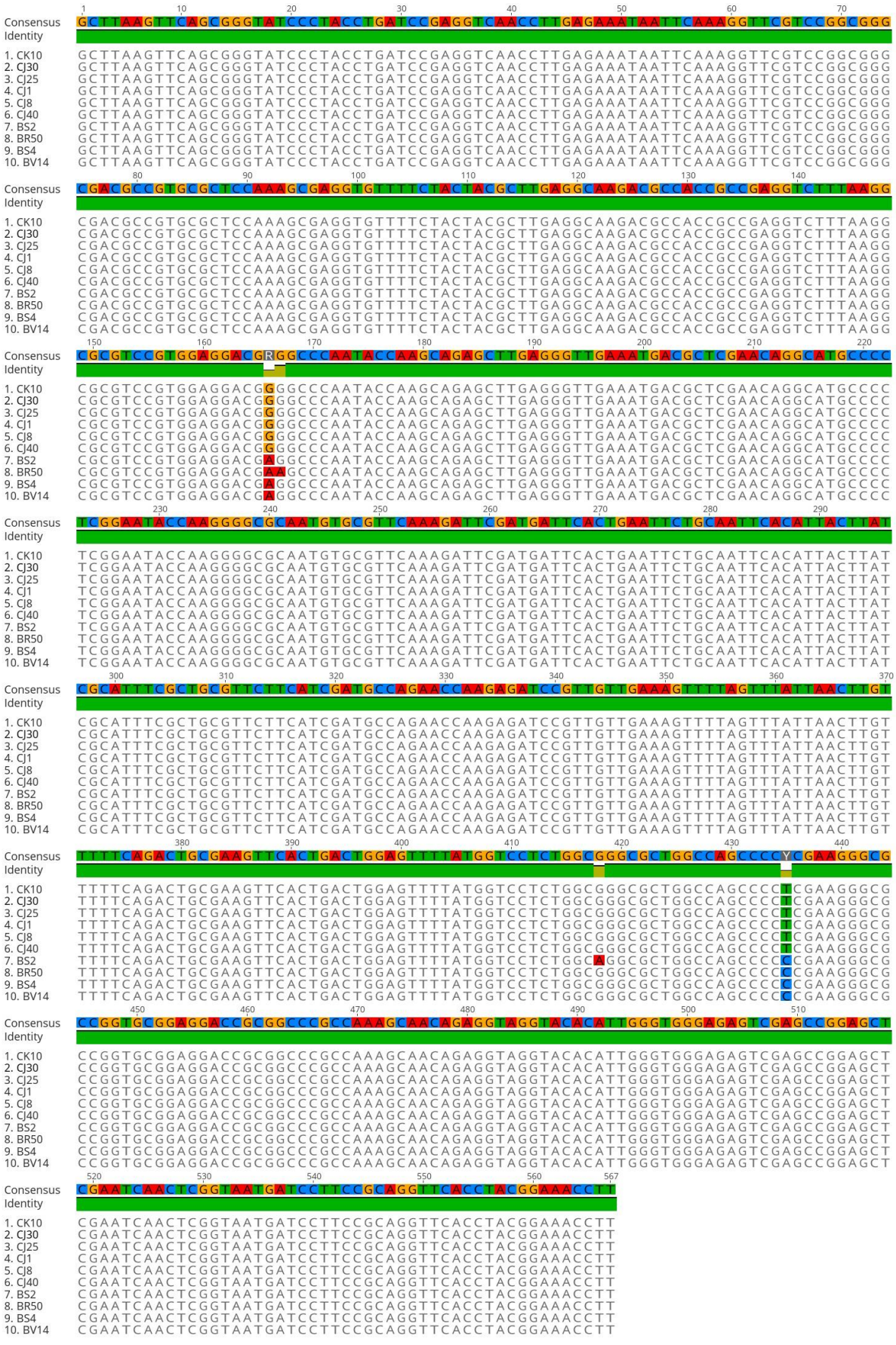

Figure 4.12: ITS sequence alignment for all WhiteGreyCotton morphotypes

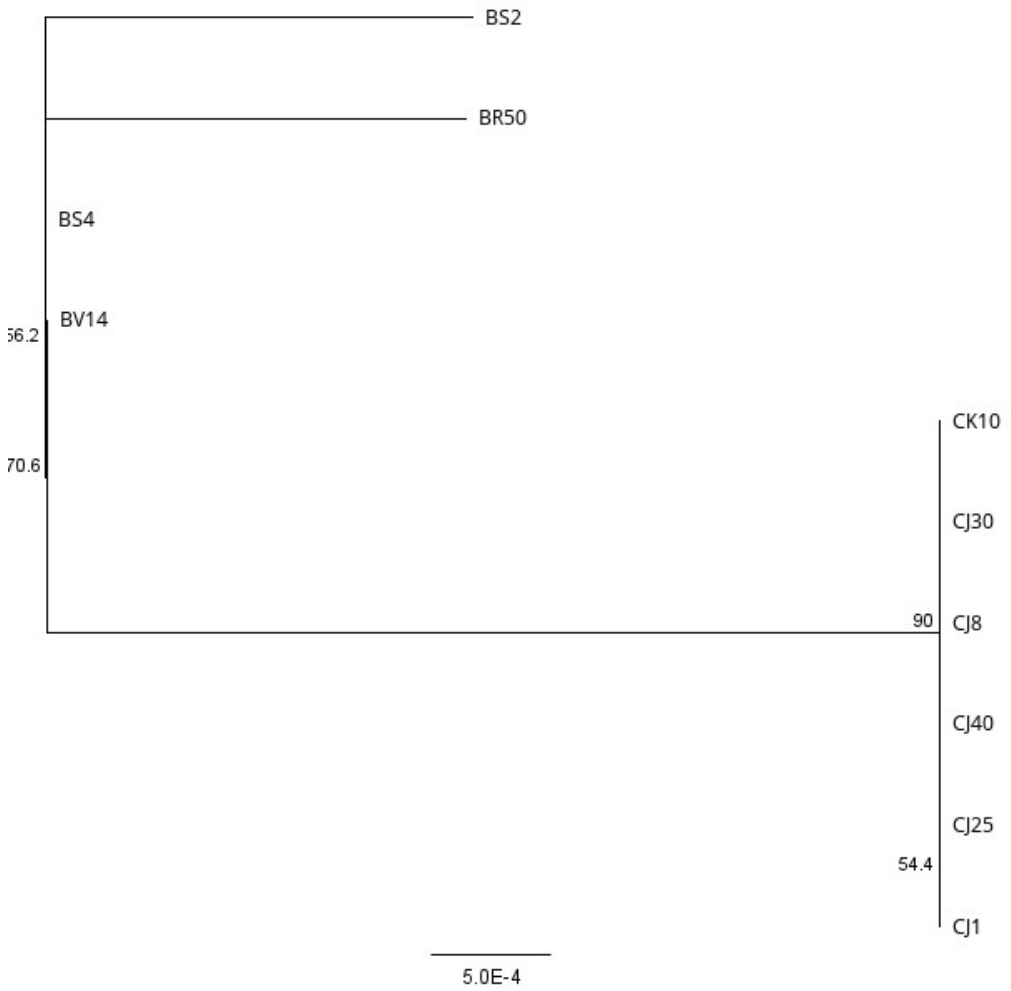

Figure 4.13: Single locus (ITS) neighbour-joining consensus phylogeny of WhiteGreyCotton (*Neofusicoccum*) samples isolated from avocado mesocarp placed on MEA, incubated at 25 ºC, and sub-cultured twice before DNA extraction and sequencing. Countries of origin for the isolates are as follows: CK and CJ isolates are from Israel, and BV, BS, and BR isolates are from Mexico. Bootstrap support of 500 replicates shown above nodes identify four clades.

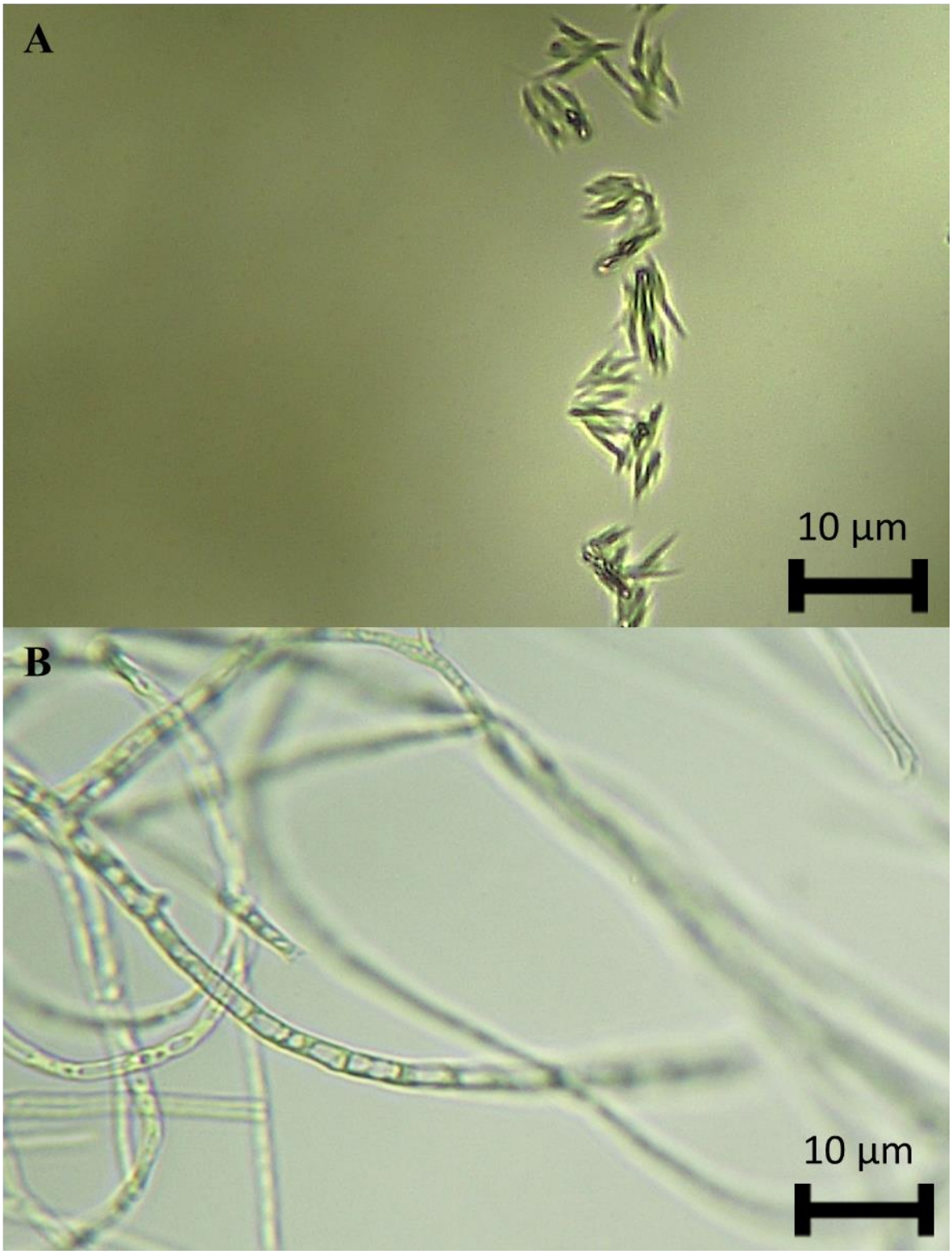

Figure 4.14: Spores collected from the WhiteGreyCotton (*Neofusicoccum*) morphotype cultured on MEA at 25 ºC for five days (A), and fungal mycelia from the same sample (B)

4.3.5 WHITELAYERED [*Neopestalotiopsis*]

All the WhiteLayered morphotype samples in Figure 4.15 produced consistent and well aligned ITS sequence regions (Figure 4.16). These sequences corresponded to three species of *Neopestalotiopsis* in Table 1. These species were more neatly organised into clades in the phylogeny than WhiteGreyCotton (Figure 4.17), but BS14 and BT30 did not follow this pattern. The sequence alignment shows that BS14 more closely matches the other *N. eucaypticola* sequences, and BT30 matches those of *N. aotearoa*, although the BLAST results in Table 1 yielded a high species identity match for *N. aotearoa* and *N. aucaypticola*, respectively. It is possible that this ITS region was not long enough to confidently identify the species of this morphotype using BLAST. The conidia morphology shown in Figure 18 is consistent with that of *Neopestalotiopsis*, (Maharachchikumbura et al., 2014) but these are indistinguishable from *Pestalotiopsis*.

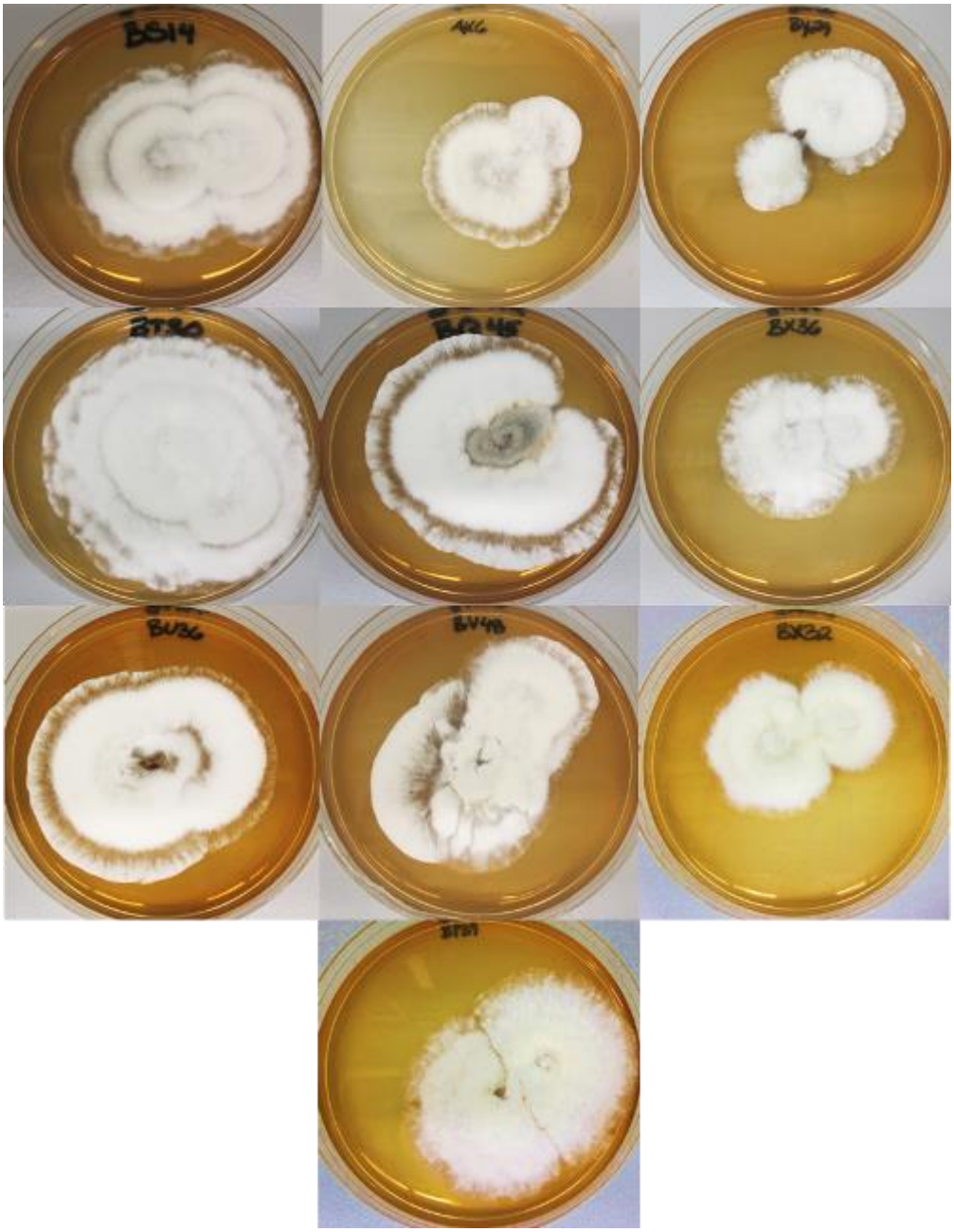

Figure 4.15: Representative WhiteLayered (*Neopestaloiopsis*) morphotypes chosen for ITS sequencing. Isolates were cultured from avocados originating from different countries.

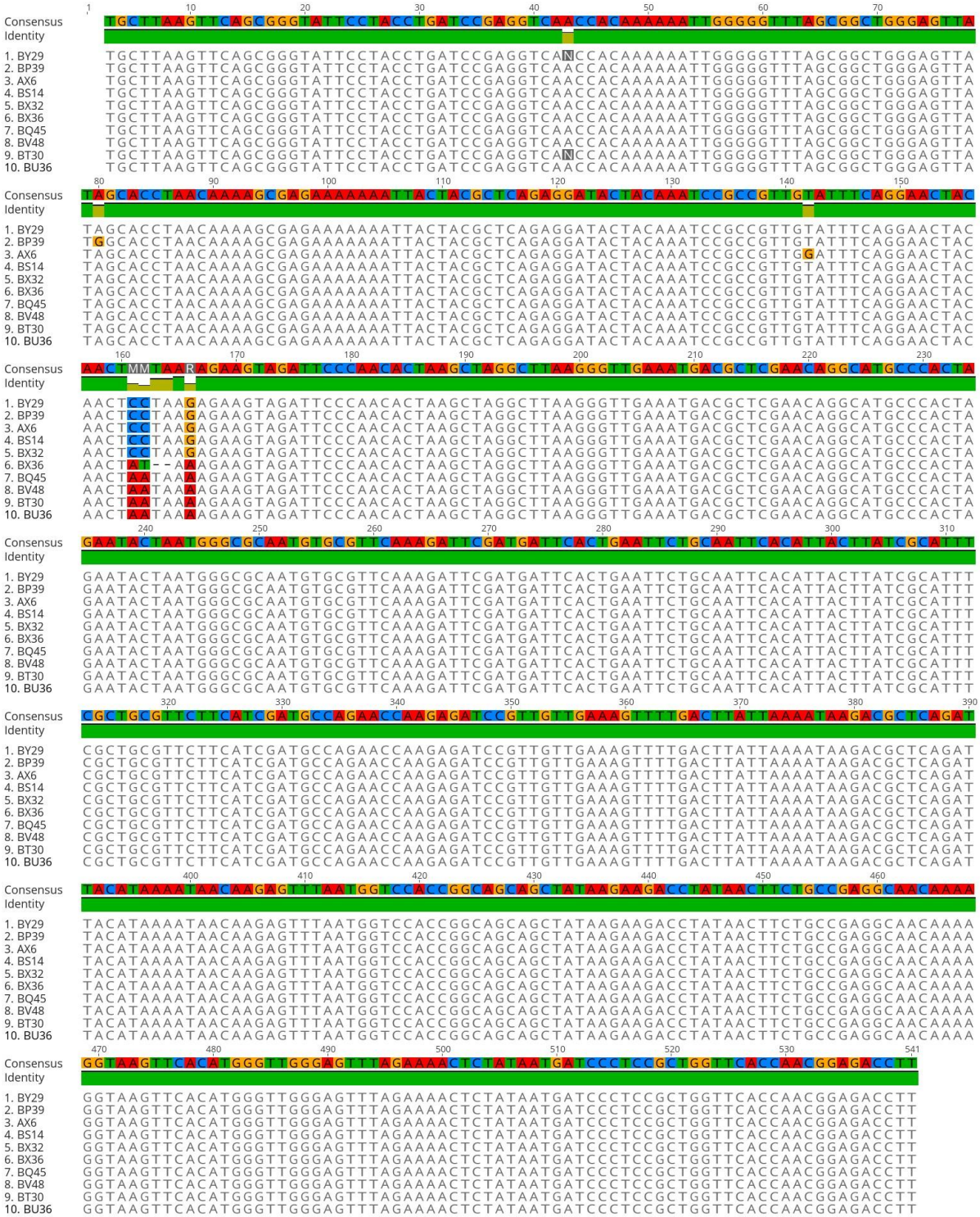

Figure 4.16: ITS sequence alignment of all WhiteLayered morphotypes

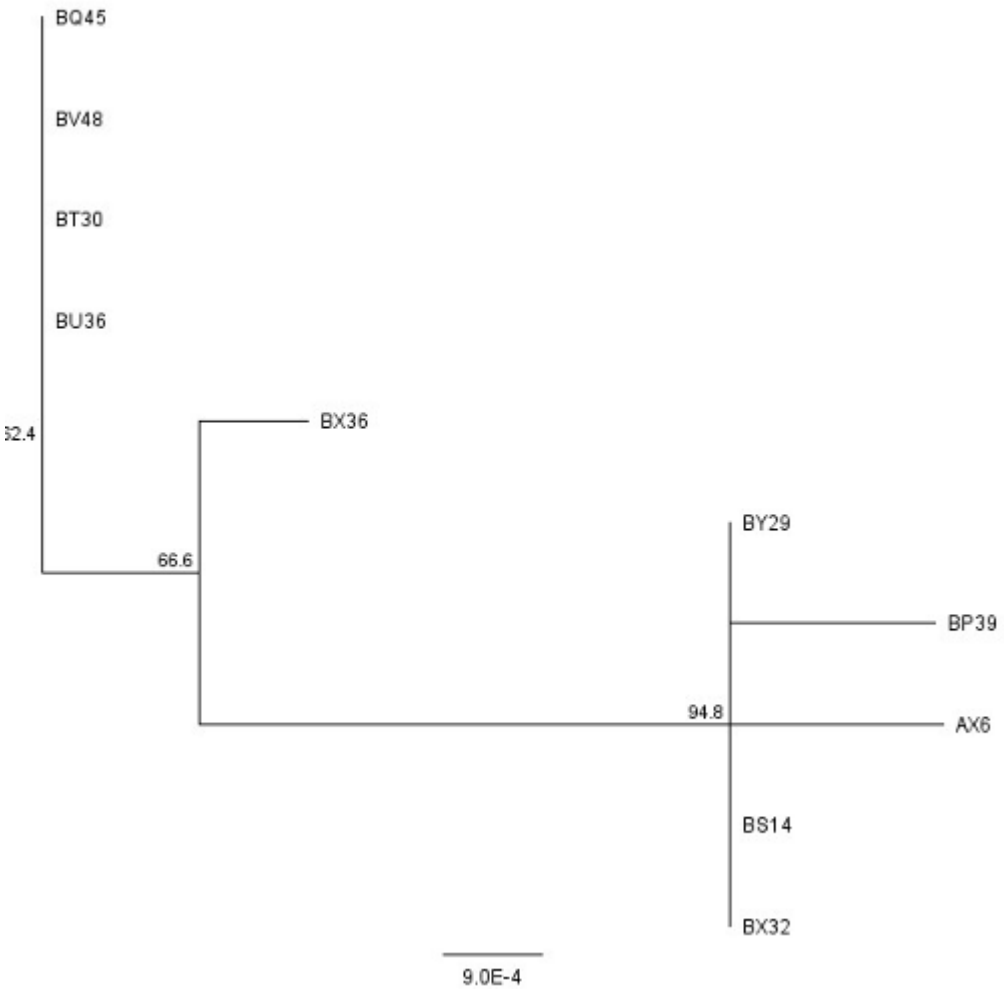

Figure 4.17: Single locus (ITS) neighbour-joining consensus phylogeny of WhiteLayered (*Neopestaloiopsis*) samples isolated from avocado mesocarp placed on MEA, incubated at 25 ºC, and sub-cultured twice before DNA extraction and sequencing. Countries of origin for the isolates are as follows: All isolates are from Mexico except for AX6, which is from South Africa. Bootstrap support of 500 replicates shown above nodes identify three clades.

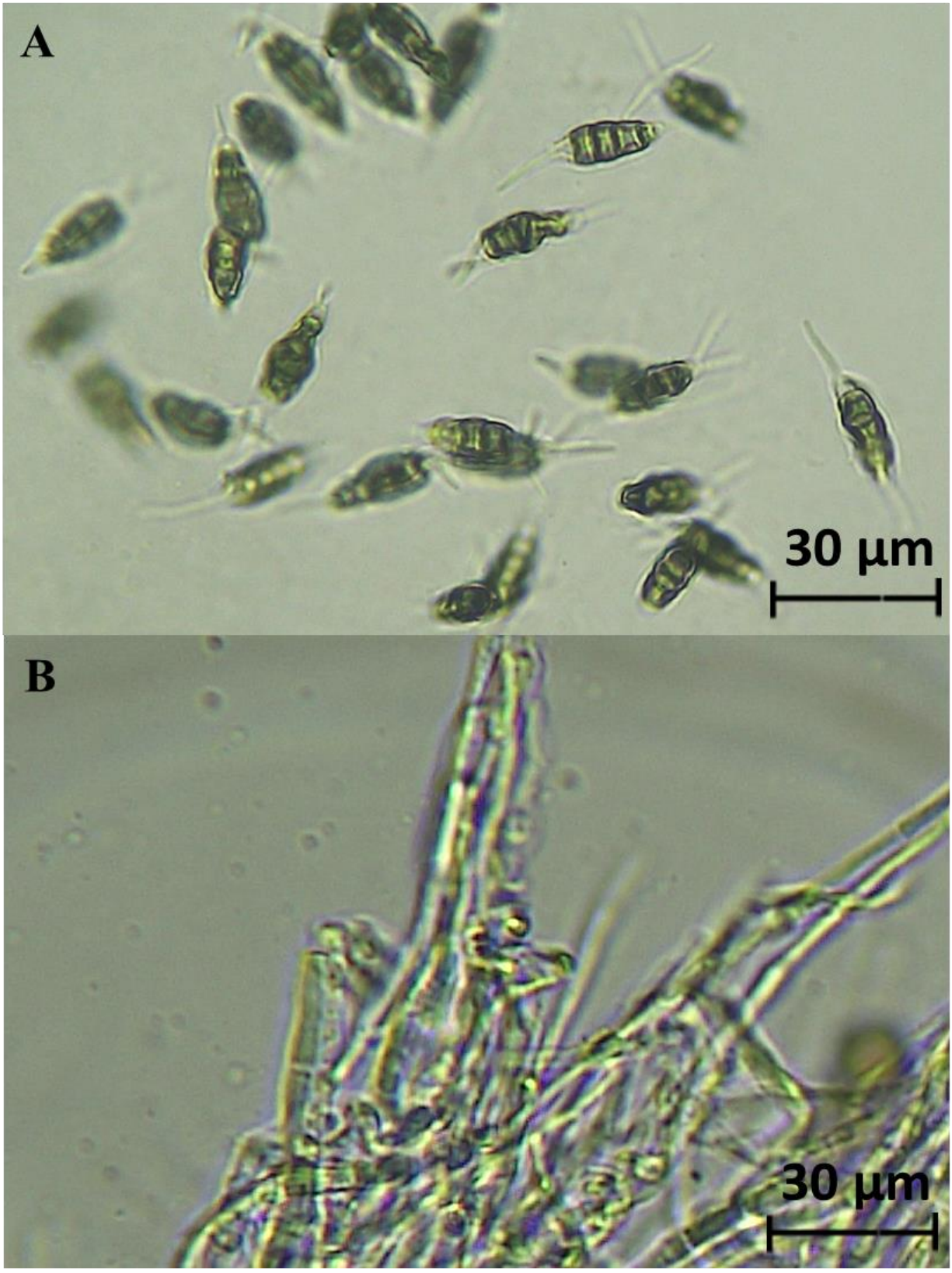

Figure 4.18: Spores collected from the WhiteLayered (*Neopestalotiopsis*) morphotype cultured on MEA at 25 ºC for five days (A), and fungal mycelia from the same sample (B)

4.3.6 WHITESTRIATED [*Diaporthe*]

Showcasing one of the most successful implementations of plate morphotype species identification, WhiteStriated was the most easily recognised and reliably cultured morphotype which was consistently identified as the same genera. All ten sample replicates shown in Figure 4.19 were confirmed as *Diaporthe*. The ITS regions of these representative samples also proved to be highly variable (Figure 4.20), revealing five species (Table 4.1). The species were organised into separate clades in the phylogeny (Figure 4.21), indicating that this ITS region is useful for distinguishing species of *Diaporthe*. Spore morphology was less reliable for *Diaporthe*, as it did not readily produce spores under laboratory conditions. The structures shown in Figure 4.22A resemble round *Diaporthe* spores (Lawrence et al., 2015), but an exact classification using morphology is ambiguous.

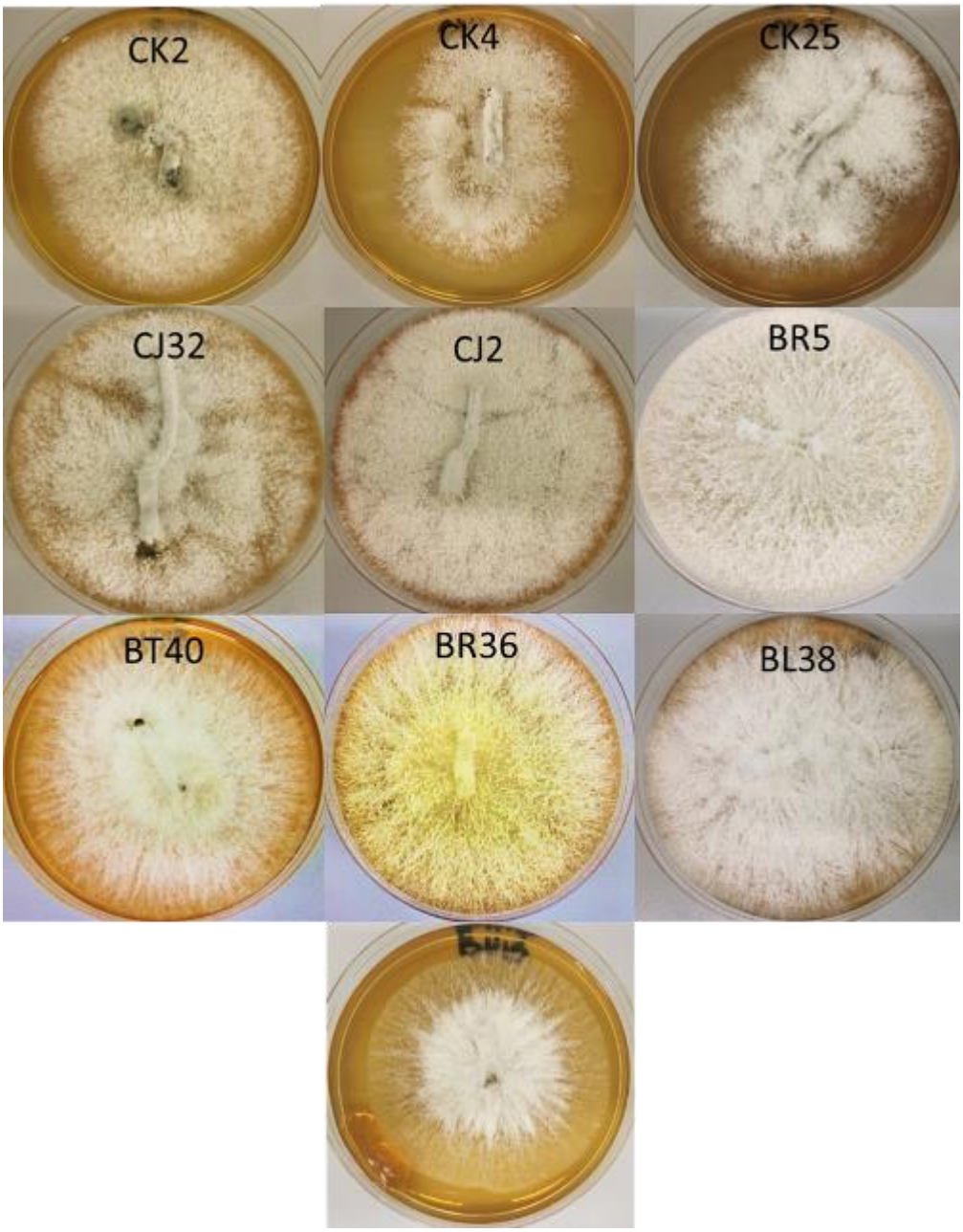

Figure 4.19: Representative WhiteStriated (*Diaporthe*) morphotypes chosen for ITS sequencing. Isolates were cultured from avocados originating from different countries.

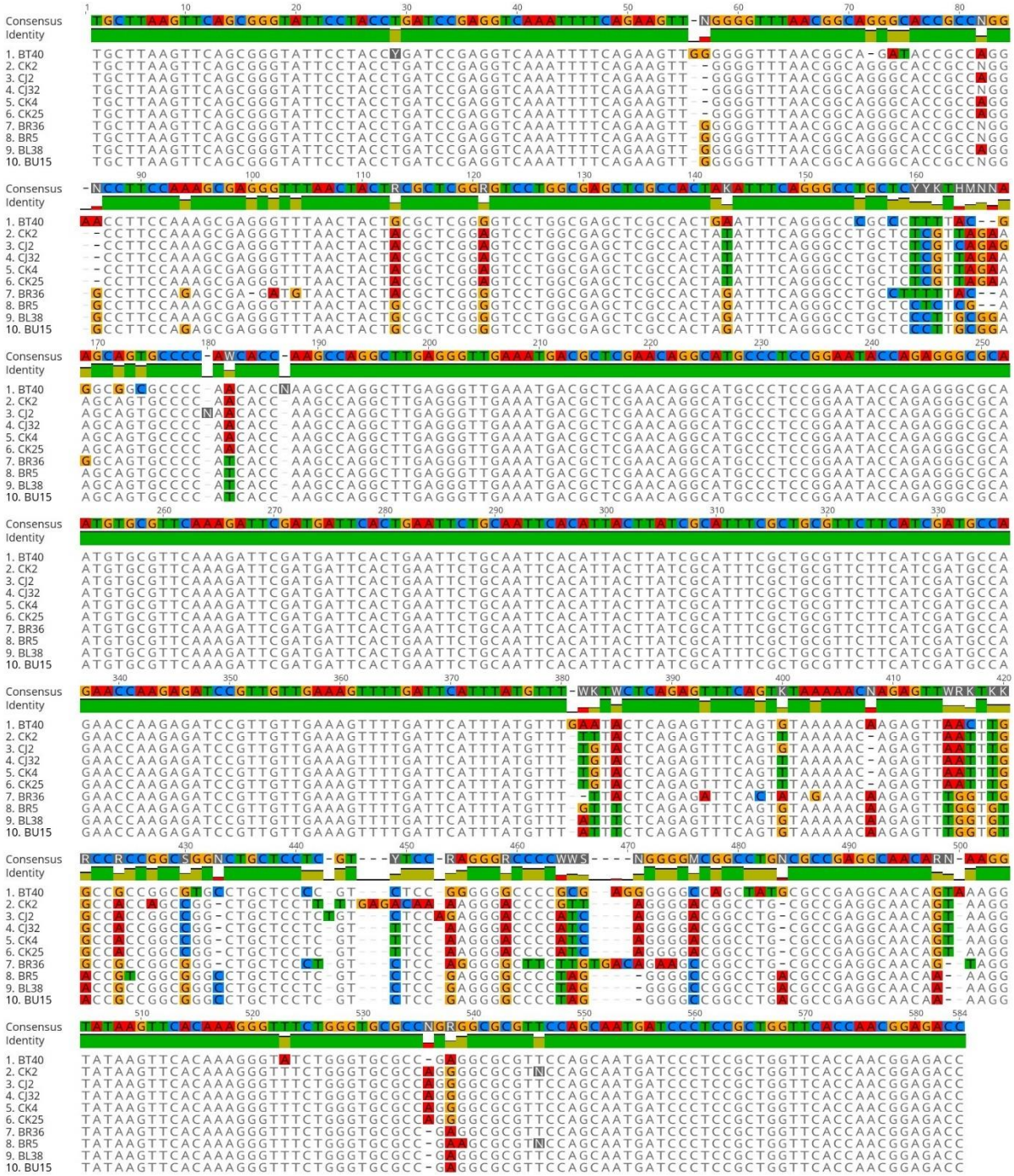

Figure 4.20: ITS sequence alignment of all WhiteStriated morphotypes

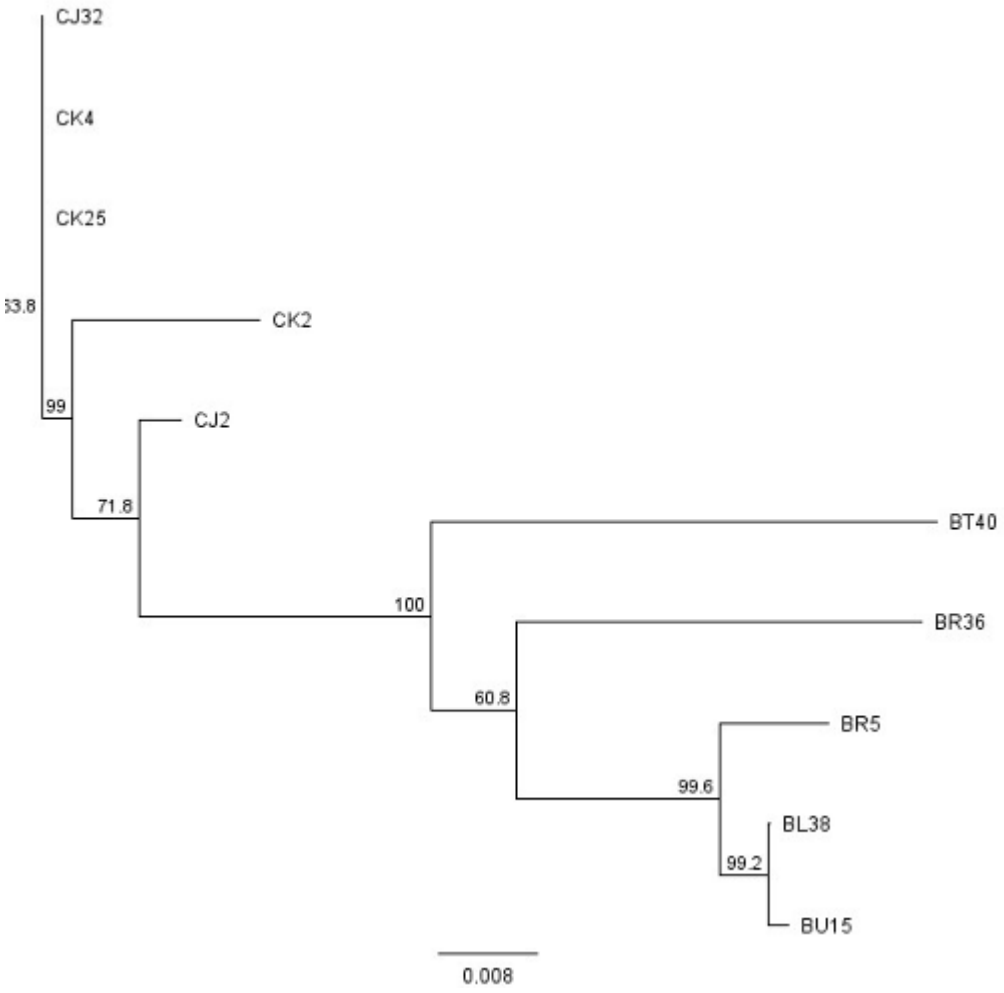

Figure 4.21: Single locus (ITS) neighbour-joining consensus phylogeny of WhiteStriated (*Diaporthe*) samples isolated from avocado mesocarp placed on MEA, incubated at 25 ºC, and sub-cultured twice before DNA extraction and sequencing. Countries of origin for the isolates are as follows: CK and CJ isolates are from Israel and all other isolates are from Mexico. Bootstrap support of 500 replicates shown above nodes identify seven clades.

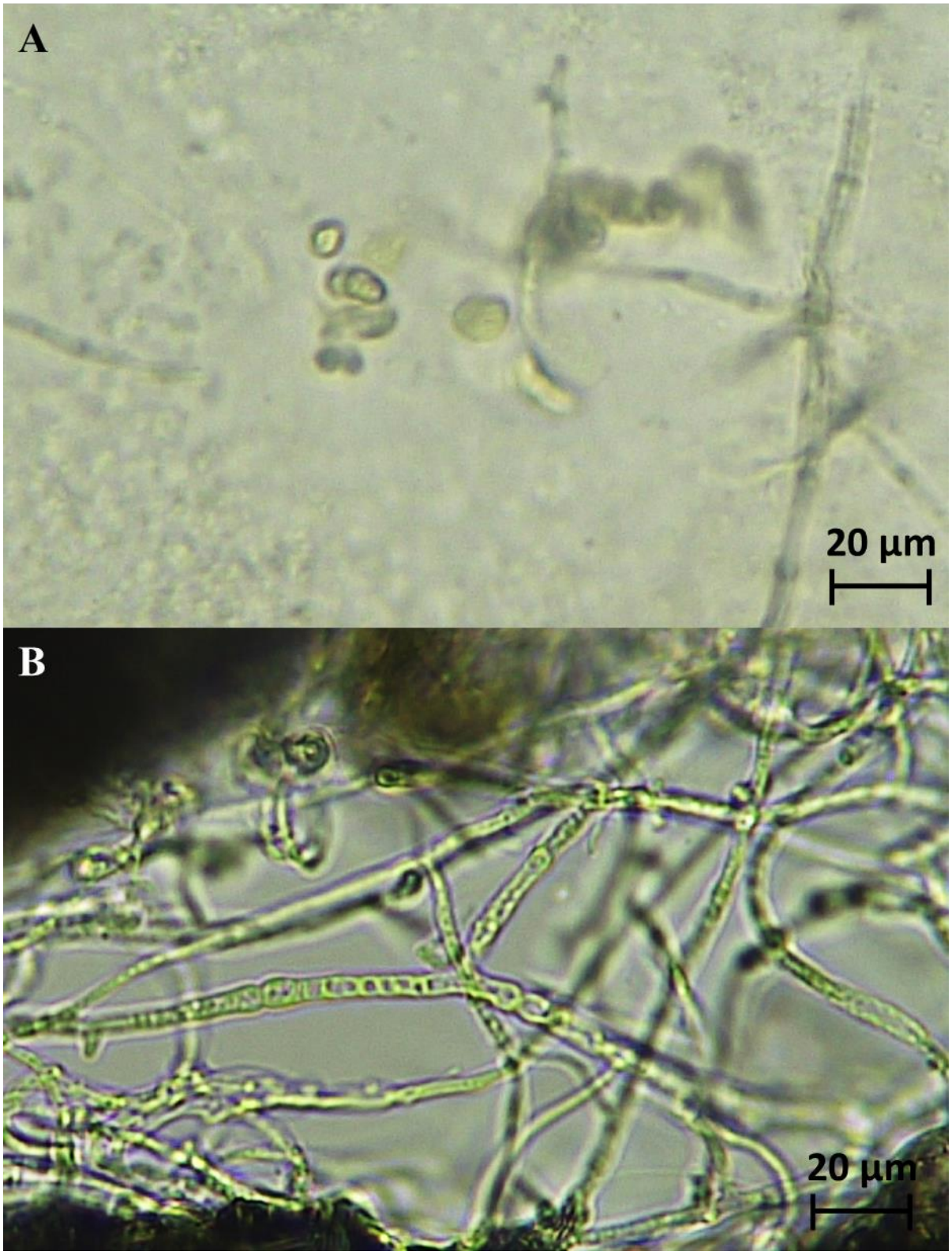

Figure 4.22: Spores collected from the WhiteStriated (*Diaporthe*) morphotype cultured on MEA at 25 ºC for five days (A), and fungal mycelia from the same sample (B)

4.3.7 WHITESLIME AND YELLOWSLIME [*Phomopsis: Diaporthe* anamorph (?)]

More than ten representative samples of WhiteSlime and YellowSlime were submitted for sequencing, and all but three failed to sequence. Figure 4.23 shows representative samples of the WhiteSlime and YellowSlime phenotypes which failed to sequence. Of the few successful sequences, a BLAST analysis indicates they are species of *Diaporthe*. This genus is known to have a reproductive anamorph, designated *Phomopsis,* and it has been shown to produce a yellow exudate. It is possible that the slime cultured from the avocados was the reproductive state of *Diaporthe*. The three available sequences, YellowSlime 1, YellowSlime 2, and P45, aligned well with the other ten *Diaporthe* samples (Figure 4.24). The phylogeny also shows that two of the samples form their own clade, as they were identified as a new species of *Diaporthe* not represented in the WhiteStriated samples (Figure 4.25). Microscopic examination of the slime proved more informative. The substance was spread across a microscope slide and photographed (Figures 4.26A, 4.26B), and the same sample was then fixed, and Gram stained (Figures 4.26C, 4.26D). The unstained sample shows small, rounded particles floating in a mass of slime, which are clearer in the stained images. The staining process removed the slime matrix and exposed the smaller granules.

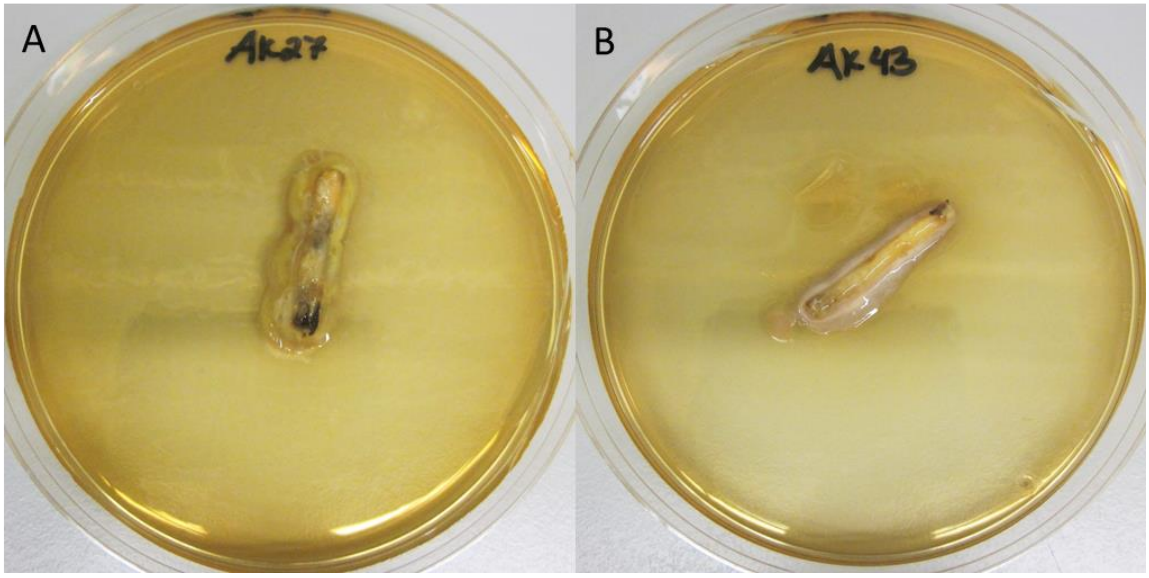

Figure 4.23: An example of the YellowSlime morphotype (A) and the WhiteSlime morphotype (B) isolated from avocado core samples from shipment AK

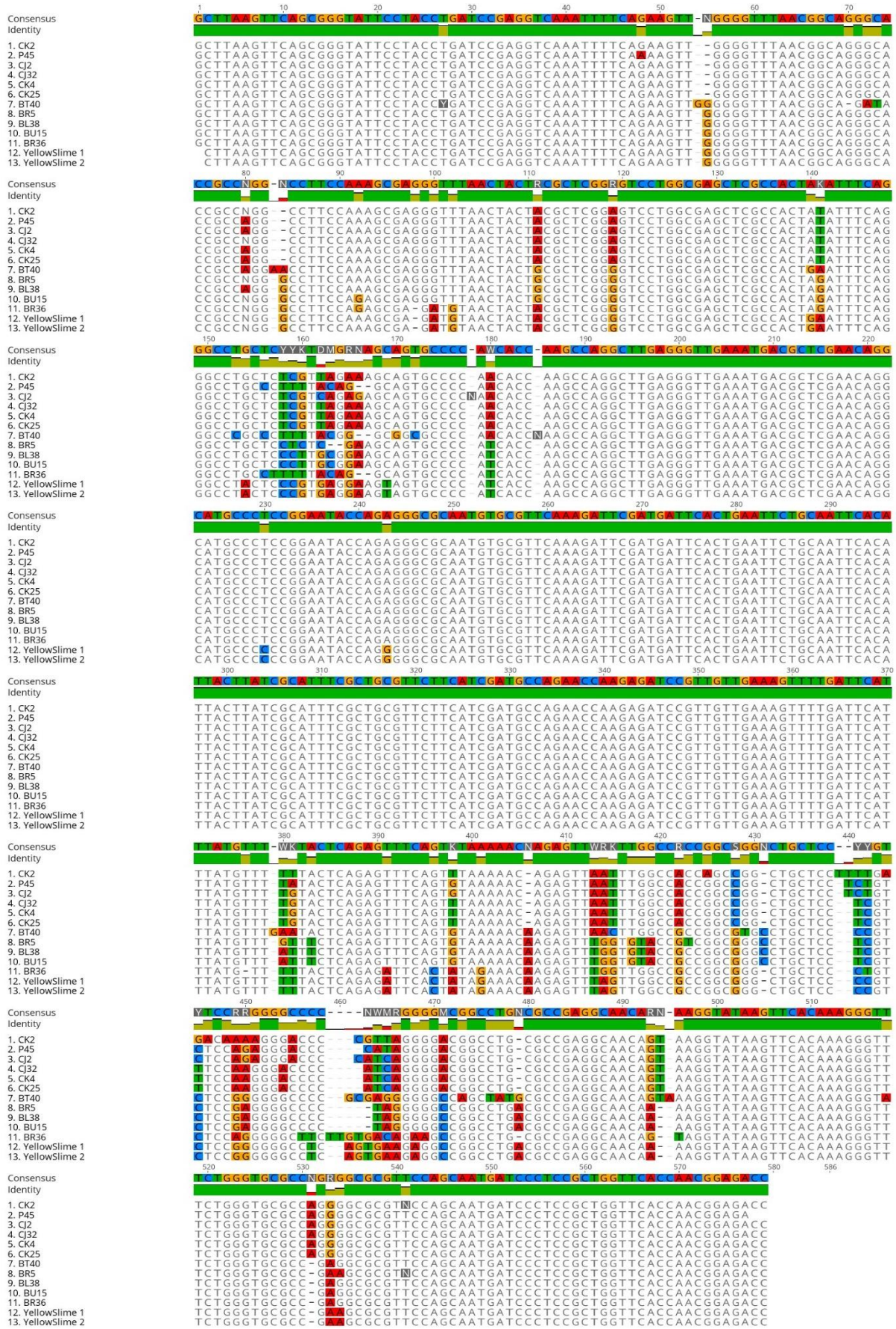

Figure 4.24: ITS sequence alignment of all WhiteSlime and YellowSlime morphotypes combined with all WhiteStriated sequences

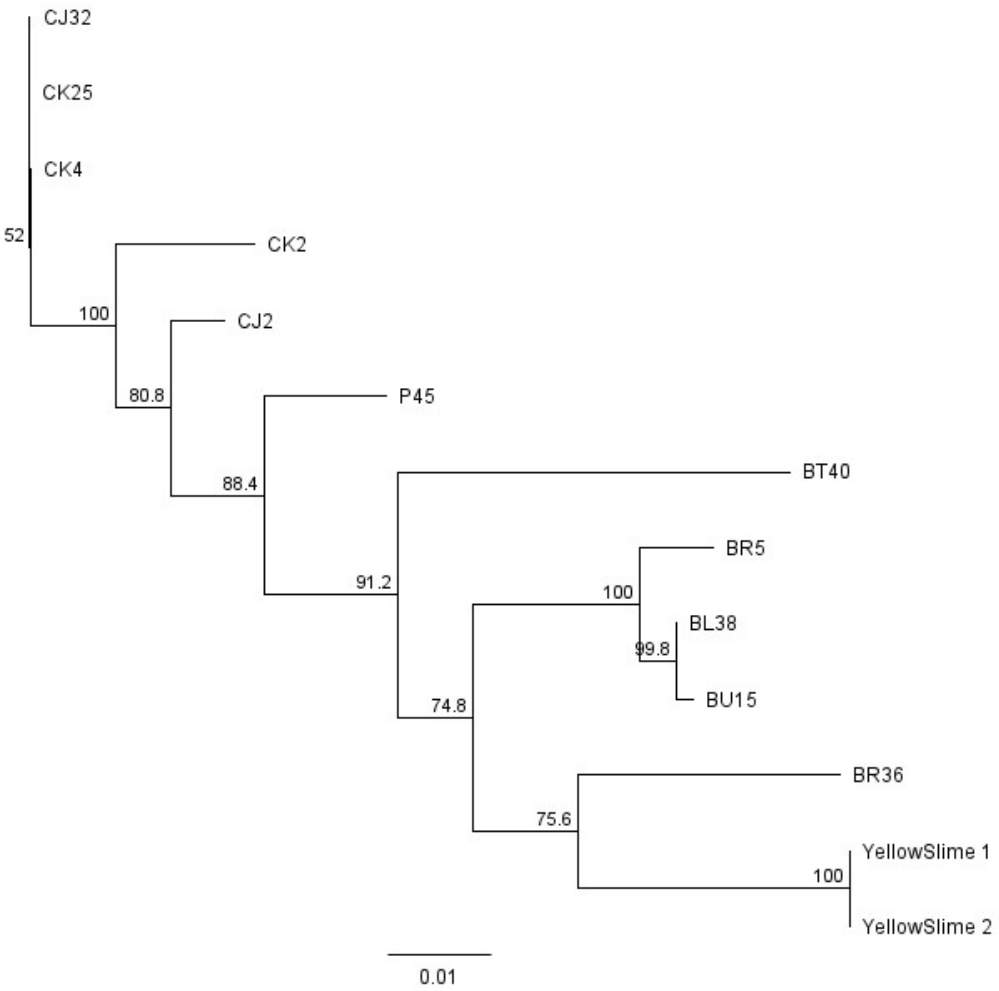

Figure 4.25: Single locus (ITS) neighbour-joining consensus phylogeny of both Slime morphotypes combined with WhiteStriated samples isolated from avocado mesocarp placed on MEA, incubated at 25 ºC, and sub-cultured twice before DNA extraction and sequencing. Countries of origin for the isolates are as follows: CK and CJ isolates are from Israel, BR5, BT40, BR36, BL38, BU15 are from Mexico, YellowSlime 1 and 2 are from Israel, and P45 is from South Africa. Bootstrap support of 500 replicates shown above nodes identify ten clades.

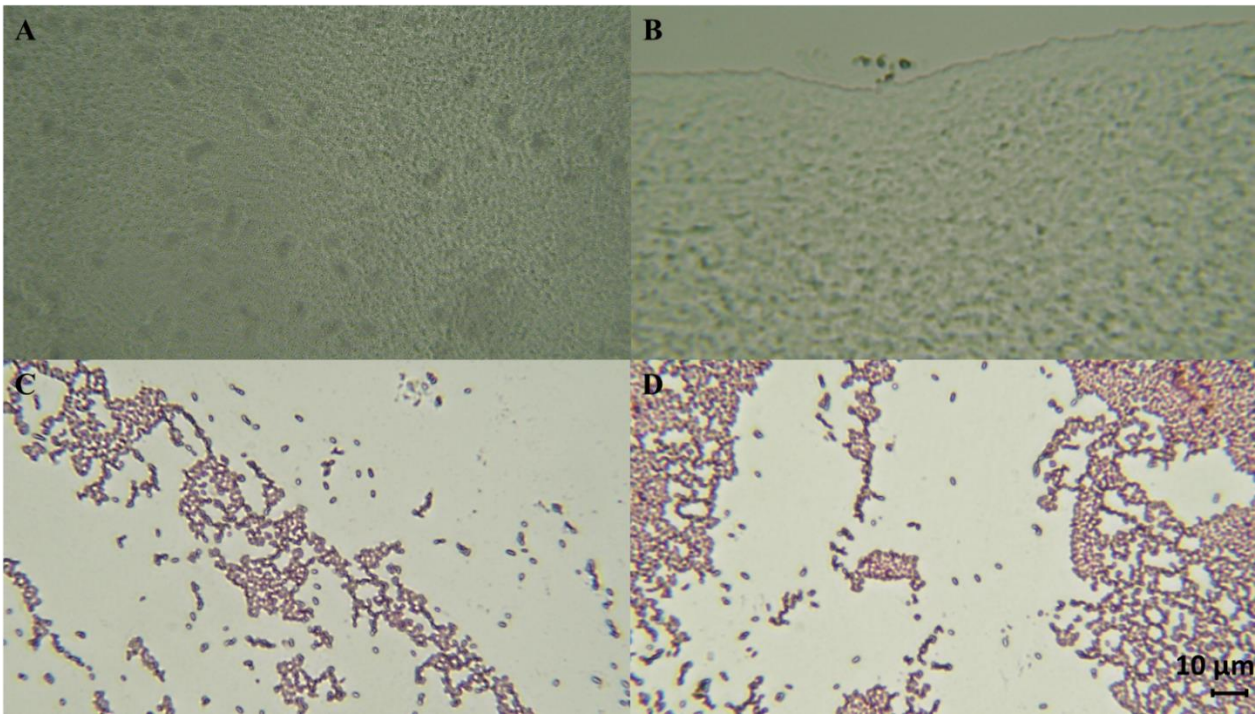

Figure 4.26: Microscope images of the YellowSlime morphotype cultured on MEA at 25 ºC for five days. A and B show the unstained material and C and D represent the same slide after Gram staining. Scale bar represents 10 µm for all images

4.4 Discussion

The short segment of the small ribosomal subunit ITS1 region consistently produced high quality sequence data and robust alignments for five of the seven most abundant morphotypes. Although only β-Tubulin and ITS1 regions were chosen for sequencing, and β-Tubulin unfortunately failed, the ITS1 region proved to be enough to positively identify most of the isolated morphotypes. Using a standard fungal culture media and consistent growth conditions, the fungal morphotypes isolated in this study were found to exhibit some reproducible patterns in their growth behaviour. It is unfortunate that the slime phenotypes were impossible to sequence. There could be several explanations for this. If the slime morphotypes were reproductive exudates of *Diaporthe*, they may not possess any genetic material. It could also be the case that the extraction techniques used were not optimised for a type of organism which produces excess polysaccharide material, or that this specific ITS region was not present. The gram staining revealed particles in the slime sample which look like yeast cells. The failure to sequence could be due to the ITS primers not being optimised for yeast samples. Although *Diaporthe* has been documented as producing a yellow exudate identical to the solitary slime morphotypes, and it may not yield genetic material, the particles revealed under the microscope suggest a yeast. If these particles are reproductive structures exuded from *Diaporthe*, they would be expected to retain some genetic material. It is possible that the slimes which were found mixed with *Diaporthe* samples were an exudate and many of the solitary slime isolations were most likely yeasts. Further study may be needed to confirm these conclusions.

Dissanayake et al. (2017) describes the genus *Diaporthe* as a worldwide generalist associated with a broad range of host species. It has been a known avocado pathogen since 1988, as well as a prolific disease-causing agent for many other plant species. *Diaporthe* was first was described in 1870, and subsequent species were

named in accordance with their host association. However, it is not host-specific, with a single species favouring many host types and multiple species often found on the same host. Morphologically, they are indistinguishable, requiring genetic identification to classify the species (Dissanayake et al., 2017). They are agriculturally important pathogens of the *Citrus* genus, with many species responsible for the peel disorder known as melanose, as well as stem end rot, in citrus growing regions around the world (Udayanga et al., 2014).

Some of the most prominent citrus pathogens are *Diaporthe citri* and *D. cytosporella*. *D. citri* was found to be a widespread generalist of citrus trees worldwide, whilst *D. cytosporella* is located mainly in Europe and California (Udayanga et al., 2014). *Diaporthe* is also responsible for twig blight and dieback of blueberry (Hilário et al., 2020). Many of the *Diaporthe* species identified in this thesis (4.3) were only recently described, and they were not previously isolated from avocado prior to this study. *D. velutina* was described in 2017 (Dissanayake et al., 2017) and *D. macadamiae* is a novel macadamia species discovered in 2020 by Wrona et al. (2020). *D. humulicola* was also recently described by Allan-Perkins et al. in (2020) after isolating it from hop. *D. caatingaensis* was discovered on *Lophosoria* by Crous et al. (2016). *D. cucurbitae* is a known avocado pathogen, but was first described by Udayanga et al. (2015) from its cucumber host, and *D. terebinthifolii* was isolated from *Schinus terebinthifolius* in 2013 by Gomes et al.

In addition to being well-known avocado pathogens, species of *Diaporthe* exist as endophytes or saprophytes on a range of economically important crop species. These include grape, soybean, dragon fruit, avocado, prune, cucurbits, and current to name a few (Dissanayake et al., 2017; Guarnaccia, et al., 2018; Huda-Shakirah et al., 2021). Species of *Diaporthe* have been isolated from healthy plant tissue as endophytes, or as

saprobes on dead plant material (Dissanayake et al., 2017).

A study of *D. citri*, by Mondal et al. (2007) revealed that *Diaporthe* typically reproduces by releasing conidia in a slimy mass on dead plant matter. The authors found that, for *D. citri* specifically, when the fungus grows saprophytically, the majority of reproductive activity occurs on the dead branches within the tree canopy. The fungal pathogen consumes the dead plant matter and then sporulates when conditions remain optimal for the desired time period, specifically 21 ºC and 70% relative humidity for 36 to 48 hours (Anco et al., 2012; Mondal et al., 2007). Interestingly, the fungus was found to increase sporulation at higher temperatures and humidity, but the resulting reproductive structures were not as viable (Mondal et al., 2007).

As an ascomycete, *Diaporthe* produces eight ascospores in a sac-like structure called an ascomata when it is in its sexual phase. During the asexual phase, it produces pycnidial conidiomata, with distinct alpha and beta conidia within the reproductive exudate. Both sexual morphs are associated with the branches and leaves of their hosts (Yang et al, 2017).

The genus *Colletotrichum* is another well-known group of plant pathogens which exist as broad range generalists. The species isolated in this project, *Colletotrichum arecicola*, was recently described as a leaf pathogen isolated from the palm tree *Areca catechu* in China (Cao et al., 2020). Talhinhas & Baroncelli (2021) describe it as a known pathogen on many host species, including strawberry, apple, citrus, stone fruits, and certain cereal crops such as maize and sorghum. The fungus can also be found on fern and pine. *Colletotrichum* is primarily a generalist anthracnose pathogen, meaning it causes skin legions on the body of the fruit as opposed to a stem end rot. It has a worldwide distribution and global relevance, whilst also existing as an endophyte. Members of this genus initially infect their host in the biotrophic phase of

their life cycle, during which they produce large intracellular primary hyphae. Once infiltrated, the fungi are able to switch to a necrotrophic growth phase, which involves narrower secondary hyphae which are able to spread through host tissue. This switch from biotropy to necrotrophy is regulated by the inner biochemical processes, but more research is needed to determine the exact triggers which facilitate the switch (Talhinhas & Baroncelli, 2021).

The genus *Fusarium* is an exceptionally widespread and important plant pathogen which threatens economically important crop plants. The majority of its members are soil-inhabiting fungi whose conidia can be disperse by rainwater splash or via irrigation systems, but they can become airborne when dried, enabling dispersal by wind over long distances (Rampersad, 2020). As such, *Fusarium* has a worldwide distribution. One of the two species isolated in this project, *F. perseae* is a known avocado pathogen, as is evident by the name. It was isolated from the trunk cankers of an infected avocado tree (Guarnaccia et al., 2018). *F. pernambucanum* is a novel species isolated from an insect host *Dactylopius opuntiae* and described by Santos et al. (2019). Like many fungi, *Fusarium* requires some moisture to reproduce, and it sporulates best at 25 ºC when the substrate maintains a pH of 6 (Cruz et al., 2019).

The genus *Pestalotiopsis* is another generalist with worldwide distribution. It includes endophytic species which can remain dormant unless its pathogenic phase is triggered. Pathogenicity can be initiated by a number of circumstances, including host stress, pathogen load or virulence, plant susceptibility, or environmental factors which favour pathogen growth. Although *Pestalotiopsis* can convert into an antagonistic form, it is considered a weak plant pathogen. Inoculum usually originates from plantations, surrounding flower fields, crop debris, diseased stock plants, used growing media, contaminated soil, or infected nursery tools. Reproductive structures are robust

and able to survive most adverse weather conditions, where they splash onto new hosts (Sajeeway et al., 2011). Their host range is vast, and they have been found primarily in China, Australia, and South Africa on trees and forbs, as well as a wide range of ornamental and agricultural crops (Jeewon et al., 2004). The two species isolated in this project, *P. grevilleae* and *P. papuana*, were not previously isolated from avocado. *P. grevilleae* was isolated from the spider flower (*Grevillea juniperina*), whereas *P. papuana* is named for its location of origin, Papua New Guinea (Maharachchiumbura et al., 2014)

Lasiodiplodia is a genus of globally distributed tree pathogens. They are not host specific, and affect many agricultural tree crops such as mango, cashew, avocado, as well as timber trees (Mehl et al., 2017). *L. pseudotheobromae*, *L. parva*, and *L. brasiliensis* were isolated in this project, with the first being a well-known avocado pathogen (Zhao et al., 2010). *L. parva* was first isolated from the coco tree *Theobroma cacao* according to Alves et al. (2008), and *L. brasiliensis* was described by Netto et al. (2014). The exact life cycle and reproductive strategy of these species have not been fully explored, but *L. theobromae* has been extensively studied as a common tree endophyte with a latent pathogenic phase. Host stress is the most likely trigger for this change (Salvatore et al., 2020).

Neofusicoccum is another well-known plant pathogen with worldwide distribution. According to Salvatore et al. (2021), members of this genus attack a wide range of woody hosts, including forest trees, orchard crops, and ornamentals. It is most widely recognised as a grape pathogen, causing leaf spot, fruit rot, and shoot dieback among other rot symptoms. Species within this genus are latent, endophytic pathogens, with a plurivorous growth habit (Salvatore et al., 2021). The two species isolated in this project, *N. italicum* and *N. ningerense*, were described by Marin-Felix et al. in 2017 and

Li G et al. in 2020, respectively. Neither has been isolated from avocado at the time of this study; *N. ningerense* was first isolated on eucalyptus. The fungus prefers 28 ºC and high UVA light exposure for optimum sporulation (Chan et al., 2020)

Neopestalotiopsis is a genus of fungal pathogens causing general fruit diseases. Their host range includes jackfruit, rose apple, mangosteen, plum, snake fruit, rambutan, strawberry, blueberry, and avocado. They are widely distributed around tropical and temperate areas (Darapanit et al., 2021; Santos et al., 2022). The three species isolated in this project were all previously unknown on avocado. Maharachchikumbura et al. (2014) first described *N. eucalypticola* on eucalyptus, *N. piceana* on spruce trees (Picea), and named *N. aotearoa* after its location. The growth habit of this genus has not been well studied, but Belisario et al. (2020) found it to sporulate best at 25 ºC following long periods of wetness up to 72 hours and a 12-hour photoperiod.

Interestingly, *Neopestalotiopsis* was also confirmed as an endophyte naturally present in the terminal branches of healthy avocado trees (Shetty et al., 2016). The study also found species of *Alternaria*, *Colletotrichum*, *Diaporthe*, *Lasiodiplodia*, *Neofusicoccum*, and *Neopestalotiopsis* to be endophytes of avocado branches, although all genera were also confirmed as known SER causal pathogens in previous experiments. It is possible that the behaviour of the fungus changes depending on which tissue it is located. Shetty et al. (2016) emphasised the beneficial contributions of endophytic fungi as part of a healthy tree microbiome, but these fungal genera are known to consume avocado mesocarp tissue, and they have been isolated from decayed fruit (Akgul et al., 2016; Montealegre et al., 2016). The question of whether SER causal pathogens grow endophytically through vascular tissue or germinate on the newly exposed abscission zone after harvest was discussed in Section 1.5. A controlled

examination of avocado branch tissue and SER incidence is needed to further explore this issue.

The goal of this study was to establish a reliable plate morphotyping procedure for the genus identification of fungal hyphae isolated from avocado mesocarp tissue. In previous studies, some effort has been made in examining the growth habits of fungi on culture plates, but this has largely been in the study of one genus (morphotype), exploring its growth forms on different plates, or an exploration of a few genera as part of a taxonomic analysis. Few of these studies used MEA. The choice of media is arbitrary, but so many different media are used across studies that the results are not comparable for plate morphotyping. There have not been many assessments of plate morphology for all common avocado SER causal species. If a plate identification method is to be applied in field research, it is important to note that the morphotype descriptions developed in this study can only apply to these genera if grown on MEA media prepared according to the manufacturer's instructions. Fungal morphotype characteristics vary when grown on alternative medias. For example, *Neofusicoccum* loses its grey appearance when grown on Potato Dextrose Agar (PDA) (Guarnaccia et al., 2016), and the layered pattern of *Lasiodiplodia* becomes obscured when overgrown.

It is also important to consider the weaknesses in plate identification if it is to be applied as a reliable genera identification system. In this study, WhiteDensefur and WhiteCotton were particularly difficult to distinguish, and WhiteLayered can have a similar appearance to either of these morphotypes depending on growth conditions. WhiteCotton can also resemble WhiteGreyCotton after seven days of growth. More replication is necessary to establish a reliable set of morphological features to distinguish the hyphae, or researchers can use spore identification for these morphotypes instead. This is especially true with WhiteCotton, which was revealed to

be a group of genera indistinguishable by their hyphal growth pattern on MEA. These genera all share similar optimal growth conditions and may have evolved a comparable physiology. These would be too difficult to separate using only plate identification, prompting field researchers to use either spore morphology or DNA barcoding when encountering these morphotypes.

Overall, the ITS barcode confirmation provided by Macrogen was an important first step in developing a plate identification system for remote fungal identification in the field. Future projects may wish to develop ID barcodes specific for each genus for researchers to quickly sequence any spores they trap. However, ITS proved to be variable enough to distinguish at least seven genera.

CHAPTER 5: FUNGAL COMMUNITY ANALYSIS

5.1 Introduction

Many fungal pathogens have already been confirmed by previous research to be SER causal species. Some of the most common are members of the genera *Alternaria*, *Colletotrichum*, *Fusarium*, *Lasiodiplodia*, *Neofusicoccum*, and *Diaporthe* (Akgul et al., 2016; Darvas & Kotze, 1987; Thompson et al., 2011; Wanjiku et al., 2020). Species of these are common to most orchards, co-existing with each other because they often prefer similar temperature and moisture conditions for optimal sporulation. Fungal pathogens, beneficial fungal species, bacteria, and yeasts form the tree's diverse microbiome (Shetty et al., 2016). Fungal species often work in tangent to decay a vulnerable fruit, whereas the presence of bacteria has been shown to both antagonise or promote fungal growth (Demoz & Korsten, 2006; Diskin et al., 2017). These communities may be influenced by the location of the tree and its vulnerability to colonisation.

Avocados first evolved in what is now Guatemala and South Mexico. They are an ancient lineage of flowering plants originally adapted to a tropical ecosystem. Their commercial popularity has expanded avocado cultivation into climates that distantly resemble their native environment, such as Mediterranean. Mexico, South Africa, and Israel were chosen as specific countries of interest. As the country of origin, Mexico has the longest evolutionary history with the pathogen. South Africa's mixture of tropical and sub-tropical climates most closely resemble the native environment of the avocado, and therefore its pathogens. Israel's Mediterranean climate provides a contrasting dataset to explore pathogen dynamics far removed from their preferred ecosystem. Weather is the single most crucial factor in plant disease incidence (Chakraborty et al., 2008). The humidity, wind, and temperature needed to facilitate pathogen growth is also necessary for the plant's growth. However, it is possible that

the overall structure of the pathogen community is affected by the slight change in environmental factors.

Community diversity and species richness are of particular interest to ecologists. One of the most commonly used metrics to assess the diversity of species communities is the Simpson's Diversity Index (D) (Simpson, 1949). It measures the probability that two randomly selected individuals from a sample community will be two different species. The calculation is defined in Equation 1, where *s* is the number of species, and *p* is the relative frequency of each species (Goudarzian & Erfanifard, 2017). The Simpson's Index shifts toward more abundant species because the probability of choosing two of the same species rises as the population of that species increases within the community.

Equation 1

$$D = 1 - \sum_{i=1}^{s} (p_i)^2$$

Simpson's Evenness is a measure of relative species abundance within a community. Equation 2 is used to calculate Simpson's evenness, where D is the Simpson's Diversity Index and n is the number of species. The Simpson's Evenness calculation should yield a number closer to 1 the more even the species population numbers are to each other.

Equation 2

$$E_{1/D} = \frac{1/D}{n}$$

The length of avocado development from fruit set to harvest has been shown to affect avocado quality, as fruit left to develop on the tree have more opportunity to

metabolise preformed anti-fungal compounds naturally present in younger fruit (Cutting et al., 1992). Unlike most produce, avocados can remain on the tree for months after reaching their full size, accumulating oil and dry mass the longer they are attached to the tree. Avocados harvested early in a country's growing season are the first to mature after fruit set, but those harvested late in the growing season have been left to hang on the tree longer. It is difficult to determine the exact age of the fruit without knowing the pollination time. However, an assessment of avocados delivered early and late in the country's export season would provide insight into the variation of fruit quality at distribution.

Unripe avocados harbour antioxidants and anti-fungal dienes, which are metabolised during ripening (Bertling & Tesfay, 2011). It is possible that avocados could be more sensitive to certain fungal morphotypes depending on when they were harvested. The biochemical composition of each fruit at harvest could also predispose it to infection from other pathogens along the supply chain. Analysis of fungal communities cultured from late harvested fruit and early harvested fruit tested the hypothesis that differences in fruit maturity will affect susceptibility to infection by specific fungal pathogens.

Fungal identification to strain level is also an important step in determining pathogen adaptation to different environments. In addition to ITS species identification (Chapter 4), the more highly variable B-Tubulin and glyceraldehyde-3-phosphate dehydrogenase (GPD) regions can be used to determine how closely members of the same species of fungus are related.

The specific objectives of this study were:

1. To explore the total fungal communities emerging from specific avocado growing regions

2. To compare the fungal communities of specific sample subsets, thereby investigating the effects of chosen variables on pathogen abundance

3. To determine if members of the same fungal morphotype differ by strain within different regions of the same country

4. To examine data for patterns which may reveal when and where avocados are infected by SER causal species

5.2 Methods and Statistical Analysis

5.2.1 Culture methods

Fungal pathogens were cultured from the stem end of SCD sample avocados according to the methods described in Section 2.1. For total fungal community analysis, all avocados sampled in the project were included in the dataset. This group is distinguished from the avocados chosen for physiological factor analysis (Chapter 6), because sample quality was inconsistent during the beginning of the project. During the first year of sampling, fruit arrived at the University of Reading too soft, and therefore developing more advanced rot symptoms. This stage of ripening would be too late to examine how the avocado's natural physiological properties and defences delay SER symptom development. However, these data are useful in assessing pathogen diversity, especially secondary and opportunistic pathogens that the fruit may have acquired at the distribution stage of the supply chain. The aim of this study was to consider every fungal morphotype cultured throughout the sampling assessment, as well as every incidence of the top seven most abundant morphotypes and evaluate the factors which favoured some morphotypes over others.

5.2.2 Statistical analysis

For statistical analysis, a plate count is defined as the total number of MEA plates which cultured the morphotype. A shipment is defined as one weekly batch of

avocados arriving at the University of Reading, as described in Section 2.2. Each shipment was given an alphabetical label, from shipment A to shipment CK. Regional information on shipment origin was often missing. If grower information was provided, the same grower was seldom replicated. There were only 12 shipments originating from the same farms. Shipments AF, AL, and AM were from the same farm in Israel while AG and AK were from a different farm in Israel, as well as CA and CG. Shipments BE, BF, and BU were from the same farm in Mexico, and shipments BH and BC were from another Mexican farm. For the regional analysis, shipments originating from the same grower were combined to form one region, and the remaining shipments were treated as separate regions.

All Principal Component Analysis (PCA), Canonical Correspondence Analysis (CCA), and Chi-Squared tests were performed in Statgraphics 18 (Statgraphics Technologies Inc.) Bar graphs and scatter plots of count data were created in Microsoft Excel (Version 2106).

The harvest period for each country was approximated using a supply calendar provided by Greencell. Mexico's season begins in September and ends in April of the next year. South Africa begins exporting in March and ends around October, and Israel's season begins in November and ends around April. 'Early Season' is defined as the first 2-3 months of an export season, and 'Late Season' was defined as the last 2-3 months of an export season. Only the seven most abundant morphotypes were considered for the seasonal fungal community analysis, cultured from sample fruit according to the method described in Section 2.1. All statistical analysis was carried out using the methods described in Section 2.2.

5.2.3 Whole Genome DNA Analysis

To explore potential differences in *Diaporthe* strains within one country, two genes of interest were identified for their association with pathogenicity factors. They

include Phosphatidylglycerol phospholipase and fungal MPG1. Externally secreted phospholipases are thought to facilitate pathogen invasion by inducing host cellular lysis or cell membrane attack (Ghannoum, 2000). The MPG1 gene encodes a protein necessary for appressorium formation, with knockout mutants demonstrating a reduced ability to infect a host (Talbot et al., 1993). Reference sequences were downloaded from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/). These regions were located within the assembled whole genome sequences of five biological replicates originating from three distinct locations within Mexico (Table 5.1). The reference sequences were used as the outgroup for each phylogeny.

Table 5.1 *Diaporthe* samples isolated for whole genome sequencing analysis from avocados originating from Mexico

Sample ID	Morphotype	Country	Farm	Harvest Month	Species ID (ITS)
BL38	WhiteStriated	Mexico	HVOSELECT	Feb-20	*Diaporthe helianthi*
BT32	WhiteStriated	Mexico	4063061361615	Nov-20	*Diaporthe helianthi*
BT40	WhiteStriated	Mexico	4063061361615	Nov-20	*Diaporthe ampelina*
BU18	WhiteStriated	Mexico	HUE08140230333	Nov-20	*Diaporthe helianthi*
BU46	WhiteStriated	Mexico	HUE08140230333	Nov-20	*Diaporthe helianthi*

5.3 Results

5.3.1 Identification of the Overall Most Abundant Fungal Morphotypes

The Supply Chain Dataset (SCD) was built from the gradual accumulation of avocado physiology and disease symptom information, one weekly batch at a time, over three years. Avocados were sampled from Mexico, Israel, South Africa, Chile, and Peru, before realising better statistical replication would be achieved if only three countries were tested. Although the decision to focus on only three countries of interest

(Mexico, Israel, and South Africa) was made early in the project, a pattern of abundant morphotypes did not emerge until much later. Many species are responsible for SER, and the initial strategy was to record and preserve any new phenotype. However, ongoing accumulation of fungal community information revealed patterns in morphotype frequency and disease association. Many unique morphotypes were only recorded once, suggesting that they may have been an opportunistic infection, a contamination, or a statistically insignificant contributor to overall SER incidence. The seven most abundant morphotypes from the SCD were cultured so frequently that they provide the most influential statistical study of SER causal factors. All seven are confirmed in the literature as being pathogens associated with SER (Darvas et al., 1987; Guarnaccia et al., 2016; Johnson et al., 1992; Madhupani et al., 2017).

All fungal morphotypes cultured for the SCD are summarised in Table 5.2. This tabulation excludes the blank plates, e.g., avocado cores which did not culture a phenotype regardless of disease symptoms. A total of 4022 avocados were analysed in the SCD, with core samples from all of these contributing to the fungal morphotype community shown in Table 5.2. Overall, 1436 (36%) avocados did not culture any fungus, regardless of symptoms recorded for the mesocarp, whilst 1893 (47%) were members of the top seven most abundant morphotypes. The remaining 693 (17%) consisted of the remaining infrequent fungal morphotypes.

The top seven most abundant morphotypes were chosen as the focus of statistical study, because the difference in plate count from the WhiteDensefur to WhiteScalloped was the greatest at the time the decision was made to focus on abundant morphotypes instead of everything that could be cultured. Only when sampling finished did the difference in plate count between WhiteLayered and WhiteGreyCotton become more pronounced. Nevertheless, the seven most abundant

phenotypes were all confirmed to be known avocado pathogens associated with SER. As established in Chapter 4, WhiteCotton represents the genus *Lasiodiplodia*, WhiteDensefur was found to be a mixture of *Colletotrichum, Fusarium,* and *Pestalotiopsis*, WhiteGreyCotton is *Neofusicoccum*, WhiteLayered is *Neopestalotiopsis*, and WhiteStriated was identified as *Diaporthe*.

Table 5.2: All fungal morphotypes cultured from all avocados used in the Supply Chain analysis. The top seven most abundant morphotypes were the focus of further study. N = 2586

Morphotype	**Plate Count**		**Morphotype**	**Plate Count**
BlackCotton	1		GreenDensefur	7
BlackWhiteSlate	1		GreenWhiteDensefur	7
BlackWhiteStriated	1		WhiteGreyDensefur	7
BlueWhiteDensefur	1		BlackDensefur	18
BrownDensefur	1		YellowScalloped	22
BrownScalloped	1		BlackWhiteCotton	25
GreyStriated	1		GreyCotton	28
GreyWhiteLayered	1		GreyWhiteCotton	29
PinkCotton	1		WhiteSlate	55
WhiteBlackDensefur	1		GreyDensefur	57
WhitePinkCotton	1		GreyWhiteDensefur	116
OrangeLayered	2		BlackWhiteDensefur	127
OrangeWhiteDensefur	2		WhiteScalloped	153
PinkStriated	2		WhiteDensefur	185
WhiteGreyLayered	2		WhiteSlime	190
GreyWhiteStriated	3		WhiteCotton *(Lasiodiplodia)*	200
PinkSlime	3		WhiteLayered (*Neopestalotiopsis*)	204
YellowWhiteDensefur	3		WhiteGreyCotton (*Neofusicoccum*)	337
BlackGreyDensefur	4		YellowSlime	355
WhiteBlackThread	4		WhiteStriated (*Diaporthe*)	422
PinkDensefur	5			

Total fungal communities by country are examined in Figure 5.1 and most abundant communities by country are summarised in Figure 5.2. All plates from Mexico, Israel, and South Africa are compared to blank plates for each country. These blank plates only represent the avocado cores that failed to culture a fungus, regardless of fruit mesocarp symptoms. The relationship between symptomatic fruit which failed to culture a fungus and asymptomatic fruit which cultured a fungus is explored in Section 6.3.3. Both Mexico and South Africa are tropical and subtropical, respectfully, but Israel is Mediterranean. Figure 5.1 demonstrates that the total fungal communities differ between the countries, with the most apparent differences in the populations of YellowSlime, *Diaporthe*, *Neopestalotiopsis*, and GreyWhiteDensefur. Considering only *Neopestalotiopsis* and *Diaporthe*, the difference in abundance for Israel compared to Mexico and South Africa was significant: X^2 (2, N = 591) = 9.455, p = .0089. This is supported by the fact that *Neopestalotiopsis* (*Neopestalotiopsis*) and *Diaporthe* (*Diaporthe/Phomopsis*) prefer a cooler sporulation temperature compared to many of the other abundant morphotypes (Table 4.2).

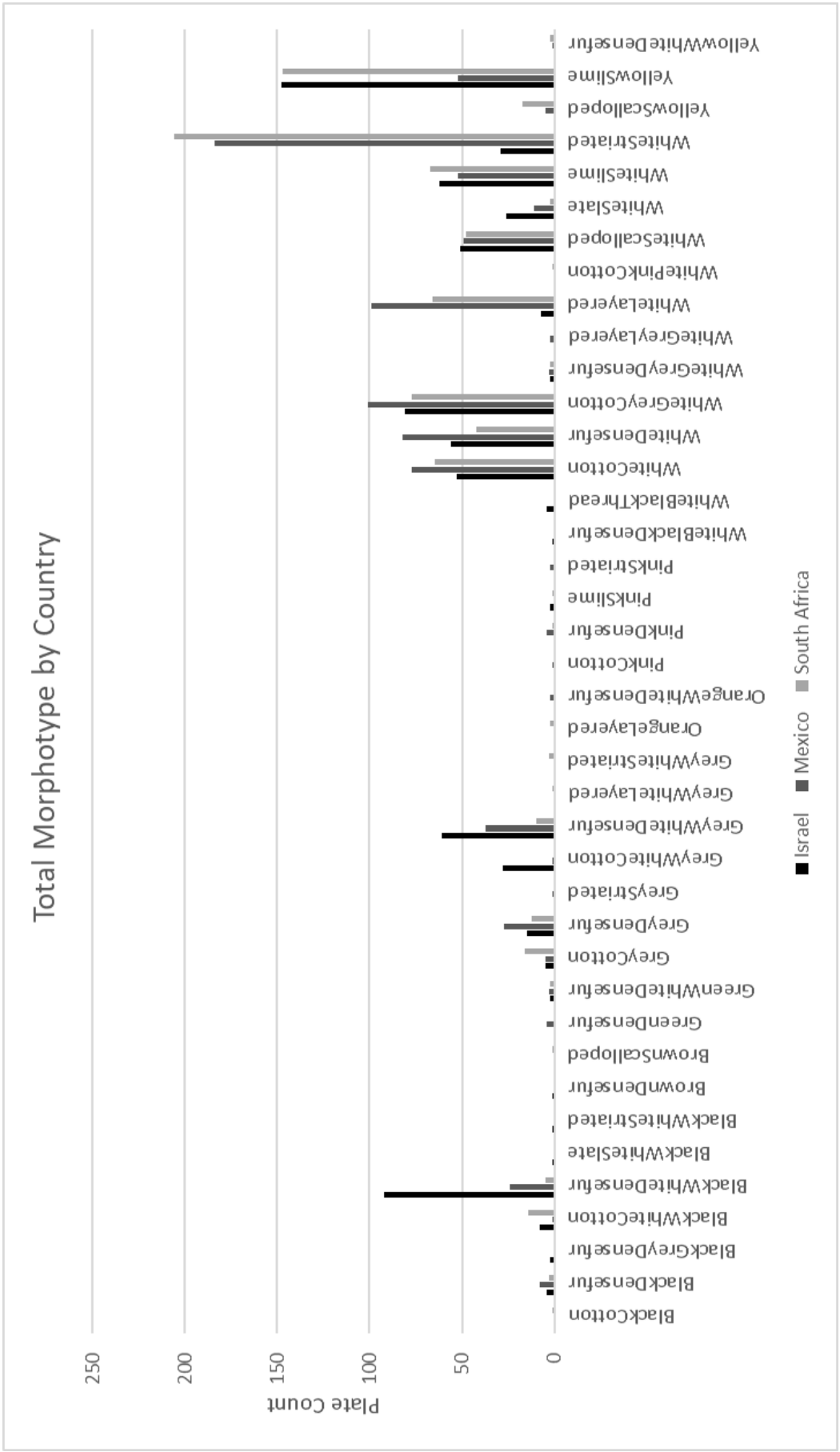

Figure 5.1: Total fungal morphotypes isolated by country. These data exclude Chile, Peru, and 1285 core samples which did not culture a fungus. Israel N = 742; Mexico N = 838; and South Africa = 814

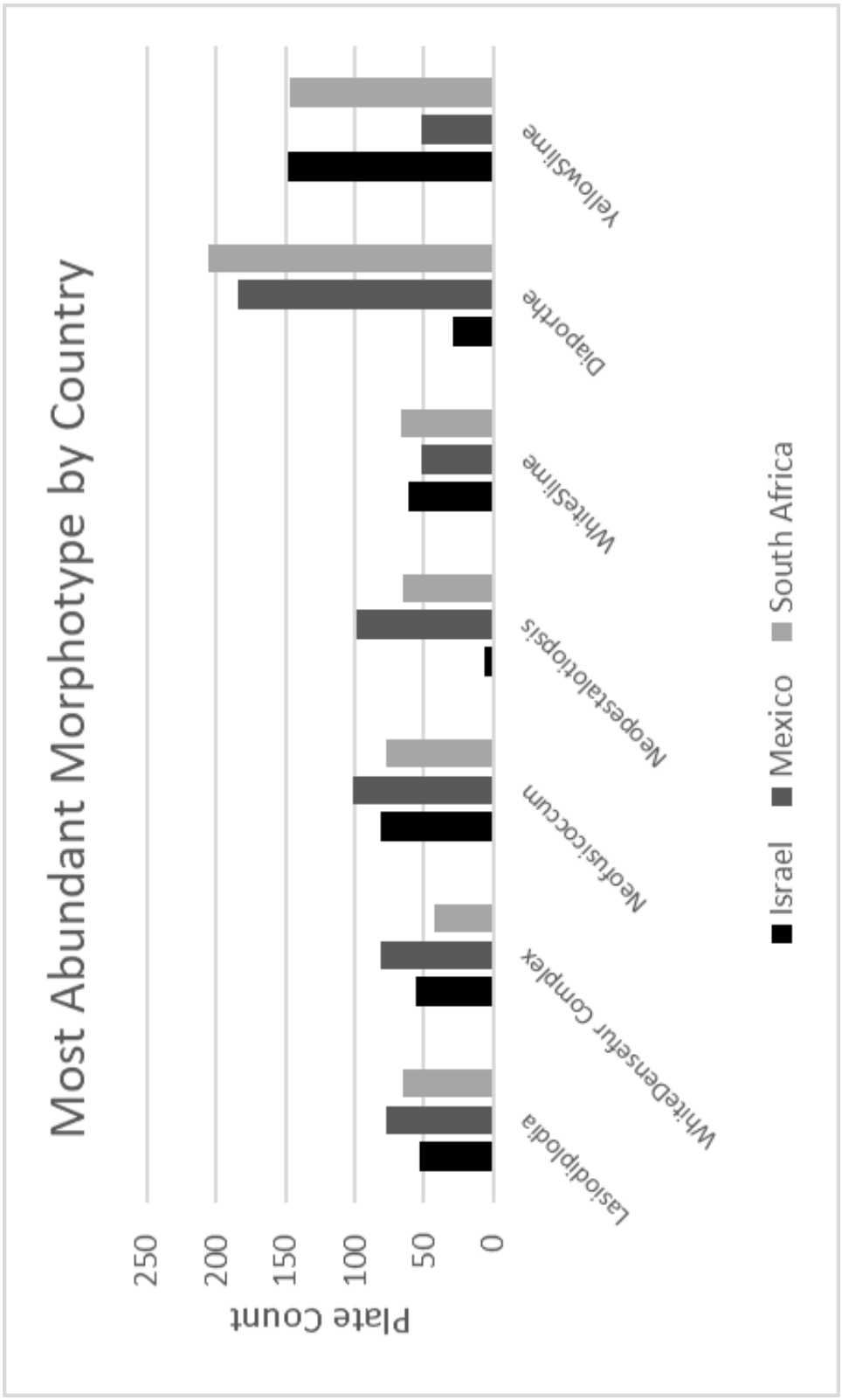

Figure 5.2: Most abundant fungal morphotypes by country. These data exclude Chile, Peru, and cultures with a plate count less than 185, as well as 1285 core samples which did not culture a fungus. Israel N = 436, Mexico N = 647, South Africa N = 670

5.3.2 Fungal Community Analysis by Country

The diversity of fungal communities by country was assessed using the Simpson's Diversity Index and Simpson's Evenness. Results are summarised in Figure 5.3. Whilst all countries were found to have similar levels of diversity and low levels of evenness, Israel's evenness score was slightly higher than South Africa. This indicates that the South African fungal community skewed toward the few most abundant species cultured. The CCA in Figure 5.4 further illustrates how South Africa is correlated with *Diaporthe*, the most abundant phenotype. The Chi-Squared analysis in Table 5.3 (X^2 (7, N = 3038) = 270.86, p = 0.00) indicates that morphotype frequency was significantly dependent on country, but the largest contributor to the overall Chi-Square score was the distance of Israel from *Diaporthe*.

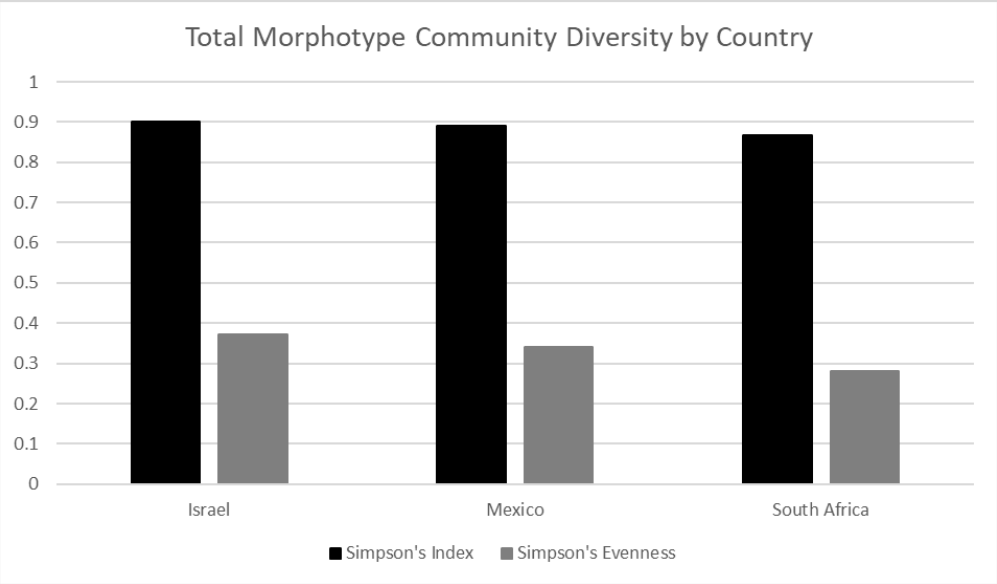

Figure 5.3: Simpson's Diversity Index and Simpson's Evenness scores for the fungal communities of Israel, Mexico, and South Africa. Israel: D = 0.9006, E = 0.3726; Mexico: D = 0.891, E = 0.3398; South Africa: D = 0.868, E = 0.2806. N = 2394

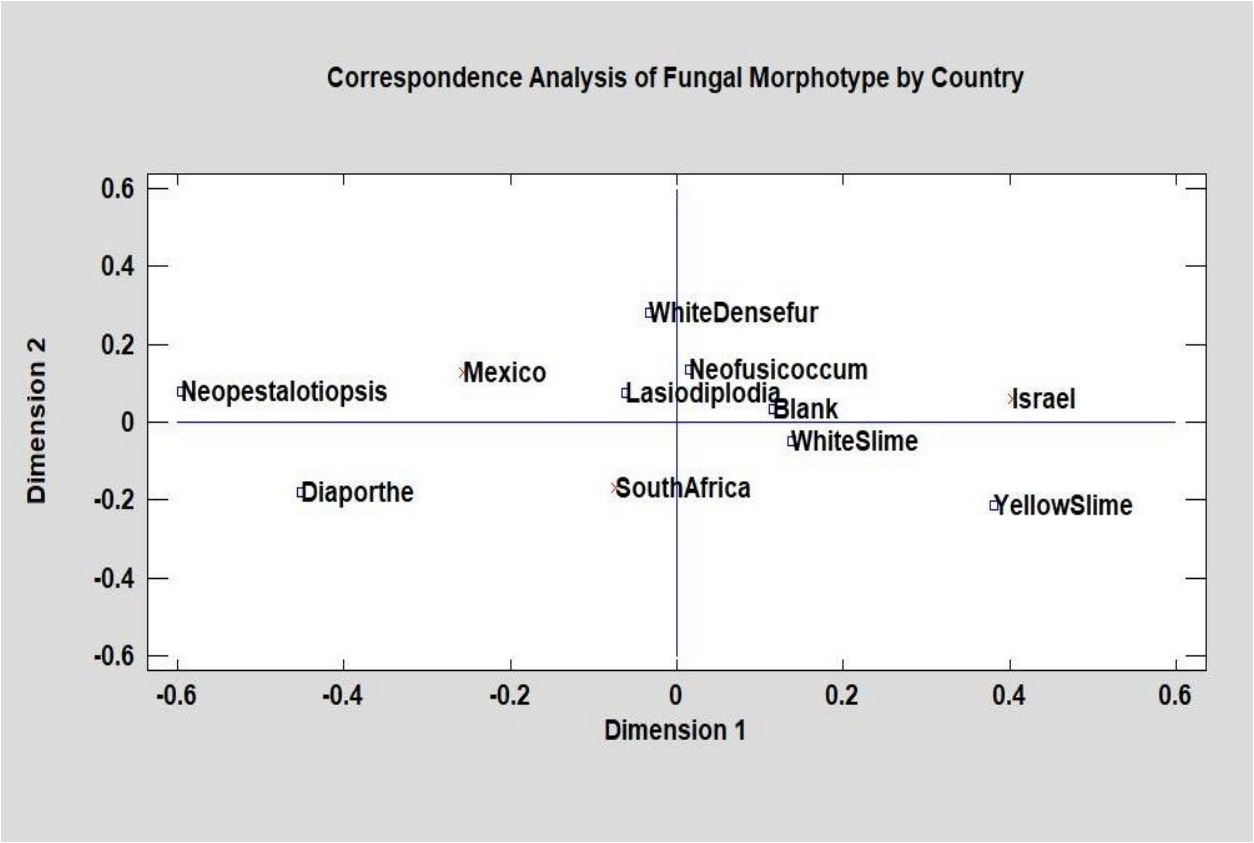

Figure 5.4: Correlation of the most abundant fungal phenotypes with the three countries of interest. N = 3038

Table 5.3: Chi-Square Distances for Fungal Morphotype and Country of Origin Individual Chi-Squared distances contributing to the total Chi-Squared statistic, which determines if the distribution of each population was significantly different from the expected random dispersal. The larger the number, the more it deviated from the expected distribution. N=3038

	Israel	Mexico	South Africa	TOTAL
Blank	13.4	2.7	2.8	18.9
Lasiodiplodia	0.2	1.2	0.4	1.8
Colletotrichum/*Fusarium*/*Pestalotiopsis*	0.3	5.8	8.2	14.3
Neofusicoccum	0.5	1.2	3.0	4.7
Neopestalotiopsis	36.7	25.2	0.2	62.2
WhiteSlime	1.8	2.0	0.0	3.8
Diaporthe	70.0	9.7	19.5	99.3
YellowSime	22.7	39.5	3.7	65.9
TOTAL	145.7	87.3	37.9	270.9

5.3.3 Fungal Community by Region

Few of the growers used in the Supply Chain Database were replicated, but those that were could be combined into one region. A total of 26 regions were identified for Israel, 30 for Mexico, and 35 for South Africa. These grower codes correspond to suppliers and not an exact location of an orchard. The mosaic graphs in Figure 5.5 show that the presence of abundant morphotypes can vary widely by grower, and the tendency of the tropical and subtropical countries to favour pathogens requiring wetter sporulation conditions emerged as an overall trend. At the region level, five areas of Israel had a high incidence of *Diaporthe*, but its occurrence was more widespread around Mexico and South Africa, with more growers making a smaller contribution to the overall abundance at the country level. The same can be said about *Neopestalotiopsis* in Israel compared to that in Mexico and South Africa. Other morphotypes, such as *Lasiodiplodia* and the *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex have a similar distribution across different regions within a country. Overall, the mosaic graphs show an increase in distribution of the abundant fungal morphotypes among growers in Mexico and South Africa compared to those of Israel.

The total number of each morphotype cultured by grower is illustrated in Figure 5.6. *Neopestalotiopsis*, *Diaporthe*, and YellowSlime were particularly abundant for just a few growers. The analysis of morphotype and genus distribution by grower illustrates how the fungal community varies between regions within a country. *Diaporthe* was particularly abundant for most of the Israeli farmers, but a small minority had no *Diaporthe* at all. Location information was not available for most of these growers, but the dissimilarity of fungal genera between growers suggests a high variability in growing conditions within the same country. These are most likely related to geography, weather, or orchard management techniques which differ between growers.

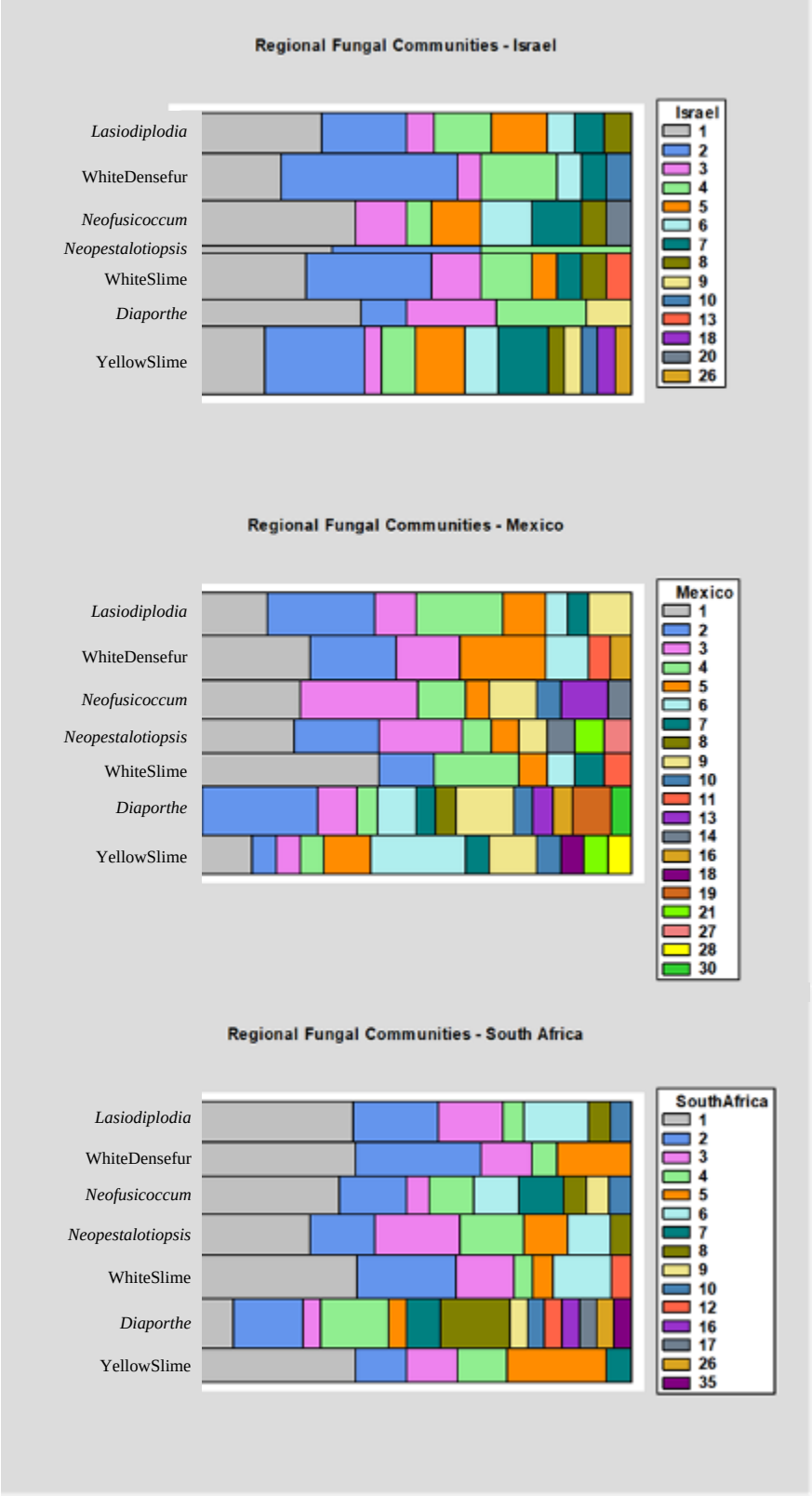

Figure 5.5: Mosaic diagrams of the most abundant morphotypes by region in Israel, Mexico, and South Africa. Each colour box in each country legend corresponds to a different region within that country. The size of the boxes indicates relative abundance. N=3038

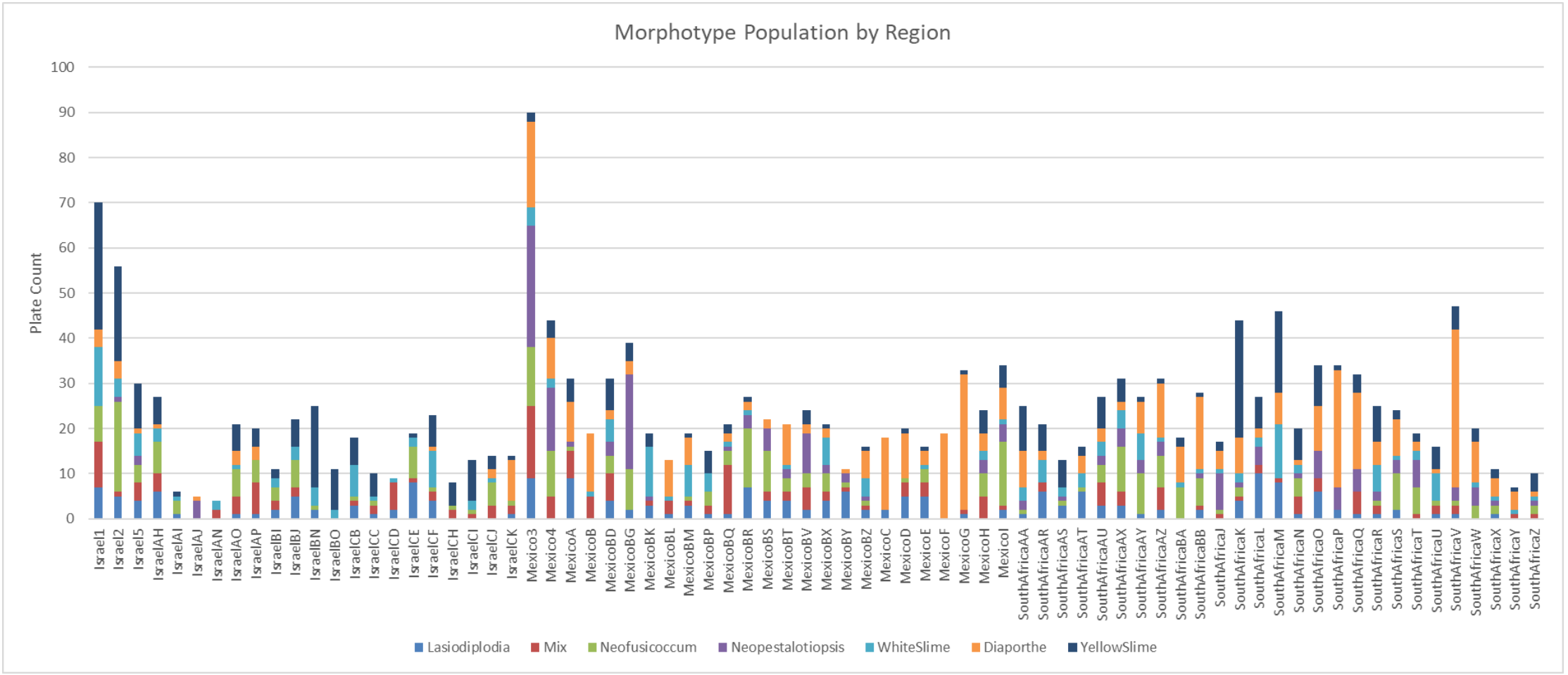

Figure 5.6: Ratio of morphotypes cultured from each grower per country. Each stacked column represents a different grower. Colours correspond to each of the seven most abundant morphotypes. N = 3038

5.3.4 Whole Genome Analysis

With reference to the ITS sequencing, Table 5.1 identified two distinct species of *Diaporthe* from three farms. *D. ampelina* was already genetically separate, but the remaining four samples of *D. helianthi* may or may not be identical in strain. Both the MPG1 alignment and maximum likelihood tree (Figures 5.7 and 5.8) and the Phosphatidylglycerol phospholipase alignment and maximum likelihood tree (Figures 5.9 and 5.10) show a similar clade organisation. BL38, BT32, and BU46 were consistently closely related to each other and more distantly related to the remaining two samples, whilst BU18 is more closely related to BT40. This is expected, because BT40 is a different species to the other four samples and is therefore most distantly related, but BL38, BT32, and BU46 all yielded closely related sequences from three separate orchards. This would suggest that these sequences are conserved in different areas within the same country. However, BU18 is the most distantly related to two samples from the same grower, suggesting that a diversity of *Diaporthe* strains may exist in a single orchard.

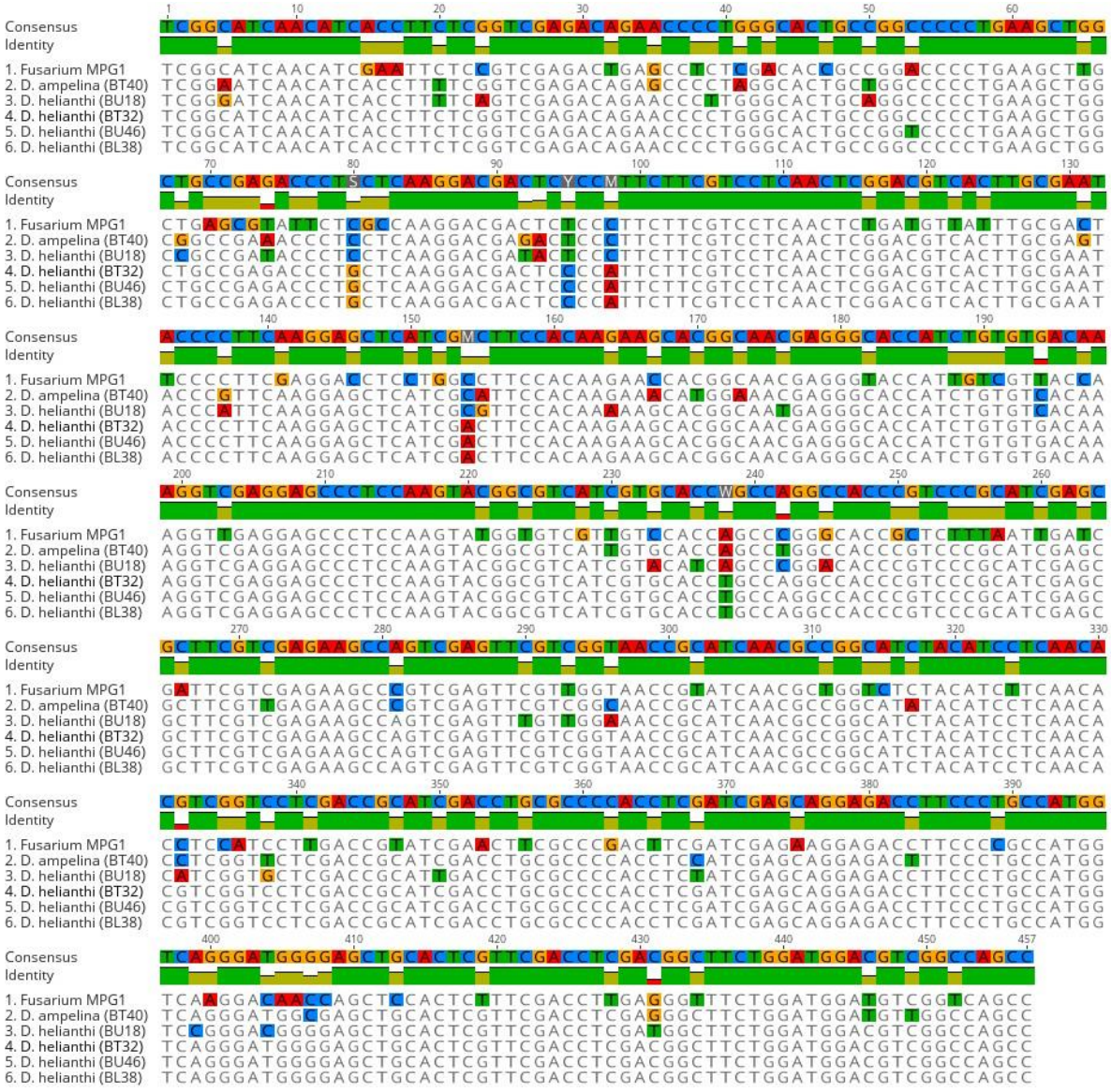

Figure 5.7 MPG1 sequence alignment of five *Diaporthe* samples identified from the whole genome sequences

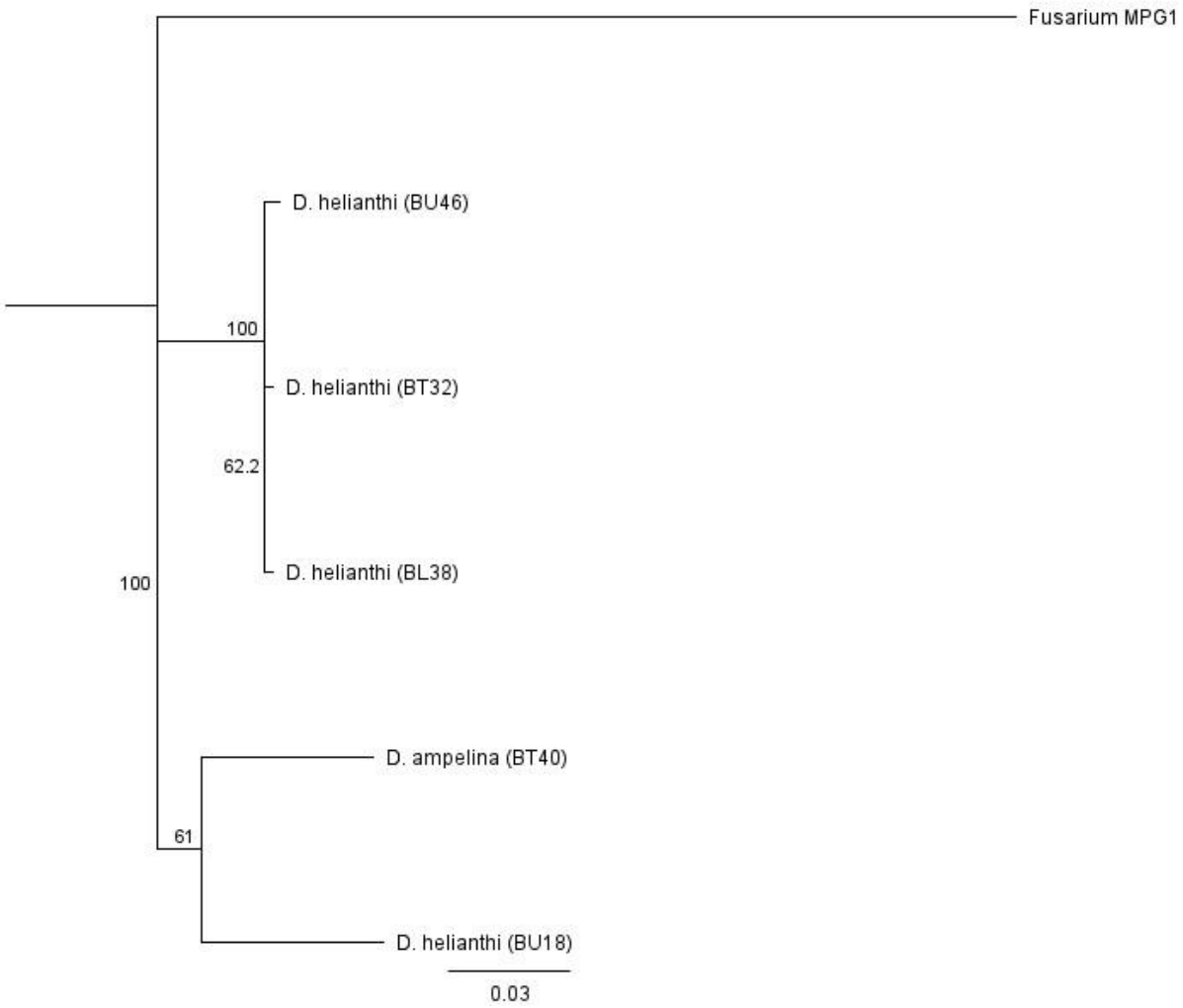

Figure 5.8 Single locus (MPG1) Maximum Likelihood consensus phylogeny of *Diaporthe* samples rooted to *Fusarium*, with bootstrap support of 100 replicates shown above nodes identifying four clades. The MPG1 gene is thought to play a role in fungal infection structures. An MPG1 sequence identified in *Fusarium* was used to locate the region in five assembled whole genome datasets of two *Diaporthe* species isolated from avocados originating from three regions of Mexico.

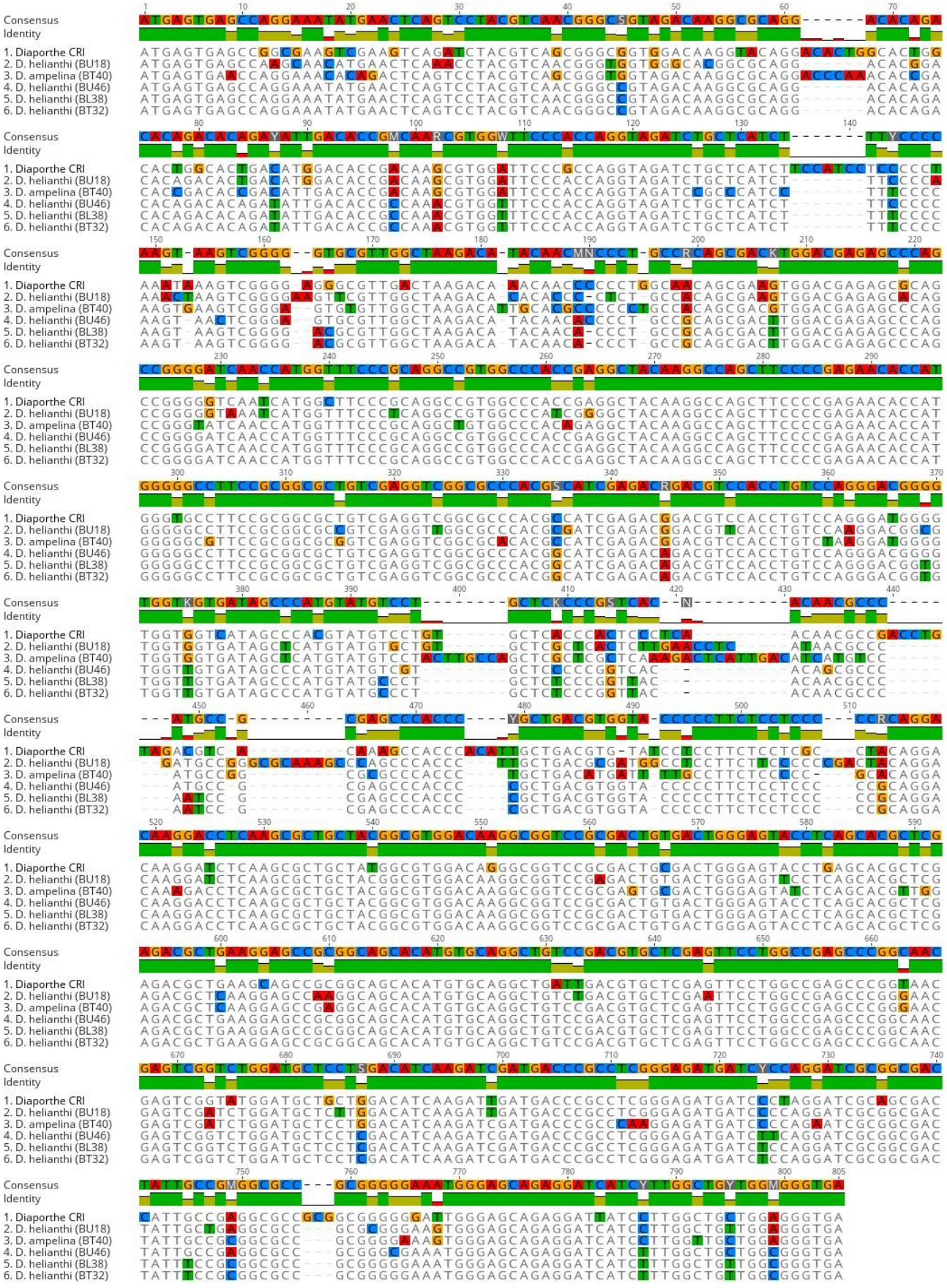

Figure 5.9 Phosphatidylglycerol phospholipase sequence alignment of five Diaporthe samples identified from the whole genome sequences

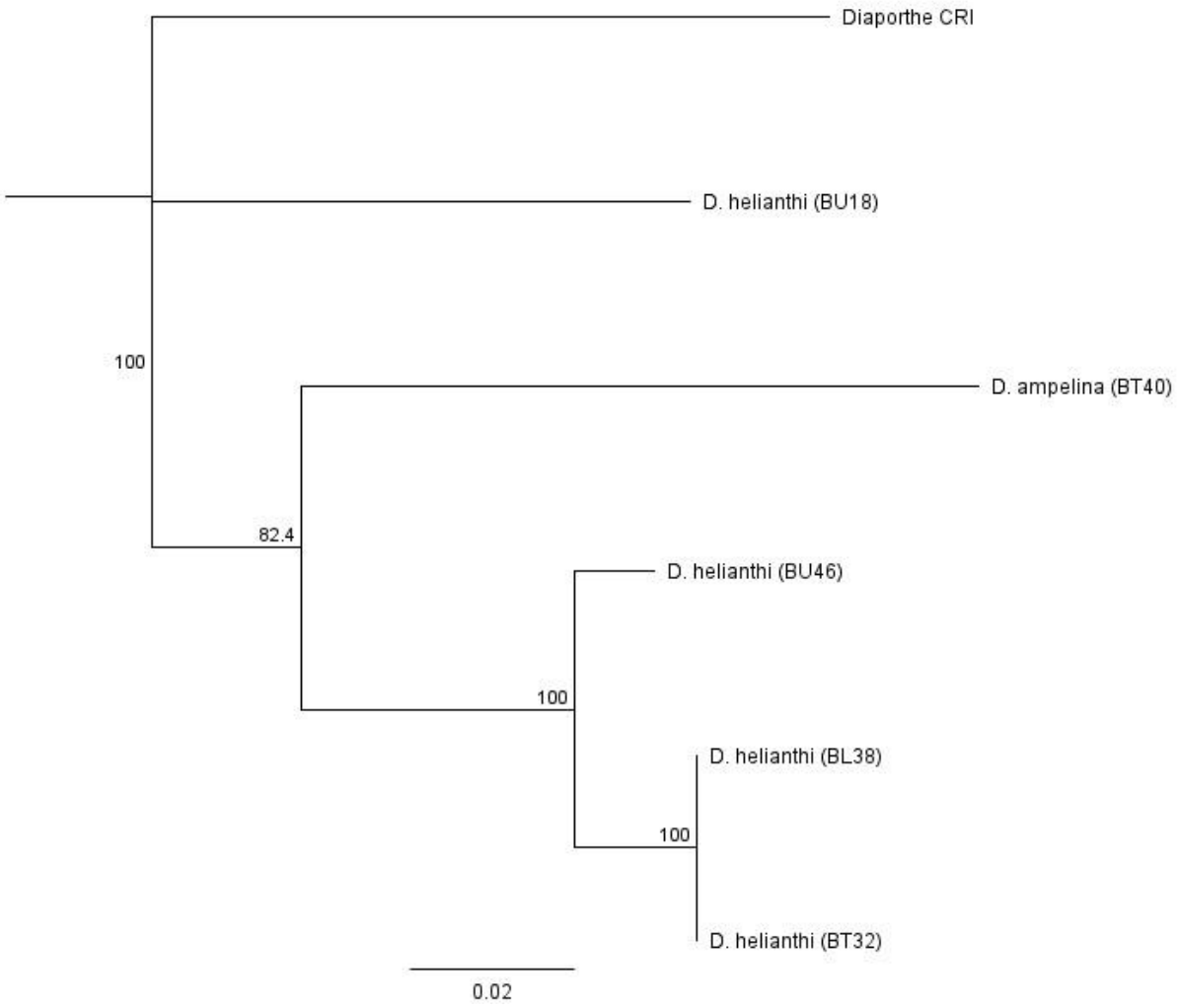

Figure 5.10 Single locus (Phosphatidylglycerol phospholipase) Maximum Likelihood consensus phylogeny of *Diaporthe* samples rooted to *Diaporthe* strain CRI, with bootstrap support of 100 replicates shown above nodes identifying four clades. Phospholipases are thought to play a role in fungal host invasion. The phosphatidylglycerol phospholipase sequence identified in the *Diaporthe* CRI strain was used to locate the region in five assembled whole genome datasets of two *Diaporthe* species isolated from avocados originating from three regions of Mexico.

5.3.5 Fungal Community by Harvest Date

The seven most abundant fungal morphotypes defined in Section 4.2.3 were found in every country, in both early and late harvest periods, at different levels of abundance (Figure 5.11). The most dramatic change is the quantity of *Diaporthe* in Mexico from the early harvest compared to the late harvest. There does not seem to be a clear pattern as to which morphotype is more or less abundant early in the harvest or later, but the overall difference in communities was statistically significant X^2 (30, N = 1752) = 503.006, p = 0.00. The PCA comparing abundant morphotype with harvest period (Figure 5.12) indicates a strong correlation of *Diaporthe* with Mexico's late harvest. This supports the fact that the increase in *Diaporthe* in Figure 5.11 from Mexico's early harvest shows the most dramatic shift. All morphotypes which cultured high quantities in Figure 5.11 such as YellowSlime during late Israel and *Neopestalotiopsis* early in Mexico's season, correlate closely in Figure 5.12 and show high Chi Squared distances in Table 5.4, indicating that the differences in fungal community composition between countries is significant. Interestingly, it is *Neopestalotiopsis* in Mexico's early season which contributes the highest Chi-Squared distance, instead of the *Diaporthe* population cultured later from the same country, meaning that early season Mexican fruit cultured *Neopestalotiopsis* more often than expected. This could be because a larger dataset from early season Mexico is skewing the statistical results.

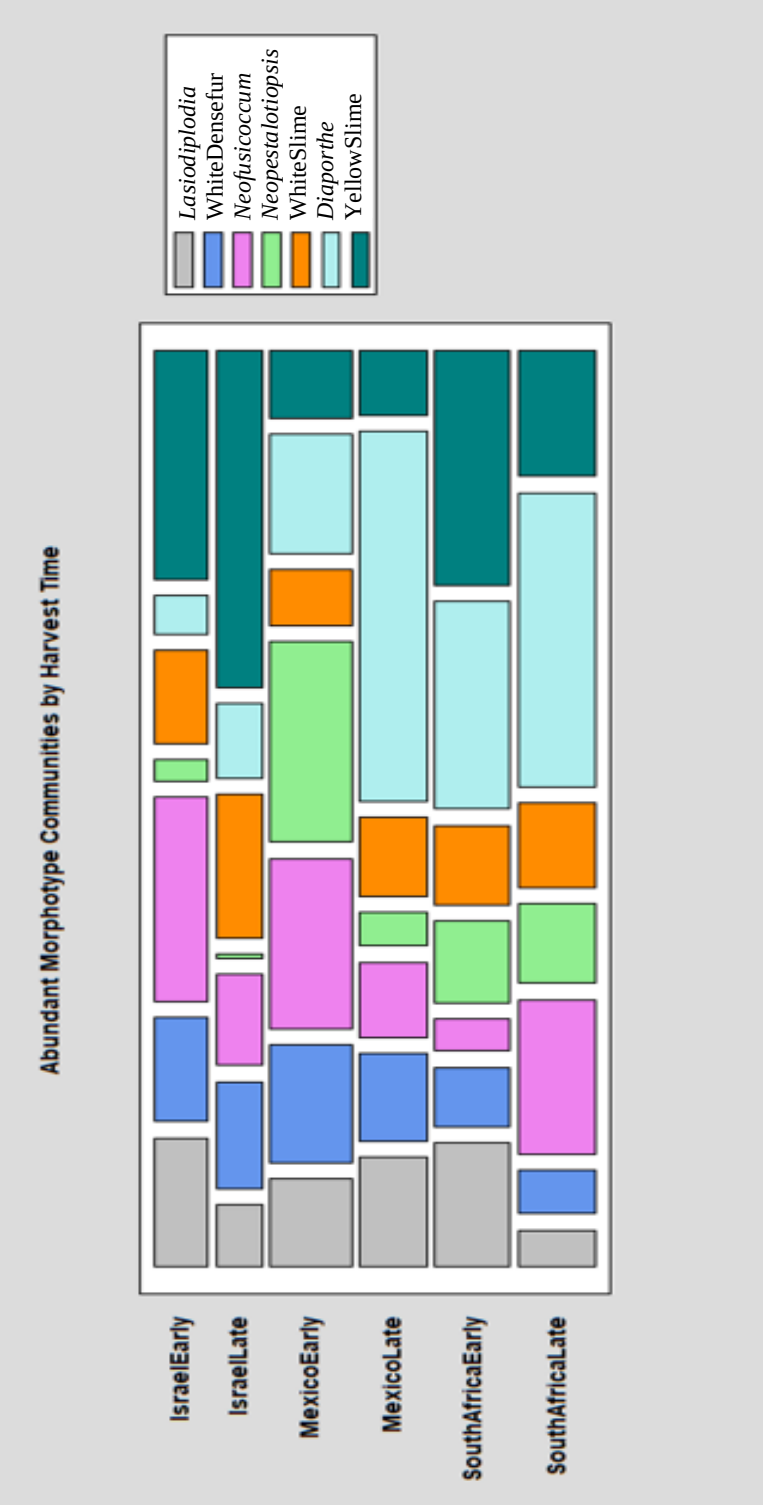

Figure 5.11: Community composition of the top seven most abundant morphotypes per harvest time for each country of interest. 'Early Season' is defined as the first 2-3 months of an export season, and 'Late Season' was defined as the last 2-3 months of an export season. The size of the square represents relative abundance. IsraelEarly N = 619, IsraelLate N = 565, MexicoEarly N = 652, MexicoLate N = 600, SouthAfricaEarly N = 531, SouthAfricaLate N = 712

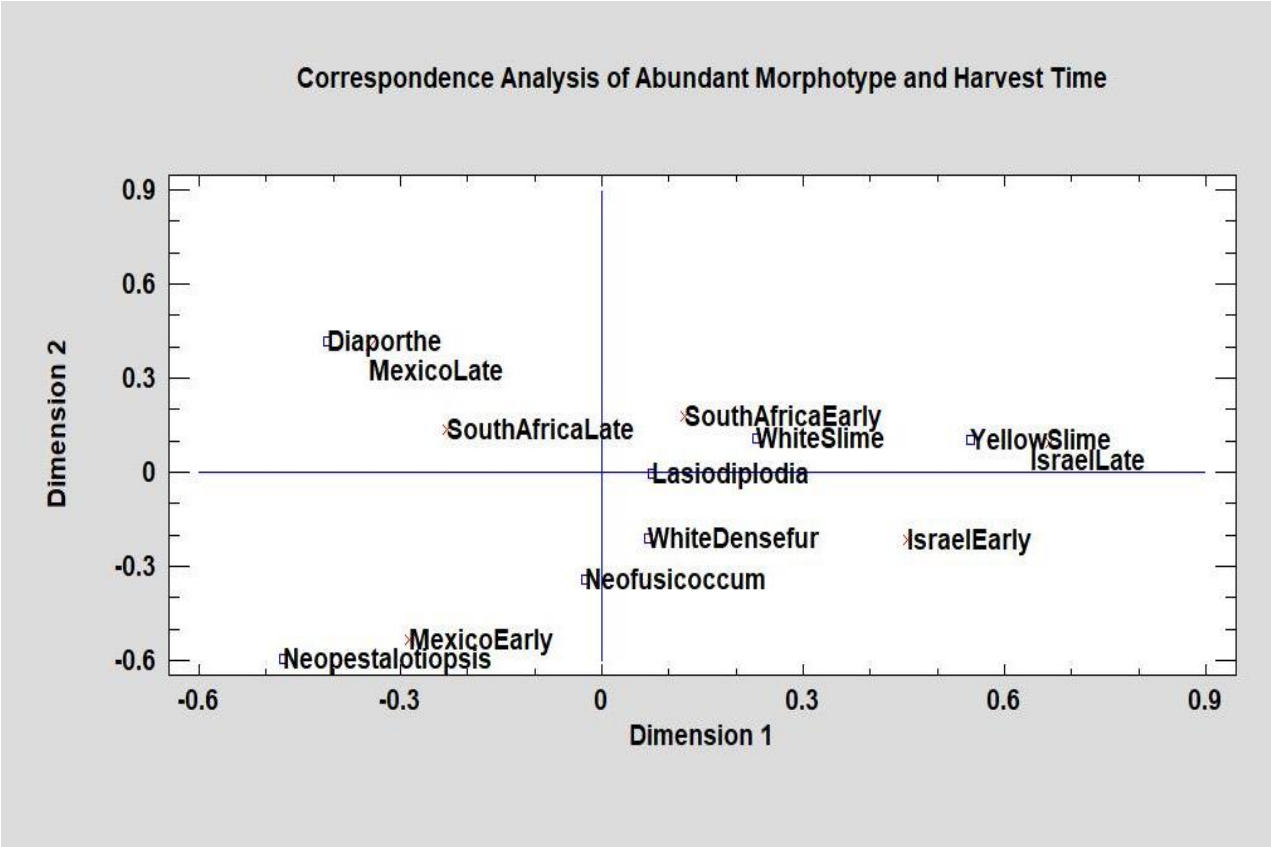

Figure 5.12: Principal Component Analysis of (PCA) of abundant morphotypes compared with harvest time for Israel, South Africa, and Mexico. Dimension 1 separates country and growing season and Dimension 2 separates morphotype. 'Early Season' is defined as the first 2-3 months of an export season, and 'Late Season' was defined as the last 2-3 months of an export season. X = Country; □ = Morphotype; N = 1752

Table 5.4: Chi-Square Distances for Fungal Morphotype and Harvest Time. Individual Chi-Squared distances contributing total Chi-Squared statistic, which determines if the distribution of each population was significantly different from the expe random dispersal. The larger the number, the more it deviated from the expected distribution. N = 1752

	IsraelEarly	IsraelLate	MexicoEarly	MexicoLate	SouthAfricaEarly	SouthAfricaLate	TOTAL
Lasiodiplodia	4.5	2.2	0.0	1.4	4.9	13.5	26.6
Pestalotiopsis/*Colletotrichum*/*Fusarium*	1.4	1.5	5.8	0.0	2.9	8.1	19.8
Neofusicoccum	16.7	1.9	8.8	6.1	26.4	3.8	63.7
Neopestalotiopsis	12.7	17.6	78.0	9.7	0.0	0.0	118.0
WhiteSlime	0.3	10.1	4.4	0.2	0.1	0.0	15.1
Diaporthe	36.6	18.4	12.7	55.3	0.3	20.7	144.0
YellowSlime	7.9	46.0	24.3	21.0	13.2	3.4	115.8
TOTAL	80.1	97.8	134.1	93.6	47.9	49.6	503.0

5.3.6 Secondary and Tertiary Culture Analysis

The stem core sample extracted from each avocado (2.1.2) occasionally cultured more than one fungal morphotype. For example, three morphotypes are growing on the stem core shown in Figure 5.13. The morphotype occupying the largest area on the plate was designated the primary culture, while others were specified as secondary or tertiary cultures by size. This distinction was made for the purpose of organising the cultured morphotypes into a dataset and it is not evidence as to which of these morphotypes, if any, is the causal species. The fact that some fungal species will grow faster on the plate does not mean they grow faster inside living plant tissue, or that they were the first to colonise the tissue. A facultative autotroph could grow on artificial media and plant material, whereas an obligate biotroph could only survive within the avocado. All morphotypes cultured in this project are facultative autotrophs by definition, and it is possible an obligate biotroph unable to survive on MEA media is a SER causal species.

The majority of avocado core samples produced only one culture, if any at all, whilst 15% of all avocados sampled cultured a secondary morphotype and 1.2% cultured a tertiary morphotype. Additional cultures on the MEA plate are most likely secondary infections or contamination. It is also possible that the secondary or tertiary culture was the primary causal agent, but it did not grow as fast on the plate compared to the additional species cultured.

The hypothesis that the secondary culture could be a causal species is supported by the fact that the most abundant secondary morphotypes are also members of the top seven most abundant primary cultures, aside from BlackWhiteDensefur (Figure 5.14). The top seven most abundant morphotypes are also most often cultured together, as illustrated in Figure 5.15. YellowSlime and *Diaporthe* were cultured together most often, further supporting the hypothesis that YellowSlime is a variation of *Diaporthe*.

The granular structures stained in Figure 4.26 could be reproductive components, excreted as a part of a propagative exudate, but more investigation is necessary to verify their identity. Tertiary morphotypes were cultured much less frequently. Table 5.5 summarises all tertiary morphotypes cultured from the three countries of interest, with many of the top abundant morphotypes appearing frequently in these populations as well. The ability of these morphotypes to cause SER symptoms cannot be confirmed with simple association; this is demonstrated using Koch analysis (Chapter 7). However, even within the secondary and tertiary communities, the seven most abundant morphotypes are still dominant.

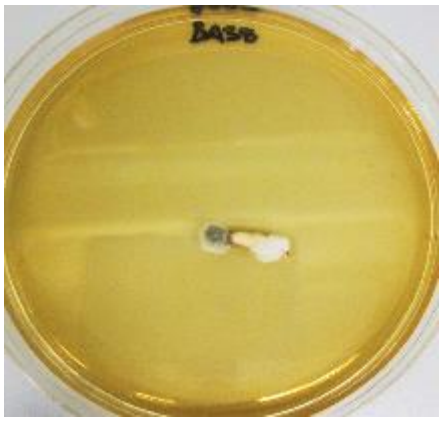

Figure 5.13: Three distinct fungal morphotypes colonising a single avocado core sample. A larger tissue sample was taken from the avocados to assess all potential fungal communities present in SER tissue

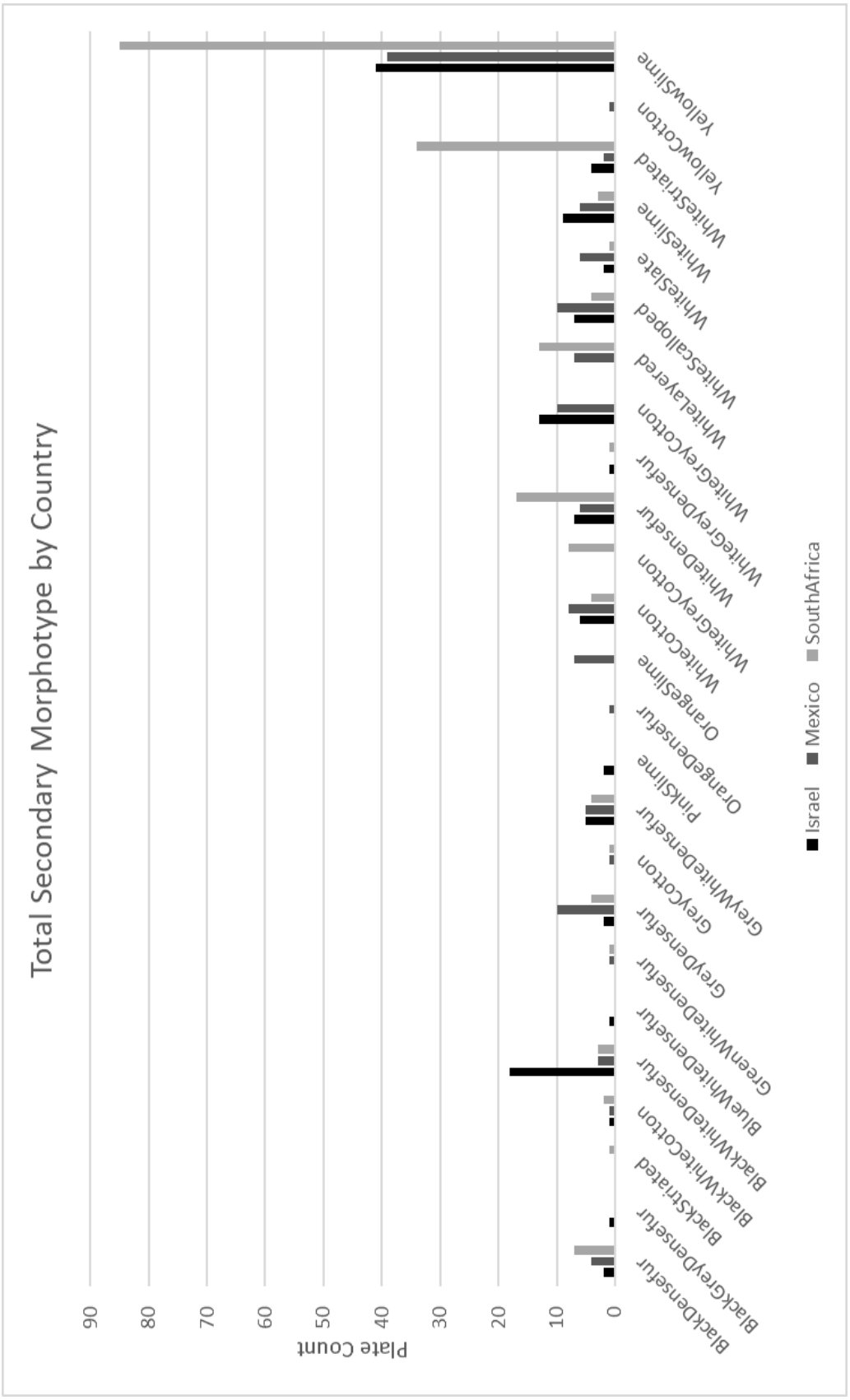

Figure 5.14: Distribution of all secondary morphotype cultures by country. N = 443

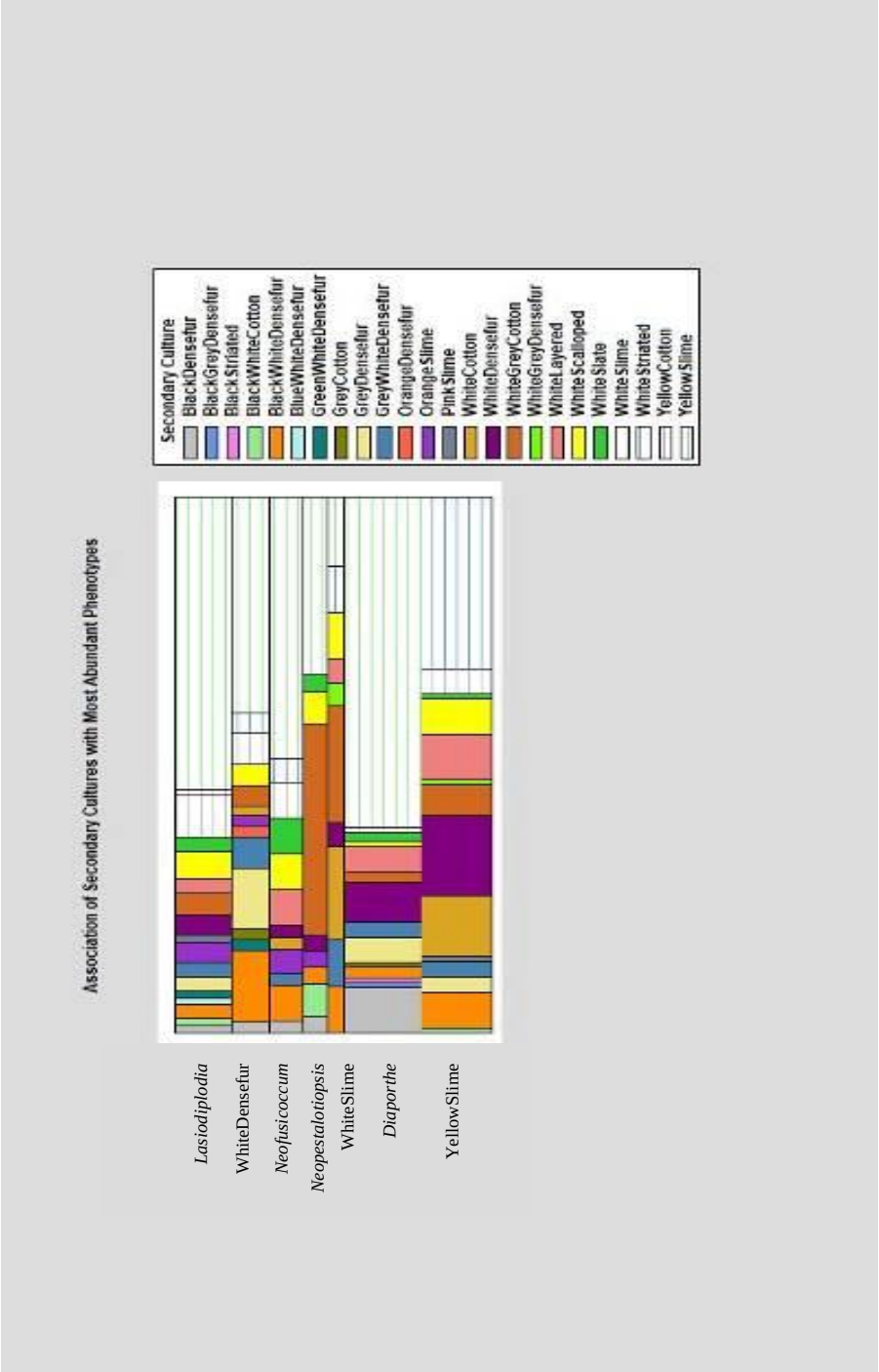

Figure 5.15: Secondary culture communities associated with each of the most abundant fungal morphotypes as primary cultures. The size of the boxes indicates relative abundance N = 443

Table 5.5: Total counts of tertiary fungal morphotypes cultured from the three countries of interest. N = 36

Israel	**Plate Count**	**Mexico**	**Plate Count**	**South Africa**	**Plate Count**
BlackDensefur	2	BlackDensefur	2	BlackDensefur	3
BlackWhiteDensefur	1	WhiteScalloped	1	BlackWhiteDensefur	2
GreenWhiteDensefur	1	YellowSlime	4	WhiteDensefur	2
GreyDensefur	1			*Neofusicoccum*	2
GreyWhiteDensefur	3			*Diaporthe*	1
Lasiodiplodia	1			YellowSlime	5
Pestalotiopsis/*Colletotrichum*/ *Fusarium*	1				
Neofusicoccum	2				
WhiteScalloped	1				
WhiteSlate	1				

5.3.7 Fungal Community Analysis Within Shipments

Each shipment of avocados was considered individually to assess how fungal communities affected SER incidence. A shipment is defined as the total weekly avocado sample arriving at the University of Reading. These data include all intake and stored avocados for each sample batch and scrutinises the difference between the harvests of all growers in all countries assessed during the study. To determine if certain fungal communities are responsible for higher rot incidence regardless of origin or harvest time, the shipments with the greatest rot incidence and least rot incidence were identified. Figure 5.16 summarises rot incidence for every shipment throughout the SCD collection period. Rot incidence fluctuates without a clear pattern.

Fungal communities within the top ten shipments with the most SER incidence and top ten shipments with the least SER incidence are illustrated in Figure 5.17. Many of the avocados from low rot shipments did not culture a fungal morphotype at all (blank). If they did, it was often YellowSlime (a possible *Diaporthe* morphotype), WhiteScalloped, or YellowScalloped. Since both WhiteScalloped and YellowScalloped were not identified as one of the top seven causal species (Section 4.2.3) they are likely to be secondary infections, or a pathogen that does not significantly contribute to overall SER incidence. However, WhiteSlime and YellowSlime are one of the top seven most abundant morphotypes. Many of the other morphotypes cultured from the shipments with high rot incidence in Figure 5.16, such as *Lasiodiplodia*, WhiteDensfur, *Neofusicoccum*, and *Diaporthe* have been shown via Koch analysis to produce rot symptoms in healthy avocado mesocarp (Chapter 7) and correlate with SER browning (Section 6.4). However, YellowSlime and WhiteSlime Koch inoculations did not produce strong SER symptoms. As the slime morphotypes are highly abundant overall, and equally abundant in shipments with high and low rot, suggests that these morphotypes are present in the orchard but not responsible for producing rot symptoms.

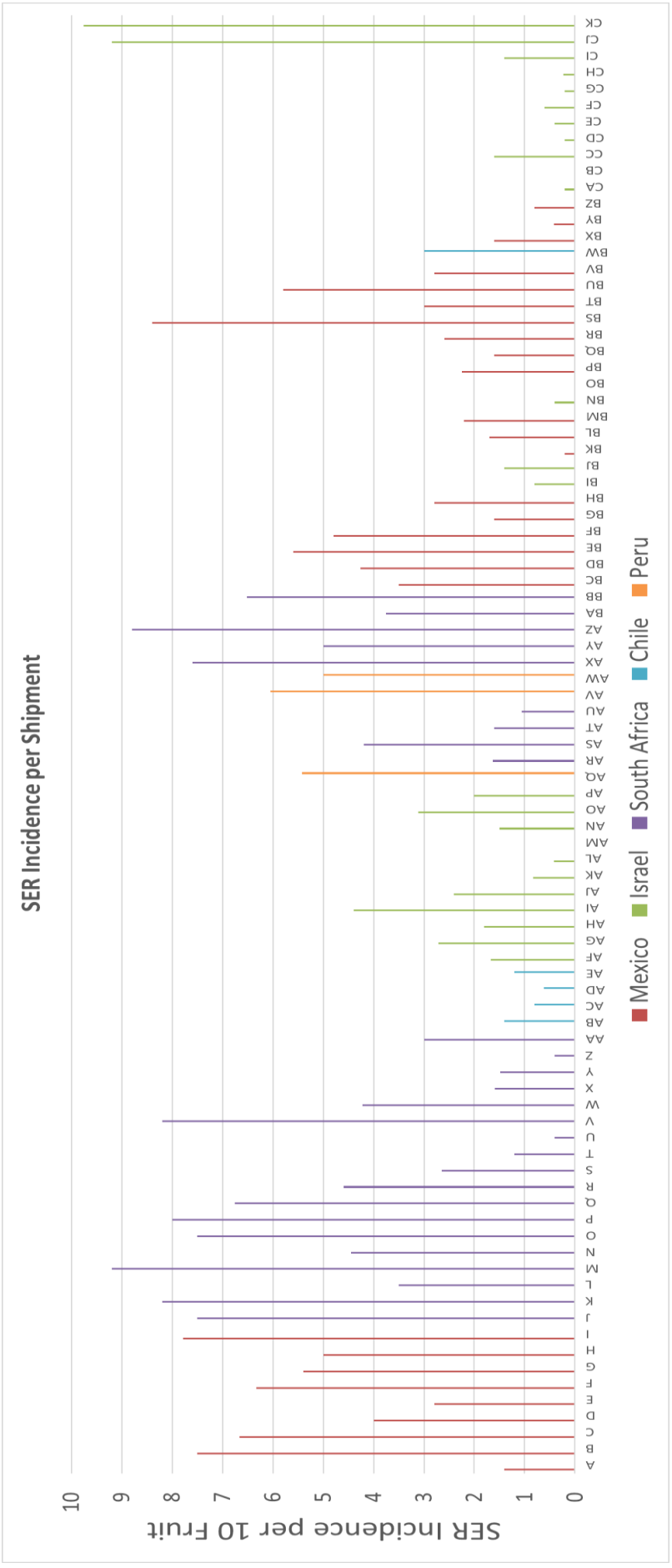

Figure 5.16: SER incidence per 10 avocados for each shipment. Each letter code represents every individual shipment assessed on each week of the supply chain analysis, and colours correspond to the country of origin. N = 4022

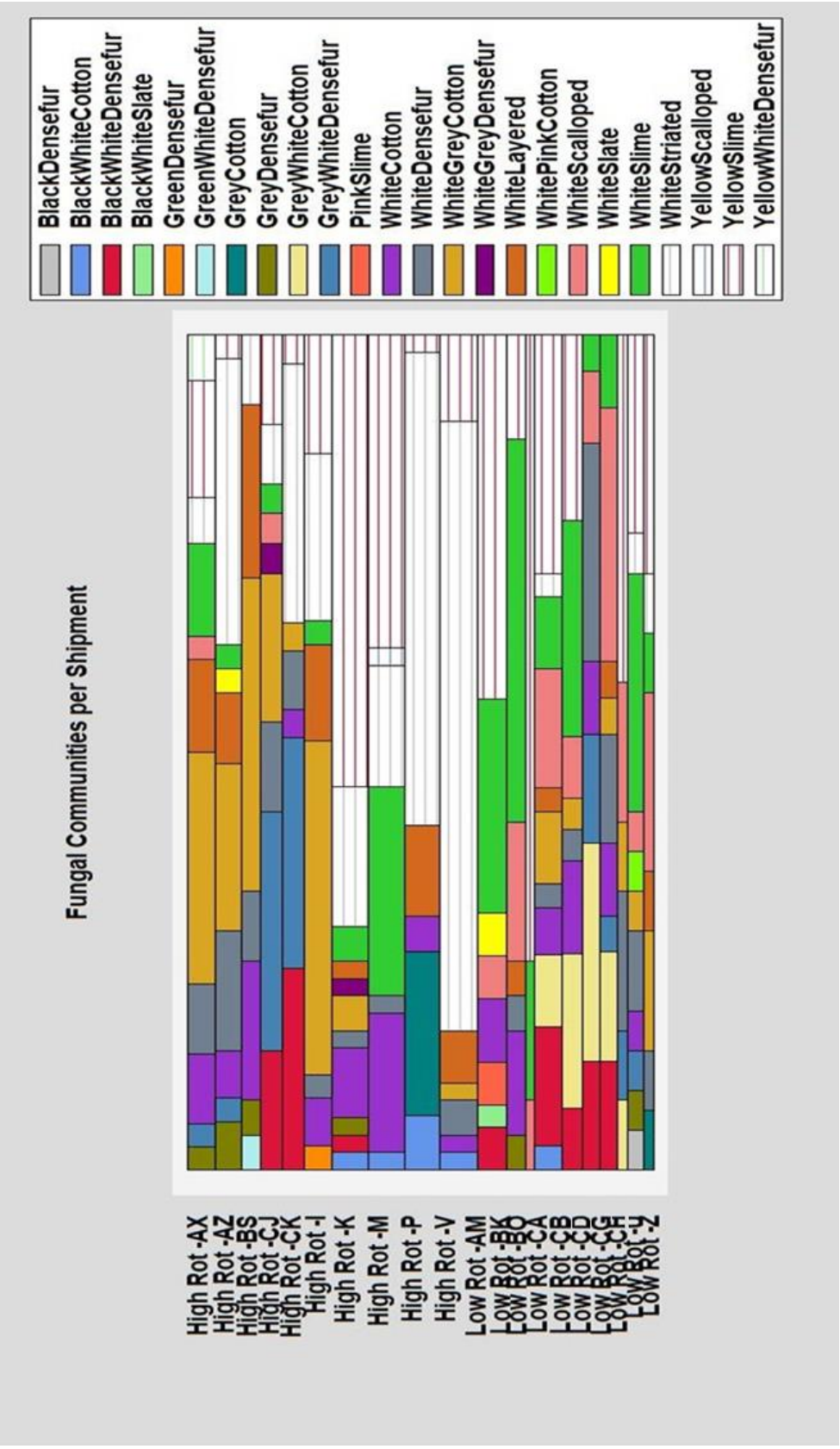

Figure 5.17: Fungal communities isolated from the top ten shipments with the highest SER incidence (High Rot) and the top ten shipments with the lowest SER incidence (Low Rot). For all shipment groups N = 50 avocados, except for BS (N = 25), CK (N = 40), I (N = 36), AM (N = 48), BO (N = 25), and CH (N = 43). The size of the boxes indicates relative abundance. Total N = 916

5.4 Discussion

The fungal community analysis uncovered a wide variety of morphotypes found in the stem end of avocados. It also revealed seven morphotypes which dominated the fungal community found in avocado mesocarp. These seven morphotypes, once genetically identified, were confirmed in the literature as known SER causal pathogens (Guarnaccia et al., 2016; Wanjiku et al., 2020). Their over representation provided the opportunity for better statistical analysis of factors affecting SER incidence and pathogen biology. It is possible that the remaining morphotypes were opportunists acquired during processing, or they were growth forms of more abundant morphotypes. Few studies to date have explored fungal infections of fruit during processing, but SER-associated pathogens have been proven to be resilient under extreme conditions. For example, some *Colletotrichum* species can overwinter within avocado tree tissues in Israel (Gunjan et al., 2017)

The warmer, humid conditions of South Africa and Mexico were expected to yield a wider variety of pathogens, because most established SER pathogens require warm, wet conditions for optimal sporulation, with humidity being an important factor in spore development for many species (Anco et al., 2012; Belisario et al., 2020; Chan et al., 2020; Cruz et al., 2019; Ranjana et al., 2010; Saha et al., 2008; Wong et al., 1983). The fact that all three countries had equal species diversity and a similar variety of secondary and tertiary pathogens suggests that either many of these statistically insignificant species may have accumulated during transport and processing or they may have just been existing as endophytes. More study is needed to confirm if the other morphotypes cultured during this project are also SER causal species individually. If so, it would suggest that any of the multiple fungal species cultured within one avocado could have been the causal pathogen.

The low abundant species were not significantly correlated with SER incidence,

but the seven most abundant morphotypes were, and they were found in all three countries in varying proportions. Mexico proved to be most vulnerable to many of the abundant hyphal pathogens requiring high germination temperatures, such as *Neofusicoccum* and *Lasiodiplodia* as indicated by Table 4.2, whilst Israel was most resistant to these (Chan et al., 2020; Saha et al., 2008). Since Mexico commands the longest evolutionary history with the pathogen, it would seem that *Diaporthe* has developed the most successful mechanism for colonising avocados by evading the fruit's defence mechanisms (Wolstenholme & Whiley, 1999). It would appear that the Mediterranean climate, characterised by dry summers, is unfavourable to *Diaporthe* spore germination, because *Diaporthe* has been shown to require long periods of wetness to sporulate (Anco et al., 2012). It is important to note that whilst members of *Diaporthe* can be found in both tropical and temperate zones, the genus as a whole is known to be predominately temperate (Taylor et al., 2000).

In contrast, Mexico and South Africa correlated well with both *Diaporthe*, and *Neopestalotiopsis*, at their tropical and sub-tropical climates can provide the wetter environments that some species of these genera prefer (Anco et al., 2012; Belisario, 2020). Although the identity of *Diaporthe* was confirmed by sequence analysis, both WhiteSlime and YellowSlime proved difficult to sequence. It is possible they are a reproductive exudate originating from the ostioles of *Diaporthe*, which retains no genetic material (Guarnaccia & Crous, 2018). However, it is also possible that they could be yeast cells, as the morphology shown in Figure 4.26 is characteristic of yeast. Experimentation with different media may yield more results, but the Slime phenotypes grew well on MEA. When placed in liquid Malt Extract, without agar, the sample died.

SER is a common and economically significant postharvest disorder of commercial tropical fruit. In addition to affecting avocado, it is a major disease of

mango, papaya, coconut, *Citrus*, mangosteen, rambutan, carambola, and banana. It has been extensively studied in mango, in which the fungus first colonises the xylem and phloem tissue before necrotising the parenchyma cells (Karunanayake & Adikaram, 2020). The most common SER causal genera for mango were endophytic *Lasiodiplodia* and *Phomopsis*, which Maqsood et al. (2014) found to prefer glucose as a carbon source. The fact that *Lasiodiplodia* was found to be abundant in the supply chain avocados, despite avocados not being a rich source of glucose, suggests that members of this genus can adapt to a wider range of hosts.

Most of the SER causal pathogens identified in Chapter 4 were found to be broad range generalists. On avocado, the majority of SER associated genera belonged to *Diaporthe*, *Colletotrichum*, *Fusarium*, *Pestalotiopsis*, *Lasiodiplodia*, *Neofusicoccum*, or *Neopestalotiopsis*, in varying proportions depending on time of year or country of origin (Chapter 4). Other tropical fruit species were also found to have a variable SER pathogen community, but it was often narrower than that of the avocados assessed in this study. The SER causal pathogens of mango are well established in the literature as growing endophytically within the tree, infecting the pedicel prior to flowering. Mango SER is most often caused by *Lasiodiplodia*, but *Colletotrichum*, *Dothiorella*, *Diaporthe*, and *Alternaria* also contribute to rot incidence depending on the fruit cultivar (Diskin et al., 2017; Karunanayake & Adikaram, 2020; Li et al., 2020).

Lasiodiplodia was also found to be a particularly virulent SER causal species for papaya and coconut (Netto et al., 2014; Rosado et al., 2016). Researchers noted that all species of *Lasiodiplodia* isolated from both coconut (Rosado et al., 2016) and papaya (Netto et al., 2014) were pathogenic, but neither study explored when or how the fruit were infected. In contrast, a study by Huang et al. (2015) identified several *Diaporthe* associated with *Citrus* as being pathogenic, saprophytic, and endophytic. Although

Diaporthe was previously thought to be host specific, more recent analysis has shown many species to be generalist, and often existing as endophytes in *Citrus* plants (Dong et al., 2021; Chepkirui & Stadler, 2017). More research is needed to determine if the *Diaporthe* species responsible for SER symptoms on *Citrus* are either external pathogens or endophytes which shifted to a pathogenic lifestyle.

Contrary to tropical fruit, when the fungal communities of commercial apples were assessed by Abdelfattah et al. (2016) via genetic sequencing, different parts of the fruit were found to harbour unique fungal communities, with *Alternaria* being most abundant in the stem end. *Alternaria* is also an established SER causal species for avocado, and some strains are known to be endophytes, although it was not one of the seven most abundant species isolated in the supply chain analysis (DeMers, 2022; Twizeyimana et al., 2013). Masangkay et al. (2000) found the optimal sporulation of *Alternaria* to be 18 ºC, which is far below the average temperature of tropical and subtropical fruit, but more common for apples grown in temperate regions. The fact that most of the fungal species associated with the avocado stem end were found to be generalists growing endophytically in many other fruits, with their community composition responding more to temperature and climate factors, suggests that they may be endophytes of avocado trees as well. The most dominant species within the endophytic community would be the best adapted to the existing environmental conditions. This end of supply chain assessment of SER fungal communities was not designed to conclusively established the SER causal species as endophytes. Further study of avocado tree material in the field would be needed to confirm if the fungal species growing endophytically within the tree are also able to produce SER symptoms, and if so, determine what factors cause them to switch to a pathogenic lifestyle.

The pattern of fungal species cultured from the avocados in the Supply Chain

Dataset indicates that high rot incidence is associated with more virulent species. *Diaporthe* correlated the most in shipments with high rot incidence, whilst YellowSlime was found in those with low incidence. The ability of *Diaporthe* to produce SER symptoms was confirmed with the Koch analysis (Chapter 7), whilst YellowSlime and WhiteSlime failed to consistently produce symptoms, and were not often reclaimed from infected tissue. Both *Diaporthe* and *Neopestalotiopsis* were most closely correlated with SER positive fruit from Mexico, which has the longest evolutionary history with the pathogen. Therefore, *Diaporthe* (*Diaporthe*) is the most statistically influential morphotype affecting SER incidence.

CHAPTER 6: PHYSIOLOGICAL FACTORS AFFECTING AVOCADO SUSCEPTIBILITY TO SER

6.1 Introduction

Physiological properties of the avocado fruit, such as mesocarp firmness and dry mass, have been shown to influence rot incidence (Hernández et al., 2016). These physical properties, along with total fresh weight and presence of a stem button, were recorded for each avocado during the SCD aggregation (Section 2.1). Although the effect of fruit softness and dry mass accumulation on SER have been previously explored, this is the first study to compare and contrast the interrelationship of four specific physiological factors (total fresh weight, presence of a stem button, average mesocarp firmness, and percentage dry mass) to assess their impact on rot incidence and severity both before and after storage.

SER incidence is expected to increase with increased fruit age and decreased firmness, because the increased ethylene produced by the softened mesocarp tissue breaks the dormancy of latent fungal pathogens within the fruit (Coates et al., 1993). It is difficult to predict whether an increase in dry mass will make an avocado more or less susceptible to developing rot symptoms. The percentage dry mass increases when an avocado is left on the tree longer after fruit set. These more fully mature avocados ripen more quickly and evenly after harvest than their early-harvested counterparts and therefore are less prone to rot (Blakey et al., 2010; Hernández et al., 2016). However, studies have also shown that immature avocado mesocarp is resistant to SER pathogens, due to its higher anti-fungal phenolic content (Cutting et al., 1992). It is unclear whether larger or smaller avocados are more prone to rot symptoms, because no previous studies have explored this in the literature. However, the presence of a stem button is expected to decrease the likelihood of contracting SER, because the stem

remnant serves as a barrier to any spores present in the orchard reaching the abscission zone wound (Madhupani & Adikaram, 2017).

Fungal rot development is dependent on the temperature and humidity conditions necessary for spore germination. Growing regions which regularly experience the environmental circumstances most conducive to fungal infection were expected to experience more SER incidence when compared to other regions. Although the exact temperature and humidity conditions required for sporulation are species specific, many recognised SER pathogens share a similar sporulation window (Section 4.2.3, Table 2). This would be expected, as a location with high rot incidence would have a fungal community adapted to its weather and climate range.

SER incidence between growing regions and countries of origin were compared over multiple seasons to determine if some regions are more conducive to rot development than others. This analysis considers avocados harvested from each country, transported through a commercial supply chain, and being analysed at both intake and after shelf-life storage. SER was measured as a binary positive (1) or negative (0) presence of disease regardless of specific rot type or severity. Mexico was expected to have the highest number of SER positive avocados, because it has the longest evolutionary history with the SER causal pathogens, and therefore more widely adapted pathogen defences (Gaillard & Godefroy, 1995). Considering harvest time, early harvested fruit were expected to have a higher rate of SER infection. This is because early harvested fruit do not have as much time to mature on the tree, and therefore do not accumulate the oil content which results in the faster and more even ripening correlated with reduced rot incidence (Hernández et al., 2016).

The specific objectives of this study were:

1. To explore factors of SER development and susceptibility with relation to the physiology of the fruit

2. To investigate the effects of fruit origin, pathogen community, and non-fungal related browning on SER incidence and susceptibility

3. To explore combinations of effects which may compound to increase fruit disease resistance or susceptibility

6.2 Materials and Methods

6.2.1 Avocado Physiology Data

The laboratory procedure used to obtain all avocado physiological data is described in section 2.1. When data collection began, the sample fruit was improperly selected, packaged, and shipped, leading to much softer fruit arriving at the university for analysis compared to the sample types requested. The aim had been to obtain fruit that were in the same condition as those offered for commercial sale in retail stores, whereas we initially were given rejected samples whose poor condition had been exacerbated by further inappropriate supply chain handling. The softer fruit were more prone to rot and bruising and were therefore excluded from the physiological data analysis to better assess what factors protect the avocado from developing rot symptoms. The dataset used to examine the fruit physiological impact on SER incidence included all avocados assessed in the SCD (Section 2.1) which were 0.5 kgf firmness and above.

The effect of firmness and dry mass on SER incidence was examined by comparing the physiology and rot symptoms of stored and intake avocados. The SCD avocado samples were split on arrival into two groups of 25, as described in section 2.1. One group, designated the ‘Intake’ avocados, was assessed on the day of arrival, whilst the other was stored under controlled retail conditions (19 °C, eight hours of fluorescent lighting, and ambient humidity) and assessed four days after arrival. The difference in

physiology and rot development between the ‘Stored’ verses ‘Intake’ avocado groups could provide insight into SER rot development over time. Avocados below 0.5 kgf firmness were excluded from the study as describe in section 5.2.2, as well as the first 26 shipments of SCD samples, because the sample quality was too variable during the beginning of the project.

6.2.2 Statistical Analysis

All data visualisation and statistical analysis was performed using Statgraphics 19 (Statgraphics Technologies Inc., The Plains, Virginia, 2021). The Statgraphics software was chosen because of its proficiency with categorical data. Much of the most important data recorded in the SCD could not avoid interpretation as a category, which means it must be recorded as a label instead of a numerical amount. The weight and firmness of an avocado are measured in number of grams or kgf, but whether SER was present in the tissues or not is a simple yes or no question, or a binary measurement. Disease incidence naturally lends itself to a binary measurement, as the avocado either has SER or it does not. The symptoms themselves were classed into groups, as well as any morphotypes cultured from the fruit, and the severity of symptoms were arranged into classifications for simplicity. Although rot severity could have been recorded continuously, finer data would not have provided additional insight into factors associated with symptom severity. This is because many of the independent variables were also categorical.

Numerous statistical tests exist for continuous variables, but few are useful for categorical variables, or a comparison of one categorical variable with one continuous variable. Statistical analysis was therefore limited to applying Canonical Correspondence Analysis (CCA), Principal Component Analysis (PCA), or Chi-Squared tests. PCA is best applied to linear data, whereas CCA assumes data is unimodal, but both are conceptually similar. They are used to visualise correlations

between categories, implying a statistical relationship, but they cannot prove statistical significance. A Chi-Squared test is one of the only available metrics for determine if differences in categorical data are statistically significant. Only the Chi-Squared test of independence was used in this work.

6.3 Results and Discussion

6.3.1 Effect of Fruit Firmness, Dry Mass, Fresh Weight, and Stem Button on Rot Incidence

The total fresh weight (Figure 6.1) and percentage dry mass (Figure 6.2) were both found to be normally distributed and similar for SER positive and SER negative fruits. SER positive fruit skewed toward a slightly higher dry mass percentage and narrower fresh weigh range compared to SER negative fruit, but both groups had similar averages for these physiological factors. Fruit firmness showed more variation between groups, as SER positive fruit skewed toward a lower firmness compared to the SER negative fruit (Figure 6.3). The difference between these two groups was statistically significant as determined by one-way ANOVA (F (1,2399) = 33.25, p = <.01). The fact that firmness was the biggest factor in rot incidence confirms what has already been established about fruit softening and rot development. In 1994, Hopkirk et al. studied the effects of temperature and ripening times on postharvest disorders of Hass avocados. Although they did not identify the specific pathogens responsible for the rot symptoms, they demonstrated the close corelation between rot severity and the ethylene-induced cellular degradation characteristic throughout the late stages of fruit ripening.

The presence of a stem button correlated with an increase in rot incidence: X^2 (1, N = 2610) = 150.84, p = < 0.01. Figure 6.4 shows how stem button retention is weakly associated with the development of rot symptoms. This is an interesting result, as it may imply that the pathogen is endophytically present within the stem button. It also

contradicts a previous study on the comparison of harvesting techniques which proved that removing the entire stem during harvest increased rot incidence (Hartill & Everett, 2002), however the two results may not be related. All of the investigations of SER incidence in relation to the stem button have been conducted before distribution, involving avocados which have either been snapped, or ripped from the tree, and those which were cut and allowed to retain part of their stem end. In the wild, fruit, leaves, and injured organs will fall from the tree once they have undergone a hormone regulated sealing process in which pectin and lignin form a plug at the abscission zone (Merelo et al., 2017; Roberts et al., 2002; Tranbarger et al., 2017; Tucker & Kim, 2015). The process of tearing fruit from the tree rips the cells at the abscission zone before they are able to naturally detach or form a seal against pathogens. The main theory as to why removing avocados from the tree without the stem button increases SER is because this damage to the stem end would create an opening for opportunistic fungal pathogens to invade the exposed mesocarp tissue.

However, fruit detachment in the wild is in contrast with commercial harvest procedures. As the fruit ripen, the stem button undergoes an abscission process and detaches naturally from the fruit, thereby sealing the stem end wound. Avocados with stem buttons were expected to develop less SER compared to those without buttons, however, the data do not support this hypothesis. At the distribution end, avocados with an intact button were found to be more prone to SER symptoms.

Although the exact origin and manifestation of postharvest rot is not fully understood in terms of all causal agents, the literature is more unanimous concerning the effects of the stem button and rot for multiple fruits. SER affects many types of fruit, but it is most often studied in citrus, mango, papaya, and avocado (Galsurker et al., 2018; Karunanayake & Adikaram, 2020). Galsurker et al. (2018) found that the

retention of a long stem resulted in the most significant decreases in rot for both mango and avocado. Mango stems contain antimicrobial properties in the sap, specifically alk(en)ylresorcinols (5-n-heptadecenylresorcinol and 5-n-pentadecylresorcinol), which are especially effective against *Alternaria alternata*. Mango fruit stored with 2 - 3 cm stems were also found to be more resistant to anthracnose body rots by retaining a higher content of resorcinol in their peel. A review by Karunanayake & Adikaram (2020), which looked at avocado, mango, and papaya also stated that removing the pedicel, or de-sapping the fruit, resulted in more rot.

Another reason for the higher rot incidence in avocados with a stem button may have to do with the nature of an end of supply chain investigation. This project is one of the few investigations of stem end rot at the distribution stage of the supply chain, and this area of research remains limited for avocados. Galsurker et al. (2018) extensively reviewed stem end rots of tropical fruit and named *Colletotrichum* and *Alternaria* as the most common genera associated with avocado SER. Other studies confirmed *Neofusicoccum* was dominant in Italy (Guarnaccia et al., 2016), *Lasiodiplodia* and *Neofusicoccum* in Kenya (Wanjiku et al., 2020), and *Diplodia*, *Lasiodiplodia*, and *Neofusicoccum* were most abundant Chile (Valencia et al., 2019). Although these genera are some of the common isolates cultured in this study, *Diaporthe* was the topmost abundant identified, despite being less abundant in the literature.

This discrepancy may indicate a shift in the pathogen community, in terms of species isolated, near the avocado stem end, although more research would be needed to confirm this. Both pathogen abundance and stem button findings in the present study contradict the published literature. The combination of a shift in microbial community, and the increase in SER symptoms of avocados which retained their button, suggests

that that antifungal compounds in the avocado stem, such as polyphenols and tannins, may break down over time, leading to an endophytic *Diaporthe* infection (Zhu et al., 2019). Avocado was shown to have antifungal Alkadienes and acetogenins in its This genus is known to grow endophytically in many hosts, entering the tree through open lesions in the wood and remaining inside the tissues. *Diaporthe* is also a saprophyte, and it may begin to necrotise the avocado once the ripening process softens the fruit (Gomes et al., 2013)

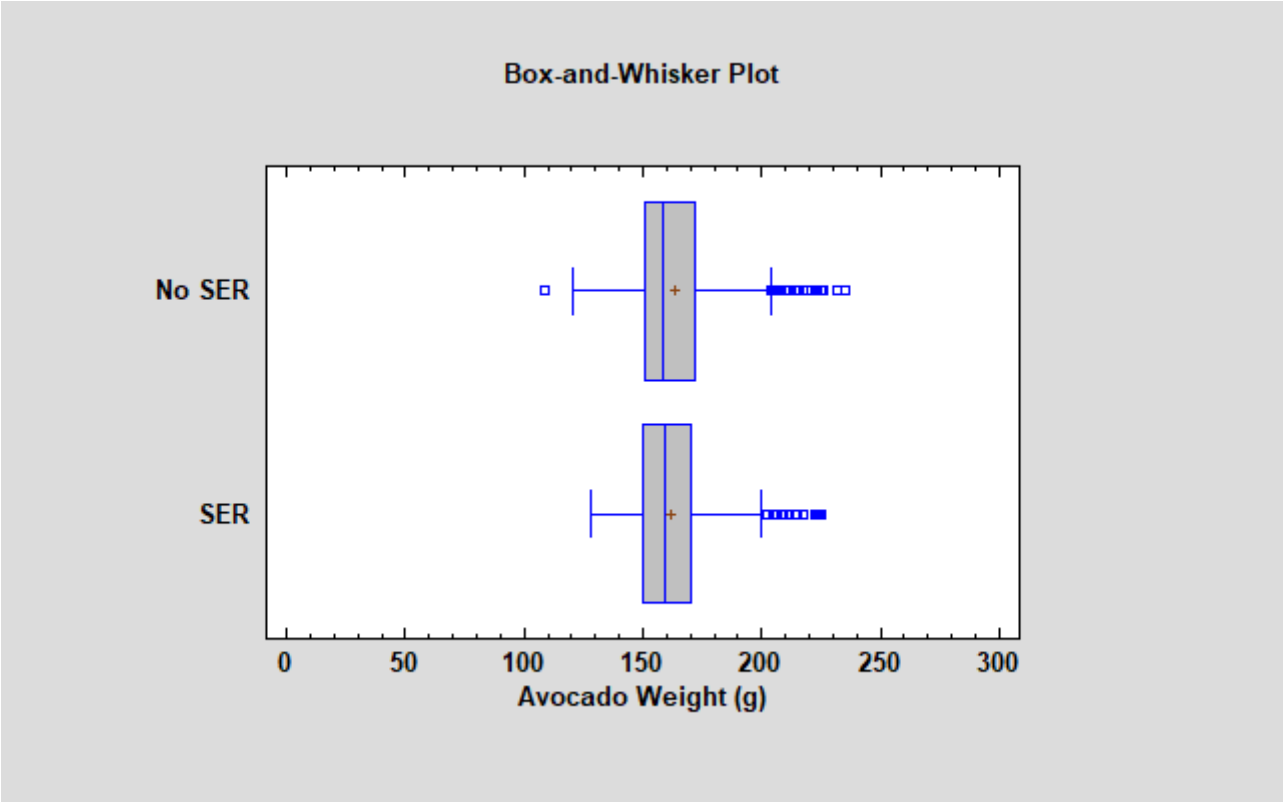

Figure 6.1: Box and whisker plot of total fresh weight in grams for all avocados above 0.5 kgf with SER symptoms and those with no symptoms. The red cross represents the average fresh weight for each group and blue boxes represent observations falling outside the 75% and 25% quartiles. N = 2610

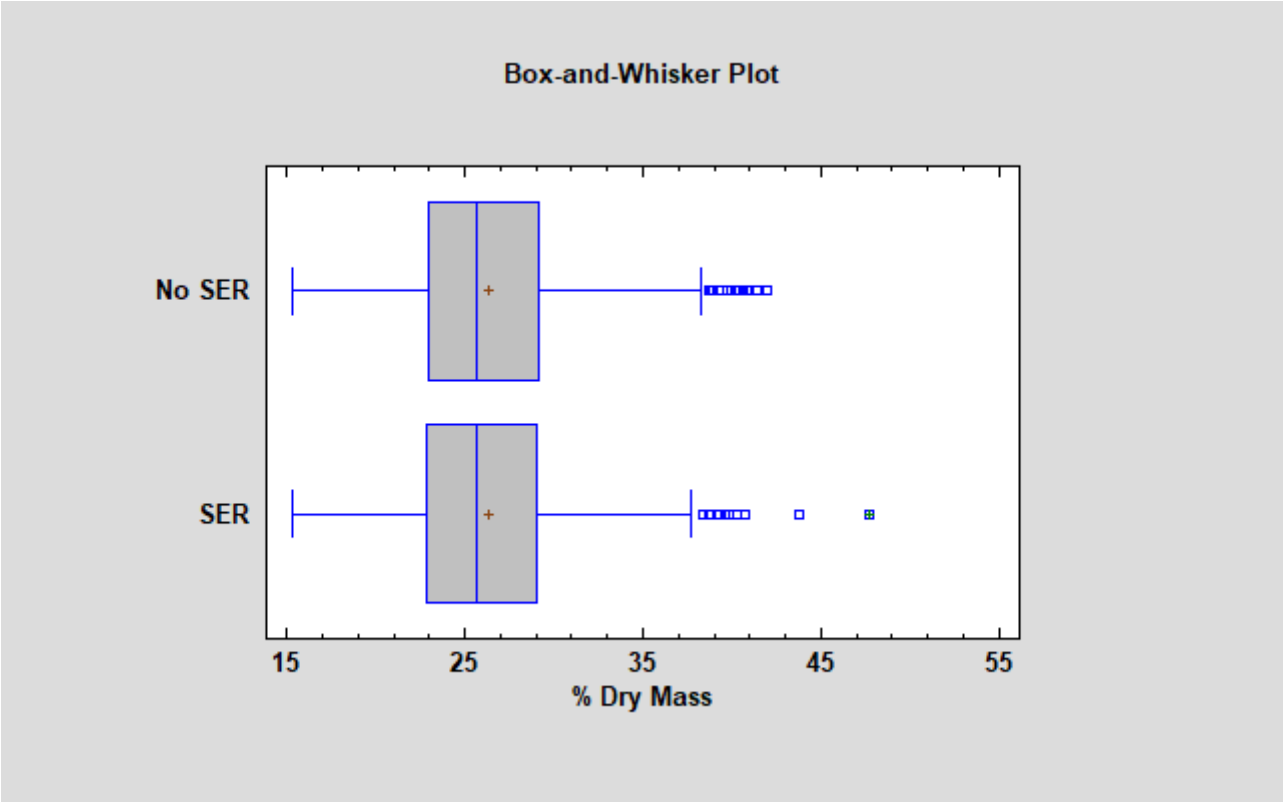

Figure 6.2: Box and whisker plot of percentage dry mass for all avocados above 0.5 kgf with SER symptoms and those with no symptoms. The red cross represents the average fresh weight for each group and blue boxes represent observations falling outside the 75% and 25% quartiles. N = 2610

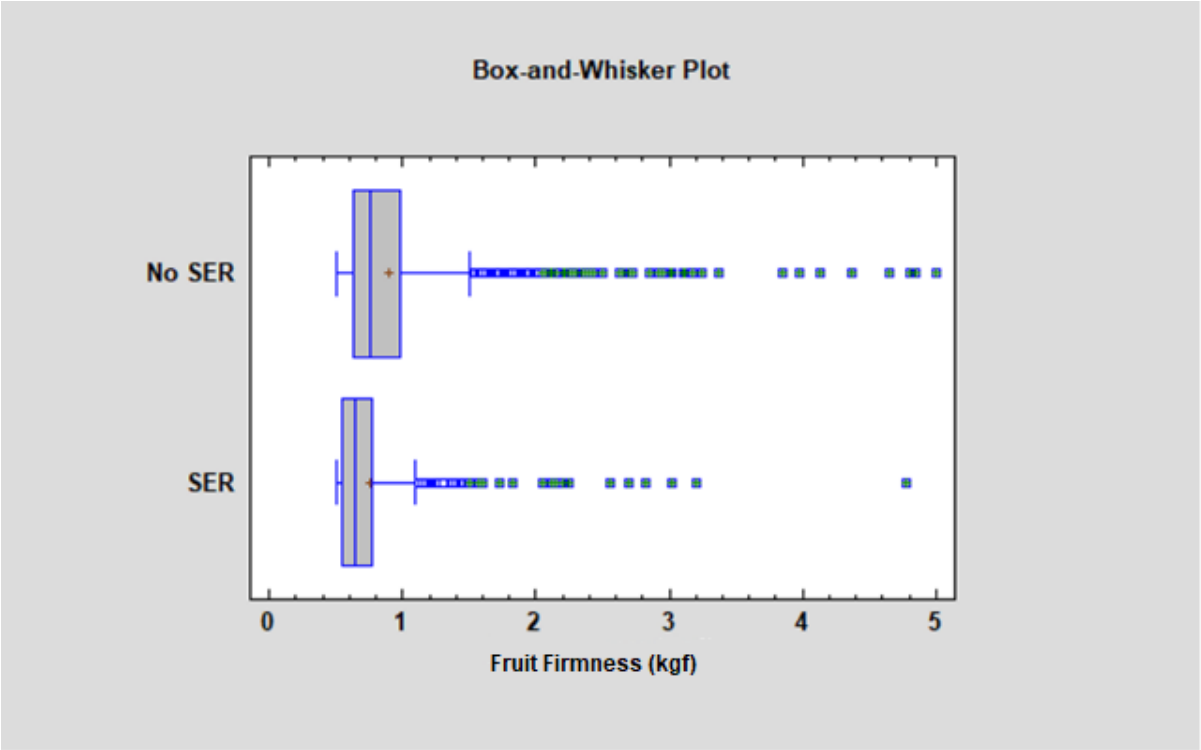

Figure 6.3: Box and whisker plot of fruit firmness for all avocados above 0.5 kgf with SER symptoms and those with no symptoms. Firmness was measured in kgf using a hand held penetrometer. The red cross represents the average fresh weight for each group and blue boxes represent observations falling outside the 75% and 25% quartiles. N = 2610

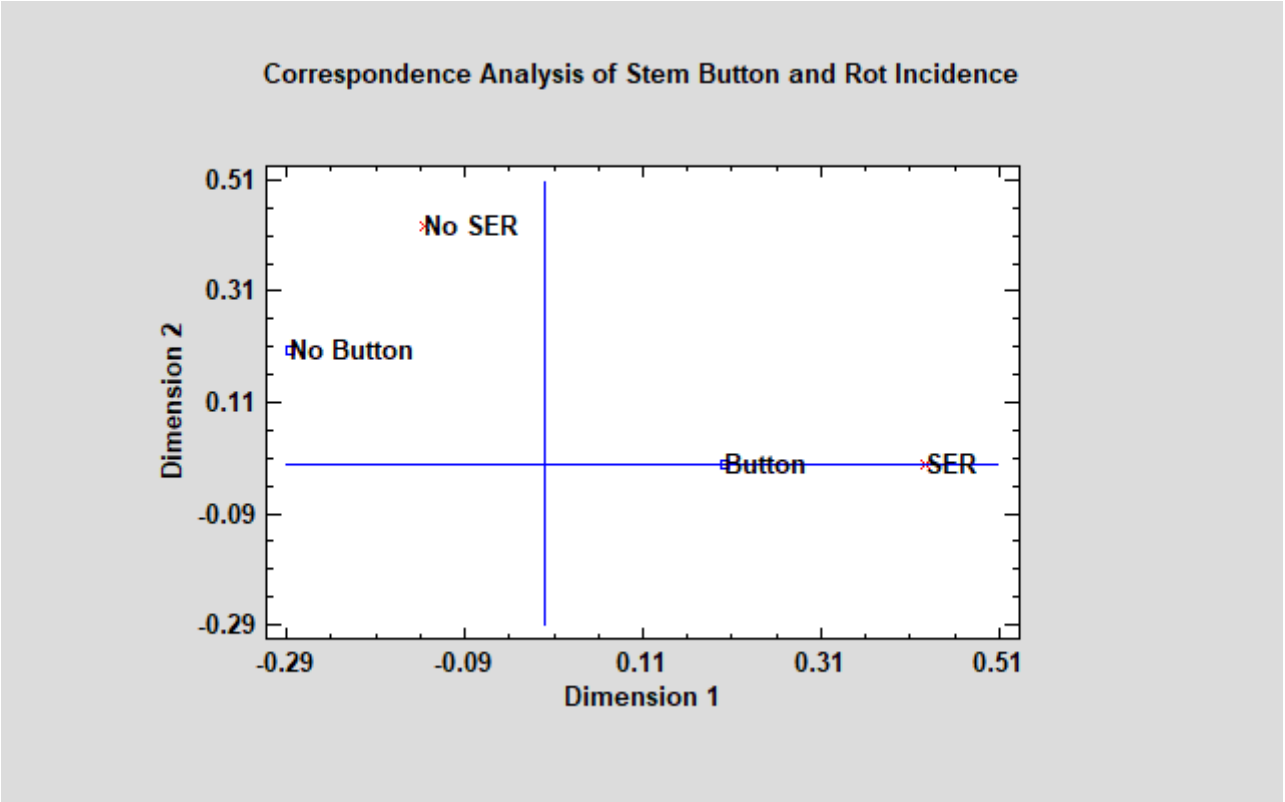

Figure 6.4: CCA Correlation of stem button with rot symptoms for all avocados above 0.5 kgf. The red X indicates presence or absence of SER symptoms, and the blue ☐ indicates the presence or absence of a remnant of the stem 'button' attached to the fruit after harvest. N = 2610

The overall influence of specific physiological factors demonstrates their contribution toward SER incidence. However, the extent of its influence may have been obscured by considering SER as a binary. Rot severity was measured on a scale of 0 to 3 (Section 2.1) to qualify rot progression. A score of 0 indicates no rot, 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% rot. Figure 6.5 illustrates that larger avocados may be prone to the most severe rot symptoms, but the severity scores of 2 and 3 both had wide margins of error, both of which overlap the entire range of avocado weights which did not have rot symptoms. Combined with the fact that the distribution of avocado weight for both SER positive and SER negative avocados were identical in Figure 6.1, the evidence suggests that total fresh weight does not influence SER susceptibility.

A high mean percentage dry mass was associated with a high rot severity score (Figure 6.6). Although the overall distribution of SER positive and SER negative avocados were also identical in their percentage of dry mass (Figure 6.2), when examined by score, it would appear that avocados that were harvested at a more mature stage, which had a higher dry mass than fruit harvested at a less mature stage, were more prone to rot. Figure 6.2 also indicates there were more outliers in the SER positive group which skewed the data toward a higher dry mass. Decreased mesocarp firmness was expected to increase rot incidence, but Figure 6.7 indicates that the most severe rot symptoms were not typically observed in the softest fruit. However, the margin of error for average firmness for avocados with a rot severity of 1, 2, and 3 all overlapped, while avocados in the 0 rot category were significantly firmer as determined by one-way ANOVA ($F(3,2397) = 12.27$, $p = <.01$). Since pathogen invasion also impacts fruit firmness, it was difficult to determine the relationship between firmness and rot incidence when the rot symptoms were extensive. Figure 6.7

implies that once avocados soften to below ~ 0.82 kgf, they are especially prone to developing SER symptoms. The analysis of stem button and rot severity in Figure 6.8 further corroborated that of Figure 6.4. The presence of a stem button correlated with SER incidence, and it was interesting to note that the stem button also correlated with the highest category of rot severity.

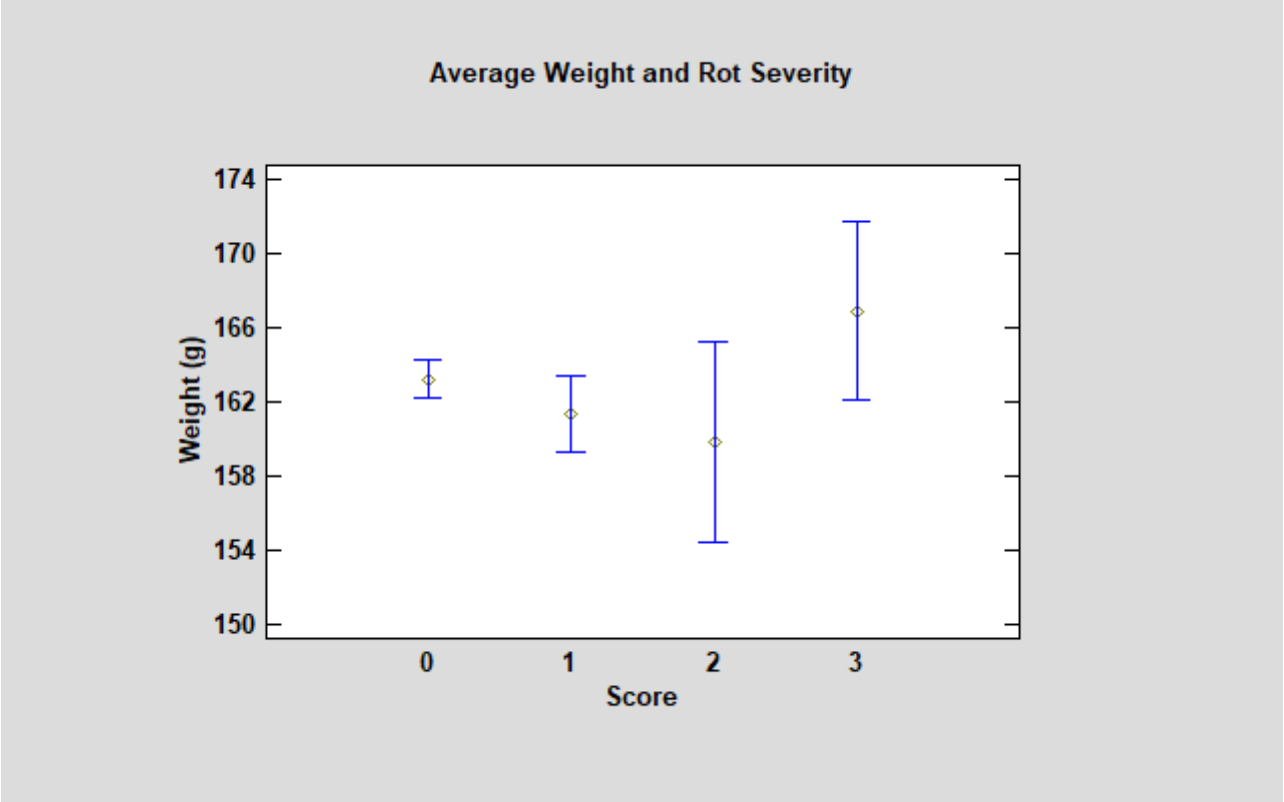

Figure 6.5: Average fresh weight in grams for each category of rot severity for all avocados above 0.5 kgf. Rot was measured on a scale of 0 – 3, where 0 indicates no rot, 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% rot. Error bars indicate 95% Least Significant Difference intervals. N = 2610

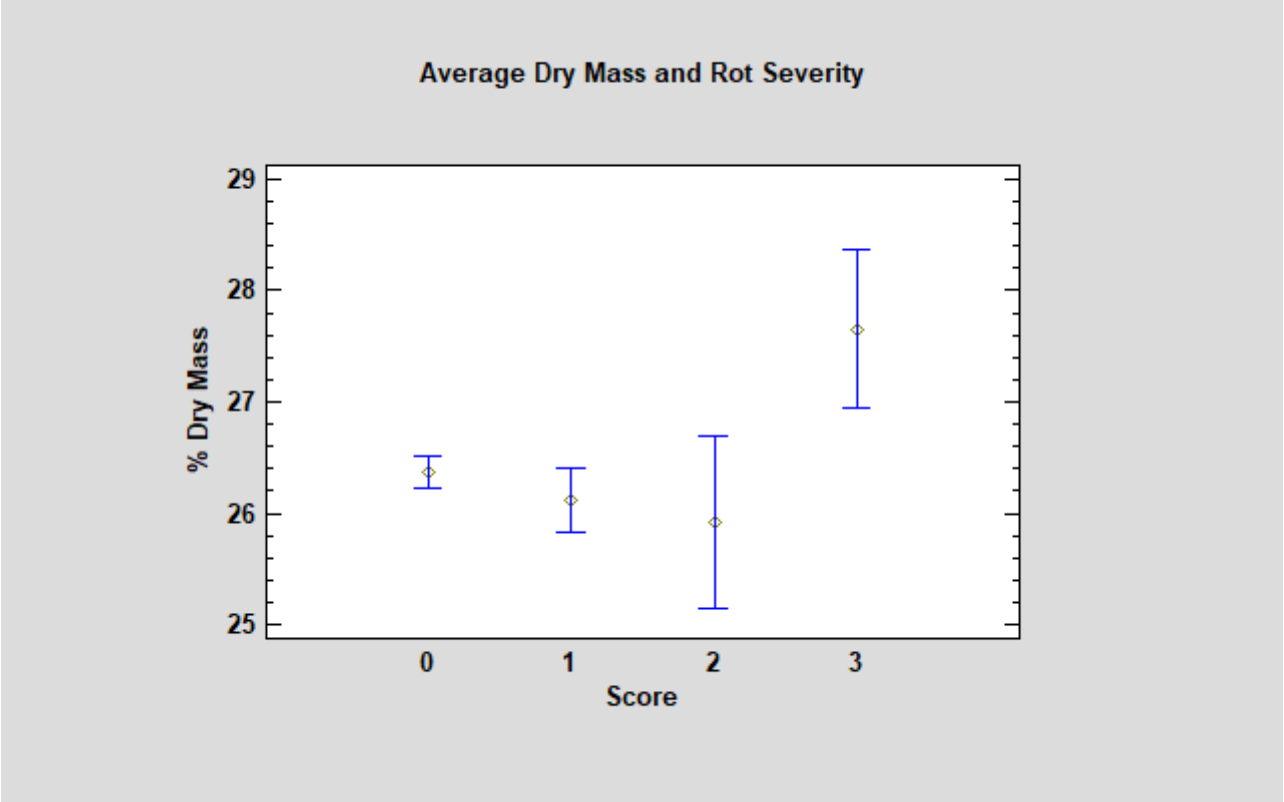

Figure 6.6: Average percentage dry mass for each category of rot severity for all avocados above 0.5 kgf. Rot was measured on a scale of 0 – 3, where 0 indicates no rot, 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% rot. Error bars indicate 95% Least Significant Difference intervals. N = 2610

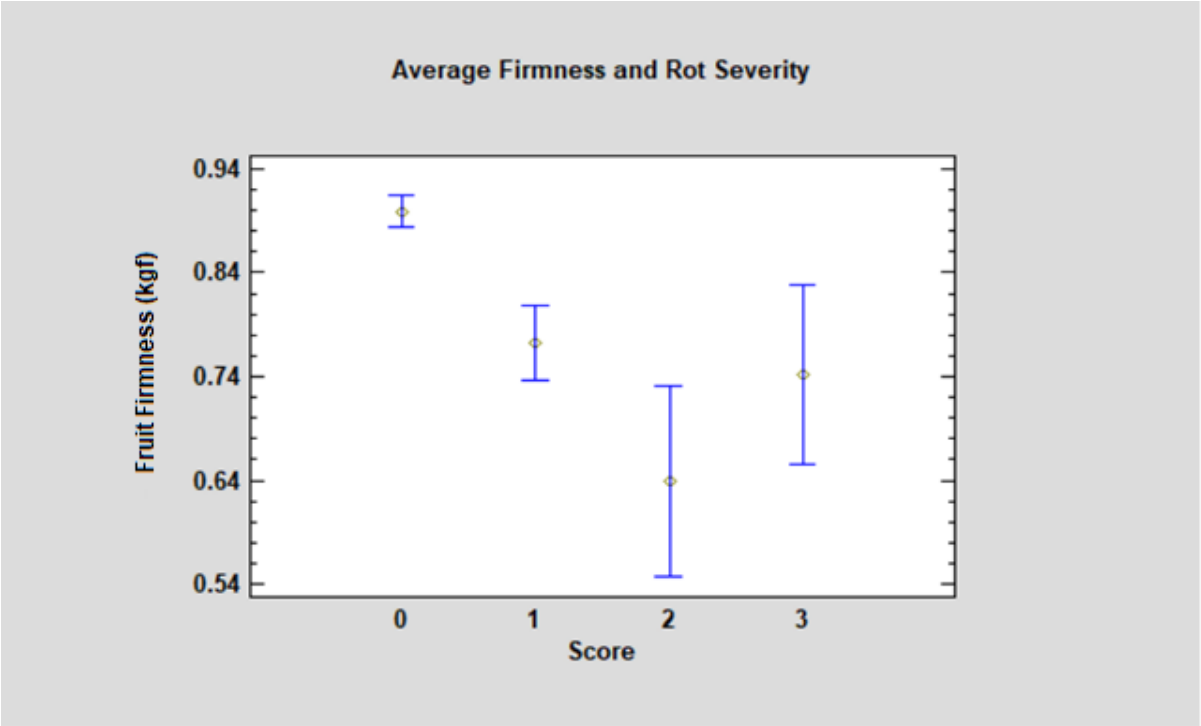

Figure 6.7: Average mesocarp firmness, measured in kgf using a handheld penetrometer, for each category of rot severity for all avocados above 0.5 kgf. Rot was measured on a scale of 0 – 3, where 0 indicates no rot, 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% rot. Error bars indicate 95% Least Significant Difference intervals. N = 2610

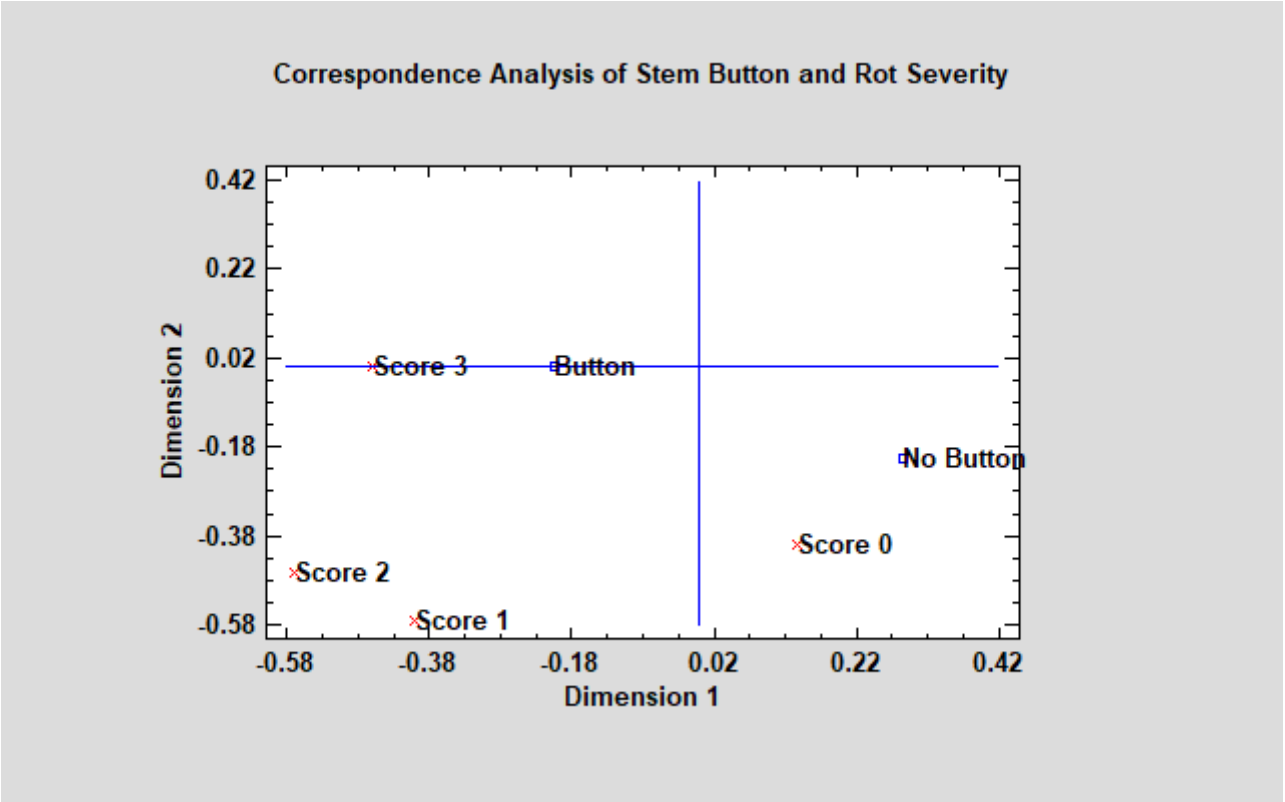

Figure 6.8: Canonical Correspondence analysis examining the correlation of a stem button with rot severity on a scale of 0 – 3 for avocados above 0.5 kgf. Dimension 1 separates rot severity and Dimension 2 separates button status. A score of 0 indicates no rot, 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% rot. N = 2610

Figure 6.9 illustrates the physiological factors measured in the SCD analysis for both ‘Stored’ and ‘Intake’ avocados. There is a clear reduction in fresh weight and fruit firmness during storage due to a measurable loss of water and increase in dry mass content. Much of this is due to cell softening and water loss as the fruit ripens. Although many outliers continue to spread past 2 kgf firmness in Figure 6.9 B, most of the samples are clustering below 1 kgf. Total dry mass is the only physiological factor that is maintained during the ripening process. Much of an avocado’s dry mass is due to its oil content, which does not evaporate or break down. In Figure 6.10, avocados stored at simulated retail conditions (19 °C, eight hours of fluorescent lighting, and ambient humidity), and ambient humidity for 72 hours were more prone to all types of browning except mycelial cavities and vascular discolouration. This is either due to physiological deterioration or injuries acquired along the supply chain.

It is possible that the pathogen is able to attack the vascular tissue or the area around the stem button first but is unable to grow into the mesocarp tissue until the fruit softens. The ‘Stored’ avocados were expected to experience increased rot severity, but Figure 6.11 shows that both ‘Stored’ and ‘Intake’ avocados were equally prone to developing severe rot symptoms. Only the category 1 rots, or the initial stages of rot development, were more frequent in the ‘Stored’ group. It is possible that avocados infected early during growth or harvest will develop symptoms as soon as the fruit begins to ripen, while avocados infected later during processing begin to develop symptoms as they age. This conclusion is supported by Figure 6.12, in which the percentage of rot for each firmness category was summarised for both ‘Stored’ and ‘Intake’ avocados. The ‘Stored’ avocados consistently experienced identical or increased SER incidence as the ‘Intake’ avocados. ‘Stored’ avocados also developed rot symptoms at higher firmness categories, where the ‘Intake’ avocados had no rot at

the same firmness. This shows how avocados can develop rot symptoms as they age, regardless of firmness, but firmness still plays a key role in overall susceptibility.

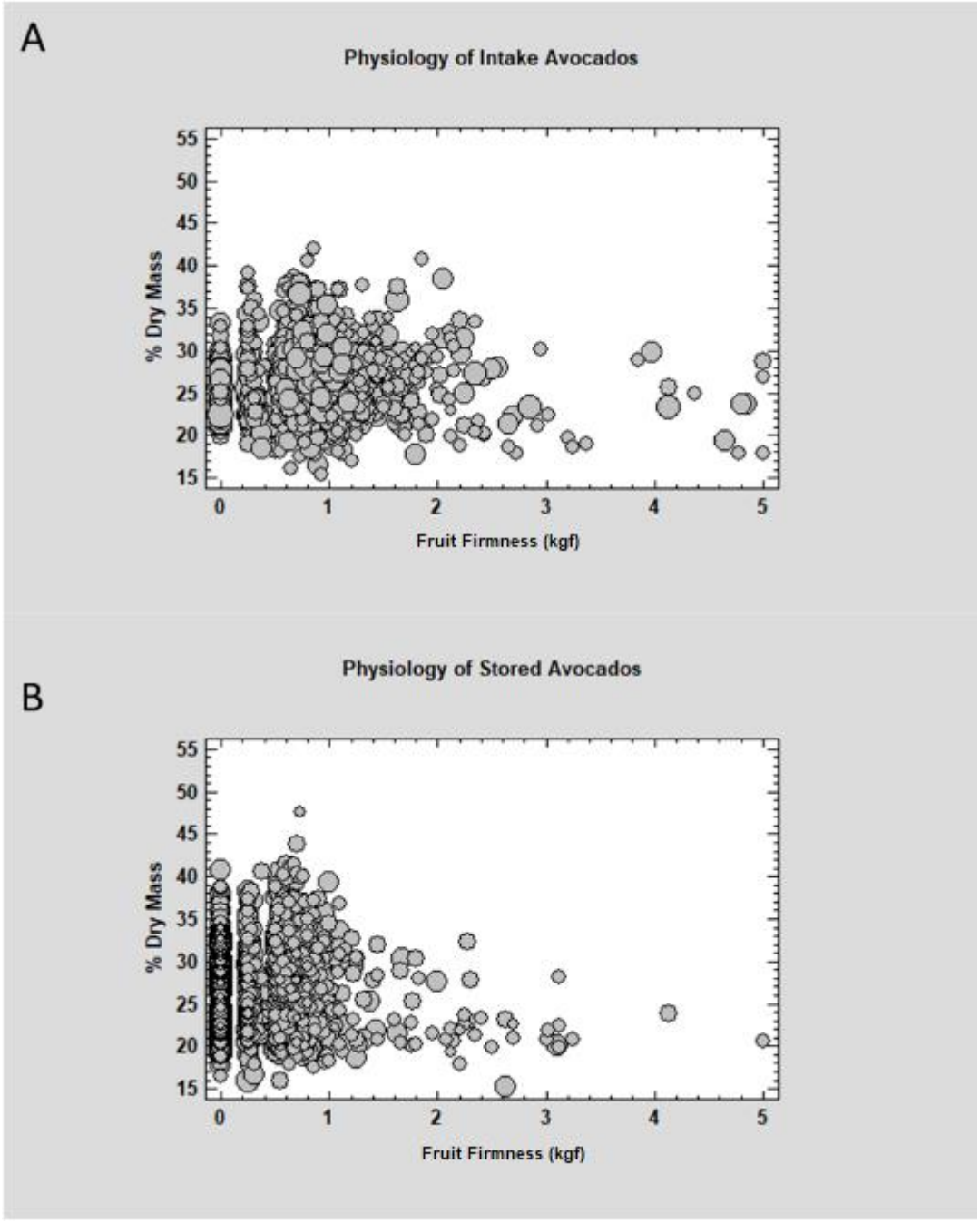

Figure 6.9: Bubble plot correlating three physiological factors of intake and stored avocados A) Fruit firmness in kgf, percentage dry mass, and fresh weight for the 'Intake' avocados. Bubble size indicates total fresh weight in grams. N = 1481. B) Fruit firmness in kgf, percentage dry mass, and fresh weight for the 'Stored' avocados. Bubble size indicates total fresh weight in grams. N = 1415

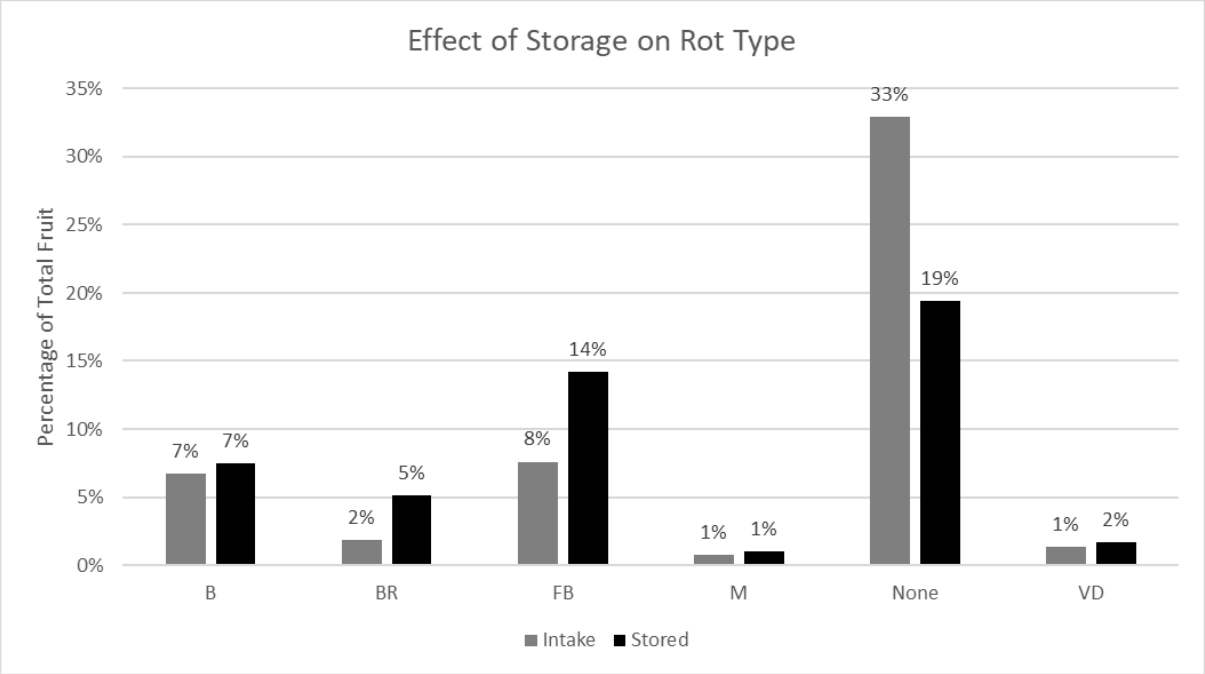

Figure 6.10: Total percentage rot and damage incidence per rot type between 'Intake' and 'Stored' avocados. 'Stored' avocados were kept at simulated retail conditions (19 °C, eight hours of fluorescent lighting, and ambient humidity) for 72 hours. B = Bruising, BR = Body Rot (Anthracnose), FB = Fungal Browning, M = Mycelial Cavity, None = No symptoms, VD = Vascular Discolouration. Bruising and Body Rot are not SER symptoms. Intake N = 1481; Stored N = 1415

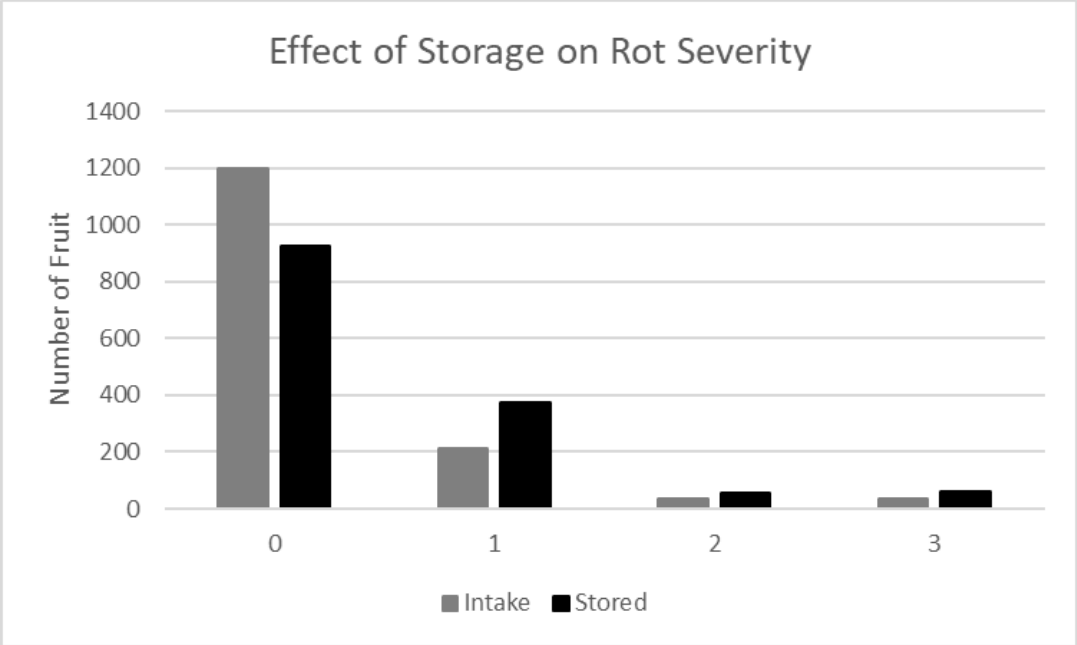

Figure 6.11: Comparison of rot severity between 'Intake' and 'Stored' avocados. 'Stored' avocados were kept at simulated retail conditions (19 ºC, eight hours of fluorescent lighting, and ambient humidity) for 72 hours. A score of 0 indicates no rot, 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% rot. Intake N = 1481; Stored N = 1415

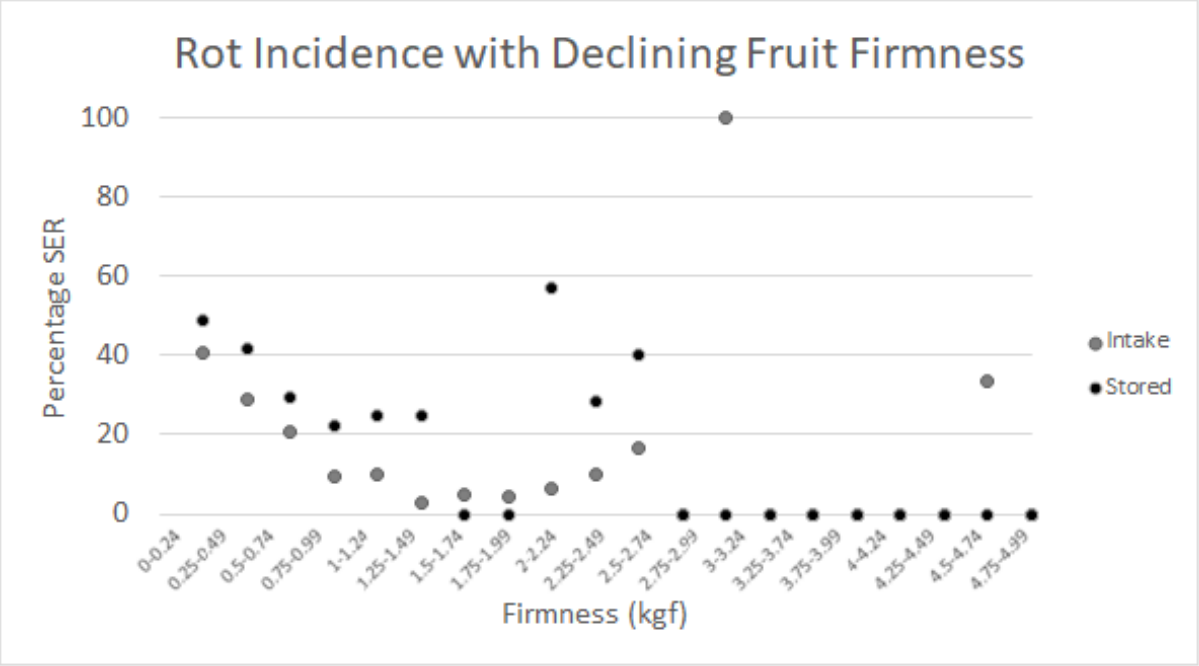

Figure 6.12: Avocado susceptibility to SER as fruit ages and softens. 'Stored' fruit were kept at simulated retail conditions (19 °C, eight hours of fluorescent lighting, and ambient humidity) for 72 hours. Fruit firmness was measured in kgf using a handheld penetrometer. The total percentage of SER positive fruit was calculated for each mean firmness range. N = 2896

6.3.2 Effects of Country, Growing Region, and Harvest Time on SER Frequency

Rot incidence between and within countries was explored for the three countries of interest, Mexico, Israel, and South Africa. Although sampling from each of the three countries was uneven in terms of total fruit number (Figures 6.13 and 6.14), South Africa had a higher proportion of SER with regard to its total sample size. A little more than half of South African avocados developed SER symptoms after four days of storage at simulated retail conditions (19 ºC, eight hours of fluorescent lighting, and ambient humidity) (Figure 6.14) whereas Israel and Mexico approximately doubled their already lower intake rot percentage after storage. Israel's 10% rot at intake increased to 22% rot after storage, and Mexico went from an initial 18% to 32% rot. Figure 6.15 shows that SER incidence fluctuated throughout the growing season of each country, with some samples from the same season and country having a high amount of rot and others lower.

Israel seems to have experienced predominantly lower rates of SER incidence for all three sample seasons, while South Africa has had two particularly bad seasons (2018 and 2019). This contradicts what was expected, with Mexico predicted to have the highest SER incidence due to its longer evolutionary history with the pathogen. The stored portion of the Supply Chain samples shows how Mexican and South Africa avocados were more prone to developing rot symptoms over time, whereas Israeli avocados remained resistant (Figure 6.16). Figure 6.17 also confirms the correlation of SER symptoms with South African avocados, while Mexico and Israel are comparatively immune. Within the sampling period of this study, Israel appears to be the least affected by SER. This could be either due to the Mediterranean growing conditions in Israel producing more resistant avocados compared to tropical and semi

tropical regions in Mexico and South Africa, or perhaps fewer avocado pathogens are present in Israel. Further study is needed to confirm either of these hypotheses.

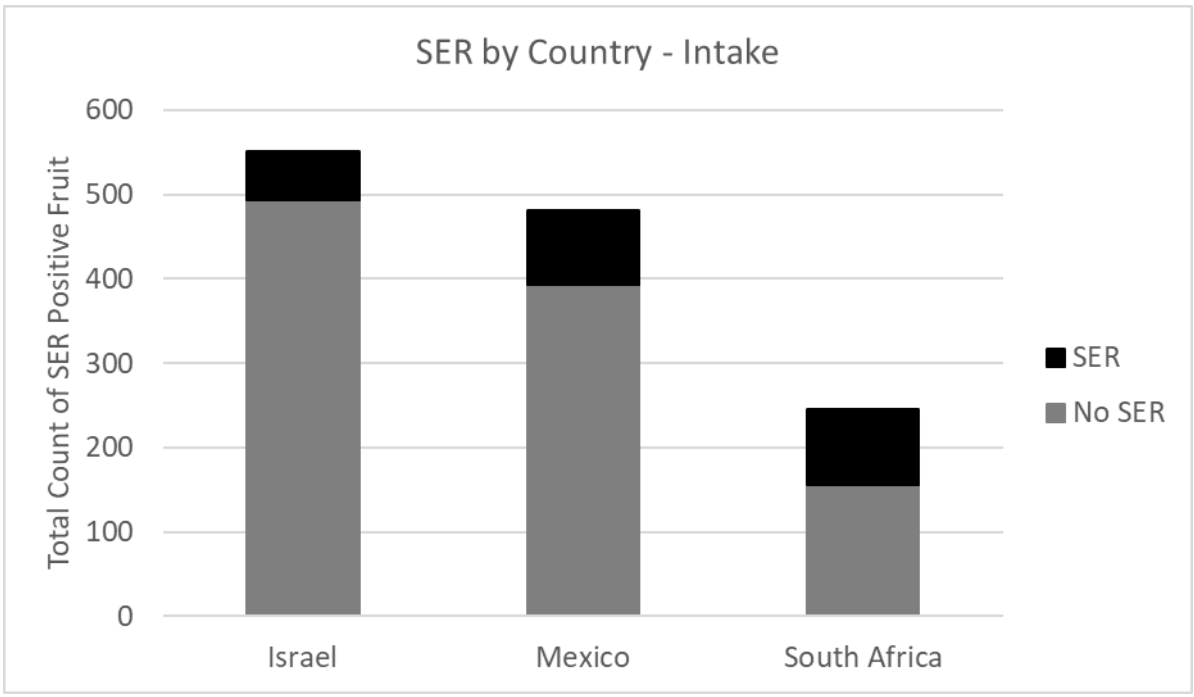

Figure 6.13: Prevalence of SER by country for avocados assessed at intake.

Israel experienced 10% overall rot, Mexico 18%, and South Africa 37% before storage.

Israel N = 551; Mexico N = 482; South Africa N = 246

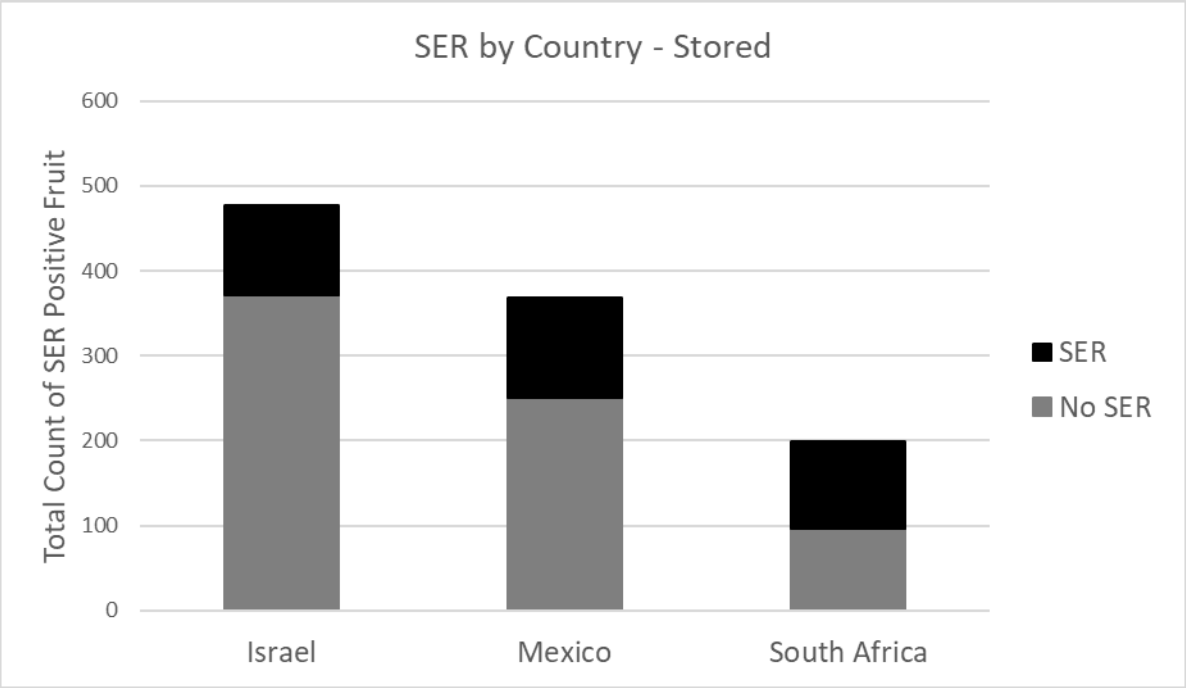

Figure 6.14: Prevalence of SER by country for Stored avocados. Israel experienced a total of 22% rot, Mexico 32%, and South Africa 48% after storage. Israel N = 477; Mexico N = 368; South Africa N = 199

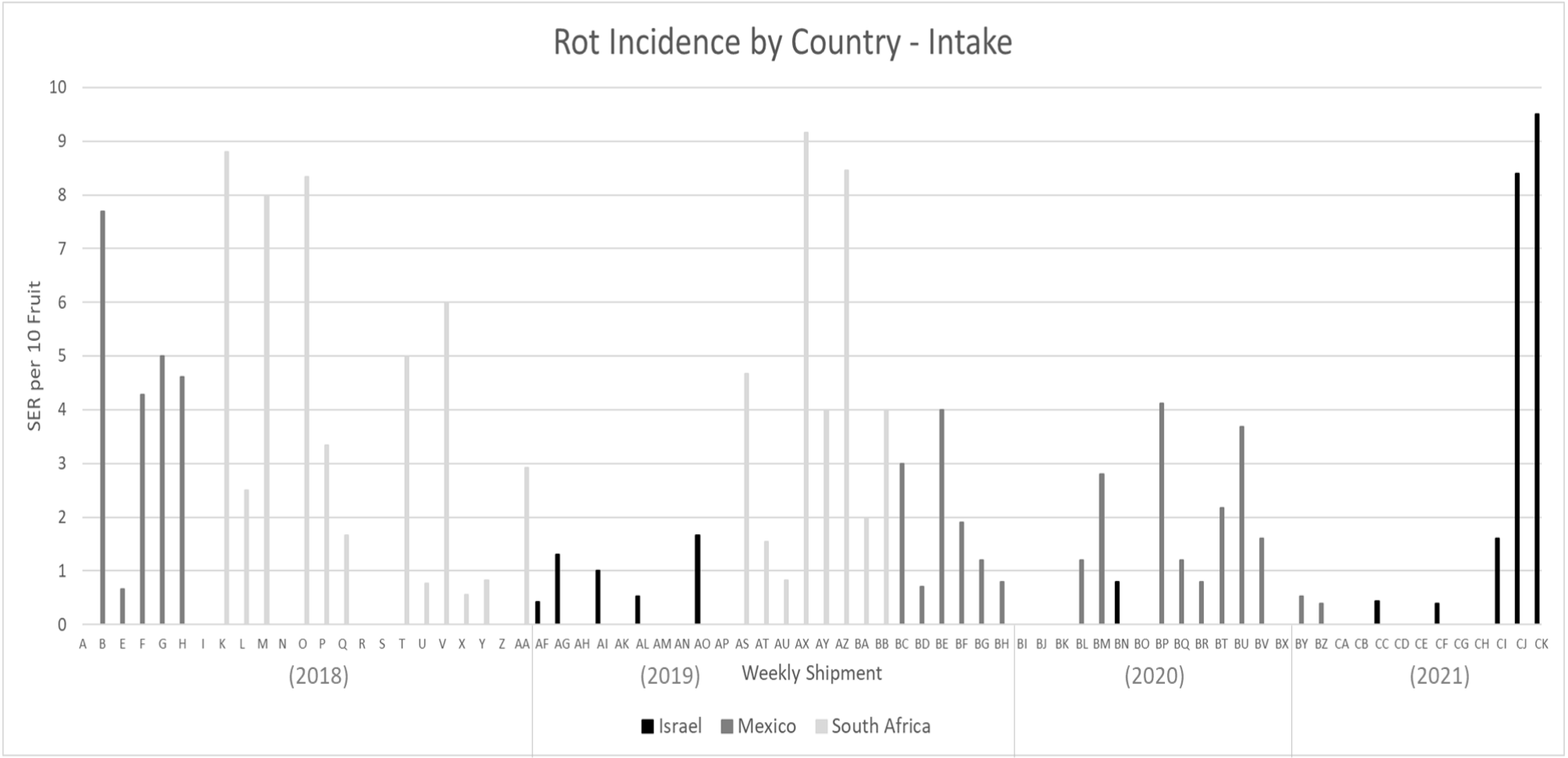

Figure 6.15: SER incidence per shipment for all intake Supply Chain samples above 0.5 kgf originating from the three countries of interest. Each weekly shipment was designated a new alphabetic code. N = 1279

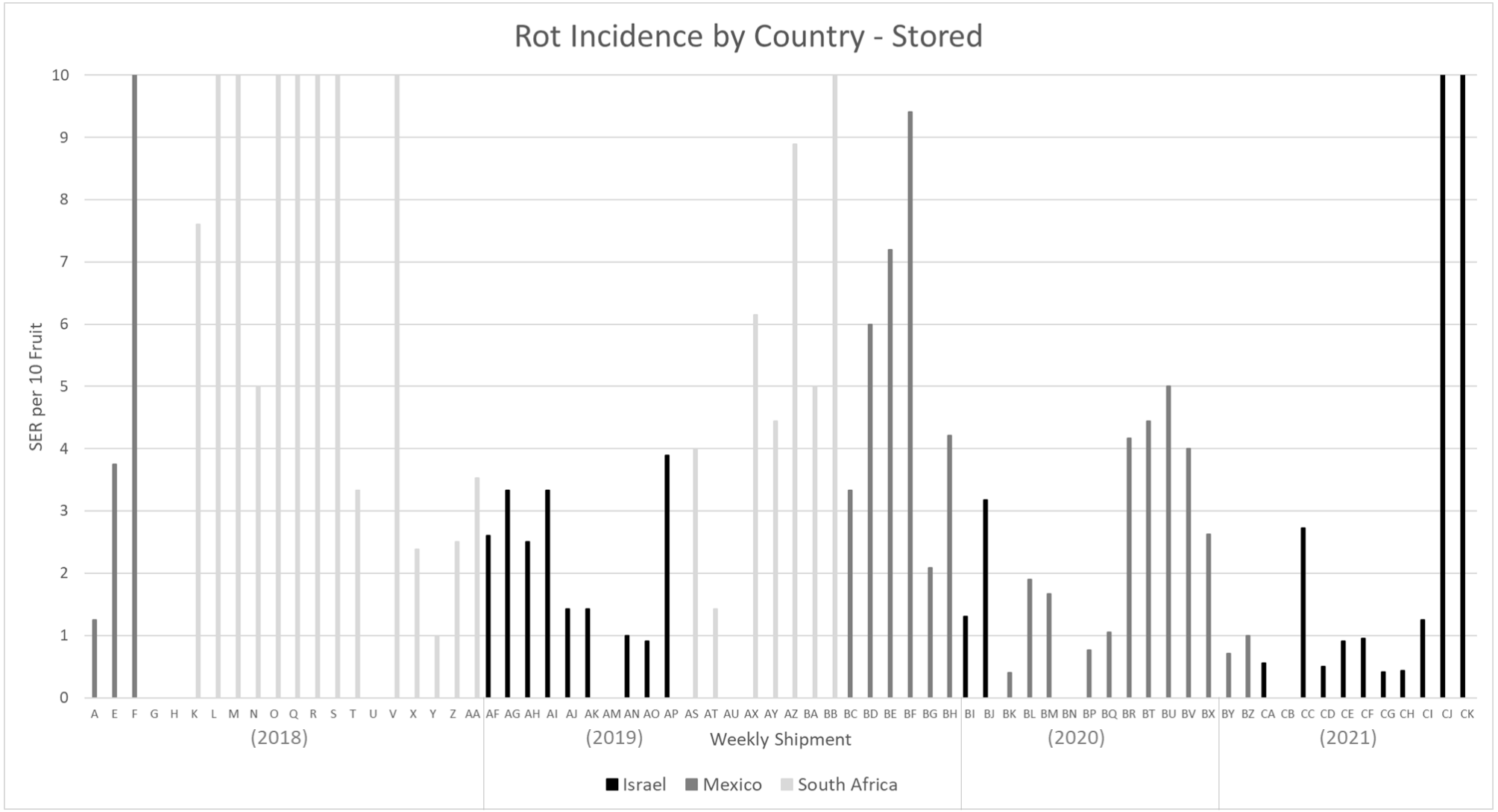

Figure 6.16: SER incidence per shipment for all stored Supply Chain samples above 0.5 kgf originating from the three countries of interest. Each weekly shipment was designated a new alphabetic code. N = 1044

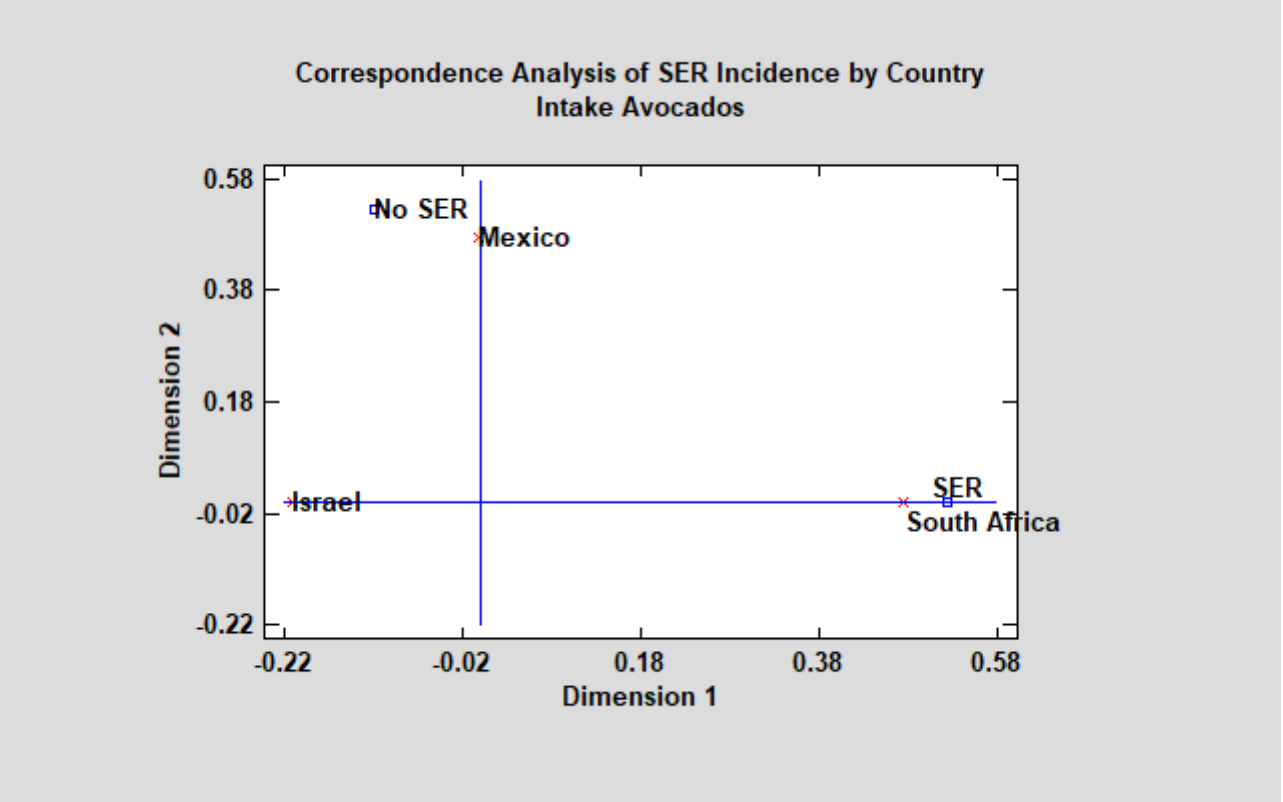

Figure 6.17: Canonical Correspondence analysis of SER incidence for intake avocados from Israel, Mexico, and South Africa above 0.5 kgf as measured by a handheld penetrometer. Dimension 1 separates rot status and Dimension 2 separates country of origin. N = 1279

6.3.3 Rot Type, Severity, and Causal Species Analysis

The seven most abundant fungal morphotypes cultured from the avocado samples were compared with SER rot symptoms and severity to assess the impact of fungal species, as characterised by morphotype, on specific types of browning. All avocados above 0.5 kgf firmness which cultured one of the seven abundant morphotypes were selected for this analysis. Eliminating extremely soft avocados excluded most opportunistic infections or contamination. Bruising and Body Rots were also included in the browning symptom analysis because non-SER mesocarp discolouration may have an effect on fruit susceptibility to pathogen attack.

The most prevalent and fastest growing fungal morphotype, *Diaporthe*, was expected to correlate with high SER incidence and disease severity. Figures 6.18A and 6.18B show a high correlation of *Diaporthe* with Vascular Discolouration and Mycelial cavity formation for both intake avocados, while both slime morphotypes correlate with no symptoms. Figures 6.19A and 6.19B show that mostly the morphotypes which produce hyphae are cultured from avocados with mycelial cavities, the hyphae-filled spaces described in Figure 2.1. Although WhiteSlime was never associated with a mycelial cavity, YellowSlime sometimes produced them, suggesting that it may be a by-product of a hyphal morphotype. The fact that the PCA correlations and proportions of symptoms produced by each morphotype was similar for both stored and intake avocados suggest that the proportion of pathogens, based on isolation and morphotype identification, does not change during simulated marketing periods. If the avocados were colonised by additional pathogens during retail storage, the fungal morphotype communities between Figures 6.18B and 6.19B would be different.

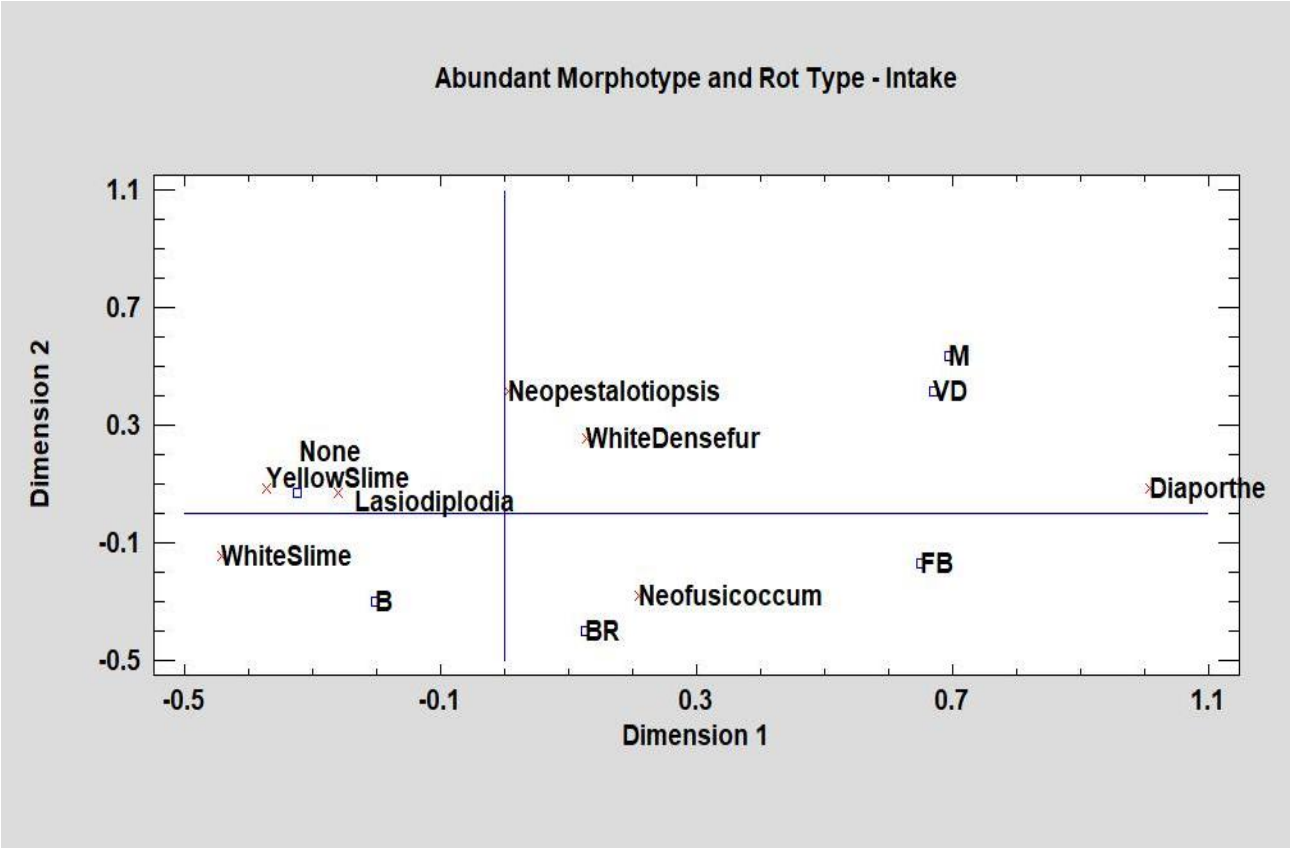

Figure 6.18A: CA examining abundant morphotype category with type of mesocarp discolouration. Dimension 1 separates mesocarpal browning symptom and Dimension 2 separates morphotype identity. None = no symptoms, B = Bruise, BR = Body Rot, FB = Fungal Browning, VD = Vascular Discolouration, and M = Mycelial cavity. The avocado mesocarp symptoms are described in Figure 2.1. N = 566

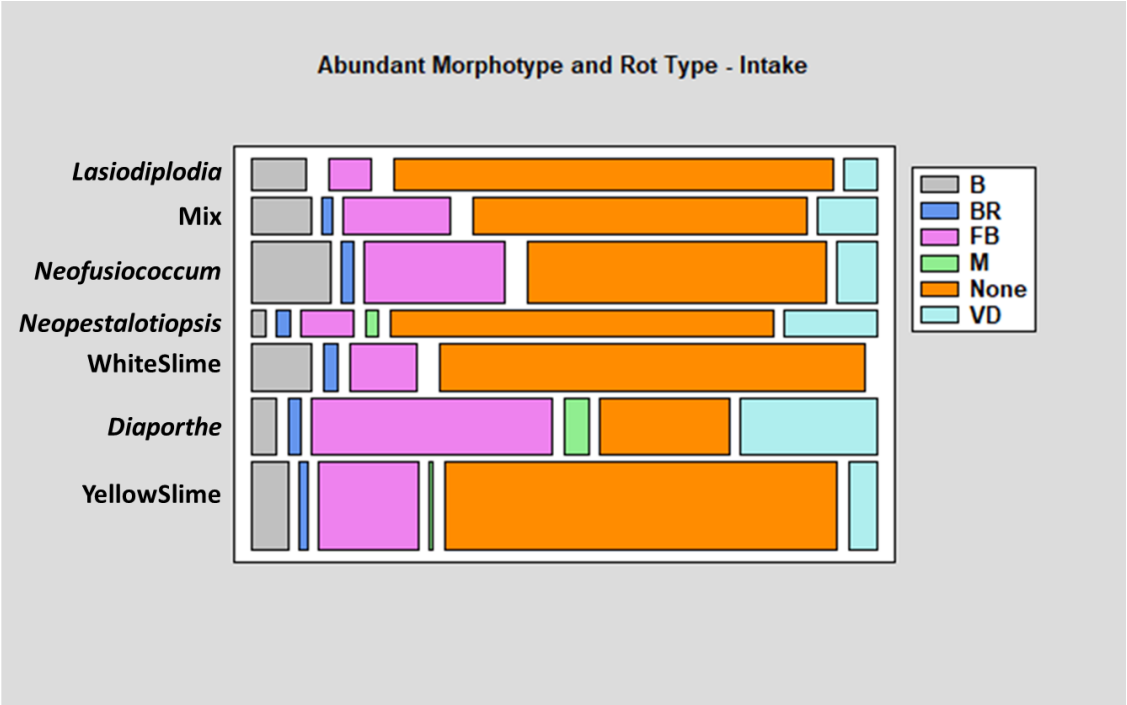

Figure 6.18B: Proportion of browning symptoms associated with each abundant morphotype cultured at intake. *Lasiodiplodia* N = 53, the *Pestalotiopsis*, *Colletotrichum*, *Fusarium* Complex (Mix) N = 58, *Neofusicoccum* N = 101, *Neopestalotiopsis* N = 43, WhiteSlime N = 76, *Diaporthe* N = 92, YellowSlime N = 143. Browning symptoms are defined as Bruising (B), Body Rot (BR), Fungal Browning (FB), Mycelial Cavities (M), and Vascular Discoloration (VD) as illustrated in Figure 2.1. The size of the boxes indicates relative abundance. FB, M, and VD are SER related browning, whilst B and BR are not fungal related.

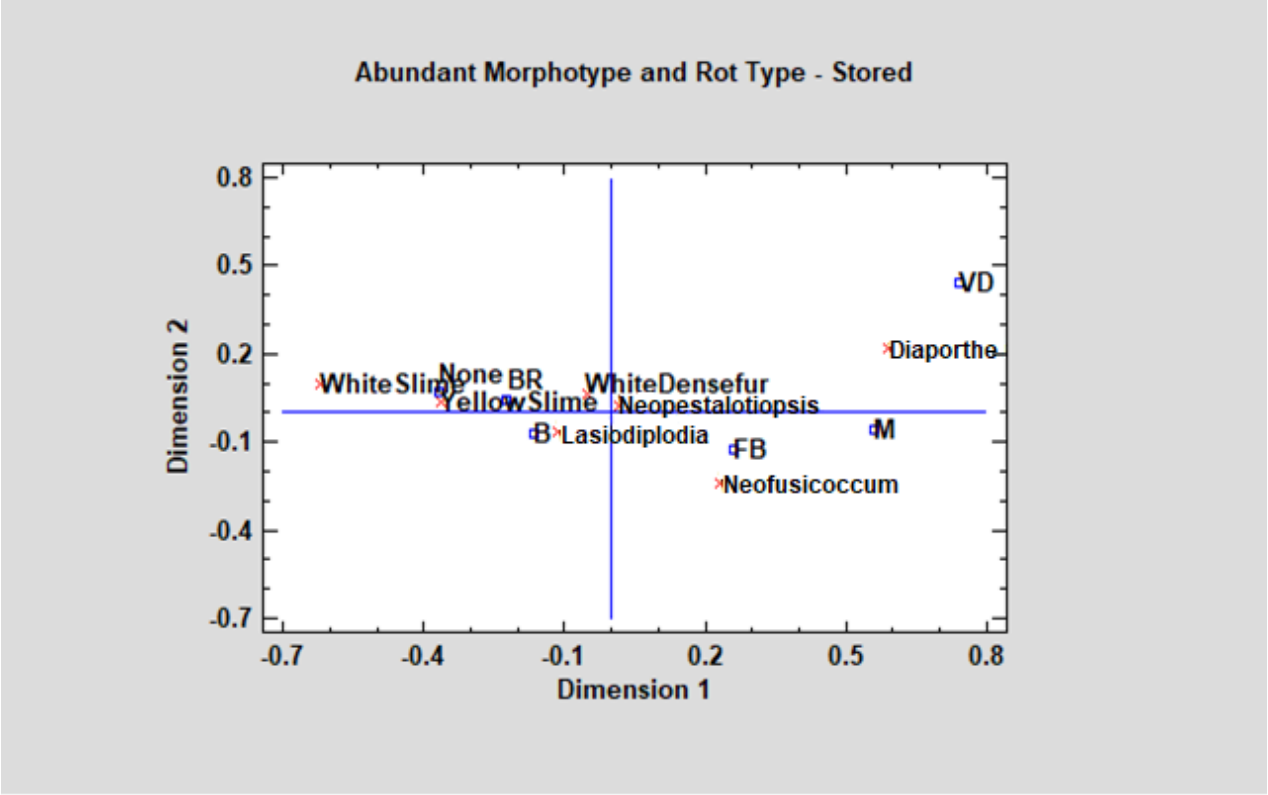

Figure 6.19A: CA of abundant morphotype with type of mesocarp discolouration. Dimension 1 separates the types of mesocarpal browning and Dimension 2 separates fungal morphotype. None = no symptoms, B = Bruise, BR = Body Rot, FB = Fungal Browning, VD = Vascular Discolouration, and M = Mycelial cavity. N = 555

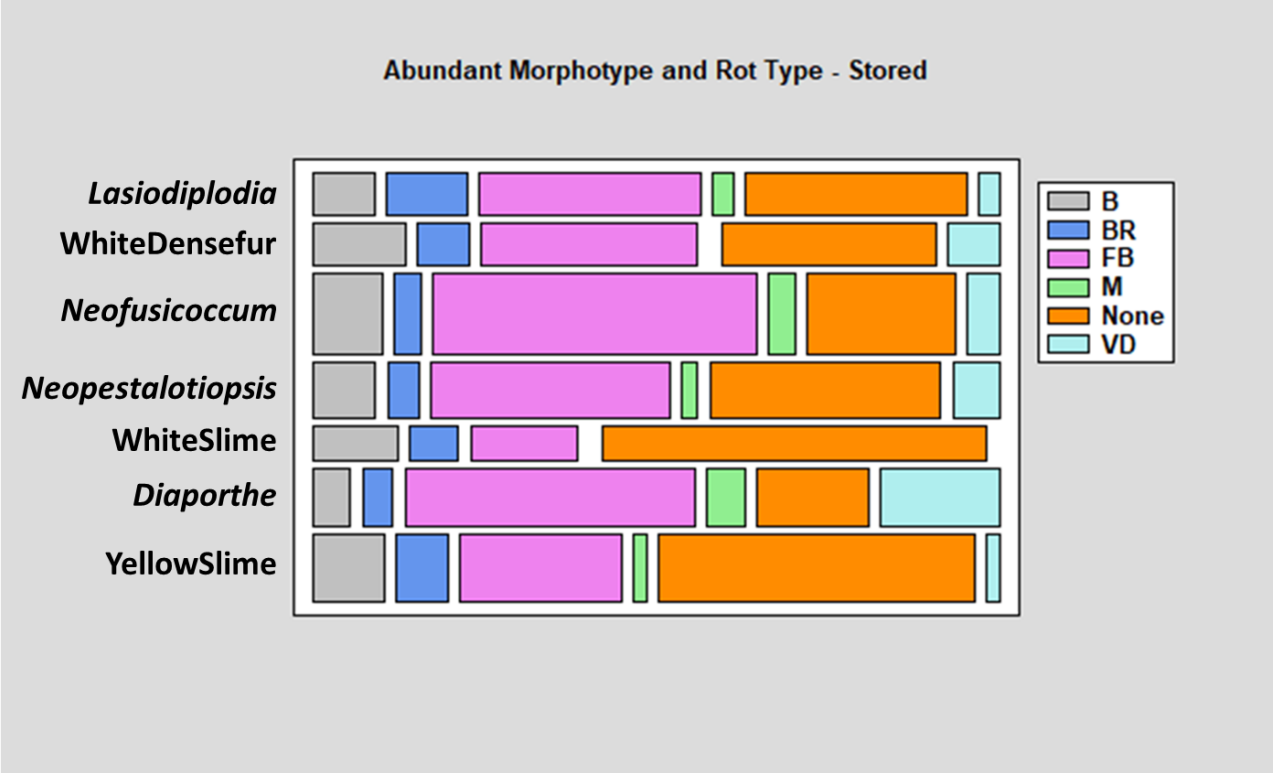

Figure 6.19B: Proportion of rot symptoms associated with each abundant morphotype. *Lasiodiplodia* N = 62, the *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex N = 61, *Neofusicoccum* N = 118, *Neopestalotiopsis* N = 81, WhiteSlime N = 52, *Diaporthe* N = 84, YellowSlime N = 97. Browning symptoms are defined as Bruising (B), Body Rot (BR), Fungal Browning (FB), Mycelial Cavities (M), and Vascular Discoloration (VD) as illustrated in Figure 2.1. The size of the boxes indicates relative abundance.

To explore whether the seven most abundant morphotypes colonise avocados with specific physiological factors, the mesocarp firmness, dry mass, and total fresh weight were examined for each avocado population which cultured a specific morphotype. Figures 6.20 A - G show no clear pattern of pathogen preference. *Neopestalotiopsis* appears to be able to colonise larger and firmer fruit, but this is only shown in a few outliers. Overall, all fungal morphotypes successfully infected fruit within the same range of firmness and dry mass.

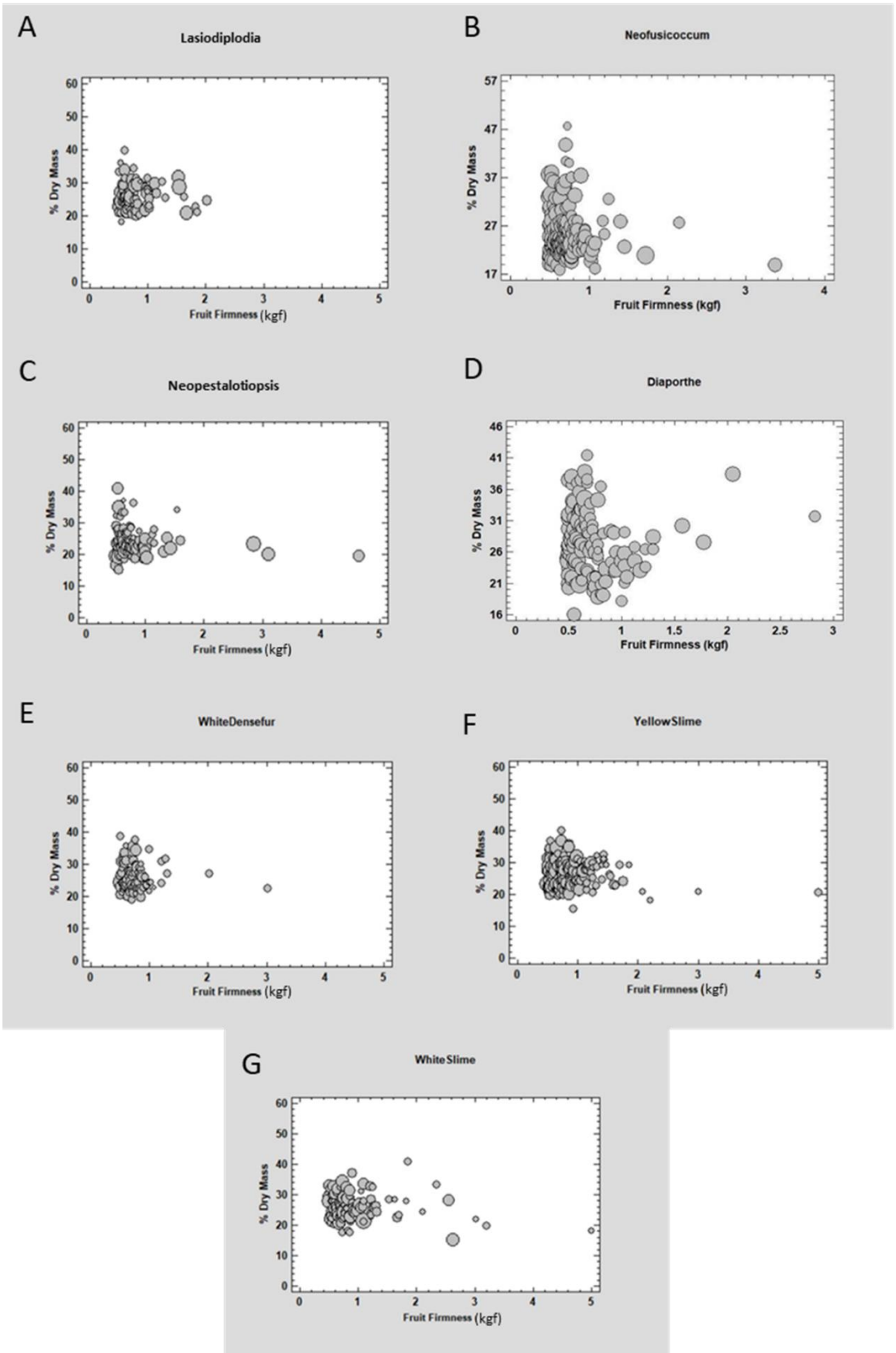

Figure 6.20: Physiological factors of all avocados above 0.5 kgf firmness which cultured (A) *Lasiodiplodia*, (B) *Neofusicoccum*, (C) *Neopestalotiopsis*, (D) *Diaporthe*, (E) *Pestalotiopsis/Colletotrichum/Fusarium* Mix, (F) YellowSlime, and (G) WhiteSlime. The size of the circles represents avocado fresh weight in grams. N = 115, N = 119, N = 219, N = 124, N = 128, N = 176, N = 240.

Over the course of the SCD aggregation, an interesting pattern emerged where some avocados with no mesocarpal browning symptoms would culture a fungus on the MEA plate (Asymptomatic Cultures) and other avocados with browning symptoms would not culture a pathogen (Symptomatic Blanks). These two groups were separated into enriched datasets to assess fruit resistance and pathogen activity. Figure 6.21 illustrates that asymptomatic avocados tend to be firmer and have a slightly higher dry mass percentage when compared to the physiology of avocados which cultured the seven most abundant morphotypes in Figures 6.20 A-G, but this could be due to the Asymptomatic Cultures comprising a larger dataset, and therefore more variation within the samples. The physiology of Symptomatic Blanks appears to have produced the same pattern as Asymptomatic Cultures (Figure 6.22), with a wide range of dry mass percentage and mesocarp firmness skewed toward higher values. This result suggests that there is no clear physiological pattern for infected avocados which resist symptom development or diseased avocados which don't culture a pathogen. Figure 6.23 illustrates that most of the asymptomatic avocados cultured one of the seven most abundant morphotypes, implying that the known SER pathogens were the cause of browning, but they sometimes do not establish themselves under *in vitro* conditions. Figure 6.24 also shows that many of the Symptomatic Blanks did not suffer from severe browning, so it is possible that there was not enough pathogen within the avocado mesocarp core sample to establish the culture *in vitro*.

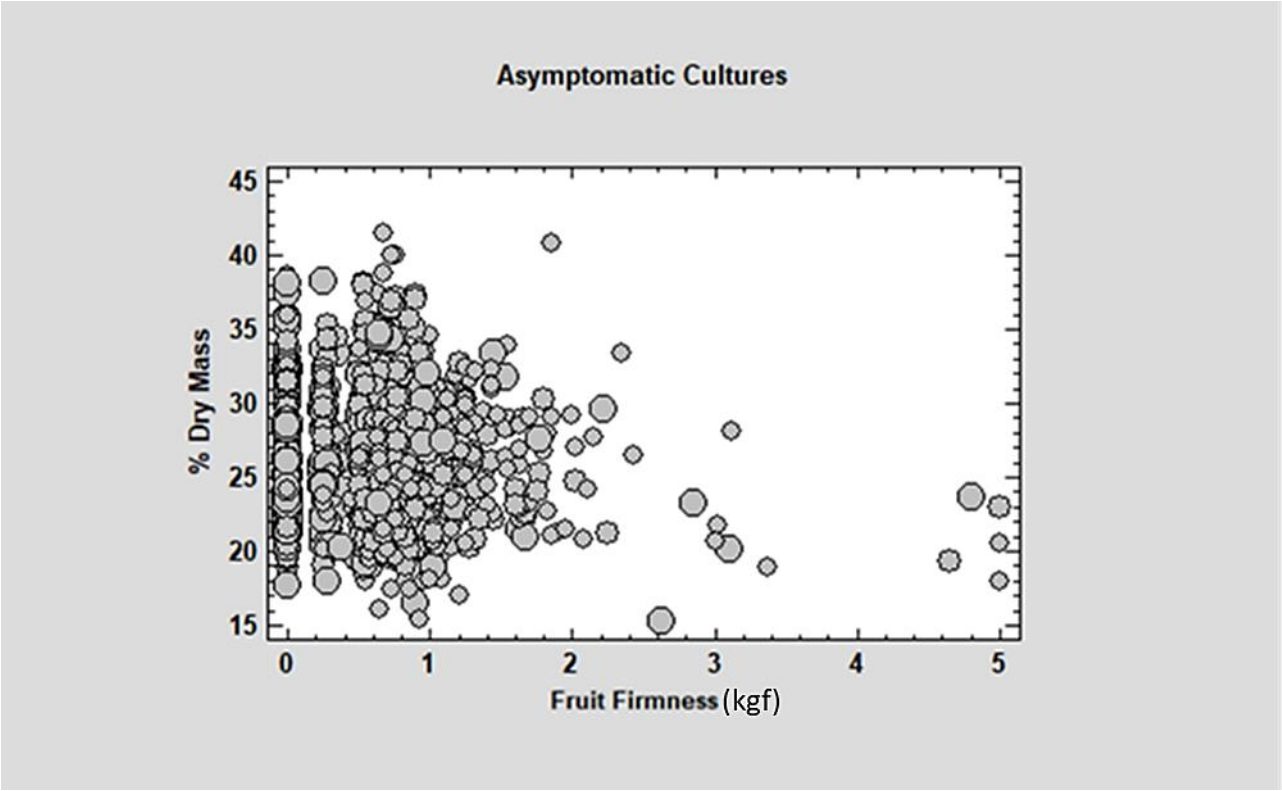

Figure 6.21: Physiological factors of avocados which cultured a fungus from the stem core but did not show any SER browning symptoms. Avocado firmness and percent dry mass are mapped to the x any y axis, respectively. Circle size represents relative fresh weight in grams. N = 1452

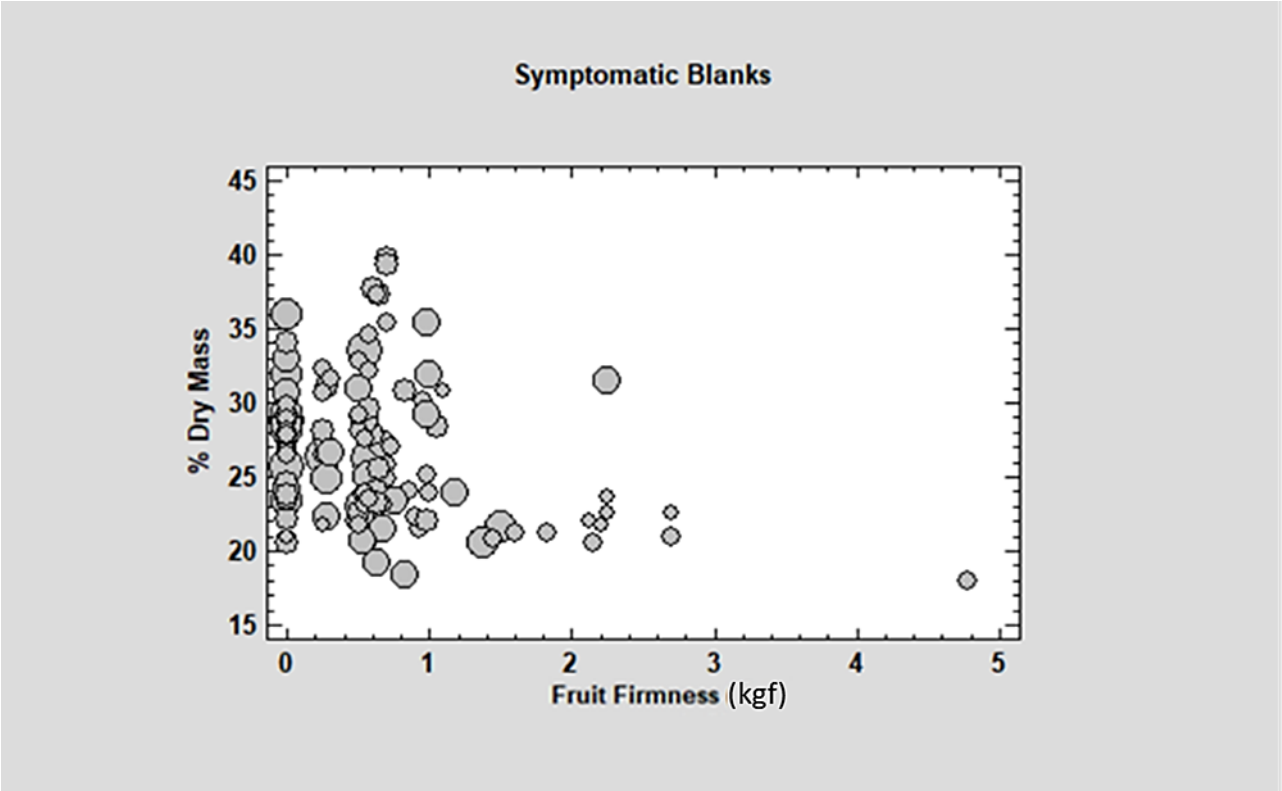

Figure 6.22: Physiological factors of avocados which exhibited SER browning symptoms but did not culture a fungus on MEA. Avocado firmness and percent dry mass are mapped to the x any y axis, respectively. Circle size represents relative fresh weight in grams. N = 158

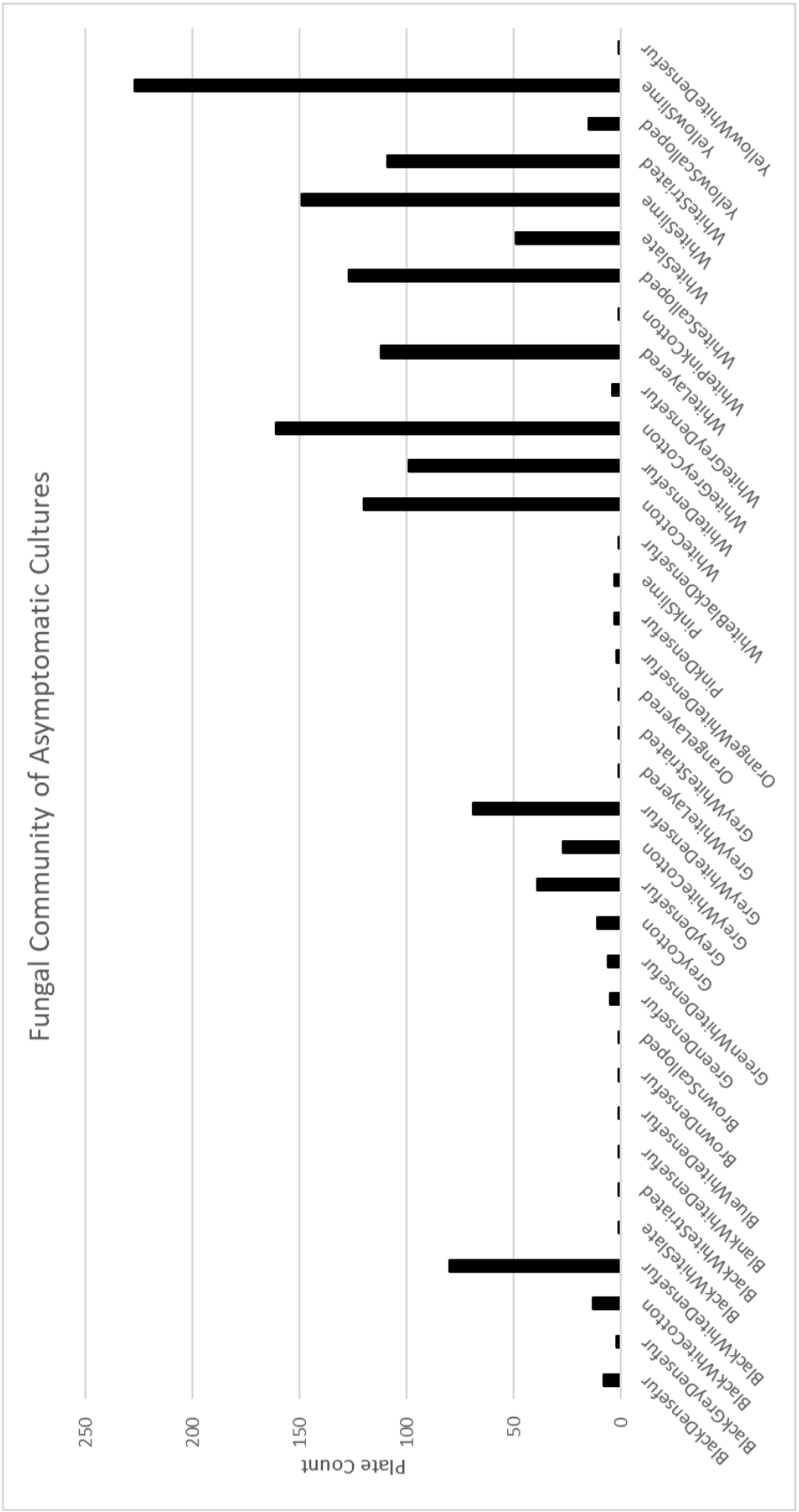

Figure 6.23: Total fungal community breakdown of all asymptomatic avocados which cultured a morphotype on MEA. N = 1452

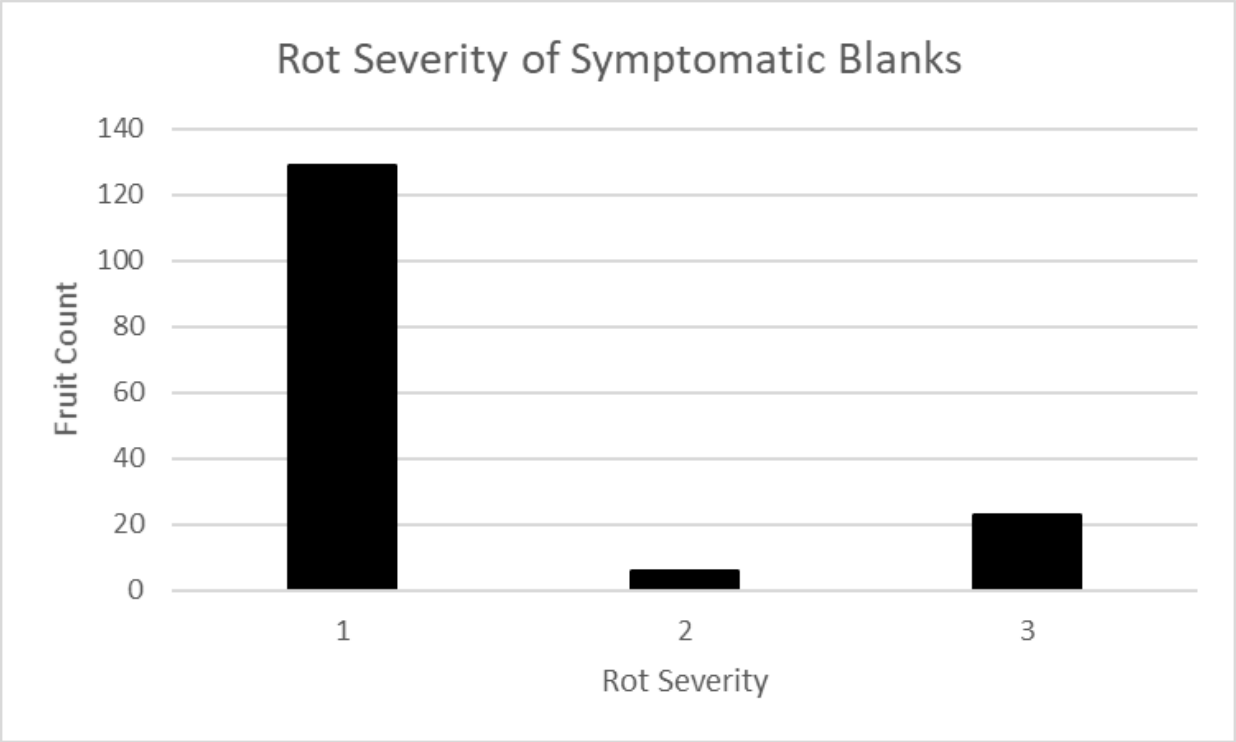

Figure 6.24: Severity of rot symptoms for avocados which showed SER related browning but did not culture a fungal morphotype on MEA. A score of 1 indicates 1 - 33% rot, a score of 2 indicates 34 - 66% rot, and a score of 3 indicates 67 - 100% mesocarp browning. N = 158

6.3.4 Fungal Communities and Physiological Factors

Fungal communities associated with specific avocado physiological factors were assessed by examining only the seven most abundant morphotype communities cultured from avocados above 0.5 kgf firmness. The presence of a stem button was expected to protect avocados against invasion by a SER pathogen, because it creates a natural barrier from airborne spores. Although presence or absence of a stem button had statistically significant effect on pathogen culture (X^2 (6, N = 1121) = 119.968, p = <.01), the correspondence analysis shown in Figure 6.25 demonstrates a correlation between the presence of a stem button and producing a *Neofusicoccum* culture.

Figures 6.26, 6.27, and 6.28 show that dry mass percentage, fruit fresh weight, and fruit firmness had little influence on the fungal morphotype cultured. It is possible that *Diaporthe* can infect avocadoes with a range of dry mass content, or the slime phenotypes prefer firmer avocados, but these fluctuations are most likely due to a small sample set. Other morphotypes, such as the slimes, *Lasiodiplodia*, and the *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex were more commonly associated with the absence of a stem button, whilst *Neopestalotiopsis* and *Diaporthe* appear to be unaffected. This suggests that some avocado pathogens may be endophytic, and others may infect the newly exposed avocado abscission zone during harvest. It is also possible that *Diaporthe* and *Neopestalotiopsis* are more aggressive pathogens that can invade secondary cell wall thickened structures such as stem buttons, whereas the *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex, YellowSlime, and WhiteSlime may be more opportunist and require damaged tissue to germinate and penetrate tissues.

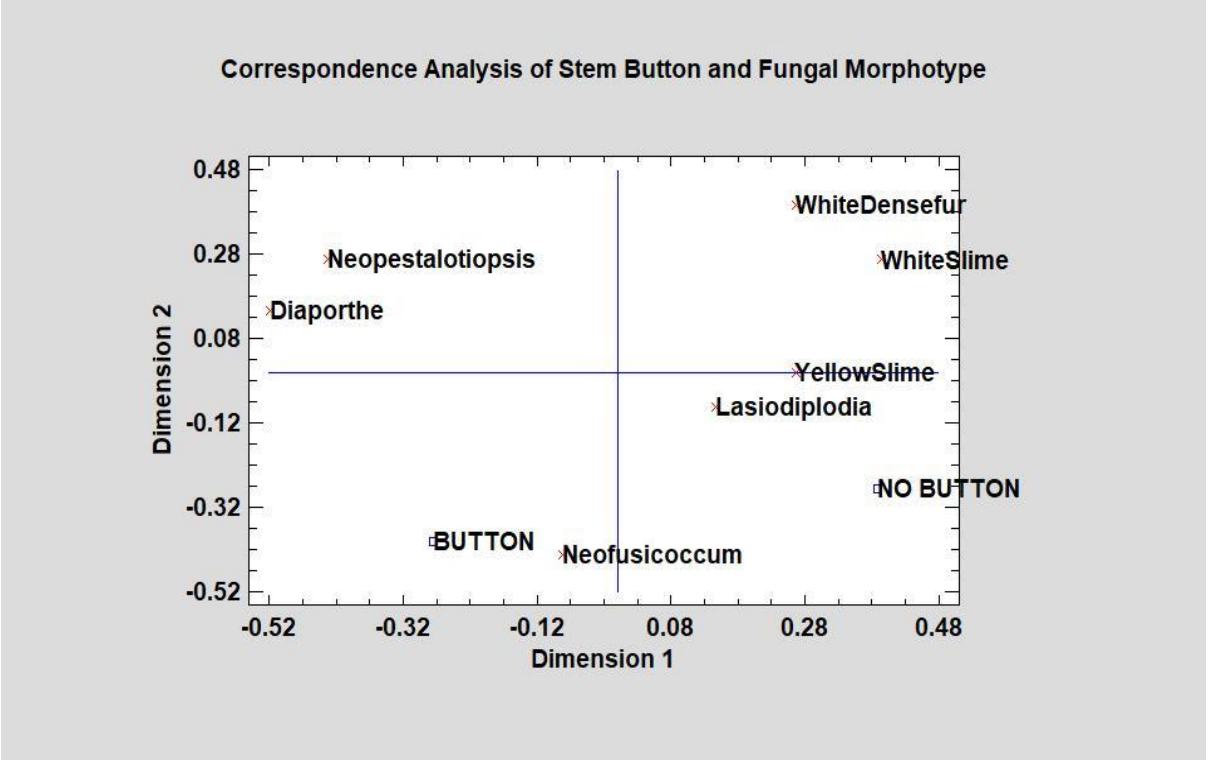

Figure 6.25: Canonical Correspondence analysis of most abundant morphotypes and the presence or absence of a stem button. Dimension 1 separates button status and Dimension 2 separates fungal morphotype identity. N = 1121

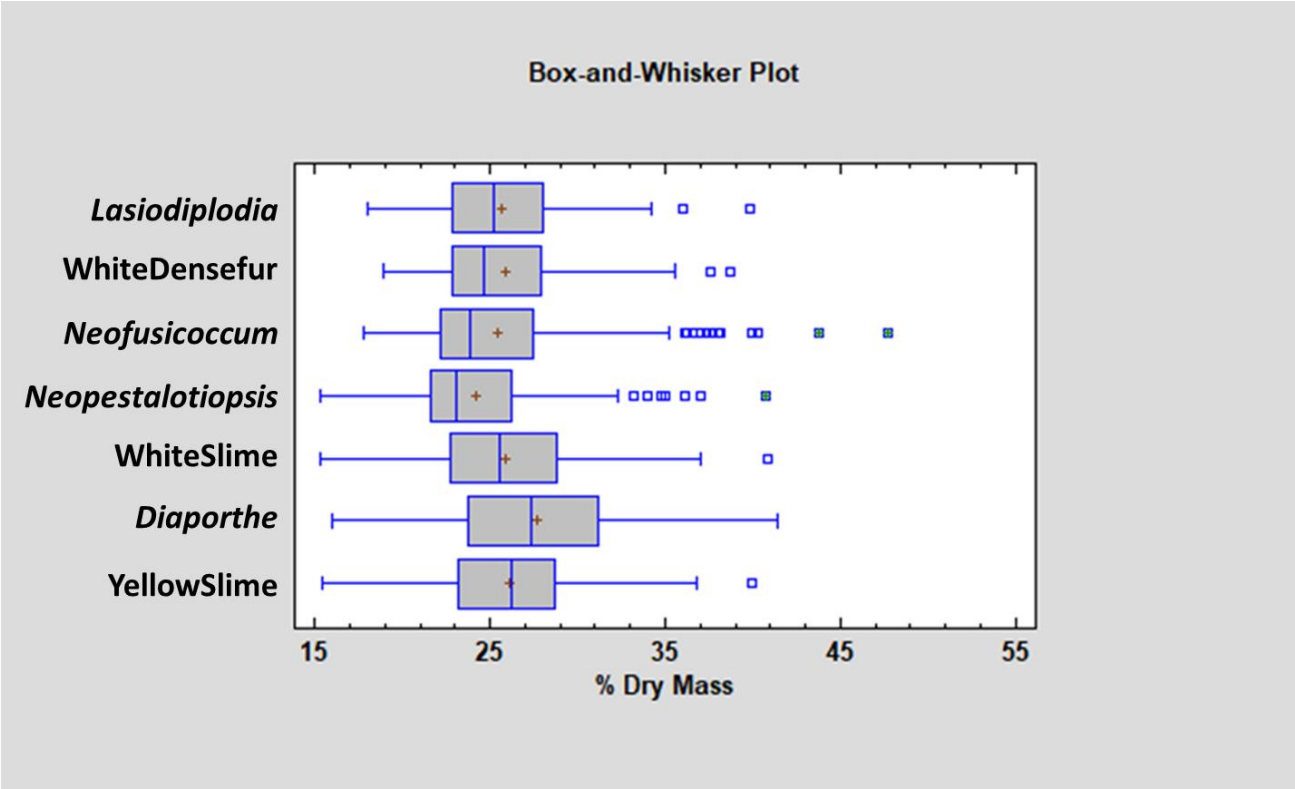

Figure 6.26: Distribution of % dry mass for avocados culturing each of the most abundant morphotypes. Mean is expressed as a red cross, the horizontal lines define the maximum and minimum, the box represents the lower and upper bounds of the lower and upper quartiles respectively, the median is designated by a vertical line, and the blue squares are outliers. *Lasiodiplodia* N = 115, *Pestalotiopsis*/*Colletotrichum*/*Fusarium* N = 119, *Neofusicoccum* N = 219, *Neopestalotiopsis* N = 124, WhiteSlime N = 128, *Diaporthe* N = 176, YellowSlime N = 240

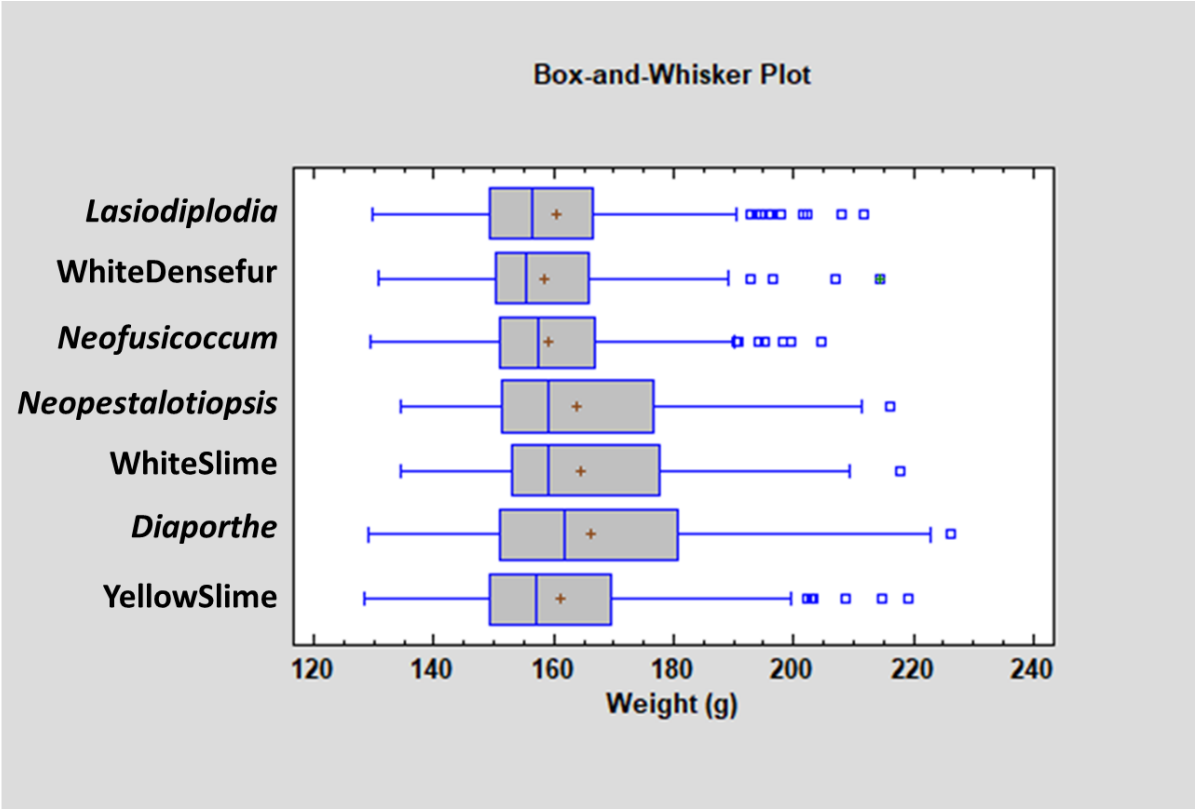

Figure 6.27: Distribution of fresh weight for avocados culturing each of the most abundant morphotypes. Mean is expressed as a red cross, the horizontal lines define the maximum and minimum, the box represents the lower and upper bounds of the lower and upper quartiles respectively, the median is designated by a vertical line, and the blue squares are outliers. *Lasiodiplodia* N = 115, *Pestalotiopsis*/*Colletotrichum*/*Fusarium* N = 119, *Neofusicoccum* N = 219, *Neopestalotiopsis* N = 124, WhiteSlime N = 128, *Diaporthe* N = 176, YellowSlime N = 240

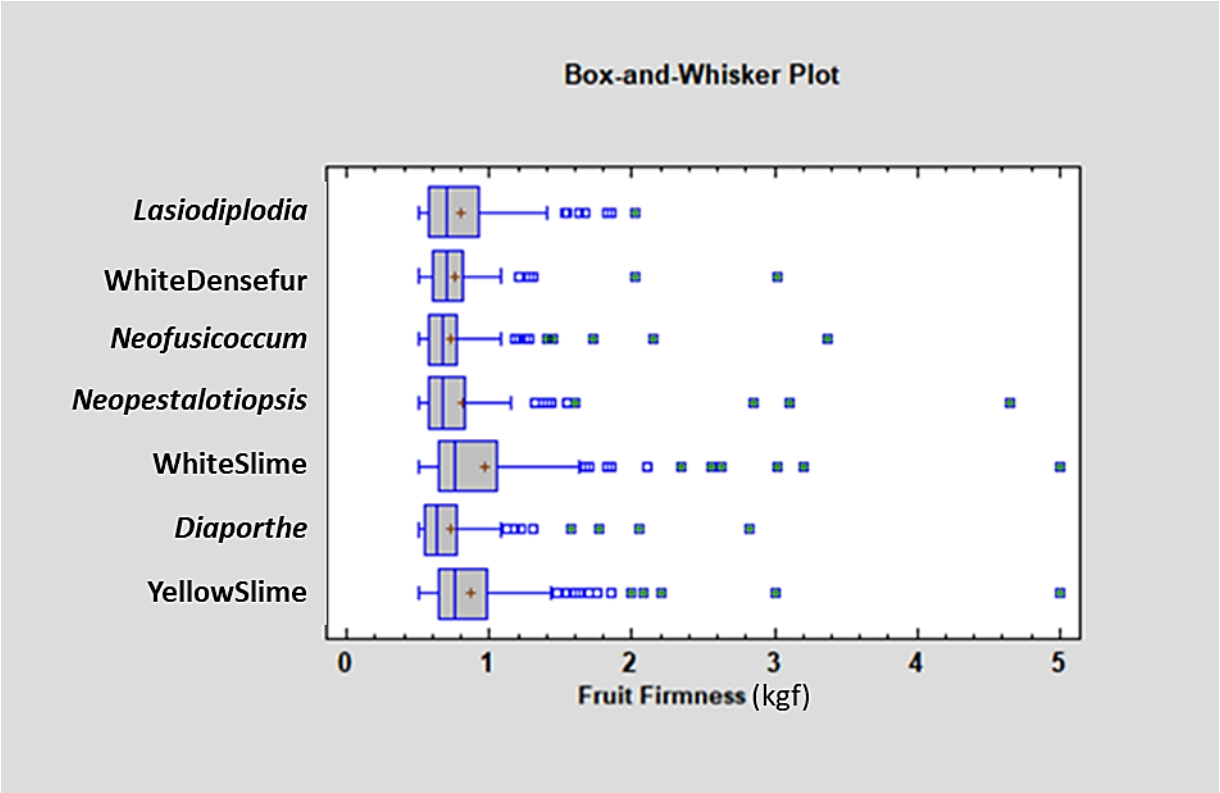

Figure 6.28: Distribution of mesocarp firmness of avocados culturing each of the most abundant morphotypes. Mean is expressed as a red cross, the horizontal lines define the maximum and minimum, the box represents the lower and upper bounds of the lower and upper quartiles respectively, the median is designated by a vertical line, and the blue squares are outliers. *Lasiodiplodia* N = 115, *Pestalotiopsis*/*Colletotrichum*/*Fusarium* N = 119, *Neofusicoccum* N = 219, *Neopestalotiopsis* N = 124, WhiteSlime N = 128, *Diaporthe* N = 176, YellowSlime N = 240

To assess whether the fungal community changes as avocados ripen, the mesocarp firmness and pathogen community breakdown of Intake and Stored avocados above 0.5 kgf firmness were studied independently. Figures 6.29 and 6.30 show that all seven abundant morphotypes have the ability to infect avocados at any firmness. The exact ratios of the total fungal communities are explored in Figure 6.31 for Intake avocados and Figure 6.32 for Stored avocados. A higher diversity of fungal morphotypes was cultured from Stored avocados (Figure 6.32) compared to Intake avocados (Figure 6.31), suggesting that the older, softer fruits attract pathogens more readily. This conclusion is supported in Figure 6.33, where softer fruit was more likely to culture multiple fungal morphotypes on the plate.

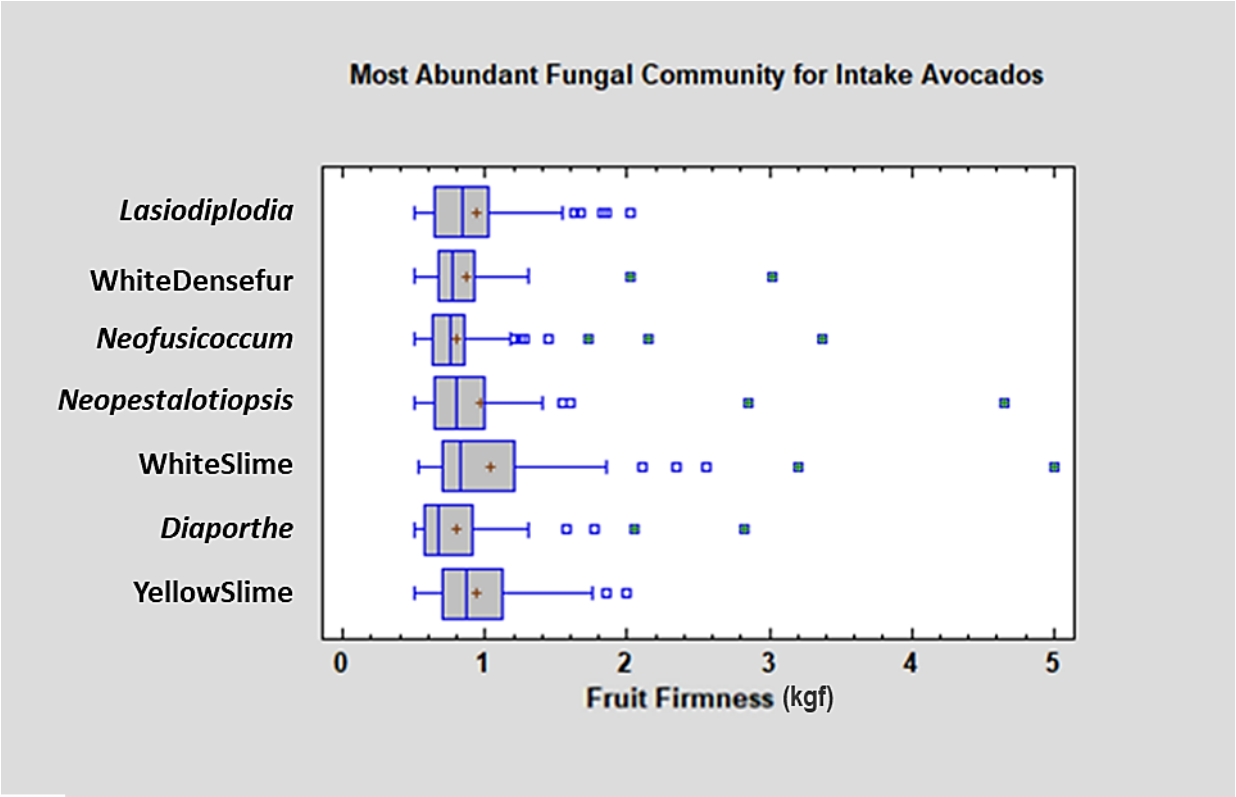

Figure 6.29: Distribution of mesocarp firmness of Intake avocados culturing each of the most abundant morphotypes. Mean is expressed as a red cross, the horizontal lines define the maximum and minimum, the box represents the lower and upper bounds of the lower and upper quartiles respectively, the median is designated by a vertical line, and the blue squares are outliers. *Lasiodiplodia* N = 53, *Pestalotiopsis*/*Colletotrichum*/*Fusarium* N = 58, *Neofusicoccum* N = 101, *Neopestalotiopsis* N = 43, WhiteSlime N = 76, *Diaporthe* N = 92, YellowSlime N = 143

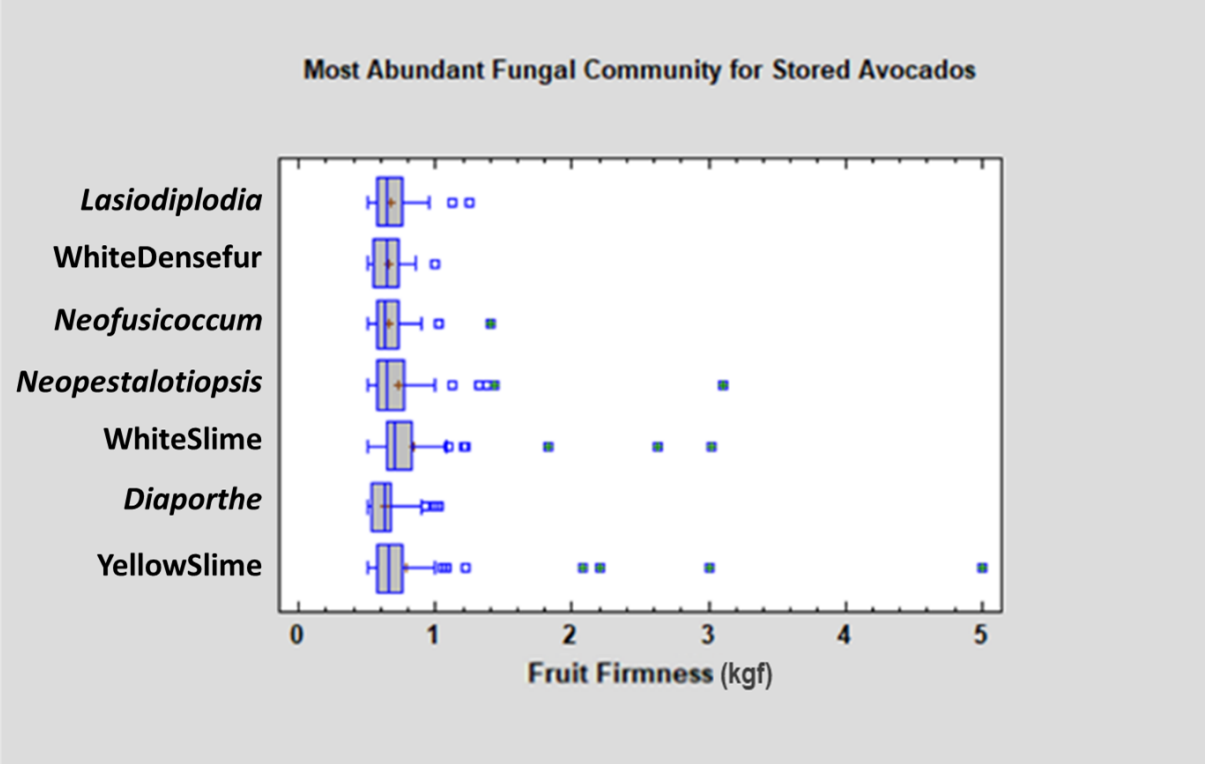

Figure 6.30: Distribution of mesocarp firmness of Stored avocados culturing each of the most abundant morphotypes. Mean is expressed as a red cross, the horizontal lines define the maximum and minimum, the box represents the lower and upper bounds of the lower and upper quartiles respectively, the median is designated by a vertical line, and the blue squares are outliers. *Lasiodiplodia* N = 62, *Pestalotiopsis*/*Colletotrichum*/*Fusarium* N = 61, *Neofusicoccum* N = 118, *Neopestalotiopsis* N = 81, WhiteSlime N = 52, *Diaporthe* N = 84, YellowSlime N = 97

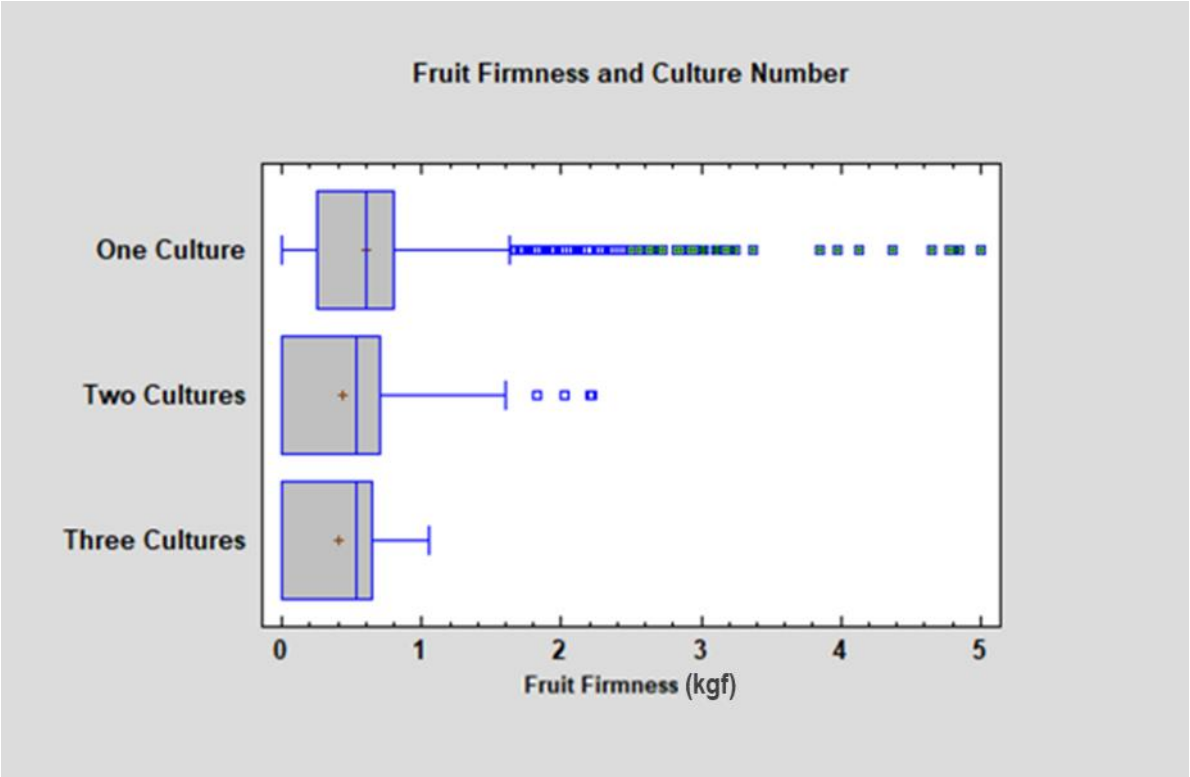

Figure 6.31: Distribution of mesocarp firmness (kgf) of avocados which cultured more than one pathogen *in vitro*. Mean is expressed as a red cross, the horizontal lines define the maximum and minimum, the box represents the lower and upper bounds of the lower and upper quartiles respectively, the median is designated by a vertical line, and the blue squares are outliers. One Culture N = 3429, Two Cultures N = 543, Three Cultures N = 50

6.3.5 Body Rot and Bruising Analysis

During the data accumulation for the SCD, other internal mesocarp disorders were recorded, if present, when the avocados were SER negative. Non-fungal mesocarp discolouration, such as bruising and internal chilling disorders, were recorded as a bruise. Anthracnose body rots, or fungal infections entering anywhere through the avocado skin, were recorded separately as body rots. Only avocados above 0.5 kgf firmness were included in this study, to eliminate opportunistic infections, but this also excluded many bruised avocados, as softer avocados are more prone to bruising. Figures 6.34 and 6.35 explore the physiology of bruised and body rot avocados. Interestingly, the body rot avocados tended to be softer, with few outliers above 1 kgf firmness. This suggests that avocados of any firmness can develop a bruise, likely due to mishandling along the supply chain. The bruised avocados also exhibited a wider range of dry mass, but similar size distribution as body rot avocados. The body rot avocados were expected to tend toward higher fresh weights, because a larger skin surface area would increase the probability of infection from airborne fungal spores, but Figure 6.35 indicates that relatively small avocados can also frequently be affected by body rots.

Since the avocados included in the bruise and body rot analysis were not positive for SER by definition, the fungal communities explored in Figures 6.36 and 6.37 are subsets of the asymptomatic cultures explored in Section 5.4. All avocados positive for SER were recorded as a specific SER symptom type, regardless of additional bruising or body rot. Both bruised and body rot avocados were equally prone to carry latent pathogens and the community breakdown for each was almost identical. Both groups cultured all seven abundant phenotypes as well as many of the same lesser morphotypes. Compared to the overall asymptomatic culture analysis in Section 6.3.4, it appears that bruising or body rots do not have an effect on the type of latent pathogen

an avocado could potentially carry. The Asymptomatic Culture breakdown showed a higher inclination toward culturing the seven most abundant phenotypes, whilst bruised and body rot avocados cultured a more even number of each morphotype.

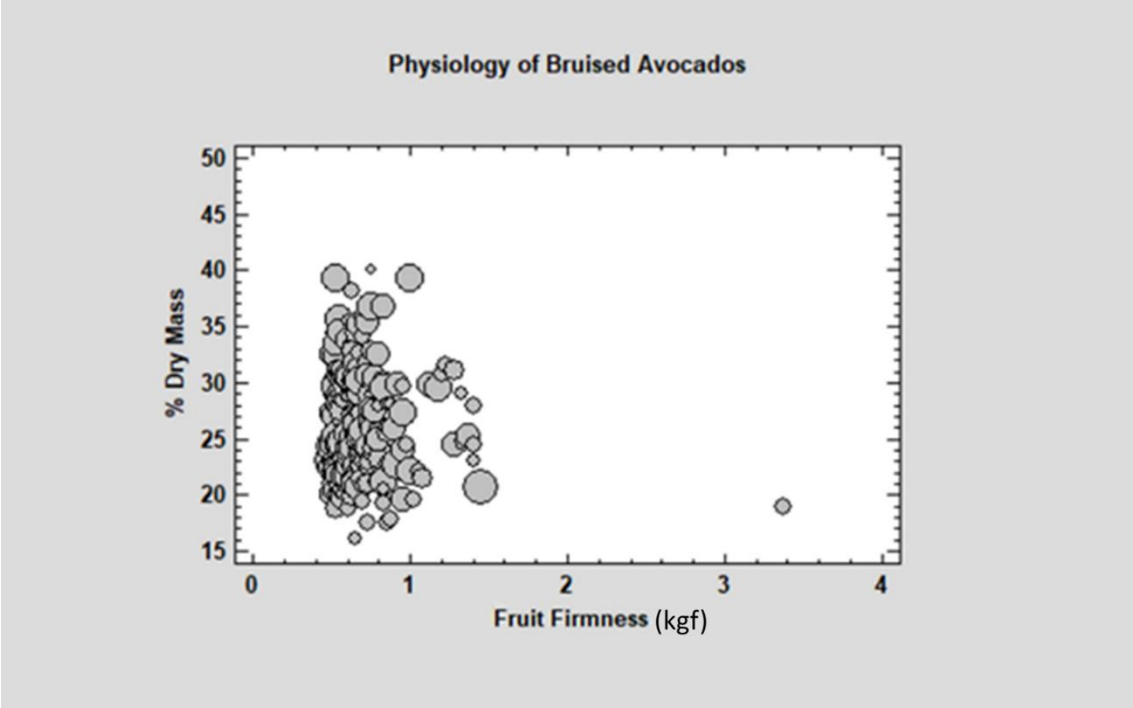

Figure 6.32: Physiological properties of avocados with any non-SER related mesocarpal discolouration, such as bruising or internal chilling. Circle size represents total fresh weight in grams. N = 271

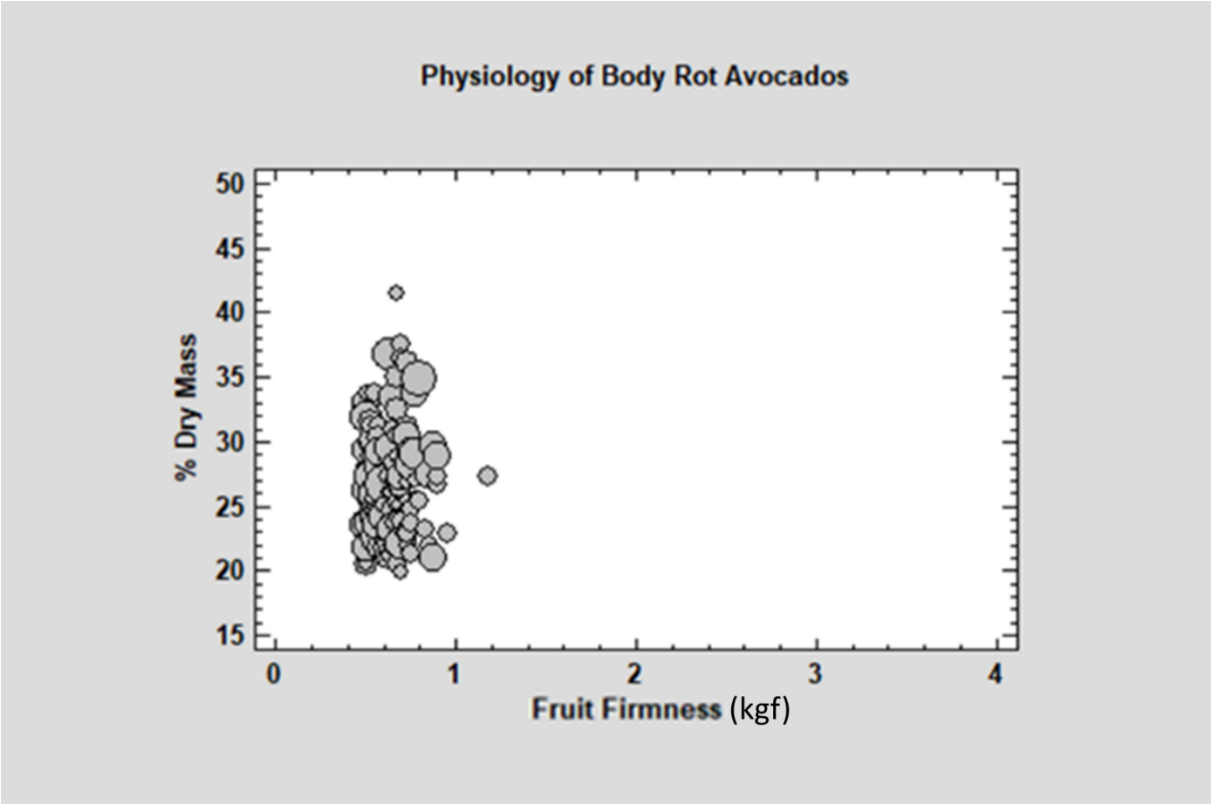

Figure 6.33: Physiological properties of avocados without SER symptoms and positive for Anthracnose body rot. N = 146

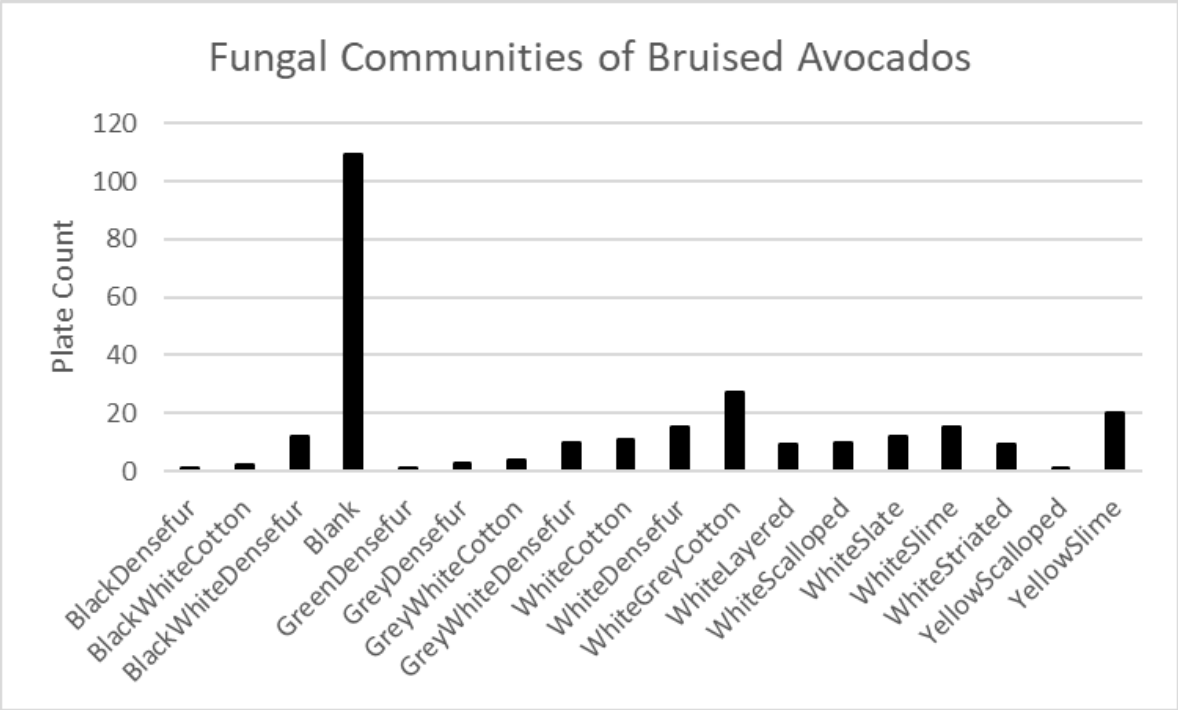

Figure 6.34: Fungal community breakdown of bruised avocados. Bruised avocados are defined as avocados with any non-SER related mesocarpal browning. N = 271

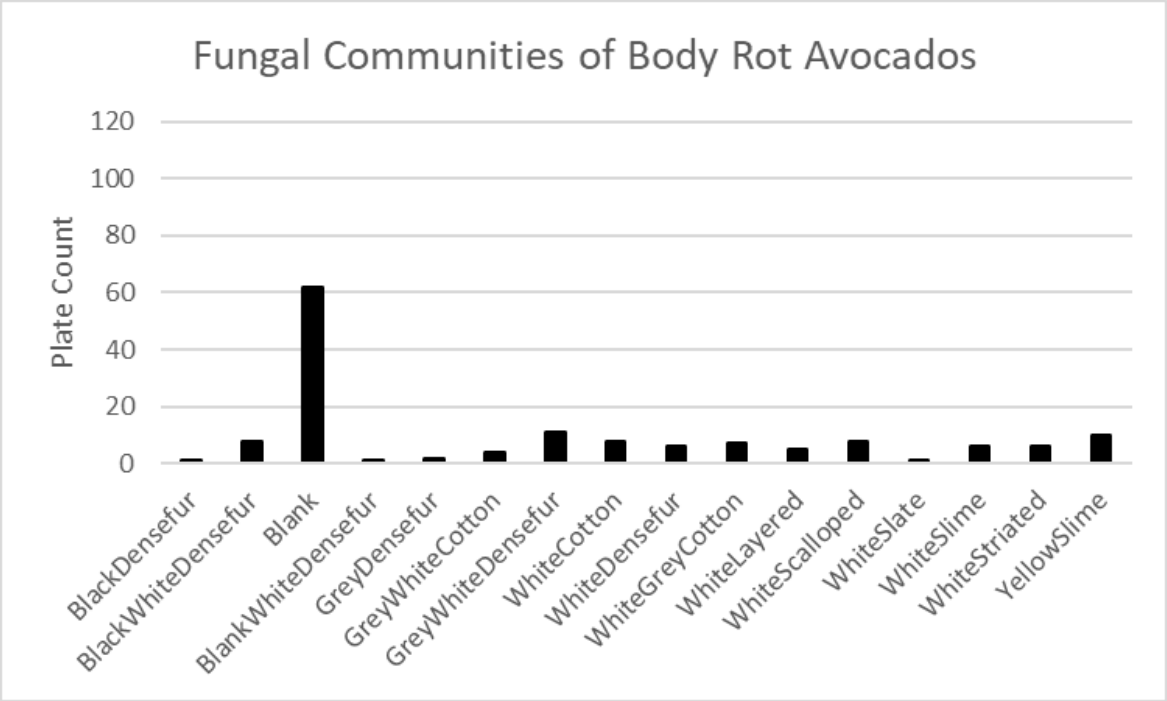

Figure 6.35: Fungal community breakdown of Anthracnose body rot avocados. These avocados did not have SER symptoms but were positive for a fungal infection through the skin. N = 146

6.4 Discussion

The exploration of avocado physiological factors affecting SER incidence generated some unexpected results. Since this project represents one of the first attempts to study SER susceptibility using avocado samples at the end of the supply chain, it provides an interesting comparison to many of the orchard studies which examined fruit shortly after harvest. For example, avocado fresh weight and dry mass were found to have no effect on disease incidence, although the literature indicates an increase in dry mass and oil content reduces SER incidence (Hartill & Everett, 2002; Hopkirk, 1994; Peterson, 1978; Schaffer et al., 2013). This is due to how the developmental maturity of the fruit affects ripening heterogeneity. However, all avocados intended for retail sale are already selected for a specific dry mass, fresh weight, and oil content. The market does not tolerate excessive deviation from set parameters. This explains why the dry mass and fresh weight were similar for all avocados in this assessment. A higher dry mass of freshly harvested fruit might also reflect a more accurate measure of fruit oil content and its effect on SER. At the distribution end, dry mass will be skewed by the amount of moisture loss that has occurred along the supply chain, and the bioactivity of antifungal oils may decline during storage. Without a sufficiently differentiated experimental group, this study was not adequate to conclusively determine the effects of these two physiological factors. It was, however, in a unique position to assess the significance of age and fruit softening.

Fruit firmness is known to be one of the most influential factors affecting rot incidence and severity overall, because SER is an age-related disorder (Hernández et al., 2016). It is possible that the other physiological factors affect SER incidence, but this study failed to show their contribution. However, the association of SER incidence and increased severity with the presence of a button was unexpected, as previous research indicated that a longer stem button delays the development of SER symptoms

(Madhupani & Adikaram, 2017). Other morphotypes, such as the slimes, *Lasiodiplodia*, and the *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex were more commonly associated with the absence of a stem button, whilst *Neopestalotiopsis* and *Diaporthe* appear to be unaffected. This suggests that some avocado pathogens may be endophytic, and others may infect the newly exposed avocado abscission zone during harvest. It is also possible that *Diaporthe* and *Neopestalotiopsis* are more virulent, and unperturbed by physical boundaries, whereas the *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex, YellowSlime, and WhiteSlime may be more opportunist and require the presence of damaged tissue to enter fruit.

The fact that increased dry mass was associated with higher rot severity was also contradicted in the literature. Older, more mature avocados tend to accumulate more dry mass and oil content, and higher dry mass was associated with more even ripening and less SER development in previous studies (Hopkirk, 1994). Perhaps the slight increase in dry mass due to water evaporation is not enough to prevent rot development of fruit softening beyond the point of consumer purchase. This illustrates how examination of fruit at the end of the supply chain reveals patterns not apparent in newly harvested fruit. Dry mass is one of the most important industry standards in assessing avocado quality, but this study demonstrated that total dry mass is not a great predictor of SER infection susceptibility. However, this could also be due to experimental error in measuring dry mass.

Mexican avocados share the longest evolutionary history with the causal pathogens and an ideal climate for fungal development, but subtropical South Africa also has a similar environment to Mexico. The data revealed South Africa as the most prone to SER incidence for this particular sampling. Rot incidence per country may be more influenced by natural fluctuations in pathogen populations within the countries,

local orchard management practices, and local pathogen survival on plant material. More study will be needed to assess the impact of factors in the orchard compared to evidence available in an outturn analysis such as this. The fact that Israel experienced the least rot could be indicative of its Mediterranean climate, which is less ideal for the fungal pathogen growth of the species studied. However, another factor could be that the orchards are younger and have had less opportunity to accumulate pathogens.

Morphotype patterns explored in Section 6.3.4 provide further evidence that WhiteSlime and YellowSlime are likely to be a reproductive form of *Diaporthe* (*Diaporthe*). Neither slime phenotype was correlated with disease symptom development, but *Diaporthe* was, and both slimes were the most cultured morphotypes from asymptomatic fruit. This suggests that another fungus, or another growth form, is causing the disease, and the slime form was cultured out of the fruit. It is also possible that latent pathogens could exist in a quiescent phase within the fruit and culturing on MEA breaks their dormancy. A similar phenomenon was discovered in *Botrytis* infection of lettuce, which was found to retain infectious material in asymptomatic tissue (Sowley et al., 2010). If many pathogens have this ability, it could explain the variety of the fungal community cultured from asymptomatic avocados.

Studies on how the presence or absence of a stem button affects SER disease incidence in avocado are still conflicted. However, an experiment by Galsurker et al. (2020) found that harvesting mangos with a short stem significantly reduced SER incidence. The SER causal pathogens of mango are well established in the literature as endophytes (Karunanayake & Adikaram, 2020), so it would follow that removing more of the infected tissue would reduce rot, either by reducing pathogen load or removing the pathogen before it has a chance to invade the ripening fruit. Like avocado, SER symptoms on mango appear once the fruit softens during the ripening process.

However, it has not been conclusively established yet whether the SER causal pathogens of avocado are endophytes or externally dispersed pathogens, and therefore removing the stem button may or may not have an effect. In general, the Galsurker et al. (2020) study suggested that removing the stem end button would have the greatest impact on disease incidence if the causal species were endophytes.

According to Zhang (2014), *Citrus* fruit are infected with SER causal pathogens, most often *Lasiodiplodia* species, inside the necrotic tissues of the fruit button, such as the desiccated calex and disk. The latent pathogen then invades the abscission zone after harvest. Removing the decayed stem button tissue from *Citrus* fruit before the pathogen has a chance to invade the newly formed abscission wound may reduce stem end rots. An older study by Brown (1986) found removing the stem button from oranges significantly reduced SER incidence, but the results were also dependent on the concentration of ethylene used to ripen the fruit.

Banana crown rot is another economically devastating postharvest disorder caused by many of the same fungal generalists decaying other commercial fruit, primarily *Fusarium* (Kamel et al., 2015). It is well established that the banana flower remnant is a key source of fungal inocula, which infect during harvest, and removing this tissue prior to harvest is already an important step in the packaging process (De Lapeyre de Bellaire & Mourichon, 1997; Kamel et al., 2015).

Removing infected tissue and sources of inocula had the greatest effect on disease incidence for mango, *Citrus*, and banana because the location and dispersal strategy of the pathogen was well established. For endophytic fungi or pathogens located within the dried calyx, removal of the infected tissues would be expected to reduce rot. To the author's knowledge, no investigation has been conducted so far as to whether SER causal species are harboured within the calyx. Determining if avocado

SER pathogens are primarily endophytic would be an important step toward targeted mitigation.

For avocados, many of the physiological factors assessed during the supply chain analysis were found to have no effect on SER disease incidence. Bruising and body rots within shipments did not impact fungal communities or SER incidence. Avocado is a climacteric fruit, which means its ripening corresponds to rising levels of ethylene within the fruit and it is sensitive to external sources of ethylene. As such, it was expected to respond to the ethylene produced by bruised tissue. Perhaps the slight increase in ripening speed had no effect on avocados which were not infected by a pathogen. The overall physiology of the avocado, as a combination of fresh weight, firmness, and dry mass also had little effect on the morphotype cultured. This could be due to all samples being standardised by this point in the supply chain, and the avocado pathogens are well adapted to infecting avocados within a wide range of physiological parameters. Overall, some fruit physiology factors were found to have an effect on rot incidence, but it is more likely that the presence of the pathogen, combined with ideal growing conditions, has a greater impact on infection and rot development.

CHAPTER 7: KOCH POSTULATE ANALYSIS

7.1 Introduction

Initially, this project explored what could be cultured out of commercial avocado fruit at the end of the supply chain. The abundant representation of several specific morphotypes generated new opportunities to explore the patterns associated with a select group of possible SER pathogens. Genetic identification (Chapter 4) confirmed these to be known SER causal pathogens or endophytes (Akgul et al., 2016; Darvas et al., 1987; Guarnaccia et al., 2016; Johnson et al., 1992; Madhupani et al., 2017; Montealegre et al., 2016; Shetty et al., 2016). However, all experiments conducted so far can only prove that these morphotypes can be cultured out of avocado tissue. To establish whether the material is able to cause disease symptoms, inoculation of healthy tissue by the cultured pathogens is necessary. An established method for accomplishing this would be to prove that the fungal morphotypes of interest fulfil Koch's postulates.

Developed in the 1800s by Robert Koch, Koch's postulates are a set of four criteria designed to relate a potential causative pathogen with the disease symptoms observed in a host organism (Koch, 1890). To fulfil these causal criteria, a pathogen must be isolated from a symptomatic host, inoculated into healthy host tissues, produce identical disease symptoms in the new host, and the same causal pathogen must be re-isolated from the new host. Updated versions have expanded the original principles to include genetic confirmation of the re-cultured pathogen (Byrd & Segre, 2016). Ideally, the pathogen species must then be isolated from the second host and be genetically identical to the originally isolated pathogen. Koch's postulates have already been applied to confirm the pathogenicity of suspected SER causal species isolated from avocados in previous research, and they should be employed any time a new potential causal species is isolated (Nilmini et al., 2020; Twizeyimana et al., 2013).

This experiment will apply these principles to determine if the fungal morphotypes cultured during the SCD aggregation were able to cause SER disease symptoms in healthy avocado mesocarp tissue.

The specific objectives of this study were:

1. To investigate the infection potential of the seven most abundant fungal morphotypes defined in Section 4.2.3 and genetically identified in section 4.1

2. To establish the abundant fungal morphotypes as SER causal species by demonstrating their association with brown mesocarp tissue and then culturing the same phenotype out of the inoculated tissue

3. To confirm the re-cultured morphotype as genetically identical to the hyphae innocula

7.2 Methods

Fungal inocula used in this experiment were originally isolated from core samples extracted from SCD avocados, placed on MEA and incubated at 25 °C for 3 - 4 days as described in Section 2.1.2. Additional Hass avocados, unaffected by any visible SER symptoms, were sourced from Israel and inoculated with isolated pathogen hyphae according to the methods described in Section 2.5. The top two cm of the avocado was cut off to remove any potential latent infection located in the stem end. Removing this much avocado also allowed for an examination of the mesocarp tissue deeper in the fruit to confirm it was not already brown. Five of the seven most abundant morphotypes were the focus of the Koch analysis, as the slime morphotypes were not easily cultured back out of the avocados. These experiments were conducted before the ITS barcode data from Section 4.1.2 revealed that YellowSlime and WhiteSlime could not be sequenced.

The Koch inoculation experiments were carried out in sets of five avocados. The five avocados within a set were inoculated with the same pathogen, therefore

serving as five technical replicates of one biological replicate. Multiple sets of avocados were inoculated at once, with only one set of controls serving as the control for the group. There were three control sets in total, for 14 fungal pathogen biological replicates with five technical replicates each.

Samples were prepared for DNA sequencing as described in Section 2.1.2. Sequencing of the ITS barcode region was performed by Macrogen, using in-house primers (2.3.2) targeting the ITS1 region of the small RNA subunit. All bioinformatics analysis was performed using Geneious Prime software (Geneious Prime 2021.1.1). Neighbor-joining trees were constructed after aligning all sequences using MUSCLE alignment, and then Geneious Prime tree building software with 500 bootstrap replicates. The maximum likelihood tree was calculated using PhyML and 100 bootstrap replicates.

7.3 Koch Analysis Results

Fungal morphotype samples selected for Koch analysis are summarised in Table 7.1. Although five avocados were inoculated for each fungal pathogen sample, the morphotype was not always cultured back out of all five avocados, as indicated by the '# Recovered'. The control avocados, inoculated with a 'blank' autoclaved piece of filter paper, remained clean, aside from avocado 2 in the topmost set (Figure 7.1). However, the plate cultures reveal at least two mesocarp samples which cultured a fungus from an uninoculated avocado. This could represent the standard level of quiescent infection present in otherwise clean avocados.

Figures 7.2 – 7.15 illustrate the results of all inoculation trials. The *Pestalotiopsis*/*Colletotrichum*/*Fusarium* Complex, the morphotype classification category which included *Fusarium*, *Colletotrichum*, and *Pestalotiopsis*, produced SER symptoms in at least 3 - 4 avocados in two inoculation sets (Figures 7.2 and 7.3). Although the symptoms were not apparent in Figure 7.4, the fungal pathogen was

cultured back out of four of the avocados within this set. *Lasiodiplodia* (*Lasiodiplodia*) and *Neofusicoccum* (*Neofusicoccum*) shared a similar morphology, as *Lasiodiplodia* can be indistinguishable from *Neofusicoccum* when overgrown. All three sets shown in Figures 7.5, 7.6, and 7.7 successfully produces SER symptoms in clean avocados and the *Lasiodiplodia*/*Neofusicoccum* morphotypes were cultured back out of all inoculated fruit. Unfortunately, one re-cultured fungal sample failed to sequence. Avocados inoculated with *Neopestalotiopsis* (*Neopestalotiopsis*) exhibited the most pronounced SER symptoms, but the same morphotype was rarely cultured back out of the fruit (Figures 7.8, 7.9, and 7.10). The inoculation set with least severe SER symptoms recovered the most *Neopestalotiopsis* morphotype (Figure 7.10). The *Diaporthe* (*Diaporthe*) inoculation sets shown in Figures 7.11 and 7.13 were the most successful. All five avocados re-cultured the *Diaporthe* morphotype in both sets, and many inoculations produced severe SER symptoms. The set depicted in Figure 7.12 did not always culture a *Diaporthe* and only two avocados had obvious SER symptoms. Two YellowSlime inoculation sets are summarised in Figures 7.14 and 7.15. These are the only slime morphotype inoculations which produced any symptoms at all, and the re-cultures were often contaminated.

Sequences from all fungal morphotypes cultured out of inoculated avocados, as well as the initial pathogen, were aligned and analysed into phylogenies. The neighbor-joining phylogeny in Figure 7.16 shows every initial fungal inoculum formed a distinct clade with all successful re-cultures associated with it. The clades were closely related by genus, as summarised in Table 7.1, with initial fungal inoculum and re-cultures of all *Neopestalotiopsis* and *Diaporthe* sets forming two clades. The rearrangement of a single BG17 sample in the AX37 clade indicates possible contamination between *Lasiodiplodia* and *Neofusicoccum*. The maximum likelihood tree shows the same

pattern, but it differs in the overall relationships of the clades to each other (Figure 7.17).

Table 7.1 Fungal morphotype samples chosen for Koch inoculation

Morphotype	**Genus**	**Morphotype Sample**	**# Recovered**
Pestalotiopsis/*Colletotrichum*/*Fusarium*	*Colletotrichum*	CK3	3
Pestalotiopsis/*Colletotrichum*/*Fusarium*	*Fusarium*	CK6	5
Pestalotiopsis/*Colletotrichum*/*Fusarium*	*Fusarium*	BV2	4
Lasiodiplodia	*Lasiodiplodia*	AX37	5
Neofusicoccum	*Neofusicoccum*	BV14	4
Neofusicoccum	*Neofusicoccum*	BG17	5
Neopestalotiopsis	*Neopestalotiopsis*	BQ45	2
Neopestalotiopsis	*Neopestalotiopsis*	BR13	3
Neopestalotiopsis	*Neopestalotiopsis*	BS18	4
Diaporthe	*Diaporthe*	BR5	4
Diaporthe	*Diaporthe*	BU15	3
Diaporthe	*Diaporthe*	BL38	5
YellowSlime	*Phomopsis (?)*	BW29	2
YellowSlime	*Phomopsis (?)*	M10	2

Figure 7.1: Control sets from the three inoculation trials with corresponding mesocarp plate cultures to the right of each set. All avocados were inoculated with autoclaved filter paper, sealed with paraffin wax, and incubated at 25 ºC for four days

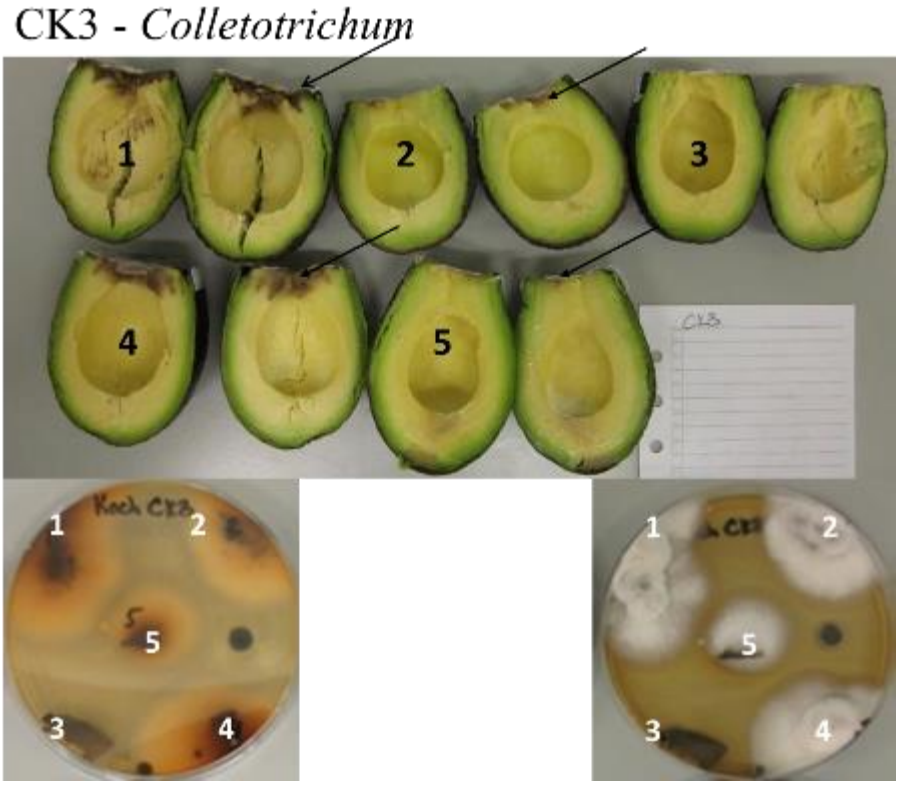

Figure 7.2: Avocados inoculated with *Colletotrichum*-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Four out of five inoculations were recovered, whilst #3 failed to culture. Black arrows indicate SER symptoms

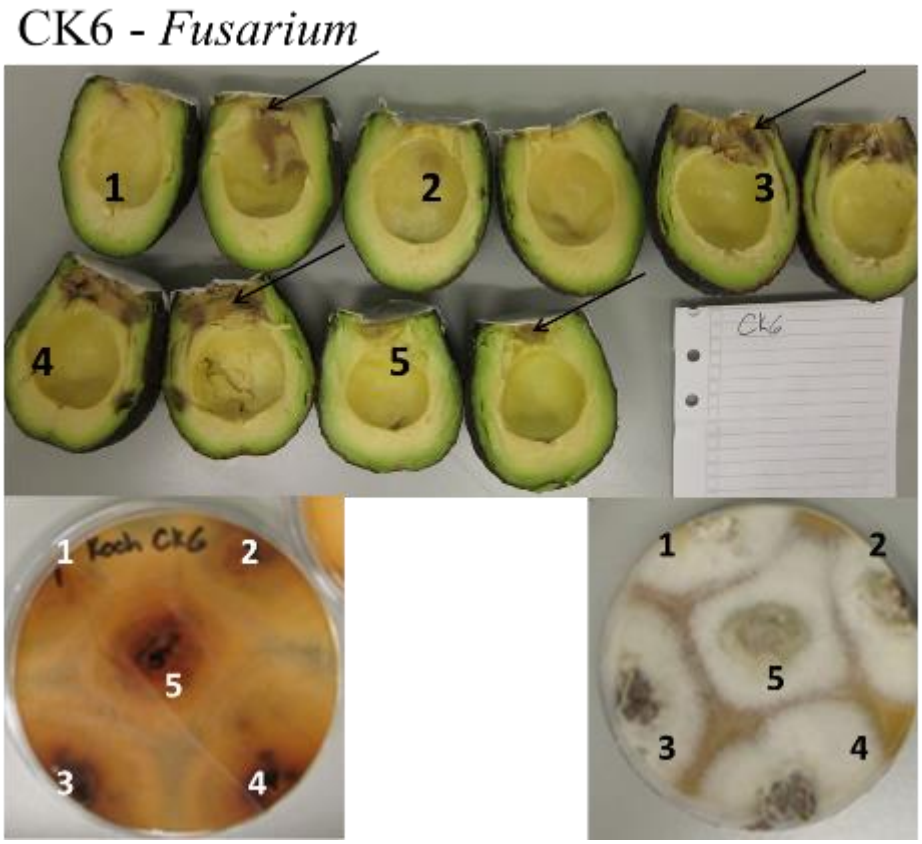

Figure 7.3: Avocados inoculated with *Fusarium*-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. All five inoculations were recovered. Black arrows indicate SER symptoms

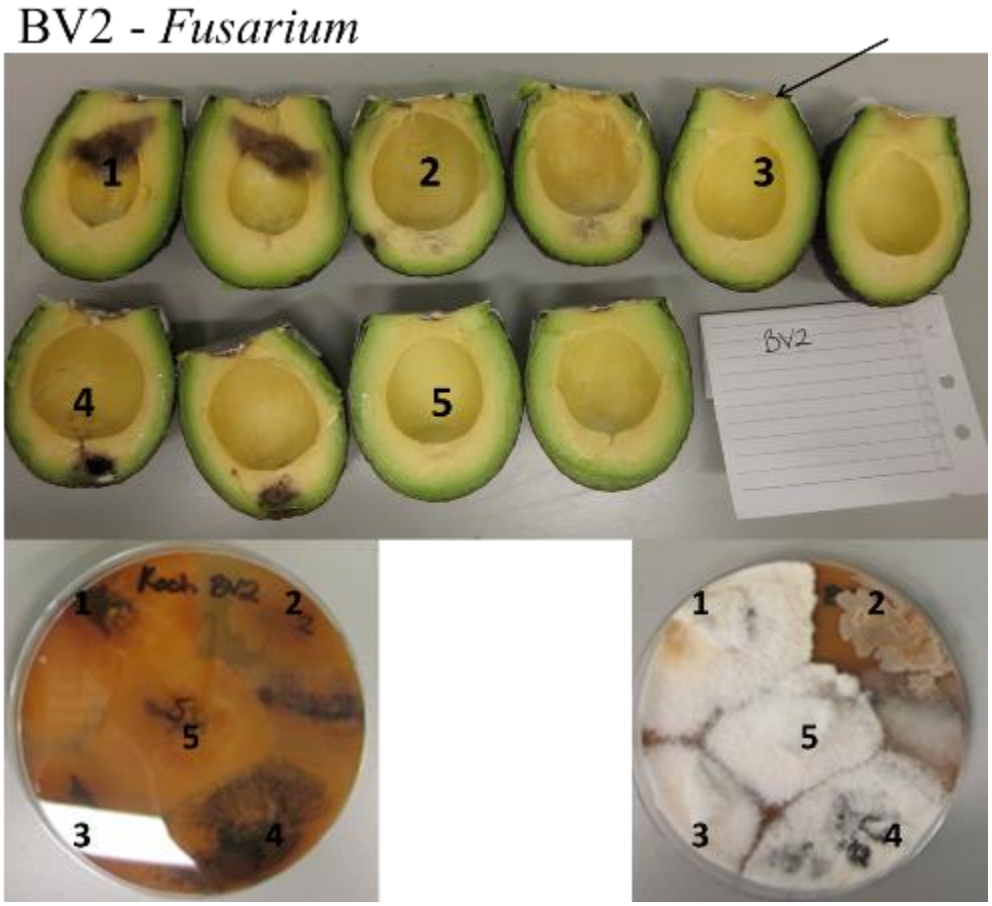

Figure 7.4: Avocados inoculated with *Fusarium*-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Four out of five inoculations were recovered whilst #2 was contaminated with an unrelated culture. Black arrows indicate SER symptoms

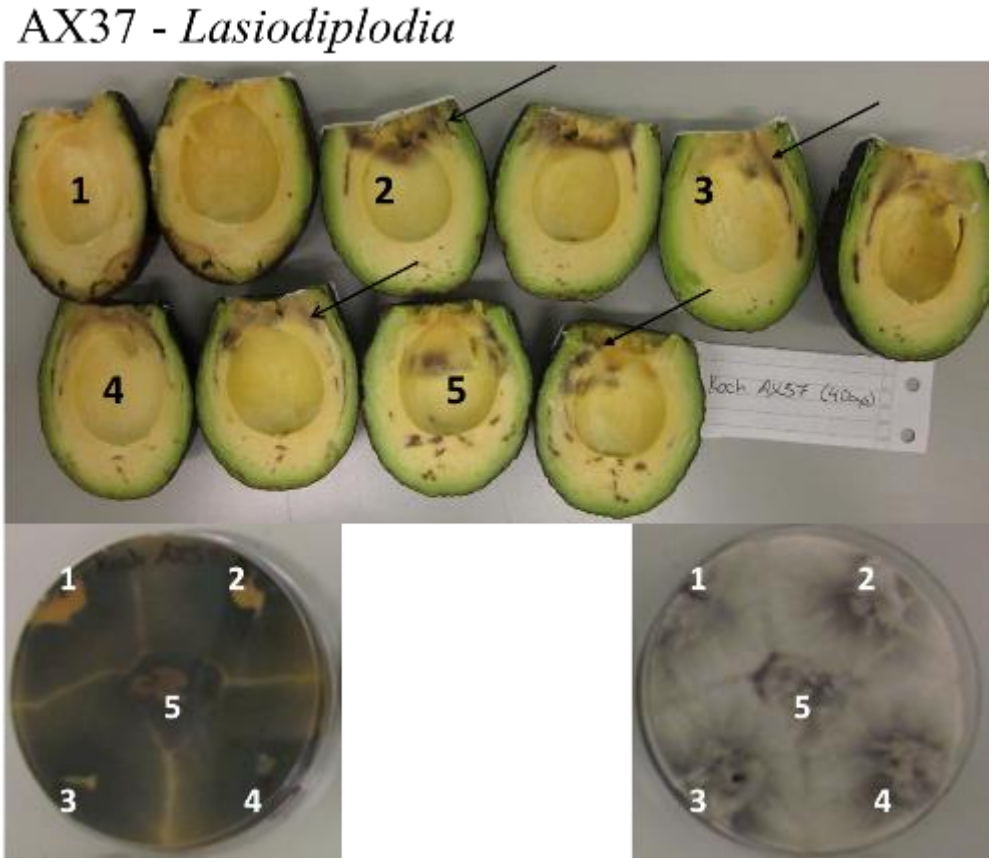

Figure 7.5: Avocados inoculated with *Lasiodiplodia* (*Lasiodiplodia*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. All five inoculations were recovered. Black arrows indicate SER symptoms

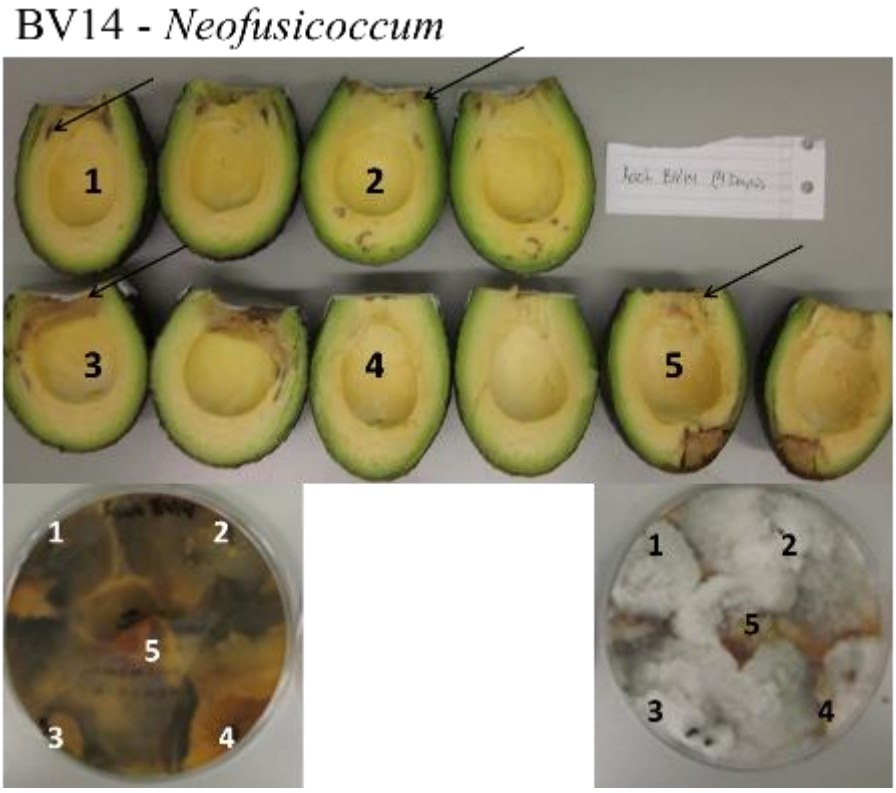

Figure 7.6: Avocados inoculated with *Neofusicoccum* (*Neofusicoccum*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. All five inoculations were recovered. Black arrows indicate SER symptoms

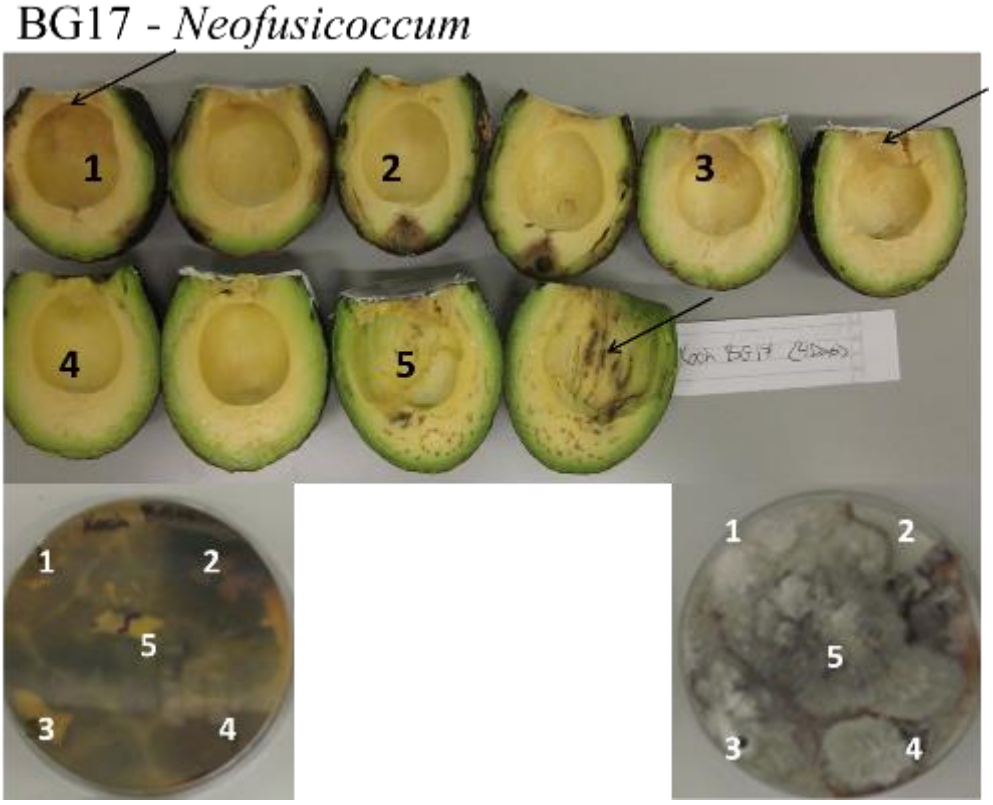

Figure 7.7: Avocados inoculated with *Neofusicoccum* (*Neofusicoccum*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. All five inoculations were recovered, although individual cultures merged on the plate due to the fast growth of the fungus. Samples from the recovered cultures were taken toward the edge of the plate. Black arrows indicate SER symptoms

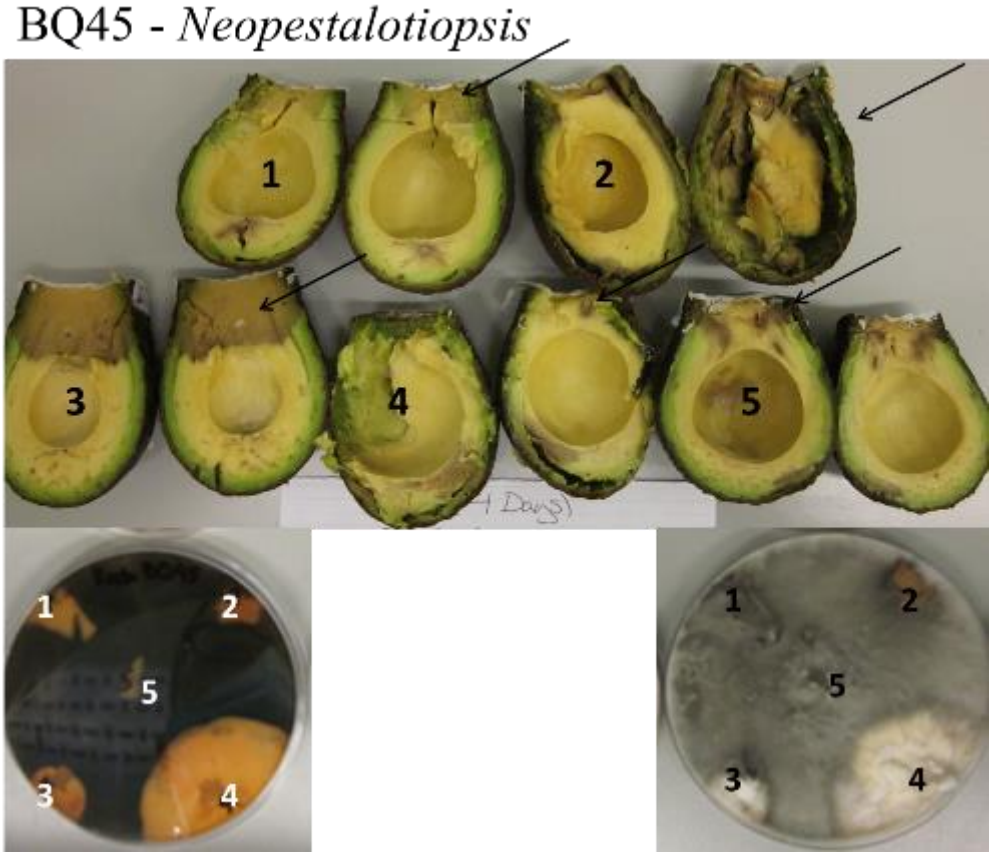

Figure 7.8: Avocados inoculated with *Neopestalotiopsis* (*Neopestalotiopsis*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Two inoculations were recovered, whilst #1, #2, and #5 were contaminated with an unrelated culture. Black arrows indicate SER symptoms

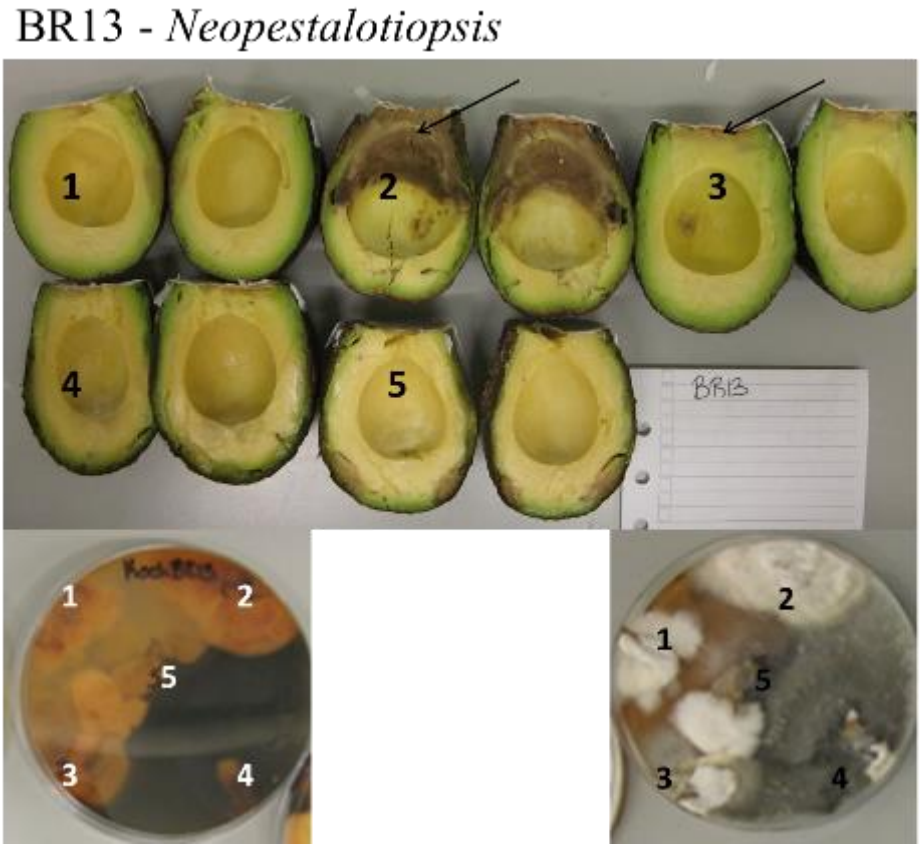

Figure 7.9: Avocados inoculated with *Neopestalotiopsis* (*Neopestalotiopsis*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Two inoculations were recovered, whilst #3, #4, and #5 were contaminated with an unrelated fungus. Black arrows indicate SER symptoms

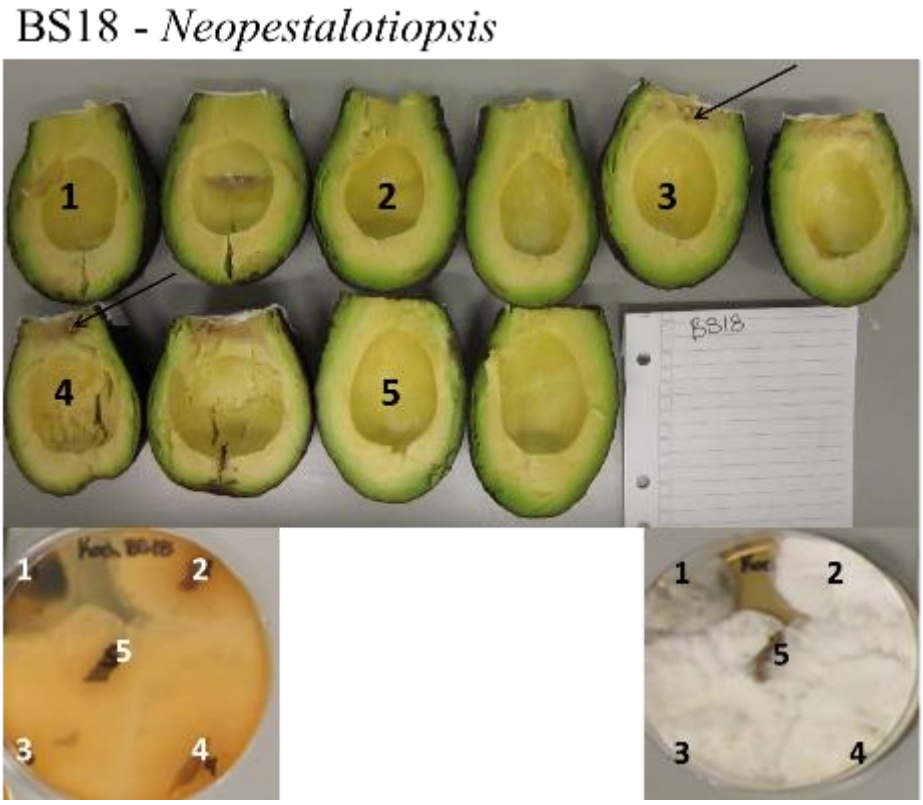

Figure 7.10: Avocados inoculated with *Neopestalotiopsis* (*Neopestalotiopsis*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Four inoculations were recovered, whilst #1 was contaminated with an unrelated fungus. Black arrows indicate SER symptoms

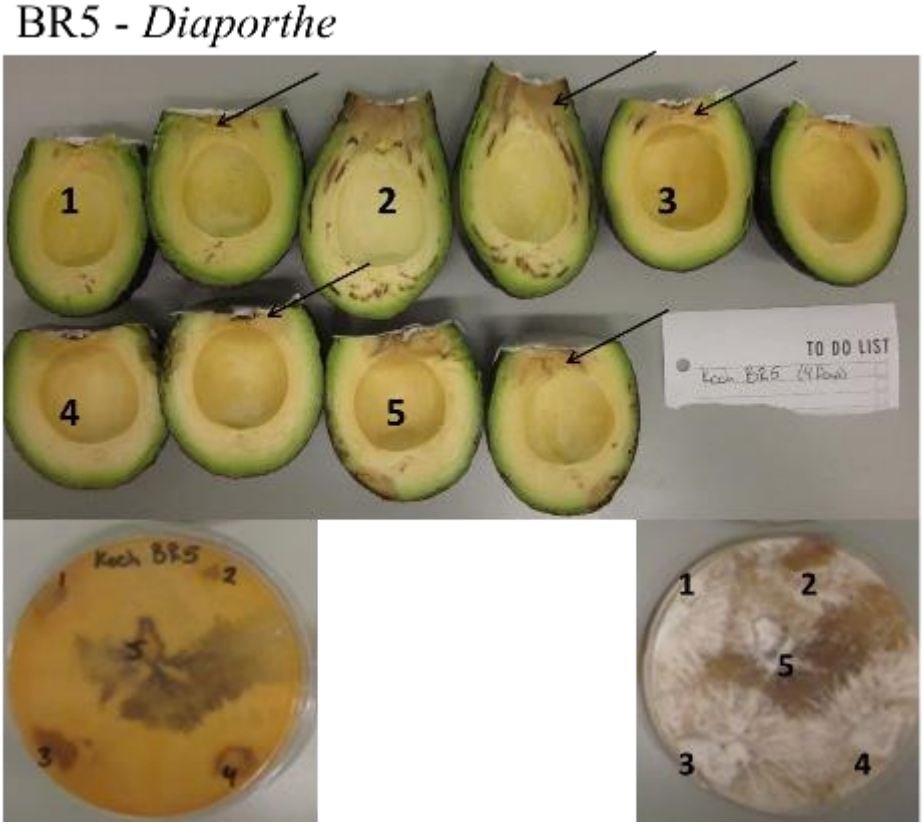

Figure 7.11: Avocados inoculated with *Diaporthe* (*Diaporthe*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. All five inoculations were recovered. Black arrows indicate SER symptoms

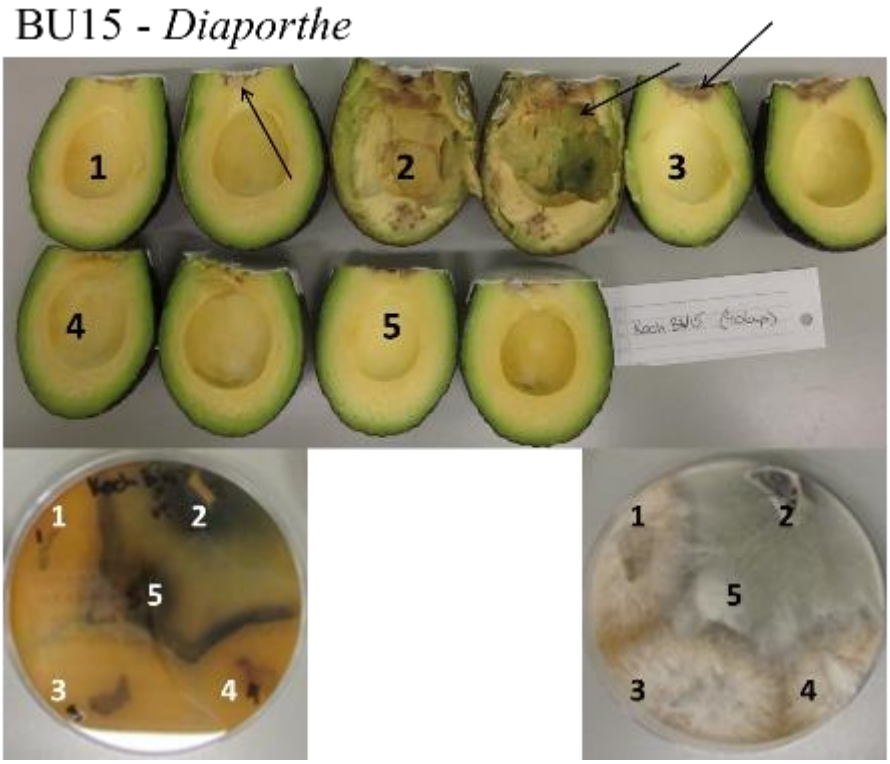

Figure 7.12: Avocados inoculated with *Diaporthe* (*Diaporthe*)-infused filter paper with (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Three inoculations were recovered, whilst #2 and #5 were contaminated with an unrelated fungus. Black arrows indicate SER symptoms

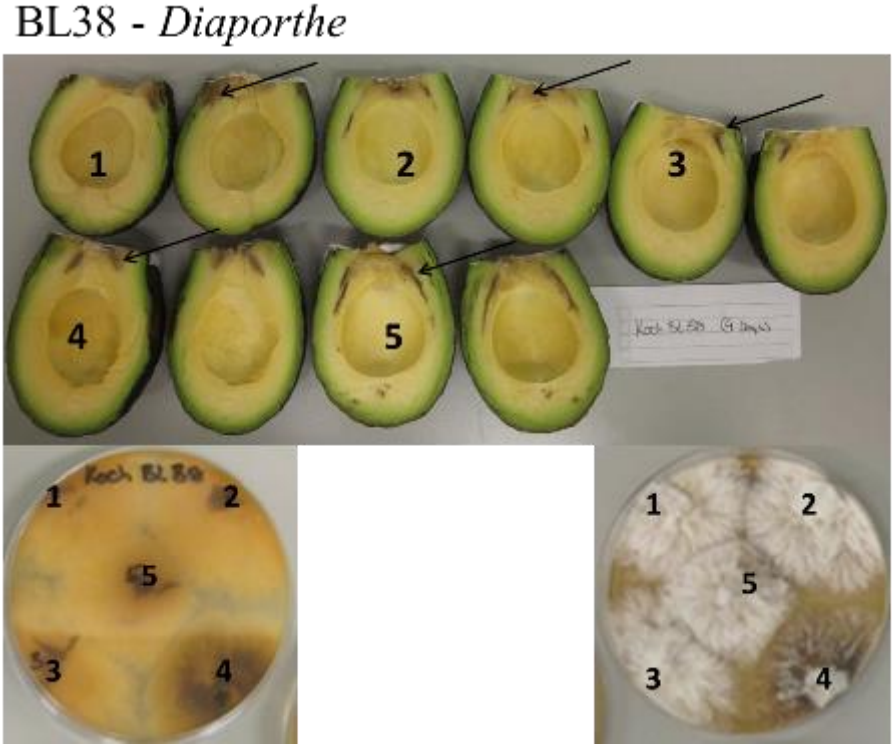

Figure 7.13: Avocados inoculated with *Diaporthe* (*Diaporthe*)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. All five inoculations were recovered. Black arrows indicate SER symptoms

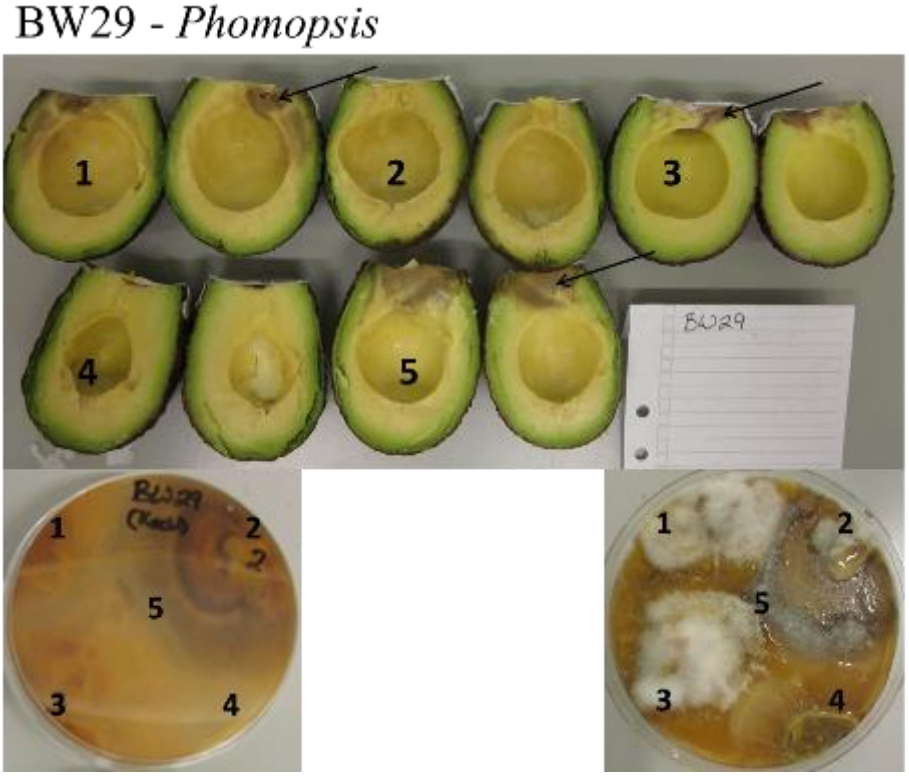

Figure 7.14: Avocados inoculated with YellowSime (*Diaporthe/Phomopsis* complex?)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Culture #4 appears to be the purest recovered inoculation, but it failed to produce a sequence. Black arrows indicate SER symptoms

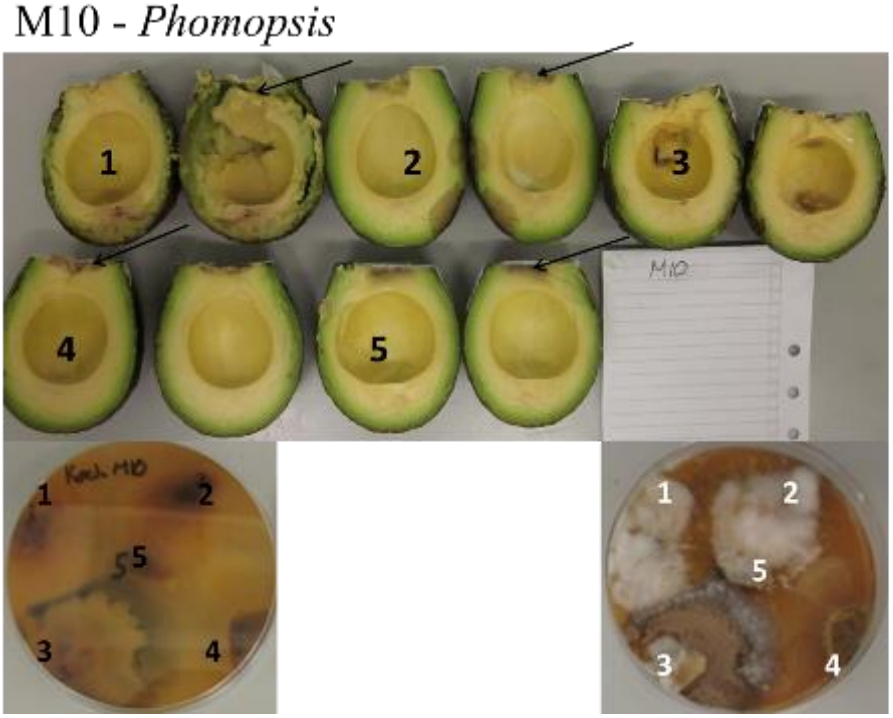

Figure 7.15: Avocados inoculated with YellowSlime (*Diaporthe/Phomopsis* complex?)-infused filter paper (top) with front and reverse plate culturing pathogens recovered from the mesocarp of the inoculated avocados. All avocados were inoculated under sterile conditions, sealed with paraffin wax, and incubated at 25 ºC for four days. Culture #4 appears to be the purest recovered inoculation, but it failed to produce a sequence. Black arrows indicate SER symptoms

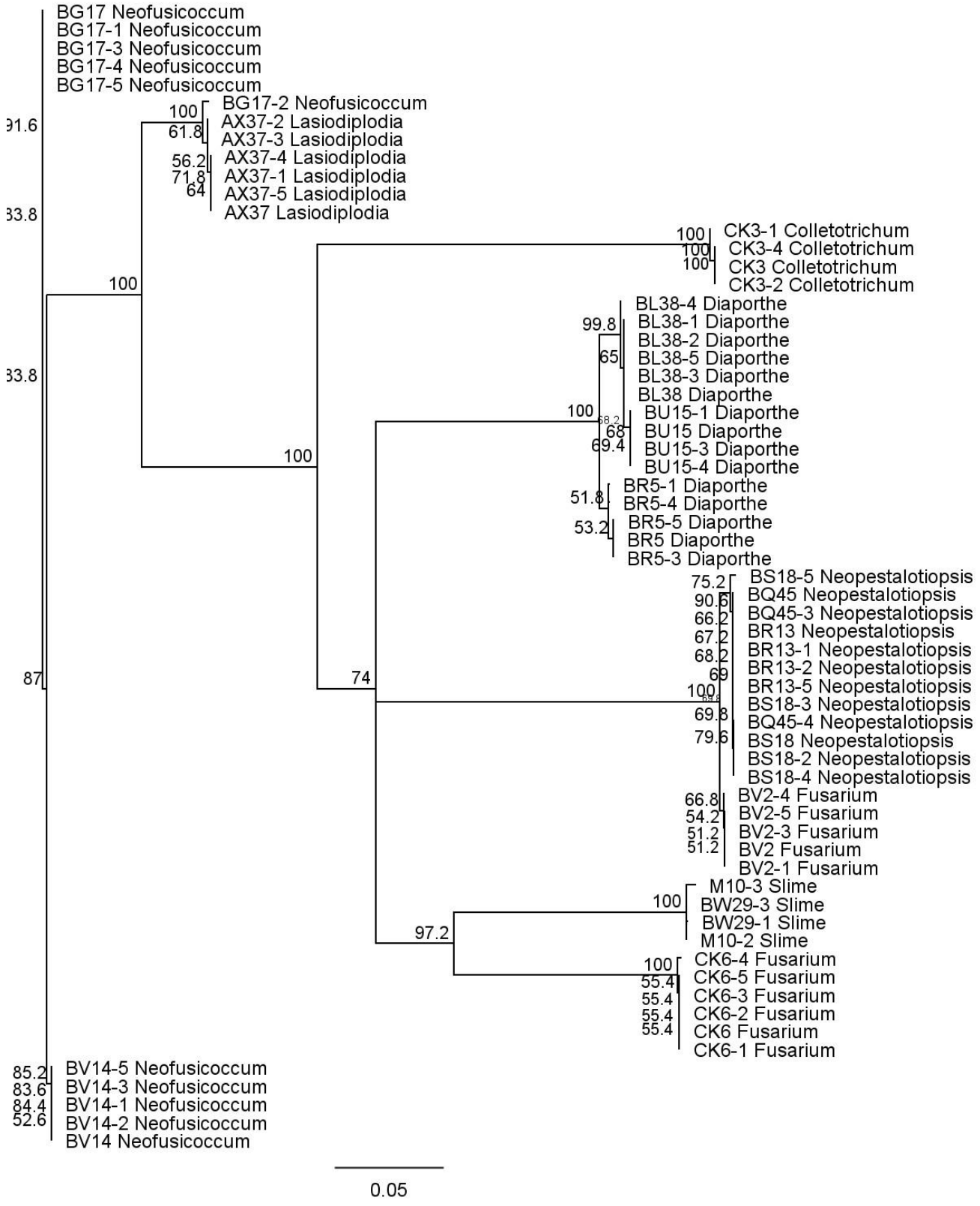

Figure 7.16: Single locus (ITS) neighbour-joining consensus phylogeny of all fungal morphotypes recovered from inoculated avocado samples, with bootstrap support of 500 replicates shown above the nodes. Most inoculated sets organised into distinct clades

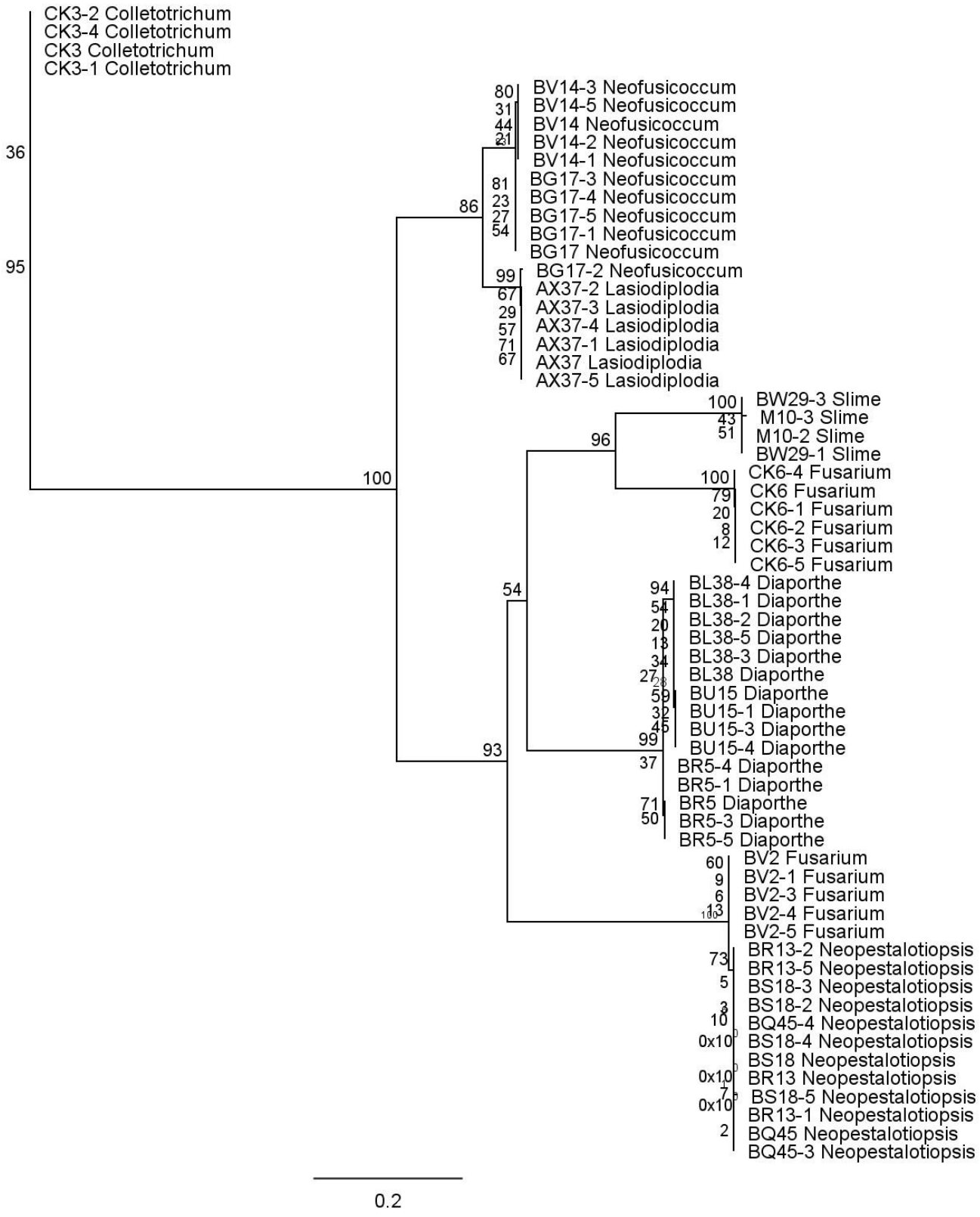

Figure 7.17: Single locus (ITS) maximum likelihood consensus phylogeny of all fungal morphotypes recovered from inoculated avocado samples, with bootstrap support of 100 replicates shown above the nodes. Most inoculated sets organised into distinct clades

7.4 Discussion

This experiment sought to demonstrate the infection capacity of fungal morphotypes isolated from avocados at the end of the supply chain. Although the morphotypes were all members of genera known to be associated with SER, much of the prevailing research focuses on fungal pathogens cultured in the orchard or shortly after harvest (Akgul et al., 2016; Darvas et al., 1987; Guarnaccia et al., 2016; Johnson et al., 1992; Madhupani et al., 2017; Montealegre et al., 2016; Shetty et al., 2016). This is one of the only attempts to apply the same parameters used to establish the infection capability of SER causal pathogens found in the orchard to potential pathogens found at the retail stage. These results also show that at least some morphtypes are still viable after processing and shipping.

Data on the impact of postharvest processing on pathogen viability is limited, but it is a growing area of study. Increased understanding of microbial communities is now possible due to advances in genomics technology. The complex interplay between pathogen species and the natural microbiome found on produce was explored by Buchholz et al. (2018) in a literature review examining the role of microbiota in postharvest food waste. Many of the examined studies reported a shift in the microbiome of the plant surface directly preceding the development of infection symptoms. Mutualistic, pathogenesis, and commensal species were found to colonize the surface of produce. A complex shift in resources, pathogen/endophyte volatiles, metabolites, or other environmental conditions favouring the growth of one species at the expense of another, may create the opportunity for the pathogens to overtake the microenvironment (Buchholz et al., 2018).

There has not been much study on the microbial populations found on avocado skin, but postharvest handling procedures, such as fungicide washes and cold chain storage, almost certainly affect this outer microbiome. Treating harvested avocados

with fungicides is intended to eliminate or at least drastically reduce the pathogenic organisms. Maintaining the cold chain, or consistent storage and transport at 4 ºC, is designed to restrict fugal pathogen development. Some filamentous fungi, such as *Penicillium*, are prone to reactive oxygen species production when exposed to cold temperatures, so it is possible the cellular toxicity caused by oxidative stress may impact pathogen viability, but this has not been specifically explored (Miteva-Staleva et al., 2015). If the SER causal fungi lost pathogenicity due to their exposure to this artificially created 'hostile environment', it would confirm the effectiveness of the current standard practice. Although this Koch study was not designed to test the viability of pathogens at the end of the supply chain compared to the same pathogen isolated at the beginning of the chain, it did demonstrate that most isolated pathogens retained their ability to attack exposed avocado mesocarp tissue.

All morphotypes assessed in the Koch analysis had the capacity to produce SER symptoms in at least one inoculated avocado. This study also confirmed previous literature which stated that *Colletotrichum* did not typically infect vascular tissue (Schaffer et al., 2013). This was especially interesting considering that the vascular bundles of the inoculated avocados were severed before introducing the test pathogen, which should have given the fungus easier access in theory. Mesocarp browning could be the preferred strategy for at least some members of the *Colletotrichum* genus, but additional study would be needed to confirm this. In contrast, *Diaporthe* consistently attacked vascular tissue, and *Neopestalotiopsis* produced some especially severe fungal browning. The exact physiological mechanism as to why *Diaporthe* prefers vascular tissue as opposed to mesocarp decay has not been widely explored in the literature at the time of this study, however a plausible theory would be that *Diaporthe* is simply a fast-growing pathogen genus, and it is able to move through the vascular tissue more

rapidly than other genera. It is possible that the vascular discoloration of *Diaporthe* infection is observed before the same pathogen also moves through the mesocarp tissue.

The vascular tissue is a common entry point for many filamentous fungal pathogens. The movement of hyphae through the hollow xylem tubes may be faster and unimpeded compared to the necrotic decay of solid mesocarp tissues. When vascular tissue is attacked by SER causal fungi, it is most often the xylem elements which are decayed (Hartill & Everett, 2002). Members of *Fusarium* in particular have been known to erode the vessel secondary walls in tomato plants. The fungus was found to use vascular parenchyma cells, the living component of xylem tissue, as a substrate when moving along the vessels. As vascular parenchyma surrounds the xylem tracheids and vessels, they likely provide a nearby source of nutrients to the growing fungal hyphae as it works its way down the more open cell structure of the xylem tubes (Bishop et al., 1983).

All vascular tissue within the developing avocado connects back to the stem, as it functions to deliver nutrients and water from the tree to the fruit. Pathogen movement through the vascular tissue would originate either from endophytic growth through the petiole or from exposure of the severed vascular tissue bundles at the abscission zone when the fruit is harvested (Everett & Hallett, 2005). In either case, vascular infection is unlikely to occur through the skin unless the fungal browning margin reaches the xylem parenchyma cells. Bishop et al. (1983) also found that *Fusarium* and *Verticillium* species typically eroded the secondary walls of tomato vessels.

Although SER symptoms manifested in avocados inoculated with YellowSlime, the slime morphotypes cultured out of the avocados were so heavily contaminated with other morphotypes that it is impossible to determine what caused the SER symptoms. Avocado number 4 in Figure 7.14 appears to have produced the only pure re-culture of

a YellowSlime from an inoculated sample, and it had no SER symptoms. These data suggest that YellowSlime may not be capable of producing SER symptoms, but it is often associated with other SER causal species.

The fact that all re-cultures clustered with the original fungal pathogen used to inoculate the avocados on both trees proves that the Koch inoculations successfully re-cultured the exact same fungal species that was inoculated into the fruit. The YellowSlime samples (M10 and BW29) were of the same species, but both were more closely related to *Fusarium* in the phylogenies, suggesting contamination. Slime phenotypes isolated for morphotype identification (Chapter 4) were found to be more closely related to *Diaporthe*, which implies that either the Koch samples, the morphotype identification samples, or both groups, were contaminated. Despite the failure of the slime morphotypes the Koch analysis was conclusive in its objective to establish the ability of all other isolated pathogens to cause SER symptoms and maintain their genetic identity with the original inoculated specimen.

CHAPTER 8: GENERAL DISCUSSION

8.1 Weather and Orchard Susceptibility

The lack of high-quality weather data from weather stations located within the orchard makes it difficult to draw conclusions from the historical commercial SER data analysis. Many potential relationships between the optimal sporulation temperature, wind speed, and rainwater spore dispersal, and their effects on SER incidence were likely obscured from the lack of accurate data, or incorrectly combining the data that was available. While the effects of weather on disease incidence had been extensively studied, further investigation is needed to bring together the correct weather data with the orchard from which the avocados were harvested (Anco et al., 2012; Bourke, 1970; Shah et al., 2019; Twizeyimana et al., 2013). Although the orchard data was inconclusive for this specific study, the experiment design highlights a potential for future research, especially with regard to transit times.

All avocados are shipped in their unripened state, but their total travel time differs depending on the country of origin. For avocados arriving at Greencell, the main distributer for the United Kingdom, avocados originating from South and Central America can take around six weeks before they reach their final destination. Shipping from South Africa are in transit for around four weeks, and from Israel, only 1 week. Avocados shipped from Spain arrive within a few days. Therefore, the Mexican avocados assessed in this project had a longer shipping time compared with those from South Africa and Israel.

Although the fruit had not softened, the added time in transit may have impacted disease incidence if it can be proven to change the species composition of fungal communities (Bill et al., 2014). A more recent study by Bill et al. (2022) confirmed that fungicide application during the supply chain had the greatest impact on fruit

fungal community composition. One of the main research questions posed by this thesis was if the supply chain and shipping process was enough to change the pathogenic fungal community on or within the avocado fruit. The fact that the fungal communities isolated from avocados originating in Mexico, South Africa, and Israel differed from each other could be attributed to the hugely varying transit times, but more study would be needed to confirm this. Ideally, one would need to isolate and measure fungal communities on avocados at the orchard and from the same avocado harvest at the destination to determine if the supply chain alone had any effect. Local climate is likely most responsible for the differences between the three fungal communities. The experiments in this thesis also demonstrated that a similar set of known, plant pathogenic fungi are found in the orchard as well as the distribution end of the supply chain. It is likely that the effect of transit time on avocado pathogen community is minimal.

8.2 Fungal Morphotype Identity and Pathogenicity

Establishing a species identity for the seven morphotypes of interest using ITS barcode sequencing and applying Koch's postulates to confirm their ability to cause SER symptoms, was one of the most successful outcomes of the project. Firstly, it achieved one of the most important aims of this investigation, which was to examine fungal community composition at the end of the supply chain. The experiment also revealed the further potential of plate identification. The consistent species identification, with only a single ITS barcode, proved that a narrow set of morphological characteristics, observable without a microscope, are all that is necessary to identify a fungal plate culture to genus level. This technique can be useful for scientists working in the field without access to DNA identification technology or microscopy equipment. Overall, the plate identification was more successful with some

genera compared to others, but it was most reliable for genera such as *Neopestalotiopsis* and *Diaporthe*.

Reoccurring cultures of known SER pathogens both confirmed previous research on known SER pathogens and challenged it. *Neopestalotiopsis* was one of the most abundant genera cultured during the SCD collection. However, its identity as an endophyte or a pathogen is controversial. Kateel et al. (2016) observed that the endophytic growth of *Neopestalotiopsis* could compose part of the tree's natural microbiome, and a healthy endophytic population may prevent disease incidence by competing for resources with pathogenic species. The Koch results show *Neopestalotiopsis* does have the ability to infect avocado mesocarp as readily as the other isolates. This further complicates the relationship an avocado tree may have with its mutualistic, endophytic microbiome. Further study is needed to elucidate under what conditions the members of *Neopestalotiopsis* are pathogenic or mutualistic, or if only certain species fall into either category.

The established literature is also contentious about resolving the taxonomy of the *Diaporthe/Phomopsis* complex. A recent study by Dhanushka et al. (2011) argued that *Phomopsis* should be combined with *Diaprothe* as the same genus. Previous classification of each new species relied on a combination of morphological traits, host identity, biochemistry, and other factors. Genetic identification is slowly resolving the phylogeny of the *Diaporthe/Phomopsis* complex, but it must be conducted species by species.

The reproductive exudate associated with *Diaporthe* in the literature was a point of contention in distinguishing fungal isolates by morphology alone. The possibility that the slime phenotypes were bacteria was eliminated because they did not Gram stain. A literature search revealed that one of the most abundant morphotypes,

Diaporthe, produces a yellow exudate that is identical in appearance to the YellowSlime morphotype (Huda-Shakirah et al., 2021). However, all of the observations made in this project concerning the Slime morphotypes, and YellowSlime in particular, were contradictory.

The slime morphotypes often cultured independently, grew well on solid media, and died in liquid media. They could be sub-cultured, they often smell like yeast, and they were able to sometimes re-infect avocado mesocarp. Microscopic imaging revealed what could be yeast cells, but genetic identification is necessary to confirm this. Lastly, they failed to sequence when using primers optimized for Ascomycetes. Although yeast is taxonomically classified as Ascomycota, the primers may have been optimised for filamentous Ascomycetes.

All these traits suggest that the slime morphotypes may be yeasts. However, the yellow slime morphotype was often associated with *Diaporthe,* where it formed a mixed culture. When sequencing was successful, the BLAST results confirmed *Diaporthe*. These slimes didn't always re-infect avocado tissue, and they resembled the reproductive exudate established in the literature for *Diaporthe*. These two contradictory sets of observations imply that each slime culture could be either a yeast or *Diaporthe* exudate. It is likely that the total community of slime isolates comprised a mixture of yeast colonies and reproductive exudate. It is also reasonable to conclude that the independent slime cultures were more likely to be yeasts and the cultures mixed with *Diaporthe* were more likely reproductive exudates.

Although conclusive genetic evidence as to the identity of the slime morphotypes was not acquired in this project, the ‘slime’ exudate of *Diaprothe* may have been classified as a separate morphotype before DNA technology. The WhiteSlime and YellowSlime morphotypes were most commonly associated with

Diaporthe, and they did not antagonise each other on the plate (Section 5.3.5). Further research is necessary to determine exactly what WhiteSlime and YellowSlime are, but the results of this study suggest that the Slime morphotypes alone are not SER causal pathogens, they are only associated with one.

All abundant morphotypes isolated in this project, aside from the slimes, were defined in the literature as broad range generalists, causing post-harvest disorders in a wide variety of fruits and vegetables (Darapanit et al., 2021; Mehl et al., 2017; Rampersad, 2020; Sajeewa et al., 2011; Salvatore et al., 2021; Talhinhas & Baroncelli, 2021; Udayanga et al., 2015). Many of these broad pathogens were generalists targeting a variety host species, and if they had any speciality at all, it was toward fruit or trees specifically.

Many previous studies found most of the isolated pathogens to be endophytes or naturally occurring opportunist decomposers. Specifically, *Colletotrichum*, *Lasiodiplodia*, *Neofusicoccum*, and *Pestalotiopsis* have all been observed as endophytes of woody species (Mehl et al., 2017; Sajeewa et al., 2011; Salvatore et al., 2021; Talhinhas & Baroncelli, 2021). One important exception was *Fusarium*, of which most members are soil inhabiting fungi spread by rainwater (Rampersad, 2020). Even some species of the more aggressively pathogenic *Diaporthe* genus were found in the literature to be endophytes, or saprophytes on healthy plant tissue (Allan-Perkins et al., 2020; Dissanayake et al., 2017). *Colletotrichum* is more often associated in the literature with body rots, but it may also be a non-specific plant tissue pathogen which found a way to circumvent many of the avocado's defences (Talhinhas & Baroncelli, 2021). The fact that the majority of isolated pathogens are known endophytes suggests that a more pertinent course of investigation would be to examine the complex set of physiological and environmental factors which influence an endophytic fungus to adopt

a pathogenic lifestyle. If fewer SER causal species are airborne or soilborne microbes than those growing endophytically, spore dispersal events such as rainfall may have less effect on SER incidence than tree stress.

The SER causal genera identified during this project were so widespread and generalist, they have been cultured extensively on host species other than avocado. However, for many of the individual fungal species, this thesis project marks the first time they were isolated from avocado. It is doubtful that these fungal species are newly evolved or novel, but more likely avocado was not yet examined as a host. They have all been long established on other host plant species. Of all the fungal species isolated and identified in this project, there were 16 which are at this point associated with avocado for the first time, to the authors knowledge. These are *Diaporthe velutina* (Dissanayake et al., 2017), *Diaporthe macadamiae* (Wrona et al., 2020), *Diaporthe humulicola* (Allan-Perkins et al., 2020), *Diaporthe caatingaensis* (Crous et al., 2016), *Diaporthe terebinthifolii* (Gomes et al., 2013), *Colletotrichum arecicola* (Cao et al., 2020), *Fusarium pernambucanum* (Santos et al., 2019), *Pestalotiopsis grevilleae* (Maharachchikumbura et al., 2014), *Pestalotiopsis papuana* (Maharachchikumbura et al., 2014), *Lasiodiplodia parva* (Alves et al., 2008), *Lasiodiplodia brasiliensis* (Netto et al., 2014), *Neofusicoccum italicum* (Marin-Felix et al., 2017), *Neofusicoccum ningerense* (Li et al., 2020), *Neopestalotiopsis eucalypticola* (Maharachchikumbura et al., 2014), *Neopestalotiopsis piceana* (Maharachchikumbura et al., 2014), *Neopestalotiopsis aotearoa* (Maharachchikumbura et al., 2014).

8.3 Fungal Community and Physiology

The fungal community and fruit physiology analyses were the main focus of this project. Slight differences in fungal communities between countries and growing regions were found, but overall, SER was mostly associated with pathogens already

established to cause postharvest rot disorders (Akgul et al., 2016; Darvas & Kotze, 1987; Thompson et al., 2011; Wanjiku et al., 2020). The fungal communities isolated from the avocados resembled those found in the orchard (Guarnaccia et al., 2016). Although no orchard samples were available to directly compare fungal species found at harvest with those found at distribution, the most abundant fungal species isolated were of genera already established in the literature to be present in the orchard. The composition of species and strain may differ, but data on these was beyond the scope of this research. Future study may benefit from collecting spore samples from the orchard and extracting fungal pathogens from avocados at the end of the supply chain to determine if the species and strain matches that of the orchard pathogens. This project established a robust SER culture method which could be applied to future research.

The physiology study endeavoured to explore all factors which may contribute to increased SER incidence. Like many of the other experiments in the project, the findings confirmed what was already established in the literature. Fruit age and softness had the greatest impact on disease incidence, and other physiological factors had no impact (Blakey et al., 2010). The fact that avocados selected for market sale must be within a narrow range of physical parameters impacted variation of sample size, weight, and dry mass percentage. Therefore, testing whether fresh weight or dry mass affects disease incidence is not ideal at the end of the supply chain, because very few avocados of abnormal weight or dry mass percentage can be included in the study. For example, perhaps higher than average dry mass percentage decreases SER incidence, but the marked only exports avocados whose dry mass percentage is between 20 – 30 %. It would be impossible to confidently determine the rate of SER incidence on avocados below 20% or above 30% without an adequate sample size.

Further study is needed, but this project concluded that it is likely SER is caused by pathogens found in the orchard, and not along the supply chain. The avocado processing industry is efficient at keeping fruit at clean and optimum conditions for transport to minimise injury and infection. Only after the fruit has ripened do avocados begin to develop rots which they were most likely already carrying (Prusky et al., 2013). As the fruit ages, secondary pathogens and rots can easily infect tissue, but by that point, the consumer would not find the product appealing.

8.4 Future Work

This research laid the foundation for substantial future work. By establishing a small list of abundant fungal morphotypes of interest, further research can focus on determining if the species or strain isolated at the end of the supply chain matches the fungal species found in the orchard from which the avocado was harvested. This would elucidate whether the fungus originated from the orchard or was picked up along the supply chain. So far, fungal communities at the end of the supply chain resemble those of the orchard, as established in previous research, but this project did not explore whether the exact fugal community isolated at the end of the supply chain matched the orchard from which the avocados originated, or if there was a difference in fungal strain between the two communities. Future research could also position weather stations within the orchard, or measure microclimates within trees, to investigate the exact relationship between weather conditions, orchard management practices, and disease incidence.

The plate identification of fungal morphotypes confirmed successful groundwork for easy identification of common SER fungal pathogens. Further research could be carried out to establish more accurate morphological characteristics to differentiate the hard to distinguish morphotypes, such as *Lasiodiplodia*,

Colletotrichum, *Fusarium*, and *Neofusicoccum*. This may involve the examination microscopic structures, but it still has the potential to deliver positive identification without the use of DNA barcoding. This research did prove that a short ITS region was all that was necessary to distinguish several common SER causal pathogens down to species level. Future work can apply this ITS barcode sequencing to the other morphotypes isolated in this project and expand on the standardised plate identification method.

DNA analysis of the fungal pathogens could have been improved in many ways. It is likely that the primers used to isolate the ITSII section were not optimised for the specific target genre or species. However, despite the failures in amplification, this research project did work toward optimising a fungal extraction protocol for filamentous Ascomycetes which proved to be successful.

Many abundant avocado pathogens, and known fungal pathogens identified in the literature, were successfully cultured on MEA. However, if given more time, it would have been interesting to explore other culture media. Much of the previous research cited used PDA, however, most of the pathogens isolated in this project and others were proven to grow equally well on both PDA and MEA (De Wet et al., 2008; Twizeyimana et al., 2013). The choice of in vitro media was found to have no effect on the growth or biomass production of filamentous fungi (Singh & Kamal, 2011). It may be interesting to see if repeating the experiments presented in this thesis with PDA would yield a significantly different fungal community, but due to the fluctuation of avocado pathogens on a year-to-year basis, it may be impossible to compare the results to this project or from one year to the next. A much more relevant line of experimentation would be to incorporate avocado puree into the growth medium. This

may encourage difficult to culture morphotypes which do not tolerate *in vitro* growth conditions.

All experiments described in the current literature regarding SER-related fungal community analysis have been, to the author's knowledge, conducted using *in vitro* culture media with no avocado puree added. To analyse the fungal community which emerged under a specific set of circumstances, experiments should be conducted using only one type of growth media. Testing different types of media would require double plating of each sample from the beginning of the project. However, this project was an exploratory examination of end of supply chain pathogens, and there was no way to determine if MEA was uniquely better than PDA at the very start of the project. Adding a new type of media later on in the project may have altered the fungal community composition and produced results incomparable to the previous years. In the literature, both MEA and PDA are used almost interchangeably, as if budget or supply access was a more important factor in media choice.

Also, it is useful to note that if some fungal isolates are difficult to culture *in vitro*, then it would be reasonable to assume that the choice of *in vitro* media is irrelevant for a morphotype which prefers *in vivo* conditions. The only relevant experiment for these types of microbes would be the incorporation of avocado puree into the media. One could argue that the autoclave process would likely destroy the delicate and heat sensitive avocado, which explains why there are no experiments using avocado puree in media to the author's knowledge. Although some examples, such as V8 media, contain vegetable puree, the plant material used is likely less heat sensitive than avocado. There is also little information as to whether the avocado nutrient components required to culture these *in vitro* pathogens are heat sensitive. Another challenge associated with incorporating avocado puree into media and using it to test

the composition of a variable and ever-changing fungal community, is that the percentage of avocado puree required is unknown. A fungus that does not grow *in vitro* would prefer 100% avocado substrate, but how little would it tolerate? Is 75% avocado enough for it, and why would it be? There has to be enough of a balance to allow the plates to solidify, which limits how much avocado can be incorporated into a culture plate. This ratio could be established using a gradient, proof of concept experiment, so it is not an issue. The real issue is heat. There may be no way to prove if an unknown in vivo pathogen simply does not exist or if the sterilisation process destroyed the nutrient it required to grow.

Additional avocado physiology work is also possible, as only a few were explored in this study. For example, anti-fungal immunities at the cellular level offer a new area of research into avocado defence mechanisms and fruit susceptibility. A phenolic study of decayed mesocarp tissue, compared to healthy tissue, could reveal which compounds are broken down during pathogen invasion. So far, the mesocarpal browning mechanism is poorly understood, and investigation into this area could lead to further research in breeding more resistant avocado cultivars better adapted to the orchard. Future research to decrease pathogen attack, increase fruit resilience, and prevent rot along the supply chain will help reduce waste and provide healthy, sustainable avocadoes.

APPENDIX

Stem Button Plating

Many of the morphotypes collected from the avocados used in this research have been known to grow endophytically. Plating a core sample of the fruit mesocarp did not provide evidence as to whether the fungal pathogen infected the petiole wound during harvest, or if the hyphae entered the fruit endophytically through the stem whilst the avocado was still attached to the tree. To examine the possibility that any of the abundant fungal morphotypes may be located in the remnant petiole tissue which remains attached to the fruit postharvest, the "stem button", these remnant petiole sections were plated on MEA prepared according to manufacturer's instructions (Oxoid CM0059), with no antibiotic.

Eight avocado fruit, originating from South Africa, were obtained from the University of Reading Co-op. The stem buttons were removed using flame sterilized tweezers and placed on MEA plates; the avocados were not sterilized. Plates were incubated for 72 hours at 25 °C and then photographed (Figure 1). Every incubated stem button cultured a slime or filamentous fungus. This suggests that the woody remnant of the avocado pedicel is not sterile and contains fungal species which were either retained from the orchard environment or accumulated along the supply chain. More experimentation is needed to determine the origin and identity of these species and whether they can infect avocado mesocarp.

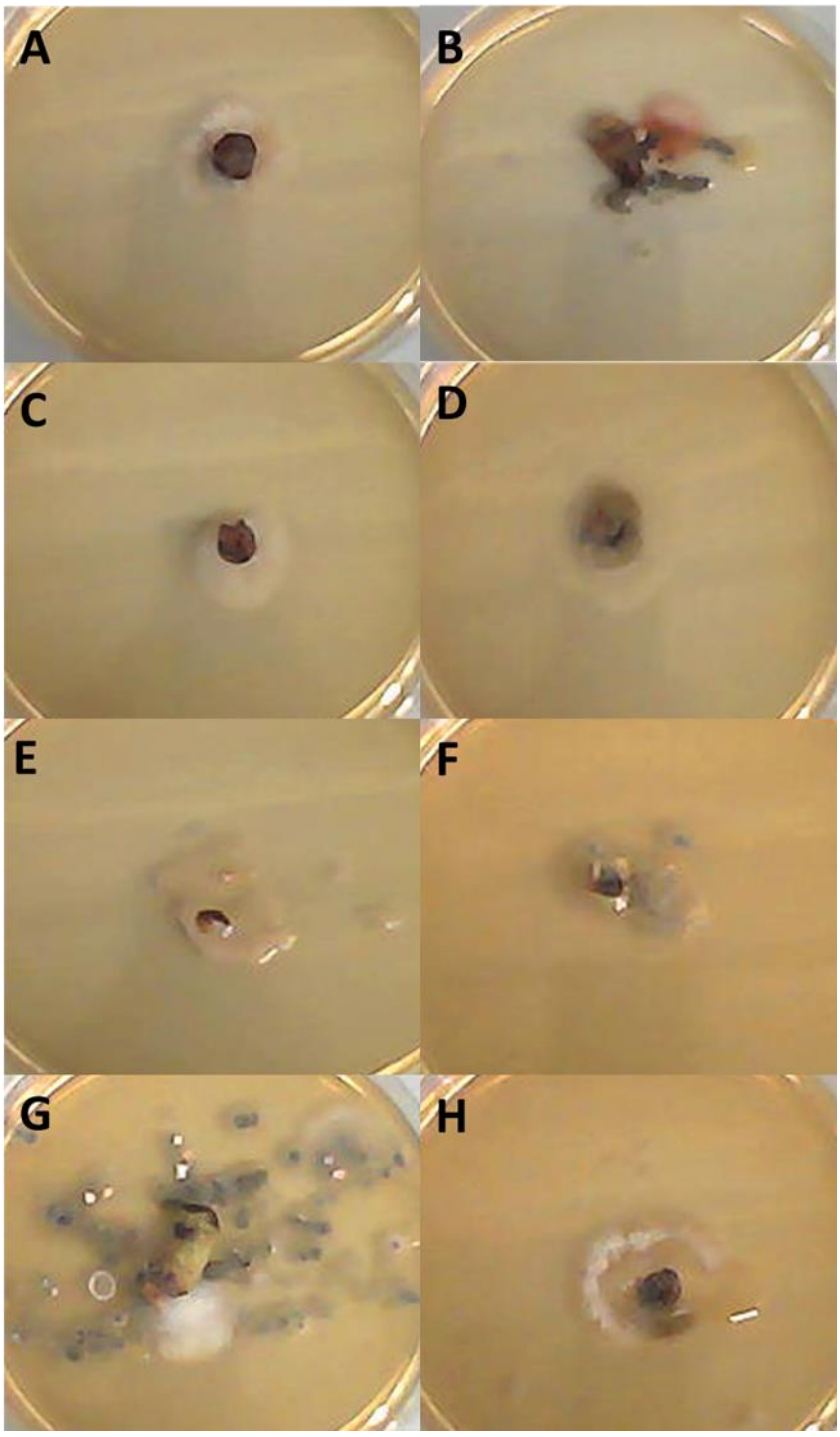

Figure Annex 1: Avocado stem button remnants collected from avocados at the retail stage of the supply chain, plated on prepared MEA and incubated for 72 hours at 25 ℃

BIBLIOGRAPHY

Abdelfattah, A., Wisniewski, M., Droby, S., & Schena, L. (2016). Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. *Horticulture research*, *3*.

Abera, A., Lemessa, F., & Adunga, G. (2016). Morphological characteristics of *Colletotrichum* species associated with mango (*Mangifera indica* L.) in Southwest Ethiopia. *Food Science and Quality Management*, *48*, 106–115.

Adkins, M. F., Hofman, P. J., Stubbings, B. A., & Macnish, A. J. (2005). Manipulating avocado fruit ripening with 1-methylcyclopropene. *Postharvest Biology and Technology*, *35*(1), 33–42.

Akgül, D. S., Awan, Q. N., Güler, P. G., & Önelge, N. (2016). First report of anthracnose and stem end rot diseases caused by *Colletotrichum gloeosporioides* and *Neofusicoccum australe* on avocado fruits in turkey. *Plant Disease*, *100*(8), 1792.

Alder, Greg. "The Gem Avocado Tree: A Profile." Greg Alder's Yard Posts: Southern California Food Gardening, 8 July 2021, https://gregalder.com/yardposts/the-gem-avocado-tree-a-profile/.

Allan-Perkins, E., Li, D. W., Schultes, N., Yavuz, S., & LaMondia, J. (2020). The identification of a new species, *Diaporthe humulicola*, a pathogen causing *Diaporthe* leaf spot on common hop. *Plant Disease*, 104(9), 2377-2390.

Alothman, M., Bhat, R., & Karim, A. A. (2009). Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry*, *115*(3), 785–788.

Altieri, M. A. (1999). The ecological role of biodiversity in agroecosystems. *Invertebrate Biodiversity as Bioindicators of Sustainable Landscapes* (pp. 19–31). Amsterdam: Elsevier.

Alves, A., Crous, P. W., Correia, A., & Phillips, A. J. L. (2008). Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal diversity*, *28*, 1-13.

Anco, D. J., Madden, L. V., & Ellis, M. A. (2012). Effects of temperature and wetness duration on the sporulation rate of *Phomopsis viticola* on infected grape canes. *Plant Disease*, *13*(1), 579–589.

Anderson, M. D. (1986). Omega (Prochloraz), a fungicide for post-harvest control of anthracnose, the *Dothiorella/Colletotrichum* complex and stem-end rot in avocados. *South African Avocado Grower's Association Yearbook*, *9*, 27–30.

Armitage, A. D., Barbara, D. J., Harrison, R. J., Lane, C. R., Sreenivasaprasad, S., Woodhall, J. W., & Clarkson, J. P. (2015). Discrete lineages within *Alternaria alternata* species group: Identification using new highly variable loci and support from morphological characters. *Fungal Biology*, *119*(11), 994–1006.

Arpaia, M. L. (2004). Avocado postharvest quality. In *Proceedings of the California Avocado Research Symposium* (pp. 112–125). University of California, Riverside. California Avocado Commission.

Ayalon, O., Avnimelech, Y., & Shechter, M. (2001). Solid waste treatment as a high-priority and low-cost alternative for greenhouse gas mitigation. *Environmental Management*, *27*(5), 697–704.

Aziz, A. B. A., Desouki, I., & El-Tanahy, M. M. (1975). Effect of nitrogen fertilization on yield and fruit oil content of avocado trees. *Scientia Horticulturae*, *3*(1), 89–94.

Bangerth, G. (2000) Abscission and thinning of young fruit and their regulation by plant hormones and bioregulators. *Plant Growth Regulation*, 31: 43-59.

Bazzicalupo, A. L., Bálint, M., & Schmitt, I. (2013). Comparison of ITS1 and ITS2 rDNA in 454 sequencing of hyperdiverse fungal communities. *Fungal Ecology*, *6*(1), 102–109.

Beesman. "Lamb Hass Avocado." Citrus and Fruit Trees | Moon Valley Nurseries, 26 Jan. 2022, https://www.moonvalleynurseries.com/lamb-hass-avocado.

Bekker, T. F., Labuschagne, N., Aveling, T., Kaiser, C., & Regnier, T. (2007). Accumulation of total phenolics due to silicon application in roots of avocado trees infected with *Phytophthora cinnamomi*. *South African Avocado Growers' Association Yearbook, 30*, 57–64.

Belisário, R., Aucique-Pérez, C. E., Abreu, L. M., Salcedo, S. S., Oliveira, W. M. d., & Furtado, G. Q. (2020). Infection by *Neopestalotiopsis spp*. occurs on unwounded eucalyptus leaves and is favoured by long periods of leaf wetness. *Plant Pathology*, *69*, 194–204.

Bennett, E. J., Brignell, C. J., Carion, P. W. C., Cook, S. M., Eastmond, P. J., Teakle, G. R., Wagstaff, C. (2017). Development of a statistical crop model to explain the relationship between seed yield and phenotypic diversity within the *Brassica napus* genepool. *Agronomy*, *7*(31), 1026.

Beno-Moualem, D., & Prusky, D. (2000). Early events during quiescent infection development by *Colletotrichum gloeosporioides* in unripe avocado fruits. *Phytopathology*, *90*(5), 553–559.

Bergh, BO. (1992). The origin, nature, and genetic improvement of the avocado. *California Avocado Society Yearbook*, 76, 61-75.

Berrocal-Lobo, M., Molina, A., & Solano, R. (2002). Constitutive expression of Ethylene-Response-Factor1 in *Arabidopsis* confers resistance to several necrotrophic fungi. *The Plant Journal*, *29*(1), 23–32.

Bertling, I. and Tesfay, S. Z. (2011). Avocado sugars – key to postharvest shelf life? In *Proceedings VII World Avocado Congress 2011 (Actas VII Congreso Mundial del Aguacate 2011)*. Cairns, Australia.

Bezuidenhout, J. (1983). Die voorkoms van mesokarpverkleuring by Fuerte avokado's op die Rungismark gedurende 1982. *South African Avocado Grower's Association Yearbook*, *6*, 24–27.

Bhuyan, D. J., Alsherbiny, M. A., Perera, S., Low, M., Basu, A., Devi, O. A., ... & Papoutsis, K. (2019). The odyssey of bioactive compounds in avocado (*Persea americana*) and their health benefits. *Antioxidants*, *8*(10), 426.

Bill, M., Gokul, J. K., Viljoen, F., & Korsten, L. (2022). Fungal microbiome shifts on avocado fruit associated with a combination of postharvest chemical and physical interventions. *Journal of Applied Microbiology*.

Bill, M., Sivakumar, D., Thompson, A. K., & Korsten, L. (2014). Avocado fruit quality management during the postharvest supply chain. *Food Reviews International*, *30*, 169–202.

Binyamini, N., & Schiffmann-Nadel, M. (1972). Latent infection in avocado fruit due to *Colletotrichum gloeosporioides*. *Phytopathology*. Tel Aviv, Israel.

Bishop, C. D., & Cooper, R. M. (1983). An ultrastructural study of vascular colonization in three vascular wilt diseases I. Colonization of susceptible cultivars. *Physiological Plant Pathology*, 23(3), 323-343.

Blakey, R. J., Bower, J. P., & Bertling, I. (2010). Post-harvest avocado physiology. *South African Avocado Growers Association Yearbook*, *33*, 56–60.

Blakey, R., & Wolstenholme, N. (2014). 35 Years of the SAAGA Yearbook : A Review.

Bourke, Austin, P. M. (1970). Use of weather information in the prediction of plant disease epiphytotics. *Annual Review of Phytopathology*, *8*(1), 345–370.

Bower, J. P., & Cutting, J. G. M. (1987). Some factors affecting post-harvest quality in avocado fruit. *South African Avocado Growers' Association Yearbook*, *10*, 143–146.

Brecht, J. K., Sargent, S. A., Brecht, P. E., Saenz, J., & Rodowick, L. (2019). Protecting perishable foods during transport by truck and rail. *EDIS*, *2019*(2).

Broekaert, W. F., Delauré, S. L., De Bolle, M. F. C., & Cammue, B. P. A. (2006). The role of ethylene in host-pathogen interactions. *Annual Review of Phytopathology*, *44*(1), 393–416.

Brown, G. E. (1986, June). Diplodia Stem-end Rot, A Decay of *Citrus* Fruit Increased by Ethylene Degreening Treatment and Its Control. In *Proceedings of the Florida State Horticultural Society* (Vol. 99, pp. 105-107).

Brown, J. K. M., & Hovmøller, M. S. (2002). Aerial dispersal of fungi on the global and continental scales and its consequences for plant disease. *Science*, *297*(5581), 537–541.

Brown, J. K., Hovmoller, M. S., Wyand, R. A., & Yu, D. (2002). Oases in the desert: dispersal and host specialization of biotrophic fungal pathogens of plants. In

Dispersal Ecology: 42nd Symposium of the British Ecological Society (pp. 395–409). Cambridge University Press.

Buchholz, F., Kostić, T., Sessitsch, A., & Mitter, B. (2018). The potential of plant microbiota in reducing postharvest food loss. *Microbial Biotechnology*, 11(6), 971-975.

Bustos, M. C., Mazzobre, M. F., & Buera, M. P. (2015). Stabilization of refrigerated avocado pulp: Effect of Allium and Brassica extracts on enzymatic browning. *LWT - Food Science and Technology*, *61*(1), 89–97.

Byrd, A. L., Segre, J. A. (2016). Adapting Koch's postulates. *Science*, *351*(6270), 224–226.

Cao X. R., Xu X. M., Che H. Y., West J. S., Luo D. Q. (2020). Eight *Colletotrichum* species, including a novel species, are associated with areca palm Anthracnose in Hainan, China. *Plant Disease*, 104:1369–1377.

Chakraborty, S., Luck, J., Hollaway, G., Freeman, A., Norton, R., Garrett, K. A., … Karnosky, D. F. (2008). Impacts of global change on diseases of agricultural crops and forest trees. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*(054).

Chepkirui, C., & Stadler, M. (2017). The genus *Diaporthe*: a rich source of diverse and bioactive metabolites. *Mycological Progress*, *16*(5), 477-494.

Clarkson, J. P., Fawcett, L., Anthony, S. G., & Young, C. (2014). A model for sclerotinia sclerotiorum infection and disease development in lettuce, based on the effects of temperature, relative humidity and ascospore density. *PLoS ONE*, *9*(4), 1–12.

Coates, L. M., Muirhead, I. F., Irwin, J. A. G., & Gowanlock, D. H. (1993). Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit. *Mycological Research*, *97*(11), 1363–1370.

Coates, L., Willingham, S., Pegg, K., Cooke, T., Dean, J., & Langdon, P. (2002). Field and postharvest management of avocado fruit diseases. *Queensland Horticulture Institute, Department of Primary Industries*, 2–11.

Cowan, A. (2004). Metabolic control of avocado fruit growth: 3-hydroxy-3-methylglutaryl coenzyme a reductase, active oxygen species and the role of C7 sugars. *South African Journal of Botany*, 70: 75-82.

Crous P. W., Wingfield M. J., Burgess T. I., et al. (2016). Fungal Planet description sheets: 469-557. *Persoonia* 37: 218-403.

Crozier, A., Clifford, M. N., & Ashihara, H. (2006). Plant secondary metabolites occurrence, structure, and role in the human diet (pp. 1–9). Oxford: Blackwell Publishing Ltd.

Cruz, D. R., Leandro, L. F. S., & Munkvold, G. P. (2019). Effects of temperature and ph on fusarium oxysporum and soybean seedling disease. *Plant Disease*, *103*(12), 3234–3243.

Cutting, J. G. M., Wolstenholme, B. N., & Hardy, J. (1992). Increasing relative maturity alters the base mineral composition and phenolic concentration of avocado fruit. *Journal of Horticultural Science*, *67*(6), 761–768.

Darvas, J. (1979). Ecology of avocado root pathogens. *South African Avocado Grower's Association Research Report*, *3*(31–32).

Darvas, J. M., & Kotze, J. M. (1987). Avocado fruit diseases and their control in South Africa. *South African Avocado Growers' Association Yearbook*, *10*, 117–119.

Darvas, J. M., Kotzé, J. M., & Wehner, F. C. (1990). Effect of treatment after picking on the incidence of post-harvest fruit diseases of avocado. *Phytophylactica*, *22*(1), 93–96.

Darvas, J., & Kotze, J. (1987). Fungi associated with pre-and postharvest diseases of avocado fruit at Westfalia Estate, South Africa. *Phytophylactica*, *19*(1), 83–85.

Darvas, J., Kotze, J., & Wehner, F. (1987). Field occurrence and control of fungi causing postharvest decay of avocados. *Phytophylactica*, *19*(4), 453–456.

Darvas, Jozsef, M. (1982). Etiology and control of some fruit diseases of avocado (*Persea americana* Mill.) at Westfalia estate. University of Pretoria.

Das, R., Chutia, M., Das, K., & Jha, D. (2010). Factors affecting sporulation of *Pestalotiopsis disseminata* causing grey blight disease of *Persea bombycina* Kost., the primary food plant of muga silkworm. *Crop Protection - CROP PROT*, *29*, 963–968.

Davis, D. R., Epp, M. D., & Riordan, H. D. (2004). Changes in usda food composition data for 43 garden crops, 1950 to 1999. *Journal of the American College of Nutrition*, *23*(6), 669–682.

De Lapeyre de Bellaire, L., & Mourichon, X. (1997). The pattern of fungal contamination of the banana bunch during its development and potential influence on incidence of crown-rot and anthracnose diseases. *Plant Pathology*, 46(4), 481-489.

DeMers, M. (2022). *Alternaria alternata* as endophyte and pathogen. *Microbiology*, 168(3).

Demoz, B. T. (2006). Alternative application methods of antagonists to avocado flowers to control stem-end rot pathogens. University of Pretoria.

Demoz, B. T., & Korsten, L. (2006). *Bacillus subtilis* attachment, colonization, and survival on avocado flowers and its mode of action on stem-end rot pathogens. *Biological Control*, *37*(1), 68–74.

De Wet, J., Slippers, B., Preisig, O., Wingfield, B. D., & Wingfield, M. J. (2008). Phylogeny of the Botryosphaeriaceae reveals patterns of host association. *Molecular Phylogenetics and Evolution*, *46*(1), 116–126.

Diskin, S., Feygenberg, O., Maurer, D., Droby, S., Prusky, D., & Alkan, N. (2017). Microbiome alterations are correlated with occurrence of postharvest stem-end rot in mango fruit. *Phytobiomes Journal*, *1*, 117–127.

Dixon, J., Pak, H. A., Mandemaker, A. J., Smith, D. B., Elmsly, T. A., & Cutting, J. G. M. (2003). Fruit age management: the key to successful long distance export of New Zealand avocados. In *Proceedings V World Avocado Congress (Actas V Congreso Mundial del Aguacate)* pp. 623–628.

Dong, Z., Manawasinghe, I. S., Huang, Y., Shu, Y., Phillips, A. J., Dissanayake, A. J., ... & Luo, M. (2021). Endophytic *Diaporthe* associated with *Citrus grandis* cv. tomentosa in China. *Frontiers in Microbiology*, *11*, 609387.

Donkin, D. (2018). The South African Avocado Growers' Association. Retrieved from https://www.avocado.co.za

Dor, R. (2022). "Israel's Avocado Industry - Overview" Agronet, 28 Mar. 2022, https://www.agronet-eng.com/israels-avocado-industry-overview/

Downer, A. J., Crohn, D., Faber, B., Daugovish, O., Becker, J. O., Menge, J. A., & Mochizuki, M. J. (2008). Survival of plant pathogens in static piles of ground green waste. *Phytopathology*, *98*(5), 547–554.

Eckert, J. W., & Ogawa, J. M. (1985). The chemical control of postharvest diseases: subtropical and tropical fruits. *Annual Reviews of Phytopathology*, *23*, 421–454.

Einax, E., & Voigt, K. (2003). Oligonucleotide primers for the universal amplification of β-tubulin genes facilitate phylogenetic analyses in the regnum fungi. *Organisms Diversity and Evolution*, *3*, 185–194.

El Jarroudi, M., Karjoun, H., Kouadio, L., & El Jarroudi, M. (2020). Mathematical modelling of non-local spore dispersion of wind-borne pathogens causing fungal diseases. *Applied Mathematics and Computation*, *376*(March).

EPPO. (n.d.). Diaporthe vaccinii. *Data Sheets on Quarantine Pests*, 1–5. Retrieved from https://www.eppo.int/QUARANTINE/fungi/Diaporthe.../DIAPVA_ds.pdf

Eskalen, A., Faber, B., & Bianchi, M. (2013). Spore trapping and pathogenicity of fungi in the *Botryosphaeriaceae* and *Eiaporthaceae* associated with avocado branch canker in california. *Plant Disease*, *97*(3), 329–332.

Everett, K. R. (1999). Infection of unripe avocado fruit by stem end rot fungi in New Zealand. *Revista Chapingo Serie Horticultura*, *5*, 337–339.

Everett, K. R., & Hallett, I. C. (2005). Stem end rots - how, where, and what do they infect? New Zealand and Australian Avocado Grower's Conference '05, (September), 11.

Everett, K. R., Boyd, L. M., Pak, H. A., & Cutting, J. G. M. (2007). Calcium, fungicide sprays and canopy density influence postharvest rots of avocado. *Australasian Plant Pathology*, *36*(1), 22–31.

Feild, T. S., & Arens, N. C. (2007). The ecophysiology of early angiosperms. *Plant, Cell & Environment*, 30(3), 291-309.

Fitzell, R. (1987). Epidemiology of anthracnose disease of avocados. *South African Avocado Growers' Association Yearbook, 10*, 113–116.

Flaishman, M. A., & Kolattukudy, P. E. (1994). Timing of fungal invasion using host's ripening hormone as a signal. *Proceedings of the National Academy of Sciences, 91*(14), 6579–6583.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture, 80*(13), 1925–1941.

Fuchs, Y., & Zauberman, G. (1981). Effect of wounding on 'Fuerte' avocado ripening. *HortScience, 16*(4), 496–497.

Fuentes-Aragón, D., Juárez-Vázquez, S. B., Vargas-Hernández, M., & Silva-Rojas, H. V. (2018). *Colletotrichum fructicola*, a member of *Colletotrichum gloeosporioides* sensu lato, is the causal agent of anthracnose and soft rot in avocado fruits cv."Hass." *Mycobiology*, *46*(2), 92–100.

Gage, S. H., Isard, S. A., & Colunga-G, M. (1999). Ecological scaling of aerobiological dispersal processes. *Agricultural and Forest Meteorology*, *97*(4), 249–261.

Gaillard, J. P., & Godefroy, J. (1995). *The Tropical Agriculturalist: Avocado*. London: Macmillan Education LTD.

Galsurker, O., Diskin, S., Maurer, D., Feygenberg, O., & Alkan, N. (2018). Fruit stem-end rot. *Horticulturae*, 4(4), 50.

Galsurker, O., Diskin, S., Duanis-Assaf, D., Doron-Faigenboim, A., Maurer, D., Feygenberg, O., & Alkan, N. (2020). Harvesting mango fruit with a short stem-end altered endophytic microbiome and reduce stem-end rot. *Microorganisms*, 8(4), 558.

Gamble, J., Harker, F. R., Jaeger, S. R., White, A., Bava, C., Beresford, M., … Marques, R. (2010). The impact of dry matter, ripeness and internal defects on consumer perceptions of avocado quality and intentions to purchase. *Postharvest Biology and Technology*, *57*(1), 35–43.

Gao, Y., Liu, F., Duan, W., Crous, P. W., Cai, L., Vhulrxv, W. R., … Orvvhv, L. (2017). Diaporthe is paraphyletic. *International Mycological Assocaition*, *8*(1), 153–187.

García-Alonso, M., De Pascual-Teresa, S., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2004). Evaluation of the antioxidant properties of fruits. *Food Chemistry*, *84*(1), 13–18.

Ghannoum, M. A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews*, 13(1), 122-143.

Gisi, U., Binder, H., & Rimbach, E. (1985). Synergistic interactions of fungicides with different modes of action. *Transactions of the British Mycological Society*, *85*(2), 299–306.

Githinji, P., Gitu, L., Marete, E., Githua, M., Mugo, M., & Mbaka, E. N. (2013). Quantitative analysis of total phenolic content in avocado (persia americana) seeds in eastern province of Kenya. *Chemistry and Materials Research*, *3*(10), 48–52.

Glass, N. L., & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, *61*(4), 1323–1330.

Glowacz, M., Roets, N., & Sivakumar, D. (2017). Control of anthracnose disease via increased activity of defence related enzymes in 'Hass' avocado fruit treated with methyl jasmonate and methyl salicylate. *Food Chemistry*, *234*, 163–167.

Golan, A., Kahn, V., & Sadovski, A. Y. (1977). Relationship between polyphenols and browning in avocado mesocarp. Comparison between the Fuerte and Lerman cultivars. *Journal of Agricultural and Food Chemistry*, *25*(6), 1253–1260.

Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., & Crous, P. W. (2013). *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, 31(1), 1-41.

Goudarzian, P., Erfanifard, S. Y. (2017). The efficiency of indices of richness, evenness and biodiversity in the investigation of species diversity changes (case study: migratory water birds of Parishan international wetland, Fars province, Iran). *Biodiversity International Journal*, *1*(2), 41–45.

"Green Avocados." (1996). Specialty Produce: Information, Recipes and Facts, https://specialtyproduce.com/produce/Green_Avocados_17071.php.

Grote, U., & Sartorius von Bach, H. (1994). An investigation of avocado trading in Europe. *Agrekon*, 33(3), 135-140.

Guarnaccia, V., & Crous, P. W. (2018). Species of *Diaporthe* on *Camellia* and *Citrus* in the Azores Islands. *Phytopathologia Mediterranea*, *57*(2), 307–319.

Guarnaccia, V., Groenewald, J. Z., Woodhall, J., Armengol, J., Cinelli, T., Eichmeier, A., … Crous, P. W. (2018). *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, *40*(February), 135–153.

Guarnaccia, V., Vitale, A., Cirvilleri, G., Aiello, D., Susca, A., Epifani, F., … Polizzi, G. (2016). Characterisation and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. *European Journal of Plant Pathology*, *146*(4), 963–976.

Guindon S., Dufayard J. F., Lefort V., Anisimova M., Hordijk W., Gascuel O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology*. 59(3):307–321.

Gustafson, D. (1976). 1976 World avocado production. *Yearbook California Avocado Society*.

Hartill, W. F. T. (1992). Post-harvest rots of avocados in New Zealand and their control. *Brighton Crop Protection Conference Pests and Diseases* (pp. 1157–1162).

Hartill, W. F. T., & Everett, K. R. (2002). Inoculum sources and infection pathways of pathogens causing stem-end rots of 'Hass' avocado (*Persea americana*). *New Zealand Journal of Crop and Horticultural Science*, *30*(4), 249–260.

Hartill, W. F. T., Everett, K. R., & Pak, H. A. (2002). Stem-end rots: the infection portal. *New Zealand Avocado Growers Association Annual Research Report*, *2*.

Hernández, I., Fuentealba, C., Olaeta, J. A., Lurie, S., Defilippi, B. G., Campos-Vargas, R., & Pedreschi, R. (2016). Factors associated with postharvest ripening heterogeneity of 'Hass' avocados (Persea americana Mill). *Fruits*, *71*(5), 259–268.

Hershkovitz, V., Friedman, H., Ben-Arie, R., Feygenberg, O., Pesis, E., & Goldschmidt, E. E. (2010). The role of ethylene receptors in avocado ripening. *Acta Horticulturae*, *858*, 143–147.

Hershkovitz, V., Friedman, H., Goldschmidt, E., & Pesis, E. (2009). The role of the embryo and ethylene in avocado fruit mesocarp discoloration. *Journal of Experimental Botany*, *60*, 791–799.

Hershkovitz, V., Saguy, S. I., & Pesis, E. (2005). Postharvest application of 1-MCP to improve the quality of various avocado cultivars. *Postharvest Biology and Technology*, *37*(3), 252–264.

Hodgkiss, I. J., & Harvey, R. (1969). Spore discharge rhythms in pyrenomycetes. *Transactions of the British Mycological Society*, *52*(3), 355–363.

Hofman, P. J., Jobin-Decor, M., Meiburg, G. F., Macnish, A. J., & Joyce, D. de C. (2001). Ripening and quality responses of avocado, custard apple, mango and papaya fruit to 1-methylcyclopropene. *Australian Journal of Experimental Agriculture*, *41*(4), 567–572.

Hofman, P. J., Vuthapanich, S., Whiley, A. W., Klieber, A., & Simons, D. H. (2002). Tree yield and fruit minerals concentrations influence "Hass" avocado fruit quality. *Scientia Horticulturae*, *92*, 113–123.

Holliday, P. (1995). *Fungus Diseases of tropical crops*. Courier Corporation.

Hopkirk, G., White, A., Beever, D. J., & Forbes, S. K. (1994). Influence of postharvest temperatures and the rate of fruit ripening on internal postharvest rots and disorders of New Zealand 'Hass' avocado fruit. *New Zealand Journal of Crop and Horticultural Science*, *22*(3), 305–311.

Hosseini, B., El-Hasan, A., Link, T., & Voegele, R. T. (2020). Analysis of the species spectrum of the *Diaporthe*/*Phomopsis* complex in European soybean seeds. *Mycological Progress*, *19*(5), 455–469.

Huang, F., Udayanga, D., Wang, X., Hou, X., Mei, X., Fu, Y., ... & Li, H. (2015). Endophytic *Diaporthe* associated with *Citrus*: A phylogenetic reassessment with seven new species from China. *Fungal Biology*, 119(5), 331-347.

Humber, R. A. (1997). Fungi: preservation of cultures. In L. A. Lacey (Ed.), *Manual of Techniques in Insect Pathology* (pp. 269–280). New York: Academic Press.

Husak, V. (2015). Copper and copper-containing pesticides: metabolism, toxicity and oxidative stress. *Journal of Vasyl Stefanyk Precarpathian National University*, *2*(1), 38–51.

Hyde, K. D., & Soytong, K. (2008). The fungal endophyte dilemma. *Fungal Diversity*, *33*, 163–173.

Jacques, J. "Products: Avocados." Greencell, 19 May 2017, https://www.greencell.com/avocados/.

Jahanban-Esfahlan, A., Ostadrahimi, A., Tabibiazar, M., & Amarowicz, R. (2019). A comparative review on the extraction, antioxidant content and antioxidant potential of different parts of walnut (*Juglans regia* l.) fruit and tree. *Molecules*, *24*, 1–40.

Jankowski, P., & Masny, S. (2019). Comparison of mathematical models of maturation rate of the airborne *Venturia inaequalis* (Cooke) Wint. ascospores in central Poland. *Journal of Plant Diseases and Protection*, *126*(4), 269–279.

Janzen, D. H., & Martin, P. S. (1982). Neotropical anachronisms: the fruits the gomphotheres ate. *Science*, *215*(4528), 19–27.

Johnson, G. I., Highley, E., & Joyce, D. C. (1997). Disease resistance in fruit. In *Disease Resistance in Fruit* (pp. 1–233).

Johnson, G. I., Mead, A. J., Cooke, A. W., & Dean, J. R. (1991). Mango stem end rot pathogens — Infection levels between flowering and harvest. *Annals of Applied Biology*, *119*(3), 465–473.

Johnson, G. I., Mead, A. J., Cooke, A. W., & Dean, J. R. (1992). Mango stem end rot pathogens - Fruit infection by endophytic colonisation of the inflorescence and pedicel. *Annals of Applied Biology*, *120*(2), 225–234.

Johnson, P. R., & Ecker, J. R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. *Annual Review of Genetics*, *32*(1), 227–254.

Jonkers, H. (1979). Biennial bearing in apple and pear: A literature survey. *Scientia Horticulturae*, 11: 303-317.

Kähkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J.-P., Pihlaja, K., Kujala, T. S., & Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, *47*(10), 3954–3962.

Kahn, V. (1975). Polyphenol oxidase activity and browning of three avocado varieties. *Journal of the Science of Food and Agriculture*, *26*(9), 1319–1324.

Kahn, V. (1977). Latency properties of polyphenol oxidase in two avocado cultivars differing in their rate of browning. *Journal of the Science of Food and Agriculture*, *28*(3), 233–239.

Kamel, M., Cortesi, P., & Saracchi, M. (2015). Source and spread of fungal pathogens causing crown rot disease in organic bananas. *III International Symposium on Postharvest Pathology: Using Science to Increase Food Availability 1144* (pp. 253-258).

Karunanayake, K., & Adikaram, N. (2020). Stem-end rot in major tropical and sub-tropical fruit species. *Ceylon Journal of Science*, 49(5), 327-336.

Kaundal, R., Kapoor, A. A., & Raghava, G. P. S. (2006). Machine learning techniques in disease forecasting: A case study on rice blast prediction. *BMC Bioinformatics*, *7*(485), 1–16. https://doi.org/10.1186/1471-2105-7-485

Kimaru, S. K., Monda, E., Cheruiyot, R. C., Mbaka, J., & Alakonya, A. (2018). Morphological and molecular identification of the causal agent of anthracnose disease of avocado in Kenya. *International Journal of Microbiology*, 1–10. https://doi.org/10.1155/2018/4568520

Kobiler, L., Prusky, D., Midland, S., Sims, J. J., & Keen, N. T. (1993). Compartmentation of antifungal compounds in oil cells of avocado fruit mesocarp and its effect on susceptibility to *Colletotrichum gloeosporioides*. *Physiological and Molecular Plant Pathology*, *43*, 319–328.

Köhne, J. S., & Kremer-Köhne, S. (1995). Picking Hass avocados without pedicel. *South African Avocado Growers' Association Yearbook*, *18*(66).

Kremer-Kohne, S. (1987). Vegetative growth and fruit retention in avocado as affected by a new plant growth regulator (Paclobutrazol). *South African Avocado Grower's Association Yearbook*, *10*, 64–66.

Kremer-Köhne, S., & Köhne, J. S. (1992). Yield and fruit quality of Fuerte and Hass on clonal rootstocks. *South African Avocado Grower's Association Yearbook*, *15*(69).

Kremer-Köhne, S., Duvenhage, J. A., & Mailula, S. M. (2001). Breeding and field testing of new avocado rootstocks for increased Hass yields and resistance to root rot. *South African Avocado Growers Association Yearbook, 24*, 33–34.

Kupfer, B. & Schieder, U. (2022). "Avocados." Transport Informations Service, https://www.tis-gdv.de/tis_e/ware/obst/avocado/avocado-htm/.

Lalancette, N., Foster, K. A., & Robison, D. M. (2003). Quantitative models for describing temperature and moisture effects on sporulation of *Phomopsis amygdali* on peach. *Phytopathology*, *93*(9), 1165–1172.

Lange, D. L., & Kader, A. A. (1997). Elevated carbon dioxide exposure alters intracellular pH and energy charge in avocado fruit tissue. *Journal of the American Society for Horticultural Science*, *122*(2), 253–257.

Lawrence, D., Travadon, R., & Baumgartner, K. (2015). Diversity of *Diaporthe* species associated with wood cankers of fruit and nut crops in northern California. *Mycologia*, 107:5, 926-940.

Lebanon, I. (2016). *Fruits and vegetables market of South Africa*. Beirut. Retrieved from http://investinlebanon.gov.lb/Content/uploads/CorporatePageRubric/160624121620358~Fruit and Vegetable Market of South Africa (2014).pdf

Li, G., Slippers, B., Wingfield, M. J., & Chen, S. (2020). Variation in Botryosphaeriaceae from Eucalyptus plantations in YunNan Province in southwestern China across a climatic gradient. *IMA Fungus*, 11(1), 1-49.

Li, L., Mohd, M. H., & Zakaria, L. (2020). Colletotrichum species associated with mango (*Mangifera indica L.*) stem-end rot. *Journal of Plant Pathology*, *102*(2), 505-509.

Louw, M. (2022). "Avocado Production: Fruit Farming in South Africa." South Africa Online, https://southafrica.co.za/avocado-production.html

Luo Lijuan, Pinggen, X., Zide, J., & Peikun, Q. (2004). Sporulation conditions of Phomopsis in pure culture. *Mycosystema*, *23*(2), 219–225.

Madhupani, Y. D. S., & Adikaram, N. K. B. (2017). Delayed incidence of stem-end rot and enhanced defences in Aureobasidium pullulans-treated avocado (Persea americana Mill.) fruit. *Journal of Plant Diseases and Protection*, *124*, 227–234.

Maharachchikumbura, S. S., Hyde, K. D., Groenewald, J. Z., Xu, J., & Crous, P. W. (2014). *Pestalotiopsis* revisited. *Studies in Mycology*, 79(1), 121-186.

Mailafia, S., Okoh, G. R., Olabode, H. O. K., & Osanupin, R. (2017). Isolation and identification of fungi associated with spoilt fruits vended in Gwagwalada market, Abuja, Nigeria. *Veterinary World*, *10*(4), 393–397.

Mandemaker, A. J., Elmsly, T. A., & Smith, D. B. (2006). Effects of drop heights and fruit harvesting methods on the quality of "Hass" avocados. *New Zealand Avocado Growers' Association Annual Research Report*, *6*, 97–104.

Maqsood, A., Rehman, A., Ahmad, I., Nafees, M., Ashraf, I., Qureshi, R., ... & Hussain, T. (2014). Physiological attributes of fungi associated with stem end rot of mango (*Mangifera indica L.*) cultivars in postharvest fruit losses. *Pakistan Journal of Botany*, *46*(5), 1915-1920.

Masangkay, R. F., Paulitz, T. C., Hallett, S. G., & Watson, A. K. (2000). Characterization of sporulation of *Alternaria alternata* f. sp. sphenocleae. *Biocontrol Science and Technology*, *10*(4), 385-397.

McCallan, S. E. A. (1949). The nature of the fungicidal action of copper and sulfur. *The Botanical Review*, *15*(9), 629–643.

Meir, S., Naiman, D., Akerman, M., Hyman, J. Y., Zauberman, G., & Fuchs, Y. (1997). Prolonged storage of `Hass' avocado fruit using modified atmosphere packaging. *Postharvest Biology and Technology*, *12*(1), 51–60.

Merelo, P., Agustí, J., Arbona, V., Costa, M. L., Estornell, L. H., Gómez-Cadenas, A., ... & Tadeo, F. R. (2017). Cell wall remodelling in abscission zone cells during ethylene-promoted fruit abscission in citrus. *Frontiers in Plant Science*, 8, 126.

Meyberg, M. (1988). Selective staining of fungal hyphae in parasitic and symbiotic plant-fungus associations. *Histochemistry*, *88*(2), 197–199.

Michailides, T., Felts, D. G., Luo, Y., Brisach, C. A., Moral, J., Puckett, R., … Cunningham, C. (2015). Epidemiology and management of *Botryosphaeria*/*Phomopsis* canker and blight and anthracnose blight of walnut in California. *California Walnut Board*, 349–376.

Miles, T. D., Hancock, J. F., Callow, P., & Schilder, A. M. C. (2012). Evaluation of screening methods and fruit composition in relation to anthracnose fruit rot resistance in blueberries. *Plant Pathology*, *61*(3), 555–566.

Miteva-Staleva, J., Krumova, E., Stefanova, T., & Angelova, M. (2015). Age-related changes in reactive oxygen species production in the filamentous fungus *Penicillium rugulosum* T35 under cold stress conditions. *Comptes rendus de l'Académie bulgare des Sciences*, 68(9), 1123-1129.

Mitiku, M. (2017). Assessment of Important Diseases of Major Fruit Crops (Mango, Avocado, Papaya, and Orange) in South Omo Zone of Ethiopia. *Journal of Natural Sciences Research*, *7*(5), 68–71.

Miyara, I., Shafran, H., Kramer Haimovich, H., Rollins, J., Sherman, A., & Prusky, D. (2008). Multi-factor regulation of pectate lyase secretion by Colletotrichum gloeosporioides pathogenic on avocado fruits. *Molecular Plant Pathology*, *9*(3), 281–291.

Mondal, S. N., Vicent, A., Reis, R. F., & Timmer, L. W. (2007). Saprophytic colonization of citrus twigs by *Diaporthe citri* and factors affecting pycnidial production and conidial survival. *Plant Disease*, 91(4), 387-392.

Montealegre, J. R., Ramírez, M., Riquelme, D., Armengol, J., León, M., & Pérez, L. M. (2016). First report of *Neofusicoccum australe* in Chile causing avocado stem-end rot. *Plant Disease*, *100*(12), 2532.

Morrissey, J. P., & Osbourn, A. E. (1999). Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiology and Molecular Biology Reviews : MMBR*, *63*(3), 708–724.

Muirhead, I. F., Fitzell, R. D., Davis, R. D., & Peterson, R. A. (1982). Post-harvest control of anthracnose and stem-end rots of Fuerte avocados with prochloraz and

other fungicides. *Australian Journal of Experimental Agriculture and Animal Husbandry*, *22*(119), 441–446.

National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. 1988. Available from: https://www.ncbi.nlm.nih.gov/

Netto, M. S., Assunção, I. P., Lima, G. S., Marques, M. W., Lima, W. G., Monteiro, J. H., ... & Câmara, M. P. (2014). Species of *Lasiodiplodia* associated with papaya stem-end rot in Brazil. *Fungal Diversity*, *67*(1), 127-141.

Nilmini, R. K., Panapitiya, D., Abeywickrama, K. and Kuruppu, M. (2020). Morphological and molecular identification of fungal species associated with postharvest stem-end rot disease of avocado in Sri Lanka. *Sri Lanka Journal of Food and Agriculture*, 6(1), 47–56.

Obianom, C., & Sivakumar, D. (2017). Natural plant volatiles as an alternative approach to control stem-end rot in avocado cultivars. *Journal of Phytopathology*, *166*(August), 1–9.

Oetiker, J. H., & Yang, S. F. (1995). The role of ethylene in fruit ripening. In *Acta Horticulturae* (pp. 167–178). International Society for Horticultural Science (ISHS), Leuven, Belgium.

Oksanen, J. (2015). Multivariate analysis of ecological communities in R: vegan tutorial. *R Documentation*, 43.

Ortiz-Viedma, J., Rodriguez, A., Vega, C., Osorio, F., Defillipi, B., Ferreira, R., & Saavedra, J. (2018). Textural, flow and viscoelastic properties of Hass avocado (*Persea americana* Mill.) during ripening under refrigeration conditions. *Journal of Food Engineering*, *219*, 62–70.

Palou, L., Crisosto, C. H., Garner, D., & Basinal, L. M. (2003). Effect of continuous exposure to exogenous ethylene during cold storage on postharvest decay development and quality attributes of stone fruits and table grapes. *Postharvest Biology and Technology*, *27*(3), 243–254.

Papademetriou, M. K. (1975) Percentage fruit set in avocados (*Persea americana* Mill.). *California Avocado Society Yearbook* 58: 99-102.

Papadopoulou-Mourkidou, E. (1991). Postharvest-applied agrochemicals and their residues in fresh fruits and vegetables. *Association of Official Analytical Chemists*, *74*(5), 745–765.

Park, S. Y., Jang, S. H., Oh, S. O., Kim, J. A., & Hur, J. S. (2014). An easy, rapid, and cost-effective method for DNA extraction from various lichen taxa and specimens suitable for analysis of fungal and algal strains. *Mycobiology*, *42*(4), 311–316.

Peleg, K., Ben-Hanan, U., & Hinga, S. (1990). Classification of avocado by firmness and maturity. *Journal of Texture Studies*, *21*(2), 123–139.

Peres, N. A. R., Kuramae, E. E., Dias, M. S. C., & De Souza, N. L. (2002). Identification and characterization of *Colletotrichum spp.* affecting fruit after harvest in Brazil. *Journal of Phytopathology*, *150*, 128–134.

Pérez-Jiménez, R. M. (2008). Significant avocado diseases caused by fungi and oomycetes. *The European Journal of Plant Science and Biotechnology*, *2*(1), 1–24.

Peterson, R. A. (1978). Susceptibility of Fuerte avocado fruit at various stages of growth, to infection by anthracnose and stem end rot fungi. *Australian Journal of Experimental Agriculture and Animal Husbandry*, 18: 158-160.

Peterson, R. A. (1978). Susceptibility of Fuerte avocado fruit at various stages of growth, to infection by anthracnose and stem end rot fungi. *Australian Journal of Experimental Agriculture*, *18*(90), 158–160.

Piper, R. B. & Gardner, F. E. (1943). Comparative seasonal development of avocado fruits and seeds. *Proceedings of the Florida State Horticultural Society*, 56:122-124.

Podila, G. K., Rogers, L. M., & Kolattukudy, P. E. (1993). Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. *Plant Physiology*, *103*(1), 267–272.

Porat, R., Weiss, B., Cohen, L., Daus, A., Goren, R., & Droby, S. (1999). Effects of ethylene and 1-methylcyclopropene on the postharvest qualities of "Shamouti" oranges. *Postharvest Biology and Technology*, *15*(2), 155–163.

Prusky, D. (1996). Pathogen quiescence in postharvest diseases. *Annual Reviews of Phytopathology*, *34*, 413–434.

Prusky, D. (2009). Mechanisms of resistance and susceptibility of mango fruits to fungal attack, and their modulation by post-harvest handling and storage. In *Acta Horticulturae* (pp. 487–492). International Society for Horticultural Science (ISHS), Leuven, Belgium.

Prusky, D., Alkan, N., Mengiste, T., & Fluhr, R. (2013). Quiescent and necrotrophic lifestyle choice during postharvest disease development. *Annual Review of Phytopathology*, *51*(1), 155–176.

Prusky, D., Keen, N. T., Sims, J. J., & Midland, S. L. (1982). Possible involvement of an antifungal diene in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Phytopathology*, *72*(12), 1578–1582.

Prusky, D., Kobiler, I., & Jacoby, B. (1988). Involvement of epicatechin in cultivar susceptibility of avocado fruits to *Colletotrichum gloeosporioides* after harvest. *Journal of Phytopathology*, *123*(2), 140–146.

Prusky, D., Wattad, C., & Kobiler, I. (1996). Effect of ethylene on activation of lesion development from quiescent infections of *Colletotrichum gloeosporioides* in avocado fruits. *Molecular Plant-Microbe Interactions*, *9*(9), 864–868.

Ramírez-Gil, J. G., Castañeda-Sánchez, D. A., & Morales-Osorio, J. G. (2017). Production of avocado trees infected with *Phytophthora cinnamomi* under different management regimes. *Plant Pathology*, 66(4), 623-632.

Ramírez-Gil, J. G., Henao-Rojas, J. C., & Morales-Osorio, J. G. (2021). Postharvest diseases and disorders in avocado cv. Hass and their relationship to preharvest management practices. *Heliyon*, 7(1).

Rampersad, S. N. (2020). Pathogenomics and management of *Fusarium* diseases in plants. *Pathogens*, *9*(5), 340.

Rendón-Anaya, M., Ibarra-Laclette, E., Méndez Bravo, A., Lan, T., Zheng, C., Carretero-Paulet, L., … Herrera-Estrella, L. (2019). The avocado genome informs deep angiosperm phylogeny, highlights introgressive hybridization, and reveals pathogen-influenced gene space adaptation. *BioRxiv*.

Report, F. (2013). *Simplification of the EU Pesiticides Regulatory Regime*.

Rice-evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., & Pridham, J. B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*, *22*(4), 375–383.

Roberts, J. A., Elliott, K. A., and Gonzalez-Carranza, Z. H. (2002). Abscission, dehiscence, and other cell separation processes. *Annual Reviews of Plant Biology*, 53, 131–158.

Robinson P. W., Mickelbart, M. V., Liu, X., Adams, C., Witney, G., & Arpaia, M. L. (2002). Development of a phenological model of avocado tree growth in California. *Acta Horticulurae*, 575: 859-864.

Rosado, A. W. C., Machado, A. R., Freire, F. D. C. O., & Pereira, O. L. (2016). Phylogeny, identification, and pathogenicity of *Lasiodiplodia* associated with postharvest stem-end rot of coconut in Brazil. *Plant Disease*, *100*(3), 561-568.

Saha, A., Mandal, P., Dasgupta, S., & Saha, D. (2008). Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. *Journal of Environmental Biology*, *29*(3), 407.

Sanders, G. M., Korsten, L., & Wehner, F. C. (2000). Survey of fungicide sensitivity in *Colletotrichum gloeosporioides* from different avocado and mango production areas in South Africa. *European Journal of Plant Pathology*, *106*, 745–752.

Santos, A. C. D. S., Trindade, J. V. C., Lima, C. S., Barbosa, R. D. N., da Costa, A. F., Tiago, P. V., & de Oliveira, N. T. (2019). Morphology, phylogeny, and sexual stage of *Fusarium caatingaense* and *Fusarium pernambucanum*, new species of the *Fusarium incarnatum-equiseti* species complex associated with insects in Brazil. *Mycologia*, 111(2), 244-259.

Schaffer, B. A., Wolstenholme, B. N., & Whiley, A. W. (Eds.). (2013). The avocado: botany, production and uses.

Schumann, G. L., & J D'Arcy, C. (2010). *Essential plant pathology* (2nd ed.). American Phytopathological Society Press.

Sedgley, M. and Annellls, CM. (1981). Flowering and fruit-set response to temperature in the avocado cultivar 'Hass'. *Scientia Horticulturae*, 14: 27-33.

Self, G. K., Ordozgoiti, E., Povey, M. J. W., & Wainwright, H. (1994). Ultrasonic evaluation of ripening avocado flesh. *Postharvest Biology and Technology*, *4*(1–2), 111–116.

Sellamuthu, P. S., Sivakumar, D., & Soundy, P. (2013). Antifungal activity and chemical composition of thyme, peppermint and citronella oils in vapor phase against avocado and peach postharvest pathogens. *Journal of Food Safety*, *33*, 86–93.

Shah, D. A., Paul, P. A., De Wolf, E. D., & Madden, L. V. (2019). Predicting plant disease epidemics from functionally represented weather series. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *374*(1775).

Shahbandeh, M. (2022). "Avocado Production Worldwide 2020." *Statista*, 21 Jan. 2022, https://www.statista.com/statistics/577455/world-avocado-production/

Sharma, G., Maymon, M., & Freeman, S. (2017). Epidemiology, pathology and identification of *Colletotrichum* including a novel species associated with avocado (*Persea americana*) anthracnose in Israel. *Scientific Reports*, *7*(1), 1–16.

Sharma, R. R., Singh, D., & Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control*, *50*(3), 205–221.

Shetty, K. G., Rivadeneira, D. V., Jayachandran, K., & Walker, D. M. (2016). Isolation and molecular characterization of the fungal endophytic microbiome from conventionally and organically grown avocado trees in South Florida. *Mycological Progress*, *15*, 977–986.

Shorter, B. (2015). "Food Transport." WinACC, Winchester Action on the Climate Crisis, https://www.winacc.org.uk/wp-content/uploads/2015/11/Laymans-Food-Transport.pdf.

Singh, M., & Kamal, S. (2011). Validity of mycelial growth on malt extract agar and compost as selection criteria for initial screening of genotypes for yield and quality in *Agaricus bisporus*. In *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7), Arcachon, France* (pp. 71-16).

Slippers, B., Smit, W. A., Crous, P. W., Coutinho, T. A., Wingfield, B. D., & Wingfield, M. J. (2007). Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. *Plant Pathology*, *56*(1), 128–139.

Smilanick, J. L., Margosan, D. A., Arpaia, M. L., Sievert, J. R., Fjeld, K., & Collin, S. (2001). Management of Postharvest Decay of Avocado Fruit. In *Proceedings of the California Avocado Research Symposium*, (pp. 105–112).

Sommaruga, R., & Eldridge, H. M. (2021). Avocado Production: Water Footprint and Socio-economic Implications. *EuroChoices*, 20(2), 48-53.

Sowley, E. N. K., Dewey, F. M., & Shaw, M. W. (2010). Persistent, symptomless, systemic, and seed-borne infection of lettuce by Botrytis cinerea. *European Journal of Plant Pathology*, *126*(1), 61–71.

Spadaro, D., & Droby, S. (2016). Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. *Trends in Food Science and Technology*.

Stirling, A. M., Stirling, G. R., Pegg, K. G., & Hayward, A. C. (1999). Effect of copper fungicide on *Colletotrichum gloeosporioides* and other microorganisms on avocado leaves and fruit. *Australian Journal of Agricultural Research*, *50*(8), 1459–1468.

Swinburne, T. R. (1978). Post-infection anti-fungal compounds in quiescent or latent infections. *Annals of Applied Biology*, *89*, 322–325.

Swinburne, T. R. (1993). *Colletotrichum*: biology, pathology and control. *The Journal of Agricultural Science*, *121*(1), 136–137.

Talbot, N. J., Ebbole, D. J., & Hamer, J. E. (1993). Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *The Plant Cell*, 5(11), 1575-1590.

Taylor, J. E., Hyde, K. D., & Jones, E. B. G. (2000). The Biogeographical Distribution of Microfungi Associated with Three Palm Species from Tropical and Temperate Habitats. *Journal of Biogeography*, 27(2), 297–310.

Tesfay, S. Z., Magwaza, L. S., Mbili, N., & Mditshwa, A. (2017). Carboxyl methylcellulose (CMC) containing moringa plant extracts as new postharvest organic edible coating for Avocado (*Persea americana* Mill.) fruit. *Scientia Horticulturae*, *226*, 201–207.

Thompson, S. M., Tan, Y. P., Young, A. J., Neate, S. M., Aitken, E. A. B., & Shivas, R. G. (2011). Stem cankers on sunflower (*Helianthus annuus*) in Australia reveal a complex of pathogenic *Diaporthe* (*Phomopsis*) species. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, *27*(December), 80–89.

Thor, K. (2019). Calcium—nutrient and messenger. *Frontiers in Plant Science*, 10, 440.

Thynne, E., Mead, O. L., Chooi, Y. H., Mcdonald, M. C., & Solomon, P. S. (2019). Acquisition and loss of secondary metabolites shaped the evolutionary path of three emerging phytopathogens of wheat. *Genome Biology and Evolution*, *11*(3), 890–905.

Tian, Y., Zhao, Y., Sun, T., Wang, L., Liu, J., Ma, X., & Hu, B. (2018). Identification and characterization of *Phomopsis amygdali* and *Botryosphaeria dothidea* associated with peach shoot blight in Yangshan, China. *Plant Disease*, *102*(12), 2511–2518.

Tieman, D. M., Taylor, M. G., Ciardi, J. A., & Klee, H. J. (2000). The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. *Proceedings of the National Academy of Sciences*, *97*(10), 5663–5668.

Tingwa, P. O., & Young, R. E. (1975). Studies on the inhibition of ripening in attached avocado (Persea americana Mill.) fruits. *J Am Soc Hort Sci*, *100*, 447–449.

Tingwa, P. O., & Young, R. E. (1975). Studies on the inhibition of ripening in attached avocado (*Persea americana* Mill.) fruits. *Journal of the American Society for Horticultural Science*, 100: 447-449.

Tranbarger, T. J., Tucker, M. L., Roberts, J. A., & Meir, S. (2017). Editorial: Plant Organ Abscission: From Models to Crops. *Frontiers in Plant Science*, 8, 196.

Tucker, M. L., and Kim, J. (2015). Abscission research: what we know and what we still need to study. *Stewart Postharvest Review*, 2:1.

Twizeyimana, M., Forster, H., McDonald, V., Wang, D. H., Adaskaveg, J. E., & Eskalen, A. (2013). Identification and pathogenicity of fungal pathogens associated with stem-end rot of avocado in California. *Plant Disease*, *97*(12), 1580–1584.

Udayanga D., Castlebury L. A., Rossman A. Y., Chukeatirote E., Hyde K. D. (2015). The *Diaporthe sojae* species complex, phylogenetic re-assessment of pathogens associated with soybean, cucurbits, and other field crops. *Fungal Biology*, 119, 383–407.

Udayanga, D., Liu, X., McKenzie, E. H. C., Chukeatirote, E., Bahkali, A. H. A., & Hyde, K. D. (2011). The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. *Fungal Diversity*, *50*(September), 189–225.

Valencia, A. L., Gil, P. M., Latorre, B. A., & Rosales, I. M. (2019). Characterization and pathogenicity of Botryosphaeriaceae species obtained from avocado trees with branch canker and dieback and from avocado fruit with stem end rot in Chile. *Plant Disease*, 103(5), 996-1005.

Valencia, A. L., Torres, R., & Latorre, B. A. (2011). First report of *Pestalotiopsis clavispora* and *Pestalotiopsis spp.* causing postharvest stem end rot of avocado in Chile. *Plant Disease*, *95*(4), 492.

Van Rooyen, Z., & Bower, J. P. (2005). The role of fruit mineral composition on fruit softness and mesocarp discolouration in “Pinkerton” avocado (*Persea americana* Mill.). *Journal of Horticultural Science and Biotechnology*, *80*(6), 793–799.

Van Woensel, G., Wouters, A., & De Baerdemaeker, J. (1987). Relation between mechanical properties of apple fruit and sensory quality. *Journal of Food Process Engineering*, *9*(3), 173–189.

Van Zwieten, L., Rust, J., Kingston, T., Merrington, G., & Morris, S. (2004). Influence of copper fungicide residues on occurrence of earthworms in avocado orchard soils. *Science of the Total Environment*, *329*(1–3), 29–41.

Wang, X., Kobiler, I., Lichter, A., Leikin-Frenkel, A., Pesis, E., & Prusky, D. (2006). 1-MCP prevents ethylene-induced accumulation of antifungal diene in avocado fruit. *Physiological and Molecular Plant Pathology*, *67*(3–5), 261–267.

Wanjiku, E. K., Waceke, J. W., Wanjala, B. W., & Mbaka, J. N. (2020). Identification and pathogenicity of fungal pathogens associated with stem end rots of avocado fruits in Kenya. *International Journal of Microbiology*, 1–8.

Weaire, P. J., & Kekwick, R. G. (1975). The synthesis of fatty acids in avocado mesocarp and cauliflower bud tissue. *The Biochemical Journal*, *146*, 425–437.

Wharton, P. S., Julian, a M., & O'Connell, R. J. (2001). Ultrastructure of the infection of *Sorghum bicolor* by *Colletotrichum sublineolum*. *Phytopathology*, *91*(2), 149–158.

White, A., Woolf, A., Hofman, P., & Lu Arpaia, M. (2009). *The international avocado quality pocketbook*. Auckland, New Zealand: The New Zealand Institute for Plant and Food Research Limited.

Witney, G. W., Hofman, P. J., & Wolstenholme, B. N. (1990). Mineral distribution in avocado trees with reference to calcium cycling and fruit quality. *Scientia Horticulturae*, *44*(3–4), 279–291.

Wolstenholme, B. N. (2013). Developments in the world avocado industry, and their relevance to the South African and African industries. In *Acta Horticulturae* (pp. 865–871). International Society for Horticultural Science (ISHS), Leuven, Belgium.

Wolstenholme, B. N., & Whiley, A. W. (1999). Ecophysiology of the avocado (*Persea americana* Mill.) tree as a basis for pre- harvest management. *Revista Chapingo Serie Horticultura*, *5*, 77–88.

Wolstenholme, B. N., & Whiley, A. W. (1999). Ecophysiology of the avocado (*Persea americana* Mill.) tree as a basis for pre-harvest management. *Revista Chapingo Serie Horticultura*, *5*, 77–88.

Wong, C. F. J., Nik, W. Z., & Lim, T. K. (1983). Studies of *Colletotrichum dematium* f. sp. *truncatum* on soybean. *Pertanika*, *6*(1), 28–33.

Wrona, C. J., Mohankumar, V., Schoeman, M. H., Tan, Y. P., Shivas, R. G., Jeff-Ego, O. S., & Akinsanmi, O. A. (2020). *Phomopsis* husk rot of macadamia in Australia and South Africa caused by novel *Diaporthe* species. *Plant Pathology*, 69(5), 911-921.

Yan, J. Y., Xie, Y., Zhang, W., Wang, Y., Liu, J. K., Hyde, K. D., … Li, X. H. (2013). Species of Botryosphaeriaceae involved in grapevine dieback in China. *Fungal Diversity*, *61*, 221–236.

Yang, Q., Fan, X. L., Du, Z., & Tian, C. M. (2017). *Diaporthe juglandicola* sp. nov.(Diaporthales, Ascomycetes), evidenced by morphological characters and phylogenetic analysis. *Mycosphere*, 8(5), 817-826.

You, M. P., & Sivasithamparam, K. (1995). Changes in microbial populations of an avocado plantation mulch suppressive of *Phytophthora cinnamomi*. *Applied Soil Ecology*, *2*(1), 33–43.

Yuen, J. E., & Hughes, G. (2002). Bayesian analysis of plant disease prediction. *Plant Pathology*, *51*, 407–412.

Zauberman, G., Schiffman-Nadel, M., & Yanko, U. (1977). The response of avocado fruits to different storage temperatures. *Horticulture Science*, *12*(4), 353–354.

Zauberman, G., Schiffman-Nadel, M., Yanko, U., Sarig, I. & Alper, I. (1969). Factors causing injury during transportation of avocado pears to packing houses. *The National and University Institute of Agriculture; The Volcani Institute of Agricultural Research*. Preliminary Report 705.

Zentmyer, G. A. (1984). Avocado diseases. *Tropical Pest Management*, *30*(4), 388–400.

Zhang, J. (2014). *Lasiodiplodia theobromae* in Citrus fruit (*Diplodia* stem-end rot). In *Postharvest Decay* (pp. 309-335). Academic Press.

Zhao, J. P., Lu, Q., Liang, J., Decock, C., & Zhang, X. Y. (2010). *Lasiodiplodia pseudotheobromae*, a new record of pathogenic fungus from some subtropical and tropical trees in southern China. *Cryptogamie-Mycologie*, 31(4), 431.

Zhu, C., Lei, M., Andargie, M., Zeng, J., & Li, J. (2019). Antifungal activity and mechanism of action of tannic acid against *Penicillium digitatum*. *Physiological and Molecular Plant Pathology*, 107, 46-50.