Postharvest Decay of Blueberry Fruit in New Zealand

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Abstract

Postharvest decay limits the storage life of fresh blueberries and is unacceptable in export markets. Currently, no surveys have been conducted to determine the microorganisms causing decay of blueberry fruit during postharvest storage in New Zealand. Identification of the major microorganisms associated with postharvest decay is critical to effective management of decay within the supply chain. This thesis identifies the microorganisms associated with postharvest decay of blueberry fruit with the aim of observing the prevalence and nature of these rots after cold storage.

Four combinations of temperature and relative humidity (RH) were used to store blueberry fruit. These treatments were obtained by a factorial combination of two temperatures, 1°C and 15°C, and two relative humidity (RH) conditions of high RH (98-100% RH) or low RH (80% RH). The 1°C storage at either RH was implemented for 42 d, while the 15°C storage had a duration of 14 d. Following storage, the fruit was stored at 20°C for 7 days to simulate shelf life (retail or home use), and decay prevalence evaluated. From the decay signs observed, microorganisms were isolated and identified through morphological and molecular analysis.

The major microorganisms isolated from blueberry fruit decay were identified as *Botrytis cinerea*, *Colletotrichum acutatum* species complex, and *Pestalotiopsis* spp. Both *B. cinerea* and *C. acutatum* spp. complex are causal agents of decay signs, responsible for grey mould and anthracnose, respectively. *Pestalotiopsis* spp. was associated with decay of blueberry fruit, but it is unclear whether this is a causal agent of decay.

The postharvest storage conditions influenced the prevalence and behaviour of decay. At 1°C, *B. cinerea* was the dominant causal agent of decay, leading to grey mould sign (extensive grey/tan mycelium) at 98-100% RH. When blueberries were stored at low RH (80%), *B. cinerea* infections were mostly restricted to the fruit stem scar.

During shelf life, after 1°C storage for 42 days, decay showed a higher accumulation rate than when the fruit was stored at 15°C prior to shelf life. In addition, after shelf life, *B. cinerea* and the *C. acutatum* spp. complex were the most predominant causal organisms isolated from decayed fruit.

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Chapter 1: Introduction

Blueberries are a fruit crop growing in popularity worldwide. Consumption is increasing due to growing consumer awareness of the health properties of blueberries, leading to their incorporation into healthy lifestyles. Blueberries have been shown to improve brain activity (Bowtell et al., 2017), memory (Whyte et al., 2016), neuroplasticity (Casadesus et al., 2004), reduce the risk of heart disease (Cassidy et al., 2016), and improve muscle recovery (McLeay et al., 2012). These findings have contributed to the recognition of blueberries as a superfood (Bell & Williams, 2021). Health benefits, along with their enjoyable taste and attractive appearance, are drivers of global demand.

The New Zealand (NZ) blueberry industry is an $80 million industry as of 2020, with 680 ha in production (Plant and Food Research, 2021). The main species cultivated are Highbush (*Vaccinium corybosum* L.) and Rabbiteye (*Vaccinium ashei* Reade). The nature of these species and the cultivars used provide an extended harvest window in NZ from October to March. In 2020, export revenue from NZ blueberries was $44 million, an increase of 275% from 2010. In comparison to the overall value of horticultural production in NZ of $6.7 billion, mostly comprising kiwifruit, apples, and wine, blueberries represent 0.6% of this value. However, the value of the blueberry industry is growing fast, with a 50% increase in export value since 2010.

Given the recent growth of the NZ blueberry industry, a major aim is to improve supply chain efficiency (Coriolis Research, 2020). At present, blueberries are shipped via sea to Australia, and air freight is used to export to high-value Asian markets. Air freight is a significant expense, which can make up 50% of the landed cost, but it is time efficient and ensures fruit arrive with high quality (Coriolis Research, 2020). The alternative is marine transport, which is substantially cheaper but of longer duration, taking up to four weeks to reach Asia. The problems stemming from the distance to market and the cost of air freight provide challenges to the blueberry industry, which are compounded by the short storage life of the fruit.

Blueberries have a short storage life, making quality maintenance during long distance exportation a central concern. Storage life is determined largely by preharvest factors, including cultivar, maturity stage, and harvest technique (Forney, 2009). Fruit quality cannot be improved after harvest, so technologies are applied to preserve at harvest quality for as long as possible. Refrigeration applied two hours after harvest at 0°C with 85–95% humidity and high carbon dioxide controlled atmosphere conditions provide optimal conditions to support fruit quality during long distance oceanic travel (Madrid & Beaudry, 2020; Retamales & Hancock, 2018). If these postharvest conditions are applied, storage life can be 3–8 weeks, depending on cultivar, presence of disease, and fruit quality at harvest (Chen et al., 2015; Hancock et al., 2008; Madrid

& Beaudry, 2020). Therefore, maritime transport to Asian markets can be undertaken, however, there is a critical risk of quality deterioration if optimal management techniques are absent.

Postharvest losses are a result of quality deterioration indicated by decay, softening, shrivel, and physical damage (Forney, 2009; Tournas & Katsoudas, 2005). Decay is the most important cause of postharvest losses and is caused by pathogenic and opportunistic microorganisms (Vicente et al., 2007). Softening is a result of cell wall degradation and weight loss; it leads to increased pathogen susceptibility and decreased consumer acceptance (Chen et al., 2015; Paniagua et al., 2013). Shrivel is a result of transpiration mainly through the cuticle and stem scar (Moggia et al., 2017a; Yan & Castellarin, 2022). The result of shrivel is softening and a corrugated appearance (Paniagua et al., 2013). Mechanical damage is a consequence of poor handling, leading to bruising and subsequent softening and decay (Opara & Pathare, 2014; Xu et al., 2015). These issues are detrimental to fruit quality and limit the storage life of fresh blueberries. However, decay remains the major cause of postharvest losses throughout the supply chain, responsible for significant losses and wastage of fresh produce (Agrios, 2005).

Decay of fresh produce is indicated by the presence of signs or symptoms of decay resulting from microorganism infection. It is an important indication of quality loss from the consumer perspective (Prusky, 2011). The major genera reported to affect blueberry fruit are *Botrytis, Colletotrichum,* and *Alternaria* (Cappellini et al., 1982). Signs of decay are direct evidence of microorganism presence. These include mycelium and other fungal bodies and exudates. Symptoms are the effects of infection on the host, for blueberry fruit, these include leakage of juice, collapsed fruit tissue, and slip skin (Latorre et al., 2014; Paniagua et al., 2014).

Plants are continually exposed to potentially pathogenic microorganisms, yet disease remains relatively infrequent among plants (Ferreira et al., 2006). This is because decay requires the coincidence of pathogen presence and virulence, a susceptible host, and conducive environmental conditions in order to produce decay signs or symptoms (Agrios, 2005; Ferreira et al., 2006). These requirements for decay are variable throughout the production system and in the postharvest environment, resulting in variable decay incidence.

When considering the causes of decay, a schematic representation of the factors influencing the presence of plant disease is helpful for determining causal factors and implementing control measures. The disease triangle (**Fig. 1.1**) consists of three sides, each representing either environmental, pathogen, or host factors influencing disease (Agrios, 2005). The length of each side relates to the sum of factors affecting disease incidence within each heading. The area of the triangle is an estimate of the quantity of disease

experienced. This method of visually quantifying the factors affecting disease incidence is relevant for determining the sources and compounding factors resulting in total disease incidence.

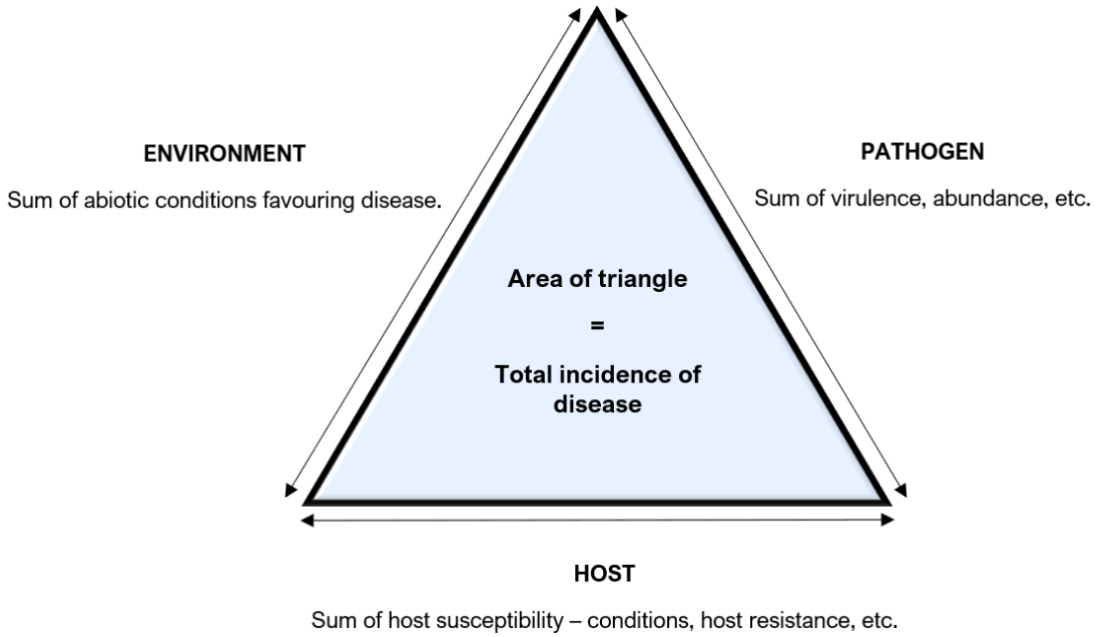


Figure 1.1. The disease triangle. Adapted from Agrios (2005).

As the disease triangle factors must be filled for the development of decay, control of decay is possible through the restriction of one or more of these factors. In the context of postharvest blueberry fruit, the main technology used is cold storage at 0–2°C. This can provide a 3–8 week storage life, depending on cultivar (Hancock et al., 2008). This technology changes the environment, slowing the decline of host resistance and limiting the pathogenicity of microorganisms. This leads to these factors being unfulfilled and decay being less prevalent (Stretch & Davis, 1960). Other control measures manipulate these factors, including through chemical, biological, and breeding methods. The disease triangle is central to understanding the origins of disease and the potential for control. It also indicates the sources of variability from all sides of the disease triangle in the expression of postharvest decay.

Currently, in New Zealand, a detailed and current survey of the postharvest pathogens affecting blueberry postharvest storage is absent. Conducting a survey of the decays occurring during postharvest storage and shelf life and identifying the microorganisms responsible is the first step in developing control strategies to improve blueberry fruit quality in the supply chain.

1.1 Research Objectives

This research intends to provide information on the microorganisms related to the decay of fresh NZ grown blueberry fruit under various postharvest conditions. The specific objectives are:

1. To determine the microorganisms associated with the postharvest decay of blueberry fruit using morphological and molecular techniques.
2. To evaluate the prevalence of postharvest decay and associated microorganisms during postharvest storage under different temperature and relative humidity conditions.

With the completion of this investigation, the author hopes to support the ability of blueberry exporters to maintain fruit quality when exporting fruit to markets requiring long distance maritime transportation. This work will provide insight into the nature of decay related microorganisms of blueberry fruit in commercial postharvest conditions. If the objectives are met, this research will facilitate targeted control measures to improve blueberry quality in the postharvest supply chain.

Chapter 2: Literature Review

This literature review chapter will provide an overview of postharvest blueberry fruit decay in relation to the disease triangle factors of host susceptibility, favourable environmental conditions, and virulent microorganisms. In addition, this chapter will review the morphological and molecular methods used to identify causal microorganisms.

2.1 The New Zealand Blueberry Industry

Blueberries were first grown in New Zealand (NZ) in the 1950s on acidic soils in the Waikato region. Since then, the industry has bred cultivars specific to NZ conditions and adopted a variety of international cultivars for domestic consumption and exportation, mostly as fresh blueberries (Retamales & Hancock, 2018). The cultivars grown enable a main production season from November to March (Retamales & Hancock, 2018). Blueberries are mostly grown in the Waikato, with production also occurring in Hawkes Bay and the Bay of Plenty (Plant and Food Research 2021). These regions experience a temperate climate with mild winters, warm summers, and regular rainfall (Plant and Food Research, 2021). Blueberry production in NZ is enabled by multiple production systems, including unprotected outdoors, under netting, tunnel houses, and indoor cropping. Soil-less systems have enabled the blueberry industry to expand into new regions and increase production. The New Zealand blueberry industry is small on the global scale but is innovative and forward thinking, with numerous cultivars grown in a variety of production systems to enable fruit availability for most of the year.

2.2 Blueberry Fruit Biology

Blueberry fruit are harvested from a multitude of cultivars, including both Highbush (*Vaccinium corymbosum* and interspecific hybrids) and Rabbiteye (*Vaccinium ashei*) blueberries, which offer a variety of growing and postharvest characteristics. Blueberries are botanical berries with a roughly spherical shape and range in surface skin colour from light to dark blue, depending on cultivar. Blueberries have an outer epidermis featuring a wax layer known as bloom and feature a stem scar and a calyx scar (Retamales & Hancock, 2018). The epidermal cells have thickened cell walls, which form the cuticle. Fruit tissue, known

as the mesocarp, consists of parenchyma cells connected by the pectin–rich middle lamella. At the centre of the fruit is the endocarp, which contains the seeds.

Blueberry fruit begin as a simple floral bud differentiated from one year old wood late in the preceding season. Flower buds burst in the spring, opening over a three to four week period during which pollination occurs. The inflorescence is a raceme of six to ten flowers, each with a four to five-lobed united white and pink corolla (Retamales & Hancock, 2018). Within each flower are eight to ten stamens featuring pollen-bearing pores. The stigma lies centrally within the corolla and is receptive to pollen for five to eight days (Retamales & Hancock, 2018). Once pollen is deposited, pollen tubes develop, and the pollen is delivered to fertilise the ovaries. From this point, the inflorescence begins to senesce, leaving it susceptible to infection and decay (Elmer & Michailides, 2007). Simultaneously, cell division begins to take place in the ovaries to develop the seeds and subsequent fruit.

2.2.1 Blueberry Fruit Development

Blueberry fruit develop over a period of 42 to 135 days after full bloom, depending on species and cultivar (Retamales & Hancock, 2018). The growth curve is double sigmoidal, with three distinct stages (Edwards et al., 1970). Initially, rapid cell division occurs, increasing fruit size and dry matter. The second phase features seed development and little gain in fruit size. The third stage involves the increase in fruit volume to its mature size and fruit ripening. Ripening involves the processes of anthocyanin accumulation, leading to a colour change from green to blue and an increase in sugars and the accumulation of volatiles. Simultaneously, organic acids decrease in concentration, which provides the balanced sweet flavour. Softening of fruit tissue due to depolymerisation and solubilisation of cell wall components also occurs during fruit development (Vincente et al., 2007). These changes create a palatable, sweet tasting, soft textured fruit (Retamales & Hancock, 2018). This process is closely related to physiological changes within fruit tissue, including respiration and ethylene production.

2.2.1.1 Ripening Physiology and Fruit Quality

The respiratory and ethylene biosynthesis behaviours of ripening blueberry fruit have been subject to controversy regarding the climacteric state. According to Bergman (1929), a climacteric peak was demonstrated. However, subsequent studies did not confirm this finding, leading to controversy (Forsyth & Hall, 1969; Frenkel, 1972). Findings indicating a respiratory peak in alignment with Bergman (1929) were found for Highbush (Wang et al., 2022; Windus et al., 1976), Lowbush (Ismail & Kender, 1969), and

Rabbiteye (Shimura et al., 1986; Wang et al., 2022). The timing of this respiratory peak varies between species, with Highbush experiencing this between the green and pink stages, Lowbush when fruit is at the pink red stage, and Rabbiteye at the green stage (Bergman, 1929; Ismail & Kender, 1969; Shimura et al., 1986; Windus et al., 1976). Additionally, there are differences in the respiratory peak of different cultivars, with the Highbush cultivars 'Lateblue' and 'Bluecrop' showing different respiration rates throughout ripening (**Fig. 2.1**) (Windus et al., 1976). This shows that ripening is a distinct process that varies between blueberry cultivars and species.

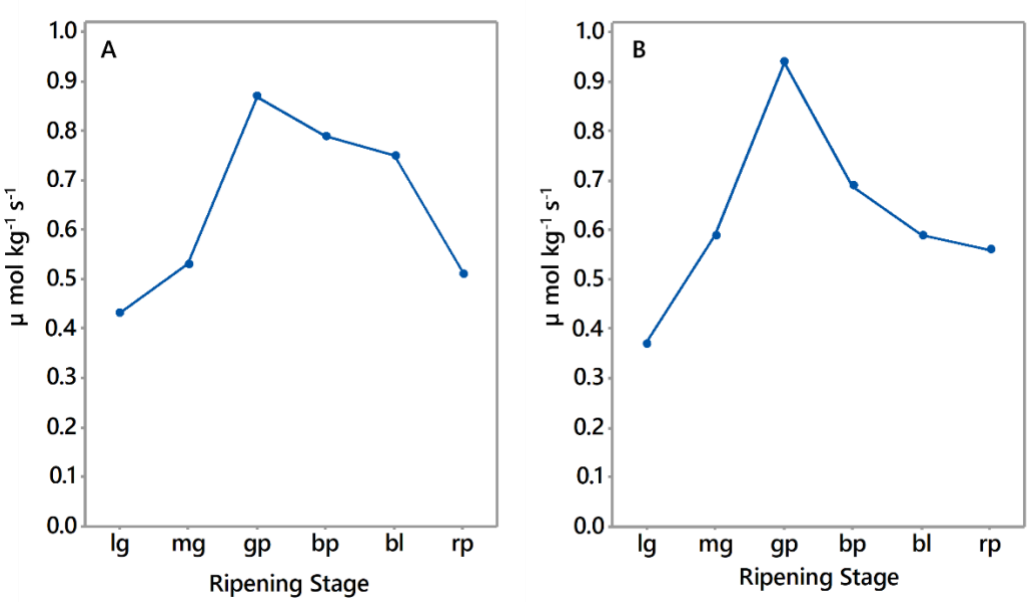


Figure 2.1. Respiration rate (CO_2 production) of Highbush blueberry cultivars 'Lateblue (A) and 'Bluecrop' (B) throughout ripening stages. CO_2 production rate measured at 23°C. Ripening stage is described by skin colour: light blue (lg), mature green (mg), green pink (gp), blue pink (bp), blue (bl), and blue ripe (rp). Adapted from Windus et al. (1976).

Simultaneous to this peak in respiration, Windus et al. (1976) reported an ethylene production peak occurring at the green pink stage of ripening (**Fig. 2.2**). The ethylene production peak observed for blueberries may also indicate autocatalytic ethylene production, which is characteristic of climacteric fruit (Lipe, 1978; Shimura et al., 1986; Windus et al., 1976). However, further study considering the response of ethylene production to exogenous ethylene application is required to demonstrate the climacteric behaviour of blueberries. Ethylene production rate also varies by species, with Rabbiteye blueberry fruit having a higher production rate of 122.15 pmol kg^{-1} s^{-1} compared to Highbush with a range of 6.11–24.43 pmol kg^{-1} s^{-1} (El-Agamy et al., 1982). This further suggests that there are differences in the ripening of these blueberry species and cultivars.

The rate and timing of ethylene production vary between cultivars (**Fig. 2.2**), with the Highbush cultivars 'Lateblue' and 'Bluecrop' showing differences in ethylene production and evolution through ripening (Windus et al., 1976). Alternatively, other studies have shown a second ethylene production peak at the

mature stage (El-Agamy et al., 1982; Shimura et al., 1986). This may be a concern for postharvest storage when blueberries are stored along with other fruits.

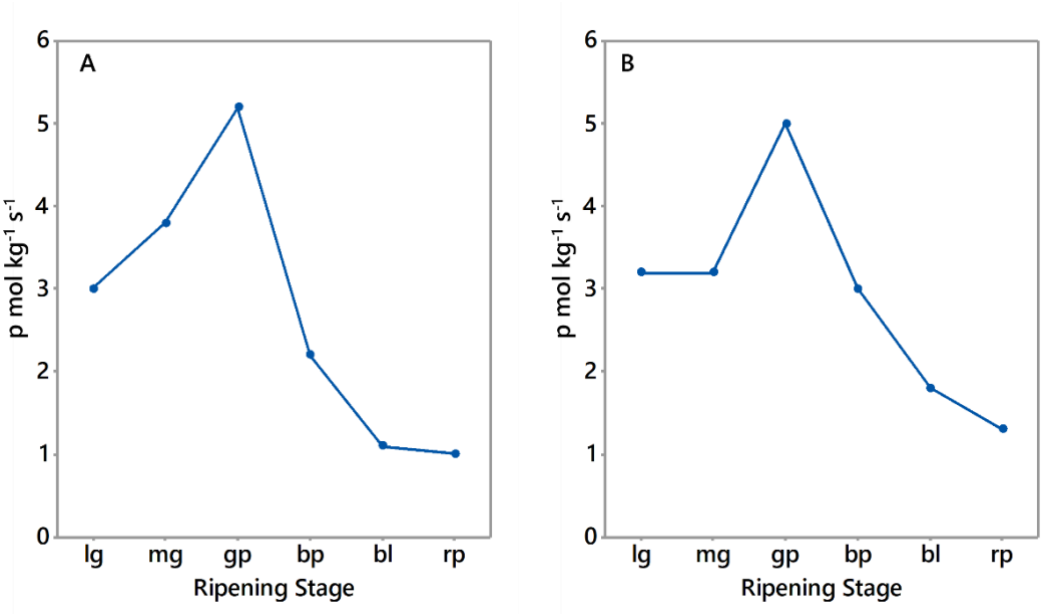


Figure 2.2. Ethylene production rate of Highbush blueberry cultivars 'Lateblue' (A) and 'Bluecrop' (B) at different ripening stages measured at 23°C. Ripening stages are described by skin colour: light blue (lg), mature green (mg), green pink (gp), blue pink (bp), blue (bl), and blue ripe (rp). Adapted from Windus et al. (1976).

2.2.1.2 Colour

As blueberry fruit develop and ripen, they undergo a colour change from green to blue. This is positively associated with an accumulation of anthocyanins and an increase in total soluble solids to acidity ratio (Ballinger & Kushman, 1970). As this ratio is an important attribute of harvest maturity, berry colour is used as an indicator of fruit maturity, with 100% blue colouration commercially used (Moggia et al., 2018). Unripe blueberry fruit are green because they have a high chlorophyll content; over time, anthocyanins accumulate in the fruit, beginning at the calyx end (Ballinger et al., 1972). This develops to give green-pink, pink-blue, and blue colours of the fruit as it matures. Anthocyanins are accumulated in the skin of blueberries within epidermal and hypodermal cells; this gives a dark blue exterior and a pale–coloured mesocarp. At harvest, anthocyanin content is variable depending on cultivar and location (Stevenson & Scalzo, 2012). Anthocyanin content accumulates after harvest, with an increase of up to 55% possible during 21 days of storage at 5°C (Mitcham, 2007). This allows fruit harvested before reaching 100% blue ripeness to continue to accumulate anthocyanins and develop blue colouration during storage (El-Agamy et al., 1982).

Also contributing to the blue colouration of the fruit are epicuticular waxes present on the surface. These provide the 'bloom' on the fruit and alter the blue colouration. Light blue and blue cultivars have waxes deposited as rodlets and upright platelets, which more effectively scatter light than the flat platelets and

annealed wax patches of dark blue and black cultivars (Sapers et al., 1984). This light scattering creates the light blue appearance of mature fruit of some cultivars. The darker blue colours associated with some cultivars and with overripe fruit is associated with the transformation of upright rodlets to flat platelets, leading to less scattering of light and thus a darker colour (Sapers et al., 1984). This wax layer is also important for postharvest performance, offering reductions in water loss and decay after harvest (Chu et al., 2018). Therefore, for optimum colour and postharvest storability, the wax layer is crucial.

2.2.1.3 Firmness

Firmness is another major component of blueberry quality, with implications for storage life and decay incidence (Vicente et al., 2007). Blueberry fruit soften as they ripen due to the enzymatic degradation of cell wall pectin, hemicellulose, and cellulose (Proctor & Peng, 1989). Most softening occurs before harvest between the green and blue stages; however, softening continues after harvest (Ballinger et al., 1973; Moggia et al., 2018). Cell wall degradation increases susceptibility to pathogen infection, and, subsequently, softer fruit are more likely to decay (Mehra et al., 2013). Postharvest firmness is also important for storage potential and consumer acceptance, with firmer fruit being preferred (Retamales & Hancock, 2018). Firmness is related to cell wall structure and composition. The major components include cellulose, hemicellulose, and pectin, which construct the cell wall as it expands during fruit growth (Keegstra, 2010). Fruit tissue softening is the result of processes including hemicellulose depolymerisation and solubilisation, arabinose solubilisation, and pectin depolymerisation and solubilisation (Vicente et al., 2007). These changes dismantle the cell wall and lead to overall fruit softening during ripening; these are visualised in **Fig. 2.3**.

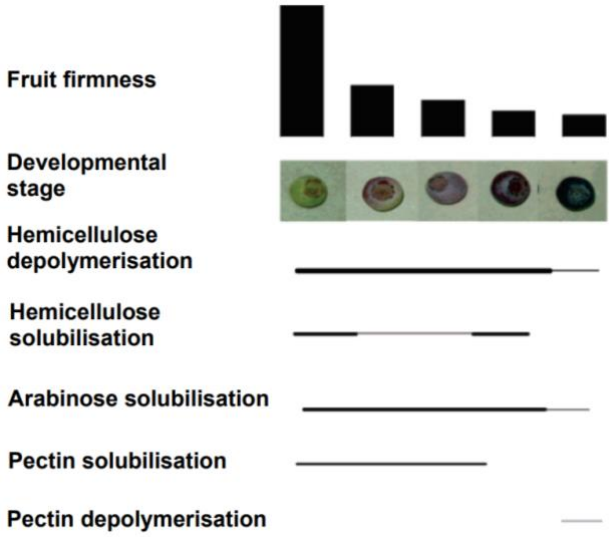


Figure 2.3. Blueberry fruit cell wall modifications occurring at different fruit development and firmness stages. Reprinted with permission from Vicente, A. R., Ortugno, C., Rosli, H., Powell, A. L., Greve, L. C., & Labavitch, J. M. (2007). Temporal sequence of cell wall disassembly events in developing fruits. 2. Analysis of blueberry (*Vaccinium* species). Journal of Agricultural and Food Chemistry, 55(10), 4125-4130. Copyright 2007 American Chemical Society.

The cell wall changes occurring during blueberry ripening are split into five developmental stages: 100% green, 25% blue, 75% blue, 100% blue, and blue ripe (Vicente et al., 2007). The most extensive softening occurs during ripening between the 100% green and 75% blue stages; this is thought to be linked mostly to the depolymerisation of hemicellulose (Chea et al., 2019). Hemicellulose degradation contributes to the loss of cell wall strength and integrity due to its role as microfibrils binding the cellulose/hemicellulose network, while pectin solubilisation reduces cell to cell cohesion and cell wall integrity (Chea et al., 2019; Zheng et al., 2020). These processes occur from the 100% green to 75% blue or 100% blue stages (Vicente et al., 2007). The initial composition at 100% green features well-structured cell walls. Hemicellulose decreases through the ripening process from 100% green to 100% blue, a result of depolymerisation and solubilisation (Chea et al., 2019; Vicente et al., 2007). Arabinose is a neutral sugar in the pectin gel, associated with the pectin egg box structure (Zheng et al., 2020) Arabinose is solubilised during the colour change from 100% green to 100% blue, leading to thinning of cell walls and eventual deconstruction of the pectin egg box structure (Vicente et al., 2007; Zheng et al., 2020). Pectin solubilisation occurs from the 100% green stage to the 75% blue stage, leading to an increase in water soluble pectin (Chea et al., 2019; Vicente et al., 2007). This leads to swelling of the cell wall, increased porosity, and reduced cell to cell adhesion (Brummell, 2006; Hématy et al., 2009). After harvest maturity at the 100% blue stage, pectin depolymerisation occurs. This is the dismantling of pectin polymers now unattached to the cell wall after solubilisation of arabinose and pectin. This leads to further softening and reduction in cell–to–cell adhesion (Loix et al., 2017; Vicente et al., 2007). These changes result in flesh firmness suitable for consumption. However, following harvest, these changes result in the continued deterioration of fruit quality and increased susceptibility of fruit tissue to pathogen infection.

Cell wall degradation increases the susceptibility of fruit tissue to pathogen infection. Ripe fruit tissue is more susceptible to infection than unripe tissue due to reduced resistance to infection (Forlani et al., 2019). Cell wall degradation facilitates the dispersal of seeds but eventually leads to unmarketable fruit (Gillaspy et al., 1993). Cell wall integrity is critical for fruit tissue to withstand pathogen attack. As cell walls continue to deteriorate after ripening, fruit tissue porosity increases, providing more potential sites for infection to occur (Brummell, 2006). The presence of host cell wall degrading enzymes also triggers pathogenicity in major decay causing microorganisms (Cantu et al., 2007). Thus, cell wall degradation leads to increased susceptibility to decay, and after harvest, the continuation of this process leads to overripe fruit which are more susceptible to decay (Rivera et al., 2013).

2.2.1.4 Total Soluble Solids

The sugar content of blueberries is measured as total soluble solids (TSS). Sugars in blueberry fruit are mainly glucose and fructose; these occur in roughly equal amounts (Darnell et al., 1994). Sugars are

accumulated through the transport of photosynthates from leaves to fruit, where they are metabolised to glucose and fructose (Li et al., 2020). During ripening, the sugar content (mg g^{-1} FW) of blueberries increases by up to 3.5–fold between the green stage and 100% blue maturity (Forney et al., 2012). After harvest, TSS remains consistent during postharvest cold storage as sugar accumulation stops because the fruit is detached from the plant (Chiabrando et al., 2009). Total soluble solids is linked to decay susceptibility, as fruit with a high TSS : acidity ratio have a poorer keeping quality (Galleta et al., 1971).

2.2.1.5 Titratable Acidity

Organic acids present in blueberry fruit include citric, malic, quinic, and ascorbic acids. Citric acid comprises the majority of total acid content with 77–87% (Forney et al., 2012). During ripening from the green to blue stages, total acidity decreases by 64% (Forney et al., 2012). This decrease corresponds with fruit maturity and provides a sweet taste (Woodruff et al., 1960). After 100% blue maturity, acidity continues to decline whether fruit is harvested or not (Fukumori et al., 2004; Moggia et al., 2017b). After harvest, acidity continues to decline but is affected by water loss, which can cause an increase in acidity during storage (Chiabrando et al., 2009). Acidity may be responsible for providing some host resistance against decay, with acidic conditions within fruit tissue related with better keeping quality of blueberries (Galletta et al., 1971). Therefore, acidity is an important consideration for fruit quality.

2.3 Blueberry Fruit Susceptibility to Decay

2.3.1 Host Defence System

Plants have evolved numerous defences against pathogen infections as they are continually exposed to potentially pathogenic microorganisms. Plants use constitutive defences, which provide passive protection against pathogens, and induced defences, which are active responses to pathogen detection. Both forms of defence are required as plants are non-circulatory organisms; thus, immunity is innate to each cell, with complex cross talk and signalling used to respond to and prepare other cells in response to pathogen attack. Host susceptibility is a major factor of the disease triangle, and it is important to understand the mechanisms of host defence in order to apply them to the postharvest supply chain.

2.3.1.1 Constitutive Defences

Constitutive defences are permanent mechanisms of the fruit host that aim to prevent the establishment of pathogen infections. Structural constitutive defences include the cell wall and fruit cuticle layer. These are often the first line of defence against pathogens and form a physical barrier which is insurmountable to most microorganisms.

The cell wall encompasses a plant cell and provides strength, support, and protection from biotic and abiotic stress (Malinovsky et al., 2014). Cell walls consist of a cellulose network bound with hemicelluloses and embedded in a pectin matrix (Loix et al., 2017). Thickness is important for the efficacy of its defensive function, as thicker cell walls are more difficult for pathogens to break through (Guest & Brown, 1997). It follows that cell wall degradation during fruit ripening and senescence leads to a weakening of this barrier and a subsequent increase in susceptibility to infection. The cell wall also plays a role in induced responses, with damage to the cell wall being recognised as a signal of potential pathogen attack, leading to induced defence responses (Hématy et al., 2009).

The cuticle is a waxy, hydrophobic coating on the surface of aerial primary plant tissue that provides strength to plant organs and forms a barrier to transpiration and pathogen infection (Ziv et al., 2018). The cuticle is comprised of cutin and waxes formed into two layers. A cuticle layer that contains cutin and polysaccharides lies beneath the cuticle proper, which is comprised of cutin and waxes (Serrano et al., 2014). The cuticle forms a further barrier between the cell wall and the outside of the plant, offering a thicker and more complex barrier to pathogen infection (Guest & Brown, 1997). This barrier is hydrophobic, so it prevents waterborne pathogens from accumulating (Ziv et al., 2018). The cuticle is also involved in signalling of pathogen presence, with damage to the cuticle recognised and responded to through induced responses (Malinovsky et al., 2014). In harvested blueberry fruit, the cuticle is compromised at the stem scar due to the picking wound, leaving the fruit exposed to decay causing microorganisms in this region. This leaves this region of fruit tissue more susceptible to infection.

Constitutive defences also include chemical compounds permanently present within plant tissue which offer anti-microbial properties. These are known as phytoanticipins and are present in plants before pathogen attack (van Etten et al., 1994). These compounds include flavonoids, tannins, saponins, alkaloids, and terpenoids (Tiku, 2020). Phytoanticipins are present throughout plant tissue, but important locations are within the cuticle, where they form an antimicrobial barrier to pathogen attack, and in the vacuole, from which they can be delivered to the site of infection (Tiku, 2020; Ziv et al., 2018). In blueberry fruit, fructose is considered a phytoanticipin for its effect on *Escherichia coli* (Tiku, 2018). Other phytoanticipins of blueberry fruit are unclear, but organic acids have been suggested to have an antifungal role in ripe wild

blueberry fruit (Cipollini & Stiles, 1992). Consequently, as acidity declines during fruit maturation, there may be a link between the loss of antifungal properties and blueberry ripening.

2.3.1.2 Induced Defences

The plant defence system is able to respond to detected threats through induced defence. Embedded within cell membranes are pattern recognition receptors (PRR), which recognise pathogen–associated molecular patterns (PAMPs) such as fungal chitin, lipopolysaccharides, β-glucan, ergosterol, and flagellin (Boller & Felix, 2009; Schwessinger & Zipfel, 2008). This induces PAMP-triggered immunity (PTI), which aims to prevent the establishment of infection and damage through a broad-spectrum series of control measures. Also triggering PTI are damage–associated molecular patterns (DAMPs). These are components of host cell walls, cuticles, cell membranes, cell contents, and DNA broken into fragments by attacking microorganisms. These are recognised and interpreted as damage caused by an infecting microorganism and lead to the induction of PTI.

PTI features short–term and longer–term defensive responses to pathogen attack perceived as PAMPs and DAMPs. Immediate responses to perceived attack include a short release of calcium ions and reactive oxygen species (ROS), protein kinase activation, signal hormone production, and transcription of defence related genes (Malinovsky et al., 2014). Occurring in the days after detection, responses include induced systemic resistance, callose deposition in the cell wall, production of phytoalexins, anti-fungal compounds, and other secondary metabolites, and upregulation of pathogenesis–related genes (Boller & Felix, 2009; Malinovsky et al., 2014). These strategies aim to disrupt the progress of microorganism infection and are generally effective against most invaders. However, some pathogens have evolved to disrupt these PTI responses and create conditions suitable for their growth.

Sophisticated pathogens utilise effectors to disrupt PTI and cause effector–triggered susceptibility (ETS). This allows pathogens to avoid the elicitation of PTI, enabling them to establish an infection of plant tissue before other control measures are triggered. Some fungi can disguise chitin, a common PAMP, by converting it to chitosan, evading detection by PRR (Gong et al., 2020). Another strategy is for a microorganism to disrupt the defence responses of PTI by secreting proteins which restrict the recognition of PAMPs and DAMPs (Kim et al., 2005). Due to microorganisms developing these mechanisms to avoid detection by plants, countermeasures have evolved, which are more specific resistance (*R*) genes. These include effector–triggered immunity (ETI), which is the use of *R* gene–encoded products to recognise pathogen effectors causing ETS and respond to them through stronger defensive action (Jones & Dangl, 2006). ETI leads to defence responses including the production of ROS, protein kinases, nitric oxide, accumulation of jasmonic acid and salicylic acid to induce systemic resistance, upregulation of defence

genes, and programmed cell death (Cui et al., 2015). Further evolution has resulted in some microorganisms being able to circumvent ETI through novel effectors (Jones & Dangl, 2006). However, as is the nature of co-evolution, or human intervention through plant breeding, host resistance has developed to meet this threat in some plants.

In the context of postharvest blueberry fruit, the mechanisms of host defence respond to perceived threats as PAMPs and DAMPs. Due to postharvest decay often resulting from quiescent infections developing into aggressive infections, defence mechanisms protect the host from decay prior to senescence. The defence mechanisms employed can be successful and result in quiescence of infections or resistance. For example, the blueberry cultivar 'Elliot' is resistant to *C. acutatum* due to earlier detection of pathogen infection, enabling an effective defence response (Miles et al., 2011). For other decay causing organisms, particularly necrotrophs, defence relies on containment of the pathogen through erecting structural barriers such as callose and wax (Agudelo-Romero et al., 2015; Tian et al., 2016). Therefore, resistance is possible, but it requires a sufficient degree of host resistance to be able to respond with effective mechanisms to limit infection.

2.3.2 Senescence

Senescence is the active decline of fresh produce quality after harvest through programmed cell death (Greenberg, 2003). In blueberry fruit, senescence represents a continuation of ripening after harvest, potentially involving ethylene (Wang et al., 2020). As senescence progresses, the likelihood of decay incidence increases due to declining host resistance (Woodruff & Dewey, 1959). Senescence encompasses physiological processes driven by fruit tissue metabolism, leading to softening, the development of off flavours and aromas, and eventually programmed cell death as available metabolites are depleted. The incidence of decay increases as pathogens recognise fruit maturity, exploit cell wall degradation, and exploit the reduced effectiveness of induced defences (Häffner et al., 2015; Prusky & Lichter, 2007). During the advanced stages of senescence, characterised by programmed cell death, susceptibility to decay is very high, enabling infection by saprophytic microorganisms and other opportunistic microorganisms.

2.3.3 Fruit Damage

Damage is incurred by blueberry fruit from the moment of harvest through the stem scar. The stem scar is a wound created at harvest at the point of abscission from the bush. This creates a moist, exposed area of fruit tissue, without effective constitutive defences, and exposes direct links to vascular tissue as blueberry

fruit do not form a true abscission zone (Beaudry, 1992). Consequently, the stem scar exhibits very high susceptibility to decay. It has been reported that the stem scar attracts 90% of decay occurring on blueberries during a six day period at 21°C (Cappellini & Ceponis, 1977). Therefore, blueberry breeders prioritise fruit with a small, dry stem scar to limit decay (Retamales & Hancock, 2018). As constitutive defences at the stem scar are minimal, major pathogens, weak pathogens, and opportunistic microorganisms are all able to infect this tissue. This increased host susceptibility and the pathogenicity of numerous microorganisms lead to the fulfilment of the disease triangle factors, and establishment of infections under suitable environmental conditions.

Bruising significantly impacts postharvest softening and decay incidence in blueberry fruit. The prevalence of fruit decay increases with the severity of bruising inflicted (Ballinger et al., 1973). Additionally, bruising accelerates softening in the supply chain, leading to a shorter storage life (Ballinger et al., 1973). Bruising occurs through the supply chain as a result of mechanical impact, compression, and vibration resulting from harvest method, packhouse operations, and transportation (Beaudry, 1992; Mainland et al., 1975; O'Brien et al., 1963). By damaging cell walls and inducing fluid release into the extracellular matrix, bruising diminishes host resistance to decay. This directly leads to softening and increased decay susceptibility. In severe cases, mechanical damage can result in the splitting of fruit, resulting in leakage of juice and formation of wounds. This damage provides substrate for numerous decay–causing microorganisms.

Physiological damage also contributes to the susceptibility of blueberry fruit to decay. Postharvest conditions and intrinsic tissue processes can result in browning, pitting, shrivel, and splitting, all of which increase host susceptibility to pathogen attack and reduce consumer acceptability (Cappellini et al., 1982). Blueberries are susceptible to damage after removal from cold storage, exhibiting symptoms such as internal browning, pitting, and decay (Zhou et al., 2014a; Zhou et al., 2014b). Shrivel has been positively correlated with decay incidence, as reported Cantín et al. (2012). Fruit splitting further enhances fruit susceptibility to decay by exposing fruit tissue to microorganisms, facilitating infection. Thus, physiological issues arising during storage can also promote decay.

2.4 Postharvest Environmental Conditions

2.4.1 Temperature

The temperature of fresh produce is the main influence on the rate of quality deterioration and resulting storage life (Wills & Golding, 2016). Temperature regulates physiological processes within the tissue of fresh produce, including respiration rate, ethylene production, enzymatic reactions, and the host defence system (Sommer, 1985; Wills & Golding, 2016). Temperature also influences physiological processes within microorganisms associated with fresh produce, and for some species, it can limit their ability to infect fresh produce (Agrios, 2005). Also affected by temperature is the water holding capacity of air, which impacts the relative humidity of the atmosphere surrounding fresh produce, having effects on water loss and subsequent quality issues. Therefore, temperature control is a key mechanism for delaying fresh produce quality loss and the development of decay in the postharvest environment.

Blueberry fruit, as is characteristic of berry fruit, are very susceptible to quality deterioration after harvest. Low temperature storage is considered critical to delaying senescence and the development of decay (Retamales & Hancock, 2018). A key process influenced by cold storage is respiration rate. Carbon dioxide production was shown by Mitcham et al. (1998) to be considerably lower at 0°C with 3 mL CO_2 kg $^{-1}$ hr $^{-1}$, compared to 10°C and 20°C, which resulted in 9 and 34 mL CO_2 kg $^{-1}$ hr $^{-1}$, respectively, Further data observed by NeSmith et al. (2005) confirms this, with a 79% reduction in respiration rate between 22°C and 1°C. These data indicate the respiration rate of harvested blueberry fruit is much lower under refrigerated conditions, which has the effect of slowing the quality loss effects associated with fruit senescence. As respiration rate comprises enzymatic reactions, these are also slowed by low temperature, and hence affect the rate of cell wall degradation. As blueberry fruit are susceptible to decay, water loss, and off flavour development during postharvest storage, low temperature storage also offers a solution to minimise these quality issues (Forney, 2009; Nunes et al., 2004).

The storage life of blueberry fruit is extended by refrigerated storage; the optimum temperature for this is 0–2°C (Hancock et al., 2008). Within this temperature range, storage can be three to eight weeks depending on cultivar, enabling long storing blueberry cultivars to be exported by sea (Hancock et al., 2008). It is well known that low temperature storage extends the storage life of blueberry fruit by reducing decay incidence. This was first reported by Woodruff and Dewey (1959), who observed a lower rate of decay accumulation on blueberries at 2°C compared to 10°C. Subsequent studies have confirmed this observation, with Ballinger et al. (1978) reporting considerably less decay and a lower rate of decay accumulation at 1.1°C than at 10°C and 22.2°C. Similarly, Nunes et al. (2004) confirmed this for several temperatures between

0°C and 20°C. At low temperatures, slight differences in temperature have been proven to impact decay development. An experiment by Paniagua et al. (2014) observed a significantly greater incidence of decay at 4°C than 0°C after five weeks of storage. This indicates that a small variation in storage temperature can have significant consequences for fruit quality. Thus, for optimal blueberry storage, low temperatures within the range 0–2°C is recommended, and, additionally, uniformity of temperature within a consignment is crucial to consistency of quality.

Another impact on the retention of postharvest quality of blueberry fruit is the cooling delay experienced between the moment of harvest and the fruit reaching the optimal storage temperature. Given the data shown previously, high temperatures result in an increased rate of fruit quality deterioration. Thus, ensuring rapid postharvest packing and cooling of blueberry fruit is critical to extending storage life. The duration and temperature of cooling delays have considerable impacts on decay incidence during storage and shelf life. A study by Hudson and Tietjen (1981) indicated that achieving 10°C within two hours of harvest resulted in significantly less decay than blueberries which were cooled to 10°C within 24 and 48 hours of harvest. Corroborating this data, Forney (2009) presents data that shows delaying cooling by up to eight hours resulted in softer fruit, greater weight loss, and more decay after six weeks of storage. Therefore, best practise is to cool blueberry fruit as rapidly after harvest as is feasible to the storage temperature in order to avoid decay and quality deterioration.

The impact of cold storage on postharvest pathogens is that it acts as an inhibitor of the growth of some microorganisms. The mechanisms delaying the quality deterioration of blueberry fruit also operate within living microbial material. Slowed respiration rates and enzymatic reactions limit the ability of some major pathogens to cause infection and slow the growth and infection of others (Sommer, 1982). Cold storage can also impact the composition of the fruit microflora, with the species of fungi present changing in response to cold storage duration (Abdelfattah et al., 2020). The impact of this is that fungi which can tolerate low temperatures can develop infections during storage. Of particular concern is *B. cinerea*, which can develop at 0°C (Droby & Lichter, 2007). Thus, low temperature storage is a beneficial method for maintaining blueberry fruit quality by avoiding decay due to the slowed metabolic rate of decay causing microorganisms and the fruit itself.

2.4.2 Relative Humidity

Relative humidity (RH) is defined as the water vapour content of air compared to the maximum water vapour capacity of air (Callahan et al., 2019). RH is a key factor in preserving the quality of stored fresh produce. This variable impacts fruit quality through water loss and interacts with microorganisms to influence the

expression of decay. For fresh blueberry fruit, RH is recommended to be 85–95% (Retamales & Hancock, 2018). Due to the low temperatures used to store fresh produce, there is potential for fluctuations in RH caused by minor variations in temperature due to interactions of psychometric parameters (Callahan et al., 2019). Thus, consistency in temperature and RH must be provided in order to prevent condensation or low RH conditions from forming.

A major impact of RH is on the pathogenicity of decay causing microorganisms present on fresh produce. Decay is a key quality issue, and its incidence is increased at high RH (Forney, 2009). This increase is due to high RH creating optimal conditions for conidia germination and infection (Eckert & Sommer, 1967). A study using 100% RH and 25°C storage for five days observed up to 29% decay incidence of blueberry fruit (Smith et al., 1996). RH also influences the nature of decay signs produced by microorganisms; the common necrotrophic pathogen, *B. cinerea,* produces a more extensive mycelium above 94% RH in table grape fruit (Thomas et al., 1988). Another aspect of RH thought to favour pathogen infection is the presence of surface water on fresh produce (Sommer, 1982). Condensation can form on fruit surfaces due to fluctuations in air temperature at high RH (Paull, 1999). Therefore, storage at RH levels above 95% is not recommended if the pathogen load is high.

Low RH can induce other fruit quality defects such as water loss and excessive softening. Weight loss is mostly attributable to the evaporation of moisture from the fruit into the atmosphere through the cuticle and stem scar, and has economic consequences when fruit are sold by weight. The impact of weight loss on fruit quality depends on the degree of weight loss observed. For example, weight loss of $\leq$1.34% was shown to produce firmer fruit, while weight loss over 3.47% reduced fruit firmness (Paniagua et al., 2013). Another impact of weight loss is the development of shrivel symptoms. These are described as the corrugation of fruit skin and are influenced by RH and the duration of storage (Rivera et al., 2021; Schotsmans et al., 2007). Weight loss of over 6.9% was observed by Paniagua et al. (2013) to produce shrivel symptoms in cv. Centurion. However, weight loss as low as 5% produced shrivel in cv. ‘Burlington’, but in cv. ‘Maru’, shrivel was only reported to occur above approximately 8% weight loss (Forney et al., 1998; Schotsmans et al., 2007). Thus, the propensity to shrivel varies by cultivar; this is influenced by cuticle permeance and stem scar size (Moggia et al., 2017a). Consequently, to limit weight loss and subsequent quality issues, RH needs to be balanced with the more extensive decay at high RH. Therefore, the recommended RH for postharvest storage of blueberry fruit is 85–95% RH to mitigate both weight loss and decay incidence (Retamales & Hancock, 2018).

2.5 Fungi and the Fruit Microbiome

The fungal kingdom features a diverse array of species with a range of relationships with plants, from symbionts to pathogens and saprotrophs. Fungi have both beneficial and negative relationships with plants. Beneficial ecosystem services include mycorrhizal fungi, which facilitate nutrient transfer and signalling between plant roots, the decomposition of organic matter, and nutrient cycling. However, fungi are better known for their role as causal agents of plant disease and the decay of fresh produce. In crop production systems, they lead to economic consequences from crop failures and the cost of control. In postharvest horticulture, the cost of postharvest decay is immense, involves substantial food wastage, and enlarges the carbon footprint of supply chains. The benefits of fungi are incalculable; however, it is important to control their impacts on food crops to maintain food security and the value of horticultural products from the field to the consumer.

2.5.1 The Fruit Microbiome

Within horticultural systems, fungi feature in relationships with plants from seeds to fruit. The fruit microbiome is a complex and diverse community of largely non-pathogenic fungi, bacteria, and archaea living within fruit tissue endophytically or on fruit tissue epiphytically (Droby & Wisniewski, 2018). The fruit microbiome is an extension of the microbiome of the phyllosphere, and as the fruit develops, it is colonised by the endophytic and epiphytic microbiota of the plant and from environmental sources. The plant microbiota has co-evolved with plant species to display numerous synergies and be highly specific to the plant (Berg et al., 2016; Zilber-Rosenberg & Rosenberg, 2008). A plant's microbiome is inherited from its parent plant and from the environment. The inheritance of microbiota through generations of plants is known as vertical colonisation and allows both beneficial and pathogenic organisms to be transmitted through generations (Faddetta et al., 2021; Mitter et al., 2017). Inoculation of microorganisms from environmental sources is known as horizontal colonisation and is a common method of pathogen inoculation in orchard environments (Bright & Bulgheresi, 2010; Jarvis, 1962). Inoculation of plants through vertical and horizontal colonisation creates a complex and unique ecology involving mutualistic, commensal, competitive, or pathogenic relationships between fungi, bacteria, and the plant host (Bastías et al., 2020). This diverse microflora is specific to an individual plant and is subject to influence from a variety of factors.

2.5.2 Influences on the Fruit Microbiome

The composition of the fruit microbiome varies in relation to pre and postharvest factors. As the microflora of fresh produce is an extension of the phyllosphere of the host plant, the microbiome is provided as the fruit develops. Additions are made through horizontal colonisation of new organisms. On–orchard practises are influential on the composition of the microbiome at harvest. Influences include climate, cultivar, landscape context, and production method. Variations in these result in differences in the composition of the fruit microbiome (Abdelfattah et al., 2016; Bösch et al., 2021; Schaeffer et al., 2021). Organic production results in a significantly wider range of organisms present in the microbiome of fruit (Abdelfattah et al., 2016). Additionally, the microbiome is vastly different between different regions of the same fruit; for example, in apples, the calyx and stem end are favoured by *Alternaria* spp., while wounded tissue and the epidermis are favoured by *Penicillium* spp. (Abdelfattah et al., 2016). When fruit become decayed, the diversity of the fruit microbiota decreases, with a few organisms becoming dominant, as shown in strawberries by Olimi et al. (2022) and kiwifruit by Wu et al. (2019). After harvest, the fruit microbiome is influenced by storage technology, storage duration, and humidity, which have the potential to favour certain organisms and influence decay development (Bösch et al., 2021). This indicates the malleability of the fruit microbiome in relation to horticultural practises and decay.

2.5.3 Plant Pathogens

The nature of horizontal colonisation by microorganisms from the environment facilitates the distribution of pathogens into the fruit microbiome. Organisms with the potential to cause decay are termed pathogens (Agrios, 2005). Opportunism is a central part of the mode of nutrition of most pathogens; these are largely saprophytes and feed on dead tissue. They are indistinguishable from non-pathogenic organisms until nutrition is freely available (Abdelfattah et al., 2016). Economically important pathogens are effective at acquiring nutrition while the host remains alive and are therefore more prevalent causes of decay. These pathogens use strategies to gain nutrition, including biotrophy, hemibiotrophy, and necrotrophy, requiring them to overcome plant defences to infect host tissue and acquire nutrition.

A requirement for fungal growth is a source of nutrition. The ability of pathogens to access sufficient nutrition for infection is influenced by the disease triangle factors of environmental conditions, host resistance, and microorganism pathogenicity. If these factors inhibit the pathogen from acquiring nutrition in the short term, it can exist in a state of dormancy until conditions become suitable (Prusky, 1996). Therefore, pathogenic microorganisms within the fruit microbiome only cause decay if the disease triangle factors are fulfilled.

2.5.4 Biotrophic Fungi

Plant–pathogenic fungi are classified by their mode of acquiring nutrition into biotrophic, hemi-biotrophic, and necrotrophic lifestyles. Biotrophs require the host cell to remain alive for them to receive nutrition. Examples of biotrophic fungi include organisms responsible for rust, mildew, and smut diseases (Perfect & Green, 2001). Because biotrophs require a living host, they are important preharvest plant pathogens, and are responsible for substantial crop losses. In the context of horticultural products, biotrophs are not pathogens due to declining host resistance, concomitant with host tissue that is moving through ripening to senescence, which enables more aggressive pathogens to cause decay as produce senesces. However, visual defects resulting from biotrophs such as sooty mould cause cosmetic issues of fruit (Sullivan et al., 2021). Biotrophy is a life stage of important postharvest pathogen genera, including *Colletotrichum,* which take up a necrotrophic lifestyle once ripening related changes occur (Kramer-Haimovich et al., 2006). As biotrophy requires a living host for pathogen success, pathogens with only this lifestyle are not causal agents of postharvest decay.

2.5.5 Necrotrophic Fungi

Necrotrophic fungi are important postharvest pathogens. Their infection strategies are to kill host cells and feed saprophytically on dead tissue. Examples of major necrotrophs are *Botrytis* spp., *Alternaria* spp*.,* *Penicillium* spp*.,* and *Rhizopus* spp*.* Necrotrophs are important postharvest pathogens because of their ability to remain quiescent on plant tissue until conditions become suitable for their infection. This means they can be inoculated at any time, from flowering to postharvest, but only begin to cause decay once host resistance has declined. This can be during senescence, or as a result of damage such as wounding (Doehlemann et al., 2018). The mechanisms of entry to plant tissue are through an appressorium in some cases, or the cell wall is dissolved using cell wall–degrading enzymes. Once inside the cell, toxins and effectors are released, which rapidly kill the host cell (Horbach et al., 2011). Another method used by necrotrophs to cause decay is by inducing the hypersensitive response (Govrin & Levine, 2000). This results in a reactive oxygen species (ROS) burst intended as a host defence mechanism; however, it is interpreted by the pathogen as a signal to release toxins, including ROS released from the pathogen, to cause the death of the host cell (Heller & Tudzynski, 2011). Following host cell death, necrotrophs utilise their extensive array of hydrolytic enzymes to digest the host cell contents and acquire nutrition (Horbach et al., 2011). As necrotrophs infect host tissue and cause cell death, lesions form, and mycelial development over the infected tissue produces characteristic signs or symptoms of decay.

2.5.6 Hemi–biotrophic Fungi

Hemibiotrophs utilise a combination of biotrophy and necrotrophy to complete their lifecycles. *Colletotrichum* is an important genus in this group, affecting fresh horticultural produce (Prusky & Lichter, 2007). The infection begins with a biotrophic lifestyle varying in length from hours to days depending on the microorganism species and environmental conditions (Ghozlan et al., 2020). These pathogens often produce haustoria and hyphae to obtain nutrition from the host (Doehlemann et al., 2018). Following this lifestyle, the pathogen detects changes within produce tissue related to ripening and senescence; from this point, necrotrophy becomes the mode of nutrition (Kleemann et al., 2012). Hyphae undergo differentiation, with toxins, hydrolytic enzymes, and specialised pathogen effectors released that kill the host cells and allow the necrotrophic pathogen to feed on the cell contents (Doehlemann et al., 2018; Yi & Valent, 2013). The subtle nature of the biotrophic stage of this lifestyle enables pathogens to advance infection through fruit tissue and obtain nutrition before producing decay signs or symptoms. Resulting in the development of an extensive infection before becoming necrotrophic.

2.5.7 Saprotrophic Fungi

Saprotrophs are opportunistic fungi that acquire nutrition from dead host tissue. They are widespread in the environment and are important decomposer fungi (Boddy & Hiscox, 2016). The decay of plant tissue by saprotrophs is caused by endophytic or epiphytic fungi present at host death or from chance arrivals from sporulating fungi within the environment (Boddy & Hiscox, 2016). There is a high degree of competition and antagonism between saprotrophs (Woodward & Boddy, 2008). Success is largely mediated by environmental conditions and the virulence of the fungi present. Due to the non-pathogenic nature of microorganisms with this lifestyle, they are not responsible for postharvest decay in ripe fruit. However, once fruit has senesced to a degree where host defences are negligible, saprotrophs begin to cause signs and symptoms of decay (Promputtha et al., 2007). Therefore, they can be present in the postharvest supply chain but only produce decay during the later stages of fruit senescence and in the absence of more aggressive competition. As saprotrophs feed on non-living tissue, areas of exposed or damaged fruit, such as wounds, bruising, the stem scar, or areas weakened by other pathogens, are regions where saprotrophs can cause decay (Doehlemann et al., 2018). Hence, saprotrophs are potential causal agents of decay but require unique conditions.

2.5.8 Preharvest Infection

Preharvest infection of blueberry fruit occurs from flowering to fruit maturity. Pathogen conidia are dispersed by wind, on windblown debris, or through rain splash, so their deposition on the surface of plant organs is dependent on environmental conditions (Harrison & Lowe, 1987; Jarvis, 1962). Conidia land on aerial plant organs, including the surfaces of fruit and flowers, where they germinate. Initially, a germ tube is produced, which seeks an entrance to plant tissue through wounds, lenticels, stomata, and the floral stigma (Prusky & Lichter, 2007). An appressoria is developed by some pathogens to create an entrance into plant tissue. Once conidia land and germinate, they interpret signals from the host and environment, which determine whether they take an aggressive or quiescent approach. These signals can be chemical (for example, ethylene production) or physical barriers such as cuticle wax (Adie et al., 2007; Prusky, 1996). Environmental conditions affecting germination and infection of conidia include prolonged surface wetness, high humidity, and warm temperatures (Miles et al., 2013; Rivera et al., 2013). If these conditions are met, the pathogen infection progresses.

If fruit tissue is susceptible to infection, an aggressive approach is taken to further the infection. The nature of this depends on the pathogen, with necrotrophs such as *Botrytis cinerea* acting to dissolve cell walls and feed on host tissue (van Kan, 2006). Hemibiotrophs, including *Colletotrichum acutatum,* use comparatively subtle methods to penetrate host tissue, using an appressorium followed by transition to necrotrophy (Wharton & Schilder, 2008). On the other hand, if the host is not susceptible to infection, a quiescent strategy is followed, featuring dormancy or non-pathogenic epiphytic or endophytic growth within fruit tissue as it grows and matures (Peres et al., 2005; Sowley et al., 2010). Once the fruit reaches maturity and begins senescence, these quiescent infections become aggressive and produce decay signs and symptoms.

2.5.9 Postharvest Infection

Postharvest inoculation of blueberry fruit can occur during handling, storage, retail, and consumer stages (Droby & Lichter, 2007). The same pathogens that are present in the orchard are brought into the postharvest supply chain with the fruit. The mechanisms of postharvest infection are similar to those before harvest; however, the infection quiescence period is reduced due to fruit senescence. Important mechanisms of inoculation and infection relate to contamination of surfaces, physical contact with decaying fruit, and human handling. Surface contamination occurs from fruit with advanced infections depositing conidia on surfaces such as picking buckets, graders, and packing lines. Other fruit subsequently pass over this surface and become inoculated. This is particularly important with microorganisms producing conidia–

rich exudates, such as *Colletotrichum* spp., which cause anthracnose decay (Cline, 1996). Similarly, physical contact between sound fruit and decaying fruit can facilitate transmission of conidia, resulting in infection. Areas of fruit-to-fruit contact, including harvest containers, and packing clamshells, can lead to the transmission of infection. The dispersal of conidia through forced air cooling may also lead to the spread of infections. Human contact with fruit has the potential to cause the transmission of microorganisms that pose a risk to public health (Calder et al., 2003; Miller et al., 2013). However, the transmission of decay–causing microorganisms by human contact is not known. Thus, there is potential for blueberry fruit to become infected in the postharvest supply chain. This highlights the need to remove fruit displaying signs or symptoms of decay to avoid further transmission.

2.6 Postharvest Pathogens of Blueberry Fruit

Commonly reported postharvest pathogens of blueberry fruit are *Botrytis cinerea*, *Colletotrichum* spp., and *Alternaria* spp. (Cappellini et al., 1982; Smith et al., 1996). Other species include *Pestalotiopsis* spp., *Diaporthe* spp., *Penicillium* spp., *Epicoccum* spp., *Neofusicoccum* spp., and numerous other opportunistic organisms (Oliver & Ali, 2021; Tournas & Katsoudas, 2005).

2.6.1 *Botrytis cinerea*

The genus *Botrytis* is of the class *Leotiomycetes,* the order *Helotiales,* and the family *Sclerotiniaceae*. It contains 38 species of necrotrophic plant pathogens able to collectively infect over 200 crop species (Dewey & Grant-Downton, 2016; Garfinkel, 2021). Most of the species in this genus have evolved a limited host range and are specific to monocotyledons or dicotyledons. However, the most important species for postharvest horticulture are generalist necrotrophic pathogens, as they are able to cause the decay of a variety of horticultural products. The major pathogen of concern is *B. cinerea,* which is documented to cause decay in a wide variety of crops with an estimated economic impact of billions of dollars (Carisse, 2016). The decay caused by this genus is known by the common name grey mould. Other species implicated in the postharvest decay of blueberries include *B. pseudocinerea* and *B. californica;* however, these are relatively minor (Saito et al., 2016). *B. cinerea* is a major concern for blueberry growers and exporters as it is responsible for blossom blight during flowering and fruit decay during postharvest storage (Hancock et al., 2008). Additionally, it is difficult to control during postharvest storage as it can grow at 0°C (Elmer & Michailides, 2007). This makes *B. cinerea* a pathogen of considerable concern to the blueberry industry.

Table 2.1. A selection of published incidences of *Botrytis cinerea* rots of blueberry fruit. N.S = not specified.

Blueberry Cultivar(s)	Location	Year	Storage Duration (days)	Temperature (°C)	Grey Mould Incidence (%)	Reference
'Scintilla', 'Star', 'Farthing', 'Sweetcrisp'	Georgia, USA	2009	21	2	0.25	Mehra et al., 2013
		2010			1.9	
'Brigitta'	Antuco, Chile	2011	45, then 3 at 20°C	0	24.8	Rivera et al., 2013
'Duke'					10.8	
'Liberty'					20.3	
'Duke'	San Clemente, Chile				7.4	
'Liberty'					17.1	
'Brigitta'	Virquenco, Chile				17.6	
'Duke'					2.3	
'Brigitta'	Yerbas Buenas, Chile				5.9	
'Duke'					4.4	
N.S.	California, USA	2013	35	0–2	20.1	Xiao & Saito, 2016
		2014			5.1	
'Legacy'	California, USA	2017	28	0–1	35.8	Saito et al., 2022
'Jewel'					30.0	
'Snowchaser'	California, USA	2018	28	1	2.5	Saito et al., 2021
'Jewel'					27.9	
'Snowchaser'		2020			27.9	
'Jewel'					55.8	

2.6.1.1 *Botrytis cinerea* Lifecycle

Botrytis cinerea is found perennially within the orchard environment as dormant sclerotia, infectious conidia, or mycelium. As the sexual stage of *B. cinerea* is very rarely observed in the environment, asexual reproduction is the primary method of proliferation of *B. cinerea* infections (Elmer & Michailides, 2007).

Asexual reproduction enables rapid completion of the pathogen lifecycle if suitable conditions persist. Infections begin as conidia, which originate from sclerotia or mycelium, and these go on to infect susceptible plant tissue and develop mycelium and new conidia, thus perpetuating the cycle.

Multiple infection cycles have the potential to occur during the blueberry growing season. Primary infections are the first of the new growing season, arising in late winter and early spring resulting from sclerotia–germinated asexual conidia or sexual ascospores. These target plant organs susceptible at this time, resulting in infection of flowers known as *Botrytis* flower blight. Blueberry flowers are susceptible to necrotrophic infection from the pink or white bud pre-bloom stage through to sensed corolla after flowering, including the full bloom period (Hildebrand et al., 2001). Other targets of primary infection are pollen and cell exudates, which often occur during the later stages of flowering. Additionally, *B. cinerea* can remain dormant on decaying floral tissue to infect adjacent fruit later in the season (Pezet et al., 2003). The development of *B. cinerea* infection is dependent on weather conditions. Under an optimal temperature of 15-20°C, a surface wetness period of over 4-8 hours is enough to produce infection in grape berries (Broome et al., 1995), strawberries (Bulger et al., 1987), blueberries (Rivera et al., 2013), and blueberry flowers (Hildebrand et al., 2001).

Importantly, infection of floral organs and adjacent tissue support the presence of inoculum in the orchard and are responsible for subsequent *B. cinerea* infections. Secondary infections are caused by inoculum produced asexually from primary infections. This includes asexual conidia produced resulting from infection of corollas, ovaries, and peduncles of decayed flowers, pollen and cell exudate surface infections, and non-pollinated ovaries (Hildebrand et al., 2001). The primary infection on flowers can rapidly develop and produce conidia, resulting in exponential growth of pathogen presence and infection of other plant organs. Once flowering is complete, *B. cinerea* infections rely on decaying plant tissue, including floral organs, leaf litter, and orchard debris, to continue to produce new conidia. Alternatively, germinated conidia present on plant surfaces enter an endophytic quiescent phase.

2.6.1.1.1 Conidia

The spread of *B. cinerea* is enabled by conidia, which are lightweight, short–lived, single–celled spores (Dewey & Grant-Downton, 2016). These attributes allow conidia to spread over kilometres (Carisse, 2016). Despite this potential distance, the most important source of *B. cinerea* inoculum is within 1.6 m of the orchard canopy, with 95% of inoculum landing within this radius in vineyards with a summer canopy (Seyb, 2004). Conidia are borne from the tips of branch-like structures known as conidiophores. The survival of these propagules in the environment is determined by environmental conditions, including moisture, sunlight, and microbial activity (Holz et al., 2007). Conidia are produced for up to 12 weeks after germination

from sclerotia (Nair & Nadtotchei, 1987). Conidia are dispersed through wetting and drying cycles resulting from rainfall or dew, which dislodge them from conidiophores. Conidia are picked up by air currents and carried through the air, where they can land on suitable substrate and germinate to produce infection.

2.6.1.1.2 Infection

Botrytis infection occurs from the moment conidia are deposited on a suitable host. The relatively heavy weight of conidia in the air leads to them falling out of suspension and impacting with obstacles in the environment, including plant tissue (Holz et al., 2007). Germ tubes are produced which explore the surface of the adjacent plant cells to determine an entry point; either a naturally occurring opening or an existing wound is found, or an appressorium is formed to force entry to the host, assisted by cell wall–degrading enzymes (Williamson et al., 2007). Once inside a host cell, the production of pathogenic phytotoxic metabolites, including botryoidal and botcinolides occurs. In response to this attack, the host produces a ROS burst from the host cell as a defence response to potentially kill the pathogen (Amselem et al., 2011; van Kan, 2006). If the host cell is unable to overcome the pathogen, the hypersensitive response occurs, and the infected cell dies, allowing *B. cinerea* to obtain nutrition from the dead cell. This process repeats with adjacent cells, and the necrotic area expands, and *B. cinerea* develops a network of hyphae known as mycelium.

B. cinerea does not always initiate necrotrophic infection immediately when it is deposited on a host. When the host is perceived to be resistant, *B. cinerea* conidia germinate, form endophytic hyphae, and enter a low metabolic state, remaining dormant until the host becomes susceptible (Wenneker & Thomma, 2020). This constitutes a quiescent phase where dormant infections are present on the fruit but remain undetected by the host. These infections occur when conidia land on a presently unsusceptible host; they can wait in a quiescent state until conditions are suitable for their development (Elmer & Michailides, 2007). These quiescent infections are often initiated during flowering, when *B. cinerea* inoculum is present from infections of decaying floral organs and through inoculum being deposited during fruit development. Quiescent infections exist as an equilibrium between host, pathogen, and environment (Wenneker & Thomma, 2020). If the environment changes through direct sunlight or UV light exposure, the germination of conidia can be reduced (Rotem & Aust, 1991). On the other hand, warm temperature, high RH, and declining host resistance encourage infection (Elmer & Michailides, 2007). Quiescent infections remain dormant until the environment changes or host resistance declines, both of which occur postharvest. In the postharvest environment, *B. cinerea* infections occur from quiescent infections or infections inoculated during postharvest handling. Infections of *B. cinerea* only become pathogenic once host resistance wanes to a critical level during the postharvest senescence process. This initiates necrotrophic attack, and the asexual cycle resumes. This aspect of *B. cinerea* is of significant concern for horticultural produce quality, as

produce is harvested asymptomatic, but once host resistance declines, infection occurs and makes the produce unmarketable.

2.6.1.1.3 Mycelium

Mycelium is the visually observed fungal tissue (hyphal threads) associated with moulds and the decay of fresh produce. In *B. cinerea,* this is a characteristic tan or grey colour, giving rise to the common name of grey mould. Grey mould is indicative of infected, necrotic tissue beneath the mycelium. The composition of mycelium is a network of hyphae covered by an extracellular matrix mainly comprised of β-1,3 D-glycan (Bar-Nun et al., 2007). The development and density of this network, and hence its appearance, are greatly influenced by relative humidity, with shelter from wind and high relative humidity (94% RH) crucial for mycelium growth as described in vitro on grape berries by Thomas et al. (1988). At high (94%) RH, the mycelium tends to be less dense and more extensive, as shown in **Fig. 2.4**, while at lower (90%) RH the mycelium is denser, and restricted to sheltered sites on the fruit, so it is less extensive (Thomas et al., 1988). Once a mycelium is established, it begins to produce conidiophores and large quantities of conidia, which are released into the atmosphere to produce new infections of susceptible tissue. Growth media most similar to fruit tissue produce conidia more profusely than other media, indicating the potential of this inoculum source to affect multiple fruit once infection of fruit is initiated (Ciliberti et al., 2016). Mycelium is reported to survive in vivo for up to 30 weeks among pruning debris of grape vines left in vineyards, depending on sward management and temperature (Thomas et al., 1983). This makes the mycelial stage a key part of the pathogenicity of *B. cinerea*.

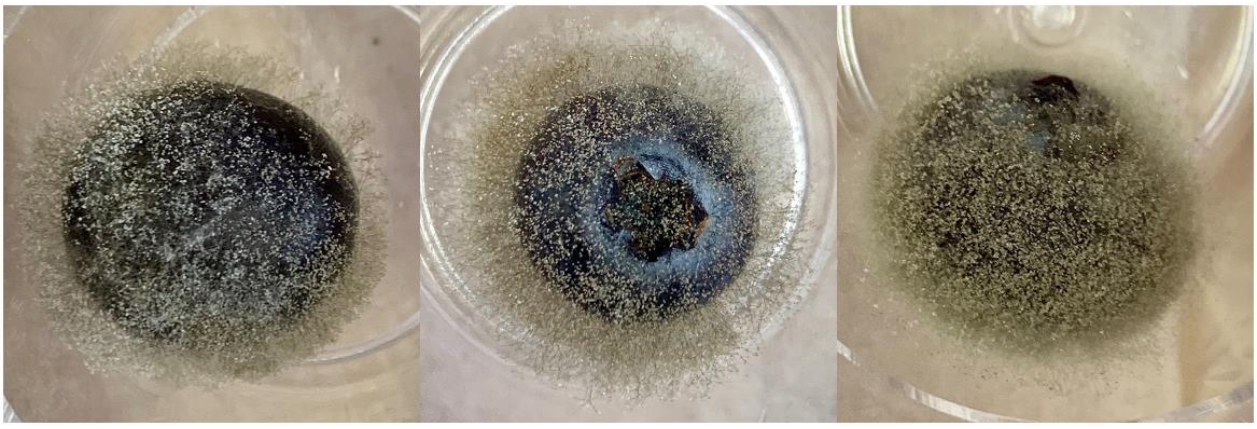


Figure 2.4. Grey mould decay caused by *Botrytis cinerea* on blueberry fruit.

2.6.1.1.4 Sclerotia

Sclerotia are the overwintering bodies of *B. cinerea.* Ageing mycelium forms sclerotia as survival structures once conditions become unsuitable for continued growth, generally due to diminishing resources (Dewey & Grant-Downton, 2016). They are melanised structures able to store nutrition and remain viable for between five and nine months in the environment (Dewey & Grant-Downton, 2016; Thomas et al., 1983). They can withstand adverse conditions to germinate when conditions become favourable and act as the primary source of *Botrytis* inoculum in late winter and early spring (Hsiang & Chastagner, 1992). On germination, mycelium, conidiophores, and conidia are produced to distribute conidia into the environment and infect susceptible plant organs. Conditions favouring sclerotia germination are wetting and drying cycles following rainfall or dew in late winter and spring (Dewey & Grant-Downton, 2016). The size of sclerotia varies between species, but they are generally irregular in shape and dark in colour; they inhabit the top layer of soil amongst decaying plant material. The sclerotial life stage facilitates the sexual cycle of *Botrytis*.

In blueberry orchards, sclerotia are found within decaying plant material in the top layer of soil or within other sources of decayed plant debris, including within the canopy (Elmer & Michailides, 2007). Plant debris includes residual fruit, leaves, pruning debris, mulches, and weeds; these offer substrate for sclerotia and overwintering mycelium (Braun & Sutton, 1987). These provide an opportunity for *B. cinerea* material to overwinter and germinate in late winter/early spring to infect new plant tissue.

2.6.1.1.5 Sexual Cycle

The sexual cycle of *B. cinerea* is rarely seen in the environment, with this thought to be due to misidentification of fruiting bodies (Dewey & Grant-Downton, 2016). The sexual cycle begins with sclerotial structures being fertilised by microconidia, which are produced from asexual mycelium (Fukumori et al., 2004). Apothecia are produced as a result of fertilisation; these utilise the nutrition stored in the sclerotia to grow phototrophically and produce apothecia (Rodenburg et al., 2018; Urbasch, 1983). On the upper surface of the apothecia, asci are borne, containing ascospores, which are released into the air. These can be primary inoculum in the early spring season, causing initial infection of plant organs before asexual reproduction from conidia becomes prevalent.

2.6.1.2 Morphology

2.6.1.2.1 Macromorphology

Isolates of *B. cinerea* on potato dextrose agar (PDA) form a white/grey colony with grey or tan-coloured conidiophores, which are a key distinguishing feature (Saito et al., 2016). Sclerotia are black, grey, or dark brown and have an irregular shape (**Fig. 2.5**). The growth rate was observed by Saito et al. (2016) to be 14.7 ± 0.6 mm/day.

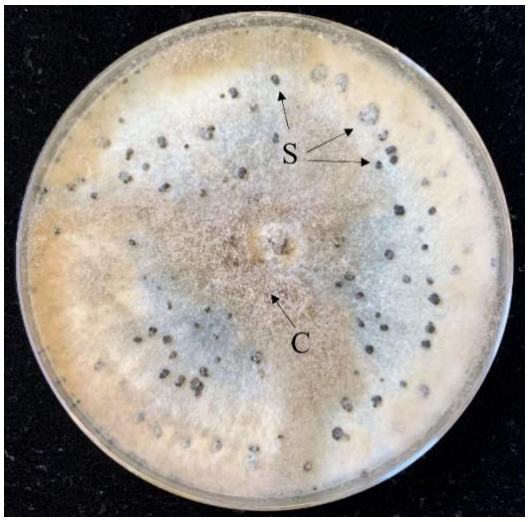


Figure 2.5. Image of *Botrytis cinerea* culture on PDA. Annotations indicate sclerotia (**S**) and conidiophores (**C**).

2.6.1.2.2 Micromorphology

The conidia of *B. cinerea* are reported to be approximately 1.4 mm long (Saito et al., 2016). The conidia of *B. cinerea* were observed by Jarvis (1977) to have a length of 8–14 µm and a width of 6–9 µm. Conidia are hyaline, ellipsoid, obovoid, or globoid with smooth edges, and are borne from near the apex of branching conidiophores, and feature an abscission scar when detached (Jarvis, 1977).

2.6.2 *Colletotrichum* spp.

The genus *Colletotrichum* is of the order *Glomerallales*, and the family *Glomerellaceae*. It contains over 200 species, which are classified into 12 species complexes. This genus causes anthracnose disease in many crops in various climates, making it a globally important pathogen (Dean et al., 2012). Anthracnose, also known as ripe rot, characteristically produces lesions that exude pink to orange-coloured liquid containing conidia (acervuli) on the surface of affected fruit (Wharton & Schilder, 2008).

In blueberries grown in the USA, the *Colletotrichum* spp. that cause fruit decay are within the *C. gloeosporioides* species complex and the *Colletotrichum acutatum* species complex (Cappellini et al., 1972; Smith et al., 1996). These species complexes also cause anthracnose disease of vegetative blueberry tissue; however, fruit anthracnose is the greater concern (Xu et al., 2013; Yoshida & Tsukiboshi, 2002). Of these species' complexes, the *C. acutatum* spp. complex is more commonly reported to cause anthracnose decay of blueberry fruit (Wharton & Schilder, 2008). This decay has been reported to cause fruit losses before and after harvest, with postharvest losses able to reach extreme levels (Wharton & Schilder, 2008). This makes *Colletotrichum* spp. one of the major pathogens of blueberry fruit.

Table 2.2. Incidence of anthracnose decay caused by *Colletotrichum* spp. reported in the literature.

Blueberry Cultivar(s)	**Location**	**Year**	**Storage Duration (days)**	**Temperature (°C)**	**Anthracnose Incidence (%)**	**Reference**
‘Scintilla’, ‘Star’, ‘Farthing’, ‘Sweetcrisp’	Georgia, USA	2009	21	2	0.63	Mehra et al., 2013
		2010			5.2	
‘Jersey’	Michigan, USA	1997	7	2	3.7	Hanson et al., 2000
Highbush	Michigan, USA	2003	42	2	17.2	Hancock et al., 2008
		2004			20.3	
‘Bluecrop’	Michigan, USA	1999	7–10	Ambient	20	Schilder et al., 2000
‘Jersey’						
‘Rubel’						
‘Elliott’						

2.6.2.1 *Colletotrichum* spp. Lifecycle

Colletotrichum pathogens affecting blueberries are present perennially in the environment. As blueberry fruit are mostly affected by the *C. acutatum* spp. complex, the lifecycle of this species complex will be the subject of this description. The asexual lifecycle is the only form of reproduction found in the field, and the sexual stage has only been observed in vitro (Guerber et al., 2003; Wharton & Diéguez-Uribeondo, 2004). The asexual lifecycle consists of conidia being distributed by rain splash, being deposited, and germinating on host surfaces (Dowling et al., 2020). These then penetrate the epidermis and grow throughout plant tissue, finally producing acervuli and conidia, which are dispersed to cause new infections (Wharton & Diéguez-Uribeondo, 2004). This infection cycle is continuous, enabling infections to spread and develop quickly on susceptible material.

Primary infections of Colletotrichum in blueberry orchards originate from remaining tissue infected in the previous season, including twigs, fruit trusses, and most commonly, buds (Wharton & Diéguez-Uribeondo, 2004). These tissues harbour infections from the previous growing season, existing as appressoria, mycelium, and conidia within these areas (Milholland, 1995; Peres et al., 2005). Once host dormancy breaks in late winter and early spring, *C. acutatum* infections become active and infect vegetative tissue adjacent to infections. This often leads to flowers and young leaves becoming infected and dying, enabling the spread of conidia to other host tissue (Wharton & Diéguez-Uribeondo, 2004). These primary infections distribute conidia in alignment with flowering, fruit set, and early fruit development (Wharton & Diéguez-Uribeondo, 2004). The conidia create new infections, resulting in significant economic damage.

2.6.2.1.1 Conidia

Conidia initiate infections of anthracnose disease. Conidia are produced prolifically from the acervuli of existing infections. As conidia are present in a mucilaginous liquid, they are dispersed by rain splash or direct contact with other tissue (Yang et al., 1990). Conidia can also be produced on the surface of symptomless living leaves and spread through rain splash (Leandro et al., 2001). As conidia are spread through rain splash, periods of high rainfall and wind are favourable to conidia dispersal. The mucus-like liquid within which conidia are exuded offers protection from desiccation; this enables conidia to survive on surfaces for a short time if they do not land on plant tissue (Perfect et al., 1999). This offers another avenue for infections to spread through infested surfaces in the postharvest environment and through fruit-to-fruit contact (Cline, 1996). Following conidia production and dispersal, if a suitable host is found, infection can commence.

2.6.2.1.2 Infection

Infection by *C. acutatum* of blueberry fruit conforms to a hemibiotrophic lifestyle consisting of biotrophy, then switching to necrotrophy as fruit ripen (De Silva et al., 2017). Infections begin with conidia landing on aerial tissue of the host plant and germinating to produce germ tubes which anchor the conidia to the host (Peres et al., 2005). Germ tubes develop appressoria, which are specialised tissue capable of penetrating the host cell (Perfect et al., 1999). Appressoria utilise pressure to push a penetration peg into the host tissue. If the host is resistant, the appressoria will penetrate the host cuticle and develop in the subcuticular region. If the host is susceptible, the cell wall is penetrated, and infection hyphae develop intracellularly (Wharton & Schilder, 2008). *Colletotrichum* can also enter through natural openings and wounds in host tissue (Wharton & Diéguez-Uribeondo, 2004). Once the infection is established and remains undetected by host defence, *C. acutatum* continues its biotrophic phase (De Silva et al., 2017). The infection can enter a quiescent period if unfavourable host resistance or environmental conditions persist. Once fruit ripen and host defences decrease to a threshold level, biotrophic or quiescent *C. acutatum* infections begin to produce secondary infection hyphae, which undertake a necrotrophic lifestyle, killing host cells and aggressively expanding the infection to produce anthracnose sign (De Silva et al., 2017). This switch to necrotrophy occurs when fruit are ripe and host resistance and environmental conditions are conducive to decay.

The ideal conditions for infection are warm, humid conditions, as conidia are spread through rain splash (Brook, 1977). High humidity is important for infection as the glycoprotein mucilage conidia are dispersed with offers limited protection against desiccation, but high humidity conditions enable greater survival of conidia by reducing desiccation and can activate quiescent infections (Perfect et al., 1999; Wharton & Diéguez-Uribeondo, 2004). For germination of conidia and development of appressoria, 100% RH is considered optimum, or uninterrupted surface water presence for over eight hours (Miles et al., 2013). Thus, periods of uninterrupted rainfall or dew offer a greater chance of infection. Temperature is also important; the lowest temperature at which *C. acutatum* is active was recorded as 7°C in vitro by Verma et al. (2006). This is relevant to postharvest storage as low temperature can inhibit activity and thus anthracnose decay caused by *C. acutatum*. The optimum temperature for growth is reported as 26°C, so warm and humid conditions can lead to greater infection incidence (Miles et al., 2013).

2.6.2.1.3 Decay

Anthracnose caused by *C. acutatum* is formed following penetration of host tissue and initiation of the necrotrophic phase. Hyphae existing intercellularly or intracellularly develop a network within plant tissue. The function of these hyphae once the necrotrophic stage is initiated is to kill host tissue in advance of the

expanding infection. Once the infection becomes necrotrophic and sufficient resources are acquired from the host, acervuli are produced, which exude orange or salmon-coloured mucilage containing conidia. If the infection has been unable to effectively obtain nutrition from the host due to host resistance, acervuli contain fewer conidia and occur less frequently (Wharton & Schilder, 2008). Anthracnose decay results in fruit displaying orange-coloured droplets and is considered unmarketable, as shown in Error! Reference source not found.**6**. Also considered part of this decay is the mycelium associated with secondary infection, which can be occasionally observed on the surface of severely infected fruit. While *C. acutatum* is a significant cause of decay; it can be observed in non-pathogenic forms and isolated from non-symptomatic plants, suggesting colonisation as an epiphyte or endophyte without producing decay (Peres et al., 2005). This shows that *C. acutatum* is an adaptable and prevalent fungus in the orchard environment and can cause severe decay of susceptible hosts.

Figure 2.6. Anthracnose decay of blueberry fruit caused by *Colletotrichum acutatum*.

2.6.2.1.4 Overwintering

The survival of *C. acutatum* between growing seasons occurs as mycelium, appressoria, or conidia in the environment or on host plants (Milholland, 1995; Peres et al., 2005). In blueberry orchards, residual infected tissue such as mummified fruit, twigs, fruit trusses, and bud scales offer habitat for *C. acutatum* to reside in dormancy (Wharton & Diéguez-Uribeondo, 2004). Non-symptomatic tissue also offers the opportunity for *C. acutatum* to remain present as an endophyte or epiphyte through the winter (Yoshida et al., 2007). A degree of control can be obtained through the removal of affected twigs, fruit trusses, and other tissue present in the bush through the winter (Wharton & Diéguez-Uribeondo, 2004). By removing these sources in the bush, the pathogen presence is reduced, and dispersal of conidia is limited in the new season.

2.6.2.1.5 Sexual Cycle

The sexual cycle of the *C. acutatum* species complex has not been observed and is poorly understood (De Silva et al., 2017). Hence, the general sexual cycle of *Colleotrichum* spp. is described. Infected tissue provides a host for overwintering and sexual reproduction. Within this tissue, fruiting bodies known as perithecia are produced, which develop ascospores (De Silva et al., 2017). These are then released into the environment and infect nearby tissue, producing anthracnose and subsequent asexual conidia.

2.6.2.2 Morphology

2.6.2.2.1 Macromorphology

The macromorphology of *C. acutatum* is a multi-coloured culture featuring orange and green sectors of mycelium with orange–coloured acervuli on oatmeal agar (Damm et al., 2012). Another species within the *C. acutatum* spp. complex is *C. fioriniae*, which is reported to cause blueberry fruit anthracnose (Castro et al., 2022). On PDA, this species produces grey, cottony mycelium with tufts of aerial mycelium. Orange coloured conidiomata/acervuli are sparse (Shivas & Tan, 2009). Also present within the *C. acutatum* spp. complex is *C. godetiae*. This species produces a light grey mycelium which becomes darker grey with age. Acervuli are dark coloured and yellow/orange spore masses become present (Baroncelli et al., 2014). Another relevant species is *C. salicis*, which has a pale amber mycelium covered by pale white/olivaceous aerial mycelium and pale salmon acervuli (Damm et al., 2012).

2.6.2.2.2 Micromorphology

The conidia of *C. acutatum* are ellipsoid or fusiform in shape, with at least one end being ellipsoid (Peres et al., 2005). Their dimensions are 11–14.5 µm x 3.5–4 µm (Damm et al., 2012). *C. fioriniae* has conidia of dimensions 13.5–16.5 µm x 4–5 µm. These are hyaline, smooth walled, fusiform, and have acute ends (Damm et al., 2012). Conidiophores are also hyaline, smooth walled and branched on synthetic nutrient poor agar (SNA) (Damm et al., 2012). *C. godetiae* has conidia which vary between one acute end and both ends acute, the conidia are hyaline with smooth walls and a fusiform shape. Their dimensions are 10.5–15 µm x 4–5 µm (Damm et al., 2012). The conidia of *C. salicis* are hyaline, straight, with smooth walls; they are 10.5–15.5 µm x 3–4.5 µm, with one end acute (Damm et al., 2012). The conidiophores are also hyaline and smooth walled, with some branching on SNA (Damm et al., 2012). These species are within the same

species complex, so they have genetic differences but are morphologically similar. Hence, molecular analysis is the most accurate means of distinguishing these species.

2.6.3 Other Pathogens Affecting Harvested Blueberry Fruit

Other pathogens have been reported to be associated with the postharvest decay of blueberry fruit. These have varying lifestyles but generally feature an endophytic or epiphytic stage, leading to the initiation of decay as a necrotroph or saprophyte. These include *Alternaria* spp., *Pestalotiopsis* spp., *Epicoccum* spp., *Neofusicoccum* spp., *Penicillium* spp., and others.

2.6.3.1 *Alternaria* spp.

Alternaria spp. are pathogens of postharvest blueberry fruit. The important species are *Alternaria alternata*, *Alternaria tenuissma*, and *Alternaria arborscens* (Zhu & Xiao, 2015). *Alternaria* spp. are reported to be opportunistic pathogens of natural openings and wounds, but they are also able breach the cuticle after ripening (Troncoso-Rojas & Tiznado-Hernández, 2014). The most common occurrence of *Alternaria* spp. decay follows damage to host tissue such as mechanical damage, environmental stress, or overripening (Swart et al., 1995). *Alternaria* decay prevalence is reported to be a significant problem for blueberry production, with losses ranging from 0.8–2.3% (Cappellini et al., 1982) to 2.6–9.5% (Xiao & Saito, 2016). Importantly, blueberry fruit decay from *Alternaria* spp. has been reported by Schilder et al. (2000) to be more prevalent in the early season and decrease in occurrence as the season progresses. This indicates the limited ability of this microorganism to produce multiple infection cycles.

As this genus is not observed to exhibit a sexual stage and has no overwintering spores, infections survive as mycelium or spores in the environment (Rotem, 1994). The infection process of *Alternaria* spp. consists of conidia adhesion to the host cuticle, followed by germination and germ tube production (Troncoso-Rojas & Tiznado-Hernández, 2014). At this stage, the infection can remain quiescent as an epiphytic infection or continue to invade host tissue, depending on the level of host resistance (Prusky et al., 2002). Once conditions become suitable for necrotrophic infection, the germ tube differentiates to become an appressorium and produces a penetration peg which is extended into the host cuticle. Once sufficient melanin accumulation has occurred in the appressorium cell wall to provide strength to withstand the pressure required to penetrate the cuticle, the penetration peg is forced through (Troncoso-Rojas & Tiznado-Hernández, 2014). Following penetration of the host, either through an appressorium, natural

opening, or wound, hyphae extend intercellularly through the infected tissue and excrete pathogenic enzymes, toxins, and polysaccharides to become necrotrophic and degrade the host cell wall (Troncoso-Rojas & Tiznado-Hernández, 2014). This results in a necrotrophic infection of host tissue, resulting in decay featuring lesions with green-coloured mycelium.

The conditions required for the establishment of *Alternaria* spp. infections of fruit tissue are surface water or very high humidity (100% RH) for 3–72 hours and temperatures above 0°C, with an optimum of 25°C (Prusky et al., 1981; Troncoso-Rojas & Tiznado-Hernández, 2014). These conditions make *Alternaria* spp. a potentially significant pathogen during prolonged fruit wetness and cold storage, especially if fruit are wounded or damaged.

2.6.3.1.1 Morphology

Cultures of *A. alternata* feature light green to olive green mycelium with a white margin. The conidia form branches of 6–15 conidia, with secondary chains present. The dimensions of individual conidia are 14.6–36.2 µm x 5.2–15.0 µm, their shape is ovate to obclavate (Zhu & Xiao, 2015). *A. tenuissima* features mycelium similar in colour to *A. alternaria*, with conidia dimensions of 13.9–43.0 µm x 5.8–13.7 µm, ovate to obclavate shaped and form branched chains (Zhu & Xiao, 2015). *A. arborescens* features darker green and grey coloured mycelium with a wavy margin and orange pigmentation and crystals present in PDA. The conidia are 11.7–35.3 µm x 6.3–15 µm and of ovate shape (Zhu & Xiao, 2015).

2.6.3.2 *Pestalotiopsis* spp.

The genus *Pestalotiopsis* is infrequently observed as a pathogen of fresh fruit, instead being reported mainly as non-specific endophyte and pathogen of numerous plant species (Maharachchikumbura et al., 2014). It is an opportunistic pathogen reported to occur rarely on blueberry fruit (Mehra et al., 2013). *Pestalotiopsis* spp. have been observed to cause decay of other fruits, including grapes (Deng et al., 2013), avocados (Valencia et al., 2011), kiwifruit (Li et al., 2016), loquat (Palou et al., 2013), and feijoa (Naeimi et al., 2015). The decay observed features a fine white mycelium and necrosis of host tissue in some species, resulting in the formation of black conidiomata (Lorenzini & Zapparoli, 2018; Valencia et al., 2011; Wharton, 2003). The endophytic presence of *Pestalotiopsis* is thought to become pathogenic when host plants become susceptible through overripening or wounding; however, it is considered a weak pathogen and is not reported to produce appressoria (Maharachchikumbura et al., 2011).

The infection process of *Pestalotiopsis* spp. begins with conidia being dispersed by rain splash or wind and deposited on the surface of fruit tissue. Here they are loosely attached and can enter an endophytic phase. If conidia are deposited near or within a natural opening or wound, they germinate and become firmly attached to the host (Watanabe et al., 2000). Following this, a germ tube is produced, which produces infection hyphae and the characteristic white mycelium. Once nutrition is obtained, a pycnidium or perithecium is formed to facilitate reproduction. Conidiomata or acervuli then form, which contain conidiophores and conidia. Pycnidia are developed from perithecia and produce large quantities of conidia (Maharachchikumbura et al., 2012). These conidia are dispersed and facilitate further infections. The required conditions for infection are temperatures above 5°C (Espinoza et al., 2008), and RH is suggested to be optimum at 70% RH on vegetative material (Das et al., 2010). The development of fruit decay occurs above 90% RH (Lorenzini & Zapparoli, 2018).

2.6.3.2.1 Morphology

The morphology of *Pestalotiopsis* spp. varies by species; the species most related to plant pathogenicity and fruit decays will be detailed. *Pestalotiopsis disseminata* conidia are cylindrical, concolourous, and five–celled, with two apical appendages and one basal appendage. Their dimensions are 18–26 µm x 5–8 µm (Maharachchikumbura et al., 2011). *Pestalotiopsis clavispora* conidia are versicolorous, five–celled, cylindrical, with two apical appendages, one basal appendage and are 19–26 µm x 6–8.5 µm (Maharachchikumbura et al., 2011). *Pestalotiopsis microspora* conidia are concolorous, five–celled, cylindrical, and have two apical appendages and one basal appendage. The dimensions of these conidia are 18–26 µm x 5–8 µm (Maharachchikumbura et al., 2011). The conidia of *Pestalotiopsis bicilita* are concolourous fusoid, ellipsoid, straight to slightly curved, five-celled, 22–28.5 µm x 6–7.5 µm. Conidia have two basal appendages and two to three apical appendages without knobs (Maharachchikumbura et al., 2014). Cultures of these species are relatively similar, with white/yellow mycelium featuring conidiomata/acervuli under suitable conditions.

2.6.3.3 *Diaporthe* spp.

Diaporthe is a genus of plant pathogens, non-pathogenic endophytes, and saprobes (Gomes et al., 2013). *Diaporthe* is also known by its anamorph, *Phomopsis*, which represents the asexual stage of this genus (Udayanga et al., 2012). Fruit rots caused by *Diaporthe* spp. have been reported in blueberry (Yu et al., 2018), kiwifruit (Díaz et al., 2014), pear (Bertetti et al., 2018), dragon fruit (Wang et al., 2022), avocado (Torres et al., 2016), mango (Ajitomi et al., 2020), and peach (Zhang et al., 2021). The variety and geographical spread of this genus make it an important plant pathogen. The decay observed on blueberry fruit from *Diaporthe nobilis* features localised softening (Yu et al., 2018). On grape berries, a whitish

mycelium was observed caused by *Diaporthe eres* (Lorenzini & Zapparoli, 2018). The asexual form of *Diaporthe vaccinii* is known to cause decay of blueberry fruit, featuring cream-coloured droplets (pycnidia) (Gomes et al., 2013; Wharton, 2003). Interestingly, *Diaporthe* spp. complete a single infection cycle over the growing season of the host, making this microorganism a less frequent pathogen (Anco et al., 2012). This microorganism can infect fruit tissue at 0°C, with an optimum of 20–30°C (Abramczyk et al., 2020; Díaz et al., 2014).

The infection process of *Diaporthe* spp. on blueberry fruit begins with penetration of the host cuticle (Cardinaals et al., 2018). Host penetration by conidia can occur at any time throughout flowering and fruit growth, but the pathogen often remains dormant until favourable conditions occur (Milholland & Daykin, 1983). This leads to the occurrence of decay signs at fruit maturity or after harvest (Pscheidt & Pearson, 1991). *Diaporthe* spp. feature two forms of conidia: alpha conidia, which are more common, and beta conidia, which are rarer. Alpha conidia are produced from the infection of fruit tissue or vegetative material and are the primary source of infections (Arciuolo et al., 2021). These conidia are formed within cirrhi in mature infections producing pycnidia (Pscheidt & Pearson, 1991). When wet conditions occur, the cirrhi open to disperse the α conidia through rain splash (Pscheidt & Pearson, 1991). This results in infection of fruit and stems within a localised area of the host.

2.6.3.3.1 Morphology

The morphology of *Diaporthe* spp. varies by species. Generally, α conidia are fusoid to elliptical in shape, while β conidia are long and thin with a spindle shape (Arciuolo et al., 2021). *D. eres* produces α conidia of 4.4–4.8 µm x 1.4–3.3 µm; these are ellipsoid to fusiform in shape and hyaline in colour (Liu et al., 2021). *D. nobilis* is reported to produce α conidia of dimensions 6.8–8.7 µm x 1.9–3.9 µm; these are also hyaline with an ellipsoid to fusiform shape (Lawrence et al., 2015). Both of these species feature concentric rings of white to grey and yellow mycelium on PDA after eight days at 25°C in darkness (Lawrence et al., 2015). *D. vaccinii* α conidia have dimensions of 5.9–11.3 µm x 2.1–3.9 µm; they are hyaline with a fusiform shape (Farr et al., 2002). Colonies of this species after eight days at 25°C are white with a yellow/grey to brownish-grey colour, and a felt to cottony texture (Farr et al., 2002).

2.6.3.4 *Epicoccum nigrum*.

Epicoccum nigrum is reported to cause fruit decay of blueberry fruit (Wharton, 2003). This decay features an orange/brown mycelium. This genus is also reported as one of the main organisms of the blueberry microflora (Munitz et al., 2013). Interestingly, species of *Epicoccum* have an antagonistic relationship with *B. cinerea,* leading to some strains being used as biocontrol agents (Auda, 2021; Sanhueza et al., 2022).

Given this organism's prevalence in the blueberry microbiome, it is a minor decay–causing organism of blueberry fruit (Oliver & Ali, 2021). *Epicoccum nigrum* has been shown to infect grape berries at low relative humidity and is suggested to exist as an epiphyte or endophyte until becoming a saprophyte on senescent fruit (Lorenzini & Zapparoli, 2015; Taguiam et al., 2021). This makes *E. nigrum* a weak pathogen, and it is not a prolific decay causing organism in the postharvest supply chain (Taguiam et al., 2021).

The infection process of pathogenic strains of *E. nigrum* requires an opening in plant tissue to gain entry (Taguiam et al., 2021). Natural openings and wounds including the stem scar, offer a means of entry for this weak pathogen, as it does not produce an appressorium (Taguiam et al., 2021; Wu et al., 2017). Following entry, infection hyphae develop and acquire host resources; these develop through dead host tissue and produce the distinct orange/brown mycelium (Taguiam et al., 2021; Wharton, 2003). Given these attributes, *E. nigrum* is considered a weak pathogen (Taguiam et al., 2021).

2.6.3.3.1 Morphology

The morphology of *E. nigrum* is distinct compared to other postharvest fungi. Within this species, there are distinct differences between isolates. The colouration of mycelium in PDA is white or yellow/brown, with pigmentation of PDA and an irregular shape. Conidia are spherical with a diameter of 10–20 µm (Li et al., 2022).

2.6.3.4 *Neofusicoccum* spp.

Neofusicoccum spp. are pathogens associated with blueberry stem canker and dieback (Espinoza et al., 2009). This genus is present in the microflora of blueberry fruit and can cause fruit decay (Tennakoon et al., 2022). The species causing decay of blueberry fruit are *Neofusicoccum parvum* and *Neofusicoccum austral.* These species can be present as endophytes, and wounding of host tissue or stress provide an opportunity for pathogenicity (Nazarpour et al., 2022). Infection consists of the excretion of necrotic elicitors and cell wall–degrading enzymes, which produce necrosis of susceptible host tissue (Nazarpour et al., 2022). This enables the acquisition of host resources and the production of pycnidia to complete the pathogen lifecycle. The decay features a necrotic lesion leading to mummified fruit and pycnidia production. This decay results in necrosis after 3–6 days on wounded fruit and 9–12 days on non-wounded fruit, followed by mummification 14–25 days after inoculation (Tennakoon et al., 2022). This extended time frame for infection means that other pathogens within the microflora are likely to outcompete these organisms.

2.6.3.4.1 Morphology

Neofusicoccum spp. features a grey mycelium with a fluffy texture on PDA and produces pycnidia on plant material (Berraf-Tebbal et al., 2014). The micromorphology of *N. australe* features sub-fusiform, hyaline, and aseptate conidia with dimensions of 18.4–19.5 µm x 6.4–6.8 µm (Lazzizera et al., 2008). *N. parvum* conidia are ellipsoid shaped with a sub-obtuse apex and a truncate base. They are hyaline and have thin walls. The dimensions are 18–21.5 µm x 4.5–7.5 µm (Golzar & Burgess, 2011).

2.6.3.5 *Penicillium* spp.

Penicillium is one of the most important decay–causing organisms of fresh produce (Errampalli, 2014). In blueberry fruit, it causes decay infrequently as it is mostly a wound pathogen, so fruit must be damaged before infection can occur (Amiri & Bompeix, 2005). *Penicillium expansum* can grow slowly in cold storage (-1–4°C), making it a threat to fresh produce quality through the supply chain (Errampalli, 2014). *Penicillium* encompasses the important pathogens *P. expansum* (blue mould) and *Penicillium digitatum* (green mould). These decays are virulent on wounded fresh produce tissue and can be prevalent on overripe fruit (Ahmadi-Afzadi et al., 2013). These microorganisms produce characteristic decays of their respective colours, causing water soaking and necrosis of host tissue.

The important *Penicillium* pathogens, *P. expansum* and *P. digitatum,* are necrotrophic fungi that require damage or wounding of host tissue to gain entry, or overripening (Amiri & Bompeix, 2005). Points of entry include the stem scar, senescent tissue, or tissue damaged through wounding or bruising. Following entry, the pathogen excretes cell wall degrading enzymes, and germ tubes proliferate deep into host tissue to further the infection (Wang et al., 2021). Once entry is gained, pathogenicity factors are released to further the infection and disrupt host defence (Wang et al., 2021). This results in the disintegration of host tissue, leading to water soaking symptoms and a sunken appearance (Errampalli, 2014). Within this damaged tissue, hyphae continue to grow, accumulating to develop the characteristic decay signs and conidia.

2.6.3.5.1 Morphology

The conidia of *P. expansum* are elliptical in shape, with dimensions of 4–5 µm x 2.5–3.5 µm. Colonies on PDA are blue-green with a white margin and velvety texture; the obverse is pale yellow (Errampalli, 2014). *P. digitatum* conidia are ellipsoid to cylindrical in shape with smooth walls; their dimensions are 3.5–8 µm x

3–4 µm (Palou, 2014). Colonies of *P. digitatum* are olive green with a light yellow/brown obverse and a velutinous texture (Palou, 2014).

2.6.3.6 Others

Other microorganisms reported to cause decay of postharvest blueberry fruit are *Aspergillus* spp., *Auerobasidium pullans*, *Cladosporium* spp., *Fusarium* spp., *Hainesia lythri*, *Neopestalotiopsis* spp., *Rhizopus* spp., *Rhizomucor* spp., and *Trichoderma* spp. (Mehra et al., 2013; Wang et al., 2016; Wharton, 2003; Xiao & Saito, 2016). These organisms occur sporadically and are often associated with fruit as endophytes or epiphytes, which undertake opportunistic strategies during blueberry fruit senescence to produce decay.

2.7 Identification of Microorganisms

2.7.1 Morphological

Morphological identification of microorganisms does not provide conclusive identification but can be used to obtain detail about the observable characteristics of microorganisms. Attributes of microbes can be recorded and analysed using macroscopic and microscopic techniques to characterise them and potentially inform identifications. Macroscopic attributes include colour, texture, growth rate, presence of organs, and pigmentation of the substrate. These are often distinct between fungal genera, enabling the grouping of cultures by these attributes. However, further analysis is required to obtain greater detail and a stronger identification. Microscopy is used to obtain detailed information on the microscopic attributes of microorganisms. For fungi, the nature of organs including conidia, conidiophores, and hyphae are used to distinguish species (Damm et al., 2012; Maharachchikumbura et al., 2014). However, species identification is more reliably confirmed through molecular techniques. The purpose of morphological analysis is to provide detailed information on the fungi and examples of cultures, which can inform casual identification in industry and non–laboratory settings.

2.7.2 Molecular

Molecular identification of microorganisms is a rapid, reliable, and accurate process for identifying species. The key techniques involved in identification are the extraction of genomic DNA, amplification of genes of interest through the polymerase chain reaction (PCR), and sequencing the PCR products to attain a DNA sequence (Spring & Thines, 2010). This sequence is then compared to previously reported sequences to gain an identification. This technology is precise and can be targeted to organisms of interest through specific genes. For identification of a wide range of fungi, genes of interest are selected based on their unique differences between genera and species, enabling DNA barcoding to be used.

DNA barcoding is a technique that targets a standardised region of the genome to accurately identify species, genera, or species complexes. For fungi, the major DNA barcoding gene is the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA cluster. The gene differs between fungal genera and species, allowing for accurate identification of fungal pathogens. In some cases, ITS alone is sufficient to reliably differentiate species; however, in some genera, including important postharvest pathogens, other genes are beneficial for more accurate identification (Kashyap et al., 2017). Other genes include *β-tubulin* (Bt2), *translation elongation factor 1 alpha* (TEF-1α), and *RNA polymerase* genes (RPB1 and RPB2) (Kashyap et al., 2017). By undertaking the analysis of multiple genes for identification of fungi, a more accurate and detailed species identification can be attained.

Multi–locus sequencing enables more detail to be obtained in order to identify fungi. This is useful when barcoding attempts are unsuccessful or limited barcoding data exists for the microorganism of interest. Multi–locus sequencing is commonly used to provide comprehensive identification of species and inform distinctions between species of important postharvest pathogens (Damm et al., 2012; Saito et al., 2016). This data can be applied to advanced downstream applications, including construction of phylogenetic trees and the identification of species. However, single locus analysis is accurate for the identification of fungi to the genus level, with species level identification being possible (Xiao & Saito, 2016). Therefore, the molecular tools to identify species can be optimised for the level of detail required for downstream purposes.

2.8 Conclusions

Decay of fresh blueberries is the result of the interaction of the disease triangle factors including host susceptibility, environmental conditions, and pathogen virulence. These factors vary greatly throughout the

supply chain, leading to inconsistent decay expression in the marketplace. Experimentation on these factors and determining their impact on decay prevalence, particularly postharvest temperature and relative humidity, is useful to understand for the growth of blueberry exports from NZ. Accurate identification of microorganisms through morphological and molecular techniques is also critical to enabling an understanding of these relationships with various postharvest environmental conditions.

The microorganisms related to postharvest decay of blueberry fruit have not been recently determined in NZ. This research will offer an improved understanding of the microorganisms causing decay of blueberry fruit and their interactions with postharvest temperature and relative humidity conditions. This research could provide a starting point for growers and marketers to better control decay in the supply chain.

Chapter 3: Identification of Microorganisms Associated with Decay of Blueberry Fruit

3.1 Introduction

Blueberry (*Vaccinium* spp.) fruit quality loss is principally caused by postharvest decay (Cappellini & Ceponis, 1977). The quality of blueberry fruit deteriorates from the moment of harvest due to senescence processes involving degradation of fruit physical and chemical quality, which increases susceptibility to decay (Wills & Golding, 2016). Postharvest decay of blueberry fruit is mainly caused by *Botrytis cinerea*, *Colletotrichum* spp., and *Alternaria* spp. (Schilder et al., 2000). These organisms infect fruit prior to harvest and can cause infection in the postharvest environment, leading to damage and unacceptable quality (Forney, 2009). Given the recent expansion of the New Zealand (NZ) blueberry industry and a focus on export markets for future growth, the determination of the major postharvest decay species of NZ blueberry fruit is required to improve the efficiency of the supply chain (Coriolis Research, 2020).

The quality of blueberry fruit is defined by the consumer, so visual appearance, flavour, and texture are critical for marketability (Chiabrando et al., 2009). Blueberry fruit quality peaks at harvest and subsequently deteriorates as softening, shrivel, flavour decline, and decay become prevalent; this irreversible process results in unmarketable fruit (Forney, 2009). Of these factors, decay is the major cause of quality loss; it is intricately linked to host susceptibility, pathogen pressure, and environmental conditions (Agrios, 2005). After harvest, host susceptibility to decay increases as senescence processes deteriorate host defences. Pathogen pressure is determined at harvest, as latent and quiescent infections initiated at flowering and throughout fruit development are present within the fruit microbiome and cause infection after harvest when suitable conditions are met. The environmental conditions experienced by the fruit have effects on postharvest decay, particularly temperature, of which low temperature is an effective control measure (Nunes et al., 2004). Invariably, due to fruit senescence, decay develops eventually after harvest.

Postharvest decay of blueberry fruit is reported to be associated with numerous plant pathogens, including *Alternaria* spp*., Aspergillus spp., Aureobasidium pullans, Botrytis* spp., *Cladosporium* spp., *Colleotrichum* spp., *Diaporthe* spp., *Epicoccum nigrum, Fusarium* spp*., Hainesia lythri, Penicillium* spp*., Pestalotia vaccini, Pestalotiopsis* spp., *Phomopsis vaccinii*, *Rhizopus* spp., and *Trichoderma* spp. (Mehra et al., 2013; Wang et al., 2016; Wharton, 2003; Xiao & Saito, 2016; Yu et al., 2018). In NZ there are few reports, but postharvest decay is reported to be caused by *B. cinerea, Colletotrichum* spp*.,* and *Alternaria tenuissima* (Johnston & McKenzie, 1982). However, due to the developing nature of the NZ blueberry industry and similarities with Australian and USA blueberry producers, the pathogens present are likely to be similar to species reported

overseas. It is also expected that postharvest decays of other fruit crops will be found due to the diverse land uses in NZ.

To identify microorganisms, multi–locus molecular analysis is the gold standard. This research will rely on this technique when other methods prove insufficient, as it is more resource intensive. Observation of macroscopic and microscopic features can provide genus-level identification, and single–locus gene sequencing alongside this is sufficient to obtain species level identification in most cases (Watanabe et al., 2011). Multi–locus molecular analysis provides further confidence in species identification and is a superior technique for molecular analysis in the case of poor–quality results from the initial analysis. These methods provide a wealth of data on the isolates related to decay and provide a confident identification to the species level of the organisms associated with decay.

The fruit microbiome is a diverse flora of microorganisms; host, pathogen, and environmental factors result in some organisms producing decay while others do not. There is a wide range of fungi present on blueberry fruit; often environmental or non–pathogenic organisms are found from fruit without decay signs or symptoms (Munitz et al., 2013; Watanabe et al., 2011). However, by isolating organisms from decaying fruit, there is a high likelihood of collecting the organism responsible for the decay. To confirm whether the organism is a causal agent of decay, pathogenicity testing is required. However, if an organism is consistently isolated from fruit producing a common decay sign, and is considered a causal agent in the literature, a confident call on the pathogenic relationship can be made. This is facilitated by categorising decay signs or symptoms and the isolates they are associated with. For example, common blueberry postharvest decays, grey mould, and anthracnose are caused by *B. cinerea* and *Colletotrichum* spp., respectively (Cappellini & Ceponis, 1977; Wharton & Schilder, 2008). The fact that these organisms are well–documented to cause decay means that there is a high probability that if these organisms are isolated from the characteristic decay, they are the causal agents. This provides a high degree of certainty that the organisms isolated from blueberry fruit decay are responsible for the reported decay.

The microorganisms associated with blueberry fruit decay in NZ are undetermined. Postharvest decay of blueberries is a limiting factor of postharvest storage life and subsequent export growth. By determining the microorganisms associated with this decay and the likely causal relationships with decay signs and symptoms, improvements to the efficiency of the supply chain can be made through the implementation of control measures. Through using morphological and molecular techniques to analyse microorganisms associated with decay, accurate species identifications can be provided, along with informative characterisations of these microorganisms.

3.1.1 Aim and Objectives

The aim of this chapter is to determine the species identity of microorganisms associated with the decay of fresh blueberry fruit in New Zealand through sequencing of genomic DNA, supplemented by morphological data. This will provide a confident species identification, which will inform a better understanding of the organisms involved in postharvest decay of blueberry fruit in this country. This is expected to offer numerous avenues for future research into these organisms and their relationships with horticultural practises. This research could be used to inform target species to implement control measures that aim to improve blueberry fruit quality through the supply chain.

The objectives of this experiment are as follows:

1. To gather morphological data from microorganisms associated with blueberry fruit decay to inform identification.
2. To conduct molecular analysis of selected microorganisms isolated from blueberry fruit decay to confirm species identification.
3. To compile morphological and molecular data to provide an informative characterisation of microorganisms isolated from blueberry fruit decay and the decay they are likely responsible for.

3.2 Materials and Methodology

3.2.1 Selection of Blueberry Fruit with Microbial Signs and/or Damage Symptoms Putatively Caused by Pathogenic Microbes

Blueberry fruit with microbial signs and/or damage symptoms putatively caused by pathogenic microbes were selected weekly at the conclusion of successive postharvest experiments for microbe isolation (see Section 4.2). Individual plastic cups (60 mL sample cup, Jadcup, Auckland, NZ) were used to contain one fruit and allow expression of signs and/or symptoms independently. Here, fruit with a specific sign and/or symptom appearing more than three times within a batch (45 fruit of one origin and with a postharvest treatment completed) were isolated at a rate of three per week per batch, while fruit with a specific sign and/or symptom appearing three or fewer times per week within a batch box were isolated as often as they appeared. This weighted the number of selections more evenly and prevented oversampling of fruit with

signs and/or symptoms of high incidence while enabling sensitivity to recognise fruit with signs and/or symptoms of low incidence.

3.2.2 Isolation of Microbes from Blueberry Fruit to Nutrient–Rich Agar for Growth in Culture

Blueberry fruit selected for microbe isolation in Section 3.2.1 were immediately taken to the Molecular Plant Pathology Laboratory at Massey University, New Zealand. Fruit were then photographed and notes taken to record the types of signs and/or symptoms observed.

Microbe isolation was carried out under a class II biological safety cabinet (Clyde-Apac BH2000 Series, Minto, NSW, Australia). Fruit remained individually in their plastic cups and were soaked in a sodium hypochlorite solution (10%) for 2 minutes to remove any contaminating surface associated microbes (it was assumed that if the microbe was pathogenic, it would not purely be surface associated). Then, the fruit were rinsed using sterile reverse osmosis (RO) water. Following this, individual fruit were removed from their plastic cup into independent sterile Petri dishes (90x15 mm, Labserv® Thermo Fisher Scientific) for excision of the microbe itself as small pieces of mycelium/spores with fruit tissue or infected tissue with no visible fungal mycelium/spores using a scalpel and tweezers. The excised material was placed onto a fresh Petri dish containing approximately 25 mL of potato dextrose agar (PDA; Millipore® Merck KGaA, Darmstadt, Germany). In between fruit samples, the scalpel and tweezers were sterilised using a Bunsen flame, while nitrile gloves were worn to minimise contamination. Following transfer, the Petri dishes were sealed using Parafilm® M (Pechiney Plastic Packaging; Chicago, IL, USA) in preparation for growth in culture.

3.2.3 Incubation of Material Isolated from Blueberry Fruit

The material excised from blueberry fruit in Section 3.2.2 was incubated at 23°C for 5 days under continuous 30–watt blue/red grow lights (Sylvania GRO-LUX T8, Wilmington, MA, USA) before being inspected for microbe development and contamination with non-target microbes. From day 5, a photo was taken each day from the top of the plate, and the diameter of the colony was recorded longitudinally and horizontally to give an approximate area and growth rate through time. Plates, if free from contamination and multiple microbes, were monitored for 28 days before final photos were taken using contrasting backgrounds.

3.2.4 Purification of Microbes Isolated from Blueberry Fruit

Petri dishes containing more than one microbe were purified by re-isolating the microbes onto fresh PDA plates in the same class II biological safety cabinet described above (i.e., to limit contamination with non–target microbes). Purification was completed six days after isolation to allow the development of the microbes present. The microbes were differentiated by observation of colours and textures; often there were clear borders between different organisms, and targeted microbes always originated from the location of the original fruit sample. Contamination was distinguished as random locations of microbe growth coherent with airborne spores or as contamination from the edges of the plates where condensation frequently built up. For purification, plates with multiple microbes were unsealed and opened as required. A scalpel and tweezers were then used to scrape a small piece of pure mycelium or to cut out a small piece of pure culture on PDA and place it in the centre of the fresh PDA plate. During this process, like isolation in Section 3.2.2, the scalpel and tweezers were sterilised with a Bunsen flame in between samples, and nitrile gloves were worn. After purification, the sub–cultures were sealed with Parafilm® M, incubated as per Section 3.2.2, and re-evaluated for the presence of multiple microbes or further contamination after 7 days.

3.2.5 Morphological Analysis of Microbes Purified from Blueberry Fruit

Once the microbial cultures from Section 3.2.3 were 28 days old, they were grouped by morphology. Cultures were segregated based on their macromorphological features of colour, texture, and presence of fungal organs; a key for this can be found in Appendix A. Microscopic features of a sample from each group were analysed for variation within the groups using brightfield microscopy. For groups with more than 10 plates, three samples were randomly selected and for groups with fewer than 10, no plates were sampled. From these samples, microscope slides were produced using a piece of mycelium fixed to a glass slide with ethanol, stained with lactophenol blue, and a glass coverslip placed over top.

3.2.6 Microbe Imaging and Analysis

The Manawatu Microscopy and Imaging Centre (MMIC) was used to obtain brightfield microscopy images of the representative samples (Olympus BX51). Images were captured using the QImaging Micropublisher 5.0 RTV (Surrey, BC, Canada) and visualised using the Lecia Software Application Suite X (LAS X). The

images were analysed using Fiji to determine asexual conidia size and shape (Schindelin et al., 2012). To obtain size and shape measurements of asexual conidia, maximum and minimum Feret values were obtained using the Fji function 'Feret's diameter' (Ross, 2017). The ratio of the maximum Feret diameter (length) to the minimum Feret diameter (width) was calculated to infer shape (Gilchrist et al., 2014). Statistical analysis was completed on these parameters using Minitab® 18 (version 18.1) with a one–way ANOVA completed using Fisher's LSD (least significant difference) to determine differences in means between isolates and between plate groups.

$$Length : Width\ Ratio = \frac{Maximum\ Feret\ Diameter}{Minimum\ Feret\ Diameter} \quad \text{Eq. 3.1}$$

3.2.7 Molecular Analysis of Microbes Purified from Blueberry Fruit

Microbial cultures were selected for molecular analysis based on the relative proportion of morphological sub–group incidence to give 30 microbial cultures weighted according to the appearance of macro and micromorphological features. Microbial cultures in sub–groups with a higher frequency of occurrence were sampled at a higher rate, and samples with a lower proportion were sampled at a lower rate. A threshold of over 1.5% occurrence was used to exclude groups of microbial cultures with very low occurrences from molecular analysis.

3.2.7.1 Genomic DNA Extraction

Cultures identified in 3.2.6 as representative of the signs and/or symptoms on the blueberry fruit were used for genomic DNA extraction. A scraped mycelium sample was isolated from each microbial culture, one culture at a time, using a sterile looped wire in a class II biological safety cabinet and placed into a sterile microcentrifuge tube. Next, DNA was extracted from these mycelial samples using an AllPrep® Fungal DNA/RNA/Protein Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. An exception was made for the elution buffer, in that this was added at a volume of 20 µL rather than 100 µL and left to incubate at room temperature for 5 min instead of 1 min to increase the concentration of eluted genomic DNA. This gave a 20 µL DNA sample suitable for the downstream analyses: quantification of concentration, assessment of DNA sample quality, and polymerase chain reactions (PCRs) (see Section 3.2.6.3).

3.2.7.2 Genomic DNA Concentration

Genomic DNA concentration was measured with a DeNovix DS-11 Spectrophotometer (DeNovix, Delaware, USA) according to the manufacturer's instructions. The spectrophotometer was blanked using the base solution of the sample and a concentration given in ng/µL.

3.2.7.3 PCR Amplification

PCRs were set up with internal transcribed spacer (ITS) 4 and ITS5 primers and *β–Tubulin* (Bt2) forward (Bt2a) and reverse (Bt2b) primers (Glass & Donaldson, 1995; Schoch et al., 2012). A PCR master–mix was prepared for each sequence to be amplified by PCR in a 0.2 mL PCR tube on ice to a final volume of 50 µL. This contained 2.5 µL of each (10 µM) forward and reverse primer, 10 µL of 5X Phusion HF Buffer, 1 µL dNTPs (10 mM), 2.5 µL forward primer (10 µM), 2.5 µL reverse primer (10 µM), 0.5 µL Phusion® High–Fidelity Polymerase (New England BioLabs Inc. Ipswich, MA, USA), and 1 µL of genomic DNA (1–50 ng/µL). The remaining volume was made up with RO water. Thermocycling was completed using a Mastercycler gradient (Eppendorf, Hamburg, Germany) with the following conditions as indicated by the manufacturer (New England Biolabs): initial denaturation at 98°C for 30 sec, followed by 25–35 cycles of 5–10 sec at 98°C, 10–30 sec at 55–62°C, and 30 sec per kb at 72°C, and a final extension for 5–10 min at 72°C.

3.2.7.4 Agarose Gel Electrophoresis

Gel electrophoresis was conducted to confirm the outcome of the PCR amplification and assess the quality of the PCR products before PCR amplicon isolation and DNA sequencing. Agarose (HyAgarose™, HydraGene, Xiamen, China) gel, 1% (w/v) in 1x Tris/acetic acid/EDTA (TAE) buffer, pH 8.3 (190 mM Tris, 342 mM acetic acid [Emsure®, Merck, New Jersey, USA], 2.5 mM EDTA), was used to make the gel, and the TAE buffer was used to run the electrophoresis. A total of 5 µL of PCR product DNA was mixed with 1 µL of 6x loading dye (Thermo Fisher Scientific, Waltham, MA, USA) and loaded into wells with a pipette. A total of 5 µL of 1 kb plus DNA ladder (Invitrogen, Massachusetts, USA) was used as a size marker, and a well with RO water and dye was used as a negative. The gel was run at 80 V for 40 min, then stained using ethidium bromide (1 µg/mL) for 20 min. PCR amplicons were visualised using a Molecular Imager® Gel Doc™ XR system and a digital photo taken with Image Lab™ Software (Bio-Rad, California, USA).

3.2.7.5 PCR Amplicon Isolation from Agarose Gel

A fresh agarose gel electrophoresis was completed as described above (3.2.7.4) with the following modifications. Agarose gel was made to a concentration of 0.8% w/v. A total of 20 µL of PCR product DNA was used. Electrophoresis was run at 80 V for 32 min. Following completion of the process in 3.2.7.4, the gel was placed over a black light and the visible bands cut out with a sterile scalpel and placed into 1.5 mL microcentrifuge tubes.

3.2.7.6 Extraction of PCR Amplicon from Agarose Gel

The extracted gel slice from section 3.2.7.5 underwent DNA extraction using a Wizard® SV Gel and PCR Clean–Up System (Promega Corporation, Madison, WI, USA). The gel slice was dissolved in 300 µL of Membrane Binding Solution (Promega) using an AccuBlock™ digital dry bath (Labnet International Inc. Edison, NJ, USA) set to 65°C. The manufacturer's instructions were followed apart from the addition of 20 µL of nuclease–free water (Promega) rather than 50 µL at the penultimate step, which was added to increase the DNA concentration of the extracted PCR amplicon in the final product. The DNA concentration was then determined by the method described in 3.2.7.2.

3.2.8 Sequencing

DNA Sequencing reactions were made using 1 µL of primer (10 µM), 20ng/µL DNA, and autoclaved RO water to make up a 20 µL total volume in a 0.2 mL PCR tube. Two solutions for each sample were made, one with the forward primer and the other with the reverse primer (described in Section 3.2.7.3). The samples were sent to the Massey Genome Service (Palmerston North, New Zealand) for DNA sequencing.

3.2.9 Sequence Analysis

Gene sequences resulting from the DNA sequencing process described in Section 3.2.7 were visualised using Geneious Prime v2022.2.2 (Biomatters Ltd. Auckland, New Zealand). This software was used to reverse the reverse sequence and pairwise align with the forward sequence for each isolate using MUSCLE (v3.8.425) for a maximum of eight iterations (Edgar, 2004). The aligned sequences then had poor quality ends trimmed using the function within the software. The aligned sequences were used to generate

consensus sequences, which were then searched using the Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) UniVec database. A BLAST search for nucleotides (BLASTn) was completed of the nucleotide collection (nr/nt) database to return similar sequences. The resulting percentage identity, maximum score, query cover, and accession number of the top hit were recorded. The top hit was considered an identification and applied to the plate group it represented (Ross et al., 2008).

3.2.10 Phylogenetic Analyses

The consensus sequences generated from Section 3.2.8 were grouped by morphological group at the genus level. Only ITS sequences were used due to the limited availability of Bt2 sequences. A pairwise alignment, as described in 3.2.8, was conducted across each morphological group if multiple consensus sequences were present. These alignments were trimmed as in 3.2.8 and then used to generate a consensus sequence for the morphological group. To construct a phylogenetic tree, a pairwise alignment of the relevant morphological consensus sequences and relevant comparative sequences were obtained from GenBank. These sequences were aligned and used to construct a neighbour-joining consensus tree, using Tamura–nei, resampled using bootstrapping with 1000 replicates, and an outgroup was added to show scale (Tamura & Nei, 1993). Trees were displayed in a proportional format, with branch labels indicating substitutions per site.

3.3 Results

3.3.1 Genomic DNA Sequencing Results

Table 3.1. DNA Sequencing Results

Plate Group	Primer	Sequence Length	Max Score	Query Cover	% Identity	Top BLASTn hit[a]	GenBank Accession no. of top hit
1a	ITS4/5	295	545	-	-	Inconclusive	-
2a	ITS4/5	475	878	100.00%	100.00%	*Botrytis cinerea*	MT5734790.1
2b	ITS4/5	568	1,048	99.00%	100.00%	*B. cinerea*	EF207413.1
2c	ITS4/5	421	778	100.00%	100.00%	*B. cinerea*	MT573470.1
2e	Bt2a/b	695	1195	100%	100.00%	*B. cinerea*	MN159914.1
2f	ITS4/5	338	625	100%	100.00%	*B. cinerea*	LC514949.1
3a	ITS4/5	630	1162	99.00%	100.00%	*Pestalotiopsis disseminata*	HQ607992.1
3b	ITS4/5	599	1107	100.00%	100.00%	*P. disseminata*	HQ607992.1
3d	ITS4/5	631	1107	100.00%	98.42%	*Pestalotiopsis* sp.	HQ608093.1
3e	Bt2a/b	746	1352	99.00%	99.60%	*Diaporthe salicicola*	KF170923.1
4a	Bt2a/b	517	955	100.00%	100.00%	*D. nobilis*	KT163359.1
5a	Bt2a/b	553	1020	99.00%	100.00%	*Colletotrichum fioriniae*	MT409125.1
5b	ITS4/5	510	941	99.00%	100.00%	*C. fioriniae*	MT466533.1
5c	ITS4/5	563	1040	100.00%	100.00%	*C. fioriniae*	MT133292.1
8d	ITS4/5	597	1103	100.00%	100.00%	*Penicillium expansum*	KX243329.1
9a	ITS4/5	283	523	100.00%	100.00%	*Neofusicoccum australe*	OP142416.1
11c	ITS4/5	530	977	100.00%	100.00%	*Colletotrichum salicis*	KU743959.1
14a	ITS4/5	573	1053	100.00%	99.83%	*Neopestalotiopsis clavispora*	MN519192.1
14b	ITS4/5	582	1068	99.00%	99.83%	*Pithomyces chartarum*	MH860227.1
17a	ITS4/5	571	1055	100.00%	100.00%	*Epicoccum nigrum*	MW486023.1

[a] Further sequencing results of ITS and BT2 can be found in Appendix B.

3.3.2 Microorganism Identification

3.3.2.1 *Botrytis cinerea*

Botrytis cinerea was found through genomic DNA sequencing to correspond with plate groups 2a, 2b, 2c, 2e, and 2f. High quality ITS sequences were used for phylogenetic analysis; these showed greater similarity to *B. cinerea* than other *Botrytis* species reported on blueberry fruit. Of these, a *B. cinerea* isolate (HQ455788.1) found on Korean blueberry fruit was the closest relative among the evaluated ITS sequences (Kwon et al., 2011).

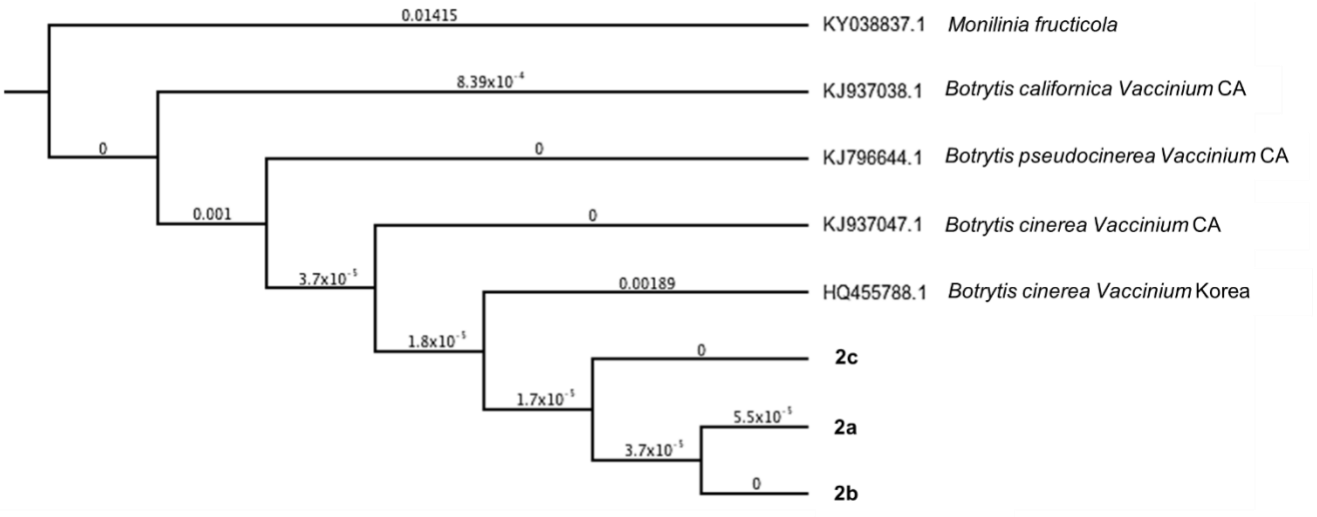


Figure 3.1. Phylogenetic tree obtained through alignment of ITS sequences of isolates and morphological groups of *Botrytis cinerea*. Values are distances between branches. *Monilinia fructicola* KY038837.1 is used as an outgroup. CA = California, USA.

The macromorphology of the *B. cinerea* isolates was similar, with common features including tan conidiophores with branching conidia at the tips, a white mycelial base, and a fast growth rate. These groups had macromorphological similarities, and DNA sequencing results corroborate this, with all sequences returning as *B. cinerea* through a BLASTn search. Group 2d was not sequenced but displayed similar morphology to the identified *B. cinerea* groups; this was deemed to be *B. cinerea*. The growth rates of 2a, 2b, and 2f were 1.7mm/day^{-1}, 2c was 1.42mm/day^{-1}, 2d was 0.71mm/day^{-1}, and 2e was 0.77 mm/day^{-1}.

Some slight differences were observed between these groups, with group 2b presenting aerial white mycelium, 2c with irregularly shaped black sclerotia, 2d displaying both white aerial mycelium and black sclerotia, 2f did not have conidiophores and conidia, and 2e producing a yellow exudate alongside aerial white mycelium. The slight differences in macromorphology are possibly due to environmental conditions

during growth, culture age, conditions experienced prior to isolation, and strain to strain variation. These features occur with the *B. cinerea* species as reported by Jarvis (1977); an outlier is group 2e with a yellow exudate, but molecular analysis confirmed this group as *B. cinerea*. Indicative *B. cinerea* macromorphological images are below. The occurrence of these plates was dominated by 2a with 9.69% of total plates, 2b with 7.93%, and 2c with 5.29%. Less frequently obtained was 2d with 1.32%, 2e with 3.74%, and 2f with 1.10% of total plates isolated. Combined, these give a total of 29.07% of all plates which were identified as *B. cinerea.*

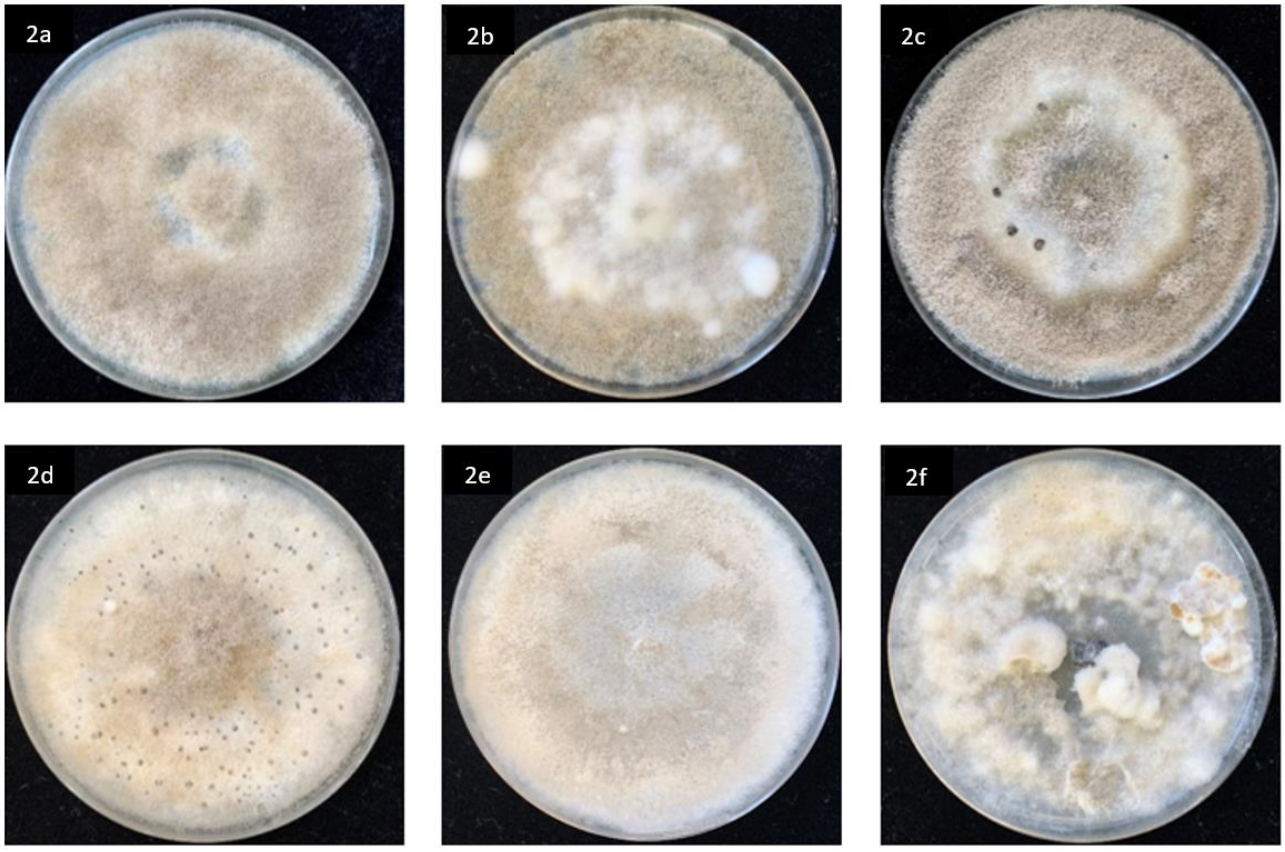


Figure 3.2. *Botrytis cinerea* macromorphology of isolates from decaying blueberry fruit. The plates shown are a representative sample of each plate group.

The micromorphological characteristics of *B. cinerea* were evaluated for groups 2a, 2b, and 2c. The shape and length to width ratio of the conidia of these groups were identical. The predominant form was symmetrical ovoid, with some ellipsoid conidia present, as shown in the images below (**Fig. 3.3**). The conidia were hyaline, and an abscission scar was present in some conidia. Lengths ranged from 18.791 ± 3.934 to 15.417 ± 3.890. Conidia lengths were significantly different between plate groups. Conidia width was also significantly different between groups, ranging from 10.879 ± 2.857 to 13.184 ± 2.431. These differences are attributed to error in the determination of dimensions through analysis and microscopy techniques. However, the ratio of length to width was constant among the plate groups, and these values

are within the range given in the classical literature (**Table 3.2**) (Jarvis, 1977). This provides confidence that these plate groups are *B. cinerea* based on sequence data and morphological analysis.

Table 3.2. *Botrytis cinerea* conidia and conidiophore micromorphology.

Plate ID	Conidia Length (µm)	Conidia Width (µm)	Length : Width Ratio	Conidia Shape
2a	18.791 ± 3.934 a	13.184 ± 2.431 a	1.432 ± 0.213 a	Ovoid or ellipsoid
2b	15.417 ± 3.890 c	10.879 ± 2.857 c	1.443 ± 0.255 a	Ovoid or ellipsoid
2c	17.331 ± 3.456 b	12.313 ± 2.143 b	1.416 ± 0.215 a	Ovoid or ellipsoid
Jarvis (1977)	8 – 14	6 – 9	1.33 – 1.55	Ovoid, ellipsoid, globose

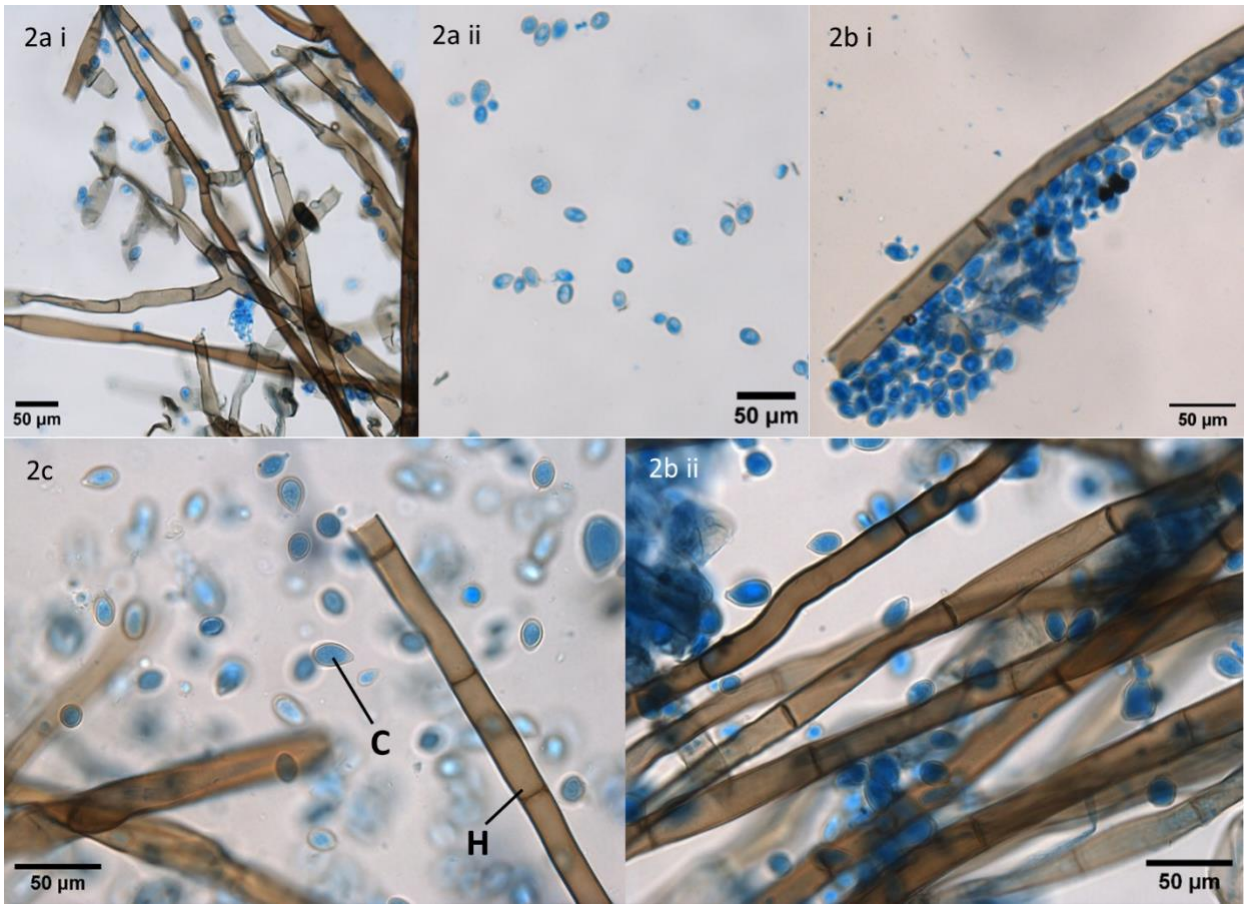


Figure 3.3. Micromorphology of *Botrytis cinerea*. **2ai = 2a**. Branching conidiophores present, 40x. **2aii = 2a**. Ovoid and ellipsoid conidia, 40x. **2bi = 2b**. Conidiophore and conidia of similar form to 2a, 40x. **2bii = 2b** Detail of conidiophores and conidia, abscission scar observed, ovoid/ellipsoid shape, 100x. **2c = 2c** Conidia displaying an external wall and an abscission scar with a symmetrical ovoid/ellipsoid shape of varying sizes and uncommon spherical conidia are shown. Segmented conidiophores are also present. Conidia are indicated by **C**. Hypha are indicated by **H**.

The fruit decay signs observed from which *B. cinerea* was isolated was largely grey mould. The plate groups contributing most frequently to this were 2a, 2b, and 2c. Another sign frequently isolated as *B. cinerea* was grey stem scar, which also often produced plate groups 2a, 2b, and 2c. The other plate groups were isolated less frequently. Fruit with these decays are shown in **Fig. 3.4**. The composition of total decay by plate group and the decay signs from which they were isolated can be found in Appendix C.

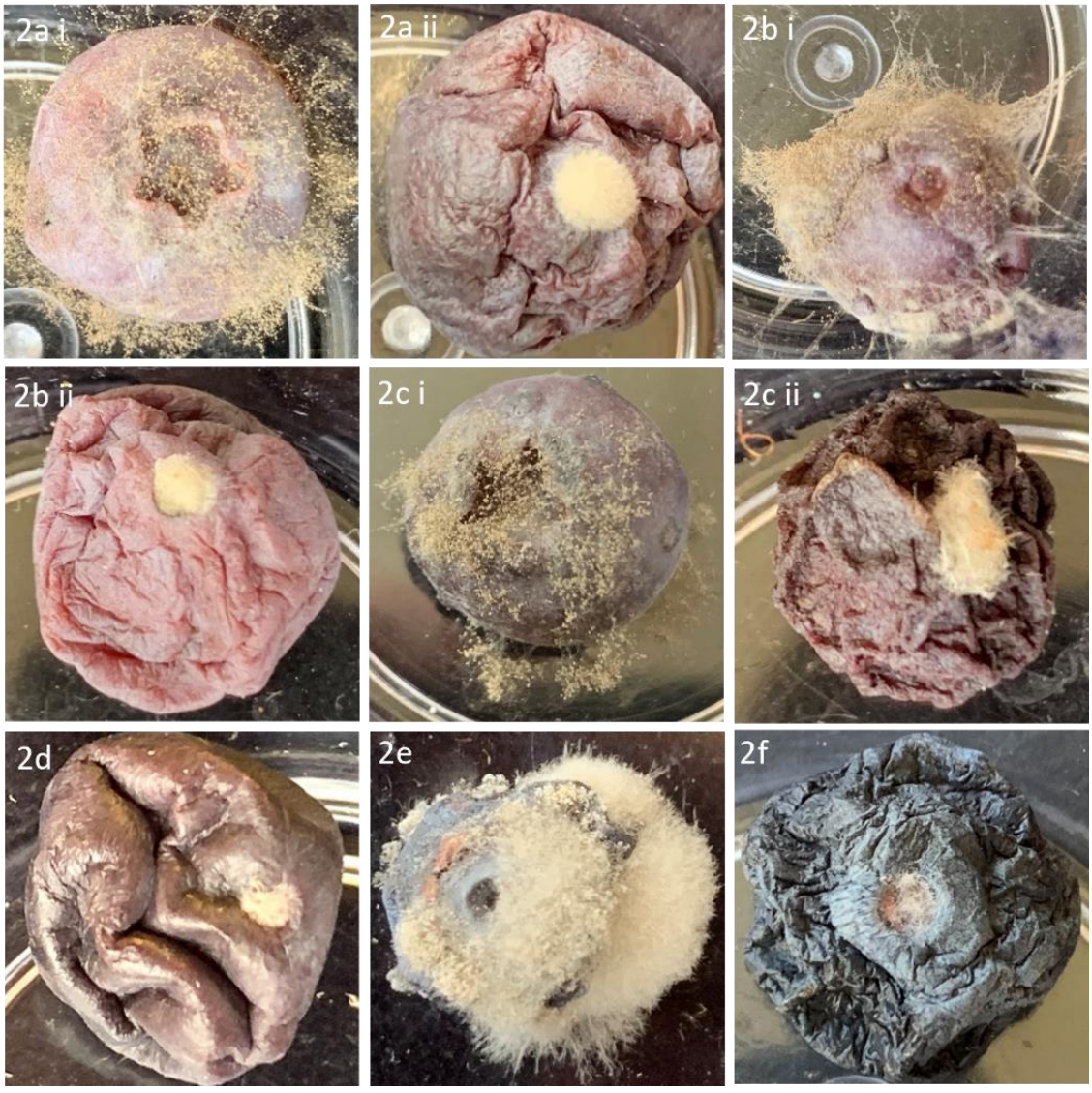


Figure 3.4. *Botrytis cinerea* plate groups linked to fruit decay from which they were isolated. **2a i, 2b i, 2c i**, and **2e** are classified as grey mould. **2a ii, 2b ii, 2c ii, 2d**, and **2f** are classified as grey stem scar.

3.3.3.2 *Pestalotiopsis* spp.

Pestalotiopsis spp. were found through DNA sequencing to align with plate groups 3a, 3b, 3c, and 3d. Plate groups 3a and 3b were closely related to *P. disseminata* through ITS sequencing. Group 3d was found to relate closely to multiple *Pestalotiopsis* spp. A high–quality sequence was not obtained for group 3c. The phylogenetic tree below indicates several organisms closely related to known *Pestalotiopsis* species in New Zealand. Within this tree, group 1a was grouped with *Pestalotiopsis* but distinct from the other groups. Group 14a was closely related to *Neopestalotiopsis rosae*, recorded on roses in NZ by Maharachchikumbura et al. (2014). However, no confirmed species identification was obtained; therefore, genus-level identification of *Pestalotiopsis* spp. will be used.

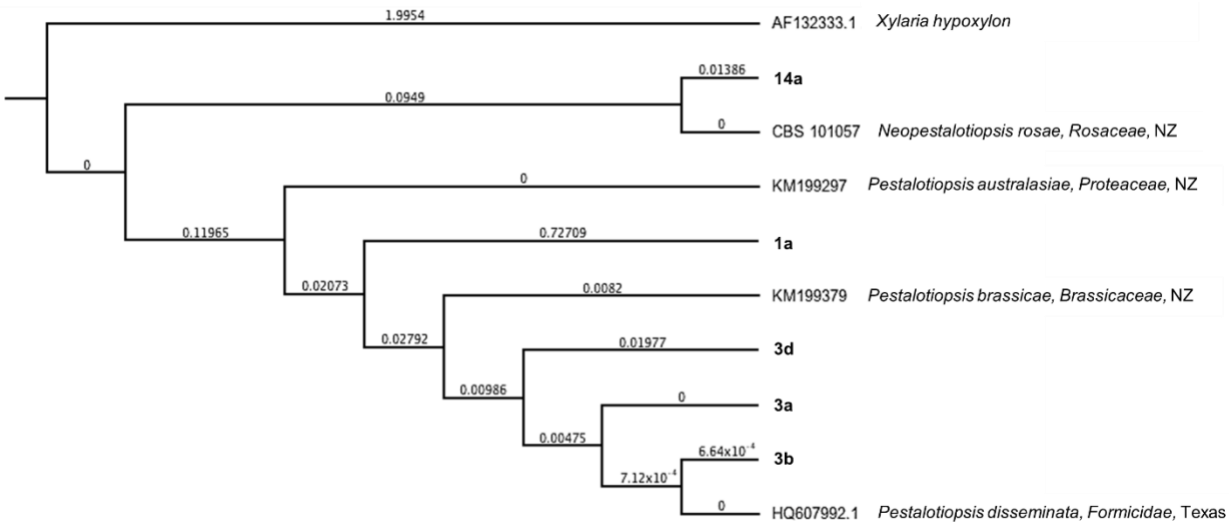


Figure 3.5. Phylogenetic tree obtained through alignment of ITS sequences of isolates and morphological groups of *Pestalotiopsis* spp. Values are distances between branches. *Xylaria hypoxylon* AF132333.1 is used as an outgroup. NZ = New Zealand.

The macromorphology of these plate groups was similar, with yellow-coloured mycelium being a common observation. The intensity of this yellow colouration varied between the groups, from light yellow in 3a and 3c to more intense yellow in 3b and 3d (**Fig. 3.6**). Colour was a key distinguishing factor of each group, appearing as a spectrum of intensities with the addition of black fruiting bodies in 3c and 3d. The growth rate of 3a was 1.4mm/day^{-1}, 3b was 0.7mm/day^{-1}, 3c was 0.94mm/day^{-1}, 3d was 0.7mm/day^{-1}, 1a was 0.77mm/day^{-1}.

Due to the diversity of colour and presence or absence of fungal bodies, there is wide variation between these groups (**Fig. 3.6**). The sequencing data confirms this observation, with a variety of species being isolated from blueberry fruit. Additionally, group 1a is also considered *Pestalotiopsis* following

micromorphological analysis, with conidia features consistent with this genus observed. The frequency of these plate groups was as follows, 3b with 9.03%, 3a with 4.19%, 3c with 2.86%, 3d with 1.98%, and 1a with 0.88%.

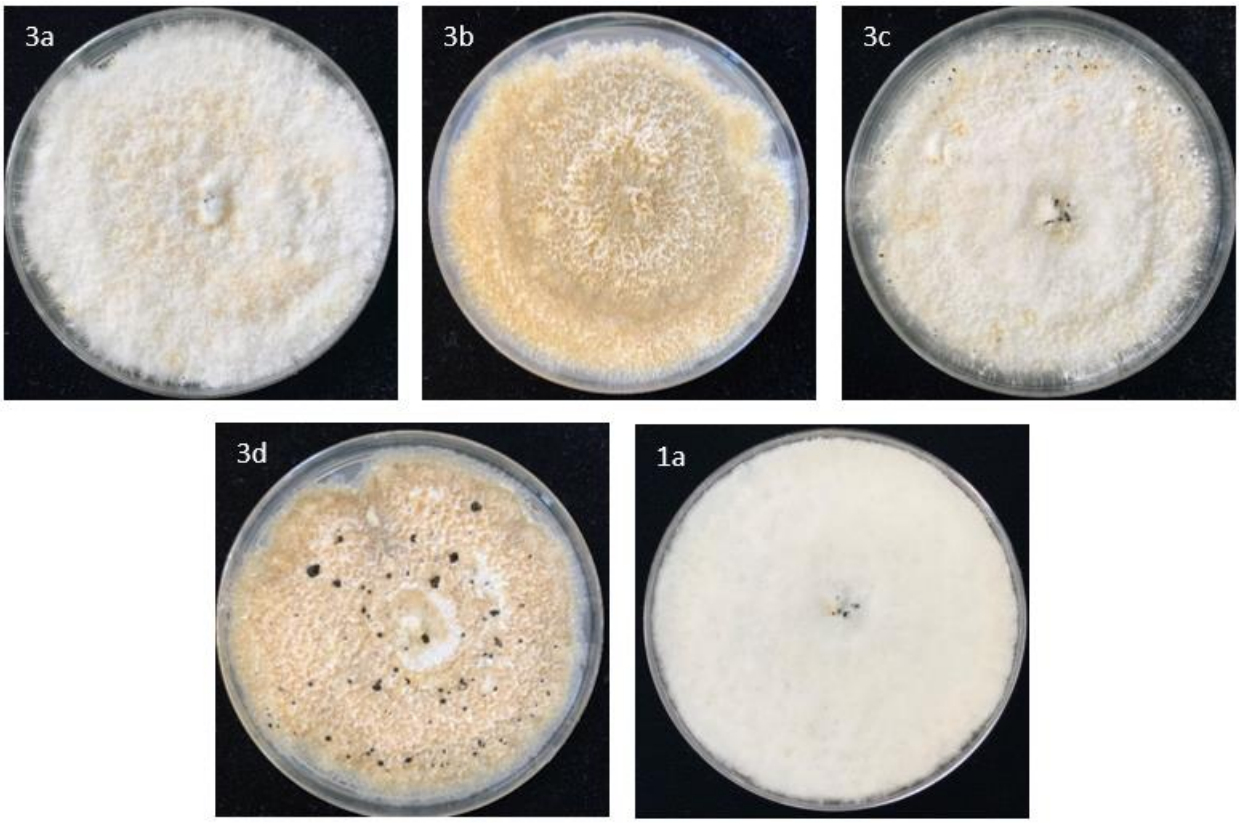


Figure 3.6. Macromorphology of *Pestalotiopsis* species isolated from decaying blueberry fruit.

The micromorphological characteristics observed were mainly distinct five–celled conidia with a cylindrical shape (**Fig. 3.7**). This form was consistent across 3b, 3c, 3d, and 1a. Conidia were not observed for group 3a. The conidia length was different across all groups analysed ranging from 18.205 ± 2.288 to 34.930 ± 7.05 (**Table 3.3**). The conidia width was the same between 3b and 3b, but 3c was significantly different. The measurements ranged from 7.409 ± 1.636 to 13.991 ± 1.947. The ratio between length and width was the same between 3b and 3c, but 3d was significantly different. The range observed was 2.219 ± 0.386 to 2.528 ± 0.562. As there is a degree of variability between conidia length, width, and the ratio of these, this likely reflects a range of species, as indicated through the molecular analysis. This genus is known to display a degree of variation within species in terms of condia length and the presence of appendages (Jeewon et al., 2003). All groups were five–celled with cylindrical conidia, but differences in appendages existed. These morphological measurements are inconclusive for the identification of these plate groups.

Table 3.3. Micromorphological characteristics of *Pestalotiopsis* spp. conidia. Significant differences determined through Fisher's LSD are indicated by a different letter within columns (P<0.05).

Plate ID	Mean Conidia Length (µm)	Mean Conidia Width (µm)	Mean Length : Width Ratio (µm)	Conidia Characteristics
3b	34.93 ± 7.050 a	13.991 ± 1.947 a	2.528 ± 0.562 a	Five-celled, cylindrical, 2 apical appendages, 1 basal appendage
3c	18.205 ± 2.288 b	7.409 ± 1.636 b	2.501 ± 0.354 a	
3d	29.383 ± 4.892 c	13.341 ± 1.704 a	2.219 ± 0.386 b	
1a	44.066 ± 4.800 a	14.885 ± 0.991 a	2.980 ± 0.452 a	Five–celled, cylindrical, 4 knobbed apical appendages, 1 knobbed basal appendage.
P. disseminata **(Naeimi et al., 2015)**	18 – 26	5 – 8	3.25 – 3.6	Five–celled, straight, or slightly curved, fusiform, 2–3 apical appendages. 1 basal appendage.

Conidia characteristics are shown in **Fig. 3.7**. Groups 3b, 3c, and 3d had two apical appendages attached to the top of the apical cell. A single, short basal appendage was observed in these groups. Group 1a was distinct in that there were four apical appendages attached to the top of the apical cell, a single, short basal appendage was observed, and knobs were present at the tips of all appendages. This indicates the likeness of 1a to the *Pestalotiopsis* genus; however, it is distinct from the other isolates.

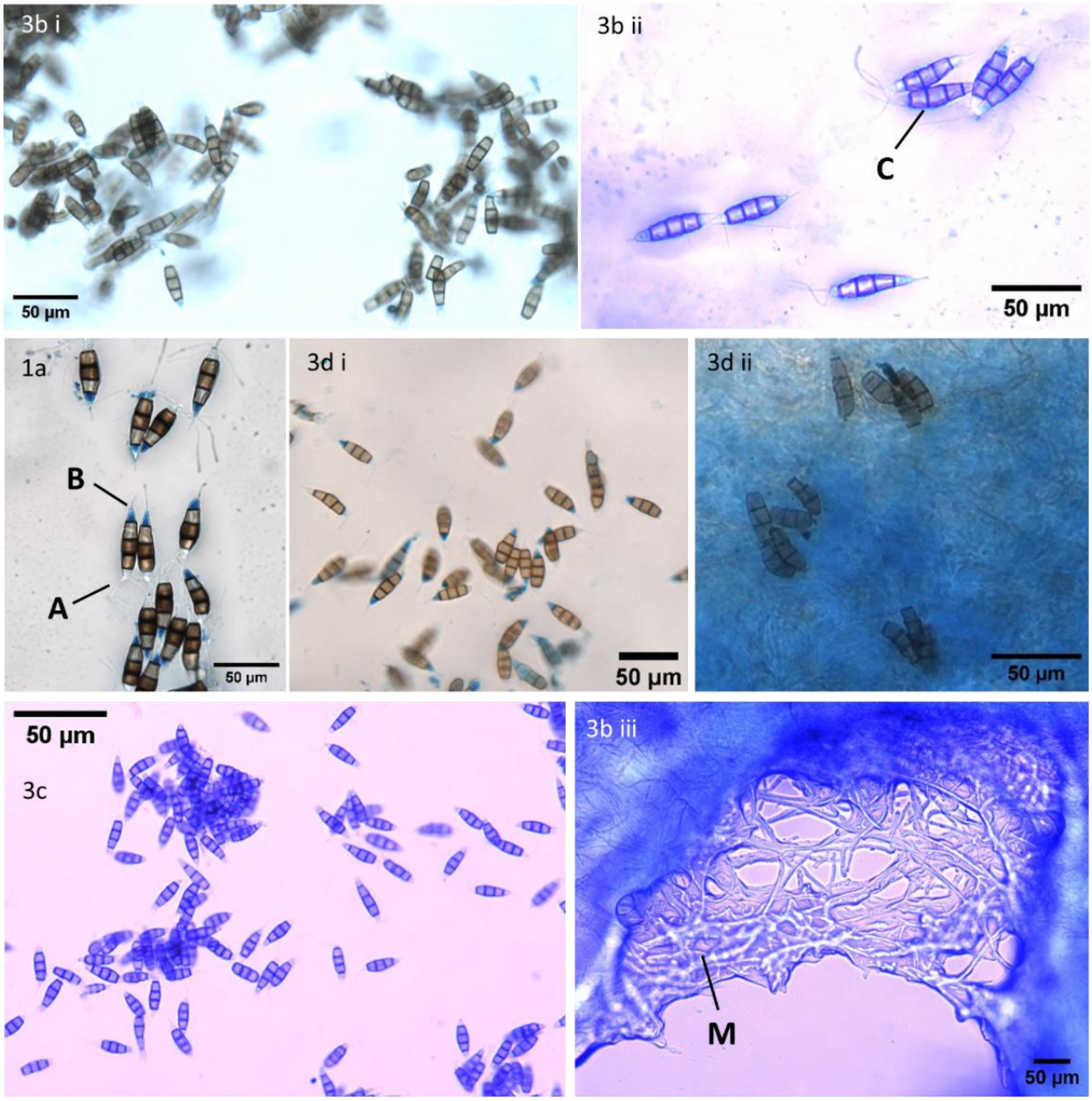


Figure 3.7. Micromorphology of *Pestalotiopsis* spp. **3bi = 3b**. Three median cells, with some apical and basal cells visible, 40x. **3bii = 3b**. Five-celled conidia visible; two apical appendages and one basal appendage observed, 100x. **1a = 1a**. Versicolourous five-celled conidia, with four apical appendages and one basal appendage; appendages are knobbed, 100x. **3di = 3d**. Five-celled conidia with a visible apical cell with two appendages, the basal cell has one appendage, 40x. **3dii = 3d**. Three celled conidia visible within the mycelium, 100x. **3c = 3c.** Five-celled cylindrical conidia, mostly straight with some curved conidia, 40x. **3biii = 3b**. Mycelium 40x. Annotations: **C** = conidia; **B** = basal appendage; **A** = apical appendage; **M** = mycelium.

Pestalotiopsis was isolated from distinct decay signs on fruit. A white mycelium was commonly associated with this decay. The location, extent, and density of this mycelium was variable, and the presence of other structures was erratic. This reflects the molecular identification, with multiple species observed within the *Pestalotiopsis* genus. Of particular interest is 3b, which displayed black spore masses. As shown in **Fig. 3.8**, the decay is similar in appearance to *Pestalotia vaccinii*; however, the molecular data does not support this (Wharton, 2003). The assignment of these microorganisms to categories of decay signs was led by the

grey mycelium in stem scar sign, with white mycelium in stem scar, and white mycelium signs also related to *Pestalotiopsis* spp. The plate group and decay sign results can be found in Appendix C.

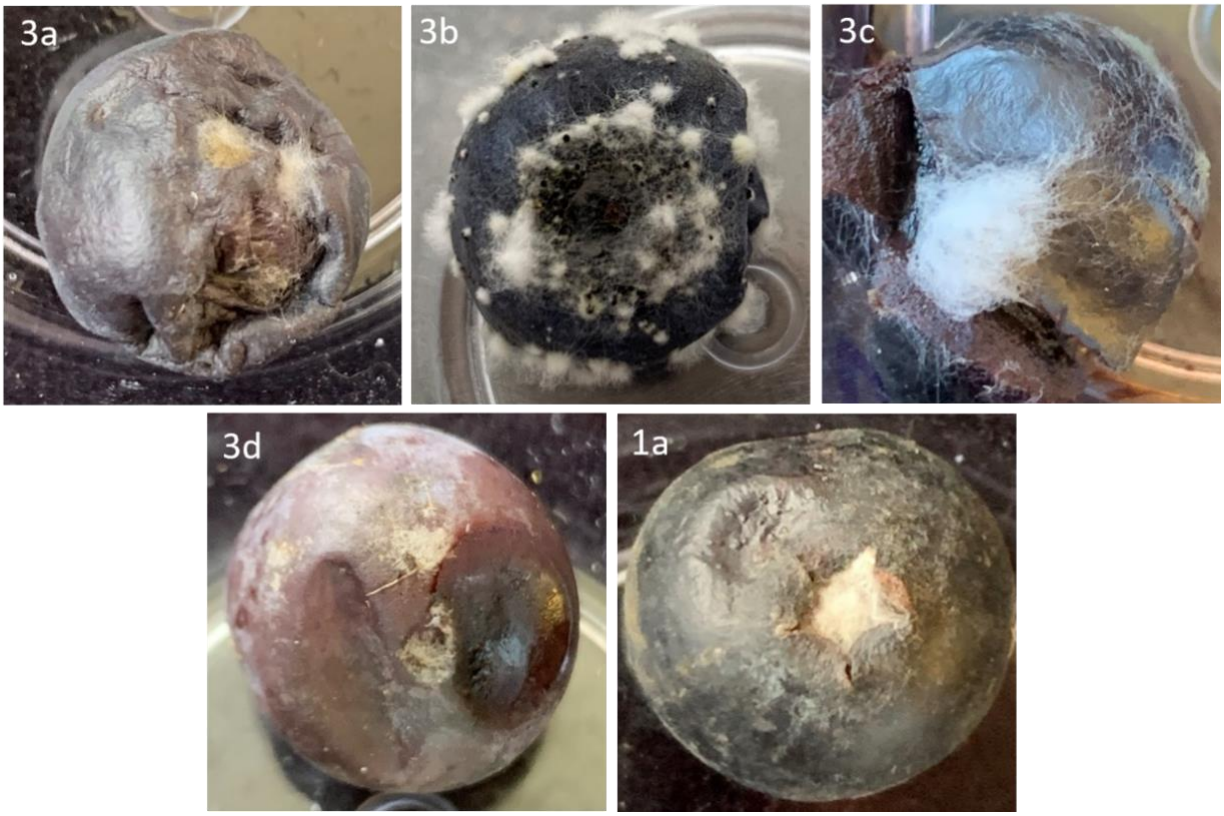


Figure 3.8. Fruit decays related to *Pestalotiopsis* spp. Image labels relate to plate groups.

3.3.3.3 *Colletotrichum acutatum* Species Complex

The *Colletotrichum acutatum* species complex was found to be linked to sequencing results from plate groups 5a, 5b, 5c, and 11c. The sequences were all identified as species within the *Colletotrichum acutatum* species complex. Associated with 5a and 5b was *Colletotrichum fioriniae*. Group 5c was related to *C. fioriniae* and *Colletotrichum godetiae*. Plate group 11c was identified as *Colletotrichum salicis* through ITS sequencing; however, the Bt2 sequence was most similar to *C. rhombiforme* (Appendix B) but had a weaker similarity. This places these species closely on the phylogenetic tree. Both 5c and 5b were closely related to previously recorded *C. fioriniae* on blueberry vegetative tissue in NZ (Damm et al., 2012).

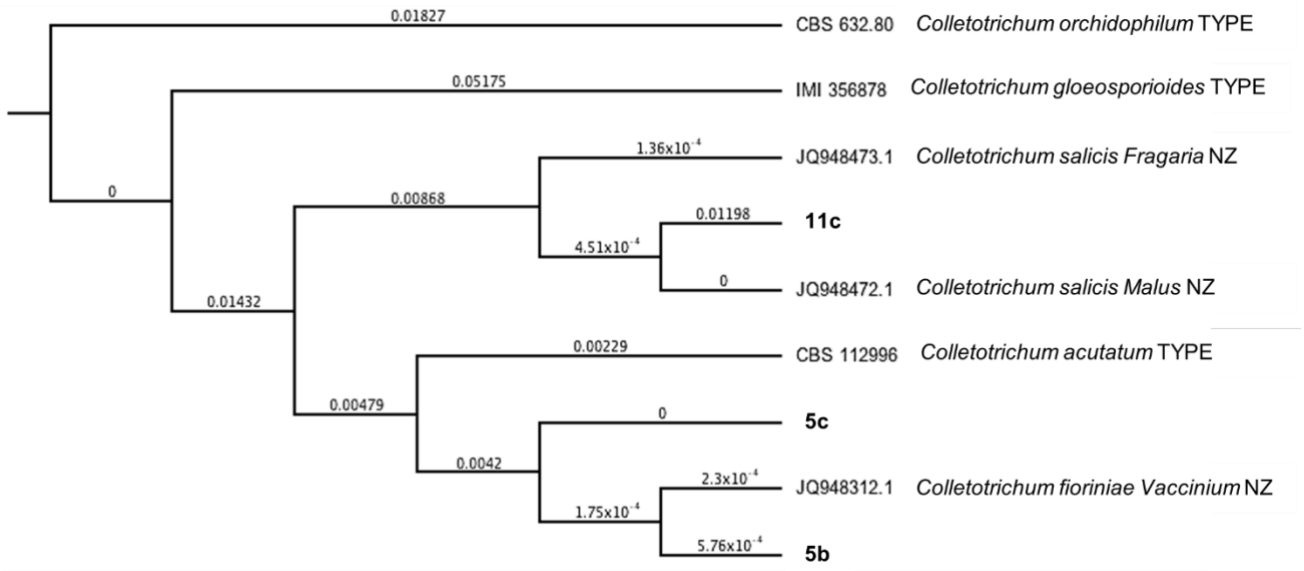


Figure 3.9. Phylogenetic tree of *Colletotrichum* spp. obtained through alignment of ITS sequences of isolates and morphological groups. Values are distances between branches. *Colletotrichum orchidophilum* CBS 632.80 is used as an outgroup. NZ = New Zealand.

The macromorphology of these groups was highly variable, with a range of colours and fungal organs present. Plate group 5a produced mainly dark colours, greys, and green shades. Plate group 5b produced orange conidiomata with pink, white, and green mycelium. Plate group 5c was dark, but with orange acervuli. Plate group 5d, not sequenced due to low frequency, had identical colours to 5b but without acervuli; this was placed within the *C. acutatum* species complex based on the macromorphology. Plate group 11c was morphologically distinct, with dark grey rings of mycelium and a depression at the centre of the culture, and no acervuli were observed. The growth rate of 5a was 0.44 mm/day^{-1}, 5b was 0.71 mm/day^{-1}, 5c was 0.43 mm/day^{-1}, 5d was 0.81 mm/day^{-1}, and 11c was 0.61mm/day^{-1}. The occurrence of the *Colletotrichum acutatum* spp. complex was mainly from 5c, with 9.03%; 5a made up 4.63%; 5b was 1.54%; 5d was 1.10%; and 11c was 1.54%.

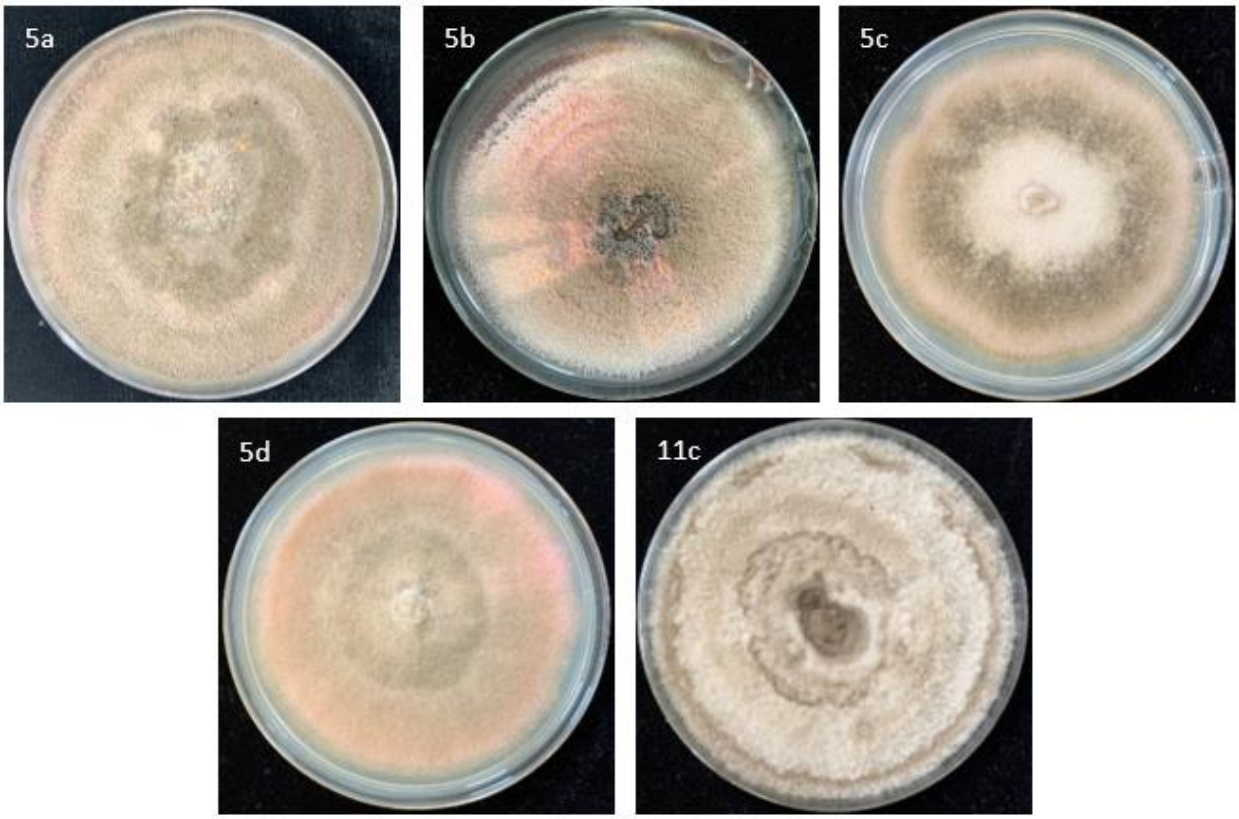


Figure 3.10. Macromorphology of the *Colletotrichum acutatum* species complex isolates obtained from blueberry fruit. **5a**, **5b** identified as *C. fioriniae*, **5c** identified as *C. godetiae*, **5d** identified as *C. acutatum* spp. complex, **11c** identified as *C. salicis*.

Table 3.4. Micromorphological characteristics of *Colletotrichum acutatum* spp. complex conidia. Significant differences are indicated by a different letter within the same column (P<0.05, Fisher's LSD).

Plate ID	Mean Conidia Length (µm)	Mean Conidia Width (µm)	Mean Length : Width Ratio (µm)	Conidia Characteristics
5a *C. fioriniae*	26.825 ± 3.395 a	8.476 ± 2.210 a	3.269 ± 0.575 a	Single celled, fusiform
5b *C. fioriniae*	24.780 ± 3.712 b	9.010 ± 2.163 b	2.851 ± 0.603 b	Single celled, fusiform
5c ***C. fioriniae***	15.323 ± 3.390 d	6.827 ± 1.89 c	2.306 ± 0.446 c	Single celled, fusiform
5c ***C. godetiae***	20.891 ± 4.143 c	6.325 ± 4.24 c	3.36 ± 1.353 a	Single celled, fusiform, rounded ends
C. fioriniae **(Damm et al., 2012)**	13.5–16.5	4–5	3.3–3.4	Single celled, fusiform

Micromorphology was also distinct between plate groups, with significant differences observed between cultures for conidia length, width, and L:W ratio (**Table 3.4**). As groups 5a, 5b, and 5c were identified as *C. fioriniae,* the differences in micromorphology are unreliable indicators for species differentiation based on the methods used. Of interest is the discovery of *C. godetiae* within group 5c; this species produced some conidia with rounded ends rather than the acute ends observed from *C. fioriniae*; this is displayed in Error! Reference source not found.. The conidia of *C. fioriniae* were symmetrical, hyaline, straight, fusiform and had acute ends. The conidia of *C. godetiae* were identical to those of *C. fioriniae,* but some conidia had rounded ends. Conidia of *C. salicis* were not observed due to the lack of acervuli present.

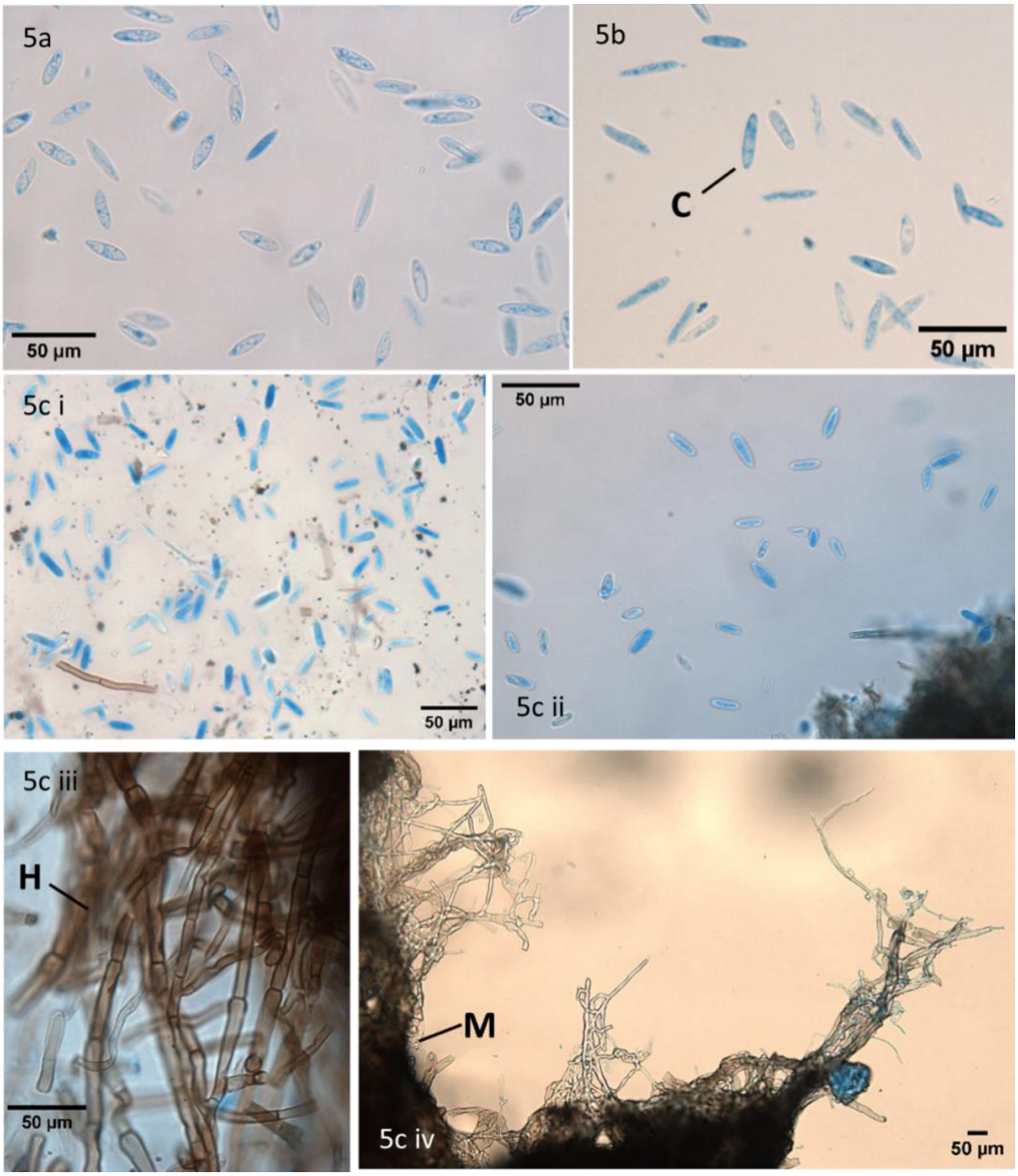


Figure 3.11. Micromorphology of *Colletotrichum acutatum* species complex isolates on PDA. **5a = 5a**. *C. fioriniae* single celled fusiform conidia with acute ends 100x. **5b = 5b** *C. fioriniae* single celled, fusiform conidia with acute ends 100x. **5c i = 5c**. *C. godetiae* single celled fusiform conidia, some rounded ends present 40x. **5c ii = 5c**. *C. fioriniae* single celled fusiform conidia 100x. **5c iii = 5c**. Mycelium of *C. godetiae* 100x. **5c iv = 5c**. Mycelium of *C. fioriniae* 20x. Annotations **C** = conidia, **H** = hyphae, **M** = mycelium.

Anthracnose was the major decay sign from which the *C. acutatum* spp. complex was isolated. All morphological groups were strongly associated with anthracnose. This is well supported in the literature, with *C. acutatum* being a causal agent of anthracnose in blueberry fruit (Wharton & Schilder, 2008). The anthracnose decay sign consisted of acervuli production from the surface of the fruit, with a distinctive orange colour. This was sometimes associated with other decays, including grey stem scar decay.

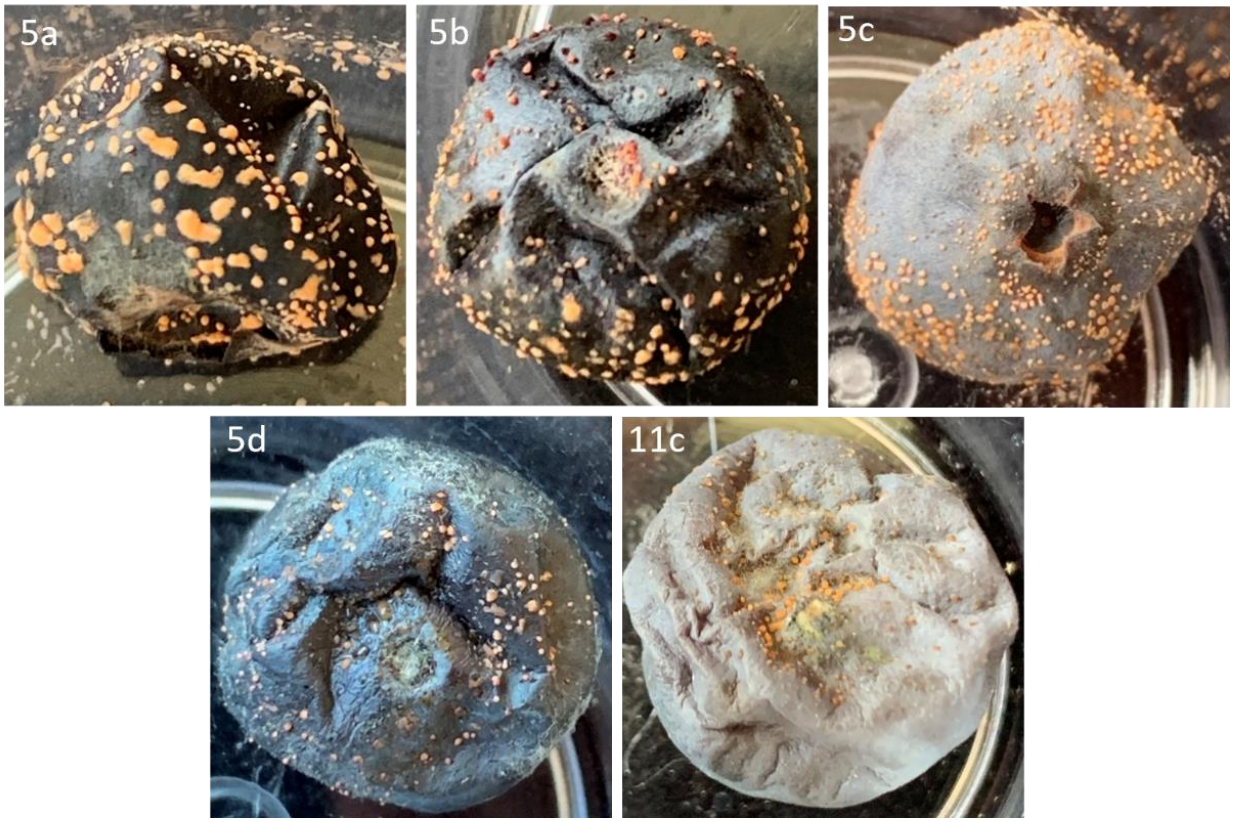


Figure 3.12. Anthracnose decay associated with molecular identifications of *Colletotrichum acutatum* species complex microorganisms. Image labels refer to plate groups.

3.3.3.4 *Diaporthe* spp.

Diaporthe spp. display distinct macromorphological differences between the plate groups identified as this genus. Molecular identification of group 3e returned a species level identification as *D. salicicola*, while the morphologically distinct group 4a was found to be *D. nobilis*. The macromorphology of 3e includes white, buff, and brown mycelium with scattered sunken spots (**Fig. 3.13**). The growth rate was 0.57 mm/day^{-1}. On the other hand, group 4a features a white mycelium with spherical aerial mycelium with a light buff colour and dark spots and a growth rate of 1.2 mm/day^{-1}. These plates were isolated rarely; 1.54% of total plates were identified as belonging to these groups. As no conidia were obtained from these isolates, analysis of

these cannot be completed; however, the mycelium of these isolates was examined using microscopy. Hyphae of both groups were septate; 3e had brown colouration, and 4a hyphae were hyaline (**Fig. 3.14**).

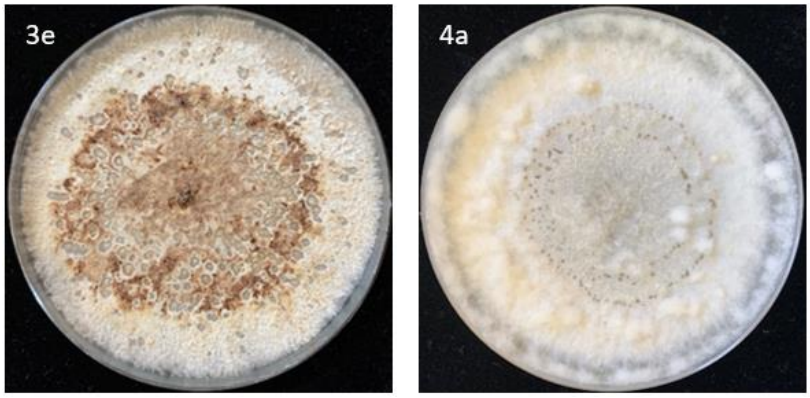


Figure 3.13. Macromorphology of *Diaporthe salicicola* (**3e**) and *D. nobilis* (**4a**).

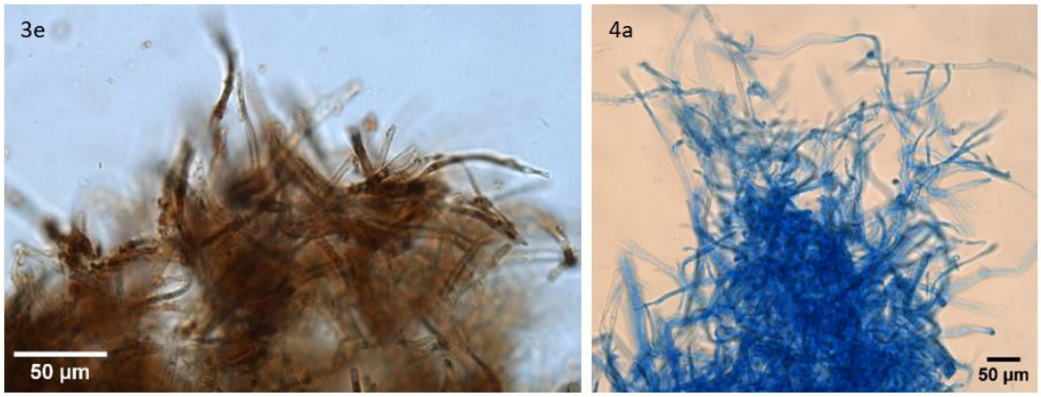


Figure 3.14. Micromorphology of *Diaporthe salicicola* **(3e)** displaying brown mycelium. **4a**, *D. nobilis* with hyaline mycelium. No conidia were observed.

Figure 3.15. Decay associated with *Diaporthe* plate groups.

Decay *Diaporthe* spp. was isolated from included a diverse group of decay signs. *D. salicicola* was isolated from fruit producing a small grey stem scar decay; this was observed infrequently. *D. nobilis* was isolated from a grey or white stem scar decay shown in **Fig. 3.15, 4a i**, or a white mycelium decay as in **Fig. 3.15, 4a ii**. Both of these decays are similar, with a fine hyphal structure and grey/white colour. This is similar to the decay observed by Lorenzini and Zapparoli (2018) on grape berries as *D. eres*. However, *D. nobilis* has been reported to produce a grey/white mycelium decay on blueberry fruit (Yu et al., 2018). The molecular identification obtained as *D. nobilis* and the similarity of the decay sign with decay reported in the literature give confidence that *D. nobilis* is the organism responsible for this decay.

3.3.3.5 *Neofusicoccum australe*

Group 9a was identified through DNA sequencing as relating to *Neofusicoccum australe*. The macromorphology of these isolates consisted of fluffy, textured dark grey mycelium (**Fig. 3.16, 9a i**). The growth rate was 0.43 mm/day^{-1}. Some isolates produced light grey or white tipped mycelium. No conidia were found from the isolates, but mycelium was observed to be thin, septate, dense, and dark brown (**Fig. 3.16, 9a ii**). The occurrence of these plates was low, with 1.54% of plates producing this morphology.

Figure 3.16. Morphology of *Neofusicoccum australe*. **9a i**, fluffy grey mycelium with a dark centre and lighter areas at the tips of the mycelium. **9a ii:** microscopy imagery of *N. australe* displaying dark brown, thin mycelium.

The decays from which *Neofusicoccum* was isolated were non–extensive decays. A fine, grey mycelial filament is observed in both images of **Fig. 3.17**. As this is superficial and there are no symptoms of decay, this is likely not a major pathogen of postharvest blueberry fruit.

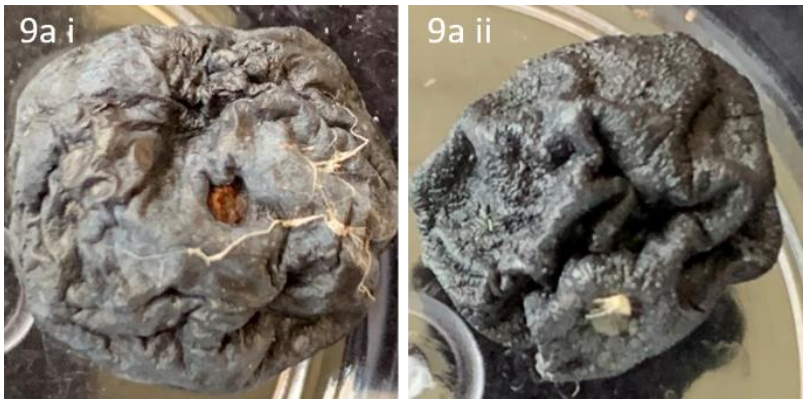


Figure 3.17. *Neofusicoccum* spp. fruit sign.

3.3.3.6 *Epicoccum nigrum*

Sequencing results found *Epicoccum nigrum* to be associated with group 17a. These plates were observed as 1.03% of all plates. There were some macromorphological differences between plates within this group, with different colours, sizes, and textures observed. However, significant commonalities remained, as all cultures produced a pigment in the agar, most often orange, but red was also observed. Colonies were slow growing at 0.2 mm/day^{-1} with a very dense texture, an irregular shape, and the formation of a central feature of a contrasting colour (**Fig. 3.18, 17a i**). Common colours were pink, yellow, white, brown, and green. Micromorphologically, no conidia were found; however, the dense mycelium was observed and indicated a fine network of tightly packed hyphae (**Fig. 3.18, 17a ii**). The fruit decay this organism was associated with was a rarely observed orange stem scar decay (**Fig. 3.18, 17a**). Since *Epicoccum nigrum* was irregularly isolated, it is unclear whether this species is the causal agent of this decay.

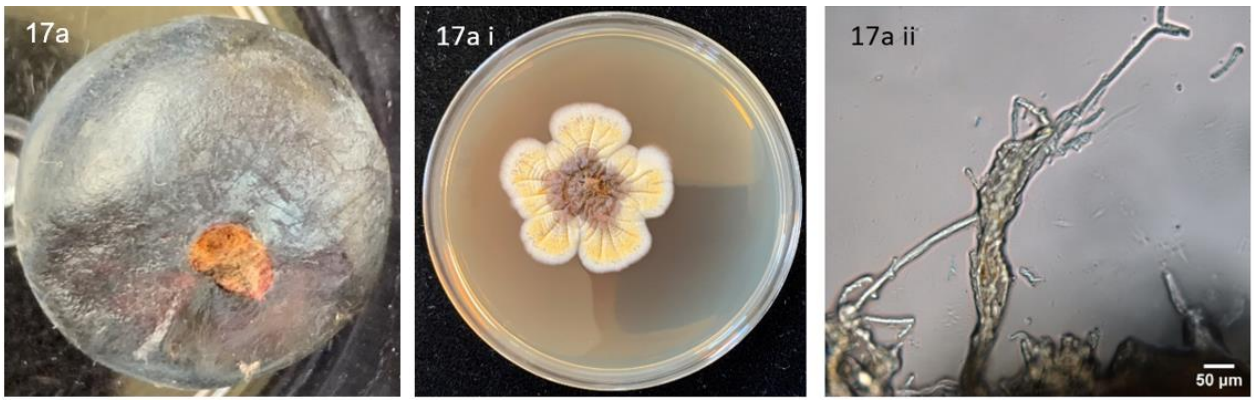


Figure 3.18. Morphology of *Epicoccum nigrum*. 17a, orange stem scar fruit decay. **17a i**, Irregular shaped colony featuring yellow and purple dense mycelium with orange pigmentation of the agar. **17a ii**, features dense mycelium of *E. nigrum* 40x.

3.3.3.7 *Penicillium*

Penicillium expansum was observed infrequently, with 0.31% of plates within group 8d. Colonies in this group shared common features, including blue mycelium with yellow or white margins. Multiple colonies of the same form appeared on all plates in this group. The texture of the mycelium was velvety, and conidia were easily dislodged (**Fig. 3.19, 8d i**). The growth rate was 0.71 mm/day^{-1}. The micromorphology of these isolates showed characteristic *Penicillium* spore structures, with elliptical/spherical conidia often grouped in chain like structures (**Fig. 3.19, 8d ii**). These spores had dimensions of 5.831 ± 1.015 x 4.592 ± 0.822, and the length : width (L:W) ratio was 1.276 ± 0.110. Compared to the literature, these spores are large, being 4–5 x 2.5–3.5 µm, with a L:W ratio of 1.4–1.6, as reported by Errampalli (2014). The fruit decays observed to be *Penicillium* had distinctive bright green or blue mycelium (**Fig. 3.20**). These are commonly reported as *P. digitatum* and *P. expansum*. Both are common postharvest decays. These decays were isolated and produced the plate groups 8c and 8d. Group 8d was identified as *P. expansum.*

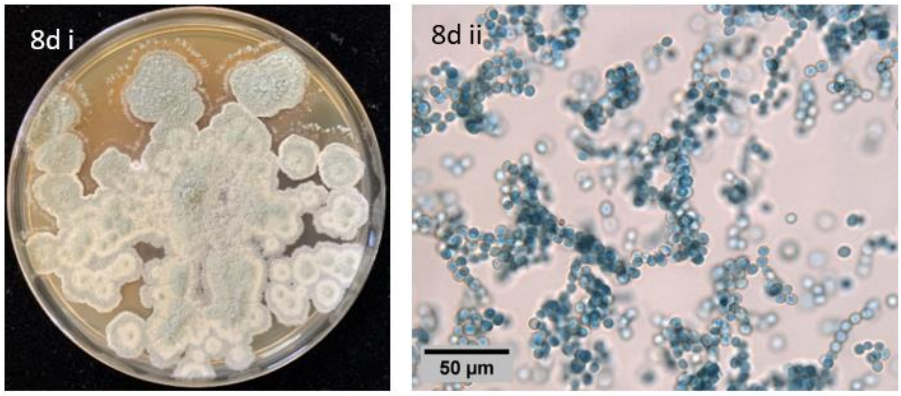


Figure 3.19. Morphology of *Penicillium expansum*. **8d i**. Culture showing velvety texture with multiple blue colonies with pale yellow margins and yellow pigmentation of agar. **8d ii**. Microscopy of *P. expansum* showing circular spores linked into chain structures 100x.

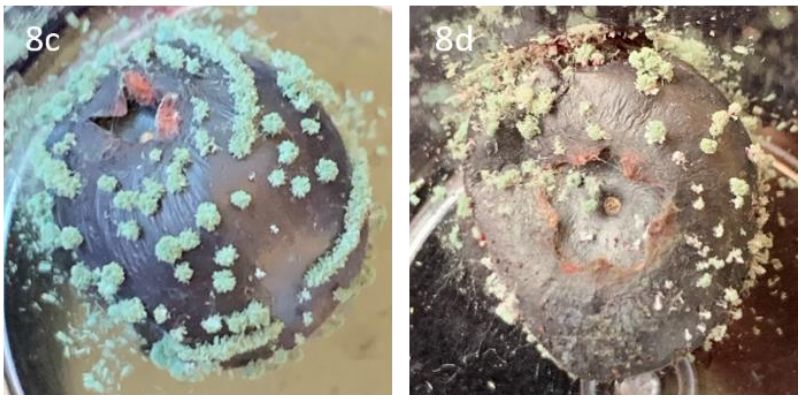


Figure 3.20. *Penicillium* fruit decay.

3.3.3.8 *Neopestalotiopsis clavispora*

Sequencing results for group 14a returned an identification of *Neopestalotiopsis clavispora.* This group occurred infrequently, accounting for 2.86% of total plates. The macromorphology of this group featured a grey mycelium with several striations originating from a darker grey centre (**Fig. 3.21, 14a i**). The growth rate was 0.65 mm/day^{-1}. No aerial mycelium or conidiomata were observed. The micromorphological analysis was limited by a lack of conidia. Hyphae were thin and densely packed, with some branching occurring (**Fig. 3.21, 14a ii**). The fruit decay this species was isolated from consisted of a grey stem scar decay; it is unclear whether *N. clavispora* is a causal agent of this decay sign (**Fig. 3.21, 14a**).

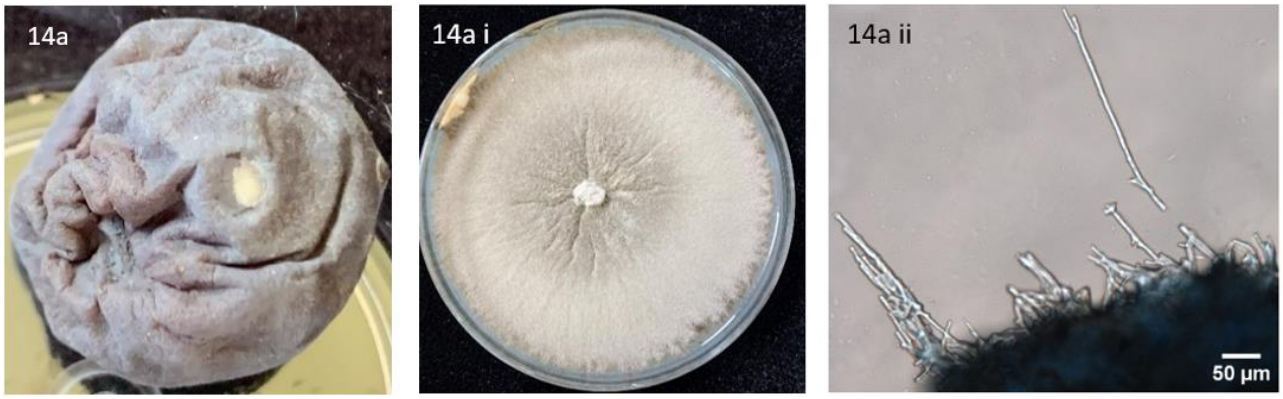


Figure 3.21. Morphology of *Neopestalotiopsis clavispora*. **14a**. Fruit decay from which this isolate was obtained. **14a i**, grey mycelium with a dark centre and striations leading to a lighter grey outer. **14a ii**, fine branching mycelium arranged densely without conidia, 40x.

3.3.3.9 Pithomyces chartum

Group 14b was identified through molecular analysis as *Pithomyces chartum*. This group was isolated from 2.20% of plates. The macromorphology displayed a light grey to cream yellow-coloured mycelium featuring a lighter centre with a darker ring towards the margin (**Fig. 3.22, 14b i**). The growth rate was 0.625 mm/day^{-1}. The micromorphology features pigmented muriform conidia with three transverse septa (**Fig. 3.22, 14b ii**). The mycelium was dense, and conidia were found embedded within the mycelium. The fruit decay from which *P. chartum* was obtained was a grey stem scar decay (14b). This is unlikely to be caused by *P. chartum*, which was more likely isolated as a cosmopolitan organism.

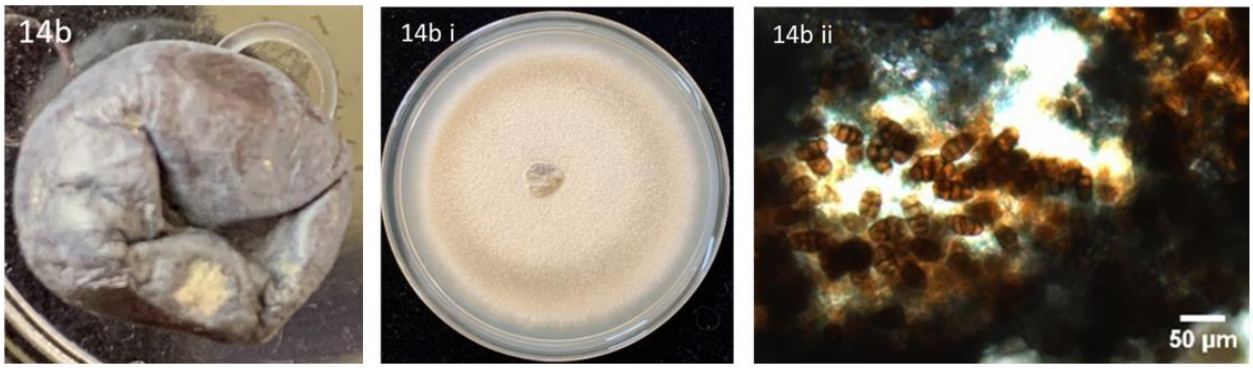


Figure 3.22. Morphology of *Pithomyces chartum*. **14b**. Fruit decay from which this isolate was obtained. **14b i**, cream mycelium with a darker ring towards the margin. **14b ii**, muriform conidia featuring three transverse septa within a dense mycelium. 40x.

3.4 Discussion

3.4.1 Discussion of Methods

Molecular analysis was the preferred method for microorganism identification. A high level of accuracy was achieved in attaining species identification at the ITS level, and for low quality ITS results, a Bt2 assay was completed, which gave further identification at the species level and confirmed species calls obtained from ITS. For simple species identification, ITS provides a reliable level of species identification if high quality sequences are obtained. However, this method relies on species having sufficient data in GenBank to complete species identification. For *B. cinerea*, an ITS type accession does not exist due to the variation observed within ITS sequences of this species (Saito et al., 2016). Therefore, ITS is not accurate for this species to determine strong phylogenetic relationships between isolates, but it is adequate for molecular species identification. For more detailed species identification and isolate phylogeny, other *Botrytis* specific genes offer more detailed results, especially when multi–locus analysis is used (Plesken et al., 2021).

The morphological analyses used showed a good level of distinction between genera at the macroscopic level. The plate groups were effective for distinguishing different genera, with a minor degree of misidentification in the groups of lower frequency, mostly for the groups without specific structures or with unique morphology, e.g., 14a and 14b. Micromorphology was able to provide distinctions between these groups as well, with unique features observed among different groups. Some groups produced very similar microscopic features, e.g., 3a, 3b, 3c, and 3d. These *Pestalotiopsis* species were found to be of the same genus through molecular analysis; however, detailed microscopy is a viable tool for identifying species of

the *Pestalotiopsis* genus (Maharachchikumbura et al., 2014). Variations within plate groups were also observed, as in 5c, with features of both *C. fioriniae* and *C. godetiae* observed through microscopy and confirmed through molecular analysis. From these analyses, there is a level of identification able to be achieved from each method; however for confident species identification, molecular analysis combined with these visual methods is more informative, especially when multi–locus molecular analysis is used. A drawback of morphological analyses is the high degree of knowledge required to distinguish species based on morphology.

A misalignment with the literature was observed in the measurements of spore length and width. The measurement of the minimum and maximum 'Feret' diameter and interpreting these as the length and width of the spores was very efficient and able to be adapted to samples; however, the measurements obtained were greater than those reported in the literature. This is likely due to the 'Feret' diameter being taken from the absolute extremities of each shape. These shapes appeared bigger in the grayscale images used for analysis than in RGB images due to the conidia boundary creating a shadow or a displacement of fluid that was absorbed into the shape of the conidia when images were converted to greyscale. This added area to the material, which obscured the accurate determination of length and width and did not prove as accurate as measuring techniques used in classical literature. An improvement would be to increase the volume of images used for analysis and compare these with traditional techniques for measuring conidia size to develop a calibration factor for 'Feret' dimensions. Redeeming features of this method were the relative accuracy of the length to width ratio and the efficiency of measuring a large number of spores.

3.4.2 Discussion of Results

3.4.2.1 *Botrytis cinerea*

The phylogenetic tree produced for *B. cinerea* indicates distinct differences between the morphological groups. The closest grouping of the *B. cinerea* isolates obtained in this work with isolates recorded from blueberry fruit in the literature was with a *B. cinerea* isolate from South Korea. No isolates from New Zealand blueberry fruit were found with an ITS accession to use. All isolates used in the tree except the outgroup were from blueberry fruit. A study by Saito et al. (2016) was used to obtain *Botrytis* species isolated from Californian blueberry fruit to compare with the isolates acquired in the present data. A multi–locus analysis of the isolates obtained would be of interest to achieve a superior comparison to *B. cinerea* isolates from New Zealand and other recorded decay of blueberry fruit. Genes of interest would be *Botrytis* specific genes as used by Plesken et al. (2021). However, using the limited ITS data, a tree has been obtained that

shows the grouping of *B. cinerea* obtained in this study compared with *Botrytis* isolates recorded on blueberry fruit in the literature.

Six macromorphological groups were identified as *B. cinerea* within the population of isolates, with variation in the presence of mycelial features and sclerotia. A study by Martinez et al. (2003) utilised eight similar groups and found that the presence of sclerotia on the isolate differed based on where the isolate was taken from on the plant. Sclerotial forms were less frequently isolated from blossoms and fruit, while mycelial forms were not isolated from grape vine canes. In the present study, groups 2a and 2b were more frequently isolated from grey stem scar decay than the other *B. cinerea* groups. These non–sclerotial *B. cinerea* isolates also formed a closer genetic distance in the phylogenetic tree than with the sclerotial group 2c. This indicates a potential difference between these morphological groups from ITS analysis, and further investigation is suggested using multi–locus analysis. Another distinction between the morphological groups is the presence of yellow exudate in group 2f, but this isolate was molecularly identified as *B. cinerea*. Therefore, there is some variability in the appearance of *B. cinerea* isolates, but molecular analysis has provided identification which overcomes this variability.

There were variations in the microscopic characteristics observed, specifically the length and width values obtained. These values are likely variable due to error in the digital method used to obtain them. However, the ratio between length and width was consistent across all groups evaluated, and the values obtained fit within the range observed by Jarvis (1977). Shape was also consistently ovoid, with abscission scars frequently observed; ellipsoid and globose conidia were also present. It is uncertain whether these other shapes represent various orientations of the conidia.

3.4.2.2 *Pestalotiopsis* spp.

Pestalotiopsis spp. have been reported to cause disease of the stems and leaves of horticultural crops, but fruit decay has only been reported on blueberry caused by *Pestalotia vaccinii* (Wharton, 2003). This species was not found in this work; however, *Pestalotiopsis disseminata* was found, as well as other *Pestalotiopsis* species. The organism *P. disseminata* has been recorded to cause fruit rot of feijoa in Iran (Naeimi et al., 2015). Other *Pestalotiopsis* spp. are recorded to cause fruit decay of grape (Deng et al., 2013; Jayawardena et al., 2015; Lorenzini & Zapparoli, 2018), avocados (Valencia et al., 2011), loquat (Palou et al., 2013), and kiwifruit (Li et al., 2016). This indicates that *Pestalotiopsis* species have the potential to cause decay of blueberry fruit. Given the prevalence of this microorganism in this work, *Pestalotiopsis* is suggested to be a cosmopolitan organism or an endophyte, as previously shown by Brookes (2017) on maize in NZ, and on blueberry in Romania by Barbu et al. (2018).

The isolates analysed in this work displayed some variation within the *Pestalotiopsis* genera, and only some were related to *Pestalotiopsis* decay reported in the literature (Lorenzini & Zapparoli, 2018). The characteristic white fluffy mycelium decay sign was isolated (groups 3a and 3b) and sequenced, with results associating this decay with *P. disseminata*, *P. brachiata*, or *P. bicilita (*Appendix B*)*. Of these, *P. brachiata* is reported as an endophyte of camelia leaves (Liu et al., 2017), so it is unlikely to be associated with fruit rot. *P. bicilita* has been reported as a postharvest fruit rot causal agent, so it is more probable that this species is associated (Lorenzini & Zapparoli, 2018). However, the molecular data is inconclusive, with ITS and Bt2 sequences being most similar to *P. disseminata* and *P. bicilita/P.brachiata,* respectively or being of low quality. Thus, further molecular analysis is required to confirm the species associated with the white mycelium decay observed. *Pestalotiopsis* spp. were also isolated from fruit without this decay, indicating the non–pathogenic presence of this organism on and within fruit tissue. Therefore, *Pestalotiopsis* spp. have the potential to cause decay, but this is rarely observed.

It is apparent from the decay *Pestalotiopsis* was isolated from that there is a mix of endophytic and pathogenic species found on NZ blueberry fruit. The macromorphological key was not useful to determine the species of *Pestalotiopsis*, as multiple species produced the same macromorphology. However, the distinct yellow colour of the mycelium distinguished this genus from the other microorganisms found. Of greater use to determine species within this genus are detailed microscopy images and multi–locus molecular analysis, as species within this genus can be reliably differentiated through both of these techniques (Maharachchikumbura et al., 2014).

3.4.2.3 *Colletotrichum acutatum* Species Complex

The C*olletotrichum acutatum* species complex is associated with anthracnose of blueberry fruit (Wharton & Schilder, 2008). Within this species complex, the major species reported in this study was *C. fioriniae*, less frequently observed were *C. godetiae* and *C. salicis*. Anthracnose is considered a key threat to blueberry production, given that most blueberry cultivars are susceptible to anthracnose and losses can be extreme (Milholland, 1995; Polashock et al., 2005). Anthracnose is commonly attributed to *C. acutatum and C. gloeosporides* (Hartung et al., 1981; Polashock et al., 2005). Recent molecular and phylogenetic advances have enabled the delineation of new species within these species complexes (Damm et al., 2012; Weir et al., 2012). This includes *C. fioriniae*, which has recently been reported to cause blueberry fruit anthracnose in Chile and was the most frequently observed microorganism isolated from anthracnose in this work (Castro et al., 2022).

The groups represented on the phylogenetic tree (**Fig. 3.9**) from the data presented in this research indicate two groupings of *Colletotrichum* species being associated with anthracnose of blueberry fruit. Both are within the *C. acutatum* spp. complex; however, they are different species. Both *C. fioriniae* and *C. salicis* were closely related to other isolates of these species obtained from fruit crops in New Zealand. The third species, *C. godetiae,* was not included due to a poor quality ITS sequence. A *Vaccinium* related *C. fioriniae* sequence was very closely related to the *C. fioriniae* sequences obtained in this research (Damm et al., 2012). From *Malus* and *Fragaria* crops, a *C. salicis* sequence was found in GenBank and observed to be closely related to *C. salicis* from this work. Both of these species fall within the *C. acutatum* species complex (Damm et al., 2012). This indicates that multiple species of *Colletotrichum* are associated with anthracnose decay of NZ blueberry fruit.

Colletotrichum commonly produces diverse isolate morphologies. This was observed in the isolates obtained, with four distinct macromorphological groups established. The identification of species making up these groups was likely representative for groups 5a and 5b. However, given the molecular and micromorphological results from group 5c, which contains both *C. fioriniae* and *C. godetiae*, there is some ambiguity within these groups. Therefore, molecular identification is the preferred method for identifying species within this genus.

Micromorphological identification using the methods described in this report is not recommended for species identification due to the inaccuracy of measurement. However, conidia characteristics can be used to differentiate species (Damm et al., 2012). The presence of acute or rounded ends on *Colletotrichum* conidia can indicate different species. As seen in these results, acute ends were seen for *C. fioriniae* in 5a and 5b, but for *C. godetiae*, some conidia were observed with one rounded end. Molecular analysis is the preferred method to differentiate these species due to the low requirement for specific knowledge and the high level of accuracy achievable.

The fruit decays from which the *Colletotrichum acutatum* spp. complex was isolated were mostly anthracnose fruit rot. In some instances, *Colletotrichum* was isolated from fruit not displaying anthracnose, indicating the presence of this genus as a quiescent inhabitant of the fruit microbiome. Fruit with multiple decay signs, including anthracnose and grey stem scar, were common. This indicates that the pathogenicity threshold of *Colletotrichum* is similar to that of pathogens related to grey stem scar, including *B. cinerea* and *Pestalotiopsis* spp.

3.4.2.4 *Diaporthe* spp.

Interestingly, *Diaporthe salicicola* and *D. nobilis* were associated with decay of blueberry fruit. The *D. salicicola* isolates were obtained from distinct signs on fruit with a grey/brown stem scar and juice leakage. This species is potentially a non–pathogenic species, with a low number of isolates obtained from this sign and no literature found reporting this as pathogenic to harvested fruit. This species was first isolated from *Salix purpurea* in Australia, which is also present in New Zealand (Tan et al., 2013). Therefore, *D. salicocla* is potentially a non–pathogenic organism associated with blueberry fruit. The decay observed may be attributed to another species; a pathogenicity test would confirm this hypothesis.

A delicate white mycelium was observed from *D. nobilis*, covering the stem scar, or spreading across the whole fruit. This decay sign has been associated with a greyish white mycelium decay on blueberry fruit in China (Yu et al., 2018). This is the first association of *D. nobilis* with decay on blueberry fruit in New Zealand. It has been previously reported to be present on the bark of apple (*Malus pumila*) and on Asian pear (*Pyrus pyrifolia*) in NZ (Gomes et al., 2013). It is also closely related to *D. vaccinii,* which is an important pathogen of blueberry plants in the USA (Gomes et al., 2013), and the *D. eres* pathogen of grape berries in Italy (Lorenzini & Zapparoli, 2018). The white mycelium observed in this study is very similar to that reported by Yu et al. (2018) and Lorenzini and Zapparoli (2018). Therefore, this species is a potential causal agent of blueberry fruit decay.

3.4.2.5 Others

Neofusicoccum is a well reported causal agent of blueberry stem and twig dieback and canker (Espinoza et al., 2009). The isolates obtained here did not produce a specific decay sign, so they were possibly non–pathogenic fruit endophytes or epiphytes. Two species were obtained: *N. parvum*, and *N. austral*. Both are pathogenic to blueberry plants but have not been recorded to cause fruit decay.

Another observed microorganism was *Epicoccum nigrum*, which was associated with an orange stem scar decay occurring on blueberry fruit. This decay was very infrequently observed and only occurred in the absence of other decays. It was readily identified to a genus level through the distinct macromorphology, and the molecular data provided a species identification as *Epicoccum nigrum*. Micromorphology was not particularly useful due to the very dense mycelium and lack of aerial mycelium or spores observed. These colonies were very slow growing, which is reported in the literature (Taguiam et al., 2021).

3.5 Conclusion

The identification of microorganisms in this experiment was successful, with the major decay causing fungi identified to a species level. Key findings include the presence of multiple species within the *C. acutatum* spp. complex being associated with anthracnose and grey stem scar decays, including *C. fioriniae*, *C. godetiae*, and *C. salicis*. The molecular identification of multiple morphological groups as *B. cinerea* is another useful finding and distinguishes *B. cinerea* as a major causal organism of postharvest blueberry decay. Other microorganisms, including *Pestalotiopsis* spp., *Diaporthe* spp., *Penicillium* spp., *Neopestalotiopsis clavispora*, and *Epicoccum nigrum*, were also found to be associated with blueberry fruit decay; however, their pathogenicity is unclear. Non–pathogenic microorganisms isolated from decaying blueberry fruit included *Neofusicoccum parvum* and *Pithomyces chartum*. These results indicate that the microflora associated with postharvest decay of blueberry fruit is diverse. The genera related to blueberry fruit rots in this study and the literature were similar (Oliver & Ali, 2021; Schilder et al., 2000; Wharton, 2003; Xiao & Saito, 2016). Microorganisms follow numerous lifestyles, with necrotrophic, hemibiotrophic, saprotrophic, opportunistic, and benign fungi identified in this work. This implies that relationships exist between these organisms, which may influence the expression of decay signs or symptoms.

The methodology utilised in the identification of these microorganisms could be improved through more extensive molecular identification and the development of digital tools to accurately determine spore length and width from light microscopy images. Also useful would be a digital measuring tool for more accurate determination of the growth rate of cultures in petri dishes. The data generated from these improved methodologies would offer a more detailed resource on the nature of the microorganisms associated with postharvest decay of blueberry fruit and to facilitate more simple morphological identification. This would also offer an enriched comparison with other reported microorganisms in NZ and overseas and would better inform the use of control measures to reduce postharvest decay. Therefore, the methodology used in this experiment has been adequate to obtain significant findings, but the level of detail could be improved to increase the applicability of the data.

Chapter 4: Effects of Storage Conditions on Postharvest Decay of Blueberry Fruit

4.1 Introduction

Decay during postharvest storage is the main cause of quality loss and subsequent consumer rejection of blueberry fruit (Forney, 2009). Decay is the infection of fruit tissue by microorganisms which cause unacceptable damage to fruit quality. Decay caused by fungal microorganisms accounted for >50% of fruit losses in the Eastern USA and >90% of rots in California (Cappellini et al., 1982; Xiao & Saito, 2016). The most common fungal pathogens previously described to affect blueberry quality are *Botrytis cinerea*, *Colletotrichum* spp., and *Alternaria* spp. Some of these pathogens are acknowledged to cause infection even under cold storage (0–5°C) conditions. These rots cause considerable waste and inefficiencies within the supply chain by causing fruit to be rejected in the marketplace. Therefore, determining the prevalence of these rots under storage conditions is of interest to the export blueberry industry.

Temperature is an important control factor for mitigating the postharvest decay of fruit. Low temperature (<5°C) limits the pathogen species that can cause infection and the rate of decay development. Cold storage alters the disease triangle factors at play by altering the environment, having the effect of reducing the pathogenicity of microorganisms by slowing their metabolic rate, and inducing dormancy in some microorganisms (Sommer, 1982). This environmental change also reduces the rate at which host susceptibility increases, having the overall effect of reducing the prevalence of decay. The recommended cold storage temperature for blueberry fruit is 0–2°C (Forney, 2009; Hancock et al., 2008). Low temperature storage decreases blueberry metabolic rate and maintains fruit firmness, which confers resistance to decay (Beaudry, 1992; Chen et al., 2015). Firmness is retained by low temperature by slowing the enzymatic cell wall degradation processes associated with softening (Chen et al., 2015). Cold storage limits the pathogen species able to cause decay. At 1°C, *Botrytis cinerea*, *Alternaria* spp., *Diaporthe* spp., and *Penicillium expansum* can cause infections (**Table 4.1**). The growth rate of these organisms is slowed by low temperature, which contributes to the reduced rate of decay observed (Stretch & Davis, 1960). Therefore, low temperature is an important control measure for limiting the postharvest decay of blueberry fruit.

Table 4.1. Minimum and optimum growth temperatures of important postharvest fungi.

Microorganism	Minimum Growth Temperature (°C)	Optimum Growth Temperature (°C)	Reference
Botrytis cinerea	0	20	Droby & Lichter, 2007
Alternaria alternata	0	25	Troncoso-Rojas & Tiznado-Hernández, 2014
Colletotrichum acutatum	7	26	Miles et al., 2013; Verma et al., 2006
***Pestalotiopsis* spp.**	5	20–25	Espinoza et al., 2008
***Diaporthe* spp.**	0	20–30	Abramczyk et al., 2020; Díaz et al., 2014
Epicoccum nigrum	5	20	Christova & Slavov, 2021
Penicillium expansum	-1	25	Baert et al., 2007; Errampalli, 2014

Relative humidity (RH) is another key variable to consider for maintaining blueberry fruit quality through the supply chain. It is recommended that RH be >95% for long term storage (Forney, 2009). For the blueberry supply chain, RH is important to control because it has a significant influence on moisture loss and subsequent shrivel and softening of fruit, which are important fruit quality attributes for consumer acceptability (Paniagua et al., 2013). The use of high humidity through postharvest storage limits moisture loss from fruit tissue and offers reduced shrivel and softening, which maintains fruit quality (Paniagua et al., 2013). For example, the rate of water loss from blueberry fruit is high (approximately 5%) when the fruit is stored at a RH of 90% for 50 days (Liu et al., 2019). However, high RH (>95%) and free water (condensation) on the fruit surface offers a favourable environment for decay, causing pathogens to develop infections of the fruit. Consequently, a RH of 95% is recommended to maintain fruit quality, but it is also suitable for the development of infection by major decay causing fungal pathogens, including *B. cinerea* (Thomas et al., 1988), *Colletotrichum* spp. (Estrada et al., 2000), and *Alternaria* spp. (Troncoso-Rojas & Tiznado-Hernández, 2014). Therefore, high RH is a suitable method for maintaining postharvest fruit quality, but it incurs an increased risk of decay. Thus, identifying the microorganisms related to decay under high RH conditions is important to understand in order to manage decay.

Decay development in the postharvest supply chain varies by temperature and RH conditions. However, within export containers and individual clamshells, there is variation in temperature and RH, thereby impacting the expression of decay observed within the supply chain (Paniagua et al., 2013). A study to

determine the effects of temperature and RH throughout storage and shelf life is required to ascertain the decay signs, symptoms, and causal pathogens occurring in different conditions.

This study will imitate conditions potentially experienced by blueberry fruit through the supply chain (Ktenioudaki et al., 2021). By evaluating the prevalence of decay and relating this to causal pathogens, a further understanding of the main pathogens involved in postharvest decay will be achieved. By completing this experiment, control measures can be targeted towards pathogens of greater frequency, leading to greater fruit acceptance in export markets.

4.1.1 Aims and Objectives

The aim of this experiment is to evaluate the prevalence of postharvest decays under temperature and RH variations during cold storage and shelf life of blueberry fruit. In addition, the causal microorganisms and microorganisms associated with decay identified in Chapter 3 are related to their prevalence under each environmental condition in this experiment.

The objectives of this experiment are as follows:

1. Evaluate the frequency of decay signs at 1°C or 15°C in combination with low (80%) or high (99%) RH during postharvest storage and shelf life.
2. Relate the pathogens identified as associated with decay in Chapter 3 to the decay sign prevalence observed in postharvest temperature and relative humidity treatments.

4.2 Materials and Methodology

4.2.1 Fruit Material

The fruit used in this experiment were blueberries grown in New Zealand, purchased weekly over eight intervals from late February to mid–April from three retail sources in Palmerston North City, New Zealand. Five clamshells without apparent signs or symptoms of decay were bought from each store and transported to the Massey University Postharvest Lab for evaluations and experiments. 120 clamshells with a total weight of 15 kg (4,320 fruit) were used in this investigation. This sample size is large relative to the objectives of this investigation. This sample is believed to be representative of the fresh blueberry fruit

available on the NZ domestic market during the sampling period. The author is confident that the fruit obtained was grown in a variety of regions, including the main production areas of Waikato and Hawkes Bay. This is known because the same distinct brands of fruit were consistently purchased from the same retailers and featured consistent packing locations on the packaging. It is believed these fruit were grown near their respective packing locations, thus providing a representation of the available blueberry fruit produced in NZ during this time. Any fruit with a sign or symptom of decay (**Tables 4.1, 4.2**) upon receipt was excluded from the experiments.

4.2.2 Fruit Evaluation Before Initiation of the Storage Treatments

Blueberries were evaluated for quality and maturity through mechanical parameters, total soluble solids (TSS), acidity, and the ratio of TSS to acidity. Samples of 60 blueberries from each set of 5 clamshells of the same origin and purchase date were set aside for one hour to reach 20°C before evaluations began. The fruit temperature was determined using a digital probe thermometer inserted into the centre of the berry.

4.2.2.1 Mechanical Parameters

Blueberry mechanical parameters were evaluated through a penetration test using a TX.AT texture analyser (Stable Micro Systems, Surrey, UK) equipped with a needle probe (SMS P/2 N, Stable Micro Systems) and a 5 kg load cell. The trigger force was 0.01 N, a pre–test speed of 5 mm s^{-1}, and a test speed of 1 mm s^{-1} were used. The penetration distance target (strain) was 30% of the berry's equatorial height. The fruit was placed on a flat metal ring with a 10mm internal diameter during the test to avoid unbalanced movement that can affect mechanical data. The berry was oriented with the stem–calyx axis parallel to the platform, exposing the equatorial region to the probe. Data were collected at a frequency of 250 points per second using Exponent software (Version 6.1.14.0, Stable Micro Systems). The penetration tests were used to determine mechanical parameters of force at skin break (N) and skin break slope (N mm^{-1}), as previously reported by Rivera et al. (2022).

4.2.2.2 Total Soluble Solids and Acidity

Fruit maturity was determined by measuring total soluble solids (TSS) and acidity with a digital Brix-acid meter (PAL-BX I ACID F5, Atago, Tokyo, Japan). Groups of 10 berries were crushed into a beaker using a

spoon and a strainer to collect pulp–less juice. A sample of 0.3 mL was taken using a pipette and placed onto the refractometer lens to determine TSS. This was repeated for all 60 fruit sampled per origin.

Acidity was determined by weighing 1 g of blueberry juice using a digital balance (TW423L 0.001 g, Shimadzu, Japan) and diluting the sample in 50 mL of reverse osmosis (RO) water. The solution was gently swirled to homogenise the liquid, and a 0.3 mL sample of this solution was pipetted onto the digital acid meter (PAL-BX I ACID F5, Atago) lens to give an acidity reading. The acid meter was set on the blueberry setting to optimise for citric acid, which is the most abundant acid in blueberry fruit (Forney et al., 2012).

4.2.2.4 Total Soluble Solids to Acidity Ratio

The ratio of TSS to acidity (TSS : acidity) was calculated using equation 4.1.

$$TSS : Acidity\ Ratio = \frac{TSS\ (\%\ Brix)}{Acidity\ (\%)} \quad \text{(Eq. 4.1)}$$

4.2.3 Postharvest Experiment Design

Fruit were placed individually into 60 mL plastic cups (60mL sample cup, Jadcup, Auckland, NZ) to allow independent expression of decay and avoid cross–contamination associated with fruit-to-fruit contact. The cups were sterilised before use by soaking with 10% bleach solution in hot water for 10 minutes. A cardboard box of 1,363 cm^3 (commercial kiwifruit tray, Zespri®, New Zealand) was assembled, containing 45 cups, with one box from each source per treatment (**Fig. 4.1**). This gave 12 boxes per week and 96 boxes over the course of the experiment. Sound blueberries were individually placed into cups randomly from a set of 5 clamshells from the same source to give 45 fruit per box, with the cups sitting loosely in an acrylic plastic sheet supported in the box; an example is presented in **Fig. 4.2**.

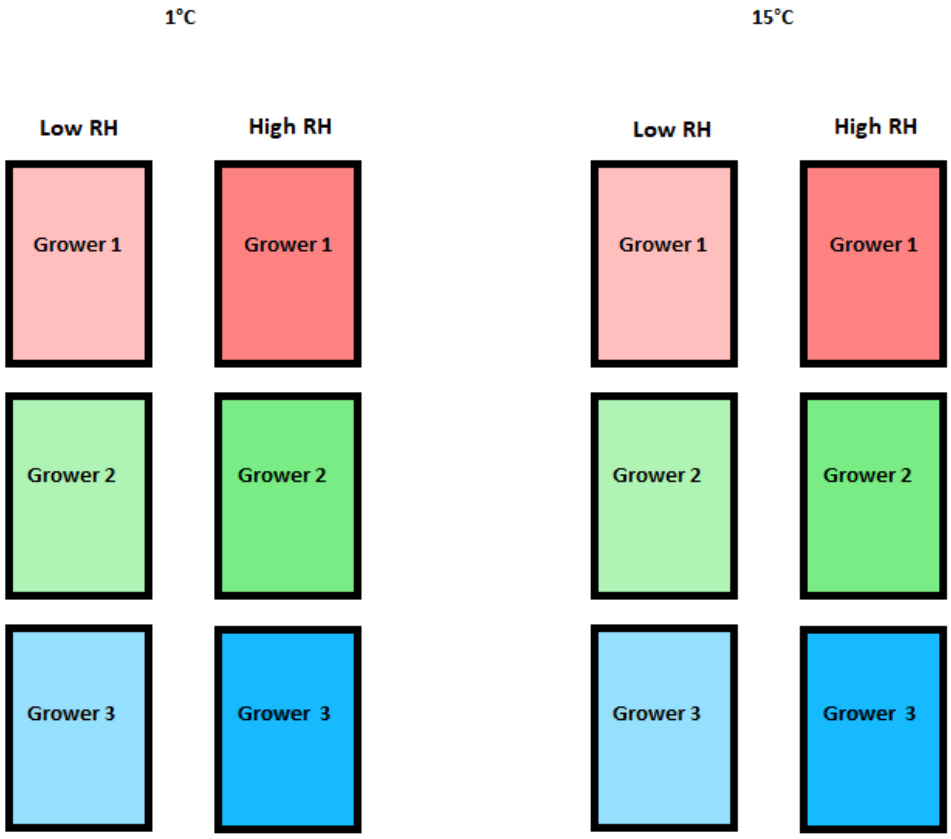


Figure 4.1. Experimental design: 3 sources of blueberry fruit across 4 temperature and RH treatments (1°C high RH, 1°C low RH, 15°C high RH, 15°C low RH).

4.2.4 Storage Treatments

Four treatments were obtained using two temperatures (1°C and 15°C) in combination with two RH conditions (99% and 80%). Each of these treatments was applied to three boxes, one per each of the three sources (n = 45 fruit) each week, giving 540 fruit treated per week over all treatment and origin combinations (**Fig. 4.1**). Both temperature (°C) and RH (%) were monitored throughout the experiment using button sized loggers (iButton®, Maxim Integrated, CA, USA). Concentrations of atmospheric gases (CO_2 and O_2) were not recorded and were assumed to be at regular atmosphere (0.04% CO2 and 21% O2).

The 1°C temperature treatment was applied to reproduce best practice cold storage conditions. This represents the minimum growth temperature of fungal species affecting blueberry (Madrid & Beaudry, 2020). This treatment was measured as 1.5°C ± 0.5°C (**Fig. 4.3A**), encompassing a range of storage temperatures from 1°C to 2°C. Although referred to as 1°C in this text for simplicity, it is important to note the actual temperature range was wider than a precise 1°C. The duration was six weeks, which represents

the maximum duration of sea–freight exportation or storage of blueberries (Paniagua et al., 2014). This temperature was achieved using a temperature–controlled room.

The 15°C temperature treatment was measured as 13.8°C ± 0.5°C (**Fig. 4.3B**) for 2 weeks to provide close to optimal conditions for the growth of fungal species affecting blueberries. Due to the temperature range and associated measurement error, this treatment is referred to as 15°C throughout this text. This was applied using a fridge thoroughly cleaned with hot water, bleach, and 70% ethanol.

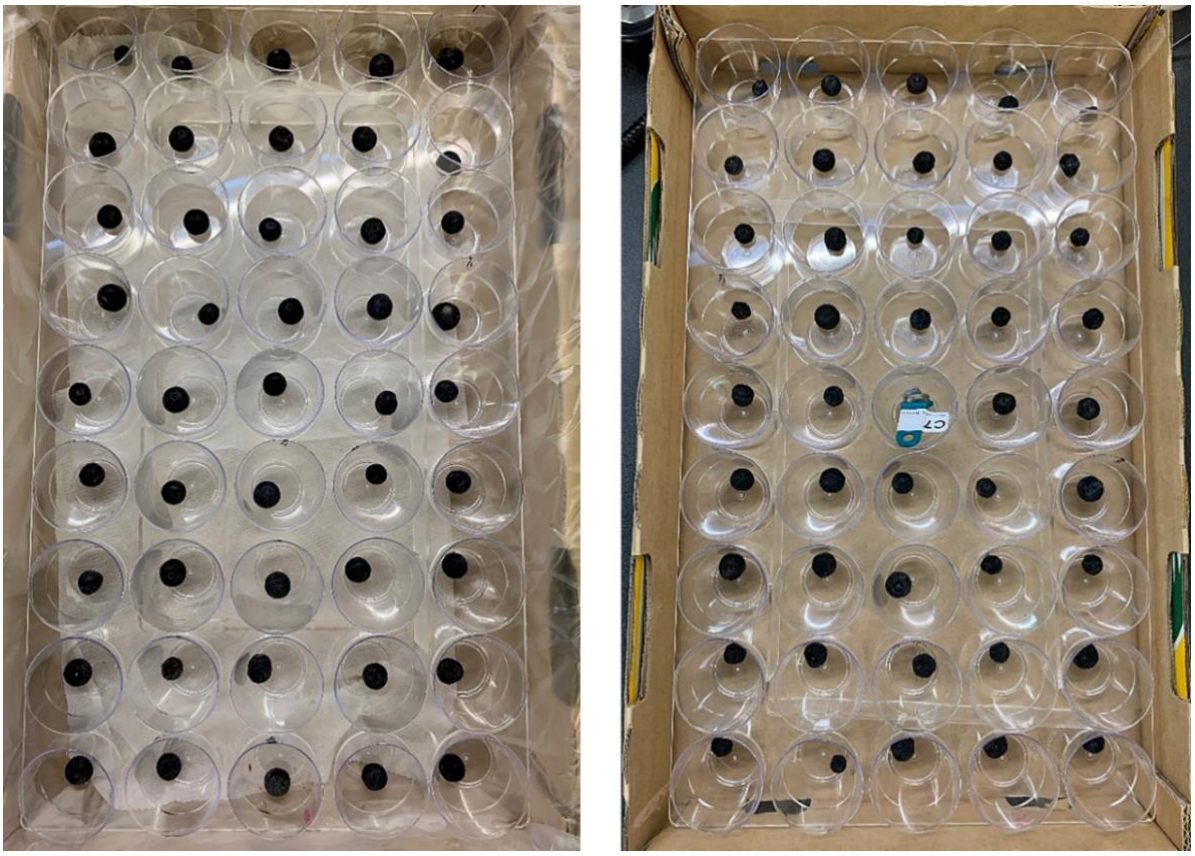


Figure 4.2. Treatment boxes used in the present experiment. **Left**. High RH treatment box. Polyliners and moist paper towels included. **Right**. Low RH treatment Box: ibutton shown in centre cup. Note the absence of polyliners and moist paper towels.

A high RH treatment was applied by creating a closed system around the fruit and building initial humidity (**Fig. 4.2**). This involved lining the inside of the box with two polyethylene polyliners (10 µm) and laying a damp, UV-sterilised paper towel at the bottom of the box to provide initial humidity. The acrylic plastic support was placed on the polyliner, and the cups and fruit were added. The polyliners were carefully folded over the cups and tucked into the sides, and the lid was closed to give the system a loose seal. This held a consistently high RH of 98–100% from initiation throughout the treatment at 1°C and 15°C (**Fig. 4.3C**).

A low RH treatment was applied by leaving the box without polyliners. The acrylic support and fruit were added, the box lid was closed, and the vents were unmodified. This gave a low relative humidity of 80–85% at 1°C and 15°C (**Fig. 4.3D**). These treatments were employed to imitate the variability in humidity blueberries experience in the export supply chain (Paniagua et al., 2013).

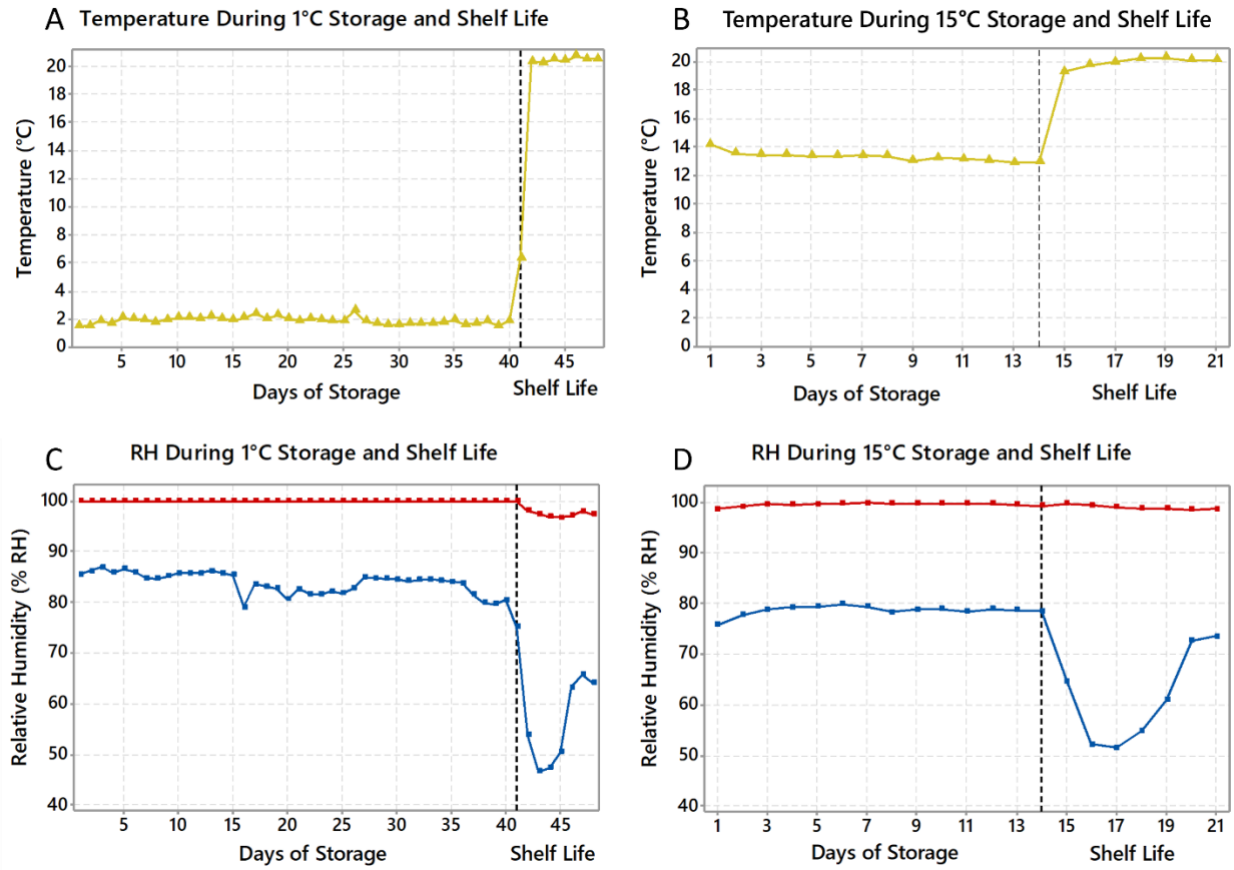


Figure 4.3. Temperature and relative humidity conditions recorded in this experiment. RH = relative humidity.

4.2.5 Evaluation of Decay

Once the duration of each treatment had elapsed, the fruit was removed from the temperature–controlled environments and exposed to shelf life at 20°C for 7 days (**Fig. 4.3 A–D**). Over this period, visual inspections of the fruit were conducted on days 0, 2, 5, and 7 to observe the incidence and prevalence of decay signs or symptoms. The humidity treatments were maintained throughout this period.

4.2.5.1 Decay Incidence and Prevalence

The visual evaluation of decay incidence and prevalence consisted of observing all sides of the fruit and recording the location, sign, and symptom of decay. Decay incidence is the frequency of decay signs and symptoms after cold storage. Decay prevalence is the cumulative total of decay incidence after shelf life. A photo was also taken of each evaluation period in order to track decay expression after postharvest storage and during shelf life. The decay prevalence was calculated for each box as the cumulative decay incidence over time. The number of fruit per batch was always 45.

$$Deacy\ Prevalence\ (\%) = \left(\frac{Sum\ of\ Day\ 0\ incidence}{No.\ Fruit\ per\ batch} + \frac{Day\ 2\ incidence}{No.\ Fruit\ per\ batch} + \frac{Day\ 5\ incidence}{No.\ Fruit\ per\ Batch} + \frac{Day\ 7\ incidence}{No.\ Fruit\ per\ Batch}\right) \times\ 100$$

(Eq. 4.2)

4.2.5.2 Location

Decay was recorded relative to the location of the fruit where it was observed. These were the stem scar, blossom end, wounds, shrivelled areas, whole fruit, or patches on the fruit.

4.2.5.3 Signs and Symptoms of Decay

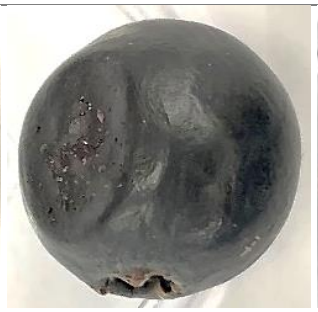
Signs are the confirmed visual presence of a pathogen (Sholberg & Conway, 2004). The signs recorded in this experiment were described using colours and textures, or commonly used terms for specific decays (e.g., grey mould). Images of the signs and their corresponding groups are listed in Table 4.2.

Table 4.2. Decay sign key

Grey mould	Anthracnose	Grey Mycelium in Stem Scar	Green Mycelium
			
White Mycelium in Stem Scar	**Fluffy White Mycelium**	**Dense Orange/Brown Mycelium**	**Bright Blue/Green Mycelium**
			
Dense Green Mycelium	**Thin White Threads of Mycelium**	**Fluffy Dark Grey Mycelium**	**Dark Grey Droplets**
			
Cream Droplets	**Pink Droplets**	**Dense White/Green Mycelium**	**Pink Mycelium**
			

Symptoms are the responses of the host to pathogenic organisms (Sholberg & Conway, 2004). In this experiment, symptoms were considered to be visible quality disorders until a decay sign was observed. These symptoms recorded were juice leakage, slip skin, fruit collapse, and split fruit. Examples of these are indicated in **Table 4.3**.

Table 4.3. Symptoms of decay

Juice Leakage	Slip Skin	Fruit Collapse	Split Fruit
			

To apply the species identifications obtained in Chapter 3 to the observed fruit decays, matrices were created, aligning plate groups with decay signs. Examples of plate groups can be found in Appendix A. Instances of contamination, and plate groups associated with fruit decay reported in the literature to be caused by other species were removed from the dataset. The identification of the plate group informed the identification of the microorganism isolated from decaying blueberry fruit.

4.2.6 Analysis of Microorganisms

After seven days at 20°C, fruit displaying representative or novel signs or symptoms were selected for isolation and identification of species through morphological and molecular techniques. These methods are detailed in Chapter 3.2.

4.2.7 Data Analysis

The data were analysed through mixed effects models using untransformed data for each decay sign and total decay prevalence. The models were constructed using temperature and RH as fixed factors and fruit origin and week of purchase as random factors. A Fisher LSD (least significant difference) test was used to

determine differences between means at a 5% significance level. Separate models were constructed for each major decay sign (when prevalence is >5%) for shelf life of 0 d and 7 d. The statistical software used for analysis was Minitab® 18 (version 18.1, Minitab Inc., PA, USA). Plots were constructed using Minitab® 18 (version 18.1). Tables were constructed in Microsoft Excel (version 2209).

4.3 Results

4.3.1 Fruit Maturity and Quality

The fruit used in this experiment, as sourced from multiple origins over eight weeks from local retailers, were not of uniform cultivar, maturity, or quality. The fruit had a total soluble solids (TSS) range of 8.7%–17.7% with an average of 12.3% and a coefficient of variation (CV) of 13.3%. The acidity varied between 0.16% and 0.78%, with a mean of 0.44% and a CV of 34.1%. The range of TSS : acidity was 12.2–84.2 with a mean of 32.4 and a CV of 43.6% (**Table 4.4**). There was some variation among TSS values, but higher variation for acidity and consequently TSS : acidity. This suggests that within the fruit population, there were differences in harvest maturity, length of cold storage period, and cultivar (Moggia et al., 2018).

Fruit mechanical properties were determined using skin break force (N), which had a mean of 0.185 N, a minimum of 0.066 N, and a maximum of 0.565 N (CV = 36.84%), and skin break slope averaged 0.267 N mm^{-1} with a minimum of 0.014 N mm^{-1} and a maximum of 0.623 N mm^{-1} (CV = 37.04%).

Table 4.4. Fruit quality and maturity descriptive statistics.

Variable	Mean	Range (Min – Max)	CV (%)[c]
TSS (%)[a]	12.29 ±1.63	8.70 – 17.70	13.26
Acidity (%)[a]	0.44 ±0.15	0.16 – 0.78	34.09
TSS : Acidity (-)[a]	32.38 ±14.11	12.18 – 84.21	43.58
Skin Break Force (N)[b]	0.19 ±0.07	0.066 – 0.565	36.84
Hardness Slope (N mm^{-1})[b]	0.27 ±0.10	0.014 – 0.623	37.04

[a] Mean ± standard deviation (n = 6 samples of 10 berries from each batch (24 batches).

[B] Mean ± standard deviation (n = 60 samples from each batch (24 batches).

[c] Coefficient of variation (%) calculated using $\left(\frac{\text{Standard deviation}}{\text{Mean}}\right) \times 100$.

The variability in quality and maturity observed between fruit sources is expected as it is influenced by cultivar, growing location, harvest timing, and time after harvest, which vary from orchard to orchard. In addition, supply chain factors before purchase, including temperature, relative humidity, and storage duration, can contribute to the observed variability.

The blueberry producers or trading companies purchased from each retailer (fruit source) in this study were likely to be consistent. This was recognised from the packaging of the fruit and the associated businesses, which were always obtained from the same local retailers at different times throughout the blueberry season. This allowed grouping by grower, maturity, and quality (**Table 4.5**). Differences were observed between producer companies for all attributes evaluated.

Table 4.5. Average of maturity and quality indicators for three blueberry retail brands. 480 fruit per grower, sampled in sets of 60 fruit per week from late summer (19 February) to mid–autumn (13 April) 2022. Fisher LSD to 95% confidence. Means that do not share a letter are significantly different (P <0.05). Retailers are believed to be representative of fresh blueberries available on the NZ market during the sampling period.

Fruit source (Retailer)	TSS (% Brix)	Acidity (%)	TSS : Acidity (-)	Skin Break Force (N)	Hardness Slope (N mm^{-1})
A	12.41^{ab}	0.51^{a}	29.41^{b}	0.20^{a}	0.30^{a}
B	11.80^{b}	0.34^{b}	36.58^{a}	0.17^{c}	0.23^{c}
C	12.66^{a}	0.48^{a}	30.81^{b}	0.18^{b}	0.27^{b}

4.3.2 Postharvest Storage

4.3.2.1 Post Storage Decay Prevalence

Blueberry decay signs and symptoms occurred during storage under all conditions observed. Decay during storage was higher for blueberries stored at a high RH and temperature (15°C). This treatment also produced a higher decay range (0–53% decay). No significant differences were observed between fruit sources.

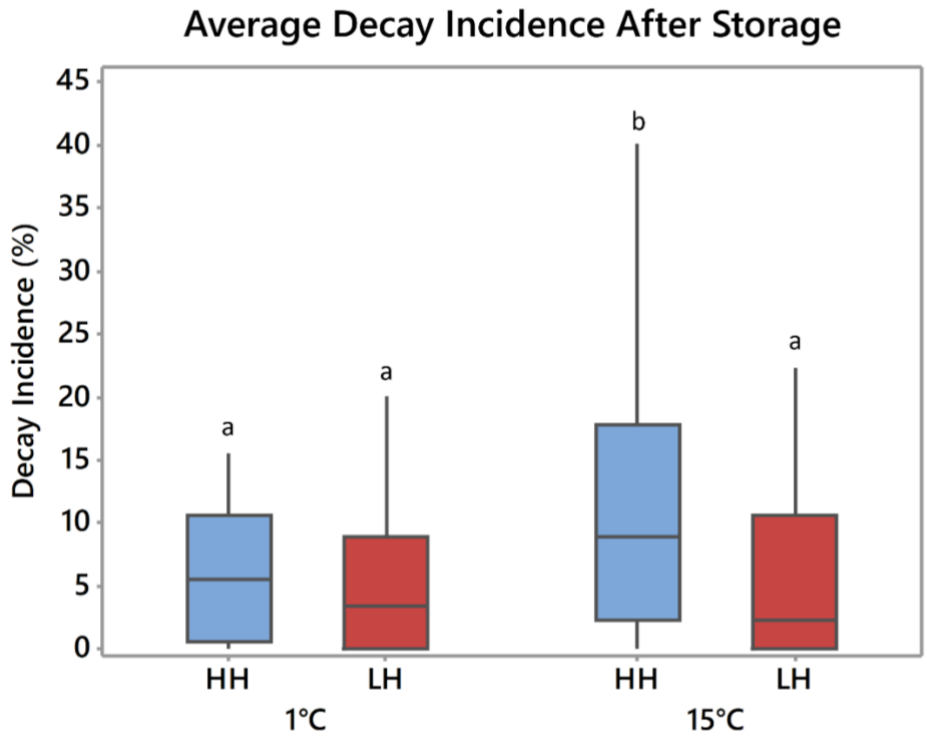


Figure 4.4. Average decay incidence per tray of blueberry fruit immediately after cold storage for 42 days at 1°C or 14 days at 15°C. Means that do not share a letter are significantly different (P <0.05) across all treatment combinations. Sample size of n = 45 fruit/tray, 24 trays per treatment combination. Outliers are not shown. HH = high RH, LH = low RH.

Decay incidence was greatest in the high RH and 15°C treatment, with a mean of 13.6% (CV = 100%). This treatment produced a significantly higher decay incidence compared to low RH (mean 6.2%, range 0–22%, CV = 118%) and the 1°C treatments. This is likely due to the high RH and 15°C conditions being more conducive to the development of decay. The higher occurrence of decay at 15°C and high RH is enabled by the activity of a greater number of fungal species at this temperature, the improved ability to infect host tissue at high RH, and a more rapid deterioration of host defence than at 1°C (Forney, 2009; Thomas et al., 1988). There was high variability within these decay incidence data, reflecting the inconsistent nature of decay incidence within the population.

Storage at 1°C reduced decay development when compared to 15°C storage at high RH. The mean decay incidence was 5.9% for high RH (CV = 90%) and 5.5% for low RH (CV = 106%). No difference was observed between RH treatments at 1°C (**Fig. 4.4**). However, the range of decay incidence within the 1°C treatments was high, indicating the inconsistency of decay incidence during storage. These results suggest that high temperature (15°C) in combination with high RH are critical for decay development, and consequently, a storage temperature of 1°C is an effective tool for restricting the expression of postharvest decay of blueberry fruit (Forney, 2009).

4.3.2.2 Decay Incidence After Cold Storage by Decay Sign Expressed

After storage, total decay incidence was divided into the decay signs and symptoms observed (**Fig. 4.5**). This produced some important differences in the distribution of decay sign between temperature and RH treatments. Decay symptoms were not included, as these quickly developed decay signs or were present simultaneously with decay signs. However, the split fruit symptom (**Table 4.3**) was categorised as a quality disorder as it did not develop any decay sign and occurred rarely (0.37%).

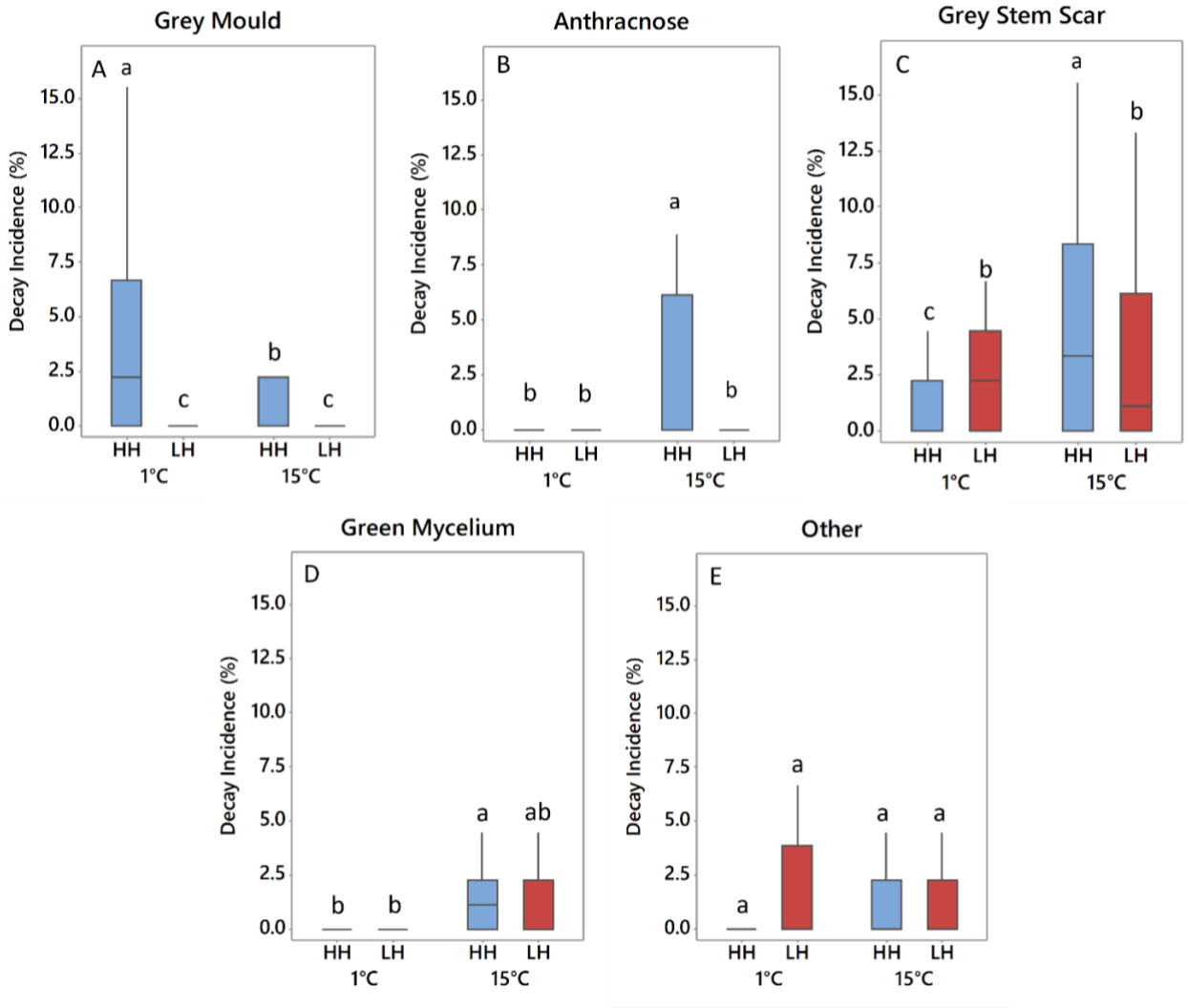


Figure 4.5. Decay incidence immediately after cold storage described by decay sign. Means that do not share a letter are significantly different using Fisher's LSD (P <0.05) within decay sign. HH = high RH, LH = low RH.

Grey mould (**Fig. 4.5A**), identified as *Botrytis cinerea* (Chapter 3), was most prevalent in blueberries stored at 1°C in combination with high RH, with an average incidence of 3.8% (range: 0–15.5%). This was similar for blueberries stored at 15°C, with the high RH treatment producing a significantly greater incidence of

grey mould. These results suggest that grey mould decay of blueberry fruit is predominantly produced at high RH.

Anthracnose (**Fig. 4.5B**) was identified as being associated with *Colletotrichum fioriniae, Colletotrichum godetiae,* and *Colletotrichum salicis* within the *Colletotrichum acutatum* species complex (Chapter 3). This decay occurred during storage at 15°C but not at 1°C (**Fig. 4.5B**). *C. acutatum* has previously been reported to be unable to infect blueberry fruit at temperatures below 7°C (Verma et al., 2006). In addition, the RH level at 15°C significantly influenced ($p<0.05$) anthracnose incidence, being higher (2.8%) for high RH compared to low RH (0%) (**Fig. 4.5B**). A high RH environment (>95%) has been reported as a favourable environment for anthracnose infection (Miles et al., 2013).

The incidence of grey mycelium in the stem scar (**Fig. 4.5C**) was molecularly identified as *Pestalotiopsis* spp.*, Colletotrichum* spp.*, B. cinerea, and Diaporthe* spp.*,* with an isolation frequency of 37%, 29%, 29%, and 7%, respectively (**Table 4.6**). Grey mycelium in the stem scar was observed most frequently on blueberries stored at 15°C in combination with high RH, with an average of 4.72% (**Fig. 4.5C**). These results are likely due to the variety of causal organisms able to produce decay under these storage temperatures. However, more interestingly, this decay was the most prevalent at low RH, which can restrict the development of pathogens, specifically *B. cinerea* and *Diaporthe* spp. (Díaz et al., 2017; Romanazzi & Droby, 2016). It is thought that at high RH, these organisms were able to produce more extensive visible decays, such as grey mould and white mycelium, instead of being restricted to the stem scar.

Green mycelium (**Fig. 4.5D**) was most prevalent at high RH during 15°C storage. This sign displayed a higher incidence at 15°C compared to 1°C, and this decay was favoured by the high humidity treatment at 15°C. Other decays (**Fig. 4.5E**) included all other fungal signs that were less than 5% of total decay. Other decay signs were not significantly different between treatments.

4.3.3 Decay Prevalence During Shelf Life

After storage, the accumulation of decay was recorded through a 7–day shelf life period at 20°C. The accumulation of decay over this period shows an increasing trend across all temperature and RH treatments. The average decay prevalence is displayed and indicates the accumulation of decay through the shelf life period (**Fig. 4.6**).

After 7 d of shelf life, the average prevalence of decay showed no significant effect between RH levels within each postharvest storage temperature. However, the 1°C storage treatment produced a more rapid increase in the prevalence of decay during shelf life compared to 15°C storage (**Fig. 4.6**). These data suggest that temperature has a greater impact on decay prevalence than humidity. The rate of increase after 1°C storage averages 2.7%/day^{-1} in high RH and 2.3%/day^{-1} in low RH. Whereas after 15°C storage, decay increases at an average rate of 0.8%/day^{-1} in high RH and 1.4%/day^{-1} in low RH.

The duration of 1°C storage was longer (6 weeks) than 15°C (2 weeks). It is possible that a longer storage duration at 1°C led to blueberries experiencing more advanced senescence than blueberries stored at 15°C, leading to a more rapid decline in fruit quality and a subsequent increase in host susceptibility upon removal from cold storage. Whether the advanced fruit senescence after storage for 6 weeks at 1°C compared to 2 weeks at 15°C contributes to the overall higher decay prevalence requires further investigation.

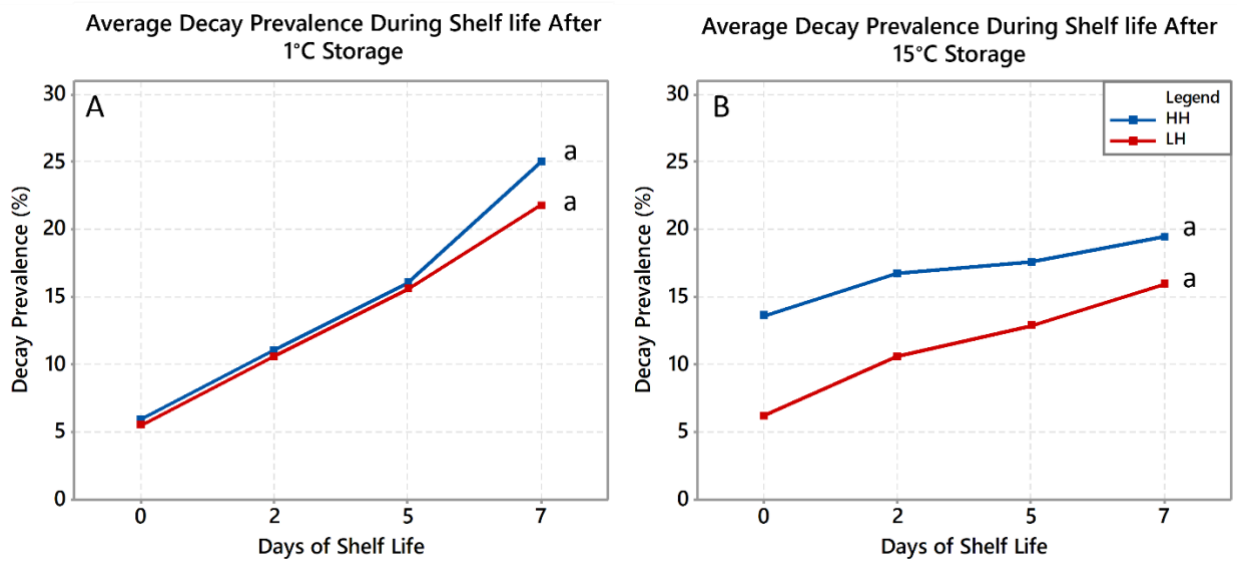


Figure 4.6. Average decay prevalence during shelf life after storage at 1°C for 6 weeks (**A**) or 15°C for 2 weeks (**B**). Means that do not share a letter are significantly different (P <0.05) within each panel. HH = high RH, LH = low RH.

4.3.3.1 Average Decay Prevalence During Shelf Life by Decay Sign

Expression of decay signs during shelf life (7 d 20°C) can be found in Appendix D. Significant differences occurred in grey mould and grey stem scar decay prevalence. Grey mould after 1°C storage at high RH produced a significantly higher ($p<0.05$) decay prevalence than low RH (**Fig. D1 A,G**). Grey stem scar prevalence was significantly higher after high RH 15°C storage than after 1°C storage (**Fig. D1 C,I**). The other treatments and decay signs did not produce any statistically significant differences.

The relatively high prevalence observed of grey mould after 1°C storage is the only significant trend occurring on day 7. This gave a mean of 11% within a range of 0–31%, comparatively higher than low RH with a mean of 1.6% with a range of 0–11%. Grey mould prevalence at high RH 7 days after removal from 1°C storage was also significantly greater than both RH treatments after 15°C storage. These had a mean of 2% for high RH and 0% for low RH, within the ranges 0–11% and 0–2%, respectively. This indicates that grey mould proliferation after storage is most rapid after 1°C storage and high RH conditions.

Anthracnose decay, caused by the *Colletotrichum acutatum* species complex, increased in prevalence throughout the shelf life of all RH treatments. The day 7 decay prevalence values were not statistically different ($p<0.05$). Interestingly, the rate of accumulation after 1°C storage was greater than the accumulation after 15°C storage. After 1°C storage, anthracnose accumulates gradually until day two, then begins to increase rapidly, reaching a peak of 2.5%/day^{-1} at high RH and 2%/day^{-1} at low RH. These data support the idea that *C. acutatum* is dormant during 1°C storage and becomes active upon removal to 1°C, where dormant infections can develop and conidia germinate to cause infections. It is suggested that due to the delayed increase in decay prevalence observed for *C. acutatum* after 1°C storage (**Fig. D1 B**), infections take at least 2 days to cause anthracnose sign from dormancy at 1°C.

Grey stem scar produced a linear increase in prevalence after both temperature treatments. After 15°C storage, prevalence at high RH was significantly greater than both RH treatments after 1°C. The rate of decay accumulation was 0.9%/day for 15°C high RH, 0.8%/day for 15°C low RH, 0.6% for 1°C high RH, and 0.6%/day for 1°C low RH. These data indicate that 15°C is slightly more favourable to the organisms responsible for this decay than 1°C, and that RH has little effect on decay prevalence through shelf life.

Green mycelium and other decays increased through shelf life without significant differences between humidity or temperature.

4.3.4 Fungi Associated with Decay Sign

From **Table 4.6**, it is observed that a variety of microorganisms are associated with each decay sign, except for the well documented grey mould and anthracnose signs. The microorganisms identified for the grey stem scar sign are numerous, indicating a wide array of species associated with this decay sign. Importantly, the common pathogens *B. cinerea* and *C. acutatum* species complex are aligned with this decay sign,

alongside the pathogenic *Diaporthe* spp. and largely non–pathogenic *Pestalotiopsis* spp. (Maharachchikumbura et al., 2014; Yu et al., 2018).

Table 4.6. Decay signs as percent of total decay and the composition of decay by fungi identified in Ch. 3 as associated with decay sign observed. Unidentified groups are characterised by plate group. Plate groups are described in Appendix A. Identifications of decay signs and plate groups as microorganisms can be found in Appendix C.

Decay Sign	% Of Total Decay	Microorganism Associated with Decay	% Microorganism Isolated from Decay Sign
Grey Mould	18.04	*Botrytis cinerea*	100.00
Anthracnose	15.35	*Colletotrichum acutatum* spp. complex	100.00
Grey Mycelium in Stem Scar	40.25	*Pestalotiopsis* spp.	37.21
		Botrytis cinerea	29.07
		Colletotrichum acutatum spp. complex	19.77
		Diaporthe spp.	6.98
		Colletotrichum salicis	6.98
Green Mycelium	9.81	*Botrytis cinerea*	50.00
		Pestalotiopsis spp.	33.33
		Colletotrichum acutatum spp. complex	16.67
White Mycelium in Stem Scar	4.06	*Botrytis cinerea*	42.86
		Neofusicoccum spp.	23.81
		Pestalotiopsis spp.	19.05
		Epicoccum nigrum	14.29
Grey Mycelium in Stem Scar and Anthracnose	3.83	*Colletotrichum acutatum* spp. complex	66.67
		Unidentified (15b)	16.67
		Pestalotiopsis spp.	8.33
		Botrytis cinerea	8.33
Fluffy White Mycelium	2.37	*Pestalotiopsis* spp.	42.11
		Botrytis cinerea	21.05
		Colletotrichum acutatum spp. complex	21.05

		Diaporthe spp.	15.79
Thin White Threads of Mycelium	1.35	*Pestalotiopsis* spp.	42.86
		Botrytis cinerea	28.57
		Unidentified (1b)	28.57
Dense Orange/Brown Mycelium	1.13	*Epicoccum* spp.	40.00
		Pestalotiopsis spp.	40.00
		Diaporthe spp.	20.00
Bright Blue/Green Mycelium	0.56	*Penicillium expansum*	40.00
		Unidentified (8c)	40.00
		Other	20.00
Other	3.25	-	-

The microorganisms are isolated at varying frequencies from numerous decay signs, indicating the prevalence of different microorganisms within the fruit microbiome and their potential involvement in multiple decay signs. Grey mould and anthracnose were strongly associated with their causal agents, *B. cinerea* and the *C. acutatum* species complex respectively. Green mycelium was associated mostly with *B. cinerea* and *Pestalotiopsis* spp., which is not typical for this decay sign (Wharton, 2003). The other decay signs observed were a mix of these microorganism, with the association of *Epicoccum nigrum*, *Neofusicoccum* spp*.,* and *Penicillium expansum* also found. This indicates further diversity of fungi present from the infrequently observed decay signs of blueberry fruit.

These results show that *B. cinerea*, the *C. acutatum* spp. complex, and *Pestalotiopsis* spp. are frequently present on decaying blueberry fruit, even when the decay signs are not characteristic of each organism. This indicates the presence of conidia of these microorganisms on fruit without the causation of characteristic signs or symptoms.

4.3.5 Impact of Storage Treatments on Decay Associated Microorganisms

Through molecular and morphological analysis detailed in Chapter 3, the identified microorganisms were associated with the decay signs they were isolated from. Isolates occurring as less than 1% of a decay sign

were regarded as contamination errors. If a decay sign was associated with a microorganism over 80% of the time and the relationship was well documented in the literature, the microorganism was deemed to be a causal agent (e.g., grey mould caused by *B. cinerea* (Williamson et al., 2007)). Hence, 100% of the decay sign was associated with the microorganism.

Table 4.7. 1°C high RH decay sign prevalence and associated microorganisms with frequency of microorganism isolated.

1°C High RH	Prevalence of Decay Sign (%)	Associated Microorganism	% of Decay Sign Associated with Microorganism[a]
Grey Mould	44.81	*Botrytis cinerea*	100.00
Grey Mycelium in Stem Scar	23.33	*Pestalotiopsis* spp.	47.37
		Colletotrichum acutatum spp. complex	21.05
		Botrytis cinerea	10.53
		Diaporthe nobilis	10.53
		Other	10.53
Anthracnose	14.81	*Colletotrichum acutatum* spp. complex	100.00
Green Mycelium	6.30	*Neofusicoccum* sp.	50.00
		Botrytis cinerea	25.00
		Colletotrichum acutatum spp. complex	25.00
Other	11.11		

[a] Summation of associated microorganisms represents 100% of prevalence of decay sign within this treatment.

After 1°C storage and a 7 d shelf life at 20°C, the high RH treatment is dominated by the grey mould sign, caused by *Botrytis cinerea*, with 44.8% of the total decay (**Table 4.7**). Also, the grey mycelium in the stem scar sign was shown to be related to different causal organisms, including *Pestalotiopsis spp., Colletotrichum acutatum* spp. complex*, B. cinerea, Diaporthe nobilis*, and others. Also present in this treatment after storage and shelf life was anthracnose caused by the *Colletotrichum acutatum* spp. complex. Green mycelium was observed less than 10% of the time and was related to *Neofusicoccum* sp., *B. cinerea*, and the *C. acutatum* spp. complex.

Table 4.8. 1°C low RH decay sign prevalence and associated microorganisms with frequency of microorganism isolated.

1°C Low RH	Prevalence of Decay Sign (%)	Associated Microorganism	% of Decay Sign Associated with Microorganism[a]
Grey Mycelium in Stem Scar	68.03	*Botrytis cinerea*	51.61
		Colletotrichum acutatum spp. complex	9.68
		Pestalotiopsis spp.	6.45
		Diaporthe spp.	6.45
		Neofusicoccum sp.	6.45
		Epicoccum nigrum	6.45
		Other	12.90
Green Mycelium	11.48	*Botrytis cinerea*	44.44
		Pestalotiopsis spp.	22.22
		Colletotrichum acutatum spp. complex	11.11
		Other	22.22
Grey Mould	4.10	*Botrytis cinerea*	100.00
Anthracnose	4.10	*Colletotrichum acutatum* spp. complex	100.00
Other	15.57		

[a] Summation of associated microorganisms represents 100% of prevalence of decay sign within this treatment.

Low RH and 1°C storage produced different prevalences of decay signs and associations with microorganisms compared to high RH and 1°C storage (**Table 4.8**). Grey stem scar was the most dominant decay sign and was mostly associated with *B. cinerea*, with 51% of this decay identified as *B. cinerea*. Other microorganisms were observed at a much lower rate and were not recorded more than 10% of the time. Green mycelium was also associated at a high rate with *B. cinerea*. Interestingly, the characteristic *B. cinerea* decay sign – grey mould, was not observed frequently, accounting for only 4% of decay in this treatment. These results suggest that *B. cinerea* causes grey stem scar at low RH rather than grey mould. It also suggests that *B. cinerea*, when restricted to the stem scar environment at low RH and after 6 weeks of 1°C storage, outcompetes other microorganisms for host resources and decay expression. Anthracnose was also rarely observed in this treatment due to the inhibition of the *C. acutatum* spp. complex below 7°C and low RH limiting decay expression (Miles et al., 2013).

Table 4.9. 15°C high RH decay prevalence and associated microorganisms with frequency of microorganism isolated.

15°C High RH	Prevalence of Decay Sign (%)	Associated Microorganism	% of Decay Sign Associated with Microorganism[a]
Grey Mycelium in Stem Scar	37.46	*Pestalotiopsis* spp.	38.00
		Colletotrichum acutatum spp. complex	10.00
		Diaporthe spp.	8.00
		Botrytis cinerea	5.00
		Other	39.00
Anthracnose	23.53	*Colletotrichum acutatum* spp. complex	100.00
Green Mycelium	11.46	*Botrytis cinerea*	43.00
		Pestalotiopsis spp.	28.00
		Colletotrichum acutatum spp. complex	7.00
		Other	22.00
Grey Mould	10.22	*Botrytis cinerea*	100.00
Other	19.20		

[a] Summation of associated microorganisms represents 100% of prevalence of decay sign within this treatment.

After 15°C storage, high RH resulted in grey stem scar being the dominant decay sign (**Table 4.9**). The composition was similar to that obtained in the 1°C high humidity treatment, with *Pestalotiopsis* species being dominant alongside the *C. acutatum* spp. complex, *Diaporthe*, and *B. cinerea*. However, 39% of this decay was associated with other microorganisms, including *Epicoccum nigrum*, *Neofusicoccum* sp., *Pithomyces chartum*, and *Neopestalotiopsis clavispora*.

Anthracnose was also relatively prevalent in this treatment, with 23% of decay associated with the *C. acutatum* spp. complex. Green mycelium decay occurred at a typical rate for this decay of 11%; this was associated mostly with *B. cinerea*, but *Pestalotiopsis* and the *C. acutatum* spp. complex were also isolated. Grey mould occurred as 10% of decay, which is much less frequent than after 1°C storage but more frequent than low RH after 15°C storage.

Table 4.10. 15°C low RH decay prevalence and associated microorganisms with frequency of microorganism isolated.

15°C Low RH	Prevalence of Decay Sign (%)	Associated Microorganism	% of Decay Sign Associated with Microorganism[a]
Grey Mycelium in Stem Scar	55.23	*Colletotrichum acutatum* spp. complex	32.00
		Pestalotiopsis spp.	29.00
		Botrytis cinerea	17.00
		Epicoccum nigrum	3.00
		Neofusicoccum sp.	3.00
		Other	16.00
Anthracnose	12.79	*Colletotrichum acutatum* spp. complex	100.00
Green Mycelium	11.05	*Botrytis cinerea*	50.00
		Other	50.00
Grey Mould	0.58	*Botrytis cinerea*	100.00
Other	20.35		

[a] Summation of associated microorganisms represents 100% of prevalence of decay sign within this treatment.

The low RH treatment and 15°C storage produced a majority of grey stem scar decay, similar to the 15°C low RH treatment (**Table 4.10**). However, the species associated with this decay were varied; the *C. acutatum* spp. complex was the most commonly isolated microorganism, with *Pestalotiopsis* spp. and *B. cinerea* also present. These results suggest that during 15°C storage, the *C. acutatum* spp. complex is the dominant pathogen in the stem scar of blueberry fruit producing grey stem scar decay. At 1°C, this microorganism is dormant; hence, *B. cinerea* is more common. It appears these three organisms are the major competitors associated with stem scar decay, and temperature conditions select which species become more dominant.

Anthracnose occurred in 12% of the decays associated with this treatment, which is less than high RH storage but more than in the fruit stored at 1°C and low RH. This result suggests that the *C. acutatum* spp. complex is a prevalent microorganism associated with the decay of blueberry fruit at 15°C. Green mycelium was also observed to occur in blueberries stored at low RH and 15°C, and it is dominated by *B. cinerea* and other microorganisms.

Interestingly, grey mould was very rarely observed in this treatment, comprising 0.58% of decay. This is indicative of the effect of low RH to limit grey mould sign. Other decays were 20% of decay in this treatment, the highest observed through this experiment.

4.3.6 Prevalence of Microorganisms Isolated from Blueberry Fruit Decay

From the isolates obtained from blueberry fruit decays and the assignment of identified microorganisms to decay signs in Section 4.3.5, the overall prevalence of the common microorganisms was calculated. **Table 4.11** details these observations. There are distinct differences in the prevalence of major microorganisms between storage and RH treatments. At 1°C storage, *B. cinerea* is the dominant pathogen associated with decay, with over 50% for both RH treatments. Whereas at 15°C, decay is spread through the major species of *B. cinerea*, *C. acutatum* spp. complex, and *Pestalotiopsis* spp.

Table 4.11. Summary of microorganism composition of decay by storage and humidity treatment. Values are displayed as a percentage of total decay for each treatment. Decay prevalence is after 7 d shelf life at 20°C.

Microorganism	1°C High RH	1°C Low RH	15°C High RH	15°C Low RH
Botrytis cinerea	51.08 %	53.33 %	18.47 %	23.27 %
***Colletotrichum acutatum* species complex**	26.40 %	14.15 %	32.02 %	25.22 %
***Pestalotiopsis* spp.**	12.76 %	6.94 %	21.15 %	22.75 %
***Diaporthe* spp.**	2.46 %	5.21 %	3.79 %	1.09 %
***Neofusicoccum* sp.**	3.15 %	4.39 %	3.11 %	7.12 %
Other Fungi	4.15 %	15.98 %	21.46 %	20.54 %
Average Decay Prevalence After 7d at 20°C	25.00 %	19.44 %	21.76 %	15.93 %

Differences in humidity were more pronounced in the 1°C storage treatments. Both *C. acutatum* spp. complex and *Pestalotiopsis* spp. were more prevalent at high RH than low RH; this was also observed at 15°C for *C. acutatum* spp. complex but not for *Pestalotiopsis* spp.. The less frequently isolated *Diaporthe* spp. were more prevalent at 1°C and low RH than at 15°C; this is possibly due to the ability to develop

infection at 1°C, as shown on kiwifruit (Díaz et al., 2017). Also isolated at low frequency was *Neofusicoccum* sp., which was isolated at the highest rate at low RH and 15°C. Other microorganisms were more prevalent at 15°C and in the 1°C low RH treatment. These decays are likely more prevalent at 15°C due to this temperature allowing the growth of most fungal organisms.

These results indicate that *B. cinerea* and *C. acutatum* spp. complex are the major microorganisms associated with postharvest decay of blueberry fruit in NZ.

The microorganism most frequently isolated from decaying blueberry fruit was *B. cinerea,* with 38.41% of total decay being isolated as this species. The *Colletotrichum acutatum* spp. complex produced 28.39% of decay. *Pestalotiopsis* spp. was isolated from 22.04% of total decay; this is significant for an organism not commonly known as a postharvest pathogen. In contrast, *Diaporthe* spp. were only observed in 3.55% of decay. This is a known postharvest pathogen of fruit species including blueberries (Yu et al., 2018) and kiwifruit (Díaz et al., 2017), but to the authors knowledge, this is the first report of this organism being isolated from blueberry fruit in New Zealand. The other organisms totalled 4.81% of decayed blueberries; these included *Neofusicoccum* sp., *Epicoccum nigrum*, *Penicillium* sp., *Neopestalotiopsis clavispora*, and *Pithomyces chartum*.

These findings may indicate that the majority of decay found on blueberries sampled in New Zealand is linked to *B. cinerea, Colletotrichum acutatum* spp. complex, and *Pestalotiopsis* spp. Minor organisms such as *Diaporthe* spp., *Neofusicoccum*, *Epicoccum nigrum*, and *Penicillium* sp. were detected but are insignificant. Likely, these minor species cause decay very rarely, potentially when the major species are not present.

4.4 Discussion

4.4.1 Fruit Maturity and Quality

It is expected that the differences in maturity and quality observed at the initiation of the experiment support that this study generated blueberry pathological data that can be associated with a range of commercial scenarios within the NZ blueberry industry. Fruit maturity and quality at the beginning of the experiment could have been influenced by different pre and postharvest factors, including blueberry cultivar, orchard environment and practises, harvest maturity, and supply chain environmental conditions and duration

(Retamales & Hancock, 2018). These factors vary between growing operations and through the supply chain and likely affected the quality of the fruit used for this experiment. However, cultivar is expected to have had a major effect on the quality and maturity values obtained in this experiment. Considering that the data was collected between the months of February and April in NZ, it is expected that the fruit collected included a variety of both Highbush and Rabbiteye cultivars.

Blueberry cultivars vary in TSS, acidity, and TSS : acidity to a significant extent within Northern and Southern Highbush and Rabbiteye cultivars (Gündüz et al., 2015). Fruit firmness is shown to vary at commercial harvest between cultivars, as observed by Hancock et al. (2008) for Highbush and NeSmith et al. (2005) for Rabbiteye. These differences in quality can have an impact on decay prevalence, as higher fruit firmness provides greater resistance to decay (Mehra et al., 2013). While TSS : acidity ratios greater than 32 have poor storage performance and a higher rate of decay (Galletta et al., 1971; Retamales & Hancock, 2018). It is expected that cultivar was the major determinant of the variation in TSS, acidity, and firmness, but other factors may also have contributed to variation.

Other factors potentially involved in fruit quality and maturity variation are harvest maturity and duration of the supply chain. Harvest maturity is defined as 100% blue surface skin colour of the berry (Beaudry, 1992). Once blueberry fruit reach this colouration, ripening on the plant continues through sugar accumulation (from photosynthesis), acidity reduction due to metabolization and softening caused by cell wall degradation (Moggia et al., 2018). Once the fruit is harvested, sugar accumulation stops, but acidity decline and softening continue regardless of whether the fruit remains attached to the bush.

It has been previously suggested that firmer fruit have a lower rate of decay from both hand and machine harvested fruit (Mehra et al., 2013). This was observed in the present study, but the correlation was very weak, as indicated in **Fig. 4.7**. Thus, this does not confirm this finding for the fruit used in this study. However, it is possible that the firmness evaluation technique used in the present study compared to Mehra et al. (2013) led to an incomparable measurement of firmness. The measurement of blueberry firmness depends on the characteristics aiming to be determined; thus, no standard method exists (Rivera et al., 2022a). However, low firmness is expected to promote decay due to weakening of the constitutive defences, mainly the cell wall, which reduces host resistance to decay (Liu et al., 2019; Malinovsky et al., 2014). Further work is required to elucidate the relationship between the various blueberry firmness parameters and the prevalence of decay.

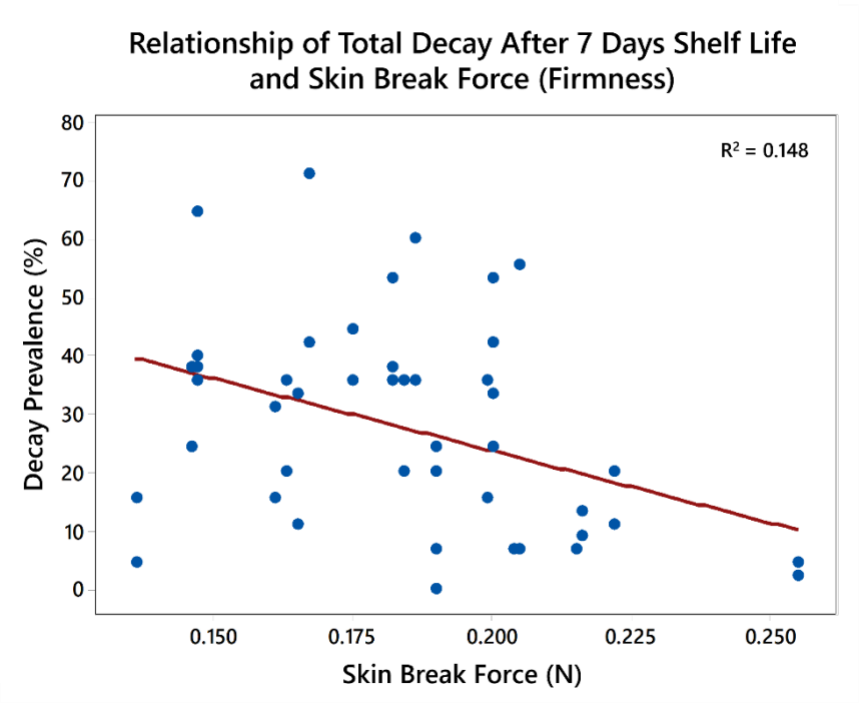


Figure 4.7. Relationship between total decay prevalence after 7 d shelf life and skin break force (firmness) for blueberry fruit. No relationship was observed through linear regression analysis (R^2 = 0.148).

Given that fruit maturation varies within blueberry bushes at different times, this can lead to a range of maturities within harvested fruit. It was observed by Moggia et al. (2018) that 100% blue fruit left unharvested for 5 days had an 18.5% lower firmness than newly 100% blue fruit for cv. 'Duke', and a 15.4% lower firmness for cv. 'Brigitta'. As overripe fruit are more susceptible to decay due to lower firmness and acidity, this is an important consideration for this experiment (Retamales & Hancock, 2018). Given that blueberry fruit clusters ripen unevenly, it is possible that fruit reaching 100% blue at different times were included in this experiment due to growers waiting for blue fruit to accumulate before harvesting. This may account for some of the differences in quality and maturity observed.

To improve the reliability and consistency of fruit sourcing and minimise variation in quality and maturity the above factors should be controlled. Directly harvesting fruit, controlling the postharvest supply chain, and using known cultivars would have minimised the variation observed and enabled relationships to be drawn between the known factors and decay sign prevalence.

The experiment conducted utilised fruit existing within the supply chain, so this experiment is a representation of conditions within the commercial supply chain. This variance enables the application of these results to the wider blueberry industry.

4.4.2 Decay Prevalence During Storage

The cold storage (1°C) conditions of low and high RH for a six-week duration in this experiment was an ideal experimental design for the expression of a range of decay signs and symptoms. The mean decay prevalence achieved in this experiment at 1°C was 5.9% for high RH and 5.5% for low RH. These align with the decay values obtained by Paniagua et al. (2014), who observed 6% decay after six weeks for cv. 'Maru' and 3.5% decay for cv. 'Brigitta' at 1°C and 90% RH in air. Similarly, 2°C storage for three weeks resulted in a decay incidence of 5.72% for blueberries 'Farthing' and 4.49% for 'Sweetcrisp' (Mehra et al., 2013). A New Zealand study by Schotsmans et al. (2007) using 1.5°C storage for 28 days observed 8% decay for 'Centurion' and 5% for 'Maru'. These studies indicate that the decay prevalence data obtained in the present study at 1°C are within the range of previously reported results.

Data from Harb and Streif (2004) indicates a 0.7% decay prevalence after storage for five weeks at 0–2°C with ambient RH in air, increasing to 8.5% after seven weeks. This indicates the decay prevalence increases as storage time progresses, which is consistent with other blueberry studies. Also considered is the temperature and the decay extent. Storage at 1°C for six weeks produced significantly less decayed fruit compared to 4°C in cv. 'Brigitta' and 'Maru' (Paniagua, 2012). While at 5°C, Smittle and Miller (1988) found that six weeks of storage resulted in 30% of blueberry 'Climax' fruit having decay. These data suggest that the 1°C treatment, measured as 1–2°C, produced comparable decay to other studies, and the temperature used led to decay similar to that observed at 1–2°C storage for six weeks.

The 15°C storage treatment used in the experimental design was set to stimulate maximum decay expression under more optimal conditions for fungal development potentially found within the blueberry retail stage of the supply chain (Miller et al., 1988). The decay prevalence of blueberries stored at 15°C was greater than that of blueberries stored at 1°C. This was expected due to the greater number of pathogenic and opportunistic fungal species able to cause decay at this higher temperature.

At 15°C, the decay prevalence observed averaged 13.6% in the high RH treatment and 6.2% in the low RH treatment. The high RH treatment was significantly greater than the 15°C low RH and 1°C treatments. This is an interesting finding as it demonstrates the greater propensity of fungal organisms to infect fruit under more suitable environmental conditions.

As 15°C storage is unlikely to occur during transportation and storage but may occur during shelf life in retail and in consumer possession, it is important to consider these results to ascertain the nature of decay at this temperature. As high RH had a significant effect on decay prevalence at this temperature, RH is a

potential control, but low RH is likely to cause blueberries to become commercially unacceptable due to weight loss, shrivel, and softening (Paniagua et al., 2013; Rivera et al., 2021). Therefore, to maintain fruit quality at 15°C, it is recommended to select an RH that will limit shrivel and weight loss and avoid extensive decay development all together. Whether a RH of 85–95% can be used for this purpose requires further investigation.

A key point of difference is that the fruit in this experiment were assessed in individual cups, which prevented fruit to fruit contact and subsequent transmission of disease. A study by Xiao and Saito (2016) using blueberry fruit in commercial clamshells at 0–2°C for five weeks produced averages of 25% and 43% decay prevalence in two consecutive years. The greater decay prevalence observed is possibly due to decay spreading through multiple fruit within clamshells. Compared to the current experiment, decay originating from one fruit is restricted to the subject fruit, and decay of multiple fruit by the same infection is avoided. This resulted in the comparatively lower rates of decay observed in the present study. This is especially relevant in the postharvest handling and storage environment.

In the postharvest environment, pathogens can be inoculated through physical contact with decaying fruit or contaminated surfaces. This is particularly important with *Colletotrichum* spp., which produce orange-coloured acervuli on infected fruit. These liquid droplets of conidia can be deposited onto any surfaces infected fruit come into contact with, contaminating the surface and inoculating subsequent fruit that contact the surface (Cline, 1996). This can rapidly increase pathogen presence within a population of blueberry fruit in the postharvest environment.

Another source of pathogen inoculum postharvest is fruit-to-fruit contact with decaying fruit. This offers the potential for decay to spread from infected fruit to sound fruit. This is of greater concern when fruit are stored in bulk, where the chance of contamination is higher than with smaller volumes of fruit. Also of concern is the ability of conidia to be spread from sporulating infections through wind currents, which may be enhanced if a pre-cooling process is used (Jarvis, 1962). This has been observed in kiwifruit, with a higher *B. cinerea* decay incidence in precooled fruit (Lallu & Webb, 1995). However, the extent of this issue has not been investigated for blueberry fruit.

4.4.3 Decay Prevalence During Shelf Life

The shelf life period (7 d at 20°C) was used to gain insights into the prevalence of decay after cold storage in a retail or consumer setting, and as expected, decay prevalence increased throughout shelf life. The effect of cold storage to suppress decay development was clearly shown in the data presented (**Fig. 4.4**).

With all decays suppressed through this period except decays linked to *B. cinerea* or *Diaporthe* spp., which have been previously reported to grow and develop infections at temperatures as low as 0–1°C (Díaz et al., 2014; Droby & Lichter, 2007). Upon removal from the cold storage (1°C) treatments, decay resulting from these organisms as well as numerous other organisms proliferated. This agrees with previously reported findings that 20°C is a suitable temperature for the expression of decay signs linked to numerous pathogenic and opportunistic organisms in blueberry.

After 1°C storage, there was a linear increase in the decay prevalence recorded through the shelf life period (**Fig. 4.6A**). However, the decay prevalence also showed a linear increase during shelf life for blueberries stored at 15°C (**Fig. 4.6B**). The author suggests that numerous factors are at play here:

1. The activation of quiescent infections present within the fruit before shelf life. Quiescent infections may be made dormant by cold storage conditions and become active when the fruit is exposed to shelf life temperatures. This leads to the rapid increase in decay prevalence shown in **Fig 4.6A** and as shown by Miceli et al. (1999) in table grapes.
2. Blueberry fruit senescence increases fruit susceptibility to rots (Rivera et al., 2013). The duration of the 1°C storage treatment was 6 weeks, compared to 2 weeks for 15°C. The longer duration of the 1°C treatment is hypothesised to have led to weakened host resistance to a greater extent than during the 15°C treatment due to the continuation of senescence. An evaluation of fruit quality and maturity (TSS, acidity, and firmness) upon removal from the storage treatments would confirm this idea. The weakened host resistance after 6 weeks at 1°C is thought to have been seized upon by pathogens during shelf life to cause an enhanced rate of decay accumulation after 1°C compared to 15°C. Alternatively, 1°C storage has previously been reported to induce physiological disorders associated with chilling injury in different horticultural products, including kiwifruit (Lallu, 1995), apples (Bramlage, 1982), and peaches (Lurie & Crisosto, 2005). However, chilling injury has not previously been reported in blueberries.
3. The composition of decay after 1°C storage was dominated by *B. cinerea* related grey mould and grey stem scar decays. It is well known that this pathogen can cause decay during 1°C storage (Droby & Lichter, 2007). This combined with the long duration of the storage treatment is suggested to have provided *B. cinerea* with an advantage over the other pathogens as it was able to infect fruit throughout cold storage with very limited competition from other microorganisms. When other pathogens became active during shelf life, *B. cinerea* was in a superior position to cause decay symptoms due to its activity during 1°C storage. As *B. cinerea* is an effective pathogen at this temperature, it was able to cause a greater frequency of grey mould and grey stem scar decays.

In comparison to other studies investigating decay prevalence through shelf life at 20°C, the observed increases in decay prevalence were greater in the present study than in other studies. This experiment observed a decay increase rate of 2.7% per day during shelf life of fruit stored for six weeks at 1°C and high

RH (98–100%). This was the greatest rate of increase observed and is considerably more rapid than that observed by Lang and Tao (1992), who achieved an increase of 0.43% per day during shelf life at 21°C for 4 days for cv. 'Sharpblue' after 7 days storage at 2°C. Another study by Miller et al. (1988) obtained a rate of increase of 0.5% for cv. 'Bonita' during shelf life of 3 days at 16°C after 21 days at 1°C. Also in this study, decay accumulation of cv. 'Bonita' was 1.8% per day during shelf life for 3 days at 21°C after 1°C storage for 21 days. This last study is comparable to the results obtained in the present study.

The fact that the decay accumulation after cold storage in this experiment was substantially greater than that observed in similar studies is possibly due to the longer storage period used in this experiment. This enabled the senescence process and active infections to progress to a greater extent during cold storage, leading to a more rapid decay accumulation on removal due to weakened host resistance. Both humidity treatments used in 1°C storage for this experiment produced greater decay accumulation during shelf life than the studies mentioned previously. It is then suggested that RH conditions do not have a significant effect on decay prevalence and that storage temperature and duration are the main determinants of decay accumulation.

In comparison to the 6–week at 1°C storage, the 2–weeks at 15°C storage showed a lower rate of decay, with 0.8% per day in high RH and 1.4% per day in low RH. These results may be explained by the effect of storage duration on host resistance and the competition between microorganisms to cause decay. Given that 15°C does not inhibit important decay causing microorganisms, infections were able to develop unconstrained by low temperature inhibition. This resulted in decay continuing with a linear trend during shelf life, as no lag phase caused by low temperature inhibition was present. Another possibility for the lower decay accumulation rate after 15°C storage is competition between the major decay causing organisms *B. cinerea* and the *C. acutatum* spp. complex, and the associated *Pestalotiopsis spp.* There is a possibility for competition between these decays for dominance over fruit tissue and key infection sites such as the stem scar, which may restrict their pathogenicity. In addition, it is also possible that interactions with other organisms, including *Pestalotiopsis* spp., led to different decay signs.

4.4.4 Effect of Relative Humidity on Decay

The data obtained in this experiment suggests that RH has no statistically significant effect on decay prevalence during storage at 1°C and during shelf life. This is a key finding as it indicates that overall decay frequency does not differ in relation to RH during 1°C storage and subsequent shelf life at 20°C. Within a commercial setting, it is important to consider the other effects of RH on blueberry fruit, including weight

loss, shrivel, and softening. A weight loss of 8% is reported by Paniagua et al. (2013) to be the maximum weight loss before excessive softening occurs. In relation to RH conditions, a range of 89–97% RH was reported by Rivera et al. (2021) to meet this objective. From the current work, it is suggested that an upper limit of 97% may produce a lower grey mould prevalence than 99–100%, however, below 95% RH a limiting effect on extensive development of *B. cinerea* mycelium is present (Thomas et al., 1988). Therefore, an optimum range for blueberry postharvest storage to limit grey mould decay and weight loss is suggested as 89–95% RH.

An interesting side note is the effect duration of postharvest storage has on water loss, evaluated by Rivera et al. (2021) to reach 8% water loss after four weeks. Whether the RH could be used dynamically in the supply chain in relation to declining host resistance to decay remains to be studied. For example, an experimental design may consider beginning the storage period with the RH at the high end of the recommended range (95% RH) to limit weight loss. Then decreasing RH through storage as host resistance declines and decay susceptibility increases, arriving at the lower end of the range (89–90% RH) by the end of storage. The author hypothesises that this RH management could offer less weight loss of fruit and limit the spread of grey mould between fruit.

4.4.5 Variability of Decay Prevalence

There was a high degree of variability between fruit sources in the decay prevalence observed in both 1°C and 15°C storage. The decay prevalence of blueberries stored at 1°C and high RH ranges between 0 and 15.5%, with a CV of 90%. Similarly, blueberries stored at 1°C with low RH treatment showed a decay prevalence range of 0–20% and a CV of 106%. At 15°C, the decay prevalence variation was even greater, with a range of 0–53% and a CV of 100% for high RH and a range of 0–22% and a CV of 118% for low RH.

The variation observed in the decay prevalence is considerable and indicates that the prevalence of decay during storage is inconsistent; some fruit batches (weekly retail sources) express decay while others do not, regardless of postharvest conditions. Similar results were reported by Rivera et al. (2022b) after storage for 42 days at 4.6°C, reporting an average decay prevalence below 7.5% and very high CV values up to 200%. This agrees with the result that decay prevalence can vary greatly within stored blueberry fruit. Given that the sample size of this experiment was relatively large, with 4,320 fruit obtained from three growers, this data has good reliability and applicability to the industry as a whole. Thus, it is expected that in commercial storage, decay prevalence is similarly variable.

Decay prevalence variability aligns with changes in disease triangle factors. Pathogen pressure, environmental factors, and host resistance have the potential to vary throughout the supply chain and thus lead to variable decay prevalence. In this experiment, postharvest environmental conditions were controlled through the experimental design but pathogen pressure, the environmental conditions prior to the initiation of the experiment and host susceptibility were uncontrolled, which could have impacted decay prevalence variation.

4.4.5.1 Pathogen Pressure

Pathogen pressure refers to the abundance of pathogens and the pathogenicity of these microorganisms. The abundance of pathogens is affected by orchard practises and environmental conditions. The number of orchards sampled, the exact location of the orchards, and the growing conditions are unknown. Hence, it is uncertain what effects these had on the prevalence of decay. However, it is known from the literature that inoculum presence in the orchard and humid conditions increase the risk of postharvest decay (Elmer & Michailides, 2007). From this, it is suggested that pathogen abundance in the orchard environment and subsequently on fruit was variable between orchards and led to the variation in decay prevalence observed.

The pathogenicity of the microorganisms is also considered an important source of variability due to the inconsistency in decay causing organisms observed. The decay associated with *Pestalotiopsis* spp. was linked to multiple species within this genus, some being pathogenic and others not. This is important as *Pestalotiopsis* spp. was frequently isolated from other blueberry fruit decays, and it is uncertain what effect this organism had on the decay observed and any interactions with the major pathogenic species of *B. cinerea* and *C. acutatum* spp. complex. Microorganism pathogenicity is also affected by host resistance, with the quiescent period being concluded when host resistance declines to a threshold level.

4.4.5.2 Environmental Conditions Prior to the Initiation of the Experiment

Environmental factors implicated in the variability observed in decay prevalence include temperature and RH conditions prior to harvest. High RH is critical to the effective growth, sporulation, and attachment to fruit of the major pathogens (Droby & Lichter, 2007; Wharton & Schilder, 2008). Warm temperatures (≥20°C) also enable the infections to progress and spread at a greater rate (**Table 4.1**). Pre–harvest RH is expected to be a key source of this variability, as blueberry orchards in NZ use a range of structures to protect crops, from overhead netting to plastic polytunnels, which have effects on RH (Retamales & Hancock, 2018). Another factor is the varied location and local weather conditions of the orchards from which fruit were harvested. NZ has a varied temperate climate, and regions are subject to different levels

of rainfall and wind (Plant and Food Research, 2021). To overcome these variables, a suggested solution is to repeat the experiment over multiple years to control for field weather conditions. Alongside this, obtaining information on the structures used on the orchard and their effect on RH will provide clarity on the RH conditions occurring before harvest and at key points during the growing season. This will enable the use of targeted control measures during periods of high infection probability (Rivera et al., 2013).

Postharvest environmental factors are important for minimising the development of decay after harvest. Low temperature (0–2°C) storage is critical for the control of decay and inhibits the growth of some major decay causing organisms while slowing the rate of host senescence and subsequent pathogen susceptibility. Reaching cold storage temperature as soon as possible after harvest is also important for reducing decay prevalence during storage (Hudson & Tietjen, 1981). High RH (≥95%) promotes decay development, but it is recommended to use high RH to minimise shrivel, weight loss, and softening (Paniagua et al., 2013). As the nature of the postharvest temperature and RH conditions experienced by the fruit before the experiment are unknown because the fruit was obtained from the retail stage of the supply chain, this is treated as a source of variance.

4.4.5.3 Host Susceptibility

Host resistance was also not able to be quantified during the present experiment and may have contributed to the variation in decay prevalence observed. Host resistance is the inherent resistance to decay of the fruit and is linked to intrinsic properties such as epidermal thickness, firmness, phytochemical content, and physical attributes such as stem scar size (Elad & Evensen, 1995; Retamales & Hancock, 2018). These attributes differ between cultivars, with some being resistant to certain pathogens; e.g., cv. ‘Elliot’ is resistant to *C. acutatum* (Wharton & Schilder, 2008). As host resistance of blueberry fruit declines over time, the duration of the supply chain and cold storage period likely contributed to host susceptibility in the present experiment and thus decay prevalence. Specifically, cultivar will have played a role, as some cultivars perform better in cold storage than others, giving a range of storage potential from 4 to 8 weeks due to the host resistance properties above (Hancock et al., 2008). Cultivars with shorter storage potential and less host resistance decay faster than cultivars with greater host resistance and longer storage potential. Given the fruit used were sold on the local market, the storage duration was likely short, and host resistance decline was not a significant factor if cold chain integrity was maintained.

Also contributing to potential variation in host resistance decline is damage incurred through impact, compression, or vibration through the supply chain. Damaged fruit lose firmness faster than sound fruit, making them more susceptible to decay (Moggia et al., 2017b; Xu et al., 2021). As the firmness measured in this experiment was variable, it is possible that some fruit were damaged at some point in the supply

chain. However, as these fruit were deemed saleable, the damage, if any, passed quality control checks within the supply chain. Thus, these fruit are representative of the commercial supply chain.

Another host factor influencing decay prevalence is fruit maturity. TSS, acidity, and firmness impact decay incidence, according to the literature. High TSS and low acidity (TSS : acidity >32) favour decay development (Galletta et al., 1971; Retamales & Hancock, 2018). While low firmness promotes decay and is associated with more mature fruit (Moggia et al., 2018). These observations were not confirmed in the present study, likely because fruit was harvested at commercial maturity for each cultivar. The maturity and quality of the fruit used in this study was variable due to a range of unknown factors, including cultivar, maturity, and supply chain conditions and duration. The fact that the quality and maturity of the fruit before entering the experiment was not associated with an effect on decay prevalence after storage or during shelf life indicates that fruit quality and maturity did not predispose fruit to decay before entering storage. The changes in quality and maturity associated with senescence and occurring during storage and shelf life were not evaluated and may have affected decay prevalence.

Overall, variability in decay prevalence is a clear phenomenon that occurs in blueberry storage. The factors mentioned above point to potential sources of this variability in the supply chain. However, due to the seasonal nature of blueberry production, decay prevalence also varies from year to year. Differences in the microorganisms associated with decay and the prevalence of decay signs have been observed (Mehra et al., 2013; Xiao & Saito, 2016). Seasonal variation is generated by the differences in factors leading to variability, as discussed previously. Central to these is the impact of growing conditions on blueberry quality and pathogen presence in the orchard.

4.4.6 Decay Signs

4.4.6.1 Grey Mould

Grey mould decay is caused by *B. cinerea*. This decay was most prevalent in the 1°C high RH treatment, averaging 3.8% of fruit after storage. This treatment produced the greatest grey mould decay because of the ability of *B. cinerea* to effectively cause infection at 1°C, and high RH (98–100%) enabled the fine mycelium of grey mould to form (Droby & Lichter, 2007; Thomas et al., 1988). In this treatment, *B. cinerea* was the only major decay able to cause infection at 1°C apart from *Diaporthe* spp., which was isolated very rarely in this experiment. Hence, there was little competition from other organisms, resulting in grey mould decay being the dominant decay during high RH 1°C storage. This decay is of particular importance for the

blueberry industry due to its ability to develop during cold storage. The data gathered in this experiment suggest that this decay cannot be completely controlled through low temperature storage in the postharvest environment. Therefore, it is suggested to reduce pathogen presence by implementing control techniques prior to harvest.

Grey mould decay is limited by RH (<94% RH). Extensive grey mould decay develops at and above 94% RH; below this level, a denser mycelium forms, corresponding with the grey stem scar decays observed in the present study (Thomas et al., 1988). The high RH treatments at 1°C and 15°C resulted in the proliferation of grey mould decay. This agrees with the findings of Thomas et al. (1988) and Ciliberti et al. (2016), who observed more sporulation and more extensive mycelial development of *B. cinerea* at high RH (90–100 %RH).

B. cinerea is most likely inoculated before harvest during flowering or on mature fruit (Rivera et al., 2013). The resulting infections can remain dormant through fruit development and maturation to become pathogenic during postharvest storage. This presents a significant problem for blueberry quality as preharvest infections cannot be graded out before they cause decay, resulting in an inconsistent occurrence of postharvest infections, as demonstrated by the variability in this decay observed in the data from the present experiment (0–15.5% range). As *B. cinerea* is inoculated from environmental sources within orchards and is thus influenced by environmental conditions, it is important to consider the potential effect of the growing system on the prevalence of grey mould decay. High humidity growing conditions favour the development of *B. cinerea* in the orchard (Elmer & Michailides, 2007). As the nature of weather is variable, it is suggested that specific control measures be taken during periods of high RH and when fruit are more vulnerable to *B. cinerea* infection.

4.4.6.2 Anthracnose

Anthracnose decay is reported to be caused by the *C. acutatum* and *C. gloeosporidoes* spp. complexes (Cappellini et al., 1972; Smith et al., 1996). This experiment found only species within the *C. acutatum* spp. complex causing anthracnose, including *C. fioriniae*, *C. godetiae*, and *C. salicis.* Anthracnose decay occurred only during the 15°C high RH storage treatment but developed in all treatments during shelf life. This observation is due to the temperature requirement of ≥7°C for *C. acutatum* to grow (Verma et al., 2006). This was repeated in this experiment, with no anthracnose decay being observed at 1°C and subsequent rapid proliferation during shelf life at 20°C. This may be a result of anthracnose being inhibited during storage and breaking quiescence upon removal to shelf life at 20°C.

The *C. acutatum* spp. complex takes advantage of the deteriorated host resistance after 6 weeks cold storage, leading to rapid decay accumulation when the temperature is increased from 1°C to 20°C. Comparatively, after 15°C storage, anthracnose decay accumulates through storage and during shelf life at a slower rate than after 1°C storage. This is thought to be due to there not being a dormancy period enforced by low temperature, allowing decay to form as infections naturally progress. Therefore, anthracnose decay can be controlled by low temperature, as shown by this data and confirmed by Miles et al. (2013).

High RH (>95%) favours the development of anthracnose decay, as shown by the results obtained, with the high RH treatment producing more anthracnose decay irrespective of either storage temperature of 1°C or 15°C. This finding is confirmed by Miles et al. (2013), who found that increasing RH resulted in greater infection frequency by *C. acutatum* on mature blueberry fruit. However, low RH (≤84%) does not completely inhibit anthracnose decay; hence, low RH is not suggested as a reliable control measure (Miles et al., 2013).

There appears to be a competitive relationship between anthracnose and *B. cinerea* linked decays. In **Table 4.9**, the high RH at 15°C treatment produces more anthracnose than grey mould and grey stem scar decay, even when considering that this temperature and RH is considered optimal for decay expression from both species (*B. cinerea* and *C. acutatum* spp. complex). This finding suggests that the *C. acutatum* spp. complex infections were more advanced, aggressive, or frequent, leading to greater prevalence of anthracnose than grey mould. Conversely, when the *C. acutatum* spp. complex is unable to produce decay (i.e., at 1°C), *B. cinerea* becomes the dominant pathogen through storage and shelf life. A possible explanation is that the *C. acutatum* spp. complex is more prolific in the orchard and therefore is more present postharvest. Another idea is that the *C. acutatum* spp. complex can outcompete *B. cinerea* when in suitable conditions due to its hemibiotrophic lifestyle prior to causing anthracnose. This may allow the *C. acutatum* spp. complex to expand through the fruit without triggering host defence, and once ripening occurs, this microorganism becomes necrotrophic and causes anthracnose decay (Wharton & Schilder, 2008). This undetected infection of fruit tissue is not possible in *B. cinerea* which uses only necrotrophic or saprotrophic lifestyles, so it may remain quiescent until fruit senescence (Elad & Evensen, 1995). This fact may provide the *C. acutatum* spp. complex with an advantage over *B. cinerea* in causing decay at suitable temperatures in postharvest storage. Consequently, the *C. acutatum* spp. complex can be considered a more damaging microorganism in high RH storage above 7°C and when fruit is held at shelf life conditions.

The prevalence of anthracnose was lower than in comparable studies. Anthracnose was reported by Hancock et al. (2008) to infect 17–20% of fruit after 42 d storage at 2°C under modified atmosphere conditions. While Mehra et al. (2013) reported anthracnose incidence of 0.63–5.2% after 21 d storage at 2°C. Given that this study used a storage temperature within a range of 1–2°C, the occurrence of

anthracnose in this research is low in comparison to both previously reported studies. Hence, it is suggested that the cold storage temperature used in the present study had an inhibitory effect on anthracnose prevalence, which was not observed in the previously reported literature. However, other factors, including cultivar susceptibility and orchard factors, are likely to play a role in the greater occurrence of anthracnose.

4.4.6.3 Grey Stem Scar

Grey stem scar decay is an ambiguous decay caused by multiple microorganisms, mainly *B. cinerea, Pestalotiopsis* spp., the *C. acutatum* spp. complex, and *Diaporthe* spp. This decay occurred frequently in all treatments except 1°C high RH. Given the numerous species that are related to this decay, it is difficult to determine a consistent causal agent, especially when the organisms isolated vary by postharvest treatment (**Tables 4.7, 4.8, 4.9,** and **4.10**). Interestingly, *B. cinerea* is considerably more dominant at the 1°C low RH treatment. This shows that temperature and RH conditions have an impact on the microorganisms isolated from this decay.

The stem scar is the most susceptible area to postharvest decay (Cappellini & Ceponis, 1977). Given that it is a fresh wound with direct vascular links into the berry, this is a common entry point for microorganisms (Beaudry, 1992). Colonisation of the stem scar region occurs from organisms present epiphytically on surrounding tissue or endophytically in the stem scar. As the disease triangle factors are often fulfilled at the stem scar, decay is common, being up to 10–fold greater than fruit with attached stems (Cappellini & Ceponis, 1977). Due to the variable origins of decay causing organisms, it is possible that this is a major source of variation observed in the microorganisms obtained from grey stem scar decays in this study. The major species observed can grow endophytically within plant tissue, including the *C. acutatum* spp. complex during its hemibiotrophic phase and *Pestalotiopsis* spp. (De Silva et al., 2017; Maharachchikumbura et al., 2014). *B. cinerea* is not known to exist as an endophyte in blueberry fruit tissue, so it is likely inoculated onto the stem scar as conidia from adjacent tissue. The ability of weak pathogens to exist endophytically facilitates their infection of the stem scar; however, they are often outcompeted by more aggressive microorganisms, especially when temperature conditions favour certain organisms.

The nature of the stem scar is that it is surrounded by a raised surface, which offers some shelter from air currents, and the stem scar is initially moist following harvest. These attributes offer some protection to microorganisms from low RH conditions. This combination of attributes enables filamentous fungi such as *B. cinerea* to develop in this location under low RH conditions (Thomas et al., 1988). This was the result of the low RH treatment in this experiment, with grey stem scar decay being most prevalent at low RH conditions in both temperature storage treatments.

4.4.6.4 Green Mycelium

Green mycelium was observed infrequently and was related to *B. cinerea, C. acutatum* spp. complex, and *Pestalotiopsis* spp. This was a surprising result, as green mycelium is often related to *Alternaria* spp. (Zhu & Xiao, 2015). This result shows that microorganisms are often present when they are assumed not to be due to uncharacteristic decay signs, which reinforces the importance of molecular and morphological analysis of infected fruit to obtain accurate causal organism identification.

Previous studies have observed *Alternaria* spp. and *Cladosporium* spp. associated with blueberry fruit decay and producing a green mycelium (Mehra et al., 2013; Xiao & Saito, 2016). The present experiment in NZ did not observe these microorganisms. *Alternaria* spp. was likely not observed because it is commonly found in wounds, and wounded fruit were not used in this experiment (Troncoso-Rojas & Tiznado-Hernández, 2014). Another potential explanation is that *Alternaria* spp. is more prevalent early in the growing season and its incidence decreases as the season progresses (Cappellini et al., 1972; Schilder et al., 2000). As this experiment was conducted in the mid–late period of the blueberry season, this factor may have limited the presence of *Alternaria* spp. Additionally, *Cladosporium* spp. has been observed infrequently in other studies, so it could be expected to not be observed in the present study in NZ (Mehra et al., 2013; Xiao & Saito, 2016).

4.4.6.5 Other Decays

Other decays were observed more commonly after 15°C storage. This is expected as higher temperatures favour the growth and infection of a large number of potentially pathogenic fungi. The higher prevalence of these decays at high RH was also expected, as these conditions favour fungal development. Therefore, at these conditions, a proportion of other decays can be expected. Other decays were also present at 1°C, but at lower rates. The decays deemed as ‘other’ were associated with *Pestalotiopsis* spp., *Diaporthe* spp., *Epicoccum* sp., *Neofusicoccum* sp., *Penicillium* sp., and *Neopestalotiopsis* sp., and their prevalence varied between postharvest conditions.

4.5 Conclusion

This chapter confirms that temperature is the most important factor in limiting the postharvest decay of blueberry fruit. In addition, *B. cinerea* remains the most important pathogen of blueberry fruit, even when fruit is stored at 1°C. On the other hand, anthracnose caused by the *C. acutatum* spp. complex is more of a concern during shelf life at 20°C and when storage temperature is >7°C. Another key finding was that the decay sign caused by *B. cinerea* varied based on the RH conditions during storage. High RH (98-100%) produced a grey mould sign over the entire fruit, while low RH (80%) led to a grey mycelium in the stem scar only.

Low temperature storage at 1-2°C is effective in reducing infection and the expression of most pathogenic fungi for up to 42 days. However, the results of this experiment have shown that *B. cinerea* persists to cause infections even at low temperature (1°C) and is the dominant microorganism related to decay at low RH (80%) and high RH (98-100%). At 1°C, high RH did not have any effect on total decay prevalence but was shown to lead to more extensive grey mould decay development from *B. cinerea*. Based on these findings, RH is not suggested as an effective control measure for decay within the range of 80-100% RH. Further research considering a range of RH management strategies is suggested to elucidate the relationship between *B. cinerea* decays and other quality disorders, including weight loss and shrivel of blueberry fruit in commercial storage conditions.

The 14 d 15°C storage treatment resulted in a greater diversity of decay signs and fungal organisms isolated, including anthracnose caused by the *C. acutatum* spp. complex and decay related to *Pestalotiopsis* spp. The differences in decay signs and microorganisms isolated between storage temperatures indicate the importance of temperature for the expression of decay signs. Also of note was the variety of fungi isolated from the fruit, as shown in the inconsistent composition of grey stem scar and green mycelium decays.

Relative humidity is shown to have little effect on the overall prevalence of decay, more strongly influencing the decay signs expressed. High RH led to more decay after 15°C storage than low RH or 1°C storage, promoting the growth and infection of numerous fungal organisms, mainly the *C. acutatum* spp. complex and *Pestalotiopsis* spp.

The decay prevalence during shelf life (7 d at 20°C) was shown to be significantly affected by the temperature during storage (prior to shelf life), but not considerably by the RH used. It is suggested that

this is possibly the result of the enforced dormancy of some microorganisms (and slowing of metabolism) at 1°C. Following 1°C storage, decay increases much faster than after 15°C storage due to the breaking of this temperature enforced dormancy and the enhanced senescence of the fruit after 42 days of storage compared to 14 days at 15°C. This is an important consideration for the NZ blueberry export industry, as once fruit arrive in the market, cold chain integrity may be broken, which has been shown in this experiment to lead to considerable proliferation of decay.

From this work, it is suggested to minimise the duration of the export supply chain to reduce the progress of senescence. This could take the form of airfreight or more efficient oceanic transportation. Ensuring cold chain integrity in export destinations is also critical. Based on the results of this experiment, these strategies would reduce the prevalence of anthracnose in the supply chain, but *B. cinerea* would remain a risk due to its ability to infect fruit at cold storage temperatures (0-2°C). Another strategy is to limit the inoculum presence on exported fruit through preharvest controls, potentially taking the form of an integrated pest management approach.

Chapter 5: General Conclusions and Recommendations

The objectives of this thesis were met.

1. The microorganisms associated with postharvest decay of blueberry fruit were accurately determined using molecular analysis, with morphological techniques providing additional information. These results offer information on the major decays occurring after postharvest storage of NZ blueberry fruit, as well as minor and unexpected microorganisms. The identification of these microorganisms also resulted in two new associations of *C. fioriniae* and *C. godetiae* with anthracnose of blueberry fruit in NZ.
2. The prevalence of decay and microorganisms associated with decay during various temperature and RH storage and shelf life conditions was evaluated and resulted in some interesting and relevant observations. The confirmation of *B. cinerea* as the major microorganism affecting storage at 1°C is very relevant to the NZ blueberry industry. Also, the identification of the *C. acutatum* spp. complex as a major pathogen above 7°C is relevant for the retail and consumer stages of the supply chain.

This work has demonstrated that the prevalence of decay and the microorganism composition isolated from blueberry fruit decays varies by temperature and relative humidity. Temperature had the greatest effect on decay prevalence and the microorganisms causing and associated with decay, while RH was more closely related to the decay signs observed. The results confirm that blueberries stored at 1–2°C are less likely to show decay compared to those stored at higher temperatures (13–15°C), and relative humidity (RH) has little effect on overall decay prevalence. In relation to the microorganisms obtained from decaying fruit, there were differences between temperatures and RH treatments. However, the major organisms isolated after 7 days of shelf life were *B. cinerea*, *C. acutatum* spp. complex, and *Pestalotiopsis* spp. Of these, *Pestalotiopsis* spp. are considered endophytes or weak pathogens. Thus, *B. cinerea* and the *C. acutatum* spp. complex are the major microorganisms causing decay of blueberry fruit in NZ.

The fungal microflora of decaying blueberry fruit was shown to be very diverse. This is indicative of variation from preharvest and postharvest sources of inoculation. The microorganisms identified and associated with decay comprised both pathogenic and non–pathogenic species. This shows that a range of microorganisms are present on blueberry fruit. The results suggest that the range is greater at 15°C, with this study reporting more ‘other’ decay at 15°C. This was compounded by the dominance of *B. cinerea* at 1°C, indicating that temperature may be a strong influence on the microorganisms that can develop and the degree of

competition between microorganisms. Thus, during commercial storage at 0–2°C, *B. cinerea* is expected to be the dominant pathogen. Considering that blueberries are commercially stored within this temperature range, the results suggest that NZ control strategies should focus on grey mould (*B. cinerea*) to reduce fruit loss during the cold storage stage of the supply chain. However, once the fruit is exposed to shelf life conditions of 20°C (for example, in non-refrigerated retail displays or at the consumer's home), other fungal microorganisms and decays such as anthracnose (*Colletotrichum* spp.) proliferate rapidly. This is likely due to the increased host susceptibility and broken dormancy of the fungal infection.

During the storage period, RH did not influence the overall decay prevalence of blueberry fruit, but it did impact the decay signs and symptoms and, consequently, the causal microorganisms found. On one hand, suppression of fungal growth and decay incidence may not be achieved through commercial manipulation of storage RH, but the extent of decay can be limited with a lower RH (80%). This was a key finding of this work, as decays caused by *B. cinerea* differed by RH conditions, with grey mould decay occurring mainly under high RH (98-100%) and being restricted to the stem scar at low RH (80%). This is suggested to reduce the virulence of the pathogen by limiting mycelial growth and the opportunity for fruit to fruit transmission of infection. On the other hand, manipulation of storage RH is highly relevant for other fruit quality variables, such as weight loss, shrivel symptoms, and excessive softening (Paniagua et al., 2013; Rivera et al., 2021). Given the ability of microorganisms to produce decay under relatively low RH conditions (80%) and the higher weight loss expected at low RH, the RH suggested by Retamales and Hancock (2018) of 85–95% RH is recommended for storing blueberries. Alternatively, control efforts should instead focus on reducing inoculum presence and storing fruit under optimal low temperature conditions to slow senescence and reduce the number of microorganism species able to cause decay.

This work has highlighted several areas for potential further research. To improve the results of this experiment, data collection over several seasons would provide a more reliable picture of the microorganisms related to the decay of blueberry fruit. By using multiple growing seasons and consistent methods, variation from weather, orchard practises, and fruit quality and maturity could be accounted for. This would provide more universal decay prevalence data in relation to storage treatments. In addition, other improvements of the experimental design would be to better simulate commercial postharvest storage conditions by storing fruit within clamshell packaging and using other storage technologies such as modified atmosphere packaging, controlled atmosphere storage, or ozone. Furthermore, research into the effects of breaks in cold chain temperature control on the prevalence of decay would be of relevance to the blueberry industry.

The post storage or shelf life period used (20°C for 7 d) in the experimental design allowed evaluation of the rapid proliferation of decay after cold storage. Whether the same rapid proliferation occurs under

different shelf life conditions of temperature and time remains to be studied. For example, an experiment using a range of temperature conditions during the post storage period could be studied. In addition, a useful piece of data for this would be the quality status of the fruit post cold storage. This would determine the condition of the fruit after storage, enabling a more accurate picture of host resistance decline to be attained, and evaluation of consumer acceptability. This potential experiment would clearly indicate the outcomes of low temperature storage and the responses of blueberry fruit to various shelf life treatments.

The determination of the relationship between firmness and decay prevalence using a greater range of blueberry genotypes is a potential area for further work. This would build on the results obtained in this study and may offer interesting insights into the specific mechanical properties that impact blueberry susceptibility to decay. This could also produce more detailed relationships between fruit firmness and decay prevalence than the current study. Other factors affecting host susceptibility could also be studied, including skin properties, cell density, biochemical profile including phytoalexins, and the progress of senescence.

As the molecular identification used in this study was limited, it would be of interest to complete further identification of the microorganisms less frequently isolated from blueberry fruit. This could uncover evidence of unreported microorganisms associated with the decay of blueberry fruit and offer a more complete picture of the microorganisms present within the fruit microbiome. With these microorganisms, pathogenicity tests and Koch's postulates could be used to determine pathogenicity under various postharvest conditions. Also of interest is using a greater number of primers to enable more detailed molecular analysis, which would provide more reliable identification of species and the production of multi-gene phylogenetic trees.

Finally, the author of this thesis recommends maintaining a low temperature throughout the blueberry supply chain, with a focus on limiting *B. cinerea* inoculum present on fruit at harvest. In addition, control strategies for the *C. acutatum* spp. complex are also recommended to decrease the expression of anthracnose decay during the shelf life period.

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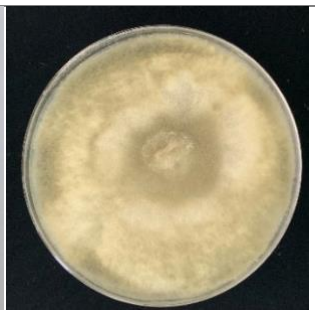
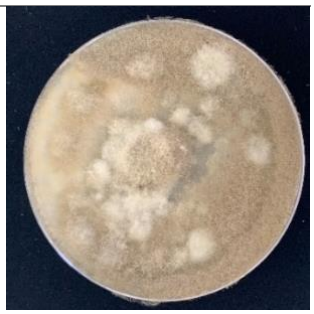
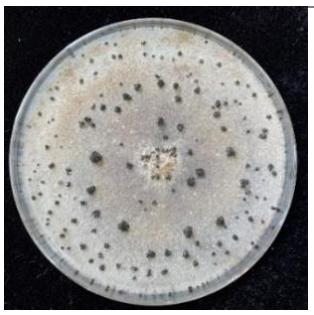
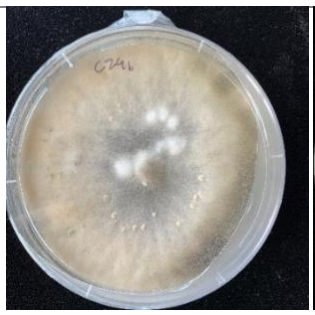
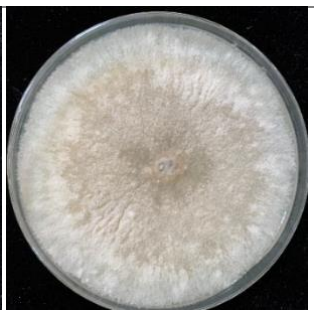
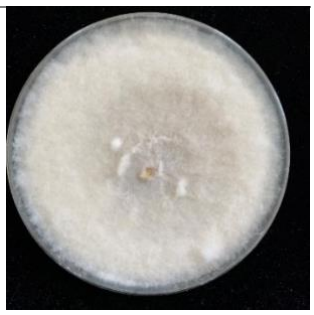
Appendices

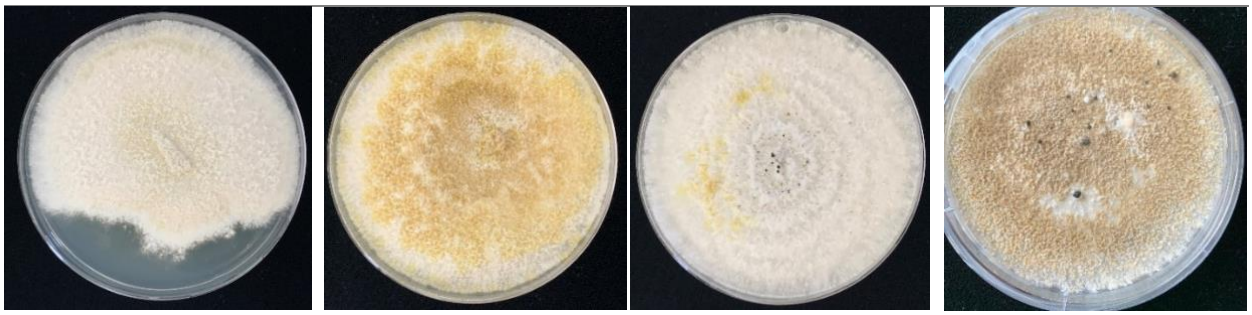
Appendix A: Macro-Morphological Analysis Key

Table A.1. Macromorphological analysis key.

1a	1b	2a	2b
White with black sclerotia	White mycelium	White base with grey/tan spore structures	White base with grey/tan spore structures and aerial white mycelium
			

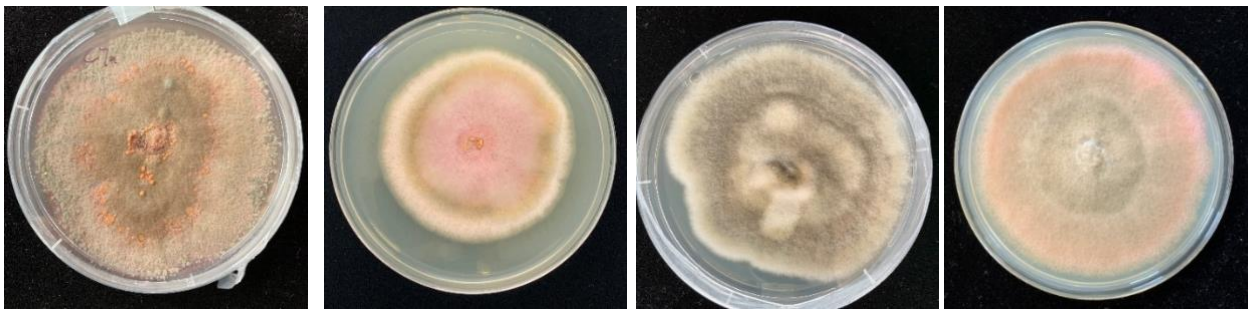
2c	2d	2e	2f
White base with grey/tan spore structures and sclerotia	With aerial white mycelium and sclerotia	Grey with white edge, no aerial mycelium	Fluffy white/grey mycelium, yellow liquid
			

3a	3b	3c	3d
White and yellow	White with intense yellow/gold	White and yellow with black fruiting bodies	White with intense yellow/gold and sclerotia

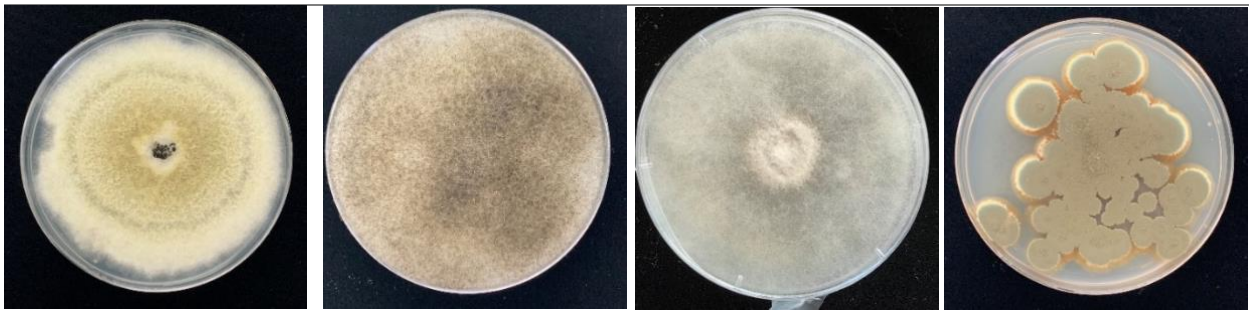


3e	3f	4a	4b
White mycelium with yellow and gold spots	White mycelium with concentric pattern	White aerial bubbles	Grey aerial bubbles

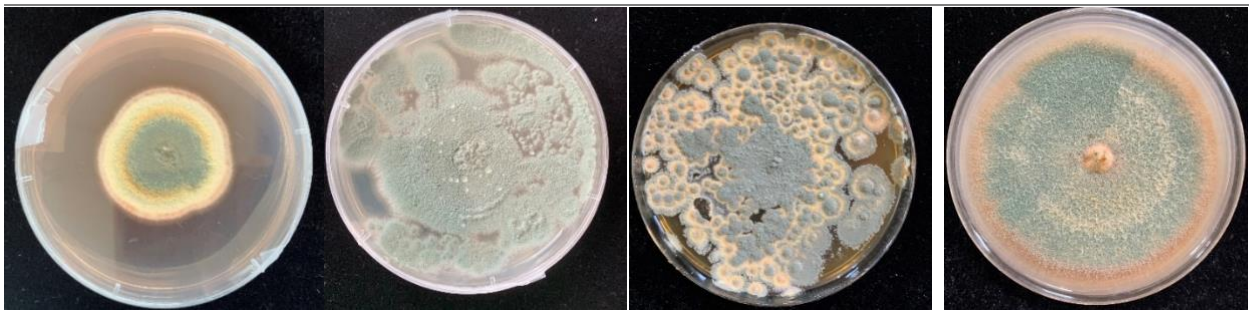
5a	5b	5c	5d
Grey/green with orange acervuli	Pink/green/white with orange acervuli	Dark grey/brown Without orange acervuli	Bright without orange acervuli



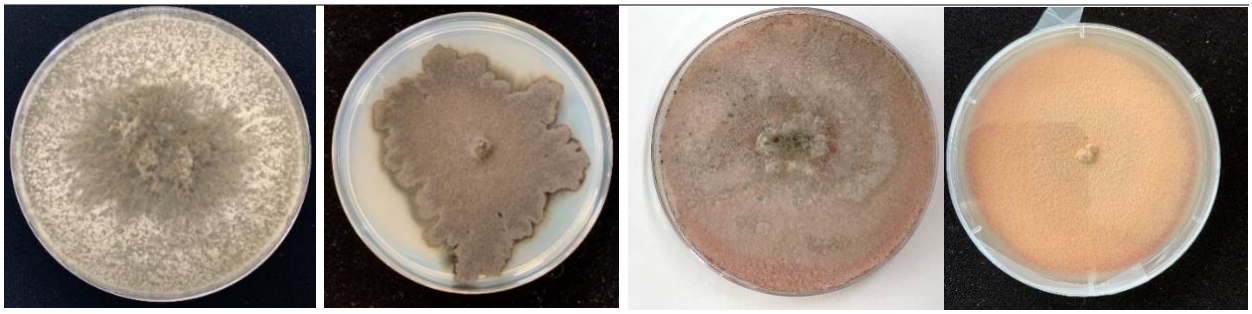
6a	7a	7b	8a
Light green to yellow with a darker ring	White mycelium with black spores interspersed	White mycelium without black spores interspersed	Green with white margins



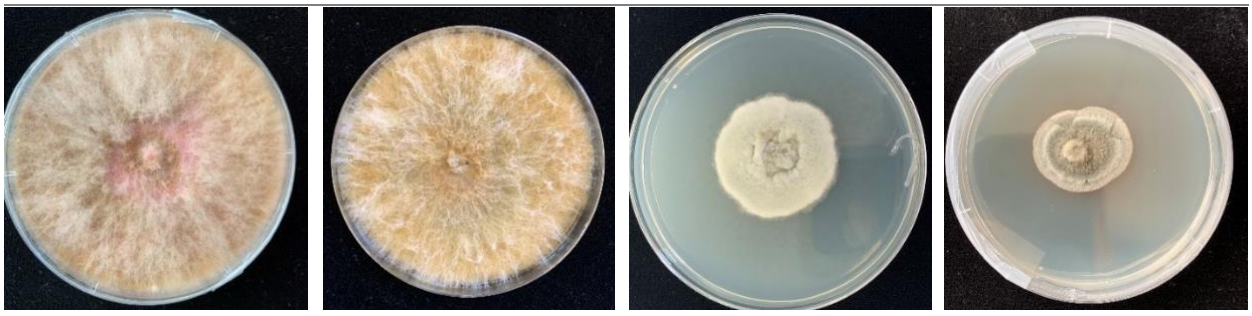
8b	8c	8d	8e
Blue with large white margin	Blue with white margins	Blue with yellow margins	Other bright colours



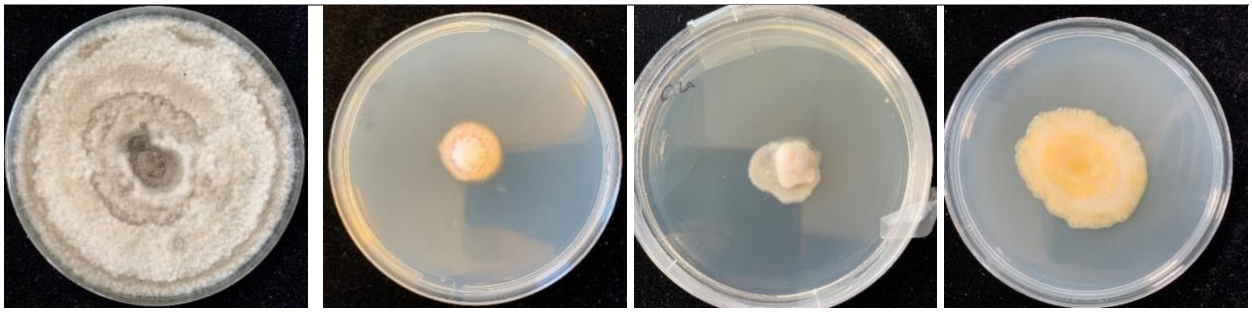
9a	9b	10a	10b
Dark grey	Dense dark grey	Pink with sclerotia	Other pink



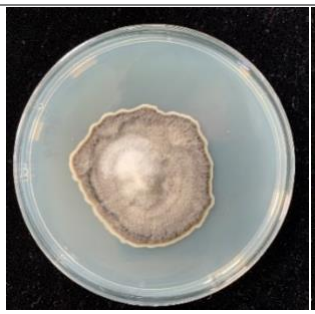
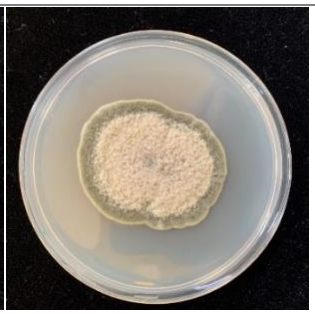
10c	10d	11a	11b
Fluffy pink	Fluffy yellow	Grey/green slow growing with white margin	Grey slow growing, segmented



11c	11d	12a	12b
Light grey, sunken centre	Other slow growing	White slime	Yellow slime



13a	14a	14b	15a
Orange/grey	Grey	Light grey	Green segmented

15b	16a	16b	17a
White segmented	Grey, slow growing, white margin	Grey, green slow growing	Irregular shape with purple/yellow rings
			

Appendix B: Sequencing Results

Internal Transcribed Spacer (ITS)

Table B.1. ITS sequencing results.

Plate Morphology	Isolate	Sequence Length	Max Score	Query Cover	Per. Ident	Top BLAST hit	GeneBank Accession no. of top hit
1a	11E23	295	545	0.00%	0.00%	Inconclusive	-
2a	12D39	475	878	100.00%	100.00%	*Botrytis cinerea*	MT5734790.1
2a	11B33a	530	957	99.00%	99.62%	*B. cinerea*	MN.589849.1
2a	17B35	0	0	0.00%	0.00%	Inconclusive	-
2b	15A25	530	699	98.00%	100.00%	*B. cinerea*	MN589852.1
2b	11A34	464	817	99.00%	98.92%	*B. cinerea*	MT150132.1
2b	16E19	568	1,048	99.00%	100.00%	*B. cinerea*	EF207413.1
2c	4C39	421	778	100.00%	100.00%	*B. cinerea*	MT573470.1
2c	16A7	514	950	100.00%	100.00%	*B. cinerea*	MN891765.1
2c	16B2	505	900	100.00%	99.01%	*B. cinerea*	MN589849.1
2e	11C36	0	0	0.00%	0.00%	Inconclusive	-
2f	3F37	338	625	100.00%	99.80%	*B. cinerea*	LC514949.1
3a	16D28a	630	1162	99.00%	100.00%	*Pestalotiopsis. disseminata*	HQ607992.1
3b	8A8	599	1107	100.00%	100.00%	*P. disseminata*	HQ607992.1
3d	5D38	630	1140	100.00%	99.37%	*Pestalotiopsis* sp.	KP689121.1

3d	6A25	576	1046	98.00%	100.00%	Fungal Endophyte sp.	EU686111.1
3e	3A3a	576	1055	99.00%	99.83%	*Diaporthe salicicola*	NR_137106.1
4a	16A18	517	955	100.00%	100.00%	*Diaporthe nobilis*	KT163359.1
5a	4A21	256	126	32.00%	92.86%	*Colletotrichum fioriniae*	MT254962.1
5b	3A20	510	941	99.00%	100.00%	*C. fioriniae*	MT466533.1
5c	11C1	563	1040	100.00%	100.00%	*C. fioriniae*	MT133292.1
5c	5F19	562	1031	99.00%	99.82%	*Colletotrichum godetiae*	MT133295.1
5c	5F30	516	941	100.00%	99.61%	*C. fioriniae*	MW497230.1
5c	11C19	482	891	100.00%	100.00%	*C. fioriniae*	MT607651.1
8d	5A19	597	1103	100.00%	100.00%	*Penicillium expansum*	KX243329.1
9a	5D30	283	523	100.00%	100.00%	*Neofusicoccum australe*	OP142416.1
9a	4D13	547	558	99.00%	85.20%	*Neofusicoccum parvum*	KF798191.1
11c	4F8	530	977	100.00%	100.00%	*Colletotrichum salicis*	KU743959.1
14a	4F33	573	1053	100.00%	99.83%	*Neopestalotiopsis clavispora*	MN519192.1
14b	6A7bi	582	1068	99.00%	99.83%	*Pithomyces chartum*	MH860227.1
17a	2C31a	571	1055	100.00%	100.00%	*Epicoccum nigrum*	MW486023.1

β-tubulin 2 (Bt2)

Table B.2. Bt2 sequencing results.

Plate Group	Isolate	Sequence Length	Max Score	Query Cover	Per. Ident	Top BLAST hit	GenBank Accession no. of top hit
2a	11B33a	0	0	0	0	Inconclusive	-
2e	11C36	650	1195	99%	100%	*Botrytis cinerea*	MN159914.1
3a	16D28a	728	1345	100%	100%	*Pestalotiopsis brachiata / P. biciliata*	KY464163.1 / KM199401.1
3b	8A8	0	0	0	0	Inconclusive	-
3d	5D38	726	1341	100%	100%	*P. brachiata / P. biciliata*	KY464163.1 / KM199401.1
3d	6A25	794	1186	98%	94%	*P. portugallica*	KX895335.1
3e	3A3a	746	1352	99%	100%	*Diaporthe salicicola*	KF170923.1
4a	16A18	517	955	100%	100%	*Diaporthe nobilis*	KT163359.1
5a	4A21	553	1020	100%	100%	*Colletotrichum fioriniae*	MT409125.1
5b	3A20	702	1297	100%	100%	*Glomerella fioriniae*	GU183269.1
5c	5F30	705	1303	100%	100%	*G. fioriniae*	GU183269.1
5c	11C19	698	1290	100%	100%	*C. fioriniae*	MT409125.1
5c	5F19	747	1380	100%	100%	*C. godetiae*	ON241124.1
11c	4F8	732	1295	97%	99%	*C. rhombiforme*	KY581595.1

Appendix C: Plate Group Frequency and Associated Decay Sign Results

Table C.1. *Botrytis cinerea* plate groups expressed by total occurrence and decays from which isolates were obtained.

Plate Group	Molecular Identification	Occurrence of Plate Group as % of Total Plates	Decay Sign Observed on Fruit	% of Sign Isolated as Plate Group
2a	*Botrytis cinerea*	13.32	Grey mould	66.67
			Grey stem scar	20.00
			No decay	2.22
			Green mycelium	2.22
			White stem scar	2.22
			White mycelium	2.22
			Other	4.44
2b	*B. cinerea*	11.52	Grey mould	45.95
			Grey stem scar	24.32
			White stem scar	10.81
			Green mycelium	5.41
			Other	13.51
2c	*B. cinerea*	6.41	Grey mould	60.87
			Green mycelium	8.70
			Grey stem scar	4.35
			White mycelium	4.35
			Other	21.75
2d	*B. cinerea*	1.36	Grey mould	40.00
			Grey stem scar	20.00
			White stem scar	20.00
			Other	20.00

2e	*B. cinerea*	4.07	Grey mould	31.25
			Grey stem scar	25.00
			White stem scar	12.50
			White mycelium	12.50
			Other	18.75
2f	*B. cinerea*	1.74%	Grey mould	25.00
			Grey stem scar	25.00
			Green mycelium	25.00
			White stem scar	25.00

Table C.2. *Pestalotiopsis* spp. plate groups expressed by total occurrence and decays from which isolates were obtained.

Plate Group	Molecular Identification	Occurrence of Plate Group as % of Total Plates	Decay Sign Observed on Fruit	% of Sign Isolated as Plate Group
3a	*Pestalotiopsis disseminata*	6.33	Grey stem scar	64.71
			No decay	5.88
			Green mycelium	5.88
			Other	23.52
3b	*P. disseminata*	9.11	Grey stem scar	48.28
			White mycelium	20.69
			Dense orange/brown	6.90
			No decay	3.45
			Green mycelium	3.45
			Other	17.24
3c	*Pestalotiopsis* sp.	3.26	Grey stem scar	40
			No decay	10

			Green mycelium	10
			White mycelium	10
			Other	20
3d	*Pestalotiopsis* sp.	2.42	Grey stem scar	50
			No decay	16.67
			Green mycelium	16.67
			White mycelium	16.67
1a	*Pestalotiopsis* sp.	0.91	White stem scar	44.44
			No decay	22.22
			Dense orange/brown	22.22
			Grey stem scar	11.11

Table C.3. *Colletotrichum acutatum* species complex plate groups expressed by total occurrence and decays from which isolates were obtained.

Plate Group	**Molecular Identification**	**Occurrence of Plate Group as % of Total Plates**	**Sign Isolated From**	**% of Sign Isolated as Plate Group**
5a	*Colletotrichum fioriniae*	7.79	Anthracnose	36.84
			Grey stem scar	31.58
			Anthracnose and grey stem scar	15.79
			No decay	5.26
			Green mycelium	5.26
			Other	5.26
5b	*C. fioriniae*	2.55	Anthracnose	66.67
			Grey stem scar	16.67
			Anthracnose and grey stem scar	16.67

5c	*C. fioriniae / C. godetiae*	15.82	Grey stem scar	22.50
			Anthracnose	50.00
			White mycelium	10.00
			Anthracnose and grey stem scar	10.00
			Green mycelium	2.50
			Other	5.00
5d	*C. fioriniae*	2.23	Anthracnose	80.00
			Grey stem scar	20.00
11c	*C. salicis*	2.81	Grey stem scar	100.00

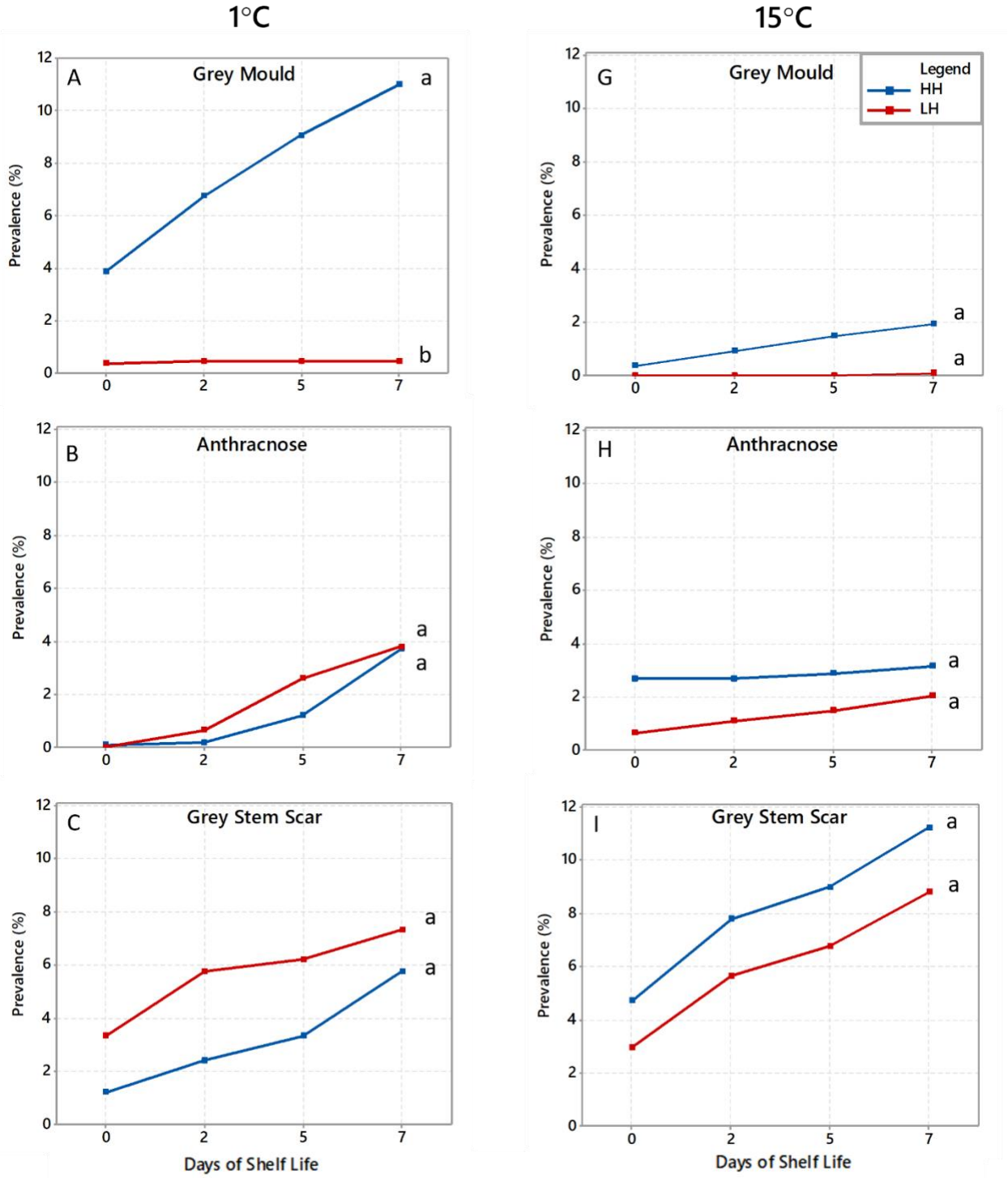
Table C.4. *Diaporthe* spp. plate groups expressed by total occurrence and decays from which isolates were obtained.

Plate Group	**Molecular Identification**	**Occurrence of Plate Group as % of Total Plates**	**Sign Isolated From**	**% of Sign Isolated as Plate Group**
3e	*Diaporthe salicola*	1.42	No decay	28.57
			Grey stem scar	28.57
			White mycelium	14.29
			Other	28.58
4a	*Diaporthe nobilis*	2.12	Grey stem scar	66.67
			White mycelium	33.33

Table C.5. *Penicillium* and *Neofusicoccum* spp. plate groups expressed by total occurrence and decays from which isolates were obtained.

Plate Group	Molecular Identification	Occurrence of Plate Group as % of Total Plates	Sign Isolated From	% of Sign Isolated as Plate Group
8d	*Penicillium expansum*	0.17	Bright blue/green mycelium	100.00
9a	*Neofusicoccum* spp.	1.54	White stem scar	50.00
			No decay	10.00
			Other	40.00
17a	*Epicoccum* sp.	1.03	Dense orange/brown	66.67
			White stem scar	33.33

Appendix D: Supplementary Postharvest Results



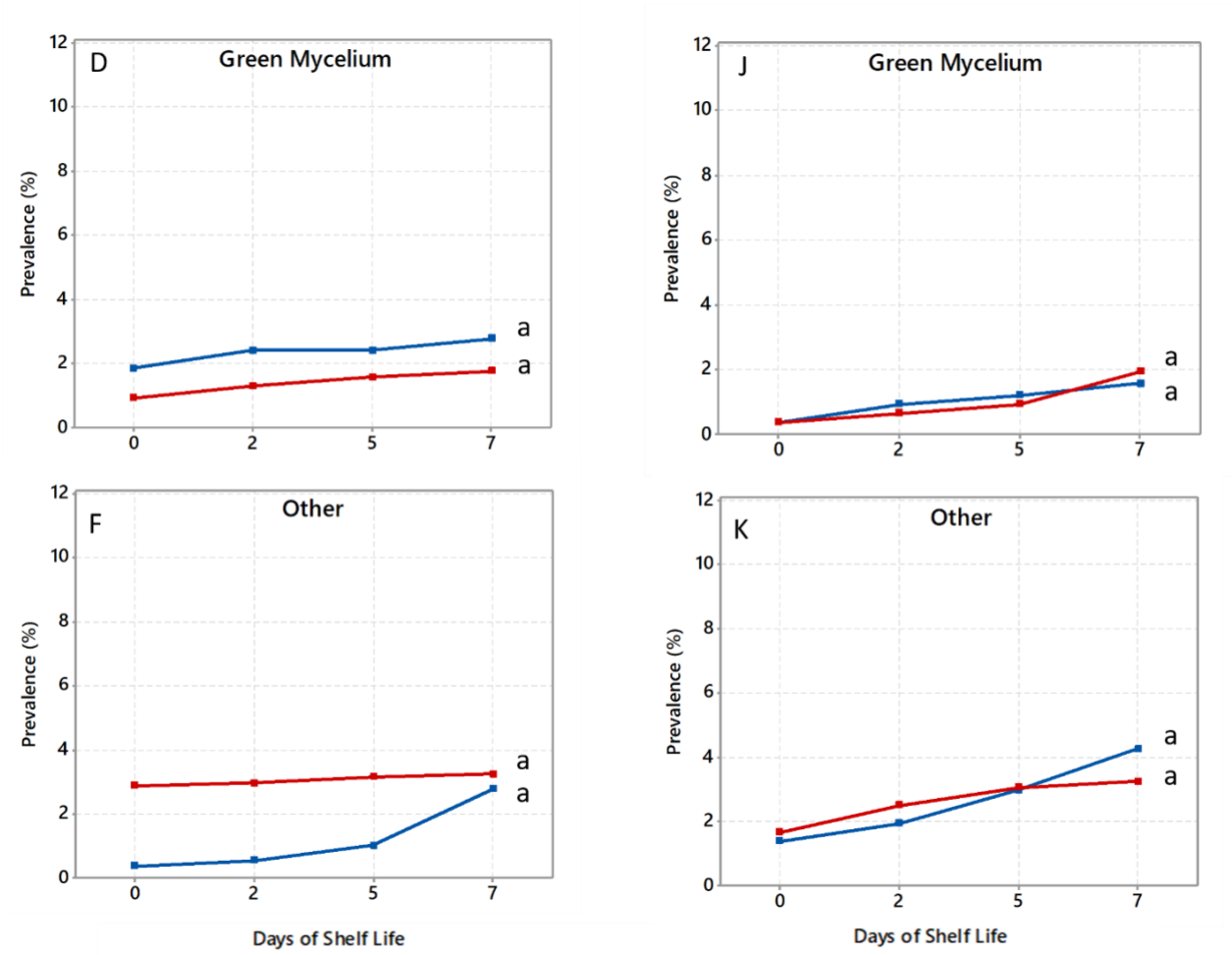


Figure D.1. Prevalence of major decay signs (>5% of total decay) occurring during 7–day shelf life after storage treatments of 1°C for 42 days or 15°C for 14 days. RH treatments are maintained as 98–100% RH as high RH, and 80% RH for low RH. HH = High RH, LH = Low RH. Significant differences are indicated by different letters adjacent to RH treatments (P>0.05). Significant differences are between temperature and humidity within the same decay sign.