Biological Control of Brown Rot Disease Caused by *Monilinia laxa* in Cherries and Plums

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No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of Learning.

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List of Abbreviations

Analysis of Molecular Variance	AMOVA
Analysis of Variance	ANOVA
Base pair	bp
Basic local alignment search tool	BLAST
Biological control agents	BCAs
Centre for Agricultural Bioscience International	CABI
Centimetre	cm
Colony forming unit/millilitre	CFU/ml
Cultivar	cv.
Day	d
Degree Celsius	°C
Deoxyribonucleic acid	DNA
et alia	*et al.*
Gram	g
Hour	hr
id est	*i.e.*
Litre	L
Malt extract agar	MEA
Malt extract broth	MEB
Metre	m
Microgram	µg
Microlitre	µl
Micromolar	µM
Millilitre	ml
Millimolar	mM
Minute	min
Nanogram	ng
National Centre for Industrial, Food and Marine Bacteria	NCIMB
Natural logarithm	ln
Normal saline solution	NSS
Nutrient agar	NA
Nutrient broth	NB
Paraquat chloramphenicol agar	PCA
Percent	%
Phosphate buffer solution	PBS
Polymerase chain reaction	PCR
Potato dextrose agar	PDA
Potato dextrose broth	PDB
Power of hydrogen	pH
Round per minute	rpm
Second	s
species (singular)	sp.
species (plural)	spp.
Ultra violet	UV
Water agar	WA
Weeks	wks
Volatile organic compounds	VOCs

Abstract

Monilinia laxa is the causal agent of brown rot disease on stone fruits, and also causes blossom wilt and twig canker. The common practice used to manage this disease is through fungicide treatments, especially during the flowering and fruiting period. However the demand to reduce fungicide input has been increasing and there is a growing number of reports of *M. laxa* strains that are resistant to fungicides. This study is based on the development of biological control agents (BCAs) as an alternative strategy for disease control using either existing BCA products or new indigenous isolates from UK orchards. Efficacy of exotic commercial BCAs against *M. laxa* was investigated using *in vivo* tests on cherries and plums. Serenade partially inhibited *M. laxa* while the other four BCA products (BioPK, BoniProtect, Prestop and Trianium) had no effect against the pathogen.

Indigenous BCAs were isolated from healthy leaves, intact fruits and mummified fruits of cherries and plums collected from orchards in Kent. A total of 217 isolates were screened against two strains of *M. laxa* in a series of *in vitro* tests using a dual culture technique. From these tests, 12 isolates were selected for further screening. The final screening based on *in vivo* tests on cherries and plums narrowed these down to two isolates with good potential for development. They were further tested under post-harvest conditions by dipping cherries and plums in suspensions of cells of the individual control agents and storing treated fruit under standard storage conditions. The two BCAs did not control brown rot on cherries under these conditions, and the low incidence of natural infection by *M. laxa* in plums also meant that significant control could not be demonstrated.

The two BCAs were identified as *Bacillus amyloliquefaciens/subtilis* (isolate B91) and *Aureobasidium pullulans* (isolate Y126). Their modes of action were investigated. *Bacillus* sp. B91 was shown to produce soluble and volatile organic compounds which inhibited *M. laxa,* while *A. pullulans* Y126 apparently competes with the pathogen for nutrients and did not produce inhibitory compounds. The capability of B91 and Y126 to grow and survive at low temperatures was studied. *Bacillus* sp. B91 was shown to be a mesophillic bacterium that could grow at 10-25°C but suffered significant mortality at 0 and 5°C, while *A. pullulans* Y126 was both mesophilic and psychrotolerant because it grew between 0-25°C, although 20°C was the optimum temperature. Once all nutrients were removed, Y126 was able to survive for several weeks in all test temperatures (0-25°C) but showed significant mortality at 25°C. The capability of B91 to survive at 20 and 25°C was higher than low temperatures (0-15°C).

Molecular studies were used to show that *M. laxa* populations on mummified fruits were likely to be responsible for fruit rot infection in the same orchards the following season. Geographical origin and host also influenced the population structure of *M. laxa*. Field trials were conducted in which mummified fruits were either treated with a commercial fungicide (Indar) or BCAs to investigate whether these treatments would significantly reduce sporulation of the pathogen in the field thus reducing the infection load in the spring. Spraying in Winter alone was not sufficient to significantly suppress sporulation of *M. laxa* but the efficacy was improved when the control agents were applied in Spring either as a single spray or in combination with a Winter spray. Sprays of Indar or *A. pullulans* Y126 greatly suppressed sporulation on mummified fruits if the fruits were treated on both occasions. It was concluded that the two novel, indigenous BCAs have potential to control *M. laxa* but more research is necessary to develop these two potential BCAs for field use.

Chapter 1
Introduction and aims

1.1 Stone fruits

Stone fruits are fruits with an outer fleshy part (skin and flesh) which surrounds a shell of hardened seed (stone). It is assumed that they originated from central Asia. Since they were introduced to Europe, Australasia, North and South America, many countries have become leading stone fruit producers *e.g.* Italy, Spain, Australia, US and Chile (Byrde and Willetts, 1977; George, 1999; Snowdon, 1990). Stone fruits are produced by many genera, but the genus *Prunus* is planted worldwide and consumed widely. Stone fruits are mainly produced for the fresh fruit market. They are also important for processing into conserves and liqueurs. Some stone fruits which are of lower quality can be processed as preserved or canned fruits. Important species of *Prunus* are peach (*P. persica* L. Batsch var. *persica*), nectarine (*P. persica* var. *nucipersica* (Suckow) C. Schneider), apricot (*P. armeniaca* L.), plum (*P. domestica* L.) and sweet cherry (*P. avium* L.).

Among European Union countries, Poland, Italy, Greece and Spain are significant plum and cherry producers (NCFAP, 2003; USDA, 2010). There are various cultivars of plums and cherries, which vary in suitability for different regions. In the UK, the plum harvest season is between June to August depending on varieties. The harvest season for British cherries, however, is significantly shorter than other stone fruits with a peak season around the two weeks of mid-July. Therefore demand for these stone fruits for the premium markets exceeds production, thus driving extensive recent plantings (Allen and Silver, 2010).

Stone fruit trees need intensive management as they are susceptible to several pests and pathogens, as well as to physical damage caused by harvest and handling. Plums and cherries are particularly susceptible to brown rot caused by *Monilinia* spp. via wounds caused either naturally or by man during picking and handling. Intact plums and cherries are also susceptible to the disease. Even a small amount of brown rot can lead to significant crop loss within a short period of time. For example McLaren *et al.* (1996) reported that losses due to brown rot disease in apricots and nectarines reached 50% when the fruits were stored at 20°C for eight days.

In term of economic impact, Holmes *et al.* (2011) reported that in 2006 and 2007 entire shipments of fresh peaches and nectarines from Australia were rejected on arrival in the UK due to brown rot. In recent years, brown rot disease has been so severe

in the field that some stone fruit crops were abandoned before harvest (Holmes *et al.*, 2011). The average annual loss due to brown rot of stone fruits in Australia was estimated to be $19 million AUD nationally plus the costs of control were approximately $25 million AUD. Commercial value for cherries in the UK market was estimated for around £40 million GBP (CALU, 2009) while wholesale and retail prices for first class cherries during 2009 were £5.00/kg and £6.00/kg, respectively.

1.2 The pathogen, *Monilinia*

1.2.1 Geographical distribution

The genus *Monilinia* Honey consists of 35 species (Mycobank, 2013), only a few of which have been studied in detail. Members of the genus are generally regarded as pathogens of the Rosaceae and Ericaceae (Holst-Jensen *et al.*, 1997). This genus contains several species which cause severe and destructive diseases such as blossom wilt, twig canker and fruit brown rot worldwide. They are most significant as agents of brown rot of fruit. Most research has been focused on *M. fructicola* (G. Winter) Honey and *M. fructigena* (Aderh. & Ruhland) Honey (Whetzel, 1945). In contrast, *M. laxa* (Aderh. & Ruhland) Honey has been studied rarely in spite of the fact that it can devastate stone fruit crops. *Monilinia fructicola* and *M. laxa* attack mainly stone fruits, such as plums and cherries, whilst *M. fructigena* also attacks these hosts but is found more frequently on pome fruits such as apples and pears. *Monilinia fructigena* causes damage on wounded fruits but *M. fructicola* and *M. laxa* infect blossom, twig, wounded fruits and intact fruits.

There are few reports on the geographical distribution of *M. laxa* in spite of the fact that these are important in epidemiological studies. Nowadays plum and cherry fruits are imported and exported across different continents, which can rapidly increase the epidemic level of brown rot disease. There are differences in the distribution of the three pathogens (*M. laxa, M. fructigena* and *M. fructicola*) in different geographical areas. In North America and the southern hemisphere (Australia and New Zealand) cherries and plums are mainly attacked by *M. fructicola* (CABI, 2010; Chen *et al.*, 2012; Wherrett *et al.*, 2001), a species which is more virulent than the others. In China, the largest stone fruit producer country in Asia, six *Monilinia* species have been detected, with *M. fructicola* the most prevalent (Hu *et al.*, 2011a; Zhu *et al.*, 2011). This species is also commonly reported in many South American countries (Keske *et al.*, 2011; May-De Mio *et al.*, 2011; Mitidieri *et al.*, 2006).

In Europe, brown rot is caused mainly by *M. laxa* and *M. fructigena*. However *M. fructicola* was reported in the south-east of France in 2001 (EPPO, 2002) and more

recently in many European countries. *Monilinia fructicola* has been isolated from various host plants, for example blackberry in Germany (Hinrichs-Berger and Mueller, 2010), plums in Poland (Poniatowska *et al.*, 2013), peaches in Spain (Villarino *et al.*, 2010) and nectarines in Italy (Pellegrino *et al.*, 2009) and Serbia (Hrustic *et al.*, 2012). The European Food Safety Authority concluded that the dispersal routes of *M. fructicola* to EU territory are plant materials used for propagation and fruits which are susceptible hosts for the disease (EFSA, 2011).

Monilinia laxa and *M. fructigena* are common in Europe and Asia as agents of brown rot, but also have a world-wide distribution. In contrast, although brown rots caused by *M. fructicola* have been reported throughout the US, *M. laxa* has yet to be detected in much of the East coast of US. Villani and Cox (2010) have reported brown rot caused by *M. laxa* in New York, a major state on the East coast. More recently, based on a variety of characters, a novel species *Monilinia yunnanensis* M.J. Hu & C.X. Luo has been described from peach in China (Hu *et al.*, 2011b) and Japanese isolates, formerly classified as *M. fructigena,* were re-designated as a new species, *M. polystroma* G. van Leewen (van Leeuwen *et al.*, 2002a). This species now also has been found in Europe, for example in Hungary (Petroczy and Palkovics, 2009).

1.2.2 Morphology of *M. laxa*

The anamorphic fungus, *M. laxa*, was firstly proposed by Ehrenberg (1818) as *Oidium laxum*. The name was later changed to *Monilinia laxa* Saccardo and Voglino (1886) before the teleomorphic stage *Sclerotinia laxa* was described by Aderhold and Ruhland (1905). *Monilinia laxa* can be distinguished from *M. fructicola* and *M. fructigena* based on microscopic and macroscopic characters from axenic cultures and is the common form of the fungus in nature. The teleomorphic stage is rarely found, in contrast to *M. fructicola* (teleomorph: *Sclerotinia fructicola*) (Boesewinkel and Corbin, 1970; Jerebzoff and Jacques, 1972; Willetts, 1968a, b) and *M. fructigena* (teleomorph: *S. fructigena*) (Callonge *et al.*, 1969; Fawcett and Spencer, 1966) where they have been reported worldwide. To form an apothecium, soil moisture, pH and climate conditions must be favorable (Holtz *et al.*, 1998).

The major distinguishing characters for these three species are their natural occurrence, colours and appearance of conidial pustules and hosts and plant parts which are infected (Byrde and Willetts, 1977; EPPO, 2009; Hughes *et al.*, 2000). For example, conidia of *M. laxa* can be produced at low temperatures (5°C) while *M. fructigena* and *M. fructicola* produce conidia at higher temperatures (15-25°C) in humid conditions. This difference provides an opportunity for these pathogens to spread and infect plants

in different conditions. Conidial dimensions of *M. laxa* produced in winter (11.5x8.0 μm) are significantly smaller than ones produced in summer (17.0x11.0 μm) (Wormald 1920).

Furthermore, conidia formed by *M. laxa* are normally smaller than *M. fructigena* and *M. fructicola* (22.0x13.0 and 18.0x12.5 μm, respectively) (Byrde and Willetts, 1977). In addition, distinctive morphotypes of these species are produced on agar media (Figure 1.1). Margins of colonies of *M. laxa* are typically lobed, and the older mycelium becomes pigmented, while colony margins of the other two species are entire. The methods relying on morphological data require a long incubation period because these fungi are slow-growing (2.0-11.0 mm/24 hours) (EPPO, 2009). They may take at least two weeks to produce a mature colony on an agar medium at 25°C.

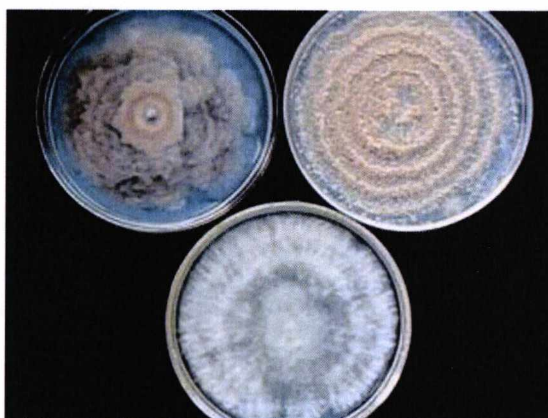


Figure 1.1 Morphologies of cultures of *Monilinia laxa* (Left), *M. fructicola* (Right), and *M. fructigena* (Bottom) on potato dextrose agar (Ritchie, 2000).

1.2.3 Molecular studies of *M. laxa*

Although these important species of *Monilinia* (*M. laxa, M. fructigena* and *M. fructicola*) can be easily distinguished from each other on the basis of colony morphology on solid agar media, this method may take several days. Molecular methods have been developed to speed up identification (Cote *et al.*, 2004; Forster and Adaskaveg, 2000; Gell *et al.*, 2007a; Ioos and Frey, 2000). As fruits are traded and moved rapidly across the world, non-indigenous pathogenic isolates are being introduced to other countries within a matter of days. Rapid detection of these fungal isolates in imported and exported fruits is crucial for plant disease quarantine. Molecular techniques have provided powerful tools to identify strains within plant pathogenic species and provide new taxonomic insights

1.2.4 Species detection

Earlier studies used SDS-PAGE profiles of protein extracts to identify strains of *Monilinia* species (Belisario *et al.*, 1999), but these have been superseded by DNA-based methods (Fulton and Brown, 1997; Fulton *et al.*, 1999; Gell *et al.*, 2007a; Holst-Jensen *et al.*, 1997; Hughes *et al.*, 2000). Sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA can be used to differentiate *Monilinia* species but not to distinguish isolates within the same species (van Brouwershaven *et al.*, 2010). Species-specific primers have been successfully used to generate different-sized amplicons and identify various species of *Monilinia* (Cote *et al.*, 2004; Forster and Adaskaveg, 2000; Hughes *et al.*, 2000; Snyder and Jones, 1999). This technique can be used to identify *M. laxa*, *M. fructigena* and *M. fructicola* directly from naturally infected fruits, whilst Forster and Adaskaveg (2000) used two sets of primers to detect *Monilinia* spp. in diseased cherries showing no visible symptoms.

1.2.5 Molecular analysis at intra-species level

Genetic diversity within *Monilinia* species is less well understood. Random amplified polymorphic DNA (RAPD) analysis of 21 isolates of *M. laxa* collected from different hosts and locations in Spain from 1996-1998 was used to construct a phylogenetic tree (Gell *et al.*, 2007b), which showed that there was considerable diversity within clusters of *M. laxa* isolates that came from same source. Forster and Adaskaveg (2000) showed that a RAPD analysis of eight primers used as genetic markers revealed large genetic diversity of seven isolates of *M. fructicola* isolated from California. Out of eight primers, polymorphisms of *M. fructicola* were detected from seven primers. However this same set of primers did not reveal polymorphisms in seven isolates of *M. laxa*. The primers may not have sufficient stringency to distinguish isolates of *M. laxa,* or it may be that larger sample sizes of *M. laxa* isolates are required.

Amplified fragment length polymorphism (AFLP) analysis has been successfully used to reveal differences among *M. laxa* and *M. fructicola* isolates (Gril *et al.*, 2010). Within *M. laxa*, AFLP analysis has shown that there were significant intra-specific differences in isolates from different host plants (Gril *et al.*, 2008). The results discriminated *M. laxa* isolates from apples from isolates originating from other host plants, but no further groupings according to other host plants were obtained. This indicated some host specialisation and suggested that this host specificity may influence genetic diversity of fungal pathogens. Thus an analysis based on other variable DNA regions is needed to improve our understanding of genetic diversity within *M. laxa*

isolates from a wide range of host plants and locations - microsatellite DNA may offer this potential.

Microsatellites are non-coding tandem repeats of one to six nucleotides found in genomic DNA of most organisms, also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) or short tandem repeats (STR). These repeat sequences mutate frequently by slippage and proofreading errors during DNA replication, with the result that alleles corresponding to these sequences differ in length and can be differentiated by gel electrophoresis (Selkoe and Toonen, 2006). SSR Analysis using five SSR markers of *M. fructicola* isolated from four European countries (Switzerland, Spain, Italy and France) revealed an origin and population structure possibly derived from at least two independent invasion events from the US (Jansch *et al.*, 2012).

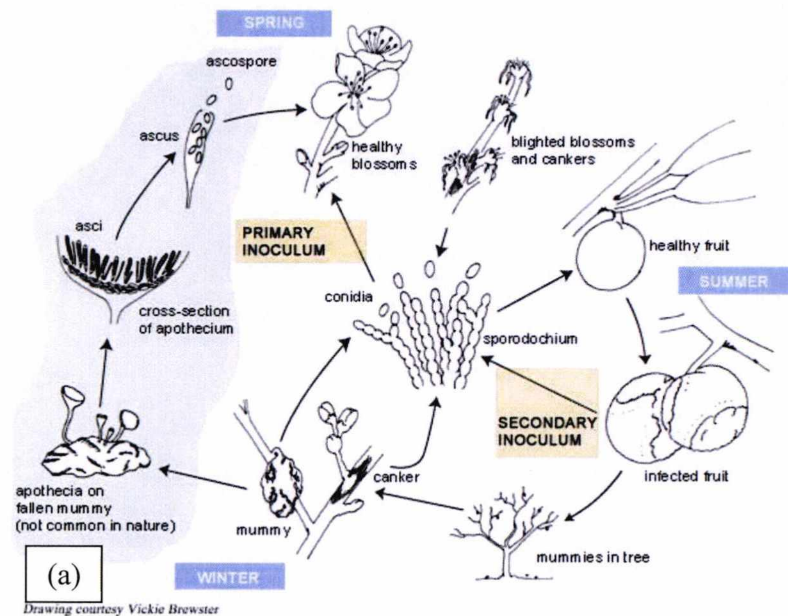
Likewise hundreds of isolates of *M. fructicola* isolated from four provinces in China were compared with those from the US and New Zealand (Fan *et al.*, 2010). SSR analysis based on 12 out of 35 SSR markers tested again revealed that the Chinese population of *M. fructicola* was very similar to that of America and they could have been introduced into China via stone fruit cultivars received from the US. To date, studies on genetic diversity of *Monilinia* spp. using molecular techniques have been reported from many countries which are major stone fruit producers including the US (Lehman and Oudemans, 2000; Snyder and Jones, 1999; Wasilwa *et al.*, 2001), Spain (Gell *et al.*, 2007b), Switzerland (Jansch *et al.*, 2012), Italy (Pizzuolo *et al.*, 2006), France (Ioos and Frey, 2000), Slovenia (Gril *et al.*, 2008), Japan (Fulton *et al.*, 1999) and China (Fan *et al.*, 2010) but there have been no reports of the genetic diversity of *Monilinia* spp. in the UK.

1.3 Epidemiology of *M. laxa*

1.3.1 Inoculum sources

The life cycle of brown rot comprises three stages (1) blossom blight and twig canker (early spring), (2) fruit brown rot (late spring and summer) and (3) mummified fruit (autumn and winter) (Figure 1.2). Overwintered mummified fruit is the most significant source of inoculum for the new season in the form of large amounts of conidia which are easily dispersed by wind, rain and insects. An optimum temperature for mycelial growth of *M. laxa* is 24.8°C but *M. laxa* produced the highest number of conidia within 15 days at 10°C and low temperatures seemed to increase conidial production (Tamm and Fluckiger, 1993). These conidia infect blossom and twigs of susceptible plants during periods of moderate temperature and humid weather.

Symptoms of blossom blight can appear rapidly, whilst twig canker appears more slowly. Infected blossom then fails to develop or produce fruits thus decreasing crop yield. Asexual spores produced from both blossom blight and twig canker also provide the main inoculum of the brown rot of young and mature fruits. However these stages of infection are frequently overlooked as *M. laxa* can also cause latent infections of developing fruit which is manifested later as brown rot as the fruit matures in summer. Mycelium contact also serves as inoculum when infected parts of plants come into physical contact with nearby healthy plant tissues. Villarino *et al.* (2010) reported that the teleomorphic stage of *Monilinia* species were not found from many growing seasons and all orchards sampled in Spain, but the disease still occurred. It is likely that inoculum for brown rot was produced only from the anamorphic stage.



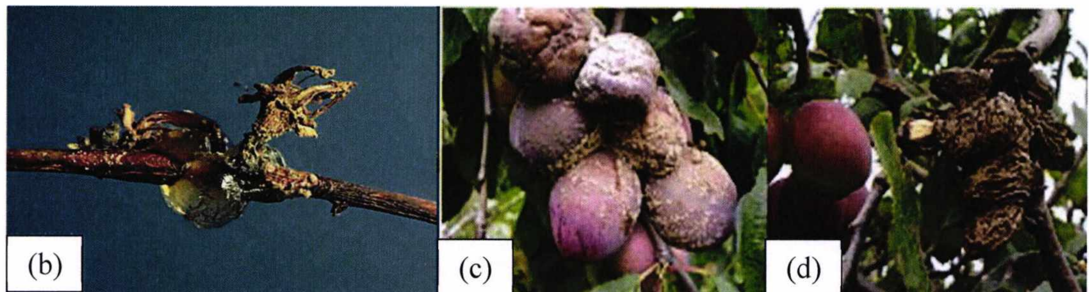


Figure 1.2 Diseases caused by *Monilinia* species. (a): Life cycle of *Monilinia* spp., (b): Blossom blight and twig canker (Ritchie, 2000), (c): Rotting plums with brown rot symptoms covered with conidia of *M. laxa* and *M. fructigena* (greyish and creamy colours, respectively) and (d): A cluster of mummified plums hanging on the tree.

The number of mummified fruits still attached to trees was significantly higher than the number of mummified fruit found on the orchard floor (Villarino *et al.*, 2010),

suggesting that ground level inoculum is not the most important source. Ninety per cent of *Monilinia* isolates from mummified fruit collected from Spanish peach orchards were *M. laxa* and this served as the main source of inoculum. Villarino *et al.* (2010) also reported that there were relationships between (1) the number of mummified fruit left on the tree and the incidence of post-harvest brown rot, (2) the number of mummified fruit and non-abscised fruits and (3) the number of conidia on the fruit surface and the incidence of latent infection. Gell *et al.* (2009) showed a significant positive relationship between the numbers of conidia of *Monilinia* spp. on the peach surface and the incidence of latent infections, but not with overall brown rot at harvest.

1.3.2 Infection sites

Understanding the infection court is key to controlling disease, as treatments need to prevent *Monilinia* conidia from establishing initial infections. Initially it was thought that *M. laxa* was a wound pathogen, but early work showed that intact fruit surfaces could also be infected by conidia (Xu *et al.*, 2007). Nevertheless, damaged fruit surfaces still provide the majority of infection sites. Conidia of *M. laxa* produced from overwintered mummified fruits can infect through cuticles on healthy blossom and fruits or via tiny injuries on the fruit surface caused by insects, natural splitting or fruit pickers during harvest, leading to latent infection. When fruits are ripening, environment conditions with high humidity in orchards or storage conditions are favourable to disease development and latent infections are activated and spread rapidly among fruit causing considerable losses. For blossom blight, the stamens and stigma are the usual sites of initial entry, although high humidity facilitates entry via all floral parts except for sepals (Weaver, 1950).

1.3.3 Factors affecting infection

Conidial germination of *M. laxa* is an important step in disease initiation, and can be seen as early as two hours after incubation at 15-25°C (Tamm and Fluckiger, 1993). Casal *et al.* (2010e) showed that the lag phase prior to germination of three *Monilinia* species (*M. laxa, M. fructigena* and *M. fructicola*) was clearly influenced by temperature and water activity (a_w). Generally, when conditions of temperature and a_w were suboptimal, the lag phase and percentage of germination was longer and lower, respectively. More than 80% of conidia of *M. laxa* germinated within four hours at 25°C and 0.99 a_w. At the lowest a_w (0.87) or the highest temperature (38°C), none of the *Monilinia* spp. were able to germinate. Tamm *et al.* (1995) showed the importance of two factors (temperature and post-inoculation wetness duration) on infection of *M. laxa*

on sweet cherry blossoms in a controlled environment. The blossoms of individual trees were inoculated by spraying with a spore suspension of *M. laxa*. The trees were covered immediately after the inoculation with polyethylene bags for zero to 24 hours. Under dry conditions at 15°C *M. laxa* was able to infect up to 40% of cherry blossoms, while infection under dry conditions at other temperatures (5, 10 and 20°C) was lower. However when post-inoculation wetness duration was extended from four to 24 hours, incidence of infected blossoms increased up to 70-90% in all temperatures tested.

Xu *et al.* (2007) demonstrated that many factors (wounding, fruit age and wetness duration) could influence the development of cherry brown rot and confirmed that *M. laxa* was able to infect intact fruits. Results from field monitoring and controlled inoculation in a polyethylene tunnel showed that the susceptibility of fruits to infection by *M. laxa* dramatically increased with fruit maturity, but infection of attached intact fruits by *M. laxa* was not affected by the length (3-24 hours) of the wetness duration tested. Physiological status of the fruit also affected susceptibility (Mari *et al.*, 2003; Mari *et al.*, 2004a; Singh *et al.*, 2012). Generally stone fruits are more susceptible to infection when they ripen, and *Monilinia* species secrete plant cell wall degrading enzymes as the mycelium spreads and form a stroma in the fruit surface resulting in mummification (Holst-Jensen *et al.*, 1997). Peach also becomes more susceptible to *M. laxa* at ripening (Thomidis *et al.*, 2007). Fourie and Holz (2003) demonstrated a similar result for nectarines sprayed with dry conidia of *M. laxa*. The hardening stage (*i.e.* green nectarines) was resistant to *M. laxa,* but resistance decreased when nectarines ripened in cold storage. The degree and duration of wetness also influenced the success of penetration of the nectarine surface and disease incidence.

1.3.4 Latent infection

Most stone fruits are normally kept in cold storage in order to reduce respiration and extend shelf life, but cherries are often directly sold from farms to markets and shelf-stored for a couple of days. Sometimes to maintain fruit firmness and to extend shelf life, cherries may be stored at 0°C for one week, their optimal storage condition. The storage life of plums is longer than cherries and they can be kept at 0°C for up to four weeks (Romanazzi *et al.*, 2003; Snowdon, 1990). However, under these conditions, latent infection by *M. laxa* develops over time resulting in brown rot. Disease spread in cold storage is usually through contact spread. In addition, low temperatures such as 5-10°C are optimal for conidial production by *M. laxa* (Byrde and Willetts, 1977; Tamm and Fluckiger, 1993) which exacerbates disease spread under such conditions when

control using fungicide applications is not permissible. Thus brown rot disease via latent infections can cause critical losses during favourable post-harvest conditions.

Latent infections of stone fruits by *Monilinia* species have been reported in plums and cherries (Borve *et al.*, 2000; Forster and Adaskaveg, 2000; Fourie and Holz, 2006; Kloutvorova and Kupkova, 2003; Szodi *et al.*, 2008; Villani and Cox, 2010; Wittig *et al.*, 1997). These infections follow a typical pattern of subcuticular infection of immature fruit followed by rapid cessation of growth of the pathogen. As the fruit matures, then growth resumes and post-harvest brown rot thus results from pre-harvest infections. Luo *et al.* (2005) demonstrated correlations between blossom blight and incidence of latent infection of *M. fructicola* on immature stone fruits, as well as between incidence of brown rot at harvest and latent infection. Therefore estimating the incidence of the latent infection is crucial for effective disease management. Failing to detect *Monilinia* species in mature fruits may lead to serious post-harvest losses and infected fruits can bear a new strain of the pathogen which can be introduced to other destinations. However detection of latent infection in commercial orchards may be difficult because an assessment of latent infection requires a period of time for incubation and needs large sample sizes.

1.4 Chemical control

1.4.1 Use and resistance of fungicides

Chemical methods which are currently used to control pre-harvest brown rot involve regular spraying with conventional fungicides. Fungicides are regularly sprayed to blossom (in early spring). This can be problematic, as overenthusiastic spraying may cause phytotoxic effects and skin damage on blossom and bud. In addition, it may lead to selection of fungicide–resistant strains of the pathogen. Fungicide resistance has been reported for *M. laxa* (Ma and Michailides, 2005; Ma *et al.*, 2005; Malandrakis *et al.*, 2013; Malandrakis *et al.*, 2012; Thomidis *et al.*, 2009). In the 1970s, two benzimidazole fungicides, benomyl and thiophanate-methyl, were widely used to control brown rot at the blossom stage. These fungicides effectively controlled the disease (Ramsdell and Ogawa, 1972) until benomyl-resistant *M. laxa* isolates were detected in the US (Ogawa *et al.*, 1984) and benomyl was withdrawn from the markets a few years later.

Fruit growers have changed to use thiophanate-methyl fungicides instead, but strains of *M. laxa* resistant to this fungicide have now been reported. Low resistant strains of *M. laxa* were isolated from different hosts *i.e.* nectarines, peaches, prunes and almonds, collected from California (Ma *et al.*, 2005). These isolates can grow on PDA amended with five mg/L of thiophanate-methyl. Isolates of *M. laxa* from rotten peaches

collected from orchards in Greece in 2005 and 2006 were able to grow on PDA supplemented with 100-200 mg/L of thiophanate-methyl and some isolates could grow on media with up to 400-500 mg/L of the fungicide (Thomidis *et al.*, 2009). Although benzimidazole-resistant *M. laxa* isolates are still susceptible to other fungicides, for example tebuconazole and iprodione, resistance may spread to these other classes of fungicides. Malandrakis *et al.* (2012) reported that one third of isolates of *M. laxa* from stone fruits in Greece were highly resistant to benzimidazoles benomyl and thiophanate-methyl.

Sensitivity of *M. fructicola* collected from Brazil to three fungicides (tebuconazole, azoxystrobin and thiophanate-methyl) has been investigated (May-De Mio *et al.*, 2011). Isolates were sensitive to azoxystrobin but resistant to the other two fungicides. Resistance to tebuconazole corresponded to its constant and repeated use within orchards. Resistance of *M. fructicola* isolated from the US (Southern California) and Europe (Italy, France, Spain and Switzerland) to some fungicides was related to a mutation in the β-tubulin gene (Weger *et al.*, 2011; Zhu *et al.*, 2010). Likewise resistance of *M. laxa* isolated from stone fruits and almond to benzimidazole was due to a point mutation, and a consequential substitution of an amino acid, in the β-tubulin gene (Ma *et al.*, 2005).

Fenhexamid, a hydroxyanilide fungicide, has been found to be highly effective against field isolates of *M. laxa* (Malandrakis *et al.*, 2013). However, appropriate strategies for detecting fungicide resistance are crucial to ensure the commercial use of this fungicide in the long run. Studies on fungicide resistance of *M. fructicola* and *M. laxa* clearly show that use of fungicides is not a sustainable method of control as brown rot pathogens, in common with many fungal pathogens, can evolve rapidly and become resistant to fungicides. Ideally fungicides should be replaced by non-toxic organic substances or biological control agents, or combined with these alternatives at significantly lower application rates. Increasingly, the restricted use of fungicides in some countries has meant that the development of alternative disease management is urgently needed.

1.4.2 Use of other chemicals

Other chemicals have been applied to stone fruit to control brown rot diseases. For example, flavipin (an antibiotic produced from several *Aspergillus, Epicoccum* species and other fungi) affects many target sites in *M. laxa, i.e.* cellular membrane permeability, ATP synthesis and protein synthesis (Madrigal and Melgarejo, 1994). When flavipin was applied to conidial suspensions of *M. laxa*, their physiological

activity suddenly dropped and malfunctioned. Simpler compounds, such as peracetic acid and potassium sorbate reduced infections by *M. laxa* in sweet cherry without damaging fruit (Mari *et al.*, 2004b). Mycelial growth of *M. laxa* was inhibited on PDA supplemented with boron (Thomidis and Michailides, 2010). This element was tested as a post-harvest dip: it significantly reduced the development of *M. laxa,* reducing losses in fruit weight and improved fruit firmness one month after storage, thus improving peach maintenance in cold storage. Mitre *et al.* (2008) also showed that copper-ammoniac phosphate can control *M. laxa* in plums, sweet cherry and sour cherry, and this chemical was long lasting and compatible with other agrochemicals.

Plant extracts from the perennial Mediterranean weeds, *Dittrichia viscose* and *Ferula communis* collected from Italy, had bioactivity against many post-harvest fungi including *M. laxa* and *M. fructigena* by reducing mycelial growth and conidial germination (Mamoci *et al.*, 2011). Low concentrations of essential oils from laurel (*Laurus nobilis*) inhibited mycelial growth of *M. laxa* following application on the fruit surface before and after inoculation, resulting in 9% inhibition of decay (De Corato *et al.*, 2010). Mari *et al.* (2008) reported that 4-methylthiobutyl-isothiocyanates showed good activity in inhibiting *M. laxa* conidial germination and mycelial growth. Furthermore two chemical substances derived from isothiocyanates reduced brown rot more than 85% on artificially-inoculated nectarines and peaches, but phytotoxic effects caused by one of these derivatives were observed. There are a few reports showing bioactivities of a variety of chemicals tested on fruits in glasshouse or in field trials. However, the efficacy of these chemicals in these conditions is variable and untried on a large scale.

1.5 Physical control

Physical treatments may be used to control post-harvest brown rot disease or even replace chemical treatments altogether. The possible mechanisms underlying physical treatments are (1) reducing fruit respiration, (2) disinfecting pathogens on fruit surface, (3) suppressing latent infection and (4) stimulating induced resistance (Balla and Holb, 2007; Holb, 2009; Mari *et al.*, 2010). Most physical treatments have been targeted at the post-harvest environment.

1.5.1 Heat treatments

Hot water dipping at 55 and 60°C for 60 and 30 seconds, respectively, reduced disease incidence and lesion diameter on peaches, nectarines and plums artificially infected with *M. fructicola* (Karabulut *et al.*, 2010), but heating injury was reported

when the fruits were treated at temperatures of 65°C or higher. Zhang *et al.* (2010a) demonstrated that conidial germination and germ tube elongation of *M. laxa* were prevented by treatments of 50 and 60°C for more than 40 and 10 seconds, respectively, whilst conidial germination was also reduced by treatment at 48°C for 12 minutes. Jemric *et al.* (2010) showed that when artificially-inoculated peaches and nectarines were treated with this method 24 hours after inoculation, the incidence of infection and visual symptoms caused by *M. laxa* were significantly reduced. A heat treatment generated from radio frequency has been recently reported to reduce brown rot disease. Casals *et al.* (2010d) found that radio frequency treatment, for 18 minutes to generate a temperature rise within fruit, decreased the disease incidence on peaches, but not on nectarines, artificially inoculated with *M. fructicola*. Likewise this heat treatment also totally inhibited natural infection by *Monilinia* spp. on peaches but not on nectarines.

1.5.2 Other physical treatments

Research on other physical treatments, including controlled atmosphere with low oxygen or high carbon dioxide concentration, has been done on *M. fructicola*. Atmospheres containing 30% of carbon dioxide and incubation at 0°C completely prevented brown rot disease on sweet cherries (Tian *et al.*, 2001). Akbudak *et al.* (2009) also showed that sweet cherries could be stored for 60 days in a controlled atmosphere of 20% CO_2 and 5% O_2 plus a plant-resistance inducer. These conditions reduced the incidence of rotten cherries caused by various post-harvest pathogens, including *M. fructicola* from 24% to 4%. Balla and Holb (2007) conducted an experiment on storage conditions in ultra-low oxygen (ULO) atmospheres, containing less than 1 kiloPascal of O_2. These conditions decreased fruit decay and brown rot caused by *M. fructigena* on apples, despite a brown-rotted apple being placed among healthy apples, ULO conditions eliminated brown rot infection while incidence of rot decay in traditional storage conditions was very high (18%-35%). Disease incidences for artificially injured apples with or without a brown-rotted apple stored under ULO conditions were significantly lower than those stored under the traditional storage conditions.

Ultra violet (UV) irradiation also has potential in disease control. A 16-hour exposure of UV each day was shown to reduce *in vitro* mycelial growth of *M. laxa, M. fructigena* and *M. fructicola* (De Cal and Melgarejo, 1999), but it failed to control the disease on *in vivo* experiments when applied to *M. fructigena* on cherries (Marquenie *et al.*, 2002) and *M. fructicola* on peaches (Sautter *et al.*, 2011). γ-Irradiation at 1.5 and 3 kilogray delayed disease development on apples inoculated with *M. fructigena* (Marcaki, 1998). There have been no reports on radiation treatment on *M. laxa*.

Covering sweet cherries on the tree with plastic rain shields reduced fruit decay caused by post-harvest fungi including *M. laxa, Botytis cinerea, Mucor piriformis* and *Colletotrichum gloeosporioides* (Borve and Stensvand, 2003). The light-transmitting shield prevented fruit cracking and splitting which were caused naturally by rainfall as well as reducing access to pathogen inoculum. Whilst these physical treatments have shown potential to inhibit *Monilinia* spp. in test trials, their use has been limited in commercial fruit production due to physical and heating injuries caused to fruit surface, labour costs, a lack of facilities and the time constraints during harvest (Casals *et al.*, 2010d; Holb, 2009; Karabulut *et al.*, 2010).

1.6 Biological control

Biological control agents (BCAs) offer an alternative to chemical control. The mode of actions of BCAs may involve (1) competition for nutrients and space, (2) production of antibiotics, (3) direct parasitism and (4) induced resistance (Sharma *et al.*, 2009). The control of post-harvest diseases of fruits is one area where BCAs have had successes (Janisiewicz and Korsten, 2002; Janisiewicz *et al.*, 2013; Nunes, 2012; Wisniewski *et al.*, 2007). However, the difficulties in commercialisation of BCAs have meant that the use of BCAs is not well-established in horticulture. Nevertheless, the stable environmental parameters in post-harvest storage conditions suggest that BCAs have more potential for post-harvest treatment and may offer protection against wound pathogens in particular (Ippolito and Nigro, 2000; Magan, 2006). A range of commercial BCAs have been available over the past ten years (Table 1.1) (McSpadden Gardener and Fravel, 2002; Wisniewski *et al.*, 2007), but few field trials testing them against brown rot have been reported. There is some doubt whether this approach would be effective against latent infections and thus pre-harvest treatments may also be necessary.

1.6.1 Bacterial biological control

Bacteria have become one of the most attractive BCAs because many bacterial genera are widely reported to have diverse bioactivity (Feng *et al.*, 2012; Janisiewicz and Buyer, 2010; Kohl *et al.*, 2011; Verschuere *et al.*, 2000; Weller, 1988). Inhibition tests using aqueous suspensions of *Pseudomonas syringae* and two strains of *Bacillus* species against *M. fructicola* on peaches showed that they reduced brown rot incidence as well as lesion diameter (Zhou *et al.*, 2008). Two bacteria *P. syringae* and *Erwinia herbicola* completely inhibited mycelial growth of *Monilinia oxycocci*, the cranberry

pathogen (Voland *et al.*, 1999). Lactic acid bacteria have also been reported to show bioactivity against *M. laxa* (Trias *et al.*, 2008).

(A) *Bacillus* species as biological control agents

Bacillus comprises many species, some of which are well recognised for bioactivity. The genus is found in a wide range of substrata and environments reflecting its capability to adapt to different habitats. *Bacillus* species have been used as BCAs for decades. For example, *Bacillus thuringiensis* is reported to have various bioactivities, including insecticidal activity against moths (Lentini *et al.*, 2012; Paul, 2008), nematodes (Seo *et al.*, 2012; Sharma, 1994) and mosquito larvae (Boyer *et al.*, 2012; Paris and Despres, 2012; Santos *et al.*, 2012; Tanapongpipat *et al.*, 2003) as well as antifungal activity against plant pathogens (Reyes-Ramirez *et al.*, 2004). Capability of *B. thuringiensis* beyond these activities include reports as a biostimulator and biofertilizer (Raddadi *et al.*, 2007). *Bacillus thuringiensis* SFC24 directly antagonised the pathogen and induced resistance of the host plant to reduce late leaf spot disease in groundnut caused by *Phaeoisariopsis personate*. Isolate SFC24 was cloned and the gene for the elicitor harpin of *P. aeruginosa* was inserted into its chromosome. The genetically modified isolate was able to promote plant growth, increase shoot length and control leaf spot disease (Anil and Podile, 2012).

In other trials, *Bacillus* isolate 'SE' reduced black rot lesion caused by *Xanthomonas campestris* by foliar spray or a combination of seed soak and soil drench. Two enzymes (the autolysin beta-N-acetylglucosaminidase and AHL-lactonase) played an important role (Mishra and Arora, 2012) in biological control. Poleatewich *et al.* (2012) used pre-harvest treatments on apple trees with three *Bacillus* species (*B. megaterium, B. mycoides* and *B. cereus*) applied at different times at pre-harvest (May or May and June) to significantly reduce fruit and foliar apple scab severity over two years of study. For post-harvest studies, artificially-wounded apples were treated with these *Bacillus* spp. and then the pathogen. A combination of two pre-harvest BCA treatments (May and June) followed by a post-harvest treatment showed greatest suppression of bitter rot on two apple cultivars.

Huang *et al.* (2012) conducted a pot experiment using cucumbers to show that *Bacillus pumilus* SQR-N43 induced hyphal deformation, enlargement of cytoplasmic vacuoles and cytoplasmic leakage of *Rhizoctonia solani*, a pathogen causing damping-off disease. This isolate also reduced the inoculum levels of *R. solani*. Activity of *B. pumilus* SQR-N43 was increased when it was mixed with a fermented organic fertiliser in soil which stabilised its population. *Bacillus subtilis* and *B. cereus* from China

controlled root disease caused by *G. graminis* and *R. solani* causing take-all disease and root rot disease of wheat, respectively (Ryder *et al.*, 1999). These results showed that different types of soil affected the inhibitory activity of the *Bacillus* species. *Bacillus cereus* A47 and *B. subtilis* B908 reduced severity of take-all of wheat grown in a sodic acid soil, while *B. subtilis* B931 had activity against *R. solani* in a calcareous sandy loam soil. These strains gave positive growth responses including gains in wheat seedling root weight, shoot weight and shoot length.

Table 1.1 Examples of BCAs those are commercially available in the agro-industry [modified from McSpadden Gardener and Fravel (2002) and Wisniewski *et al.* (2007)].

BCAs	Microorganism	Company	Targeted disease	Crop
AQ10	*Ampelomyces quisqualis* M-10	Ecogen, Inc, USA	Powdery mildew	Apples, curcubits, grapes, strawberries and tomatoes
Aspire	*Candida oleophila* I-182	Ecogen, Inc, USA	Fruit decay	Citrus
Bio-Save	*Pseudomonas syringae* ESC-11	Village Farms LLC, USA	*Botrytis cinerea*, *Penicillium* spp., *Mucor pyriformis* and *Geotrichum candidum*	Pome fruits, citrus, cherries and potatoes
BlightBan	*Pseudomonas fluorescens* A506	NuFarm, Inc., USA	*Erwinia amylovora* russet-inducing bacteria	Pome fruits, stone fruits, potatoes, tomatoes
BoniProtect	*Aureobasidium pullulans*	Bio-ferm, Germany	*Penicillium*, *B. cinerea*, *Monilinia* and *Gloeosporium*.	Pome fruits
Companion	*Bacillus subtilis* GB03	Growth Products, USA	*Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*	All plants in greenhouse and nursery
Galltrol	*Agrobacterium radiobacter* 84	AgBioChem, Inc., USA	Crown gall caused by *Agrobacterium tumefaciens*	Fruit, nut and ornamental nursery stock
Mycostop	*Streptomyces griseoviridis* K61	Kemira Agro Oy, Finland	*Fusarium*, *Alternaria brassicola*, *Phomopsis*, *Botrytis*, *Pythium* and *Phytophthora*	Ornamental and vegetable crops
Prestop	*Glicocladium catenulatum* J1446	Verdera, Finland	*Pythium*, *Rhizoctonia* and *B. cinerea*	Vegetables, herbs and ornamentals
RootShield	*Trichoderma harzianum* T-22	BioWorks, USA	*Pythium*, *Rhizoctionia*, *Fusarium*, *Thielaviopsis* and *Cylindrocladium*.	Greenhouse, nursery and vegetable crops
Serenade	*Bacillus subtilis* QST713	AgraQuest, USA	Powdery mildew, downy mildew, early blight, brown rot, fire blight	Cucurbits, grapes, vegetables, pome fruits, stone fruits
YieldShield	*Bacillus pumilus* GB34	Gustafson, Inc. USA	Soil pathogens	Soybean

(B) *Bacillus subtilis*

Bacillus subtilis has been extensively studied as a BCA due to its broad spectrum of inhibition against various plant pathogens as well as a tolerance to a wide range of environments. For example, *Bacillus subtilis* 14B produced a novel bacteriolytic, antimicrobial peptide called Bac14B which had a broad range of inhibition toward Gram-positive and Gram-negative pathogens (Hammami *et al.*, 2012). It also stimulated the seedling vigour of tomatoes and muskmelons. When plant seeds were treated with Bac14B, percentage seed germination, shoot weight, shoot height and root length of the plants were significantly enhanced. It was also effective as a disinfectant against seed-borne disease and in controlling damping-off diseases. *Bacillus subtilis, Pseudomonas cepacia* and *Pseudomonas corrugata* were reported to have an inhibitory effect against *M. fructicola* on peach with *B. subtilis* as the most effective isolate (Bosch *et al.*, 1992).

Zhang *et al.* (2011b) studied two strains of *B. subtilis* (SB01 and SB24) on soybean under field conditions against *S. sclerotiorum* and showed that the activity of the cell suspension was better than cell-free culture filtrates. Cell suspensions applied 10 days before inoculation of the pathogen reduced disease by 20%-90% when assessed five days after inoculation of the pathogen. Efficacy and longevity of the effect of these BCAs was increased when soybean was treated with BCAs less than three days before inoculation and assessed after 15 days. However, Zhang *et al.* (2011b) cautioned that the cell density of *B. subtilis* was affected by weather conditions and was reduced, for example, when it rained after application. An endophytic *B. subtilis* was shown to inhibit *Gaeumannomyces graminis* var. tritici E1R-j (Liu *et al.*, 2009). This strain also inhibited *Coniothyrium diplodiella*, *Phomopsis* sp. and *S. sclerotiorum*. In a greenhouse experiment, a soil drench with cells of this isolate reduced take-all disease in seedings when assessed at four weeks after sowing.

Four strains of *B. subtilis* showed antifungal activity against cucurbit powdery mildew and two of these also had antibacterial activity against *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum* (Zeriouh *et al.*, 2011). Yang and Sung (2011) showed that three commercial BCAs of *B. subtilis* were able to increase seed germination of bitter gourd and to reduce mildew infection by *R. solani*. Wen *et al.* (2011) studied bioactivity of an endophytic *B. subtilis* SEBS05. Fermentation broth and cell-free culture filtrate of *B. subtilis* reduced wheat sharp eyespot disease by *Rhizoctonia cerealis*. There was no difference in inhibition between the fermentation broth and cell-free culture broth suggesting secreted compounds played a major role. These broths were subjected to chemical separation and results showed that the bioactive

compounds were mixtures of β-hydroxyl C12-C15-Leu^7 surfactin A isomers which differed in molecular weight. The same strain of *B. subtilis* completely inhibited *M. laxa* and *M. fructigena* in *in vivo* tests on stone fruit compared with 70% and 90% infection in control treatments, respectively (Yanez-Mendizabal *et al.*, 2011). Serenade, a commercially-available agrochemical containing living cells of *B. subtilis* QST713, suppressed blossom infection of blueberry caused by *Monilinia vaccinii-corybosi* (Ngugi *et al.*, 2005).

(C) *Bacillus amyloliquefaciens*

A genomic analysis of *B. amyloliquefaciens* strain FZB42 showed that it harboured giant gene clusters involved in the synthesis of lipopeptides and polyketides, important classes of organic compounds showing antifungal, antibacterial and nematicidal activities (Chen *et al.*, 2009). Nihorimbere *et al.* (2012) also reported that the production of lipopeptides by *B. amyloliquefaciens* S449 inoculated into roots of tomato was qualitatively and quantitatively induced by the specific nutritional context of the rhizosphere and the formation of biofilm structures on root hairs.

Arrebola *et al.* (2010a) reported that iturin A was the principle inhibitor produced by *B. amyloliquefaciens* PPCB004. Iturin A extracted and purified from culture broth of isolate PPCB04 was able to inhibit seven citrus post-harvest pathogens including *Alternaria citri, Botryosphaeria* sp., *Colletotrichum gloeosporioides, Fusicoccum aromaticum, Lasiodiplodia theobromae, Penicillium crustosum* and *Phomopsis persea.* For citrus fruit trials, oranges were artificially inoculated with three pathogens including *A. citri, C. gloeosporioides* and *P. crustosum.* The contribution of iturin A to biological control was confirmed by comparison of isolate PPCB004 (an iturin A producer) with *B. amyloliquefaciens* PPCB004itu-, a mutant strain which cannot produce iturin A as well as with *B. subtilis* UMAF6639 and *B. subtilis* PPCB001 used as positive and negative controls (iturin A producer and non-producer, respectively). The two strains which were iturin A producers significantly decreased disease incidence on oranges caused by the three pathogens.

Bacillus amyloliquefaciens PPCB004 also inhibited three other pathogens, *Botrytis cinerea, Penicillium expansum* and *Rhizopus stolonifer* on peaches (Arrebola *et al.*, 2010b). Isolate PPCB004 was combined with essential oil extracted from lemongrass. The combined treatment completely inhibited hyphal growth of the pathogens *in vitro* on agar plates. Peaches artificially inoculated with the pathogens were incubated at 25°C for five days, the combination treatment with isolate PPCB004, lemongrass essential oil and a

modified atmosphere packaging all resulted in lower disease levels than either the individual treatments or the control treatment. In a naturally infected peach experiment, the combination treatment showed an absence of disease and retained its overall appearance during storage at 4°C for fourteen days plus market shelf conditions at 20°C for two days.

A culture filtrate from *B. amyloliquefaciens* RC-2 (originally isolated from healthy mulberry leaves) had activity against *Colletotrichum dematium*, the mulberry anthracnose fungus (Yoshida *et al.*, 2001). The treatment had to be applied before inoculation of the pathogen, which showed that the culture filtrate had a preventive rather than curative effect. Isolate RC-2 also inhibited other plant pathogens including *Rosellinia necatrix, Pyricularia oryzae, Agrobacterium tumefaciens* and *X. campestris* pv. *campestris* when tested *in vitro*. One of the major products was identified as iturin A2. Alvarez *et al.* (2012) reported that two strains of *B. amyloliquefaciens* (MEP218 and ARP23) produced lipopeptides showing bioactivity against *Sclerotinia sclerotiorum*, causal agent of stem rot disease of soybean. When the pathogen was treated with the lipopeptide-containing supernatant from cultures of *B. amyloliquefaciens*, the mycelial morphology and sclerotial germination were negatively affected. When these bacteria were sprayed on leaves of intact soybean plants prior to infection of *S. sclerotiorum*, they protected soybean from stem rot disease.

(D) *Bacillus* species antagonistic to *Monilinia* spp.

Bacillus species have been shown to control brown rot disease on stone fruits. Blossoms of apricots were artificially inoculated with *M. laxa* and then treated with *Bacillus* sp. OSU142 to reduce disease levels relative to control treatments (Altindag *et al.*, 2006). Following artificial inoculation of *M. laxa* on peach and nectarine, a combination of hot water treatment at 60°C for 40 seconds, followed by 2% (w/v) sodium bicarbonate for 40 seconds and *B. subtilis* CPA-8 ($1x10^7$ cells/ml) reduced brown rot under two different storage conditions: (1) after incubation at 20°C for five days, brown rot infection was significantly reduced in combination treatments, (2) after incubation at 0°C for 21 days plus 20°C for five days, although the low temperature reduced the efficacy of individual and combined treatments. Casals *et al.* (2010c) also conducted an experiment on peaches using natural inoculum. Both individual and combined treatments reduced brown rot incidence when peaches were incubated at 20°C for five days, but *B. subtilis* CPA-8 alone or in the combined treatment failed to control brown rot when the fruits were also incubated at low temperatures. These results showed that that efficacy and degree of protection by *B. subtilis* CPA-8 was related to storage temperature. Yanez-Mendizabal *et*

al. (2012) assayed cell-free culture broths and chemical extracts from isolate CPA-8 against *M. fructicola* and *M. laxa*. An agar plug of the fungal pathogen was placed at the centre of Petri dishes and wells made into the same agar plate were filled with 100 μl of cell-free supernatant or extract. Antifungal compounds (the lipopeptides fengycin, iturin and surfactin) were isolated from the cell-free supernatant to establish individual antifungal activity. Thin layer chromatography (TLC) showed fengycin was a major contributor of antifungal activity.

Treatment at 50°C for two hours and 95%-99% relative humidity (RH) had been shown to reduce pre-harvest infection of *Monilinia* species already established in the field (Casals *et al.*, 2010a; Casals *et al.*, 2010b). These treatments were combined with treatment with isolate CPA-8 in further studies (Casals *et al.*, 2012). When artificially inoculated peaches were incubated at 20°C for five days, *B. subtilis* CPA-8 alone or combined with 50°C for two hours and 95%-99%RH significantly reduced *M. fructicola* brown rot infection compared with control treatments. However when the inoculated peaches were stored at 0°C for seven days followed by 20°C for five days, the efficacy of isolate CPA-8 was markedly decreased. Treatment with CPA-8 alone and the combined treatment were unable to reduce brown rot infection when the fruits were stored at low temperature. These results clearly show that efficacy of *B. subtilis* CPA-8 is temperature-dependent.

Both bacterial culture broth and cell-free culture filtrates of *Bacillus amyloliquefaciens* C06 inhibited brown rot caused by *M. fructicola* on peaches (Zhou *et al.*, 2008). Bacterial cells alone failed to control the disease showing that inhibitory activity came from organic compounds produced and secreted into the broth. Liu *et al* (2011) reported that cell-free culture filtrates of *B. amyloliquefaciens* C06 inhibited mycelial growth and conidial germination of *M. fructicola* in an *in vitro* test. They extracted and purified the antifungal compounds bacillomycin D and fengycin from the culture broth and showed that the biosynthesis of the compounds was regulated by two genes (bmyC and fend, respectively) on the chromosome of *B. amyloliquefaciens* C06. Conidial germination of *M. fructicola* was inhibited by the joint contribution of bacillomycin D and fengycin but its mycelial growth was supressed by fengycin. In addition Liu *et al.* (2010) reported that an isolate C06 produced extracellular mucilage as γ-polyglutamic acid which promoted colonisation and maintained a mass of cells on the plant surface.

(E) Inconclusive taxonomy of *B. subtilis* and *B. amyloliquefaciens*

Bacillus amyloliquefaciens is closely related to *B. subtilis* and used to be classified within this latter species (Breed *et al.*, 1957; Logan and Berkeley, 1984). It was elevated

to a new species based on physiological and molecular characteristics (Nakamura, 1987; Priest *et al.*, 1987). However the lack of distinctive phenotypic characteristics can lead to misidentification between these two species. *Bacillus amyloliquefaciens* can be distinguished from *B. subtilis* using (1) biochemical tests, (2) chemical profiles from gas liquid chromatography, (3) DNA-DNA hybridisation and (4) phylogenetic analysis using DNA sequences coding for recombination protein (rec A) or enzyme gyrase (gyr) (Chun and Bae, 2000; O'Donnell *et al.*, 1980; Wang *et al.*, 2007).

For identification of an unknown isolate, it must be compared with type strains. For example *Bacillus* isolate BCRC14193, originally identified as *B. velezensis* was later reassigned to *B. amyloliquefaciens* based on gyr DNA analysis by comparing with type strains of *B. velezensis* and *B. amyloliquefaciens* (Wang *et al.*, 2008). Likewise *Bacillus* strain S499 was originally identified as *B. subtilis* based on conventional methods, but re-classified as *B. amyloliquefaciens* based on gyr sequencing (Nihorimbere *et al.*, 2012).

1.6.2 Fungal biological control

(A) Yeasts

Yeasts or yeast-like fungi are attractive candidates as BCAs, as many fruit surfaces have a natural 'bloom' of these organisms on the mature fruit surface. Janisiewicz *et al.* (2010) point out that the resident fruit microflora may be more acceptable to the public and regulatory agencies, as they have been consumed with fruit for millennia without any known adverse effects. In their study of yeasts associated with nectarines and their potential as BCAs, these authors suggested that *Cryptococcus, Rhodotorula* and *Sporidiobolus* might be the genera to focus on as they colonise fruit throughout the growing season, as does the yeast-like fungus *Aureobasidium pullulans* which has also been reported as an effective BCA (Ippolito and Nigro, 2000; Lidon *et al.*, 2012; Mari *et al.*, 2012a; Mari *et al.*, 2012b; Robiglio *et al.*, 2011).

Aureobasidium pullulans is a cosmopolitan and ubiquitous yeast which occurs commonly in diverse environments from damp stones, clinical laboratories, food, plants and human surfaces. It has been developed and used as a BCA for decades. Some of these strains have been studied and produced on an industrial scale - for example 'BoniProtect' from the company Bioferm (Bio-ferm, 2011; Trapman *et al.*, 2010). Living cells of *A. pullulans* were effective against *B. cinerea* and *P. expansum* on stored apples (El Hamouchi *et al.*, 2007; Mounir *et al.*, 2007). An isolate of *A. pullulans* reduced the incidence and lesion diameter of blue mould disease caused by *P. expansum* on pears (Ferreira-Pinto *et al.*, 2006). Vero *et al.*(2009) suggested that *A. pullulans* was a favourable

and potential candidate for commercial development for post-harvest treatment. Their study showed that nine of 10 strains of epiphytic *A. pullulans* isolated from apples had bioactivity against blue and grey mould disease when tested under storage conditions. The one showing highest activity was able to grow in a wide range of temperatures and was resistant to fungicides commonly used for post-harvest treatment.

The production of lytic enzymes can be important for antagonistic activity by fungal BCAs, but *A. pullulans* may also show activity against plant pathogens via competition on nutrients. Bencheqroun *et al.* (2007a) showed that *A. pullulans* strain Ach I-I greatly reduced spore germination of *P. expansion* when nutrients were limited in low concentrations of apple juice (0.5%), but spore germination of the pathogen was higher when nutrient levels were increased by adding 5% apple juice. Zhang *et al.* (2010c) demonstrated the effect of inoculum size of *A. pullulans* PL5 on antagonistic activity. At higher concentrations ($1x10^8$ cells/ml), the isolate PL5 greatly reduced spore germination and germ tube elongation of *M. laxa*, whereas lower concentrations ($1x10^6$ and $1x10^7$ cells/ml) only inhibited *M. laxa* moderately.

Thirty-five isolates of *A. pullulans* originally obtained from sweet cherry flesh tissue showed a wide range of control against two post-harvest pathogens, *M. laxa* and *B. cinerea* in artificially-wounded sweet cherries and table grapes (Schena *et al.*, 2003). When applied at post-harvest, two of these isolates (533 and 547) gave the highest reduction of rot incidence in both sweet cherries and table grapes over 2 years. Isolate 547, applied at pre-harvest, also reduced post-harvest rot. In addition to the bioactivity, isolate 547 was capable of surviving under field conditions, and could reproduce during cold storage and penetrate the flesh of cherry blossom - all important attributes for biological control.

Efficacy of *A. pullulans* against *M. laxa* has been reported on plums and peaches under two storage conditions (Zhang *et al.*, 2010b). *Aureobasidium pullulans* PL5 was able to reduce disease incidence on plums and peaches which were stored at 1.2°C for 28 days and 1.0°C for 21 days, respectively. Mari *et al.* (2012a) found two strains of *A. pullulans* inhibited three brown rot pathogens including *M. laxa, M. fructicola* and *M. fructigena* tested on peaches and nectarines. Living cells of these BCAs completely inhibited rot development caused by *M. laxa* and *M. fructicola* and reduced infection caused by *M. fructigena* by 70%-90% in *in vivo* tests. To assess post-harvest activity, nectarines were treated with two BCAs and stored at 0°C for 21 days followed by seven days at 20°C. When assessed after 21 days, the low temperature had reduced brown rot infection in all treatments. However when nectarines were assessed again after seven days at 20°C, the treatment with *A. pullulans* significantly reduced *Monilinia* infection compared to

untreated nectarines. Zhang *et al.* (2010a) demonstrated that *A. pullulans* PL5 reduced brown rot incidence caused by *M. laxa* to 25.8% on peaches relative to 74.2% for untreated controls. When peaches were dipped in hot water at 55°C for 50 seconds and then cooled at 1°C for 10 minutes and then treated with isolate PL5, infection was reduced further to 17.5%. These results showed that *A. pullulans* is a potential candidate for commercial production by having activity against a broad spectrum of plant pathogens and being adaptable to combination with other treatments.

(B) Filamentous fungi

A mycological approach has been used by Spanish scientists in seeking fungal BCAs with activity against *M. laxa* (De Cal *et al.*, 2009; Guijarro *et al.*, 2008a; Larena *et al.*, 2005; Larena *et al.*, 2010). Initial progress in controlling twig blight was made using a strain of *Penicillium frequentans* isolated from healthy peach twigs (Melgarejo *et al.*, 1986). *Penicillium frequentans* may inhibit disease development through the production of antibiotics which suppress melanin synthesis in *M. laxa*. Unmelanized hyphae produce fewer conidia, leading to lower pathogenicity (De Cal and Melgarejo, 1994). For post-harvest control, conidia of *P. frequentans* were produced by liquid and solid state fermentation for testing as a BCA. Treatment of plum fruits with fresh conidia of *P. frequentans* reduced the incidence and lesion diameter of brown rot caused by *M. laxa* (De Cal *et al.*, 2002; Larena *et al.*, 2004). More effective control was achieved when additives such as sodium alginate or carboxymethyl cellulose were included to increase adhesion of conidia to fruit surfaces (Guijarro *et al.*, 2008a).

Twig canker and brown rot of peaches caused by *M. laxa* has also been controlled using conidial suspensions of *Epicoccum nigrum* (De Cal *et al.*, 2009; Madrigal *et al.*, 1994). Wettable powder formulations were made from conidia produced in solid-state fermentation systems and then dried using a fluid-bed drier to preserve viability for over 12 months (Larena *et al.*, 2003). When 2.5% methylcellulose was incorporated into the conidial formulation (Larena *et al.*, 2010), the adhesion of *E. nigrum* to peach surfaces improved with corresponding increased biocontrol efficiency. Applications of *A. pullulans* and *E. nigrum* (cited as the pseudonym *E. purpurescens*) to cherry blossoms have been shown to reduce the incidence of latent infections by *M. fructicola* (Wittig *et al.*, 1997).

1.7 Combination treatments

Combining chemicals with other treatments may increase control efficacy: for example adding chitosan in a combination with a short hypobaric treatment can reduce

brown rot in sweet cherry (Romanazzi *et al.*, 2003). Combination of essential oils with heat treatment by immersion in 48 or 52°C for three minutes and then spraying with 0.5% essential oil extracted from thyme (*Thymus vulgaris*) was an effective method to control *M. laxa* on peaches although the higher water temperature induced a colour change in peaches (Lopez-Reyes *et al.*, 2011). Hot water dipping at 48°C for two minutes increased residues of fludioxonil, a fungicide by reducing the dissipation rate from the fruit surface of nectarines, apricots and peaches (D'Aquino *et al.*, 2007; Schirra *et al.*, 2005). Combination of immersion of fruits into water and radio frequency significantly reduced brown rot in peaches and nectarines (Sisquella *et al.*, 2013).

Treatment with a high concentration of fludioxonil (100 mg/L) with 20°C of water was more effective than dipping in 48°C alone. However a combination of quarter strength fludioxonil (25 mg/L) with 48°C further improved fungicide performance, hence reducing fungicide inputs. A combination of physical, chemical and biological treatments was tested on nectarines and peaches in France, Italy and Spain. Treatment with hot water (40°C for two minutes or 60°C for 20 seconds) reduced brown rot in all cases (Mari *et al.*, 2007). Treatment with fresh and formulated cells of *E. nigrum* also reduced brown rot incidence on nectarines. Pre-harvest treatment with either *E. nigrum* or a fungicide and followed by post-harvest physico-chemical treatment (dipping in 1% sodium bicarbonate at 60°C for 20 seconds) reduced rot caused by *Monilinia* species on peaches and nectarines. This shows that combination treatments at different handling times may improve brown rot disease control.

Integrated control involving use of a BCA in combination with a fungicide has been used successfully to control *M. fructicola* on cherry (Spotts *et al.*, 2002), and *M. laxa* on peaches and nectarines (Casals *et al.*, 2010c; De Cal and Melgarejo, 1992), but non-chemical methods are preferred as many countries have now banned the post-harvest use of fungicides. Each of three antagonists, *Pseudozyma fusiformata, Metschnikowia fructicola* and *A. pullulans,* was combined with a treatment with hot water dipping to control *M. laxa* on peaches (Zhang *et al.*, 2010a). When these antagonists were individually applied alone (without hot water treatment), the antagonists reduced brown rot incidence in untreated controls from 74.2% to 28.3%, 30.0% and 25.8%, respectively. When antagonists were applied and followed by hot water treatment (55°C for 50 seconds), control was further improved to incidences of 16.7%, 15.8% and 17.5%, respectively. This result emphasised that a combination treatment is a good candidate to replace fungicide treatment if it could be developed economically for commercial post-harvest use.

1.8 Objectives of the study

The main objectives of this project were:

(1) To search for biological control agents (BCAs) against *Monilinia laxa* causing brown rot disease on cherries and plums.

(2) To develop the most promising BCAs for use under field and storage conditions.

(3) To use molecular analyses of the pathogen, *M. laxa*, to understand population epidemiology and inoculum sources.

The first objective was tackled using a dual culture technique to screen microbes for their bioactivity against *M. laxa*. A number of microbes went onto secondary screening using an *in vivo* test of BCAs against *M. laxa* on cherries and plums. Combinations of BCAs and physical treatment (storage at low temperature) to reduce brown rot infection were also investigated. To achieve these objectives the following studies were conducted:

1.8.1 To isolate and test indigenous BCAs for their potential against *M. laxa* using an *in vitro* technique (Chapter 3)

1.8.2 To assess efficacy of indigenous BCAs and existing BCAs against *M. laxa* in an *in vivo* test on cherries and plums (Chapter 4)

BCAs giving the highest bioactivity and consistent results were selected. These BCAs were identified at generic and species level. As *M. laxa* is able to infect cherries and plums over a wide range of temperatures and at different stages of stone fruit production, the capability of BCAs to reproduce and survive as similar temperatures to *M. laxa* were studied. Mechanisms of action of BCAs against *M. laxa* were also investigated.

1.8.3 To identify isolates and study the capability of selected BCAs to grow and survive at different temperatures (Chapter 5)

1.8.4 To examine the mode of action of BCAs against *M. laxa* (Chapter 5)

To effectively control brown rot disease, it is critically important to understand whether the fungal populations of *M. laxa* at different stages of infection are same. If the principal source of inoculum is the population over-wintering on mummified fruits, then eliminating mummified fruits should be sufficient to reduce fruit rot infection and latent infection at pre- and post-harvest, respectively. Morphological characteristics fail to differentiate species variation within *M. laxa*. Simple sequence repeat (SSR) molecular

markers have been developed and used to distinguish and compare the populations of *M. laxa* isolated from different stages of infection. In addition to control *M. laxa* at flowering and fruiting, an ability of BCAs to control *M. laxa* on mummified fruits was studied to reduce this important source of inoculum in the field.

1.8.5 To compare the population of *M. laxa* originally isolated from two different stages of infection including mummified fruits and fruit brown rot (Chapter 6)

1.8.6 To assess efficacy of indigenous BCAs and existing BCAs to suppress sporulation of *M. laxa* on mummified plums (Chapter 7)

The ultimate goal was to assess whether the selected BCAs had potential to reduce *M. laxa* infection on cherries and plums, either by pre-harvest or post-harvest application, and to assess their potential for commercial development.

Chapter 2
Materials and methods

2.1 Isolation of microbes and primary screening

2.1.1 Isolation of microbes from plant samples

Microbes, including bacteria and yeasts, were isolated from mummified cherries, mummified plums, green plums, and healthy leaves of cherries and plums collected from orchards within Kent. The details of plant samples are given in the Table 2.1. Microbes were isolated from samples of ten mummified cherries, five mummified plums, five green plums, five plum leaves and five cherry leaves. Plant materials were submerged in 100 ml of 1% Triton X-100 contained in a 250 ml Duran bottle. The bottle was shaken at 200 rpm for 1 hr using a shaking incubator at room temperature. Isolation was done using four sets of plant samples per collecting site. Serial dilutions (from 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were prepared and a 200 μl of aliquot of each dilution was spread on isolation plates. Plates for isolation of bacteria contained nutrient agar (NA) amended with cyclohexamide to suppress fungal growth, whilst plates for isolation of yeasts contained malt extract agar (MEA) amended with streptomycin and chloramphenicol to prevent growth of bacteria. The details of microbiological agar and antibiotics are given in Appendix 1. The droplets were spread over the plate using a sterile glass rod and then allowed to dry in a flow cabinet. The plates were then incubated at 25°C for 3 d. Each dilution was done in duplicate. Colonies which grew on the media were randomly selected, although preference was given to isolates from similar colonies which appeared in large numbers. Around 20-30 bacterial and yeast isolates per collecting site were picked up and streaked on fresh NA and MEA plates without antibiotics and incubated at 25°C for 3 d.

2.1.2 Culture maintenance of microbes

Bacterial and yeast isolates were maintained in liquid nutrient broth (NB) and malt extract broth (MEB), respectively. Single colonies from agar media without antibiotics from Section 2.1.1 were transferred into 2 ml screw cap tubes containing 0.5 ml of the liquid media. The tubes were shaken at 200 rpm on a shaking incubator for 6 hr to ensure that they had entered log phase. After this time, 0.5 ml of 30% glycerol was added to the cultures and the tubes were mixed thoroughly using a vortex mixer before being placed at -80°C for storage.

Table 2.1 Codes, collecting sites and date of isolation of plant samples used for microbe isolation.

Code	Original code	Substrate	Collecting site	Date of isolation
1M-CH	R35/10	Mummified cherries	A.R. Neaves & Sons Limited Little Sharsted Farm Dottington, Sittingbourne, Kent	17 May 2010
1M-PL	R51/10	Mummified plums	A.R. Neaves & Sons Limited Little Sharsted Farm Dottington, Sittingbourne, Kent	24 May 2010
2M-PL	R118/10	Mummified plums	John Myatt Eende-Koy Decoy Hill Road, High Halston, Rochester, Kent	22 Jun 2010
2M-CH	R21/1	Mummified cherries	A.R. Neaves & Sons Limited Little Sharsted Farm Dottington, Sittingbourne, Kent	29 Jun 2010
3GP	-	Green plums	A former test plot, East Malling Research Station New Road, East Malling, Kent	29 Jul 2010
1PL-P	-	Leaves of plums	A former test plot, East Malling Research Station New Road, East Malling, Kent	6 Sept 2010
1CH-L	-	Leaves of cherry	A former test plot, East Malling Research Station New Road, East Malling, Kent	13 Sept 2010

2.1.3 Primary screening on malt extract agar

A dual culture technique was used to determine the initial inhibition by the potential BCAs against *M. laxa*. This rapid method was used to screen the large number of isolates from the initial isolation process. An inoculating loop was used to pick up three to four colonies of either bacterial or yeast isolates grown for 3 d at 25°C on NA and MEA (for bacteria and yeasts, respectively) and microbes were inoculated on MEA at 1 cm away from the margin of the Petri dish (Figure 2.1). The plates were incubated for 3 d at 25°C to allow the microbes to grow.

Two test isolates (A, B in Figure 2.1) were placed on the same plate and a third area left without any inoculum as a control (C in Figure 2.1). An agar plug of *M. laxa* cut from an actively-growing colony margin was taken using a cork borer no. 2 (5 mm diam), and placed culture-side down in the centre of the MEA plate. There were three replicate plates per test. The plate was then incubated for 14 d at 25°C. Mycelial growth of *M. laxa* was measured using a digital Vernier calliper. 'Radius of test' was the distance from the mycelial front of *M. laxa* (M) to A or B and 'Radius of control' was distance from the mycelial front of *M. laxa* (M) to C. The percentage inhibition was calculated using the equation below.

$$I = \left[\frac{(D_2 - D_1)}{D_2}\right] \times 100\%$$

I = Percentage of inhibition D_1 = Radius of test (mm) D_2 = Radius of control (mm)

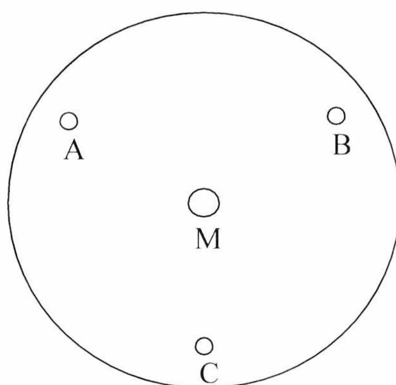


Figure 2.1 Diagram showing the dual culture on MEA, A and B (inoculum of test microbes), C (control) and M (*M. laxa*).

2.1.4 Secondary screening on potato dextrose agar

To confirm bioactivity and its consistency on different media, bacterial and yeast isolates showing bioactivity against *M. laxa* from the previous section were tested on potato dextrose agar (PDA) using the method described above with slight modification. As before, a potential antagonist was inoculated 1 cm away from the margin of the Petri

dish, but in this case only a single test isolate was inoculated per plate. The plates were incubated at 25°C for 3 d and an agar plug of *M. laxa* was inoculated 1 cm away from the margin of Petri dish on the opposite site (Figure 2.2). A sterile agar plug was used as a control.

Mycelial growth of *M. laxa* in the absence and presence of a microbe was measured using a digital Vernier caliper at 14 d after the inoculation with *M. laxa*. 'Radius of test' was the distance from the mycelial front of *M. laxa* (M) to A (left diagram in Figure 2.2) and radius of control was distance from the mycelial front of *M. laxa* (M) to C (right diagram in Figure 2.2). The percentage inhibition was calculated as before. There were five replicate plates per treatment.

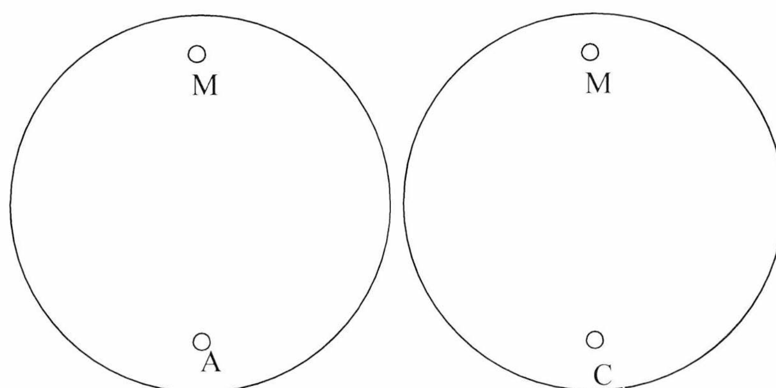


Figure 2.2 Diagram showing the dual cultures on PDA, A (a test microbe), C (control) and M (*M. laxa*).

2.1.5 Comparison of inhibition on MEA and PDA

Inhibitory activities against *M. laxa* obtained on MEA and PDA was compared. The threshold inhibition for isolates to progress to the next screening was based on bioactivity of potential microbes. Scatter plots were used to illustrate the relationship of percentage inhibition conducted on the two media.

2.1.6 Inhibition test of known BCAs

Three BCAs which were previously screened and shown to have inhibitory activity against plant pathogens were tested against *M. laxa*. Table 2.2 shows the details of these three BCAs. The first was *Epicoccum nigrum* ATCC96794, kindly provided by Dr. Antonieta De Cal y Cortina from the Instituto Nacional de Investigacion y Techologia Agraria y Alimentaria (INIA), Spain, and currently being used there successfully as a BCA of *M. laxa* (De Cal et al., 2009). In addition, two bacteria previously isolated at East Malling Research were included: *Bacillus pumilus* NCIMB13374 and *Pseudomonas fluorescens* NCIMB13373 originally isolated by

Swadling and Jeffries (1998). A dual culture technique described in Section 2.1.4 was used to test the inhibitory activity of these BCAs but the test was conducted on MEA. There were three replicate plates per BCA. The percentage inhibition was calculated as before.

Table 2.2 BCAs selected to test against *M. laxa*.

Culture	Sources of culture	Target pathogen	Reference
E. nigrum ATCC96794	INIA	*Monilinia* spp.	De Cal *et al.* (2009)
B. pumilus NCIMB13374	NCIMB	*Botrytis cinerea*	Swadling and Jeffries (1998)
P. fluorescens NCIMB13373	NCIMB	*Botrytis cinerea*	Swadling and Jeffries (1998)

2.2 *In vivo* screening

2.2.1 Production of *M. laxa* inoculum

Intact plums were surface-sterilised by submerging in 70% (w/v) ethanol for 5 min then dried in a flow cabinet for 1 hr. Fruit were wounded on the shoulders in a triangle shape of 1 cm width and 0.5 cm depth using a sterile blade. An agar plug with mycelium (5x5 mm^2) from the margin of a 2-wk old culture of *M. laxa* was inoculated into each wound. Inoculated fruit were then placed in plastic boxes (two fruits per box). In spring and summer the inoculated plums were incubated at room temperature for 2-4 wks. In the autumn and winter, inoculated plums were incubated in a glasshouse at a constant temperate of 20°C. During the incubation period, lids were opened once a week for 30 min to increase air circulation.

Conidiophores of *M. laxa* were harvested *en masse* using sterile forceps and conidia were suspended in 5 ml of sterile distilled water in a screw-capped tube. Spore concentration was estimated using a haemocytometer and adjusted to $1x10^5$ spores/ml. A spore suspension was freshly prepared for each *in vivo* study. Germination of *M. laxa* spores was quantified by dropping 10 µl of the spore suspension on PDA plates and allowing the inoculum to dry on the agar. Plates were then sealed with Parafilm to maintain high humidity. There were four replicate plates and two inoculum drops per plate. Percentage spore germination was recorded after 4-12 hr.

2.2.2 Fruit picking and maintenance

Apparently healthy cherries and plums with stalks were picked by hand from commercial orchards in Kent. Fruits were randomly picked from different trees in different rows. Fruits were brought back to East Malling Research on the same day. Fruits were kept in plastic bags tied with rubber bands and stored at 4°C before experiments (Figure 2.3). There were 200-300 cherries per bag and 100-120 plums per

bag to avoid cracking and squashing of the fruits at the bottom of the bags. Cherries and plums were used in experiments within 2 and 4 wks of picking, respectively.

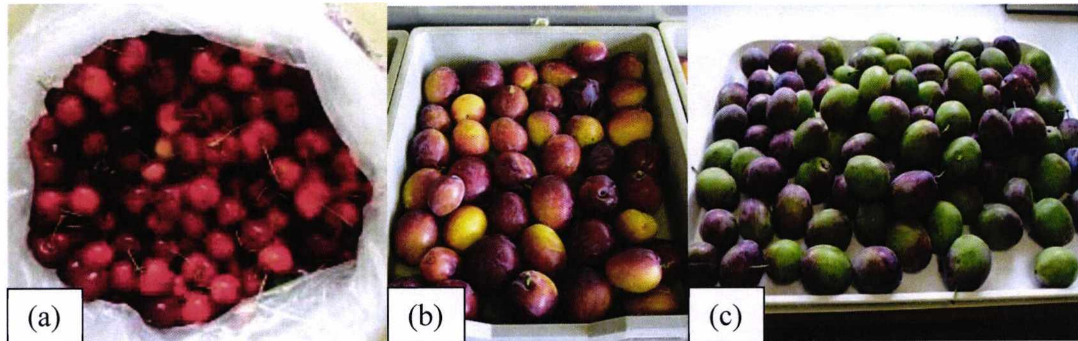


Figure 2.3 Stone fruits collected from orchards used in this study. (a): Cherries cv. Merchant, (b): Plums cv. Victoria and (c): Plums cv. Marjorie.

2.2.3 Efficacy of indigenous BCAs

Potential BCAs showing good bioactivity from Chapter 3 were further screened in *in vivo* tests. There were 12 isolates including one bacterium (isolate B91) and 11 yeasts (isolates Y51, Y52, Y69, Y80, Y89, Y100, Y101, Y104, Y106, Y10 and Y126). Potential BCAs were removed from low temperature and streaked onto NA or PDA, respectively. Plates were incubated at 25°C for 3 d in the dark. Single colonies were transferred into NB and PDB, respectively, and incubated at 25°C on a shaker incubator at 200 rpm for 4 d. Viable cell counts were determined using the serial dilution technique. An approximate concentration of BCAs between 10^7-10^8 CFU/ml was used in this study. There were 14 treatments including 12 BCAs. The fungicide Indar and sterile distilled water (DW) served as positive and negative controls, respectively. Cherries Section 2.2.2 were surface-sterilised by submerging in 1.5 L of 0.5% (w/v) of sodium hypochlorite for 15 min and then washed with 1.5 L of sterile distilled water for 15 min. After air-drying in a flow cabinet for 1 hr, cherries were stabbed with a sterile 200 µl-tip (one wound per cherry, ca. 2 mm depth). Five µl each of a cell suspension of the potential BCAs (as prepared above) was pipetted into wounds. Treated cherries were left to dry in a flow cabinet for 1 hr. Then 5 µl of the *M. laxa* conidial suspension ($1x10^5$ spores/ml) (as prepared in Section 2.2.1) was pipetted into the same wounds.

Treated cherries were placed in round plastic pots lined with filter paper (7 mm diam, Fisher brand) soaked with sterile distilled water to maintain high humidity and provide conducive conditions for infection by *M. laxa*. A small distance was left between cherries to avoid direct contact between artificially-infected wounds and the fruit surface of neighbouring cherries. Pots containing treated cherries were incubated at

room temperature on a bench in a completely randomised design. Cherries were assessed between 4-7 d after inoculation with *M. laxa* depending on development of disease. When brown rot or contamination was first seen, infected cherries were removed from the pots to avoid cross-contamination between fruits. The experiment was repeated once. There were ten and six replicates (plastic pots) per treatments, respectively, with five cherries per replicate.

The same set of treatments was tested on plums using a similar protocol with some modification. Plums cv. Victoria were used without surface sterilisation to observe natural infection. Plums were stabbed on two opposite sites on fruit shoulders using a sterile 200 µl-tip resulting in two wounds per plum (ca. 2 mm depth). Applying BCAs and inoculation with the *M. laxa* were carried out using a similar protocol used for the cherry experiment, using 10 µl of the cell suspension of potential BCAs and the spore suspension of *M. laxa*. Treated plums were placed in Phytatrays (Sigma Aldrich) without filter paper and incubated at room temperature. Boxes containing treated plums were incubated at room temperature on a bench in a completely randomised design. The experiment was repeated once. There were ten replicates (Phytatray boxes) per treatment with two plum fruits per replicate. Disease development (expressed as a percentage of plums infected) and lesion size of rotten wounds on plum were assessed between 4-7 d after inoculation of the pathogen. Lesion size (mm) was measured using a digital Vernier calliper. The lesion size of rotten wounds was assessed from every wound on the left-hand side of the plum fruits. If the wound on the left-hand side was not infected, the wound on the right-hand side was measured instead. The lesion size was the average of the vertical and horizontal diameters.

For percentage infection in cherries and plums, data were analysed separately. Lesion size on plums was analysed separately from percentage infection. Fruit containers of cherries and plums *i.e.* plastic pots and Phytratray boxes, respectively were treated as experimental units. Percentage infection was transformed using an angular transformation, while lesion size was analysed without transformation. For percentage infection, statistical analysis was performed and data presented based on transformed data. The data were analysed using one-way ANOVA, using treatment as a factor and percentage infection as a variate. For lesion size on plums, the data was analysed using one-way ANOVA using treatment as a factor, lesion size as a variate and repeat as block overtime.

2.2.4 Necrosis test on stone fruits

Results from the experiments in the previous section showed that isolates B91 and Y126 had high bioactivity against *M. laxa*. However some necrosis was observed around artificially wounded fruit surfaces on both cherries and plums treated with these two isolates. Therefore a necrosis test on intact fruits was studied. Cherry cv. Lanpins (from the US) and plum cv. Victoria were used in the test. Cherry cv. Lanpins were bought from a supermarket because British cherries were not available at that time in the year, while the plums were picked from a commercial orchard.

Intact cherries and plums fruits were sprayed with 20 ml of a cell suspension of the treatments (inocula prepared as in Section 2.2.3) until runoff and the fruits were allowed to dry for 1 hr. The treated cherries and plums were placed in plastic pots and Phytatray boxes, respectively. The fruit containers were incubated at room temperature with a completely randomised design and assessed 1 wk after treatment. There were three treatments; (1) distilled water, as control treatment, (2) B91 and (3) Y126. There were six and 15 replicates per treatment with four cherries and two plums per box, respectively. The percentage necrosis was calculated as the proportion of affected fruits. The experiments on cherries and plums were independently performed and data were analysed separately using angular transformation. The experiment on cherries was performed once while the experiment on plums was performed twice.

2.2.5 Effect of commercial BCAs, application times and cultivars

Several agrochemicals and commercially available BCAs are claimed to have activity against various plant pathogens including *Monilinia* species. However, there have been no published studies on their efficacy against *M. laxa* under field conditions or in post-harvest conditions. These agrochemicals and BCAs were evaluated for their activity against *M. laxa* on cherry and plum fruits. Rates of use and preparation followed the manufacturers' guidelines (Table 2.3). Two chemicals were tested, XzioX and KBV, and five BCAs, BioPK, BoniProtect, Trianum, Prestop and Serenade.

The commercially-available agrochemicals and BCAs were prepared at recommended rates. The products were suspended in sterile distilled water to minimise water-borne contamination. Each product suspension was made up in a hand-held sprayer, which was rinsed with absolute ethanol for 1 min and left to dry at room temperature for 15 min. The suspension was manually shaken for 10-15 s to ensure that active ingredients were homogenised. All treatments were used within 3 hr of preparation.

Table 2.3 Details of treatments, concentrations and rates of use of chemicals and BCAs used in the *in vivo* study.

Product name	Active ingredient	Targeted disease and pathogens	Stock concentration	Category	Company	Country of origin	Rate of use
Indar	Fenbuconazole	Apple scab, rusts, powdery mildew, blossom blight, fruit brown rot, peach scab and cherry leaf spot in stone fruit, mummy berry in blueberries	5%	Chemical control	Dow AgroScience	United States of America	2 ml/L
XzioX	Chlorine dioxide	*Escherichia coli, Pseudomonas* and *Legionella*. Waterborne diseases such as brown rot.	99.99%	Chemical control	Ximax Water solution	United Kingdom	0.35% (3,500 ppm)
KBV	Potassium bicarbonate		85%	Chemical control	Koppert Biological Systems	The Netherlands	3 g/L
BioPK + surfactant	*Trichoderma viride, T. harzianum* and *T. longibrachiatum*	Neck rot, crown rot, brown rot	30%	Biological control	Avance Biotechnologies	Chile	6 g/L + 0.5 ml of surfactant
BoniProtect	*Aureobasidium pullulans*	Storage pathogens; *Penicillium* sp., *Botrytis* sp., *Monilia* sp. and *Gloeosporium* sp. in pome fruit production (apple, pear and quince).	5.0×10^{9} CFU/g	Biological control	Bio-ferm GmbH	Germany	1 g/L
Trianum	*T. harzianum* strain T-22	Soil-borne diseases; *Fusarium, Pythium, Rhizoctonia* and *Sclerotinia.*	1.0×10^{9} spores/g	Biological control	Koppert Biological Systems	The Netherlands	10 g/L
PreStop	*Gliocladium catenulatum* strain J1446	Soil and foliar pathogens; *Pythium* sp., *Rhizoctonia* sp., *Botrytis* grey mould and stem cancer and *Didymella* gummy stem blight	1.0×10^{8} CFU/g	Biological control	Verdera	Finland	1.6 oz./1 gallon of water
Serenade	*Bacillus subtilis* strain QST 713	Fire blight, *Botrytis*, Sour Rot, Rust, *Sclerotinia*, powdery mildew, bacterial spot and white mold.	1.0×10^{9} CFU/ml	Biological control	AgraQuest, Inc.	United States of America	10 ml/L

In addition to efficacy of BCAs to inhibit *M. laxa*, timing in application plays an important role to delivery of target BCAs to protect plants. In this experiment, two application times, applying either before or after the inoculation with the pathogen, were compared. Cherries from Section 2.2.2 were used without surface-sterilisation. There were 14 treatments: seven control agents [distilled water (DW), Indar, Serenade, BoniProtect, Bio-PK, KBV and XzioX] and two application times (before and after the inoculation with the pathogen). Seven trays of cherries were sprayed with the seven individual treatments. The spraying was performed in plastic bags while another seven trays of cherries were put in plastic bags without spraying. All plastic bags were then tied with rubber bands to contain the moisture and humidity before being incubated in a controlled environment (CE) cabinet programmed with 20°C at day and 15°C at night with 12 hr light. After 24 hr, a spore suspension of *M. laxa* (Section 2.2.1) was sprayed onto all treatments and the trays of cherries in plastic bags were put back into the CE cabinet. The fruit that had not been initially sprayed with control agents was taken out 24 hr after the inoculation with *M. laxa*, and then treated with the individual seven control agents and put back into the CE cabinet. The assessment of brown rot infection was made visually 7 d after the inoculation with the pathogen. There was one replicate (tray) per treatment with 100 cherry fruits per replicate. The experiment was performed only once.

A similar method to the cherry experiment was used for the plum experiment with some modifications, using the same set of 14 treatments. Two plums cultivars, Victoria and Marjorie, were used in this study. Plums of each cultivar were sorted into 14 trays. The first set of seven trays of plums were sprayed until run-off with 100 ml of appropriate agrochemical or BCA treatment. Different treatments were performed independently in plastic bags; latex gloves were worn and changed each time when applying BCAs to avoid cross-contamination. Treated plums were then dried in a flow cabinet for 2 hr. In each treatment two plums were randomly selected and put in a Phytatray box (Sigma Aldrich). For the second set of seven treatments (for treatment with control agents after inoculation), plums were also put in Phytatray boxes. All plums were wounded 24 hr later using a sterile tip on the two opposite shoulders of each plum. After 1 hr air-drying in the flow cabinet, 10 μl of spore suspension of *M. laxa* ($1x10^5$ spores/ml; Section 2.2.1) was inoculated into each wound. The second set of plums were treated 24 hr after the inoculation with *M. laxa* with the seven control treatments and plums were put back into the boxes. The boxes containing inoculated plums were incubated at room temperature in a completely randomized design. Disease

development (percentage of infection) and lesion size (mm) were assessed between 4-7 d after the inoculation of *M. laxa*. There were six replicates (boxes) per treatment, with two plums per replicate (box). Experiments for each plum cultivar were repeated once. The experiment for each plum cultivar were performed independently, as these two plum cultivars ripen at different times.

Lesion size on plums was analysed separately from percentage infection. Boxes of cherries and plums were treated as experimental units. Percentage infection was subjected to angular transformation, while lesion size was analysed without transformation. For the plum experiments, the data were analysed using three-way ANOVA using treatments, application times and cultivars as factors, percentage infection (or lesion size) as a variate and repeat as block over time.

2.2.6 Effect of commercial BCAs and wound age

Although *M. laxa* can infect intact stone fruits, wounds are still a major route for brown rot infection. Therefore, the effect of wound age on brown rot infection was studied by comparing between fresh and one-day-old wounds. There were 14 treatments: seven control agents [distilled water (DW), Indar, Serenade, BoniProtect, BioPK, Prestop and Trianum] and two wound types (fresh and one-day-wounds). Cherries (Section 2.2.2) were surface-sterilised as before (Section 2.2.3). After air-drying in a flow cabinet for 1 hr, cherries were laid in a plastic tray, and sprayed with 100 ml of the appropriate treatment suspension using a hand-held sprayer until run-off. Each treatment was done in a plastic bag, the bag and tray were changed for each treatment to avoid cross-contamination between treatments. After treatment, the tray of cherries was taken out from the bag and cherries were dried for 2 hr in a flow cabinet.

Half of the cherries were wounded using a sterile tip on a shoulder to provide cherries with one-day-old wounds (one wound per cherry, 2 mm depth). All cherries were placed within round plastic pots on filter paper soaked with sterile distilled water to maintain high humidity and left at room temperature for 24 hr. The other half of the cherries were wounded the following day using a same method just before inoculation with *M. laxa*, resulting in fresh wounds. Five µl of an inoculum of *M. laxa* ($1x10^5$ spores/ml; Section 2.2.1) was inoculated into the fresh and one-day-old wounds. The inoculated cherries were incubated at room temperature in a completely randomised design. The experiment was repeated once. There were ten and six replicates (boxes) per treatment respectively, with five cherry fruits per replicate (box). Cherries were assessed for brown rot 7 d after the inoculation with the pathogen. When brown rot or

other symptoms were found, infected fruits were removed from the pots in order to avoid cross-contamination.

A similar method was used for a plum experiment, with slight modifications. Plums were not surface-sterilised in order to observe the natural infection. Ten μl of the inoculum of *M. laxa* was used and plums were put in Phytatray boxes (Sigma Aldrich). Plums were wounded using a sterile tip on the two opposite shoulders to provide plums with one-day-old wounds (two wounds per fruit). After the plums were kept at room temperature for 24 hr, the rest of plums were wounded using the same method just before the inoculation with *M. laxa*. The boxes containing inoculated plums were incubated at room temperature in a completely randomised design. Disease development (expressed as percentage infection) and lesion size were assessed between 4-7 d after inoculation. There were ten replicates (boxes) per treatment, with two plums per replicate (box). The experiment was repeated once. The data were analysed as described previously with two-way ANOVA using treatments and wound types as factors, percentage infection (or lesion size) as a variate and repeat as block over time.

2.2.7 Post-harvest treatment using semi-commercial storage conditions

Cherries were surface-sterilised using previously described protocols. There were 12 treatments; six treatments of control agents [untreated, distilled water (DW), Indar, Serenade, B91 and Y126]; and 2 incubation temperatures (room temperature and 1°C). After air-drying in a flow cabinet for 1 hr, 40 cherries were treated by spraying with 50 ml of the individual treatments with a hand-held sprayer. Each operation was done in a plastic bag to avoid cross-contamination. Cherries were dried for 2 hr, then stabbed with a tip (one wound per cherry). Five μl of a spore suspension of *M. laxa* was pipetted into the wounds. Inoculated cherries were placed in plastic boxes lined with soaked filter paper to maintain high humidity. The experiments were repeated three times. There were eight replicates (boxes) per treatment with five cherries per replicate (box). Boxes of cherries were incubated at room temperature or 1°C.

For plums, a similar method was used with some modifications. There were 12 treatments: six treatments with control agents [untreated, distilled water (DW), Indar, Serenade, B91 and Y126] and two incubation temperatures as before. Plums were treated first by spraying control agents, then 10 μl of a spore suspension of *M. laxa* was inoculated into the artificial wounds. The treated plums were put into Phytatray boxes. The first set of boxes was incubated at room temperature and the second set was

incubated at 1°C. There were ten replicates per treatment, with two plums per replicate. The experiment was repeated once.

For assessment after incubation at room temperature, the brown rot infection in cherries and plums was assessed within 7 d after inoculation with *M. laxa*. For treatments incubated at 1°C, the treatments were assessed at 4 wks after inoculation. The brown rot infection was assessed immediately after fruits were taken out from cold storage. Cherries and plums were left at room temperature and reassessed for infection 1 wk after they were taken out from cold storage. The data were analysed as described previously with two-way ANOVA using incubation temperature and treatments as factors, percentage infection (or lesion size) as a variate and repeat as block over time.

2.2.8 Post-harvest treatment using commercial storage conditions

(A) Production of BCA inocula for post-harvest treatment

The BCA isolates B91 and Y126, which were able to inhibit *M. laxa* on cherries and plums *in vitro* and *in vivo*, were selected for post-harvest studies. The BCAs were taken from storage at -80°C and sub-cultured onto NA and MEA for B91 and Y126, respectively. Plates were incubated at 25°C for 3 d. Single colonies were transferred by loop into 50 ml of liquid broth (NB and PDB, respectively) in a 100 ml Duran bottle. The bottle was incubated at 25°C on a shaker incubator at 200 rpm for 24 hr before 2 ml of this inoculum was transferred to each of 20 Duran bottles containing 500 ml of fresh liquid broth. These were shaken at room temperature on a shaker incubator at 150 rpm, and after 4 d of incubation, the cultures for each BCA were pooled. The resulting 10 L of inoculum was diluted with 15 L of sterile distilled water (25 L in total). Viable cell counts were made using the serial dilution technique. The concentrations of BCAs used in this study were between 10^7-10^8 CFU/ml.

(B) Dipping treatment

The two BCA isolates B91 and Y126 were compared to Serenade and Boni Protect, respectively. Apparently-healthy cherries were freshly picked from a commercial orchard and kept overnight in cold storage as described in Section 2.2.2. Cherries were randomly sorted into seven treatments: (1) Untreated, (2) Tap water, (3) Rovral, a standard fungicide for post-harvest control, (4) Serenade, (5) B91, (5) BoniProtect and (7) Y126. Rovral and tap water served as positive and negative controls respectively, while one batch of cherries was left untreated to indicate the natural levels of infection by *M. laxa*. The details of the seven treatments are given in Table 2.4.

Table 2.4 Details of post-harvest treatments.

Treatment	Product category	Manufacturer	Product rate
Untreated	Negative control	-	
Tap water	Negative control	-	
Rovral	Fungicide	Bayer Cropscience, UK	1.3 g/L
B91	BCA	From this study	$1 x 10^8$ CFU/ml
Y126	BCA	From this study	$1 x 10^7$ CFU/ml
Serenade	BCA	AgraQuest Inc	10 ml/L
BoniProtect	BCA	Bio-Firm	1 g/L

Cherries were stored in plastic trays (36 long x 26 wide x 11 depth cm^3). The trays were filled with cherries until nearly full to avoid any squashing when trays were stacked (Figure 2.4). There were four replicates (four trays) per treatment, with 400-500 cherries per tray. Tap water and Rovral were the first and last treatments to be applied, respectively, to avoid possible contamination of other treatments with fungicide residues. Trays of cherries were submerged into 25 L of liquid for each individual treatment for 1 min and gently shaken to ensure that all cherries were completely submerged. After drying at room temperature for 3 hr, all treated cherries were stored at 1°C for 6 wks at cold storage at the Jim Mount Building, EMR.

During storage, trays of cherries were covered with black plastic bags to maintain high humidity and to avoid dehydration of the cherries. Trays of cherries were arranged in a randomised block design of four blocks. In each block, there was one replicate from each treatment. The four blocks were allocated to the four corners of the cold store. Every week, the trays of cherries in the same block were shuffled before each block was rotated clockwise to ensure that all treatments had a similar exposure to the cold temperature. After 6 wks, the trays of cherries were taken out from cold storage and cherries were assessed for five different types of symptoms, including (1) brown rot caused by *M. laxa;* (2) brown rot caused by *M. fructigena;* (3) rot caused by *Botrytis cinerea;* (4) rot caused by *Mucor* spp. and (5) other rot. The fruit with the first four of these rot symptoms were counted and discarded from the trays. Those fruit with other types of rot were put separately in a punnet and kept for further assessment. All fruits were left for 1 further week in a glasshouse compartment at a constant temperature of 20°C and then assessed again. Percentage infection of cherries was calculated as:

$$I_1 = \left[\frac{N_1}{N_3}\right] \times 100\%$$

$$I_2 = \left[\frac{N_1+N_2}{N_3}\right] \times 100\%$$

I_1 = Percentage of infection at four weeks

I_2= Percentage of infection after one week

N_1= Number of infected cherries assessed immediately after they were taken out from cold storage

N_2= Number of newly infected cherries assessed at 1 wk after they were taken out from cold storage

N_3= Number of cherries in each tray

Experiments on plums cv. Victoria were carried out using a similar protocol with 80-100 plums per tray. Due to the high fruit price, the large number of fruits required and the limited harvest time, experiments on cherries and plums were carried out once only.

Figure 2.4 Cherries and plums used in post-harvest treatment. (a): Stacks of trays of cherries (b): Stacks of trays of plums, (c): A tray of cherries being dipped into a treatment and (d): Trays of cherries were left in a glasshouse for one week.

2.3 Molecular and ecological characterisation of BCAs

2.3.1 Molecular identification using ribosomal DNA sequences

(A) DNA Extraction

The two most successful BCAs (B91 and Y126) from *in vivo* screening (Chapter 4) were taken from storage at -80°C and streaked onto NA and MEA, respectively. The plates were incubated at 25°C for 3 d. Single colonies of each isolate were randomly picked and suspended in 1 ml of sterile distilled water in a 1.5 ml Eppendorf tube. The tubes were submerged in boiling water for 10 min to break the cell wall to extract the DNA (Fang and Hedin, 2003; Holmes and Quigley, 1981). The tubes were mixed using a vortex mixer. Crude DNA extract was put on ice and used as a DNA template.

(B) Primers

Primers were from Integrated DNA Technologies (IDT, Belgium). References of bacterial and fungal primers used in this study are given in Table 2.5 Primers in a lyophilised form were dissolved by adding double-processed sterile-filtered water also known as PCR water (Sigma-Aldrich) into the tubes containing the primers with the volume adjusted to 100 μM. These were stored as stock primers for PCR. The stock primers were diluted 1 in 50 for use as a working solution (2 μM). Stock and working primers were kept at -20°C and working primers were thawed on ice for a few minutes before use.

(C) PCR amplification

The ~1,000 bp small subunit (SSU) region of B91 was amplified using the two universal bacterial primers 27F and 1525R (Lane, 1991). A summary of concentration and volume of PCR reagents is given in Table 2.6. The amplification was carried out under the PCR conditions (Table 2.7) using a DNAEngine DYAD Thermocyler PTC-220 (MJ Research, Watertown, USA). Concentrations and volumes of the PCR mixture for fungal PCR amplification of Y126 DNA were similar to those used for B91 but the volumes of the DNA template and PCR water were adjusted to 2.5 and 24.3 μl, respectively.

(D) PCR product visualisation

Amplified PCR products were visualised using electrophoresis. Five μl of the PCR product was mixed with 5 μl of PCR water and 2 μl of loading dye. The mixture was loaded into a 3 mm deep well in a 1.6% (w/v) agarose gel. A DNA ladder (size

ranging from 100-1,500 bp) was used to size PCR products. The gel was placed in a gel tank containing TE buffer and run at 100 V for 90 min before being taken out and stained in ethidium bromide solution (0.5 μg/ml) for 20 min. Gel band patterns of PCR products were recorded using Image Lab Version 2.0.1, Bio-Rad Laboratory.

(E) Purification and DNA sequencing of PCR products

PCR products were purified using a QIAquick PCR purification kit (Qiagen Cat. No. 28104) following the standard protocol. PCR products with an initial volume of 45 μl were purified and eluted to a final volume of 35 μl. The purified PCR products together with the same primers from Table 2.5 used in the PCR amplification were sequenced by the Qiagen Sequencing Services, Germany.

(F) BLAST Search and sequence analysis

The DNA sequences of the two BCAs from the Qiagen Sequencing Services were first checked for their quality. The sequence regions for primer capture at the two terminal ends of the sequence were removed. Sequences in the same region were consented and assembled into a single strand DNA sequence from 5' to 3'. The consensus sequences were compared with sequences deposited in the GenBank DNA database using the BLAST search tool. Thirty sequences showing closest relationships were selected and downloaded into the FASTA format. The sequences were aligned using BioEdit Version 7.1.3 (Hall, 1999) and all sequences that differed in length were trimmed to the same length. A sequence similarity matrix was computed and identification was based on the sequence with the highest DNA similarity with the two BCA DNA sequences.

Table 2.5 Universal primers used for molecular identification of two BCAs.

Primer	Primer	Position	Region	Sequence	T_m (°C)	Reference
Bacterial primers	27F	Forward	SSU	5'-AGA GTT TGA TCM TGG CTC AG-3'	53.0	Lane (1991)
	1525R	Reverse	SSU	5'-AAG GAG GTG WTC CAR-3'	46.4	Lane (1991)
	P515FPL	Forward	SSU	5'-GTG CCA GCM GCC GCG GTA A-3'	63.8	Relman (1992)
Fungal primers	LR3R	Forward	LSU	5'-GTC TTG AAA CAC GGA CC-3'	50.6	Vilgalys and Hester (1990)
	LR7	Reverse	LSU	5'-TAC TAC CAC CAA GAT CT-3'	45.6	Vilgalys and Hester (1990)
	ALF01	Forward	LSU	5'-GGA AAG ATG AAA AGA ACT TTG AAA AGA G-3'		Clapp *et al.* (2001)
	NDL22	Reverse	LSU	5'-TGG TCC GTG TTT CAA GAC G-3'		Van Tuinen *et al.* (1998)
	ITS1	Forward	ITS	5'-TCC GTA GGT GAA CCT GCG G-3'	59.5	White *et al.* (1990)
	ITS4	Reverse	ITS	5'-TCC TCC GCT TAT TGA TAT GC-3'	52.1	White *et al.* (1990)

Table 2.6 Concentrations and volumes of PCR reagents used in amplification (final volume 50.0 μl).

PCR Master mix	Formula for 1 reaction (total 50.0 μl)
PCR Water	23.8 μl
25 mM $MgCl_2$	4.0 μl
2.5 mM dNTPs	4.0 μl
Thermophilic DNA polymerase buffer (10X)	5.0 μl
Forward primer	5.0 μl
Reverse primer	5.0 μl
1.0 U *Taq* polymerase	0.20 μl
DNA Template	3.0 μl

Table 2.7 PCR conditions for amplification of bacterial and fungal DNA.

PCR Cycle	Bacterial primers	Fungal primers	Fungal primers
	27F/1525R	ITS1/ITS4	ALF01/NDL22 and
Reference	In this study	Martini *et al.* (2009)	LR3R/LR7 In this study
(1) Initialisation step	94°C (4 min)	94°C (4 min)	94°C (4 min)
(2) Denaturisation step	94°C (30 s)	94°C (1 min)	94°C (1 min 30 s)
(3) Annealing step	50°C (30 s)	54°C (1 min)	53°C (1 min 30 s)
(4) Extension step	72°C (2 min)	72°C (1 min)	72°C (2 min 30 s)
(5) Cycles (2)-(4)	40x	40x	40x
(6) Final extension	72°C (10 min)	72°C (10 min)	72°C (10 min)
(7) Final hold	4°C	4°C	4°C

2.3.2 Confirmatory traditional identification

(A) Bacterial morphological identification

The bacterial isolate B91 was grown on NA and incubated for 3-7 d at 25°C in the dark before being examined for shape and size. Macroscopic colony morphology of B91 was photographed under a stereo microscope. These characters were compared to the descriptions in Bergey's Manual of Systematic Bacteriology (Logan and De Vos, 2009).

(B) Bacterial biochemical tests

Molecular identification of isolate B91 was confirmed using recommended biochemical tests (Logan and De Vos, 2009; Norris *et al.*, 1981; Priest, 1989). The tests were (1) salt tolerance at 7% and 10% NaCl, (2) casein hydrolysis on milk agar, (3) antibiotic resistance to chloramphenicol and streptomycin and (4) growth at different temperatures (4, 10, 15, 40, 50 and 55°C).

(C) Yeast morphological identification

The morphological identification of yeast Y126 was based on the Atlas of Clinical Fungi (De Hoog *et al.*, 2000) and the study by Zalar *et al.* (2008). For macroscopic study, isolate Y126 was grown on PDA and MEA to determine its growth rate and phenotype. The cultures were incubated at 25°C for 14 d in the dark. The growth rate was recorded on day 7 and 14. The colour of the colony surface was rated using the colour chart of Kornerup & Wanscher (1978).

For microscopic study, isolate Y126 was grown on PDA using the slide culture technique (Zalar *et al.*, 2008). Potato Dextrose Agar blocks (0.5x0.5 cm^2) were cut out from a PDA plate using a sterile blade. The agar blocks were placed on sterile microscope slides and inoculated with Y126 along the upper four edges of the agar blocks. The inoculated blocks were covered with sterile cover slips. The slides with agar blocks were placed on a V-shape glass rod and incubated in a Petri dish moist chamber (achieved by laying the rods on a piece of filter paper soaked with sterile water). The cultures were incubated at room temperature under UV light for 7 d. Cover slips with hyphae growing out from the agar blocks were carefully taken out from agar blocks, mounted on a new slide and stained with cotton blue in lactophenol. The morphological characters of hyphae were examined at magnifications of 400x and 1000x on intact slides under a microscope.

(D) Yeast biochemical tests

Molecular identification of Y126 was confirmed using recommended biochemical tests (Domsch *et al.*, 1980). The tests were (1) salt tolerance at 7% and 10% NaCl and (2) growth PDA and MEA at different temperatures (0, 4, 10, 15, 25 and 35°C).

2.3.3 Culture registration

The two selected BCAs were deposited in international culture collections. The details are given in Table 2.8. *Bacillus* sp. B91 was deposited at the National Centre for Industrial, Food and Marine Bacteria (NCIMB) and *Aureobasidium pullulans* Y126 was deposited in the National Collection of Fungus Cultures, Centre for Agricultural Bioscience International (CAB International). The DNA sequences of the two BCAs were deposited in the GenBank database.

Table 2.8 Detail and registration number of two BCA strains.

BCAs	Source of culture	Date of isolation	Culture collection	Deposition code	GenBank accession number
Bacillus sp. B91	Green plum	29 Jul 2010	NCIMB	NCIMB30287	KC161970 (SSU)
Aureobasidium pullulans Y126	Leaf of cherry	13 Sep 2010	CAB International	IMI501717	KC161971 (ITS) KC161972 (LSU)

2.3.4 Effect of temperature on growth of BCAs

Inoculum of isolate B91 was transferred into 250 ml Duran bottles containing 100 ml of NB and placed on a shaking incubator at 25°C for 4 d at 200 rpm; by day 4 the culture was in log phase. This culture was then inoculated into fresh liquid medium by pipetting 0.5 ml of the log phase culture into Duran bottles containing 100 ml of NB. The initial concentration of the cell suspension in the bottles containing fresh medium was measured by a dilution method. The initial concentration was between $1x10^5$-10^6 CFU/ml. The cultures were incubated at six different temperatures (0, 5, 10, 15, 20 and 25°C) without shaking. There were triplicate bottles for each temperature (18 bottles in total). The cultures were sampled weekly for 6 wks.

The cultures were maintained under a static condition therefore before sampling the bottle was manually shaken 15-20 s in order to disperse cells and ensure that the liquid culture was homoginised. Forty µl of liquid culture from each bottle was taken and serially diluted with 360 µl of NSS (*i.e.* a 1 in 10 dilution). All samples were spread onto duplicate NA plates. Plates were incubated at 25°C for 3 d and resultant colonies were counted using a digital counting pen. Cell numbers were expressed as colony forming units per ml (CFU/ml). Isolate Y126 was characterised using the same protocol except that MEA and MEB were used as media. All experiments were repeated once. Data on numbers of cells (CFU/ml) were transformed using natural logarithms (ln). The data were subjected to repeated measure ANOVA using temperature and time as factors, number of CFU as a variate and repeat as block over time.

2.3.5 Effect of temperature on survival of BCAs

Inoculum of isolate B91 was initially prepared as above. B91 was inoculated into 6 Duran bottles containing 100 ml of NB and incubated at 25°C at 200 rpm. After 4 days growth, the liquid cultures were centrifuged at 14,000 rpm for 10 min. Supernatant was discarded and the cell pellets were washed twice using phosphate buffer solution (PBS) at pH 7.2 and mixed thoroughly using a vortex mixer. Cell pellets from six different bottles were re-suspended using PBS and pooled together. The final volume of cell suspension was adjusted to 20 ml and the concentrated cell suspension was

aliquoted (1 ml) into 18 Duran bottles each containing 100 ml of PBS (*i.e.* no nutrients were provided). The bottles were incubated at six temperatures (0, 5, 10, 15, 20 and 25°C); three bottles were randomly assigned to each temperature.

Samples were taken to estimate number of viable cell counts weekly for 6 wks. Before sampling, the bottle was manually shaken in order to disperse the cells in the PBS. Forty µl of liquid culture from each bottle was taken and serially diluted with 360 µl of NSS. The samples were spread-plated and incubated at 25°C for 3 d. The samples were plated immediately to prevent cell multiplication or any effects of temperature change. Colonies on NA were counted and converted into cell concentration (CFU/ml). Isolate Y126 was studied using the same protocol but MEA and MEB were used as solid and liquid media respectively. Data of numbers of cells (CFU/ml) were transformed using natural logarithms (ln). The data were subjected to repeated measure ANOVA using temperature and time as factors, number of CFU as a variate and repeat as block over time.

2.3.6 Inhibtion of spore germination

Activity of the two BCA strains on spore germination of *M. laxa* was studied in a number of ways to investigate whether inhibitory activity needed living cells or came from soluble organic compounds produced and released into the liquid broth. There were five treatments: living cells of each BCA, culture filtrates of each BCA and sterile PDB as a control. Living cells were separated from culture broths by centrifugation. Tubes containing 50 ml of a 4 day-old culture broth were centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a new tube and filter-sterilised using a 0.2 µM filter and a sterilised disposable syringe to remove remaining cells from the culture broth. Cell pellets were washed twice with PBS and re-suspended in 5 ml of PBS. Spore suspensions (0.5 ml) of *M. laxa* at $1x10^5$ spores/ml (prepared as described in Section 2.2.1, Chapter 2) were mixed with either 0.5 ml of living BCA cell suspension (10^8-10^9CFU/ml) or cell free-culture broth in a sterile 15-ml screw cap polystyrene tube. One ml of PDB was added the tubes containing spore suspension and treatment and the contents were mixed with a vortex mixer. A droplet of the mixture (100 µl) of each treatment was transferred onto water agar (WA).

The tubes containing the rest of the mixture were inclined and the caps were loosened to increase surface area and air circulation, respectively. The plates and tubes were incubated at 25°C in the dark. There were five replicate plates per treatment (one drop per plate). Germination of spores was assessed after 12-14 hr by counting 100

spores per plate. The experiment was repeated once. Percentage data of spore germination were subjected to angular transformation. Statistical inferences were drawn from the transformed data. Homogeneity of variance and normality were tested before statistical analysis using a Bartlett's test and Shapiro-Wilk test, respectively.

The first objective of this study was to test whether BCAs, as a whole group, reduced spore germination of *M. laxa*. Therefore the first group (the cell-free culture broth and the living cells of the two BCAs that had been growing) was compared to the control treatment. Percentage germination was analysed using one-way ANOVA with treatment as a factor. The second and third objectives were to determine the possible mechanisms of inhibition of germination and to study on the effect of media on bioactivity. This study focused on three factors including (1) BCAs (B91 and Y126), (2) sources of bioactivity (culture broth and living cells) and (3) media (PDB and WA). The data were subjected to angular transformation and subjected to three-way ANOVA using BCAs, sources and media as factors.

2.3.7 Production of volatile organic compounds (VOCs)

This test was carried out in a closed system. A co-culture technique was adapted from the method described by Swadling (1994). Spread plates of isolates B91 and Y126 were prepared on NA and PDA, respectively, by pipetting 0.5 ml of a 24 hr culture onto agar plates before spreading using a sterile triangular glass rod. The plates were incubated at 25°C for 2 d to ensure the confinement growth of B91 and Y126. Plates of PDA were inoculated centrally with an agar plug cut from the edge of an actively growing mycelial culture of *M. laxa* on PDA using a sterile cork borer (cork borer No. 2, 5 mm diam.). The lids were removed from the Petri dishes and the PDA plate inoculated with *M. laxa* was inverted and placed on top of either the NA or PDA plate seeded with isolates B91 or Y126 respectively, and the plates were taped together and incubated at 25°C for 2 wks. The control was a plate of PDA inoculated with a sterile agar plug.

Diameters of *M. laxa* colonies were measured with a digital vernier calliper. There were 8 replicate (plates) per treatment, and the diameter for each replicate plate was an average from two measurements across the vertical and horizontal angles. The experiment was repeated once. Homogeneity of variance and normality were tested before statistical analysis using a Bartlett's test and Shapiro-Wilk test, respectively. The diameter was normally distributed and was analysed therefore without transformation.

Colony diameter of *M. laxa* was analysed using two-way ANOVA using BCAs and isolates of *M. laxa* as factors and colony diameter as a variate.

2.4 Population biology of *M. laxa*

2.4.1 Collection of isolates

The *M. laxa* isolates used in this study were isolated by Dr Angela Berrie and Mrs Karen Lower at East Malling Research from mummified fruits and green fruits. Isolation from mummified fruits was straightforward. A sterile needle was used to pick off conidia of *M. laxa* from sporing pustules on mummified fruit and the conidia were streaked onto PDA. The PDA plates were incubated at 25°C. After 3-5 d any *Monilinia*-like colonies were transferred to fresh PDA and the plates incubated for 2 wks. To isolate *M laxa* from green fruits, the fruits were first washed in tap water and surface-sterilised by immersing in a solution of 0.5% sodium hypochlorite for 1 min, followed by rinsing in sterile water three times to reduce other contaminants. The sterilised fruit were then placed in pots on paraquat chloramphenical agar (PCA) and incubated at ambient temperature (20-25°C) under UV light to encourage sporulation of *M. laxa* present as latent infection in the fruit. Once sporing pustules of *M. laxa* were present on the fruit, isolations were conducted as for mummified fruit. Once clean cultures of *M. laxa* were obtained the mycelium was harvested and stored in Eppendorf tubes at -80°C until required.

Table 2.9 describes the sources of isolates of *M. laxa* collected from commercial orchards that were used in this study. Populations needed to comprise of at least 15 isolates for genetic analysis and there were not a sufficient number of *M. laxa* isolates from blossom wilt and twig canker in the orchards sampled during two consecutive years of collection (2009-2010) to include this phase of the life cycle within the analysis. Isolates of *M. laxa* were divided into several groups based on the stage of life cycle, season of collection, sources of cultures (mummified fruits and green fruits), locations (two from Herefordshire and three from Kent) and hosts (cherries and plums). Isolates were from two different stages of life cycle for all locations except the East Malling Research location where only isolates from mummified cherries were available. There was only one location in Kent where *M. laxa* isolates were sampled from both plums and cherries.

2.4.2 Genomic DNA Extraction

Genomic DNA of *M. laxa* was extracted using a rapid method modified from Cenis (1992). Fungal mycelia of *M. laxa* isolates were maintained in a -80°C freezer. A small amount of fungal mycelia (~10 mg) was taken out from the stock mycelium collection using sterile forceps and added to 100 µl of lysis buffer in a 1.5 ml Eppendorf tube. Mycelium was crushed with a pellet pestle and cordless motor (Sigma-Aldrich). A further 200 µl of lysis buffer was added into the tube and the contents mixed. Then 150 µl of 3 M sodium acetate was added and mixed using a vortex mixer and the tubes were kept at -20°C for 10 min. Tubes were then centrifuged in a table-top microcentrifuge Mikro-200 (Hettich Zentrifugon) at 14,000 rpm for 5 min. Clear supernatant was transferred to a new Eppendorf tube and the volume of the supernatant was recorded. An equal volume of isopropanol was added and the contents were mixed gently and let to stand at room temperature for 5 min. DNA was collected by centrifugation at 14,000 rpm for 5 min. Supernatant was carefully discarded and the pellet was washed twice with 70% ethanol. The DNA pellets were dried under a flow cabinet for 2 hr. Fifty µl of TE buffer was added to the dried DNA pellets and mixed gently. DNA Concentration was measured using a NanoDrop 100 Spectrophotometer (ThermoScientific). Stock DNA was maintained at -20°C.

Table 2.9 Location of origin, date of isolation, stage of infection of *M. laxa* isolates collected from orchards in Kent and Herefordshire.

Host	Location	Post code	Date of isolation	Original code	Stage of infection	No. of Isolates	Code for analysis
Cherries	The Little Sharsted Farm	ME9 0JT	21 Jan 10	R35/10	Mummified fruit	26	MMF-CH-ME9
	Sittingbourne, Kent		15 Jul 10	R130/10	Green fruit	43	GRF-CH-ME9
	The South Park	ME19 6BJ	2 Dec 09	R238/09	Mummified fruit	24	MMF-CH-ME19
	East Malling, Kent						
	The Lower Hope Farm	HR1 3JF	19 Feb 10	R92/10	Mummified fruit	39	MMF-CH-HR1
	Hereford, Herefordshire		27 Jul 10	R131/10	Green fruit	43	GRF-CH-HR1
	The Man of Ross Ltd	HR9 6AU	23 Mar 10	R116/10	Mummified fruit	18	MMF-CH-HR9
	Herefordshire		27 Jul 10	R132/10	Green fruit	30	GRF-CH-HR9
Plums	The Little Sharsted Farm	ME9 0JT	28 Jan 10	R51/10	Mummified fruit	15	MMF-PL-ME9
	Sittingbourne, Kent		01 Aug 10	R141/10	Green fruit	38	GRF-PL-ME9
	The Decoy Farm	ME3 8SR	10 Apr 10	R118/10	Mummified fruit	29	MMF-PL-ME3
	Rochester, Kent		02 Aug 10	R142/10	Green fruit	31	GRF-PL-ME3
Total						336	

2.4.3 PCR amplification

A summary of concentrations and volume of PCR reagents is given in Table 2.10. For DNA templates of high concentration (> 50 ng/µl), the templates were diluted with PCR water in a 1:5 ratio before use. The PCR amplification of each primer pair was performed individually. The amplification was carried out with the PCR conditions given in Table 2.11, using a DNAEngine DYAD Thermocyler PTC-220 (MJ Research, Watertown, USA). Genomic DNA of *M. laxa* was amplified with species-specific SSR primers (Table 2.12).

In a preliminary study, amplified PCR products were visualised by electrophoresis. Ten µl of the PCR product was mixed with 2 µl of loading dye. The mixture was loaded into a 3 mm deep well in a 1.6% agarose gel. The gel was placed in a gel tank contained TE buffer and run at 100 V for 90 min. The gel was taken out and stained in ethidium bromide solution (0.5 µg/ml) for 20 min. PCR Products were visualised using a camera and software (Image Lab Version 2.0.1, Bio-Rad Laboratory). A DNA ladder (size ranging from 100-1,500 bp) was used to size PCR products.

Table 2.10 Concentrations and volumes of PCR reagents used in amplification (final volume 12.5 µl).

PCR Master mix	Formula for 1 reaction (total 12.5 µl)
PCR Water	4.7 µl
25 mM $MgCl_2$	1.0 µl
2.5 mM dNTPs	1.0 µl
Thermophilic DNA polymerase buffer (10X)	1.25 µl
Forward primer	1.25 µl
Reverse primer	1.25 µl
1.0 U *Taq* polymerase	0.05 µl
DNA Template	2.0 µl

Table 2.11 PCR Condition for amplification of SSR primers.

PCR Cycle	PCR Condition
(1) Initialisation step	94°C (4 min)
(2) Denaturisation step	94°C (30 s)
(3) Annealing step	55°C (30 s)
(4) Extension step	72°C (30 s)
(5) Cycles (2)-(4)	35x
(6) Final extension	72°C (10 min)
(7) Final hold	4°C

Table 2.12 SSR Primers used to amplify genomic DNA of *Monilnia* spp. (developed at EMR by Dr. Nick Harvey; unpublished data).

Locus*	Sequence	Motif	T_m (°C)	Fluorescent label	Approximate size (bp)
MF10F	ATGGAAGTTCCCCTTGTGTG	[CAG]6	57	Fam	200
MF10R	GCTGCCATTGTTCCGATACT		57		
ML5F	GGGAGATTGCCGGTTAGTTT	[TG]8	57	Fam	185
ML5R	CCCAAAACATTGCCTTTTGT		53		
ML6F	GAAAGCGAAAGCGAGGAGTA	[AG]7	57	Fam	203
ML6R	TTTGGTTGGGAAGGGAAAAT		53		
ML15F	TATGATGTTGCGGCGTAGAA	[AAG]7	55	Fam	234
ML15R	TAATCCAGCGGTCGAGGTAG		59		
ML16aF	AGGGAAGGTATTTGGGCAAC	[GCA]6	57		164
ML16aR	AGAAGAGTGGCTGATGCTGA		57		
ML16bF	AGGGAAGGTATTTGGGCAAC	[GCA]6	57	Fam	105
ML16bR	TGGGAGATATTGAGTTGTTGTTATTG		58		
ML17F	GGATAGAGATCGAGGGGGTTT	[GGAT]5	59	Fam	215
ML17R	GATTTCCGCATCCAGTGC		56		
ML18F	GCTCCGCCATGTATGGAATA	[TGTGTGAG]4	57	Fam	256
ML18R	GAGACAGAAAACGGCGACAT		57		

*MF = *M. fructigena* and ML = *M. laxa*

2.4.4 Genotyping

To save time and cost, PCR products of the same fungal isolates amplified with different primers were pooled together because fragment lengths of PCR products amplified by some pairs of primers differ by at least 50 bp (Table 2.12). PCR products generated from three pairs of primers were thus mixed; (1) ML15 and ML16b, (2) ML16a and ML17 and (3) ML6 and ML18. PCR product was diluted in 1:50 ratio in a 96-well plate. Two µl of PCR products from each two SSR primers was added into 96 µl of PCR water. Twenty four µl of ladder was added to 900 µl of HiDi. The mixture of ladder and HiDi was aliquoted into a 96-well plate using 9 µl of the mixture per well. A drop of diluted PCR products (1.3 µl) was added into each well. The plate was centrifuged at 14,000 rpm for 30 s and denatured at 90°C for 3 min using the thermocycler. DNA fragments were analysed using an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Fragment size was determined using the software GenScan and Genotyper (Applied Biosystems).

2.4.5 Population comparison

The collection of isolates from the various orchards was split into a series of populations (Table 2.9). A population with at least 15 isolates from each group was included in the analysis. To investigate the population structure of *M. laxa*, AMOVA developed by Excoffier *et al.* (1992) was used to statistically compare variation between populations of *M. laxa* as implemented in Arlequin Version 3.5.1.2 (Excoffier and Lischer, 2010). The significance level of the AMOVA was set to 0.05 based on 1023 permutations.

Groups of isolates were defined to test three main hypotheses.

(1) H_0 = There is no difference of populations among *M. laxa* isolated from mummified fruits and green fruits

(2) H_0 = There is no difference of populations among *M. laxa* isolated from different locations

(3) H_0 = There is no difference of populations among *M. laxa* isolated from cherries and plums

2.5 Application of BCAs to mummified fruits

2.5.1 Efficacy of BCAs on sporulation of *Monilinia* spp.

(A) Mummified plums from a commercial orchard

Mummified plums were collected from an orchard in Kent in November 2010 (Figure 2.5) and brought back to East Malling on the same day. The fruits were put in plastic bags and stored in a glasshouse for 2-4 wks. If fruits were densely attached in the same cluster, they were torn into individual mummified fruits.

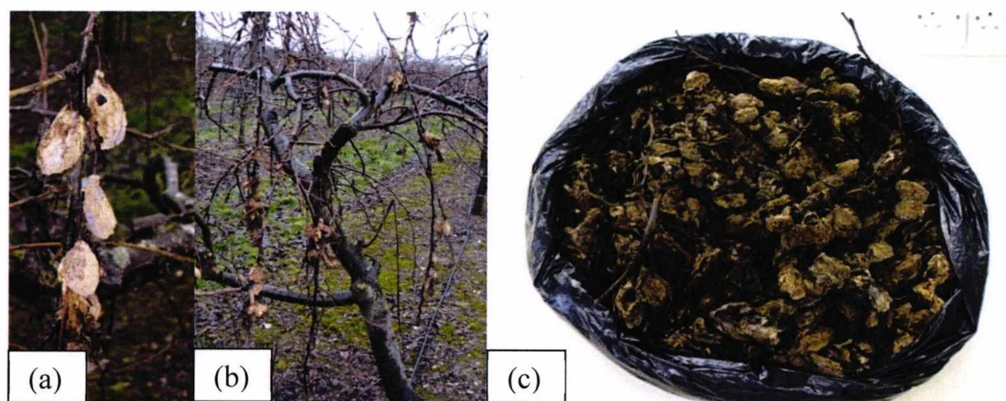


Figure 2.5 Mummified plums. (a) and (b): Mummified plums attached on the tree and (c): Clusters of mummified plums were kept in a plastic bag before being treated with different treatments.

(B) Treatments

A group of BCAs including one bacterial BCA (B62) and four yeast BCAs (Y35, Y52, Y80 and Y90) that performed well in the initial screens against *M. laxa* described in Chapter 2 were used in a preliminary study of sporulation inhibition on mummified plums. Two potential BCAs (*E. nigrum* ATCC96794 and *P. fluorescens* NCIMB13373) described in earlier studies (De Cal *et al.*, 2009; Swadling and Jeffries, 1998), and two commercial products, the BCA Serenade and the fungicide Indar were also included in this study. The aim of this experiment was to determine whether BCAs can suppress sporulation of *Monilinia* species in the field during the overwintering period and to compare these BCA treatments with a fungicide.

The capability of the five indigenous BCAs, the other known BCAs and the fungicide to suppress sporulation on mummified plums was tested under under field conditions. The details of treatments used in this study are given in Table 2.13. *Epiccocum nigrum* ATCC96794 was kindly provided by Dr. De Cal (INIA, Spain) and was grown on PDA incubated at 25°C with a 16: 8 hr, light: dark regime for 2 wks (Larena *et al.*, 2004). Spores were harvested by scraping the surface of colony using sterile forceps and the spores were suspended in sterile distilled water to a final concentration of 1.0×10^6 spores/ml. *Pseudomonas fluorescens* NCIMB13373 was grown on NA and incubated at 25°C for 3 d in the dark. Single colonies were inoculated in a

500 ml-Duran bottle containing 250 ml of NB. The indigenous BCAs, B62, Y35, Y52, Y80 and Y90, were grown using the same protocol as for *P. fluorescens* NCIMB13373, but MEA and MEB were used for the yeast BCAs. The Duran bottles were then incubated at 25°C for 4 d on a shaking incubator at 200 rpm. Indar and Serenade were prepared according to manufacturers' recommendation.

Table 2.13 Treatments of BCAs on anti-sporulation on mummified fruits.

Treatment	Category	Final concentration
Tap water	Negative control	-
Indar	Fungicide	5% (w/v)
E. nigrum ATCC96794	Known BCA	$1x10^6$ spores/ml
P. fluorescens NCIMB13373	Known BCA	$1x10^8$ CFU/ml
Serenade	Commercial BCA	10 ml/L
B67	Indigenous BCA	$1x10^8$ CFU/ml
Y35	Indigenous BCA	$1x10^7$ CFU/ml
Y52	Indigenous BCA	$1x10^7$ CFU/ml
Y80	Indigenous BCA	$1x10^7$ CFU/ml
Y90	Indigenous BCA	$1x10^7$ CFU/ml

(C) Sporulation inhibition on mummified plums using indigenous and known BCAs

The field trial was set up at EMR in Kent between December 2010 - February 2011. There were ten treatments (Table 2.13). Mummified plums (from Section 2.5.1 A) were split into ten treatment groups. They were sprayed with individual treatments using a handheld sprayer until run-off. All operations were performed in polyethylene bags to avoid cross-contamination between treatments. Figure 2.6 shows the individual treatments used in this study prior to spraying onto fruits. After mummified plums were allowed to dry in a flow cabinet for 1 hr, the fruits were put in green net bags (15x20 cm^2). A randomised block design was used with six blocks (one bag per block) of ten treatments. There were five mummified fruits per block (bag). A green net bag represented an experimental unit. Four replicates were hung on cherries trees in an orchard at the same height (around 1.5 m above the ground) and on the same side of trees, while the other two replicates were put 30 cm above the ground by tying the bags inside plastic boxes. The experiment was repeated once. The dates of experiment are shown in Table 2.14.

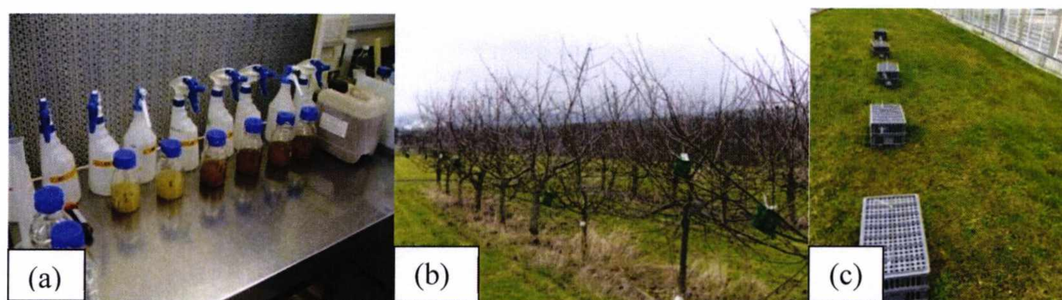


Figure 2.6 Experiment on anti-sporulation on mummified plums (a): Treatments made up in handheld sprayers, (b): Bags of mummified plums hanging on trees and (c): Bags of mummified fruits placed above the ground covered with plastic boxes.

Table 2.14 Date of spraying treatments, collecting mummified plums.

Experiment	Date of spray	Collection of mummified plums	Spore harvest
First trial	6 Dec 2010	3 Feb 2011	16 Feb 2011
Second trial	20 Dec 2010	17 Feb 2011	4 Mar 2011

(D) Assessment of sporulation

Two months after treatment, mummified plums were collected from the orchard (February – March 2011), the dates of collection of mummified plums are given in Table 2.14. The fruits from each replicate were put in plastic containers containing filter paper soaked with sterile water. Fruits were sprayed with sterile distilled water until run-off before the lids were put on (Figure 2.7). The containers were incubated at 10°C for 7 d with 12:12 hr light:dark.

After 7 d, 50 ml of sterile distilled water was added into the plastic containers and the container was shaken gently for 10-15 s to release spores from surface of mummified plums. The spore suspensions were collected into 50 ml-centrifuge tubes and centrifuged at 14000 rpm for 10 min. The supernatant was discarded and 5 ml of sterile distilled water was added into the pellet. A drop of phenol (0.05 ml) was added to the spore suspension to kill spores and prevent spore germination. The spore suspension was stored at 4°C until assessment. Spores of *Monilinia* spp. were counted using a haemocytometer. There were four drops per replicate, and two counts made per drop. The number of spores/ml was log transformed using natural logarithm (ln) and subjected to one-way ANOVA using treatments as a factor and trials as block over time.

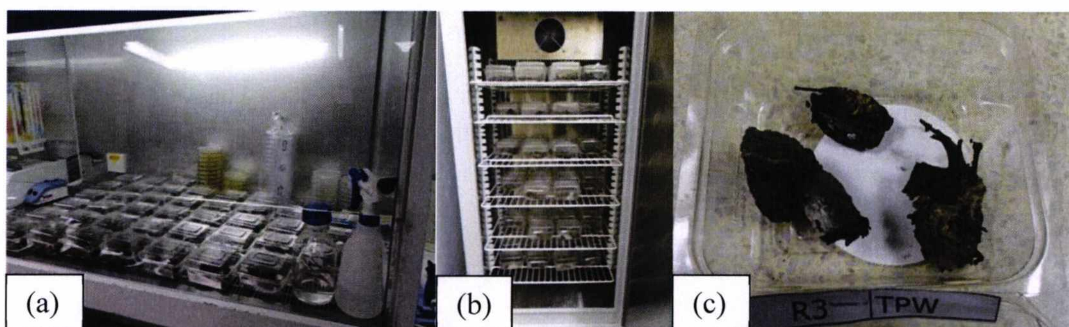


Figure 2.7 Spores assessment (a): Boxes of mummified plums sprayed with sterile distilled water, (b): Incubation at 10°C for one week and (c): An example of sporulating mummified plums before the spores were harvested.

2.5.2 Efficacy of BCAs and time of spray on sporulation of *M. laxa*

The experiment described in Section 2.5.1 and conducted during 2010-2011 was an initial trial using commercial products and some of the BCAs showing early potential in initial screens. However, the two similar experiments on mummified fruits in 2012 and 2013 were carried out using a similar protocol but used the 2 BCAs identified as the leading contenders during the later screens (B91 and Y126; see Chapters 4 and 5). The objectives of this experiment were to (1) to determine efficacy of two BCAs in comparison with Serenade and Indar for suppression of sporulation of *M. laxa* on mummified fruits and (2) to test whether a single spray or double spray affects the efficacy of suppression of sporulation.

(A) Production of mummified plums

In order to ensure that sufficient mummified fruits, infected only with *M. laxa*, were available for experimentation, it was necessary to produce artificially-infected fruit under laboratory conditions rather than rely on field infection later in the season. Infected plums from experiments described in Chapter 4 provided some of this material, but plums that had been treated with a fungal BCA, Trianum or the fungicide Indar were excluded to ensure that no residual control agents were carried over. In addition, a number of fresh plums were inoculated with *M. laxa* to provide additional materials for mummification. Plums from both these sources were mixed together and laid in plastic trays about 5 cm away from each other (Figure 2.8). The mummification took place in a glasshouse compartment at 20°C where trays of plums with brown rot symptoms were kept for 6-8 wks. An electric fan was switched on for 6 hr a day to increase air circulation. Mummifying plums were checked weekly to assess whether they were

became heavily colonised by air-borne fungi such as *Aspergillus* and *Penicillium*, in which case they were removed from the trays.

Figure 2.8 *Monilinia laxa*- inoculated plums being mummified in a glasshouse.

(B) Treatments

The concentrations of the five treatments are given in Table 2.15. For the two indigenous BCAs, single colonies of *Bacillus* sp. B91 grown on NA for 3 d were transferred to a 500-ml Duran bottle containing 250 ml of NB. The culture was incubated on a shaking incubator at 25°C for 4 d at 200 rpm. *Aureobasidium pullulans* Y126 was cultured using the same procedure as *Bacillus* sp. B91, but PDA and PDB were used as culture media.

Table 2.15 Treatments for the anti-sporulation on mummified plums.

Treatment	Category	Stock concentration	Rate of use
Tap water	Control	-	-
Indar	Fungicide	5% (w/v)	2 ml/L
A. pullulans Y126	BCA	$1x10^8$ CFU/ml	Whole culture
Bacillus sp. B91	BCA	$1x10^8$ CFU/ml	Whole culture
Serenade	BCA	$1x10^9$ CFU/ml	10 ml/L

(C) Sporulation inhibition on mummified plums

There were 15 treatments: five treatments of chemical and BCAs (Tap water, Indar, *A. pullulans* Y126, *Bacillus* sp. B91 and Serenade) and three spraying times (November, February and November+February) (Table 2.16). The exact dates of spraying treatments, collecting mummified fruit back to the lab and harvesting spores of *M. laxa* are shown in Table 2.17. Around mid-November, the mummified plums (Section 2.5.2A) were sorted into 15 treatment groups by lying them down in trays. The first spray was conducted in a glasshouse. Trays of mummified plums from Treatments 1-10 were sprayed with individual treatments listed in the Table 2.16, while trays of

mummified plums for Treatments 11-15 were left without spraying. All trays were left overnight in a glasshouse.

One day later, all mummified plums were put into green net bags (15x20 cm^2), which were hung on cherry trees growing in an orchard at EMR. Each bag was hung around 1.5 m above the ground and the same side of each tree. A randomised block design was used with four blocks (one bag per block) of 15 treatments. There were five mummified fruits per block (bag). A bag containing five fruits represented an experimental unit. Three months after the first spray (around mid-February), the second spray was conducted in the field. Treatment of chemical and BCAs were made up using the same concentration as the first spray. Bags containing mummified plums for Treatment 6-15 were untied from the cherry trees. Mummified plums were placed in plastic containers and sprayed with individual treatments until run-off. Mummified plums were put back to the same bags and re-hung on the same cherry trees.

Table 2.16 Treatments of chemical and BCAs used in anti-sporulation on mummified plums.

Treatment		November	February
1	Tap water	✓	
2	Indar	✓	
3	*A. pullulans* Y126	✓	
4	*Bacillus* sp. B91	✓	
5	Serenade	✓	
6	Tap water	✓	✓
7	Indar	✓	✓
8	*A. pullulans* Y126	✓	✓
9	*Bacillus* sp. B91	✓	✓
10	Serenade	✓	✓
11	Tap water		✓
12	Indar		✓
13	*A. pullulans* Y126		✓
14	*Bacillus* sp. B91		✓
15	Serenade		✓

Table 2.17 Dates of spraying treatments, collecting of mummified fruits and harvest of spores.

Experiment	Winter spray	Spring spray	Collection of mummified plums	Spore harvest
First trial	23 Nov 2011	20 Feb 2012	5 Mar 2012	20 Mar 2012
Second trial	28 Nov 2011	23 Feb 2012	8 Mar 2012	23 Mar 2012
Third trial	15 Nov 2012	25 Feb 2013	11 Mar 2013	18 Mar 2013

(D) Assessment of sporulation

Two weeks after the second spray (early March), treated mummified plums were collected from the trees. The spore assessment was conducted as described previously (Section 2.5.1D) with some modifications. Spores were harvested by soaking the five mummified plums with 30 ml of distilled water and manually shaking them for 15-20 s. The spore suspension was collected into a 50 ml screw-cap polystyrene tube (Fisherbrand) and the final volume of spore suspension was adjusted to 25 ml. A drop of phenol was added to kill spores. All samples were stored at 4°C until numbers of spores were counted using a haemocytometer. Four drops were taken for each replicate and two counts made per drop. The experiment was repeated three times and these three trials were conducted separately. The dates of experiments are given in Table 2.17. Numbers of spores/ml produced by each treatment were log transformed using natural logarithm (ln) and analysed using two-way ANOVA. Treatments and spraying times were main factors and trials as block over time.

Chapter 3

Isolation of microbes and primary screening

3.1 Introduction

Monilinia laxa causes brown rot diseases on stone fruits and is reported worldwide causing serious economic losses in Europe (CABI, 1991; Chen *et al.*, 2012; Wherrett *et al.*, 2001). This pathogen is capable of infecting stone fruits throughout the process of fruit production from blossom production to post-harvest packaging. Fungicides are the most effective method of disease control and are applied on blossom and young fruit pre-harvest. They are not permitted for use on ripening fruits or post-harvest (Adaskaveg *et al.*, 2005; Holb *et al.*, 2006; Yoshimura *et al.*, 2004). This leaves a high risk gap for disease development. Fruits become more susceptible to the pathogens when they are ripening (Mari *et al.*, 2004a). Repeated and constantly use of fungicides can also lead to fungicide resistance (Hily *et al.*, 2011; Zhu *et al.*, 2010). Thus, there is an urgent need for alternative methods of disease control.

To fill this gap, both physical and biological control methods have been developed and proven to be useful in reducing disease development (Bonaterra *et al.*, 2003; Sautter *et al.*, 2011; Sharma *et al.*, 2009). However, physical control methods such as cooling, heating or controlling atmospheric storage conditions require investment in infrastructure and may incur high operating and maintenance costs. Biological control agents (BCAs) offer an alternative. There are, however, few BCAs available on the market, especially any developed to use specifically against *M. laxa*. Moreover, most of the BCAs used to control *Monilinia* species are non-native (*i.e.* originally isolated, produced or manufactured from outside the UK), for example Serenade (AgraQuest, USA) and BoniProtect (Bio-ferm, Germany). The registration of new products, their testing in field trials and launching into UK fruit markets can be complicated due to environmental concerns. Introducing alien species usually has an impact on ecological systems and native species (Keller and Perrings, 2011; Lankau, 2011; Pysek *et al.*, 2012); therefore searching for indigenous BCAs is preferred.

Isolation of potential BCAs was based on the hypothesis that antagonistic organisms might be found where they are in competition with *M. laxa* in the same ecological niche. Natural plant epiphytic microbes were targeted because they are likely to be non-pathogenic to host plants and would be able to multiply rapidly and survive in a similar habitat as the pathogen as well as having similar nutrient needs (Wang *et al.*, 2009; Wittig *et al.*, 1997). Therefore, infected cherry and plum fruits as well as intact

fruits and health leaves were used as sources of potential BCAs in the present study. A standard dilution plate technique was used to isolate microbes from plant materials using basic agar media instead of selective media in order to obtain a variety of common species which are fast-growing and competitive. Bacteria and yeasts are favoured as BCAs because they have been widely reported as a rich source of bioactive compounds with various modes of actions, as well as having minimal growth requirements, making them potential candidates for development on an industrial scale (Gaur *et al.*, 2010; Holb, 2009; Janisiewicz and Korsten, 2002; Sharma *et al.*, 2009).

Various modes of actions have been reported by which BCAs inhibit target pathogens. These include (1) production of organic compounds such as antibiotics and lytic enzymes, (2) competition on nutrients and space, (3) induction of natural plant defense mechanisms and (4) mycoparasitism. Taking these into account, an appropriate screening method corresponding to these modes of action must be used. Screening for induction of plant defense mechanisms is complex, involving large scale trails on intact plants, so it is the first two mechanisms that are usually targeted. A dual culture technique is often used because this technique allows for a rapid screening of a large number of potential microbes with minimal expense. This technique also allows observation of the interaction between pathogens and antagonists which can also detect mycoparasitism. Inhibition activity is nutrition-dependent. The culture medium should favour the growth and organic compound production by the microbes being screened as well as growth of the pathogen (Dhingra and Sinclair, 1985). Therefore, an inhibition test should be conducted on at least two agar media to ensure that the bioactivity is consistent and does not rely on a specific source of nutrients - this could be problematic when they are deployed in the field.

3.2 Objectives

The objectives of this chapter were (1) to isolate indigenous BCAs from plant materials collected within the UK and (2) to test the inhibitory activity of these isolates against *M. laxa* using a dual culture technique. A number of isolates were selected for *in vivo* study.

3.3 Result

3.3.1 Primary screening of microbes against *M. laxa* on MEA

A total of 217 microbes consisting of 92 bacterial strains, 100 yeast strains and 25 filamentous fungi were isolated from plant materials collected from six locations in

the county of Kent during 2010-2011. These microbes were tested for inhibition of mycelial growth of two strains of *M. laxa* (one originally isolated from cherries and one from plums). However 25 strains of filamentous fungi were dismissed after these fungal isolates were identified as *Monilinia* species. The data of these 25 strains were excluded from the analysis. The percentage inhibition of *M. laxa* is shown as a histogram of the number of microbes and the percentage inhibition (Figure 3.1).

An initial threshold of percentage inhibition at 50% was chosen as a cut-off for further screening of these microbes. The number of microbes which were able to inhibit *M. laxa* by more than 50% (32 isolates) was less than the number inhibiting by less than 50% (160 isolates). Therefore the histogram is asymmetric with a right-skewed distribution. Most microbes inhibited growth of the pathogen by 20%-50% (103 isolates). Considering the isolates with more than 50% inhibition (the right part of the histogram), 36 isolates of microbes inhibited *M. laxa* from cherries (Figure 3.1, black column) while 43 isolates inhibited *M. laxa* from plums (grey column). Thirty two microbes were able to inhibit both strains of *M. laxa,* and there were 57 microbes in total showing at least 50% inhibition of one or both strains of *M. laxa.* The details of inhibition from primary screening are given in Table A2.1 Appendix 2. These 57 isolates were taken forward for testing on MEA and PDA to confirm their bioactivity.

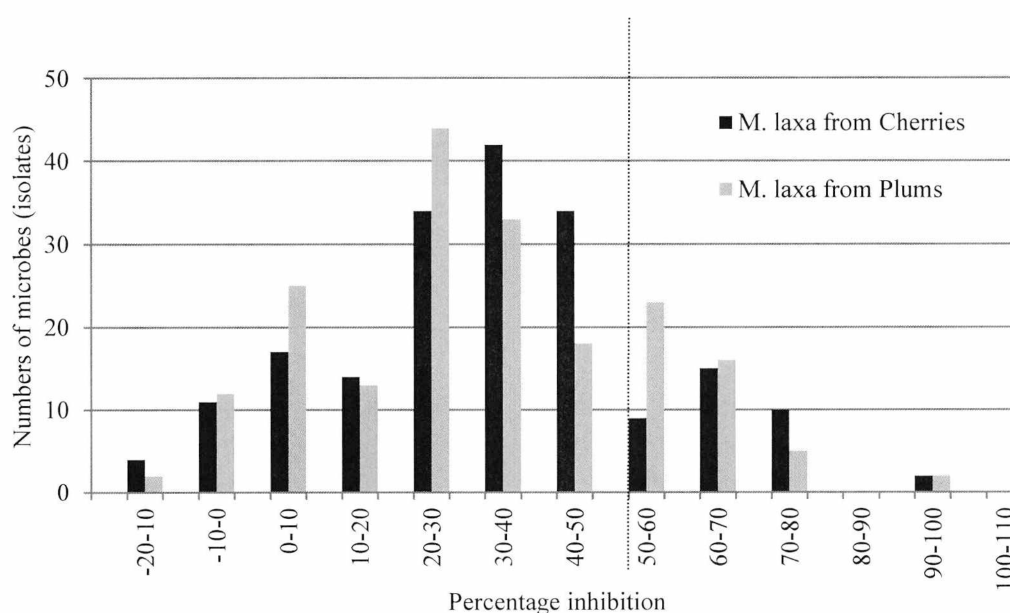
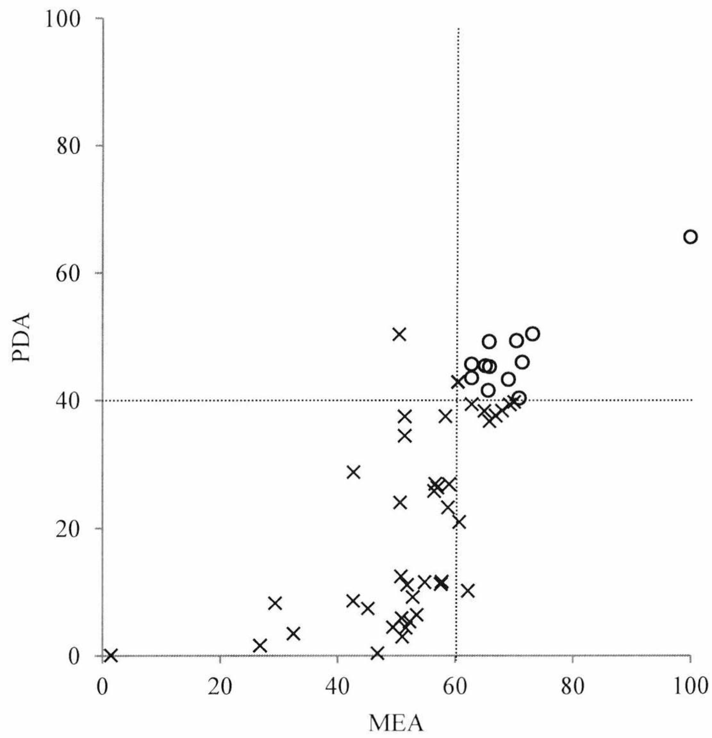


Figure 3.1 A histogram of percentage inhibition and number of microbes tested against two strains of *M. laxa*. The screening was conducted on MEA incubated at 25°C for 14 days.

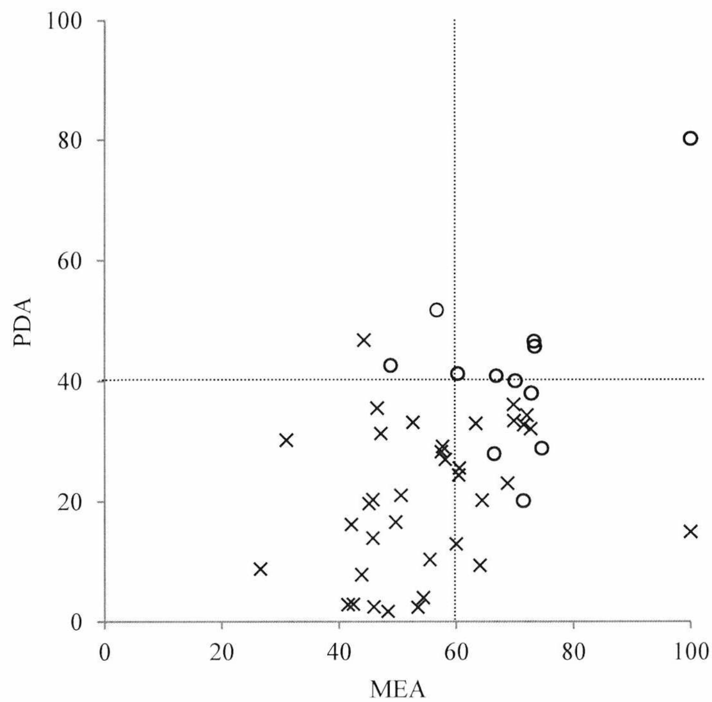
3.3.2 Comparison between MEA and PDA on bioactivity

The 57 isolates from the previous section were tested on PDA and again on MEA. Scatter plots (Figure 3.2) illustrate the percentage inhibition conducted on the

two media. The bioactivity on MEA and PDA against two strains of *M. laxa* isolated from cherries and plums are shown in Figure 3.2a and 3.2b respectively. The results shown on scatter plots were non-linear. Therefore the Pearson correlation coefficient (*r*) was not calculated. In general there was a positive relationship between bioactivity on MEA and PDA. The percentage inhibition data on MEA were higher (around 40%-80%) than those on PDA (20%-60% inhibition). In order to select the most promising BCAs, a decision was made to use threshold levels of inhibition on MEA and PDA of 60% and 40%, respectively. These values corresponded to the mean inhibition on each medium. In this study we are interested in isolates having consistent bioactivity across media and strains of *M. laxa*. Therefore only the 12 microbes that surpassed threshold levels on both media and fungal strains were selected for further study (Chapter 4). The detail of inhibition from MEA and PDA are given in Table A2.2 Appendix 2.



(a)



(b)

Figure 3.2 A plot of percentage inhibitions conducted on MEA and PDA against a *M. laxa* isolate from cherries (a) and plums (b) using a dual culture technique. The cultures were incubated at 25°C for two weeks. O = 12 isolates that were selected for *in vivo* screening.

3.3.3 Inhibition test of known BCAs

Of the three known BCAs that were tested, *E. nigrum* ATCC96794 performed the best in the primary screen (Table 3.1). It inhibited the growth of the two strains of *M. laxa* from cherries and plums by 49% and 47%, respectively. The others were less effective in inhibiting the growth of the pathogen. The percentage inhibition of these two BCAs was between 26% to 40%. However none of the three passed the cut-off threshold used for further testing and so these isolates were not carried forward for further screens.

Table 3.1Inhibition of three BCAs against *M. laxa* tested on MEA.

BCAs	Percentage inhibition (%)	
	M. laxa from cherries	*M. laxa* from plums
E. nigrum ATCC96794	49	47
B. pumulis NCIMB13374	26	34
P. fluorescens NCIMB13373	34	40

3.4 Discussion

3.4.1 Isolation and sources of microbes

The Convention of Biological Biodiversity (1992) sets restrictions on the international exploitation of organisms, and maintains that the rights of donor countries should be respected. Thus in searching for potential BCAs, it is better that the microbes should be isolated and used within the country of origin in order to avoid any conflicts. Indigenous microbes are also preferred to avoid complications in product registration and to avoid any negative impact of using alien species in the UK environment. It is also expected that indigenous microbes will already be well adapted to prevailing environmental conditions. Therefore our samples were collected from the UK rather than from foreign countries.

Microbes with bioactivity can be isolated from various sources, for example soil, crop residues, air, marine sediments and plant roots (Nikapitiya, 2012; Subramani and Aalbersberg, 2012; Zhang *et al.*, 2005). However resident microbes from the fruit surface have been the richest and most attractive source of BCAs (Janisiewicz *et al.*, 2013). Microbes should be collected from similar ecological niches where the targeted plant disease is present. A collection of resident microbes from the same niche are likely to have potential to control plant disease because these microbes are likely to be able to compete with pathogens. On the other hand, isolation of BCA candidates from samples only taken from a specific location or a single host leads to a biased collection of BCAs, therefore the samples in this study were collected from different orchards and two host

plants. Nutrient agar and MEA, plain and inexpensive media, were used as isolation media instead of selective media or enrichment media in order to minimise bias and avoid pre-selection of BCAs that grow on specific media.

For several decades, BCAs against plant diseases have not impressed public and crop growers due to ineffectiveness and inconsistency when they were applied in uncontrolled conditions outside a glasshouse environment. Selection of potential BCAs needs a careful strategy. Microbe isolation and screening is the first step and has a huge impact on the types of BCAs that are taken forward. Nunes (2012) suggested that there are many criteria that need to be taken into consideration. Firstly the BCA must have mechanisms to inhibit the growth of the target pathogen; this might be through antibiosis, nutrient competition, direct parasitism, or via indirect mechanisms such as the triggering of natural plant defences.

In addition, ideal BCAs should have their own fundamental characteristics: genetically stable, resistant to fungicides, an ability to survive adverse conditions, have simple nutrient requirements, be non-pathogenic, easy to use and inexpensive to produce. Screening based on all these criteria is costly and time-consuming. These criteria can be compromised by selecting the most important criteria. In this work, selection was based on two criteria: a mechanism of action via antibiosis and/or competition for nutrients, and simple nutrient requirements. These criteria can be evaluated at a low cost per isolate and are amenable for mass production.

3.4.2 Primary screening

Although *Monilinia* species can infect intact fruits, wounds are the major route for brown rot infection. Therefore some researchers have directly screened the efficacy of microbes on artificially wounded fruits instead of using a preliminary *in vitro* screen, for example for 59 yeast isolates tested on nectarines (Janisiewicz *et al.*, 2010), 210 isolates of yeasts or yeast-like microbes tested on peaches (Zhang *et al.*, 2010c), 173 bacterial isolates tested on plums (Janisiewicz *et al.*, 2013) and 86 isolates on peaches and nectarines (Mari *et al.*, 2012a). However, under these circumstances only a small number of isolates were able to inhibit brown rot pathogens. Nevertheless, this approach is relevant and gives impressive results for potential post-harvest use, but this test is limited in scale and laborious as it requires a large number of fruits and can be done only once a year during the fruit harvest season.

For cherry, the harvest season is shorter than most other stone fruits (two weeks in June in the UK). With this restriction, a simple dual culture technique was used here

as a primary screen conducted on agar media. This technique is also suitable and practical for direct effects of the BCA against the fungal pathogen and for giving an indication of the mechanism of action. Plain and simple growth media were used rather than selective media as the latter contain selective and enrichment components which might favour or interfere with the physiological activity of the BCAs or the target pathogen.

3.4.3 Effect of media on bioactivity

In general, primary screening has been solely conducted on one agar medium (Berg *et al.*, 2001; Raspor *et al.*, 2010; Sowndhararajan *et al.*, 2013; Wang *et al.*, 2009). However testing on at least two media is encouraged to ensure that their bioactivity is nutrient independent and selected media should be based on the prospective mechanisms of target BCAs. Different media also reflect the effect of nutrients which is a major factor for bioactivity. Pastor *et al.* (2012) tested 10 bacterial strains against three fungal pathogens using three media, trypic soy agar, King's B medium and PDA. The effect of bacterial BCAs depended on the composition of culture media used.

Whipps (1987) studied the growth and antagonistic activity of five fungal BCAs against six fungal pathogens. The inhibition test was conducted on three complex media, including soil extract agar, PDA and tap water agar. The different media had significant effects on growth rate of the fungi, production of antibiotics and competition for nutrients. However there was no single medium which can reflect all possible mechanisms that can occur. Whipps (1987) suggested that the use of chemically-defined media of known content and ingredients rather than complex media would be a good way to screen BCAs, therefore the analysis of bioactivity is reproducible and this would indicate essential components of media affecting on bioactivity of BCAs. This clearly shows the importance of the sources of nutrients that need to be considered.

In this study, inhibitory activity was firstly screened on MEA and then bioactivity was confirmed on PDA, a medium widely used for screening for bioactivity against *Monilinia* species (Ma *et al.*, 2005; Pinto *et al.*, 2011; Voland *et al.*, 1999; Zhu *et al.*, 2010). The inhibitory activity recorded for isolates screened on MEA was often slightly higher than on PDA. This may be because the organisms were originally isolated from MEA and may grow better on MEA than PDA. On the other hand, PDA might favour growth of *M. laxa,* PDA is normally used as a basal medium for selection and spore induction of *M. laxa* (Amiri *et al.*, 2009; Pascual *et al.*, 1990). Daayf *et al.* (2003) also emphasised that screening on a single medium may not be adequate. A

number of bacterial isolates provided moderate inhibition (2%-52%) against *Phytophthora infestans* on rye agar but this group of bacteria did not inhibit the pathogen on V8-PDA. If screening was conducted on either rye agar or V8-PDA alone, this might have led to a biased group of BCAs. Ideally, BCAs should be able to inhibit across different isolates of a pathogen and their bioactivity should not be regulated by sources of nutrients. Therefore only BCAs showing consistent inhibitory activity on both media and isolates were selected.

3.4.4 Bioactivity of known BCAs against *M. laxa*

Three BCAs which were previously reported to have bioactivity against plant pathogens were tested for inhibition against *M. laxa* using the same methodology used here to select new BCAs. *Epiccocum nigrum* ATCC96794 was reported to inhibit *Monilinia* spp. by a team of Spanish plant pathologists (De Cal *et al.*, 2009; Larena *et al.*, 2005; Madrigal *et al.*, 1991; Madrigal *et al.*, 1994). This BCA has been tested in laboratories and field trials and has proven to be one of the most promising to control *Monilinia* spp. in Spain. However when this BCA was tested here against *M. laxa* originally isolated from the UK, it only showed moderate inhibition. This may be due to differences between Spanish and UK isolates of *M. laxa.* Genetic variation of *M. laxa* has been reported among isolates from Spain (Gell *et al.*, 2007b) or isolates from different host plants (Gril *et al.*, 2008). Although *E. nigrum* can inhibit *Monilinia* in Spanish orchards, this particular strain of *E. nigrum* might not be able to control across an entire population of *Monilinia* species in different countries.

The two other BCAs selected for testing here *i.e. B. pumilus* NCIMB13374 and *P. fluorescens* NCIMB13373 from a preceding project (Swadling and Jeffries, 1998) had known bioactivity against grey mould disease caused by *B. cinerea.* They had not been tested against *M. laxa.* These two BCAs inhibited growth of *B. cinerea* via antibiotics which had fungicidal and fungistatic effects on conidial germination of *B. cinerea.* However these BCAs only slightly reduced mycelial growth of *M. laxa* and hence did not pass through the primary screen used in this investigation. Strains of *Bacillus pumilus* have often been selected as a BCA against various plant pathogens including *R. solani* (Cottyn *et al.*, 2009; Huang *et al.*, 2012), *Fusarium* (Recep *et al.*, 2009), *B. cinerea* (Essghaier *et al.*, 2009; Swadling and Jeffries, 1998). In fact, *Bacillus pumilus* QST2808 is produced commercially under trade name Sonata (AgraQuest), which is claimed to control several plant diseases including downy and powdery mildews and rusts. Similarly, strains of *P. fluorescens* are frequently reported to have

bioactivity against various plant pathogens such as *Rastonia solanacearum, F. oxysporum* and *R. solani* (Couillerot *et al.*, 2009; Gao *et al.*, 2012). Other species of *Pseudomonas* have been shown to have bioactivity against *M. fructicola* (Smilanick *et al.*, 1993; Zhou *et al.*, 2008) and *M. laxa* (Altindag *et al.*, 2006). However, in our tests, it seems that the antibiotic effect demonstrated against *B. cinerea* is not effective against *M. laxa.*

3.5 Conclusions

The methodology used in this primary screen was inexpensive and simple to do and enabled a large number of isolates to be screened for bioactivity against *M. laxa*. However, the results will be biased towards antagonists which either compete for nutrients or produce diffusible antibiotics which inhibit the growth of *M. laxa* in agar media. Nevertheless, these criteria will be relevant for BCAs that need to antagonise the growth of *M. laxa* in the infection court and thus a number of microbes were selected from *in vitro* screening that showed good inhibitory potential against *M. laxa*. These microbes were taken forward for secondary screening.

Chapter 4

In vivo screening

4.1 Introduction

Secondary screening for BCAs should follow and relate to the mechanisms of inhibition favoured by primary screening (Wilson *et al.*, 1993). In this study, two types of inhibition were favoured (1) production of inhibitory organic compounds and (2) competition for nutrients and space. Although some BCAs perform well in *in vitro* tests, their performance *in vivo* can be different because there are numerous other factors that affect their activity. When stone fruits are ripening on trees or during post-harvest storage, there are dramatic changes in their physiology, for example in moisture content, pH, respiration and fruit nutrients (Famiani *et al.*, 2012; Serradilla *et al.*, 2012; Singh *et al.*, 2012). These factors also significantly affect their susceptibility to *Monilinia* species (Emery *et al.*, 2000). *In vivo* tests on the fruit surface have been used in this study because this method allows for specific selection of BCAs for post-harvest control of fruit diseases (Droby *et al.*, 2009). This screening method is usually conducted on artificially-wounded fruits which are treated with agrochemicals or BCAs and then inoculated with a pathogen. It favours selection of BCAs having a protective effect rather than a curative effect. It also allows for competition between the pathogen and potential BCAs within nutrients leaked from wound injuries.

Cultivars of cherries and plums vary from country to country. For example, cherry cultivars Bing, Lambert Tartarian, Royal Ann and Napoleon are among the most popular sweet cherries grown in the US, while important cultivars of plum are Italian, French Sugar and Imperial Epineuse (Sallunkhe and Desao, 1984). In the UK, it is Merchant and Lapins in cherry, and Marjorie and Victoria in plums, that are the most widely planted cultivars. All commercial stone fruit cultivars are susceptible to *Monilinia* spp., but the level of susceptibility varies from cultivar to cultivar. Due to their pre-harvest tendency to crack, swell and split during ripening, nectarines are more susceptible than peaches, and sweet cherries are more susceptible than sour cherries (Northover and Biggs, 1990). Peach cultivars Elberta, Glohaven and Babygold No. 5 have been reported to be less susceptible to brown rot, but other cultivars are highly susceptible (Bush *et al.*, 2009; Ziems, 2009). *In vivo* tests of BCAs should be ideally conducted on cultivars planted in the target countries.

BCAs can be used either pre- or post-harvest to protect stone fruits from *Monilinia* infection. Infection by *M. laxa* usually occurs via blossom or through the

developing fruit surface. Although in the present study potential BCAs were isolated from healthy plant materials, which showed no visible symptoms, they were multiplied and reintroduced into plants in high concentrations. These conditions may enable a potential BCA to become an opportunistic pathogen. Many BCAs are, or are closely related to, opportunistic pathogens of humans and plants. Examples of opportunistic BCAs in this category are *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Trichoderma viride* and *Bacillus cereus* as some strains of these species are potential threats to humans and plants by causing disease, allergy or food poisoning (Brodeur, 2012; Handelsman, 2002). There is a need to ensure that BCAs selected in this project are not opportunistic pathogens of plants, so an *in vivo* screening also provides an opportunity to investigate the potential threat of selected BCAs to the fruits themselves.

Few BCAs have been reported to have activity against *Monilinia* spp., but some of the agrochemicals and commercial BCAs are claimed to control related diseases or were recommended for other diseases of fruits. Trianum (*Trichoderma harzianum* T-22) has an indirect effect on disease by promoting plant growth and increasing plant resistance to diseases (Koppert, 2012). Prestop (*Gliocladium catenulatum* J1446) is claimed to have activities against various pathogens including *Alternaria, Botrytis, Cladosporium, Didymella, Fusarium, Penicillium, Pythium, Rhizoctonia, Phytophthora* and *Verticillium* (Verdera, 2006). BoniProtect is reported to have activity against *Penicillium*, *Botrytis*, *Monilinia* and *Gloeosporium* on pome fruit and stone fruit (Bio-ferm, 2011; Weisshaupt *et al.*, 2011). Moreover these BCAs may have been tested against strains of *M. laxa* from other countries rather than UK isolates. The experiments described in this thesis aimed to determine whether these products could inhibit *M. laxa* from the UK.

Timing of application of agrochemicals and BCAs plays an important role in disease control (Carisse and Rolland, 2004; Savchuk and Dilantha, 2004). Applying treatments before arrival of an inoculum might prevent fruits from infection by a pathogen while applying treatments after inoculation might not eliminate the disease in an infected fruit but might reduce the spread of the disease into surrounding fruits. Applying treatment at the best timing would help to enhance efficacy of treatments. Studies of timing of application have been reported for example on the effect of application timing of BCAs against *Colletotrichum acutatum* on apples (Poleatewich *et al.*, 2012) and timing of fungicide application against *C. acutatum* on cherries (Borve and Stensvand, 2006) and *M. laxa* on peaches (Sharma, 2005). However effect of timing of treatment application on cherries and plums infected with *M. laxa* is not known.

Therefore effect of timing of application was studied by combining with the study of the effect of treatments of agrochemicals and commercial BCAs.

In the present study, experiments were conducted on artificially-wounded fruits which were then artificially inoculated with the pathogen. However, under commercial conditions, fruits are usually stored post-harvest and further trials of potential BCAs need to be conducted under these conditions as they may present different challenges than the *in vivo* screening. Post-harvest storage conditions may differ from laboratory conditions such as temperature, fruit arrangement and humidity. Poor handling at harvest can injure fruits, and growers may fail to remove fruits with brown rot prior to storage. Both these factors can favour brown rot development during post-harvest storage. Even when fruits with brown rot symptoms are removed prior to storage, latent infection can lead to brown rot a few weeks after storage due to natural pre-harvest infection.

Control of brown rot in storage from latent sources can be achieved through either removing fruits with latent infection or protecting uninfected fruits from the pathogen. The former is difficult to achieve because latent infection is invisible and a large sample size would be required for assaying latent infection by culture methods. Chemical control is prohibited at post-harvest to reduce pesticide residues at consumption. Thus the post-harvest environment presents an opportunity to deploy BCAs as an alternative control method as a component of an integrated strategy. Experiments investigating post-harvest control are reported in this chapter.

4.2 Objectives

The objectives of the experiments described in this chaper were (1) to assess efficacy of newly isolated indigenous BCAs using *in vivo* tests on artificially-wounded stone fruits, (2) to determine the efficacy of commercially available BCAs and the effect of application times, cultivars and wound age on infection caused by *M. laxa* and (3) to study the potential for use of indigenous and commercial BCAs to control *M. laxa* on stone fruits in post-harvest conditions. A summary of experiments is shown in Table 4.1.

Table 4.1 Summary of experiments of *in vivo* studies in this chapter

Topic	Experiment
4.3.1	Efficacy of indigenous BCAs
4.3.2	Phytotoxicity
4.3.3	Effect of commercial BCAs, application times and cultivars
4.3.4	Effect of commerical BCAs and wound age
4.3.5	Post-harvest treatment using semi-commercial storage conditions
4.3.6	Post-harvest treatment using commercial storage conditions

4.3 Results

4.3.1 Efficacy of indigenous BCAs

In the cherry experiment, there was significance difference in percentage infection between treatments ($p < 0.001$). Treatments with Indar, and isolates B91 and Y126 were significantly different from treatment with distilled water (DW) (Figure 4.1). The percentage infection after treatment with isolates Y101 and Y106 was significantly lower than distilled water, but significantly higher than Indar, B91 or Y126. The percentage infection of fruit treated with the other eight isolates was not significantly different from distilled water.

Percentage infection on plums of different treatments also showed significant differences ($p < 0.001$). Indar, isolates B91 and Y126 significantly reduced infection compared to distilled water (Figure 4.2), while isolates Y101 and Y110 partially reduced percentage infection. Percentage infection of the fruit treated with the other eight isolates was not significantly different from distilled water.

Lesion sizes on plums were assessed in order to quantify the degree of disease development. There was a significant difference in lesion sizes on plums among different treatments ($p < 0.001$). When brown rot occurred in the wounds, lesion sizes on fruits treated with Indar, B91 or Y126 were significantly smaller than distilled water (Figure 4.3). Although the lesion sizes of the other ten BCAs showed some statistical differences, the sizes of lesion were very close to the distilled water treatment. An example of lesion of brown rot disease caused by *M. laxa* on plums are shown in Figure 4.4.

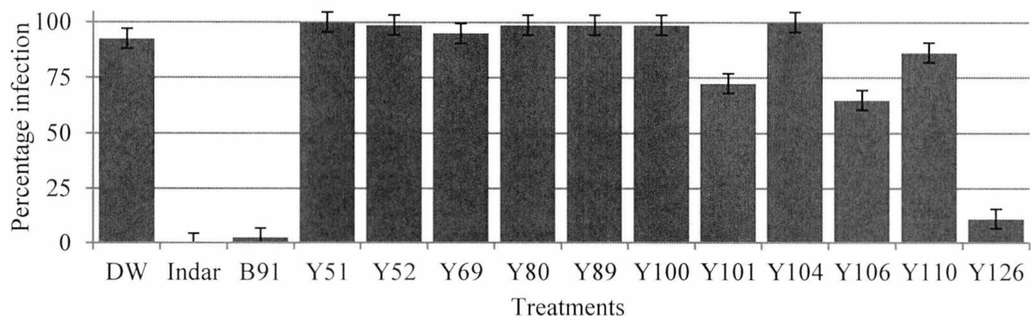


Figure 4.1 Mean values of percentage infection on cherries for different treatments. Bars represent standard error of the mean. DW: distilled water.

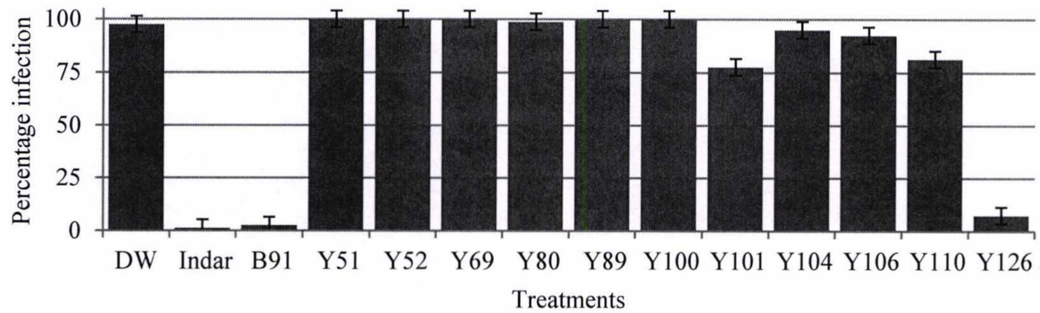


Figure 4.2 Mean values of percentage infection on plums for different treatments. Bars represent standard error of the mean. DW: distilled water.

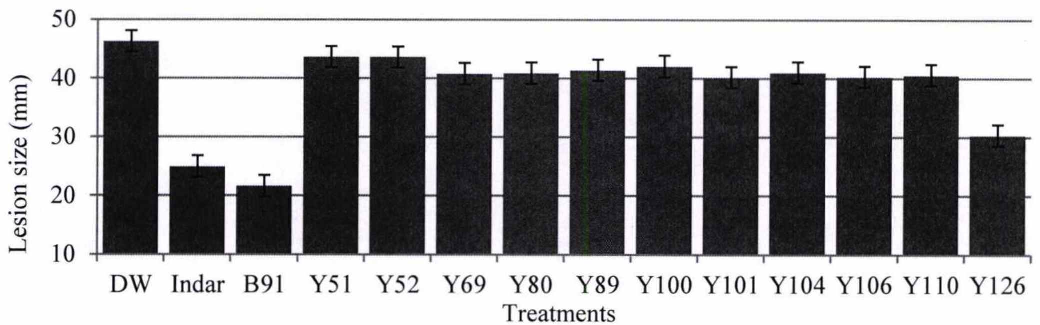


Figure 4.3 Mean values of lesion sizes on infected plum fruits for different treatments. Bars represent standard error of the mean. DW: distilled water.

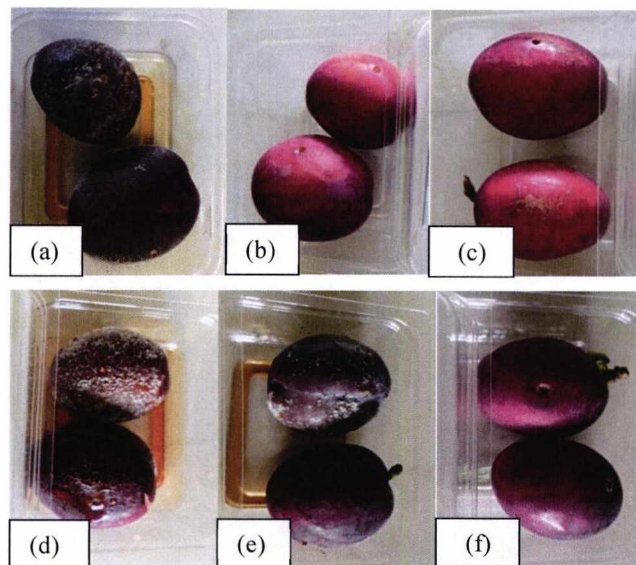


Figure 4.4 Rot symptom on plums treated with treaments and then artificially inoculated with *M. laxa*. (a): distilled water, (b): Indar, (c): B91, (d): Y52, (e): Y69 and (f): Y126

4.3.2 Phytotoxicity

There was no fruit rot observed on cherries and plums treated with distilled water or isolate B91. However, 10% of plums treated with isolate Y126 showed some symptoms of rot, although there was no rot on cherries treated with Y126. Lesions on plums treated with Y126 were soft with circular spots which later became sunken.

4.3.3 Effect of commercial products, application times and cultivars

This initial screening experiment of commercially-available BCAs took place in 2010, using unwounded cherries that were not surface-sterilised. As a result, there was heavy contamination with *Aspergillus* and *Penicillium* and infection with *M. laxa* was very low resulting in data that could not be analysed. In contrast, in the plum experiment, infection by *M. laxa* was facilitated using artificial wounds, resulting in sufficient infections for data to be analysed.

All three factors (treatments, application times and cultivars) had a significant effect on percentage of disease ($p < 0.001$), with treatments being the most important factor. There was an interaction effect between the three main factors ($p = 0.001$). Indar was the best treament for control of *M. laxa* and significantly reduced infection across both plum cultivars and both application times (Figure 4.5). It completely inhibited infection if plums were treated with the fungcide 24 hour before inoculation with *M. laxa*. When Indar was applied 24 hour after inoculation, it still greatly reduced infection. If Serenade was applied before inoculation with *M. laxa*, it significantly reduced the infection and this effect of Serenade was greater when it was applied on plum cv. Majorie than on cv. Victoria. When Serenade was applied after inoculation, it was unable to reduce infection on cv. Victoria but still partially reduced infection on cv. Majorie. The other four treatments (BoniProtect, BioPK, KBV and XzioX) did not reduce disease levels.

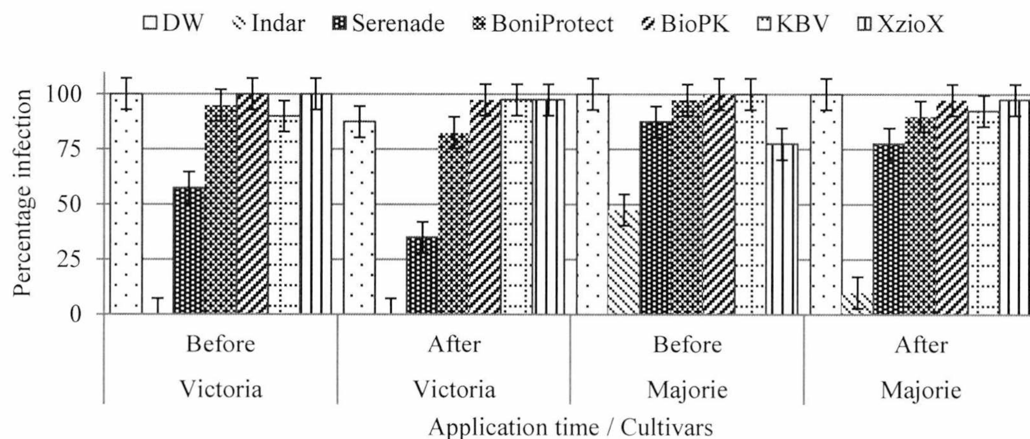


Figure 4.5 Mean values of percentage infection for different treatments, application times and plum cultivars. Bars represent standard error of the mean. DW: distilled water.

In addition to percentage infection, lesion sizes on plums were also analysed. The three main factors also had a siginificant effect on lesion sizes: treatments ($p < 0.001$), application times ($p = 0.049$) and cultivars ($p < 0.001$). Cultivar was the most

important effect and cv. Victoria was more susceptible to *M. laxa* than cv. Marjorie. In all cases, Indar completely inhibited infection by *M. laxa*, therefore this treatment was excluded from this analysis. There was an interaction between treatments, application times and cultivars ($p < 0.044$). Neither treatments nor application times affected lesion size on plum cv. Victoria. (Figure 4.6), except XzioX if it was applied after inoculation. However, skin damage was present as a result of this treatment.

In general, the lesion size on cv. Marjorie was smaller than that on cv. Victoria. If Serenade was applied on plum cv. Marjorie before the inoculation, this treatment reduced lesion size from >35 mm (control treatment) to less than 30 mm. BoniProtect and BioPK applied before the inoculation also reduced lesion size to a more limited extent, while the other two treatments had no effect. These treatments did not affect lesion size when applied to plum cv. Marjorie after the inoculation.

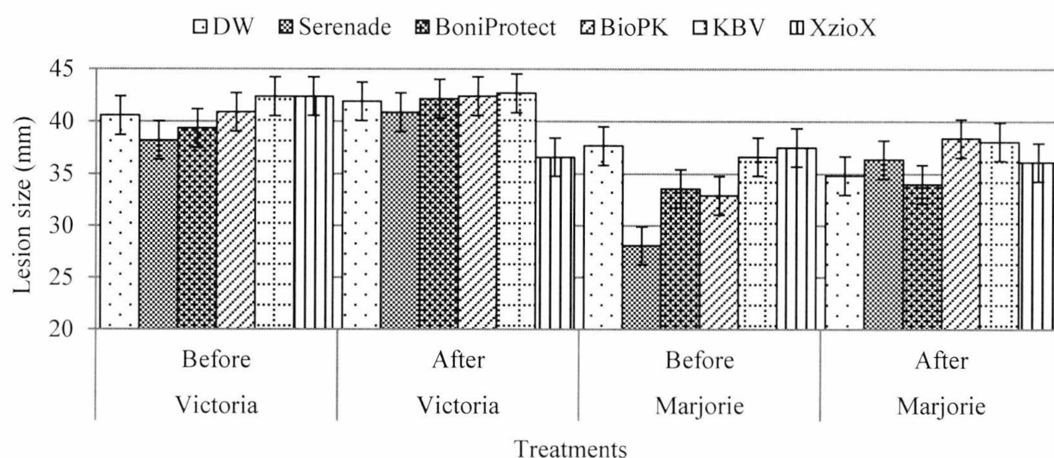


Figure 4.6 Mean values of lesion sizes for different treatments, application times and plum cultivars. Bars represent standard error of the mean. DW: distilled water

4.3.4 Effect of commercial BCAs and wound age

In later experiments after 2010, cherries were surface-sterilised and artificially wounded which resulted in high infection levels of *M. laxa* such that the data could be analysed. There was a significant difference between the treatments ($p < 0.001$) but there was no significant difference between fresh and one-day-old wounds ($p = 0.575$). There was no interaction between treatments and wound types ($p = 0.058$). Figure 4.7 shows the treatment effect, whereby Indar completely inhibited the disease while Serenade significantly reduced infection by nearly 40% compared to distilled water (DW). Percentage infection after treatment with the other four commercial BCAs was not significantly different from the control treatment.

For plum experiments, two factors, treatments and wound types, were significant factors influencing percentage infection by *M. laxa*. The treatment effect was far greater than the wound effect ($p < 0.001$ and $p = 0.032$, respectively), and there was no interaction ($p = 0.485$). Figure 4.8 shows the treatment effect on percentage infection. Infection after treatment with Indar was zero, while Serenade again partially inhibited *M. laxa* infection and significantly reduced percentage infection compared to the control treatment. The other four treatments did not reduce infection.

As Indar completely inhibited infection of *M. laxa,* it was excluded from the analysis of lesion sizes. Lesion sizes from the different treatments were not significantly different ($p = 0.310$) while wound type had a significant effect on lesion size ($p < 0.001$) whereby lesions of *M. laxa* in one-day-old wounds were smaller than those from fresh wounds. Figure 4.9 shows an interaction effect between treatment and wound type ($p = 0.003$). Lesion sizes in treatments with Serenade, BoniProtect and BioPK were not significantly different from the control treatment. Lesion sizes of fresh wounds treated with Prestop and Trianum were not significantly different from the control treatment but Prestop and Trianum had a significant effect in reducing lesion size of one-day-old wounds.

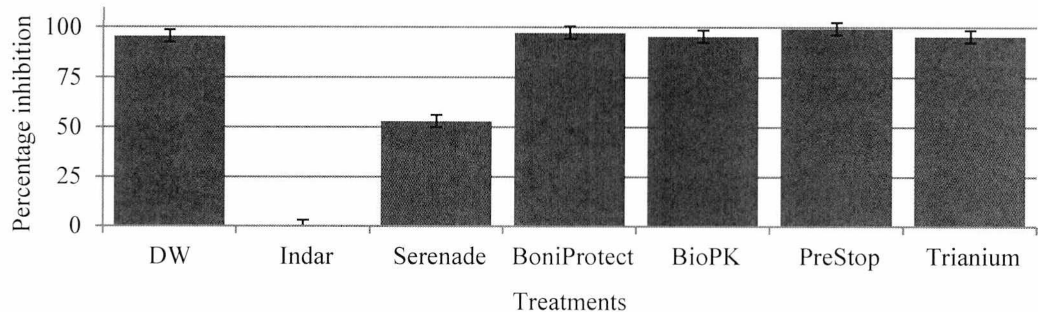


Figure 4.7 Mean values of percentage infection on cherries for different treatments. Bars represent standard error of the mean. DW: distilled water.

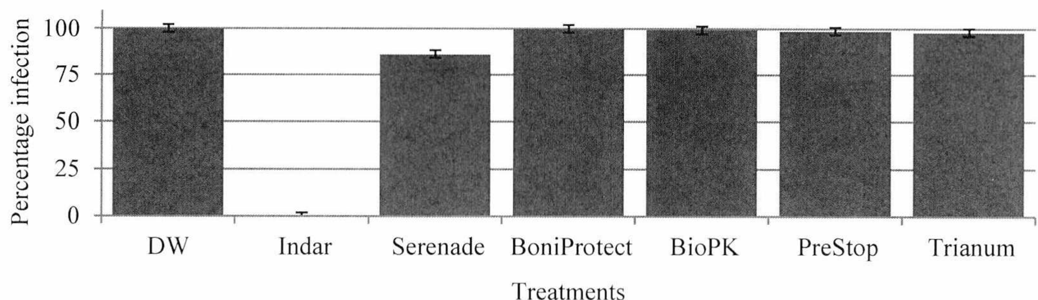


Figure 4.8 Mean values of percentage infection on plums for different treatments. Bars represent standard error of the mean. DW: distilled water.

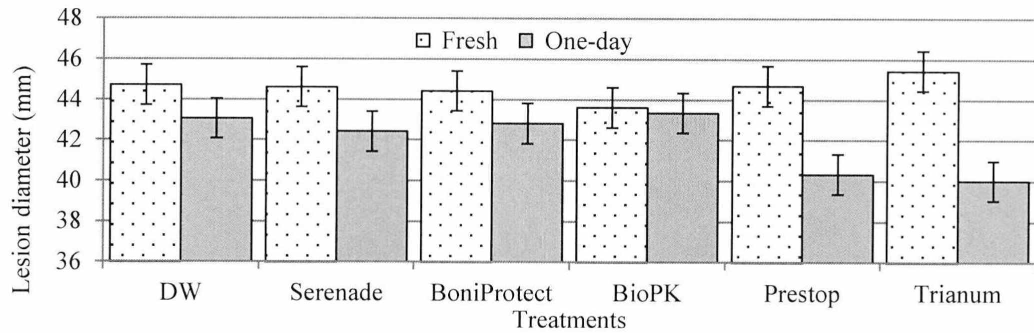


Figure 4.9 Mean values of lesion sizes for different treatments and wounds. Bars represent standard error of the mean. DW: distilled water.

4.3.5 Post-harvest treatment using semi-commercial storage conditions

Both main factors (treatments and temperature) had an effect on percentage infection of cherries infected with *M. laxa* ($p < 0.001$). Figure 4.10 shows the interaction effect between the two main factors ($p < 0.001$). There was a very low level of background level of natural infection in untreated fruits. Indar completely inhibited infection by *M. laxa* under all test conditions. After cherries were inoculated with *M. laxa* and incubated at room temperature, isolate B91 also reduced infection while Serenade and Y126 did not. When cherries were kept at 1°C for four weeks, infection levels were always lower than the treatment with distilled water (DW). However when cherries were then left at room temperature for an additional week, the infection levels increased again to high levels with no difference between treatments and the control.

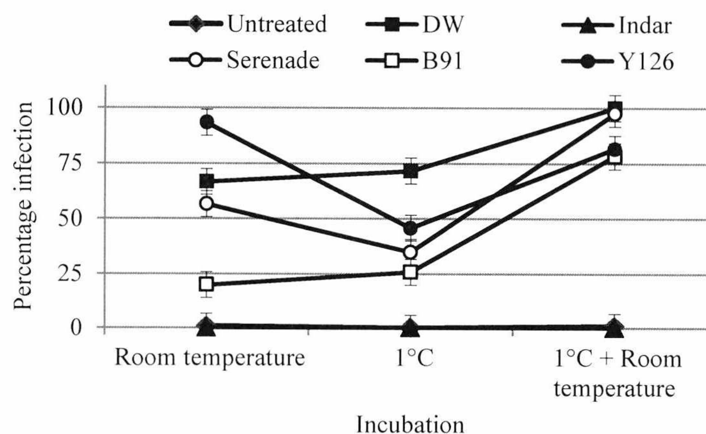


Figure 4.10 Mean values of percentage infection on cherries under semi-commercial storage conditions. Bars represent standard error of the mean. DW: distilled water.

Results from plum experiments showed similar results in that treatments and incubation time had an effect on percentage infection ($p < 0.001$). An interaction

between these two factors was present ($p < 0.001$). In general, Indar was the most effective treatment to control the disease (Figure 4.11) and often eliminated the disease completely. Efficacy of Indar was not affected by incubation temperature. Serenade slightly reduced the percentage infection if plums were incubated at room temperature, but had no effect when plums were incubated at 1°C. At room temperature, isolate B91 had a greater effect than the other biological treatments, resulting in inhibition levels similar to Indar. When plums were incubated at 1°C, both B91 and Y126 reduced infection levels of *M. laxa* almost to zero. However, if plums were then left at room temperature for one week, the percentage infection of fruits treated with B91 and Y126 increased but still remained significantly lower than control treatments.

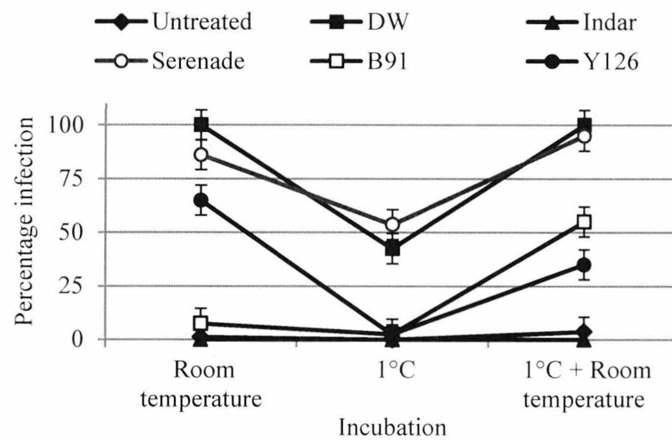


Figure 4.11 Mean values of percentage inhibition on plums under semi-commercial storage condition. Bars represent standard error of the mean. DW: distilled water.

Treatments and temperature had a significant effect on lesion size in plums ($p < 0.001$), but there was no interaction between these two factors ($p = 0.537$). Indar treatments were excluded from analysis as no lesions resulted from this treatment. Serenade had no effect on lesion sizes plums (Figure 4.12), while isolates B91 and Y126 reduced lesion size from 37 mm (in control treatment) to 30 mm. Lesion sizes of plums measured immediately after incubation at 1°C for four weeks was significantly smaller than lesion sizes in fruit subjected to a further week of incubation at room temperature (Figure 4.13).

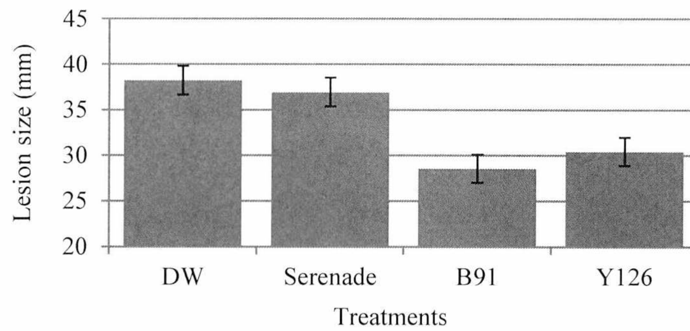


Figure 4.12 Mean values of lesion size on plums treated with different treatments. Bars represent standard error of the mean. DW: distilled water.

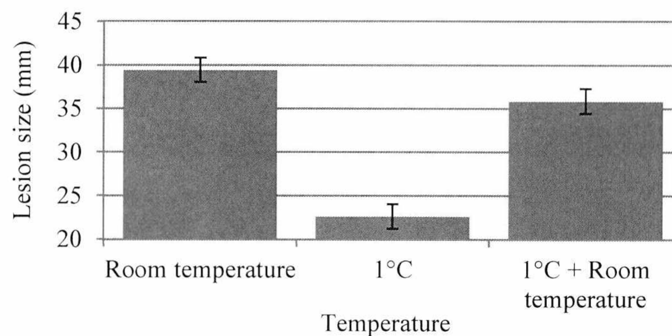


Figure 4.13 Mean values of lesion size on plums incubated under semi-commercial conditions. Bars represent standard error of the mean.

4.3.6 Post-harvest treatment using commercial storage conditions

These fruits were not artificially inoculated and natural infection on plums was very low in all treatments or even absent in some untreated replicates. Infection levels were thus too low for analysis of effects of treatments. In contrast, the level of natural infection of cherries was high and data analysis could be carried out. When rots were assessed immediately after cherries were taken out from cold storage, four different types of rots were found: *M. laxa, M. fructigena, Botrytis* and *Mucor*. Rovral, a standard fungicide, significantly reduced rot caused by *M. laxa* ($p = 0.015$, Table 4.2) but there was no difference between treatments for other types of rot. For total rot, there were no significant differences between treatments.

After the initial assessment, cherries were left at room temperature for seven days and re-assessed. Rovral was still the best treatment, and the fungicide significantly reduced brown rot caused by *M. laxa* ($p = <0.001$). However rot caused by *Mucor* was significantly higher when cherries were treated with Rovral ($p = 0.002$). The two commercial BCAs and our two indigenous BCAs had no effect on rot infection of cherries in these experiments.

Table 4.2 Mean of losses due to rots in cherries following treatment with a fungicide and BCAs. Cherries were stored at 1°C in cold storage. Rots were assessed twice at four weeks after cold storage and one week after at room temperature.

1°C	*M. laxa*	*M. fructigena*	*Botrytis*	*Mucor*		Total rot
Untreated	9.7	0.5	14.3	3.0		27.5
Tap water	8.0	0.6	10.3	4.0		22.9
Roval	**0.8**	0.1	13.5	4.4		18.8
Serenade	7.6	0.7	12.8	1.9		23.0
B91	8.0	0.6	10.7	1.5		20.8
BoniProtect	9.7	1.0	11.9	1.7		24.3
Y126	5.0	0.7	11.8	0.7		18.2
F Prob	**0.015**	0.077	0.743	0.344		0.151
SED	2.309	0.263	2.691	1.767		3.386
LED	4.851	0.554	5.654	3.711		7.114

Room temperature	*M. laxa*	*M. fructigena*	*Botrytis*	*Mucor*	Unknown rot	Total rot
Untreated	46.0	0.9	14.1	29.2	3.3	93.5
Tap water	47.1	1.1	14.0	34.8	3.0	100.0
Roval	**1.9**	0.1	21.2	**60.0**	**16.8**	100.0
Serenade	43.5	1.7	16.8	32.5	5.5	100.0
B91	43.1	1.3	15.9	34.6	5.1	100.0
BoniProtect	43.3	2.0	18.2	32.7	3.8	100.0
Y126	42.4	1.3	18.2	26.6	11.5	100.0
F Prob	**< 0.001**	0.121	0.534	**0.002**	**< 0.001**	0.451
SED	6.252	0.610	3.848	6.744	2.003	3.523
LED	13.136	1.282	8.084	14.168	4.208	7.401

4.4 Discussion

4.4.1 Efficacy of indigenous BCAs

The results support the idea that resident microbes are a good source of BCAs to be used against plant pathogens that live in a similar environment (Janisiewicz and Buyer, 2010; Janisiewicz *et al.*, 2013; Swain and Ray, 2009). The *in vitro* screens indicated that 12 isolates had potential for biological control, including 11 yeasts of similar morphological appearance and a single bacterium. However, only one of these yeast isolates (Y126) was found to have significant bioactivity in the subsequent *in vivo* tests. Two potential BCAs (Y100 and Y101) showed some inhibition, but this was not deemed sufficient to be taken forward for further study.

The failure of the isolates to pass the *in vivo* screens may reflect differences in the conditions under which the screens were conducted. For example, nutrients leaked from artificial wounds on fruit surfaces may have affected the mechanism of inhibition used by the BCAs. The results showed that isolates B91 and Y126 were able to inhibit mycelium growth *in vitro* and reduce infection initiated from spore suspensions applied to wounds *in vivo*. Therefore they can be used to protect blossom, young fruits and fruits in post-harvest stores from air-borne spore infections, as well as from mycelial invasion from adjacent infected fruits either on the tree or in post-harvest storage conditions. Similar results have been reported elsewhere. For example, Zhang *et al.* (2010c) isolated 120 strains of epiphytic yeasts and yeast-like fungi from fruits. After six rounds of *in vivo* screening on peaches against *M. laxa*, three isolates with high efficacy were selected for further study. The three isolates significantly reduced the severity of brown rot lesions but failed to reduce the percentage infection. Although potential BCAs were selected, their methods required a large amount of fruits and screening can be performed only during the harvest season.

In the present study, 217 strains of epiphytic microbes were screened firstly with *in vitro* tests and 12 strains were selected. *In vivo* tests on cherries and plums were then carried out with 12 strains and two BCAs were selected for further study. Testing fewer strains could help to save time and resources. *In vivo* tests are critically important for screening of BCAs because they provide crucial and informative results that can be used for characterisation and improvement of BCAs but they are usually time-consuming and more complex than *in vitro* studies. Thus, screening of BCAs should start with *in vitro* screens followed by *in vivo* tests.

4.4.2 Efficacy of commercial products

In most cases, four commercial BCAs (BioPK, BoniProtect, Prestop and Trianium) had no activity against *M. laxa* when they were tested here on cherries and plums while Serenade had partial activity. In addition, two potential chemicals that were tested, KBV and XzioX, did not reduce infection by *M. laxa* and skin damage was found on plums treated with XzioX. This may have been caused by chlorine dioxide, an active ingredient in XzioX. There might be understandable explanations for the failure of these commercial products in the tests described here. For example, these chemicals and BCAs were specifically screened against a variety of plant pathogens, but brown rot pathogens may not have been included. BoniProtect is recommended for use in protecting blossom of stone fruit from blossom blight and to prevent fruit rot, and it is recommended that this product should be applied up to three times before harvest (Bio-ferm, 2008).

Elmhirst *et al.* (2011) studied a variety of commercial BCAs under greenhouse conditions and showed that Prestop provided the best control of *Botrytis* blight of geranium. In addition, Prestop, Serenade and Rhapsody provided significant control of powdery mildew of roses. In these circumstances, the BCAs might also be able to inhibit brown rot pathogens. However in our study, the *in vivo* test was conducted on fruits under laboratory conditions and the BCAs were applied with a single spraying on fruit surfaces. Therefore the BCAs were used under very different conditions which might have affected the efficacy of BCA products.

Of the commercial BCAs tested here, Serenade showed some promising results by reducing infection of *M. laxa* in both cherries and plums, as well as reducing lesion size. Serenade has been widely reported for showing bioactivity against plant pathogens such as *Glomerella cingulata* (Everett and Machin, 2005), *P. infestans* (Olanya and Larkin, 2006), *Xanthomonas euvesicatoria* and *X. perforans* (Roberts *et al.*, 2008). Serenade has also been tested on stone fruits. It significantly reduced mould rot caused by *Penicillium crustosum* and *Mucor circinelloides* on artificially wounded peaches (Restuccia *et al.*, 2006). Yanez-Mendizabal *et al.* (2012) showed that if Serenade was applied just after inoculation with *M. fructicola* and *M. laxa*, it reduced brown rot in peaches.

In the present study, Serenade was applied as a single spray 24 hours before inoculation. Under these conditions, Serenade partially inhibited *M. laxa* although it was not as good as a fungicide. According to the manufacturer's recommendation Serenade is recommended for use on blossom and green fruit and this BCA should be

sprayed regularly before harvest (AgraQuest, 2009). This suggests that timing and frequency of spray and different stage of plants have influence on efficacy of Serenade.

4.4.3 Effect of application time

In most cases described here, applying the chemical or BCA treatments before or after the inoculation with *M. laxa* made no difference for infection. Indar was the best treatment for control of *M. laxa* and eliminated the visible symptoms of disease, but if it was applied 24 hours after inoculation with the pathogen, rot infection could be found on plums. Efficacy of Serenade depended on application timing. It worked better if it was applied before inoculation with the pathogen suggesting that this BCA might have protective effect rather than curative effect. Our results are supported by Stephan *et al.* (2005) who showed that Serenade was most effective when applied 24 hours before inoculation with *P. infestans* on potato leaves.

4.4.4 Level of susceptibility of plum cultivars

Most varieties of stone fruit are susceptible to brown rot disease to some degree. Due to limitations on harvest season and the high cost of cherries, only one cultivar of cherries was tested here so that discussion on susceptibility of different cherry cultivars cannot be made. Plum cultivars also have different levels of susceptibility to disease (Castejon *et al.*, 2010; Garcia-Galavis *et al.*, 2009). For brown rot, Vitanova *et al.* (2004) showed that two out of ten Bulgarian local cultivars were tolerant of *M. laxa* whilst the rest were resistant to this pathogen, except for cv. Debeljaca from Eastern Serbia, which was highly susceptible (Petrovic *et al.*, 2002).

In the experiments described here, lesion size on cv. Marjorie was usually smaller than those on cv. Victoria. It may be that different plum cultivars contain different levels of nutrients and organic compounds (Gomez-Plaza and Ledbetter, 2010; Ismail *et al.*, 1981) which might lead to a different level of susceptibility to plant pathogens. Plum cultivars also influenced efficacy of BCAs, particularly Serenade. Wounds on plum cv. Marjorie treated with Serenade were smaller by almost 10 mm compared with those on cv. Victoria treated with the same BCA preparation.

4.4.5 Effect of wound age

Although *M. laxa* can cause disease on intact fruits, wounds are still a major route for brown rot infection (Fourie and Holz, 2006; Xu *et al.*, 2007). Wounding dramatically increases the chances of infection by airborne spores of *M. laxa*. The

results described here have shown that wound age did not affect infection of *M. laxa* on cherries. However there was a significant effect of wound age on infection of plums. One-day-old wounds had a lower percentage of infection than fresh wounds. This effect of wound age also affected results obtained with BCAs. When one-day-old wounds of plums were treated with Prestop and Trianium, the lesions were smaller than when fresh wounds were treated, but these two BCAs nevertheless failed to reduce the infection potential of *M. laxa*.

These results are supported by Filonow (2005) who studied the importance of wound age on apples. Three apple cultivars were wounded at different times and then inoculated with either of two pathogens, *B. cinerea* and *P. expansum*. The results showed that fresh wounds were more susceptible to these two pathogens and one-day-old wounds and three-day-old wounds had significantly less disease and severity than fresh wounds. Likewise Wade and Cruickshank (1992) reported that fresh wounds on green apricot were susceptible to *M. fructigena* but became resistant to the pathogen after 6 hours. On fresh wounds, there are abundant free nutrients, especially sugars, which favour infection by a pathogen. However, if fruits were wounded many hours before arrival of inoculum of a pathogen, these nutrients may diffuse and be re-absorbed by the living cells surrounding wounds, or alternatively may promote the growth of saprotrophic microbes that will inhibit the growth of the pathogen when it eventually arrives at the infection court.

4.4.6 Semi-commercial treatment

It is difficult to translate the market potential shown by BCAs in the laboratory to the field because the effectiveness depends largely in application in the uncontrolled environment of the field at pre-harvest. The post-harvest environment therefore offers an better alternative for use of BCAs where a controlled environment exists during processing, packaging and storage (Magan, 2006). This stable condition is conducive for application of BCAs although testing on a large scale can be expensive. Despite chemical treatments being applied in the orchard at the appropriate time, brown rot still causes considerable losses post-harvest due to latent infections already established in the field prior before storage. Post-harvest treatment is desirable. There are various approaches to control brown rot disease at post-harvest.

Physical and biological control offer alternatives to chemicals to control the disease. Physical treatments have been successfully used to control brown rot disease on sweet cherries in controlled atmosphere conditions (Akbudak et al., 2009), on peaches

and nectarines using radio frequency heating (Casals *et al.*, 2010d), and on peaches, plums and nectarines by hot water dipping (Jemric et al., 2010; Karabulut et al., 2010). Although these physical controls may significantly reduce brown rot disease, these methods are not likely be adopted by fruit growers due to complexity and investment in infrastructure. Cherries are too delicate to be treated by physical treatments. Biological control offers an alternative. Spraying and dipping are common practices for BCA applications due to their low cost of operation and ease of delivery.

In order to save time and cost, the BCA candidates identified here were first tested under semi-commercial conditions. BCAs were applied by spraying, which is a common practice for pre- and post-harvest commercial applications. Storage at room temperature was compared to 1°C as the latter is often recommended for stone fruit storage. However fruits were artificially wounded and infected with *M. laxa* to ensure the success of infection.

Zhang *et al.* (2010c) compared efficacy of three BCA candidates at three storage temperatures (20, 8 and 1°C) on peaches artificially infected by *M. laxa*. They found that when peaches were stored at 20°C, three BCAs significantly reduced lesion size caused by *M. laxa* but the best efficacy occurred at 1°C. We also found that efficacy of our two BCAs was better when cherries and plums were stored at 1°C. Serenade was unable to reduce infection of *M. laxa*. Storage temperature played a critical role in the suppression of infection by *M. laxa* when stone fruits were treated with our two BCAs. Isolate B91 greatly reduced infection if the fruits were stored at room temperature, while the efficacy of Y126 was improved if the fruits were stored at 1°C. However when fruits were left at room temperature for checking for a period of one week after cold storage, infection of *M. laxa* dramatically increased. It is worth noting that the two BCA candidates performed better in plums. When lesion size on plum was considered, both of them were able to reduce lesion size. In comparison, the efficacy of Indar, a standard fungicide, was consistent and reliable.

These results indicate that low temperature during handling, storage and shipping can extend the market by reducing infection by *M. laxa* and potentially increasing efficacy of BCAs. Low temperature may affect germinability of spores of *M. laxa*. Casals *et al.* (2010e) showed that at 25°C spores of *M. laxa* needed four hours to reach > 80% germination but at 0°C only 30% of spores germinated within 48 hours. Tian and Bertolini (1999) reported that if *M. laxa* spores were incubated at 0°C, it took around four to six days to reach 80% germination while it took only four to six hours to reach the same level at 20°C. It may be that low temperature has an effect on infection

by *M. laxa* by slowing down spore germination and therefore allowing more time for BCAs to grow, colonise and compete with *M. laxa*. Indar again showed consistent control under all conditions. It is likely that this fungicide completely inhibited the pathogen resulting in no infection on either cherries and plums.

4.4.7 Dipping treatment

For the commercial trial, a dipping treatment was chosen in this study because it required little infrastructure and can accommodate a large amount of fruits at post-harvest. Our results showed that a fungicide was still the best treatment to reduce brown rot infection, and the indigenous BCAs and exotic commercial BCAs failed to control the disease. It may be that BCAs which were not yet formulated might sharply decrease in viability or might not survive during a long period post-harvest. This could follow from the results in Chapter 5 which showed that the viability of BCAs sharply dropped when they were kept at low temperature and nutrients were removed. There are few reports on post-harvest studies which were set in real commercial storage conditions, especially those depending on natural inoculation and using fruit arrangements in trays.

Zhang *et al.* (2010b) showed that when peaches and plums, which were treated by dipping into cell suspensions of BCAs were stored under commercial conditions at 1°C for three weeks, the BCAs were able to significantly reduce infection on the fruits. However the fruits were not wounded but were disinfected in sodium hypochlorite and then artificially inoculated with spore suspensions of *M. laxa*. Smilanick *et al.* (1993) suggested that there was a significant difference between natural infection and artificial infection in terms of infection characteristics on commercially-grown fruits. Disinfection of fruits can remove natural inoculum of antagonists as well as resident competitive microbes on fruit surfaces, both of which can affect fruit infection. Removing the resident microbes from fruit skin might increase the opportunity for BCAs to adhere and colonise the fruit surface thus giving them a competitive advantage over an incoming pathogen. However post-harvest disinfection of fruits is not likely to be used at a commercial scale because this might have caused skin colour changes (Bermudez-Aguirre and Barbosa-Canovas, 2013) and reducing microflora on fruit surface (Fan *et al.*, 2006; Samish *et al.*, 1963).

Cherries and plums picked from commercial orchards in this study were used without surface sterilisation and this may have significantly affected results compared to the use of sterilised fruit in earlier screens. The naturally occurring rots in cherries in this study were mainly caused by *M. laxa, M. fructigena, B. cinerea* and *Mucor* which

have all been commonly reported (Ippolito et al., 2005; Romanazzi et al., 2008). Ippolito et al. (2005) reported the successful use of *A. pullulans* L47 in post-harvest treatments. Sweet cherries picked from an organic farm were dipped into cell suspensions of *A. pullulans* L47, and the treated cherries were then kept in trays covered with plastic sheet and stored at 0°C for 15 days followed by room temperature for seven days. The efficacy of this BCA was increased when it was used in combination with calcium chloride or sodium bicarbonate. However rot was mainly caused by *Botrytis*. This raises the possibility of using these two salts to improve the efficacy of the indigenous BCAs used in this study against *M. laxa*.

The success of BCAs partly depends on the formulation. Product formulation of BCAs plays an important role for (1) maintaining their bioactivity, (2) providing extension of shelf life and (3) allowing products to be applied with existing equipment (Lumsden et al., 1995; Mokhtarnejad et al., 2011; Nunes, 2012). A formulated product is defined as a product composed with a BCA and ingredients that improve survival and efficacy of the BCA (Schisler et al., 2004). Guijarro et al. (2007) used conidia of *P. frequentans* formulated into a wettable power to reduce *M. laxa* infection on peaches. In our study, indigenous BCAs were used without formulation. Although the BCAs were freshly prepared and instantly used, they failed to control rots in cherries. Formulation by adding stabilizers, nutrients, binders or surfactants might improve the efficacy of the indigenous BCAs.

4.5 Conclusions

Efficacy of commercial BCAs was investigated using *in vivo* tests. Serenade partially inhibited *M. laxa* while the other exotic BCAs had no effect against the pathogen. From new 12 indigenous BCAs isolated from UK orchards, the *in vivo* tests on cherries and plums narrowed these down to two isolates with good potential for development.

Chapter 5
Molecular and ecological characterisation of BCAs

5.1 Introduction

When BCAs are introduced into environments, they might have unforeseen impacts on humans and the environment. Before any BCA can be used in commercial agriculture, they must be approved for use by the relevant UK authority, the Chemicals Regulation Directorate (CRD, 2012) and also comply with the EU Regulation 1107/2009/EC (EU, 2009). This necessitates a thorough study of the BCAs in terms of their identity, ecology, biochemistry and potential impact on the environment. In order to begin this process, we have carried out relevant studies to identify them and investigate their mode of action as a BCA, and their capability to grow and survive under relevant environmental conditions. This chapter describes these studies.

Conventional identification techniques are based on morphology and laboratory tests. For example, identification and characterization of yeast species are usually based on morphological characteristics and their physiological capabilities in culture. In contrast, bacterial identification has relied more on biochemical tests. These traditional methods require evaluation of some 60 to 90 separate tests which are time-consuming and laborious (Arias *et al.*, 2002; Janda and Abbott, 2007). In some cases, similar phenotypic characters can be misleading and confusing; scientists may thus need a lengthy period of experience before they can be confident in their identifications.

More recently, molecular identification using universal primers designed to amplify genomic regions within a broad range of microbes can be used to circumvent these problems of identification objectively. After potential microbes are initially identified using molecular techniques, they should also be confirmed with a limited set of conventional identification methods. Identification that relies only on molecular methods may also lead to misidentification, which enters the molecular databases and proliferates the error (Janda and Abbott, 2002). Therefore identification based on a combination of conventional and molecular methods is essential and in this study an initial molecular identification was confirmed by comparing morphological characters with those expected from the tentative molecular identification.

BCAs have various modes of actions; some BCAs are able to inhibit a pathogen using more than one mode of action. For example, modes of action within *Trichoderma* species, known to be amongst the best sources of BCAs, include mycoparasitism, antibiosis, competition for nutrients, tolerance to stress through enhanced root and plant

development, induced resistance and inactivation of the enzymes produced by pathogens (Kamala and Indira, 2011; Scala *et al.*, 2007). For successful biocontrol, BCAs must be able to exert its inhibitory effect in field conditions where it will reduce pathogen activity. Failure to do this gives rise to inconsistency of bioactivity, which is exacerbated by the complexity of the interactions between BCA, pathogen and the environment (Elad, 2003).

Brown rot can be initiated by hyphal infection from adjacent infected fruit or *de novo* by air-borne infection via spores. The hyphal infections can be reduced by removal of rotten fruits which have visible symptoms during harvest. Infection by air-borne spores is more difficult to control as individual spores are invisible to the naked eye and once spores have germinated and invaded the fruit surface, the fungus becomes established as a latent infection at pre-harvest and is thus difficult to detect. Spore germination is an initial step for fungal infection. Understanding the interaction between a BCA and the germinating spores of the pathogen is needed to improve its potential. The ability of selected BCAs to inhibit spore germination of the pathogen is reported in this chapter. Studies on the bioactivity of BCAs mostly focus on bioactive compounds produced and secreted in a soluble form, especially antibiotics and enzymes (Raaijmakers *et al.*, 2002; Sharma *et al.*, 2009; Yuan *et al.*, 2012). However, an alternative bioactivity may come from volatile organic compounds (VOCs), which are produced and volatilise at room temperature (Arrebola *et al.*, 2010c; Fernando *et al.*, 2005; Pimenta *et al.*, 2012). This mechanism of action of BCAs was also investigated here.

Monilinia species can be found in many countries worldwide. These pathogens also grow, infect and survive at different conditions varying from season to season (Hu *et al.*, 2011b; Villani and Cox, 2010; Wherrett *et al.*, 2001). Potential BCAs must also be able to survive and compete under these conditions. There are many factors affecting the use of BCAs in field conditions, including exposure to UV radiation, tolerance to temperature and persistance to rainfall. Temperature tolerance has been prioritised in this study because temperature is a major factor which affects host plants in the field as well as fruits in storage. Temperature also plays a critical role in fungal infection by influencing spore germination and hyphal growth of *Monilinia* species (Koball *et al.*, 1997; Marquenie *et al.*, 2002; Tamm and Fluckiger, 1993). BCAs originally isolated from other climates might have a different capability to grow in the UK climate, thus indigenous BCAs are preferred. The capability of the selected BCAs to tolerate a range of temperature is reported in this chapter.

5.2 Objectives

The objectives of this chapter were (1) to identify candidate BCAs using molecular and morphological data, (2) to study the capability of the candidate BCAs to reproduce and survive at low temperature and (3) to investigate the modes of action of candidate BCAs particularly in relation to inhibition of spore germination and mycelial growth.

5.3 Results

5.3.1 Bacterial identification: *Bacillus* sp. B91

(A) Molecular identification

The SSU sequence of isolate B91 (1,148 bp, accession number KC161970) was compared to sequences available from the GenBank database. The sequence showed 100% DNA similarity to SSU sequences from three *Bacillus* species including *B. licheniformis, B. subtilis* and *B. amyloliquefaciens*. There were no differences in SSU sequence among these three *Bacillus* species.

(B) Macro and microscopic description

The cells stained Gram-positive. The organism was a facultative anaerobe. The culture did not sporulate on NA. Cells were rod-shaped and not in chains. Colonies on NA at 25°C (Figure 5.1) were creamy to light yellow, opaque, dull, spreading, velvety, smooth to slimy at a young stage (three to five days) but become rough and wrinkled by 14 days.

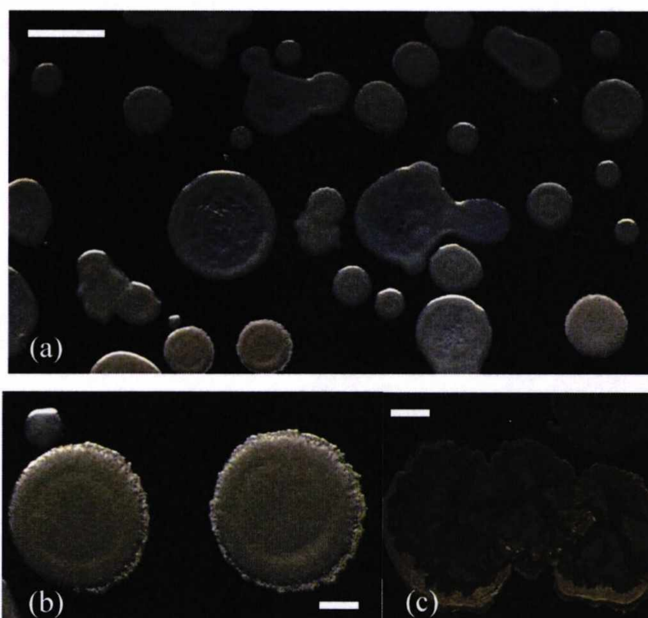


Figure 5.1 Colony morphology of *Bacillus* sp. B91 on NA at 25°C. (a): Creamy colour and slimy colonies, (b): Round and smooth colonies at young stage and (c): Rough colonies at 14 days. Scale bars: (a)= 5 mm; (b,c)= 2.5 mm.

(C) Biochemical description

Salt tolerance: There was good growth in salt broth up to 7% (w/v) NaCl but no growth at 10% (w/v) NaCl. There was no growth on media amended with chloramphenicol or streptomycin. There was positive evidence of casein hydrolysis on relevant test media. Growth on solid medium (NA): Optimum growth occurred between 20-25°C. There was growth at 37 and 40°C but no growth at 50 or 55°C. There was growth was 15°C but no growth at or below 10°C.

5.3.2 Yeast identification: *Aureobasidium pullulans* Y126

(A) Molecular identification

The LSU sequence of isolate Y126 (770 bp, accession number KC161971) showed a close relationship (97%-100% DNA similarity) with various fungal taxa, for example *Aureobasidium, Botryosphaeria, Cladosporium, Discosphaerina, Dothidea, Mycosphaerella* and *Sydowia* (the Dothideomycetes, a type Class within the Pezizomycotina, Ascomycota). This confirms the taxonomic placement of this taxon in the Kingdom Fungi. DNA similarity of ITS sequence of Y126 (518 bp, accession number KC161972) with sequences of *Aureobasidium pullulans* (accession numbers, JN886796, JN886797, JN886798, HM849057, HM849619, EF690466 and AM160630) was between 99%-100%. By using the BLAST search tool, *A. pullulans* was only species showing this close affinity with Y126 which suggested that the identity of isolate Y126 is *A. pullulans*.

(B) Macro and microscopic description

Cultural characteristics: Colonies of Y126 on PDA and MEA at 25°C attained 18 and 23 mm diameter after seven days. Colonies were smooth, slimy and round [Figure 5.2 (a) and (b)]. After seven days growth at 25°C on PDA, the colony colours of Y126 were compared to the colour chart of Kornerup & Wanscher (1978). The colour of the front of the colonies was light orange to pale orange (colour code 6A3/5A4), with the reverse orange white (colour code 5A6); the colour on MEA was pinkish-white to orange white (colour code 7A2/6A2) from the front, with the reverse pale yellow (colour code 4A3). The colour of the colony margin was slightly darker or remained the same after 14 days. There was no aerial mycelium.

The vegetative hyphae were hyaline, and stained in cotton blue in lactophenol. Pseudohyphae were smooth, thin-walled and septate. Conidia were ellipsoidal or ovoidal, one-celled, smooth and produced in dense groups at terminal and subterminal

ends of hyphae [Figure 5.2 (c), (d) and (e)]. Budding of conidia was observed, with secondary conidia usually smaller than primary conidia. These characters confirm that the isolate is *Aureobasidium pullulans* and the tendency for the colony to darken with age suggests it is *A. pullulans* var. *pullulans*.

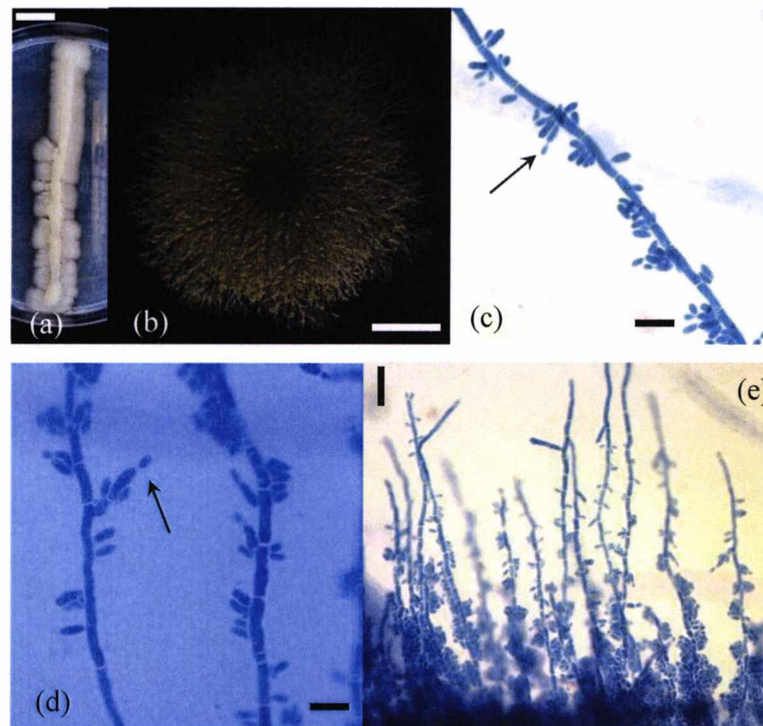


Figure 5.2 Morphology of *Aureobasidium pullulans* var. *pullulans* Y126 (a): Colony on PDA at seven days at 25°C, (b): Close-up colony with lateral mycelia on PDA, (c): Septate hyphae with branching conidia (arrow), (d): Ellipsoidal conidia and budding conidium (arrow) and (e): Growing hyphae with branches of conidia stained with cotton blue in lactophenol. Scale bars: (a)=10 mm, (b)= 1 mm, (c,d)=20 μm, (e)=40 μm.

(C) Biochemical description

Salt tolerance: Growth in NaCl broth, good growth up to 7% (w/v) NaCl but no growth at 10% (w/v) NaCl. Based on visual observation, the optimum temperature for growth on solid agar (PDA and MEA) was 25°C, with no growth occurring at 35 and 37°C. There was no growth at 0 and 4°C.

5.3.3 Effect of temperature on growth of BCAs

(A) Growth of *Bacillus* sp. B91

Colony forming unit (CFU), an estimate of viable microbe numbers, was used to quantify the numbers of BCAs in this study instead of direct microscopic count which all cells either dead or living are counted. The CFU numbers represented living cells of the BCAs in original liquid cultures which the cultures were maintained under a static

condition. Temperature had a highly significant effect on growth of *Bacillus* sp. B91 ($p < 0.001$). An interaction between time and temperature was present ($p < 0.001$). Figure 5.3 shows the viability of *Bacillus* sp. B91 illustrating the nature of this interaction.

At the beginning, the CFU number started at 13 ln CFU/ml. The bacterial growth behaviour was divided into three groups based on temperature. Firstly, at low temperatures (0 and 5°C), the numbers of viable cells of *Bacillus* sp. B91 sharply decreased from 13 ln CFU/ml to less than 8 ln CFU/ml after three weeks and remained at 7 ln CFU/ml. Secondly, at 10°C the CFU numbers of *Bacillus* sp. B91 slowly decreased for two weeks but the CFU number gradually increased from week two to week six. Finally at 15, 20 and 25°C, the CFU number of *Bacillus* sp. B91 rose from 13 to 15 ln CFU/ml within one week and remained steadily between 14 and 16 ln CFU/ml until the final week of the observation.

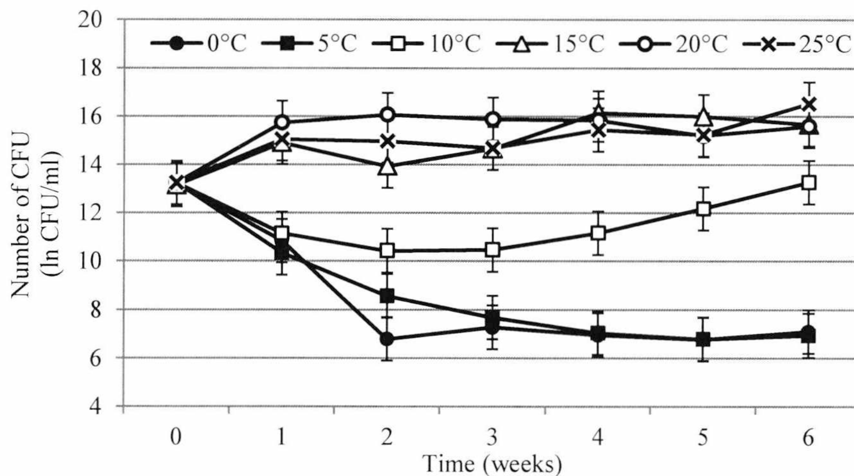


Figure 5.3 Viability of *Bacillus* sp. B91 when the cultures were maintained in NB and incubated at different temperatures for six weeks under a static condition. Bars represent standard error of the mean.

(B) Growth of *A. pullulans* Y126

Under a static condition, the nature of the growth of *A. pullulans* Y126 is shown in Figure 5.4. Temperature had significant effect on growth of Y126 ($p < 0.001$). An interaction between temperature and time was present ($p < 0.001$). The CFU number of *A. pullulans* Y126 started at 14 ln CFU/ml. At 20 and 25°C, the CFU number of *A. pullulans* Y126 sharply rose from 14 to 17 ln CFU/ml while the growth at 15°C also increased but to a lesser extent. By week two the CFU number of *A. pullulans* at 20 and 25°C sharply dropped, whilst the CFU number at 15°C also dropped but at a slower rate. However the CFU number continued to significantly increase at 2 weeks at 10°C although it did not reach the week-one numbers of the cultures at 20 and 25°C. After 2 weeks, these numbers also began to fall. In contrast, the CFU number gradually

increased at 0 and 5°C until the final week of the observation. In general, the CFU numbers among these six temperatures were not statistically different from week three to week six.

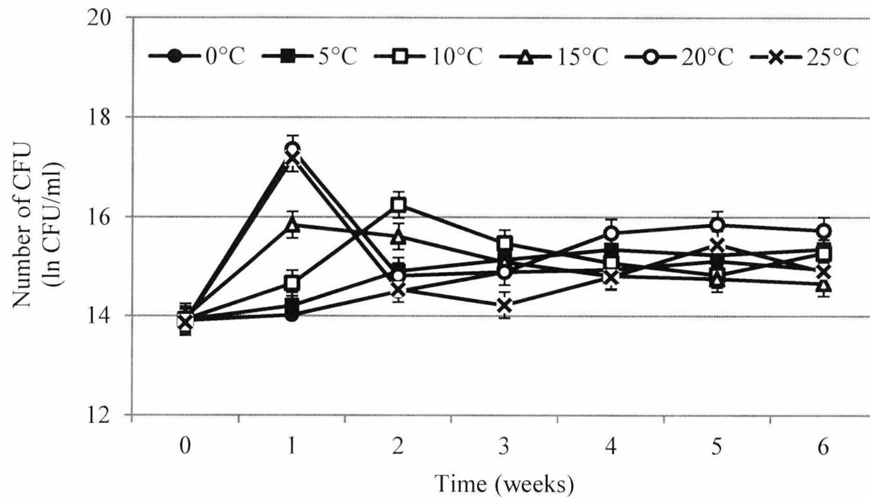


Figure 5.4 Viability of *A. pullulans* Y126 when the fungal cells were maintained in PDB and incubated at different temperatures for six weeks under a static condition. Bars represent standard error of the mean.

5.3.4 Effect of temperature on survival of BCAs

(A) Survival of *Bacillus* sp. B91

Temperature and time had significant effects on survival of *Bacillus* sp. B91 ($p < 0.001$). There was an interaction between temperature and time ($p < 0.001$). The seed cultures of *Bacillus* sp. B91 all started with a concentration of 17 ln CFU/ml (Figure 5.5). After one week, the CFU number had sharply dropped at all test temperatures from 17 to 14 ln CFU/ml. The CFU number of *Bacillus* sp. B91 at 20 and 25°C then remained steady for the next 4 weeks with no statistical difference between each sampling time. However the CFU number at lower temperatures (at 0, 5, 10 and 15°C) continued to drop to less than 12 ln CFU/ml and then remained steady until the final week.

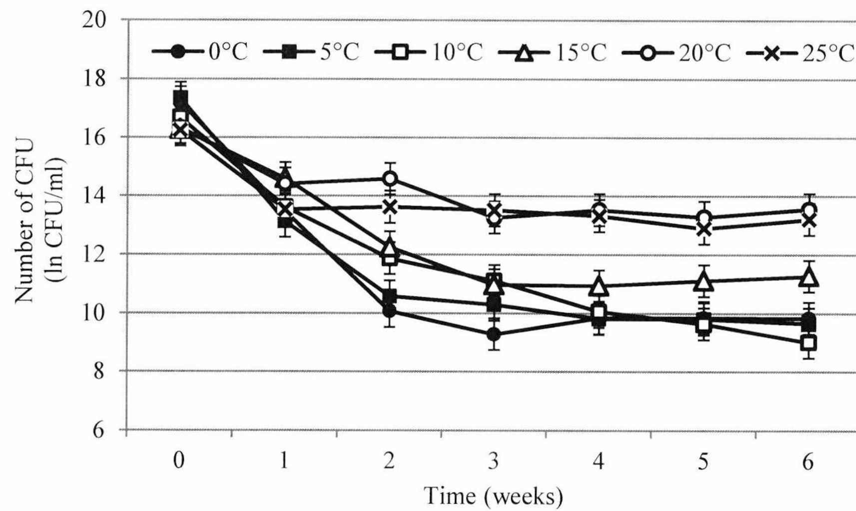


Figure 5.5 Survival of *Bacillus* sp. B91 when the bacterial cells were kept in PBS and incubated at different temperatures for six weeks under a static condition. Bars represent standard error of the mean.

(B) Survival of *A. pullulans* Y126

Temperature had a significant effect on survival of cells of *A. pullulans* Y126 ($p < 0.001$). The seed cultures of Y126 all started with a concentration of 18 ln CFU/ml (Figure 5.6). After an initial drop in the first week, the CFU number maintained high concentrations (around 14 ln CFU/ml) with initially lesser decline at 0°C. The survival fingures for *A. pullulans* at 5, 10, 15 and 20°C were not significantly different from each other throughout the study. The viability at 25°C steadily declined over the whole 6 week period such that there were significantly fewer cells surviving at this temperature than the lower temperatures at the end of the experiment.

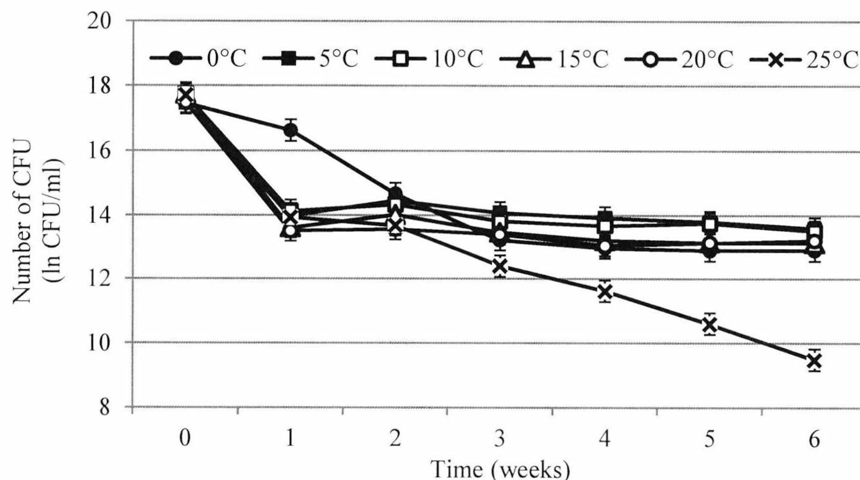


Figure 5.6 Survival of *A. pullulans* Y126 when the fungal cells were kept in PBS and incubated at different temperatures for six weeks under a static condition. Bars represent standard error of the mean.

5.3.5 Inhibition of spore germination

There was a significant difference in the percentage of spore germination of M. laxa between the control and treatments with BCAs ($p < 0.001$). Percentage germination (after angular transformation) for the control and BCA treatments were 88.5% and 20.3% respectively. This clearly shows that BCAs had reduced spore germination. BCAs and sources were two factors that had significant effects on the percentage of germination (Figure 5.7 $p < 0.001$). The interaction between BCAs and sources of bioactivity was present ($p < 0.001$).

The culture broth and living cells of *Bacillus* sp. B91 reduced the germination suggesting that bioactivity of *Bacillus* sp. B91 did not depend on living cells being present. However bioactivity of *A. pullans* Y126 was dependent on living cells being present, as the cell-free extract of isolate Y126 did not inhibit spore germination. For the effect of media, spore germination in PDB was not significantly different from the germination on WA ($p = 0.224$). There was no interaction between media and BCAs or media and sources ($p = 0.312$ and 0.749, respectively). It was concluded that efficacy of BCAs did not depend on media used in this study and different types of media (PDB and WA as liquid and solid media, respectively) did not affect the test.

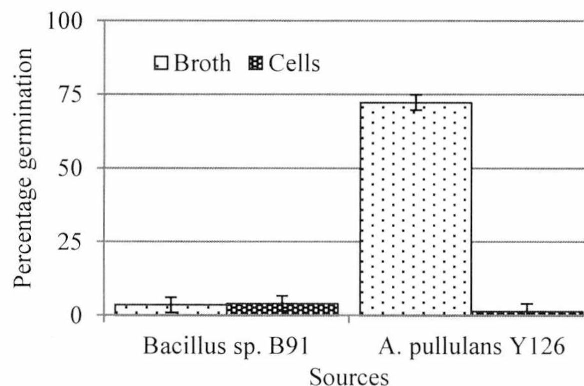


Figure 5.7 Mean values of percentage germination of *M. laxa* spores treated with cell cultures and cell-free extracts from two BCAs. Bars represent standard error of the mean.

5.3.6 Production of VOCs

The co-culture between confinement culture of the BCAs and actively growing culture of *M. laxa* showed that colonies of *M. laxa* co-cultured with either B91 or Y126 were significantly smaller than the colonies in the control treatment (sterile PDA; Figure 5.8). For the effect of the two main factors, BCAs and isolates of *M. laxa* had a significant effect on the final colony diameter of *M. laxa* ($p < 0.001$). An interaction between BCAs and isolates of *M. laxa* was present ($p < 0.001$), *i.e.* the effect of the BCAs against different strains of *M. laxa* was not equal.

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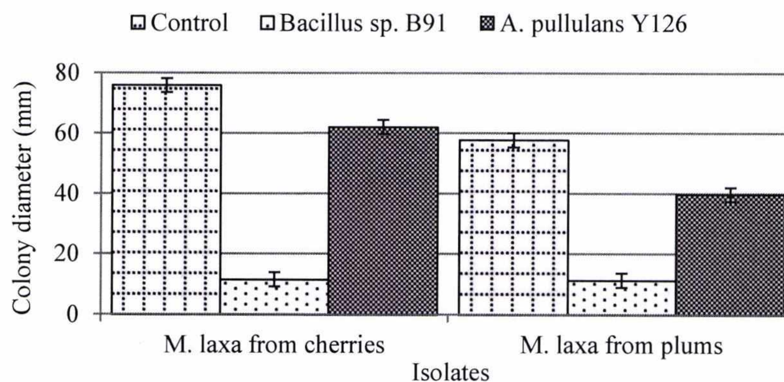

Figure 5.8 Effect of VOC production produced by two BCAs against two isolates of *M. laxa*. Mean values for radius of *M. laxa* colony (mm) on PDA incubated for two weeks tested with *Bacillus* sp. B91 and *A. pullulans* Y126. Bars represent standard error of the mean.

The effect of VOCs produced by *Bacillus* sp. B91 on two isolates of *M. laxa* was similar and highly inhibitory, while the inhibitory activity of *A. pullulans* Y126 varied across the 2 isolates of *M. laxa.* Although *A. pullulans* Y126 reduced colony diameters of both *M. laxa* isolates compared to the control treatment, this effect was not as pronounced as that with *Bacillus* sp. B91 and both isolates were able to grow to some extent (Figure 5.9).

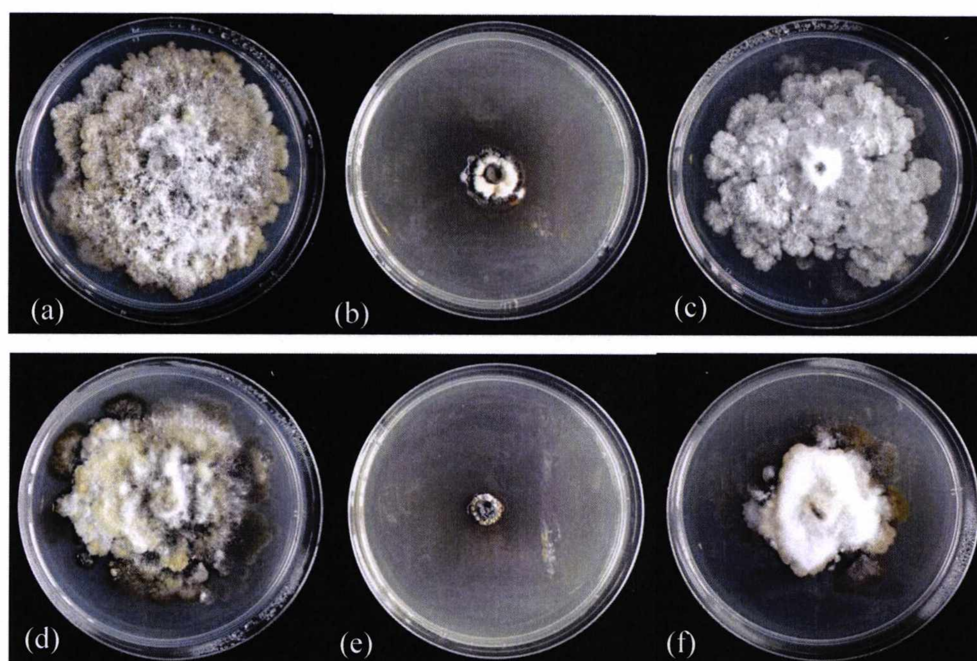

Figure 5.9 Mycelial growth of *M. laxa* isolates from cherries (upper row, a-c) and plums (lower row, d-f) when they were co-cultured with sterile PDA (left column), *Bacillus* sp. B91 (middle column) and *A. pullulans* Y126 (right column). The plates were incubated at 25°C for two weeks.

5.4 Discussion

5.4.1 Bacterial identification

In recent years, a large numbers of bacterial SSU primers have been developed. Some of these primers are designed to be taxon-specific while others are designed to universally amplify all prokaryotic taxa (Baker *et al.*, 2003). In this study, SSU sequence analysis revealed that isolate B91 had a strong affinity with several *Bacillus* spp. which have identical SSU sequences. The SSU region, or 16S ribosomal DNA region, is generally regarded as the most common genetic marker for bacterial identification (Janda and Abbott, 2007). Analysis based on the SSU has replaced the labour-intensive DNA-DNA hybridisation for determination of new species and assigning identities to unknown strains. In most cases, sequence comparison of the DNA from the SSU region is sufficient to identify bacterial isolates. Mignard and Flandrois (2006) demonstrated the success of SSU sequences for routine bacterial identification of clinical specimens. From 683 clinical isolates, the majority of specimens (83%) were identified to species level and 16% of specimens were identified to genus level. Only 1% SSU sequences were unassignable at genus level. For isolates identified to genus level only, three possible reasons for failure to assign to species level were put forward: (1) failure to differentiate between two species, (2) lack of related and relevant sequences to compare and (3) presence of undetermined positions in the sequences. In this study, the identification of *Bacillus* sp. B91 was limited to genus level due to the first reason. As a consequence, a set of biochemical tests was used to further determine the species identity of *Bacillus* sp. B91. However, these tests were still inconclusive and showed that B91 was closely related to two *Bacillus* species: *B. subtilis* and *B. amyloliquefaciens*.

Results from further biochemical tests showed that isolate B91 was susceptible to chloramphenicol and streptomycin, which suggests it is *B. amyloliquefaciens*. On the other hand, isolate B91 grew in 7% NaCl but not at 10% NaCl and the cells did not form chains. This suggests it is closer to *B. subtilis,* although it did not grow above 50°C which most strains of *B. subtilis* do. Thus it appears that the large variation between species and presence of intermediate strains may have obscured the clear distinction between these two species (Logan and Berkeley, 1984; Norris *et al.*, 1981) and the exact identity of B91 cannot easily be determined. *Bacillus amyloliquefaciens* used to be classified as sub-group of *B. subtilis* but it was proposed as a new species by using (1) biochemical tests, (2) gas liquid chromatography, (3) DNA-DNA hybridisation and (4) sequence analysis of recA and gyreA genes (Borriss *et al.*, 2011; Chun and Bae, 2000;

Nakamura, 1987; O'Donnell *et al.*, 1980; Priest *et al.*, 1987; Wang *et al.*, 2008). To differentiate these two species, at least two of these techniques should be used and the type species of both species would need to be included and analysed. This was deemed beyond the scope of this study but it clearly shows that *B. amyloliquefaciens* cannot be separated from *B. subtilis* using conventional biochemical tests and SSU sequence comparison.

Alvarez *et al.* (2012) also reported the difficulty and confusion of identification of these two species. Based on SSU sequence analyis, two strains of *Bacillus* sp. were initially identified as *B. subtilis* but recA gene sequencing and a set of biochemical tests focussing on these two particular species identified one of the isolates as *B. amyloliquefaciens*. Yuan *et al.* (2012) also noted difficulties when they identified an isolate of *Bacillus* NJN-6 as *B. amyloliquefaciens* solely using SSU sequences (accession number GQ452909) as it showed 100% identity to *B. amyloliquefaciens* CAU-B946, accession number HE617159 deposited by Blom *et al.* (2012). However the identity of accession number GQ452909 on the GenBank database is displayed as *B. subtilis* N-6. This clearly shows that information on the public databases is sometimes unreliable and identification based on a single method may lead to misunderstanding and confusion.

5.4.2 Yeast identification

Two regions of ribosomal DNA were sequenced and gave different levels of taxonomic information. The sequence from the LSU region was used to determine the taxonomic position within the Kingdom Fungi and confirmed that Y126 is an ascomycetous yeast with a close relationship within the Dothideomycetes. The sequence from the ITS region further revealed the identity of this isolate at species level to be *Aureobasidium pullulans* with more than 99% sequence similarity. In order to confirm this specific identity, the colony description including colours, growth rate on PDA, MEA as well as microscopic structures *i.e.* conidia shape, hyphae, were also found to match the description of *A. pullulans* in the Atlas of Clinical Fungi (De Hoog *et al.*, 2000) and a recently published paper which redefined the species concept of *A. pullulans* (Zalar *et al.*, 2008).

One aspect of the study is worth noting with respect to the production of conidia, which is used as a taxonomic character for identification of *A. pullulans*. Descriptions usually state that conidia are usually produced under 'normal conditions', for example on PDA or MEA incubated at 25°C in the dark. In this study, isolate Y126 did not

produce conidia under such conditions, but did so if cultured on PDA and incubated under UV light for seven days. In conclusion, a combination of molecular identification and microscopic observation, was sufficient to identify this BCA as *A. pullulans*.

There are varieties of *A. pullulans* recognised, based on geographical distribution and characterisation of pigmentation (Zalar *et al.*, 2008). The var. *namibiae* is isolated from Namibia, Africa and the var. *subglaciale* is exclusively known from sub-glacial ice and sea water in the Arctic. Two varieties which are characterised by the pigmentation are var. *pullulans* and var. *melanogenum*. The var. *pullulans* is phenetically characterised by rapidly expanding and pinkish cultures which can develop to dark brown while the var. *melanogenum* is black from beginning and produces dark conidia. The var. *pullulans* is mostly isolated from sugary or osmotically fluctuating habitats, fruit surfaces and phyllosphere while the sources of the var. *melanogenum* are low nutrient, low-strength environments such as moist metal, glass surfaces and showers. In the present study, isolate Y126 produced orange to pinkish colonies which gradually became darker. The colonies remained pinkish after 14 days. The source of this culture was leaves of cherries. Therefore we concluded that isolate Y126 is *A. pullulans* var. *pullulans*.

5.4.3 Growth of BCAs at low temperatures

When BCAs are applied to blossom and green fruits in spring and summer, BCAs should be able to grow over time and to colonise expanding plant tissues. Studying capability of BCAs to grow at different temperatures provides crucial information for BCA development. Yanez-Mendizabal *et al.* (2012) studied on population dynamics of *B. subtilis* by inoculating the BCA in wounds of peaches and showed that number of cells of *B. subtilis* increased and remained constant throughout five days of the incubation period. Likewise the CFU counts of two strains of *A. pullulans* increased significantly in wounds of apples and nectarines when the fruits were incubated for 7 and 28 days, respectively (Mari *et al.*, 2012a; Mari *et al.*, 2012b). Their results might show the possibility to use BCAs to control spread of fruit rot at post-harvest. In this study, our objective was to use BCAs to compete with *M. laxa* on mummified fruits and to protect blossom and green fruits pre-harvest. Therefore the growth study was conducted in culture broth instead of in wounds of stone fruit.

The two BCAs were able to grow over a wide range of temperatures, with the optimum temperature of *Bacillus* sp. B91 over a range of 15-25°C, and the optimum temperature of *A. pullulans* Y126 between 20-25°C. However the temperature range of

active growth of *Bacillus* sp. B91 was narrower than that for *A. pullulans* Y126. When nutrients were present, *Bacillus* sp. B91 grew well if it was incubated at 15-25°C, but it grew poorly at low temperatures such that the reproduction rate was lower than the mortality rate resulting in a significant loss of viable cells. There was some recovery, however, of cell counts at 10°C. This might have been an adaptation of bacterial cells in order to cope with low temperature. It is generally accepted that there are two phases of cold stress response: (1) an immediate response and (2) subsequent delayed response (Klein *et al.*, 1999). At 10°C, *Bacillus* sp. B91 took around two weeks to adapt to cold then it was able to grow gradually over time.

In contrast, growth of *A. pullulans* Y126 was limited by lower temperatures but not prevented and, even at the lowest test temperature (0°C), it still grew slowly. The growth rate of Y126 was highest at 20 and 25°C for the first week, and then sharply dropped. These two temperatures are the optimum temperatures for Y126 therefore it multiplied rapidly and nutrients were possibly exhausted, which resulted in the decline in numbers over the following weeks. Subsequently the numbers of Y126 cells were slightly increased suggesting that it survived by utilising potentially dead cells and left over organic matter in liquid culture.

Differences in the growth of *A. pullulans* Y126 at 0°C on two types of media are worth noting, in that it grew in liquid broth but there was no growth on solid agar. It may be that on solid agar, growth was limited by a low diffusion rate of an essential nutrient through solid media. Conversely if *A. pullulans* Y126 was in liquid broth, it could easily and quickly absorb nutrients which were dissolved in a liquid form into the cells.

According to the ability to grow at high, intermediate and low temperature, microbes are divided into three categories: thermophiles, mesophiles and psychrophiles, respectively (D'Amico *et al.*, 2006; Hebraud and Potier, 1999). We can conclude that *Bacillus* sp. B91 is a mesophilic bacterium because it only multiplies at intermediate temperatures, while *A. pullulans* Y126 is both mesophilic and psychrotolerant because *A. pullulans* Y126 grows optimally around 20-25°C, but is able to reproduce and grow at 0°C.

5.4.4 Survival of BCAs at low temperatures

Application of BCAs largely focuses on blossom and green fruits, but post-harvest treatments to fruits and applying BCAs to mummified fruits have been studied less. Temperatures in storage conditions and during winter are usually low and fairly

constant, and the environments have low levels of nutrients. In order to study the potential for use of BCAs in these two environments, the capability of BCAs to survive under such conditions was studied. Potential BCAs should be able to survive and maintain competitive cell numbers relative to the pathogen under these harsh conditions.

Bacillus species are more likely to tolerate high temperatures than low temperatures as they produce endospores which are heat-resistant (Warth, 1978). However there is less information on cold-tolerance of spores but these experiments suggest they offer little advantage. The ability of BCAs to grow or survive growth of BCAs at different temperatures, especially low temperatures, is crucial for their successful pre- and post-harvest use. Both *B. subtilis* or *B. amyloliquefaciens* are known to live epiphytically on plant surfaces and in the upper layers of soil, and thus it might be expected that they are adapted to field conditions.

In the present study, although low temperatures initially reduced the viable counts of *Bacillus* sp. B91, it was still able to survive and maintain a lower but stable concentration for many weeks in all test temperatures. This result is supported by Tokuda *et al.* (1995) who studied the survival of *B. subtilis* in sterile soil incubated at 15 and 25°C. They showed that cells of *B. subtilis* initially declined in the first week and then stabilised over the 50 day incubation period. Survival was higher if *Bacillus* sp. B91 was incubated at room temperature (20-25°C), but began to decline faster at temperatures of 15°C and below. It is likely that 15°C is a critical temperature that triggers a cold response for *B. subtilis* (Budde *et al.*, 2006).

Aureobasidium pullulans Y126 was tolerant to a wide range of temperatures only if this BCA was kept between 0-20°C. In the growth study when nutrients were provided, *A. pullulans* Y126 grew well at 25°C. However when nutrients were removed, viability of cells of *A. pullulans* Y126 gradually deteriorated over time showing that this BCA could not cope with this temperature because Y126 was unable to utilise solely dead cells as a nutrient souce. To survive at 25°C, it is likely that some basic nutrients have to be provided for *A. pullulans* Y126. This highlights an issue for product formulation in that if *A. pullulans* Y126 is to be applied in the orchards where field temperatures are 25°C or above; a source of nutrients is a must in the formulation of *A. pullulans* Y126.

Some strains of *A. pullulans* could cope with higher temperature which might be related to climates and their countries of origin, for example 30°C and 25°C were preferred for strains of *A. pullulans* isolated from India and Thailand, respectively

(Punnapayak *et al.*, 2003; Sugumaran *et al.*, 2013). In the present study, *A. pullulans* Y126 was isolated in the UK where the climate is colder, therefore this BCA is less capable of survival at 25°C. Nevertheless *A. pullulans* managed to maintain a high concentration of cells at 0°C throughout the study. This emphasises the psychrotorerant nature of *A. pullulans*. It is supported by strains of *A. pullulans* have repeatedly isolated from cold environments such as Arctic ice (Gunde-Cimerman *et al.*, 2003; Zalar *et al.*, 2008).

5.4.5 Inhibition of spore germination

Spore germination is important for fungal infection. After spores land on flowers or a fruit surface, spores germinate if environmental conditions permit, and germ tubes grow on or into the surface and initiate disease. To minimise infection, control measurements should inhibit spore germination as early as possible. BCAs can affect spore germination through direct competition for nutrients and by the production of inhibitory compounds ('antibiotics'). When studied *in vitro*, the age of cultures seems to be a factor in determining inhibitory activity. When tests are based on an assumption of antibiosis, experimenters should give enough time to ensure that secondary metabolites are produced and secreted from cells; this process usually starts when cells are in a stationary phase or the growth rate is sub-optimal. For example *B. subtilis* CPA-8 had inhibitory activity against *M. fructicola* and *M. laxa* after it had been cultured for 3 days (Yanez-Mendizabal *et al.*, 2012). For the two BCAs investigated here, inhibitory activity was tested when they were cultured for 4 days to ensure that target bioactive compounds were produced and secreted from the cells.

5.4.6 Possible modes of action

Results clearly show that both BCAs inhibited spore germination. The sources of bioactivity were studied to test whether culture filtrates alone or also living cells are needed for bioactivity – to indicate whether diffusible antibiotics are involved. Our study revealed that cell-free culture filtrates of *Bacillus* sp. B91 were able to reduce spore germination, but living cells of *A. pullulans* Y126 were needed in addition to inhibit the spore germination of *M. laxa*. This result confirmed the involvement of antibiotics produced by *Bacillus* sp. B91 in the inhibition of spore germination of *M. laxa*. Nutrient competition might also have a contribution but this cannot easily be confirmed unless all target antibiotics are identified and excluded from the tests. Yanez-Mendizabal *et al.* (2012) suggested that antibiosis was a major factor for bioactivity of

B. subtilis CPA-8 in controlling brown rot disease on peaches. They showed that lipopeptides produced by CPA-8 were responsible for inhibitory activity. If biosynthesis of these compounds was disrupted by mutagenesis, *B. subtilis* CAP-8 lost the ability to control brown rot disease. At this stage, it can only be stated that there were bioactive compounds in the culture broth of *Bacillus* sp. 91 that inhibited spore germination of *M. laxa* but their characterisation has not been attempted. The role of antibiotics cannot be confirmed unless targeted bioactive compounds are isolated, identified and tested separately.

On the other hand, living cells of *A. pullulans* Y126 were essential for bioactivity. Liquid cultures of *A. pullulans* Y126 suppressed spore germination of *M. laxa* but the cell-free culture broth had no effect. This does not mean that this BCA does not produce bioactive metabolites but such compounds had no effect against spore germination of *M. laxa*. Alternatively it might be that any bioactive compounds were not stable or deteriorated after they were secreted and kept at room temperature. Zhang *et al.* (2010b) reported that *A. pullulans* PL5 at a concentration at 1.0×10^8 CFU/ml completely inhibited spore germination of *M. laxa* tested in PDB, whereas dead cells or cell-free broth had no effect. They emphasised an importance of inoculum concentration because the inhibitory activity significantly dropped when an inoculum of 1.0×10^6 CFU/ml was used. In this study, the higher concentration of cells (1.0×10^8 CFU/ml) was used, which suppressed spore germination to less than 5%.

In previous studies, bioactivity of *A. pullulans* has been assigned to two mechanisms: (1) secretion of lytic enzymes and (2) competition for nutrients (Bencheqroun *et al.*, 2007b; Janisiewicz *et al.*, 2010; Spadaro *et al.*, 2011; Zhang *et al.*, 2010b). β-1,3-glucanase and chitinase produced by *A. pullulans* were intensively studied for their biological role in inhibiting pathogens. Zhang *et al.* (2010b) studied enzyme activity of *A. pullulans* by measuring the activity when *A. pullulans* was co-cultured with *M. laxa* that was used as a sole carbon source. The enzymes broke down the hyphal wall of the pathogen. They found that the maximum level of two enzymes was reached after 48 hours of co-culture at 25°C. However these enzymes were enriched using optimum conditions to maximise the quantity of enzymes produced - which it is not likely to happen in a natural situation. Castoria *et al.* also (2001) found that the enzyme activity of β-1,3-glucanase from *A. pullulans* was higher when cultured with hyphae of *Penicillium expansum*.

However Vero *et al.* (2009) suggested that enzymes did not seem to play a major role in inhibiting pathogens or their spore germination because these enzymes took at

least 48 hours to produce in log phase. Moreover they found that *A. pullulans* took 7 and 10 days to produce β-1,3-glucanase and chitinase at 5°C, respectively. This would be too late to control infection, which usually occurs within the first few hours, even at low temperatures. These enzymes may have an impact in the longer term protection of wounds or fruit skins. In this study, experiments on enzyme activity were not initiated as the bioactivity of *A. pullulans* Y126 probably came from competition for nutrients as inhibition was manifested within 12 hours during which time these enzymes might not have been induced. Some residual enzyme activity would also have been manifested in cell-free extracts – which was not the case.

Based on competition for nutrients, Bencheqroun *et al.* (2006) showed that *A. pullulans* Ach1-1 was able to inhibit spore germination of *P. expansum* when tested in low concentrations of apple juice. When the concentration of apple juice was much higher, *A. pullulans* Ach1-1 and the pathogen were no longer competing and spores of *P. expansum* were able to germinate. Likewise, Zhang *et al.* (2010b) reported that in low concentrations of peach juice, *A. pullulans* was more effective in inhibiting mycelial growth of *M. laxa*. In order to maximise efficacy of *A. pullulans* against spores of pathogens, nutrients should be in low concentration – a situation common in the natural environment. Media used for screening process should contain enough nutrients to maintain growth of a BCA and the pathogen but should be a minimal medium in order to a favour mechanisms based on nutrient competition. Therefore relatively simple agar such as PDB and WA were used in this study.

Takesako *et al.* (1991) found that antibiotics from *A. pullulans*, the so-called 'aureobasidins', had antifungal activity against *Candida* spp., but Castoria *et al.* (2001) reported that this was not the main source of bioactivity. Extracts from *A. pullulans* failed to inhibit growth of all four pathogens including two bacteria, *Pseudomonas corrugate* and *B. subtilis* and two fungi, *P. expansum* and *Alternaria alternata* (Castoria *et al.* 2001). Our findings support the assumption that competition for nutrients plays an important role in bioactivity of *A. pullulans*, while other mechanisms such as production of enzymes and antibiotics, are less important.

5.4.7 Effect of media on spore germination

Studies on spore germination are mostly conducted on a single medium. Zhang *et al.* (2010b) studied effect of *A. pullulans* on the spore germination of *P. expansum* using PDB while Arrebola *et al.* (2010a) used PDA as a medium for inhibition of spore germination of *P. crustosum* by *B. amyloliquefaciens*. In this study, two different media

(PDB and WA) were compared. Our results have shown that there was no difference in the germination of *M. laxa* on the two media. Therefore experimenters can choose either of them, although WA might be a closer match to the natural environment.

5.4.8 Production of VOCs

Both BCAs investigated in this study were shown to produce VOCs. The VOCs from *Bacillus* sp. B91 were very inhibitory to growth of *M. laxa* and this effect was consistent for both strains of the pathogen tested. In contrast, the VOCs from *A. pullulans* Y126 were less inhibitory and the relative effects varied for the two *M. laxa* strains. Microbial VOCs against *Monilinia* species have been previously reported. Neri *et al.* (2007) studied nine VOCs, originally isolated from plants but also available as pure compounds from Sigma-Aldrich, and tested them against *M. laxa.* Three of them had strong antifungal activity against spore germination and mycelial growth of the pathogen. They also conducted experiments to test VOCs on brown rot on four stone fruits including apricots, nectarines, peaches and plums. Trans-2-hexenal, one of the selected three VOCs, showed the best result by significantly reducing brown rot disease in all cultivars of the stone fruits. Silveira *et al.* (2010) showed that when hexenal was applied at either the moment of inoculation or 24 hours after inoculation with the pathogen, it was able to reduce brown rot disease on two cultivars of peaches *i.e.* it had both a protective and curative effect. *Muscodor albus*, the best known VOC-producing filamentous fungus, reduced brown rot caused by *M. fructicola* on peaches when a live culture of *M. albus* was put inside a bag containing peaches (Schnabel and Mercier, 2006). Pimenta *et al.* (2012) showed that endophytic fungi isolated from plums were able to produce VOCs against *M. fructicola* and five VOCs were identified. These results illustrate the potential for use of VOCs post-harvest.

Mari *et al.* (2012b) reported that two strains of *A. pullulans* significantly reduced mycelial growth of three pathogens including *Botrytis cinerea, Colletotrichum acutatum* and *P. expansum.* There was no direct physical contact between the BCAs and pathogens so they concluded that the antifungal effect was due to production of VOCs generated by the BCAs. However, as the inhibitory effects were not strong and varied across the two strains of *M. laxa*, it might be that *A. pullulans* reduced growth of *M. laxa* by better competing for oxygen used for growth and reproduction rather than by production of VOCs (Rho *et al.*, 1988; Wecker and Onken, 1991). Thus the significance of VOCs from *A. pullulans* Y126 in this study is less obvious than those of *Bacillus* B91.

From our study, inhibitory VOCs produced by *Bacillus* sp. B91 were apparently present but the properties and chemical structures of the VOCs were not identified. Each individual VOC should be isolated, purified and retested to confirm the bioactivity. Once identified, the production of the VOCs at industrial scale might be easier and quicker than using VOCs produced from plants and moulds such as *M. albus* because bacteria generally require less nutrients and grow faster than plants.

5.5 Conclusions

Our two BCAs have been identified as *Bacillus amyloliquefaciens/subtilis* and *Aureobasidium pullulans* var. *pullulans*. They have distinctive modes of action. Competition for nutrients is a major mechanism of inhibition by *A. pullulans* Y126 while *Bacillus* sp. B91 involes producing inhibitory compounds and potentially competition for nutrients. Understanding the mechanisms of action involved in the bioactivity of BCAs is crucial for product commercialisation and registration. *Bacillus* sp. B91 is less able to grow and survive at low temperatures than *A. pullulans* Y126, whilst the latter can reproduce and survive at a wider range of temperatures thus exhibiting potential for use at the post-harvest environment or during the winter months.

Chapter 6

Population biology of *M. laxa*

6.1 Introduction

There are three *Monilinia* species, *M. laxa, M. fructigena* and *M. fructicola,* which are mainly responsible for brown rot disease on stone fruit. The phenotypes and genotypes of these species are clearly different from each other. Phenotypically, it is the size of spores and colony colours that can be used to differentiate these species (Byrde and Willetts, 1977). Molecular techniques based on DNA sequence analysis can also be used to separate the three species (Cote *et al.*, 2004; Hughes *et al.*, 2000; Zhu *et al.*, 2011).

Monilinia laxa can infect different tissues of host plants including blossom, twig and fruit. This pathogen is also found in a range of climates and regions (Fan *et al.*, 2010; Jansch *et al.*, 2012). The life cycle of *M. laxa* comprises three stages (1) survival within mummified fruits, (2) early infections causing blossom blight and twig canker and (3) infection of fruit causing brown rot. In order to manage brown rot disease phase, it is important to understand the relationships between the fungal populations that are prevalent at all these three stages. It is impossible to exclude the brown rot pathogen from commercial orchards as they are open environments with no physical barrier to prevent infection by inoculum from outside. There are also reservoirs of pathogen inoculum inside the orchard within mummified fruit and other substrates where *Monilinia* spores can proliferate and survive. It is not clear whether new infections are initiated from inoculum coming from these external or internal sources. Thus an understanding of the life cycle and population structure of *M. laxa* is necessary to identify possible sources of inoculum. Once the main source of inoculum is known, then control methods can be targeted more effectively to reduce or eliminate them.

Relationships between populations from mummified fruits and blossom blight, and blossom blight and fruit brown rot have been reported (Gell *et al.*, 2009; Villarino *et al.*, 2010). However, neither the origin of the inoculum source nor a dispersal route for *M. laxa* has been thoroughly studied using molecular tools. For example, how important are the populations of spores produced on mummified fruit in the subsequent infection of blossom and fruit in the following season? Are the brown rot infections caused by genotypes from external sources of inoculum blown in by the wind or solely caused by genotypes already present in the orchards? If the fungal populations at different epidemic stages are similar and there is no an external source of inoculum

involved in the infection, elimination of mummified fruits might be sufficient to control brown rot disease. However if the populations of *M. laxa* causing fruit rot are significant different from those on mummified fruits or blossom blight, then it is likely that there are also external inoculum sources that cause fruit infection.

Morphotypes of *M. laxa* isolated from the three stages of infection or different hosts are not sufficiently different to distinguish isolates. Hence, molecular techniques have been used to address this problem. Gril *et al.* (2008) showed that AFLP analysis revealed intra-species differences among isolates of *M. laxa* originating from apples and those from other host plants. However, no further grouping according to host or life cycle was found. In an earlier study, RAPD analysis showed genetic variation within *M. fructicola* but this technique failed to reveal genetic variation within *M. laxa* (Forster and Adaskaveg, 2000). More recently, simple sequence repeat (SSR) analysis has successfully been used to reveal possible origins and epidemic routes of the disease caused by *M. fructicola* (Fan *et al.*, 2010; Jansch *et al.*, 2012). Therefore SSR analysis may also offer the potential to study the population biology of *M. laxa*. Results generated from studies on genetic variation of *M. laxa* will provide a better understanding of pathogen development and may assist in managing the disease. The study will also provide biodiversity information. If there is high genetic variation within the targeted population of *M. laxa*, an effective BCA will require broad spectrum of activity against the entire population of *M. laxa*.

6.2 Objectives

The main objective of this chapter was to study molecular diversity within populations of *M. laxa* within UK orchards and use the information to identify possible sources of inoculum of brown rot disease within individual orchards. To achieve this goal, populations of *M. laxa* from mummified fruits and green fruits were compared using Analysis of Molecular Variance (AMOVA) in order to clarify the involvement of local versus distant sources of inoculum, as well as influence of host.

6.3 Results

6.3.1 The origins and locations of *M. laxa*

Isolates of *M. laxa* in this study were kindly provided by Dr. Angela Berrie of East Malling Research. Isolates of *M. laxa* came from two hosts: cherries (CH) and plums (PL) at two stages: mummified fruits (MMF) and green rotten fruits (GRF). Isolates from mummified fruits were collected during winter and spring (December

2009-April 2010) while isolates from green fruits were collected during summer of the same year (July-August 2010) (Figure 6.1).

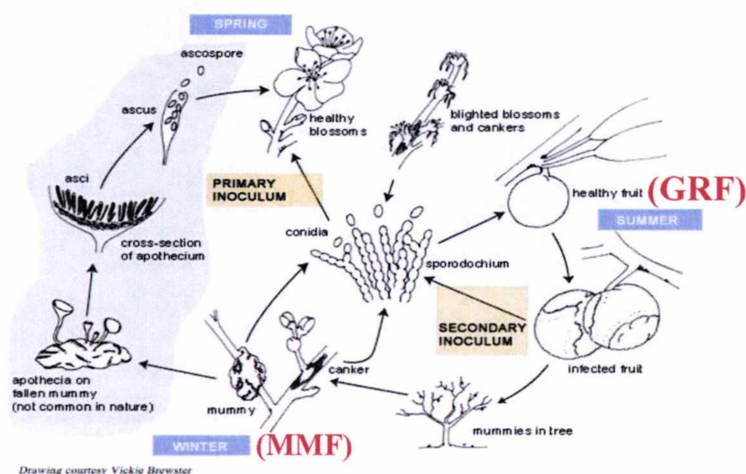

Figure 6.1 Life cycle of *Monilinia* spp. (Ritchie, 2000). Two stages of infection of *M. laxa* were compared in this study. MMF: mummified fruits, GRF: green fruits.

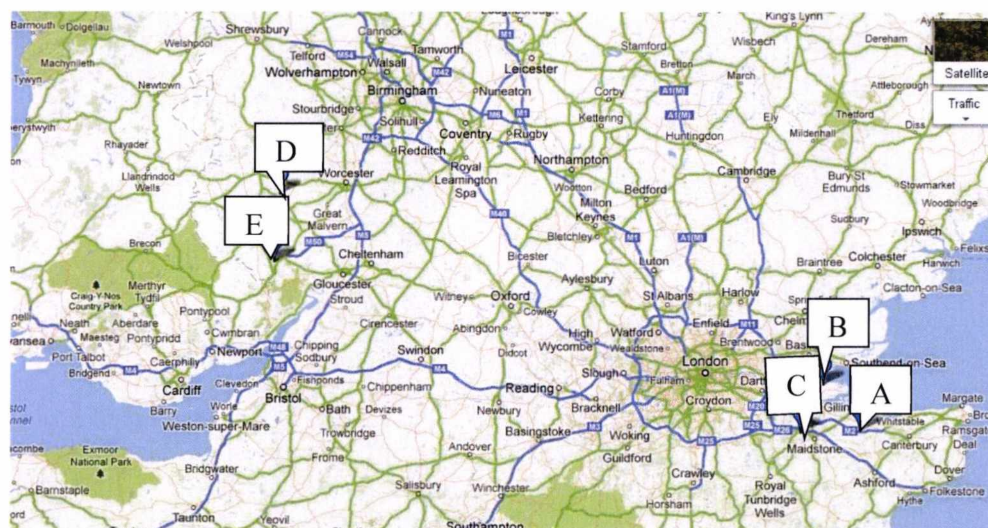

Figure 6.2 A map showing locations of five commercial orchards where *M. laxa* were isolated and used in this study (modified from Google Maps). A: The Little Sharsted Farm, B: The Decoy Farm, C: The South Park (in Kent), D: The Lower Hope Farm and E: The Man of Ross Farm (in Herefordshire).

Figure 6.2 shows five locations from which isolates of *M. laxa* were collected, including three orchards in Kent, (A) the Little Sharsted Farm, (B) the Decoy Farm and (C) the South Park, and two orchards in Herefordshire (D) The Lower Hope Farm and (E) the Man of Ross Farm. Isolates of *M. laxa* from plums only came from two locations in Kent (Figure 6.2, locations A and B), while isolates of *M. laxa* from cherries were obtained from two locations in Kent (Figure 6.2, locations A and C) and two locations in Herefordshire (Figure 6.2, locations D and E). There was only one

location (Figure 6.2, location A) where *M. laxa* was isolated from both plums and cherries. Isolates derived from the two stages of fungal life cycle for all locations except the South Park at East Malling Research (Figure 6.2, location C) where isolates from only mummified cherries were isolated.

6.3.2 Preliminary PCR amplification with seven isolates

A group of *M. laxa* isolates, consisting of four and three isolates from cherries and plums, respectively, were chosen for a preliminary study to optimise PCR conditions for the eight SSR primers (Table 6.1). A *M. fructigena*-specific primer, MF10, was also used to check for specificity. There was no amplification with MF10, confirming that there was no contamination or cross reaction from *M. fructigena* within the PCR amplifications.

Six of the seven SSR primers, which were putatively *M. laxa*-specific, were able to amplify SSR regions from genomic DNA of the seven isolates of *M. laxa*. Only primer ML5 failed to amplify SSR regions using standard PCR conditions. More isolates of *M. laxa* were used to optimise the PCR conditions for ML5, but despite many attempts to amplify ML5 under a variety of different PCR conditions, no PCR products were detected and the ML5 was excluded from this study.

Table 6.1 SSR loci of *M. laxa*, number of alleles per locus and sizes of alleles.

Locus	No. of alleles	Size of alleles (bp)
MF10	-	-
ML5	-	-
ML6	1	198
ML15	1	230
ML16a	7	134, 146, 147, 152, 153, 154, 160
ML16b	8	92, 93, 97, 98, 99, 100, 101, 103
ML17	5	215, 219, 220, 223, 235
ML18	5	235, 237, 241, 247, 256

6.3.3 PCR Amplification of SSR

After the optimal PCR conditions were identified, a total of 425 isolates of *M. laxa* were amplified using the SSR primers. PCR products were not detected in 89 isolates, despite PCR being repeated several times and they were excluded from this study. Finally, a total of 336 isolates of *M. laxa* were used for further analysis. Two primers, ML6 and ML15, produced no polymorphisms with a single allele of size of 198 and 230 bp, respectively (Table 6.1). Therefore these two primers were excluded.

6.3.4 Allele frequency of *M. laxa*

The allele frequency of 336 isolates of *M. laxa* is shown in Table 6.2. Primer ML16b produced the most genetic variation with eight alleles ranging from 92-103 bp, with the allele size of 98 being the most frequent. For primer ML16a, there were seven alleles, with size ranging 134-160 bp, and allele size of 153 being the most frequent. Primers ML17 and ML18 produced an equal number of alleles with five alleles per locus. The sizes of the alleles of these two loci were 215-235 and 235-256 bp, respectively. Allele size of 219 bp was the most frequent for locus ML17 while 256 bp was the most frequent for ML18. SSR data from these four loci were subjected to AMOVA study.

Table 6.2 Allele frequency in 336 isolates of *M. laxa* analysed at four SSR loci.

ML16a		ML16b		ML17		ML18	
Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
134	0.009	92	0.015	215	0.268	235	0.003
146	0.003	93	0.003	219	0.637	237	0.003
147	0.071	97	0.003	220	0.003	241	0.095
152	0.006	98	0.705	223	0.054	247	0.021
153	0.896	99	0.247	235	0.039	256	0.878
154	0.003	100	0.009				
160	0.012	101	0.006				
		103	0.012				

6.3.5 Comparison of sources of inoculum of *M. laxa*

For the initial study, the comparison between two stages of the life cycle, *i.e.* isolates from either mummified fruits or green fruits, was made at individual locations. Isolates from cherries and plums were separately analysed. There were three comparisons of *M. laxa* from cherries at (1) the Little Sharsted Farm (Kent), (2) the Lower Farm and (3) the Man of Ross Farm (Herefordshire). There were two comparisons of *M. laxa* from plums at (1) the Little Sharsted Farm and (2) the Decoy Farm. Table 6.3 shows the summary of comparisons between *M. laxa* isolated from mummified fruits and green fruits.

Comparison two sources of inoculum at individual locations

The molecular diversity of the population of *M. laxa* isolated from mummified cherries was not significantly different from population from green cherries at the Little Sharsted Farm (p = 0.623, Table 6.3). For the second location, the Lower Hope Farm,

there was a statistically significant difference between the genetic diversity of the populations of *M. laxa* isolated from mummified and green cherries ($p = 0.047$). At the third location, there was a highly significant difference between populations of *M. laxa* from mummified cherries and green cherries at the Man of Ross Farm ($p < 0.001$). The variation between these two sources of inoculum (Va) at this location accounted for 21.49% of total variation. For comparison between genetic variation of *M. laxa* in plums, there was no difference between fungal populations of *M. laxa* isolated from mummified and green plums collected from the Little Sharsted Farm ($p = 0.087$). Likewise populations of *M. laxa* from mummified and green plums from the Decoy Farm were not statistically different ($p = 0.258$).

Comparison between sources of inoculum on cherries across locations

Isolates of *M. laxa* from mummified and green cherries collected from three locations were compared. The isolates were divided into two groups (and then analysed on two levels, source and location), those isolated from mummified cherries and those from green cherries. The difference between populations of *M. laxa* from mummified and green cherries (among groups) was not significant ($p = 0.303$). The difference between locations (among populations within each group) was highly significant ($p < 0.001$). With all comparisons, the vast majority of variation was within populations (91.96% and $p < 0.001$).

Comparison of sources of inoculum on plums across locations

From two farms sampled, the Little Sharsted Farm and the Decoy Farm, there was no significant different between populations of *M. laxa* isolated from mummified and green plums ($p = 1.000$), but the difference between locations (among populations within the groups) was highly significant ($p < 0.001$). Variation within populations accounted for 96.64% of total variation.

Comparison between two sources of inoculum across hosts and locations

All *M. laxa* isolates from cherries and plums were combined and grouped into two sources. There was no significance between isolates from mummified and green fruits ($p = 0.487$), but the difference among populations within the groups was highly significant ($p < 0.001$).

Table 6.3 Summary of results of AMOVA with four SSR loci of *M. laxa* using stages of infection as a factor.

Test	Comparison	Variation (%)			*P* value		
		Va	Vb	Vc	Va	Vb	Vc
Mummified cherries vs green cherries	The Little Sharsted Farm	-0.94	100.94	-	0.623	-	-
Mummified cherries vs green cherries	The Lower Hope Farm	4.65	95.35	-	0.047	-	-
Mummified cherries vs green cherries	The Man of Ross Farm	21.49	78.51	-	< 0.001	-	-
Mummified plums vs green plums	The Little Sharsted Farm	4.91	95.09		0.087		
Mummified plums vs green plums	The Decoy Farm	0.73	99.27		0.258		
Mummified cherries vs green cherries	Across locations	1.06	6.98	91.96	0.303	< 0.001	< 0.001
Mummified plums vs green plums	Across locations	-4.04	7.40	96.64	1.000	< 0.001	0.004
Mummified fruits vs green fruits	Across locations and hosts	-0.92	12.00	88.91	0.487	< 0.001	< 0.001

6.3.6 Other factors that affect fungal populations of *M. laxa*

The effect of location on populations of *M. laxa* was studied. For comparison of mummified cherries, there were significant differences between populations of *M. laxa* from mummified cherries from different orchards ($p < 0.001$) (Table 6.4). Variation within populations accounted for the most variation (Vb = 91.78%). There was a significant difference among populations of *M. laxa* from green cherries collected from the three different orchards ($p < 0.001$). The populations of *M. laxa* from mummified plums at the Little Sharsted farm and the Decoy farm in Kent were not significantly different ($p = 0.517$). There was a significant difference, however, between the populations of *M. laxa* on green plums collected from the two orchards ($p < 0.001$).

The effect of different hosts (i.e. cherries and plums) on fungal population structure at a single location was also studied. The previous results showed that a location effect was present in this study. There was only one location, the Little Sharsted Farm (Table 6.4) that contained the isolates from both cherries and plums and these two populations were compared. The population of *M. laxa* from mummified cherries at Little Sharsted Farm were significant different ($p < 0.001$) from that collected from mummified plums and the variation between cherries and plums accounted for 18.15% of the total variation. Populations of *M. laxa* from green cherries were significantly different from green plums ($p < 0.001$), accounting for 18.71% of the total variation. For the overall comparison, the difference between the *M. laxa* populations isolated from these two stages of life cycle (mummified and green fruits) was not significant ($p = 0.656$). However there was significant difference between the two hosts, ($p < 0.001$) accounting for 20.32% of the total variation.

Table 6.3 Summary of results of AMOVA with four SSR loci of *M. laxa* using locations as a factor.

Test	Comparison	Variation (%)			*P* value		
		Va	Vb	Vc	Va	Vb	Vc
4 Locations (the Little Sharsted vs the Man of Ross vs the Lower Hope vs the South Park)	Mummified cherries	8.22	91.78	-	< 0.001		
3 Locations (the Little Sharsted vs the Lower Hope vs the Man of Ross)	Green cherries	8.90	91.10	-	< 0.001		
2 Locations (the Little Sharsted vs the Decoy)	Mummified plums	-0.84	100.84	-	0.517		
2 Locations (the Little Sharsted vs the Decoy)	Green plums	11.44	88.56	-	< 0.001		

Table 6.4 Summary of results of AMOVA with four SSR loci of *M. laxa* using hosts as a factor.

Test	Comparison	Variation (%)			*P* value		
		Va	Vb	Vc	Va	Vb	Vc
Mummified cherries vs mummified plums	The Little Sharsted farm	18.15	81.85	-	< 0.001		
Green cherries vs green plums	The Little Sharsted farm	18.71	81.29	-	< 0.001		
Mummified fruits vs green fruits	The Little Sharsted farm	-9.60	20.32	89.28	0.656	< 0.001	< 0.001

6.4 Discussion

6.4.1 SSR primers

Fungi generally have fewer morphological characters than plants and animals and it is generally accepted that rate of morphological changes will be slower for microbes with less elaborate development and fewer cells (Taylor *et al.*, 2006). In contrast, the rate of genetic evolution of fungi is faster than morphological evolution, thus from an evolutionary perspective, molecular approaches seem to be a better alternative for studying populations of fungi. Molecular studies based on SSR markers offer an opportunity for studying genetic diversity within *M. laxa* because SSR markers have already been reported to successfully differentiate populations of *Monilinia* spp. (Everhart *et al.*, 2012; Fan *et al.*, 2010; Jansch *et al.*, 2012; Villarino *et al.*, 2012).

For example, Fan *et al.* (2010) showed that of 35 SSR primers developed by their colleagues (Ma *et al.*, 2003; Ma *et al.*, 2005), 12 primers revealed polymorphic alleles within 165 isolates of *Monilinia* spp. isolated from China. Everhart *et al.* (2012) reported that 16 out of 40 SSR primers consistently amplified polymorphic alleles within 47 isolates of *M. fructicola* from Greece. In the present study, 6 SSR primers were used to amplify genomic DNA of *M. laxa*, and only two of them failed to produce polymorphisms. Although the number of primers used in this study were fewer than those used in the aforementioned studies, this set of primers was still sufficient to reveal genetic variation and unveil the fungal populations of *M. laxa*. Theoretically, in order to detect a statistical significant value in a haploid species, a sample size of 20-30 individual organisms per population is needed (Hale *et al.*, 2012; Selkoe and Toonen, 2006; Xu, 2006). Over 20 isolates were used for most populations analysed in this study, although two populations had less than 20 isolates (15 and 18 isolates).

The number of alleles produced and allele frequency vary from species to species. For example the entomopathogenic fungus *Paecilomyces fumosoroseus* was amplified with 9 SSR primers which produced 1-5 alleles per locus (Gauthier *et al.*, 2007) and, as noted above, *M. fructicola* was amplified with 16 SSR primers which produced 2-16 alleles per locus (Everhart *et al.*, 2012). In this study, 4 of 6 SSRs produced polymorphisms, with high numbers of allelic diversity ranging from 5 to 8 alleles per locus i.e. these *M. laxa*-specific SSR primers produced sufficient allele polymorphisms to use for population comparisons.

6.4.2 Sources of inoculum of *M. laxa*

Understanding the role of different population origins within the life cycle of a target pathogen is a key for disease management. The connection between populations

of *M. laxa* found on mummified fruits and fruit rot is discussed in this section. There was only one orchard in Herefordshire, the cherry orchard at the Man of Ross Farm, where there was an indication that there might be an external source of inoculum contributing to infection. In all other orchards there was no evidence to support that there was an external source of inoculum, and infections were probably initiated from inoculum already present within the orchard population of *M. laxa*.

For *M. fructicola*, an occasional source of inoculum can be sexual spores (ascospores) produced by apothecia developed on mummified fruits in winter which are later normally discharged and dispersed to neighbouring orchards in spring (Holtz *et al.*, 1998; Villarino *et al.*, 2010). This may increase possibility of external sources of inoculum of brown rot caused by *M. fructicola* through genetic recombination via sexual reproduction. If *M. fructicola* reproduces sexually, it will pass on only half of genes to its progeny. This process generates mixes of genes and might increase genetic variation of *M. fructicola*.

However apothecia of *M. laxa* are rarely found in the field, have never been produced in culture, and never been reported in Britain (Byrde and Willetts, 1977). Therefore, this supports the conclusion above that *M. laxa* is likely to reproduce and infect plants via asexual reproduction within the orchard. The sources of this inoculum could be from tree cankers or mummified fruits that were left on trees or on the ground or, perhaps less likely, saprotrophic growth on *M. laxa* on other substrates within the orchard. This hypothesis is supported by the results of Everhart *et al.* (2011) who monitored disease incidence within sour cherry trees. Their results showed that twig cankers formed in the previous year may have been the most important source of inoculum for blossom blight, shoot blight and twig canker in the following year. Their finding emphasised that local inoculum is more likely to cause brown rot disease than airborne conidia dispersed over a long distance. In this study the link between fungal populations on mummified fruits and the subsequent season's fruit rot was demonstated and confirms that populations of *M. laxa* on mummified fruits which survive overwinter are likely to be an inoculum source for fruit rot within the same orchards the following year.

In the exceptional case of the cherry orchard at the Man of Ross Farm in Herefordshire, it might be that an external source of inoculum might have been brought into the orchard possibly via naturally airborne spores by wind, or infected plant materials or seedlings brought into the nursery as a result of grower practice. Thus these results confirm the widely-held belief that mummified fruits should be removed in order

to reduce and minimise the spore load of *M. laxa* on blossom and fruits the following season. Managing orchards for disease control should focus on eliminating mummified fruits from the trees or spraying chemicals onto mummified fruits rather than focusing on spraying blossom and green fruits. Reducing the source of inoculum ought to be more effective than combating that inoculum once it is dispersed from point sources within the orchard. Spraying trees during fruiting has the added disadvantage that it may result in residues on fruits.

6.4.3 Other factors affecting fungal populations of *M. laxa*

In the early mycological literature, it was generally believed that fungi were free-living microbes that will grow anywhere if conditions were right for them. It was believed that fungal populations were largely unstructured and geographic dispersal played no role in establishing new populations (Berkeley, 1863; De Candolle, 1820). However recent molecular studies in biogeography, ecology and population genetics of fungi have shown that fungi are capable of long distance dispersal, such that the distribution of most fungi were affected and regulated by major geographical barriers, for example oceans, mountains and continents (Lumbsch *et al.*, 2008; Taylor *et al.*, 2006). Ecological determinants of fungal distribution, especially of human and plant pathogens, are important because they provide the basic knowledge on disease epidemiology which is vital for management. However, once the fungi have been distributed over long distances, it is often local factors that then influence the local population diversity.

In this study, we found that populations of *M. laxa* were structured by location. Although the majority of variation within populations (*i.e.* at the same location) were larger than variation between populations (*i.e.* between locations), biogeography was still one of major evolutionary forces that influences population diversity of *M. laxa*. Jansch *et al.* (2012) compared populations of *M. fructicola* isolated from Switzerland, Spain, Italy, France and the US. Their study suggested that *M. fructicola* (a quarantine fungal pathogen in Europe) were apparently-derived from the US population. However several groups of *M. fructicola* isolated from commercial orchards from these four European countries produced different haplotypes which were not found from those originating from the US, suggesting the effect of climate and geography may drive genetic changes in *M. fructicola* or that local sexual reproduction was more important in driving variation than at first thought.

Fan *et al.* (2010) used SSR makers to study variation among populations of *M. fructicola* from different regions in China and the US. They showed that 93% of genetic variation was found within regional populations, whist 7% of genetic variation was due to geographical separations (among the regional populations). Likewise Gell *et al.* (2007b) studied genetic variation of *M. laxa* isolates from 7 commercial orchards in Spain and found that local variation within these populations accounted for 97% of total genetic variation, while only 3% represented genetic variation between populations (*i.e.* between Spanish orchards). The low genetic variation between Spanish orchards might be caused by two reasons: firstly few isolates of *M. laxa* from each of the 7 orchards were used (21 isolates in total); secondly the study was based on RAPD makers which might generate less genetic variation compared to SSR markers (Powell *et al.*, 1996). In the present study using SSR markers, our finding showed locations play an important role in population structure of *M. laxa*. The majority of the genetic variation was within populations (88-91%) while 8-11% of the genetic variation was due to location.

In addition to the effect of geography on population diversity of *M. laxa*, the effect of hosts has also been reported in this study. The three common *Monilinia* species (*M. fructicola, M. fructigena* and *M. laxa*) infect a wide range of pome fruits and stone fruits. Although *M. fructigena* mainly causes disease on pome fruits, it can cause fruit brown rots in stone fruits (Zhu *et al.*, 2011). Likewise, although *M. laxa* usually infects blossoms and fruit rot of stone fruits, it also causes considerable losses in pome fruits (Byrde and Willetts, 1977). However, the effect of host plant on populations of *M. laxa* has not been well studied despite the fact that understanding the relationship between hosts and the pathogen is crucial for disease management. The results reported here demonstrated that there were significant differences between the *M. laxa* populations isolated from cherries and those isolated from plums at the same location. This analysis was only possible at this one site, though, and comparison between hosts at other locations is required in order to verify the effect of host on the fungal populations of *M. laxa*.

Gril *et al.* (2008) used AMOVA of results using AFLP markers with 36 isolates of *M. laxa* (18 from sweet cherries and 18 from apricots) to show that populations of *M. laxa* from the two hosts were not different. Gerber *et al.* (2000) compared AFLP and SSR markers and concluded that SSR approaches were better tools to determine genetic variation than AFLP markers. In the present study, AMOVA based on SSR markers with 122 isolates of *M. laxa* (69 and 53 from cherries and plums, respectively) revealed that there was a significant difference in populations isolated from cherries and plums.

Evolutionary forces driving genetic variation in populations are recombination, migration, selection, mutation and population sizes (Hartl and Clark, 1997; McDonald and McDermott, 1993). In this study, the processes of migration and selection via locations and hosts, respectively have been shown to be important. However, other forces should be taken into consideration and could be studied using a similar approach, for example temperature and farm management. Temperature strongly influences growth of the pathogens and host plant, and thus affects infection indirectly. Farm management of disease may use three different approaches (1) conventional management with agrochemicals, (2) IPM and (3) organic farming. The regulation and use of chemicals and fungicides in these management strategies are considerably different but offer the opportunity for further studies of the populations of *M. laxa* to gain further knowledge of the influence of management on the epidemiology of *M. laxa*.

6.5 Conclusions

Monilinia laxa found on overwintered mummified fruits was likely to be responsible for the fruit brown rot found in the same orchards the following year. Although the majority of the genetic variation of *M. laxa* was within local populations, there was molecular evidence to support the involvement of external factors such as geography and host in governing and shaping populations of *M. laxa*. Thus although local factors seem most important, geographical distribution and hosts also play important roles in determining the population structures of *M. laxa*.

Chapter 7
Application of BCAs to mummified fruits

7.1 Introduction

Although mummified fruits play a major role in brown rot epidemiology in providing air-borne spore inoculum of the pathogen for infections on flowers and fruit, managing the inoculum produced from mummified fruits has not been well studied. Disease control treatments usually focus on blossom and immature fruits (Holb and Schnabel, 2005; Percival and Beaton, 2012) while mummified fruit is overlooked in spite of the fact that mummified fruits are an important source of overwintering mycelium (Biggs and Northover, 1985; Villarino *et al.*, 2010). Nevertheless, Benomyl and benzimidazole fungicides have been shown to be the most effective fungicides in suppressing sporulation of *M. fructicola* on mummified peaches and peach twigs (Kable, 1970, 1976).

During harvest season, fruits with visible rot symptoms are usually ignored and left to mummify on the trees or sometimes thrown on the ground. Without proper management, these mummified fruits become an important source of inoculum in the next season. This source of inoculum is difficult to deal with due to (1) the complexity of the mummified fruit structure and (2) the fact that mummified fruits can produce a large number of spores which are then dispersed by wind, insects and rain (Gell *et al.*, 2009; Villarino *et al.*, 2010). In addition, mummified fruit can also be a source of survival for other fruit rot pathogens (van Leeuwen *et al.*, 2002b).

There are several ways to control the infection of *Monilinia* spp. caused by mummified fruits, including physical, chemical and biological control. For physical control, infected plant materials should, in theory, be removed from the trees to reduce sources of inoculum (Byrde and Willetts, 1977; Tamm *et al.*, 2004; van Leeuwen *et al.*, 2002b). Orchard sanitation by removing blighted twigs and mummified fruits is widely recommended against brown rot disease of stone fruit crops (Tamm *et al.*, 2004; van Leeuwen *et al.*, 2002b). However this method is labour-intensive and raises the cost of fruit production. Thus infected fruits often mummify during autumn and winter and remain in the orchard as a source of inoculum the following spring. When these spores land onto buds and blossom of cherries and plums and environmental conditions are favourable, they germinate and infect blossoms. Suppression of sporulation or decreasing the number of spores produced by mummified fruits thus offers a way to reduce infection of blossom wilt and fruits.

Mummified fruits are present in orchards for three to four months from late autumn to early spring, when they begin to rot away as temperature rises. If living BCAs were to be applied to mummified fruits, they must be able to survive and reproduce over this period of time in order to compete with the pathogen and to reduce pathogen inoculum production. Spraying chemicals and BCAs at an early stage of mummification might eliminate *Monilinia* species, but any BCAs applied in early winter, must be resilient enough to survive in conditions of low nutrients and in extreme conditions of low temperature and high UV exposure. Alternatively, applying BCAs in early spring might increase the chance of survival as conditions improve. Spraying BCAs onto mummified fruits in early spring might also prevent or reduce the spore germination of *Monilinia* species. This chapter reports results from a study of the effect of BCA sprays onto mummified fruit.

7.2 Objectives

The first objectives of in this chapter was to determine the efficacy of indigenous BCAs, existing BCAs and a fungicide to reduce the inoclum level of *Monilinia* spp. on mummified plums collected from a commercial orchard. Indigenous BCAs used in this experiment were the isolates showing good inhibitory activity from the primary screening (Chapter 3). The second objective was to study effect of spraying time and to assess the efficacy of candidate BCAs to reduce the incolum level on mummified plums artificially inoculated with *M. laxa*. Two candidate BCAs used in this experiment were selected from the secondary screening (Chapter 4)

7.3 Results

7.3.1 Efficacy of BCAs against *Monilinia* spp. on mummified plums

In the preliminary study in 2010, some treatments had a significant effect in suppressing sporulation on mummified plums ($p < 0.001$). As this experiment was conducted on field-collected mummified fruit, the plums might have been infected with either *M. laxa* or *M. fructigena*. Therefore interpretation and discussion of this experiment are based on *Monilinia* spp. rather than on *M. laxa*. Indar was the best treatment in reducing the spore number from mummified plums (Figure 7.1). *Epiccoccum nigrum* ATCC96794 slightly reduced the spore numbers, whilst *P. fluorescens* NCIMB13373 and Serenade failed to suppress the sporulation. The spore number produced on mummified fruit treated with BCAs (B62, Y35 and Y80) were significantly lower than control treatments, while the other two BCAs (Y52 and Y90) had no effect on sporulation of mummified plums.

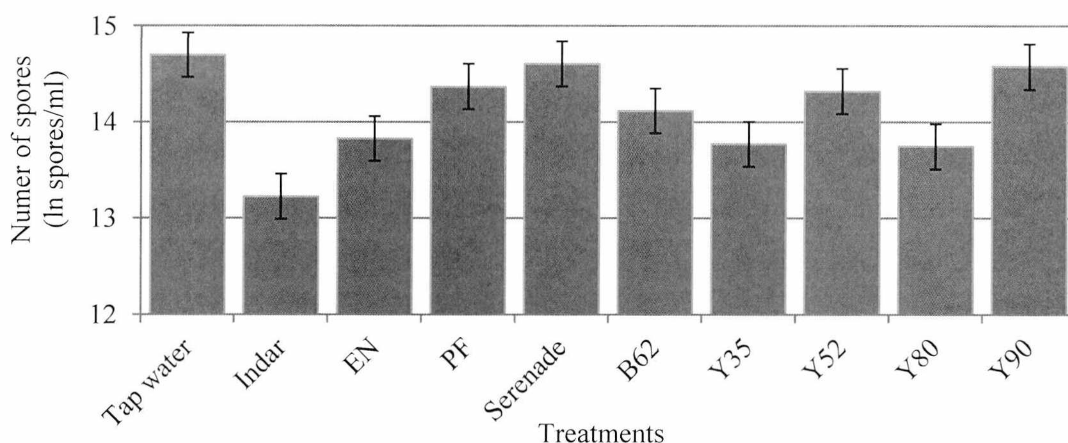

Figure 7.1 Number of spores/ml of *Monilinia* spp. produced on mummified plums after they were sprayed once with individual treatments. EN: *E. nigrum* ATCC96794, PF: *P. fluorescens* NCIMB13373. Bars represent standard error of the means.

7.3.2 Efficacy of BCAs against *M. laxa* on mummified plums

The results from two-year experiment conducted in 2011 and 2012 show that treatments and spraying times both had significant effects in reducing sporulation on mummified plums ($p < 0.001$). An interaction between these two main factors was present ($p < 0.001$) (Figure 7.2) In general, the spore number recorded from the control treatment (12 spores/ml on the ln scale) was higher than other treatments. When treatments were applied in November alone, Indar and *A. pullulans* Y126 significantly reduced the spore number of *M. laxa* on mummified plums, while the spore numbers for treatments with *Bacillus* sp. B91 and Serenade were not significantly different from the control. When treatments were applied in February alone, all treatments suppressed sporulation on mummified plums. For the treatments involving a double spray (November+February), Indar greatly reduced the spore number on mummified plums from 12 spores/ml (on the ln scale) in the control treatment to 7 spores/ml (on the ln scale). Double application of *A. pullulans* Y126 also reduced the spore number considerably compared to the control, whereas double treatments with *Bacillus* sp. B91 and Serenade also reduced sporulation on mummified plums but to a lesser extent than either Indar or Y126.

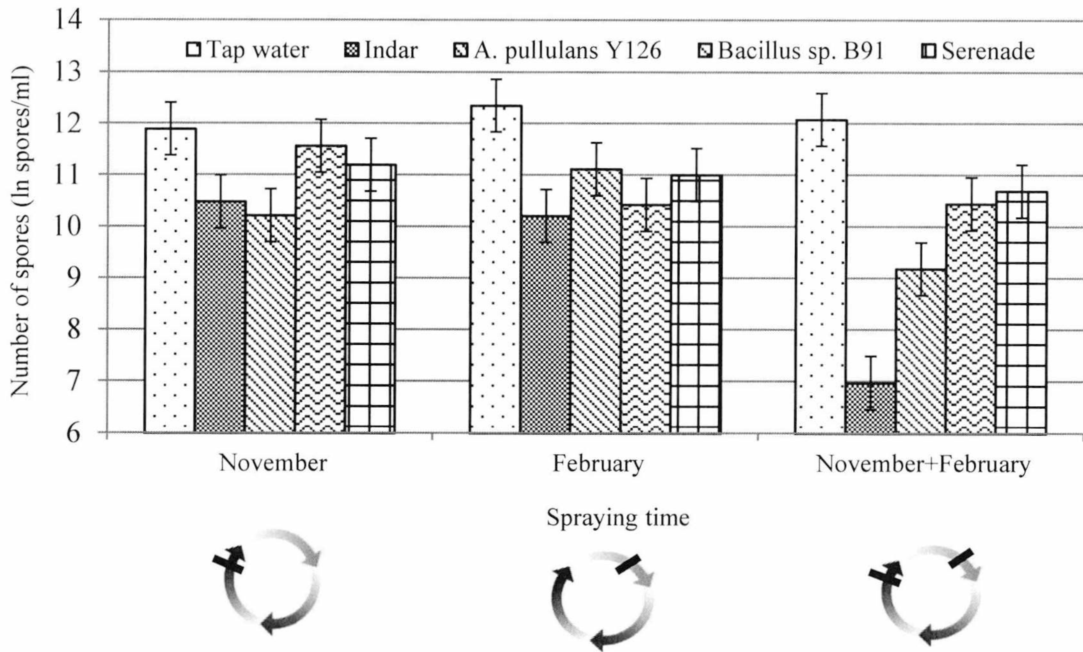

Figure 7.2 Numbers of spores produced from mummified plums when they were treated with different treatments and left in the orchard for three months. Bars represent standard error of the mean.

7.4 Discussion

7.4.1 Initial trial of suppression of sporulation of *Monilinia* spp.

In the first year of study, mummified plums were collected from a commercial orchard, and Indar, a fungicide, was the best treatment to suppress sporulation of *Monilinia* spp. on these naturally mummified plums, although *E. nigrum* ATCC96794 and three of the indigenous BCAs had significant but lower activity. However none of these treatments completely prevented sporulation by *Monilinia* spp. Sprays of the BCA *E. nigrum* ATCC96794 have previously been used successfully in Spain to decrease the number of conidia produced in the field by *Monilinia* spp. (De Cal *et al.*, 2009; Larena *et al.*, 2005; Madrigal and Melgarejo, 1995). Conidial suspensions of *E. nigrum* ATCC96794 were applied three to six times on stone fruit trees starting from flowering until pre-harvest (*i.e.* on blossom, twig and fruits). These workers have not reported its use on mummified fruit, and here it has been shown that *E. nigrum* ATCC96794 can also be used to partially control *Monilinia* spp. on mummified plums when this fungal BCA was sprayed as a single spray in winter.

In contrast, two other BCAs did not show potential when used on mummified fruit. *Pseudomonas fluorescens* NCIMB13373 was originally selected after screening against *B. cinerea* (Swadling and Jeffries, 1998). This BCA did not pass the screening tests for further testing described in Chapter 3 and again the results from this Chapter show that *P. fluorescens* NCIMB13373 was unable to control sporulation of *Monilinia*

spp. Serenade also failed to suppress sporulation of *Monilinia* spp. This commercial BCA is recommended to use on blossom and green fruits but no recommendations are made for its use on mummified fruit. The indigenous three BCAs that showed some control in this initial trial were not taken forward, as other isolates (Y126 and B91) were found to have a superior potential in the later laboratory screens in 2011 (see Chapter 4).

There were a number of issues that arose from the experiment that had an influence on the subsequent field experiments on mummified fruit:

(1) In the first year, conidia of *E. nigrum* ATCC96794 were produced in culture on PDA with a 16:8 hour light:dark regime at 25°C, which is the recommended culture method for this BCA (Larena *et al.*, 2003). Although *E. nigrum* ATCC96794 was cultured under the same conditions, it did not sporulate the following year despite many attempts. Induction for conidia production of *E. nigrum* ATCC96796 can be conducted by culturing in liquid- or solid-fermentation using a complex mineral liquid medium or mixtures of solid substrates (*i.e.* peat, vermiculite and lentil), respectively (De Cal *et al.*, 2009; Larena *et al.*, 2004). These more complex techniques to induce sporulation of *E. nigrum* ATCC96796 were not attempted because it was beyond the scope of this study, and *E. nigrum* ATCC96794 was excluded from the subsequent experiments.

(2) Mummified plums used in this experiment were collected from a commercial orchard. These mummified plums could be potentially infected with either *M. laxa* or *M. fructigena* and there might be infection from other air-borne fungi. Distinguishing the spores of *M. laxa* from these others is not easily possible. Thus the use of collected mummified fruit means that the starting material is inconsistent and the results are not solely related to the target pathogen. To overcome this problem for 2011 and 2012, plums were artificially inoculated with *M. laxa* and if plums which were naturally infected by *M. fructigena* or other air-borne fungi were found, they were removed.

(3) A single spray which was applied in winter, resulting in a small decrease in the spore numbers of *Monilinia* spp. As the spray did not eliminate the pathogen, there is a chance that new mycelial growth could give rise to more spores later in the cycle so a second spray timed in late winter might be more effective.

7.4.2 Effect of treatments using B91 and Y126 and spraying time against sporulation of *M. laxa* on mummified fruit

When sprayed once in winter, *A. pullulans* Y126 failed to supress sporulation but its efficacy was greater when it was applied once in spring. The results from Chapter 5 suggest that whilst Y126 can survive low temperatures, its population density might

sharply decrease. Alternatively, when *A. pullulans* Y126 was applied in early spring, it might have been at a disadvantage in competing with *M. laxa* which had already colonised and dominated the mummified fruit. However, if this BCA was sprayed both in November and in February, the efficacy of Y126 was much greater, as it may have prevented some colonisation by *M. laxa* in winter and then competed more effectively in spring. It is likely that maintaining the populations of Y126 at a high level plays an important role in increasing the biocontrol efficacy.

It is not surprising that the single Winter spray of *Bacillus* sp. B91 was not very effective as the results from Chapter 5 showed that this BCA was particularly sensitive to low temperatures at or below 15°C. Conversely, applying *Bacillus* sp. B91 in early spring when temperature was milder, resulted in a significant reduction of sporulation. The double spray, however, did not increase the efficacy of *Bacillus* sp. B91. Serenade, a trade name of a preparation of *B. subtilis* QST713, also had some efficacy in suppressing sporulation of *M. laxa* after the Spring application and had a similar pattern of efficacy to B91. Zhong *et al.* (2008) studied the survival and spore germination of *M. fructicola* on mummified peaches in Beijing, China. Germination of spores of *M. fructicola* from mummified peaches collected in autumn was 64%. The germination decreased to 24% from mummified peaches collected in winter and then further down to 2-5% when mummified fruit was collected in early spring.

A single spray of Indar in either November (Winter spray) or February (Spring spray) did not completely control *M. laxa*. Treatments might not be able to persist on surfaces of mummified fruits and become washed off by rainfall. A single spray in February significantly reduced sporulation of *M. laxa* but the degree of reduction was low and possibly uneconomic. However, Indar worked well when the spray was repeated and the efficacy was far better than either single spray. Thus this might be economic and effective as part of an IPM strategy and avoids any risk of carry-over onto fresh fruit surfaces.

As discussed earlier, *Bacillus* spp. and *A. pullulans* have been used to control *Monilinia* spp. on various stages of fruit production during the Spring-Summer season *i.e.* blossom (Altindag *et al.*, 2006; Wittig *et al.*, 1997), pre-harvest (Ippolito and Nigro, 2000; Poleatewich *et al.*, 2012) and post-harvest (Ferreira-Pinto *et al.*, 2006; Schena *et al.*, 2003; Yanez-Mendizabal *et al.*, 2011). However, this is the first report to use BCAs to control *M. laxa* on mummified fruits and expands our knowledge of the use of BCAs to control brown rot disease during this other stage of the life cycle of *M. laxa*.

Although the fungicide treatment was still the most effective way to control *M. laxa*, the search for other alternatives to suppress sporulation of brown rot pathogens should continue. Fungicide-resistance strains of *Monilinia* spp. have emerged due to continual use of fungicides (Holb and Schnabel, 2007; Ma *et al.*, 2003; Weger *et al.*, 2011). To minimise and avoid the emergence of new fungicide-resistant strains of *Monilinia* spp. studies of fungicides applied at reduced rates in an integrated treatment with a BCA should be carried out to determine whether sporulation of *M. laxa* on mummified fruits can be suppressed effectively under these circumstances.

7.5 Conclusions

Winter spray alone was not sufficient to suppress sporulation of *M. laxa* on mummified fruits. Capability of Indar and BCAs to suppress the sporulation of *M. laxa* was improved when the treatments were applied in Spring. Indar and *A. pullulans* Y126 greatly suppressed sporulation of *M. laxa* if the fruits were treated on both occasions.

Chapter 8
General discussion and conclusions

8.1 Overview

The overall objective of this project was to develop biological control agents (BCAs) against brown rot disease of stone fruits caused by *M. laxa*. Fungicide control is currently used but is not a sustainable long-term answer due to possible negative impact of fungicide residues on human health and the environment. Biological control offers an alternative and has been increasingly used to control a variety of plant diseases with a varying degree of successes. The Sustainable Use Directive on pesticides (2009/128/EC) was adopted by the EU in 2009 and sets a long-term policy direction for the use of pesticides in member states. The Directive places considerable emphasis on Integrated Pest Management (IPM) and will make IPM mandatory for farmers by 2014. The Directive (Annexe III) sets out the principles of IPM and states that sustainable biological, physical and other non-pesticidal controls must be preferred over pesticides if they provide satisfactory control.

However, the process of development of BCAs may take many years or decades to be achieved. This entire process includes choosing sources of BCAs, screening for bioactivity, improving efficacy of BCAs, formulation, registration of BCAs and finally launching BCAs to the market. Due to limited time, this project could only address the first stage of this process and provides the basic information for future research. The findings and main conclusions are discussed and summarised here.

8.2 The importance of indigenous BCAs

The clearance required for testing BCAs in field trials varies between countries. The EU Regulation no. 1107/2009 (EU, 2009), which applies in all European countries, lays down rules for the authorisation of plant protection products in commercial form and for their placing on the market, use and control witin the Community. It ensures a high level of protection of both human and animal health and the environment, as well as enhancing the competitiveness of EU Community agriculture.

The release of BCAs which are genetically modified organisms (GMOs) is much more strictly controlled and subjected to various legal authorities (EU, 1991, 2001). However non-genetically modified BCAs are not subjected to such legislations, but if the intention is to use BCAs which are not indigenous to the country (*i.e.* 'exotic') where they are to be used, then other considerations need to be taken into account. Even

if exotic BCAs are approved and registered in other countries, post-approval monitoring following controlled release might be needed for risk assessment to ensure that there is no consequent impact on non-target organisms. Therefore the preferred approach for developing BCAs is to study and deploy indigenous BCAs within a country. This is especially true for the UK, with its geographical separation from continental Europe. If these indigenous BCAs are not effective, then searching for exotic BCAs may be necessary.

8.3 Sources of BCAs

The term 'phyllosphere' is used to describe the surface of the aerial parts of plants which is colonised by a diverse group of microbes (mainly bacteria, yeasts and filamentous fungi). Microbes inhabiting the phyllosphere are called epiphytes (Lindow and Brandl, 2003; Whipps *et al.*, 2008). Magan (2006) pointed out that the phyllosphere environment has been considered as a harsh and hostile environment for epiphytic microbes because the plant surfaces are exposed to rapid fluctuations of temperature, humidity and UV radiation, as well as limited amounts of nutrients. The capability to tolerate such harsh conditions is a prerequisite for candidate BCAs. Thus the microflora that live epiphytically on healthy plant materials have long been considered as one of the good sources of BCAs (Janisiewicz and Buyer, 2010; Janisiewicz *et al.*, 2010; Janisiewicz *et al.*, 2013; Raaijmakers *et al.*, 2002).

In this study, microbes were isolated from both infected and healthy plant materials from stone fruit trees. No particular niche for collection was selected, as the microflora of stone fruits has been only sparsely described previously (Janisiewicz *et al.*, 2013). *Bacillus* sp. B91 and *A. pullulans* Y126 were isolated from green plums and cherry leaves, respectively, neither of which showed any symptoms of disease. It may be that these two BCAs, together with other resident microbes, might have an important role in protecting hosts from plant pathogens.

8.4 Are the selected BCAs also opportunistic pathogens?

8.4.1 Impact of bacterial BCAs

Bacteria often feature strongly in biological control studies as they are widespread, easy to isolate and culture, and lend themselves to mass-production and formulation. However, they suffer a disadvantage in that there are many known human pathogenic bacteria and the public perception of bacteria is negative (*e.g.* as 'germs'). Any bacterium selected for use as a BCA must not have been implicated in human

diseases. *Bacillus subtilis* and *B. amyloliquefaciens* are ubiquitous bacteria, commonly isolated from diverse environments and substrata such as soil, water, food, compost etc. The potential risk of these bacteria as human pathogens is very low, as they are considered non-pathogenic and non-toxic to human, animals and plants (EBI, 2012; Logan and De Vos, 2009). These two species are reported worldwide for having potential use as BCAs and there are commercial BCA products involving these species available on the markets.

Bacillus amyloliquefaciens strain D747 has been previously risk assessed for humans (EPA, 2011). The report showed that there was no evidence of infectivity, pathogenicity and toxicology when tested on rats and mammalian cells. Likewise, Serenade, the trade name for a preparation of *B. subtilis* QST713, was assessed for impact on humans and was approved for commercial production (AgraQuest, 2009). Serenade has been widely used in many countries against various plant pathogens and there have been no reports of a negative impact on humans or environments (Dedej *et al.*, 2004; Elmhirst *et al.*, 2011; Restuccia *et al.*, 2006; Yanez-Mendizabal *et al.*, 2012).

8.4.2 Impact of fungal BCAs

In contrast to bacteria, yeasts do not have a negative public image and rarely cause human diseases. *Aureobasidium pullulans* is found commonly from diverse isolation sources such as soil, plant debris, other living organisms, industrial materials and extreme environments. The fungus is reported globally and has been used in the biotechnological industries for the production of biomaterials such as pullulan, an extracellular polysaccharide widely used in the food and pharmaceutical industries (Cheng *et al.*, 2011; Donot *et al.*, 2012; Gaur *et al.*, 2010; Singh *et al.*, 2008; Zhang *et al.*, 2011a). The species has also been reported as a promising BCA against various plant pathogens (Janisiewicz *et al.*, 2010; Mari *et al.*, 2012a; Mari *et al.*, 2012b; Trapman *et al.*, 2010). Thus the impact of this fungus on humans has been well studied. For example, two strains of *A. pullulans* (DSM 14940 and 14941), registered for use within BoniProtect (a commercial BCA produced by Bio-ferm, Germany), were tested during the registration procedures for this product (Bio-ferm, 2011). Toxicological results showed that there was no toxicity when tested on rabbits and no carcinogenicity and mutagenicity when tested in mammalian cells. This BCA did not have a negative ecological impact when tested against duckweed, algae and rainbow trout.

However the criteria mentioned above were set by the US and German authorities as the BCAs were first registered in their respective countries. Different

countries have different criteria and guidelines. If they were to be used commercially in the UK, *Bacillus* sp. B91 and *A. pullulans* Y126 would need to be evaluated against the risk assessments required by the UK authority, the Chemical Regulation Directorate (CRD, 2012) which follows those set out in EU Regulation (EC) no 1007/2009 (EU, 2009).

8.5 Product registration

The registration of plant protection products in the European Union has been harmonised and is now under the same legislation which is regulated by the Regulation 1107/2009 passed by the European Parliament and the council of the European Union (EU, 2009). To register prospective BCAs, there are nine pieces of information needed (1) identity of the microbes, (2) biological properties of the microbes, (3) further information on mass production, handling and storage conditions, (4) Analytical methods, (5) effects on human health, (6) residues on treated products, food and feed, (7) fate and behaviour in the environment, (8) effects on non-target organisms and (9) summary and evaluation of environment impact. The information provided in this thesis addresses the first two parts of this registration requirement.

The two BCAs have been identified at generic and species level, using morphological and molecular data. Their bioactivity toward *M. laxa* has been demonstrated, and various biological properties have been reported including inhibition of mycelial growth, production of inhibitory organic compound, and inhibition of spore germination and sporulation on mummified fruit. In order to achieve the remaining information, collaboration with fruit growers and industrial partners is needed because this involves product formulation, field trials and assessment of impact on human and environment.

8.6 Product formulation

Mass production of BCAs must optimise product quantity without compromise of product efficacy, and formulation of BCAs is an important aspect of commercialisation (Burges and Jones, 1998; Schisler *et al.*, 2004). There are numerous factors that need to be taken into account, for example mode of action, basic nutrients required, optimum temperature for growth etc. The fact that efficacy of BCAs is not consistent in the field is possibly explained by two factors (1) fluctuation of environmental conditions and (2) instability of the BCAs. The first factor is difficult to deal with as it is naturally unpredictable except in closed environments such as

glasshouses, while the second factor can be addressed by the formulation of BCAs into products which are more stable and retain viability longer than unformulated cells. Product formulation can also provide the basic nutrients to BCAs in order to favour and initiate the growth of BCAs under suitable conditions such that BCAs can establish and colonise plant tissues in advance of pathogens. Formulation can help to maintain BCAs at high concentrations, which is important because competition is one of the major biological control mechanisms.

There are two types of formulations *i.e.* dry products and liquid products (Boyetchko *et al.*, 1998; Schisler *et al.*, 2004). Consideration of an appropriate type of formulation is critically important to maintain and enhance efficacy of BCAs by helping to deliver effective BCAs to target plant tissues. In this study, the mechanism of action of *Bacillus* sp. B91 was via the production of soluble and volatile organic compounds, and potentially also competition for nutrients, thus formulation of *Bacillus* sp. B91 should focus on preserving the bacterial cells and maximising production of bioactive compounds. In contrast, as competition for nutrients and space seems to play a central role for *A. pullulans* Y126, and the living cells are essential for bioactivity, basic nutrients should be provided for the formulation of *A. pullulans* Y126 in order to initiate the growth of the BCA and allow it to build up the population rapidly.

Carriers and protectants are essential components for formulation. Adding compounds which help BCAs to attach on plant surfaces and persist during rainfall is vital to extend the efficacy of BCAs. Applying BCAs on different plant tissues needs appropriate chemical adjuvants. For example, McGuire and Shasha (1995) used a flour formulation with *B. thuringiensis*. This formulation resulted in water-soluble granules that were adherent and resistant to wash-off by rain. Adding a similar type of carrier and adjuvants might have increased adhesion of *Bacillus* sp. B91 onto target plant tissue.

8.7 Pre- or post-harvest BCAs?

Post-harvest fungicide application on stone fruit has been well studied in many countries, for example on plums in Canada (Northover and Cerkauskas, 1998), and on peaches, nectarines and plums in the US (Forster *et al.*, 2007). This method is, however, not likely to be adopted in European countries due to EU restrictions of post-harvest chemical use. Fungicides must be applied as early as possible and a certain period of time must be left from final fungicide treatment before harvest to ensure that there are no fungicide residues on fruit surfaces. However, this restriction does not apply to BCAs, so BCAs have the advantage that they can be used at pre- or post-harvest.

From the experiments presented in this thesis, the choice between pre- or post-harvest treatment on stone fruit is straightforward. Treatment of plums with *A. pullulans* Y126 caused some adverse symptoms, although these were not seen on cherries. Thus, Y126 should be used on mummified fruits and possibly blossom as well, which is supported by the results from Chapter 7 showing that it is capable of supressing sporulation of *M. laxa* on mummified fruits.

Bacillus sp. B91 can be used on immature and mature fruits at pre- and post-harvest, because it did not cause fruit damages. However, the results from Chapter 5 showed that it is sensitive to low temperature, and should therefore be applied in the milder conditions at pre- and post-harvest. Furthermore, Isolate B91 is capable of producing VOCs that inhibit the mycelium of *M. laxa,* and thus may have potential as a biofumigant at post-harvest. Instead of choosing just one BCA, there may also be the possibility that these two BCAs can be integrated by applying them at different times to tackle *M. laxa* at various parts of its life cycle.

8.8 Disease management

In addition to being able to establish on plant tissue and survive in fluctuating conditions, BCAs will have to be compatible with other commercial practices in stone fruit production, especially the use of agrochemicals. As stated previously, the environmental factors are uncontrollable but the exposure to agrochemicals makes the selection of BCAs very difficult if their spectrum of activity includes the BCAs. Therefore some compatibility of BCAs and the agrochemicals is desirable if a true IPM regime is to be implemented.

8.9 Applying BCAs on mummified fruits

Current disease control focuses on blossom and fruits because when the disease occurs at these stages of fruit production, it causes significant direct net loss. Mummified fruits are overlooked, in spite of the fact they play a critical role in infection the following season. In this study, it has been shown that the two BCAs were able to reduce inoculum levels of *M. laxa* mummified fruit. This is the first report to use BCAs to control *M. laxa* on mummified fruit and results suggested that there is potential to use BCAs on mummified fruit. To adopt and integrate these BCAs into a disease management programme, there are a number of further studies that need to be taken into consideration including compatibility of BCAs fungicides, and further investigations of

the population dynamics and persistence of the BCAs on plant tissue after application in field conditions.

8.10 Can BCAs replace fungicides?

There are numerous ways to reduce brown rot development in commercial orchards without using fungicides. These ways include removing mummified fruits, pruning out blossom wilt, removing infected fruits prior storage and treating fruits at post-harvest. However the methods mentioned above do not prevent infection of stone fruits by *Monilinia* spp., they only reduce inoculum levels with the consequential decreased level of crop losses. It is undeniable that chemical control of brown rot is still a key component of integrated pest management and that fungicide treatments are more reliable than BCAs. Nevertheless, there has been an increased interest in BCAs and a number have been tested at different stages of stone fruit production, including mummified fruits (in this study), blossom (Guijarro *et al.*, 2008b; Loncaric *et al.*, 2006), fruits pre- and post-harvest (Ippolito *et al.*, 2005; Larena *et al.*, 2005; Poleatewich *et al.*, 2012). However, from the reports and hence the fruit growers' perspective, the efficacy of BCAs is still not consistent, especially in open field use.

Will BCAs ever replace fungicides? The answer to the question is no – but that biological control should be used in combination with chemical control. For example, chemicals could be applied regularly on mummified fruit to reduce inoculum levels while BCAs could be applied at blossom and pre-harvest and potentially also on mummified fruit. Physical and biological control can be used post-harvest to suppress latent infection and reduce losses. It is generally accepted that plant disease cannot be eradicated from orchards because disease can re-emerge on following years (Gottwald, 2000). However with integration of these all measures we might be able to control the disease and marginalise the pathogen.

In the meantime, the biological control industry will continue to grow in line with increasing restrictions on chemical usage. Overall there are now about 1400 'biopesticide' products being sold, with around 90 of these being registered in the EU and over 300 in the US. The number of new registrations in the EU is growing steadily but they currently represent only 2.5% of the pesticide market (CPL, 2010). There is a pressing need to determine ways in which BCAs can be integrated into the existing IPM strategies.

8.11 Conclusions

The overall aims of this study were:

(1) *To isolate and test indigenous BCAs for their potential against M. laxa using an in vitro technique.* Chapter 3 describes the successful isolation of over 200 micro-organisms from stone fruit sources in Kent. The chapter also describes how *in vitro* screening tests were used to select the 12 isolates with most potential for further study.

(2) *To assess efficacy of indigenous BCAs and existing BCAs against M. laxa in an in vivo test on cherries and plums.* Chapter 4 describes how these 12 isolates were compared with a commercial fungicide or a commercial BCA preparation in relation to their ability to inhibit development of brown rot on harvested stone fruits. Two isolates in particular, a bacterium B91 and a yeast Y126, showed good activity, although they did not give results that equalled those obtained with a commercial fungicide.

(3) *To identify isolates and study the capability of selected BCAs to grow and survive at different temperatures.* Chapter 5 describes how isolate B91 was identified as either *Bacillus amylofaciens* or *B. subtilis*, and isolate Y126 was identified as *Aureobasidium pullullans*. Their growth characteristics under a range of temperatures likely to be experienced under field conditions or in cold storage were studied and the effects of temperature on growth and survival were reported.

(4) *To examine the mode of action of BCAs against M. laxa.* Chapter 5 also included a study of the modes of action of both BCAs, and revealed that competition for nutrients is a major biocontrol mechanism by *A. pullulans* Y126, while *Bacillus* sp. B91 potentially produced inhibitory compounds and also competed for nutrients.

(5) *To compare populations of M. laxa originally isolated from two different stages of infection (mummified fruits and fruit brown rot).* Chapter 6 described how molecular techniques have been used to study the biodiversity of populations of *M. laxa* found in stone fruit orchards at several locations in the UK. The information obtained allowed speculation on epidemiology of the pathogen and showed that locations and hosts play important roles for population of *M. laxa*. Therefore these two factors should be taken into account for disease management of brown rot disease. It seemed likely that the source of most fruit infections came from populations of *M. laxa* already present in orchards rather populations brought in by wind or human agencies.

(6) *To evaluate efficacy of BCAs to suppress sporulation of M. laxa on mummified fruits.* Chapter 7 built on this information by using a novel strategy of biological control and spraying mummified fruit in the field in the overwinter period to suppress the levels of inoculum available to colonise the developing fruit later in the season. Results indicated that sporulation was reduced but more work is needed to refine this potential strategy for use of these BCAs.

8.12 Future work

To expand the knowledge of these BCAs and provide relevant information for commercialisation, a number of studies should be carried out including (1) optimisation of formulations for enhanced biocontrol activity, (2) potential for reduction of inoculum on blossom, (3) combinations of chemical and biological control and (4) a more detailed comparison of inoculum sources. This thesis has shown the potential use of BCAs against *M. laxa*. However there was a failure of control in some experiments, for example the dipping treatment in the post-harvest study. Whichever strategy is used for biological control, the candidate BCAs should be formulated in order to maximise and enhance the efficacy of the BCAs before being applied at post-harvest and in the field.

Aureobasidium pullulans Y126 was able to reduce inoculum of *M. laxa* when it was applied onto mummified fruits. A further treatment by spraying Y126 on blossom might further control the inoculum levels of the pathogen and thus result in lower disease levels in fruit. Nevertheless, at present chemical control is still the best treatment to control brown rot disease as it is highly effective and reliable. However rather than entirely rely on chemical control, a study of a combination of biological control with reduced chemical inputs is needed. Finally this study has shown that mummified fruit is a significant source of inoculum for infection on green fruit the following season. To gain further knowledge of this aspect of the epidemiology and life cycle of *M. laxa*, isolates of *M. laxa* collected from blossom wilts should be compared to these two sources of inoculum.

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Appendix 1

A1.1 Microbiological growth media

All media were shaken well and the pH was adjusted before autoclaving. The media were sterilised at 121°C for 15 min. For solid agar, they were cooled down to 50°C in water bath before pouring into Petri dishes.

Malt extract agar (MEA) pH 5.4

Malt extract (Fluka)	30.0 g
Mycological peptone (Oxoid)	5.0 g
Agar technical no. 3 (Oxoid)	12.0 g
Distilled water	1000 ml

Malt extract agar amended with antibiotics (MEA+) pH 5.4

Malt extract (Fluka)	30.0 g
Mycological peptone (Oxoid)	5.0 g
Agar technical no. 3 (Oxoid)	12.0 g
Distilled water	1000 ml

MEA was sterilised and after cooling to 50°C the following antibiotics were added.

Streptomycin (Sigma)	0.05 g
Chloramphenicol (Sigma)	0.05 g

Malt extract broth (MEB) pH 5.4

Malt extract (Fluka)	17.0 g
Mycological peptone (Oxoid)	3.0 g
Distilled water	1000 ml

Milk agar (MA), pH 7.2

Agar	15.0 g
Peptone	5.0 g
Yeast extract	3.0 g
Milk (solid or fresh milk)	1.0 g or 10.0 ml

Nutrient agar (NA) pH 7.4

Nutrient broth (Fluka)	25.0 g
Agar technical no. 1 (Oxoid)	15.0 g
Distilled water	1000 ml

Nutrient agar amended with an antibiotic (NA+) pH 7.4

Nutrient broth (Fluka)	25.0 g
Agar technical no. 1 (Oxoid)	15.0 g
Distilled water	1000 ml

NA was sterilised and after cooling to 50°C the following an antibiotic was added.

Cycloheximide (Sigma)	0.05 g
or	
Chloramphenicol (Sigma)	0.05 g
or	
Streptomycin (Sigma)	0.05 g

Nutrient broth (NB) pH 7.4

Nutrient broth (Fluka)	25.0 g
Distilled water	1000 ml

Paraquat chloramphenicol agar (PCA)

Paraquat (methyl viologen, Sigma)	20.0 mg
Chloramphenicol (Sigma)	200.0 mg
Agar (Oxoid)	20.0 g
Distilled water	1000 ml

Potato dextrose agar (PDA) pH 5.6

Potato dextrose broth (Fluka)	24.0 g
Agar technical no. 1	15.0 g
Distilled water	1000 ml

Potato dextrose broth (PDB) pH 5.6

Potato dextrose broth (Fluka)	24.0 g
Distilled water	1000 ml

Water agar (WA)

Agar technical no. 1	15.0 g
Distilled water	1000 ml

A1.2 Chemical reagents

Phosphate buffer solution (PBS), pH 7.4

Sodium chloride (NaCl, Fluka)	8.0 g
Potassium chloride (KCl, Sigma)	0.2 g
Sodium phosphate (Na_2HPO_4, Sigma)	1.44 g
Potassium phosphate (KH_2PO_4, sigma)	0.24 g

Normal saline solution (NSS)

Sodium chloride (NaCl, Fluka)	9.0 g
Distilled water	1000 ml

1% Triton X

Triton X (BDH)	0.1 ml
Distilled water	100 ml

A1.3 Molecular regents

1.0 M Tris

Tris (hydroxymethyl) aminomethane	60.57 g
Milli Q water	500 ml

Dissolve 60.57 g of Tris in 400 ml of Milli Q water. Adjust pH to 7.5 using HCl. Final volume was adjusted to 500 ml.

0.5 Ethylenediaminetetraacetic acid (EDTA)

EDTA	18.6 g
Milli Q water	100 ml

Add 18.6 g of EDTA to 80 ml of Milli Q water. The pH was adjusted to 8.0 using NaOH. EDTA will not be soluble until pH reaches 8.0 and the solution must be stirred on a magnetic stirrer for hours and moderate heated. Final volume was adjusted to 100 ml.

Ethidium bromide (EtBr) (10.0 mg/ml)

EtBr	10.0 mg
Distilled water	10.0 ml

Add 10.0 g of EtBr to 10.0 ml. Stir on a magnetic stirrer for hours to ensure that EtBr completely dissolves. Keep EtBr solution in a glass or plastic container wrapped with aluminium foil or a dark bottle and stored at room temperature. The container must be clearly labelled as a carcinogen and kept separate from other molecular reagents.

Working concentration (0.5 µg/ml)

Add one drop (50 µl) of EtBr (10.0 mg/ml) to 1,000 ml of distilled water. Ethidium bromide solution was kept in a plastic container covered with a lid to protect from the light.

70% Ethanol

Absolute ethanol	70.0 ml
Sterile distilled water	30.0 ml

Appendix 2

Additional result tables for Chapter 3

A2.1 Inhibition test on MEA

Table A2.1 Radii of *M. laxa* originally isolated from cherries and plums and percentage inhibition when tested against microbes which *M. laxa* grown on MEA in a 90 mm diameter Petri dish incubated in the dark at 20°C for two weeks.

Isolate	*M. laxa* from cherries			*M. laxa* from plums		
	Radius (mm)	Control (mm)	% inhibition	Radius (mm)	Control (mm)	% inhibition
B39	31.52	42.00	24.96	22.33	37.83	40.97
B40	24.73	42.00	41.11	24.58	37.83	35.02
B41	19.38	39.63	51.09	26.65	38.63	31.02
B42	27.17	39.63	31.46	22.63	38.63	41.42
B43	37.58	40.48	7.16	36.68	39.28	6.62
B44	38.50	40.48	4.90	34.90	39.28	11.16
B45	24.27	41.42	41.41	27.52	36.62	24.85
B46	22.43	41.42	45.84	21.95	36.62	40.05
B47	26.38	41.28	36.09	19.35	36.40	46.84
B48	23.75	41.28	42.47	18.40	36.40	49.45
B49	16.25	29.70	45.29	10.67	10.83	1.54
B50	21.82	29.70	26.54	11.17	10.83	-3.08
B51	40.20	36.89	-8.96	30.42	39.18	22.37
B52	33.02	36.89	10.51	28.73	39.18	26.67
B53	29.82	40.98	27.25	32.05	35.18	8.91
B54	39.27	40.98	4.19	33.63	35.18	4.41
B55	39.70	41.97	5.40	37.30	39.82	6.32
B56	33.00	41.97	21.37	34.38	39.82	13.65
B57	38.60	41.20	6.31	39.85	40.23	0.93
B58	36.80	41.20	10.68	38.98	40.23	3.11
B59	37.30	41.28	9.63	38.68	39.15	1.19
B60	39.35	41.28	4.66	38.28	39.15	2.21
B61	22.88	40.70	43.78	19.53	36.83	46.97
B62	20.98	40.70	48.44	17.80	36.83	51.67
B63	39.20	39.13	-0.19	39.23	39.45	0.55
B64	35.23	39.13	9.97	34.25	39.45	13.18
B65	27.52	39.95	31.12	19.47	39.68	50.94
B66	22.68	39.95	43.22	20.67	39.68	47.92
B67	25.40	40.67	37.54	25.95	36.50	28.90
B68	24.48	40.67	39.80	22.65	36.50	37.95
B69	26.13	40.30	35.15	25.17	34.57	27.19
B70	23.40	40.30	41.94	23.32	34.57	32.55
B71	25.75	41.38	37.78	27.90	35.03	20.36
B72	25.77	41.38	37.74	25.88	35.03	26.12
B73	28.65	41.02	30.15	25.07	36.65	31.61
B74	25.65	41.02	37.46	22.47	36.65	38.70
B75	28.73	37.83	24.05	26.80	35.23	23.94
B76	28.62	37.83	24.36	31.73	35.23	9.93
B77	41.55	40.17	-3.44	35.13	38.23	8.11
B78	29.95	40.17	25.44	28.78	38.23	24.72
B79	41.20	38.48	-7.08	28.67	30.73	6.72
B80	39.58	38.48	-2.86	34.57	30.73	-12.47
B81	38.33	37.83	-1.32	28.08	27.43	-2.37
B82	35.27	37.83	6.78	26.68	27.43	2.73
B83	27.35	28.40	3.70	41.50	40.38	-2.77
B84	30.35	28.40	-6.87	40.60	40.38	-0.54
B85	30.65	39.42	22.24	22.90	25.77	11.13

Continue…

Table A2.1 (continued) Radii of *M. laxa* originally isolated from cherries and plums and percentage inhibition when tested against microbes which *M. laxa* grown on MEA in a 90 mm diameter Petri dish incubated in the dark at 20°C for two weeks.

Isolate	*M. laxa* from cherries			*M. laxa* from plums		
	Radius (mm)	Control (mm)	% inhibition	Radius (mm)	Control (mm)	% inhibition
B86	41.58	39.42	-5.50	24.20	25.77	6.08
B87	28.58	37.03	22.82	26.58	26.68	0.37
B88	27.58	37.03	25.52	26.30	26.68	1.41
B89	23.00	24.00	4.17	38.43	34.57	-11.16
B90	22.13	24.00	7.81	24.23	34.57	29.89
B91	0.00	0.00	100.00	0.00	0.00	100.00
B92	0.00	0.00	100.00	0.00	0.00	100.00
B93	25.88	26.83	3.54	29.73	39.95	25.57
B94	26.75	26.83	0.28	41.52	39.95	-3.92
B95	27.75	30.50	9.02	41.57	40.22	-3.36
B96	26.78	30.50	12.21	38.88	40.22	3.32
B97	30.02	39.50	24.01	29.77	29.63	-0.45
B98	27.19	39.50	31.16	23.00	29.63	22.38
B99	25.72	37.49	31.40	22.82	39.90	42.79
B100	43.71	37.49	-16.61	35.37	39.90	11.34
B101	43.33	38.94	-11.26	39.73	38.25	-3.86
B102	40.87	38.94	-4.94	30.74	38.25	19.63
B103	35.71	39.07	8.60	34.03	34.94	2.59
B104	30.71	39.07	21.41	24.74	34.94	29.18
B105	26.70	40.04	33.31	43.55	39.60	-9.99
B106	32.47	40.04	18.91	42.29	39.60	-6.80
B107	35.72	40.91	12.69	37.42	39.75	5.85
B108	35.72	40.91	12.68	31.58	39.75	20.54
B109	43.73	39.56	-10.55	31.63	40.36	21.62
B110	42.34	39.56	-7.03	28.07	40.36	30.45
B111	37.58	32.13	-16.96	32.48	37.43	13.22
B112	20.33	32.13	36.73	28.05	37.43	25.06
B113	21.45	27.04	20.65	25.22	38.49	34.48
B114	27.78	27.04	-2.74	41.03	38.49	-6.59
B115	25.57	36.08	29.13	28.69	37.35	23.20
B116	29.89	36.08	17.16	38.34	37.35	-2.65
B117	17.32	33.49	48.27	27.73	39.31	29.44
B118	22.84	33.49	31.79	24.52	39.31	37.61
B119	27.92	41.51	32.73	27.91	39.44	29.22
B120	26.92	41.51	35.14	25.87	39.44	34.40
B121	25.26	40.11	37.04	29.16	35.42	17.68
B122	25.66	40.11	36.03	26.07	35.42	26.41
B123	26.57	39.81	33.26	29.12	37.26	21.85
B124	24.83	39.81	37.64	26.68	37.26	28.39
B125	21.21	33.35	36.39	24.08	36.97	34.87
B126	29.15	33.35	12.59	23.51	36.97	36.40
B127	23.64	39.28	39.83	28.05	38.01	26.20
B128	26.99	39.28	31.30	25.83	38.01	32.04
B129	32.39	40.58	20.20	28.65	38.05	24.71
B130	35.85	40.58	11.66	27.82	38.05	26.88
Y31	24.45	42.18	42.04	23.07	36.27	36.40
Y32	19.97	42.18	52.67	19.87	36.27	45.22
Y33	28.10	38.82	27.61	27.17	36.70	25.98
Y34	21.47	38.82	44.70	22.80	36.70	37.87
Y35	17.72	39.85	55.54	9.58	25.35	62.23

Continue...

Table A2.1 (continued) Radii of *M. laxa* originally isolated from cherries and plums and percentage inhibition when tested against microbes which *M. laxa* grown on MEA in a 90 mm diameter Petri dish incubated in the dark at 20°C for two weeks.

Isolate	*M. laxa* from cherries			*M. laxa* from plums		
	Radius (mm)	Control (mm)	% inhibition	Radius (mm)	Control (mm)	% inhibition
Y36	18.15	39.85	54.45	14.53	25.35	42.70
Y37	30.47	40.87	25.45	25.72	36.70	29.93
Y38	21.57	40.87	47.23	22.57	36.70	38.51
Y39	24.00	37.65	36.25	19.70	38.58	48.94
Y40	28.35	37.65	24.70	23.72	38.58	38.53
Y41	21.73	40.13	45.85	15.38	36.47	57.82
Y42	21.78	40.13	45.72	19.87	36.47	45.52
Y43	22.58	39.70	43.12	22.75	40.08	43.24
Y44	22.63	39.70	42.99	23.10	40.08	42.37
Y45	27.20	40.00	32.00	24.53	33.13	25.96
Y46	40.15	41.00	2.07	32.85	33.13	0.83
Y47	19.63	35.95	45.39	28.45	38.27	25.65
Y48	21.00	35.95	41.59	18.42	38.27	51.87
Y49	23.27	40.17	42.07	24.17	37.92	36.26
Y50	30.52	40.17	24.02	18.57	37.92	51.03
Y51	11.37	37.73	69.88	12.53	31.75	60.52
Y52	10.25	37.73	72.84	9.07	31.75	71.44
Y53	22.03	39.20	43.79	14.80	15.60	5.13
Y54	16.28	39.20	58.46	14.80	15.60	5.13
Y55	22.20	39.87	44.31	19.05	38.77	50.86
Y56	20.05	39.87	49.71	18.28	38.77	52.84
Y57	24.93	41.15	39.41	17.73	39.30	54.88
Y58	24.58	41.15	40.26	21.13	39.30	46.23
Y59	26.80	41.38	35.24	24.55	41.10	40.27
Y60	22.32	41.38	46.07	22.15	41.10	46.11
Y61	23.93	41.38	42.17	18.33	38.45	52.32
Y62	22.10	41.38	46.60	19.97	38.45	48.07
Y63	24.95	40.37	38.19	24.22	39.25	38.30
Y64	22.65	40.37	43.89	16.62	39.25	57.66
Y65	29.40	38.30	23.24	30.03	37.08	19.02
Y66	26.83	38.30	29.96	25.80	37.08	30.41
Y67	21.62	40.03	46.00	17.78	38.27	53.53
Y68	21.95	40.03	45.17	18.57	38.27	51.48
Y69	11.57	40.58	71.50	9.93	29.08	65.85
Y70	12.65	40.58	68.83	9.28	29.08	68.08
Y71	28.62	37.37	23.42	25.08	31.22	19.65
Y72	27.65	37.37	26.00	23.38	31.22	25.09
Y73	26.18	39.12	33.06	22.58	29.52	23.49
Y74	26.40	39.12	32.51	21.22	29.52	28.12
Y75	23.77	37.42	36.48	25.33	34.45	26.46
Y76	24.73	37.42	33.90	23.50	34.45	31.79
Y77	27.53	37.00	25.60	24.33	32.08	24.16
Y78	25.95	37.00	29.86	25.40	32.08	20.81
Y79	14.60	39.90	63.41	12.03	24.78	51.46
Y80	11.93	39.90	70.11	7.33	24.78	70.43
Y81	29.12	37.87	23.11	22.83	24.62	7.24
Y82	25.62	37.87	32.35	23.88	24.62	2.98
Y83	31.15	35.75	12.87	28.10	33.42	15.91

Continue...

Table A2.1 (continued) Radii of *M. laxa* originally isolated from cherries and plums and percentage inhibition when tested against microbes which *M. laxa* grown on MEA in a 90 mm diameter Petri dish incubated in the dark at 20°C for two weeks.

Isolate	*M. laxa* from cherries			*M. laxa* from plums		
	Radius (mm)	Control (mm)	% inhibition	Radius (mm)	Control (mm)	% inhibition
Y36	18.15	39.85	54.45	14.53	25.35	42.70
Y84	30.13	35.75	15.71	28.00	33.42	16.21
Y85	29.23	39.43	25.87	13.45	28.30	52.47
Y86	31.78	39.43	19.40	23.58	28.30	16.67
Y87	15.08	38.02	60.32	10.20	29.95	65.94
Y88	12.58	38.02	66.90	11.12	29.95	62.88
Y89	13.33	39.08	65.90	9.25	26.93	65.65
Y90	13.75	39.08	64.81	8.05	26.93	70.10
Y91	24.29	38.59	37.05	27.08	39.72	31.81
Y92	22.41	38.59	41.94	31.30	39.72	21.18
Y93	24.75	32.56	23.99	30.08	38.54	21.95
Y94	20.37	32.56	37.46	24.47	38.54	36.51
Y95	26.82	31.58	15.07	28.40	37.41	24.09
Y96	22.68	31.58	28.17	26.56	37.41	29.01
Y97	28.43	36.85	22.85	29.18	37.46	22.12
Y98	23.40	36.85	36.50	27.75	37.46	25.92
Y99	13.85	34.68	60.05	16.99	34.34	50.53
Y100	9.85	34.68	71.60	11.71	34.34	65.89
Y101	12.26	36.62	66.52	13.32	35.81	62.81
Y102	10.21	36.62	72.12	13.33	35.81	62.79
Y103	8.86	32.57	72.79	12.30	35.15	65.01
Y104	8.65	32.57	73.43	10.22	35.15	70.92
Y105	12.63	35.56	64.47	14.64	35.23	58.45
Y106	9.48	35.56	73.33	10.89	35.23	69.08
Y107	12.36	34.46	64.14	16.15	39.25	58.87
Y108	10.36	34.46	69.92	12.98	39.25	66.94
Y109	28.20	40.63	30.59	26.70	38.25	30.19
Y110	10.31	40.63	74.62	13.32	38.25	65.16
Y111	22.09	28.96	23.74	29.76	39.08	23.85
Y112	25.03	28.96	13.58	27.51	39.08	29.61
Y113	25.90	37.99	31.83	25.25	38.24	33.96
Y114	28.28	37.99	25.57	24.92	38.24	34.82
Y115	26.41	40.89	35.41	25.59	39.01	34.40
Y116	28.17	40.89	31.11	25.55	39.01	34.50
Y117	29.53	41.01	27.98	25.39	37.65	32.56
Y118	36.60	41.01	10.75	27.55	37.65	26.83
Y119	23.63	40.29	41.36	24.71	38.87	36.44
Y120	23.97	40.29	40.51	24.50	38.87	36.98
Y121	15.15	35.87	57.76	15.94	40.66	60.80
Y122	15.22	35.87	57.57	17.60	40.66	56.71
Y123	15.89	40.23	60.49	17.79	36.08	50.69
Y124	15.85	40.23	60.59	15.69	36.08	56.51
Y125	10.82	30.39	64.41	11.77	38.44	69.37
Y126	8.77	30.39	71.15	10.31	38.44	73.19
Y127	17.89	36.23	50.63	15.37	37.51	59.04
Y128	15.15	36.23	58.19	16.12	37.51	57.04
Y129	26.55	37.59	29.38	24.74	36.87	32.88
Y130	23.27	37.59	38.10	24.74	36.87	32.90

A2.2 Inhibition test on MEA and PDA

Table 2.2 Percentage inhibition of BCAs when tested against *M. laxa* grown on MEA and PDA in a 90 mm diameter Petri dish incubated in the dark at 20°C for two weeks.

Isolate	Percentage inhibition			
	M. laxa from cherries		*M. laxa* from plums	
	MEA	PDA	MEA	PDA
B41	26.64	8.80	31.02	-1.90
B46	45.84	20.31	40.05	-2.40
B47	36.09	-10.92	46.84	0.39
B48	42.47	2.97	49.45	4.51
B49	45.29	-10.50	1.54	0.08
B50	26.54	-3.25	-3.08	3.87
B62	48.44	1.75	51.67	4.39
B65	31.12	30.22	50.94	5.94
B70	41.94	-10.55	32.55	3.47
B91	100.00	80.24	100.00	65.60
B92	100.00	14.98	100.00	-1.45
B99	44.34	46.81	42.79	28.80
B117	53.53	2.43	29.44	8.30
B130	11.66	-3.39	26.88	1.58
Y32	52.67	33.19	45.22	7.43
Y35	55.54	10.39	62.23	10.23
Y36	54.45	4.06	42.70	8.63
Y41	45.85	13.93	57.82	11.70
Y48	41.59	2.85	51.87	11.13
Y50	24.02	-2.86	51.03	2.97
Y51	69.88	36.13	60.52	42.84
Y52	72.84	38.05	71.44	45.99
Y55	44.31	-1.62	50.86	12.49
Y56	49.71	16.60	52.84	9.28
Y57	39.41	-0.05	54.88	11.61
Y61	42.17	16.19	52.32	5.36
Y64	43.89	7.89	57.66	11.28
Y67	46.00	2.52	53.53	6.44
Y68	45.17	19.66	51.48	34.49
Y69	71.50	20.17	65.85	49.17
Y70	68.83	23.02	68.08	38.38
Y79	63.41	32.98	51.46	37.45
Y80	70.11	40.09	70.43	49.32
Y85	25.87	-1.18	52.47	-0.34
Y87	60.32	41.3	65.94	36.76
Y88	66.90	40.95	62.88	39.40
Y89	48.85	42.66	65.65	41.55
Y90	47.22	31.3	70.1	39.73
Y99	60.05	12.97	50.53	50.30
Y100	71.60	32.79	65.89	45.28
Y101	66.52	28.00	62.81	45.67
Y102	72.12	34.35	62.79	43.52
Y103	72.79	32.13	65.01	38.33
Y104	73.43	45.83	70.92	40.33
Y105	64.47	20.22	58.45	37.50
Y106	73.33	46.66	69.08	43.30
Y107	64.14	9.42	58.87	23.27
Y108	69.92	33.43	66.94	37.60
Y110	74.62	28.87	65.16	45.41
Y121	57.76	29.17	60.80	21.01

Continue...

Table 2.2 (continue) Percentage inhibition of BCAs when tested against *M. laxa* grown on MEA and PDA in a 90 mm diameter Petri dish incubated in the dark at 20°C for two weeks.

	Percentage inhibition			
	M. laxa from cherries		*M. laxa* from plums	
Isolate	MEA	PDA	MEA	PDA
Y122	57.57	28.31	56.71	26.92
Y123	60.49	24.4	50.69	24.03
Y124	60.59	25.56	56.51	25.83
Y125	46.61	35.53	69.37	39.37
Y126	56.73	51.85	73.19	50.39
Y127	50.63	21.04	59.04	26.88
Y128	58.19	27.03	57.04	26.38

Appendix 3

Additional result tables for Chapter 4

A3.1 Indigenous BCAs

Table A3.1.1 Analysis of variance for percentage infection on cherries and indigenous BCA using treatment as the factor and percentage infection as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	2880.0	2880.0	20.19	
Repeat.*Units* stratum					
Treatments	13	238568.5	18351.4	128.68	<.001
Residual	209	29806.9	142.6		
Total	223	271255.4			

Table A3.1.2 Analysis of variance for percentage infection on plums and indigenous BCAs using treatment as the factor and percentage infection as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	2090.1	2090.1	15.98	
Repeat.*Units* stratum					
Treatment	13	319189.0	24553.0	187.71	<.001
Residual	265	34663.7	130.8		
Total	279	355942.8			

Table A3.1.3 Analysis of variance for lesion sizes of plums and indigenous BCAs using treatments as the factor and lesion sizes as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum					
Treatments	13	895.81	68.91		
Residual	-12	0.00			
Repeat.*Units* stratum					
Treatments	13	2439.42	187.65	14.39	<.001
Residual	213	2778.20	13.04		
Total	227	6113.43			

A3.2 Commercial BCAs

A 3.2.1 Effect of treatments, application times and cultivars

Table A3.2.1a Analysis of variance for percentage infection on plums using treatments, application time and cultivars as factors with repeat as a block and percentage infection as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	1700.4	1700.4	7.97	
Repeat.*Units* stratum					
Treatments	6	178838.0	29806.3	139.68	<.001
Application time	1	4888.9	4888.9	22.91	<.001
Cultivars	1	3088.9	3088.9	14.48	<.001
Treatments.Application time	6	12717.3	2119.6	9.93	<.001
Treatments.Cultivars	6	4414.8	735.8	3.45	0.003
Application time.Cultivars	1	3.2	3.2	0.02	0.902
Treatments.Application time.Cultivars	6	4935.5	822.6	3.85	0.001
Residual	251	53559.6	213.4		
Total	279	264146.8			

Table A3.2.1b Analysis of variance for lesion size on plums using treatments, application time and cultivars as factors with repeat as a block and percentage infection as the variates.

Change	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat	1	246.13	246.13	14.74	<.001
Repeat.Box	8	119.93	14.99	0.90	0.519
Treatments	5	300.88	60.18	3.60	0.004
Application_time	1	69.77	69.77	4.18	0.042
Cultivars	1	1751.72	1751.72	104.93	<.001
Treatments.Application_time	5	474.66	94.93	5.69	<.001
Treatments.Cultivars	5	132.91	26.58	1.59	0.164
Application_time.Cultivars	1	27.71	27.71	1.66	0.199
Treatments.Application_time.Cultivars	5	194.03	38.81	2.32	0.044
Residual	204	3405.49	16.69		
Total	236	6723.21	28.49		

A3.2.2 Effect of treatments, wound types

Table A3.2.2a Analysis of variance for percentage inhibition on cherries using treatments and wounds as factors, with repeat as block and percentage inhibition as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	1173.3	1173.3	9.21	
Repeat.*Units* stratum					
Treatments	6	213803.0	35633.8	279.84	<.001
Wounds	1	40.1	40.1	0.31	0.575
Treatments.Wounds	6	604.8	100.8	0.79	0.577
Residual	209	26613.4	127.3		
Total	223	242234.5			

Table 3.2.2b Analysis of variance for percentage infection on plums using treatments, wounds as factors, with repeat as block, and percentage infection as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	39.38	39.38	0.52	
Repeat.*Units* stratum					
Treatments	6	265016.25	44169.38	582.06	<.001
Wounds	1	354.37	354.37	4.67	0.032
Treatments.Wounds	6	416.25	69.37	0.91	0.485
Residual	265	20109.38	75.88		
Total	279	285935.62			

Table 3.2.2c Analysis of variance for lesion size on plums using treatments and wounds as factors, with repeat as block, and lesion sizes as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	71.474	71.474	7.29	
Repeat.*Units* stratum					
Treatments	5	58.844	11.769	1.20	0.310
Wounds	1	400.501	400.501	40.86	<.001
Treatments.Wounds	5	184.743	36.949	3.77	0.003
Residual	227	2225.260	9.803		
Total	239	2940.822			

A3.2.3 Semi-commercial storage conditions

Table A3.2.3a Analysis of variance for percentage inhibition on cherries using treatments, incubation temperature as the factors and percentage inhibition as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	2	872.5	436.2	1.40	
Repeat.*Units* stratum					
Treatments	5	360830.0	72166.0	231.50	<.001
Temperatures	2	50663.2	25331.6	81.26	<.001
Treatments.Temperatures	10	53962.0	5396.2	17.31	<.001
Residual	412	128432.5	311.7		
Total	431	594760.2			

Table 3.2.3b Analysis of variance for percentage inhibition using treatments, incubation temperature as the factors and percentage inhibition as the variates.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	2480.6	2480.6	6.46	
Repeat.*Units* stratum					
Treatments	5	307150.6	61430.1	159.88	<.001
Incubation_temperature	2	54005.0	27002.5	70.28	<.001
Treatments.Incubation_temperature	10	52225.0	5222.5	13.59	<.001
Residual	341	131023.1	384.2		
Total	359	546884.4			

Table 3.2.3c Analysis of variance by an unbalanced design for lesion sizes on plums using treatments, incubation as the factors and lesion sizes as the variates.

Change	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat	1	232.40	232.40	8.54	0.004
Repeat.Boxes	18	551.58	30.64	1.13	0.334
Treatments	3	1388.35	462.78	17.01	<.001
Incubation	2	6216.26	3108.13	114.28	<.001
Treatments.Incubation	6	138.03	23.00	0.85	0.537
Residual	127	3454.23	27.20		
Total	157	11980.84	76.31		

Appendix 4

Additional result tables for Chapter 5

A4.1 Anti-spore germination of *M. laxa*

Table 4.1a Analysis of variance for anti-spore germination of *M. laxa* treated with sterile PDB or BCAs.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	447.3	447.3	0.89	
Repeat.*Units* stratum					
Control vs Treated	1	44553.6	44553.6	88.92	<.001
Residual	97	48602.6	501.1		
Total	99	93603.5			

Table 4.1b Analysis of variance for spore germination using BCAs, Sources and media as factors and transformed percentage germination as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	293.71	293.71	4.64	
Repeat.*Units* stratum					
BCAs	1	10697.14	10697.14	168.86	<.001
Sources	1	16224.25	16224.25	256.11	<.001
Media	1	95.46	95.46	1.51	0.224
BCAs.Sources	1	14140.16	14140.16	223.21	<.001
BCAs.Media	1	65.72	65.72	1.04	0.312
Sources.Media	1	6.54	6.54	0.10	0.749
BCAs.Sources.Media	1	152.92	152.92	2.41	0.125
Residual	71	4497.76	63.35		
Total	79	46173.65			

A4.2 Production of VOCs

Table A4.2a Analysis of variance for production of VOCs by two BCAs using treatments and isolates as the factors and colony diameter of *M. laxa* as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	17.92	17.92	0.41	
Repeat.*Units* stratum					
Treatments	2	52176.79	26088.39	597.05	<.001
Isolates	1	4415.14	4415.14	101.04	<.001
Treatments.Isolates	2	2210.22	1105.11	25.29	<.001
Residual	89	3888.88	43.70		
Total	95	62708.94			

Tables 4.2b Mean values of with standard error of the difference of means.

	Colony diameter (mm)	
Treatments	*M. laxa* from cherries	*M. laxa* from plums
Control	75.87	57.67
Bacillus sp. B91	11.39	11.19
A. pullulans Y126	61.91	39.62
s.e.d.	2.337	

A4.3 Effect of temperatures on growth of BCAs

Table 4.3a Analysis of variance for growth of Bacillus sp. B91 using temperatures and times as the factors and ln CFU/ml as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum	2	0.294	0.147	0.01	
Replicate.Subject stratum					
Temperature	5	2199.427	439.885	40.62	<.001
Residual	28	303.206	10.829	10.55	
Replicate.Subject.Time stratum					
d.f. correction factor 0.4527					
Time	6	71.775	11.963	11.65	<.001
Time.Temperature	30	531.637	17.721	17.26	<.001
Residual	180	184.825	1.027		
Total	251	3291.165			

Table 4.3b Analysis of variance for growth of *A. pullulans* Y126 using temperatures and times as the factors and ln CFU/ml as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Repeat stratum	1	1.4621	1.4621	32.21	
Replicates stratum	2	0.3934	0.1967	4.33	
Repeat.Replicates stratum	2	0.0908	0.0454	0.27	
Repeat.Replicates.Subject stratum					
Temperature	5	15.9437	3.1887	18.73	<.001
Residual	25	4.2553	0.1702	0.78	
Repeat.Replicates.Subject.Time stratum					
d.f. correction factor 0.4478					
Time	6	56.4810	9.4135	43.13	<.001
Time.Temperature	30	82.3550	2.7452	12.58	<.001
Residual	180	39.2861	0.2183		
Total	251	200.2675			

A4.4 Effect of temperatures on survival of BCAs

Table 4.4a Analysis of variance for survival of *Bacillus* sp. B91 using temperatures and times as the factors and ln CFU/ml as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicates stratum	2	0.0841	0.0420	0.01	
Replicates.Subject stratum					
Temperature	5	300.8436	60.1687	12.96	<.001
Residual	28	130.0228	4.6437	4.97	
Replicates.Subject.Time stratum					
d.f. correction factor 0.2956					
Time	6	934.3504	155.7251	166.53	<.001
Time.Temperature	30	171.6526	5.7218	6.12	<.001
Residual	180	168.3168	0.9351		
Total	251	1705.2703			

Table 4.4b Analysis of variance for survival of *A. pullulans* Y126 using temperatures and times as the factors and ln CFU/ml as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicates stratum	2	0.0946	0.0473	0.06	
Replicates.Subject stratum					
Temperature	5	86.5915	17.3183	22.70	<.001
Residual	28	21.3618	0.7629	2.87	
Replicates.Subject.Time stratum					
d.f. correction factor 0.3015					
Time	6	629.4639	104.9107	394.39	<.001
Time.Temperature	30	104.9808	3.4994	13.16	<.001
Residual	180	47.8811	0.2660		
Total	251	890.3737			

Appendix 5

Additional result tables for Chapter 6

A5.1 Comparison between sources of inoculum

Table A5.1.1 AMOVA for two levels of populations (mummified cherries and green cherries) at the Little Sharsted Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	0.526	-0.00705 Va	-0.94	0.623
Within populations	67	50.589	0.75507 Vb	100.94	
Total	68	51.116	0.74801		

Table A5.1.2 AMOVA for two levels of populations (mummified cherries and green cherries) at the the Lowere Hope Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	1.585	0.02582 Va	4.65	0.047
Within populations	80	42.354	0.52942 Vb	95.35	
Total	81	43.939	0.55524		

Table A5.1.3 AMOVA for two levels of populations (mummified cherries and green cherries) at the Man of Ross Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	3.300	0.12618 Va	21.49	< 0.001
Within populations	46	21.200	0.46087 Vb	78.51	
Total	47	24.500	0.58705		

Table A5.1.4 AMOVA for two levels of populations (mummified plums and green plums) at the Little Sharsted Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	1.296	0.03171 Va	4.91	0.087
Within populations	51	31.326	0.61424 Vb	95.09	
Total	52	32.623	0.64595		

Table A5.1.5 AMOVA for two levels of populations (mummified plums and green plums) at the Decoy Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	0.808	0.00486 Va	0.73	0.258
Within populations	58	38.442	0.66279 Vb	99.27	
Total	59	39.250	0.66765		

Table A5.1.6 AMOVA for two levels of populations (mummified cherries and green cherries) across different locations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among groups	1	2.790	0.00683 Va	1.06	0.303
Among populations within groups	4	8.158	0.04490 Vb	6.98	< 0.001
Within populations	193	114.143	0.59141 Vc	91.96	< 0.001
Total					

Table A5.1.7 AMOVA for two levels of populations (mummified plums and green plums) across different locations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among groups	1	0.593	-0.02674 Va	-4.04	1.000
Among populations within group	2	3.922	0.04900 Vb	7.40	< 0.001
Within populations	109	69.768	0.64007 Vc	96.64	0.003
Total	112	74.283	0.66233		

Table A5.1.8 AMOVA for two levels of populations (mummified fruits and green fruits) across different hosts and locations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among groups	1	2.308	-0.00627 Va	-0.92	0.487
Among populations within group	8	25.089	0.08222 Vb	12.00	< 0.001
Within populations	302	183.911	0.60898 Vc	88.91	< 0.001
Total	311	211.308	0.68492		

A5.2 Other factors that affect population structures of *M. laxa*

Table A5.2.1 AMOVA for four levels of populations (four locations) for mummified cherries.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	3	5.865	0.05258 Va	8.22	< 0.001
Within populations	103	60.471	0.58710 Vb	91.78	
Total	106	66.336	0.63968		

Table A5.2.2 AMOVA for three levels of populations (three locations) for green cherries.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	2	5.841	0.06031 Va	8.90	< 0.001
Within populations	113	69.797	0.61767 Vb	91.10	
Total	115	75.638	0.67799		

Table A5.2.3 AMOVA for two levels of populations (two locations) for mummified plums.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	0.560	-0.00558 Va	-0.84	0.517
Within populations	42	28.145	0.67011 Vb	100.84	
Total	43	28.705	0.66453		

Table A5.2.4 AMOVA for two levels of populations (two locations) for green plums.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	3.362	0.08028 Va	11.44	< 0.001
Within populations	67	41.623	0.62124 Vb	88.56	
Total	68	44.986	0.70152		

Table A5.2.5 AMOVA for two levels of populations (two hosts) of mummified fruits at the Little Sharsted Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	3.848	0.16351 Va	18.15	< 0.001
Within populations	39	28.762	0.73748 Vb	81.85	
Total	40	32.610	0.90099		

Table A5.2.6 AMOVA for two levels of populations (two hosts) of green fruits at the Little Sharsted Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	1	6.920	0.15484 Va	18.71	< 0.001
Within populations	79	53.154	0.67284 Vb	81.29	
Total	80	60.074	0.82768		

Table A5.2.7 AMOVA for two levels of populations (two hosts) across stages of infection at the Little Sharsted Farm.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
Among groups	1	1.095	-0.07463 Va	-9.60	0.656
Among populations within groups	2	10.768	0.15799 Vb	20.32	< 0.001
Within populations	118	81.916	0.69420 Vc	89.28	< 0.001
Total	121	93.779	0.77756		

Appendix 6

Additional result tables for Chapter 7

A6.1 Anti-sporulation on mummified plums conducted on 2010-2011.

Table A6.1 ANOVA for number of spores of *Monilinia* spp. using treatment as the factors and ln spores/ml as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Blocks stratum	1	118.380	118.380	5.30	
Blocks.Rows stratum	10	223.431	22.343	3627.68	
Blocks.Rows.Replicates stratum	36	0.222	0.006	0.00	
Blocks.Rows.Replicates.*Units* stratum					
Treatments	9	97.439	10.827	8.20	<.001
Residual	423	558.571	1.320		
Total	479	998.042			

A6.2 Anti-sporulation on mummified plums conducted on 2011-2013.

Table A6.2 ANOVA for number of spores of *Monilinia laxa*. using treatment as the factors and ln spores/ml as the variate.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Blocks stratum	2	5571.324	2785.662	39.92	
Blocks.Rows stratum	9	628.096	69.788	26.19	
Blocks.Rows.Replicate stratum	36	95.940	2.665	0.42	
Blocks.Rows.Replicate.*Units* stratum					
Treatments	4	650.928	162.732	25.80	<.001
Time of spray	2	219.660	109.830	17.41	<.001
Treatments.Time of spray	8	286.804	35.851	5.68	<.001
Residual	658	4150.136	6.307		
Total	719	11602.888			

Appendix 7

Presentations arising from work within this thesis

A7.1 Oral presentations

Rungjindamai, N., Xiang-Ming X. and Jeffries, P. 2012. Studies of biological control agents against the brown rot pathogen of stone fruits, *Monilinia laxa*. 8th International Conference on Integrated Fruit Production. 7-12 October 2012. Pinebay Holiday Resort, Kusadasi, Izmir, Turkey.

Rungindamai, N. Xiang-Ming, X. and Jeffries, P. 2011. Selection and characterisation of an antagonistic yeast for biocontrol of the brown rot pathogen, *Monilinia laxa*. 16th Congress of European Mycologists. 19-23 September 2011. Porto Carras Resort, Halkidiki, Thessaloniki, Greece.

A7.2 Poster presentation

Rungindamai, N. Xiang-Ming, X. and Jeffries, P. 2011. Biocontrol of brown rot in cherries. 12 October 2011. AAB Advance in Biological Control. The Olde Barn Hotel, Marston, Lincolnshire, the UK.

A7.3 Peer reviewed publication

Rungindamai, N. Xiang-Ming, X. and Jeffries, P. 2013. Identification and characterisation of new microbial strains for biocontrol of *Monilinia laxa*, the causal agent of brown rot on stone fruit. Agronomy. (submitted).