Hypobaric treatment: An innovative approach to control postharvest diseases of strawberries

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Abstract

Fungal decay is one of the major causes of postharvest losses in strawberries. Conventionally fungicides have been used to control fungal decay. However, fungicides have been questioned as a sustainable and safe method. Pre-storage application of short-term hypobaric treatment is one of the potential non-chemical methods for reducing fungal decay. In this study efficacy of postharvest hypobaric treatments to control natural rot development in strawberries was evaluated and analysed for the possible induction of resistance. Treatment of strawberries with hypobaric pressure (50 kPa_a for 4 h at 20 °C) consistently reduced rot development in subsequent storage at 20 or 5 °C. An *in vitro* study found that the rate of radial growth of colonies of *Botrytis cinerea* and *Rhizopus stolonifer* was unaffected by hypobaric treatment. Moreover, post-hypobaric treated strawberries inoculated immediately or 12 h after treatment with corresponding fungi led to significant ($P< 0.05$) rot reduction during storage at 20 °C. Bio-chemical analysis of strawberries suggested that hypobaric treatment upregulated the activities of defence-related enzymes with phenylalanine ammonia-lyase (PAL) and chitinase peaking 12 h after treatment, while peroxidase (POD) increased immediately after treatment. These results provide evidence that the mechanism of action of hypobaric treatment is induction of the defence system within the fruit rather than a direct effect on fungal viability. Furthermore, the 4 h delay in cooling required for hypobaric treatment did not cause greater rots compared to immediate cooling at-harvest. Therefore treatment is recommended to be applied at low temperature immediately after harvest. Despite these scientific results, a combination of hypobaric treatment and cold storage may not be sufficient for extending commercial storage life of strawberries. For more effective application, probably hypobaric treatment should be regarded as a hurdle technology to be used in conjunction with other physical treatments such as (UV-C, heat and ultrasound) and/or volatile chemical treatments.

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In the loving memory of my beloved mother

Table of contents

List of figures

List of abbreviations

1-MCP	1-methylcyclopropene
ANOVA	analysis of variance
CA	controlled atmosphere
° C	degree Celsius
d	day (s)
ETI	effector-triggered immunity
g	gram (s)
g	gravitational acceleration
h	hour (s)
JA	jasmonic acid
kPa_a	kilo Pascal absolute pressure
kg	kilogram (s)
L	litre (s)
MSCs	mechanosensitive ion channels
MeJA	methyl jasmonate
µL	microlitre (s)
mL	millilitre (s)
mm	millimetre (s)
mmol	millimole (s)
min	minutes
MA	modified atmosphere
MAP	modified atmosphere package
nmol	nanomole (s)
N	Newton
OD	optical density

PAMP	pathogen-associated molecular patterns
PR	pathogenesis-related
%	percent
POD	peroxidase
PAL	phenylalanine ammonia-lyase
PPO	polyphenol oxidase
PDA	potato dextrose agar
PCD	programmed cell death
PTI	PAMP-triggered immunity
RH	relative humidity
SA	salicylic acid
s	second (s)
SAR	systemic acquired resistance
UV	ultraviolet
WVP	water vapour pressure

Chapter 1 Introduction

Strawberry (*Fragaria x ananassa*) is a non-climacteric fruit usually harvested at full maturity (Cordenunsi et al., 2003). At room temperature the storage life of fresh strawberry is limited to 1-2 days (Zhang et al., 2006). Apart from high physiological activity, postharvest fungal decay is the main cause of reduction in storage life of strawberries (Nigro et al., 2000; Perdones et al., 2012). As a general rule, appearance of noticeable symptoms of decay indicates end of strawberry acceptability (Bower, 2007).

Grey mould caused by *Botrytis cinerea* is the principal fungal decay agent in strawberry and is ubiquitous in the environment (Ceponis et al., 1987; Tao et al., 2010). *B. cinerea* infects strawberry during flowering and becomes quiescent in the fruit (Babalar et al., 2007). When the conditions become favourable for disease development (i.e. during ripening and senescence) necrotrophic activity initiates (Prusky and Lichter, 2008). *Rhizopus stolonifer* is one of the main causal agents of strawberry leak (Siefkes-Boer et al., 2009). This disease is characterised by soft and watery rot with brown spots on the fruit, leading to juice leakage and tissue collapse (Maas, 1998), usually prevalent in cold-stored fruit (Siefkes-Boer et al., 2009).

Traditionally, application of fungicides has reduced fruit rot incidence efficiently for extended periods of time (Terry and Joyce, 2000). However, increased global concerns over the negative effects of residue and development of fungicide resistance (Mengiste et al., 2010) have led to the investigation of alternative options to mitigate

postharvest fungal decay of fresh produce. Several novel techniques are being investigated in contemporary research including use of biological control; natural resistance inducers (Romanazzi et al., 2013) and physical treatments (Mari et al., 2010). Among the physical methods, controlled atmosphere (Harker et al., 2000), heat (Civello et al., 1997; Dotto et al., 2011) and UV treatments (Nigro et al., 2000; Pombo et al., 2011) have been trialled for controlling postharvest decay of strawberries.

Pressure treatments (hypobaric or hyperbaric) have also been studied for increasing storability of fresh produce (Liplap et al., 2013a; Romanazzi et al., 2003, 2008; Romanazzi et al., 2001). In contrast to other physical treatments (heat and UV), a possible benefit of pressure treatment is the homogeneity of application during treatment (Vigneault et al., 2012). Previously Romanazzi et al. (2001) reported that hypobaric treatments (25.3, 50.6 and 76 kPa of absolute pressure, i.e. kPa_a) for 2-24 h reduced fungal decay in strawberries, sweet cherries, and table grapes. Recently Hashmi et al. (2013) observed that hypobaric treatment (50 kPa_a, 4 h) slightly reduced natural rots in blueberries stored at 20 °C for 7 d. However, the mechanism by which hypobaric treatment exerts this benefit remains unknown.

One possible mechanism of reduced decay development might be a direct effect of treatment on the fungus as reported by Liu et al. (2012) and Pan et al. (2004) in heat and UV treatments respectively. Apelbaum and Barkai-Golan (1977) reported that pressure below 6 kPa_a at 23 °C reduced the growth of *B. cinerea*. However, fungi continued growth after shifting to normal condition (Barkai-Golan, 2001). Previous studies suggested that hypobaric treatment may trigger a stress response in fruit

tissue causing increased natural disease resistance (Romanazzi et al., 2001). Reduction of fungal decay in UV-C treated strawberries (Pombo et al., 2011) and heat treated mangoes (Benitez et al., 2006) has been attributed to the mild stress which stimulates defence-related enzymes.

The objectives of this thesis are:

- To validate the effectiveness of hypobaric treatment in reducing strawberry decay.
- To test whether hypobaric treatment has a direct effect on fungal growth.
- To determine the role of hypobaric treatment in inducing fruit resistance against fungi
- To evaluate the usefulness of hypobaric treatment at low temperature and to study the significance of cooling delay on efficiency of hypobaric treatment.

Chapter 2 Literature review

2.1 Background

Horticultural commodities are an important part of the global economy and postharvest management of fresh produce has always been a challenge for growers. Most efforts in the past and current postharvest research have focused on devising different methods to extend storage life of fresh produce. Horticultural commodities deteriorate rapidly after harvest and sometimes are unable to reach consumers in good quality. Fungi and bacteria cause a significant portion of postharvest losses of fresh produce (Barkai-Golan, 2001). Controlling microbial spoilage can significantly reduce storage losses. Postharvest technology is the field of developing different practices to minimize losses by either complementing existing methods with new techniques or developing new cost-effective solutions (Valero and Serrano, 2010). In addition to traditional means such as low temperature storage, chemical treatments and heat treatments, there are a suite of new approaches that are currently at embryonic stage but have the potential to augment or replace existing techniques. These new technologies include UV, ozone, hyperbaric and hypobaric treatments. To understand the phenomena of hypobaric treatment in controlling fungal decay, existing methods and some of the new techniques for extending storage life of fresh produce are reviewed in this chapter. In addition, the role of plant defence systems in improving storage life of fresh produce is also discussed.

2.2 Strawberry

Strawberry is a herbaceous perennial plant belonging to family Rosaceae. The edible portion has multiple fruit consisting of red receptacle tissue and numerous achenes (seeds, or true fruit) (Mitcham, 2004). In 2007, global strawberries production was more than 4 million tonnes. The United States is the primary producer with more than 1 million tonnes of produce each year (Wu et al. 2012). The main strawberry producing states are California, Florida and Oregon. California produces about 80% of the total strawberry production in the United States. Strawberry production in New Zealand was estimated to be 6,500 tonnes in 2007 (Aitken and Hewett, 2012). 'Camarosa' is the most popular commercial cultivar grown around the world, while other cultivars grown in New Zealand are 'Diamante', 'Seacape', 'Selva', 'Pajaro' and 'Gaviota'. Commercial strawberry is produced in Auckland, Canterbury, Waikato, Horowhenua and Hawke's Bay region. Strawberry decay caused by *Botrytis cinerea* (grey mould), *Rhizopus stolonifer* (strawberry leak) and *Colletotrichum acutatum* (anthracnose) is the major cause of postharvest losses in New Zealand (Timudo-Torrevilla et al., 2005). Strawberry industry in New Zealand experiences about 20% losses per annum due to fungal diseases (Timudo-Torrevilla et al., 2005), which can be reduced by efficient temperature management.

2.2.1 Types of plants

Strawberry plants are classified into two types on the basis of flowering time, bud initiation and fruiting. Short-day type usually initiates buds during short days of autumn and spring (Hancock, 1999). Generally flower bud initiation takes place in temperatures below 16 °C or when days are shorter than 14 h (Hancock, 2000). Day-

neutral or everbearer types are not extensively used and grown only in colder regions with high elevations. They are susceptible to high temperatures, but produce crowns and buds around the season (Anon, 2013).

2.2.2 Anatomy and morphology

The strawberry plant has a central stem known as a crown from where stolons (runners), leaves and roots emerge (Figure 2.1; Anon, 2013). The leaves are trifoliate with spiral arrangement around crown (Strik, 2007) and produced around the season, however leaf growth is decreased below 0 °C and above 30 °C (Hancock, 2000). Proper leaf canopy is necessary for suitable flower bud development (Anon, 2013). Runners are the source of vegetative propagation of plant. In most species runners comprise two nodes and the daughter plant emerges from second node (Hancock, 1999).

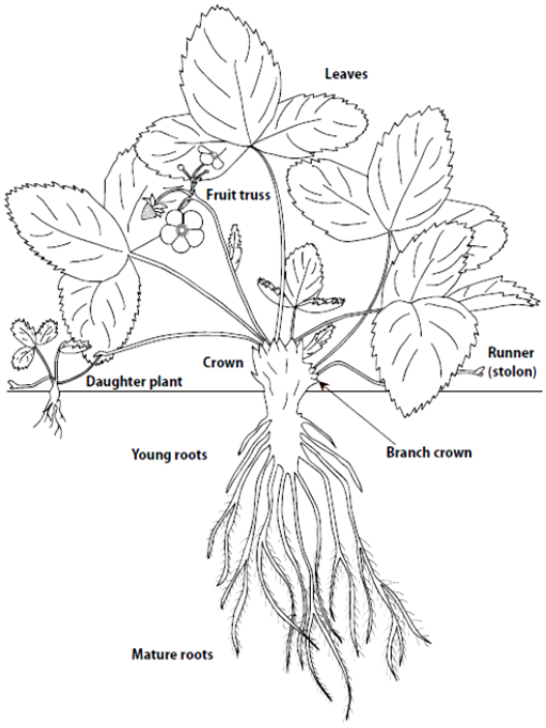

Figure 2.1 The strawberry plant (source: Anon, 2013).

2.2.3 Strawberry flowering and fruit

Flowers develop in the form of a cluster in which the terminal blossom is termed as primary or king flower. This flower emerges into the primary fruit followed by two secondary and four tertiary flowers (Hancock, 1999). The primary flower bears the largest fruit and is more vulnerable to frost (Anon, 2013). The strawberry flower has 10 sepals, 5 petals, 20-30 stamens and 60-600 pistils (Figure 2.2; Hancock, 1999). There is a cone shaped structure within the ring of stamens known as a receptacle. This receptacle is the extension of the pedicel or flower stem and this part develops into the berry (Anon, 2013). Strawberries are mostly insect pollinated, with bees (honey bee and bumble bee) as the most common pollinators (Darrow, 1966). Maturation of pollens occurs before anthers open, however pollen does not release before flower opening. Stigma remains responsive to pollen for 8-10 days, whereas pollens are viable for about 2-3 days (Hancock, 1999). Strawberry fruit is the combination of multiple ovaries developing into seeds known as achenes, which are the true fruit of strawberry (Darrow, 1966), embedded in the swollen receptacle. From pollination to ripening, fruit develop in about 20 to 50 days, depending on temperature and cultivar (Strik, 2007).

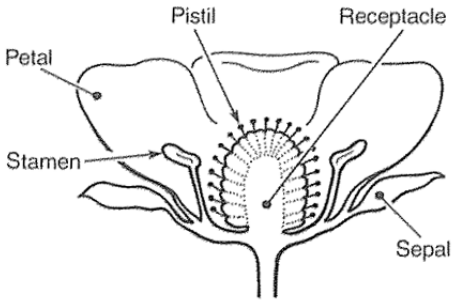

Figure 2.2 Typical strawberry flower (source: Hancock, 1999).

Fruit maturity is influenced by air temperature. Higher temperatures during fruiting negatively affect fruit size and quality. Fruit temperature can surpass air temperature by about 8 °C on sunny days at 26.5 °C which may cause surface breakdown, tissue damage and excessive softening (Hancock, 2000). Similarly cool temperatures also have a negative effect on fruit development by restricting pollen production (Hancock, 1999).

2.2.4 Cultural systems

There are two major types of strawberry production systems in the world, matted rows and annual hills (Hancock, 1999). The matted rows system exploits runners as the prime yield component. This system is used in climates with cold winters and short summers and combines spring planting, straw mulch, flat beds, overhead irrigation with 3-5 production seasons (Hancock, 2000).

For matted rows system, short-day plants are grown in spring and the flowers that appear in first year are usually discarded to increase runner production and enhance plant vigour. Plants are grown on raised or flat beds. The height of raised beds is generally 15-25 cm with two or four rows, plant spacing is approximately 30-35 cm and space between four-row beds is around 150-170 cm (Anon, 2013). The annual hills system employ crowns as the major yield component and is used in areas with hot or moderate summers and warm winters. This system is particularly favourable in regions with extreme climate during part of year. In hills system short-day plants are grown in early/late winter or summer and plant to plant distance is 20-30 cm. Although the majority of strawberries are grown in open fields, greenhouses and plastic tunnels are

very popular for off-season production. Combination of open fields and greenhouses can provide strawberries throughout the year (Hancock, 1999).

2.2.5 Irrigation

Irrigation is essential for commercial strawberry production. Proper irrigation improves optimal plant growth and ensures sufficient moisture supply to the plant, therefore sites with adequate amount of water should be selected for strawberry cultivation. For strawberries overhead sprinklers, furrows, drip or a combination of drip and sprinkler types of irrigation systems are used (Hancock, 2000). Commercially drip irrigation is the most popular system (Hancock, 1999), however in cold areas overhead irrigation is used for effective frost protection (Domoto et al., 2008). Rate of water application depends on cultural system and climate (Hancock, 1999).

2.2.1 Ripening and senescence

Strawberry fruit ripening can be divided into four stages such as green, white, pink and red (Huber, 1984). White stage starts about 21 days after anthesis, which become red in the next 10 to 20 days (Dennis, 1984). Strawberry softening occurs to a great extent between green and white stage and steadily increase with the progression of colour (Perkins-Veazie and Huber, 1987). Increase in the pectin content and degradation of parenchyma cells are correlated with softening of fruit. In addition, softening has also been associated with depolymerisation and solubilisation of cell wall contents (Vicente et al., 2005), which ultimately contributes to the fruit senescence – the phase where synthesis of biochemical processes leads to the degradation and eventually program cell death (PCD) of plant tissue (Wills et al., 2007). In addition, increase in protein

metabolism and membrane permeability of fruit tissue during senescence make way for fungal invasion. Therefore delay in solubilisation of pectin results in extension of senescence process of the fruit (Vicente et al., 2005).

Fruit can achieve fully red colour even if harvested at partially ripe stage, though volatiles, sugars and acids synthesis does not occur properly, resulting in poor texture (Van de Poel et al., 2014). Auxin is considered to be the most important hormone for strawberry ripening, which is produced in achenes (true fruit) and triggers fruit growth (Perkins-Veazie, 2010). Auxin inhibits ripening and senescence of the receptacle in strawberry. Fruit development result in decline of the auxin levels, which consequently allows the fruit to ripen (Trainotti et al., 2005).

The soluble solids of strawberry increases during ripening, green fruit has 5% soluble solids increasing to about 7.3% in dark red fruit. However soluble solids may vary in different cultivars and environmental conditions (Perkins-Veazie, 2010). The soluble solids of strawberries mostly consist of sugars (80-90%), whereas total sugars range from 4 to 10% in fully ripe strawberries (Kader, 1991). In strawberries, citric acid is the major acid which contributes to the fruit pH and is necessary for controlling enzyme activity. Citric acid content decreases as maturation and ripening progress becoming minimal at fully ripe stage (Perkins-Veazie, 2010). Similarly pH of the green fruit (3.5-4.6) initially decreases (3.1-3.3) during white phase due to production of organic acids, but increases (3.5-3.7) at the fully ripe stage (Spayd and Morris, 1981).

Strawberry fruit contain many phenolic compounds such as flavonols, anthocyanins, proanthocyanins polyphenols, peroxidases, cinnamic acid derivatives, catechin and simple phenols (Perkins-Veazie, 2010). Some of these compounds (such as proanthocyanins and catechin) are also termed as antifungal compounds (phytoanticipins), which tend to decrease with ripening (Terry et al., 2004). Proanthocyanins concentration was directly associated with increase in resistance against *Botrytis cinerea* (Hébert et al., 2000). However these antifungal compounds are more pronounced in green stage and minimal at fully ripe stage (Terry et al., 2004). As compared to green berry, the concentration of total soluble phenols and peroxidases decreases by 100% and 80% respectively in red fruit (Perkins-Veazie, 2010). Similarly reaction of polyphenol oxidase with catechin results in degradation of anthocyanins (Spayd and Morris, 1981).

The colour of strawberry fruit is due to anthocyanin pigments (Bakker et al., 1994). Generally flavonoids are responsible for the synthesis of anthocyanins through phenylpropanoid pathway (Harborne, 1973). Similarly phenylalanine ammonia lyase (PAL) also regulates anthocyanin formation (Perkins-Veazie, 2010). Development of anthocyanins in strawberry continues even after harvest, especially when the temperature is above 5 °C. The colour difference among strawberry cultivars is mainly due to variation in concentration of anthocyanins (Nunes, 2009). Colour degradation usually occurs due to reduction in anthocyanins causing development of red-brown pigments (Bakker et al., 1992). Storage at 5 °C causes a two to threefold loss in colour as compared to 0 °C (Nunes, 2009).

Strawberry fruit contains around 35-200 volatile compounds, developing a characteristic aroma (Perez et al., 1993). However, most of the aroma of ripe fruit is associated with seven different volatiles such as 2-hexenyl acetate, ethyl butanoate, ethyl hexanoate, ethyl heptanoate, ethyl propionate, methyl butanoate and methyl hexanoate (Perkins-Veazie, 2010). Moreover the unique aroma of strawberry is attained only in fully ripe berries and strawberries harvested at green or pink stage fail to develop the characteristic flavour (Van de Poel et al., 2014).

2.2.2 Harvesting and postharvest handling

Strawberries are mostly harvested manually and the maturity of fruit is determined by surface colour. The US minimum maturity standard for harvest is 75% red fruit (Mitcham, 2004). Fruit are usually picked manually and put directly into small crates or baskets for fresh market or packed in trays or clamshells for wholesale market. Due to the short storage life of strawberry, good postharvest temperature management practice is needed. A delay of 1 h in pre-cooling of strawberry results in diminishing storage life by 1 day (Mitchell et al., 1996). Therefore, in an internationally efficient system (such as the United States), strawberry field heat is removed by forced air cooling immediately (within 30-60 min) after harvest. After prompt pre-cooling, strawberries are maintained at refrigerated temperature (0 or 1 °C) during the whole distribution process (Pelletier et al. 2011). The average shelf life of strawberry stored at 0 °C and 90-95% RH is up to 7 days depending upon the fungal spore load (Mitcham, 2004). Increase in temperature from 0 to 10 °C may accelerate deterioration by 2-4 fold. Similarly keeping strawberries at 29.4 °C caused a quick decrease in marketable fruit (Mitchell et al., 1996). Thus to avoid strawberry decay and quality losses pre-

cooling should be carried out as soon as after harvest as possible (Nune et al., 1995a). Commercially, strawberries are shipped from field to distribution centre using refrigerated trailers within 24 h.

In New Zealand, strawberries are manually picked at 95% red colour (Anon, 2011). Generally strawberries are pre-cooled before grading. However there is need of improvement in organising pre-cooling process, because many growers still do not follow standard operation procedures regarding pre-cooling (Crawford, 2012).

Storage life of strawberry depends upon many factors, such as pre-harvest environmental condition, cultivar differences (Rahman et al., 2014), at-harvest maturity stage and postharvest storage temperature (Nunes et al., 2002). Recently Rahman et al. (2014) studied storage life of three different cultivars (‘Sweet Charlie’, ‘Festival’ and ‘Camarosa’) at three different maturity stages. ‘Camarosa’ strawberries harvested at 75% red colour demonstrated a longer storage life as compared to other cultivars. However strawberry harvested earlier does not develop the characteristic aroma (Van de Poel et al., 2014). On the other hand Phillips and Reid, (2008) found that ‘Gaviota’ strawberry is more tolerant to rain, anthracnose crown rot and powdery mildew, but the yield of ‘Gaviota’ strawberries is significantly lower than ‘Camarosa’ strawberries (Bussell et al., 2005).

Strawberries are susceptible to physical damage and fungal attack due to its soft nature and hence have a very short storage life (Romanazzi et al., 2013). Physical damage during handling in the field may cause losses in postharvest quality of fruit.

This damage may result in fruit leakage, and as a consequence making the damaged fruit even more vulnerable to fungal decay. Fungal decay is the major cause of postharvest losses in berries (Bower, 2007). *Botrytis cinerea* causes grey mould which is one of the main diseases of strawberries. Other important diseases of strawberries are watery soft rot (strawberry leak) and green mould; caused by *Rhizopus stolonifer* and *Penicillium* spp respectively (Maas, 1998).

2.3 Fungal decay

Fungal and bacterial decay cause an estimated postharvest loss of 50% in developing countries mainly due to improper handling and shortage of cold storage facilities (Droby and Lichter, 2007). The perishable nature of fresh produce and their high water content make them very susceptible to physiological deterioration and microbial decay (Eckert and Ogawa, 1988). The most common rots are caused by a few fungal species in fruit including *Botrytis*, *Penicillium*, *Alternaria*, *Rhizopus*, *Monilinia* and *Fusarium* (Narayanasamy, 2006). Bacteria such as *Erwinia* and *Pseudomonas* are the main sources of bacterial damage in fresh produce (Barkai-Golan, 2001). Fungal infection is more prevalent in fruit as their low pH prevents bacterial spoilage (Moss, 2002), while vegetables are prone to both fungal and bacterial infection (Moss, 2008). Pathogens that depend on living hosts are termed as biotrophic while those attacking dead cells are called necrotrophic pathogens (Agrios, 2005).

Postharvest fruit decay is caused by pre-harvest contamination or postharvest infection, which becomes noticeable during transportation or storage of fruit (Droby and Lichter, 2007). In most cases pathogens attack the host fruit or vegetable during

its ripening and senescence, as the resistance against pathogens starts to diminish after harvest (Barkai-Golan, 2001).

Appropriate temperature, relative humidity (RH), pH and availability of nutrients are the main factors affecting growth of microorganisms (Korsten and Wehner, 2003). The optimal temperature for fungal growth is 20-25 °C (Valero and Serrano, 2010). Variation in temperature may cause delay in germination and mycelial growth, which ultimately extends the appearance of decay symptoms (Barkai-Golan, 2001).

Fungal pathogens exploit different means to penetrate fruit, either by infiltrating through natural openings or through wounds caused during postharvest handling (Agrios, 2005). It is not known what stimulates spores to germinate, but it may be due to contact with host and availability of nutrients on the host surface. Agrios (2005) stated that there are some mechanisms which restrain spore germination until a specific action of stimulation. Spores germinate by producing a specialized mycelium outgrowth referred to as a germ tube. The germ tube develops a well-defined swollen tip called an appressorium. This appressorium penetrates into the host cell through an appressorial peg (Korsten and Wehner, 2003). After the appressorium make their way into the host tissue, pathogens can behave according to the conditions by invading the fruit immediately or remaining latent/quiescent until the fruit ripen (Prusky et al., 2010). Latent or quiescent infection is used alternatively in the literature for the dormant phase of fungal spores, which start growth upon a favourable environment. However Michailides et al. (2010) differentiated the latent and quiescent infections in the following manner:

> "*A true latent infection involves a parasitic relationship that eventually induces macroscopic symptoms (Verhoeff, 1974). Quiescent infection, however, is microscopically visible although mycelial development is arrested after infection and resumes only as the host plant reaches maturity and/or senescence (Sinclair and Cerkauskas, 1996)*" (Michailides et al., 2010, p. 70).

Botrytis cinerea has the ability to grow in a range of environmental conditions. As a result *B. cinerea* can infect a wide variety of hosts. The spores of *B. cinerea* can germinate in a drop of water on the flower of strawberry, developing into a quiescent mycelium. It enters into the active stage and starts decay development when fruit reaches maturity (Arya, 2010). This disease affects fruit both in pre-harvest and postharvest conditions; however it has a larger impact during shipment and cold storage (Droby and Lichter, 2007).

Rhizopus stolonifer are not capable of entering healthy fruit tisues due to unavailability of enzyme cutinases. Therefore they penetrate the host through injury or wounds caused by physiological or mechanical damage (Korsten and Wehner, 2003). *R. stolonifer* has the ability to utilize wounds caused by other organisms for its growth (Barkai-Golan, 2001). Pectinolytic enzymes secreted by the hyphae inside the infected tissue degrade middle lamella causing soft rot, initially develops into cottony white mycelia, which later on becomes darker with sporulation (Agrios, 2005).

2.4 How to manage postharvest fungal decay?

Controlling postharvest fungal decay remains a challenge for researchers. Different

approaches have been adapted to manage fungal decay, such as pre-harvest horticultural practices, chemical treatments, biological control, cold storage and physical treatments. There is no single solution to tackle fungal decay, however a combination of different techniques are used to control postharvest fungal rots of strawberry. Commercially good cultural practices, pre-harvest chemical treatments, postharvest cold storage and controlled or modified atmosphere are collectively used for controlling postharvest diseases of strawberries. Currently, the strawberry industry is relying on extensive usage of pre-harvest synthetic fungicides to control postharvest fungal decay, whereas the usage of CA is limited to export market only (Terry, 2012).

2.4.1 Pre-harvest horticultural practices

Selection of disease resistant cultivars and good cultural and handling practices are necessary steps to ensure the quality of fruit (Korsten, 2006). Poor use of fertilizers, availability of water and variation in weather conditions can directly affect disease resistance and postharvest quality of fruit (Grobler et al., 2002; Michailides et al., 2010). In the case of strawberry, eradication of infected fruit from the field is essential as Botrytis rot is a direct source of inoculum for infecting nearby flowers (Droby and Lichter, 2007).

2.4.2 Chemical treatments

Chemical treatments are the most effective means of managing pathogens and controlling pest infestation in crops (Sudheer and Indira, 2007). In 1885, the discovery of Bordeaux mixture is considered to be the first significant milestone in the history of chemical control (Ragsdale and Sisler, 1991). Waard et al. (1993) extensively reviewed

the role of chemical treatment in plant diseases; the authors divided chemical developments into four generations. Bordeaux mixture was termed to be the first generation of fungicides which also includes other inorganic chemicals. In 1934, the development of organic chemicals (dithiocarbamates, organotins, quinones and captan) was the second generation of fungicides lacking penetrability into the plant tissues (Byrde et al., 1980). Third generation fungicides were comprised of benzimidazoles, carboxamides, phenyl amides, foetal-AI, azoles, morpholines and 2-aminopyrimidines. These chemicals have the additional capability of penetrating plant tissue and circulating within it to control infection (Lyr and Braun, 1987), in other words they could be termed as systemic compounds. The fourth generation of fungicides consists of tricyclazole and probenazole – non-toxic to fungi in *in vitro* conditions, but still effective in reducing fungal diseases by inducing plant resistance resulting in delay in the processes of fungal penetration into the plant (Waard et al., 1993).

Botrytis infection initiates during flowering in strawberry, therefore this disease should be controlled in the field (Arya, 2010). Captan is considered to be the industrial standard for controlling Botrytis infection and its weekly application (2.3 to 3.4 kg a.i./ha) for up to 24 times per season is allowed (Blacharski et al., 2001). In addition thiram, cyprodinil, fenhexamid, pyrimethaniland and iprodione pyraclostrobin also effectively reduce incidence of grey mould in strawberry (Adaskaveg et al., 2010; Mercier et al., 2010).

Chemical treatment remains the major way to reduce grey mould incidence in many

crops (Leroux, 2007). However recent research has revealed that chemical fungicide residues can lead to the formation of carcinogenic and toxic compounds (Sharpe et al., 2009). In addition, extensive use of fungicides also developed resistance in pathogens against chemicals (Adaskaveg and Förster, 2010; Sánchez-Torres and Tuset, 2010). Consequently, the increased public concerns over the harmful effects of chemicals have driven industries and researchers to look for novel and safe technologies of extending storage life of the fresh produce (Adaskaveg and Förster, 2010).

Recently natural compounds such as chitosan, which has potential antifungal properties are getting attention (Terry and Joyce, 2004). Chitosan is extracted from the outer shell of crustaceans (shrimps, krills and crabs). Previous studies demonstrated the effectiveness of chitosan as a pre-harvest (Bhaskara Reddy et al., 2000) or postharvest (Liu et al., 2007) treatment to reduce fungal rots. The efficacy of chitosan has been credited to directly exhibiting fungicidal attributes (Feliziani et al., 2013), delaying fruit ripening (Ghaouth et al., 1991) and inducing defence-related enzymes in the fruit (Romanazzi et al., 2002).

2.4.3 Biological control

The application of antagonistic microorganisms (yeasts, fungi and bacteria) is one of the promising methods to control postharvest fruit decay (Korsten, 2006). Antagonistic microorganisms (also called biological control agents) are used to inhibit growth of plant pathogens without affecting the host plant. Sharma et al. (2009) thoroughly reviewed the role of microbial antagonists in controlling postharvest diseases of fresh produce. Several microbial antagonists including *Aureobasidium pullulans*, *Bacillus*

subtilis and *Cryptococcus laurentii* have been tested in different fruits such as apple (Ippolito et al., 2000), strawberry (Zhao et al., 2007) and stone fruit (Wang et al., 2004) against green mould, grey mould and brown rot respectively. Several possible mechanisms have been suggested to be involved in the process of rot reduction due to the activity of biological control agents which include competition for space and nutrients (Mateescu et al., 2006), direct fungicidal effects (Singh and Sharma, 2007) and inducing fruit resistance (Yu et al., 2012). However, commercial testing of biological control agents indicated variation in diseases control. Therefore for consistent and efficient performance, antagonistic microorganisms are used in conjunction with physical methods or antimicrobial elicitors (Droby et al., 2009).

2.4.4 Physical methods

Physical methods like low temperature storage, controlled/modified atmosphere (CA/MA), heat, ultraviolet (UV), hyperbaric and hypobaric treatment have been used for quality maintenance in fresh commodities (Goyette et al., 2007). Most of these treatments directly inhibit fungal growth; however physical methods can create mild stress that may activate natural defence systems in fruits and vegetables (Terry and Joyce, 2004).

2.4.4.1 *Low temperature storage*

Low temperature storage is the classical and most effective method for delaying senescence and extending storage life of fresh produce (Wills et al., 2007). Low temperature storage can reduce fruit metabolism which consequently delays fruit ripening and quality loss. Additionally, low temperature storage is the primary method

to suppress decay while other methods in combination with refrigeration improve the shelf life of fresh produce (Eckert and Sommer, 1967; Wills et al., 2007). Thus, low temperatures have the ability to delay disease development in fresh produce in two ways; either by delaying ripening and senescence processes of the host plant that extends the period to maintain resistance to disease and/or by controlling development of pathogens through an unfavourable temperature for its growth.

Cold storage is more effective in crops such as kiwifruit, grapes, strawberry, apple and carrot as these products are tolerant of low temperatures that delay fungal or bacterial growth. However, low temperature causes physiological disorder (chilling injury) in commodities like mature green tomatoes (Biswas et al., 2012), peaches (Crisosto et al., 2004), banana (Wang et al., 2012) and sweet potatoes (Lukatkin et al., 2012). Different strategies have been used to tackle chilling injury problems, such as temperature management (Woolf et al., 2004), heat treatment (Luengwilai et al., 2012), intermittent warming (Biswas et al., 2012) and CA or MA storage (Singh and Pal, 2008).

2.4.4.2 *Controlled/Modified atmosphere (CA/MA)*

Controlled atmosphere (CA) and modified atmosphere (MA) are the terms used for alteration in gas composition of the atmosphere (reduced O_2 with or without increased CO_2 concentrations) during storage, which influence storage life of fresh commodities (Wills et al., 2007). These techniques are usually used in combination with low temperature storage. CA is relatively costly as compared to MA, because a precise gas concentration is artificially maintained in a sealed chamber; while in MA

storage, atmospheric composition is allowed to alter by the product's own respiration. Plastic packages can be used to generate MA, in which case the term MAP is used (Sandhya, 2010). Manipulation of CO_2 and O_2 concentration in the atmosphere around fresh commodities retards metabolic activity, ethylene production rate and ripening processes. In addition, this also reduces fungal decay in many fresh products (Erkmen, 2012). Strawberry is tolerant of high CO_2 concentrations and MA has been used to reduce rots during transportation (Wills et al., 2007). The lower O_2 (2-5 kPa O_2) and higher CO_2 (10-15 kPa) level decreases grey mould in strawberry (Brandenburg and Zagory, 2010), therefore low density polyethylene (LDPE) modified atmosphere packages are commercially used in different countries for controlling grey mould (Droby and Lichter, 2007).

In addition, CA also reduces plant sensitivity to ethylene by manipulating O_2 and CO_2 concentration (CA). Increasing CO_2 concentration to 1-2 kPa (CA) can decrease the detrimental effect of ethylene and reduce the respiratory activity. Unlike CA, in hypobaric storage, the modified atmosphere conditions are achieved just by decreasing pressure, which reduces the partial pressure of O_2 without the introduction of other gases (Burg, 2004). Similarly, low oxygen atmosphere (2-5 kPa O_2) in CA storage, also inhibits fungal rots by increasing host resistance (Barkai-Golan, 2001). These conditions maintain fruit firmness and as a result prevent fungal penetration (Elhadi and Peter, 2009) by extending the quiescent stage of fungal spores (Barkai-Golan, 2001). However, O_2 levels from 21 to 5% has no effect on fungal development and spore germination. Therefore, for an effective fungal reduction, less than 1% O_2 is needed in many species. Similarly, hypobaric storage of 13, 7 and 3 kPa_a with oxygen

partial pressure of approximately 2.7, 1.38 and 0.69 kPa_a respectively inhibited the mycelial growth of *B. cinerea* for 5 d. However *in vitro* fungal growth resumed after the fungi were transferred to atmospheric pressure (Apelbaum and Barkai-Golan, 1977). These studies suggest that both CA and hypobaric condition (with very low level of O_2) can only temporarily reduce fungal development.

Nielsen and Leufvén (2008) reported that MAP retained sensory characteristics and reduced weight loss of strawberries during storage at 5 °C. However the authors suggested that different cultivars of strawberry might behave differently to MAP, therefore effectiveness of MAP could not be generalized for all cultivars of strawberries. Shamaila et al. (1992) observed MAP reduced sensory quality when compared with air packaged strawberries stored at 5 °C. Nevertheless unpackaged strawberries showed fungal growth after 6 d while MAP and air packaged fruit did not develop fungal decay even after 10 d. Recently Cunha Junior et al. (2012) demonstrated that 40% CO_2 and 20% O_2 delayed decay incidence and maintained firmness of strawberries. However higher CO_2 level (80%) caused anaerobic respiration, making strawberries unsuitable for consumption.

2.4.4.3 *Heat treatment*

Postharvest heat treatment is an effective method to control postharvest fungal disease and pest infestation in fresh produce (Schirra et al., 2000). Heat treatment can be used by different ways: hot water, vapour and hot air treatment (Lurie, 1998). Hot water treatment is the classical method for fungal control and vapour heat was

devised for insect management only, whereas hot air can be utilized for both insect and fungal control (Sivakumar and Fallik, 2013).

Postharvest hot water dip (53 °C for 3 min) controlled decay in muskmelon stored at 22 °C for 18 d (Yuan et al., 2013). As higher temperature leads to heat damage, therefore 50-60 °C for up to 10 minutes is the tolerance temperature for most fruit (Valero and Serrano, 2010). Mild pre-storage hot water treatment also protects certain phytochemicals in tomato, melon and mango (Fallik, 2004) and increases the storage life of fresh produce (Mulas and Schirra, 2007). In addition, short-term hot water treatment can reduce microbial load on fruit skin (Fallik, 2004).

The vapour heat method is commonly used for killing insect larvae and eggs by utilizing hot air saturated with water vapour at 40-50 °C (Lu et al., 2007). Heat is transferred through condensation on the surface of fruit (Vigneault et al., 2012). However Shellie and Mangan (2000) reported that fruit surface temperature is much higher than surrounding water vapour. Therefore high humidity during vapour heat treatment may damage fruit surface (Vigneault et al., 2012).

Advanced forced hot air treatment is the more recent method developed as a result of improvements in moisture and temperature monitoring (Tang et al., 2007). The main drawbacks of hot air application are the relatively long treatment time and the complicated equipment required. In addition, this method is also not suitable for all fresh produce, because some fruits are sensitive to higher temperature for longer time (Lurie, 1998).

Vicente et al. (2002) demonstrated that heat treated (45 °C, 3 h) strawberries showed reduced fungal decay only up to 24 h at 20 °C after subsequent storage at 0 °C for 7 d. However no beneficial effect of heat treatment was observed after 14 d of cold storage. Recent studies demonstrated that hot water rinsing and brushing (60 °C, 20 s) of 'Feng xiang' strawberries significantly reduced fungal decay without affecting external quality (Jing et al., 2010). Similarly Lara et al. (2006) observed inhibition of fungal infection in 'Pajaro' strawberries immersed in hot water (45 °C, 15 min), but caused external damage and reduced firmness. In contrast Garcia et al. (1995) demonstrated that hot water treatment (45 °C, 15 min) retained firmness of 'Tudla' strawberries. Alternatively a hot air treatment (45 °C, 60 min) partially reduced fungal infection and maintained firmness with no effect on external appearance.

Vicente et al. (2005a) observed that heat treatment reduced depolymerisation and solubilisation of cell wall contents (α- and β-galactosidase and polygalacturonase) accompanied by delayed ripening of strawberries. Heat treatment (38 °C for 96 h) inhibited spore germination and mycelial growth of *Penicillium expansum*. Pan et al. (2004) reported that a combination of UV-C (4.1 kJ m^{-2}) and heat (45 °C, 3 h) treatment was more effective than individual treatment in reducing spore germination of *B. cinerea* and *R. stolonifer*. However, all three treatments only delayed fungal growth and did not eliminate the fungal spore population. In other words the mode of action of these treatments is fungistatic (inhibit fungi) rather than fungicidal. However, recently Zhao et al. (2013) demonstrated that heat treatments (43 °C for 10, 20 or 30 min) caused oxidative damage to the spores of *B. cinerea* resulting in inhibition of spore germination.

Currently postharvest heat treatment is commercially used in many fruit (Fallik, 2004), however from the above studies it could be concluded that there is no standard heat treatment which could be useful for all strawberry cultivars and feasibility of heat treatment for a particular cultivar would need to be studied.

2.4.4.4 *UV treatment*

UV light is a useful tool for disinfecting surfaces and controlling postharvest diseases. It is considered as an alternative to chemical application in fresh produce and is a new approach that extends the storage life of fresh horticultural crops by controlling microbial growth (Ben-Yehoshua, 2003). UV irradiation can be classified according to wavelength into near-ultraviolet radiation (UV-A) ranging from 315 to 400 nm, mid-range UV (UV-B) from 280 to 315 nm and far-UV (UV-C) ranging from 100 to 280 nm. As most of the studies were conducted on UV-C, therefore usually UV usually means UV-C (Valero and Serrano, 2010). Low UV doses (254 nm) increase fruit resistance against pathogens, reduce decay in many fresh produce and improve quality attributes in horticultural crops (Ben-Yehoshua, 2003).

Pre-storage application of UV effectively controlled postharvest decay in several horticultural crops (Shama and Alderson, 2005). Shama (2007) stated that UV treatment at 260 nm is the most effective in controlling fungal decay. The modes of action UV-C treatment are both fungicidal and induction of fruit resistance against pathogens (Shama and Alderson, 2005). Gardner and Shama 2000 suggested that UC wavelengths have the ability to be absorbed by the DNA of organisms which may directly inactivate pathogens on the fruit surface. In addition, this treatment also

stimulates defence-related enzymes and stress response genes in strawberries (Nigro et al. 2000; Pombo et al. 2011). Most commodities require only one dose, while some require a range of doses. UV treatment also delays colour change in broccoli (Costa et al., 2006) and pepper (Vicente et al., 2005b) and delays softening in peach, strawberry, apple, pepper and tomato (Charles and Arul, 2007). Pan et al. (2004) found that combination of UV-C (4.1 kJ m^{-2}) and heat (45 °C, 3 h) treatments was more effective in reducing strawberry rots than individual treatments subsequently stored for 6 d at 20 °C.

High UV doses are harmful to living cells and can cause negative effects on produce quality, such as increased susceptibility of peach to brown rot and browning and drying in mango and strawberry (Goyette et al., 2012). Extended treatment to tomato resulted in skin discolouration and accelerated ripening (Shama and Alderson, 2005). UV treatments have an adverse impact on vitamins B, C and carotenoids, especially lycopene in pepper (Vicente et al., 2005b) and tomato (Jagadeesh et al., 2009). Due to these negative effects on the most important phytochemicals, UV treatment has not been recommended in tomato (Goyette et al., 2010). Additionally, because of the low penetrability and lack of homogeneity, UV treatments can only be beneficial for surface disinfection of fresh produce (Shama, 2005) and its effectiveness is dependent on the smoothness of surface, shape and skin type of the commodity (Charles and Arul, 2007). Similarly the defence system of the produce will only be induced in tissues that are directly exposed to UV light (Shama, 2007). Finally, before UV technology can be used commercially, different aspects of its hazards and risks to human health should be taken into consideration.

2.4.4.5 *Ozone treatment*

Ozone (O_3) is an unstable triatomic reactive form of oxygen that produces hydroxyl and other free radicals either by spontaneous decomposition or by contacting other oxidizing surfaces. The high oxidative potential of ozone makes it very effective against contaminants in air and water (Perez et al., 1999).

Sharpe et al. (2009) observed that ozone treatment of horticultural produce at 20 °C inhibited the germination of *B. cinerea* spores more effectively than at 5 °C, while viability of *B. cinerea* spores was reduced by about 99.5% with 450 ppb of ozone in air. However, ozone treatment was unable to kill all fungal spores (Ames et al., 2013). Similarly, Nadas et al. (2003) observed that an ozone enriched atmosphere (1500 ppb) retarded the *in vitro* growth of *B. cinerea* at 2 °C and also reduced the occurrence of fungal decay in strawberry. Similarly Metzger et al., (2007) demonstrated that ozone treatment reduced the growth of *Penicillium* in citrus, but its use was recommended in conjunction with traditional methods such as heat treatment and low temperature storage. Ozone application temporarily affected fruit aroma, which recovered after 48 h storage at ambient temperature.

Apart from direct toxicity against fungi, ozone treatments also induce plant resistance against pathogens. Sandermann et al. (1998) stated that ozone can activate plant defence reactions and application of 0.15 ppm ozone for 5 h stimulated production of pathogenesis-related (PR) protein in tobacco leaves. The effectiveness of ozone treatment is dependent on several factors such as temperature and humidity (Salvador et al., 2006). Since the low penetrability is a limiting factor of this treatment,

type of package can influence the efficacy (Palou et al., 2001). Salvador et al. (2006) reported a higher weight loss in ozone-treated persimmon as compared to the un-treated samples after 30 d storage at 15 °C.

The high reactive nature of ozone is the main drawback of this treatment. This gas cannot be stored and must be produced during its application. In addition there is also likelihood of oxidizing the equipment, which makes it expensive method due to frequent replacement of equipment for treatments. Similarly improper doses and contact time of ozone can cause loss of sensory quality of fruit (Karaca and Velioglu, 2007).

2.4.4.6 *Pressure treatment*

The application of pressure other than atmospheric pressure to fresh or processed foods can be termed as a pressure treatment (Ahmed and Ramaswamy, 2006). The aim of a pressure treatment technique is to ensure microbiologically safe food with extended storage life (Patterson, 2005). Unlike UV treatment or heat processing, pressure application is homogeneous throughout the product irrespective of the size, shape or composition (Vigneault et al., 2012). Pressure treatments in range of 0 to 1000 kilo Pascal absolute (kPa_a) (hypobaric or hyperbaric) are applied to horticultural produce, while high pressure above 10^5 kPa_a is applied to processed food (Figure 2.3). Higher pressure is not recommended for fresh produce because it may cause physical injury to the product (Ahmed and Ramaswamy, 2006), whereas hypobaric treatments are not effective for inactivation of enzymes and elimination of microorganisms required for processed food (Goyette et al., 2007).

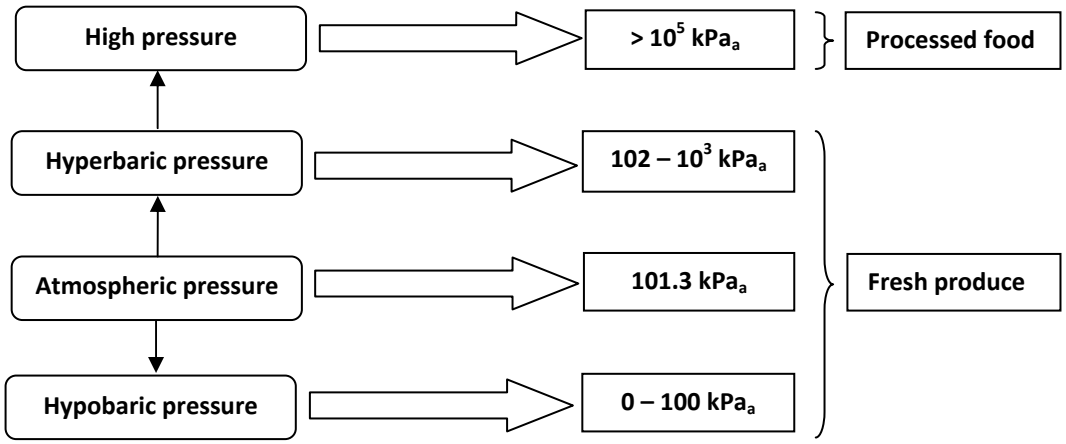

Figure 2.3 Ranges of pressure treatments and their respective applications.

2.4.4.6.1 Hyperbaric pressure treatment

Hyperbaric pressure means pressure greater than atmospheric pressure, which is ≈ 101 kPa_a. Hyperbaric treatments improve storage quality of fresh produce like lettuce (Liplap et al., 2014), mume fruit (Baba et al., 2008), tomato (Goyette et al., 2012; Liplap et al., 2013a), sweet cherries and grapes (Romanazzi et al., 2008). Liplap et al. (2014) reported that hyperbaric treatment (850 kPa_a, 5 d) significantly reduced decay in lettuce stored at 20 °C. Hyperbaric atmospheres with (>21 kPa_a O_2) may affect postharvest physiology of fresh produce, as indicated by changing ethylene and CO_2 production rates (Kader and Ben-Yehoshua, 2000). Baba et al. (2008) demonstrated that hyperbaric storage (500 kPa_a) of mume fruit for 10 d at 4 °C significantly reduced weight loss, chilling injury and production of ethylene and CO_2 with no adverse effect after transferring to atmospheric pressure at 25 °C. The same treatment in sweet basil leaves caused accelerated chilling injury causing browning after 3 d storage at 4 °C. Similarly Baba and Ikeda (2003) found that application of 500 kPa_a to mume fruit decreased ethylene and CO_2 production up to 48% and 65% respectively and reduced

chilling injury and weight loss during storage for 5 d at 4 °C. However, the mechanism of hyperbaric treatments by which reduction in chilling injury and ethylene production occurred was not identified. Goyette et al. (2012) reported that application of 709 and 912 kPa_a for 15 d reduced respiration rate and delayed ripening but resulted in irreparable physiological damage in tomato. However, treatments of 304 or 506 kPa_a for 5 d significantly reduced weight loss in tomato. Additionally treatments of 304, 506 or 709 kPa_a for 10 d, or 506 kPa_a for 5 d maintained firmness of tomato.

In another study Yang et al. (2009) compared the effect of hyperbaric, UV and CA treatments on peach volatiles. They concluded that application of 414 kPa_a for 4 weeks at 4 °C significantly reduced total volatiles and esters. Hyperbaric treatment had a greater undesirable effect on peach volatiles than CA storage (3 kPa O_2 and 7 kPa CO_2). Similarly in comparison to control, there was an adverse affect from the combined hyperbaric treatment and CA on peach volatiles.

Romanazzi et al. (2008) tested efficacy of short-term hyperbaric treatment on fruit storage life. They observed reduced fungal rot in hyperbaric treated (152 kPa_a for 4 and 24 h) sweet cherries and inoculated table grapes respectively, storing sweet cherries at 0 °C for 14 d, while table grapes were stored for 3 d at 20 °C. This treatment differs from these previously discussed in literature due to slightly higher pressure than atmosphere and the brief time of treatment. There is no available literature regarding effect of short-term hyperbaric treatment on fruit physiology and quality.

2.4.4.6.2 Hypobaric pressure treatment

Hypobaric pressure also known as sub-atmospheric pressure refers to pressure below atmospheric conditions (≈ 101 kPa_a). Burg and Burg (1966) introduced the concept of hypobaric or low pressure storage (LPS) as a new method of storing fresh produce. Subsequently the effects of LPS on the physiology and quality of fresh produce have been extensively studied (Apelbaum et al., 1977a; Dilley, 1977; Lougheed et al., 1978; Wu et al., 1972). Similar to vacuum cooling, there is a risk of high weight loss in low pressure storage; therefore controlling relative humidity in LPS is very important (Burg, 2010). The hypobaric system is similar to a vacuum cooling chamber having a vessel for treating fresh produce. During treatment the chamber is hermetically sealed and a vacuum is generated using a vacuum pump. Among the various options of vacuum pumps, an oil-sealed rotary pump is the one most commonly used (Sun and Zheng, 2006).

Hypobaric conditions speed up the diffusion of gases from fruit tissue. Apelbaum et al. (1977b) investigated hypobaric pressure storage effect on mango fruit. A pressure of 13.33 and 6.66 kPa_a at 13 °C delayed ripening of mango fruit for about 9 and 19 d respectively. Application of 13.33, 9.99, 6.66 kPa_a pressure significantly reduced weight loss in comparison to control (101 kPa_a), and pressure above 33.33 kPa_a did not affect the ripening process. During ethylene synthesis, transformation of 1-aminocycloprpane carboxylic acid (ACC) to ethylene involves an oxidative reaction. Therefore low O_2 partial pressure in hypobaric condition may reduce ethylene production, thus delaying the ripening process of fresh produce (Beaudry, 1999). Similarly, low O_2 partial pressure in LPS reduces aerobic respiration rate of fresh

produce (Li et al., 2006), however further study is needed to understand the effect of short-term hypobaric treatment on respiration rate of fresh produce.

Gao et al. (2006) reported that application of 40-50 kPa_a reduced fruit browning and flesh leatheriness in loquat stored at 2-4 °C for 49 d. In addition hypobaric pressure (35-40 kPa_a) delayed ripening and extended storage life of asparagus by twofold at 3 °C (Li et al., 2006). Recently, Laurin et al. (2006) studied low pressure effect in cargo aircraft simulated system. Cucumbers stored at 70.9 kPa_a for 6 h at 20 °C had increased weight loss compared to atmospheric storage. Stomata of treated cucumbers remained open even after 96 h after returning to normal pressure (101 kPa). Perhaps hypobaric pressure caused stress, delaying closure of stomata resulting in higher weight loss. The authors proposed spraying water before hypobaric treatment to reduce weight loss.

Romanazzi et al. (2001) introduced a new application for hypobaric pressure by studying the outcome of short-term low pressure treatments on the postharvest fungal rot of strawberries, sweet cherries and table grapes. Hypobaric pressure treatments of 25, 50 and 76 kPa_a for 2-24 h inhibited fungal decay by *R. stolonifer* and *B. cinerea* compared to control at 101 kPa_a. The best results for decay reduction were obtained in 25 and 50 kPa_a treatments for 4 h in sweet cherries. Nevertheless, the authors did not study the effect of hypobaric treatment on weight loss and firmness. In addition, there is also a need to investigate the effect of hypobaric treatment on respiration rate and ethylene production of fruit. Respiration rate is an important indicator of fruit physiology, which responds to different stresses (Barkai-Golan, 2001).

Tovar et al. (2011) reported an increase in respiration rate of hypobaric treated (34 kPa_a for 20 min) mangoes and attributed this to a stress response of the fruit (Plaxton and Podestá, 2006). However there are no other published data on short-term hypobaric treatment effects on respiration rate of fruit. Recently Hashmi et al. (2013) demonstrated that hypobaric treatment (50 kPa_a, 4 h) reduced fungal rots in blueberries stored at 20 °C for 7 d, with no effect on weight loss, firmness and respiration rate. This study indicated that short-term hypobaric treatment did not affect fruit physiology. However, there is a need to further explore the effect of short-term hypobaric treatment on fruit physiology.

One of the possible advantages of hypobaric treatment over other physical treatments (heat and UV) is the homogeneity of application during treatment irrespective of the size, shape or composition. Furthermore, unlike other physical treatments, the mechanisms through which hypobaric treatment affect fruit decay are not known. Therefore, the current study focused on the efficacy of hypobaric treatment in controlling strawberry decay and its possible mechanisms.

2.5 Gas Exchange

Movement of gases between plant cells and environment occurs according to Fick's first law of diffusion (Kader and Saltveit, 2003). A simplistic representation of Fick's law interpreted for gas diffusion in a plant cell and atmosphere is provided in equation 2.1.

$$J = \frac{A \,.\, \Delta C}{R} \qquad \text{(Eq . 2 .1)}$$

Where J = gas flux (m^3 s^{-1}); A = surface area (m^2); ΔC = concentration gradient of gas across the barrier and R = resistance to diffusion of gas (s m^{-1})

Fick's law means that gas movement in a plant system is directly proportion to the surface area and concentration difference of gas across the barrier and inversely proportion to barrier resistance (Ben-Yehoshua and Rodov, 2003). During respiration, CO_2 production increases in the plant tissue and results in outward diffusion of CO_2 into the intercellular spaces where the concentration is lower. Finally CO_2 moves out from the intercellular spaces through cuticle or other openings (Burton, 1982). Exchange of O_2 between plant tissues and external environment also occurs through the reverse process.

Pressure is important factor in gas diffusion into a liquid. According to Henry's law "the solubility of a gas is directly proportional to the partial pressure of that gas over the solution" (Myers, 2003). Mathematically Henry's law can be presented as:

$$c = kP \qquad \text{(Eq. 2.2)}$$

Where c = the concentration of gas (mol L^{-1}), P = partial pressure of the gas (Pa) and k = Henry's law constant (mol L^{-1} Pa^{-1}).

The classical example of Henry's law is illustrated in the carbonation of beverages. During the process of carbonation, CO_2 is mixed in the beverage under high pressure to increase solubility. After sealing the container, partial pressure of CO_2 in the space above the beverage is much higher than that of atmosphere. After opening the container, partial pressure of CO_2 instantly decreases; consequently the solubility of gas reduces inside the beverage and CO_2 bubbles out of the beverage.

2.5.1 Gas diffusion under hypobaric condition

During the process of reducing pressure in a vacuum chamber, the partial pressure of gases (O_2 and CO_2) in the chamber falls. According to Fick's law of diffusion, the gases inside the plant tissue will move outward (in the direction of lower concentration) to maintain the gases equilibrium between the plant tissue and surrounding environment. Consequently the partial pressure of gases in the plant cell will decrease. As per Henry's law, this will diminish the solubility of gases inside the cell fluid. Similar to the example to carbonated beverages, under hypobaric conditions the gases will escape from cell fluid to the surrounding environment. In other words hypobaric conditions cause the outward movement of gases from plant tissue (Burg, 2004).

2.5.2 Gaseous composition under hypobaric pressure

At atmospheric condition (101 kPa_a) the composition of air is approximately 78.9% N_2, 20.9% O_2 and 0.03% CO_2 with partial pressure of 78.9, 20.9 and 0.03 kPa_a respectively. Reducing pressure in hypobaric condition reduces the partial pressure of each gas but the mixing ratio of these gases remains constant. For example, decreasing atmospheric pressure from 101 kPa_a to 50 kPa_a in a vacuum chamber will reduce the partial pressure of major gases to 39.45 kPa_a N_2, 10.45 kPa_a O_2 and 0.015 kPa_a CO_2 approximately.

2.5.3 Conditions after releasing hypobaric pressure

Transpiration is caused by the change in water vapour pressure (WVP) between a plant organ and the surrounding environment (Wills et al., 2007). Moisture content of the air is expressed in terms of relative humidity (RH), which is "the ratio of actual

WVP in the air to the saturation WVP possible at a given temperature, expressed in percent" (Ben-Yehoshua and Rodov, 2003). The hypobaric condition returns to atmospheric pressure after releasing vacuum, consequently the process of gas exchange reverses. Gases and water vapour move from surrounding environment to the vacuum chamber and from chamber to plant tissues till the equilibrium is maintained. Hashmi et al. (2013) demonstrated that blueberries kept for short time (4 h) in a non-ventilated hypobaric chamber (at 50 kPa_a) did not suffer accelerated weight loss. After the pressure inside vacuum chamber returns to normal, WVP also rises and water vapour may infuse into the plant tissues. In case of vacuum infusion the vapour of ethanol (Ratanachinakorn et al., 1999) or 1-methylcyclopropene (1-MCP) (Chen et al., 2010) are infiltrated (under hypobaric pressure) to maintain the quality of fresh produce. In this case water vapour in the surrounding environment may infuse into the fruit tissue to maintain equilibrium relative humidity. However, ventilation of hypobaric chamber may cause higher water loss due to air movement (Burg and Kosson, 1983), therefore high RH is required to be maintained.

2.6 Mechanisms involved in decay reduction

Different methods to control postharvest decay involve different modes of action. Some methods directly affect pathogen or host plant, while others may act indirectly through stimulation of host resistance. These two modes of action may also work simultaneously.

2.6.1 Direct effect

2.6.1.1 *Effect on the pathogen*

Ripening and senescence processes are accelerated at higher temperatures, while the same environment is conducive to pathogen attack on fresh produce. Variation in temperature through a physical treatment can result in reduced spore germination. Tian and Bertolini (1995) reported that low temperature storage (-2 and -4 °C) significantly reduced the spore germination and mycelial growth of *Botrytis allii* in garlic bulbs.

Recent research demonstrated that hyperbaric treatments (200-850 kPa_a for 7 d) had a direct inhibitory effect on bacterial growth in fresh produce (Liplap et al., 2013b). However hyperbaric treatment response was dependent on bacterial species, with *Pseudomonas cichorii* the most pressure sensitive bacterium. Romanazzi et al. (2001) observed that direct exposure of *B. cinerea* to hypobaric pressure (25 kPa_a for 3 d) did not affect fungal growth. Nevertheless many authors have reported the inhibitory effect of LPS on *in vitro* growth of pathogens (Adams et al., 1976; Burg and Kosson, 1983; Lougheed et al., 1978). Adams et al. (1976) demonstrated that 26 kPa_a treatment was most effective in limiting the development of fungi (*P. expansum* and *P. patulum*). However 60.8 and 47.6 kPa_a treatment had no effect on mycelial growth, while the amount of sporulation was decreased with each reduced pressure treatment. Apelbaum and Barkai-Golan (1977) observed that mycelial growth of *B. cinerea*, *Alternaria alternata*, *Diplodia natalensis* and *Penicillium digitatum* were inhibited by 13.33, 6.66, and 3.33 kPa_a after 5 d. However *in vitro* fungal growth

resumed after the fungi were transferred to atmospheric pressure (101 kPa_a). The effect of short-term hypobaric treatment on fungal growth needs further study.

2.6.1.2 *Effect on the host*

Postharvest infections influence physiological and biochemical systems of fresh produce. In most cases fungal spores germinate in fruit after endogenous resistance declines during ripening and senescence (Vilanova et al., 2014). Therefore any delay in ripening and senescence processes inhibits fungal growth, consequently extending storage life of fruit. Respiration rate and ethylene production are the main parameters which indicate the metabolic activity of fresh produce (Valero and Serrano, 2010). Respiration rate and ethylene production depend on a wide range of factors, of which temperature is the most important (Kader, 2002). Hence low temperature storage is used to reduce respiration rate and ethylene production, ultimately delaying deterioration of fresh produce (Wills and Kim, 1995).

Vicente et al. (2002) demonstrated that heat treatment (45 °C, 3 h) delayed ripening and softening of strawberries subsequently stored at 0 °C for 7 d followed by 48 h at 20 °C. However heat treatment did not show any beneficial effect in strawberries subsequently stored for 14 d. Similarly Vicente et al. (2005a) observed that heat treatment reduced cell wall degrading enzymes (α- and β-galactosidase and polygalacturonase) accompanied by delayed ripening of strawberries.

Fruit can be classified into climacteric and non-climacteric on the basis of their physiological pattern (Symons et al., 2012). Climacteric fruit such as apple, avocado,

banana and tomato display a pronounced upregulation in respiratory activity and ethylene production during ripening process (Seymour et al., 1993). Increase in ethylene biosynthesis during ripening trigger an autocalatalytic production of ethylene (Klee and Clark, 2004). This results in a coordinated ripening process and regulation of key genes responsible for colour development, cell wall degradation, and fruit softening (Alexander and Grierson, 2002). Therefore to reduce fruit ripening process, the techniques to block ethylene production and reduce respiratory activities are commercially utilized in different fruit (Watkins, 2008). For this purpose 1-methylcyclopropene (1-MCP) is widely used in apple industry for improving the storage life (Watkins et al., 2000).

Unlike climacteric fruits (in which ethylene play a major role during ripening), ethylene has a limited role in ripening of non-climacteric fruit such as strawberry (Wills et al., 2007). However recent research found new molecular evidence regarding significance of ethylene in the ripening of strawberry (Merchante et al., 2013; Sun et al., 2013). Wills and Kim (1995) demonstrated that reducing ethylene in commercial punnets extended the storage life of strawberries. Similarly Modares et al. (2013) observed that infusion of ethylene inhibitor 1-MCP (1 µL L^{-1}) under low pressure (20 kPa_a) enhanced quality and physicochemical characteristics of strawberry during cold storage. Burg, (2004) reported that strawberries produces about 0.1 mL kg^{-1} h^{-1}ethylene, which increases the internal concentration to 30-360 nL L^{-1} in a commercial package (punnets) at 20 °C. Tthe storage life of strawberries can be improved by reducing the ethylene in packages to 50 nL L^{-1} or 5 nL L^{-1} at 20 °C and 5 °C respectively (Wills and Kim, 1995). Salicylic acid (SA) is a natural organic elicitor which has the

ability to reduce rots in fresh produce. Babalar et al. (2007) reported that salicylic acid application decreased ethylene production, inhibited fungal rots and reduced metabolic activity in strawberry. On the other hand Bower et al. (2003) found that removal of ethylene from strawberry is not a cost-effective solution for extending storage life. Current literature indicates that ethylene's role in non-climacteric fruit needs to be further studied.

2.6.2 Indirect effect or inducing host resistance

Plants are prone to a broad range of pathogens such as fungi, bacteria and viruses in the environment. For the sake of their survival, pathogens produce virulent factors in the plant cell to cause disease. In response, plants use their complex multi-layer defence system to cope with a variety of pathogens in an effective manner (Amil-Ruiz et al., 2011). In addition to pre-existing physical and chemical barriers for these microbes, plants develop a vast array of induced resistance mechanisms. Plants can recognize different pathogens by pattern recognition receptors (PRRs) through highly specialized pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). Similarly at cellular level plants evolve PAMP-triggered immunity (PTI) as an initial defence against pathogenic infections (Boller and Felix, 2009). For a successful infection, the pathogens inject virulent protein (effectors) into the plant cells. In response plant cells develop resistance protein (R) to detect effectors and its activity results in activation of effector-triggered immunity (ETI) making the pathogen harmless (avirulent) (Jones and Dangl, 2006). ETI is associated with localized hypersensitive response to the infection caused by programmed cell death (PCD) (Greenberg and Yao, 2004). The avirulent pathogen stimulates the signals of defence

hormones such as salicylic acid (SA) (Fu and Dong, 2013), leading to a systemic expression of pathogenesis-related (PR) genes in unaffected tissues (Durrant and Dong, 2004). This phenomenon of induction of resistance in whole plant as a result of localized infection is termed as systemic acquired resistance (SAR). Unlike ETI, SAR is associated with cell survival and not with PCD, however ETI may stimulate SAR through synthesis of SA (Gaffney et al., 1993). Similarly, studies suggest that phenylalanine ammonia-lyase (PAL) is involved in the synthesis of SA in plants (Chen et al., 2009). During SAR, resistance reactions take place in the non-infected parts of the plants (Metraux et al., 2002). Generally, this response includes synthesis of pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999), modifications of the cell wall and production of defence-related enzymes (Hammerschmidt, 1999). Based on indirect experiments, Amil-Ruiz et al. (2011) suggested that infection in strawberry may result in similar responses as other plants involving PRRs for the recognition of pathogen-associated molecular patterns (PAMPs). However the characterisation and pathways of these receptors in strawberry against *B. cinerea* are not well understood.

Fruit and vegetables always remain under certain types of stress. This stress is either created by pathogens (biotic) or caused by changes in environment (abiotic) (Terry and Joyce, 2004). Fruit produce defence-related enzymes as a result of biotic and abiotic stress (Ferreira et al., 2007). These include PAL, chitinase, peroxidase (POD), polyphenol oxidase (PPO) and β-1,3-glucanase. To understand the role of induced resistance in decay control of fresh produce, defence-related enzymes have previously been studied in different treatments (Jin et al., 2009; Pombo et al., 2011; Wang et al., 2010a; Zeng et al., 2006). Additionally, molecular studies of these enzymes have also

been used to investigate the mechanism of induced resistance (Dotto et al., 2011; Pombo et al., 2009). Romanazzi et al. (2008) suggested that short-term hyperbaric treatment (152 kPa_a, 4-24 h) may not be enough for inhibiting fungal growth, and hence the observed reduction of rot might be caused by the induced resistance of fruit following the mild stress produced by hyperbaric pressure treatment.

Burg and Kosson (1983) stated that reducing atmospheric pressure decreased cellular hydrostatic pressure, thus lowering cellular water potential. Plant cells have a pressure sensing system termed as mechanosensitive ion channels (MSCs) or stretch-activated ion channels (SACs) which are activated as a result of pressure stress (Sachs, 2010). These pressure sensing systems aim to return the cell back to the original hydrostatic pressure. As a result, cellular permeability and transport of potassium ions adjust to re-establish original turgor (Burg and Kosson, 1983). Disturbance in turgor pressure causes alteration in cell wall properties triggering restoration and reinforcement of cell wall through defence enzymes. This indicates that stress created by hypobaric treatment may stimulate defence-related enzymes in fruit, which may potentially induce fruit resistance against fungal attack.

Plant hormones such as ethylene, jasmonates and salicylic acid also play an important role in responding to biotic and abiotic stresses (Fujita et al., 2006). Ethylene and jasmonates are involved in plant defence response against necrotrophic pathogens, whereas salicylic acid is associated with defence against biotrophic pathogens (Bari and Jones, 2009). Nigro et al. 2000 demonstrated that higher doses of UV-C treatment resulted in transient increase in PAL activity (defence-related enzyme), which

coincided with the increase in ethylene levels of strawberry fruit. The authors suggested that increase in ethylene level might be the result of abiotic stress caused by UV-C treatment.

Preformed antifungal compounds (phytoanticipin) play an important role in natural disease resistance of strawberry against infections. Terry et al. (2004) observed that presence of preformed fungal inhibitors decreased with maturity in strawberry. This decline in antifungal compounds was associated with the natural disease resistance of strawberry against *B. cinerea*. Similarly, chemical or physical elicitors stimulate induction of resistance in fruit.

Jasmonates (jasmonic acid (JA) and methyl jasmonate (MeJA)) are important plant growth regulators (Saniewski et al., 2009). Application of MeJA vapour effectively reduces postharvest rots in peach (Jin et al., 2009), loquat (Cao et al., 2008), tomato (Zhu and Tian, 2012), chinese bayberries (Wang et al., 2010b) and strawberries (Ayala-Zavala et al., 2005). Jasmonates play a significant role in inducing defence-related enzymes in fruit (Jin et al., 2009). Similarly rot reduction in UV-C (Nigro et al., 2000), heat (Benitez et al., 2006) and ozone (Sandermann et al., 1998) treated fresh produce has been associated with an increase in defence-related enzymes. Similarly Zeng et al. (2006) reported that infusion of SA (1 mmol L^{-1}) under hypobaric pressure (20 kPa_a) reduced rot incidence in mango fruit and associated this reduction with the increase of defence-related enzymes (PAL, chitinase, POD, PPO and β-1,3-glucanase).

Pombo et al. (2011) observed that UV-C treated strawberries inoculated with *B. cinerea* showed significantly reduced rots during storage, which indicated the induced response of host (strawberry) to the corresponding infection. Furthermore, treated strawberries also resulted in higher level of defence-related enzymes and increased expression of stress response genes, suggesting that UV-C treatment induced strawberry resistance against *B. cinerea*.

2.6.2.1 *Phenylalanine ammonia-lyase (PAL)*

Two major groups of metabolites occur in plants i.e. primary metabolites and secondary metabolites. Primary metabolites contribute in cellular development, such as amino acids, proteins, carbohydrates and lipids. Whereas, secondary metabolites play important role in defence against biotic and abiotic stresses (Mazid et al., 2011). Secondary metabolites can be divided into terpenes, phenylpropanoids and their derivatives, alkaloids and isoprenoids (Korkina, 2007). Phenylpropanoids are the largest group of secondary metabolites produced through deamination of phenylalanine by PAL (Fraser and Chapple, 2011). Increase in PAL activity is an excellent indicator of plant resistance to different stresses (Vogt, 2010). Previous studies demonstrated the involvement of PAL in plant defence (Kruger et al., 2002) as application of the PAL inhibitors (2-amino-2-indanophonic acid (AIP) and α-aminooxy-β-phenylpropionic acid (AOPP)) resulted in reduced resistance of grape berries (Chen et al., 2006) and barley (Prats et al., 2007). Similarly increase in PAL due to UV-C (Nigro et al., 2000), heat (Yuan et al., 2013), methyl jasmonate (MeJA) (Cao et al., 2008), salicylic acid (SA) (Chen et al., 2006) and chitosan (Romanazzi et al., 2002) treatment have been associated with a reduction in rots.

2.6.2.2 *Chitinase*

Chitinase (E.C 3.2.2.14) enzymes are involved in hydrolytic breakdown of glycoside bonds of chitin (Kasprzewska, 2003), which is an important constituent of fungal cell walls and insect exoskeleton (Sharma et al., 2011). Chitinases are present in a wide range of plants, flowers, seeds, stems and tubers. Plant chitinases are triggered as a result of pathogenic attack or abiotic stresses (i.e. cold, high salt concentration and drought) (Hamid et al., 2013), whereas stimulation of chitinases in healthy plants during germination and ripening is associated with induced defence against potential pathogen attack (Grover, 2012). Therefore stress caused by different physical treatments such as heat, UV-C, hyperbaric and hypobaric treatments may stimulate chitinase synthesis. Chitinase, in combination with β-1,3-glucanase causes lysis (rupture) of chitin-containing fungal hyphal tips (Van Loon et al., 2006) resulting in the inhibition of fungal germination.

Chitinase activity has been studied in different fruits to investigate possibility of postharvest induced resistance (El Ghaouth et al., 2003; Ippolito et al., 2000; Wang et al., 2010a; Yu et al., 2012). Ippolito et al. (2000) demonstrated that inoculation of biological control agent (*Aureobasidium pullulans*) in apple resulted in threefold rise in chitinase activity after 96 h and a reduction in Botrytis rots by 89%. Similarly El Ghaouth et al. (2003) observed that UV-C (7.6 kJ m^{-2}, 10 min) upregulated chitinase activity by twofold in peach 96 h after treatment. The authors associated this increase with the induction of resistance due to UV-C treatment; however the increase in chitinase was not compared with a corresponding rot reduction in peach. Likewise Wang et al. (2010) reported that hot air treatment (48 °C, 3 h) increased chitinase

activity of bayberry fruit by 60% after 48 h of inoculation with *Leptographium abietinum* resulting in about 70% decay reduction.

2.6.2.3 *Peroxidase (POD)*

Plants contain two classes of peroxidases, intercellular peroxidases (termed as class I) and peroxidases secreted into cell walls (class III). Class II peroxidases are secreted by fungi (Welinder, 1992). Plant peroxidases class III (POD; EC 1.11.1.7) play an important role in many metabolic processes (Almagro et al., 2009). POD activity occurs throughout the life cycle of plants – from germination to senescence (Passardi et al., 2005). Previous studies have demonstrated that pathogenic infections and abiotic stresses induce POD activity triggering defence against pathogens, and cause lignification and/or cell wall cross-linking in the plant (Almagro et al., 2009).

Lignin is an important component of the plant cell wall and considered to be among the initial defence line against pathogen invasion (Bhuiyan et al., 2009). Lignification is essential for plant growth and is crucial for structural cohesion of the cell wall (Passardi et al., 2004). The process of lignification makes the plant cell wall more resistant in the event of fungal invasion or environmental stress (Almagro et al., 2009). Similarly peroxidases catalyse cross-linking of cell wall components during biotic or abiotic stress (Passardi et al., 2005) making the cell wall more challenging for pathogenic attack (Passardi et al., 2004).

As increase in POD activity due to abiotic stress indicates stimulation of plant defence system against pathogens (Djebali et al., 2007), therefore it is considered to be one of

the excellent indicators to predict induced resistance in fruit. Previous studies investigated POD activity to understand the possibility of induced resistance in fresh produce. Increase in POD activity due to application of microbial antagonists has been associated with reduction in peach decay (Yu et al., 2012). Similarly reduction in rots due to heat (Wang et al., 2010a) and UV-C treatment (Pombo et al., 2011) has been related to the upregulation of POD activity in chinese bayberry and strawberry respectively.

2.6.2.4 *Polyphenol oxidase (PPO)*

Polyphenol oxidase (PPO) is a copper containing enzyme and is present in most of the plants (Mayer, 2006). PPO is well known for its role in enzymatic browning in plant products. PPO oxidises phenolic compounds into quinone, causing brown pigments (Holderbaum et al., 2010). In addition, PPO is also known for its role in plant resistance to pathogens and stresses. However, most studies regarding involvement of PPO in inducing resistance are correlative rather than mechanistic. Li and Steffens (2002) observed that increased PPO levels resulted in higher resistance of tomato against *Pseudomonas syringae*. Similarly Thipyapong et al. (2004) reported that reduced PPO results in increased susceptibility of tomato to the same pathogen. Although these findings demonstrated the involvement of PPO in plant defence, the mode of action of this association was not studied. Yoruk and Marshall (2003) hypothesized that synthesis of quinone by PPO may be toxic to pathogens. Similarly quinones produced by wound stress may cause localized programmed cell death (PCD) (Li and Steffens, 2002), restricting pathogenic progression which may further lead to systemic acquired resistance (SAR).

PPO activity has been studied in assessing the induction of resistance due to different postharvest treatments. Hot water dipping (53 °C for 3 h) of muskmelon fruit upregulates PPO activity resulting in reduced rot incidence (Yuan et al., 2013). Likewise Wang et al. (2004) reported that increase in PPO activity of peach inoculated with biological control agent (*Cryptococcus laurentii*) reduced fungal decay. However several studies suggested that PPO activity may not always respond to the corresponding stress treatments (Liu et al., 2010; Shao et al., 2010; Zhao et al., 2009).

2.7 Research opportunity

This chapter has reviewed the importance of controlling fungal decay in increasing storage life of strawberry. In addition, different techniques used for controlling fungal decay have been examined. The available literature suggests that there is a need for a non-chemical treatment which could effectively improve storability without compromising the quality of strawberry. As discussed in this chapter, currently there are some innovative treatments (heat, UV, ozone and pressure treatments) used to increase storage life of fresh produce, however the treatments vary in their effectiveness and ease of application. Similarly the mechanisms of heat, UV and ozone treatments have been well studied. Nevertheless further study is needed to assess the effectiveness of short-term pressure treatments (hyperbaric and hypobaric) in reducing fungal decay and increasing storage life of fresh produce. Additionally the modes of action of short-term pressure treatments are not well understood.

This research evaluates the efficacy and mechanisms of short-term hypobaric treatment in controlling fungal decay of strawberry. Chapter 3 focuses on assessing

the effectiveness of different hypobaric treatments in controlling fungal decay and its effect on physiological and quality parameters of strawberry. In addition the impact of delay in hypobaric treatment and influence of duration and intensity of hypobaric treatments have also been studied.

Chapter 4 investigates the likelihood of a direct impact of hypobaric treatment on *in vitro* fungal growth. This chapter also analyses the possibility of fruit response to the hypobaric treatment and involvement of low O_2 partial pressure in strawberry rot reduction.

Chapter 5 describes the role of hypobaric treatment in stimulating defence-related enzymes in strawberry. This chapter further investigates the potential mechanism of pre-storage hypobaric treatment in reducing fungal decay.

Chapter 6 examines the efficacy of hypobaric treatment during subsequent storage at low temperature. Additionally this chapter investigates the impact of cooling delay (caused by hypobaric treatment) on the effectiveness of the hypobaric treatment. Finally chapter 7 presents the overall conclusions and recommendations of this research work.

Chapter 3 Efficacy of pre-storage hypobaric treatments of strawberry fruit to reduce fungal decay (*)

3.1 Introduction

Postharvest fungal decay is one of the main causes of reduced strawberry storage life. As discussed in chapter 2, hypobaric pressure treatments have been studied for increasing storability of fresh produce (Romanazzi et al., 2003; Romanazzi et al., 2001). Unlike other physical treatments (heat, gamma irradiation and UV), a potential advantage of pressure treatment is the homogeneity of application during treatment (Vigneault et al., 2012). In order to validate the feasibility of hypobaric treatment as a new physical treatment, its efficiency in reducing fungal decay needs to be confirmed. Additionally, there is no available literature regarding the effect of hypobaric treatment on other quality attributes of strawberries.

Previous research demonstrated that hypobaric treatments (25.3, 50.6 and 76 kPa_a) for 2-24 h reduced disease incidence in sweet cherries, strawberries and table grapes (Romanazzi et al., 2001). In order to enable hypobaric treatment adaptation into a commercially acceptable treatment the optimum pressure/time combination needs to be studied for each product.

Respiration rate is an important indicator of fruit physiology, which responds to different stresses (Barkai-Golan, 2001). Tovar et al. (2011) reported an increase in respiration rate of hypobaric treated (34 kPa_a for 20 min) mangoes and attributed this

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Hashmi, M.S., East, A.R., Palmer, J.S., & Heyes, J.A. (2013). Pre-storage hypobaric treatments delay fungal decay of strawberries. Postharvest Biology and Technology 77, 75-79.

to a stress response of the fruit (Plaxton and Podestá, 2006). However there are no other published data on short-term hypobaric treatment effects on respiration rate of fruit.

Another essential factor in strawberry postharvest storage life is the time between harvest and treatment/cold storage (Nunes et al., 2005). In a commercial scenario delay between harvest and cool storage occurs, leading to the reduction in storage life (Nunes et al., 1995a). For commercially applicable hypobaric treatment, an acceptable delay time prior to hypobaric treatment needs to be established.

The intensity and duration of any physical treatment influences its effectiveness (Fallik, 2004; Shama and Alderson, 2005). Cote et al. (2013) demonstrated that UV-C doses with higher radiation intensity (33 W m^{-2}) reduced strawberry rots more efficiently than lower radiation intensity (3 W m^{-2}). In similar manner intensity and length of hypobaric treatment are important parameters that must be considered in hypobaric treatment of fruit and vegetables.

The objectives of this study were to:

1. Confirm the efficacy of hypobaric treatments in inhibiting fungal decay of strawberries.
2. Assess the effect of hypobaric treatment on strawberry quality parameters (firmness and weight loss) in subsequent storage.
3. Investigate if a respiratory response of fruit to hypobaric treatment exists as an indicator of a potential stress response.

4. Evaluate the effect of delay in hypobaric treatment on quality of strawberry.
5. Determine whether the efficacy is influenced by length and intensity (including repeated pressure cycling) of hypobaric treatment.

In combination, these objectives aim to establish a reliable decay response to hypobaric treatment. As a result of these objectives a robust and effective treatment could be used in further experiments to explore the mechanism of hypobaric treatment.

3.2 Materials and methods

3.2.1 Fruit source

Six different batches of strawberries obtained on different occasions were used to assess the potential effect of hypobaric treatments in controlling fungal rots (Table 3.1). Two experiments were conducted using 'Gaviota' strawberries obtained from a commercial grower in Whanganui, New Zealand. Further experiments used 'Camarosa' strawberries from a commercial grower in Hawkes Bay, New Zealand. In all cases, fruit were harvested near to maturity stage (> 95% red colour), with calyx and pedicel, and sourced on the day of harvest. Strawberries were transported to Massey University, Palmerston North within approximately 2 h of harvest. On arrival at the lab, fruit with visible rot and blemishes were removed from the population.

Table 3.1 Details of all experiments

S.No.	Cultivar	Date	Number of replicates	Type of Treatment
1	Gaviota	Nov-2010	3	50 kPa_a for 4 h
2	Gaviota	Dec-2010	3	50 kPa_a for 4 h
3	Camarosa	Feb-2011	3	25, 50 and 75 kPa_a for 4 h each
4	Camarosa	Feb-2013	4	50 kPa_a for 4 h with delay of 0, 4, 8 and 12 h
5	Camarosa	Jan-2013	4	50 kPa_a for 2 and 6 h each
6	Camarosa	Nov-2012	4, 6	50 kPa_a for 4 h slow or fast release and single or multiple pull

3.2.1.1 *Effect of hypobaric treatment on fungal decay, physiology and quality of strawberries*

In two experiments the sorted fruit were randomized to fill 96 commercial vented clamshells (250 ± 10 g). These were divided into two treatments: 50 kPa_a for 4 h in a vacuum vessel, or atmospheric conditions (≈ 101.3 kPa_a refer to as 101 kPa_a hereafter) for the same time at 20 °C. In a third experiment a total of 84 units of commercial vented clamshells (250 ± 10 g) were filled (12-15 fruits per replicate), weighed and randomized into four treatments: 4 h treatment at 25, 50, 75 or 101 kPa_a (control) and subsequently stored at 20 °C. These experiments were performed to confirm the feasibility of hypobaric treatment in rot reduction and to select the most effective treatment for further experiments.

3.2.1.2 *Effect of delay prior to hypobaric treatment*

In a fourth experiment 80 clamshells (250 ± 10 g) were filled with sound strawberries and divided into 5 treatments: 4 h treatment at 50 kPa_a with a time delay of 0, 4, 8 and 12 h at 20 °C or 101 kPa_a (control). The aim of this experiment was to evaluate the influence of delay time on hypobaric treatment efficacy. In addition, as only one pressure chamber was available for use in this work, other experiments evaluating different pressure treatments required different delay times. Hence this investigation was necessary, as treatment differences in those experiments may be attributed to either the differences in the treatment or the delay time.

3.2.1.3 *Effect of length and intensity of hypobaric treatments*

In a fifth experiment 72 clamshells of strawberries were divided into 3 treatments: treatment at 50 kPa_a for 2 or 6 h and a control at 101 kPa_a. In this experiment, the pressure chamber was first loaded with strawberries for 6 h treatment and then with strawberries for 2 h treatment. The experiment was repeated with changing the order of treatments. The purpose of this experiment was to assess the suitability of different pressure treatment application time.

In a sixth experiment 60 clamshells of strawberries were divided into 3 treatments: 4 h treatment at 50 kPa_a with slow pressure release (2 min), fast pressure release (30 sec), and a control at 101 kPa_a. Pressure was released after 4 h slowly within 2 min or fast within 30 seconds. In another experiment 72 clamshells of strawberries were divided into 3 treatments including: 4 h treatment at 50 kPa_a with ‘single pull’ or ‘multiple pull’ (24 times in 4 h) and a control at 101 kPa_a. Hypobaric pressure (50 kPa_a) created only

once and maintained for 4 h was termed as 'single pull', while 50 kPa_a pressure repeatedly released after each 8 min and re-built in next 2 min was termed as 'multiple pull'. The process in multiple pull was repeated 24 times for 4 h (10 X 24 = 240 min). These experiments were conducted to establish the role of pressure intensity (rather than pressure application) on the observed response. Details of all experiments are presented in Table 3.1.

3.2.2 Hypobaric treatment

Hypobaric pressures were generated with a vacuum pump attached to a hermetically sealed tank (Figure 3.1). Clamshells of strawberries were placed inside the vessel at 20 °C. Vacuum generation during treatments was measured with a recently calibrated pressure gauge (EN 837-1, Nuova Fima, Invorio, Italy) (0-100 ± 1.6 kPa_a). Clamshells placed inside the same room at atmospheric pressure (101 kPa_a) were used as control. A single pressure chamber was used for hypobaric treatments. Consequently, since clamshells were used as the experimental units, strictly speaking these were pseudo-replicates, as they were all treated in the same chamber at the same time. However on occasions the experiments were repeated to ensure that results could be consistently reproduced.

Pressure was monitored with a digital electronic manometer (610-03, LI-COR Inc., Lincoln, USA). A voltage output from the electronic manometer was recorded at 5 s intervals with a data logger (Squirrel 1200, Grant Instruments, Cambridge, UK) and later converted to pressure through a linear calibration curve. Temperature and

relative humidity (RH) inside the pressure vessel were monitored with a data logger (TGU-1500, Tinytag Ultra Gemini, West Sussex, UK).

After pressure treatment, samples were stored in their clamshells at 20 °C and 80–90% RH until substantial rot incidence (> 40%) had occurred. Observations were recorded daily at 20 °C. At each sampling day independent clamshells (3 to 6 per treatment) were assessed destructively.

Figure 3.1 Hypobaric pressure chamber.

3.2.3 Fruit assessment

3.2.3.1 *Rot incidence*

Rot incidence was evaluated by visually examining each berry. Fruit with visible decay were counted with the results recorded as percent rot incidence per replicate.

3.2.3.2 *Respiration rate*

Respiration rate was determined from the rate of CO_2 production. From each replicate clamshell three sound fruit of a known weight were placed into a 540 mL airtight glass jar fitted with a rubber septum. Headspace samples of 1 mL were collected at 0 and 15 minutes at 20 °C and injected into a CO_2 analyzer equipped with a miniature infrared CO_2 transducer (Analytical Development Company, Hoddesdon, UK) at 20 °C. Nitrogen was used as the carrier gas at a flow rate of 35 mL min^{-1}. The carbon dioxide sensor was calibrated by injecting 1 mL β-standard 0.49% ± 0.01% CO_2 (BOC, Auckland, New Zealand). Output signals were recorded with an integrator (HP 3396A, Hewlett Packard, California, USA).

3.2.3.3 *Firmness*

Firmness of strawberries was determined using a TA-XT2i texture analyzer (Stable Micro Systems Ltd. Surrey, UK) using a 3 mm cylindrical flat end probe and a 50 N load cell. Each fruit was measured twice at opposite points on the equatorial zone. The probe was driven to a depth of 5 mm at a speed of 100 mm min^{-1}. The maximum force measured during penetration was recorded in Newton (N). Firmness of 5 sound fruit was measured per replicate at each storage interval.

3.2.3.4 *Weight loss*

Initial net weight of each clamshell was recorded to 0.001 g accuracy using a balance (Mettler Toledo PG 503-S, Columbus, USA) at 20 °C. Clamshells were reweighed at each storage interval and percentage weight loss from the initial was calculated.

3.2.4 Data Analysis

A Completely Randomized Design was used in all experiments. All the data were analysed for homogeneity of variance and then subjected to overall analysis of variance (ANOVA) with Minitab Version 16 (Minitab Inc, State College, PA, USA). In the case of respiration, one-way ANOVA was also applied at each point. Non-homogeneous data of percent weight loss were transformed by using square root transformation. Means were compared by a Tukey's test at a significance level of 0.05.

3.3 Results

3.3.1 Effect of hypobaric treatment on fungal decay, physiology and quality of strawberries

Hypobaric treatment of 50 kPa_a consistently reduced rot incidence ($P< 0.05$) in strawberries stored at 20 °C, becoming statistically significant when decay incidence became high (≈ 40%, Figure 3.2A-C). In 'Gaviota' strawberries maximum rots (>60 %) were observed after 7 and 5 d (Figure 3.2A and B), decay reduction became statistically significant ($P< 0.05$) after 7 d and 5 d with a reduction of 34% and 57% respectively in two separate experiments.; while 'Camarosa' strawberries showed maximum rots (>80%) after 4 d. A 50 kPa_a treatment was most effective after 4 d, reducing ($P< 0.05$) rot incidence by 52% followed by 75 kPa_a treatment with 43% rot reduction (Figure 3.2C). Hypobaric treatment of 25 kPa_a also appear to reduce rot incidence by 21% after 4 d when compared with control, but was not statistically ($P>0.05$) different from control (Figure 3.2C).

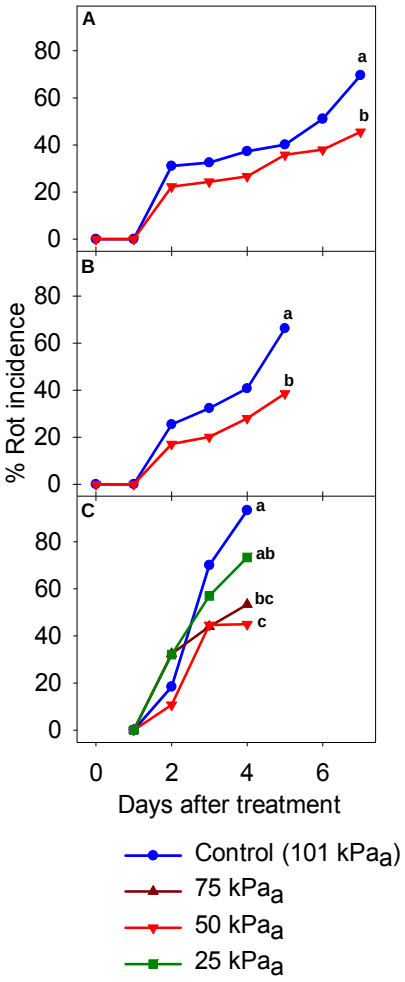

Figure 3.2 Effect of hypobaric treatment on the percentage of rot incidence of 'Gaviota' strawberry (A, B) and 'Camarosa' strawberry (C) stored at 20 °C. Each data point represents the mean value of three replicate clamshells. Values with different letters show overall significant difference (*P*< 0.05) from each other as determined by Tukey's test.

Respiration rate differed considerably between batches of strawberries ranging from 430 to 1020 nmol kg^{-1} s^{-1} (Figure 3.3). Immediately after treatment the 50 kPa_a treated samples consistently showed higher (*P*< 0.05) respiration rate than control strawberries (Figure 3.3A and B), while in 'Camarosa' strawberries 50 kPa_a treatment resulted in an initial increase in respiration rate as compared to 25 kPa_a and 75 kPa_a

treated samples at 20 °C (Figure 3.3C). Prior hypobaric treatment had no effect on respiration rate of strawberries during subsequent storage at 20 °C.

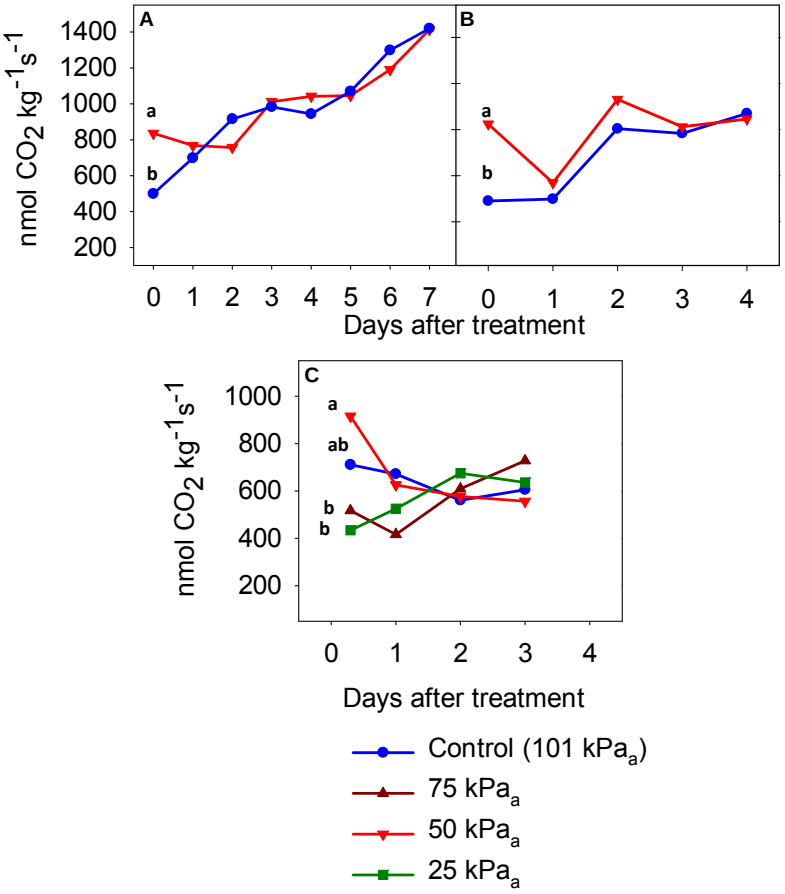

Figure 3.3 Effect of hypobaric treatment on respiration rate of 'Gaviota' strawberries (A, B) and 'Camarosa' strawberries (C) subsequently stored at 20 °C. Each data point represents the mean value of three independent measurements of 3 fruit. Different letters on initial points show significant difference (*P*< 0.05) from each other as determined by Tukey's test.

Firmness of all strawberries decreased (*P*< 0.05) with storage time (Figure 3.4A). Hypobaric treatments did not affect firmness loss of strawberries during 3 d of storage (Figure 3.4A).

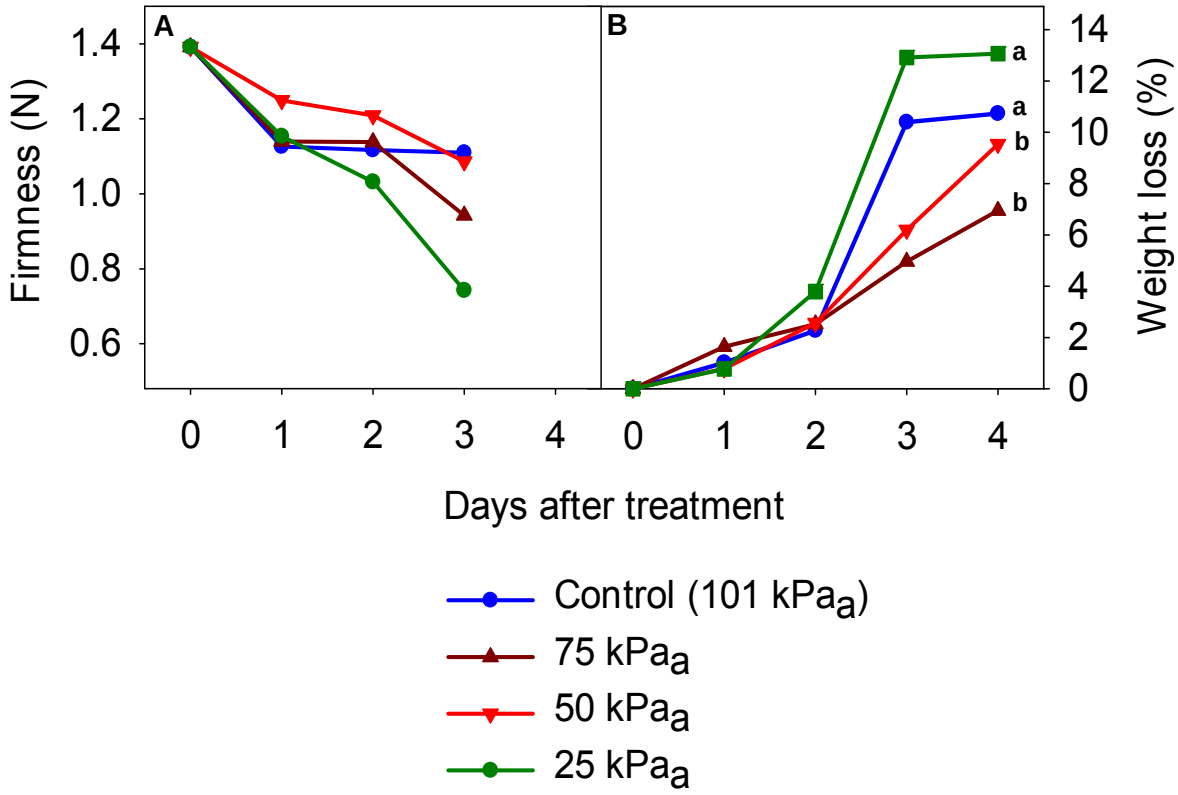

Figure 3.4 Effect of hypobaric treatments on firmness (A) and percent weight loss (B) in 'Camarosa' strawberries subsequently stored at 20 °C. Each data point represents the mean value of 15 fruit for firmness and three independent clamshells for the percentage of weight loss. Values with different letters show overall significant difference (*P*< 0.05) from each other as determined by Tukey's test.

Weight loss increased in all the samples during storage. There was no short-term effect of brief hypobaric treatment on weight loss (Figure 3.4B). Differences between treatments were observed after several days but these results are likely to be an indirect measurement of rot incidence as samples with the highest weight loss (Figure 3.4B) were the samples with the most damage from rots (Figure 3.2C).

3.3.2 Effect of delay in hypobaric treatment on rot development and quality of strawberry

Hypobaric treatments after a delay of up to 8 h effectively reduced rot development, while treatment after 12 h delay was comparable with control (Figure 3.5, Figure 3.6). Previously, the experiment assessing different pressure treatments (Figure 3.2-4) was carried out using a single pressure chamber and there was a delay of 8 h between the treatments (75 kPa_a treatment for 4 h was subsequently followed by 50 kPa_a and 25 kPa_a). Therefore it was essential to assess the effect of delay time in that experiment. The results of this experiment indicate that 8 h delay may not have influenced the efficacy of hypobaric treatment.

Firmness was not affected by a 12 h delay before treatment, despite a decrease in firmness being observed during storage for 3 d at 20 °C (Figure 3.7A). Similarly, a delay prior to hypobaric treatment for 12 h did not affect weight loss of strawberries, with weight loss increasing ($P< 0.05$) in all treatments after storage at 20 °C for 3 d (Figure 3.7B).

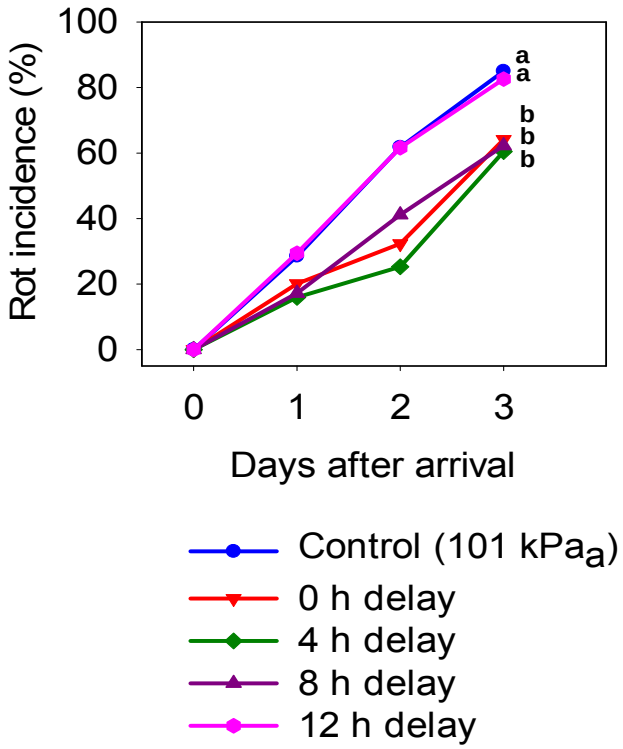

Figure 3.5 Effect of delay before onset of hypobaric treatment (50 kPa$_a$, 4 h) on rot development in strawberries subsequently stored at 20 °C. Each data point represents the mean value of four replicate clamshells. Values with different letters show overall significant difference (*P*< 0.05) from each other as determined by Tukey's test. The data for day 0 collected on arrival in lab about 2 h after harvest.

Figure 3.6 Effect of delay time prior to 4 h, 50 kPa_a treatment on rot development in strawberries. Control (A), immediate treatment (B), 4 h (C), 8 h (D) and 12 h (E) treatment delay after subsequent storage for 3 d at 20 °C.

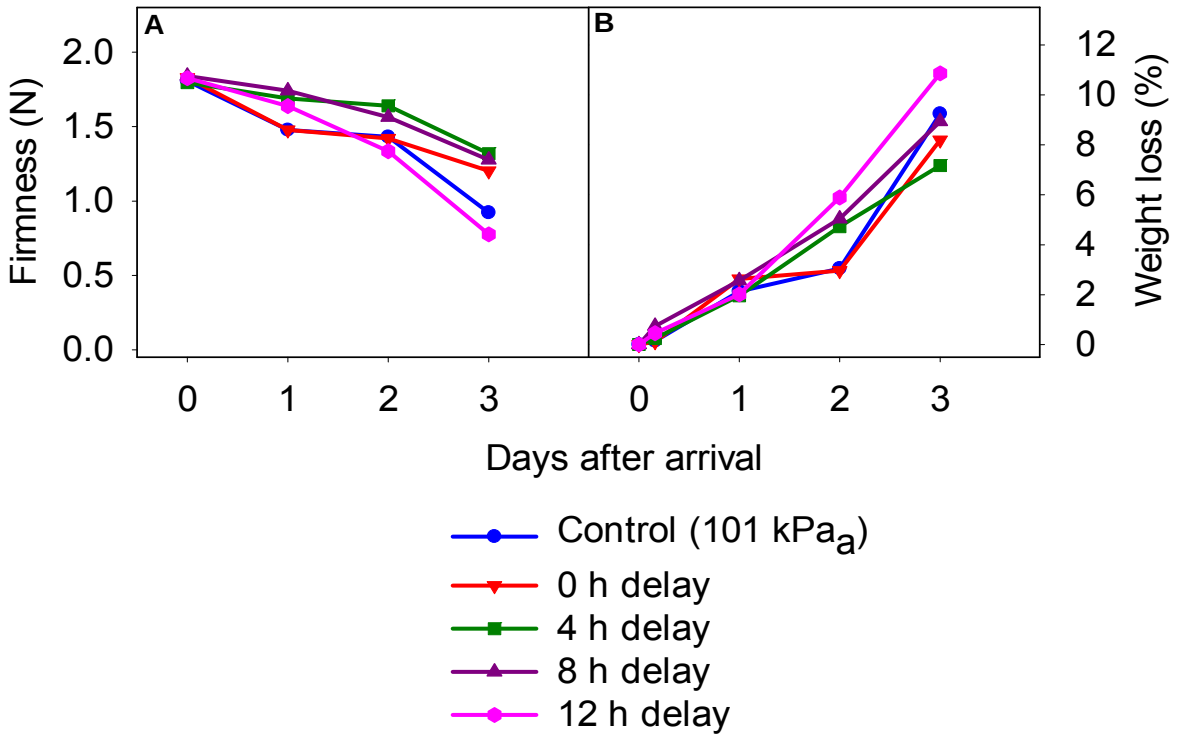

Figure 3.7 Effect of after-harvest delay prior to hypobaric treatment (50 kPa$_a$, 4 h) on the firmness (A) and weight loss (B) of strawberries subsequently stored at 20 °C. Each data point represents the mean value of 20 fruit for firmness and four independent clamshells for the percentage of weight loss. There was no significant difference (*P*> 0.05) between treatments. The data for day 0 collected on arrival in lab about 2 h after harvest.

3.3.3 Effect of length and intensity of hypobaric treatments on rot development in strawberry

Hypobaric treatment (50 kPa$_a$) for 2 h resulted in rot reduction only after day 4, but 6 h treatment at 50 kPa$_a$ was not effective in rot reduction (Figure 3.8A). A trial comparing fast and slow pressure release demonstrated a similar decrease in fungal rots (Figure 3.8B). Hypobaric treatments with multiple pull were comparable with control (Figure 3.9A), while a single 50 kPa$_a$ treatment demonstrated a reduction in rots (Figure 3.10)

agreeing with previous results (Figure 3.2). Multiple pressure release caused an increase in weight loss ($P< 0.05$) as compared to control and pressure treatment (50 kPa_a) with single pull (Figure 3.9B).

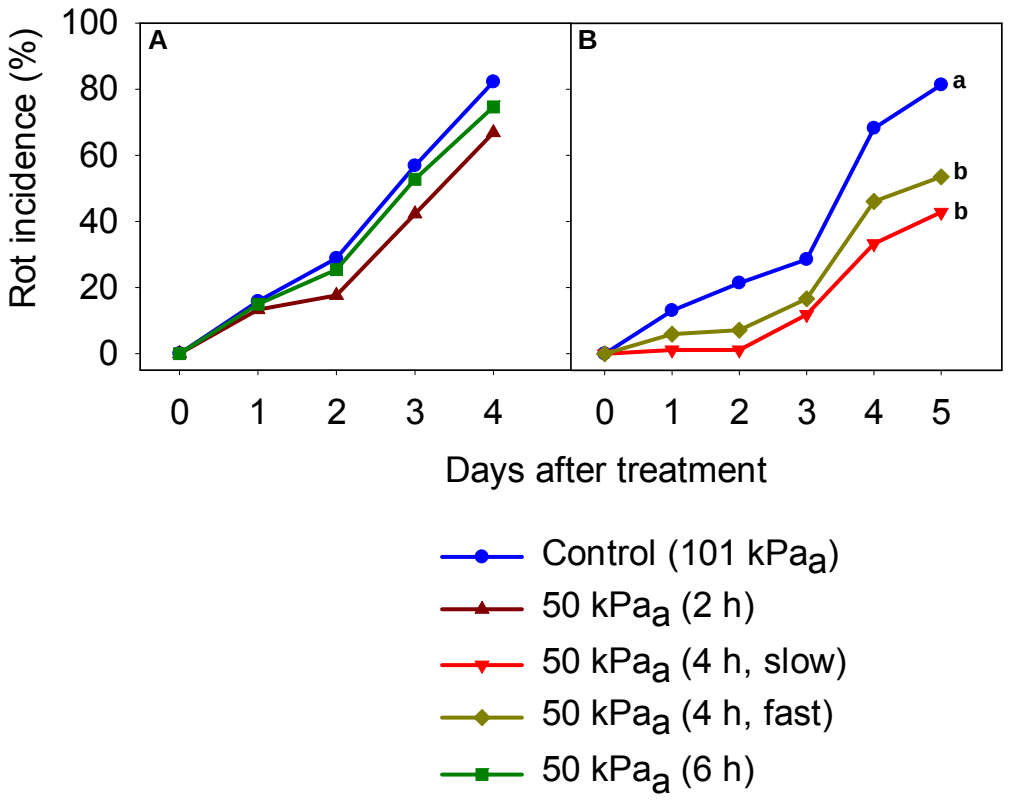

Figure 3.8 Effect of length of hypobaric treatment (A) and rate of pressure release (B) on rot development in strawberries subsequently stored at 20 °C. Each data point represents the mean value of four replicate clamshells. Values with different letters show overall significant difference ($P< 0.05$) from each other as determined by Tukey's test. The data for day 0 was collected about 2 h after harvest.

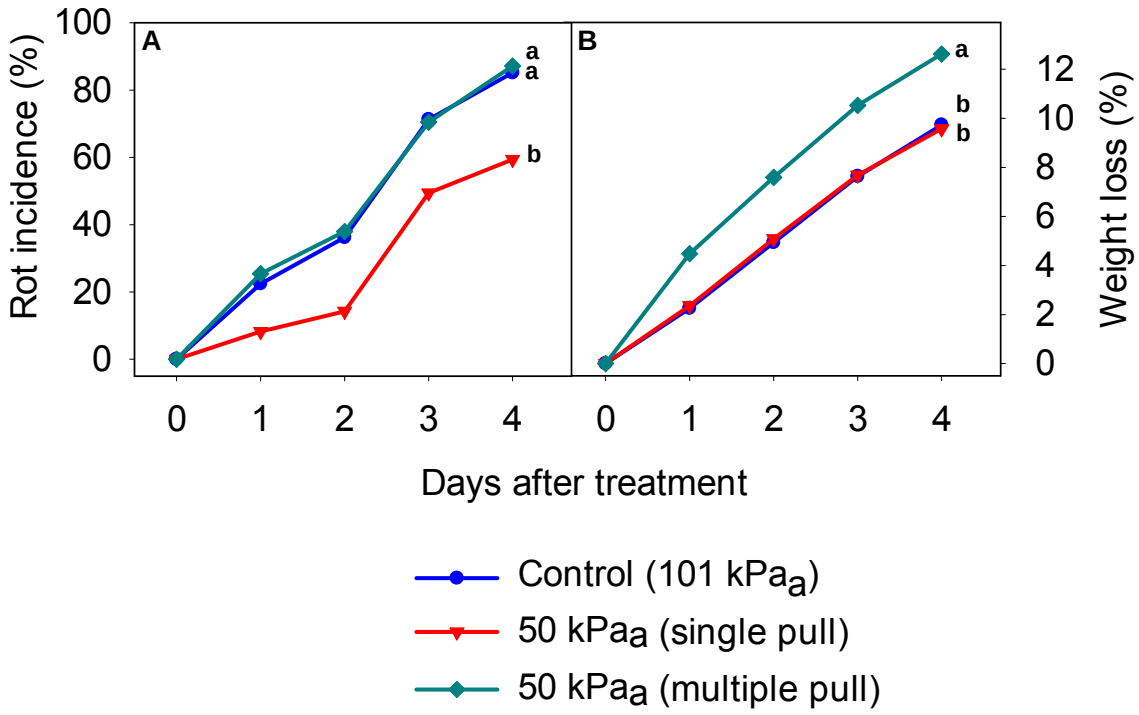

Figure 3.9 Effect of single and multiple hypobaric pressure release on rot incidence (A) and weight loss (B) in strawberries subsequently stored at 20 °C. Each data point represents the mean value of six replicate clamshells. Values with different letters show significant difference (*P*< 0.05) from each other on 4 d as determined by Tukey's test. The data for day 0 was collected about 2 h after harvest.

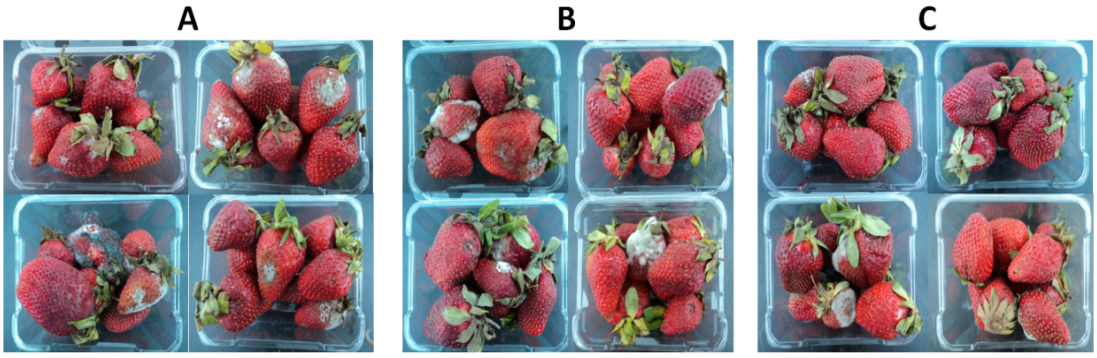

Figure 3.10 Effect of single and multiple hypobaric pressure release on rot incidence in strawberries subsequently stored at 20 °C for 4 d. (A) Control (B) 50 kPa$_a$ with multiple pull (C) 50 kPa$_a$ with single hypobaric pressure release.

3.4 Discussion

Symptoms of strawberry decay indicated that natural decay was mainly caused by *Botrytis cinerea* and *Rhizopus stolonifer* at 20 °C (Figure 3.6). In most cases initially small lesions of *B. cinerea* appeared with tan spots on fruit and later on developed into grey conidia (Droby and Lichter, 2007). Soft and juicy rots of *R. stolonifer* were also observed which resulted in leaky strawberries due to tissue collapse (Maas, 1998).

The rate of rot development was rapid due to the 20 °C conditions, with visible fungal rots first observed after either 2 d (Figure 3.2) or 1 d of storage (Figure 3.5; Figure 3.8 and Figure 3.9A). This research demonstrated a variation in prevalence of rots across different experiments (Figure 3.11) as observed by Jabbar et al. (2010). Many factors could be responsible for variation of quality in strawberry, including genetic, environmental and cultural practices (Kader, 1991). In present case variation in rot development might be due to different cultivars, harvesting time and pre-harvest climatic conditions. In this work the progression of rot in 'Gaviota' was slower than 'Camarosa' strawberries (Figure 3.2). Pre-harvest field temperatures also affect the postharvest storage life of fruit (Woolf and Ferguson, 2000). Furthermore, Hancock (2000) stated that higher air temperature in the field may accelerate softening and damage berry surface. Therefore apart from cultivar difference, pre-harvest field temperature may also be an important factor in influencing postharvest storage life of strawberry.

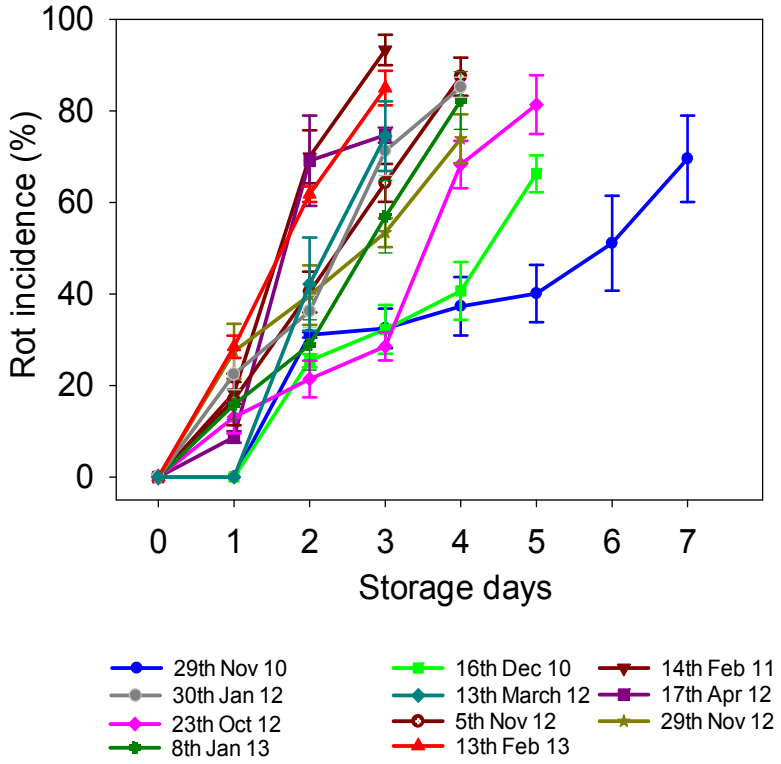

Figure 3.11 Percent rot incidence of strawberry in different batches at 20 °C. 'Gaviota' strawberries were used in experiments on 29th Nov 2010 and 16th Dec 2010, while all other experiments were carried out using 'Camarosa' strawberries. Vertical bars represent standard errors of means of 3-6 replicate clamshells.

Results of this chapter suggest that 50 kPa_a treatment for 4 h was the most effective in rot reduction (Figure 3.2), while 6 h treatment did not differ from control. Similarly, Laurin et al. (2003) reported that 70 kPa_a treatment for 8 h did not affect strawberry decay severity. Romanazzi et al. (2001) previously reported a decrease in rots following low pressure treatment (50 kPa_a), but suggested that 25 kPa_a might be more effective. In this work, fruit treated with 25 kPa_a did not differ from the control at 20 °C, whereas 50 kPa_a and 75 kPa_a delayed fungal rot development (Figure 3.2C). In summary, this work established that 50 kPa_a treatments for 4 h were repeatedly effective in reducing strawberry decay (Figure 3.2, Figure 3.5, Figure 3.8, Figure 3.9A).

Due to the consistency of results, 50 kPa_a for 4 h was used for further research in this thesis. In addition, as the strawberry industry relies on temperature management as their prime tool to extend the storage life of strawberry. Therefore effectiveness of hypobaric treatment under low storage temperature has been studied in chapter 6.

Hypobaric treatment was found to be useful in reducing rot if applied within 8 h of arrival in lab, while a 12 h delay before treatment resulted in ineffective hypobaric treatment (Figure 3.5A). The non-efficacy after 12 h delay may be due to a decrease in resistance of the harvested fruit with time (Wang et al., 2012). Gabler et al. (2006) reported reduced grey mould incidence with biofumigation of grape berries for 7 d at 20 °C within 24 h of inoculation, but with a 48 h interval between fumigation and inoculation the efficacy of biofumigation decreased. In this work, results from delay before hypobaric treatment experiment confirm that a short treatment delay (8 h) (section 3.2.1.1) did not affect the efficacy of hypobaric treatment (Figure 3.5A).

Plaxton and Podestá (2006) proposed that variation in respiratory metabolism may improve the capability of the plant to adapt to biotic and abiotic stresses including pathogenic infections. The transient increase in respiration rate observed immediately after 50 kPa_a treatments (Figure 3.3) may indicate that the tissue perceived hypobaric treatment as a hormetic stress, while less successful treatments (25 kPa_a, 75 kPa_a) did not lead to similar increase (Figure 3.2). Tovar et al. (2011) also observed an increase in respiration rate during subsequent storage after 34 kPa_a, 20 min treatment of mango.

It has been suggested that hypobaric (8 kPa_a) storage of horticultural crops delays senescence and decreases microbial infection (Apelbaum et al., 1977a; Lougheed et al., 1978). An et al. (2009) reported significantly reduced respiration rate during hypobaric storage (25.3 kPa_a for 4 d at 3 °C) of strawberries. However in the above studies, low pressure was applied during storage for many days, while in the present work, exposure time to low pressure treatment was very short (4 h) after which strawberries were subsequently stored at atmospheric pressure (101 kPa_a). Since short-term hypobaric treatment (4 h) did not affect respiration rate in subsequent storage (Figure 3.3), it is reasonable to conclude that there was no lasting impact on tissue physiology.

Firmness is an important quality parameter of strawberry (Chen et al., 2011). In all experiments, firmness was not significantly different from control. According to Gao et al. (2006), the mode of action of hypobaric storage (40-50 kPa_a, 49 d) was to delay senescence and ripening, but in the present case, the short time of treatment did not influence firmness reduction, suggesting that the mode of action for hypobaric treatments is different to that of hypobaric storage. Another possible explanation for the lack of firmness response observed is that strawberries were sourced at near to ripe stage; hence fruit softening was already advanced leaving little opportunity for hypobaric treatment to have an effect. In order to observe an effect of hypobaric treatment on strawberry firmness, it would be interesting to repeat this work with less ripe fruit (i.e. 75% red).

Hypobaric treatment (50 kPa_a, 4 h) did not affect weight loss in strawberries. In contrast, a 70 kPa_a pressure treatment for 6 h increased the water loss in Beit Alpha-type cucumbers in subsequent storage, attributed to stomatal opening due to low pressure stress (Laurin et al., 2006). However strawberry fruit have very few stomata (1-3 per mm^2; Blanke, 2002) in comparison to cucumber (20-30 per mm^2; Adams and Ho, 1995) which may explain the lack of response observed for strawberry in this case. Goyette et al. (2012) demonstrated that a hyperbaric pressure treatment (900 kPa_a) for 15 d damaged the appearance of tomato and causes severe weight loss due to high pressure stress. Similar to high pressure, longer time of treatment (6 h) and multiple pull of hypobaric treatments may have damaged cell structure of strawberry due to severe stress caused by low pressure, creating a conducive environment for increased rot development (Figure 3.8A) and higher weight loss (Figure 3.9B) respectively. The lack of impact on weight loss indicates that 50 kPa_a for 4 h with a single pull was not a severe stress for tissues.

Romanazzi et al. (2001) suggested that hypobaric treatment may instigate a stress response within the tissue of the fruit resulting in the enhancement of natural disease resistance without damaging tissue structure (i.e. a mild stress). Plant tissues respond to different stresses by stimulation of natural defence systems (Kuc, 1995). UV treatment created a stress condition that resulted in increased expression of defence-related enzymes and consequently reduced fungal rot in strawberry (Pombo et al., 2011). In this work, the reduction in rot development may be due to a similar mechanism. This possibility is investigated in chapter 5 of this thesis. Another possibility is direct effect of hypobaric treatment on the growth of fungi (Apelbaum

and Barkai-Golan, 1977). Effect of hypobaric treatment on *in vitro* fungal growth is described in the next chapter.

3.5 Conclusion

Among different hypobaric treatments, 50 kPa_a for 4 h demonstrated a consistent reduction in postharvest fungal decay of strawberry without affecting quality parameters. However, extended delay prior to treatment (12 h), longer treatment time (6 h) and multiple pull negatively influenced the efficacy of hypobaric treatment (50 kPa_a). Firmness and weight loss remained unaffected by 50 kPa_a, 4 h treatment. An immediate increase in respiration rate of 50 kPa_a treated fruit may suggest a physiological response to hormetic stress. This increase in respiration rate may be indicative of potential for activation of defence-related enzymes and phytochemicals that later aid strawberry to restrain fungal decay during storage.

Chapter 4 Elucidating mechanisms for hypobaric treatments (*)

4.1 Introduction

Botrytis cinerea and *Rhizopus stolonifer* are the main causal organisms for rots in strawberries (Romanazzi et al., 2013). *B. cinerea* causes grey mould which is the most common disease of strawberries (Tao et al., 2010). Babalar et al. (2007) stated that infection starts during flowering and remains dormant until favourable conditions prevail. *R. stolonifer* causes strawberry Rhizopus rot and is most prominent during cold storage (Timudo-Torrevilla et al., 2005). This disease causes soft rots and brown spots on fruit resulting in tissue collapse and juice leakage (Maas, 1998). In chapter 3, it was demonstrated that hypobaric treatment reduced fungal rots in strawberries at 20 °C. Similarly previous work on blueberries (Hashmi et al., 2013) also showed the effectiveness of hypobaric treatment in decreasing fungal rots, but the mechanisms involved in rot reduction due to hypobaric treatment are still unknown.

One potential mechanism of the observed reduced decay development in strawberries could be a direct effect of low pressure on the fungi contaminating the strawberries. Pan et al. (2004) and Liu et al. (2012) found that UV and heat treatments reduced the viability of fungi contaminating fruit respectively. In hypobaric storage Apelbaum and Barkai-Golan (1977) demonstrated pressure application at below 6 kPa_a reduced mycelial growth of *B. cinerea*. Pressures below 3.33 kPa_a resulted in growth being

(*) This chapter includes material published in the papers:
Hashmi, M.S., East, A.R., Palmer, J.S., & Heyes, J.A. (2013). Pre-storage hypobaric treatments delay fungal decay of strawberries. Postharvest Biology and Technology 77, 75-79.

Hashmi, M.S., East, A.R., Palmer, J.S., & Heyes, J.A. (2013). Hypobaric treatment stimulates defence-related enzymes in strawberry. Postharvest Biology and Technology 85, 77-82.

Hashmi, M.S., East, A.R., Palmer, J.S., & Heyes, J.A. (2013). Reduced fungal decay in strawberries inoculated after hypobaric treatment suggests induced resistance. Acta Horticularae (in press).

completely inhibited, but normal growth of fungi resumed after transferring to atmospheric pressure (Barkai-Golan, 2001).

Another possible mode of action of hypobaric conditions could be the modification of oxygen partial pressure (An et al., 2009). As a result of applying hypobaric treatment (50 kPa_a, 4 h), the oxygen partial pressure decreases from ≈ 21 kPa_a (≈ 21% at 101 kPa_a) to ≈ 10 kPa_a (or ≈ 21% at 50 kPa_a). This reduction in oxygen partial pressure may be responsible for a mild stress that results in induced resistance within the strawberries and thus causes a delay in fungal decay of the strawberries. A study of low oxygen concentration treatment at atmospheric pressure is needed to determine if low oxygen partial pressure in hypobaric treatment contributes to the potential stress response.

Romanazzi et al. (2001) suggested that hypobaric treatment of fruit may initiate a stress response within the fruit tissue resulting in the enhancement of natural disease resistance. Normally plant tissue reacts to different stresses by activation of natural defence systems (Kuc, 1995). To evaluate the response of fruit to the treatment, it is necessary to inoculate fruit with challenge fungi after treatment (Jin et al., 2009). For strawberries, UV treatment created a stress condition that resulted in increased expression of defence-related enzymes and consequently reduced fungal rot (Pombo et al., 2011).

The aims of this chapter are to:

1. Evaluate the possibility of direct hypobaric treatment effect on *in vitro* growth of *B. cinerea* and *R. stolonifer* spores and hyphae.
2. Investigate if low O_2 partial pressure achieved during hypobaric treatment contributes to the reduced decay development of hypobaric treated strawberries
3. Assess the response of previously treated fruit upon fungal inoculation at different time intervals.

These objectives would enable differentiation between potential mechanisms involved in the responses observed after applying a short-term hypobaric treatment to strawberries.

4.2 Materials and methods

4.2.1 *In vitro* fungal growth

B. *cinerea* and *R*. *stolonifer* isolated from diseased strawberries were grown on PDA (Potato Dextrose Agar) for 10-14 days at 20 °C. After 10-14 days growth, 5 mL of sterile peptone water was pipetted onto the surface of the fungal colony and a sterile spreader was used to mix fungal spores (conidia) into the peptone water, creating a spore suspension. One mL of the spore suspension was then added to Microbank® beads (Pro-Lab Diagnostics, Austin, Texas, USA) and stored at -75 °C. When required, one bead was removed and placed on PDA and incubated for 10-14 days at 20 °C. Pure cultures were prepared by transferring a small proportion of mycelium with the use of a straight wire to PDA in Petri dishes, and incubated at 20 °C for 14 days. From these two cultures 40 PDA coated Petri dishes were inoculated with the use of a straight wire and incubated at 20 °C for 24 h to develop fungal colonies. In each case, after

24 h, twenty (20) Petri dishes were kept at atmospheric pressure (101 kPa_a) while the other 20 were subjected to 50 kPa_a for 4 h. All Petri dishes were subsequently returned to 20 °C, with the mean radial growth was observed by measuring colony diameter in two orthogonal directions using callipers. The experiment was completed when the cultures reached the edges of Petri dishes. For *B. cinerea*, 8 measures were conducted over 48 h, while for *R*. *stolonifer*, 10 measures were analysed over 12 h. This experiment would help in understanding if there is any long-term inhibition of fungal germination due to short hypobaric treatment.

For spore germination study, conidia from pure cultures were harvested by suspending in sterile water containing 0.03% (v/v) Tween20. This conidial suspension was then filtered through a sterile cheese cloth. The concentration of the filtrate was adjusted with the help of haemocytometer to around 10^4 spores mL^{-1} by adding sterile water, 10 µL of this suspension was spread on each Petri slide and incubated at 20 °C for 2 h (*B. cinerea*) and 5 h (*R. stolonifer*). In each case, after 1-2 spores showed signs of germination, twenty (20) Petri slides were kept at atmospheric pressure (101 kPa_a), whereas the other 20 were subjected to 50 kPa_a for 4 h. All Petri slides were subsequently incubated at 20 °C. Approximately 200 spores per replicate were counted for germination rate under microscope after 4 h of treatment.

4.2.2 Oxygen treatment experiment

Field grown ‘Camarosa’ strawberries were obtained from a commercial grower in Whanganui region, New Zealand. On arrival at Massey University, bruised and

damaged fruit were removed from the population. Fruit with calyx and pedicel were selected for experimental work.

Fifty six clamshells (≈ 250 g each) of about 12–15 sound fruit were selected and were divided into two treatments: ≈ 10% O_2 treatment for 4 h, or atmospheric conditions (≈ 21% O_2) for the same time at 20 °C. Strawberries in clamshells were kept in 7 L airtight Perspex boxes (6 clamshells in each box) at 20 °C and exposed to 10% O_2 (at 101 kPa_a) or control (21% O_2) by supplying the desired humidified gas mixture in a flow through system. The 10% O_2 treatment was achieved by reducing the oxygen content of air through mixing with nitrogen (BOC, Auckland, New Zealand). The flow rate of both treatments was maintained (0.5 L min^{-1}) for 4 h, to ensure no modification of the gas environment due to strawberry respiration.

For natural rot, 4 clamshells per treatment were assessed destructively on each sampling day, with rot incidence determined by visually examining each berry. Fruit with visible decay were counted and the results recorded as percentage of rot incidence per clamshell. Observations of decay development were recorded daily.

4.2.3 Inoculation of strawberry with *B. cinerea* and *R. stolonifer*

In a third experiment strawberries were inoculated with fungal spores in order to investigate the possibility of induced resistance by hypobaric treatment. *B*. *cinerea* and *R*. *stolonifer* were isolated from diseased strawberries, grown on PDA and incubated at 20 °C until conidia developed. Cultures were prepared by transferring a small proportion of mycelia, with the use of a straight wire to PDA. After 7 days the conidia

from each pure culture were harvested by suspending in sterile water containing 0.03% (v/v) Tween20. This conidial suspension was then filtered through a sterile cheese cloth. The concentration of the filtrate was adjusted to approximately 10^4 spores mL^{-1} with the addition of sterile water by estimating fungal spore concentration through the use of a haemocytometer.

Four hundred (400) hydroponically grown 'Camarosa' strawberries were obtained from Massey University Plant Growth Unit, Palmerston North, New Zealand. Strawberries were disinfected by dipping in commercial grade NaClO solution (2%) for 5 seconds. After drying the fruit at room temperature, each fruit was placed in a cylindrical plastic container (Figure 4.1) with a porous lid to avoid cross-contamination and ensure respiration. Half of the fruit were treated with hypobaric pressure (50 kPa_a for 4 h), with the others used as a control (101 kPa_a). Each strawberry fruit from both groups was inoculated by spreading 10 µL of spore suspension (10^4 mL^{-1}) of *B. cinerea* or *R. stolonifer* on the surface 0, 6, 12, 18 or 24 h after hypobaric treatment.

For each inoculation time, 20 individual strawberries were repeatedly assessed daily for rot severity. The extent of macroscopic fungal development of individual strawberry fruit was externally analysed by estimating the affected area percentage of each fruit to nearest 10%. From these figures, mean percentage severity of infected strawberry fruit was calculated.

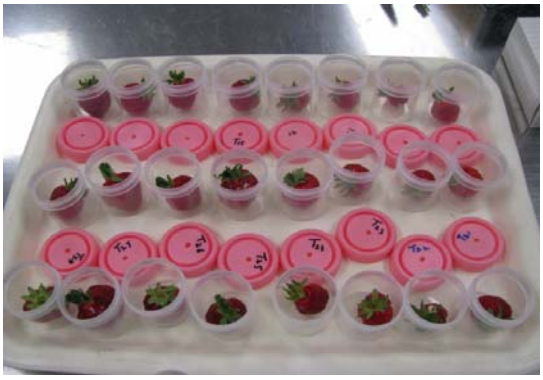

Figure 4.1 Each strawberry placed in a separate container to avoid cross contamination.

4.2.4 Hypobaric treatment and subsequent storage

Hypobaric pressure was generated in a hermetically sealed tank with a vacuum pump. Clamshells/containers of strawberries were placed inside the tank at 20 °C as described in section 3.2.2. Samples were subsequently stored after pressure treatment at 20 °C and 80–90% RH.

4.3 Data analysis

The data for *in vitro* fungal study was analysed by Student's t-test. Data for rot incidence of oxygen treatment was analysed by overall ANOVA, while fungal inoculation was analysed by one-way ANOVA at each measurement time using Minitab 16 (Minitab Inc, State College, PA, USA) and means were separated by Tukey's test.

4.4 Results and discussion

4.4.1 Response of *B. cinerea* and *R. stolonifer* to hypobaric treatment

A linear increase in *B. cinerea* and *R. stolonifer* colony diameter was observed for both control and treated colonies (Figure 4.2). Colonies reached the edges of Petri dishes

(70 mm) after 48 h and 12 h at 20 °C for *B. cinerea* and *R. stolonifer* respectively. There was no difference in colony diameter between 50 kPa_a treated and control samples throughout the experiment for either fungus (Figure 4.2 and 4.3). In a similar study Romanazzi et al. (2001) also observed no effect of hypobaric treatment (25 kPa_a for 3 d) on the growth of *B. cinerea*. Earlier studies on the *in vitro* growth of fungi under very low pressure storage (3.33-6.66 kPa_a for 3-4 d) reported delays in growth during treatment with normal growth resuming after transferring to atmospheric conditions (Apelbaum and Barkai-Golan, 1977).

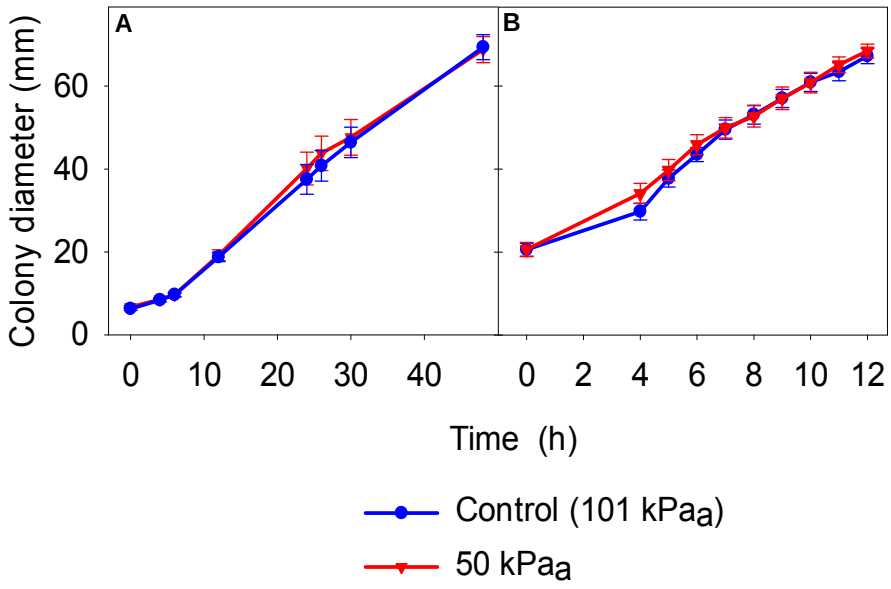

Figure 4.2 Effect of hypobaric treatment on the *in vitro* growth of (A) *B. cinerea* and (B) *R. stolonifer* colonies at 20 °C. Petri dishes were pre-seeded with centrally-applied spores for 24 h and colonies were exposed to 50 kPa_a pressure for 4 h. Initial data point represents colony diameter before treatment. Each data point represents the mean value of 20 Petri dishes. Vertical bars represent standard errors of means.

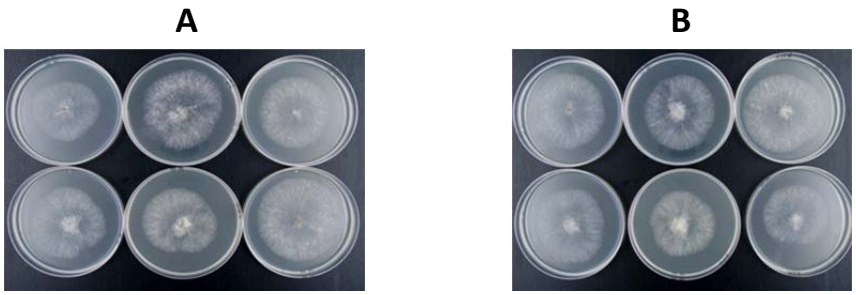

Figure 4.3 Effect of hypobaric treatment on the *in vitro* growth of *R. stolonifer* after 8 h of treatment. Six representative Petri dishes for (A) Control (101 kPa_a) and (B) 50 kPa_a treated.

In another experiment conidia of *B. cinerea* and *R. stolonifer* on agar-coated microscopic Petri slides were put through a standard 4 h, 50 kPa_a hypobaric treatment and were found to germinate rapidly and uniformly, confirming that the hypobaric treatment did not affect spore viability (Table 4.1).

Table 4.1 Effect of hypobaric treatment (50 kPa_a, 4 h) on spore germination of *B. cinerea* and *R. stolonifer* spores. Values in each column are not significantly different.

Treatment	Germination (%) (*B. cinerea*)	Germination (%) (*R. stolonifer*)
Control (101 kPa_a)	93.22	82.97
50 kPa_a	91.17	82.22

Contrary to the results of this work, Liplap et al. (2013b) reported that 200-850 kPa_a treatment for 7 d inhibited bacterial growth in fresh produce. Likewise Hernández-Lauzardo et al. (2008) observed significant reduction in *R. stolonifer* colony diameter

and conidia germination after chitosan treatment, indicating a fungicidal effect of chitosan. Similarly Pan et al. (2004) found that both UV-C (4.1 kJ m^{-2}) or heat treatment (45 °C, 3 h) inhibited spore germination of *B. cinerea,* but were unable to inhibit *R. stolonifer* spores. However, Liu et al. (2012) demonstrated that heat treatment (40 °C for 5 or 10 min) only delayed germination of *Monilinia fructicola*, but did not have a lethal effect on conidia germination. Unlike all the above treatments, current work indicated that short-term (4 h) hypobaric treatment (50 kPa_a) has neither a direct toxic effect nor an inhibitory effect on fungal spore germination. These results suggest that an indirect mechanism involving induction of host resistance might be responsible for the delay in rot development observed after a short-term hypobaric treatment (chapter 5).

The results of this work demonstrated that hypobaric treatment had no effect on fungal growth. However the growth of fungi on nutritious media (PDA) may be quite different from that of a fruit surface due to different intrinsic factors (i.e. pH, water activity); therefore it would be interesting to further study *in vitro* growth and sporulation of the corresponding treated fungi with manipulated intrinsic factors of the growing media.

4.4.2 Effect of 10% oxygen on rot development in strawberries

Rot development in strawberries treated with 10% O_2 showed no difference from control during subsequent storage (Figure 4.4). In a similar study Laurin et al. (2003) demonstrated that 70 kPa_a with low oxygen (14 kPa_a O_2) treatments for 8 h was comparable to control (101 kPa_a) in subsequent storage at 1 °C. In the current work

reduced O_2 (10%) was not effective in reducing rot. This indicates that reduction in rots by hypobaric treatment may not be due to low oxygen partial pressure but to some other mechanism that triggers the response of fruit to the low pressure stress.

Laurin et al. (2003) further reported that strawberry stored at 70 kPa_a for 8 h with 21% O_2 was similar in decay severity of strawberry and weight loss to the corresponding control (101 kPa_a) in subsequent storage at 1 °C, but previous results (Figure 3.2) demonstrated the positive effect of hypobaric treatment (50 kPa_a, 4 h). The reason for ineffectiveness of hypobaric treatment in decay reduction in this case might be the different combination of pressure (70 kPa_a) and time (8 h).

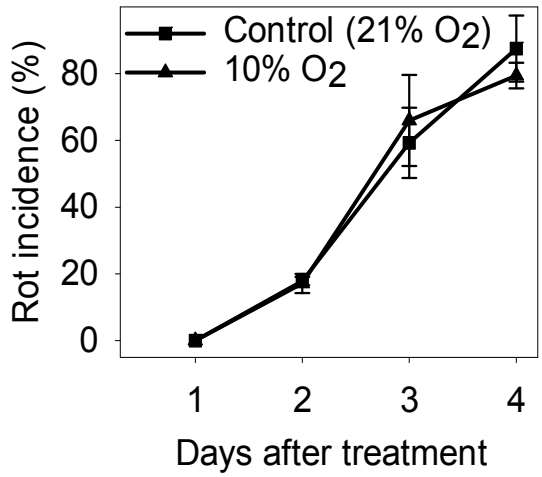

Figure 4.4 Effect of 10% O_2 treatment for 4 h on rot incidence of strawberry subsequently stored at 20 °C. Each data point represents the mean value of four replicate clamshells. Vertical bars represent standard errors of means.

4.4.3 Response of hypobaric treated strawberry to fungal inoculation

Control strawberries inoculated immediately demonstrated visible *B. cinerea* and *R. stolonifer* infection after 2 and 3 days respectively (Figure 4.5A and Figure 4.6A).

Hypobaric treated strawberries inoculated immediately or 12 h after treatment demonstrated significant ($P< 0.05$) reduction in rot severity of *B. cinerea* (Figure 4.5A and C) and *R. stolonifer* (Figure 4.6A and C) after 5 d and 8 d respectively as compared to control, while the treated strawberry samples inoculated after 6 or 18 (Figure 4.5B and D; Figure 4.6B and D) and 24 h (data not shown) were comparable with control.

The rot severity in samples inoculated 18 and 24 h after treatment may be influenced by the reduced resistance of fruit due to ripening (Guidarelli et al., 2011). Terry et al. (2004) reported that fruit maturity significantly affect strawberry resistance against diseases. The current study suggests that due to advanced maturity stage hypobaric treatment may not be able to stimulate natural disease resistance in fruit inoculated 18 and 24 h after treatment. Strawberries inoculated 12 h after hypobaric treatment showed reduced rot severity by twofold for both pathogens (Figure 4.5C and Figure 4.6C). Pombo et al. (2011) observed a similar twofold reduction in rot severity of UV-C treated strawberries inoculated 8 h after UV-C treatment.

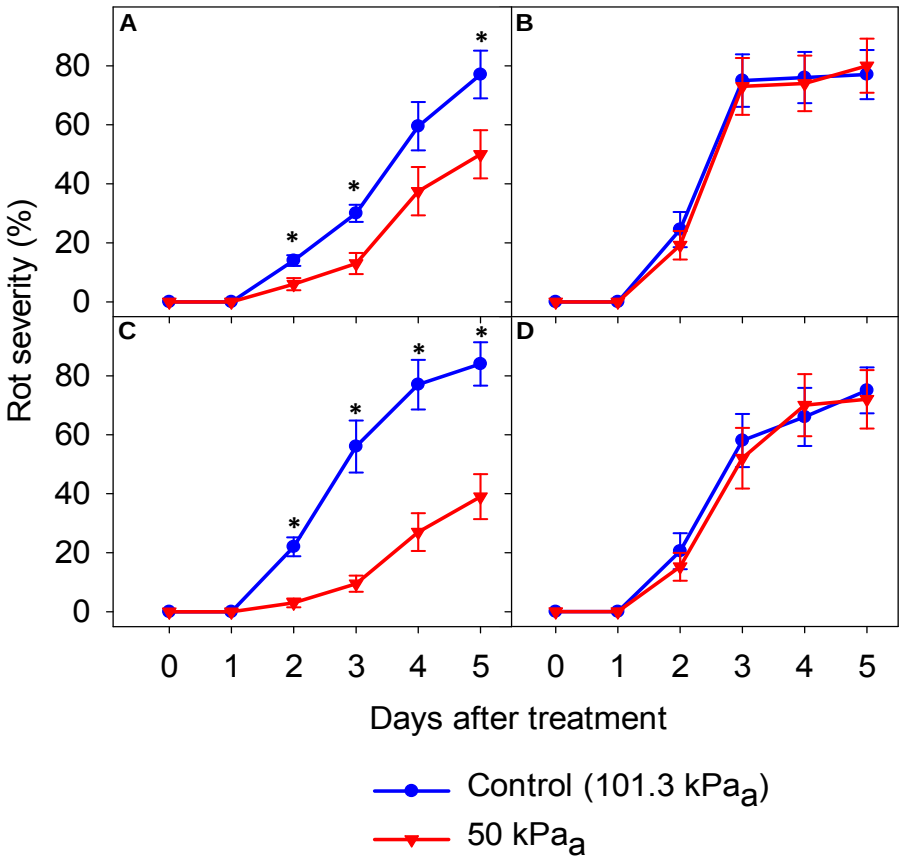

Figure 4.5 Strawberry rot severity after inoculation of *B. cinerea* (A) immediately, (B) 6 h, (C) 12 h and (D) 18 h after hypobaric (50 kPa$_a$, 4 h) treatment respectively and subsequently stored for 5 d at 20 °C. Each data point represents the mean value of 20 individual fruit. Significant differences (P< 0.05) at each measurement time are represented by * as determined by Tukey's test. Vertical bars represent standard errors of means. Data for day 0 were collected immediately after treatment.

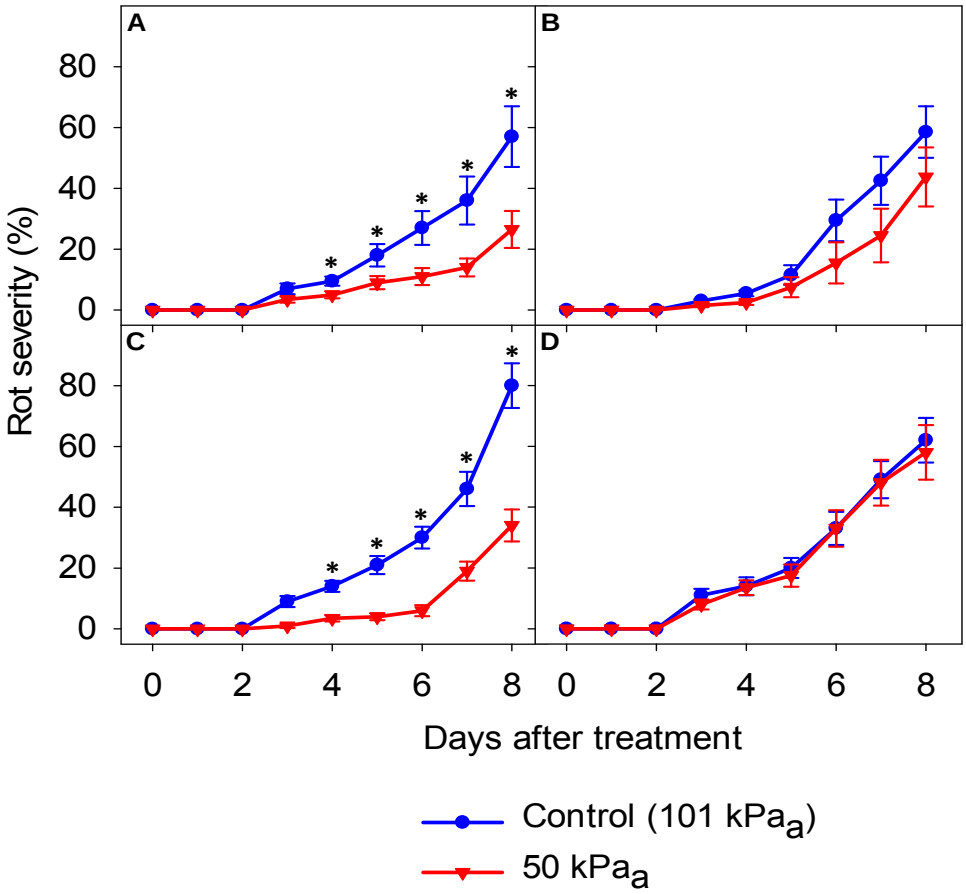

Figure 4.6 Strawberry rot severity after inoculation of *R. stolonifer* (A) immediately, (B) 6 h, (C) 12 h and (D) 18 h after hypobaric (50 kPa$_a$, 4 h) treatment respectively and subsequently stored for 8 d at 20 °C after treatment. Each data point represents the mean value of 20 individual fruit. Significant differences (P< 0.05) at each measurement time are represented by * as determined by Tukey's test. Vertical bars represent standard errors of means. Data for day 0 were collected immediately after treatment.

As hypobaric treatment occurred before inoculation, this decrease in rot must be attributed to an effect of treatment on the fruit rather than a direct fungicidal or fungistatic effect. The reduced decay in samples inoculated immediately and 12 h after treatment suggests a possible induction of some toxic chemicals or enzymatic

inhibitors that result in fungicidal or fungistatic activity after treatment. As the reduction in rot severity was observed only in treated samples inoculated 0 and 12 h after the treatment and not at 6, 18 and 24 h, this response tends to suggest involvement of time dependent defence-related compounds.

A number of other authors have found similar time dependant and labile responses when investigating physical treatments effect on postharvest decay reduction. Jin et al. (2009) showed that different inoculation times after methyl jasmonate treatment of peach affected *B. cinerea* and *R. stolonifer* growth pattern. Inoculation 12 h after methyl jasmonate treatment resulted in maximum reduction for both *B. cinerea* and *R. stolonifer*. Nigro et al. (2000) demonstrated that time of inoculation after UV-C treatment significantly affected the development of rots with strawberries inoculated 12 h after UV-C treatment (0.5 kJ m^{-2}) resulting in minimal rots in comparison to other inoculation times (0, 24 and 48 h). The current study shows a similar dependency of rot development on inoculation time.

Nigro et al. (2000) further observed that UV-C treatment resulted in the activation of a defence-related enzyme (PAL) 12 h after treatment, which may be the reason for the reduction in fungal rot. Likewise, Pombo et al. (2011) demonstrated that UV-C treatment of strawberries enhanced the activity of defence-related enzymes (PAL, PPO, POD, Chitinase and β-1,3-glucanase). Comparable to hormetic effect of UV-C, hypobaric treatment might have generated the hormetic stress and induced resistance thus delaying rot development in strawberries without affecting fruit quality. To further explore the potential induced resistance mechanism of hypobaric treatment in

delaying fungal rots of strawberries, changes in defence-related enzymes subsequent to hypobaric treatment is the focus of the following chapter.

4.5 Conclusion

An *in vitro* fungal study demonstrated that hypobaric treatment (50 kPa_a, 4 h) had no direct effect on fungal growth rate or spore germination of *Botrytis* or *Rhizopus*. Furthermore, reduction in rots of hypobaric treated strawberries was not attributable to the low oxygen partial pressure created during treatment. Hypobaric treated strawberries inoculated immediately or 12 h after treatment resulted in subsequent rot reduction in comparison to non-treated fruit, indicating a response of fruit to the hypobaric treatment. Together, the results of this chapter suggest that the mechanism of rot reduction due to hypobaric treatment may be stimulation of antifungal compounds or defence-related enzymes.

Chapter 5 Hypobaric treatment stimulates defence-related enzymes in strawberry (*)

5.1 Introduction

Different approaches have been practised to understand the mechanism of induced resistance in plants. In chapter 4, strawberries inoculated after hypobaric treatment demonstrated reduced fungal rot in comparison to un-treated fruit (Figure 4.5 and Figure 4.6). It is believed that fruit produce defence-related enzymes upon biotic and abiotic stress (Ferreira et al., 2007). These enzymes serve as a first defence line against pathogen invasion (Sun et al., 2008). Creating stress conditions by application of hypobaric treatment could lead to the stimulation of defence-related compounds. Defence enzymes have previously been studied to understand the mechanism of strawberry decay control in different treatments (Civello et al., 1997; Pombo et al., 2011). These defence enzymes include phenylalanine ammonia-lyase (PAL), chitinase, peroxidase (POD), polyphenol oxidase (PPO) and β-1,3-glucanase. In addition, molecular studies of these enzymes have been used to investigate the mechanism of induced resistance in strawberry (Dotto et al., 2011; Pombo et al., 2009).

PAL plays a key role in the phenylpropanoid pathway (Kruger et al., 2002). Synthesis of phytoalexins and lignin which are related with induced resistance to pathogen invasion (Wang et al., 2011) directly involves PAL (Baarlen et al., 2007; Sticher et al., 1997). Chen et al. (2009) reported that PAL is the precursor of salicylic acid in plants, which plays a vital role in stimulating resistance against pathogens. Other evidence of the involvement of PAL in plant defence is that application of α-aminooxy-β-

(*) This chapter includes material published in the paper:
Hashmi, M.S., East, A.R., Palmer, J.S., & Heyes, J.A. (2013). Hypobaric treatment stimulates defence-related enzymes in strawberry. Postharvest Biology and Technology 85, 77-82.

phenylpropionic acid (AOPP) as phenylpropanoid biosynthesis inhibitor resulted in reduced resistance of barley to *Blumeri graminis* (Kruger et al., 2002) and *Puccinia hordei* (Prats et al., 2007). It has previously been reported that UV-C (Pombo et al., 2011) and heat (Yuan et al., 2013) treatments triggered PAL activity resulting in the reduction of postharvest decay in strawberry and muskmelon respectively.

The importance of plant chitinases in defence against pathogens is well documented (Grover, 2012; Van Loon et al., 2006). Chitin is the main cell wall constituent of fungi and insect exoskeleton (Sharma et al., 2011). Chitinases in combination with β-1,3-glucanases are pathogenesis-related proteins and hydrolyse chitin present in the fungal cell wall resulting in inhibition of fungal growth (Grover, 2012). Increase in chitinase activity due to UV-C treatment in strawberry (Pombo et al., 2011) and heat treatments in peach (Liu et al., 2012) have been associated with reduced rot.

POD is an anti-fungal enzyme (Ferreira et al., 2007), which plays an important role in biosynthesis of lignin and fortification of the cell wall (Gulen and Eris, 2004). This enzyme is considered to be a biochemical marker for disease resistance (Bi et al., 2006). Different treatments such as tea tree oil vapour (Shao et al., 2013), chitosan (Sun et al., 2008), and methyl jasmonate (Zhu and Tian, 2012) treatments demonstrated activation of POD and a reduction in postharvest decay in strawberry, potato and tomato respectively.

Likewise PPO is involved in several catalytic reactions in formation of quinones (Shao et al., 2013). Synthesis of quinone is one of the first responses after fungal invasion of

plants (Zhang et al., 2012). Pombo et al. (2011) associated post-UV-C treatment increase in PPO to delayed fungal rots in strawberries. Increase in PPO activity observed in oxalic acid treated peaches was also ascribed to induced resistance (Zheng et al., 2007).

The objective of this chapter is to assess the effect of hypobaric treatment on activity of defence-related enzymes in strawberry. As a whole this chapter further explores the possible modes of action for decay reduction as caused by pre-storage hypobaric treatments.

5.2 Materials and methods

5.2.1 Strawberry fruit

In January, March and April 2012, field grown 'Camarosa' strawberries were obtained from a commercial grower in Whanganui region, New Zealand. On arrival at Massey University, bruised and damaged fruit were removed from the population. Fruit with calyx and pedicel were selected for experimental work. In each experiment 56 clamshells (250 ± 10 g) of 12-15 sound fruit were prepared and were divided into two treatments: 50 kPa_a for 4 h, or atmospheric conditions (101 kPa_a) for the same time at 20 °C. After treatment, 32 clamshells (16 / treatment) were used for storage, while 24 clamshells (12 / treatment) were used for collection of samples for enzyme analysis 0, 6, 12, 18, 24 and 48 h after hypobaric treatment.

Strawberry samples from different hypobaric treatment application options were also analysed for PAL activity. These variants include:

- 50 kPa_a for 4 h, pressure released either slowly within 2 min or fast within 30 seconds. This treatment was termed as single pull treatment.
- Pressure (50 kPa_a) was released after each 8 min and re-built in next 2 min. The process was repeated 24 times for 4 h (10 X 24 = 240 min). This treatment was termed as multiple pull treatment.
- Hypobaric treatment (50 kPa_a) for 2 h or 6 h.

Full details of the differences of application of these treatments are provided in section 3.2.1.3.

5.2.2 Hypobaric treatment

Hypobaric pressure was generated as described in section 3.2.2

5.2.3 Storage and decay development

After pressure treatment clamshells were subsequently stored at 20 °C and 80–90% RH. Observations of decay development were recorded daily for 4 days. Four clamshells per treatment were assessed destructively at each sampling day, with rot incidence visually assessed by examining each berry. Fruit with visible decay were counted and the results recorded as percentage of rot incidence per clamshell.

5.2.4 Enzyme assay

Four independent replicates were used for PAL and chitinase activities while 3 independent replicates were used for POD and PPO. For each replicate 5 fruit with no sign of decay were cut into small pieces with a sharp blade, immediately frozen with liquid nitrogen and stored at -30 °C for later analysis.

5.2.4.1 *Phenylalanine ammonia-lyase (PAL) activity*

PAL activity determination method was modified from Civello et al. (1997) and Pombo et al. (2011). Frozen strawberries (≈ 5 g) were homogenized in a high-performance dispersing instrument (Ultra-Turrax® T25 Basic) for 2 min with 20 mL of enzyme extraction buffer (0.1 mol L^{-1} $Na_2B_4O_7 \cdot 10H_2O$, 5 mmol L^{-1} 2-mercaptoethanol, 2 mmol L^{-1} EDTA, 3% (w/v) polyvinylpolypyrrolidone (PVPP), pH 8.8). The mixture was left to stir for 1 h at 4 °C and centrifuged at 10,000 × *g* for 20 min. The supernatant was immediately used for PAL assay. The following reaction mixture was employed: 2550 µL 0.03 mol L^{-1} $Na_2B_4O_7 \cdot 10H_2O$ (pH 8.8), 450 µL 0.01 mol L^{-1} L-phenylalanine in 0.03 mol L^{-1} $Na_2B_4O_7 \cdot 10H_2O$ (pH 8.8) and 1500 µL of enzyme extract. The optical density (OD) of reaction mixture was measured at 290 nm after 0, 0.5, 2 and 3 h while incubating at 37 °C in a water bath. The OD change with time was linear; therefore specific enzymatic activity was calculated as increase in optical density per kilogram of fruit per second (ΔOD kg^{-1} s^{-1}) between 0 and 3 h (Eq. 5.1).

5.2.4.2 *Chitinase activity*

Chitinase activity was determined by the method of Pombo et al. (2011) with some modifications. Frozen strawberries (≈ 5 g) were homogenized (Ultra-Turrax® T25 Basic) with 15 mL of 10 mmol L^{-1} $CH_3COONa.\ 3H_2O$ buffer, 2% (w/v) Polyvinylpyrrolidone (PVP), pH 5. The homogenate was stirred for 3 h at 4 °C and centrifuged at 12,000 × *g* for 30 min. The pellet was discarded and chitinase activity was assayed from supernatant using following reaction mixtures as employed by Pombo et al. (2011): 739 µL of 2 g L^{-1} chitin azure (Sigma) and 2210 µL of enzyme extract. The reaction mixture was kept at 37 °C while stirring, 710 µL of extract was taken at 0, 2, 4 and 6 h

and the reaction was stopped by adding 178 µL of 2 M HCl at each time interval. The samples were kept on ice for 5 min and increase in absorbance at 575 nm was measured between 0 and 6 h (Eq. 5.1). Chitinase activity data of only one experiment (January 2012) is presented in this chapter.

5.2.4.3 *Peroxidase (POD) activity*

The method for determination of POD activity was modified from Civello et al. (1995). The extraction buffer consisted of 0.02 mol L^{-1} Na_2HPO_4, 0.08 mol L^{-1} NaH_2PO_4, 0.1% (v/v) Triton X-100, 1 mol L^{-1} NaCl, 30 g L^{-1} PVPP, at pH 6. Frozen strawberries (≈ 5 g) were mixed with extraction buffer (1:4) and homogenized with a high-performance dispersing instrument (Ultra-Turrax® T25 Basic) for 2 min. The mixture was left to stir for 1 h and then centrifuged at 12,000 × *g* for 20 min. The supernatant was immediately used as enzyme extract. The reaction mixture consisted of: 500 µL of 0.02 mol L^{-1} Na_2HPO_4/0.08 mol L^{-1} NaH_2PO_4 buffer at pH 6, 200 µL of 2 mmol L^{-1} pyrogallol, 200 µL 4 mmol L^{-1} H_2O_2 and 100 µL enzyme extract. The mixture was kept at 20 °C and the enzymatic activity determined by calculating the increase in OD at 470 nm between 0 and 15 min (Eq. 5.1).

5.2.4.4 *Polyphenol oxidase (PPO) activity*

Enzyme extract was prepared according to Pombo et al. (2011) with some minor modifications as described in section 5.2.4.3. The resulting supernatant was used in the enzyme assay described below.

The following reaction mixture was used: 950 µL 0.02 mol L^{-1} Na_2HPO_4/0.08 mol L^{-1} NaH_2PO_4 buffer at pH 6, 400 µL of enzyme extract (section 5.2.4.3) and 150 µL of 20 mmol L^{-1} pyrocatechol. The mixture was kept at 20 °C and increase in optical density (OD) at 410 nm was measured as the enzyme activity over a 90 minute period. The enzyme activity was determined by calculating the increase in absorbance between 0 and 90 min (eq. 5.1).

In all cases distilled water was used as solvent and pH was adjusted to the desired value by using 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl. Control reactions were employed in buffer without enzyme extract and no increase in OD was observed. The enzyme activity was expressed as ΔOD kg^{-1} s^{-1} as described in equation 5.1. Optical density was measured with Shimadzu UV 160A UV-VIS spectrophotometer.

$$\Delta OD = \frac{\frac{(OD_f - OD_i)}{m}\left(\frac{V_s}{V_e}\right)}{t} \qquad \text{(Eq. 5.1)}$$

Where OD_f = final optical density; OD_i = initial optical density at time (t); m= fruit sample mass (kg); V_s = volume of sample (mL); V_e = volume of enzyme extract (mL); t = time between initial and final measurements (s).

5.2.5 Data Analysis

All data were subjected to analysis of variance (ANOVA) with Minitab Version 16 (Minitab Inc, State College, PA, USA). For percent rot incidence one-way ANOVA was used at each time point. Data were tested for normality and homogeneity of variance. Means were compared by a Tukey's test at a significance level of 0.05.

5.3 Results

5.3.1 Rot incidence

Hypobaric treated (50 kPa_a, 4 h) strawberries showed significant reduction in natural rot incidence after subsequent storage for 4 d at 20 °C as compared to control (P< 0.05, Figure 5.1). Visible fungal rots were observed 2 days after treatment in both treated and control samples (data not shown). Hypobaric treatment reduced fungal rot by 36, 28 and 27% respectively in three harvests after 4 days at 20 °C.

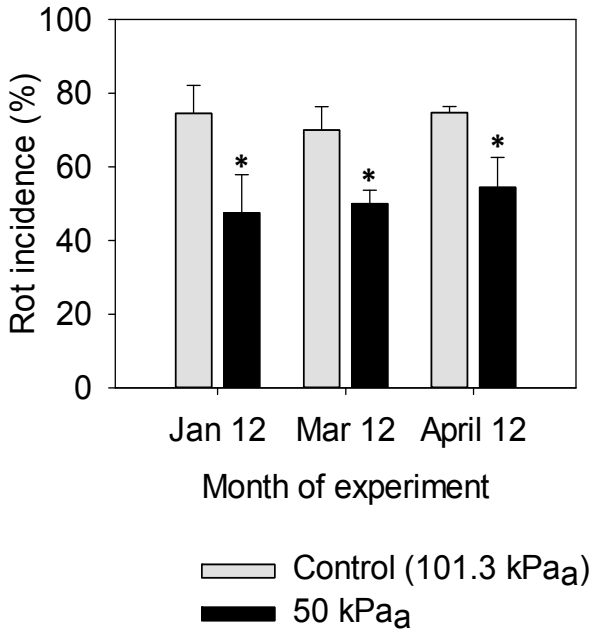

Figure 5.1 Effect of hypobaric treatment (50 kPa_a, 4 h) on percentage rot incidence of strawberries after subsequent storage for 4 d at 20 °C. Each bar represents the mean value of four replicate clamshells. Significant differences (P< 0.05) are represented by * as determined by Tukey's test. Error bars represent standard errors of means.

5.3.2 PAL activity

In all 3 experiments hypobaric treatment consistently increased PAL activity of strawberries as compared to control 12 h after treatment (P< 0.05, Figure 5.2). Enzyme

activity of treated and control strawberries remained similar up to 6 h after treatment. The increase in PAL activity of treated samples 12 h after treatment was 40% (Figure 5.2A), 31% (Figure 5.2B) and 32% (Figure 5.2C). PAL activity differed noticeably among batches of strawberries ranging from 0.04 to 0.08 ΔOD kg^{-1} s^{-1} at harvest (Figure 5.2).

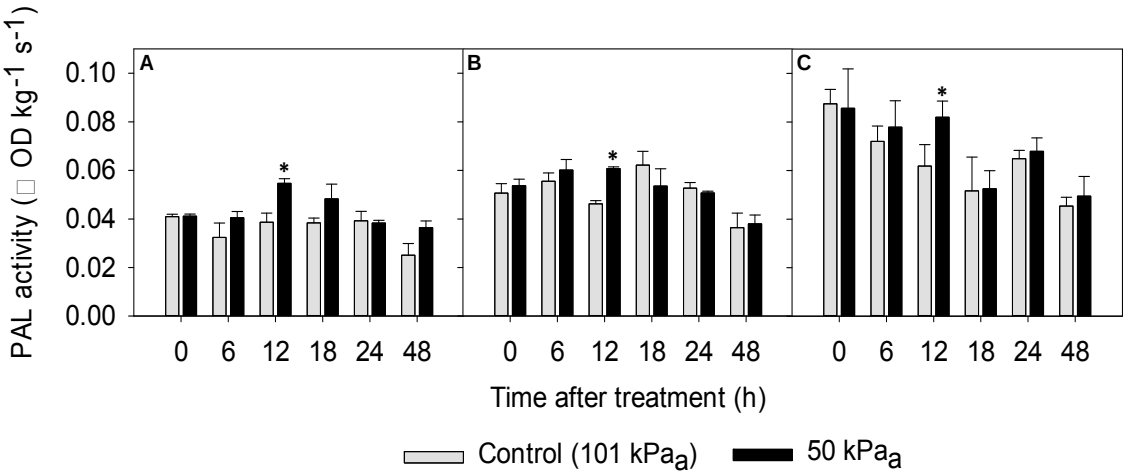

Figure 5.2 Effect of hypobaric treatment (50 kPa_a, 4 h) on PAL activity of strawberries harvested in (A) January, (B) March and (C) April 2012. Each bar represents the mean value of four independent measurements. Error bars represent standard errors of means. Significant differences (*P*< 0.05) at each measurement time are represented by *.

Data from January displayed a significant rise (*P*< 0.05) in treated samples 12 h after treatment. PAL activity of treated samples was slightly higher 18 h after treatment but started subsiding to a minimum level 48 h after treatment (Figure 5.2A). Likewise results from experiment in March reveal comparable PAL value immediately after treatment but with higher levels than data observed in January (Figure 5.2B). PAL activity of treated samples started increasing 6 h after treatment with a significantly higher (*P*< 0.05) activity 12 h after treatment as compared to control. Both control and

treated samples showed a gradual decrease 18 h after treatment with a lowest activity observed 48 h after treatment.

In April, the initial PAL activity of both treated and control was almost twofold of samples observed in January. Control samples showed gradual decrease 6 h after treatment with a dramatic decline observed 12 h after treatment, while treated samples maintained PAL activity for 12 h (Figure 5.2C). Minimal value was observed in both treated and control samples 48 h after treatment.

PAL activity of other treatments (50 kPa_a, single, multiple pull; slow, fast treatments and treatment for 2 h, 6 h; section 3.2.1.3) was also analysed. A 50 kPa_a treatment with multiple pull demonstrated significantly ($P< 0.05$) reduced PAL activity immediately after treatment as compared to control and 50 kPa_a (single pull) (Figure 5.3A). The 50 kPa_a (single pull) treatment repeated the increase in PAL activity 12 h after treatment observed 3 times previously (Figure 5.2). A 2 h or 6 h hypobaric treatment did not increase PAL activity of strawberries (Figure 5.3B). In addition, 6 h treatment did not delay rot development during subsequent storage at 20 °C, whereas 2 h treatment showed significant difference only on day 4 when the rot incidence was above 60% (Figure 3.8A).

Hypobaric treatment with slow or fast pressure release were equally effective in increasing PAL activity 12 h after treatment (Figure 5.3C), though in this experiment control also showed increase when compared with control data in other experiments (Figure 5.3A and B).

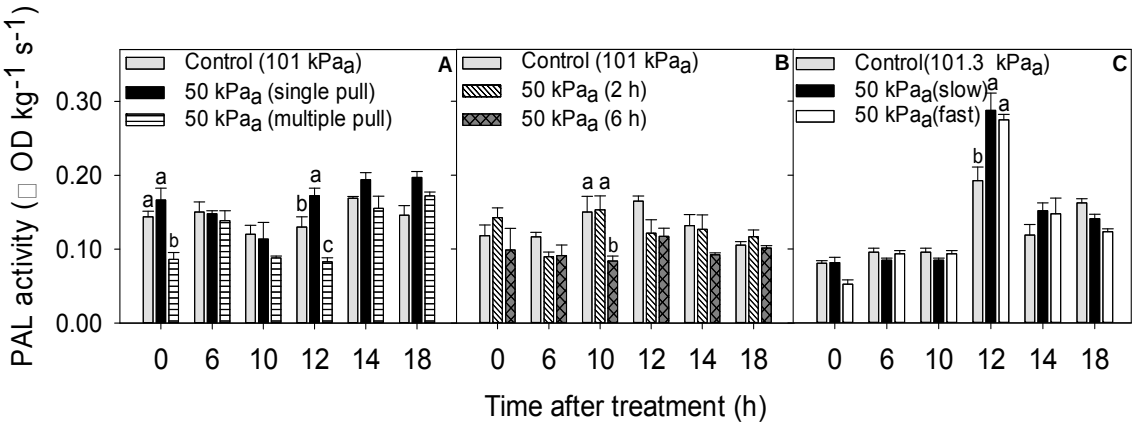

Figure 5.3 Effect of repeated application (A), duration of hypobaric treatment (B) and releasing pattern of hypobaric treatment (C) on PAL activity of strawberries. Each bar represents the mean value of four independent measurements. Error bars represent standard errors of means. Different letters at each measurement time show significant differences (*P*< 0.05) from each other as determined by Tukey's test.

5.3.3 Chitinase activity

Chitinase activity remained higher in hypobaric treated samples as compared to control during subsequent storage for 48 h (*P*< 0.05, Figure 5.4). Activity of control samples remained consistent through 48 h, while in treated fruit the peak value was observed 12 h after hypobaric treatment. Treated samples showed consistent activity for 24 h before gradually decreasing 48 h after treatment.

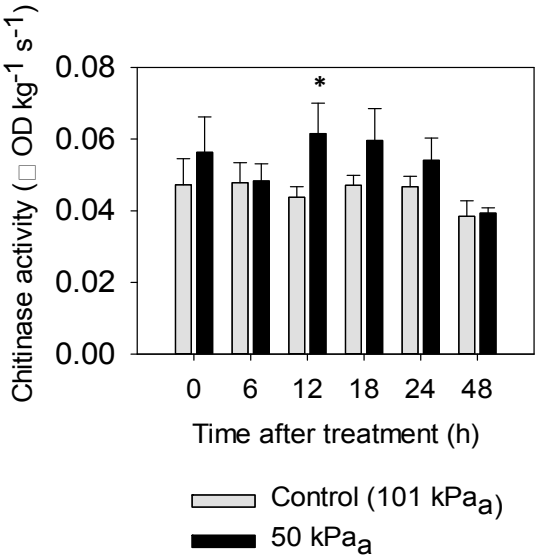

Figure 5.4 Effect of hypobaric treatment (50 kPa_a, 4 h) on chitinase activity of strawberries. Each bar represents the mean value of four independent measurements. Error bars represent standard errors of means. Significant differences (P< 0.05) at each measurement time are represented by *.

5.3.4 POD activity

An increase in POD activity of hypobaric treated samples was consistently observed immediately after treatment, reverting to the same activity as control from 6 h after treatment (P< 0.05, Figure 5.5). Initial increase in POD activity of treated samples was 80% (Figure 5.5A), 19% (Figure 5.5B) and 35% (Figure 5.5C) in three experiments carried out at different times of year. Similarly a significant (P< 0.05) increase (29%) was observed 12 h after hypobaric treatment only in first experiment (Figure 5.5A). Apart from immediate increase, the POD activity of both treated and control samples remained inseparable in second and third experiments (Figure 5.5B and C).

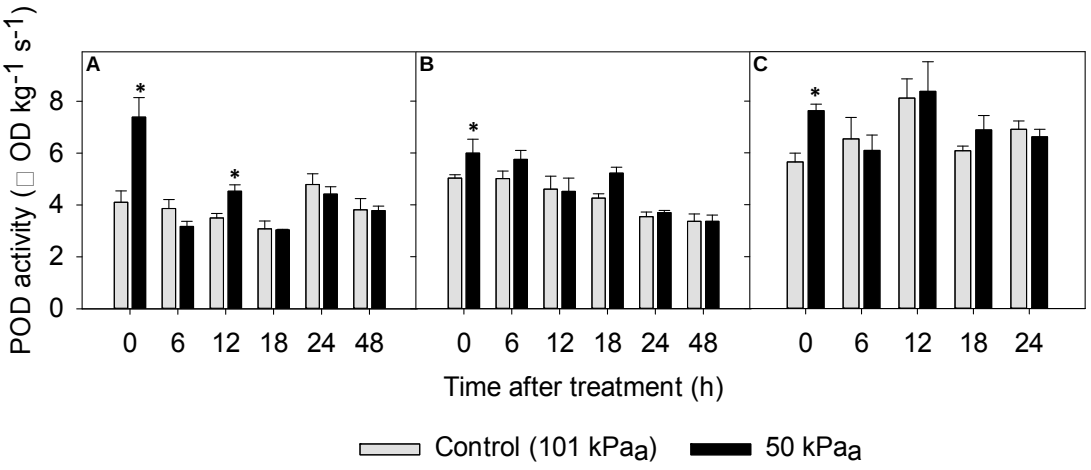

Figure 5.5 Effect of hypobaric treatment (50 kPa_a, 4 h) on POD activity of strawberries harvested in (A) January, (B) March and (C) April 2012. Each data point represents the mean value of three independent measurements. Vertical bars represent standard errors of means. Significant differences (*P*< 0.05) at each measurement time are represented by *.

5.3.5 PPO activity

PPO activity of both control and hypobaric treated strawberries was similar during subsequent storage at 20 °C for 48 h (Figure 5.6), indicating that 50 kPa_a treatments did not induce a change in PPO. In addition, PPO activity of both treated and control samples remained stable during subsequent storage for 24 h.

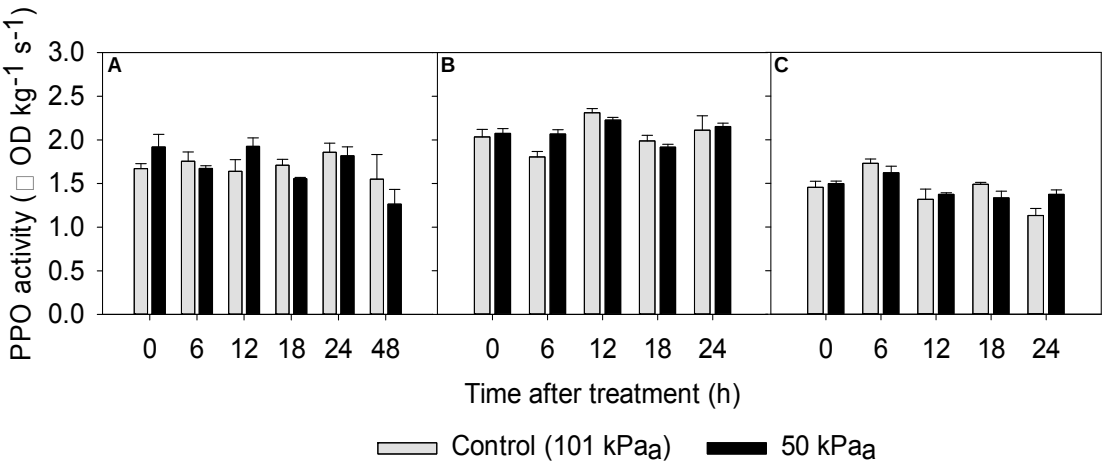

Figure 5.6 Effect of hypobaric treatment (50 kPa_a, 4 h) on PPO activity of strawberries harvested in (A) January, (B) March and (C) April 2012. Each bar represents the mean value of three independent measurements. Error bars represent standard errors of means. There were no significant differences (*P*> 0.05) between the treatments in all cases.

5.4 Discussion

In this study hypobaric treatment significantly delayed postharvest fungal decay in strawberries obtained during different seasonal timings. These data sets are additional to that presented previously (Figure 3.2 and Figure 3.5). Hence these data further demonstrate a consistent delay in development of decay for hypobaric treated strawberries irrespective of seasonal timing. Similar results have previously been reported for hypobaric treated sweet cherries, grapes, strawberries (Romanazzi et al., 2001) and blueberries (Hashmi et al., 2013).

The experiment on post-hypobaric treatment fungal inoculation (immediately and 12 h after treatment) demonstrated a reduction in strawberry decay (Figure 4.5A and C; 4.6A and C). The observed immediate increase in POD (Figure 5.5) and delayed (12 h

after treatment) rise in PAL (Figure 5.2) and chitinase (Figure 5.4) may indicate a causative correlation between these enzyme activities and a decrease in strawberry decay.

Previous studies investigated time ranges of induced resistance in different treatments (Pombo et al., 2011; Shao et al., 2013; Yu et al., 2012). Nigro et al. (2000) observed a twofold increase in PAL activity 12 h after mild UV-C treatment, while Pombo et al. (2011) reported a threefold rise in PAL activity immediately after higher UV-C treatment doses of strawberries. Across the 3 replicate experiments, the current results demonstrate up to 34% increase in PAL activity 12 h after hypobaric treatment (Figure 5.7), suggesting that hypobaric treatment may create a milder stress than UV-C treatment. However one potential reason for the lower response in this work is the treatment of near to ripe strawberry while the previous studies were carried out on 75% red strawberries. As described earlier (section 2.3), the mycelial development remains quiescent till full maturity of fruit (Sinclair and Cerkauskas, 1996). In addition, Terry et al. (2004) demonstrated that higher fruit maturity significantly affects strawberry antifungal compounds, (proanthocyanins and catechin), as a result decreasing fruit resistance against diseases. Moreover, higher levels of sucrose, glucose and fructose (non-structural carbohydrates) in ripe strawberries also provide favourable environment for fungal development. Therefore induced resistance may be dependent on the ripening stage of fruit (Yao and Tian, 2005). Perhaps the partially ripe strawberry has mycelia in quiescent stage and has higher concentrations of PAL and higher levels of antifungal compounds; thus a higher response to induced treatment results. Either way, the elevated PAL activity reported in the current study

signifies an ability of hypobaric treatment to induce resistance in commercially mature stage.

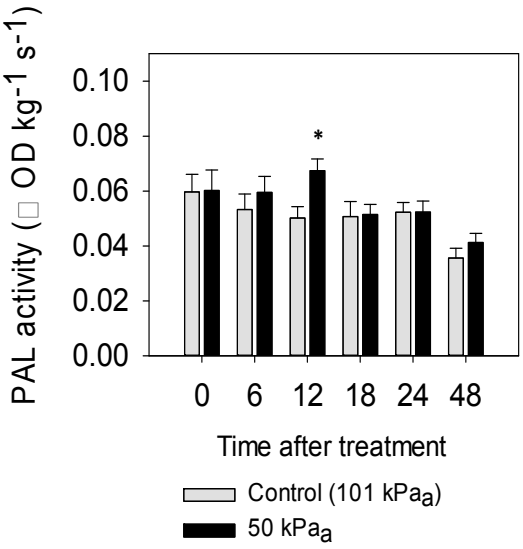

Figure 5.7 PAL activity determined from the combination of three experiments as presented in figure 5.2. Each bar represents the mean value of 4 independent measurements of 3 replicate experiments (Figure 5.2). Error bars represent standard errors of means. Significant differences (*P*< 0.05) at each measurement time are represented by *.

In another study Shao et al. (2013) found a 50% rise in PAL activity of inoculated strawberries 24 h after tree oil (TTO) vapour treatment, which also resulted in 50% rot reduction. A 50% rot reduction in hypobaric treated strawberries inoculated 12 h after treatment was observed in this work (Figure 4.5-6).

Results of experiment on application of 50 kPa_a (multiple pull) show that repeated variation in pressure caused decrease in PAL activity 12 h after treatment (Figure 5.3A). The rot incidence in these samples (87%) was not statistically different from control (85%) in subsequently storage for 4 d at 20 °C (Figure 3.9A). Similarly treatment of 50 kPa_a for 6 h indicated reduction in PAL activity 10 h after treatment (Figure 5.3B), with 75% rots as compared to control (82%) in subsequent storage for

4 d at 20 °C (Figure 3.8A). This implies that 6 h treatment might impose more stress on fruit, counteracting the effectiveness of hypobaric treatment in reducing postharvest fungal rots. Across all experiments the 50 kPa_a treated samples (single pull) showed higher PAL activity 12 h after treatment and were also most effective in reducing rots when compared with multiple pull and 6 h hypobaric treatment (Figure 5.8). This indicates that PAL activity might be playing an essential role in rot reduction of hypobaric treated strawberries. However there was a big variation in the PAL activities of different experiments, for example the lowest PAL activity recorded in April (Figure 5.2C) was almost equal to the highest levels reported in January (Figure 5.2A). Different factors such as pre-harvest weather, temperature and seasonal variation might be contributing to fluctuation in PAL activity of strawberries.

Contrary to the results found in this work, Civello et al. (1997) observed a reduction in PAL activity and fungal rots of heat treated (48 °C for 3 h) strawberry. This decline was correlated with delayed fruit softening and surface colour change, suggesting it may have been related with ripening. Hypobaric treatment did not affect firmness during storage (Figure 3.4A) and the observed rot reduction in treated fruit (Figure 5.1) was due to the response of fruit resistance (Figure 5.2, Figure 5.4 and Figure 5.5) to hypobaric treatment stress.

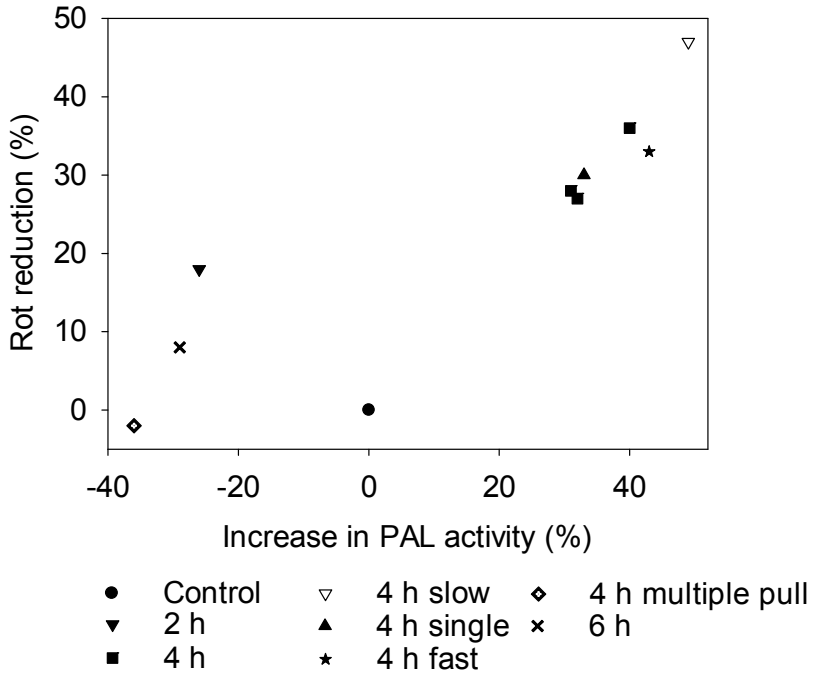

Figure 5.8 Comparison of percent increase in PAL activity and rot reduction of hypobaric treated (50 kPa_a) strawberries in comparison to non-treated control (101 kPa_a). Percent increase in PAL activity was calculated from the data taken 12 h after hypobaric treatment, whereas percent rot reduction data represent reduction on the day when maximum rots (> 60%) were visible.

In this study a 38% increase in chitinase activity was observed 12 h after hypobaric treatment (Figure 5.4), coinciding with a rot reduction by 36% (Figure 5.1). Previously, individual strawberries inoculated 0 and 12 h after treatment also showed significant reduction ($P< 0.05$) in rots (Figure 4.5A and C). Ippolito et al. (2000) reported that inoculation of *Aureobasidium pullulans* in apple wounds caused threefold transient increase in chitinase activity after 96 h which reduced fungal rot development. Similarly Wang et al. (2010) observed 70% rot reduction in hot air treated (48 °C, 3 h) bayberry fruit, correlating it to a 60% rise in chitinase activity after 48 h of inoculation with *Leptographium abietinum*.

This research observed an 80, 19 and 35% increase in POD activity (Figure 5.5) in strawberries immediately after treatment corresponding with a significant decrease in rots (Figure 5.2). Yu et al. (2012) demonstrated that yeast saccharide treatment induced the activities of POD, PAL and chitinase causing reduction in decay of peach fruit. Wang et al. (2010) observed that hot air treatment of inoculated chinese bayberry fruit increased POD activity by 38%, reducing rots by 70%. In the current work, the immediate rise in POD activity (Figure 5.5) after treatment may be correlated with rot reduction observed in treated strawberries inoculated immediately after treatment (Figure 4.5A and Figure 4.6A). Rise in POD activity is associated with the response of fruit against different stresses (Matamoros et al., 2003). Pre-infection increase in POD stimulates plant defence system against pathogens and catalyses reinforcement of cell wall proteins (Djebali et al., 2007) which may limit the diffusion of pathogenic toxin into the host cell (Brisson et al., 1994). In the current work, perhaps the initial increase in POD after hypobaric treatment (Figure 5.9) may have strengthened cell wall and restricted pathogen entry, resulting in reduced rot incidence.

In contrast, Gao et al. (2006) reported a reduction in POD activity of loquat during hypobaric (40-50 kPa_a) storage for 49 d at 2-4 °C. This reduction was correlated with diminished ethylene production and respiration rate, which indicated that long-term hypobaric storage influenced ripening and senescence of fruit. However, previous results have demonstrated that hypobaric treatment (50 kPa_a, 4 h) does not affect respiration rate (Figure 3.3) and firmness (Figure 3.4A) of strawberries during subsequent storage at 20 °C. In addition, loquat is a climacteric fruit with high

production of ethylene and respiration rate during ripening while current study is about strawberry which is a non-climacteric fruit. Furthermore study on loquat was about hypobaric storage for a longer time (49 d) at 2-4 °C, while current study is about the effect of short-term (4 h) hypobaric treatment during subsequent storage at 20 °C, therefore this work could not be compared with the above study on loquat.

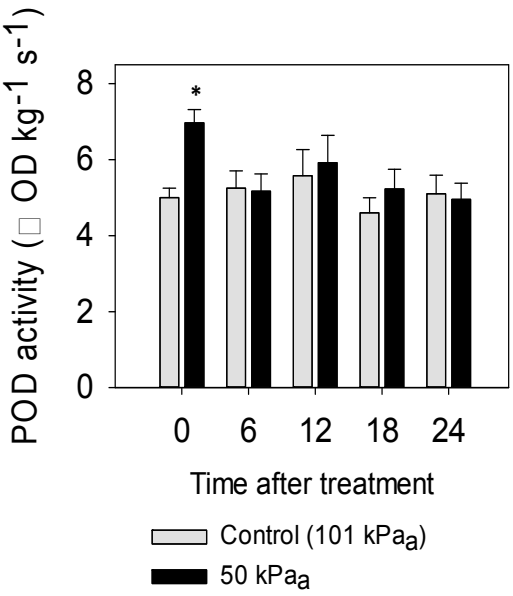

Figure 5.9 POD activity determined from the combination of three experiments as presented in figure 5.5. Each bar represents the mean value of 4 independent measurements of 3 replicate experiments (Figure 5.5). Error bars represent standard errors of means. Significant differences (*P*< 0.05) at each measurement time are represented by *.

This work demonstrated that hypobaric treatment did not stimulate PPO activity during subsequent storage (Figure 5.6). Previously Liu et al. (2010) and Shao et al. (2010) have suggested that PPO may not be involved in disease resistance in loquat and apple respectively. In contrast, Yuan et al. (2013) reported an increase in PPO activity of muskmelon fruit after hot water dipping at 53 °C for 3 min causing reduction in rot incidence. Similarly Wang et al. (2004) demonstrated a rise in PPO activity of

peach 3 d after inoculation with *Cryptococcus laurentii*. This transitory rise in PPO was correlated with induction of resistance resulting in reduction of peach decay. Results of this study indicate that the potential induced resistance caused by hypobaric treatment in strawberry may not be associated with PPO activity

In this study hypobaric treatments were applied to create a stress condition at cellular level to potentially activate the defence system of fruit. It is well documented that stress causes plant cells to defend themselves by increasing the activity of defence-related enzymes, strengthening of cell walls and production of fungitoxic substances (Wang et al., 2006). Previous studies suggested that cellular hydrostatic pressure is decreased with reduction of atmospheric pressure (Eckert and Ratnayake, 1983), affecting the forces near the plasma membrane resulting in activation of mechanosensitive ion channels (Zonia and Munnik, 2007). As a result, transport of material and passive permeability are altered leading to thickening of the cell wall (Eckert and Ratnayake, 1983). Accumulation of lignin is one of the important steps in cellular level defence in plants. PAL is one of the key enzymes in lignifications of the cell wall, while POD activates dehydrogenation and polymerization for cell wall reinforcement (Van Loon et al., 2006). In the present study, the surge in PAL activity (Figure 5.2) and POD activity (Figure 5.5) may be involved in the immediate cell wall reinforcement through catalyzing lignification and initiating phenylpropanoid pathway. These processes might be responsible for prolonged maintenance of resistance in fruit tissue (Barkai-Golan, 2001) resulting in strawberry rot reduction (Figure 5.1).

In this study chitinase activity peaked 12 h after hypobaric treatment (Figure 5.4), while experiments on inoculating hypobaric treated strawberries 12 h after treatment also showed reduced rots (Figure 4.5C and Figure 4.6C). In contrast, previous studies suggested that increase in chitinase activity as a result of biotic and abiotic stresses may not be directly associated with early host-pathogen interactions (Punja and Zhang, 1993). However, recently Grover (2012) stated that accumulation of chitinases after fungal attack was the outcome of induced resistance and thus plays an important role in plant resistance to pathogen invasion. Therefore the involvement of chitinase in early stage of fungal attack in this case could not be ruled out.

Despite the fact that this work demonstrates a consistent increase in the defence-related enzymes of hypobaric treated strawberries, the process of increase in these enzymes was also labile. One might expect a requirement for a prolonged alteration in enzyme activities to impact fungal decay. Nevertheless there are previous examples where brief increases of defence-related enzymes influenced rot development in fruit. Yao and Tian (2005) reported that a 90% increase in PAL activity 4 d after salicylic acid (SA) treatment resulted in 60% rot reduction in sweet cherries after subsequent storage for 15 d at 25 °C. Similarly Pombo et al. (2011) observed a 44% decrease in rots of inoculated UV-C treated strawberries after 9 d at 20 °C, which was correlated with a 61% increase in POD activity 4 h after UV-C treatment. In the current study increase in POD activity (80, 19 and 35%; Figure 5.5) and PAL activity (40, 31 and 32%; Figure 5.2) immediately and 12 h after treatment respectively could be correlated with 36, 28 and 27% (Figure 5.1) reduction in strawberry rots after subsequent storage for 4 d at 20 °C.

5.5 Conclusion

Post-hypobaric treatment transient increase of defence-related enzymes (POD, PAL and chitinase) might be collectively or individually associated with stimulation of induced resistance limiting fungal development in strawberry.

Chapter 6 Effectiveness of hypobaric treatment at low temperature (*)

6.1 Introduction

Strawberries are a highly perishable fruit with a storage life of 1-2 days at room temperature (Cao et al., 2010a). High respiration rate and weight loss; physical injury during handling, transportation and storage; and susceptibility to fungal rots make strawberry extremely perishable (Wills and Kim, 1995; Zhang et al., 2006).

Temperature management is one of the important factors in reducing fruit decay and extending storage life (Ayala-Zavala et al., 2004; Li and Kader, 1989). Takeda et al. (1990) observed 35% decay in strawberries after 2 d storage at 18 °C, while samples stored at 5 °C showed slight fungal decay after 13 d of storage (Ayala-Zavala et al., 2004). In chapter 3, it was demonstrated that hypobaric treatment (50 kPa_a, 4 h at 20 °C) significantly reduced strawberry decay during subsequent storage at 20 °C. However the strawberry industry uses temperature management as their primary tool to extend the storage life of strawberry. It is possible that the benefit of hypobaric treatment observed at 20 °C may not be commercially significant during subsequent storage at low temperature. Thus to evaluate the practical application of hypobaric treatment, research is required to be conducted under low storage temperature.

Previous research demonstrated that a postharvest delay in cooling (4-8 h at 20-30 °C) reduced the storage quality of strawberry (Nunes et al., 1995b; Pelletier et al., 2011).

(*) This chapter includes some material published in the paper:
Hashmi, M.S., East, A.R., Palmer, J.S., & Heyes, J.A. (2013). Pre-storage hypobaric treatments delay fungal decay of strawberry. Postharvest Biology and Technology 85, 77-82.

In the current work a 4 h hypobaric treatment at 20 °C would consequently cause a delay in shifting to cold storage and may reduce storage life of strawberry. Therefore the choice of applying a hypobaric treatment should be compared with alternatively rapidly cooling the strawberries. An alternative supply chain which removes the extended delay in cooling required to apply hypobaric treatment at 20 °C, is to conduct hypobaric treatment at low temperature. This alternative supply chain also needs to be explored.

The aims of this study were to:

1) Evaluate the effectiveness of hypobaric treatment (50 kPa_a, 4 h at 20 °C) on strawberries subsequently stored at 5 °C.
2) Assess the effect of delay in cooling due to hypobaric treatment at 20 °C on rot development, quality and physiology of strawberries subsequently stored at 5 °C.
3) Investigate the effectiveness of hypobaric treatment at 5 °C.

6.2 Materials and methods

6.2.1 Fruit source

Three batches of 'Camarosa' strawberries were obtained from a commercial grower in Hawkes Bay, New Zealand in February 2011, February 2013 and March 2013. In all cases, strawberries were harvested at near to maturity stage (> 95% red fruit) with calyx and pedicel and transported to Massey University within approximately 2 h of harvest. On arrival at the lab, fruit with visible rot and blemishes were removed from the population.

6.2.1.1 *Effect of hypobaric treatment on strawberries subsequently stored at low temperature*

In one experiment 42 units of commercial vented clamshells (250 ± 10 g) were filled (12-15 fruit per replicate), weighed and randomized. These were divided into two treatments: 50 kPa_a for 4 h in a vacuum vessel, or atmospheric conditions (101 kPa_a) for the same time at 20 °C and subsequently stored at 5 °C. Three independent clamshells per treatment were evaluated destructively on every 3 d for 16 d. The aim of this experiment was to investigate the efficacy of hypobaric treatment (50 kPa_a, 4 h at 20 °C) in reducing strawberry decay during subsequent storage at 5 °C.

6.2.1.2 *Effect of 4 h cooling delay on rot development in hypobaric treated strawberries*

In a second experiment 45 clamshells (250 ± 10 g) were filled with sound strawberries and divided into 3 treatments: 4 h hypobaric treatment at 50 kPa_a at 20 °C, atmospheric conditions (101 kPa_a) for the same time at 20 °C or atmospheric conditions at 5 °C. All the samples were subsequently stored at 5 °C after treatment. Four independent clamshells were assessed destructively on every 3 d for 12 d. This experiment was performed to investigate the effect of 4 h cooling delay on efficacy of hypobaric treatment. In previous experiment (section 6.2.1.1) hypobaric treatment (50 kPa_a, 4 h) was applied at 20 °C. This experiment would help to compare treatment at 20 °C for 4 h with an un-treated control directly stored at 5 °C.

6.2.1.3 *Hypobaric treatment at 5 °C*

In a third experiment 40 clamshells of strawberries were divided into two treatments: 50 kPa_a for 4 h in a pressure chamber or atmospheric conditions (101 kPa_a) for the same time, with both treatments applied at 5 °C and subsequently stored at 5 °C. Four independent clamshells were evaluated on each 3 d for 9 d. Internal temperature of individual strawberry was monitored by Squirrel (SQ 1000 series) temperature data logger. Thermocouples were inserted in individual strawberries placed in the middle of each of 3 instrumented clamshells. Fruit pulp temperature became uniform (5 °C) in about 2 h after shifting to cold storage. Earlier studies have focused on hypobaric treatment at 20 °C. In the light of results from previous experiment (section 6.2.1.2), this study was conducted to evaluate the effectiveness of hypobaric treatment (50 kPa_a, 4 h) at 5 °C.

6.2.2 Hypobaric treatment

Hypobaric pressure was generated as described in section 3.2.2. For hypobaric treatment at 5 °C, the pressure chamber was placed in cold storage room one day before experiment. Temperature and relative humidity (RH) inside the pressure vessel were monitored with a data logger (TGU-1500, Tinytag Ultra Gemini, West Sussex, UK).

6.2.3 Fruit assessment

6.2.3.1 *Rot incidence*

Rot incidence was evaluated by visually examining each berry. Fruit with visible decay were counted with the results recorded as percent rot incidence per replicate.

6.2.3.2 *Respiration rate*

Respiration rate was determined from the rate of CO_2 production. From each clamshell two replicate sound fruit of a known weight were each placed into a 540 mL airtight glass jar fitted with a rubber septum. Headspace samples of 1 mL were collected at 0 and 30 minutes at 5 °C and injected into a CO_2 analyzer equipped with a miniature infrared CO_2 transducer (Analytical Development Company, Hoddesdon, UK) at 20 °C. Nitrogen was used as the carrier gas at a flow rate of 35 mL min^{-1}. The carbon dioxide sensor was calibrated by injecting 1 mL β-standard 0.49 ± 0.01% CO_2 (BOC, Auckland, New Zealand). Output signals were recorded with an integrator (HP 3396A, Hewlett Packard, California, USA).

6.2.3.3 *Firmness*

Firmness of strawberries was determined using a TA-XT2i texture analyzer (Stable Micro Systems Ltd. Surrey, UK) as described in section 3.2.3.3. Firmness of 5 sound fruit was measured per replicate at each storage interval.

6.2.3.4 *Weight loss*

Initial net weight of each clamshell was recorded to 0.001 g accuracy using a balance (Mettler Toledo PG 503-S, Columbus, USA) at 20 °C or 5 °C. Clamshells were reweighed at each storage interval and percentage weight loss from the initial was calculated.

6.2.4 Data Analysis

A Completely Randomized Design was used in all experiments. All the data were analysed for homogeneity of variance and then subjected to overall analysis of

variance (ANOVA) with Minitab Version 16 (Minitab Inc, State College, PA, USA). Means were compared by a Tukey's test at a significance level of 0.05.

6.3 Results

6.3.1 Effect of low temperature storage on hypobaric treated strawberries

The onset of the rot development was slow in both treated and control fruit due to low temperature. Visible fungal rots were observed after 4 d in both treated and control fruit. Rots developed steadily during storage at 5 °C, reaching >60% on day 16 in both treatments, but hypobaric treatment (50 kPa_a, 4 h at 20 °C) significantly (P< 0.05) reduced percent rot incidence of strawberries after 16 d (Figure 6.1A).

Overall firmness of hypobaric treated strawberries was comparable with control during storage (Figure 6.1B). Nevertheless hypobaric treatment led to significantly firmer fruit with 28% reduction in firmness as compared to control (33%) on day 16 at 5 °C. Respiration rate and percent weight loss of treated fruit remained similar to control through 16 d storage at 5 °C (Figure 6.1C and D). Respiration rate of both treated and control samples increased on day 4, but after a dramatic decrease in control fruit on day 7, steady respiration rate was observed through 16 d.

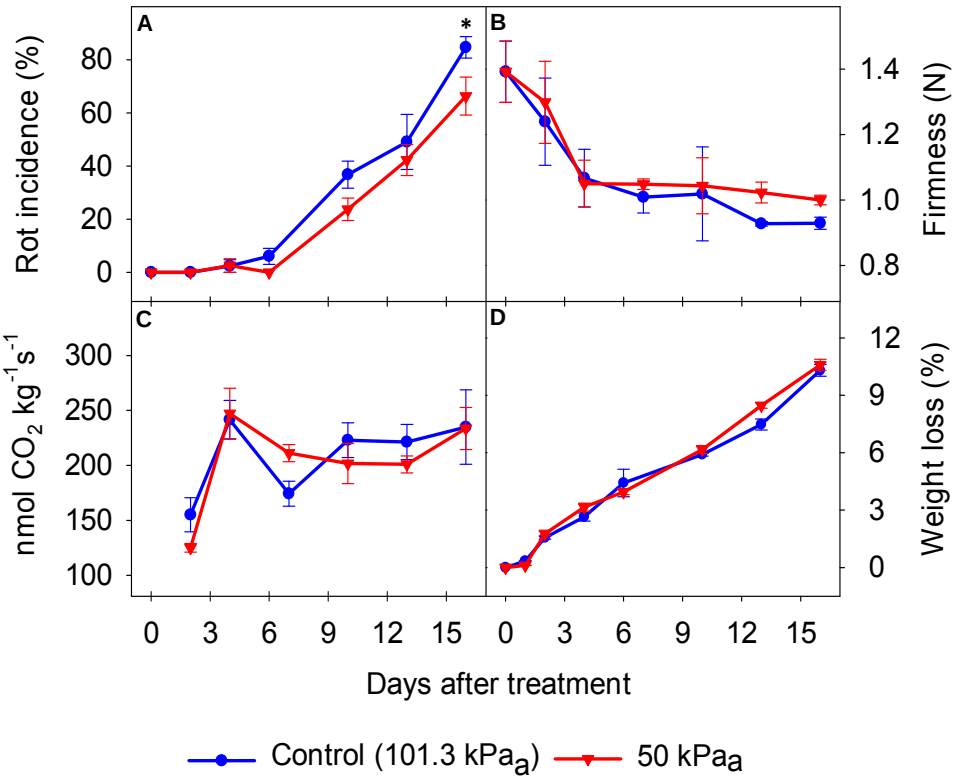

Figure 6.1 Effect of hypobaric treatment (50 kPa$_a$, 4 h at 20 °C) on percent rot incidence (A), firmness (B), respiration rate (C) and percent weight loss (D) in 'Camarosa' strawberries subsequently stored at 5 °C. Overall significant difference (*P*< 0.05) is shown by * as determined by Tukey's test. The data for day 0 was collected immediately after hypobaric treatment. Each data point represents the mean value of three replicate clamshells. Vertical bars represent standard errors of means.

6.3.2 Effect of cooling delay on strawberry rot development, physiology and quality

Rot incidence in hypobaric treated (50 kPa$_a$, 4 h at 20 °C) strawberries subsequently stored at 5 °C was similar to control samples stored immediately at 5 °C for 12 d (Figure 6.2A). However the treatment significantly (*P*< 0.05) reduced rots by 36% on day 12 when compared with control kept at 20 °C for 4 h and subsequently stored at 5 °C.

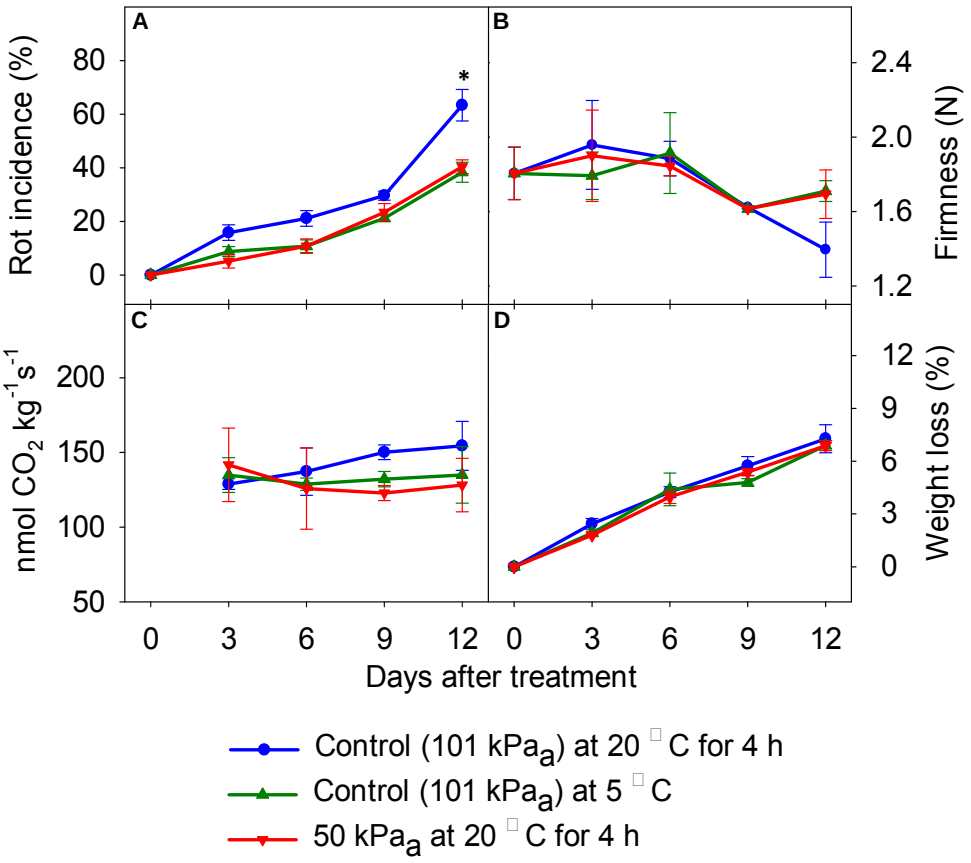

Figure 6.2 Effect of cooling delay caused by hypobaric treatment (50 kPa_a, 4 h at 20 °C) on percent rot incidence (A), firmness (B), respiration rate (C) and percent weight loss (D) in 'Camarosa' strawberries subsequently stored at 5 °C. Control (blue line) at 20 °C for 4 h was followed by a subsequent storage at 5 °C. Overall significant difference (*P*< 0.05) is shown by * as determined by Tukey's test. The data for day 0 was collected immediately after hypobaric treatment. Each data point represents the mean value of four replicate clamshells. Vertical bars represent standard errors of means.

Firmness of all samples significantly (*P*< 0.05) reduced during subsequent storage at 5 °C (Figure 6.2B). Hypobaric treatment did not affect the firmness of fruit, though the control kept at 20 °C for 4 h before subsequent storage at 5 °C was slightly softer than treated and control samples at 5 °C.

Respiration rate of all treatments remained consistent during subsequent storage at 5 °C (Figure 6.2C). Control at 20 °C for 4 h and subsequently stored at 5 °C has slightly higher respiration rate than other two treatments in subsequent storage at 5 °C after 12 d. Percent weight loss increased to 7% in subsequent storage at 5 °C after 12 d, with no difference among treatments (Figure 6.2D).

6.3.3 Effectiveness of hypobaric treatment at low temperature

In this experiment hypobaric treatment (50 kPa_a, 4 h) at 5 °C significantly reduced rot incidence in comparison to control at atmospheric pressure (101 kPa_a) subsequently stored at 5 °C for 9 d (Figure 6.3A). A consistent increase of decay in control fruit was observed, while the progression of fungal rot was slow in treated strawberries.

Firmness of treated strawberries remained consistent for 3 d while control fruit steadily lost firmness (Figure 6.3B). Overall firmness was reduced ($P< 0.05$) in both treated and control samples (26 and 30% respectively) during subsequent storage at 5 °C for 9 d. No significant differences between the treatments during storage were found.

Respiration rate of hypobaric treated strawberries remained constant, while control samples showed a significant increase in respiration rate during subsequent storage at 5 °C for 9 d (Figure 6.3C). However respiration rate of treated strawberries remained comparable with control during storage. Similarly percent weight loss was unaffected by hypobaric treatment during storage (Figure 6.3D).

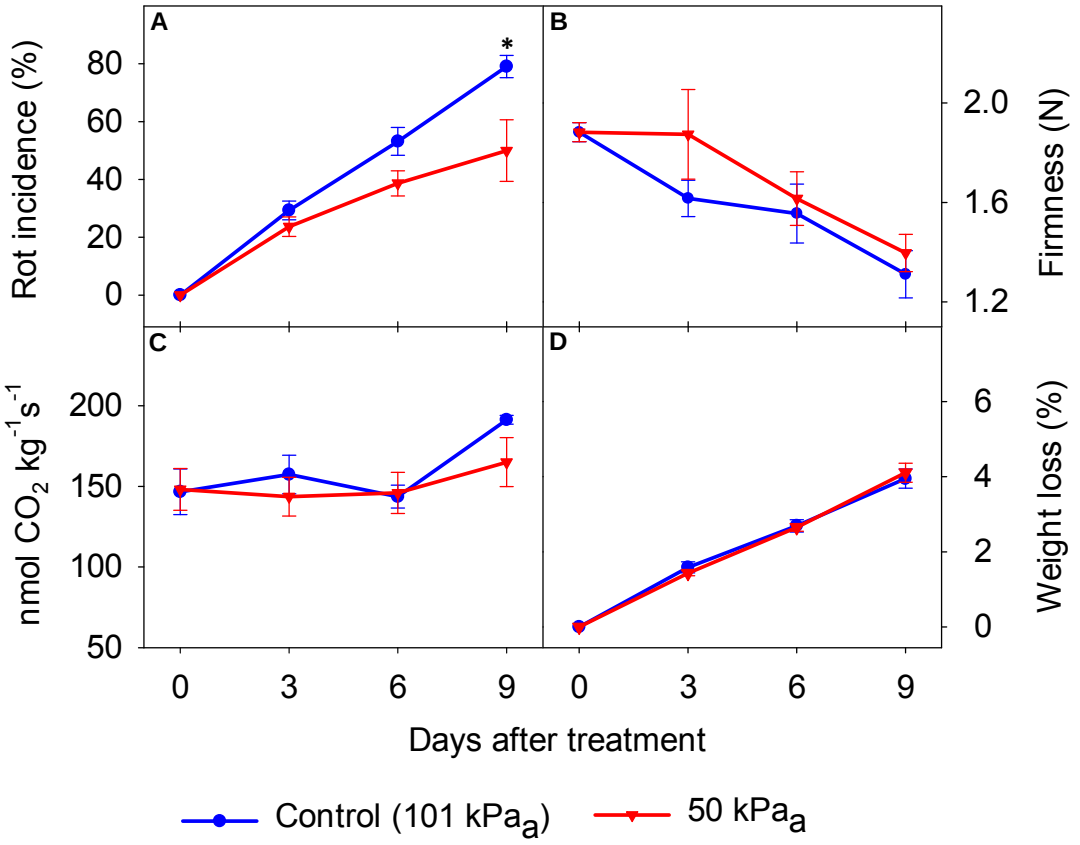

Figure 6.3 Effect of hypobaric treatment (50 kPa_a, 4 h at 5 °C) on percent rot incidence (A), firmness (B), respiration rate (C) and percent weight loss (D) in 'Camarosa' strawberries subsequently stored at 5 °C. Overall significant difference (P< 0.05) is shown by * as determined by Tukey's test. The data for day 0 was collected immediately after hypobaric treatment. Each data point represents the mean value of four replicate clamshells. Vertical bars represent standard errors of means.

6.4 Discussion

Storage temperature is an imperative factor in maintaining the storage life of fruit due to its effects on fruit quality and microbial growth (Ayala-Zavala et al., 2004). A 50 kPa_a treatment at 20 °C reduced (P< 0.05) rot incidence by 22% after 16 d at 5 °C (Figure 6.1A). Similar to earlier results at 20 °C (Figure 3.2), grey mould and strawberry leak were the main rots observed, however in this case the onset of fungal development

was delayed due to low storage temperature (5 °C). Nunes (2009) reported that grey mould germination was retarded at 5 °C and expansion of grey mould lesions further reduced at 2 °C, while no decay was noticed on 0.5 °C in strawberry (Shin et al., 2007). Storage temperature in this work was 5 °C; therefore rots were visible after a delay as compared to warmer temperature (20 °C).

Comparable to these results, Cao et al. (2010b) demontrated that ultrasound treatment (40 kHz, 10 min for 20 °C) inhibited strawberry decay in subsequent storage at 5 °C for 8 d. However unlike the findings in this thesis (Figure 4.2) the authors also observed reduced *in vitro* microbial population signifying a direct effect of ultrasound treatment on fungal growth.

In this work, hypobaric treatment reduced fungal rot to 66% in comparison to control 85% after subsequent storage at 5 °C for 16 d (Figure 6.1A). Karabulut et al. (2004) demonstrated that hot water treatments (55 and 60 °C for 30 s) reduced strawberry rots to 23.6 and 14.3% in comparison to control (91.3%) after 3 d at 1 °C plus 2 d at 20 °C. In the current study, strawberries were not been tested in a simulated retail chain (i.e. returning to 20 °C after cold storage). In experiments, where treated fruit are hardly showing rots after 3 d (Figure 6.1 and Figure 6.2), hypobaric treatment would probably make a major difference to marketable fruit quantities in these simulated retail chain regimes. Potentially if fruit had been taken out of cool storage after 3-5 d and exposed to warm temperatures (20 °C) for 2 d, this may have further emphasised the effectiveness of hypobaric treated strawberries.

Jing et al. (2010) reported 81% rot reduction in heat treated (60 °C for 20 s) strawberries after subsequent storage for 12 d at 0 °C. The effectiveness of heat treatment was attributed to both direct effect on fungal germination and induction of resistance. The current study indicates that hypobaric treatments only induce fruit resistance (Figure 5.2-5) without any direct effect on fungi (Figure 4.2).

A 4 h cooling delay due to hypobaric treatment at 20 °C nullified the effectiveness of treatment in subsequent storage at 5 °C (Figure 6.2A). Likewise Nunes et al. (2005) reported that a short-term cooling delay (6 h) increased fungal decay up to 30% in comparison to prompt cooling of strawberry. Similarly Nunes et al. (1995b) observed that a 6-8 h delay in shifting to cold storage reduced marketability of strawberries. Results of this study demonstrate that hypobaric treatment (at 20 °C) was not more useful in reducing rots than control fruit with prompt cooling (Figure 6.2A). This indicates that a 4 h cooling delay (due to hypobaric treatment at 20 °C) may have minimized the efficacy of the treatment. In order to test this possibility, hypobaric treatment was carried out at low temperature (5 °C), immediately after harvest (section 6.2.1.3).

Hypobaric treatment (50 kPa_a, 4 h at 5 °C) augmented low temperature storage by further delaying rot development in strawberry (Figure 6.3A). Nunes et al. (2005) suggested that prompt cooling alone may not be sufficient to manage fungal decay in strawberry. The current study indicated that application of hypobaric treatment in combination with low temperature storage improved the storage life of strawberry. However the magnitude of rot reduction in this work was only 36% as compared to

control on 9 d at 5 °C, this may be due to the use of near to ripe strawberries. Nunes et al. (2002) demonstrated that strawberries harvested at three-quarter maturity performed well in cold storage in comparison to fully ripe fruit. As results from chapter 5 indicate, the mode of action of hypobaric treatment is induction of fruit resistance, while fruit resistance is higher during development stage (Guidarelli et al., 2011). Therefore early application of hypobaric treatment may stimulate defence system before fully ripe stage. Hence the use of three-quarter ripe strawberry for hypobaric treatment may further improve its efficacy.

This research demonstrated a variation in prevalence of rots across different experiments. High rot incidence (> 60% in control) was observed after 14 d (Figure 6.1A), 12 d (Figure 6.2A) and 7 d (Figure 6.3A) in subsequent storage at 5 °C. Pre-harvest factors such as temperature, light and moisture affect the chemical composition and texture of fruit (Lee and Kader, 2000; Sams, 1999). Previously Woolf and Ferguson (2000) stated that high field temperature can affect the internal quality and skin properties of the fruit. In this work, pre-harvest temperature data suggested that high field temperature may have resulted in a reduced postharvest storage life of strawberry (data not shown). Perhaps high pre-harvest temperature may have accelerated ripening and senescence process resulting in early development of postharvest fungal growth.

Strawberry firmness is influenced by cell wall constituents (Ali et al., 2011), which starts degradation after ripening (Nunes et al., 2002). Low temperature storage can retard solubilisation and depolymerisation of cell wall contents. Cooling delays of 6

and 8 h (30 °C) resulted in reduction of firmness in strawberries (Nunes et al., 1995b). However, reduction in firmness and increase in respiration rate during storage might be influenced by the presence of rots. Treatments with lower firmness and higher respiration rate were also higher in rots (Figure 6.2 and Figure 6.3). Although sound fruit were selected for analysis, fungal progression inside the fruit may have altered firmness and respiration rate.

Nunes et al. (1995a) demonstrated that 6 h cooling delay (at 30 °C) resulted in 50% more weight loss in strawberries as compared to control after subsequently stored for 7 d at 1 °C and kept for one day at 20 °C. Similarly strawberry kept at 25 °C for 8 h before shifting to 1 or 5 °C significantly increased weight loss of strawberries (Collins and Perkins-Veanzie, 1993). However in current work, cooling delay for 4 h did not affect the weight loss of strawberries during subsequent storage (Figure 6.2D), because in previous work higher temperature (30 and 25 °C) and longer cooling delay (6 and 8 h) was used.

Together, these results suggest that the statistical significance observed in hypobaric treated strawberries may not be commercially significant, as reduction in rots was detectable only after long-term storage. However in commercial conditions, fruit could be stored for only a few days, before a rot threshold of perhaps 10% is reached. Therefore current data supports only the scientific significance of hypobaric treatment. Presumably, in addition to cold storage, synergy of hypobaric treatment with other physical treatment like UV-C, heat and ultrasound or resistance inducers (methyl jasmonates and chitosan) may improve commercial storability of strawberries.

6.5 Conclusion

In summary, these results suggest that hypobaric treatment (50 kPa_a, 4 h at 20 °C) effectively reduced decay development during subsequent storage at 5 °C. However, the same benefit in rot reduction can be found simply by ensuring speedy removal of field heat, without any postharvest delay in cooling. Therefore hypobaric treatment is suggested to be applied at low temperature immediately after harvest. Furthermore the combination of hypobaric treatment and cold storage may not be sufficient for substantially increasing storage life of strawberries.

Chapter 7 Overall discussion and recommendations

7.1 Introduction

Postharvest fungal decay is a key cause of reduction in storage life of fruit. Pre-harvest fungicide application is considered to be one of the most effective methods for controlling postharvest fungal rot. In contemporary research, pre-harvest application of chemicals has been questioned, while postharvest chemical treatments are prohibited due to residues remaining on the fruit (Romanazzi et al., 2013). In order to further improve the postharvest storability of fruit, modern research is focusing on the development of physical methods that have the potential to influence fungal growth. Physical methods may act directly, inhibiting or delaying fungal growth or alternatively, may retain or stimulate the innate defence system of fruit, enabling the maintenance or production of fungistatic or fungicidal substances.

This study focused on evaluation and development of short-term hypobaric treatment as a new and innovative postharvest physical technique for controlling fungal decay. Prior to this work only Romanazzi et al. (2001) had studied the short-term hypobaric treatments effect on fruit and decay response. Therefore the initial aim of this study was to confirm the efficacy of hypobaric treatments. Once this was determined, efforts were made to understand the mechanisms involved in controlling fungal rot. Furthermore, this research also tested hypobaric treatment integrated into a simulated commercial supply chain (i.e. including low temperature storage).

Strawberry was chosen as a model fruit to study hypobaric treatment. Strawberry is one of the major berry fruits in New Zealand with an estimated production of 47.6% of the overall berry fruit production (Timudo-Torrevilla et al., 2005). Moreover, due to the short storage life and the fast onset of fungal rots, strawberry is considered to be an ideal fruit for testing a new postharvest technique.

7.2 Summary of results

This study confirmed the effectiveness of hypobaric treatment (50 kPa_a, 4 h) in delaying fungal decay of strawberries (section 3.3.1). Furthermore, results of this work also indicated that hypobaric treatment is effective even in strawberries treated after a delay of up to 8 h (3.3.2). This is important because in commercial scenario there is always a delay between harvest and corresponding treatment.

However, no direct toxic effect of treatment on fungi was observed (section 4.4.1). Moreover, results demonstrated that hypobaric treatment stimulated defence-related enzymes (sections 0-5.3.5) possibly leading to the observed reduction in fungal rots (section 5.3.1). In simulating application of hypobaric treatments to a commercial supply chain, this study found that they should be applied at low temperature as soon as possible after harvest (section 6.3.3). However, hypobaric treatment olone may not be sufficient for extending storability by a commercially relevant magnitude. Thus integration of hypobaric treatment with other treatments (jasmonates, ethanol, ozone or chitosan) may be a good combination in improving shelf-life of strawberry.

7.3 Mechanisms of rot reduction

7.3.1 *In vitro* fungal growth

In order to study the direct effect of a treatment on fungal growth, *in vitro* growth subsequent to treatment is studied. In UV-C (Pombo et al., 2011), ultrasound (Cao et al., 2010b) and heat (Wang et al., 2010a) treatment studies, inhibitory effect of these treatments on fungal growth were observed. In the current study hypobaric treatment neither affected colony growth nor spore germination of *Botrytis cinerea* and *Rhizopus stolonifer* (section 4.4.1). However, inoculating hypobaric treated fruit with target fungi resulted in reduced decay development in comparison to non-treated fruit (section 4.4.3). These results suggest that hypobaric treatments affect fungal growth by activating fruit defences rather than killing fungal spores or arresting fungal growth directly.

Previously Adams et al. (1976) reported that 26 kPa_a treatment inhibited the growth of fungi (*P. expansum* and *P. patulum*), while 61 and 48 kPa_a treatment did not affect mycelial growth. Similarly Apelbaum and Barkai-Golan (1977) demonstrated that hypobaric conditions (13, 7 and 3 kPa_a for 5 d) hindered mycelial growth of *Alternaria alternata*, *B. cinerea*, *Diplodia natalensis* and *P. digitatum*. But *in vitro* fungal growth recommenced after transferring to atmospheric pressure (101 kPa_a). This indicates that severe hypobaric condition for longer time created fungistatic environment rather than causing irreparable damage. The hypobaric pressure (13, 7 and 3 kPa_a) resulted in a reduction of oxygen partial pressure to approximately 2.7, 1.38 and 0.69 kPa_a respectively, which might have decreased the respiratory activities of fungal spores. In contrast, this work demonstrated that short-term hypobaric treatment (50 kPa_a, 4 h)

did not affect fungal growth (section 4.4.1) and fungi have not been affected by the low oxygen partial pressure (10 kPa_a) stress created by hypobaric conditions (50 kPa_a) for a short period (Table 4.1).

7.3.2 Fruit response to fungal inoculation

In this study, hypobaric treated strawberry inoculated immediately or 12 h after treatment demonstrated significant reduction in fungal rots as compared to un-treated fruit (Figure 4.5 and Figure 4.6). The response was observed to be time dependent, since fruit inoculated 6, 18 or 24 h after hypobaric treatment did not display a rot reduction (Figure 4.5 and Figure 4.6). A similar time-dependency was observed in UV-C treated strawberry (Nigro et al., 2000) and methyl jasmonate treated peach (Jin et al., 2009). In both cases inoculation 12 h after treatment led to maximum rot reduction. Recently Vilanova et al. (2014) established that defence response of apple depends on maturity of fruit. The authors further stated that immature and commercially mature wounded apple produced lignin (defence compound) more actively than over-mature fruit, which resulted in lower rot incidence. In current work, strawberry inoculated 18 and 24 h after treatment may have become over-mature therefore may not be able to synthesis defence compounds for controlling rots.

7.3.3 Induction of resistance in fruit

Modern research is focusing on understanding the ability of fruit to develop resistance against pathogenic microorganisms. In induced resistance the treatment influences fruit defence enzymes or gene expression, which inhibits growth or development of

the pathogenic microorganisms. Both biotic and abiotic stresses cause activation of induced resistance in plants (Terry and Joyce, 2004).

7.3.3.1 *Mechanosensitive ion channels (MSCs) / pressure-sensitive ion channels*

Plant cells have a specialized system to sense internal (membrane deformation and osmotic pressure) and external (mechanical force, gravity and touch) changes in environment (Haswell et al., 2011). Ion channels are protein complexes which act as gated macromolecular sensors in the cell membrane (Wilson et al., 2013), whereas mechanosensitive ion channels (MSCs) are force-sensing structures of plant cells (Haswell et al., 2011). These channels are also termed as pressure-sensitive ion channels (Martinac et al., 1987), pressure-induced or stretch-activated ion channels (Sachs, 2010). As a result of signals produced by MSCs, plant cell maintain cellular viability, regulate production of enzymes and synthesise osmoprotective molecules in the event of changes in osmotic or pressure stress (Martinac et al., 1987).

In this work, hypobaric treatments generated a stress that stimulated the defence system of fruit. Reduced pressure causes stretching in cell membrane (Sachs, 2010), which may have instigated the activation of MSCs / pressure-sensitive ion channels (Zonia and Munnik, 2007). Consequently the production of PR proteins (PAL and POD) may have been upregulated, as part of a response to strengthen cell wall structure (Amil-Ruiz et al., 2011).

7.3.3.2 *Hypobaric treatment and role of PAL and POD in rot reduction*

PAL has an important role in phenylpropanoid pathway (Fraser and Chapple, 2011) and POD plays a key role in lignification (Almagro et al., 2009). Lignin is considered to be the primary line of defence against pathogen invasion (Bhuiyan et al., 2009). Therefore in event of a pathogen attack or wound, the lignin content of plant increases (Vilanova et al., 2014). Pombo et al. (2011) demonstrated that UV-C treatment caused transient increase in PAL and POD activity coupled with the amplified expression of defence-related genes (*FaPAL* and *FaBG2*). Hypobaric treatment may also result in elevated expression of defence and stress response genes.

Previously Djebali et al. (2007) stated that pre-infection upregulation of POD may strengthen cell wall, making plant tissue more resistant to infection. In this work, hypobaric treatment resulted in transient increase in PAL and POD activity. These processes may have resulted in delayed penetration of fungi and prolonged maintenance of resistance causing reduced strawberry rots (Figure 7.1). Cell wall maintenance of fruit tissue is also related to fruit firmness (Vicente et al., 2005a). However, in this work, hypobaric treatment did not lead to any significant effect on fruit firmness (Figure 3.4A and Figure 3.7A).

Alternatively, PAL in also involved in the synthesis of salicylic acid (SA) (Chen et al., 2009), while stimulation of SA results in the systemic expression of PR genes in healthy tissues (Durrant and Dong, 2004). Perhaps the higher PAL activity 12 h after hypobaric treatment (Figure 5.2) may have stimulated SA synthesis, activating systemic acquired

resistance (SAR), protecting the non-infected parts of the fruit (Metraux et al., 2002), and consequently assisting hypobaric treated fruit to be more resistant to fungal invasion (Hammerschmidt, 1999).

7.3.3.3 *Role of ethylene as stress response phytohormone*

Previous studies suggest that ethylene play an important role in responding to biotic and abiotic stresses (Fujita et al., 2006). Nigro et al. 2000 observed that hormetic stress caused by UV-C treatment resulted in transient increase in ethylene levels and PAL activity (defence-related enzyme) of strawberry. Similar to UV-C, hypobaric treatment stress may have stimulated ethylene production, which might be one of the reasons to trigger defence-related enzymes and reduce fungal decay in strawberry.

7.3.3.4 *Potential role of reduced CO_2 in hypobaric condition*

According to Henry's law, solubility of a gas in a liquid is directly proportion to the partial pressure of the surrounding environment (Myers, 2003). In the case of hypobaric condition, the intercellular partial pressure in fruit tissue reduces which decreases solubility of CO_2 in the cell. Previously Bown (1985) found that alteration in intercellular CO_2 concentration results in perturbation of pH. An increase in CO_2 yielded H_2CO_3 and consequently decreased pH. Therefore reduction in CO_2 partial pressure due to hypobaric condition would raise cellular pH. In response, plant cell regulates its pH through specialized proton pump (Sanders et al., 1981). However CO_2 induced disturbance in pH leads to stress, causing the upregulation of defence enzymes (Bown, 1985). In this case the temporary rise in POD and PAL activity might be indicative of stress caused by alteration in pH of the cell (Figure 7.1).

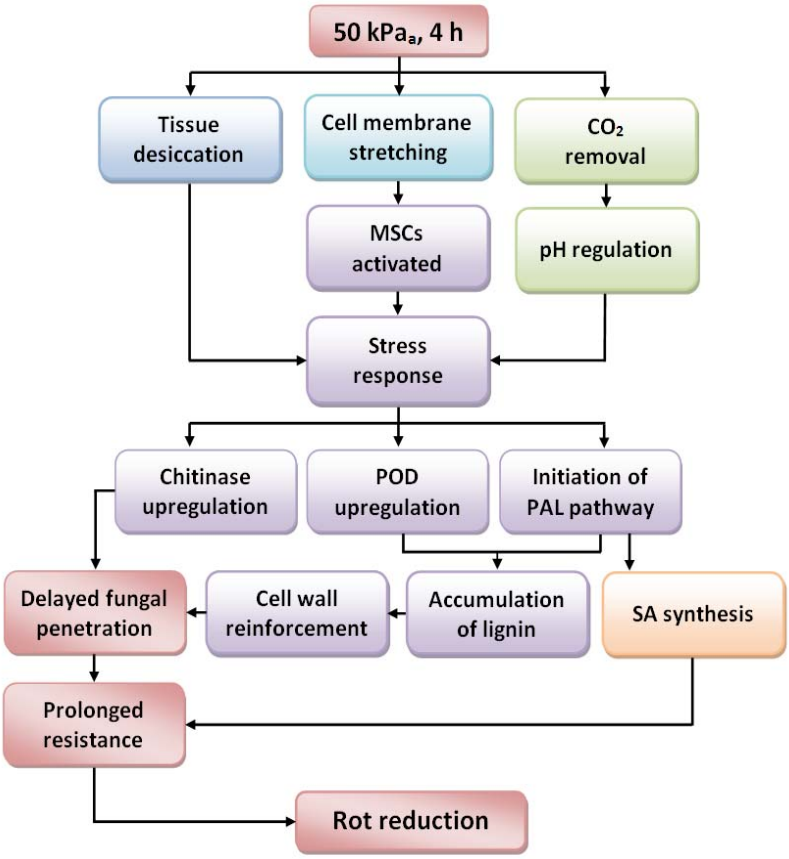

Figure 7.1 Possible mechanisms involved in rot reduction due to hypobaric treatment.

7.3.3.5 *Role of chitinase*

Plant chitinases are associated with lysis of fungal hyphae resulting in fungal growth inhibition (Van Loon et al., 2006). Mauch et al. (1988) reported that fungal growth inhibition by chitinase is more pronounced in conjunction with β-1,3-glucanases. Expression of chitinase is not only induced due to fungal attack but also as a result of an abiotic stress (Van Loon et al., 2006). In current work, the observed transient surge in chitinase activity (Figure 5.4) was caused by hypobaric treatment (abiotic stress). Consequently the presence of high chitinase activity before development of fungal mycelia may have extended the quiescent stage, ultimately prolonging storage life of strawberry (Figure 7.1).

7.3.3.6 *Limitations of induced resistance*

While induced resistance has the potential to contribute positively in improving the storage life of horticultural commodities, it is also important to understand the limitations of the induced resistance. Unlike fungicides, induced resistance due to physical treatment does not target the pathogen directly. Therefore induced resistance must act before infection so that the plant has enough time to respond to the infection (Dann, 2003). Additionally, induced resistance can only suppress pathogens and cannot lead to pathogen death. Moreover, physical, physiological and environmental factors also affect the efficacy of induced resistance in different crops (Dann, 2003). The current work indicated that hypobaric treatment induced fruit resistance against pathogens (sections 0-5.3.5) without directly affecting fungal growth (section 4.4.1). Therefore this treatment may slightly improve the storage life; however for a commercially significant effect, hypobaric treatment should be evaluated as a hurdle technique in combination with other treatments which can directly target the fungal growth (UV and ozone).

7.4 Efficacy of different hypobaric treatments

In this work, different pressure applications (75, 50 and 25 kPa_a for 4 h) were tested for effectiveness against fungal rots. Among all options, 50 kPa_a treatments were more effective than others. It was observed that 75 kPa_a slightly reduced rot as compared to control but was less effective than 50 kPa_a, while 25 kPa_a treatment was not different from control. This signifies that, although rot reduction might be due to low pressure stress, perhaps the lower pressure (25 kPa_a) might have imposed more severe stress, which resulted in tissue damage of strawberry. Similarly 75 kPa_a may not be enough to

create the required stress for influencing rot; perhaps 50 kPa_a might have created mild stress which reduced rots in strawberry (Figure 3.2). In addition, 25 kPa_a treated strawberries showed abundance of strawberry leaks caused by *R. stolonifer*, which caused tissue collapse resulting in high weight loss. This indicates that 25 kPa_a may prompt the growth of *R. stolonifer*. In contrast, Romanazzi et al. (2001) demonstrated that 25 kPa_a was more effective than 50 kPa_a in reducing fungal rots, however this study was carried out on 'Pajaro' strawberries while current work is on 'Camarosa'. Probably the difference in efficacy of pressure ranges may be due to cultivar differences in susceptibility to low pressure damage. Studies on heat treatment of strawberries also indicated variation in effectiveness of treatment in different cultivars of strawberries (Garcia et al., 1995; Jing et al., 2010; Lara et al., 2006).

7.5 Variation of rot incidence in different cultivars

Fungal decay is the major cause of postharvest losses in strawberry. In this work *B. cinere*a and *R. stolonifer* were identified as the main rot causing organisms. The prevalence of rots increased rapidly at 20 °C as compared to 5 °C, however there was a wide variation in rot incidence amongst different experiments (Figure 3.11). More importantly, the difference of rot occurrence in 'Camarosa' and 'Gaviota' strawberries was substantial (Figure 3.2). Previous research demonstrated that 'Gaviota' strawberries are relatively resistant to damage caused by excessive rain. In addition 'Gaviota' plants are also tolerant to anthracnose crown rot and powdery mildew (Phillips and Reid, 2008). However, Bussell et al. (2005) reported that yield of 'Gaviota' is significantly lower than 'Camarosa' strawberries. Nevertheless there is a need to

compare the yield and marketable fruit of both cultivars, because the higher postharvest losses may jeopardize the benefit of more yield.

7.6 Effect of intensity of hypobaric treatments on rot reduction

Experiments on pressure treatment (50 kPa_a for 4 h) with different intensity (slow and fast; single and multiple pull) showed that single pull (either slow or fast) was more effective than 50 kPa_a multiple pull (repeated pressure cycling). According to Fick's law, gases diffuse from higher concentration to lower concentration (Kader and Saltveit, 2003). During hypobaric condition, O_2, CO_2 and water vapour move out of strawberry tissue to vacuum chamber to maintain the gas equilibrium. Probably this may cause temporary tissue desiccation. In single pressure pull the water vapour and O_2 would move inside the tissue when the vacuum is released after 4 h, however the tissue would need some time for restoration of equilibrium. Whereas in multiple pull, there would be an increased chance of water loss, since in this work 50 kPa_a pressure was repeatedly released every 8 min and re-built in the next 2 min (as explained in section 3.2.1.3). Consequently, there was not enough time for strawberry tissue to restore equilibrium, as a result causing excessive water loss. This is evident from weight loss data (Figure 3.9B), where multiple pull resulted in excessive water loss even more than control. Therefore, temporary tissue desiccation (for 4 h) caused by single pull hypobaric treatment (50 kPa_a for 4 h) may have stimulated strawberry defence against fungal rots (Figure 7.1).

7.7 Effect of delay before hypobaric treatment

Delay between harvest and corresponding treatment also increases the incidence of strawberry rots (Nunes et al., 2005). This work demonstrated that hypobaric treatments (50 kPa_a, 4 h) applied within 8 h of arrival in lab effectively delayed fungal rots in comparison to un-treated control (section 3.3.2). However, treatment after a 12 h delay was not effective. The reason for ineffectiveness after 12 h delay might be the loss of fruit resistance due to high maturity. Vilanova et al. (2014) found that disease resistance of over-mature apple was less than commercially mature and immature apple. Most likely strawberry kept for 12 h at 20 °C before treatment might have increased its maturity, which may have reduced the resistance against fungal rots.

7.8 Future opportunities

7.8.1 Study of defence-related enzymes at low temperature

Hypobaric treated strawberries showed stimulation of defence-related enzymes (PAL, POD and chitinase) in subsequent storage at 20 °C (Figure 5.2-5.5). Peak enzyme activities were observed immediately (POD) and 12 h after treatment (PAL and chitinase). Low storage temperature reduces the physiological activity in strawberry (Figure 6.2C). Presumably enzyme activity may peak at different time points at low temperature than observed at 20 °C. It is suggested that defence-related enzymes should be studied in strawberries treated and stored at 5 °C, as the timing of rise in enzymes may be different due to difference in temperature. This would confirm the involvement of hypobaric treatment in inducing resistance of strawberry.

7.8.2 Investigation of defence-related stress response genes

Previous studies suggested that biotic and abiotic stresses in plants could increase synthesis of defence-related phytohormones (Peña-Cortés et al., 2005) and enzymes (Wang et al., 2006). In addition, these stresses may trigger stress response genes that could induce resistance in plants. This study indicated that hypobaric treatments stimulate defence-related enzyme activity and that could be the possible reason in reducing rot development (chapter 5). Another approach could be studying the effect of hypobaric treatment on the expression of stress response genes that induce plant resistance. Currently there is no published work about the hypobaric treatment effect on stress response genes. Therefore similar to studies of Pombo et al. 2011 in UV treatment, expression of pathogenesis-related (PR) genes should be studied in hypobaric treated strawberries. The results of this study will confirm the data of defence-related enzymes. In addition, unlike enzyme activity where each enzyme is studied separately, in molecular study there is an opportunity to analyse multiple genes at once. Furthermore, there is also a potential to study more fine time course for defence-related genes.

7.8.3 Study of hypobaric treatment in simulated retail chain

Another possibility for testing the feasibility of hypobaric treatment could be the use of a simulated retail chain (keeping strawberry at 20 °C after cold storage). In this work, cold stored hypobaric treated strawberries hardly showed rots after 3 d (Figure 6.1 and Figure 6.2). It could be hypothesized that hypobaric treated fruit taken out of cool storage after 3-5 d and then exposed to warm temperatures (20 °C) for 2 d may show more promising results as observed by Karabulut et al. (2004) for heat

treatment. Therefore hypobaric treatment should be tested in simulated storage regime, which may possibly make a major difference to marketable fruit.

7.8.4 Effect of fruit maturity on efficacy of hypobaric treatment

Near to ripe fruit were used for this study, although others have shown that three-quarters-coloured (75% red) strawberries store for a longer time with a better visual quality (Nunes, 2009). In this research near to ripe (>95% red) fruit were used because this represents commercial harvest conditions in New Zealand (Anon, 2011). Vilanova et al. (2014) reported that less mature fruit has higher resistance against fungal infection than mature or over-mature fruit. Therefore, testing hypobaric treatment on 75% red strawberries may show more promising results.

7.8.5 Effect of field temperature on postharvest storage

Pre-harvest field temperature may affect the postharvest storage life of fruit (Woolf and Ferguson, 2000). In current work, there were some indications that warmer pre-harvest field temperatures may have reduced the storage life of strawberries (data not shown). Therefore it is suggested that field temperature for a few days preceding harvest should be considered in testing the efficacy of any treatment. In addition, a study should be carried out to compare the effect of field temperature on the storage life of strawberry.

7.8.6 Integrated approaches

7.8.6.1 *Infusion of volatile chemicals and gases*

Methyl jasmonate (MeJA) treatment led to improvement in storage life of strawberries (Ayala-Zavala et al., 2005). Jin et al. (2009) demonstrated that treatment of MeJA (1 µmol L^{-1}) decreased fungal decay and stimulated defence-related enzymes in peach. Likewise, the ethanol vapour (Ratanachinakorn et al., 1999) and 1-MCP (Chen et al., 2010; Modares et al., 2013) infiltration (under hypobaric pressure) improved the quality of fresh produce. As described in chapter 2, ozone is very effective in controlling moulds (Boonkorn et al., 2012) and its application kills spores of *B. cinerea* within 1 h of treatment (Ozkan et al., 2011). In creating hypobaric condition, intercellular gas and water vapour are temporarily removed. On return to atmospheric pressure (101 kPa_a) after releasing the vacuum, water vapour and gases return to the intercellular space, but composition takes time to return to normal. Hypobaric treatment could be combined with vapour treatment of volatile chemicals and used to increase absorption.

7.8.6.2 *Chitosan treatment*

Chitosan is an important resistance inducer. Recently Romanazzi et al. (2013) reported effectiveness of chitosan in controlling strawberry decay. Likewise Dang et al. (2010) observed induction of defence enzymes and maintenance of quality in chitosan treated sweet cherries. In addition Feliziani et al. (2013) reported that both pre- and postharvest chitosan treatments are useful in reducing sweet cherries decay. The authors presented chitosan an alternative to synthetic fungicides. Earlier studies suggested positive impact of synergy between hypobaric treatment and chitosan in

reducing sweet cherry rots (Romanazzi et al., 2003). Integration of hypobaric treatment with chitosan application (either pre- or postharvest) could lead to a useful treatment to control fungal decay. In this work hypobaric treatment has been tested in commercially grown strawberries with pre-harvest application of synthetic fungicides. Hypobaric treatment may be useful in strawberries which are sprayed with chitosan instead of synthetic fungicides.

7.9 Industrial application

Due to the short storage life of strawberry, the industry needs approaches which can be applied immediately after harvest. Although research in many innovative techniques is in progress, currently cold storage is the only practical solution for extending the postharvest storage life of strawberry. Hypobaric treatment has the potential of immediate postharvest application, because it involves only a vacuum chamber and could be applied during transportation of strawberry with the help of mobile refrigerated vacuum chambers. As mentioned earlier (section 2.2.2) there is a need of improvement in New Zealand strawberry supply chain, since the industry faces losses of about 20%. Therefore to extend the findings of this research, it is suggested to integrate hypobaric treatment with volatile chemicals during or after pre-cooling. Infusion of MeJA or ethanol vapour upon release of vacuum would lead to greater uptake, in this way these vapour may reinforce the defence system of fruit more effectively in combination with hypobaric treatment than as a standalone treatment. This may enhance efficacy of hypobaric treatment. Furthermore, the current work was carried out on pilot scale; nevertheless more extensive studies are needed for industrial feasibility of hypobaric treatment.

7.10 Final conclusion

This research investigated the effect of hypobaric treatment on the strawberry physiology (respiration rate) and quality (firmness). Furthermore, the mechanisms involved in rot reduction due to hypobaric treatment were also studied; this included the direct effect of hypobaric treatment on fungal growth and the indirect effect by inducing fruit resistance. In addition, efficacy of hypobaric treatment at low temperature was also investigated. However, further research is needed to determine the commercial efficacy of hypobaric treatment in combination with volatile chemical treatment. Should hypobaric treatment be found commercially viable, this could eventually facilitate the development of an innovative hurdle technology to control postharvest strawberry decay.

This thesis confirmed the efficacy of hypobaric treatment in reducing strawberry decay. Furthermore, it was found that there was no direct effect of the treatment on fungal growth. In addition, hypobaric treatment stimulated the defence-related enzymes. Therefore the mode of action of hypobaric treatment is the induction of fruit resistance. Moreover, treatment at 20 °C reduced the effectiveness of hypobaric treatment in subsequent cold storage. Thus treatment is suggested to be applied at low temperature immediately after harvest. Although the findings of this thesis are statistically significant, they may be commercially less important. Significant differences in treatments were observed only after long-term storage with high incidence of rots; but in commercial scenario, fruit could only be stored till the rot threshold of perhaps 10% is reached. Thus present data supports the scientific importance of hypobaric treatments, but for commercial application further study is

needed. Perhaps hypobaric treatment might be considered as a hurdle technology in combination with other physical treatments (UV-C, heat and ultrasound) and/or resistance inducers (chitosan), and facilitating infusion of volatiles (MeJA, ethanol, 1-MCP).

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