OZONE FUMIGATION EFFECTS ON BACTERIAL AND ANTHRACNOSE DEVELOPMENT ON BELL PEPPER (*Capsicum annuum* L.) AND ITS EFFECT ON FRUIT QUALITY

By:

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philisophy

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ABSTRACT

OZONE FUMIGATION EFFECTS ON BACTERIAL AND ANTHRACNOSE DEVELOPMENT ON BELL PEPPER (*Capsicum annuum* L.) AND ITS EFFECT ON FRUIT QUALITY

Bacterial contamination and anthracnose development on bell pepper pose a threat to food safety and food security. Bacteria contamination by pathogenic species can be a fatal outbreak and risking worldwide population. Meanwhile, anthracnose development on bell pepper can contribute to substantial product loses which will substantially affect world economy and food availability. Current postharvest treatment such as the use of chlorine and fungicide poses harmful effects on human and environment due to the production of carcinogenic by-products. This leads to urgency to develop a safe postharvest treatment which leads to the objective of this study to develop a new postharvest treatment; ozone fumigation which has high potential to reduce bacterial contamination and anthracnose development on bell pepper. Ozone fumigation treatment is safe to human and environment and very practical. This technology is promising hence, worth to study.

This study investigated the effect of ozone fumigation on 1) growth of *Escherichia coli* O157, *Salmonella enterica* sv. Typhimurium and *Listeria monocytogenes*; selected pathogens that contributed to food poisoning in fruit 2) development of anthracnose disease on bell pepper caused by *Colletotrichum capsici* 3) activity of defense related enzymes 4) antioxidant capacity of bell pepper 5) physico-chemical, physiology and

sensory qualities of bell pepper. Antibacterial studies was conducted on fresh cut bell pepper with treatments of 0, 1, 3, 5, 7 and 9 ppm ozone for 0.5, 3, 6 and 24 h at 18 - 20˚C, 95% RH. The results showed that ozone reduced growth of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations where optimal dosage was 9 ppm ozone for 6 h. This ozone dosage resulted in 2.89, 2.56 and 3.06 log reduction of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations, respectively. Scanning electron micrograph showed that the bacterial population was inactivated by disrupting the cell structure which leads to cell lysis.

Ozone also reduced anthracnose development on bell pepper. *Colletotrichum capsici*, the causal agent of anthracnose on bell pepper was treated with 0, 1, 3, 5, 7 and 9 ppm ozone for 24, 72 and 120 h. The results showed that exposure to 7 ppm ozone for 72 h had the highest inhibition in disease incidence (34.8%) and disease severity (41.2%). This inhibition was non-significantly different to fruit exposed to 3, 5 and 9 ppm ozone for 72 h. The inhibition was due to effect of ozone on mycelia morphology where ozone inhibited mycelia development by inducing hyphae branching. Besides, the ozone dosage also significantly reduced spore production (31.6%) and spore germination (100%). Increasing ozone dosage by prolonging the exposure to 120 h induced fungal sporulation and had no significant effect on disease development.

Reduction in anthracnose disease development was correlated with activity of plant defense enzymes. Increase in activity of plant defense enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD) and β-1,3-glucanase was found to reduce variation in disease incidence on bell pepper. Optimal enzyme activity was observed from exposure to 3 ppm ozone for PAL, PPO

and β-1,3-glucanase and 3 and 5 ppm ozone for POD. This showed that ozone inhibit disease incidence on fruit by stimulating the activity of plant defense enzymes as well as reducing mycelia elongation, spore germination and spore production.

Analysis on antioxidant content and antioxidant capacity of bell pepper showed ozone dosage of 3 ppm ozone for 72 h was the most effective dose to induce fruit ascorbic acid (26.6%) and total phenol content (15.2%) which reflect antioxidant capacity (15.3%) of bell pepper. Further increase in ozone concentration reduced fruit antioxidant content and its capacity. Analysis on fruit β-carotene content showed negative correlation with fruit antioxidant capacity hence, suggested that β-carotene may not be the major antioxidant in the bell pepper under study.

The increase in fruit antioxidants from exposure to 3 ppm ozone for 72 h reduced fruit oxidative status (malondialdehyde (MDA) content) and resulted in no oxidative damage. This maintained fruit ripening progress similar to control as indicated by fruit respiration, colour development, soluble solid concentration and titratable acidity. The ozone dosage also maintained fruit water content similar to control hence, maintaining its firmness during storage. Meanwhile, exposure to higher ozone dosage; 7 and 9 ppm ozone for 72 h; increased cell oxidative status which resulted in oxidative damage as observed in high MDA content and increase in membrane permeability. This enhanced ripening progress as indicated by progressive colour development, increase in soluble solid concentration and reduction in titratable acidity and firmness. This quality deterioration negatively affected fruit flavour hence, not preferred by the panellist.

Thus, under current observation, this study showed exposure to 3 ppm ozone for 72 h reduced populations of foodborne pathogen, decreased anthracnose development,

increased plant defense enzyme as well as enhanced its antioxidant capacity. It can be used as an alternative to chlorine and fungicide and eliminate the risk of producing harmful by-products. Ozone treatment is also very practical where it can be installed in truck or shipping container which allows the treatment to be carried out during transport. This reduces fruit handling time and labour cost.

ACKNOWLEDGMENT

Up and above, everything, all gratitude to **Almighty Allah**, the compassionate, the merciful, who enabled me to elucidate a drop form the existing ocean of knowledge, and the Holy Prophet Muhammad (peace be upon him) the most perfect and exalted among and even born on the surface of earth, who, is far ever a torch of guidance and knowledge for the humanity as a whole.

I am highly obliged to my worthy supervisor, **Dr. Asgar Ali**, Professor and Director of Centre for Postharvest Biotechnology (CEPB), School of Biosciences, The University of Nottingham Malaysia Campus for his pleasing temperament, personal interest, inspiring guidance, enlightened supervision and constructive criticism throughout the course of research endeavour.

In addition, I would like to thank **Dr. Ajit Singh**, Associate Professor, School of Biosciences, The University of Nottingham Malaysia Campus who introduced me to the statistical programme and taught me from the basics of statistics

I like to articulate my humble gratitude to my internal assessor **Dr. Lim Yin Sze**, Associate Professor, School of Biosciences, The University of Nottingham Malaysia Campus for her advice and sympathetic disposition which enabled me to concentrate during my research and write-up.

I would also like to thank the Chief Lab Technician, **Mr. Wan Ghani** and other lab technicians, **Ms. Radha, Ms. Siti Norazlin, Ms. Shankari**, my exquisite fellows, **Dr. Mehdi Maqbool, Dr. Noosheen Zahid, Dr. Ong Mei Kying, Ms. Maysoun Mustafa,**

Mr. Yeoh Wei Keat, Ms. Azhani, Mr. Paak Kwesi Bordoh and Faculty Office Staff, **Ms. Sabariah, Ms. Salma** for their assistance and help during my PhD programme.

The most profound thanks go to the **Medklinn Sdn. Bhd** for the financial support. I also would like to thank to **Mr. Danielle Yap** and **Mr. Lim Boon Han** for their kind advise and assist in establishing ozone fumigation system.

I do not have words at command to express my gratitude and profound admiration to my dearest parents and family for their spiritual inspiration and moral support which made it possible for me to achieve the present knowledge in my life and wished to see me glittering in the skies of success.

SUPERVISORY COMMITTEE

The thesis submitted to the School of Biosciences, Faculty of Science, The University of Nottingham Malaysia Campus has been accepted as fulfilment of the requirement for the degrees of Doctor of Philosophy. The members of the Supervisory Committee are as follows;

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DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degrees at The University of Nottingham Malaysia Campus or other institutions

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NURUL ALYAA ALWI

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Bell pepper is one of the important commercial vegetables and it is consumed for its high potential of health functionality. It has high antioxidant content such as ascorbic acid, carotenoids and phenolic compounds (Alvarez-Parrilla *et al.*, 2010; Tan *et al.*, 2012). Consumption of bell pepper reduces the risk of degenerative diseases such as cancer, cardiovascular disease, cataract, diabetes and neurological disorder such as Alzheimer's and Parkinson's (Deepa *et al.*, 2007; Sun *et al.*, 2007). Bell pepper is extensively used as salad and condiment and its demand is growing due to its wider use in fast food industry (Castro *et al.*, 2011). Besides, consumer motivation to consume high nutritive fruit and vegetable as healthy diet also increased bell pepper demand (Castro *et al.*, 2011; Tan *et al.*, 2012). This is coherent with its growing production where it reached 31 million tonnes, valued at USD 14 billion in 2012 (FAOSTAT, 2014). This indicates the significant influence of bell pepper in agricultural industry.

Bell pepper cases related to foodborne poisoning is increasing coherent with its growing demand. Contamination by pathogenic bacteria was reported on whole fruit (Larsen, 2013), fresh cut fruit (Clark, 2016) as well as frozen bell pepper (Grabowski, 2016). Bacterial contamination on bell pepper has sickened 14 people in 2013 and has resulted in withdrawal of 30,200 pounds of fresh bell peppers (Larsen, 2013).

Contaminated fresh cut and frozen bell pepper were also withdrawn from markets (Grabowski, 2016). Without immediate detection and action taken to stop distribution of the contaminated products, these contaminations would cause fatal outbreaks which can result in miscarriages and deaths. As bell pepper is preferably consumed raw or fresh cut, fresh cut bell pepper has high risk of bacterial contamination which therefore poses a threat to food safety. Therefore, this requires an effective measure to control the contamination.

Another major problem encountered by bell pepper is fungal disease development, particularly anthracnose. The disease is caused by *Colletotrichum* species, being *C. capsici* is the most prevalent in Asia (Harp, 2008). It infects immature bell pepper in field and remains latent (Edirisinghe *et al.*, 2012). Disease symptom of anthracnose appears during postharvest storage as the fruit ripen (Edirisinghe *et al.*, 2012). Therefore, early disease prevention is difficult to be implemented.

Anthracnose development on bell pepper is currently controlled by application of fungicide such as Maneb (manganese ethylenebisdithiocarbamate) (Lewis Ivey *et al.*, 2004). It is however, had carcinogenic, mutagenic and teratogenic effects hence, has been banned in Korea and Russia (United Nations, 2005). Meanwhile, bacterial contamination on fruit is currently controlled by application of chlorinated water (López-Gálvez *et al.*, 2010). It is however, poses high risk of producing carcinogenic by-products such as trihalomethanes (THMs)(Stauffer, 2004) (Gibbons and Laha, 1999). Therefore, development of safe and effective postharvest treatment to control both fungal disease and bacterial contamination is needed for a sustainable agriculture.

A novel approach to control bacterial and fungal growth on fruit is by the use of ozone. It is a triatomic oxygen molecule (O_3) which can be commercially produced from the reaction of oxygen molecules (O_2) with atomic oxygen (O) using corona discharge (Forney, 2003). It has high oxidizing capacity which is 1.5 times higher than chlorine (Forney, 2003). It has the potential to oxidize microbial cell membrane, proteins, DNA and other cell components due to its high affinity towards compounds containing oxygen (O), nitrogen (N), sulphur (S), phosphorus (P) and carbon-carbon (C-C) double bonds (Forney, 2003). This affects cell differentiation and energy production hence, leads to cell death. In comparison to fungicide and chlorine, ozone produces environmental friendly by-product which is oxygen molecules (Gabler *et al.*, 2010). Its application has been approved by the U.S. Food and Drug Administration (FDA) in 2001 to be utilized during food treatment and food storage either in aqueous or gaseous form (Lake, 2001).

Published studies have reported on the effect of ozone on several bacterial species such as *Salmonella* sp. (Selma *et al.*, 2008b), *Listeria innocua* (Fan *et al.*, 2007), *Staphylococcus aureus* and *Bacillus subtilis* (Thanomsub *et al.*, 2002). The reports showed that the effectiveness of ozone treatment is depending on bacterial species. Therefore, it is crucial to specifically study the effectiveness of ozone on selected pathogenic foodborne bacteria such as *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes*. The insights help to establish a postharvest treatment to control bacterial contamination on bell pepper.

Published studies have also reported on the effect of ozone on several fungal species such as *Penicillium* sp. (Palou *et al.*, 2003), *Botrytis cinerea* (Ozkan *et al.*, 2011), *Alternaria alternata* (Tzortzakis *et al.*, 2008) and *Aspergillus* sp. (Antony-Babu

and Singleton, 2009). Antifungal effect of ozone was also reported on *Colletotrichum* sp., the anthracnose causal agent, including *Colletotrichum acutatum* (Yun *et al.*, 2006) *Colletotrichum gloeosporioides* (Barbosa-Martinez *et al.*, 2002) and *Colletotrichum lindemuthianum* (Treshow *et al.*, 1969). These reports showed different effectiveness of ozone where it is largely influenced by the fungal species and the fungal response toward oxidative stress of ozone. To our knowledge, there has no report on the effect of ozone on *C. capsici*, the main causal agent of anthracnose in bell pepper. Therefore, there is a need to study the effect of ozone on the fungal species considering its large influence on postharvest loss of bell pepper.

Ozone also has the potential to elicit plant defense enzymes such as PAL, PPO, POD and β-1,3-glucanase. Published studies showed ozone induced activity of PAL in bell pepper (Chen *et al.,* 2016), PPO in hot pepper (Sachadyn-Król *et al.*, 2016), POD in pear (Zhao *et al.,* 2013b) and β-1,3-glucanase in tobacco (Ernst *et al.*, 1992). The response towards ozone however, varies depending on the fruit species and ozone dosage. Exposure to inappropriate ozone dosage may reduce the activity of these enzymes hence, weaken plant defense against disease. For example, Chen *et al.* (2016) reported that exposure to high ozone dosage, 6420 ppm ozone for 15 min, reduced PPO activity in bell pepper. This suggests each commodity has to be applied with an appropriate ozone dosage. Therefore, the response of bell pepper towards ozone has to be investigated.

Ozone also has the potential to enhance fruit phytochemical content and antioxidant capacity. The effects of ozone on fruit phytochemical content such as phenolic compounds, ascorbic acid and β-carotene has been studied on kiwi (Minas *et*

al., 2010), tomato (Rodoni *et al.*, 2010), guava and pineapple (Alothman *et al.*, 2010). The studies reported that the fruit showed variation in response where ozone can be stimulating or reducing fruit phytochemical content (Alothman *et al.*, 2010; Rodoni *et al.*, 2010; Minas *et al.*, 2010). The response largely depended on fruit commodity and ozone dosage. To our knowledge, there are limited studies on the effect of ozone on phytochemical content of bell pepper. Therefore, it is crucial to investigate the effect of ozone on phytochemical content and antioxidant capacity of bell pepper. This will give insights on the fruit response towards ozone exposure and its potential to enhance phytochemical content in bell pepper.

Considering the potential of ozone to reduce bacterial growth and fungal development as well as its potential to stimulate fruit phytochemical content, it is a positive approach to study the effect of ozone on bacterial contamination, anthracnose development and phytochemical content of bell pepper. This would investigate its potential to reduce microbial development on fruit and the fruit response towards ozone in terms of phytochemical content, physical and chemical qualities. The outcome from this study would give insights on the potential of ozone treatment to reduce disease development and maintain quality of bell pepper during storage. This will provide a potential alternative to the farmers to use a safe and sustainable postharvest treatment to replace chlorine and fungicides. This leads to the objectives of this study:

- to determine the effect of ozone on pathogenic foodborne bacteria, *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* on fresh cut bell pepper
- to investigate the effect of ozone on *C. capsici* and anthracnose development on bell pepper

- to study the effect of ozone on plant defense enzymes such as PAL, PPO, POD and β-1,3-glucanase
- to evaluate the effect of ozone on antioxidant content of bell pepper such as ascorbic acid, phenolic compounds and β-carotene
- to investigate the effect of ozone on physical, biochemical and physiological quality of bell pepper

CHAPTER 2

REVIEW OF LITERATURE

2.1 Overview of bell pepper

2.1.1 Taxonomy of *Capsicum* genera

In 1700, a French botanist, Joseph Pitton de Tournefort described pepper species and named the genus as *Capsicum. Capsicum* which derived from Latin words of 'capsa' means satchel (Bosland and Votava, 2000). *Capsicum* genus belongs to *Solanaceae* family, a flowering plant which also comprises of other economically important crops such as potatoes, tomatoes and tobacco (Figure 2.1).

Modern science described *Capsicum* genus as flowering plants with three or more pedicles, smooth or toothed calyx margin, non-pulpy fruit and have pungency characteristic of capsaicin components (Andrews, 1995). It is categorized into five domesticated species which are purple flowered species; *pubescens* and white flowered species; *annuum, chinense, baccatum* and *frutescens* (Andrews, 1995) (Figure 2.2). In exception for bell pepper, a single mutation in capsaicin gene losses its ability to produce capsaicin compounds which have pungency characteristic (Bosland and Votava, 2000). This therefore, contributes to the 'sweet' flavour of bell pepper.

Kingdom	: Plantae
Division	: Angiospermae
Class	: Dicotyledonese
Subclass	: Metachlamydeae
Order	: Tubiflorae
Family	: Solanaceae
Tribe	: Solaneae
Subtribe	: Solaninae
Genus	: *Capsicum*
Species	: *pubescens* / *annuum / chinense / baccatum / frutescens*

Figure 2.1: Taxonomy of Capsicum genera (Adapted from: Andrews, 1995)

C. annuum is unique compared to other species by the presence of 2 pairs of acrocentric chromosomes in their DNA structure unlike other *Capsicum* species with 1 pair of acrocentric chromosome (Andrews, 1995). *C. annuum* can be divided into two major groups; 1) the pungent species such as jalapeno, cayenne and serrano peppers 2) the non-pungent or sweet species such as bell, Cuban and pimiento peppers (Bosland and Votava, 2000) (Figure 2.3).

Bell group of *C. annuum* has a characteristic of rectangular pod. It has the largest number of cultivar such as 'California wonder', 'Waki', 'Walter', 'Zamboni', 'Robusta', 'Camelot' and 'King Arthur'(Bosland and Votava, 2000; Fox *et al.*, 2005; Villavicencio *et al.*, 1999) . It can be found in various of colours including green, purple, yellow and

Capsicum annuum

Capsicum chinense

Capsicum baccatum

Capsicum frutescens

Capsicum pubescens

Figure 2.2: Five domesticated species of *Capsicum* genera (McMullen and Livsey, 2012)

white which ripen to red, orange, green and brown, depending on cultivar (Bosland and Votava, 2000). It has sweet or non-pungent taste and valued for its bright colour, flavour and phytochemical content (Fox *et al.*, 2005).

Pungent *Capsicum annuum*

Cayenne

Jalapeno

Serrano

Non-pungent *Capsicum annuum*

Bell

Cuban

Pimiento

Figure 2.3: Pungent and non-pungent group of *C. annuum* (Hultquist and Hultquist, 2011)

2.1.2 Physiology of bell pepper

C. annuum varies in terms of their physiology. It can either be climacteric or non-climacteric, depending on the cultivar. For example, *C. annuum* species of chili

(cv. Chooraehong) has climacteric physiology where it shows a dramatic increase in respiration rate (Gross *et al.*, 1986). On the other hand, chili (cv. Changjiao) has non-climacteric physiology with no climacteric increase in respiration pattern and it does not respond to ethylene application (Lu *et al.*, 1990).

Variation in *C. annuum* physiology is also observed in bell pepper. For instance, bell pepper (cv. Yolo Wonder) has non-climacteric pattern in both respiration and ethylene production (Villavicencio *et al.*, 1999) while bell pepper (cv. Maor) displays no climacteric pattern in respiration but climacteric pattern in ethylene production (Lurie, 1986). Furthermore, some cultivars displayed climacteric peak at mature green stage while other cultivars has the climacteric peak as ripened. Therefore, it is difficult to clearly classify the physiology pattern of bell pepper.

Bell pepper undergo changes in colour during ripening which turn from green to variety of colours such as red, orange and yellow, depending on cultivar (Figure 2.4). Bell pepper are commonly harvested at physiologically mature green stage when the fruit pod reaches typical size with thick pericarp wall and will not significantly increase in size if left to ripe on plant (Fox *et al.*, 2005). Fruit harvested at physiologically mature stage only undergo slight changes in colour after harvest, producing fruit with chocolate or partially colour fruit (Fox *et al.*, 2005). Full colour fruit are obtained when left to ripe on plant and have sweeter taste, improved aroma and higher nutritional attributes. However, harvesting fruit at ripe stage would add extra cost to farmers due to extended ripening time and risk of damage from disease or insect (Fox *et al.*, 2005). Besides, ripe fruit also has shorter shelf life and more susceptible to physical injury than green fruit

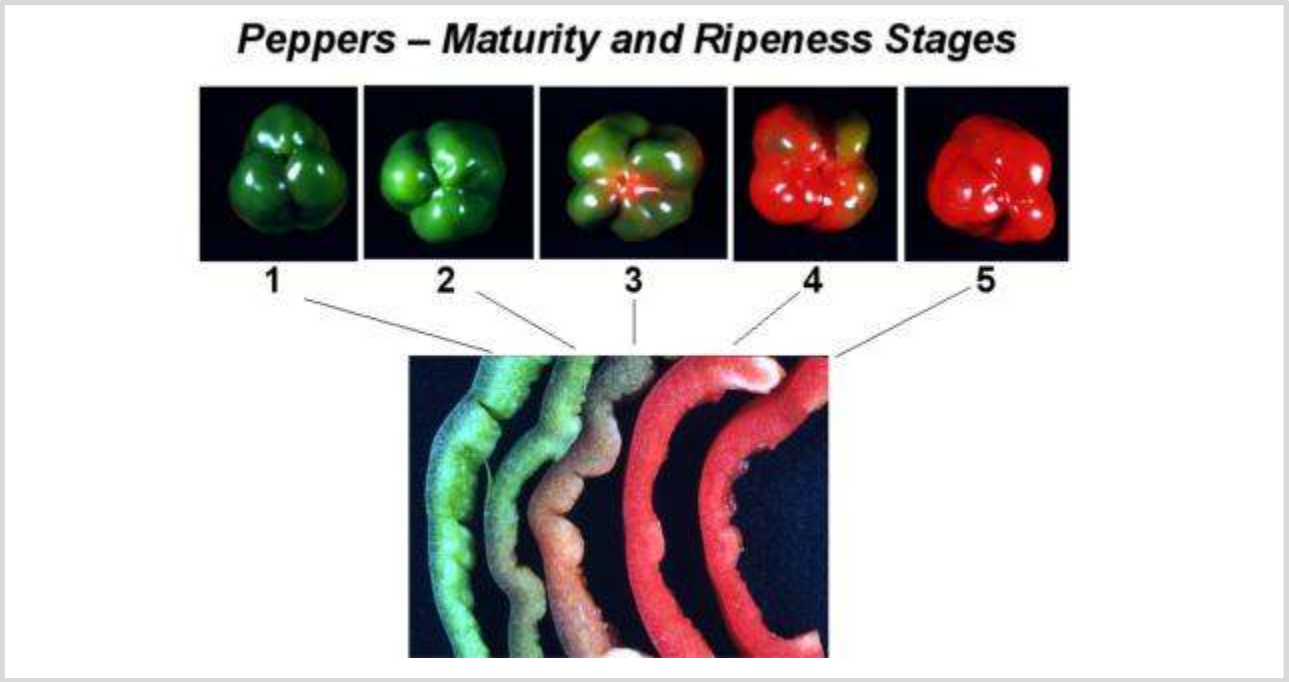

Figure 2.4: Ripeness stage of bell pepper (Source; Cantwell, 1996)

(Fox *et al.*, 2005). Therefore, bell pepper is commonly harvested at physiologically mature green stage (stage 1 – Figure 2.4).

2.1.3 Pepper production and consumption

Pepper is mostly produced in Asia, primarily in China which accounted for 52% of world total production (Figure 2.5). This is followed by Mexico, Turkey and Indonesia. Pepper production has positive growth every year where it increased from 10 million

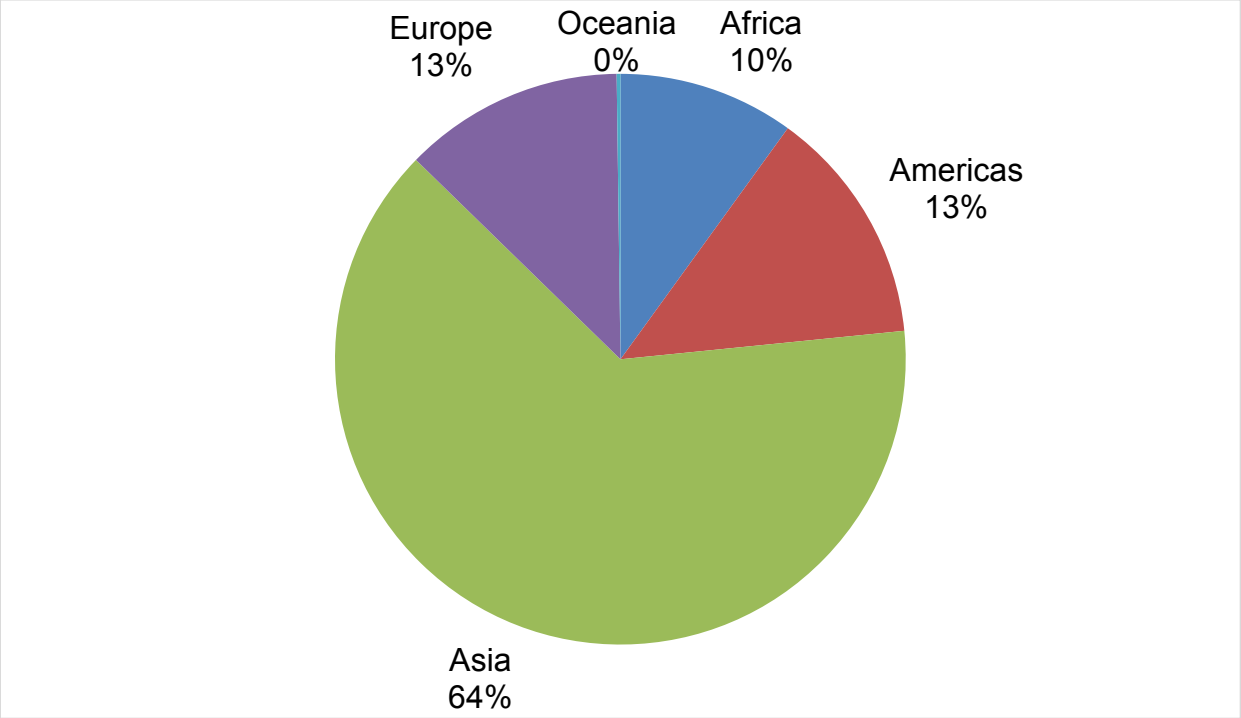

Figure 2.5: Production share of peppers by region (Source: FAOSTAT, 2014)

tonnes, valued at USD 5 billion in 1990 to 31 million tonnes, valued at USD 14 billion in 2012 (Figure 2.6) (FAOSTAT, 2014). This shows the increasing demand of pepper throughout the years which possibly due to increase use of chillies and peppers in fast food industry and as a replacement for artificial flavour. Besides, it is also due to awareness for a healthy diet by consuming pepper as a source of vitamin C.

Trading pattern of peppers is also increasing since year 1990 to 2011 (Figure 2.7) being Mexico is the largest exporter with 699,000 tonnes in 2011 and United States is the largest importer with 779,000 tonnes. Meanwhile, India was reported to has the highest consumption pattern followed by Bangladesh, United States and China (TIPS and AusAID, 2005).

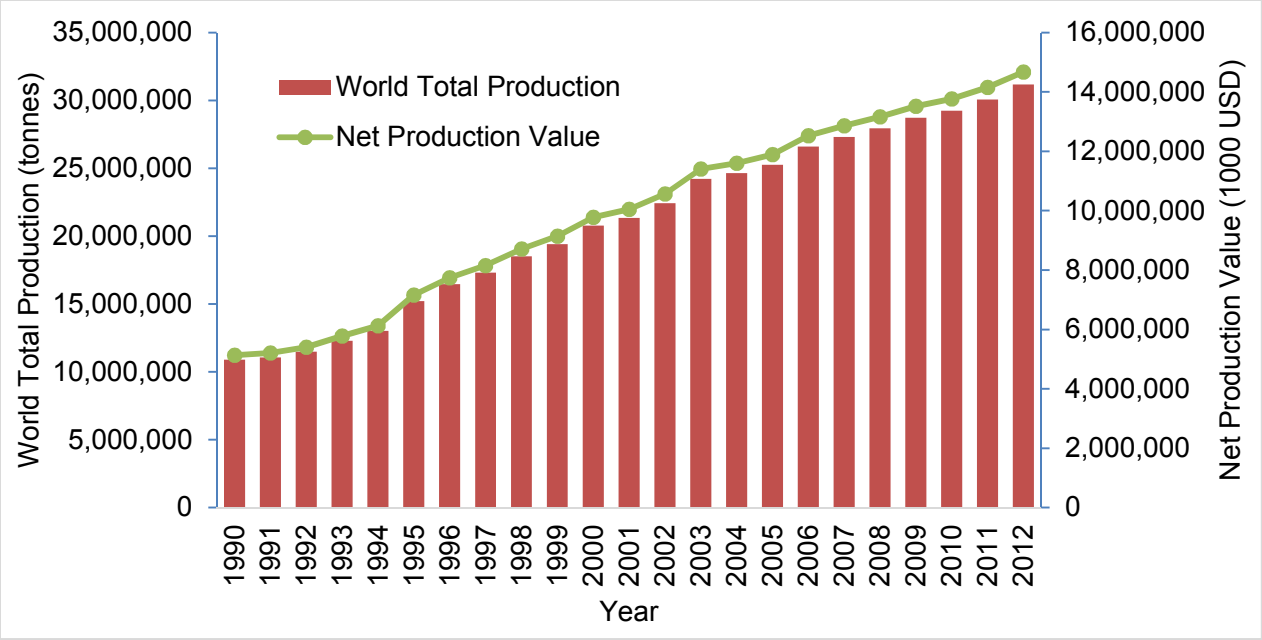

Figure 2.6: World total production and net production value of peppers (Source: FAOSTAT, 2014)

Malaysia is also a producer of peppers. Its production is however, relatively small compared to its neighbours, China and Indonesia. The highest production of peppers in Malaysia was reported in 2009 with production of 35,000 tonnes. The production decreased to 28,000 tonnes in 2011 and increased to 29,000 tonnes in 2012. Export of peppers from Malaysia started in 2008 with 7,800 tonnes and increased to 9,000 tonnes in 2012. Meanwhile, Malaysia imported 42,000 tonnes of peppers in 2008 and 40,000 tonnes in 2012 to support its high consumption pattern which has the highest annual growth in consumption after United States (TIPS and AusAID, 2005). This shows Malaysia has a large market for peppers and it would be a valuable sector for agricultural industry to invest. Apart from fulfilling the local demand, this would enhance its export potential hence can be a source of income for the country.

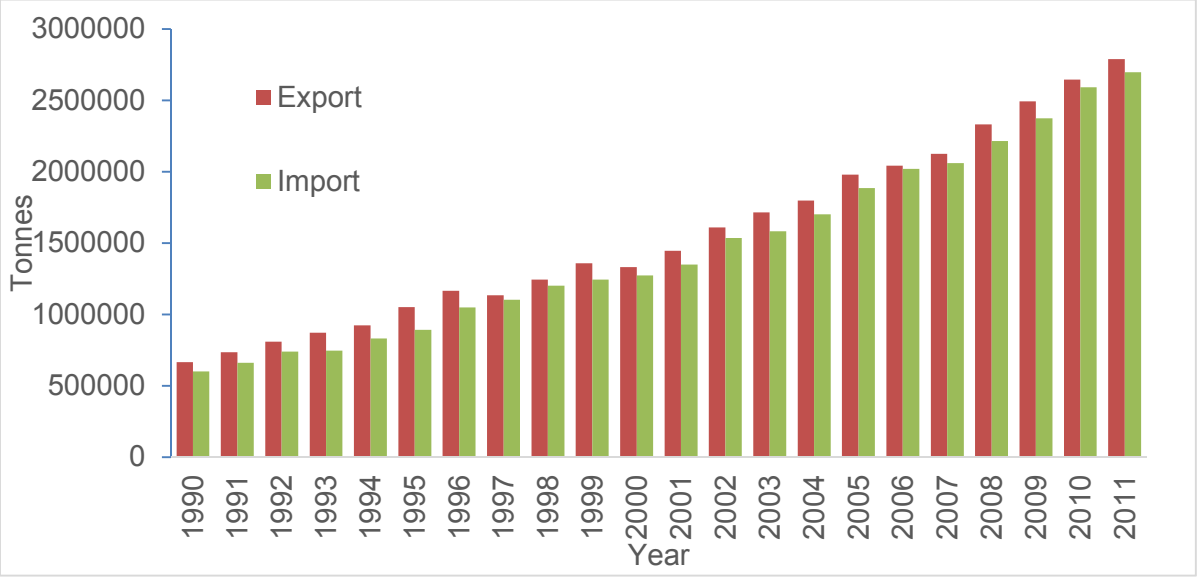

Figure 2.7: World import and export quantity of peppers (Source: FAOSTAT, 2014)

2.1.4 Nutritive quality of bell pepper

Bell pepper is extensively consumed for its high antioxidant content. It contains high level of antioxidant such as vitamin A and C as well as other vitamins including vitamin B_1 (thiamine), B_2 (riboflavin), B_3 (niacin) and K (Table 2.1) (Bosland and Votava, 2000). In comparison to other vitamin C-rich fruit, bell pepper provides up to six times more vitamin C compared to orange (Bosland and Votava, 2000). Besides, serving of a medium size bell pepper provides 180% of Vitamin C recommended daily allowance (RDA) (Bosland and Votava, 2000). Bell pepper is also a good source of vitamin A where daily requirement of vitamin A can be obtained from half tablespoon of red

Table 2.1: Nutritional constituents in 1.9 kg of green bell pepper (Bosland and Votava, 2000)

Nutritional constituents	Quantity	Nutritional constituents	Quantity
Water (%)	93	Na (mg)	3.0
Energy (kcal)	25	Vitamin K (mg)	195
Protein (g)	0.9	Vitamin A (IU)	530
Fat (g)	0.0	Vitamin B_6 (mg)	0.16
Carbohydrate (g)	5.3	Vitamin B_1 (mg)	0.09
Fibre (g)	1.2	Vitamin B_2 (mg)	0.05
Ca (mg)	6.0	Vitamin B_3 (mg)	0.55
P (mg)	22.0	Vitamin C (mg)	128
Fe (mg)	1.33		

pepper (Bosland and Votava, 2000). In addition to the antioxidant content, bell pepper also provides carbohydrates, fibre, potassium, iron and calcium (Bosland and Votava, 2000).

Bell pepper is used in culinary for its bright colour as well as to enhance food flavour (Sun *et al.*, 2007; Tan *et al.*, 2012). However, its application in cooking reduced its Vitamin C content by 30% (Bosland and Votava, 2000). Meanwhile, for dried bell pepper, it may loss all of its phytochemical contents (Bosland and Votava, 2000). Therefore, it is best to be consumed raw. Concurrently with the awareness to consume

fresh and minimally processed fruit and vegetables, consumption pattern of bell pepper increased and commonly consumed salad (Wright, 2002). This allows the consumers to obtain optimal antioxidant benefit from bell pepper.

Consumption of bell pepper helps to reduce the risk of various degenerative diseases such as cancer, cataract, cardiovascular, diabetes, Alzheimer's and Parkinson's (Deepa *et al.*, 2007). This is due to its high antioxidant content which potentially detoxifies harmful oxidative compounds in the body (Tan *et al.*, 2012). Therefore, dietary with bell pepper is a natural measure to prevent the degenerative diseases.

2.2 Bell pepper postharvest issues

2.2.1 Bacterial contamination

Bacterial contamination on fruit has been an issue for more than a century. The contamination source could be faeces from wild and domestic animals which contaminated irrigation water or unhygienic cultivation area (Yuk *et al.*, 2006). Pathogenic bacteria in improperly processed manure could also contribute to contamination during preharvest stage. An example of bacterial contamination at preharvest stage was reported by Ganeshan and Neetoo (2015) where 37% of the harvested bell pepper was contaminated with *Salmonella* sp. At postharvest stage, contamination could occur from unhygienic handling procedures and improper storage area. Fruit flies could be a vector in transmitting the pathogenic bacteria at both pre- and postharvest stage (Heaton and Jones, 2008). Leon-Felix *et al* (2010) reported that 15% of packed bell pepper were contaminated with fecal coliforms. This shows bacterial contamination could occur at every stage; from cultivation until storage stage; hence a proper treatment is required to disrupt the contamination pathway before the produce reaches consumer.

Bacterial contamination was reported in whole, fresh cut and frozen fruit. Irregular fruit surface such as carrot and cantaloupe can harbor bacterial growth hence escalating the risk of food poisoning (Stine *et al.*, 2005). Fruit with smooth surface such as bell pepper and tomato could also contribute to food poisoning as certain bacterial species such as *Salmonella* sp. is capable to attach to the fruit surface (Fernandes *et*

al., 2014). In addition, the presence of wounds, crevices and cracks on fruit surface, allows bacterial cells to multiply inside fruit tissues hence escalating the risk of food poisoning (Selma *et al.*, 2007). Fresh cut fruit posed higher risk of food poisoning as the porous fruit tissues has high tendency to harbour bacteria and presence of fruit juices would promote bacterial growth. Contamination on cut fruit could be from cross-contamination during cutting process and handling and storage procedures (Selma *et al.*, 2008a). A major bacterial contamination on cut fruit was reported recently where 30,000 cases of fresh cut products including bell pepper was withdrawn from market due to contamination by *Listeria* sp. (Staff, 2016). This had substantial negative economic impact on farmers, wholesalers, retailers and posed a significant threat to consumers.

2.2.2 Fungal disease

Fungal disease is a major problem affecting bell pepper and can result in massive postharvest loss. The main fungal disease affecting bell pepper is anthracnose, which is caused by *Colletotrichum* sp. including *C. capsici* (Syd.) Butl. & Bisby, *C. gloeosporioides* (Penz) Sacc. *C. coccodes* (Wallr.) and *C. acutatum* Simmonds (Tomás-Callejas *et al.*, 2012), being *C. capsici* is the most prevalent in Asia (Harp, 2008). Anthracnose is acquired from source of inoculum such as fungal spores on plant debris and infested seed (Elizaquível *et al.*, 2012). The fungal pathogenesis is initiated by germ tube and apressoria formation which penetrates fruit tissues and allows hyphae development. The hyphae then secrets thermostable toxin that further degrades the fruit

tissues and contributes to its symptoms; circular, sunken and water soaked lesions (Figure 2.8) (Chanchaichaovivat *et al.*, 2007; Phoulivong, 2011) . The lesion then expands throughout the fruit producing soft lesions with dark colour (Phoulivong, 2011). Fungal acervuli then develops on the lesion and produces mass of black conidia (Chanchaichaovivat *et al.*, 2007). As the disease symptoms only develop during ripening stage, particularly during postharvest storage period, the disease development is difficult to control.

Figure 2.8: Anthracnose symptom on bell pepper (Source: Ontario Ministry of Agriculture Food and Rural Affairs, 2009)

Prevalence of anthracnose on bell pepper has caused massive loss to its production. In Unites States, anthracnose was reported to be contributing agent for production loss in bell pepper where it was reported in several states including Ohio and

Florida (Lewis Ivey *et al.*, 2004). Anthracnose was also reported in Trinidad, Brazil and was claimed to contribute to 50% product loss (Ramdial and Rampersad, 2015). Meanwhile, bell pepper in Japan was also infected by anthracnose and it involved multi-prefectures including Shimane, Hyogo, Chiba, Toyama, and Nagano (Kanto *et al.*, 2014). The prevalence of bell pepper worldwide is increasing hence, an effective postharvest treatment is required to reduce the postharvest loss.

2.3 Current postharvest technology

2.3.1 Application of chlorine

Bacterial contamination on fruit is commonly controlled by the use of chlorine, in the form of sodium hypochlorite (NaClO) (López-Gálvez *et al.*, 2010). Chlorine inactivates bacterial cells by the action of hypochlorous acid (HOCl) which disrupts bacterial cellular processes such as nucleic acid activity, molecule transport system and respiration (Gibbons and Laha, 1999). Chlorine also inactivates viruses by disrupting virus' protein coats (Gibbons and Laha, 1999).

Chlorinated water of sodium hypochlorite is used at concentration of 100 ppm to sanitize fresh cut fruit (An *et al.*, 2007). The solution has limited efficacy and increase in its concentration to improve effectiveness is limited by regulation (An *et al.*, 2007). Besides, sodium hypochlorite also produces carcinogenic by-products such as trihalomethanes (THMs) which includes trichloromethane ($CHCl_3$), tribromomethane ($CHBr_3$), bromodichloromethane ($CHCl_2Br$) and dibromochloromethane ($CHClBr_2$) (Stauffer, 2004). These by-products are produced from chemical reaction of chlorine with organic compounds such as humic and fulvic acid present in water (Gibbons and Laha, 1999). They were reported to promote rectal and bladder cancer and may also contribute to spontaneous abortions and low birth weight in pregnancy (Gibbons and Laha, 1999). These major health issues limit the application of sodium hypochlorite as a postharvest treatment.

Chlorine in the form of chlorine dioxide (ClO_2) was developed as an alternative to sodium hypochlorite. Chlorine dioxide is allowed at be used up to 3 ppm and it has higher efficacy compared to sodium hypochlorite (López-Gálvez *et al.*, 2010). It was reported to be effective against *E. coli*, *Salmonella* sp., *L. monocytogenes*, moulds and yeasts on fruit (Fu *et al.*, 2007). Its application was reported to produce negligible level of THMs by-products (López-Gálvez *et al.*, 2010), but the risk of producing the carcinogenic by-products cannot be omitted. Besides, chlorine dioxide was reported to cause discolouration on lettuce after its application at concentration of 1 ppm for 15 min (López-Gálvez *et al.*, 2010). This negatively affects cosmetic look of the vegetables hence not suitable to be used as a postharvest treatment. These drawbacks therefore, limit chlorine dioxide application.

2.3.2 Application of fungicides

Fungal disease on bell pepper is controlled by application of fungicide where in the case of anthracnose on bell pepper, fungicides such as Maneb (manganese ethylenebisdithiocarbamate), Flint (trifloxystrobun), Quadris (azoxystrobin) and Cabrio (pyraclostrobin) are used (Lewis Ivey *et al.*, 2004). Maneb (Group M3 fungicide) is an ethylene bisdithiocarbamates (EBDCs) fungicide. It attacks multiple fungal biochemical sites that disrupts fungal biochemical processes hence, inhibits its proliferation (Fungicide Resistance Action Comittee, 2012). Meanwhile, Flint, Quadris and Cabrio are strobilurin fungicides. They attack fungal mitochondria and interfere with fungal respiration system (Fungicide Resistance Action Comittee, 2012). This inhibits fungal

disease development. Quadris was reported to be effective to control anthracnose in peppers (Harp, 2008). Its efficacy was however, found to be inconsistent (Lewis Ivey *et al.*, 2004).

Application of fungicide can lead to development of fungal resistance species, particularly from the use of specific target fungicide such as strobilurin fungicide. This fungicide specifically attacks Quinone binding site of fungal mitochondria and was reported to induce resistance in *Alternaria solani* (Pasche *et al.*, 2004). Besides, Benomyl was also reported to induce fungal resistance such as in *Colletotrichum gloeosporioides* (Maymon *et al.*, 2006) and *Botrytis cinerea* (Washington *et al.*, 1992). This requires the use of fungicide at higher concentration which is costly and harmful to the environment.

Fungicide also negatively affects human and the environment. For example, Triazole inhibits fungal development by disrupting fungal sterol synthesis. However, it also reacts with male reproduction system (Goetz *et al.*, 2009). This causes reduction in testosterone production level hence, contributes to infertility (Goetz *et al.*, 2009). Meanwhile, Captafol has threatening effect on freshwater invertebrates and bird reproduction system. It is therefore has been banned in United States, Australia, Thailand and Korea (United Nations, 2005). Besides, Maneb is banned in Korea and Russia due to its carcinogenic, mutagenic and teratogenic effects (United Nations, 2005). These negative effects posed from the application of fungicides limits its application. This also shows application of fungicide is not a sustainable approach for agriculture industry.

2.3.3 Low temperature storage

Low temperature storage is the most common postharvest treatment applied on bell pepper by storing the fruit at 7 – 12°C (Biosecurity Australia, 2009; Bosland and Votava, 2000). This postharvest treatment is applied during fruit storage and transportation where trucks with cold storage facility are used for domestic transportation and refrigerated containers are used for international distribution (Biosecurity Australia, 2009).

Low temperature storage improves fruit shelf life by reducing fruit enzymatic activities. This includes decreasing the rate of starch and sucrose degradation in carbohydrate metabolism as well as pectin degradation in cell wall hydrolysis (Bosland and Votava, 2000). Besides, low temperature also decreases fruit respiration and transpiration hence, reduces water loss (Bosland and Votava, 2000). This delays fruit ripening and preserves fruit firmness and colour degradation. Furthermore, low temperature may inhibit fungal and bacterial growth hence would reduce disease development.

Storage at temperature lower than its optimal (7 – 12°C) induces imbalanced metabolism and results in chilling injury (Bosland and Votava, 2000). This causes development of sunken discoloured spots, pitting and pericarp softening which increase its susceptibility to decay (Bosland and Votava, 2000; Vicente *et al.*, 2005). Meanwhile, storage at temperature below fruit freezing point causes freezing of tissue water and formation of intracellular ice. The loss of free water causes desiccation and imbalance

cellular reaction. Besides, the intracellular ice may disrupt fruit cell structure and the fruit unable to restore its metabolism or structures even after thawing (Wills *et al.*, 2007). With a proper control of temperature, storage at 7-12°C is a good postharvest treatment for bell pepper. This postharvest treatment may extend its shelf-life for up to 2 to 3 weeks (Bosland and Votava, 2000).

2.3.4 Modified Atmosphere Packaging (MAP)

Modified Atmosphere Packaging (MAP) is another postharvest treatment used to maintain bell pepper quality during storage. It is used to export bell pepper into United State (Wills *et al.*, 2007). It uses polyethylene bags to create low oxygen environment around the fruit which inhibits fruit respiration (Wills *et al.*, 2007). Low oxygen environment also inhibits fungal growth hence, reduces postharvest decay. Besides, MAP also reduces fruit transpiration and reduces water loss (Wills *et al.*, 2007). This delays fruit ripening and hence, prolongs its shelf life.

Application of MAP needs to be carefully monitored by considering fruit respiration rate and level of oxygen and carbon dioxide used and released by the fruit. Appropriate bag needs to be used for the right commodity where diffusion of oxygen into the bag should be higher than its consumption by fruit. Meanwhile, carbon dioxide should diffuses out from the bag at higher rate than its production by the fruit (Kader, 2002). In order to apply this treatment, parameters such as thickness of bag, temperature storage and arrangement of the fruits need to be carefully monitored. This is to ensure appropriate oxygen and carbon dioxide content to avoid anaerobic

respiration and carbon dioxide injury which could deteriorate fruit quality hence reduce its storage life.

2.3.5 Other postharvest technology in development

Limitations and safety issues pose by current postharvest treatment lead to development safe and green alternatives. This includes utilization of antagonistic microorganisms such as yeasts. The antagonistic microorganisms compete with spoilage fungus for space and nutrients hence, inhibits fungal development (Nantawanit *et al.*, 2010). It also secrets hydrolytic enzymes which disrupt fungal cell wall (Nantawanit *et al.*, 2010). Besides, the antagonists also induce fruit natural defense system and increase fruit antioxidant capacity (Nantawanit *et al.*, 2010). This increases fruit resistance against pathogen. Examples of antagonist are *Pichia guilliermondii* and *Aureobasidium pullulans* which were used to control proliferation of *C. capsici* (Nantawanit *et al.*, 2010) and *Botrytis cinerea* and *Peniillium expansum,* respectively (Abano and Sam-Amoah, 2012). Its application is safe, biodegradable and cost effective. However, antagonists are inefficient to inhibit wide-range of spoilage fungus and may not be able to survive during postharvest storage (Abano and Sam-Amoah, 2012). Therefore, this limits its application as a postharvest treatment. Besides, the idea to apply microorganism on fruit is not well accepted by consumers.

Another potential alternative to replace current postharvest treatment is by the use of organic fungicides such as chitosan, seed powder of yam bean and plant derived biochemical components such as hinokitiol and saponin which are derived from Hinoki

tree and pepper cayenne, respectively (Bautista-Banos and Lucca, 2004). These natural fungicides inhibit microbial growth by oxidizing microbial cell membrane, disrupting their biochemical process and altering their DNA (Bautista-Banos and Lucca, 2004). Efficacy of these natural fungicides was reported against a variety of spoilage fungus including *C. gloeosporioides*, *Aspergillus niger*, *B. cinerea* and *Monilinia fructicola* (Bautista-Banos and Lucca, 2004). However, organic fungicide such as plant essential oil is ineffective at low concentration and exhibits phytotoxic effect at high concentration (Plotto *et al.*, 2003). This reduces its efficiency hence limits its application as a postharvest treatment.

2.4 Novel postharvest technology: Ozone fumigation

2.4.1 Overview of ozone fumigation technology

Ozone, triatomic molecule of oxygen (O_3) is a naturally occurring gas that accommodates the stratosphere layer of atmosphere (Forney, 2003). It is a potent oxidizing agent with an oxidation potential of 2.09 V which is the highest oxidizing potency after fluorine (F), chlorine trifluoride (ClF_3), atomic oxygen (O) and hydroxyl free radical (•OH) (Forney, 2003). In comparison to chlorine, ozone has 1.5 times higher oxidizing capacity suggesting better efficacy in disease control (Forney, 2003).

Ozone can be commercially produced by applying high voltage energy through narrow gap of electrode (Gabler *et al.*, 2010) (Figure 2.9). This energy movement discharges corona which subsequently excites electrons in molecular oxygen (O_2) and results in splitting of the oxygen molecule to atomic oxygen (O). The reactive atomic oxygen then reacts with molecular oxygen (O_2) producing triatomic oxygen molecule or ozone (O_3) (Figure 2.9) (Forney, 2003). Ozone is a highly unstable compound where it decomposes back to oxygen and no harmful by-products are produced (Gabler *et al.*, 2010). Therefore, ozone was approved by U.S. Food and Drug Administration (FDA) in 2001 to be utilized during food treatment and food storage either in aqueous or gaseous form (Lake, 2001).

Stability of ozone is influenced by several factors such as temperature, organic matter and pH. Ozone is more stable at low temperature where its shelf-life can be extended to 6 min at 4℃ compared to 3 min at 20℃ (Forney, 2003). Ozone shelf-life is

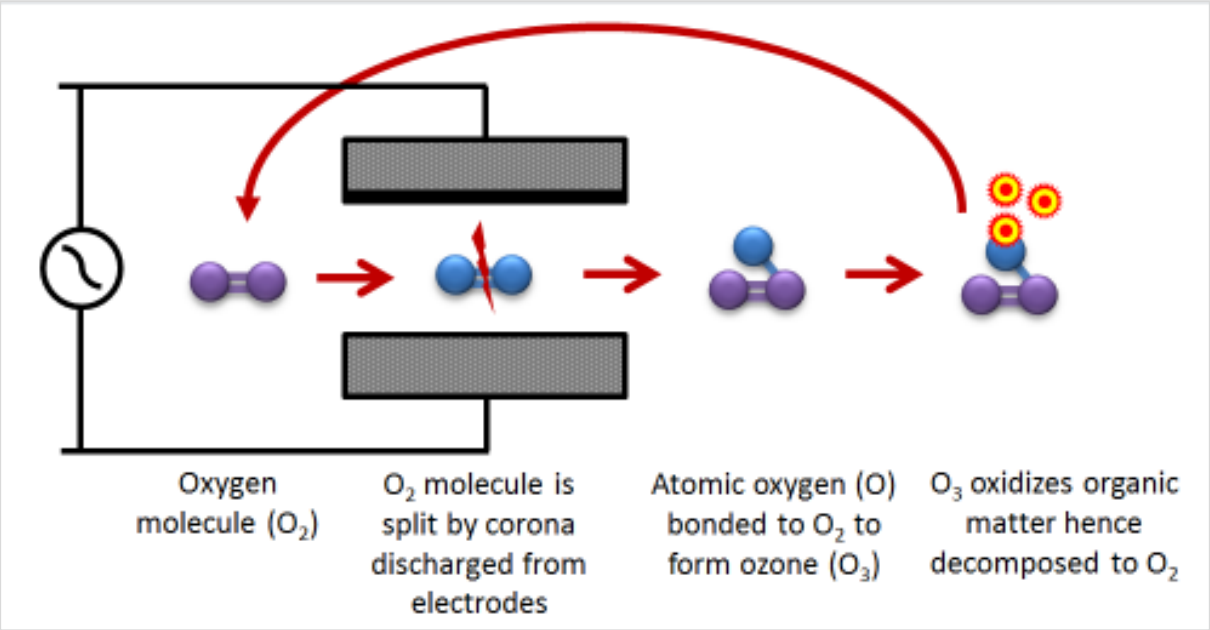

Figure 2.9: Schematic diagram of oxygen transformation into ozone molecule by corona and its decomposition to oxygen after reaction with organic matter

also influenced by availability of organic matter which serves as a reactant and decomposes the ozone (Palou *et al.*, 2001). Besides, surrounding pH also affects ozone shelf life where pH 10 causes instantaneous ozone decomposition (Forney, 2003).

Ozone can be applied either in aqueous or gaseous form. Application of aqueous ozone was reported to reduce *Shigella sonnei* (Selma *et al.*, 2007) and *Escherichia coli* O157:H7 (Ölmez and Akbas, 2009) populations on lettuce. However, its efficacy is highly susceptible towards pH change and presence of organic matter in the water (Forney, 2003). This contributes to inconsistency in its efficacy hence, limits its application. Besides, the use of water may introduce cross-contamination when used with large volumes of produce (Tomás-Callejas *et al.*, 2012). The risk of cross-contamination is escalated by reconditioning and recycling of water as recommended by U.S Department of Agriculture (USDA), because of economic and environmental factors

(Tomás-Callejas *et al.*, 2012). This leads to development of gaseous ozone treatment which could alleviate some of these challenges.

Application of ozone in gaseous form is more promising due to its higher stability compared to aqueous ozone. The use of air in gaseous ozone omitted the influence of pH and organic content of water in its application. However, the efficacy of gaseous ozone is influenced by relative humidity of air where humid environment improves its efficacy (Han *et al.*, 2002).

Application of gaseous ozone is practical where ozone generators can be installed in an enclosed chamber where gaseous ozone can be directly exposed to the fruit (Forney *et al.*, 2007). Besides, gaseous ozone also can be distributed through Teflon tube which allows its application in a large storage room (Palou *et al.*, 2003). Gaseous ozone can be applied at low temperature (7°C) hence, it is expected to improve its efficiency in extending fruit storage life. The practicality of gaseous treatment also allows this technology to be installed in storage container during transport. This will create a conducive environment during transportation which can inhibit disease development and ripening progress. As this treatment can be applied during transportation, it reduces postharvest processing time as well as labour.

2.4.2 Ozone fumigation and microbial proliferation

Ozone has the potential to inhibit bacterial growth and reduce fungal development due to its strong oxidizing property. It reactively attacks compounds

containing oxygen (O), nitrogen (N), sulphur (S), phosphorus (P) and carbon-carbon (C-C) double bonds such as microbial cell membrane, amino acid, protein and reducing agent such as NADPH (Forney, 2003; Keutgen and Pawelzik, 2008). This degrades microbial cell membrane integrity and leads to cell lysis. Besides, ozone also affects microbial cellular metabolism by inactivating biologically important enzymes such as thiokinases, acyl-CoA-thioesterase, and acyltransferases (Forney, 2003). Ozone also affects microbial cellular redox potential by altering glutathione enzyme function hence, hampering microbial proliferation (Forney, 2003). This multiple mode of action of ozone to inactivate microorganism allows its use on wide range of microbial species. This minimizes the risk of developing microbial resistance species. This is an advantage of using ozone in comparison to fungicides such as Flint, Quadris and Cabrio which has the potential to develop microbial resistance species.

2.4.2.1 Bacterial proliferation

Ozone has the potential to inactivate wide range of bacterial species due to its capability to attack bacterial cell membrane. Its antibacterial activity was reported on various bacterial species including *Salmonella* sp. (Selma *et al.*, 2008b), *Listeria innocua* (Fan *et al.*, 2007), *Staphylococcus aureus* and *Bacillus subtilis* (Thanomsub *et al.*, 2002). This allows ozone to be a universal antibacterial agent which is an advantage property for a postharvest treatment.

The efficacy of ozone however, varies depending on bacterial species. For example, exposure to 2.2 ppm ozone for 1 min effectively reduced 5.6 $\log_{10}$ colony forming unit (CFU) of *Shigella sonnei* population (Selma *et al.*, 2007) but only reduced 1.2 $\log_{10}$ CFU of *L. monocytogenes* population on lettuce (Ölmez and Akbas, 2009). The differences in the efficacy could be due to differences in cell membrane components of different bacterial cells as ozone uses cell membrane as an entry pathway. Besides, this is also could be due to different response by the bacteria towards oxidative stress of ozone.

Antibacterial activity of ozone is also influenced by other factors such as surrounding relative humidity (RH). Ozone is more effective at high RH where exposure to 2 ppm ozone for 25 min at 90% RH reduced 1.94 $\log_{10}$ CFU of *L. monocytogenes* population while treatment at 60% RH only reduced 0.97 $\log_{10}$ CFU of the population (Han *et al.*, 2002). Efficacy of ozone is also affected by other factors such as inoculum concentration, temperature, pH and ozone demand status where low inoculum concentration (Thanomsub *et al.*, 2002), high temperature (Steenstrup and Floros, 2004), high pH (Fan *et al.*, 2007) and low ozone demand status (Kim *et al.*, 1999) increase ozone efficacy. Therefore, these factors have to be carefully controlled while applying ozone treatment in order to get the optimal efficacy.

2.4.2.2 Fungal proliferation

Ozone also has the potential to inactivate wide range of fungal species. This includes blue mold, *Penicillium italicum*, green mold, *Penicillium digitatum* (Palou *et al.*, 2003), grey mold, *Botrytis cinerea* (Minas *et al.*, 2010), brown rot causal agent, *Monilinia fructicola* (Palou *et al.*, 2002) and food spoilage agent, *Aspergillus* sp. (Antony-Babu and Singleton, 2009). Ozone was also reported to reduce anthracnose disease caused by *C. acutatum* (Yun *et al.*, 2006) *C. gloeosporioides* (Barbosa-Martinez *et al.*, 2002), *C. coccodes* (Tzortzakis *et al.*, 2008) and *C. lindemuthianum (Treshow et al., 1969).* This allows ozone to be applied to various commodities with different fungal diseases.

Efficacy of ozone in reducing fungal disease varies depending on fungal species. For example, exposure to 0.3 ppm ozone for 144 h inhibited approximately 50% of *B. botrytis* mycelia growth (Minas *et al.*, 2010) but had no significant effect on *Alternaria alternate* (Tzortzakis *et al.*, 2008). Meanwhile, exposure to 0.05 ppm ozone effectively inhibited *C. coccodes* spore germination (Tzortzakis *et al.*, 2008) but stimulated *Sphaerothecafuliginea* spore germination (Khan and Khan, 1998). The variation in antifungal effect can be due to variation in cell membrane components or fungal response towards oxidative stress of ozone.

2.4.3 Ozone fumigation and chemical contaminants

Ozone has the potential to disinfect chemical contaminant on fruit such as fungicide and pesticide residues. This is due to its strong oxidizing property which oxidizes and decomposes the toxic chemicals into unstable compounds which then undergo isomerisation, dimerization or protonation in order to achieve a stable state (Ikehata and Gamal El-Din, 2005). The unstable products may be further oxidized by ozone which transforms the products into lower oxidation state. This oxidation process may occur repetitively until the toxic chemicals are completely oxidized and decomposed into harmless compounds (Ikehata and Gamal El-Din, 2005).

The efficacy of ozone to decompose chemical contaminants was reported on grapes where exposure to 10 ppm ozone for 1 h reduced fenhexamid, pyraclostrobin, pyrimethanil and cyprodinil content on fruit surface by 68.5, 100.0, 83.7 and 75.4%, respectively (Gabler *et al.*, 2010). Meanwhile, ozone was also reported to decompose insecticides such as imidacloprid (Bourgin *et al.*, 2011). Therefore, the application of ozone on fruit as a postharvest treatment not only inhibits microbial proliferation but also sanitizes the fruit from toxic chemicals. This is an advantage of ozone compared to other postharvest treatment such as low temperature and MAP.

2.4.4 Ozone fumigation and ripening processes

Oxidative effect of ozone also affects fruit respiration, ethylene production and ripening process, depending on the commodity. For example, in carrot, exposure to 10

ppm ozone for 10 min reduced its respiration rate during storage hence prolonged the storage life (Chauhan *et al.*, 2011). The ozone treatment however, caused transient increase in respiration of tomatoes (Rodoni *et al.*, 2010). The variation in response is possibly due to different susceptibility of the commodity toward ozone exposure. The effect of ozone on fruit respiration and ethylene production has to be carefully monitored as it will affect fruit ripening progress.

Oxidation action of ozone is capable to decompose ethylene molecules produced by fruit. This was reported by Palou *et al.* (2001) as ozone application reduced 57.1% of ethylene content in export container. This prevents stimulation action of ethylene on fruit ripening hence prolongs fruit shelf-life. This is very crucial as accumulation of ethylene in storage box highly stimulate fruit ripening process. This is very important particularly for climacteric fruit where ethylene influences its ripening progress.

2.4.5 Ozone fumigation and fruit antioxidant content

Oxidizing effect of ozone has stimulating effect on fruit antioxidant content such as phenolic compounds, ascorbic acid and β-carotene. This was reported by Alothman *et al.* (2010), where exposure to 0.72 mmol ozone for 20 min enhanced fruit phenolic content of pineapple and banana by 15.7 and 14.7%, respectively. The stimulating effect of ozone was also reported in kiwi after exposure to 0.3 ppm of ozone for 144 h (Minas *et al.*, 2010). This could be due to increase in the activity of PAL which responsible for flavonoids or phenol biosynthesis (Emiliani *et al.*, 2009).

Ozone was also reported to enhance fruit ascorbic acid content where exposure to 0.35 ppm ozone for three days increased ascorbic acid content in strawberry (Pérez *et al.*, 1999). This could be due to the inhibition action of ozone on ascorbate peroxidase and ascorbate oxidase enzymes; the main enzymes involve in ascorbic acid decomposition (Pérez *et al.*, 1999). In contrary, ozone reduced ascorbic acid content in lettuce (Ölmez and Akbas, 2009), pineapple, guava and banana (Alothman *et al.*, 2010). This could be due to the scavenging effects of ozone and its induced reactive compounds on ascorbic acid (Alothman *et al.*, 2010).

Variation in the response of fruit antioxidant content towards ozone suggested that this parameter has to be carefully controlled. Ozone can be a stimulant to the antioxidant content hence can improve fruit phytochemical content. This improves its potential phytochemical transfer. However, ozone can also have negative effect on the antioxidant content which reduced its photochemical value. This undesirable effect has to be avoided when applying a postharvest treatment.

2.4.6 Ozone fumigation and fruit defense system

Ozone also influences fruit defense system by affecting activity of certain enzymes such as PPO and POD. For example, ozone was found to inhibit PPO activity in celery where exposure to 0.18 ppm ozone for 5 min reduced the PPO activity by 70.1% (Zhang *et al.*, 2005). Decrease in PPO activity reduces fruit defense against pathogen attack as PPO is responsible for production of antimicrobial compounds such

as quinone and lignin (Zheng *et al.*, 2011). This is an undesirable effect of ozone. However, decrease in PPO activity reduces fruit browning which is a positive attribute to the fruit quality (Ölmez and Akbas, 2009). Therefore, the level of PPO in a fruit has to compromise between defence towards fungal disease and its cosmetic look.

On the other hand, ozone was reported to increase POD activity where exposure to more than 1 ppm ozone enhanced POD activity in pear (Zhao *et al.,* 2013). This shows that ozone has triggered fruit defense mechanism. This increases biosynthesis of quinone and lignin (Wang *et al.*, 2009). Quinone is toxic to fungal cell wall while lignin provides as a physical barrier to inhibit fungal proliferation in fruit tissue (Zheng *et al.*, 2011). This reduces disease development and subsequent quality degradation.

Ozone was also reported to affect activity of pathogenesis-related enzymes such as chitinase (CHI) and β-1,3-glucanase where exposure to 0.15 ppm ozone for 5 h significantly increased CHI and β-1,3-glucanase activity in tobacco (Ernst *et al.*, 1992). Similar stimulating effect of ozone on CHI and β-1,3-glucanase was reported in spring barley plants (Plessl *et al.*, 2005). The pathogenesis-related enzymes; CHI and β-1,3-glucanase, pose antimicrobial property where the enzymes hydrolyze fungal chitin and β-1,3-glucan of fungal cell wall, respectively (Schraudner *et al.*, 1992). This prevents fungal proliferation on fruit hence improves fruit quality.

Variation in the effects of ozone on the different defense related enzymes suggested that ozone can either improve or weaken fruit defense system. The variation in the fruit response depends on the fruit species in encountering the oxidative stress of ozone. Therefore, each commodity has to be carefully studied in their response towards

ozone treatment. A treatment which can improve fruit defense system would consequently reduce disease development hence, extends fruit storage life.

2.4.7 Ozone fumigation and fruit physico-chemical quality

The effect of ozone on diseases, ethylene, respiration, antioxidant level and defense related enzymes influences fruit physical and biochemical qualities. Antifungal and antibacterial effect of ozone reduced disease development on fruit hence improves fruit physical quality (Tzortzakis *et al.*, 2008). Meanwhile, the effect of ozone on defense related enzymes improves fruit defense against fungal invasion which also restricts disease development (Schraudner *et al.*, 1992). This improves fruit physical quality and extends fruit storage life.

The effect of ozone on ethylene and respiration influences fruit acidity and sugar content. Degradation of ethylene and reduction in fruit respiration by ozone delays fruit ripening hence, reduces degradation of starch into sugars (Hong *et al.*, 2012). Meanwhile, it also reduces utilization of organic acid in fruit hence, preserves fruit biochemical property (Hong *et al.*, 2012). This delays fruit senescence hence reduces postharvest losses.

Ozone may also negatively affect fruit physical quality, particularly from exposure to excessive dosage of ozone which could contribute to physical injuries such as rind discolouration, bleaching or browning. These ozone induced injuries were observed in carrot where exposure to 0.05 ppm for 2 months induced discolouration of fruit tissue

(Hildebrand *et al.*, 2008). Further increase in ozone dosage resulted in brown discolouration near to its periderm and subsequently pitting and bleaching of the carrot tissues (Hildebrand *et al.*, 2008). Ozone induced injury was also observed by the appearance of rachis injury in grape (Gabler *et al.*, 2010) and by epidermis browning in papaya (Ong *et al.*, 2012). This suggested each commodity has different susceptibility towards ozone and produces different toxicity symptoms. Therefore, ozone with the right dosage has to be applied to a specific commodity in order to avoid ozone induced injuries.

In conclusion, with the increasing demand of bell pepper worldwide and its high value for phytochemical content, a sustainable postharvest treatment are needed to reduce postharvest loss and extend its storage life. Ozone treatment is a promising technology with the potential to reduce fungal diseases and bacterial contamination as well as stimulate phytochemical content of the fruit. This reduces senescence progress, extends fruit storage life and increases the potential phytochemical transfer to consumer. This multiple benefits of ozone is an advantage of ozone compared to other postharvest treatment such as low temperature refrigeration and MAP. Meanwhile, in terms of safety aspect, ozone provides an obvious benefit compared to fungicide and chlorine where it does not produce carcinogenic by-products, does not induce microbial resistant species and safe for environment. These advantages of ozone provide a promising technology to have a sustainable treatment for agricultural industry.

Practicality of gaseous ozone treatment allows the treatment to be applied to a large number of fruit as the gas can be easily distributed either to a container or a room. This reduces the processing time and labour hence, reduces the processing cost. This

will definitely benefits farmers, distributors and agricultural industry. The practicality of gaseous ozone treatment also allows the treatment to be incorporated into transportation system by installing ozone fumigation system into truck or shipping container. This allows ozone treatment to be applied during transportation hence, reduces processing time as well as labour. The development of this technology will definitely improve postharvest treatment system by reducing postharvest loss and improving food security.

CHAPTER 3

EFFECT OF OZONE FUMIGATION ON BACTERIAL CONTAMINATION ON FRESH CUT BELL PEPPER

3.1 Introduction

Bacterial contamination on fruit can contribute to food poisoning which can be a fatal outbreak. It has been an issue for more than a century. It affected more than 48 million people per year in United States, involving 3,000 deaths and 128,000 hospitalizations, at the cost of USD 50 billion (Bermúdez-Aguirre and Barbosa-Cánovas, 2013; Scharff, 2012). This is a massive threat to food safety and jeopardizing consumer's health. It also contributed to economic loss as the contaminated products were recalled from market hence affected farmers, distributor and supplier (Bermúdez-Aguirre and Barbosa-Cánovas, 2013).

Incidence of food poisoning is associated with consumption of minimally processed or fresh cut fruit and vegetables (Alexopoulos *et al.*, 2013). Its prevalence is increasing in coherent with consumer preference for healthy diet and ready-to-eat (RTE) products (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). Particularly for fresh cut fruit, bacterial contamination introduced by pickers, packagers or during any stage of food production can multiply inside fruit tissues hence, poses high risk of food poisoning. This is a major concern for bell pepper with increasing demand to be consumed raw or fresh cut

Bacterial species that are commonly associated with food poisoning are *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes.* This is due to prevalence of the former two and fatality of the latter (Elizaquível *et al.*, 2012). The contamination is due to animal and human sources along the pre- and postharvest processes including contaminated irrigation water, improperly processed manure and unsanitized handling area (Yuk *et al.*, 2006). Outbreak of *E. coli* O157 and *Salmonella* Typhimurium was reported every year since 2009 where it was associated to consumption of organic spinach, romaine lettuce, hazelnut, cantaloupe and tomatoes (Centers for Disease Control and Prevention, 2012). These incidences led to hospitalizations and deaths due to severe diarrhea, abdominal cramp and vomiting. Outbreak of *L. monocytogenes* was less prevalent but accounted for the highest percentage of hospitalization and death due to its fatality (Elizaquível *et al.*, 2012).

Pepper is a recognized vehicle for foodborne pathogens. Similarly to tomato, its smooth surface allows attachment of certain pathogenic bacteria hence permits their transmission. This contributed to Salmonella outbreak in Mexico on chilli peppers in 2008 which has sickened 1400 people (Gage, 2008). Bell pepper was also reported to be a vehicle for foodborne pathogens as *Salmonella* sp. was detected on red and green bell peppers in 2013 (Larsen, 2013). Besides, *L. monocytogenes* was detected on fresh cut bell pepper in United States recently hence, leads to withdrawal of the products in 9 states (Clark, 2016). The prompt actions of the related bodies have prevented potential injuries and deaths. However, the risk of food poisoning carried by bell peppers cannot be omitted.

The risk of food poisoning caused by these pathogens being bell peppers as the transmission vehicles substantially affected food safety. This leads to the urgency of a safe and effective sanitization treatment to reduce microbial load on fruit hence, reduces the risk of food poisoning. This leads to the objectives of this study to:

1. assess the effect of ozone fumigation on *in vitro* population of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes*
2. investigate the effect of ozone fumigation on *in vivo* population of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* on fresh cut bell pepper
3. to evaluate the effect of ozone fumigation on the pathogens' morphology

3.2 Materials and Methods

3.2.1 Ozone fumigation chamber

Ozone fumigation was conducted in air-tight polycarbonate chambers (112 x 47.5 x 42.5 cm) equipped with 12 V fans (Figure 3.1). Ozone was generated using MedKlinn Professional Series Ozone Generator and its concentration was monitored using an ozone sensor (Model OEM-2 Eco-Sensor, Inc.), controlled using a process controller (Model K3MA-J, OMRON Corp.) and calibrated against an ozone analyser (Model IN2000-L2-LC, In USA, Inc.) (Ong *et al.*, 2012). Temperature and relative humidity inside the chambers were monitored using a data logger (Model U14 LCD Logger, HOBO®, USA) and were maintained at 18 - 20°C and 95% relative humidity (RH), respectively. Ozone concentration was set using the process controller (1.0 ppm to 10.0 ppm) and ozone concentration was monitored continuously using the ozone sensor. The chambers were placed in a room equipped with a charcoal ozone scrubber. Ozone generator was turned off prior opening and chambers were only opened after ozone concentration was less than 0.3 ppm, the Threshold Limit Value - Short Term Exposure Limit (TLV-STEL) set by the United States Occupational Safety and Health Administration (US-OSHA) (Palou *et al.*, 2002). Protective mask (Half Facepiece Respirator, Brand 3M, USA) was used in case of ozone exposure.

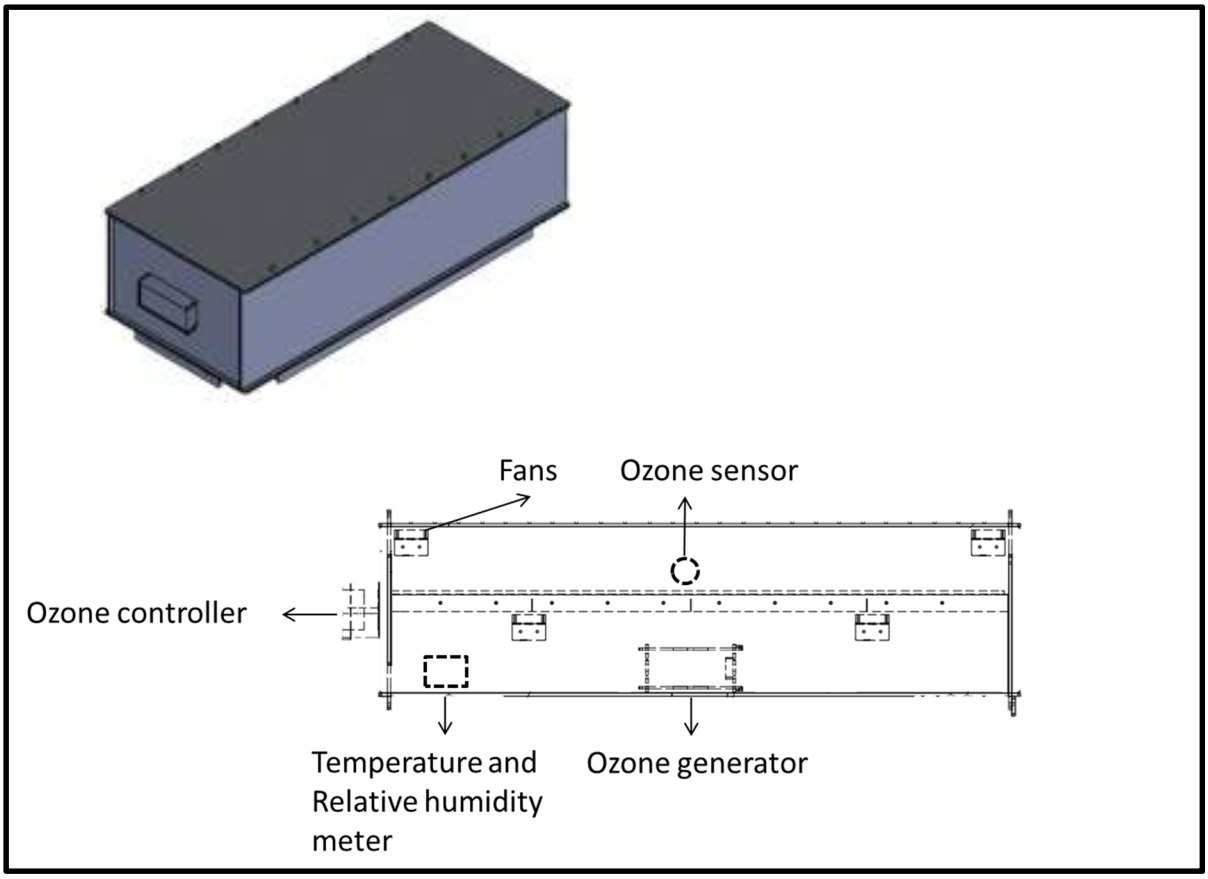

Figure 3.1: Schematic diagram of air-tight ozone chamber equipped with fans, ozone generator, ozone sensor, ozone controller and temperature and humidity meters

3.2.2 Fruit material

Bell pepper (*Capsicum annuum* cv. 'Zamboni') were harvested at physiologically matured, green stage (maturity index 1 (Figure 2.4)) from commercial farm in Cameron Highland, Pahang. Fruit of uniform size (≈150 g fruit^{-1}), free of physical damage and fungal infection were cleaned and rinsed with distilled water three times and air dried to remove surface water.

3.2.3 Preparation of inocula

Clinical isolates of *E. coli* O157 and *L. monocytogenes* obtained from Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) and commercially available *Salmonella* Typhimurium ATCC® 14028 were used in this study. The identity of the bacterial species was re-confirmed through Gram staining and inoculation onto selective and differential media such as MacConkey Agar with Sorbitol (SMAC, Pronadisa), Xylose Lysine Deoxycholate Agar (XLD, Oxoid) and PALCAM Listeria Selective Agar (PC, Fluka Analytical), respectively.

Growth curves for *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* were prepared by inoculating 1 ml of 1 x 10^7 colony forming units (CFU) ml^{-1} cultures into 50 ml of nutrient broth (NB, Merck, pH 7.0). The cultures were statically incubated at 37°C for 24 h and bacterial concentration was estimated using a Biochrom Libra S12 Spectrophotometer at A_{600} (Appendix A 3.1 – 3.3). Cultures at early stationary stage were used for *in vitro* and *in vivo* assays.

3.2.4 Screening of antibacterial activity of ozone

Freshly prepared bacterial cultures were serially diluted with ¼ strength of Ringer's solution (RS, Merck, pH 7.0) to achieve a range from 200 to 300 CFU $plate^{-1}$ on nutrient agar (NA, Merck, pH 7.0) using spread plate method. The inoculated plates were incubated at room temperature for 1 h at room temperature to allow bacterial

attachment before ozone exposure. Screening of antibacterial activity of ozone was determined by exposing the inoculated agar plates (with lids removed) to ozone at concentrations of 0 (control), 0.1, 0.5, 1.0, 5.0 and 9.0 ppm, for exposure times of 0.25, 0.5, 3, 6, 18 and 24 h at 18 - 20°C and 95% RH. Following ozone exposure, the plates were incubated at 37°C for 24 h for *E. coli* O157 and *Salmonella* Typhimurium and 48 h for *L. monocytogenes*. Number of colony forming unit (CFU) for each treatment was determined using a Galaxy 230 colony counter (Rocker Scientific Co. Ltd.) and antibacterial activity of ozone towards each bacterial strain was calculated and presented in percentage as below (Fan *et al.*, 2002). The results presented indicated the percentage of bacterial colonies inactivated by ozone under the current set of experiment.

$$\text{Antibacterial activity (\% CFU)} = \left(\frac{N_0 - N}{N_0}\right) \times 100$$

N = microbial load of samples

N_0 = microbial load of control

3.2.5 *In vitro* antibacterial assay

Based on the screening result, *in vitro* antibacterial assay was conducted by exposing the inoculated plates (with lids removed) to ozone at concentrations of 0 (control), 0.1, 0.3. 0.5 and 1.0 ppm, for exposure times of 0.5, 3, 6 and 24 h at 18 - 20°C and 95% (RH). The treated plates were incubated as in section 3.2.4 and antibacterial activity of ozone (%) towards each bacterial strain was calculated as in section 3.2.4.

3.2.6 *In vivo* antibacterial assay

Fruit of bell pepper as described in section 3.2.2 were used. Bell pepper plugs (1.13 cm^2, approximately 1 g) were prepared using a 1.2 cm cork borer. The plugs were used as an experimental form of fresh cut fruit (Abadias *et al.*, 2011). Fresh cut were used in this experiment as bacterial contamination is more prevalent in fresh cut rather than whole fruit. The plugs were dipped into freshly prepared bacterial inoculum (1 x 10^4 CFU ml^{-1}) for 1 min and air-dried in the biosafety cabinet for 1 h to allow bacterial attachment.

The inoculated bell pepper plugs were then aseptically transferred to ozone chambers and exposed to ozone at concentrations of 0.0 (control), 0.1, 0.3, 0.5 and 1.0 ppm for 0.5, 3, 6 and 24 h at 18 - 20°C and 95% RH. Following ozone exposure, the bell pepper plugs were transferred to control chambers (0.0 ppm ozone) until the end of incubation period of 24 h.

Bacterial cells were recovered from the bell pepper plugs by homogenizing with 9 ml of sterile ¼ strength Ringer's solution (RS, Merck, pH 7.0) in a sterile plastic bag and aliquots were serially diluted (10-fold dilution) and spread plated onto selective and differential media; SMAC, XLD and PALCAM media for *E. coli, Salmonella* Typhimurium and *L. monocytogenes,* respectively. The plates were incubated at 37°C for 24 h for *E. coli* O157 and *Salmonella* Typhimurium and 48 h for *L. monocytogenes*. Number of CFU for each treatment was quantified using the colony counter and microbial reduction was expressed in log reduction using the following equation modified from Alexandre *et al.* (2011)

Microbial Log Reduction (log CFU ml^{-1}) = log (N / N_0)

N = microbial load of samples

N_0 = microbial load of control

In vivo antibacterial assay was repeated using higher ozone concentration. The inoculated bell pepper plugs were exposed to ozone at concentrations of 0.0 (control) 1.0, 3.0, 5.0, 7.0 and 9.0 ppm for 0.5, 3, 6 and 24 h at 18 - 20°C and 95% RH. Bacterial cells were recovered and microbial reduction (log reduction) was calculated.

3.2.7 Microscopic evaluation: Environmental scanning electron microscopy (ESEM)

1 ml of 1 x 10^3 CFU ml^{-1} of the bacterial cultures were freshly prepared and their extracellular matrix was removed by washing the cultures with 1 x Phosphate Buffer Saline pH 7.2 for 3 times. The bacterial cells were inoculated onto a sample stub and exposed to 0.5 and 1.0 ppm ozone for half hour. The cells were then viewed using a Scanning Electron Microscope (Quanta 400F ESEM) in ESEM mode using Gaseous Secondary Electron Detector (GSED) at 20.0 kV and photographs were taken with 30,000X magnification.

3.2.8 Statistical analysis

The experiments were carried out using a Completely Randomized Design (CRD) with three replicates using three ozone chambers. *In vitro* experiment was conducted using five technical replicates (agar plates) while *in vivo* experiment comprised of three technical replicates of 20 fresh cut plugs. The experiment was repeated thrice and data for antibacterial activity were transformed prior to analysis. Data were analyzed using Statistical Analysis Software (SAS, version 9.1.3, SAS Institute Inc., USA). Analysis of variance (ANOVA) was performed with $P < 0.05$ significance level and differences in data means were analyzed using Duncan's Multiple Range Test (DMRT).

3.3 Results and Discussion

3.3.1 Screening of antibacterial activity of ozone

Screening of antibacterial activity of ozone was conducted to select a range of effective ozone concentration and exposure time to reduce bacterial colonies on agar plates under current set of experiment. Table 3.1 - Table 3.3 showed the percentage of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* colonies inactivated by ozone. The results showed that interaction of ozone concentration and exposure time significantly reduced ($P < 0.0001$, Appendix B 3.1 – 3.3) colonies of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* inoculated on agar plates. Increase in ozone concentration and exposure time increased antibacterial activity. This result was supported by Fan *et al.* (*2007)* where increase in ozone concentration and exposure time also increased antibacterial activity on *Listeria innocua*. This could be due to the effect of ozone on cell membrane which subsequently leads to cell death (Forney, 2003). In comparison to *L. monocytogenes* colony in this study, the ozone antibacterial activity on *L. innocua* was reported to be lower. This was demonstrated from exposure to 0.1 ppm ozone for three hour where the treatment reduced 57% of *L. innocua* colony on agar plates but reduced 75% reduction of *L. monocytogenes* colony (Fan *et al.*, 2007). This could be due to differences in bacterial resistancy towards ozone.

Table 3.1: The effect of ozone concentration and exposure time on *E. coli* O157 colonies at 18 - 20℃, 95% RH.

Ozone concentration (ppm)	Exposure time (hour)							
	0	0.25	0.5	1	3	6	18	24
	---------- Antibacterial activity of ozone on *E. coli* O157 colonies (%) ----------							
0.0 (control)	0.0i	0.0i	0.0i	0.0i	0.0i	0.0i	0.0i	0.0i
0.1	0.0i	57.0i	56.0i	42.0h	42.3h	66.9g	67.4g	69.3g
0.5	0.0i	44.7h	46.0h	93.3a-f	94.7a-e	97.7a	96.7ab	97.2ab
1.0	0.0i	88.8f	90,.6d-f	95.8a-d	96.2ab	97.1ab	97.5a	97.5a
5.0	0.0i	89.9e-f	90.8c-f	95.6a-d	95.7a-d	96.6ab	96.6ab	96.5ab
9.0	0.0i	89.1f	91.7b-f	96.8ab	95.0a-d	96.6ab	96.0a-c	97.1ab

Antibacterial activity of ozone is the percentage of bacterial colonies compared to control after ozone treatment. The data presented is the data after transformation.

Values are interaction effect of ozone concentration and exposure time on antibacterial activity. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

Table 3.2: The effect of ozone concentration and exposure time on *Salmonella* Typhimurium colonies at 18 - 20℃, 95% RH.

Ozone concentration (ppm)	Exposure time (hour)							
	0	0.25	0.5	1	3	6	18	24
	----- Antibacterial activity of ozone on Salmonella *Typhimurium* colonies (%) -----							
0.0 (control)	0.0g	0.0g	0.0g	0.0g	0.0g	0.0g	0.0g	0.0g
0.1	0.0g	0.7g	1.0g	9.72f	10.2ef	24.1d	23.3d	24.0d
0.5	0.0g	13.1ef	15.3e	73.7c	74.7c	92.5a	93.6a	94.1a
1.0	0.0g	82.3b	84.4b	90.3a	91.3a	94.9a	95.8a	95.6a
5.0	0.0g	81.4b	80.3b	91.2a	93.9a	95.0a	94.0a	94.4a
9.0	0.0g	82.5b	82.9b	94.2a	92.9a	95.0a	95.6a	95.5a

Antibacterial activity of ozone is the percentage of bacterial colonies observed after ozone treatment. The data presented is the data after transformation.

Values are interaction effect of ozone concentration and exposure time on antibacterial activity. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

Table 3.3: The effect of ozone concentration and exposure time on *L. monocytogenes* colonies at 18 - 20ºC, 95% RH.

Ozone concentration (ppm)	Exposure time (hour)							
	0	0.25	0.5	1	3	6	18	24
	---------- Antibacterial activity of ozone on *L. monocytogenes* colonies (%) --------							
0.0 (control)	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d
0.1	0.0d	42.3c	44.6c	74.0b	74.8b	94.2a	94.5a	96.5a
0.5	0.0d	90.9a	93.5a	95.3a	95.0a	95.3a	95.4a	95.6a
1.0	0.0d	93.0a	95.0a	95.8a	95.7a	95.6a	94.1a	96.1a
5.0	0.0d	92.2a	95.2a	96.3a	94.2a	94.7a	96.2a	95.8a
9.0	0.0d	94.3a	95.2a	.95.7a	95.6a	93.8a	95.8a	95.6a

Antibacterial activity of ozone is the percentage of bacterial colonies observed after ozone treatment. The data presented is the data after transformation

Values are interaction effect of ozone concentration and exposure time on antibacterial activity. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

Results from this screening experiment showed that optimal antibacterial activity on *E. coli* O157 colony was achieved from exposure to 0.5 ppm ozone for 1 h. Meanwhile, the optimal antibacterial activity was achieved from exposure to 0.5 ppm ozone for 6 h for *Salmonella* Typhimurium. Increase in ozone concentration to 1.0 ppm ozone for shorter exposure time, 1 h, resulted in comparable antibacterial activity. Lower ozone dosage; 0.1 ppm for 6 h, resulted in optimal antibacterial activity on *L. monocytogenes* colonies. This is comparable to antibacterial activity from exposure to 0.5 ppm ozone for 0.25 h. These optimal dosages resulted in high antibacterial activity which is 93.3, 92.5 and 94.2% antibacterial activity for *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* respectively. Increase in ozone concentration or exposure time from these optimal ozone dosages had no significant effect on the antibacterial activity. Therefore, based on the optimal dosage obtained for each bacteria species in this experiment, ozone concentrations of 0.0 (control), 0.1, 0.3. 0.5 and 1.0 ppm, for exposure times of 0.5, 3, 6 and 24 h were selected to determine the effect of ozone on the three different bacterial species.

3.3.2 Effect of ozone on *in vitro* bacterial population

In vitro assay showed that colonies of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* on agar plates were significantly reduced ($P < 0.0001$, Appendix B 3.4 – 3.6) by interaction of ozone concentration and exposure time (Figure 3.2). Increase in ozone concentration and exposure time for three to six hours increased ozone antibacterial activity. Effectiveness of ozone reached a plateau when exposure

time was prolonged to 24 hours. This could be due to the presence of dead bacterial cells layer on top of living bacterial cells which protected the living cells from oxidation action of ozone (Mossel *et al.*, 1996). This would prevent the ozone molecules to have in contact with the living cells hence allow the living cells to grow and multiply. This reduced the effectiveness of ozone. These results were in line with Fan *et al.* (2007) where the highest death rate of *L. innocua* cells was observed in less than 2 h and the rate reached a plateau after 4 h. This suggested that optimum effect of ozone on agar plate was achieved with short term exposure of less than 6 h.

The results showed optimal antibacterial activity was achieved from exposure to 0.3 ppm for 3 h for *E. coli* O157, 0.3 ppm for 6 h for *Salmonella* Typhimurium and 0.1 ppm for 3 h for *L. monocytogenes.* Further increase in ozone concentration and exposure time had no significant effect ($P > 0.05$). Different ozone dosages were required to achieve the optimal activity where *Salmonella* Typhimurium required the highest dosage. This showed that the bacterial species had different resistance to ozone where *Salmonella* Typhimurium was more resistant, followed by *E. coli* O157 and *L. monocytogenes*.

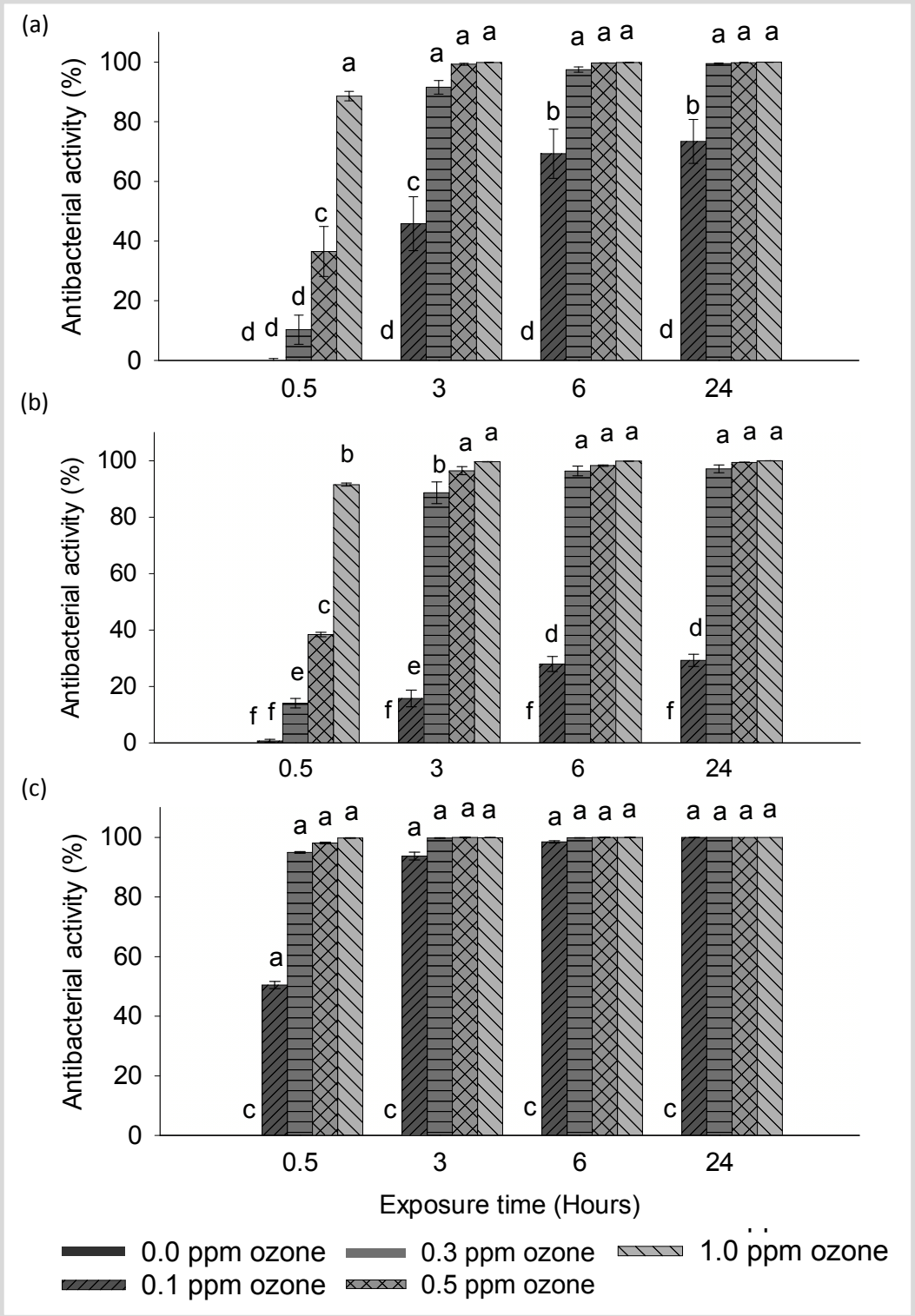

Figure 3.2: The effect of ozone concentration and exposure time (*c* x *t*) on colonies of (a) *E. coli* O157, (b) *Salmonella* Typhimurium and (c) *L. monocytogenes*. Bars with the same letters are not significantly different using DMRT, (n = 3, $P < 0.05$).

3.3.3 Effect of ozone on *in vivo* bacterial population

Ozone concentration (0.1, 0.3, 0.5 and 1 ppm ozone) significantly reduced (P < 0.0001, Appendix B 3.7 – 3.9) *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* population on fresh cut bell pepper (

Figure *3.3*). Increase in ozone concentration resulted in higher reduction in bacterial population. The highest reduction was observed from exposure to 1 ppm ozone for 6 h which reduced 1.69, 1.59 and 2.01 log reduction of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations, respectively. The reduction on *E. coli* O157 and *Salmonella* Typhimurium populations did not meet requirement for an antimicrobial agent with minimal reduction of 2 log microbial population (Torlak *et al.*, 2013). Therefore, the treatment was repeated using higher ozone concentration; 1, 3, 5, 7 and 9 ppm ozone; to achieve better control of microbial population.

Application of higher ozone concentration, 1, 3, 5, 7 and 9 ppm ozone for 0.5, 3, 6 and 24 h significantly reduced (P < 0.0001, Appendix 3.10 – 3.12) *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations on fresh cut bell pepper (Figure 3.4). Increase in ozone concentration increased the reduction of bacterial population where exposure to 9 ppm ozone resulted in the highest antibacterial activity. Antibacterial activity of ozone was also enhanced as exposure time increased up to 6 h. Further increase to 24 h had no effect on the antibacterial activity. Optimal bacterial reduction was achieved from exposure to 9 ppm ozone for 6 h which reduced 2.89, 2.56

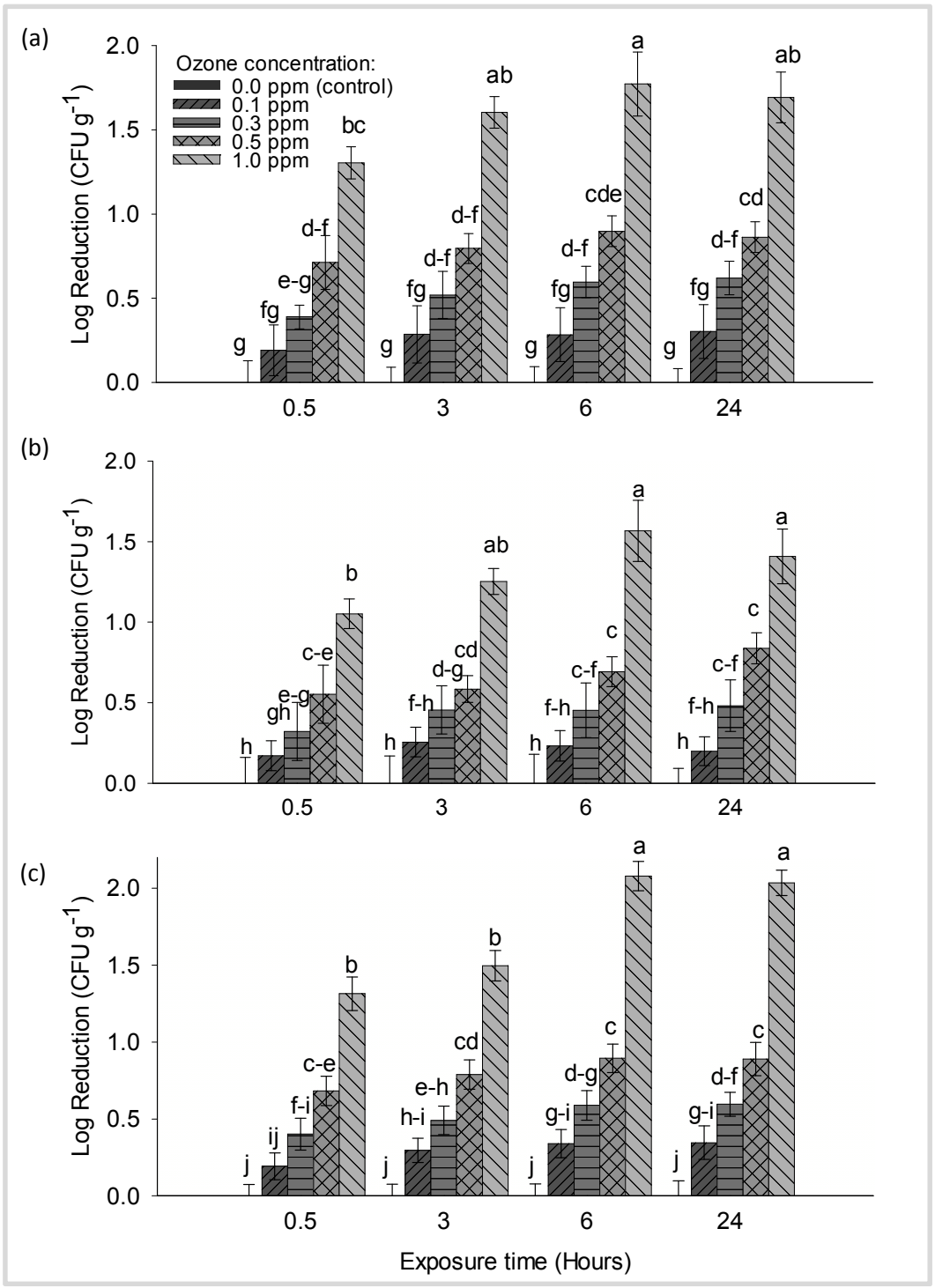

Figure 3.3: The effect of ozone concentration and exposure time (*c* x *t*) on populations of (a) *E. coli* O157, (b) *Salmonella* Typhimurium and (c) *L. monocytogenes* on bell pepper plugs. Bars with the same letters are not significantly different using DMRT, (n = 3, $P < 0.05$).

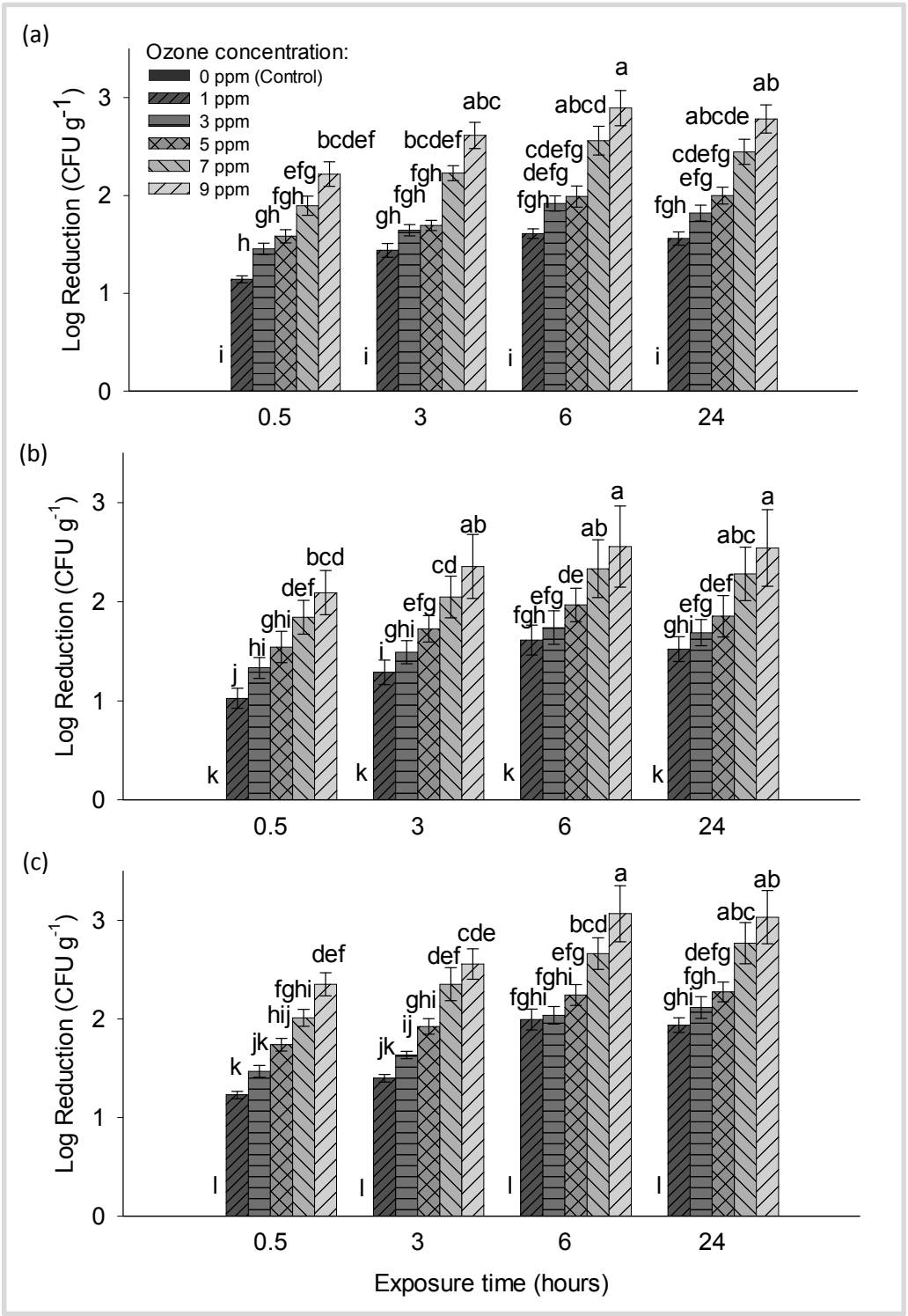

Figure 3.4: The effect of ozone concentration and exposure time (*c* x *t*) on populations of (a) *E. coli* O157, (b) *Salmonella* Typhimurium and (c) *L. monocytogenes* on bell pepper plugs. Bars with the same letters are not significantly different using DMRT, (n = 3, $P < 0.05$).

and 3.06 log reduction of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations, respectively. This meets the requirement for an antimicrobial agent (Torlak *et al.*, 2013). In comparison to *in vitro* study, higher ozone dosages were required to achieve optimal antibacterial activity in fresh cut bell pepper. This could be due to the structure of fruit cells which protects the bacterial cells from oxidation action of ozone and allows the bacteria to multiply inside the cells. Besides, fresh cut bell pepper may have organic juices and natural antioxidant which may protect the bacterial population from ozone oxidation. These factors reduces the effectiveness of ozone hence, higher ozone dosages were required to achieve optimal antibacterial activity in fresh cut bell pepper compared to *in vitro* study.

In line with *in vitro* assay, *Salmonella* Typhimurium showed the highest resistance to ozone followed by *E. coli* O157 and *L. monocytogenes*. *Salmonella* Typhimurium showed the highest resistance towards ozone could be due higher density of its cell membrane with higher content of phospholipid components, such as phosphatidylethanolamine and phosphatidylglycerol, compared to that of *E. coli* O157 (Ames, 1968; Heinrichs *et al.*, 1998). This increased the rigidity of the cell wall and provided extra protection for the cell membrane against ozone (Alexopoulos *et al.*, 2013).

In spite of the presence of a peptidoglycan layer in *L. monocytogenes* cell walls, a Gram positive bacterium, the cell wall was very susceptible to ozone action. This was due to low degree of cross-linking of the cell wall (Navarre and Schneewind, 1999), which reduced its rigidity hence increased its susceptibility to ozone. This was supported by Restaino *et al.,* (1995) who found *L. monocytogenes* was more

susceptible to ozone than Gram negative bacteria, such as *E. coli*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa*.

Reduction in bacterial population on fresh cut bell pepper was also reported by Horvitz and Cantalejo (2012) where exposure to 0.7 ppm ozone for 5 mins resulted in 0.1 log reduction in aerobic mesophilic bacteria population. This reduction is very minimal compared to our results. This could be due to the lower ozone concentration and shorter exposure time used. Increase in ozone concentration and exposure time would increase microbial reduction in the fresh cut bell pepper.

When compared the optimal antibacterial activity achieved in this study to other commodities, higher efficacy of microbial reduction was observed from ozone treatment on whole tomato where a 2 log reduction was achieved from application of 5 ppm ozone for 3 min (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). This could be due to the smooth surface of whole tomato compared to the porous surface of cut bell pepper (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). Besides, the effectiveness of ozone in reducing bacterial population on cut fruit may be reduced due to organic juices (Alexopoulos *et al.*, 2013), lignin and suberin (Aquino-Bolaños and Mercado-Silva, 2004), which are released or produced by fruit at cut surface. These compounds negated antibacterial activity of ozone hence, reduced its effectiveness to penetrate inner tissue of the fruit.

Optimal antibacterial activity observed for *E. coli* O157 in this study was similar to the reduction in *E. coli* O157 population on lettuce (2.31 log reduction), from application of 1 ppm chlorine dioxide gas for 15 min (Singh *et al.*, 2002). This chlorine gas application, however, caused discoloration to the lettuce (Singh *et al.*, 2002).

Meanwhile, similar reduction in *L. monocytogenes* population was achieved with the use of a lower ozone dosage in aqueous ozone treatment; exposure to 4.5 ppm aqueous ozone for 3.5 min on lettuce, where it resulted in 2.5 log reduction of total population (Ölmez and Akbas, 2009). This showed that the aqueous ozone treatment has higher efficacy compared to gaseous treatment conducted in this study. This could be due to the nature of the leafy vegetable used, which has smooth surfaces compared to porous surface of cut bell pepper.

The antibacterial activity of gaseous ozone obtained in this study demonstrated the potential for its application for fruit sanitization without the use of water, hence eliminating the high possibility of cross contamination in the aqueous treatment. In comparison to aqueous treatment, gaseous ozone treatment is more stable as it is not affected by the presence of organic matter and pH of the water used in aqueous treatment (Forney, 2003).

3.3.4 Effect of ozone on bacterial morphology

ESEM study revealed that morphology of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* was affected by ozone (Figure 3.5). Bacterial cells treated with ozone had disrupted structure which is indicated by angular and irregular structure. This is in contrast to control cells which has smooth and oval structure. Similar results were also reported on *Geobacillus stearothermophilus*, *Bacillus stearothermophilus*

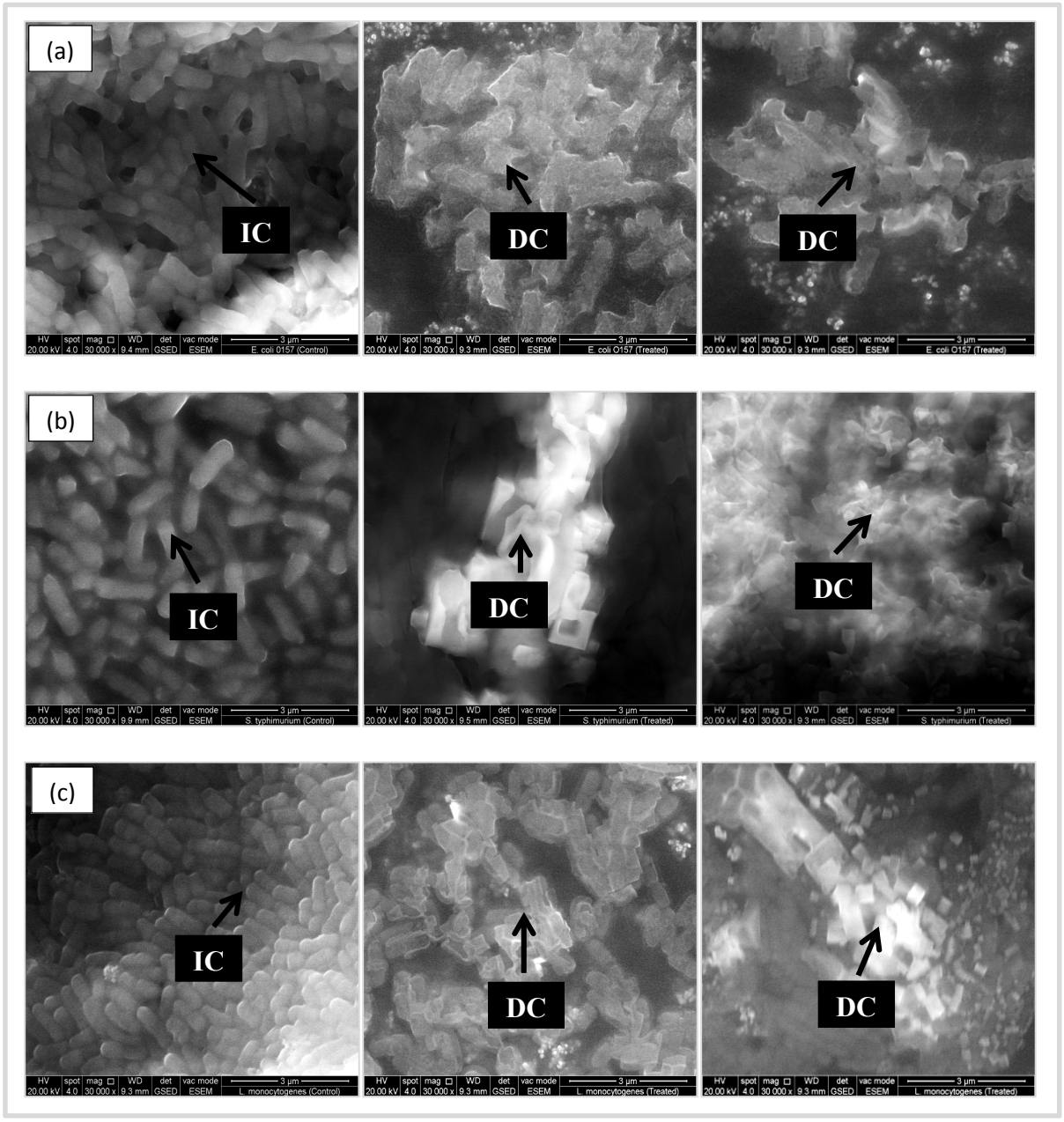

Figure 3.5: Scanning electron micrographs of control (a) *E. coli* O157, (b) *Salmonella* Typhimurium and (c) *L. monocytogenes*. Micrographs shows bacterial cells exposed to 0 (left), 0.5 (middle) and 1.0 (right) ppm ozone for 0.5 hour. Note that the cells of control bacterial cultures were intact (IC) while the cells of treated bacterial cultures were disrupted (DC).

(Mahfoudh *et al.*, 2010) and *Staphylococcus aureus* (Thanomsub *et al.*, 2002) where ozone disrupted the bacterial structure. This shows that ozone attacked bacterial cell membrane and may result in cell death observed in *in vitro* and *in vivo* experiments.

The irregular bacterial structure observed in ozone treated cells could be due to oxidative action of ozone and its decomposition products such as •OH, •OOH and H_2O_2 on double bonds of unsaturated phospholipid of cell membrane (Forney, 2003). These oxidative agents are usually neutralized by defence enzymes such as catalase produced by bacteria to protect the cells from oxidative damage (Buchmeier *et al.*, 1995). High oxidative stress however, perturbed the equilibrium between the defence enzymes and oxidative stress. This led to accumulation of reactive oxygen species, which progressively attacked the cell membrane. The lipid peroxidation by ozone led to formation of lipid hydroperoxides (LOOH), which leads to subsequent chain reaction of lipid degradation (Forney, 2003). This increased membrane fluidity, reduced its integrity, disrupted cell osmotic balance and lead to cell wall rupture, cellular leakage, excessive nutrient loss and subsequent cell death (Al-Haddad *et al.*, 2005; Torlak *et al.*, 2013). Inactivating bacterial cells by disrupting cell membranes is an effective mechanism as it does not lead to microbial resistance and ozone or its induced reactive products do not need to permeate into the cells to be effective (Torlak *et al.*, 2013).

Ozone and its decomposed products also inactivated bacterial cell by oxidizing sulfhydryl groups and amino acids of enzymes or proteins (Al-Haddad *et al.*, 2005; Torlak *et al.*, 2013). It decomposed the proteins to smaller peptides, changed its conformation and subsequently inactivated the protein (Torlak *et al.*, 2013). Ozone also disrupted bacterial DNA by changing its secondary structure (Hunt and Marinas, 1999)

or introducing mutation (Torlak *et al.*, 2013), which altered genetic coding. The inactivation of protein and disruption of DNA altered bacterial cellular metabolism and subsequently lead to cell death.

In conclusion, the *in vitro* and *in vivo* experiments showed ozone reduced *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations on agar plates and fresh cut bell pepper by disruption of the bacterial cell membrane which leads to cell lysis. From the *in vivo* experiment, the optimal dosage to reduce the bacterial populations on fresh cut bell pepper was 9 ppm ozone for 6 h (Figure 3.4). This ozone dosage resulted in 2.89, 2.56 and 3.06 log reduction of *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations, respectively. This showed the potential of ozone fumigation to be used as a sanitization treatment to reduce microbial load on fresh cut fruit before reaching the consumer.

CHAPTER 4

EFFECT OF OZONE FUMIGATION ON ANTHRACNOSE DEVELOPMENT ON BELL PEPPER

4.1 Introduction

Anthracnose is a devastating disease affecting bell pepper which resulted in huge economic loss to producing countries. It is commonly observed on ripe fruit (usually red in bell pepper, depends on cultivar) hence, classified as ripe-rot pathogen (Alexopoulos *et al.*, 2013). After 1998 however, anthracnose outbreak was observed on bell pepper harvested at green stage and the disease was reported every year since then (Lewis Ivey *et al.*, 2004). This disease spread profusely and contributed up to total loss in bell production in Ohio, United States (Lewis Ivey *et al.*, 2004). Besides, anthracnose also caused massive loss in bell pepper production in Trinidad, Brazil (Ramdial and Rampersad, 2015) and Japan (Kanto *et al.*, 2014).

Anthracnose in bell pepper is caused by several *Colletotrichum* species including *C. capsici. C. gloeosporioides*. *C. coccodes* and *C. acutatum* (Tomás-Callejas *et al.*, 2012) where *C. capsici* is the most prevalent species in Asia (Harp, 2008). Occurrence of *C. capsici* on bell pepper was first reported in Mississippi, United States in 1994 (Roy *et al.*, 1997). The prevalence of this pathogen is increasing and was reported to be the main causal agent (72%) for bell pepper anthracnose in Trinidad (Ramdial and Rampersad, 2015). This *Colletotrichum* species is the most severe species due to its

resistant genotype (Ratanacherdchai *et al.*, 2007). Therefore, an effective postharvest treatment is required to control its proliferation in order to reduce fruit losses. This leads to the objectives of this study to:

1. evaluate the effect of ozone on mycelia growth, spore production and spore germination of *C. capsici in vitro*
2. evaluate the effect of ozone on disease incidence, disease severity and spore production caused by *C. capsici* on bell pepper

4.2 Materials and Method

4.2.1 Isolation and identification of *Colletotrichum capsici*

4.2.1.1 Isolation of *C. capsici* from bell pepper

Bell pepper with anthracnose symptoms (black-brown, circular and sunken lesions) were collected from Pasar Semenyih, Semenyih, Selangor, Malaysia. Diseased tissues were inoculated onto Potato Dextrose Agar (PDA, Merck, pH 7.0) and incubated at 25°C for 9 days.

4.2.1.2 Macro- and microscopic identification of *C. capsici*

Putative *C. capsici* (white greyish radial mycelia that turned to a grey greenish colour with age; aerial mycelium was white or grey and acervulus was dark brown or black) was sub-cultured to obtain pure cultures. The cultures were further identified under an optical microscope (Model: Eclipse 80i, Nikon Corp.). Cultures produced fungal hyphae that were hyaline, septate and branched and spores that were one-celled, hyaline, smooth walled and sickle shaped ranging from 17 - 18 x 3 - 4 µm were selected (Chanchaichaovivat *et al.*, 2007). The *C. capsici* culture was maintained on PDA and continuously sub-cultured onto fresh PDA to maintain pathogenicity of the culture.

4.2.1.3 Pathogenicity assay

Fruit (section 3.2.2) were surface sterilised with 0.5% sodium hypochlorite and rinsed with sterile distilled water. Equatorial of the fruit was artificially injured and applied with 50 µl of 1 x 10^5 spores ml^{-1}, which were estimated using a 0.0025 mm^2 Neubauer Improved haemocytometer (Hirschmann EM Techcolor, Germany). Control fruit was inoculated with sterile distilled water. The inoculated fruit were then incubated at room temperature and symptoms were recorded after four days. Pathogenicity of *C. capsici* was confirmed as black brownish, circular and sunken lesions were observed. The *C. capsici* cultures were re-isolated onto PDA to confirm according to Koch's postulates (Roy *et al.*, 1997).

4.2.2 Screening of antifungal activity of ozone

Fungal mycelia plugs (5.0 mm diameter) from periphery of actively growing *C. capsici* cultures were inoculated onto PDA, pH 7.0. The mycelium was exposed to ozone at concentrations of 0 (control), 0.1, 0.5, 1.0, 5.0 and 9 ppm for exposure times of 0.5, 1, 3, 6, 12, 24, 48, 72 and 120 h at 18 - 20°C and 95% RH with Petri plate lids removed to allow air circulation. Following ozone exposure, the cultures were incubated in duplicate clean air chambers (0 ppm ozone) at 18 - 20°C, 95% RH until the end of incubation period (total of 9 days). Radial mycelia growth was recorded and antifungal activity was calculated by comparing the growth of treated mycelium with the control and expressed in percentage (Minas *et al.*, 2010).

$$\text{Antifungal activity (\%)} = \left(\frac{M_c - M_t}{M_c}\right) \times 100$$

M_t = Mycelia growth of treated cultures

M_c = Mycelia growth of control cultures

4.2.3 *In vitro* antifungal assay on *C. capsici*

4.2.3.1 Antifungal assay on mycelia of *C. capsici*

4.2.3.1.1 Measurement of radial mycelia development

The effect of ozone on radial mycelia development was carried out based on screening result. Fungal mycelia plugs (5.0 mm diameter) from periphery of actively growing *C. capsici* cultures were inoculated onto PDA, pH 7.0 and the mycelium was exposed to ozone at concentrations of 0 (control), 1, 3, 5 and 7 and 9 ppm for exposure times of 24, 72 and 120 h at 18 - 20°C and 95% RH with Petri plate lids removed to allow air circulation. Following ozone exposure, the cultures were incubated in duplicate clean air chambers (0 ppm ozone) at 18 - 20°C, 95% RH until the end of incubation period (total of 9 days). Radial mycelia growth was recorded and antifungal activity was calculated by comparing the growth of treated mycelium with the control and expressed in percentage as in section 4.2.2 (Minas *et al.*, 2010).

4.2.3.1.2 Analysis of mycelia structure using Environmental scanning electron microscopy (ESEM)

Following the period for treatment and clean-air incubation (total of 9 days), agar plugs (19.63 mm^2) adjacent to the mycelium colony (with mycelia developed under ozone) were carefully removed using a 5.0 mm cork borer without disturbing the fungal structure. The culture plugs were viewed under a scanning electron microscope (Model: Quanta 400F ESEM, FEI, USA) using ESEM mode to study the effect of ozone on fungal mycelium development (Antony-Babu and Singleton, 2011).

4.2.3.2 Quantification of *C. capsici* spore production

C. capsici sporulation was determined according to Antony-Baby and Singleton (2011). Following the period of treatment and clean-air incubation (total of 9 days), agar plugs (117.78 mm^2) adjacent to the mycelial colony (mycelia grown under ozone treatment) were carefully sampled using a 5.0 mm cork borer. The plugs were incubated in 5 ml distilled water with continuous agitation at 250 rpm for 18 hours to produce spore suspension. Concentration of the spore suspension was estimated using a 0.0025 mm^2 Neubauer Improved haemocytometer.

4.2.3.3 Antifungal assay on spore of *C. capsici*

4.2.3.3.1 *C. capsici* spore germination test

Spore suspensions were prepared by agitating 8 days old *C. capsici* cultures with sterile distilled water and detached fungal hyphae fragments were removed by filtering the suspension through a cheese cloth. Concentration of the spore suspensions was adjusted to 1 x 10^5 spores ml^{-1} using a 0.0025 mm^2 Neubauer Improved haemocytometer and 100 µl were then evenly spread onto PDA pH 7.0. The plates with removed lids were exposed to ozone treatment at 18 - 20°C and 95% RH with Petri plate lids removed to allow air circulation. Following ozone treatment, the spores were incubated in a clean air chamber (0 ppm ozone) at 18 - 20°C and 95% RH for 72 hours and viewed under a Universal Zoom Microscope (Model: Multi-Purpose Zoom Microscope Multizoom AZ100, Nikon Corp.) using 40X magnification. For each treatment, 100 spores were viewed and number of germinated spores were recorded (Minas *et al.*, 2010). The number of germinated spores obtained was compared with the control and spore germination percentage was calculated as below;

$$\text{Spore germination (\%)} = \left(\frac{N_t}{N_c}\right) \times 100$$

N_t = Number of germinated spore in treated cultures

N_c = Number of germinated spore of control cultures

4.2.3.3.2 Analysis of spore structure using Environmental scanning electron microscopy (ESEM)

A spore suspension (1 x 10^5 spores ml^{-1}) of *C. capsici* was prepared and treated as previously described in section 4.2.3.3.1. The fungal spores were exposed to ozone at concentrations of 0 (control), 1, 3, 5, 7 and 9 ppm for 24 h at 18 - 20°C and 95% RH. Following the ozone exposure, agar plugs (19.63 mm^2) were carefully removed and viewed using a scanning electron microscope (Model: Quanta 400F ESEM, FEI, USA) under ESEM mode to determine the effect of ozone on spore morphology.

4.2.3.3.3 Quantification of spore intracellular H_2O_2

Intracellular H_2O_2 is an ozone decomposition product. Quantification of spore intracellular H_2O_2 gives an indication of ozone oxidation level on the spore cells. Intracellular H_2O_2 was measured according to Chen and Dickman (2005). A spore suspension (1 x 10^5 spores ml^{-1}) of *C. capsici* was prepared and treated as described in section 4.2.3.3.1. The spores were then washed with 10 mM potassium phosphate buffer, incubated in 10 µM 2,7-dicholorodihydrofluorescein diacetate (DCHF-DA) for 5 min, washed with potassium phosphate buffer twice and analysed under a fluorescence microscope (Model: Multi-Purpose Zoom Microscope Multizoom AZ100, Nikon Corp.). For each treatment, 100 spores were viewed and the number of fluorescence spores (indicating the presence of H_2O_2) was compared with the control and the percentage of spores with intracellular H_2O_2 was calculated as below;

$$\text{Spore with intracelllular } H_2O_2\ (\%) = \left(\frac{N_t}{N_T}\right) \times 100$$

N_t = Number of fluorescence spore in treated cultures

N_T = Total number of spore of control cultures

4.2.4 *In vivo* antifungal assay on artificially inoculated bell pepper

4.2.4.1 Assessment of anthracnose severity and incidence

In vivo analysis was carried out according to Chanchaichaovivat *et al.,* (2007). Fruit (section 3.2.2) were artificially injured with 5.0 mm cork borer and 50 µL of 1 x 10^5 spore ml^{-1} spore suspension as described in section 4.2.3.3.1, were inoculated onto the injured tissues, ensuring uniform inoculation on each wound.

The inoculated fruit were then exposed to ozone at concentrations of 0 (control), 1, 3, 5 and 7 and 9 ppm for exposure times of 24, 72 and 120 h at 12°C and 95% RH. Following ozone treatment, fungal cultures were incubated in duplicate clean air chambers (0 ppm ozone), at 12°C (Controlled Environment Chamber, Model ECR, Brand RISHA) until the end of storage period, 21 days. Disease incidence (percentage of fruit with anthracnose symptoms out of total number of fruit) and disease severity (wound diameter (lesion diameter)) were recorded at the end of storage period. The ability of ozone to inhibit fungal growth was compared.

$$\text{Disease incidence (\%)} = \left(\frac{N_t}{N_T}\right) \times 100$$

N_t = Number of fruit with anthracnose symptoms

N_T = Total number of fruit

4.2.4.2 Quantification of *C. capsici* spore production on fruit

In vivo spore production analysis was conducted according to Tzortzakis *et al.,* (2008). Following ozone treatment and clean air storage as previously described, fruit were agitated with 100 mL Tween 80 (0.1%) at 120 rpm for 20 min to displace *C. capsici* spores from fruit tissues. The spore suspension was then centrifuged at 2000×*g* (Model: 5810 R, Eppendorf, UK) for 10 min and pellet of *C. capsici* spores were recovered and diluted into 1 ml distilled water. Concentration of the spore suspensions was then estimated using a 0.0025 mm^2 Neubauer Improved haemocytometer.

4.2.5 Statistical analysis

The experiments were carried out following a Completely Randomized Design (CRD) with three replicates using three different ozone chambers. *In vitro* experiments was conducted using five technical replicates (agar plates) while *in vivo* analysis was conducted three technical replicates each containing 20 fruits. The experiment was repeated thrice and data was analysed separately to check for homogeneity. Data of *in vitro* spore germination and intracellular H_2O_2 were transformed prior to analysis. Data

were analysed using Statistical Analysis Software (SAS, version 9.1.3, SAS Institute Inc., USA). Analysis of variance (ANOVA) was performed with $P < 0.05$ significance level and differences in data means were analysed using DMRT.

4.3 Results and Discussion

4.3.1 Isolation and identification of *C. capsici*

C. capsici was isolated from infected bell pepper and the macroscopic and microscopic morphology are as described by Hang (2008). The isolated *C. capsici* has white greyish radial mycelia that turned to grey greenish with age. Meanwhile, aerial mycelium was grey and acervulus was dark brown. Microscopic observation showed that the fungal hyphae was hyaline, septate and branched while the spores were one-celled, hyaline, smooth walled and sickle shaped with size ranging from 3 – 4 µm x 16 – 25 µm (Figure 4.1). This characteristic microscopic features of *C. capsici* is a distinct characterization from other *Colletotrichum* species; *C. gloeosposoides* and *C. acutatum*, which has long elongated shape spores with blunt end (former) and sharp end (later) (Than *et al.*, 2008). Therefore, it is reliable to characterize *Colletotrichum* species based on their microscopic spore. In this experiment, *C. capsici* was successfully re-isolated hence proved that it was the causal agent of anthracnose on the bell pepper.

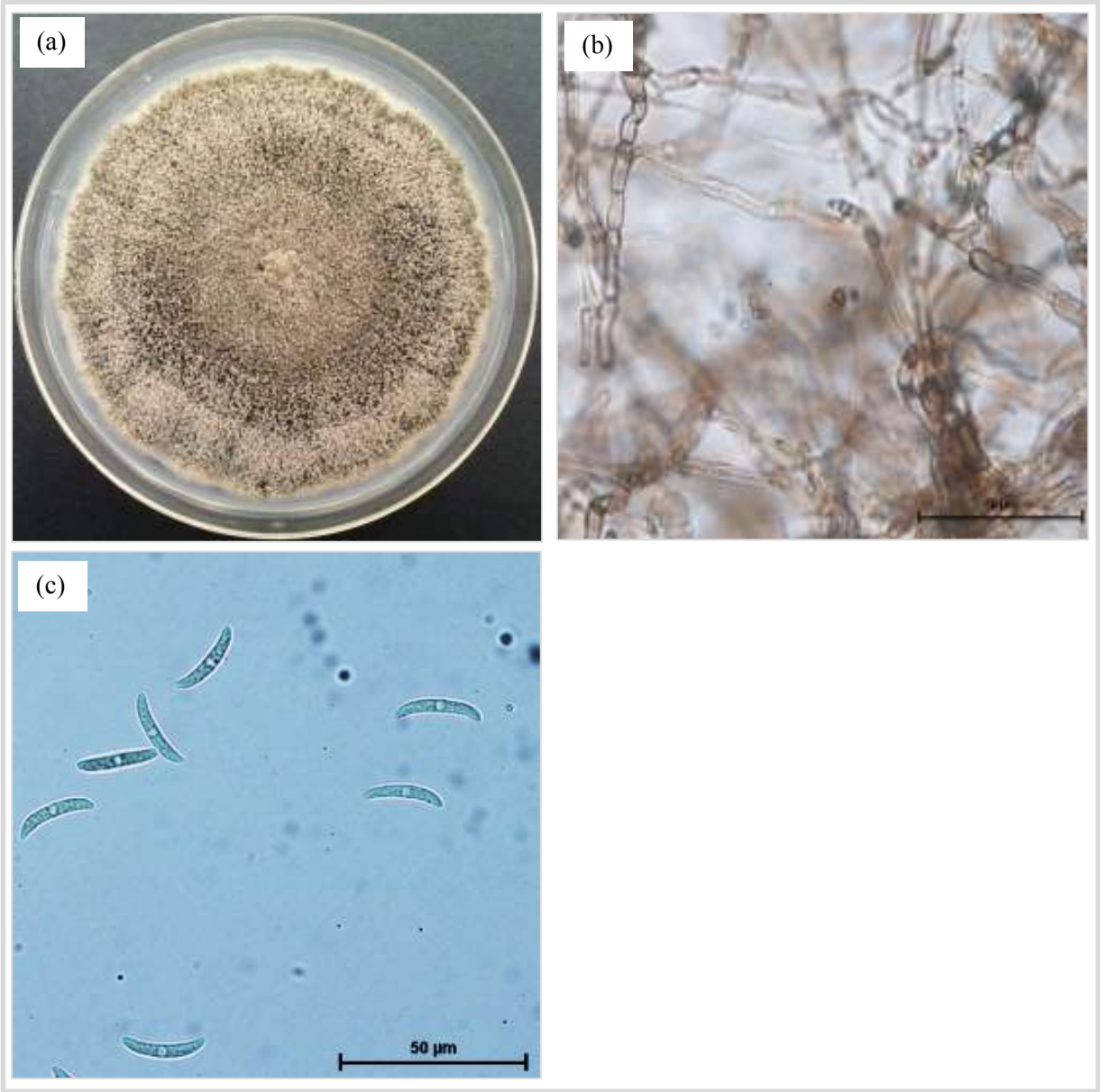

Figure 4.1: Macro- and microscopic morphology *C. capsici* (a) grey mycelia with dark brown acervuli on PDA; (b) hyaline, branched and septate hyphae under optical microscope (Magnification: 400X); and (c) one celled, sickle shaped spores with size ranging from 3 – 4 µm x 16 – 25 µm under optical microscope (Magnification: 400X)

4.3.2 Pathogenicity of the isolated *C. capsici*

Pathogenicity of the isolated *C. capsici* was confirmed as bell pepper inoculated with *C. capsici* spores produced anthracnose symptoms; black brownish sunken lesions (Figure 4.2). In contrast to control fruit which inoculated with sterile distilled water where no disease symptoms were observed. The sunken lesion of *C. capsici* infected fruit was observed on day 1 and lesion was continuously growing to cover the fruit. On day 12 after inoculation, the fruit tissues were soft and watery. Koch's postulates were confirmed as *C. capsici* was recovered from the infected fruit (Roy *et al.*, 1997).

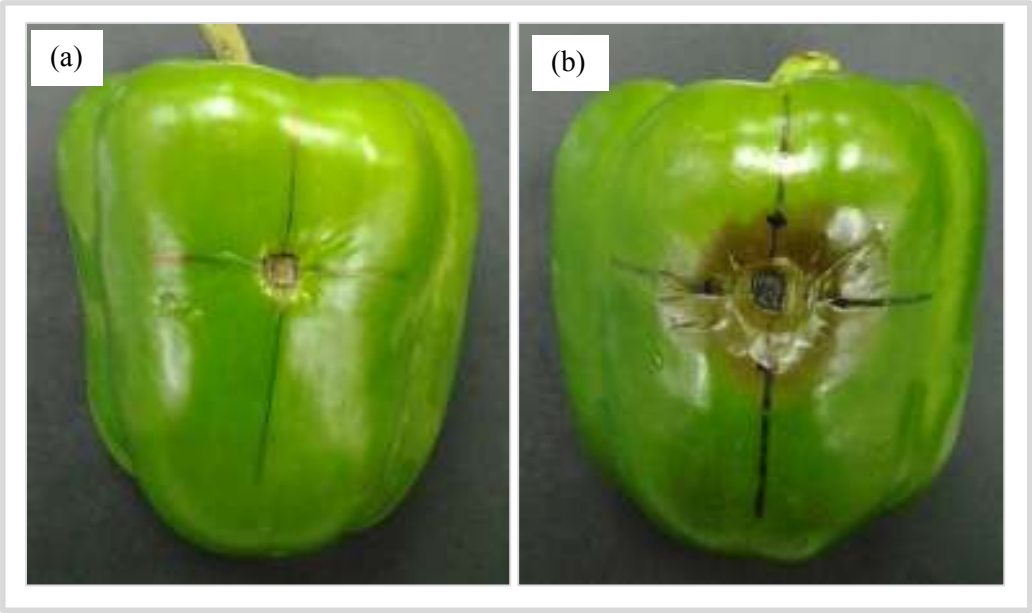

Figure 4.2: Pathogenicity assessment of the isolated *C. capsici*. The isolated *C. capsici* was pathogenic as (a) bell pepper inoculated with sterile distilled water (control) showed no anthracnose symptom while (b) bell pepper inoculated with *C. capsici* spore showed anthracnose symptom.

4.3.3 Screening of antifungal activity of ozone

Screening of antifungal activity showed ozone treatment reduced fungal mycelia growth (Table 4.1). Interaction of ozone concentration and exposure time had significant effect ($P < 0.0001$, Appendix B 4.1) on the antifungal activity. The interaction effect of ozone concentration and exposure time showed exposure to 9.0 ppm ozone for 72 h had the highest antifungal activity on *C. capsici* radial mycelia growth. Referring to this screening result, a range of ozone concentration; 0.0 (control), 1.0, 3.0, 5.0, 7.0 and 9.0 ppm, for exposure times of 24, 72 and 120 h; was selected to conduct an *in vitro* assay on the effect of ozone on *C. capsici* growth. These ozone dosages were selected to obtain optimal antifungal activity of ozone. Exposure time was selected from 24 to 120 h to suit the potential of ozone treatment to be incorporated into transportation or storage system. This allows ozone treatment to be carried out during transportation hence, reduces the fruit handling time. This would be a new development for ozone treatment in postharvest technology.

Table 4.1: Antifungal activity of ozone on *C. capsici* mycelia growth

Ozone concentration (ppm)	Exposure time (hour)									
	0	0.5	1	3	6	12	24	48	72	120
	----- Antifungal activity of ozone on *C. capsici* mycelia growth (%) -----									
0.0 (control)	0.0j	0.0j	0.0j	0.0j	0.0j	0.0j	0.0j	0.0j	0.0j	0.0j
0.1	0.0j	2.6h-j	2.7h-j	1.8ij	2.8h-j	3.0h-j	3.2h-j	3.9g-j	4.7d-j	4.9d-j
0.5	0.0j	1.8ij	2.3h-j	3.5h-j	2.9h-j	3.5h-j	3.9g-j	4.6d-j	7.2c-i	5.5c-j
1.0	0.0j	3.0h-j	3.3h-j	3.6h-j	6.2c-j	6.0c-j	7.5c-h	9.1c-h	10.9b-d	3.9g-j
5.0	0.0j	3.1h-j	4.1f-j	4.4d-j	5.7c-j	6.5c-j	12.1bc	10.9b-e	15.6b	3.0h-j
9.0	0.0j	3.3h-j	2.6h-j	3.9g-j	5.1d-j	4.8d-j	10.6b-f	12.0bc	29.9a	3.8g-j

Antifungal activity of ozone is the percentage of mycelia growth compared to control after ozone treatment.
Values are interaction effect of ozone concentration and exposure time on antibacterial activity. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

4.3.4 Effect of ozone on *in vitro C. capsici* growth

4.3.4.1 Effect of ozone on mycelia of *C. capsici*

4.3.4.1.1 Effect of ozone on mycelia development

Interaction of ozone concentration and exposure time significantly affected ($P < 0.0001$, Appendix B 4.2) *C. capsici* mycelia growth (Figure 4.3). The highest mycelia inhibition was achieved from exposure to 7 ppm ozone for 72 h. Increase in ozone concentration or exposure time showed a reduction in mycelia inhibition. This could be due to oxidation effect of ozone and its induced reactive compounds on fungal cell membrane (Forney, 2003). Ozone and its induced reactive compounds such as hydrogen peroxide and superoxide radicals may reactively oxidize lipids and proteins components of fungal cell membrane (Keutgen and Pawelzik, 2008). This disrupted the fungal cell membrane hence restricted mycelia development.

Exposure to ozone dosage higher than the optimal dosage (7 ppm for 72 h), resulted in reduction in mycelia inhibition. This could be fungal response towards high ozone dosage where the ozone dosage stimulated mycelia growth. This finding was supported by Minas *et al*. (2010) where reduction in mycelia inhibition was also observed in *Botrytis cinerea* exposed to ozone longer than 24 h. Meanwhile, Oktarina *et al.* (2012) also showed reduction in mycelia inhibition as *Rhizopus* species; *Rhizopus oryzae*, *Rhizopus stolonifera* and *Rhizopus microsporus var. chinensis* were exposed to higher ozone dosage. The study reported that the fungal species respond differently towards

ozone where reduction in mycelia inhibition was observed after ozone exposure was exposed to more than 4, 9 and 13 days, respectively (Oktarina *et al.*, 2012). This suggested that different fungal species respond differently towards ozone and an optimal ozone dosage is required to obtain maximum mycelia inhibition.

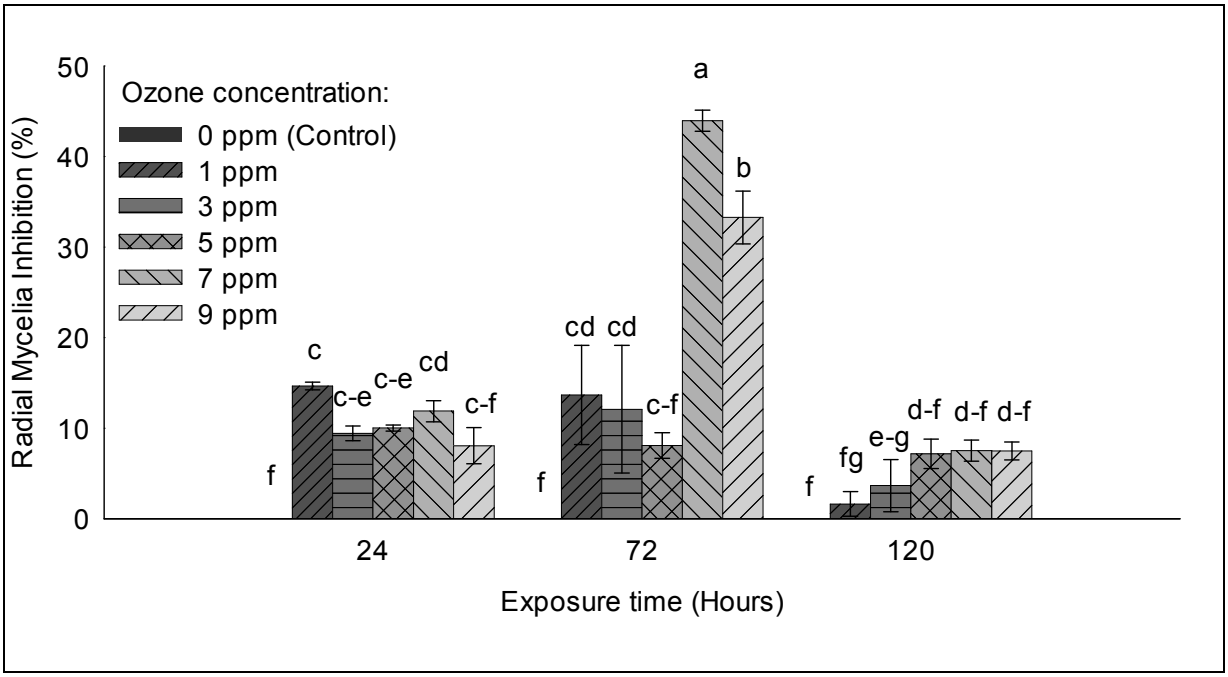

Figure 4.3: The effect of different ozone concentration and exposure time on *in vitro C. capsici* radial mycelia growth after 9 days incubation at 18 - 20°C, 95% RH, under ozone treatment and subsequent clean air incubation. Error bars are the standard error values. Bars with different letters are significantly different using DMRT ($P < 0.05$). Values are mean of three replicates.

4.3.4.1.2 Effect of ozone on structure of *C. capsici* mycelia under ESEM

Scanning electron micrograph of *C. capsici* mycelia showed ozone affected the mycelia structure (Figure 4.4 – Figure 4.5). Exposure to ozone for 24 h resulted in more

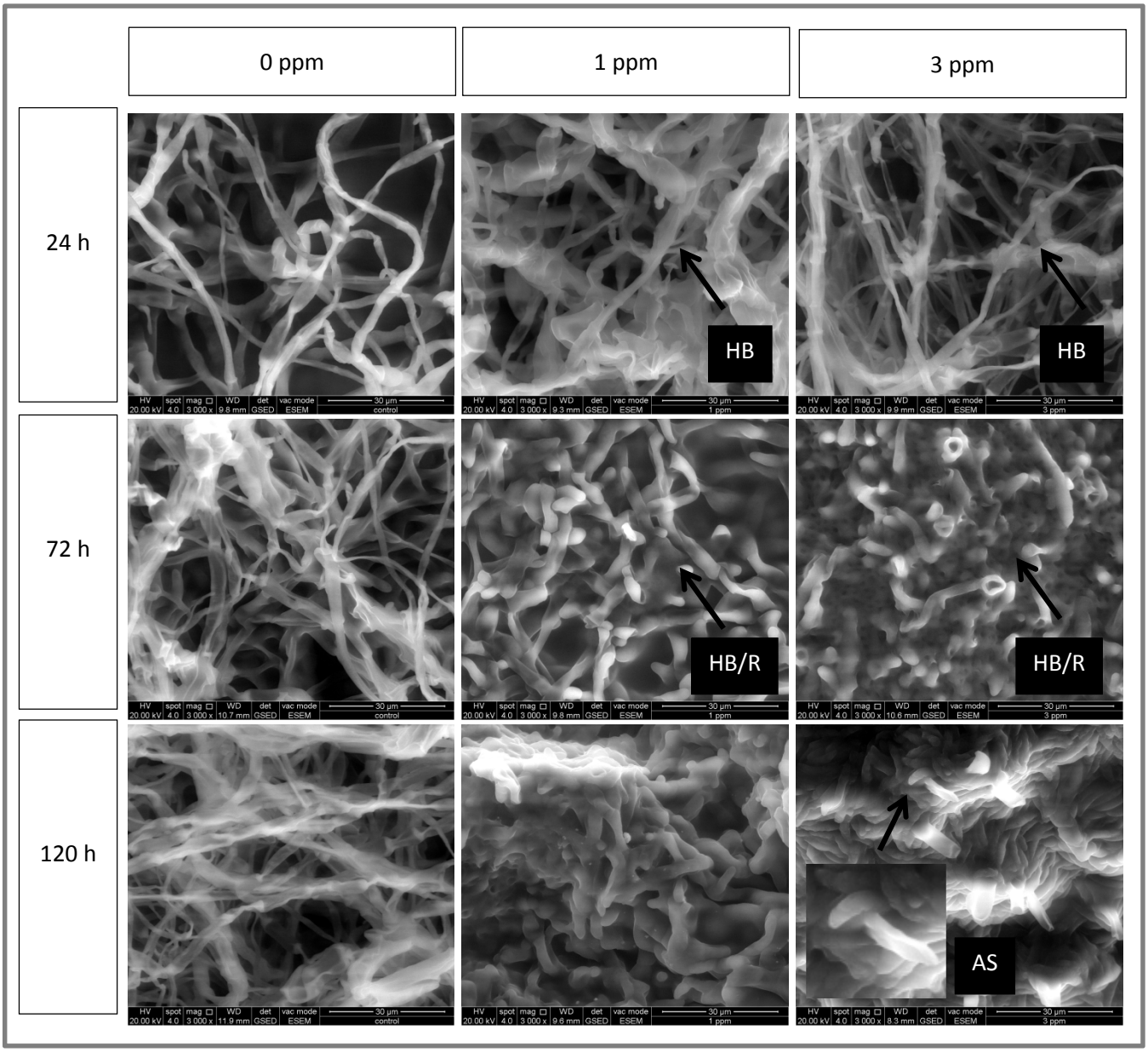

Figure 4.4: Scanning electron micrograph (magnification: 3000X) of *C. capsici* mycelia exposed to 24, 72 and 120 hours to different ozone concentration; (from left to right) 0 (control), 1 and 3 ppm ozone. I) Note the mycelium was highly branched (HB) and has more complex mycelia network with exposure to ozone. II) Note exposure to 72 hours of ozone resulted in highly branched mycelia with retarded growth (HB/R). III) Note exposure to 3 ppm ozone for 120 hours induced sporulation with abundance of spore acervuli on mycelium. The spores produced however, has abnormal morphology (AS).

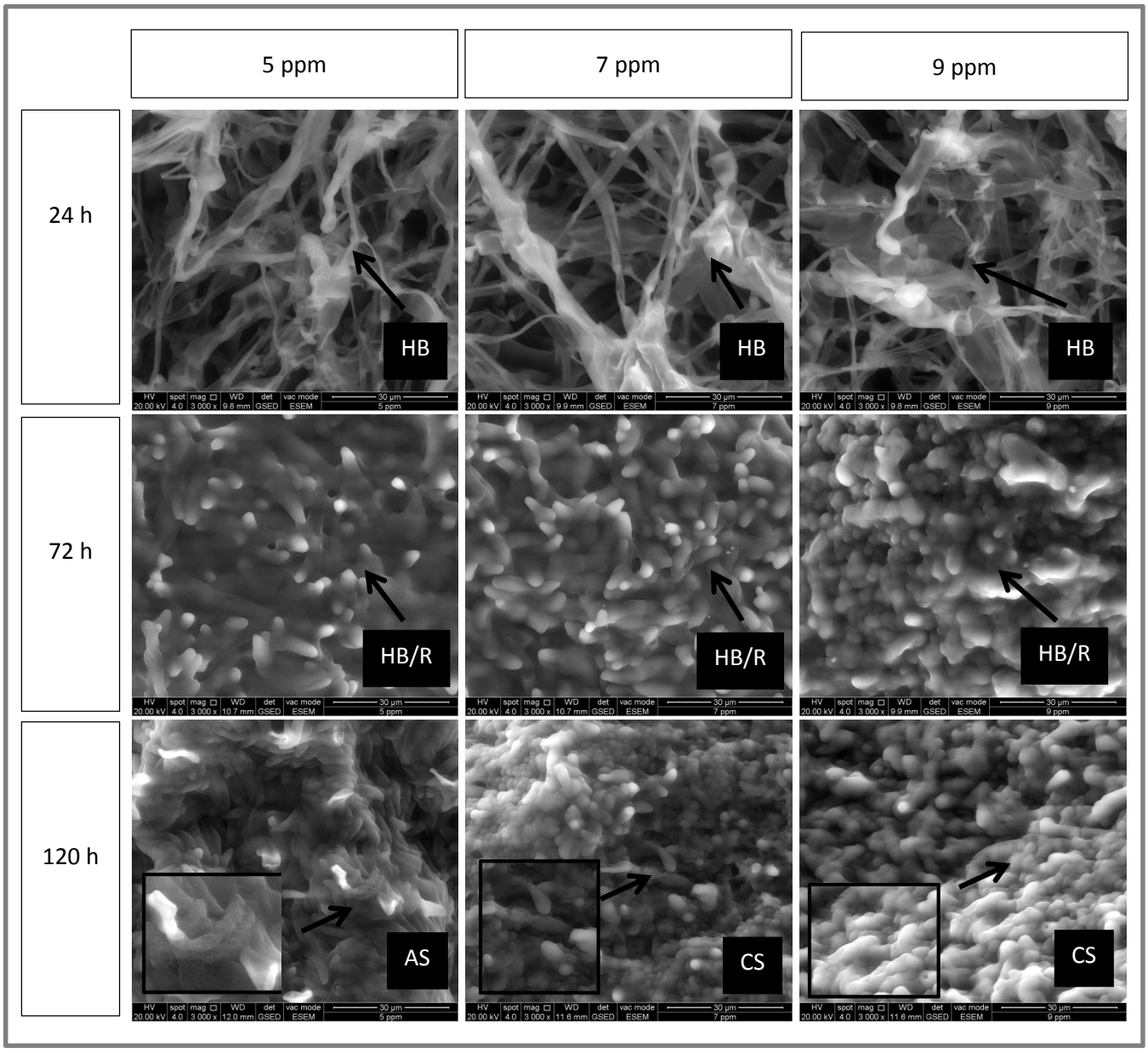

Figure 4.5 Scanning electron micrograph (magnification: 400X) of *C. capsici* mycelia exposed to 24, 72 and 120 hours to different ozone concentration; (from left to right) 5, 7 and 9 ppm ozone. I) Note the mycelium was highly branched (HB) and has more complex mycelia network with exposure to ozone. II) Note exposure to 72 hours of ozone resulted in highly branched mycelia with retarded growth (HB/R). III) Note exposure to 5 ppm ozone for 120 hours induced sporulation with abundance of spore acervuli on mycelium. The spores produced however, has abnormal morphology (AS). IV) Note exposure to 7 and 9 ppm ozone for 120 hours produced clumped structures (CS), presumably fungal spores severely disrupted by ozone.

dense and highly branched mycelia compared to control. The effect of ozone on mycelia structure was more apparent as the mycelia were exposed to ozone for 72 and 120 h. This is evident from the extremely compact and highly branched structure of the mycelia. Similar finding was observed on *Aspergillus nidulans* exposed to 0.2 ppm ozone for 8 days (Antony-Babu and Singleton, 2009) and *A. niger* exposed to H_2O_2 where compact and retarded growth of mycelia was observed (Kreiner *et al.*, 2003). This could be due to oxidation of fungal cell membrane by the oxidative agents, ozone and H_2O_2. Besides, it could also be due to fungal response to reduce hyphae surface area (Kreiner *et al.*, 2003). This would decrease its oxygen intake hence reduce the toxicity effect of ozone on the cells. This reduced hyphae elongation hence restricted fungal growth. This could reduce fungal growth on fruit hence reduced disease development and subsequently prolonged fruit storage life.

ESEM micrograph also revealed that exposure to 3, 5, 7 and 9 ppm ozone for 120 h induced spore production as observed from abundance of acervuli and spores on the mycelia. The increase in spore production was also observed in *Alternaria oleraceae* cultures exposed to 0.6 ppm ozone (Treshow *et al.*, 1969) and certain fungi species exposed to 110 ppm ozone (Kuss, 1950). This could be the fungal survival response upon exposure to high ozone dosage. This study also revealed that the spores produced during ozone treatment had disrupted or abnormal structures as evident from their irregular shape. This was most prevalent on cultures exposed to 7 and 9 ppm ozone for 120 h where abundance of clumped masses was observed. This is probably due to the oxidation action of ozone and its reactive decomposition products on spore cell membrane which disrupted the spore integrity. The mycelia under this condition may continuously sporulate

under clean air storage and produce viable fungal spores. These fungal spores may germinate during clean air storage and result in reduction in mycelia inhibition as observed in section 4.3.4.1.1.

4.3.5 Effect of ozone on *C. capsici* spore production

C. capsici spore production was significantly affected ($P < 0.0001$, Appendix B 4.3) by interaction of ozone concentration and exposure time (Figure 4.6). Exposure to ozone for 24 and 72 h had no effect on *C. capsici* spore production. Increase in ozone concentration to 3 ppm ozone for 120 h increased spore production where it resulted in the highest spore production. Further increase in ozone dosage to 5, 7 and 9 ppm ozone for 120 h however, reduced *C. capsici* spore production.

Figure 4.6 showed that exposure to high ozone dosage (3, 5, 7 and 9 ppm ozone for 120 h) induce *C. capsici* spore production. This is in agreement to ESEM micrograph of *C. capsici* mycelia (Figure 4.5) where abundant of spores were observed in cultures exposed to 3, 5, 7 and 9 ppm for 120 h. The increase in spore production when exposed to high ozone dosage was also reported in *A. nidulans* and *A. ochraceus* where exposure to 200 ppm ozone increased the fungal spore production compared to exposure to lower ozone concentration, 0.2 ppm ozone (Antony-Babu and Singleton, 2009). This indicated that the fungal cultures sporulate at higher rate when exposed to high oxidative stress. This could be due to increase in conidiophore respiration which results in premature sporulation (Roushdy *et al.,* 2011). This increased spore production as observed in the result.

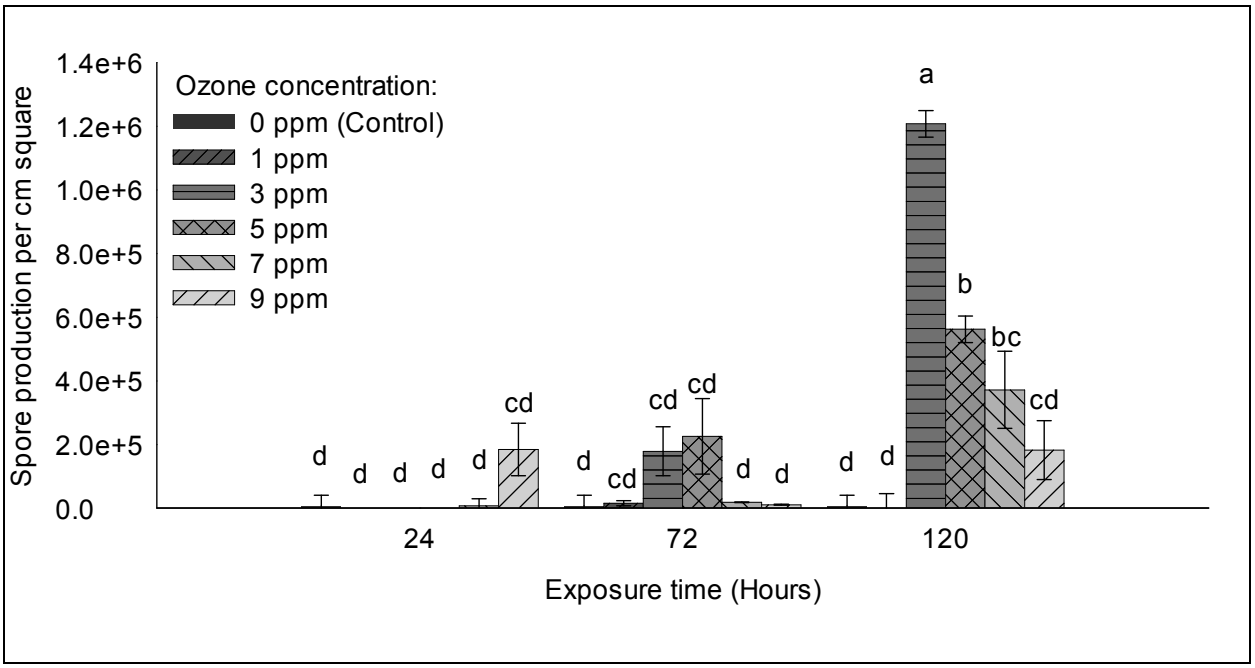

Figure 4.6: The effect of different ozone concentration and exposure time (*c* x *t*) on *in vitro C. capsici* spore production after 9 days incubation at 18 - 20°C, 95% RH, under ozone treatment and subsequent clean air incubation. Error bars are the standard error values. Bars with different letters are significantly different using DMRT ($P < 0.05$). Values are mean of three replicates

In this study, reduction in spore production was observed in mycelia exposed to 5, 7 and 9 ppm ozone for 120 h compared to 3 ppm ozone for 120 h. This could be due to the disrupted and abnormal shape of spores as observed in ESEM. The disrupted spore structure resulted in clumped masses under strong ozone dosage (7 and 9 ppm ozone for 120 h) and did not have the unique sickle shape of *C. capsici* spores which therefore was not considered in the spore production analysis. This contributed to the decrease in number of spore observed in cultures exposed to 5, 7 and 9 ppm ozone for 120 h compared to mycelium exposed to 3 ppm ozone for 120 h.

The mycelia exposed to ozone for 120 h may continuously sporulate under clean air incubation after ozone exposure. This resulted in hyphae elongation and mycelia growth. This contributed to reduction in mycelia inhibition observed in cultures exposed to ozone for 120 h in mycelia development analysis (Figure 4.3). Therefore, these results suggested that an appropriate ozone dosage is required to reduce mycelia growth but not induce sporulation. In this study, we found that exposure to 7 and 9 ppm ozone for 72 h decreased *C. capsici* mycelia growth but did not induce sporulation. This could be the best treatment for anthracnose on bell pepper.

4.3.5.1 Effect of ozone on spore of *C. capsici*

4.3.5.1.1 Effect of ozone on germination of *C. capsici* spore

Germination or viability of *C. capsici* spores was significantly affected ($P < 0.0001$, Appendix 4.4) by ozone concentration (Table 4.2). Increase in ozone concentration up to 3 ppm ozone significantly reduced spore germination where it resulted in nearly total inhibiton. Further increase in ozone concentration to 5, 7 and 9 ppm ozone or exposure time to 72 and 120 h had no effect on the spore germination.

This experiment showed that *C. capsici* spores were highly susceptible to ozone as exposure to 1 ppm ozone for 24 h reduced 95.1% of spore germination. Further increase in ozone dosage resulted in total inhibition. Lower inhibition effect of ozone was observed in *C. coccodes* and *A. alternata* spores as exposure to 1 ppm ozone for 24 h only reduced approximately 88 and 60% of the spore germination (Tzortzakis *et al.*, 2008). This

Table 4.2: The effect of ozone concentration and exposure time (*c* x *t*) on *in vitro C. capsici* spore germination percentage after 72 h incubation at 18 - 20°C, 95% RH, under ozone treatment and subsequent clean air incubation.

Ozone concentration	Exposure time			
	24 hours*	72 hours*	120 hours*	Mean†
	-*In vitro C. capsici* spore germination (%)-			
0 ppm (control)	8.2 ± 0.2 a	8.2 ± 0.2 a	8.2 ± 0.2 a	8.2 ± 0.0 a
1 ppm	0.4 ± 0.2 b	0.0 ± 0.0 c	0.0 ± 0.0 c	0.1 ± 0.0 b
3 ppm	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
5 ppm	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
7 ppm	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
9 ppm	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
Mean‡	1.4 ± 0.0 a	1.4 ± 0.0 a	1.4 ± 0.0 a	

In vitro C. capsici spore germination percentage is the percentage of *C. capsici* spores germinated after 72 hours incubation at 18 - 20°C, 95% RH. The data presented are data after transformation.

*Values are interaction effect of ozone concentration and exposure time on *C. capsici* spore germination. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

†Values are effect of ozone concentration on *C. capsici* spore germination. Means across rows followed by same letter are not significantly different using DMRT ($P < 0.05$).

‡ Values are effect of exposure time on *C. capsici* spore germination. Means across columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

variation in spore susceptibility towards ozone could be due to differences in spore membrane composition (Palou *et al.*, 2001; Tzortzakis *et al.*, 2008). In comparison between *C. capsici* and C*. coccodes* and *A. alternata*, *C. capsici* spore membrane may have lower degree of cross-linking and less density of phospholipid content which may contribute to its high susceptibility towards oxidation of ozone.

4.3.5.1.2 Effect of ozone on *C. capsici* spore under ESEM

ESEM micrograph of *C. capsici* spore showed ozone affected the spore structure. Spores exposed to ozone (1, 3, 5, 7 and 9 ppm ozone) were unviable and unable to germinate (Figure 4.7). Besides, the treated spores had disrupted structure as indicated by their crooked surface. In contrast to the treated spores, untreated spores had germinated and produced mycelia.

The disrupted structure of ozone treated spores showed that ozone oxidized the spore cell wall, reduced integrity of the cell and may contribute to the unviability of the spore. This finding was supported by Roushdy *et al.* (2011) where similar spore disruption was observed on *Aspergillus brasiliensis* spore where ozone damaged the spore cell wall which could result in leakage of the spore cellular contents. Besides, ozone also disrupted cell wall structure of *Fusarium fujikuroi* spore which resulted in crooked surface as reported in this study (Kang *et al.* 2015). The mode of action of ozone on the spore was possibly by attacking lipid contents of spore cell wall (He and Häder, 2002). The disruption of spore structure affected its viability and contributed to inhibition of spore germination

observed in Table 4.2. These results suggest that ozone works as an antifungal by inhibiting spore germination. This mechanism would reduce fungal source of inoculum hence restrict further infection.

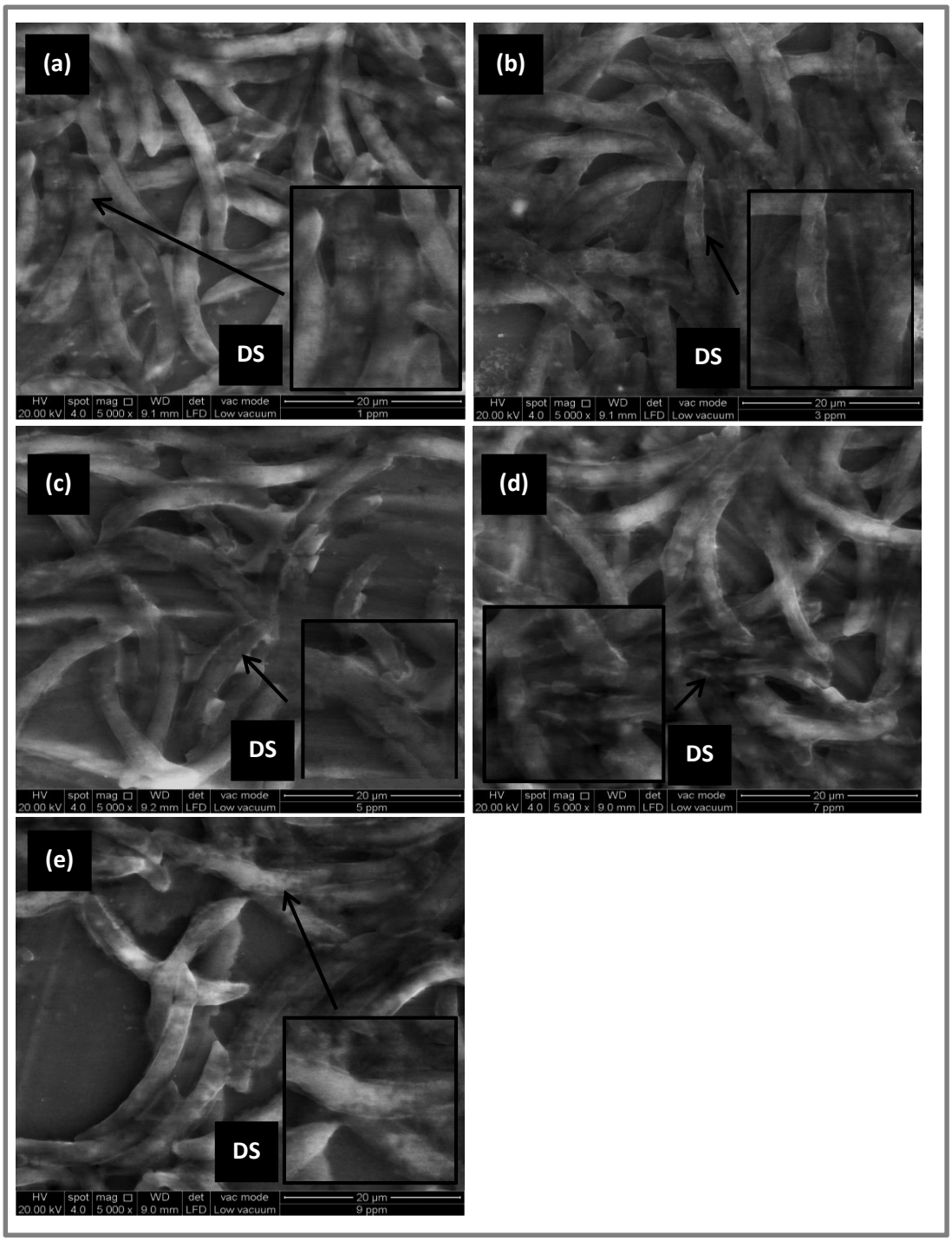

Figure 4.7: Scanning electron micrograph (magnification: 5000X) of *C. capsici* spores exposed for 24 h to (a) 0 (control), (b) 1, (c) 3, (d) 5, (e) 7 and (f) 9 ppm ozone. Control spores have germinated producing mass of mycelia hence, picture of the spores cannot be obtained. Thus, not included in the result. Note that control spores germinated producing mycelia while ozone-treated spores did not germinate and has disintegrated and disrupted structure (DS).

4.3.5.1.3 Effect of ozone on intracellular H_2O_2 of *C. capsici* spore

Effect of ozone on *C. capsici* spore was further confirmed by production of intracellular H_2O_2, an ozone decomposition product. This would indicate the level of ozone oxidation on the spore cells. Intracellular H_2O_2 of *C. capsici* spore was significantly affected ($P < 0.0001$, Appendix 4.5) by ozone concentration where exposure to 1 ppm ozone caused 98% of the spore to produce intracellular H_2O_2 (Table 4.3). Increase in ozone concentration to 3, 5, 7 and 9 ppm ozone and exposure time to 72 and 120 h did not further increase production of intracellular H_2O_2.

Production of intracellular H_2O_2 was detected using oxidant-sensitive probe DCHF-DA. It is a product of ozone decomposition and therefore can be used as an indication of ozone oxidation on fungal cells (Chen and Dickman, 2005). Table 4.3 showed that spores exposed to ozone dosage of 1 ppm ozone for 24 h or higher, increased production of intracellular H_2O_2. Similar result was reported on *C. gloeosporoides* exposed to 1.5 ppm ozone for 24 h where it increased 82.9% of spore intracellular H_2O_2 compared to untreated spores (Ong and Ali, 2015). This indicated that ozone oxidized the spore cell wall (as observed in Figure 4.7) and permeated into the spore cell. This allowed ozone to oxidize fungal cellular components which could result in cell death. This contributed to spore unviability obtained in Table 4.2.

Table 4.3: The effect of ozone concentration and exposure time (*c* x *t*) on *in vitro C. capsici* spore with intracellular H_2O_2 at 18 - 20°C, 95% RH

Ozone concentration	Exposure time			
	24 hours*	72 hours*	120 hours*	Mean†
	- *C. capsici* spore with intracellular H_2O_2 (%)-			
0 ppm (control)	13.4 ± 2.3c	15.4 ± 4.6b	18.8 ± 2.4b	16.3 ± 0.9 b
1 ppm	97.5 ± 5.1a	99.8 ± 5.4a	98.2 ± 3.6a	98.0 ± 0.9 a
3 ppm	99.8 ± 3.1a	96.1 ± 3.9a	96.1 ± 4.2a	96.8 ± 0.9 a
5 ppm	98.4 ± 5.3a	99.0 ± 4.6a	97.2 ± 4.5a	97.8 ± 0.9 a
7 ppm	99.1 ± 3.8a	99.8 ± 5.2a	98.5 ± 3.1a	98.1 ± 0.9 a
9 ppm	97.2 ± 5.1a	96.5 ± 4.3a	97.3 ± 4.4a	97.0 ± 0.9 a
Mean‡	83.7 ± 0.6 a	83.9 ± 0.6 a	84.5 ± 0.6 a	

In vitro C. capsici spore with intracellular H_2O_2 is the percentage of fluorescence spores after ozone treatment

*Values are interaction effect of ozone concentration and exposure time on *C. capsici* spore germination. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

†Values are effect of ozone concentration on *C. capsici* spore germination. Means across rows followed by same letter are not significantly different using DMRT ($P < 0.05$).

‡ Values are effect of exposure time on *C. capsici* spore germination. Means across columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

4.3.6 Effect of ozone on *in vivo C. capsici* growth

4.3.6.1 Effect of ozone on disease incidence and disease severity

Ozone concentration and exposure time significantly reduced ($P < 0.0013$, Appendix B 4.6) disease incidence on bell pepper (

Table 4.4). Ozone concentration of 1, 3, 5, 7 and 9 ppm ozone reduced anthracnose incidence on bell pepper. Increase in ozone concentration to more than 1 ppm however, had no effect on disease incidence. Meanwhile, exposure time of 72 h had the highest inhibition in disease incidence. Increase in exposure time to 120 h increase disease incidence.

Severity of anthracnose on bell pepper significantly affected ($P < 0.05$, Appendix B 4.7) by interaction of ozone concentration and exposure time (Table 4.5). The highest inhibition in disease severity was observed from exposure to 7 ppm ozone for 72 h (41.2%). The inhibition is comparable to exposure to 1, 3, 5 and 9 ppm ozone for 72 and 9 ppm ozone for 24 h. Increase in ozone dosage by prolonging exposure time to 120 h increased disease severity.

In agreement to *in vitro* results, exposure to 7 ppm ozone for 72 hours resulted in the highest inhibition in disease incidence (34.8%) and disease severity (41.2%). Reduction in fungal disease incidence by ozone was also reported on kiwi exposed 0.3 ppm ozone for four months where stem-end rot incidence was reduced by 56%. The higher percentage of disease control achieved, compared to our results, could be due to

Table 4.4: The effect of concentration and exposure time (*c* x *t*) on disease incidence percentage (%) on bell pepper after 21 days storage at 12°C, 95% RH, under ozone treatment and subsequent clean air incubation.

Ozone concentration	Exposure time			
	24 hours*	72 hours*	120 hours*	Mean†
	---------- Disease incidence (%) ----------			
0 ppm (control)	94.4±5.6a	94.4±5.6a	94.4±5.6a	94.4±3.9a
1 ppm	88.9±5.6a	72.2±5.6abc	77.8±5.6abc	79.6±3.9b
3 ppm	83.3±9.6ab	61.1±5.6bc	77.8 ±5.6abc	74.1±3.9b
5 ppm	77.8±5.6abc	61.1±11.1bc	83.3±9.6ab	74.1±3.9b
7 ppm	77.8±5.6abc	55.6±5.6c	77.8±5.6abc	70.4±3.9b
9 ppm	72.2±5.6abc	61.2±5.6bc	83.3±9.6ab	72.2±3.9b
Mean‡	82.4±2.8a	67.6±2.8b	82.4±2.8a	

Disease incidence is expressed as percentage of anthracnose incidence on inoculated bell pepper after 21 days storage at 12°C, 95% RH.

*Values are interaction effect of ozone concentration and exposure time on *C. capsici* spore germination. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

†Values are effect of ozone concentration on *C. capsici* spore germination. Means across rows followed by same letter are not significantly different using DMRT ($P < 0.05$).

‡ Values are effect of exposure time on *C. capsici* spore germination. Means across columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

Table 4.5: The effect of ozone concentration and exposure time (*c* x *t*) on disease severity (lesion diameter (mm)) on bell pepper after 21 days storage at 12°C, 95% RH, under ozone treatment and subsequent clean air incubation.

Ozone concentration	Exposure time			
	24 hours*	72 hours*	120 hours*	Mean†
	---------- Disease severity (mm)----------			
0 ppm (control)	38.8± 0.2a	38.8±0.2a	38.8±0.2a	38.7±0.8a
1 ppm	35.1±1.6ab	29.3±1.6cde	31.3±1.5ab	32.9±0.8b
3 ppm	33.6±1.6bc	27.5±1.6de	33.6± 0.9ab	31.9±0.8bc
5 ppm	31.6±1.6bcd	25.8±1.5e	33.1± 1.9ab	30.6±0.8bc
7 ppm	31.3±1.6bcd	25.3±1.6e	31.6± 0.3ab	30.4±0.8bc
9 ppm	29.3±1.6cde	26.3±1.7e	35.1± 1.6ab	30.2±0.8c
Mean‡	33.2±0.6a	28.8±0.6b	35.3±0.6c	

Disease severity is anthracnose lesion diameter (mm) on inoculated bell pepper after 21 days storage at 12°C, 95% RH.

*Values are interaction effect of ozone concentration and exposure time on *C. capsici* spore germination. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

†Values are effect of ozone concentration on *C. capsici* spore germination. Means across rows followed by same letter are not significantly different using DMRT ($P < 0.05$).

‡ Values are effect of exposure time on *C. capsici* spore germination. Means across columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

longer exposure time applied. Besides, *Botrytis cinerea*, the causing agent for stem-end rot disease in kiwi may have lower resistancy towards ozone compared to *C. capsici* in this study. Therefore, it resulted in higher inhibition in disease incidence. The inhibition in disease incidence and disease severity could be due to oxidation action of ozone on fungal mycelia as observed in *in vitro* assay. Exposure to ozone resulted in compact and highly branched mycelia which retarded the mycelia growth. The ozone dosage also reduced fungal spore germination rate hence, contributed to the reduction in disease incidence and disease severity observed.

Increase in exposure time to 120 h resulted in reduction in disease control. This could be stimulating effect of ozone mycelia growth and spore production as observed in *in vitro* experiments. Besides, it could also be due to enhanced ripening progress as indicated by progressive colour change (from green to red) in the treated fruit (Figure 4.8). This could be due to strong oxidative stress from the ozone treatment. The strong oxidative stress enhanced ripening and senescence progress and weakened fruit disease resistance hence reduced disease control (Nigro *et al.*, 2000; Terry and Joyce, 2004). Similar findings were observed on papaya exposed to 1.6 ppm ozone where higher disease incidence was obtained when exposed to 144 h compared to 96 h (Ong *et al.*, 2012). The strong ozone dosage disrupted papaya cell structure hence reduced its resistance towards disease. This negative effect of oxidative stress on fruit disease resistance was also reported in a study using UV-C in strawberry. The study reported that higher disease incidence in fruit exposed to higher UV-C dose (1.0 KJ m^{-2}) compared to lower dose (0.5 KJ m^{-2}) (Nigro *et al.*, 2000).

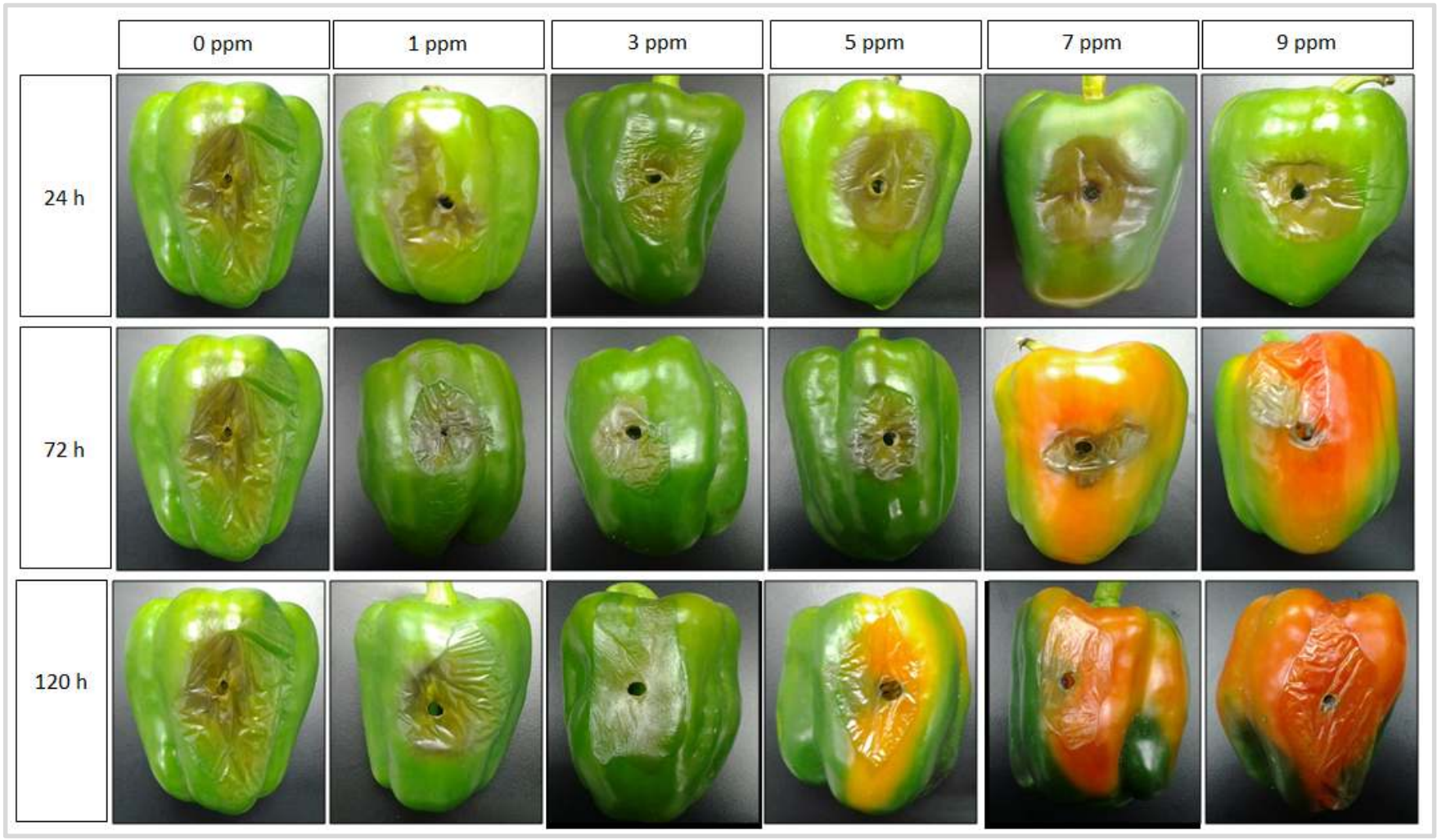

Figure 4.8: The effect of ozone fumigation at different ozone concentration; 0, 1, 3, 5, 7, and 9 ppm ozone for 24, 72 and 120 h; on anthracnose disease development on bell pepper on day 21 at 12°C, 95% RH.

4.3.6.2 Effect of ozone on *C. capsici* spore production on bell pepper

Ozone treatment significantly affected (P = 0.023, Appendix B 4.8) *C. capsici* spore production on bell pepper (Table 4.6). The highest reduction in fungal spore production was observed from exposure to 5 ppm ozone for 72 h (37.4%). The reduction is comparable with spore production from exposure to 3, 7 and 9 ppm ozone for 72 h. Meanwhile, exposure to high ozone dosage, 7 and 9 ppm for 120 h induced spore production.

Reduction in fungal spore production was also observed peach inoculated with *Monilinia fructicola and Botrytis cinerea* where exposure to 0.3 ppm ozone for 4 weeks reduced the fungal sporulation rate (Palou *et al.*, 2002). The reduced sporulation rate would inhibit disease progression hence extended its storage life. This is coherent with findings in Table 4.5 where reduction in disease severity was observed in fruit exposed to 3, 5, 7 and 9 ppm ozone for 72 h.

Enhanced sporulation rate observed in this experiment (exposure to 7 and 9 ppm ozone for 120 h) could be due to increase in conidiophore respiration under high oxidative stress which results in premature sporulation. This was in line with spore production assay on agar plate (section 4.3.5). The high rate of spore production could provide a source of infection for disease progress. The spores could germinate to produce hyphae and mycelia which enhance disease severity and could infect nearby fruits hence increase disease incidence. This suggests that ozone dosage of 3, 5, 7 and 9 ppm ozone for 72 h is an effective to reduce *C. capcisi* sporulation but not ozone dosage of 7 and 9 ppm ozone for 120 h.

Table 4.6: The effect of ozone concentration and exposure time (*c* x *t*) on *C. capsici* spore production on bell pepper after 21 days storage at 12°C, 95% RH, under ozone treatment and subsequent clean air incubation.

Ozone concentration	Exposure time			
	24 hours*	72 hours*	120 hours*	Mean†
	-*C. capsici* spore production ($1x10^6$ spores ml^{-1})-			
0 ppm (control)	1.23±0.09bcd	1.23±0.09bcd	1.23±0.09bcd	1.23±0.06a
1 ppm	1.25±0.11bcd	1.00±0.11def	1.53±0.11ab	1.25±0.06a
3 ppm	1.21±0.08bcd	0.85±0.10ef	1.46±0.11abc	1.17±0.06a
5 ppm	1.15±0.11cde	0.77±0.10f	1.54±0.10ab	1.15±0.06a
7 ppm	1.00±0.09def	0.84±0.10ef	1.59±0.09a	1.14±0.06a
9 ppm	1.09±0.08def	0.88±0.11ef	1.62±0.11a	1.20±0.06a
Mean‡	1.15±0.04a	0.9±0.04b	1.15±0.04c	

In vivo C. capsici spore production is expressed as 1 x 10^6 spores per ml of spore suspension prepared, produced on inoculated bell pepper after 21 days storage at 12°C, 95% RH.

*Values are interaction effect of ozone concentration and exposure time on *C. capsici* spore germination. Means across rows and columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

†Values are effect of ozone concentration on *C. capsici* spore germination. Means across rows followed by same letter are not significantly different using DMRT ($P < 0.05$).

‡ Values are effect of exposure time on *C. capsici* spore germination. Means across columns followed by same letter are not significantly different using DMRT ($P < 0.05$).

In conclusion, under the current set of experiment, ozone treatment reduced anthracnose development on bell pepper where exposure to 7 ppm ozone for 72 h resulted in lowest disease incidence (34.8%) and disease severity (41.2%). This inhibition was comparable to fruit exposed to 3, 5 and 9 ppm ozone for 72 h. The reduction in disease development was due to synergistic effect of ozone action on mycelia morphology, spore production and spore germination as the ozone dosage inhibited mycelia development and reduced spore production and spore germination.

CHAPTER 5

EFFECT OF OZONE FUMIGATION ON ACTIVITY OF DEFENSE RELATED ENZYMES

5.1 Introduction

Plant has evolved a defense mechanism in response to harmful effect of biotic (fungal, bacterial and viral attacks) and abiotic stresses (low temperature, osmotic and oxidative stresses). A part of plant defense mechanism involves induction of defense related enzymes such as PAL, PPO, POD and β-1,3-glucanase (Asghari and Aghdam, 2010). These enzymes have direct or indirect involvement to encounter the stresses. For example, β-1,3-glucanase inhibits fungal development by hydrolyzing fungal cell wall (Cota *et al.*, 2007). Meanwhile, POD decomposes hydrogen peroxide to reduce fruit oxidative stress (Wang, 1995). PAL involves indirectly in plant defense by being the key enzyme to produce secondary metabolites with antifungal and antioxidant properties (Nigro *et al.*, 2000).

Enzyme regulation in respond to biotic and abiotic stresses improves plant defense to maintain fruit quality. For instance, the induction of PAL and β-1,3-glucanase activities during pathogen invasion restricts pathogen development in fruit tissue hence, decreases disease development (Cota *et al.*, 2007; Nigro *et al.*, 2000). Meanwhile, high expression of PAL, PPO and POD activities during storage at low temperature detoxifies fruit tissue from oxidative stress hence, prevents chilling injury (Asghari and Aghdam, 2010; Wang, 1995). Therefore, regulation of these inducible

enzymes is important in order to reduce fruit quality deterioration and extends its storage life.

Strengthening plant defense system by manipulating the inducible enzymes is a promising strategy to maintain fruit quality. Elicitors such as active microorganisms (i.e: yeast) (Xu *et al.*, 2008) and chemical treatment (i.e: methyl jasmonate) (Fung *et al.*, 2004) were introduced to induce the enzyme activity hence, improved fruit quality. Ozone is one of the promising treatments to induce the defense related enzyme and was reported in pear (Zhao et al., 2013b) and tobacco (Schraudner *et al.*, 1992). The fruit response to ozone varies depending on commodity as well as maturity stage and ozone dosage (Salvador *et al.*, 2006). The knowledge on the effect of ozone on the inducible enzymes is scarce while their potential to improve fruit quality by improving fruit resistance against pathogen is promising. This leads to the objectives of this study to:

1. evaluate the effect of ozone on bell pepper antioxidant enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD)
2. investigate the effect of ozone on bell pepper pathogenesis-related enzyme which is β-1,3-glucanase
3. evaluate the effect of inducible defense enzyme on anthracnose development on bell pepper

5.2 Materials and Method

5.2.1 Fruit material

Fruit material was prepared as described in section 3.2.2.

5.2.2 Ozone exposure

Fruit was inoculated as described in section 4.2.4.1 and exposed to ozone at concentrations of 0 (control), 1, 3, 5 and 7 and 9 ppm for exposure time of 72 h at 12°C and 95% RH. Following the ozone treatment, fruit were incubated in duplicate clean air chambers (0 ppm ozone) until the end of storage period, 21 days.

5.2.3 Assessment of anthracnose incidence

Disease incidence was assessed according to the method in section 4.2.4.1.

5.2.4 Determination of phenolic metabolism enzymes

5.2.4.1 Crude enzyme preparation

Enzyme assay was conducted by the method described by Zheng et al. (2011). Briefly, 1 g of fruit tissue was homogenized in 5 ml 0.05 M phosphate buffer (pH 6.8) and centrifuged at 10 000×*g* for 5 min at 4ºC. The crude enzyme was then subjected to PAL, PPO and POD assays.

5.2.4.2 Phenylalanine ammonia lyase (PAL) assay

PAL (E.C 4.3.1.5) activity was determined using L-phenylalanine as a substrate. Briefly, a reaction mixture containing 500 µl of enzyme extract, 2 ml of 0.2 M boric acid buffer and 1 ml of 0.02 M L-phenylalanine was prepared. The enzyme reaction was carried out at 30ºC for 1 h and absorbance at 290 nm was measured using UV-Vis Spectrophotometer. PAL activity was expressed as U g^{-1} FW.

5.2.4.3 Polyphenol oxidase (PPO) assay

PPO (E.C 1.10.3.1) activity was determined using catechol as a substrate. Briefly, a reaction mixture containing 200 µl of enzyme extract, 2 ml of 0.05 M phosphate buffer, pH 6.8 and 1 ml of 0.1 M catechol. Absorbance at 420 nm was measured using UV-Vis Spectrophotometer. PPO activity was expressed as U g^{-1} FW.

5.2.4.4 Peroxidase (POD) assay

POD (E.C 1.11.1.7) activity was determined according to (Liu *et al.*, 2007). The activity was determined using guaiacol as a substrate. A reaction mixture containing 0.1 ml enzyme extract and 2 ml of 8 mM guaiacol, pH 6.4 was prepared. The enzyme reaction was carried out at 30ºC for 30 min and stopped by the addition of 24 mM hydrogen peroxide. The production of tetraguaiaccol by POD was determined by measuring absorbance at 460 nm using UV-Vis Spectrophotometer. POD activity was expressed as U mg^{-1} FW.

5.2.5 Determination of pathogenesis-related (PR) enzyme

5.2.5.1 β-1,3-glucanase assay

β-1,3-glucanase (E.C 3.2.1.39) assay was determined using laminarin as a substrate. Briefly, enzyme extract was prepared by homogenizing 1 g of fruit tissue with 5 ml of 0.1 M acetic acid buffer, pH 5.0 and centrifuged at 10,000×*g* for 5 min at 4ºC. A reaction mixture containing 100 µL of enzyme extract and 50 µL of 0.4% laminarin was prepared and the reaction was conducted at 37ºC for 1 h. The reaction was stopped by addition of 200 µl of 1% 3,5-dinitrosalicylic acid and boiled for 5 min. The reaction mixture was cooled in ice and absorbance at 500 nm was measured using UV-Vis Spectrophotometer. β-1,3-glucanase activity was expressed as U g^{-1} FW.

5.2.6 Statistical analysis

The experiments were carried out with a Completely Randomized Design (CRD) and three replicates per treatment using three different ozone chambers, each containing 20 fruits. The experiments were repeated thrice and data was analysed separately to check for homogeneity. The results were analyzed using Statistical Analysis Software (SAS, version 9.1.3, SAS Institute Inc., USA). Analysis of variance (ANOVA) was performed with $P < 0.05$ significance level and differences in data means were analyzed using DMRT.

5.3 Results and Discussion

5.3.1 Effect of ozone on disease development

Anthracnose development was monitored during storage until maximum acceptable weight loss of 7% was reached by the fruit. This was reported after 21 days in preliminary results, thus the experiment was kept at 21 days. Storage after this time period produced fruit below the acceptable market standard. The results showed the disease development was significantly affected ($P < 0.0001$, Appendix B 5.1) by ozone concentration and storage time (Figure 5.1). Disease incidence increased with time in storage where it reached 94% in control fruit at the end of storage period. Significant reduction in disease development was observed from day 6 where slower rate of disease development was observed in the treated fruit. Increase in ozone concentration reduced disease development where exposure to 7 ppm (38.8%) ozone had the lowest disease incidence. It is comparable to fruit exposed to 3 (33.3%), 5 (33.33%) and 9 (33.2%) ppm ozone.

Inhibition of disease development observed in this experiment could be due to direct effect of ozone on fungal development as discussed in previous Chapter 4. This includes the effect of ozone on mycelia elongation, spore production and spore germination. The antifungal effect of ozone on *C. capsici* inhibited disease development in the treated fruit compared to control fruit. However, reduction in disease progress in fruit could also be due to induction of plant defense enzymes such as PAL, PPO, POD and β-1,3-glucanase. The effects of ozone on these enzymes are discussed in the next section.

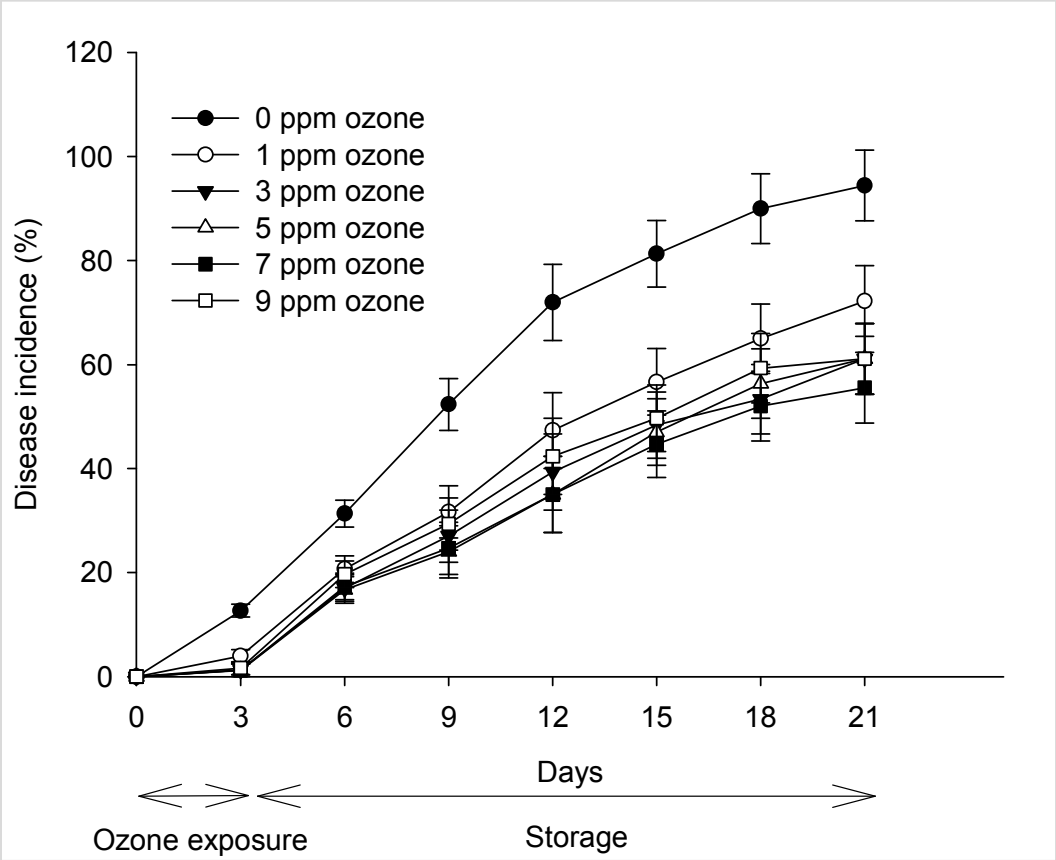

Figure 5.1: Effect of different ozone exposure on disease incidence on bell pepper during three days treatment and 18 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

5.3.2 Effect of ozone on phenolic metabolism enzymes

5.3.2.1 Phenylalanine ammonia lyase (PAL) activity

Activity of PAL of bell pepper significantly increased ($P < 0.0001$, Appendix B 5.2) after exposure to 1, 3 and 5 ppm ozone (Figure 5.2). The highest PAL activity was observed from exposure to 3 ppm ozone which increased by 44.1% of PAL

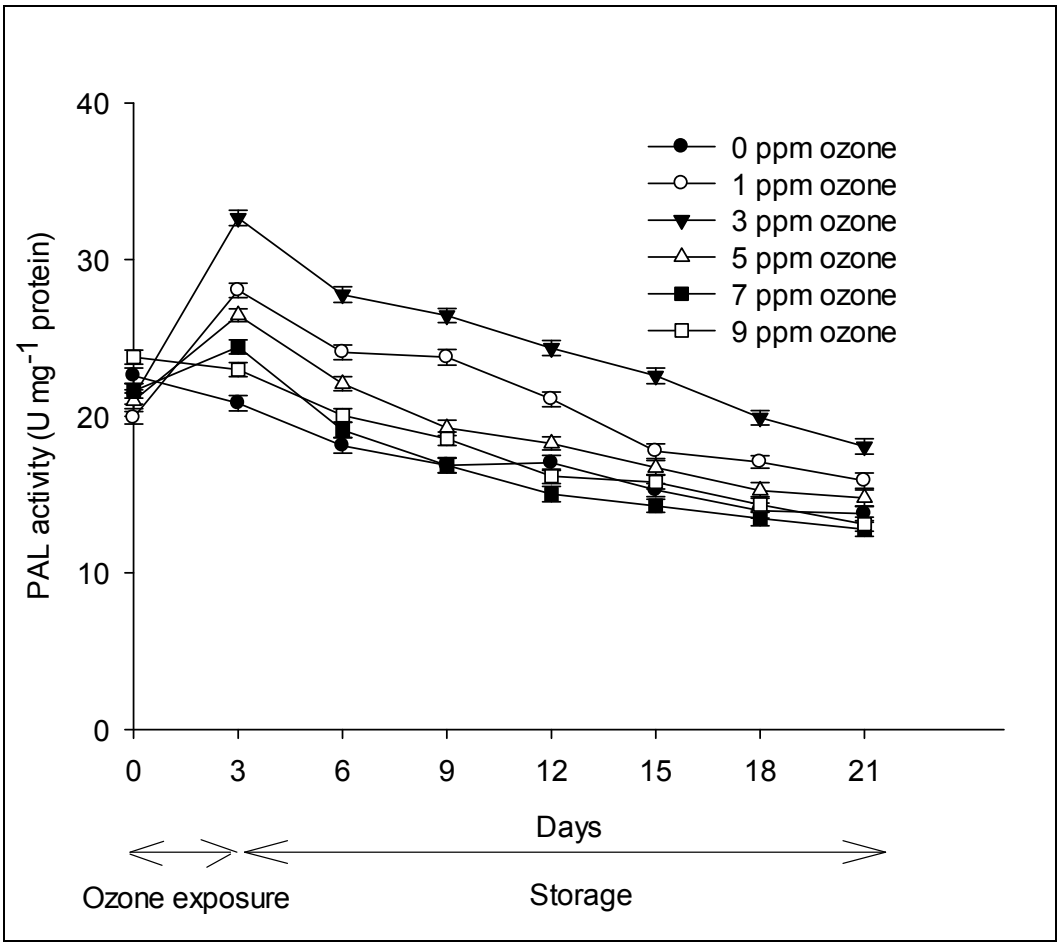

Figure 5.2: The effect of different ozone exposure on phenylalanine ammonia lyase (PAL) activity of bell pepper during three days treatment and 18 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$

activity compared to control, immediately after exposure. Exposure to 7 and 9 ppm ozone minimally increased PAL activity with no significant difference with control. Fruit exposed to 1 and 3 ppm ozone had higher PAL activity until the end of storage period.

PAL is a key enzyme of phenylpropanoid pathway which catalyzes the production of trans-cinnamate from L-phenylalanine (Benkeblia, 2000). This step is the first process for downstream processing of secondary metabolites such as

phenolic compounds, lignin and phytoalexin, which involve in plant defense response and restriction of pathogen growth (Wang *et al.*, 2009). Activity of PAL is influenced by several factors such as hormone (Nigro *et al.*, 2000; Terry and Joyce, 2004), wounding (An *et al.*, 2007), pathogen attack (El Ghaouth *et al.*, 2003; V. Ramamoorthy *et al.*, 2002) and abiotic stress (El Ghaouth *et al.*, 2003; Nigro *et al.*, 2000).

Exposure to abiotic stress such as ozone induced PAL activity as observed in this study. This was also reported in bell pepper exposed to 6420 ppm ozone for 15 mins (Chen *et al.,* 2016). This showed that ozone induced formation of reactive oxygen species in fruit cells causing an oxidative burst which triggered fruit first line of defense and induced PAL activity (Asghari and Aghdam, 2010; Zhao *et al.*, 2013b). The activation of fruit first line of defense through stimulation of PAL activity was also reported in mango irradiated with 2.5 and 4.9 kJ m^{-2}UV-C (González-Aguilar *et al.*, 2007), peach irradiated with 7.6 kJ m^{-2}UV-C (El Ghaouth *et al.*, 2003) and pear irradiated with 5.0 kJ m^{-2}UV-C (Li *et al.*, 2010). Stimulation of oxidative stress through application of 0.5 mM salicylic acid also induced PAL activity as reported in sweet cherry (Qin *et al.*, 2003) and kiwi fruit (Poole *et al.*, 1996). This showed that oxidative stress of ozone may have similar effects with UV-C and salicylic acid on fruit where it involves induction of PAL activity.

Figure 5.2 showed the threshold dose of ozone on PAL activity was 3 ppm ozone. Exposure to higher ozone dosage, 5, 7 and 9 ppm ozone has reduced PAL activity compared to 3 ppm ozone. The diminish PAL activity after exposure to a certain threshold dose was also reported in strawberry irradiated with UV-C stronger than 0.05 kJ m^{-2} (Nigro *et al.*, 2000). This showed that exposure to oxidative stress beyond its threshold limit reduced fruit capacity to stimulate PAL activity. This could

be due to the strong oxidative stress which may have weaken fruit defense system (Nigro *et al.*, 2000). Therefore, exposure to 3 ppm ozone for 72 h is the most effective dosage to induce PAL activity in bell pepper.

PAL activity in bell pepper was negatively correlated ($r = -0.6743$, $P < 0.0001$) with disease incidence (Figure 5.3). Using coefficient of determination value ($r^2 = (-0.6743)^2 \times 100 = 45\%$), it was observed that PAL activity contributed to 45% of the variation in disease incidence in Figure 5.1. This may be due to the induction of fruit resistance by PAL where increase in PAL activity resulted in decrease in variation of disease incidence. This showed that in addition to the effect of ozone on fungal elongation and spore germination as discussed in Chapter 4, the decrease in disease incidence in fruit exposed to 3 ppm ozone (33.3%) could also be due to the stimulating effect of ozone on PAL activity. Meanwhile, reduction in disease incidence in fruit exposed to 7 ppm ozone could be mainly due to the effect ozone on fungal growth or other enzymes but not PAL as PAL activity was not induced in this fruit. Similar pattern between PAL and disease incidence was also observed in strawberry irradiated with 0.25 and 0.5 kJ m^{-2} UV-C where the treatment increased fruit PAL activity to 25% and 119% and resulted in reduction in disease incidence for 30% and 60% (Nigro *et al.*, 2000). Besides, increase in PAL activity was also reported to reduce *B. cinerea* development on tomato (Zheng *et al.*, 2011) and pear (Li *et al.*, 2010). The induction in fruit resistance by PAL was probably due to production of antifungal compounds such as phytoalexin and phenol which inhibited fungal growth hence reduced disease development (Li *et al.*, 2010; Nigro *et al.*, 2000). Subsequently, this would increase fruit quality and extend fruit storage life.

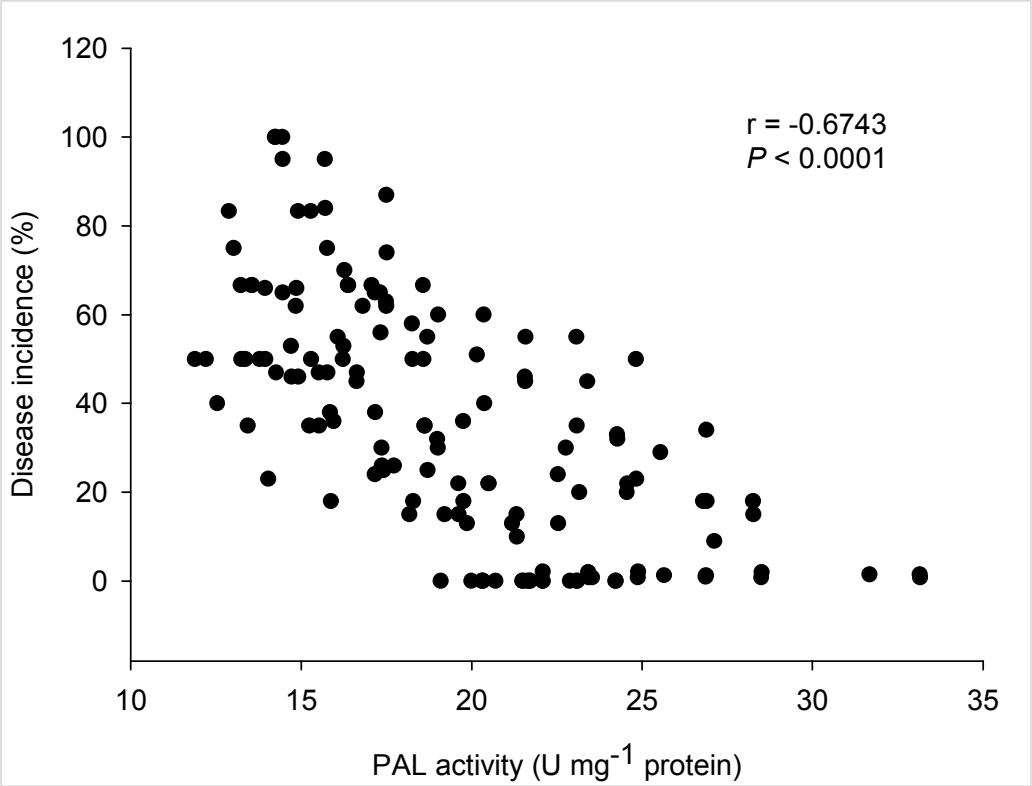

Figure 5.3: Relationship between PAL activity and disease incidence (%) in bell pepper

The effect of ozone on PAL activity could be due to changes in gene expression level. The effect could be similar to the effect of UV-C irradiation on peach. As reported by El Ghaouth *et al.* (2003), increase in PAL activity after exposure to 7.6 kJ m^{-2} UV-C was due to upregulation of PAL gene. This resulted in accumulation of PAL RNA transcript which translated into increase in PAL activity (El Ghaouth *et al.*, 2003).

Under the current observation, it can be suggested that the induced activity of PAL involved in resistance against anthracnose. The increase in PAL activity is responsible for the reduction in disease development in the treated bell pepper.

5.3.2.2 Polyphenol oxidase (PPO) activity

Activity of PPO in bell pepper was significantly affected ($P < 0.0001$, Appendix B 5.3) by ozone treatment. Immediately after treatment, exposure to 1 and 3 ppm ozone enhanced fruit PPO activity where fruit exposed to 3 ppm ozone had the highest PPO activity where it was increased by 43.9% (Figure 5.4). The increase in PPO activity after ozone treatment resulted in elevated level of PPO activity until the end of storage period. The induction effect of ozone on PPO activity was also observed in hot pepper exposed to 2 ppm ozone for 3 h where PPO activity was increased by 9.7% (Sachadyn-Król *et al.*, 2016). The induction is lower compared to our results obtained in this study as the fruit were only exposed for 3 h compared to 3 days in our study. Similarly to the results obtained in this experiment (Figure 5.4), the induction was probably fruit first line of defense in response to oxidative burst of ozone (Asghari and Aghdam, 2010). This first line of defense or termed as ROS avoidance genes increased PPO activity through induction of alternative oxidase (AOX) enzyme (Fung *et al.*, 2004). As an antioxidant enzyme, the induction in PPO activity increased fruit antioxidant capacity to neutralize reactive oxygen species such as H_2O_2 resulted from ozone decomposition (Asghari and Aghdam, 2010).This would consequently reduce cell oxidative state hence minimizes oxidative damage.

Our results showed that exposure to 7 and 9 ppm ozone reduced the PPO activity where significant reduction in PPO activity was observed from day 3 and day 6, respectively. This resulted in reduced PPO activity during storage. This negative effect of high ozone concentration on PPO of bell pepper was also reported by Chen *et al*. (2016) where the author exposed bell pepper to a very high ozone

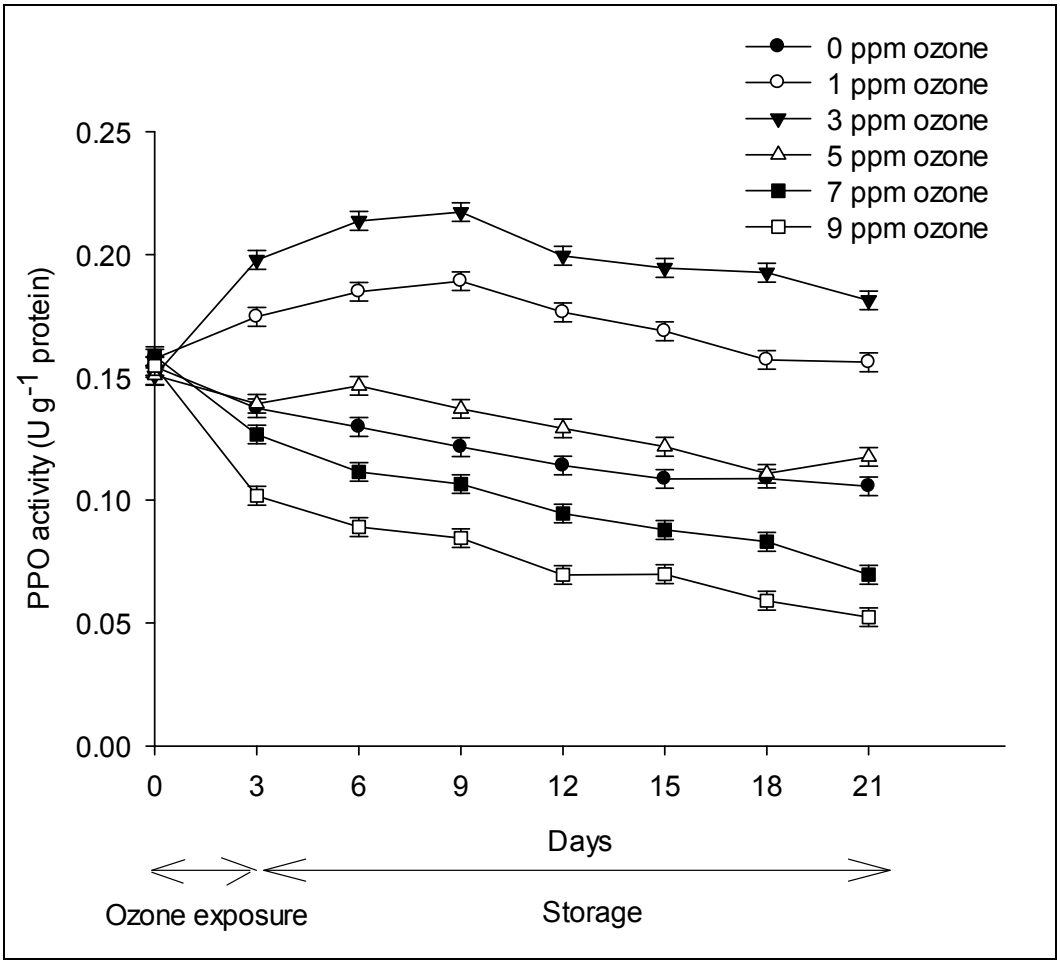

Figure 5.4: The effect of different ozone exposure on polyphenol oxidase (PPO) activity of bell pepper during three days treatment and 18 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

concentration, 6420 ppm ozone for 15 min. This reduced the fruit PPO activity by 14.3%. The reduction is however, lower than bell pepper exposed to 9 ppm ozone for 3 days (26.0%). This could suggest that high ozone concentration but short exposure time would cause less PPO activity reduction compared to low ozone

concentration but long exposure time. The reduction in PPO activity observed in the fruit could be due to oxidation of amino acids or disulfide bonds of PPO by reactive oxygen species induced by ozone (Fu *et al.*, 2007). The strong ozone dosages produced high level of reactive oxygen species which altered PPO conformation hence resulted in enzyme inactivation (Fu *et al.*, 2007). Besides, reduction in PPO activity may also be due to the effect of ozone on availability of its precursor such as flavonoid. The reduction in PPO activity reduced fruit capacity to produce quinone, an antimicrobial compounds which therefore, weakened plant defense system against diseases. However, for other commodity such as apple, the reduction in PPO activity is desirable as it reduced production of quinone, a browning compound which reduced the fruit cosmetic look.

PPO activity in bell pepper was negatively correlated ($r = -0.2866$, $P = 0.0005$) with disease (Figure 5.5). Using coefficient of determination value ($r^2 = (-0.2866)^2 \times 100 = 8\%$), it was observed that PAL activity contributed to 8% of the variation in disease incidence in Figure 5.1. This indicated that increase in PPO activity slightly resulted in decrease in variation in disease incidence, particularly for fruit exposed to 3 ppm ozone where PPO activity was increased by 43.9%. PPO may reduce disease incidence by catalyzing oxidation reaction of phenolic compounds, commonly produced in polypropanoid pathway to quinone (Nguyen *et al.*, 2004). Quinone covalently modified cellular molecules of pathogen which resulted in cell death hence, limiting pathogen progression in fruit tissue (Thipyapong *et al.*, 2007). Quinone also modified plant protein via alkylation which reduced its bioavailability to pathogen (Thipyapong *et al.*, 2007). Besides, PPO enhanced formation of lignin

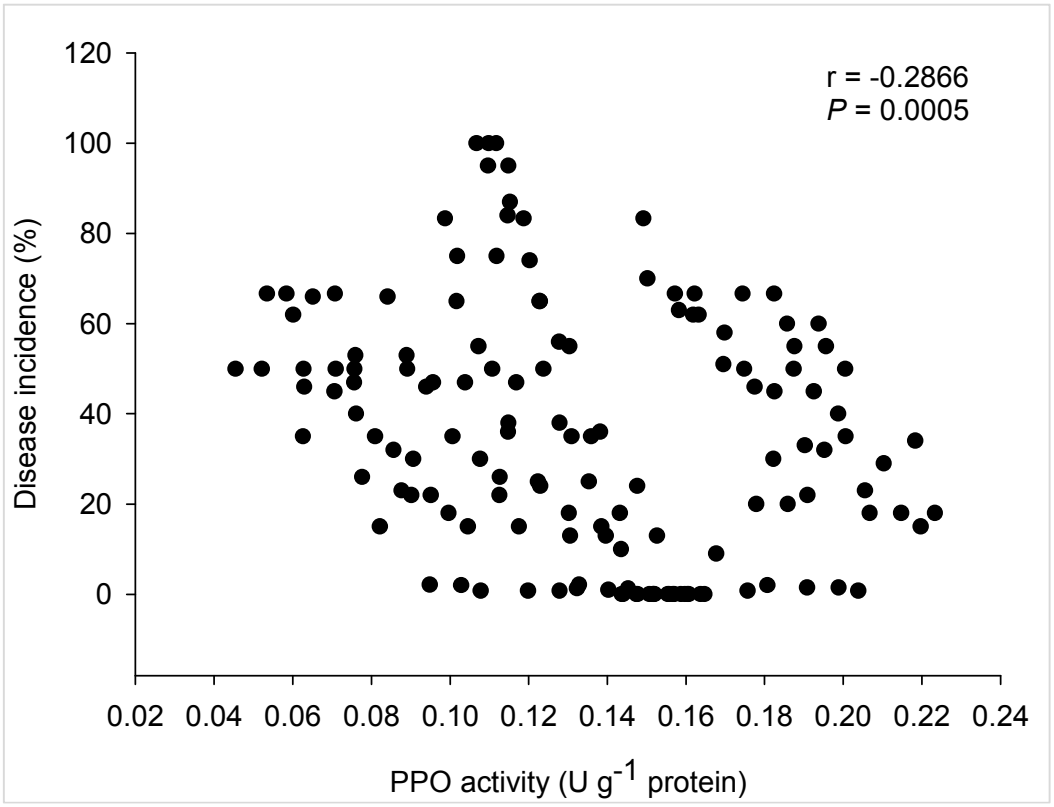

Figure 5.5: The relationship between PPO activity and disease incidence (%) in bell pepper

which provides as a physical barrier in inhibiting pathogen proliferation (Zheng *et al.*, 2011). Therefore, the reduction in disease development in bell pepper could be due to induction of PPO activity.

The negative relationship between PPO activity and disease incidence (Figure 5.5) also indicated that the reduction in PPO activity in fruit exposed to 7 and 9 ppm ozone should increase the disease incidence. However, no increment in disease incidence was observed in fruit exposed to 7 and 9 ppm ozone (Figure 1). This could be due to the effect of ozone on fungal growth and spore germination as discussed in Chapter 4 which compensates reduction in PPO activity. This shows that both,

antimicrobial activity of ozone and its effects on PPO activity influence disease reduction in bell pepper.

5.3.2.3 Peroxidase (POD) activity

Ozone treatment significantly affected ($P < 0.0001$, Appendix B 5.4) fruit POD activity where exposure to 1, 3 and 5 ppm ozone significantly increased POD activity, immediately after ozone exposure (Figure 5.6). The increase in POD activity resulted in elevated level of POD activity throughout the storage period. The highest POD activity was observed from exposure to 3 and 5 ppm ozone on day 9 and the POD activity gradually decreased during subsequent storage. The result showed threshold dose on POD activity is at 5 ppm ozone as increase in ozone concentration to 7 and 9 ppm ozone showed a reduction.

Induction in POD activity by ozone was also observed in hot pepper exposed to 2 ppm ozone for 3 h (Sachadyn-Król *et al.*, 2016) where PPO activity was induced by 48.0%. Ozone also induced POD activity in pear where exposure to 1.9 ppm ozone for 1 h every day for 8 days induced around 50% of the fruit PPO activity (Zhao *et al.*, 2013b). The increase in POD activity in response to ozone was also fruit first line of defense to reduce H_2O_2 level in fruit tissue induced by ozone (Asghari and Aghdam, 2010; Wang, 1995). POD catalyzes decomposition of H_2O_2 and produces free radicals such as hydroxyl radicals, OH^- (Apel and Hirt, 2004; C. Y. Wang, 1995). The reaction is accompanied by oxidation of phenolic compounds to quinone and formation of lignin which improved fruit defense against pathogen attack

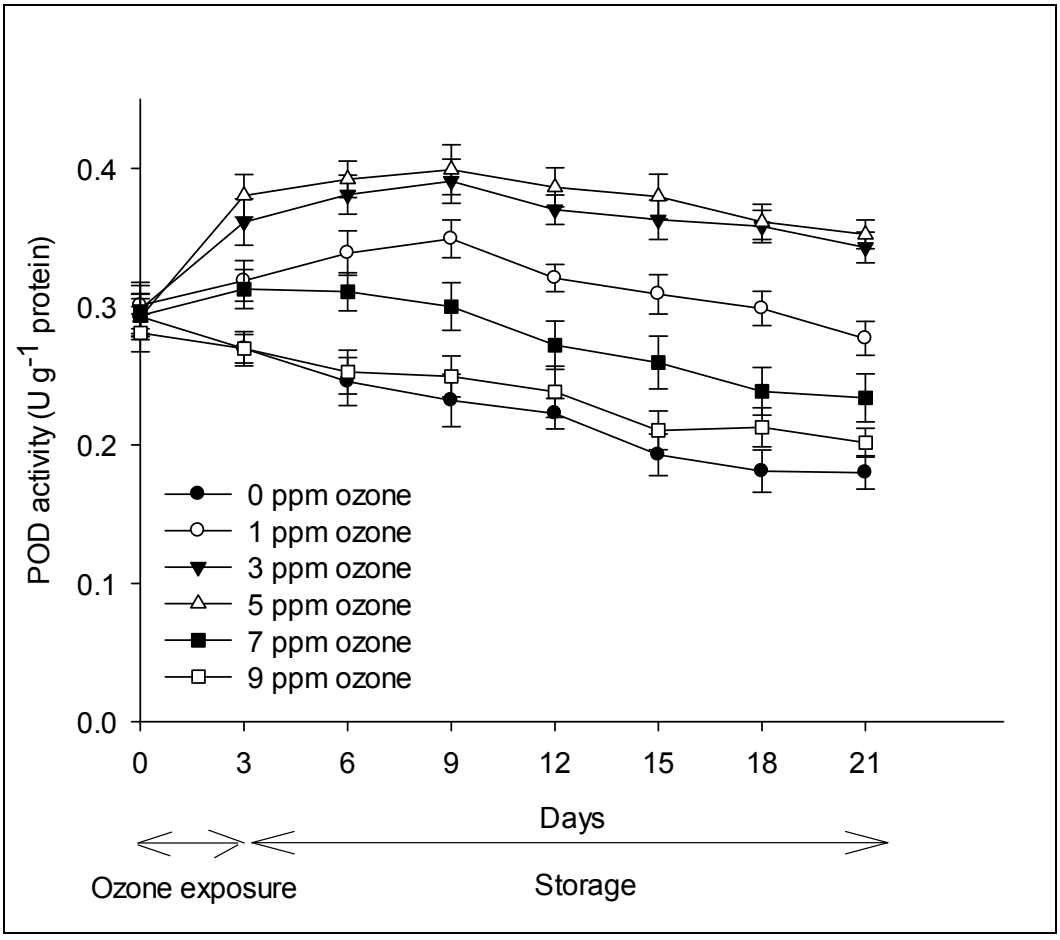

Figure 5.6: The effect of different ozone exposure on peroxidase (POD) activity of bell pepper during three days treatment and 18 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

(Apel and Hirt, 2004). The increase in POD activity also could protect the fruit from oxidative damage such as peroxidation due to high level of H_2O_2 induced by ozone. This would maintain fruit quality hence extending fruit storage life.

POD activity in bell pepper was negatively correlated ($r = -0.3507$, $P < 0.0001$) with disease incidence (Figure 5.7). Using coefficient of determination value [$(-0.3507)^2 \times 100 = 12\%$], it was observed that POD activity contributed to 12% of the

variation in disease incidence in Figure 5.1. This suggested that increase in POD activity, particular in fruit exposed to 3 ppm ozone may slightly reduce variation in disease development in the fruit. The role of POD to reduce pathogen development was also reported in chitosan treated tomato where the treatment increased fruit POD activity for 36.6% and decreased *Botrytis cinerea* development on fruit by 28.6%. (Wang *et al.*, 2009). POD may reduce disease development by catalyzing the production of quinone and induces formation of lignin (Wang *et al.*, 2009). This would restrict pathogen growth in fruit tissue.

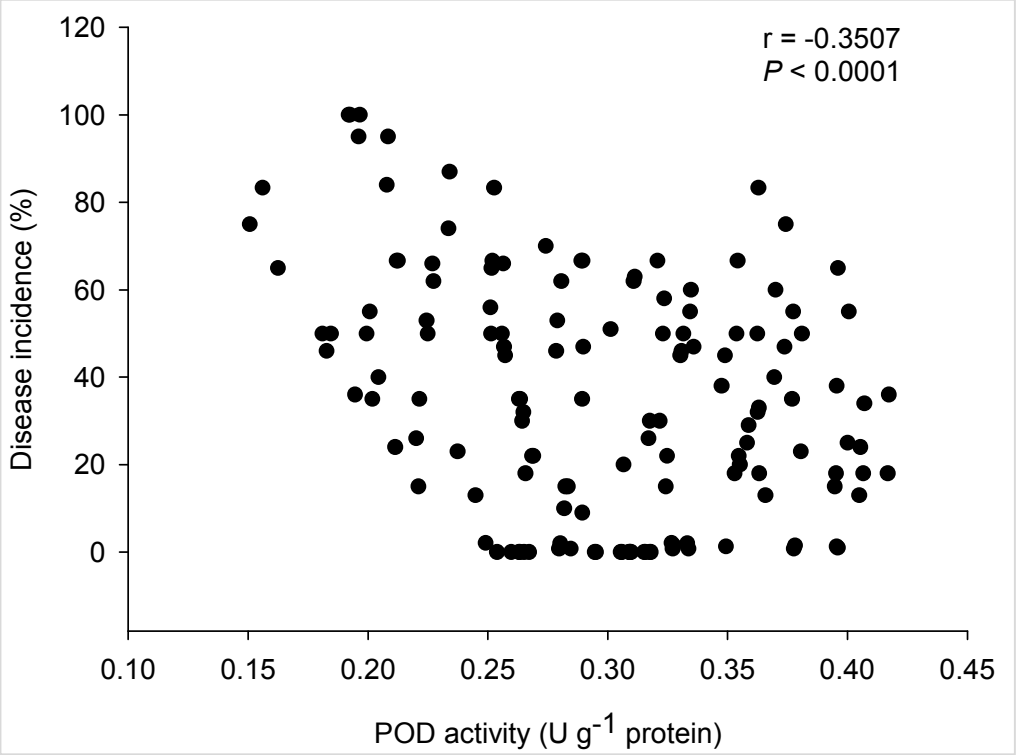

Figure 5.7: Relationship between POD activity and disease incidence (%) in bell pepper

The changes in POD activity observed after ozone treatment could be regulated at gene expression level. The reactive oxygen species produced from

ozone decomposition may oxidize components of POD signaling pathway hence alters the gene expression (Apel and Hirt, 2004). Besides, the reactive oxygen species may also alter gene expression by modifying activity of POD transcription factors (Apel and Hirt, 2004). The correlation between POD gene expression and its enzyme activity was reported in peach (Xu *et al.*, 2008).

5.3.3 Pathogenesis-related (PR) enzyme

5.3.3.1 β-1,3-glucanase activity

Activity of β-1,3-glucanase was significantly affected ($P < 0.0001$, Appendix B 5.5) by ozone treatment (Figure 5.8). Immediately after ozone exposure, β-1,3-glucanase activity was induced in all treatments; 1, 3, 5, 7 and 9 ppm ozone. The increase in β-1,3-glucanase activity was optimal after exposure to 3 ppm ozone where further increase in ozone concentration to 5, 7 and 9 ppm had no significant effect. The stimulating effect of ozone on β-1,3-glucanase activity was transient where the increase in β-1,3-glucanase activity was only observed until day 6 (1 ppm ozone), day 9 (3 ppm ozone) and day 12 (5, 7 and 9 ppm ozone). Further storage had no significant effect on β-1,3-glucanase activity.

β-1,3-glucanase belongs to PR-2 family and one of the most well-characterized pathogenesis-related enzyme in plant (Goñi *et al.*, 2010). It is a hydrolytic enzyme which hydrolyzes β-1,3-glucan, polymer constructing fungal cell wall(Cota *et al.*, 2007). This inhibits fungal progression in fruit tissue. It is commonly associated with fruit hypersensitive response (HR) which induced by pathogen

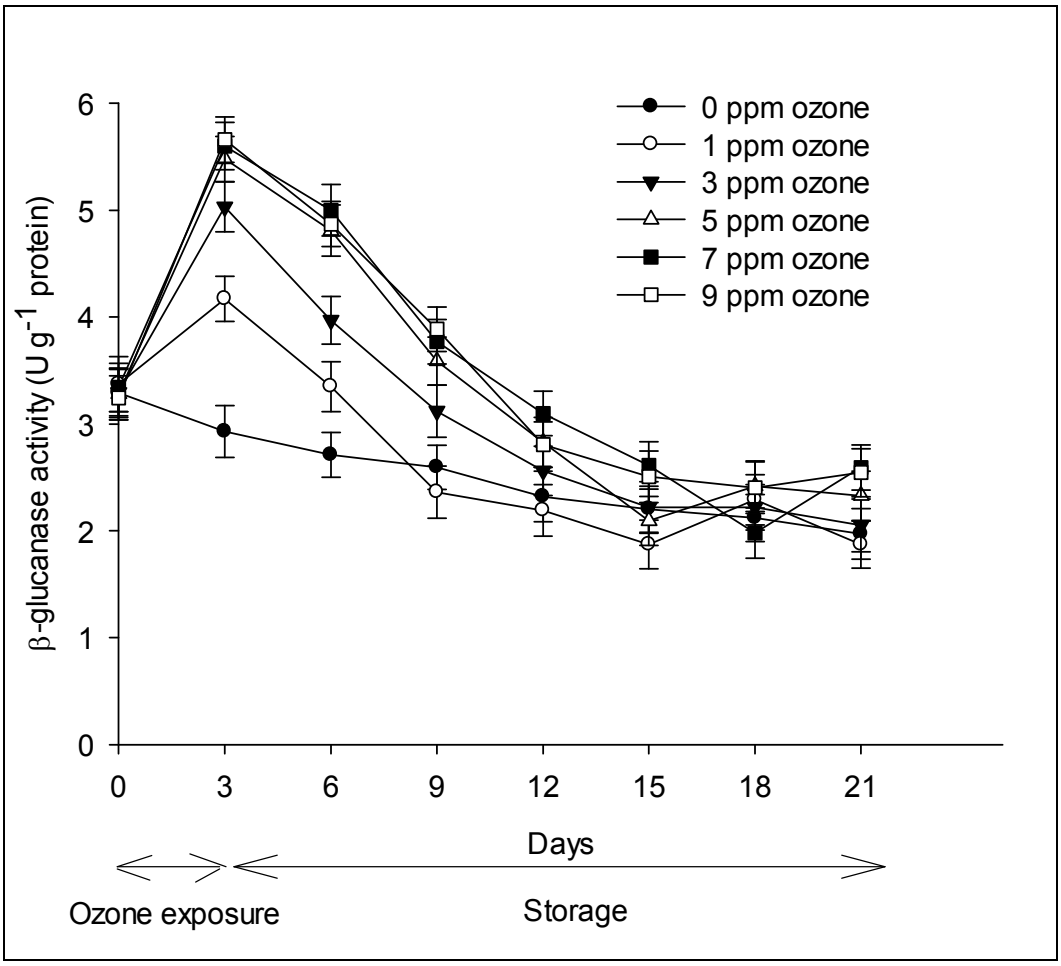

Figure 5.8: The effect of different ozone exposure on β-1,3-glucanase activity of bell pepper during three days treatment and 18 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

invasion in fruit tissue (Van Loon and Van Strien, 1999). It is also induced in response to abiotic stress such as chemical treatment and oxidative stress (Kesari *et al.*, 2010; Schraudner *et al.*, 1992).

The induction in β-1,3-glucanase activity observed in this study showed that oxidative stress of ozone acted as an elicitor to increase β-1,3-glucanase activity. The eliciting effect of ozone on β-1,3-glucanase activity was also observed in tobacco exposed to more than 0.1 ppm ozone for 5 h (Schraudner *et al.*, 1992). The

author also showed that the stimulating effect of ozone on β-1,3-glucanase activity was cultivar dependent where β-1,3-glucanase activity in resistant cultivar (Bel W3) was induced to a lesser extent than sensitive cultivar (Bel B) (Schraudner *et al.*, 1992). Besides, oxidative stress of UV-C irradiation also induced β-1,3-glucanase activity as reported in peach and pear treated with 7.6 (El Ghaouth *et al.*, 2003) and 5 kJ m^{-2} UV-C (Li *et al.*, 2010), respectively. This suggested that UV-C triggers similar defense mechanism as ozone where it involves upregulation of β-1,3-glucanase activity. The induction of β-1,3-glucanase could be the fruit hypersensitive response towards oxidative stress. This would increase fruit resistance against pathogen attack hence reduced disease development.

The transient pattern of eliciting effect of ozone on β-1,3-glucanase activity observed in this study was also reported in tobacco exposed to 0.15 ppm ozone for 5 h (Ernst *et al.*, 1992) and pear exposed to 5 kJ m^{-2} UV-C (Li *et al.*, 2010). This showed that fruit β-1,3-glucanase activity was only induced in response to stress. The persistency of β-1,3-glucanase activity can be improved by prolonging the treatment exposure time. This was reported in tobacco which had more persistent level of β-1,3-glucanase activity in tobacco exposed for 48 hours than 5 hours (Ernst *et al.*, 1992).

β-1,3-glucanase activity in bell pepper was negatively correlated ($r = -0.7245$, $P < 0.0001$) with disease incidence (Figure 5.9). Using coefficient of determination [$(-0.7245)^2 \times 100 = 52\%$] it was observed that β-1,3-glucanase activity contributed to 52% of the variation in disease incidence in Figure 5.1. This suggested that β-1,3-glucanase activity observed in the treated fruit is moderately associated with

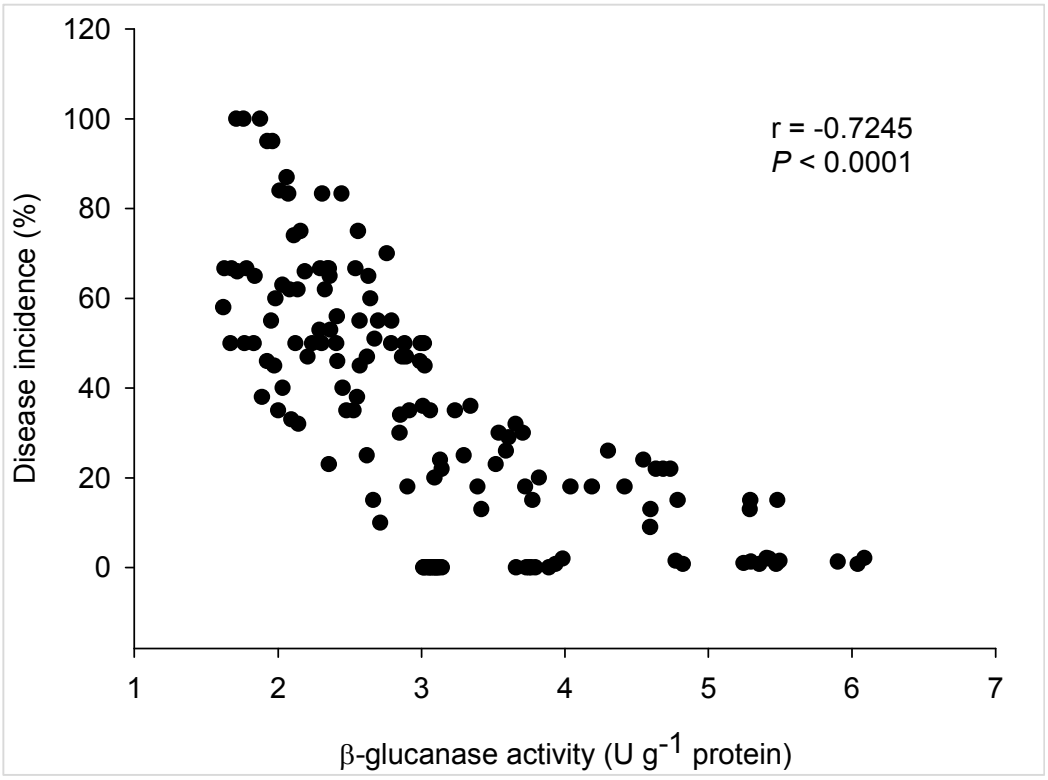

Figure 5.9: Relationship between β-1,3-glucanase activity and disease incidence (%) in bell pepper

reduction in variation in disease development of anthracnose. The high level of β-1,3-glucanase activity in fruit exposed to 3, 5, 7 and 9 ppm after exposure to ozone may help in reducing anthracnose development in Figure 5.1. Similar pattern was observed in barley grown at double ambient ozone where the treatment induced β-1,3-glucanase in barley by 66.7% but reduced fungal growth by 45.5% (Plessl *et al.*, 2005). Besides, treatment of UV-C (5 kJ m^{-2}) also reported to increase β-1,3-glucanase activity in pear which associated with decrease of fungal disease caused by *Monilinia fructicola* (Li *et al.*, 2010). The enzyme may reduce disease development by hydrolyzing β-1,3-glucan of fungal cell wall hence, lead to fungal cell death.

The stimulating effect of ozone on β-1,3-glucanase activity could be regulated at gene expression level. Ozone may affect gene expression similarly to UV-C irradiation where oxidative stress of UV-C has activated the corresponding genes and resulted in upregulation of β-1,3-glucanase mRNA. The upregulation of β-1,3-glucanase mRNA was observed in peach irradiated with 7.6 kJ m^{-2} UV-C (El Ghaouth *et al.*, 2003). This increased the fruit β-1,3-glucanase activity in respond to UV-C irradiation.

Oxidative stress of ozone may also affect activity of other enzyme such as superoxide dismutase (SOD), catalase (CAT), gluthathione peroxidase (GPX), and ascorbate peroxidase (APX). These enzymes are part of ROS avoidance gene which is fruit second line of defense against oxidative stress (Fung *et al.*, 2004). These enzymes react in respond to ozone as SOD dismutate superoxide anion, O_2^-, the reactive oxygen species induced by ozone decomposition, to H_2O_2 while CAT, GPX and APX detoxify H_2O_2 to water (Apel and Hirt, 2004). However, SOD, CAT and APX of bell pepper was not responsive to oxidative stress of chilling injury, methyl jasmonate and methyl salicylate. This was reported by (Fung *et al.*, 2004) where the oxidative stresses dramatically induced ROS avoidance gene (AOX) which involve in fruit first line of defense (PAL, PPO, POD and β-1,3-glucanase), but not ROS scavenging gene (SOD, CAT and APX). This indicated that bell pepper mainly used its first line of defense (ROS avoidance gene) to encounter oxidative stress enzymatically.

In conclusion, ozone treatment affected fruit PAL, PPO, POD and β-1,3-glucanase where the effect was dose dependent. Threshold limit was observed in the effect of ozone on PAL (3 ppm ozone), PPO (3 ppm ozone) and POD activities (5 ppm ozone) where increase in ozone dosage beyond their threshold reduced the

enzyme activities. Meanwhile, optimal β-1,3-glucanase activity was observed after exposure to 3 ppm ozone where further increase in ozone dosage had no significant effect on the enzyme activity. The results also showed β-1,3-glucanase had the highest correlation with anthracnose disease incidence in bell pepper, where it contributed to 52% of the variation in disease incidence. This is followed by PAL, POD and PPO.

CHAPTER 6

EFFECT OF OZONE FUMIGATION ON ANTIOXIDANT CONTENT AND CAPACITY

6.1 Introduction

Bell pepper is a highly nutritive fruit which is consumed for its high vitamin C content. It also contains other phytochemicals including carotenoid and phenolic compounds (Alvarez-Parrilla *et al.*, 2010). It has low sodium and calories contents hence, increases its efficiency in supplying the effective antioxidant to consumer (Wright, 2002). Therefore, bell pepper can be a major source of antioxidant in order to prevent diseases such as cancer, cardiovascular disease and neurological disorder.

Application of postharvest treatment such as UV-C (Promyou and Supapvanich, 2012), salicylic acid (Huang *et al.*, 2008) and ozone (Zhao *et al.*, 2013b) was reported to have stimulating effect on fruit antioxidant content. This approach can be used to increase antioxidant content in bell pepper hence, improve its potential antioxidant transfer to consumer. The effect of the postharvest treatment on fruit antioxidant varies depending on the response of fruit commodity towards the postharvest treatment applied. This is related to fruit defense mechanism in response to oxidative stress.

The effect of ozone on antioxidant activity of fruit greatly depends on ozone dosage. Previous studies reported that different commodity respond differently

towards ozone dosage where ozone can have positive or negative effects on fruit antioxidant content (Jin-Hua *et al.*, 2007; Keutgen and Pawelzik, 2008; Minas *et al.*, 2010). This greatly influenced fruit functionality which is an important criteria for bell pepper. This leads to the objectives of this study to:

1. evaluate the effect of different ozone concentration on antioxidant of bell pepper such as phenolic compound, ascorbic acid and β-carotene
2. investigate the effect of different ozone concentration on bell pepper antioxidant capacity during storage
3. determine major antioxidant of bell pepper which is correlated with its antioxidant capacity

6.2 Materials and Method

6.2.1 Fruit material

Fruit material was prepared as described in section 3.2.2.

6.2.2 Ozone exposure

Fruit was exposed to ozone as described in section 5.2.2

6.2.3 Determination of antioxidant

6.2.3.1 Total phenol content

Total phenol content was determined using Folin Ciocalteau (FC) spectrophotometric method, adopted from (P. K. Ramamoorthy and Bono, 2007). Briefly, 5 g of fruit tissues were homogenized in 50 ml of 80% methanol, centrifuged at 10,000 rpm for 15 min at 5ºC and filtered using Whatman filter paper (pore size 11 µm). 0.1 ml of filtrate was then mixed with 0.5 ml of Folin reagent, 1.5 ml of 7% Na_2CO_3 and 7.9 ml of distilled water. The reaction mixture was incubated at 37ºC for 2 h and absorbance at 765 nm was measured using UV-Vis Spectrophotometer. The absorbance obtained was measured against a blank at 765 nm. A standard curve was prepared using Gallic acid stock solution (Appendix B 6.1). Total phenolic content was expressed in mg of gallic acid equivalent g^{-1} of fresh weight of fruit sample.

6.2.3.2 Ascorbic acid concentration

Ascorbic acid concentration was determined according to the method of Ranggana (1986). 10 g of fruit tissue from equatorial region was homogenized with 90 ml of 3% HPO_3 + 8% glacial acetic acid, filtered using Whatman filter paper (pore size 11 µm) and 100 ml of filtrate were titrated with 2,6-dichlorophenol indophenol (DCPIP) dye (containing 2.49 mM $NaHCO_3$) until pink colour persisted for 15 sec. Standardization of DCPIP dye was determined prior to analysis by titrating 1 g l^{-1} ascorbic acid (dissolved in 3% HCl) against the prepared DCPIP dye to obtain the dye factor (Dye factor = 0.5 per titre value). Ascorbic acid content was calculated using the following equation and expressed in mg of ascorbic acid in 100 g of fruit sample.

$$AA\ (mg\ 100\ g^{-1}) = \frac{\text{Dye factor} \times T_s \times V_s \times 100}{W_s \times A_s}$$

T_s = Titre value of standard (ml)

W_s = Weight of sample (10 g)

V_s = Volume of sample (100 ml)

V_a= Volume of aliquot (100 ml)

6.2.3.3 β-carotene concentration

β-carotene concentration was determined according to (Georgé *et al.*, 2011). Briefly, 10 g of fruit tissue were homogenized in 50 ml acetone, filtered using Whatman filter paper (pore size 11 µm). The fruit tissues were re-extracted with 30

ml acetone and the filtrates were combined as an extract. The extract was mixed with 75 ml petroleum ether and washed three times with distilled water to remove water content. The remaining water was removed with anhydrous sodium sulphate and final volume of the extract was made up to 100 ml with petroleum ether. Absorbance of the extract was measured at 450 nm and 503 nm using UV-Vis Spectrophotometer. The absorbance obtained was measured against a blank at 450 and 503 nm. β-carotene concentration was determined using the following equation and result was expressed in μg ml^{-1}.

$$\beta\text{-carotene } (\mu g\ ml^{-1}) = 4.624 \times A_{450} - 3.091 \times A_{503}$$

6.2.3.4 Determination of antioxidant capacity using Ferric Reducing/Antioxidant Power Assay (FRAP Assay)

Antioxidant capacity using FRAP assay was estimated according to (Alothman *et al.*, 2010). Briefly, 20 μL of methanolic extract from total phenolic analysis was added with 200 μL of freshly prepared FRAP reagent (2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer, pH 3.6) and incubated for 4 min in dark. Absorbance of the reaction mixture was measured at 593 nm using UV-Vis Spectrophotometer. The absorbance obtained was measured against a blank at 593 nm. A standard curve was prepared using $Fe(II)SO_4$ stock solution (Appendix B 6.2). Antioxidant capacity was expressed as the concentration of antioxidant having a ferric reducing activity equivalent to 1 mg g^{-1} $FeSO_4$ of fresh weight of fruit sample.

6.2.4 Statistical analysis

The experiments were carried out with a Completely Randomized Design (CRD) and three replicates per treatment using three different ozone chambers, each containing 20 fruits. The experiment was repeated thrice and data was analyzed separately to check for homogeneity. The results were analyzed using Statistical Analysis Software (SAS, version 9.1.3, SAS Institute Inc., USA). Analysis of variance (ANOVA) was performed with $P < 0.05$ significance level and differences in data means were analyzed using DMRT.

6.3 Results and Discussion

6.3.1 Effect of ozone on fruit antioxidant capacity

Ozone treatment significantly affected (P = 0.0335) fruit antioxidant capacity to reduce ferric ions to ferrous ions (Figure 6.1, Appendix B 6.1). Immediately after ozone treatment, exposure to 3 ppm ozone enhanced fruit antioxidant capacity by 26%. The antioxidant capacity gradually decreased during storage but maintained at elevated level compared to control. Increase of ozone dosage to 5, 7 and 9 ppm ozone resulted in diminish antioxidant capacity compared to 3 ppm ozone.

Exposure to 1 and 3 ppm ozone acted as an elicitor which enhanced fruit antioxidant capacity and maintained the elevated level during storage. This stimulating action of oxidative stress was also observed in kiwi fruits treated with 0.3 ppm ozone for 3 days where the antioxidant activity was increased by 2.8% (Minas *et al.*, 2010). This is lower compared to results obtained in our study where exposure to 3 ppm ozone for 3 days induced 26.0% of bell pepper antioxidant capacity. This could be due to higher ozone concentration used and different response between kiwi and bell pepper towards ozone. The increase in antioxidant capacity could be due to oxidative stress of ozone and its decomposed products which triggered synthesis of antioxidants. This is a mechanism to reduce and balance oxidative stress and critical for cell survival (Lim *et al.*, 2009). Exposure to the stimulating doses of ozone, 1 and 3 ppm ozone, reduced antioxidant capacity loss during storage, producing fruit with 33.0 and 46.7% higher antioxidant capacity compared to control.

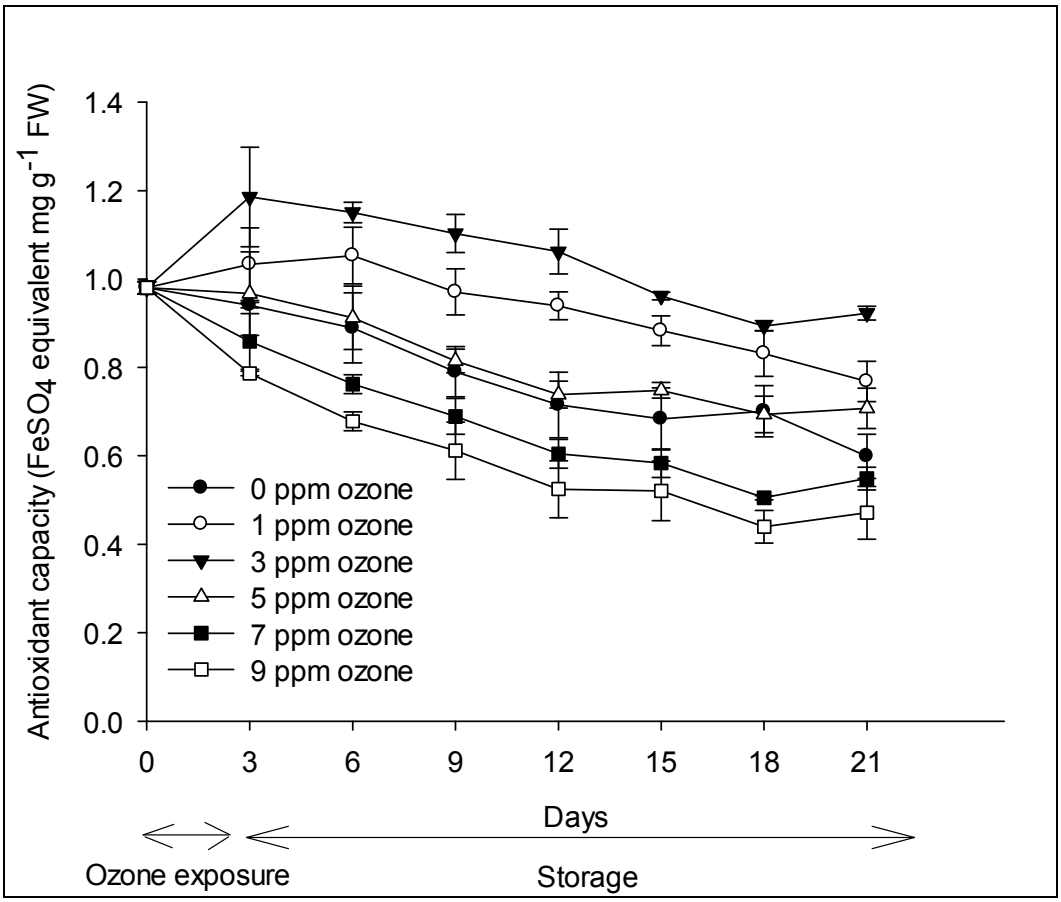

Figure 6.1: Effect of different ozone exposure on antioxidant activity of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$

Reduced level of fruit antioxidant capacity in fruit exposed to 7 and 9 ppm ozone indicated reduction in fruit antioxidant content. This could be a toxicity symptom of ozone on bell pepper. Similar symptom was observed in tomato where reduction in antioxidant capacity was observed when exposed to UV-C higher than 8 kJ m^{-2} (Liu *et al.*, 2012). This indicates that the threshold dose for antioxidant capacity in tomato is 8 kJ m^{-2} UV-C while the threshold dose for antioxidant capacity in bell pepper under study is 3 ppm ozone, The reduction in antioxidant capacity in fruit exposed to ozone higher than its threshold dose could be due to the strong oxidative stress caused by the high dosage of ozone and UV-C. This perturbed

antioxidant-oxidative stress equilibrium hence resulted in reduced level of antioxidant. The reduction in fruit antioxidant content and capacity may reduce fruit defense against oxidative stress during ripening hence, accelerating senescence.

5.3.1 Effect of ozone on fruit antioxidant content

5.3.1.1 Total phenolic content

Total phenol analysis showed ozone treatment significantly affected (P < 0.0001, Appendix 6.2) fruit phenolic content (Figure 6.2). Similarly to the effect of ozone on antioxidant capacity, 3 ppm ozone had a stimulating effect on phenolic content of bell pepper where it was increased by 15.6% immediately after treatment. The phenolic content gradually decreased during storage but maintained at higher level compared to control. Increase in ozone concentration reduced fruit phenolic content and exposure to 9 ppm ozone significantly reduced fruit phenolic content by 11.6%. This resulted in fruit with low phenolic content at the end of storage period.

Fruit phenolic compounds are fruit secondary metabolites including antioxidants, structural polymers and compounds involved in non-specific defense mechanisms (Cuadra-Crespo and del Amor, 2010). This study showed that exposure to 3 ppm ozone stimulated the production of phenolic compounds in bell pepper. This finding was supported by Minas *et al*. (2010) where phenolic content in kiwi fruit increased by 5.1% after exposure to 0.3 ppm ozone for 3 days. This increase is lower compared to bell pepper exposed to 3 ppm ozone for 3 days in this study. This was possibly due to lower ozone concentration applied and different response of different commodity towards ozone exposure. Ozone also induced phenolic compounds in tomato where exposure to 10 ppm for 6 days was reported to increased phenolic content by 45.5% (Rodoni *et al.*, 2010). The increment is higher

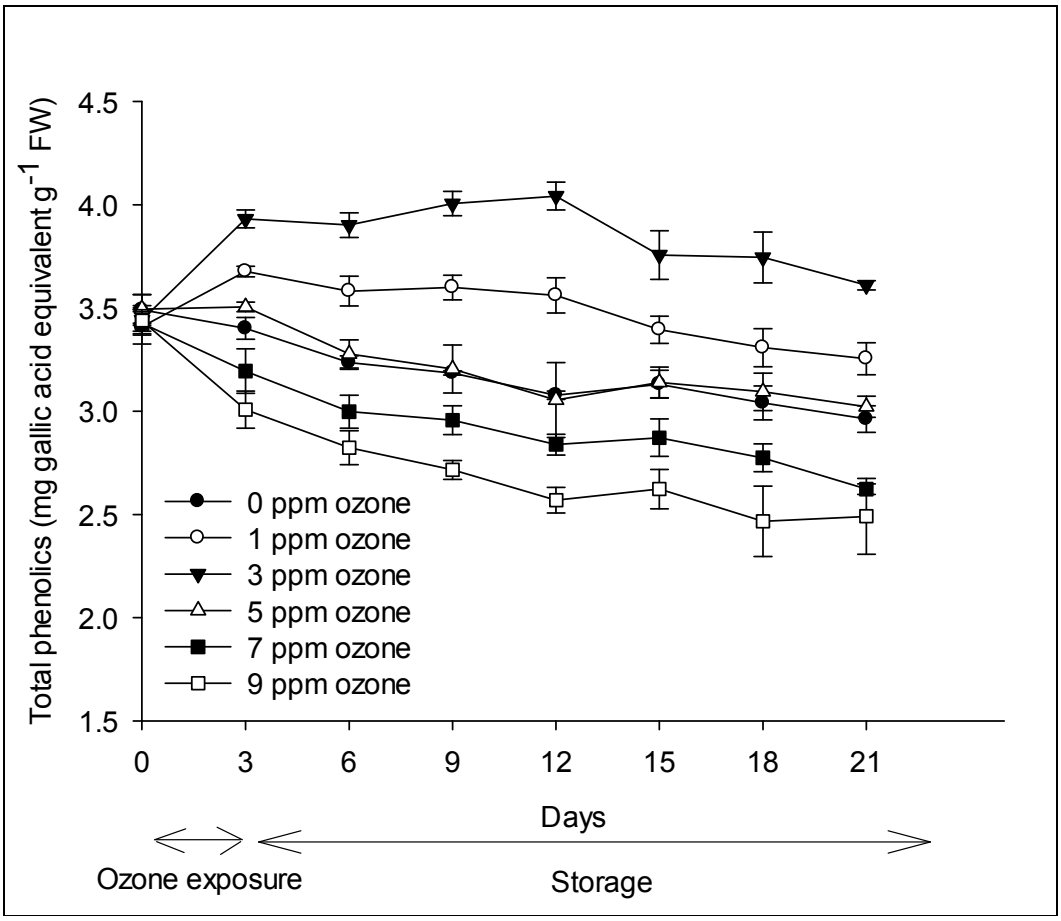

Figure 6.2: Effect of different ozone exposure on total phenol content of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

compared to our results. This could be due to higher ozone concentration and longer exposure time applied. The stimulating effect of ozone on fruit phenolic content could be due to the increase in phenolic acids and flavonoids contents which is triggered by high oxidative state of fruit apoplast caused by ozone (Tan *et al.*, 2012). The phenolic compounds may scavenge reactive compounds of ozone to protect the cells from oxidative chain reaction (Tan *et al.*, 2012). This was due to their structure with high tendency to donate a single electron to free radicals (Huang *et al.*, 2008;

Tan *et al.*, 2012). The upregulation of phenolic antioxidant maintained equilibrium between production and scavenging activity of reactive oxygen species in fruit cell. This is crucial to prevent oxidative damage such as lipid autoxidation and subsequent cell death.

Reduction in fruit total phenol content after exposure to 9 ppm ozone indicated that ROS equilibrium in the cells was perturbed by the strong ozone dosage. Fruit phenolic antioxidants may have been negated by ozone-reactive compounds hence, resulted in its depletion. This could be a toxicity symptom of ozone on bell pepper. Similar toxicity symptom was also observed on fresh cut guava where reaction with 0.77 ppm ozone reduced 46.0% of its total phenolic content (Alothman *et al.*, 2010). The higher reduction in the total phenolic content reported could be due to fresh cut fruit was used in that experiment compared to whole fruit in our study. The fresh cut fruit may be more susceptible to ozone oxidation hence, resulted in higher reduction in total phenolic content. This weakened fruit defensive response against oxidative stress during ripening hence, further reduced fruit total phenol during storage (Tan *et al.*, 2012). This resulted in significant reduction ($P < 0.05$) in fruit phenol content at the end of storage period.

The effect of ozone on fruit total phenol content was similar to its effect on antioxidant capacity. Phenolic content in bell pepper was positively correlated (r – 0.8291, $P < 0.001$) with antioxidant capacity (Figure 6.3). Using coefficient of determination value [$(-0.8291)^2 \times 100 = 69\%$], it was observed that PAL activity contributed to 69% of the variation in antioxidant capacity in Figure 6.1. This

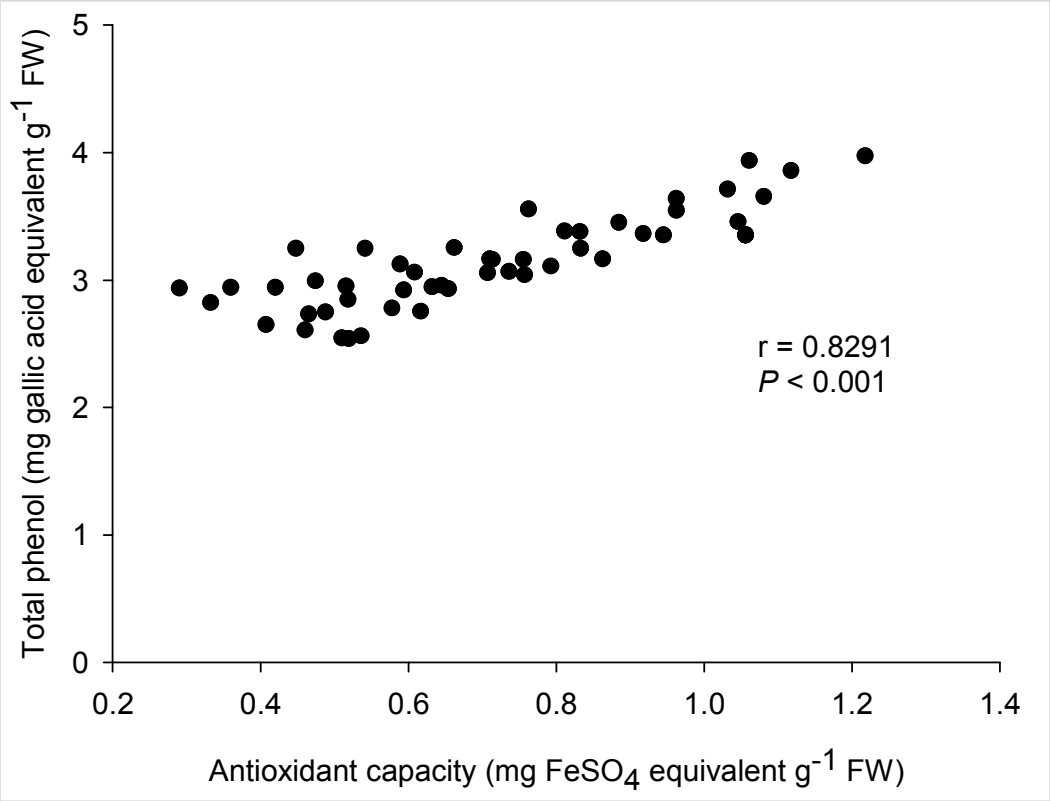

Figure 6.3: The correlation between antioxidant capacity of bell pepper measured by FRAP assay and its total phenol content

suggested that the increase in antioxidant capacity could be due to the increase in phenolic content in the fruit. This also suggested that phenolic compound is one of the major antioxidant in bell pepper under study.

5.3.1.2 Ascorbic acid content

Ascorbic acid content in bell pepper was significantly affected ($P < 0.0177$, Appendix B 6.4) by ozone treatment. Immediately after treatment, exposure to 3 ppm ozone increased fruit ascorbic acid content by 15.9% (Figure 6.4). The ascorbic acid

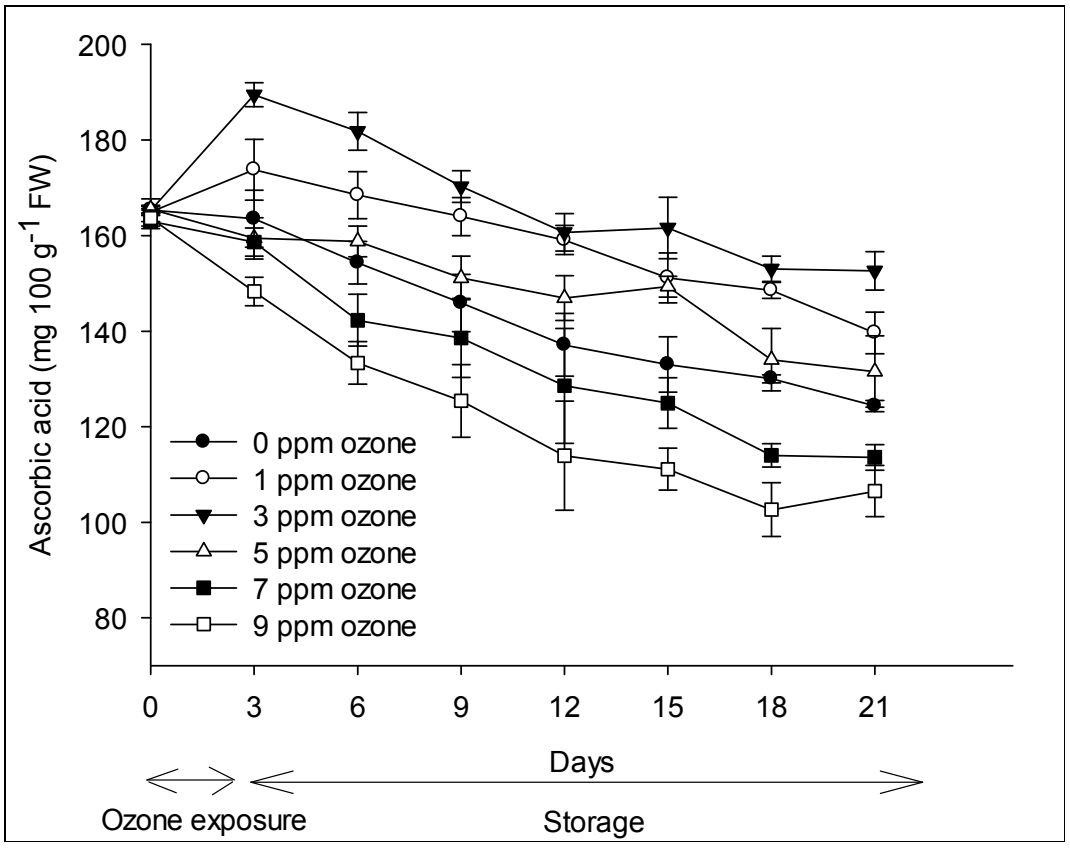

Figure 6.4: Effect of different ozone exposure on ascorbic acid content of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of six replicates; DMR test at $P < 0$

content gradually decreased during storage. The stimulating effect on fruit ascorbic acid content observed in this study was also reported in pepper treated with 1 mM H_2O_2 (Bayoumi, 2008), UV-C at 254 nm for 10 min (Sakaldaş and Kaynaş, 2010) and 1.0% chitosan (Xing *et al.*, 2011) where the ascorbic acid content was increased by approximately 100.0, 51.9 and 54.5%, respectively. This showed that ozone may have reacted similar to the other oxidizing agents (H_2O_2, UV-C and chitosan) where it induced defense response of pepper by inducing ascorbic acid content. Ozone may cause oxidative burst and trigger nonenzymatic ROS scavenging mechanism.

This may induce the level of ascorbic acid by upregulating ascorbate-gluthathione cycle enzymes such as monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) (Apel and Hirt, 2004; Forney, 2003). This increased the level of reduced ascorbate to neutralize ozone and its induced reactive compounds which therefore, maintained the cell oxidative status. This also increased fruit ascorbic acid content and its phytochemical value where in our case, fruit exposed to 1 and 3 ppm had high ascorbic acid content by 12.3% and 22.7%, respectively.

Exposure to 9 ppm ozone displayed toxicity effect where reduction in ascorbic acid content was observed on day 6. Further reduction was observed during subsequent storage. The toxicity effect of ozone was also reported on fresh cut pineapple and guava after exposure to 0.76 ppm ozone which reduced 46 and 67% of their ascorbic acid content, respectively (Alothman *et al.*, 2010). High oxidative stress of the ozone dosage perturbed the equilibrium of ascorbate-gluthathione cycle where oxidized form of ascorbate exceeded the reduced form (Apel and Hirt, 2004). This reduced ascorbic acid content and resulted in its depletion.

The low level of ascorbic acid in fruit may act as a pro-oxidant which produced H_2O_2 in a reaction with oxygen (Fry, 1998). The H_2O_2 then produced hydroxyl radical (OH•) from its reaction with traces of metal compounds such as Fe^{2+} and Cu^{2+} through Haber-Weiss reaction (Apel and Hirt, 2004; Gaetke and Chow, 2003; Sakihama *et al.*, 2002). This pro-oxidant property was also observed in fruit phenolic compounds which undergo similar reaction in contact with metal (Sakihama *et al.*, 2002). Hydroxyl radical is a highly reactive radical and its accumulation increased

fruit oxidative stress. This leads to subsequent oxidative chain reaction such as lipid and DNA degradation (Apel and Hirt, 2004; Sakihama *et al.*, 2002).

Ascorbic acid obtained in this study was positively correlated (r = 0.972, *P* = < 0.0001) with antioxidant capacity (Figure 6.5). Using coefficient of determination value [$(0.972)^2$ x 100 = 94%], it was observed that ascorbic acid activity contributed to 94% of the variation in disease incidence in Figure 6.1. This suggested that the increase in antioxidant capacity could be strongly due to the increase in ascorbic acid content. This suggested ascorbic acid is also the major antioxidant in bell pepper.

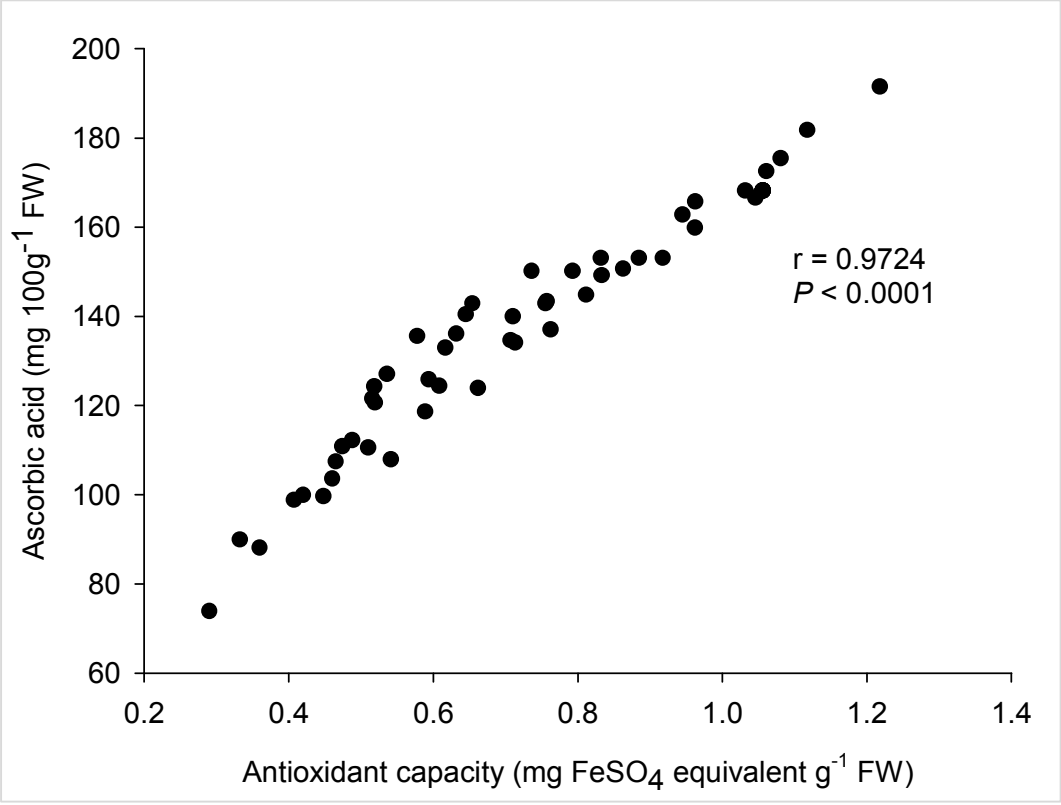

Figure 6.5: The correlation between antioxidant capacity of bell pepper measured by FRAP assay and its ascorbic acid content

Our results (Figure 6.3 and Figure 6.5) showed that fruit phenolic compounds and ascorbic acid are the major antioxidant in bell pepper and may worked co-operatively to detoxify harmful effect of ozone and its induced reactive compounds. Phenolic compounds may protect ascorbic acid from oxidative decomposition (Forney, 2003; Huang *et al.*, 2008; Sakihama *et al.*, 2002). Meanwhile, ascorbic acid may restore phenolic compounds oxidized by free radicals by reducing oxidized form of phenolic compounds such as phenoxyl radical of tocopherol (Forney, 2003). This restored availability of phenolic compounds in cells. Due to the dependent characteristic of phenolic compound and ascorbic acid on one another, similar responses to ozone were observed for both compounds .

5.3.1.3 β-carotene content

β-carotene content analysis showed ozone concentration and exposure time had significant ($P < 0.0001$, Appendix B 6.5) effect on fruit β-carotene content while no significant effect was observed from their interaction (Figure 6.6). Exposure to 1 ppm ozone increased fruit β-carotene content while exposure to 5, 7 and 9 ppm ozone reduced β-carotene content. No significant effect was observed from exposure to 3 ppm ozone.

β-carotene is orange and red pigments which are localized in chloroplast and chromoplast. It is an antioxidant with scavenging property to neutralize free radicals in order to balance cell oxidative status (Wolbang *et al.*, 2010). It is also a precursor in biosynthesis of vitamin A (Wolbang *et al.*, 2010). Our results (Figure 6.6) showed

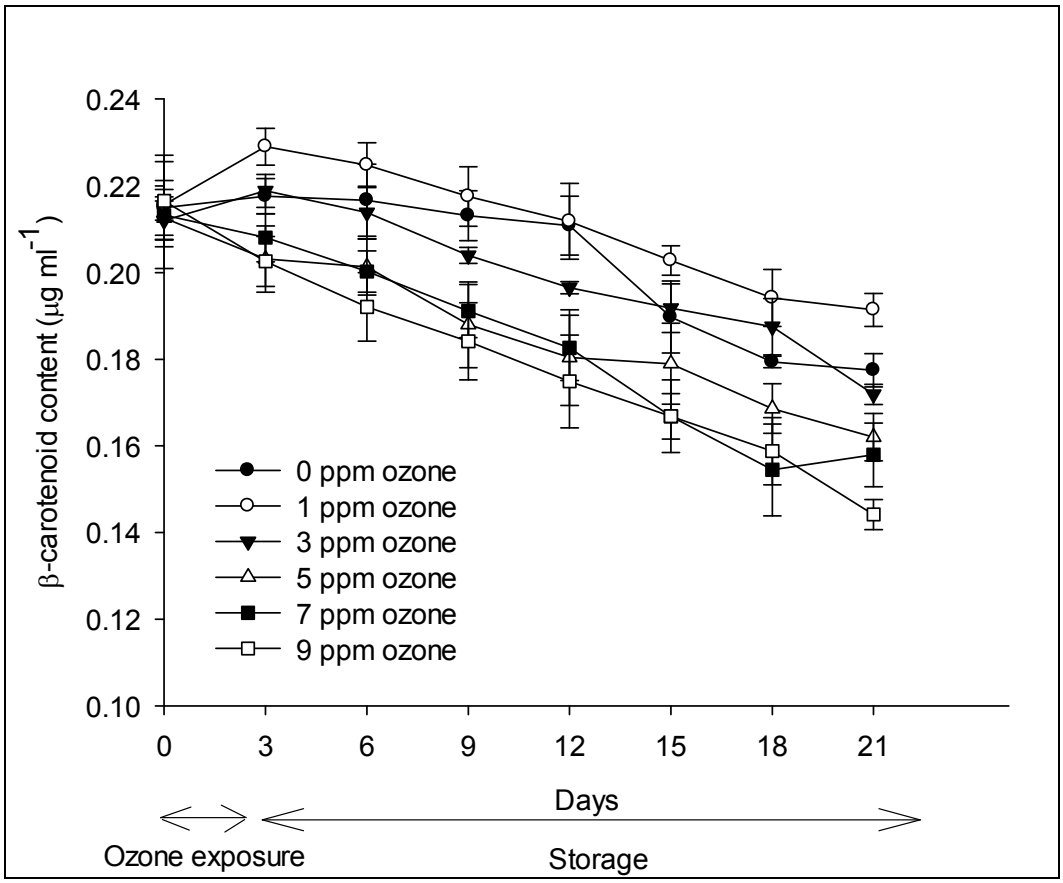

Figure 6.6: Effect of different ozone exposure on β-carotene content of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

that ozone induced β-carotene content in bell pepper treated with 1 ppm ozone. The stimulating effect on β-carotene content was also reported in bell pepper exposed to 6.6 kJ m^{-2} UV-C where the β-carotene content was increased by 57.1% (Promyou and Supapvanich, 2012). This showed that ozone and UV-C triggered similar response on bell pepper where β-carotene was induced. As an antioxidant, increase in β-carotene content is a result of ROS scavenging mechanism in fruit second line of defense. The antioxidant scavenged oxidative burst caused by oxidative agent such as ozone hence, protecting the cells from oxidative damage. This also

increased fruit β-carotene content hence added the phytochemical value to the fruit. This improves potential phytochemical transfer to consumer hence reduces the risk of oxidative related disease. Besides, it also improved vision and immune system as a result from the benefit of vitamin A.

Reduction in fruit β-carotene content in fruit exposed to 5, 7 and 9 ppm ozone could be a phytotoxic effect of ozone. Similar phytotoxic effect was also observed in bell pepper treated with 7 kJ m^{-2} UV-C where the fruit β-carotene content was reduced by 17.1% (Vicente *et al.*, 2005). The author only determined the effect of UV-C at one concentration (7 kJ m^{-2}) and no comparison was made to lower UV-C dosage. However, in comparison to a study by Promyou and Supapvanich (2012), exposure to lower UV-C dosage, 6.6 kJ m^{-2} increased β-carotene in bell pepper. This could indicate that UV-C and ozone affect β-carotene of bell pepper in similar pattern where it increased when exposed up to a threshold dose and decreased when exposed to higher dose. In comparison to another commodity, ozone was also reported to reduce lutein, another naturally occurring carotenoid, content in tomato (Tzortzakis *et al.*, 2007). The author reported that exposure to 0.05 ppm ozone for 1 day increased tomatoes lutein content approximately by 45%. Increase in ozone dosage either by increase of ozone concentration to 1.0 ppm or extending the exposure to 6 days however, reduced the fruit lutein content by approximately 45 and 66%, respectively (Tzortzakis *et al.*, 2007). Similarly to our results, this suggested that the carotenoid content was induced at low ozone dosage but reduced as ozone dosage exceeded its threshold.

β-carotene content obtained in this study was negatively correlated ($r = -0.4704$, $P = 0.0003$) with antioxidant capacity (Figure 6.7). This suggested that the

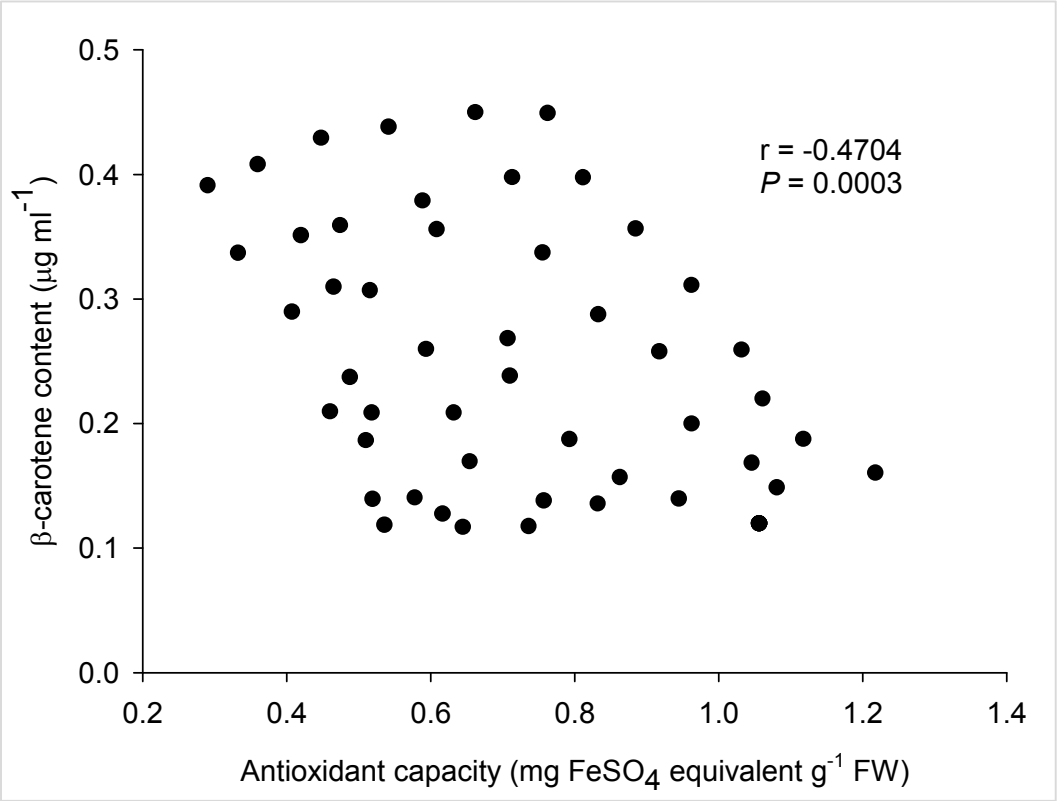

Figure 6.7: The correlation between antioxidant capacity of bell pepper measured by FRAP assay and its β-carotene content

increase in variation in fruit antioxidant capacity was not due to increase in β-carotene content. Therefore, β-carotene may not be the major antioxidant in bell pepper under study.

This study showed phytotoxic effect of ozone on β-carotene was observed at lower dosage (5 ppm ozone) than total phenolic (7 ppm ozone) and ascorbic acid content (9 ppm ozone). This suggested that β- carotene was more susceptible to ozone oxidation than ascorbic acid and phenolic compound. The high susceptibility of β-carotene was due to its molecular structure which has high affinity towards hydroxyl radical (OH•), the most reactive radical compound (Henry *et al.*, 2000).

Oxidation of β-carotene by hydroxyl radical produced β-carotene radical which then degraded to unsaturated and conjugated aldehydes and dialdehydes (Henry *et al.*, 2000).

In conclusion, this study showed that bell pepper triggered its defense system by utilizing ROS avoidance and ROS scavenging mechanism to encounter oxidative stress of ozone. This resulted in the increase in fruit antioxidants such as phenolic compounds, ascorbic acid and β-carotene. This subsequently enhanced fruit antioxidant capacity which benefits the consumer. Besides, we also found that the stimulating dosages of ozone on fruit antioxidants are 1 and 3 ppm for total phenolic content and 1 ppm ozone for β-carotene. The antioxidants then underwent natural reduction during storage. This therefore, suggesting that bell pepper is best consumed immediately after ozone treatment with the stimulating dosage in order to obtain optimal antioxidant content. In the case of fruit total phenol and ascorbic acid contents, the stimulating effect of ozone compensated their natural reduction during storage hence, producing fruit with higher antioxidant content during storage compared to untreated fruit. This increased the fruit functionality and its efficacy of antioxidant transfer to consumer.

Referring to the effect of ozone on fruit inducible enzymes which are PAL, PPO, POD and β-glucanase (Chapter 5), this showed bell pepper utilized its ROS avoidance and non-enzymatic ROS scavenging mechanisms to encounter oxidative stress of ozone. These mechanisms work cooperatively in response to oxidative stress of ozone. The eliciting effect of ozone on fruit defense system increased its resistance to biotic and abiotic stress during storage.

CHAPTER 7

EFFECT OF OZONE FUMIGATION ON PHYSICO-CHEMICAL, PHYSIOLOGY AND SENSORY QUALITIES

7.1 Introduction

Physical quality, biochemical quality and physiology of fruit are an important aspect which determines the overall quality of a fruit hence, influencing consumer selection process. Apart from diseases and blemishes, the main problem affecting bell pepper is the loss of texture and firmness. This is one of the main criteria influences consumer selection where a firm and crispy texture are preferred than soft texture (Wright, 2002). Colour of bell pepper also influences consumer selection where solid colour fruit is preferred than partially colour fruit (Fox *et al.*, 2005).

These physical qualities are correlated with fruit biochemical status, physiology and ripening progress. Parameters such as respiration rate, soluble solid concentration and titratable acidity reflect fruit physiology and biochemical quality. Postharvest treatments which induced abiotic stress in fruit has the potential to influence the parameters hence may reduce fruit ripening progress and improve fruit quality. For example, chlorine dioxide (ClO_2) (Jin-Hua *et al.*, 2007), hydrogen peroxide (H_2O_2) (Bayoumi, 2008) and UV-C (Vicente *et al.*, 2005) were reported to improve quality of peppers.

By manipulating the strong oxidizing property of ozone, gaseous ozone treatment has the potential to influence fruit ripening progress and subsequently its physical quality. Therefore, this chapter evaluates the effect of ozone on fruit physico-chemical quality. The objective includes to;

1. determine the effect of the ozone dosage on bell pepper chemical quality such as titratable acidity, total soluble solid, chlorophyll content and cell oxidative level
2. investigate the effect of the ozone dosage on bell pepper respiration and ethylene production
3. evaluate the effect of the ozone dosage on consumer perception on quality of bell pepper in terms of appearance, colour, aroma, flavour and overall acceptability

7.2 Materials and Method

7.2.1 Fruit material

Fruit material was prepared as described in section 3.2.2.

7.2.2 Ozone exposure

Fruit was exposed to ozone as described in section 5.2.2

7.2.3 Determination of physical quality

7.2.3.1 Fruit colour

Fruit colour at two opposite sides of the fruit equator was measured using a colourimeter (Model: Miniscan XE Plus, HunterLab, USA), which was calibrated with standard black and white tiles. Values for L^*, a^* and b^* were recorded every three days and values for chroma (C^*) and hue angle ($h°$) were calculated using the following equations (Xing *et al.*, 2011):

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$h° = \tan^{-1} b^* / a^*$$

7.2.3.2 Weight loss

Fruit weight loss was determined by weighing the fruit on a digital balance (Model GF-6100, A&D Co. Ltd., Japan) before the ozone treatment and every three days until the end of the storage period. Same fruit was used throughout the experiment. Percentage of weight was then calculated.

$$\text{Weight loss } (\%) = \frac{(W_i - W_f)}{W_i} \times 100$$

W_i = Initial fruit weight

W_f = Final fruit weight

7.2.3.3 Fruit firmness

Fruit firmness was determined using an Instron texture analyzer (Instron 2519-104, MA), equipped with an eight mm plunger tip. Fruit pieces, 2.5 x 2.5 cm, were cut from the equatorial region and firmness was determined by applying force with a constant speed of 20 mm min^{-1}. Two fruit pieces were sampled per fruit. Sampling was done every three days and maximum force required to penetrate through the fruit was recorded and results were expressed in Newton (N).

7.2.3.4 Membrane permeability

Membrane permeability was determined according to the method of (Xing *et al.*, 2011). Briefly, ten fruit discs, including epidermis (≈ 0.059 cm^3) were sampled from equatorial region using 0.5 cm cork borer, washed with distilled water, dried on

filter paper, placed in a flask with 30 ml distilled water, shaken at 25°C on an orbital shaker (Model S1500, Bibby Scientific Limited, UK) for 30 min and boiled for 10 min. Electrical conductivity of the solution was measured using a conductivity meter (Model: Eutech Cond 6+, Thermo Fisher Scientific Inc., USA) before and after boiling and percentage leakage was calculated. Sampling was done every three days.

$$\text{Relative leakage rate (\%)} = \frac{EL_i - EL_f}{EL_f} \times 100$$

EL_i = Initial electrolyte leakage

EL_f = Final electrolyte leakage

7.2.4 Determination of chemical quality

7.2.4.1 Soluble solids concentration

Soluble solid concentration (SSC) was determined using a Palette digital refractometer (Model: PR-32α, Atago Co, Ltd. Japan). 10 g of fruit tissue from equatorial region were homogenized in 40 ml distilled water, centrifuged at 5500 rpm for 5 min and SSC of the supernatant was determined using the refractometer calibrated with distilled water. Sampling was done every three days. The reading was recorded and multiplied by the dilution factor.

7.2.4.2 Titratable acidity

Titratable acidity (TA) was determined based on malic acid content according to the method of Fox et al. (2005). Two drops of phenolphthalein were added to 5 mL

of supernatant from the SSC analysis and titrated with 0.1 % NaOH until the solution turned pink. The malic acid content was determined by measuring volume of NaOH used and multiplying it by the dilution factor and molecular weight of malic acid (134.09 g mol^{-1}). Sampling was done every three days.

$$\text{Titratable acidity (\%)} = \frac{V_{NaOH} \times V_f \times MW_{malic\ acid}}{W_s \times V_u \times 1000} \times 100$$

V_{NaOH} = Volume of 0.1% NaOH used (ml)

V_f = Volume of fruit juice made in SSC analysis (50 ml)

V_u = Volume of sample used in TA (5 ml)

$MW_{malic\ acid}$ = Molecular weight of malic acid (134.09 g mol^{-1})

W_s = Weight of sample used in SSC analysis (10 g)

7.2.4.3 Chlorophyll content

Chlorophyll concentration was determined according to the method of (Xing *et al.*, 2011). 1 g of fruit tissue from equatorial region was homogenized with 10 ml 80% acetone, centrifuged at 5500 rpm for 5 min and absorbance of the supernatant was measured at 646.6, 663.6 and 750 nm using a UV-Vis spectrophotometer. Absorbance at 646.6 and 663.6 nm were corrected by subtracting with absorbance at 750 nm. Sampling was done every three days and chlorophyll content was calculated using the following equation. Results are expressed as µg g^{-1} fresh weight of fruit sample.

$$\text{Total chlorophyll (µg g}^{-1}) = (17.76 \times A_{646.6}) + (7.34 \times A_{663.6})$$

7.2.4.4 Malondialdehyde (MDA) content

MDA content which represents TBARS content was determined according to (Xing *et al.*, 2011). Briefly, 5 g of fruit tissues were homogenized in 50 ml of 10% trichloroacetic acid (TCA) and centrifuged at 5579×*g* for 30 min at 4°C. 1 ml of the supernatant was mixed with 3 ml of 0.5% thiobarbituric acid (TBA) (dissolved in 10% TCA), incubated at 95°C for 30 min and quickly cooled in ice bath. Absorbance of the reaction mixture was measured at 532 nm and subtracted from absorbance at 600 nm using UV-Vis Spectrophotometer. Sampling was done every three days and MDA content was calculated using the following equation. The results were expressed in µmol MDA equivalents g^{-1} of fresh weight of fruit sample.

$$\text{MDA content } (\mu\text{mol g}^{-1}) = \frac{(A_{532} - A_{600}) \times V_e \times V_f \times 1000}{V_i \times W_s \times 155}$$

Ve = volume of extract (50 ml)

Vf = volume of reaction mixture (4 ml)

Vi = volume of extract in reaction mixture (1 ml)

Ws = weight of sample used (5 g)

7.2.5 Determination of respiration and C_2H_4 production

7.2.5.1 Respiration

Fruit respiration or CO_2 production was determined according to (Forney *et al.*, 2007). Briefly, two fruit were sealed in 500 ml container and incubated for one hour at room temperature (25-26º). 1 ml of head space gas was then sampled and

injected into a gas chromatograph (Model: Clarus 500, Perkin Elmer Inc, USA) equipped with a stainless steel column, 30 m x 0.530 mm (Porapak R 80/100) and a thermal conductivity detector (TCD). Helium was used as a carrier gas at a flow rate of 20 ml min^{-1} and temperature of oven, injector and TCD was set at 60°C, 100°C and 200°C respectively. 1 ml of CO_2 gas standard (Scotty gases, Beltifonte, USA) was used for calibration. Fruit respiration was measured every three days and the CO_2 production was expressed in ml kg^{-1} h^{-1}.

7.2.5.2 C_2H_4 production

C_2H_4 production by fruit was determined according to the method of Forney et al. (2007) with modifications. Briefly, two fruit were sealed in a 500 ml container and incubated for one hour at room temperature (25-26º), after which 1 ml of head space gas was sampled and injected into a gas chromatograph (Model: Clarus 500, Perkin Elmer Inc, USA) equipped with a stainless steel column, 30 m x 0.530 mm (Porapak R 80/100) and a flame ionization detector (FID). Nitrogen was used as a carrier gas and temperature of the oven, injector and FID was set at 150°C, 200°C and 200°C respectively. One ml of C_2H_4 gas standard (Scotty Gases, USA) was used for calibration. Ethylene production was measured every three days and expressed as µl kg^{-1} h^{-1}.

7.2.6 Sensory evaluation

Sensory evaluation of bell pepper was performed at day 19 of storage using a Hedonic scale rating (Whangchai *et al.*, 2006). Fruit tissues (5 cm x 5 cm) from

equatorial region were sampled and coded in random numbers. 20 panelists were asked to evaluate the criteria by allotting values; 1-extreme dislike, 3-dislike, 5-acceptable, 7-good and 9-excellent. The samples were rated based on appearance, colour, texture, aroma, flavour and overall acceptability.

7.2.7 Statistical analysis

The experiments were carried out with a Completely Randomized Design (CRD) and three replicates per treatment using three different ozone chambers, each containing 20 fruits. The experiment was repeated thrice and data was analysed separately to check for homogeneity. The results were analyzed using Statistical Analysis Software (SAS, version 9.1.3, SAS Institute Inc., USA). Analysis of variance (ANOVA) was performed with $P < 0.05$ significance level and differences in data means were analyzed using DMRT.

7.3 Results and Discussion

7.3.1 Bell pepper physical quality

7.3.1.1 Colour

Ozone concentration and storage time significantly affected (P < 0.0001, Appendix B 7.1) colour lightness (L^*) of bell pepper (Figure 7.1). Significant change in colour lightness was observed from day 12 where the epidermis colour changed to lighter colour until the end of storage period. Exposure to 1 and 3 ppm ozone retained fruit colour lightness similar to control where no significant changes were observed. Increase in ozone concentration to 5, 7 and 9 ppm enhanced colour lightness producing fruit with lighter colour.

Colour vividness or chroma (C^*) was significantly affected (P = 0.0022, Appendix B 7.2) by interaction of ozone concentration and storage time (Figure 7.2). At the end of storage period, fruit exposed to 1 and 3 ppm ozone had comparable colour vividness compared to control. Meanwhile, exposure to 5, 7 and 9 ppm ozone enhanced fruit colour vividness producing fruit with more vivid colour.

Perceived colour or hue angle ($h°$) was significantly affected (P < 0.0001, Appendix B 7.3) by ozone concentration and storage time (Figure 7.3). Significant changes in colour hue angle were observed from day 6 where reduction in colour hue angle was observed until the end of storage period. Exposure to 7 and 9 ppm ozone enhanced progression of colour which significantly reduced colour hue angle producing fruit with yellowish hue. Exposure to 1, 3 and 5 ppm ozone retained colour hue angle similar to control.

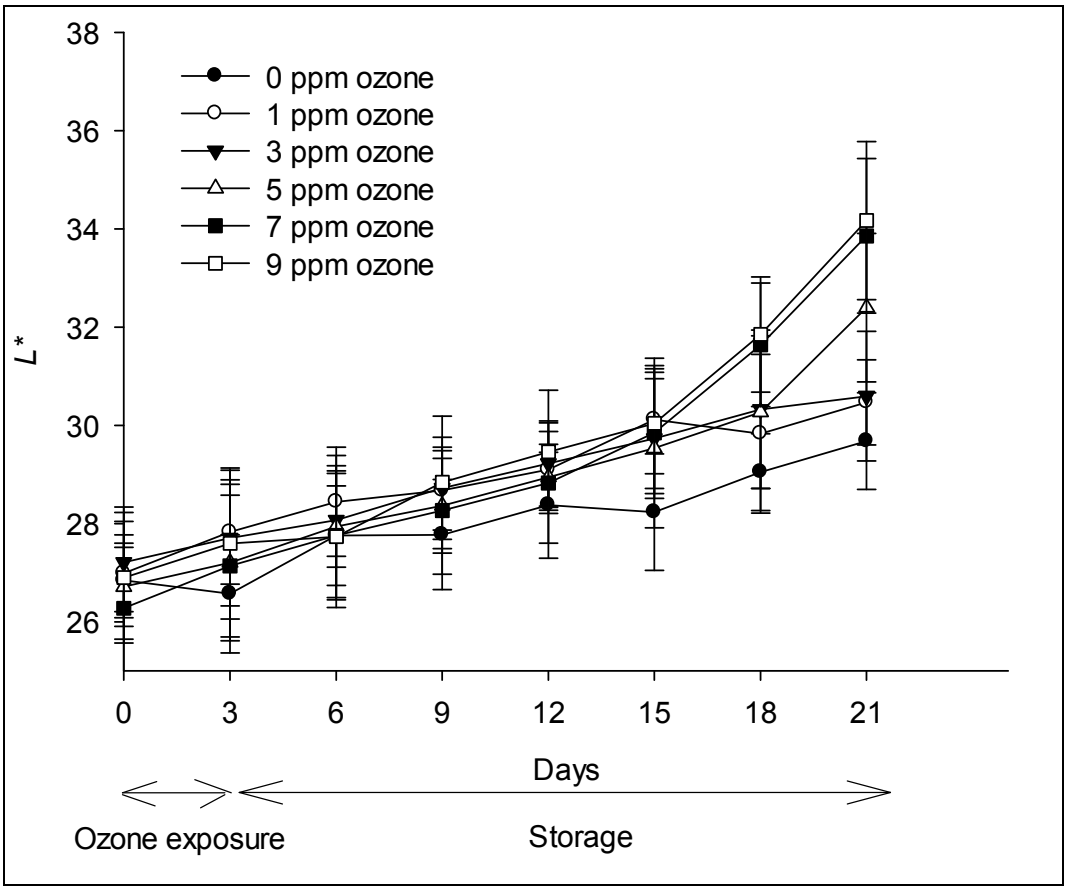

Figure 7.1: Effect of different ozone exposure on *L** value of fruit colour during three days treatment and 18 days storage at 12°C, 95% RH. Vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

Fruit epidermis colour is measured using a colorimeter to quantify colour using CIELAB system by measuring three colour coordinates; *L** (lightness), *a** (red/green) and *b** (yellow/blue) (Pathare *et al.*, 2013). These colour coordinates was expressed in CIELCH system to quantify chroma (*C**), vividness of a colour and hue angle (*h*°), the perceived colour which is ranging from 0°/360° (red), 90° (yellow), 180° (green) to 270° (blue) (Pathare *et al.*, 2013). These attributes are easily

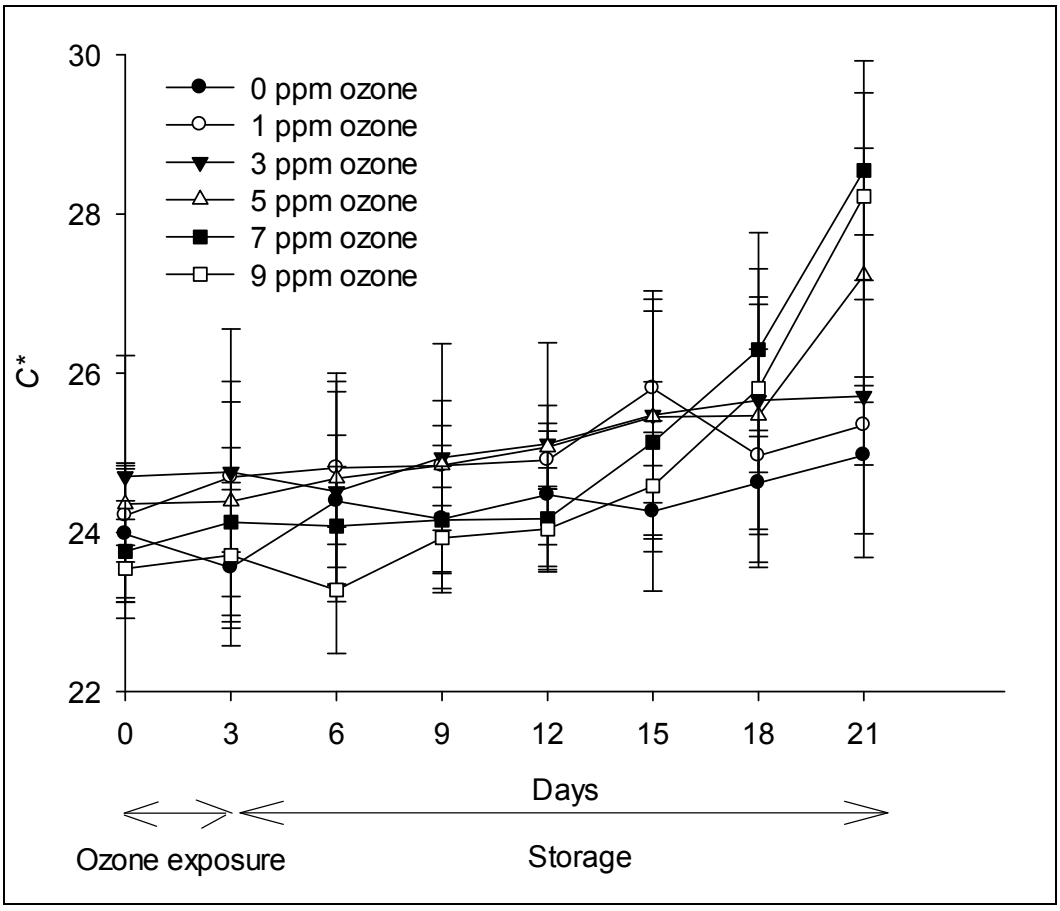

Figure 7.2: Effect of different ozone exposure on *C** value of fruit colour during three days treatment and 18 days storage at 12°C, 95% RH. Vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

correlated with human perception towards colour. Bell pepper (cv. Zamboni) used in this study is a red variety bell pepper. The fruit was harvested at mature green stage which turned to yellow and then red during ripening. Figure 7.1 – 7.3 show *L**, *C** and h^{o} values of fruit exposed to 1 and 3 ppm ozone was similar to control. This is similar to the findings reported by Glowacz *et al*. (2015) where

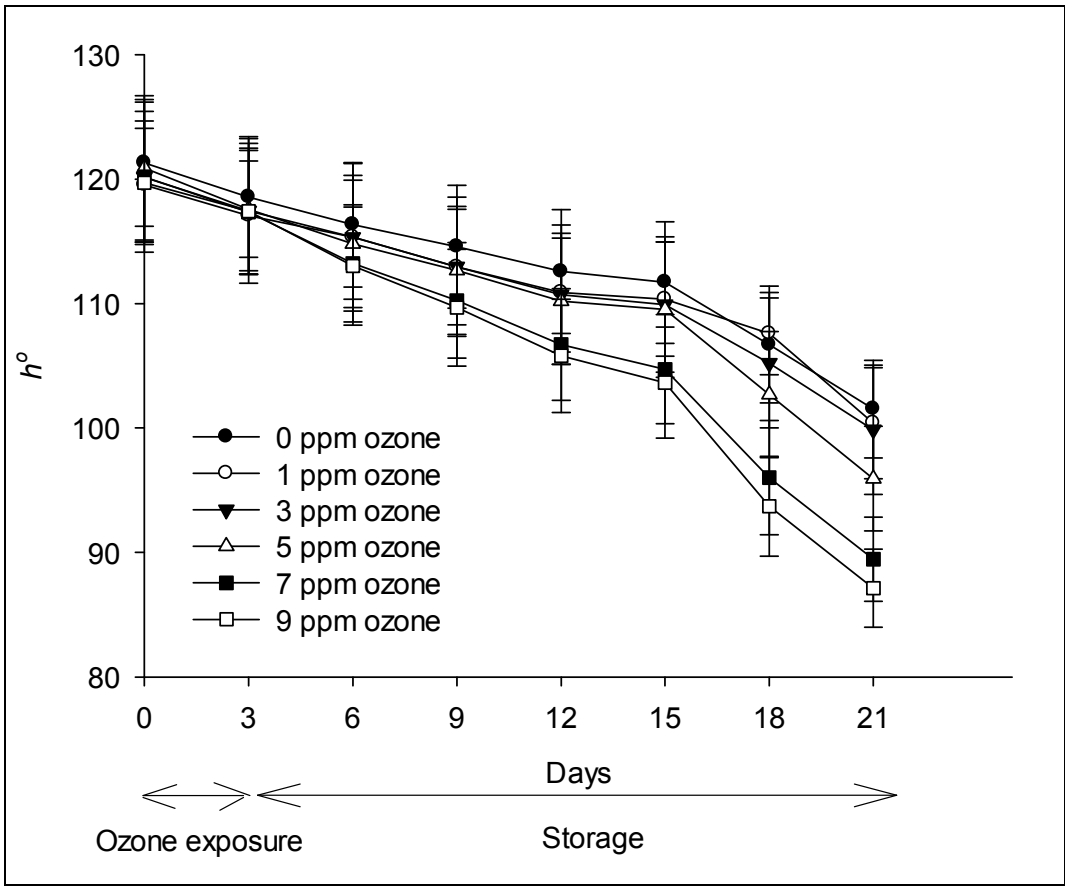

Figure 7.3: Effect of different ozone exposure on *h*° value of fruit colour during three days treatment and 18 days storage at 12°C, 95% RH. Vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

exposure to 0.1 and 0.3 ppm for 7 and 14 days had no effect on *L**, *C** and *h*º values of the fruit. This suggested the treatments had no effect on fruit colour development, pigmentation or ripening progress. The treatments maintained fruit ripening progression similar to control during storage.

Significant progression of colour (*L**, *C** and *h*º values) was observed in fruit exposed to high ozone dosage, 7 and 9 ppm ozone. It resulted in fruit with yellow and reddish hue (62.16 – 63.79º). The increase in colour progression was also observed in bell pepper treated with 1 ppm aqueous ozone when the exposure time was increased from 1 min to 3 and 5 min (Horvitz and Cantalejo, 2012). This is an

indication of stimulated progression of ripening. Exposure to higher ozone dosage (either by increase in ozone concentration or exposure time) may produce stronger oxidative stress hence, increase fruit respiration and lipid peroxidation (Hodges, 2003). This increased ripening and colour development as observed.

Enhanced progression of colour in fruit treated with 7 and 9 ppm ozone could be a phytotoxic effect of ozone on bell pepper. This symptom differs from phytotoxic symptoms observed on papaya (Ong *et al.*, 2012) and carrot (Hildebrand *et al.*, 2008) which resulted in epidermis browning after exposure to 4 ppm ozone for 6 days and 0.05 ppm ozone for 2 months, respectively. The epidermis browning could be resulted from necrosis of cells near to lenticels which are the main entry of ozone into the cells (Forney, 2003). In contrast to bell pepper with less lenticels (Torlak *et al.*, 2013), ozone has no selective entry into the cells hence, oxidation activity occurred evenly on the epidermis and resulted in the enhanced colour progression. Besides, epidermis of bell pepper is protected by surface cuticle layer (Maalekuu *et al.*, 2006), which prevented the cells from strong oxidation activity and subsequent cell necrosis. This reduced susceptibility of bell pepper to ozone.

Minimal changes observed in the perceived colour (h^{o}) of fruit treated with 1, 3 and 5 ppm ozone retained most of their green colour (Figure 7.4). These fruit are more preferred by consumer than partially coloured fruit (7 and 9 ppm ozone treated fruit) (Fox *et al.*, 2005). This concluded that exposure to 1, 3 and 5 ppm ozone retained consumer perception or buying preference toward bell pepper while exposure to higher ozone concentration reduced their buying preference.

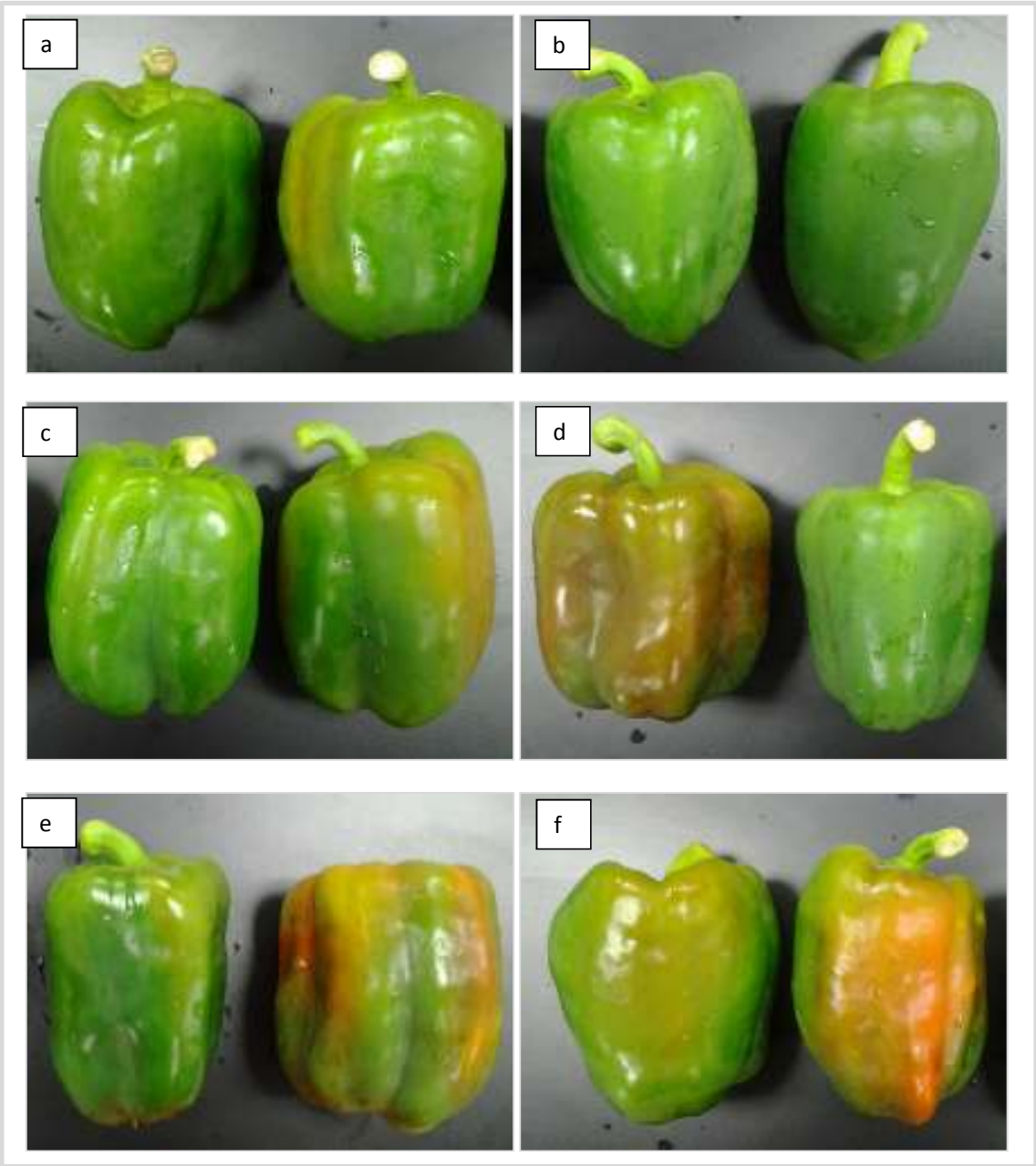

Figure 7.4: Epidermis colour of bell pepper after exposure to (a) 0 ppm (control) (b) 1 ppm (c) 3 ppm (d) 5 ppm (e) 7 ppm and (9) ppm ozone. The pictures were taken on day 21, in storage at 12°C, 95% RH.

7.3.1.2 Weight loss

Ozone treatment significantly affected ($P < 0.0001$, Appendix 7.4) fruit weight loss (Figure 7.5). Exposure to 5, 7 and 9 ppm ozone increased fruit weight loss where significant weight loss was observed from day 18 (5 ppm ozone) and day 3 (7 and 9 ppm ozone). At the end of storage period, the treatments increased fruit weight loss by 18.5, 36.6 and 55.3%, respectively. Meanwhile, exposure to lower ozone concentration, 1 and 3 ppm ozone, had no effect on fruit weight loss where the fruit weight was similar to control until the end of storage period.

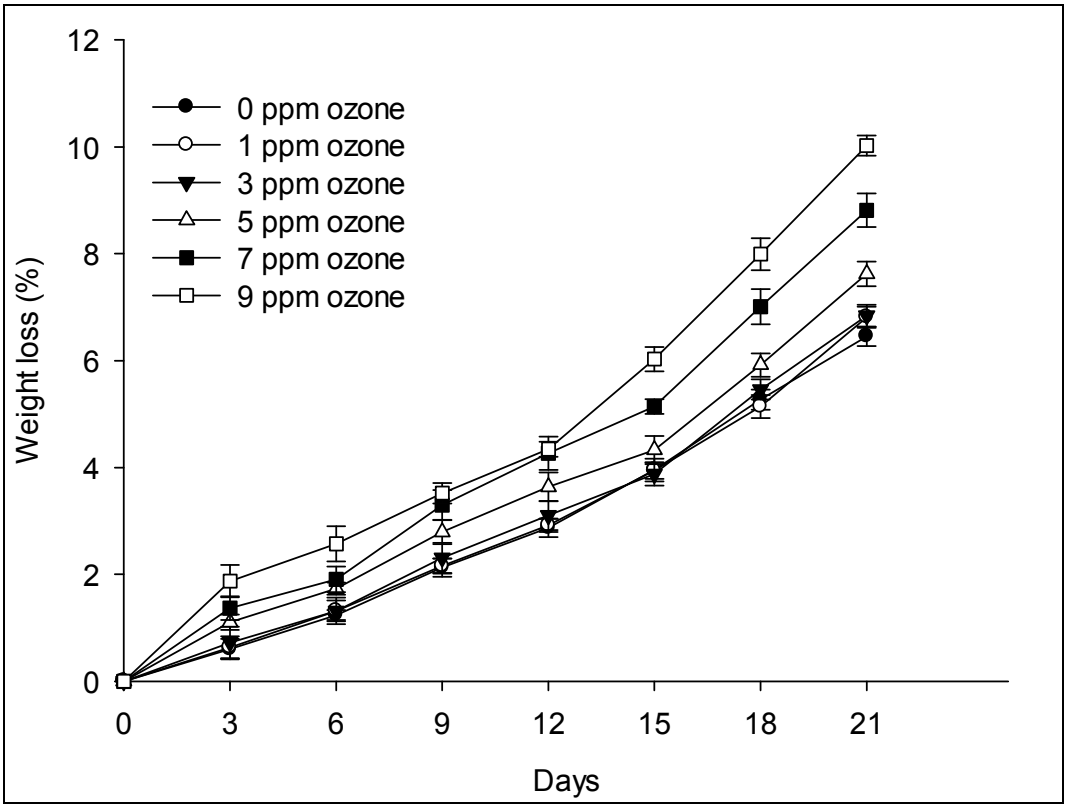

Figure 7.5: Effect of different ozone exposure on weight loss of bell pepper during three days treatment and 24 days of storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

Weight loss during storage is crucial for fruit physical quality, particularly bell pepper. Weight loss occurred due to water loss in transpiration through stomata and lenticels on fruit epidermis (Díaz-Pérez *et al.*, 2007). The rate of transpiration is influenced by environmental factors such as temperature and relative humidity (Díaz-Pérez *et al.*, 2007). Weight loss also occurred due to fruit respiration, a process of converting organic reserves to energy (Hong *et al.*, 2012). Respiration occurs in all living cells to sustain energy requirement and affected by factors such as temperature, pathogen attack and oxidative stress.

Bell pepper is highly susceptible to water loss due to its hollow structure which limits its capacity to store water (Maalekuu *et al.*, 2005). Besides, its large surface area relative to weight increased water loss in transpiration (Díaz-Pérez *et al.*, 2007). This is in contrast to fruit with low surface area to weight ratio such as guava. Transpiration in fruit can be reduced by storing at low temperature, where in the case of bell pepper is at 7 to 12ºC (Tan *et al.*, 2012). Storage at temperature below 7ºC induces chilling injury which leads to rapid weight loss and subsequent shriveling and quality degradation (Xing *et al.*, 2011).

Exposure to 1 and 3 ppm ozone maintained fruit weight loss comparable to control during storage. These findings are similar to bell pepper exposed lower ozone concentration but longer exposure time, 0.1 and 0.3 ppm for 14 days (Glowacz *et al.*, 2015). This showed that prolong exposure time to 14 days have similar effect on fruit weight loss. The treatments maintained fruit transpiration similar to control. This could be due to the presence of cuticle layer on bell pepper surface which protect the epidermis from oxidation activity of ozone (Maalekuu *et al.*, 2005).

The fruit reached maximum acceptable weight loss (7%) on day 21, where prolonged storage exceeded the threshold and not suitable for market.

Increased weight loss in fruit exposed to 5, 7 and 9 ppm ozone reduced its storage life. The treated fruit reached their maximum acceptable weight loss three days earlier than control. This could be due to oxidation of surface cuticle layer by the strong ozone doses (Forney, 2003; Skog and Chu, 2001). Degradation of the protective layer exposed fruit cell to ozone oxidation. This may lead to lipid peroxidation which reduces cell membrane integrity and cell water retention hence, increases transpiration and weight loss. Ozone and its induced free radicals also increased respiration by upregulating electron transport chain at mitochondria which subsequently increased the loss of water (Tiwari *et al.*, 2002). This resulted in wrinkle and surface pitting which were observed at the end of storage period.

Effects of ozone on weight loss were also observed in peach. This was reported by Palou *et al*. (2002) where exposure to 0.3 ppm ozone for 5 weeks increased 22.2% of weight loss. In comparison to bell pepper, peach does not have cuticle layer to protect the cell epidermis from the action of ozone hence, more susceptible to water loss. In contrast to bell pepper which has hollow structure, peach however has solid structure hence has higher capacity to retain its water content. These factors may influence their response to ozone in terms of weight loss.

7.3.1.3 Fruit firmness

Ozone treatment significantly affected ($P < 0.0001$, Appendix B 7.5) fruit firmness (Figure 7.6). Exposure to 1 and 3 ppm ozone had no significant effect on fruit firmness. The fruit firmness was maintained similar to control until the end of

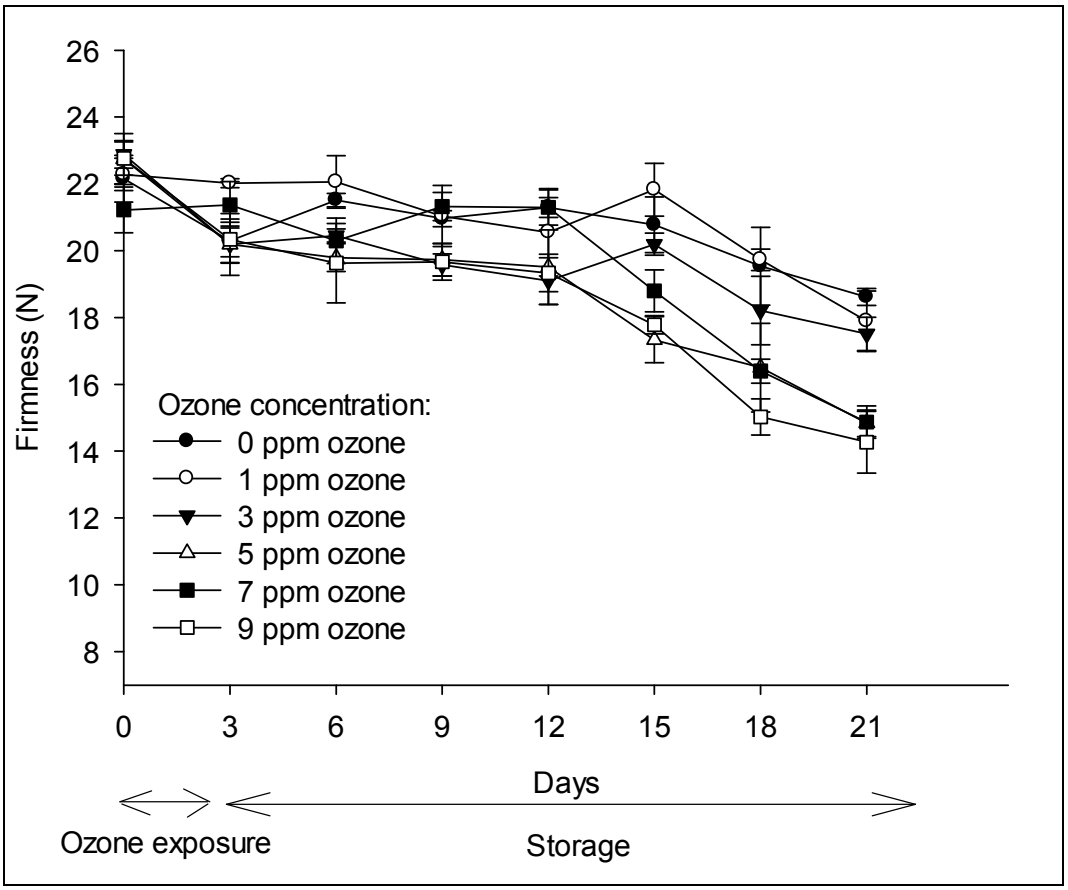

Figure 7.6: Effect of different ozone exposure on firmness of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

storage period. Exposure to 5, 7 and 9 ppm ozone reduced fruit firmness. Significant reduction in fruit firmness was observed from day 18 (5 and 7 ppm ozone) and day 15 (9 ppm ozone) until the end of storage period.

Fruit firmness was quantified using a texture analyser which measured force required to penetrate the fruit per surface area. It is related to fruit texture hence, influence fruit storage life and consumer acceptance. Firmness of fruit naturally decreased during storage. This was due to the loss of water content in cells during transpiration and respiration hence, cell turgidity and firmness.

Treatment of 1 and 3 ppm ozone had no effect on fruit firmness. Similar response was also observed in bell pepper exposed to 0.1 and 0.3 ppm for 14 days (Glowacz *et al.*, 2015). This indicated that prolong ozone exposure to 14 days with lower ozone concentration, 0.1 and 0.3 ppm, produces similar oxidation stress on bell pepper where it had no effect on fruit firmness. Similar findings was reported in carrot, where ozone dosage of 2.2 ppm ozone for 15 min had no effect on the fruit firmness (Barbosa-Martinez *et al.*, 2002). This suggested that oxidative stress of the treatments had no effect on cell membrane integrity. This maintained fruit capacity to retain water hence, maintained fruit weight loss comparable to control (Figure 7.5).

Exposure to higher ozone concentration, 5, 7 and 9 ppm ozone reduced fruit firmness. Decrease in fruit firmness as exposed to higher ozone dosage was also observed in bell pepper exposed to 1 ppm aqueous ozone when the exposure time was increased from 1 min to 3 min (Horvitz and Cantalejo, 2012). The higher oxidative stress from high ozone dosage reduced fruit firmness. This could be due to the increase in loss of water as discussed in section 7.3.1.2. Besides, reduction in fruit firmness could be due to oxidizing effect of ozone and its decomposition products (hydrogen peroxide, single oxygen, hydroxyl and other free radicals) on unsaturated fatty acids of cell membrane and sulfhydryl groups of membrane proteins (Forney, 2003). This lipid peroxidation led to formation of lipid hydroperoxides (LOOH) which lead to subsequent chain reaction of lipid degradation (Forney, 2003). This further reduced cell membrane integrity and cell water retention which hence, reduced its firmness.

7.3.1.4 Membrane permeability

Ozone concentration and storage time significantly affected ($P < 0.0001$, Appendix B 7.6) fruit membrane permeability (Figure 7.7). Fruit membrane permeability increased during storage, producing fruit with high electrolyte leakage. The effect of ozone concentration on membrane permeability was dose dependent. Exposure to low ozone concentration, 1 and 3 ppm ozone had no effect to membrane permeability while exposure to high ozone concentration, 5, 7 and 9 ppm ozone increased membrane permeability.

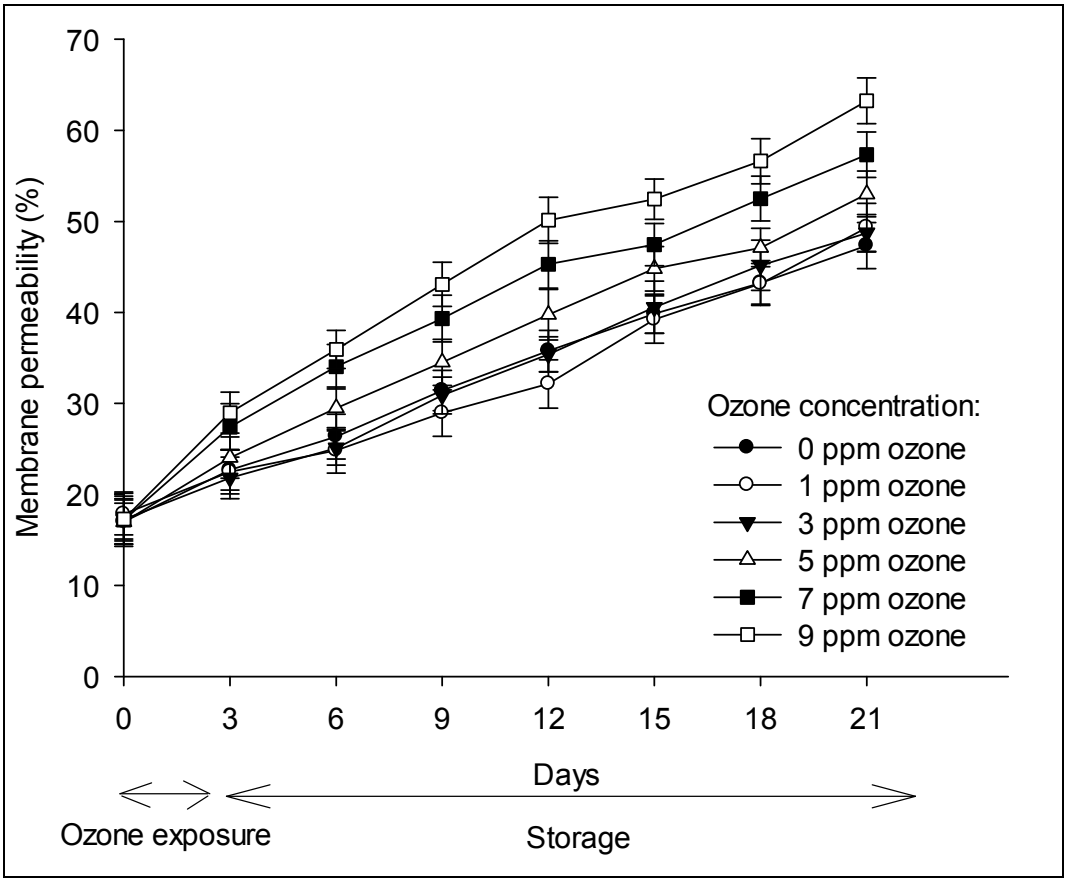

Figure 7.7: Effect of different ozone exposure on membrane permeability of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

Fruit membrane permeability quantified relative ion content in apoplastic space of fruit cell (Xing *et al.*, 2011). It indirectly measured cell leakage which indicates cell membrane integrity or damage as a result of lipid peroxidation (Xing *et al.*, 2011). Fruit membrane permeability gradually increased during storage. This was an indication of ripening which due to increase of cell oxidative stress (Hodges, 2003). Increase of membrane permeability during ripening was also observed in pepper (Xing *et al.*, 2011) and melon (Hodges, 2003).

Membrane permeability of fruit exposed to 1 and 3 ppm ozone was not affected by the treatments. This suggested the oxidative stress of the treatment did not affect cell membrane lipid integrity hence, indicating a balanced oxidative status in the cell. This could be due to enhanced production of fruit antioxidants such as ascorbic acid and phenolic compounds as discussed in Chapter 6. The antioxidants neutralized oxidative activity of ozone and its induced free radical, reduced cell oxidative level and subsequent lipid peroxidation. This maintained the fruit membrane integrity and permeability similar to control. Therefore, the cell maintained its capability to retain water and preserved its firmness similar to control (Figure 7.6).

Increase in membrane permeability in fruit exposed to 5, 7 and 9 ppm ozone indicated the high level of cell oxidative stress as a result of the treatments. This is in agreement with findings reported by Plazek *et al*. (2000) where increase in ozone dosage by prolonging exposure time from 6 days 12 days increased membrane permeability of barley leaves exposed to 180 ppm ozone. The stronger ozone dosage may has encountered physical barrier of cuticle layer of fruit or leaves and

diffused into cell apoplast (Forney, 2003; Skog and Chu, 2001). The high reactivity of ozone and its induced free radicals disrupted antioxidant-oxidative stress balance, resulted in depletion of antioxidants such as ascorbic acid and phenolic compounds (Chapter 6) and excess of reactive free radicals. This led to decomposition of unsaturated fatty acids and degradation of membrane integrity. Subsequently, it increased cell membrane permeability and resulted in loss in cellular compartmentation (Skog and Chu, 2001). This will affect other physical qualities such as firmness and weight loss.

7.3.2 Bell pepper chemical quality

7.3.2.1 Soluble solids concentration (SSC)

Ozone concentration and storage time significantly affected ($P < 0.0023$, Appendix B 7.7) fruit soluble solid concentration (SSC) (Figure 7.8). Fruit SSC increased during storage where significant increase was observed from day 6 until the end of storage period. In the effect of ozone concentration, exposure to 1, 3 and 5 ppm ozone had no effect on fruit SSC. Increase in ozone concentration to 7 and 9 ppm significantly enhanced accumulation of soluble solid producing fruit with higher SSC.

Soluble solid concentration are concentration of total sugar, soluble mineral and organic acid in fruit (Barboni *et al.*, 2010). SSC of bell pepper gradually increased during storage The increase in fruit SSC was a result of biosynthesis of polysaccharides and sugar (Antoniali *et al.*, 2007). Being a non-climacteric fruit, no climacteric peak was observed in SSC of bell pepper.

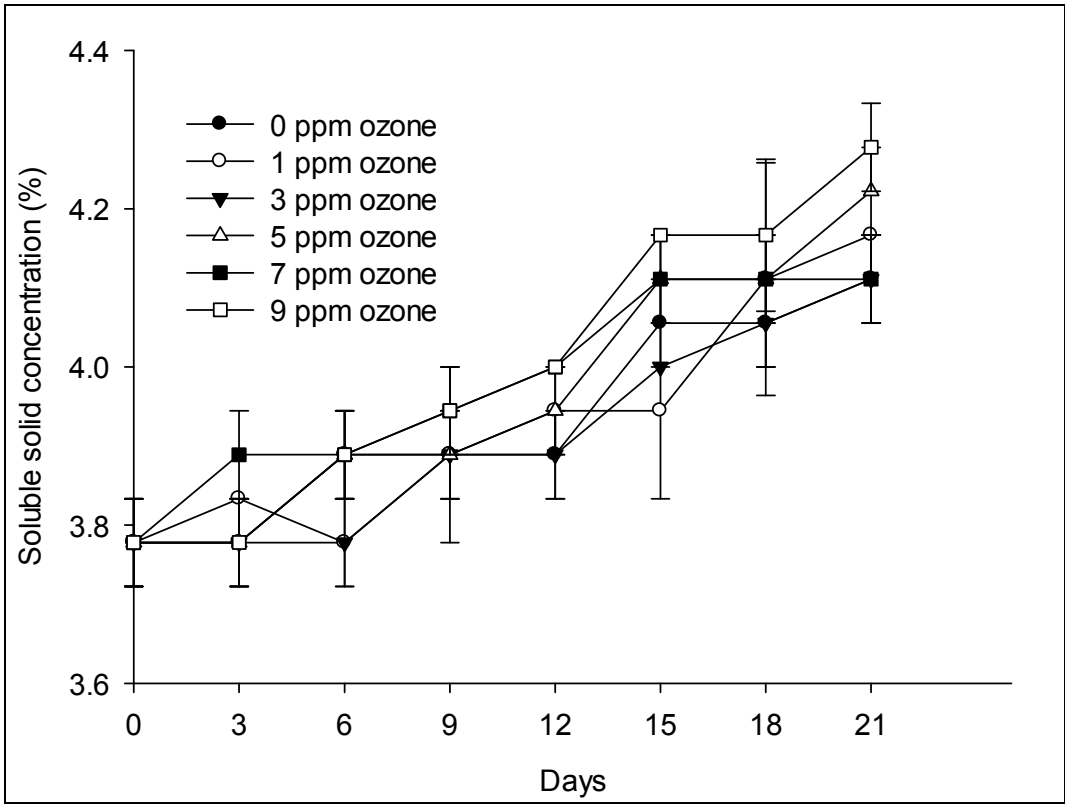

Figure 7.8: Effect of different ozone exposure on soluble solid concentration of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

No significant changes in SSC of fruit exposed to 1, 3 and 5 ppm indicated that oxidative stress of the ozone treatment had no effect on biosynthesis of sugar during storage. Similar findings was also observed from application of 0.3 ppm ozone for 14 days on bell pepper (Glowacz *et al.*, 2015) and 2.2 ppm ozone for 45 min on mango (Barbosa-Martinez *et al.*, 2002). Application of other oxidizing agent was also reported to have no effect on SSC of bell pepper as observed from application of 50 ppm ClO_2 on bell pepper (cv. Longrum) for 40 days (Jin-Hua *et al.*, 2007), application of 14 kJ m^{-2} UV-C on bell pepper (cv. Zafiro) (Vicente *et al.*, 2005)

and application of 15 mM H_2O_2on white pepper (Bayoumi, 2008). These results suggested that the particular dosage of oxidative agents did not affect fruit biosynthesis of polysaccharides. This may maintain fruit ripening progress and other associated physical and chemical qualities such as weight loss, firmness, respiration and titratable acidity.

Increase in SSC in fruit exposed to 7 and 9 ppm ozone indicated high rate of polysaccharide biosynthesis. Increase in SSC upon exposure to ozone was also observed in strawberry exposed to 0.7 ppm ozone for 7 days (Kute *et al.*, 1995). This leads to accumulation of sugar and was an indication of high progression of ripening. In our case, the high progression of ripening is in agreement with enhanced colour development observed in fruit exposed to 7 and 9 ppm ozone observed in Figure 7.1 – Figure 7.3

7.3.2.2 Titratable acidity

Fruit titratable acidity was significantly affected ($P < 0.0001$, Appendix 7.8) by interaction of ozone concentration and storage time (Figure 7.9). Fruit titratable acidity decreased during storage. Significant decrease was observed in fruit exposed to 5, 7 and 9 ppm on day 15 (5 ppm ozone) and day 12 (7 and 9 ppm ozone). This produced fruit with lower acidity compared to control. Exposure to 1 and 3 ppm ozone had no effect on fruit titratable acidity where it was maintained similar to control during storage.

Titratable acidity measured organic acid content such as malic acid, citric acid and tartaric acid in fruit. Malic acid, the main organic acid in bell pepper gradually decreased during storage as a result of its utilization in respiration. Significant

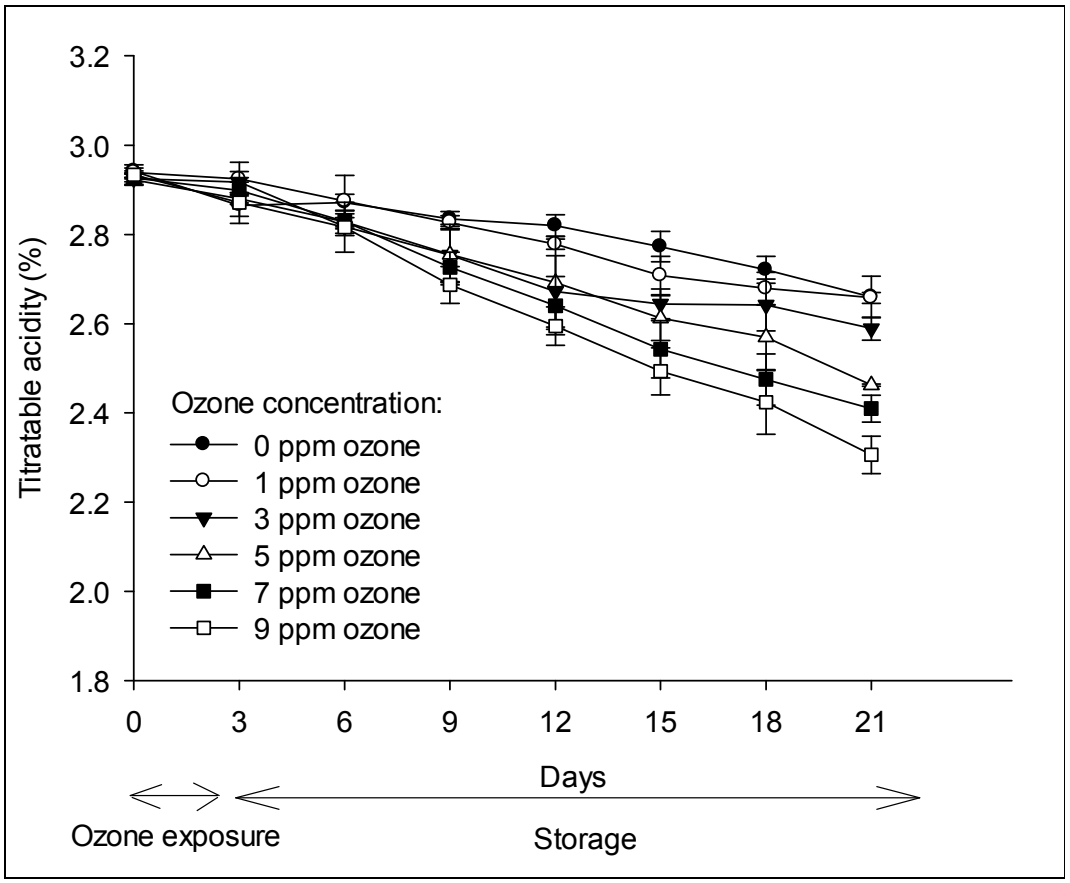

Figure 7.9: Effect of different ozone exposure on titratable acidity of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$

reduction in titratable acidity of fruit exposed to 5, 7 and 9 ppm ozone could be due to oxidative stress. This finding was supported by Horvitz and Cantalejo (2012) where exposure to 200 ppm ClO_2 increased titratable acidity of bell pepper. The strong oxidative stress by ClO_2 and ozone in this study may cause tissue injuries which subsequently increase fruit respiration and increased reduction in organic acid, the substrate in respiration process (Horvitz and Cantalejo, 2012). This stimulated ripening progress in 5, 7 and 9 ppm ozone treated fruit hence explained their enhanced colour development (Figure 7.1 – Figure 7.3). This leads to faster

senescence and quality deterioration as the fruit has higher weight loss (Figure 7.5) and softer texture (Figure 7.6).

Exposure to lower ozone dosage (1 and 3 ppm ozone) maintained fruit titratable acidity similar to control, indicating comparable ripening progress. Similar results were also observed in bell pepper exposed to 0.7 ppm ozone for 5 mins where no significant changes were observed (Horvitz and Cantalejo, 2012). This suggested that the ozone dosages maintained fruit respiration rate and the treatment had no effect on reduction in organic acid and ripening progress. This is in agreement with colour development (Figure 7.1 – Figure 7.3) and firmness (Figure 7.6) of the fruit, which are comparable to control. This slower reduction in titratable acidity of fruit exposed to 1 and 3 ppm may also responsible to maintain the elevated level of ascorbic acid observed in Chapter 6 (Huang *et al.*, 2008).

In contrast to our results, oxidative effect of ClO_2 was reported to have inhibitory effect on pepper ripening progress where exposure to 50 ppm ClO_2 for 40 days was reported to decrease the reduction of titratable acidity in bell pepper (Jin-Hua *et al.*, 2007). The author claimed that this could be due to the inhibitory effect of ClO_2 on methionine (Met) metabolism which reduced bell pepper respiration (Jin-Hua *et al.*, 2007). This pattern was however not observed in ozone in this study. This suggested that the decrease in reduction of titratable acidity could be a characteristic effect of ClO_2 on bell pepper but was not observed from exposure to ozone.

7.3.2.3 Chlorophyll content

Fruit chlorophyll content was significantly affected ($P < 0.0001$, Appendix B 7.9) by ozone concentration and storage time (Figure 7.10). Fruit chlorophyll content

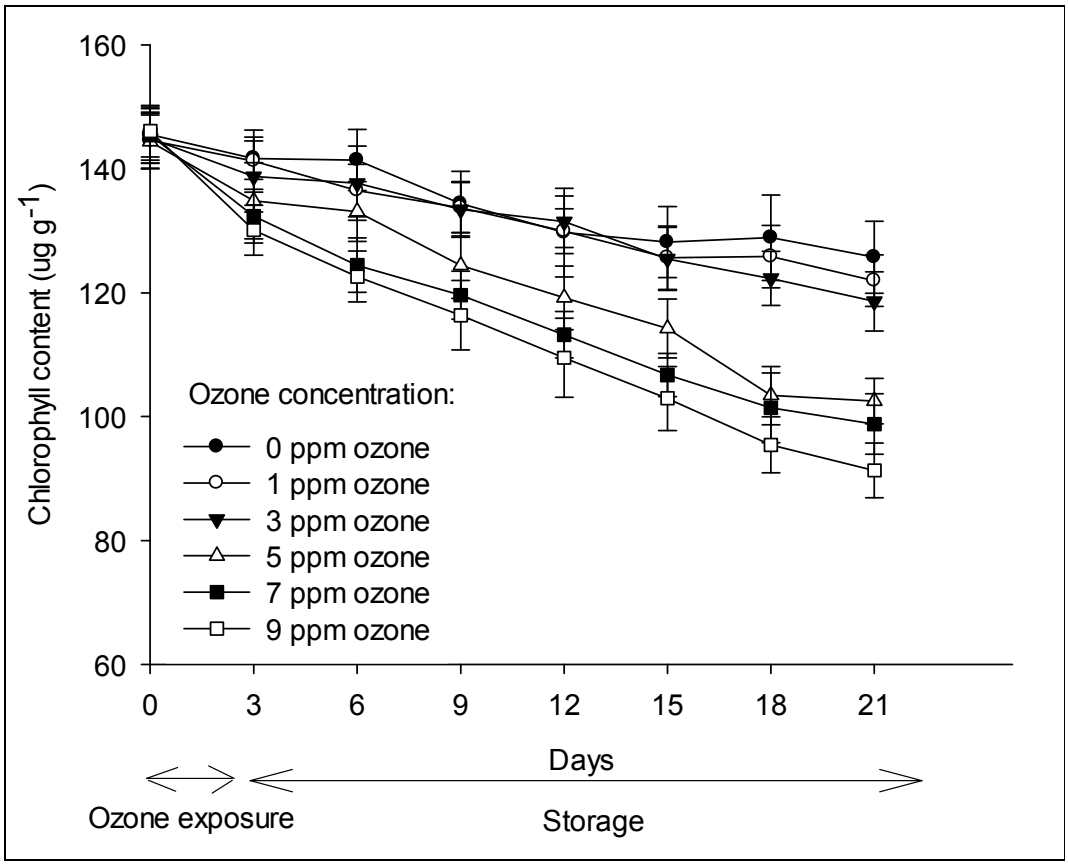

Figure 7.10: Effect of different ozone exposure on chlorophyll content of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

reduced during storage where significant reduction was observed from day 9 till the end of storage period. Effect of ozone concentration of chlorophyll content was dose dependent. Exposure to 1 and 3 ppm had no effect on fruit chlorophyll content while exposure to higher ozone concentration, 5, 7 and 9 ppm ozone reduced fruit chlorophyll content.

Fruit chlorophyll is a green pigment localized in chloroplast (Sun *et al.*, 2007). It is an important molecule to absorb light energy in photosynthesis process. It also acts as a radical scavenger which increase fruit total antioxidant capacity (Alvarez-

Parrilla *et al.*, 2010). During ripening, chlorophyll is degraded with the synthesis of chromoplast pigments (Deepa *et al.*, 2007). This is correlated with the loss of green pigmentation during ripening. (Fox *et al.*, 2005)

Chlorophyll content obtained in this study was positively correlated (r = 0.854, *P* = < 0.0001) with colour hue angle (Figure 7.11). Using coefficient of determination value [$(0.854)^2$ x 100 = 72%], it was observed that chlorophyll content contributed to 72% of the variation in colour hue angle in Figure 7.3. This suggested that reduction in colour hue angle or colour development observed in bell pepper could be due to reduction in chlorophyll content. This finding was supported by Fox *et al.* (2005) who studied the correlation between colour and antioxidant content of bell pepper.

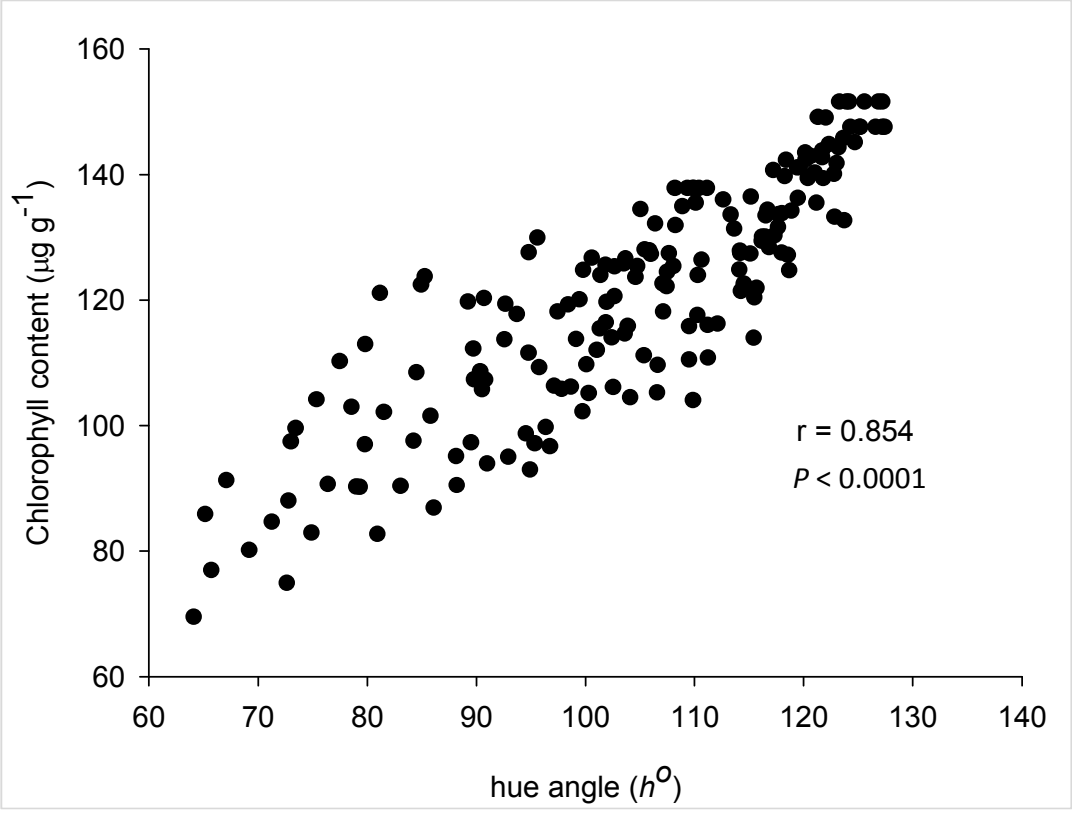

Figure 7.11: The correlation between hue angle (*h*°) of fruit colour and its chlorophyll content

Exposure to 1 and 3 ppm ozone had no significant effect on fruit chlorophyll content and colour development. This indicated that the treatments had no effect on the fruit ripening progress and did not cause significant tissue injury which can lead tissue browning. Meanwhile, significant loss of chlorophyll content in fruit exposed to 5, 7 and 9 ppm ozone explained their enhanced colour changes observed in colour analysis (Figure 7.1 – Figure 7.3). Similar finding was reported by Nunes and Emond (1999) where increase in chlorine dosage from 50 to 200 ppm chlorine resulted in reduction in chlorophyll content of bell pepper. High chlorophyll degradation was also observed from application of UV-C higher than 10 ppm (Jin-Hua *et al.*, 2007). This could be due to ozone and UV-C oxidation by-products such as superoxide radical (O_2^-) produced by the oxidizing agents (ozone, chlorine and UV-C) which was reported to involve in chlorophyll *a* degradation (Jin-Hua *et al.*, 2007; Sakaki *et al.*, 1983). Therefore, our results suggested that ozone treatment higher than 5 ppm ozone is not suitable for bell pepper which can result in colour degradation.

The enhanced chlorophyll loss in fruit exposed to 7 and 9 ppm ozone could also due to their low antioxidant content as reported in Chapter 6. The fruit has low capability in scavenging free radicals induced by ozone hence, led to accumulation of free radicals in the cells. This could lead to high progress of chlorophyll degradation. In contrast to fruit treated with 1 and 3 ppm ozone, the stimulating effect of ozone on the fruit antioxidant content reduced free radicals content in the cells which indirectly protecting chlorophyll from degradation.

7.3.2.4 Malondialdehyde (MDA) content

Fruit malondialdehyde (MDA) was significantly affected ($P < 0.0001$, Appendix 7.9) by ozone concentration and storage time (Figure 7.12). Fruit MDA gradually increased during storage where significant increase was observed immediately after ozone treatment until the end of storage period. In the effect of ozone concentration, exposure to 1 and 3 ppm ozone reduced fruit MDA content. Meanwhile, increase in ozone concentration to 5, 7 and 9 ppm ozone enhanced production of MDA in fruit cells.

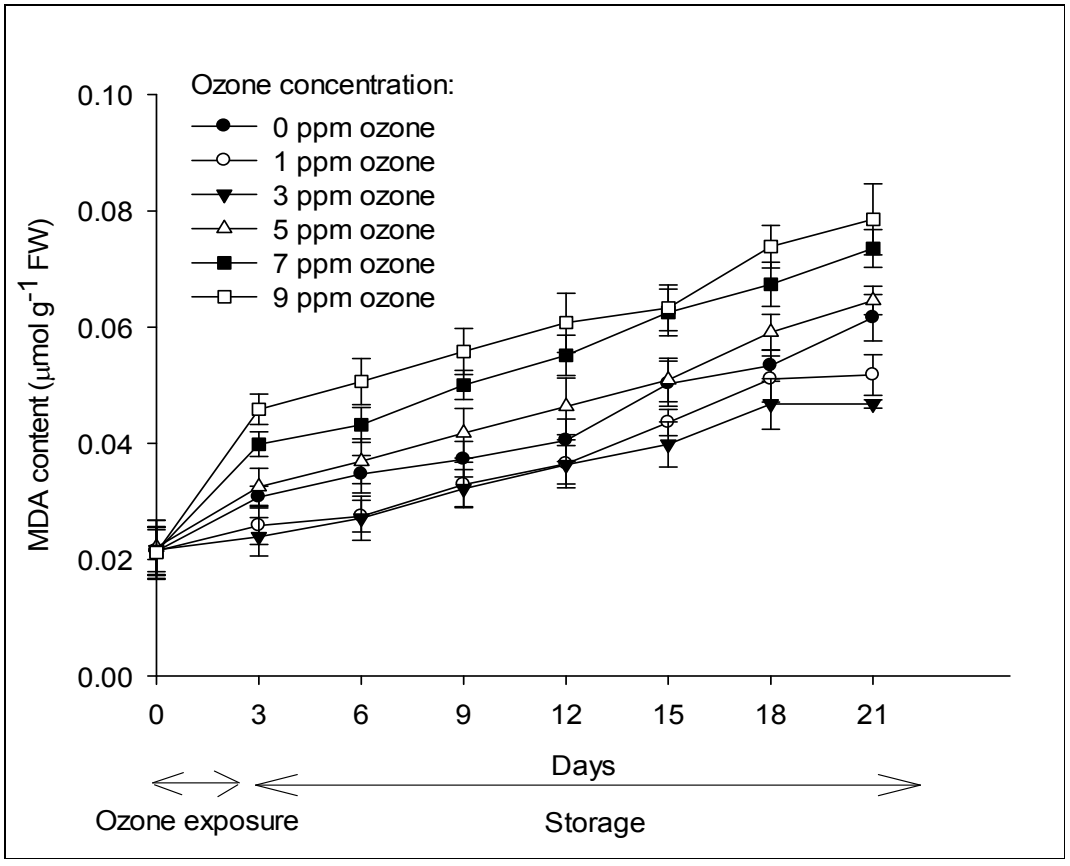

Figure 7.12: Effect of different ozone exposure on TBARS content of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

MDA is a degradation product of unsaturated phospholipid by reactive oxygen species (Tan *et al.*, 2012). It is used as an indicator of membrane injury and membrane integrity due to lipid peroxidation (Hong *et al.*, 2012). MDA reacted with thiobarbituric acid (TBA) which yielded a chromophore and quantified spectrophotometrically (Xing *et al.*, 2011).

Reduction in MDA content in fruit exposed to 1 and 3 ppm ozone suggested the reduced extend of lipid peroxidation. This could be due to the high antioxidant capacity in 1 and 3 ppm ozone treated fruit (Chapter 6) which was induced in response to oxidative stress of ozone. The antioxidants protected membrane phospholipid from oxidative action of ozone and its induced free radicals hence, reduced the production of MDA.

Exposure to high ozone concentration, 5, 7 and 9 ppm ozone enhanced production of MDA. Similar results were observed in strawberry (cv. Elsanta) grown under exposure to 140 ppm ozone where the MDA content was enhanced by 42.4% (Keutgen and Pawelzik, 2008). This could be a result of lipid peroxidation by ozone or its induced radicals on unsaturated phospholipid of cell membrane (Hong *et al.*, 2012). The reaction also produced lipid hydroperoxides which lead to a series of redox reaction hence, contributed to further degradation of cell membrane (Forney, 2003). This increased membrane fluidity and integrity which subsequently increased water loss and fruit firmness. In contrast to strawberry (cv. Elsanta), exposure to 140 ppm ozone had no effect on its MDA content (Keutgen and Pawelzik, 2008). This could be due to stimulation of fruit antioxidant content in response to oxidative stress of ozone as observed in fruit exposed to 1 and 3 ppm ozone in this study. This

suggested that the effects of ozone on fruit antioxidant content influence cell membrane integrity and subsequently physical quality of the fruit.

7.3.3 Bell pepper gaseous exchange response

7.3.3.1 Respiration

Fruit respiration was significantly affected ($P < 0.0010$, Appendix B 7.11) by ozone concentration and storage time. Immediately after ozone exposure, fruit respiration was increased. The increment was transient where it gradually decreased during storage. Effect of ozone concentration on fruit respiration was found to be dose dependent. Exposure to 1 and 3 ppm ozone had no effect on fruit respiration while increase in ozone concentration to 5, 7 and 9 ppm significantly enhanced fruit respiration.

CO_2 is a product of respiration process which can be an indicator of fruit respiration. Being a non-climacteric fruit, respiration rate of bell pepper gradually decreased and no climacteric peak was observed during storage. No significant effect observed in respiration of 1 and 3 ppm ozone treated fruit. This was also reported on peach exposed to 0.3 ppm for 3 weeks (Palou *et al.*, 2002). This suggested that the ozone treatments maintained fruit respiration similar to control and had no effect fruit ripening.

Increment in respiration of fruit exposed to 5, 7 and 9 ppm ozone was transient where the respiration rate reduced to normal rate during storage. A similar transient increase was also observed in tomato exposed to 10 ppm ozone for 10 min (Rodoni *et al.*, 2010) and carrot exposed to 1 ppm ozone for more than 5 days in

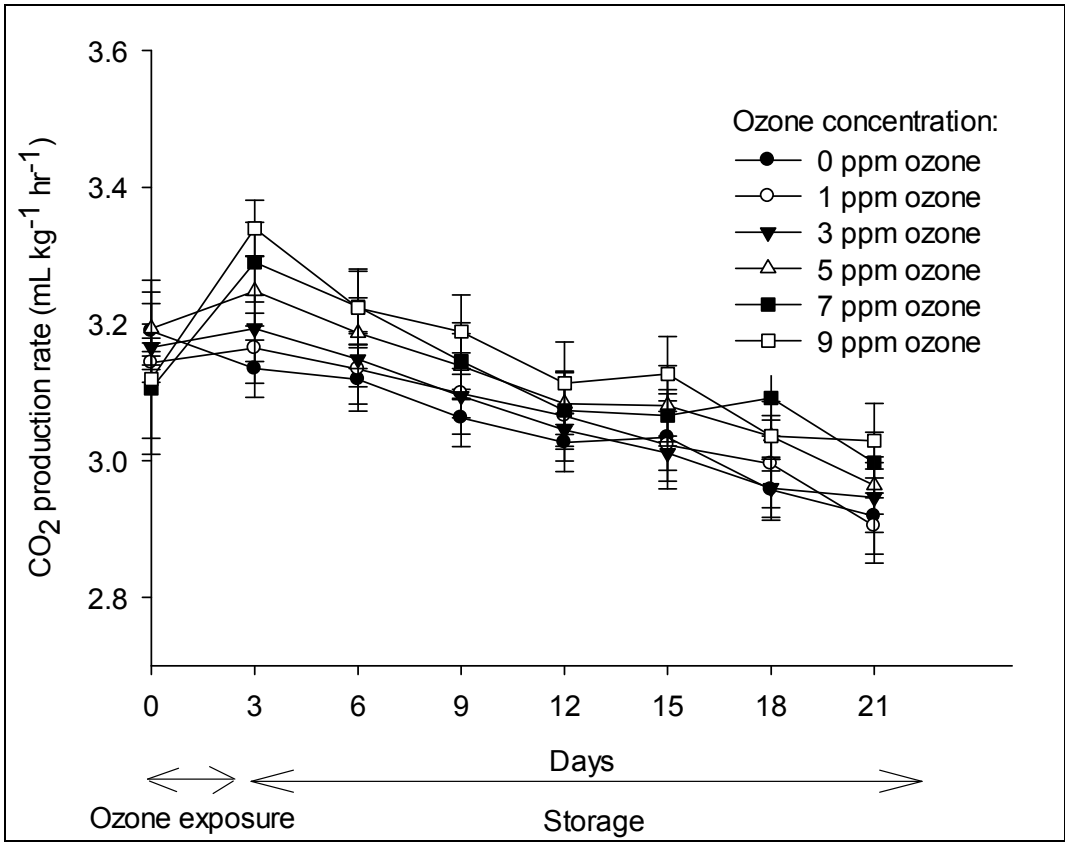

Figure 7.13: Effect of different ozone exposure on respiration rate of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

combination with 1-MCP (Forney *et al.*, 2007) where the fruits recovered their normal respiration rate after the treatments were stopped. This suggested that the particular dosage of oxidative stress induced by ozone and its free radicals increased fruit respiration rate during treatment but the effect did not retain during subsequent storage.

The increase in fruit respiration rate could be due to upregulation of electron transport chain at mitochondria (Tiwari *et al.*, 2002). This would also increase

production of H_2O_2 by mitochondria which may lead to accumulation of the radicals which further increase the cell oxidative level (Tiwari *et al.*, 2002). This could negatively affect membrane integrity, water retention and subsequently fruit physical quality of the fruit as previously discussed. This explained the loss of firmness, weight loss and significant colour change in fruit exposed to 7 and 9 ppm ozone. Besides, the increase in respiration also increased organic acid degradation hence resulted in decrease in organic acid content as observed in titratable acidity assay (section 7.3.2.2).

7.3.3.2 Ethylene production

Ethylene production in bell pepper was significantly affected ($P < 0.0142$, Appendix 7.12) by ozone concentration and storage time while their interaction had no significant effect (Figure 7.14). Ethylene production decreased during storage and significant reduction was observed from day 12 until the end of storage. In the effect of ozone concentration, exposure to 7 and 9 ppm increased fruit ethylene production. Meanwhile, exposure to 1, 3 and 5 ppm ozone had no effect on fruit ethylene production.

Ethylene is a plant hormone which is responsible in regulating fruit maturation and ripening. It stimulates biochemical processes resulting in fruit colour development, reduction in firmness and other physico-chemical changes associated with ripening (Saltveit, 1999). Fruit such as bell pepper has low ability to produce ethylene and may have no response to ethylene exposure. The results showed that the effect of ozone on fruit ethylene production was dose dependent. Exposure to 1,

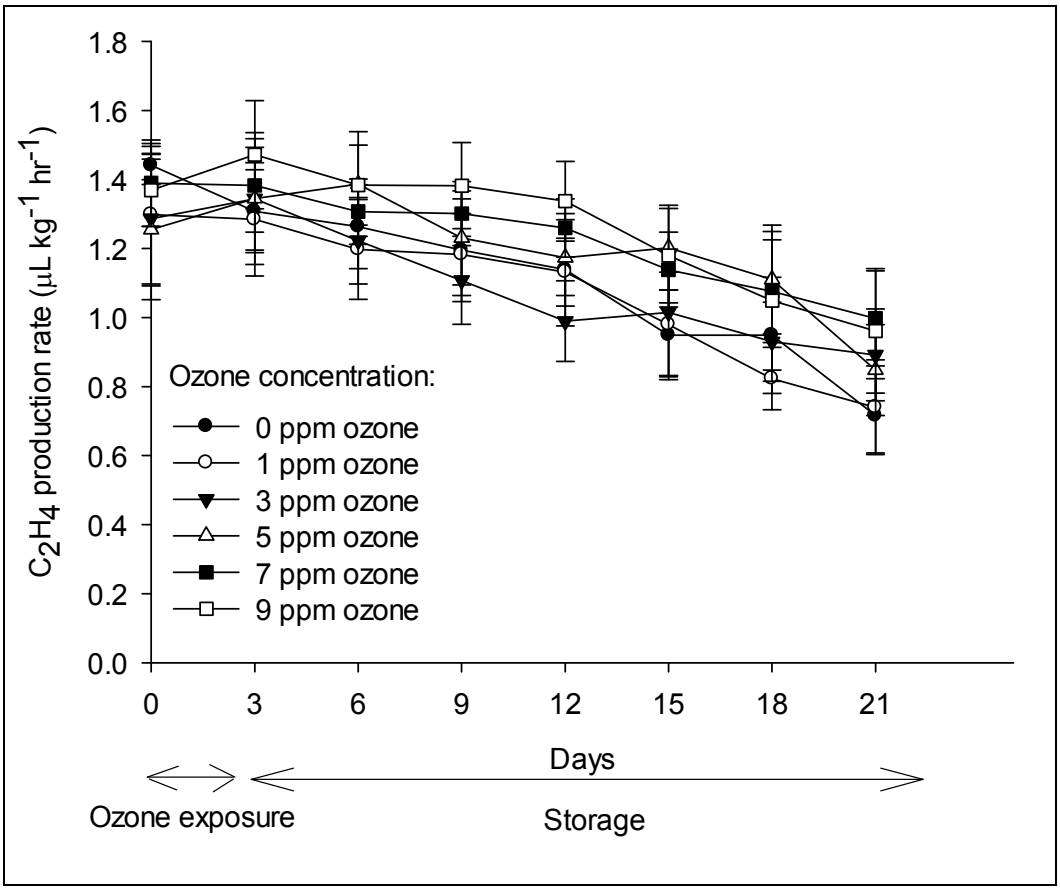

Figure 7.14: Effect of different ozone exposure on ethylene production of bell pepper during three days treatment and 24 days storage at 12°C, 95% RH; vertical bars indicate standard errors; means of three replicates; DMR test at $P < 0.05$.

3 and 5 ppm ozone had no effect on ethylene production. This was also observed in peach exposed to 0.3 ppm ozone for 3 weeks (Palou *et al.*, 2002). The ozone dosage may not trigger fruit ethylene production pathway hence, had no effect on total ethylene production.

Increase in ozone concentration to 7 and 9 ppm stimulates production of ethylene. The stimulating effect of oxidative stress on ethylene production was also

observed in strawberry exposed to UV-C higher than 0.5 kJ m^{-2} UV-C (Nigro *et al.*, 2000). The oxidative stress level induced by the treatments may have triggered ethylene production in bell pepper and strawberry, respectively. However, the increase in ethylene production did not influence fruit ripening progress as strawberry and bell pepper are both non-climacteric fruit.

Meanwhile, in tomato, ozone was found to have inhibitory effect on its ethylene production after exposure to 1 ppm ozone and 10^4 cm^{-3} negative ions (Jin *et al.*, 1989). The author claimed that it could be due to inhibition action of ozone on ethylene biosynthesis (Jin *et al.*, 1989). This inhibition pattern was not observed in bell pepper under study. This could be due to differences in ethylene production pathway of different fruit and nature of fruit in response to oxidative stress of ozone.

7.3.4 Sensory evaluation

Sensory evaluation was performed to evaluate consumer perception on appearance, colour, aroma, flavour and overall acceptability on control and treated bell pepper. The results showed no significant ($P > 0.05$) difference was observed from panelist perception on the appearance, colour, aroma, flavour and overall acceptability of the treated fruit compared to control (Figure 7.15). Fruit exposed to 9 ppm ozone however, received the lowest rating in terms of all attributes (appearance, colour, aroma, flavour and overall acceptability). This could be due to the increase in colour changes, decrease in firmness and high progression of fruit ripening progress.

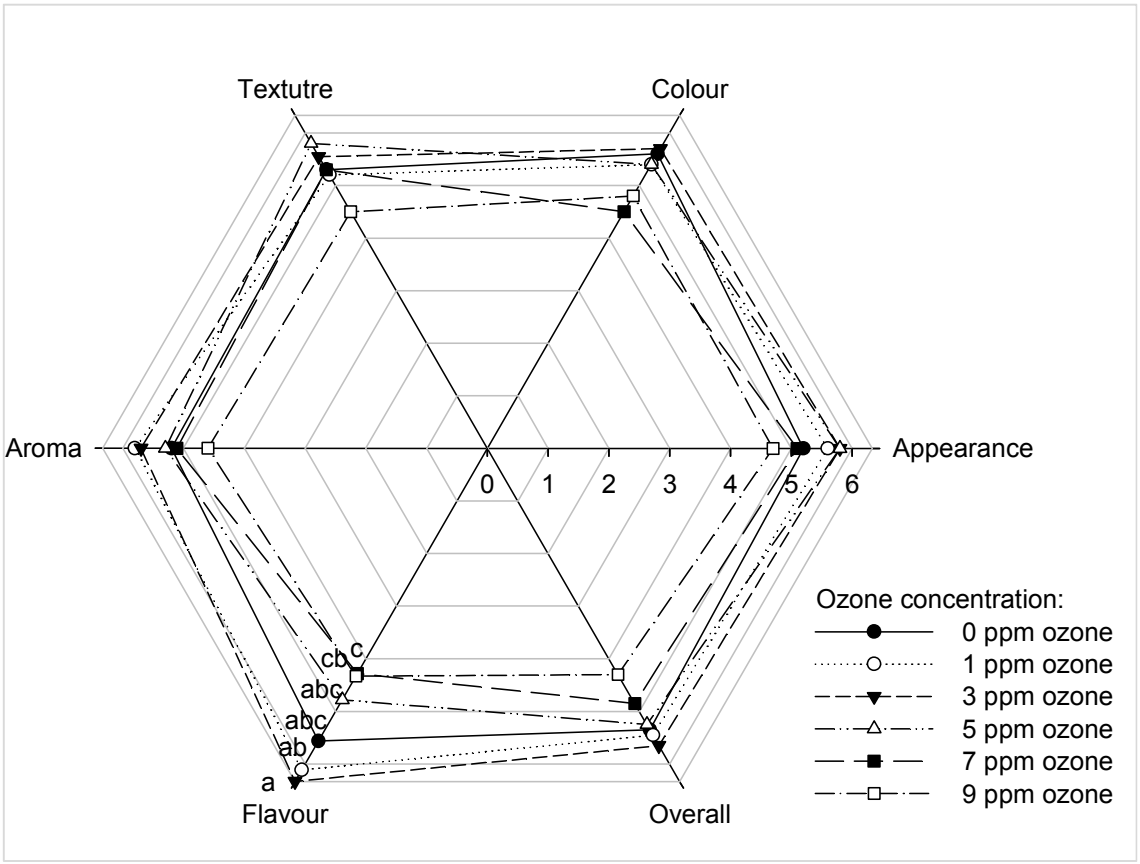

Figure 7.15: Effect of different ozone exposure on fruit appearance, colour, texture, aroma, flavour and overall acceptability after three days treatment and 19 days storage at 12°C, 95% RH.

Significant difference ($P < 0.05$) was observed in flavour of fruit exposed to 1 (6.11-good) and 3 (6.33-good) ppm ozone compared to fruit exposed to 9 ppm ozone (4.33-fair). The significant deterioration in flavor of fruit exposed to 9 ppm could be due the loss of its sweetness and fruity taste. This could be due to oxidation of ozone or its induced radicals on sugar and several lipid derivatives such as (E)-2-hexen-1-ol which associated with flavour of bell pepper (Eggink *et al.*, 2012). This reduced its sensory quality hence less preferred by the panelists.

Fruit exposed to 3 ppm ozone had slightly higher rating compared to control in all attributes; appearance, colour, aroma, flavour and overall acceptability. This showed that stimulating effect of ozone on the fruit antioxidant content (total phenol and ascorbic acid) had no negative effect on the fruit quality. This therefore, did not affect consumer preference. Meanwhile, Tzortzakis *et al*. (2007) reported that the panelist significantly preferred ozone treated fruit (0.15 ppm ozone for 7 days) based on appearance and sensory, compared to non-treated fruit. This suggested that ozone may have improved the fruit physical quality and sensory quality hence, more preferred by the panelist.

In conclusion, the effect of ozone on fruit physico-chemical quality was dose dependent and related to cell oxidative level and fruit defense system in encountering the oxidative stress of applied ozone dosage. Application of low ozone dosage (1 and 3 ppm) induced fruit defense system to neutralize the applied oxidative stress which resulted in no significant symptom of oxidative damage (MDA content and cell membrane permeability). This maintained fruit ripening progress similar to control as indicated by fruit respiration, colour development and titratable acidity. The ozone dosage also maintained fruit water content similar to control hence, maintained its firmness during storage.

Strong oxidative stress from application of high ozone dosage, particularly 7 and 9 ppm ozone, resulted in cell oxidative damage as observed in high MDA content and increase in membrane permeability. This enhanced ripening progress of the fruit as indicated by progressive colour development and reduction in titratable acidity, weight loss and firmness. The progressive colour change was also due to progressive chlorophyll degradation by ozone. This quality deterioration negatively affected fruit flavour hence, not preferred by the panelist.

CHAPTER 8

CONCLUSION

Ozone fumigation showed multiple effects on bell pepper which are by reducing microbial growth as well as increasing its antioxidant property. The effect of ozone on microbial growth was observed from significant reduction in *E. coli* O157, *Salmonella* Typhimurium and *L. monocytogenes* populations on fresh cut bell pepper. Optimal antibacterial activity of ozone was achieved from exposure to 9 ppm ozone for 6 h where it meets the criteria for an antimicrobial agent to reduce a minimum of 2 log microbial population. Ozone reduced the bacterial populations by introducing cell lysis which subsequently resulted in cell death.

The effect of ozone on microbial growth was also observed from its antifungal property which reduced anthracnose development on bell pepper. Optimal antifungal property of ozone was achieved from exposure to 3, 5, 7 and 9 ppm ozone for 72 h. The reduction was due to synergistic effect of ozone action on mycelia morphology, spore production and spore germination as the ozone dosage restricted mycelia development and reduced spore production and spore germination.

The reduction in disease development was correlated to eliciting effect of ozone on plant defense enzymes such as PAL, PPO, POD and β-1,3-glucanase. Increase in the enzyme activity may contribute to the decrease in disease incidence. Exposure to 3 ppm ozone for 72 h was the most effective dosage where it enhanced activity of all of the tested enzymes; PAL, PPO, POD and β-1,3-glucanase activities. These enzymes were induced as a fruit first line of defense mechanism to encounter

oxidative stress introduced by ozone. The increase in production of these enzymes may help in reducing fungal growth on fruit.

Ozone dosage of 3 ppm ozone for 72 h was also the most effective dosage to induce fruit antioxidant property. The ozone dosage enhanced fruit ascorbic acid and total phenolic contents by 15.9 and 15.6%, respectively. This was due to activation of both, first and second line of fruit defense mechanisms. The increase in antioxidant contents enhanced fruit antioxidant capacity hence, increased its functionality and efficacy of antioxidant transfer to consumer.

The stimulating effect of the effective ozone dosage (3 ppm ozone for 72 h) on fruit defense system reduced fruit oxidative status and resulted in no cell oxidative damage indicated by low MDA content and cell membrane permeability. This maintained fruit ripening progress similar to control as indicated by fruit respiration, colour development and titratable acidity. The ozone dosage also maintained fruit water content similar to control hence, maintained its firmness during storage.

Application of higher ozone dosage; 7 and 9 ppm ozone for 72 h; perturbed the equilibrium between ozone-induced reactive compounds and fruit antioxidant. This reduced fruit ascorbic acid and total phenol contents and hence, fruit antioxidant capacity. The imbalance redox potential increased cell oxidative status which resulted in oxidative damage as observed in high MDA content and increase in membrane permeability. This enhanced fruit ripening progress as indicated by progressive colour development and reduction in titratable acidity and firmness. This quality deterioration negatively affected fruit flavour hence, not preferred by the panelist.

Thus, under current observation, the findings of these studies showed ozone dosage of 3 ppm for 72 h was effective to reduce microbial growth as well as

increase fruit antioxidant capacity. These findings however, need commercial scale study to validate its efficiency at larger scale. Besides, study of the effect of ozone on enzyme and antioxidant activities on fungal infected fruit should be conducted to give insight on fruit mechanism in response to simultaneous stress from both, biotic and abiotic stresses. Gene expression study also should be conducted to understand gene regulation in response to oxidative stress of ozone.

Considering safety and environmental friendly aspect of ozone, ozone treatment has the potential to be used as an alternative to chlorine and fungicide to control bacterial contamination and anthracnose development. The treatment also improves phytochemical content of the fruit hence, increases phytochemical transfer content of the fruit. Besides, ozone treatment is very practical where it can be incorporated into truck or shipping container. This allows the treatment to be carried out during transportation hence, reduces time and labour for postharvest treatment. However, a study comparing the effectiveness of ozone and chlorine or fungicide in reducing disease development and maintaining fruit quality during storage needs to be conducted.

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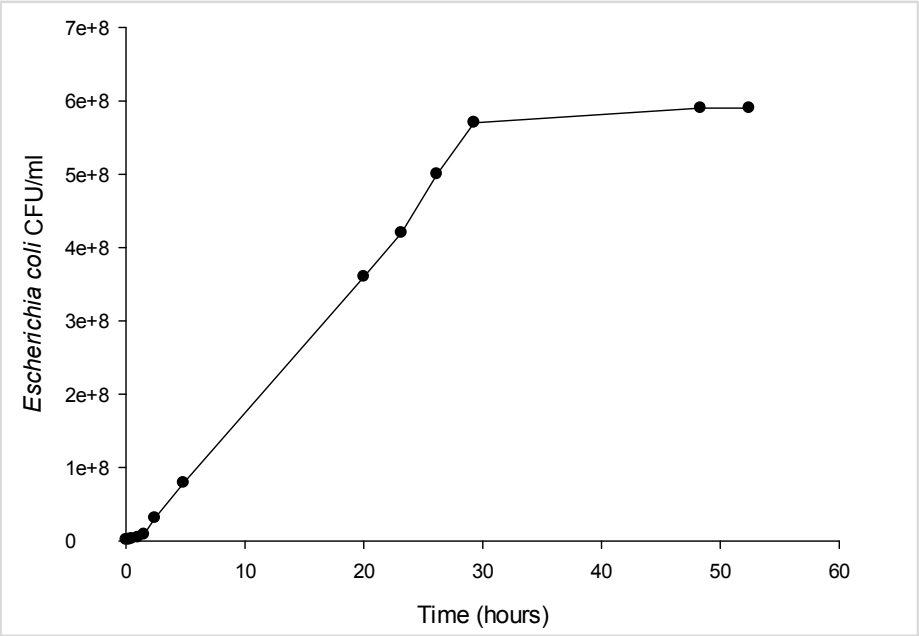
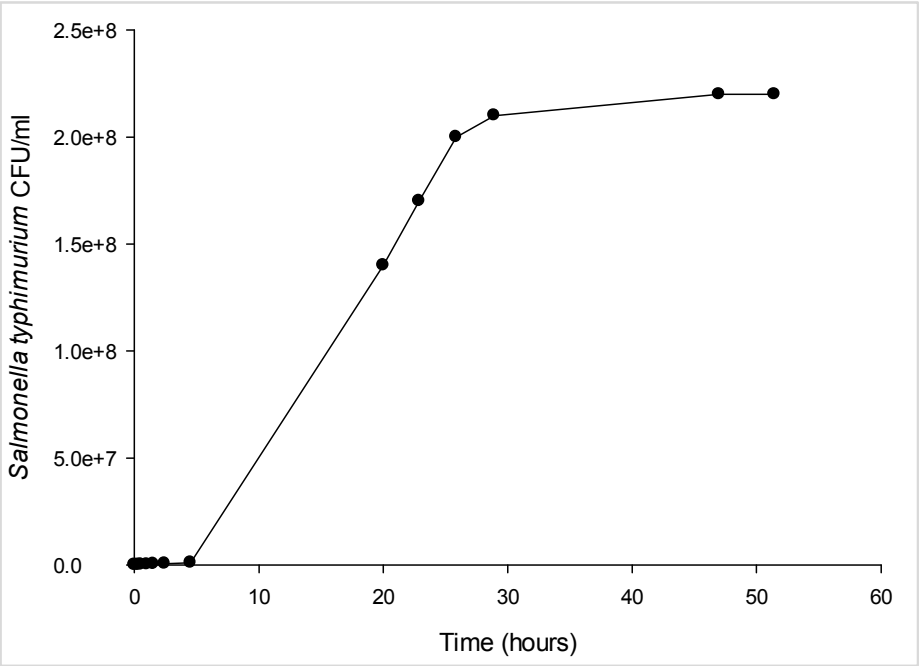
APPENDICES

APPENDIX-A

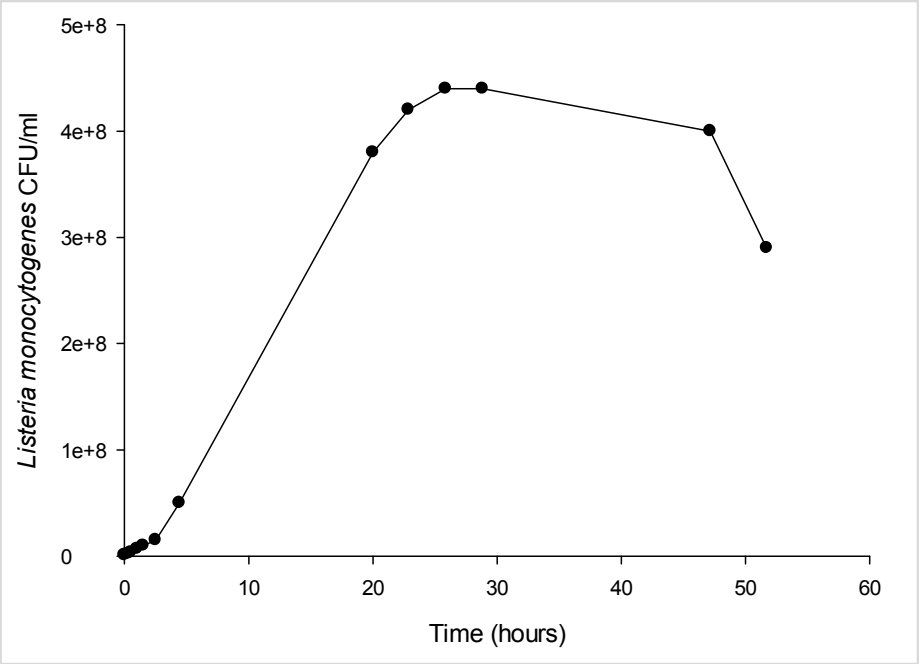
A 3.1: Standard curve of *E. coli* O157 growth used for bacterial inoculum preparation



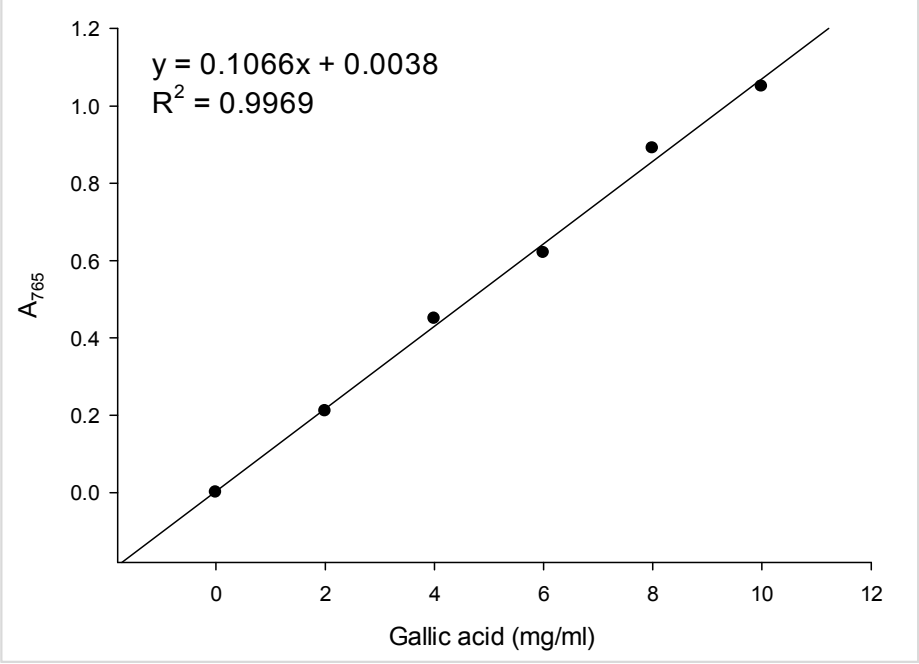
A 3.2: Standard curve of *Salmonella* Typhimurium growth used for bacterial inoculum preparation



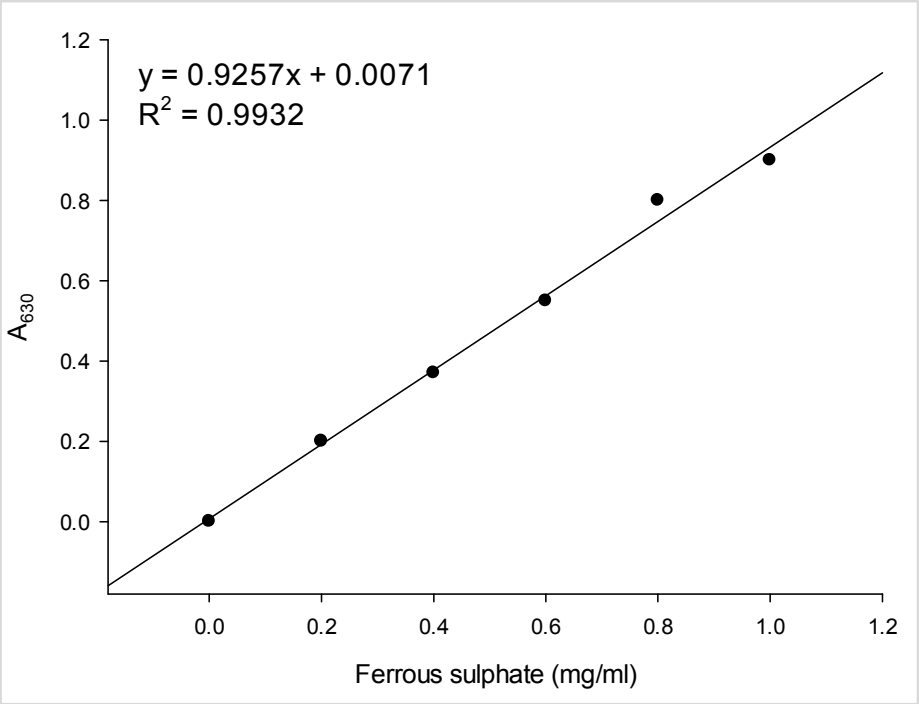
A 3.3: Standard curve of *Listeria monocytogenes* growth used for bacterial inoculum preparation



A 6.1: Gallic acid standard curve for total phenolic



A 6.2: Ferrous sulphate standard curve for FRAP analysis



APPENDIX –B

B 3.1 Analysis of variance for screening of antibacterial activity using different concentration of ozone and exposure time on *E. coli* O157

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	140527.65	28105.53	3663.17	< 0.0001
Exposure time (T)	7	84389.52	12055.65	1571.29	< 0.0001
C x T	35	35884.70	1025.28	133.63	< 0.0001
Error	96	736.56	7.67		
Corrected Total	143	261538.43			

B 3.2 Analysis of variance for screening of antibacterial activity using different concentration of ozone and exposure time on *Salmonella* Typhimurium

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	165202.77	33040.55	3694.291	< 0.0001
Exposure time (T)	7	64274.208	9182.01	1026.262	< 0.0001
C x T	35	40729.47	1163.70	1031.11	< 0.0001
Error	96	858.61	8.94		
Corrected Total	143	27106.93			

B 3.3 Analysis of variance for screening of antibacterial activity using different concentration of ozone and exposure time on *L. monocytogenes*

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	132393.70	26478.74	2780.81	< 0.0001
Exposure time (T)	7	92246.98	13178.14	1383.97	< 0.0001
C x T	35	26553.02	258.66	79.67	< 0.0001
Error	96	914.11	9.52		
Corrected Total	143	252107.81			

B 3.4 Analysis of variance for *in vitro* antibacterial activity using different concentration of ozone and exposure time on *E. coli* O157

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	4	71202.77	17800.69	386.41	< 0.0001
Exposure time (T)	3	23151.14	7717.11	167.52	< 0.0001
C x T	12	13424.29	1118.69	24.28	< 0.0001
Error	40	1842.66	46.07		
Corrected Total	59	109621.05			

B 3.5 Analysis of variance for *in vitro* antibacterial activity using different concentration of ozone and exposure time on *Salmonella* Typhimurium

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	4	88140.47	22035.12	3158.20	< 0.0001
Exposure time (T)	3	13479.85	4493.28	644.00	< 0.0001
C x T	12	10817.95	901.49	129.21	< 0.0001
Error	40	279.08	6.98		
Corrected Total	59	112717.36			

B 3.6 Analysis of variance for *in vitro* antibacterial activity using different concentration of ozone and exposure time on *L. monocytogenes*

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	4	90081.12	22520.28	952.89	< 0.0001
Exposure time (T)	3	1273.93	424.64	17.97	< 0.0001
C x T	12	3595.60	299.63	12.68	< 0.0001
Error	40	945.35	23.63		
Corrected Total	59	95896.01			

B 3.7 Analysis of variance for *in vivo* antibacterial activity after exposure to 0, 0.1, 0.3, 0.5 and 1 ppm ozone for 0.5, 3, 6 and 24 h on *E. coli* O157

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	4	12.41	3.10	60.42	< 0.0001
Exposure time (T)	3	0.29	0.10	1.86	0.1521
C x T	12	0.36	0.03	0.58	0.8441
Error	40	2.05	0.05		
Corrected Total	59	15.11			

B 3.8 Analysis of variance for *in vivo* antibacterial activity after exposure to 0, 0.1, 0.3, 0.5 and 1 ppm ozone for 0.5, 3, 6 and 24 h on *Salmonella* Typhimurium

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	4	18.02	4.50	135.70	< 0.0001
Exposure time (T)	3	0.34	0.11	3.39	0.0270
C x T	12	0.22	0.02	0.55	0.8676
Error	40	1.33	0.03		
Corrected Total	59	19.9			

B 3.9 Analysis of variance for *in vivo* antibacterial activity after exposure to 0, 0.1, 0.3, 0.5 and 1 ppm ozone for 0.5, 3, 6 and 24 h on *L. monocytogenes*

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	4	21.13	5.29	286.86	< 0.0001
Exposure time (T)	3	0.73	0.24	13.28	< 0.0001
C x T	12	0.80	0.07	3.64	0.0010
Error	40	0.74	0.02		
Corrected Total	59	23.4			

B 3.10 Analysis of variance for *in vivo* antibacterial activity after exposure to 0, 1, 3, 5, 7 and 9 ppm ozone for 0.5, 3, 6 and 24 h on *E. coli* O157

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	42.24	8.45	338.77	< 0.0001
Exposure time (T)	3	1.72	0.57	22.99	< 0.0001
C x T	15	0.40	0.03	1.07	0.4082
Error	48	1.20	0.02		
Corrected Total	71	45.55			

B 3.11 Analysis of variance for *in vivo* antibacterial activity after exposure to 0, 1, 3, 5, 7 and 9 ppm ozone for 0.5, 3, 6 and 24 h on *Salmonella* Typhimurium

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	49.88	9.98	83.84	< 0.0001
Exposure time (T)	3	2.15	0.72	6.03	0.0004
C x T	15	0.57	0.04	0.32	0.9903
Error	48	5.71	0.12		
Corrected Total	71	58.32			

B 3.12 Analysis of variance for *in vivo* antibacterial activity after exposure to 0, 1, 3, 5, 7 and 9 ppm ozone for 0.5, 3, 6 and 24 h on *L. monocytogenes*

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	55.81	11.16	239.40	< 0.0001
Exposure time (T)	3	4.02	1.34	28.77	< 0.0001
C x T	15	0.95	0.06	1.36	0.2051
Error	48	2.24	0.05		
Corrected Total	71	63.03			

B 4.1 Analysis of variance for screening of ozone antifungal activity using different concentration of ozone and exposure time on radial mycelial growth of *C. capsici*

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	6	1276.82	212.80	19.15	< 0.0001
Exposure time (T)	8	1389.14	173.64	15.63	< 0.0001
C x T	48	1471.79	30.66	2.76	< 0.0001
Error	126	1400.24	11.01		
Corrected Total	188	5537.98			

B 4.2 Analysis of variance for ozone antifungal activity using different concentration of ozone and exposure time on *in vitro C. capsici* radial mycelial growth

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	2467.09	493.42	38.92	< 0.0001
Exposure time (T)	2	1828.69	914.34	72.12	< 0.0001
C x T	10	2278.52	227.85	17.97	< 0.0001
Error	36	456.40	12.68		
Corrected Total	53	7030.71			

B 4.3 Analysis of variance for ozone antifungal activity using different concentration of ozone and exposure time on *in vitro C. capsici* spore production

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	1.23	0.25	9.01	< 0.0001
Exposure time (T)	2	1.31	0.65	23.99	< 0.0001
C x T	10	2.08	0.21	7.63	< 0.0001
Error	36	0.99	0.27		
Corrected Total	53	5.61			

B 4.4 Analysis of variance for ozone antifungal activity using different concentration of ozone and exposure time on *in vitro C. capsici* spore germination

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	535.14	101.03	7276.88	< 0.0001
Exposure time (T)	2	0.05	0.02	1.73	0.1911
C x T	10	0.24	0.02	1.73	0.1106
Error	36	0.50	0.01		
Corrected Total	53	505.98			

B 4.5 Analysis of variance for production of intracellular H_2O_2 by *C. capsici* spore after exposure to different concentration of ozone and exposure time

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	49584.31	9916.86	1433.02	< 0.0001
Exposure time (T)	2	6.68	3.34	0.48	0.6211
C x T	10	66.55	6.65	0.96	0.4921
Error	36	249.13	6.92		
Corrected Total	53	49906.67			

B 4.6 Analysis of variance for anthracnose incidence on bell pepper after exposure to different concentration of ozone and exposure time

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	3544.24	708.85	5.10	0.0012
Exposure time (T)	2	2633.74	1316.87	9.48	0.0005
C x T	10	1131.69	113.17	0.81	0.6164
Error	36	5000.00	138.89		
Corrected Total	53	12309.67			

B 4.7 Analysis of variance for anthracnose severity on bell pepper after exposure to different concentration of ozone and exposure time

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	4.73	0.95	16.01	< 0.0001
Exposure time (T)	2	3.95	1.97	33.43	< 0.0001
C x T	10	1.25	0.13	2.12	0.0488
Error	36	2.13	0.06		
Corrected Total	53	12.05			

B 4.8 Analysis of variance for *C. capsici* spore production on bell pepper after exposure to different concentration of ozone and exposure time

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.08	0.02	0.54	0.7460
Exposure time (T)	2	2.98	1.49	48.84	< 0.0001
C x T	10	0.75	0.08	2.47	0.0230
Error	36	1.10	0.03		
Corrected Total	53	4.91			

B 5.1 Analysis of variance for disease incidence of anthracnose on bell pepper during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	10937.81	2187.56	26.57	< 0.0001
Storage time (T)	7	85379.30	12197.04	148.13	< 0.0001
C x T	35	3313.61	94.67	1.15	0.2923
Error	96	7904.45	82.34		
Corrected Total	143	107535.18			

B 5.2 Analysis of variance for fruit phenylalanine ammonia lyase (PAL) activity during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	855.85	171.17	263.05	< 0.0001
Storage time (T)	7	1728.66	246.95	379.50	< 0.0001
C x T	35	319.38	9.13	14.02	< 0.0001
Error	96	62.47	0.65		
Corrected Total	143	2966.36			

B 5.3 Analysis of variance for fruit polyphenol oxidase (PPO) activity during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.20	0.04	920.52	< 0.0001
Storage time (T)	7	0.03	0.00	90,83	< 0.0001
C x T	35	0.03	0.00	22.70	< 0.0001
Error	96	0.00	0.00		
Corrected Total	143	0.26			

B 5.4 Analysis of variance for fruit peroxidase (POD) activity during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.42	0.08	129.72	< 0.0001
Storage time (T)	7	0.06	0.01	12.76	< 0.0001
C x T	35	0.07	0.00	3.02	< 0.0001
Error	96	0.06	0.00		
Corrected Total	143	0.61			

B 5.5 Analysis of variance for fruit β-1,3-glucanase activity during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	21.37	4.27	27.52	< 0.0001
Storage time (T)	7	116.49	16.64	107.16	< 0.0001
C x T	35	20.05	0.57	3.69	< 0.0001
Error	96	14.91	0.16		
Corrected Total	143	172.83			

B 6.1 Analysis of variance for fruit antioxidant capacity measured by FRAP during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	3.69	0.74	100.62	< 0.0001
Storage time (T)	9	3.09	0.34	46.78	< 0.0001
C x T	45	0.51	0.01	1.54	0.0335
Error	120	0.88	0.01		
Corrected Total	179	8.17			

B 6.2 Analysis of variance for fruit total phenol content during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	22.57	4.51	216.82	< 0.0001
Storage time (T)	9	9.39	1.04	50.10	< 0.0001
C x T	45	3.20	0.07	3.42	< 0.0001
Error	120	2.50	0.02		
Corrected Total	179	37.66			

B 6.4 Analysis of variance for fruit ascorbic acid content during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	35656.17	7131.23	105.23	< 0.0001
Storage time (T)	9	56654.48	6294.94	92.89	< 0.0001
C x T	45	4996.67	111.04	1.64	< 0.0177
Error	122	8267.35	67.77		
Corrected Total	181	107974.46			

B 6.5 Analysis of variance for β-carotene content during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.02	0.00	35.82	< 0.0001
Storage time (T)	9	0.08	0.01	66.40	< 0.0001
C x T	45	0.00	0.00	0.82	0.7734
Error	120	0.02	0.00		
Corrected Total	179	0.12			

B 7.1 Analysis of variance for fruit colour lightness (*L**) during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	171.94	34.39	6.3	< 0.0001
Storage time (T)	9	1087.52	120.84	23.29	< 0.0001
C x T	45	298.60	6.64	1.28	0.1475
Error	120	622.61	5.19		
Corrected Total	179	2180.66			

B 7.2 Analysis of variance for fruit colour vividness (C*) during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	120.01	24.00	4.95	0.0004
Storage time (T)	9	877.22	97.47	20.11	< 0.0001
C x T	45	424.91	9.44	1.95	0.0022
Error	120	581.49	4.85		
Corrected Total	179	2003.64			

B 7.3 Analysis of variance for fruit colour hue angle (*h*°) during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	1881.76	376.35	5.94	< 0.0001
Storage time (T)	9	33669.27	3741.03	59.03	< 0.0001
C x T	45	1064.81	23.66	0.37	0.9998
Error	120	7605.17	63.38		
Corrected Total	179	44221.01			

B 7.4 Analysis of variance for fruit weight loss during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	107.99	21.60	153.78	< 0.0001
Storage time (T)	9	2291.95	254.66	1813.21	< 0.0001
C x T	45	33.54	0.75	5.31	< 0.0001
Error	120	16.85	0.14		
Corrected Total	179	2450.34			

B 7.5 Analysis of variance for fruit firmness during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	203.10	40.62	31.93	< 0.0001
Storage time (T)	9	1081.93	120.21	94.51	< 0.0001
C x T	45	158.90	3.53	2.78	< 0.0001
Error	120	152.64	1.27		
Corrected Total	179	1596.57			

B 7.6 Analysis of variance for fruit membrane permeability during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	3629.83	725.97	42.43	< 0.0001
Storage time (T)	9	34199.47	3799.94	222.10	< 0.0001
C x T	45	680.28	15.12	0.88	0.6761
Error	120	2053.14	17.11		
Corrected Total	179	40562.73			

B 7.7 Analysis of variance for fruit soluble solid concentration (SSC) during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.20	0.04	3.99	0.0022
Storage time (T)	9	5.33	0.59	58.19	< 0.0001
C x T	45	0.23	0.01	0.51	0.9943
Error	120	1.22	0.01		
Corrected Total	179	6.99			

B 7.8 Analysis of variance for fruit titratable acidity during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.96	0.19	36.05	< 0.0001
Storage time (T)	9	6.21	0.69	129.23	< 0.0001
C x T	45	0.46	0.01	1.93	0.0025
Error	120	0.64	0.01		
Corrected Total	179	8.28			

B 7.9 Analysis of variance for fruit chlorophyll content during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	14705.92	2941.18	40.67	< 0.0001
Storage time (T)	9	34042.05	3782.45	52.30	< 0.0001
C x T	45	3888.26	86.41	1.19	0.2223
Error	120	8678.14	72.32		
Corrected Total	179	61314.36			

B 7.10 Analysis of variance for fruit malondialdehyde (MDA) content during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.01	0.00	61.21	< 0.0001
Storage time (T)	9	0.05	0.00	128.11	< 0.0001
C x T	45	0.00	0.01	1.01	0.4644
Error	120	0.00	0.00		
Corrected Total	179	0.06			

B 7.11 Analysis of variance for fruit respiration during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.20	0.04	4.50	0.0009
Storage time (T)	9	1.94	0.22	24.89	< 0.0001
C x T	45	0.13	0.00	0.33	1.0000
Error	120	1.04	0.01		
Corrected Total	179	3.31			

B 7.12 Analysis of variance for fruit ethylene production during storage after exposure to different ozone concentration

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Ozone concentration (C)	5	0.87	0.17	2.99	0.0141
Storage time (T)	9	9.08	1.01	17.29	< 0.0001
C x T	45	0.50	0.01	0.19	1.0000
Error	120	7.00	0.06		
Corrected Total	179	17.45			

APPENDIX-C

C 7.1: Evaluation form for sensory analysis

Hedonic Scale Rating

Product: _____Variety: _____Date: _____

Name of panelist: _____Signature:_____

Instructions: (Please read the instructions carefully before filling blanks.)

1. This is sensory analysis form for the evaluation of different treatments.
2. Please follow the numerical system for scoring the samples.

1……	Extreme dislike
3…….....	Dislike
5……..	Acceptable
7……..	Good
9……..	Excellent

3. Please do not disturb the sequence of the samples provided.
4. Please rinse your tongue before testing next sample, with the water provided.

Sample No.	Appearance	Colour	Texture	Aroma	Flavour	Overall acceptability
336						
352						
358						
375						
421						
429						
600						
664						
753						