EFFECT OF OZONE ON ANTHRACNOSE, PHYSICOCHEMICAL RESPONSES AND GENE EXPRESSION IN PAPAYA (*CARICA PAPAYA* L.)

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By

ONG MEI KYING

Thesis Submitted to The University of Nottingham Malaysia Campus, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

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Dedication of love and gratitude to:

My caring parents and loving husband
whose endless support,
understanding and timely encouragement
inspired me to
strive and fulfil this goal.

ABSTRACT

EFFECT OF OZONE ON ANTHRACNOSE, PHYSICOCHEMICAL RESPONSES AND GENE EXPRESSION IN PAPAYA (*CARICA PAPAYA* L.)

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Chairman : Associate Professor Asgar Ali, PhD

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A study was conducted to investigate the effects of varying levels of ozone (0, 1.5, 2.5, 3.5 or 5.0 ppm) for 96 h on 1. the *in vitro* and *in vivo* growth of *Colletotrichum gloeosporioides*, the causal organism of anthracnose; 2. the reactive oxygen species generation and spore mitochondria of *C. gloeosporioides* using transmission electron microscope, fluorescence microscope and laser scanning confocal microscope; 3. the production of defence-related enzymes in papaya; 4. microbiological analysis on ozone-treated and non-treated papaya; 5. the biochemical, physiological, gas exchange and sensory characteristics of papaya fruit during storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days; 6. the changes in total phenols, total carotenoids and antioxidant activity; and 7. gene expression of ozone-fumigated papaya fruit. Data were analyzed using analysis of variance and differences among treatment means were separated by Duncan Multiple Range Test (DMRT). The results of antifungal studies showed that mycelial

growth of *C. gloeosporioides* was reduced significantly ($p < 0.05$) at all concentrations compared to the control. The maximum inhibition in mycelium growth (41.2 %) was obtained at 5.0 ppm ozone. Similarly, conidial germination inhibition was 100 % for 5 ppm ozone. *In vivo* analysis revealed that 2.5 ppm ozone was the optimal concentration for controlling anthracnose disease incidence (72.5 %) and disease severity after 10 days of storage, showing that a moderate concentration of ozone is effective in the reduction of *C. gloeosporioides* in artificially inoculated papaya fruit without affecting the quality aspect of the fruit.

The results of scanning electron microscopy (SEM) also confirmed that ozone fumigated fungus at levels above 3.5 ppm deformed and disintegrated spore and mycelia structure. Further to that, transmission electron microscopy (TEM) illustrated that the mitochondria of ozonized fungus was disintegrated and had ruptured membrane. In spores treated with 3.5 ppm ozone, mitochondrial cristae were distorted, whereas the mitochondria were almost completely degraded in spores treated with 5.0 ppm. Meanwhile, the results from microscopy studies using laser scanning confocal microscope and fluorescence microscope showed that ozone treatment caused production of reactive oxygen species (ROS) in mitochondria of *C. gloeosporioides*. With increased concentration of ozone, higher levels of ROS were induced in the spores.

Besides its direct antifungal activity, the study strongly suggested that ozone induces a series of defense reactions through production of compounds such

as total phenols, polyphenol oxidase (PPO), peroxidase (POD) and phenylalanine ammonia-lyase (PAL) in ozone-fumigated papaya. Likewise, content of ascorbic acid, β-carotene, lycopene and antioxidant activity of papaya increased as fruit ripened and was further enhanced by exposure to ozone for 96 hours from day 4 until day 8. Twenty-four hours of ozone treatment at the level of 0.5, 2, 3.5 and 5.8 ppm reduced the total mesophilic microorganism counts of fruit with initial values of 4.48 to 2.18 log $cfug^{-1}$. In addition, no coliform bacteria were initiated after 24 hours at all levels of ozone exposure.

In addition, 2.5 ppm ozone treated fruit showed maximum beneficial effects in reducing weight loss, maintaining firmness, reduced rate of respiration, delaying changes in peel colour and containing the highest soluble solids concentration (SSC) as compared to the control. The titratable acidity declined throughout the storage period with slower rate in ozone-fumigated fruits. Overall sensory assessment of quality after ripening showed fruit were significantly better in quality when fumigated with 2.5 ppm ozone which were assigned highest sensory score in terms of appearance, sweetness, pulp colour, texture, aroma and overall acceptability than the control.

The discovery of the gene expression of papaya in defense response induced by ozone fumigation has further clarified the understanding on how specific gene involved in controlling its expression when the plant changes during stress or in any plant lifecycle event. Among those genes, some involved in ethylene biosynthesis, generation of reactive oxygen species and stress

responses of plant defense were found (mitochondrion, chloroplast, heat shock proteins, polygalacturonase-inhibiting protein, hydroxyproline-rich glycoprotein, ethylene responsive factor and acyl-CoA oxidase).

Thus, the findings from all the experiments carried out during this study showed that 2.5 ppm ozone reduced anthracnose incidence and extended the storage life for up to 12 days while maintaining acceptable quality of papaya fruit. Ozone exposure at 1.5 ppm resulted in poorer quality fruit as compared to 2.5 ppm ozone treated fruit. Higher concentration of ozone exposure at 3.5 ppm and 5 ppm ozone seems non-physiological and caused phytotoxic effect on the quality of papaya fruit. As a non-toxic, biodegradable product, eco-friendly and safe sanitizer, ozone has the potential to become a natural preservative for prolonging the shelf life and retaining quality of papaya by combating fungal disease, particularly fungus *C. gloeosporioides*, thus promoting the marketability of the crop and minimizing postharvest losses in the papaya industry.

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SUPERVISORY COMMITTEE

The thesis submitted to the School of Biosciences, Faculty of Science, The University of Nottingham Malaysia Campus has been accepted as fulfillment of the requirement for the degree 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 acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at the University of Nottingham Malaysia Campus or other institutions.

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ONG MEI KYING

Date:

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LIST OF ABBREVIATIONS AND NOTATIONS

ANOVA	Analysis of Variance
°C	degree centigrade
DMRT	Duncan's Multiple Range Test
h	hour (s)
HPO_3	metaphosphoric acid
NaOH	sodium hydroxide
H_2O_2	hydrogen peroxide
DCPIP	2,6-dichlorophenol-indophenol
l	litre
V	volt
M	Molar
mM	miliMolar
µg	microgram
min	minute
sec	second
ml	millilitre
µl	microlitre
cm	centimetre
mm	millimetre
nm	nanometre
µm	micrometre
µM	micromolar (10^{-6} M)
%	percentage

mt	metric ton
ft	feet
N	Newtons
O_3	ozone
C_2H_4	ethylene
CO_2	carbon dioxide
cv.	cultivar
ppm	part per million
ppb	part per billion
SAS	Statistical Analysis System
UPM	Universiti Putra Malaysia
UV	ultraviolet
UV/Vis	ultraviolet / visible
v/v	volume per volume
w/v	weight per volume
RH	relative humidity
FRAP	ferric reducing antioxidant power
DPPH	2,2-diphenyl-1-picrylhydrazyl
TPTZ	2,4,6-tripyridyl-s-triazine
PVPP	polyvinyl polypyrrolidone
GAE	gallic acid equivalent
APHA	American Public Health Association
EPA	Environmental Protection Agency
PCR	polymerase chain reaction

RT-PCR	reverse transcriptase PCR
qPCR	real time quantitative PCR
bp	base pair
L*	Lightness
h°	hue angle
C*	Chroma
SSC	soluble solids concentration
GRAS	generally recognized as safe
CA	controlled atmosphere
EDTA	Ethylene Diamine Tetra Acetic acid
SEM	scanning electron microscopy
TEM	transmission electron microscopy
cfu	colony forming unit
Ct	threshold cycle
ABA	abscisic acid
GC	gas chromatography
TE	Trolox equivalent
TCD	thermal conductivity detector
FID	flame ionization detector
AFLP	amplified fragment length polymorphism
PAL	phenylalanine ammonia lyase
POD	peroxidase
PPO	polyphenol oxidase
PDA	potato dextrose agar

ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RAPD	random amplified polymorphic DNA
SSR	simple sequence repeat
RFLP	restriction fragment length polymorphism

CHAPTER 1

INTRODUCTION

Papaya (*Carica papaya* L.) like many climacteric fruits undergoes a variety of physical and chemical changes after harvest (Shiota, 1991). Climateric fruit exhibits a characteristic rise in ethylene production and respiration at the onset of ripening (Tucker and Grierson, 1987; Abeles *et al*., 1992). Ethylene plays a major role in the ripening process by stimulating the ripening attributes such as colour, texture, aroma and flavour. Part of the ripening process is the softening of the fruit cell wall. The ripeness stage determines the final quality of fruits, which explains why many studies have attempted to define the ripening parameters for commercial fruit (Peterson, 1990; Wills *et al*., 2007). Papaya like other tropical fruits has a very short shelf life, which limits its export potential. Extension of shelf life will help open the way to potential new markets that do not need to depend on refrigerated transportation of papaya fruits and subsequently reduce postharvest losses.

Papaya is a tropical crop and is considered to be one of the most economically important and nutritious fruits, being rich in antioxidants such as carotenes, vitamins, flavonoids and industrially important enzyme papain (Nakasone and Paull, 1998). The fruit has a strong and characteristic aroma. Ripe fruit is served as dessert and drink. Papain is used in the pharmacological industry to treat arteriosclerosis and in food industries as a meat tenderizer (Sanchez, 1994). Papaya has an excellent potential as an

export crop. Production of papaya occurs in more than 60 countries worldwide, with the vast majority grown in developing countries. In 2010, global production was estimated to reach 11.22 million metric tons (mt) (FAOSTAT, 2012a). Asia is the leading papaya-producing region, accounting for 52.2 % of global production, followed by South America (23.8 %), Africa (13.16 %), Central America and the Caribbean (10.92 %), North America (0.13 %), and Oceania (0.12 %) (FAOSTAT, 2012a). The main papaya-producing countries are India, Brazil, Indonesia, Nigeria, and Mexico. The top two producing countries accounted for more than 55 % of global production during the period from 2008 to 2010. Mexico is the largest exporter of papaya and the United States is the largest single-country importer (Evans *et al*., 2012). The next largest importers are China and Singapore with 16.8 % and 9.1 % of the total world import, respectively. The major supplier to these markets is Malaysia (ISAAA, 2012).

In addition, postharvest disease during storage and transportation has significantly lowered the quality and value of this commodity. Papaya shipments arriving at the New York terminal markets were reported to have a range of disorders associated with mechanical injury, over-ripeness and parasitic disease (Cappellini *et al*., 1988). Anthracnose (*Colletotrichum gloeosporioides*) rot affected 62 % of shipments, bruise damages 22 % and overripe fruit 48 %. Other causes of loss included chilling injury (2 %), soft fruit (17 %) and other diseases (stem end rot, *Rhizopus* and grey mould) in 35 % of shipments inspected (Cappellini *et al*., 1988). According to Paull *et al*. (1997), the major postharvest diseases are anthracnose, R*hizopus*, stem end

rot and black spot. Postharvest diseases, especially anthracnose, become a problem when fruit have 25 % or more skin yellowing (Wardlaw *et al*., 1939; Alvarez and Nishijima, 1987).

The most common postharvest disease observed in the market chain is caused by the fungus *Colletotrichum gloeosporioides* Penz. Sacc.(Paull *et al*., 1997) and is a major disease in tropical countries (Snowdon, 1990). This disease is initiated on developing fruit in the field, but the symptoms do not appear until the fruit ripens (Alvarez and Nishijima, 1987). Field sprays are required to prevent fruit infection. Postharvest heat (20 minutes at 48-50 °C) and fungicide treatments such as thiabendazole and Prochloraz (Sportak®) can reduce but rarely eliminate these infections once they are established (Couey and Farias, 1979; Couey *et al*., 1984; Glazener *et al*., 1984). Rainy weather favours the development and spread of the disease. A single hot water dip after vapour heat treatment has been shown to slightly reduce postharvest disease (Nishijima *et al*., 1992).

In the early part of the 20th century, heat treatment was often employed commercially to curb fungal and bacterial contamination along with insect infestation. But, by the 1950s, this practice had been superseded by the introduction of cheap and effective synthetic fungicides, used in combination of pre-pack sanitation treatments employing chlorine- (or bromine-) based disinfectants (Eckert and Ogawa, 1988). However, in papaya, hot water dip treatment affects the ripening process (Paull, 1990) and the use of fungicides for extended periods may cause the emergence of strains of fungus resistant

to these fungicides. Further the residues of fungicides present on the fruits may be harmful to consumers. These current practices are commonly ineffective (Sapers, 1998) and there is growing legislative pressure on traditional activities due to health concerns over pesticide residues and treatment by-products (United States Environmental Protection Agency, 1993).

Ozone is a strong oxidant which is effective in controlling bacteria, moulds, protozoa, and viruses (Kim *et al*., 2003). In 1997, ozone was granted Generally Recognized as Safe (GRAS) status (US-FDA, 1997) and has since received full US-FDA approval as a direct contact food sanitizing agent (US-FDA, 2001). Since then, the number of studies evaluating different uses in the food industry has rapidly increased (US-FDA, 1997). Ozone might have different applications, such as cleaning surfaces or equipment and disinfecting water for recycling (Kim *et al*., 1999). More recently, there has been interest in the evaluation of ozone treatments during processing and storage of fruit and vegetables (Palou *et al*., 2001; Palou *et al*., 2002; Perez *et al*., 1999, Tzortzakis *et al*., 2007a). Continuous exposure to low concentrations (0.1−0.3 μl l^{-1}) in storage areas can oxidize ethylene (Smilanick *et al*., 1999), and treatments with ozone gas have been shown to elicit the accumulation of antioxidants (Gonzales-Barrio *et al.*, 2006). Achen and Yousef, 2001 reported that the use of ozone-containing water for washing apples decreased the counts of *E. coli* O157:H7. This has raised commercial interests in the deployment of ozone for various applications in the food industry, including the administration of gaseous ozone during storage (Palou *et al*., 2003).

Ozone is one of the most powerful oxidants known to man (Lide, 1991), leaves no detectable residues in/on treated produce and is a powerful antimicrobial agent (Graham *et al*., 1997; Droby *et al*., 1998; Geering, 1999; Rice, 1999). Moreover, it is safer to use [lower Threshold Limit Value-Long Term Exposure Limit (TLV-LTEL)] than many other chemical treatments adopted for similar purposes. The use of gaseous ozone treatments as a non-thermal processing is a means of enhancing food safety without compromising quality and desirability of food products. According to a review by Guzel-Seydim *et al*. (2004) on ozone, the food industry is always seeking a better method for maintaining food safety.

Several studies reveal ozone exposure prevents microbial growth and extends the shelf-life of treated produce. For example, Barth *et al*. (1995) observed that treatment of blackberries with 0.3 µmol mol^{-1} of ozone effectively suppressed fungal development, similar findings reported by Sarig *et al*. (1996) for grapes, Liew and Prange (1994) for carrots, Jin *et al*. (1989) and Aguayo *et al*. (2006) for tomatoes, Spalding (1968) and Palou *et al*. (2002) for peaches, Rice *et al*. (1982) for potatoes and Baranovskaya *et al*. (1979) for beetroot and onions. In addition, several studies reported reductions in spore production and viability following ozone-treatment (Krause and Weidensaul, 1978; Palou *et al*., 2002, 2003), although effects have commonly been observed to be restricted to situations where the gas is present, spore production resumes when treated fruit are removed from the ozone-enriched atmosphere (Smilanick, 2003). Khadre and Yousef (2001) compared the effectiveness of ozone and hydrogen peroxide against *Bacillus*

spores, and they showed that ozone was more effective against *Bacillus* spores than hydrogen peroxide.

Kim *et al*. (1999) concluded that ozone is more efficient at lower concentrations and treatment times than more standard sanitizers including chlorine. Singh *et al.* (2002) were able to reduce *E. coli* O157:H7 on lettuce by 1.79 log $cfug^{-1}$ after 15 min of treatment with gaseous ozone, indicating that gaseous treatments were generally more effective than aqueous. Bialka and Demirci (2007) were able to show similar reductions of *E. coli* O157:H7 and *Salmonella enterica* inoculated on blueberries treated with gaseous ozone, among other treatments. Akbas and Ozdemir (2008) reduced *E. coli* and *Bacillus cereus* cells by 3.5 log $cfug^{-1}$ after 360 min of treatment with gaseous ozone at low concentrations.

The demand for safe food with high nutrients by the consumer has prompted research to discover and evaluate novel antimicrobial agents as an alternative to fungicides. The use of different ozone treatments on the microbiological population, physicochemical and organoleptic properties of papaya has not been studied. An earlier study by Cia *et al*. (2007) used gamma and UV-C radiation on postharvest control of papaya anthracnose. Most recent publication on effect of gaseous and aqueous ozone in the postharvest of papaya cv. *Maradol-red* has shown positive control of fungal pathogens growth such as *Curvularia* spp., *Fusarium* spp., *Aspergillus* spp., *Sclerotium rolfsii*, *Phoma* spp., *Gliocladium* spp., and *Rhizoctonia solana* (Bataller *et al*.,

2012). With reasonable justification for this research, this study was carried out with the following objectives:

1. To study antifungal activity of ozone against *C. gloeosporioides* isolated from papaya fruit.
2. To study the mode of action of ozone in controlling anthracnose.
3. To evaluate the effect of ozone on production of plant defense inducible enzymes in papaya fruit during storage.
4. To examine the effect of ozone treatment on changes in microbial flora of papaya fruit.
5. To investigate the effect of ozone treatment on quality, physiological behaviour and sensory characteristics of papaya fruit during storage.
6. To study the gas exchange characteristics of ozone-treated papaya fruit.
7. To assess the effect of ozone treatment on changes in total phenols, total carotenoids and antioxidant activity of papaya fruit.
8. Quantitative analysis of genes expression in papaya fruit in response to ozone.

CHAPTER 2

LITERATURE REVIEW

2.1 Papaya *(Carica papaya L.)*

2.1.1 Origin and distribution

The papaya, *Carica papaya* L., is a member of the small family *Caricaceae* allied to the *Passifloraceae*. In some parts of the world, especially Australia and some islands of the West Indies, it is known as *papaw*, or *pawpaw* (Morton, 1987).

The papaya is an exotic tropical fruit native to Central America, and was first cultivated in Mexico. The flesh of the ripe papaya is bright orange-pink. The sweet-tart, musky flavor is likened to that of apricots and ginger (Filippone, 2007). They bring an exotic touch to savoury dishes and desserts. The deliciously sweet papaya was called the 'fruit of angels' by Christopher Columbus for its heavenly taste, but today, the fruit is easily found in markets around the world (Samantha, 2011).

The papaya plant is commonly and erroneously referred to as a "tree" (Figure 2.1). The plant is a large herb and grows at the rate of 6 to 10 ft (1.8-3.0 m) in the first year and reaches to 20 or even 30 ft (6-9 m) in height, with a hollow green or deep-purple stem becoming 12 to 16 inches (30-40 cm) or more thick at the base and roughened by leaf scars. The 5-petalled flowers are fleshy, waxy and slightly fragrant. Some plants bear only short-stalked

pistillate (female) flowers, waxy and ivory-white; or hermaphrodite (perfect) flowers (having female and male organs), ivory-white with bright-yellow anthers and borne on short stalks; while others may bear only staminate (male) flowers, clustered on panicles, 5 or 6 ft (1.5-1.8 m) long (Morton, 1987).

Figure 2.1: Papaya tree, flower and fruit (Köhler, 1887).

2.1.2 Nutritional composition

Generally, the fruit is melon-like, oval to nearly round, or elongated club-shaped, 6 to 20 inches (15-50 cm) long and 4 to 8 inches (10-20 cm) thick, weighing up to 20 lbs (9 kg). Semi-wild (naturalized) plants bear miniature fruits 1 to 6 inches (2.5-15 cm) long. The skin is waxy and thin but fairly tough. When the fruit is green and hard it is rich in white latex. As it ripens, it becomes light or deep-yellow externally and the thick wall of succulent flesh becomes aromatic, yellow, orange or various shades of salmon or red. It is then juicy, sweetish and somewhat like a cantaloupe in flavour; in some types quite musky (Morton, 1987).

The papaya is regarded as a fair source of iron and calcium, and is a good source of vitamins A, B and an excellent source of vitamin C (ascorbic acid). The carotenoid content of papaya (13.8 mg $100g^{-1}$ dry pulp) is low compared to mango (19.0 mg $100g^{-1}$), carrot and tomato. The major carotenoid is cryptoxanthin (Morton, 1987). The ascorbic acid content of papaya may range from 47 to 72 mg $100g^{-1}$ fruit fresh weight in green papaya, and from 80 to 145 mg $100g^{-1}$ fruit fresh weight in half-ripe papaya (Imungi and Wabule, 1990; Proulx *et al*., 2005).

Ripe papayas are most commonly eaten fresh, merely peeled, seeded, cut in wedges and served with a half or quarter of lime or lemon. Sometimes a few seeds are left attached for those who enjoy their peppery flavour but not many should be eaten. The flesh is often cubed or shaped into balls and served in fruit salad or fruit cup. Firm-ripe papaya may be seasoned and baked for

consumption as a vegetable. Ripe flesh is commonly made into sauce for shortcake or ice cream sundaes, or is added to ice cream just before freezing; or is cooked in pie, pickled, or preserved as marmalade or jam (Morton, 1987).

2.1.3 Papaya production and postharvest handling

Papaya is one of the promising tropical fruits traded in the world market. Global production of tropical fruits (excluding bananas) reached 73.02 million (M) metric tonnes (t) in 2010. Gaining in popularity worldwide, papaya is now ranked third with 11.22 Mt, or 15.36 % of the total tropical fruit production, behind mango with 38.6 Mt (52.86 %) and pineapple with 19.41 Mt (26.58 %) (FAOSTAT, 2012a). Global production in 2010 was 7.26 % higher than 2009, and 34.82 % higher than 2002, with an estimated value of about $197.2 million (FAOSTAT, 2012b). Malaysia, as one of the major exporters of papaya accounted for US$ 22.5 million in 2004 (Rabu and Mat Lin, 2005). The demand for of tropical fruits was stimulated by consumer preference, rising health consciousness, rising income, better postharvest technologies and the globalization of the supply chain.

Several varieties of papaya are grown in Malaysia: Subang 6, Taiping 3, Serdang 1, Setiawan and other foreign cultivars like the Honey Dew and Sunrise Solo. In Malaysia, the Eksotika II Papaya was released in 1991. It is a F1 hybrid developed from a cross between the Exotica and Line 19 (Chan, 1993). This hybrid is an improvement over the Exotica because of its smooth fruit skin and reduced blemishes (freckles). The fruit is also 20 % larger and

has a better keeping quality. The fruits are also very sweet (Brix 13-16 %), with orange red flesh and a pleasant aroma. The yield is 50-70 tonnes per hectare over a two-year crop cycle. The Eksotika II is also recommended as a table variety for the local market (MARDI, 2009). In 1986, the year before 'Eksotika' was released; the export revenue of papaya was a mere RM 3 million. The export revenue climbed steadily every year since then and by 2004, the year when export earnings were at its peak, the value was RM120 million (Anonymous, 2006a).

Long foot papaya is known locally as Papaya Sekaki or Hong Kong papaya. This papaya cultivar is the second most popular cultivated variety in Malaysia after Exotica. It is a cross-pollinated variety with medium sized fruit of 1.5-2.0 kg. Sekaki fruit is attractive with smooth, even-coloured and freckle-free skin. The flesh is red and firm but sugar content is not high, Brix 10 % or less (DOA, 2007).

According to Morton (1987), studies in Hawaii have shown that the flavour of papaya is at its peak when the skin is 80 % coloured. For the local market, in winter months, papayas may be allowed to colour fairly well before picking, but for the local market in summer and for shipment, only the first indication of yellow is permissible. The fruits must be handled with great care to avoid scratching and leaking of latex which stains the fruit skin. Home growers may twist the fruit to break the stem, but in commercial operations it is preferable to use a sharp knife to cut the stem and then trim it level with the base of the fruit.

Good and proper postharvest handling of papaya is important as the fruit is very susceptible to handling and storage damages before it reach to the consumer. In a tropical country, like Malaysia, with hot and humid conditions hasten ripening and are conducive to the development of fungal diseases, the main diseases attacking fruits and vegetables (Sepiah, 1993).

Papaya can be held at 85 °F (29.4 °C) and high atmospheric humidity (> 90 %) for 48 hours to enhance colouring before packing. Standard decay control has been a 20 minutes submersion in hot water at 120 °F (49 °C) followed by a cool rinse. In India, dipping in 1,000 ppm of aureofungin has been shown to be effective in controlling postharvest rot. In Philippine trials, thiabendazole reduced fruit rot by 50 %. In 1979, Hawaiian workers demonstrated that spreading an aqueous solution of carnauba wax and thiabendazole over harvested fruits gives good protection from postharvest diseases and can eliminate the hot-water bath. In Puerto Rico, gamma irradiation (25-50 krads) delayed ripening up to 7 days. Treatment at 100 krads slightly accelerated ripening in storage. Even at the lowest level irradiation inhibited fungal growth. Carotenoid content was unaffected but ascorbic acid was slightly reduced at all exposures. Partly ripe papayas stored below 50 °F (10 °C) will never fully ripen. This is the lowest temperature at which ripe papayas can be held without chilling injury (Morton, 1987).

2.2 Papaya anthracnose *(Colletotrichum gloeosporioides)*

Papaya anthracnose is caused by the fungus *Colletotrichum gloeosporioides* Penz. It has been considered a postharvest disease, implying that the

pathogen enters papaya tissue through wounds created during postharvest handling (Dickman *et al*., 1982). Indeed, the recent paper on the subject (Stanghellini and Aragaki, 1996) concluded that *C. gloeosporioides* was primarily a wound pathogen. However, an epidemiological study (Alvarez *et al*., 1977) has suggested that infection occurs in presumably intact tissues in the orchard. Furthermore, recent histological and ultrastructural studies (Chau, 1981) have shown that the infection hyphae penetrate the host's cuticular layer directly. This observation suggested that *C. gloeosporioides* might penetrate papaya cuticle by enzymatic means.

The fungus *Colletotrichum gloeosporioides* results in three types of symptoms, with different names: anthracnose, chocolate spots and grey-depressed lesion (Table 2.1). The initial symptoms are watersoaked, sunken spots from 8 to 25 mm in diameter on fruit. The centres of these spots later turn black and then pink when the fungus produces spores. Lesions may become as large as 50 mm in diameter. Pinkish-orange areas are formed by the conidial masses that cover the lesion centre and are frequently produced in a concentric ring pattern (Dickman, 1993). Symptoms may also appear as irregular to circular spots 1 to 10 mm in diameter, sharply defined, occasionally slightly depressed and reddish-brown in colour. These lesions are referred to as "chocolate spots." As the fruit ripens, these spots rapidly enlarge (up to 20 mm in diameter), to form the characteristic circular sunken lesions. *Colletotrichum gloeosporioides* also causes anthracnose on papaya leaves (Dickman and Alvarez, 1983).

Table 2.1: Major postharvest diseases of papaya (Paull *et al*., 1997).

Disease (s) and responsible organism	Symptom (s)
Anthracnose, chocolate spot; grey-depressed lesion; *Colletotrichum gloeosporioides* (Penz.) Sax.	Small, round, depressed areas on ripening portions of the fruit. The spots enlarge rapidly during fruit ripening forming circular, slightly sunken lesions. Green portions of the papaya may become affected and the disease appears as small, water-soaked lesions. These lesions enlarge very slowly and rarely become larger than 12 mm in diameter as long as the fruit remains green.
***Rhizopus* fruit rot (*Rhizopus stolonifer* (Ehr ex. Fr.) Lind.)**	*Rhizopus* invades injured mature fruit only, and does not usually cause rot in sound uninjured immature fruit.
Stem-end rot; various fungi	A dry, firm, dark rot extends into the fruit starting at the stem end.
Black spot (*Alternaria alternate* (Fr. Keisoler))	Tiny, dark, raised spots which turn black and enlarge to 0.75-3 mm in diameter. The tissue just beneath the epidermis of the fruit becomes corky but the spot does not develop into a fruit rot.

In the climate of south Texas, cultural control measures should be sufficient for control of this disease in the home garden. These measures include removing the fruit as soon as it matures, removing all dead leaves and fruit from the vicinity of the plants, and removing infected fruits from the trees. Under conditions of severe disease pressure that would be found in more rainy and humid climates, fungicides may be used. This disease is regularly seen in the field on ripe or overripe fruits and they are not a serious problem with unrefrigerated fruit sold in the local markets.

2.2.1 Morphology of *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides Penz. a facultative parasite belongs to the order *Melanconiales.* The fungus produces hyaline, a single cell, ovoid to oblong, slightly curved or dumbbell shaped conidia, 10-15 µm in length and 5-7 µm in width. Masses of conidia appear pink or salmon coloured (Figure 2.2). The waxy acervuli that are produced in infected tissue are subepidermal, typically with setae, and simple, short, erect conidiophores (Dickman, 1993). Isolations from the two different lesion types result in isolates of *C. gloeosporioides* indistinguishable culturally from one another. Each isolate is capable of producing the two different lesion types.

The pathogen initially infects intact, non-wounded immature green fruit in the field. Spores germinate and form appressoria on the fruit surface. The fungus, using its appressorium, enzymatically penetrates the cuticle and then remains as sub-cuticular hyphae until the post climacteric stage of fruit growth is attained. At this point, for reasons that are not understood, the fungus

resumes growth and causes the characteristic symptoms. Thus, papaya anthracnose has a latent stage in its development that is similar to many other anthracnose diseases of tropical fruits. Primary inoculum can be disseminated by wind or rain.

Figure 2.2: Basic morphology of *Colletotrichum gloeosporioides*

2.2.2 Control of papaya anthracnose

2.2.2.1 Physical control

Physical control involves handling procedures that minimise postharvest injury and hence the entry of microorganisms into the commodity, and also creating an external environment unfavourable to the growth of microorganisms. An important benefit of packaging is the protection it provides against damage during handling, transportation and cross contamination.

The use of low temperature during handling and storage is the most important physical method of controlling postharvest wastage. Heat treatments, using either moist hot air or hot water dips, are used commercially to control postharvest wastage in papaya, mango, stone fruits and cantaloupe (Wills *et*

al., 2007). The beneficial effect is at least partly due to the enhanced formation of lignin and related compounds, which prevent invasion by germinating mould spores. Hot water dips at 48 °C for 20 min is an effective method for reducing anthracnose incidence. Although hot water dips do not completely eliminate anthracnose but reduce the disease which is economically significant to the farmers.

UV radiation or ozone treatment can be directly used against postharvest pathogens. Irradiation of fruit, particularly citrus by UV light with 254 nm has been found to effectively inhibit mould growth. It appears to stimulate the fruit to increase its production of lignin in the surface layers and of endogenous phytoalexins, such as scoparone, that are the natural defence system of the fruit against microbial invasion (Wills *et al.,* 2007). The other important factors in minimising microbial growth are the usage of modified atmospheres with elevated carbon dioxide, reduced oxygen and reduced ethylene levels, and better control over humidity conditions.

Edible coatings may be used on the surface of fresh fruits to modify the internal atmosphere, decrease the transpiration loss, and delay ripening during postharvest storage and handling. Meanwhile, certain coating materials, such as chitosan have a strong antifungal ability (Zhao, 2010). Therefore, edible coating technology, when designed and applied correctly, is an effective means to control decay and extend shelf-life of some fruits (Ali *et al*., 2010). In addition, edible coatings may provide an excellent vehicle to further enhance the health benefit of fruits where the lack of some important

nutraceuticals, such as vitamin E and calcium, may be compensated by incorporating them into the coatings (Zhao, 2010).

2.2.2.2 Chemical control

The success of a chemical treatment depends on several factors, such as spore load, depth of the infection within the host tissues, growth rate of the infection, temperature and humidity, and depth to which the chemical can penetrate the host tissues. Moreover, the applied chemical must not be phytotoxic (i.e. must not injure the host tissues) and must be permitted for use by the local food regulations. The range of chemicals include alkaline inorganic salts (sodium tetraborate), ammonia and aliphatic amines (*sec*-butylamine), aromatic amines (dicloran), benzimidazole (benomyl), triazoles (prochloraz), hydrocarbons (biphenyl), oxidising substances (iodine), organic acids (sorbic acid), aldehydes (formaldehyde), inorganic compounds (sulphur, salicylanilide), fungicides (azoxystrobin, pyrimethanil), organic compounds (captan) and endogenous plant metabolites (essential oil). Chlorine and iodine are commonly added to wash-water to kill bacteria and fungi, and sulphur dioxide is lethal to *Botrytis* on grapes (Wills *et al*., 2007).

For papaya, with an appropriate protective fungicide, orchard sprays applied at 14 - 28 day intervals, depending on rainfall, is commonly recommended. Although no known cultivars of papaya offer complete resistance to anthracnose, the Hawaiian cultivar, Sunrise Solo, is more resistant than Kapoho Solo. Postharvest fungicides, applied as a spray or dip, with a food-

grade wax have also shown to be effective in reducing anthracnose. This is a common practice especially for fruits shipped to overseas markets.

2.2.2.3 Biological control

Biological control methods are often the main tools used to prevent the degradation of food crops. Interest in biological control methods for postharvest diseases has been increasing in recent years due to consumer dislike for synthetic chemicals applied to foods. Many biological agents including fungi, yeasts (*Cryptococcus magnus*) and bacteria may potentially be useful in controlling postharvest diseases. Biological control agents can suppress pathogens by a range of or combination of mechanisms. Direct attack, competition for space and nutrients and eliciting heightened host defence mechanisms are generally considered acceptable mechanisms (Wills *et al*., 2007).

2.3 Ozone

2.3.1 Definition, source and structure

Ozone is the highly unstable tri-atomic form of oxygen (O_3), formed by the addition of an oxygen atom to a molecular diatomic oxygen (O_2) (Figure 2.3) that was first identified in 1840 by Schönbein. It can be generated readily and economically for application to several commodities, including processed horticultural products (Artes *et al*., 2009). Ozone is a very pungent, naturally occurring gas with strong (highly reactive) oxidizing properties.

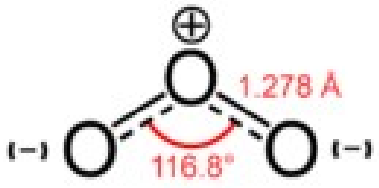


Figure 2.3 : Ozone structure

Ozone is generated naturally by ultraviolet irradiation from the sun and from lightning. It can be generated artificially and commercially by UV light (at 185 nm) or corona discharge. If a high concentration of ozone is desired, corona discharge is commonly used (Xu, 1999). Production of ozone occurs between two electrodes operated at a particular voltage, frequency, and geometry. The preferred method of generation is the dielectric barrier discharge (Becker *et al.*, 2005). Reactive oxygen species are generated which react with each other as well as oxygen molecules resulting in the formation of ozone, heat and light. The reaction can be summarized as shown in Figure 2.4. The most oxidative species in air and oxygen gas include ozone (O_3), singlet oxygen (O^-), superoxide (O_2^-), peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Gaunt *et al.*, 2006). Most of these species have very short half-lives (in the range of milliseconds) making them difficult to work with, however, ozone has a much longer half-life ranging from minutes to days depending on conditions (MKS Instruments, Inc., 2004).

Figure 2.4 : Ozone formation reaction

2.3.2 Properties and application of ozone

In clean, potable water free of organic debris and soil particulates, ozone is a highly effective sanitizer at a concentration of 0.5 to 2 ppm (1 mgl^{-1} = 1 ppm). Ozone's disinfectant activity is only marginally affected at a water pH from 6 to 8.5. Ozone is highly corrosive to equipment and is lethal to humans with prolonged exposure at concentrations above 4 ppm. Ozone is readily detectable by human smell at 0.01 to 0.04 ppm (Suslow, 1998).

The United States Federal Occupational Safety and Health Administration (OSHA) limit of exposure is 0.1 ppm threshold for continuous exposure during an 8-hour period and 0.3 ppm for a 15-minute period. At the concentration of 1 ppm ozone has a pungent disagreeable odour and is irritating to the eyes and throat. For high-dose applications, disinfection of recirculating postharvest water used in an open-flume, off-gas containment and destruction (conversion back to oxygen) is essential (Suslow, 1998). Each system design must be carefully evaluated to protect worker comfort and safety.

Gaseous ozone may be blown into the air space of a properly constructed postharvest storage facility or storage unit (Suslow, 1998). Introducing

gaseous ozone into postharvest storage facilities or refrigerated shipping and temporary storage containers is reported to be optimal at cooler temperatures and higher relative humidity (85 to 95 %). For aqueous applications, ozone is pulled into a water stream under the negative pressure created by a Venturi injection system. Excess ozone not dispersed in water must be captured and neutralized to prevent corrosion and personal injury. One method of neutralizing ozone is by using UV light at a longer wavelength (254 nm) combined with the use of a catalytic agent such as granular activated charcoal.

The half life of ozone in water at room temperature is only 20 minutes and it decomposes into oxygen, with no safety concerns about consumption of residual ozone in the treated food product (Graham *et al*., 1997). Moreover, ozone is not characterized as a carcinogen or mutagen. It does not accumulate in fatty tissue or cause long-term chronic effects (Pryor, 1998).

Ozone is a strong antimicrobial agent and is now approved for use on food by the United States of Food and Drug Administration (USFDA) (Federal Register, 2001). The potential use of ozone in the produce industry depends on the fact that as an oxidizing agent, it is 1.5 times stronger than chlorine and is effective over a much wider spectrum of microorganisms (Xu, 1999). Ozone can also destroy pesticides and chemical residues, such as chlorinated by-products (Langlais *et al*., 1991). Besides, it also has a unique property of auto-decomposition and will leave no toxic residues (Neff, 1998). It can eliminate undesirable flavour produced by bacteria and chemically

remove ethylene gas to slow down the ripening process (Rice *et al*., 1982). Ozone is very effective in removing ethylene through chemical reaction (Figure 2.5) to extend the storage life of many fruits and vegetables (Rice *et al*., 1982).

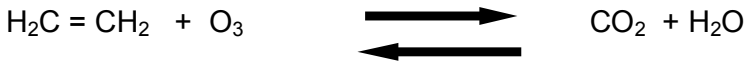


Figure 2.5 : Reaction of ozone with ethylene

Ewell (1940) carried out several studies on the application of gaseous ozone treatment to strawberries, raspberries and grapes, which doubled the shelf life when 2-3 ppm ozone was applied. Kuprianoff (1953) on prolonging apple shelf life to several weeks and Baranovskaya *et al*. (1979) on prolonging potato shelf life to as long as 6 months with 3 ppm ozone. Ozone has been evaluated as a sanitizer in the food industry, aiming at inactivating microorganisms (Karaca and Velioglu, 2007), as a removal agent of toxic substances such as mycotoxins (McKenzie *et al*., 1997) and pesticide residues (Ong *et al*., 1996), as well as for the extension of postharvest life of horticultural products through the ozone-mediated oxidation of ethylene (Skog and Chu, 2001). At doses required for effective disease control, phytotoxicity (localized product damage) has been identified in table grapes (fruit and rachis damage), carrots (bleaching) and tomatoes (calyx and vine / cluster desiccation and browning), which were stored in ozonated air.

2.3.3 Mode of action of ozone

Ozone has been reported to reduce the decay of some fruit and vegetables, but the results have been variable (Forney, 2003). The antimicrobial activity of ozone is obtained either directly through the progressive oxidation of vital cell components leading to an inhibition of microbial growth (Parish *et al*., 2003) or indirectly through the induction of plant defence mechanisms (Kangasjärvi *et al*., 1994).

The effects of ozone, and its primary decomposition metabolites, are associated with multiple reactions, including the inactivation of enzymes, alteration of nucleic acids, and oxidation of membrane lipids (Kim *et al.*, 2003). However, a higher inhibitory effect on mould development was observed when the treatments were done in inoculated fruit, relative to treatments of isolated fungi (Tzortzakis *et al*., 2008; Tzortzakis *et al*., 2007b). This suggests that at least part of the decay control observed in ozonated produce is related to fruit-mediated responses and is consistent with previous work showing the activation of defensive pathways in ozone-treated plants (Langerbartels *et al*., 2002).

In a study by Khadre and Yousef (2001), electron microscopic study of ozone-treated *B. subtilis* spores suggests that the outer spore coat layers are a probable site of action of ozone. Inspecting these micrographs revealed damage to the surface layer, the outer spore coat, and to some extent to the inner spore coat layer in ozone-treated spores, which may have lead to exposing the cortex to the action of ozone.

2.3.4 Antifungal properties of ozone

The efficacy of ozone has been evaluated against *Alternaria alternata* and *Colletotrichum coccides* on tomatoes (Tzortzakis *et al*., 2008), *Rhizopus stolonifer* on table grapes (Sarig *et al*., 1996), *Penicillium italicum* and *P. digitatum* on citrus fruit (Palou *et al*., 2002, 2003) and *Monilinia fructicola* on peaches (Palou *et al*., 2002).

Ozone treatments resulted in reduction of *B. cinerea* decay incidence on strawberries (Nadas *et al*., 2003), lesion development on tomatoes (Tzortzakis *et al*., 2007a) or carrots (Hildebrand *et al*., 2008), while ozone treatments had no effect on the development of grey mould on table grapes when applied at a concentration of 0.3µl l^{-1} (Palou *et al*., 2002).

2.3.5 Physiological and biochemical plant defense response of ozone

Ozone (O_3) is one of the most widespread and phytotoxic air pollutants to which vegetation is exposed as its concentration in the troposphere has dramatically increased over the past few decades. Sensitivity to ozone differs between species and even between cultivars and ecotypes (Heggestad, 1991 and Nali *et al*., 1998), but the underlying mechanisms responsible remain poorly understood. Selection of plant species which have shown differences in ozone sensitivity proved to be useful for investigating ozone responses. Scebba *et al*. (2003) focused on ozone responses, in terms of physiological and biochemical modifications of two different clover species, namely white clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.), that were exposed to an acute dose of ozone (3 h, 150 ppb).

Plants grown in elevated atmospheric ozone are known to undergo several biochemical changes before any actual damage can be detected. These reactions include increases in the activities of enzymes associated with general plant defence mechanisms. In addition to the direct effect of ozone on moulds and bacteria, it can trigger the accumulation of phytoalexins or activate other defense mechanisms (Tzortzakis *et al*., 2007b, Langerbartels *et al*., 2002 and Sandermann *et al.,* 1998). Ozone exposure often causes a surge in the production of the plant hormone ethylene, as well as changes in polyamine metabolism and increases in the activities of several phenylpropanoid and flavonoid pathway enzymes. The activities of superoxide dismutase and peroxidases that protect cells from the oxidative damage caused by hydroxyl radicals, H_2O_2 and superoxides also increase. However, ozone-induced changes in plant cells at the gene level are almost unknown (Kangasjärvi *et al*., 1994).

Plants have evolved a complex defence response mechanism to respond to various environmental stresses from morphological, biochemical and physiological changes triggered by ozone. Molecular and biochemical studies have suggested that the air pollutant ozone also stimulates phenol metabolism and biosynthesis of lignin or substances partly derived from coniferyl alcohol. Ozone stress or injury to plants can stimulate the production of phenolic compounds, including lignin and suberin. Ozone increases the salicylic acid level by participating in the regulation of ozone-induced phenylalanine ammonia-lyase (PAL) expression (Sgarbi *et al*., 2003).

In a study on tomato, ozone treatments induced the accumulation of phenolic compounds (Rodoni *et al.,* 2010) and this rapid response suggests that the modifications might have been due to the activation of pre-existing enzymes. Booker and Miller (1998) also found that ozone treatments induced phenylalanine ammonia-lyase (PAL), a key regulatory enzyme in the biosynthesis of phenolic compounds, and resulted in increased accumulation of caffeic and p-coumaric acid. The correlation between phenolic compounds and reduced fruit damage might favour hypotheses related to the involvement of fruit defense responses in preventing the spread of fungal pathogens.

2.3.6 Effect of ozone on spore mitochondria

Mitochondria are a primary source as well a principal target of reactive oxygen species within cells. Electron microscopy revealed a loss of matrix density and disorganization of inner membrane cristae upon oxidative stress. In an ultrastructural examination of control and hydrogen peroxide-treated cells, mitochondria from untreated cells contained a dense matrix and well-organized cristae, predominantly oriented transverse to the long axis of the mitochondria. In a study done by Cole *et* al. (2010), mitochondria from cells treated with glucose oxidase appeared swollen with decreased electron density within the matrix. The cristae were noticeably thinner and often misoriented and/or decreased in number. The swollen nature of the mitochondria suggests that osmotic effects may be one cause of this morphology. In addition, intra-mitochondrial amorphous deposits that likely represent matrix precipitates and degenerating cristae, that is, flocculent or ‘‘woolly” densities, were common (Chiba *et al*., 2005; Oberley *et al*., 2008).

The reduced number of cristae and their misorientation after oxidative stress is consistent with alteration of the inner membrane. Functional changes likely parallel these structural changes, and may alter the flux of ions, particularly potassium, across the inner mitochondrial membrane, resulting in osmotic dysregulation and matrix swelling. Conditions that cause increased production of ROS within mitochondria may permit signalling proteins, peptides, or other molecules to move from the mitochondria to the nucleus. Chronic or excessive oxidative stress may induce an excessive or prolonged increase in mitochondrial permeability (Cole *et al*., 2010).

Mitochondria are critical for cellular adenosine triphosphate (ATP) production; however, recent studies suggest that these organelles fulfil much broader range of tasks. For example, they are involved in the regulation of cytosolic Ca^{2+} levels, intracellular pH and apoptosis, and are the major source of reactive oxygen species (ROS). There has been increasing interest in the development of tools that simulate mitochondrial function, and the refinement of techniques that allow for real time monitoring of mitochondria, particularly within their intact cellular environment. Innovative imaging techniques are especially powerful since they allow for mitochondrial visualization at high resolution, tracking of mitochondrial structures and optical real time monitoring of parameters of mitochondrial function (Foster *et al*., 2006).

Mitochondria constitute the major source of superoxide (O_2^-) and other ROS within cells, generating approximately 85 % of total cellular O_2^-, via unusual O_2 reactions (Dröge, 2002). During enhanced mitochondrial activity or

respiratory chain inhibition, either chronic or acute, the generation of O_2^- may markedly increase, causing oxidative damage.

MitoTracker probes demonstrate binding to and fluorescent labeling of mitochondria. These probes mainly serve to mark mitochondrial structures, without yielding detailed functional information regarding dynamic changes in mitochondrial metabolism or membrane potential (Poot *et al*., 1996). MitoTracker dyes are available for various excitation and emission wavelength ranges, spanning from green to orange to red, and have been used for both one- and multiphoton excitation. Due to their rapid membrane permeability, mitochondrial labeling is easily performed by simple cell / tissue incubation. Once reaching the mitochondria, they bind covalently to peptidergic sulfhydryl groups (Buckman *et al*., 2001).

2.4 Scanning electron microscopic (SEM) studies

The scanning electron microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a faster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity.

Microscopic studies of the surface of fresh fruits and vegetables reveal structures which may be important in disease resistance and postharvest storage stability of the product. Transpiration, gas exhange, entry of

microorganisms, loss of aroma, and temperature reception depend upon the nature and distribution of epidermal structures (Pantastico, 1975). Scanning electron microscopy revealed the presence of stomata, trichomes, scars left by detached trichomes, and epidermal cells on the surface of fresh fruit (Smith and Fleming, 1979). Several scanning electron microscope studies have been carried out to evaluate the chemical properties and functional characteristics of unripe and ripe plantain (Osundahunsi, 2009), infection sites and lesion development on tomato fruit (Getz *et al*., 1983), floral structures (Chin and Phoon, 1982), apple and apricot (Bolin and Huxsoll, 1987).

Scanning electron microscopic study can be carried out to analyse the effect of ozone on fungal spore structure, mycelial distribution and on fruit surface. It has the ability to extend previous cytological information and open new avenues for additional research. For a better understanding on mechanism of ozone and relation of fungal resistance upon ozone exposure, it is necessary to solve questions concerning changes in fungus and mesocarp cells during ozone application.

2.5 Transmission electron microscopic (TEM) studies

The statement 'seeing is believing' expresses the importance of electron microscopy (EM) in basic and applied research. As documented by modern biology textbooks, transmission electron micrograph is a key instrument to most of the pictorial representations that foster our understanding of tissues, cells, organelles and biological nanomachines. Indeed, without electron micrograph it would be difficult to assemble the biochemical and atomic scale

structural information provided by X-ray crystallography and NMR into a meaningful model and place it in a cellular context (Müller *et al*., 2008).

Sample preparation in transmission electron microscopy was achieved by preventing dehydration of the specimen in the high vacuum of the microscope, and to preserve it in a close-to-native state. In these novel techniques, macromolecular complexes are vitrified in a liquid layer by rapid freezing (Dubochet *et al*., 1988), while cells and tissue samples are high-pressure frozen and cryo-sectioned (Al-Amoudi *et al*., 2004).

TEM provides a powerful tool capable of giving deep insight into protein structure. TEM can also be used to investigate tissues and the organization of organelles within large cells at nanometer scale resolution. This is done by combining classical embedding and sectioning with electron topography (Hess, 2007), or by cryo-EM of vitreous sections (Al-Amoudi *et al*., 2004). Since electrons interact strongly with matter it is possible to depict thin objects such as 2D protein crystals, viruses or single protein complexes. However, multiple scattering concurrent with loss of signal and image blurring, impose further limitations when imaging thick objects such as entire cells (Koster *et al*., 1997).

The only technique that allows for the visualization of mitochondrial ultrastructure is electron microscopy, applied as either classical transmission electron microscopy or scanning electron microscopy. Despite yielding the highest resolution of all imaging techniques, down to 0.2 nm, a major

drawback is that electron microscopy remains only applicable to fixed / dehydrated samples, as proper function of an electron microscope requires the material of interest to be analyzed under high vacuum condition. Labeling of cells and organelles, including mitochondria, for electron microscopy requires staining with electron-dense metal ions such as uranyl acetate or lead citrate (Foster *et al*., 2006).

Electron microscopy has been applied successfully to both isolated mitochondria and mitochondria still enclosed in their host cell environment to analyze organelle interactions, mitochondrial ultrastructure under normal and pathological conditions, and protein expression at the single organelle level (Foster *et al*., 2006). On the structural level, electron microscopy is useful for mapping the distortion of mitochondrial ultrastructure during metabolic or excitotoxic insults. Anoxia in isolated rat brain mitochondria caused shrinkage of the matrix space (Fujii *et al*.,2004), while hypoxia combined with increased Ca^{2+} concentrations disrupted mitochondrial integrity (Schild *et al*., 2003). Even though electron microscopy does not allow for functional studies or the dynamic probing of living mitochondria, it is indispensable in terms of its detailed morphological analysis providing unique insights into ultrastructure and protein expression of single organelles.

2.6 Gene expression

Gene expression is the process by which information from a gene is used or interpreted in the synthesis of a functional gene product. Gene expression is the most ultimate level at which genotype gives rise to the phenotype. The properties of the expression products give rise to the organism's phenotype.

2.6.1 cDNA-AFLP

In an adaptation of the amplified fragment length polymorphism (AFLP) technique, whole-genomic DNA is replaced with reverse transcriptase polymerase chain reaction (RT-PCR)–derived cDNA preparations and this is termed cDNA-AFLP. This technique is also fragment-based and assays genome-wide gene expression. Theoretically, it is capable of analyzing all genes involved in a particular biological process or expressed under certain conditions. Unique transcript tags of expressed genes are PCR amplified and visualized on polyacrylamide gels. This is used principally for transcript profiling and permits the display and quantification of transcripts based on AFLP fingerprinting of double-stranded cDNA. This is applied in the identification of differentially expressed messenger RNA (mRNA).

A critical part of any assay is data management. This is true also for AFLPs, particularly because an appreciable number of data points are generated on a typical gel. For fluorescent-based AFLPs, the outputs from GeneScan are exportable to the analytical software, Genotyper, which is capable of identifying and measuring bands within the size range of 50–500 bp. The GeneScan software itself has the capacity for analyzing four fluorescent labels that have the pseudo-colors of blue, green, yellow, and red. In the case of autoradiograms or silver stained displays, the bands could be manually read off the X-ray film, photographic paper, the gel, or a scanned image of the gel. The AFLP-Quantar and the update AFLP-Quantar-Pro from Keygene, developed specifically for AFLP image analyses, are the most widely used AFLP data management software. In addition to scoring for

presence / absence of bands, this software is also capable of using the band intensities to score for heterozygosity or homozygosity of the bands. Such other software as Gene ImagIR and SAGA GT (both from LI-COR, Inc., Lincoln, NE) and RFLP Scan (Scanalytics, Inc., Fairfax, VA) also may be used for collecting and scoring AFLP data. In general, a binary matrix is constructed based on scoring the migrating bands as present or absent (Tohme *et al*., 1996).

One of the greatest advantages of the AFLP technique is its capacity to generate many polymorphic bands per assay. Robinson and Harris (1999) cited many studies showing that in general, the AFLP technique produced up to four times more polymorphic loci per primer combination than the comparable techniques of RAPDs, RFLP, and SSRs. In addition, where the genome of the organism being assayed has not been characterized sufficiently so as to have target sequence information, AFLP will remain the molecular marker of choice. It shares this advantage with RAPDs and RFLP, but the high reproducibility of the polymorphisms (as compared with RAPDs) and the ease of use, particularly its being PCR-based (as compared with RFLP), make this technique quite superior to the other ones.

AFLP analysis of phenetic organization and genetic diversity has also been used to study domestication trends in crops such as cowpea (Coulibaly *et al*., 2002) and Omami banana cultivars (Opara *et al*., 2010). Furthermore, Tohme *et al*. (1996) had applied this technique in analyzing the gene pools of a wild bean collection. Information on genetic diversity usually based on ecotypic

differences was also generated using AFLP assays for wild barley (Turpeinen *et al*., 2003), alfalfa (Sergovia-Lerma *et al*., 2003), *Brassica juncea* (Srivastava *et al*., 2001), *Carica papaya* (Kim *et al*., 2002), *Curcubita pepo* (Ferriol *et al*., 2003), aromatic grapevines (Fanizza *et al*., 2003), *Arabidopsis thaliana* (Erschadi *et al.,* 2000), plantain (Ude *et al*., 2003), wheat (Barrett and Kidwell, 1998), faba beans (Zeid *et al*., 2003), chicory (van Custem *et al*., 2003), rhodesgrass (Ubi *et al*., 2003), rapeseed (Lombard *et al*., 2000), *Caladium bicolor* (Loh *et al*., 1999), and sugar beet (de Riek *et al*., 2001).

Although a gene does not have to be up- or down-regulated to play a key role in a certain process, screening for differentially expressed genes is one of the most straightforward approaches to unravel the molecular basis of a biological system. As a differential screening method, cDNA-amplified fragment length polymorphism (cDNA-AFLP) is more stringent and reproducible than many others and can amplify low-abundance transcripts (Lievens *et al*., 2001). In contrast to most hybridization-based techniques, it can distinguish between highly homologous genes from gene families without any prior knowledge of the sequence (Qin *et al*., 2001). A combination of bulked segregant analysis (BSA) (Michelmore *et al*., 1991) and the 'double pseudotest cross' (Hemmat *et al*., 1994) theory, cDNA-AFLP is an effective method to screen differentially expressed genes related to apple fruit acidity.

The possibility to discover large amounts of expressed (i.e., protein coding) sequence information (expressed sequence tags, ESTs) in non-model organisms offers an unique chance to screen and detect molecular variation

of genes at a genome-wide level, and to discover the polymorphisms that affect the success of populations / species in natural environments (Vera *et al*., 2008). The cDNA-AFLP is commonly used in conducting genome-wide transcription profiling to explore genes involved in various biological phenomena and to detect variation in gene expression between individuals and groups. The standard AFLP (amplified fragment length polymorphism) DNA fingerprinting method, which is commonly applied to study variation in genomic DNA, involves restriction endonuclease digestion of genomic DNA, adaptor ligation to the DNA fragments, and pre- and selective amplifications using primers containing adaptor sequences with zero to three arbitrary nucleotides, followed by electrophoretic separation of the amplification products (Vos *et al*., 1995). When applied to cDNA, it is capable of finding genes that have not been cloned but have interesting expression patterns under the studied conditions. The method is also known to have high sensitivity, particularly the ability to detect low-abundance transcripts (Fukumura *et al*., 2003).

2.6.2 Quantitative analysis of gene expression (qPCR)

Quantitative real-time polymerase chain reaction (QRT-PCR) has become an extensively applied technique. It enables quantitative analyses of gene expression applicable to basic molecular biology, medicine, and diagnostics. Nowadays, it is broadly used to describe messenger RNA (mRNA) expression patterns and to compare the relative levels of mRNA within distinct biological samples. The scope of the QRT-PCR technique makes it applicable across a

wide range of experimental conditions and allows experimental comparison between normal and abnormal tissue (Jozefczuk *et al*., 2010).

Quantitative real-time polymerase chain reaction (QRT-PCR) enables the continuous monitoring of the amplification process as it occurs and uses fluorescent reporter dyes to merge the amplification and detection steps. The assay is based on measuring the increase in fluorescent signal, which corresponds to the amount of DNA produced during each PCR cycle. A single PCR reaction is characterized by the PCR cycle at which fluorescence first rises above threshold background levels (threshold cycle, Ct). Hence, higher the messenger RNA (mRNA) concentration of a target gene, lower is the Ct value. The process of QRT-PCR consists of three steps: reverse transcriptase-based conversion of RNA to cDNA, the amplification of cDNA by PCR, and the detection and quantification of amplified products-referred as amplicons. The sensitivity of QRT-PCR has enabled the investigation of gene expression during oogenesis and embryogenesis in mouse, bovine, and human (Adjaye, 2005, 2007; Adjaye *et al*., 2005; Kues *et al*., 2008; Zuccotti *et al*., 2008).

As QRT-PCR is now a common method for quantifying gene expression, it is imperative to be aware of the various options available to enable the execution of a successful reaction (Wong and Medrano, 2005). A well designed experiment carried out with the appropriate controls can be the most sensitive, efficient, and reproducible assay to measure gene expression.

Realtime PCR, sometimes referred to as kinetic PCR, is a process by which a fluorescent signal is generated as the target sequence is amplified, and this fluorescent signal is measured in the reaction tube, during the PCR process (Heid, 1996; Gibson, 1996). There are two fundamental techniques used to generate this fluorescent signal. One is to include a dye, such as SYBR® Green, which binds double-stranded DNA. In this technique, any amplicon that is synthesized will bind the dye and generate a fluorescent signal that is detected by the instrument. The second technique, more widely used, uses a sequence specific fluorescent probe that hybridizes to the target sequence during the annealing and extension phases of the reaction.

2.7 Summary and concluding remarks

Papaya is a tropical crop with significant export potential. Nutritionally, it is a good source of iron, calcium, pro-vitamin A, ascorbic acid and antioxidants. Papaya like other tropical fruits has a very short shelf life, which limits its export potential. Extension of shelf life will help open the way to potential new markets that do not need to depend on refrigerated transportation of papaya fruits and subsequently reduce postharvest losses. The most common postharvest disease observed in the market chain is caused by the fungus *Colletotrichum gloeosporioides* and is a major disease in tropical countries.

Moreover, the incidence of foodborne illness attributable to food pathogens and chemicals has greatly increased from time to time. This has been the main concern for the public and government. As a result and given the health conscious consumers demand for “fungicide-free fruits”, the development of

safe approaches for controlling postharvest disease is ongoing. Research and commercial applications have indicated that ozone can replace chlorine and fungicide with additional benefits. Ozone is a strong oxidant effective in controlling bacteria, moulds, protozoa, and viruses. Also, it does not exhibit any negative sensory attributes on produce and does not leave toxic residues in the environment.

Therefore, based on the literature to date and the key issues affecting the papaya industry, this study was conducted to evaluate the direct antifungal effect of ozone for the preservation of tropical fruit, in particular papaya. The effectiveness of ozone in controlling the fungal anthracnose disease was studied using scanning electron microscope, transmission electron microscope and laser scanning confocal microscope. The indirect antifungal effect of ozone was also assessed by studying the production of defence-related enzymes in papaya fruit.

This ozone technology could be adopted to protect freshly harvested papaya for the effective control of anthracnose. However, little is known on the effect of ozone on the nutritional value and eating quality of the fruit. Therefore, further research is needed to examine the effect of ozone on physical and biochemical quality, physiological behaviour, antioxidant activity and organoleptic properties of papaya during storage. Further to that, quantitative analysis of gene expression by cDNA-AFLP and qPCR were proposed to analyse potential genes expressed in ozone-treated and non-treated papaya fruit. Screening and exploring the potential genes in papaya fruit involved in

response to ozone is vital because the molecular diagnostic is more sensitive and can provide a clearer picture of what could be induced by ozone treatment.

In short, this study is aimed to investigate ozone as a potential antifungal agent to maintain quality and overcome fungal disease of papaya during storage, particularly of the fungus *Colletotrichum gloeosporioides* in order to prolong the shelf life and marketability of the crop and can be used as an alternative to synthetic fungicides.

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter describes the general methods that are common to all experiments. The specific methods for each experiment are mentioned in the respective chapters.

3.1 Plant material

Mature-green papaya cv. 'Sekaki' with colour index 2 (green with trace of yellow) (Figure 3.1) were obtained on the day of their harvesting from a local fruit wholesaler at Pasar Borong Selangor, Seri Kembangan, Malaysia. Fruit of uniform size (800-1200 g), shape, maturity, free from any sign of mechanical injury, and insect or pathogenic infection free were selected for the studies. They were washed with distilled water and air-dried at ambient temperature (25-28 °C) before ozone treatment.

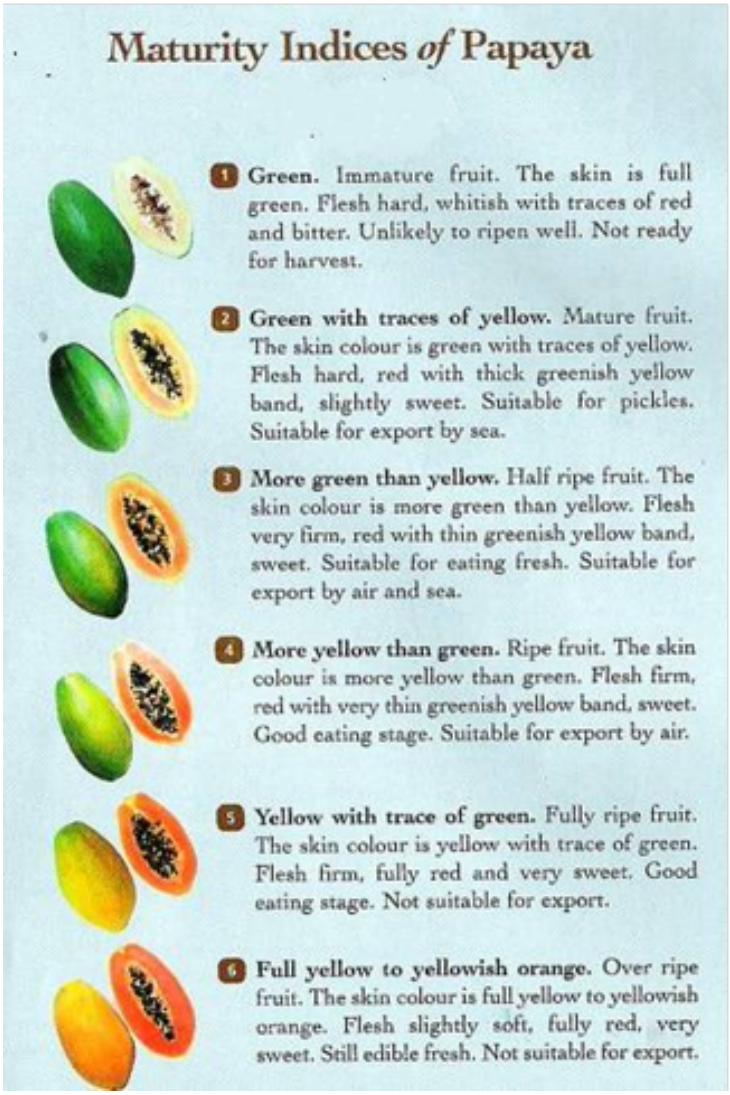


Figure 3.1: Maturity indices of papaya (Anonymous, 2006b)

3.2 Ozone fumigation

The fumigation system, comprised of chambers constructed from 5 mm thick polycarbonate (112.0 cm length x 47.5 cm width x 42.5 cm height) equipped with four 12 V fans positioned directly below the sample platform to ensure a well mixed atmosphere, was set up in the postharvest laboratory, School of Biosciences, University of Nottingham, Malaysia Campus (Figure 3.2). Ozone was introduced to each chamber by an ozone generator (Model MedKlinn Professional Series, MedKlinn International Sdn. Bhd., Malaysia) and the ozone concentration was controlled automatically by OEM-2 sensor (Eco-Sensor, MedKlinn International Sdn. Bhd., Malaysia). The ozone concentration in each chamber was recorded via an ozone analyzer (Model IN-2000 L2-LC, IN USA Incorporated, USA). The chambers were maintained at 25 ± 3 °C and 70 ± 5 % relative humidity (RH), in a controlled environment laboratory with the aid of a portable dehumidifier (Edenaire, Model ED 50106S, Enmark (M) Sdn. Bhd., Malaysia), 24 hours air-conditioned, and monitored by temperature/humidity sensors (Model U14-001, HOBO LCD Data Logger, Onset Computer Corporation, USA).

A replicate of 10 fruits were placed in a single layer of the chamber. Each chamber was prepared as a replicate. There were four replications for each ozone treatment. Fruit were fumigated with ozone concentrations of 1.5, 2.5, 3.5 or 5.0 ppm for 96 h.

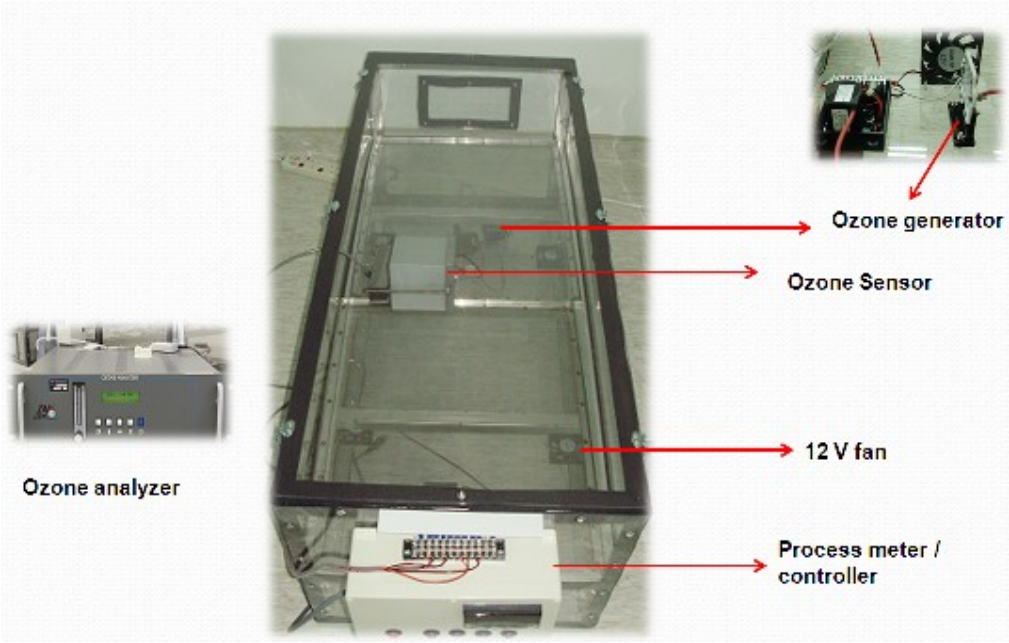


Figure 3.2: Ozone chamber

3.3 Experimental design and statistical analysis

Treatments were arranged in a completely randomized design. Experimental data were analyzed using the analysis of variance (ANOVA) procedure in SAS 9.1 software. Significant differences among treatment means were analyzed using Duncan's Mutiple Range Test (DMRT, at $p < 0.05$ level). All percentage data were arcsine transformed before analysis. There were four replications per treatment. The entire experiment was repeated twice, and data were pooled before analysis.

CHAPTER 4

EFFECT OF OZONE ON ANTHRACNOSE CAUSED BY *C. gloeosporioides*

4.1 Introduction

Papaya (*Carica papaya* L.) belongs to the family Caricaceae and is rapidly becoming an important fruit internationally, both as fresh as well as a processed product (Paull *et al*., 1997). Papaya (*Carica papaya* L.) is a climacteric fruit susceptible to postharvest losses due to the ethylene-induced ripening. The fruits of papaya cv. 'Sekaki' were found to be highly susceptible to postharvest fungal diseases. Among them, anthracnose caused by *Colletotrichum gloeosporioides* was the most prevalent, where disease incidence and severity was recorded as 90-98 % and 25-38 %, respectively (Rahman, 2008).

Currently there are several postharvest technologies adopted to control anthracnose in papaya which include the use of prochloraz or propiconazole (Sepiah, 1993), hot water treatment at 43-49 °C for 20 min (Couey *et al.,* 1984), heat treatment in combination with fungicides (Couey and Farias, 1979), edible coatings (Ali *et al*., 2010) and use of gamma rays in combination with UV-C irradiation (Cia *et al*., 2007).

One approach to improve safety, quality and shelf life is to identify alternative postharvest treatments to replace the traditional ways of using chlorine or fungicides. The ideal treatment would be non-toxic, biodegradable, and non-residual with a high efficacy as a microbial agent while retaining the nutritional value of the food. Previous research and commercial applications have indicated that ozone could replace chlorine treatment and fungicides. Ozone is a strong oxidant effective in controlling bacteria, moulds, protozoa, and viruses (Kim *et al*., 2003). In 1997, ozone was granted 'Generally Recognized as Safe (GRAS)' status (US-FDA, 1997) and has since received full US-FDA approval (Kim *et al*., 1999).

To the best of our knowledge, in spite of its growing importance, there is no published work on the potential of ozone fumigation against anthracnose disease of papaya. Therefore, the present study was designed to investigate the efficacy of gaseous ozone on *in vitro* growth of *C. gloeosporioides, in vivo* control of papaya anthracnose as well as to study the mode of action of ozone in controlling anthracnose.

4.2 Materials and Method

4.2.1 Isolation and identification of *C. gloeosporioides* from papaya fruits

4.2.1.1 *C. gloeosporioides* source, collection and fungal isolation

Papaya fruit showing typical anthracnose symptoms or lesions of rot were examined under a NIKON Eclipse 80i optical microscope (Nikon Instruments Inc., USA). Small pieces of tissue were excised from the border of an actively growing lesion and surface sterilized with 1.0 % sodium hypochlorite (NaOCl) for 2-3 min, followed by double washing with sterile distilled water.

4.2.1.2 Preparation of *C. gloeosporioides* inoculums on PDA

The tissues were dried on a sterilized filter paper, plated on Potato Dextrose Agar (PDA) (Difco Brand, USA), and incubated at 25 $\pm$ 3 °C for 8 days.

4.2.1.3 Microscopic identification of *C. gloeosporioides*

Once mycelial growth was observed, the colonies were re-isolated on fresh PDA plates to obtain pure cultures. The isolates were identified based on their morphological and cultural characters (Barnett and Hunter, 1972). Cultures identified as *C. gloeosporioides* were maintained on PDA slants for further use.

4.2.2 *In vitro* antifungal assay of ozone against *C. gloeosporioides*

4.2.2.1 Antifungal assay

The *in vitro* antifungal activity of ozone was determined based on inhibition of radial mycelia growth and conidia germination of *C*. *gloeosporioides* using the

poisoned food technique (Kiran, *et al*., 2010). Mycelia plugs (5 mm) obtained from 8-days old cultures of *C. gloeosporioides* were transferred to the centre of petri dishes containing 20 ml of PDA. Subsequently, the lids were left open under aseptic conditions in order to allow exposure to ozone enriched atmospheres with a concentration of 0, 1.5, 2.5, 3.5 or 5.0 ppm for 96 h of exposure. A replicate of 40 petri dishes were placed in a single layer of the chamber. Each chamber was prepared as a replicate and there are four replications for each ozone treatment.

4.2.2.2 Radial mycelia growth

Daily radial measurements of mycelia growth were recorded until the fungus reached the edge of the control plate. The percentage inhibition in radial growth (PIRG) was recorded after 8 days of incubation according to the formula described by Sivakumar *et al.* (2002).

$$\% \text{ Inhibition} = \frac{R1 - R2}{R1} \times 100$$

where, R1 = Radial growth in control;

R2 = Radial growth in treatment

4.2.2.3 Conidial germination test

The *in vitro* conidial germination inhibition test was carried out using the cavity slide technique (Cronin *et al.,* 1996). An aliquot of 10 µl of a freshly harvested conidial suspension of *C. gloeosporioides* (adjusted to 10^5 conidia ml^{-1} using a hemocytometer) was pipetted into the cavity and exposed to ozone concentrations of 0, 1.5, 2.5, 3.5 or 5.0 ppm for 24 hours in the dark environment. Cavity slides without exposure to ozone served as controls.

After 24 hours of incubation, the conidia were killed by adding 10 µl 2 % sodium azide to each cavity. Approximately 100 conidia were observed for germination in each treatment. A conidium was said to be germinated when its germ tube was half the length of the conidium. The percentage of inhibition in germination was calculated:

% Germination inhibition = 1- (Gr/Gc) x 100

where, Gr = Number of spore germination in the treatment;

Gc = Number of spore germination in the control

4.2.2.4 Morphology studies

The shape, size and colour of the conidia were observed under an optical microscope before and after incubation at 25 °C for 10 days on PDA and images of the conidia were taken using camera attached to a microscope. In addition, a drop of conidial suspension (10^5 conidia ml^{-1}), was inoculated on cellophane membrane on a slide glass and incubated for 24h at 25 °C to induce appressoria formation.

4.2.3 *In vivo* antifungal assay of ozone against C*. gloeosporioides*

4.2.3.1 Pathogen inoculum preparation

Pathogen inoculum was prepared by pouring 10 ml sterilized distilled water onto a two-week-old culture of *C. gloeosporioides*. The conidia were dislodged from the surface with a sterile bent glass rod. The conidial suspension obtained was filtered through a double layered sterilized muslin cloth. The conidial counts were adjusted to 10^5 conidia ml^{-1} using a hemocytometer (Sivakumar *et al*., 2002). Surface cleaned papaya fruit were

inoculated artificially by dipping in a 1 litre of conidial suspension of *C. gloeosporioides* with 0.2 % (v/v) of Tween-80 for 1 min. The fruit were then allowed to air dry for one hour, followed by ozone treatments with a concentration of 0, 1.5, 2.5, 3.5 or 5.0 ppm for 96 hours. A replicate of 10 fruits were placed in a single layer of the chamber. Each chamber was prepared as a replicate for each ozone concentration. There are four replications for each ozone treatment.

4.2.3.2 Disease incidence

For each treatment, fruit were packed in corrugated cardboard boxes and stored at ambient storage (25 ± 3 °C and 70 ± 5 %RH) for 14 days. Disease incidence (DI) was recorded based on the anthracnose symptoms on fruit surfaces. The effect of ozone on disease incidence and severity for 40 fruits from each treatment were evaluated at 2 days intervals for 14 days during ambient storage. Disease incidence was expressed as the number of fruit showing anthracnose out of the total number of fruits in each treatment (Sivakumar *et al*., 2002).

$$DI\ \% = \frac{\text{Number of infected fruit}}{\text{Total number of fruit assessed}} \times 100$$

4.2.3.3 Disease severity

Disease severity was scored at 2 day intervals as per the method of Sivakumar *et al.* (2002) with some modification. A rating of 1 was scored when there was no sign of anthracnose disease on the fruit surface. A rating of 2 was scored when up to a quarter (1-25 %) of the fruit surface showed anthracnose symptom. If 26 % to 50 % of the fruit surface exhibited

anthracnose symptom, a rating of 3 was scored. If anthracnose symptom appeared on 51 % to 75 % of the fruit surface, then a rating of 4 was scored. A rating of 5 was recorded when more than 76 % of the fruit surface was infected with anthracnose disease.

4.2.4 Scanning Electron Microscopy (SEM)

4.2.4.1 SEM on *C. gloeosporioides* spore structure

Four spore samples from each treatment were mounted on aluminum stubs and viewed and photographed under the scanning electron microscope (Model Quanta 400 FESEM, FEI Company, USA). Any changes in spore structure and shape were visualized. The results obtained were compared with 'control samples', the spore samples that did not receive ozone treatment.

4.2.4.2 SEM on *C. gloeosporioides* mycelial structure

Four mycelial samples from each treatment were mounted on aluminum stubs and viewed and photographed under the scanning electron microscope (Model Quanta 400 FESEM, FEI Company, USA). Any changes in mycelial structure and shape were visualized. The results obtained were compared with 'control samples', the mycelial samples that did not receive ozone treatment.

4.2.4.3 SEM on papaya fruit surfaces

Images of four fruit from each treatment were viewed under the scanning electron microscope. Samples of 5 mm^2 of papaya surfaces were taken from

the equatorial section of each fruit (twenty samples in total). The samples were mounted on aluminum stubs and viewed and photographed under the scanning electron microscope. Changes to the cuticular surface of papayas were visualized.

4.2.5 Effect of ozone on generation of reactive oxygen species in *C. gloeosporioides*

The oxidant-sensitive stain 2,7-dichlorodihydrofluorescein diacetate (DCHF-DA) was used to assess the intracellular ROS levels in *C. gloeosporioides* according to the method of Chen and Dickman (2005). An aliquot of 100 µl of a freshly harvested spore suspension of *C. gloeosporioides* (adjusted to 10^5 spores ml^{-1} using a hemocytometer) was pipetted onto PDA, distributed evenly with a spreader and exposed to ozone concentrations of 1.5, 2.5, 3.5 and 5.0 ppm for 24 h at 25 °C. The control comprised of PDA inoculated with spore suspension of *C. gloeosporioides* without exposure to ozone. After ozone exposure, the spores were washed with 10 mM potassium phosphate buffer, incubated for 5 min in the same buffer containing 10 µM DCHF-DA (dissolved in dimethyl sulfoxide), washed twice with potassium phosphate buffer and examined under a NIKON AZ100 fluorescence microscope (Nikon Instruments Inc., USA) to determine the percentage of spores stained by DCHF-DA in each treatment. DCHF-DA is commonly used to determine intracellular ROS, particularly H_2O_2 (Chen and Dickman, 2004). In each treatment, approximately 100 spores were examined. Each treatment contained three replicates.

4.2.6 Effect of ozone on mitochondria of *C. gloeosporioides*

4.2.6.1 Laser scanning confocal microscopy

The spore suspension (10^5 spores ml^{-1}) was incubated on PDA medium and exposed to ozone concentrations of 1.5, 2.5, 3.5 and 5.0 ppm for 24 h at 25 °C. Spores without ozone exposure served as the control. MitoTracker® Orange CMTMRos probes (Invitrogen) were added to the fungal inoculum at final concentrations of 500 nM for 5 min. Fluorescence of MitoTracker® Orange CMTMRos stained spores was detected using a Zeiss LSM 5 PASCAL Exciter laser scanning confocal microscope (LSCM) (Germany). At least 20 spores were examined for each treatment with three replications.

4.2.6.2 Transmission electron microscopy (TEM)

For TEM analysis, the spores treated with 0, 1.5, 2.5, 3.5 and 5.0 ppm ozone for 24 h at 25 °C as described above, were fixed overnight in 4 % glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB) pH 7.2, and then centrifuged (5,000 ×g for 10 min at 4 °C.). The supernatant of SCB was discarded and 1–2 mm^3 of serum gel was added to the pellet. After thorough rinsing with 0.1 M SCB, the gels were post-fixed with 1 % osmium tetroxide in 0.1 M SCB for 4 h at 4 °C, and dehydrated with 15 min stages in an ascending acetone series. The samples were embedded in Spurr's resin in beam capsules. The beam capsules were polymerized in an oven at 60 °C for 48 h before sectioning. Ultrathin sections were obtained using a diamond knife, stained by soaking in a 2 % uranyl acetate for 15 min and post-stained in lead citrate for 10 min. The sections were examined in a Hitachi H7100 transmission electron microscope (Japan) at 80 kV.

4.3 Results

4.3.1 *In vitro* antifungal assay of ozone against *C. gloeosporioides*

4.3.1.1 Radial mycelia growth

Mycelia growth of *C. gloeosporioides* was significantly ($p < 0.05$) affected by all ozone treatments as compared to the control following the 8 day incubation period. Maximum inhibition of 41.2 % in mycelia growth was observed at 96 hours with 5 ppm ozone exposure. Mycelia plugs that were exposed for 96 hours to ozone at concentrations of 3.5 ppm (14.1 % inhibition), 2.5 ppm (9.4 % inhibition) and 1.5 ppm (7.1 % inhibition) suppressed the mycelia growth compared to the control (Figure 4.1). Growth in the control plates was almost 1.7 times higher than the 5 ppm ozone at the end of the incubation period (Figure 4.2). This has shown that percentage inhibition in radial mycelia growth of *C. gloeosporioides* was significantly increased with ozone concentration.

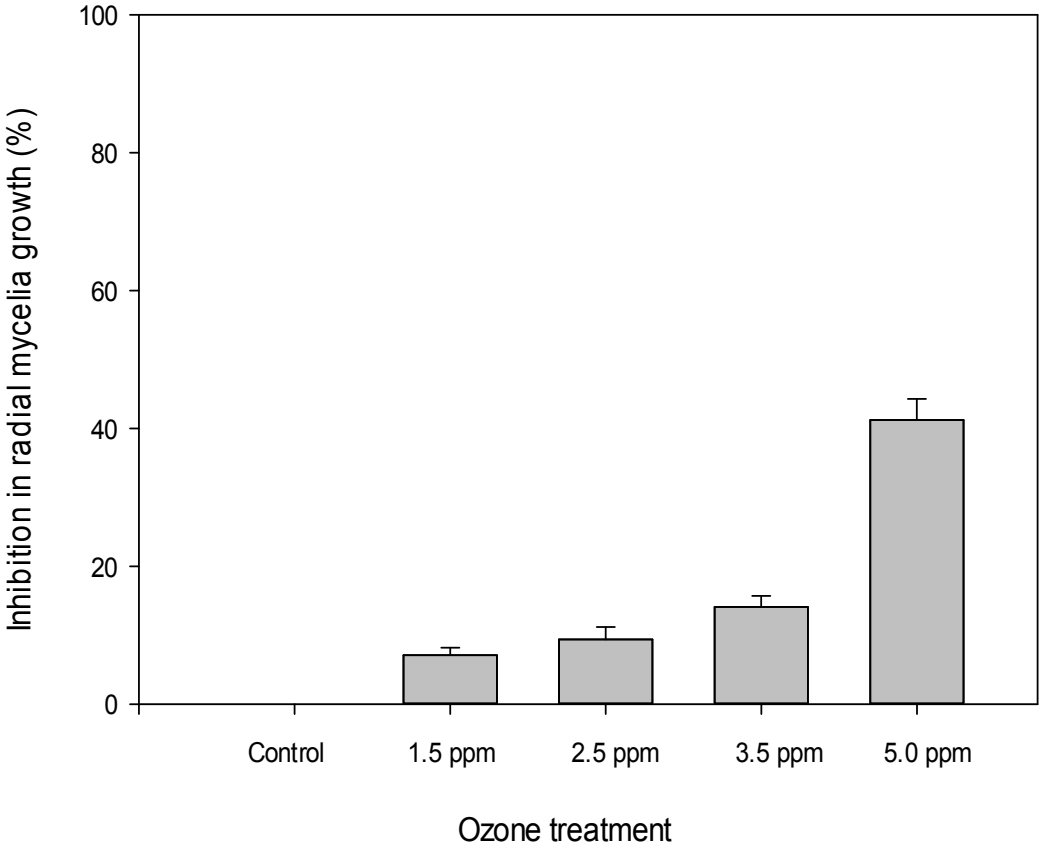


Figure 4.1 : Percentage inhibition in radial mycelia growth of *C. gloeosporioides* in all ozone treatments as compared to control following an 8 d incubation period at room temperature (25 ± 3 °C). Values are mean ± SE.

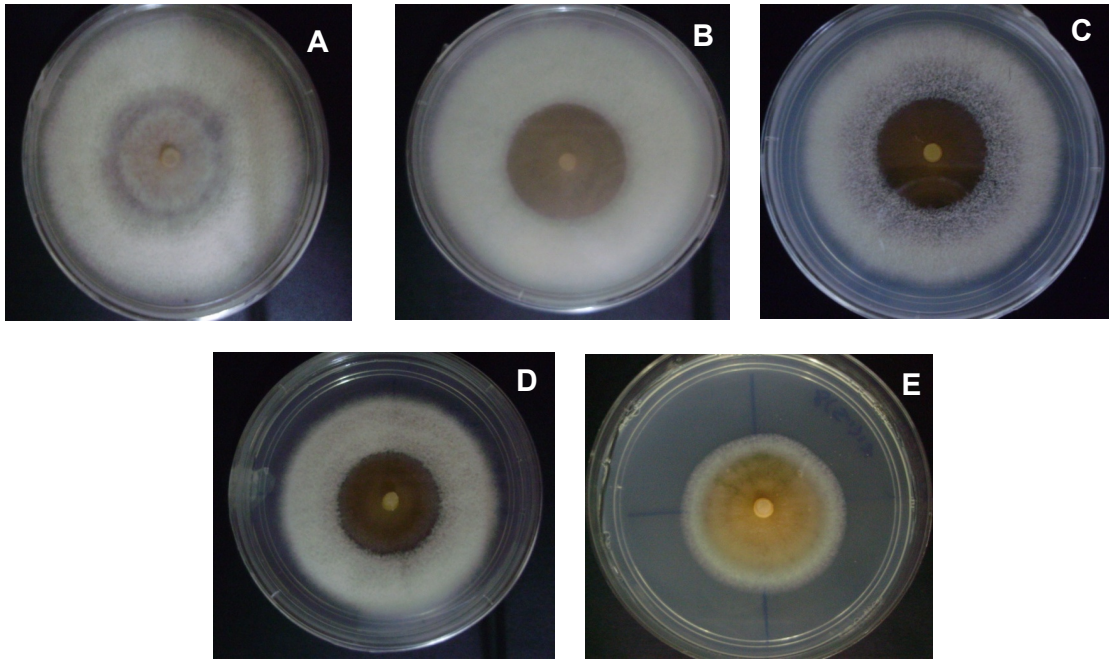


Figure 4.2: Effect of different concentrations of ozone on mycelial growth of *C. gloeosporioides* after eight days of incubation at 28 ± 2 °C. Control (A), 1.5 ppm (B), 2.5 ppm (C), 3.5 ppm (D) and 5 ppm (E) ozone.

4.3.1.2 Conidial germination test

The ozone treatments significantly ($p < 0.05$) inhibited conidial germination as compared to the control after 24 hours of incubation. All ozone treatments at 1.5, 2.5, 3.5 and 5.0 ppm completely inhibited conidial germination by 100 % (Figure 4.3). However, if the spores were incubated following the ozone treatments, they were able to germinate and the ozone treatment was merely inhibitory. The control (untreated) spores of *C. gloeosporioides* germinated with the emergence of germ tube (Figure 4.4).

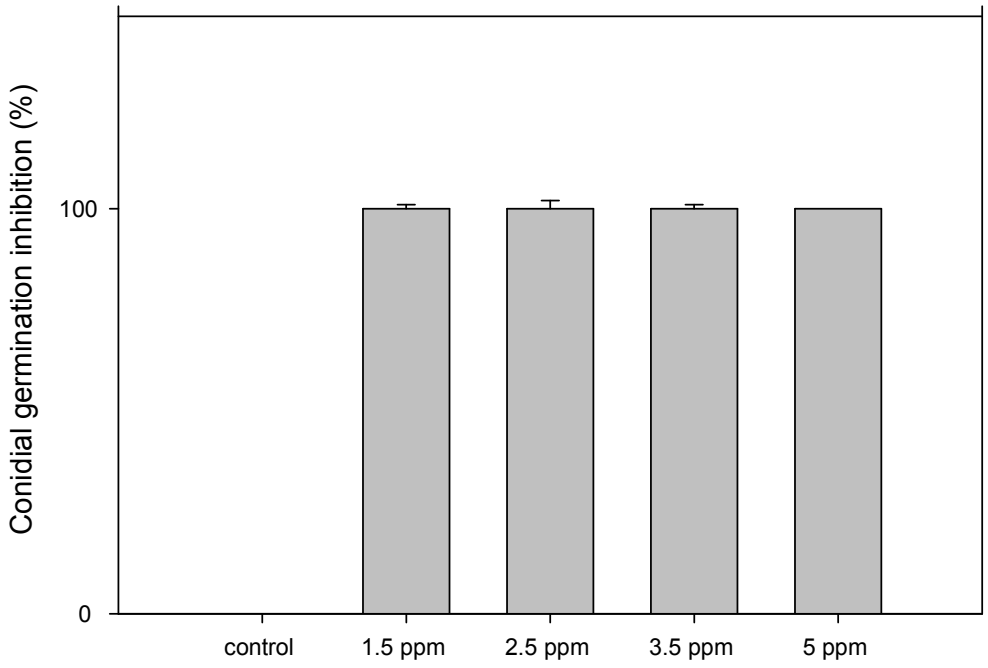


Figure 4.3: Effect of different concentrations of ozone on conidial germination inhibition (%) of *C. gloeosporioides.*

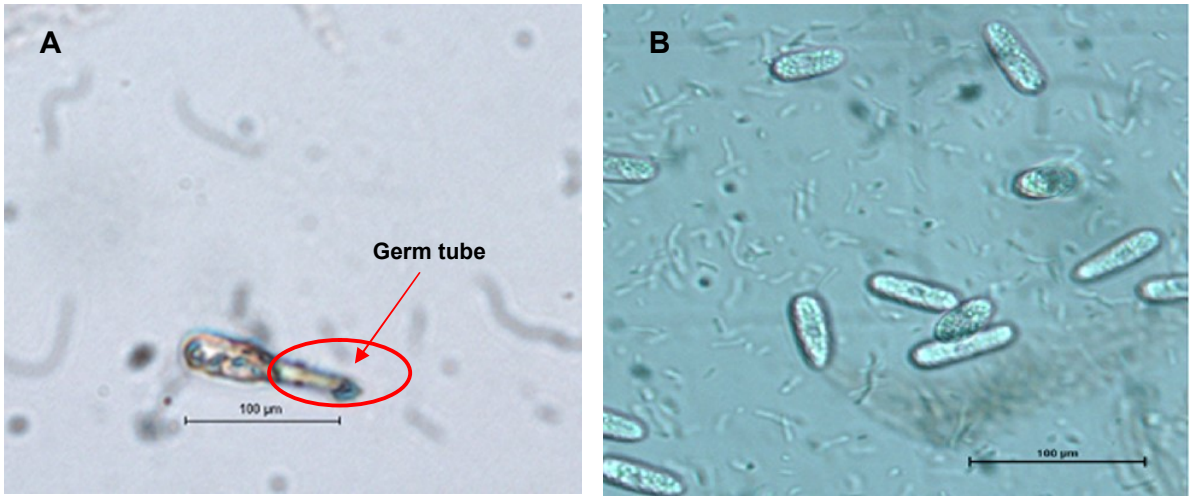


Figure 4.4: Conidial germination of *C. gloeosporioides* in control plates (A); Ungerminated spores of *C. gloeosporioides* in treated plates with ozone exposure (5 ppm) after 24h incubation in the dark (B).

4.3.1.3 Morphological studies

Morphology study showed that the untreated spores of *C. gloeosporioides* germinated with the emergence of germ tube gradually developed into long hyphae. Then, appresoria grew from the hyphal branch (Figure 4.5). Appresoria are capable in penetrating their host by its peg and infect the whole fruits. In contrast, all the ozone-treated spores remained dormant and did not show any sign of germination. Disintegration of spore structure was identified for spores which are treated with 5 ppm ozone only (Figure 4.6).

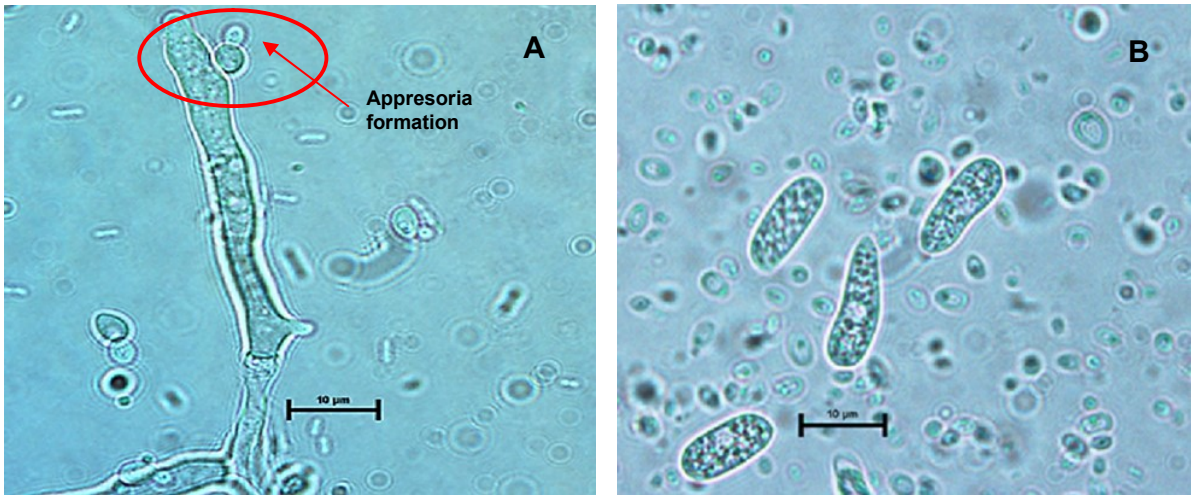


Figure 4.5: Effect of different concentrations of ozone on morphology of *C. gloeosporioides* after 24 h incubation in the dark. Formation of appresoria in control (A), Ungerminated spores in treated plates with 5 ppm ozone exposure (B).

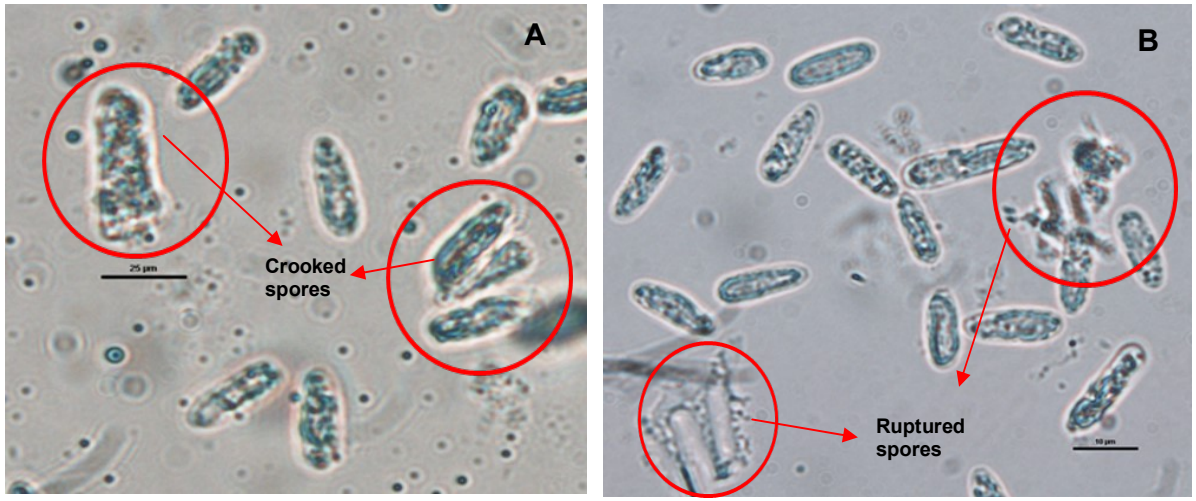


Figure 4.6: Disintegration of spore structure with crooked spores (A) and ruptured (lysis) spores (B) when treated with 5 ppm ozone exposure after 24 h incubation in the dark.

4.3.2 *In vivo* antifungal assay of ozone against C*. gloeosporioides*

4.3.2.1 Disease incidence

As shown in Figure 4.7, there was a significant ($p < 0.05$) delay in anthracnose disease development in both non-inoculated and artificially inoculated papaya fruit, after 96 hours of ozone exposure during the 14 days of ambient storage (25 ± 3 °C, 70 ± 5 %RH). The disease incidence in the controls (for both non-inoculated and inoculated fruit) increased gradually from day to day and reached 100 % after 14 days of storage. The symptoms were visible as early as two days of storage. Different concentrations of ozone exposure not only delayed the onset of anthracnose disease but also maintained the freshness of papaya during the first 6 days of storage. The lowest disease incidence was observed in artificially inoculated papayas treated with 2.5 ppm ozone for 96 hours. In non-inoculated fruits, disease incidence of 67.5 % was observed after 10 days of storage when treated with 2.5 ppm ozone for 96 hours. Meanwhile, the lowest disease incidence of 72.5 % in inoculated fruits was also obtained with 96 hours of exposure to 2.5 ppm ozone, after 10 days of incubation at 25 ± 3 °C and 70 ± 5 %RH.

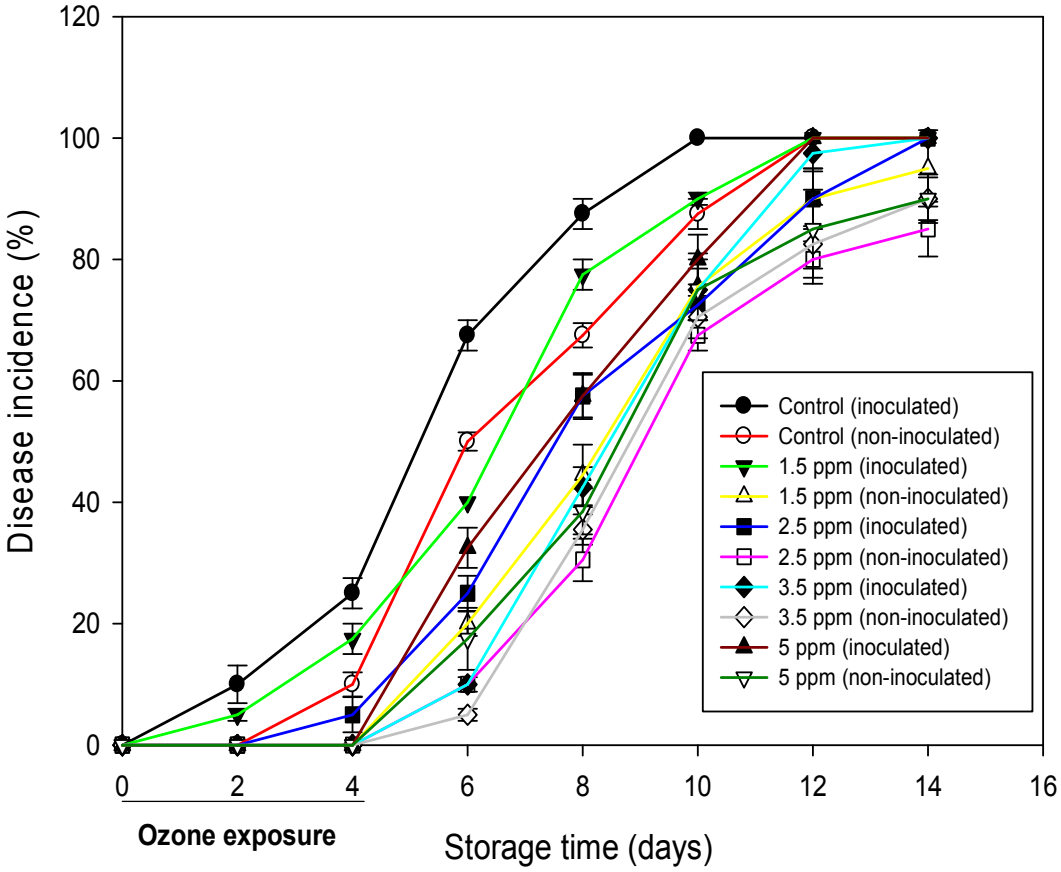


Figure 4.7: Effect of different concentrations of ozone with 96 h exposure on anthracnose disease incidence (%) on inoculated and non-inoculated papaya fruit during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Values are the means ± SE.

4.3.2.2 Disease severity

The disease severity in the controls increased gradually from day to day and reached score 3.5 and 3.8 in non-inoculated and inoculated fruit, respectively, after 14 days of storage. Most papayas (in control) were spoiled due to severe disease infection after 8 days of storage compared to ozone-treated fruit which showed minimal signs of anthracnose. The highest fungistatic effect was observed in non-inoculated papayas treated with 2.5 ppm ozone for 96 hours. In non-inoculated fruits, disease severity scale of 1.5 was

observed after 10 days of storage when treated with 2.5 ppm ozone for 96 hours. Meanwhile, the lowest disease severity score of 2.0 in inoculated fruits was also obtained with 96 hours of exposure to 2.5 ppm ozone, after 10 days of incubation at 25 ± 3 °C and 70 ± 5 %RH (Figure 4.8).

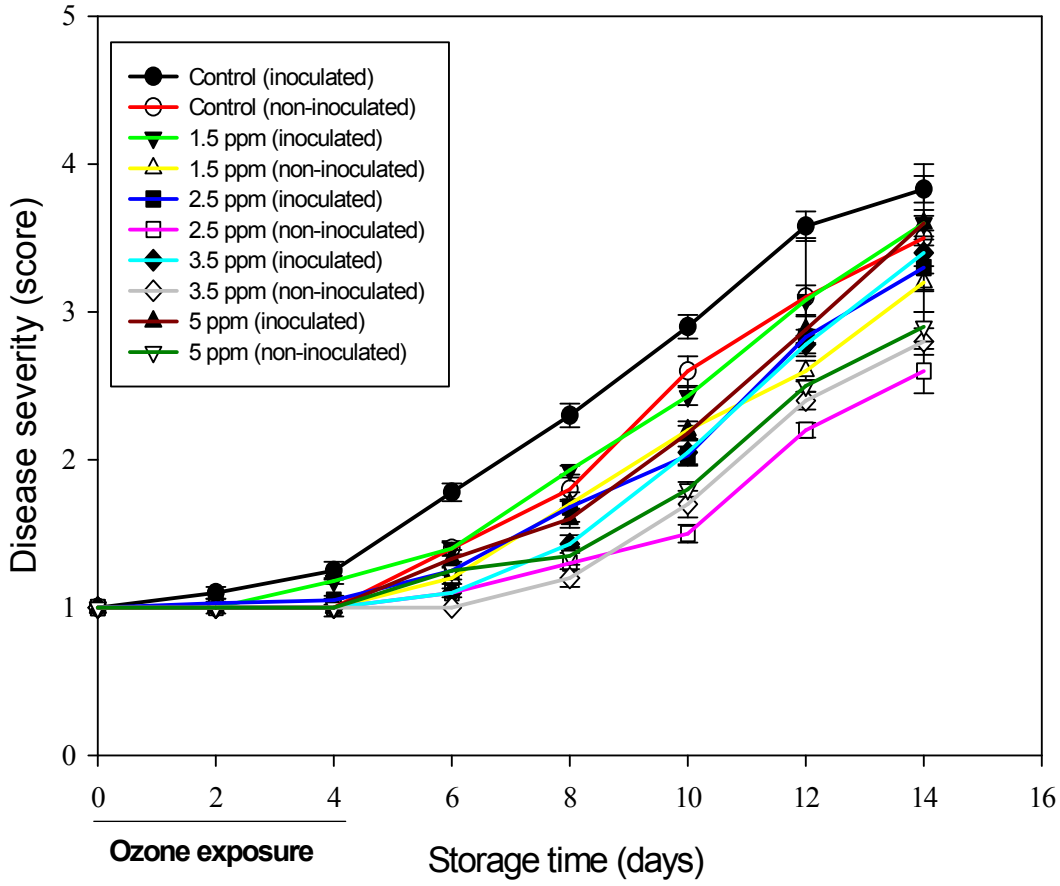


Figure 4.8: Effect of different concentrations of ozone with 96 h exposure on anthracnose disease severity (score) on inoculated and non-inoculated papaya fruit during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Values are the means ± SE.

4.3.3 Scanning Electron Microscopy (SEM) of spore, mycelial and papaya surfaces

Scanning electron micrographs showed morphology of treated and untreated *C. gloeosporioides* spores. The SE micrographs showed that spores treated with 5.0 ppm ozone (Figure 4.9E) were malformed and swollen compared to treatment with 1.5 ppm (Figure 4.9B), 2.5 ppm (Figure 4.9C) and 3.5 ppm ozone (Figure 4.9D). No apparent erosion of the spore coat was observed with 1.5 ppm, 2.5 ppm and 3.5 ppm ozone exposure. There was regularity, smoothness and integrity in unexposed (control) spores and those spores exposed to 1.5, 2.5 and 3.5 ppm ozone. However, spores in the 1.5, 2.5 and 3.5 ppm ozone treatments appeared smaller in width than the control spores. Even though no apparent structural damage was visualized, the spore inner structure may have been considerably weakened. The SE micrographs showed damage to the spore external structure in those subjected to 5 ppm ozone. Some spores were seriously deformed or had lost their original shape and extrusion of their inner material was also observed (Figure 4.9E). Some of the spores had disintegrated, showing fragments of their initial structure.

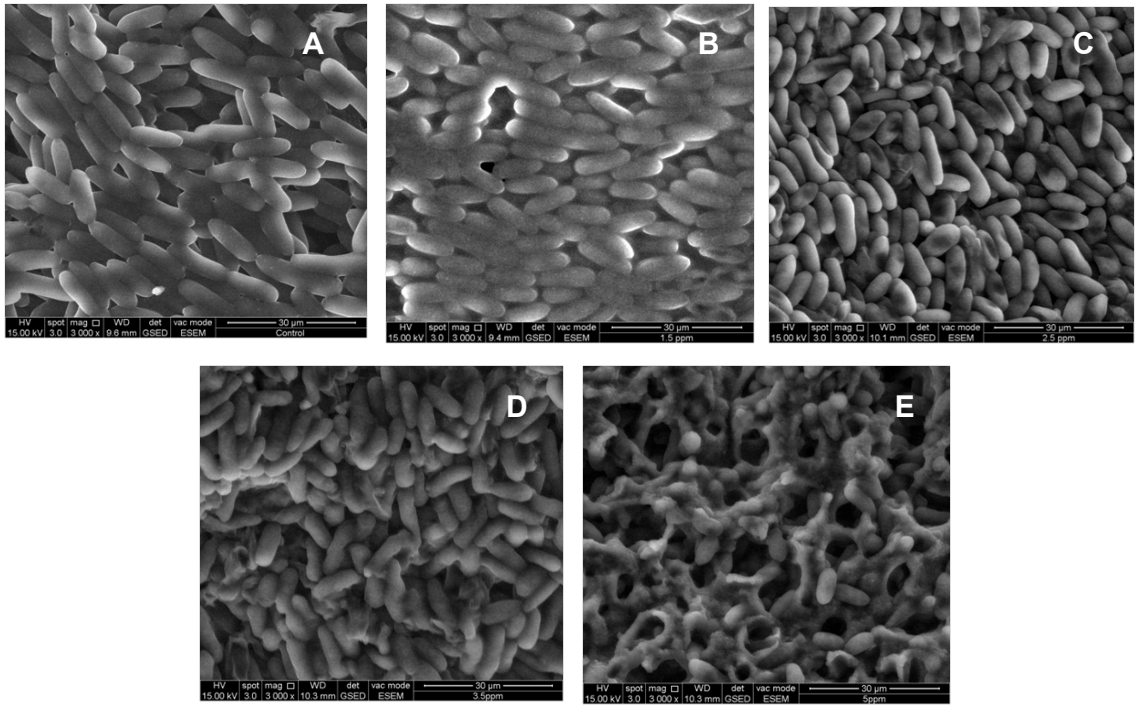


Figure 4.9: SE micrographs (30µm) of untreated spores (A) and *C. gloeosporioides* spores treated with 1.5 ppm (B), 2.5 ppm (C), 3.5 ppm (D) and 5.0 ppm (E) ozone after 24 h exposure (25 ± 3 °C, 70 ± 5 %RH).

SEM of *C. gloeosporioides* mycelia exposed to ozone fumigation revealed that all ozone treatments (Figure 4.10) showed ozone affected the morphology of mycelium. It was very evident on mycelia treated with high concentration. Control mycelia (Figure 4.10A) were smooth as compared to treated one. The effect was more prominent when mycelia treated with higher concentration. The mycelia appeared crooked, rough and loose integrity than the control mycelia. Therefore, apparent structural damage was visualized for all the treated mycelia. The SE micrographs show disintegration of the mycelia makeup or internal structure, despite apparent external fragmented damages in those subjected to 5.0 ppm ozone (Figure 4.10E). These micrographs clearly show that the structure of *C. gloeosporioides* mycelia was affected. The actual degree of suppression in fumigated mycelia may have been greater than indicated, since much of the mycelia analyzed grew within the substrate and was not directly subjected to ozone.

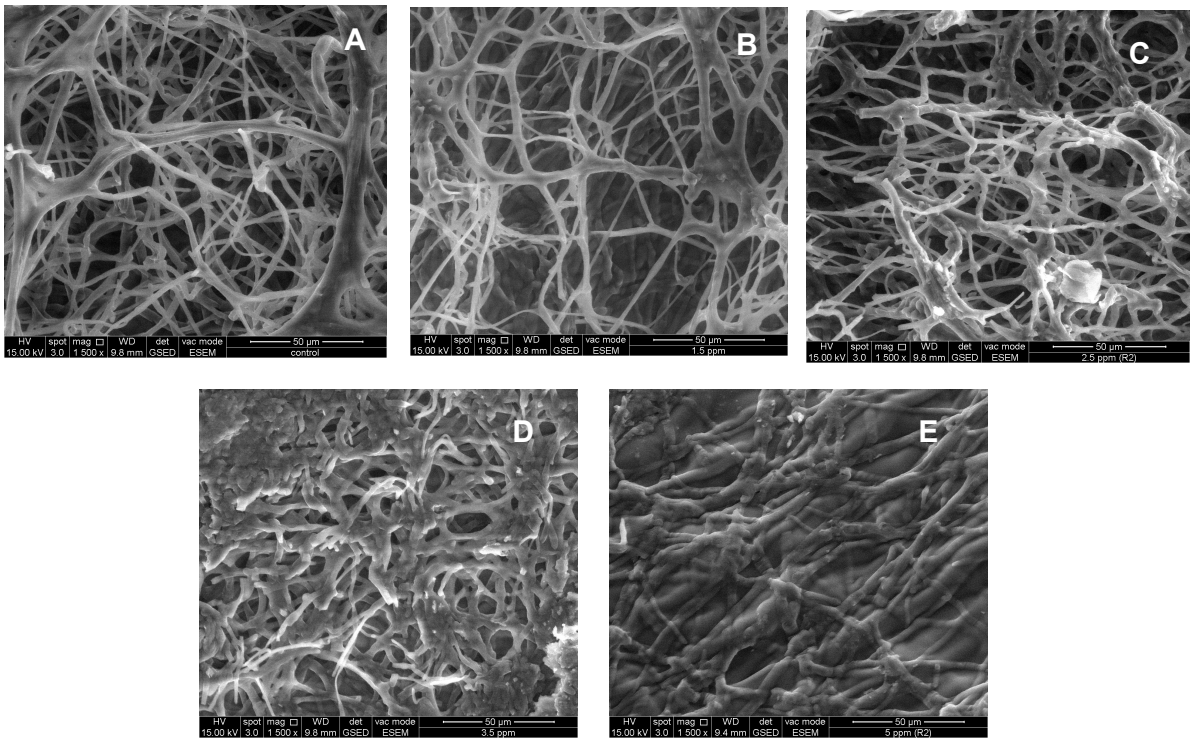


Figure 4.10: SE micrographs (50 µm) of untreated mycelial (A) and mycelial treated with 1.5 ppm (B), 2.5 ppm (C), 3.5 ppm (D) and 5 ppm (E) ozone after 24 h exposure (25 ± 3 °C, 70 ± 5 %RH).

The SE micrograph of control samples of the papaya cuticles illustrated the typical surface morphology of papaya fruit (Figure 4.11A). The SEM images of papaya fruit treated with 1.5, 2.5, 3.5 and 5.0 ppm ozone are illustrated in Figures 4.11B, 4.11C, 4.11D and 4.11E, respectively. It can be observed that cuticles of fruit treated with 1.5, 2.5 and 3.5 ppm ozone had similar wax covering as those in the control sample (Figure 4.11A). Nevertheless, the 5.0 ppm treated fruit (Figure 4.11E) appears to be smoother, less granular than the control or the 1.5, 2.5 and 3.5 ppm treated fruit. This indicates that these ozone treatments below 5.0 ppm did not affect this layer. These results revealed no major variation in cuticle morphology between control and treated fruits below 5.0 ppm ozone. No external wax accumulation or wax crystals could be detected.

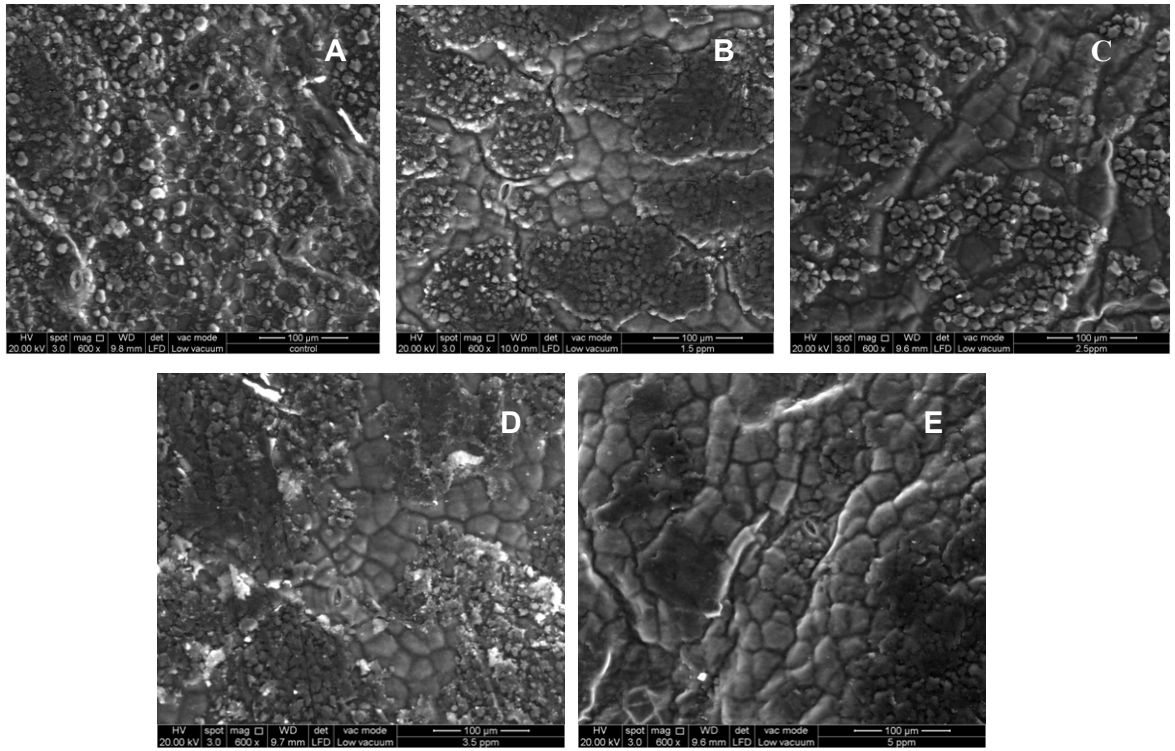


Figure 4.11: SE micrographs (100µm) of untreated papaya fruit surface (A) and fruit surfaces treated with 1.5 ppm (B), 2.5 ppm (C), 3.5 ppm (D) and 5 ppm (E) ozone after 24 h exposure (25 ± 3 °C, 70 ± 5 %RH).

4.3.4 Effect of ozone on reactive oxygen species generation

For the control treatment, ROS generation was poor with only 24 % spores stained by DCHF-DA (Figure 4.12). However, in all ozone treatments, a higher percentage of spores were stained. With increased concentration of ozone, higher levels of ROS were induced in the spores. The highest percentage of stained spores was observed with 5.0 ppm ozone exposure on the fungus at 95 %.

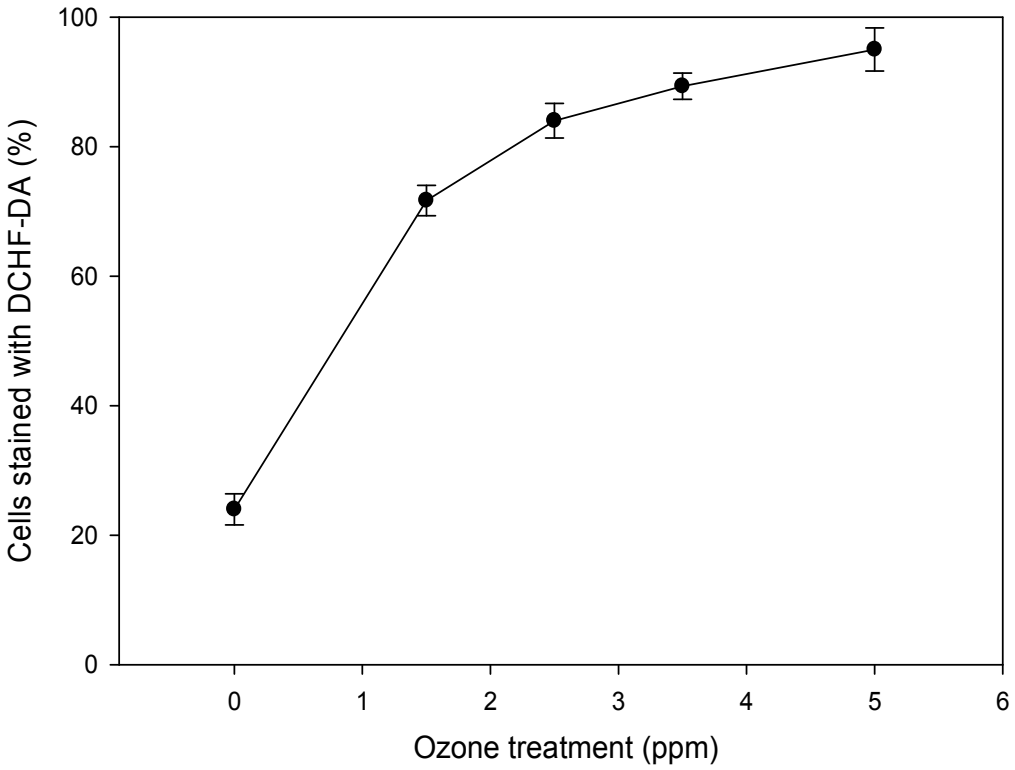


Figure 4.12: Effect of ozone on the accumulation of reactive oxygen species in spores of *C. gloeosporioides*. Values are the means ± SE.

4.3.5 Effect of ozone on spore mitochondria

4.3.5.1 Effect of ozone on distribution of mitochondria

Laser scanning confocal microscopy revealed that mitochondria in untreated spores were present throughout the intracellular space during incubation (Figure 4.13). In contrast, irregular distribution of mitochondria was observed in ozone-treated spores. After exposure to ozone (1.5 - 5.0 ppm), the spores had many spaces void of mitochondria and mitochondria were only found around the cell periphery.

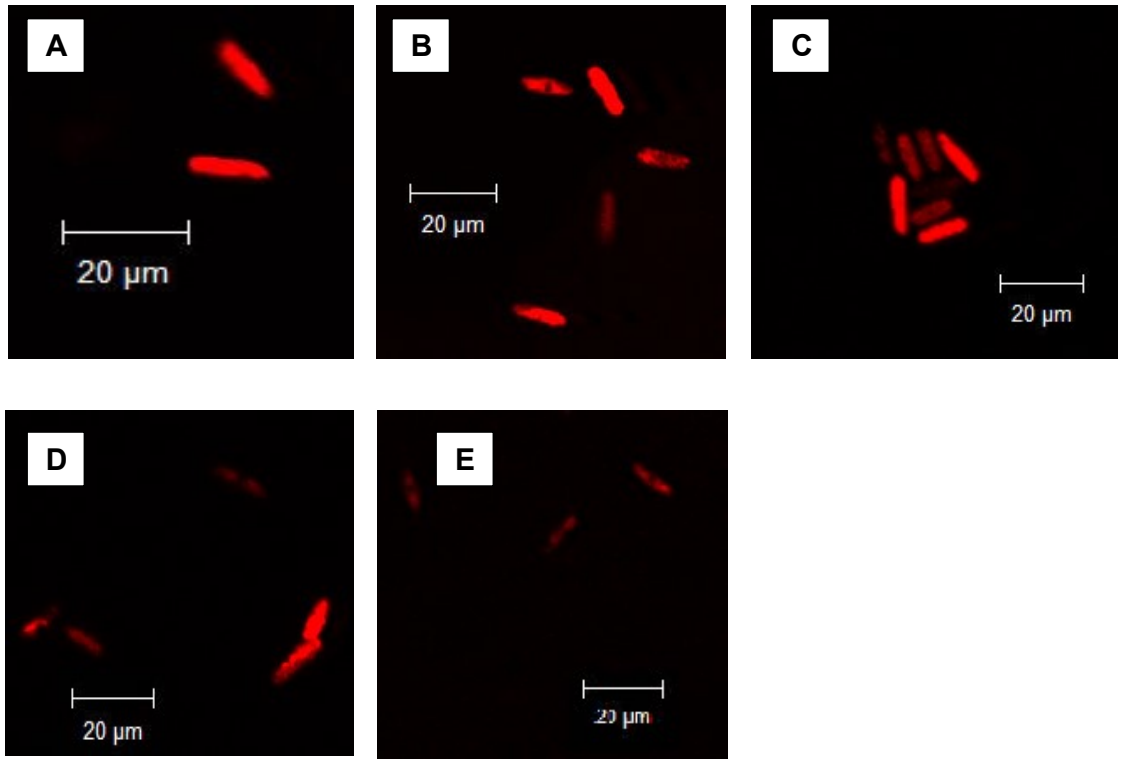


Figure 4.13: Effect of ozone on the distribution of mitochondria in spores of *C. gloeosporioides*. Control spores (A) and spores treated with ozone at 1.5 ppm (B), 2.5 ppm (C), 3.5 ppm (D) and 5.0 ppm (E) were stained with MitoTracker®.

4.3.5.2 Effect of ozone on mitochondria analysed by TEM

Non-ozone exposed mitochondria were obvious in sections examined by TEM, and possessed normal morphology (Figure 4.14A). With increased concentration of ozone, fewer mitochondria were present and many small vacuoles were visible (Figure 4.14B – 4.14E). In spores treated with 2.5 ppm and 3.5 ppm ozone, mitochondrial cristae were distorted (Figure 4.14C and 4.14D), whereas the mitochondria were almost completely degraded in spores treated with 5.0 ppm ozone (Figure 4.14E).

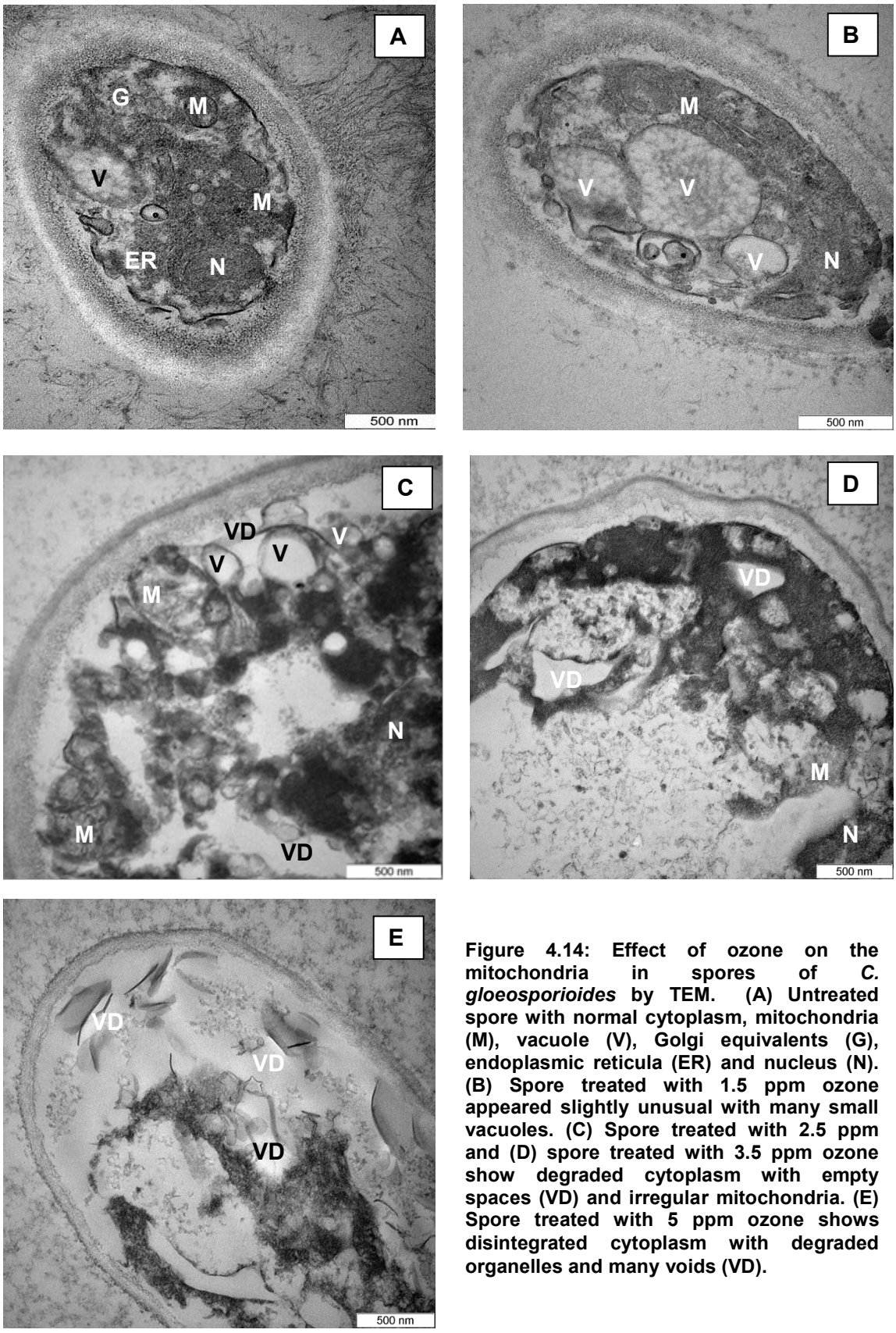


Figure 4.14: Effect of ozone on the mitochondria in spores of *C. gloeosporioides* by TEM. (A) Untreated spore with normal cytoplasm, mitochondria (M), vacuole (V), Golgi equivalents (G), endoplasmic reticula (ER) and nucleus (N). (B) Spore treated with 1.5 ppm ozone appeared slightly unusual with many small vacuoles. (C) Spore treated with 2.5 ppm and (D) spore treated with 3.5 ppm ozone show degraded cytoplasm with empty spaces (VD) and irregular mitochondria. (E) Spore treated with 5 ppm ozone shows disintegrated cytoplasm with degraded organelles and many voids (VD).

4. 4 Discussion

Mycelia growth and conidia germination were clearly affected at various concentrations of ozone exposure. Among all the ozone treatments tested, the 5.0 ppm ozone treatment was found to be the most effective in controlling fungal growth and spore germination *in vitro*. However, 96 hours of 2.5 ppm ozone exposure showed the best suppression of disease incidence and severity *in vivo*.

In vitro inhibition was directly related to the ozone concentration. These results are in agreement with the findings of Tzortzakis *et al.* (2008), who showed that ozone was highly inhibitory to mycelia growth and spore production of *Alternaria alternata* and *Colletotrichum coccodes* in tomato fruits, and that the inhibitory effect was directly related to the concentration and duration of ozone exposure. Ozkan *et al*. (2011) also confirmed that the inhibitory effect of ozone on spore germination of *Penicillium digitatum*, *Penicillium italicum* and *Botyris cinerea* of table grapes was higher with an increase in the concentration of ozone and exposure time.

On the other hand, the same relationship with ozone concentration was not apparent in the *in vivo* study. Fruit treated with 3.5 ppm and 5.0 ppm ozone had increased fruit decay, indicating that higher doses of ozone (3.5 ppm and above) reduced the disease resistance to anthracnose. This could be due to degradation of structural components and membrane structures. Papayas treated with 3.5 ppm and 5.0 ppm ozone continuously for 96 h developed distinct surface browning as a result of surface oxidation from excessive use

of ozone. Kim *et al*. (1999) stressed that ozone may promote oxidative spoilage.

Exposure to 2.5 ppm ozone delayed the onset of disease incidence by up to 10 days in papayas during storage, while in control fruit the anthracnose symptoms appeared within 2 days of storage. The actual mechanism of action of ozone is not clear and needs to be further investigated. However, anthracnose is a latent infection and begins with the germination of conidia, and therefore ozone may have had fungistatic effects (Ranasinghe *et al*., 2005). Furthermore, ozone has been shown to induce natural resistance to disease caused by *Botyris cinerea,* a type of latent infection in carrot (Forney *et al*., 2007). Krause and Weidensaul (1978) reported that exposure to low ozone concentrations did not kill but reduced the subsequent virulence of conidia in *Botyris cinerea*.

Reports describing the doses of ozone required to inactivate fungal conidia are few and conclusions differ (Ozkan *et al*., 2011). It is generally reported that ozone may delay the appearance of disease symptoms of infected fruit. Several studies have reported reductions in spore production and viability following ozone-treatment (Krause and Weidensaul, 1978; Palou *et al*., 2002; Aguayo *et al.,* 2006; Karaca and Velioglu, 2007; Tzortzakis *et al*., 2007b), but spore production can resume when treated fruit are removed from the ozone-enriched atmosphere (Smilanick, 2003). Reports on other microbes indicate that very high ozone doses may be required for control. Currier *et al*. (2001)

reported that exposure to 9000 ppm of ozone for 15 hours was required to kill the spores of *Bacillus globigii* var. *niger*.

The major factor determining spore resistance to biocidal agents appears to be the spore coat (Komanapali and Lau, 1996; Young and Setlow, 2004). This was supported with TEM micrographs by Kim *et al*. (2003), where aqueous ozone treatments caused damage to the surface layer as well as to the outer and inner coats. Such damage facilitates the action of ozone on the cortex, and finally causes spore inactivation through intracellular damage (Kim *et al*., 2003; Young and Setlow, 2004). Besides, ozone as a biocidal agent may have several targets such as proteins, enzymes, and even DNA (Swadeshi *et al*., 1986). Mahfoudh *et al*. (2010a) working on *Bacillus atrophaeus* and *B. pumilus* spores observed severe alterations to spore integrity, although the morphology of a minority of them was apparently unaffected. The progressive degradation of these structures involves changes in permeability and cell integrity, and is often followed by cell lysis (Broadwater *et al*., 1973; Kim and Yousef, 2000; Khadre and Yousef, 2001; Thanomsub *et al.,* 2002). Recent findings by Mahfoudh *et al*. (2010b) suggest that ozone molecules diffuse through the spores and then react (oxidation process) with targets that are essential to spore survival such as water. Besides, the presence of water in the core of the spore can initiate chemical reactions with ozone and their reaction by-products may provide further oxidant species such as radicals and oxidant molecules which participate in the spore inactivation process.

Scanning electron micrographs of Kechinski *et al.* (2012) revealed that cuticles of papaya fruits treated with 4 ppm ozonized water for 2 minutes had the same wax covering as those in the control samples. Thus, ozone application did not affect the fruit's cuticular surface or wax arrangement on fruit. According to Forney (2003), harvested fruits and vegetables have closed stomata that limit the penetration of ozone into harvested plant tissues.

Ozone is a strong oxidative agent as well as a highly toxic gas (Alothman *et al*., 2010) and is known to produce oxidative stress in fungi. Its toxicity is related to its potential to form reactive oxygen species (ROS) inside cells. ROS may cause oxidative damage to cellular compounds and lead to cellular dysfunction or cell death (Angelova *et al*., 2005; Circu and Aw, 2010). In this study, we used an oxidant-sensitive probe DCHF-DA to investigate ROS generation in *C. gloeosporioides* spores, and observed that ozone-treated spores showed a higher ratio of stained cells than in the control. Additionally, ozone induced ROS generation in *C. gloeosporioides* spores. ROS accumulation can cause oxidative damage of the fungal spore and result in cell death.

MitoTracker® Orange CMTMRos is a mitochondrion-selective stain, and has been widely used for studying mitochondrial distribution and functionality (Chida *et al*., 2004; Czarna *et al*., 2010). Accumulation of the stain in cells is dependent upon membrane potential which is one of the most important parameters indicating mitochondrial functionality.

The ultrastructural changes observed in mitochondria and endoplasmic reticula are probably due to the effect of toxin or oxidative stress. Smooth and rough endoplasmic reticulum was usually found in close association with mitochondria. From the TEM analysis, it was observed that the mitochondria were swollen, had disorganized cristae and contained numerous small osmophilic vacuoles. Some mitochondria had ruptured membranes. It is well known that the mitochondrial respiratory chain is the major endogenous source of ROS, particularly when the mitochondria are damaged (Osiewacz, 2002; Shi *et al*., 2011).

Based on confocal microscopy and TEM, we found irrregular distribution and serious degradation of mitochondria in the ozone-treated spores, indicating that ozone treatment can lead to mitochondrial damage. This illustrated that the higher the ozone concentration, the more degradation of organelles and disintegration of cytoplasm occurred. Shi *et al*. (2012) reported abnormal mitochondria in spores of *C. gloeosporioides* isolated from mango fruit after incubation for 6 h in borate (20 mM of potassium tetraborate), and borate was considered as a potential antifungal agent. This suggests that mitochondrial degradation of fungal spores may be one of the important modes by which antifungal compounds inhibit fungal growth.

Although several studies have reported the effect of ozone on plant mitochondria and elucidated the specific mode of action (Lee, 1968), to our knowledge there is no information regarding the effect of ozone on *C. gloeosporioides* pathogen ultrastructure. Although accumulating evidence

has increased our understanding of oxidative stress and antioxidant defenses in ozone responses (Kangasjärvi *et al*., 1994; Sandermann *et al*., 1998; Overmyer *et al*., 2003), the mechanisms involved in ozone induced cell death are still unclear. Due to the strong chemical reactivity of ozone, its toxicity has previously been attributed to an ability to form toxic ROS that directly damage membranes (Heath and Taylor, 1997). However, the view of ozone has recently shifted, where it is now regarded in many cases not as a toxin but rather as an elicitor of cell death (Sandermann *et al*., 1998). This study has provided evidence that ozone alters the ultrastructure of *C. gloeosporioides* conidia, leading to disintegration of the cytoplasm and degradation of mitochondria. Higher concentrations of ozone disrupted the cell membrane of *C. gloeosporioides*, eventually leading to leakage of cytoplasmic materials and the death of the fungal pathogen.

On the other hand, the same relationship with ozone concentration was not apparent in the *in vivo* study. Logically, 5.0 ppm ozone treatment should have the highest fungistatic effect and lowest disease severity scale, however appropriate application of ozone dosage such as 2.5 ppm has proven its effectiveness against growth of *C.* g*loeosporioides*. Therefore, fruit treated with 3.5 ppm and 5.0 ppm ozone had increased fruit decay, indicating that higher doses of ozone (3.5 ppm and above) reduced the disease resistance to anthracnose. The stage of fungus growth also affect its sensitivity to ozone indicating why low concentration of ozone for long duration is effective in killing microorganisms as the microorganisms go through several growth cycles (Nagy, 1959). Several studies have reported reductions in spore

production and viability following low concentration of ozone-treatment (Palou *et al*., 2002; Aguayo *et al*., 2006; Tzortzakis *et al*., 2007b).

The demand for safe and good quality foods with high nutrients by the consumer has prompted research to discover and evaluate novel antimicrobial agents as an alternative postharvest technology. Many studies have proven that ozone is a safe and effective antimicrobial agent in many food processing applications. It is concluded from the present study that ozone exposure at the concentrations of 2.5 and to a lesser extent 3.5 ppm for 96 hours is a safe and effective method for treating papayas to control anthracnose disease. Ozone treatment inhibited mycelia growth and spore germination effectively and delayed the onset of decay of artificially inoculated fruits.

Ozone treatment can promote ROS generation in fungal spores, resulting in mitochondrial damage, which may act as the antifungal mechanism of ozone inhibiting spore growth of *C. gloeosporioides* and controlling anthracnose incidence in papaya fruit. Furthermore, ozone treatment has excellent potential to be used as an effective eco-friendly antifungal agent and a substitute for synthetic fungicides currently used in postharvest papaya disease control caused by *C. gloeosporioides*.

CHAPTER 5

EFFECT OF OZONE ON PRODUCTION OF PLANT DEFENSE INDUCIBLE ENZYMES IN PAPAYA

5.1 Introduction

The constitutive defenses of plants include structural barriers, such as the plant cell wall, as well as inhibitory compounds including phenolics (Nürnberger *et al*., 2004). Phenolic compounds can be formed in response to the ingress of pathogens and their appearance is considered as part of an active defense (Nicholson and Hammerschmidt, 1992). Phenylalanine ammonia lyase (PAL) is a key enzyme of phenylpropanoid metabolism in plants. PAL activity in plant tissue may rapidly change under the influence of various factors, such as pathogen attack and treatment with elicitors (Dixon and Lamb, 1990). However, peroxidase (POD) is a phenol oxidizing enzyme. The activities of PAL and POD may rapidly be enhanced under the influence of elicitors or pathogen attack. Polyphenol oxidase (PPO) is a copper containing enzyme with molecular oxygen as co-substrate. The role of PPO has been demonstrated in phenol metabolism and in defense mechanisms against pathogens (Lax and Cary, 1995).

Ozone is a strong antimicrobial agent with high reactivity, penetrability and spontaneous decomposition to a non-toxic product (Grass *et al*., 2003; Kim *et al*., 1999). Ozone triggers plant responses similar to those triggered by fungal and viral pathogens, including the formation of cellular barriers to

pathogens by the formation of lignins, extensins, and callose (Sandermann,1998). Ozone-induced 1,3-glucanase activity in tobacco is associated with increased cell wall callose formation (Ernst *et al*., 1992; Schraudner *et al*., 1992). In soybean leaves, ozone-induced changes are believed to stabilize cell walls against microbial attack (Booker and Miller, 1998). These include increases in peroxidase activity and changes in the cell walls including impregnation with phenolic esters, suberization, and lignification (Forney, 2003).

Ozone treatment has been shown to increase the activities of PAL (Rosemann *et al*., 1991; Eckey-Kaltenbach *et al*., 1994) enzyme controlling the phenylpropanoid biosynthesis pathway. This pathway plays a significant role in plant defence responses because it synthesizes protective compound including flavonoids (UV protectants and phytoalexines). Its induction under environmental stress conditions such as wounding, pathogen attack, UV light and ozone is well characterized at both the biochemical and gene levels. Induction of PAL gene transcription in ozone-exposed plants has been observed in parsley (Eckey-Kaltenbach *et al*., 1994).

The enzymes PAL, PPO and POD are very important in plant disease resistance. They are involved in the formation of lignin and phytoalexins, which induce resistance in plants and related to management of plant diseases (Graham and Graham, 1996). However, there have been no report on induction of resistance in papaya against anthracnose disease by ozone through the changes in the activities of the phenolic enzymes such as

phenylalanine ammonia-lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO). In this study, defense enzyme activation on PAL, PPO and POD activities of papaya fruit affected by ozone were compared with the untreated fruits.

5.2 Materials and Method

5.2.1 Enzymatic assays

5.2.1.1 Crude enzyme preparation

Fruit pulp samples from each replication (2 g) were homogenized at 4 °C using mortar and pestle in 10 ml of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g polyvinylpolypyrrolidone (PVPP). The fruit pulp was sampled from the equatorial region of the whole piece of fruit and was a pooled sample from each replicate. The homogenates were centrifuged at 12,000 rpm for 30 min at 4 °C and the supernatant was used as the crude enzyme extract for determining the activity of polyphenol oxidase and peroxidase enzymes. The crude enzyme extracts were kept on ice during the experiment (Liu *et al.,* 2007).

5.2.1.2 Polyphenol oxidase (PPO) activity

Crude enzyme extract (0.1 ml) was added to 3 ml of catechol substrate (500 mM in 100 mM sodium phosphate buffer, pH 6.4). PPO (EC 1.14.18.1) activity was assayed according to the method of Liu *et al*. (2007) by measuring the conversion of catechol to quinine mediated by PPO. The increase in absorbance at 398 nm was automatically recorded for 3 min, using a spectrophotometer (Model: Biochrom Libra S12; Biochrom Ltd., Cambridge, UK). The PPO activity was expressed as unit (U) ml^{-1} enzyme, where one unit of the enzyme activity is defined as the amount that causes a change of 0.001 in absorbance per minute.

5.2.1.3 Peroxidase (POD) activity

Crude enzyme extract (0.1 ml) was mixed with 2 ml of guaiacol (8 mM in 100 mM sodium phosphate buffer (pH 6.4) and incubated for 30 min at 30 °C and then 1 ml of hydrogen peroxide (24 mM) was added. POD (EC 1.11.1.7) activity was assayed by measuring the conversion of guaiacol to tetraguaiacol as described by Liu *et al.* (2007). The increase in absorbance at 460 nm was recorded for 3 min using a spectrophotometer (Model: Biochrom Libra S12; Biochrom Ltd., Cambridge, UK). The POD activity was expressed as unit (U) ml^{-1} enzyme, where one unit of the enzyme activity is defined as the amount that causes a change of 0.001 in absorbance per minute.

5.2.1.4 Extraction and assay of Phenylalanine ammonia lyase (PAL) activity

Crude enzyme extract was prepared by the method described by Morelló *et al*. (2005) with minor modifications. Pulp tissue of papaya fruit (0.5 g) from each treatment was homogenized for 30 sec at 4 °C using mortar and pestle in 12.5 ml of 0.05 M potassium phosphate buffer (pH 6.6) containing 0.1 g of Triton X-100. Polyvinylpolypyrrolidone (PVPP) (12.5 mg) was added and the suspension was centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant were collected as crude enzyme and stored on ice.

PAL (EC 4.3.1.5) activity was assayed using slightly modified method of McCallum and Walker (1990) using a reaction mixture of 4.1 ml of 0.06 M sodium borate buffer (pH 8.8) and 0.4 ml crude enzyme. The reaction was initiated by addition of 1 ml of 11 mM L-phenylalanine. Glass tubes were

incubated at 37 °C for 1 h, and the reaction stopped by adding 35 % (w/v) trichloroacetic acid (0.5 ml). The tubes were centrifuged for 5 min at 5000 rpm to pellet the denatured proteins, and the absorbance at 290 nm was measured by a spectrophotometer (Model: Biochrom Libra S12; Biochrom Ltd., Cambridge, UK). The PAL activity was expressed as unit (U) ml^{-1} enzyme, where one unit of the enzyme activity is defined as the amount that causes a change of 0.001 in absorbance per minute.

5.3 Results and Discussion

5.3.1 Production of plant defense inducible enzymes as an indicator of disease resistance in papaya

In papaya fruits treated with ozone, the activity of PAL started to increase 2 days after treatment (day 6), and peaked at day 10 (Figure 5.1). PAL activity increased slightly from 8 to 10 days of storage in ozone treated fruits while PAL activity for untreated fruits was the lowest. The highest PAL activity in untreated fruits and 5 ppm ozone treated fruits are 70 U ml^{-1} enzyme and 2010 U ml^{-1} enzyme, respectively. PAL activity for untreated fruits peaked at day 8 during storage.

PAL activity of the ozone treated fruit underwent a high activation during ambient storage. The elicitation of PAL in citrus by other stresses such as wound (Ismail and Brown, 1979), gamma radiation and exogenous ethylene (Riov *et al*., 1969) has been reported. This study supports the involvement of PAL in the response of papaya fruit to other stresses such as ozonation. PAL activity has been associated with a defense mechanism operating in the stress-affected cells (Dixon and Paiva, 1995). PAL, the first committed enzyme in the phenylpropanoid and flavonoid pathways, is involved in biosynthesis of phytoalexins (antimicrobial), lignins and salicylic acid associated with disease resistance expression (Mauch-Mani and Slusarenko, 1996). Activation of PAL could directly affect accumulation of secondary toxic compounds, such as phytoalexins, which might be released from the inoculated plants to inhibit fungal spore germination and growth.

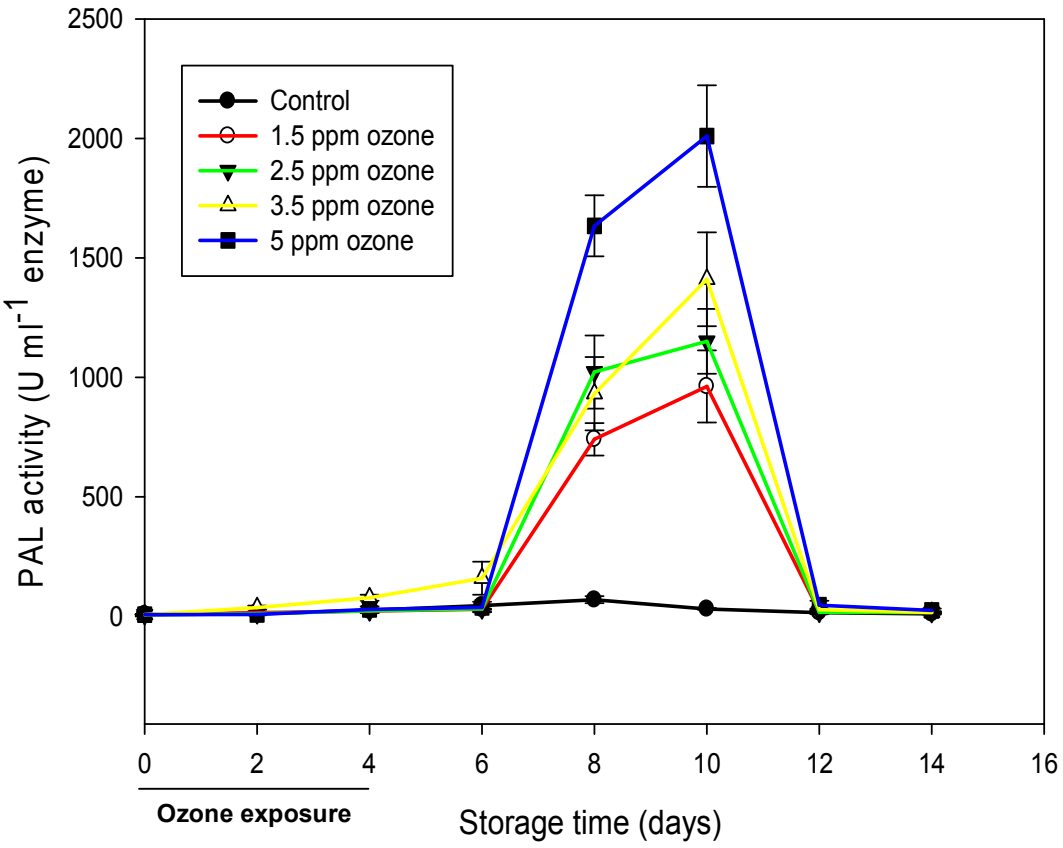


Figure 5.1: Effect of various concentrations of ozone on the changes of PAL activity of 'Sekaki' papaya during ambient storage (25 ± 3°C, 70 ± 5 % RH) for 14 days.
Each value is the mean of four replicates ± SE.

The induction of PAL activity in response to stressful conditions has been considered to be the defensive mechanism of plants against stress. PAL have been associated with some physiological disorders related to altered phenol metabolism (Hyodo *et al*., 1978; Sarkar and Phan, 1979). Koseki and Isobe (2006) observed that the PAL activity was significantly higher and the development of browning was faster in 5 ppm ozone treated lettuce samples than in water and chlorine washed samples. Therefore, it is apparent that the effect of ozone treatment on quality of produce is concentration dependent. It may be advantageous up to a certain level to ozone application, whereas after

a critical level, it may speed up the browning reactions resulting in an inferior quality as compared with the control sample. However, the effects may vary with the commodity.

Plants submitted to ozone generally respond with a stimulation of enzymes involved in the phenylpropanoid pathway. Both the activity and transcript level of Phenylalanine Ammonia Lyase (PAL), the first enzyme of the phenylpropanoid pathway, have been reported to rapidly increase under ozone exposure in various herbaceous plants and forest species (Rosemann *et al*.,1991). The activation of phenylpropanoid metabolism also has been reported for other biotic and abiotic stresses (Dixon and Paiva, 1995) such as wounding, pathogen attack, UV irradiation, heavy metals, or drought. Ozone triggers plant responses similar to those triggered by fungal and viral pathogens (Forney, 2003). Stress-induced modifications of the metabolism seem to be associated with a rapid oxidative burst similar to ozone stress (Langebartels *et al*., 2002). The stress response could be initiated by the accumulation of reactive oxygen species exacerbating damage or signaling the activation of defense responses (Neill *et al*., 2002).

Treatment with ozone induced the activities of PPO and POD (Figure 5.2 and Figure 5.3) in papaya fruit stored at 25 °C. PPO activity in papaya fruit reached its highest value at 10 days in the 5 ppm ozone-treated fruit, and the level was almost 2.25 fold that of control fruit at the same time (Figure 5.2). Data presented in Figure 5.2 indicate that the effect of ozone on PPO activity was significant. The activity of this enzyme in all ozone treated fruit was

higher than the untreated fruit stored at ambient condition. However, after day 10, the enzyme activity in the ozone treated fruits declined and on day 14 was similar to that in the untreated fruit. The rapid decline of enzymes activities was recorded after 10 days because the fruit showed drastic senescence and over ripeness.

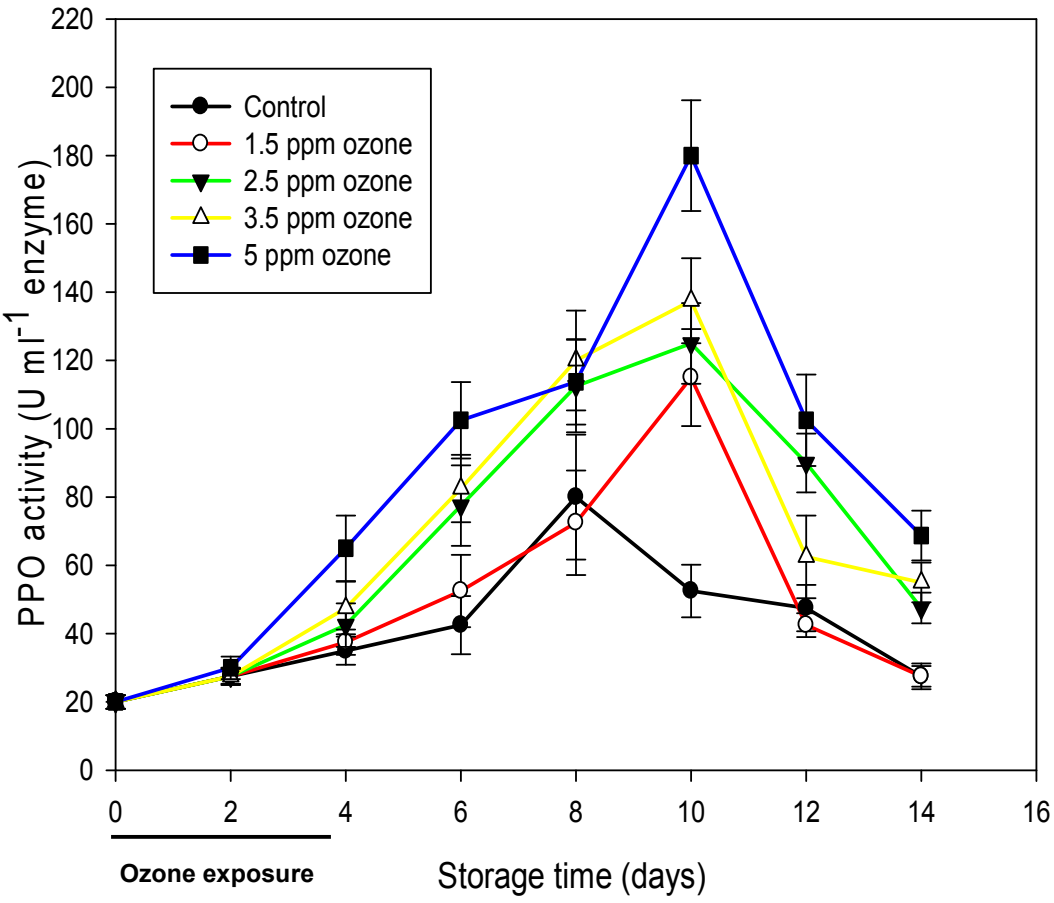


Figure 5.2: Effect of various concentrations of ozone on the changes of PPO activity of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days.
Each value is the mean of four replicates ± SE.

POD activity increased in all ozone-treated fruits stored at ambient condition, but POD activity in higher ozone concentration treated fruit was higher than that in lower ozone concentration treated fruit (Figure 5.3). POD activity increased gradually and peaked at 10 days in ozone-treated fruits. The level of POD activity in 5.0 ppm ozonated fruit was approximately 2.6 fold than in control fruit at day 10. POD activity in untreated fruit decreased during ambient storage after day 8 (Figure 5.3). The rate of increase in POD activity for 5.0 ppm ozone treated fruit was the highest compared to all others ozone treatments. This showed that ozone treatment delayed an increase of POD activity in stored fruits.

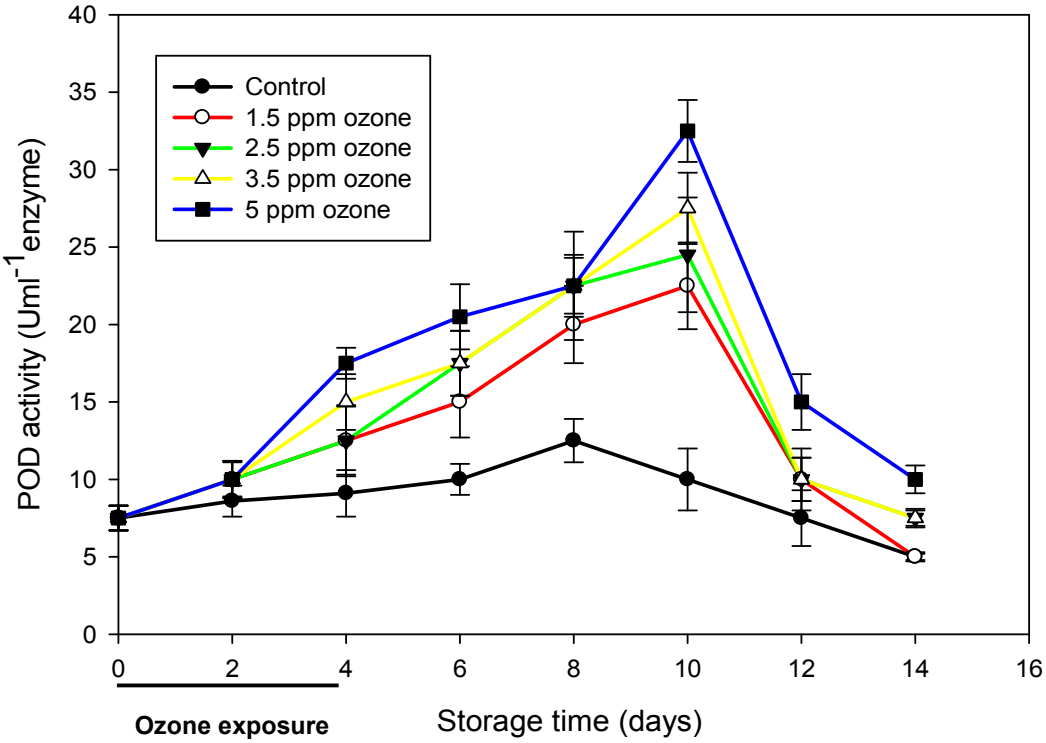


Figure 5.3: Effect of various concentrations of ozone on the changes of POD activity of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days.
Each value is the mean of four replicates ± SE.

POD may play key roles in controlling the level of oxygen free radicals induced by stress conditions (Martínez-Téllez and Lafuente, 1997). The activity of the phenolic enzymes peroxidase (POD) and polyphenol oxidase (PPO) may also increase in response to biotic and abiotic stresses (Siegel and Siegel, 1986; Cohen *et al*., 1988). These enzymes have also been involved in the development of physiological disorders (Cohen *et al*., 1988; Ke and Saltveit, 1988). In other crops, deterioration and browning have been associated with decreased PPO and POD activities during storage (Odebode and Oso, 1995). POD activity also declined progressively as drought stress-induced senescence developed in alfalfa leaves (Irigoyen *et al*., 1992).

Polyphenol oxidase is a copper-containing enzyme which can be either latent or active in many plants (Whitaker, 1995). PPO can be found in both soluble and membrane-bound forms in chloroplasts, mitochondria, microsomes, peroxisomes and the cytoplasm (Subramanian *et al*., 1999). Different names have been associated with this enzyme, including tyrosinase, catecholase, diphenolase and phenolase. It is the enzyme which catalyses two different reactions in the presence of molecular oxygen: the hydroxylation of monophenols to *o*-di-phenols (monophenolase or hydroxylase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase, catecholase or oxidase activity) (Sánchez-Ferrer *et al*., 1995). The *o*-quinones non-enzymatically polymerise and give rise to heterogenous black, brown or red pigments commonly called melanins (Zawistowski *et al*., 1991). In this study, some surface browning effect was observed on the papaya treated with 3.5 ppm and 5.0 ppm ozone after 8 days of storage. Similar effect of surface

browning on papaya was reported by Ong *et al*. (2012) when the fruit was exposed with 4.0 ppm ozone. Cell disruption (bruises, wouding) leading to decompartmentalisation involves a surge of events, including the activation of latent PPO and / or its systemic *de novo* induction (Thipyapong *et al*., 1995) as well as an induction of POD (Hyodo *et al*., 1991; Ketsa and Atantee, 1998). Increases of PPO activity can promote lignin synthesis and this process of lignification not only forms physical barriers against pathogen intrusion, but its precursor materials can also be oxidized by PPO to generate quinones that act as strong poisons to pathogens (Ramiro *et al*., 2006).

Other enzymes with polyphenol oxidase activity are peroxidases, which perform single-electron oxidation on a wide variety of compounds in the presence of hydrogen peroxide (Dunford and Stillman, 1976). POD can be found either soluble in the cytoplasm or cell wall-bound (Ingham *et al*., 1998). Their involvement in slow processes such as internal browning is possible. It has been proposed that PPO could act as a promoter of POD activity, which could be due to the generation of hydrogen peroxide during the oxidation of phenolic compounds in PPO-catalysed reactions (Richard-Forget and Gaulliard, 1997). The antioxidant status of fruits and vegetables can also decrease by the direct oxidation of antioxidants catalysed by both PPO and POD enzymes (Jiménez and García-Carmona, 1999; Espín and Wichers, 2000). Appropriate level of ozone has the inhibitory effect on antioxidant promoted by the browning-related enzymes, namely polyphenol oxidase (PPO) and peroxidase (POD) as reported previously (Rico *et al*., 2006).

PPO and POD are able to oxidize phenolic compounds and have been related to fruit deterioration. Changes in PAL, PPO and POD activities in papaya fruits during 96 hours exposure to ozone were significant and increased markedly the tolerance of fruit to oxidative stress. Hence, ozone fumigation inhibited or delayed the increase in POD or PPO activity in fruits stored at ambient temperature.

As conclusion, the inductive effects of ozone may be associated with its degree of oxidative burst in the plant species, which acts as an antimicrobial agent to fungal infection in plant or fruit. Ozone treatment induced a significant increase in the activities of PAL, PPO and POD, and enhanced the content of phenolic compounds in papaya, thus providing protection against anthracnose disease. Ozone, as a natural substance potently induces defence reactions in papaya fruit. This suggests that ozone improves resistance of papaya fruit against abiotic or biotic stress like fungal invasion and implies that ozone is a promising natural fungicide, a potential substitute for the utilization of synthetic fungicides in fresh horticultural produces. These results also indicate that ozone treatments may improve the ability of the antioxidative defence mechanism in papaya fruit and result in a better control of fruit decay. However, further study on the mechanism of ozone against fungal pathogens or oxidative stress at the molecular level is needed.

CHAPTER 6

EFFECT OF OZONE ON MICROBIAL FLORA OF PAPAYA DURING STORAGE

6.1 Introduction

It is estimated that foodborne diarrheal diseases cause about 4 to 6 million deaths per year, with most of these occurring in young children (WHO, 2003). Although chemical preservatives have been used for years, there is much controversy because they have been shown to cause respiratory or other health problems (Fleming-Jones and Smith, 2003). Therefore, it is necessary to find a novel way to reduce or eliminate food-related microorganisms during the shelf life of food products. In the meantime, the increasing demand of consumers for natural products has led to research on new antimicrobial agents to improve the safety of products.

Ozone (O_3) or triatomic oxygen, is naturally produced from oxygen as a result of lightening or UV light interaction (Kim *et al*., 1999). The possibility of using ozone as an alternative sanitizing technology has been engineered to substitute regular sanitizers such as chlorine. Ozone at concentrations of 0.15-5.0 ppm has been shown to inhibit the growth of spoilage bacteria as well as yeasts (Jay *et al*., 2005). In 2001, the U.S. Food and Drug Administration approved the use of ozone as an antimicrobial agent in the treatment, storage, and processing of meats and horticultural produce (Anonymous, 2001). Ozone destroys microorganisms by the progressive

oxidation of vital cell components (Beuchat, 1992), preventing the microbial growth and extending the shelf-life of many fruits, as Gane (1936) found in banana (1.5–7.0 μl l^{-1}), Baranovskaya *et al*. (1979) in potatoes, onions and beetroot (3 μl l^{-1}), Jin *et al*. (1989) in tomatoes and mandarins, and Barth *et al*. (1995) in blackberries (0.3 μl l^{-1}). Ewell (1950) showed that 2–3 μl l^{-1} of ozone applied continuously for a few hours per day doubled the storage life of strawberries, raspberries and grapes. Singh *et al*. (2002) showed that ozone (combinations of 2.1, 5.2 or 7.6 ppm for 5, 10 or 15 min) reduced the microbial counts in lettuce and carrot inoculated with *Escherichia coli*.

Microorganisms, especially bacteria normally occur on the tissues of fresh fruit and vegetables. They include mesophilic bacteria, lactic acid bacteria, coliforms, and yeasts and moulds (Nguyen-The and Carlin, 1994). Numbers of microbial counts reported on fresh fruit and vegetables are within the range 10^1-10^9 $cfug^{-1}$, depending upon the fruit or vegetable. The native microbial flora of fruit is mostly composed of mould and yeasts. Fungi such as *Botrytis cinerea* and *Aspergillus niger* and yeasts such as species of *Candida*, *Cryptococcus*, *Fabospora*, *Saccharomyces* and *Zygosaccharomyces* are present in most fresh fruits (Chen, 2002).

Microbial spoilage appears to be one of the major reasons for quality loss in fresh fruit and vegetables through the formation of off-flavours, fermented aromas, and tissue decay. The basic principle of shelf-life prediction involves the quantification of the populations of microbes present on the food product (Zhuang *et al*., 2003). In the search for useful disinfectant treatments for fresh

fruit and vegetables, decontamination methods include physical (Ukuku *et al*., 2006; Ozkan *et al*., 2011; Tzortzakis *et al,.*2008), chemical (Wang *et al*., 2007; Maqbool *et al*., 2011), and biological processes (Rouse *et al*. 2008; Spadaro *et al*., 2008). The most versatile treatment is processing with ionizing radiation. Sanitation of food with ionizing radiation such as ozone is safe, efficient, environmentally clean and energy-efficient. Recently, there has been a high level of interest in postharvest applications of ozone for decay control and as a potential sanitizer against human pathogens because ozone has been affirmed the status of Generally Recognized As Safe (GRAS) as a food processing aide and as compliant with EPA Disinfection by Products Rule (US-FDA, 1997).

The application of ozone at the postharvest stage has been studied by many researchers especially for the prevention of fungal decay (Palou *et al.,* 2002; Perez *et al*., 1999), inactivation of bacteria (Achen and Yousef, 2001; Kim and Yousef 2000; Sharma *et al*., 2002; Xu, 1999), destruction of pesticides and chemical residues (Ong *et al*., 1996; Hwang *et al.,* 2001), and the control of storage pests (Kells *et al*., 2001; Mendez *et al.,* 2002).

The study of the effect of ozone on papaya fruit is of great importance to determine the potential of ozone as a disinfecting agent and, most importantly, to enhance food safety. The aim of this work was to evaluate the efficacy of ozone in gaseous form on microbial flora disinfection of papaya fruits.

6.2 Materials and Method

6.2.1 Microbial evaluation

Fruit were exposed to ozone enriched atmospheres of 0.05, 0.5, 2.0, 3.5 or 5.8 ppm for durations of 0, 0.5, 1, 3, 5 and 24 h. The experiments were conducted in a completely randomized design and a sample of 10 fruits in each treatment was used. Eight fruits from each treatment were used for the microbiological analysis.

6.2.1.1 Standard plate counts

Microbiological analysis of papaya samples was carried out aseptically according to American Public Health Association (APHA, 1992) procedures by mixing 25 g of papaya fruit skin with 225 ml sterile Ringer solution in sterile stomacher bag. The samples were massaged by hand for 1 min. The pour plate method was used for total count mesophilic bacteria (Plate count agar, Merck) at 35 °C for 24 h. Microbial counts were expressed as log $cfug^{-1}$.

6.2.1.2 Yeast and mould counts

The surface spread method (APHA, 1992) was used to count the number of yeast and mould (Yeast Extract Glucose Chloramphenicol Agar, YGC, Merck) at 25 °C for 3 to 5 days. Microbial counts were expressed as log $cfug^{-1}$.

6.2.1.3 Total coliform counts

The surface spread method (APHA, 1992) was used for coliform bacteria (Mcconkey agar, Merck) at 37 °C for 48 hours. Microbial counts were expressed as log $cfug^{-1}$.

6.3 Results and Discussion

6.3.1 Microbial evaluation

6.3.1.1 Standard plate count

Treatment of fruit with ozone at 0.5, 2, 3.5 and 5.8 ppm for 24 h reduced the total mesophilic microorganism counts from 4.48 to 2.18 log $cfug^{-1}$over 24 hours (Table 6.1) ($p < 0.05$) for both the concentration of ozone and the exposure time. These bacteria are commonly found on crop surfaces. Many microbes are capable of colonizing the produce by producing pectin-degrading enzymes which enhance tissue softening and breakdown (Watada *et al.*, 1996). Ozone can be useful for maintaining quality and reducing microbial populations in tomato (Aguayo *et al*., 2006), cucumber (Khan and Khan, 1999), table grapes (Gabler *et al*., 2010), strawberries (Allende *et al.,* 2007), fresh-cut cantaloupe (Selma *et al*.,2008), packaged spinach (Klockow and Keener, 2009) and carrots (Hildebrand *et al.,* 2008).

Microbial load in fresh-cut produce is greater than that in intact produce because tissue damage caused by cutting breaks cells and promotes the release of nutrients used by microflora (Toivonen and DeEll, 2002). However, high reductions of bacterial populations were detected in other studies conducted on ozone treated fresh-cut fruits and vegetables. For instances, fresh-cut carrot treated with 10 ppm of aqueous ozone for 10 minutes has resulted in a total reduction of 2.17 and 1.24 log $cfug^{-1}$ in mesophilic and coliform populations after treatment and further reduction after 30 days of storage (Chauhan *et al*., 2011). Also, Olmez and Akbas (2009) have reported

that the mesophilic bacteria in green leaf lettuce were reduced by 1.5 log $cfug^{-1}$ upon exposure to 2 ppm of gaseous ozone for 2 minutes.

Table 6.1: Effect of exposure time and ozone concentration on microorganism count on 'Sekaki' papaya fruit (log $cfug^{-1}$)

	Exposure time (hour)	Ozone concentration (ppm)				
		0.05	**0.5**	**2**	**3.5**	**5.8**
Total coliforms	0	4.13^{Aa}	4.13^{Aa}	4.13^{Aa}	4.13^{Aa}	4.13^{Aa}
	0.5	4.01^{Bb}	3.48^{Cb}	3.33^{Db}	3.02^{Eb}	2.98^{Fb}
	1	3.56^{Bc}	3.31^{Cc}	2.54^{Fc}	2.93^{Dc}	2.88^{Ec}
	3	2.98^{Bd}	2.81^{Dd}	2.4^{Fd}	2.93^{Cc}	2.54^{Ed}
	5	2.98^{Bd}	2.65^{De}	2.18^{Ee}	2.93^{Cc}	1.7^{Fe}
	24	1.7^{Be}	1.7^{Bf}	1.7^{Bf}	1.7^{Bd}	1.7^{Be}
Yeast and mould	0	3.48^{Aa}	3.48^{Aa}	3.48^{Aa}	3.48^{Aa}	3.48^{Aa}
	0.5	3.22^{Bb}	3.16^{Cb}	3.06^{Db}	2.81^{Eb}	2.54^{Fb}
	1	3.16^{Bc}	2.93^{Dc}	2.98^{Cc}	2.65^{Ec}	1.7^{Fc}
	3	2.88^{Bd}	2.54^{Cd}	1.7^{Dd}	1.7^{Dd}	1.7^{Dc}
	5	2.81^{Be}	2.4^{Ce}	1.7^{Dd}	1.7^{Dd}	1.7^{Dc}
	24	2.18^{Bf}	1.7^{Cf}	1.7^{Cd}	1.7^{Cd}	1.7^{Cc}
Total mesophilic bacteria	0	4.48^{Aa}	4.48^{Aa}	4.48^{Aa}	4.48^{Aa}	4.48^{Aa}
	0.5	4.39^{Bb}	3.94^{Cb}	3.8^{Db}	3.7^{Eb}	3.55^{Fb}
	1	4.3^{Bc}	3.75^{Cc}	3.24^{Fc}	3.61^{Dc}	3.48^{Ec}
	3	4.0^{Bd}	3.67^{Cd}	3.19^{Ed}	3.27^{Dd}	2.98^{Fd}
	5	3.86^{Be}	3.19^{Ce}	3.13^{De}	3.02^{Ee}	2.18^{Fe}
	24	3.7^{Bf}	2.18^{Cf}	2.18^{Cf}	2.18^{Cf}	2.18^{Ce}

*Data represent average value of three colony counting.

^{ABCD} means within row with the different letters indicate significant difference ($p < 0.05$) between treatments.

^{abcd} means within column with the different letters indicate significant difference ($p < 0.05$) between ozone exposure time.

6.3.1.2 Yeast and mould counts

The initial yeast and mould count was 3.48 log $cfug^{-1}$ (Table 6.1). It decreased significantly as ozone concentration increased. One hour of ozone at 0.05, 0.5, 2, 3.5 and 5.8 ppm reduced the yeast and mould count to 3.16, 2.93, 2.98, 2.65 and 1.7 log $cfug^{-1}$, respectively. A decrease in yeast and mould count with longer treatment on dried figs has been reported by Oztekin *et al*. (2006).

The moulds commonly associated with the spoilage of fruits and vegetables include *Botrytis cinerea* and *Aspergillus niger*. They produce a cocktail of enzymes such as hydrolases that are able to break down cell walls of produce, causing spoilage (Walton, 1994). The anthracnose symptom which commonly occurs in papaya is caused by the fungus *Colletotrichum gloeosporioides.*

To reduce yeast and mould activity, ozone should either be applied for longer periods at low concentration, or for short periods at higher concentrations (Najafi and Khodaparast, 2008). At 5.8 ppm, no yeasts and moulds were found after 1 h of exposure. Likewise, no yeasts and moulds were seen in fruit samples exposed for 3 h to 2.0, 3.5 and 5.8 ppm. Some yeasts are very effective at fermenting single sugars to produce alcohol and other volatiles that affect fruit quality (Barnett *et al.,* 2000). The destruction of yeasts and moulds just after harvesting reduces the possibility of aflatoxin formation before the next processing steps (Ahmed and Ahmed, 1997; Ahmed and

Robinson, 1999) which is a better preventive strategy than a subsequent complicated detoxification process.

6.3.1.3 Total coliforms counts

The initial mean value of coliform bacteria was 4.13 log $cfug^{-1}$ (Table 6.1). After 5 h of ozone at 0.05, 0.5, 2.0, 3.5 and 5.8 ppm, coliform counts were reduced to 2.98, 2.65, 2.18, 2.93 and 1.7 log $cfug^{-1}$, respectively. In addition, there was no increase in coliform bacteria after 24 h at all concentrations of ozone.

Biological factors such as the type of fruit or vegetable may result in different reduction of microbial populations after treatment with ozone. This is because the nature and composition of fruits and vegetables' surfaces and the type and load of microbial contaminants can affect the degree of bacteria attachment and hence overall performances of ozone treatment (Yuk *et al.,* 2006).

In conclusion, treatment of papaya fruit with gaseous ozone reduced significantly the total count of mesophilic microorganisms, coliform bacteria, yeasts and moulds. Significant differences between exposure times were found for all treatments. A minimum of 5 h of ozone treatment at 5.8 ppm could be successfully used for eliminating the coliforms, total mesophilic bacteria, yeasts and moulds. However, longer exposure times (up to 24 h) were required to reduce total counts of mesophilic bacteria, coliform bacteria, yeasts and moulds for all the ozone concentrations tested.

CHAPTER 7

EFFECT OF OZONE ON QUALITY AND PHYSIOLOGICAL BEHAVIOUR OF PAPAYA DURING STORAGE

7.1 Introduction

The term quality denotes the degree of excellence of a product or its suitability for a particular use. Quality of produce includes sensory properties (appearance, texture, taste and aroma), nutritive values, chemical constituents, mechanical properties, functional properties and defects (Abbott, 1999). Quality of fresh produces has been reported to be influenced by factors such as cultivar, post-harvest handling, fruit maturity, environmental factors and storage period (Van der Sluis *et al*., 2001; Ruiz-Rodriguez *et al*., 2008). Key determinants of the physicochemical and nutritional quality of fresh produce include colour, texture, soluble solids content (SSC) and acidity (Wu *et al*., 2007).

Post-harvest respiration and transpiration cause quality deterioration of fresh fruit, limiting shelf life. In addition, bioactive compounds may degrade rapidly during post-harvest storage, partly due to the oxidation of polyphenolics with exposure to light and oxygen (Connor *et al*., 2002). Several preservation technologies, including cold storage, vapour heat treatment, UV irradiation, edible coating, controlled and modified atmosphere storage have been used to reduce deterioration, prolong shelf life, and retain the nutritional value of

papaya. New technologies are needed to reduce decay in storage while maintaining quality.

Although the antimicrobial capacity of ozone has been widely reported (Beuchat, 1998; Aguayo *et al*., 2006; Tzortzakis *et al*., 2007b), only a few studies have reported the effects of ozone on product quality (Baur *et al*., 2004; Garcia *et al*., 2003; Rico *et al*., 2006 and Zhang *et al*., 2005). According to Skog and Chu (2001), an ozone concentration of 0.4 ml l^{-1} was effective in removing ethylene from the atmosphere in an apple and pear storage room, from 1.5–2 ml l^{-1} to a non-detectable level. Ozone treatment may affect fruit quality. The total soluble solids levels steadily increased in ozonized fruit, reaching significantly higher levels than in controls by the end of the week (Kute *et al*., 1995; Aguayo *et al*., 2006; Tzortzakis *et al*., 2007a). Maguire and Solverg (1980) reported that tomatoes ripened in 3.7 µl l^{-1} ozone had a more pronounced typical tomato aroma than those ripened in air. The same behaviour was early found in strawberries by Ewell (1950).

The objective of this study was to evaluate the efficacy of ozone treatment in gaseous form on physiological and compositional changes in papaya, and characterize physical and chemical changes that may impact fruit quality. In short, the aim of this work was to investigate the effects of exposure to ozone on the physical quality parameters (weight loss percentage, fruit firmness, peel colour), chemical composition (titratable acidity, soluble solids concentration), gas exchange evolution and sensory evaluation of papaya.

7.2 Materials and Methods

Quality evaluation

All treatments (0 (control), 1.5, 2.5, 3.5 and 5.0 ppm) with 96 h exposure were kept up to 14 days of ambient storage (25 ± 3 °C, 70 ± 5 %RH). The quality attributes of papaya fruit were evaluated at two days intervals. In each treatment, twelve fruits from 4 replications were randomly selected for physicochemical analyses.

7.2.1 Determination of physical quality

7.2.1.1 Weight loss percentage

Fruits in each replication were marked and weighed (using a digital balance, EK-600H, Japan) before storage for weight loss determination. The fruits were then weighed at 2-day intervals over the storage period. The results were expressed as percentage weight loss relative to the initial weight.

7.2.1.2 Fruit firmness

Fruit firmness was analysed using the Instron Universal Testing Machine (Model 5540, USA). Three fruits in each replication were penetrated using the 50 mm diameter probe, at a speed of 50 mm min^{-1} on three points in the equatorial region of the whole piece of fruit. The compression force measured at the maximum peak of the recorded force on the chart was expressed in Newtons (N).

7.2.1.3 Peel colour

Fruit peel colour on three points of the whole piece of fruit was determined using the Minolta CR-300 Chroma Meter (Minolta Corp., Japan). The colour determination made on papaya peels was expressed in chromaticity values of L*, C* and h°. The values of lightness (L*), forms the vertical axis with values ranging from 0 = black to 100 = white, a* (red-green axis) and b* (yellow-blue axis) which represent coordinates in the colour chart indirectly reflecting chroma (C*) and hue angle (h°). The C* which refers to the vividness of colour was computed from values of a* and b*, i.e. $C^* = (a^{*2} + b^{*2})^{½}$, which represents the hypotenuse of a right angled triangle with values ranging from 0 = least intense, to 60 = most intense. The h° was referred to as colour, and was the angle of tangent^{-1} b*/a*, where 0° = red purple, 90° = yellow, 180°= bluish-green and 270° = blue.

7.2.2 Determination of chemical quality

7.2.2.1 Soluble solids concentration

Fruit pulp samples from each replication (10 g) were homogenized using a kitchen blender (Model HR2094, Philip, Malaysia) with 40 ml of distilled water, and filtered through muslin cloth. The fruit pulp was sampled from the equatorial region of the whole piece of fruit and was a pooled sample from each replicate. A drop of the filtrate was then used to determine the SSC (°Brix) using Palette Digital Refractometer (Model: PR-32α) from Atago Co, Ltd (Japan), which were calibrated with distilled water prior to taking readings. The readings were multiplied by the dilution factor to obtain the original SSC (%) of the papaya pulp.

7.2.2.2 Titratable acidity

The titratable acidity (TA) was analysed using the titration method described by Ranganna (1999). Pulp tissue (10 g) was homogenized with 40 ml distilled water using a kitchen blender (Model HR2094, Philip, Malaysia) for two minutes. The mixture was then filtered through muslin cloth. An aliquot (5 ml) of filtrate with one to two drops of 0.1 % phenolphthalein as a pH indicator was titrated against 0.1 N sodium hydroxide (NaOH) to a pink endpoint (at least for 10 sec.). Titratable acidity was expressed as percentage of citric acid equivalents.

7.2.3 Gaseous exchange analysis

7.2.3.1 Determination of respiration rate

Fruit from each replication was placed in a sealed oblong storage container (13.5 cm diameter x 26.5 cm height). After 1 h of storage, 1 ml of gas sample was withdrawn from the container headspace and analyzed for CO_2 in a gas chromatograph (GC) (Claru-500, Perkin-Elmer, USA), equipped with thermal conductivity detector (TCD) fitted with a stainless steel column packed with Prorapack R of size 80/100 mesh. Helium was used as carrier gas with a flow rate of 20 ml min^{-1}. Temperatures were 60 °, 100 ° and 200 ° for the oven, injector and thermal conductivity detector, respectively. One milliliter of CO_2 gas (1.0 %) (Scotty Gases, Bellefonte, PA) was used as the external standard for calibration. Independent CO_2 samples were taken from each replication and the result expressed as means (ml kg^{-1} h^{-1}).

7.2.3.2 Determination of ethylene evolution

To determine the rate of ethylene production, papaya fruit were removed from treatment chambers every 2 days and placed in a sealed oblong storage container (13.5 cm diameter x 26.5 cm height). After 1 h of storage, a 1 ml gas sample was withdrawn from the container headspace and quantified using a gas chromatograph (Claru-500, Perkin-Elmer, USA), equipped with a flame ionization detector (FID) and a Poropack R column. Nitrogen, hydrogen and air flow rates were 20 ml min^{-1}. Nitrogen served as the carrier gas. Temperatures were 150 °, 200 ° and 200 ° for the oven, injector and FID, respectively. One milliliter of ethylene gas (10 µl ml^{-1}) (Scotty Gases) was used as an external gas standard for calibration. Independent ethylene samples were taken from each replication and the results expressed as means (µl kg^{-1} h^{-1}). The free headspace of each container was estimated by subtracting the fruit volume from the total volume of each sealed storage container.

7.2.4 Sensory evaluation of ripe papaya

Sensory qualities of fruit stored 12 days in ambient storage were evaluated by an untrained twenty member panel. The evaluation was done using a hedonic scale of 1 - 9, where 9 = Excellent, 7 = Good, 5 = Acceptable, 3 = Dislike and 1 = Extreme Dislike for appearance, pulp colour, aroma, sweetness, texture and overall acceptability (Appendix A). Scores of 5 and above were considered to be acceptable for commercial purpose. For sensory evaluation, sample of 20 fruit in each treatment were stored separately and used for evaluations.

7.3 Results and Discussion

7.3.1 Physical quality changes in papaya

7.3.1.1 Weight Loss

Concentration of ozone had little effect on weight loss of papaya as shown in Figure 7.1. The weight loss for the control and all treated papaya were 25.58 % (control), 25.14 % (1.5 ppm), 24.60 % (2.5 ppm), 24.21 % (3.5 ppm) and 24.83 % (5.0 ppm) at the end of the storage period (day 14).

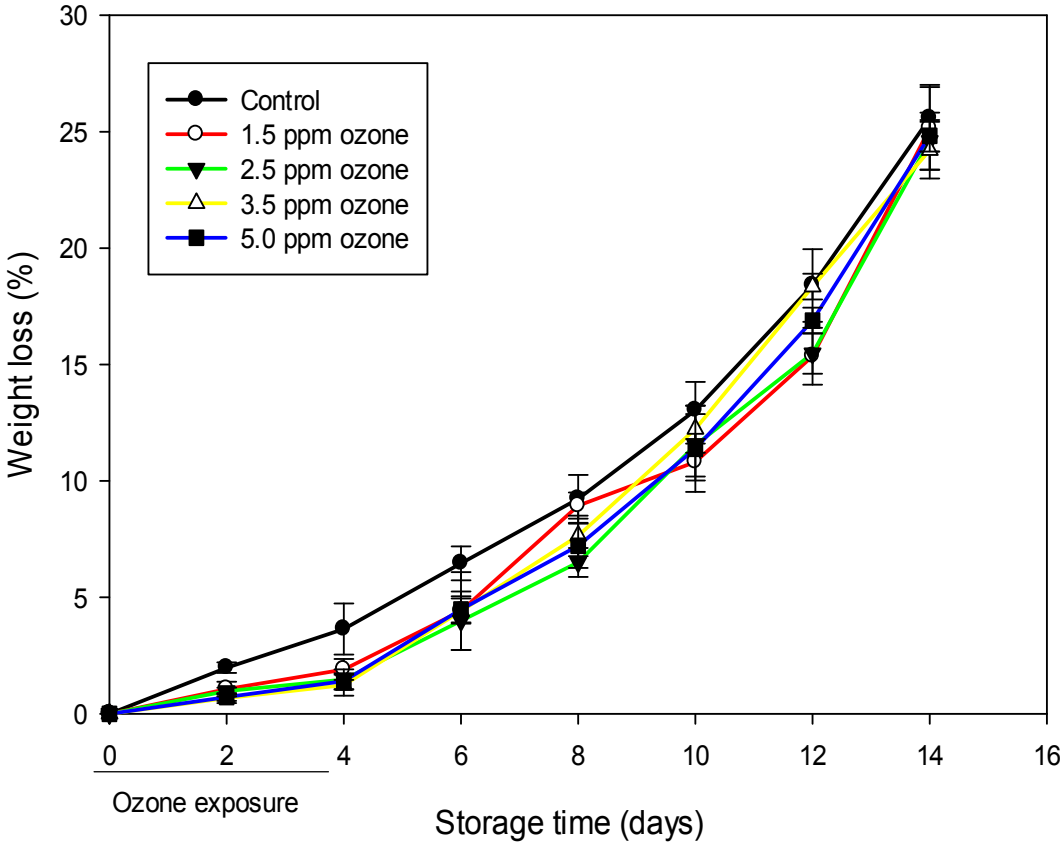


Figure 7.1 : Effect of ozone concentration on weight loss of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days at ambient storage (25 ± 3 °C, 70 ± 5 % RH). Each value is the mean of four replicates ± SE.

Fruit weight loss increased during storage in both control and treated fruit. After 4 days at 25 °C, all ozone-exposed papaya fruit had lower weight loss compared to the control (om average 2.43% lower). The weight loss for the control and all treated papaya were 3.64 % (control), 1.90 % (1.5 ppm), 1.47 % (2.5 ppm), 1.23 % (3.5 ppm) and 1.40 % (5.0 ppm) after four days of storage period. Weight loss was observed to be gradually increased during the storage period.

This is contrary to other investigations that have reported increased rates of water loss in ozone-treated 'Zee Lady' peaches under continuous ozone exposure at 0.3 ppm after 5 weeks of storage at 5 °C and 90 %RH (Palou *et al*., 2002). Weight loss of fresh commodities was found to be unaffected by low-level ozone treatment, but increased when exposed to higher concentrations of ozone, which was observed in 'Howes' cranberries (Norton *et al*., 1968), 'Zee Lady' peaches (Palou *et al*., 2001, 2002), onions (Song *et al*., 2000) and tomatoes (Shah *et al*., 2004). It has been suggested that higher levels of ozone may result in damage to the cuticle and/or epidermal tissues (Palou *et al*., 2002). The greater thickness of papaya cuticle compared to other fruits such as tomato and cranberries could be the reason for the reduced weight loss in ozone-treated papaya demonstrated in this study. Paull and Chen (1989) suggested that major pathway for weight loss in papaya was mainly water lost through stem scar, stomata and the cuticle. Consequently, the amount of water lost by a papaya fruit may differ depending on the cuticle thickness, which is cultivar and maturity dependent.

7.3.1.2 Fruit firmness

Firmness of papaya significantly ($p < 0.05$) declined with storage time for all treatments. At the end of the storage day 14, papaya treated with 2.5 ppm ozone clearly displayed the highest firmness (16.54 N) followed by papaya treated with 3.5 ppm ozone (14.43 N), 1.5 ppm ozone (12.52 N), 5.0 ppm ozone (11.74 N) and the lowest firmness in control papaya (10.93 N). During storage, samples treated with ozone maintained a good firmness. There was significant differences in firmness between the ozone treated fruits with the control fruit. All ozone treated fruit except fruit treated with 5 ppm ozone, were firmer at day 10 than control fruit, which suggests that the ozone treatments delayed ripening compared to controls as shown in Figure 7.2. This showed that fruits which were fumigated with 2.5 - 3.5 ppm ozone have delayed ripening process compared to control. Whereas, firmness of fruit which were treated with 5.0 ppm ozone exhibited deteriorated quality and high ozone level has caused the degradation of plant cuticle during storage. When the natural defenses in the plant cells can not neutralize the oxidative stress imposed by ozone, cellular damage occurs (Forney, 2003). Exposure of grapes to 4,000 ppm ozone for more than 40 minutes also results in the degradation of the cuticle seen as microscopic vein-like cracks on the epidermis (Sarig *et al*., 1996).

Flesh firmness is the parameter of greatest concern in papaya fruit storage and marketing, because flesh softening is associated with senescence and increases the fruit's susceptibility to injuries during handling. Tzortzakis *et al*. (2007a) also reported that tomato fruit stored in ozone-enriched atmosphere

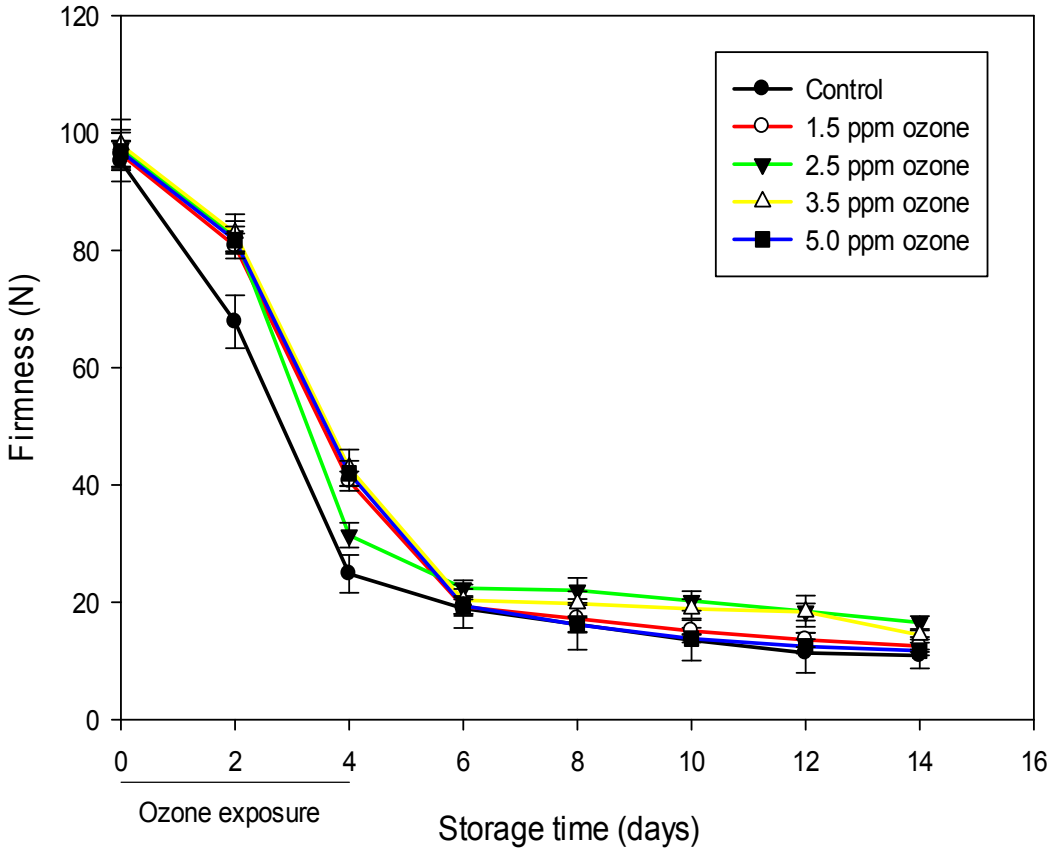


Figure 7.2 : Effect of ozone concentration on firmness of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days at ambient storage (25 ± 3 °C, 70 ± 5 %RH). Each value is the mean of four replicates ± SE.

(0.05 – 1 ppm) remained substantially firmer than fruit subjected to traditional (without ozone) storage conditions.

Ozone reacts rapidly with ethylene, and for those commodities that benefit from ethylene removal during storage (such as banana, papaya, persimmon), ozone is considered a potential tool to extend storage life (i.e. fruit ripening delay / firmness retention) with the added advantage of controlling disease proliferation (Jin *et al*., 1989; Aguayo *et al*., 2006; Salvador *et al*., 2006).

Similarly, Rodoni *et al*. (2010) mentioned that ozone treatments could delay some ripening-associated processes which maintained firmness of fruits during storage.

7.3.1.3 Peel colour

Colour is one of the major visual attributes of papaya and the change in colour from green to yellow continues over the storage period. The result on determination of peel colour showed that the lightness value of papaya increased when it starts to ripen, from 40.97 to 61.46 (Figure 7.3). Lightness value gradually reduces when it turns overripe. This means that fruit treated with 2.5 ppm (52.08) and 3.5 ppm (52.56) ozone were fully ripe at day 8, whereas control fruit (60.96) and fruit treated with 1.5 ppm (64.09) showed higher lightness value at day 6. This illustrated that fruit treated with 2.5 ppm and 3.5 ppm ozone have delayed ripening for 2 days and underwent slower changes in their peel colour, as indicated by the slower increase in L* value. Normally, luminosity or brightness of fruit was higher in ripened fruit, where the loss of chlorophyll makes yellow and red tones more evident with carotenoids and other pigments are responsible for these colours (Yahia and Ornelas-Paz, 2009). Ozone may cause physiological injury of produce due to its strong oxidizing activity. For instance, bananas treated with ozone developed black spots after 8 days of exposure to 25-30 ppm gaseous ozone (Parish *et al.,* 2003). Lightness (L*) parameter were slightly increased and decreased, after ozone treatments.

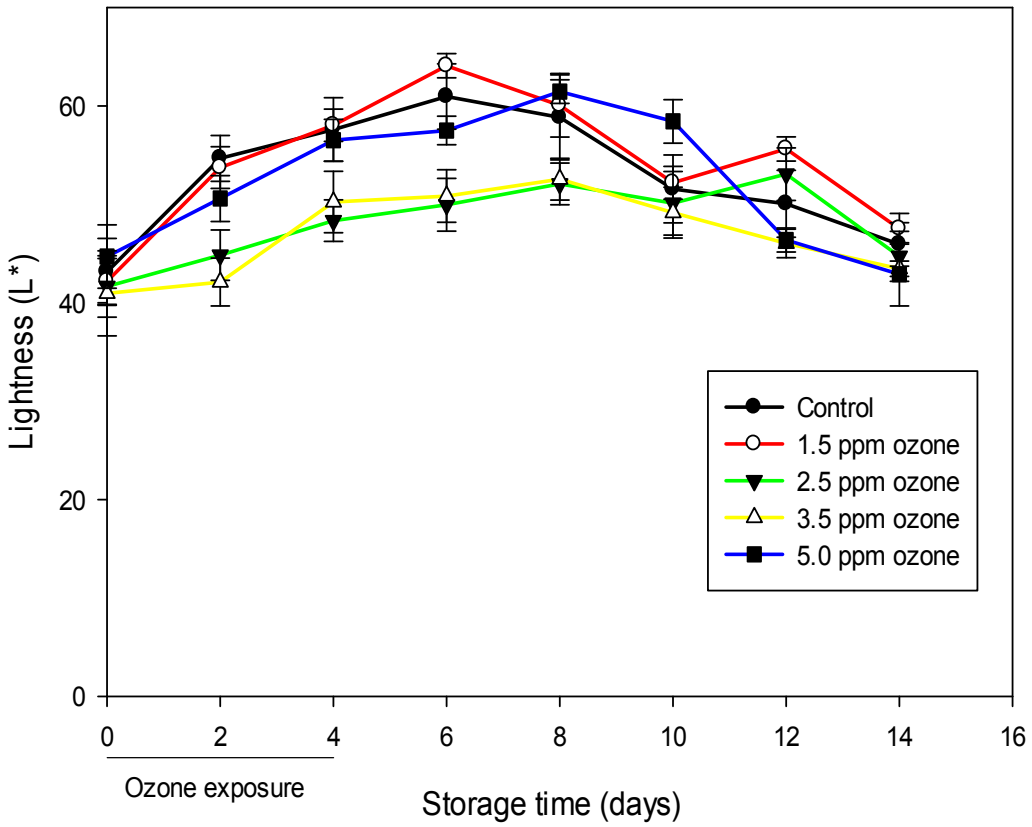


Figure 7.3 : Effect of ozone concentration on development of lightness (L*) of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days at ambient storage (25 ± 3 °C, 70 ± 5 %RH). Each value is the mean of four replicates ± SE.

The hue angle (h°) of papaya showed reduction, when fruit firmness was reduced. The hue angle measured for papaya was reduced from 123.82 to 65.66 (Figure 7.4). Higher hue was observed in 2.5 ppm treated papaya followed by 3.5 ppm treated papaya as compared to control. Basically, reduction of fruit firmness observed in papaya was correlated with the reduction of h° because ripened fruit which is less turgid has less hue (Gayosso-García Sancho *et al*., 2010). In contrary, the h° measured in the skin of whole tomatoes did not show significant changes over the ozone exposure time by a study done by Aguayo *et al*. (2007).

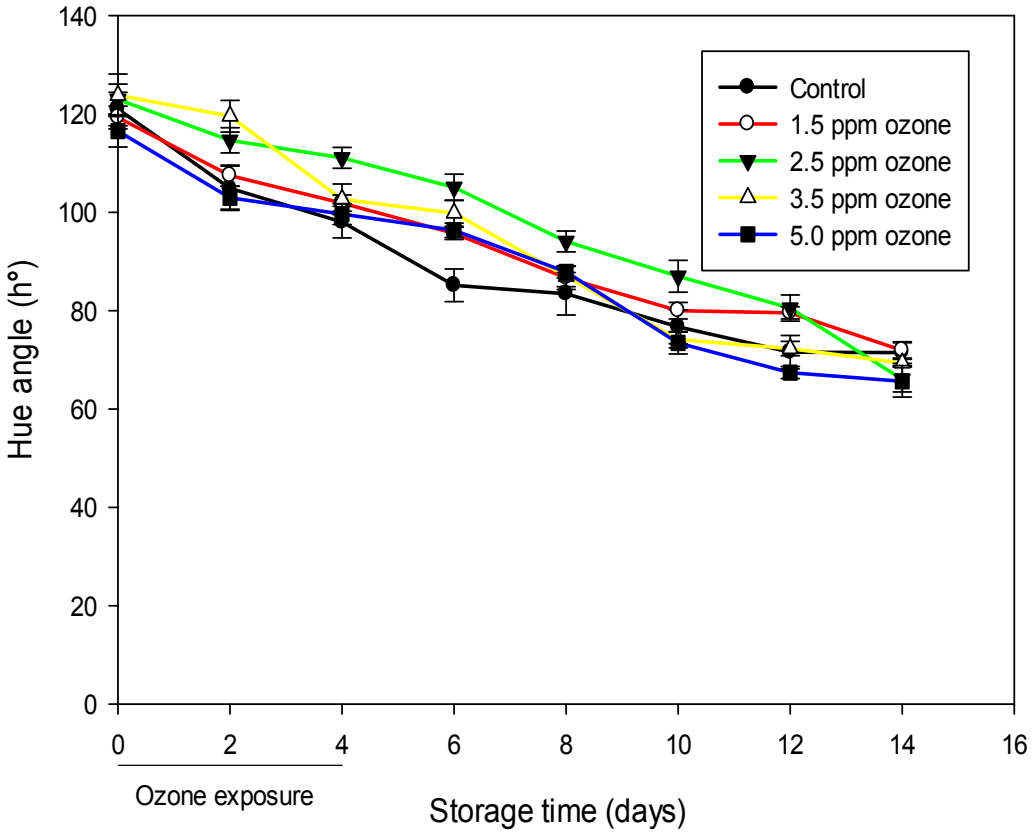


Figure 7.4 : Effect of ozone concentration on development of hue angle (h°) of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days at ambient storage (25 ± 3 °C, 70 ± 5 % RH). Each value is the mean of four replicates ± SE.

Chroma levels describe the degree of saturation or intensity of colour and the papaya chroma increased their colour intensity from the lowest, 22.76 to 53.17 when it starts to ripen (Figure 7.5). Lower increased of chroma resulted from 2.5 ppm and 3.5 ppm ozone treated papaya fruit demonstrated that intensity colour of that ozonated papaya is lower compared to the control. It also illustrated delayed process of ripening for those fruit treated with 2.5 ppm and 3.5 ppm ozone.

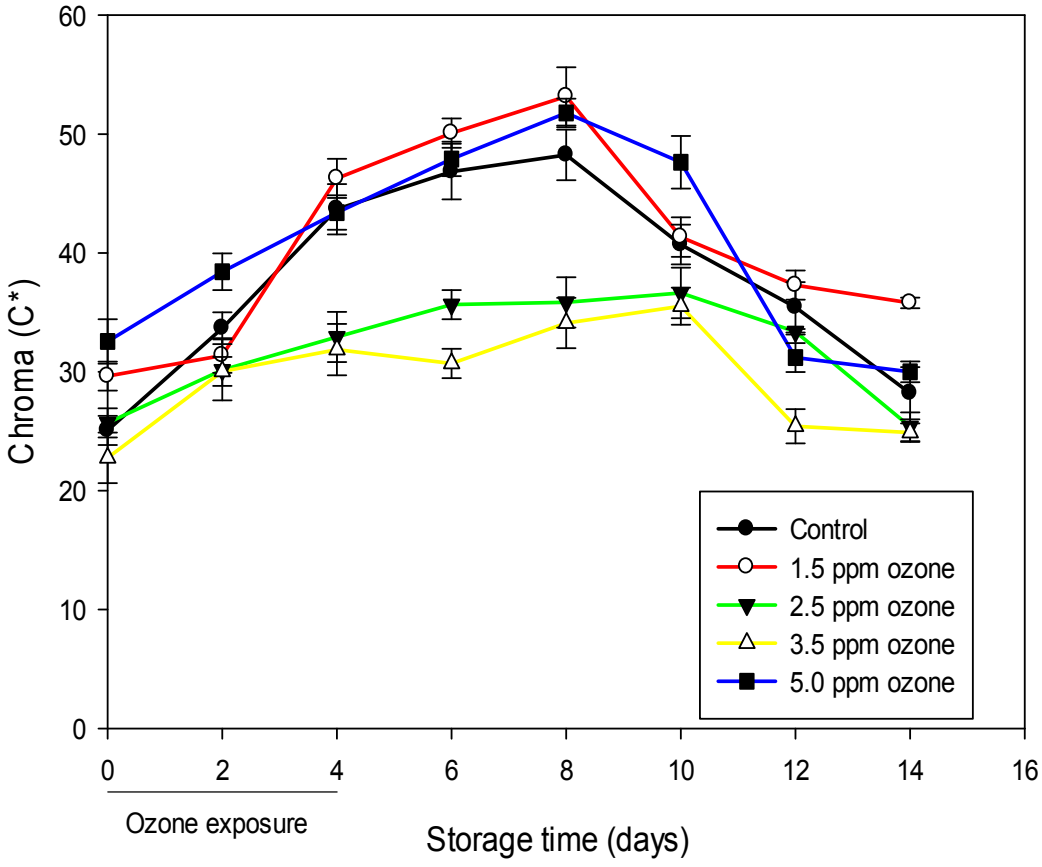


Figure 7.5 : Effect of ozone concentration on development of chroma (C*) of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days at ambient storage (25 ± 3 °C, 70 ± 5 % RH). Each value is the mean of four replicates ± SE.

In the present study, fruit exposed to 1.5 ppm and 2.5 ppm ozone for up to 96 hours showed no phytotoxic effects. As shown in Figure 7.6, some distinct surface browning developed on papaya treated with 3.5 ppm and 5.0 ppm ozone continuously for 96 hours (after 8 days of storage at 25 ± 3 °C) as a result of surface oxidation from excessive use of ozone. Ozone may promote oxidative spoilage (Kim *et al*., 1999).

Under high concentrations of ozone and after long term storage, fruit decay increases, indicating potential physiological damage to fruit (Baker, 1933). Ozone has been shown to change the surface colour of some fruits and vegetables such as carrots (Liew and Prange, 1994) and broccoli florets (Lewis *et al*., 1996). In contrast, treatments with lower concentrations of ozone showed no phytotoxic effects and had better physical appearance (uniform colour with less fungal spoilage and blemishes).

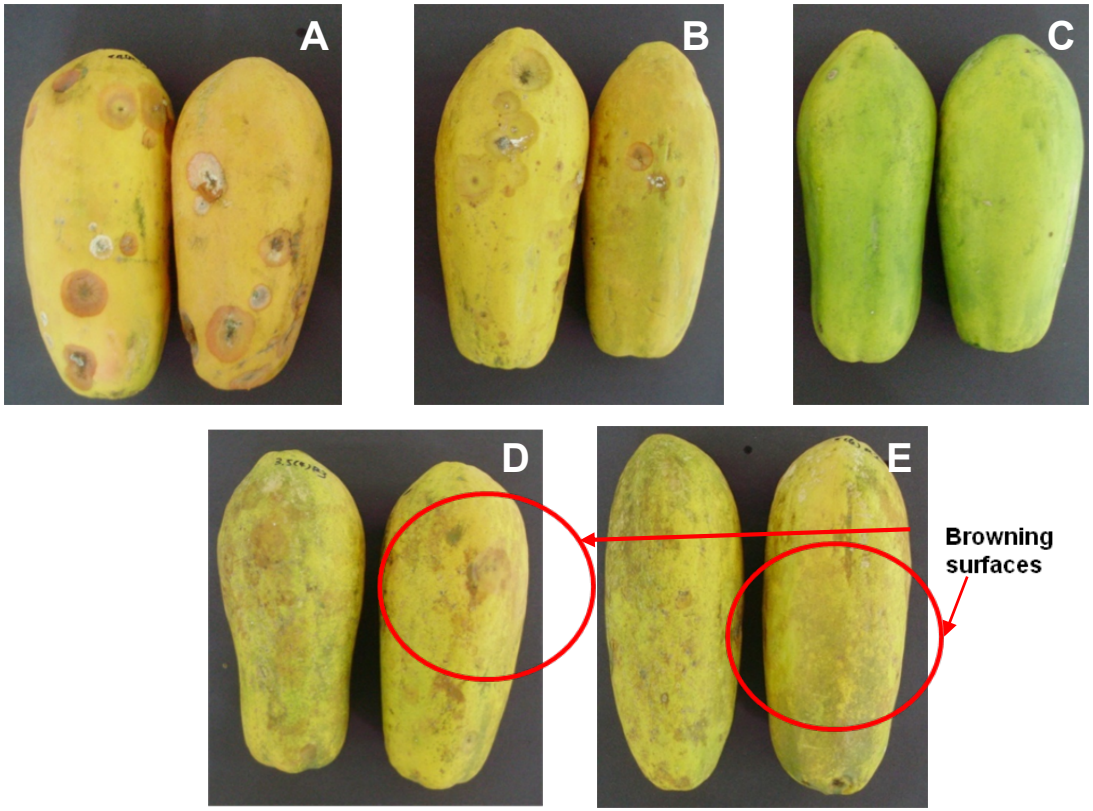


Figure 7.6: Papaya inoculated with spore suspension of *C. gloeosporioides* as control (A), and after treated for 96 h with 1.5 ppm (B), 2.5 ppm (C), 3.5 ppm (D) and 5 ppm (E) ozone during eight days of ambient storage (25 ± 3 °C, 70 ± 5 % RH).

7.3.2 Chemical quality changes in papaya

7.3.2.1 Soluble solids concentration (SSC)

Sugar content is another important quality attribute of papaya fruits. Fruit with high SSC are generally acceptable to consumers. Soluble solids concentration in whole papaya tended to increase with fruit maturation (Corral-Aguayo *et al*., 2008). The SSC for control (9 %) peaked at day 8 when the fruit ripened. Moreover, when the storage time was prolonged, a slight increase in SSC was observed being more evident in samples treated with the higher ozone dose. Soluble solids concentration increased during storage with significant difference between treated and untreated samples (Figure 7.7). The SSC at the end of storage period at day 14 was considerably highest ($p < 0.05$) in 2.5 ppm ozone-treated papaya (9.38 %) followed by 1.5 ppm ozone-treated papaya (8.50 %), 3.5 ppm ozone-treated papaya (8.38 %), 5.0 ppm ozone treated papaya (8.00 %) and the lowest value in control fruit (6.75 %). The highest SSC (11.25 %) in papaya fruits was found at day 10 after treated with 2.5 ppm ozone, whereas, the untreated fruits contained 9 % at day 8. Tzortzakis *et al.* (2007a) also reported that the soluble sugar content of tomato fruits previously stored in ozone-enriched atmosphere (0.05 – 1 ppm) was significantly greater than that of control fruit.

The delayed peak in SSC for the ozone treated papaya at day 10 was associated with an ozone-induced delay in ripening compared to the untreated fruit. However, both the SSC for the control and ozone treated papaya decreased after 8 days and 10 days, respectively and was associated with overripeness or senescence. Overall, ozone treated papaya was

sweeter or had higher SSC in comparison with non-treated fruit. As explained by Tzortzakis *et al.* (2007a), the levels of the dominant non-structural carbohydrate fractions (fructose and glucose) were sustained following ozone treatment. This is consistent with the recognized influence of non-structural carbohydrate and organic acid balance on the taste and flavor of ozone-treated fruit perceived as the degree of sweetness more than sourness.

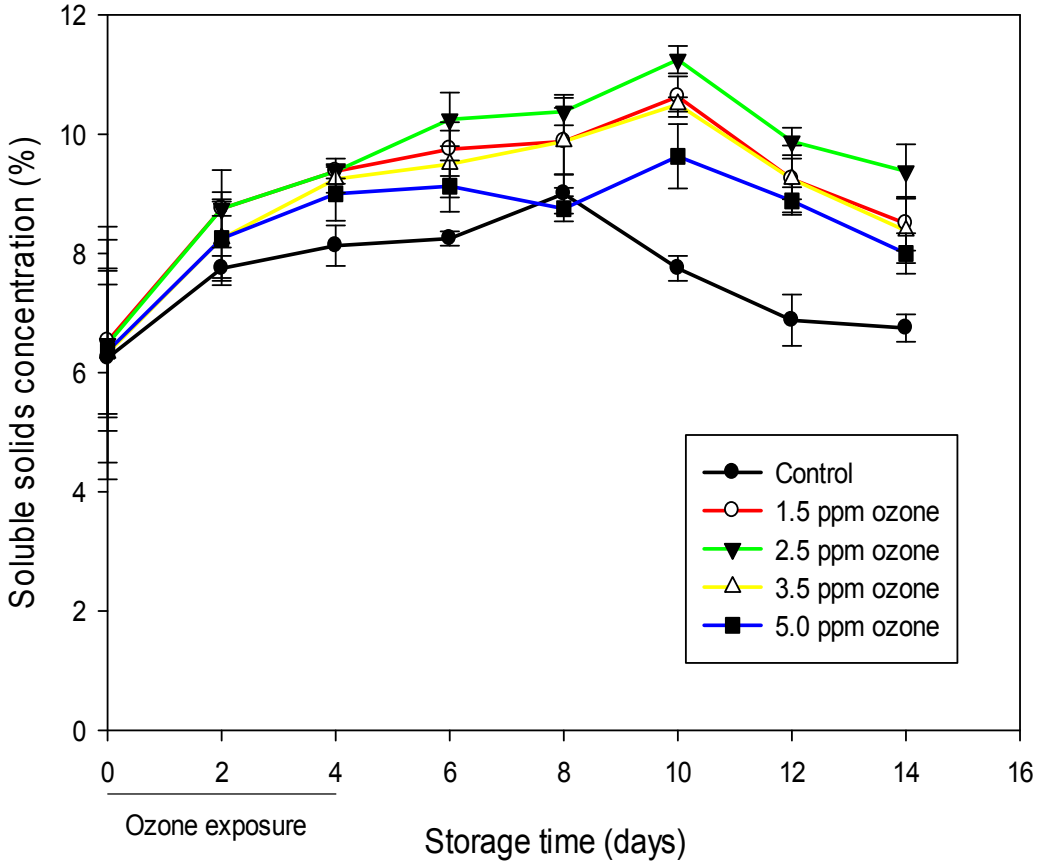


Figure 7.7 : Effect of ozone concentration on soluble solids concentration of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days at ambient storage (25 ± 3 °C, 70 ± 5 %RH). Each value is the mean of four replicates ± SE.

Finding was consistent with the reported SSC increase in strawberry (Kute *et al*., 1995) fruit in response to ozone exposure. The effect of a humidified flow of ozone-enriched air applied cyclically (4 ± 0.5 µl l^{-1} of O_3 for 30 min every 3 h) on metabolic behaviour and sensorial and microbial quality of whole and fresh-cut 'Thomas' tomatoes stored up to 15 days at 5 °C, measured that in ozone treated whole and sliced tomatoes contained a higher sugar (fructose and glucose) and organic acid (ascorbic and fumaric) content (Aguayo *et al.,* 2006).

7.3.2.2 Titratable acidity (TA)

Ozone treatments employed in this work caused significant effect on the titratable acidity of papaya fruit as shown in Table 7.1. The TA of 'Sekaki' papaya fruit ranged from 0.51 to 1.57 %, and those fruits exposed to ozone with 2.5 ppm and above resulted in lower reduction of titratable acidity compared to control fruit and fruits subjected to lower ppm of ozone. The differences were observed during day 6, 8 and 10. In contrast, no significant effect of ozone on TA was reported by Perez *et al.* (1999) for the storage of strawberry. Low-level ozone enrichment has been reported to have no effect on organic acid composition of treated tomatoes and citrus (Smilanick, 2003). In general, levels of acids decline during ripening, presumably due to their consumption in respiration. Organic acids provide most of the hydrogen ion and normally decrease with ripening. TA in whole papaya tended to decrease with fruit maturation (Corral-Aguayo *et al*., 2008). However, all ozone-treated papaya reached the same level of titratable acidity at the last day of ambient storage.

Table 7.1: Effect of ozone concentration on titratable acidity (% citric acid equivalents) of 'Sekaki' papaya after treated for 4 days and later stored up to 14 days under ambient conditions (25 ± 3 °C and 70 ± 5 % RH).

Storage time (days)	Ozone concentration (ppm)				
	0	1.5	2.5	3.5	5.0
0	1.57^{a}	1.55^{ab}	1.54^{ab}	1.54^{ab}	1.52^{b}
2	1.51^{a}	1.43^{b}	1.43^{b}	1.48^{b}	1.48^{b}
4	1.38^{a}	1.28^{b}	1.38^{a}	1.38^{a}	1.28^{b}
6	1.08^{a}	1.08^{a}	1.02^{b}	1.02^{b}	0.98^{c}
8	1.02^{a}	1.02^{a}	0.98^{b}	0.98^{b}	0.67^{c}
10	0.77^{a}	0.72^{b}	0.64^{c}	0.72^{b}	0.64^{c}
12	0.67^{a}	0.64^{a}	0.61^{a}	0.64^{a}	0.61^{a}
14	0.51^{a}	0.51^{a}	0.51^{a}	0.51^{a}	0.51^{a}

Values are means of three experiments with four replicates determinations per experiment.

^{abcd} means within row with the different letters indicate significant difference ($p < 0.05$) between treatments.

7.3.3 Gaseous exchange analysis

7.3.3.1 Determination of respiration rate

Changes in respiration rate of ozone treated and untreated papaya fruits kept for 14 days at ambient conditions (25 ± 3 °C and 70 ± 5 %RH) are shown in Figure 7.8. The respiration rate for the control fruit is higher than the ozonated fruits. This showed that the ozonated fruits have slower metabolism and have delayed ripening compared to the control. Besides, the peak of respiration rate of all ozone-treated fruits were delayed for 2 days compared to control fruit, which have respiration rate peak at day 8. This may be caused by the abiotic stress imposed by ozone treatments which reduced the respiration rate. Hence, ozone may stimulate respiration in a way similar to a stress, in which after day 10, the metabolic activity decreased to levels lower

than in control. Results in respiration rate showed a transient increase after day 10, but slightly lower than that of control fruit. This behavior pattern of whole fruit treatment was similar with respiration rate in treated whole tomato fruit by Aguayo *et al*. (2006).

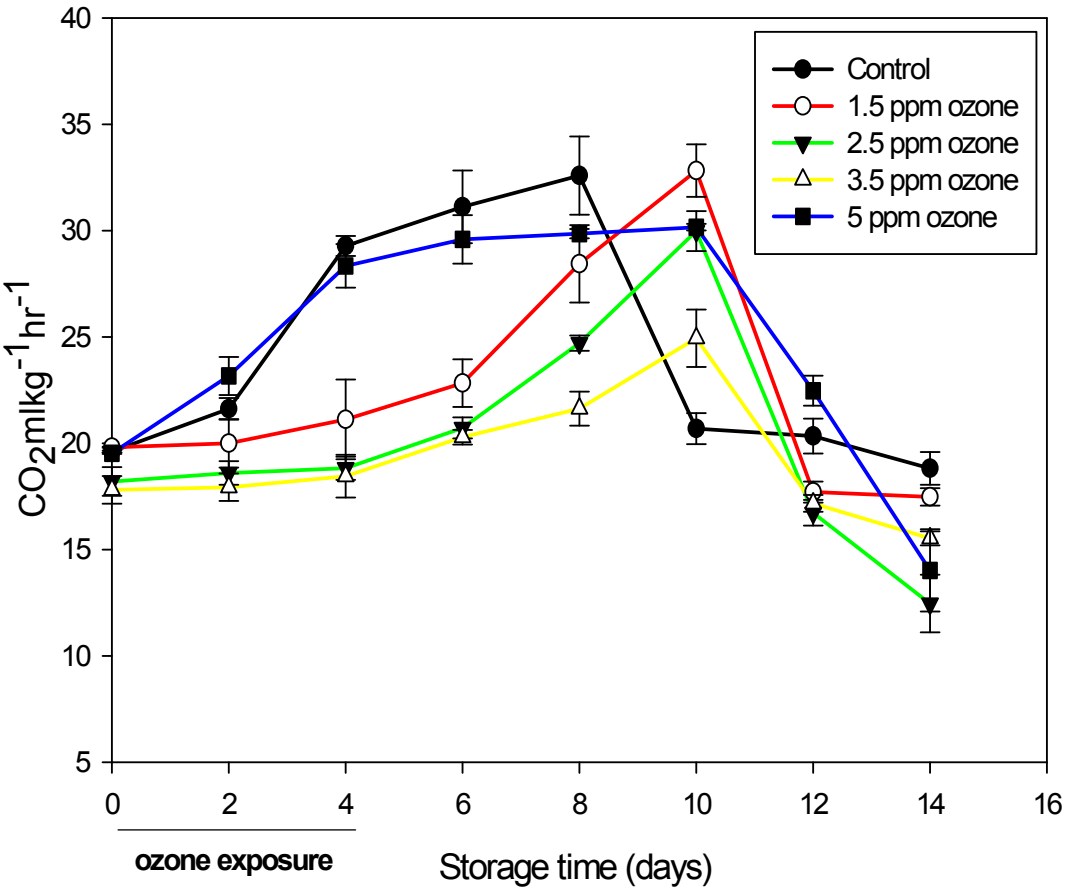


Figure 7.8: Effect of ozone concentration on CO_2 production of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

Low-level ozone enrichment (0.3 ppm / < 1.0 ppm) has been reported to have no effect on respiration and ethylene production in grapes and peaches (Smilanick, 2003). Low-level of ozone enrichment atmosphere resulted in no significant change in the rate of tomato fruit respiration or ethylene production during or following storage (Tzortzakis *et al*., 2007a). This difference may be caused by ethylene-insensitivity of non-climateric fruits such as grapes and peaches. Ozone treatments were able to delay some ripening-associated processes (Rodoni *et al*., 2010). Several studies on tropical fruit such as mango and papaya observed that the higher the level of ripeness and / or storage temperature, the higher the respiration rate (Rivera-Lopez *et al*., 2005). Climateric fruits such as papaya are characterized by an increment in their respiration and ethylene biosynthesis patterns during the ripening process (Lelievre *et al.*, 1997). Jin *et al*. (1989) found longer shelf life in tomatoes under ozone due to lower respiration rate and ethylene emission. In a study done by Chauhan *et al*. (2011), fresh-cut carrots were ozonized in water (1:2 w/v; @ 200 mg O_3/h) for 10 min and stored under controlled atmosphere (CA) conditions (2 % O_2, 5 % CO_2 and 93 % N_2) at 6 ± 1 °C and 85 % RH for up to 30 days. The maximum decrease in respiration and ethylene emission rates were obtained by the combination of CA with ozone followed by CA alone and ozonation compared with the control samples kept under low temperature (6 ± 1 °C).

Gane (1936) observed that the exposure of ripe bananas to 1.5-1.7 ppm ozone did not change the respiration rate and extended the shelf life but only when the fruit were nearly ripe. Palou *et al.* (2002) also detected a similar

CO_2 and ethylene emission in 'O' Henry' peaches under ozone exposure. A study by Craker (1971) showed conflicting evidence in which the ozone treated tomato, tobacco and bean plants have increased rates of ethylene production. This result indicated that some responses of plant tissue to ozone fumigation may be due to active production of ethylene by the injured tissue. Similarly, Liew and Prange (1994) found in carrot a slight increase in CO_2 production under ozone treatments, depending on doses and storage time.

7.3.3.2 Determination of ethylene

Changes in ethylene production of ozone treated and untreated papaya fruits kept for 14 days at ambient conditions (25 ± 3 °C and 70 ± 5 %RH), are shown in Figure 7.9. During ozone exposure for 4 days, the ethylene production of the ozone treated fruits was significantly lower than the untreated fruits. This explained that ozone treatment delay ripening of papaya fruit. The ethylene production of untreated fruits increased gradually and then reached its maximum production (45.05 µl kg^{-1} hr^{-1}) after 8 days of storage. However, following ozone fumigation, ethylene production of papaya fruit increased. On day 10 of storage, the ethylene production of 5 ppm ozone treated fruits (72.89 µl kg^{-1} hr^{-1}) was about 1.62 times higher than that of the untreated fruits. On day 10, the ethylene production of ozone treated fruits was directly proportionate with the concentration of the ozone treatment. The fruit receiving higher concentrations of ozone fumigation gave off more ethylene. In all cases ethylene production was higher than control fruits receiving no ozone treatment.

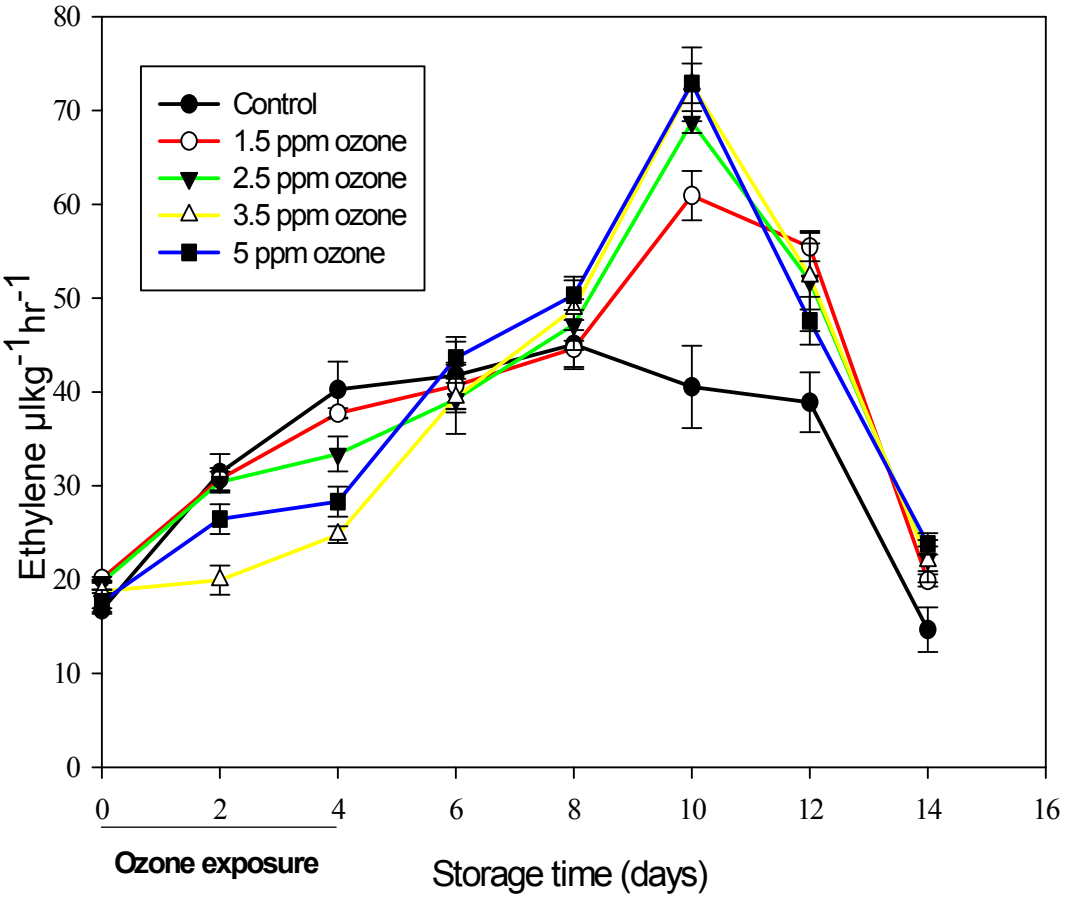


Figure 7.9: Effect of ozone concentration on ethylene production of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

Results of these studies indicate that ozone injury to plant tissue can induce ethylene production. Apparently, structural damage on papaya surface was visualized when the fruit were treated with 3.5 ppm and 5.0 ppm ozone. This is similar to other reports where physical or chemical stress to plants caused increased ethylene production (Burg, 1962; Pratt and Goeschl, 1969). Aguayo *et al*. (2006) also reported the same trend of ethylene emission in whole tomato fruit. The ethylene emission of tomato fruit was slightly

increased by ozone in comparison to the control. Forney (2003) observed similar increase in ethylene production in broccoli exposed to injurious ozone treatments of 700 ppb, which resulted in increased water loss and tissue browning.

Ethylene production from ozone-treated fruit appeared to be related to the extent of visual injury. The papaya fruit in this study was visually injured by ozone treatment on day 10 of storage. The fruit receiving higher concentrations of ozone fumigation gave off more ethylene. These results would be expected if the ethylene formation is induced by ozone injury to the cell. More severe plant injury is a result of more cells being damaged by pollutants like ozone and thus would induce more cells to evolve ethylene. However, some physiological responses observed and reported after ozone treatment have been previously reported as ethylene responses (Pratt & Goeschl, 1969), and they may be a result of induced ethylene from oxidant injury.

According to Kangasjärvi *et al.* (1994) and Sandermann (1998), ozone exposure induces the emission of ethylene in various plant species. The ethylene emission was positively correlated with ozone sensitivity, indicating that ethylene may be involved in the control of ozone damage (Langerbartels *et al*., 1991; Mehlhorn *et al*., 1991). Tuomainen *et al*. (1997) also reported that ozone causes a rapid and highly selective activation of genes involved in ethylene biosynthesis resembling plant-pathogen interactions.

A rapid reaction between ethylene and ozone in air occurs (Dickson *et al*., 1992), and for those commodities that benefit by ethylene removal, ozone may be of use to delay the ripening process and maintain quality. Several researches proved the ability of ozone to abate ethylene in empty storage rooms (Palou *et al*., 2001; Rice *et al*., 1982). However, controversy has arisen about the potential of ozone to scrub ethylene as ozone could alter the plant physiological response to ethylene and in some circumstances could even induce the production of ethylene by the commodity (Forney, 2003).

7.3.4 Sensory evaluation of ripe papaya

Sensory evaluation of ozone treated and untreated papaya fruits after storage for 12 days at ambient temperature revealed significant ($p < 0.05$) differences in sweetness and texture (Table 7.2). There was no significant difference in term of appearance, pulp colour, aroma and overall acceptability. Fruit treated with 2.5 ppm ozone attained the highest scores by the panelists in sweetness tests, as also demonstrated from the previous SSC. The control fruits were not presentable to panelists for sensory evaluation at the end of storage period (14 days) because they started rotting; therefore this evaluation was done when the fruits were stored up to 12 days. The sensory attributes of papaya treated with 2.5 ppm and 3.5 ppm ozone were overall superior after 12 days of ambient storage. These results suggest that ozone treatment at 2.5 ppm can be used successfully as a sanitizing and preservation tool for prolonging shelf life and improving papaya fruit quality during ambient storage.

Table 7.2: Sensorial parameters of ozone treated and untreated 'Sekaki' papaya after treated for 4 days and later stored up to 12 days under ambient conditions (25 ± 3 °C and 70 ± 5 %RH).

Treatment	Appearance[a] (1-9)	Pulp Colour[a] (1-9)	Aroma[a] (1-9)	Sweetness[a] (1-9)	Texture[a] (1-9)	Overall acceptability[a] (1-9)
Control	5.40^{A}	5.30^{A}	4.63^{A}	5.33^{C}	5.20^{C}	5.15^{C}
1.5 ppm	5.90^{A}	5.95^{A}	4.75^{A}	5.35^{C}	5.53^{BC}	5.45^{B}
2.5 ppm	6.15^{A}	6.20^{A}	5.15^{A}	6.43^{A}	6.03^{A}	6.00^{A}
3.5 ppm	5.43^{A}	5.30^{A}	4.75^{A}	6.00^{B}	5.78^{AB}	5.88^{A}
5 ppm	4.95^{A}	4.88^{A}	4.50^{A}	5.65^{BC}	5.80^{AB}	5.60^{B}

[a] Hedonic scale where 1 = Extreme dislike , 3= Dislike , 5 = Acceptable , 7= Good and 9 = Excellent

^{ABCD} means within column with the different letters indicate significant difference ($p<0.05$) between treatments.

At the market level, only produce that corresponds to the expectations of the consumers is acceptable. Thus, it is vital to assess the effects of potential innovative practices on sensory and organoleptic properties of fruits and vegetables (Tzortzakis *et al.,* 2007a). This is particularly so in the case of ozone-enrichment, as effects on quality-related characteristics seem highly dependent on the commodity and storage conditions (Liew and Prange, 1994).

In some other studies, researchers reported that ozone treatment improved and did not alter the sensory quality of some fruits and vegetables significantly (Kute *et al*., 1995, Lewis *et al.,* 1996). Hence, changes in the sensory attributes depend on the chemical composition of food, ozone dose and treatment condition (Kim *et al.,* 1999).

Abbot (1999) reported that general appearance is the main sensory attribute that consumers use to evaluate the quality of fresh fruits and vegetables and a cut-off score above 5 is regarded as acceptable. For papaya, lack of sweetness and texture are accepted as the critical factors in perceived loss of quality. Sweetness is an important quality parameter as consumers associate it with high product quality. °Brix or soluble solids content, which is associated with sweetness, is considered to be an important factor in terms of eating quality (Lallu *et al*., 1989; Tavarini *et al*., 2008). All the samples exceeded the acceptability cut-off score at day 12. There were no significant differences in the sensory attributes among the samples except for the control sample, which was in general inferior in overall acceptability. The 2.5 ppm ozone treated samples had significantly ($p < 0.05$) better scores in all the sensory parameters compared with the other ozone treated fruits and control sample at day 12. All the ozone treated samples degraded more slowly compared with the control samples. Sensory results of tomatoes as conducted by Tzortzakis *et al*. (2007a) also revealed that fruit subjected to ozone-enrichment were perceptibly sweeter and retained their firmness in comparison with fruit subject to traditional storage. It is apparent that the effect of ozone treatment on quality of produce is concentration dependent. Ozone treatments may be advantageous up to a certain level, whereas higher concentrations may speed up browning reactions resulting in inferior quality as compared with controls. It is common that effect of air pollutants such as sulfur oxide and ozone can reduce photosynthesis and cause formation of lesions, chlorosis and discolouration which reduced the product quality (Kays, 1997).

In conclusion, these results indicate that gaseous ozone treatment from 1.5 to 2.5 ppm ozone for 96 hours would be useful in enhancing quality of papaya fruits, but it would have a negative effect on the aesthetic value if being treated with 3.5 to 5.0 ppm ozone. Overall, the application of 2.5 ppm of ozone fumigation for 96 hours was found to be the optimum treatment for maintaining and improving the fruit sensory quality in terms of taste and firmness during ambient storage.

CHAPTER 8

EFFECT OF OZONE ON MAJOR ANTIOXIDANT COMPONENTS OF PAPAYA DURING STORAGE

8.1 Introduction

Fruit and vegetables contain significant levels of biologically active components with physiological and biochemical functions which have health benefits to human. Fruit is an excellent food characterised by a low content of calories and a high amount of antioxidant substances which are able to prevent a wide range of chronic diseases, such as cancer, cardio-vascular diseases, and degenerative illnesses connected to the aging processes (Jang *et al*., 1997; Arai *et al*., 2000). Consumers are becoming increasingly aware of the nutrient quantity and composition of many essential minerals, vitamins and phytonutrients from fruit and vegetables, and the healthy benefit of regular intake of fruit and vegetables as part of the diet (Arscott and Tanumihardjo, 2010; Lindsay, 2000).

Papaya is one of the tropical fruits with important antioxidant properties and is in high demand in international markets. Papaya is ranked first on nutritional scores among 38 common fruits, based on the percentage of the United States Recommended Daily Allowance for vitamin A, vitamin C, potassium, folate, niacin, thiamine, riboflavin, iron and calcium, plus fibre (Ming *et al*., 2008). High amounts of antioxidants, both of hydro-soluble and lipid-soluble varieties, including vitamin C, E and A in papaya have numerous health

benefits, ranging from reduced risks of developing cardiovascular diseases, macular degeneration to protection against colon and prostate cancer. According to FAOSTAT (2012), about 11.2 million metric tonnes of papaya produced in 2010 from 433,500 hectare in various countries. In 2010, Malaysia produced papaya for domestic and export market with about 46,000 metric tonnes (FAOSTAT, 2012). It is noted that agronomic conditions, variability among cultivars, divergence among seasons, stage of ripeness and postharvest manipulation, all could be possible factors explaining the differences of antioxidant capacity and quality of fruit (Kevers *et al*., 2007).

In recent years, there has been a growing interest in the effect of ozone on inducing antioxidant in plants, as well as on the compounds responsible for such activity. Ozone also induces oxidative stress in plant tissues, which induces a wide array of physiological responses, including increased concentrations of antioxidants, polyamines, phenolic compounds, ethylene, and other secondary metabolites (Forney, 2003). Other beneficial effects of ozone have been reported in potatoes by Kolodyaznaya and Suponina (1975) and in strawberries by Perez *et al.* (1999) who found a 3-fold increase of ascorbic acid content in ozone-treated fruit. Tzortzakis *et al.* (2007a) reported that an ozone-enriched atmosphere (concentration up to 1.0 μmol mol^{-1} at 13 °C) did not change the antioxidant activity of tomatoes (*Lycopersicon esculentum* L. cv. Carousel) despite the two- to three-fold increase in beta-carotene, lutein, and lycopene contents due to the treatment.

In relation to the importance of phytochemicals and antioxidant power for functional aspect of papaya, the objective of this study was to determine changes in total phenols, ascorbic acid content, β-carotene content, lycopene content and antioxidant activity of papaya following ozone treatment and subsequent storage.

8.2 Materials and Methods

8.2.1 Determination of major antioxidant components

8.2.1.1 Ascorbic acid (Vitamin C)

Ascorbic acid was determined by using the 2,6 dichlorophenol-indophenol (DCPIP) dye titration method (AOAC, 1990). A solution containing 3 % metaphosphoric acid (HPO_3) was prepared by dissolving 30.0 g HPO_3 in 1000 ml of distilled water. The DCPIP dye solution was prepared by dissolving 50 mg of DCPIP in 150 ml of hot water containing 42 mg sodium bicarbonate. Samples were prepared by using 10 g of fruit blended with 90 ml of 3 % HPO_3 solution and filtered and poured into a 100 ml volumetric flask. The volume was made up to 100 ml using 3 % HPO_3. An aliquot (10 ml) of the sample was taken for titration against 2,6 dichlorophenol-indophenol dye until a pink colour persisted for 15 sec.

A standard ascorbic acid solution was also prepared by dissolving 100 mg of L-ascorbic acid in 100 ml of 3 % metaphosphoric acid (HPO_3) solution. An aliquot (1 ml) of the standard was pipetted out and a further 9 ml of 3 % metaphosphoric acid (HPO_3) solution was added to the 1 ml standard to create a standard solution with the concentration of 1 ml = 0.1 mg ascorbic acid.

An aliquot (5 ml) of the standard solution was then mixed with 5 ml of HPO_3 and the solution was titrated with DCPIP until the appearance of a persistent faint pink colour for 15 seconds. The dye factor was then determined using the formula below. The dye factor was calculated by titrating the standard

ascorbic acid against 2,6 dichlorophenol-indophenol. The ascorbic acid was calculated using the following formula:

Dye factor = 0.5 / Titre value (ml)

Ascorbic Acid (mg $100g^{-1}$):

$$\frac{\text{Dye factor x Titre value (ml) x Volume made up (100 ml) X 100}}{\text{Sample weight (10 g) x Aliquot taken for estimation (10 ml)}}$$

8.2.1.2 β-carotene and Lycopene content

Carotenoids were determined by the method described by George *et al.* (2011), which involved extracting the sample with acetone. Briefly, ground papaya pulp tissues (10 g) were mixed with 50 ml acetone for 20 min in the dark and the mixture filtered through cotton wool. Carotenoids from the remaining material were subsequently extracted in the same way twice by mixing with 30 ml acetone for 5 min and combining the filtrates in a 250 ml separating funnel. An aliquot of 75 ml petroleum ether was added and the organic phase washed three times with 50 ml of distilled water. Remaining water was removed with anhydrous sodium sulphate, and the volume was made up to 100 ml with petroleum ether. The optical density of the layer was measured with a spectrophotometer (Biochrom, Libra S12, UK) at 450 nm and 503 nm using petroleum ether as a blank. In this experiment, all the equipment used was wrapped with aluminium foil to avoid exposure to the light. The concentrations of lycopene and β-carotene were determined spectrophotometrically using the equations (Lime *et al*., 1957):

[C] β-carotene = 4.624 x A_{450} - 3.091 x A_{503}

[C] lycopene = 3.956 x A_{450} - 0.806 x A_{503}

Where [C] is the concentration of carotenoid expressed in mg $100g^{-1}$ FW, and A_{450} and A_{503} represent the absorbance at 450 nm and 503 nm, respectively.

8.2.1.3 Extraction of polyphenols

The fruit extract was prepared using the modified method of Chew *et al*. (2011). Papaya pulp tissues (2 g) from each replication were homogenized in a glass tube with 10 ml methanol (80 %) as extraction solvent. The glass tube was sealed with parafilm and wrapped with aluminum foil to prevent solvent loss and exposure to light. The mixture was then incubated at 45 °C for 1 hour using a water bath (Model BH-230, Yih Der, Taiwan). The papaya extract was then filtered through Whatman No.1 filter paper (Whatman International Ltd., England) to obtain a clear crude extract solution. Subsequently, this crude extract was stored in aluminium foiled tubes and kept at -80 °C until further analysis of total phenolic contents and antioxidant activity.

8.2.1.4 Total phenolic content

The phenolic content in papaya fruit was determined using the method of Singleton and Rossi (1965). The Folin-Ciocalteu assay which is an electron transfer based method was used in this experiment to detect the total phenolic contents of ozone treated samples. A blue coloured complex was formed and easily detected at wavelength A_{765nm} upon the oxidation of phenolic compounds by the phosphomolybdate and phosphotungstate present in Folin-

Ciocalteu reagent (Prior *et al*., 2005). An aliquot (0.1 ml) of crude extract solution was placed in a test tube. An aliquot of 0.1 ml water in a test tube served as the control (blank). Water (6 ml) was added to the sample or blank, followed by 0.5 ml undiluted Folin-Ciocalteau reagent. After 30 seconds to 8 minutes, 1.5 ml of 7 % (w/v) sodium carbonate was added. An aliquot of 1.9 ml water was added to the solutions to give a final volume of 10 ml and the solution was vortexed. The mixture was incubated for two hours at 40 °C. The absorption of total phenolics was determined at 765 nm using a spectrophotometer (Biochrom, Libra S12, UK). A standard curve (R^2 = 0.999) was established using 0.1 ml of different concentrations of gallic acid solution in 80 % ethanol. Briefly, 10 mg of gallic acid was dissolved in 2 ml 80 % methanol and the volume made up to 10 ml with water to give a final concentration of 1000 µg ml^{-1}. The concentration of stock solution of gallic acid was diluted and mixed to 100, 200, 400, 600, 800 and 1000 µg ml^{-1} respectively (Appendix B1). Total phenolic content was determined against the standard gallic acid calibration curve and the absorbance value was converted to mg of gallic acid equivalents (GAE) per gram of fresh weight (mgGAE g^{-1} FW).

8.2.1.5 Determination of total antioxidant activity

8.2.1.5.1 FRAP Assay

For antioxidant activity, the Ferric Reducing Antioxidant Power (FRAP) assay was conducted as described in Benzie and Szeto (1999). There are two mechanisms involved in the deactivation of free radicals by antioxidants which are hydrogen atom transfer (HAT) and Single Electron Transfer (SET). The

potential of an antioxidant to quench free radicals by hydrogen donation is known as HAT; whereas, the ability of antioxidants to reduce any compounds for example, metals, carbonyls and radicals by donating their electrons is known as SET (Prior *et al*., 2005). The FRAP assay which measures the reducing power of antioxidants through SET mechanisms was used to detect the antioxidant capacities of the polyphenol extract. The redox reactions changed the FRAP reagent into intense blue which was detected at wavelength $A_{593\ nm}$ as shown in the equation below:

Fe^{3+}-TPTZ + reducing antioxidants ⟶ Fe^{2+} - TPTZ (intense blue at 593 nm)

To conduct the assay, 500 ml of 300 mM acetate buffer (pH 3) was prepared by weighing 0.94 g anhydrous sodium acetate, dissolving in water and adding 8 ml glacial acetic acid in a fume hood. Hydrochloric acid (40 mM) was prepared by adding 1.8 ml concentrated HCl to 500 ml distilled water in a fume hood. TPTZ (2,4,6-Tripyridyl-s-triazine) (15.6 mg) was dissolved in 5 ml 40 mM HCl to give a 10mM solution. A 20 mM solution of iron (III) chloride was prepared by weighing 27.1 mg iron (III) chloride hexahydrate and dissolving in 5 ml water. The FRAP reagent was pre-warmed at 37 °C and was freshly prepared by mixing 2.5 ml of 10 mM TPTZ (2,4,6-Tripyridyl-5-triazine) solution in 40 mM HCl with 2.5 ml of 20 mM iron (III) chloride hexahydrate and 25 ml of 0.3 M acetate buffer at pH 3.6. Briefly, a 40 µl aliquot of the fruit extract was mixed with 3 ml of FRAP reagent. The solution was vortexed and incubated at 37 °C for 5 minutes. The absorbance value of the blue-coloured complex formed was measured against a blank that was

prepared using distilled water and incubated for 1 hour instead of 5 min, at A_{593nm} using a spectrophotometer (Biochrom, Libra S12, UK). The experiment was performed similarly in the preparation of standard curve (R^2= 0.998) by using 0.1 ml of different concentration of iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_20$). Briefly, 2.8 mg of $FeSO_4 \cdot 7H_20$ was dissolved in distilled water to give a final concentration of 1000 µM. The concentration of stock solution of $FeSO_4 \cdot 7H_20$ was diluted to 100, 150, 200, 500 and 1000 µM respectively (Appendix B2). FRAP values were expressed on a fresh weight basis as micromolar of ferrous equivalent Fe (II) per gram of sample (µMFe(II) g^{-1} FW).

8.2.1.5.2 DPPH Assay

The ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals was determined based on the method described by Guorong *et al.* (2009) with some modifications. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to detect both hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms of antioxidants. The ability of antioxidants to scavenge free radicals of DPPH results in a colour loss of DPPH which can be detected at A_{517nm}. The equation below shows the scavenging of DPPH radicals by antioxidants (Brand-Williams *et al*., 1995):

DPPH● + antioxidant ⟶ DPPH-H + A●

A stock solution of DPPH (0.3 mM) was freshly prepared prior to analysis by dissolving 11.8 mg of DPPH in 100 ml of pure methanol. DPPH reagent (0.5 ml) was added to 0.4 ml of 0.1 M Tris-Hydrochloride and 0.1 ml of crude

extract. The solution was vortexed and incubated in the dark at room temperature (25 ± 3 °C) for 20 minutes. Aliquots (200 µl) of reaction mixtures for each treatment were pipetted out into the 96 flat test plate (Orange Scientific, Belgium). A blank was prepared by replacing 0.1 ml of extract with 0.1 ml of 80 % (v/v) methanol. The absorbance value was measured against the blank at A_{517nm} using the 96-well microplate reader (Thermo Scientific, Varioskan Flash, US). The experiment was performed similarly in the preparation of a standard curve (R^2= 0.990) by using 0.1 ml of different concentrations (100-1000 µM) of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Briefly, 2.5 mg of Trolox was dissolved in pure methanol to give a final concentration of 1000 µM. The concentration of stock solution of Trolox was diluted to 100, 150, 200, 500 and 1000 µM respectively (Appendix B3). DPPH value was expressed as micromolar of Trolox Equivalent per gram of sample (µMTE g^{-1}).

8.3 Results and Discussion

8.3.1 Major antioxidant components

8.3.1.1 Ascorbic acid (Vitamin C)

Figure 8.1 shows the ascorbic acid content of control and ozone-treated fruits. Ascorbic acid content of both control and ozone-treated fruits increased as the fruit ripens. The control fruit presented the highest ascorbic acid content at day 8, whereas the ozone treated fruit reached the highest value at day 10, which it reflects a 2-days delay in ripening. The initial concentration of ascorbic acid in papaya fruits was 862.5 mg $100g^{-1}$. Upon exposure to 3.5 ppm ozone for 96 hours, the concentration of ascorbic acid increased significantly ($p < 0.05$) to 2490 mg $100g^{-1}$ after 10 days of storage compared to the control (~1600 mg $100g^{-1}$). The results obtained were significantly different for each treatment at ($p < 0.05$) by Duncan's test. The content of ascorbic acid in all ozone-treated papaya increased gradually up to 10 days after storage and then decreased. Whereas, the content of ascorbic acid of control papaya increased gradually up to 8 days after storage and was lower than all the ozone-treated papaya.

The increase of ascorbic acid content in ozone-treated papaya fruit was also observed in ozone-treated strawberries (Perez *et al.*, 1999) and tomato (Aguayo *et al*., 2006). Perez and colleagues (1999) have reported that the content of ascorbic acid in strawberries increased significantly by 0.15 mg $100g^{-1}$ after exposure to 0.35 ppm ozone for 3 days at 2 °C. Even though that no significant changes of ascorbic acid content in fresh-cut celery treated with 0.18 ppm of aqueous ozone for 10 minutes, it has been observed that its concentration was significantly increased by 1.76 mg $100g^{-1}$ after 3 days of

storage (Zhang *et al*., 2005). It has been proposed that the increase in ascorbic acid in ozone treated fruits and vegetables may be due to the inhibitory roles of ozone on the activity of several enzymes such as ascorbate peroxidase and ascorbate oxidase. Ascorbate oxidase is a copper-containing enzyme and responsible for enzymatic degradation of ascorbic acid (AA). Ascorbate oxidase oxidizes ascorbic acid to dehydroascorbic acid (DHA). In contrast, high ozone at injurious concentrations for the commodity may reduce ascorbic acid by promoting activity of ascorbate oxidase.

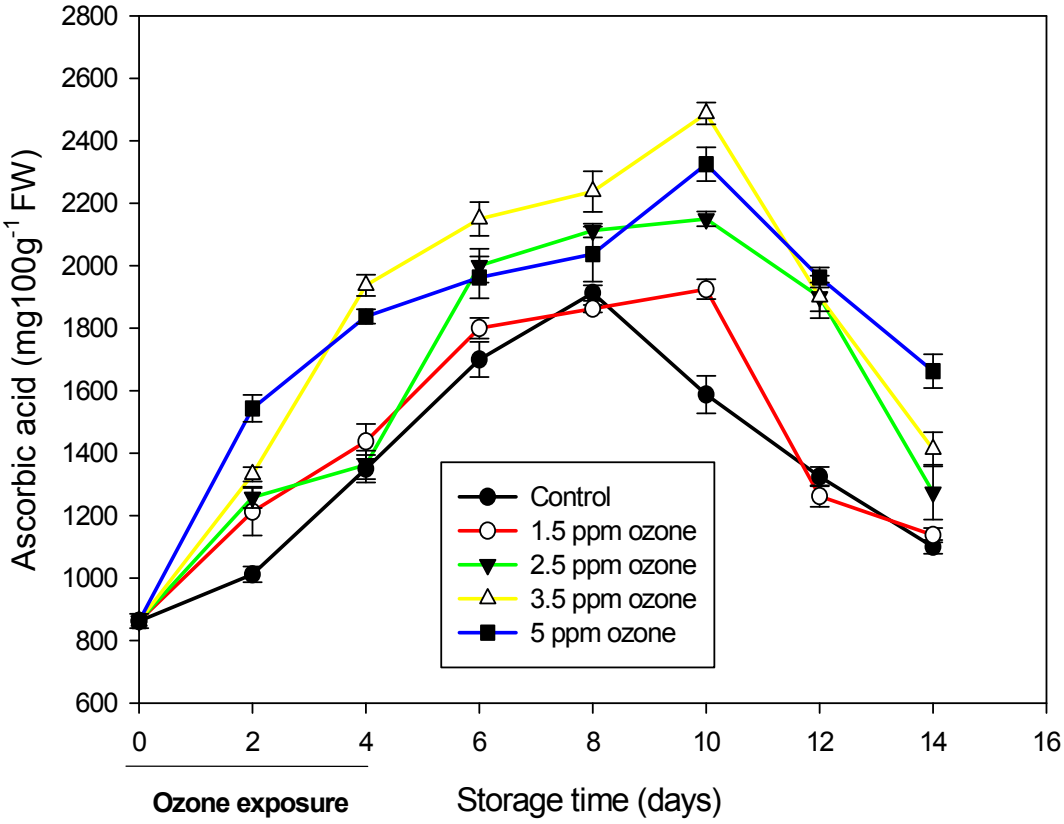


Figure 8.1: Effect of various concentrations of ozone on ascorbic acid content of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

Dehydroascorbic acid can be further degraded due to its instability and hence results in reduction of total vitamin C (Beltran *et al*., 2005; Silveira *et al*., 2011). In this case, ascorbic acid content significantly increased in ozonated papaya during storage.

Moreover, Perez *et al*. (1999) have suggested that ozone stress may lead to the biosynthesis of ascorbic acid by utilizing carbohydrates reserves. The toxicity of ozone is related to its potential to form different reactive oxygen species (ROS) inside the cell. As an antioxidant, ascorbic acid plays a significant role in the detoxification process that results from the formation of different ROS, such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radicals ($OH^\bullet$) inside the plant cell (Moldau, 1998). However, Vitamin C content declines concurrently with the degradation of fruit tissues, when fruit becomes overripe (Kalt, 2005).

8.3.1.2 β-carotene and Lycopene content

The β-carotene and lycopene contents of 2.5 ppm ozone-treated fruits showed increased value by 19.6 % (Figure 8.2) and 52.1 % (Figure 8.3) at day 10, respectively, compared to the fruits that were not exposed to ozone. Tzortakis *et al*. (2007a) reported that an ozone-enriched atmosphere (concentration up to 1.0 ppm) resulted in a two- to three-fold increase in β-carotene, lutein and lycopene contents of tomato. A similar transient increase in anthocyanin content and colour intensity has been observed in blackberry and strawberry fruit subjected to storage in an ozone-enriched atmosphere (Barth *et al*., 1995; Perez *et al*., 1999).

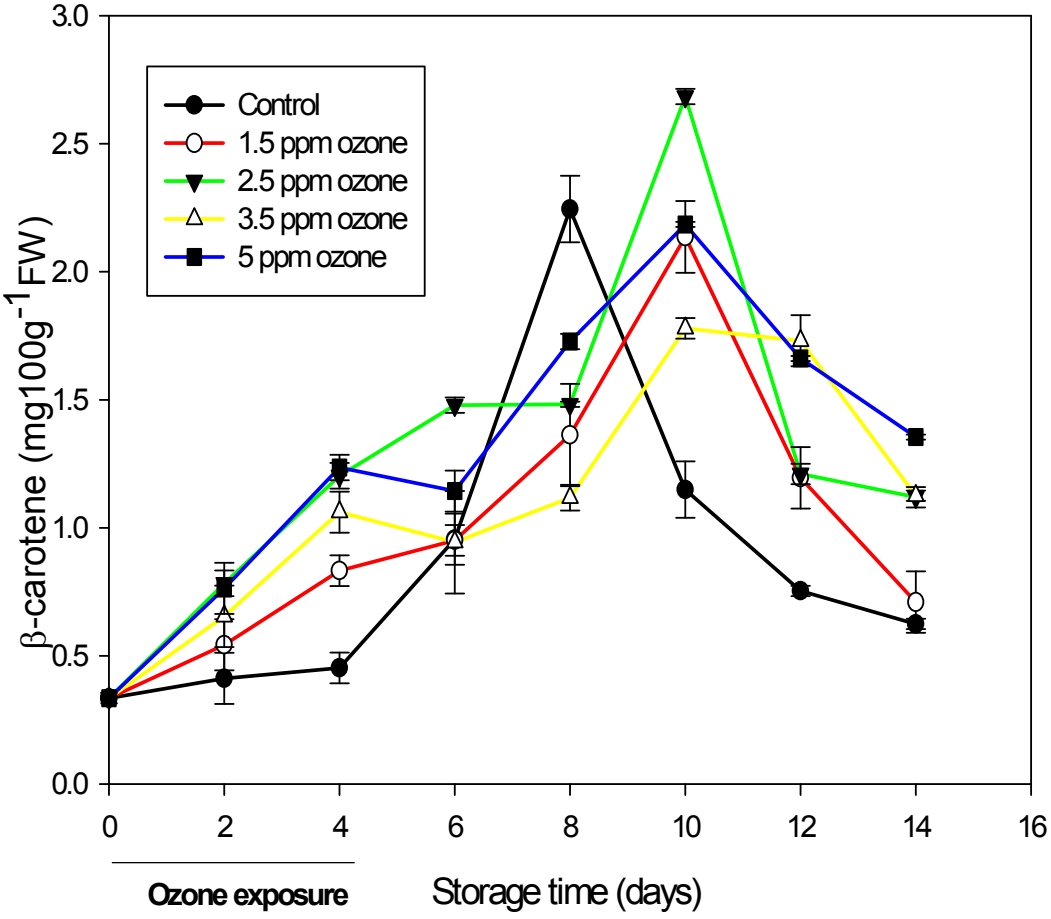


Figure 8.2: Effect of various concentrations of ozone on β-carotene content of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

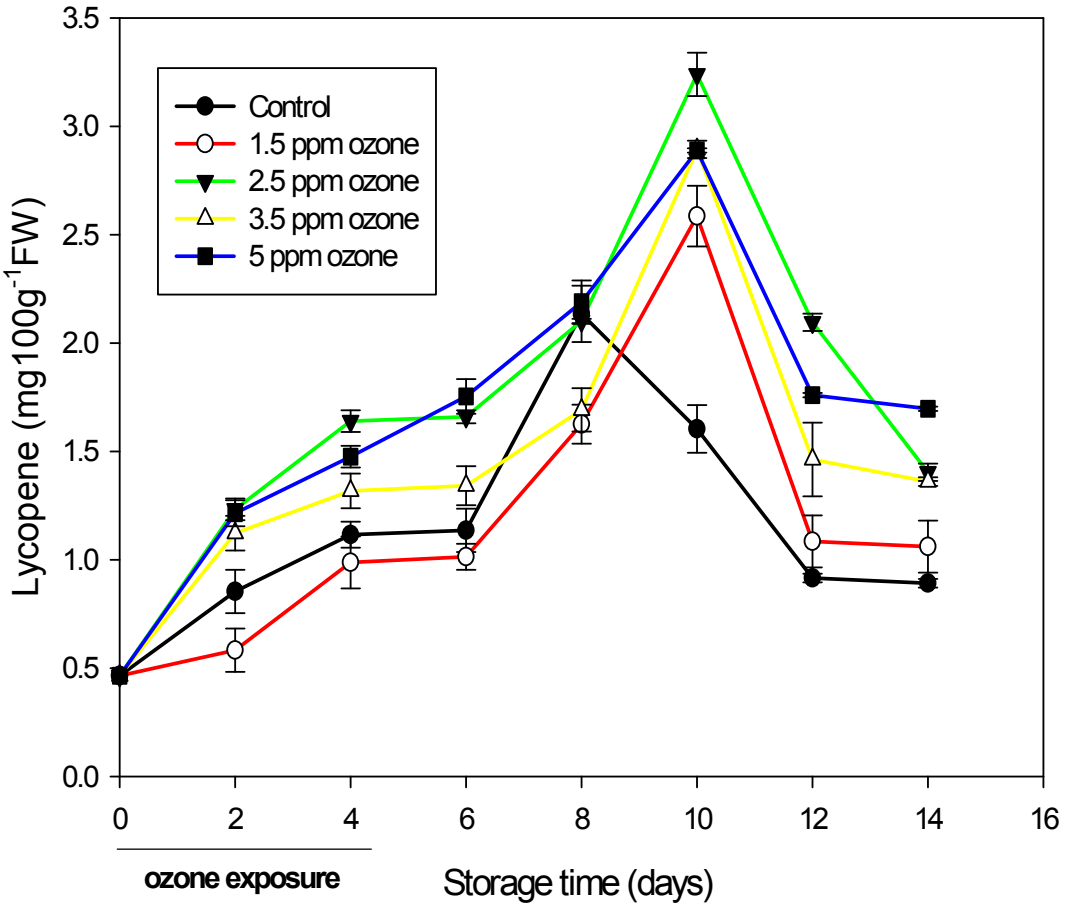


Figure 8.3: Effect of various concentrations of ozone on lycopene content of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

Results obtained were similar to preliminary work conducted by Minas and colleagues (2010) who reported that the total carotenoids content of kiwifruits was increased by 2.1, 2.5, 2.8, and 6.8 % upon exposure to 0.3 ppm gaseous ozone for 8, 24, 72 and 144 hours, respectively. Also, tomatoes stored under 1.0 ppm of ozone enrichment atmosphere for 1 day have significantly increased the total carotenoids content 2 to 3-fold (Tzortzakis *et al*., 2008). However, the concentration of β-carotene in fresh-cut lettuce was decreased by approximately 35 % upon exposure to 2 ppm of gaseous ozone for 2 minutes (Olmez and Akbas, 2009). Chauhan and workers (2011) also have reported the total carotenoids content of ozone treated fresh-cut carrot decreased by 1.7 % when compared to that of control.

Meanwhile, the reduction of β-carotene and lycopene in fruit and vegetables subjected to high concentration and long exposure of ozone may be triggered by the oxidative cleavage of carotenoids that lead to the production of abscisic acid (ABA). It is worth taking note that the phytohormone ABA is produced when plants are subjected to abiotic stresses such as drought, salinity and ozone (Auldridge *et al*., 2006; Seki *et al*., 2007). Therefore, it is possible that the oxidative enzymes, 9-cis-epoxycarotenoid dioxygenase (NCED) which is responsible for the oxidative cleavage of xanthophylls into ABA is enhanced during prolonged exposure to high concentration of ozone for 30 minutes. Furthermore, the increase of β-carotene and lycopene may be due to their antioxidant mechanisms to scavenge free radicals by quenching singlet oxygen and peroxide, neutralizing thiol radicals and suppressing the formation of free radicals (Guorong *et al*., 2009) when exposed to ozone stress.

8.3.1.3 Total Phenolic Content

Figure 8.4 shows the changes in the total phenolic content of papaya fruits during storage for 14 days. Total phenolic content decreased after 2 days of storage for all treatments because total phenolic content of papaya decreased with fruit ripening. Compared to the untreated fruits (0.193 mgGAE g^{-1}), total phenolic content of all treated fruits increased significantly after 4 days in response to ozone treatments for up to 10 days of ambient storage. The phenolic content of papaya at day 10 increased significantly by 13.98 %, 24.35 %, 21.76 % and 23.32 % when exposed to 1.5 ppm, 2.5 ppm, 3.5 ppm and 5.0 ppm of gaseous ozone for 4 days (96 h), respectively. The highest total phenolic content of 0.240 mgGAE g^{-1} was found in 2.5 ppm ozone-treated papaya at day 10 of ambient storage.

Tzortakis *et al*. (2007a) also reported that an ozone-enriched atmosphere tended to increase fruit total phenol content. Similarly, application of 10 ppm ozone for 10 minutes on tomato fruit induced the accumulation of phenolic compounds with a 50 % increase relative to control fruits after 6 days of storage at 20 °C (Rodoni *et al*., 2010).

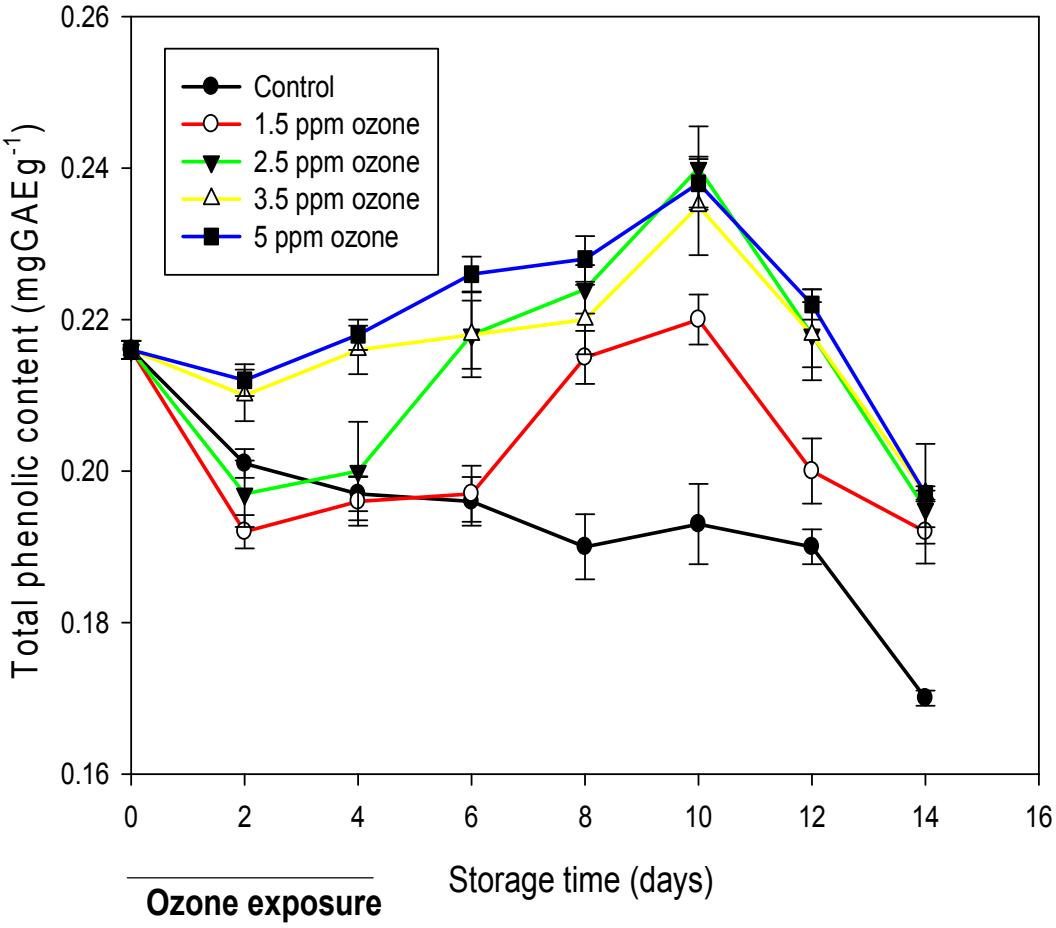


Figure 8.4: Effect of various concentrations of ozone on total phenolic content of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

This reaction to ozone treatment could be attributable to the activation of phenylalanine ammonia lyase (PAL: EC 4.3.1.5). PAL is one of the key enzymes used in the synthesis of phenolic compounds in plant tissues. González-Aguilar *et al.* (2007) found that the activation of PAL in mango 'Haden' fruits was strongly correlated with the increase in the phenolic content of the fruits. According to Camm and Towers (1973), PAL can be stimulated by different stimulants rather than wounding. The increase in the phenolic content of the fruit also might have been caused by cell wall modification that

occurred during ozone exposure. Cell wall modification may release some of the conjugated phenolic compounds in the cell wall.

The accumulation of phenolic compounds under ozone treatment in stored papaya fruit is consistent with their ability to scavenge oxygen radical and avoid oxidative stress (Klopotek *et al*., 2005). Previous studies have shown that several phytoalexins have been accumulated under ozone treatments in grapes (Sarig *et al*., 1996; Gonzaléz-Barrio *et al*., 2006) or in tomato (Maharaj *et al*., 1999). Often associated with the accumulation of total phenols is an increase of phytoalexins. Phytoalexins are low molecular weight compounds of phenolic nature that accumulate locally in response to microbial challenge or stress exhibiting antifungal activity, and thus constitute an important part of the plant defense reactions (Wurms *et al*.,1999). Moreover, another possible factor is the induction of pathogenesis-related proteins (PR-proteins) in the host tissues for which is well established that they can be elicited by ozone (Sandermann *et al*., 1998).

Alothman and colleagues (2010) have reported that the total phenolic content of fresh-cut pineapple and banana increased significantly by 15.7 % and 14.7 % upon exposure to ozone for 20 minutes. Similarly, the phenolic content of kiwifruits was increased significantly by 5.6 %, 6.8 % and 13.7 % when exposed to 0.3 ppm of gaseous ozone for 24, 72 and 144 hours respectively (Minas *et al*., 2010). Also, the total phenolic content of tomato treated with 1.0 ppm of ozone for 1 day was slightly increased although the changes were not significant (Tzortzakis *et al*., 2007a). Detailed analysis of bioactive phenolic

compounds using high performance liquid chromatography (HPLC) in table grapes upon flushing of 8 ppm of ozone for 30 minutes every 2.5 hours have been carried out by Artes-Hernandez and colleagues (2007). In the study, the individual phenolic compounds such as piceid, resveratrol and stillbenoids were increased by 18.64, 1.59 and 1.79 μg g^{-1} after treated with 8 ppm of ozone and stored for 38 days under cold storage for 0 °C.

Phenolic compounds possess several properties that make them effective protectants against oxidative damage (Larson, 1995). When phenolic compounds react with oxidizing agents, phenoxy radicals are formed, which are relatively stable due to the resonance delocalization of the unpaired electron in the ring structure. Therefore, phenoxy radicals tend not to initiate further free-radical reactions. Ozone tolerance in plants is associated with the presence of phenolic compounds and ozone induces or enhances the production of phenolics in plants (Forney, 2003).

8.3.1.4 Total antioxidant activity

Antioxidant activity of papaya fruit increased as fruit ripened and was further enhanced by exposure to ozone for 96 hours from day 4 until day 8, but the activity tended to decrease after day 10 (Figure 8.5 and Figure 8.6). The antioxidant activity of papaya, as estimated by the FRAP assay increased with the increase of ozone concentrations. Papaya fruit exposed to various ozone concentrations of 1.5, 2.5 and 3.5 ppm after 10 days of storage, increased the antioxidant activity by 0.03 %, 30.9 % and 21.9 %, respectively (Figure 8.5). On the other hand, when the antioxidant activity of papaya fruit was

determined with the DPPH method, a significant increase ($p < 0.05$) of 6.3 % was measured at day 10 in the 2.5 ppm treated fruit compared to control fruit (Figure 8.6).

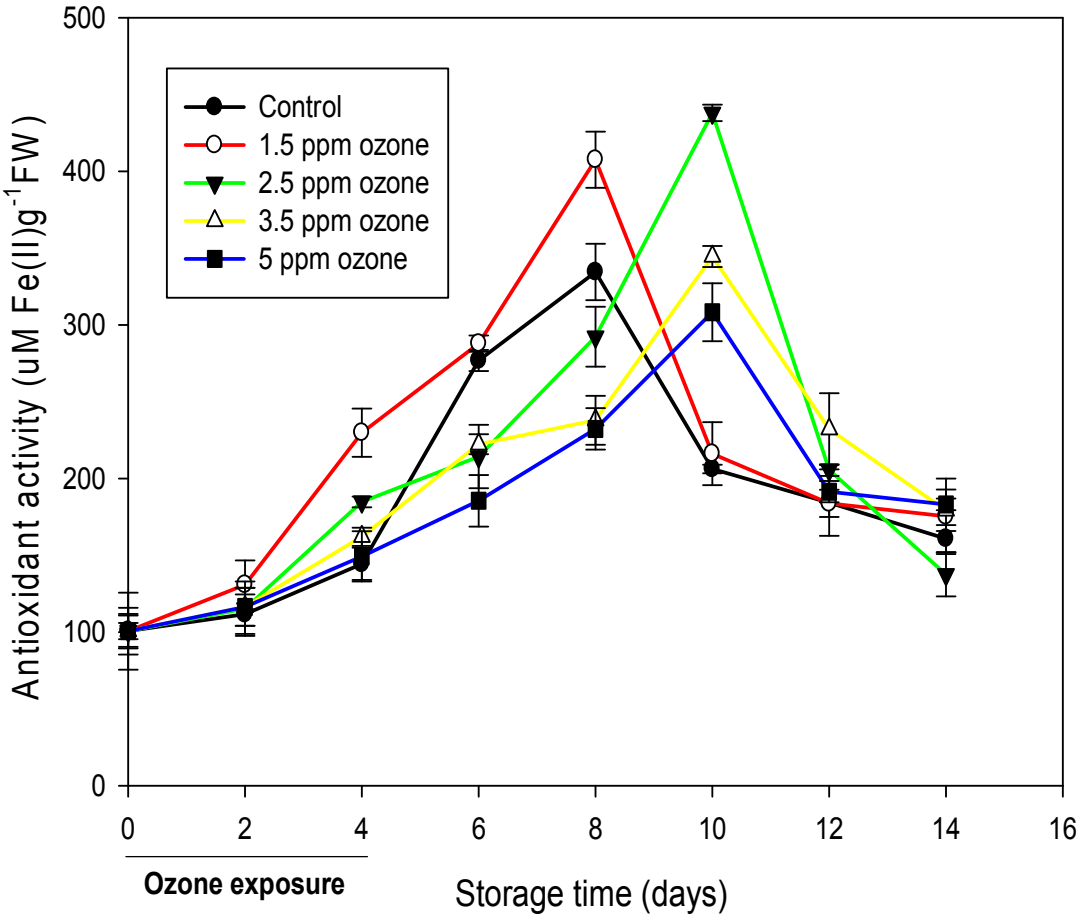


Figure 8.5: Effect of various concentrations of ozone on antioxidant activity through FRAP of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days.
Each value is the mean of four replicates ± SE.

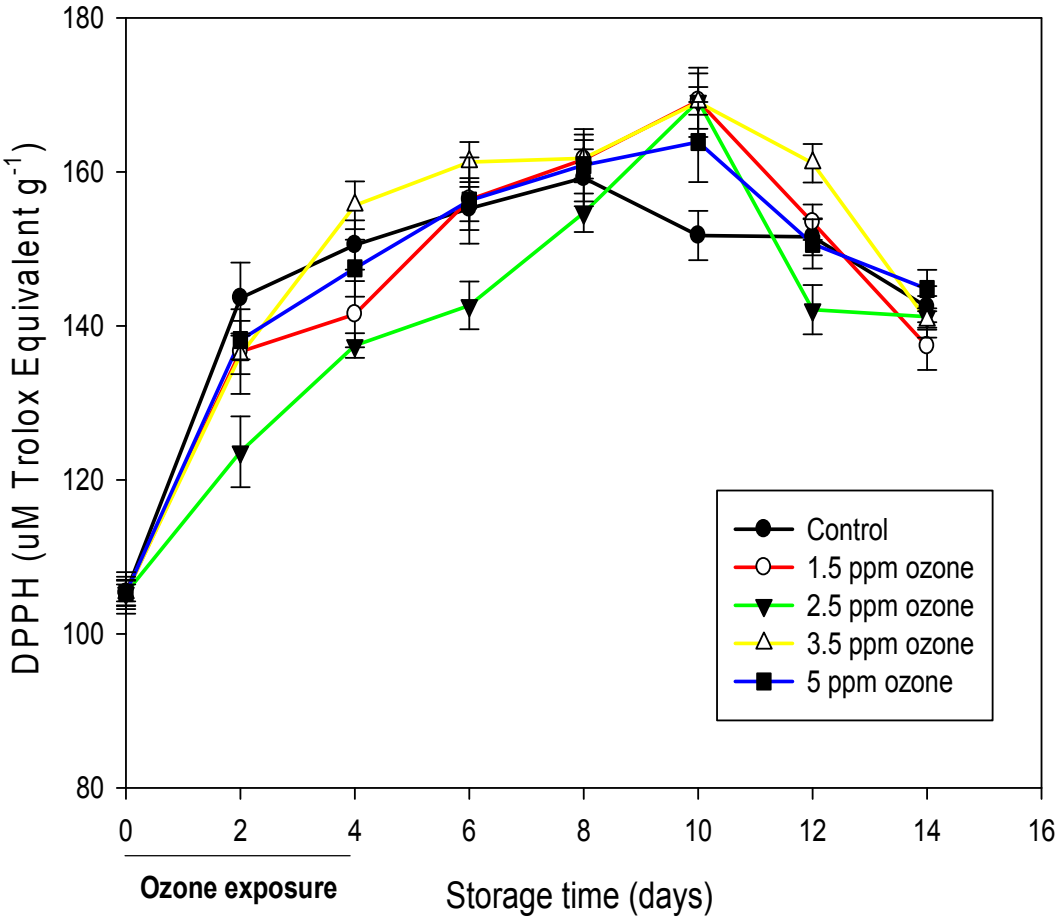


Figure 8.6: Effect of various concentrations of ozone on antioxidant activity through DPPH of 'Sekaki' papaya during ambient storage (25 ± 3 °C, 70 ± 5 %RH) for 14 days. Each value is the mean of four replicates ± SE.

The trend obtained from both DPPH and FRAP assays was similar to that of total phenolics. Antioxidant capacity increases when total phenolic content increased. Phenolic compounds can act as antioxidants and their activity is determined according with the chemical structures that possess depending on types of fruits (Wang *et al*., 2008). This was expected because there was a strong correlation between antioxidant capacity and total phenolic content.

Furthermore, Tzortzakis *et al*. (2007a) found that the tomato pulp tissue exhibited a transient increase in antioxidant activity after 1 day exposure to ozone (0.05 – 1 ppm at 13 °C), but the effect did not persist. Treatments with ozone gas have been shown to elicit the accumulation of antioxidants in strawberry (Perez *et al*., 1999).

The FRAP assay can measure only hydrophilic antioxidants, so compounds such as lipophilic carotenoids cannot be detected (Minas *et al*., 2010). On the other hand, the DPPH assay can detect antioxidants soluble in organic solvents such as alcohols or hydro-alcohol mixture, however it is characterized by steric inaccessibility and a narrow linear range of absorbance versus concentration (Apak *et al*., 2007). Therefore, both protocols were utilized to determine papaya antioxidant capacity. In this study, methanol was used as an extracting solvent as it has been shown to exhibit potentially high antioxidant activities (Khattak *et al*., 2008; Ndhlala *et al*., 2008).

Several studies have suggested that ascorbic acid and carotenoids are general sources of antioxidant in fruits. However, it should be noted that both antioxidants are less likely to contribute to the antioxidant capacity as measured by DPPH and FRAP in this study. This is because the content of ascorbic acid and total carotenoids can be degraded due to high temperature and long duration of extraction. Also, hydrophilic solvent [80 % (v/v) methanol] used in this study cannot be used to extract lipophilic compounds such as carotenoids.

Overall, this study showed that increased concentration of ozone exposure for 96 hours increased the chemical compositions such as total phenolic content, antioxidant activity, ascorbic acid content, β-carotene and lycopene. Further ripening processes, physiological changes and carotenoids content were effectively delayed in fruits due to 2.5 ppm ozone treatment for 96 hours. These results indicate that gaseous ozone treatment from 1.5 to 2.5 ppm ozone for 96 hours would be useful in enhancing the nutritional properties of papaya fruit. Moreover, both FRAP and DPPH values increased significantly with the increase in ozone concentration.

CHAPTER 9

GENE EXPRESSION IN PAPAYA FRUIT IN RESPONSE TO OZONE

9.1 Introduction

Ozone appears to act by inducing a hypersensitive response in plant tissue mimicking biochemical and molecular events induced by pathogens and other stresses (Rao *et al*., 2000). The specific mechanisms by which ozone causes changes in plant gene expression are not known, but it is generally accepted that ozone initiates an oxidative burst in plants which results in the accumulation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) (Rao *et al*., 2000). Furthermore, the rapid generation of ROS and changes in transcript accumulation of defence-related genes resemble the responses to pathogen attack (Sandermann, 1996; Sharma *et al.*, 1996; Schraudner *et al.*, 1998).

Ming *et al*. (2008) have drafted the genome sequence of papaya, which showed that the papaya genome is three times the size of the *Arabidopsis* genome, but contains fewer genes, including significantly fewer disease-resistance gene analogues. When faced with pathogen invasion or stress condition, the host plant activates a multi-component physical and biochemical response, which involves radical changes in the pattern of gene expression (Hill *et al.*, 1999). Gene expression data is available for papaya–fresh cut stress condition as reported by Karakurt and Huber (2007). Their study found the partial cDNAs showed significant homologies to signaling

pathway genes, membrane proteins, cell-wall enzymes, proteases, ethylene biosynthetic enzymes, and enzymes involved in plant defence responses. Besides, identification and expression analysis of benzothiadiazole (BTH) induced gene in papaya was conducted by Qiu *et al*. (2004). Gene expression during papaya fruit ripening has also been studied by several researchers. Othman and Nuraziyan (2010) have studied subtilisin-like serine proteases (EC 3.4.21) and obtained the full-length cDNA and the corresponding genomic DNA for papaya subtilase using rapid amplification of cDNA ends and PCR primer walking techniques, respectively. Fabi *et al.* (2010) also did transcript profiling of non-induced and ethylene-induced fruit samples, and 71 differentially expressed genes were identified. Among those genes some involved in ethylene biosynthesis, regulation of transcription, and stress responses or plant defence were found (heat shock proteins, polygalacturonase-inhibiting protein, and acyl-CoA oxidases).

There have been many similar studies in other plant pathogen interactions to examine changes in gene expression during stress, for example on genes expressed during compatible interactions between leaf rust (*Puccinia triticina*) and wheat (Zhang *et al*., 2003), low-level atmospheric ozone exposure induces protection against *Botrytis cinerea* with down-regulation of ethylene-, jasmonate- and pathogenesis-related genes in tomato fruit (Tzortzakis *et al*., 2011), modulation of transcription factor and metabolic pathway genes in response to water-deficit stress in rice (Ray *et al*., 2011), heat stress in cowpea nodules (Simões-Araújo *et al*., 2002) and barley transcript profiles under dehydration shock and drought stress (Talame *et al*., 2007). The

present study is aimed to shed light on papaya fruit defence systems through analysis of gene expression in response to ozone stress condition. The gene expression profiles of ozone treated fruit have been compared to those of untreated fruit stored under identical conditions.

9.2 Materials and method

9.2.1 Plant materials and ozone fumigation

Various ozone concentration (1.5, 2.5, 3.5 and 5.0 ppm) were applied to the fruit for 96 hours. The fruits were left for 4 days to ripen at ambient storage (25 ± 3 °C, 70 ± 5 %RH). The untreated fruit served as controls. A replicate of 3 fruit were placed in a single layer of the chamber. Each chamber was prepared as a replicate. There were three biological replications for each ozone treatment. After removal of the peel and seeds, the sliced pulp of each fruit was frozen in liquid N_2 and stored at −80 °C. The samples were further freeze-dried to obtain dry samples.

9.2.2 RNA isolation

Total RNA was extracted from fresh (100 mg) or lyophilized (10 mg) plant material using the InnuPREP Plant RNA Kit (Analytikjena, Germany) following manufacturer recommendations. The concentration of total RNA was determined by spectrophotometry (Nanodrop 2000 Spectrophotometer, Thermo Scientific, UK) and the integrity was checked on an agarose gel.

9.2.3 cDNA synthesis

First-strand cDNA was synthesized from total RNA extracts from plant material using the Tetro cDNA Synthesis Kit (Bioline, UK).

9.2.4 Amplified Fragment Length Polymorphism (AFLP) analysis

9.2.4.1 Preparation of DNA digests and adapter-ligated fragments

Amplified fragment length polymorphism (AFLP) analysis for cDNA was performed principally as described in Vos *et al.* (1995) and Bachem *et al.* (1996), with some modifications. Preparation of cDNA digests and adapter-ligated fragments were done using the AFLP® Core Reagent Kit (Invitrogen, UK). Genomic DNA (500 ng) was digested with restriction enzymes (*EcoR*-I*/Mse*-I*)* (Invitrogen, UK) in appropriate restriction enzyme buffer in a total volume of 25 µl at 37 °C for 2 hours. The reactions were then heated at 70 °C for 15 min to inactivate the restriction endonucleases, before annealed double-stranded adapters were ligated to the digested fragments as follows: Adapter / Ligation Solution and T_4 DNA ligase were added to each digest and incubated at 20 °C for 2 hours. The products were stored at -20 °C.

9.2.4.2 Pre-amplification

For the pre-selective amplification, 5 µl of diluted ligation mixture (10 fold) was mixed with *Eco*R-I universal primer (5'-CGT AGA CTG CGT ACC AAT TC-3') (50 ng μl^{-1}, approximately 5 pmol total), *Mse*-I universal primer (5'-GAC GAT GAG TCC TGA GTA A-3') (50 ng/µl, approximately 5pmol total), *Taq* DNA polymerase (0.5 U), dNTPs (250 µM), 10X PCR buffer and $MgCl_2$ (1.25 mM) in a final volume of 20 µl. The fragments were amplified once at 72 °C for 2 min, then amplified for 30 cycles with the following protocol: 94 °C for 30 sec; 56 °C for 1 min; 72 °C for 1 min and a final elongation step at 60 °C for 10 min. The PCR products were checked on a 1 % (w/v) agarose gel for a smear

(approx. 0.1-1 kb) then diluted 1:20 in 10 mM Tris-HCl, 0.1 mM EDTA for selective amplification and stored at -20 °C.

9.2.4.3 Selective amplification

The second selective amplification was carried out using primers with 1, 2 or 3 selective nucleotides at the 3' end. Eighteen primers combination were used and the sequences are shown in Table 9.1. Diluted pre-amplification product (5 µl) was mixed with *Eco*R-I selective primer (100 or 200 ngµl^{-1}, approximately 10 or 20 pmol total), *Mse*-I selective primer (100 or 200 ngµl^{-1}, approximately 10 or 20 pmol total), *Taq* DNA polymerase (0.5U), dNTPs (250 µM), 10XPCR buffer and $MgCl_2$ (1.25 mM) in a final volume of 20 µl. The initial PCR programme was an 11-cycle touchdown programme: 1 cycle of 94 °C for 30 sec; 69 °C for 1 min; 72 °C for 1 min followed by 10 cycles where the annealing temperature decreased by 0.9 °C each cycle. This was followed by 25 cycles of the following PCR conditions: 94 °C for 40 sec; 56 °C for 1 min; 72 °C for 2 min and a final elongation step at 72 °C for 15 min.

Table 9.1: AFLP selective primers (E_1 and M_1)

No	Primer name	Oligonucleotide sequence (5'→3')
1	EcoR-I – GAG	GAC TGC GTA CCA ATT CGA G
2	EcoR-I - ACG	GAC TGC GTA CCA ATT CAC G
3	EcoR-I - AGC	GAC TGC GTA CCA ATT CAG C
4	Mse-I - CTG	GAT GAG TCC TGA GTA ACT G
5	Mse-I - CAA	GAT GAG TCC TGA GTA ACA A
6	Mse-I - CAC	GAT GAG TCC TGA GTA ACA C
7	Mse-I - CAG	GAT GAG TCC TGA GTA ACA G
8	Mse-I - CAT	GAT GAG TCC TGA GTA ACA T
9	Mse-I - CTA	GAT GAG TCC TGA GTA ACT A

9.2.4.4 Analysis by electrophoresis and identification of the AFLP-amplified products

The PCR products or amplicons from the selective amplification were analysed on 2.5 % (w/v) agarose gels containing ethidium bromide (0.1 µg ml^{-1}), run at 90 volts for 1 hour in TBE (Tris-borate, EDTA) buffer, or at 80 volts for 16 hours (960 minutes) in the case of the 8 % polyacrylamide gels, until the loading dye reached the bottom of the gel. The vertical gel electrophoresis was performed using dual vertical slab gel system (Model DSG-200-02, C.B.S. Scientific Company, Inc., San Diego, CA, USA). The gels were then visualized under UV light (Bio-Rad Quantity One, Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software, CA, USA). The PCR was repeated until the levels of product or banding patterns were consistent between the replicate samples. Differentially expressed bands were excised from the gels by aligning the film on the top of the gel and re-

amplified with the appropriate amplification primers. PCR products were checked on a 1 % agarose gel, purified using the Gel Band Purification kit (Amersham Biosciences) and sequenced with one of the universal primers by Applied Biosystems 3730xl DNA Analyzer, USA. Sequences were processed using Applied Biosystems Sequencing Analysis software v 5.2.0 and homology searches were performed with internet-based BLASTN (Altschul *et al*., 1990) using the *non-redundant* (*nr*) database (http://blast.ncbi.nlm.nih.gov). The TDFs were classified according to the E-values generated in the BLAST search. E-values less than 1e-10 were deemed to indicate significant homology.

9.2.5 Quantitative analysis of gene expression by qPCR

9.2.5.1 Primers design and selection of target sequence for qPCR

For quantitative analysis of gene expression by qPCR (Real-Time Quantitative PCR), the 18S rRNA (GenBank accession no. U42514) was used as an internal control (forward primer 5'- AAA CGG CTA CCA CAT CCA AG-3'; reverse primers 5'-CGA AGA GCC CGG TAT TGT TAG GG - 3'). Thirteen primers were used as designed by Fabi *et al*., (2010) and the sequences are shown in Table 9.2. There were three biological replications and three technical replicates for each ozone treatment.

Table 9.2: Nucleotide sequences used in qPCR analyses (Fabi *et al*., 2010)

No	Primer name	Sequence (5'→3')	Size (bp)	Identity (tBlastx)
	Primary and secondary metabolism			
1	Pap18sf	AAACGGCTACCACATCCAAG		
	Pap18sr	CGAAGAGCCCGGTATTGTTAGG		
2	Enolf	AGGTGGCCATTGACAACTTC	446	Enolase(*ENOL*)[*Gossypium barbadense*]
	Enolr	GCAAACTGCGAGAGATACAG		
3	Aactf	GCTATCATTGATGGGATGCTC	341	Acetyl CoA acetyltransferase (*AACT*)[*Hevea brasiliensis*]
	Aactr	ATTCCACGCTCAAAGCTTCG		
4	Spsf	TGTCAGGAACATCAGCCTGT	203	Sucrose phosphate synthase (SPS) [*Vicia faba*]
	Spsr	CCTCTGTTCTTCTCTCAGCA		
5	Mdhaf	TTGTTAAAGCCGGTCTCTCG	774	Monodehydroascorbate reductase (MDAR) [*Picrorhiza kurrooa*]
	Mdhar	AGGTAATGCTGCGGGTTATG		
6	Invf	AGGGAGGTCTGCCATTACAT	225	Invertase (INV)[*V. faba*]
	Invr	GCAGAATTCATTTGCCAGACTC		
	Responsive to stress and plant defence			
7	Hsp91f	GGTTGTATGAAGATGGTGAGG	342	Heat Shock Protein 91 (*HSP91*)[*A.thaliana*]
	Hsp91r	CCGTTCTTCAATTGGGTCAC		
8	Pgif	CATGCTTGTGGGTGAAACAC	433	Polygalacturonase-inhibiting protein (*Pgi*)[*Gossypium hirsutum*]
	Pgir	GAGCGCCATGAAGTACGATT		
9	Acx3f	ACATTGCTGCAGTGAGGAAAG	404	Acyl-CoA oxidase 3 (*ACX3*) [*A.thaliana*]
	Acx3r	CCAAGAATTCGCTTCAATCCAG		
	Cell wall-related proteins			
10	Hydroxf	GAGTTTGCAGAGACGTGGA	416	Hydroxyproline-rich glycoprotein (*HRP*) [*A.thaliana*]
	Hydroxr	AGGTGACCACCTGCTGAAGG		
11	Pmef	TATCTTGGTAGGCCCTGGA	305	Pectinesterase(*PME*) [*Phaseolus vulgaris*]
	Pmer	AGGCCAGTGTTTCGGTACT		
	Ethylene biosynthesis			
12	Metsf	ACAACTTCCATGCCCATCTC	531	Methionine synthase (*MET*) [*C. papaya*]
	Metsr	CGGGTTGATGAGGGTAATTG		
	Transcription regulation			
13	Erff	ATACTGGGGAGATGCGTGA	259	Ethylene responsive factor (*ERF7*)gene [*P.trichocarpa*]
	Erfr	CTCTCGCTCCGTTCTTCTT		

9.2.5.2 PCR-amplification

All real time PCR reactions were carried out in 48 well reaction plates using KAPA™ SYBR® FAST qPCR Master Mix (2X) Universal (Kapa Biosystems, Inc., Boston, USA) in a Illumina Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) with real-time data collection. Amplification was carried out under the following conditions: 50 °C for 2 min followed by an initial denaturation step at 95 °C for 2 min and 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec, 72 °C for 30 sec and a final elongation step at 40 °C for 30 sec.

9.2.5.3 Data analysis

The threshold cycle (Ct) values of the cDNA triplicate reactions were obtained using the Illumina Eco Real-Time PCR Software (Illumina, Inc., San Diego, CA, USA), and quantification was performed using the relative standard curve method (Pfaffl, 2001) using the untreated papaya fruit as the control value (ratio value = 1). Data were analyzed against the control (untreated papaya) by one-way ANOVA, and means were separated using Duncan's Mutiple Range Test (DMRT) at ($p < 0.05$).

9.3 Results

9.3.1 Possible gene expression of ozone-treated papaya by cDNA-AFLP

Amplicons of non-induced and ozone-induced papaya fruit using 18 different EcoR-1 +3 / Mse-1 +3 primer combinations for selective amplification were obtained. The size of the selective amplified products ranged from 100 to 1000 bp, which was highly reproducible in three biological replications. Based on the fragment distribution, obvious difference in fingerprint was observed between ozone treated and untreated fruit (Figure 9.1). It is interesting to note that none of the same fragments was detected from the ozone-treated samples.

These amplicons were excised from the gels, and were successfully re-amplified and sequenced. These sequences were used for BLASTN search against the NCBI Genbank non-redundant nucleotide sequence database and amplicons matched to some extent (85-100 %) with papaya genome sequences were tabulated (Table 9.3). Based on the BLASTN results the amplicons were mostly related to mitochondrion, chloroplast, ethylene biosynthesis, cell wall structure and metabolism, and response to stress and plant defence.

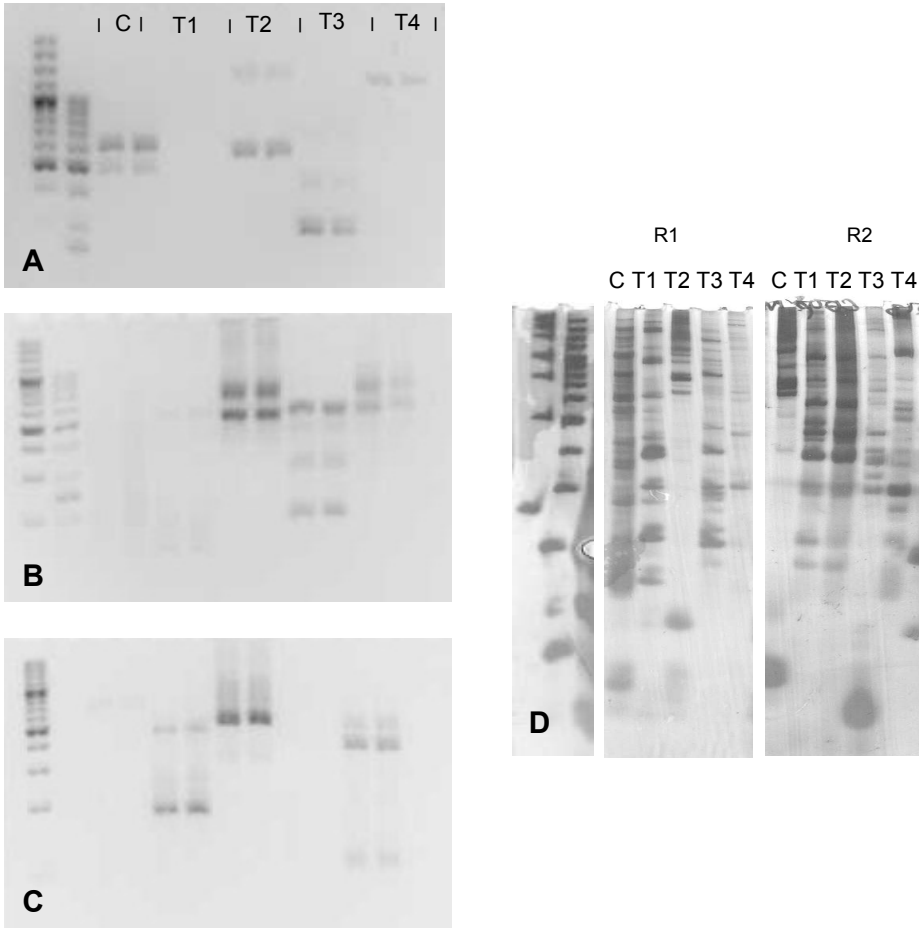


Figure 9.1: Partial result of cDNA-AFLP analysis in ozone-treated (T1:1.5 ppm, T2:2.5 ppm, T3:3.5 ppm, T4:5.0 ppm) and untreated (C) papaya fruit. (A) Amplicons from selective amplification with EcoR-I+GAG and Mse-I+CTA primer combination on 2.5 % agarose gel (Marker : 100 bp Plus and 50 bp DNA ladder); (B) Amplicons from selective amplification with EcoR-I+ACG and Mse-I+CTA primer combination on 2.5 % agarose gel; (C) Amplicons from selective amplification with EcoR-I+ AGC and Mse-I+CAT primer combination on 2.5 % agarose gel (Marker : 100 bp Plus DNA ladder); (D) TDFs fragments (R1: biological replicate 1, R2: biological replicate 2) from selective amplification with EcoR-I+GAG and Mse-I+CAG primer combination on 8 % denatured polyacrylamide gel (Marker : 1 kb and 100 bp Plus DNA ladder).

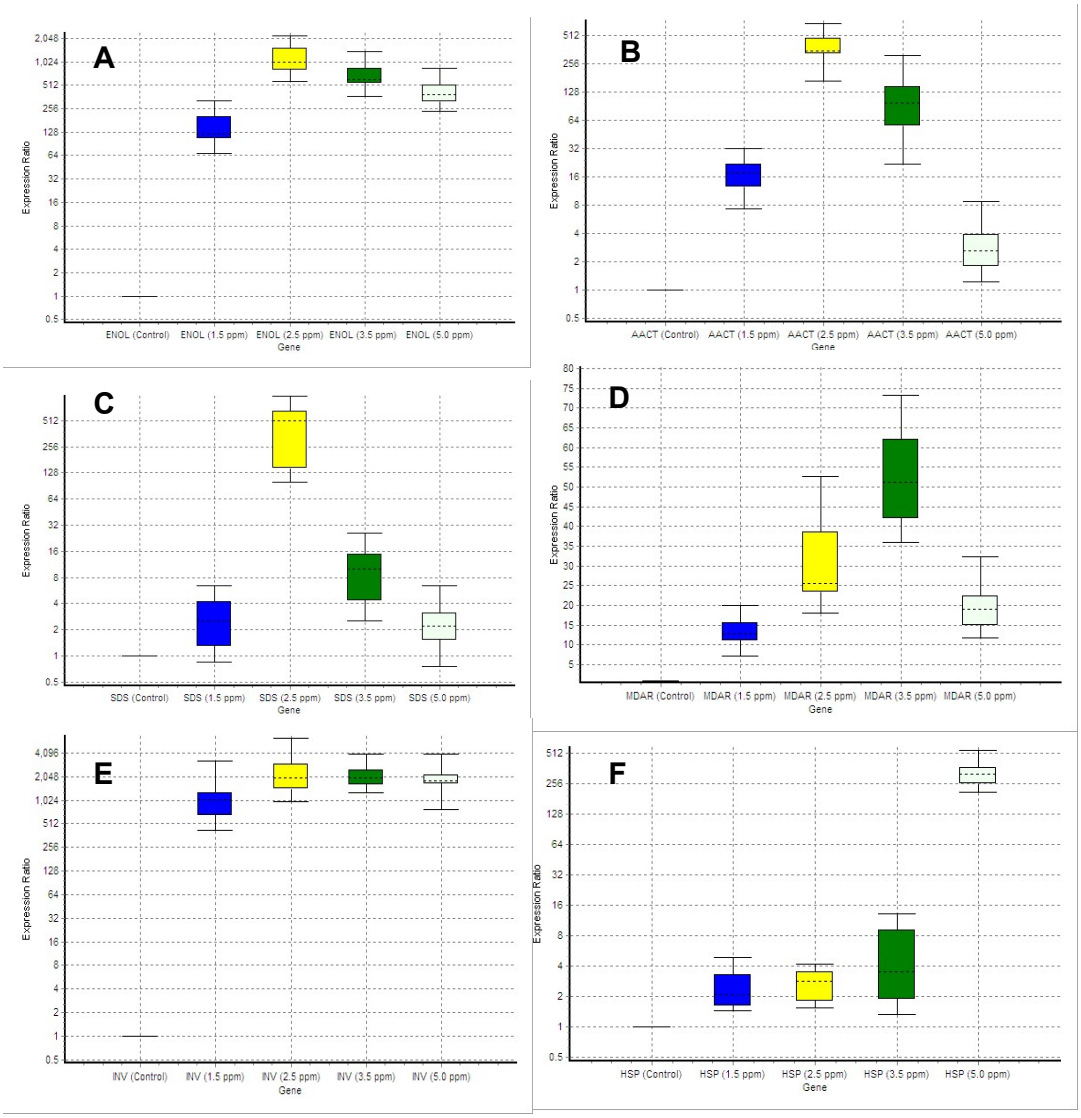
Table 9.3: Differentially expressed transcripts of papaya fruit identified in response to ozone fumigation

R[a]	Accession number	Size (bp)	Identity (Blastn)	Identity (%)	E-Value
↑	EU431224.1	250	*Carica papaya* mitochondrion, complete genome	99	3e-23
↑	EU293620.1	250	*Bacillus subtilis* strain NRRL B-941 AFLP marker MCA-941-16 genomic sequence	100	9e-9
↑	EU431224.1	120	*Carica papaya* chloroplast, complete genome	97	9e-19
↑	HM367685.1	800	*Vigna radiata* mitochondrion, complete genome	95	9e-30
↑	JF412792.1	800	*Cucumis melo* subsp. melo mitochondrial sequence	94	4e-23
↑	BA000042.1	800	*Nicotiana tabacum* mitochondrial DNA, complete genome	91	1e-28
↑	AC246010.3	600	*Solanum lycopersicum* strain Heinz 1706 chromosome 1 clone sle-34k18 map 1, complete sequence	86	1e-120
↑	AC254062.7	600	*Solanum lycopersicum* strain Heinz 1706 chromosome 9 clone sle-45a15 map 9, complete sequence	85	6e-119

R[a] Transcripts response to ozone treatment: up-regulated (↑) or down-regulated (↓).

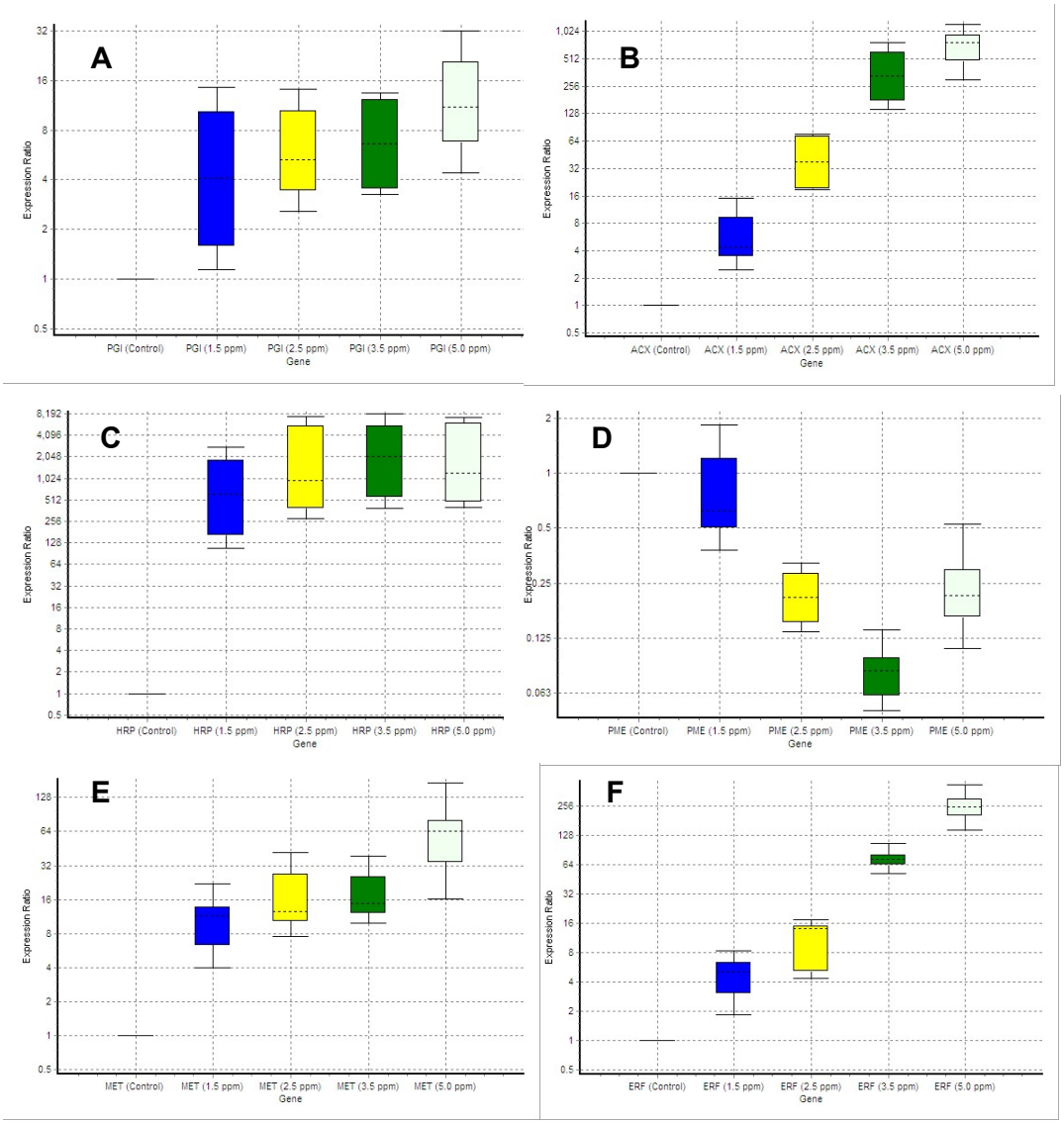
9.3.2 Gene expression of ozone-treated papaya by qPCR

The expression of 12 representative transcripts which were found by Fabi *et al*. (2010) were analysed by qPCR during defence response of papaya induced by ozone (Figure 9.2, Figure 9.3 and Appendix C). According to qPCR results, Pectinesterase (PME) was negatively affected during ozone fumigation, clearly decreased when the ozone concentration increased. In contrast, Heat Shock Protein 91 (HSP91), Polygalacturonase-inhibiting protein (Pgi), Acyl-CoA oxidase (ACX 3), Acetyl CoA acetyltransferase (AACT), Ethylene responsive factor (ERF 7), Methionine synthase (MET), Sucrose phosphate synthase (SPS), Enolase (ENOL), Hydroxyproline-rich glycoprotein (HRP), Monodehydroascorbate reductase (MDAR) and Invertase (INV) were clearly induced, as suggested by their up-regulation after ozone treatment.



Legend:
Boxes represents the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 9.2: Expression of papaya transcripts namely (A) ENOL, (B) AACT, (C) SPS, (D) MDAR, (E) INV and (F) HSP. Real-time quantitative PCR (qPCR) was used to analyze TDF expression patterns for ozone-treated and untreated fruit. Column height indicates relative mRNA abundance; expression values in untreated fruit were set to 1. All data were normalized to the 18S rRNA expression level. Values are the means ± SE from three technical replications.



Legend:
Boxes represents the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 9.3: Expression of papaya transcripts namely (A) PGI, (B) ACX, (C) HRP, (D) PME, (E) MET and (F) ERF. Real-time quantitative PCR (qPCR) was used to analyze TDF expression patterns for ozone-treated and untreated fruit. Column height indicates relative mRNA abundance; expression values in untreated fruit were set to 1. All data were normalized to the 18S rRNA expression level. Values are the means ± SE from three technical replications.

9.4 Discussion

The results showed that papaya is sensitive to ozone treatment and that ozone effects are manifested at the level of gene expression. Examining DNA information expression is different and more powerful than examining enzyme activity (Heath, 2008). It has been suggested that active oxygen species generated during pathogen attack and oxidative stress conditions such as elevated ozone levels act as signal molecules leading to induction of gene expression (Ernst *et al.*, 1992). The formation of oxygen radicals may interact directly with a signal-transduction pathway that ultimately controls plant defence gene expression. For example, lipoxygenase activities (LOX) in soybean and mRNA levels were shown to be increased during ozone stress (Maccarone *et al.,* 1992). Methionine synthase (MET) is one transcript directly related to ethylene biosynthesis. The up-regulation of the transcript suggests the stimulation of methionine synthase which is increased for ethylene demand during ozone treatment.

Tuomainen *et al*. (1997) also reported that ozone causes a rapid and highly selective activation of genes involved in ethylene biosynthesis resembling plant-pathogen interactions. Papaya fruit undergoes solubilisation of cell wall polysaccharides during ripening. The transcript similar to a pectinesterase (PME) was not stimulated by ozone treatment. Lignification and reinforcement of the cell wall play an important role in plant growth and defence against pathogen attack or stress response. In the present study, several genes involved in cell wall morphology and composition were up-regulated. On the other hand, Hydroxyproline-rich glycoprotein (HRP) was stimulated by ozone

treatment. Insolubilization of HRP in cell walls through oxidative cross-linking which is elicited by stress, is an important process to strengthen the cell walls that contributes to plant defence reactions. Oxidative phenolic coupling products formed from amino acid residues within HRP are known to be one of the important factors that contribute to the strengthening of cell walls (Deepak *et al*., 2010). HRP transcripts also accumulated in maize upon induction with an elicitor from *Fusarium moniliforme* (Garcia-Muniz *et al*. 1998).

Heat shock proteins (HSPs) are involved in stress responses and participate in protein folding as chaperones (Wegele *et al*., 2004). This protein was also up-regulated in ozone induced papaya. Heat-shock proteins (HSPs)/chaperones are responsible for protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membranes, and can assist in protein refolding under stress conditions. They can play a crucial role in protecting plants against stress by reestablishing normal protein conformation and thus cellular homeostasis (Wang *et al*., 2004). Molecular HSPs/chaperones are located in both the cytoplasm and organelles, such as the nucleus, mitochondria, chloroplasts and endoplasmic reticulum (Waters *et al*., 1996; Boston *et al*., 1996; Vierling, 1991). Mitochondria, like the nucleus, are enclosed within a double membrane and its mitochondrial matrix contained RNA, DNA and enzymes of the tricarboxylic acid cycle and electron transport system. The primary function of mitochondria is in cellular respiration. Moreover, chloroplast contained proteins, lipids, starch grains, DNA, RNA and various organic compounds (Kays, 1997).

HSPs / chaperones are known to be expressed in plants not only when they experience high temperature stress but also in response to a wide range of other environmental insults, such as water stress, salinity and osmotic, cold and oxidative stress (Waters, *et al*., 1996). For example, heat-shock transcription-factor-dependent expression of antioxidants ascorbate peroxidases in *Arabidopsis* (Panchuk *et al*., 2002) suggested that heat shock factors might be involved not only in HSP synthesis but also in oxidative stress regulation of antioxidant gene expression. It was shown in *Arabidopsis* that several HSP genes were up-regulated under high light stress (Rossel *et al*., 2002).

An assumed antifungal protein gene (polygalacturonase inhibitor protein-Pgi) was also expressed in ozone induced papaya. Polygalacturonase-inhibiting protein is a cell wall protein that inhibits fungal polygalacturonases (PGs) and retards the invasion of plant tissues by phytopathogenic fungi (Spadoni *et al*., 2006). The inhibition of PGs is thought to be an important event during plant defense against fungi (Ferrari *et al*., 2003; Manfredini *et al*., 2005). The inhibition of PGs by Pgi is also thought to cause the accumulation in the plant apoplast of oligogalacturonides, which act as elicitors of a wide range of defense responses (De Lorenzo *et al*., 2001). Meanwhile, Acyl-CoA oxidases (ACXs) promote the peroxisomal β-oxidation of fatty acyl-CoA, which is a first step in the biosynthesis of jasmonic acid (Schilmiller *et al*., 2007), an important compound in plant response to biotic stress (Kang *et al*., 2006.). Acyl-CoA oxidase 3 (ACX3) was stimulated by ozone treatment. In various plants, acyl-CoA oxidase exists as a family of isoenzymes (Hooks *et al*.,

1999). It has been suggested that the ACX isoforms have specialized involvement of β-oxidation in the wound response of *Arabidopsis thaliana* (Castillo *et al*., 2004) and *Solanum lycopersicum* (Li *et al*., 2005).

The positive effect of ozone treatment on Enolase (ENOL) expression reflects the possible increase in glycolysis and tricarboxylic acid cycle (TCA cycle). This transcript derived fragment is induced during ripening, and is similar to an enolase from cotton, which is an enzyme responsible for a dehydration step in the glycolytic pathway (Hannaert *et al*., 2000). Further to its primary importance to plant tissues, metabolism of carbon compounds during fruit ripening is also relevant to fruit quality. The concentrations of soluble sugars, volatiles, carotenoids, and other secondary compounds determine the taste, flavour, colour and nutritional value of papaya fruit (Fabi *et al*., 2010). Sucrose accumulates during papaya fruit development and is converted to glucose and fructose at ripening (Selvaraj *et al*., 1982). The stimulation of Sucrose phosphate synthase (SPS) by ozone treatment and its increase during ripening, is compatible with SPS playing a role in sugar accumulation in developing papaya. Transcript fragment Invertase (INV) is similar to a vacuolar acid invertase from fava bean and identical to a putative papaya acid invertase (Paull *et al*., 2008). It is induced by ozone treatment and qPCR results show a significant increase in expression.

A putative acetyl-CoA acetyltransferase (AACT), a transcript related to the synthesis of precursors for volatiles and carotenoids is up-regulated after ozone treatment. Carotenoid levels increase during papaya ripening (Devitt *et*

al., 2010), and this enzyme is responsible for the synthesis of mevalonate (Chemler *et al*., 2006). Some other transcripts related to secondary metabolism such as Monodehydroascorbate reductase (MDAR) could be relevant to fruit quality. This is related to the metabolism of ascorbate (vitamin C) and is positively affected by ozone treatment. This transcript expression agrees with the observation that this vitamin appears to be synthesized during ozone-induced papaya development.

In addition, the ripening-repressed protein is similar to ethylene responsive factors from poplar (ERF7). In ozone-induced papaya, ERF7 is positively affected by ozone treatment and is up-regulated in its expression. ERF proteins modulate the expression of many defence-related genes through the interaction with a consensus nucleotide sequence AGCCGCC (GCC box) present in their promoters (Ohme-Takagi and Shinshi, 1995; Chakravarthy *et al*., 2003). Several ERFs have been recognized to be important for plant adaptation to various environmental stresses (Champion *et al*., 2009; Jin and Liu, 2008).

Most of the differentially expressed transcripts of papaya fruit identified in response to ozone fumigation were from the mitochondrion genome sequence. It is well known that the mitochondrial respiratory chain is the major endogenous source of ROS, particularly when the mitochondria are damaged (Osiewacz, 2002; Shi *et al*., 2011). Several studies have reported the effect of ozone on plant mitochondria and elucidated the specific mode of action (Lee, 1968). The specific mechanisms by which ozone causes

changes in plant gene expression are not known, but it is generally accepted that ozone initiates an oxidative burst and an active production of reactive oxygen intermediates (ROIs) (Schraudner *et al*., 1998; Rao and Davis, 1999). Rapid generation of ROIs and changes in transcript accumulation of defence-related genes resemble the responses to pathogen attack (Sandermann, 1996; Schraudner *et al*., 1998). Several signal molecules like ethylene (Tuomainen *et al*., 1997), jasmonic acid (Örvar *et al*., 1997) and salicylic acid (SA) (Rao and Davis, 1999) have been hypothesized to act as a second or third messenger for ozone-induced gene expression.

In summary, this study has shown that ozone fumigation of papaya fruit allowed us to identify genes that are positively or negatively regulated by this external gaseous application in postharvest technology. The most significant outcome in controlling ripening or resistance to pests are those genes involved in ethylene biosynthesis, stress responses or plant defence, accumulation of antimicrobial phytoalexin or pathogenesis-related protein and generation of reactive oxygen species (oxidative burst). Similarly, genes related to cell wall structure and metabolism, primary and secondary metabolisms are pertinent in fruit quality determination.

CHAPTER 10

GENERAL CONCLUSION

Ozone is a highly effective antimicrobial agent that oxidizes organic matter, bacteria, fungi, viruses, pesticides and chemical residuals. It can react faster than chlorine with organic matter, and disintegrate microbial cell membranes and bacteria spore coat. It has potential applications in the agriculture, food industries and postharvest treatment of fresh fruit and vegetables for sanitation and control of postharvest disease. Ozone application emerges as a popular alternative to chlorine because ozone is eco-friendly, biodegradable, safe and not leaving any toxic residues in the environment. In this current work, gaseous ozone of 2.5 ppm has shown effectiveness in delaying anthracnose of papaya and extending the storage life for up to 12 days at ambient condition (25 ± 3 °C, 70 ± 5 %RH). This is attributed to the fungitoxic and fungistatic activity of ozone which protects the fruit against the growth of *C. gloeosporioides* in papaya. *In vitro* inhibition was directly related to the ozone concentrations. However, higher concentration of ozone exposure at 3.5 ppm and 5 ppm ozone seems non-physiological and caused phytotoxic effect on the quality of papaya fruit.

Using scanning electron microscope, transmission electron microscope and laser scanning confocal microscope, this study has also provided evidence that ozone involved in controlling anthracnose disease via fungal study. Ozone has the ability to alter the ultrastructure of *C. gloeosporioides* conidia,

leading to disintegration of the cytoplasm and degradation of mitochondria. Additionally, ozone induced the generation of reactive oxygen species (ROS) in *C. gloeosporioides* spores and caused oxidative damage of the fungal spore. Mitochondria had ruptured membranes and is a major endogenous source of ROS production. This finding is further supported by the result of gene expression using cDNA-AFLP method to discover transcripts that were mostly expressed is related to mitochondrion, chloroplast, ethylene biosynthesis, cell wall structure and metabolism, and response to stress and plant defense.

In addition, multiple modes of action were found involved in the suppression of pathogens with ozone. Ozone can induce the resistance in papaya fruits by eliciting activities or production of total phenolic compounds, phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD). Generally, plant cells exposed to ozone respond with a stimulation of enzymes involved in the phenylpropanoid pathway, as a defense mechanism operating in the stress-affected cells. Plant defense inducible enzymes activities such as PAL, PPO and POD in higher ozone concentration treated fruit was higher than that in lower ozone concentration treated fruit and consequently, have an important role in plant disease resistance. Likewise, antioxidant activity of papaya fruit increased as fruit ripened and was further enhanced by exposure to ozone for 96 hours from day 4 until day 8. The antioxidant activity of papaya, as estimated by the FRAP and DPPH assays increased with the increase of ozone concentrations. It has been proposed that the increase in ascorbic acid, β-carotene and lycopene in ozone treated

fruit may be due to the inhibitory roles of ozone on the activity of several enzymes such as ascorbate peroxidase and ascorbate oxidase. Ozone stress may lead to the biosynthesis of major antioxidants by utilizing carbohydrates reserves in papaya as a significant role in the detoxification process against the toxicity of ozone, which is related to the formation of different reactive oxygen species inside the plant cell. This result was further demonstrated with the findings of the up-regulation of Acetyl CoA acetyltransferase (AACT) and Monodehydroascorbate reductase (MDAR) in ozonated fruit.

The maintenance of firmness in papaya treated with ozone could be due to the slower activity of cell wall degrading enzymes, thus delaying the ripening process during storage. Ozone fumigation led to increase SSC in papaya fruit and the highest content of SSC was found in 2.5 ppm ozonated fruit. Sensory evaluation of quality revealed that fruit treated with 2.5 ppm ozone received significantly higher score for sweetness, and texture, as also demonstrated from the quantitative gene expression results by using qPCR with the up-regulation of Sucrose phosphate synthase (SPS), Enolase (ENOL) and Invertase (INV). Furthermore, the reduction in weight loss, delay in colour changes could be due to the effect of ozone on delayed ripening process. This was further supported by the results of respiration rate, which showed that control fruit has higher respiration rate than the ozonated fruit. This may be caused by the abiotic stress imposed by ozone treatments which reduced the respiration rate and metabolism of ozonated fruit. Even the ripening process of ozonated fruit were delayed, the ethylene production of ozone

treated fruit was directly proportionate with the concentration of the ozone treatment and was higher than control fruit. This indicated that ozone injury to plant tissue can induce ethylene production in which structural damage on papaya surface was visualized when the fruit were treated with 3.5 ppm and 5 ppm ozone.

Overall, the findings of this study suggest that the application of 2.5 ppm of ozone fumigation for 96 hours at 25 ± 3 °C and 70 ± 5 %RH were the optimum conditions for ozone disinfection of papaya fruit, in terms of reducing the microbial and fungus load and maintaining the sensory quality in terms of taste and firmness during 14 days of ambient storage. Studies at the genetic level also have shown that ozone application in postharvest technology may positively or negatively regulate genes. With the finding of ozone-affected genes, fresh and clear understanding about effect of ozone treatment on climacteric fruit would propose candidate genes to serve as an important tool for genetic control in retaining fruit quality and prolonging shelf life, thus assisting the global aim of food security and sustainable agriculture.

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APPENDICES

Appendix A

Hedonic Scale Rating

Product: _____Variety: _____Date: _____

Name of panelist:

_____Signature:_____

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

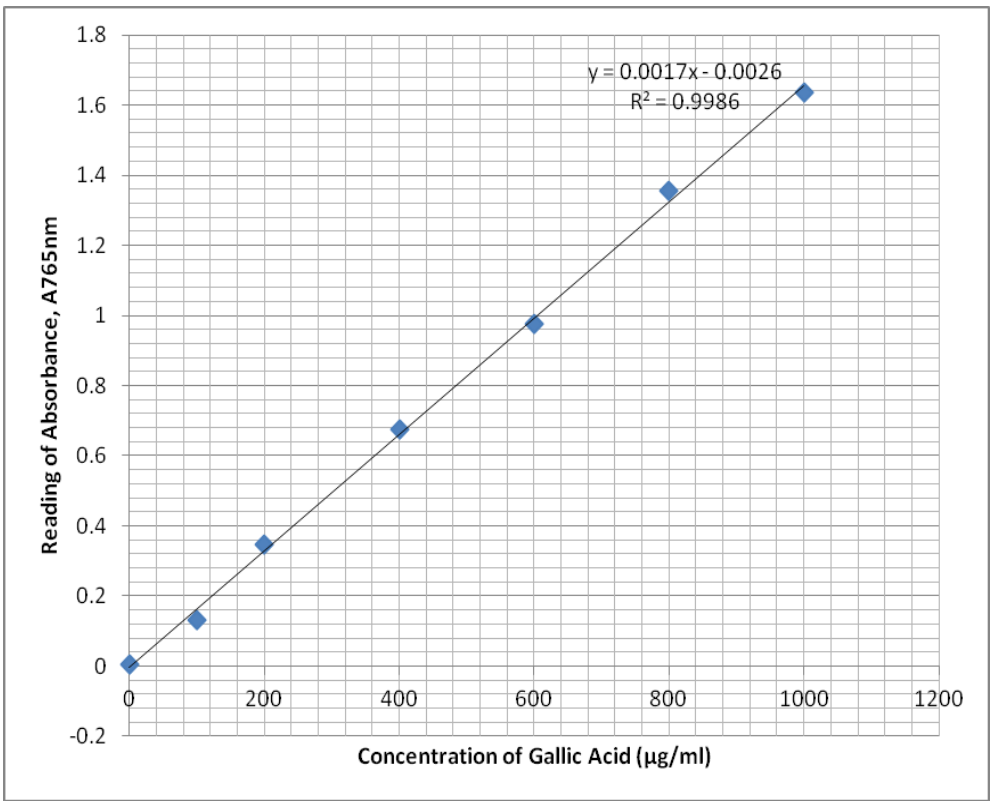
1. This is sensory analysis form for the evaluation of different treatments.
2. Please follow the numerical system for scoring the samples.

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

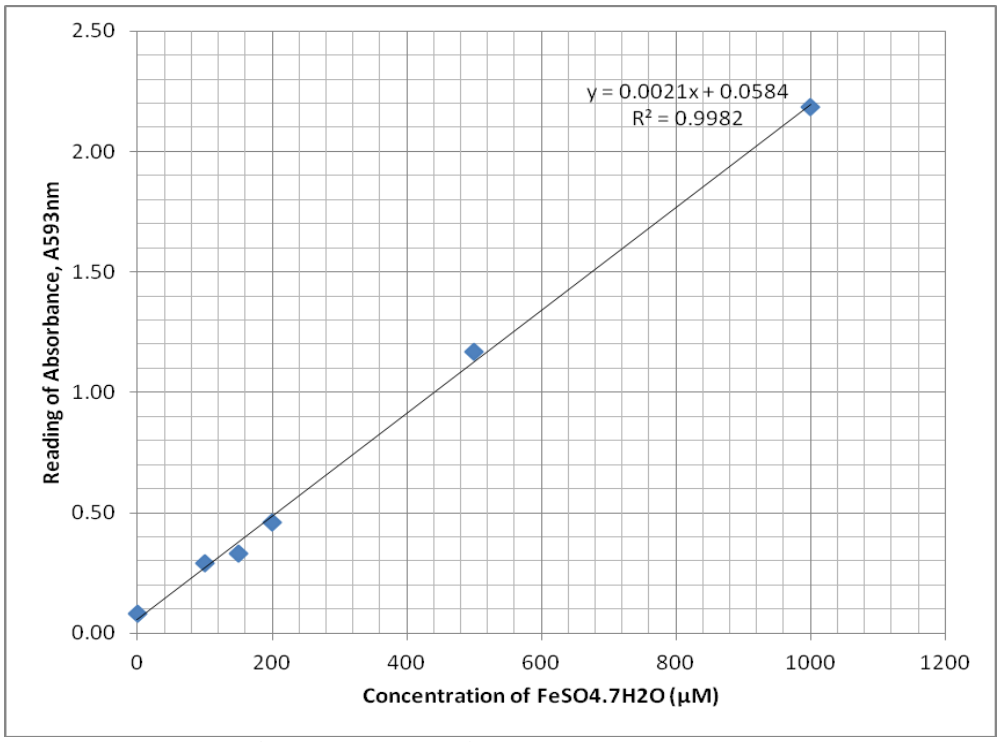
3. Please do not disturb the sequence of the samples provided.
4. Please rinse your tongue before testing next sample, with the water provided.

Sample #	Appearance	Pulp Colour	Aroma	Sweetness	Texture	Overall acceptability
124						
567						
488						
618						
342						
422						
426						
987						
896						
633						

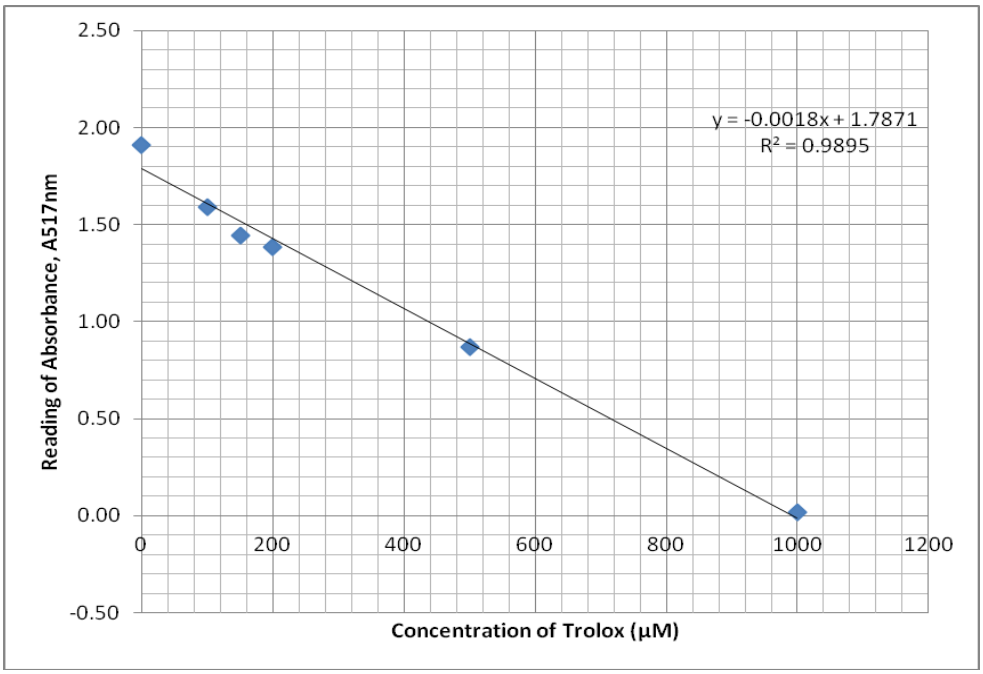
Appendix B: Standard curve



Appendix B1: Standard curve for total phenolic contents illustrating the linear regression between absorbance at 765 nm and gallic acid concentration ($\mu g ml^{-1}$).



Appendix B2: Standard curve for FRAP assay illustrating the linear regression between absorbance at 593 nm and ferrous sulphate concentration (µM).



Appendix B3: Standard curve for DPPH assay illustrating the linear regression between absorbance at 517 nm and Trolox concentration (µM).

Appendix C

Relative Expression Report

(Report produced by REST 2009 V2.0.13)

Legend:
P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.
TRG - Target
REF – Reference

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
ENOL (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
ENOL (1.5 ppm)	TRG	1.0	138.582	81.624 - 218.285	68.731 - 305.049	0.069	
ENOL (2.5 ppm)	TRG	1.0	1,093.313	671.849 - 1,670.532	578.336 - 2,130.021	0.000	UP
ENOL (3.5 ppm)	TRG	1.0	659.063	420.603 - 883.343	372.008 - 1,299.923	0.041	UP
ENOL (5.0 ppm)	TRG	1.0	423.769	295.751 - 627.939	245.729 - 806.618	0.000	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
AACT (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
AACT (1.5 ppm)	TRG	1.0	16.718	12.065 - 27.317	8.176 - 31.449	0.000	UP
AACT (2.5 ppm)	TRG	1.0	372.217	259.059 - 593.835	180.972 - 667.417	0.057	
AACT (3.5 ppm)	TRG	1.0	92.411	48.584 - 221.265	26.486 - 298.467	0.000	UP
AACT (5.0 ppm)	TRG	1.0	2.815	1.542 - 5.381	1.255 - 8.086	0.057	

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
SDS (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
SDS (1.5 ppm)	TRG	1.0	2.411	1.173 - 5.297	0.909 - 6.323	0.172	
SDS (2.5 ppm)	TRG	1.0	368.085	139.940 - 873.524	107.476 - 970.594	0.000	UP
SDS (3.5 ppm)	TRG	1.0	8.707	3.311 - 16.747	2.602 - 24.264	0.000	UP
SDS (5.0 ppm)	TRG	1.0	2.212	1.195 - 4.188	0.826 - 6.034	0.134	

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
MDAR (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
MDAR (1.5 ppm)	TRG	1.0	12.660	9.649 - 17.124	7.608 - 19.491	0.055	
MDAR (2.5 ppm)	TRG	1.0	29.071	20.969 - 41.968	18.535 - 50.839	0.040	UP
MDAR (3.5 ppm)	TRG	1.0	51.254	39.781 - 66.119	36.520 - 72.005	0.000	UP
MDAR (5.0 ppm)	TRG	1.0	18.952	14.243 - 24.700	12.235 - 31.056	0.000	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
HRP (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
HRP (1.5 ppm)	TRG	1.0	569.549	150.140 - 2,396.198	115.727 - 2,738.936	0.083	
HRP (2.5 ppm)	TRG	1.0	1,270.765	320.351 - 6,024.584	285.374 - 7,298.229	0.071	
HRP (3.5 ppm)	TRG	1.0	1,882.492	551.352 - 7,325.618	423.282 - 8,115.383	0.071	
HRP (5.0 ppm)	TRG	1.0	1,534.072	450.801 - 6,586.830	411.332 - 7,216.875	0.071	

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
ERF (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.263	
ERF (1.5 ppm)	TRG	1.0	4.379	2.537 - 6.914	1.939 - 8.053	0.036	UP
ERF (2.5 ppm)	TRG	1.0	10.270	4.799 - 15.996	4.444 - 17.270	0.030	UP
ERF (3.5 ppm)	TRG	1.0	72.813	59.779 - 89.137	52.753 - 102.816	0.000	UP
ERF (5.0 ppm)	TRG	1.0	247.556	185.045 - 331.955	151.882 - 406.444	0.000	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
ACX (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
ACX (1.5 ppm)	TRG	1.0	5.506	3.255 - 11.559	2.631 - 14.582	0.000	UP
ACX (2.5 ppm)	TRG	1.0	37.457	18.656 - 75.203	18.593 - 75.461	0.017	UP
ACX (3.5 ppm)	TRG	1.0	333.176	164.643 - 675.788	146.577 - 758.742	0.000	UP
ACX (5.0 ppm)	TRG	1.0	684.211	409.718 - 1,055.494	319.238 - 1,197.117	0.000	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
HSP (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
HSP (1.5 ppm)	TRG	1.0	2.283	1.561 - 3.605	1.457 - 4.571	0.000	UP
HSP (2.5 ppm)	TRG	1.0	2.612	1.681 - 3.799	1.549 - 4.121	0.042	UP
HSP (3.5 ppm)	TRG	1.0	3.925	1.635 - 10.563	1.366 - 12.622	0.000	UP
HSP (5.0 ppm)	TRG	1.0	317.417	241.658 - 398.224	216.128 - 513.504	0.000	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
PGI (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
PGI (1.5 ppm)	TRG	1.0	4.061	1.406 - 11.787	1.178 - 14.060	0.011	UP
PGI (2.5 ppm)	TRG	1.0	5.771	2.830 - 10.708	2.568 - 13.506	0.000	UP
PGI (3.5 ppm)	TRG	1.0	6.643	3.445 - 12.812	3.303 - 13.363	0.000	UP
PGI (5.0 ppm)	TRG	1.0	11.600	5.508 - 23.341	4.546 - 30.382	0.030	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
PME (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
PME (1.5 ppm)	TRG	1.0	0.758	0.478 - 1.517	0.397 - 1.803	0.408	
PME (2.5 ppm)	TRG	1.0	0.208	0.145 - 0.299	0.136 - 0.318	0.044	DOWN
PME (3.5 ppm)	TRG	1.0	0.079	0.060 - 0.101	0.052 - 0.131	0.043	DOWN
PME (5.0 ppm)	TRG	1.0	0.232	0.150 - 0.371	0.117 - 0.502	0.037	DOWN

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
INV (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
INV (1.5 ppm)	TRG	1.0	1,036.040	556.159 - 1,866.331	437.618 - 3,025.148	0.000	UP
INV (2.5 ppm)	TRG	1.0	2,196.970	1,267.340 - 4,037.925	1,022.667 - 5,885.293	0.000	UP
INV (3.5 ppm)	TRG	1.0	2,019.755	1,368.127 - 3,049.001	1,269.937 - 3,847.900	0.069	
INV (5.0 ppm)	TRG	1.0	1,848.750	1,144.754 - 3,295.471	806.165 - 3,935.008	0.000	UP

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PAP	REF	1.0	1.000				
MET (Control)	TRG	1.0	1.000	1.000 - 1.000	1.000 - 1.000	0.000	UP
MET (1.5 ppm)	TRG	1.0	9.936	5.811 - 18.204	4.292 - 21.466	0.031	UP
MET (2.5 ppm)	TRG	1.0	15.778	9.566 - 31.877	7.938 - 39.785	0.069	
MET (3.5 ppm)	TRG	1.0	17.455	11.494 - 30.161	10.121 - 37.586	0.031	UP
MET (5.0 ppm)	TRG	1.0	56.573	30.386 - 128.553	18.740 - 166.794	0.031	UP

BIODATA OF THE AUTHOR

Ong Mei Kying was born in Taiping, Perak. She obtained her primary education at Sekolah Rendah Kebangsaan Convent Klian Pauh, Taiping. Her secondary education was at Sekolah Menengah (P) Treacher Methodist, Taiping. In 1997, she enrolled as a tertiary science student at Sekolah Menengah Jenis Kebangsaan (C) Hua Lian, Taiping. After two year of schooling, she continued her study at Faculty of Food Science and Technology, UPM where she graduated with second class upper Bachelor of Science Degree in Food Science and Technology in 2002.

In July 2001, she obtained a scholarship from PASCA graduates fund sponsored by The Ministry of Science, Technology and Environment of Malaysia (MOSTE) to pursue her study in Master's Science Degree in the field of Plant Biotechnology at Faculty of Biotechnology and Biomolecular Sciences, UPM under the supervision of Assoc. Prof. Dr. Norihan Mohd. Saleh along with co-supervisor, Assoc. Prof. Dr. Azizah Abdul Hamid.

Once graduated, she worked as Quality Control Executive at Oceanbest Sdn. Bhd., a dried and frozen seafood company in Balakong, Selangor, Malaysia. In September 2007, she was offered to join as Food Safety Auditor at Malaysian Agrifood Corporation Berhad, a food supply chain management and distribution company. In year 2008, she was married to Andrew Chong and they are blessed with one daughter born in December 2009. She also served as Technical Manager (Fresh Produce) at Tesco Stores (Malaysia) Sdn. Bhd. at Mutiara Damansara for a several months.

Due to her keen interest in food safety, quality and postharvest physiology of tropical fruits and fresh produces, she decided to pursue further study. In August 2010, she was offered a scholarship for her doctorate programme from MedKlinn International Sdn. Bhd., represented by the School of Biosciences, Faculty of Science, The University of Nottingham Malaysia Campus. During her research period, she also obtained MyBrain scholarship from the Ministry of Higher Education, Government of Malaysia (MOHE), represented by The University of Nottingham Malaysia Campus. During her postgraduate studies in the present university, she has published research papers in highly reputable international refereed journals and also presented (oral, poster) in many conferences, seminars and symposiums.

LIST OF PUBLICATIONS

Refereed Journal's

1. Ali, A., Chow, W. L., Zahid, N. and **Ong, M. K.** 2014. Efficacy of propolis and cinnamon oil coating in controlling postharvest anthracnose and quality of chilli (*Capsicum annuum* L.) during cold storage. Food and Bioprocess Technology, DOI: 10.1007/s11947-013-1237-y.

2. Ali, A., **Ong, M. K.** and Forney, C. F. 2014. Effect of ozone pre-conditioning on quality and antioxidant capacity of papaya fruit during ambient storage, *Food Chemistry*, 142: 19-26. doi: http://dx.doi.org/10.1016/ j.foodchem.2013.07.039.

3. **Ong, M. K.**, Forney, C. F., Alderson, P. G. and Ali, A. 2013. Postharvest profile of a Solo variety 'Frangi' during ripening at ambient temperature. *Scientia Horticulturae,* 160: 12-19. http://dx.doi.org/10.1016/j.scienta.2013.05.026.

4. **Ong, M. K.,** Kazi, F. K., Forney, C. F. and Ali, A. 2012. Effect of gaseous ozone on papaya anthracnose. *Food and Bioprocess Technology*, DOI: 10.1007/s11947-012-1013-4.

5. **Ong, M. K.**, Kazi, F. K. and Ali, A. 2012. Effect of gaseous ozone exposure on the control of *Colletotrichum gloeosporioides* of papaya during ambient storage. *Acta Horticulturae* (ISHS) 1012: 727-734. http://www.actahort.org/books/1012/1012_97.htm. 7th International Postharvest Symposium (IPS 2012), organized by MARDI Malaysia and ISHS, PWTC Kuala Lumpur, Malaysia. June 25-29, 2012.

Oral / Poster Presentations

1. **Ong, M. K**., Chow, W. L., Ali, A. 2013. Effect of natural products in controlling postharvest anthracnose and quality of chilli (*Capsicum annuum* L.) during cold storage. Postgraduate Symposium on Plant Protection (PSPP) 2013, organized by Malaysian Plant Protection Society, Residence Hotel, UNITEN Bangi, Malaysia. August 26, 2013. (Poster). Won the Best Poster Presenter Award.

2. **Ong, M. K.**, Kazi, F. K., Ali, A. 2012. Effect of gaseous ozone exposure on the control of *Colletotrichum gloeosporioides* of papaya during ambient storage. 7th International Postharvest Symposium (IPS 2012), organized by MARDI Malaysia and ISHS, PWTC Kuala Lumpur, Malaysia. June 25-29, 2012. (Poster).

3. **Ong, M. K**., Forney, C. F., Ali, A. 2012. Physicochemical changes in 'Frangi' papaya (*Carica papaya* L.) during ambient storage. 7th International Postharvest Symposium (IPS 2012), organized by MARDI Malaysia and ISHS, PWTC Kuala Lumpur, Malaysia. June 25-29, 2012.(Poster).

4. **Ong, M.K**., Ali, A. 2012. Ozone, solution for missing food. Postgraduate Research Showcase 2012, organized by The Graduate School, The University of Nottingham Malaysia Campus, Malaysia, May 18, 2012. (Poster). Won the Judge's Choice Award.

5. **Ong, M. K**. 2012. Ozone towards safe and effective postharvest treatment for enhancing shelf life and quality of fresh produce. Postgraduate Student Conference, organized by the School of Biosciences, Faculty of Science, The University of Nottingham Malaysia Campus, Malaysia, May 2, 2012. (Oral).

6. **Ong, M. K**., Kazi, F. K., Ali, A. 2011. Antifungal activity of gaseous ozone against *Colletotrichum gloeosporioides* of papaya *in vitro*. National Horticulture Conference 2011, organized by MARDI Malaysia, Renaissance Melaka Hotel, Malaysia, October 18-20, 2011. (Poster).

7. **Ong, M.K**., Ali, A. 2012. Cerafusion Technology, an Innovation in Postharvest Technology. Postgraduate Research Showcase 2011, organized by The Graduate School, The University of Nottingham Malaysia Campus, Malaysia, May 18, 2011. (Poster).