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Gestione dell'innovazione nei sistemi agro-alimentari della regione mediterranea

International PhD Course in:

Management of the Innovation in the Agricultural and Food Systems of the Mediterranean Region

OZONE AND ELECTROLIZED WATER APPLICATION TO PRESERVE QUALITY OF CITRUS FRUIT AND EFFECT OF OZONE ON GENE EXPRESSION RELATED TO PLANT DEFENSE MECHANISMS

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XXIX CICLO

A.Y. 2016-2017

Dedication

It is my pleasure to dedicate this modest work

To my great father …

To my mother with my great appreciation …

To my wife … Thanks for your patience, support, love …

To my little angels SILINA

To my brothers and sisters.

EYAD

Acknowledgments

I would like to express my cordial thanks and profound gratitude to all MAIB staff, particularly to the CIHEAM General secretary Dr. C. LACIRIGNOLA and to the MAIB Director Dr. M. RAELI for giving me the opportunity to study and work in MAIB and for their great support.

Special thanks to the Department team at University of Foggia

My sincere thanks and gratitude to my supervisor Prof. Giancarlo Colelli for his suggestions help and precious advises.

It is difficult to express my enormous appreciation to my supervisor Dr. Thaer Yaseen, for her supervision, advices, and gentleness, friendly, respectful and unlimited support.

Special thanks to Dr. Raied Abou Koubaa for his great help and support from the very beginning of my study and help and efforts from the preliminary to the concluding stage of this study.

I thankfully acknowledge my Italian family, Finally, I would like to thank all my friends and colleagues.

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%	Percent
°C	Degrees celsius
µg	Micrograms
AMV	Avian Myeloblastoma Virus
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony forming units
CFU	Colony Forming Unit
CHI1	Chitinase gene
CRO	Conventional refrigeration
CT	Threshold Cycle
CTRL	Control
cv	Cultivar
DNA	Deoxyribonucleic Acid
Ds-DNA	Double-Stranded DNA
E	Amplification Efficiency
FDA	United States Food And Drug Administration
g	Gram
GNS1	B-1,3-Glucanase gene
GRAS	Generally Recognized As Safe
h	Hour
HCl	Hydrogen chloride
IAMB	Istituto Agronomico Mediterraneo di Bari
Kg	Kilogram
L	Liter
min	Minutes
MMLV	Moloney Murine Leukemia Virus
Ng	Nanograms
nm	Nanometer
O_3	Ozone
PAL1	Phenylalanine Ammonia-Lyase gene
PCR	Polymerase Chain Reaction
PDA	potato Dextrose agar
POD1	Peroxidase gene
ppm	Parts per million
PRO	Passive refrigeration
RFU	Relative Fluorescence Unit
RNA	Ribonucleic Acid
Rnase H	Hydrolytic Ribonuclease
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
T	Temperature
t	Time
Tm°	Melting Temperature
US	United States
UV	Ultra-Violet
µl	Microliter

1 Chapter 1

1.1 Introduction

The production of fruits and vegetables represents an important sector in the total world agricultural output. Although an accurate evaluation of economic contribution from these segments around the world is difficult to obtain because of the reporting systems, it is estimated that the contribution from these segments could be over US$600 billion (Paliyath *et al.*, 2008).

Fruits and vegetables are subject to qualitative and quantitative losses, starting from the field continuing with the whole postharvest period, since most fruits and vegetables are highly perishable commodities. The extent of the loss of horticultural produce after harvest can vary in different countries. In the parts of the world where the methods of agricultural production and storage employ advanced technology, postharvest losses may be minimal, and most of it occurs during the transit of produce from the production site to the destination along the consumer chain. The losses can range from 10% to 20% but in tropical areas and where storage facilities are limited losses can reach 50% or over (Paliyath *et al.*, 2008).

Among the treatments used to control postharvest diseases, we find Synthetic Chemical treatments which are still largely used, like bicarboximide fungicides, sulphur dioxide (SO_2) and benzimidazole compounds, the use of these synthetic chemicals, is still the primary means for controlling plant diseases. However, particularly during postharvest storage and handling, the need to reduce the use of chemicals, concern about environmental pollution and the inability to control fungal diseases due to the development of fungicide-tolerant strains of pathogens have encouraged the research to find alternative approaches to plant disease control (Barkai-Golan, 2001).

The antimicrobial and antifungal activity of different plant products has been assessed in some studies (Wedge *et al.*, 2000; Paranagama *et al.*, 2003; Proestos *et al.*, 2006). These compounds are usually low toxic to mammals and safe to the environment, and thus they may serve as substitutes for synthetically produced fungicides or to develop integrated strategies to manage and control contaminations.

In this category of natural elements several compounds are used, like essential oils and plant extracts which are often characterized by antifungal activity against a wide range of fungi mainly due to the presence of volatile compounds like 1,8-Cineole, α-Pinene and p-Cymene (Celikel and Kavas, 2008) and Chitosan which is a bio-polymer formed by de-acetilation of chitin that constitutes the supporting material of invertebrates and of fungal cell walls (Barkai-Golan, 2001).

Another alternative strategy in the control of postharvest diseases is biological control. The term “Biological control" or “Biocontrol" refers to the use of naturally found microorganisms (and/or their metabolites) which antagonize the postharvest pathogens we wish to suppress or are able to control their proliferation.

Antagonism between microorganisms is a ubiquitous phenomenon involving fungi, yeasts and bacteria which naturally inhabit the soil and the surfaces of various plant organs. It is assumed that bio-control of plant diseases occurs naturally on aerial plant surfaces and may be one of the main reasons why crops are protected to some extent during their cultivation (Barkai-Golan, 2001).

Interest to physical treatments that could serve as alternatives to fungicides has been increased in last decades. Cold storage and controlled or modified atmospheres are physical means that are widely used in the management of postharvest diseases. Among the techniques used we mention heating, ionizing radiation and ultraviolet illumination. Several studies have demonstrated that physical treatments like hot water treatments elicit resistance responses in harvested commodities, increasing enzymatic activity (e.g. phenylalanine ammonia lyase, and peroxidase) related to the host’s resistance against the pathogens (Paliyath *et al.*, 2008).

Generally recognized, as safe (GRAS) compounds are substances, having a common knowledge of safety within the expert community (FAD Rules and Regulations, 2001).

Fungicides that leave low or non-detectable residues in the commodity, these are actively sought in research programs. Compounds are selected that rapidly degrade on the host surface or metabolize quickly in the tissue (Barkai-Golan, 2001).

Among this type of compounds, we distinguish Chlorine, acetic acid, bicarbonate and carbonate salts, ozone and others. These compounds have a broad spectrum of activity against food-borne bacteria and yeasts, and are Generally Recognized As Safe (GRAS) compounds for many applications, by the US Food and Drug Administration (FDA) (Barkai-Golan, 2001).

Recently ozone (O_3) and electrolyzed water (EW) have received a particular consideration as antimicrobial agents to inactivate bacteria, fungi, viruses and protozoa for disinfecting water, in the food industry, and for reuse of waste water (Guzel-Seydim B.Z. *et al.*, 2003). Recent studies indicates that ozone is an effective treatment for increasing shelf-life and control fungal deterioration of fresh fruit such as table grapes (Gabler *et al.*, 2010a; Yaseen *et al.*, 2015; Yaseen *et al.*, 2016) citrus and stone fruit (Crisosto *et al.*, 2002) during postharvest, leaving any residues potentially toxic.

Ozone was also used for the reduction of pesticides residue in food industry, in particular the effect of ozone on the reduction of pesticide residue in baby corn (Zea mays L.,) (Whangchai *et al.*, 2010a) and vegetables like lettuce, cherry tomatoes, and strawberries (Ikeura *et al.*, 2011) was studied and It was also shown that fumigation with ozone can effectively reduce fungicide residues on table grapes: Residues of fenhexamid, cyprodinil, pyrimethanil, and pyraclostrobin were reduced by 68.5, 75.4, 83.7, and 100.0%, respectively, after a single fumigation of table grapes with 10,000µL xL^{-1} ozone for 1h (Gabler *et al.*, 2010a).

Electrolyzed water (EW) is generated by the electrolysis of sodium chloride solution in an electrolysis chamber where nonselective membranes are separating the anode and cathode. Water collected at the anode has unique oxidation properties due to its content of hypochlorous acid (HOCl) and low pH (Buck *et al.*, 2002). More HOCl which is related with a strong antimicrobial activity is available in relatively low pH (Kim *et al.*, 2000). EW water is widely used for disincentive purposes in Japan hospitals and its use in aquaculture is also under estimation (Tanaka *et al.*, 2006). In agricultural and food industrial processes (Kim *et al.*, 2000), (EW) has been widely applied as well as postharvest disease control (Hong and Michailides, 1998; Al-Haq *et al.*, 2002).

Among physical means, passive refrigeration proved to be a good strategy to obtain an effective and sustainable in storage and transportation chambers. This

technology depends on: the isolated walls of PRS-units contain a specially designed fluid, which can absorb large amounts of heat over a long period of time. After charging, the unit will maintain a stable internal temperature for up to 30 days (depending on unit specifications). During this time, the system does not use any external energy supply (100% autonomy). Therefore, no need of forced ventilation, with its high EMS Ship Supply Group energy use and noise, and the relative humidity remains constant (Marrone *et al.*, 2009). New promising techniques show an efficacy in controlling fungal storage disease, ozone, passive refrigeration and electrolyzed water (EW). They are applied in wide group of aspects related to cleaning, sanitation, disinfection and other industrial purposes. Even though the evidences of antifungal effect of all of them have been shown, there are still some disadvantages lead to think in making combination of their applications in complementary approach. The aim of this study was to define:

1. The effect of ozone and EW on microorganism population on the surface of harvested fruits treated for different periods, and to investigate the improvement of the quality and shelf life of stored orange fruits.

2. The effects of Ozone in inducing disease resistance of orange fruit after harvest in response to pathogen attack, and on the gene expression levels that might be involved in induced resistance; primer design and validation of genes related to plant defines response: β-1,3-endoglucanase (GNS1), Chitinase (CHI1), phenylalanine ammonia-lyase (PAL1), Peroxidase (POD1). The second part of the study investigated the capacity of ozone as an abiotic elicitor in terms of triggering the natural disease response mechanisms. Quantitative reverse transcriptase PCR (qRT-PCR) was used to evaluate the influence of ozone in citrus fruit (ozone either treated alone or challenged together with *P. digitatum*) on expression of genes involved in different responses such as SAR, ROS and ISR. (Systemic acquired resistance, production of Reactive Oxygen Species, and Induced Systemic Resistance respectively).

2 Chapter 2- General introduction

2.1 Use of ozone during the postharvest period in fresh fruits and vegetables

2.1.1 History of ozone use in fresh fruits

The name “ozone” is derived from the Greek word ozein, meaning, “to smell”, It was not until 1840 that Sch ö nbein reported the pungent odor as a new substance he called ozone. In 1886, the first experimental use of ozone in water for disinfection was in de Meritence, France (Alex, 2008).

In the United States, ozone was first used in 1908. Ozone has been utilized for more than a century for water treatment. In 1936 Ozone was used to treat shellfish in France, six years after in the United States O_3 was used in egg storage rooms and in cheese storage facilities. In 1982, Ozone was declared GRAS (Generally Recognized as Safe) for bottled water in the United States, twenty years after, FDA recognizes ozone as a secondary direct food additive. (Federal Register, Vol. 66, no. 123, Tuesday, June 26, 2000. Rules and Regulations) (FDA, 2009).

Over the years, attempts have been made to use the reactive properties of ozone to preserve the quality of fresh fruits and vegetables. Most of these attempts have focused on the antimicrobial effects of ozone and its usefulness to inhibit or prevent decay, although, ozone may induce many physiological changes in horticultural products, both positive e.g. effect on decay and delaying fruit shelf-life (Smilanick *et al.*, 1999) and negative e.g. alteration of product quality (Torres *et al.*, 2011).

2.1.2 Decay control in fruits by ozone

In efforts to control postharvest decay, a wide range of ozone concentrations have been tested in storage environments, as continuous or intermittent treatments. In general, fruit are more tolerant to ozone than vegetables and therefore higher concentrations of ozone can be used on fruit without risk of damage. Ozone has been added to the storage environment of a wide variety of fruits and vegetables with mixed results. In general, ozone is effective in inhibiting mycelium growth and the resulting nesting, as well as sporulation, which may reduce the spread of decay during storage. However, its effectiveness to slow or prevent decay is variable, depending on crop, cultivar, decay organism, and storage conditions (Forney, 2003).

The effectiveness of ozone in the control of post-harvest pathogens was demonstrated by several researchers. Spores of *Monilinia fructicola*, *Geotrichum citriaurantii*, *Penicillium italicum*, and *Botrytis cinerea* are killed in about one minute when exposed to 16.5°C water containing 1.5 mg/L ozone, while *Penicillium digitatum*, *Penicillium expansum*, and *Rhizopus stolonifer* require an exposure of two to three minutes (Smilanick *et al.*, 1999; Yaseen *et al.*, 2015; Romanazzi *et al.*, 2016; Yaseen *et al.*, 2016). Gabler *et al.* (2010b) demonstrated that ozone is an effective treatment for increasing shelf-life and decreasing fungal deterioration in the postharvest treatment of table grapes.

Ozone gas also inhibited the normal aerial growth of the mycelia and greatly prevented sporulation of *P. italicum* and *P. digitatum* from lesions among infected fruit once lesions developed. However, aerial mycelia growth and sporulation resumed afterward in ambient atmosphere (Palou *et al.*, 2001).

Also ozone gas penetration through packaging materials and its effectiveness in controlling sporulation of *Penicillium digitatum* and *P. italicum* on artificially inoculated and commercially packed oranges was studied and sporulation of both *P. digitatum* and *P. italicum* was significantly inhibited by ozone exposure on oranges packed (Palou *et al.*, 2003). The effects of gaseous ozone exposure on development of post-harvest green and blue molds (*Penicillium digitatum* and *Penicillium italicum*) in artificially inoculated oranges was studied and the use of ozonized air during the storage period reduced the number of fruits affected by mould but not reduce *Penicillium mold* incidence in no-washed fruit (Di Renzo *et al.*, 2005).

Ozkan *et al.* (2011) demonstrated that at 95% RH, 99% of the conidia of *P. digitatum*, *P. italicum*, and *B. cinerea* were incapable of germination after O_3 exposures of 817, 732, and 702 $\mu L.L^{-1}\times h$, respectively. At 75% RH, similar inhibition required exposures of 1781, 1274, and 1262 $\mu L.L^{-1}\times h$, respectively (Ozkan *et al.*, 2011). Hildebrand *et al.* (2008b) observed that mycelial growth of *Penicillium expansum* and *Botrytis cinerea* is progressively reduced with increasing ozone concentrations of 0.10 to 0.50$\mu L.L^{-1}$ (Hildebrand *et al.*, 2001; Romanazzi *et al.*, 2016).

It was demonstrated that exposure of conidiophores of *Alternaria solani* to 0.1 $\mu L.L^{-1}$ ozone for two hours or 1.0 $\mu L.L^{-1}$ ozone for four hours stops elongation,

and causes swelling and collapse of the cell wall at the apical tips (Rich and Tomlinson, 1968).

Smilanick *et al.* (2002) reported that green mold and sour rot on citrus fruit, caused by *P. digitatum* and *Geotrichum citri-aurantii*, respectively, were not reduced by 20 min immersion in 10 ppm ozone. The failure of O_3 in aqueous applications might be related with the low solubility of ozone in water (Smilanick *et al.*, 2002).

2.1.3 Effect of ozone in fruits physiological quality

Ozone treatment applied on fresh carrots had no effect on fresh weight loss, sprouting of carrot crowns, or on concentrations of glucose, fructose, sucrose or galactose (Hildebrand *et al.*, 2008a).

Sucrose, glucose, and fructose are reduced by about 20 percent in strawberry fruit exposed to 0.35 $\mu L.L^{-1}$ ozone at 2°C for three days, but after an additional two or four days in air at 20°C, these differences are less apparent and the ozone-treated fruit actually have higher concentrations of sucrose (Perez *et al.*, 1999). The concentrations of fructose, galactose and sucrose sugars increased in treated kiwi fruits during storage in both air at 0°C and ozone enriched (Barboni *et al.*, 2010).

Aguayo *et al.* (2006) demonstrated that ozone treatment of whole and sliced tomatoes increase the rate of sugar (fructose and glucose) and organic acid (ascorbic and fumaric), and in whole tomatoes, O_3 maintained the tissue firmer than in control fruit (Aguayo *et al*., 2006). Sarig *et al.* (1996) demonstrated that ozone treatment can significantly control storage grape cluster decay for 6 days at 20°C probably for both its direct fungicidal activity on the decay organism tested (*R. stolonifer*) and for its ability to elicit stilbene phytoalexins in grapes (Yaseen *et al.*, 2016).

Alothman *et al.* (2010) observed that total phenol and flavonoid contents of pineapple and banana increased significantly when exposed to ozone (0.44-0.59 m mol depending on fruit treated) for up to 20 min, and decreased in guava fruits, he also confirmed that Ozone treatment significantly decreased the vitamin C content of all three fruits.

The removal of ethylene from the storage environment may have beneficial effects in extending storage life of fresh produce by delaying ripening and senescence (Forney, 2003). Since ozone is able to low the amount of ethylene in

packinghouse during the storage of some studies were performed in order to measure ethylene destruction by ozone in storage environments.

When ozone concentrations are maintained constant at 0.10 $\mu L.L^{-1}$ in a storage room containing 6 $\mu L.L^{-1}$ ethylene, it was found the ethylene destruction rate to be 0.28 $\mu L.L^{-1}$ per hour (Forney, 2003). Ethylene destruction by ozone has been attributed to inhibiting banana ripening and broccoli yellowing (Skog and Chu, 2001). Palou *et al.* (2001) claimed that ozone is effective in removing ethylene from export containers.

2.1.4 Effect of ozone on pesticides residues

The worldwide overuse of pesticides increased public concern about possible health risks from residues on agricultural products, raising consciousness against hazardous effects of chemicals make many people pay higher prices for organically produced foods. Ozone, with its high oxidation potential, seems to be a good alternative that can substitute for many toxic pesticides. Ong *et al*., (1996) demonstrated the effectiveness of chlorinated and ozonized water dips in the dissipation of azinphos-methyl, captan and formetanate hydrochloride in solution and on fresh and processed apples, all three pesticides in model systems solution decreased 50%-100% with chlorine and ozone treatment (Ong *et al.*, 1996).

Recent work indicated that ozone was used for the reduction of pesticides residue in food industry. Effect of ozone on the reduction of pesticide residue in baby corn (Zea mays L.,) (Whangchai *et al.*, 2010b) and vegetables like lettuce, cherry tomatoes, and strawberries (Ikeura *et al.*, 2011) was studied and the results showed that O_3 is effective. It was also shown that fumigation with ozone could effectively reduce fungicide residues on table grapes: Residues of fenhexamid, cyprodinil, pyrimethanil, and pyraclostrobin were reduced by 68.5, 75.4, 83.7, and 100.0%, respectively, after a single fumigation of table grapes with 10,000$\mu L.L^{-1}$ ozone for 1 h (Gabler *et al.*, 2010b).

Degradation of the four pesticides: methylparathion, parathion, diazinon and cypermethrin by dissolved ozone was investigated in order to establish the effect of operational parameters. Wu *et al.* (2007) demonstrated that dissolved ozone (1.4 $mg.L^{-1}$) was effective to oxidize 60–99% of methyl-parathion, cypermethrin, parathion and diazinon in aqueous solution in 30 min and the degradation was mostly completed in the first 5 min.

2.2 Use of electrolyzed water during the post-harvest process for fresh fruits and vegetable

2.2.1 History of electrolyzed water use in fresh fruits

Electrolyzed water (EW) was first used around 1900 in the soda industry, including in the production of sodium hypochlorite (Japan Soda Industry Association, 1982). In 1980, the technology was introduced into the market as sanitary supervision of stored water in an automatic dispenser. With improvement and miniaturization of the equipment, electrolysis technology has been applied in various fields and is now regarded as a promising non-thermal treatment for hygiene control (Koseki *et al.*, 2004b). EW is generated by the electrolysis of sodium chloride solution in an electrolysis chamber where non-selective membranes are separating the anode and cathode. Water collected at the anode has unique oxidation properties due to its content of hypochlorous acid (HOCl) (Koseki *et al.*, 2004a) and low pH (Buck *et al.*, 2002). More HOCl which is related with better antimicrobial activity is available in relatively low pH (Kim *et al.*, 2000), EW water is widely used as a sanitizer in Japan hospitals and its use in aquaculture is currently being assessed (Tanaka *et al.*, 2000). In agricultural and food industrial processes as well as in post-harvest disease control (Kim *et al.*, 2000; Al-Haq *et al.*, 2002; Hong *et al.*, 1998) EW has been widely applied.

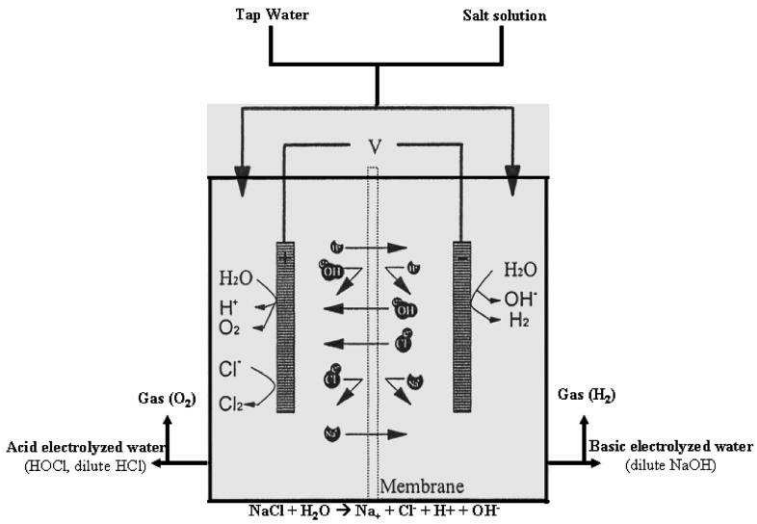

Figure 1. Basic electrolyzed water-generation schema.

Izumi (1999), showed that EW containing 15-50 ppm of available chlorine was effective in reducing microbial flora in several fresh-cut vegetables; The effect of EW is strongly related to contact time. During this bibliographic review, we find that, just one articles describe the effect of electrolyzed water in combination with

ozone to control post-harvest decay of Tangerine fruits, by Whangchai *et al.* (2010a); he proved a complete inactivation of *Penicillium digitatum* spore germination occurred in all concentration of EW when the exposure time increased to 16 min. The duration of exposure time is the major factor effect of EW, It was found that the immersion of the citrus fruits for 8 min in the EW was more effective to reduce disease incidence. Moreover, washing the fruits with EW and kept in refrigerated chamber at 5°C with continues ozone exposure at concentration of 200 mg xL^{-1} for 2 hours /day it was more effective to reduce disease incidence until 28 days (Whangchai *et al.*, 2010a).

It have been demonstrated that the agitation has an impact on the effectiveness of EW treatment, in the same work of Park *et al*. (2002) reported that EW in static conditions was less effective than with agitation due to the limited ability of chlorine in EW to penetrate microbial cell layers. Vorobjeva *et al*. (2004) reported that EW water possess antimicrobial activity on Pseudomonas aeruginosa. Buck *et al*. (2002) mentioned that EW water has a positive effect by reducing fungal

Buck *et al*., (2002) study the germination of species such as *Alternaria* spp., *Botrytis* spp., *Fusarium* spp.. and other 22 fungal species treated with EW *in vitro* and reported thin walled species (e.g. *Botrytis*, *Monilinia*) were killed by incubation times of 30s or less but thicker-walled, pigmented fungi (e.g. *Curvularia, Helminthosporium*) required 2min or longer for germination to be reduced significantly. Bonde *et al*. (1999) observed that treatment of wheat seed for 20 min with EW eliminated contamination by fungi such as *Aspergillus* spp., *Cladosporium* spp., and *Penicillium* spp. Hypochlorous acid in EW water can harm *Penicillium digitatum* by oxidizing nucleic acids and proteins, causing lethal damage (Acher *et al.*, 1997). It was approved that, EW water application can extend postharvest storage of table grapes and completely killed *B. cinerea* conidia at 10 g xL^{-1}, and decreased the gray mold incidence on artificially inoculated, single table grape berries, compared to samples dipped in water (Guentzel *et al.*, 2010), furthermore, EW pH can influence bactericidal properties of EW (Iwasawa and Nakamura, 1996), in particular when pH is more acidic (Lin *et al.*, 2013). Sch *et al*., (1999) used EW for controlling powdery mildew in cucumber and he found that it apparently reduced powdery mildew for about two weeks from 18 days after

planting. He also found that fungal decay was delayed for about two days in peaches treated with EW (Sch *et al.*, 1999).

2.2.2 Decay control in fruits by electrolyzed water

Hypochlorous acid damages the microbial cell by oxidizing nucleic acids and proteins, causing lethal damage (Acher *et al.*, 1997), its acidic pH has an inhibiting role on the fungal development because it sensitizes the outer membrane of the cells, thereby allowing hypochlorous acid to enter the cells more efficiently (Whangchai *et al.*, 2010a). Leyer and Johnson (1997) reported that the sensitivity of acid-adapted cell of Samonella typhimurium to hypochlorous acid was higher than non-adapted cells.

Al-Haq *et al*., (2001) found that EW water suppressed the incidence and disease severity on wounded European pears inoculated with *Botryosphaeria berengenriana*. (McDonnell and Russell, 1999) suggested that hypochlorous acid can penetrate microbial cell membranes and exert its antimicrobial Action through the oxidation of key metabolic system like suspensions of Escherichia coli decreases their ability to accumulate 14C-labeled glutamine, proline, thiomethyl galactoside, and leucine in leading to a loss of cell viability. Kim *et al*. (2000) mentioned that the ORP (oxides reduction potential) of the treatment solution was the primary factor affecting microbial inactivation. Liao *et al.* (2007) found that the high ORP of the solution that affects fungi in disrupting the outer membrane and facilitates the transfer of HOCl across the cell membrane, resulting in further oxidation of intracellular reactions and respiratory pathways such as the glutathione disulfide-glutathione cellular redox couple. Hypochlorous acid (HOCl) can inactivate bacterial cells by inactivation of enzymes which participate in metabolism such as in active transport (Hurst *et al.*, 1991), by the inhibition of ATP generation (Hurst *et al*., 1991) and oxidation of cell surface sulfhydryl compounds (Leyer and Johnson, 1997; Park *et al.*, 2002).

2.3 Effect of electrolyzed water in fruits physiological quality

Application of EW on harvested sugarcane during summer months leads to a relatively less decline as sucrose content and purity of juice compared to untreated and water treated control (Solomon and Singh, 2009). Furthermore, another effect of electrolyzed water on total on microbial count on several fresh-cut vegetables as well as decontamination of food borne microorganisms in food industry (Izumi, 1999; Subrota *et al.*, 2012). The efficacy of EW is directly correlated to the pH of

the final available chlorine; therefore the Electrolyzed water containing 50 ppm available chlorine had a stronger bactericidal effect than that containing 15 or 30ppm chlorine for fresh-cut carrots, spinach, or cucumber. Electrolyzed water did not affect tissue pH, surface colour, or general appearance of fresh-cut vegetables. While, acidic EW had decontaminative effects on the surfaces of lettuce by reducing the aerobic bacteria when applied after 7 d at 1–2°C. In the same way EW containing 60mgxL^{-1} free chlorine (pH 6.5) resulted as effective as chlorine 50% solutions in washing lettuce samples, showing good quality retention (Rice *et al.*, 2004). Other researchers prove that EW does not affect the texture, scent, flavour, etc. of treated fruits, which are brought about by heat-treatment (Koseki *et al.*, 2003). In addition, Vandekinderen *et al.* (2009) reported that high doses of EW did not affect the nutritional and sensory qualities of fresh-cut iceberg lettuce after washing for 10 min.

2.4 Effects of combined treatment with electrolyzed water and ozone on agricultural commodities

Whangchai *et al*., (2010a) found that containing 10^5 *Penicillium digitatum* spore suspension conidia were activated in all concentration of EW when the exposure time increased to 16 min. The duration of exposure time is the major factor effect of EW, when the fruits inoculated with *P. digitatum* where washed in EW for 4,8 and 16 min stored at 5°C for 18 days, It was found the immersion of the fruits for 8 min in the EW was more effective to reduce disease incidence. Moreover washing the fruits with EW and kept in refrigerated chamber at 5°C with continues ozone exposure at concentration of 200 mg xL^{-1} for 2 hours/day it was more effective to reduce disease incidence until 28 days (Whangchai *et al.*, 2010a).

2.5 Passive Refrigeration

Starting in the early 1960s there were several studies into freezing large masses of brine using passive cooling tubes. These brine freezing systems, one in Frobisher Bay, Canada and one in Savoonga, Alaska, had some success but both suffered from brine storage problems due to corrosion of the holding tanks and thawing of permafrost under the tanks (Ringer, 1958; Zarling and Burdick, 1981).

Thermosyphons are used in Alaska to stabilize permafrost under buildings, roads, and pipelines. They are large tubes, partially filled with fluid and vacuum sealed, that operate passively when the ground temperature is greater than the ambient air temperature. A variety of fluids are used in the permafrost thermosyphons:

ammonia, carbon dioxide, and synthetic refrigerants. Heat in the ground heats the fluid, causing it to evaporate. The vapor rises to the fins at top of the tube, usually above ground, where it condenses due to the colder temperatures, releasing the absorbed heat to the heat exchanger and then into the air. The condensed fluid then flows back to the bottom of the tube for the cycle of phase change to continue. A thermosyphon needs to be orientated with a downward slope so that the fluid can flow down. (There are newer tubes called heat pipes which can be orientated any direction because capillary mesh directs the flow of the liquid). Thermosyphons move heat passively whenever there is enough of a temperature difference between the ground and the air. When there is no temperature difference or the air is warmer than the ground, they are not active.

A recent study (Peterson and Wendler, 2011) modeled the use of thermosyphons and extra insulation to maintain the thawing ice cellars on the North Slope. They concluded that a combined approach of thermosyphons and insulation could keep the cellars sufficiently cool to maintain frozen food year round. This study is currently on hold pending funding to test a prototype installation.

In addition to the aforementioned large-scale community cold storage systems designed to store and freeze large quantities of subsistence and commercial meats, there have been attempts at selling residential-scale passive refrigeration systems. In the 1980s Sun Frost, a company in California, designed and built hybrid passive residential refrigerators. The DC units ran efficient electric compressors to cool the units in the summer and refrigerant filled thermosyphons to cool the units in the winter using outdoor air as a heat sink. The company only made 10 due to low demand (Sun Frost, n.d.).

The system is designed for commercial size freezers and would be hard to scale down for residential freezer use (it would not work for a refrigerator compartment). A University of Alaska Fairbanks project looked at running glycol cooling coils in a residential refrigerator and pumping the glycol outside to cool it (Gustafson *et al.*, 2009). The prototype system suffered from mechanical problems but the idea worked. It was not completely passive but has potential even in the summer if they route their exterior piping underground.

The recently developed Passive refrigeration system (PRS) is a system developed for the preservation and transport of perishable products. It is based on thermal

accumulation ("Thermal Charge"). The system guarantees perfect preservation through the maintenance of optimal temperature and relative humidity. The system works without ventilation thus assuring shelf life which is better than the active refrigeration system equipment. The thermal autonomy allows the storage and transport without use of power during operations (Nomos, 2008).

2.6 Postharvest fungal diseases of studied fruits

Citrus fruits are an important economic resource; unfortunately, losses from post-harvest diseases caused by various pathogens can reach 50% of the entire product. Infections and contaminations can occur at different stages in the field and after harvest (Ladaniya, 2008). Post-harvest disease of citrus depends on the variety, weather conditions during cultivation, post-harvest handling and conditions. The high incidence of post-harvest disease is primarily due to green mold *Penicillium digitatum*, blue mold *Penicillium italicum* and rots caused by Alternaria and Rhizopus (Naqvi, 2004). Green mold, caused by *Penicillium digitatum* is one of the most important post-harvest diseases of citrus (Holmes and Eckert, 1999). It affects 5–8% of the total fruit handled, and results in a significant economic loss to both growers and packers. To maintain shelf life and fruit quality in the market, control of postharvest diseases is critical also because citrus fruits can be stored for a long period (several months) before being marketed.

2.6.1 Penicillium Rots

Penicillium digitatum and *Penicillium italicum* are the two most significant and widely reported postharvest pathogens in citrus. They occur in all citrus growing countries, worldwide and may attack the fruits in packinghouses, in transit, in storage and in the market. The major menace of these pathogens is due to their spores, which appear as fine powder and are airborne (Ladaniya, 2008). In infected fruit, very profuse sporulation can be observed; fruit is completely covered by white mycelium followed by green or bluish spores of *Penicillium digitatum* and *Penicillium italicum* respectively. The two fungi may appear together in the same lot or even on the same fruit (Ladaniya, 2008). Blue mold is more harmful because it spreads in the box and healthy fruits are directly attacked, regardless of injury. Green mold is a nesting-type pathogen, meaning that it produces enzymes that soften the adjacent fruit and thus allow fungus to enter. Green mold does not spread by nesting; thus, if a single fruit is affected it remains as such without contaminating adjacent fruit. However, spores lead to soiling of fruits and thus

require repacking with a box change (Ladaniya, 2008). At an early, stage both fungi cause a soft rot of the peel. Following this stage, white mycelium develops from the centre of the affected soft area and later starts sporulation from the centre of the colony. The sporulation part becomes olive green in the case of *P. digitatum* and blue in the case of *P. italicum* (Barkai-Golan, 2001) *P. italicum* is able to germinate at lower temperatures than *P. digitatum*, and even at 0°C (Wyatt and Parish, 1995). The optimal growing temperature range for both fungi is 20-27°C, which the fruits may rot within a few days. Although fungal growth is reduced at lower temperatures, a very slow rate has still been recorded at 4.5-10°C, allowing the fungi to progress under these conditions when storage is extended or in overseas shipments. At 0-1°C the growth of the two *Penicillium* is arrested, but these temperatures can result in chilling injury, expressed in pitting and internal physiological injury (Barkai-Golan, 2001; Beber-Rodrigues *et al.*, 2015).

2.7 Gene expression

2.7.1 Elicitors

Enhanced protection of host plant tissue during periods of susceptibility through induced/acquired resistance is considered a preferred strategy for achieving integrated pest management (IPM) (Lucas, 1999; Kuć, 2000). Preformed and/or inducible defense mechanisms are important in enhancing or boosting the natural defense response in horticultural crops. The factors that bring about this defense response are known as elicitors.

2.7.2 Elicitors definition

An elicitor (derived from the verb "to elicit" meaning provoke or induce) is a substance capable of triggering a cascade of events leading to the expression of defense responses in plants. This term, originally restricted to molecules capable of stimulating the synthesis of phytoalexins, is now assigned to all molecules capable of inducing one or many defense reactions (Ebel and Cosio, 1994; Boller, 1995).

This expanded definition is used to include two large groups of inducers: biotic and a biotic elicitors. Elicitors, also known as stimulators of Natural Disease Resistance (NDR), have a broad spectrum of action on various diseases and promote, in some cases, tolerance to climatic stress (e.g. water stress) (Bray, 1997). With the advance of knowledge about elicitors, the idea that they could be

used as natural pesticides to protect plants against diseases progressively emerged. The first product developed in the 1980s, and claiming a mode of action type NRD, is an analogue of salicylic acid, acibenzolar-S-methyl (ASM), better known under the name of Benzothiadiazole (BTH) and marketed by Syngenta to the United States under the trade name Bion®.

The general scheme summarizing the main families of elicitors is shown in Figure 3. The biotic elicitors are divided into two categories: exogenous elicitors, which include molecules of various origins (oligosaccharide, glycoprotein, peptide or lipid), and endogenous elicitors represented by the released pectic fragments of the plant cell wall under the effect of microbial endopolygalacturonases. A biotic elicitors are very diverse (ultraviolet radiation, ozone, heavy metals, etc.).

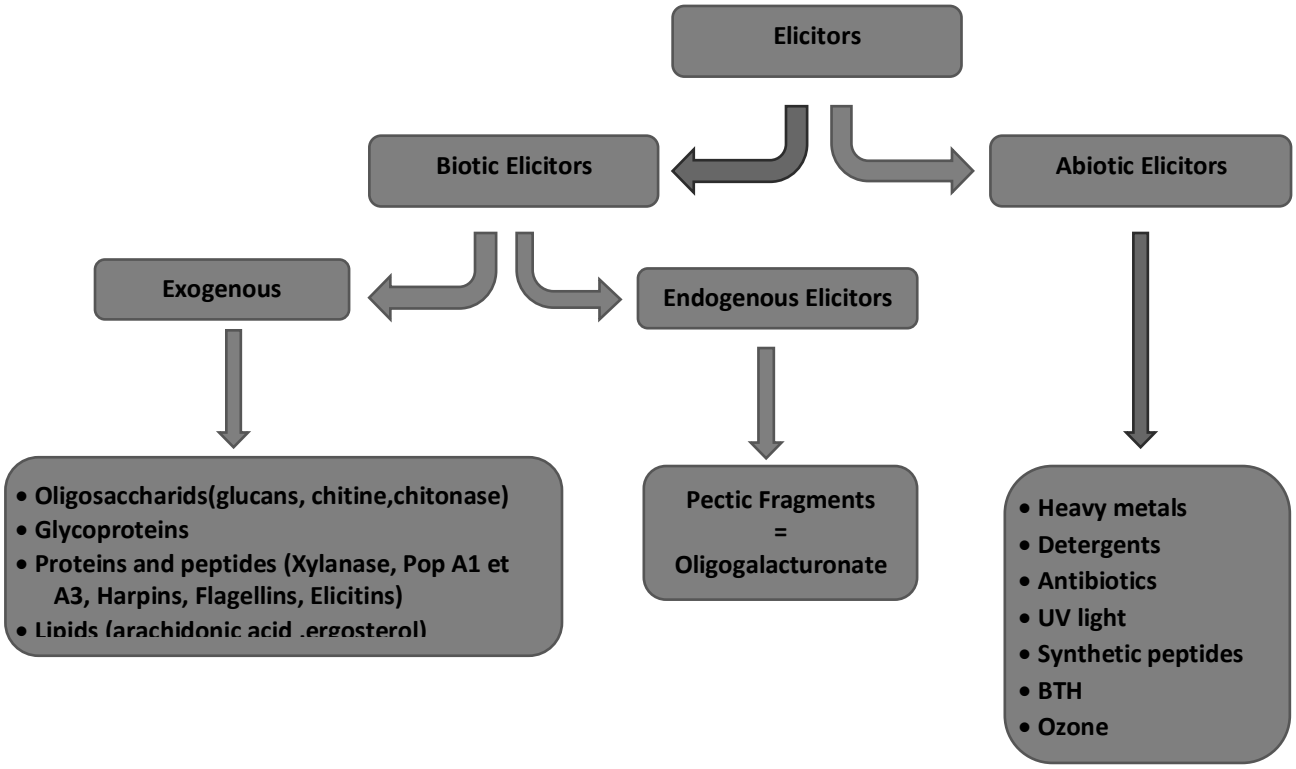

Figure 2. Principal families of general elicitors.

2.7.3 biotic elicitors:

2.7.3.1 Exogenous biotic elicitors

Exogenous biotic elicitors are many and varied. Although most of them are of fungal or bacterial origin, some elicitor molecules derive from viruses and even from phytophagous insects (Table1). Among the best characterized elicitors in insects we can cite the example of a fatty acid-amino acid complex produced by the caterpillar Manduca sexta (L.). This latter induces the synthesis of volatile compounds, which activate in turn defense plant genes and act indirectly on attracting natural predators of the caterpillar (Kessler and Baldwin, 2002).

Table 1. Examples of biotic elicitors of host plant resistance in horticultural products.

Species	Elicitor	Pathogen	Experiment type	Authors
Ananas comosus (pineapple)	*Penicillium funiculosum* (non-pathogenic)	*P. funiculosum*	Postharvest	Lim and Rohrbach (1980)
C. annum (bell pepper)	*Glomerella cingulata*	*B. cinerea*	Postharvest	Adikaram et al. (1988)
C. paradisi (grapefruit)	*Candida oleophila*	*P. digitatum*	Postharvest	Droby et al. (2002)
D. carota (carrot)	*B. cinerea*	*B. cinerea*	Postharvest	Harding and Heale (1980)
	B. cinerea and Sclerotinia sclerotiorum	As above	As above	Mercier and Arul (1993)
	B. cinerea	As above	As above	Mercier et al. (2000)
F. ananassa (strawberry)	*Aureobasidium pullulans*	*B. cinerea*	Field/postharvest and postharvest	Adikaram et al. (2002)
M. domestica (apple)	Messanger™ (harpin)	*P. expansum*	Postharvest	de Capdeville et al. (2003)
	Candida saitoana	*B. cinerea*	As above	El Ghaouth et al. (2003a)
Musa spp. (banana)	*Phyllosticta musarum*	*Colletotrichum musae*	Postharvest	Abayasekara et al. (1998)
P. americana (avocado)	*Colletotrichum magna* (non-pathogenic)	*C. gloeosporioides*	Postharvest	Prusky et al. (1994)

2.7.3.2 Endogenous biotic elicitors:

Endogenous elicitors are components of the intercellular signal transduction system of plants. In the late 1970s, when they had just discovered and described the existence of resistance elicitors oligosaccharides in the wall of some fungi, Albersheim 1965 and his collaborators found that pectic fragments of the cell wall itself produced a similar effect (Hahn *et al.*, 1989). These fragments, generated essentially by pectic compounds under the action of endopolygalacturonases (endoPGs) induce a cascade of events leading to the expression of the resistance instantly (Côté and Hahn, 1994; Vorwerk *et al.*, 2004). Unlike the high degree of structural specificity of fungal elicitors, the oligosaccharides plant inducers appear to be less defined since no precise structure has been identified (Aldington and Fry, 1993).

2.7.4 Abiotic elicitors:

Many physical and chemical compounds are effective elicitors. Examples include salts of heavy metals such as cadmium, silver and lead, detergents, antibiotics, synthetic peptides, salicylic acid derivatives such as benzol (1,2,3) - thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Kessmann *et al.*, 1994; Benhamou and Bélanger, 1998; Strange, 2006) and environmental stresses such as water stress

(Bray, 1997). Prolonged exposure to ultraviolet radiation or high doses of atmospheric ozone can also induce defense reactions in plants (Mercier *et al.*, 1993; Baier *et al.*, 2005; Charles *et al.*, 2008). Moreover, strengthening structural barriers with inlay of lignin and suberin is commonly observed in plants and fruits exposed to ultraviolet light response (Charles *et al.*, 2008). Ozone, in turn, interacts with components of the plasma membrane as ion channels and calcium channels, resulting in severe membrane disruption and immediate and massive production of Reactive Oxygen Species (ROS) and certain enzymes such as superoxide dismutase and peroxidases (Oksanen *et al.*, 2004). Indeed, ozone has been found to resemble fungal elicitors (Sandermann *et al.*, 1998). Ozone can easily be generated and applied, so that it is a convenient model elicitor that supplements existing preparations (intact pathogens, fungal elicitors or signaling substances). Among these, ozone and ethylene are the only model elicitors that can easily be removed at any time after application (Sandermann *et al.,* 1998).

2.7.5 Effects of ozone on plant defense response:

Plants have evolved systems capable of sensing and responding to environmental changes, including increases in ozone levels. The initial site of ozone reactions in plants is not yet completely understood. Indeed, attempts have been made to use the reactive properties of ozone to preserve the quality of fresh fruits and vegetables at certain concentrations but most of these attempts have focused on the antimicrobial effects of ozone. However, ozone may induce many physiological changes in horticultural products, including changes in proteins and enzymes related to plant defense mechanisms (Thalmair *et al.*, 1996; Baier *et al.*, 2005; Yaseen *et al.*, 2015). Several genes are expected to be involved in natural defense response in plants after ozone exposure (Sandermann *et al.*, 1998). We present here a summary about some genes expected to be sensitive to ozone exposure.

-CHI1: Chitinases are well-known antifungal proteins. They belong to the pathogenesis-related (PR) group of proteins responsible for Systemic Aquired Resistance (SAR) pathways. The CHI1 gene encodes a predicted polypeptide of 231 amino acids with a predicted molecular mass of 25.1 kDa and an Isoelectric Point (pI) of 9. 15. The CHI1 protein shares 60, 58, and 56% identity with the basic chitinase proteins of rice, grape and maize, respectively. Southern blot analysis indicates that CHI1 is present as a low-copy gene. RNA gel blot hybridizations

reveal that CHI1 gene expression is markedly induced by various treatments that induce fruit resistance against the green mold pathogen *P. digitatum*. These treatments include elicitation of fruit pathogen resistance by UV irradiation, hot water brushing, and application of *β* aminobutyric acid (BABA) and *Candida oleophila* antagonist yeast cells (Porat *et al.*, 2001).

-GNS1: *β*-1,3-endoglucanase belongs to Systemic Aquired Resistance (SAR) group of proteins and has been purified from Citrus sinensis (L) Osbeck cv. `Valencia' orange callus. Specific antibodies raised against the purified protein are used to screen Valencia' callus complemantery DNA (cDNA) expression libraries, and to isolate its corresponding cDNA, designated as GNS1. The GNS1 gene encodes a predicted polypeptide of 336 amino acids with a molecular mass of 37.3 kDa and a basic pI of 9.19, and shares 55–65% identity with several other plant β-1,3 endoglucanase proteins (Porat *et al.*, 2002a; Porat *et al.*, 2002b).

-PAL1: Phenylalanine ammonia-lyase (PAL) are involved in the defence of citrus fruit against biotic and abiotic stresses and have been studied for their Induced Systemic Resistance activities (Sanchez-Ballesta *et al.*, 2000; Sanchez *et al.*, 2000; Ballester *et al.*, 2006). In addition, induction of PAL activity has been reported in response to wounding (Ismail and Brown, 1979), UV light (Droby *et al.*, 1993b), gamma irradiation (Riov *et al.*, 1968), heat treatment (Martinez-Tellez and Lafuente, 1997) and ethylene (Riov *et al.*, 1969).

-POD1: Peroxidases are a variety of enzymes that catalyze the breakdown of H_2O_2 with the concomitant dependent oxidation of a wide variety of substrates. They are heme proteins involved

in numerous physiological roles in plant tissues, including lignin biosynthesis, indole-3-acetic acid degradation, wound healing and pathogen defense via Reactive Oxygen Species pathways (ROS). Several studies confirm that hydrogen peroxide is a signaling molecule that mediates responses to abiotic and biotic stresses in plants (Levine *et al.*, 1994; Vanlerberghe and McIntosh, 1996; Van Breusegem *et al.*, 2001; Neill *et al.*, 2002).

2.7.6 Gene Expression Profiling and Quantitation: Methods and Techniques

Researchers studying gene expression employ a wide range of molecular biology techniques. Gene expression analysis studies can be broadly divided into four

areas: RNA expression, promoter analysis, protein expression, and post-translational modification.

2.7.6.1 Promoter Analysis

2.7.6.1.1 Expression of reporter genes

Reporter genes (promoters) are genes that enable the detection or measurement of gene expression. They can be fused to regulatory sequences or genes of interest to report expression location or levels. Reporter genes include genes that code for fluorescent protein and enzymes that convert invisible substrates to luminescent or colored products. Promoter activity (transcription rate) is measured in vivo by introducing fusions of various promoter sequences with a gene encoding a product that can be readily measured to monitor activity levels (Ma, 2007).

2.7.6.1.2 In vitro transcription (nuclear run-on assays)

Transcription rates are measured by incubating isolated cell nuclei with labeled nucleotides, hybridizing the resultant product to a membrane (slot blot), and then exposing this to film or other imaging media (Murphy, 1993).

2.7.6.1.3 Gel shift assays

Also called electrophoretic mobility shift assays, they are used to study protein-DNA or protein-RNA interactions. DNA or RNA fragments that are tightly associated with proteins (such as transcription factors) migrate more slowly in an agarose or polyacrylamide gel (showing a positional shift). Identifying the associated sequences provides insight into gene regulation (Hellman and Fried, 2007).

2.7.6.1.4 Chromatin immunoprecipitation (ChIP)

Protein-binding regions of DNA can be identified in vivo. In living cells, DNA and proteins are chemically cross-linked, and the resulting complex is precipitated by antibody-coated beads (immunoprecipitation). Following protein digestion and DNA purification, the sequences of the precipitated DNA are determined (Carey *et al.*, 2009).

2.7.6.2 Protein Expression

2.7.6.2.1 Western blotting

Quantification of relative expression levels for specific proteins is accomplished by electrophoretically separating extracted cell proteins, transferring them to a membrane, and then probing the bound proteins with antibodies (targeted to antigens of interest) that

are subsequently detected using various chemistries or radiolabelling (Mahmood and Yang, 2012).

2.7.6.2.2 2-D Gel Electrophoresis

Protein expression profiling is achieved by separating a complex mixture of proteins in two dimensions and then staining to detect differences at the whole-proteome level (Rabilloud and Lelong, 2011).

2.7.6.2.3 Immunoassays

Proteins are quantitated in solution using antibodies that are bound to color-coded beads (as in the Bio-Plex® supension array system) or immobilized to a surface (ELISA), which is subsequently probed with an antibody suspension and is typically detected using a chromogenic or fluorogenic reporter (Lequin, 2005).

2.7.6.3 Post-translational Modification Analysis

2.7.6.4 Posttranslational Immunoassays

Levels of protein phosphorylation and other post-translational modifications are detected using antibodies that are specific for these adducts (BH Braakman and Umar, 2013).

2.7.6.5 Mass spectrometry

Proteins and their modifications are identified based on their mass (Parker *et al.*, 2009).

2.7.7 RNA Expression

2.7.7.1 Northern blotting

This relatively laborious technique was the first tool used to measure RNA levels. The steady-state levels of mRNA are directly quantitated by electrophoresis and transferred to a membrane. The membrane is then incubated with specific probes. The RNA-probe complexes can be detected using a variety of different chemistries or radionuclide labelling (Krumlauf, 1994).

2.7.7.2 DNA microarrays

An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done based on base pairing rules. An array experiment makes use of

common assay systems such as microplates or standard blotting membranes. The sample spot sizes are typically less than 200 microns in diameter and usually contain thousands of spots.

Thousands of spotted samples known as probes (with known identity) are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane). The spots can be DNA, cDNA, or oligonucleotides (Karakach *et al.*, 2010). These are used to determine complementary binding of the unknown sequences thus allowing parallel analysis for gene expression and gene discovery. An experiment with a single DNA chip can provide information on thousands of genes simultaneously. An orderly arrangement of the probes on the support is important as the location of each spot on the array is used for the identification of a gene (Watson *et al.*, 1998).

2.7.7.3 Reverse transcription quantitative PCR (RT-qPCR)

Reverse transcription quantitative PCR (RT-qPCR) distinguishes itself from other methods available for gene expression in terms of accuracy, sensitivity, and fast results. Because of this, the technology has established itself as the golden standard for medium throughput gene expression analysis. Due to its simplicity, inexperienced users can rapidly produce results; however, care should be taken when performing RT-qPCR as numerous critical quality issues may arise throughout the entire workflow influencing the accuracy of the results and the reliability of the conclusions. Intensive quality control is an important and necessary part that can be captured with the appropriate acronym PCR: plan/prepare, cycle and report (Figure 3) (Derveaux *et al.*, 2010). Steady-state levels of mRNA are quantitated by reverse transcription of the RNA to cDNA followed by quantitative PCR (qPCR) on the cDNA. The amount of each specific target is determined by measuring the increase in fluorescence signal from DNA-binding dyes or probes during successive rounds of enzyme-mediated amplification. This precise, versatile tool is used to investigate mutations (including insertions, deletions, and single-nucleotide polymorphisms (SNPs)), identify DNA modifications (such as methylation), confirm results from northern blotting or microarrays, and conduct gene expression profiling. Expression levels can be measured relatively to other genes (relative quantification) or against a standard (absolute quantification). Real-time PCR can be used to quantitate mRNA or

miRNA expression following conversion to cDNA or to quantitate genomic DNA directly in order to investigate transcriptional activity (Guénin *et al.*, 2009).

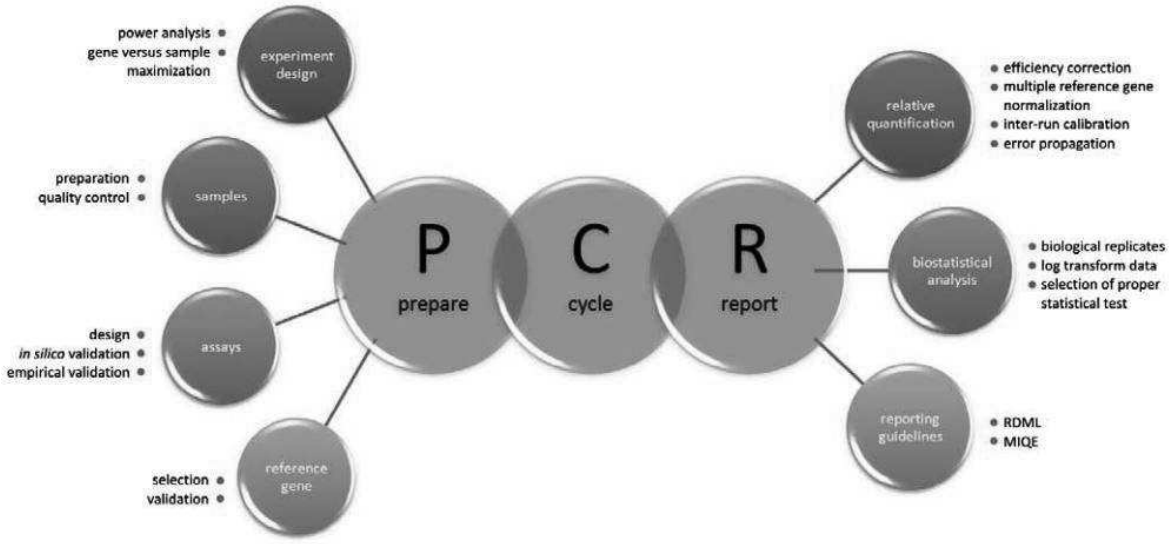

Figure 3. Quality control tools throughout the entire qPCR workflow (Derveaux et al., 2010)

In most gene expression studies, researchers are interested in determining the relative gene expression levels in test versus control samples. For example, a researcher might be interested in the expression of a particular gene in cancerous tissues versus normal tissues. This workflow, which is typical of RT-qPCR experiments, is summarized in Figure 4 (Volkov, 2012).

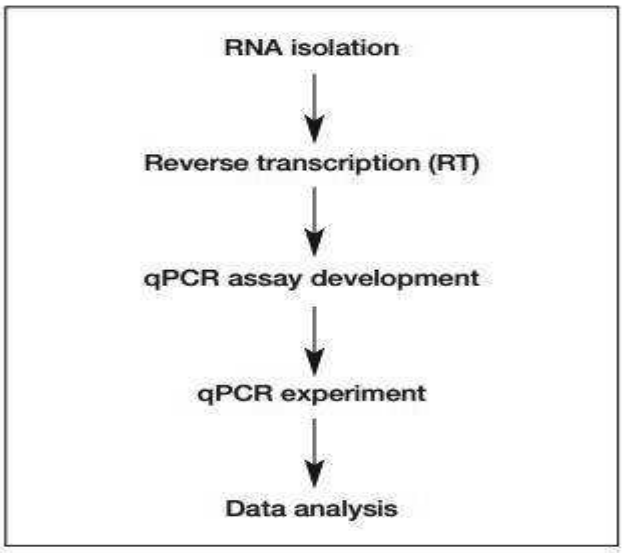

Figure 4. Gene expression analysis workflow

In our study, we mostly focused on this latter technique to perform a gene expression analysis. Thus, the key concepts of Quantitative or Real Time PCR are detailed in the next sections.

2.7.8 Key Concepts of Real-Time PCR:

2.7.9 What is Real-Time PCR?

In conventional PCR, the amplified product, or amplicon, is detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in "real time". Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal (Mackay, 2007). The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle (Dorak, 2007).

The main advantage of real-time PCR over conventional PCR is that real-time PCR allows you to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA) (Gherbawy and Voigt, 2010). Real-time PCR that is quantitative is also known as qPCR. In contrast, conventional PCR is at best semi-quantitative. Additionally, real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a closed-tube system, opportunities for contamination are reduced and the need for post-amplification manipulation is eliminated (Kostic *et al.*, 2009).

2.7.10 How does Real-Time PCR work?

In order to understand how real-time PCR works, we can examine a sample amplification plot (Figure 4). In this plot, the PCR cycle number is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to

the amount of amplified product in the tube, is shown on the y-axis. The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28–40 in Figure 5) (Hochstrat *et al.*, 2015).

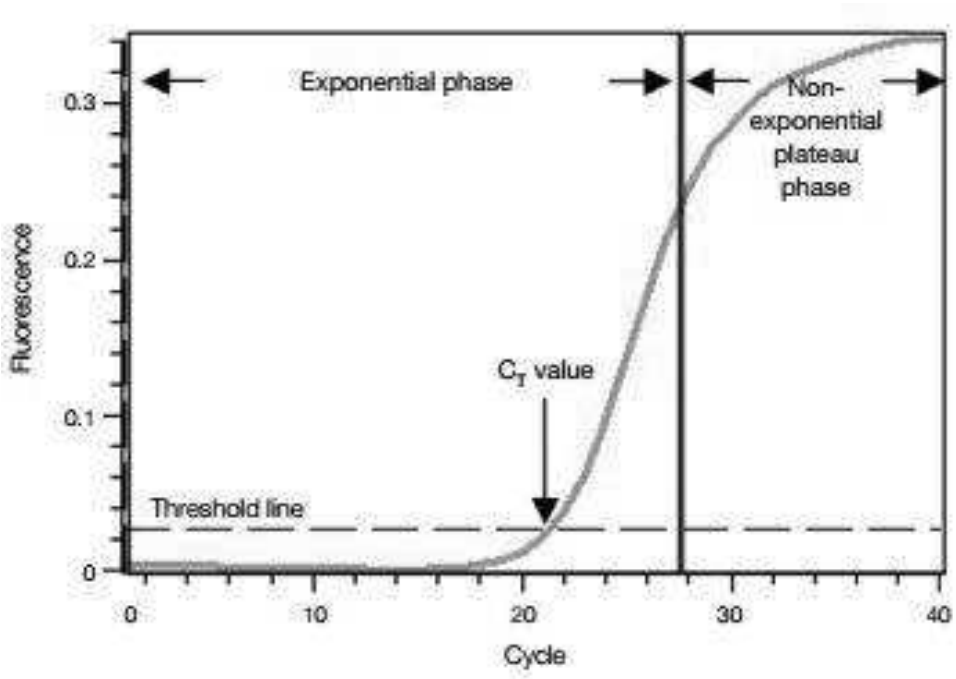

Figure 5. qPCR amplification plot (Hochstrat et al., 2015)

Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18 in Figure 27) even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or CT. Since the CT value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction (Conn, 2012).

The CT of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus,

the reaction will have a low, or early, CT. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, CT. This relationship forms the basis for the quantitative aspect of real-time PCR (Lorkowski and Cullen, 2006).

2.7.11 Gene expression analysis workflow.

2.7.11.1 RNA isolation

Sample Collection:

For quantifying gene expression, sample material should be as homogeneous as possible. If the tissue sample consists of many different cell types, pinpointing the expression pattern of the target gene may be difficult. If the sample is heterogeneous, we can use one of the many methods that are available for separating and isolating specific cell types. These methods include tissue dissection, needle biopsies, and laser capture microdissection. The collected cells can then be used to obtain the RNA samples (Sow *et al.*, 2009).

2.7.11.2 RNA Extraction:

Either total or poly (A+) RNA can be used for most real-time RT-qPCR applications. One critical consideration in working with RNA is to avoid RNases in the solutions, consumables, and labware. Ready-to-use solutions that are RNase-free can be purchased, or the solutions can be treated with diethyl pyrocarbonate (DEPC), and then autoclaved. RNases on can also be inactivated by DEPC treatment, or by baking it at 250°C for 3 hours (Bustin, 2002). RNase-free consumables are available for purchase from many commercial sources.

Prepared RNA samples may need DNase treatment to prevent potential amplification of any contaminating genomic DNA, which could lead to overestimation of the copy number of an mRNA. When starting material is limited, however, DNase treatment may be inadvisable, because the additional manipulation could result in loss of RNA. The amplification of potential contaminating genomic DNA can be precluded by designing transcript-specific primers, for example, primers that span or amplify across splice junctions (Hansen, 2002).

2.7.11.3 Analysing Nucleic Acid Quantity and Quality

RNA concentration can be qualitatively measured by comparing the relative fluorescence intensity of the RNA bands to the ones of known RNA standards. It can also be determined quantitatively by gel densitometry. This technique relies on a sophisticated equipment that uses a software to analyze an image of the gel. General information about RNA integrity can be obtained by observing the staining intensity of the major ribosomal RNA (rRNA) bands and any degradation products. A 28S/18SrRNA ratio of 2:1 is generally representative of good-quality RNA. Genomic

DNA contamination of RNA samples can be visualized in the sample as genomic DNA typically runs much slower through the gel matrix than RNA. On the other hand, RNA degradation resulting in the loss of the 28SrRNA band and an accumulation of degraded RNA near the bottom of the gel (Figure 6).

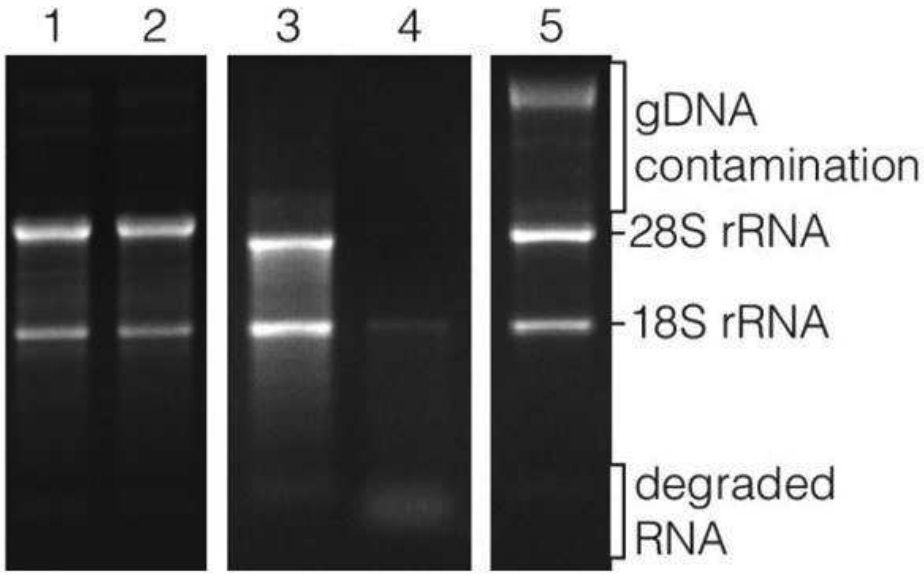

Figure 6. RNA analysis by agarose gel electrophoresis.

Accurate nucleic acid quantification is essential for gene expression analysis, especially if total RNA amounts are used to normalize target gene expression. RNA concentrations and purity are commonly determined by measuring the ratio of the UV absorbance at 260 nm and 280 nm. The overall sensitivity of this method is low, especially for relatively diluted samples, and it does not indicate the quality of the RNA (Clark, 2013).

2.7.11.4 cDNA Template Preparation (Reverse Transcription)

Two methods are available for quantification of gene expression by RT-qPCR: “two-step” RT-qPCR or “one-step” RT-qPCR. In both cases, RNA is reverse transcribed into cDNA, and the cDNA is then used as the template for quantitative PCR amplification. “One-step” and “two-step” refer to whether the reverse transcription and real-time PCR amplification are done in the same or separate tubes (Jain and Brar, 2013). In the two-step method, RNA is first transcribed into cDNA in a reaction using reverse transcriptase. An aliquot of the resulting cDNA can then be used as a template source for multiple qPCR reactions. In the one-step method, reverse transcription and qPCR are performed in the same tube. In two-step RT-qPCR, the RT step can be primed with oligo (dT) primers, random primers, a mixture of them, or gene-specific primers. One-step RT-PCR must be performed using gene-specific primers, and can be achieved either by using Thermusthermophilus (Tth) polymerase, a DNA polymerase with inherent RT activity, or by a two-enzyme system combining a reverse transcriptase with a thermostable DNA polymerase. Since Tth DNA polymerase is derived from a thermophilic bacterium, higher temperatures (> 60°C) can be used for the RT step, which can minimize secondary structure in high-%GC-content mRNAs (Dorak, 2007).

Accurate analysis of gene expression requires good reproducibility of reverse transcription reactions. The robustness of a reverse transcription is determined by the sensitivity, dynamic range, and specificity of the reverse transcriptase used. Reverse transcriptases from Moloney Murine Leukemia Virus (MMLV) and Avian Myeloblastoma Virus (AMV) are the most commonly used enzymes. When long or full-length cDNA transcripts are needed, MMLV reverse transcriptase and its derivatives are better choices than AMV reverse transcriptase due to their lower RNase H activity (van Pelt-Verkuil *et al.*, 2008). It has been observed, however, that RT reactions performed with RNase H– reverse transcriptases sometimes require subsequent RNase H treatment to effectively amplify certain templates and those at low concentration in the PCR.

Reverse transcriptase needs a primer to start the DNA synthesis. There are three alternative primer methods:

-Gene specific primers can be used to synthesize cDNA when the mRNA sequence is known. Primers like the ones used in PCR are used and only the fragment of interest is synthesized.

-Oligo(dT) primers consists of a number of thymine residues. These primers bind the 3´ poly(A) tails of the mRNA and cDNA synthesis starts at the end of the mRNA. It may be difficult to get transcripts of the entire 5´ end of the mRNA when oligo (dT) primers are used, but that depends on the length of the template.

-Random hexamers are six nucleotides put together randomly. These primers bind at several locations in the total RNA and cDNA synthesis starts from there.

2.7.12 qPCR Assay Development

A successful qPCR assay requires efficient and specific amplification of the product. Both primers and target sequence can affect amplification efficiency and specificity and thus the accuracy of qPCR assays (Derveaux *et al.*, 2010). Therefore, care must be taken when choosing a target sequence and designing primers. The use of PCR primers specifically designed and validated for qPCR assays with the target of interest is highly recommended (Hoebeeck *et al.*, 2007). Therefore, for a successful qPCR assay, these steps should be followed:

-Check the literature and databases for existing primers

-Choose a target sequence

-Design primers and probes

-Check primer specificity

-Assess primer and probe properties: melting temperature (Tm), secondary structure, and complementarity.

-Determine PCR product properties

-Validate the primers and/or probes and optimize the protocol

2.7.13 Designing Primers for a qPCR Assay

When designing primers, these guidelines should be followed (ThermoFisher, 2015):

-Design primers that have a GC content of 50–60%

-Strive for a Tm between 50 and 65°C

-Avoid secondary structure; adjust primer locations so they are located outside secondary structure in the target sequence

-Avoid repeats of Gs or Cs longer than 3 bases

-Place Gs and Cs on the ends of the primers

-Check the sequences of forward and reverse primers to ensure no 3' complementarity

(avoid primer-dimer formation).

-Verify specificity using tools such as the Basic Local Alignment Search Tool (BLAST).

2.7.14 qPCR Assay Validation and Optimization

To achieve accurate template quantification in a qPCR assay, each reaction must efficiently amplify a single product, and amplification efficiency must be independent of template concentration and the amplification of other templates. Therefore, we should validate our qPCR assay to verify that these conditions hold (Bustin *et al.*, 2010).

2.7.14.1 Determining Reaction Efficiency

A common method for validating qPCR assays involves the construction of a standard curve, enabling the determination of the efficiency, linear dynamic range, and reproducibility of a qPCR assay. The efficiency of the assay should be 90–100% and the quantification cycle (CT) values of the replicates should all be similar (Taylor *et al.*, 2010).

2.7.14.2 qPCR Assay Specificity Verification

Melt curve analysis can be used to identify different PCR products, including nonspecific products and primer-dimers when the fluorescence of the reporter chemistry depends on the presence of double stranded DNA (dsDNA), as with SYBR® Green dye. This property is valuable because the presence of secondary nonspecific products and primer-dimers can severely reduce the amplification efficiency and, ultimately, the accuracy of the qPCR assay (Bustin *et al.*, 2009). Primer-dimers can also limit the dynamic range of the desired standard curve due to competition for reaction components during amplification. After completion of

the amplification reaction, a melt curve is generated by increasing the temperature in small increments and monitoring the fluorescence signal at each step. As dsDNA in the reaction is denatured, the fluorescence decreases rapidly and significantly. A plot of the negative first derivative of the fluorescence versus temperature (–d(RFU)/dT) vs. T, the rate of change of fluorescence intensity) displays distinct peaks corresponding to the Tm of each product (BioRad, 2016). The melt peak distinguishes specific products from other products that melt at different temperatures, such as primer-dimers. Because of their small size, primer-dimers usually melt at lower temperatures than the desired product, whereas nonspecific amplification can result in PCR products that melt at temperatures above or below that of the desired product Figure 7 (Bustin *et al.*, 2009).

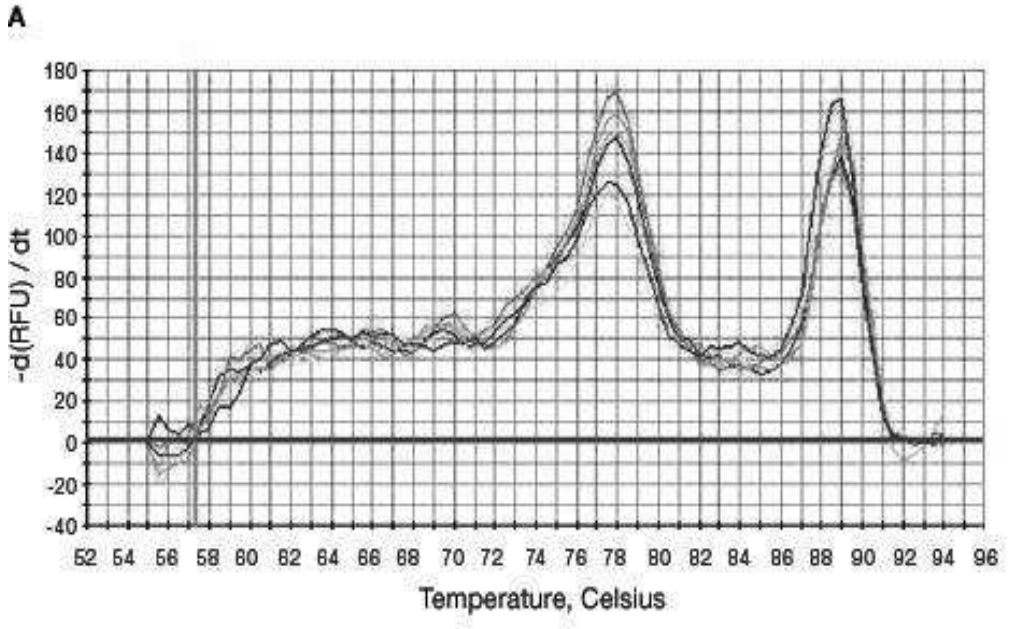

Figure 7. Melt curve analysis of products from a SYBR® Green assay. A, the negative first derivative of the change in fluorescence plotted as a function of temperature. The two peaks indicate the presence of two PCR products (BioRad, 2016).

2.7.14.3 Annealing Temperature Optimization

The annealing temperature of a qPCR assay is one of the most critical parameters for reaction specificity. Setting the annealing temperature too low may lead to amplification of nonspecific PCR products. Conversely, setting the annealing temperature too high may reduce the yield of a desired PCR product (Taylor *et al*., 2010). Even after calculating the Tm°C of a primer, you may need to determine the annealing temperature empirically by repeating a reaction at many different

temperatures. Similar time-consuming tests may also be required to optimize the denaturation temperature (Hoorfar *et al.*, 2004).

The optimal annealing temperature for an assay can easily be determined using qPCR instruments that have a thermal gradient feature. The gradient feature allows you to test a range of temperatures simultaneously, so the annealing temperature can be optimized in a single experiment. To find the optimal annealing temperature for your qPCR assay, test a range of temperatures above and below the calculated Tm of the primers. The optimal annealing temperature is the one that results in the lowest CT with no nonspecific amplification Figure 8 (McPherson and Møller, 2007).

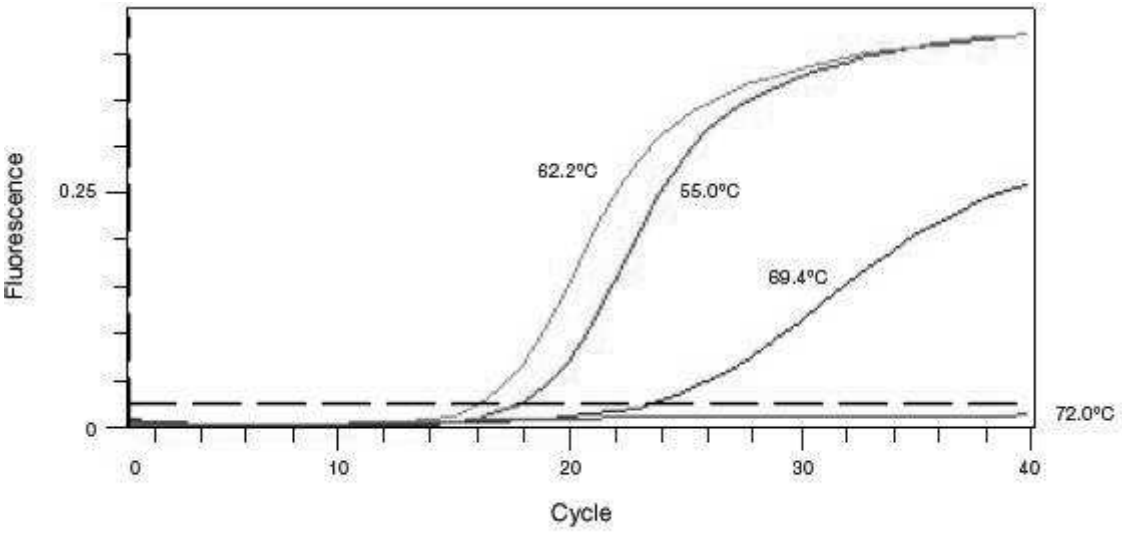

Figure 8. Annealing temperature optimization. An annealing temperature gradient from 55 to 72°C was performed. The 62.2°C reaction gave the lowest CT value and was selected as the annealing temperature for this assay (BioRad, 2016).

2.7.15 Analyzing Real-Time PCR Data:

The analysis methods that treat the Real-Time PCR data are commonly known as absolute quantification and relative quantification, respectively. The choice is based on both the experimental goals and available resources (Dorak, 2007). Absolute quantification is achieved by comparing the CT (threshold cycle) values of the test samples to a standard curve. The result of the analysis is the quantity of the nucleic acid (copy number, µg) per given amount of sample (per cell, per µg of total RNA). In relative quantification, the analysis result is a ratio: the relative amount (fold difference) of a target nucleic acid for equivalent amounts of tested and control sample A vs. B. Both cases need to address the question of what the

"amount of sample" is, and in relative quantification, to ensure that equivalent amounts of samples are compared (Dorak, 2007).

For both absolute and relative quantification methods, quantities obtained from a qPCR experiment must be normalized in such a way that the data become biologically meaningful. This is done through the use of normalizers. In absolute quantification, normalizers are used to adjust or standardize the obtained target quantity to the unit amount of sample (Filion, 2012). In relative quantification, normalizers are used to ensure that the target quantities from equivalent amounts of samples are compared (Dorak, 2007). Although the normalizer for either quantification method can be the number of cells used for template preparation, µg of nucleic acid used as PCR template, or the expression level of a reference gene, the first two are more commonly used for absolute quantification, whereas the third is typically used for relative quantification (Dorak, 2007). A reference gene is one whose expression level is constant across all test samples and whose expression is not affected by the experimental treatment under study (Lorkowski and Cullen, 2006). The use of a reference gene is advantageous in cases where the precise quantification of input RNA amount is not possible.

This can occur when only a small amount of starting template is available (Lorkowski and Cullen, 2006). Many different methods are available for performing both absolute and relative quantification

2.7.16 Absolute Quantification

In absolute quantification, the quantity (e.g., copy number or unit mass) of the unknown sample is interpolated from a range of standards of known quantity. To construct a standard curve, a template with known concentration is required. Dilution of this template is then performed and these dilutions serve as the standards. The unknown test samples are assayed with the standards in the same experimental run.

The standard curve constructed from the diluted standard template can then be used to determine the target quantity in the unknown sample by interpolation, similarly to using molecular size standards to determine the molecular size of an unknown DNA band on an agarose gel (Bustin, 2000). Common real-time PCR applications that employ absolute quantification include chromosomes or gene copy number determination and viral load determination. This method of

quantification is conceptually simple and the mathematical analysis is easy to perform. However, the method requires a reliable source of template of known concentration, and standards must be amplified in parallel with the samples every time the experiment is performed (Pfaffl, 2004).

2.7.17 Relative Quantification

2.7.17.1 Relative Quantification Normalized Against Unit Mass

The advantages of using a unit mass (such as cell number or µg of nucleic acid) rather than a reference gene as the normalizer are that the experimental design is conceptually simple and the mathematical treatment is straightforward (Dorak, 2007).

The method requires accurate quantification of the starting material, regardless of whether cell number or µg of nucleic acid is used as the normalizer. When comparing multiple samples using relative quantification, one sample is usually chosen as the calibrator (sometimes known as the control sample), and the expression of the target gene in all other samples is expressed as an increase or decrease relative to the calibrator. Customarily, the untreated or baseline sample is chosen as the calibrator (Pfaffl, 2004). The CT values for the test sample (treated sample) and the calibrator sample (non-treated sample) are then used to calculate the ratio between the two by the following equation:

Ratio (test/calibrator) = ECT (calibrator) – CT (test)

E is the efficiency of the reaction. If we assume that the assay has perfect amplification efficiency (that is, the template is doubled in each amplification cycle), then the equation above becomes:

Ratio (test/calibrator) = ECT (calibrator) – CT (test) Or

Ratio (test/calibrator) = 2 ΔCT (where: ΔCT = CT(calibrator) – CT(test))

2.7.17.2 Relative Quantification Normalized to a Reference Gene

The advantage of using a reference gene (such as GAPDH, β-actin, etc.) rather than unit mass as a normalizer is that this method circumvents the need for accurate quantification and loading of the starting material (Vandesompele *et al.*, 2002). This is especially convenient when performing relative gene expression experiments where starting material is frequently limited. The drawback is that this method requires the availability of a known reference gene or genes with constant

expression in all samples tested and whose expression is not changed by the treatment under study (Pfaffl, 2004; Vorwerk *et al*., 2004; Dorak, 2007). The identification of such a reference gene is not trivial, and recently it has been proposed that in most cases, the use of multiple reference genes may be necessary for accurate quantification (Vandesompele *et al*., 2002). To determine the relative expression of a target gene in the test sample and calibrator sample using reference gene(s) as the normalizer, the expression levels of both the target and the reference genes need to be determined using RT-qPCR. In short, we need to determine the CT values. After the CT values are measured, different methods can be used to determine the expression level of the target gene

in the test sample relative to the calibrator sample. In the following sections, we present three methods for relative quantification using a reference gene:

The Livak method, also known as the 2–ΔΔCT method:

The 2–ΔΔCT method for relative gene expression analysis is widely used and easy to perform. This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other. Before using the 2–ΔΔCT method, it is essential to verify theassumptions by determining the amplification efficiencies of the target and the reference genes (Schmittgen and Livak, 2008). Then, we have to normalize the CT of the target gene to that of the reference (ref) gene, for both the test sample and the calibrator sample:

ΔCT(test) = CT (target, test) – CT (ref, test)

Δ CT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)

After that, we have to normalize the Δ CT of the test sample to the Δ CT of the calibrator:

ΔΔ CT = Δ CT (test) – Δ CT (calibrator)

Finally, we have to calculate the expression ratio:

2–ΔΔCT = Normalized expression ratio

The result obtained is the fold increase (or decrease) of the target gene in the test sample relative to the calibrator sample. It is then normalized to the expression of a reference gene. Normalizing the expression of the target gene to that of the

reference gene compensates for any difference in the amount of sample tissue (Schmittgen and Livak, 2008).

The ∆CT method using a reference gene:

The ∆ CT method using a reference gene is a variation of the Livak method that is simpler to perform and gives essentially the same results. In contrast to the ∆CT values obtained in the Section (Relative Quantification Normalized Against Unit Mass), this method uses the difference between reference and target CT values for each sample.

The expression level of the reference gene is taken into account. The key difference in the results is that the expression value of the calibrator sample is not 1.0 (Schmittgen and Livak, 2008). If the resulting expression values obtained in this method are divided by the expression value of a chosen calibrator, the results of this calculation are exactly the same as those obtained with the

2–∆∆ CT method:

Ratio (reference/target) = 2 CT (reference) – CT (target)

2.7.18 The Pfaffl method:

The Pfaffl Method or the 2–∆∆CT method for calculating relative gene expression is only valid when the amplification efficiencies of the target and reference genes are similar. If the amplification efficiencies of the two amplicons are not the same, an alternative formula must be used to determine the relative expression of the target gene in different samples (Pfaffl, 2001). To determine the expression ratio between the sample and calibrator, the following formula should be used:

Ratio = (E target) ∆CT, target (calibrator – test)/(Eref) ∆ CT, ref (calibrator – test)

In the above equation, Etarget and Eref are the amplification efficiencies of the target and reference genes, respectively. ∆CT, target (calibrator – test) = CT of the target gene in the calibrator minus the CT of the target gene in the test sample, and ∆CT, ref (calibrator – test) is the CT of the reference gene in the calibrator minus the CT of the reference gene in the test sample. The above equation assumes that each gene (target and reference) has the same amplification efficiency in test samples and calibrator samples, but it is not necessary that the target and reference genes have the same amplification efficiency (Pfaffl et al.,

2002). The 2–ΔΔ CT method and the Pfaffl method are closely related; in fact, the 2–ΔΔ CT method is simply a special case of the Pfaffl method where Etarget = Eref = 2. If we substitute Etarget and Eref with 2, then the Pfaffl method simplifies as follows:

Ratio = 2ΔCT, target (calibrator – test) / 2ΔCT, ref (calibrator – test)

= 2–[(CT,target (test)–CT,target(calibrator)]–[(CT, ref (test) – CT, ref (calibrator)]

= 2–ΔΔ CT

3 Chapter 3- The effect of ozone and EW on microorganism population and on the quality and shelf life of stored orange fruits.

3.1 Material and Methods

3.1.1 Plant material and ozone and electrolyzed water treatment

3.1.2 Orange Fruits

Orange fruits cv "navel" used in this study, were harvested from commercial citrus orchard located in Apulia region (Ginosa Marina–TA) and provided to postharvest conservation at 4 - 5°C for 28 days; the experiment was carried out in (Palagiano – Taranto) packinghouse. Oranges were harvested at the end of January 2014 after ripening.

3.1.2.1 Inoculation

P. italicum was previously isolated from rotted fruits and unambiguously identified by morphological examination. The fungus was grown on potato dextrose agar (PDA) and incubated at 23 °C for 7 days. Spore suspension was prepared by flooding 1 week old culture with sterile distilled water containing 0,01% of Tween 20, Then 100µl were transferred to tubes containing 900 µl of sterile distilled water. Spores (conidia) count was done with a Thoma-hemocytometer chamber and adjusted to 2 X10^6conidia/mL (25 × 10^3 conidia / 20µl).

Electrolyzed water was obtained by the electrolysis process of Potassium chloride solution in an electrolysis chamber (EVA 100 DeNora Srl), where non-selective membranes are separating the anode and cathode. Water collected at the anode has unique oxidation properties due to its content of hypochlorous acid (HOCl) and to acidic pH, ozone was generated by a Corona discharge ozone generator (Air Met) MET srl. The device was installed and activated 3 hours twice per day. Ozone concentration was set at 0.3±0.05 and 0.1±0.05 µL xL^{-1} during night and day time respectively.

3.1.3 Measuring parameters

3.1.3.1 Microorganism population on fruits surface

The effect of ozone and/or electrolyzed water was determined by the quantification of fungi on fruits surface after ozone treatment according to mentioned times. One kilogram of fruits from each replicate were shacked at 270 rpm for 1hr, in the presence of 1000ml of sterile distilled water, the quantity of microorganisms were

detected using plate dilution method on selective media NYDA for yeasts and fungi adding; the selective media contained Nutrient broth (8g.L^{-1}), Yeast extract (5g.L^{-1}), D-glucose (10g.L^{-1}), Agar (18g.L^{-1}), Streptomycin sulphate (250mg) and Ampicillin (250mg), and selective media for bacteria NYDA and the addition of Cycloheximide (150mg.L^{-1}). After inoculation with a serial dilution of water suspension on the selective medium, Petri dishes were incubated at 24°C for 3 days and the number of colony forming units (CFUs) of fungi on the fruits surface was determined.

3.1.3.2 Sugar content

Ten fruits from each replicate were used for measuring sugar content directly after each sampling by using a Refractometer Brix Series (REF 103 -RFE113). Sugar content is measured by Brix degrees (°Bx). A solution containing 1% (w/w) of sucrose measures one °Bx. The refractometer determines the refractive index (R.I) of a solution by measuring the critical angle of refraction (Figure 9). The Light from a light source is directed against the

Interface between a prism and the solution. The light rays meet this surface at different angles. The reflected rays form an image (ACB), where (C) is the position of the critical angle ray. The rays at (A) are totally reflected at the interface, the rays at (B) are partially reflected and partially refracted into the process solution. In this way, the optical image is divided into a light area (A) and a dark area (B). The position of the borderline (C) between the areas shows the value of the critical angle and thus of the refractive index of the process solution. The refractive index normally increases with increasing concentration.

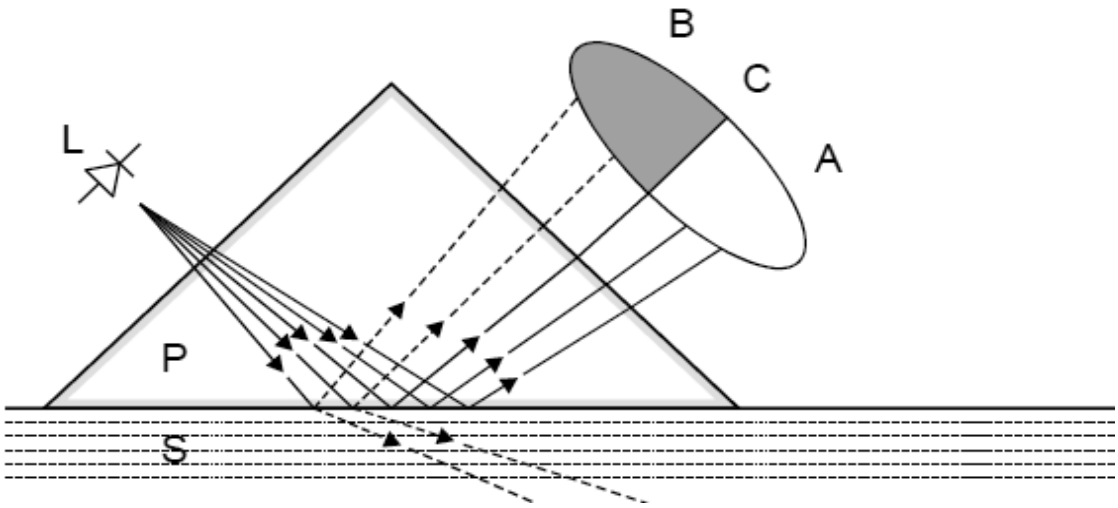

Figure 9. Refractometer scheme were (C) is the position of the critical angle ray. A Represents the rays at (A) totally reflected at the interface, (B) the rays partially reflected

3.1.3.3 Fruit firmness

Ten fruits from each replicate were used for measuring hardness directly after each sampling by using by a penetrometer (PCE-FM200). The penetrometer measures the amount of force (as gram per square centimetres) needed to penetrate the fruit through applying compression on middle side area of unpeeled fruits. It provides a quick and easy-to-use method of determining the ripeness of produce. It can be used to determine if produce is acceptable for consumption. Additionally, it allows the user to monitor shelf life as it monitors the ripening process and softening process in storage.

3.1.3.4 Shelf life

Shelf life was estimated by calculating McKinney index. Four Boxes of each treatment were transferred to 23°C chamber in order to monitor rot progress. Shelf life estimation was stopped when the control fruits were completely rotted. McKinney index was calculated by using this formula: Mi = [Σ (d × f) / (Tn × D)] × 100 Where d: Degree of infection.

f: Frequency of degree of infection.

Tn: Total number of examined fruits (healthy and diseased).

D: Highest degree of disease intensity occurring on the empirical scale:

Citrus rot scale: 0 = fruit without rots; 1 =1-20 % of decayed fruit surface; 2 = 21-40 % of decayed fruit surface; 3 = 41-60 % of decayed fruit surface; 4 =61-80 % of

decayed fruit surface; 5 = 81-100 % of decayed fruit surface. Lower McKinney index indicates higher storage tolerance and longer shelf life.

3.2 Experiment A: washing by electrolyzed water and ozonized water 2 ppm then storage in ozone enriched atmosphere

The aim of this experiment is evaluate citrus quality parameter after washing by using different concentration of electrolyzed water before storage in ozone or in conventional atmosphere chambers.

Orange fruits where wounded in four cardinal positions of fruit centre and inoculated, using a micropipette, with 20 µL of a suspension containing 2×10^6 spores/mL (25×10^3conidia / 20µl) of *P. italicum* (Figure 10).

Figure 10. The process of fruit inoculation

All fruits were divided into two groups (144 fruits per each); the first group was inoculated with *P. italicum* and the other one non-inoculated. Washing operation required 15 minutes and was conducted before storage using ozonized water 2 ppm and washed by 500ppm of active chlorine (EW 15%) and normal water as control, (A, B and C) respectively (Figure 11).

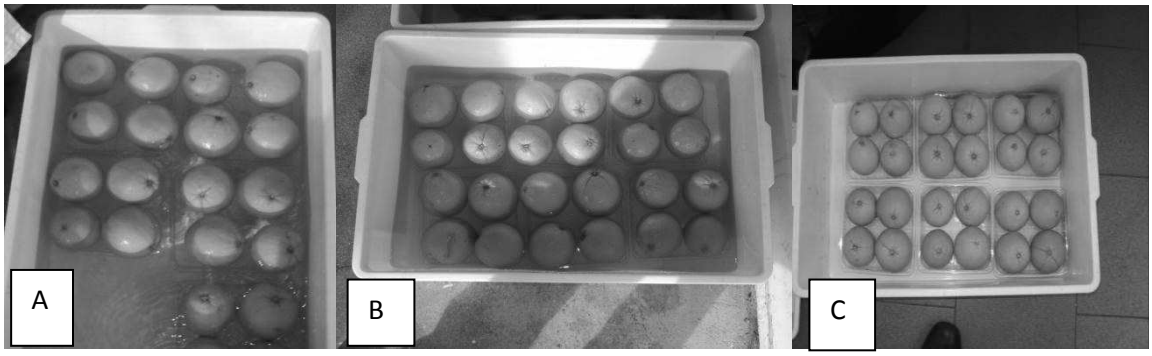

Figure 11. Citrus fruit cv «naval» immersed for 15 minutes using ozonized water 2 ppm A, or electrolyzed water 15% B, and normal water as control.

Trials were arranged in a completely randomized block design with four blocks and four fruits/blocks for each treatment with three different types of conservation were used: Conventional refrigerated (CRO) storage with ozone, passive refrigerated (PRO) storage with ozone and Conventional storage without ozone as control (Ctrl), (A, B and C) respectively (Figure 12).

Figure 12. Washed fruits ready for conservation in conventional refrigerated with ozone A, passive refrigerated with ozone B, and conventional storage without ozone as control C.

Conventional cold store chamber was used to store the fruit at 5°C and 95% relative humidity (RH). While in PRS, Low temperature (5°C) was transferred throw store walls in passive cold refrigeration (Zarling and Burdick, 1981). Storage continued for 28 days in all treatments. Control fruits were stored in the same condition of treated fruits, keeping temperature, RH and storage period as same as treatments. Sampling was carried out each ten days by choosing four fruits (1/each block) randomly for each treatment. First sampling was implemented before storage at time zero (T0) and after 7, 14, 21, 28 days from the treatments (T1, T2, T3, T4 respectively).

3.2.1 Statistical analyses

The data were analyzed using a one-way ANOVA with Statistica 7.0 fron StatSoft software for Windows (version 7.0). A significance level of 0.05 was employed for

data analysis. Treatments were arranged in a randomized complete block design, with similar results combined for statistical analysis. Means were separated according to Duncan's Multiple Range Test (DMRT). A significance level of 0.05 was employed for this analysis. Data from multi variables were assayed using Factorial ANOVA test.

3.2.2 Results of experiment A

The detected parameter (hardness, sugar content and colony forming units) of orange fruits washed by 500ppm of active chlorine (EW 15%), ozonized water 2 ppm and stored in ozone chamber were evaluated using factorial ANOVA test.

3.2.2.1 Colony forming units CFU

The amount of CFUs was evaluated by using factorial ANOVA test immediately after the treatment (T0), after 7 days (T1), after 14 days (T2), after 21 days (T3) and after 28 days (T4). Results achieved through different sampling time showed that ozonized water and EW had both a significant reduction on fungal contamination. In particular, in case of ozonized water treatment, the lowest CFU amount was obtained after 14 days, while in case of EW a significant control effect on fungal contamination has been approved until the end of the treatment after 28 days. In comparison control, fruits present a t significantly higher number of colonies (Figure 13).

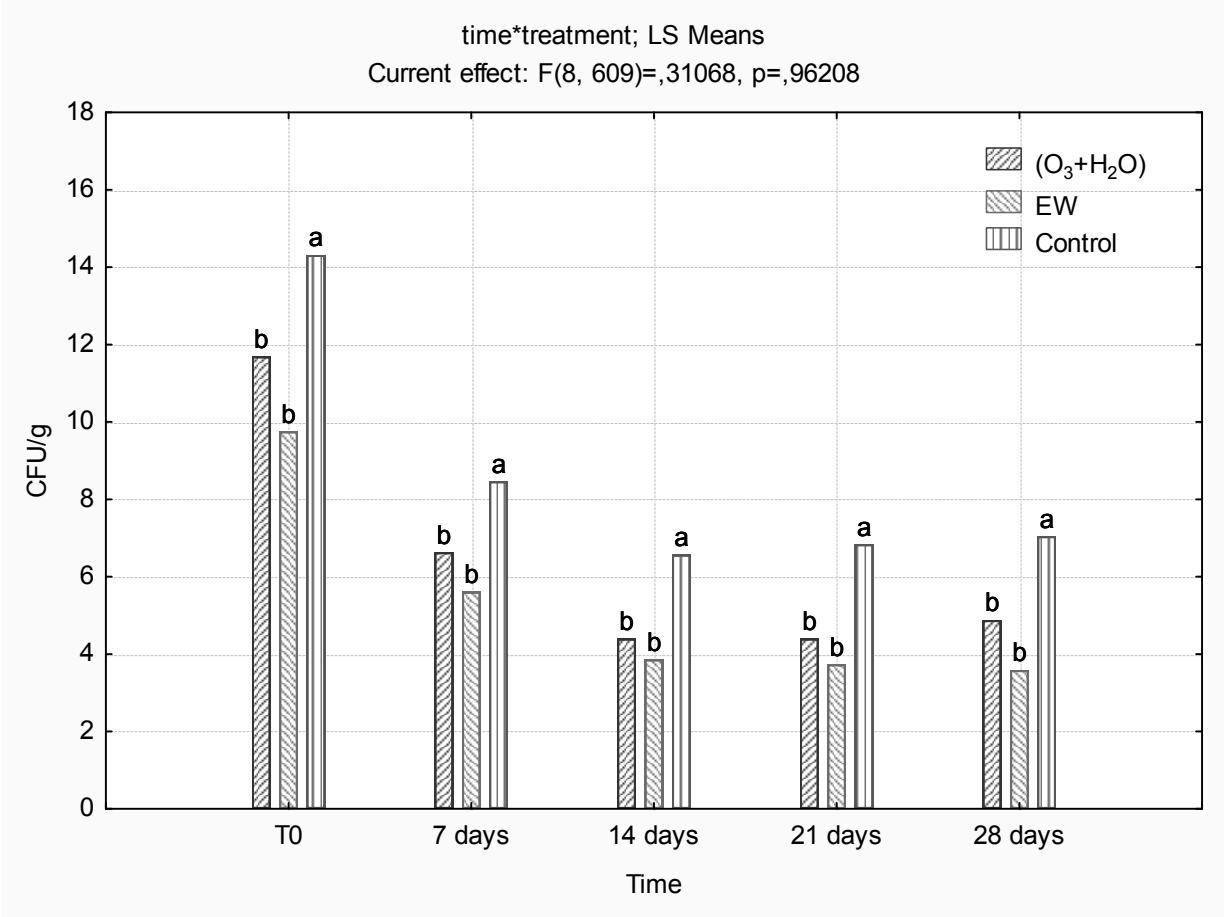

Figure 13. Colony Forming Units (CFUs) present on orange fruits treated with Ozone (O_3) in water or electrolyzed water (EW) and normal water after different storage periods at 4°C with different methods. Statistical analyses were carried out separately for each sampling time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (p≤ 0.05).

Un-inoculated and inoculated fruits treated with EW and stored in passive refrigerator with O_3 had lowest Microorganism's population followed by inoculated fruits treated with EW and stored in conventional refrigerator with O_3. Inoculated fruits in conventional storage had the highest severity and both Inoculated and un-inoculated fruits in conventional storage without O_3 are strongly affected by pathogens (Table 2).

Table 2. Colony Forming Units (CFUs) on orange inoculated and un-inoculated fruit after different storage periods at 4°C with different methods, and stored in passive refrigeration (PRS) or in conventional refrigeration (Conv.). EW= Electrolyzed Water; O_3=Ozone in water; control T1= after 7 days storage; T2= after 14 days storage; T3= after 21 days storage; T4= after 28 days storage. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (p=0.05).

Treatment/Conservation	CFUs											
	T1			T2			T3			T4		
	EW	O_3	Control	EW	O_3	Control	EW	O_3	Control	EW	O_3	Control
(Conv+O_3) inoculated	2.9^{b}	7^{c}	12.1^{d}	2.1^{b}	4^{c}	9.3^{d}	2.3^{b}	3.8^{c}	9.3^{d}	2.1^{b}	3.9^{c}	10.1^{d}
(Conv+O_3) un-inoculated	3.6^{b}	4^{c}	6.3^{d}	1.3^{b}	2^{c}	3.9^{d}	1.1^{b}	2^{c}	4^{d}	1.2^{b}	2.4^{c}	4^{d}
(PRS+O_3) inoculated	3^{b}	6^{c}	7.6^{d}	1.3^{b}	3^{c}	7.8^{d}	1.4^{b}	2.6^{c}	7.9^{d}	1.3^{b}	2.3^{c}	8.6^{d}
(PRS+O_3) un-inoculated	0.8^{a}	4^{c}	5.4^{d}	1.5^{a}	2^{c}	4.8^{d}	0.8^{a}	2.1^{c}	4.9^{d}	0.6^{a}	1.9^{c}	5^{d}
(Conv) inoculated	13^{d}	11^{d}	12^{d}	10^{d}	12^{d}	8.9^{d}	10^{d}	13^{d}	9^{d}	10^{d}	14^{d}	9^{d}
(Conv) un-inoculated	7.3^{d}	6^{d}	6.3^{d}	6^{d}	4^{d}	4.8^{d}	6^{d}	4.4^{d}	6.3^{d}	6^{d}	5.5^{d}	6.4^{d}

Orange fruits washed with EW and conserved in passive refrigeration (PRS) showed a clear reduction in CFU/g, this result was associated with an increase of fruit hardness; in particular, and the effect was statistically evident in un-inoculated fruits, followed by hardness values obtained from fruit treated with ozonized water and conserved in PRS in ozone enriched atmosphere (Table 3).

3.2.2.2 Hardness

Table 3. Orange fruit hardness measured in inoculated and un-inoculated fruit after different storage periods T1= after 7 days' storage; T2= after 14 days' storage; T3= after 21 days storage; T4= after 28 days storage; at 4°C with different methods, and stored in passive refrigeration (PRS) or in conventional refrigeration (Conv.). EW= Electrolyzed Water; O_3= Ozone in water; control. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (p=0.05).

Treatment/Conservation	Hardness											
	T1			T2			T3			T4		
	EW	O_3	Control	EW	O_3	Control	EW	O_3	Control	EW	O_3	Control
(Conv+O_3) inoculated	2.2^{b}	2^{b}	2.0^{b}	2.2^{b}	2^{b}	2.0^{b}	2.2^{b}	2^{b}	2.0^{b}	2.2^{b}	2^{b}	2.0^{b}
(Conv+O_3) un-inoculated	2.1^{b}	2^{b}	1.8^{b}	2.1^{b}	2^{b}	1.8^{b}	2.1^{b}	2^{b}	1.9^{b}	2.1^{b}	2^{b}	1.9^{b}
(PRS+O_3) inoculated	2.4^{b}	2^{b}	2.1^{b}	2.5^{b}	2^{b}	2.2^{b}	2.7^{a}	2^{b}	2.6^{a}	2.8^{a}	2^{b}	2.2^{b}
(PRS+O_3) un-inoculated	2.9^{a}	2.8^{a}	2.1^{b}	2.8^{a}	3^{a}	2.1^{b}	2.9^{a}	2^{b}	2.1^{b}	3.1^{a}	3^{a}	2.1^{b}
(Conv) inoculated	1.8^{c}	2^{c}	1.8^{c}	1.8^{c}	2^{c}	1.8^{c}	1.9^{c}	2^{c}	1.6^{c}	1.9^{c}	2^{c}	1.6^{c}
(Conv) un-inoculated	2.1^{c}	2^{c}	1.7^{c}	2^{c}	2^{c}	1.7^{c}	2^{c}	2^{c}	1.7^{c}	2^{c}	2^{c}	1.7^{c}

The important observation was that EW treatment not only maintain the firmness, but also induced fruit hardness over time during the experiment. Similar effect was obtained by washing the fruits with ozonized water in T1 and in T2, but the effect was not long lasting as in the case of EW, in the same time, was significantly higher than the control (Figure 14).

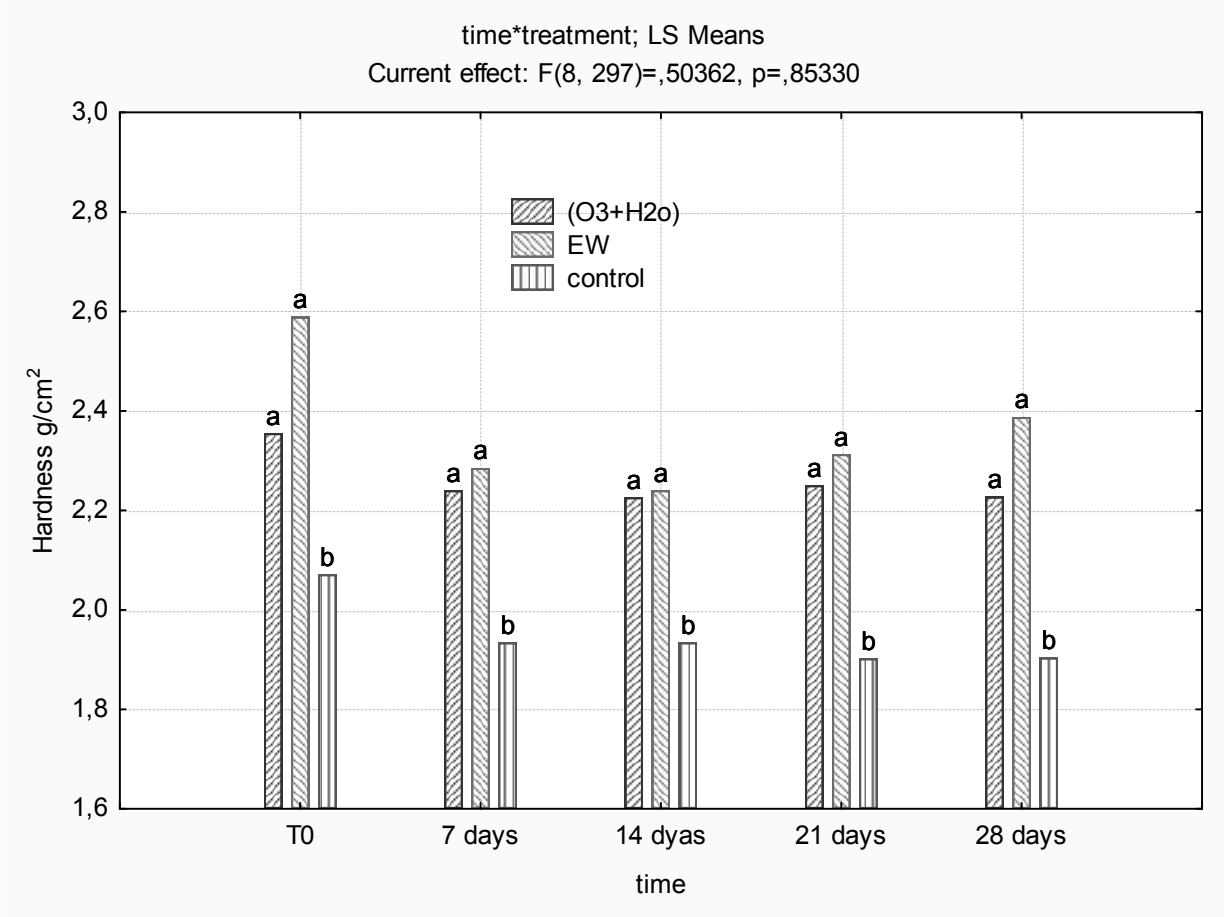

Figure 14. Effect of ozone (O_3) and/or electrolyzed water (EW) on orange fruits hardness, normal tap water was used as control. Statistical analyses were carried out separately for each sampling time. Evaluated after different storage periods (T0, 7, 14, 21 and 28 days) at 4°C. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P=0.05).

3.2.2.3 Sugar content

Both EW and ozonized water treatment led to a better maintenance of sugar content compared to the untreated fruits during the entire storage period. Sugar content of fruits did not change significantly through sampling times. In addition, EW and ozonized water did not differ in term of effect on sugar content (Figure 15). Un-inoculated and inoculated fruits treated with EW, ozonized water and stored in passive refrigerator in ozonized atmosphere had the highest level of sugar content (Table 4).

Table 4. Sugar content present on orange inoculated and un-inoculated fruit after different storage periods at 4°C with different methods, and stored in passive refrigeration (PRS) or in conventional refrigeration (Conv). EW= Electrolyzed Water; O_3=Ozone in water; control T1= after 7 days' storage; T2= after 14 days' storage; T3= after 21 days' storage; T4= after 28 days storage. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (p=0.05).

Treatment	Sugar content											
	T1			T2			T3			T4		
	EW	O_3	Control	EW	O_3	Control	EW	O_3	Control	EW	O_3	Control
(Conv+ O_3) inoculated	10^{b}	10^{b}	9.25^{c}	10^{b}	10^{b}	9.25^{c}	10^{b}	10^{b}	9.25^{c}	10^{b}	10^{b}	9.25^{c}
(Conv+ O_3) un-inoculated	10.3^{b}	10^{b}	10^{c}	10^{b}	10^{b}	9.25^{c}	10^{b}	10^{b}	10^{c}	10^{b}	10^{b}	10^{c}
(PRS+ O_3) inoculated	11.5^{ab}	11^{ab}	10	12^{a}	11^{ab}	9.25	12^{a}	11^{ab}	9.25	12^{a}	11	9.25
(PRS+ O_3) un-inoculated	11.8^{ab}	12^{a}	9.25	12^{a}	12^{a}	9.25	12^{a}	12	9.25	12^{a}	12^{a}	9.25
(Conv) inoculated	9.75^{c}	10^{c}	9.25^{c}	10^{c}	10^{c}	9.25^{c}	10^{c}	10^{c}	9.25^{c}	10^{c}	10^{c}	9.25^{c}
(Conv) un-inoculated	9.75^{c}	10^{c}	9.25^{c}	10^{c}	10^{c}	9.25^{c}	10^{c}	10^{c}	9.25^{c}	10^{c}	10^{c}	9.25^{c}

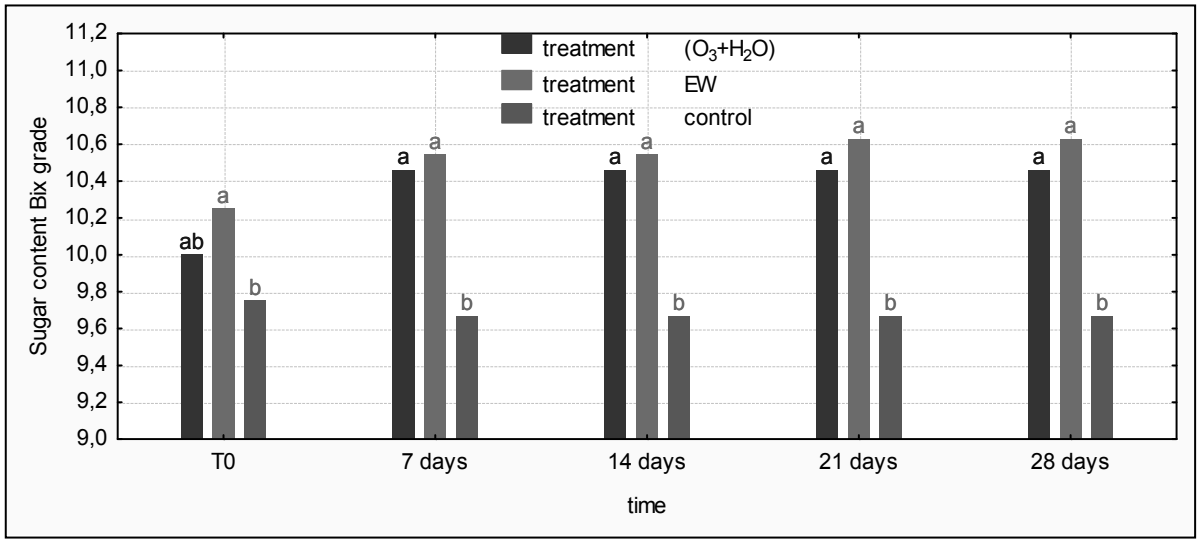

Figure 15. Effect of ozonized water and EW treatment on sugar content (Brix grade) of orange fruits during T0, 7, 14, 21 and 28 days of storage at 4°C. Statistical analyses were carried out separately for each sampling time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

3.3 Experiment B: *in vitro* ozone fumigation treatments

The aim of this experiment was to examine the direct effect of O_3 and EW on shelf life of oranges fruits. Trials were arranged in a completely randomized block

design with four blocks and four fruits/blocks for each treatment, these treatments were applied separately with different doses of ozone concentration (1 or 2 ppm) and different periods of exposure times 15, 30, 60 and 120 minutes by using ozone fumigation device (BOX). Control fruits kept at room temperature for the same duration of treated fruits were used as controls. Sampling was carried out by choosing four fruits (1/each block) randomly for each treatment. First sampling was carried out before storage at time zero (T0). Another quantity of the fruits was stored at 4°C for 10 days. Colony forming units (CFU), hardness and sugar content were measured after 10 days (Time 1). Similar quantity of the fruits where stored at 23°C, the duration of conservation time related to the completely rotted un-treated control fruits for measuring the shelf-life.

3.3.1 Results of experiment B: *in vitro* Ozone fumigation experiment

3.3.1.1 Colony forming unites CFU

Figure 16 represent the amount of CFUs measured on the surface of treated with different concentrations of gaseous ozone (1 and 2 ppm) and different exposure times 15, 30, 60, and 120min. The results showed high efficiency (decreasing microorganism's population) in correspondence with higher ozone concentration and exposures time. Increasing exposure from 1 to 2 ppm led to a better control of microorganism's population, so ozone concentration was not the only factor able to control pathogens but ozone exposure time also contributed in decreasing CFUs. The highest CFUs reduction obtained after 10 days of storage of fruit treated with 2 ppm of O_3 for 120 minutes. Nevertheless, the application of ozone at 1 or 2 ppm for 15 minutes gives a significant reduction as compared to the control (Figure 16).

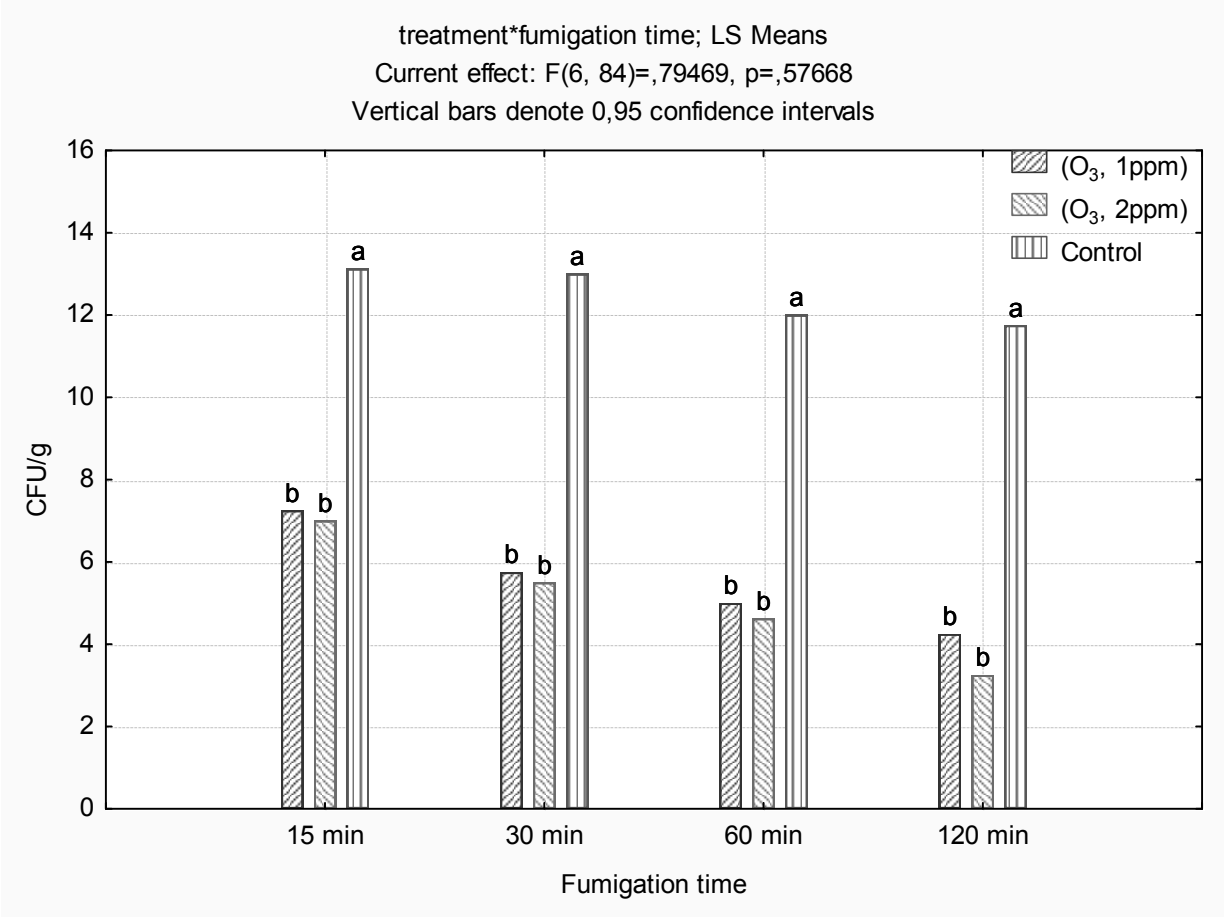

Figure 16. Colony Forming Units (CFUs) present on orange fruits after treatment with gaseous ozone (1 or 2 ppm) with different fumigation times (15, 30, 60, and 120min). Statistical analyses were carried out separately for each fumigation time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (p≤0.05).

3.3.1.2 Hardness

Both ozone concentrations 1ppm and 2ppm were significantly increased orange fruit hardness as compared to control fruits. Ozone fumigation for 120 minutes showed the highest significant increase in fruit hardness in both concentrations 1 and 2 ppm, statistical differences were obtained on hardness value at 30 and 60 min of fumigation by 1 or 2 ppm of ozone (Figure 17).

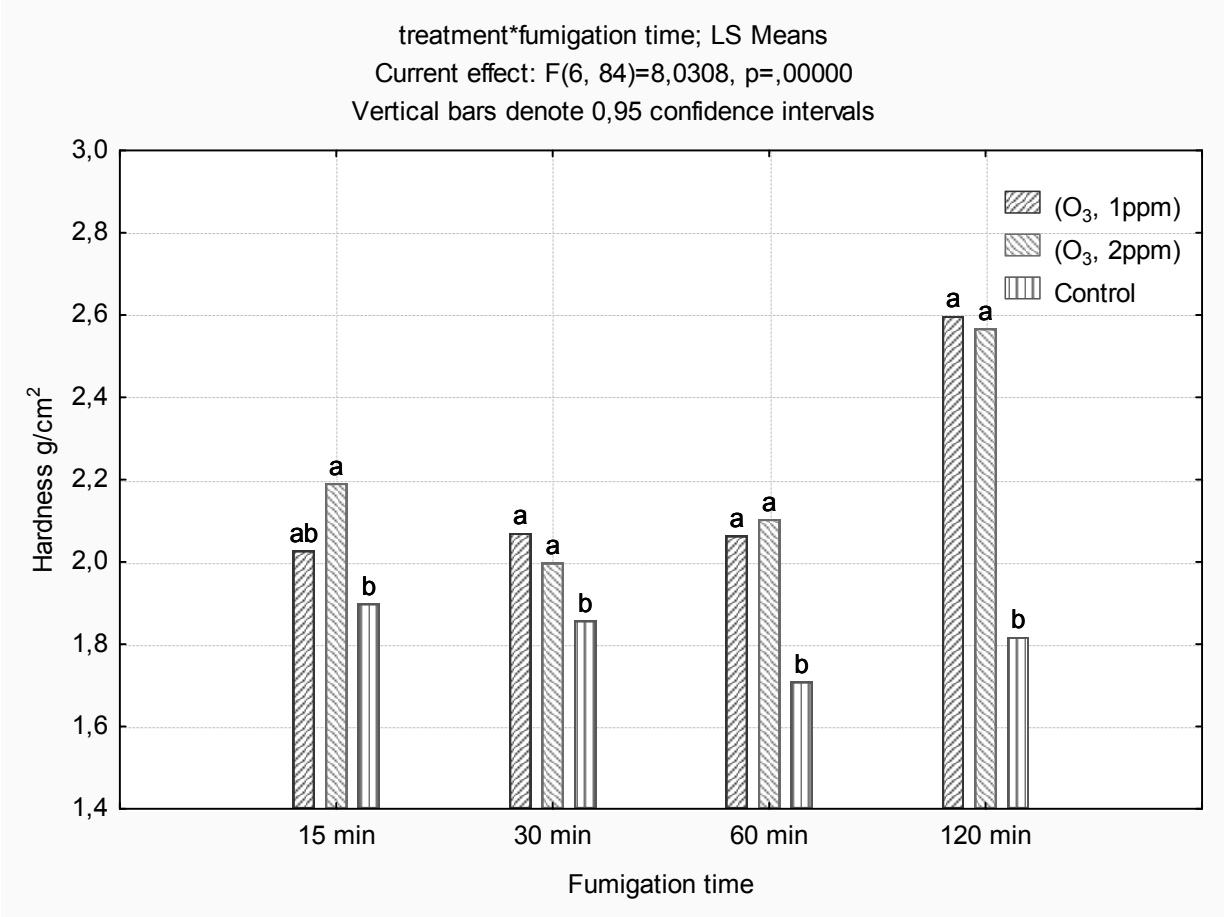

Figure 17. Effect of gaseous O_3 at different concentrations 1ppm and 2ppm on the orange fruits hardness with different fumigation period (15, 30, 60, and 120min). Statistical analyses were carried out separately for each fumigation time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

3.3.1.3 Sugar content

High sugar content was reported in orange fruits treated for 120 minutes at 1 ppm or 2 ppm (Figure 18). A treated orange fruits with 1ppm for 15 min showed an increasing of sugar content. Treatment with gaseous O_3 is generally able to maintain the concentration of soluble sugars compared to the controlled fruits.

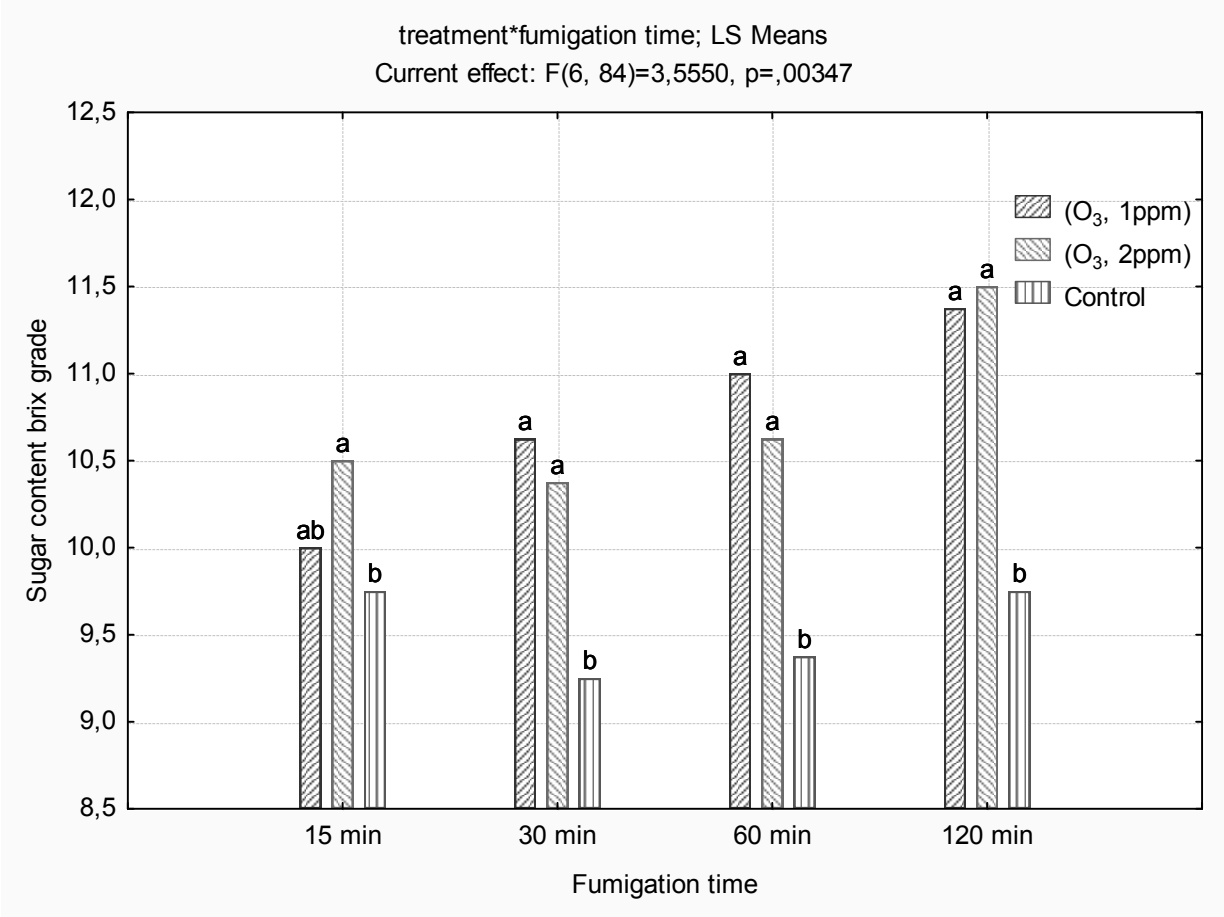

Figure 18. Effect of gaseous O_3 at different concentrations (1ppm and 2ppm) on sugar content of orange fruits after treatment with gaseous ozone (1 or 2 ppm) with different fumigation times (15, 30, 60, and 120min). Statistical analyses were carried out separately for each fumigation time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

Ozone application at 2 ppm led to a clear reduction of microorganism's population, obviously the duration of the ozone application also contributed in decreasing CFUs. The highest CFUs reduction obtained after 10 days of storage of fruit treated with 2 ppm of O_3 for 120 minutes. In the same sense, a significant reduction of disease incidence measured as McKinney index was observed in fruits treated with 2ppm O_3 for 120 min (Figure 19).

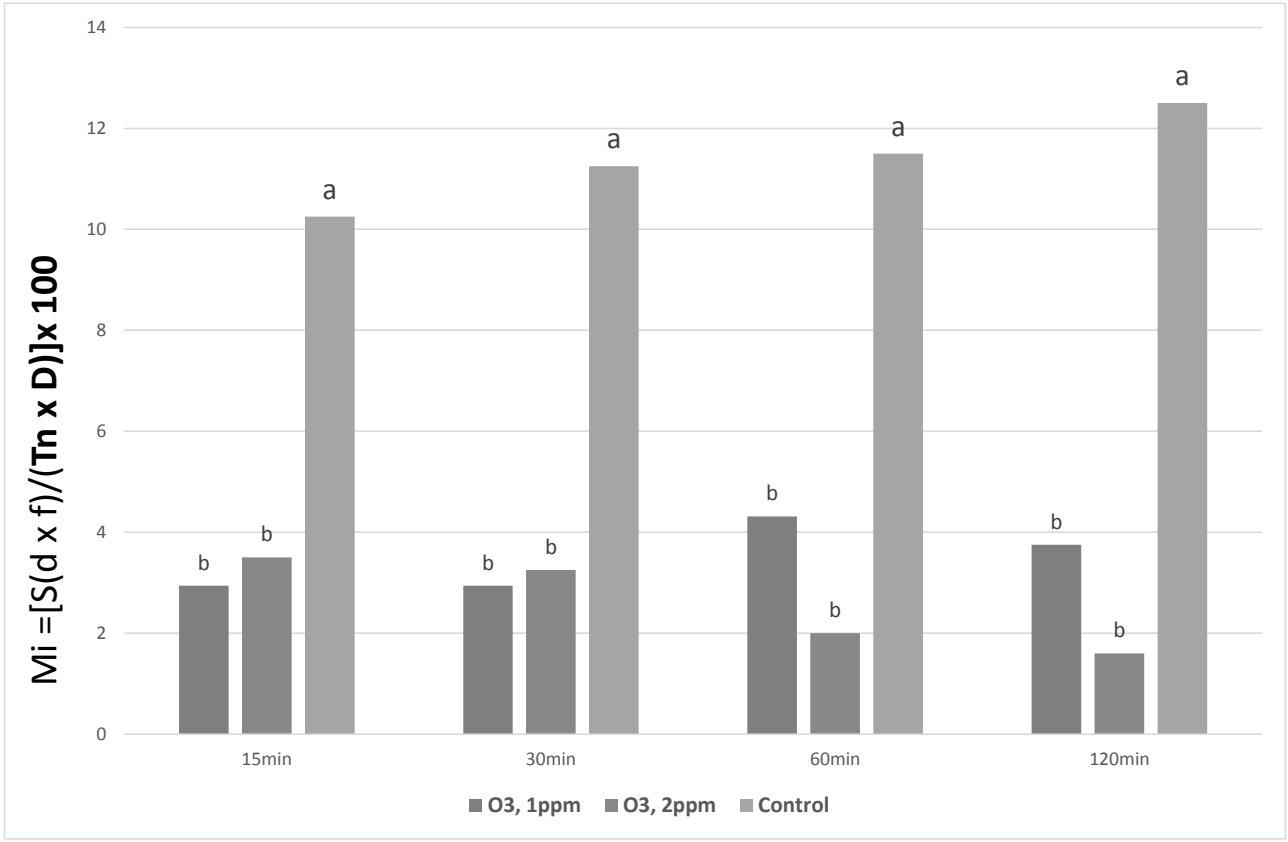

Figure 19. Effect of gaseous ozone at different concentrations 1ppm and 2ppm on disease incidence of orange fruits after application with different fumigation times 15, 30, 60, and 120min then stored at 23°C for 10 days. Statistical analyses were carried out separately for each fumigation time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

3.4 Experiment C: Electrolyzed water and ozonized water treatments

Oranges fruits where wounded in four equal positions of the fruit center with the rod and only the control fruits where inoculated, using a micropipette, with 20 µL of a suspension containing 2×10^6 spores/mL (25×10^3conidia /20µl) of *P. italicum*. Trials were arranged in a completely randomized block design with four blocks and four fruits/blocks for each treatment, washing operation required 5,10 and 15 minutes and was conducted before storage using ozonized water 2 ppm or electrolyzed water 10 or 20% and normal water as control. All fruits where transferred to 23 °C chambers, the duration of conservation time related to the completely rotted un-treated control fruits for measuring the shelf-life.

3.4.1 Results of Experiment C: Using EW 10 and 20% and O_3 in water

It has been proved that washing by ozonized water wash and dipping time for 10 and 15 min was the best treatment for the extension of shelf life by decreasing the disease incidence, as well as washing by 800ppm of active chlorine EW (20%), considering the all-dipping times. Washing by 400ppm of active chlorine EW (10%) did not show to be high efficient in the three washing times. Both the untreated inoculated and un-inoculated control had the shortest shelf life. Fifteen minutes washing gave the best results in terms of disease incidence decrease. Figure 20 represents the incidence and the severity of spoiling in the considered fruit by the determination of McKinney index. Ozonized water washing ozone gave completely different results in consequence of the application time.

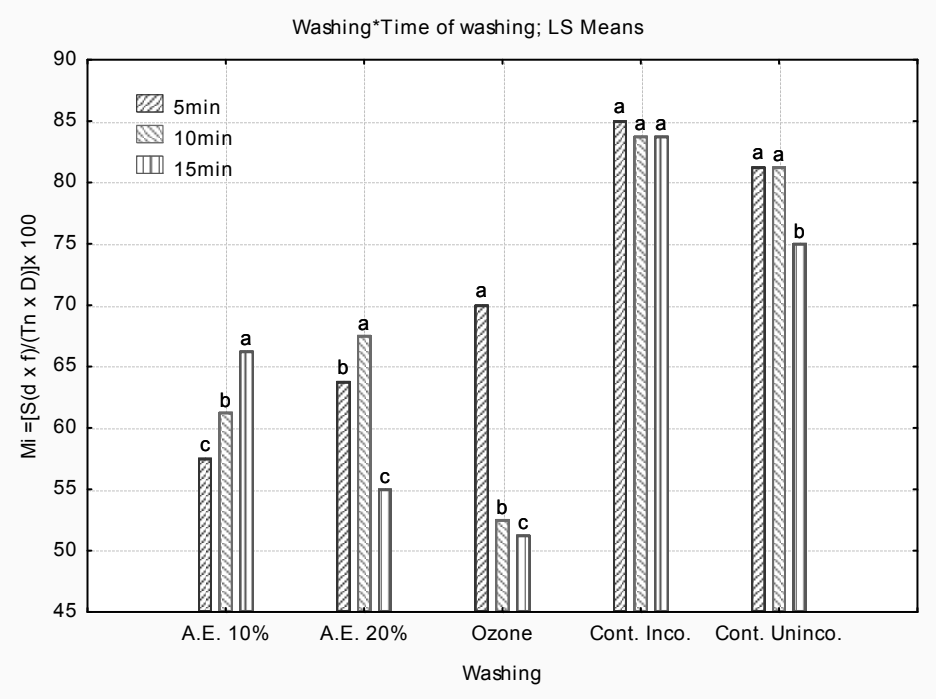

Figure 20. Effect of EW treatment at different concentrations EW 400ppm of active chlorine (10%) and EW 800ppm of active chlorine (20%) and ozone washing (2ppm) on disease incidence (McKinney index) of orange fruits. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

3.5 Experiment D: Electrolyzed water treatments

Oranges fruits where dipped in three dilution of electrolyzed water 15% (500ppm of active chlorine), 20% (800ppm of active chlorine), 30% (1200ppm of active chlorine) for different time 0.5, 1, 2, 3, 4 and 5 minutes, normal tap water as control. All fruits where stored at 23°C chamber, the duration of conservation time related to the completely rotted un-treated control fruits for measuring the shelf-life.

3.5.1 Results of Experiment D: Using EW 15, 20 and 30%

High doses of EW strongly reduced disease incidence (McKinney index). Results showed the high effect of EW 30% and 15% in decreasing disease incidence measured in McKinney index. It is obvious that increasing exposure time give a better results, but in this case we found that also 15 and 20% were significantly reduce disease incidence as compared to the control (Figure 21).

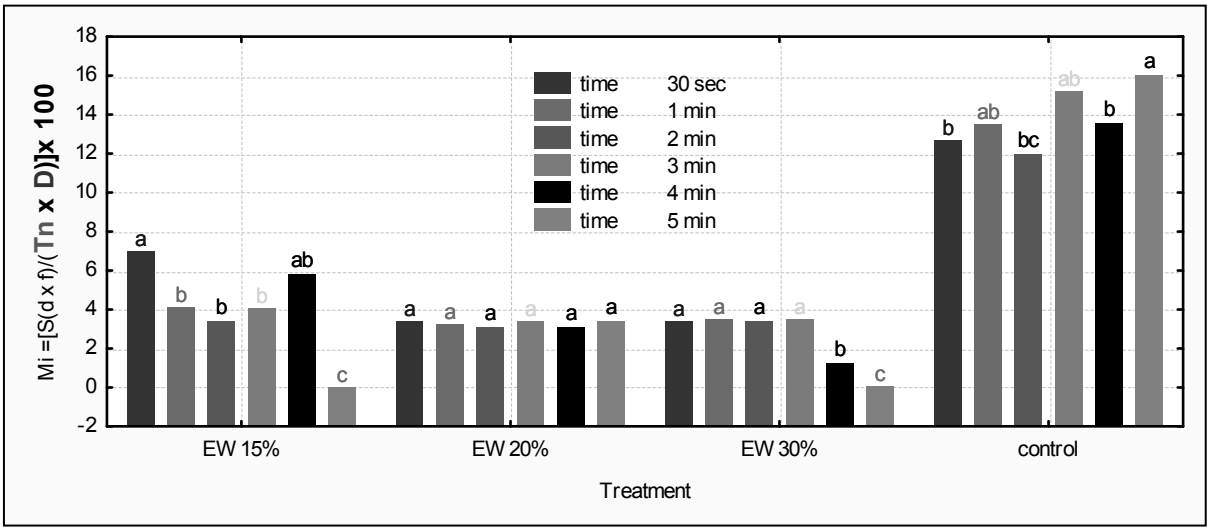

Figure 21. Effect of EW at 15%, 20% and 30% (500ppm, 800ppm, 1200ppm of active chlorine respectively) for different application time 0.5, 1, 2, 3, 4 and 5 minutes, normal tap water as control, on the control of disease incidence (McKinney index). Statistical analyses were carried out separately for each treatment. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (p≤0.05).

3.6 Experiment E: Ozone fumigation in passive refrigeration

The experiment was designed to evaluate the impact of ozone in combination with PRS to optimize postharvest conservation, reducing losses and to improve decay control of the products during handling, processing and storage has been investigated for commercial applications.

Orange fruits cv. "navel" were harvested from commercial orchards located in Apulia region (Ginosa Marina–TA) and transported to postharvest laboratory (CIHEAM-Bari). Samples were divided in 4 replicates for reach treatment, and stored in passive refrigerated champers enriched with different ozone concentrations. Ozone was provided continually in two chambers 0.1ppm and 0.3 ppm; and just 2 hours of ozone application at 1ppm at night in the third chamber, a chamber without ozone application was used as control (0ppm).

Sampling was carried out each ten days in each treatment. The first sampling time was immediately before storage (time zero), firmness (Penetrometer measures g/cm^2), sugar content (Refractometer Brix grade) and Colony Forming Unite (CFUs/g of citrus fruit) were assessed. The detected parameters were evaluated by using the one-way ANOVA test immediately after the treatment (T0), after 10 days (T1), after 20 days (T2) and after 30 days (T3). Sugar content, Fruit firmness, Colony forming units and Shelf life were evaluated during the storage period.

3.6.1 Results of experiment E: Ozone fumigation in passive refrigeration

3.6.1.1 Sugar content

Generally, a reduction of sugar content was reported in un-treated fruits during the conservation period. From Figure 22 is clearly observe that the sugar content decreasing according to the storage period.

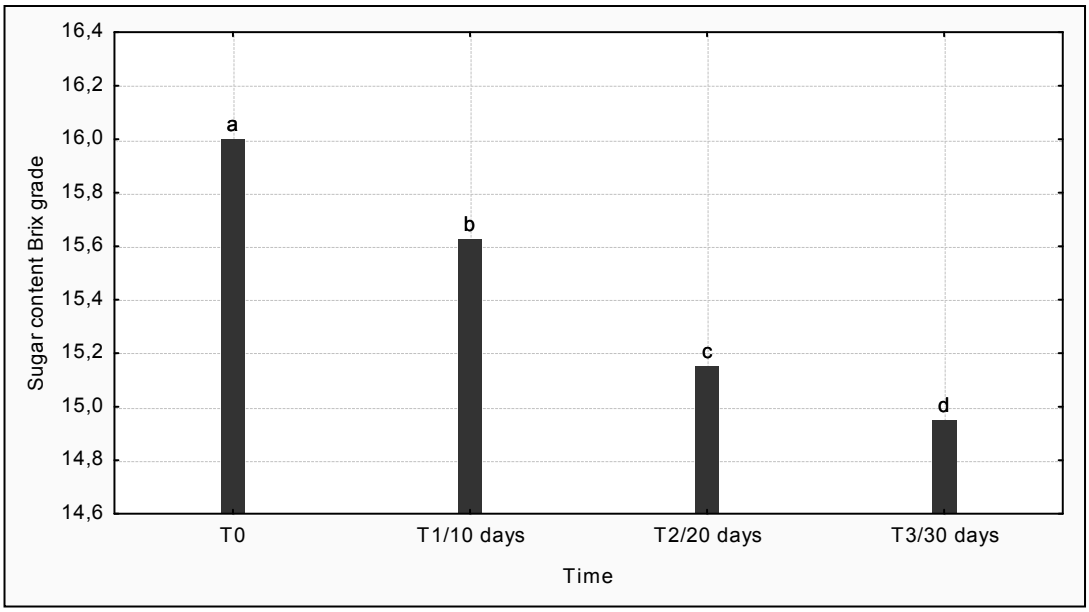

Figure 22. Orange fruit sugar content in different storage periods in PRS.

The comparative graph with different concentrations levels of ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) indicates the respective sugar content percentage. The effectiveness of ozone treatment can be seen with improvement of sugar content percentage; hence the optimized concentration level can notice, which retain the sugar content also constant (Figure 23).

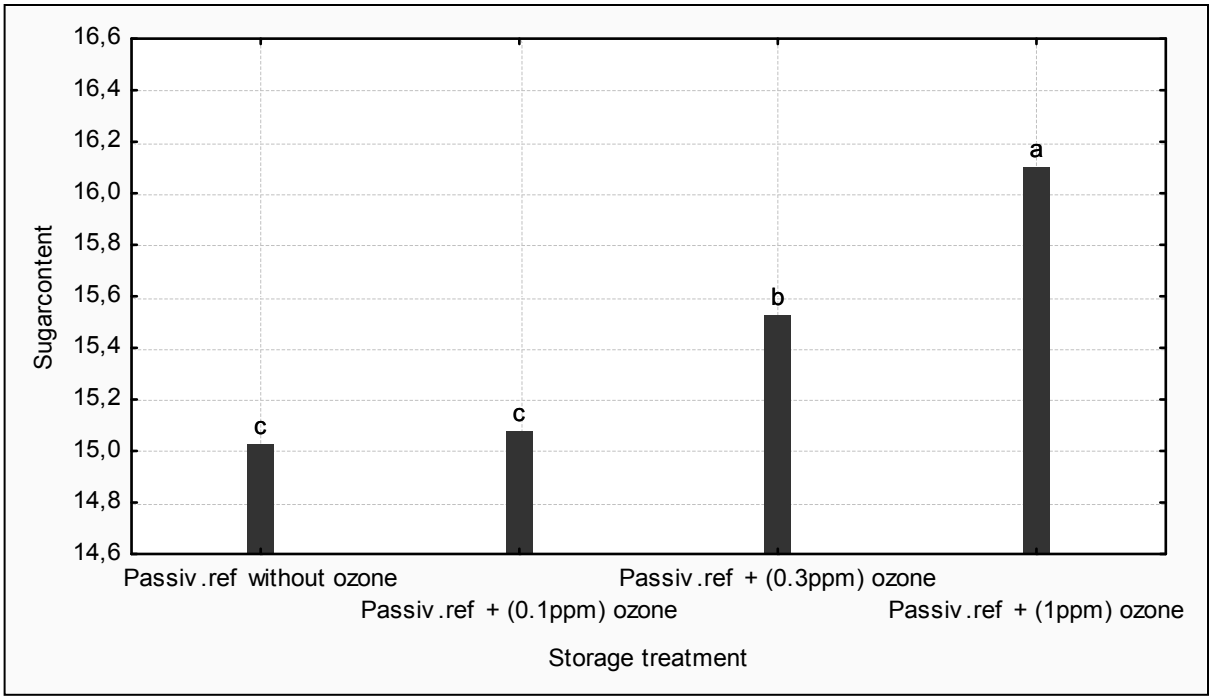

Figure 23. Orange fruit sugar content in different storage periods in PRS under ozone enriched atmosphere 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm, measures obtained at the end of the experiment (30 days of conservation).

In the light of figure 24, a reduction of sugar content was observed in fruit stored under 0ppm, 0.1ppm and 0.3ppm of ozone after 10, 20 and 30 days of conservation. In contrast, fruits conserved under 1ppm of ozone showed an increase of sugar content during storage period. We can conclude that 1ppm ozone is the optimum concentration to conserve orange quality (Figure 24)

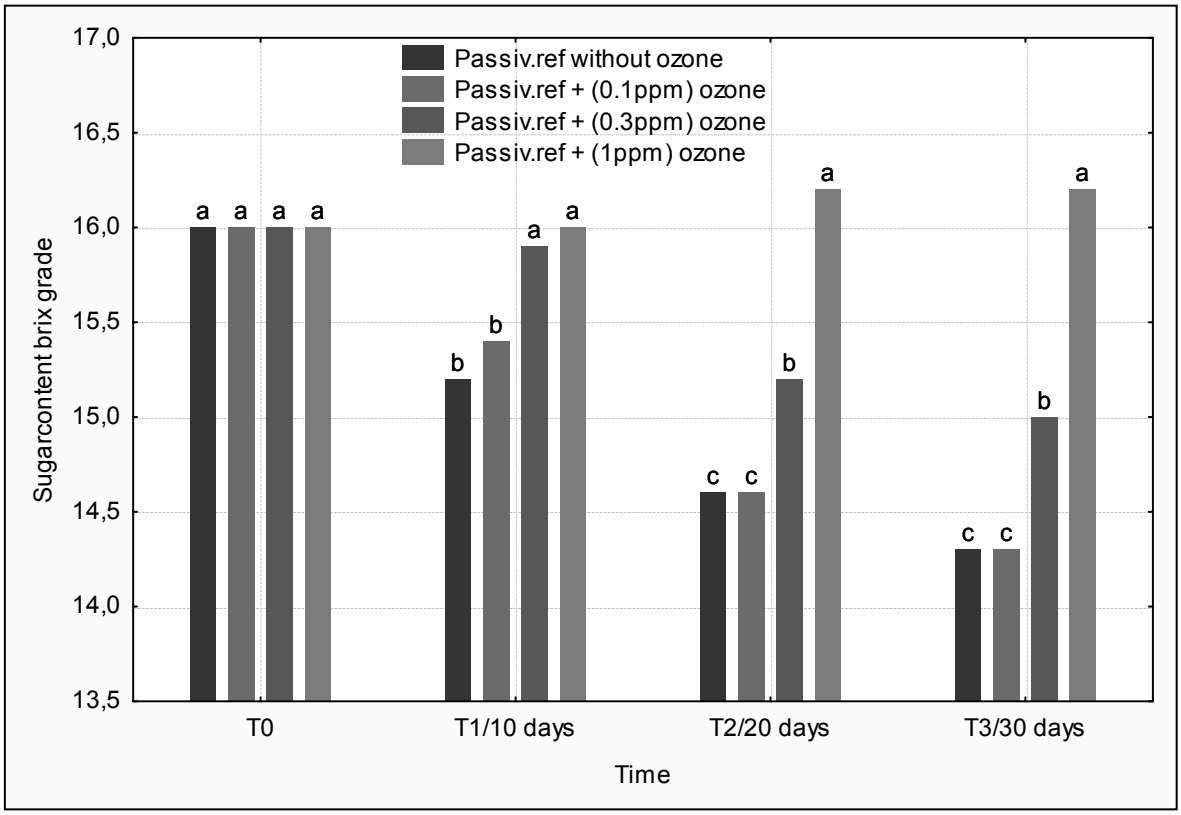

Figure 24. Comprehensive Graph Between Different Concentrations Levels of Ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) and Storage Period (i.e. 0day, 10days, 20days, and 30 days). Statistical analyses were carried out separately for each storage time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

3.6.1.2 Fruit firmness

Figure 25 show that the hardness decreases gradually with increasing storage period, as a control fruits.

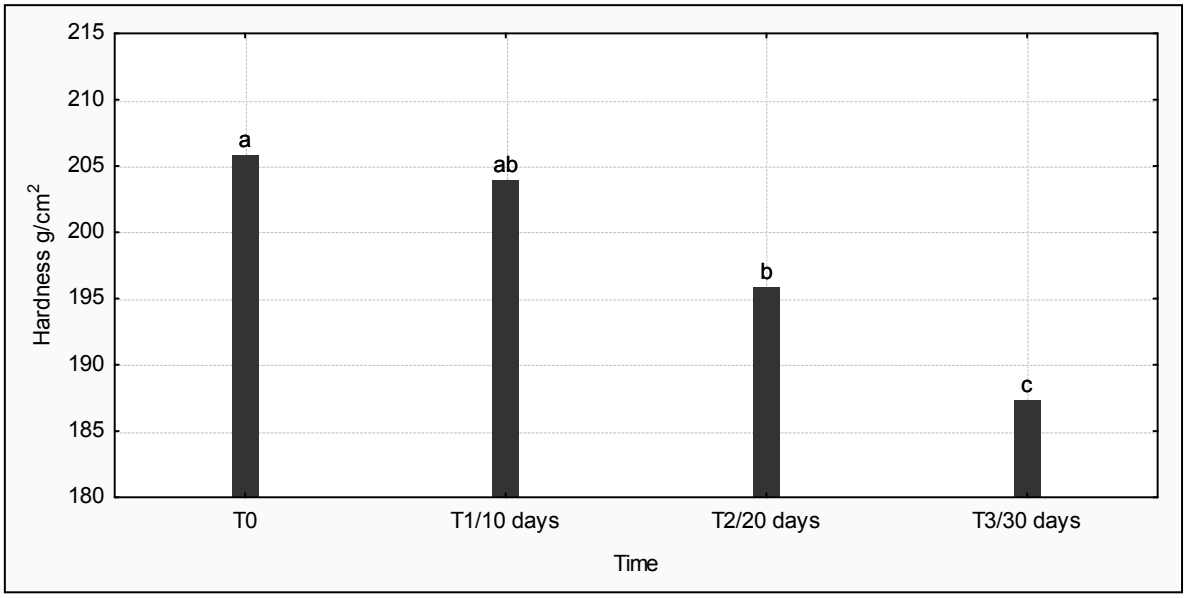

Figure 25. Citrus fruit hardness in ozone exposure experiment measured in different storage Periods (0, 10, 20 and 30 days) of storage.

The comparative graph with different concentrations levels of ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) indicates the respective hardness level. According the Figure 26, effectiveness of ozone treatment can be seen with improvement of hardness; hence the optimized concentration level can notice, which retain the hardness also constant.

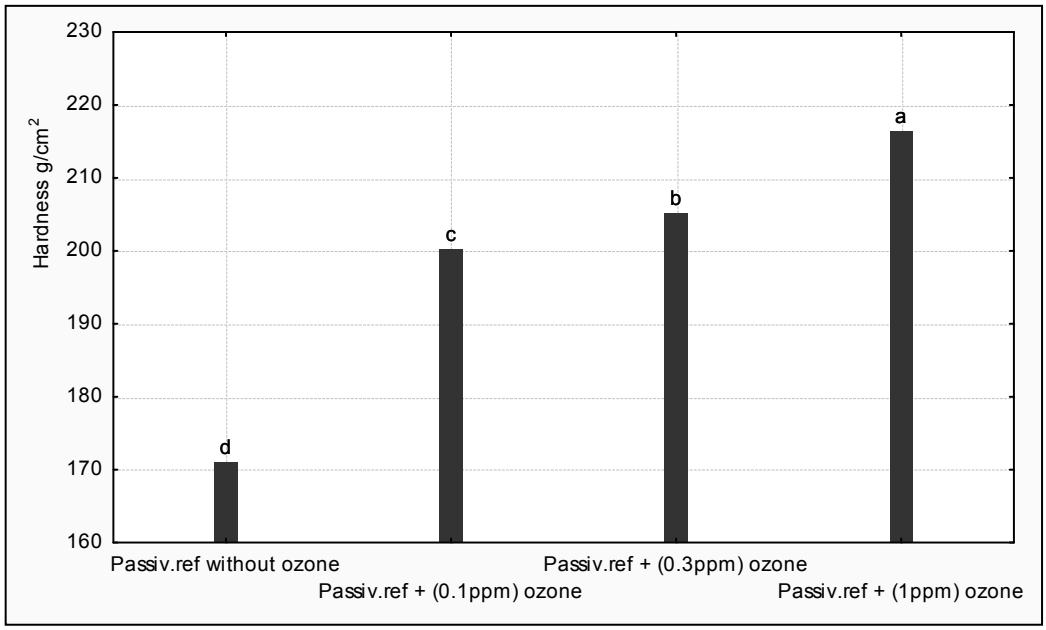

Figure 26. Comparative Graph between Hardness with Different Concentrations Levels of Ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm).

The comprehensive graph between different concentrations of ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) and storage period (i.e. 0day, 10days, 20days, and 30 days) to determine the mutual effect on hardness. An increase of fruit hardness was recorded in the fruit conserved in passive refrigeration under ozone gas exposure; higher ozone concentration (1ppm in this case) higher hardness value was detected. Even after 30 days of conservation. In the light of figure 27, we can conclude that 1 ppm ozone is the optimize level to retaining the hardness remain content over the storage period of 30 days.

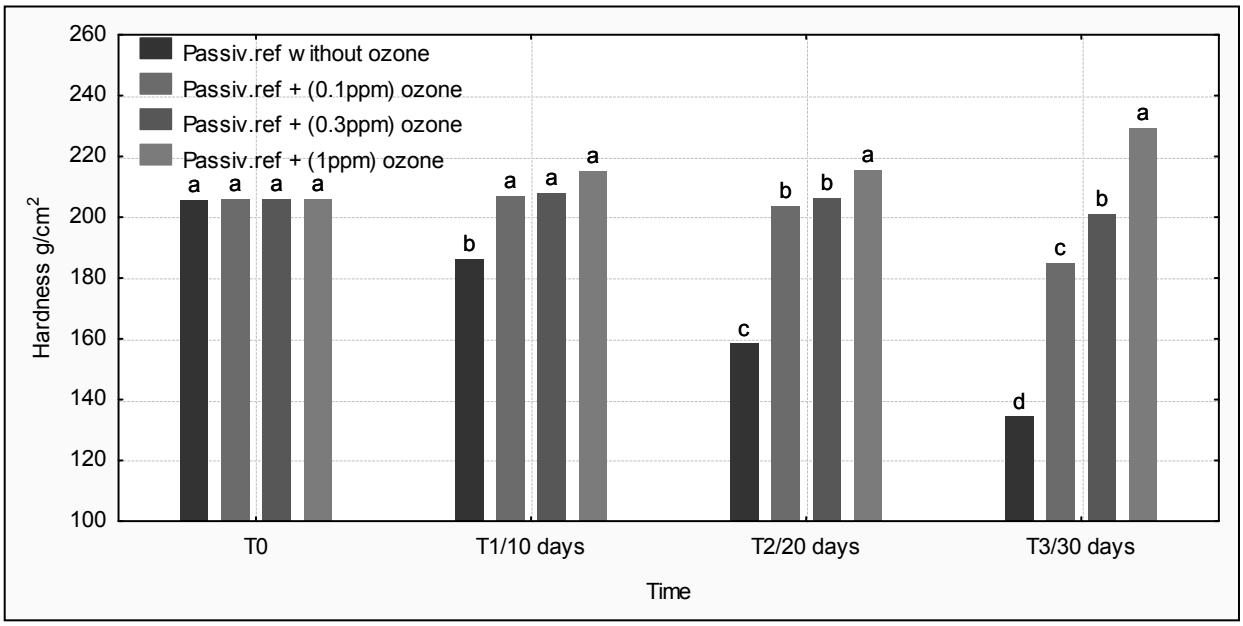

Figure 27. Comprehensive Graph Between Different Concentrations Levels of Ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) and Storage Period (i.e. 0day, 10days, 20days, and 30 days). Statistical analyses were carried out separately for each storage time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

3.6.1.3 Colony forming units:

The general evaluation of Colony forming units shows that the initials value starts with value around 29 CFU/g of fruit in T0, this amount reduce significantly during the conservation since the first sampling T1 after 10 days of conservation. From Fig. 28 is clearly observe that the colony forming units decreases gradually without any significant defences between T1, T2 and T3. This, lead us to understand ozone reduce the CFU since the first application.

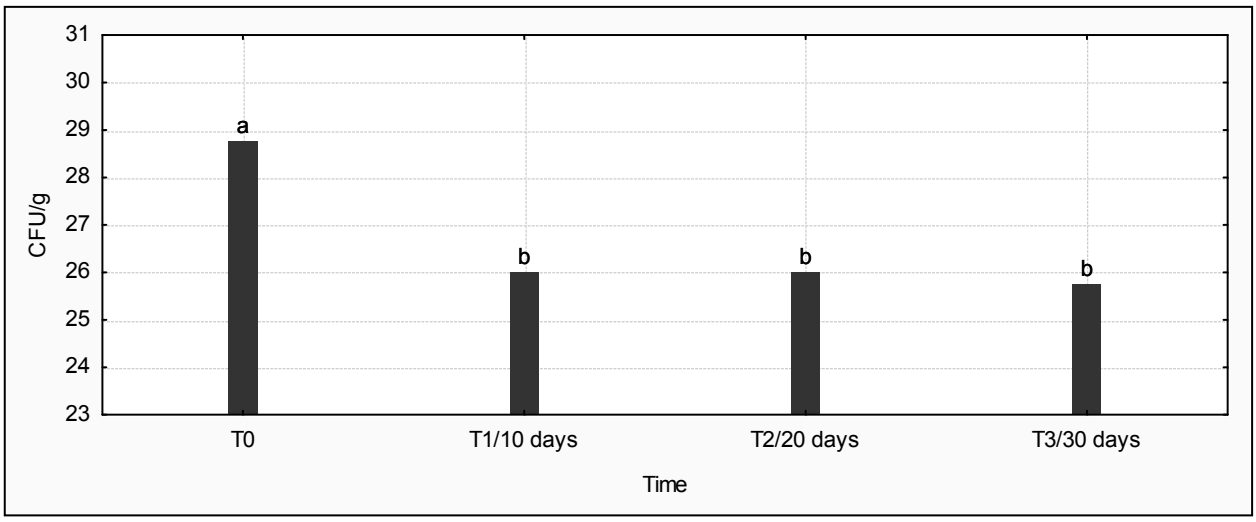

Figure 28. Colony Forming Unites (CFU) on the surface of citrus fruit during ozone exposure experiment measured in different storage Periods (0, 10, 20 and 30 days) of storage.

A significant reduction of colony forming unite (CFU) of filaments fungi was obtained with different ozone concentrations (0.1 ppm, 0.3ppm and 1ppm). Ozone concentration of 1ppm was more efficient in CFU reduction as compared with 0.1ppm and 0.3ppm. The reduction from 32 CFU up to 12 CFU without affecting fruit quality as we notice in Harness and sugar content impose that 1ppm could be applied to reduce the contaminates on fruit surface.

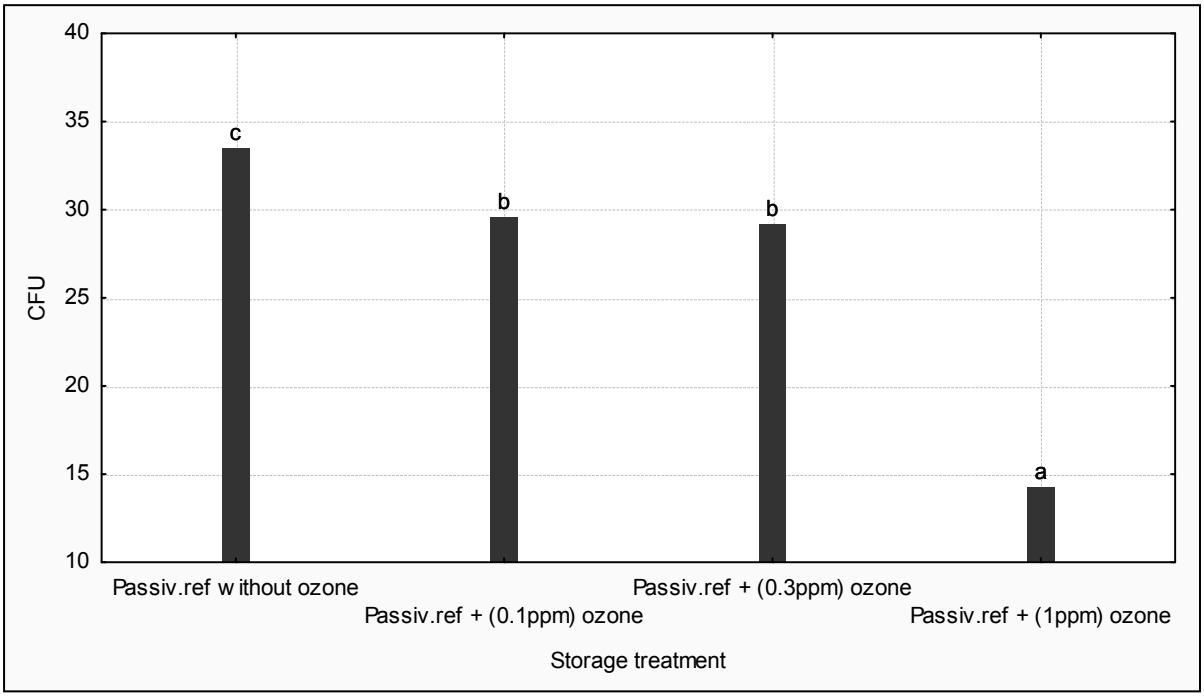

Figure 29. Comparative Graph between CFU with Different Concentrations Levels of Ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm).

The comprehensive graph between different concentrations levels of ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) and storage period (i.e. 0day, 10days, 20days, and 30 days) to determine the mutual effect on CFU. CFU reduction obtained after 30 days of conservation in passive refrigeration under 1ppm of ozone fumigation riche the lowest value. In the light of figure 30, we can conclude that 1 ppm ozone is the optimize level to return the CFU at safe level over the storage period of 30 days.

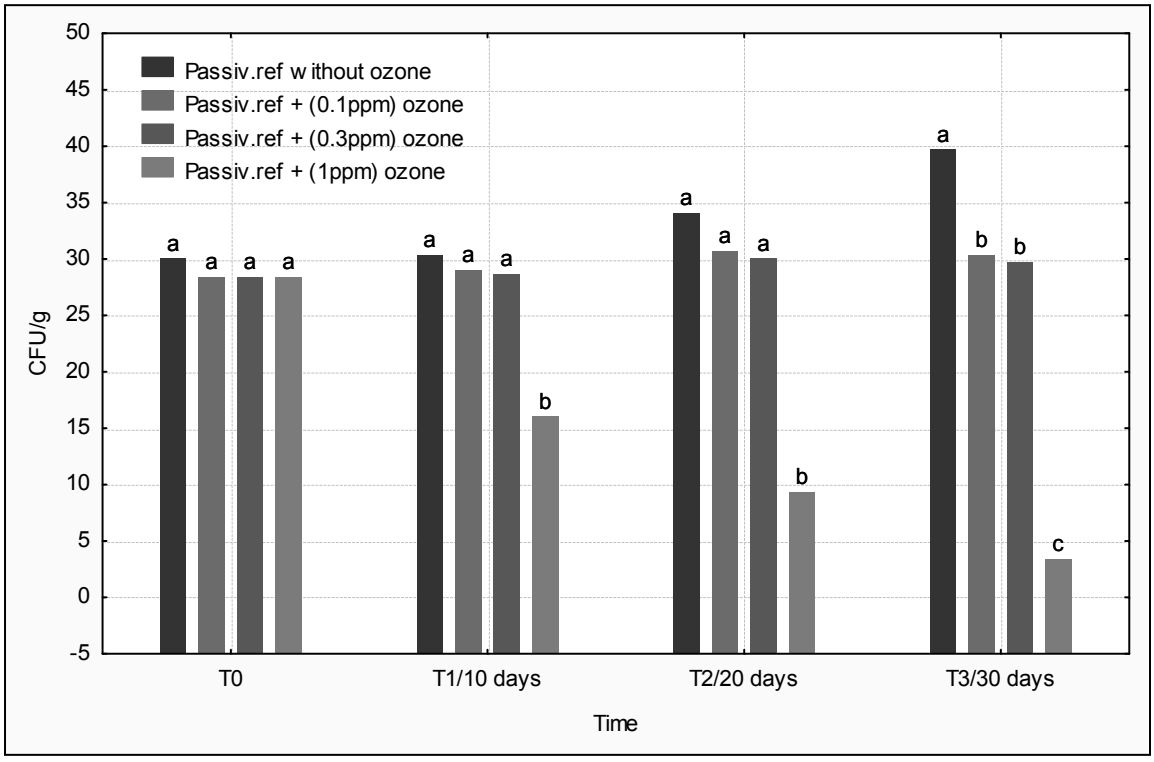

Figure 30. Comprehensive Graph between Different Concentrations Levels of Ozone (i.e. 0 ppm, 0.1 ppm, 0.3ppm, and 1ppm) and Storage Period (i.e. 0day, 10days, 20days, and 30 days). Statistical analyses were carried out separately for each storage time. Letters indicate homogeneous groups according to Duncan's LSD honest significance test (P≤0.05).

4 Chapter 4. The effect of ozone on the expression of plant related genes on citrus fruits

4.1 Materials and Methods

4.1.1 Genes selection and primer design

Four genes were selected for investigation to study their expression in response to ozone exposure and fungi challenging. This group of genes are the most commonly involved in antifungal response in citrus fruit. The four chosen candidate genes were β-1,3-glucanase (GNS1), chitinase (CHI1), phenylalanine-ammonia lyase (PAL1) and peroxidase (POD1) and their sequences were obtained from Citrus Genome Database (https://www.citrusgenomedb.org/) (Table 5). Based on previous studies , we chose 3 reference genes (18SrRNA, ACTB, rpII) as an internal control in order to normalize the mRNA levels between different samples for an exact comparison of gene expression levels (Yan *et al.*, 2012). Primer pairs for target genes were designed based on selected sequences using Primers3Plus®. The primers used for reference genes were copies of primers used by (Yan et al., 2012). All the primers pairs were assessed to determine the amplification efficiency and the optimal annealing temperature.

Table 5. Selected genes, their correspondent product and the related plant de defense mechanism

Genes	Product	Related plant defense mechanism
GNS1	β-1,3-glucanase	Systemic AcquiredResistance(SAR)
CHI1	chitinase	Systemic AcquiredResistance(SAR)
PAL1	phenylalanine-ammonia lyase	Induced Systemic Resistance (ISR)
POD1	Peroxidase	Reactive Oxygen Species (ROS)

4.1.2 Plant materials

The genotype tested was sweet orange (C. sinensis var. Navel). Fruits at 180 days after flowering were collected from the trees and transported to the t IPM laboratory (MAIB lab facility).

4.1.3 Fruit treatment

Mature intact orange fruits were surface-sterilized (2% sodium hypochlorite) and rinsed with sterile distilled water. Fruits were then wounded on their peel in the equatorial region (four wounds for each fruit). A 20µl suspension of *P. digitatum* strain (1×10^9 CFU/ml-1) was applied to each wound and fruits were incubated at 20°C (Figure 31).

Figure 31. P. digitatum suspension injected in citrus fruits

After 48 hours, challenged oranges were taken to chambers and exposed to ozone gas at a concentration of 1 ppm for 6, 12, 24 and 48 hours (Figure 10). Oranges singularly inoculated with *P. digitatum* or only treated with ozone, and fruit used as controls (untreated control fruit) were included in the assay (Table 6). Ten fruits for each treatment were arranged on trays and put on the ozone chamber (Figure 32).

Figure 32. Ozone gas treatment chamber.

Table 6. Treatment assignment of orange fruits

Fungi inoculation	Ozone exposure
Inoculated fruits with *P. digitatum*	Ozone (passive refrigeration 1 ppm)
	-
Non-inoculated fruits	Ozone (passive refrigeration 1 ppm)
	-

4.1.4 RNA preparation

About 0.5g of orange peel tissue was ground to fine powder in mortars using liquid nitrogen (Figure 33). Total RNA was isolated using ISOLATE II RNA Mini Kit by (BIOLINE, UK) according to manufacturer's instructions.

Figure 33. Grounding orange fruit peel in fine powder with liquid nitrogen

4.1.5 RNA quality and quantity assessment:

One of the most common methods of verifying the RNA integrity is performing a 1.5% agarose gel electrophoresis. Samples were loaded into precast gels, and as an electrical current was passed through the gel, nucleic acid fragments were separated on the basis of size. Larger fragments moved more slowly through the gel matrix, while smaller fragments moved more quickly. The gels containing the separated fragments were stained with a fluorescent dye that binded nucleic acids. The separated fragments then could be visualized by excitation of the fluorescent dye bound to the nucleic acid. Based on the 28S/18SrRNA bands, we only selected the samples that presented a good RNA quality for further analysis (Figure 34).

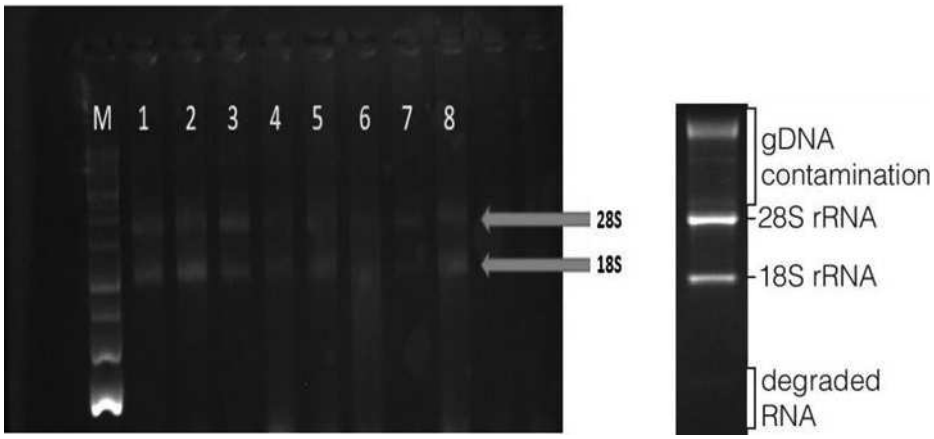

Figure 34. RNA analysis by agarose gel electrophoresis

The RNA was quantified by spectrophotometer using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), as described by the manufacturer instructions (Figure 35) The RNA samples with absorption ratios of A260/A280 = 1.9–2.1 and A260/A230 ≈ 2.0 were used for cDNA synthesis. RNA samples concentration was determined and adjusted to 10 ng µl-1. An aliquot of 7µg of total RNA was used for cDNA synthesis with a final volume of 20 µl.

Figure 35. Spectrophotometer UV-1800 Shimadzu

4.1.6 Synthesis of cDNA for qPCR

Complementary DNA, cDNA, was synthesized using reverse transcriptase (RT) in the kit M-MLV Reverse Transcriptase (200 U/μL) for RT-PCR (Invitrogen). Reverse transcriptase is an enzyme that generates cDNA from DNA. This creates one DNA (cDNA) strand complementary to the RNA strand in a DNA-RNA hybrid. The RNA strand is then degraded by RNase H, leaving single stranded cDNA ready for PCR. In this case, total RNA was isolated and used as template for cDNA synthesis. The mRNA (which make up 1-5% of the total RNA) are the interesting fragments for the expression study.

Reverse transcription was performed with 7 μg of total RNA according the following protocol:

RNA/primer mixtures:

7μl of total RNA

1μl of Random Primers (50ng/μl)

5 μl of H2O

The mixtures are incubated at 95°C for 5 minutes, and then on ice for 3 minutes.

Add a reaction mixture of the following components and amounts to each sample:

1.5 μl of 0.1M DTT- 4 μl of Buffer 5x

1 μl of dNTPs

0.5 μl of M-MLV Reverse Transcriptase

The reaction is terminated at 39° C for 60 min and then at 70° C for 10 minutes

The samples are chilled on ice.

4.1.7 qPCR protocol

Gene transcript levels for of β-1,3-glucanase (GNS1), chitinase (CHI1), phenylalanine ammonia-lyase (PAL1) ,peroxidase (POD1) and phenylalanine ammonia-lyase (PAL1) were measured the first 48 hours after P. digitatum inoculation or/and exposure to ozone gas.

The qPCR was performed on Bio-Rad iCycler Thermal Cycle in a 96-well reaction plate using the parameters recommended by the manufacturer. A 25 μl PCR mixture was prepared containing 2.5 μl of template cDNA, 12.5 μl GoTaq® qPCR Master Mix (x2) (PROMEGA), 0.5 μl forward primer, 0.5 μl reverse primer and 9μl sterile water. A negative control devoid of template (water sample) was included for each primer pair analysis to determine genomic contamination. qPCR results were analyzed with the sequence detection software Bio-Rad CFX Manager 3.1 by BioRad Laboratories, USA). Direct detection of the PCR product was measured by monitoring the increase in fluorescence caused by the binding of SYBR green dye to double-stranded DNA.

The transcript level was calculated by relative quantification of treated fruits (inoculated with P. digitatum, exposed to ozone and both) normalized against the non-treated fruits (Control). The relative quantification of target genes was performed by using the "relative quantification normalized against unit mass" or the ΔCT method comparing CT (CT target gene - CT control). The CT value of the control samples (time 6,12,24,48 hours) were used as calibrators and fold activation was calculated by the expression: 2-ΔCT, where ΔCT = CT (calibrator) – CT (test). We have reported the data as relative units.

4.2 Results

4.2.1 Primers design and validation:

The designed primer pairs flanked the length of sequence between 101 and 120 bp with the optimal Tm° at 52–67 °C and GC% between 44 and 67% (Table 7).

Table 7. Primer sequences for 4 candidate genes and 3 reference genes and their annealing temperatures and amplicon sizes.

Genes	Left primer	Right primer	Annealing T°(C)	Amplicon size	Reference
GNS1	CCTTACAGCCTTGCTTCCCA	ACTGGAGCAAAGTGGGGATG	52.1	105	This study
CHI1	TGACCAATGTGCACCCAGTT	TCCAATGCGAGCTTGAACCT	55.8	103	This study
PAL1	AAAAAGTTACATCGCCCGGC	CGCCATTCCATTCCCTGAGA	54.9	105	This study
POD1	TGATCTGGTTGCACTCTCCG	TCAGCGTTGGGTCTGGATTG	53.6	101	This study
18SrRNA	TCGGGTGTTTTCACGTCTCA	TGGATGCCGCTGGGAAGC	-	120	(Yan *et al.*, 2012)
ACTB	CCAATTCTCTCTTGAACCTGTCCTT	GAAGACCGTCAAGAGTAGTCAGT	-	120	(Yan *et al.*, 2012)
rpII	CACTGCTCTGCTTTCCTTCCAT	AACTGCCCACTATTCTCGTCTC	-	120	(Yan *et al.*, 2012)

The PCR cycles charts display that the amplification was not successful for the three reference genes (18sRNA, ACTB, rpII). However, the amplification charts of the 4 target genes (GNS1, CHI1, POD1, PAL1) was detectable (Figure 37). The primers of the target genes flanked an amplification efficiency (E) between 94 and 98% and an annealing temperature between 52.1 and 55.8°C (Table 7).

Real time PCR conditions were optimized for specific gene amplification. Melting curves profile for real time RT-PCR analysis showed absolute specificity of each primer pair (GNS1, CHI1, PAL1, POD1) for its target sequence (Figure 36).

4.2.2 q-PCR assay:

The results showed that untreated control fruits exhibited only a low basal level GNS1, CHI1, PAL1, and POD1 gene expression, which did not change during the experiment. In contrast, treatments with ozone alone, with *P. digitatum* alone, or with ozone preceded by inoculation with *P. digitatum* increased gene expressions to a different extent (Figure.36).

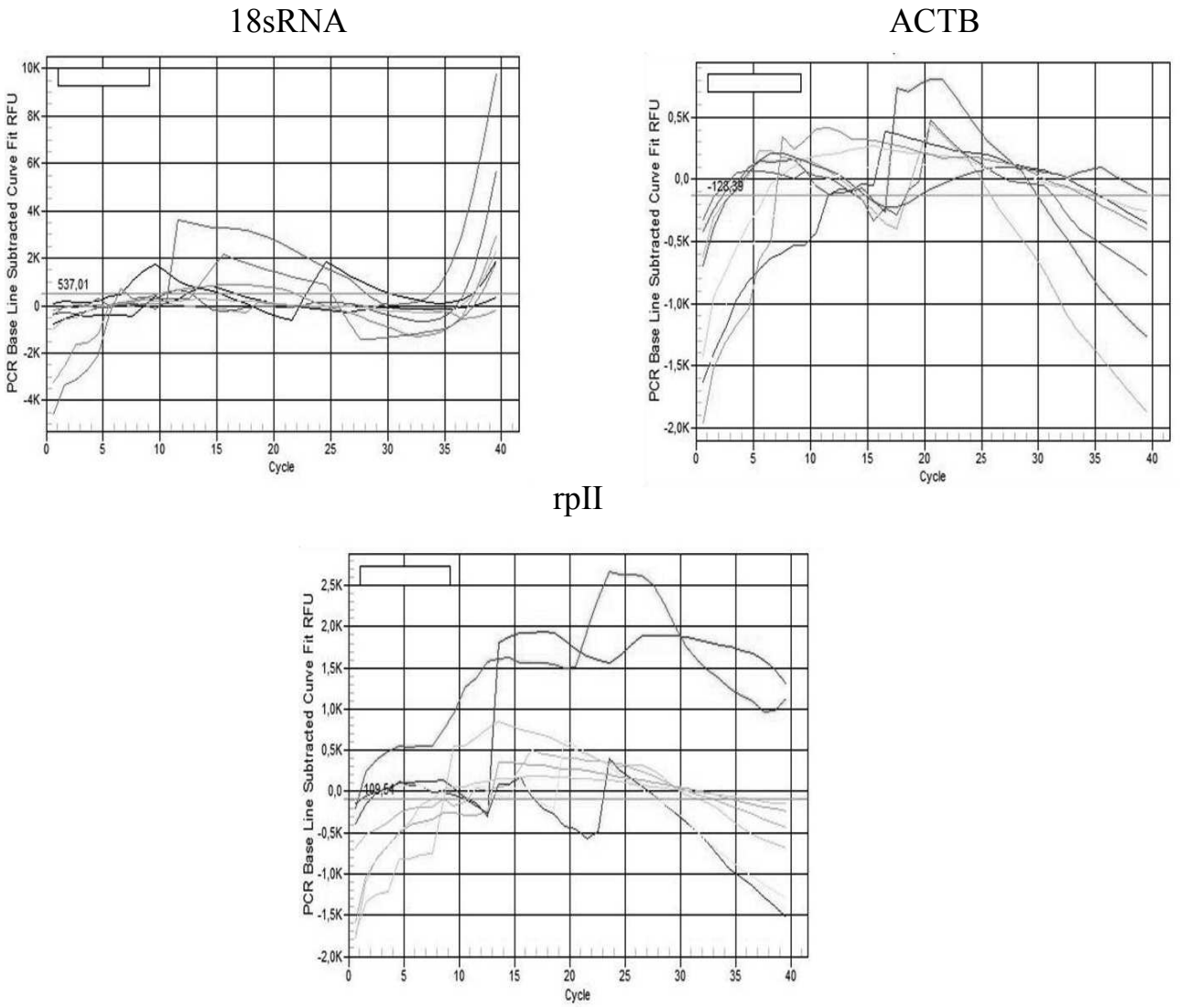

Figure 36. Amplification charts of the target genes: 18sRNA, ACTB, rpII

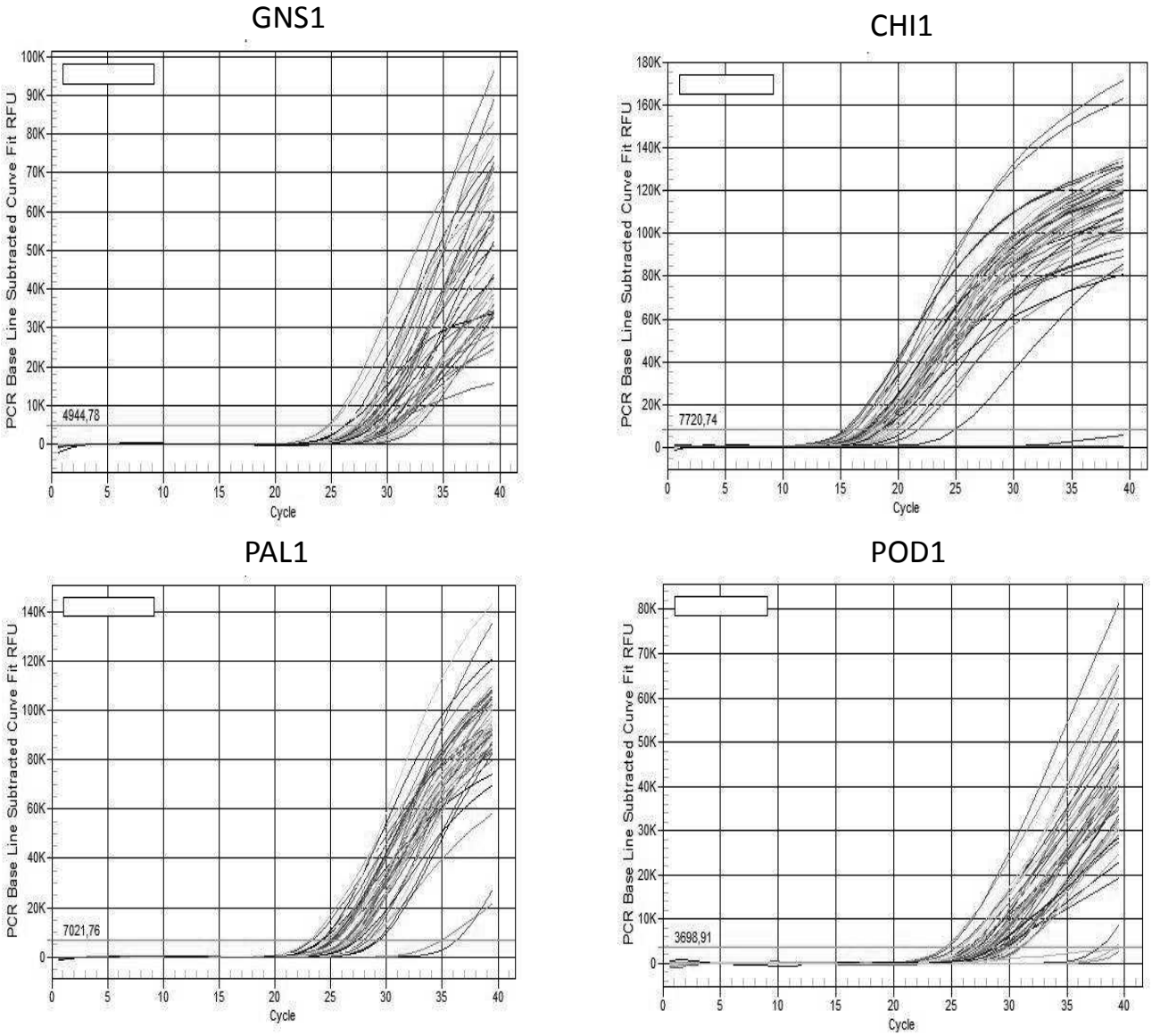

Figure 37. Amplification charts target genes: GNS1, CHI1, PAL1, POD1,

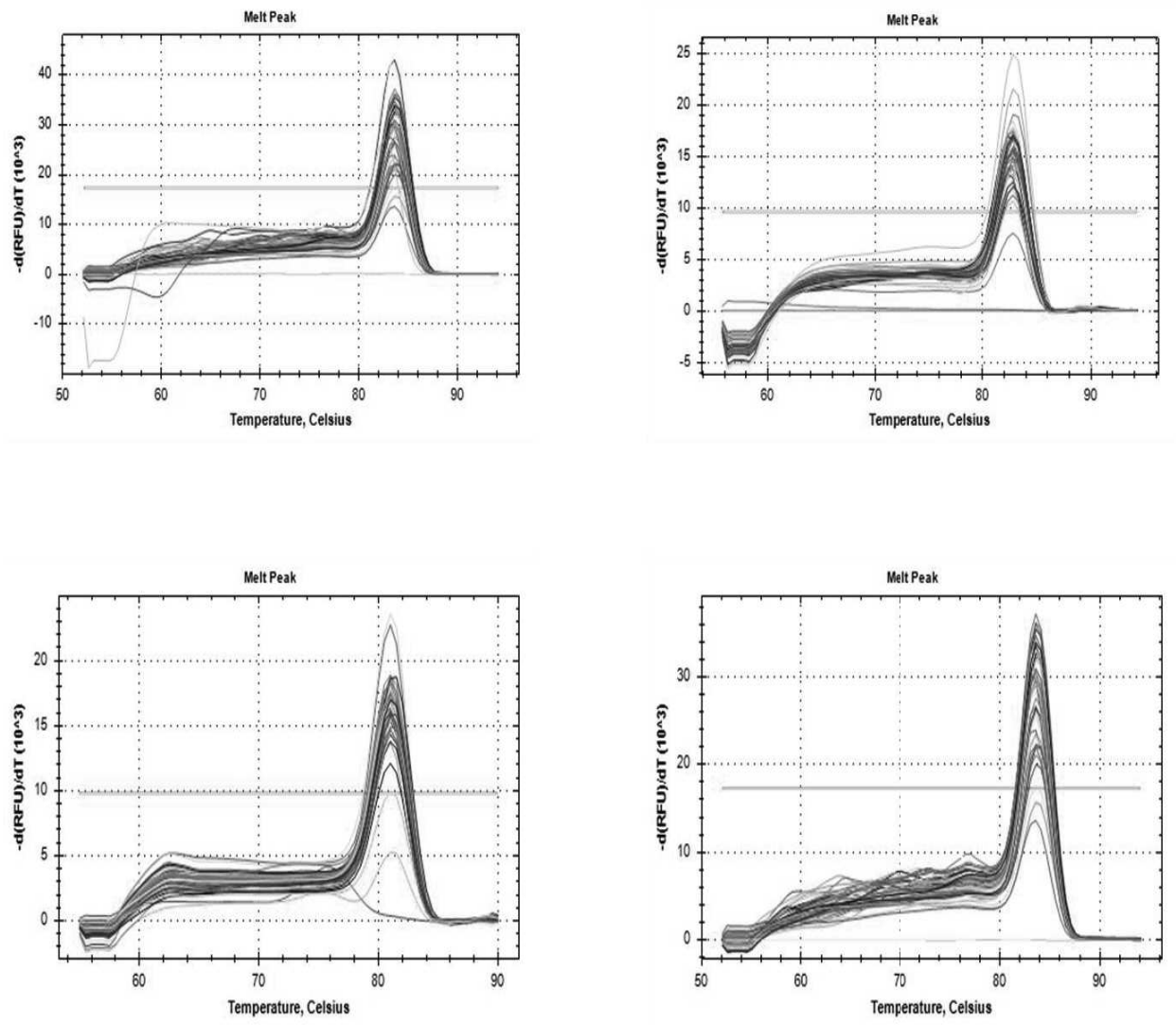

Figure 38. Melt peaks of the target genes: (A)GNS1, (B)CHI1(C) PAL1, (D)POD1

GNS1 showed an increase in gene expression level in fruits treated singly with ozone. The highest peak of gene expression is noticed at 24 hours after ozone exposure with a 22-fold change compared to the control (Figure 38).

Similarly, CHI1 gene expression showed some increase 6 h after ozone exposure, and reached its maximum expression level at 48 h (21 times higher compared to the control). The expression level of CHI1 in fruits inoculated with the pathogen *P. digitatum* peaked at 12 h (9 times higher than the control), and decreased at 24 h. We observed that CHI1 gene was expressed in a less drastic way in fruits treated by both ozone and *P. digitatum* than in fruits singly exposed to either ozone or *P. digitatum* (Figure 40)

The expression levels of POD1 and PAL1 were higher in *P. digitatum* inoculated fruits. Indeed, PAL1 expression showed a peak after 24 h with 4-fold change compared to the control whereas a strong Increase of POD1 was observed after 12 h with 25-fold change above the control (Figure 41). Additionally, we noticed also a significant peak in PAL1 expression after 12 h with 2-fold change in fruits treated with ozone and previously inoculated with *P. digitatum* (Figure 42).

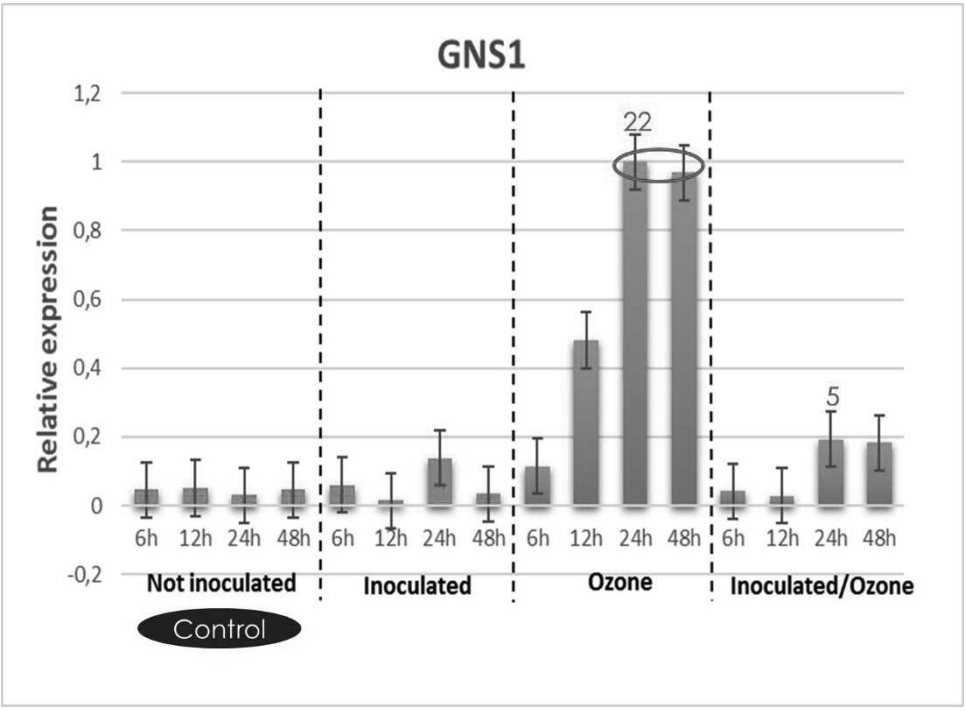

Figure 39. Relative quantification by real time RT-PCR of GNS1 gene expression in citrus fruits treated with ozone and inoculated with pathogen P. digitatum (either alone and together) during first 48 h.

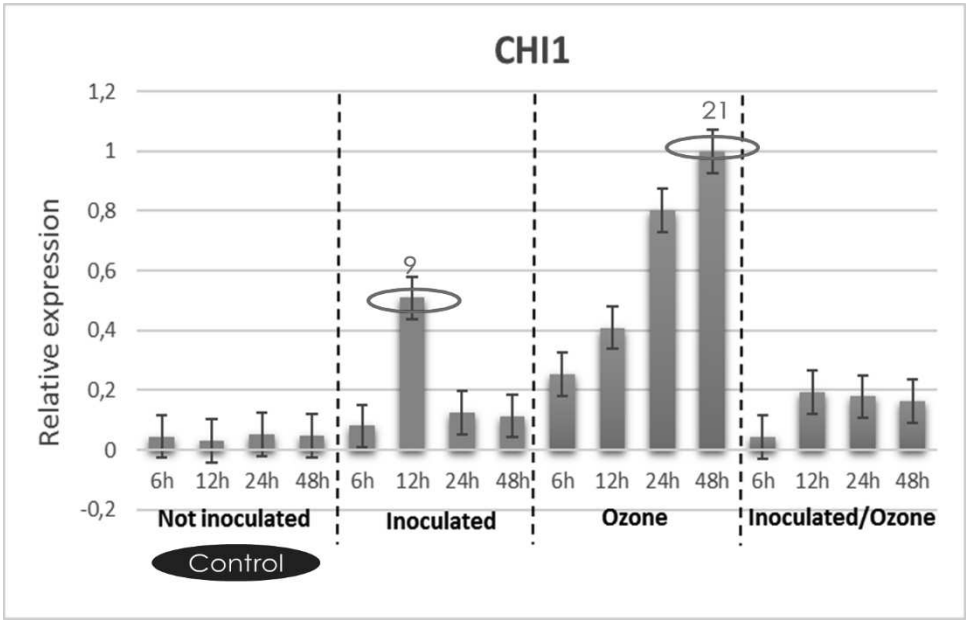

Figure 40. Relative quantification by real time RT-PCR of CHI1 gene expression in citrus fruits treated with ozone and inoculated with pathogen P. digitatum (either alone and together) during first 48 h.

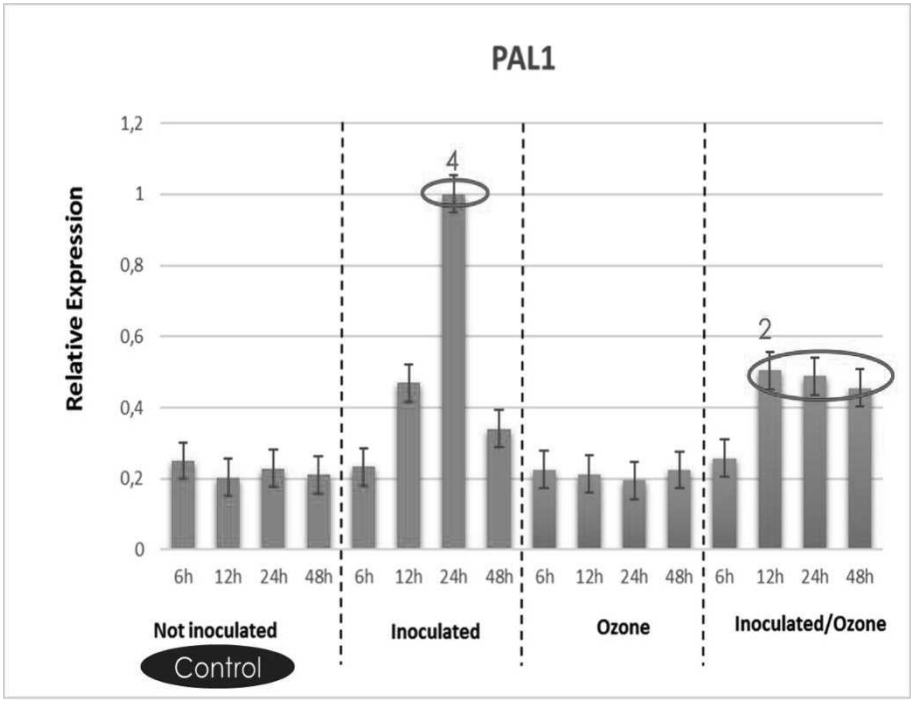

Figure 41. Relative quantification by real time RT-PCR of PAL1 gene expression in citrus fruits treated with ozone and inoculated with pathogen P. digitatum (either alone or together) during first 48 h.

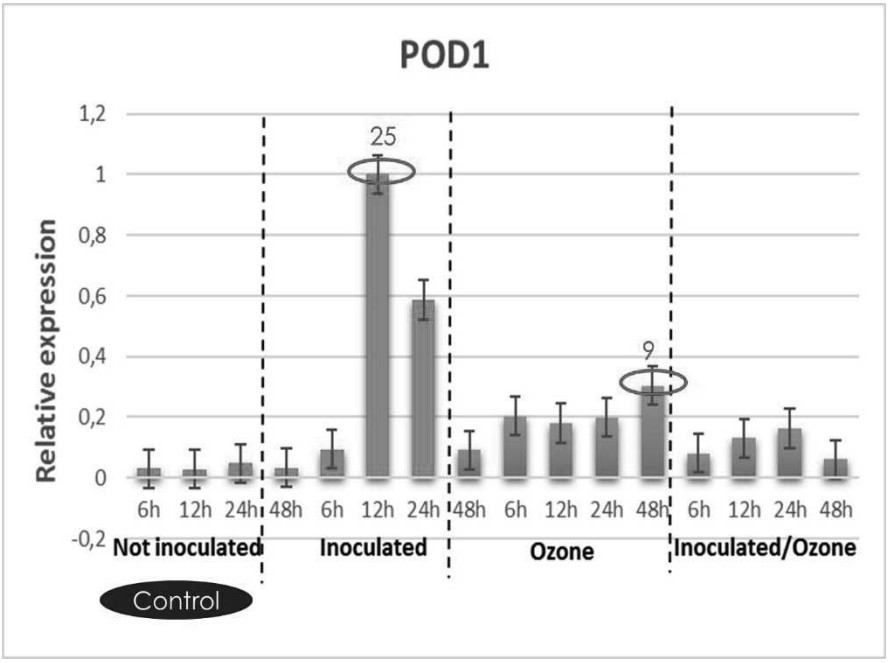

Figure 42. Relative quantification by real time RT-PCR of POD1 gene expression in citrus fruits treated with ozone and inoculated with pathogen P. digitatum (either alone or together) during first 48 h.

5 Chapter 5 - Discussion

5.1 The effect of ozone and EW on microorganism population and on the quality and shelf life of stored orange fruits

In this work, we tried to determine the effect of ozone, electrolyzed water and passive refrigeration treatment on the development of microorganism populations present on the surface of citrus fruits. Moreover, we evaluate effectiveness of studied methods on quality parameters like: hardness, sugar content and fruits shelf-life. The efficacy of ozone and electrolyzed water in the control of post-harvest pathogens was demonstrated by several researchers on many fungi and fruits. Smilanick *et al.* (1999) prove that spores of *Penicillium digitatum*, requires two to three minutes of ozone exposure to deactivate germination. It is well known that, storage conditions are the key factor in shelf life extension beside to ozone application. Therefore, relative humidity value play an important rule for the calibration of the concentration of ozone to be used in fact at 95% RH, 99% of the conidia of *P. digitatum*, *P. italicum*, and *B. cinerea* were unable to germinate after O_3 exposures to 817, 732, and 702 $\mu L x L^{-1} \times h$, respectively (Ozkan *et al.*, 2011). Ozone efficacy also depends on the ability of ozone gas to penetrate through packaging materials (Palou *et al.*, 2003) and its effectiveness in controlling sporulation of *P. digitatum* and *P. italicum* on artificially inoculated and commercially packed oranges and sporulation. Ozone have also side effects on the concentration of fructose, galactose and sucrose sugars, which increased in treated kiwi fruits during storage in both air at 0°C and ozone enriched environment (Barboni *et al.*, 2010), and the maintenance of the tissue firmer than in control fruit (Aguayo *et al.*, 2006).

Ozone fumigation applied in this work goes in the same line with the previous researches. In the experiments, we tried to use several modality of ozone application (dissolved in water or as ozone gas application). In fact, the results of experiment A demonstrate that washing orange fruit with ozonized water (2ppm) for 15min significantly reduce fruit contamination (Figure 13). Also washing the fruit by electrolyzed water proved to be efficient in reducing disease incidence, Whangchai *et al.* (2010a) has proved that complete inactivation of spore germination occurred in all concentration of EW when the exposure time increased to 16 min. The duration of exposure time is the major factor effect of EW. When the fruits inoculated with *P. digitatum* where washed in EW for 4, 8 and 16 min

then stored at 5°C for 18 days, it was found that the immersion of the fruits for 8 min in the EW was more effective to reduce disease incidence. Such long time of washing 15-16 min not practical for packinghouses, this is why in the experiment D higher EW concentrations and shorter time of washing were used.

Experiment B, in *in-vitro* trial the application of ozone gas in 1 or 2 ppm for short time 15, 30, 60,120 min were significantly reduce the contamination on fruit surface (Figure 16), induce fruit hardness (Figure 17), increase fruit sugar content (Figure 18) and reduce disease incidence (Figure 19). Indeed, the application of 2ppm for 120 min proved to be the best application at the end of storage time 10 days.

In experiment C, washing inoculated orange fruits with different concentration of EW (10% and 20%) and ozonized water (2ppm) (Figure 20), demonstrate the efficacy of ozone application by to be more powerful than washing the fruits with EW 20%, but both application reduce significantly disease incidence.

We can conclude form experiment D, that washing orange fruits by EW 500ppm of active chlorine (15%) for 15 min have the same effect as washing the fruit by EW 1200ppm of active chlorine (30%) (Figure 21). So, the effect of EW is strongly related to contact time contact time (Izumi, 1999) and by EW concentration. However, the application of EW (15%) for 1 min had the same effect like EW (30%) for 1 min, clustering in the same value. Therefore, we conclude that the application of EW (15%) for 1 min is suitable for the practical washing in packinghouses.

The combination between ozone application and passive refrigeration in experiment E showed the synergic effect of this post-harvest innovation methods, in order to maintain fruit quality for a longer time and prevent the infection by postharvest disease. In fact, quality parameter as sugar content (Figure 24) and fruit firmness (Figure 27) were induced when they conserved in passive refrigeration, under 1ppm of ozone for 30 days. In addition of a significant reduction of CFU on the fruit (Figure 30).

As a conclusion of orange storage experiments, we strongly recommend the combined use of more than one of the experimented methods in this study Ozone, passive refrigeration and electrolysed water. The synergic effect of those

advanced methodology, can permit to reduce orange fruit losses in postharvest and increase the possibility to riche distant market facing the problem of food safety and food security.

5.2 The effect of ozone on the expression of plant related genes on citrus fruits

In order to perform this gene expression study, we wanted to adopt a relative expression study based on the use of at least one reference gene which is the method mostly used in relative gene expression studies. However, the three pairs of primers designed for the three reference genes (18SRNA, ACTB, rpII) were not successful. This could be due to the genetic variability among orange varieties. Indeed, we used "Navel" orange whereas the (Yan *et al*., 2012) used "Bigtang" sweet orange variety. Consequently, we decided to implement a relative expression study normalized against a unit mass, which does not require a reference gene. This method is less accurate than the one using a reference gene because of its sensitivity to pipetting error and the amount precision of the starting RNA. However, we tried to mitigate these inconveniences by a strict measure of the starting material (RNA) and by increasing experimental replicates (Pfaffl, 2004).

Furthermore, the primers designed for the target genes GNS1, CHI1, PAL1 and POD1 are validated and can serve for further gene expression studies on citrus fruits.

Results of the gene expression study provide evidence that treatment of oranges 'Navel' with ozone caused markedly increase of GNS1 and CHI1 gene expression. Indeed, chitinases are enzymes that hydrolyze the N-acetylglucosamine polymer chitin, and they occur in diverse plant tissues over a broad range of crop and non-crop species. The enzymes may be expressed constitutively at low levels but are dramatically enhanced by numerous abiotic elicitors such as ethylene, salicylic acid, salt solutions, ozone and UV light (Punja and Zhang, 1993; Yalpani *et al.*, 1994; El Ghaouth *et al.*, 2003). El Ghaouth *et al.* (2003) found that by 96 h after UV light treatment, chitinase and beta-1,3-glucanase in peach fruits were over two fold above the gene expression levels observed for the control. This indicate that UV light and ozone may activate the same pattern of plant defense mechanism in fruits. In addition, the rapidity of the CHI1 expression (6h after ozone exposure) showed the early response of this gene to ozone. This early response was also

demonstrated in Tobacco with a peak of expression only one hour after ozone exposure (Ernst *et al.*, 1992).

Chitinases and β-1,3-glucanases are two important hydrolytic enzymes that are abundant in many plant species after infection by different type of pathogens. The amount of them significantly increase and play a main role in defense reaction against fungal pathogens by degrading their cell walls. Indeed, these latter are mainly composed of chitin and β-1,3-glucan.

Furthermore, Ebrahim *et al.* (2011) affirms that β-1,3- glucanases are coordinately expressed along with chitinases. This was confirmed by our study where we observed a peak of expression of both CHI1 and GNS1 between 24h and 48 h on fruits singly exposed to ozone. This co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat (Mauch *et al.*, 1988; Vogelsang and Barz, 1993; Jach *et al.*, 1995; Bettini *et al.*, 1998; Lambais and Mehdy, 1998; Petruzzelli *et al.*, 1999; Cheong *et al.*, 2000; Li *et al.*, 2001). However, expression levels of GNS1 and CHI1 were lower in fruits successively inoculated with *P. digitatum* and exposed to ozone. This might be due to a short ozone exposure time of inoculated fruits. We can suppose that ozone effect needs more than 48 hours to induce high expression levels.

On the contrary, gene expression of phenylalanine-ammonia lyase (PAL1) is weakly induced in fruits singly treated with ozone and strongly induced upon inoculation with the pathogen alone. This means that ozone is less involved in the pattern of Induced Systemic Resistance (ISR) mechanisms. However, the less drastic peak of expression detected in fruits exposed to ozone previously inoculated with *P. digitatum* in PAL1 can be due to the presence of endogenous elictors in the fruit tissue. These elicitors can mediate the effect of ozone on the gene expression of PAL1 which needs more time to perceive the effect of this gas (Ernst *et al.*, 1992). Moreover, a similar tendency was noticed regarding POD1 where the highest peak of expression was detected on the fruits singly inoculated with *P. digitatum*. Indeed, several studies mentioned the effect of *P. digitatum* as biotic elicitor for the peroxidases as well as the phenylalanine-ammonia lyase in citrus fruits (Droby *et al.*, 1993a; Ballester *et al.*, 2006; Ballester *et al.*, 2010).

Further study can be done by increasing the time of exposure to ozone (More than 48 hours) in order to investigate the effect of ozone on phenylalanine-ammonia lyase (PAL1) and peroxidase (POD1). Other studies, show that elicitors can trigger the plant defense mechanisms even in the inner parts of the citrus fruit (Ballester *et al.*, 2013a; Ballester *et al.*, 2013b). This can be a serious lead to investigate especially if ozone can enhance defense responses in orange pulp.

Conclusions and recommendations: Elicitors are capable of inducing several biochemical defense responses in fruits, such as expression of defense related genes. In this work we designed and tested 4 genes related pathogenesis-related proteins. Indeed, we set up a RT-qPCR assay used to evaluate the influence of ozone and *P. digitatum* (on fruits either treated with ozone, or challenged by *P. digitatum*, or treated by both ozone and *P. digitatum*) on expression of these genes in citrus fruits. These genes are believed to be involved in different responses such as SAR (Systemic acquired resistance), production of reactive oxygen species (ROS) and induced systemic resistance (ISR). We found that ozone can seriously induce genes related to Systematic Acquired Resistance which are Beta-1,3-glucanase (GNS1) and chitinase (CHI1) especially in non-inoculated fruits. This means that ozone can trigger this defense mechanism before the infection of the fruit and that it can be used as a post-harvest treatment as soon as the fruits are picked-up from the field. However, ozone shows less effect on PAL1 and POD1 which are genes related to ROS and ISR mechanisms. This means that ozone is less involved in these defense mechanisms at least in the first 48 hours.

Additionally, the gene expression experiment should be completed by a protein expression analysis of the correspondent enzymes of all genes studied in this work. This will serve to check correlations between the level of the mRNA expression and the enzymatic activity.

Finally, the validated deigned primers of our target genes (GNS1, CHI1, PAL1, POD1) can be used in further studies to investigate the effect of ozone after 48 hours of exposure as well as to study its effect on the inner parts of the fruit.

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