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INACTIVATION KINETICS OF *BOTRYTIS CINEREA* INOCULATED ON STAINLESS STEEL COUPONS BY CHLORINE DIOXIDE GAS

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INACTIVATION KINETICS OF *BOTRYTIS CINEREA*

INOCULATED ON STAINLESS STEEL COUPONS

BY CHLORINE DIOXIDE GAS

A Thesis Presented for the

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Degree

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Aruna Dhanapal

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ABSTRACT

Botrytis cinerea (B. cinerea) is a soil borne fungus that produces airborne spores and causes serious damage to crops. Inactivation of *B. cinerea* has become a difficult challenge as it has become more resistant to fungicides, disinfectants, thermal, and non-thermal inactivation techniques, such as ultraviolet light and pulsed white light. Chlorine dioxide gas (ClO_2) has proven to have promising and effective antifungal properties. The aim of this study is to determine the inactivation kinetics (D-*value* and z-*value)* of *B. cinerea* on stainless steel coupons (SS) using very low concentrations of chlorine dioxide gas, and to determine the survival rate of *B. cinerea* on stainless steel coupons. The *D-value* and *z-value* can be used to determine the appropriate combination of gas concentration and treatment time to achieve the desired log reductions of *B. cinerea. Botrytis cinerea* at initial levels of ~ 6 log spores/ml on SS coupons was treated with 36, 60, and 90 ppmv (0.1, 0.17, and 0.25 mg/l respectively), of ClO_2 gas for 15, 30, 45, and 60 min; 6, 13, 20, and 26 min and 4, 8, 16, and 24 min, respectively. The first order linear model D-*values* for 36, 60, and 90 ppmv were 22.34 ± 2.53, 10.18 ± 0.75, and 10.65 ± 0.44 min respectively with a z-*value* of 175.43 ± 0.19 ppmv. The D-*value* decreased as the ClO_2 gas concentration increased. The survival analysis determines the efficiency of *B. cinerea* to survive on the surface of the SS coupons without any added nutrients. It is important to determine the survival rate of *B. cinerea* during food processing or on food contact surfaces.

In this study, the survival rate decreased from 60% on Day 3 to 17% on Day 4.

Also, in this study, *B. cinerea* survived on the coupons without any nutrients for 7

days, which was the limit of testing.

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CHAPTER ONE

INTRODUCTION

The demand for fresh produce has increased recently because of its vital role in the human healthy diet (1). However, fresh produce is susceptible to contamination by spoilage microorganisms at any point from harvest to shelf. Unlike pathogens, they do not cause disease in humans, but they are responsible for spoilage of fruits and vegetables that cause major economic losses (2) and accounts for 20% of post-harvest loss in the United States (3). This is also a major concern in countries where there are no proper storage facilities for harvested crops (4).

Post-harvest infection can occur as early as the time period between flowering and fruit maturity, or anytime during harvesting, handling, transportation, storage, and /or after reaching consumers (5).The rate of spoilage caused by microorganisms depends on pH, water activity (a_w),and temperature of the fruits and vegetables (6), (7). Fruits are very rich in sugar and nutrients, making them highly susceptible to bacterial and fungal spoilage (8). Spoilage with mold results in development of spores and visible mycelial growth on the product (9). *Botrytis cinerea* is a necrotrophic fungus causing spoilage in more than 200 varieties of crops (10). Almost all parts of plants, i.e., leaves, stem, flowers, and fruits, are susceptible to gray mold infection caused by *B. cinerea* (11). It is hard to control or inactivate *B. cinerea* due to its ability to survive as mycelia or conidia (10). Sclerotia are an

important survival structure of *B. cinerea* that persists during unfavorable environmental conditions (5). Knowledge of survival strategies and growth characteristics of the fungus is important for effective sanitation treatment (2). Often, microorganisms that adhere to food contact surfaces as well as to the surface of food products have been found to develop resistance to sanitation treatments (12). As increased limitations of most of the effective fungicides occur and there is a decrease in residue tolerance limits, there is a critical need for better sanitation methods for food and environmental surfaces (13).

Chlorine dioxide has proven to be an effective sanitizer both in an aqueous and gaseous state (14-21). Its usage as a disinfectant has been approved by EPA and FDA in drinking water (< 1 ppm chlorite ion in treated water), for controlling bacteria in poultry processing water (< 3 ppm of residual ClO_2), brewing, sanitizing water for washing whole fruits and vegetables, and sanitizing food processing equipment (22, 23). Also, ClO_2 can be used as a sanitizing solution for water washing fruits and vegetables before processing. It can also be used for sanitizing equipment and other food contact surfaces, and as well as on the package of the finished product to extend its shelf life (2). The ClO_2 gas has already been proven to be an effective sanitizer against fungus on the surface of equipment as well as on produce (19).

In contrast to other sanitizers, ClO_2 is less corrosive due to its effectiveness at a lower concentration, unlike other chlorine-containing sanitizers. ClO_2 effectiveness is not as significantly affected by the presence of organic substances as chlorine therefore it has been studied for treating fruits and vegetables that are rich in organic matter (24). Little is known about the effect of low concentrations of ClO_2 gas on bacteria, virus, and fungi (25). Though various studies have reported the effects of a high concentration of ClO_2 gas on fungi and bacteria on produce, there has been very limited research focused on the effectiveness of ClO_2 gas on hard food contact surfaces (26). This has led to an increasing demand to develop more effective and efficient disinfectants for food contact surfaces (12). However, the inactivation kinetics of *B. cinerea* on stainless steel (SS) surface using low concentration of ClO_2 gas has not been reported.

The objectives of this study were to determine (i) the inactivation kinetics (D- *value* and z-*value)* of *B. cinerea* on an SS surface by ClO_2 gas; and (ii) the survival rate of *B. cinerea* on SS coupons without any nutrients. Results from this study will help to determine the effective combination of the lowest ClO_2 gas concentration and treatment time for inactivation of *B. cinerea.*

CHAPTER TWO

LITERATURE REVIEW

Fruits and vegetables are prone to contamination by spoilage microorganisms that are generally not harmful to humans, but are responsible for economic losses and food product losses (2). These spoilage organisms cause undesirable changes in fruits and vegetables producing off odors, flavors, and mushy or slimy textures. The growth of microorganisms depends on the intrinsic factors of that fruit or vegetable (7). Fungal contamination can also increase the pH of tissues, thereby increasing the growth of pathogenic microbes such as *Salmonella, Escherichia coli* O157:H7*, and Clostridium botulinum* (27),(28),(29)*.* Similar contamination can be observed in a food processing plant, where the surfaces that are in frequent contact with fresh produce are a high source of contamination. The surface of equipment, walls, floors, tables, and cutting boards are all potential sources of microbial contamination (30),(31).

Knowledge of the behavior and growth characteristics of fungi will help to determine suitable sanitizing technology to reduce spoilage caused by a specific fungus (2). Sanitizers are agents that help to reduce and control the growth of microorganisms to the safe level, either on the food contact surfaces or on the surface of the produce itself. On the other hand, disinfectants are chemical agents

that kill pathogens on the inert surfaces at a specific rate (32),(33). Studies on ClO_2 have demonstrated its effective antifungal properties (34).

Botrytis cinerea

Botrytis cinerea is a necrotrophic, airborne fungus that is responsible for gray mold disease in more than 200 crops worldwide (35). It is a unique fungal pathogen, which can live pathogenically as well as saprophytically. It is well known for its latent infections in plants that cause detrimental effects in fruits even before ripening (36). Gray mold disease caused by *B. cinerea,* gained attention after an outbreak on strawberry in Louisiana, U.S.A (37), (38). It is important to understand the nature and behavior of this fungus, to help in determining the most effective inactivation strategy (2). The fungus produces a mycelia, sclerotia, microconidia, macroconidia, ascospores, chlamydospores, and apothecia (5). Sclerotia and mycelia are considered to be important survival structures (5). Understanding the behavior of sclerotia and mycelia of *B. cinerea* is important to design effective control methods (39). Infections caused by *B. cinerea* are from saprophytic mycelia or spores on the surface of the fruits (38).

Botrytis cinerea affects a wide range of fruits (grape, strawberry, raspberry, blackberry), vegetables (cabbage, lettuce, broccoli) and protein-rich legumes (10). Ben et al. (11) studied the spoilage caused by *B. cinerea* on cucumber plants. The effect of chitin and chitosan on *B. cinerea* were studied. A 50% inhibition of

germination was observed with a relatively minimum level of 20-30 µg ml^{-1} of chitosan, and complete inhibition was observed at 50 µg ml^{-1}. Chitin oligomers mixture (95% DA, 2-8 degree of polymerization) at 0.1% (w/v) spray had no effect on the fungus at any concentrations, although complete inhibition was observed with chitosan (50 ppm) 1h before inoculation with *B.cinerea* .Chitosan was found to be more effective in controlling the disease (65%) in cucumber plants when the leaves are sprayed with chitosan before 1h of inoculation(11). Similarly Reddy et al, observed that pre-harvest chitosan spray had the ability to control the infection of strawberries against *B. cinerea* in the field (40). Chitosan develops glucanohydrolase activity in strawberries that is unfavorable for *B. cinerea* (41). Chitosan (6g/l) that was sprayed twice in 10 days protected strawberries from decay without compromising quality for 4 weeks at 3˚C (40). *B. cinerea* spoilage is predominant in grapes, and Keller et al. (42) determined that the common entry point for *B. cinerea* was the receptacle of grape flowers because there is lack of quercetin derivative and low concentrations of flavanols and cinnamic acid derivatives, which are present in other florals (42). In strawberries, flowers are infected by *B. cinerea* and the pathogen grows into the developing fruit. This is the important cause of decay in developed fruit after harvest (5).

Cope et al. (33) studied the dosage effect of different disinfectants (chlorazene hydrosol, hydrogen dioxide, quaternary ammonium chloride, hydrogen peroxide, iodine, sodium hypochlorite) on *B. cinerea* on various plant production surfaces

(galvanized metal, stainless steel, ground fabric, pressure-treated pine, exterior latex-painted pine, polyethylene, polyethylene pots, raw pine). The study concluded, that the dosage of disinfectants for inactivation of *B. cinerea* depended upon the growth substrate. Raw pine was observed to be the highly reactive surface and required high dose of disinfectant(33).

Disinfecting methods for *B. cinerea*

Pulsed white light and UV-C or mild heat

Pulsed white light and combinations of pulsed white light with heat or UV-C on conidia of *B. cinerea* and *Monilinia fructigena* have been studied (43). A maximum conidial reduction of 3-4 log units was observed with 30 µs of pulses at 15 Hz frequency for 1 to 250 s treatment, but inactivation of conidia was not achieved lower than 40 s. A 2 log reductions were obsereved from 40-100 s and after 100 s only minimal conidial inactivation was observed. Complete inactivation was not observed even after 250 s of treatment. However, maximal inactivation(3-4 log reductions) was achieved by a combination treatment of pulsed white light and UV-C (short-wave band from 180-280 nm with in a peak of 254 nm) or a combination of pulsed white light and heat (43). When combining UV-C and pulsed white light, a maximum inactivation was observed at the highest dose concentration of 0.10 J/cm^2 of UV-C and 120-s exposure to pulsed white light. When a combination of heat and pulsed white light was used, a 3.5 log reduction was observed at 43°C/10 min and 120-s pulsed light treatment. They observed synergism in the combination

treatment, which had a greater inactivation effect than the sum of inactivation of the individual treatments (43). Similar results of synergism were observed in another study with a combination treatment of thermal (44°C/ 4 min) and γ-irradiation (75- krad), where sequence of the treatment had an impact on the survival (%) of *B. cinerea.* A 30% survival rate was observed with irradiation followed by heat treatment, and 5% survival rate was observed when the fungus was exposed to heat treatment followed by irradiation (44).

Heat treatment

Numerous thermal inactivation studies have been conducted on *B. cinerea*. Hot water inactivation of conidia in sterile strawberry puree medium (SSP) and a synthetic medium (comprised of similar sugar and organic acid formulations of strawberry fruit in phosphate buffer solution) have been studied (45). At 42˚C, *B. cinerea* in strawberry medium was more resistant to thermal treatment than in the synthetic medium (45). In another study, D-*value*s of 30 min and 6.7 min were determined at 40˚C and 43˚C, respectively, in a phosphate buffer (43). The differences in *D-value* was attributed to media composition (45). Complete inactivation of *B. cinerea* spores was achieved at 40˚C with 30% (v/v) ethanol (47). However, only 20% (v/v) ethanol was required for complete inactivation of spores when temperature was increased to 50˚C (47). Like other studies, they found synergism in the combination treatment of ethanol and heat treatment to be more efficient for inactivation of fungal spores. The combination of ethanol and effective

heat treatment can be a useful disinfection tool for food processing equipment and packaging (47).

Ozone

Ozone (O_3) is an effective and natural antimicrobial treatment against a wide spectrum of microorganisms (48). Liew et al. (49) studied the effect of ozone (0.3-1.5 µLL^{-1}) on *B. cinerea* on carrots and determined that ozone was effective in reducing mycelial growth and sporulation at very low concentrations. Charles et al. (50) studied the effect of ozone in-vitro and in-vivo on the viability of conidia. Conidial germination had been inhibited at 0.3 ppm ozone for two 6 h periods on consecutive days, in-vitro and in-vivo (50).Sharpe et al. (51) observed the effect of temperature and ozone on mycelia and spore germination of *B. cinerea.* Spore viability was reduced by 99.5% when exposed to 450 ppb of ozone for 48 hr at 20˚C. Ozone treatment was more effective at 20˚C than at 5˚C (51). However, ozone treatment was found to be more effective for reducing population of bacteria rather than fungi (52),(53).

Chemical control

Botrytis cinerea may become resistant when repeatedly exposed to the same chemicals or fungicides (10). Inactivation of *B. cinerea* using fungicides has been limited to one dose per season in vineyards in France to maintain the MRL

(Maximum Residue Level) values within limits (5). Synthetic fungicides re categorized as (i) those affecting fungal respiration,(ii) anti-microtubule,(iii) those affecting osmoregulation,(iv) sterol biosynthesis inhibitors, and (v) fungicides whose toxicity can be reversed with amino acids (5, 10). Studies have recorded that *B. cinerea* develops resistance towards fungicides rapidly. Reavill et al. (54) studied the effect of tetra chloronitrobenzene (TCNB) vapor on *B. cinerea.* The fungus was more resistant to pentachloronitrobenzene (PCNB) than TCNB. TCNB has been observed to control the rate of sporulation (54). Bollen et al. (55) observed resistance of *B. cinerea* to benzimidazoles (55), Elad et al. (5) stated that due to the resistance of *B. cinerea*, benzimidazoles have not been registered as a control fungicide for *B. cinerea* (5). Clearly, there is a demand to develop better inactivation techniques for *B. cinerea.*

Fallik et al. (56) studied the effect of hydrogen peroxide on *B. cinerea* to prevent post-harvest contamination in eggplant and sweet red pepper. Sanosil-25 is an effective disinfectant containing 48% hydrogen peroxide and 0.05% silver ion as a stabilizing agent. Treating eggplant and red pepper at 0.5% sanosil-25 for 20 min reduced spore germination and mycelial growth of *B. cinerea* (56). Similar results were observed by Forney et al. (57) where vapor phase hydrogen peroxide was used to reduce the post-harvest decay caused by *B. cinerea* on grapes. Thompson Seedless and Red Globe grapes exposed for 10 min to 30-50% liquid hydrogen peroxide reduced the number of *B. cinerea* spores in Thompson Seedless and Red

Globe grapes to 81% and 62%, respectively. The incidence of decay was also reduced to 33% and 16%, respectively, after 8 days of storage at 10˚C (57)

Chlorine dioxide

Chlorine dioxide has proven to be an effective sanitizer, both in aqueous and gaseous states (14-21). It has been used as a disinfectant since the 1920s (23). Apart from sanitizing the surface of fruits and vegetables using ClO_2 gas, hard surfaces like belts, metal conveyors and pads are treated with ClO_2 foam as they are also one of the important transmitters of contamination during packaging or processing of fruits and vegetables (13). Since ClO_2 gas is highly soluble in water, its inactivation properties are similar to the aqueous solution (58), (59). The kill mechanism of ClO_2 is by interfering with protein synthesis (14), and by reacting with proteins and lipids, thereby increasing the permeability of the outer membrane (60), which causes loss of intracellular ions (61) and major destruction. This theory of kill mechanism of ClO_2 gas was observed in several studies. Han et al. (17) determined that when *Listeria monocytogenes* and *E. coli* O157:H7 were treated with higher gas concentration (1.8 and 3 mg/l), their resistance was similar. However, at lower gas concentration and short treatment time (0.6 mg/l for 15 min), *L. monocytogenes* was more sensitive than *E. coli* O157:H7. The difference in resistance between Gram-positive and Gram-negative bacteria, is due to the difference in cell wall structure and components (17).

Chlorine dioxide is one of the major disinfectants used for water treatment to control odor and taste as its oxidation capacity is 2.5 times greater than chlorine (62).Studies have reported that ClO_2 gas is more effective than chlorine in inactivating surface microorganisms (63). It was reported also to produce less carcinogenic substances and chlorinated by-products than chlorine (64).Chlorine dioxide gas was found to be an effective fumigant against fungus associated with sick building syndrome, but the treatment was not effective against mycotoxins (Satratoxin G produced by *Stachybotrys.chartarum*) associated with sick building syndrome. This is because the location of mycotoxin in the outer and inner layer of conidia makes it difficult to reach for inactivation (65). At 3 and 5 ppm, aqueous ClO_2 was more effective than other sanitizers (peracetic acid at 80 ppm, Chlorinated trisodium phosphate (CTP) at 100 ppm, and 200 ppm chlorine) for inactivation of *E. coli* and *L. monocytogenes* on apple, lettuce, strawberry, and cantaloupe. At 3 ppm, aqueous ClO_2 treatment of apples, lettuce, strawberry, and cantaloupe reduced populations of *E. coli* and *L. monocytogenes* to non-detectable limits when compared to other sanitizers (63). Roberts et al.(13) also studied the effect of aqueous ClO_2 on *B. cinerea* and found that it was the least sensitive fungus to ClO_2 and the minimum dose-exposure combination to achieve 100% spore mortality was 5 µg/ml for 2 min (13). Spotts et al. and Roberts et al., reported similar observations on the efficacy of ClO_2 against *B. cinerea.* They suggested that *B. cinerea* was resistant against ClO_2 at lower concentrations.

Studies have reported that both aqueous and gaseous ClO_2 have a wide range of inactivation effects on bacteria, viruses, fungi and protozoans, including *Cryptosporidium (*oocysts) and *Giardia* cysts. (23). The gas form of ClO_2 had greater penetration than the aqueous form, and the residual water after treatment with aqueous ClO_2 could further promote the growth of molds (21). A comparative study of aqueous and gaseous ClO_2 has resulted in a better outcome with gaseous chlorine dioxide being more effective on *L. monocytogenes.* A 6 log CFU/ 5g was observed with *L. monocytogenes* with 3 mg/l of gaseous ClO_2 on uninjured green peppers, while the same concentration of aqueous ClO_2 resulted in only 3.7 CFU/ 5g of uninjured peppers. When injured green peppers were treated with 0.6 mg/l of ClO_2 gas, there was a 3.5 log reduction, while aqueous ClO_2 at the same concentration resulted in only 0.4 log reduction of *L. monocytogenes* (66*).* Studies have also reported the effects of high concentrations of ClO_2 gas on fungi and bacteria on produce, as well as on environmental surfaces. The gas ClO_2 has been reported to control mycelial and conidial germination of *Alternaria. alternata* and *Stemphylium. vesicarium* (76). Under in vitro conditions, 10 mg/l of ClO_2 gas treatment for 3 min on PDA (15 ml inoculated with 10 mm plugs of fungus) reduced *A. alternate* and *S. vesicarium* mycelial growth from 77.75 ± 0.26 and 72.25 ± 0.32 mm diameter to 11.00 ± 0.05 mm. Complete inhibition of spore germination was observed after 10 mg/l of ClO_2 gas treatment for 3 min under in *vitro* conditions (76). Under *in vivo* conditions, complete inhibition of *A. alternata* and *S. vesicarium* on tomatoes was achieved only after 7 and 5 min, respectively (76).

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Factors affecting ClO₂ efficiency

Mahmoud et al. (67) determined that the type of product also affects the inactivation rate of ClO_2 gas treatment. A treatment of 5 mg/l of ClO_2 for 10 min was required to achieve a 3 log reduction of *E. coli* O157:H7 and a 2.8 log reduction for *Salmonella. enterica* from a initial concentration of $1x10^8$ CFU/ml of both the bacteria on lettuce. This was comparatively fewer reductions when compared with a 7.3 log reduction of *E. coli* on green pepper with 0.6 mg/l of ClO_2 gas for 30 min observed by Han et al. (68). The study results indicate that inactivation of the same organism in a different product requires different exposure time and concentration (68).

Han et al studied (69) the effect of gaseous ClO_2 concentration (0.1 to 0.5 mg/l), temperature (5-25°C), RH (55-95%) and treatment time (7 to 135 min) on the inactivation efficiency of ClO_2 against *E.coli on green peppers.* A linear increase in log reduction was observed with an increase in ClO_2 concentrations and relative humidity (RH). A linear increase of log reductions was observed with an increase in temperature, but it had the least significant effect when compared to RH and ClO_2 concentration. For time factor, a non-linear increase in log reductions was observed perhaps because there was a lack of ClO_2 distribution during the first 15 min (69).

Another factor that influenced ClO_2 efficiency was studied by Gomez-Lopez et al. (64) and Yuk et al. (70), i.e., "amount of produce relative to the amount of ClO_2 treatment.". On the other hand, the amount of ClO_2 is proportional to the volume of the treatment chamber and the degradation of ClO_2 is related to the amount of organic matter present in the sample. Degradation of ClO_2 reduces the amount of gas available for inactivation of microorganisms. A larger sample in a smaller treatment chamber results in faster degradation of ClO_2 than a smaller sample in a larger chamber (64).

Surface integrity of the product also affects the efficiency of ClO_2. This effect was studied by Han et al. (68) where more efficient reduction of *E. coli* was observed on uninjured bell pepper than on the injured bell pepper (69). The difference is because the injured surface hides the attached bacteria from exposure to sanitation treatment (68).

The location of the microorganism on the product also influences ClO_2 efficiency. Du et al. (72) observed that inactivation of *L. monocytogenes* on apple pulp skin using ClO_2 was more effective than on apple calyx and stem as it can hide bacteria and prevent it from exposure to proper sanitation treatment (71, 72).

Han et al. (82) studied the effect of Relative Humidity (RH) and temperature on ClO_2 gas treatment on an epoxy coated stainless steel. With an increase in RH or

temperature, the survival rate of *L. buchneri* decreased regardless of gas concentration (8 mg/l for 10 or 30 min and 10 mg/l for 10 or 30 min). The sanitation method was effective at room temperature, rather than under refrigerated conditions or lower RH.

Similar results were observed by Park et al. (80) on the effect of RH on the antimicrobial effect of ClO_2 gas to inactivate *E. coli* O157:H7*, S. Typhimurium*, and *L. monocytogenes* on spinach leaves at 10, 30, and 50 ppmv ClO_2 gas. The three pathogens were reduced to below the detection limit at 50 ppmv for 15 min at 90% RH. ClO_2 gas treatment under 90% RH showed significantly more reductions in population than 50-70% RH under the same gas concentration and treatment time. The difference in the reductions was due to the solubility rate of ClO_2 at different RH values (80).

Morino et al. (25) observed similar results with a study on a low concentration of ClO_2 gas against *influenza A virus (Flu-A), feline calicivirus (FCV),Staphylococcus aureus and E. coli* on a glass surface under wet conditions (without any drying process). The ClO_2 gas concentration (mean) of 0.05 ppmv for 3 h and 5 h had >2 log reductions of *E. coli* and *S. aureus* respectively. Similar >2 log reductions were observed for FCV at 0.05 ppmv for 4 hours, and Flu-A was reduced to below detection limit after 3 h treatment. All the inactivation treatments were carried out in a wet state. This study suggested that moisture was an essential factor for

inactivation of bacteria and viruses on a hard surface using a low concentration of ClO_2 gas (0.05 ppmv) (25).

Vandekinderen et al. (81), studied the effect of food composition on ClO_2 gas during the inactivation of foodborne microorganisms. Inactivation of *Leuconostoc mesenteroides,* responsible for spoilage of cured meats and minimally processed carrots using ClO_2 gas was studied. *Leuconostoc mesenteroide* was grown in agar plates supplemented with corn oil, starch, NaCl, fat and protein. The study *observed* that the antimicrobial activity of ClO_2 against *Leuconostoc mesenteroides* was influenced by the food components such as protein, fat and carbohydrates. Also, carbohydrate rich foods were found to be more resistant to ClO_2 treatment as these foods are the source of contamination for bacterial spores, yeast and mold which requires higher concentration of ClO_2 and longer exposure time.

Vandekinderen et al (81) also studied the effect of gas concentration of 0.08 ± 0.02 mg ClO2/L for 1 min to treat Gram-positive and Gram-negative bacteria, yeast and mold spores. They found a mean log reduction of 3.5 and 2.6 log CFU/cm^2 for Gram-negative and Gram-positive bacteria, respectively, and a mean of 1.1.log CFU/cm^2 reduction for yeast. However, the bacterial spores (*B. cereus*) and mold spores (*Aspergillus niger, Penicillium roqueforti, Botrytis cinerea)* did not

have noticeable reductions. For *B. cinerea*, only 0.9 ± 0.4 log CFU reductions were observed (81).

ClO_2 gas generating methods

ClO_2 gas being highly unstable and under pressure it is a highly explosive gas. According to EPA (Environmental Protection Agency, 1999), ClO_2 gas is not permitted to be shipped. ClO_2 should be produced only on site at the point of use. Chlorine dioxide is explosive at concentrations above 10% and it could be ignited by any form of energy such as sunlight, spark or heat (*New Jersey Department of health and senior service, Hazardous substance factsheet, 1998*). Different methods have been adopted for generating gaseous ClO_2: 4% chlorine in 96% nitrogen gas was used for reaction with sodium chlorite to generate ClO_2 in a CDG laboratory generator (CDG Technology, Inc., N.Y., U.S.) (73). Another simple method is a dry chemical pouch method (Intellectual Capital Associates [ICA] TriNova, LLC, GA, U.S.). This method utilizes activation of an equal quantity of sodium chlorite along with activating acids in a sachet. The sachet is placed inside the distilled water at room temperature (in dark) for 3 days for complete release of ClO_2 in the water. Followed by refrigeration (4°C) and stored it as a stock ClO_2 solution (pH 4.2). The amount of ClO_2 is measured by DPD method (N,N-diethyl-r-phenylenediamine) and the required concentration can be achieved by diluting the stock.(21). The third method, called Aseptrol® (Engelhard, Iselin, N.J., U.S.A), allows the generation of a stable form of ClO_2. This is in the form of a tablet

containing activators and chlorite salts; they react with each other when there is the availability of humidity to generate ClO_2 (23). The combination of chlorine and sodium chlorite generation method is considered to be safest method as it prevents the gas concentration exceeding the explosive limits.

CHAPTER THREE

MATERIALS AND METHODS

Botrytis cinerea

A culture of *B. cinerea* was obtained from diseased *Stevia rebaudiana* (B.Ownley, University of Tennessee). The culture was grown on V8/PDA agar (Difco potato dextrose agar: 39 g; V-8 Juice-200 ml; deionized water-800 ml and autoclaved at 121˚C for 30 min); plates incubated at room temperature (25 ± 2˚C) under a black light (XX-15M UV Bench Lamp, UVP, CA, USA) for 11 to 15 days until heavy sporulation was observed.

Preparation of coupons

Stainless steel (SS) coupons 1.4 x 5 cm (area 12.5 cm²) (Type-304), 2B finish (Ra ~0.25 µm) determined using a profilometer (Hommel Tester T500), were used as the substrate for inoculation of *B. cinerea* (74) (75). The coupons were prepared by soaking them in a detergent (Micro-90 soap, Cincinnati, OH) to remove surface debris. The coupons were washed with deionized water and dried, and placed on glass Petri plates with coarse filter paper (Fisher brand Filter Paper; Dia-9 cm; Qualitative- P8) in between each coupon, wrapped with aluminum foil and autoclaved at 121˚C for 30 min.

Inoculation of Botrytis cinerea on Stainless Steel (SS) coupons

Determining the initial spore population

Botrytis. cinerea (initial population of 1 x 10^7 spores/ml) was recovered from 11 to 15 day old cultures with 3 ml of water by washing with a sterile pipette. The spores were enumerated using a hemocytometer (Southern Micro Instruments, Atlanta, GA) and under a microscope (Nikon Optiphot-2, Garden city, Newyork, Nippon, Kogaku (USA)) under 10x magnification.

Inoculation of B. cinerea

To a 3ml aliquot of the harvested *B. cinerea* suspension, 1 µl of tween 20 (United States Biochemical Corporation, Cleveland, Ohio) was added to break the surface tension of the water and suspend spores in water in the centrifuge tube, thereby preventing the spores from sticking to the walls of the centrifuge tube. After vortexing the stock for 1-2 min, a 100µl (~10 drops of 10µl) suspension of *B. cinerea* spores was inoculated on each SS coupon using a sterile micropipette. The inoculated coupons were dried under the biosafety hood for 2h at room temperature (74).

Recovery of spores from SS coupons

After drying for 2 h at room temperature, the SScoupons were transferred to a sterile centrifuge tube containing 30 ml of sterile water (30 ml was sufficient to

immerse the 1.4 x 5 cm coupon) to recover the spores. The centrifuge tubes containing the coupons were vortexed for 1-2 min. One ml of sample from the 30ml centrifuge tube was used for serial decimal dilutions. Aliquots of 0.1 ml from each dilution were spread plated on PDA with Rifampicin antibiotic (8ml of Rifampicin per 800ml of PDA), (stock concentration- 1g rif/1000 ml methanol) (74). All plates were stored for 48 hrs (based on trial assays) at room temperature (25 ±2 °C) before enumeration. The CFU were enumerated under a microscope (Nikon for Hg 100W, Southern micro instruments, Atlanta, GA). Recovered *B. cinerea* from the untreated coupons had an initial population of ~6 log cfu/coupon.

The ClO$_2$ gas treatment system

The chlorine dioxide gas was generated using the Cl$_2$ gas-NaClO$_2$ solid method. Using a ClO$_2$ gas generator (Enerfab, Inc.; Cincinnati, OH), 2% chlorine gas (98% nitrogen) was passed through solid sodium chlorite cartridges. This reaction produces pure chlorine dioxide gas at a concentration of ~4% with no residual chlorine.

A large gas chamber (~0.422m^3) was used as a gas reservoir for the experiments. The reservoir was first filled with water vapor from a humidifier to a relative humidity of 85% and then sealed. Then, the generated ClO$_2$ gas was injected into the gas reservoir to the desired concentration. A programmable logic controller (PLC) (model DL06, Automation Direct.com) and Optek sensor (Model AF26, optek-Danulat, GmbH, WI, USA) were used to monitor and control the ClO$_2$ gas

concentration in the reservoir by opening and closing a solenoid valve connected to the Cl_2 gas being passed through the sodium chlorite and then connected to the reservoir (76). The gas inside the reservoir was well mixed inside using a fan (Figure 1).

A small, polypropylene box (3L) (treatment chamber) was used to treat the SS coupons under a controlled gas concentration. The chamber had an inlet valve, which was connected to the ClO_2 reservoir via a diaphragm pump, and the outlet valve which served as the pressure vent for the treatment chamber.

Treatment with ClO_2 gas

Before gas treatment, a single SS coupon was pre-humidified to 80-85% RH with a humidifier (Vicks model V5100N, Kaz, USA, INC) and held for 5 min in a Petri plate placed inside the treatment chamber. Then ClO_2 gas from the reservoir was pumped into the treatment chamber at a flow rate of 0.14 liters/sec. The target gas concentrations of 36, 60 and 90 ppmv were monitored and controlled (in the reservoir during each treatment) for 15, 30, 45, and 60 min; 6, 13, 20 and 26 min; 4, 8, 16 and 24 min, respectively. The entire treatment process was carried out in a chemical hood at room temperature ($25 \pm 2°C$).

In this study, ClO_2 gas concentration is expressed as ppmv. The gas phase conversion used was 1 mg/l = 362 ppmv (19). The concentrations used in this study were 0.1 mg/l (36 ppmv), 0.17 mg/l (60 ppmv) and 0.25 mg/l (90 ppmv).

Microbiological analysis

After each treatment, the SS coupon was immediately transferred to a sterile centrifuge tube containing 30 ml sterile water (30 ml was sufficient to immerse the 1.4 x 5 cm coupon) to prevent the gas from further reacting with the spores. After all treatments (for a single replicate) were completed, the centrifuge tubes containing the coupons were vortexed for 1-2 min. One ml of sample from the 30 ml centrifuge tube was used for serial decimal dilutions. Aliquots of 0.1 ml from each dilution were spread plated on PDA with Rifampicin antibiotic (8 ml of Rifampicin solution per 800 ml of PDA), (stock concentration = 1 g rifampicin/1000 ml methanol) (74). All plates were stored for 48 h (based on trial assays) at room temperature (25 ± 2 °C) before enumeration. The CFU were enumerated under a microscope (Nikon for Hg 100W, Southern micro instruments, Atlanta, GA). Results are reported as CFU/ml (3.33×10^4 cfu/ml = 1×10^6 cfu/coupon).

D-value and Z-value analyses

A First order kinetic model (linear model) was used to determine the change in the number of survivors per treatment time. D-*value* is the time required for 90% or

one log reduction. The D-*value* of *B. cinerea* for 36, 60 and 90 ppmv ClO_2 treatment were determined from the survival data of the respective treatments. The first order kinetic model can be represented as follows (77) (78):

$$log_{10} \frac{N_{(t)}}{N_0} = -\frac{t}{D}$$

Where $N_{(t)}$ represents the number of survivors after treatment time in CFU/coupon, N_0 is the initial population in CFU/coupon, D is the decimal reduction time (time required for 90% reduction of exposed microorganisms), t is the treatment time (min) and z-*value* is the ClO_2 concentration change required to change the D-value by 10-fold (84), which was determined by plotting the log D-*value* versus ClO_2 concentration (36, 60 and 90 ppmv).

B. cinerea survival, growth, and development

For survival analysis assays, the same methods for inoculation, recovery, and microbiological analysis as described above were used, except coupons were not treated with ClO_2 gas. The survival time of *B. cinerea* on the SS coupons was determined at room temperature ($25 \pm 2°C$) over ~ 7 days with RH (~21-64%). The results were reported as CFU/ml in the 30 ml of recovery buffer. The same method was repeated every day to determine how long *B. cinerea* survived on the surface of the SS coupon without any nutrients.

Statistical analysis

All ClO_2 gas treatment experiments were repeated thrice and assayed in duplicate. Statistical analysis was performed using SPSS version 24 statistical package (IBM Corporation, Armonk, NY). Analysis of Co-variance (ANCOVA) was used to determine significant differences between the gas treatments at different treatment times. Analysis of variance (one-way ANOVA) was used to determine the significant difference between the D-*value* of different treatments.

The survival analysis was repeated twice. Each replicate used 3 inoculated coupons per day and held up to 7 days. The number of survivors and the survival rate of *Botrytis cinerea* was determined using Microsoft Excel (Microsoft Windows 7).

CHAPTER FOUR

RESULTS AND DISCUSSION

The initial population of *B. cinerea* recovered from the stainless-steel coupons was 6 log CFU/coupon. The treatment times for each ClO_2 concentration (36, 60, and 90 ppmv) were selected to achieve approximately 1, 2 and 3 log reductions of *B. cinerea* during the 15, 30, 45, and 60 mins; 6, 13, 20, and 26 mins; 4, 8, 16, and 24 min, respectively. At the lowest gas concentration of 36 ppmv, a mean value of 1.3 ± 0.6 log CFU/coupon reduction was achieved after 15 min treatment time with RH of 80-85% (Figure 2). As treatment time increased, significant increases in log reductions were observed (*P<0.005*) *(*Figure 2). Similar increase in log reduction with increase in treatment time were observed by Mahmoud et al., 2008 (67), and Trinetta et al., 2012 (74) for inactivation of *L. monocytogenes* using ClO_2 gas on a stainless-steel surface and also in another study by Trinetta et al.,2013 (76) for inactivation of *A. alternata* and *S. vesicarium*. For *B. cinerea in this study*, a maximum of 2.9 ± 0.4 log CFU/coupon reduction was observed when treated with 36 ppmv of ClO_2 gas for 60 min (Figure 2). From the log reduction data for each replicate, a mean D-*value* of 22.34 min ± 2.53 at 36 ppmv ClO_2 gas concentration was determined (Table. 1).

When *B. cinerea* was treated with 60 ppmv of ClO_2 gas, a mean of 1.1 ± 0.1 log CFU/coupon reduction was observed at 6 min of treatment with 80-85% RH

(Figure 2b). After 13 min, a 1.5 ± 0.1 log CFU/coupon reduction was observed and followed by 1.8 ± 0.3log CFU/coupon reduction at 20 min of treatment time (Figure. 2b). .A maximum of 3.1 ± 0.4 log CFU/coupon reduction was observed when *B. cinerea* was treated for 26 min at 60 ppmv ClO2 gas (Figure 2b). Using the three replicates, a mean D-*value* of 10.18 min ± 0.75was determined (Table 1).

At 90 ppmv ClO_2 gas treatment, significant log reductions were observed for increasing treatment time (*P<0.005*). A mean of 1.2 ± 0.3 log CFU/coupon reduction was obtained when *B. cinerea* was treated with ClO_2 gas at 90 ppmv for 4 min, 80-85 % RH (Figure 2c). A 1.6 ± 0.6 and 2.0 ± 0.12 log CFU/coupon reductions was observed at 8 and 16 min of treatment, respectively. After 24 min, a 2.6 ± 0.2 log CFU/coupon reduction was obtained. The *D-value* for 90 ppmv treatment was 10.65 ± 0.44 min using a linear model (Table 1).

 A significant difference (P<0.005) in log reduction of *B. cinerea* was observed between the three treatment concentrations 36 and 60 ppmv and 36 and 90 ppmv of ClO_2 gas. However, no significant difference was observed between 60 and 90 ppmv. This data helps to determine that for inactivation of *B. cinerea* using a lower concentration of ClO_2 gas (36 ppmv for 60 min) would be sufficient to achieve a 3 log reduction on a stainless-steel surface. Also, since no significant difference was observed between the D-*values* of 60 and 90 ppmv, commercial application of 60 ppmv ClO_2 gas on a stainless-steel surface would be more economical than 90

ppmv with no loss of efficiency. As expected, the D-*value* decreased with increasing concentration of ClO_2 gas. Similar findings were observed by Mahmoud et al. (67) on inactivation of *E. coli* and *S. enterica* on lettuce using ClO_2 gas. The D-*values* decreased with an increase from 0.5-5 mg/l of ClO_2 gas for both bacteria. Similar results were observed by Mahmoud et al., (79) on inactivation kinetics of *E. coli*, *L. monocytogenes* and *S. enterica* on strawberries with ClO_2 gas. The D-*value* of *S. enterica* decreased from 4.2 mins to 2.7 mins with the increase in ClO_2 concentration from 0.5 to 5 mg/l (79). The inactivation effect of ClO_2 gas treatment against microorganisms depends on RH, temperature, gas generation methods and gas flow rate (25), (80), (81).

A Similar effect of ClO_2 gas on spore germination of *B. cinerea* to control decay of d' Anjou Pear was reported by Spotts et al., (34). Rates of 0.1, 0.5, and 1 mg/l for 10 min treatment time did not affect the conidial germination of *B. cinerea* although when treated with 10 mg/l of ClO_2 gas for 10 min the conidial germination was completely inhibited (34). Roberts et al.(13) also studied the effect of aqueous ClO_2 on *B. cinerea* and found that it was the most resistant fungus against ClO_2 and the minimum dose-exposure combination to achieve 100% spore mortality was 5 mg/l for 2 min (13). From Spotts et al. and Roberts et al., similar observations on the efficacy of ClO_2 on *B. cinerea* implied that *B. cinerea* was resistant against ClO_2 at lower concentrations.

Arango et al,(2) investigated the effect of ClO_2 gas on *B. cinerea* inoculated on strawberry. They reported that, ClO_2 gas treatment 0.63 mg/l (~228 ppmv) for 7 mins prevented the onset of *B. cinerea* in strawberry with better visual quality than 5 mg/l for 7 mins treatment which was also effective but it was observed to have bleaching effect on strawberry that had an impact on visual quality. . Complete inhibition of *B. cinerea* on strawberry cannot be achieved unless the calyx of the fruit is removed and all parts of the fruit are treated with ClO_2 gas (2). The important difference observed between the ClO_2 gas treatment on a food surface inoculated with foodborne pathogens and plant pathogens is that foodborne pathogens are artificially inoculated on the surface of the product, whereas *B. cinerea* may be present in the plant tissue even before harvest from the field. Based on this, complete inactivation of *B. cinerea* on strawberries using ClO_2 gas treatment will pose a serious challenge because the gas doesn't penetrate into the fruit to any significant depth.

While comparing the microbial inactivation results of gaseous ClO_2, the experimental design should also be taken into consideration, more specificially whether the ClO_2 increases, decrease,s or remains constant during treatment (64). Lee et al.(18) and Sy et al, (19) studied the inactivation mechanism of ClO_2 concentration from a sachet where the gas concentration increased during treatment. Gomez et al.(83) observed during injection that there was an increase in ClO_2 concentration, and the concentration decreased during the reaction with

the product (41). Du et al.(72) reported that the ClO_2 concentration remained stable due to a lower product to ClO_2 concentration ratio. In our experiments, the gas concentration was held constant during the treatment time by flowing the gas through the treatment chamber.

Survival analysis of B. cinerea on SS coupon

This objective was to determine the efficiency of *B. cinerea* to survive on the surface of the stainless-steel coupon without any added nutrients, thereby determining its survival over the period. The initial population of *B.cinerea* recovered from the stainless-steel coupons on Day 0 was 5 log CFU/coupon. The number of survivors decreased to 17% by Day 4 (Figure 4), and by Day 7 the survivors were below the limit of detection (3 log reductions of *B .cinerea*). The decrease in number of survivors may be due to lack of nutrients on the SS coupon, or unfavorable RH or temperature. However, this data tells how long the B. cinerea can survive on the SS coupon which is important for future experiments to examine the effects of very low concentrations of ClO_2 gas over several days of exposure.

Berg et al. (61) studied the effect of temperature and relative humidity on survival and growth of mycelium and conidia of *B. cinerea*. Raposo et al. (39) also analyzed the survival rate of mycelium and sclerotia of *Botrytis cinerea* when exposed to summer environmental conditions in southeast Spain. *B. cinerea* is capable of adapting to different habitats by production of different growth structures, like

mycelium, micro- and macroconidia, and sclerotia. Sclerotia are considered to be one of the survival structure for *B. cinerea* that helps to survive in hostile environmental conditions *(5).*

When mycelia and conidia were studied under non-nutrient conditions on a cover glass slide with 85-99% RH and growth rate was optimal from 0 to 20˚C (61), Berg et al. (61) found that the survival of mycelium decreased with decreasing RH in non-nutrient medium and; growth rate was better at 0°C than at 20 ˚C. Whereas, in a nutrient medium (carrot) the mycelium survived for longer period (more than 1 year) at RH of 90-100%. Contradictory results in the survival rate of mycelia on infected tomato stems was observed by Raposo et al (39) based on the location as well as the year of the experiment (39). Under controlled conditions and continuous exposure to RH=100% and constant temperature for 2520h (15 weeks) the mycelia lost its viability. However, under ambient conditions (below 100% RH and constant temperature) mycelia were viable after 2520 h. Raposo et al. (39) also observed that continuous exposure to high RH increased the loss of viability.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

The goal of this research was to determine the effective combination of lowest ClO_2 gas concentration and treatment time for inactivation of *B. cinerea* on a stainless steel surface and determine the survival rate of *B. cinerea* on a stainless-steel surface over several days.

In summary, low ClO_2 gas concentration (36 ppmv) and treatment time (60 min) were effective in significantly reducing *B. cinerea* by 3 log CFU on stainless steel coupons. The first order linear model D-*values* for *B. cinerea* at 36, 60, and 90 ppmv were 22.34 ± 2.53, 10.18 ± 0.75, and 10.65± 0.44 min respectively with z-*value* of 175.43 ± 0.19 ppmv. This data suggests that the gas treatment at higher concentrations (90 ppmv) is not significantly better than 60 ppmv. Additional research is needed to determine its application on a commercial scale and determine if even lower concentrations of ClO_2 gas can be effective in controlling growth of *B. cinerea* which may be internal to fresh produce.

Also, quantifying the rate of absorption of ClO_2 gas by fresh produce can also be considered for future study. This can help further determine the speed and concentration of gas required in any packaging design for preservation of fresh

produce. Further research can also be extended to include determining the sensory and physical attributes of the fresh produce after low concentrations of ClO_2 gas treatment for extended times

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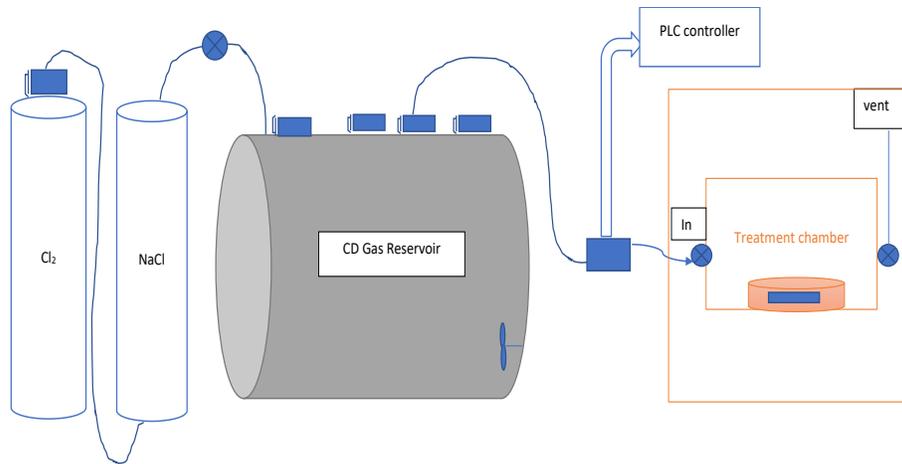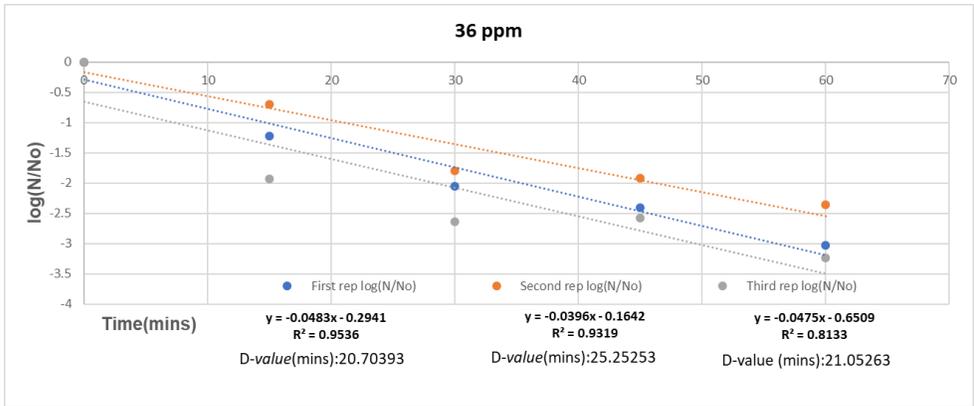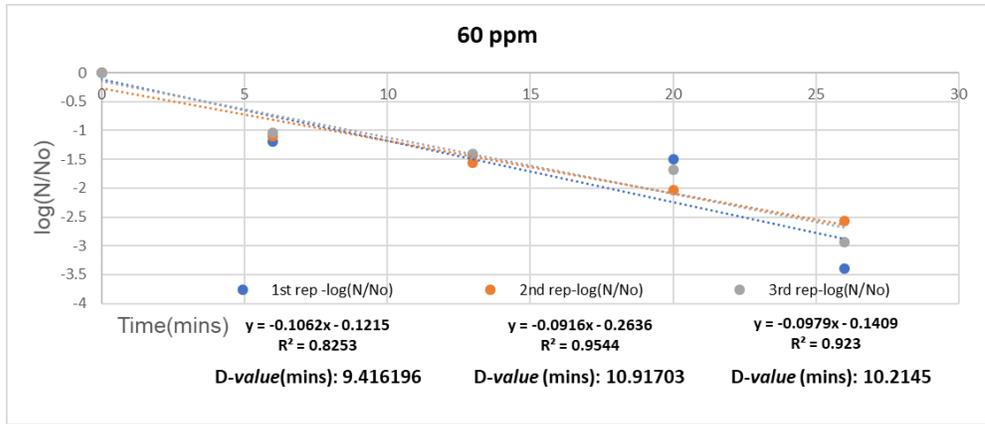
APPENDIX

Figures

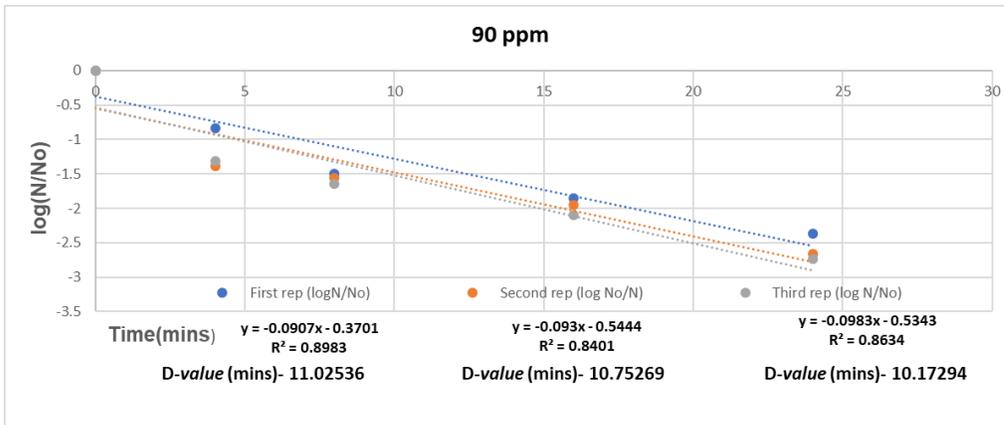**Figure 1**: Chlorine dioxide gas generator system

a) At 36 ppmv ClO$_2$ gas concentration,

b) At 60 ppmv ClO$_2$ gas concentration,

Figure 2: Survival curves for *Botrytis cinerea*.

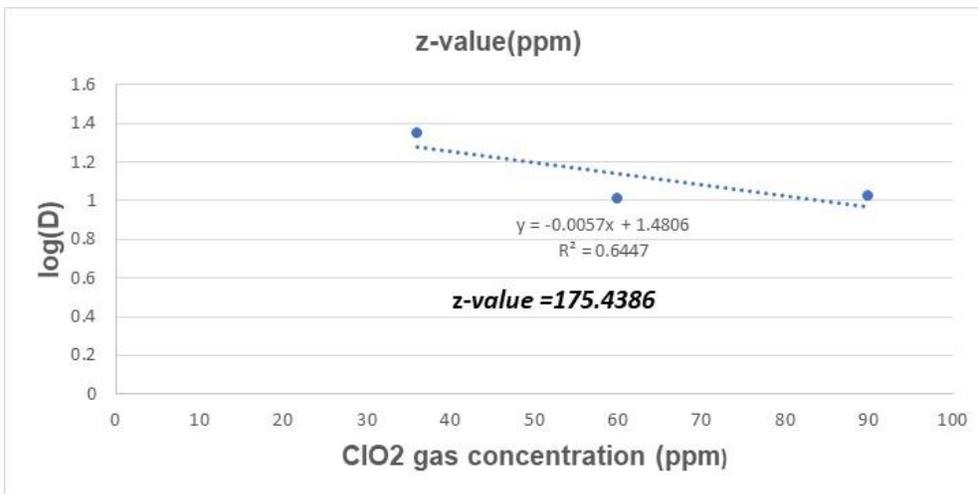c) At 90 ppmv ClO$_2$ gas concentration.

Figure 2 continued

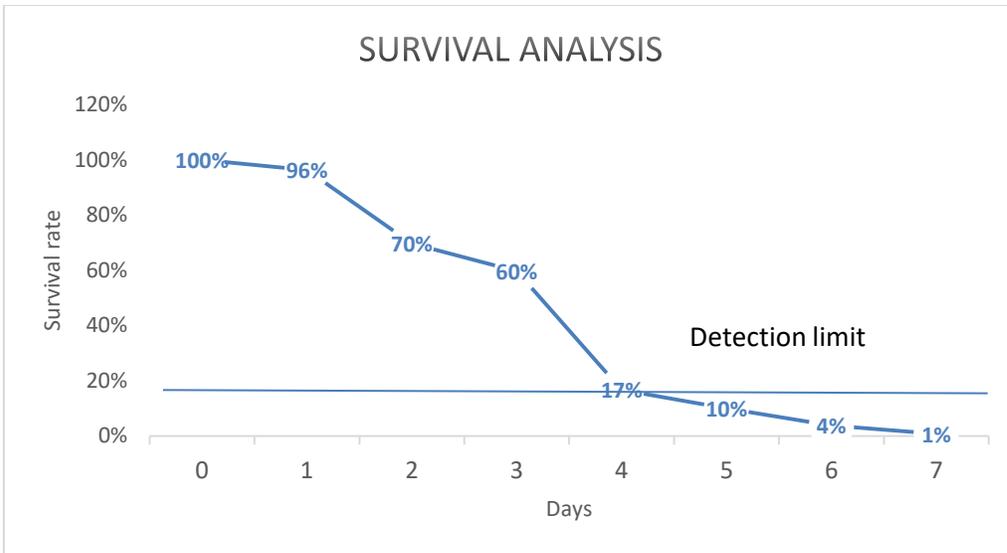**Figure 3**: z-value of *Botrytis cinerea* when treated with ClO$_2$ gas

Figure 4: Survival analysis of *Botrytis cinerea* on stainless steel coupons (room temperature (25°C ± 2˚C) at ~21-64% relative humidity)

Tables

Table 1: Chlorine dioxide gas treatment at 36, 60 and 90 ppmv

ClO$_2$ gas concentration(ppmv)	D-value(mins)	SD (±)	Z-value(ppmv)
36	22.34[a]	± 2.53	
60	10.18[b]	± 0.75	175.43± 0.19
90	10.65[b]	± 0.44	

D-values with different lowercase letters are significantly different (P<0.005)

VITA

Aruna Dhanapal was born in Tamil Nadu, India on October 31, 1988. She graduated from Avinashilingam University and received her Bachelor's degree in Food Processing and Preservation Technology and a Masters degree in Food Technology. She earned her second Master of Science degree in Food Science from the University of Tennessee, Knoxville. She will be working as a Quality Control and Food Safety Technician at Mars, Texas.