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Soil ozonation as a sustainable alternative to methyl bromide fumigation and synthetic pesticides

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Soil ozonation as a sustainable alternative to methyl bromide fumigation and synthetic pesticides

by

Nahed Msayleb

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Sustainable Agriculture

Program of Study Committee:
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Iowa State University

Ames, Iowa

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DEDICATION

I dedicate this work to Abu Hadi (Hasan AbdelKareem), and Abu Hafez

To Air, Soil and Water

To Biodiversity and the Environment

To Earth and Humanity

To those who will pick up from where I reached:

“May you handle science with passion and enthusiasm as it deserves”

TABLE OF CONTENTS

	Page
LIST OF FIGURES	vii
LIST OF TABLES	viii
NOMENCLATURE	x
ACKNOWLEDGEMENTS	xi
ABSTRACT.....	xiii
CHAPTER I GENERAL INTRODUCTION AND LITERATURE REVIEW ...	1
Root Health and Methyl Bromide.....	1
Methyl Bromide, a ‘Substance that Depletes the Ozone Layer’	2
The Conflict Between Economic Profits, and Environmental and Human Health Risks in Implementing MeBr Phaseout.....	3
Justifications for the Need to Identify Efficient and Environmentally Safe Alternatives to MeBr	4
Methyl Bromide Current Alternatives.....	5
Non-chemical alternatives	6
Chemical alternatives.....	8
Approaches to Improve Fumigants Efficacy.....	9
Ozone Characteristics and Potentials in Disinfection	11
Research Objectives and Dissertation Organization	14
Nematodes.....	14

	Page
<i>Phytophthora sojae</i>	16
<i>Fusarium oxysporum</i>	17
Ozonation	18
Ozone Generator and Accessory Equipment Cost Breakdown.....	20
Dissertation Organization.....	20
References	20
CHAPTER II SOIL OZONATION FOR SOIL NEMATODES INACTIVATION AS AN ALTERNATIVE TO METHYL BROMIDE AND NEMATICIDES.....	30
Abstract	30
Introduction	31
Methods and Materials.....	35
Soil samples	35
Ozone treatment of soil.....	36
Ozonation.....	38
Data analysis	39
Results	39
Discussion	43
Acknowledgements.....	46
References	46

	Page
CHAPTER III OZONATION EFFICACY AS ENVIRONMENT-FRIENDLY ALTERNATIVE TO TOXIC FUNGICIDES IN THE TREATMENT OF SOILBORNE PHYTOPHTHORA SOJAE.....	52
Abstract	52
Introduction	52
Methods and Materials.....	56
Inoculum preparation.....	57
Experimental design.....	58
Exmperimental treatments and incubation.....	59
Monitoring and assessment of treatments.....	59
Ozonation.....	60
Results	61
Discussion	63
Acknowledgements.....	66
References	66
CHAPTER IV OZONE INACTIVATION OF FUSARIUM OXYSPORUM CONIDIA IN HYDROPONIC NUTRIENT SOLUTIONS	74
Abstract	74
Introduction	75
Methods and Materials.....	79
Preparation of F. oxysporum spore suspensions.....	79
Ozone gas sources.....	80

	Page
Ozonation dosages	81
Temperature effect	81
Determination of viable conidia of 370TSB	81
Data analysis	83
Results	84
Discussion	89
Acknowledgements.....	91
References	91
CHAPTER V CONCLUSIONS AND RECOMMENDATIONS	96
Summary of the current research findings	96
Enhancements to the ozonation soil treatments	96
Recommendations.....	97
References.....	99

LIST OF FIGURES

	Page
CHAPTER II	
Figure 1 a. Ozone generator. b. Baermann funnels. c. Nematode counting	38
Figure 2 Filtrates from ozonated and control samples	42
CHAPTER III	
Figure 1 a. Ozone generator. b. Incubation of samples. c. Emergence of seedlings	60
Figure 2 Monitoring and evaluation of treatments	62
CHAPTER IV	
Figure 1 <i>F. oxysporum</i> isolate	80
Figure 2 Plated controls.....	80
Figure 3 Ozone generator, glass reactor and silicone tubing	81
Figure 4 Growing colonies of a dilution rank 10^{-2} after ozonation with air feed.....	83

LIST OF TABLES

	Page
 CHAPTER II	
Table 1 Experimental design with number of subsamples and replications per ozone level, dose, time of ozonation, rate of ozonation and temperature	37
Table 2 Regression statistics and ANOVA, showing the significance of ozonation level (Dose*Rate) and temperature on nematode viability	39
Table 3 Effect of soil ozonation at different Doses*Rates on nematode viability in soil samples collected from a corn field in central Iowa at 21 °C and 5 °C	40
Table 4 P values of means comparison using a t-Test: two-sample assuming unequal variances (REF) between three ozonation levels at 5 °C and 21 °C	41
Table 5 Effect of ozone in ascending doses on the soil pH and the release of bioavailable forms of P, Zn, Fe and Mn	42
 CHAPTER III	
Table 1 Texture and organic matter contents of the soil used in a study to determine the effect of ozone on <i>P. sojae</i>	56
Table 2 Experimental design with number of subsample treatments and controls per batch (ozonation treatments as a function of time)	58
Table 3 Effect of treatment with ozone at different doses on root and stem rot disease caused by <i>P. sojae</i> on susceptible Sloan soybean	61

CHAPTER IV

Table 1	Percent of viability of spores of <i>Fusarium oxysporum</i> after treatment with varying ozonation levels generated from oxygen at 21°C and 5°C	85
Table 2	Percent of viability of spores of <i>Fusarium oxysporum</i> after treatment with varying dosages of ozone generated from air at different rates and at 21°C and 5°C	85
Table 3	Percent of viability of spores of <i>Fusarium oxysporum</i> after treatment with varying levels of ozonation (Dose*Rate) from air at 21°C and 5°C	86
Table 4	P values of means comparison using a t-Test: two-sample assuming unequal variances (REF) between three ozonation levels at 5 °C and 21 °C	87
Table 5	Regression statistics and ANOVA, showing the significance of GPC on <i>Fusarium oxysporum</i> spores viability	87
Table 6	Regression statistics and ANOVA, showing the significance of ozonation level (Dose*Rate) from air, and temperature on <i>Fusarium oxysporum</i> spores viability	88

NOMENCLATURE

EPA	Environmental Protection Agency
GPC	Gas Phase Concentration
IOA	International Ozone Association
MeBr	Methyl Bromide
UNEP	United Nations Environment Programme
USDA	United States Department of Agriculture

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ABSTRACT

The goal of this dissertation was to investigate the potentials of ozone as a sustainable alternative to the soil fumigant methyl bromide and to synthetic pesticides. Three pathogens were selected for this research, given their economic importance, and the spectrum variety that they represent: Phytoparasitic nematodes, important pests that cause severe crop yield losses; *Phytophthora sojae*, a predominant soybean pathogen that causes root and stem rot, and pre- and post-emergence damping-off of soybean; and *Fusarium oxysporum*, which causes Fusarium wilt, an economically important disease in hydroponic systems.

Soil samples that were naturally infested with nematodes were treated with different levels of gaseous ozone at 21 °C and 5 °C. A medium level of ozonation (2.1 g O₃ kg⁻¹ for 10 min at a rate of ozonation 0.21 g O₃ kg⁻¹min⁻¹) and low temperature (5 °C) resulted in 96% nematode inhibition. Regression analysis showed that nematode viability was a function of the level of ozonation (P = 5.1E-07) and the soil temperature (P = 4.4E-08; Adjusted R-square = 0.65).

Assays of artificially inoculated soil samples with *P. sojae* were treated with different doses of gaseous ozone. This study showed that a dosage of 0.47 g O₃/kg soil, totally prevented root and stem rot disease symptoms caused by *P. sojae*.

Samples of conidial suspensions of *F. oxysporum* were treated with incremental doses of ozone from either oxygen feed with high gas-phase concentration (GPC) or air feed with low GPC. Trials resulted in non-viability of the pathogen at high ozone GPC with a dose of 0.84 mg O₃/L for 3 seconds. The optimal conditions for *F. oxysporum* treatment with ozone were high GPC (oxygen feed), and low temperature (5 °C).

Given these promising results, and since ozone degenerates quickly to oxygen, the findings of this research clearly indicate that ozone may be an efficient and sustainable alternative to methyl bromide and to:

1. nematicides in the treatment of nematodes in the soil,
2. fungicides in the inhibition of Phytophthora diseases in the soil, and
3. fungicides in the treatment of Fusarium wilt in hydroponic nutrient solutions.

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

Root Health and Methyl Bromide

Root health is essential for plant vigor, that can lead to higher yield with less costly agricultural inputs, hence higher profits. Soilborne diseases and pests attack plant roots, and by weakening these vital plant parts for support, nutrient and water uptake, nutrient storage, undermine plant productivity and in sever cases crop survival. Methyl bromide (MeBr) is a wide-spectrum soil fumigant, used efficiently in the treatment of soilborne phytopathogens, pests and weeds (Ragsdale et al., 1995) of many high-input, high-value crops in U.S. agriculture, including vegetables, nursery plants, ornamentals, fruit trees, strawberries and grapes (Zasada et al., 2010). It provides broad-spectrum pest control, and has higher efficacy compared to other fumigants (McKenry, 1994), since its volatility allows it to penetrate the treated soil sufficiently (Duniway, 2002). For this reason, some crop production systems such as strawberries and fresh market tomatoes are highly dependent on MeBr. Such dependence lead to reductions in crop rotation and in diversification of production practices (Braun and Supkoff, 1994). In 1992, MeBr was one of the five most used pesticides in the United States (UNEP, 2000). In the early 1990's, approximately 25,000 to 27,000 tons of methyl bromide was applied annually (USDA, 1995), with pre-plant soil fumigation constituting more than 75% of its use (UNEP, 1992). The economic viability of specific crops in Florida, California, North Carolina, and other states could be affected by the loss of this compound if no alternatives are available (Spren et al., 1995; USDA, 1993).

Methyl Bromide, a ‘Substance that Depletes the Ozone Layer’

Ozone plays a key function in moderating the climate of Earth by absorbing ultraviolet radiation from the sun below a wavelength of 320 nm. Stratospheric ozone plays the role of a reactive filter that prevents many negative environmental impacts by UV radiation like sunburn, skin cancer, crop damage, and others (Robock, 1996). Absorption of UV radiation by ozone in the Earth’s stratosphere also moderates the Earth’s temperature (World Meteorological Organization, 1994). Bromine is a potent ozone depletory, and is 50 times more reactive than chlorine in depleting ozone (Anderson et al., 1989). MeBr is the major carrier of bromine to the stratosphere (Penkett et al., 1985). When MeBr reaches the stratosphere, it breaks down to form bromine, which participates in a series of ozone-depleting cyclical reactions (Cox et al., 1995; and UNEP, 1992). Estimates of sources of MeBr from soil fumigation range from 16 to 47.3 Gg per year (World Meteorological Organization, 1994). Substantial retention and degradation of MeBr within agricultural soils is unlikely, and most of it is released into the atmosphere following soil fumigation (Gan et al., 1994; Yagi et al., 1993; and Yates et al., 1996). Concern over ozone depletion led to negotiations among countries that resulted in the 1987 drafting of the Montreal Protocol on “Substances that Deplete the Ozone Layer” (Gushee, 1996; and UNEP, 1995). An ozone depletion potential (ODP) index is used to measure a substance’s relative potential to deplete stratospheric ozone (Ristaino and Thomas, 1997). The ODP represents the amount of ozone destroyed by the emission of 1 kg of a chosen gas over a particular time scale compared with chlorofluorocarbon-11 (CFC-11), a major ozone depletory (UNEP, 1992). The UNEP calculated that MeBr had an ODP of 0.6, or 60% of CFC-11’s ODP, and the atmospheric lifetime was calculated at 1.7 years (Mellouki et al., 1992; and Solomon et al., 1992).

Accordingly, MeBr was scheduled under the Montreal Protocol to be completely phased-out by 2005 in developed countries and 2015 in developing countries. Nevertheless, as of 2008, the fumigant was still being used in the United States, Australia, Canada, and Japan (Zasada et al., 2010) because the protocol allows for critical use exemptions (CUE) to MeBr ban if “(a) there are no technically and economically feasible alternatives that are acceptable from a regulatory and bystander exposure perspective, and (b) the use is considered crucial to avoid a significant market disruption of selected commodities” (UNEP, 2000). In 2008, the United States accounted for 91% of the total global CUE approvals for high-value crops (Brennen, 2008).

The Conflict Between Economic Profits, and Environmental and Human Health Risks in Implementing MeBr Phaseout

The National Pesticide Impact Assessment Program (NAPIAP) estimated annual economic losses of \$1.3 to 1.5 billion if a ban of MeBr use occurred in the United States (USDA, 1993). Most of the losses estimated were due to loss of soil fumigation (\$800 to 900 million), mostly occurring in tomatoes and strawberries (USDA, 1993). Hence, it is expected that the most challenged productions in managing soilborne pathogens and pests without MeBr, are the U.S. high-value crops relying on its use. These loss estimates assumed that few or no efficient alternatives would be available or used (Ristaino & Thomas, 1997). In contrast to the merely economic assessment, the EPA conducted a cost-benefit analysis of the elimination of MeBr (unpublished data), taking in consideration the environmental, ecological and health implications of MeBr use, and resulted in an estimated \$1.2 to 2.3 billion in losses if the MeBr phase-out did not occur. Additionally, the EPA evaluated the

likely health effect costs of MeBr use, and estimated that between \$244 and \$952 billion in benefits would result primarily from a reduction in 2,800 skin cancer deaths over the period from 1994 to 2010. Indirect costs of MeBr use include negative human health effects from increased UV-B (Slaper et al., 1996), detrimental effects of increased UV-B on global photosynthetic rates (Robock, 1996), health effects from exposure of workers to MeBr (Ragsdale & Wheeler, 1995), increased pest control expenses resulting from pesticide-related destruction of beneficial organisms (Menge et al., 1978), yield reductions due to phytotoxicity (Menge et al., 1978), groundwater contamination (Braun & Supkoff, 1994), and governmental expenditures to reduce the environmental and societal costs of the use of the pesticide, including alternative research and development in the United States and developing countries (Ristaino & Thomas, 1997).

Justifications for the Need to Identify Efficient and Environmentally Safe Alternatives to MeBr

Since MeBr has provided a reliable and feasible treatment for soilborne pests, many high-value commodities became highly MeBr-dependent. These commodities have standardized their production practices on the use of this pesticide, hence they will be negatively impacted upon MeBr outright phase-out if effective and economical alternatives were not identified (Zasada et al., 2010). The repercussions of total MeBr ban without identifying efficient and feasible alternatives, would be reduced production levels, higher prices for consumers, and possible use of more toxic compounds by growers (Zilberman et al., 1991). In 1997, over 95% of the tomato fields in Florida were still fumigated with MeBr due to problems from soilborne diseases including the root-knot nematode, Fusarium, and

bacterial wilt diseases (USDA, 1995). If a single treatment method that is environmentally safe, efficient and feasible could be identified as alternative to MeBr, it would be rapidly adopted by growers (Ragsdale and Wheeler, 1995). Currently, there is no single alternative pesticide or treatment for all the uses of MeBr (Messenger and Braun, 2000). In addition, the non-chemical alternatives that are implemented, are considered partial alternatives to MeBr, which necessitates integrating them with complementing treatments and control measures to fully cover the efficacy spectrum of MeBr. Alternatives like cultural practices, genetic resistance, and integrated pest management, require more biological knowledge of pests than with MeBr, to achieve satisfactory results.

Methyl Bromide Current Alternatives

Due to the concerns associated with the use of MeBr on the ozone layer, human health and the environment, which enjoined its phaseout plan, this necessitated the development of alternative treatments. Alternatives vary between chemical treatments and non-chemical control measures. Chemical alternatives include chloropicrin, dazomet, 1,3-dichloropropene, methyl iodide and metam sodium. Non-chemical alternatives include steam and aerated steam, soil solarization, crop rotation, biological control, resistant plant varieties, cover crops, organic amendments and compost. Chemical and non-chemical options could be grouped under integrated pest management (IPM), to increase their efficacy and complement each other's spectrum. However, none of these alone proved as single alternative to methyl bromide. In addition, each of these alternatives has some drawbacks on the environment (especially synthetic chemicals), feasibility or efficacy.

Non-chemical alternatives

Non-chemical alternatives, such as steam and aerated steam soil treatments are efficient at controlling soil pathogens and weeds (King and Greene, 2000), but are very expensive and are only practical and feasible in greenhouse settings (Gullino and Lodovica, 1992). In addition, steaming creates a biological “vacuum” in the soil by impairing its microbial balance and/or killing beneficial microorganisms, which renders the soil readily re-infested by phytopathogens (Mus and Huygen, 1992).

Soil solarization on the other hand could be practical only in hot locations (Carpenter et al., 2000), and it is inefficient on some weeds (e.g. nut sedge) and fungi that are located deeply in the soil (i.e. *Armillaria spp.*; Anonymous, 1993). Beside these drawbacks, soil solarization is too labor-intensive, and necessitates covering the soil with tarp or plastic material for 6 to 8 weeks, leaving it unproductive for this duration (Messenger and Braun, 2000).

Crop rotation depends on alternating between susceptible and resistant crops to a pathogen, which decreases inoculum in the infested field (Cook and Baker, 1989). However, when the pathogen has long-lasting spores, such as microsclerotia of *Verticillium dahliae* (Huisman and Ashworth, 1976a; and Huisman and Ashworth, 1976b) that can survive for up to 20 years in the soil, or when it subsists in the soil as a saprophyte (survives on dead plant parts), this practice fails to control the targeted pathogen. Also, crop rotation often necessitates the rotation with non-cash crops, and requires to be applied for years to be efficient, which decreases the farm income (Mukhopadhyay, 1990).

Biological control of soilborne pathogens, hinges on controlling a pest or pathogen by the use of its antagonist organism(s), and the success of this method is limited and sometimes unpredictable, due to the complexity of the soil ecosystem.

As for the use of resistant plant varieties, its major drawback is that resistance genes are usually pathogen- and sometimes race-specific (Messenger and Braun, 2000). In addition, the development of resistant varieties takes time, yet sometimes the developed variety becomes useless when the targeted pathogen mutates and changes its virulence traits.

Genetically engineered plants to express pesticidal traits, known as “genetically modified pest-protected plants”, are associated with possible environmental and human health risks (National Research Council, 2000).

Finally, cover crops, organic amendments and compost, although efficient in some applications, have weaknesses. Cover crops for example are not omnipotent in the suppression of nematodes: a cover crop would control some nematode species, but not the others. Organic amendments are only partial alternative to MeBr (Jarvis, 1992; Linderman, 1989), and even could cause damage to some plants roots, like in the case of field and greenhouse grown lettuce seedlings (Phillips et al., 1971). Similarly, compost soil amendment provides many benefits to the soil and the planted crop, as it increases nutrient availability, enhances drainage, boosts the proliferation of beneficial microorganisms, which result in pathogens suppression and increased plant vigor. However, compost use necessitates proper preparation and usage, with special attention being given to the build-up of heavy metals and soluble salts in the soil, putting crop growth and ultimately human health at risk (Bevacqua and Mellano, 1993).

Chemical Alternatives

The main synthetic alternatives to MeBr are metam sodium, dazomet, 1,3-dichloropropene (1,3-D), chloropicrin, and methyl iodide. These pesticides have a narrower spectrum of efficacy in comparison with MeBr, hence, they are mostly used in different combinations to reach the desired broad-spectrum control (Zasada et al., 2010). In addition, they are associated with detrimental environmental and human-health concerns, and when mixed together to reach higher efficiency, the risks accrue even more.

Metam sodium is an effective nematicide, but it is not as efficient as MeBr in the control of root-knot nematodes, or *Fusarium* and *Verticillium spp.* (Anonymous, 1993b). In addition, metam sodium has limited soil penetration capacity and poor dispersal, which prevents its uniform distribution in the soil (Gullino, 1992; Martin, 2003; McKenry, M.V. 1994; Mus and Huygen, 1992), and limits its efficiency in the control of soilborne pathogens of deep-rooted trees and shrubs. Accordingly, metam sodium is applied with large quantities of water (Munnecke and van Gundy, 1979) to homogenize its dispersal in the soil, which increases the cost of its application and may result in groundwater contamination (Kim, 1988). Another limitation of metam sodium is its phytotoxicity, which necessitates waiting after its application for long period before planting (Gerstl et al., 1977). Metam sodium is carcinogen, a developmental toxin, and an air contaminant (Kelley and Reed, 1996).

Likewise, dazomet has reduced disease control and slow diffusion (Parochetti and Warren, 1970), it requires a 60-day re-entry period after application (Anonymous, 1993a) and causes groundwater contamination (Anonymous, 1992; Kim, 1988).

The fumigant 1,3-D is a nematicide that can reduce nematode populations in the soil for up to 6 years (Carpenter et al., 2000), however it does not control soil fungi or insects

(Anonymous, 1989). Beside its limited pesticidal range, dazomet is listed as a carcinogen and groundwater contaminant (Office of Environmental Health Hazard Assessment, Cal/EPA).

Conversely, chloropicrin is an effective fungicide but does not control weed and nematode (Anonymous, 1993b; Duniway, 2002). Hence, for higher pesticidal control, chloropicrin is usually mixed with MeBr and applied to the soil (Sances and Ingham, 1999). In addition, chloropicrin has a slower dispersion into soil and evaporation after application than MeBr (Smelt and Leistra, 1974), which necessitates a longer waiting time before planting to prevent phytotoxicity. Another drawback of chloropicrin is that it is a potential groundwater contaminant.

Finally, methyl iodide is comparable or even superior to MeBr in efficiency as a soil fumigant, however it could not be considered as a safe alternative to MeBr because it is a known carcinogen (Office of Environmental Health Hazard Assessment, Cal/EPA).

Approaches to Improve Fumigants Efficacy

The minimum distribution of synthetic fumigants in soil and their loss through atmospheric emission, have been addressed in numerous studies that have aimed to increase their efficacy and reduce environmental pollution resulting from their use. Fumigants tend to diffuse quickly out of the soil after application due to their high volatility. Emissions from soil fumigation can range between 20% and 90% of the fumigant after application (Yates et al., 2002). This characteristic encounters many repercussions: (i) it reduces the time of fumigant residence in the soil, which decreases its interaction with soil pests and leads to lower pest control efficacy, (ii) and it increases air pollution. Approaches found to alleviate fumigant emission include: applying the fumigant with irrigation water (Ajwa et al., 2002; Papiernik et al., 2004; Schneider et al., 2008; Sharon et al., 2004), increasing the depth of

underground application through drip irrigation from 15 to 30 cm (Schneider et al., 2008; Sharon et al., 2004), and covering the soil with virtually impermeable film (Nyezepir and Rodriguez-Kabana, 2007) or HDPE (Sharon et al., 2004). Water decreases gas-phase diffusion in the soil, which decreases the fumigant's atmospheric emission (Ajwa et al., 2002). Yates et al. (1997) showed that increasing soil water content decreased the atmospheric emission of methyl bromide. In addition, water enhances the uniformity of fumigant distribution in the soil (Ajwa et al., 2002). However, caution should be taken with the application of irrigation water, as El Hadiri et al. (2003) showed that irrigating after the fumigation with 1,3-D or methyl isothiocyanate results in the leaching of the fumigants, which might lead to the contamination of shallow groundwater.

On the other hand, the deeper application of the fumigant elongates its path length between application point and soil surface. This increases the fumigant residence in the soil, and increases its interaction with soil pathogens and pests, which improves its pest control efficacy (Sharon et al., 2004). However, Papernick et al. (2004) showed that covering the fumigated soil with a virtually impermeable film or HDPE is even more effective in containing fumigants than increasing the depth of injection.

The environmental repercussions of these soil fumigants, especially MeBr (Gan et al., 1994, Gushee, 1996, Yagi et al., 1993, and Yates et al., 1996), metam sodium (Cone et al., 1994, and Macalady et al., 1998) and chloropicrin (Gan et al., 2000) necessitate the search for more efficient, eco-friendly, and durable alternatives.

Ozone Characteristics and Potentials in Disinfection

Ozone is a potent oxidant with strong germicidal properties, and it has been implemented successfully against numerous pathogens including viruses, bacteria, fungi, protozoa and metazoa (Cullen et al., 2009; Finch and Fairbairn, 1991; Khadre et al., 2001; Mun et al., 2009; Orta de Velásquez et al., 2002 and 2004; Ramírez-Cortina et al., 2005; Restaino et al., 1995). It is often used to disinfect drinking water and wastewater (Van Leeuwen, 1996; Van Leeuwen et al., 2003), and disinfest ships ballast water (Oemcke and van Leeuwen, 2004 & 2005) due to its oxidizing properties. Ozone has also been applied in mold prevention on stored corn (White et al., 2010), and in the degradation of mycotoxins (Tiwari et al., 2010). Mycotoxins are toxins produced by stored-grain pests and are “*known to exhibit carcinogenic, teratogenic, immunosuppressive properties and cause several physiological disorders both in humans and animals*” (Fung and Clark, 2004; Leung et al., 2006; Mally and Dekant, 2009; Stockmann-Juvala and Savolainen, 2008; Wu et al., 2009). Postharvest processing of fruits and vegetables with ozone gas or ozonated water inactivates pathogens and spoilage microorganisms (Cullen et al., 2009). Sarig et al. (1996) work indicates that postharvest treatment of fresh fruit (e.g. table grapes) with ozone decreases fungal deterioration caused by *Rhizopus stolonifer* and increases shelf-life. Scanning electron microscopy showed that ozone causes damages to the surface of *Toxocara canis* ova, a nematode parasite of dogs and other canines (Ooi et al., 1998). It is also capable of diffusing across bacterial membranes and reacting with cytoplasmic biomolecules, such as DNA, which results in cell death (Ishizaki et al., 1987). Micrographs of transmission electron microscopy of ozone-treated *Bacillus* spores showed degradation of the outer spore coat layers, thus exposing the core to further oxidation by ozone (Foegeding, 1985; Khadre and

Yousef, 2001). Furthermore, ozone reacts with biomolecules such as proteins, carbohydrates and polyunsaturated fatty acids bound to albumin, dyes, and is involved in lipid peroxidation (Bocci, 2005; Zhu et al., 2013).

Ozone has been approved by the American Food and Drug Administration for direct use in human food as it was affirmed as Generally Recognized as Safe-GRAS (Graham, 1997), as well as in drugs, cosmetics and also as compounds in food contact materials such as cutting boards and other surfaces that come in contact with unprotected food (Kobayashi et al., 2011). In addition, ozone is listed by the National Organic Program under the list of “*The National List of Allowed and Prohibited Substances*” with code (§205.605) referring to: “*Nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled as “organic” or “made with organic (specified ingredients or food group(s))”*” (National Organic Program, 2005). In contrast to other disinfection methods and conventional pesticides used in soil fumigation, postharvest fruit and vegetable processing fumigants, stored-grain pest treatments, and other pesticide applications, such as methyl bromide and other fumigants, the use of ozone as a disinfection method has the advantage that it does not produce pollutants, because its rapid decomposition only produces oxygen. In addition, ozone has a short half-life in soil of an order of minutes, because it decomposes quickly into oxygen, and results in low persistent chemicals in the soil after reacting with soil components (Takayama et al., 2006).

Sopher et al. (2002), reported the successful use of gaseous ozone for soil fumigation in increasing plant yield and minimizing the damaging effects of soil pathogens for a range of crops and soils under different climatic conditions. They reported that positive effects of preplant ozone application, might be due to the decrease in soil pathogen populations and

increased nutrient availability. However, they recommended further studies to accurately predict specific responses achieved from ozonation under different soils, plants, and environmental factors (crops, soils, pathogens and climatic conditions). In contrast to other disinfection methods and conventional fumigants used in the treatment of soil pathogens, namely MeBr, metam sodium, and chloropicrin, the use of ozone as a disinfection method has the advantage because it is environmentally friendly and not a source of pollution.

Matsuo (1993), reported that the treatment of nutrient solution with 0.25 mg/L of ozone for hydroponic cucumber culture was considerably effective in reducing germination of *F. oxysporum* f.sp. *cucumerinum* microconidia, while a concentration of 0.4 mg O₃/L completely inhibited the germination of fungal spores. In another study evaluating gaseous ozone for inactivating mixtures of non-specified spores and mycelia of fungi in malting barley, Allen et al. (2005), showed that 96% of fungi were inactivated at a dosage of 0.1 mg O₃ g⁻¹ barley min⁻¹ for 5 min, without affecting germination of the barley. The referenced research on hydroponic nutrient solutions treatment of *F. oxysporum* with ozonated water showed the need for further research to optimize the ozonation procedure. Further investigation in this is needed particularly the optimization of the ozonation procedure regarding the effects of temperature, rate of ozonation, and gas phase concentration (GPC), and the effect of admitting ozone gas directly to the nutrient solution in contrast to adding it in the form of ozonated water.

The high oxidative power of ozone, its efficiency in inhibiting pathogens without leaving toxic residues in the environment, the limited research conducted on the use of ozone as a soil fumigant, the absence of research on ozone as an oomycete treatment, and the need for optimizing ozonation procedure in the treatment of hydroponic nutrient solutions and

greenhouse wastewaters infected with plant pathogens, had encouraged us to do additional research in soil fumigation targeting the oomycete *Phytophthora sojae*, and in the optimization of ozone treatment of *Fusarium oxysporum* in water suspension, as a model of treatment of hydroponic nutrient solution and agricultural wastewater collected from drainage ditches. In addition, soil ozonation for nematode treatment was added to the research to broaden the targeted spectrum of soil pathogens.

Accordingly, the objectives of the current research are: For *P. sojae*, to investigate the use of gaseous ozone in suppressing the disease caused by this pathogen in soil assay, as a model *Phytophthora* pathogen that affects a wide range of high-value crops. For *F. oxysporum* (i) to test the effect of varying dosages of ozone on the viability of *F. oxysporum* spores in suspension, (ii) to compare the effect of ozone gas-phase concentration, i.e. when ozone is produced from either oxygen (high GPC) or air (low GPC) on the viability of *F. oxysporum* spores, and (iii) to evaluate the effect of ozone on the viability of *F. oxysporum* spores in suspension at either 5°C or 21°C. For nematode, to evaluate, on bench scale, (i) the effect of varying dosages of ozone on the viability of nematodes in the soil, and (ii) the efficacy of soil ozonation in nematode treatment at low temperature.

Research Objectives and Dissertation Organization

Nematodes

Plant parasitic nematodes are microscopic, nonsegmented roundworm parasites that live in soil and attack the plants through their roots. Nematodes feed on the nutrients found in the plant roots and vascular tissues, thus weakening the plant and leading to decreased yields.

An international survey determined annual crop losses due to nematodes as follows: cotton, 10.7%; peanut, 12%; wheat, 7%; and soybean, 10.6% (Sasser and Freckman, 1987).

Nematodes can cause up to 75% yield loss in some crops, in addition to vectoring plant viruses and creating root wounds through which other pathogens can enter (Barker and Koenning, 1998). In 2000, global production losses to nematodes were estimated at US\$ 121 billion, \$9.1 billion of which in the United States (Chitwood, 2002). Most nematicides are broad-spectrum, highly volatile fumigants, able to move in the soil and reach nematodes in their confinement. Most of the efficient volatile nematicides were deregistered (e.g. ethylene dibromide and dibromochloropropane, Chitwood, 2002), because they were correlated with environmental degradation and human health risks.

Currently, there are only a handful of chemicals registered for pre-plant nematode control (Duniway, 2002 and Martin, 2003). The most important remaining nematicide used is methyl bromide (MeBr). Many commodities have become dependent on MeBr for nematode control, which necessitates identifying effective alternatives (Carpenter et al., 2000). Zasada et al., (2010) believed that it would be too difficult to manage phytoparasitic nematodes without MeBr. However, given the pesticidal ozone characteristic, it would be a justified choice as potential alternative to MeBr in the control of nematodes. To our knowledge, no previous research has used ozone targeting soil nematodes. The use of nematicides is prohibited within 100 feet of drinking-water wells to protect groundwater from potential contamination (U.S. EPA, 2001), while ozone could be used safely near groundwater bodies. Hence, this encouraged us more to investigate its nematicidal efficacy.

Phytophthora sojae

Phytophthora is the name of an important phytopathogen that means literally “plant destroyer”. With more than 80 species, Phytophthora is an oomycete that attacks a wide range of agriculturally-important plants, and results in billions of dollars in losses worldwide each year (NSF Current, 2013). *Phytophthora infestans* was behind the famous Irish famine in 1840’s, which destroyed all potato production as a result of potato late blight. *P. infestans*, the most investigated *Phytophthora spp*, causes enormous losses in potato fields with estimations of US\$3 billion in losses annually worldwide, in spite of the use of fungicides and resistant varieties, because the pathogen in many instances has adapted quickly and become resistant (Duncan, 1999). *P. sojae* is one of the important species of Phytophthora. It can infect soybeans at all growth stages and causes seed rot, pre- and post-emergence ‘damping off’ and root and stem rot of older plants, with an annual cost worldwide of US\$1–2 billion (Tyler, 2007). Seedlings infected with *P. sojae* show lesions anywhere between the root, hypocotyls and cotyledon, turn brown, wilt, and die (Dorrance et al., 2008). The estimated reduction in soybean yield due to *P. sojae* in 1994 was 560,300 metric tons, and mild symptoms, referred to as hidden damage, may reduce yield by as much as 40% (Schmitthenner, 2000; Schmitthenner and Bhat, 1994).

Chemical fungicides that are mostly used in the control of *Phytophthora spp*. in high-value crops are metalaxyl, mefenoxam, phosphite, fosetyl-al, and soil fumigants i.e. methyl bromide, metam sodium and chloropicrin. The development of resistance to metalaxyl (Dahl et al, 1995; Dowley and O’Sullivan, 1985; Sankaran et al., 2008), and to mefenoxam (Lamour and Hausbeck, 2001; Mathia, 1999; Parra and Ristaino, 1998; Pennisi et al., 1990), the limited efficiency in disease control of fosetyl-al (Browne et al., 1999) and phosphite

(Forster et al., 1998), and the environmental repercussions of these fungicides and of soil fumigants, especially MeBr (Gan et al., 1994; Gushee 1996; Yagi et al., 1993; Yates, 1996), metam sodium (Cone et al., 1994; Macalady et al., 1998) and chloropicrin (Gan et al., 2000) necessitate the search for more efficient, eco-friendly, and durable alternatives to control the “plant destroyer” especially for high-value crops.

Furthermore, the economic importance of Phytophthora, and the need for efficient and environmentally safe alternatives to the use of fungicides used in its control, has justified the choice of *P. sojae* as one of the three targeted pathogens in this research. To our knowledge, no previous research has tried ozone against an oomycete.

Fusarium oxysporum

Fusarium wilt, caused by *Fusarium oxysporum*, is one of the most widespread and destructive diseases of many major ornamental and horticultural crops (Bowers and Locke, 2000). Currently, the major control practices adopted to control wilts and other soilborne pathogens on high-value crops (such as ornamental cut-flowers and greenhouse crops) are preplant soil fumigation and fungicide applications. For field crops, control measures adopted for Fusarium wilt diseases are limited to planting resistant cultivars; However, in some cases more virulent races of the pathogen have developed and therefore necessitated the continued development of new resistant cultivars to the new pathogen race (Mace et al., 1981). Because Fusarium spores remain viable in water, spores leached out of contaminated soil into drainage ditches, ponds and other water bodies used later in irrigation, can indefinitely be a source of inoculum (Ratting, 1977). This is particularly important in the case of water aggregation bodies (ponds and ditches) used in crop irrigation, or to treat the agricultural wastewater aggregating in drainage ditches of small-scale high-value crops, and

in hydroponic cultures where contaminated nutrient solution is reused. According to Song et al. (2004), Fusarium wilt is the most serious soilborne disease in hydroponic cultivation systems. *F. oxysporum* is highly virulent in hydroponic greenhouses because it spreads easily through the nutrient solutions (Sutton et al., 2009).

With the growing environmental and health concerns over chemical pesticides, water scarcity and the need to treat agricultural wastewaters contaminated with pathogens to be safely reused, the increasing demand for residue-free produce, and the current trends towards organic farming, the search for efficient fungal control methods that address these concerns and needs has become of high importance. In contrast to other disinfection methods and conventional fungicides used in the treatment of *F. oxysporum* in hydroponic cultures, such as prochloraz and carbendazim, ozone appears to be an effective method at reducing viability of Fusarium (Kottapalli et al., 2005) without generating toxic residues in the environment.

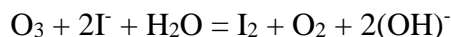
The economic importance of *F. oxysporum* especially in hydroponic cultivation systems, and the need for environmentally safe alternative treatments to the currently adopted fungicides, made it a justified choice as a target for treatment optimization with ozone.

Ozonation

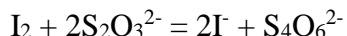
The measurement of absorbed ozone by samples was done by the iodometric wet-chemistry method (IOA, 1987). This method is based on the principle that iodide ion is oxidized by ozone to form iodine, as the carrier gas is bubbled through a solution of KI. When bubbling is stopped, the KI solution pH is adjusted with sulfuric acid to pH 2, in order to complete the reactions. The liberated iodine is reduced with a standardized 0.1N sodium thiosulfate titration to an endpoint using a starch indicator. The mass of ozone reacted is

determined based on a theoretical ozone/iodine stoichiometry of 1.0. The reactions of iodine liberation by ozone and its titration with sodium thiosulfate are described as follows:

Iodine liberation reaction:



Titration reaction:



For each O_3 molecule reacting with two of KI, 2 molecules of sodium thiosulfate are needed in the titration to the endpoint. Knowing the amount of $\text{Na}_2\text{S}_2\text{O}_3$ used, we could deduce the amount of O_3 that reacted with iodine by the multiplication with a factor of molar weight comparison as follows:

The sodium thiosulfate pentahydrate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) used has a molar mass of 248.18. The mass of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ 0.1N per ml titration is: $248.18 \times 10^{-1} \times 10^{-3} = 0.02482\text{g}$. The molar mass of ozone is 48, hence the mass of ozone (corresponding to the titrated amount of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) could be calculated by multiplying the volume of titrated $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ by a factor of: $0.02482 \times 2 \times 48 \sim 2.4$.

Before each experiment, measurement of the rate of ozone generation is tested with this method and noted, as the factors affecting the rate of ozone generation may fluctuate.

Knowing the amount of ozone (A) initially generated and subtracting the amount of unreacted ozone (B) determined by the KI method described above and multiplying by the factor of 2.4, the reacted amount of ozone (C) could be calculated using the following formula:

$$C = [(A - B) \times 2.4]/V$$

Where: V = volume of sample in liters - A and B are in mgO_3 - C is the calculated ozone dosage in mg/L .

Ozone Generator and Accessory Equipment Cost Breakdown

Ozone generators are available in different sizes and capacities according to the need. A medium-sized oxygen-source ozone generator with ozone output 5 kg O₃/h, costs \$168,000 including generator price at source (\$85,000), freight (\$2,000) and import duties (\$2,000), 3% insurance, 20% pipework, venturis and air drying equipment, 50% engineering, construction and power supply, and 2% maintenance.

Dissertation Organization

The dissertation is organized in five chapters, including a general introduction with a literature review, three papers and a conclusions chapter. Each of the papers tackles research on one of the three pathogens tested: Nematodes, *Phytophthora sojae* and *Fusarium oxysporum*.

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CHAPTER II

SOIL OZONATION FOR SOIL NEMATODES INACTIVATION AS AN ALTERNATIVE TO METHYL BROMIDE AND NEMATICIDES

A paper to be submitted to *The Journal of Plant Disease*

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Abstract

Phytoparasitic nematodes are important pests that cause severe crop yield losses. In the past, chemical compounds have been used as management practices but these practices are not environmentally sustainable. Ozonation was studied as an alternative management practice since it is highly effective against microorganisms and degenerates quickly to oxygen. Soil samples that were naturally infested with nematodes were treated with different levels of gaseous ozone at 21 °C and 5 °C. A medium level of ozonation (2.1 g O₃ kg⁻¹ for 10 min at a rate of ozonation 0.21 g O₃ kg⁻¹min⁻¹) and low temperature (5 °C) resulted in 96% nematode inhibition. Regression analysis showed that nematode viability was a function of the level of ozonation (P = 5.1E-07) and the soil temperature (P = 4.4E-08; Adjusted R-square = 0.65). These data imply that ozone may be an efficient and sustainable alternative to nematicides and the fumigant methyl bromide in the treatment of nematodes in the soil.

Additional keywords: ozone, soil ozonation, nematodes, sustainability, methyl bromide alternatives, nematicides alternatives.

Introduction

Plant-parasitic nematodes are microscopic, nonsegmented roundworm parasites that live in soil and attack the plants through their roots. Endoparasitic nematodes infect and colonize the roots of plants (e.g. lance, root-lesion, and root-knot) while ectoparasitic nematodes remain outside of the root tissue, (e.g. dagger, needle, spiral, sting, stubby root and stunt). Nematodes feed on the nutrients found in plants roots and vascular tissues, weakening the plant and leading to decreased yields. An international survey determined annual crop losses due to nematodes as follows: cotton, 10.7%; peanut, 12%; wheat, 7%; and soybean, 10.6% (36). Nematodes can cause up to 75% yield loss in some crops, in addition to vectoring plant viruses and creating root wounds through which other pathogens can enter (3). In 2000, global production losses to nematodes in all crops were estimated at US\$ 121 billion, \$9.1 billion of which in the United States (8).

Phytoparasitic nematodes survive in the soil or in plant roots, and active nematode stages are more susceptible to nematicides than resting stages (11,21). Most systemic nematicides are needed in high concentrations (e.g. 1000 ppm of Vydate) to control nematodes within plant roots, which is impractical under field conditions (11). Hence, it is difficult to deliver a nematicide in efficiently sufficient concentration directly in contact with nematodes within plant roots and root surroundings. Total eradication of nematode populations with a nematicide or fumigant is difficult to achieve due to the heterogeneous nature of soil that offers protection to some individuals or ova (8). However, management should be aimed at inhibiting or deactivating the number of phytoparasitic nematodes in the soil below their economic threshold. Most nematicides are broad-spectrum, highly volatile fumigants, that are able to move through the soil pores. Many of the most efficient volatile

nematicides have been deregistered (e.g. ethylene dibromide and dibromochloropropane) (8), because they were associated with environmental and human health risks. Ethylene dibromide was the most abundantly used nematicide in the world, until 1983 when it was prohibited in the United States because of groundwater contamination and possible carcinogenicity (13,23). Similarly, 1,3-dichloropropane was prohibited because it was classified as a probable carcinogen (1) while 1,2-dibromo-3-chloropropane (DBCP) was suspended in the U.S in the late 1980s because it was found to cause male infertility and was a probable carcinogen (40). Carbamates used as nematicides (i.e. aldicarb, carbofuran and oxamyl) are highly toxic to humans and animals (1), and organophosphates (ethoprop, fenamiphos, cadusafos, fosthiazate and phorate) have been reviewed by the U.S. EPA and several were withdrawn from use (24). Some nematicides, however, have recently undergone re-registration eligibility decisions (REDs) by the U.S. Environmental Protection Agency (44). These include, metam sodium that has limited efficiency in controlling nematodes in some circumstances (9,13,18), and the fumigants chloropicrin, metam-potassium, and dazomet.

Currently, there are only a handful of chemicals registered for pre-plant nematode control (10,19). The most important remaining nematicide, methyl bromide (MeBr), was the fourth most abundantly used pesticide in the U.S. in 1997 (1), is now under phaseout due to its degradation of the stratospheric ozone layer. Many commodities have become dependent on MeBr for nematode control, which necessitates identifying effective alternatives (7). Zasada et al. (53), believed that it would be too difficult to manage phytoparasitic nematodes without MeBr. Methyl bromide is an effective pre-plant soil fumigant used to control soil pests (weed seeds, nematodes, insects, fungi, bacteria and viruses) (31), in many high-input,

high-value crops in U.S. agriculture, including vegetables, nursery plants, ornamentals, tree fruits, strawberries and grapes (53). This broad-spectrum pest control, along with its higher efficacy compared to other fumigants (20), and its volatility that enables it to penetrate treated soil sufficiently (10), has made some crop production systems highly MeBr-dependent, e.g. strawberries and fresh market tomatoes, and led to reductions in crop rotation and in diversification of production practices (6). Approximately 25,000 to 27,000 ton of MeBr was still applied annually between 1990 and 1994 (42), with more than 75% of its use for pre-plant soil fumigation (41). In 2013, only 562 metric tons of MeBr were allowed by the EPA as “critical use exemption”, in compliance with the MeBr phaseout plan mandated by the Montreal Protocol (45) to protect the stratospheric ozone layer.

Ozone is a potent oxidant and it has been implemented successfully against numerous pathogens including bacteria, viruses, protozoa and also metazoa (28,29,32,39). Ozone is often used to disinfect drinking water and wastewater (46,47), and disinfest ships ballast water (25,26) due to its oxidizing properties. Ozone has also been applied in mold prevention on stored corn (51,52). Scanning electron microscopy showed that ozone causes damage to the surface of the ova of *Toxocara canis*, a nematode parasite of dogs and other canides (27). Ozone is also capable of diffusing across bacterial membranes and reacting with cytoplasmic biomolecules, such as DNA, which results in cell death (16). Furthermore, ozone reacts with biomolecules such as proteins, carbohydrates and polyunsaturated fatty acids bound to albumin, dyes and is involved in lipid peroxidation (4,54).

Ozone has been approved by the American Food and Drug Administration for direct use in human food, drugs, and cosmetics and also as compounds in food contact materials such as cutting boards and other surfaces that come in contact with unprotected food (17). In

addition, ozone is listed by the National Organic Program under the list of “The National List of Allowed and Prohibited Substances” with code (§205.605) referring to: “Nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled as “organic” or “made with organic (specified ingredients or food group(s))”” (22). In contrast to other disinfection methods and conventional pesticides used in the treatment of soil pests, such as soil fumigants MeBr, metam sodium and chloropicrin described above, the use of ozone as a disinfection method has the advantage that it does not produce pollutants, because its rapid decomposition produces oxygen only. The use of other nematicides is prohibited within 100 feet of drinking-water wells to protect groundwater from potential contamination (43), while ozone could be used safely near groundwater bodies.

Sopher et al. (35), reported the successful use of gaseous ozone soil fumigation in increasing plant yield and reducing the detrimental effects of soil pathogens in a range of crops and soils under different climatic conditions. They reported positive effects of preplant ozone application, theoretically attributed to the decrease in soil pathogens and increased nutrient availability. However, they recommended further studies to confirm this theory and predict specific responses achieved from ozonation under different crops, soils, pathogens and climatic conditions. Nevertheless, to our knowledge, no further studies have been done in this regard.

The high oxidative power of ozone, its effectiveness in inhibiting pathogens without leaving toxic residues in the environment, and the limited research on ozone use in the domain of soil fumigation as alternative to nematicides inspired the current research. Furthermore, the economic importance of phytoparasitic nematodes, and the need for efficient and environmentally safe alternative treatments to the currently adopted fumigant

nematicides, made treatment with ozone a realistic aim for further investigation.

We conducted studies in the laboratory to evaluate the effect of ozone on nematode viability in soil samples collected from a field in Iowa. Our objectives were to evaluate (i) the effectiveness of different ozone doses and rates at reducing the viability of nematodes in the soil, and (ii) the efficacy of soil ozonation at low soil temperature (5 °C) versus high soil temperature (21 °C).

Methods and Materials

Soil samples

Soil for this experiment was collected from the Hinds Farm (Iowa State University research farm, near Ames, Story County, Iowa). This soil belongs to the Clarion-Nicolett-Webster “principal association area”, and Zenor soil series (Iowa Soil Properties and Interpretations Database-ISPAID). The soil was analyzed for texture and organic matter content and was found to contain 79% sand, 4.9 % coarse silt, 4.7% fine silt and 10.4% clay. The soil had low organic matter content (1.4%) and low total carbon (0.7%).

The species composition of nematodes present in the soil was determined by centrifugal floatation and species identification with the aid of an inverted compound microscope, on four soil samples (100 g each). The soil contained an average of 225 non-plant parasitic nematodes, 2 spiral (*Helicotylenchus* sp.) and 0.5 ring (*Criconemoides* sp.) nematodes per 100 g of soil. Non-plant parasitic species lack for feeding stylet, a mouth part necessary in plant parasitism. These nematodes belong to the group of free-living terrestrial nematodes, constituting 25% of all nematode species. Spiral nematode is one of the most

common ectoparasites that occur in corn fields and floor of forests. Damage potential of spiral nematode is low, with a threshold of 500 – 1000 per 100 cc soil. Ring nematode is an ectoparasite with a damage threshold of 100 per 100 cc soil. Accordingly, both spiral and ring nematodes detected were well below damage thresholds.

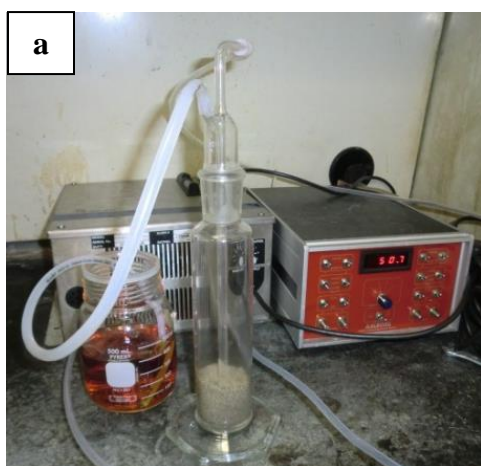
Ozone treatment of soil

Prior to ozone treatment, the soil was sieved and mixed well. Samples of 100g were treated with incrementally greater ozone doses (low, medium and high; Table 1) generated by increasing the ozone generation time (Fig. 1.a), at a flow rate of 0.1L/min. Each experiment consisted of 5 replicates of 100 g each: three ozonated at the same dose, and two nontreated control samples. Doses of ozone applied ranged from 0.39 to 3.12 g O₃/kg soil. The effect of temperature on the efficacy of ozone to reduce the viability of nematodes was also tested. Two temperatures (5°C and 21°C) were tested for each ozone dose. For experiments at 5 °C, soil was kept in a refrigerator at 5 °C until the ozonation experiments. After ozonation, the five subsamples were soaked in Baermann funnels (Fig. 1.b) (51) at room temperature. Since only viable nematodes migrate down through the soil sample, penetrate the filter and fall down into the distillate, nematode viability was easily determined by comparing nematode counts in the treated and untreated (sub) samples in the distillate after 24 h and 48 h. Nematodes were counted with the aid of an inverted compound microscope (Fig. 1.c) at x40 magnification. Viability was determined as the total number of nematodes in the treated sample divided by the total number of nematodes in the control samples as a percentage. The experiment was repeated twice as shown in the experimental design (Table 1).

Table 1. Experimental design with number of subsamples and replications per ozone level, dose, time of ozonation, rate of ozonation and temperature.

Temperature (°C)	Level of ozonation	Dose (g O ₃ kg ⁻¹ soil)	Time (min)	Rate (g O ₃ kg ⁻¹ min ⁻¹)	Samples ^a x Reps.
5 (°C)	Low	0.6	5	0.14	5 x 2
		0.8	5	0.16	5 x 2
		0.9	5	0.17	5 x 2
	Medium	1.4	7.5	0.19	5 x 2
		1.9	7.5	0.26	5 x 2
		2.1	10	0.21	5 x 2
	High	2.5	13	0.19	5 x 2
		2.5	13	0.19	5 x 2
		3	15	0.20	5 x 2
3.2		15	0.21	5 x 2	
21 (°C)	Low	0.4	1	0.39	5 x 2
		0.4	1.5	0.29	5 x 2
		0.6	2	0.31	5 x 2
		0.7	2	0.35	5 x 2
	Medium	1.1	4	0.27	5 x 2
		1.1	5	0.22	5 x 2
		1.5	7.5	0.19	5 x 2
		1.5	7.5	0.19	5 x 2
	High	2.2	8.75	0.25	5 x 2
		3.5	15	0.23	5 x 2

^a Each experiment consisted of 5 subsamples (100 g soil each), three subsamples were ozonated and two controls. Then, the experiment was repeated twice.



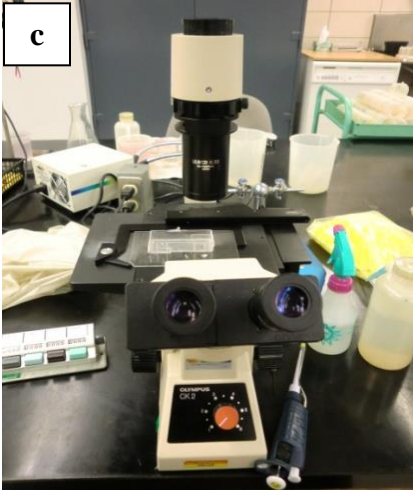


Figure 1. **a.** Ozone generator, reactor, and sample ozonation. **b.** Soaking of samples after ozonation in Baermann funnels for 24 h and 48 h. and draw-off of the filtrate to collect viable nematodes for counting and assessment of treatment **c.** Nematode counting with a dissecting microscope at x40 magnification.

Ozonation

The ozone generator used was a 1000BT-12 Triogen Model TOG C2B,—generating a maximum of 1g O₃/h from pure oxygen by corona discharge, where the conversion of oxygen to ozone occurs in a tubular cell excited by a high-voltage potential. The reactor was made of glass (Fig. 1.a), and all tubing was made of silicone material. The operating volume in the reactor was 250 cc. In each test the ozone flow rate per min was maintained at 1L min⁻¹ L⁻¹ gas-flow/liter volume of soil sample (34). The excess and unreacted ozone was captured in a solution of 2% potassium iodide (KI). The amount of absorbed ozone by the soil sample was measured by the iodometric wet-chemistry method (15). Well-established, standardized methods for ozonation and ozone measurement were used (15).

Data analysis

Mean percent viability for each ozone level was calculated and data were analyzed using RStudio software (RStudio, Inc., Boston, Massachusetts). Data is clustered in three ozone levels: low ($0.12 - 0.16 \text{ g O}_3 \text{ kg}^{-1} \text{ s}^{-1}$), medium ($0.25 - 0.31 \text{ g O}_3 \text{ kg}^{-1} \text{ s}^{-1}$) and high ($0.57 - 0.62 \text{ g O}_3 \text{ kg}^{-1} \text{ s}^{-1}$), representing each an average of Dose*Rate of about 30 samples with a marginal standard error. Dose*Rate is an interaction factor combining ozone dose in grams of ozone per kilogram of soil, and the rate of ozone generation in grams of ozone per kilogram of soil per second, indicating the speed of ozone generation and feeding the sample. Comparison of means between levels at different temperatures was done by Student's t-Test: two-sample assuming unequal variances at 95% confidence interval.

To quantify the effects of ozonation levels and temperature on nematode viability, a multiple regression analysis was conducted and a linear regression model was obtained.

Results

Before processing the linear regression model analysis, the distribution of data and homogeneity of variance were checked and found normally distributed.

ANOVA table (Table 2) shows each factor significance in the linear model.

Table 2. Regression statistics and ANOVA, showing the significance of ozonation level (Dose*Rate) and temperature on nematode viability.

Regression Statistics	
Multiple R	0.814871638
R Square	0.664015787
Adjusted R Square	0.646785827

Table 2 continued

ANOVA	Coefficients	Standard Error	t Stat	P-value
Intercept	14.11445702	2.305026305	6.123338805	3.49072E-07
Temperature	1.058978269	0.156333081	6.773859128	4.38677E-08
Dose*Rate	-32.70057066	5.445014351	-6.005598618	5.08697E-07

Regression analysis shows that both the level of ozonation (Dose*Rate) and temperature are significantly important in affecting nematode viability. The regression model explaining the relation between nematode viability and the two factors (Temp. for temperature and O₃-Level for Dose*Rate) is:

$$\text{Viability} = 14 + 1.06 \text{ Temp.} - 32.7 \text{ O}_3\text{-Level.}$$

Nematode viability was reduced with ascending ozone levels (P=5.08697E-07) and decreasing temperature (P=4.38677E-08). Ozonation at 5 °C was more effective than at 21 °C (Tables 2, 3). At low ozone levels, viability of nematodes was reduced 61% compared with 82% at 21 °C and 5 °C, respectively. At medium ozone levels, nematode viability was reduced by 76% compared with 96% at 21 °C and 5 °C respectively, and at high levels, it was reduced by 85% at 21 °C compared to 97% at 5 °C.

Table 3. Effect of soil ozonation at different Doses*Rates on nematode viability in soil samples collected from a corn field in central Iowa at 21 °C and 5 °C

Level ^x	Dose*Rate ^y (g O ₃ kg ⁻¹ s ⁻¹)	Temperature (°C)	Viability ^z (%)
Low	0.12 (0.06)	5	18 (8) a
Medium	0.31 (0.05)	5	4 (3) b
High	0.62 (0.05)	5	3 (1) b

Table 3 continued

Low	0.16 (0.03)	21	39 (6) c
Medium	0.25 (0.04)	21	24 (5) d
High	0.57 (0.12)	21	16 (1) a

^x Each level (low, medium and high) represents an average of Dose*Rate of 30 samples. Values are presented by the average followed by the standard error in parenthesis.

^y Dose*Rate is an interaction factor combining ozone dose in grams of ozone per kilogram of soil, and rate of ozone generation in grams per second (indicating the speed of ozone generation and feeding the sample).

^z Viability of nematodes was evaluated as the percent of nematodes in treatment distillate in comparison with the count in control samples. Mean viability followed by the standard error in parenthesis are followed by a letter (a, b, c, d). Values followed by the same letter were not significantly different ($P > 0.05$) by “t-Test: two sample assuming unequal variances”. Detailed means comparison and p-values are shown in table 4.

Table 4. P values of means comparison using a t-Test: two-sample assuming unequal variances (REF) between three ozonation levels at 5 °C and 21 °C

P(T<=t) two-tail	Low (5 °C)	Medium (5 °C)	High (5 °C)	Low (21 °C)	Medium (21 °C)	High (21 °C)
Low (5 °C)	-----	0.000009	0.000003	0.005	0.055	0.209*
Medium (5 °C)	0.000009	-----	0.21*	0.00457	0.003	0.000012
High (5 °C)	0.000003	0.21*	-----	0.0039	0.00268	0.0009
Low (21 °C)	0.005	0.00457	0.0039	-----	0.021	0.020
Medium (21 °C)	0.055	0.003	0.00268	0.021	-----	0.0496
High (21 °C)	0.209*	0.000012	0.0009	0.020	0.0496	-----

*P-value higher than 0.05, with no significant difference between groups.

Temperature had significant effect on treatment ($P \ll 0.05$). Ozonation at 5 °C was more efficient than at 21 °C at inhibiting nematodes.

Level of ozonation had significant effect on treatment ($P \ll 0.05$). Ozonation was more efficient at ascending levels at inhibiting nematodes.

The optimal treatment with ozone was obtained at a medium level of ozonation (a dose of 2.1 g O₃ kg⁻¹ soil for 10 min at a rate of 0.21 g O₃ kg⁻¹min⁻¹) and low temperature (5 °C) which resulted in 96% nematode inhibition. A higher dose of ozone did not result in a significantly important reduction in nematode viability. More than 50% of nematodes were inhibited at the lowest ozonation level applied (a dose of 0.4 g O₃ kg⁻¹ soil for 1 min at a rate of 0.14 g O₃ kg⁻¹min⁻¹, at 21 °C) (Table 1, 3).

It was noticed that the collected filtrate from treated samples was yellow in color (Fig. 2), unlike that from untreated samples that was colorless.



Figure 2. Filtrates from ozonated (yellow) and control (colorless) samples collected after 24 h soaking in Baermann funnels.

In order to investigate this observation, ozonated soil samples were analyzed for pH and the main oxidizable elements: P (Mehlich-3 extraction, showing P in its bioavailable form), Zn, Fe & Mn (analyses of the bioavailable forms by DTPA extraction method). Results did not show any correlation between ozonation dose (expressed in time of ozonation in min. and in dose in g O₃ kg⁻¹ soil) and any of the analyzed parameters (Table 5).

Table 5. Effect of ozone in ascending doses on the soil pH and the release of bioavailable forms of P, Zn, Fe and Mn

Time of ozonation	Dose (g O ₃ kg ⁻¹ soil)	pH ^a	M-3 P ^b (ppm)	DTPA ^c -Zn (ppm)	DTPA ^c -Fe (ppm)	DTPA ^c -Mn (ppm)
control	0	7.90	34	0.5	16	116
10 min	0.5	7.75	38	0.9	24	24 ^d
13 min	0.7 ^e	7.70	37	0.8	21	141
15 min	0.8 ^e	7.80	33	0.7	17	115
17 min	1.1 ^e	7.80	38	0.7	20	123
20 min	1.2 ^e	7.80	37	0.7	22	24 ^d
25 min	1.4 ^e	7.80	36	0.7	21	42 ^d

^a The soil ozonation did not show a correlation between ozone doses and variation in soil pH.

^b Bioavailable form of phosphorous in response to ozonation was measured with the Mehlich-3 method, and showed no correlation between ozone dose and M-3 P.

^c Bioavailable forms of zinc, iron and manganese in response to ozonation were measured using the DTPA extraction method. No correlation was detected between ozone dose and the variations in DTPA forms of Zn, Fe or Mn.

^d Differences between DTPA-Mn numbers are of an order of ppm. This is a normal and non-significant difference between soil samples from the same soil.

^e Difference in dosage increase in response to the same increase in ozonation duration (2 min) is due to the difference in ozone absorption by the soil samples. This fluctuation depended on how tightly submerged the ozone diffuser was in the soil sample. Doses presented are averages of dosage measurements of 12 replicates of the same ozonation duration.

Discussion

The overall results of this study clearly indicate that ozonating soil infected with nematodes at a medium level at 5 °C is sufficient to kill 96% of the nematodes. Also, ozonation at a low temperature (5 °C) was more efficient at killing soil nematodes than at a high temperature (21 °C), which favors the application of this treatment at the beginning of the growing season. Experiments showed that more than 50% of nematodes were inhibited at the lowest level of ozonation executed at either temperatures (Table 3). Accordingly, this level of disinfection might be enough to reduce the nematodes viability below damaging

thresholds, without harming the soil biotic balance. Biotic balance is a crucial factor in maintaining the soil health and productivity, and non-plant parasitic nematodes and other beneficial microorganisms play an essential role in maintaining that through organic and non-organic nutrients recycling, and by competing with -and suppressing, plant parasitic microorganisms. Hence, it is not recommended to use unnecessary higher ozone doses in the control of soil nematodes.

Ozone was more efficient at reducing nematode viability at lower temperature, which is similar to that reported by Patil et al. (30). This is attributed to the increasing ozone solubility ratio with decreasing temperature (2), and the slower ozone decomposition at lower temperature (33). Hence, ozone is more stable at 5°C, which prolongs its activity at oxidizing and inhibiting nematodes in the soil. Consistent with these physico-chemical ozone properties, the current study confirms a higher efficacy at a lower temperature. This effect of temperature efficacy does not occur with many nematicides (e.g. EDB and 1,3-D), (38) and fumigants (MeBr) (12), which is an advantage for ozone use, because nematicides are usually applied at the beginning of the growing season, when temperatures are usually below optimal soil temperature range for nematode development and multiplication (21 °C to 27 °C). This qualification is an advantage over nematicides and other gas fumigants, because these latter are less efficient at low temperatures.

The results in Table 5 do not show any correlation between ozonation dose or time and the analyzed soil parameters (pH, Me-3 P, and DTPA- Zn, Fe, & Mn) in response to ozonation, which does not prove the theory of Sopher et al. (35) of increased nutrient availability by soil ozonation. A plausible explanation of the yellow coloration of ozonated soil filtrate might be the oxidation of soil organic matter. By oxidizing soil organic matter,

the organic carbon content transforms from humine to humic acid then to fulvic acid, which might explain the yellowish coloration of the filtrate. Fulvic acid is the most soluble and mobile form of organic carbon, and the most active form in chelating nutrients and rendering available to plants. Hence, this could partially confirm Sopher et al. (35) theory, since fulvic acid ameliorates the soil physical-chemical properties and increases plant productivity as a consequence. The soil we used in this experiment is sandy with low organic matter content (Table 1). Ozone is known to be able to selectively oxidize colored matter and cause color changes (48, 49).

Fumigants diffusion is faster in coarse-textured soil with high moisture (5), and these become less efficient in soils with high organic matter content (35). Organic matter and metals increase the ozone demand because they are oxidizable. Hence, higher ozone doses will be required than in this research to reach similar nematode inhibition rates in heavier soils with higher organic matter and metal contents.

This study was not species-specific, since the observations were assessing the aggregate number of nematodes inhibited by the treatment unselectively amongst species. Therefore, further experimentation with species specificity is recommended, taking in consideration the significance of nematode inactivation by species. In addition, although the soil that was used in this research did not include significant numbers of phytoparasitic nematodes, the high efficiency of ozone in inactivating non-parasitic nematodes could be an indicator for comparable effect on plant-parasitic nematodes as well. Hence, this could be a plausible confirmation of Sopher et al. (35) assumption that the increased crop yield after soil ozonation was attributed in part to a decrease in soil pathogens by ozone.

Since ozone does not leave toxic residues, and given that low doses are required to inactivate nematodes by half, which would control the nematodes without harming the soil biotic balance, ozonation could be used as a sustainable alternative to the conventional treatments that have been used to manage nematodes and other soil pathogens. Thus it could play an important role in organic agriculture. Furthermore, due to the complexity of ozone generation systems required in field application and the difficulty of bringing big ozone generators on site, the application of this technique is limited to small crop-lands. Lands that are suitable for soil ozonation are those usually treated with gas fumigants, (e.g. MeBr), namely high-value crops and greenhouse crops. Finally, additional research is required to evaluate the economic feasibility of ozonation to control soil nematodes, the species-specific response to ozonation, and the application of soil ozonation at the field level.

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CHAPTER III

OZONATION EFFICACY AS ENVIRONMENT-FRIENDLY ALTERNATIVE TO TOXIC FUNGICIDES IN THE TREATMENT OF SOIL-BORNE *PHYTOPHTHORA SOJAE*

A paper to be submitted to The Journal of Bioresource Technology

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Abstract

Ozonation was studied for inactivating *Phytophthora sojae*, a predominant soybean pathogen that causes root and stem rot, and pre- and post-emergence damping-off of soybean. Assays of artificially inoculated soil samples with *P. sojae* were treated with different doses of gaseous ozone. This study showed that a dosage of 0.47 g O₃/kg soil, totally prevented root and stem rot disease symptoms caused by *P. sojae*. The findings of this research clearly indicate that ozonation is an efficient and sustainable alternative to chemical fungicides in the inhibition of Phytophthora diseases in the soil.

Additional keywords: ozone, soil ozonation, soil disinfection, *Phytophthora sojae*, sustainability, soilborne pathogens.

Introduction

Phytophthora is an important phytopathogen that means literally “plant destroyer”. With more than 80 known species, Phytophthora is an oomycete from the kingdom Chromalveolata that attacks a wide range of agriculturally-important plants, and results in billions of dollars in losses worldwide each year (29). *Phytophthora infestans* was behind the

infamous Irish famine in 1840's, which destroyed all potato production as a result of potato late blight. *Phytophthora* produces several kinds of spores to survive under different soil conditions, the most predominant of which are (i) sporangia, asexual sac-like multinucleate spores, (ii) oospores, which are non-motile sexual spores specialized for survival in the absence of a host-plant and adverse conditions, and (iii) zoospores which are dispersal spores adapted to move with water, locate the host-plant, and disseminate the pathogen (17). Asexual spores (sporangia and zoospores) are often targeted by treatments to manage *Phytophthora*, because they represent a vulnerable phase in the pathogen life cycle. Also, they are exposed to the environment and have limited nutrient reserves which prevent them from persisting for long outside a host (17).

P. sojae is one of the important species of *Phytophthora*. It can infect soybeans at all growth stages and causes seed rot, pre- and post-emergence 'damping off' and root and stem rot of older plants, with an annual cost worldwide of US\$1–2 billion (51). Seedlings infected with *P. sojae* show lesions anywhere between the root, hypocotyls and cotyledon, turn brown, wilt, and die (8). Similar to the other *Phytophthora* species, *P. sojae* persists in soils as oospores which can survive for many years without a host, either in the crop residue or in the soil after the residue decomposes (51).

Cultural practices, development of resistant varieties, organic amendments, fungicides and fumigants are all adopted in the control of *Phytophthora* diseases. However, each control measure has some drawback. Based on the biological knowledge of *Phytophthora* and understanding the ecological processes that could suppress the disease, the most important cultural practice in the control of *Phytophthora* diseases is the management of soil moisture since the pathogen's spores disperse with free moisture and through water.

However, controlling soil moisture is not always manageable, like in the case of *P. sojae*, one of the predominant soybean pathogens, in production regions with poorly drained soils and heavy rain occurrence (14). The estimated reduction in soybean yield due to *P. sojae* in 1994 was 560,300 metric tons, and mild symptoms, referred to as hidden damage, may reduce yield by as much as 40% (43,44). Organic treatments like composts and soil amendments, did not reduce soil populations of *P. capsici* causing pepper root and crown rot, although they provided some control of the disease incidence (18). The use of resistant varieties is not a durable solution, because the pathogen in many instances has adapted quickly and become resistant (10). In addition, some of the developed resistant varieties to *Phytophthora* do not possess desirable horticultural characteristics that are accepted by growers (1), or in some cultivars, they possess excellent horticultural characteristics combined with resistance to one phase of the pathogen, but do not have resistance to its other phases (5,40). Chemical fungicides that are mostly used in the control of *Phytophthora spp.* in high-value crops are metalaxyl (trade name Ridomil), mefenoxam (trade name Ridomil Gold), phosphite (salt of phosphorous acid), fosetyl-al (trade name Aliette), and soil fumigants i.e. methyl bromide, metam sodium and chloropicrin. The development of resistance to metalaxyl (7,9,36), and to mefenoxam (22,25,35,36), the limited efficiency in disease control of fosetyl-al (4) and phosphite (11), and the environmental repercussions of these fungicides and of soil fumigants, especially MeBr (13,41,55,56), metam sodium (6,23) and chloropicrin (12) necessitate the search for more efficient, eco-friendly, and durable alternatives to control the “plant destroyer” especially for high-value crops.

Ozone is a potent oxidant and it has been used successfully against numerous pathogens including viruses, bacteria, protozoa, fungi and metazoa (20,27,28,33,34,39,49).

Ozone is often used to disinfect drinking water and wastewater (52,53), and disinfest ships' ballast water (30,31) due to its oxidizing properties. Ozone has also been used in mold prevention on stored corn (54). Scanning electron microscopy showed that ozone causes damages to the surface of *Toxocara canis* eggs, a nematode parasite of dogs and other canines (32). Ozone is also capable of diffusing across bacterial membranes and reacting with cytoplasmic biomolecules, such as DNA, which results in cell death (16). Furthermore, ozone reacts with biomolecules such as proteins, carbohydrates and polyunsaturated fatty acids bound to albumin, dyes and is involved in lipid peroxidation (3,57).

In contrast to other disinfection methods and conventional fungicides used in the treatment of soil pathogens, namely metalaxyl, mefenoxam, MeBr, metam sodium, and chloropicrin, the use of ozone as a disinfection method has the advantage because it is environmentally friendly and not a source of pollution. To our knowledge, no previous research has tried ozone against an oomycete. The high oxidative power of ozone, its efficiency in inhibiting pathogens without leaving toxic residues in the environment, the limited research conducted on the use of ozone as a soil fumigant, and the absence of research on ozone as an oomycete treatment, had encouraged us to do additional research on this topic. Furthermore, the economic importance of Phytophthora, and the need for efficient and environmentally safe alternatives to the use of fungicides, has justified the need of this research.

Sopher et al. (45), reported the successful use of gaseous ozone for soil fumigation in increasing plant yield and minimizing the damaging effects of soil pathogens for a range of crops and soils under different climatic conditions. They reported that positive effects of preplant ozone application were due to the decrease in soil pathogen populations and

increased nutrient availability. However, they recommended further studies to accurately predict specific responses achieved from ozonation under different soils, plants, and environmental factors (crops, soils, pathogens and climatic conditions). Nevertheless, to our knowledge no further studies were conducted on this topic.

Therefore, the main objective of this study was to investigate the use of gaseous ozone in controlling *P. sojae* in soil assay, as a model Phytophthora pathogen that affects a wide range of high-value crops.

Methods and Materials

Experimental investigations for this study were conducted in the environmentally controlled greenhouse of the Department of Horticulture at Iowa State University (ISU). Soil for this experiment was collected from Hinds Farm (an ISU research farm, near Ames, Story County, Iowa). This soil belongs to the Clarion-Nicolett-Webster “principal association area”, and Zenor soil series according to the Iowa Soil Properties and Interpretations Database-ISPAID. The soil was analyzed for texture and organic matter contents and results of soil analysis show that the soil texture is sandy with low organic matter and organic carbon content (Table 1).

Table 1. Texture and organic matter contents of the soil used in a study to determine the effect of ozone on *P. sojae*

Total C (%)	OM (%)	% SAND	% COARSE SILT	% FINE SILT	% CLAY
0.73	1.4	79.0 ^a	4.9	4.7	10.4

The analysis of soil shows that the soil used in the current research is sandy^a in texture constituted in 4/5 of sand, with a low organic matter (<2%) and organic carbon (0.73%) content.

Inoculum preparation

To evaluate the effect of ozonation on *P. sojae*, soil was artificially infested with *P. sojae* rice inoculum, treated with ozone at various dosages, then seeded with susceptible soybean cultivar (Sloan), and incubated for two weeks. To prepare the soil samples, soil was first sterilized through autoclaving (dry heat at 170°C for 60 min) to eliminate any undesired pathogens, and then the soil was artificially inoculated with rice infested with *P. sojae* (46). The isolate of *P. sojae* R7-2a (pathotype 1d, 2, 3a, 5, 6, 7) (acquired from Dr. Anne Dorrance, Department of Plant Pathology at Ohio State University) was used in this study. For long-term storage, the isolate was first plated on DV8++ (diluted V8 juice agar plus antibiotics neomycine sulfate and chloramphenicol) and after 7 days, plugs ~2mm² of *P. sojae* mycelia were transferred to sterilized water in a tube including sterile water, at room temperature without the presence of any light (complete darkness). To prepare *P. sojae* rice inoculum, two-week old agar plugs of R7-2a were transferred to rice that had been autoclaved twice for 45 min on two consecutive days, and incubated for two weeks at room temperature, with daily break of clumps that were built in the plastic bag. The rice was dried for two consecutive days at room temperature, before it was mixed with the autoclaved soil.

Experiments were conducted in a greenhouse using 16 oz PVC pots. Each pot was first filled with 150g of sterilized soil, then 15cc of *P. sojae*-infested rice was placed in a layer, and finally the inoculum layer was covered by adding 300g of sterilized soil. The pots were flooded with deionized water for 24 h, then drained for another 24 h or until the moisture content approaches ~ 300 mb matrix potential (44). The pots were then placed in polyethylene bags and incubated in a greenhouse for a total of 2 weeks (greenhouse temperature was maintained at 25°C for 16 h to simulate day hours, and at 21°C for 8 h to

simulate night hours). Oospores will germinate and form sporangia during this period. Plastic bags from pots were removed after the two-week period, and then pots were flooded again for 24 h period and then drained for 48 h. The last flooding procedure is required to disperse zoospores, emerging from sporangia in the rice inoculum layer, throughout the soil in the pot.

Experimental design

A total of 5 runs of the experiment or “batches” were prepared. Batches consisted of 24 pots of *P. sojae*-infested soil each. For each batch, eight samples were non-treated control samples, and 16 samples were treated in quadruplicate sub-samples (4 x 4 subsamples) at different ozone doses. There were 6 treatments that were ascending doses of ozone generated by increasing the time of ozonation (10, 13, 15, 17, 20 and 25 min per 450g soil corresponding to ~ 0.47, 0.73, 0.79, 1.1, 1.2, and 1.41 g O₃/kg soil, respectively). Treatment samples (# of pots-subsamples per treatment dose) and non-treated control pots per batch are listed in Table 2. Each batch was treated separately on a different day, including 4 of the 6 treatments and one set of 8 control pots per batch. Control samples, consisting of 8 pots per batch of *P. sojae*-infested soil, sown with Sloan seeds and incubated without treating with ozone, served to confirm inoculation success by revealing disease symptoms on seeds and seedlings, and these were compared with soil samples treated with ozone.

Table 2. Experimental design with number of subsample treatments and controls per batch (ozonation treatments as a function of time)

	Control	10 min	13 min	15 min	17 min	20 min	25 min
Batch # 1	8	4	--	4	4	--	4
Batch # 2	8	--	4	4	4	--	4
Batch # 3	8	--	4	4	4	4	--
Batch # 4	8	4	4	4	--	4	--
Batch # 5	8	--	--	--	5	5	5

The experimental design consisted of 5 Batches of 24 pots each, including 8 control pots and 4 out of 6 treatment doses each, with each treatment dose ran in quadruplicate subsamples in

the first 4 batches, and 3 treatment doses with 5 replicates each in the 5th batch.
-- no treatment pots.

Experimental treatments and incubation

Soil in each pot (weighing 450 g) was ozonated at a flow rate of 0.5 L/min. Doses of ozonation in this experiment varied from 0.47 to 1.41 g O₃/kg soil by incrementally increasing the ozonation time (Fig. 1.a).

Following ozonation, pots were placed again in the greenhouse (where temperature of 25°C was maintained for 16 h during the day and, temperature of 21°C was maintained for 8 h during the night). Then, 10 soybean seeds of cultivar Sloan, which is susceptible to *P. sojae*, were placed on the surface of the soil in each pot and covered with 2.5 cm of wet coarse vermiculite (Fig. 1.b), flooded for 24 h and drained for another 24 h. Each pot was flooded separately, to avoid cross contamination between treatments if any. The germination rate of Sloan seeds used in this experiment was 96.5%. The pots were then placed into plastic bags for three days to prevent drying out during seed germination. Three days later, bags were removed and the pots were flooded again for 24 h then placed on benches to drain. Over the next 15 days, pots were monitored for symptoms.

Monitoring and assessment of treatments

Evaluation of treatment efficiency was done by monitoring the treated (ozonated) and non-treated (control) samples and assessing the symptoms of infection with *P. sojae* including: seed rot, root rot, seedling emergence, collapsed hypocotyls of emerging seedlings, and stem lesions, thus presenting the disease incidence (Fig. 1.c).

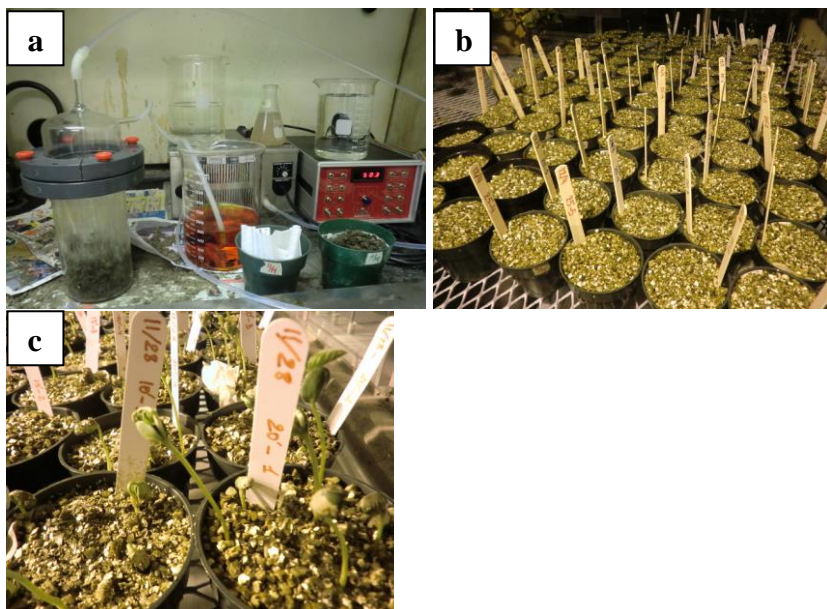


Figure 1. **a.** Ozone generator, reactor, and sample ozonation. **b.** incubation of samples after ozonation and sowing of Sloan seeds. **c.** emergence of seedlings.

Ozonation

The ozone generator used was a 1000BT-12 Triogen Model TOG C2B –generating 1g O₃/h from pure oxygen by corona discharge, where the conversion of oxygen to ozone occurs in a reaction cell excited by a high-voltage potential. The reactor was made of glass (Fig. 1.a), and all tubing was made of silicone material. The operating volume of the reactor was 1.5L (Fig. 1.a). In each test, the ozone flow rate per min was maintained at 1L min⁻¹L⁻¹ gas-flow/liter volume of soil sample (42). The feed and excess unreacted ozone were measured by the iodometric wet-chemistry method (15). The amount of ozone absorbed by the soil sample was determined by difference.

Data analysis

The data analysis was done using RStudio software (RStudio, Inc., Boston, Massachusetts). To prepare the data for statistical analysis, a data matrix was constructed,

with vectors consisting of three factors: two main factors (time in minutes, and ozone dosage value in g O₃/kg), and one interaction factor between the main factors (rate of ozonation: ozone dose/time). Then, the code of vector-binding followed by the command “leaps” and matrix analysis was implemented. For finding the best model that fitted the data best, ANOVA table was prepared to compare the means between various treatments for significance of variance.

Results

Seedling emergence (Fig. 2.a) started at day three after sowing soybean seeds in all pots except pots that received higher ozonation doses (1.09, 1.2 and 1.41 g O₃/kg) when it occurred on day 4 in these pots. All emerged seedlings in all treatments were free of any disease symptoms like root and stem rot, stem lesions, collapse of hypocotyls and damping-off (Table 3, Fig. 2.b, 2.d & 2.e), whereas seedlings in non-treated (control samples) showed different levels of disease incidence (Table 3, Fig. 2.c). In addition, it was observed that the emergence rate of seedlings was negatively correlated with the dose of ozone treatment (Table 3). Data are presented in averages of “% emergence” and “% disease incidence” related to infection with *P. sojae* of seed, root rot, stem lesions, collapse of hypocotyls, and seedling damping-off that were observed in non-ozone treated pots (Table 3).

Table 3. Effect of treatment with ozone at different doses on root and stem rot disease caused by *P. sojae* on susceptible Sloan soybean

Ozonation time (min)	Ozone dose g O ₃ kg ⁻¹	Rate of ozonation g O ₃ kg ⁻¹ min ⁻¹	% Emergence	% Disease incidence
0	0.0	0.00	48 ^a	70 ^b
10	0.5	0.05	81 ^c	0 ^d

Table 3 continued

13	0.7 ^e	0.06	82 ^c	0 ^d
15	0.8 ^e	0.05	72 ^c	0 ^d
17	1.1 ^e	0.06	66 ^c	0 ^d
20	1.2	0.06	62 ^c	0 ^d
25	1.4	0.06	56 ^c	0 ^d

^a Rates and percentages are the averages of subsamples readings. Less than 50% of seeds emerged in control pots. The non-emerged seedlings in control pots included 80% rotted seeds and 20% non-germinated seeds.

^b Control samples showed a disease incidence on 70% of seeds and seedlings, including root and stem rot, damping-off and collapse of hypocotyls, and stem lesions.

^c Treated pots showed a decline in seedling emergence as ozonation time and dose increased.

^d All treated pots showed healthy seedlings, exempt of any symptoms related to infection with *P. sojae*.

^e Difference in dosage increase in response to the same increase in ozonation duration (2 min) is due to the difference in ozone absorption by the soil samples. This fluctuation depended on how tightly submerged the ozone diffuser was in the soil sample. Doses presented are averages of dosage measurements of 12 replicates of the same ozonation duration.

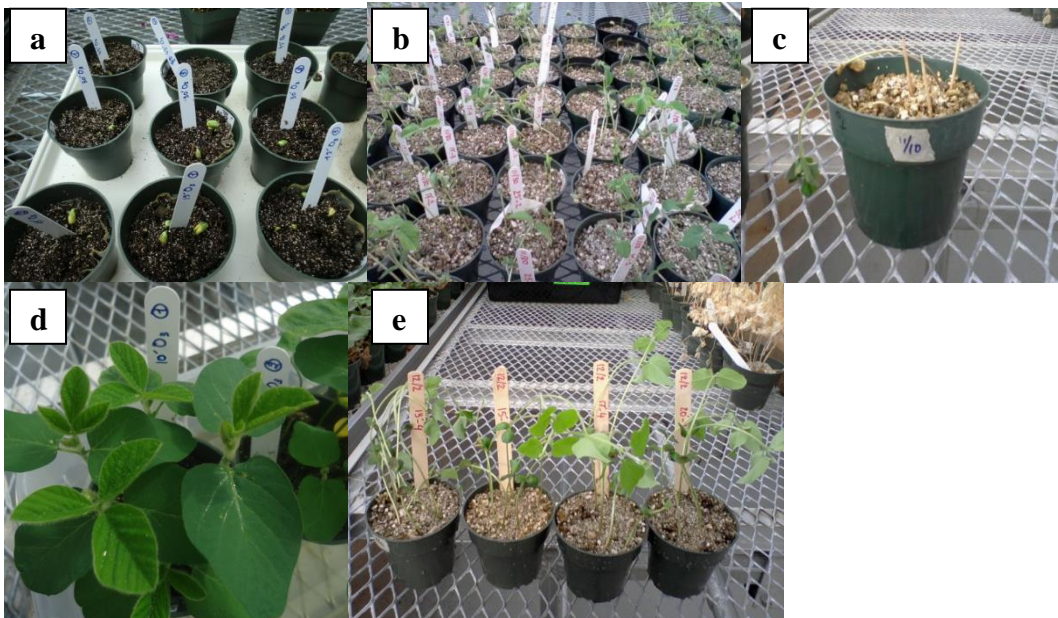


Figure 2. Monitoring and evaluation of treatments. **a.** Beginning of hypocotyls emergence after 3 days of incubation. **b.** All emerged seedlings in all treatments were free of any disease symptoms. **c.** Control pot showing damping-off of one seedling, three toothpicks marking collapsed hypocotyls, and of the rest of the sown seeds four sprouted and rotted and two non-

germinated. **d.** Treated sample showing healthy seedlings exempt of any *P. sojae*-related symptoms. **e.** Treated pots at four different ozone doses (expressed in time of ozone generation from left to right: 13 min., 15 min., 17 min., 20 min.) from the same batch, showing fewer number of seedlings from left to right as the ozonation dose increases.

The statistical data analysis resulted in the following best fit linear model:

$$\text{Emergence} = 101 - 1.83\text{Time}$$

where Emergence is percent emergence of seedlings, and Time is the duration of ozonation in min. Ozone dosage is represented in the model by Time, where the increase in the duration of ozonation resulted in an increase of ozone dosage.

Discussion

The appearance of *P. sojae* disease symptoms on control pots seedlings and the absence of these symptoms in treated pots confirmed our belief that pots not treated with ozone resulted in infected seedlings from pathogens present in the soil after artificial infestation with *P. sojae* rice inoculum. While the exemption of treated pots from any *P. sojae*-related disease symptoms, concluded that ozonation of soil resulted in healthy seedlings free from pathogen damage.

Since the variation in ozone dosage was less pronounced than that of time, only this latter was statistically revealed significant (with p-value < 0.05) in the linear model. Rate of ozonation also did not show any significance in the linear model, which could be attributed to the fact that this factor was almost constant at all dosage levels, and that seeds were sown after the treatment, which means that they were not directly subjected to the effect of rate of ozonation, and the germplasm would not be harmed. Germination is defined as “*the emergence of the radicle through the seed coat*” (24), while emergence is the superficial

outgrowth of the seedling shoot from the soil. Most non-emerged seeds in the treated pots had germinated. This observation confirms the explanation about rate of germination.

Symptoms seen in non-ozone treated pots were attributed to infection from *P. sojae* for three reasons: (i) disease-like symptoms from pathogens were observed only in non-treated pots, (ii) the soil in the pots was autoclaved at the beginning of the experiment eliminating the possibility from other diseases except from *P. sojae*, and (iii) disease symptoms matched those usually seen in *P. sojae*-infected soybean, namely seed and root rot, stem lesions, collapse of hypocotyls, and seedling damping-off that were observed (Fig. 2.c). These results also show that the ozonation of *P. sojae*-infected soil was seen highly efficient because even the pots treated with lowest dose of 0.47 g O₃/kg (10 min) resulted in an average of 81% seedling emergence rate of healthy plants (Table 3, Fig. 2.d). The non-emergence of seedlings in the treated samples, could not be attributed to the direct harm to the germplasm by ozone, since 95% of the non-emerged seeds were germinated, and the ozonation process was done in the absence of seeds. A possible explanation for the observation of lowered seed emergence in response to the increased ozonation dosages, could be that higher dosages result in lowering the viability of beneficial microorganisms responsible for many vital processes in promoting plant growth, like rhizobacteria, which could, by consequence, decrease emergence (2). Examples of mechanisms that these microorganisms promote are nutrients mineralization, solubilization and immobilization, induced plant resistance and pathogens suppression, growth promotion, and increased yield (2,19). In addition, ozonation might form oxidized products with potential deleterious properties, like the oxidized bromide ion that upon reaction with water or soil constituents

might form mildly toxic hypobromous and bromate ion or tribromomethane (56). Ozone concentrations of 0.2 – 0.3 mg/L caused root injury when immersing cucumber plant root in ozonated water (26). Kottapalli et al. (21) found that an exposure of barley seeds to 11 mg O₃ g⁻¹ barley min⁻¹ for 30 min resulted in significant reduction in barley germination energy. However, in the current research, the seeds were not directly exposed to ozone, but were sown after ozonation.

Since the lowest ozone dose (0.47 g O₃/kg) was as good as inhibiting 100% of the disease without affecting or harming seed germination, residual ozone toxicity would not be a practical limitation. The occurrence of soilborne disease and its severity depends on the populations of both the pathogen and disease-suppressing organisms in the soil. Pesticides reduce the diversity of soil microorganisms, and break the balance between beneficial and phytopathogenic organisms. In healthy soils, beneficial organisms suppress disease-causing organisms, however breaking the balance between these, fosters resistant pathogens (19,47). Accordingly, it is highly important to study the effect of soil ozonation on non-targeted organisms at the effective dose to treat the pathogen.

The overall results of this study clearly indicate that ozonating soil contaminated with *P. sojae* at a rate of 0.47 g O₃/kg is sufficient to minimize any harmful impact on seed germination and plant health. This level of ozonation rate can be considered enough to inhibit soil pathogens efficiently. In addition, given that ozone does not leave toxic residues in nature, we conclude that ozonation can be practiced as a sustainable alternative to the conventional treatment against soil pathogens such as *Phytophthora*, and could be used in organic agriculture. At the same time, future research must concentrate on the economics of ozonation to control disease effects on soil pathogens. This study focused on the feasibility of

the technique and not on the viability of ozonation for mass use in agriculture. Treating a field of soybean with ozone, would be impractical because row crops occupy large areas, needing huge amounts of oxygen and large ozone generators to generate enough ozone to treat soil to a minimum depth of 15 cm. In addition, ozone gas application to the soil would be done using irrigation system pipes or shanks (as fumigation gas), and the soil covered by tarp or impermeable nylon mulch to reduce the fumigant emission and increase ozone residence time in the soil to maximize its pesticidal activity, which is undoable for field crops. Hence, a practical application of this treatment would be in high-value cash crops like greenhouse crops. Finally, we recommend investigating the efficiency of ozone in the control of seedborne pathogens (e.g. ozonation of seedlings, potato seed tubers) based on the promising results of this work.

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CHAPTER IV

OZONE INACTIVATION OF *FUSARIUM OXYSPORUM* CONIDIA IN HYDROPONIC NUTRIENT SOLUTIONS

A paper to be submitted to Journal of Bioresource Technology

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Abstract

Ozonation was studied for inactivating conidia of *Fusarium oxysporum*, which causes Fusarium wilt, an economically important disease in hydroponic cultivation systems. Samples of conidial suspensions of *F. oxysporum* were treated with incremental doses of ozone from either oxygen feed with high gas-phase concentration (GPC) or air feed with low GPC. Trials resulted in non-viability of the pathogen at high ozone GPC with a dose of 0.84 mg O₃/L for 3 seconds. The optimal conditions for *F. oxysporum* treatment with ozone were high GPC (oxygen feed), high rate of ozonation (> 16 mg O₃ L⁻¹ min⁻¹), and low temperature (5 °C). Regression analyses showed that *F. oxysporum* spore viability is function of GPC (P = 7.7252E-11; Adjusted R-square = 0.81), of temperature (P = 2.16734E-05) and level of ozonation (Dose*Rate; P = 5.63367E-11; Adjusted R-square = 0.38). The linear model from air feed ozonation: Viability = 16.6 + 0.97 Temp. - 9.7 O₃-Level. Furthermore, LD50 of ozone at 21 °C and at 5 °C were determined as 34 mg O₃/L and 30 mg O₃/L respectively, using the linear model. The findings of this research imply that ozone is an efficient and sustainable alternative to chemical fungicides in the treatment of Fusarium wilt in hydroponic nutrient solutions, especially since it degenerates quickly to oxygen, an environmentally-safe, non-toxic residue.

Additional keywords: ozone, *Fusarium oxysporum*, gas-phase concentration, sustainability, soilborne pathogens, hydroponic

Introduction

Fusarium wilt, caused by *Fusarium oxysporum*, is one of the most widespread and destructive diseases of many major ornamental and horticultural crops (7). Over 120 *formae speciales* and races of *F. oxysporum* are known to cause vascular wilts of agricultural crops in many areas of the tropical and temperate zones (6,8). Although not all soils are conducive to Fusarium wilt, the disease may cause considerable losses in areas in which it becomes established (15). This fungus is soilborne and causes vascular wilts by infecting plants through the roots and spreading internally through the cortex to the vascular tissue (2,7,18). Currently, the major control practices adopted to control wilts and other soilborne pathogens on high-value crops (such as ornamental cut-flowers and greenhouse crops) are preplant soil fumigation and fungicide applications.

The fungus has been found in the imperfect state in three forms only: microconidia, macroconidia and chlamydospores (6,8,15,20). It is disseminated in these three forms in and on seeds, and in vegetative propagation material, as well as in soil, water, by air and human activity. Because *Fusarium* spores remain viable in water, spores leached out of contaminated soil into ditches, ponds and other water bodies used later in irrigation, can indefinitely be a source of inoculum (29). This is particularly important in the case of water aggregation bodies (ponds and ditches) used in crop irrigation, and in hydroponic cultures where contaminated nutrient solution is reused. According to Song et al. (32), Fusarium wilt is the most serious soilborne disease in hydroponic cultivation systems. *F. oxysporum* is

highly virulent in hydroponic greenhouses because it spreads easily through the nutrient solutions (33).

Song et al. (32) reported that prochloraz and carbendazim were the most effective fungicides in inhibiting *Fusarium* mycelial growth in the tomato Fusarium wilt in hydroponic system. An inspection report by the “European Commission – DG Health and Consumer Protection” entitled “Monitoring for pesticide residues in the European Union and Norway – Report 1996”, declared carbendazim as one of the 12 most commonly detected pesticides in foodstuffs (1). Concerns have been raised regarding carbendazim’s effect on human health and the environment, to the extent that “Friends of the Earth” highlighted it as one of their ‘filthy four’ pesticides (12). Similarly, prochloraz is a possible carcinogen and a suspected endocrine disruptor (26). With the growing environmental and health concerns over chemical pesticides, the increasing demand for residue-free produce, and the current trends towards organic farming, the search for efficient fungal control methods that address these concerns and needs has become of high importance.

Ozone is a potent oxidant and it has been implemented successfully against numerous pathogens including bacteria, viruses, protozoa and also metazoa (17,24,25,28,34). It is often used to disinfect drinking water and wastewater (35,36), and disinfest ships ballast water (21,22) due to its oxidizing properties. Ozone has also been applied in mold prevention on stored corn (38,39). Scanning electron microscopy showed that ozone causes damages to the surface of *Toxocara canis* eggs (a nematode parasite of dogs and other canides) (23). It is also capable of diffusing across bacterial membranes and reacting with cytoplasmic biomolecules, such as DNA, which results in cell death (11). Furthermore, ozone reacts with

biomolecules such as proteins, carbohydrates and polyunsaturated fatty acids bound to albumin, dyes, and is involved in lipid peroxidation (5,38).

Ozone has been approved by the American Food and Drug Administration for direct use in human food, drugs, and cosmetics and also as compounds in food contact materials such as cutting boards and other surfaces that come in contact with unprotected food (13). In addition, ozone is listed by the National Organic Program under the list of “*The National List of Allowed and Prohibited Substances*” with code (§205.605) referring to: “*Nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled as “organic” or “made with organic (specified ingredients or food group(s))”*”. (19). In contrast to other disinfection methods and conventional fungicides used in the treatment of *F. oxysporum* in hydroponic cultures, such as prochloraz and carbendazim, the use of ozone as a disinfection method has the advantage that it does not produce undesirable byproducts, because its rapid decomposition only produces oxygen.

Matsuo (16), reported that the treatment of nutrient solution with 0.25 mg/L residual ozone for hydroponic cucumber culture was considerably effective in reducing germination of *F. oxysporum* f.sp. *cucumerinum* microconidia, while a residual concentration of 0.4 mg O₃/L completely inhibited the germination of fungal spores. Residual concentrations of 0.2 – 0.3 mg O₃/L, however, caused root injury when immersing plant roots in ozonated water. In another study evaluating gaseous ozone for inactivating mixtures of non-specified spores and mycelia of fungi in malting barley, Allen et al. (3), showed that 96% of fungi were inactivated at a dosage of 0.1 mg O₃/g barley/min for 5 min, without affecting germination of the barley. They also suggested fungal mycelia were more susceptible to ozonation than spores. However, Kottapalli et al. (14) found that an exposure of Fusarium-infected barley to 26 mg O₃/g barley/min for 15 min resulted in 53% inhibition of Fusarium viability. Exposure

to 11 mg O₃/g for 30 min gave a higher rate of Fusarium inhibition but resulted in significant reduction in barley germination energy, an important characteristic in the malting process.

Although ozone appears to be an effective method at reducing viability of Fusarium, further investigation in this is needed particularly the optimization of the ozonation procedure regarding the effects of temperature, rate of ozonation, and gas phase concentration (GPC), and the effect of admitting ozone gas directly to the nutrient solution rather than adding it in the form of ozonated water.

The high oxidative power of ozone, its effectiveness in inhibiting pathogens without leaving toxic residues in the environment, and the limited research on ozone use in the domain of agricultural applications inspired the current research. Furthermore, the economic importance of *F. oxysporum* especially in hydroponic cultivation systems, and the need for environmentally safe alternative treatments to the currently adopted fungicides, made it a justified choice as a target for treatment optimization with ozone. The referenced research on hydroponic nutrient solutions treatment of *F. oxysporum* with ozonated water showed the need for further research to optimize the ozonation procedure. Accordingly, the goal of this work was to optimize the use of gaseous ozone in the treatment of *F. oxysporum* spores in suspension, as a representation of contaminated irrigation ponds and hydroponics nutrient solutions with fungal spores. Our objectives were to (i) test the effect of varying dosages of ozone on the viability of *F. oxysporum* spores in suspension, (ii) to compare the effect of ozone gas-phase concentration, i.e. when ozone is produced from either oxygen (high GPC) or air (low GPC) on the viability of *F. oxysporum* spores, and (iii) to evaluate the effect of ozone on the viability of *F. oxysporum* spores in suspension at either 5°C or 21°C.

Methods and Materials

Preparation of *F. oxysporum* spore suspensions

Fusarium oxysporum 370TSB (acquired from Dr. Alison Robertson, Corn Pathology Lab., Iowa State University - Fig. 1) was used in this study. For long-term storage, the isolate was stored on potato dextrose agar (PDA), at 5 °C. To prepare spore suspensions, 370TSB was sub-cultured on PDA (Fig. 1) and allowed to grow at room temperature and normal day/night light, for four to five weeks. Cultures grown on PDA sporulate within a month (20). Spores were harvested from eight Petri plates (Fig. 2) that were 3 to 5 weeks old, by washing the cultures under aseptic conditions. To each plate, 5 ml distilled sterile water was added and a sterile glass rod (hockey stick) was used to gently dislodge the fungal tissue from the media. The mycelia and spore suspension from each plate was bulked and filtered through sterile cheesecloth to remove the mycelia. The obtained spore suspension was made up to 1L with distilled sterile water. The concentration of conidia in the original conidial suspension (OS) was determined with a hemacytometer. The original suspension was divided into four approximately 250 ml aliquots. Each aliquot was diluted tenfold to prepare the following dilutions to be treated with ozone or not treated as control: 10^{-2} , 10^{-4} and 10^{-6} . Each aliquot was plated in quadruplicate control samples (non-ozonated). The prepared suspensions (OS and the three dilutions) were divided into 4 samples of 250 ml each (to have 4 replicates per treatment, a total of $4 \times 4 = 16$ samples of 250 ml each) and ozonated. Then each treated sample was plated (two plates per sample) and incubated at the same conditions as the control plates. To obtain low ozone doses (in oxygen feed at high GPC), sample volume was increased to 1L and 2L.



Figure 1. *F. oxysporum* isolate



Figure 2. plated controls

Ozone gas sources

To determine the effect of ozone generated from either oxygen or air on the viability of *F. oxysporum* spores, a 1000BT-12 Triegen Model TOG C2B ozone generator was used. This machine generates a maximum of 1g O₃/h from pure oxygen (99.9%) and 0.5g O₃/h from air by corona discharge, and the conversion of oxygen to ozone occurs in a reaction cell excited by a high-voltage potential. The reactor was made of glass (Fig. 3), and all tubing was silicone. In each test the ozone flow rate per min was maintained at 1L min⁻¹ L⁻¹ gas-flow/sample-volume (31). The unreacted ozone was captured in a solution of 2% potassium iodide (KI). The measurement of absorbed ozone by the sample was done by the iodometric wet-chemistry method (10). To test the ozone gas-phase concentration (GPC) effect, two gas sources were used in ozone generation, (i) pure oxygen for a high GPC, and (ii) air for a lower GPC.



Figure 3. ozone generator, glass reactor and silicone tubing

Ozonation dosages

A flow rate of 0.25 L/min was used to ozonate each dilution sample (250ml) with incrementally increased ozone doses that were generated by increasing the ozone generation time. The operating volume in the reactor was 250 ml, except at the lowest ozone dosages (7.7, 6.5, 4.6, 2.4, 2.6, 1.3, 0.84 mg O₃/L) where sample volume was increased to 1L – 2L to reach a lower ozone dosage. Doses of ozone that were produced ranged from 0.84 to 88 mg O₃/L with oxygen and from 9 to 31.8 mg O₃/L with air.

Temperature effect

The effect of temperature on the efficacy of ozone to reduce the viability of spores of 370TSB was tested at two temperatures: 5 °C and 21 °C (room temperature). Sterile distilled water and dilutions of conidial suspensions were kept in a refrigerator at 5 °C throughout the experiment except during the preparation of suspensions and during ozonation treatment.

All experiments were repeated twice.

Determination of viable conidia of 370TSB

The number of viable conidia was determined by plating aliquots from each dilution before and after ozonation and comparing the number of colony forming units (CFU) of ozone-treated and non-treated control. For each sample, 100 µL from the suspension was

spread onto the surface of a PDA plate using a sterile glass rod. Each plate was sealed with Parafilm (BEMIS FLEXIBLE PACKAGING, Neenah, WI) and incubated for 72 hours at room temperature, with normal day/night light. For each sample (controls and treated samples), two replicate plates were done. Colonies were counted after 72 hours.

The results shown in Table 1 are comparisons in percent conidial (CFU) viability between ozonated samples and corresponding controls (non-ozonated) by colony-counting and comparison with the treated samples of the same dilution- CFU count, from all dilution ranks (OS, 10^{-2} , 10^{-4} and 10^{-6}). In the results of ozonation with air feed, the comparison between controls and treatments is expressed with the CFU counts from dilution rank 10^{-4} , because the corresponding CFU numbers were countable (between 50 and 200 per plate). The other dilutions and OS plates were crowded with too many colonies to be counted (OS and 10^{-2} – Fig. 4), or too few to show a reliable colony count (10^{-6}) for viability determination and treatment efficiency assessment. However, in this case all other solutions (OS and dilutions) were still treated and plated as well to monitor differences in dilution responses to the treatment if any. A test was conducted to monitor spores viability in suspension between the times of samples preparation and ozonation. This test was done by plating four plates from the dilution rank 10^{-4} at the time of suspensions preparation, and again plating another four plates right before ozonation (both are non-ozonated), incubating plates for 72 hours and making colony-count comparison between the two sets of plates.

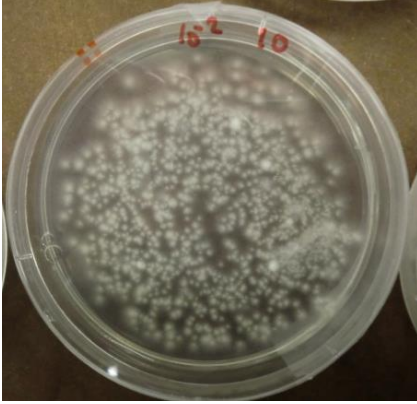


Figure 4. Growing colonies of a dilution rank 10^{-2} after ozonation with air feed.

Data analysis

Mean percent spore viability for each ozone level was calculated for data obtained from ozonation with air feed, and data were analyzed using RStudio software (RStudio, Inc., Boston, Massachusetts). Data is clustered in three ozone levels: low ($1.9 - 2.1 \text{ mg O}_3 \text{ L}^{-1} \text{ s}^{-1}$), medium ($2.9 - 3.3 \text{ mg O}_3 \text{ L}^{-1} \text{ s}^{-1}$) and high ($4.5 - 6.2 \text{ mg O}_3 \text{ L}^{-1} \text{ s}^{-1}$), representing each an average of Dose*Rate of about 40 samples with a marginal standard error. Dose*Rate is an interaction factor combining ozone dose in milligrams of ozone per liter, and the rate of ozone generation in milligrams of ozone per liter per second, indicating the speed of ozone generation and feeding the sample. Comparison of means between levels at different temperatures was done by Student's t-Test: two-sample assuming unequal variances at 95% confidence interval (29). To determine the significance of temperature and the confidence intervals for ozone LD50 at 5°C and 21°C , data from air feed only was used, since it included variability in response to temperature change, and included viability response values around 50% (which are necessary to determine LD50), unlike data from oxygen feed where response-values were all 100%.

To quantify the effects of GPC, ozonation levels and temperature on *F. oxysporum* spores viability, multiple regression and ANOVA analyses (29) were conducted and linear regression models were obtained.

To determine the linear model showing the effect of GPC on spore viability with this method, data from both oxygen and air feed were analyzed together. As for the linear model showing the effects of ozonation levels and temperature on spore viability, data from air feed alone was analyzed.

Results

Ozonation decreased the number of viable *F. oxysporum* spores in suspension (Table 1, 2, 3). A difference in efficiency between ozone generated using oxygen (Table 1) and ozone generated from air (Table 2, 3) was detected. The lowest dose of ozone generated from pure oxygen, 0.84 mg O₃/L, was 100% efficient at inhibiting growth of the spores of *F. oxysporum*. Conversely, the highest dose of ozone (31.8 mg O₃/L) generated by air as source, was only able to inhibit growth of 41% of the pathogen spores.

All of the applied ozone dosages at high ozone GPC and rate of ozonation when ozone was generated from oxygen, completely inhibited growth of *F. oxysporum* spores even at the lowest dose (0.84 mg O₃/L). At 5 °C, ozonation also resulted in complete inhibition of conidial germination (Table 1). When air was used for ozone generation, the GPC and rate of ozonation were comparatively lower as compared to ozone from oxygen, the percent viability of conidia ranged from 20 to 100%.

After several trials, no significant change in spores viability during the preparation of suspensions was noticed. Hence, the resulted decline in spores viability after treatment, is

attributed to the ozonation, not any decline in spore viability during suspension preparation time.

Table 1. Percent of viability of spores of *Fusarium oxysporum* after treatment with varying ozonation levels generated from oxygen at 21°C and 5°C

Ozonation Level (& Temperature)	Dose*Rate (mg O ₃ L ⁻¹)* (mg O ₃ L ⁻¹ s ⁻¹)	Mean CFU ^a prior to ozonation	Mean CFU ^a after ozonation	% Spore viability
High (21 °C)	66.2 (3.2)	1.7 x 10 ⁷	0	0
Medium (21 °C)	33.1 (2.1)	1.7 x 10 ⁷	0	0
Low (21 °C)	1.4 (1.1)	1.0 x 10 ⁷	0	0
Medium (5 °C)	28.7 (3.0)	1.5 x 10 ⁷	0	0

^a CFU – colony forming units per ml

Table 2. Percent of viability of spores of *Fusarium oxysporum* after treatment with varying dosages of ozone generated from air at different rates and at 21°C and 5°C

Time	Ozone dosage mg O ₃ L ⁻¹	Rate of ozonation mg O ₃ L ⁻¹ s ⁻¹	Temp. °C	Mean CFU ^a prior to ozonation	Mean CFU ^a after ozonation	% Spore viability
6 min	31.8	5.3		11 x 10 ⁶	7 x 10 ⁶	59
3 min	28.8	9.6		12 x 10 ⁶	3 x 10 ⁶	28
4 min	22.7	5.7		13 x 10 ⁶	9 x 10 ⁶	71
4 min	23.7	6	21 °C	16 x 10 ⁶	11 x 10 ⁶	72
2 min	23	11.5		2.9 x 10 ⁶	2.5 x 10 ⁶	87
2 min	19.2	9.6		15 x 10 ⁶	18.5 x 10 ⁶	123 ^b
1 min	12.6	12.6		16 x 10 ⁶	16.5 x 10 ⁶	101 ^b
45 s	9	12		18 x 10 ⁶	16 x 10 ⁶	89
10 min	60.9	6.1		16 x 10 ⁶	3 x 10 ⁶	20
5.5 min	22.1	4		14 x 10 ⁶	9 x 10 ⁶	62
4 min	22.1	5.5	5 °C	16 x 10 ⁶	11 x 10 ⁶	67
3 min	22.6	7.5		20 x 10 ⁶	13 x 10 ⁶	66
3 min	26	8.7		8.4 x 10 ⁶	50 x 10 ⁶	58
2 min	15.8	8		14 x 10 ⁶	11 x 10 ⁶	80

Table 2 continued

^a CFU – colony forming units per ml.

^b as microconidia are known to germinate but do not divide and multiply, an increase of conidial number in the treated samples would be justified as normal marginal difference in count between treated and control samples; hence, viability-response numbers above 100% are set to 100 for statistical data analysis, signifying no response at the corresponding dosage, which means a dosage falling below the treatment efficiency threshold.

Before processing the statistical data analysis, the distribution of the data and homogeneity of variance were checked and found normally distributed.

Table 3. Percent of viability of spores of *Fusarium oxysporum* after treatment with varying levels of ozonation (Dose*Rate) from air at 21°C and 5°C

Level ^x	Dose*Rate ^y (mg O ₃ L ⁻¹)* (mg O ₃ L ⁻¹ s ⁻¹)	Temperature (°C)	Viability ^z (%)
Low	1.87 (0.53)	5	69 (4) a
Medium	3.29 (0.69)	5	62 (4) a
High	6.18 (0.16)	5	20 (6) b
Low	2.09 (0.48)	21	76 (3) c
Medium	2.88 (0.35)	21	67 (4) ac
High	4.52 (0.32)	21	51 (5) d

^x Each level (low, medium and high) represents an average of Dose*Rate of 40 samples. Values are presented by the average followed by the standard error in parenthesis.

^y Dose*Rate is an interaction factor combining ozone dose in mg of ozone per liter, and rate of ozone generation in mg per liter per second (indicating the speed of ozone generation and feeding the sample).

^z Viability of spores was evaluated as the percent of viable CFU in treatment in comparison with the CFU in control samples. Mean viability followed by the standard error in parenthesis are followed by a letter (a, b, c, d). Values followed by the same letter were not significantly different ($P > 0.05$) by “t-Test: two sample assuming unequal variances”. Detailed means comparison and p-values are shown in table 4.

Table 4. P values of means comparison using a t-Test: two-sample assuming unequal variances (REF) between three ozonation levels at 5 °C and 21 °C

P(T<=t) two-tail	Low (5 °C)	Medium (5 °C)	High (5 °C)	Low (21 °C)	Medium (21 °C)	High (21 °C)
Low (5 °C)	-----	0.14	0.003*	0.098	0.739	0.032*
Medium (5 °C)	0.14	-----	0.008*	0.002*	0.428	0.187
High (5 °C)	0.003*	0.008*	-----	0.001*	0.004*	0.035*
Low (21 °C)	0.098	0.002*	0.001*	-----	0.141	0.004*
Medium (21 °C)	0.739	0.428	0.004*	0.141	-----	0.084
High (21 °C)	0.032*	0.187	0.035*	0.004*	0.084	-----

*P-value lower than 0.05, indicating significant difference between compared groups. Temperature had significant effect on treatment ($P < 0.05$). Ozonation at 5 °C was more efficient than at 21 °C at inhibiting spores viability. Level of ozonation had significant effect on treatment ($P < 0.05$). Ozonation was more efficient at ascending levels at inhibiting spores viability.

Regression analysis of data from air and oxygen feeds showed that *F. oxysporum* viability was function of GPC ($P = 7.7252E-11$), where high GPC with oxygen feed killed all spores (Table 1), but low GPC with air feed was much less efficient (Table 2, 3).

Table 5. Regression statistics and ANOVA, showing the significance of GPC on *Fusarium oxysporum* spores viability

Regression Statistics				
Multiple R	0.909781469			
R Square	0.827702322			
Adjusted R Square	0.809241856			
ANOVA	Coefficients	Standard Error	t Stat	P-value
Intercept	58.42378583	8.063574492	7.245395437	6.90675E-08
Temperature	0.823983021	0.469996062	1.753170053	0.090516351
GPC	-73.40277801	7.267687222	-10.09988126	7.7252E-11

Regression analysis shows that GPC is very significant in affecting *F. oxysporum* spores viability, with high GPC (with oxygen feed) being much more effective in decreasing spores viability than low GPC (with air feed).

ANOVA analysis of data from air ozonation (Table 6) shows the significance of ozonation level and temperature in the linear model.

Table 6. Regression statistics and ANOVA, showing the significance of ozonation level (Dose*Rate) from air, and temperature on *Fusarium oxysporum* spores viability

Regression Statistics				
Multiple R		0.623892482		
R Square		0.38924183		
Adjusted R Square		0.379062527		
ANOVA	Coefficients	Standard Error	t Stat	P-value
Intercept	16.55664041	5.530300514	2.993804833	0.003346515
Rate * Dose	-9.738912502	1.352085116	-7.20288419	5.63367E-11
Temp.	0.967588526	0.218842438	4.421393469	2.16734E-05

Regression analysis shows that both the level of ozonation (Dose*Rate) and temperature are significantly important in affecting *F. oxysporum* spores viability. The regression model explaining the relation between spores viability and the two factors (Temp. for temperature and O₃-Level for Dose*Rate) is:

$$\text{Viability} = 16.6 + 0.97 \text{ Temp.} - 9.7 \text{ O}_3\text{-Level.}$$

The model shows that as the level of ozonation increases, conidial viability decreases. The model also shows that as temperature decreases, viability of the conidia decreases, indicating that ozonation at 5 °C is more efficient at reducing conidial viability.

In table 1, all response data were equal (0% conidial viability), regardless the change in temperature, mainly due to the effect of high GPC and rate of ozonation. The ozonation with air feed showed difference in response to the treatment at different temperatures, unlike

data from table 1 where ozone generation was from oxygen and total inactivation of spores resulted at both temperatures. The difference in response at different temperatures with air feed ozone generation, allowed to reveal the significance of temperature on ozonation efficiency. Using this linear model, the confidence intervals of ozone LD50 at 21°C and 5°C under air feed setting were determined as follows:

C.I. of LD50 at 21°C: [30 – 38] mg O₃/L, with optimal LD50 = 34 mg O₃/L at 21 °C;

C.I. of LD50 at 5 °C: [27 – 33] mg O₃/L, with optimal LD50 = 30 mg O₃/L at 5 °C.

Discussion

The difference in efficiency between ozone generated using oxygen (Table 1) and ozone generated from air (Table 2) can be explained by the gas-phase concentration effect (GPC). Generally, the rate of ozonation with ozone from oxygen was about an order of magnitude higher than with ozone from air. The rate effect also is demonstrated by the difference between the upper two readings in table 2, where the higher dose (31.8 mgO₃/L) inhibits fewer spores (41% vs. 72%) than the lower dose (28.8 mg O₃/L), but where the rate of ozonation was almost twice as high as for the higher dosage. Higher rates of ozonation and also higher gas-phase concentrations in the bubbles, result in higher ozone concentrations in the liquid surrounding the bubbles. These higher concentrations are short-lived because of the ozone demand, but these higher concentrations ensure that disinfection proceeds rapidly. These results agree with the findings of Patil et al. (27).

In contrast to Matsuo's work (16), the methodology presented depends on direct ozonation of the spores in suspension, rather than adding ozonated water to the solution. The importance of this methodology is its wider applications for treatment of *F. oxysporum*-

contaminated waters in aggregation ponds for irrigation, and for the recycling of contaminated agricultural wastewaters. In addition, this methodology is more efficient and practical in the treatment of hydroponic nutrient solutions, since it expresses the action of GPC and allows to cutoff intermediary steps between ozone generation and delivery.

The difference in CFU counts (in controls - prior to ozonation) between experiments is due to the different maturation ages (3 to 5 weeks old) of culture plates used in the preparation of spore suspensions.

In comparison with a study by Hitoshi (9), where an ozone injection dosage of 1.56 mg O₃/L in a nutrient solution of hydroponic system was needed to sterilize it from *F. oxysporum*, this study showed complete conidial eradication with only 0.84 mgO₃/L at high GPC. In our study, ozone gas was directly delivered to the conidial suspension as fine bubbles, rather than injected as ozonated water, and this study treated a conidial suspension in sterile deionized water, while Hitoshi's study treated conidia present in recycled nutrient solution.

These data agree with those reported by Kobayashi et al. (13) who showed that the effect of ozone on declining the viability of phytopathogens in hydroponic culture solutions increases concomitant with increasing initial dissolved ozone (dO₃) concentration, and with those reported by Patil et al. (27) who showed that higher rates of ozonation and higher GPC in the bubbles result in higher ozone concentrations in the immediately surrounding liquid, which results in higher ozonation efficiency. However, the observation that temperature didn't show significance in the first linear model, might be due to including data from both tables (air and oxygen feed) in the data analysis, where the effect of temperature in the first table was outweighed by those of high GPC and rate of ozonation. This observation confirms

similar findings by Patil et al. (27). The linear model for data from ozonation with air feed shows that as temperature decreases, disinfection becomes more efficient. This is attributed to the increasing ozone solubility ratio with decreasing water temperature (4), and the slower ozone decomposition at lower temperature (30). Hence, ozone is more stable in water at 5°C, which prolongs its activity duration at oxidizing and inactivating the spores in suspension. Consistent with these physico-chemical ozone properties, the current study confirms a higher response at lower temperature.

The findings of this study suggest that ozonating *F. oxysporum*-contaminated waters and hydroponic nutrient solutions, at high GPC, high rate of ozonation and low temperature, are a viable management option for hydroponic production. Caution should be exercised, however, to allow enough time for decomposition of ozone to oxygen since ozone solutions may be phytotoxic to plants (16). Since ozone does not leave toxic residues that would pollute the environment or harm human health, ozonation treatment could be considered a sustainable alternative to chemical fungicides that are currently used for Fusarium wilt management in the hydroponic industry, as well as being an effective disease management practice in organic settings.

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CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Summary of the Current Research Findings

A dose of ozonation 0.47 g O₃/kg was found to inhibit the effect of *P. sojae* disease on susceptible Sloan soybean in the soil assays carried out in this research, without affecting or harming seed germination. Similarly, ozonating soil infected with nematodes at a dose 0.59 g O₃/kg at 5°C was found sufficient to kill 76% of the nematodes. The current study confirms similar works finding on ozonation of a higher response at a lower temperature. Hence, for optimal results with soil ozonation, we recommend the application of this treatment at the beginning of the growing season, when temperatures are usually low. On the other hand, the findings of the study of ozone effect on conidial suspensions of *Fusarium oxysporum*, suggest that ozonation at high GPC, high rate of ozonation and low temperature, are a viable management option for hydroponic production.

Ozonation might form oxidized products with potential deleterious properties, like the oxidized bromide ion that upon reaction with water or soil constituents might form mildly toxic bromate ion or a type of trihalomethane (Suslow, 2004). In addition, ozone solutions may be phytotoxic to plants (Matsuo, 1993). Accordingly, caution should be exercised upon ozonation treatments, to allow enough time for decomposition of ozone to oxygen.

Enhancements to the ozonation soil treatments

Several works focused on overcoming the limitations to the application of ozonation in the management of soil phytopathogens, namely the need for (i) high ozone doses enough for the treatment of big surfaces at soil depth enough to control the targeted pathogens, (ii) reducing energy consumption for the generation of ozone and increasing the process

feasibility, and (iii) reducing the dependence on fossil fuel to make ozonation more eco-friendly. Takayama et al. (2006) developed an ozonation technology using barrier discharge (DBD) by applying high voltage between electrodes (Ebihara et al., 2004; Stryczewska et al., 2004). Two types were developed: a “pyramid-type electrode” that can control ozone dosage from low ($0.1 \text{ gO}_3/\text{m}^3$ soil) to high ($12 \text{ gO}_3/\text{m}^3$ soil), and a “screw-type electrode” that can generate high ozone dosage ($20 \text{ gO}_3/\text{m}^3$ soil) with high efficiency. High ozone dosage ($20 \text{ gO}_3/\text{m}^3$ soil for 10 min.) permitted to kill 97.5% (Takayama et al., 2006) to 99.9% (Pawlat et al., 2011; Pawlat and Stryczewska, 2012) of *Fusarium oxysporum* spores in the soil. On the other hand, tackling the need to reduce energy consumption for the generation of ozone, Pawlat et al. (2011) developed fully automatic power system from photovoltaic panels that could cover up to 95 – 100% energy needs for ozone generation. This technology allows to substitute fossil fuel with renewable energy in ozone generation, with zero-emission and with reasonable cost (Pawlat et al., 2011).

Recommendations

Seed treatment can enhance stand establishment, reduce seedling disease, and increase yield and economic benefits (Bradley, 2008). Current grain and postharvest fruit and vegetable treatment of pathogens and pests include the fumigants aluminum phosphide, methyl bromide and phosphine (Tiwari et al., 2010). The persistent dependence on these fumigants resulted in the disruption of natural agents biological control systems, and has been reported with pest outbreaks, widespread resistance development, adverse effects on non-target organisms, and detrimental effects on the environment and human health (Collins et al., 2005; Islam et al., 2009; Kells et al., 2001; Pimentel et al., 2007 and 2009). These adverse effects highlight the need to search for safer alternatives (Fields and White, 2002).

Ozone was reported to be efficient in the control of phosphine-resistant strains of stored grain pests *Silophilus zeamais*, *S. oryzae*, *Tribolium castaneum* and *Rhyzopertha dominica* (Qin et al., 2003).

Ciccarese et al. (2007) showed that the treatment of wheat, barley and pea seeds with a mixture of ozone and air at a concentration of 3% by seed weight, and for 3 min. exposure was effective in seed disinfestation without affecting germination.

According to what preceded and to the promising findings of our research, we recommend the following:

- A- Conducting further research on seed and grain treatment with ozone and optimizing its processes on economically important seeds and stored grains.
- B- Adopting the above mentioned enhancements to the ozonation process to increase its feasibility and further enhance its environmental benefits.
- C- Adopting ozone treatment in pest control under organic agriculture setting, given ozone efficiency coupled with its degeneration into oxygen, an environmentally safe byproduct.
- D- Integrating ozonation with cultural practices, integrated pest management, and resistant varieties when necessary and applicable.
- E- Conducting further research on ozonating other soilborne phytopathogens, especially those with history of resistance to common fumigant pesticides, or with high virulence on economically important crops.
- F- Carry out species-specific research on ozone treatment to phytoparasitic nematodes, and species of *Phytophthora* and *Fusarium* to investigate differences in species

response to ozonation if any, and to generalize the ozonation for treatment of these pathogens in high-value cash crops where it could be applicable.

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