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Development of Biopolymer Based Diffusion System by Encapsulating Plant Derived Essential Oils (EOs) -Application in a Stored Food Product

Par

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List of Abbreviations

A

Analysis of Variance (ANV)

Adenosine Triphosphate (ATP)

Acetylcholinesterase (AChE)

Antimicrobial Diffusin Film (ADF)

C

Cellulose Nanocrystal (CNC)

Colony Forming Unit (CFU)

Chitosan (CH)

Cyclodextrins (CDs)

E

Elongation at Break (EB)

Emulsion Droplet Size (EDS)

Essential Oils (EOs)

Ethylene vinyl alcohol (EVOH)

F

Food and Drug Administration (FDA)

Fractional Inhibitory Concentration (FIC)

Food and Agricultural Organization (FAO)

Food Contact Materials (FCMs)

Film Forming Dispersion (FFD)

G

Gray (Gy)

Gallic Acid Equivalent (GAE)

I

International Atomic Energy Agency (IAEA)

K

Kilogray (kGy)

L

Low Density Polyethylene (LDPE)

M

Methyl Cellulose (MC)

Minimum Inhibitory Concentration (MIC)

N

Nanoclay (NC)

Nanoparticle (NP)

R

Relative Humidity (RH)

S

Solid lipid nanoparticles (SLNs) and

Self-emulsifying drug delivery systems (SEDDS).

U

Universal Testing Machine (UTM)

P

Poly (lactic acid) (PLA)

Potato Dextrose Agar (PDA)

Potato Dextrose Broth (PDB)

Polyvinyl alcohol (PVA)

Polyethylene (PE)

PolyPropelene (PP)

Poly Dispersity Index (PDI)

T

Tensile strength (TS)

Tensile Modulus (TM)

Total Phenol (TP)

W

Water Vapour Permeability (WVP)

World Health Organization (WHO)

Résumé

Les organismes ravageurs retrouvés dans les aliments stockés constituent une problématique mondiale majeure. Les systèmes d'emballages actifs permettant d'encapsuler des émulsions d'huiles essentielles (HEs) se présentent comme une approche innovante, et se sont avérés très efficaces pour contrôler la croissance et la prolifération des ravageurs et des moisissures dans les produits alimentaires stockés. L'utilisation de traitements combinés suscite également l'intérêt des consommateurs car elle permet une synergie entre les traitements et peut réduire la dose nécessaire de chaque traitement. Dans ce contexte, le but de cette recherche était de développer des systèmes de diffusion à base de biopolymères contenant une formulation antimicrobienne avec des HEs. La formulation d'HEs développée a été encapsulée dans des matrices polymériques telles que le chitosane (CH), la méthylcellulose (MC) et l'acide polylactique (PLA) afin de développer un large milieu qui favorise et contrôle la diffusion des composants actifs. En outre, l'effet synergique entre le film bioactif synthétisé et un traitement de pasteurisation à froid (irradiation gamma) a été étudié pour améliorer l'efficacité du traitement combiné qui aura une application et une faisabilité commerciale pour garantir l'innocuité et la qualité des céréales pendant le stockage.

Les activités antifongiques de huit (8) HEs, à savoir le basilic, la cannelle, l'eucalyptus, la mandarine, l'origan, la menthe poivrée, l'arbre à thé et leurs combinaisons, ont été évaluées pour leur capacité à inhiber la croissance d'*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus* et *Penicillium chrysogenum*. De plus, les toxicités par fumigation de ces HEs et une combinaison binaires de thym et d'origan ont été étudiées contre le charançon du riz, *Sitophilus oryzae*. À partir des résultats obtenus, des formulations d'HEs à base d'origan et de thym ont été développées pour les tests antifongiques et insecticides. Tous les films développés dans le cadre de ce projet ont montré des propriétés fongicides et insecticides. Cependant, les films composés de CH et de MC étaient plus efficaces que le film constitué de PLA. Les résultats ont montré un effet antifongique et insecticide amélioré en combinant un traitement de films bioactifs avec l'irradiation gamma contre toutes les espèces de moisissures testées.

L'ajout de nanocristaux de cellulose (CNC) en tant qu'agent de renforcement dans la composition des films a amélioré la résistance à la traction et a réduit la perméabilité à la vapeur d'eau des films nanocomposites. De plus, l'ajout de CNC dans les films nanocomposites à base de CH et de

MC a permis de maintenir une libération contrôlée des composants volatiles des HEs pendant 12 semaines de stockage. Des tests de provocation *in situ* avec des films bioactifs à base de MC/CNC contenant une émulsion d'HEs ont montré une réduction de 2 log de la croissance fongique dans du riz contaminé pendant 8 semaines de stockage à 28°C. De plus, le traitement combiné de films bioactifs avec irradiation gamma à 750 Gy a montré des propriétés antifongiques plus élevées que le traitement avec le film bioactif ou l'irradiation seuls. Le film nanocomposite à base de CH s'est révélé plus efficace. En effet, des tests *in situ* avec des films bioactifs à base de CH/CNC ont généré une réduction de 1 à 2 log de la croissance fongique dans le riz infecté pendant 8 semaines de stockage à 28°C. D'autre part, la combinaison des films de CH chargés en HEs de thym et d'origan et d'une faible dose d'irradiation (750 Gy) a significativement augmenté les effets inhibiteurs des films dans le riz inoculé avec les champignons en provoquant une réduction d'environ 4 log UFC/g de la croissance fongique. Ainsi, l'efficacité insecticide (pourcentage de mortalité à 95%) des films à base de CH a augmenté de manière significative ($p \leq 0.05$) par l'irradiation combinée de 300 kGy à 10 jours d'incubation. Les films nanocomposites de CH contenant une nanoémulsion d'HEs ont montré une libération lente des composants volatils sur une période de stockage de 12 semaines. L'évaluation sensorielle d'échantillons de riz emballés avec les films bioactifs de CH contenant la nanoémulsion de thym et d'origan n'a montré aucun changement significatif ($p > 0.05$) de l'odeur, du goût, de la couleur et de l'appréciation générale par rapport au riz non traité. Cette thèse est présentée dans un format manuscrit conforme aux directives de préparation des thèses à l'INRS.

Abstract

Stored food pest is an enormous global concern. Active packaging system with the encapsulation of EOs emulsion is an innovative approach that have proven to be very effective in controlling the growth and proliferation of stored product pests and molds. The use of combined treatments is also gaining consumer interest as it permits a synergy between the treatments and can reduce the dose needed for each treatment. In this context, the aim of this research was to develop an EO-loaded biopolymeric diffusion systems containg an antimicrobial formulation. EOs formulation developed was encapsulated into polymer matrices such as chitosan (CH), methyl cellulose (MC) and Poly Lactic acid (PLA) to develop a broad cast medium in order to facilitate and control the diffusion of active components. Furthermore, the synergistic effect between synthesized active biofilm and a cold treatment (γ-irradiation rradiation) was investigated to improve the efficiency of the treatment that will have commercial application and acceptability to ensure the safety and quality of cereals during storage.

The antifungal activities of eight EOs namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme and their combinations were evaluated for their ability to inhibit the growthof *Aspergillus niger, A. flavus, A. parasiticus* and *Penicillium chrysogenum*. In addition, the fumigant toxicities of these EOs and one binary combinations thyme and oregano were investigated against the rice weevil, *Sitophilus oryzae.* Based on the results obtained, EOs formulation based on oregano and thyme were developed for the antifungal and insecticidal tests. All the biopolymerc films developed in this project showed fungicidal and insecticidal properties. However, biopolymeric films composed of chitosan and methyl cellulose were more efficient as compared to the film made of poly (lactic acid) PLA. The results showed an enhanced antifungal and insecticidal effect achieved by combining a treatment of bioactive films and gamma radiation against all tested fungal species.

The addition of CNC as a reinforcing filler improved the tensile strength and deceased the water vapor permeability of nanocomposite films. Moreover addition of CNC into MC and CH based nanocomposite based films, maintained a sustained release of volatile EO components during 12 weeks os storage. *In situ* tests with MC/CNC based bioactive films containing EO emulsion

produced a 2 log reduction in fungal growth in infected rice during 8 weeks of storage at 28°C. In addition, combined treatment of bioactive films with an irradiation treatment at 750 Gy showed more pronounced antifungal properties than treatment with the bioactive film or irradiation alone. Chitosan based nanocomposite film found to be more efficient. *In situ* tests with CH/CNC based bioactive films containing EO emulsion produced a 1-2 log reduction in fungal growth in infected rice during 8 weeks of storage at 28°C. Combining the bioactive chitosan films loaded with thyme and oregano essential oils and low dose of irradiation (750 Gy) significantly enhanced the inhibitory effects of the films in rice inoculated with fungi by causing an approximately 4 log CFU/g reduction in fungal growth. The insecticidal efficiency (percent mortality 95%) of chitosan based films increased significantly ($p \leq 0.05$) by the combined application of 300 Gy gamma radiation and bioactive chitosan films at 10 days of incubation period. Chitosan nanocomposite films containing EOs nanoemulsion showed a slow release of volatile component over 12 weeks of storage period. Sensorial evaluation of rice samples packed with the chitosan bioactive films loaded with thyme and oregano nanoemulsions showed no significant ($p > 0.05$) change in odor, taste, color and general appreciation compared with untreated rice. This thesis is presented in a manuscript based format in accordance with the Guidelines for Thesis preparation.

Chapter 1

Literature Review

Perspectives on essential oil-loaded nano-delivery packaging technology for controlling stored cereal and grain pests

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Contribution of the authors

This review was written by the framework planned with guidance from Prof. Monique Lacroix and Dr. Peter Follett. Both professors corrected the main draft several times. I have discussed many time with Stephane Salmieri and Dr. Khanh vu regarding some section of the review to improve the Writing. Dr. Majid has corrected the main draft and gave valuable comments. Dr. Monique Lacroix and I have replied to the reviewer's comment and corrected the review.

Résumé:

Les insectes nuisibles et les moisissures d'origine alimentaire et leurs métabolites toxiques associés entraînent des pertes significatives dans les produits alimentaires stockés. Les huiles essentielles dérivées des plantes (HE) peuvent contrôler la croissance et la prolifération des insectes nuisibles et fongiques. Les HE sont respectueux de l'environnement et non toxiques, et leur application pour protéger les produits alimentaires stockés a récemment augmenté. Cependant, bien que les HE aient de nombreuses propriétés bénéfiques, leur commercialisation et leur application ont été limitées en raison de plusieurs propriétés inhérentes, notamment une volatilité élevée, une instabilité, une faible solubilité dans l'eau et une susceptibilité à l'oxydation. Ces contraintes peuvent être surmontées par diverses stratégies de libération contrôlée telles que l'encapsulation et l'emballage actives, qui protègent les HE d'une dégradation rapide. Dans la présente revue, nous discutons des avancées récentes dans le développement de nouveaux emballages actifs avec des HE issues de plantes pour protéger les produits stockés contre les moisissures et les insectes. Différentes stratégies pour la nano-encapsulation des HE sont présentées avec un accent particulier sur l'utilisation de la nanotechnologie dans les emballages alimentaires et le potentiel de migration des nanoparticules dans les matrices alimentaires.

Abstract

Insect pests and food-borne fungi and their associated toxic metabolites cause significant losses in stored food products. Plant-derived essential oils (EOs) can control the growth and proliferation of insect and fungal pests. Plant EOs are environmentally friendly and non-toxic, and their application to protect stored food products has recently increased. However, although plant EOs has many beneficial properties their commercialization and application have been limited due to several inherent properties including high volatility, instability, poor water solubility and susceptibility to oxidation. These constraints can be overcome by various controlled release strategies such as active packaging and encapsulation, which protect the EO molecules from rapid degradation. In the present review, recent advances in the development of novel active packaging with plant derived-EOs to protect stored products against fungi and insects are discussed. Different strategies for the nano-encapsulation of EOs are presented with special emphasis on the use of nanotechnology in food packaging and the potential of nanoparticle migration into food matrices.

1.1. Introduction

Protection of stored food crops against damage from insect pests and pathogens is a major concern for the food industry, farmers, public health organizations, and environmental agencies. Insect feeding causes damage to stored grains and processed products by reducing their dry weight and nutritional value (Follett *et al.*, 2013). In addition, insect infestation-induced changes in the storage environment can create warm, moist "hot spots" that provide suitable conditions for storage fungi that cause further losses (Abou-Elnaga, 2015, Sung *et al.*, 2013). Moulds may cause a decrease in the quantity of fats, carbohydrates, vitamins and proteins (Lamboni *et al.*, 2009). Reducing postharvest food losses due to stored product pests is critical to ensure food security for our rapidly expanding global population. Stored product packaging is an important component of the food processing chain and a critical step in reducing postharvest losses, maintaining quality, adding value and extending shelf-life of food commodities (Opara *et al.*, 2013).

Active and intelligent packaging are new and innovative concepts that involve the incorporation of active chemical molecules into packaging material (Khan *et al.*, 2014a). When the active chemical exhibits antimicrobial properties, the packaging is known as antimicrobial active packaging (AP). Different kinds of active substances (chemical and natural) have been tested and incorporated into packaging materials, both to improve their functioning and provide new functions (Khan *et al.*, 2014b, Salmieri *et al.*, 2014b, Sung *et al.*, 2013). However, an increase in consumer desire for natural, local and organic products is creating new challenges to the use of microbial-suppressive packaging to enhance food preservation (Prakash *et al.*, 2013). Among the various biopesticides that have been developed and commercialized, certain plant-derived essential oils (EOs) show great promise (Tripathi *et al.*, 2009, Viuda-Martos *et al.*, 2007) for incorporation in food packaging to reduce food contamination and spoilage (Balasubramanian *et al.*, 2009). Linalool, thymol, carvacrol, cinnamaldehyde, clove and basil oil are widely used into food packaging (Bilia *et al.*, 2014). Most of the research to date on active food packaging has used active biocides with broad spectrum activity and with controlled release characteristics designed to counter pathogenic bacteria such as *E. coli*, *Salmonella* and *Listeria* (Salmieri *et al.*, 2014b, Severino *et al.*, 2014, Sung *et al.*, 2013, Takala *et al.*, 2013). Adopting similar packaging approaches to inhibit fungal growth and insect infestation may prove useful for stored products.

This review focuses on EO loaded systems in active packaging intended for insecticidal and fungicidal applications with a special emphasis on nano-based strategies to develop nano bioactive packaging materials for eventual large-scale application for protecting stored grain products.

1.2. Plant-derived EOs: Potential alternatives to synthetic pesticides

In recent years, plant-derived EOs and their bioactive chemical constituents have gained increased attention due to their beneficial insecticidal (Hossain *et al.*, 2014b, Picard *et al.*, 2012) and antifungal activities (Hossain *et al.*, 2016, Hossain *et al.*, 2014a, Prakash *et al.*, 2013). EOs are mixtures of complex secondary metabolites containing a wide spectrum of strong aromatic components, such as monoterpenes and sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides) in various ratios (Ebadollahi *et al.*, 2011, Huq *et al.*, 2015, Salmieri *et al.*, 2014b). Since EOs are rich in volatile terpenoids and phenolic components, they exhibit a wide spectrum of activity against insects and various microorganisms (Ghabraie *et al.*, 2016, Lacroix *et al.*, 2015). Monoterpenes such as 1,8 cineole, camphor, carvone, linalool and geraniol can penetrate insect bodies through breathing and interfere with their physiological functions (Lee *et al.*, 2003). These monoterpenes can also act as neurotoxic compounds, affecting acetylcholinesterase activity or octopamine receptors (Tripathi *et al.*, 2009) (Figure 1.1). Other modes of action such as membrane disruption and blockage of the tracheal system may also be involved in insecticidal activity of EO components. These compounds also can act at the cellular level disrupting organelles such as mitochondria. Mansour *et al.* (2012) conducted an elaborate study to elucidate the action of allylisothiocyanate (AITC) oil, which occurs naturally in black mustard (*Brassica nigra*) and Indian mustard (*Brassica juncea*), on the mitochondria of *Sitophilus oryzae, Tribolium confusum* and *Plodia interpunctella*. They found that AITC induced significant alteration in insect mitochondrial structure, reducing the quantity of cristae and causing vacuolization and rarefaction of the mitochondrial matrix.

Phenolics and terpenes components have been reported for possible antifungal modes of action as well (Viuda-Martos *et al.*, 2007). The major target sites of EOs on fungal cells are illustrated in Figure 1.2. Abd-Aiia *et al.* (2013) reported that the cell wall of fungal pathogens is the main

target of phenolic compounds which primarily disrupt the permeability barrier of cell membranes and inhibit respiration. The hydrophobic nature of EOs and their components allow them to penetrate the lipid layer of fungal cell membranes and mitochondria, thereby disrupting their structure and causing energy depletion (Abd-Aiia *et al.*, 2013). Tian *et al.* (2012) reported that EO components like eugenol inhibit respiration and ion transport, and increase membrane permeability causing release of cellular contents. The physical nature of EOs which includes low molecular weight and pronounced lipophilic tendencies, allow them to penetrate cell membranes more quickly than other substances (Pawar *et al.*, 2006). EO components have been shown to inhibit synthesis of DNA and transcription of genes involved in aflatoxin synthesis (Yahyaraeyat *et al.*, 2013). These properties make them attractive alternatives to traditional fumigants such as phosphine and methyl bromide, which have been reported to exhibit toxic effects against human health and the environment. In addition, insect and fungal species have been shown to develop resistance against traditional fumigants over prolonged exposure , but this is less likely to occur with natural bioactive EOs (Hossain *et al.*, 2014b).

1.3. Active EO-impregnated packaging against insects

Insects are typically the most serious pests of dried, stored, durable agricultural commodities (Follett *et al.*, 2013, Follett *et al.*, 2014). Global post-harvest grain losses incurred by insect pests is estimated up to 17.7 billion dollars per year (Oliveira *et al.*, 2014a). The most destructive insect pests of stored products are taxonomically found in the order Coleoptera (beetles) and *Lepidoptera* (moths), which are worldwide distributed in various climatic conditions (Du *et al.*, 2009). The search for tools to prevent infestations is crucial and packaging represents a critical step in food quality preservation and the ultimate defence against insect pests. Therefore, considerable efforts should be made for the development of novel insect-proof packages, which are able to resist insect penetration and their potential infestation of food commodities. Active packaging, containing plant-derived EOs have been designed using many different compounds and found effective in reducing food losses resulting from insect infestations (Licciardello *et al.*, 2013). Allahvaisi (2010) investigated the repellent efficacy of EO extracted from *Prunus amygdalus* L. and *Mentha viridis* L. against *T. castaneum, S. granaries, S. paniceum,* and *R. dominica* pests by spraying the EOs onto the interior surface of polyethylene (PE) packages. The EOs from *P. amygdalus* and *M. viridis* EO were repellent against *T. castaneum,* and *S. granarius,*

reducing the contamination of the packaged food by 79 and 64%, respectively as compared to the control. Such beneficial effect has also been shown on simple paper-based packaging used in grocery stores. Wong *et al.* (2005) assessed the efficiency of such type of packaging with potential plant extracts of citronella, garlic oil, neem extract, pine oil, and pyrethrum against *Tribolium castaneum* and reported that out of the five plant extracts, citronella was the most effective in deterring infestation of carton packaging. An application of citronella at 0.2 g m^{-2} of the carton packaging reduced the beetle infestation by 50%, with the repellency activity sustained for at least 16 weeks. The promising use of citronella oil was also evidenced by Licciardello *et al.* (2013) who showed that packaging coated with EO could inhibit growth of the red flour beetle by 53-87%. This technology has potential for impregnation of carton packaging, bags, sacks and containers used for packaging food commodities such as breakfast cereals, confectionery, pet food, grains and, milk powder for food preservation. Besides EO coated PE and paper-based packaging, other approaches such as use of sachets containing active EOs inside packaging have also been developed. Jo *et al.* (2013) used sachets made of polyvinyl alcohol (PVA) loaded with cinnamon EO to test the fumigant and insect repellent activity against *Plodia interpunctella*. They showed that the cinnamon oil physically diffused throughout the PVA polymer matrix to efficiently repel the insect larvae.

These results clearly show that it is possible to utilize EO loaded active packaging to control stored food product insect pests. Successful implementation of such packaging would help to minimize loss of stored products. EO-loaded active packaging against insects opens an era of possibilities in contemporary green revolution approaches as many plant-derived natural products are comparable to or even better than synthetic pesticides. Table 1.1 provides some studies which involved application of active packaging containing EOs against insects.

1.4. Active packaging against fungi

Another important cause of grain deterioration is fungal infection, which can be caused by high moisture content in crops if they are not adequately dried after harvest. Insect pests are promoters or facilitators of fungal infections (Fathi *et al.,* 2012). Pathogenic species of *Fusarium, Aspergillus* and *Penicillium* are the frequent causal agents of food spoilage and food-borne diseases (Betts *et al.,* 1999, Viuda-Martos *et al.,* 2007). These fungi can contaminate food before

and after harvest. Fungal diseases of food crops can be hazardous as certain fungi produce mycotoxins that can cause severe health problems such as mycotoxicosis in humans (Oliveira *et al.*, 2014b) and in farm animals (Zain, 2011). Mycotoxins are highly stable compounds that are not destroyed during normal food processing. They can be carcinogenic, mutagenic, genotoxic, teratogenic, neurotoxic, and oestrogenic. The most commonly described effect of acute mycotoxin poisoning is the deterioration of liver or kidney function, which in extreme cases may lead to death (Hossain *et al.*, 2016, Perveen, 2012, Plooya *et al.*, 2009). Active food packaging can play an essential role in food processing to reduce fungal contamination and proliferation, and ensure food safety. Numerous studies have unravelled the potential of such type of packaging in controlling fungal infestations in food commodities. The use of antimicrobial packaging is more beneficial as compared to directly adding antimicrobial agents onto food. Antimicrobial agents added directly on food surfaces by sprays or drips are not effective enough to inhibit microorganisms in the long term (Turhan, 2013).

López *et al.* (2007a) designed active films made up of polypropylene (PP) and polyethylene/ethylene vinyl alcohol copolymer (PE/EVOH) and used them to incorporate EOs of cinnamon (*Cinnamomum zeylanicum*), oregano (*Origanum vulgare*), clove (*Syzygium aromaticum*), and cinnamon fortified with cinnamaldehyde. They tested these formulations against a wide range of fungi, including *Penicillium islandicum*, *P. roqueforti*, *P. nalgiovense*, *Eurotium repens* and *Aspergillus flavus.* Their results showed that the PP or PE/EVOH films with incorporated oregano or fortified cinnamon EOs at a concentration of 4% (w/w) were potentially effective as antifungal packaging materials. The packaging sustained their antifungal properties over a prolonged period of time and extended the shelf-life of the food. Gutiérrez *et al.* (2011) showed that cinnamon-loaded active packaging considerably increased the shelf-life of packaged bread and maintained quality up to several months. Their study revealed that the active packaging not only inhibited fungal growth but also maintained the high quality sensorial properties of gluten-free breads. Simple packaging material such as paper can also be converted to active packing by incorporating bioactive EOs. Rodriguez *et al.* (2008) evaluated the use of such paper packaging as a smart alternative for protecting bread from fungal infestation. They developed an active paper package based on the incorporation of cinnamon to solid paraffin wax as an active coating. A 4% (w/w) impregnated cinnamon significantly inhibited the mold species *Rhizopus*

stolonifer under *in vitro* conditions. When used with actual bread slice, almost complete inhibition was obtained with 6% cinnamon EO.

Protein-based active films have also been shown to be an option for controlling fungal growth. An active protein-based film developed by Bahram *et al.* (2014) consisting of whey protein concentrate (WPC) and incorporated cinnamon (1.5%) exhibited good inhibitory effect against *Candia albicans* species. Carbohydrate-based films are equally effective. Cassava starch composite films incorporating cinnamon and clove EO were reported to be effective against *Penicillium commune* and *Eurotium amstelodam* (Souza *et al.*, 2013). In the latter study, the release profiles of cinnamon from the starch films were monitored for 2 h, by UVvis spectroscopy at 289 nm. Released amounts of cinnamon were found to vary from 0.88-1.19 mg cinnamon g^{-1} film.

Other authors have explored the use of sachets containing bioactive agents as smart delivery systems in food packaging against fungal species. Medeiros *et al.* (2011) used sachets incorporated with EO to preserve mangoes inside paper bags, and assessed their antifungal properties. Sachets containing oregano (*Origanum vulgaris*) and lemongrass (*Cymbopogon citratus*) were tested against *Colletotrichum gloeosporides*, *Lasiodiplodia theobromae*, *Xanthomonas campestris pv. mangiferae indica* and *Alternaria alternate*. The mangoes were individually wrapped in paper bags containing antimicrobial sachets and maintained at 25 ± 2 °C and relative humidity (RH) of 80 ± 5%, for nine days. The oregano and lemongrass containing sachets were both effective in reducing the growth of the fungi by approximately 2 logs. Another study conducted by Espitia *et al.* (2011) using active sachets with 20% cinnamon incorporated in polymeric resin were used to study the antifungal effect of the developed matrix in preserving Hawaiian papayas in nonwoven fabric sacks. The treatment with the EO containing sachets presented a lower growth of filamentous fungi as compared to the control treatment. The cinnamaldehyde release from the polymeric matrix was found to be linear and gradual during the storage.

Paraffin coating of paper and board has also been evaluated in active packaging systems. Rodriguez-Lafuente *et al.* (2010) studied the antimicrobial protection and decay retardation of cherry tomatoes in this type of packaging configuration. Bark cinnamon was evaluated against *Alternaria alternata* both *in vitro* and *in vivo* using inoculated cherry tomatoes. Almost total

inhibition of the fungus was obtained when 6% of bark cinnamon was applied to the packaging material. Physicochemical parameters such as pH, weight loss, water activity and color were monitored, and no significant differences were found between the treated and control samples. The maximum transfer of trans-cinnamaldehyde and carvacrol to the food was detected after 1 or 2 days of storage. Sensorial analysis performed by expert panelists showed that there were no apparent changes in the cinnamon-based packaged tomatoes.

Active antifungal packaging has been shown to exert their effect mainly through vapor activity. A study conducted by Avila-Sosa *et al.* (2012) involved the use of Mexican oregano (*Lippia berlandieri Schauer),* cinnamon (*Cinnamomum verum*) or lemongrass (*Cymbopogon citratus*) incorporated in amaranth, chitosan, and starch edible films. The potential of vapor induced inhibition of these films was tested against *Aspergillus niger* and *Penicillium digitatum.* It was found that the EOs in the film caused fumigant toxicity against the tested fungal species. For both types of film, a significant increase in the lag phase was observed as well as a decrease in the maximum specific growth rates of the fungal species. The effectiveness such active packaging through vapor contact has also been reported by other studies (Lopez *et al.*, 2005, López *et al.*, 2007).

The results of previous reports encourage the study and application of EO loaded active packaging materials for the safeguard of packaged food. Though the potential for EO-loaded active packaging is gaining more and more recognition, comprehensive approaches and multidisciplinary researches are still necessary in order to implement and commercialize such eco-friendly active packaging technologies (Chulze, 2010, Sung *et al.*, 2013). The widespread of such packaging is currently limited by the lack of scientific studies. Therefore, these results should encourage further investigation considering active substances, doses, modes of application and target species.

1.5. Limits and challenges for the application of plant-derived EOs in active packaging

Although plant-derived EOs have been shown to exhibit highly desirable traits in terms of their insecticidal and antifungal properties, the future potential for their use in active packaging resides in the ability to develop innovative packaging materials that enable their controlled release in food products. EOs bear several features that limit their application such as their volatility,

instability, and insolubility in water (Bilia *et al.*, 2014), and their rapid diffusion into food matrices (Boumail *et al.*, 2013). While the addition of active EOs in packaging may result in immediate inhibition of non-desired microorganisms, survivors may continue to grow, especially when concentrations of the added EOs are depleted as a result of complex interactions with the food matrix or by natural degradation over time, thereby reducing their shelf-life. This can effectively lead to the proliferation of antimicrobial-resistant strains (Chi-Zhang *et al.*, 2004, Coma *et al.*, 2003). In order to overcome these potential limitations, nano delivery systems have been proposed to enhance the efficacy of EO-based formulations in active packaging. Encapsulation of EOs in a nano-based delivery system as a packaging films or diffusion films could provide an alternative issue, in such a way that only desired levels of the active component diffuse progressively and come into contact with the food (Boumail *et al.*, 2013, Severino *et al.*, 2015) .

1.6. EO-loaded nanodelivery systems

Recent advances in nanotechnology have enhanced the efficacy of controlled release biocide applications through the use of nanoparticles (Perlatti *et al.*, 2013). Nanotechnology deals with the application, production and processing of materials with sizes ranging between a single atom or molecule to particles with 100 molecular diameters (or about 100 nm) (Bilia *et al.*, 2014) . The chemical properties of nanoparticles are controlled to promote an efficient assembly of a structure. Such self-assembly or self-organization of nanoparticles ensures a better interaction and mode of action at a target site due to their tunable controlled release and larger surface area (Duncan, 2011). As smart delivery systems, they confer more selectivity without hindering the movement of bioactive compounds towards the target pathogen (Perlatti *et al.*, 2013). These features enable the use of smaller amounts of an active compound per area as long as the formulation provides an optimal concentration of delivery of the target pesticide for a given period of time (Peteu *et al.*, 2010). EO-loaded nanocarriers can be classified as polymer-based and lipid based nanoparticles. Molecular complexes such as inclusion complexes with cyclodextrins have also been reported (Perlatti *et al.*, 2013). Different strategies employed for EO entrapment in a nano-based systems are shown in Figure 1.3.

(i) Polymer-based nanocarriers are classified as (a) nanocapsules with a core that is surrounded by a shell of the matrix material and (b) nanospheres with a core that is entrapped within a continuous network of the matrix material (Augustin *et al.*, 2009).

(ii) Lipid-based nanocarriers include phospholipids and emulsions. Phospholipids are amphipathic lipids that can be categorized into liposomes and phytosomes. Liposomes are spherical bilayer vesicles with polar heads facing outward and non-polar tails pointing to the inner region. The bilayer structure of a liposome allows it to serve as a delivery vehicle for both hydrophilic and lipophilic compounds locating at the center core (Augustin *et al.*, 2009). Phytosomes, commonly referred to as phospholipid complexes, are structurally related to liposomes, but phytosomes allow higher compound loading capacity and better chemical stability. Phytosomes are composed of chemically associated molecules of phosphatidylcholine, a bi-functional compound with lipophilic phosphatidyl moiety and a hydrophilic choline moiety coupled to a natural active ingredient (Bhattacharya, 2009). Flavonoid and terpenoid constituents of plant extracts are able to bind phosphatidylcholine, producing a lipid compatible molecular complex (Bhattacharya, 2009, Ting *et al.*, 2014).

Emulsions are mixtures of two or more originally immiscible liquids, with one dispersed in the other and stabilized by amphipathic molecules. To improve physical functional stability, emulsion-based delivery systems have been designed in various structures, namely multilayer emulsions, microemulsions, nanoemulsions, solid lipid nanoparticles (SLNs) and self-emulsifying drug delivery systems (SEDDS). Multilayer emulsions are formed through layer-by-layer adsorption of oppositely charged polyelectrolytes onto a primary emulsion droplet. This enhances the toughness of the interface, and allows more control of core release compared to conventional unilayer emulsions (McClements *et al.*, 2007). Microemulsions are homogenous, thermodynamically stable isotropic dispersions of nanodroplets with a mean radius ≥100 nm. In contrast, nanoemulsions are non-equilibrium systems, possessing a relatively high kinetic stability, even for several years, due to their very small size with a mean radius of ≤ 100 nm (Bilia *et al.*, 2014). Solid lipid nanoparticles (SLNs) are a class of submicron emulsions prepared using solid or semi-solid lipid core structure. SLNs as delivery system has been reported to immobilize active elements by the solid particle structure allowing an increased chemical protection, less leakage, and sustained release (Weiss *et al.*, 2008). Self-emulsified drug delivery systems (SEDDS) are isotropic mixtures of oils, surfactants and sometimes include co-solvents.

SEDDS formulations are an advanced variation of emulsion-based delivery system that provide a physically stable environment to contain the mixture of bioactives during storage and allowing rapid emulsification upon contact with aqueous phase (Ingle *et al.*, 2013).

In addition, molecular complexes are developed by the physical association between EOs and cyclodextrins (CDs) which are natural macro cyclic oligosaccharides composed of α-(1,4) linked glucopyranose subunits. They have toroid-shaped structures with rigid lipophilic cavities and a hydrophilic outer surface, capable of enclosing highly hydrophobic molecules inside their cavity (Loftsson *et al.*, 2007). A comprehensive list of studies involving the use of nano structured EO systems applied against insects and fungi is presented in table 1.2.

1.7. EO-nanosystems in insecticidal and fungal packaging materials

Nanocomposite polymers, nanoemulsions and nanoencapsulates have been used in food packaging to enable the slow release of bioactive agents over time, and extend the shelf-life of food (Severino *et al.*, 2015). Several studies have explored the application of nanomaterials in food packaging to acquire enhanced protection against insect and fungal pests. Cindi *et al.* (2015) used chitosan boehmite-alumina nanocomposite films as packaging material in conjunction with thyme oil to control brown rot in peaches (*Prunus persica L.*) during postharvest storage. The nano-based packaging significantly reduced the incidence and severity of brown rot caused by *Monilinia laxa* in artificially inoculated peach fruits at 25 °C for 5 days. The active components of thyme oil namely thymol (56.43% RA), β-linalool (37.6% RA) and caryophyllen (9.47% RA) were preserved within the head space of the punnet. This type of packaging preserved the appearance, taste and natural flavor better as compared to ordinary packaging.

Similar observations were found against fungal species. A recent study conducted by Otoni *et al.* (2014b) involved the formulation of coarse emulsions (diameters of 1.3-1.9 μm) and nanoemulsions (diameters of 180-250 nm) of clove bud (*Syzygium aromaticum*) and oregano (*Origanum vulgare*) EOs with methylcellulose films. The EO emulsions were found to reduce the rigidity and increase the extensibility of the methylcellulose films. The effects were more pronounced for the nanoemulsions. Although the EOs decreased yeast and mold counts in sliced breads, the nanoencapsulated version of the EOs resulted in a better efficiency by increasing the

bioavailability of the EOs. Another study conducted by Heshmati *et al.* (2013) showed that nanoparticles in active packaging can also be used to extend the shelf-life of luxury and delicate food such as caviar which can easily perish at mild or high temperature conditions. They used a packaging comprising of *Zataria multiflora* EO (0.03%, 0.06% (w/w)), nisin (9.18 mg kg^{-1}), potassium sorbate (500 and 1000 mg kg^{-1}) and LDPE packaging material containing nano-ZnO. They found that the nano-based packaging was able to significantly reduce the fungal growth and preserve the original color of the caviar samples. The results showed considerable Zn migration from the package to the caviar especially after 40 days of storage. In a recent study, we found similar observations by comparing coarse emulsions (particle size of 270 nm) and nanoemulsions (particle size of 77 nm) of oregano and thyme mixtures that were used to prepare active polymeric biofilms made up of chitosan, methyl cellulose (MC) and poly lactic acid (PLA) (Hossain et al., 2016, *in preparation*). These film matrices were tested against four fungal species namely *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum*. Antifungal assays showed that the nanoemulsion-based formulated films exhibited antifungal activities up to 3 times higher as compared to the films designed with coarse emulsions. In addition, the nanoemulsion-based film matrix sustained antifungal activity over 40-72 h while the films synthesized with the coarse emulsions maintained activity over only a 24 h period. These studies show that nano-active biodegradable films embedded with active EOs can be customized to produce the next generation food packaging, which can significantly help towards improving food safety.

Nanoparticles can improve the crystallinity and thermal stability of polymeric packaging materials and, thus control the release of EOs in antimicrobial films. Efrati *et al.* (2014) prepared antifungal films by melt mixing polyethylene copolymers in the presence of oregano-modified montmorillonite nanoclay (NC) and thymol EO. It was found that addition of NC affected the structure and homogeneity of the polymeric crystals. The combination of high polymer crystallinity during film preparation and chemical affinity between the EO and NC increased the thermal stability of the EO during film processing. The whole packaging system had a multilayer structure with varied densities and polarities. An increase in polarity of the outer packaging layer reduced the desorption of EO due to chemical interactions between the EO and polymer. Such configuration improved the partitioning of the EO inside the packaging material and led to an enhanced antifungal activity of the nano-based package.

As such, studies have confirmed the beneficial properties that nanoparticles convey to active packaging materials, and the remarkable insecticidal and antifungal activities that they may produce. Nano-based packaging imparts such desirable traits by helping to overcome the limitations of EOs such as their poor solubility in water, low stability and high volatility. Nanoencapsulated EOs can also greatly help to preserve EO bearing highly volatile property during the synthesis of active packaging material itself especially at stages involving high-temperature melt processing, while preserving the insecticidal and antifungal activity of the EOs (Cindi *et al.*, 2015, Mohammadi *et al.*, 2015). In addition, as biodegradable packaging systems often lack mechanical stability and have poorer barrier properties than conventional materials, nanomaterials offer the possibility of making up for these limitations.

1.8. Release kinetics model for EOs from polymeric systems

In active packaging system, the migration of volatile active agents is mainly attributed by the mass transfer phenomenon (Otgonzul, 2010). Based on mass transfer mechanisms involved, release systems can be classified as diffusion controlled, erosion controlled, swelling controlled or a combination of these. A polymer-based controlled release device for EOs is a solid state asymmetrical diffusion system. The main method that can be used to determine the diffusivity of small molecules through solid or semisolid polymer matrices is the determination of sorption or desorption kinetics. The effective diffusivity is calculated from the non-steady part of the sorption/desorption curve, assuming a Fick's model mechanism and appropriate boundary conditions (Tramón, 2014).

Studies have shed light on the physical process of release of EOs embedded in packaging materials. The mathematical modelling of the diffusion process can enable prediction of the bioactive agents release profiles, kinetics and time during which the agent will remain above the critical inhibiting concentration. Mastromatteo *et al.* (2009) investigated the release kinetics of thymol from monolayered and multilayered zein-based composite films. The formulations loaded with spelt bran and thymol (35% w/w) (50.24 cm^2) were placed in a container with 500 ml distilled water at room temperature and the concentrations of the released thymol were measured

by means of HPLC. The results showed that the release rate of thymol was dependent on the layers, thickness and fiber amount of the polymer matrix.

Another study by Kurek *et al.* (2014) investigated the release of carvacrol from chitosan-based films and coatings at a relative humidity (RH) of 2-96% and temperature in the range of 4-37 °C) for more than 60 days by gas chromatography analysis. They found that the release of carvacrol was significantly enhanced by saturating the system with water vapour and increasing the temperature from 4 to 37 °C. After 60 days, the release was lowest at 0% RH and highest at > 96% RH.

EOs can either be released onto the surface or within the headspace between the food and packaging. Compounds released into the gaseous environment within the package can change the atmosphere to prevent ethylene production, lipid oxidation or growth of microorganisms (Balasubramanian *et al.*, 2009). Various polymer compositions can affect the release rate of the antimicrobials. In addition, incorporation of active substances into the polymer can change polymer chemistry, which in turn can affect its characteristics such as oxygen permeability, tensile strength, release rate, brittleness, flavor, color and taste. Therefore, it is important to establish an effective methodology for determining the migration potential of EO loaded nanodelivery systems from FCMs to packaged food.

To date, however, studies of nanoparticle migration from composite packaging materials to food remain scarce. According to Hamdani *et al.* (1997) and Sanches *et al.* (2008), the migration process is highly dependent on the migrant molecular structure, its ability to form hydrogen bonds with the packaging material and any additive present therein, its affinity with the food, the interaction between the food and composite material, as well as its molecular size. However, the process of nanoparticle (NP) migration can be understood as the mass transfer of NPs through polymeric materials obeying Fick's laws of diffusion. Simon *et al.* (2008) successfully modeled the migration of NPs from composite materials based on these basic laws of diffusion kinetics to study non-steady diffusion for different interfaces.

The physicochemical perspective on the potential migration of engineered nanoparticles from packaging to food is based on the average distance travelled by nanoparticles in the polymer matrix. The progress of the migration process can be described using the differential equation

below (Eq. 1), which relates to the migration of an additive from polymeric packaging film (Abreu *et al.*, 2010).

$$\frac{\partial C_p}{\partial_t} = D_P \frac{\partial^2 C_p}{\partial x^2} \qquad (1)$$

where C_p (mg kg^{-1}) represents the concentration of the migrant through the composite polymer, *P*, at time *t (s)* and position *x* in *P*, with D_p being the diffusion coefficient in *P* (cm^{2}s^{-1}).

Based on the concept of the potential chemical properties of both nanoparticles and packaging polymers, it is possible to derive the migration potential (*m*) of NPs through composite materials by taking into consideration the density of the polymer, size of the NPs, density of the NPs in the polymer and average distance of the nanoparticles from the surface of the polymer (Eq. 2) and to estimate the number of migrating particles (Eq. 3) (Wei *et al.*, 2011).

$$m = \sqrt{\frac{k_B T t}{24\,\pi^2 \eta a}} \qquad (2)$$

$$n = mSc_0 \qquad (3)$$

Where in Eq. (2), *m* is the migration potential of NPs in the polymer, K_B is the Boltzmann constant (K_B = 1.3807 × 10^{-23} JK^{-1}), *a* is the radius of NPs, and *T*, *t* and η stand for the absolute temperature, migration time and dynamic viscosity of the polymer, respectively. In Eq. (3), *n*, *S* and C_0 represent the amount of migrating nanoparticles, surface area and initial concentration in the polymer film, respectively. Based on Eq. (2) and (3), *m* and *n* for NPs with different radii and polymers with various densities can be easily computed. Theoretical calculations stemming from the above mathematical expressions indicate that the migration of NPs strongly depends on the size of the nanocomposites and density of the matrix. Smaller NPs and less dense polymers will result in higher migration potential (Simon *et al.*, 2008). However, this is based on the assumption that the interphase boundary between the polymer and food does not represent any obstacle for the movement of the NPs, while in fact some NPs may be reflected back to the polymer matrix at the interphase boundary. The mathematical equation accurately models the migration of very small NPs with a radius in the order of magnitude of 1 nm from polymer matrices that have a low viscosity and do not interact with the NPs. These conditions are readily found in the case of nanocomposites of silver with polyolefines (polyethylene and polypropylene). On the other hand, the migration of bigger NPs with relatively high dynamic

viscosities such as nanosilver composites with polyethylene terephthalate, polystyrene and surface-modified montmorillonite embedded in various polymer matrices may not be detectable (Wei *et al.*, 2011).

A study conducted by von Goetz *et al.* (2013) introduced the parameter of surface area and according to these authors, in modelling the migration of nanoparticles, constant parameters can be lumped into *a* and *b* to take in the form of a power function equation to describe the number of particles migrating from a polymer per surface area (Eq. 4):

$$\boldsymbol{f(t) = at^b} \qquad (4)$$

Where *f(t)* relates to the number of nanoparticles migrating from the polymer per surface area (*a*) and *b* is a calibrated model parameter. By estimating the parameters *a* and *b*, they attempted to fit a power function to the migrating values for the release of silver from plastic material over time based on Fickian diffusion processes.

The studies conducted so far provide a sound basis for the migration of NPs from composite materials. However, further studies are required for a more in-depth understanding of the mass transport of NPs from nanocomposite film packaging. Similarly important will be to establish an effective methodology for determining the migration potential of NPs from FCMs packaged food. In migration tests, the ability of NPs to migrate in a matrix, their dispersion and alterations in size and morphologies before and after the migration tests, should all be considered to assess the safety of packaging polymers embedded with nanomaterials. The formulation of new models and consolidation of existing ones can help to predict such migration behavior.

1.9. Conclusion

Many plant essential oils have been studied that offer novel and effective insecticidal and fungicidal compounds for control of stored products pests nano-based controlled delivery systems in packaging using EO or their components are a promising approach for the food industry. Recent investigations into the design and improvement of EO loaded nano-based active packaging systems are tantalizing, and hold great promise to control stored product pests.

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Table 1.1. Studies investigating antifungal and insecticidal active packaging

Packaging material/polymeric mixture	Essential Oil (EO)	Method of EO incorporation	Application	References
Polypropylene	*Cinnamomum zeylanicum* (cinnamon)	Coating	Antifungal	(Gutiérrez *et al.*, 2011)
Polypropylene (PP), polyethylene/ethylene	*Cinnamomum zeylanicum* (cinnamon) *Origanum vulgare* oregano *Syzygium aromaticum* (clove)	Film	Antifungal	(López *et al.*, 2007a)
Solid wax parrafin, active paper	Cinnamon	Coating	Antifungal	(Rodriguez *et al.*, 2008)
PE polymer	*Prunus amygdalus* L. and *Mentha viridis*	Spraying	Insect repellent	(Allahvaisi, 2010)
Carton board	citronella, garlic oil, neem extract, pine oil, and pyrethrum	Coating	Insect repellent	(Wong *et al.*, 2005)
Plastic	citronella, oregano and rosemary	Coating	Insect repellent	(Licciardello *et al.*, 2013)

Polyvinyl alcohol (PVA)	cinnamon (*Cinnamomum zeylanicum*)	Strip	Insect repellent	(Jo *et al.*, 2013)
Jute bags	Neem	Coating	Insect repellent	(Anwar *et al.*, 2005)

Table 1.2.a Studies involving use of nano-structured EO systems against insects

Polymer	Essential Oil (EO)	Target sp.	Nano delivery system	Test condition	References
-	*Ageratum conyzoides*, *Achillea fragrantissima* and *Tagetes minuta*	*Cowpea beetle, Callosobruchus maculatus*	Nanoemulsion	*In vitro*	(Nenaah *et al.*, 2015)
Poly Caprolactone (PCL)	*Zanthoxylum rhoifolium*	*Bemisia tabaci*	Nanospheres	*In vitro*	(Christofoli *et al.*, 2015)
Myristic acid-chitosan (MA-chitosan	*Carum copticum*	*Sitophilus granarius (L.) and Tribolium confusum (L.)*	Nanogel	*In vitro*	(Ziaee *et al.*, 2014b)
Myristic acid-chitosan	Cumin, Cyminum cuminum	*Sitophilus granarius (L.)* and *Tribolium confusum*	Nanogels	*In vitro*	(Ziaee *et al.*, 2014a)
Polyethylene glycol (PEG)	Geranium or bergamot	*Tribolium castaneum* and *Rhizopertha dominica*	Nanoparticles	*In vitro*	(González *et al.*, 2014)
Polyethylene glycol	Garlic	*Tribolium castaneum*	Nanoparticles	*In vitro*	(Yang *et al.*, 2009)

Table 1.2.b Studies involving use of nano structured EO systems against fungi

Polymer	Essential Oil (EO)	Target sp.	Nano delivery systems	Test condition	References
Chitosan (CS)-caffeic acid (CA)	*Cuminum cyminum*	*Aspergillus flavus*	Nanogel	*In vitro*	(Zhaveh *et al.*, 2015)
ZnO loaded chitosan	*Zataria multiflora*	*Botrytis cinerea Pers*.	nanoparticle	*In situ* Starwberry	(Mohammadi *et al.*, 2015)
Chitosan	Thyme	*Aspergillus flavus*	Nanogel	*In situ* Tomato	(Khalili *et al.*, 2015)
Gold nanoparticle (AuNPs)	Mentha piperita	*Aspergillus niger, Aspergillus flavus,*	Nanoparticles	*In vitro*	(Thanighaiarassu *et al.*, 2014)
Silver	Cinnamon, clove carom eucalyptus coriander mustard	*Aspergillus, Fusarium, Curvularia, Cladosporium, Phoma*	Nanoparticles	*In vitro*	(Jogee *et al.*, 2012)
chitosan-cinnamic acid	*Mentha piperita*	*Aspergillus flavus*	Nanogel	*In situ* Tomato	(Beyki *et al.*, 2014)
Chitosan	Turmeric	*Candida albicans, Trychophytol mentagrophyte*, *Fusarium oxysporum*, and *Penicilium italicum*	nanoparticles	*In vitro*	(Cuc *et al.*, 2014)

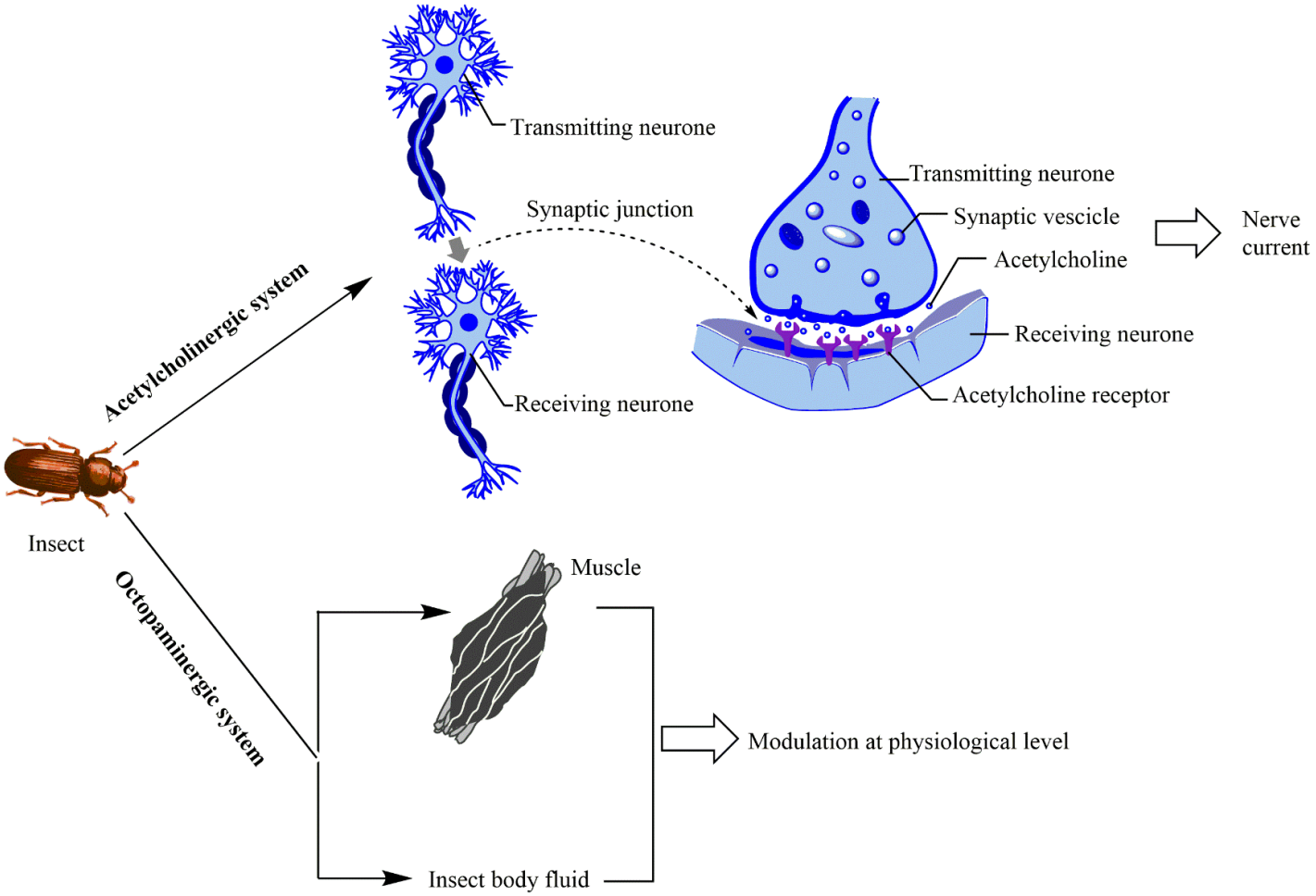

Figure 1.1. Target sites of EO components in insects. EOs act at the level of acetylcholinergic nervous system blocking the action of acetylcholinesterase, which is important in the degradation of acetylcholine neurotransmitter preventing neurones to regain their resting potential. Studies have also reported the action of EOs on octopaminergic nervous system in insects where they disrupt octopamine receptors.

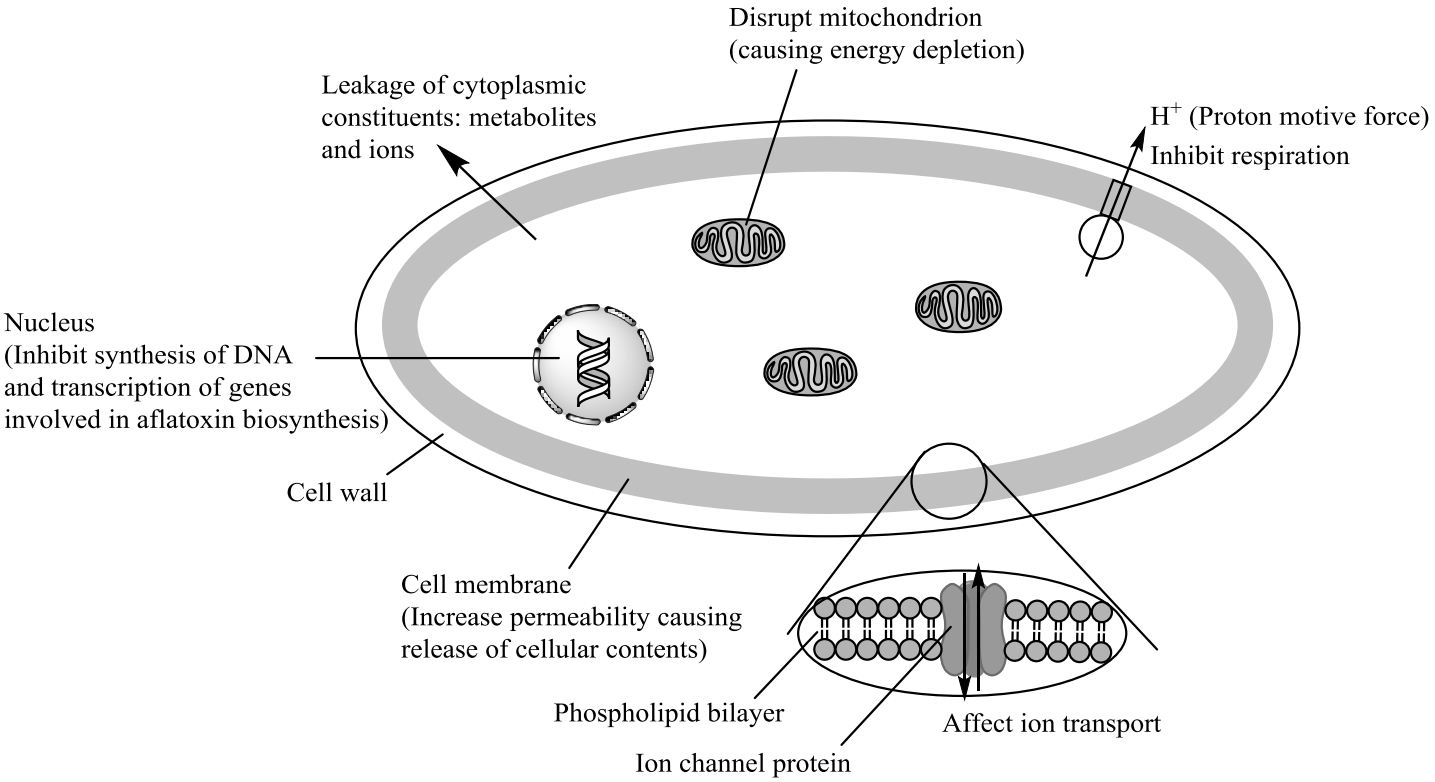

Figure 1.2. Major sites of action for EO components on fungal cell

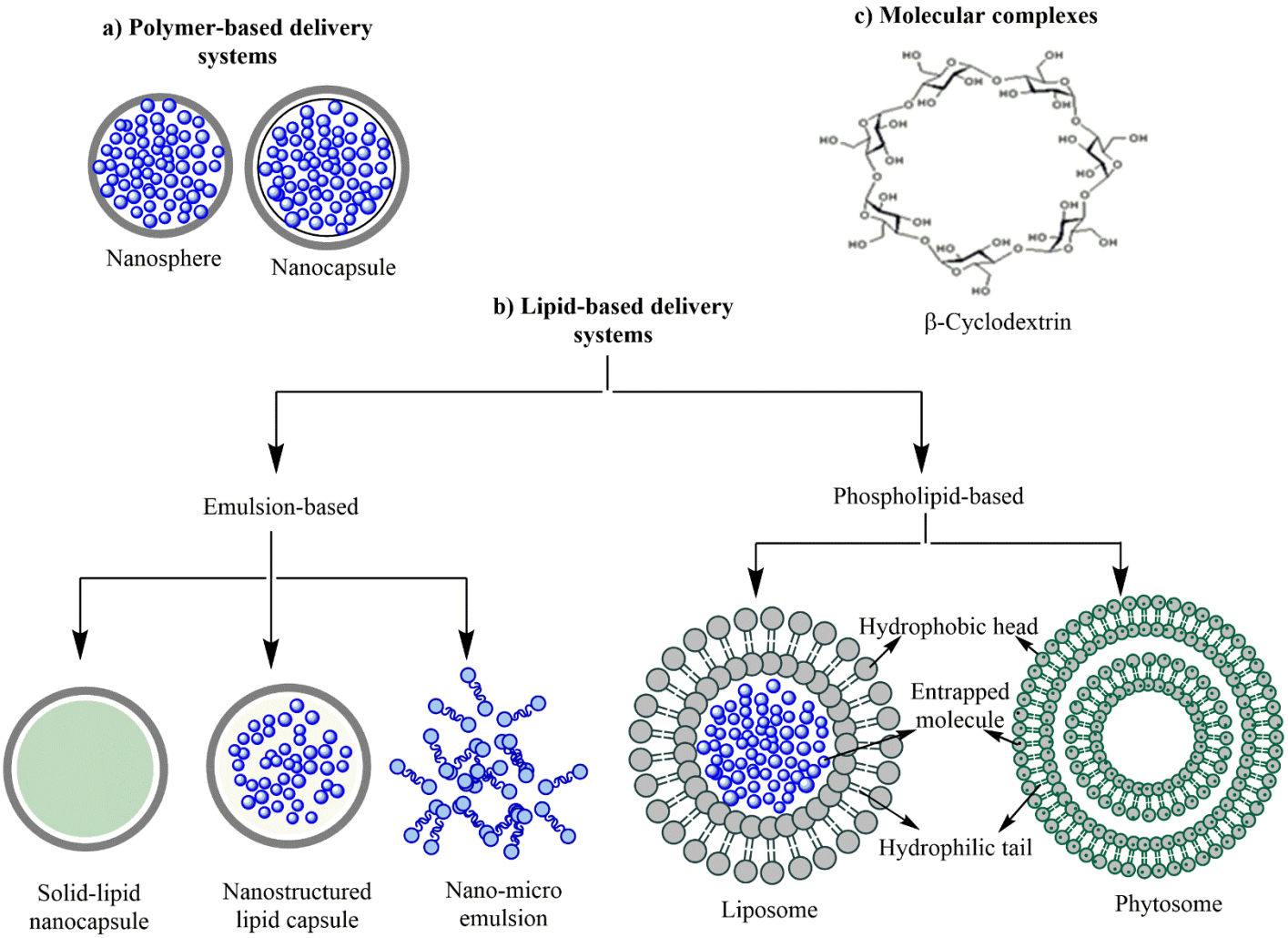

Figure 1.3. Schematic diagram showing nano-based strategies for EO entrapment. a) Polymer-based delivery systems, b) Lipid-based delivery systems, and c) Molecular complexes. Adapted from Bilia, et al. (2014); Ting, et al. (2014) with modifications.

Potential application of natural essential oils and gamma radiation for controlling stored agricultural products – (review)

Introduction

Cereal grains such as wheat, rice, and maize represent basic, staple food commodities for billions of people worldwide. Minimizing cereal losses in the supply chain can be an efficient way to strengthen food security, sustainably combat hunger, reduce agricultural land needed for crop production, develop rural areas and improve farmers' livelihoods. In developing countries, most of the crop loss occurs during postharvest or storage. In some cases, these losses can be up to 80% of the total production including direct physical losses and quality losses that reduce the economic value of crops. Postharvest loss is a complex problem and its scale varies with several factors such as varieties of crops, practices, climatic conditions, and country economics. Most of the harvested grains are susceptible to insect infestation and mold growth during storage. Integrated pest management system with improved technology interventions can play a critical role in reducing postharvest losses (Kumar *et al.*, 2017).

Increasing consumer concern has driven research into development of novel methods to eliminate food-borne pathogens in conjunction with the existing ones for controlling stored product pests. The biorational mode of action of some control measures such as natural essential oils (EOs) and ionizing radiation have been found effective and safe in controlling food pests and achieving 'Green consumerism' in the agricultural sector. Concerns about the safety of synthetic compounds have promoted research in plant resources and naturally-derived essential oil metabolites. Their application can effectively retard the growth of insect, food borne molds, mycotoxin production and oxidative deterioration of food commodities without altering the organoleptic properties. A wide range of EO-based formulations, in connection with gamma radiation, can be developed as safe, economic preservatives to control postharvest losses.

i. Fungi and insects as potent crop pests

Fungi and insects are notorious biotic agents capable of inflicting heavy losses in stored food products. Insect pests are considered to be the most serious biotic stressors that can substantially decline food production (Chaubey, 2007, Ebadollahi *et al.*, 2011). *Coleoptera* (beetles) account for three-quarters of stored product pests, and the genera *Sitophilus* and *Tribolium* are the most damaging known insect pests. On the other hand, pathogenic molds such as *Fusarium*, *Aspergillus* and *Penicillium* species have been reported as causal agents of food spoilage and food-borne diseases (Betts *et al.*, 1999, Viuda-Martos *et al.*, 2007). These fungi can contaminate foods from cultivation to harvest as well as during transportation and storage.

After crop harvest serious losses may result if the processing is done improperly. As a start, intact grain should always consider an essential item for successful storing. Improper application of post-harvest practices such as threshing, drying or transporting damage crack the grain, which leads to an entry point for infestation by insect and mold during storage. Threshing, which is the removal of grain or seed from its straw, in itself may cause a degree of physical damage to the grains. Some studies found that threshed sorghum grains were more susceptible to *Sitophilus oryz*ae infection than un-threshed grain (Wongo *et al.*, 1990). Similarly, millet is also sensitive to threshing. An adequate knowledge on the physiological and biological aspects of pests is also necessary for the safe and proper handling of post-harvest operations (Sallam, 1999).

Storage is another vital and critical post-harvest operation as crop products storage may extend over several months to years. Deterioration of the grain quality during storage can be due to improper storage conditions, which eventually leads to contamination with fungi or insects. In many cases, the infestation by microbial agents starts in the field where the crop has been cultivated. For instance, in cowpeas cultivation, a 1-2% initial field infestation by *C. maculatus* may result in 80% of the pods being attacked after 6-8 months of storage. On the other hand, several studies have demonstrated the importance of insect pests as promoters or facilitators of fungal infection (Sallam, 1999). Storage generally leads to a degree of change in the quality of the product due to seed respiration, which depletes seed nutrients over time and also promotes further fungal growth and insect infestation. In addition, insects ingest and carry spores on their bodies and their droppings and spores may spread as far as insect go.

Transportation represents another stage where physical damage, grain spilling or deterioration might occur (Makunh *et al.*, 2007, Reddy *et al.*, 2009, Sallam, 1999). In addition, some of the stored product insects such as *Tribolium castaneum, Sitophilus granarius* L., *Rhyzopertha dominica* F. and *Stegobium paniceum* are capable of penetrating food packaging. However, such losses can be avoided through proper handling, loading and packaging (Allahvaisi, 2010, Allahvaisi *et al.*, 2009).

ii. Use of plant-derived essential oils (EOs) as biopesticides

Application of chemical pesticides for the postharvest control of pests on stored products has been shown to constitute serious drawbacks such as environmental pollution, high mammalian and non- target organism toxicities, persisting pesticide residues in food items and pest resistance. This has led to the search for safe and inexpensive alternatives (Bello *et al.*, 2001, Ebadollahi *et al.*, 2011). In subsequence, plant essential oils (EOs) and their components have been found to exhibit potential antifungal and insecticidal properties (Chaubey, 2011). A pure EO is a mixture of complex secondary metabolites that contain a wide spectrum of strong aromatic components. The components of an EOs can mainly be classified into two groups such as volatile fraction (90-95% wt) and nonvolatile residue (10% wt). Volatile fraction consists of hydrocarbons and terpenes and their oxygenated derivatives, such as camphor along with acids, aliphatic aldehydes, ketons, alcohols and esters. Nonvolatile residues consist of hydrocarbons, fatty acids, carotenoids, sterols, waves, and flavonoids (Rathore, 2017). An estimated 3000 EOs are known among which approximately 300 are commercially important at present especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Essential oils are mostly formed from different parts of plants such as from flowers, leaves, buds, petals, seed, roots, resin etc. The extraction of EOs can be conventional method which includes hydro or stem distillation, solvent extraction and cold pressing or recent methods including supercritical fluid extraction and micro-wave assisted extraction. The quality and quantity of extraction method affects the chemical profile of the essential oil products not only in the number of molecules but also in the stereochemical types of molecules extracted (Bakkali *et al.*, 2008). For example it has been repoted that herbal oils extracted using hexane possess a stronger antimicrobial activity than the corresponding steam distilled EOs. Also oils produced from herbs extracted during or immediately after flowering show greater activity (Vergis *et al.*, 2015). Table I represents the bioactivity of some plant derived EOs.

iii. Antifungal properties of plant-derived essential oils (EOs)

Microscopic fungi such as *Aspergillus*, *Fusarium*, and *Penicillium* are well-known food spoilage agents capable of reducing yield and quality of crops thereby entailing significant economic losses. These fungi produce low weight metabolites which can cause mycotoxicosis and affect the health of livestock, domestic animals and humans (Ashiq, 2015, Jeswal *et al.*, 2015) Mycotoxins can be acutely or chronically toxic depending on their chemical structure of the toxin. Certain type of mycotoxins such as aflatoxins and ochratoxin A represent significant threats to human health. Aflatoxins have been reported to be potent carcinogens and, in association with hepatitis B virus, are responsible for many thousands of human deaths per annum, mostly in underdeveloped tropical countries (Richard, 2007). Some aflatoxins are secondary metabolites such as difuranocoumarins which are known to be produced by *A. flavus* and *A. parasiticus* and are commonly found in food and feeds and have been associated with various diseases such as aflatoxicosis in livestock, domestic animals and humans worldwide (Ashiq, 2015). A list of mycotoxins and their potential impacts on health are shown in Table II.

In recent years, the use of naturally occurring essential oils to control food spoilage caused by fungal species has been gaining more and more interest. Greater consumer awareness and concern regarding synthetic chemical additives have driven the formulation of a wide range of EOs as natural alternatives to food preservation. An exhaustive number of studies have uncovered the antifungal properties of a broad spectrum of EOs extracted from plants including basil, oregano, mint, sage, coriander, cinnamon (Basılico *et al.*, 1999, Chatterjee, 1990, Pawar *et al.*, 2006). Agar disc diffusion and disc volatization methods are commonly used for screening the potential antifungal activity of EOs. These two methods are useful to generate preliminary qualitative data and enable the selection of the EOs with high activity for further evaluation (Dobre *et al.*, 2011). The minimum inhibitory concentration (MIC) of EOs against fungal species are commonly determined by the broth dilution method or agar plate technique (Bansod *et al.*, 2008). Screening of EOs with potential antimicrobial activity capable of inhibiting the growth of undesirable microorganisms has paved the way for the development of product formulations and surface coated or incorporated packaging materials to limit food losses during storage.

An exhaustive number of studies have shown the successful application of EOs to reduce or eliminate fungal growth on food products. The inhibitory influence of *Citrus limon* and *Citrus*

sinensis EOs on the growth of *Aspergillus flavus* and AFB_1 production was evaluated by El Miri *et al.* (2018). Their results have shown that the extract of *Citrus limon* at 1.75 mg/mL and *Citrus sinensis* at 2 mg/mL could totally inhibit fungal growth as well as AFB_1 production. In addition, the Citrus EOs displayed a wide spectrum of fungitoxic effects against well-known fungal pests. The EO of *Origanum vulgare* and *Cinnamomum zeylanicum* and their major active constituents, carvacrol and cinnamaldehyde, were assayed for inhibiting the growth of fungal species and production of aflatoxin production in maize extract medium. The results showed that doses of 10–1000 mg l^{-1} were effective in inducing 50% (ED_{50}) and 90% (ED_{90}) growth inhibition of the test specimens. The ED_{50} of cinnamaldehyde, carvacrol, oregano and cinnamon extracts against *A. flavus* were in the range of 49–52.6, 98–145, 152–505, 295–560 mg l^{-1} and in the ranges of 46–55.5, 101–175, 260–425 and 490–675 mg l^{-1}, respectively, against *A. parasiticus* depending on environmental conditions (Gómez *et al.*, 2018). The antifungal attributes of other EOs such as those derived from *Origanum onites, Salvia officinalis*, and *Mentha piperita* EOs and their mixture against the growth of ochratoxigenic *Penicillum verrucosum* was demonstrated by Ozcakmak *et al.* (2017). Their findings confirmed the potential fungistatic and fungicidal effects of plant and spice-derived EOs in food systems.

The antifungal properties of EO extracted from flowers and leaves of *Origanum vulgare* were evaluated by Hashemi *et al.* (2016). They reported Minimum Fungicidal Concentration (MFC) values of 200 and 100 µg/ml against *A. niger* and *A. flavus*, respectively. Extracts of *Rosmarinus officinalis* (Rosemary) was also found efficient against *A. niger* (Baghloul *et al.*, 2017). The authors reported a minimum inhibitory concentration (MIC) of 0.5% against several strains of *A. niger*. In another study, the EO spearmint (*Mentha spicata* L.) was found to significantly inhibit the growth of the toxigenic strain of *A. flavus* and aflatoxin B_1 production at 1.0 and 0.9 µl ml^{-1} levels, respectively. The EO extract also showed a broad fungitoxic effect against 19 food-deteriorating molds (Kedia *et al.*, 2014). El-Habib (2012) evaluated the effect of natural food additives such as thyme, marjoram, basil, rosemary, dill, mint and coriander on the growth of *A. flavus* and its AFB1 production capacity. The results showed that the EO of dill, coriander, basil, rosemary, mint and thyme have significant *in vitro* antifungal activities against *A. flavus.* Dill extract was the most effective against aflatoxin production, while extracts of both thyme and basil significantly delayed the growth of *A. flavus* and induced complete growth inhibition at 150 µl of test extract. The antifungal activity of marjoram and clary sage EOs against the bread TRACT

spoilage molds *A. niger*, *Penicillium chrysogenum* and *Rhizopus* spp. was investigated by the reversed Petri dish method on wheat, wheat-rye mixed, and rye bread slices. The growth of investigated molds was found to be significantly reduced on EO-vapor treated bread slices (Krisch *et al.*, 2013).

iv. Insecticidal properties of plant-derived EOs

Insects include thousands of species that are considered pathogenic agents for humans as well as disastrous for plant crops. They have been reported to damage crops such as rice, cotton, corn, wheat and sugarcane (Regnault-Roger *et al.*, 2012;Regnault-Roger, 1997). For more than 60 years, man has relied extensively on synthetic chemical insecticides such as chlorinated hydrocarbons (e.g. DDT and the cyclodienes), organophosphates and carbamates (e.g., parathion and carbaryl), pyrethroids (e.g., permethrin) and neonicotinoids (e.g., imidacloprid). The use of these insecticides has been shown to induce ecological balance to varying degrees; mammalian toxicity, disruption of food chains and increasing resistance to chemical insecticides have been reported (Soujanya *et al.*, 2016). Hence, it is highly recommended that pest management diversify approaches to better control harmful insect pests. Among the various alternative strategies to conventional synthetic insecticides, ecofriendly approaches based on existing plant-insect relationships is one of the most promising methods. For centuries, plants and insects have followed a parallel and interdependent evolution. Plant EOs constitutes a relatively new class of natural insecticides that have been found effective against a wide range of insect pests. Their neurotoxic effects on insects and mites have been largely reported. Anecdotal evidence has shown that their efficacy could be attributed in part to their actions as behavior modifiers (i.e. as repellents or deterrents).

Generally three common criteria can be used to evaluate the insecticidal properties of plant-derived EOs namely fumigant toxicity (Lee *et al.*, 2001a, Park *et al.*, 2003, Tun *et al.*, 2000), contact toxicity (Abdelgaleil *et al.*, 2009, Kim *et al.*, 2001) and repellent toxicity (Baba *et al.*, 2012, Choi *et al.*, 2002, Choochote *et al.*, 2007, Gbolade, 2000, Wang *et al.*, 2006, Yoon *et al.*, 2007). Although the bioactivity of EOs may vary greatly with minor parameter changes, certain plant spices such as oregano, thyme, basil, rosemary and mint have been found consistently bioactive (Tripathi *et al.*, 2009). The toxic effect of EOs depends on the point of entry of the toxin, apart from the variability of the phytochemical patterns. Commonly, EOs can be inhaled,

ingested or skin absorbed by insects. Plant families such as Myrtaceae, Lamiaceae, Asteraceae, Apiaceae and Rutaceae are highly targeted for insecticidal activities against orders like lepidoptera, coleoptera, diptera, isoptera and hemiptera. EOs have been scrutinized for their repellent, fumigant, larvicidal and adulticidal activities (Tripathi *et al.*, 2009).

Oliveira *et al.* (2018) evaluated the lethal and sublethal effects of *Lippia* sidoides extract and its major compounds (thymol and ρ-cymene) on different populations of *maize weevil* and *Sitophilus zeamais*. The lethal concentration required to kill 50% of *S. zeamais* populations ranged from 35.48 to 118.29 μL L^{-1} air for EO of *L. sidoides*, whereas 65.00–91.23 μL L^{-1} air for thymol and 801.24 to 2188,83 μL L^{-1} air for ρ-cymene. Their results demonstrated that the *L. sidoides* extract and thymol are a promising alternative for controlling *S. zeamais*. Zaka *et al.* (2018) studied four essential oils namely *Azadirachta indica* (neem), *Ricinus communis* (castor), *Eruca sativa* (arugula), *Eucalyptus globulus* (eucalyptus) against *Tribolium confusum*. LC_{50} values of 19, 25, 33 and 35 mg/L were obtained for neem, castor, eucalyptus and arugula EOs respectively, supporting that the plant EOs are effective against stored grain insect pests. Encapsulation of EOs was studied by Skuhrovec *et al.* (2018) using combinations of plant extracts including *Rosmarinus officinalis* and *Cymbopogon, citratus,* and *Pelargonium graveolens* and *Thymus vulgaris.* Encapsulated versions of the EO mixtures were tested for their insecticidal potential for wheat protection against adults and larvae of *Oulema melanopus* (L.). The results showed the effective inhibitory effects of these EOs and their encapsulations which induced 100% mortality within 24 h. Murcia-Meseguer *et al.* (2018) tested thirteen EOs namely sandalwood, cinnamon, orange, bergamote, citronella, eucaliptus, lavender, mentha, basil, red cedar, geranium, patchouli and rosemary against *Spodoptera exigua.* All plant extracts were found to be effective against *S. exigua.* EOs of *Cinnamomum* and red cedar showed the highest toxicity (mortality above 90%) against the larvae, while cinnamon and patchouli EOs were the most harmful to adults (95% mortality). Citronella, basil and eucalyptus oils were found to significantly reduce fecundity. The fumigant toxicity of *Thymus persicus* EO was studies against two stored grains, *Tribolium* castaneum and *S. oryzae, by* Saroukolai *et al.* (2010). The LC_{50} values were 237 and 3.33 ul/L for *T. castaneum*, and *S. oryzae*, respectively. Kim *et al.* (2016) evaluated the fumigant toxicity of eight EOs and their constituents against the adult rice weevil *Sitophilus oryzae*. Out of the eight EOs tested, hyssop (*Hyssopus offcinalis*), majoram (*Origanum majorana*), and *Thymus zygis* extracts displayed strong fumigant toxicity at 25 mg/L air

concentration against *S. oryzae* adults. The study conducted by Kedia *et al.* (2014) demonstrated that the EO of spearmint (*Mentha spicata* L.) was efficent against *C. chinensis* causing 100% mortality during a fumigation test with an LC_{50} value of 0.003 µl ml^{-1} air after 24 h of treatment, and 100% repellency at 0.025 µl ml^{-1} air concentration. An air concentration of 0.1 µl ml^{-1} was found to be effective in inducing fumigant toxicity and showed 98.46% oviposition deterrence, 100% ovicidal activity, 88.84% larvicidal activity, 72.91% pupaecidal activity, and 100% antifeedant activity against *C. chinensis*. Thus EOs based pesticides have great impact in integrated Pest Management (IPM) programs due to their safety to non target organism and environment.

v. Regulatory considerations

The industrial use of EOs is a promising area but requires intensive research in order to determine their associated toxicological and safety effects and to develop novel active ingredients. Plant-derived EO-based formulations are considered as "Generally Recognized as Safe' (GRAS)" by the Food and Drug Administration (FDA) and Environment Protection Agency (EPA) in USA. According to United Nations Comtrade statistics, it was estimated that the world market for the use of EO as fragrance and flavour agents represented a value of US $ 24 billion, increasing at about 10% per year. The major consumers in the world's EOs market are the United States (40%), Western Europe (30%), and Japan (7%). EOs are routinely used in the food industry for enhancing flavors, and aromatic agents in detergents, and domestic and industrial products (Govindasamy *et al.*, 2013). The major EOs consumed worldwide are orange, corn mint and peppermint, whose consumptions are estimated at over 50, 25 and 4.5K, respectively. Some EOs are used in quantities between 100-500 tons per year and include bergamot, cassia, cinnamon leaf, sage, dill, geranium lemon grass, tea tree, pine and rosemary. Table IV provides pertinent data on world consumption of EOs that are used over 500 tons per year (Brud, 2015). Some EO-based preservatives are already commercially available. Currently 'DMC Base Natural' commercial products comprising 50% of EO from rosemary, sage and citrus are being used as a safe food additive. Carvone has been introduced under the trade name TALENT and is commercially available in the Netherlands and widely used in warehouses to inhibit the growth of

storage pathogens. EcoSMART Technologies has developed and introduced pesticide products derived from natural plant oils. Eugenol-based formulations have shown potential efficiency against apple fungal pathogens during marketing. Several formulations of neem are commercially available and sold as "Neem Products"(Sintim, 2012). Crop protection imparted by natural plant sources is considered safer, and when used in combination with other pest control techniques such as irradiation guarantee maximum protection. Fumigation of food commodities using EOs has been shown to be economically viable and effective, and leaves minimum residue. It is expected that many EO-based formulations will be developed in the near future as safe preservatives for eco-friendly management of post-harvest crop losses (Dwivedy *et al.*, 2016).

vi. Food irradiation as an emerging technology

Over the recent decades novel technologies have emerged worldwide in food production, processing and preservation. These technical innovations are in broad development as a result of modern demands for foods that are fresher, with higher nutritional values, more natural with minimum food additives, and with no toxins and allergens (Brody *et al.*, 2014, Heldman *et al.*, 2010). As a result of these emerging technologies higher-quality foods are produced with safer attributes since they have an extended shelf-life and are at a reasonable cost.

Food irradiation is a processing technique which involves exposing food to ionizing radiation such as electron beams, X-rays or gamma radiation to induce bacterial demise that can cause food poisoning, to control insect infestation, to delay fruit ripening or prevent vegetables from sprouting (Lacroix *et al.*, 2007). Studies have shown that this technology can prevent the proliferation of microorganisms which cause food spoilage, such as bacteria and moulds, by changing their molecular structure (Lacroix *et al.*, 2015). Also commonly known as "cold pasteurization" it offers a wide range of benefits to the food industry and the consumer by ensuring a hygienic quality of solid or semisolid foods, through inactivation of foodborne pathogens (Crawford *et al.*, 1996). Interest in irradiation food technologies is increasing because of persistently high food loses from infestation, contamination and spoilage by bacteria and fungi, rising concern about foodborne diseases, and growing international trade in food products that must meet strict import standards of quality and quarantine. In all these respective areas, food

irradiation has demonstrated valuable and practical benefits when integrated within an established system for the safe handling and distribution of food products (Mostafavi *et al.*, 2012). In addition, with increasingly restricted regulations or complete prohibition on the use of a number of chemical fumigants for insect and microbial control in the food industry, irradiation is becoming effective alternative to protecting food against insect damage and as a quarantine treatment for fresh produce (Follett, 2014, Follett *et al.*, 2006). As such, irradiation can help to ensure a safer and more plentiful food supply by extending food shelf-life through the control of pests and pathogens. The lethal dose of gamma radiation against various microorganisms is listed in Table III.

It has been reported in many studies that the effect of radiation varies among different organisms . The inactivation of individual seed -born fungal viability (*in vitro*) on *Oryza sativa* was found to range between 1.0 – 2.0 kGy against *A. alternata* and *A. fl*avus (Maity *et al.*, 2011). Aziz *et al.* (2002) studied the effects of gamma radiation on the fungal microflora of 100 random fruit samples and observed a progressive reduction in the fungal population. Their results showed that samples treated with irradiation at doses of 1.5 and 3.5 kGy significantly reduced the total fungal counts compared with unirradiated control after 28 days stored at refrigeration temperatures (below 10 ºC). However complete fungal growth inhibition required higher doses of 5 kGy. Irradiation is a safe and feasible method for controlling stored product insects in bulk grains. The USDA recommends a minimum dose of 400 Gy for all insect pests, except for adults and pupae of the order Lepidoptera. Radiation doses required to prevent insects from completing development or reproducing range from 0.05 kGy for beetles to 0.45 kGy for some Lepidoptera species. Several countries allow the application of a dose of up to 1 kGy against stored-product insects (Hallman, 2013).

Irradiation has proven to be an effective method for food decontamination of stored food products, especially grains as it damages the internal structure of infesting pests. Importantly, according to the World Health Organization (WHO) and Food and Agriculture Organization (FAO), it is a safe technology for processing food commodities when the appropriate radiation dose is respected (Organization, 1991, WHO, 1999). In 1997, the United Nations reported that food items could be treated with any radiation dose without any detrimental effect on the food's wholesomeness. A study group concluded that high-dose irradiation, when applied in accordance

with good manufacturing and irradiation practices, could improve hygienic quality of food commodities rendering them shelf stable. It should be noted that the FDA evaluates radiological, toxicological and microbiological safety, as well as nutritional adequacy to establish the safety of irradiated foods. Experts have found no evidence of harmful effects incurred by food irradiation and have approved their safe application to control pests (Maherani *et al.*, 2016). Food irradiation facilities are protected by various safety measures; the use of machine sources (e-beam and X-ray) entirely removes any risk of the presence of radioactive isotopes (Hallman, 2013). Foods permitted to be Irradiated under FDA's Regulation is presented in Table IV.

vii. Combined preservation techniques

Application of gamma radiation and EOs at high doses separately is expensive and time-consuming. It is desirable to implement techniques that use lower radiation dose and EO concentrations without compromising the effectiveness of the individual treatments. Combined treatment can effectively inhibit the growth microorganisms by dual action. As is the case with *S. oryzae,* fumigant application of EOs kills only adults and not individuals at their early developmental stages that are located inside the kernel. Low doses of gamma radiation have been reported to effective in killing insects at their early stage of development. In addition, the effect of EO fumigation can last for a long time after treatment. Hence, combining these two preservation methods can be beneficial due to the synergistic or additive effect to enhance the elimination of pathogenic microorganisms. Such approach will also involve less extreme use of a single treatment or dose requirement for destroying foodborne illness and preserve the sensory quality of foods (Lacroix *et al.*, 2015).

A study conducted by Ahmadi *et al.* (2013) has shown that the insecticidal effectiveness of EOs can be enhanced by irradiation treatment. The authors studied the effect of sublethal doses of *Rosmarinus officinalis* (L.) and *Perovskia atriplicifolia* (Benth) extracts in combination with gamma radiation on the mortality of adult *Tribolium castaneum*. The insects were subjected to two radiation doses and two atmospheric concentrations of EOs. The results showed an increased mortality, which was 3-6 times higher than the effect of each individual treatments. A synergistic

effect was observed in the case of *R. officinas* (L.) and was more pronounced than that of *P. atriplicifolia* (Benth). A significant synergistic correlation resulted from the exposure to gamma radiation and EO treatment on 1-7 days against old adults of *T. castaneum*.

Hence, combined application of gamma irradiation and natural plant extracts can ensure a long-term antimicrobial safety and extend the shelf-life of food. According to a study conducted on cauliflower, irradiation at a dose of 1 kGy followed by the spraying with natural antimicrobial formulations , led to a reduction of tested yeast and mold by 2 logs (formulation M) or 3 logs (formulation A) as compared to unirradiated control (Tawema *et al.*, 2016). Carboxymethyl cellulose (CMC) coatings on plum alone and in combination with gamma irradiation were tested for their ability to extend the shelf-life of plum by Hussain *et al.* (2015). Combination of CMC at 1.0% w/v and 1.5 kGy irradiation was found significantly ($p \leq 0.05$) superior to all other treatments in maintaining the storage quality of the plum and in delaying their decay. Irradiation alone at 1.5 kGy and in combination with 1.0% w/v CMC resulted in 2.0 and 1.8 log reduction in yeast and mold count after 20 and 35 days of ambient and refrigerated storage. CMC coatings alone and in combination with gamma irradiation were also tested for maintaining food storage quality and extending shelf life of pear by Hussain *et al.* (2010). Matured green pears were coated with CMC at levels 0.25% to 1.0% w/v and irradiated at 1.5 kGy. Irradiation alone at 1.5 kGy alone extended their shelf-life by 8 and 4 days while CMC coated with 1.0% w/v EO was effective prolonging the shelf-life by 6 and 2 days. . Combining the two treatments led to an extension of 12 and 6 days in the shelf life of the pears.

Conclusion

Despite the numerous and ongoing research, adoption of EOs as grain protectant is not widespread due to biological, technical, legal and commercial barriers. Sustainable and commercial uses have some drawbacks such as their relatively high application concentrations, differences in sensitivity of various insect species, varying effectiveness among different quantity of grain and current prices of natural EOs on the market. However, the demand for organic food is rapidly growing because of a desire to improve nutrition and prevent environmental contamination. The global organic food market is projected at an annual growth rate of over 16%

from 2015 to 2020 and would reach $211.44 billion by 2020 (Han *et al.*, 2017). About 91% of consumers in advanced countries opt for pesticide or fertilizer-free crops, driving growers to integrate safe pest control strategies including organic insecticides, biological control, cultural practices and physical control (Sintim, 2012). Therefore, crop protection from natural sources is safer, and when used in combination with other pest control techniques guarantees maximum protection. Fumigation with EOs and application of gamma irradiation are two ecologically safe methods that have proved to be beneficial in controlling stored-product pests and extend shelf-life of food products.

Table I. The bioactivities of some EOs

Common name of EO	Latin name of plant sources	Major components	Bioactivities	References
Rosemary	*Rosmarinus officinalis*	α –pinene Bornyl acetate Camphor 1,8-cineol	Antibacterial, antifungal, insecticidal	Bas¸er and Buchbauer (2010); Djilani and Dicko (2012)
Oregano	*Origanum vulgare*	Carvacrol Thymol γ-Terpinene p- Cymene	Immune Stimulating, Antifungal, Antibacterial, insecticidal	Bas¸er *et al.* (2010), Djilani *et al.* (2012)
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	Anti bacterial, anti fungal, insecticidal, Expectorant, Anti-Inflammatory,	Bas¸er and Buchbauer (2010); Djilani and Dicko (2012);
Basil	*Ocimum Basilicum*	Estragole, Eugenol, linalool	Anti bacterial, anti fungal, insecticidal,	Kocić-Tanackov *et al.* (2012), (Suppakul *et al.*, 2003)
Clove (bud)	*Syzygium aromaticum*	Eugenol Eugenol acetate	Antifungal, insecticidal, antibacterial	Cardiet *et al.* (2011)
Thyme	*Thymus vulgaris*	Thymol Carvacrol γ-Terpinene p- Cymene	Antifungal, anti insecticidal, anti bacterial	Vitoratos *et al.* (2013), (Ahmed, 2010)
Peppermint	*Mentha piperita*	Menthol, menthone, Pulegone	Antifungal, anti insecticidal, anti bacterial	Gibriel et al. (2011), (Saroukolai *et al.*, 2010)

Table II. Tested fungal species, produced toxin and health impact.

Name of the fungal species	**Mycotoxin Produced by that fungi**	**Health impact in human and animal**
Aspergillus niger	Ochratoxin (OTA)	Toxic to kidneys and the immune system, carcinogen (Oliveira *et al.*, 2014)
Aspergillus flavus	Aflatoxin B_1, B_2, cyclopiazonic acid	Carcinogen, neurotoxin, immunosuppressant, liver diseases, aflatoxicosis (Rajarajan *et al.*, 2013).
Aspergillus parasiticus	Aflatoxin B_1, B_2, G_1, G_2	Carcinogen, neurotoxin, immunosuppressant, liver diseases, aflatoxicosis (Rajarajan *et al.*, 2013)
Penicillium chrysogenum	Citrinin, patulin	Potent nephrotoxin, Carcinogen

Table III. Lethal doses of ionizing radiation. The table is adopted from (Aquino, 2011)

Organism	Dose (kGy)
Insect	22-93
Virus	10-40
Yeast	4-11
Mould	1.3-11

Table IV. Foods Permitted to be Irradiated under FDA's Regulation (21 CFR 179.26).

Data were adopted from (Maherani *et al.*, 2016).

Food	**Purpose**	**Dose**
Fresh, non-heated processed pork	Control of *Trichinellaspiralis*	0.3 kGy min. to 1 kGy max.
Fresh foods	Growth and maturation inhibition	1 kGy max.
Foods	Arthropod disinfection	1 kGy max.
Dry or dehydrated Enzyme preparations	Microbial disinfection	10 kGy max.
Dry or dehydrated spices/seasonings	Microbial disinfection	30 kGy max.
Fresh or frozen, uncooked poultry products	Pathogen control	3 kGy max.
Frozen packaged meats (solely NASA)	Sterilization	44 kGy min.
Refrigerated, uncooked meat products	Pathogen control	4.5 kGy max.
Frozen uncooked meat products	Pathogen control	7 kGy max.
Freshshelleggs	Control of *Salmonella*	3.0 kGy max.
Seeds for sprouting	Control of microbial lpathogens	8.0 kGy max.
Fresh or frozen molluscan shellfish[1]	Control of *Vibrio* species and other foodborne pathogens	5.5 kGy max.
[1] Data provided by FDA (Food *et al.*, 2005)		

Cellulose nanocrstals (CNCs) in biopolymeric films

Cellulose nanostructures includes cellulose nanocrystals (CNCs), cellulose nanofibrils (CNFs) and bacterial cellulose (BC), have found important applications in the food sector especially as food additives and packaging films. They have been most usually applied as reinforcing phases, but may also be used as matrices for a variety of materials including films for food packaging applications with antimicrobial properties (Abdollahi *et al*., 2013; Hubbe *et al*., 2008). CNC is an interesting class of nanomaterials that can be used in antimicrobial active packaging due to their bioavailability, bio-renewability, biocompatibility and biodegradability. CNCs are obtained from fine pulp cellulose fibers through acid hydrolysis disrupting the hydrogen bonds and cleaving the amorphous domains of the fiber to yield well-defined crystalline rods. The use of different acids results in the formation of different specific groups on the nanostructure which in turn influences their colloidal stability. Hydrochloric acid produces CNCs with poor colloidal stability while sulfuric acid produced CNCs possess negatively charged sulfate ester groups on their surface that induces electrostatic repulsion to yield a highly stable colloidal dispersion. The size of the CNC depends on the material used to generate it. CNCs derived from hard wood typically have a lateral and length dimension of 3–5 nm and 100–300 nm, respectively while CNCs extracted from tunicate have a lateral and length dimension of 15–30 nm and 1000–1500 nm, respectively (Elazzouzi-Hafraoui *et al*., 2007; Peng *et al*., 2011).

The use of biopolymers in food packaging has certain limitations like poor mechanical, thermal, and barrier properties. However, incorporation of nanocomposites can improve their structural and chemical properties and render them suitable and efficient in food packaging applications (Hubbe *et al*., 2017). Cellulose nanocrystals (CNCs) can act as reinforcement agents in biodegradable polymers by interacting with the matrix to favor mechanical and barrier properties. Water vapor permeability (WVP) is one of the most important parameters to consider while selecting films for developing food packaging applications. Studies have shown that water has a noticeable impact in deteriorative reactions and microbial growth in packaged food products (Salmieri *et al*, 2014) . Although CNCs are hydrophilic due to the presence of OH groups, CNC fillings have been shown to enhance the moisture barrier properties and decrease the WVP of biopolymers (Abdollahi *et al*., 2013). Several studies have investigated the effect of adding CNCs to a matrix on the water vapor barrier properties of polymers. Dispersion of CNCs in polymer

matrices has been known to drastically reduce the permeability of gases and water vapor making CNC a promising material for packaging applications. The diffusion pathway followed by the penetrant is greater in case of intercalated polymer/CNC nanocomposites than that in aggregated composites. Hence, nanocomposites with well-dispersed CNCs are expected to possess better barrier properties towards gases and vapors such as oxygen and water vapour. However excessive CNC (up to 10%) in naocomposites increases the WVP as CNC has high surface energy and can be easily agglomerated and can prevent preferential paths for water vapor diffusion and reduce barrier performance (Huq *et al*., 2012; Tang *et al*., 2017). Studies have shown that CNC plays an important role in enhancing the bioactivity of active component by improving dispersion into packaging surface (Dufresne 2017). Generally, nanocellulose-based antimicrobial materials are obtained by conjugation of antimicrobial agents and nanocellulose using physical and chemical approaches; as such the nanocrystalline cellulose itself has no antibacterial or antimicrobial activity (Jorfi and Foster 2015). The use of novel nanomaterial-embedded active packaging has a huge potential that open excellent prospects for the increase of their use in the design of novel high-performance packaging materials in the food and pharmaceutical industry. Therefore, the use of nanocellulose should be evaluated, monitored and controlled in order to confirm their safe applications. When a material is intended for food contact, there is the possibility of migration of components to the food. Several research works have been carried out in order to examine the biocompatibility and toxicity of cellulose nanocrystals and their nanocomposites in terms of any potential toxic effects on human health and environment. The structure–activity relationship of nanocelluloses indicate that they do not pose significant risk to human health and environment (Endes *et al*., 2016). Therefore innovations in nanotechnology related to CNC have many potential applications in many areas such as high-performance biodegradable material science, electronics, biomedical engineering, drug delivery etc.

Problematics, Hypothesis, Objectives

1. Problem Statement

Strategic measures involving development of high-yield crops, use of irrigation and fertilizers, and application of pesticides have marked the “Green Revolution” to help increase crop production to meet the world food demand (Bhonsle, 2010, Larsen, 2013). According to Perveen

(2012), arthropods pests can destroy up to 20-30% of the world's food supply every year. Therefore, considerable efforts have been harnessed to improve pest management strategies in order to ensure food security and access of people to food (Bailey *et al.*, 2010). Pesticide residues in food have been shown to have a cumulative toxic effect in humans both in the short and long-term. Growing concern about cancers, neuro-developmental disorders, higher risks of birth defects and a variety of endocrine system malfunctions have been associated with the presence of pesticide residues in food products (Gold *et al.*, 2001).

Although plant-derived essential oils (EOs) have been shown to exhibit beneficial properties and often characterized as "Green" and environmentally safe as compared to chemical pesticides, their application to protect stored food products is still limited. This is mainly due to the fact that EOs are highly volatile, unstable and susceptible to oxidation (Turek *et al.*, 2013). Other obstacles hindering the commercialization of these biopesticides include their requirement in sufficient quantity to induce the desired effect, poor water solubility, and issues related to their standardization, refinement, quality control and difficulty in securing registration (Perveen, 2012). In order to overcome these constraints and pave the way for the successful application of EOs in the large-scale preservation of stored food products, strategies should be formulated to counteract their undesired properties and render their commercial application feasible.

The immobilization of the EOs in polymer matrices may protect the EO molecules and permit a control release of the active components (Rattan, 2010). In fact, one of the recommendations proposed by Perveen (2012) is to enhance and stabilize the activity of plant extracts by means of encapsulation techniques. Polymeric nano particles such as nanocellulose, nano-starch, nano-chitosan are used for encapsulation technology as a reinforcing agent and the incorporation of nanocellulose an effect on controlled release. Based on the above, the intent of our study is to develop new films based on polymeric materials and encapsulated with nano emulsion of EOs exhibiting antifungal and insecticidal activities for eventual application to protect cereal grains during storage.

Another possible means may be to exploit the application of EOs in combination with other treatments such as radiation. If EOs have been shown to exhibit noteworthy antimicrobial and insecticidal activities (Abbasipour *et al.*, 2011), irradiation has also been shown to display potent capacity for quarantine control of stored products pests (Follett, 2009, Tuncbilek *et al.*, 2003). In

this study we propose to explore the possible application of a combined treatment of EOs, bioactive films and gamma radiation to assess their potentiality in inducing insect and fungal demise in synergy (Caillet *et al.*, 2005, Follett *et al.*, 2013a).

2. Hypothesis

i. Plant derived essential oils (basil, Cinnamon, mint, thyme) are effective against fungal and insect activities.

ii. Optimization of production of nanoemulsion of EOs (emulsifier, EOs, and CNC concentration, microfluidizer cylcle number) can increase the stability and the bioactivities of EOs.

iii. Incorporation of developing nanoemulsion of EOs into a polymeric system can further improve the controlled release of bioactive components and protect its activities during storage.

iv. The incorporation of cellulose nano crystal (CNC) will improve the physico-chemical, barrier and release properties of nanocomposite films

v. Combining the nanocomposite films with other treatments such as irradiation can give a synergistic effect and can increase the radiosensitization of insect and fungal species.

3. Objectives

i. Evaluate the possible radiosensitization of *Aspergillus niger* and *Penicillium chrysogenum* using basil essential oil and ionizing radiation for food decontamination

ii. Evaluate the possible radiosensitization of grain insect pest *Sitophilus oryzae* in combination with basil essential oil and ionizing radiation

iii. Identify effective plant-derived essential oils (EOs) against 4 mold species

iv. Identify effective plant-derived essential oils (EOs) against insect *Sitophilus oryzae*

v. Develop bio-polymeric diffusion devices for encapsulation of EOs based on biopolymeric based composite films; Evaluation of *in vitro* antifungal and insecticidal properties

vi. Evaluate *in situ* tests with nanocomposite films in combination with gamma radiation on fungal species during 8 weeks of storage period

vii. Evaluate the release/diffusion of volatile components encapsulated in biopolymeric films incubated on rice grain at 28˚ C during storage (*in situ*)

viii. Evaluate the combined effect of gamma radiation and the active films against insects with the most efficient film selected

ix. Develop trilayer/ bilayer films based on PCL (poly caprolactone) and methyl cellulose based films and evaluate their efficiency against fungi and insect species.

Simplified organogram of PhD thesis

Chapter 1

Literature review

Chapter 2: Objective 1.

Publication 1. Evaluate the possible radiosensitization of *Aspergillus niger* and *Penicillium chrysogenum* using basil essential oil and ionizing radiation for food decontamination

Chapter 3: Objective 2.

Publication 2. Evaluate the possible radiosensitization of grain insect pest *Sitophilus oryzae* in combination with basil essential oil and ionizing radiation

Chapter 4: Objective 3.

Publication 3. Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food

Chapter 5: Objective 4, 5 & 8.

Publication 4. Synergistic effects of nanocompositte films containing EOs nanoemulsions in combination with ionizing radiation against *Sitophilus oryzae*

Chapter 6: Objective 5, 6 & 7.

Publication 5. Antifungal activity of combined treatments of active methylcellulosic based films containing encapsulated nanoemulsion of EOs and γ – irradiation: *in vitro* and *in situ* evaluations

Chapter 7: Objectives 5, 6 &7.

Publication 6. Antifungal activities of combined treatments of irradiation and EO encapsulated chitosan nanocomposite films in *in vitro* and *in situ* conditions

Chapter 8

General discussion, conclusion and future perspectives

Annex A: Objective 9

Preparation of trilayer/ bilayer films and evaluation of antifungal and insecticidal properties

Chapter 2

Publication 1

Radiosensitization of *Aspergillus niger* and *Penicillium chrysogenum* using basil essential oil and ionizing radiation for food decontamination

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Key words: *Aspergillus niger*; *Penicillium chrysogenum*; basil EO; ionizing radiation; relative sensitivity.

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Contribution of the authors

Prof Lacroix is responsible of the project. She did the coordination of the research activities, she has done the corrections of the paper, participated on the scientific discussion and she has revised the article. I contributed and supervised the elaboration of the experimental design and protocols, Dr. Follett has participated to the discussion, corrections of the papers, revised the articles. Khanh Vu and Stephane Salmieri helped me with figure illustrations and statistical analysis. Senoussi Chaabane helped in conducting the experiments.

Résumé

La contamination des produits stockés par des moisissures engendre leur détérioration, la perte de leur qualité et de leur quantité. Le contrôle des moisissures peut être réalisé en utilisant des agents antifongiques naturels ou des traitements tels que l'irradiation. L'objectif de cette étude était d'évaluer les effets antifongiques de l'huile essentielle de basilic (HE) et l'irradiation appliquée seule ou en combianaison. La concentration minimale inhibitrice (CMI) de l'HE du basilic était de 0.1% (v/ v) contre *Aspergillus niger* et *Penicillium chrysogenum* après 48 h. La radiosensibilisation d'*A. niger* et de *P. chrysogenum* en présence de 1% ou 2% (v / v) d'HE de basilic a été évaluée *in vitro* et *in situ*. À 1 et 2% de l'HE de basilic, la valeur de D_{10} *in vitro* était respectivement de 0.43 et 0.31 kGy pour *A. niger* et de 0.44 et 0.34 kGy respectivement pour *P. chrysogenum*. Dans le riz inoculé, les valeurs de D_{10} pour les témoins (échantillon sans HE) étaient respectivement de 0.67 et 0.63 kGy pour *A. niger* et *P. chrysogenum*, et ces valeurs ont diminué avec l'application de concentrations plus élevées d'HE. Pour *A. niger*, HE du basilic à 2% (v / wt) seule a entraîné une réduction de 0.42 à 1.18 log aux jours 1 et 14 respectivement, alors qu'un traitement avec 2 kGy a entraîné une réduction de 2.18 log seulement. Les traitements combinés ont entraîné une réduction de 4.6 log d'*A. niger* après 14 jours de stockage. Dans le cas de *P. chrysogenum*, l'HE de basilic à 2% seule a entraîné une réduction de 0.76 et de 1.12 log aux jours 1 et 14 respectivement, alors qu'une dose de 2 kGy a entraîné une réduction de 2.41 log. Les traitements combinés ont entraîné une réduction de 5.0 log de *P. chrysogenum* après 14 jours de stockage. Les résultats ont démontré le potentiel de l'HE du basilic comme agent antifongique et son efficacité à augmenter la radiosensibilité d'*A. niger* et de *P. chrysogenum* au cours du traitement par irradiation.

Abstract

The stored products contaminated by fungi lead to their deterioration and loss of quality and quantity. Controlling fungi can be achieved using natural antifungal agents or irradiation. The objective of this study was to evaluate the antifungal effects of basil essential oil (EO) and irradiation alone or in combinations. Minimum Inhibitory Concentration (MIC) of basil EO was found to be 0.1% (v/v) against *Aspergillus niger* and *Penicillium chrysogenum* after 48 h. Radiosensitization of *A. niger* and *P. chrysogenum* in presence of 1% or 2% (v/v) basil EO was evaluated *in vitro* and *in situ*. At 1 and 2% of basil EO, the *in vitro* D_{10} value was 0.43 and 0.31 kGy respectively for *A. niger* and 0.44 and 0.34 kGy respectively for *P. chrysogenum*. In inoculated rice, D_{10} values for controls (sample without EO) were 0.67 and 0.63 kGy for *A. niger* and *P. chrysogenum* respectively, and the values were decreased at higher EO concentrations. For *A. niger*, a 2% (v/wt) basil EO alone caused a 0.42 to 1.18 log reduction on days 1 and 14 respectively, whereas treatment with 2 kGy radiation alone caused a 2.18 log reduction. The combined treatments resulted in a 4.6 log reduction of *A. niger* after 14 days of storage. For *P. chrysogenum*, 2% basil EO alone caused a 0.76 and a 1.12 log reduction on days 1 and 14 respectively, whereas a 2 kGy radiation dose caused a 2.41 log reduction. The combined treatments resulted in a 5.0 log reduction of *P. chrysogenum* after 14 days of storage. The findings demonstrated the potential of basil EO as a antifungal agent and its efficacy to increase the radiosensitivity of *A. niger* and *P. chrysogenum* during irradiation treatment.

2.1. Introduction

In spite of the huge technological progress achieved, food safety still remains a known problem worldwide (WHO, 2013). Pathogenic molds such as *Fusarium* spp., *Aspergillus* spp., and *Penicillium* spp. have been reported as causal agents of food spoilage and food-borne diseases (Betts *et al.*, 1999, Viuda-Martos *et al.*, 2007). These fungi can contaminate foods from cultivation to harvest, and during transportation and storage. Fumigation with plant essential oils (EO) and irradiation treatment are fungicidal options to control spoilage caused by disease microorganisms in food. In the case of grains, transportation may include long overseas journeys, during which the moisture content of the dried grains can increase to the levels suitable for the growth of xerophillic fungi. The metabolic moisture and heat resulting from respiration of fungi growing on grain creates an environment ideal for the growth of less xerotolerant fungi as well. This chain of reaction can result in massive colonisation by various types of fungi in bulk grain (Makunh *et al.*, 2007).

The effects of fungal invasion of grain include development of visible mold, discoloration, unpleasant odor, chemical and nutritional changes, and loss of quality and production of mycotoxins. Applying synthetic fungicides in the field can control many pathogenic and toxicogenic fungi (Chen *et al.*, 2008). However, due to adverse environmental effects and resistance development, alternative control methods are needed (Deising *et al.*, 2008, Kabera *et al.*, 2011). Plant EOs contains a wide array of aromatic compounds that give plants a distinctive odor, flavor or scent. These aromas are complex mixtures of a large number of constituents, but mainly consist of monoterpenes and sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides) in variable ratios (Boumail *et al.*, 2013 a, Ebadollahi *et al.*, 2011, Lee *et al.*, 2001a). Basil (*Ocimum basilicum L.*) EO belonging to the *Lamiaceae* family is known to exhibit antimicrobial and antifungal properties, but studies are limited (Lashowicz *et al.*, 1998).

Irradiation is another alternative to preserve food items and ensure they are free from pathogenic microorganisms. Irradiation has been considered as a safe and effective technology by the World Health Organization (WHO), Food and Agricultural Organization (FAO) and the International Atomic Energy Agency (IAEA) (IAEA, 2004). Several studies have reported that molds are sensitive to ionising radiation and their mycotoxin production decreases after irradiation

treatment (Rustom, 1997, Youssef *et al.*, 1999). The objectives of this study were 1) to determine the antifungal properties of basil EO against two molds, *Aspergillus niger* and *Penicillium chrysogenum* and 2) to evaluate the their radiosensitivity during combined treatment with basil EO and ionising radiation.

2.2. Materials and methods

2.2.1. Basil EO

Ocimum basilicum, (100% purity) was obtained from Robert & Fils, Ghislenghien, *Belgium*. It contained 77.6% estragole (methyl chavicol) and 20.30% linalool, two compounds known to have antifungal properties.

2.2.2. Fungal inocula and assay media

A. niger ATCC 1015 and *P. chrysogenum* ATCC 10106 were grown and maintained in potato dextrose broth (PDB, Difco, Becton Dickinson) containing glycerol (10% v/v). Prior to each experiment stock cultures were propagated through two consecutive 48h growth cycles in potato dextrose broth at 28˚C ± 2˚. The fungi were then pre-cultured in PDA for 3 days at 28˚C ± 2˚. Conidia were isolated from the agar media using sterile saline containing 0.05% Tween 80. Mycelia were removed by filtration through gauze, and the filtrate concentration was adjusted to 1 x 10^6 conidia/mL for *in vitro* experiments on potato dextrose broth (PDB) media and *in situ* experiments on packaged rice.

2.2.3. Preliminary study

The antifungal activity of basil EO was evaluated by the agar disc diffusion and volatilization methods (Benkeblia, 2004, Kordaly *et al.*, 2005, P. Lopez *et al.*, 2005a). Both methods showed notable antifungal efficiency of basil EO against *A. niger* and *P. chrysogenum.* The agar diffusion test using a quantity of 10 µl of 5% basil EO produced clear inhibition zones of 10 mm diameter against *A. niger* and 13 mm diameter against *P. chrysogenum*. In the volatilization assay a quantity of 30 µl of 5% basil EO was required to produce the same inhibition effect as the agar diffusion method against *A. niger and P. chrysogenum.* The diffusion assay was more sensitive than the vapor assay with the same concentration of basil EO (data not shown).

2.2.4. Determination of MIC

The agar dilution method described by Bansod *et al.* (2008) was adopted with some modifications. Dilution of basil EO ranging from 0.5% (v/v) to 0.005% (v/v) was prepared in PDA in presence of Tween 20 (0.05% v/v) to enhance oil solubility. A sample of 20 mL of the diluted EO was plated onto Petri dishes and allowed to solidify at room temperature. PDA media containing Tween 20 (without EO) was used as control. The prepared PDA plates were inoculated with 100 µl of *A. niger* and *P. chrysogenum* representing 1 x 10^6 conidia/mL. The fungal inocula were spread homogenously using glass beads and the plates were tightly sealed with sterile parafilm to prevent evaporation of EO. The plates were incubated at 28˚C ± 2˚C for 48-96h and colonies were checked twice a day. Minimum inhibitory concentrations (MICs) were determined as the lowest concentration of EO that inhibited the growth of each microorganism on the agar plate. The concentration at which no colony appeared was considered as the MIC of basil EO for the tested specimens.

2.2.5. *In vitro* radiosensitization of *A. niger* and *P. chrysogenum*

The viability of irradiated and non-irradiated spores were determined by a dilution plate method in three replicates (up to 10^{-6} dilution) after up to 72h incubation time in PDB media. A concentration of 0, 1% and 2% of basil EO was added to determine the radio sensitivity of *A. niger* and *P. chrysogenum* in presence of different concentrations of basil EO. The microbial colonies were counted following the plate count method described by (Dubey *et al.*, 2005). After 24 h, all samples were irradiated at the Canadian Irradiation Center in a underwater calibrator irradiator, UC-15A underwater calibrator (NORDION, Kanata, Canada) equipped with a ^{60}Co source and having a dose rate of 19 kGy/h. Samples were irradiated at room temperature at eight doses ranging from 0-4 kGy. Samples without basil EO were considered as control.

2.2.6. Inoculation and oil treatment on packaged rice (*In situ* test)

A quantity of 30 g of white long grain rice (Nu pak, Shah Trading Company Limited, Scarborough, Ontario) was inoculated with 200 µl of (1 x 10^6 conidia/mL) *A. niger* and *P. chrysogenum* in sterile plastic bags. A quantity of 1%, 2% or 4% (v/wt) basil EO was added to a sterile sponge cube of dimension 5x5x5 cm placed inside a cup. The sponge cubes were covered

with muslin screen to ensure that the rice grains were not in direct contact with the essential oil, in view of determining the vapor effect of basil EO. The oil infused sponges were placed in the sterile plastic bags containing rice inoculated with the fungal conidia to evaluate the vapor effect of basil EO during storage. The samples were grouped into two subsets with one undergoing irradiation and without irradiation. The samples were incubated at 30 °C for 14 days. The humidity inside the incubator was monitored and maintained constant at 65% throughout the experiment. The microbiological analyses of the stored rice grain were carried out after 1, 7 and 14 days of storage.

2.2.7. Microbiological analysis

Microbiological analyses of the samples were performed using Standard methods adopted from the International Commission of Microbiological Specification on Foods (ICMSF) (W. Braide *et al.*, 2011). A sample of 60 mL of sterile peptone water (0.1%, wt/vol) was added to 30 g of rice and homogenized for 1 min at 2000 rpm with a Lab-blender 400 stomacher (Laboratory Equipment, London, UK). All the samples were diluted decimally and adjusted to 10^4 of cells using a haemocytometer. An aliquot portion (0.1 mL) of each dilution was inoculated in triplicate onto the surface of solidified freshly prepared nutrient PDA. The plates were spread evenly with a sterile spreader and incubated for 3-5 days at 28±2 °C.

2.2.8. *In situ* radiosensitization of *A. niger* and *P. chrysogenum*

Basil EO treated and rice inoculated samples were irradiated at the Canadian Irradiation Center at room temperature using an underwater calibrator (NORDION Inc., Kanata, Canada) equipped with a ^{60}Co source at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 kGy as described previously. D_{10}-values were determined for *A. niger* and *P. chrysogenum* considering the kinetics of fungal destruction with and without basil EO and evaluated by linear regression considering the reciprocal of the slope of the line produced by plotting fungal counts (log CFU/g) against the irradiation doses. The microbial counts were normalized by applying a coefficient for both control and treatment. The D_{10} values for each fungus (*A. niger and P. chrysogenum*) were calculated in kGy based on Maity *et al.* (2011). After irradiation, samples were immediately incubated at 28±2°C. The relative sensitivity (RS) was calculated using the equation described by Caillet *et al.* (2005).

Relative radiation sensitivity (RS) = (radiation D_{10} of control samples) / (radiation D_{10} of samples treated with EO)

The D_{10} is defined as the radiation dose required reducing the fungal population by 1 log or decreasing it by 90%.

2.2.9 Statistical Analysis

A design composed of 3 × 3 × 3 × 4 randomized complete block design of 3 replicates (n = 3), 3 days of analysis (days 1, 7, 14), 3 irradiation doses (0, 1, 2 kGy) and 4 concentrations of basil EO (0, 1, 2, 4% v/wt) was used for this study. The statistical processing was performed based on the Generalized Linear Model (GLM) univariate analyses using fungal count as dependent variable, and the day, dose and concentration as the independent variables to evaluate the interaction effects between the variables i.e. day-radiation dose, day-EO concentration, radiation dose-EO concentration and day-radiation dose-EO concentration, for both *A. niger* and *P. chrysogenum* as test specimen. Comparison of means between treatments was done on the effect of dose and concentration on each day of analysis by Duncan's multiple range tests at 5% level, analysis of variance was performed using the PASW Statistics Base 18 software (SPSS Inc., Chicago, IL).

2.3. Results and Discussion

2.3.1. Determination of MIC

The MIC of basil EO against *A. niger* and *P. chrysogenum* in the current study was 0.1% (v/v) basil EO at 48 h incubation at 28˚C ± 2˚C, since no fungal colony growth was observed at this concentration. At lower concentrations (0.005% and 0.01%), fungal colonies started to appear after 8 h of incubation. A study conducted by Dube *et al.* (1989) found that basil EO exhibited a wide fungitoxic spectrum capable of suppressing the mycelial growth of 22 species of fungi by poisoned food technique using Czapek-Dox agar medium for 7 days at 26˚C ± 2˚C. They showed that a concentration of 0.1% (v/v) basil EO could completely inhibit mycelial growth of *A. niger* and *P. chrysogenum*. Zollo *et al.* (1998) reported the MIC for 10^4 CFU/mL of *A. flavus* as 0.5% (v/v) by broth dilution micro method at 25˚ C for 7 days of incubation period using Sarbouraud glucose broth. Basıˊlico *et al.* (1999) studied the inhibitory effects of several essential oils including basil EO, on the mycelial growth and ochratoxin A production by *A. ochraceus* growth

using yeast-extract-sucrose broth at 25˚ C and assessing fungal growth by drying and weighing mycelial mats. They reported that at a concentration of 1000 ppm basil EO was effective against 10^6 spores/mL of *A. ochraceus* up to 7 days. However, mold growth was detected after this incubation period. Although it may not be appropriate to compare MICs measured by different studies due to inherent variations in experimental parameters and conditions including sources of essential oils, fungal strains tested and assay protocols implemented (Jakowienko et al., 2011), our study advocates the potent mycotoxic properties of basil EO with an observed MIC as low as 0.1% (v/v) against both *A. niger* and *P. chrysogenum*.

2.3.2. *In vitro* fungal radiosensitivity in Potato Dextrose Broth (PDB)

Both *A. niger* and *P. chrysogenum* were found to be sensitive to irradiation and basil EO treatments. The D_{10} value for the controls (irradiation only) was 0.49 and 0.47 kGy for *A. niger* and *P. chrysogenum* respectively (Figures 2.1. & 2.2). The D_{10} value was 0.43 and 0.31 kGy for 1 and 2% of basil EO respectively for *A. niger,* and 0.44 and 0.34 kGy for 1 and 2% of basil EO respectively for *P. chrysogenum*. Our findings corroborate similar studies with related species of fungi. NH. Aziz *et al.* (2004) obtained a D_{10} value of 0.52 kGy for *A. flavus* isolated from spices. Blank *et al.* (1995) reported D_{10} values for five different *Aspergillus* species ranging from 0.21 – 0.32 kGy, mentioning a D_{10} for *A. niger* of 0.245 kGy. They also reported the D_{10} values for six different *Penicillium* species ranges from 0.24 – 0.33 kGy. Zeinab *et al.* (2001) found a D_{10} value of 0.50 and 0.40 kGy for *P. chrysogenum* and *P. crylophillum* respectively.

Our results showed that the D_{10} value decreased for both fungal species as the concentration of basil EO increased, resulting in an increase in RS. The RS increased from 1.14 to 1.55 for 1% and 2% basil EO for *A. niger*, and from 1.07 to 1.39 for 1% and 2% basil EO against *P. chrysogenum* as compared to the control. In the presence of 2% basil EO, no colony was observed after irradiation at 2 kGy for both fungi. These results demonstrated that basil EO can increase the RS of the tested fungal species. Although there are many studies on the relative sensitivity of pathogenic bacteria, similar studies with fungi are limited. M. Turgis *et al.* (2008) found that the addition of EOs or their constituents to ground beef before irradiation reduced the radiation dose required to eliminate *Salmonella* Typhi. Chiasson *et al.* (2004b) reported that the addition of thyme EO or its main constituents to ground beef before irradiation increased the RS of *E. coli* and *Salmonella* Typhi by up to 10 times. It is generally believed that the site of action

of EOs is principally the cell cytoplasmic membrane of microorganisms. Oussalah *et al.* (2006) showed that EOs derived from Spanish oregano (*Corydothymus capitatus*), Chinese cinnamon (*Cinnamomum cassia*), and savory (*Satureja montana*) affect the membrane integrity of bacteria and induce a depletion of intracellular ATP concentration. They also showed, through electronic microscopy, that the EOs induces significant damage to the bacterial cytoplasmic membrane. In addition, ionizing radiation initiates a series of event that impairs the cell structure which is further compounded by the addition of EO leading to the disintegration of the cytoplasmic membrane, making the cell impossible to repair the damage incurred by the complementary action of both treatments (Chiasson *et al.*, 2004a, P. N. Takala *et al.*, 2011, M. Turgis *et al.*, 2008). Hence the combined treatment showed greater efficiency against tested species than the individual treatment of basil EO or irradiation alone.

2.3.3. *In situ* relative sensitivity of *A. niger* and *P. chrysogenum*

The D_{10} values for controls (irradiation only) were 0.67 and 0.63 kGy for *A. niger* and *P. chrysogenum* respectively. The D_{10} value decreased significantly ($P \leq 0.05$) for both fungi with the addition of 4% basil EO. For *A. niger*, D_{10} values were 0.62, 0.52, and 0.49 kGy for 1, 2 and 4% of basil EO respectively, and the RS increased 1.07, 1.27 and 1.37 times respectively as compared to the control (Figure 2.3). For *P. chrysogenum*, D_{10} values were 0.46, 0.41 and 0.39 kGy for 1, 2 and 4% of basil EO respectively. Similar trend was observed with *P. chrysogenum* where the RS increased with increasing basil EO concentrations with respective values of 1.36, 1.50 and 1.60 as compared to the control (Figure 2.4)**.** The D_{10} values in inoculated rice grain were higher than the D_{10} values obtained in the *in vitro* in the PDB medium. Such difference in D_{10} values for the *in situ* and *in vitro* studies may be explained by the phase states of the basil EO. In the *in situ* experiment, basil EO diffused as vapor from the sponge to reduce fungal growth in the rice grains, whereas in the *in vitro* study basil EO was directly incorporated in the medium which provided greater surface area for the EO to induce antifungal activity. Such observation may be due to a concentration effect of the EO. In the *in vitro* experimental condition, the EO may not be subjected to major concentration variation while in the *in situ* condition the EO diffusion may result in the dilution of the EO molecules by air thereby reducing the toxicity of the active components. It has been reported that EOs are more active in direct contact methods as the active components can directly inhibit the metabolism of microbial cells

(Ikeura *et al.*, 2011). P. Lopez *et al.* (2005a) also showed that inhibition in solid diffusion tests was stronger than in vapor tests. This observation is also well explored in another study conducted by Dobre *et al.* (2011) who evaluated the *in vitro* antimicrobial activity of seven EOs against four different bacterial and five fungal strains that are involved in food poisoning using agar disc diffusion and disc volatilization methods. Using 10µl basil EO they could observe antimicrobial and antifungal activities against all the tested bacteria and fungi using the diffusion method; however, no such activity could be observed using the vapor method.

In the *in situ* study, the D_{10} values for the control were higher as compared to the *in vitro* D_{10} values against *A. niger* and *P. chrysogenum* respectively. *In situ* experiments with basil EO at a concentration of 4% had D_{10} values of 0.49 kGy against *A. niger* and 0.39 kGy against *P. chrysogenum* whereas the D_{10} values with 2% basil EO in the *in vitro* studies (0.31, 0.34 kGy respectively). This observation may cause from the fact that food components exhibit a protective nature and cause D_{10} values to be always higher as compared to culture media. According to Benkeblia (2004), a greater concentration of EO is required in case of food system to have the same efficiency as the *in vitro* assay. In addition, it can also be explained by the instability of some of the basil EO components such as limonene and α-pinene in the vapor phase as compared to their stable state conditions in the aqueous phase. In the vapor phase, it has been shown that these components cause rapid gas phase reactions with atmospheric oxidants to yield oxygenated products (Dorman *et al.*, 2000, Ikeura *et al.*, 2011).

2.3.4. Combined effect of γ-radiation and basil EO on inoculated rice grains during storage

The antifungal effects of basil EO vapor on the growth of *A. niger* and *P. chrysogenum* on rice grains up to 14 days of storage at 28˚ C ± 2° are presented in Table 2.1 & 2.2. The fungal density was highly significant ($p \leq 0.01$) for the effects of day, dose and concentration and for all interaction effects. Therefore, means separations were done on the effect of dose and concentration on each day the tests were evaluated (1, 7 and 14 days). All samples had an initial fungal load of 4 log CFU/g of rice grains at day 0.

For *A. niger* at day 1, no significant difference ($p > 0.05$) was observed between control and 1% of basil EO. Irradiating the samples with 2 kGy of radiation dose alone resulted in 2.51 log CFU/g of fungal growth representing a significant ($p \leq 0.05$) reduction of 2.18 log CFU/g as

compared to the control. On the other hand, applying 2% EO without irradiation nanoemulsion resulted in a fungal count of 4.27 log CFU/g thereby showing a decrease of 0.42 log CFU/g. At day 7, the presence of 2% EO without any radiation led to the formation of 4.10 log CFU/g of fungus which corresponded to a 0.59 log CFU/g reduction as compared to the control of day 1. This also represents a significant ($p \leq 0.05$) reduction of 1.64 log CFU/g as compared to 5.74 log CFU/g obtained in the absence of EO and radiation altogether at day 7. At day 14, the addition of 2% without any radiation caused a significant ($p \leq 0.05$) reduction of 1.18 log CFU/g as compared to the control (4.69 log CFU/g), and a significant ($p \leq 0.05$) decrease of 2.01 log CFU/g as compared to 5.52 log CFU/g obtained in the control of day 14. Considering the whole duration of the study, the addition of 2% EO caused a reduction in fungal count by 0.4 log CFU/g on day 1 to 1.18 log CFU/g on day 14. The application of 2 kGy of radiation alone led to a reduction of 1 log CFU/g in fungal count from day 1 to day 14. Combining with 2% basil EO with 2 kGy irradiation dose caused a caused a significant ($p \leq 0.05$) reduction of 5 log CFU/g at day 14 as compared to the control. This study demonstrates the efficacy of basil EO in combination with irradiation in reducing fungal growth. Previous studies have investigated the individual application of either plant-derived EOs (Aldred *et al.*, 2008, Y.A.Y. Gibriel *et al.*, 2011, Sumalan *et al.*, 2013) or radiation separately (Y. Gibriel *et al.*, 2009, Menasherov *et al.*, 1992) against various fungal species. However, our study purports the enhanced inhibitory effect that can be achieved by combining EO and ionizing radiation treatment against fungal microorganisms. Accrued inhibitory effects were observed on all the samples tested against fungal growth from day 1 using the combined treatment. At day 7 a dose of 2 kGy of radiation without any basil EO led to a decrease in 0.93 log CFU/g as compared to the control of day 1. However, combining the 2% basil EO treatment with 2 kGy of radiation resulted in the complete absence of fungal growth at day 7 and 14. Higher inhibitory activity was observed with 4% basil oil.

For *P. chrysogenum*, similar trends were observed following treatment with EO or irradiation alone or in combination. At day 1, in the presence of 2% EO alone, a reduction of 0.76 log CFU/g of fungus was observed as compared to the control. On the other hand, irradiating the samples with 2 kGy dose individually led to a decrease of 2.41 log CFU/g in fungal colonies. Combining the 2% EO and a radiation level of 2 kGy resulted in a decrease of 4.09 log CFU/g. At day 7, treatment with 2% EO alone led to a reduction of 1.84 log CFU/g while an irradiation

treatment alone caused a decrease of 2.61 log CFU/g of fungus as compared to the day 1 control (5.09 log CFU/g). Applying a 2% EO with a radiation level of 2 kGy in combination completely inhibited the fungal growth. At day 14, a reduction of 1.32 log CFU/g was observed following a 2% EO treatment alone which is significant ($p \leq 0.05$) while a 2.84 log CFU/g reduction was obtained with 2 kGy radiation treatment only. Combining a 2% EO treatment with a 2 kGy radiation level caused a complete inhibition of fungal growth.

In the present study the complete inhibition of fungal growth was observed at 2.5 kGy radiation doses for *A. niger* and *P. chrysogenum*. Treating with essential oil led to a lower radiation dose for the complete inhibition of the fungal growth. Menasherov *et al.* (1992), reported that *A. flavus* and *A. ochraceus* isolated from different cereal stop germination at 2.5 kGy. Similarly, Y. Gibriel *et al.* (2009) found that γ-radiation at a dose of 3 kGy was quite efficient to stop the growth of *A. flavus* in stored corn and aflatoxin production. In another study, N. H. Aziz *et al.* (1997) reported that the dose required for complete inhibition of fungi in different food and feed products ranged from 4 to 6 kGy.

However, all the above studies were conducted using either EO or irradiation as the inhibitory source for fungal growth. Our study shows that supplementing irradiation treatment greatly enhances the efficiency of basil EO. The combined treatment of basil EO and ionizing radiation was more efficient against fungal growth than that individual treatment with either basil EO or gamma radiation alone. These results suggest that basil EO can be used to increase the relative sensitivity of *A. niger* and *P. chrysogenum* to irradiation treatment. It has also been well established that irradiation in combination with other treatments suppress the growth of surviving micro-organism in vegetables, meat products during storage (Caillet *et al.*, 2005, H. A. Mostafavi *et al.*, 2012a, Thayer *et al.*, 1999). The present study showed the antifungal efficacy of basil EO in combination with irradiation in rice grains and represents a potential approach that could be applied commercially to protect stored grain product.

2.4. Conclusion

This study showed the enhanced antifungal effects that can be achieved by combining a treatment of basil EO and gamma radiation against *A. niger* and *P. chrysogenum*. *In situ* studies carried out showed that the basil EO in conjunction with the ionizing radiation can be effectively used to

control fungal growth in rice grains. Moreover, our data support the fact that a combined treatment of γ-radiation and basil EO can significantly increase the relative sensitivity of fungal species and result in a more pronounced inhibition of fungal growth as compared to individual treatments. The combined ionizing radiation-basil EO treatment, thus, offers a promising approach to control food contamination by fungi in ambient storage conditions. Further research may be warranted to gain understanding on the mode of action of a combined EO-γ-radiation treatment in extending the shelf life of stored products.

Acknowledgement

This research was supported by the United States Department of Agriculture and the Natural Sciences and Engineering Research Council of Canada (NSERC). Nordion Inc. is also acknowledged for the irradiation procedures.

Table 2.1. Fungal count (CFU/g) on rice grain inoculated with *A. niger* (104 conidia/g) treated with basil EO (0, 1, 2, 4% v/wt) and 0, 1 and 2 kGy during storage at 1, 7 and 14 days

Day	**Irradiation (kGy)**	**Concentration of basil oil (% v/wt)**			
		0	1	2	4
1	0	4.69 ± 0.08^{cC}	4.64 ± 0.04^{cC}	4.27 ± 0.67^{cB}	3.91 ± 0.61^{cA}
	1	3.97 ± 0.12^{bD}	3.39 ± 0.08^{bC}	2.39 ± 0.13^{bB}	2.09 ± 0.18^{bA}
	2	2.51 ± 0.10^{aD}	1.50 ± 0.0^{aC}	0.75 ± 0.10^{aB}	ND^{aA}
7	0	5.74 ± 0.05^{cD}	5.53 ± 0.05^{cC}	4.10 ± 0.08^{cB}	3.89 ± 0.03^{cA}
	1	4.28 ± 0.06^{bC}	4.23 ± 0.07^{bC}	3.50 ± 0.29^{bB}	2.04 ± 0.2^{bA}
	2	3.76 ± 0.08^{aC}	1.57 ± 0.98^{aB}	ND^{aA}	ND^{aA}
14	0	5.52 ± 0.31^{cD}	4.55 ± 0.10^{cC}	3.51 ± 0.06^{cB}	2.93 ± 0.16^{cA}
	1	3.97 ± 0.10^{bC}	3.79 ± 0.1^{bC}	3.08 ± 0.05^{bB}	1.63 ± 0.36^{bA}
	2	3.58 ± 0.06^{aC}	3.30 ± 0.46^{aB}	ND^{aA}	ND^{aA}

Values are means ± standard deviations. Within each row, means with the same uppercase letter are not significantly different ($P > 0.05$). Within each column and each day of analysis, means with the same lowercase letter are not significantly different ($P > 0.05$).

ND corresponds to non-detectable.

Table 2.2. Fungal count (log CFU/g) on rice grain inoculated with *P. chrysogenum* (104 conidia/g) treated with basil EO (0, 1, 2, 4% v/wt) and 0, 1 and 2 kGy during storage at 1, 7 and 14 days

Day	**Irradiation (kGy)**	**Concentration of basil oil (% v/wt)**			
		0	1	2	4
1	0	5.09 ± 0.13^{cD}	4.40 ± 0.06^{cB}	4.33 ± 0.08^{cAB}	4.24 ± 0.04^{cA}
	1	4.00 ± 0.611^{bD}	3.75 ± 0.20^{bC}	3.04 ± 0.08^{bB}	2.57 ± 0.23^{bA}
	2	2.68 ± 0.45^{aB}	1.25 ± 0.36^{aA}	1.00 ± 0.01^{aA}	0.75 ± 0^{aA}
7	0	4.28 ± 0.07^{bA}	4.07 ± 0.08^{bC}	3.25 ± 0.08^{cB}	3.10 ± 0.07^{cA}
	1	3.88 ± 0.05^{bD}	3.67 ± 0.12^{bC}	2.67 ± 0.1^{bB}	2.46 ± 0.11^{bA}
	2	2.48 ± 0.54^{aC}	1.25 ± 0.36^{aB}	ND^{aB}	ND^{aA}
14	0	4.45 ± 0.51^{cD}	3.99 ± 0.06^{cC}	3.77 ± 0.24^{cB}	3.22 ± 0.04^{cA}
	1	3.94 ± 0.07^{bD}	3.08 ± 0.05^{bC}	2.94 ± 0.06^{bB}	2.26 ± 0.18^{bA}
	2	2.25 ± 0.20^{aB}	1.32 ± 0.39^{aB}	ND^{aA}	ND^{aA}

Values are means ± standard deviations. Within each row, means with the same uppercase letter are not significantly different ($P > 0.05$). Within each column and each day of analysis, means with the same uppercase letter are not significantly different ($P > 0.05$).

ND corresponds to non-detectable.

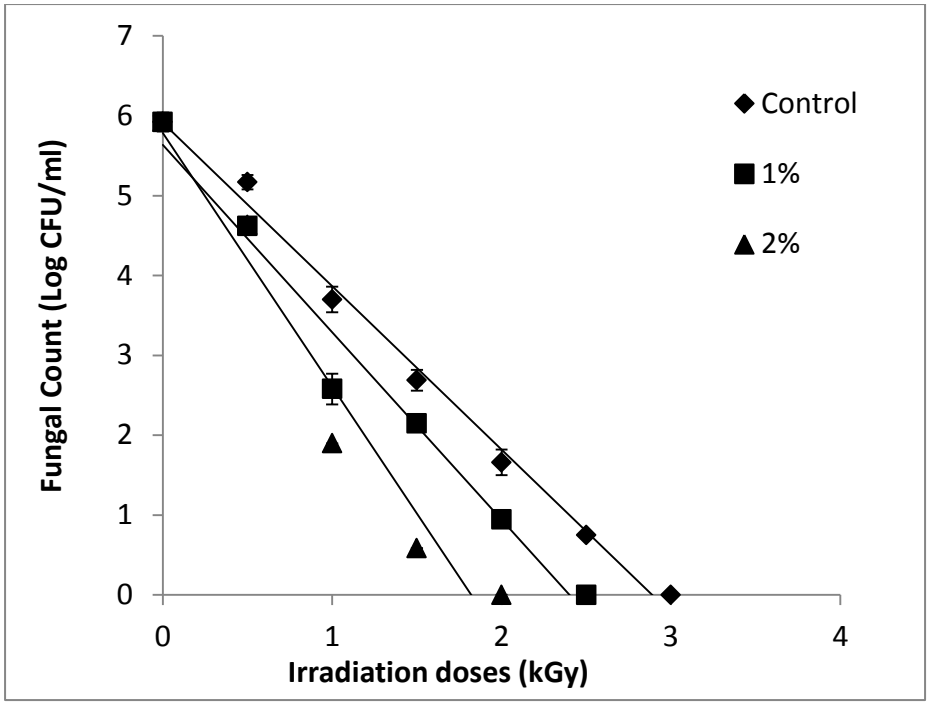

Figure 2.1. Relative sensitivity of *A. niger* in the liquid medium with different concentrations (0-2%) of basil EO.

Regression equations for plot are as follows: $y = -2.0457x + 5.91$ ($R^2 = 0.99$) for control, $y = -2.3459x + 5.6345$ ($R^2 = 0.97$) for 1% EO and $y = -3.164x + 6.4574$ ($R^2 = 0.9441$) for 2% EO. Relative sensitivity (RS) obtained for control experiment was 1, 1% EO was 1.14 and 2% EO was 1.55.

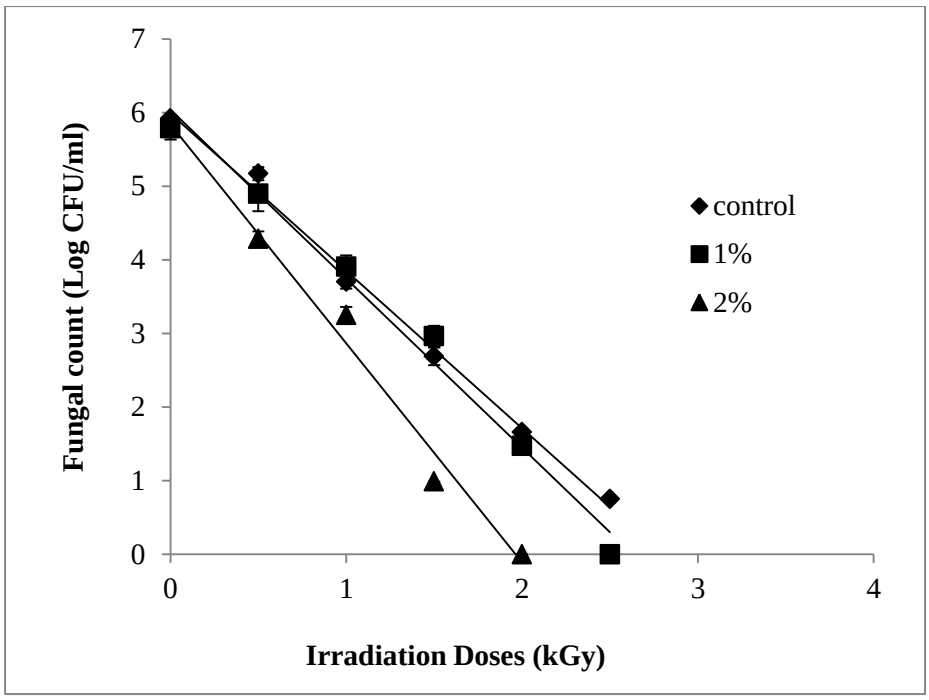

Figure 2.2. Relative sensitivity (RS) of *P. chrysogenum* in liquid medium at different basil EO concentrations (0-2%).

Equations for each regression line and RS are as follows: for Ctrl, y=-2.1366x+5.9857 (R^2 =0.99); for 1% EO, y=-2.2954x+6.0402 (R^2=0.98); for 2%, y=-3.0164x+6.1711 (R^2=0.98). Relative sensitivity (RS) obtained for control experiment was 1, 1% EO was 1.07 and 2% EO was 1.39.

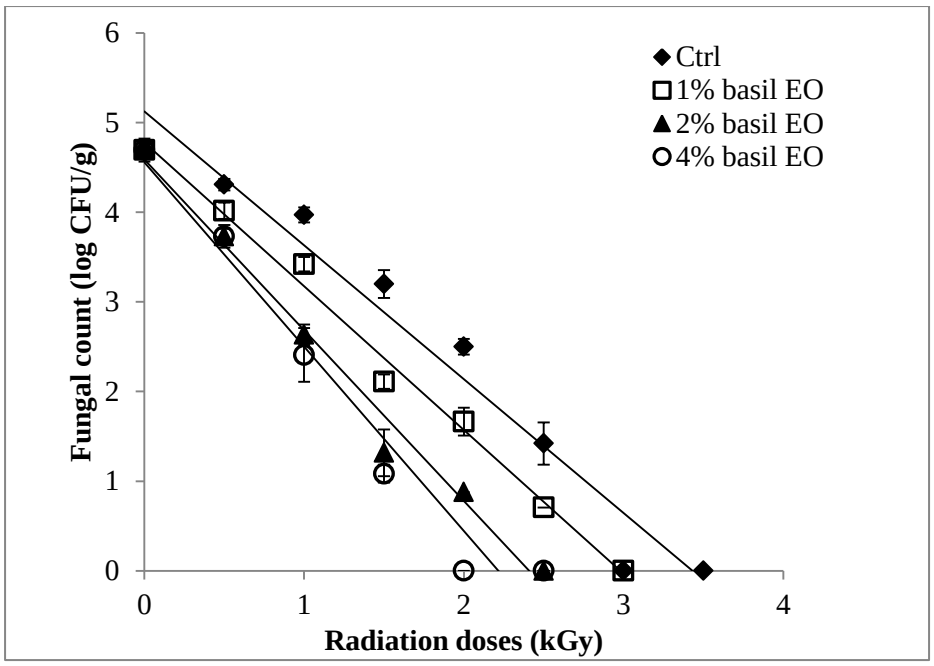

Figure 2.3. Relative sensitivity (RS) of *A. niger* at different basil EO concentrations (0-4% v/wt) on rice grain.

Equations for each regression line and RS are as follows: for Ctrl, y=-1.4943x+5.1268 (R^2 =0.98); for 1% EO, y=-1.6045x+4.7806 (R^2=0.99); for 2%, y=-1.9057x+4.5914 (R^2=0.98); for 4% EO, y=-2.0566x+4.45568 (R^2= 0.96).

Relative sensitivity (RS) obtained for control experiment was 1, 1% EO was 1.07, 2% EO was 1.27 and 4% EO was 1.37.

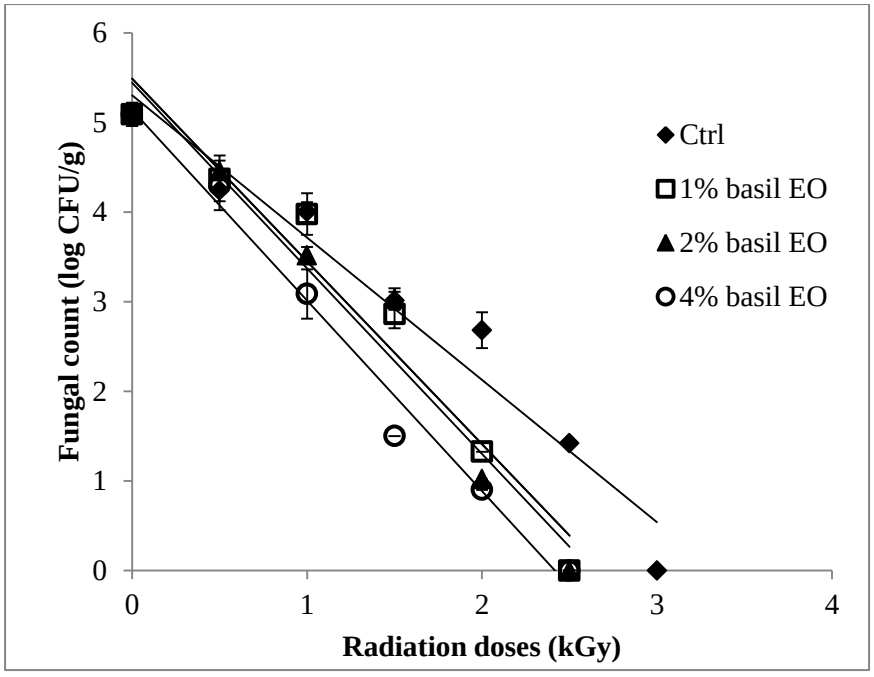

Figure 2.4. Relative sensitivity (RS) of *P. chrysogenum* at different basil EO concentrations (0-4% v/wt) on rice grain.

Equations for each regression line and RS are as follows: for Ctrl, y=-1.5879x+5.3018 (R^2 =0.96); for 1% EO, y=-2.0396x+5.4869 (R^2=0.96); for 2%, y=-2.071x+5.4404 (R^2=0.96); for 4% EO, y=-2.1272x+5.138 (R^2= 0.99).

Relative sensitivity (RS) obtained for control experiment was 1, 1% EO was 1.36, 2% EO was 1.50 and 4% EO was 1.60.

Chapter 3

Publication 2

Basil Essential Oil fumigant toxicity to the grain insect pest *Sitophilus oryzae* in combination with irradiation

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Contribution of the authors

Dr. Follett has trained us on conducting experiments with insect, he has supervised the protocols. Prof. Lacroix coordinated the research activities. I have performed the research and written the article. Both Dr. Lacroix and Dr. Follett revised the article, gave comments and corrected the paper. Khanh Vu and Stephane Salmieri helped by reviewing the data and writing interpretation of some data.

Résumé

L'activité biologique de l'huile essentielle (HE) de basilic, *Ocimum basilicum* L., avec et sans irradiation, a été testée contre le produit stocké du charançon du riz, Sitophilus oryzae (L.). Les charançons du riz adultes ont été exposés à l'HE du basilic à des concentrations de 20, 30, 40, 45, 50, 70 et 100 µL / mL et la mortalité a été évaluée après 72, 96 et 120 h d'incubation. Les charançons du riz ont également été exposés à 20 et 40 µL / mL d'HE du basilic et irradiés à 20, 40, 60, 80, 100 et 120 Gy et la mortalité a été évaluée après 72, 96 et 120 h d'incubation. L'efficacité de l'HE du basilic a augmenté avec l'augmentation du temps d'exposition et des concentrations. Les valeurs de la CL50 ont diminué avec l'augmentation du temps d'exposition à l'HE du basilic. A 72, 96 et 120 h, les valeurs de CL50 étaient respectivement de 78.0, 58.0 et 45.0 µL / mL. L'efficacité du traitement par irradiation a été améliorée par l'exposition à l'HE du basilic. Après 72 h, les charançons du riz exposés à 20 et 40 µL / mL d'HE du basilic étaient respectivement 1.4 et 2.5 fois plus sensibles à l'irradiation que les charançons témoins traités uniquement par irradiation. Après 120 h, les charançons du riz exposés à 20 et 40 µL / mL d'HE de basilic étaient respectivement 4,8 et 6,2 fois plus sensibles à l'irradiation que les charançons témoins traités seulement par irradiation. Une dose de 1.6 et 2.5 µL / g d'HE de basilic et une dose d'irradiation de 50 Gy s'est révélée avoir un effet synergique dans les sacs de riz en papier et en plastique. Dans les sacs de riz en papier, une mortalité de 100% a été observée avec 2.67 µL / g d'HE de basilic et 250 Gy de dose d'irradiation après 120 h de la période post-traitement. Dans les sacs en plastique, une mortalité de 100% a été obtenue avec 1.6 µL / g d'HE de basilic et 200 Gy de dose d'irradiation après la même période de post-traitement que les sacs en papier. Cela indique que les matériaux des sacs de riz affectaient la survie de S. oryzae. Cependant, le traitement combiné était significatif ($P \leq 0.05$) par rapport au traitement individuel dans tous les cas. Les résultats ont démontré que l'HE du basilic a un potentiel significatif de synergie pour abaisser la dose de rayonnement requise pour lutter contre les insectes responsable de l'infestation du riz et des céréales.

Abstract

The biological activity of basil essential oil (EO), *Ocimum basilicum L*., with and without irradiation was tested against the stored product pest rice weevil, *Sitophilus oryzae* (L.). Adult rice weevils were exposed to basil EO at concentrations of 20, 30, 40, 45, 50, 70 and 100 µl/mL and mortality was assessed after 72, 96 and 120 h incubation. Rice weevils were also exposed to 20 and 40 µL/mL basil EO and irradiated at 20, 40, 60, 80,100 and 120 Gy and mortality was assessed after 72, 96, and 120 h incubation period, and respective D_{10} values were calculated. The efficacy of basil EO increased with increasing exposure time and concentrations. The LC_{50} values decreased with the increased exposure time period to basil EO. At 72, 96 and 120 h the LC_{50} values were 78.0, 58.0 and 45.0 µL/mL respectively. The effectiveness of irradiation treatment was enhanced by exposure to basil EO. After 72 h, rice weevils exposed to 20 and 40 µL/mL of basil EO were 1.4 and 2.5 times more sensitive to irradiation respectively as compared to control weevils treated only with irradiation. After 120 h, rice weevils exposed to 20 and 40 µL/mL of basil EO were 4.8 and 6.2 times more sensitive to irradiation respectively compared to control weevils treated only by irradiation. A dose of 1.6 and 2.5 µl/g of basil EO at 50 Gy radiation dose was found to incur a synergistic effect in rice bags made of paper and plastic. In paper rice bags, 100% mortality was observed with 2.67µl/g basil EO and 250 Gy of irradiation dose after 120 h of post treatment period. In plastic bags 100% mortality was achieved with 1.6 µl/g basil EO and 200 Gy of irradiation dose after same post treatment period as the paper bags. This indicated that the materials of the rice bags affected the survivorship of *S. oryzae*. However, combined treatment was significant ($p \leq 0.05$) as compared to individual treatment in all cases. The results demonstrated that basil EO has significant potential as a synergistic agent to lower the required radiation dose to control quarantine pests.

3.1. Introduction

Although stored grains can be destroyed by a plethora of biotic elements, insect pests are considered most damaging worldwide and can inflict heavy losses of stored products (Chaubey, 2007, Ebadollahi *et al.*, 2011). *Coleoptera* (beetles) account for three-quarters of stored product pests, and the genera *Sitophilus* and *Tribolium* are the most damaging insect pests. *Sitophilus oryzae* (L.) (rice weevil) of the order *Coleoptera* and family *Curculionidae,* is one of the most ubiquitous and economically important pests of cereal grains, causing loss in grain appearance, weight and quality in harvested products. Postharvest control of such pests on stored products has been primarily through the use of chemicals, which unfortunately, have serious drawbacks such as environmental pollution, high mammalian and non-target organism toxicities, persisting pesticide residues in food items and pest resistance. This has led to the search for safe and inexpensive alternatives (Bello *et al.*, 2001, Ebadollahi *et al.*, 2011, Shaaya *et al.*, 1997, Shaaya *et al.*, 1991).

Plant essential oils (EO)and their components have potential as new fumigants (Chaubey, 2011). EOs are complex secondary metabolic products in plants that contain strong aromatic components, giving plants a distinctive odor, flavor, or scent. These oils are typically liquid at room temperature, lipophillic in nature and have densities lower than water. Most of them contain a number of bioactive compounds with potent antimicrobial and insecticidal activities (Adebayo *et al.*, 1999, Boumail *et al.*, 2013, Chaubey, 2007, Liu *et al.*, 2011). The biological activity of EOs depend on the chemical composition which varies with plant parts used, method of extraction, age, phenological stage of plant, harvesting season and environmental conditions (Angioni *et al.*, 2006, Chaubey, 2007). Irradiation is another feasible alternative for quarantine control of stored products pests including rice weevil and has been studied extensively (Follett, 2009, Tuncbilek *et al.*, 2003). Hoedaya *et al.* (1973) irradiated stored rice infested with rice weevils and found that 200 Gy can kill 99% of weevil after three weeks while Tilton *et al.* (1966) found that 175 Gy can kill 100% of rice weevils after three weeks. Tuncbliek (1995) irradiated adult rice weevils with 12 different doses ranging from 0 to 180 Gy and found that 130 Gy caused 90% mortality after 1 week and 90 Gy caused 100% mortality after 2 weeks. In another study, Ignatowicz (2004) irradiated immature rice weevils at different developmental stages such as eggs, larvae and pupae and observed no development to the adult stage at doses $\geq$ 80 Gy. Follett *et al.* (2013) showed that a radiation dose of 120 Gy prevented reproduction in rice weevil

and significantly reduced post-irradiation weight loss in the commodity due to weevil feeding. Immediate kill of rice weevil and other stored product insects may require much higher doses in the range of 1-3 kGy (Ignatowicz, 2004). However, Mostafavi *et al.* (2012b) reported that 0.15-1 kGy of radiation doses are recommended for the prevention of post-harvest losses by destruction of insects in stored cereals.

The objective of this study was to evaluate the effects of basil EO vapor on rice weevil survivorship, and examine the potential for combining basil EO and low-dose irradiation for control of the weevil in rice and other stored grains.

3.2. Materials and methods

3.2.1 Insects

A colony of rice weevils comprising of 200 adult individuals was obtained from the Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, Manitoba. The colony was fed and maintained on long grain white rice (Nu pak, Shah Trading Company Limited, Scarborough, Ontario) in 2 L plastic containers with ventilated covers at 24 ± 1ºC, 50-60% relative humidity, and 12:12 h light/dark cycle in an incubator. Rice samples were irradiated at 10 kGy for decontamination before use. Adult weevils were periodically transferred every month to new containers with fresh rice.

3.2.2. Basil EO

Ocimum basilicum, (100% purity, Pranarom International, Inc., Ghislenghien, Belgium) was used as bio-insecticidal agent. The basil EO batch contained 77.6% estragole, 20.30% linalool, 0.26% 1,8 cineole, and 0.02% methyl eugenol. Pure basil EO was diluted to different concentrations ranging from 10-100 µL/mL using acetone and stored at 4 ºC until used.

3.2.3. Effect of solvent

Tests were conducted to determine whether the acetone used for basil EO dilution would affect the insect. A 0.3 mL aliquot of acetone was applied to a small sponge (10x10x8 mm) and placed in a 10 mL cup wrapped with muslin cloth. The screened cup was placed in a Petri dish (95x15 mm) with 35 grains of rice and 35 adult weevils (4-7 days old), covered and sealed with

Parafilm. Samples without acetone were used as control. Mortality was determined every 24 h for 14 days. The tests were replicated three times under the same conditions. There was no observed effect of the solvent in the mortality of the *S. oryzae* and no death occured for more than three weeks of the experimental period.

3.2.4. Dose response tests with basil EO

The fumigation toxicity of basil EO was examined using dose response tests. Basil EO was diluted with acetone to concentrations of 20, 30, 40, 45, 50, 70 and 100 µL/mL. A 0.3 mL aliquot of basil EO solution was applied to a small sponge (10x10x8 mm) placed in a 10 mL cup wrapped with muslin cloth. The acetone was allowed to evaporate for 10 min (Cardiet *et al.*, 2011). The screened cup with basil EO was placed in a Petri dish (95 x 15 mm) with 35 grains of rice and 35 adult weevils (1-3 weeks old), covered and sealed with laboratory parafilm. Acetone only was used as a control. A 1:1 sex ratio was assumed (Howe, 1952). Mortality was scored daily for 120 h (5 days). Weevils showing no leg or antennal movement when prodded with a camel hair brush were scored as dead. The LD_{50} values were calculated using probit analysis after correcting the data for control mortality using Abbott's (1925) formula.

3.2.5. *In vitro* basil EO and irradiation combined treatment

Experiments were conducted to determine if basil EO could enhance the effectiveness of irradiation on rice weevil mortality. Basil EO was diluted in acetone to concentrations of 20 or 40 µL/mL. These concentrations were chosen based on the results obtained from the basil EO dose response study up to 120 h. The study showed that the LD_{50} values were 78.05 and 45.0 µL/mL respectively after 72 and 120 h of post treatment period. Based on these findings, 20 and 40 µL/mL of basil EO were chosen to allow some survivorship in order to demonstrate the combined effect of EO and irradiation on rice weevil. A 0.3 mL aliquot of basil EO solution was applied to a sponge (10x10x8 mm) and placed in a 10 mL screened cup. The acetone was allowed to evaporate for 10 min. The screened cup with basil EO was placed in a Petri dish with 10 g of rice and 35 adult weevils, covered and sealed with laboratory parafilm. Acetone only was used as a control. Half of the Petri dishes with weevils were treated with a radiation dose ranging from 20 to 120 Gy at the Canadian Irradiation Center (Laval, QC, Canada) using a cobalt-60 Gamma cell 220 irradiator (Nordion, Ottawa, Canada) with a dose rate of 0.36 Gy min^{-1}.

3.2.6 Combined basil EO and irradiation treatment in packaged rice

Different concentrations of basil EO and gamma radiation doses were applied in combination to packaged rice samples. To investigate whether the bag material has an effect on the experimental outcome, 10 bags made of paper and 10 plastic (polyethylene) bags were used. A quantity of 0.83, 1.6, 2.5, 2.67 and 3.33 µL/g aliquot of pure basil EO was applied to sponge cubes (10 x 10 x 8 mm) and placed in a 10 ml cup and sealed with muslin sheet. Four cups containing the sponge with the basil EO were placed in 300 g of rice containing 40 adult weevils and sealed. The insects were irradiated with 50, 100, 150, 200 and 250 Gy radiation doses after 24 h incubation, at the Canadian Irradiation Center (Laval, QC, Canada) using a cobalt-60 Gammacell 220 irradiator (Nordion, Ottawa, Canada) with a dose rate of 0.36 Gy min^{-1}. The experiment was conducted in six replicates for each sample. The experiment was repeated three times for reproducibility. The mortality was counted after 12 h of post treatment period.

3.2.7 Calculation of relative radiation sensitivity

Relative radiation sensitivity was calculated using the following equation: Relative radiation sensitivity (RS) = (radiation D_{10} of control samples) / (radiation D_{10} of samples treated with irradiation and basil EO) (Caillet *et al.*, 2005). The D_{10} is defined as the radiation dose required to achieve a 90% reduction of the insect population and is expressed in terms of Grays. The D_{10} value was determined by calculating the reciprocal of the slope of the line produced by plotting percentage of live insects versus the irradiation dose. Data for the percentage of live insects were normalized with the normalization coefficient for both control and treatments over different post-treatment period.

3.2.8 Synergistic effect assessment

The synergistic effect of the combined treatment with irradiation and basil EO was calculated using the formula from Ahmadi *et al.* (2013).

$$S = d / D + c / C$$

Where, S is the synergistic effect ($S > 1$ at antagonism, $S=1$ at additivity, $S < 1$ at synergism), d and c are the radiation doses and concentrations used in the combined mode, while D and C are the radiation doses and concentrations producing same activity when used separately.

3.2.9 Statistical analysis

Mortality data were adjusted using Abbott's formula (Abbott, 1925). For each experiment, adjusted percentage mortality was transformed to probits and subjected to regression analysis. Eight radiation doses were evaluated for each experiment. For the D_{10} value calculations, the percentage mortality of weevil by irradiation with or without basil EO, was evaluated by linear regression. For the dose response test with basil EO, treatments were replicated five times in a factorial design. For the basil EO and irradiation in combined treatments, each experiment was conducted in three replicates and for each replicate four samples were analyzed. Results were analyzed using the SPSS program (SPSS, Chicago, Ill.).

3.3. Results

3.3.1. Dose response tests with basil EO

The results of dose response of basil EO on rice weevils are shown in figure 3.1. Results showed that the mortality of *S. oryzae* increased as the post-treatment period and the concentration of basil EO increased. For samples containing 50 and 100 µL/mL of basil EO, the mortality increased from 9% to 65% and from 28% to 100% respectively between 48 and 120 h of post treatment period.

The results of linear regression and probit analyses showed that there was linear significant relationship between percentage mortality and duration of post-treatment periods to the basil EO vapors within all concentrations. After 72h of incubation, the LD_{50} calculated from probit analysis was 78.05 µL/mL whereas after 96h and 120h of incubation, the LD_{50} was 58.03 µL/mL and 45.0 µL/mL of basil EO respectively (Table 3.1). These values represent a reduction of 20.02 µL/mL of basil EO concentration between the 72 and 96 h incubation time period. At 120h, the reduction of LD_{50} calculated from the probit analysis was 33 and 13µL/mL of basil EO concentration as compared to the LD_{50} of 72 and 96h respectively. These decreased LD values indicate that lower concentrations of basil EO may also be efficient over a prolonged exposure period.

3.3.2. Basil EO and irradiation combined treatment on the sensitivity of rice weevil

The result of the combined treatment showed that the effect of *S. oryzae* mortality was dose and post-treatment time dependant. Higher mortality was observed for *S. oryzae* adults exposed to combined treatment than for adults exposed to gamma radiation and basil EO vapor alone, in all cases (Figure 3.2, 3.3 & 3.4).

The D_{10} value for *S. oryzae* treated with 20 and 40 µl/ml of basil EO is presented in table 3.2. The results showed that the D_{10} value for *S. oryzae* decreased significantly ($p \leq 0.05$) over exposure time which led to an increase in relative sensitivity. After 72 h of post-treatment period the D_{10} value reduced by 28% as compared to control in the presence of 2% of basil EO. This reduction in D_{10} values caused an increase of 1.4 fold in relative sensitivity as compared to the control. In the presence of 4% basil EO, the D_{10} value was reduced by 59%, which represents an increase of 2.5 fold of relative sensitivity as compared to the control samples.

At 96 h post-treatment period, the D_{10} value for samples treated with 20 and 40 µL/mL basil EO was reduced significantly ($p \leq 0.05$) by 57% and 75% respectively, as compared to the control. The decreased D_{10} values represent an increase in relative sensitivity by 2.5 and 4.2 fold respectively as compared to the control. Similar trend in D_{10} values was observed at 120 h post-treatment period. An increase of 4.8 and 6.2 fold in relative sensitivity was observed in the presence of 20 µL/mL and 40 µL/mL of basil EO respectively. The relative sensitivity also increased significantly ($p \leq 0.05$) between treatments with exposure time.

3.3.3. Basil EO and irradiation combined treatment in packaged rice

The mortality of *S. oryzae* increased significantly ($p \leq 0.05$) with an increase in basil EO concentration and radiation doses for both paper and plastic bags. Significant ($p \leq 0.05$) mortality was observed between treatments as compared to the control. Samples in plastic bags showed higher mortality as compared to the paper bags. In paper bags, 4% mortality was observed for control samples after 120h of post-treatment period increased significantly ($p \leq 0.05$) with the basil EO treatments. Increasing the irradiation dose from 50 to 250 Gy led to an increment in mortality of 28% for control samples in paper bags. Treatments with 0.27µL/g of basil EO alone caused 20% mortality, but combining with 250 Gy of radiation dose resulted in 100% mortality,

while a radiation dose of 250 Gy alone caused 38% mortality. In case of plastic bags, significant ($p \leq 0.05$) mortality was observed for all the samples. Even for the control (without basil EO and irradiation treatment) 18% mortality was observed, which is 14% higher ($p \leq 0.05$) as compared to the mortality observed for control samples in paper bags. Samples containing 1.6µl/g of basil EO at 200 Gy of radiation doses caused 100% mortality whereas samples treated individually with 1.6 µl/g basil EO and 200 Gy radiation dose caused 44 and 64% of mortality respectively.

For both paper and plastic bag, a synergistic effect was observed for a combination of 50 Gy gamma radiation dose and a basil EO concentration of 1.60 µL/g and 2.50 µL/g respectively resulting in a synergistic index of S<1 (table 3.4). At lower concentration (0.08 µL/g), an additivity effect was observed with the synergistic index equalling to 1 for both paper and plastic bags.

3.4. Discussion

Fumigation plays a very effective role in insect pest control and management in stored products. Plant EO and their components have favorable position over conventional fumigants in terms of low toxicity, fast degradation and local availability (Isman, 2000, Isman, 2006, Zettler *et al.*, 2000). In this study it was clearly found that basil EO fumigation was highly toxic to *S. oryzae* causing significant ($p \leq 0.05$) mortality over time. Basil EO used in the present study contains a total of 70 bioactive constituents such as methyl chavicol (estragole), methyl eugenol, linalool, 1,8-cineole, camphor, cinamaldehyde and methyl cinnamate in various ratios. These chemical components can be attributed for insecticidal, repellent, nematicidal, fungistatic or anitmicrobial properties. In agreement with the present study, a number of other studies have also showed the insecticidal activity of basil EO against stored products pests (Jembere *et al.*, 1995, Obeng-Ofori *et al.*, 1999, Popovic *et al.*, 2006).

The susceptibility of *S. oryzae* in this study was associated with the basil EO concentration and exposure time. Higher concentrations of basil EO were capable of killing more *S. oryzae* than lower concentrations at longer time periods (figure 3.1). Several studies also found that the insecticidal activity of volatile oils to be related to concentration of essential oils and exposure time against a range of stored product insects (Huang *et al.*, 2000, Lee *et al.*, 2003, M. Mondal *et al.*, 2010).

The LD_{50} was found to be 78.05 µL/mL of basil EO respectively after 72h of post-treatment. The value decreased to 58 and 45 µL/mL of basil EO respectively after 96 and 120h of post-treatment. Several studies also evidenced the toxicity of basil EO components against a variety of stored product pests. The basil EO used in the present study contained linalool, carvone, estragole and methyl eugenol as main components, and these ingredients have been identified as toxic fumigant against *S. oryzae*, *R. dominca* and *C. pusillus* by Lopez *et al.* (2008). Wang *et al.* (2006) observed a contact toxicity against *S. zeamais* with estragole, linalool and sabinene, and reported LD_{50} values of 17.63, 13.90 and 23.98 µg/adult respectively for these components. The LD_{50} values for linalool was found to be 14 µl/ml air against *S. oryzae* after 24 h exposure by Lee *et al.* (2001b). In another study, the LD_{50} value for linalool was found to be 39.2 µL/mL air against *S. oryzae* after 24h of exposure (Coats *et al.* (1991). The LD_{50} values obtained reported in these studies are lower than the LD_{50} values obtained in the present study. Such difference may arise from the use of different strain of *S. oryzae*, bioassay method and composition of tested oil (Abdelgaleil *et al.*, 2009b, Lee *et al.*, 2001b).

The LD_{50} were found to decrease as the weevils were exposed to the basil EO for a longer period of time (Table 3.1). Several studies are in agreement with this finding (Perry *et al.*, 1998, Robertson *et al.*, 1992, Zia *et al.*, 2013). Zia *et al.* (2013) studied the bio-efficiency of four different citrus peel extract essential oils against *Callosobruchus chinensis*, *Trogoderma granarium*, and *Tribolium castaneum*. They found that the essential oil exhibited toxicity depending on its concentration and exposure duration and resulted in LD_{50} values of 19.51, 15.66, 7.36 and 2.93% of *Citrus paradisi* essential oil after 24, 72, 120 and 168h respectively against *Callosobruchus chinensis* (pulse beetle) species. In addition, the LD_{90} values were 34.17, 29.49, 8.28, and 6.30% of *Citrus paradisi* essential oil after 24, 72, 120 and 168h respectively, showing a significant ($p \leq 0.05$) decrease of the LD values over exposure time. These observations are in accordance with the present study.

Irradiation treatment for disinfestations is currently a promising method since this technique seems to offer solutions that are desirable in many respects. It has been observed in different studies that irradiation technique can control pests like *Sitotroga cereailella*, *Callosobruchus maculates* and *Sitophilus oryzae* (Darfour *et al.*, 2012). The results obtained in this study showed that lower radiation doses at 20 and 40 Gy were not sufficient enough to induce any significant mortality in the control and treatments ($p > 0.05$). Several studies also showed that a high dose of

irradiation is required to produce the desired effect on insect mortality (Darfour *et al.*, 2012, Khaghani *et al.*, 2010, Tuncbilek *et al.*, 2003). Doses between 20 and 200 Gy killed *Tribolium castaneum* adults in 2 weeks, while doses less than 10 Gy caused insect demise in several weeks (Tuncbilek *et al.*, 2003). Khaghani *et al.* (2010) also stated that mortality could be taken into account with high dose of irradiation.The present study also showed that samples treated at 120 Gy without basil essential oil had 100% mortality after 11 days. Follett *et al.* (2013) suggested that a dose of 120 Gy can ensure the sterilization of adult weevils and prevent larval feeding and weight loss of rice kernels.

Assessment of the *in vitro* combined treatment showed that a significant ($p \leq 0.05$) effect of exposure to gamma radiation and basil EO in this study. The potential toxicity of the basil EO increased significantly ($p \leq 0.05$) when doses were in combination with irradiation treatment. In the presence of 20 µL/mL basil EO with 120 Gy of irradiation dose, the insect mortality increased by 20% between 72 and 120h of post-treatment as compared to samples treated only with 20 µL/mL of basil EO (without irradiation). Significant ($p \leq 0.05$) increment of mortality was also noticed with samples containing 40 µl/ml of basil EO and treated at 120 Gy. Mortality increased to 39 and 68% between 72 and 120 h post-treatment period as compared to samples treated only with 40 µl/ml of basil EO (no irradiation). As a result, the lethal dose to kill 90% of weevils (D_{10}) was found to decrease significantly ($p \leq 0.05$) over exposure period and concentration of basil EO. The D_{10} value for the control as was 713.6 Gy after 72h of post-treatment decreased significantly ($p \leq 0.05$) over exposure time and concentrations of basil EO. This reduction in D_{10} value led the relative radiation sensitivity of *S. oryzae* to be increased significantly ($p \leq 0.05$) during post treatment period. Our results provide the basis for successful application of gamma radiation in the presence of basil EO for controlling stored grain pests. Several studies also reported that combination of gamma radiation with other treatment like infrared, microwaves, and insecticide are economical and effective against insect pests (Ahmadi *et al.*, 2013, Ahmadi *et al.*, 2009, Sharma *et al.*, 2005). Ahmadi *et al.* (2013) showed that the mortality rate of the red flour beetle *Tribolium castaneum* treated with either gamma radiation or *R. officinalis* and *P. artiplicifolia* essential oils alone, was significantly ($p \leq 0.05$) lower than the mortality rate after the combined treatment. They concluded that the mortality was 3 to 6 times higher in combined treatment as compared to the effect of the each treatment alone.

Assessment of the interaction between the basil EO and gamma radiation in packaged rice showed that there is a synergistic effect (table 3.4) between 50 Gy of radiation dose and higher concentrations of EO (1.60 and 2.50 µl/g) respectively for both paper and plastic bags. At the same radiation dose but with lower EO concentration (0.8 µl/g) the interaction shifted to additivity with the synergistic index equalling to 1. Synergism results when the effect of the combined components is greater than the sum of the individual effects (Vasilakogloua *et al.*, 2013), and in this study such effect seems to occur at higher EO concentrations. At lower EO concentration, the blending of the EO and radiation components appears to result in an effect equalling the sum of the individual components leading to an additivity effect. Inducing radiosensitivity to the rice weevil seems to pave the way for the basil EO to induce higher mortality at high concentrations. Although the main components of the basil EO such as methyl chavicol and linaool, may play an important role in the insecticidal process, inducing radiosensitivity may enable other trace basil EO components to also participate in increasing the insect mortality rate. The inherent activity of the basil EO may not exclusively depend on the ratio of its various components but on the interactions among these different components as well as on the applied gamma radiation. Knowledge on the potential mechanisms which may govern the synergism between essential oils and radiation is practically inexistent. However, such mechanism may share commonalities with the theoretical mechanisms of antimicrobial interactions producing synergy, which according to Hyldgaard *et al.* (2012), may involve sequential inhibitions in biochemical pathways, inhibition of enzymes degrading antimicrobials and/or interaction with membranes leading to an increased uptake of antimicrobials. In accordance Ahmadi *et al.* (2009) found a synergistic effect of gamma radiation with essential oil on *T. castaneum* adults. The data obtained in their study showed that 100 Gy dose alone can caused 12.5% mortality but when irradiated insects were treated with 7.66 µl/ml air of *P. atriplicifolia* oil and incubated for 7 days after irradiation, the mortality increased to 32.5%. Ayvaz *et al.* (2002) studied the combined effect of gamma radiation and malathion pesticide on the flour beetle, *Tribolium confusum* and found that combined methods are more efficient to control *T. confusum* species.

In the current study, attempt was also made to assess insect mortality under control and treatment conditions with two types of packaging condition, paper and plastic (figure 3.5). Considering the percentage mortality between bag types showed a higher insect mortality with the plastic bag

than with the paper bag under all studied groups (control, individual EO and irradiation treatments and combined EO and radiation treatments). This may be attributed to the fact that the paper bags were not strictly a closed system enabling free movement of gases across the paper medium leading to a steady-state condition with the outside atmosphere. On the other hand, the higher insect mortality in the plastic bags as compared to the paper bags may be attributed to the plastic package material that may provide a more stringent barrier to gases either leaving or entering the package. A deficiency in atmospheric oxygen inside the plastic bags may thus have contributed to the demise of the insects in the plastic bags while such components may not have played a significant role in the paper bags. This shows that the choice of packaging material has an impact on the control of insects during storage of food products. In this study, the plastic bags seem to exert more control on the insect population than the paper bags indicative of the influential role that the bag material on the insects survivability.

Our results provide the basis for successful application of gamma radiation in the presence of basil EO for controlling stored grain pests. Although numerous studies focused on the use of plant essential oil to protect stored grains, there is a lack of sufficient data on the relative sensitivities of these essential oils against stored pests. The present data show that the basil EO vapor and irradiation can be used as an effective combined method to increase the relative sensitivity of *S. oryzae*. It also reveals that these two methods can be combined as an additional control approach to reduce the insect population on stored product. However, as the vapor pressure of essential oil is very low to enable them to penetrates into commodities to kill pests, the treatment with EO can be applied only on a small scale (Ahmadi *et al.*, 2013). In order to make a practical application these compounds can be formulated through nanoencapsulations. Nanoencapsulation is a process through which a chemical (insecticide) is slowly and efficiently released from a carrier which is generally a polymer to a particular host for insect pest control. Release mechanisms include diffusion, dissolution, biodegradation and osmotic pressure with specific pH (Owolade *et al.*, 2008, Vidhyalakshmi *et al.*, 2009). Controlled release of the active component from nanoencapsulated formulations allow the EO's to be used more effectively over a time period. Nanoencapsulated essential oils can be put into packages of products allowing the control release of the active component over a time period. Further studies are necessary for the development of control release formulations for the large scale application of plant derived EO to protect stored product pests.

3.5. Conclusion

Our study shows that basil EO can act as a potential candidate for the protection of stored rice grains. Combined with irradiation treatment, it can offer promising results and enable the reduction of applied concentration of basil essential oil and the dose of irradiation treatment. Further investigations may help to elucidate the mechanisms underlying the use of basil EO to protect grains susceptible to attacks by *S. oryzae*. However long-term effects of the combined treatment and its application at large scale, in households and for storage of industrial crop grains to reduce post-harvest losses may require further investigations.

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Table 3.1. Predicted LD_{50} and LD_{95} values after 72, 96 and 120 h of treatment from regression analysis.

Hour	**LD_{50}**	**Chi square**	**P value**
72	78.05 (65-104) µL/mL	17.05	0.004
96	58.03 (51-57) µL/mL	12.76	0.026
120	45.00 (43-46) µL/mL	3.92	0.479

Table 3.2. D_{10} (Gy) values for *S. oryzae* treated with 2 and 4% of basil EO and irradiated at 20-120 Gy of radiation doses.

		72 hour	**96 hour**	**120 hour**
$D_{10\ (Gray)}$	Control	713.6±5.2 ^{Bc}	597.3 ± 6.5 ^{Ac}	547.3 ± 8.7 ^{Ab}
	20 µl/l	510.6 ± 7.9 ^{Bb}	251.6 ± 5.8 ^{Ab}	113.6 ± 2.4 ^{Aa}
	40 µl/l	288.0 ± 4.2 ^{Ba}	147.6 ± 4.9 ^{Aa}	89.0 ± 6.0 ^{Aa}

Within each row, means with the same upper case letter are not significantly different ($p \geq 0.05$). Within each column, means with the same lowercase letter are not significantly different ($p \geq 0.05$).

Table 3.3. Relative radiosensitivity for *S. oryzae* treated with 2 and 4% of basil EO and irradiated at 20-120 Gy of radiation doses.

		72 hour	**96 hour**	**120 hour**
Relative radiosensitivity	Control	1.0 ± 0^{A}	1.0 ± 0^{A}	1.0 ± 0^{A}
	20 µl/l	1.4 ± 0.1^{Aa}	2.5 ± 0.6^{Aba}	4.8 ± 0.4^{Bb}
	40 µl/l	2.5 ± 0.1^{Aa}	4.2 ± 0.8^{Bab}	6.2 ± 0.8^{Bb}

Within each row, means with the same upper case letter are not significantly different ($P \geq 0.05$). Within each column, means with the same lowercase letter are not significantly different ($P \geq 0.05$).

Table 3.4. Combined effect of different concentrations of basil EO and irradiation (50 Gy) on mortality of adult *S. oryzae*.

Concentrations (µL/g)	**Paper bag**	**Plastic bag**
0.8	1.00*	1.00
1.6	0.95	0.88
2.5	0.97	0.93

* S > 1 at an antagonist effect, S = 1 at additivity, S < 1 at a synergistic effect.

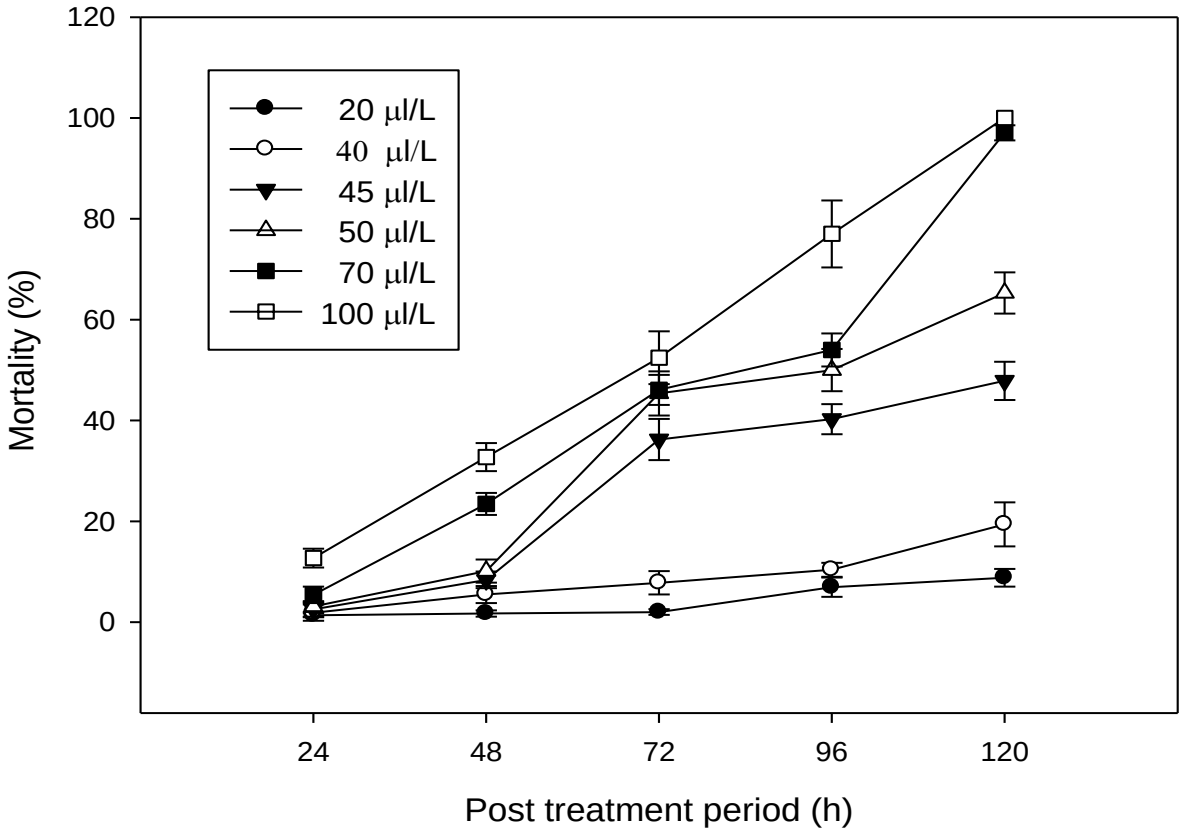


Figure 3.1. Effect of basil EO concentration and time post-treatment on percentage mortality of *S. oryzae* .

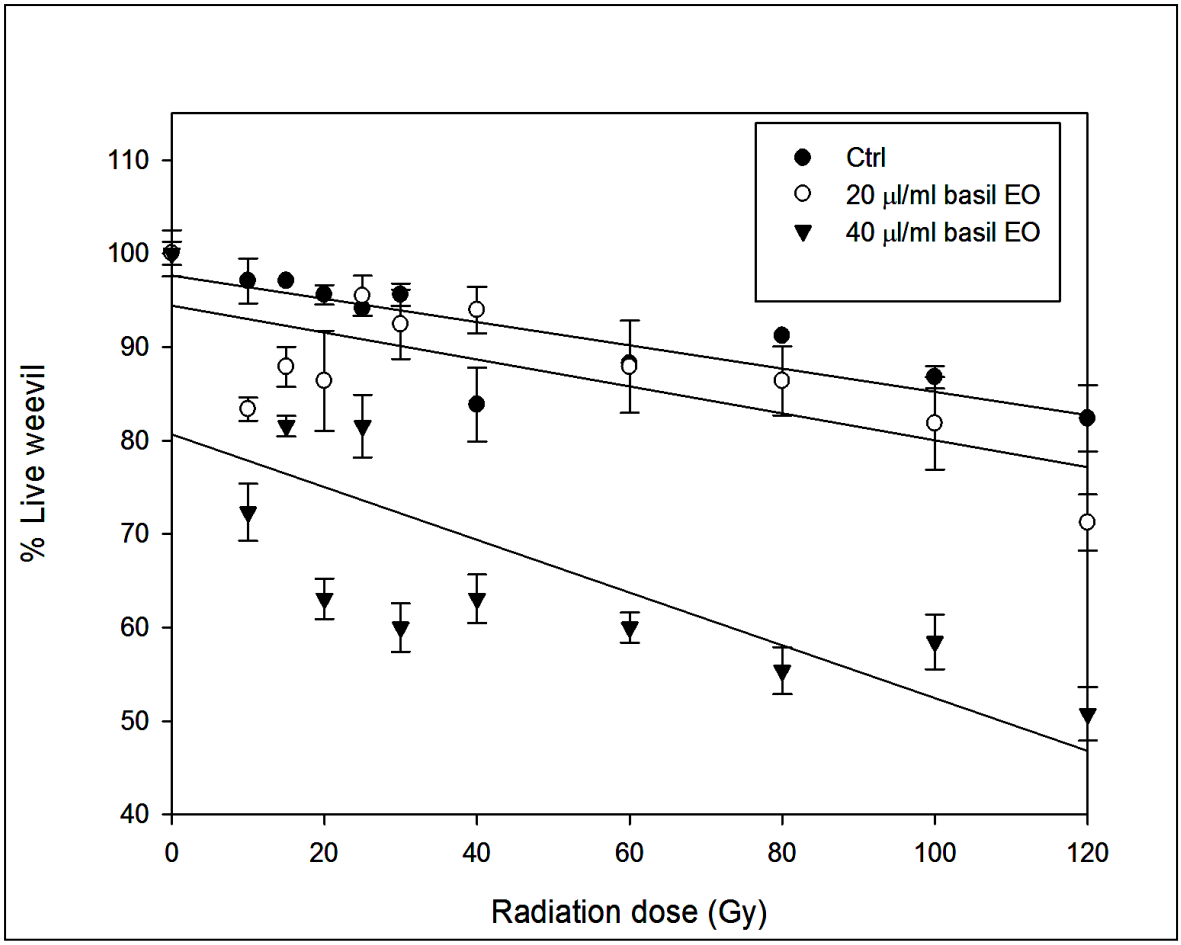


Figure 3.2. Radiosensitization of *S. oryzae* (Rice weevil) in presence of 20 µl/ml and 40 µl/ml of basil EO after 72h of the experiment. The relative sensitivity is indicated for each curve.

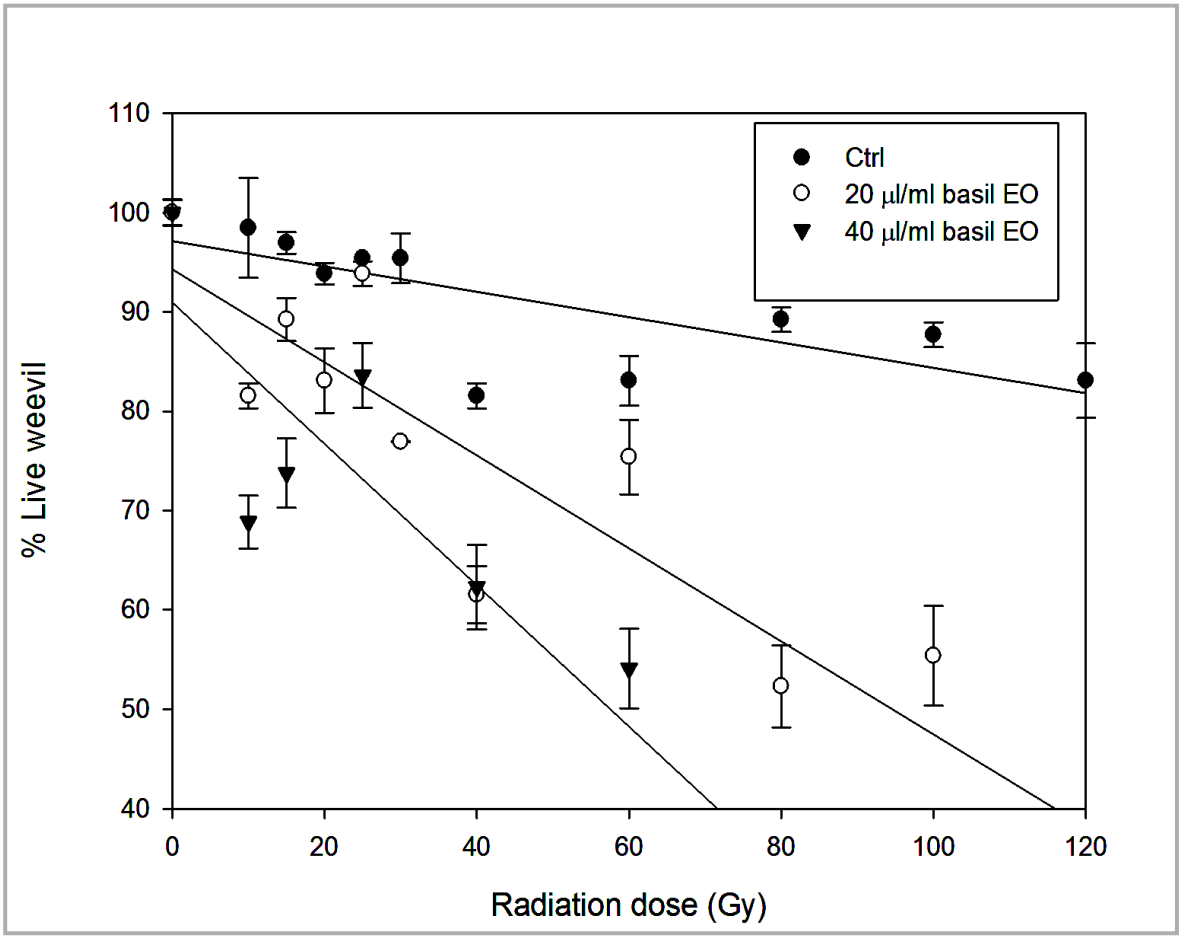


Figure 3.3. Radiosensitization of *S. oryzae* with exposure to 20 µL/mL and 40 µL/mL basil EO after 96h of the experiment. The relative sensitivity value is indicated for each curve.

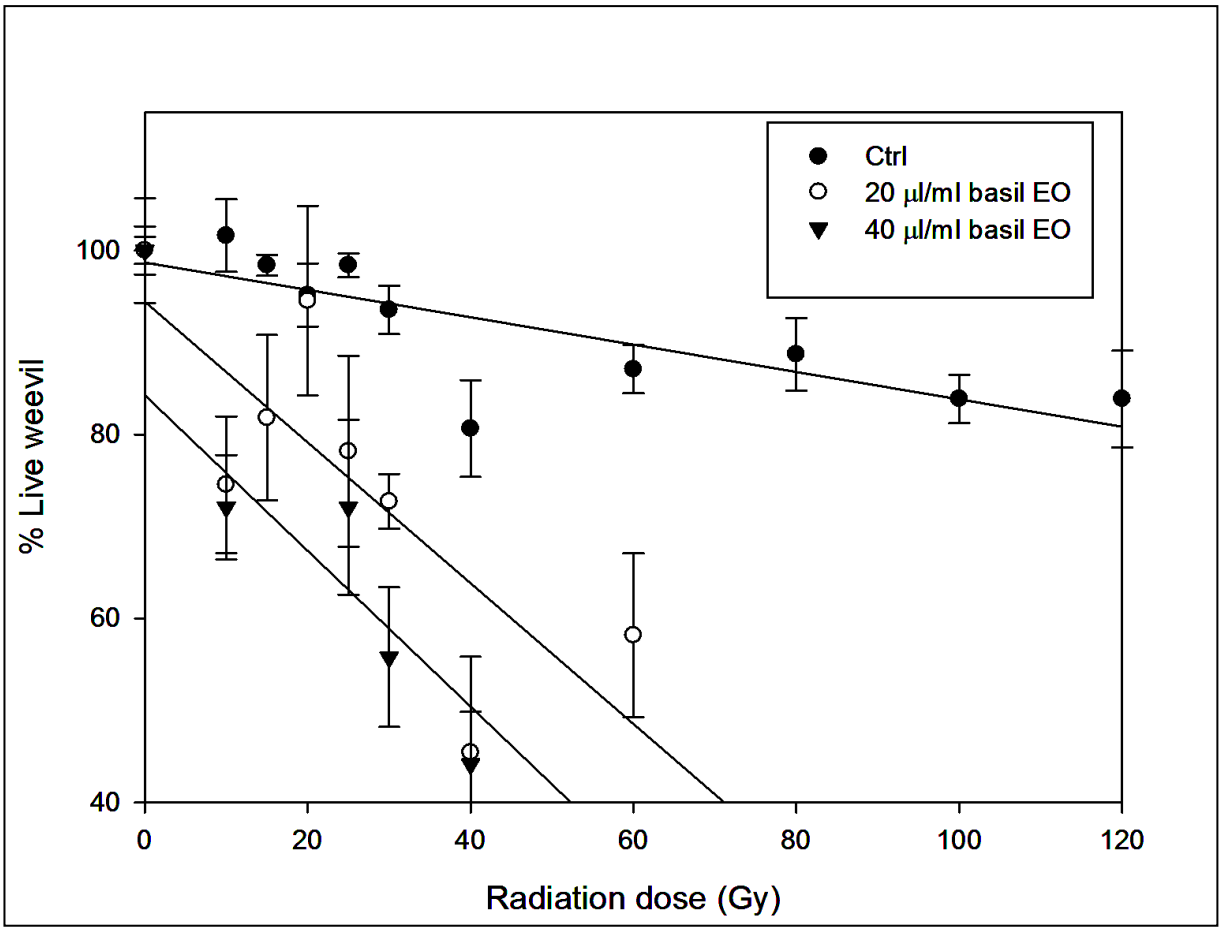
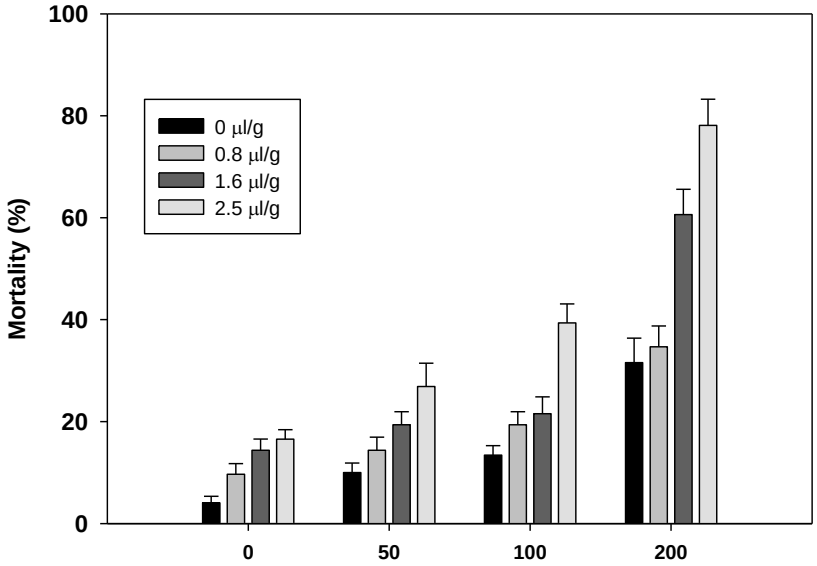


Figure 3.4. Radiosensitization of *Sitophilus oryzae* with exposure to 20 µL/mL and 40 µL/mL basil EO after 120h of the experiment. The relative sensitivity value is indicated for each curve



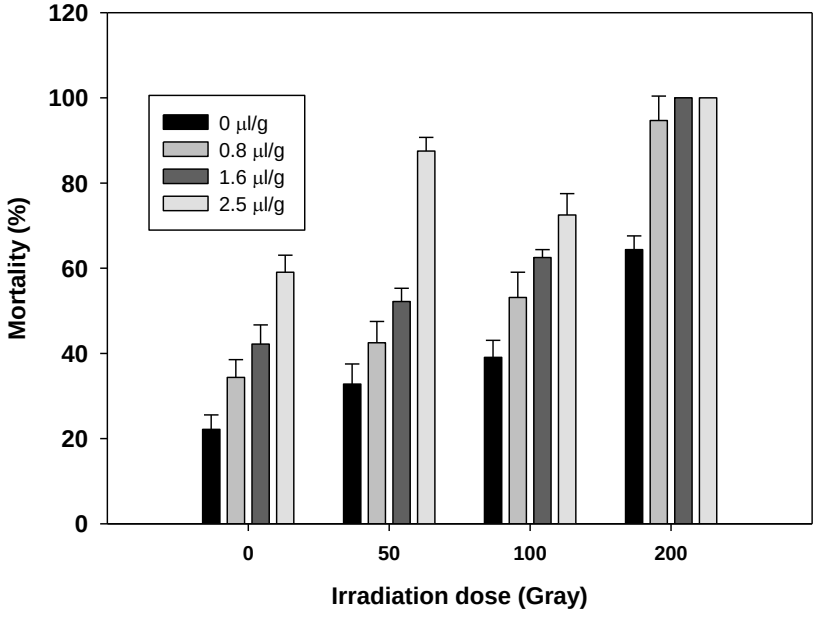


Figure 3.5. Effects of combined treatments with different concentrations of basil EO and gamma radiation on mortality of *S. oryzae* in (i) paper bag and (ii) plastic bag.

Chapter 4

Publication 3

Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food

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Contribution of the authors

Most of the experimental work was planned and performed by Farah Hossain with guidance from Prof. Monique Lacroix. The manuscript was written by Farah Hossain. Prof. Monique Lacroix and Dr. Peter Follett corrected the main draft. Stephane Salmieri helped to improve the discussion. Dr. Khan Vu helped in statistical analysis. Mehdi Harich helped by conducting experiments and preparing excel files.

Résumé

L'activité antifongique de huit huiles essentielles (HE), à savoir le basilic, la cannelle, l'eucalyptus, la mandarine, l'origan, la menthe poivrée, l'arbre à thé et le thym a été évaluée pour leur capacité à inhiber la croissance d'*Aspergillus niger*, *Aspergillus parasiticus* et de *Penicillium chrysogenum*. L'activité antifongique des HE a été évaluée en déterminant la concentration minimale inhibitrice (CMI) par la méthode de microdilution sur microplaques à 96 puits. Les interactions entre les différentes combinaisons d'HE ont également été évaluées. L'origan et le thym présentent une activité antifongique plus élevée avec des valeurs de CMI plus faibles parmi toutes les moisissures testées. Les autres HEs pourraient être classées selon l'importance de leur activité antifongique comme suit : cannelle, menthe poivrée, arbre à thé et basilic. L'eucalyptus et la mandarine ont montré une efficacité antifongique moindre car leur CMI dépasse 10 000 ppm. La combinaison de ces deux HE n'a également démontré aucune interaction sur les espèces testées. Une formulation combinée d'origan et de thym a entraîné un effet synergique, montrant une amélioration de l'efficacité antifongique contre *A. flavus*, *A. parasiticus* et *P. chrysogenum*. Le mélange de menthe poivrée et de l'arbre de thé a induit un effet synergique contre *A. niger*. L'application d'un modèle modifié de Gompertz tenant compte des paramètres de croissance fongique tels que le diamètre maximal des colonies, le taux de croissance maximal et les périodes de latence, montre que le modèle pourrait adéquatement décrire et prédire la croissance des moisissures testées dans ces conditions.

Abstract

The antifungal activities of eight essential oils (EOs) namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme were evaluated for their ability to inhibit growth of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium chrysogenum*. The antifungal activity of the EOs was assessed by the minimum inhibitory concentration (MIC) using 96-well microplate analysis. The interactions between different EO combinations were done by the checkerboard technique. The highest antifungal activity was exhibited by oregano and thyme which showed lower MIC values amongst all the tested fungi. The antifungal activity of the other EOs could be appropriately ranked in a descending sequence of cinnamon, peppermint, tea tree and basil. Eucalyptus and mandarin showed the least efficiency as they could not inhibit any of the fungal growth at 10,000 ppm. The interaction between these two EOs also showed no interaction on the tested species. A combined formulation of oregano and thyme resulted in a synergistic effect, showing enhanced efficiency against *A. flavus* and *A. parasiticus* and *P. chrysogenum*. Mixtures of peppermint and tea tree produced synergistic effect against *A. niger*. Application of a modified Gompertz model considering fungal growth parameters like maximum colony diameter, maximum growth rate and lag time periods, under the various EO treatment scenarios, showed that the model could adequately describe and predict the growth of the tested fungi under these conditions.

4.1. Introduction

Fungal infestation of food poses the greatest threat to food security and represents a serious concern during food storage (Zhaveh *et al.*, 2015). Genera such as *Aspergillus*, *Penicillium* and *Fusarium* are associated with crops like wheat, rye and corn grains under both field and storage conditions (Krisch *et al.*, 2011). Fungal growth has also been reported on raw and processed foods which result in several kinds of spoilage including textural and sensorial changes, off-flavor development and odour emission, rotting and formation of pathogenic and allergenic propagules (Dellavalle *et al.*, 2011). The deterioration of sensorial properties is often due to the production of exoenzymes during fungal growth. Once inside the food, these enzymes may continue their activities independent of the destruction or removal of the mycelium. In addition, the production of mycotoxins by fungi, in stored food commodities constitutes a serious health threat to humans and livestock. Five types of mycotoxins are deemed noxious world-wide for human health: aflatoxins, ochratoxin A, fumonisins, certain trichothecenes and zeazalenone (Pitt *et al.*, 2000). Long-term ingestion of these toxins as a result of eating contaminated foods has been associated with liver and kidney tumors in animals and humans. Some mycotoxins can cause auto-immune illnesses, while some are teratogenic, carcinogenic and mutagenic (Angelini *et al.*, 2006, Garcia *et al.*, 2009, Krisch *et al.*, 2011). Furthermore, these toxins can accelerate lipid oxidation due to the chain reaction of free radical oxidation. Certain mycotoxins have been reported to produce free radicals which certainly impose an undesirable influence on human health (Alves-Silva *et al.*, 2013). Hence, the development of multiple measures to prevent fungal growth, mycotoxin production and free radical generation has become a crucial aspect to combat food losses and preserve food quality during storage and transport.

The food industry has reacted to increasing pressure imposed by consumers and legal authorities to reduce the use of chemical preservatives bearing antifungal activity and to either completely remove these compounds or adopt more natural alternatives for the maintenance or extension of product shelf-life (Beyki *et al.*, 2014). Essential oils (EOs) represent one of these natural additives and bear potent biological activities. In recent years, numerous *in vitro* and *in vivo* studies have reported the antifungal effects of plant EOs to control food spoilage (Gibriel *et al.*, 2011, Mohammadi *et al.*, 2013, Sumalan *et al.*, 2013, Tian *et al.*, 2012). However, the biological activity of EOs varies greatly with individual EO, depending on the chemical composition which

is specific to plant parts used, method of extraction, harvesting season etc (Chaubey, 2007, Vitoratos *et al.*, 2013).

The aim of this study was to evaluate the inhibition activity, as a food control and preservation strategy, of eight EOs against *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum* by determining (i) the minimum inhibitory concentration (MIC) of the EOs and (ii) the possible synergistic effects between EO combinations, and finally iii) antifungal activities of the volatile components resulting from the EOs to better understand the inhibitory kinetics caused by EOs on fungal growth.

4.2. Materials and methods

4.2.1. Preparation of essential oil emulsion

Basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme EOs were obtained from Robert & Fils (Ghislenghien, Belgium) and stored at 4 ˚C prior to use. Each EO was prepared as an emulsion containing 2.5% (v/v) of EO and 2.5% (v/v) of Tween 20 (Laboratoire Mat, QC, Canada). The mixtures were homogenized for 5 min with an Ultra-Turrax homogenizator (model TP18/1059, Germany) at 20,000 rpm to obtain a colloidal suspension. The emulsions were aseptically filtered using a 0.45 um pore size sterile filter. The mixtures were then stored at 4 ˚C. The major component (provided by the manufacturer) of these EOs are presented in table 4.1.

4.2.2. Fungal inocula and assay media

Aspergillus niger (ATCC 1015), *A. flavus* (ATCC 9643), *A. parasiticus* (ATCC 16869), and *Penicillium chrysogenum* (ATCC 10106) were used for the assays. Each fungal species was grown and maintained in potato dextrose broth (PDB, Difco, Becton Dickinson) containing glycerol (10% v/v). Prior to each experiment, stock cultures were propagated through two consecutive 48 h growth cycles in PDB medium at 28˚C ± 2 ˚C. The cultures were pre-cultured in PDA for 3 days at 28˚C ± 2 ˚C. Conidia were isolated from the agar media using sterile saline containing 0.05% Tween 80, and the filtrate was adjusted to 1 x 10^7 conidia/mL for broth dilution and checkerboard method and 1 x 10^8 conidia/mL for vapor contact assay by using a microscope (Inouye *et al.*, 2006).

4.2.3. Determination of the Minimum Inhibitory Concentrations (MIC) using broth dilution method

The method for determination of MIC was adopted from Turgis *et al.* (2012) with slight modification. All EOs were diluted in Potato Dextrose Broth (PDB) medium to obtain serial concentrations of 10000-10 ppm. A sample of 125 µl of the serially diluted EOs was pipetted into 96 well microplates (Sarstedt, QC, Canada). Each sample well was inoculated with 15µl of fungi at a concentration of 10^7 CFU/mL in order to obtain 1 x 10^6 conidia/mL of final concentration. The microplate was incubated aerobically for 36 h at 28 °C. After incubation, the absorbance was measured at 590 nm using an Ultra Microplate Reader (Biotek instruments, VT, USA). Sterile PDB medium was incubated under the same condition and used as a negative control blank. PDB medium incubated with a specific fungal species (without EO) was used as a positive control of growth. The minimum inhibitory concentration (MIC) was determined as the lowest EO concentration showing a complete growth inhibition of the tested fungal strains. This was evaluated by measuring the absorbance that is equal to the absorbance of the blank.

4.2.4 Assessing interaction between EO mixtures by the checkerboard method

Combination assays were evaluated based on a checkerboard procedure described by Turgis *et al.* (2012). The checkerboard method was performed to obtain the fractional inhibitory concentration (FIC) index of mixtures of EO combinations against each mold species. The index was calculated by adding the FIC values of EO (a) denoted by FIC_a and (b) denoted by FIC_b. The FIC_a and FIC_b values represented the fraction of the MIC of the EO combination to the MIC of the individual EO causing inhibition of the fungal growth. The calculations were performed using the following equations

$$FIC_a = MIC_{a,combined}/MIC_{a,alone} \quad (1)$$

$$FIC_b = MIC_{b,combined}/MIC_{b,alone} \quad (2)$$

$$FIC = FIC_a + FIC_b \quad (3)$$

Based on the above, the FIC of an EO could be equated to the concentration which caused deactivation of the fungal species when used in combination with another EO divided by the concentration that had the same effect when used alone (Gutierrez *et al.*, 2009, Mamoudou *et al.*, 2010). An FIC $\leq$ 0.5 was interpreted as a synergistic effect, 0.5 $\leq$ FIC $\leq$ 1 represented as an additive effect, FIC $\leq$ 4 represented as no interactive effect and FIC > 4 indicated an antagonistic effect between two tested EOs (Gutierrez *et al.*, 2008, Krisch *et al.*, 2011).

4.2.5. Vapor Contact Assays

Vapor contact assays were performed based on the method described by Inouye *et al.* (2006). A sample of 1 mL containing 1 x 10^8 of conidial suspension of the each fungal species were added to 100 mL of agar medium containing 1% peptone, 1% glucose and 1% agarose at 50 ˚C. A 3 mL volume of the prepared mixture was overlaid onto the surface of hardened PDA medium (20 mL) in a Petri dish (83 mm in diameter) to prepare a double layered agar medium. Sterile filter paper (10 mm diameter) was placed at the center of the upper lid of the plate. A quantity of 10 µl of each EO was added at the center of individual paper filters. Since oregano and thyme showed a synergistic effect based on their computed FIC index, their combined vapor activity was investigated by adding 10 µL of oregano and thyme mixture (1:1 v/v) on paper filter. Sterile water was used as control. The resulting Petri dishes containing the double-layered agar medium were inverted and placed on the upper lid containing the EOs. The Petri dishes were then incubated at 28 ˚C ± 2 ˚C for 1 week. The inhibitory diameter zone, which showed absence of growth of the test microorganisms, was measured in mm using a Traceable® Carbon Fiber Digital Caliper (Fisher Scientific). The amount of fungal growth was assessed by subtracting any clear inhibition zone produced from the internal diameter of the Petri dish. Based on the assays, the maximum colony diameter, lag time and mold growth rate were used as variables for modeling studies described in section 2.6 below.

4.2.6. Gompertz modeling of fungal growth

Fungal growth modeling and growth parameters were fitted using the modified Gompertz model proposed by Char *et al.* (2007) and Avila-Sosa *et al.* (2012)using the following growth parameter relationship.

$$\ln (D_t/D_o) = Aexp\{-exp[\ (\upsilon_m\ .\ e/A)(\lambda\text{-}t) + 1]\} \qquad (4)$$

Where, D_t (cm) is the average colony diameter at time t (day), D_o (cm) is the average colony diameter at initial time, A is the maximum colony diameter achieved during the stationary phase, υ_m is the maximum growth rate (1/day), λ is the lag phase (day) and e = exp (1).

4.2.7. Statistical Analysis

All experiments were done in triplicate. The experiments were then repeated two more times (with each treatment performed in triplicate). Fractional Inhibitory Concentration (FIC) of combined EOs and modified Gompertz model parameters against four fungal species were analyzed with the SPSS program (SPSS, Chicago, Ill.). An analysis of variance was performed using PASW Statistics Base 18 software (SPSS Inc., Chicago, IL), and comparisons of means between treatments and target fungal species were based on Duncan's multiple range tests at 5% level.

4.3. Results and Discussion

4.3.1. Minimum Inhibitory Concentrations (MIC) of EOs

The MICs obtained for the eight EOs are presented in Table 4.2. Based on MIC values, EOs were classified into three distinct groups corresponding to i) highly effective (312.5 ppm-5000 ppm) ii) moderately effective (5000-10,000 ppm), and iii) less effective (>10,000ppm). Among the tested EOs, thyme and oregano were found to be the most effective against all the four fungal species tested, with consistently low MIC values, followed by cinnamon. *A. niger* and *P. chrysogenum* were sensitive to oregano with a MIC of as low as 625 ppm. *A. flavus* and *A. paraciticus* showed less susceptibility to oregano bearing a MIC of 2500 ppm. Previous studies also showed similar trends. Viuda-Martos *et al.* (2007) reported that oregano induced the highest degree of inhibition against *A. niger* among other tested EOs including clove and thyme.

In the present study, *A. flavus* was found to be more sensitive to thyme with a MIC of 1250 ppm, followed by oregano, with a MIC of 2500 ppm. The other tested EOs cinnamon, peppermint and tea tree showed a MIC value of 5000 ppm against *A. flavus*. The study conducted by Omidbeygi. et al. (27) also reported that thyme exhibited the highest antifungal activity against *A. flavus* followed by summer savory and clove in both *in vitro* and *in vivo* tests. In our study, *A. parasiticus* was found to be sensitive to thyme with a MIC value of 1250 ppm.

Cinnamon EOs found to be very effective with a MIC of 2500 ppm against *A. niger*, *A. parasiticus* and *P. chrysogenum* and 5000 ppm for *A. flavus.* Basil and tea tree could be grouped into the moderately effective category of EOs with MICs ranging from 5000-10,000 ppm against

all tested fungi. Peppermint showed good activity against *P. chrysogenum* with a MIC of 1250 ppm but lower activity against *A. parasiticus* and *A. flavus* with MIC values of 2500 and 5000 ppm, respectively. *A. niger* showed more resistance to peppermint in comparison to the other tested fungi, exhibiting a MIC of up to 10,000 ppm. In a similar way, *P. chrysogenum* was found to be more resistant to basil and tea tree as compared to other tested EOs. Eucalyptus and mandarin did not inhibit the growth of any of the test specimens at concentrations of up to 10,000 ppm. Safaei-Ghomi *et al.* (2010) reported that eucalyptus oil, whose major component is 1, 8 cineole, did not inhibit the growth of *A. niger* in diffusion and MIC tests and showed lower degree of antimicrobial activity compared to other EOs containing limonene, α-pinene, p-cymene, and terpineol-4-ol. Cineole-containing EOs, thus, seem to display low antifungal properties unlike phenolic compounds such as carvacrol, thymol, and eugenol which display major fungicidal effects. Etherified compounds such as anethole, methyl chavicol, and safrole exhibit low fungicidal action, while monoterpene hydrocarbons such as limonene and myrcene have almost no effect.

Our findings generally showed that the EOs had antifungal activity, however each EO showed a specific pattern of activity against the fungal species tested. The variation in the fungicidal action of the different EOs used in our study can be explained by the difference in the water solubility and lipophilic properties of the EOs. According to Knobloch, et al. (31), the higher was the water solubility property of the EO, the more significant was its antimicrobial activity. This inference can be applied in our case where cinnamon, whose major component is linalool, exhibited higher antifungal activity compared to basil and peppermint, which contain methyl chavicol and menthol as their main components. Linalool has an inherently higher water solubility compared to chavicol and menthol, and thus may exhibit higher antifungal activity (Suppakul, et al. 2003).

4.3.2. Fractional Inhibitory Concentration (FIC) of combined EOs against fungal species

The FICs of the EO combinations tested during the current study are presented in Table 4.3. The highly effective oregano and thyme combination displayed a synergistic effect against all the fungi except for *A. niger*. Stević *et al.* (2014) reported a synergistic effect between carvacrol and thymol contained in oregano and thyme against *A. flavus, A. alternata, Penicillium sp* and *Fusarium* species. Such synergism between carvacrol and thymol has been reported against the bacteria *S. typhimurium* where a combined effect resulted in enhanced reduction of the bacterial

population than when applied alone (Zhou *et al.*, 2007). Here we report similar observations for fungal species.

The mixture of oregano and thyme showed an additive effect against *A. niger*. Thyme mixed with cinnamon, however, exhibited a synergistic effect against *A. flavus*. Combination of oregano with cinnamon, tea tree, thyme and mint, and an individual mixture of thyme and mint resulted in synergistic effects against *P. chrysogenum*. Similarly, a combination of cinnamon and thyme, oregano and mint, oregano and thyme showed a synergistic effect against *A. flavus*. Mint mixed with tea tree showed a synergistic effect against *A. niger*. Other mixtures namely basil and mint, cinnamon and tea tree, eucalyptus and tea tree, cinnamon and eucalyptus showed additive effects against the four tested fungi.

In contrast, mandarin and eucalyptus, when combined with thyme and oregano respectively, produced no interaction and resulted in FIC values ranging from 1.2-2.5 against all the tested fungi. It should be noted that synergism between antimicrobial agents such as carvacrol and some hydrocarbons monoterpenes (such as α-pinene, camphene, myrcene, α-terpinene and p-cymene) has been reported previously, which on a standalone basis, typically display low antimicrobial properties (Azeredo *et al.*, 2011, Ultee *et al.*, 2000). Such synergism may stem from the capacity of hydrocarbons to interact with the microbial cell membrane thereby facilitating the penetration of carvacrol into the cell (Azeredo *et al.*, 2011, Ultee *et al.*, 2002, Ultee *et al.*, 2000). Synergy is not only influenced by major compounds of essential oils alone, since minor components may have a more critical role to this effect with the activity of the main components being modulated by other minor molecules (Sahaf *et al.*, 2008). Most of the antimicrobial activity of EOs appears to be derived from oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols. Other constituents such as hydrocarbons that typically show low activities can be used in combinations to increase their bioactivities (Bassolé *et al.*, 2012). Hence, in the present study, combination of some particular oils showed additive and synergistic activity when combined, due to the combined activities of two or more components of EOs. Such increased fungistatic activity would have the advantage in pre- and post- harvest protection because pathogens cannot easily acquire resistance to multiple components of the two or more EOs (Stević *et al.*, 2014).

4.3.3. Gompertz modeling of fungal growth

The variation in colony diameter of the four fungal species under different EO treatment is presented in Figure 4.1-4.4. The modified Gompertz model developed by Avila-Sosa *et al.* (2012) was used to compute the fungal growth in presence of the EOs, and the predictions of the model were compared with the actual observed experimental data generated by the vapor assays. Model outputs describing the lag time, maximum colony diameter, and maximum mold growth during the stationary phase are shown in Table 4.4. The proposed model adequately described the growth curves exhibited by the fungi in presence of the eight EOs, and the mixture of oregano and thyme. The maximum colony diameter achieved during the stationary phase (*A*), the maximum exponential growth rate (υ_m) and the lag time (λ) values obtained from the growth curves provided useful quantitative comparison among the various EOs tested against the four fungal species. Concurrently, the modified Gompertz model was used to fit the data to predict the model parameters of the fungal species. No significant difference ($P>0.05$) was found between the Gompertz predicted model growth parameter values and the actual observed experimental values.. Oregano and thyme induced the highest growth inhibition for the four tested fungal species. Oregano and thyme also had the highest negative impact on the growth of the tested molds by decreasing their maximum growth rates (υ_m). Eucalyptus and mandarin were found to be the least effective EOs in limiting the maximum growth rate of the fungi. Cinnamon and tea tree significantly ($P \leq 0.05$) reduced the maximum specific growth rate of *P. chrysogenum.* Regarding the lag phase (λ), thyme was found to be the most effective against *A. niger* and *A.parasiticus,* increasing their lag time up to 3 days, followed by *P. chrysogenum* (2.6 d) and *A. flavus* (2.1 d). Oregano was found to be the most effective against *P. chrysogenum* in extending its lag phase time to 2.83 d. As shown in table 4.4 and figure 4.5, a combination of oregano and thyme was as well very effective against all the four tested fungal species; this combination significantly ($P \leq 0.05$) limited the colony diameter and the maximum growth rate of the fungal species and extended their lag times. These findings provide valuable insights on the potential of the tested EOs in exerting antifungal activities by altering the growth kinetics of fungi. Avila-Sosa *et al.* (2010) demonstrated how growth parameters derived from a modified Gompertz model affected the growth kinetics of *A. niger* in presence of the Mexican oregano added to amaranth, chitosan and starch edible films. This study also illustrates the potential of EOs in restricting the growth of fungal species by modifying their growth kinetics.

4.4. Conclusion

The present research highlights the potential antifungal effects of eight EOs against four mold species. Based on MIC assays, the EOs were broadly grouped as having high, moderate and low effective groups with oregano and thyme showing the highest antifungal activity. Checkerboard analyses showed that some EO combinations displayed synergistic effects as was the case for oregano and thyme, while others such as mandarin and eucalyptus mixed with thyme and oregano respectively, showed no interaction at all. Studying the effect of the EOs against the fungi using the modified Gompertz model provided valuable insights on their growth parameters. In general, the EOs were effective in reducing the colony diameter and exponential growth rate while increasing the lag time of the fungi tested. Further research on the antifungal activity of EOs and their main components are required together with a better understanding of the mode of action in order to evaluate their usefulness in extending the shelf-life of packaged foods.

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Table 4.1. List of EOs and their major active components

Essential oil	**Scientific name**	**Major components**
Basil	*Ocimum basilicum*	Estragole, Eugenol, linalool
Cinnamon	*Cinnamomum zeylandicum*	Linalool, trans-cinnamaldehyde
Eucalyptus	*Eucalyptus Globus*	1,8 cineole
Mandarin	*Citrus reticulate*	Limonene, γ terpinene
Oregano	*Origanum vulgare*	Carvacrol, thymol
Peppermint	*Mentha piperita*	Menthol, menthone,
Tea tree	*Melaleuca alternifolia*	Terpinol
Thyme	*Thymus vulgaris*	Thymol, carvacrol, γ-terpinene

Table 4.2. Minimum Inhibitory Concentration (MIC) (ppm) of tested EOs against 4 fungal species after 36 h of incubation using broth dilution method.

Essential oil	**Minimum inhibitory concentration (MIC) (ppm)**			
	A. niger	***A. flavus***	***A. parasiticus***	***P. chrysogenum***
Basil	10000	10000	5000	10000
Cinnamon	2500	5000	2500	2500
Eucalyptus	10000	> 10000	>10000	> 10000
Mandarin	> 10000	10000	10000	> 10000
Oregano	625	2500	2500	625
Peppermint	10000	5000	2500	1250
Tea tree	10000	5000	5000	10000
Thyme	1250	1250	1250	312.5

Table 4.3. Fractional Inhibitory Concentration (FIC) of combined EOs against target fungal species

EO Mixtures	*A. niger*		*A. flavus*		*A. parasiticus*		*P. chrysogenum*	
	FIC activity	**Effect**	**FIC activity**	**Effect**	**FIC activity**	**Effect**	**FIC activity**	**Effect**
Basil+ Cinnamon	$2.00\pm 0.28^{efgh,B}$	I	$2.16\pm 0.16^{ijk,B}$	I	$4.00\pm 0.16^{h,C}$	I	$0.83\pm 0.07^{abc,A}$	AD
Basil + Eucalyptus	$2.17\pm 0.20^{efgh,A}$	I	$3.16\pm 0.28^{l,A}$	I	$3.33\pm 0.33^{g,B}$	I	$2.33\pm 0.44^{fgh,A}$	I
Basil+ Mandarin	$2.50\pm 0.28^{hi,A}$	I	$2.00\pm 0.21^{hijk,A}$	I	$2.33\pm 0.18^{f,A}$	I	$2.67\pm 0.16^{h,A}$	I
Basil + Oregano	$1.00\pm 0.1^{abcd,A}$	AD	$1.50\pm 0^{efghi,AB}$	I	$1.25\pm 0.13^{cd,AB}$	I	$1.00\pm 0.00^{bc,A}$	AD
Basil+ Peppermint	$0.83\pm 0.08^{abc,AB}$	AD	$0.83\pm 0^{abcdef,AB}$	AD	$1.00\pm 0.12^{cd,A}$	AD	$0.58\pm 0.06^{ab,A}$	AD
Basil + Tea tree	$1.67\pm 0.33^{defg,A}$	I	$1.33\pm 0.12^{defgh,A}$	I	$1.00\pm 0.16^{abcd,A}$	I	$1.00\pm 0.00^{bc,A}$	AD
Basil + Thyme	$1.41\pm 0.08^{bcde,A}$	I	$0.99\pm 0.12^{abcdef,A}$	AD	$1.04\pm 0.11^{abcd,A}$	I	$1.25\pm 0.12^{cd,A}$	I
Cinnamon + Eucalyptus	$1.00\pm 0.13^{abcd,A}$	AD	$0.70\pm 0^{abcd,A}$	AD	$0.99\pm 0.10^{abcd,A}$	AD	$0.83\pm 0.04^{abc,A}$	AD
Cinnamon + Mandarin	$2.33\pm 0.16^{gh,C}$	I	$1.58\pm 0.12^{fghij,B}$	I	$1.16\pm 0.16^{bcd,A}$	I	$2.00\pm 0.28^{ef,BC}$	I
Cinnamon + Oregano	$4.00\pm 0.14^{kl,D}$	A	$3.16\pm 0.16^{l,C}$	I	$2.00\pm 0.18^{ef,B}$	I	$0.33\pm 0.04^{a,A}$	S
Cinnamon + Peppermint	$4.16\pm 0.16^{ghi,B}$	A	$1.83\pm 0.16^{ghij,B}$	I	$2.35\pm 0.25^{f,B}$	I	$1.00\pm 0.12^{bc,A}$	AD
Cinnamon + Tea tree	$1.00\pm 0.12^{abcd,B}$	AD	$0.54\pm 0.11^{abc,A}$	AD	$0.54\pm 0.08^{abcd,A}$	AD	$0.75\pm 0.06^{abc,AB}$	AD
Cinnamon + Thyme	$2.00\pm 0.16^{fgh,B}$	I	$0.41\pm 0^{a,A}$	S	$1.00\pm 0.00^{abcd,B}$	AD	$1.00\pm 0.00^{bc,B}$	AD
Eucalyptus + Mandarin	$2.33\pm 0.33^{ghi,A}$	I	$2.30\pm 0.12^{jk,A}$	I	$2.08\pm 0.16^{ef,A}$	I	$2.50\pm 0.16^{gh,A}$	I
Eucalyptus + Oregano	$1.33\pm 0.08^{bcde,A}$	I	$1.50\pm 0.12^{fghij,A}$	I	$2.41\pm 0.23^{f,B}$	I	$1.25\pm 0.07^{cd,A}$	I

Eucalyptus + Peppermint	1.50± 0^{cdef,C}	I	1.25± 0.12^{cdefg,B}	I	1.20± 0.14^{bcd,B}	AD	0.62± 0.00^{ab,A}	AD
Eucalyptus + Tea tree	1.00± 0.16^{abcd,C}	AD	0.79± 0^{abcde,B}	AD	0.60± 0.02^{abc,A}	AD	1.00± 0.0^{bc,C}	AD
Eucalyptus + Thyme	2.17± 0.17^{fgh,A}	I	1.67± 0.16^{fghij,A}	I	2.00± 0.28^{ef,A}	I	2.16± 0.18^{ef,A}	I
Mandarin + Oregano	3.00± 0^{ij,B}	I	2.67± 0.10^{kl,B}	I	1.33± 0.16^{d,A}	I	1.66± 0.16^{de,A}	I
Mandarin + Peppermint	2.33± 0.17^{hi,C}	I	1.50± 0.12^{efghi,B}	I	1.00± 0.00^{abcd,B}	AD	1.00± 0.01^{bc,A}	AD
Mandarin + Tea tree	3.50± 0.50^{jk,B}	I	2.00± 0.18^{hijk,A}	I	2.00± 0.19^{ef,A}	I	1.25± 0.13^{cd,A}	I
Mandarin + Thyme	2.67± 0.16^{hi,C}	I	1.83± 0.14^{ghij,B}	I	1.25± 0.14^{cd,A}	I	1.66± 0.11^{de,A}	I
Oregano + Peppermint	0.92± 0.08^{ab,B}	AD	0.50± 0.06$^{ab,\ A}$	S	0.99± 0.10^{abcd,B}	AD	0.50± 0.00^{ab,A}	S
Oregano + Tea tree	1.00± 0.10^{abcd,A}	AD	3.00± 0.12^{l,B}	I	2.33± 0.26^{f,A}	I	0.79± 0.00^{abc,A}	AD
Oregano + Thyme	0.75± 0.16^{ab,A}	AD	0.47± 0.04$^{ab,\ A}$	S	0.41± 0.00^{a,A}	S	0.41± 0.00^{a,A}	S
Peppermint + Tea tree	0.43± 0.06^{a,A}	S	1.16± 0.12$^{bcdefg,\ C}$	I	0.54± 0.04^{ab,A}	AD	0.83± 0.01^{abc,B}	AD
Peppermint + Thyme	0.79± 0.11^{abc,A}	AD	0.79± 0^{abcde,A}	AD	1.50± 0.19^{de,B}	I	0.33± 0.00^{a,A}	S
Tea tree + Thyme	1.16± 0.08^{bcd,C}	I	1.00± 0.1^{abcdef,BC}	AD	0.83± 0.09^{abcd,AB}	AD	0.75± 0.00^{abc,A}	AD

Activity: FIC≤0.5: synergic effect (S); 0.5bFIC≤1: additive effect (AD); 1bFIC≤4: no interactive effect (I); FIC>4: antagonistic effect (A)

Values are means ± standard error. Within each row, means with the same uppercase letter are not significantly different (P > 0.05). Within each column means with the same lowercase letter are not significantly different (P > 0.05).

Table 4.4. Modified Gompertz model parameters (means ± standard error) derived for four fungal species subjected to eight EOs and one mixture of EO through vapor assay

EO	*A. niger*			*A. flavus*			*A. parasiticus*			*P. chrysogenum*		
	A (cm)	v_m **(d^{-1})**	**λ (d)**	**A (cm)**	v_m **(d^{-1})**	**λ (d)**	**A (cm)**	v_m **(d^{-1})**	**λ (d)**	**A (cm)**	v_m **(d^{-1})**	**λ (d)**
Basil	2.12±0.0 ^{d}	2.20±0.0 ^{c}	1.00±0.0 ^{b}	2.12±0.0 ^{c}	2.21±0.1 ^{e}	1.00±0.1 ^{b}	2.12±0.0 ^{d}	2.75±0.0^{e}	0.50±0.0 ^{a}	2.12±0.0 ^{c}	2.17±0.0^{d}	1.00±0.0
Cinnamon	2.12±0.0 ^{d}	2.10±0.1 ^{c}	1.00±0.0 ^{b}	2.12±0.0 ^{c}	1.86±0.2 ^{d}	1.00±0.0 ^{b}	2.12±0.0 ^{d}	2.32±0.2^{d}	1.00±0.0 ^{b}	2.12±0.0 ^{c}	1.74±0.2^{c}	1.00±0.0
Eucalyptus	2.12±0.0 ^{d}	2.63±0.1 ^{d}	0.50±0.0 ^{a}	2.12±0.0 ^{c}	3.45±0.3^{f}	0.50±0.1 ^{a}	2.12±0.0 ^{d}	4.34±0.3^{g}	0.50±0.0 ^{a}	2.12±0.0 ^{c}	4.56±0.1^{f}	0.50±0.0
Mandarin	2.12±0.0 ^{d}	4.02±0.1 ^{e}	0.50±0.0 ^{a}	2.12±0.0 ^{c}	4.24±0.1 ^{g}	0.50±0.1 ^{a}	2.12±0.0 ^{d}	3.97±0.2^{f}	0.50±0.0 ^{a}	2.12±0.0 ^{c}	2.90±0.1^{e}	1.00±0.1
Oregano	1.47±0.1 ^{c}	0.72±0.1 ^{a}	2.00±0.0 ^{c}	1.66±0.1 ^{b}	0.73±0.0 ^{a}	2.00±0.1 ^{c}	1.43±0.1 ^{b}	0.58±0.0^{b}	2.00±0.3 ^{c}	1.41±0.2 ^{b}	0.64±0.0^{b}	2.83±0.2
Peppermint	2.12±0.0 ^{d}	1.55±0.1 ^{b}	1.00±0.0 ^{b}	2.12±0.0 ^{c}	1.65±0.1 ^{c}	1.00±0^{b}	2.12±0.0 ^{d}	2.12±0.0^{d}	1.00±0^{b}	2.12±0.0 ^{c}	1.82±0.1^{e}	1.00±0.1
Tea tree	2.12±0.0 ^{d}	1.45±0.1 ^{b}	2.00±0.0 ^{c}	2.12±0.0 ^{c}	1.47±0.1 ^{b}	1.00±0^{b}	2.12±0.0 ^{d}	2.24±0.1^{d}	2.00±0^{c}	2.12±0.0 ^{c}	1.90±0.1^{c}	2.00±0.0
Thyme	1.21±0.0 ^{b}	0.60±0.1 ^{a}	3.00±0.2 ^{d}	1.46±0.1 ^{a}	0.62±0.0 ^{a}	2.16±0.1 ^{c}	1.63±0.0 ^{c}	0.82±0.0^{c}	3.00±0.2 ^{d}	1.44±0.1 ^{b}	0.70±0.0^{b}	2.66±0.3
Oregano + Thyme	1.12±0.0 ^{a}	0.54±0.0 ^{a}	3.66±0.3 ^{c}	1.41±0.0 ^{a}	0.60±0.0 ^{a}	3.16±0.2 ^{d}	1.31±0.1 ^{a}	0.20±0.0^{a}	3.33±0.1 ^{e}	1.24±0.1 ^{a}	0.19±0.0^{a}	4.66±0.3

Foot note. A: maximum colony diameter during stationary phase; $\upsilon_{m:}$ maximum growth rate; λ: lag time

Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different (P > 0.05).

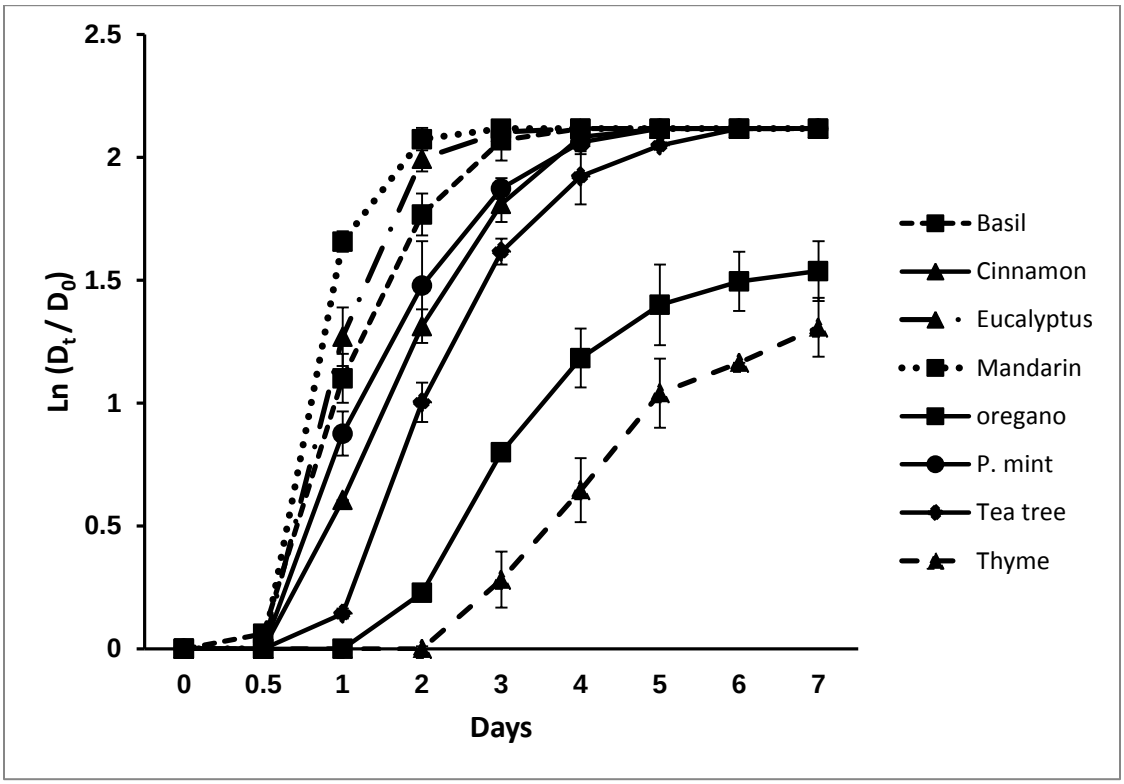

Figure 4.1. Effect of different EOs on the maximum colony diameter, Ln (Dt/D_0), of *A. niger* over time.

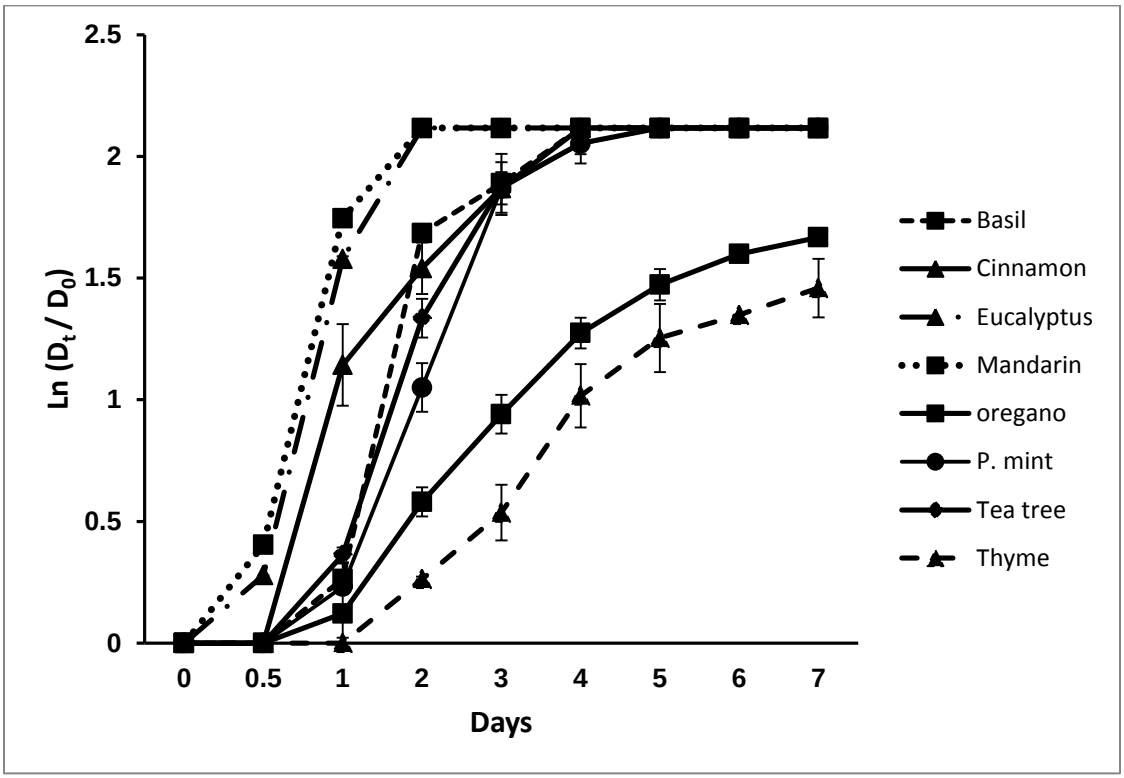

Figure 4.2. Effect of different EOs on the maximum colony diameter, Ln (Dt/D_0), of *A. flavus* over time.

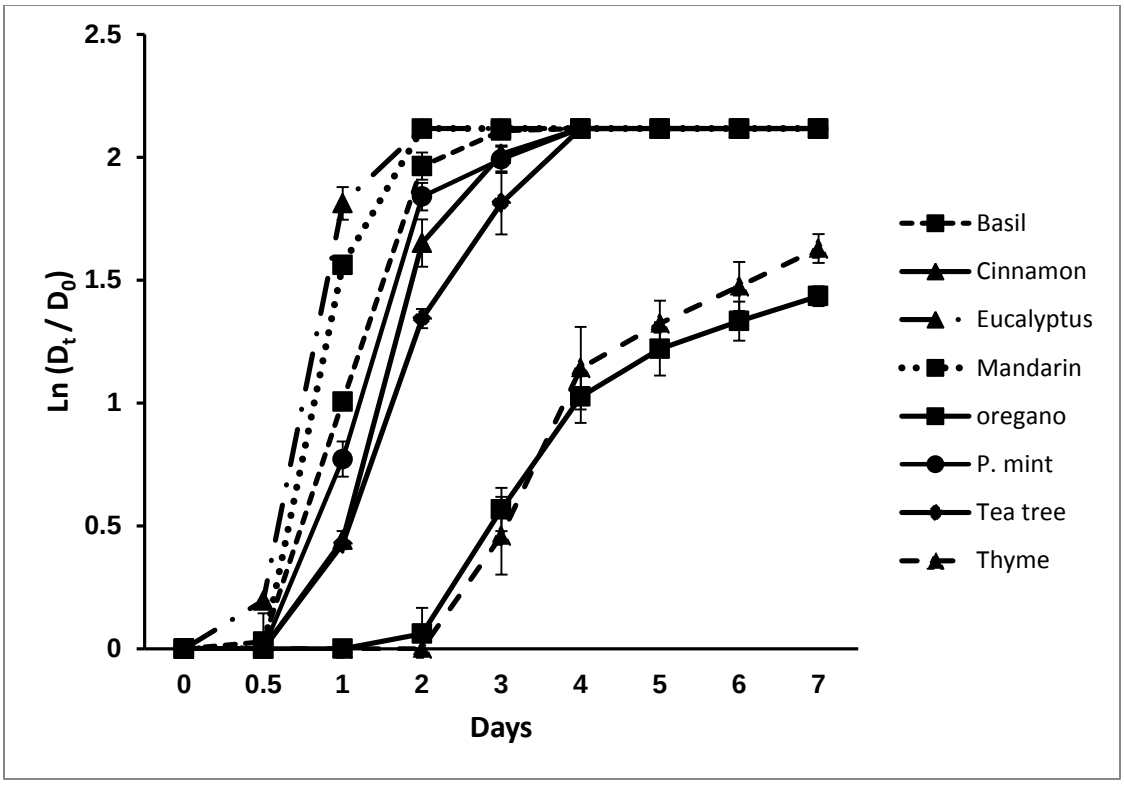

Figure 4.3**.** Effect of different EOs on the maximum colony diameter, Ln (Dt/D_0), of *A. parasiticus* over time.

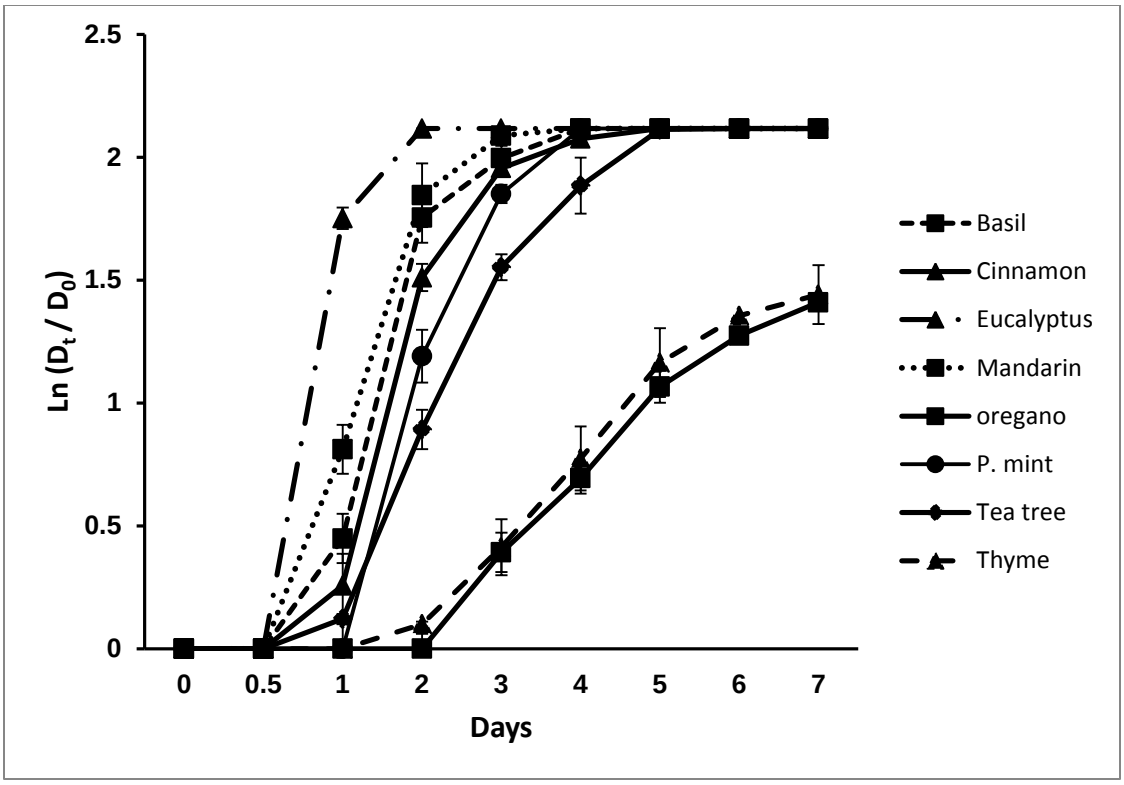

Figure 4.4. Effect of different EOs on the maximum colony diameter, Ln (Dt/D_0), of *P. chrysogenum*.

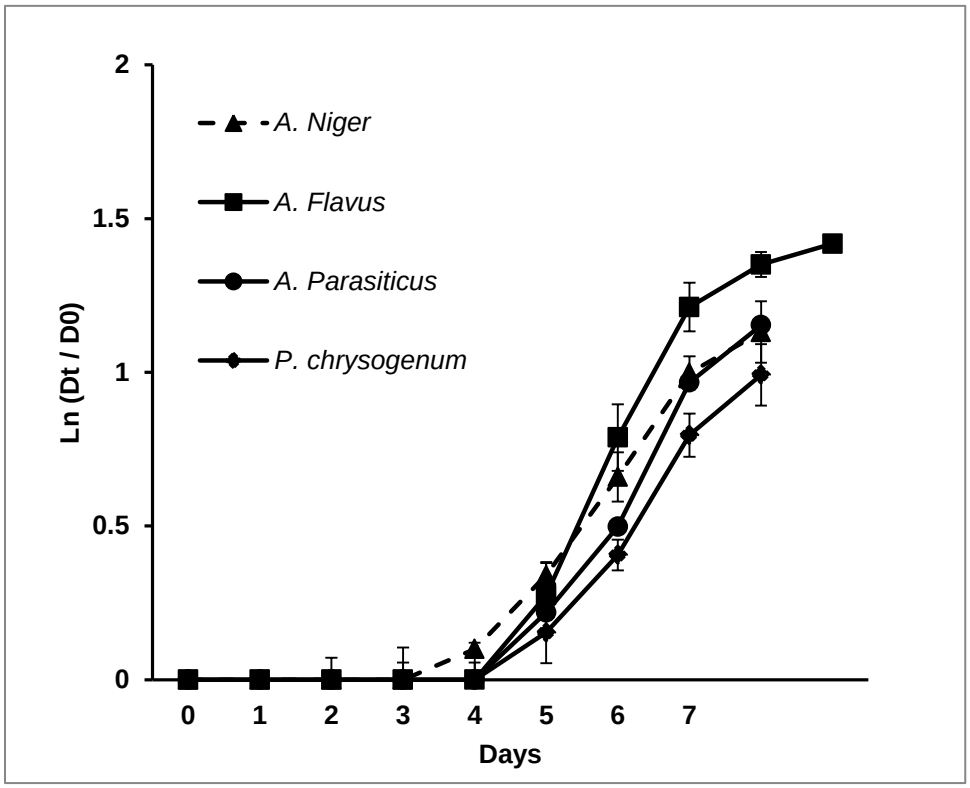

Figure 4.5. Effect of different EOs on the maximum colony diameter, Ln (Dt/D_0), of *A. niger, A. flavus, A. parasiticus and P. chrysogenum* over time.

Chapter 5

Publication 4

Insecticidal and synergistic effects of active biopolymers containing plant essential oils and γ-irradiation against the rice weevil, *Sitophilus oryzae*

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Keywords: *Sitophilus oryzae*, plant essential oils, fumigant toxicity, acetylcholinesterase activity, bioactive film, gamma radiation

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Contribution of the authors

Most of the experiments work was planned, performed and analyzed by Farah Hossain with guidance of Prof. Lacroix and Dr. Follett. Both Professors corrected the main draft and gave valuable suggestions to improve the discussion. Mehdi Harich helped to conduct some experiments. Dr. Khanh showed some data analysis. Stephane Salmieri coordinated research activities to perform on time.

Résumé

La toxicité de la phase vapeur de huit huiles essentielles, HE (basilic, cannelle, eucalyptus, mandarine, origan, menthe poivrée, thé et thym) et une combinaison binaire (thym et origan) ont été étudiées pour évaluer leur effet sur le charançon du riz, *Sitophilus oryzae*. Toutes les huiles essentielles appliquées seules ou en combinaison étaient toxiques pour le charançon du riz. L'activité de la phase vapeur a montré que le taux de mortalité de *S. oryzae* a significativement augmenté ($p \leq 0,05$) avec l'augmentation de la concentration en huiles essentielles et de la période d'exposition. Parmi les HE testées, l'eucalyptus présente la toxicité la plus élevée en induisant une mortalité complète des insectes (100%) au bout de 24 heures suivant l'exposition et ceci à une concentration minimale de 40 µL. La combinaison des HE d'origan et de thym a montré une amélioration de l'activité inhibitrice de la phase vapeur et de l'acétylcholinestérase (AChE) que les traitements individuels (origan et thym seul). L'inhibition de l'AChE n'était pas nécessairement liée à la toxicité des huiles essentielles, ce qui implique que les divers composants des huiles essentielles peuvent exercer leurs effets par des mécanismes autres que la perturbation du système cholinergique. L'efficacité insecticide des HE origan: thym incorporées dans trois matrices différentes, chitosan (CH), méthylcellulose (MC) et poly (acide lactique) PLA renforcé avec nanocristal de cellulose (CNC) a été analysée. Une activité insecticide allant de 4 à 42% a été confirmée avec des films à base de biopolymères chargés d'HE (0.75%) pendant 10 jours d'incubation. La mortalité était significativement plus élevée par rapport aux films à base de chitosane. En outre, la mortalité a augmenté jusqu'à 95% par l'application combinée de l'irradiation gamma à une dose de 300 Gy et de films de chitosane bioactifs à 10 jours de la période d'exposition. Ces résultats suggèrent que la combinaison d'HE (origan: thym) dans des films polymères pourrait être prometteuse comme modèle pour développer de nouveaux insecticides qui pourraient être appliqués pour le contrôle du charançon du riz pendant le stock.

Abstract

The fumigant toxicities of eight individual essential oils (basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme) and one binary combinations (thyme and oregano) were investigated against the rice weevil, *Sitophilus oryzae.* Results showed that all individual and combined essential oils were found toxic to the rice weevil. Fumigant activity have shown that *S. oryzae* mortality rate increased significantly ($p \leq 0.05$) with the increase in essential oils concentration and incubation period. Eucalyptus EO exhibited the highest toxicity by inducing complete insect mortality (100%) at a minimum concentration of 40 µl within 24 h of exposure among all tested essential oils. Combination treatments of oregano and thyme EO displayed higher fumigant and acetylcholinesterase (AChE) inhibitory activity than individual treatment (oregano and thyme alone) applications. AChE inhibition was not necessarily linked to essential oil toxicity levels, implying that the various components of the essential oils may be exerting their effects through mechanisms other than disruption of the cholinergic system. The insecticidal effectiveness of oregano:thyme essential oils (EOs) incorporated into three different matrices, Chitosan (CH), methyl cellulose (MC) and poly (lactic acid) PLA reinforced with cellulose nanocrystal (CNC) was analysed. Insecticidal activity was confirmed ranging between 4-42% with EOs (0.75%) loaded biopolymeric films during 10 days of incubation period. Mortality percentage was significantly greater when compared with those of the chitosan based films. In addition, the percent mortality increased to 95% by the combined application of 300 Gy gamma radiation and bioactive chitosan films at 10 days of incubation period. These results suggested that combination of EOs (oregano: thyme) into polymeric films could be promising as models to develop new insecticides that might be applied into the integrated management of rice weevil during grain storage.

5.1. Introduction

The rice weevil, *Sitophilus oryzae* (L.), is an important pest of stored cereals worldwide (Kim *et al.*, 2001, Kim *et al.*, 2016, Swany *et al.*, 2014). Infestation by this species causes severe quantitative and qualitative losses on grain products, alter nutritional and aesthetic value and contaminate food commodities with insect bodies, excrements and most importantly by mycotoxins, as a result of insect-promoted fungal growth during storage (Mondal *et al.*, 2016, Seada *et al.*, 2016). The control of stored-product insect pests depends mainly on the use of fumigants and broad-spectrum contact insecticides, which are encountering legislative limitations and consumer reluctance due to their environmental impact and potential human health concerns (Kiran *et al.*, 2015). The repeated use of these insecticides for decades has disrupted natural biological control systems and led to outbreaks of stored-product insect pests (Kiran *et al.*, 2015, Pimentel *et al.*, 2007). Therefore, the development of novel insect-proof packages may play an important role to resist insect penetration and their potential infestation in stored product commodities (Hossain *et al.*, 2017)

Active packaging, containing plant-derived EOs is one of the promising innovations to control stored food product insect pests. Many plant extracts and essential oils are known to possess insecticidal activity against various stored product insects (Kedia *et al.*, 2014, Regnault-Roger *et al.*, 2012). Recent studies have shown that EOs possess insecticidal and repellent properties, the capacity to delay development, adult emergence and fertility and deterrent effects on oviposition (Koul *et al.*, 2008). EOs provide novel modes of action against insects with reduced risk of cross-resistance, and offer new prospects for the design of target-specific molecules (Rajashekar *et al.*, 2013, Zhou *et al.*, 2012). Several authors reported that essential oils mainly poison insects by a neurotoxic mode of action (Kang *et al.*, 2013, Rajashekar *et al.*, 2014). Acetylcholinesterase (AChE) is a key enzyme involved in insects' nervous system, responsible for nerve impulse transmission (Rana *et al.*, 2015). It has been recently reported that plant extracts exhibit acetylcholinesterase (AChE) inhibitory activity (Dohi *et al.*, 2009, Prakash *et al.*, 2015). However, EOs bear several drawbacks that limits their application such as their volatility, instability, and insolubility in water (Bilia *et al.*, 2014), and their rapid diffusion into food matrices (Boumail *et al.*, 2013). Encapsulation of EOs in a bio-based delivery system as a packaging films or diffusion films could provide an alternative issue that ensures a better

interaction and mode of action at a target site due to their controlled release of active agent over time (Hossain *et al.*, 2017).

Biopolymers from renewable sources have vast diversity, and their applications in food packaging provide unique solutions to enhance product shelf-life (Khan *et al.*, 2014a). Biopolymers such as chitosan (CH) (Elsabee *et al.*, 2013), methyl cellulose (MC) (Boumail *et al.*, 2013) and Poly (lactic acid) (PLA) (Salmieri *et al.*, 2014a) have proven to be very promising packaging materials, but their application on rice weevil has never been demonstrated. Developing EO-loaded bioactive packaging against insects opens an era of possibilities in green revolution by reducing the use of synthetic chemicals as pesticide and plastic materials as packaging.

In addition, food irradiation is an efficient and emerging technology for disinfestations since it seems to offer solutions in many respects. A minimum dose of 400 Gy is recommended by the USDA treatment manual for disinfestation of stored product insects while several countries allow a dose of 1 kGy (Anonyme, 2009). Banks *et al.* (1995) reported an irradiation doses of 3–5 kGy were effective in controlling insects. Enu *et al.* (2014) demonstrated that a dose of 300 Gy and 500 Gy was required to disinfest both *Sitophilus zeamais* and *Callosobuchus maculatus* within 3 weeks after the gamma irradiation. Das *et al.* (2013) reported 0.6 kGy irradiation dose was effective to sterilize rusty grain beetles whereas saw-toothed grain beetles and red flour beetles required a 2.0 Gy dose. However the use of combined treatment such as active film in combination with gamma radiation also permits a synergy between the treatments and can reduce the dose needed for each treatment.

The aim of the present work was to i) study the fumigant toxicities of eight EOs namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme against *S. oryzae*, alone and one EO combination oregano and thyme. ii) Determine the inhibitory effects of individual and combined EOs on AChE activity *in vitro* to explore the mode of action of these individual and combined EO applications. iii) Develop EOs formulation encapsulated into polymer matrices such as chitosan (CH), methyl cellulose (MC) and Poly Lactic acid (PLA) to develop a broad cast medium in order to facilitate and control the diffusion of active components and iv) evaluate the combined effect between most efficient bioactive film and

irradiation treatment to improve the efficiency of the treatment that will have commercial application and acceptability to ensure the safety and quality of cereals during storage.

5.2. Materials and methods

5.2.1. Insect population

A colony of *Sitophilus oryzae* was obtained from the Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, Manitoba. The colony was fed and maintained on long grain white rice (Nu pak, Shah Trading Company Limited, Scarborough, Ontario) in 2 L plastic containers with ventilated covers in an environment-controlled chamber at 28 ± 1 ºC, 50-60% relative humidity, and a 12:12 h light/dark cycle. Adult weevils in the colony were periodically transferred to new containers with fresh rice to perpetuate the colony.

5.2.2. Essential oils (EOs)

Essential oils of basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme (100% purity, Pranarom International, Inc., Ghislenghien, Belgium) were used as bio-insecticidal agents. Based on our previous work (Hossain *et al.*, 2016) one EO combination consisting of oregano and thyme was prepared in a ratio of 1:1 and tested against *S. oryzae* to evaluate their combined efficacy. A list of the major active components in each EO, as provided by the manufacturer Pranarôm International, Belgium, is presented in Table 5. 1.

5.2.3. Dose-response tests with individual EOs

The fumigation toxicity of the EOs was examined using dose-response tests. Based on preliminary tests, an aliquot of 0.2, 0.4, 0.8, 1.6 and 2.4 µL/mL of basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree, thyme and the combination of oregano and thyme were applied to a small sponge (10 x 10 x 8 mm) and placed in a 10 mL cup wrapped with muslin screen. The screened cups with the EOs were placed in Petri-dishes (95 x 15 mm) containing 10 g of rice and 25 individual *S. oryzae* adults (1-3 weeks old), and sealed with Parafilm. The petri dishes were incubated at 28± 2˚C at 65% RH. The method was similar to the one described by Hossain *et al.* (2014b). Weevils showing no leg or antennal movement when prodded after 72 h of treatment were considered as dead. Percent mortality were calculated for each EO as below

(Number of dead insects/ Number of total insects) x 100 (Eq. 1)

5.2.4. Inhibition of acetylcholinesterase (AChE) activity

Inhibition of AChE was determined for tested eight EOs and one combined oregano and thyme EO, using the colorimetric method described by Ali-Shtayeh *et al.* (2014) and Abdelgaleil *et al.* (2009a), with some modifications. Acetylthiocholine iodide (ATChI) and 5, 5′-dithio-bis (2-nitrobenzoic) acid (DTNB) were purchased from Sigma–Aldrich Chemical Co., USA. This assay is based on the hydrolysis of ATChI substrate to thiocholine and acetic acid by AChE. Resulting thiocholine is made to react with DTNB to produce a yellow color, the intensity of which is proportional to the enzyme activity. A quantity of 0.5 g of a whole body preparation of *S. oryzae* was homogenized in 5 mL of 0.1 M ice-cold phosphate buffer (pH 7.0). The resulting homogenates were centrifuged at 5,000 rpm for 20 min at 0 ºC, and the supernatants were retained for determining AChE activity. EO dilutions were prepared in 99% methanol. A reaction mixture comprising of 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in buffer, and 25 µL of EO diluted in 99% methanol was loaded onto 96-well plates and the absorbance was measured at 405 nm. Thereafter, 25 µL of AChE solution was added to each well and incubated for 10 min at 37 °C. The stabilized absorbance was read at 405 nm 10 times at 1 min intervals. Control treatments were prepared by using 25 µL of 99% methanol in place of the EOs. The percentage inhibition of the enzyme activity by the test solution was calculated using equation 2.

$$\text{Inhibition (\%)} = 1 - (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100 \quad \text{(Eq. 2)}$$

5.2.5. Preparation of EO loaded biopolymeric film

Chitosan, methyl cellulose and poly (lactic acid) PLA based composite diffusion matrix containing cellulose nanocrystal (CNC) were developed following Khan *et al.* (2012c) and Boumail *et al.* (2013 a) and (Salmieri *et al.*, 2014a). In brief, a quantity of 2 g chitosan was added to the 0.1 g crystalline nanocellulose (CNC) suspension followed by 2 g acetic acid in 100 mL distilled water. The CH solution was then homogenized with IKA RW-20 mechanical homogenizer at 1,500 rpm for overnight. A quantity of 0.5% ethylene glycol was added during homogenization. A quantity of 2 g methyl cellulose (MC) was solubilized in distilled water, under stirring at 40-50°C (for pre-gelatinization) and was then cooled in an ice bath to ensure complete solubilization. 0.15% CNC was added to the solution. The MC solution was then magnetically stirred overnight followed by homogenization with IKA RW-20 mechanical

homogenizer at 1,500 rpm for 5 hrs. A quantity of 0.5% glycerol was added during homogenization.

Then a quantity of 0.75% (w/v) oregano: thyme EO combination was added to CNC containing CH and MC matrix and allowed to mix well. Composite film was cast by applying 12 mL of CH/CNC and MC/CNC film forming suspension onto Petri dishes (95 × 15 mm; Fisher Scientific, Ottawa, ON, Canada) and allowing it to dry for 24 h under a chemical hood at room temperature. Bioactive CH/CNC and MC/CNC films were peeled off and placed in plastic bags.

Poly (lactic acid) PLA film was prepared by solvent casting method. A quantity of 2% (w/v) of PLA-NCC granules, were dissolved in solvent (Chloroform), under magnetic stirring, for 40 min at room temperature. Then a quantity of 0.75% oregano: thyme EO combination was added into the solution under magnetic stirring for another 20 min, at room temperature to prepare bioactive films. A quantity of 0.5% glycerol was added to the oil dispersions. The resulting formulation was poured into polytetrafluoroethylene (PTFE) Petri dishes (100 mm diameter; 1.5 cm depth; 15 mL/dish). The film solution was allowed to evaporate under chemical hood for 2 h, at room temperature. The resulting films were peeled off manually using a spatula and placed in plastic bags prior to analysis.

5.2.6. Insecticidal activities of biopolymeric film and in combination with gamma radiation

The insecticidal activities of the bioactive CH/CNC, MC/CNC and PLA/CNC films were evaluated in rice samples against *S. oryzae*. A quantity 50 *S. oryzae* was added in a plastic bag containing 30 g of rice. Two pieces of each bioactive films containing 0.75% (w/w) of (Oregano and thyme) EO mixture were cut in to 4 pieces and placed in the rice samples. The mortality of the insects was assessed at day 3, 7 and 10. The rice samples without bioactive films were considered as control samples.

To evaluate the combined effect of active film and gamma radiation, CH/CNC based films were selected. The rice bags containing 30 gm of rice and 50 weevil and bioactive CH/CNC based films were irradiated at 100, 200 and 300 Gy radiation doses after 24 h incubation, at the Canadian Irradiation Center (Laval, QC, Canada) using a cobalt-60 Gammacell 220 irradiator (Nordion, Ottawa, Canada) with a dose rate of 0.36 Gy min^{-1}. The mortality was counted at day 3, 7 and 10 days of post treatment period.

5.2.7. Statistical analysis

Treatment mortality with EOs was calculated at 24, 48 and 72 h. The experiment of fumigant toxicity and acetyl cholinesterase inhibition assay of individual EOs and oregano: thyme EO combination was conducted three times and for each experiment 4 replicates for each treatment were performed. The experiment with biopolymeric films and γ irradiation was conducted in four replicates for each sample. The experiment was repeated three times for reproducibility. All levels were subjected to analysis of variance and means separations were performed using a Duncan's multiple range tests at the 5% level. Analysis of variance was performed using the PASW Statistics Base 18 software (SPSS Inc., Chicago, IL).

5.3. Results and Discussion

5.3.1. Bioactivity of individual EOs against *S. oryzae*

Insecticidal activities of different concentrations of eight EOs and one combination of oregano: thyme EOs was evaluated against *S. oryzae* during 72 hour of storage period (table 5.2). The results showed that the percentage of mortality (%) linearly increased with concentrations and incubation time. After a 24 h of incubation period, EOs of eucalyptus found most efficient inducing 17% and 57% mortality with a concentration 0.2 and 0.4 µl/mL respectively as compared to other tested EOs ($p \leq 0.05$). A quantity of 0.8 µl/mL of eucalyptus EOs found to be efficient to induce 100% mortality whereas a maximum of 18% mortality was observed with this concentration with mandarin EOs. This concentration of 0.8 µl/mL was not effective against other tested EOs. At highest concentration of 120 µl caused ≥ 40% mortality with peppermint, mandarin, tea tree and thyme: oregano EOs respectively with 24 h incubation period.

After 48 h incubation period, 100% mortality was observed with 10 µl of tea tree and 1.6 µl/mL of peppermint and basil EOs. At same incubation period highest tested concentrations of 2.4 µl/mL caused 77, 64, 44 and 65% of mortality with cinnamon, mandarin thyme and combination of oregano: thyme EOs. However, oregano EOs showed significantly ($p \leq 0.05$) least efficiency (14%) as compared to other tested EOs. After 72 h incubation period, 100% mortality was observed with 0.8 µl/mL of basil and peppermint EOs and 120 µl of cinnamon and oregano: thyme EOs. At 72 h incubation period, mandarin, thyme and oregano EOs caused 77, 80 and 33% mortality respectively at 2.4 µl/mL concentration.

The biological activity of EOs is generally related to the levels and volatility of various major and minor chemical constituents (Bachrouch *et al.*, 2015). The major volatile components of eucalyptus, tea tree and peppermint are primarily 1, 8 cineole 79.18%, terpineol 38.4% and menthol 33.38%, respectively. These components have been reported to exhibit high toxicity against a number of stored insect pests (Abdelgaleil *et al.*, 2009b, Michaelraj *et al.*, 2008, Padalia *et al.*, 2015). Abdelgaleil *et al.* (2009b), Nattudurai *et al.* (2012) showed in two separate studies, that the oxide monoterpene 1-8-cineole possesses strong fumigant toxicity against *S. oryzae*. The high mortality induced by eucalyptus, tea tree and peppermint treatments in present study may thus be linked to these major bioactive constituents. In addition the biological activity of EOs may be attributed to individual and/or blend effects of the biochemical constituents contained in the EOs. This has been explored by various authors such as Bakkali *et al.* (2008), Ogendo *et al.* (2008). The efficient biological activity of eucalyptus could have resulted from a single secondary metabolite but is most likely to have been induced by the combined effect of its major chemical constituents such as 1,8-cineole, terpineol and methol. These chemical components with others such as camphor, linalool, thymol, borneol, limonene α-pinene p-cymene, and γ-terpinene, have indeed been associated with strong fumigant toxicity against various insect pests in previous studies (Rozman *et al.*, 2007, Shaaya *et al.*, 1991)

Nonetheless, the binary mixture of oregano: thyme showed potential fumigant toxicity against *S. oryzae* as compared to individual treatment (oregano and thyme alone) during 72 h incubation period. No interactions ($P > 0.05$) were observed between lower concentrations of (0.2, 0.4, 0.8 and 1.6 µl/mL) oregano and thyme individually or in combinations after 24 h. However, the activity increased significantly ($P \leq 0.05$) with time and concentrations. A quantity of 2.4 µl/mL of combinations of thyme: oregano caused 40% mortality which is significantly ($P > 0.05$) higher as compared to individual effect of thyme (19%) and oregano (11%) EOs at same concentration, after 24 h. After 48 h of incubation period a quantity of 0.4, 0.8 and 1.6 µL/mL showed enhanced ($P \leq 0.05$) mortality against *S. oryzae* than individual effect of thyme and oregano EOs alone. Similar enhanced activity was observed with 0.2-2.4 µL/mL of combinations of thyme: oregano at 72 hr as compared to individual effect at same concentrations. Combination of thyme: oregano caused 100% mortality at a concentration 2.4 µL/mL as compared to 80 and 33% mortality with thyme and oregano EOs respectively at same concentration when applied alone. According to Koul et al. (2013), combination of compounds may show a broader spectrum of insecticidal

activity due to the presence of a wider range of bioactive components. An oregano and thyme combination treatment also showed significant synergistic activity against *Spodoptera littoralis* and *Culex quinquefasciatus* larvae (Pavela, 2015). Koul *et al.* (2013) reported that alcohols and phenols are more active in combinations than in their individual forms. Hence, it can be deduced that not all components in EOs have the ability to display synergistic effects when applied in combinations. Pavela (2014) tested thirty aromatic compounds for acute toxicity against *Spodoptera littoralis* larvae, and found that only γ-terpinene, limonene, p-cymene, trans-anethole, borneol and camphor, were able to induce a synergistic effect on mortality. According to Faraone *et al.* (2015), synergism between naturally-occurring EOs is not uncommon in plants growing in their natural habitats. Plants have been shown to defend themselves against herbivores using a suite of secondary metabolites rather than individual ones. Hence, even minor EO constituents present at low concentrations may be potentially very active in combination and even assist major constituents in enhancing their toxic effects through a variety of mechanisms (Bassolé *et al.*, 2012, Hyldgaard *et al.*, 2012). Hence, in the present study, binary combination of oregano: thyme EOs showed synergistic activity when combined, due to the combined activities of two or more components of EOs. However, the biological activity of EOs varies greatly on the chemical composition of EOs, which is specific to harvesting and extraction condition (Hossain *et al.*, 2016).

5.3.2. Inhibition of acetylcholinesterase (AChE) activity

Although research in the field is ongoing, little is known about the physiological actions of essential oils and their constituents on insects. However, application of plant-derived EOs or their constituents induces symptoms that suggest a neurotoxic mode of action (Kostyukovsky *et al.*, 2002, Rana *et al.*, 2010). In the present study the inhibitory effects of the individual and binary EO combination (oregano:thyme) were investigated on the activity of AChE extracted from *S. oryzae*. All individual EOs and EO combination showed AChE inhibitory activity (Fig. 5.1). Among all the tested EOs, broadly three groups displaying different degree of inhibition could be distinguished. Combinations of oregano: thyme, eucalyptus, oregano and thyme were found to exert the highest inhibitory effect on the catalytic activity of AChE (26-37%). Another group comprising of mandarin (22%) and tea tree (20%) showed relatively moderate inhibitory effect while basil, cinnamon and peppermint (5-11%) showed the least inhibitory effect on the

enzymatic activity of AChE. Binary mixtures of oregano: thyme and eucalyptus showed higher inhibitory effect indicating AChE inhibition was not always related to insect mortality. A previous study has shown that 1,8 cineole, which is a major component of eucalyptus, exhibited the highest toxicity and AChE inhibitory properties against *S. oryzae* and *T. castaneum* among a list of 11 common screened monoterpenes (Abdelgaleil *et al.* (2009b). The inhibitory effect showed in the current study by the tested EOs advocate their role in exerting a neurotoxic mode of action disrupting neurotransmission in insects. Ryan *et al.* (1988) showed that the toxic effect of EO constituents was due to the reversible competitive inhibition of acetylcholinesterase by occupation of the hydrophobic site of the enzyme's active center. Plant-derived EOs exhibit lipophilic properties and can be inhaled, ingested or skin absorbed by insects. Lee *et al.* (2003) provided evidence that contact toxicity was mainly induced through the insect cuticle while fumigant toxicity mainly occurred through the respiratory and digestive systems. Low molecular weight terpenoids are very lipophilic and may have low solubility in the hemolymph once they cross the cuticle (Veal, 1996) hence passage through the trachea appears to be a more plausible route of entry. Once absorbed in the body, the EO molecules can bind to protein receptors and interrupt normal neurotransmission causing paralysis and death (Priestley *et al.*, 2006). However, the high AChE inhibitory activity observed in this study was not necessarily correlated with high fumigant mortality. For example, oregano showed relatively low fumigant toxicity against *S. oryzae* but displayed high AChE inhibitory activity alone and in combination with other EOs. Although some studies have reported a relationship between insecticidal and AChE inhibitory activities of EO components such as terpenoids (Ryan *et al.*, 1988), others found no direct correlation between insect toxicity and AChE inhibition by plant-derived EOs (Picollo *et al.*, 2008). Hieu *et al.* (2012) studied the insecticidal effect of *Zanthoxylum* plant extracts on the fly *Stomoxys calcitrans* (L.), and found that although strong AChE inhibition was produced by citronellyl acetate, alpha-pinene, thymol, carvacrol and alpha-terpineol, no direct correlation could be observed between fly toxicity and AChE inhibition by the test compounds. This suggests that, in addition to inducing AChE inhibition, various EO components may have other target sites. Ketoh *et al.* (2002) and Lee *et al.* (2001b) showed that monoterpenes may interfere with cytoplasmic membrane functioning and affect cytochrome P450-dependent monooxygenases. Enan (2001) showed that octopamine receptors of insects were more sensitive than AChE to some oils, e.g. pulegone. EOs have also been found to increase cAMP production

and disrupt the octopaminergic and circulatory systems in insects. The toxicity of various EOs on insects may be attributed to the nature of their inherent chemical constituents. As shown by Ayvaz *et al.* (2010) EOs from oregano (*Origanum onites*), savory (*Satureja thymbra*) and myrtle (*Myrtus communis*) induced different degrees of toxicity against the Mediterranean flour moth (*Ephestia kuehniella*), the Indian meal moth (*Plodia interpunctella*) and the bean weevil (*Acanthoscelides obtectus*) depending on their chemical constituents. The extent of induced activity may also result from the relative adsorption of the EO components on the insect surface particularly for insects at the larval stage (Enan, 2001). Furthermore, it has been shown that different insecticidal efficacies of the same oil for the same insect can vary against different strains of the insects (Lee, 2002, Lee *et al.*, 2000). However in present study, all tested EOs showed showed AChE inhibitory activity.

5.3.3. Insecticidal effect of biopolymeric film alone and in combination with gamma radiation

The results of EO loaded bio polymeric films are presented in Fig 5.2. The results showed that CH/CNC, MC/CNC and PLA/CNC based polymer matrices containing the oregano:thyme EO exhibited insecticidal activities to varying extent against *S. oryzae*. Bioactive chitosan based films showed highest insecticidal activities among other tested films. Bioactive PLA and MC based films caused 4, 13, 20% and 5, 20 and 27% mortality at day 3, 7 and 10 respectively whereas CH based films caused 8, 27 and 43% mortality during respective days. Bioactive PLA, MC and CH based films induced 72, 80 and 87% higher mortality as compared to control samples after 10 days of incubation period. Biopolymers such as PLA, MC and CH films are excellent matrices for incorporating a wide variety of functional additives. The encapsulated EOs (oregano and thyme) diffused progressively from developed polymeric matrices showed strong insecticidal efficiencies against *S. oryzae*. The insecticidal effect of biopolymer based films incorporated with EO nanoemulsions may have been due to chemical interactions between functional groups of the polymeric matrices and the constituents of EO mixtures.

Active packaging, containing plant-derived EOs have been investigated by many researchers found effective in reducing insect infestations (Licciardello *et al.*, 2013). A study conducted by Allahvaisi (2010) investigated the repellent efficacy of EO of *Prunus amygdalus* L. and *Mentha viridis* L. against four types of stored product named as *T. castaneum, S. granaries, S. paniceum,*

and *R. dominica* by spraying the EOs onto the interior surface of polyethylene (PE) packages. The EOs from *P. amygdalus* and *M. viridis* EO found effective, by reducing the contamination of the packaged food by 79 and 64%, against *T. castaneum*, and *S. granarius*, respectively as compared to the control. These results clearly show that it is possible to utilize EO loaded active packaging to control stored food product insect pests.

The results of EO loaded CH based bio polymeric films in combination of gamma radiation presented in Fig 5.2. The results showed that the mortality increased significantly while applying gamma radiation ranging between 100-300 Gy. Chitosan-based films containing 0.75% of EO alone induced 42.5% mortality after 10 days of incubation. The mortality increased to 9, 28 and 55% with combination of 100, 200 and 300 Gy of gamma radiation respectively as compared with bioactive chitosan film. The combined treatment of bioactive films and ionizing radiation was more efficient against *S. oryzae.* It has also been well established that irradiation in combination with other treatments has an enhanced effect on stored product pest. In accordance Ahmadi *et al.* (2009) reported a synergistic effect of gamma radiation and *P. atriplicifolia* essential oil on stored pest *T. castaneum* adults. They found that 100 Gy dose alone can caused 12.5% mortality but when combined with 7.66 µL/mL air of oil the mortality increased to 32.5% after 7 days of incubation period. The combined effect of gamma radiation and malathion pesticide on flour beetle, *Tribolium confusum* was studied by Ayvaz *et al.* (2002) and found that combined methods are more efficient to control *T. confusum* species. Hossain *et al.* (2014b) evaluated the combined effects of basil EO vapor and gamma radiation on rice weevils. The data obtained in their study showed that rice weevils exposed to 20 and 40 µL/mL of basil EO were 4.8 and 6.2 times more sensitive to irradiation respectively compared to control weevils treated only by irradiation after 120 h exposure period (*in vitro* tests). *In situ* tests showed that100% mortality was observed with 2.67µL/g basil EO and 250 Gy of irradiation dose and 1.6 µL/g basil EO and 200 Gy of irradiation dose in paper and plastic bags containg rice grains respectively after 120 h post treatment period.

Our results provide the basis for successful application of gamma radiation in the presence of active films for controlling stored grain pests. Although numerous studies focused on the use of plant essential oil to protect stored grains, there is a lack of sufficient data on the relative sensitivities of these bioactive films against stored pests. The present data show that bioactive films loaded with EO and irradiation can be used as an effective combined method to increase

the radio sensitivity of *S. oryzae*. It also reveals that these two methods can be combined as an additional control approach to reduce the insect population on stored product.

5.4. Conclusion

The current study showed the insecticidal activity of eight individual EOs and oregano: thyme EO combinations against *S. oryzae* through fumigant toxicity assays. While some EOs displayed strong toxicities against the test specimen, like eucalyptus, others showed moderate activity. All eight individual and one EO combination inhibited AChE activity. However, AChE inhibition was not always related to insect mortality. This implies that EO components may exert their effects through other mechanisms in addition to disrupting the cholinergic system of the insects. The findings of this study highlight the insecticidal potential of EOs, and support the use of plant-derived EOs as alternatives to synthetic fumigants to control stored grain products in a sustainable way. In addition successful implementation of oregano: thyme EO encapsulation was demonstrated in CH, MC and PLA polymer matrices. The bioactive films showed significant insecticidal toxicity during 10 days of incubation period. The combined treatment of bioactive films and ionizing radiation has an enhanced effect on stored product pest.

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Table 5.1. Plant essential oils (EOs) and their major active components provided by the manufacturer Pranarôm International, Belgium.

Common name	**Latin name**	**Origin**	**Distilled part**	**Main composition (Area %)***
Basil	*Ocimum basilicum*	Belgium	Flower top	77.60% Methyl carvacrol, 20.30% linalool
Cinnamon	*Cinnamomum zeylandicum*	China	Wood	98% linalool
Eucalyptus	*Eucalyptus globus*	China	Leaf	79.18% 1,8 cineole 6.33% limonene
Mandarin	*Citrus reticulate*	Brazil	Zest	71.43% limonene 17.70% γ terpinene
Oregano	*Origanum vulgare*	Bel ium	Flower top	50.9% Carvacrol 16.8% Thymol 11.6% γ-Terpinene 14.7% p- ymene
Peppermint	*Mentha piperita*	India	Aerial part	33.38% menthol 34.31% menthone, 6.34% 1,8 ineole
Tea tree	*Melaleuca alternifolia*	Belgium	Leaf	38.4% Terpinen-4-ol 22.6% γ terpinen 8.1% α terpinen
Thyme	*Thymus vulgaris*	Spain	Flower top	26.04% thymol 16.69% γ-terpinene 26.36% p- Cymene

*According to the gas chromatography analysis of essential oils provided by the manufactu

1 5. 2. Insecticidal activities of different concentration of eight EOs against *S. oryzae* during 72 hour of storage period.

Hr	µl/mL	% mortality								
		Basil	Cinnamon	Eucalyptus	Mandarin	Oregano	Peppermint	Tea tree	Thyme	Thyme:oregano
24	0.2	$0\pm0^{a,A}$	$0\pm0^{a,A}$	$17.33\pm2.82^{c,A}$	$3.55\pm0.25^{b,A}$	$0\pm0^{a,A}$	$2.22\pm0^{b,A}$	$0\pm0^{a,A}$	$0\pm0^{a,A}$	$0\pm0^{a,A}$
	0.4	$1.77\pm0^{a,A}$	$0\pm0^{a,A}$	$57.33\pm3.85^{d,B}$	$11.11\pm0.85^{c,B}$	$0\pm0^{a,A}$	$5.33\pm0.95^{b,A}$	$2.22\pm0^{a,A}$	$2.22\pm0^{a,A}$	$0\pm0^{a,A}$
	0.8	$16.00\pm2.82^{a,B}$	$4.00\pm0^{a,B}$	$100\pm0^{e,C}$	$18.66\pm2.82^{d,C}$	$2.66\pm0^{a,A}$	$13.33\pm1.23^{b,B}$	$2.66\pm0^{a,A}$	$4.88\pm0.56^{a,B}$	$0\pm0^{a,A}$
	1.6	$24.88\pm3.12^{c,C}$	$6.22\pm0.85^{a,B}$	$100\pm0^{e,C}$	$25.77\pm2.90^{bc,D}$	$6.22\pm0.85^{a,B}$	$34.22\pm2.05^{d,C}$	$25.33\pm2.82^{bc,B}$	$4.00\pm0^{a,B}$	$3.55\pm0^{a,B}$
	2.4	$32.88\pm0.85^{d,D}$	$7.55\pm0.85^{a,BC}$	$100\pm0^{h,C}$	$57.33\pm4.23^{f,E}$	$11.55\pm0.85^{b,C}$	$64\pm3.46^{g,D}$	$44.88\pm4.37^{e,C}$	$19.11\pm2.66^{c,C}$	$40.00\pm3.89^{e,C}$
48	0.2	$4.00\pm0^{c,A}$	$0\pm0^{a,A}$	$100\pm0^{e,A}$	$5.77\pm0.85^{d,A}$	$0\pm0^{a,A}$	$2.22\pm0^{b,A}$	$100\pm0^{e,A}$	$3.11\pm0^{bc,A}$	$0\pm0^{a,A}$
	0.4	$15.11\pm2.66^{e,B}$	$0\pm0^{a,A}$	$100\pm0^{f,A}$	$11.11\pm0.85^{d,B}$	$2.22\pm0^{b,A}$	$4.00\pm0^{bc,A}$	$100\pm0^{f,A}$	$5.33\pm0.85^{c,A}$	$0\pm0^{a,A}$
	0.8	$30.66\pm3.30^{e,C}$	$29.33\pm4.22^{e,B}$	$100\pm0^{f,A}$	$21.77\pm2.90^{d,C}$	$6.66\pm2.82^{a,B}$	$17.33\pm0.85^{c,B}$	$100\pm0^{f,A}$	$13.77\pm0.85^{b,B}$	$15.11\pm0.85^{c,B}$
	1.6	$100\pm0^{e,D}$	$45.33\pm3.85^{d,C}$	$100\pm0^{e,A}$	$38.66\pm2.82^{c,D}$	$8.44\pm3.12^{a,B}$	$100\pm0^{e,C}$	$100\pm0^{e,A}$	$22.22\pm0.85^{b,C}$	$23.33\pm0^{b,C}$
	2.4	$100\pm0^{e,D}$	$77.77\pm4.05^{d,D}$	$100\pm0^{e,A}$	$64.44\pm3.12^{c,E}$	$14.22\pm2.90^{a,C}$	$100\pm0^{e,C}$	$100\pm0^{e,A}$	$44.88\pm3.33^{b,D}$	$65\pm0^{c,D}$
72	0.2	$30.22\pm4.52^{e,A}$	$10.66\pm0.85^{c,A}$	$100\pm0^{f,A}$	$6.66\pm0.85^{b,A}$	$0\pm0^{a,A}$	$13.22\pm085^{d,A}$	$100\pm0^{f,A}$	$4.88\pm0.85^{b,A}$	$0\pm0^{a,A}$
	0.4	$54.22\pm3.52^{f,B}$	$23.11\pm3.33^{e,B}$	$100\pm0^{g,A}$	$14.66\pm2.82^{c,B}$	$2.22\pm0^{a,A}$	$19.11\pm3.33^{d,B}$	$100\pm0^{g,A}$	$5.77\pm0.85^{b,A}$	$7.11\pm0.33^{b,B}$
	0.8	$100\pm0^{e,C}$	$45.77\pm4.05^{d,C}$	$100\pm0^{e,A}$	$28.44\pm1.77^{c,C}$	$8.33\pm0^{a,B}$	$100\pm0^{e,C}$	$100\pm0^{e,A}$	$13.77\pm0.85^{b,B}$	$29.33\pm2.82^{c,C}$
	1.6	$100\pm0^{e,C}$	$63.55\pm3.17^{d,D}$	$100\pm0^{e,A}$	$50.77\pm3.57^{b,D}$	$21.77\pm3.52^{a,C}$	$100\pm0^{e,C}$	$100\pm0^{e,A}$	$57\pm4.0^{c,C}$	$59.25\pm0^{d,D}$
	2.4	$100\pm0^{d,C}$	$100\pm0^{d,E}$	$100\pm0^{d,A,A}$	$77.77\pm3.05^{b,E}$	$33.33\pm2.00^{a,D}$	$100\pm0^{d,C}$	$100\pm0^{d,A}$	$80.00\pm3.50^{c,D}$	$100\pm0^{d,E}$

2

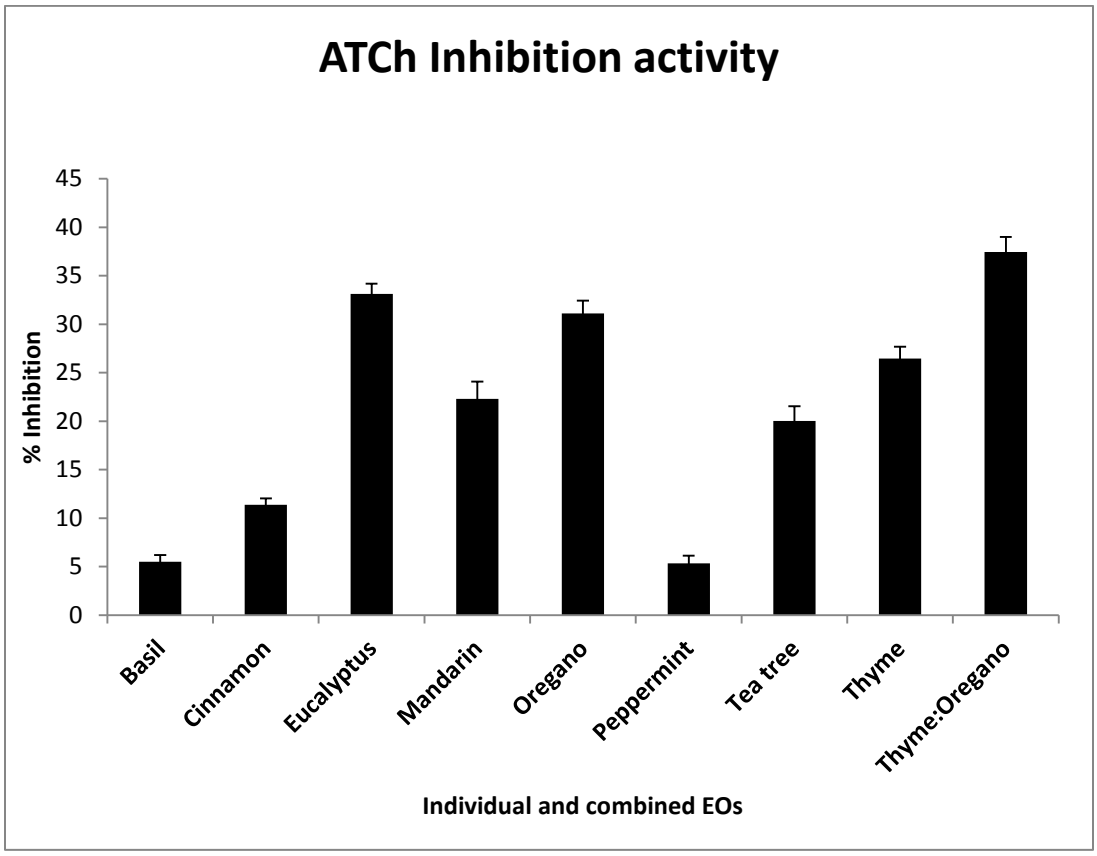
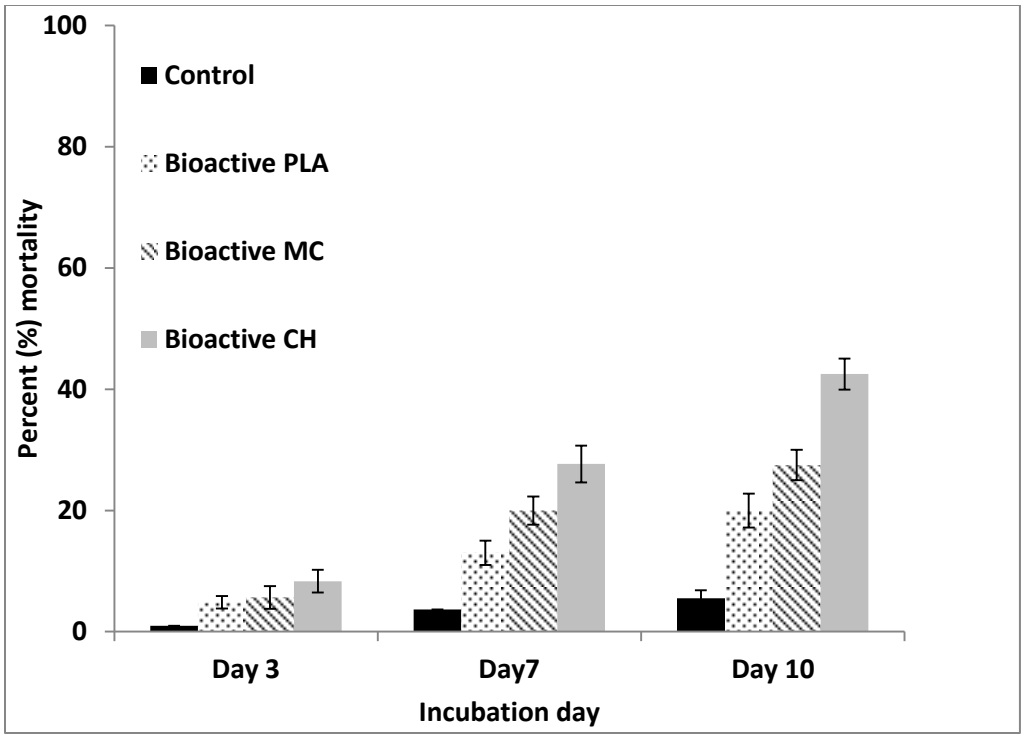


Figure 5.1. Inhibition (%) of *S. oryzae*-derived AChE activity by individual and combined EOs.



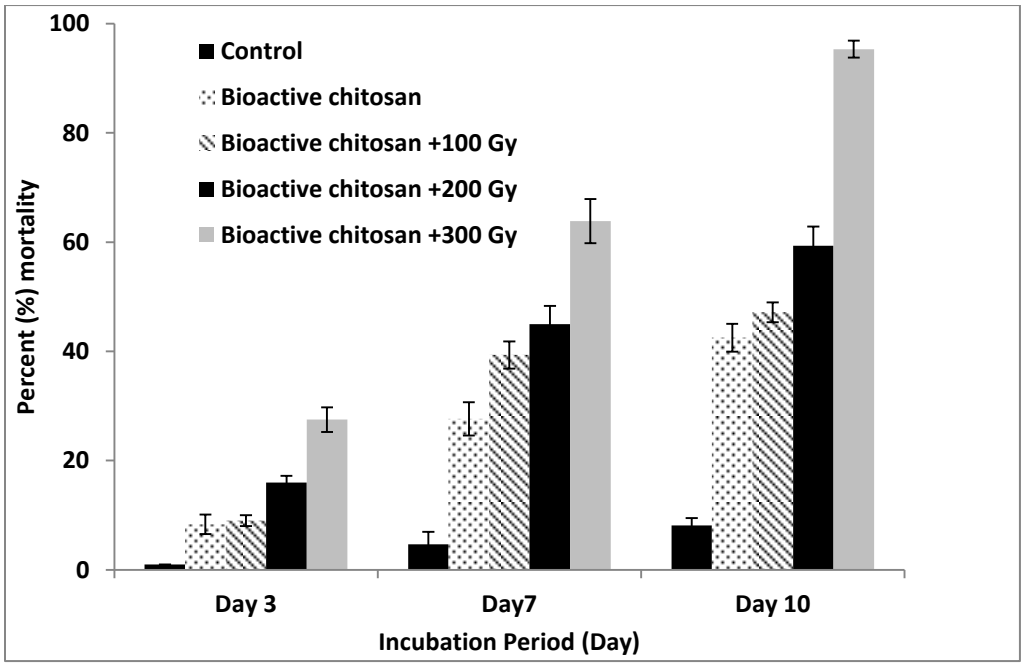


Figure 5.2. Percent (%) mortality of *S. oryzae* in rice grain treated with i) bioactive CH, MC and PLA film ii) bioactive CH film and gamma radiation at doses of 100, 200 and 300 Gy

Chapter 6

Publication 5

Antifungal activity of combined treatments of active methylcellulosic based films containing encapsulated nanoemulsion of essential oils and γ –irradiation: *in vitro* and *in situ* evaluations

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Contribution of the authors

Most of the research work was planned and performed by Farah Hossain. Prof. Lacroix contributed and supervised the elaboration of the experimental design protocols, she has done the corrections of the paper, participated on the scientific discussion. Dr. Khanh Vu helped in experimental design and statistical analysis. Stephane Salmieri and Dr. Majid helped to develop the protocol of release properties. Dr. Follett has participated to the discussion, corrections of the papers, revised the articles.

Résumé

Une technique d'homogénéisation à haute pression, la microfluidisation, a été utilisée pour développer un nanocristal de cellulose (CNC) hautement dispersé comme agent de renforcement dans des films nanocomposites à base de méthylcellulose contenant une émulsion de mélange d'huiles essentielles végétales (origan: thym). Un modèle expérimental à trois facteurs a été utilisé pour optimiser systématiquement la pression de microfluidisation en fonction de la taille et de l'activité antifongique de l'émulsion préparée. Les résultats ont montré que la microfluidisation des dispersions formant un film (FFD) fournit une nouvelle approche pour le développement de films de bionanocomposite de haute résistance par une distribution homogène des nanoémulsions CNC et HE dans les matrices polymères. L'incorporation de 7.5% de CNC dans MC contenant 0.50 -0.75% d'HE et l'application d'une pression de 15,000 psi crée une nanoémulsion avec une taille de particule ≤ 100 nm qui présente une activité antifongique significative *in vitro* contre *Aspergillus niger*, *A. flavus*, *A. parasiticus* et *P. chrysogenum*. Des essais *in situ* avec des films bioactifs à base de MC / CNC contenant une émulsion d'HE ont permis une réduction de 2 log de la croissance fongique dans du riz inoculé pendant 8 semaines de stockage à 28 °C. De plus, le traitement combiné de films bioactifs et un traitement d'irradiation à 750 Gy a montré des propriétés antifongiques plus prononcées que le traitement avec le film bioactif ou l'irradiation seul. L'ajout de CNC comme charge renforçante améliore la résistance à la traction des films à base de nanocomposite de 30% et réduit les propriétés de barrière à l'eau de 4%. Des films de nanocomposite de méthylcellulose contenant de la nanoémulsion d'HE ont montré une libération lente (35%) de composant volatil sur une période de stockage de 12 semaines. En outre, la libération de composants volatils du film était 25% plus lente avec l'ajout de CNC. Ces résultats montrent le potentiel des films à base de méthylcellulose chargés en HE en tant qu'emballage bioactif pour limiter la détérioration des aliments et de prolonger leur durée de conservation.

Abstract

A high-pressure homogenization technique, microfluidization, was used to develop highly dispersed cellulose nanocrystal (CNC) as a reinforcing agent in methyl cellulose (MC) based nanocomposite films containing a plant essential oil (EO) blend (oregano: thyme) emulsion. A three factorial experimental design was used to systematically optimize the microfluidization pressure based on size and antifungal activity of the prepared emulsion. Results showed that microfluidization of the film forming dispersions (FFD) provides a novel approach for the development of high strength bionanocomposite films by homogenous distribution of CNC and EO nanoemulsions into polymer matrices. Incorporation of 7.5 % CNC into MC containing 0.50 -0.75 % EO and application of a pressure of 15000 psi created a nanoemulsion with particle size ≤ 100 nm which exhibited significant antifungal activity *in vitro* against *Aspergillus niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum*. *In situ* tests with MC/CNC based bioactive films containing EO emulsion produced a 2 log reduction in fungal growth in infected rice during 8 weeks of storage at 28°C. In addition, combined treatment of bioactive films with an irradiation treatment at 750 Gy showed more pronounced antifungal properties than treatment with the bioactive film or irradiation alone. The addition of CNC as reinforcing filler improved the tensile strength of the nanocomposite based films by 30% and decreased water barrier properties by 4%. Methyl cellulose nanocomposite films containing EOs nanoemulsion showed a slow release (35%) of volatile component over 12 weeks of storage period. In addition the release of volatile component from film was 25% slower with the addition of CNC. These results show the potential for EO-loaded methyl cellulose-based films as bioactive packaging to limit food spoilage and prolong shelf life.

6.1. Introduction

Cereal grains and their processed food products are frequently contaminated with fungi and their associated toxic metabolites. The genus *Aspergillus* and *Penicillium* are well-known storage fungi and have ability to produce various kinds of mycotoxins that can cause acute and chronic health problems in humans and in farm animals (Oliveira *et al.*, 2014b). Mycotoxins are extracellular metabolites of filamentous fungi that can be carcinogenic, mutagenic, genotoxic, teratogenic, neurotoxic, and oestrogenic. Long term ingestion of contaminated food can deteriorate liver and kidney functions and in extreme cases may lead to death (Hossain *et al.*, 2017, Lee *et al.*, 2017, Oh *et al.*, 2007). Active and intelligent packaging is an essential component of the food processing chain in reducing fungal contamination and proliferation, ensuring food safety, maintaining quality, and extending shelf-life of stored food commodities (Opara *et al.*, 2013).

One of the current trends in food packaging research involves the development of bioactive packaging containing additives such as antioxidants, antimicrobials, vitamins, flavours and colorants with the goal of extending the shelf-life of food products while maintaining the nutritional, sensorial and microbiological standards during storage (Moura *et al.*, 2012, Cozmuta *et al.*, 2015, Ramos *et al.*, 2014). Designing a novel and eco-friendly bioactive packaging involves the use of natural polymers and additives that have no negative impact on human health or the environment and reduce the carbon foot-print associated to packaging production (Atarés *et al.*, 2016, Cozmuta *et al.*, 2015).

The effectiveness of essential oils (EO) derived from plant origin against spoilage microorganisms and food-borne and post-harvest pathogens are well documented. EO have great potential in replacing synthetic preservatives to satisfy consumers' requirements for natural products (Calo *et al.*, 2015, Prakash *et al.*, 2015). Our previous studies showed that an EO combination of oregano and thyme has strong activity against four fungal species namely *Aspergillus niger*, *A. flavus*, *A. parasiticus* and *Penicillium chrysogenum*. The main component of these EO are carvacrol and thymol (Hossain *et al.*, 2016). These components have exhibited antifungal properties in many studies (Nostro *et al.*, 2012, Santonja *et al.*, 2013). Despite the

high efficacy of the EO, their use in food products is often limited due to their strong flavor and rapid degradation after exposure to heat, humidity, light, or oxygen (Avila-Sosa *et al.*, 2012, Ben-Fadhel *et al.*, 2017, Bilia *et al.*, 2014, Ruiz-Navajas *et al.*, 2013). Encapsulation of EO in an edible packaging or diffusion films (Boumail *et al.*, 2013, Severino *et al.*, 2015) could serve as carriers to release the active agents over time and avoid problems with rapid degradation (Avila-Sosa *et al.*, 2012).

The most common materials for the formulation of edible/biodegradable films and coatings are polysaccharides, proteins and lipids, and blends of these compounds may allow for improved functionality (Atarés *et al.*, 2016). Polysaccharide based packaging such as cellulose, cellulose derivatives, carrageenan, agar, chitosan, pectin, starch and alginate have been used for making biodegradable antimicrobial packaging films and they are attractive due to their better film forming properties, moderate oxygen and moisture permeability, and unique colloidal nature. Methylcellulose (MC) is a derivative of cellulose produced from cotton cellulose, wood, or annual plant pulps. As a food grade polymer, MC has excellent film forming properties and the ability to carry active agents (Campos *et al.*, 2014, Otoni *et al.*, 2014b). Nevertheless, some of their properties must be improved to position them as potential candidates that can compare with fossil fuel derivatives. The blending of biopolymers and/or adding of nanofillers represents an effective way to improve the properties of biopolymers, and thereby broaden their application (Reddy *et al.*, 2013; Rhim *et al.*, 2013). Bio-nanocomposite technology with low loading of nanofillers (5 wt %) has already been proven as an effective way to produce new biomaterials with specific properties and high performances for packaging applications (Miri *et al.*, 2015). Cellulose nanocrystals are recognized as being more effective than their microsized counterparts to reinforce polymers due to interactions between the nanosized elements that form a percolated network connected by hydrogen bonds, providing good dispersion of antimicrobial compounds in the matrix and better efficiency, stability, and controlled release of the active agents to the food surface (Huq *et al.*, 2012, Khan *et al.*, 2014a, Khan *et al.*, 2012b).

Irradiation has another option to reduce stored products pathogens, and, in combination with other treatments such as EO, can suppress the growth of surviving micro-organism during

storage (Ben-Fadhel *et al.*, 2017, Hossain *et al.*, 2014a, Lacroix *et al.*, 2015). Here we report to 1) optimize the homogenization pressure, reinforcing agent (CNC) and EO (binary mixture of oregano and thyme) concentrations to prepare methyl cellulose based edible films through the characterization of the stability related parameters such as particle size, zeta-potential of the film forming dispersions and the antifungal properties against four fungal species *A. niger*, *A. flavus*, *A. parasiticus* and *P.chrysogenum* of the films; and 2) explore the possible application of a combined treatment of irradiation and EO-encapsulated films to protect bagged cereal grains during storage against these fungi.

6.2. Materials and Methods

6.2.1. Essential oils (EOs)

Essential oil of oregano (*Origanum compactum*; Moroccan oregano) and thyme (*Thymus vulgaris*) were obtained from Robert & Fils, (Ghislenghien, Belgium) and stored at 4 ˚C prior to use. The chromatogram of EO obtained from the manufacturer showed that oregano oil contained 46.37% carvacrol, 13.7% thymol, 13.33% *p*-cymene, 12.32% ɣ-terpinene and thyme oil contained thymol 26.04%, *p*-Cymene 26.36%, γ- terpinene 16.69%.

6.2.2. Film ingredient

Methyl Cellulose (MC) (M_n ~ 40,000; viscosity 400 cP, 2% in water at 20°C) was obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Crystalline nanocellulose (CNC) was provided by FPInnovations (Pointe Claire, QC, Canada). Glycerol, used as a plasticizer, Tween®80 and soy lecithin, used as an emulsifier, were purchased from LabMat (Beauport, QC, Canada).

6.2.3. Preparation of film forming dispersion

6.2.3.1. Preparation of CNC suspension

At first 1% CNC suspension was prepared by dispersing spray dried CNC powder (FPInnovations, Pointe-Claire, QC, Canada) in distilled water with magnetic stirring. The

suspension was then subjected to ultra-sonication of CNC at 1000 j/g. The CNC suspension was then allowed to stir under magnetic stirrer for 2 hour before it was stored at 4˚C.

6.2.3.2. Preparation of MC matrix

A quantity of 2.5% MC was solubilized in distilled water, under stirring at 40-50°C (for pre-gelatinization) and was then cooled in an ice bath to ensure complete solubilization. The MC solution was then magnetically stirred overnight followed by homogenization with IKA RW-20 mechanical homogenizer at 1,500 rpm for 5 hrs. A quantity of 0.5% glycerol was added during homogenization. After homogenization, a paraffin film was wrapped on top of the beaker and kept for few hours to remove all the bubbles.

6.2.3.3. Preparation of Essential oil emulsion

A quantity of 5% of tested essential oil (oregano and thyme) by Robert et Fils (Montreal, Quebec, Canada) were prepared using lecithin and tween 80 as an emulsifier. The EO: Tween 80: lecithin ratio were 1:0.75: 0.25with respect to EO concentration. To obtain a homogenous colloidal suspension the mixture was homogenized by using Ultra- Turrax homogenizator at 15,000 rpm for 2 min.

6.2.3.4. Dispersion of antimicrobials in the CNC and MC dispersion

Desired quantitites of CNC, MC and antimicrobial emulsions were then homogenized at room temperature, using a digital Ultra-Turrax T25 disperser (IKA® Works Inc., Wilmington, NC, USA), at 15,000 rpm for 2min. The resulting solutions were kept in a beaker prior to further homogenization or microfluidization.

6.2.3.5. Non-microfluidized film forming dispersion

Samples without microfluidization were prepared by mixing the film forming solution using a digital Ultra-Turrax T25 disperser, at 15,000 rpm prior to obtaining a homogenous suspension. After that, a paraffin film was wrapped on top of the beaker and kept at 4˚ C for few hours to remove all the bubbles.

6.2.3.6. Microfluidized film forming dispersion

The three level full factorial design was made by considering three levels of independent factors (variables) in the experimental design are presented in the Table 6.1. The independent factors were pressure, EO and CNC concentrations. The experimental design consisted of 27 runs (formulations) and the data of particle size, Zeta-potential and antifungal activity against 2 target fungi are presented in Table 2. The experimental runs were conducted in the microfluidizer and to obtain a homogenous film forming dispersion, the microfluidizer cycles were fixed at 5 after making some preliminary tests. The antimicrobial CNC/MC suspensions were introduced in the inlet reservoir of the microfluidizer (Microfluidics Inc., Newton, MA, USA). Ice was placed in the cooling jacket in order to negate overheating of the suspensions due to microfluidization. According to the experimental design, the CNC/MC suspensions were subjected to different microfluidization pressures and number of cycles in a continuous operation mode. The resulting solutions were kept at 4˚ C for few hours to set the film forming solution before casting the films.

6.2.3.7. Preparation of films

Composite films were cast by applying 12 mL of the film-forming suspension onto Petri dishes (95 × 15 mm; Fisher Scientific, Ottawa, ON, Canada) and allowed to dry for 24 h under chemical hood at room temperature. MC-based films containing no antimicrobials were designated as MC-control.

6.2.4. Characterization of the film-forming dispersions

Particle size, polydispersity index and Zeta-potential of nanoemulsions

The average particle size, size distribution and ζ-potential of nanoemulsions prepared in different experimental runs in the experimental design were determined by dynamic light scattering using a photon correlation spectroscopy (Malvern Zetasizer Nano-2S, Model ZEN3600). Measurements were carried out with a scattering angle of 173° at a constant temperature (25 °C). All samples were diluted approximately 10 times with distilled water prior to each measurement to avoid multiple light scattering effects. The particle size of the nanoemulsions was described by the mean (z-average) diameter, and the size distribution was indicated by the polydispersity

index (PdI). The oil droplet size (nm) was characterized by distribution curves in intensity (%), average droplet size and polydispersity index. A polydispersity index near to 1 indicates a heterogeneous distribution between sizes of droplets. The z-potential of oil droplets was measured to determine the surface charge at the interface of the droplets dispersed in the biopolymeric solution.

6.2.5. Fungicidal characterization of the films

6.2.5.1. Fungal Inocula and Assay media

Aspergillus niger (ATCC 1015), *A. flavus* (ATCC 9643), *A. parasiticus* (ATCC 16869), and *Penicillium chrysogenum* (ATCC 10106) were used for antifungal assays. Each fungal species was grown and maintained in potato dextrose broth (PDB, Difco, Becton Dickinson) containing glycerol (10% v/v). Prior to each experiment, stock cultures were propagated through two consecutive 48 h growth cycles in PDB medium at 28˚C ± 2 ˚C. The cultures were pre-cultured in PDA for 3/4 days at 28 ˚C ± 2 ˚C. Conidia were isolated from the agar media using sterile saline containing 0.05% Tween 80. Mycelia were removed by filtration through a 40 µm cell strainer (Fisher Scientific), and the filtrate concentration was adjusted to 1 x 10^8 conidia/mL by using a microscope (Inouye *et al.*, 2006).

6.2.5.2. Diffusion Assays

Diffusion assays were performed based on the method described by Inouye *et al.* (2006). A sample of 1 mL containing 1 x 10^8 of conidial suspension of the four fungal species was added to 100 mL of agar medium containing 1% peptone, 1% glucose and 1% agarose at 50 ˚C. A volume of 3 mL of the prepared mixture was overlaid onto the surface of hardened PDA medium (16 mL) in a Petri dish (83 mm in diameter) to prepare a double layered agar medium. Film pieces were cut (1 cm^2) and placed at the center of the inoculated plates. All Petri dishes was sealed by laboratory parafilm and then incubated at 28˚C ± 2˚. The inhibitory diameter zone, which showed absence of growth of the test microorganisms, was measured in mm using a Traceable® Carbon Fiber Digital Caliper (resolution: 0.1 mm/0.01; accuracy ± 0.2 mm/ 0.01; Fisher Scientific). When no growth occurred, the inhibition zone was recorded as 83 mm, which is the inside diameter of the Petri dish (positive control). The percentage (%) of inhibition will be

calculated from the equation, Inhibition (%) = C-T /C ×100. Where, C is the mean of fungal growth of three replicates (mm) of controls and T is the mean of fungal growth of three replicates treated with EO.

6.2.6 Fungal inoculation of rice grains

An inoculation bath was prepared with peptone water containing 10^5 conidia/mL of *A.niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum*. A quantity of 500 g of rice grain was added to the inoculation bath and stirred gently for 30 s. After inoculation, the rice grains were dried under sterile conditions on a sheet of sterile aluminum foil for 2 h. A quantity of 30 g of the inoculated rice was packaged in a plastic bag. Two types of bioactive films containing 0.5% (MC-A) and 0.75% (MC-B) EOs nanoemulsion were inserted in each rice bag (1 gm/ cm^2). The rice samples were grouped into two subsets with one receiving irradiation at 750 Gy and one which was not irradiated. The samples were incubated at 28 ˚C for 8 weeks. The humidity inside the incubator was monitored and maintained constant at 65% throughout the experiment. Microbiological analyses of the stored rice grain were carried out on a weekly basis during storage.

6.2.7. Microbiological analyses

Microbiological analyses were performed using standard methods adopted from Hossain *et al.* (2014a). A sample of 60 mL of sterile peptone water (0.1%, w/v) was added to 30 g of rice and homogenized for 1 min at 2000 rpm using a Lab-blender 400 stomacher (Laboratory Equipment, London, UK). The resulting homogenate was serially diluted using sterile peptone water and an aliquot of 0.1 mL of each dilution was inoculated in triplicate onto the surface of freshly prepared solidified Potato Dextrose Agar (PDA). The plates were spread evenly using sterile glass spreader and incubated for 3- 4 days at 28 ˚C.

6.2.8. Release/diffusion of volatile component encapsulated in biopolymeric films during storage (*in situ*)

The controlled release of volatile component from active component was evaluated by a method described by Tunc *et al.,* (2010). Bioactive methyl cellulose based films were kept in rice grains (1 g/cm^2) incubated (at 28˚ C and 65% RH) for 12 weeks. Two types of films were tested: i) MC

based film ii) MC reinforced with CNC based film both containing 0.75% oregano: thyme EO nanoemulsion. Films were removed from the rice grains every week and cut into known sizes to maintain a constant weight sample (500 mg). Then, the films were placed into 10 ml of ethanol for 4 hr with constant agitation for the extraction of volatile componentsfrom the film matrix. The release of volatile components was measured spectrophotometrically at 274 nm. It should be noted that the initial concentrations for each type of film were determined before they were put into the chamber. Moreover, the decreased concentration in the film samples was considered as release of volatile components. By studying the plot of the UV visible absorption profile for release at 274 nm, the kinetics of the EO release was analyzed by assuming a first order release kinetics following a method described by Patil *et al.* (2016). The equation for calculation of first order velocity constant K is given below

$$K = \frac{2.303}{t} \log (A_\infty - A_0)/ (A_\infty - A_t)$$

Where A_∞, A_0, A_t refers to absorption values of infinity, initially and at time (t).

6.2.9. Physicochemical Characterization of the films

6.2.9.1. Mechanical properties of the film

The mechanical properties of the films were determined following Salmieri *et al.* (2014a). The tensile strength (TS), tensile modulus (TM) and elongation at break (Eb) of the composite films were measured using a Universal Testing Machine (UTM) (model H5KT; Tinius Olsen Testing Machine Co. USA), with a 100 N-load and 1.5 kN-specimen grips. Tensile strength (TS, maximum stress, MPa), tensile modulus (TM, elastic modulus, MPa) and elongation at break (Eb, %) values were collected after film break due to elongation, using Test Navigator® program. Film thickness was measured using a Mitutoyo Digimatic Indicator (Type ID-110Ewith resolution: 1 µm; Mitutoyo MFG Co. Ltd, Tokyo, Japan), at five random positions around the film. Film width was measured using a Traceable® Carbon Fiber Digital Caliper (resolution: 0.1 mm/0.01; accuracy: ± 0.2 mm/0.01; Fisher Scientific).

6.2.9.2. Water vapor permeability (WVP) of irradiated and no irradiated bioactive films

The WVP tests were conducted following a procedure described by Salmieri *et al.* (2014b). Briefly, films were sealed mechanically onto Vapometer cells (model 68-1; Twhing-Albert Instrument Co., USA) containing 30 g of anhydrous calcium chloride to maintain 0% RH storage condition and placed in a Shellab 9010L controlled humidity chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA). The humidity chamber was maintained at 25 ˚C and 60 % RH for 24 h. The assemblies were weighed initially and after 24 h for all samples. The weight gain of the cell determined the amount of water vapor transferred through the film and absorbed by the desiccant (anhydrous $CaCl_2$).

The values of WVP were calculated according to the combined Fick and Henry's laws of gas diffusion through coatings and films, according to the equation WVP (g mm m^{-2} day $^{-1}$ kPa $^{-1}$) = $\Delta w \times / A\ \Delta P$

Where, Δw is the weight gain of the cell (g) after 24 h, x is the film thickness (mm), A is the area of exposed film (31.67 X 10^{-4} m^2), and ΔP is the differential vapor pressure of water through the film (ΔP = 3.282 kPa at 25 ˚C) (Salmieri *et al.*, 2014b).

6.2.10. Statistical Analysis

Regression analysis was conducted to build polynomial equations based on different independent factors that affecting the size, zeta potential of nano emulsion and antifungal activity of active films. The results of the 3 factorial experimental design were analysed by STATISTICA 12 (STATSOFT Inc., Tulsa, US). The equations were expressed as:

- $Y = B_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} B_{ij} X_i X_j$

Where Y, predicted response (size; stability; or antimicrobial activity); B_0, the constant coefficient; *Xi* and *Xj*, values of various levels of the independent factor; *Bi*, the linear coefficient of each independent factor; *Bii*, the quadratic coefficient of each factor; and *Bij*, the interactive coefficient between two independent factor. All experiments were repeated three times with three replicates.

6.3. Results and Discussion

6.3.1. Microfluidization and film Quality

Microfluidization was used to fabricate the CNC reinforced bionanocomposite films containing an EO nanoemulsion. There was a clear visible difference between the microfluidized and nonmicrofluidized suspensions for each CNC concentration under processing conditions. The films prepared from nonmicrofluidized suspensions were opaque and a non-homogenous distribution of EO throughout the film surface was visible, whereas films prepared with microfluidized suspension appeared to be transparent, homogenous and even. Also, the nonmicrofluidized nanocomposite suspension exhibited aggregation and coagulation at the bottom when kept in a falcon tube for more than 1 week. Similar observations were found by Khan *et al.* (2014d). Moreover the antifungal activity of nonmicrofluidized found to be two times less (12 mm inhibition zone) than the antifungal activities of microfluidized films (20-40 mm inhibition zone) at higher EO concentration (0.75%) when tested against *A. niger*.

6.3.2. The analysis of variances (ANOVA) of the models of experimental design

6.3.2.1. The ANOVA analysis for the particle size and Zeta-potential of nanoemulsion

The results of the particles sizes and zeta potential of methylcellulose film forming dispersions and the antifungal activity of the films in the experimental design containing 27 runs is presented in table 6.2. The ANOVA analysis for the particles sizes and Zeta-potential of nanoemulsion of methylcellulose (MC) containing EO and CNC is presented in Table 3. This table includes only the linear, quadratic and interactive effects that are important for the models. The coefficients of determination (R^2) of the model for prediction of particle size and Zeta-potential were 0.98 and 0.85, respectively (Table 6. 3). These are high values which indicate that 98 % and 85 % of the variation in the size and Zeta-potential, respectively, could be explained by the combination of the response in the models.

The model for size prediction showed that only the linear effect of EO did not have significant effect ($P > 0.1$) which was excluded from the model. The linear effects of CNC ($P = 0.0001$) and pressure ($P = 0.0000$) were very importance for the size. The quadratic effect of EO was important ($P = 0.088$) but less than those of CNC ($P = 0.0165$), and pressure ($P = 0.0029$). These results indicate that the change in CNC concentrations and pressure levels will have a greater

impact than an increase in EO concentration on the particle size of nanoemulsion. It is of interest to observe that the interactive effects between two independent factors were also significant ($P \leq 0.05$) for the model to predict the particle size (Table 6.3).

The model for Zeta-potential prediction showed that the linear effects of EO, CNC are significant. It was also found that the quadratic effect of EO and CNC are not important ($P > 0.1$) for Zeta-potential and were excluded from the model, and only the quadratic effect of pressure is important ($P = 0.0018$) (Table 3). The interactive effects between EO and CNC or between EO and pressure have significant impacts ($P \leq 0.05$) on Zeta-potential.

The final regression coefficients for the equations to predict the particle size (equation 1) and Zeta-potential (equation 2) of MC nanoemulsion containing EO and CNC are as follow:

Size = $481.62 + 65.25\ X_1^2 - 46.71X_2 + 4.57\ X_2^2 + 0.02\ X_3 - 0.000001X_3^2 + 21.83\ X_1X_2 - 0.019\ X_1X_3 - 0.002\ X_2X_3$ (Eq. 1)

Zeta-potential = $-38.16 + 58.42\ X_1 + 1.17\ X_2 + 0.052\ X_3 - 0.00000X_3^2 - 4.15\ X_1X_2 - 0.004\ X_1X_3$ (Eq. 2)

6.3.2.2. ANOVA analysis for the antifungal activity of MC films against four fungi

The ANOVA analysis for the antifungal activity of prepared films containing EO nanoemulsion and CNC against 4 fungal species is presented in table 4. The ANOVA analysis for the antifungal activity of these films prepared with 27 formulations against 4 fungi is presented in Table 4. This table included only the linear and quadratic effects that are important for the model for each target fungi. The coefficient of determination (R^2) of the model for prediction of antifungal activity against *A. niger, A. flavus, A. parasiticus and P. chrysogenum* were 0.90, 0.95, 0.94 and 0.85, respectively (Table 4). These are high values which indicate that at least 85 % of the variation in antifungal activity, irrespective of fungi, could be explained by the combination of the response in the models.

Table 6.4 showed that EO is significantly important factor. It is reasonable since the EOs used in this study already demonstrated their antifungal activity (Hossain *et al.*, 2016). The effect of CNC was significant ($P \leq 0.05$) on the growth of *A. niger*; however, since CNC is one of 3 independent factors in the experimental design, its effect was kept in the models to ensure the predictable properties against tested fungi (Table 4). The pressure had linear significant impact

on the antifungal activity of MC films against 4 fungi ($P \leq 0.0005$). It is also observed that there was significantly interactive effect ($P \leq 0.005$) between EO and pressure on the antifungal activity of MC films against tested fungi.

The final regression coefficients for the equations to predict the inhibition of fungi by MC films containing EO and CNC

A. niger inhibition (mm) = $-39.22 + 163.11 X_1 - 143.11X_1^2 + 0.98X_2 - 0.00X_3 + 0.002X_1X_3$ (Eq. 3)

A. flavus inhibition (mm) = $-45.55 + 182.76 X_1 - 156.53X_1^2 + 0.344X_2 - 0.00X_3 + 0.002X_1X_3$ (Eq. 4)

A. parasiticus inhibition (mm) = $-37.96 + 166.36 X_1 - 143.73X_1^2 + 0.271X_2 - 0.00X_3 + 0.002X_1X_3$

(Eq. 5)

P. chrysogenum inhibition (mm) = $-39.54 + 195.11 X_1 - 198.4 X_1^2 + 0.636X_2 - 0.001X_3 + 0.005 X_1X_3$ (Eq. 6)

6.3.3. Response surface plots

To evaluate the impact of different interactive effects among two independent factors on the size, Zeta-potential of nanoemulsion, and antifungal activity of prepared bioactive films, the response surface plots of these effects are presented in figures (6.1a,b,c). Figure 1a present the effect of CNC and pressure on particle size when EO were fixed at 0.5 %. It can be observed that at high CNC concentration (10 %) and lower pressure of homogenization (5000 psi), a large size of particle (around 500 nm) can be formed. With the same CNC concentration (10 %) but in combination with the pressure of 10000 psi, a smaller particle size of around 300 nm can be obtained (Figure 1a). This indicates the important role of pressure (both linear and quadratic effects) on the size formation as mentioned above in ANOVA analysis. It is of interest to see that when CNC concentrations are from 5.0 to 7.5 % and in combination of pressure of 15000 psi, a smaller particle size of less than 100 nm can be formed (EO concentration was fixed at 0.5 %). Since CNC plays an important role in the mechanical properties of the polymeric films (El Miri *et al.*, 2015, El Miri *et al.*, 2016), it is of interest to see the impact of EO concentrations on particle size when combining with CNC at the concentration of 7.5 %. This effect is presented in

Figure 1b. For example, at a pressure of 5000 psi, the particle size is around 400 nm and at a pressure of 15000 psi, the particle size is around 100 nm. Thus, it can be concluded that incorporation of 7.5 % CNC into MC containing 0.25 -0.75 % EO and application of a pressure of 15000 psi can create a nanoemulsion with particle size ≤ 100 nm. This could be due to the fact that low pressures may not be able to create even homogenization or dispersion of CNC into the suspension of MC containing EO. In accordance with a recent study Khan *et al.* (2014d) found that the increased pressure and number of cycles allowed to breakdown the CNC-Chitosan aggregates. This provides an enhanced mechanical property of the film by allowing a homogenous distribution of CNC within the chitosan matrix

Figure 6.2 presents the effect of EO and pressure on Zeta-potential properties of EO nanoemulsion containing MC and 7.5 % CNC. It can be found that higher pressure of microfluidizer (15000 psi) and higher concentrations of EO (0.5-0.75%), a Zeta-potential (from -25 to -35 mV) of nanoemulsion can be obtained. The zeta potential and charge density is important for the evaluation of the stability of the colloidal suspension (Asmawati *et al.*, 2014, Wen *et al.*, 2016). For electrostatically stabilized dispersions, it has been demonstrated that the higher is the value of zeta potential, the more stable the dispersion is. It is considered that nanoemulsions with very low (-30 mV) or very high (+ 30 mV) zeta-potential are the most stable nanoemulsion (Asmawati *et al.*, 2014). A study conducted by Wen *et al.* (2016) showed that increasing the CNC concentration during the PAA (Poly Acrylic Acid) grafting process had a positive impact on the charge characteristics. It was seen that the zeta potential increased from -43 mv to -50 mv when the CNC concentration was increased from 0.8 to 10% wt/v. In accordance with this observation our study showed that the incorporation of 7.5 %-10% of CNC into MC containing 0.5 -0.75 % EO and the application of a pressure of 15000 psi in a microfluidizer created a stable nanoelumsion with Zeta-potential of around -30 mV.

Figure 6.3 (a, b, c, d) represents the combined effect of EO and pressure on the inhibition against *A. niger*, *A. flavus* , *A. parasiticus* and *P. chrysogenum* respectively, at a fixed CNC concentration of 7.5 % (w/w). It can be seen that at a low concentration of EO (0.25%) and the increase of the pressure from 5000 to 15000 psi did not improve the antifungal activity of nanoemulsion against *A. niger* (less than 10 mm zone of inhibition). At 0.5% EOs concentration,

an increased pressure of the microfluidizer resulted in a higher antifungal activity of bioactive films against *A. niger* (20-30 mm); 15000 psi pressure is required for proper dispersion of EOs under same conditions. This is consistent as mentioned above in our ANOVA analysis showing that the EO concentration was the most important factor affecting the growth of fungi. Increasing EO concentrations from 0.5 to 0.75 % did not significantly increase the antifungal activity of the nanoemulsion against *A. niger* (around 30 mm zone of inhibition) at a pressure of 15000 psi (Figure 6.3). Thus, incorporation of 0.5-0.75 % EO into MC nanoemulsion containing 7.5 % CNC and application of a pressure of 15000 psi and cycle (number of passes) 5 in a microfluidizer can be considered as optimized conditions for creating MC based bio composite films.

Similar results were obtained in terms of antifungal activity of nanoemulsions against *A. flavus, A. parasiticus* and *P. chrysogenum* as compared to that of antifungal activity against *A. niger*. In general, *P. chrysogenum* was found to be more sensitive to bioactive films than those of three species of *Aspergillus* (Figure 6.2). The highest zones of antifungal inhibition of bioactive films ranged between 35-40 mm when EO concentrations were from 0.5-0.75% and a pressure of microfluidizer at 15000 psi. Based on the analysis of regression, 15000 psi pressure, 7.5% (wt/wt) CNC concentration were optimized to obtain a film forming dispersion with particle size of 90-104 nm. Films prepared following the optimal conditions containing 0.5-0.75% of EO showed 30-35 mm zone of inhibition against the four tested fungal species. However, the results of antifungal activity of active films against 4 fungi need to be evaluated *in situ* conditions.

6.3.4. *In situ* experiment with CNC-reinforced bioactive methyl cellulose (MC) based films combined with gamma radiation against four fungal species during 8 weeks of storage period.

In situ experiments with CNC reinforced MC based bioactive films are shown in Fig 6.4. Bioactive MC/CNC films containing 0.5 and 0.75% EO were considered as Bioactive MC film A and B respectively. The results showed that MC based bioactive films added with EO emulsion were very efficient in reducing the growth of the mold species during storage. The initial

inoculation for all mold species was 3 log conidia/g. For *A. niger* the fungal growth increased from 2.97 log to 6.20 log after 8 weeks of storage. However, samples treated with bioactive film A and B, reduced the growth by 2.00 and 2.60 log CFU/g, respectively, after 8 weeks of incubation period as compared to the control samples. Similarly, samples treated with A and B bioactive films reduced the growth of *A. flavus* by 1.47 and 2.41 log CFU/g and by 1.38 and 2.41 log cfu/g for *A. parasiticus* respectively. Samples treated with bioactive films A and B reduced the growth of *P. chrysogenum* by 2.00 and 2.81 log CFU/g, respectively, after 8 weeks of incubation period as compared to the control samples.

The main components of the EO based formulations are carvacrol and thymol. The antifungal effects of these components have been studied by many authors (Nostro *et al.,* 2012, Ramos *et al.,* 2013). A recent study conducted by Otoni *et al,*. (2014b) involved the formulation of EO emulsion with methylcellulose films. The EO emulsions were found to reduce the rigidity and increase the extensibility of the methylcellulose based films. The presence of EO decreased yeast and mold counts in sliced bread. However, the nanoencapsulation of the EO based formulation resulted in a better antimicrobial efficiency by increasing the bioavailability of the EO in the food system. Therefore, the chemicals responsible for the inhibition of the mold species must have been the natural volatile compounds (carvacrol, thymol) that had released from the polymer matrix and present in the headspace packaging. Results present in this study showed the sustained release of active components from EOs emulsion by prolonging the efficacy of bioactive films during 8 weeks of storage.

Irradiation of samples at 750 Gy caused a 2.40 log CFU /g reduction of *A. niger* growth after 8 weeks as compared to control samples. Combined treatment with 750 Gy gamma radiation and bioactive film A and B led to a 3.20 and 3.60 log CFU/g reduction, respectively, after 8 weeks of incubation as compared to the control samples. Similar decreases of fungal counts were observed with samples against *A. flavus* and *A. parasitucus*. Irradiation of samples at 750 Gy caused approximately 1.94 log CFU /g reduction in *A. flavus* and *A. parasiticus.* Combining with 750 Gy gamma radiation and bioactive film A and B led to ~ 2.61 and ~ 3.86 log CFU/g reduction, respectively, after 8 weeks of incubation as compared to the control samples (Without bioactive films and irradiation). Irradiation of samples at 750 Gy caused a 2.80 log CFU/g reduction of *P.*

chrysogenum. Combined treatment with 750 Gy gamma radiation and bioactive film A and B, caused a 3.15 and 3.84 log CFU/g reduction, respectively, after 8 weeks of incubation as compared to the control samples. Combined treatment showed a pronounced inhibition of fungal growth for both tested species. It has been demonstrated that the active compounds present in natural antimicrobials can significantly improve the radiosensitivity of various food borne pathogens. Studies conducted by Hossain *et al.* (2014a) showed that basil EO in conjunction with ionizing radiation controlled the growth of *A. niger* and *P. chrysogenum* in rice grains. Their results showed that the fungal count reached to 5.52 log CFU/g after 14 days of incubation. Irradiating the samples with 2 kGy of radiation dose alone or applying 2% EO without irradiation resulted in respective fungal growth of 3.58 and 3.51 log CFU/g after 14 days of incubation period. On the other hand combining 2% basil EO with a 2 kGy irradiation dose caused a significant ($p \leq 0.05$) reduction of 5 log CFU/g at day 14 as compared to the control. Fruit coatings based on carboxymethyl cellulose prolonged the shelf life of pears and plums treated at 1.5 kGy (Hussain *et al.*, 2015). In the case of plum, the combination of irradiation and coating resulted in a 2 log reduction of yeast and mold after one month of storage at room temperature. It is believed that the addition of EOs destabilizes the cytoplasmic membrane and ionizing radiation impairs the cell structure through a series of event including action on DNA. Combining treatments makes it impossible the cell to repair the damage incurred by both treatments (Chiasson *et al.*, 2004a, Takala *et al.*, 2011, Turgis *et al.*, 2008). Similarly in this study, the combination of γ irradiation and EO-encapsulated MC bio-films was found to provide a more enhanced treatment than the individual treatment applications against tested fungal species from week 1. Treating with active films led to a lower radiation dose for controlling the growth of tested fungal species for 8 weeks.

6.3.5. Release/diffusion of volatile component from active MC based matrix

To obtain the release of EOs from MC film matrix a standard curve was obtained for oregano: thyme EO combination at 274 nm. For the construction of the standard reference curve, the absorption peaks of the oregano: thyme EO were first determined from spectra of pure oregano: thyme EO obtained using a UV-VIS spectrophotometer following a method adopted from Barzegar *et al.* (2016) and Partheniadis *et al.* (2017). A major peak which appeared at

wavelength 274 nm was used for the determination of released EO. The extracted solvent was subjected to analysis of absorbance to obtain the release of volatile components during storage. The result (Fig 6.5) showed a gradual release of volatile component (oregano: thyme) from methyl cellulose based film matrix. The percentage of residual volatile component inside the film decreased significantly ($p \leq 0.05$) from 62.39% to 48.83% at week 1 and 4 respectively which represents 21% active component released during the 4 weeks storage period. The reduction or decrease of volatile component was found to be 46.97% (representing 24% release of active component) at 8 weeks of storage. The decrease of active component was found 43.67 and 40.23% representing 29 and 35% of release during week 10 and 12 respectively. The result shows that the release was faster (8-24%) in the first few weeks and then it becomes slower over week 8-12. In addition, the release of volatile component was even slower with the film containing crystalline nanocellulose (CNC) as a reinforcing agent. It is clearly seen from the figure 6.5 that CNC exhibits a significant ($P \leq 0.05$) effect in the maintenance of active component in the film. The release of volatile components from CNC reinforced MC film was 3, 9 and 11% at weeks 1, 4 and 6 respectively. The release was found between 16-26% between week 8 and 12 respectively. The release of volatile components from CNC reinforced MC based film was 25% slower ($P \leq 0.05$) as compared to MC based film matrix after 12 weeks of storage. These results suggest that the addition of CNC in MC polymer matrices may protect the volatile component of EO and permit a controlled release of the active components during storage.

Previous studies also showed that CNC allows a homogenous distribution of EOs emulsion within CH matrix to control EOs release through nanocomposite films (Boumail *et al.*, 2013). A study conducted by Boumail *et al.* (2013 a) developed novel trilayered antimicrobial diffusion films (ADFs) with two external layers of polycaprolactone and one internal layer of CNC-reinforced methyl cellulose (MC) matrix. Two natural EOs mixtures (formulation A and B) were incorporated in the MC layer. To evaluate the release of active component they found the Total Phenols (TP) availability decreased from 68.3 to 57.0 μg GAE/mg for MC-A and from 68.2 to 59.0 μg GAE/mg for MC-B from day 0 to day 13. These represent a diffusion level of only 21.2 and 14.0%, respectively for MC-A and MC-B films after 14 days of storage. The TP release was 19.0 and 10.2% for ADF-A and ADF-B formulations, between day 2 and 8 respectively,

suggesting a slower release during this second subperiod, which is in agreement with present study. The results indicated that the trilayered ADFs containing CNC allowed a slow controlled release of the antimicrobial compounds during storage.

Release of active component from bioactive packaging is an important aspect for food preservation and extends the shelf life of food product. Nanocomposite polymers containing CNCs have been demonstrated successfully into food packaging to enable the slow release of bioactive agents over time, and extend the shelf-life of food (Boumail *et al.*, 2013 a, Salmieri *et al.*, 2014 b). Salmieri *et al.* (2014 b) prepared PLA–CNC– oregano films by incorporating oregano EO as antimicrobial agent and found a controlled release of total phenols (TP) from the film matrix. They found TP release was only 16.6 % at day 14 with the developed PLA–CNC–oregano film system. The results indicated a low diffusion rate from CNC reinforced film matrix during storage. The incorporation of CNC appears to modify the structure of the active films and enable a more controlled release of the active agent. This property extends the lifetime of the bioactive films which allows a sustained release over a prolonged period (Fortunati *et al.*, 2013, Yang *et al.*, 2016). Hence the addition of CNC in MC matrix greatly helped to preserve EO bearing highly volatile properties, while preserving the biological activity of the EOs during storage. The release of EO nanoemulsion from the MC matrix can be explained by first order release kinetics as the regressions of the developed model was found to be 0.80 and 0.87 respectively, (Fig 6.6 i and ii) indicating suitability of the data for studying the release kinetics during 12 weeks of storage.

6.3.6. Physicochemical Characterization of irradiated and non-irradiated composite films

The mechanical properties (tensile strength, tensile modulus, elongation break and water vapor permeability) of the developed chitosan films were evaluated alone and impregnated with other agents such as formulations A (0.5% EO emulsion) and B (0.75% EO emulsion) and cellulose nanocrystals (CNC), without and with gamma irradiation at 750 Gy (table 6.5). The results showed that CNC and gamma radiation had a significant effect ($p \leq 0.05$) on tensile strength of methyl cellulose based films. The tensile strength of MC films increased significantly from 54.21 to 71.67 MPa with CNC which is an increment of approximately 30%. Similarly, the tensile

strength of bioactive films A and B increased by 20 and 31% respectively with the addition of 7.5% (w/w) CNC as compared to respective films without CNC. The tensile modulus of Film A and Film B was significantly ($p \leq 0.05$) lower as compared to the control MC based film indicating a less dense film matrix for the incorporation of EO emulsion. Loading of 7.5% (w/w) CNC nanofiller allowed 15 and 13% ($p \leq 0.05$) increment of TM values with MC (no EOs) and Bioactive- A films respectively. However no significant change ($p > 0.05$) in the TM properties was observed with bioactive B films containing CNC (7.5%w/w). Bioactive filma A and B possessed higher elongation at break (EB) corresponding to significantly higher film flexibility ($P \leq 0.05$) than control MC based film. Generally, the presence of EOs play a role as plasticizer in films by reducing intermolecular forces between adjacent polymer chains, decreasing TS and increase film flexibility. Addition of CNC allows a decrease of EB significantly ($p \leq 0.05$) for bioactive MC based Film-A (25%) and B (16%) respectively as compared to respective films without CNC. The improvement of mechanical properties of the nanocomposite is related to a mechanical percolation phenomenon of cellulose nanocrystals which form interfacial interactions (*i.e.* tight hydrogen bond networks) between CNC and biopolymer matrix. The efficiency of CNC as a filler (reinforcing agent) is due to its high crystallinity by forming a stiff continuous network linked through hydrogen bonding has been widely demonstrated (Dufresne, 2008, El-Wakil *et al.*, 2015).

In the present study, gamma radiation treatment significantly improved the mechanical and the water vapor properties of the film. The tensile strength of MC based films increased by 8% only by applying 750 Gy radiation doses, which further increased by 43% when CNC was added as compared to MC control film (non-irradiated and without CNC). The maximum tensile strength (77 MPa) and tensile modulus 919 MPa was found with the irradiated methyl cellulose films containing CNC. Similarly, the water vapor permeability decreased significantly ($p \leq 0.05$) in films containing CNC and treatment of gamma irradiation (table 6.5). Irradiation treatment alone caused a reduction of WVP by 4%. However, a combined treatment of irradiation (750 Gy) and the addition of CNC (7.5% w/w) decreased by 9% of WVP on MC based films. Saurabh *et al.* (2013) investigated the mechanical and water vapor barrier properties of biodegradable films based on guar gum (GG) prepared from irradiation process. Their results showed that films

prepared from GG irradiated treatment at doses up to 500 Gy resulted in 32.6% increase in tensile strength and improved by 15% the WVP as compared to non-irradiated control films. Improved functional properties of bio based films due to γ irradiation was attributed to the increase of the ordering of polymer structures. Previous studies have also shown that processing the films by gamma radiation involves high energy radiations in a controlled manner which led to better crosslinking among polymeric chains thus improving the mechanical and barrier properties of the films (Akter *et al.*, 2012, Jo *et al.*, 2005). Hence, in the present study, improved mechanical and barrier properties of irradiated (750 Gy) MC composites were observed as compared to control (No irradiated) films.

6.4. Conclusion

The results obtained in this study showed that incorporation of 0.5-0.75 % EO nanoemulsion into MC based films containing 7.5 % CNC and the application of a pressure of 15000 psi by a microfluidizer at cycle 5, which is the number of passes was considered as optimized condition for creating MC based composite films. *In vitro* tests showed that the films prepared under these conditions produce a 30-35 mm zone of inhibition against tested four fungal species. The *in situ* tests showed ~2-3 log reduction of fungal species in rice during 8 weeks of storage at 28°C. Therefore, microfluidization provides an innovative approach to improve CNC and EO nanoemulsion distribution in the polymer matrix and develop bionanocomposites with high mechanical and bioactive properties. Combined treatment of bioactive films with 750 Gy irradiation showed enhanced antifungal and physicochemical properties. The integration of CNC in to MC matrix for the preparation of bioactive films enhanced the physicochemical properties of the matrix and allowed a sustained release of EOs over 12 weeks of storage period. These results show the potential application of CNC reinforced MC nanocomposite films for eventual application to protect cereal grains during storage

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Table 6.1. Level of factor in the experimental design ($3^{(3-0)}$)

Factors	**Symbol (unit)**	**-1**	**0**	**+1**
Essential oils (EO)	X_1 (%, v/v)	0.25	0.50	0.75
Cellulose NanoCrystals (CNC)	X_2 (%, w/w polymer)	5	7.5	10
Pressure	X_3 (psi)	5000	10000	15000

Table 6.2. The particle size, Zeta-potential of methylcellulose film forming dispersions and the antifungal activity of the films in the experimental design

Independent variables			**Dependent variables**					
			Emulsion characterization		**Antifungal activity of bioactive film (inhibition in mm)**			
Pressure (psi)	**CNC (%)**	**EO (%)**	**Size (nm)**	**Zeta-potential (mV)**	***A. niger***	***A. flavus***	***A. parasiticus***	***P. chrysogenum***
5000	5	0.25	386.32	-4.32	0	0	0	0
5000	5	0.50	410.10	-3.76	14	11	10	18
5000	5	0.75	426.42	-3.18	16	18	18	22
5000	7.5	0.25	401.24	-9.43	0	0	0	0
5000	7.5	0.50	430.10	-5.53	13	15	12	18
5000	7.5	0.75	444.59	-6.68	22	19	16	22
5000	10	0.25	440.15	-4.73	8	0	0	8
5000	10	0.50	501.9	-6.82	16	15	14	20
5000	10	0.75	595.7	-6.68	20	18	15	0
10,000	5	0.25	319.6	-5.85	0	0	0	0
10,000	5	0.50	280.4	-5.78	16	18	17	15
10,000	5	0.75	265.1	-2.54	20	24	23	10

10,000	7.5	0.25	300.1	-8.74	0	0	0	0
10,000	7.5	0.50	289.4	-4.57	20	23	22	28
10,000	7.5	0.75	270.6	-8.97	22	25	23	32
10,000	10	0.25	389.5	-6.45	10	0	0	0
10,000	10	0.50	305.2	-6.80	22	23	26	26
10,000	10	0.75	404.5	-12.1	28	30	28	28
15,000	5	0.25	124.07	-10.6	0	0.0	0	0
15,000	5	0.50	99.98	-17.6	28	31.7	27.4	33.8
15,000	5	0.75	95.81	-18.5	28	32.2	31.7	36.3
15,000	7.5	0.25	90.63	-6.65	0	0.0	0	0
15,000	7.5	0.50	98.31	-20.5	35	29.5	26.2	39.3
15,000	7.5	0.75	104.13	-30.5	37	32.0	25.9	41.1
15,000	10	0.25	115.83	-5.39	0	0	0	0
15,000	10	0.50	101.37	-31.5	32	34.1	29.8	38.7
15,000	10	0.75	110.90	-32.4	30	30.3	26.5	40.0

Table 6.3. Analysis of variance for the particle size and Zeta-potential of MC nanoemulsion

Factor	**Particle size**				**Factor**	**Zeta-potential**			
	Sum of square	***df***	**F value**	***p* value**		**Sum of square**	***df***	**F value**	***p* value**
EQ (L) *	N.I.**				EQ (L) *	195.9	1	13.25	0.0016
EO (Q)	2285.4	1	3.26	0.0880	EO (Q)	N.I.			
CNC (L)	17251.5	1	24.59	0.0001	CNC (L)	92.2	1	6.24	0.0213
CNC (Q)	4904.9	1	6.99	0.0165	CNC (Q)	N.I.			
Pressure (L)	532336.6	1	759.03	0.0000	Pressure (L)	833.8	1	56.39	0.0000
Pressure (Q)	8344.0	1	11.89	0.0029	Pressure (Q)	189.5	1	12.82	0.0018
EO x CNC	3615.0	1	5.15	0.0357	EO x CNC	80.9	1	5.47	0.0298
EO x Pressure	5576.7	1	7.95	0.0113	EO x Pressure	307.0	1	20.77	0.0001
CNC x Pressure	7837.2	1	11.17	0.0036	CNC x Pressure	N.I.			
Total SS	594775.5	26			Total SS	1995.2	26		
Model R^2	0.98				Model R^2	0.85			

* L, Q represent linear and quadratic effect, respectively of the independent factors on the particle size or Zeta-potential.

** Factor that is **n**ot **i**ncluded in the model.

Table 6.4. Analysis of variance for antifungal activity of MC films

		A. niger			*A. flavus*			*A. parasiticus*			*P. chrysogenum*		
Factor	***df***	**SS****	**F value**	***p* value**	**SS**	**F value**	***p* value**	**SS**	**F value**	***p* value**	**SS**	**F value**	***p* value**
EO (L)*	1	1760.2	107.0	0.00000	2228.9	217.0	0.00000	1889.1	192.2	0.00000	2733.1	68.4	0.00000
EO (Q)	1	480.0	29.3	0.00002	574.3	55.9	0.00000	484.2	49.3	0.00000	922.6	23.1	0.00009
CNC (L)	1	107.5	6.	0.01812	13.3	1.3	0.26714	8.3	0.8	0.36941	45.4	1.1	0.29846
Pressure (L)	1	280.0	17.1	0.00047	448.0	43.6	0.00000	378.1	38.5	0.00000	686.9	17.2	0.00045
EO x Pressure	1	102.0	6.2	0.02091	105.0	10.2	0.00432	102.7	10.4	0.00399	512.2	12.8	0.00176
Total SS**	26	3426.0			4156.			3562.4			5705.4		
Model R^2		0.90			0.95			0.94			0.85		

* L, Q represent linear and quadratic effect, respectively of the independent factors on the inhibition of fungi. ** Sum of square

Table 6.5. Physicochemical properties of irradiated and nonirradiated composite films.

Treatment	Samples	Tensile Strength TS (MPa)	Tensile Modulus, TM (MPa)	Elongation of Break (EB%)	Water Vapor Permeability g mm m^{-2} day^{-1} kPa^{-1}
Without gamma radiation	MC	54.2 ± 6.3^{d}	796.3 ± 75.7^{f}	19.3 ± 3.4^{a}	4.4 ± 0.4^{b}
	MC+ formulation A	44.3 ± 4.3^{b}	516.0 ± 47.0^{c}	32.4 ± 3.1^{c}	5.8 ± 0.5^{def}
	MC+ Formulation B	32.4 ± 4.0^{a}	435.0 ± 43.5^{b}	37.6 ± 6.0^{f}	6.0 ± 0.5^{f}
	MC +CNC	71.6 ± 3.9^{g}	920.7 ± 78.4^{g}	20.0 ± 3.6^{a}	4.0 ± 0.4^{ab}
	MC+ CNC+ formulation	53.0 ± 4.3^{cd}	582.6 ± 46.3^{d}	24.7 ± 5.7^{b}	5.6 ± 0.3^{cdef}
	MC+ CNC+ Formulation B	42.5 ± 2.9^{b}	420.9 ± 31.4^{b}	32.2 ± 4.5^{c}	5.9 ± 0.3^{ef}
With 750 Gy gamma radiation	MC	59.1 ± 5.5^{e}	871.3 ± 94.7^{g}	20.8 ± 3.1^{a}	4.3 ± 0.4^{b}
	MC+formulation A	49.5 ± 5.5^{c}	708.2 ± 45.5^{e}	26.1 ± 2.9^{b}	5.5 ± 0.5^{cde}
	MC+ Formulation B	44.2 ± 5.6^{b}	379.3 ± 33.2^{a}	34.5 ± 4.0^{cd}	5.80 ± 0.3^{cdef}
	MC +CNC	77.6 ± 5.3^{h}	919.2 ± 74.5^{g}	19.8 ± 3.2^{a}	3.9 ± 0.3^{a}
	MC+ CNC+ formulation A	63.8 ± 3.4^{f}	585.0 ± 55.8^{d}	26.1 ± 2.4^{b}	5.3 ± 0.3^{c}
	MC+ CNC+ Formulation B	53.4 ± 4.4^{cd}	351.4 ± 35.4^{a}	31.3 ± 3.2^{c}	5.4 ± 0.3^{cd}

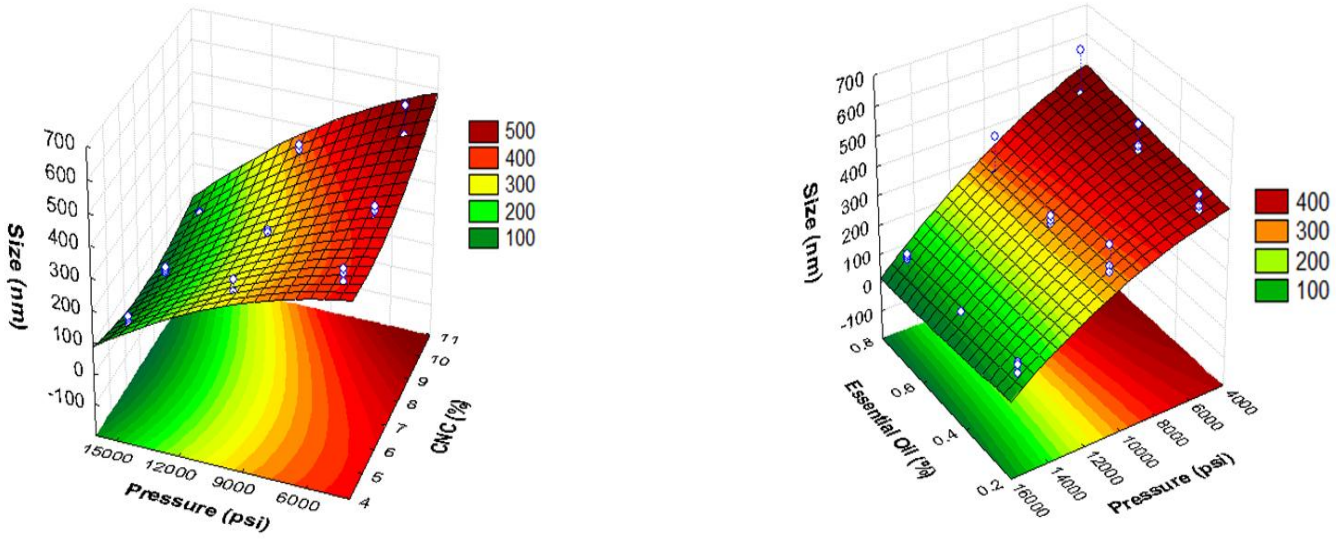


Figure 6.1. Combined effect of a) CNC and pressure (concentration of EO was fixed at 0.05 % (w/v)) and b) EO and pressure (concentration of CNC was fixed at 7.5 % (w/w)) on the particle size of nanoemulsion

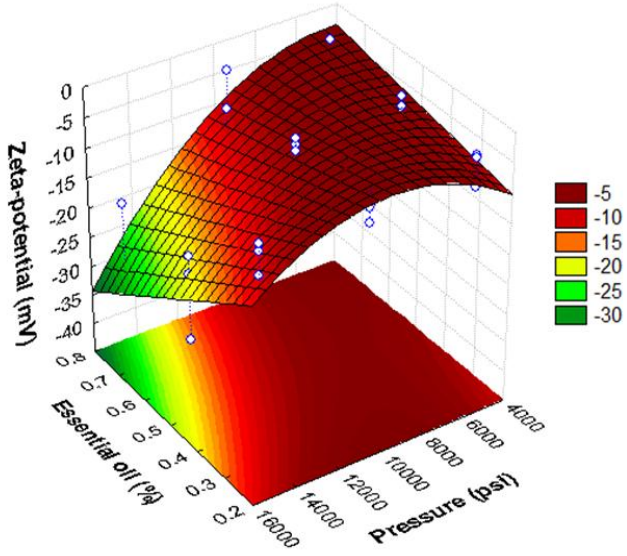


Figure 6.2. Combined effect of EO and pressure on the Zeta-potential of nanoemulsion (concentration of CNC was fixed at 7.5 % (w/w))

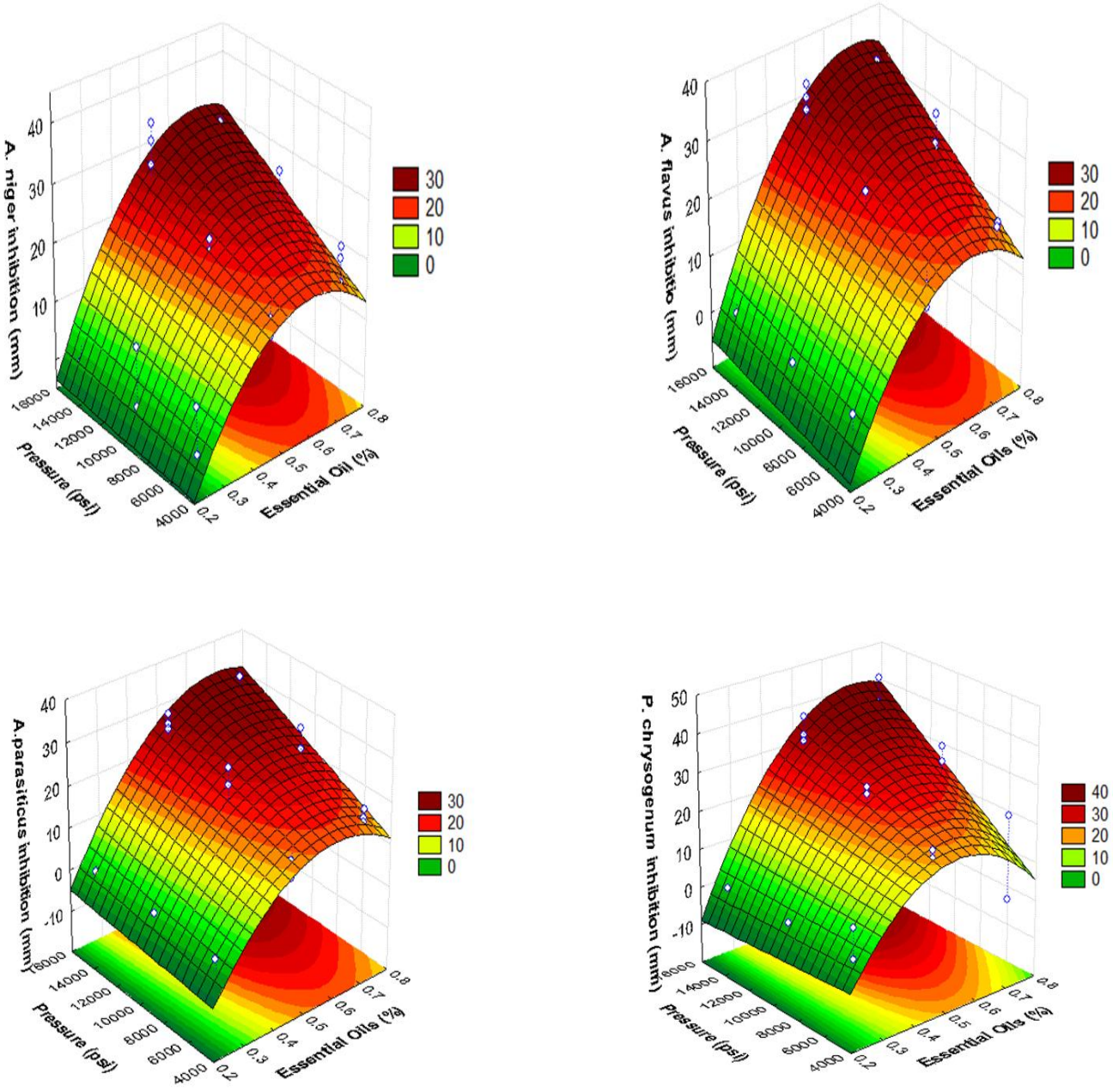


Figure 6. 3 Combined effect of EO and pressure on the inhibition against *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum* (concentration of CNC was fixed at 7.5 % (w/w)

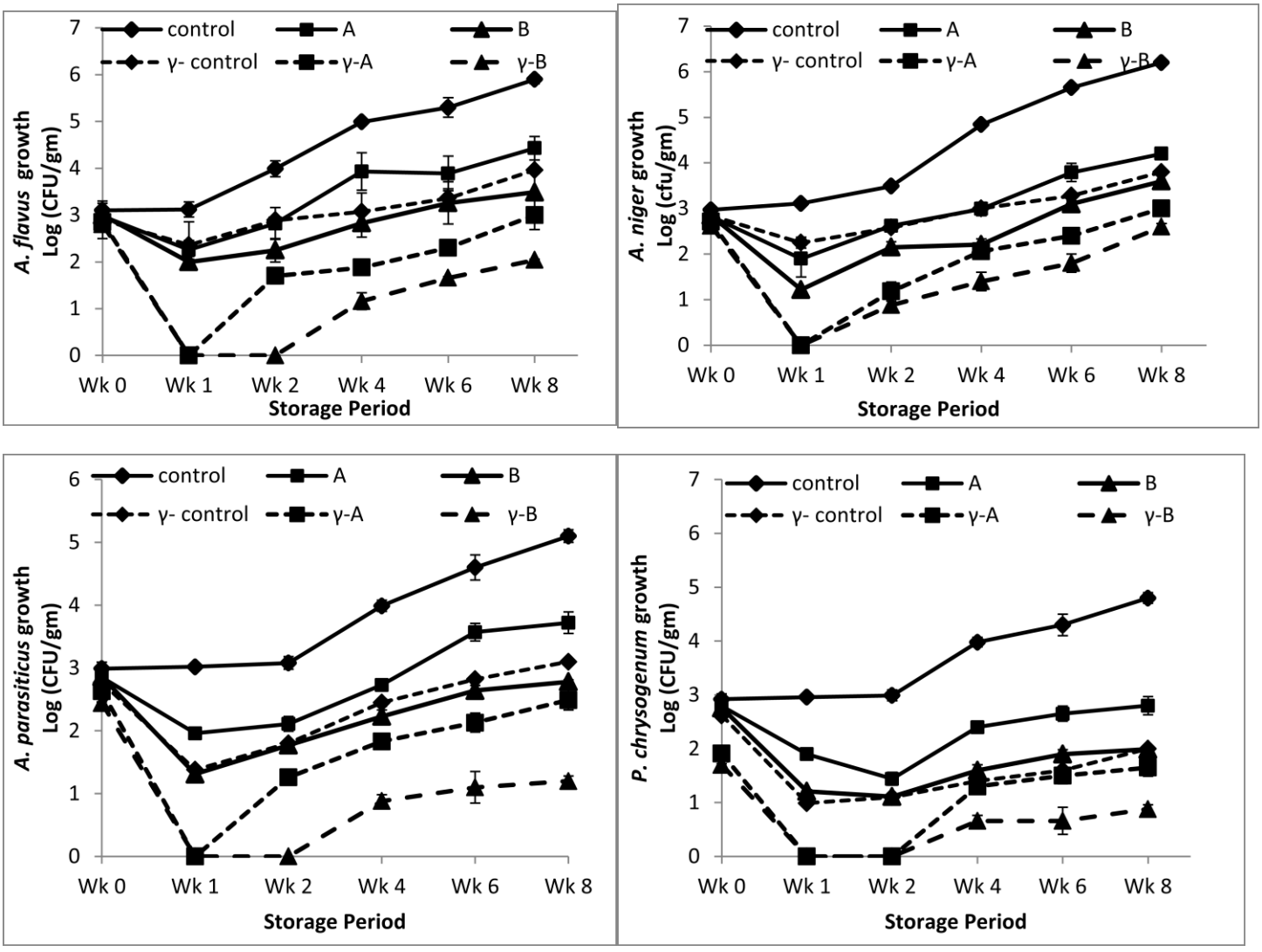


Figure 6.4. Fungal growth profiles of *A. flavus A. niger, A. parasiticus and P. chrysogenum* following in situ tests with bioactive methylcellulose (MC) nanocomposite films over 8 weeks of storage period. Abbreviations: Wk-week; γ-gamma irradiated sample; a-biofilm impregnated with 0.13% EO; b-biofilm containing 0.19% EO.

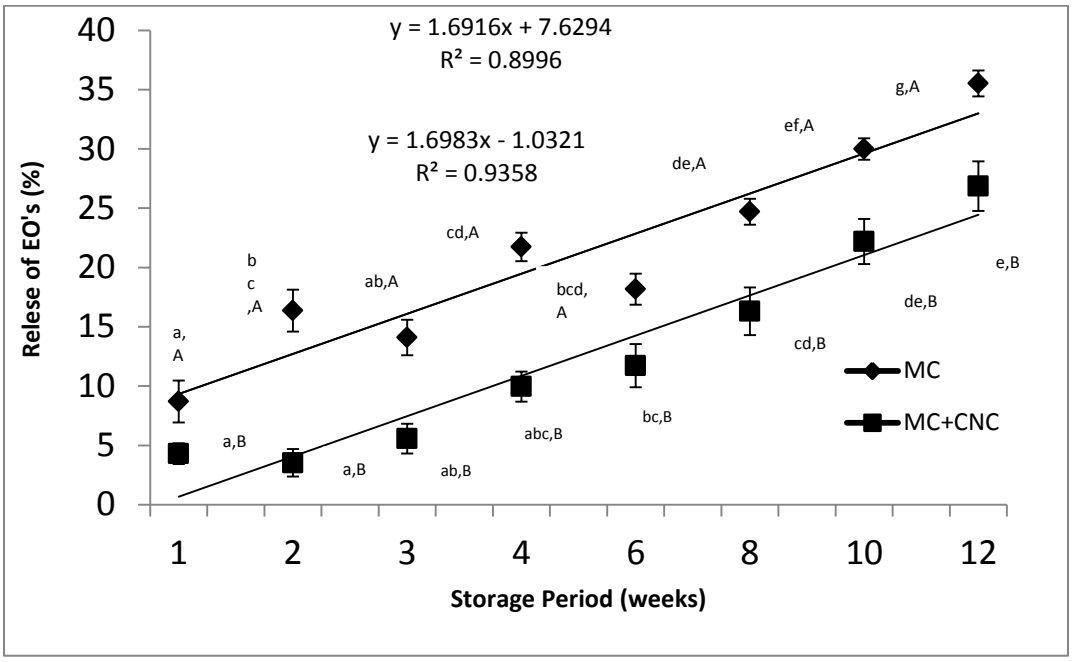
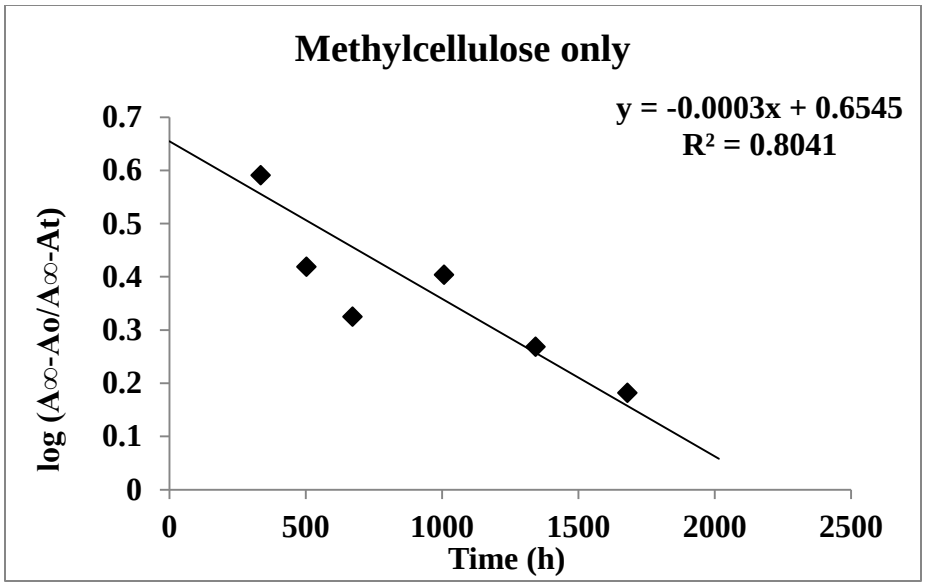


Figure 6.5. Release (%) of EOs from bioactive MC and MC+ CNC based films during storage



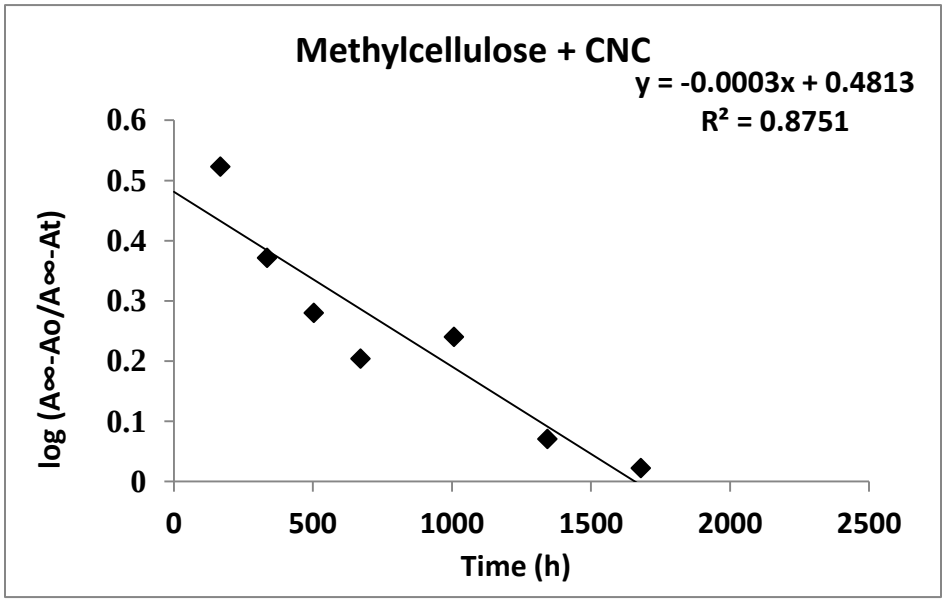


Figure 6.6. Kinetics for release study for i) MC ii) MC + CNC nanocomposite films containing EO nanoemulsion.

Chapter 7

Publication 6

Antifungal activities of combined treatments of irradiation and EO encapsulated chitosan nanocomposite films in *in vitro* and *in situ* conditions'

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Contribution of the authors

Most of the research work was planned and performed by Farah Hossain. Prof. Lacroix contributed and supervised the elaboration of the experimental design protocols, she has done the corrections of the paper, participated on the scientific discussion. Stephane Salmieri helped to develop the protocol of release properties. Dr. Follett has participated to the discussion, corrections of the papers, revised the articles.

Résumé

L'application des nanoémulsions pour la conservation des aliments retient beaucoup l'attention de la communauté scientifique. Avec l'augmentation de la demande des consommateurs pour des produits alimentaires plus sains et plus sûrs il est nécessaire de développer des matériaux comestibles capables d'encapsuler, de protéger et de conserver les produits alimentaires. La présente étude vise le développement et l'évaluation d'une formulation de films à base de nanocomposite de chitosane chargé d'huiles essentielles (HE) ayant une biodisponibilité améliorée pour la protection des aliments contre les microorganismes fongiques responsables de la détérioration des aliments. Des films minces de chitosane bioactifs capables de protéger la biodisponibilité des HE sans affecter les propriétés mécaniques des films. Le traitement des films bioactifs à base de polymère de chitosane à une faible dose d'irradiation gamma (750 Gy) a permis d'améliorer significativement ($p < 0.05$) la résistance à la traction (TS) et le module de traction (TM) du matériau biopolymérique. L'incorporation de nanocristaux de cellulose (CNC) avec le chitosane a amélioré la matrice biopolymérique, en améliorant la résistance à la traction et la rupture d'allongement. La CNC a également joué un rôle important dans la stabilisation des propriétés physico-chimiques et la libération des films nanocomposites. Les essais en phase vapeur des films nanocomposites à base de chitosane chargés de mélanges d'huiles essentielles de thym-origan, de thym- théier et de thym-menthe poivrée ont montré une activité antifongique significative contre *Aspergillus niger*, *A. flavus*, *A. parasiticus* et *Penicillium chrysogenum* en réduisant leur croissance de 51- 77%. La combinaison des films bioactifs de chitosane chargés d'huiles essentielles de thym et d'origan et une faible dose d'irradiation (750 Gy) ont significativement amélioré l'efficacité des films à inhiber les moisissures dans le riz inoculé démontrant une réduction d'environ 3 log UFC / g de croissance fongique. Des films de nanocomposite de chitosane contenant une nanoémulsion d'HE ont montré une libération lente du composant volatil sur une période de stockage de 12 semaines. L'évaluation sensorielle d'échantillons de riz emballés avec des films bioactifs de chitosane chargés de nanoémulsions de thym et d'origan n'a montré aucun changement significatif ($P < 0.05$) de l'odeur, du goût, de la couleur et de l'appréciation globale par rapport au riz non traité. Ces films bioactifs ont de

nombreuses applications potentielles pour améliorer la sécurité et prolonger la durée de conservation des aliments emballés.

Abstract

The application of nanoemulsions for food preservation is receiving considerable attention from the scientific community. The demand of consumers for healthier and safer food products is increasing, and there is a need for designing edible materials capable of encapsulating, protecting, and preserving food commodities. The current study focuses on the development and assessment of film formulations based on a chitosan nanocomposite loaded with essential oils (EOs) with improved bioavailability for protection of food items against fungal spoilage organisms. Thin, bioactive chitosan films are able to protect the bioavailability of EOs without affecting the mechanical properties of the films. Treatment of the active films based on chitosan polymer with low gamma irradiation dose (750 Gy) was able to significantly improve ($P \leq 0.05$) the tensile strength (TS) and tensile modulus (TM) of the biopolymeric material. Incorporation of cellulose nanocrystals (CNC) with the chitosan improved the biopolymeric matrix, by enhancing tensile strength and elongation break. The CNC also played an important role in stabilizing the physicochemical and release properties of the nanocomposite films. Vapor phase assays of the chitosan-based nanocomposite films loaded with thyme-oregano, thyme-tea tree and thyme-peppermint essential oil mixtures showed significant antifungal activity against *Aspergillus niger, A. flavus, A. parasiticus,* and *Penicillium chrysogenum*, reducing their growth by 51-77%. Combining the bioactive chitosan films loaded with thyme and oregano essential oils and low dose of irradiation (750 Gy) significantly ($P \leq 0.05$) enhanced the inhibitory effects of the films in rice inoculated with fungi by causing an approximately 3 log CFU/g reduction in fungal growth. Chitosan nanocomposite films containing EO nanoemulsions showed a slow release of volatile components over a 12 week storage period. Sensorial evaluation of rice samples packed with the chitosan bioactive films loaded with thyme and oregano nanoemulsions showed no significant ($P > 0.05$) change in odor, taste, color and general appreciation compared with untreated rice. These bioactive films have many potential applications for improving the safety and prolonging the shelf life of packaged foods.

7.1. Introduction

Protection of food crops against storage pathogens is a major concern for the food industry, farmers, public health organizations, and environmental agencies. In 2010, about 133 billion pounds of food, representing 31% of the global food available, was wasted at the retail and consumer level in the United States due to spoilage caused by storage food pests (Buzby *et al.*, 2014). One of the main foods affected by pest infestation are cereal crops, which bear the highest calorific content among food commodities (Kumar *et al.*, 2017). Worldwide, cereal grains are commonly (25%) contaminated by mycotoxins produced by storage fungi (Smith *et al.*, 2016). Aflatoxins, produced as secondary metabolites by two fungal species namely *Aspergillus flavus* and *A. parasiticus*, are considered the most dangerous group of mycotoxins, as they deteriorate liver and kidney functions and increase the risk of cancer. High concentrations of aflatoxin can lead to aflatoxicosis, a condition causing severe illness, and in extreme cases, death (Kumar *et al.*, 2017, Kumar *et al.*, 2007, Magan *et al.*, 2007, Tefera *et al.*, 2011). Major challenges to food preservation necessitate the search for novel technologies that satisfy the global market with current regulations including environmental and health concerns, longer shelf-life, authenticity, and waste treatment (Azeredo *et al.*, 2017, Realini *et al.*, 2014). Active food packaging containing subsidiary constituents such as plant-derived essential oils (EOs) is currently playing an important role in reducing fungal contamination and proliferation in processed food. The development of bioactive food packaging combined with nano-based technology has not only increased the shelf-life of foods, but also improved their safety and quality. Several studies have reported the potential of active packaging containing EO nanoemulsions in controlling fungal infestations to ensure food safety. (Donsì *et al.*, 2011, Lu *et al.*, 2016, Otoni *et al.*, 2014a).

EOs are more efficiently used in foods when encapsulated in appropriate delivery systems to overcome dosage limitations and increase the biological stability of active compounds. When encapsulation is at the nanoscale level, the bioactivity of EOs can be enhanced through the activation of passive mechanisms of cell absorption or tissue infusion, thereby enabling the reduction of the EO doses required to ensure antimicrobial activity (Bilia *et al.*, 2014). Such low

doses minimize the impact of the bioactive compounds on the natural aroma, flavor and taste of the food (Acevedo-Fani *et al.*, 2015, Lu *et al.*, 2016, Severino *et al.*, 2014).

Biopolymeric films are ideal matrices for incorporating a wide variety of functional additives such as antioxidants, antifungal agents, antimicrobials, colors, and nutrients (Atarés *et al.*, 2016, Lee *et al.*, 2015, Cozmuta *et al.*, 2015, Salmieri *et al.*, 2014a). The most common type of biopolymers for food packaging includes carbohydrates such as starch, cellulose and chitosan, and proteins like gelatin, gluten, alginate, whey protein and collagen (Ferreira *et al.*, 2016, Othman, 2014). Among biopolymers, chitosan is the second most abundant naturally occurring polysaccharide after cellulose, and is commonly found in the chitinous exoskeletons of crustaceans. It is mainly composed of 2-amino-deoxy-β-D-glucopyranose and 2-acetamido-deoxy-β-D glucopyranose residues, and can be derived from chitin by deacetylation in an alkaline media. Chitosan can also be obtained from the insect cuticle and the cell wall of some fungi (Elsabee *et al.*, 2013, Khan *et al.*, 2014b, Sun *et al.*, 2014). Increasing interest in chitosan-based polymers is driven by several unique properties such as their biodegradability and biocompatibility, and inherent antimicrobial properties in conjunction with their cationicity, and excellent adsorption and film-forming properties (Ferreira *et al.*, 2016, Reddy *et al.*, 2013). Chitosan has been approved by the Food and Drug Administration (FDA) as food ingredient (Avila-Sosa *et al.*, 2012, Ma *et al.*, 2016, Zemljič *et al.*, 2013), and chitosan films and coatings have potential as carriers for plant-derived EOs (Yuan *et al.*, 2016).

Several research studies have attempted to improve the mechanical and functional properties of chitosan by combining or blending it with other polymers and reinforcing agents (Ferreira *et al.*, 2016, Khalil *et al.*, 2016). Loading of nanofillers (5 wt%) has been shown to be an effective way to produce high performance chitosan matrix with additional functionalities (Khan *et al.*, 2012a). Nanocrystaline cellulose (CNC), which can be extracted from lignocellulosic materials, bacteria or algae, have reinforcing properties that make them potential nanofillers for chitosan-based films (Khan *et al.*, 2012a, Vilarinho *et al.*, 2016). Well-dispersed CNC has been shown to reinforce polymers by forming a percolation network connected by hydrogen bonds. Reinforcement with CNC can also improve the thermal, mechanical and barrier properties or polymers, as well as their surface wettability, and controlled release of active compounds/drugs (Boumail *et al.*, 2013, Salmieri *et al.*, 2014a).

Combined preservation treatments with synergistic or additive effects may have applications in certain cases where a high rate of a single treatment alters the sensory or nutritional properties of the food (Lacroix *et al.*, 2015, Severino *et al.*, 2014). Food irradiation or "cold pasteurization" is a disinfection treatment option, and involves exposing food to ionizing radiation to reduce the microbial load, particularly for control of food borne pathogens. Irradiation is an effective kill step when integrated within an established system for the safe handling and distribution of food products (Maherani *et al.*, 2016). Combining irradiation with other treatments such as active films can increase microbial radiosensitivity (Lacroix *et al.*, 2015, Vu *et al.*, 2012)

The aim of this current study was to develop chitosan-based nanocomposite films with enhanced antifungal properties, alone and in combination with gamma radiation. The objectives of the research were to (i) prepare and characterize EO nanoemulsions for three EO combinations (ii) prepare chitosan-based active films containing the EO nanoemulsions (iii) evaluate the antifungal activity of the bioactive films *in situ* on rice in combination with gamma radiation, iv) Evaluate the release of EOs during storage v) assess the physico-chemical properties of irradiated and non-irradiated films.

7.2. Materials and Methods

7.2.1. Essential oils

Essential oil of oregano (*Origanum compactum*; Moroccan oregano), thyme (*Thymus vulgaris*), Tea tree (*Melaleuca alternifolia*), and peppermint (*Mentha piperita*) were obtained from Robert & Fils, (Ghislenghien, Belgium) and stored at 4 ˚C prior to use. Chromatograms of the EOs provided by the manufacturer showed that the oregano oil contained 46.37% carvacrol, 13.7% thymol, 13.33% *p*-cymene and 12.32% γ- terpinene. Thyme oil contained 26.04% thymol, 26.36% *p*-Cymene, and 16.69% γ- terpinene. Tea tree oil contained 38.4% terpinene-4-ol, 22.6% γ terpinene, 8.1% α terpinene and peppermint oil contained 33.38% menthol, 34.31% menthone, and 6.34% 1,8 cineole.

7.2.2. Preparation of combined EO emulsions

Three EO mixtures—thyme and oregano, thyme and tea tree, thyme and peppermint—were selected based on previous antifungal tests (Hossain *et al.*, 2016). A coarse emulsion was

prepared by blending 5% (w/v) mixed oil phase (1:1 ratio) with an aqueous phase (water) containing 1.25% lecithin and 3.75% tween 80 as an emulsifier. The mixture was homogenized using an Ultra-Turrax homogenizer at 15,000 rpm for 2 min. The resulting coarse emulsion was passed through a Microfluidizer (Microfluidics Inc., Newton, MA, USA) to produce Eos nanoemulsion. The emulsion preparation parameters were tested at different homogenization pressures (5000, 10,000, 15000) and numbers of cycles (1-5) (results not shown). Based on the results, optimized nano-sized emulsions were prepared using 3 cycles at 15,000 psi. The average particle size, Poly Dispersity Index (PDI) and ζ-potential of the prepared emulsions were determined by dynamic light scattering using photon correlation spectroscopy (Malvern Zetasizer Nano-ZS, Model ZEN3600). Measurements were carried out with a scattering angle of 173° and at a constant temperature of 25 °C.

7.2.3. Preparation of chitosan-based bioactive composite films containing EO emulsion

7.2.3.1. Preparation of chitosan nanocomposite suspension

A quantity of 5% (w/w) crystalline nanocellulose (CNC) suspension (0.1 g in 100 ml) was prepared by dispersing dried CNC powder (FPInnovations, Pointe-Claire, QC, Canada) in distilled water with magnetic stirring. The CNC suspension was then subjected to ultra-sonication at 1000 j/g of CNC, followed by mixing using a magnetic stirrer for 2 h. A quantity of 2 g chitosan was added to the CNC suspension followed by 2 ml acetic acid in 100 ml water. The CH/CNC suspension was stirred overnight until complete solubilization was achieved. The chitosan solution was then mixed with 0.5 g ethylene glycol and homogenized with an IKA RW-20 mechanical homogenizer at 1,500 rpm for 5 h. After homogenization, a paraffin film was wrapped on top of the beaker to allow removal of air bubbles from the suspension. The prepared CH/CNC suspension was referred as film forming dispersion (FFD).

7.2.3.2. Dispersion of EO emulsion in CNC and chitosan suspensions

Three EO emulsions, prepared as described in the section 2.2 at two concentrations (0.13 and 0.19% w/w), were mixed and homogenized with the Film Forming Dispersion at room temperature using a digital Ultra-Turrax T25 disperser (IKA® Works Inc., Wilmington, NC, USA) at 15,000 rpm for 2 min. The resulting homogenates were kept in a beaker wrapped with parafilm at 4 °C for 3 hours to enable the removal of any air bubble formed during the

homogenization process. The CNC/CH suspensions were introduced in the inlet reservoir of the microfluidizer (Microfluidics Inc., Newton, MA, USA). Ice was placed in the cooling jacket to negate overheating of the suspensions during the microfluidization process. The CH/CNC suspensions were subjected to microfluidization pressures of 5,000 psi and 3 number of cycles to obtain a homogenous FFD.

The resulting solutions were kept at 4 ˚C for 3 hours to enable the film-forming solutions to set before the casting process. Composite films were cast by applying 12 mL of the film-forming suspension onto Petri dishes (95 × 15 mm; Fisher Scientific, Ottawa, ON, Canada) and allowing it to dry for 24 h under a chemical hood at room temperature. Chitosan (CH)-based films containing no antimicrobial were used as a control. Chitosan films containing the EO nanoemulsions were labeled as film 1 (thyme and oregano), film 2 (thyme and tea tree) and film 3 (thyme and peppermint).

7.2.4. Fungicidal activity of biopolymeric films

7.2.4.1. Fungal inocula and assay media

A.niger (ATCC 1015), *A. flavus* (ATCC 9643), *A. parasiticus* (ATCC 16869), and *P. chrysogenum* (ATCC 10106) were used for the bioassays. Each fungal species was grown and maintained in potato dextrose broth (PDB, Difco, Becton Dickinson) containing 10% v/v glycerol. Prior to each experiment, stock cultures were propagated through two consecutive 48 h growth cycles in PDB medium at 28 ˚C ± 2 ˚C. The fungal species were pre-cultured in potato dextrose agar (PDA) for 3 days at 28 ˚C ± 2 ˚C. Conidia were isolated from the agar media using sterile saline containing 0.05% Tween 80. Mycelia were removed by filtration through a 40 µm cell strainer, and the filtrate concentration was adjusted to 1 x 10^8 conidia/mL for subsequent *in vitro* and *in situ* assays (Inouye *et al.*, 2006).

7.2.4.2. *In vitro* Micro-atmosphere assays

Micro-atmosphere assays were performed based on the method described by Inouye *et al.* (2006) and Hossain et al. (2014). A sample of 1 mL containing 1 x 10^8 conidia/ml suspension of each fungal species was added to 100 mL of agar medium containing 1% peptone, 1% glucose and 1% agarose at 50 ˚C. A volume of 3 mL of the prepared mixture was overlaid onto the surface of

hardened PDA medium (20 ml) in a Petri dish (83 mm in diameter) to prepare a double layered agar medium. For assessing the antifungal activity of nanoemulsion, sterile filter paper (10 mm diameter) was placed at the center of the upper lid of the plate. A quantity of 10 µl of each EO emulsion was added at the center of individual paper filters. For evaluating the bioactivity of the prepared films, 10 mm diameter of each film pieces was placed at the center of the upper lid of the petri dishes containing the double-layered agar medium. The Petri dishes were inverted and placed on the upper lid containing the EOs and incubated at 28 ˚C ± 2 ˚C for 24-72 h. The inhibitory diameter zone, which showed absence of growth of the test microorganisms, was measured in mm using a Traceable® Carbon Fiber Digital Caliper (Fisher Scientific). The percentage (%) of inhibition was calculated from the equation below.

$$\text{Inhibition } (\%) = \frac{(\mathrm{C} - \mathrm{T})}{\mathrm{C}} \times 100$$

Where, C and T are the mean of fungal growth of three (3) replicates (mm) for the control and treated sample, respectively. The vapor activity of the nanoemulsion was tested against one fungal species, *A. niger*, and the vapor activity of the three designed films was tested against *A. niger, A. flavus, A. parasiticus* and *P. chrysogenum*.

7.2.4.3. *In situ* antifungal activity of bioactive films combining with gamma radiation

An inoculation bath was prepared with peptone water containing 10^5 conidia/mL of *A. niger, A. flavus, A. parasiticus* and *P. chrysogenum*. A quantity of 500 g of rice grain was added to the inoculation bath and stirred gently for 30 s. After inoculation, the rice grains were dried on a sheet of sterile aluminum foil for 2 h under sterile condition. A quantity of 30 g of the inoculated rice was packaged in a plastic bag. The most efficient bioactive films were selected from the *in vitro* tests at two concentrations (0.13 and 0. 19% w/w) (CH-film A and CH film B) and were placed in each rice bag (1 gm/ cm^2). The rice samples were grouped into two subsets with one receiving irradiation at 750 Gy and one without irradiation. The samples were incubated at 28 ˚C for 8 weeks. The humidity inside the incubator was monitored and maintained constant at 65% throughout the experiment. Microbiological analyses of the stored rice grain were carried out on a weekly basis during incubation period.

7.2.4.4. Microbiological analyses

Microbiological analyses were performed based on the method from Hossain *et al.* (2014a). A sample of 60 mL of sterile peptone water (0.1%, w/v) was added to 30 g of rice and homogenized for 1 min at 2000 rpm using a Lab-blender 400 stomacher (Laboratory Equipment, London, UK). The resulting homogenate was serially diluted using sterile peptone water. An aliquot of 0.1 mL of each dilution was inoculated in triplicate onto the surface of freshly prepared solidified Potato Dextrose Agar (PDA). The plates were spread evenly using a sterile glass spreader and incubated at 28 ˚C for 2-4 days.

7.2.5. Release/diffusion of volatile component encapsulated in biopolymeric films during storage (*in situ*)

The controlled release of volatile component from active component was evaluated by a method described by Tunc et al., (2010). Bioactive chitosan based films were kept in rice grains (1 g/cm^2) incubated (at 28˚ C and 65% RH) for 12 weeks. Two types of films were tested. i) CH based film ii) CH reinforced with CNC based film containing oregano: thyme EO nanoemulsion. Films were taken out from the rice grains every week and cut into known sizes to keep the weight of samples as constant as possible (500 mg). Then, the films were placed into 10 ml of ethanol for 4 hr with constant agitation for the extraction of volatile components from the film matrix. The release of volatile components was determined by spectrophotometer method at 274 nm. It should be noted that the initial concentrations for each type of film were determined before they were put into the chamber. Moreover, the decreased concentration in the film samples was considered as release of volatile component.

By studying the plot of the UV visible absorption profile for release at 274 nm, the kinetics of the EO release was analyzed by assuming a first order release kinetics following a method described by Patil *et al.* (2016). The equation for calculation of first order velocity constant K is given below

$$K = \frac{2.303}{t} \log (A_\infty - A_0)/ (A_\infty - A_t)$$

Where A_∞, A_0, A_t refers to absorption values of infinity, initially and at time (t).

7.2.6. Physicochemical characteristics of irradiated and non-irradiated composite films

Physicochemical properties were evaluated for six types of irradiated and non-irradiated films. These were i) CH film (Control) ii) CH-bioactive film A containing 0.13 (w/w%) of thyme and oregano nanoemulsion ii) CH-bioactive film B containing 0.19 (w/w%) of thyme and oregano nanoemulsion iv) CH-CNC film (control film) v) CH- CNC- bioactive film A containing 0.13 (w/w%) of thyme and oregano nanoemulsion, and vi) CH- CNC-bioactive film B containing 0.19 (w/w%) of thyme and oregano nanoemulsion

7.2.6.1. Mechanical properties of biopolymeric films

Film thickness was measured using a Mitutoyo Digimatic Indicator (Type ID-110E; resolution: 1 µm; Mitutoyo MFG Co. Ltd, Tokyo, Japan), at five random positions around the film. Film widths were measured using a Traceable® Carbon Fiber Digital Caliper (resolution: 0.1 mm/0.01; accuracy: ± 0.2 mm/0.01; Fisher Scientific). The mechanical properties namely tensile strength (TS), tensile modulus (TM) and elongation at break (Eb) of the composite films were measured using a Universal Testing Machine (UTM) (model H5KT; Tinius Olsen Testing Machine Co., Inc., Horsham, PA, USA), equipped with a 100 N-load cell (type FBB) and 1.5 kN-specimen grips. Measurements were carried out following the ASTM D638-99 method. UTM parameters were set up for "plastics tensile from position" test type with the selections of 25 mm effective gauge length, flat specimen shape, 1 number of entries and minimum type. The position rate of machine control was fixed to 50 mm/min. Y- and X-axes were assigned to load (100 N-range) and position (500 mm-range) coordinates, respectively. Tensile strength (TS, maximum stress, MPa), tensile modulus (TM, elastic modulus, MPa) and elongation at break (Eb, %) values of the films were measured after film break due to elongation, using the Test Navigator® program for two sets of films, without gamma radiation and treated with irradiation at 750 Gy.

7.2.6.2. Water vapor permeability (WVP)

WVP tests were conducted following the procedure described by Salmieri *et al.* (2014b). The films were mechanically sealed onto Vapometer cells model 68-1; Twhing-Albert Instrument Co., West Berlin, NJ, USA) containing 30 g of anhydrous calcium chloride (0 % relative humidity, RH). Cells were initially weighed and placed in a Shellab 9010L controlled humidity

chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA) maintained at 25 ˚C and 60 % RH for 24 h. The assemblies were weighed initially and after 24 h for all samples. The weight gain of the cell represented the amount of water vapor transferred through the film and absorbed by the desiccant (anhydrous $CaCl_2$).

WVP was calculated according to the combined Fick and Henry's laws of gas diffusion through coatings and films, according to the equation below.

WVP (g mm m^{-2} day^{-1} kPa^{-1}) = $\Delta w. x /A. \Delta P$

Where, Δw is the weight gain of the cell (g) after 24 h, x is the film thickness (mm), A is the area of exposed film (31.67 X 10^{-4} m^2), and ΔP is the differential vapor pressure of water through the film (ΔP = 3.282 kPa at 25 ˚C).

7.2.7. Sensory analysis

The most efficient chitosan-based bioactive film containing 0.19% of EO emulsion (highest concentration) was chosen for an evaluation of its effect on sensorial properties of cooked rice. A panel comprising of 12 individuals evaluated the odor, the taste and the color independently, and provided global appreciation of the samples stored with bioactive films for two months. Rice samples were cooked for 20 min in steam rice cookers at a 1:2 (v/v) rice-to-water ratio (Meullenet *et al.,* 1998). The samples were served in separate cups with closed lids and were identified by 3 random digits. The evaluation was held on a 9-point hedonic scale: 9 = Like extremely, 8 = Like very much, 7 = Like moderately, 6 = Like slightly, 5 = Neither like nor dislike, 4 = Dislike slightly, 3 = Dislike moderately, 2 = Dislike very much, 1 = Dislike extremely.

7.2.8. Statistical Analysis

All experiments were done triplicate and each replicate include analysis of three samples. Data were subjected to analysis of variance and mean separations were performed using the Duncan's multiple-range test. Differences between means were considered significant when the confidence interval was smaller than 5 % ($P \leq 0.05$). All analyses was performed using PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

7.3. Results and Discussion

7.3.1. Characteristics of combined EO nanoemulsions

Emulsions are heterogeneous systems comprised of two immiscible liquids (generally oil and water), where one of the phases, termed as the dispersed phase, is distributed in the other one as a continuous phase (Juttulapa *et al.*, 2017). Emulsions with droplet sizes ranging between 20-200 nm are referred as nanoemulsions. High pressure homogenization allows more flexibility in nanoemulsion formulation (Jasmina *et al.*, 2017, Wang *et al.*, 2015). In this study three types of EO mixtures were used to produce EO nanoemulsions using high-pressure microfluidization. The size, polydispersity index (PDI), zeta potential and antifungal activity of the prepared nanoemulsions were evaluated. Results showed that a pressure of 15,000 psi and 3 cycles resulted in the formation of nanoemulsions with droplet size of less than 100 nm, and exhibited a higher antifungal activity as compared to the coarse emulsions (Table 7.1).

The sizes of the coarse emulsions prepared with formulation 1, 2 and 3 was 219, 262 and 279 nm respectively, while the size of the nanoemulsions prepared from the same formulations was significantly smaller (77, 70 and 59 nm, respectively) ($P \leq 0.05$). The nanoemulsion based on thyme and peppermint combination had smallest size (57.9 nm) as compared to the nanoemulsions 1 and 2. The particle size distribution was also narrower when compared with that obtained by low pressure homogenization. Hence, the PDI was lower for nanoemulsions as compared to coarse emulsions ($P \leq 0.05$) rendering the emulsion less heterogenous. As such, the PDI measures the distribution of molecular mass in a polymer or indicates the degree of heterogeneity of sizes of the polymer molecules. Microfluidization of the coarse emulsions decreased the PDI between 0.4-0.2, and dispersed the particles with narrower particle size distributions due to the high shear stresses developed in the microchannels of the interaction chamber. In addition, the microfluidization process had a stabilizing effect on the nanoemulsions as indicated by the zeta-potential measurements. Zeta-potential indicates the stability of colloidal systems due to electrostatic repulsion. If all the particles in suspension have a large negative or large positive zeta potential, then they will tend to repel each other, reducing their tendency to flocculate. Low zeta potentials mean that particles will likely tend to aggregate and flocculate. Studies have shown that emulsions with zeta potential of -11 to -20 mV were close to the

threshold of agglomeration, while emulsions with zeta potential of -41 to -50 mV had good stability (Lu *et al.*, 2010). The zeta-potential of the prepared emulsions (coarse and nanoemulsions) ranged between -43 to -56 mV and thereby displayed higher stability. The tendency observed in these average values indicated that the particles were of smaller size as a result of high pressure application, which caused the clusters to segregate into individual particles. Similar results have been obtained by Pereda *et al.* (2007) when applying homogenization pressure to cow milk emulsion systems.

The antifungal properties were found to be significantly ($P \leq 0.05$) dependent on the size of the emulsions. As particle size decreased following microfluidization, the percentage inhibition of *A. niger* increased. The percent inhibition induced by the coarse and nanoemulsions was 39, 36, 43% and 83, 75 and 87%, respectively, for the thyme and oregano, thyme and tea tree, and thyme and peppermint mixture. The antifungal activity was also found to decrease significantly over time ($p \leq 0.05$). After 72 hours of incubation, the percent inhibition induced by the coarse and nanoemulsions was 27, 16, 22% and 64, 43 and 51%, respectively, for thyme and oregano, thyme and tea tree, and thyme and peppermint mixture. However, the decrease in bioactivity was significantly less ($p \leq 0.05$) for the nanoemulsions as compared to the coarse emulsions for all the EO formulations. Smaller particles have greater surface area-to-volume ratios, which considerably increases the dissolution rate of the particles, enabling them to overcome solubility-limited bioavailability thereby exhibiting a higher biological activity. Such size-dependent antimicrobial activity has previously been observed by other authors (Goldberg *et al.*, 2007, Jeong *et al.*, 2014). Jeong *et al.* (2014) incubated *Methylobacterium* spp with water-soluble silver nanoparticles of 10 and 100 nm for two days. The results showed that the 10 nm nanoparticles displayed higher antimicrobial activity than the 100 nm sized particles. We hypothesize that the enhanced antimicrobial activity of the smaller nanoparticles is due to their ease in penetrating cell membranes and cell walls as compared to larger nanoparticles. Floury *et al.* (2000) reported that high-pressure homogenization increases the surface activity of emulsifying molecules and improve their efficiency. The nanoemulsions of the three EO mixtures in the current study exhibited between 15-20% inhibition depending on the formulation; however, the coarse emulsions did not display any antifungal activity after 1 week (data not shown).

7.3.2. *In vitro* antifungal activity of chitosan-based nanocomposite films

The vapor phase antifungal properties of the films were evaluated by measuring the clear inhibitory zone around the Petri dishes after 24 h incubation (Table 7.2). The chitosan film without any EO mixture exhibited no inhibition of fungal growth, whereas the bioactive films containing the three EO mixtures showed significant inhibition of fungal growth. All three formulations displayed the highest percentage inhibition against *P. chrysogenum* at the concentration of 0.13% (wt/wt). With 0.19% EO containing films, *P. chrysogenum* was also found to be the most sensitive to the thyme-oregano, and thyme-tea tree mixtures while the growth of *A. niger* was most inhibited by the thyme-peppermint mixture. The difference in the inhibitory activity of the EO formulations against the tested fungal species may be attributed to factors such as the nature and composition of the oils, the possible interactions between the polymeric material of the film and the active compounds, and the diffusion rate of active components through the film matrix (Sánchez-González *et al.*, 2011). This may explain why the films displayed antifungal properties with varying extent against the fungal species. Based on the micro-atmosphere assay, *A. flavus* and *A. niger* were found to be the most resistant fungal species towards the tested bioactive films, followed by *A. parasiticus* and *P. chrysogenum*, respectively. All films significantly reduced the growth of *P. chrysogenum* by 51-77% ($P \leq 0.05$). The antifungal potential of the chitosan/EO films may result from electrostatic interactions of Van der Waals forces and hydrogen bonds between the negatively charged components present in the cell membrane of the tested fungi and the positive charge of the bionanocomposites used in this study. It has been shown that electrostatic interactions between positively charged nanoparticles and microbial cell membranes can cause membrane destabilization, altering the properties thereof and causing the death of the microorganisms (Luque-Alcaraz, et al., 2016).

The bioactivity of various plant EOs embedded in different polymeric matrices has been investigated by several researchers and shown inhibit a variety of microorganisms including bacteria, yeasts and molds. Avila-Sosa *et al.* (2012) studied bioactive amaranth, chitosan, and starch edible films by incorporating Mexican oregano (*Lippia berlandieri Schauer)*, cinnamon (*Cinnamomum verum*) or lemongrass (*Cymbopogon citratus*). The potential of vapor activity of

these films was tested against *A. niger* and *P. digitatum.* A significant increase in the lag phase as well as a decrease in the maximum specific growth rates were observed against both fungal species. López *et al.* (2007a) designed active films made up of polypropylene (PP) and polyethylene/ethylene vinyl alcohol copolymer (PE/EVOH), and incorporated EOs of cinnamon (*Cinnamomum zeylanicum*), oregano (*Origanum vulgare*), clove (*Syzygium aromaticum*), and cinnamon fortified with cinnamaldehyde. The formulated bioactive films were found to exhibit significant activity against a wide range of fungi, including *P. islandicum, P. roqueforti, P. nalgiovense, Eurotium repens* and *A. flavus*

7.3.3. *In situ* analysis with CNC-reinforced Chitosan (CH)

In situ analyses were performed using CNC-reinforced chitosan (CH/CNC) containing oregano and thyme nanoemulsions in view of evaluating their inhibitory effects on fungal growth on packaged rice during 8 weeks of storage. The fungal growth profiles for the control (no film) and CH/CNC-containing the oregano-thyme nanoemulsion are shown in Fig.7.1. The initial inoculation was 3 log CFU/g for the tested fungal species. The growth of *A. niger* in the control samples reached 6.49 log CFU/g after 8 weeks of incubation (Fig 1a). For the samples incubated with film A (0.13% EO) and B (0.19% EO), the fungal growth found to be 4.94 and 4.22 log CFU/g, respectively, representing a reduction of 1.26 and 1.99 log CFU/g, respectively, of the fungal growth after 8 weeks of incubation as compared to the control samples. The *A. niger* growth was found 3.54 log CFU/g after 8 weeks of incubation when applied gamma radiation at 750 Gy alone, reduced the growth of *A. niger* by 2.54 log CFU/g as compared to the control sample. Combining the bioactive films with a treatment of gamma irradiation at 750 Gy caused an enhanced reduction in *A. niger* growth. Irradiation of the inoculated samples and application of the bioactive film A and B incurred a reduction of 4.18 and 4.88 log CFU/g, respectively, after 8 weeks of incubation as compared to the control samples.

The growth of *A. flavus* in the control samples reached 7.48 log CFU/g after 8 weeks of incubation (Fig 7.1b). For the samples incubated with film A (0.13% EO) and B (0.19% EO), the fungal growth found to be 4.61 and 5.02 log CFU/g, respectively, representing a reduction of 2.87 and 2.46 log CFU/g, respectively, of the fungal growth after 8 weeks of incubation as compared to the control samples. By the application of 750 Gy of gamma radiation, the growth

of *A. flavus* was found to be 4 log CFU/g causing a reduction of 2.48 log CFU/g as compared to the control sample after 8 weeks of incubation. Combining the bioactive film A and B with a treatment of gamma irradiation at 750 Gy incurred a reduction of 4.35 and 4.93 respectively, after 8 weeks of incubation as compared to the control samples.

Similarly, samples treated with A and B bioactive films alone reduced the growth by 1.07 and 2.08 log CFU/g for *A. parasiticus* and 2.49 and 3.11 log CFU/g for *P. chrysogenum*, respectively, as compared to the control samples. By the application of 750 Gy of gamma radiation, the growth of *A. parasiticus* and *P. chrysogenum* was found to be 3.8 log CFU/g and 2.70 log CFU/g causing a reduction of 2.77 log CFU/g and 3.19 log CFU/g as compared to the control sample after 8 weeks of incubation. Combining the bioactive film A and B with a treatment of gamma irradiation at 750 Gy incurred a reduction of 4.37 and 5.27 log CFU/g respectively for *A. parasiticus* and 3.40 and 4.53 log CFU/g respectively for *P. chrysogenum* after 8 weeks of incubation as compared to the control samples. Figure 7.5 shows photographic images of rice grains inoculated with *A. niger* under different treatment conditions. No visible fungal growth could be observed for the samples with the bioactive A and B films combined with gamma irradiation treatment during 8 weeks of storage period (Fig. 7.5 v, vi).

Based on the results, it is found that CH/CNC films loaded with oregano and thyme, and combined with gamma radiation can represent an attractive means for storing food commodities and improving food safety for months. Carvacrol and thymol are the major components of thyme and oregano EOs. These compounds have been shown to exhibit broad antimicrobial effects and possess heat stability during processing (Majeed *et al.*, 2015, Ramos Santonja *et al.*, 2013). CH/CNC polymeric matrices offer a great potential as a diffusion matrix for bioactive agents and protecting their efficiencies during storage (Deng *et al.*, 2017). It has been reported that CNC stabilizes encapsulated bioactive compounds in polymers while allowing better control in their release into food (Azizi *et al.*, 2014, Boumail *et al.*, 2013). The results obtained in this current study suggest that EO nanoemulsions incorporated within the CH-CNC polymeric matrix were progressively released from the film surface to protect the rice grains over a longer period of time. Moreover, the composite matrix protected the incorporated nanoemulsions from

interactions with environmental factors and limited their rapid loss and consumption into the surrounding surface.

Combining the encapsulated bioactive films with γ radiation exerted a more accrued inhibitory effect on the growth of *A. niger*. Applying a low concentration of (0.19%, w/w) oregano-thyme mixture with a low irradiation (750 Gy) dose caused an almost 70-80% reduction in the fungal growth during the 8 weeks of storage. A recent study by Severino *et al.* (2015) on a bioactive coating formulation involving modified chitosan-based coatings containing carvacrol nanoemulsion, gamma irradiation, modified atmosphere packaging (MAP), alone or in combinations, against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium was evaluated on inoculated green bean samples during a storage period of 13 days. Samples subjected to combined treatments of MAP, gamma irradiation and bioactive coating showed a significant reduction of in the levels of the two bacteria. The combined treatment reduced the *E. coli* population to a level not detectable after 7 days of storage. Such treatments also led to a significant reduction ($P \leq 0.05$) of 2.07 log CFU/g of *S.* Typhimurium as compared to the control after 13 days of storage, exhibiting a strong synergistic antimicrobial effect (Severino *et al.*, 2015). The authors suggested that microorganisms that are able to survive after irradiation treatment may be more sensitive to adverse conditions such as antimicrobial or other preservative techniques. Similar observations were also made by Hossain *et al.* (2014a), Lacroix *et al.* (2015). Hence, a combined treatment with EO-containing bioactive films and irradiation is not only effective against bacteria, but also fungal species as shown in the current study. Such combined treatments are more efficient at inducing fungal demise during food storage than individual treatment.

7.3.4. Release/diffusion of volatile component from active CH based matrix

To obtain the release of the volatile components of encapsulated EOs from CH film matrix a standard curve was obtained for oregano: thyme EO combination at 274 nm. The standard curve was plotted by the absorption peaks of the oregano: thyme EO from spectra of pure oregano: thyme EO obtained using a UV-VIS spectrophotometer. A major peak appeared at wavelength 274 nm was used for the determination of released EO from CH film matrix. The extracted eluents were subjected to analysis of absorbance to obtain the release of volatile components

during storage. This is in agreement with Barzegar *et al.* (2016) and Partheniadis *et al.* (2017). The result showed (Fig 7.2) a gradual release of volatile components (oregano: thyme) from the chitosan based film matrix. The release was 8% and 29% with chitosan based film matrix at weeks 1 and 12 respectively. The result shows that the release was faster (8%) in the first week and then it becomes constant over week 2 and 3 (11%). Between week 4 and 12 the release of EOs was 21-29%. The release was even slower ($P \leq 0.05$) with the film containing crystalline nanocellulose (CNC) as a reinforcing agent. It is clearly seen from the figure that CNC exhibits a significant ($P \leq 0.05$) effect in the sustained release of active components in the film. The release of volatile components from CNC reinforced chitosan film was 3 and 17% at week 1 and week 12 respectively. The release of volatile components in CNC reinforced CH based nanocomposite film was 40.8% slower as compared to CH based nanocomposite film. Hence the addition of CNC in CH matrix greatly helped to preserve the EO bearing highly volatile property, while preserving the biological activity of the EOs during storage. The release of EOs nanoemulsions from the CH matrix can be explained by first order release kinetics as the regressions of the developed model was found to be 0.85 and 0.84 respectively (Fig 7.3 i and ii) indicating suitability of the data for studying the release kinetics during a 12 week of storage period.

CNC has important applications in the food sector especially as food additives and packaging films by enhancing potential structural and chemical properties and the efficacy of controlled delivery in active packaging systems (Fortunati *et al.*, 2013). Comparative studies showed that CNC application with chitosan-based films have shown to accrue delay in fruit ripening by Deng *et al.* (2017). Gelatin coatings containing CNC were found to extend the shelf-life of strawberry fruit (*Fragariaananassa*) over 8 days (Fakhouri *et al.*, 2014). The strawberry samples covered with GEL/CNC displayed significant extension in their shelf-life by retaining ascorbic acid (AA) (vitamin C) in the strawberries, as compared to the control samples which experienced a fast decay in AA content. Huq *et al.* (2014) developed edible nisin- beads microencapsulated on alginate–CNC matrices, which exhibited a better antimicrobial effect as compared to free nisin over 28 days of storage period. Similarly, Wang *et al.* (2017) found chitosan-cellulose nanocrystal microencapsulation improved the encapsulation efficiency and stability of entrapped

fruit anthocyanins. Their results indicated that CNC incorporation into chitosan functioned as macro-ion crosslinking agent and as fillers for chitosan matrix. The chitosan-CNC matrices generate more rigid and stable microcapsules. Hence this study demonstrated the integration of CNC in to CH based nanocomposite films enabled a better protection and retention of the encapsulated EO molecules over storage period. These results were further confirmed by the antifungal effectiveness of the films during a storage period.

7.3.5. Physico chemical properties of non-irradiated and irradiated films

The physico chemical properties of the developed chitosan films, including tensile strength, tensile modulus, elongation break and water vapor permeability, were evaluated alone and impregnated with other agents such as EO formulations A (0.13%) and B (0.19%), cellulose nanocrystals (CNC), and without and with gamma irradiation at 750 Gy (table 7.3). For the polymeric films without irradiation, the tensile strength of CH films (thickness~ 45 µ) was 59.6 MPa. Addition of both formulation A (thickness~ 54 µ) and B (thickness~ 61 µ) reduced the tensile strength to 50 and 43 MPa, respectively ($P \leq 0.05$). Such reduction in tensile strength may be attributed to the plasticizing effect of EOs (Salmieri *et al.*, 2014a, Yanwong *et al.*, 2015). Incorporating the films with 5% (w/w) CNC enhanced their tensile strength by 23, 19 and 20% for CH, CH-A and CH-B films respectively ($P \leq 0.05$). The increment in tensile strength is an indication of strong reinforcement of the chitosan matrix by the CNC. The high reinforcing effect of CNC is due to a mechanical percolation phenomenon which form a stiff continuous network of cellulosic nanoparticles linked through hydrogen bonding (Abou-Zeid *et al.*, 2015).

In terms of tensile modulus (TM) the higher TM value is indicative of a more rigid material. The tensile modulus of the CH -Film A and CH Film B was less as compared to the control film indicating a less dense film matrix. Loading of 5% CNC nanofiller caused a 9, 24 and 17% increment of the TM. Bioactive CH-Film A and CH Film B films displayed a higher elongation of break (EB) corresponding to a higher film flexibility than the control. The EB values of the film improved significantly ($P \leq 0.05$) from 29 to 41% with the incorporation of EOs. Generally, plasticizers weaken intermolecular forces between adjacent polymer chains, resulting in a decreased TS and increased film flexibility. Decreases in TS and increases in EB are common results of essential-oil incorporation which is consistent with the present results (Ghasemlou *et*

al., 2013, Zivanovic *et al.*, 2005). Addition of CNC to the biopolymer significantly decreased the EB between 21 and 34% for CH and bioactive CH films. Similar observations have been reported by other authors who reinforced polymers with nanofillers (Dhar *et al.*, 2015, Khan *et al.*, 2012a). Incorporation of CNCs into the polymer matrix has been shown to result in a strong interaction between the matrix and filler restricting the motion of the matrix and hence lowering EB (Azeredo *et al.*, 2010).

The water vapor permeability (WVP) of biopolymeric films was investigated since water has a noticeable impact in deteriorative reactions as it acts as a carrier to cause texture degradation, and chemical and enzymatic reactions in polymers (Table 4). The WVP of the control and bioactive CH films were not significantly different ($P > 0.05$) with respective values ranging between 9.77-10.66 g mm m^{-2} day $^{-1}$ kPa $^{-1}$. However incorporating 5% CNCs decreased the WVP significantly ($P \leq 0.05$) from 9.84 -7.61 g mm m^{-2} day $^{-1}$ kPa $^{-1}$. No significant difference ($P > 0.05$) was observed for the WVP between bioactive formulations ($P \leq 0.05$). Water vapor properties of bioactive films are dependent on the polymer to EO ratio, and the homogenization technique implemented (Bonilla *et al.*, 2011a). Bonilla *et al.* (2011b) studied the influence of homogenization conditions and basil EO content on the physical properties of chitosan films, and found that the WVP increased when the chitosan to oil ratio was 1:1, but did not cause any improvement with the ratio of 1:0.5. The low EO concentration used in present study may not have significantly affected the WVP of the films. However, loading of 5% CNC decreased the WVP by 23% as compared to the control film.

According to Belbekhouche *et al.* (2011) and Huq *et al.* (2012) CNC does not have the ability to form a film by itself; however, it can play an important role as a filler for strengthening other polymeric matrices. In addition, CNC forms a mechanical, steric barrier in oil/water interface capable of protecting emulsion droplets against coalescence (Dickinson, 2012). Sampath *et al.* (2017) also found that integrating CNC to chitosan remarkably improved the latter's mechanical properties and sensitivity towards pH changes. Studies with other polymeric materials have shown that CNC can have similar effects (Azeredo *et al.*, 2010, Khan *et al.*, 2012b). Recently, the effects of nanocellulose on different types of hydrogels, such as gelatin, alginate, poly (vinyl alcohol), and collagen, have been investigated for various applications such as drug delivery,

organic pollutant absorption, and tissue engineering (Li *et al.*, 2014, Tanpichai *et al.*, 2016, Yin *et al.*, 2015). Our results concur with previous findings and show that CNC can effectively stabilize and strengthen chitosan polymeric matrix thereby enhancing its mechanical properties. This may be due to the strong interactions creating a crosslinking effect, thus decreasing the free volume and molecular mobility of the polymer (Pereda *et al.*, 2012). The improved mechanical properties the CNC-CH formulation can effectively expand their range of applications in food packaging, and various other fields such as tissue engineering, pharmaceuticals, drug delivery.

For chitosan films combined with irradiation, a net improvement in the mechanical properties was observed (Table7. 3). The application of gamma irradiation on chitosan alone significantly improved its tensile strength and modulus, and decreased it water permeability. Similar observations were also found with the chitosan films impregnated with formulations A and B. However, the highest improvement was obtained with the chitosan films mixed with CNC, where the tensile strength and modulus reached 94 MPa and 987, respectively. This was accompanied by a notable reduction in water permeability. Hence, it appears that gamma irradiation induces cross-linking of the chitosan films and its effect is more accrued in the presence of CNC. Such increased cross-linking, as induced by irradiation, may be responsible for further reinforcing the chitosan polymeric matrix. These findings were in agreement with Khan *et al.* (2012a) and Nasreen *et al.* (2016). Hence, gamma-irradiated chitosan films were found more resistant to mechanical stress and their structure is likely more conducive in protecting the activity of antimicrobial agents and extending the shelf-life of food products.

7.3.6 Nanocomposite films in cooked rice

A sensorial evaluation of rice samples incubated with 0.19% (w/w) of thyme-oregano EO nanoemulsion loaded chitosan-based films was performed in view of evaluating any sensorial alteration of the treated rice grains during storage. The results showed (fig 7.4) that all the control and treated samples were acceptable, and received high scores by the panelists. The scores obtained ranged between 7.4-8.0 for odor, taste, color and general appreciation. No negative effect (“Dislike a little” or “Lower appreciation”) was reported on the organoleptic properties of any samples. Fragrance related to the presence of the EOs was expected to induce a high aromatic intensity over a prolonged period. However, incorporation of EOs to edible films

requires very low concentrations as compared to direct applications (Badr *et al.*, 2013). Further, nano encapsulating systems represent a viable and efficient approach to increase the physical stability of EOs, protection from evaporation, and gradual release with enhanced bioactivity (Bilia *et al.*, 2014). In the present study, a low concentration as 0.19 % (w/w) of oregano-thyme nanoemulsions incorporated into the chitosan matrix remained highly acceptable after cooking until the end of the two-month storage period and did not induce any alteration to the organoleptic properties of the food. This aspect is extremely important for consumer acceptance in the global market.

7.4. Conclusion

Chitosan-based nanocomposite films loaded with EOs were designed and tested in the current study. Vapor phase assays showed that the developed chitosan films were very efficient at inhibiting the growth of fungi, and resulted in fungal control to varying extent depending on the EO combination. Application of gamma irradiation significantly improved the tensile strength and modulus of the designed films. Incorporation of CNC into the chitosan biopolymeric matrix even reinforced its structure to a higher extent in terms of tensile strength and elongation break and a controlled release of active component. In addition, higher fungal inhibitory effect was observed by combining the chitosan bioactive polymeric films with low dose irradiation (750 Gy). Sensorial evaluation of rice samples containing CH-CNC films loaded with EO nanoemulsions showed no significant ($p > 0.05$) alteration in odor, taste, color and overall appreciation. Such bioactive films bearing enhanced structure and antifungal properties hold significant potential for controlling fungal growth during storage of food items.

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Table 7.1. Average droplet diameter, polydispersity index (PDI), zeta-potential and antifungal activity of coarse and nanoemulsions.

Combination of EOs	**Type of emulsion**	**Size (nm)**	**PDI**	**Zeta (mV)**	**Percentage (%) inhibition of *A. niger***		
					24 h	**48 h**	**72 h**
Formulation 1: Thyme-Oregano	Coarse	219.0±1.62^{d}	0.45^{e}	-56.2^{a}	39.75±1.70^{ab,C}	31.32±1.70^{b,B}	27.71±1.70^{c,A}
	Nano	76.58±0.35^{c}	0.25^{b}	-51.0^{c}	83.73±2.55$^{d, C}$	71.08±1.70^{e,B}	64.45±2.55^{f,A}
Formulation 2: Thyme-tea tree	Coarse	262.4±0.70^{e}	0.37^{d}	-43.7^{e}	36.71±0.85^{a,C}	22.28±0.85^{a,B}	16.26±2.55^{a,A}
	Nano	69.9±1.13^{b}	0.21^{a}	-50.7^{c}	75.60±1.27^{c,C}	55.42±3.40^{c,B}	43.97±4.25^{d,A}
Formulation 3: Thyme-peppermint	Coarse	279.0±0.35^{f}	0.46^{e}	-48.0^{d}	43.07±0.42^{b,B}	28.91±3.40^{b,B}	22.59±2.98^{b,A}
	Nano	57.9±0.70^{a}	0.32^{c}	-53.3^{b}	87.95±6.81^{c,D}	66.26±6.^{81c,D}	51.20±0.85^{e,A}

Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different (P > 0.05). Within each row means with the same uppercase letter are not significantly different (P > 0.05).

Table7.2. *In vitro* antifungal property of CH/CNC bioactive films containing EO nanoemulsions

Formulation 1: Thyme-Oregano	**EO Conc. (%)**	***A. niger*** **% inhibition**	***A. flavus*** **% inhibition**	***A. parasiticus*** **% inhibition**	***P. chrysogenum*** **% inhibition**
Formulation 2: Thyme-tea tree	0.13	52.74±2.67 [b, A]	58.88±3.65 [d, B]	54.08± 3.26 [b, A]	69.61±3.89 [c, C]
	0.19	69.34±2.95 [d, B]	61.31±4.14 [d, A]	71.35± 4.25 [d, B]	77.37±4.85 [d, C]
	0.13	42.83±3.49 [a, B]	33.88±2.89 [a, A]	42.30± 5.12 [a, B]	51.67±3.58 [a, C]
	0.19	52.34±4.34 [b, B]	44.17±5.55 [b, A]	51.40± 4.89 [b, B]	62.51±2.20 [b, C]
Formulation 3: Thyme-peppermint	0.13	58.24±3.13 [c, B]	42.71±3.68 [b, A]	55.02± 4.62 [b, B]	64.25±3.01 [b, C]
	0.19	73.62±3.87 [e, D]	49.93±1.71 [c, A]	63.85± 4.21 [c, B]	69.07±4.74 [c, C]

Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different (P > 0.05). Within each row means with the same uppercase letter are not significantly different (P > 0.05).

Table 7.3. Mechanical properties and Water Vapor Permeabilities (WVP) of non-irradiated and irradiated films

Treatment	**Samples**	**Tensile Strength TS (MPa)**	**Tensile Modulus, TM**	**Elongation of Break (EB%)**	**Water Vapor Permeability g mm m^{-2} day^{-1} kPa^{-1}**
Non-irradiated	CH	59.67 ± 3.35^{ef}	852.0 ± 82.72^{c}	29.52 ± 4.16^{bc}	9.84 ± 0.31^{e}
	CH+ film A	50.07 ± 3.80^{b}	595.65 ± 40.34^{a}	35.24 ± 4.13^{ef}	9.77 ± 0.65^{e}
	CH+ Film B	43.81 ± 2.56^{a}	578.53 ± 70.70^{a}	41.65 ± 5.27^{g}	10.66 ± 0.47^{e}
	CH +CNC	77.12 ± 3.19^{h}	943.14 ± 135.6^{d}	21.91 ± 2.19^{a}	7.61 ± 0.23^{c}
	CH+ CNC+ Film A	62.50 ± 2.16^{fg}	790.86 ± 115.81^{c}	34.52 ± 2.00^{ef}	9.53 ± 0.26^{e}
	CH+ CNC+ Film B	54.16 ± 5.17^{cd}	703.21 ± 86.87^{b}	34.04 ± 2.99^{f}	9.95 ± 0.85^{e}
Irradiated	CH	83.96 ± 5.49^{i}	994.74 ± 73.33^{d}	30.22 ± 1.98^{cd}	6.93 ± 0.16^{b}
	CH+ Film A	64.66 ± 2.75^{g}	795.64 ± 67.26^{c}	33.90 ± 3.33^{ef}	8.03 ± 0.82^{c}
	CH+ Film B	51.22 ± 4.62^{bc}	708.96 ± 59.14^{b}	36.96 ± 3.35^{f}	8.60 ± 0.37^{d}
	CH +CNC	94.26 ± 4.95^{j}	986.68 ± 65.59^{e}	26.63 ± 3.05^{b}	6.46 ± 0.29^{ab}
	CH+ CNC+ Film A	66.05 ± 2.82^{g}	810.75 ± 87.61^{c}	32.95 ± 2.65^{de}	6.14 ± 0.51^{a}
	MC+ CNC+ Film B	57.02 ± 4.14^{de}	732.28 ± 51.43^{b}	35.95 ± 4.09^{ef}	7.63 ± 0.32^{c}

Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different ($P \leq 0.05$)

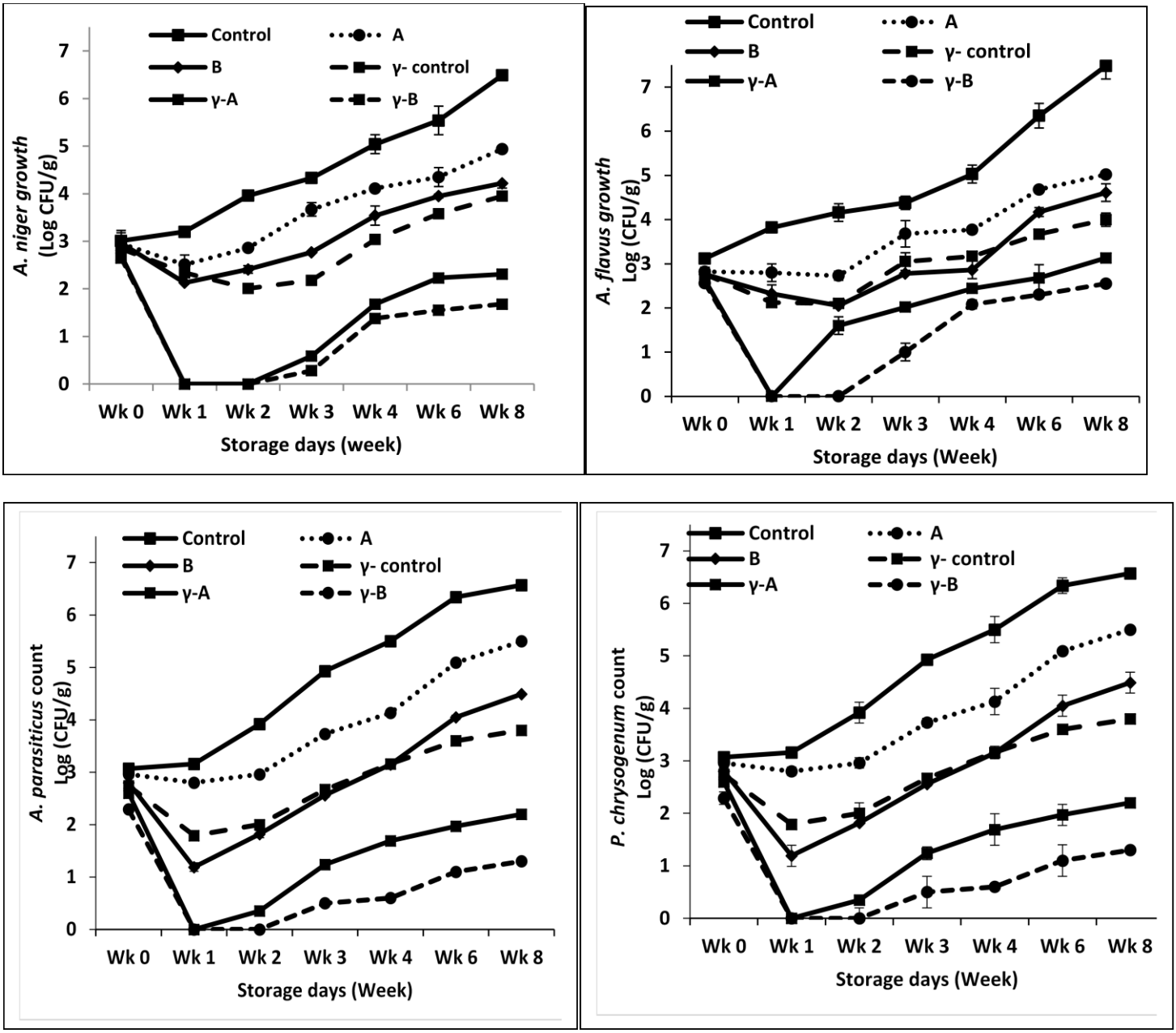


Figure 7.1. Fungal growth profiles of *A. flavus, A. niger*, *A. parasiticus* and *P. chrysogenum* following in situ tests with bioactive chitosan (CH) nanocomposite films over 8 weeks of storage. Abbreviations: Wk-week; γ-gamma irradiated sample; A-biofilm impregnated with 0.13% EO; B-biofilm containing 0.19% EO.

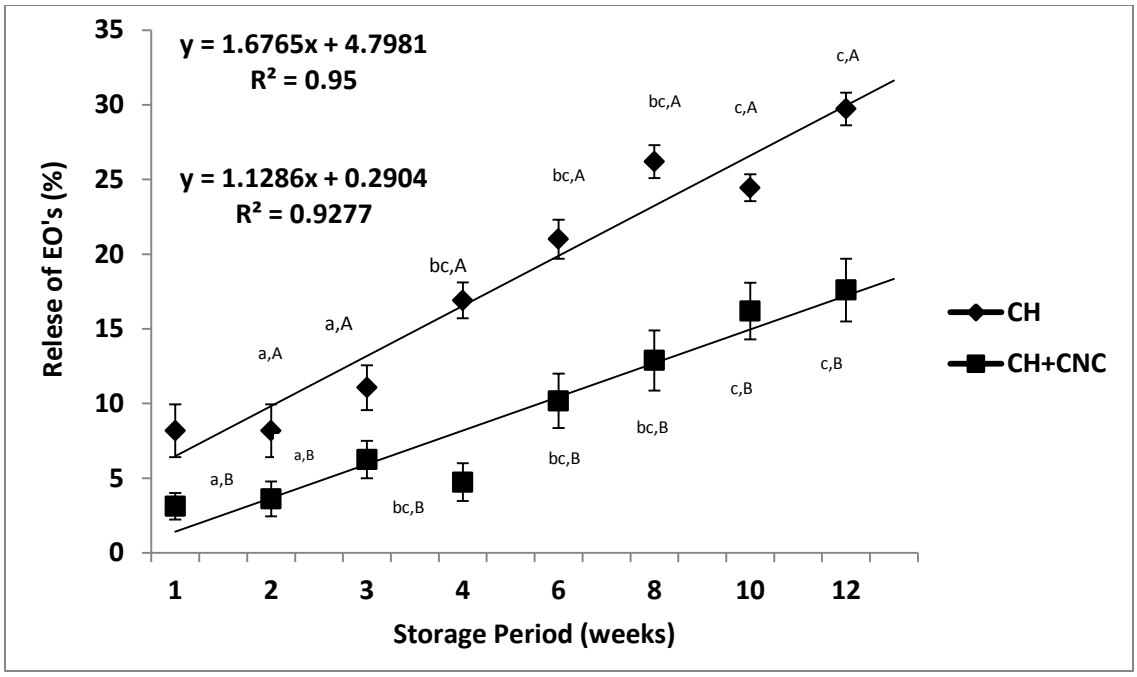
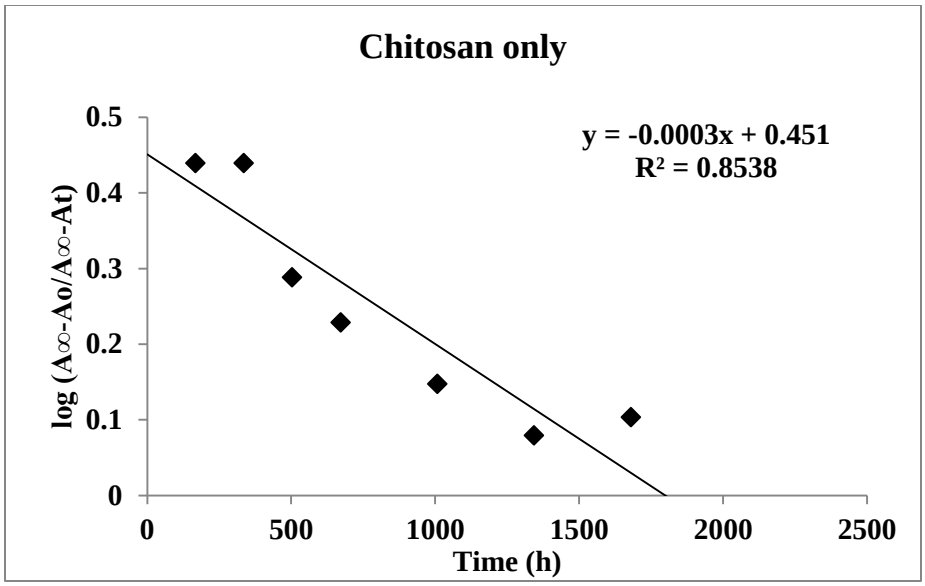


Figure 7.2. Release (%) of EOs from bioactive CH and CH+ CNC based films during storage.



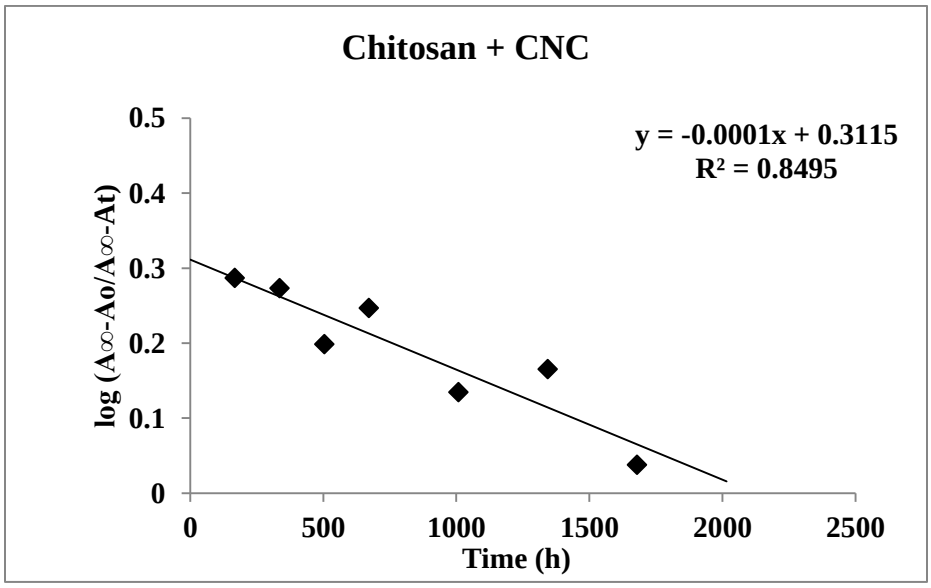


Figure 7. 3. Kinetics for release study for i) CH ii) CH+ CNC nanocomposite films containing EO nanoemulsion.

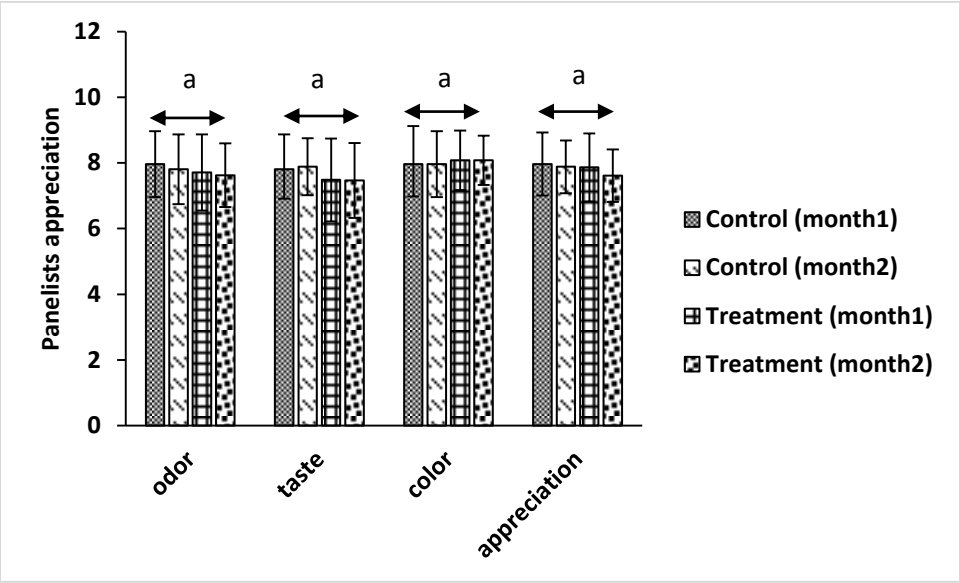


Figure 7.4. Sensorial evaluation of rice grain containing chitosan based bioactive nanocomposite films during 2 months of storage period. Means followed by the same lower case letter are not significantly different at the 5% level.

Figure 7.5. Photographic images of rice samples inoculated with *A. niger* with control samples i) CH-CNC (no EOs) ii) Bioactive film A ii) Bioactive film B. iv) γ- CH-CNC (no EOs) v) γ Bioactive film A vi) γ Bioactive film B after 8 weeks of storage.

Chapter 8

General Discussion, conclusion and future perspectives

Although considerable research efforts have been made on the pesticide properties of essential oils and their constituents throughout the world, few pest control products in the marketplace are based on plant derived EOs. This could be due to a consequence of regulatory barriers as discussed on the literature review. Our team developed two (2) biopolymeric diffusion systems, impregnating plant-derived essential oil nanoemulsions for application in bagged cereal grain preservation during storage against fungi and insects. Plant EOs contain a wide array of aromatic compounds that give plants a distinctive odor, flavor or scent. The strong odor of EOs limits its application on food products. One possible means to overcome these constraints is by the use of combined treatments that permit a synergy between the treatments and can reduce the dose needed for each treatment. Although numerous studies focused on the use of EOs to protect stored grains, there is a lack of data on the relative sensitivities of these essential oils against pests encountered during storage. In this context, the radiosensitivity of basil EOs was tested against *A. niger* and *P. chrysogenum* (publication 1) and rice weevil *S. oryzae* (publication 2) on rice grains during storage.

Relative radiosensitization of EOs

Our study from publication 1 confirmed an enhanced inhibitory effect by combining EO and ionizing radiation treatment against fungal microorganisms. Both *A. niger* and *P. chrysogenum* were found to be sensitive to irradiation and basil EO treatments. The D_{10} value for the controls (irradiation only) was 0.49 and 0.47 kGy for *A. niger* and *P. chrysogenum* respectively. The RS increased significantly for increased EOs concentration. In the presence of 2% basil EO, no colony was observed after irradiation at 2 kGy for both fungi. These results demonstrated that basil EO can increase the RS of the tested fungal species. The D_{10} values in inoculated rice grain were higher than the D_{10} values obtained in the *in vitro* in the PDB medium. Accrued inhibitory effects were obtained from day 1 using the combined treatment on all the samples tested. The complete inhibition of fungal growth was observed at 2.5 kGy radiation doses for *A. niger* and *P. chrysogenum*. However, combining the 2% basil EO treatment with 2 kGy of radiation resulted in the complete absence of fungal growth at day 7 and 14. Treating with essential oil led to a lower radiation dose for the complete inhibition of the fungal growth. It is believed that the EOs induces significant damage to the cytoplasmic microorganism membrane. Further ionizing

radiation impairs the cell structure, making the cell unable to repair the damage incurred by the dual action of both treatments. Hence, combined treatment reduces the dose of individual treatment required to inactivate the growth of microorganisms.

Again, the biological activity of basil essential oil (EO), with and without irradiation was tested against the stored product pest rice weevil, *Sitophilus oryzae* (L.) and the data is presented in publication 2. The results showed that the D_{10} value for *S. oryzae* decreased significantly ($p \leq 0.05$) over exposure time which led to an increase in relative sensitivity. After 72 h of post-treatment period the D_{10} value reduced by 28% as compared to control in the presence of 2% of basil EO. This reduction in D_{10} values caused an increase of 1.4 fold in relative sensitivity as compared to the control. In the presence of 4% basil EO, the D_{10} value was reduced by 59%, which represents an increase of 2.5 fold of relative sensitivity as compared to the control samples. After 120 h, rice weevils exposed to 20 and 40 µl/ml of basil EO were 4.8 and 6.2 times more sensitive to irradiation respectively compared to control weevils treated only by irradiation. The results obtained in this study showed that lower radiation doses at 20 and 40 Gy were not sufficient enough to induce any significant mortality in the control and treatments ($p > 0.05$). Plant EO and their components play a very effective role in insect pest control over conventional fumigants in terms of low toxicity and fast degradation. On the other hand irradiation has proven to be an effective method as it damages the internal structure of infesting pests. Hence, these results shows that EO in combination with gamma radiation can act as synergystically and offers promising results and allows the reduction of the applied concentration of essential oil and the dose of irradiation treatment. In accordance, recent studies have also shown that combining irradiation with other treatments can increase microbial radiosensitivity (Lacroix and Follett 2015; Vu *et al*., 2012).

i) Screening of EOs

Based on the literature survey, eight EOs were selected to evaluate the most efficient EOs against both molds and insects (Hossain *et al*., 2016; Korunić *et al*., 2008). The antifungal activities of eight essential oils (EOs) namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme were evaluated for their ability to inhibit the growth of *A. niger*, *A. flavus*,

A.parasiticus and *P.chrysogenum* (publication 3). The interactions between 28 different EO combinations were done using the checkerboard technique. The highest antifungal activity was exhibited by oregano and thyme which showed lower MIC values amongst all the tested fungi. The antifungal activity of the other EOs could be appropriately ranked in a descending sequence of cinnamon, peppermint, tea tree and basil. Eucalyptus and mandarin were the least effictive as they could not inhibit any fungal growth at 10,000 ppm. The interaction between these two EOs also showed no interaction on the tested species. Other mixtures namely basil and mint, cinnamon and tea tree, eucalyptus and tea tree, cinnamon and eucalyptus showed additive effects against the four tested fungi. Hence, in the present study, the combination of some particular oils showed additive and synergistic activity when combined, due to the combined activities of two or more components of EOs. A combination of oregano and thyme was also very effective against all the four tested fungal species; this combination significantly ($P \leq 0.05$) limited the colony diameter and the maximum growth rate of the fungal species and extended their lag times. The biological activity of EOs is generally related to the levels and volatility of various major and minor chemical constituents (Bachrouch *et al*., 2015). The major volatile components of oregano and thyme EOs consists of carvacrol and thymol respectively (Hossain *et al*., 2016). These components have exhibited antifungal properties in many studies (Nostro *et al*., 2012; Santonja *et al*., 2013). The inhibitory effect of these components was explained by permeability alteration in the cell membrane of microorganisms in relation with the hydrophobicity of the compound. In this study the application of a modified Gompertz model of fungal growth parameters under the various EO treatment scenarios, provided valuable insights on the potential of the tested EOs in exerting antifungal activities by altering the growth kinetics of fungi.

Based on the above findings these eight EOs and the combination of oregano and thyme EOs were tested against rice weevil *S. oryzae* (article 4). The study showed the insecticidal activity of eight individual EOs and oregano:thyme EO combinations against *S. oryzae* through fumigant toxicity assays. While some EOs displayed strong toxicities against the test specimen, like eucalyptus, others showed moderate activity. Eucalyptus EO exhibited the highest toxicity by inducing complete insect mortality (100%) at a minimum concentration of 40 µl within 24 h of exposure among all tested essential oils. The biological activity of EOs may be attributed to

individual and/or blended effects of the biochemical constituents contained in the EOs. Hence, combination treatments of oregano and thyme EOs displayed higher fumigant and acetylcholinesterase (AChE) inhibitory activity than individual treatment (oregano and thyme alone) applications. According to Koul *et al*., (2013), combinations of compounds may show a broader spectrum of insecticidal activity due to the presence of a wider range of bioactive components. An oregano and thyme combination treatment also showed significant synergistic activity against *Spodoptera littoralis* and *Culex quinquefasciatus* larvae (Pavela, 2015). Koul *et al*., (2013) reported that alcohols and phenols are more active in combinations than in their individual forms. Hence, in the present study, the binary combination of oregano:thyme EOs showed synergistic activity when combined, due to the combined activities of two or more components of EOs.

ii) Encapsulation of EO emulsion in to film matrices

Combined EOs of oregano:thyme were encapsulated into biopolymeric matrices as carriers to release the active agents over time and to avoid problems with rapid degradation. A high-pressure homogenization technique, microfluidization, was used to develop highly dispersed cellulose nanocrystal (CNC) as a reinforcing agent in methyl cellulose (MC) and chitosan based nanocomposite films containing a plant essential oil (EO) blend (oregano: thyme) nanoemulsion. Biopolymers have been approved by the Food and Drug Administration (FDA) as food ingredients and chitosan and biopolymeric films and coatings have potential as carriers for plant-derived EOs (Yuan *et al*., 2016). *In situ* tests with MC/CNC and CH/CNC based bioactive films containing EO nanoemulsion produced~2 log reduction in fungal growth in infected rice during 8 weeks of storage at 28°C. The nano encapsulation of the EO based formulation resulted in an increased antimicrobial efficiency by increasing the bioavailability of the EO in the food system. Moreover encapsulation allowed a sustained release of active components from EOs emulsion by prolonging the efficacy of bioactive films during 8 weeks of storage. Combined treatment of these bioactive films with an irradiation treatment at 750 Gy showed enhanced (3-4 log reduction) antifungal properties than treatment with the bioactive film or irradiation alone. Treating with active films led to a lower radiation dose for controlling the growth of tested fungal species for 8 weeks. It has been demonstrated that the active compounds present in natural

antimicrobials can significantly improve significantly the radiosensitivity of various food borne pathogens which is consistent with this present study (Hossain *et al*., 2014; Hussain *et al*., 2015). These results show the potential application of CNC reinforced MC or CH nanocomposite films for eventual application to protect cereal grains during storage

iii) The effect of CNCs

The addition of CNC as reinforcing filler in to MC and CH based films significantly ($p \leq 0.05$) improved the physico chemical properties and release properties of the active components. Cellulose nanocrystals (CNC), which can be extracted from lignocellulosic materials, bacteria or algae, have reinforcing properties that make them potential nanofillers for various biopolymeric films (Khan *et al*., 2012 a; Vilarinho *et al*., 2016). The addition of CNC as reinforcing filler improved the tensile strength of the methylcellulose nanocomposite films by 30% and decreased the water barrier properties by 4% as compared to the films without CNC. The release of volatile components from methyl cellulose based film was 25% slower with the addition of CNC. Similarly, CNC played an important role in physico chemical and release properties in chitosan based nanocomposite films. Loading of 5% CNC in to CH based films increased the TS by 9% and decreased the WVP by 23% as compared to the control film. The release of active component was 40% slower with CNC incorporated CH based films as compared to the control (only CH film). Well-dispersed CNC has been shown to reinforce polymers by forming a percolation network connected by hydrogen bonds. Reinforcement with CNC also improved the thermal, mechanical and barrier properties or polymers, as well as their surface wettability, and controlled release of active compounds/drugs (Boumail *et al*., 2013; Khan *et al*., 2012 a,b ; Salmieri *et al*., 2014). The diffusion pathway followed by the penetrant is greater in case of intercalated polymer/CNC nanocomposites than that in aggregated composites. Hence, nanocomposites with well-dispersed CNCs are expected to possess better barrier properties towards gases and vapors such as oxygen and water vapour. However excessive CNC (up to 10%) in naocomposites increases the WVP as CNC has high surface energy and can be easily agglomerated and can prevent preferential paths for water vapor diffusion and reduce barrier performance (Huq *et al*., 2012). Moreover CNC allows a homogenous distribution of EOs emulsion within polymer matrix to control EOs release. The integration of CNC in to polymeric

matrix enhanced the physico chemical properties of the matrix and allowed a sustained release of EOs over 12 weeks of storage period. CNC is an interesting class of nanomaterials that can be used in antimicrobial active packaging due to their bioavailability, bio-renewability, biocompatibility and biodegradability. Studies have shown that CNC plays an important role in enhancing the bioactivity of active component by improving dispersion into packaging surface. Moreover the structure–activity relationship of nanocelluloses indicate that they do not pose significant risk to human health and environment (Endes *et al*., 2016). Therefore innovations in nanotechnology related to CNC embedded active packaging has a huge potential that open excellent prospects for the increase of their use in the design of novel high-performance packaging materials in the food and pharmaceutical industry.

Insecticidal activities

Based on the above results the insecticidal effectiveness of oregano:thyme essential oils (EOs) incorporated into three different matrices, chitosan (CH), methyl cellulose (MC) and poly (lactic acid) PLA reinforced with cellulose nanocrystal (CNC) was analysed. Insecticidal activity was confirmed ranging between 4-42% with EOs (0.75%) loaded biopolymeric films during 10 days of incubation. The encapsulated EOs (oregano and thyme) diffused progressively from developed polymeric matrices showed strong insecticidal efficiencies against *S. oryzae*. The insecticidal effect of biopolymer based films incorporated with EO nanoemulsions may have been due to chemical interactions between functional groups of the polymeric matrices and the constituents of EO mixtures. Bioactive chitosan based films showed highest insecticidal activities among other tested films. The diffusion effectiveness of a bioactive film depends on the chemical nature and structure of the molecule, the formation of cross-links between molecules, and on the degree of crystallinity. The extra methyl group in the side chain of PLA makes this polymer more hydrophobic, leading to less water absorption and therefore slower degradation rate. On the other hand, MC and CH are relatively more hydrophillic in nature as compared to PLA matrix, allows faster diffusion of EO from the film matrix (Avila-Sosa *et al*., 2012). The percentage of mortality was significantly greater when compared with those of the chitosan based films. In addition, the percent mortality increased to 95% by the combined application of

300 Gy gamma radiation and bioactive chitosan films at 10 days of incubation period. The results are in consistent with our previous studies.

iv) Sensorial evaluation of rice samples

The sensory evaluation of food is an important aspect of new product development since it offers insights into consumer's acceptance and quality assurance. Hence sensorial analysis was carried out with rice samples packed with the CH based films loaded with thyme and oregano nanoemulsions after 8 weeks of storage period. The result showed no significant ($p \leq 0.05$) change in odor, taste, color and general appreciation compared with untreated rice. In the context of food industry, sensory evaluation is one of the most vital attributes that marketing management can use in order to identify the most important features of a product, eliminate wasted effort during product development, deal with the degree of excellence of food such as taste, appearance, and nutritional quality issues, compare their brands to others and try to ensure long shelf life. Hence the developed bioactive films have many potential applications for improving the safety and prolonging the shelf life of packaged food as well as keeping the quality.

v) Preparation of bi layer/ tri layer films

Bilayer/trilayer bioactive films were prepared in order to allow a gradual release of loaded essential oils (EOs). Bioactive films were prepared using two types of film matrices based on i) methylcellulose (MC) and ii) Poly (caprolactone) (PCL). Trilayer films were not efficient against tested fungal species *A. niger* and *P. chrysogenum* even at lower fungal concentrations (10^4 conidia/mL). As fungi are more resistant and grow faster as compared to bacteria, 0.75 and 1.5% EOs concentration from bilayer films were not effective to control the growth of tested fungal species. However bilayer films were found to exhibit some efficiency at concentration of 2% against 10^4 conidia/ml of fungal growth. *In situ* tests showed that samples treated with 2% bioactive films reduced growth by 0.60 log CFU/g and 1.16 CFU/g for *A. niger* and *P. chrysogenum* respectively after 4 weeks of incubation as compared to the control samples. Insecticidal activities showed 22% mortality of *S. oryzae* after 21 days of incubation. The results obtained with bilayer films were not as efficient as the CH/CNC or MC/CNC based films. Bi

layer films allow a slower diffusion of EOs as they are protected by an external layer. The results are presented in Annex A.

Conclusion

Consumer demand nowadays focuses not only on microbiological safe and stable food products but also demands high quality products with enhanced attributes. Therefore, food technologists and the food industry have combined their efforts in research to develop novel technologies to improve the safety and quality of food products. In this research our team developed two (2) biopolymeric diffusion systems, impregnating plant-derived essential oil nanoemulsions for application in bagged cereal grain preservation during storage against fungi and insects. One system was made up of a cellulose nanocrystal (CNC)-reinforced methylcellulose (MC) matrix while the other one was designed with cellulose nanocrystal (CNC)-reinforced chitosan based polymer. The novelty of this technology lies in the design and formulation of the polymeric materials itself, where we reinforced them with cellulose nanocrystals (CNC), which not only significantly enhanced their mechanical and physicochemical properties (e.g. tensile strength, tensile modulus, controlled release) but also the bioactivity of these materials. Studies have shown that grains damaged by insects are more likely to be contaminated with fungi during storage. Hence, the other innovative aspect of our technology is that it can provide a dual protection against both insects and fungi at the same time over a more prolonged period. The binary mixture of oregano and thyme EOs were most efficient among all tested EOs against 4 mold and 1 insect species. A stable and efficient oil/water nanoemulsion was developed using high pressure micro fluidization technique. The EOs nanoemulsion was encapsulated into biopolymers in order to develop a bioactive patch-like film to be inserted into packaging to eliminate fungi and insects from stored products. Chitosan based films showed higher efficiencies compared to other films. They were able to reduce 1-2 log CFU/g of *A. niger* growth on rice grain during 8 weeks of storage period. Moreover, chitosan based films caused 53% mortality against *S. oryzae* as compared to control samples (no film) after 10 weeks of storage. The controlled release of volatile components was also studied. The developed films were more

efficient by allowing a slower release of EOs during 12 weeks of storage. Only 29% of the volatile components were released from CNC reinforced chitosan based films after 12 weeks of storage. Organoleptic and sensorial evaluation of rice grains covered with chitosan film was acceptable after 1 and 2 months of storage. Moreover combining this technology with gamma radiation resulted in a more enhanced efficiency against tested fungi and insect species. Other technologies are rather either specific for insects or fungi, unlike our biopolymeric systems which target both pests thereby imparting better protection for bagged cereal grains. Therefore this technology of EO loaded nano-based active packaging systems hold great promise to control stored product pests.

FUTURE PERSPECTIVES

The present study has shown the insecticidal and antifungal properties of a range of plant-derived essential oils (EOs), and their potential applications in extending the shelf-life of food products. In addition, the design of packaging materials using EO nanoemulsions has provided promising results, and insights to overcome limitations preventing their application such as low water solubility, high volatility and strong odor. EOs contain unstable volatile compounds that can be easily degraded by oxidation, volatilization, thermal treatment and light among others. This is why, research should be continued to develop protection methods to increase their action duration and control their release into food matrix for optimum results. Today's challenges in EO application are multifold and the following future perspectives may provide additional research insights to address them.

a) The current study focused on the insecticidal activity against the rice weevil as well as the antifungal properties of the EOs. Such bioactive property can be extended against other food spoilage insects and fungi.
b) The EOs may be screened for their antibacterial, antiseptic, antioxidant and antiviral properties in view of identifying potential sources of novel antimicrobial compounds, agents to promote food preservation and alternatives to treat infectious diseases. With the rise in microbial resistance to antibiotics, interest for using plant-derived EOs has emerged as a potential alternative to reduce bacterial resistance. EOs have been shown to

contain a huge range of complex and structurally diverse compounds which may also have radical-scavenging properties and be useful in combatting pathologies such as cancer and neurodegenerative diseases.

c) It may be appropriate to explore the mechanism of individual essential oil components, along with an initiation in systematically investigating the synergy mechanisms among different components. Various mechanisms of action are possible view the plethora of compounds contained in EOs and may include cellular leakage, destruction of membrane integrity, cell wall damage and changes in cytoplasm. It may be important to investigate the mechanism of action in view of optimizing their application in food preservation.
d) Nano-encapsulation of the EOs has been investigated to enhance their insecticidal and antifungal effects. Other methods such as coating, nanogel, polymer-based nanocarriers such as nanocapsules and nanospheres, liposomes and other molecular complexes can be investigated to prevent thermo-oxidation reactions and enable a controlled release of the EOs into food matrix. Research in developing such systems to increase their dispersibility in the food areas where microorganisms grow and proliferate, to reduce the impact on the quality attributes of the food products, as well as to enhance their bioactivity, is most desirable.
e) It may be also important to address any issues related to flavor defects or deviations induced by EOs in food package systems. Also, it has become imperative to overcome flavor defects in healthy food, with low salt and sugar, monosodium glutamate (MSG) and fat; the use of natural EOs or high-potency taste modulation systems for the delivery of authentic flavor signatures may be looked into.

Future research is needed to validate the efficiency of EO-systems to prolong the shelf life of different food commodities and to study their impacts on product acceptance by consumers as well as to assess the feasibility of scaling-up such approach to large-scale scenarios. Food products are complex matrices and packaging parameters can be highly product-specific. Thus, to achieve an optimal activity or capacity of the desired active packaging, it may be necessary to implement product-tailored systems. Henceforth, it is crucial to consider all influential factors such as the physical, chemical and physiological properties of the food, packaging material and

size, and storage conditions. Scaling-up and industrialization of the active EO-impregnated packaging technologies could be challenging and, therefore, should be taken into consideration at early development stage for successful commercialization. The cost of the implementation of the technology has to correspond with the benefit gained by the particular food product. In addition, it is equally important to access legislative and regulatory issues relating to the use of innovative EO-loaded packaging systems as well as consumer acceptance. A successful collaboration and implementation between research institutes and industry, including commercial functions, is required to overcome these challenges and contribute in accelerating their potential commercial adoption.

Annex A

Preparation of trilayer/ bilayer films and evaluation of antifungal and insecticidal properties

Résumé

Le but de cette e'tude e'taite d'améliorer la libération contrôlée du composant actif en utilisant une couche externe de films. Des films bioactifs ont été préparés en utilisant deux types de matrices i) méthylcellulose (MC) et ii) poly (caprolactone) (PCL). Des films bicouches / tricouches ont été préparés afin de permettre une libération prolongée des huiles essentielles chargées (HE). Les films tricouches n'étaient pas efficaces contre les moisissures testées (*A. niger* et *P. chrysogenum)* même à une concentration plus faible (10^4 conidias / mL). Cependant, le film bicouche à 2% a montré une certaine efficacité. Les tests *in situ* ont montré que pour les échantillons traités avec un film bioactif à 2%, la croissance fongique a diminué pour atteindre respectivement 0.60 log UFC / g et 1.16 UFC / g pour *Aspergillus niger* et *Penicillium chrysogenum* après 4 semaines d'incubation par rapport au contrôle. Les activités insecticides ont montré un taux mortalité de 22% de *S. oryzae* après 21 jours d'incubation. Cependant, les résultats obtenus avec les films bicouches n'étaient pas aussi efficaces que les films monocouches CH / CNC ou MC / CNC.

Abstract

The aim of the experiment was to improve the controlled release of active components by one external layer of films. Bioactive films were prepared using two types of film matrices based on i) methylcellulose (MC) and ii) Poly (caprolactone) (PCL). Bilayer/trilayer films were prepared in order to allow a gradual release of loaded essential oils (EOs). Trilayer films were not efficient against tested fungal species *Aspergillus niger* and *Penicillium chrysogenum* even at lower concentration (10^4 conidia/ml). However bilayer films were shown to exhibit some efficiency at concentration of 2%. *In situ* tests showed that samples treated with 2% bioactive films, showed growth to be reduced by 0.60 log CFU/g and 1.16 CFU/g for *A. niger* and *P. chrysogenum* respectively after 4 weeks of incubation as compared to the control samples. Insecticidal activities showed 22% mortality of *S. oryzae* after 21 days of incubation period. However the results obtained with bilayer films were not as efficient as the CH/CNC or MC/CNC based unilayer films.

Introduction

Although considerable interest in biopolymer-based films has been renewed, they possess some drawbacks. Therefore, many studies are now attempting to overcome these drawbacks to meet the expectations of world market. Petroleum-based synthetic polymers have several advantages as packaging materials due to their excellent thermo-mechanical properties. The performance of biopolymers must be tailored in order to increase the competitiveness of biopolymers. Modifications like plasticization, the incorporation of fillers and reinforcements, blending with other biopolymers is a way to improve properties and achieve property combinations required for specific applications.

Methylcellulose (MC), cellulose ether is more economical and more readily available than other cellulose derivatives possesses good film-forming properties. MC is currently used in the pharmaceutical and food industry for its remarkable gelling but also emulsifying properties as compared to other food-grade cellulose derivatives. Studies have already reported significant improvements of the physicochemical properties of MC-based films when mixed to CNC reinforcing fillers (Takala *et al.*, 2013).

Poly (*ε*-caprolactone) (PCL), is a semi-crystalline synthetic biodegradable aliphatic polyester obtained from chemical synthesis from crude oil PCL-based films that show good water resistance, processability and is compatible with various polymer blends (Sharmin *et al.*, 2012). It has been shown that PCL exhibits desirable characteristics including biodegradability, biocompatibility, commercial availability, and affordability as a controlled release system (Khan *et al.*, 2012b). Films prepared from PCL thermoplastic polymer demonstrate good mechanical properties and are currently being investigated for use in medical devices, pharmaceutical and biodegradable packaging films. Moreover studies have shown that multilayer films composed by polycaprolactone matrix and one internal layer of nanocellulose (NCC)-reinforced methylcellulose (MC) allowed a slow release of active component during storage. It was also shown that the film released only 17% of its active content by diffusion after 14 days under normal storage conditions. In addition it also inhibited the growth of *L. monocytogenes, E. coli* and *S.* Typhimurium (Boumail *et al.*, 2013).

In this study, bioactive films were prepared using two types of film matrices based on i) methylcellulose (MC) and ii) Poly (caprolactone) (PCL). Bilayer/trilayer films were prepared in order to allow a gradual release of loaded essential oils (EOs). The aim of the experiment was to study their effects in extending the shelf- life of food products and to design a novel MC films by improving their physicochemical properties.

Materials and methods

Preparation of PCL/MC/PCL trilayer and PCL/ MC bilayer films

Biofilms comprising of a MC and a PCL layer were prepared by compression molding according to a procedure developed by Boumail *et al*. (2013). A quantity of 500 mg of PCL granules were placed on a release film. Another release film was placed on the PCL and the upper plate was fixed on it. First, melting of PCL (without compression) was performed for 2 min, and immediately followed by the application of 2 tons of pressure for 1 min at 120 °C. The mold was then immersed in an ice bath for 2 min for cooling after which the PCL was taken out of the mold. Methyl cellulose films were prepared as an internal layer following the same protocol. The concentration of EOs in the MC film matrices were 0.5, 0.75, 1.0 and 2.0%.

For the synthesis of the trilayer composite films, one layer of MC film (with and without antimicrobial formulations) was sandwiched between two (2) layers of PCL films of the same dimension. The temporary trilayer construction was placed between stainless steel plates in a compression molding machine. Adhesion of the three layers was achieved at 2 metric tons pressure for 3 min at 80 °C. The trilayer formulation was cooled in an ice bath for 30 s. Bilayer PCL/ MC films were prepared similarly to the compression molding process of PCL films described above. PCL/MC/PCL trilayer and PCL/MC bilayer films were used in *vitro* and *in situ* bioassays. The fabrication of trilayer/ bilayer films are shown in figure 1.

Determination of antifungal activities *(in vitro)*

In vitro testes were carried out to evaluate the antifungal activities of tri layer / bilayer bioactive films against *A. niger* as described in chapter 6 and 7.

Determination of release properties

The release properties of EOs from bilayer films were determined as described in chapter 6 and chapter 7.

Determination of *in situ* antifungal and insecticidal activities

The *in situ* antifungal activities of bilayer bioactive films were determined against *A. niger* as described in chapter 6 and 7. The insecticidal activities of the the bioactive films were evaluated in rice samples against *S. oryzae*. A quantity of 30 g of rice was placed in a plastic bag inoculated with 50 adult *S. oryzae*. Two pieces of PCL/ MC based bi-layer nanocomposite bioactive films were cut in to 4 pieces and placed in the rice samples. The mortality of the insects was assessed at day 3, 7, 14 and 21.

Results and Discussion

***In vitro* tests with trilayer films**

The synthesized trilayer films loaded with EOs were used in *in vitro* tests against *A.niger* and *P. chrysogenum* at fungal concentrations of 10^4-10^6 conidia/ml (table 1). No area of inhibition was observed even at highest concentration of EOs tested (i.e. 2%). Diffusion assays showed that the trilayer films were not effective against *A. niger* at all three EO concentrations (0.75, 1.5 and 2%). On the other hand, bilayer films were not effective at higher concentrations of 10^5 and 10^6 conidia/ ml of *A. niger* but showed inhibition at the concentration of 10^4 conidia/ mL of *A. niger* at 2% EO concentration.

According to Boumail *et al.* (2013) and Takala *et al.* (2013) trilayer films were found to be effective against bacteria. In general, trilayer films allow a slow release of EOs as they slowly diffuse from the internal impregnated layer. In our case, *A. niger* proved to be more resistant than bacteria to the applied EOs. A recent study conducted by Ben-Fadhel *et al.*, (2017) showed that the minimum inhibitory concentrations (MICs) of different EOs were higher against *A. niger* compared to three tested bacteria. Therefore, in our study tri layer films were not able to inhibit fungal growth at high fungal concentrations even using higher EOs concentrations (0.75-2%). Similarly, bilayer films were not effective at higher concentrations of fungal populations (10^6, 10^5 conidia/mL) but effective when the fungal concentration was lower (10^4 conidia/mL). Based

on these results, a fungal concentration of 10^4 conidia/ ml was chosen as the working culture for *in vitro* and *in situ* tests with bilayer films. Result from diffusion assays are shown in Figure .2. Bilayer films containing 0.75-2% of EOs showed 13-24% and 16-41% inhibition against *A. niger* (10^4 conidia/mL) and *P. chrysogenum* (10^4 conidia/mL), respectively. Bilayer films containing 2% EOs were found to be the most efficient against *A. niger* and *P. chrysogenum*.

Release of EOs from bilayer film

The release of EOs at three concentrations (0.75, 1.5 and 2%) were obtained using a UV –VIS spectrophotometer at 274 nm. First 10 mg of film pieces were cut and dissolved in 10 ml of ethanol for 24 hours. The extracted eluents were subjected to analysis of absorbance to obtain the release of volatile components during 0, 2, 4 and 24 hour of storage. The result (Fig 1) showed that the maximum release of EOs was observed within 4 hr. There was not much difference in the amount of released EOs in films containing 0.75 and 1.5% EOs. A quantity of 0.042 and 0.045 mg/ml of volatile components were released with the films containing 0.7 and 1.5% of EOs respectively after 24 hr of incubation. On the other hand the release of 0.068 mg/ml of volatile components were observed with films containing 2% EOs initially (after 5 mins of incubation). This value is 3 and 1.5 times higher as compared to 0 hr and 24 hr of incubation period with films containing 0.75 and 1.5% of EOs. After 24 hours the release of EOs was 0.18 mg/ml, which is 4 times higher as compared to release of EOs (0.045 mg/mL) obtained with 1.5% EOs. After 24 hours, only 2% EO release was obtained with films containing 0.75 and 1.5% of EOs. The release of EOs was 14% only with films containing 2% of EOs.

As fungi are more resistant and grow faster as compared concentration of 0.75 and 1.5% EOs from bilayer films were not effective in controlling the growth of tested fungal species. However bilayer films with a concentration of 2% EOs were effective against 10^4 conidia/ml of fungal growth as compared to films containing 0.75 and 1.5% EOs. Hence bilayer films containing 2% EOs were considered for testing *in situ* antifungal and insecticidal efficiencies.

***In situ* tests with PCL/MC bilayer biofilm on rice grains against fungal species**

In situ tests were done with bio active films containing 2% of EOs. The result of *in situ* tests with bi-layer bioactive film is shown in figure 3. The results showed that PCL/MC based bilayer films

were not efficient as monolayer films to reduce the growth of the mold species during storage. The initial inoculations for all mold species were 3 log conidia/g. For the control assays which involved incubation without EO loaded biofilms, the growth of *A. niger* increased from 3.13 log to 5.40 log at 4 weeks. For samples treated with bioactive films, the growth was found to be reduced by 0.60 log CFU/g after 4 weeks of incubation as compared to the control samples. *P. chrysogenum* was more sensitive to the encapsulated biofilms than *A. niger*. The fungal growth increased from 3.08 log to 5.86 log at 4 weeks for the control. Samples treated with PCL/MC films showed a reduction in the growth of *P. chrysogenum* by 1.16 CFU/g after 4 weeks of incubation as compared to the control samples.

The findings showed that bilayer films reduced fungal growth on rice grains, but not to the same extent as monolayer films. The gradual release of active components from the bilayer films might affect the efficacy of the film to control fungal growth. Takala *et al*. (2013) studied the antimicrobial effects of PCL/MC/PCL trilayer bioactive films containing EOs and organic acids. The films showed a significant reduction of two bacterial species, *E. coli* and *salmonella* Typhimurium in broccoli, but could not inhibit the growth of *Listeria monocytogenes*. The authors hypothesized that the action of the bioactive agent was not due to a single component but rather comprised of a synergetic action of different bioactive agents, especially through indirect action of volatile substances as compared to their diffusion by contact with the food system. Moreover, in the bilayer film the direct diffusion of active volatile components in food matrix would be too slow, thereby enhancing the growth of the fungal species.

Evaluation of the insecticidal activities of PCL/MC bilayer nanocomposite films

Evaluation of bilayer films showed insecticidal activities (figure 4) against *S. oryzae* during storage as compared to control samples. Samples treated with bilayer films caused 4, 8, 14 and 22% mortality at day 3, 7, 14 and 21 days of incubation, respectively. Bi layer films allow a slower diffusion of EOs as they are protected by an external layer. As a result, higher mortality was observed with longer incubation period. Hence, novel bioactive films were developed in the current study and evaluated for their potential fungal and insecticidal properties.

Conclusion

Bilayer films containing 2% EOs inhibited low dense (10^4 conidia/mL) fungal growth to some extent against *A. niger* and *P. chrysogenum* (0.60 log CFU/g and 1.10 log CFU/g respectively) and caused slow mortality (only 22%) over 21 days of incubation period. Therefore, they were not as efficient as unilayer films (chapter 6 and 7) for commercial application.

Table 1. Release of volatile component from bilayer films containing different concentration of EOs during 24 hr of storage period.

EO Concentration in bi layer film (%)	**Released EOs (mg/ml)**			
	0 (hr)	**2 (hr)**	**4(hr)**	**24(hr)**
0.75	0.021	0.039	0.042	0.042
1.50	0.021	0.041	0.042	0.045
2	0.068	0.163	0.174	0.18

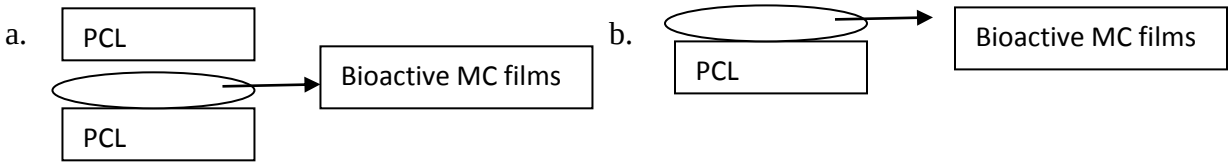


Figure 1. Schematic presentation of a. Tri layer and b. bilayer films

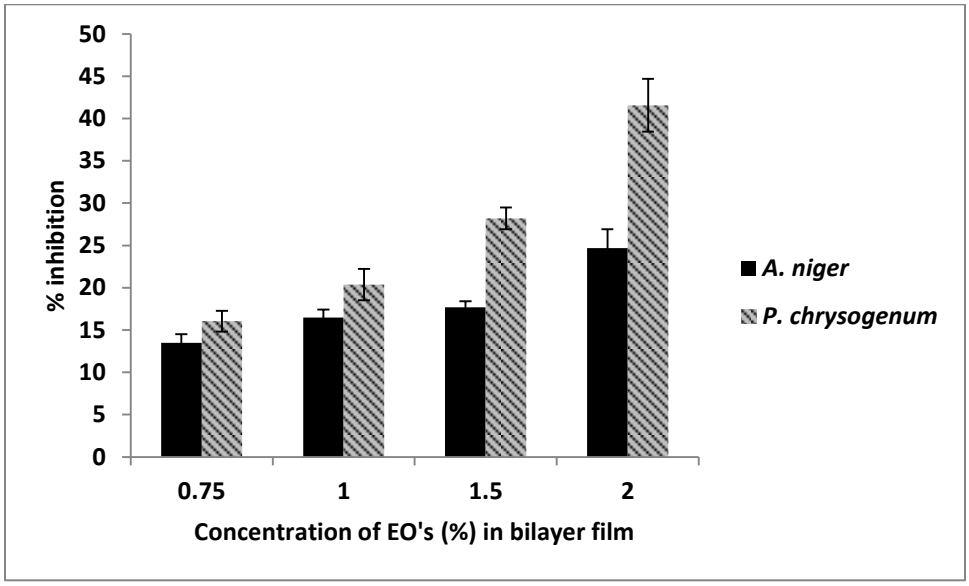
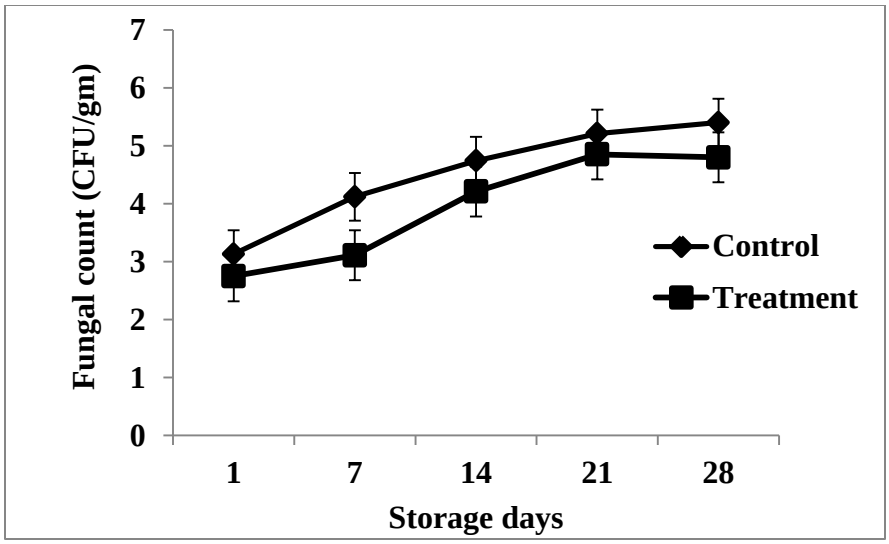


Figure 2. *In vitro* tests of bi-layer films against 104 conidia/mL of *A. niger* and *P. chrysogenum.*



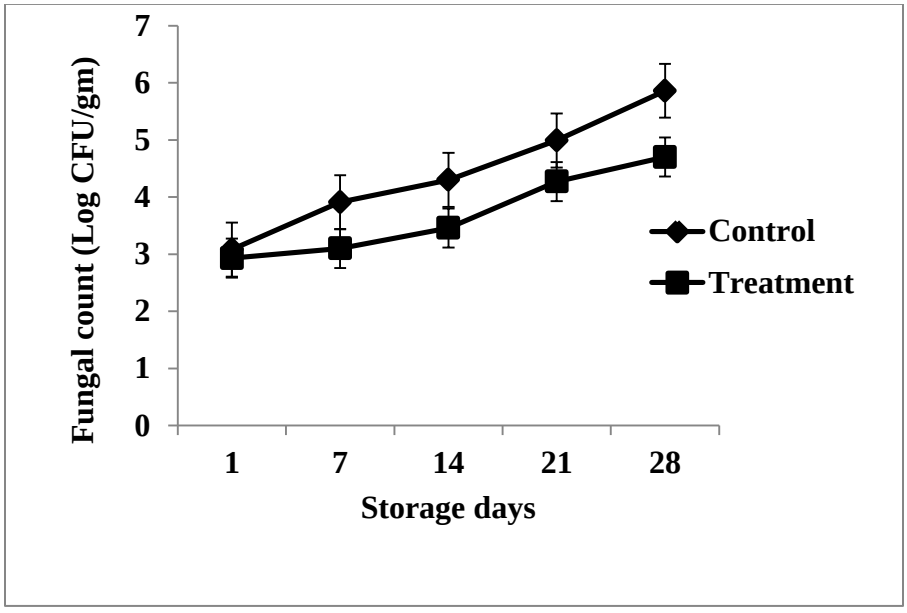


Figure 3. Fungal growth profiles of (a) *A. niger* and (b) *P. chrysogenum* following in situ tests with bioactive PCL/ MC bilayer nanocomposite bioactive films over 4 weeks of storage period on rice grain.

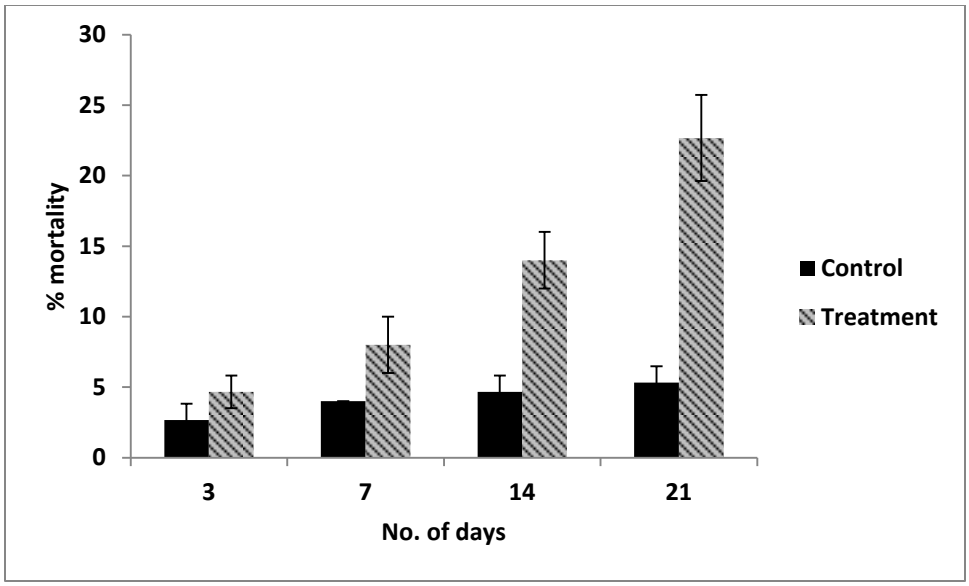


Figure 4. *In situ* insecticidal tests of bioactive PCL/MC bilayer on rice grains during 3 weeks of storage period.

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Annex B

Other Contribution

1. Salmieri Salmieri, Islam Farhana, Khan Ruhul, Hossain Farah, Ibrahim HM, Miao C, Hamad W & Lacroix Monique, Antimicrobial nanocomposite films made of poly(lactic acid)-cellulose nanocrystals (PLA-CNC) in food applications: part A—effect of nisin release on the inactivation of *Listeria monocytogenes* in ham 2014, *Cellulose* 21(3):1837-18502.

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