FABRICATION AND CHARACTERIZATION OF ANTIFUNGAL ESSENTIAL OIL-IN-WATER NANOEMULSION DELIVERY SYSTEM TARGETING *FUSARIUM GRAMINEARUM* IN VITRO AND DURING THE MALTING PROCESS

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ABSTRACT

Fusarium mycotoxins represent the most food safety concern for the malting industry. The complete prevention of *Fusarium* mycotoxins in the grains by limiting toxigenic fungi in the field and during storage is not practical. The common way to control *Fusarium* mycotoxins in malting industry is to avoid infected grains. However, avoidance is not always possible. To ensure the quality and safety of food products, development of food-grade antifungal strategies that can be applied in food processing, would benefit growers and the food industry. Recently, plant-based essential oils (EOs) have received considerable attentions in the food industry due to broad-spectrum of antifungal activities and inhibitory effect against mycotoxin biosynthesis. However, direct application of EOs during the malting process is impractical.

In this project, parameters that impact on the formation of EO-in-water nanoemulsions and functional properties including antifungal and mycotoxin inhibitory efficacy were evaluated *in vitro*. The proper-designed EO-in-water nanoemulsions were then applied in micro-malting process. Results indicated that physically stable EO-in-water nanoemulsions can be fabricated by incorporating either ≥75 wt% of corn oil or ≥50 wt% of medium chain triacylglycerol (MCT) into EO before homogenization and homogenized under optimized processing conditions (68.95 MPa and 2 passes). In general, the mycotoxin inhibitory efficacy of EO was enhanced considerably in nanoemulsion form than bulk oil. Among all selected five EOs, thyme and clove oil-in-water nanoemulsions had the greatest antifungal and mycotoxin inhibitory activities. In terms of emulsifiers, the antifungal activity was mainly dominated by EO rather than emulsifier to alter mycelial and spore cell membrane integrity. At last, clove oil-in-water nanoemulsions stabilized by three different emulsifiers (Tween 80, bovine serum albumin, quillaja saponins) were selected to apply in micro-malting process according to our germinative energy test of

barley seeds. All clove oil-in-water nanoemulsions had the capability to inhibit fungal growth and DON production during the micro-malting process. Among the three emulsifiers, Tween 80-stablized clove oil nanoemulsion displayed largest reduction of mycotoxin and least flavor impact on the final malt. The overall project showed a great potential for utilization of EO-in-water nanoemulsion as antifungal agent and mycotoxin inhibitor in the food industry.

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DEDICATION

I dedicate this work to my dearest husband Dr. Xuntao Yin:

Your inspiration, deep love, wisdom make me to be here. Your passion on your own career and your positive attitude towards life make you a role-model for me. Thank you for all your love, support, and sacrifice.

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INTRODUCTION

Barley (*Hordeum vulgare L.*) is the principal source grain used in the malting industry for various applications, such as brewing. Unfortunately, barley is susceptible to Fusarium head blight (FHB), a severe disease mainly caused by *Fusarium graminearum* in the field, which can further produce various mycotoxins in barley (Morcia et al., 2013). In order to minimize the yield and quality losses of FHB-contaminated barley, farmers have applied breeding, crop rotation, and fungicide application to prevent the occurrence of the FHB on barley in the field, which however cannot guarantee safe grains especially under unexpected climate. In order to prevent the damage on malting performance and health threaten by mycotoxins, maltsters have developed various chemical methods, which nevertheless were either so harsh as to impair food quality or to be repelled by the food industry for the chemical residues.

Moreover, there is a growing consumer trend towards more natural food products so that the natural preservatives are preferable over chemically synthetic materials by both food consumers and manufactures. Essential oils (EOs), naturally occurring plant metabolites, have been considered as one of the most promising natural preservatives due to their strong antifungal and antimicrobial activities. Previous studies have shown that EOs can effectively inhibit *Aspergillus flavus*, *Fusarium verticillioides*, and *Fusarium graminearum* (Da Silva Bomfim et al., 2015; Kordali et al., 2005; Singh et al., 2012). The chemical compositions of EOs are variable and complex but can be roughly divided into terpene, terpenoid, and phenolic groups, which have an antifungal role by destroying fungal mycelium growth and spores germination. Although EOs have shown great potential to replace chemical preservatives against fungal and mycotoxin contamination in cereals, high volatility and low water solubility of EOs are the critical challenges limiting the direct usage of EOs in aqueous food systems.

In order to overcome the application limitations of EOs, nanoemulsions displayed promising advantages by encapsulating lipophilic EOs to the oil-in-water nanoemulsion delivery systems. EOs gain higher physical stability and enhanced antifungal activity due to the nanometric (≤ 200 nm) EOs particles dispersed in the nanoemulsion form. The fabrication and stabilization of essential oil-in-water nanoemulsions depend on the emulsifier type, oil composition, and homogenization conditions. Nanoemulsion delivery system can be formulated by natural emulsifiers at the low ratio to the oil phase and under various homogenization methods, such as high-pressure homogenization, giving nanoemulsion priority when choosing the delivery system for EOs to be applied in the food matrix.

Though essential oil-in-water nanoemulsions have been studied as replacements of chemical preservatives in various food systems, study about their antifungal effects on *Fusarium* and inhibition of their mycotoxin biosynthesis was rare. The goal of this project was to develop antifungal essential oil-in-water nanoemulsions to effectively mitigate the fungal and mycotoxin contamination on malting barley during the malting process. In order to meet this goal, work was done *in vitro* and during the micro-malting process. In order to fabricate the long-term stability of essential oil-in-water nanoemulsions with boosted antifungal and mycotoxin inhibitory activity, the impact of essential oil type, emulsifier type, and homogenization conditions were evaluated *in vitro*. Then, essential oil-in-water nanoemulsions screened in the laboratory were applied in the micro-malting process to explore their potential application in food processing.

LITERATURE REVIEW

Fusarium head blight (FHB) in barley

Fungi species inducing FHB and *Fusarium* mycotoxins production in barley

Barley (*Hordeum vulgare L.*) is the fourth most widely grown cereal crop in the world and is mainly used for feed and malting purpose. Unfortunately, this important cereal crop is susceptible to Fusarium head blight (FHB, also called Scab) in the field, an economically devastating plant disease caused by the genera of Fusarium fungi (Wegulo, Baenziger, Hernandez Nopsa, Bockus, & Hallen-Adams, 2015).

Fusarium graminearum is the major pathogen infecting barley with FHB and followed by other *Fusarium* species, such as *F. avenaceum*, *F. culmorum*, *F. poae*, *F. cerealis*, *F. equiseti*, *F. sporotrichioides* and *F. tricinctum*. The infection generally occurs on the plant during the flowering and early grain-filling stage experiencing relatively humid weather, which can cause yield loss due to floret sterility and shriveled grains. Furthermore, with the pathogenesis and colonization of FHB on barley, *Fusarium* pathogens also produce various trichothecene mycotoxins. These trichothecenes can accumulate at high levels on the harvested barley grains so that the usage of barley in feed and food can be heavily devalued.

Among 170 types of trichothecenes identified and classified (Marin, Ramos, Cano-Sancho, & Sanchis, 2013), DON is the most prevalent and detected one with high levels in cereals and cereal-based foods (Murphy, Hendrich, Landgren, & Bryant, 2006; Wu, Groopman, & Pestka, 2014). DON is mainly produced by *F. graminearum* and *F. culmorum* in the field. The optimal conditions for the growth of *F. graminearum* require the temperature of 25 ℃ and water activity of 0.88, while the conditions of temperature of 21 ℃ and water activity of 0.87 are for *F. culmorum*. As for DON production, besides the abovementioned environmental conditions, the

timing of FHB incidence and rainfall, cultivar susceptibility, and agricultural practices collectively determine the DON contents in barley grains (Gautam & Dill-Macky, 2012).

Toxicity and regulation of deoxynivalenol (DON)

DON is a chemically stable compound with a high melting point of 151-153℃, so that DON can be survive in many food processing operations and be detected in various food products, such as malts, beer and flour. Therefore, the usage of DON contaminated cereals increases the health risks to human beings and animals. The accumulation of DON in humans and animals after ingestion of contaminated food can result in both acute and chronic effects (Pestka, 2007). For acute DON exposure, DON promotes leukocyte apoptosis through associated immune suppression. Prolonged dietary exposure to DON, induces symptoms of chronic DON toxicity are weight loss, anorexia, and reduced nutritional efficiency (Pestka, 2007). There is a guideline (2010) from the U.S. Food and Drug Administration (FDA) that the advisory level of DON is 1.0 ppm (mg/kg) in the finished wheat products intended for human consumption. In malting barley, the acceptable DON level is below 0.5 ppm (Bai & Shaner, 2004). The European Union (EU) Regulation (2013) also set maximum level of DON for unprocessed barley is 200 $\mu g/kg^{-1}$ (Beccari, Caproni, Tini, Uhlig, & Covarelli, 2016). DON was classified in Group 3 by the International Agency for Research on Cancer (IARC).

Safety concern during malting of FHB-infected barley

Fate of deoxynivalenol (DON) during the malting process

Malting is the most value-adding food processing operations for barley. About 90% of malted barley were used for beer making and the remainder were added in various foods, like cakes, bread and biscuits. The malting process basically includes steeping, germination, and kilning. During the steeping step, cereal grains were soaked in water at 15-17℃ to reach

moisture level of 43-45%. This step can reduce the levels of water-soluble mycotoxins due to the rinsing by steeping water. Studies have found that the DON levels were largely reduced after the steeping step due to its water solubility (Lancova et al., 2008; Maul et al., 2012; Vaclavikova et al., 2013). However, during the germination step (humidity >95%, 15-17℃, 4-6 d), the pericarp of barley kernel becomes soft so that fungi can easily access to nutrients. In addition, because the germination temperature and high relative humidity are suitable for fungal growth and mycotoxin synthesis, DON levels tend to increase over the germination stage (Medina et al., 2006). Kilning is the final stage to decrease the moisture of malts less than 5% moisture by drying. Kilning extends malt storage and develops flavor, color of malts while maintaining enzymatic activity of malts by increasing temperature in a stepwise manner from 40 to 100 ℃ or even higher depending on the malt types. Although most fungal growth can be ceased, DON and other mycotoxins cannot be fully inactivated depending on the kilning temperatures. For example, the DON level of barley was reduced to 30-49% after steeping, increased to 250-300% after germination, and dropped to 170-190% compared with initial DON content in the starting materials (Habler et al., 2016). Studies on the FHB contaminated barley during malting process, it observed that the growth of *Fusarium* fungi is closely related to the DON levels. DON level increased eight fold after the whole malting stage due to the accumulated fungal biomass (Oliveira, Mauch, Jacob, Waters, & Arendt, 2012). In addition, DON level increased during kilning stage probably due to the hydrological activity of enzyme (Vegi, Schwarz, & Wolf-Hall, 2011). From above, the malting process impact the transfer of DON in both the positive and negative way, ascribed to the physicochemical properties of the mycotoxin and the applied operation circumstance.

Impact of deoxynivalenol (DON) on the malting quality of barley

Besides food safety concern, barley with DON contamination can also damage the quality performance of barley during the malting process due to the change of chemical composition and enzyme activity in the contaminated barley grains. For example, “gushing”, the rapid air releasing out of the bottle, was significantly related with the DON infection (Deckers et al., 2011; Schwarz, Beattie, & Casper, 1996). Malt qualities, such as enzymatic activity, nitrogen availability and foam stability, were negatively impacted by DON and the other mycotoxin infection (Nielsen, Cook, Edwards, & Ray, 2014; Oliveira, Mauch, Jacob, Waters, & Arendt, 2012), probably by the expression modification of key malting-quality genes (Hofer et al., 2016). Barley grains infected with DON displayed weak germination energy and low levels of proteolytic enzymes and gibberellins, which can result in poor malt quality (Schwarz et al., 1996). Mycotoxin contaminated malts tend to have a high friability so that the contents of soluble nitrogen and free amino nitrogen were increased, which can cause the darker color in beer. Besides, the lower wort pH, beta-glucan content and wort viscosity were also accounted by mycotoxin infection in cereal grains (Oliveira et al., 2012).

Fusarium mycotoxin management during the malting process

Chemical methods

Sodium bisulfite, alkaline solutions of sodium hypochlorite, and sodium carbonate have been used to decontaminate DON in cereal grains and showed high efficiency (Wolf-Hall, 2007). However, these harsh treatments are either too severe damage on the grain quality or leave undesired residues on the grain. Gaseous ozone and hydrogen peroxide treatments inhibited the *Fusarium* growth in *Fusarium*-contaminated barley but also decreased the germination rate of barley grains (Kottapalli, Wolf-Hall, & Schwarz, 2005). Modified gaseous ozone treatment (26

mg/cm^3 for 120 min) applied after 2 and 6 h of steeping significantly inhibited the *Fusarium* growth in the germinated barley seeds and final malts without the negative influence on malt quality (Dodd et al., 2011). However, the effect of ozone treatment on the DON level was inconclusive due to the low concentration of DON in the original barley seeds (1.4 mg/kg).

Physical methods

Electron-beam irradiation and hot water have been evaluated for their effects on *Fusarium* infection, DON contents, and malting quality properties, like germination rate of barley seeds during the malting process. Hot-water treatment at 45/50℃ for 1, 5, 12, or 20 min showed potential to control mildly FHB infected malting barley. However, the longer treatment duration at higher hot-water temperature also affect the malting quality of barley (Kottapalli & Wolf-Hall, 2008). Although electron-beam was an effective measure to reduce the Fusarium infection and DON levels on malts, the effective dose should be controlled at low level (6-8 kGy) to sustain the acceptable malt qualities (Kottapalli, Wolf-Hall, & Schwarz, 2006).

Biological methods

Biological treatment also called bio-preservation, the control of one organism by another, has received much attention in the last decade (Zhang, Schisler, Boehm, & Slininger, 2007). Among all the natural bio-preservatives, lactic acid bacteria (LAB) presents the greatest potential for both fungal growth inhibition and detoxification of mycotoxins due to its naturally occurrence in foods or pure cultures added to various food products (Franco, Garcia, Hirooka, Ono, & dos Santos, 2011). Currently, research has mainly been directed towards identifying different antimicrobial or antifungal substances (e.g., organic acid) in simple *in vitro* systems, but there is little known about the overall mechanisms of complex preservation systems within food due to complex food system and commonly synergistic interactions between different

compounds (Schnürer & Magnusson, 2005; Peyer et al., 2016). There are two possible mechanisms that might explain the antimicrobial or antifungal efficiency of LAB. (i) The competition for nutrients in the medium between LAB and microorganism; and (ii) Production of organic acid, antagonistic compounds and other metabolized compounds. The detoxification capability of LAB could be related to the adsorption of mycotoxins by the bacterial cell structure or by degradation through its metabolism (Franco et al., 2011). LAB also retarded *Fusarium* contamination during the malting process by adding the LAB starter cultures to barley during the steeping step (Laitila, Alakomi, Raaska, Mattila-Sandholm, & Haikara, 2002). From the current studies, LAB has the potential as an effective measure for combating the problem of *Fusarium* infected malting barley. However, the safety issue related with the metabolites of LAB itself is still unclear, which poses more challenges for the commercial application of LAB in the food industry.

Essential oils (EOs) as natural antifungal agents

Although multiple chemical, physical, and biological strategies have been exploited to mitigate or eliminate the fungal and mycotoxin contamination on cereals, few of them meet the requirements of commercial usage in food industry due to the high cost, safety concern, and limited efficacy. In addition, consumers and food industries demonstrate an increased demand for natural agents as food preservatives. Essential oils (EOs) are a class of plant secondary metabolites containing a complex mixture of nonvolatile and volatile compounds (Gracia-Valenzuela, Orozco-Medina, & Molina-Maldonado, 2012), which are originally intended for the protection of the plants from the external damages, such as light, insects and pathogen (Seow, Yeo, Chung, & Yuk, 2014). Due to their wide-spectrum biological functions, such as antioxidant, antiradical, and antimicrobial properties (Gracia-Valenzuela et al., 2012), they have

been widely used as functional ingredients in foods, cosmetic, and pharmaceutical applications (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). As plant-based natural aroma compounds with multiple biological functions have been studied for their use on natural food preservatives. For example, thyme oil and clove oil have been shown to exhibit antimicrobial and antifungal activity against several bacteria and fungi species (Friedman, Henika, Levin, & Mandrell, 2004). Moreover, EOs have been found to be capable of inhibiting the formation of spores (Sharma & Tripathi, 2006). Considering the fact that EOs are natural original components instead of synthetic additives, food industries have high desire to develop natural food preservative system using EOs.

EOs are a group of plant secondary metabolites, which contain a complex mixture of more than 20 different nonvolatile and volatile compounds with low molecular weight at variable content. EOs are produced and secreted by glandular trichomes and secretory tissues mainly on the surface organ of plants, such as flowers and leaves, which can protect plant from external damage, such as light, insects and pathogen (Seow, Yeo, Chung, & Yuk, 2014b). Generally, EOs were mainly composed by monoterpenes and sesquiterpenes, followed by diterpenes and phenylpropanoids at certain concentrations. Among these compounds, only few of them constitute the main content account for 70% and therefore represent the biological activity of the EOs.

Among more than 3000 explored EOs, only a few were studied against cereal related mycotoxigenic fungi. These studies generally explored the antifungal activity of EOs in aspects of mycelium growth, sporulation, and mycotoxin production by mycotoxigenic fungi, but a few included all these aspects in one study. Some studies only tested the antifungal activity of EOs against fungal mycelium growth. For example, oregano oil, mint oil, and rosemary oil were

effective against mycelium growth of *Aspergillus flavus*, *Penicillium oxalicum*, and *P. minioluteum* isolated from corn seeds (Camiletti, Asensio, Pecci, & Lucini, 2014). Five EOs extracted from Cameroon local aromatic plants displayed strong antifungal activity against mycelium growth and mycotoxin production by three common cereal pathogens: *F. moniliforme*, *Aspergillus flavus*, and *A. fumigatus* (Nguefack, Leth, Amvam Zollo, & Mathur, 2004). Some studies further evaluated the inhibitory activity of EOs against mycotoxin production in certain cultural media. Ten types of EOs and cinnamaldehyde oil, citral oil, and eugenol oil were screened to effectively inhibit the fungal growth and Ochratoxin (OTA) production by *A. ochraceus* (Hua et al., 2014). Since the above studies explored the antifungal and mycotoxin inhibition activity of EOs *in vitro*, it is necessary to further prove the conclusions in planta study because mycotoxigenic fungi perform differently in culture media and cereal grains. Cinnamon oil and oregano oil were applied on *F. proliferatum* infected corn grains under preharvest condition and inhibited the fungal growth and FB1 production (Velluti, Sanchis, Ramos, Egido, & Marín, 2003).

Moreover, there are some interesting studies. Bergamot oil, lemon EOs and five main EOs components can promote the fungal growth and production of T-2 toxin, HT-2 toxin, and DON by three FHB causing pathogen: *F. sporotrichioides*, *F. graminearum*, *F. langsethiae* at sub-lethal concentrations (Morcia et al., 2017). EOs can directly degrade Fumonisin B1 (FB1) by incubating with FB1 compound, but the mechanism is unclear (Xing, Hua, Selvaraj, Yuan, et al., 2014). Lemongrass oil highly inhibited the sporulation, mycelium growth and aflatoxins production by *Aspergillus flavus* in rice, but the surfactant (Tween 20) was also employed to evenly disperse lemongrass oil in cultural media, which can alter the physicochemical property of EOs, such as hydrophobicity. Thus, it is not precise to ascribe the strong antifungal and

mycotoxin inhibition effect to lemongrass oil as pure oil form (Paranagama, Abeysekera, Abeywickrama, & Nugaliyadde, 2003).

For decades, researches have revealed that the EOs components with phenolic structures or aldehydes were highly active against bacteria, microorganism and fungi, such as thymol, carvacrol, eugenol and cinnamaldehyde. For example, three common components of EOs: thymol, eugenol, and carvacrol were highly effective in inhibiting the mycelium growth of *A. parasiticus* (Pillai & Ramaswamy, 2012). The mechanism of antifungal activity in EOs depends on the molecular hydrophobicity of EOs, which strongly interact with the lipids of cell membrane. This interaction disrupts the cell membrane permeability, breaks the cell structures, disturbs the intracellular homeostasis, and finally results in the leakage of essential ions and cytoplasm (Seow et al., 2014b; Sharifi-Rad et al., 2017). Besides, the active compounds of EOs have the capability to destroy the existing mycelia and spores of fungi (Tripathi, Sharma, & Sharma, 2009). Moreover, EOs can inhibit the mycotoxin production by downregulating the genes which have an important role in the mycotoxin biosynthetic pathway. For example, cinnamaldehyde inhibited the OTA biosynthesis by downregulating OTA biosynthesis related genes in *A. ochraceus* (Wang et al., 2018).

Nanoemulsion delivery system to improve the antifungal efficacy of essential oils

The use of EOs as natural preservatives in food industry has raised awareness in the past decade, mainly motivated by the negative perception of chemical preservation techniques by the consumers. However, EOs will confront a lot of challenges when being directly applied in foods. EOs are sparse solubility in water, have low stability against physicochemical degradation and highly volatile, and can interact with the food matrix, which jointly weaken the antimicrobial activity of EOs and limit their application. In addition, EOs may also negatively influence food

organoleptic quality by the strong flavor. Thus, it is necessary to explore delivery systems to mitigate these limitations of EOs.

Nanoemulsion formation and their properties

Oil-in-water nanoemulsion is a colloid system with relatively small oil droplets (mean particle size 10-200 nm) dispersed in aqueous medium. Like conventional emulsion, this system is also thermodynamically unstable and tends to break down over time due to various physicochemical mechanisms (Sagalowicz & Leser, 2010). However, the nanometric size gives nanoemulsion advantages over conventional emulsion: (i) In general, nanoemulsion has better stability against droplet aggregation and gravitational separation except for Oswald ripening (Rao & McClements, 2010). Ostwald ripening is processing whereby the growth of larger droplets at the expense of the smaller droplets through continuous phase. The driving force for Ostwald ripening is the located oil in smaller droplets has the higher solubility than that of larger droplets because of the difference in Laplace pressures (Wooster, Golding, & Sanguansri, 2008); and (ii) Nanoemulsion has enhanced biological activity due to the increased contacting area between EOs and surface area (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015).

In general, there are two types of fabrication methods: low energy methods and high-energy methods. Low energy methods rely on spontaneous formation using different formulation or temperature to decrease the interfacial tension. In contrast, high-energy methods use mechanical devices (high pressure homogenization) such as microfluidizer, two stage homogenizer and sonication method to break the larger droplets into small droplets (Donsì & Ferrari, 2016). At present, high-energy approaches are the most common methods used to prepare nanoemulsion in industrial food operations because they are capable of large-scale

production, and they can be used to prepare nanoemulsion from a variety of different starting materials (McClements & Rao, 2011).

Antifungal activity of EOs-in-water nanoemulsion

Plant essential oils have been widely reported for their antimicrobial and antifungal properties in the form of nanoemulsion delivery system (Donsì, Annunziata, Sessa, & Ferrari, 2011). Eugenol oil-in-water nanoemulsion was found to achieve an efficient antifungal activity against *F. oxysporum* on cottonseed (Abd-Elsalam & Khokhlov, 2015). Thyme oil-in-water nanoemulsions also have an important role against yeast (*Zygosaccharomyces bailii*, ZB) (Chang, McLandsborough, & McClements, 2012) and other four acid-resistant spoilage yeasts (Ziani, Chang, McLandsborough, & McClements, 2011). Peppermint oil-in-water nanoemulsion worked well against *Listeria monocytogenes Scott A* and *Staphylococcus aureus* two foodborne pathogenic microorganisms (Liang et al., 2012). However, limited study has explored the antifungal activity of encapsulated EOs in cereal related pathogens as mentioned above, although encapsulation of EOs into emulsion, nanoemulsion, and microemulsion-based delivery system has been proven to enhance the antimicrobial activity of EOs against common food spoilage microorganisms. Thyme oil was encapsulated into a self-assembly microemulsion system which was able to sustain the antifungal activity of thymol oil against *Fusarium graminearum* (Gill, Li, Saenger, & Scofield, 2016). Cinnamon, clove and thyme oil encapsulated in chitosan stabilized microparticles maintained the similar antifungal activity of bulk essential oils against *F. verticillioides* and *A. parasiticus* in terms of mycelium growth, sporulation, and production of aflatoxins and fumonisins in corn grains (Villegas-Rascón et al., 2017). Recently, we successfully formulated several EOs (clove oil, thyme oil, lemongrass oil, peppermint oil, and cinnamon oil)-in-water nanoemulsions stabilized by food-grade surfactants which displayed

strong antifungal activity against mycelium growth, DON and its derivatives (3ADON and 15ADON) production by *F. graminearum* (Wan, Zhong, Schwarz, Chen, & Rao, 2018a, 2019b).

The antifungal activity of essential oil-in-water nanoemulsion is due to bioactive compounds of EOs to be incorporated into the lipid membrane of microbial cells, thereby disrupting normal cellular function, such as increasing membrane permeability, cell structures disrupting, and cytoplasmic content leakage (Seow et al., 2014). It has been demonstrated that the level of hydrophobicity of EOs influences the antimicrobial efficacy (Goñi et al., 2009). The EO-in-water nanoemulsion based delivery system can improve the interaction between EOs and cell membranes by four approaches: (i) the droplets with nanometric size increase the surface area so that it brings more EOs to membrane surface. The arrived EOs possibly break the phospholipid bilayer integrity and act with embedded transport proteins (Moghimi, Ghaderi, Rafati, Aliahmadi, & Mcclements, 2016); (ii) Specific emulsifiers also act with the phospholipid bilayer in order to increase the release of EOs on the action sites (Li et al., 2015); (iii) EOs formed partition between droplets and water will prolong the release of EOs from nanoemulsions (Majeed et al., 2016); and (iv) The electrostatic interaction between positively charged nanoemulsion and negatively charged microbial membrane enhances the EOs concentration in active site (Chang, McLandsborough, & McClements, 2015).

CHAPTER 1. INFLUENCE OF OIL PHASE COMPOSITION ON ANTIFUNGAL AND MYCOTOXIN INHIBITORY ACTIVITY OF CLOVE OIL-IN-WATER NANOEMULSIONS[1]

Abstract

The influence of oil compositions on the physical properties, antifungal and mycotoxin inhibitory activity of clove oil-in-water nanoemulsion were investigated. The physically stable clove oil-in-water nanoemulsions could be fabricated by incorporating either ≥ 75 wt % of corn oil, or ≥ 50 wt % of medium chain triacylglycerol (MCT) into clove oil before homogenization to prevent Ostwald ripening. The clove oil-in-water nanoemulsions with mean diameters <150 nm showed high physical stability over 30 days of storage time. The antifungal activity of physically stable clove oil-in-water nanoemulsions were further evaluated using effective concentration (EC) and inhibitory activity towards mycotoxin production in two chemotypes of *Fusarium graminearum* isolates. The composition of oil phase, i.e., ripening inhibitor type and concentration, in clove oil-in-water nanoemulsions had a remarkable impact on antifungal activity as well as inhibition of mycotoxin production. In general, under the same clove oil concentration in oil phase, the addition of MCT decreased the antifungal and mycotoxin inhibitory activity of clove oil more than corn oil. Compared with bulk clove oil, this study also indicated that mycotoxin inhibitory activity of clove was significantly enhanced when encapsulated in nanoemulsions. These results have important implications for the design of essential oil based nanoemulsions as effective antifungal and detoxification delivery system in food or other industries.

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Introduction

The Food and Agricultural Organization (FAO) has estimated that up to 25% of the world's cereal grains are contaminated by molds in the field and during storage, some of which are known to produce mycotoxins (Kabak, 2009). Toxigenic molds are produced by certain phytopathogenic and food spoilage fungi, such as *Aspergillus, Penicillium,* and *Fusarium* species (da Cruz Cabral, Fernández Pinto, & Patriarca, 2013). Mycotoxins are secondary metabolites which are produced by fungi and exert detrimental toxic effects on animals and humans. Deoxynivalenol (DON, also known as vomitoxin) and its 3-acetyl and 15-acetyl derivatives (3ADON and 15ADON, respectively) are the most common mycotoxins found in *Fusarium* infected grains, such as wheat, rye, barley, corn, rice and oats (Placinta, D'Mello, & MacDonald, 1999). Deoxynivalenol can be produced not only during the development of the grains in the field but also in post-harvest and during storage. In general, DON is chemically stable to resist thermal processing, which can persist into the final food products (e.g., flour, bread, noodles, and beer) through contaminated grains (Bullerman & Bianchini, 2007). The ingestion of DON has been reported to alter the intestinal, immune, endocrine, and nervous systems. The acute exposure of DON can cause severe illnesses associated with vomiting, anorexia, abdominal pain, diarrhea, malnutrition, headache and dizziness (Escrivá, Font, & Manyes, 2015). The reduction of such mycotoxins in food production is thus of primary importance and there is great interest in developing efficient and safe prevention strategies in terms of food safety.

In recent years, the "clean-label" is on the rise in food industry which requires foods without artificial food additives including widely used chemical preservatives. Consequently, natural antimicrobial or antifungal agents that could be used as potential alternatives to combat foodborne pathogens or fungal pathogens have received lots of attention. Plant essential oils

(EOs) have been shown to be effective in controlling food spoilage and pathogenic bacteria in food safety and preservation applications (Gutierrez, Barry-Ryan, & Bourke, 2008). It has been reported that some EOs, such as clove oil, thyme oil, lemongrass oil, and cinnamon oil, have broad-spectrum antimicrobial and antifungal properties. Plant essential oils are usually the mixtures of hundreds of chemical compounds. Phenolics, phenolic acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids are the major compounds to display antimicrobial and antifungal activity (Burt, 2004). For instance, eugenol, a phenolic component accounting for more than 80% of clove oil, has been shown to exhibit antifungal activity against several fungi. However, there are technological limitations with regards to the antimicrobial or antifungal efficacy of EOs in aqueous food products due to their low solubility in water and high volatility. In order to maintain antifungal activity, EOs should be restrained from interacting with food materials, and kept stable against environmental stress during food processing. Nanoemulsion based delivery systems, which have been widely applied in food and pharmaceutical industry to encapsulate lipophilic bioactive compounds such as vitamins, natural colors and antimicrobials, are the type of optimal system for essential oil protection (Donsì et al., 2011). Such delivery systems have two advantages. The lipophilic antimicrobial or antifungal compounds, such as EOs, can be easily incorporated into aqueous foods after being encapsulated into nanoemulsion based delivery systems. In addition, the mass transfer efficacy of lipophilic bioactive compounds to certain sites of action is promoted by virtue of their increased water solubility in nanoemulsions.

Nanoemulsions are thermodynamically unstable systems that typically consist of oil, surfactant, and water. The small particle size (d < 200 nm) of nanoemulsions results in either a translucent or slightly turbid appearance. It is believed that nanoemulsions have a number of

potential advantages over conventional emulsions for encapsulating lipophilic bioactive compounds (McClements & Rao, 2011). In general, nanoemulsions have good stability against gravitational separation, flocculation and coalescence due to their small particle size. Besides, the antimicrobial activity of the encapsulated EOs in nanoscale droplet might be increased when compared with the bulk essential oils due to an increased total surface to volume ratio (Donsì et al., 2011). However, nanoemulsions are more prone to encounter droplet growth with time due to Ostwald ripening (McClements & Rao, 2011). The Ostwald ripening rate increases with the increase of oil solubility in aqueous phase. The smaller molar volume of relatively polar constituents in essential oils have appreciable solubility in water resulting in destabilization of essential oil-in-water nanoemulsions by Ostwald ripening. In contrast, larger molar weight of medium chain triacylglycerol (MCT) or long chain triacylglyceride type of oils such as corn oil are less water soluble, and therefore can be incorporated into oil phase and act as inhibitors to prevent Ostwald ripening in nanoemulsions (Chang, Mclandsborough, & Mcclements, 2012). However, the antifungal activity of EOs might be altered by the addition of ripening inhibitors (Liang et al., 2012).

Over the last decade numerous studies on physiochemical stability and antimicrobial activity of essential oil-in-water nanoemulsions have been reported (Benjemaa et al., 2018; Char, Cisternas, Pérez, & Guerrero, 2016; Clavijo-Romero, Quintanilla-Carvajal, & Ruiz, 2018; Ryu, McClements, Corradini, & McLandsborough, 2018). However, very few of the studies were aimed at investigating the effect of essential oil-in-water nanoemulsion compositions (e.g., Ostwald ripening inhibitors) on antifungal activities, and particularly the inhibition of mycotoxin production by *Fusarium graminearum*. In this study, clove oil was selected as a model essential oil to form food grade clove oil-in-water nanoemulsions using either MCT or corn oil as Ostwald

ripening inhibitor. The impact of Ostwald ripening inhibitors (i.e., MCT and corn oil) on particle size and long-term stability of clove oil-in-water nanoemulsions was assessed. Moreover, the role of oil phase composition (i.e., Ostwald ripening type and concentration) in clove oil-in-water nanoemulsions on antifungal activities against *Fusarium graminearum* isolates were evaluated. Finally, the effect of clove oil-in-water nanoemulsions on the inhibition of Fusarium mycotoxins production using rice culture was examined. The results of this study will provide useful information for design and utilization of the essential oils as antifungal delivery systems in food industry.

Materials and methods

Materials

Polyoxyethylene (20) sorbitan monooleate (Tween 80), clove oil (purity≤100%), Mirex, and Bis(trimethylsilyl)acetamide (BSA)/trimethylchlorosilane (TMCS)/Trimethylchlorosilane (TSIM) kit were purchased from MilliporeSigma Co. (St. Louis, MO, USA). Corn oil mung beans and white basmati rice were obtained from a local supermarket (Fargo, ND, USA). Medium-chain triacylglyceride (MCT, NEOBEE M-5) was kindly provided by Stepan Company (Bordentown, NJ, USA). The manufacturer reported that the MCT used was mainly composed of 50-65% caprylic acid (C8:0) and 30-45% of capric acid (C10:0) in terms of its fatty acid profile. Potato dextrose agar (PDA) was purchased from AMRESCO (Solon, OH, USA). Potato dextrose broth was purchased from BD Biosciences (Franklin Lakes, NJ, USA). All solutions were prepared using ultrapure distilled de-ionized water (DDW, 18.2 MΩ cm, Barnstead ultrapure water system, Thermo Fisher Scientific, USA).

Nanoemulsion preparation

The aqueous phase used to prepare clove oil-in-water nanoemulsions consisted of 0.5 wt % Tween 80 dispersed in 94.5 wt % of buffer solution (10 mM phosphate buffer, pH 7.0). Oil phase (5 wt %) was prepared by mixing different mass ratio of the clove oil and ripening inhibitors (MCT or corn oil, 0, 25, 50, 75, and 100 wt %) prior to homogenization. The oil phase was then mixed with the aqueous phase by a high-speed hand blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min. The mixture was further homogenized using a high-pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) at 103.42 MPa for three passes. The nanoemulsions were kept on ice over the whole procedure. After homogenization, the nanoemulsions were collected and stored at 4 and 25 °C for long term storage stability study.

Particle size measurement

The mean particle diameters (Z-average) of nanoemulsions were measured at 0, 1, 2, 3, 4, 5, 6, 7, and 30-day using a dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK). The instrument determines the particle size from intensity-time fluctuations of a He–Ne laser beam (633 nm) scattered from a sample at a fixed angle of 173°. The data is reported as the mean droplet diameter and particle size distribution.

Determination of antifungal activity using effective concentrations (EC)

Fusarium graminearum isolates can be identified as one of three discrete chemotypes, i.e.,3-acetyl-deoxynivalenol (3ADON), 15-acetly-deoxynivalenol (15ADON), and nivalenol (NIV) (Puri & Zhong, 2010). Two *Fusarium graminearum* isolates (F8-1 and 10-124-1) were selected to evaluate the antifungal efficiency of clove oil-in-water nanoemulsions. Isolate F8-1 is a representative of deoxynivalenol (DON) and 3ADON producers and isolates 10-124-1 is a

representative of DON and 15ADON producers. These isolates were stored at -80°C and refreshed on PDA plates. The PDA cultures were incubated at 25°C avoiding light for 4 days prior to usage.

The physically stable clove oil-in-water nanoemulsions with oil phase containing 50 wt % MCT (50M), 75 wt % MCT (75M), and 75 wt % corn oil (75C) were chosen to assess its antifungal activity using following methods. Firstly, the selected nanoemulsions were diluted by phosphate buffer (10 mM, pH 7.0) to create desired gradient clove oil concentrations in the final nanoemulsions (0.125, 0.625, 1.25, 3.75, 6.25, 12.5, 25.0 mg/g nanoemulsions). Diluted nanoemulsions were then filtered through a Whatman sterile filter (0.45 μm, 25 mm cellulose acetate filtration medium, Catalog # 28138-406, GE Healthcare) to remove the microorganisms before adding to PDA media. The PDA plates were prepared by pouring the autoclaved medium to Petri dishes (10 cm diameter). After solidification of PDA, 500 μl nanoemulsions containing a series concentration of clove oil were introduced to the surface of PDA media, whereas the control dish was prepared by adding the same volume of distilled water. Then a square mycelial plug (5 mm side length) of *Fusarium* isolates was placed at the center of media and incubated at 25°C avoiding light for 4 days prior to measurement of fungal growth. The diameter of mycelial colony was measured and compared to control dish. The mycelial growth inhibition (MGI) rate was calculated as MGI rate (%) =100 × (mycelial colony's diameter of control – mycelial colony's diameter of treatment)/mycelial colony's diameter of control (Patzke, Zimdars, Schulze-Kaysers, & Schieber, 2017). MGI rates were fitted to cubic regression model and EC values were calculated by the regression equation. For example, EC_{50} was calculated when MGI was observed in 50% inhibition.

Fungal morphological study

This assay was aimed at the observation of potential morphological changes of *Fusarium graminearum* isolates when exposed to clove oil-in-water nanoemulsions. Mung bean agar (MBA) plates were prepared by boiling and filtering of 40 g mung beans and mixing with 15 g agar in 1 L double distilled water. After sterilization, the medium was poured into small Petri dishes (4 cm diameter). EC_{50} concentration of nanoemulsions were added on the surface of the MBA plates and a mycelial plug (3 mm side length) from the 4-day old *Fusarium graminearum* isolates was placed at the center of medium for mycelia growth and conidia production. The plates were incubated for 11 days at 25°C under an ultraviolet light. After eluting by double distilled water, conidia were observed by phase contrast microscope (Olympus EX51TF, Olympus Optical CO, Japan) and images were taken at 400× magnification.

Determination of mycotoxin production in rice culture

Preparation of Fusarium graminearum conidial suspension

Mung bean agar (MBA) media were autoclaved and poured into Petri dishes (10 mm diameter). After cooling, mycelial plugs were cut from 4-day old cultures of *Fusarium graminearum* isolates (i.e., 10-124-1 and F8-1) and used to inoculate MBA plates by gently rubbing the plugs on the surface of the plates. All the inoculated MBA plates were stored under ultraviolet light (light on: light off = 12h: 12h) at ambient temperature for 9 days. Then, conidial suspensions were made from the MBA plates and filtered through autoclaved Miracloth (pore size 22-25 µm, MilliporeSigma, St. Louis, MO, USA) to remove hyphae. The concentration of conidial suspensions was calculated using a Levy Ultraplane Hemocytometer (CA Hausser & Son, PA, USA) and diluted to 1×10^6 spore/ml.

Preparation of rice culture for mycotoxins production

The physically stable clove oil-in-water nanoemulsions with oil phase containing 50 wt % MCT (50M), 75 wt % MCT (75M), and 75 wt % corn oil (75C) were selected to evaluate the inhibitory effect of EO on mycotoxins production of *Fusarium graminearum* isolates *in vitro*. Rice (25 g) and water (10 ml) were added to a 125 ml Erlenmeyer flask, which was then autoclaved for 25 min. After cooling, a mixture of 700 µl conidia suspension (1×10^6 spore/ml) with 700 µl of series of clove oil-in-water nanoemulsions or 17.5 µl bulk clove oil was added to the rice culture, and then shaken for 10 s. For the control group, the 700 µl conidia suspension and 700 µl double distilled water were added. The final clove oil concentrations in the rice culture when treated with bulk clove oil, 50M, 75M and 75C were 700, 700, 350, and 350 mg/kg rice, respectively. The rice cultures were incubated in dark at 25 ℃ for 9 days.

Extraction and detection of mycotoxins in rice culture by GC-MS

The procedure to extract mycotoxins including DON, 15ADON, and 3ADON in rice culture was conducted using the method described by Rishi et al. with some modifications (Burlakoti et al., 2008). The inoculated rice cultures were frozen at -80℃ prior to freezing drying (Lyophilizer, SP scientific, Gardiner, New York) for two days. The dried rice cultures were ground with a Perten laboratory mill (model 3600, Perten Instruments, Hagersten, Sweden), and 2 g of rice flour were extracted using 20 ml of acetonitrile:water (84/16, v/v) solution by shaking at 180 rpm (Eberbach Corporation, Ann Arbor, MI, USA) for 1 h. Then, 4 ml of supernatant was filtered through a siliaprep C18/alumina solid phase extraction column (Chrom Tech Inc, MN, USA). After filtration, 2 ml solution was transferred to sample tube (15×150 mm) and concentrated by drying in an evaporator at 50 ℃ along with air flush for 1 h. Then, 100 µl of BSA : TMCS : TMSI (3:2:3, v:v:v) was added into each sample tube and derivatized for 30 min.

One milliliter of isooctane consisting of 0.5 µg/ml Mirex as internal standard was added into the sample tube before the termination of derivatization by adding 1 ml $NaHCO_3$ (3 %) solution. The derivatized mycotoxins were extracted into the supernatant by shaking for 10 min, and then transferred to 2 ml GC vial. Trichothecene mycotoxins were measured by GC-MS as previously described (Jin et al., 2018). The system consisted of an Agilent 6890N gas chromatography coupled with 5973 mass selective detector and a 35% phenyl siloxane column (30.0 m × 250 µm × 0.25 µm film) (Agilent HP-35). Two microliters of the derivatized extract were injected and carried out in splitless mode at 300℃. The oven temp was initially kept at 150℃ for 1 min, then raised to 280℃ at a rate of 10 ℃/min, further ramped to 310 ℃ at a rate of 30 ℃/min, and finally maintained for 5 min at 310 ℃. The energy was −70 eV in electron impact mode. The following fragment ions (m/z) were used for the qualification of trimethylsilyl ether derivatives of mycotoxins, as well as Mirex: 295.20 for DON; 392.20 for 15ADON; 377.20 for 3ADON; and 271.90 for Mirex. The limits of quantitation (LOQ) and detection (LOD) for all the mycotoxins were 0.20 and 0.10 mg/kg, respectively.

Statistical analysis

All measurements were performed at least triplicate using freshly prepared samples (*i.e.*, new samples were prepared for each series of experiments) and were reported as mean ± standard deviation. One-way analysis of variance (ANOVA) was conducted, and significant difference of mean value was defined at $p < 0.05$ by Tukey's test (Minitab 18.0).

Results and discussions

Pure clove oil-in-water nanoemulsions formation

The primary goal of this research was to fabricate food-grade clove oil-in-water nanoemulsions as antifungal and detoxification agents in food systems. Consequently, it is

important to ensure that clove oil can be encapsulated in the nano-size range emulsions with diameter less than 200 nm and good initial physical stability as well.

Initially, 5 wt % of pure clove oil, as the solo oil phase, was dispersed into the aqueous phase containing 0.5 wt % Tween 80 before homogenizing using microfluidizer. However, the resulting fresh emulsions with the mean particle diameter of 784 nm were highly unstable to droplet growth. Prompt phase separation of emulsions was visualized 1.5 h after preparation and the mean particle diameter was around 876 nm (Fig. 1-1A). The measurements of the evolution of particle size showed that there were two main size classes with peaks around 100 and 1000 nm in diameter in pure clove oil-in-water emulsions soon after homogenization, suggesting that droplet growth occurred very rapidly in this system (Fig. 1-1B). The oil droplets continued to grow very fast during storage and after 1 h, the population of small-sized droplets had disappeared and only a population of larger droplets was observed (Fig. 1-1B). The instability of bulk clove oil-in-water emulsions and the growth of large size population can be explained by the occurrence of Ostwald ripening, the process whereby large droplets grow at the expense of smaller ones through the intervening continuous phase. Ostwald ripening is a common problem responsible for the instability of EOs emulsions or nanoemulsions due to the relatively high water-solubility of EOs leading to the mass transport of dispersed phase from one droplet to another. The phenomenon observed in the current study was in good agreement with other published papers. For example, emulsion made by pure peppermint oil exhibited very larger droplets around 4 μm right after homogenization (Liang et al., 2012).

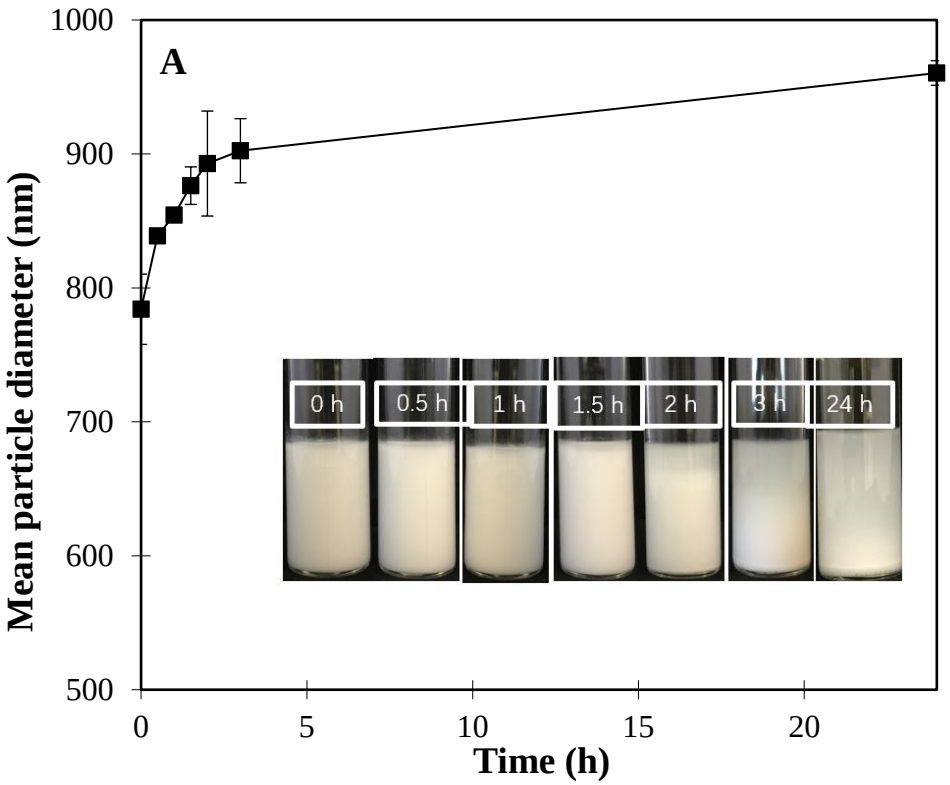


Figure 1-1. Time-dependence of (A) mean particle diameter; (B) particle size distribution of 5 wt % bulk clove oil-in-water emulsions stored at 25 ^{o}C (0.5 wt % Tween 80, 94.5 wt % of 10mM phosphate buffer, pH 7; the inserted pictures were the visual observation of emulsions).

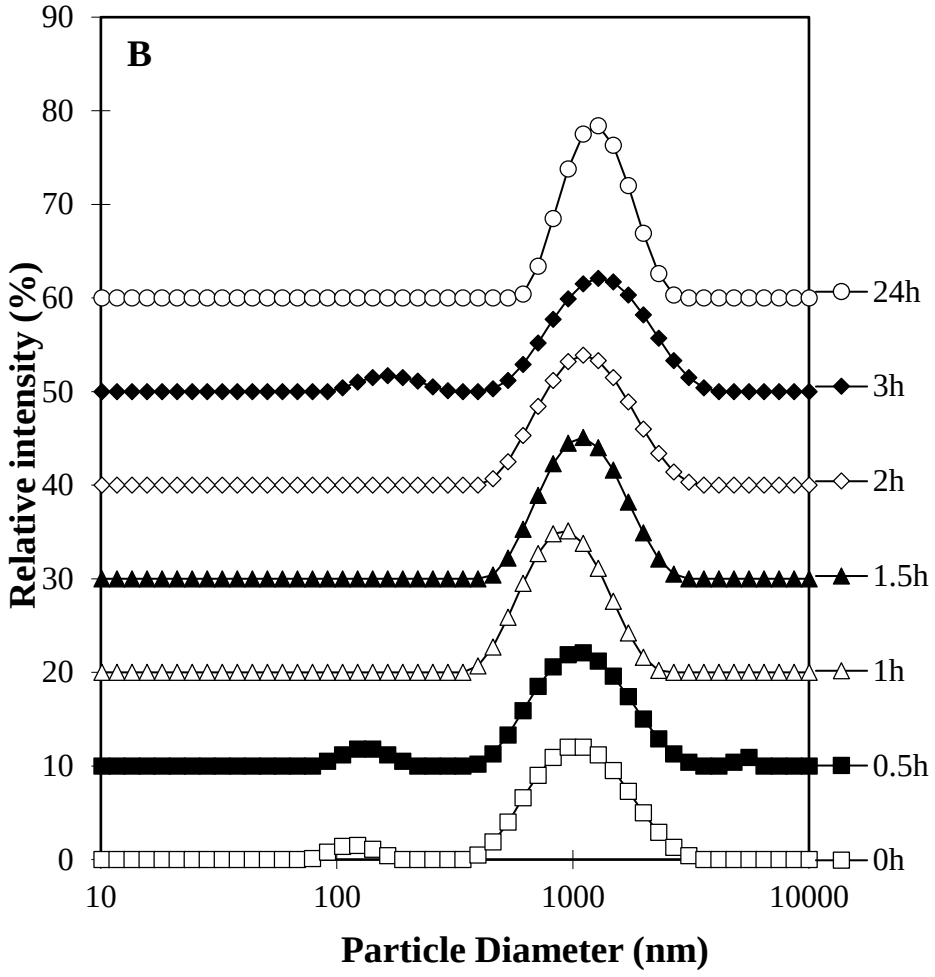


Figure 1-1. Time-dependence of (A) mean particle diameter; (B) particle size distribution of 5 wt % bulk clove oil-in-water emulsions stored at 25 ^{o}C (0.5 wt % Tween 80, 94.5 wt % of 10mM phosphate buffer, pH 7; the inserted pictures were the visual observation of emulsions) (continued).

Influence of Ostwald ripening inhibitors on clove oil-in-water nanoemulsions formation

Previous studies have shown that Ostwald ripening can be retarded or inhibited by incorporating highly hydrophobic component, such as medium chain triacylglycerol (MCT) and long chain triacylglycerol (LCT), in the essential oil phase prior to homogenization. It is corroborated that molecules with low water solubility might not only inhibit Ostwald ripening by generating entropy of mixing effect to counterbalance the interfacial curvature effect, but also

facilitate the size reduction of droplets to the desired nano-size range (McClements & Rao, 2011). In this study, we examined whether food grade corn oil as the LCT representative or MCT was more effective to mitigate Ostwald ripening in clove oil-in-water nanoemulsion systems. A series of nanoemulsions with different clove oil concentrations (100, 75, 50, 25 wt %) mixed with different type and amounts of ripening inhibitor (corn oil or MCT) were prepared to examine the effect of ripening inhibitor on the stability of clove oil-in-water nanoemulsions. The ripening inhibitors were mixed with clove oil prior to homogenization. After homogenization, the nanoemulsion samples were stored for 24 h at 25 °C prior to measure the particle size (Fig. 1-2).

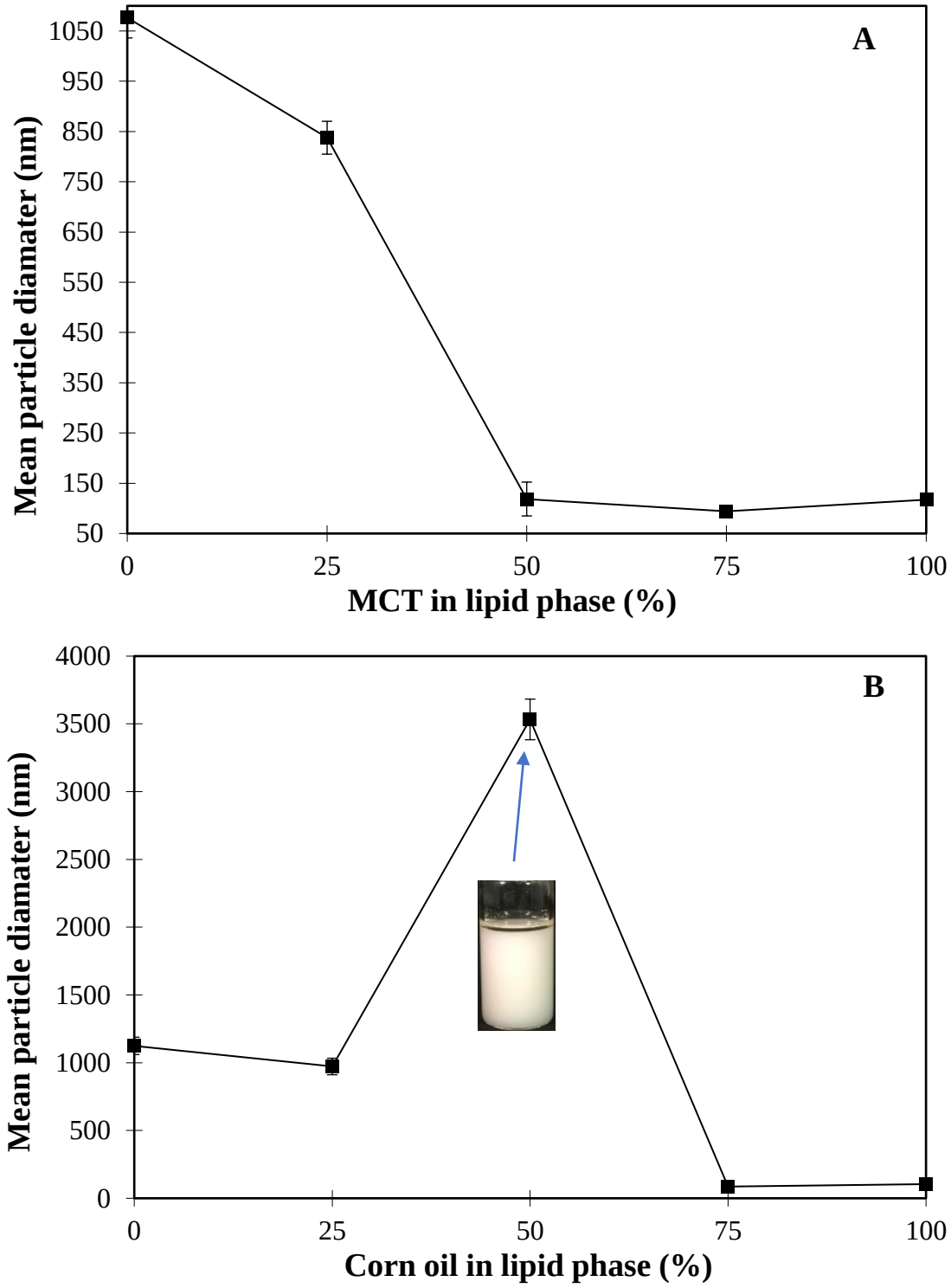


Figure 1-2. Dependence of oil phase Ostwald ripening inhibitor (A) Medium Chain Triacylglycerol (MCT); (B) corn oil on mean particle diameter of 5 wt % clove oil-in-water emulsions after 24 h storage at 25 ^{o}C (0.5 wt % Tween 80, 10 mM phosphate buffer, pH 7; the inserted picture was the visual observation of oiling off in emulsion).

For the MCT mixed with clove oil system, the trend of decreasing droplet diameter was found with increasing concentration of MCT (Fig. 1-2A). The droplet size decreased dramatically to 118 nm when 50 wt % of MCT was mixed with clove oil in oil phase, which can be attributed to the ability of MCT to inhibit Ostwald ripening. A further increase in MCT concentration did not change the mean droplet diameter steeply. The smallest mean droplet diameter of 94 nm was obtained in the system produced by 75 wt % of MCT in oil phase. For the system containing more than 50 wt % of MCT, it could be considered as nanoemulsions, that is, $d \leq 200$ nm. In the system containing corn oil in the oil phase, there was a slightly decrease in droplet diameter (972 nm) of clove oil emulsion when 25 wt % of corn oil was incorporated in oil phase. Surprisingly, as corn oil increased to 50 wt %, a highly unstable dispersion system was appeared accompanied with a visible oiling off soon after homogenization (Fig. 1-2B). Further increase corn oil content to 75 wt % in oil phase yielded a stable clove oil-in-water nanoemulsions with mean droplet diameter of 86 nm. The turning point as 50 wt % of corn oil was present can be explained by the formation of relatively small droplets under high pressure homogenization, followed by a quick droplet growth, presumably because of the entropy of mixing in 50 wt % of corn oil is lower than that of interfacial curvature. Our results demonstrated that 50 wt % MCT is the threshold to retard Ostwald ripening, whereas 75 wt % of corn oil was needed to prevent droplet growth in clove oil-in-water nanoemulsions.

Interestingly, one would expect that MCT is less effective to prevent Ostwald ripening since the water solubility of MCT is somewhat higher than that of corn oil. However, our results indicated that MCT is a highly effective ripening inhibitor in the performance of enhancing physical stability of clove oil-in-water nanoemulsions than corn oil. Similar findings were also reported by Chang et al. (Chang et al., 2012), whose results also demonstrated that the addition

of corn oil could inhibit Ostwald ripening in thyme oil-in-water nanoemulsions at pH 3.5 more efficient than MCT. The discrepancy implies that solubility is not the only factor to determine the efficacy of inhibitors to prevent Ostwald ripening. Overall, mixing sufficient amount of MCT or corn oil with clove oil phase before high pressure homogenization was a useful tool to inhibit Ostwald ripening of clove oil-in-water nanoemulsions.

Storage stability of clove oil-in-water nanoemulsions

As mentioned earlier, clove oil-in-water nanoemulsions are anticipated to be used as effective antifungal and detoxification agents in food systems. Therefore, a good long-term stability of nanoemulsion is critical to ensure the activity of encapsulated clove oil to be retained during storage. However, the successful fabrication of clove oil-in-water nanoemulsions with good initial physical stability cannot guarantee a long-term storage stability, especially under different storage temperatures. In this case, three clove oil-in-water nanoemulsions that were found to be stable to droplet growth during the first 24 h storage, i.e., the oil phase containing 50 wt % MCT (50M), 75 wt % MCT (75M), and 75 wt % corn oil (75C), were selected for the long term storage study. The change of particle size within 30 days storage at different storage temperature (4 and 25℃) were measured as shown in Fig. 1-3.

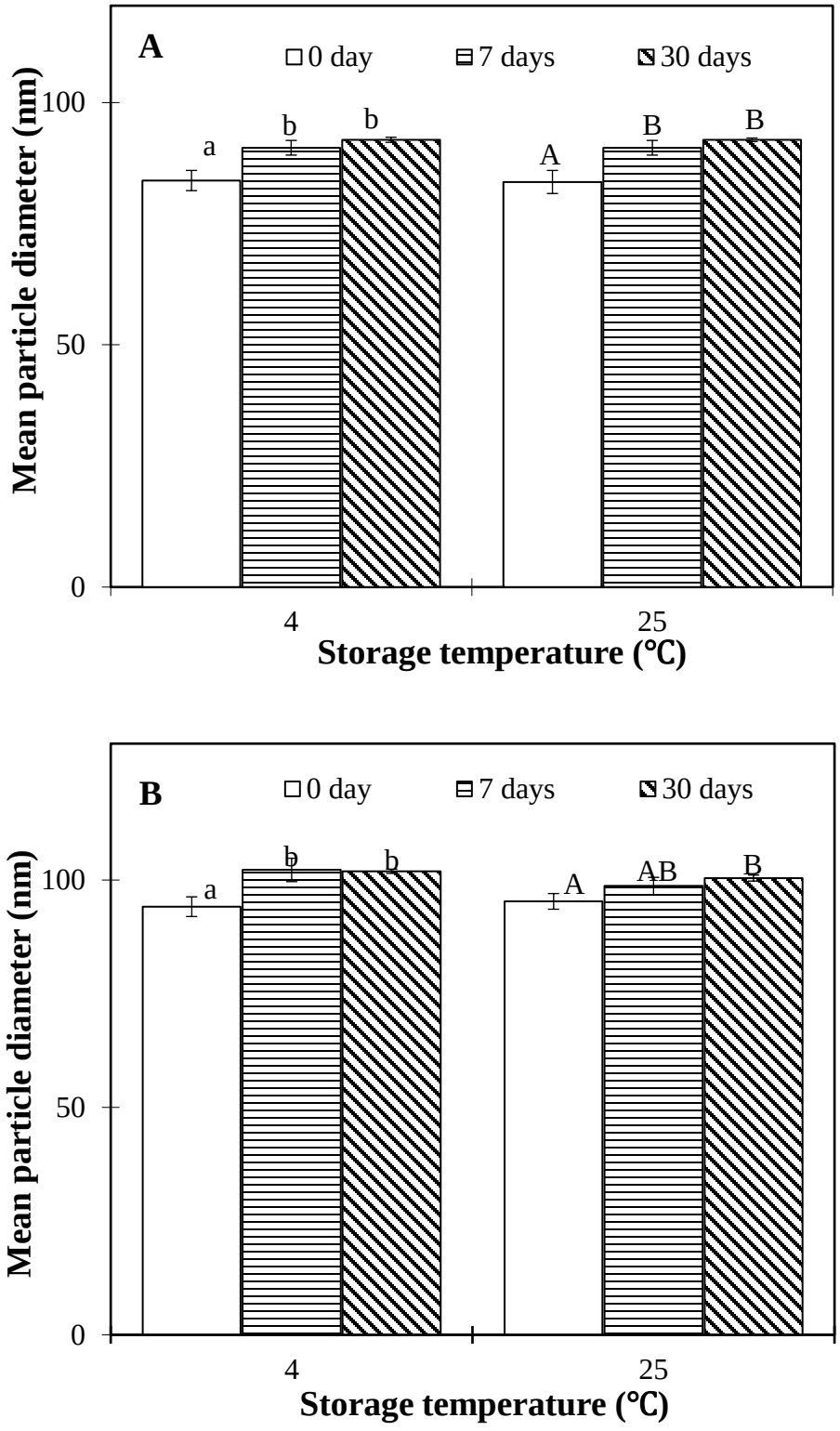


Figure 1-3. Evolution of mean particle diameter of 5 wt % oil-in-water nanoemulsions with oil phase containing (A) 75 wt % corn oil (75C); (B) 75 wt % MCT (75M); (C) 50 wt % MCT (50M) upon 30 days storage at 4 and 25 ℃.

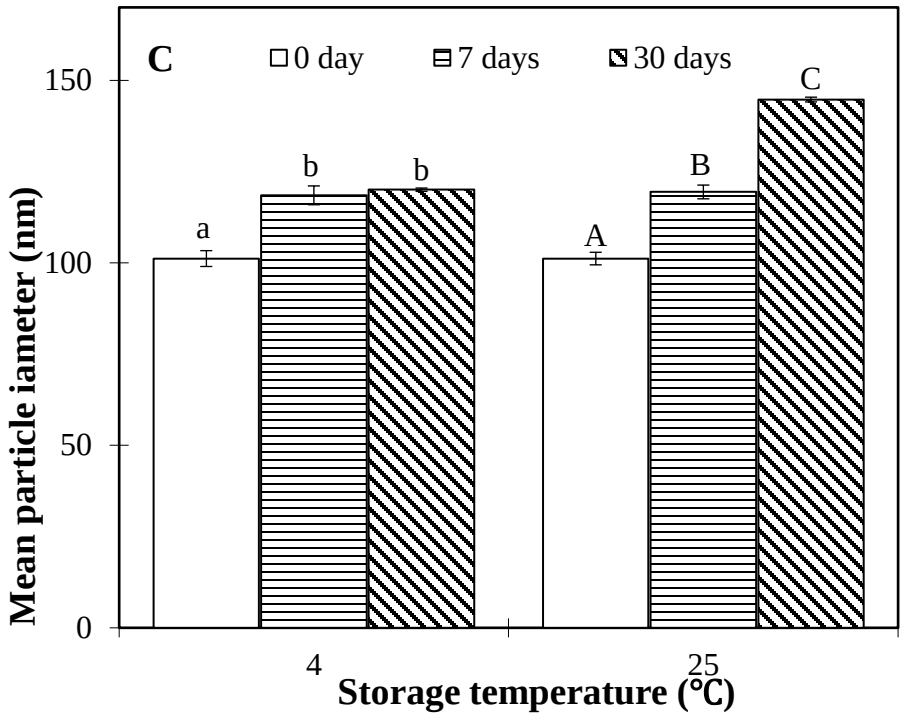


Figure 1-3. Evolution of mean particle diameter of 5 wt % oil-in-water nanoemulsions with oil phase containing (A) 75 wt % corn oil (75C); (B) 75 wt % MCT (75M); (C) 50 wt % MCT (50M) upon 30 days storage at 4 and 25 ℃ (continued).

Under both storage temperature, there was a slight increase in mean particle diameter of clove oil-in-water nanoemulsions from ~84 and ~ 94 nm to ~90 and ~102 nm, respectively, with oil phase containing either 75 wt % corn oil or 75 wt % MCT in first 7 days storage, after which maintained constantly over the course of 30 days storage (Fig. 1-3A & B). The mean particle diameter of nanoemulsions containing 50 wt % MCT in oil phase was increased from ~101 nm to ~118 nm after 7 days storage and subsequently remained constant over storage time when stored at 4℃; however, higher storage time promoted the growth of particle size to 144 nm, still remaining in nanometric range, after 30 days storage (Fig. 1-3C). In the meantime, no phase separation or oiling off was observed after 30 days storage at both storage temperatures, strongly manifesting its good long-term stability.

The particle size distribution, rather than just the mean particle diameter, is an important factor for monitoring stability of nanoemulsions. We therefore plotted particle size distribution of nanoemulsion during storage time (Fig. 1-4).

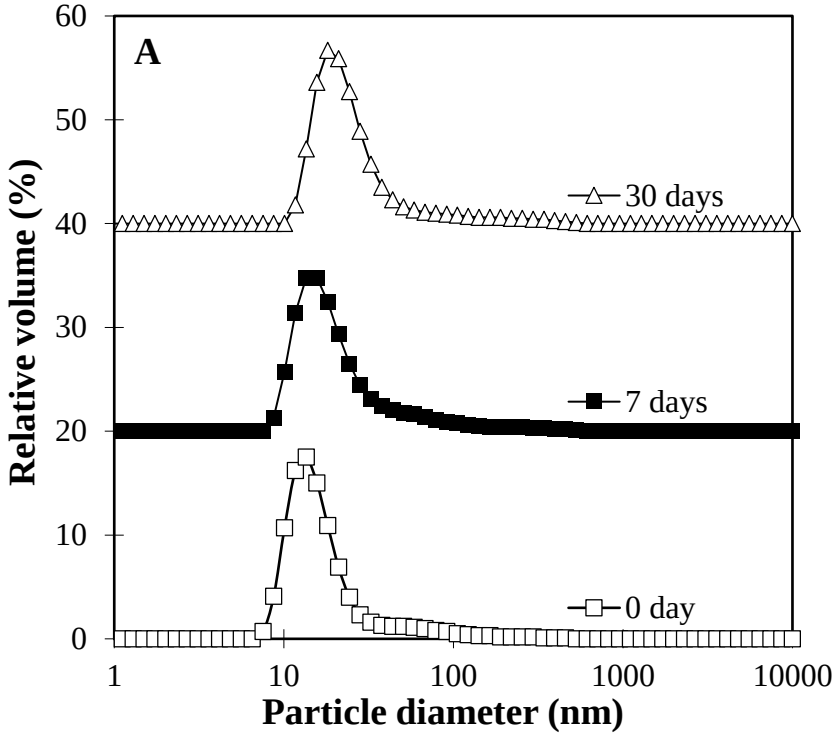


Figure 1-4. Particle size distribution of 5 wt % oil-in-water nanoemulsions with oil phase containing (A) 75 wt % corn oil (75C); (B) 75 wt % MCT (75M); (C) 50 wt % MCT (50M) upon 30 days storage (the inserted pictures were visual appearance of nanoemulsions during storage at 25 ℃).

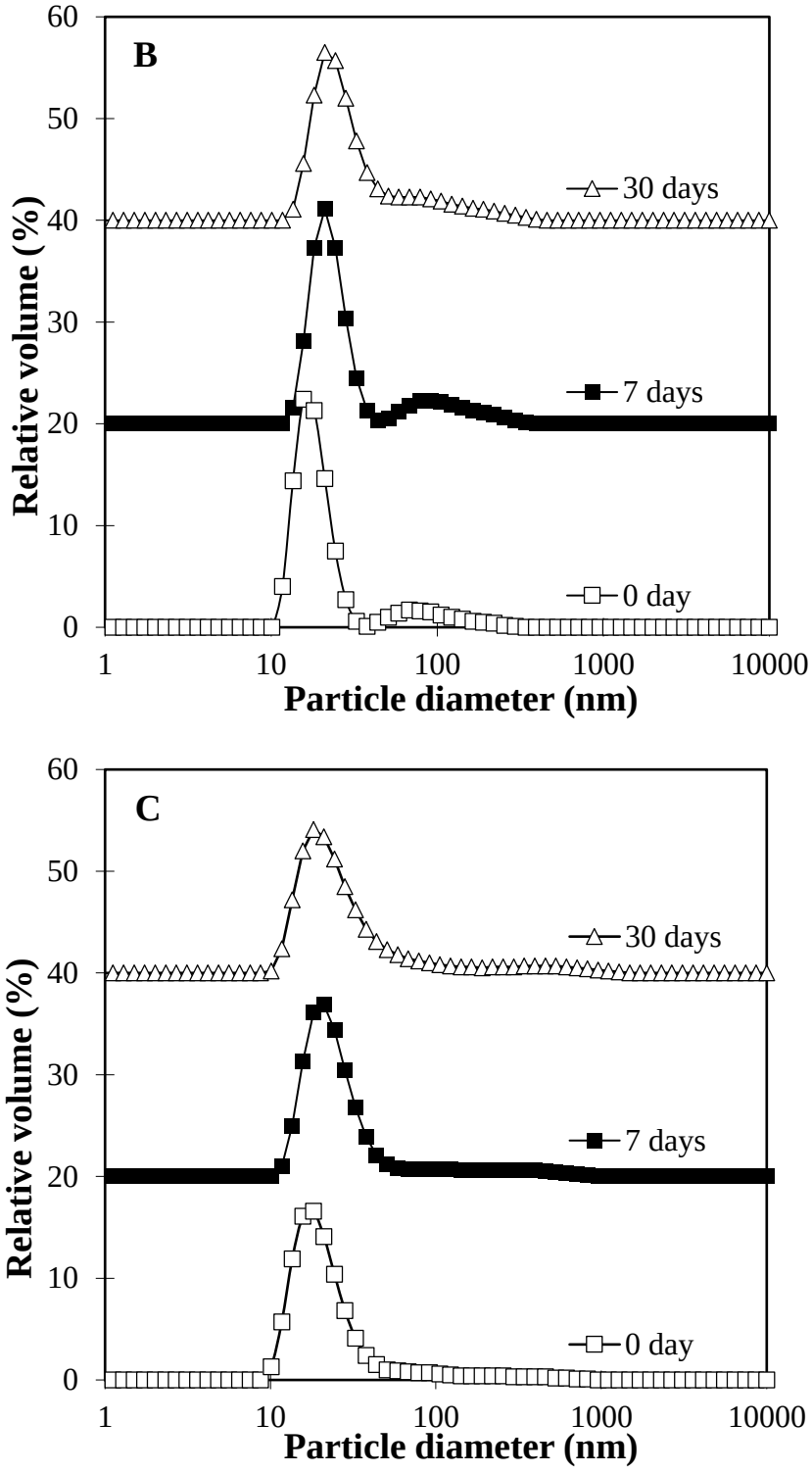


Figure 1-4. Particle size distribution of 5 wt % oil-in-water nanoemulsions with oil phase containing (A) 75 wt % corn oil (75C); (B) 75 wt % MCT (75M); (C) 50 wt % MCT (50M) upon 30 days storage (the inserted pictures were visual appearance of nanoemulsions during storage at 25 ℃) (continued).

The particle size distribution of clove oil-in-water nanoemulsions (75C & 50M) had no shift and maintained monomodal pattern within 30 days, again indicating that Ostwald ripening has been largely inhibited (Fig. 1-4A & C). However, a slightly difference among size distribution were observed in the nanoemulsions prepared by different concentration (50M & 75M) of Ostwald ripening inhibitor, with the higher concentration in the oil phase generating longer stability (Fig. 1-4B & C). The particle size distribution of 50M nanoemulsions had a slightly shift towards larger region after 7 days storage; still, no phase separation was observed upon 30 days storage (Fig. 1-4B).

Overall, the consistent mean diameter of the three types clove oil-in-water nanoemulsions over storage time implies that the nanoemulsions are highly stable against droplet growth across the whole measurement temperature and storage time.

Influence of oil phase composition on antifungal activity of clove oil-in-water nanoemulsions

The antifungal activity and inhibition of mycotoxins production of clove oil might be affected by the oil composition in nanoemulsion systems. The antifungal activity of the three types clove oil-in-water nanoemulsions (MCT or corn oil ≥ 50 wt % in oil phase) that exhibited good long-term physical stability was then evaluated against two common chemotypes (3ADON and 15ADON) of *Fusarium graminearum* isolates in USA using agar dilution method. The mycelial growth inhibition (MGI) was used to compare the antifungal activity of nanoemulsions carrying different concentrations of clove oil (0.125 to 12.500 mg/g) and higher MGI rate represents greater activity.

Emulsions in the absence of clove oil (i.e., 100 wt % of corn oil or MCT in oil phase) against *Fusarium graminearum* was also examined and neither of them exhibited any antifungal

activity (data not shown), indicating that it was the clove oil which exclusively generates antifungal activity against *Fusarium graminearum*. We did not examine the antifungal activity of nanoemulsions prepared by bulk clove oil due to its extremely unstable physical nature. The mycelial growth inhibition (MGI) rate of nanoemulsions loaded with different concentrations of clove oil and ripening inhibitors (corn oil or MCT) in oil phase was shown in Fig. 1-5.

The results clearly showed that MGI rate increased with increasing the concentration of clove oil-in-water nanoemulsions. At the lower clove oil concentrations, nanoemulsions with corn oil in oil phase showed stronger antifungal activity than that containing MCT across all tested *Fusarium graminearum* isolates (Fig. 1-5). For instance, when *Fusarium graminearum* isolate F8-1 was treated with nanoemulsions containing same concentration of clove oil (i.e., 2.500 mg/g), the MGI rate was 34.63% and 26.32% for ripening inhibitor corn oil and MCT, respectively, proving our hypothesis that different Ostwald ripening inhibitor had varying effects on clove oil antifungal activity (Fig. 1-5A). As the total concentration of clove oil in nanoemulsions was increased from 7.500 to 12.500 mg/g, ripening inhibitor (corn oil and MCT) had no significant influence on the antifungal activity of clove oil-in-water nanoemulsions. Similar trend was also found in *Fusarium graminearum* isolate 10-124-1 (Fig. 1-5B).

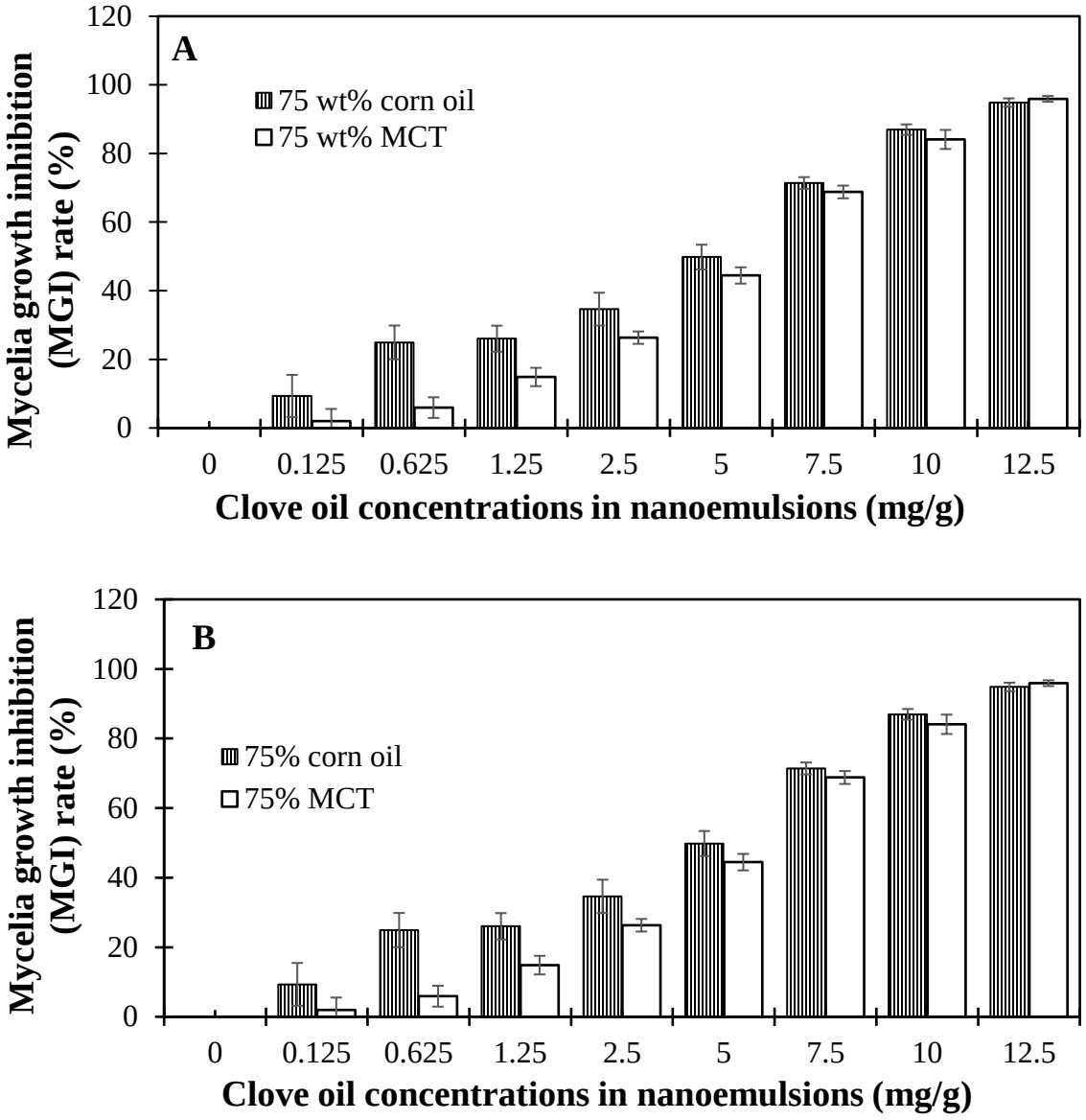


Figure 1-5. Influence of clove oil concentrations and Ostwald ripening inhibitor type on mycelia growth inhibition rate (MGI) in (A) *Fusarium graminearum* isolate F8-1; (B) *Fusarium graminearum* isolate F 10-124-1 after 4 days of incubation. The mycelial growth inhibition (MGI) rate was calculated as MGI rate (%) =100× (mycelial colony's diameter of control – mycelial colony's diameter of treatment)/mycelial colony's diameter of control (the inserted images were the appearance of mycelia growth inhibition zone).

EC values of the two tested *Fusarium graminearum* isolates were calculated by the cubic regression model to establish the relationship between MGI rate and clove oil concentration in nanoemulsions (Table 1-1). The results showed that increasing the level of ripening inhibitor in

oil phase reduced the antifungal activity of clove oil-in-water nanoemulsions. For instance, clove oil-in-water nanoemulsions with 50 wt % MCT in oil phase (50M) received the smallest EC values (e.g., EC_{50}= 3.569 and 4.140 mg/g in F8-1 and 10-2124-1, respectively) due to the highest net clove oil concentration (25.000 mg/g nanoemulsion) compared with the other two systems with only 12.500 mg/g nanoemulsion of net clove oil existed in oil phase when the isolates was treated by the same volume (i.e., 500 μl) of nanoemulsions. For this reason, the concentration of clove oil in the nanoemulsion delivery systems had essential impact on the antifungal activity. When same concentration of clove oil was loaded in oil phase (75C and 75M), nanoemulsions using corn oil as ripening inhibitor displayed stronger antifungal activity than the ones using MCT in oil phase as reflected by both EC_{50} and EC_{70} against two isolates (p<0.05). This study demonstrated that net clove concentration and ripening inhibitor type had a profound influence on the antifungal activity of clove oil-in-water nanoemulsions against *Fusarium graminearum*. This can be explained by the higher oil-water partition coefficient of clove oil when mixing with MCT than with corn oil. Accordingly, higher amount of clove oil will be dissolved and physically trapped in MCT than in corn oil under same initial clove oil concentration. As a result, the incorporation of MCT in oil phase of nanoemulsions renders a relatively lower antifungal activity of clove oil by attenuating the efficient amount of clove oil to be delivered to the site at which it acts as antifungal agent. Similar results had also been reported that MCT decreased the antimicrobial ability of thyme oil-in-water nanoemulsions larger than that of corn oil against an acid-resistant spoilage yeast (Chang et al., 2012). In contrast, there was no distinctive differences between 75C and 75M in EC_{90} value, which reveals that the limited impact of oil-water partition coefficient of clove oil in oil phase is no longer an important factor to influence the antifungal activity of nanoemulsions at such higher clove oil concentration. From the result described

above, the antifungal activity of clove oil-in-water nanoemulsions was determined not only by the concentration of active compounds in oil phase, but also by the location of active compounds in the system being determined by the type Ostwald ripening inhibitor.

Table 1-1. Antifungal activity against *Fusarium graminearum* isolates on PDA media. Effective concentration (EC) was expressed by the concentration of clove oil in nanoemulsions. For example, EC_{50} was calculated when isolate mycelia growth was observed in half inhibition. For each EC value against a certain isolate, means with different letters are significantly different (p<0.05).

Fusarium graminearum isolates	Nanoemulsions	EC_{50}	EC_{70}	EC_{90}
			(mg/g)	
F8-1	75C	4.674b	7.731b	11.174a
	75M	5.814c	9.013c	11.785a
	50M	4.140a	6.764a	10.760a
10-124-1	75C	4.150B	7.260B	11.249B
	75M	5.300C	7.866C	11.103B
	50M	3.569A	6.125A	10.339A

In order to better understand the mechanism by which clove oil-in-water nanoemulsions inhibit fungal growth, the morphology of *Fusarium graminearum* isolate F 8-1 treated with clove oil-in-water nanoemulsions was examined by phase contrast light microscope (Fig. 1-6). In the control group, prevailing germ tubes from spores were observed, some of which became branched hyphae, representing the fast growth of fungi (Fig. 1-6A). Such morphology was in consistent with normal spore germination and hyphae growth in *Fusarium graminearum* (Chandranayaka et al., 2016). Conversely, no spore germination and hypha growth were observed in clove oil-in-water nanoemulsions treated *Fusarium graminearum*, indicating that clove oil-in-water nanoemulsions had remarkable effect on retardation of *Fusarium graminearum* growth (Fig. 1-6B & C). Based on light microscopy studies along with agar

dilution method, clove oil-in-water nanoemulsions could inhibit *Fusarium graminearum* growth by retarding the mycelial growth.

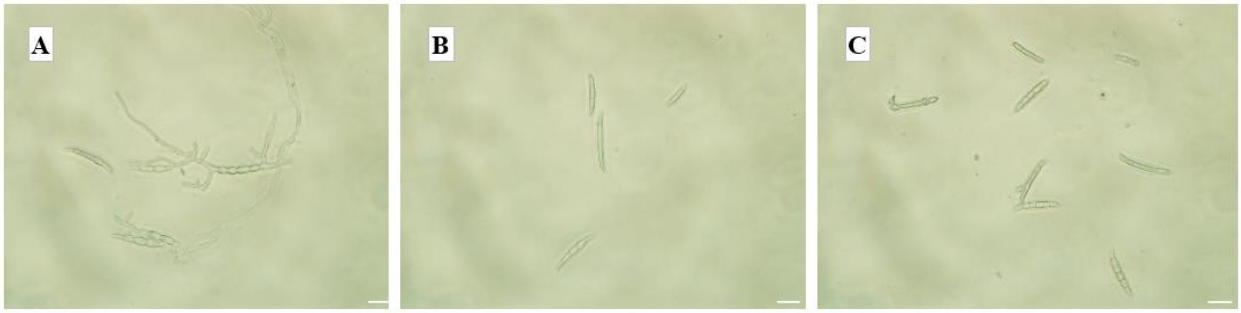


Figure 1-6. Light microscope images (400 × magnification) of spores in *Fusarium graminearum* isolate F8-1 grown on MBA after 11 days of incubation (A) in control group; (B) treated with 4.674 mg clove oil/g nanoemulsions (EC_{50}) of 75C; (C) treated with 5.814 mg clove oil/g nanoemulsions (EC_{50}) of 75M. Spore germination was only observed in control group. Scale bar indicates 10 μm.

Influence of oil phase composition on inhibition of mycotoxin production

In terms of food safety, the main issue that needs to be addressed is the consumption of mycotoxin contaminated food. The inhibitory activity of clove oil-in-water nanoemulsions to *Fusarium graminearum* growth cannot be extrapolated to the inhibition of mycotoxins production because antifungal agents might trigger the production of secondary metabolites and mycotoxins as a response to environmental stress (da Cruz Cabral et al., 2013). Thus, it is crucial to evaluate the effect of clove oil-in-water nanoemulsions on the production of mycotoxin in *Fusarium graminearum* isolates. The effect of clove oil-in-water nanoemulsions on mycotoxins production by two chemotypes of *Fusarium graminearum* isolates (10-124-1 and F8-1) in rice culture was studied upon incubation at 25 ℃. The isolate10-124-1 of *Fusarium graminearum* used in this study produces DON, Fig. 1-7A and 3ADON, Fig. 1-7B, whereas isolate F8-1 produces deoxynivalenol DON, Fig. 1-7C and 15ADON, Fig. 1-7D.

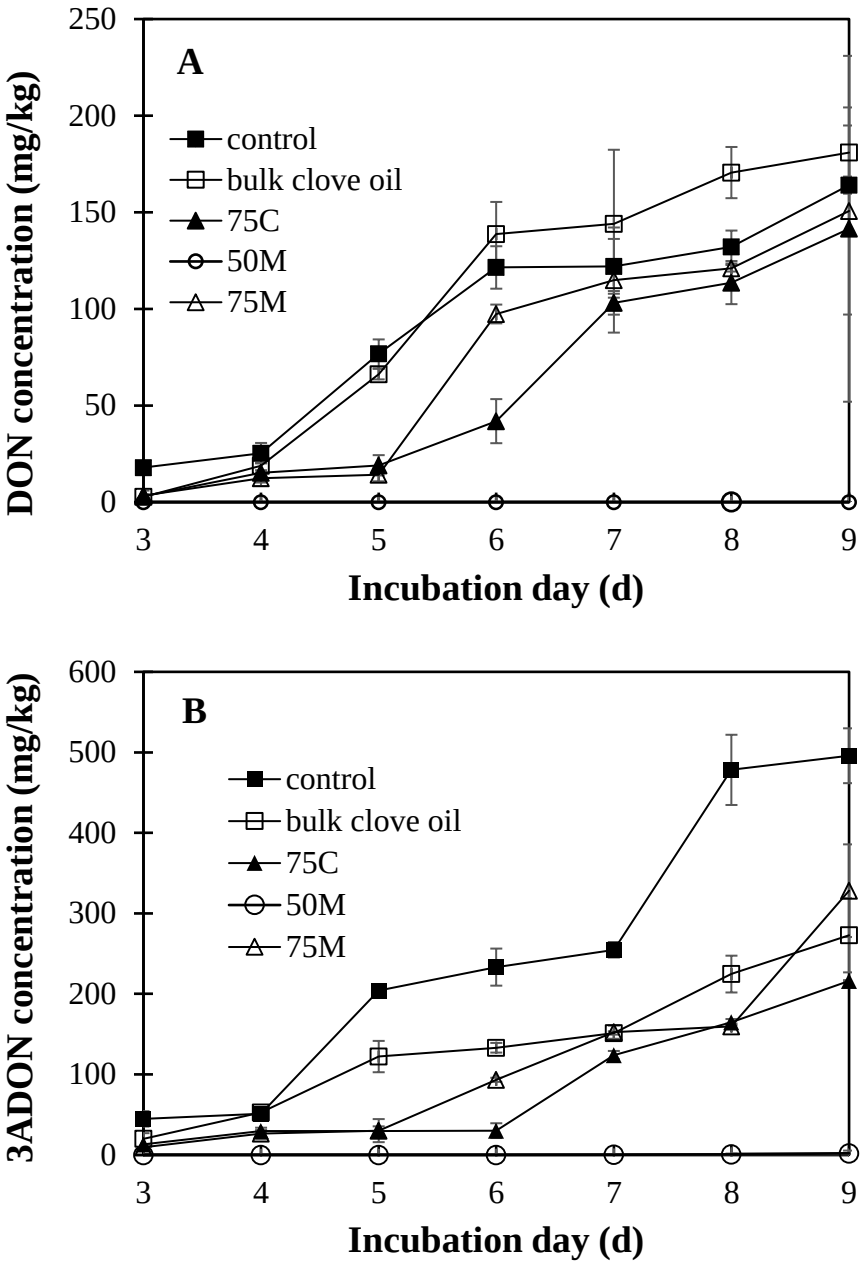


Figure 1-7. Mycotoxins production behavior of *Fusarium graminearum* isolates in rice culture during 9 days of incubation after treatment with different clove oil-in-water nanoemulsions. (A) DON produced from isolate F8-1; (B) 3ADON produced from isolate F8-1; (C) DON produced from isolate 10-124-1; (D) 15ADON produced from isolate 10-124-1.

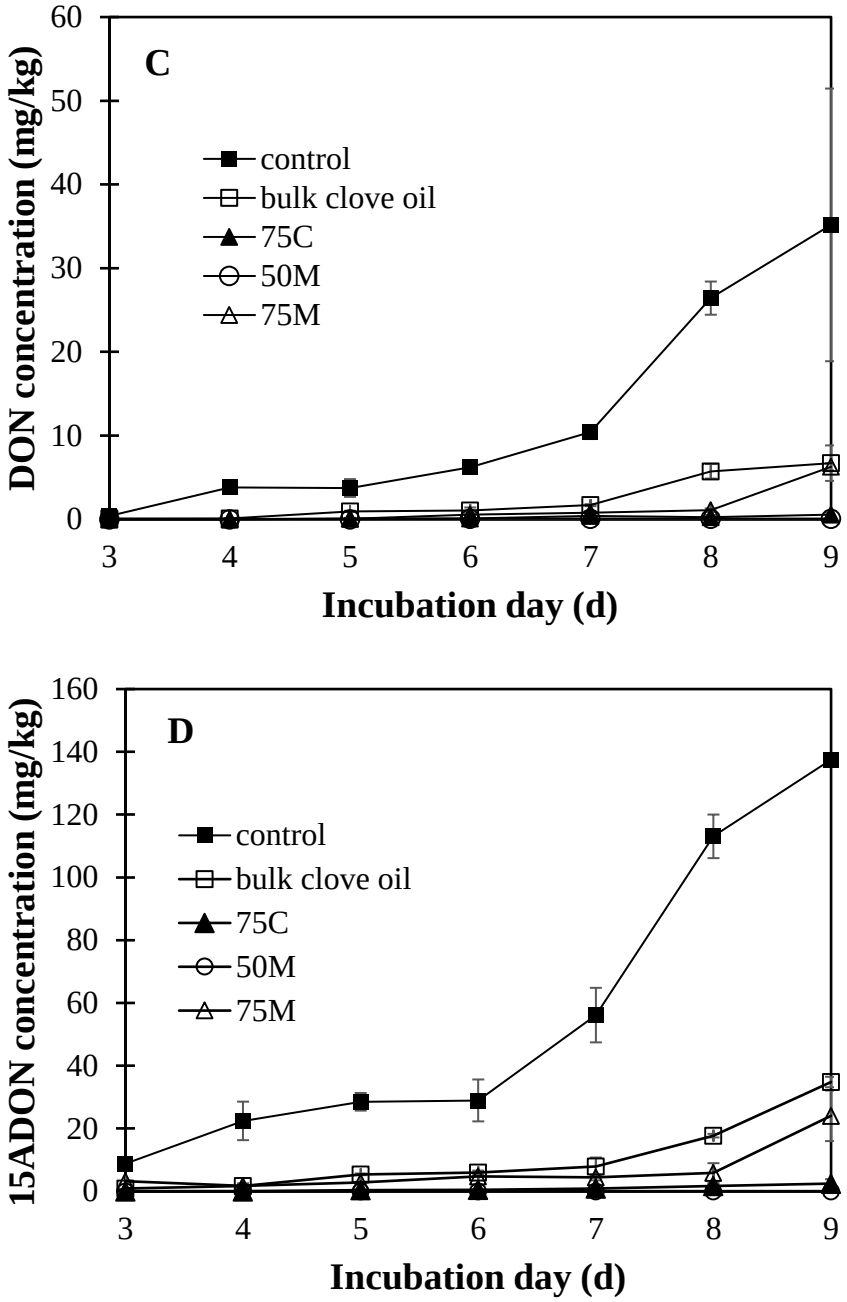


Figure 1-7. Mycotoxins production behavior of *Fusarium graminearum* isolates in rice culture during 9 days of incubation after treatment with different clove oil-in-water nanoemulsions (continued). (A) DON produced from isolate F8-1; (B) 3ADON produced from isolate F8-1; (C) DON produced from isolate 10-124-1; (D) 15ADON produced from isolate 10-124-1.

In general, all clove oil-in-water nanoemulsions showed an inhibition on mycotoxins production over incubation times. The oil phase composition (i.e., ripening inhibitor type and clove oil concentration) had an appreciable influence on the mycotoxin inhibition of *Fusarium graminearum* isolates. The inhibitory activity of clove oil-in-water nanoemulsions increased with increasing clove oil concentrations (50M > 75M). At the same concentration of clove oil in oil phase (75C and 75M), clove oil-in-water nanoemulsions with corn oil as ripening inhibitor in oil phase performed stronger inhibitory activity on mycotoxins production than those with MCT in oil phase for both fungal isolates studied (Fig. 1-7). The possible reason for this phenomenon was again due to the higher amount of clove oil being physically trapped in MCT than in corn oil at the same initial clove oil concentration. As a result, the existence of MCT in oil phase causes a bigger reduction in the mycotoxin inhibitory effect of clove oil than that of corn oil. Furthermore, mycotoxin inhibition of bulk clove oil (700 mg clove oil/kg rice) was significantly lower than that of nanoemulsions with equivalent amount of clove oil. For example, a complete inhibition (100%) of all three mycotoxins were achieved in the two isolates across the entire incubation time with the addition of 50M nanoemulsions bearing 700 mg clove oil/kg rice; however, DON was only reduced by ~ 80% in *Fusarium graminearum* isolate F8-1 upon the addition of 700 mg bulk clove oil /kg rice after 9 days incubation (Fig. 1-7C). Interesting, it is also noticed that bulk clove oil had shown some inhibition of DON production in the first 5 days of incubation, after which it promoted the production of DON in *Fusarium graminearum* isolate 10-124-1 (Fig. 1-7A). Presumably, the nanoemulsion systems would be useful to increase the stability and solubility of clove oil in the rice culture medium to further control the release of bioactive components in clove oil during incubation, resulting in the extending of mycotoxin inhibition effect.

Among the two *Fusarium graminearum* isolates, isolate 10-124-1 was more sensitive to the action of three types of clove oil-in-water nanoemulsions in comparison with isolate F8-1 towards the inhibition of mycotoxins production. For *Fusarium graminearum* isolate 10-124-1, both DON and 15ADON level gradually increased during the first 6 days of incubation, and subsequently increased to high levels in control group. With addition of clove oil either in bulk oil form (700 mg clove oil/kg rice) or in nanoemulsion forms (50M, 75C and 75M), a strong inhibition of both DON and 15ADON production were observed (Fig. 1-7C & D). For example, only 0.55±0.24 mg/kg level of DON produced in *Fusarium graminearum* isolate 10-124-1 by adding 75C nanoemulsions after 9 days of incubation (Fig. 1-7C). In contrast, DON was completed suppressed by 50M nanoemulsions containing 50 wt % MCT (700 mg clove oil/kg rice) in oil phase, while for 75C nanoemulsions containing 75 wt % corn oil (350 mg clove oil/kg rice) in oil phase, over 140 mg/kg level of DON could be detected after 9 days of incubation for *Fusarium graminearum* F8-1 (Fig. 1-7A). Similar trend was observed in the inhibition of 3ADON (Fig. 1-7B). On the basis of the results described above, it is clear that clove oil-in-water nanoemulsions exerted higher inhibitory activity on mycotoxins production of *Fusarium graminearum* isolates 10-124-1 and F8-1 than bulk clove oil under the same concentration.

Some of possible mechanisms behind the effects of EOs on fungal growth and mycotoxin production against fungal pathogen have been proposed. For instance, it is suggested that the fungi respond by limiting secondary metabolite mycotoxins production with respect to the stress induced by certain active compounds from EOs such as phenolic compounds (da Cruz Cabral et al., 2013). A recent study has also indicated that EOs components decreased the mRNA levels encoding proteins in fungi, which is prerequisite for DON biosynthesis (Yaguchi et al., 2009). The mechanism of bulk clove oil to inhibit the growth and mycotoxins production of *Fusarium*

graminearum isolates 10-124-1 and F8-1 may be largely due to the abovementioned reasons because bulk clove oil contained bioactive phenolics eugenols. The striking enhancement of antifungal and detoxification activity of clove oil when incorporated in nanoemulsions could be attributed to the increased solubility and controlled release of clove oil.

Conclusion

Physical stable clove oil-in-water nanoemulsions could be fabricated by mixing clove oil with either ≥ 50 wt % of MCT or ≥ 75 wt % of corn oil. The particle size of clove oil-in-water nanoemulsions containing appropriate amount of ripening inhibitors such as MCT or corn oil remained stable during 30 days storage under 4 and 25 ℃. The ripening inhibitor type and concentration had a remarkable influence on antifungal activity and mycotoxins inhibitory activity of the clove oil-in-water nanoemulsions. In general, the incorporation of corn oil in oil phase enhanced antifungal activity and mycotoxins inhibitory activity of clove oil-in-water nanoemulsions compared to MCT. The antifungal activity and mycotoxins inhibitory effect decreased with increasing ripening inhibitor levels in oil phase. Our study has also shown that nanoemulsions based delivery system substantially increased the mycotoxins inhibitory activity of the clove oil-in-water nanoemulsions containing 350 mg clove oil/kg rice was required for a complete inhibition of mycotoxins production from *Fusarium graminearum*, whereas a double concentration (i.e., > 700 mg clove oil/kg rice) of bulk clove oil was needed to achieve the same efficacy. This effect may be attributed to: (i) the nanoemulsion based delivery systems could significantly increase the stability and solubility of the essential oil in medium; (ii) the controlled release of essential oil bioactive constituents in rice culture medium, thus extending the mycotoxin inhibitory activity. The results reported in this study have important implications for the design and utilization of nanoemulsions as effective antifungal and efficient detoxification

delivery systems in food or other industries. For example, essential oils often have a strong flavor profile. The addition of low concentrations of the nanoemulsions encapsulated essential oils might be able to completely inactivate fungi and mycotoxins production, while minimizing the impact on the organoleptic properties of the foods.

CHAPTER 2. PHYSICAL PROPERTIES, ANTIFUNGAL AND MYCOTOXIN INHIBITORY ACTIVITIES OF FIVE ESSENTIAL OIL-IN-WATER NANOEMULSIONS: IMPACT OF OIL COMPOSITION AND PROCESSING PARAMETERS

Abstract

The influence of homogenization condition on selected essential oil (thyme, lemongrass, cinnamon, peppermint, and clove)-in-water nanoemulsion formation and stability was investigated. Physically stable essential oil-in-water nanoemulsions could be fabricated by microfluidizer under optimized processing conditions (68.95 MPa and 2 passes). The chemical compositions of EOs was characterized using GC-MS. The antifungal activity and mycotoxin inhibitory activity of essential oils in both bulk and nanoemulsion forms were determined using two isolates of *Fusarium graminearum*. Major chemical components of essential oil had a remarkable impact on long term physical stability, antifungal activity, and inhibition of mycotoxin production. With regard to inhibition of mycotoxin production, the mycotoxin inhibitory activity of essential oils was enhanced considerably in nanoemulsion form, which was attributed to greater solubility of the essential oils. It was also noticed that the same essential oils exhibited significant difference in inhibition of mycotoxin production in the two isolates of *Fusarium graminearum*.

Introduction

Cereals are important sources of nutrients for human food and animal feed worldwide. In terms of food safety, cereal based food can be contaminated by toxic secondary metabolites produced by a range of fungal species in the field. Molds and mycotoxins are a dominant reason for the food loss and have caused the contamination of up to 25 % of the world's harvested cereal

grains (Marin, Ramos, Cano-Sancho, & Sanchis, 2013). The genus *Fusarium* and pathogens such as *Fusarium graminearum* causes devastating diseases on cereal grains including wheat, barley, and corn (Kazan, Gardiner, & Manners, 2012). The mycotoxins deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), and 15- acetyldeoxynivalenol (15ADON) are common contaminants of cereal products arising from *Fusarium graminearum* infected cereal grains. As these mycotoxins are relatively stable under the common food processing conditions, they can be transferred into the final products(Voss & Snook, 2010). For example, *Fusarium* mycotoxins (DON) can be transferred from *Fusarium spp.* contaminated barley grains to malt and then to the finished beer (Schwarz, 2017). With regard to human health, DON can cause both acute and chronic toxicity when the contaminated grains are consumed (Bhat, Rai, & Karim, 2010). The US Food and Drug Administration (FDA) has established DON Advisory Levels to ensure the safety of food and feed products (FDA, 2010).

The application of antifungal preservatives to inhibit mold growth and mycotoxin production during food process has been considered as one of the effective strategies for improving the safety of the food supply chain. Natural antifungal agents such as essential oils (EOs) have received increasing attention such as to be as potential alternatives to combat fungi (Hyldgaard, Mygind, & Meyer, 2012; Prakash, Singh, Kedia, & Dubey, 2012). EOs are naturally occurring ingredients that are concentrated from plants including flowers, buds, leaves and bark. Typically, they are complex mixtures of volatile substances including terpenes, terpenoids, and phenols. In addition, EOs, such as clove oil, thyme oil, cinnamon oil, lemongrass oil, and peppermint oil, have been reported to possess a broad spectrum of antifungal properties and as well mycotoxin inhibitory activities (Bluma & Etcheverry, 2008; Xing, et al., 2014). Despite the potential application of EOs as natural antifungal agent, the direct incorporation of the EOs in

food has been limited by several factors. First, the antifungal efficacy of EOs mainly depends on the targeted fungi and chemical composition of the EOs which varies in different oil types. Previous research reported that variable chemical composition was found within the same oil type but different geographic regions or using different extraction methods (Stević et al., 2014). Second, lower water solubility, high volatility, and lower antifungal activity in food matrices can also diminish the applications. Thus, a number of researchers have investigated the incorporation of EOs into appropriate delivery systems for improving their water solubility and antifungal efficacy in foods (Hill, Gomes, & Taylor, 2013; Salvia-Trujillo et al., 2015; Zhu, Zhang, Tian, & Chu, 2018). Among the delivery systems, there is a growing interest in the development of EO-in-water nanoemulsion due to its greater physical stability than conventional emulsion, as well as its potency to increase antifungal activity of EOs rather than applying bulk EOs on foods directly (Donsì et al., 2011).

Generally, nanoemulsions consist of two immiscible liquids, with one of the liquids being dispersed as nano-sized particles (20 nm- 200 nm) in another (McClements & Rao, 2011). Previous results have shown that the antimicrobial and antifungal activity of EO-in-water nanoemulsion is considerably improved over that of bulk EOs due to the reduced particle size resulting in an increased total surface area of particles (Donsì et al., 2011; Wan, Zhong, Schwarz, Chen, & Rao, 2018). In general, two main methods have been developed to form nanoemulsions, high energy and low energy approaches. High energy approaches (e.g., high pressure microfluidization) have the capability to generate intense disruptive forces that breakup the oil phase into tiny oil droplets during homogenization processing. The final smaller particle size of EO-in-water nanoemulsions achieved by microfluidization is important because it determines the stability and functional performance (e.g., antifungal activity and mycotoxin inhibitory activity)

of the EO-in-water nanoemulsions. Previous studies have shown that the formation and stability of the minimum size of the nanoemulsion using the high energy approach depends on homogenizer operating conditions (e.g., pressure and pass time) and sample composition (e.g., oil type and concentration) (Qian & McClements, 2011).

The objectives of the current research were to develop physically stable nanoemulsions containing five EOs including clove, lemongrass, thyme, peppermint, and cinnamon oils, and then to evaluate their antifungal efficacy and mycotoxin inhibitory activity. Our hypothesis was that homogenizer operating conditions would impact the physical stability of EOs-in-water nanoemulsions. In addition, the chemical compositions of EOs would determine both antifungal and mycotoxin inhibitory activities of EOs-in-water nanoemulsions. The influence of number of passes, homogenization pressure, and EOs type on the formation and stability of EO-in-water nanoemulsions using a microfluidizer were investigated. The effect of five encapsulated essential oils on inhibition of *Fusarium graminearum* growth and mycotoxin production, including DON, 3ADON, and 15ADON were also assessed. The results of this study have important implications for the design and utilization of EO as antifungal agents and mycotoxin inhibitors in the food industry.

Materials and methods

Materials

Thyme oil (FEMA # 3065, FG grade), peppermint oil (FEMA # 2848, FG grade), clove oil (FEMA # 2323, FG grade), cinnamon oil (FEMA # 2292, FG grade), and lemongrass oil (FEMA # 2624, FG grade), Polyoxyethylene (20) sorbitan monooleate (Tween 80), Mirex and Bis(trimethylsilyl)acetamide (BSA)/trimethylchlorosilane (TMCS)/Trimethylchlorosilane (TSIM) kit were purchased from Millipore Sigma Co. (St. Louis, MO, U.S.). Medium chain

triacylglyceride (MCT, NEOBEE M-5) was kindly donated by the Stepan Company (Bordentown, NJ, U.S.). The manufacturer reported that the fatty acid profile of MCT was 50-65% caprylic acid (C8:0) and 30-45% of capric acid (C10:0). Potato dextrose agar (PDA) was purchased from AMRESCO (Solon, OH, U.S.). Potato dextrose broth was purchased from BD Biosciences (Franklin Lakes, NJ, U.S.). Mung beans and long grain white basmati rice were obtained from local market (Fargo, ND, U.S.). All solutions were prepared using ultrapure distilled de-ionized water (DDW, 18.2 MΩ cm, Barnstead ultrapure water system, Thermo Fisher Scientific, U.S.).

Nanoemulsion preparation

In total, five EOs including clove, lemongrass, thyme, peppermint, and cinnamon, were selected for nanoemulsion formation. With the exception of oil type, the formula for the nanoemulsion remained the same. The coarse nanoemulsion was prepared by mixing 0.5 wt % Tween 80, 5 wt % of total oil phase, and 94.5 wt % phosphate buffer solution (10 mM, pH 7.0), and then mixed using a high-speed blender (Model 985370, Biospec Products, Bartlesville, OK, U.S.) for 2 min. The oil phase was prepared by mixing 50 wt % of EO (clove, cinnamon, thyme, peppermint, and lemongrass oil) and 50 wt % MCT at ripening inhibitor to prevent the Ostwald ripening based on the results from Chapter 1. The resulting coarse EO-in-water nanoemulsion was then passed through a LM20-20 high pressure Microfluidizer (Malvern Instruments, Worcestershire, UK) at four homogenization pressures (34.47, 68.95, 103.42, and 137.90 MPa) for three different number of passes (1, 2 and 3 times). The finished nanoemulsions (0.5 wt% Tween 80, 2.5 wt % of EO, 2.5 wt % of MCT, and 94.5 wt % of buffer) were stored in test tube sealed with the screw cap. The samples were stored at 4 °C and 25 °C for 30-days as part of a

long-term storage stability study. According to this formula, the total concentration of each EO in nanoemulsion was 25 mg/g.

Particle size measurements

The mean particle diameter (Z-averages) of nanoemulsions were measured at 0, 1, 2, 3, 4 ,5 ,6, 7, and 30-day using a dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK). The data were reported as the mean particle diameter and particle size distribution.

Chemical composition analysis of five essential oils

The chemical composition of the five EOs were analyzed using Agilent 7890B/5977A GC-MS system. The sample (1.0 μl) was injected with a PAL RSI 120 at 250 °C with a split ratio 5:1. Volatiles were separated on a ZB-Wax column (60 m × 0.25 mm i.d., 0.25 μm film thickness). Oven temperature program was set as: initial temperature 40 °C held for 1 min, followed by a rate of 4 °C/min to 250 °C, and then held for 10 min. Helium carrier gas was used at a flow rate of 1 mL/min. The column effluent was transferred into MS detector that was operated in electron impact (EI) ionization mode at 70 eV. Ion source temperature was 230 °C. Scan time segments were set from 40 to 350 amu.

Raw data acquired by GC-MS were analyzed using Agilent MassHunter Unknown Analysis (B.07.01) coupled with MS libraries NIST 14. Absolute area > 5000 counts and min match score > 80 were the main criteria applied for validating the entities and filtering the target compounds. In addition, the retention index (RI) of each compound was calculated using a series of *n*-alkanes (C8-C40). Calculated RI was matched with the reference value according to NIST database (https://webbook.nist.gov) to further confirm the compound.

Determination of antifungal activity using effective concentration (EC)

Two *Fusarium graminearum* isolates 10-124-1 and 10-125-1 were employed to evaluate the antifungal efficiency of five EO-in-water nanoemulsions. *Fusarium graminearum* 10-124-1 belongs to the 15-ADON chemotype, which mainly produces DON and 15ADON; whereas *Fusarium graminearum* 10-125-1 belongs to the 3ADON chemotype mainly for DON and 3ADON production. Both isolates were stored at -80 ℃ and refreshed on potato dextrin agar (PDA) media for hyphal growth. A round PDA plug (diameter of 0.5 cm) with freshly growing mycelium was incubated on a new PDA cultural plate treated with EOs-in-water nanoemulsions for detection. The antifungal efficiency of five EOs incorporated in nanoemulsions were determined by measuring the EC values against the *Fusarium graminearum* isolates as previously described by Wan, Zhong, Schwarz, Chen, & Rao (2018). All the EOs-in-water nanoemulsions were diluted with phosphate buffer (10 mM and pH 7) to create desired gradient EO concentrations in the final nanoemulsions. The concentration gradients for the peppermint oil-in-water nanoemulsion were 0, 2.5, 5, 10, 15, 20, 25 mg/g, and 0, 2, 4, 6, 8, 10 mg/g for the thyme, lemongrass, clove, and cinnamon oil-in-water nanoemulsions, respectively. Each treatment was replicated at least twice. The mycelial growth inhibition (MGI) rate was calculated as MGI rate (%) =100 × (mycelial diameter of control colony – mycelial of treatment colony) / (mycelial diameter of control colony). MGI rate was fitted to cubic regression model and the EC values were calculated. For example, EC_{50} was when a MGI rate of 50% was observed.

Impact of nanoemulsions on mycotoxin production in rice culture

Preparation of Fusarium graminearum macroconidia suspension

Macroconidia suspensions of the *Fusarium graminearum* isolates 10-124-1 and 10-125-1 were prepared as previously described by Wan, Zhong, Schwarz, Chen, & Rao (2018). The

concentration of macroconidia was measured by hemacytometer (Levy Ultraplane, PA, U.S.) and adjusted to 1.0×10^6 spores/mL with sterile carboxymethyl cellulose (CMC) media.

Preparation of rice culture for mycotoxin production

Mycotoxins were produced in solid rice culture according to Puri and Zhong (2010) with some modifications. Briefly, long grain rice was soaked in deionized water for 10 h in a beaker and the excess water was then decanted off. Twenty grams of the soaked kernels were loaded into 125 mL Erlenmeyer flask and autoclaved at 121 °C for 1 h. After cooling to 25 °C, the sterile rice culture was inoculated with the mixture consisting of 1 mL of macroconidia solution at 1.0×10^6 spores/mL and 1 mL variable EO treatment (i.e., bulk EOs, EOs-in-water nanoemulsions, and 10 times diluted EOs-in-water nanoemulsions). Rice culture treated with the same amount of autoclaved water was considered as a control treatment. For bulk essential oil group, 25 mg essential oil was mixed with 975 μl water, and then with 1 mL macroconidia suspension. The final EO concentrations in rice culture were 1250, 1250, and 125 mg/kg rice for the treatment of bulk EOs, EO-in-water nanoemulsions, and 10 times diluted EOs-in-water nanoemulsions, respectively. Each treatment had three replicates and incubated at 25 °C for 5 days.

Detection of mycotoxins in rice culture by GC-MS

The method reported by Wan and coworkers (2018) was followed to extract and quantify mycotoxins (DON, 3ADON, and 15ADON) using GC-MS.

Statistical analysis

All measurements were carried out at least twice using freshly prepared samples (i.e., fresh samples were prepared for each series of experiments) and reported as mean ± standard

deviation. One-way analysis of variance (ANOVA) was conducted and Tukey's test was used to define significant difference of mean value at the 5% of probability level (MINITAB 18.0).

Results and discussions

Chemical composition of the essential oil

The chemical composition of EOs is influenced by extraction method, cultivar, plant tissue, the environment, and geographical origin. Knowing the chemical composition of the EO is critical to understand their other activities. Therefore, the chemical constituents representing more than 1% of the total oil (calculated as % peak area), along with their water solubility at 21 °C are listed in Table 2-1.

Table 2-1. Major chemical constituents and water solubility of thyme, lemongrass, cinnamon, peppermint, and clove oils. Water solubility of each compound was obtained from the data banks of SciFinder (www.scifinder.cas.org), ChemSpider (www.chemspider.com) and Pubchem (https://pubchem.ncbi.nlm.nih.gov/).

NO.	Compounds	Persentage (%)	Water Solubility (mg/L)
Thyme oil			
1	Thymol	21.69	900.00
2	p-Cymene	21.36	23.40
3	γ-Terpinene	13.87	3.62
4	Linalool	10.08	1590.00
5	α-Pinene	3.90	1.70
6	Caryophyllene	3.85	<0.01
7	Terpinen-4-ol	3.27	81.10
8	D-Limonene	3.06	7.57
9	Endo-Borneol	3.02	738.00
10	Caryophyllene oxide	2.45	<0.01
11	Eucalyptol	2.22	3.80
12	α-Terpineol	1.26	70.70
Lemongrass oil			
1	β-Citral	31.33	<0.01
2	α-Citral	14.65	1.34
3	D-Limonene	5.85	7.57
4	Geraniol	5.17	100.00
5	Geranyl acetate	4.15	<0.01
6	6-Methyl-5-hepten-2-one	2.75	3.02
7	Endo-Borneol	1.90	738.00
8	Caryophyllene	1.84	<0.01
9	Linalool	1.76	1590.00
10	Citronellal	1.69	70.20
11	Camphene	1.62	4.60
12	Humulene	1.54	0.01
13	Caryophyllene oxide	1.49	<0.01
14	Trans-Isoeugenol	1.38	810.00
15	Rosefuran	1.29	17.32
16	β-Cedrene	1.18	0.15
17	α-Pinene	1.02	1.70

Table 2-1. Major chemical constituents and water solubility of thyme, lemongrass, cinnamon, peppermint, and clove oils (continued). Water solubility of each compound was obtained from the data banks of SciFinder (www.scifinder.cas.org), ChemSpider (www.chemspider.com) and Pubchem (https://pubchem.ncbi.nlm.nih.gov/).

NO.	Compounds	Persentage (%)	Water Solubility (mg/L)
Cinnamon oil			
1	Eugenol	37.13	2460.00
2	Caryophyllene	9.87	<0.001
3	Benzyl Benzoate	9.30	15.40
4	Linalool	6.98	1590.00
5	Safrole	4.86	121.00
6	Cinnamaldehyde	4.74	<0.01
7	o-Cymene	3.82	23.30
8	α-Phellandrene	3.63	<0.01
9	α-Copaene	3.09	1.80
10	Caryophyllene oxide	2.30	<0.01
11	D-Limonene	1.39	7.57
12	Camphene	1.31	<0.01
13	β-Pinene	1.03	4.89
Peppermint oil			
1	Menthol	23.54	420.00
2	L-Menthone	13.07	688.00
3	Eucalyptol	7.94	3500.00
4	dl-Menthol	5.82	456.00
5	L-Menthol	4.90	490.00
6	Menthofuran	4.69	73.00
7	Caryophyllene	3.83	<0.01
8	D-Limonene	3.48	7.57
9	D-germacrene	2.28	0.01
10	Terpinen-4-ol	2.15	81.10
11	β-Pinene	2.10	4.89
12	Pulegone	2.06	276.00
13	α-Pinene	1.70	2.49
14	p-Cymene	1.41	23.40
15	β-Bourbonene	1.13	2.40
16	γ-Terpinene	1.06	8.68
Clove oil			
1	Eugenol	34.42	2460.00
2	Eugenol acetate	24.53	<0.01
3	Caryophyllene	21.30	<0.01
4	α-humulene	5.84	0.13
5	Caryophyllene oxide	2.42	<0.01

The number of volatiles components were identified in each EO ranging between 7 and 22, comprising 80.6–90.0% of the total EO volatile detected constituents. In general, any chemical compound accounting for above 5 % of the total oil is considered as a major chemical constituent. From Table 2-1, it can be seen that peppermint oil is particularly rich in terpene alcohol (menthol), which accounted for 29.4%, of the total, followed by L-menthone, and eucalyptol. Cinnamon and clove oils share some similar major chemical constituents such as eugenol and caryophyllene. The major types of components in cinnamon oil included phenols

(eugenol), sesquiterpenes (caryophyllene), esters (benzyl benzoate), and terpene alcohol (linalool), whereas phenol (eugenol), sesquiterpene (caryophyllene, α-humulene), and ester (eugenol acetate) were dominant in clove oil. Thyme and lemongrass oils are high in the content of terpenoids, monoterpene, and terpene alcohol. Thyme oil is mainly composed of thymol, p-cymene, γ-terpinene, and linalool. In lemongrass oil the major components include β-citral, α-citral, D-limonene, and geraniol. Similar chemical constituents of five EOs have be reported, albeit to different proportions (Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007).

Impact of homogenization conditions on mean particle size of EO-in-water nanoemulsion

In general, pure EO-in-water nanoemulsions are highly unstable due to Oswald ripening (OR), but stability can be increased by mixing with nonpolar medium or long chain triacylglyceride oils. Based on our previous work (Wan, Zhong, Schwarz, Chen, & Rao, 2018), 50% of the oil phase in the current study was MCT , which was included as a study as OR inhibitor. The impact of microfluidization pressure (34.47, 68.95, 103.42, and 137.90 MPa) and the number of passes (1, 2, and 3 times) through the microfluidizer on the mean particle size (Table 2-2) and particle size distribution (Fig. 2-1) of EO-in-water nanoemulsions was evaluated.

Table 2-2. Droplet diameter (nm) of 5 wt % EOs-in-water nanoemulsions produced by microfluidization at different processing pressures and number of passes (2.5 wt % EOs, 2.5 wt % MCT, 0.5 wt % Tween 80, 10 mM phosphate buffer, pH 7). Values are reported as the mean ± standard deviation. For each EOs-in-water nanoemulsion type, values in each column with capital letters indicate differences among cycles, while means in each row with lower case letters indicate differences among pressures. Values in the same column or row do not share a same letter are significantly different (p<0.05).

Essential oil type	Pass	Pressure (MPa)			
		34.47	68.95	103.42	137.90
		……d.nm……			
Thyme oil	1	151.67±8.96 ABCD	142.00±8.06 ABCD	163.48±2.85 AB	173.94±0.62 A
	2	122.30±5.61 BCD	112.47±14.09 D	145.13±10.04 ABCD	161.97±18.90 ABC
	3	118.4±6.27 CD	109.66±13.12 D	136.60±15.88 ABCD	159.53±15.28 ABC
Lemongrass oil	1	157.22±17.94 A	121.72±18.55 A	121.22±10.73 A	119.58±25.25 A
	2	134.00±1.27 A	111.48±15.44 A	117.87±12.92 A	114.37±22.86 A
	3	126.87±1.13 A	105.40±13.44 A	108.93±9.76 A	108.44±11.49 A
Cinnamon oil	1	130.79±17.23 A	112.02±3.23 AB	108.66±11.38 AB	113.27±0.52 AB
	2	101.71±1.97 AB	91.35±1.65 B	90.11±13.15 B	102.53±1.93 AB
	3	103.35±3.18 AB	93.07±1.64 B	88.57±12.47 B	101.71±3.47 AB
Peppermint oil	1	173.45±6.43 A	142.45±14.73 AB	138.82±12.85 AB	140.08±12.24 AB
	2	154.53±2.55 AB	131.47±11.45 AB	131.60±19.66 AB	128.69±11.33 AB
	3	145.17±5.71 AB	117.42±7.80 B	118.69±14.16 B	117.37±12.73 B
clove oil	1	116.68±7.29 A	106.25±10.16 A	119.02±6.20 A	112.73±12.58 A
	2	96.43±4.57 A	85.64±3.46 C	91.49±0.10 BC	97.78±3.61 ABC
	3	95.42±2.13 ABC	83.91±6.55 C	91.62±0.95 BC	96.60±0.96 ABC

As shown in Table 2-2, mean particle diameter tended to decrease with increasing homogenization pressure from 34.47 to 68.95 MPa and number of passes from one to two was observed. For example, the mean particle diameter of thyme oil-in-water nanoemulsion decreased from 15.67 to 112.47 nm as homogenization pressure and number of passes was increased from 34.47 to 68.95 MPa and one to two respectively. This is in agreement with previous studies (Qian & McClements, 2011; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2013). The reduction of particle size was attributed to the consistently high shear rate generated by microfluidizer. However, no significant reduction in mean particle diameter was observed when pressure was further increased from 68.95 to 137.90 MPa, suggesting that the reduction of average particle diameter reached up to a certain limit as the pressure is beyond certain level with 68.95 MPa in this case. Regarding different EOs such as cinnamon, thyme, and

clove oil, it was found that an increase in microfluidizer pressure beyond 68.95 MPa caused a slight increase in the resultant mean droplet diameter (Table 2-2). Similar results have previously been reported that higher pressures and more pass times during nanoemulsion formation using homogenizer may lead to "over-processing", which leads to an increase in the particle size of nanoemulsion (García-Márquez, Higuera-Ciapara, & Espinosa-Andrews, 2017; Ricaurte, Hernández-Carrión, Moyano-Molano, Clavijo-Romero, & Quintanilla-Carvajal, 2018). This phenomenon can be explained by an enhanced droplet re-coalescence rate than that of droplet disruption rate under higher pressure (Tang, Shridharan, & Sivakumar, 2013). Under the same pressure, mean particle diameter decreased as nanoemulsions were passed through the homogenizer with an increasing number of times, but the further reductions were fairly modest from pass 2 times to 3 times. The mean droplet diameter did not change remarkably after three passes and over 68.95 MPa.

The overall particle size distribution is an important indicator for homogeneity of nanoemulsion droplets. We therefore plotted the change in particle size distribution with number of pass 1 and 2 at 68.95 MPa (Fig. 2-1). In general, the width of the particle size distribution decreased appreciably, and the fraction of large droplets disappeared with increasing number of passes. Monomodal size distributions were observed in all EOs-in-water nanoemulsion with 2 passes at 68.95 MPa. This attributed to the larger droplets that were not disrupted in a single pass submitted to a higher shear rate generated in reaction chamber, thus reducing its size and leading to a more uniform nanoemulsion. In conclusion, homogenization conditions of 68.95 MPa with 2 passes were selected in the remainder of the experiments.

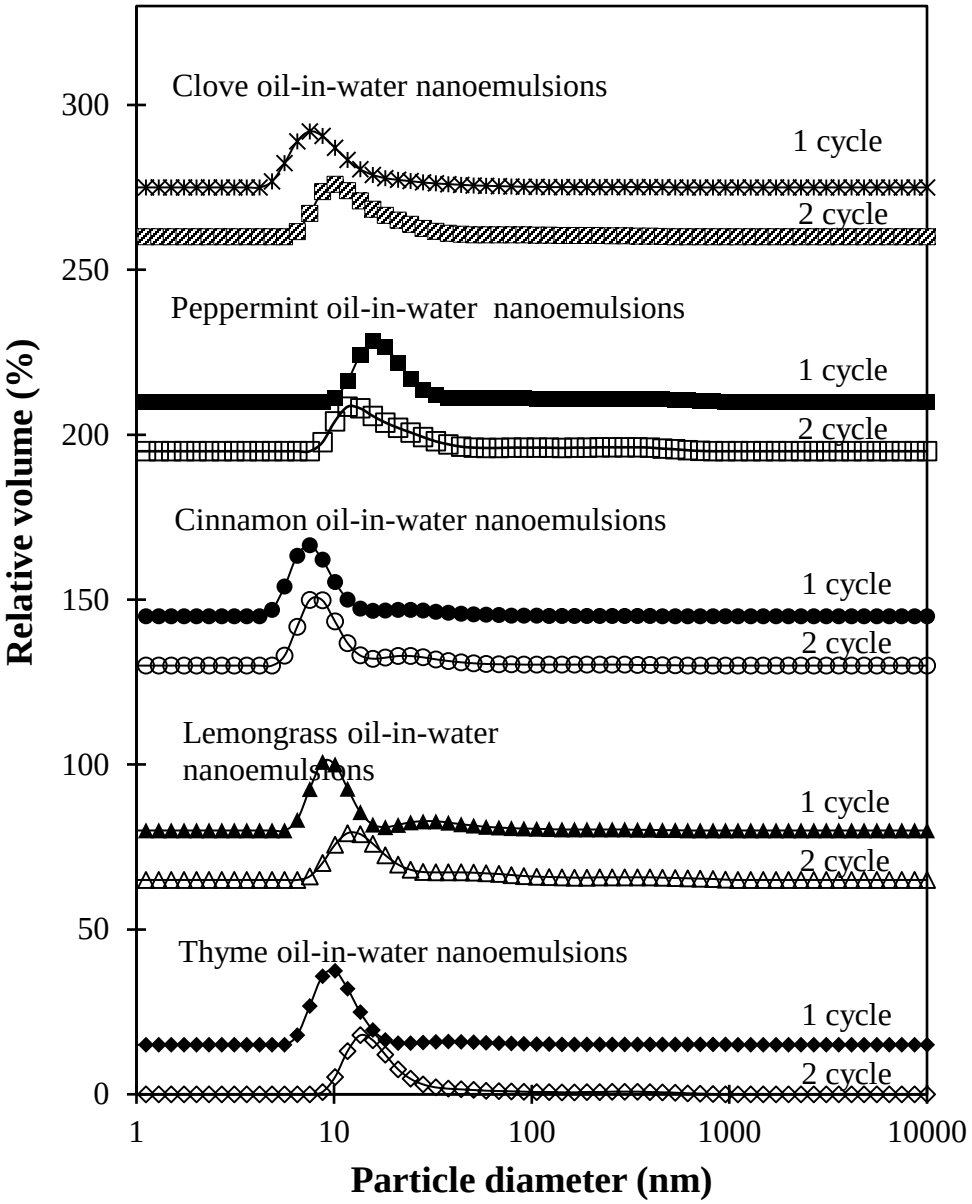
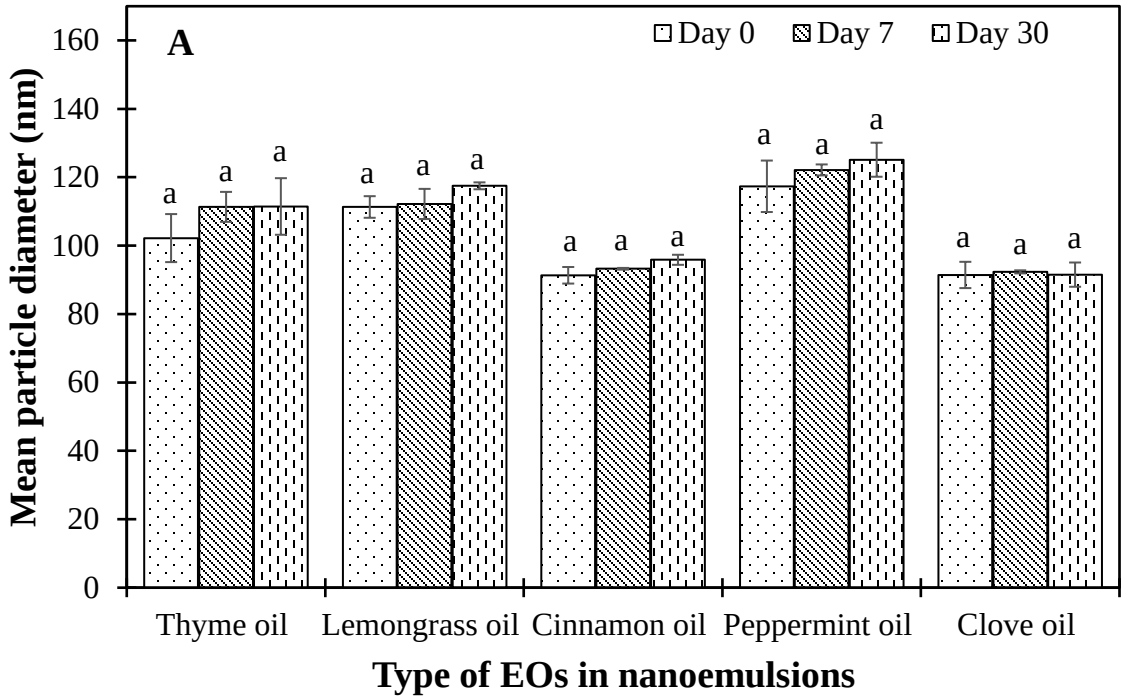


Figure 2-1. Changes in particle size distribution of 2.5 wt % EO-in-water nanoemulsions containing 0.5 wt % Tween 80 with number of passes (68.95 MPa).

Physical storage stability of nanoemulsions

For practical applications, it is essential to ensure the long-term stability of EOs-in-water nanoemulsions. Therefore, changes in the mean particle diameter of five EOs-in-water nanoemulsions were measured over 30 days of storage time at both 4 °C and 25 °C (Fig. 2-2).



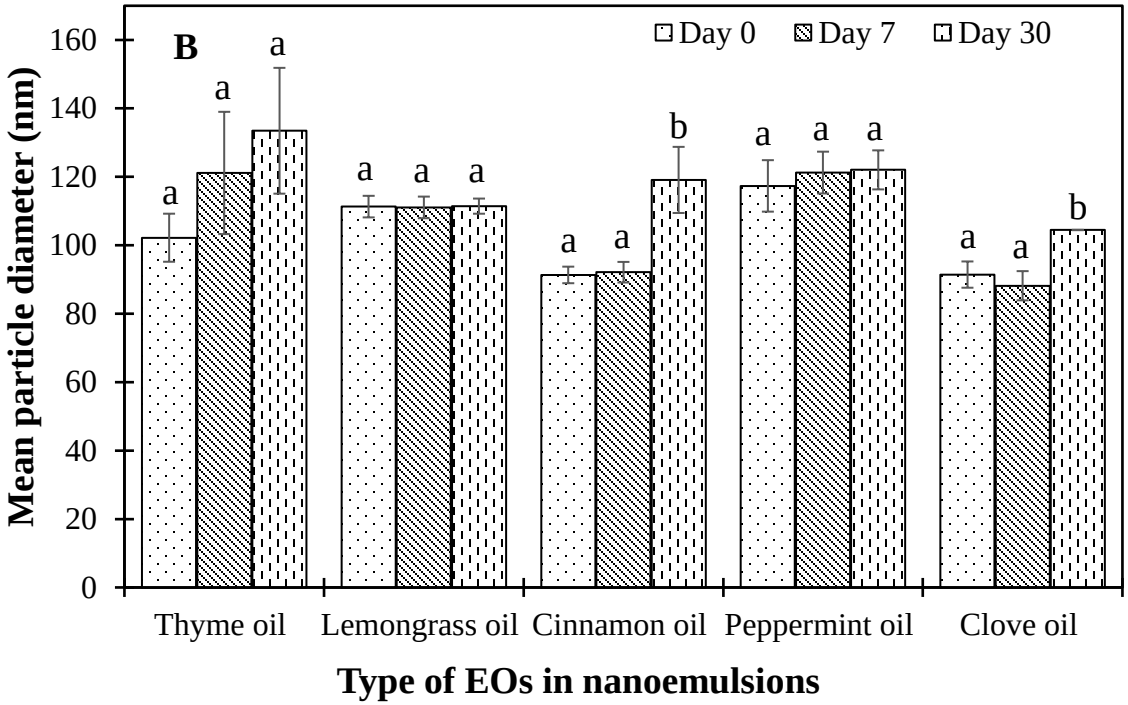


Figure 2-2. Evolution of the mean particle diameter of 2.5 wt % EO-in-water nanoemulsions after 30 days storage time at 4 ℃ (A) and 25 ℃ (B). Columns within an EO type with different letters are significantly different ($p<0.05$).

Overall, the five EOs-in-water nanoemulsions displayed good stability during long-term storage at both 4 and 25 °C. The mean particle diameter of the EO-in-water nanoemulsions still remained in the nanometric range after 30 days storage at both temperatures, and no phase separation or oiling off occurred. At 4 °C (Fig. 2-2A), there was no significant changes (p> 0.05) in mean particle diameter during storage for all the samples, indicating their excellent physical stability. For example, the mean particle diameter of clove oil-in-water nanoemulsion kept the same nanometric size (91.44 nm) after 30 days of storage. Conversely, we observed differences in nanoemulsion stability in terms of oil type stored at 25 °C after 30 days. For example, there was a significant increase (p<0.05) in the mean particle diameter of cinnamon oil-in-water nanoemulsions from 91.33 nm to 119.10 nm (Fig. 2-2B). The thyme, peppermint, and lemongrass oil-in-water nanoemulsions were relatively stable within the whole measurement time at 25 °C. Since the formula of each nanoemulsions was identical except the type of EOs, the observed differences in nanoemulsion stability might be derived from Ostwald ripening and can be explained by the differences in the chemical composition. The change in mean droplet radius (r) with time (t) due to Ostwald ripening of a single-component emulsified lipid in the steady regime can be depicted by the following equation (Rao & McClements, 2011):

$$\omega = \frac{\mathrm{d}r^3}{\mathrm{d}t} = \frac{8}{9}\left(\frac{C_\infty \gamma V_m D}{RT}\right) \quad (1)$$

where, r is the number average droplet radius, t is the storage time, C_∞ is the water solubility of EOs, γ is the interfacial tension, V_m is the molar volume of the lipid, D is the translational diffusion coefficient of the EOs through the water, R is the gas constant, and T is the absolute temperature. EOs with relatively high amount of water soluble compounds owns greater C_∞, thus tending to promote nanoemulsion droplet growth due to Ostwald ripening (Rao & McClements, 2012). In the current nanoemulsion system, eugenol, the major constituent of cinnamon oil

(37.13%) and clove oil (34.42%), has a relatively high water-solubility (≈2460 mg/L), which would account for the increase in droplet diameter observed during storage at 25 °C temperature (Table 2-1). Conversely, the major constitutes of thyme, lemongrass, and peppermint oil have relatively poor water solubility, which may have inhibited droplet growth through a compositional ripening effect. Such results were also reported in different chemical composition of lemon oils in nanoemulsion systems (Rao & McClements, 2012).

The effect of EO type in nanoemulsions on mycelial growth

Antifungal activities of the five EOs-in-water nanoemulsions prepared under optimized homogenization conditions were tested against two chemotype isolates of *Fusarium graminearum* (10-124-1 and 10-125-1) using the agar dilution method. EC value is defined as the concentration of EOs in nanoemulsion that can effectively inhibit the percentage of mycelial growth. Results for antifungal activity of the EOs-in-water nanoemulsions are presented in Table 2-3.

Table 2-3. Antifungal activity of EOs-in-water nanoemulsions against *Fusarium graminearum* isolates on PDA media. Effective concentration (EC) was expressed by the concentration of EO in nanoemulsions. For example, EC_{50} was calculated when isolate mycelia growth was observed in half inhibition rate. Values with lower case letter indicate differences between EOs-in-water nanoemulsions against isolate 10-124-1, while capital letters indicate differences between EOs-in-water nanoemulsions against isolate 10-125-1. Values in the same column do not share a same letter are significantly different (p<0.05)

Essential oil types	*Fusarium graminearum* 10-124-1			*Fusarium graminearum* 10-125-1		
	EC_{50}	EC_{70}	EC_{90}	EC_{50}	EC_{70}	EC_{90}
	……mg g^{-1}……					
Thyme oil	4.34±0.03 a	5.82±0.02 a	7.61±0.09 a	3.75±0.87 A	5.28±0.74 A	7.25±0.43 A
Lemongrass oil	5.80±0.32 b	7.09±0.28 c	8.54±0.21 b	6.70±0.42 B	8.01±0.22 B	9.29±0.04 B
Cinnamon oil	6.71±0.59 b	8.36±0.09 b	9.56±0.14 b	7.22±0.76 B	8.60±0.34 B	9.59±0.06 B
Peppermint oil	14.75±0.07 c	19.57±0.52 d	23.67±0.23 c	15.67±0.39 C	20.17±0.17 C	23.84±0.08 C
Clove oil	6.34±0.23 b	7.86±0.14 bc	9.33±0.03 b	6.48±0.50 B	7.74±0.27 B	8.82±0.89 AB

The five EOs displayed considerable variations in antifungal activity against the two *Fusarium graminearum* isolates. As shown in Table 2-3, the thyme oil-in-water nanoemulsion

had the strongest antifungal activity against mycelium growth of both selected isolates, whereas peppermint oil-in-water nanoemulsion exhibited the lowest antifungal activity among the 5 selected EOs. For instance, the EC_{90} of thyme oil-in-water nanoemulsion against isolate 10-124-1 was 7.61 ± 0.09 mg/g. The EC_{90} of peppermint oil-in-water nanoemulsion (23.672 ± 0.234 mg/g) was 3 times higher than that of thyme oil-in-water nanoemulsion. This phenomenon indicated that chemical composition of individual EO constituents and their concentrations exert a significant impact on the antifungal activity of EOs. General antifungal activity of essential oils components has been reported to be phenol > alcohols > aldehydes > ketones > ethers > hydrocarbons (Dambolena et al., 2008). From Table 2-1, the most prevalent component in peppermint oil was menthol, whereas the most abundant chemical constituent of thyme oil was thymol. Terpenoid phenols such as thymol, exhibit remarkable antifungal activity against a number of genera when compared to menthol (Abbaszadeh et al., 2014; Marchese et al., 2016). The presence of a free hydroxyl group in phenols and a system for electron delocalization is essential for EOs to have antifungal activity. Therefore, it can be deduced that the main component thymol (21.69%) in our studied thyme oil was responsible for the enhanced antifungal activities against *Fusarium graminearum*, which is in agreement with other studies (Dambolena et al., 2008). The antifungal mechanism of phenols includes disruption of ergosterol biosynthesis and membrane integrity, which could affect pH homeostasis and equilibrium of inorganic ions (Ahmad et al., 2011).

The other three EO-in-water nanoemulsions (cinnamon, clove, and lemongrass oil) showed intermediate antifungal activity, and the order of their antifungal efficacy in mycelium growth was slightly different for isolate 10-124-1 as compared to isolate 10-125-1. However, there were no statistically significant differences in terms of EC values amongst the three EOs-

in-water nanoemulsions ($p > 0.05$). The antifungal effects of cinnamon and clove oil-in-water nanoemulsions may result primarily from the main phenol compound (eugenol) as suggested earlier. Indeed, the major chemical components of cinnamon oil and clove oil are quite similar (Table 2-1). Consequently, one would expect that the antifungal activity of cinnamon-in-water nanoemulsions should be fairly similar to that of clove oil-in-water nanoemulsions. Interesting, several studies have shown that clove oil and cinnamon oil, owing to their principle components such as eugenol, cinnamaldehyde, caryophyllene, possessed superior activities against many filamentous fungi when compared to lemongrass oil (Sharma, Rajendran, Srivastava, Sharma, & Kundu, 2017; Sharma et al., 2018). This was not the case in the current study, which might be due to the distinct responses of different fungi to the same EO (Velluti et al., 2003).

The effect of EO type in nanoemulsions on mycotoxins production

The ultimate goal of the present study was to develop a method that would control the production of DON and its derivatives by *Fusarium graminearum* in contaminated cereal grains and other food samples intended for consumption. The inhibitory activity of EOs against *Fusarium graminearum* mycelial growth cannot be directly extrapolated to their inhibitory activities for mycotoxin production. Thus, we examined the impact of EO type, concentrations (1250 and 125 mg/kg rice), and the encapsulation form (bulk and nanoemulsion) on mycotoxin production in rice culture. DON, 3ADON, and 15ADON, produced by *Fusarium graminearum* isolate 10-125-1 and 10-124-1, respectively, were quantified after culturing for 5 days. Results are expressed in mg toxin/kg rice culture (Fig. 2-3). Inoculated rice cultures treated with autoclaved water were used as controls. The final EO concentrations were 1250, 1250, 125 mg/kg rice for bulk EOs, EO-in-water nanoemulsions, and 10× diluted EOs-in-water nanoemulsions, respectively.

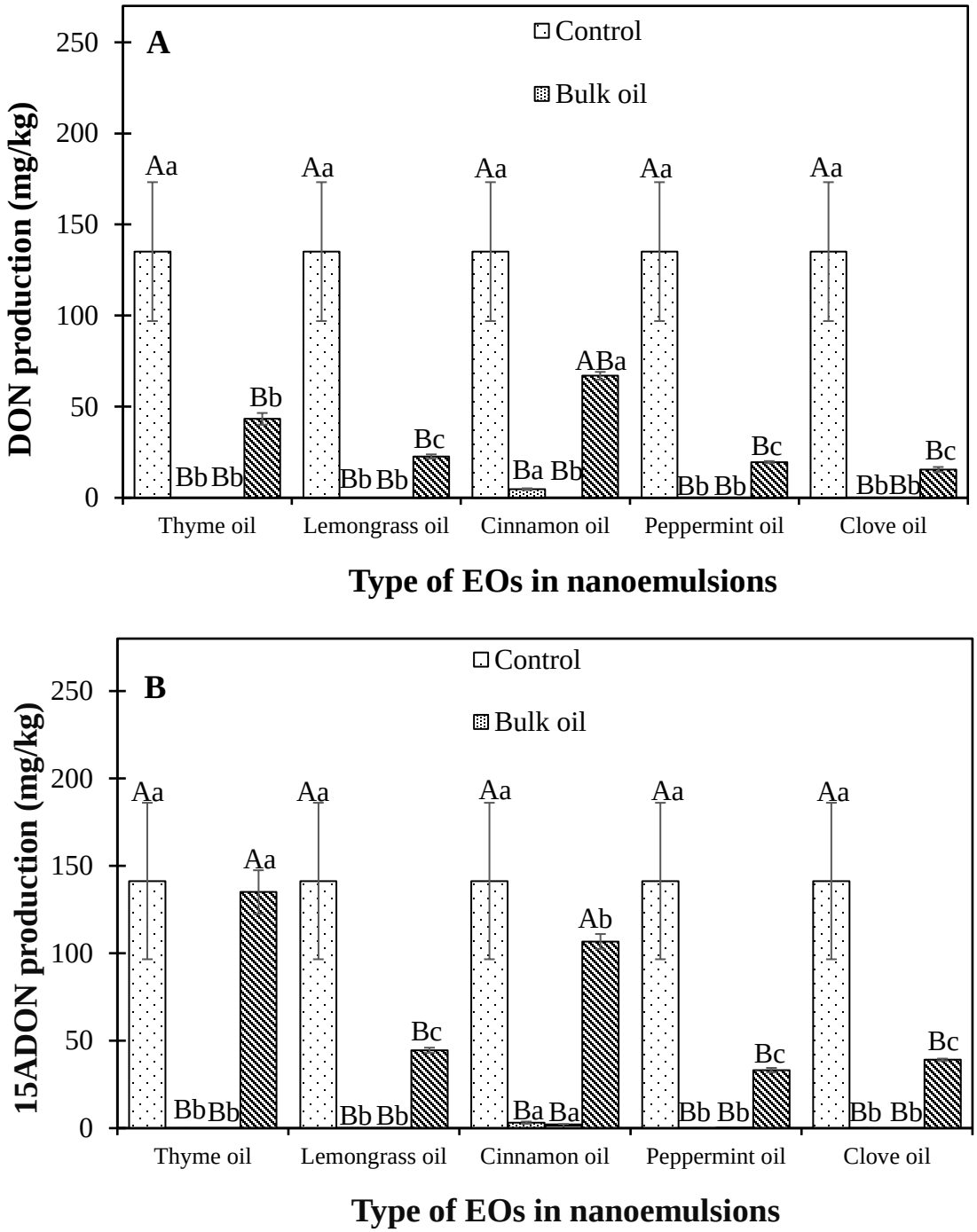


Figure 2-3. Mycotoxins production by *Fusarium graminearum* isolates in rice culture following treatment with EOs-in-water nanoemulsions and EOs and 5 days incubation. (A) DON produced by isolate 10-124-1 (B) 15ADON produced by isolate10-124-1 (C) DON produced by isolate 10-125-1 (D) 3ADON produced by isolate 10-125-1. Columns with capital letters compared control, bulk oil, nanoemulsion treatment within one type of EOs. Columns with low-case letter compared the same form of EOs types, such as differences in DON production in rice cultures treated with the five EOs in bulk oil form. Columns with different letters are significantly different (p<0.05).

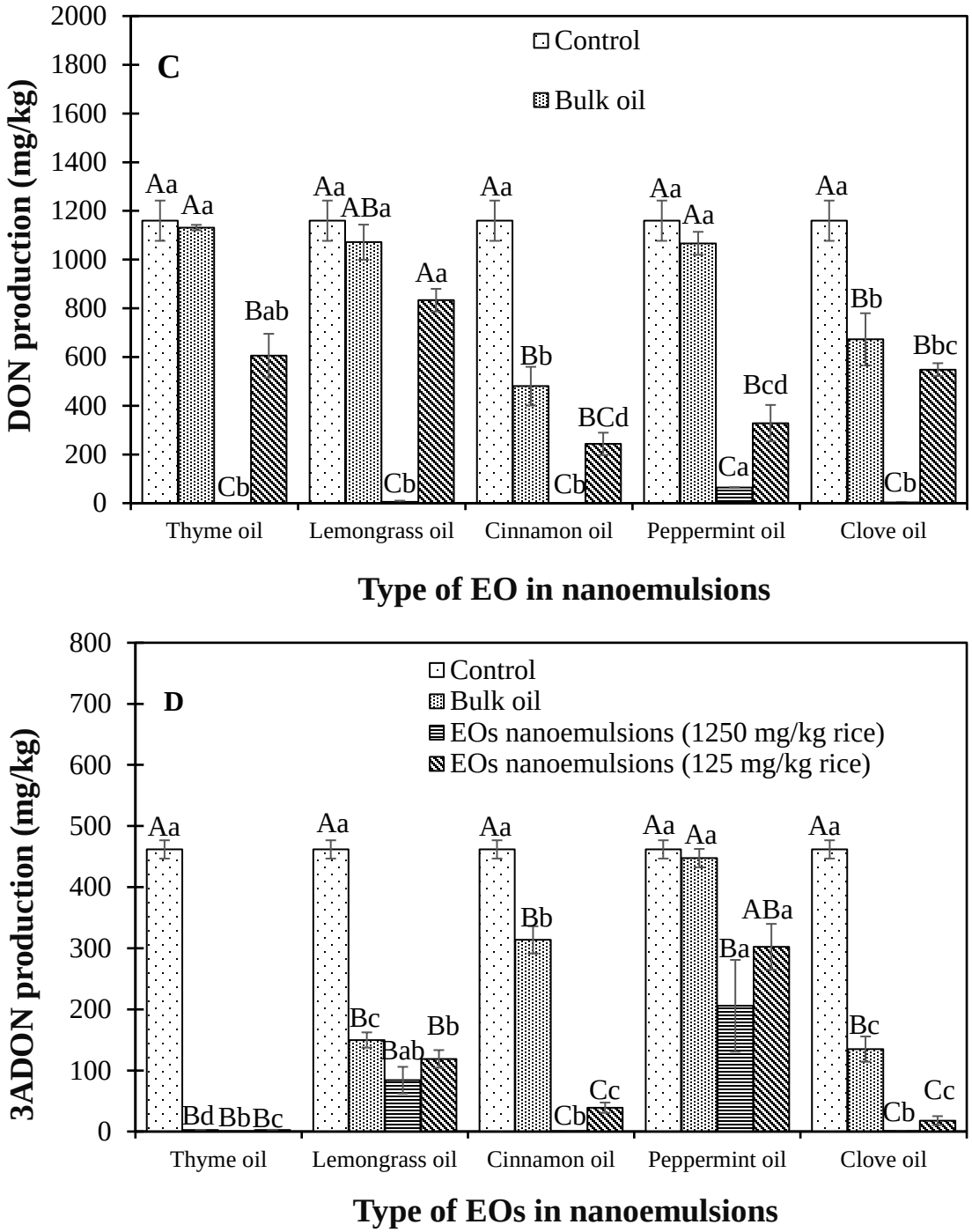


Figure 2-3. Mycotoxins production by *Fusarium graminearum* isolates in rice culture following treatment with EOs-in-water nanoemulsions and EOs and 5 days incubation (continued). (A) DON produced by isolate 10-124-1 (B) 15ADON produced by isolate10-124-1 (C) DON produced by isolate 10-125-1 (D) 3ADON produced by isolate 10-125-1. Columns with capital letters compared control, bulk oil, nanoemulsion treatment within one type of EOs. Columns with low-case letter compared the same form of EOs types, such as differences in DON production in rice cultures treated with the five EOs in bulk oil form. Columns with different letters are significantly different (p<0.05).

The effects of EOs on fungal growth and mycotoxin production of some toxigenic fungal genera like *Fusarium spp*., *Aspergillus spp*., and *Penicillium spp*. have been reported (Ferreira et al., 2013; Prakash, Kedia, Mishra, & Dubey, 2015; Prakash et al., 2012). The results of the present study not only support the previous finding that the essential oils possess antifungal activity against *Fusarium graminearum*, but also give a clear indication of the extent of inhibition for mycotoxin production. In general, bulk EOs and EOs-in-water nanoemulsions with two concentrations displayed a variable degree of mycotoxin inhibitory activity after 5 days incubation (Fig. 2-3). These results clearly indicate that all treatments led to a reduction of mycotoxin production and mycotoxin inhibitory activity is dose dependent. For instance, complete inhibition (100%) of majority mycotoxins was achieved in the two isolates across the selected five studied nanoemulsions containing 1250 mg EOs /kg rice. However, DON was only reduced by ~68.0 % in *Fusarium graminearum* isolate 10-124-1 upon the addition of 10×times diluted thyme oil-in-water nanoemulsions (125 mg/kg rice) (Fig. 2-3A). It was also noticed that mycotoxin inhibitory activity of EOs encapsulated in nanoemulsions was always greater or equal to bulk EOs in the two tested isolates. In particular, the encapsulation of EOs greatly enhanced mycotoxin inhibitory activity as compared to the oil in the bulk form (1250 mg/kg rice) on isolate 10-125-1 under the same concentration (Fig. 2-3 C & D). For instance, the DON contents in rice culture treated with bulk lemongrass oil and non-diluted lemongrass oil-in-water nanoemulsion were 1071.40 and 5.44 mg/kg, respectively, which declined by 7.5% and 99.5% as compared to the control. As one can see from Table 2-1, some of bioactive antifungal terpenoids and monoterpenes from lemongrass oil such as citral, caryophyllene, and geranyl acetate have extremely low water solubility. The improved mycotoxin inhibitory activity of lemongrass oil in the form of nanoemulsion may be due to the enhanced solubility. Previous studies have

suggested that the antifungal activity of essential oils is limited by the solubility of chemical constituents and only those dissolved molecules in the aqueous phase are available for interaction with cells (Donsì et al., 2011). It is presumed that the nanoemulsion systems would be useful to increase the solubility of EOs in the rice culture media, which facilitates the transportation of EOs through the cell membrane of the target fungal spores, resulting in the extending of the mycotoxin inhibitory activity (Liang et al., 2012).

Interestingly, the mycotoxin inhibitory activity of the five bulk EOs and their nanoemulsion forms did not follow a similar pattern with isolates 10-124-1 and 10-125-1. Firstly, isolate 10-124-1 was more sensitive to the action of all studied EOs in terms of inhibition of mycotoxin production when compared with isolate 10-125-1. As shown in Fig. 2-3A & B, a similar mycotoxin inhibitory activity of all EOs was observed, which was independent of EO forms treated (bulk oil or non-diluted nanoemulsions). On the other hand, the majority of bulk EOs did not exhibit any mycotoxin inhibitory activities towards DON and 3ADON production at a concentration of 1250 mg/kg rice (Fig. 2-3C & D). Furthermore, among the five EOs, lemongrass, peppermint, and clove oil expressed the greatest mycotoxin inhibitory activity against DON and 15ADON production in isolate 10-124-1. Conversely, isolate 10-125-1 was more sensitive to the action of cinnamon oil-in-water nanoemulsion in terms of DON and 3ADON production. For instance, a strong inhibition of both DON and 3ADON was observed with the addition of cinnamon oil in the 10-fold diluted nanoemulsion forms (125 mg/kg rice). Different responses of *Fusarium graminearum* isolates against the same antifungal agent have been previously reported. For example, Pagnussatt and coworkers found that phenolic extract had different inhibition activities for mycelia growth and mycotoxin productions between the

two *Fusarium graminearum* isolates used (Pagnussatt, Del Ponte, Garda-Buffon, & Badiale-Furlong, 2014).

Conclusion

Five physically stable EOs-in-water nanoemulsions (d < 200 nm) were fabricated using a microfluidizer and optimizing homogenization conditions (68.95 MPa and two passes). The particle size of the EOs-in-water nanoemulsions remained relatively stable, and the size distribution remained monomodal for 30 days storage at both 4 and 25 °C. Among the five selected EOs-in-water nanoemulsions, the particle size of cinnamon oil-in-water nanoemulsion slightly increased during storage at 25 °C probably due to the relatively higher water solubility of eugenol, which accelerated Ostwald ripening. The EO type and concentration were found to have a remarkable influence on the antifungal activity in terms of mycelia growth. The thyme oil-in-water nanoemulsion exhibited the strongest antifungal efficacy on inhibition of mycelium growth, which was attributed to the major chemical constituents such as thymol. With regard to the inhibition of mycotoxin production in rice culture, our study showed that a nanoemulsion based delivery system increased the mycotoxin inhibitory activity of all tested essential oils. The two chemotype isolates of *Fusarium graminearum* showed different responses in terms of mycotoxin production against the presence of the five EOs evaluated. This study has important implications for optimizing EO based nanoemulsion systems to be as safe alternatives to synthetic preservatives in food industry.

CHAPTER 3. ENHANCEMENT OF ANTIFUNGAL AND MYCOTOXIN INHIBITORY ACTIVITIES OF FOOD-GRADE THYME OIL-IN-WATER NANOEMULSIONS WITH NATURAL EMULSIFIERS

Abstract

Thyme oil-in-water nanoemulsions stabilized by three different sources of natural emulsifiers (bovine serum albumin, quillaja saponins, and soy lysolecithin) were prepared as potential natural antifungal agents. The thyme oil-in-water nanoemulsion containing bovine serum albumin or quillaja saponins with mean particle diameters < 200 nm showed high physical stability during 28 days of storage. The antifungal and mycotoxin inhibitory activities of thyme oil-in-water nanoemulsions stabilized with three natural emulsifiers against *Fusarium graminearum* isolates were then investigated. Mycelial growth, spore germination, and mycotoxin production results indicated that the antifungal activity was mainly dominated by thyme oil rather than emulsifier to alter mycelial and spore cell membrane integrity. The mycotoxin inhibitory efficacy of thyme oil was enhanced considerably in nanoemulsion form as compared to bulk oil. Among the three natural emulsifiers, bovine serum albumin stabilized thyme oil-in-water nanoemulsion exhibited higher mycotoxin inhibitory activity due to a joint function of thyme oil and emulsifier.

Introduction

Cereal grains can become contaminated by fungi while in the field, and during storage, transportation and processing. Mycotoxins are fungal secondary metabolites that exert toxic effects on animals and humans (Bai & Shaner, 2004). In general, mycotoxins are stable chemical compounds that persist during food processing operations such as cleaning, milling, brewing, cooking, baking and extrusion (Voss & Snook, 2010). Consequently, they may contaminate

finished cereal based foods (Malachova et al., 2011). For example, the mycotoxin deoxynivalenol (DON) and its derivatives 15-acetyl-deoxynivalenol (15ADON) and 3-acetyl-deoxynivalenol (3ADON) are the most common *Fusarium* mycotoxins detected in cereal grains (Oliveira, Zannini, & Arendt, 2014). The occurrence of DON in barley beer illustrates that mycotoxin contamination can occur at various stages of food processing operation, as well as being transferred from the field barley to the final product (Paul B. Schwarz, 2017). The consumption of mycotoxin contaminated food has been linked to adverse effects ranging from acute toxicities to modulation of the immune system (Wu, Groopman, & Pestka, 2014). Currently, strategies for controlling the growth of *Fusarium* and the synthesis of DON focus on pre- and/or post-harvest treatments (Neme & Mohammed, 2017). However, there is also a need for efficient, cost-effective methods to inhibit fungal growth and for the management of mycotoxins during food processing. The use of chemically synthesized preservatives to control fungal spoilage has become a common practice in the last decades. With the increasing consumer trends towards more natural and healthy food products, food manufactures have started to find natural preservatives to replace synthetic materials. Plant essential oil is one of the most promising classes of natural preservatives (Burt, 2004; da Cruz Cabral, Fernández Pinto, & Patriarca, 2013b; Prakash et al., 2015). For instance, essential oils were found to inhibit the growth of a large variety of fungi, such as *Aspergillus flavus*, *Fusarium verticillioides*, and *Fusarium graminearum* (Da Silva Bomfim et al., 2015; Kordali et al., 2005; Shukla, Singh, Prakash, & Dubey, 2012). Among the many types of essential oils, thyme oil has been shown to have superior antimicrobial properties against the growth of various bacteria and fungi (Šegvić Klarić, Kosalec, Mastelić, Piecková, & Pepeljnak, 2007; Ziani et al., 2011). However, the antifungal efficacy of bulk thyme oil in food systems often gets substantially suppressed due to

the presence of interacting components in conjunction with the lower diffusion rate of bulk oil to the target action site of an organism. One promising strategy is to incorporate thyme oil into nanometric delivery systems that would potentially increase the mass transfer rate, thus increasing the antifungal activity of essential oils (Donsì et al., 2011; Moghimi et al., 2016). Among the nanometric delivery systems, nanoemulsions are particularly suitable for encapsulation of essential oils, because they can be fabricated with food grade emulsifiers through scalable top-down approaches using high-pressure homogenization. In the past decade, the antimicrobial activities of bulk thyme oil and thyme oil-in-water nanoemulsions prepared with synthetic emulsifiers have been well documented (Chang et al., 2015; Chang et al., 2012; Xue & Zhong, 2014). Although synthetic emulsifiers such as Tween 80 are usually very effective in reducing the size of oil droplets needed to form nanoemulsions, their applications in food industry are often limited. Alternatively, natural biopolymer-based emulsifiers and biosurfactants (such as protein, polysaccharide, lecithin, and saponins) have been shown to be effective surface-active agents for forming and stabilizing essential oil-in-water nanoemulsions (Donsì, Annunziata, Vincensi, & Ferrari, 2012; McClements & Gumus, 2016). In addition, previous studies have shown that essential oil-in-water nanoemulsions formulated with natural emulsifiers such as quillaja saponin or whey protein isolate had greater antimicrobial activity than those formulated with synthetic emulsifiers (e.g., Tween 80) (Ribes et al., 2017; Ryu, McClements, Corradini, Yang, & McLandsborough, 2018). Nevertheless, up to now little systematic research has been carried out to evaluate the impact of essential oil-in-water nanoemulsions stabilized with natural emulsifiers on antifungal activity and the inhibition of mycotoxin production by filamentous fungi, such as *Fusarium graminearum*. In this study, we compared the ability of three different sources of natural emulsifiers, including bovine serum albumin (BSA), quillaja

saponins (Q-Naturale), and soy lysolecithin, to form and stabilize thyme oil-in-water nanoemulsions, as well as their antifungal efficacy. BSA is an animal source globular protein emulsifier that has been widely used in the food industry. Quillaja saponins, as emerging plant based natural emulsifiers, are surface active amphipathic glycosides. In this emulsifier, saponins are the major surface active components, and they contain hydrophobic triterpenoid rings and hydrophilic carbohydrate groups which allow the formation of relatively small emulsion droplets ($d < 200$ nm) (Yang, Leser, Sher, & McClements, 2013). It has also been shown to inhibit the growth of *Escherichia coli* and has the potential to modulate microbial growth. (Sen, Makkar, Muetzel, & Becker, 1998). Soy lecithin is another plant derived natural emulsifier. It belongs to the class of complex lipids and contains a polar head group and two non-polar tails. It can be converted to lysolecithin which has improved emulsification capacity, by removing one non-polar tail from lecithin. As the type of emulsifier is critical to the characteristic of nanoemulsions, it is important to understand how the different emulsifiers might impact their antifungal activities. This understanding is necessary in order to effectively design nanoemulsion delivery systems that incorporate thyme oil as a natural means to inhibit fungal growth and mycotoxin production.

The objective of this study was to evaluate the efficacy of thyme oil-in-water nanoemulsion fabricated using three abovementioned natural emulsifiers to control the growth of *Fusarium graminearum* and subsequently its mycotoxin production. The physical properties of thyme oil-in-water nanoemulsions including particle size, charges, and long-term stability were examined. The antifungal activities of three nanoemulsions against *Fusarium graminearum* were evaluated in terms of the inhibition of mycelium growth and spore germination, as well as on the morphological damage caused to fungal hyphae and spores. Moreover, the effect of natural

emulsifiers on the inhibition of mycotoxins produced by two chemotypes of *Fusarium graminearum*, including 3ADON and 15ADON producing isolates was assessed. The results of this study have important implications for the design and utilization of essential oils as natural antifungal agents and mycotoxin inhibitors in the food industry.

Materials and methods

Materials

Thyme oil (FEMA # 3065, FG grade) was kindly provided by Kalsec Inc. (Kalamazoo, MI). Quillaja saponin (Q-Naturale®200) was kindly given by Ingredion Inc. (Westchester, IL, USA). Soy lysolecithin (L) was kindly donated by American Lecithin Company (Oxford, CT, USA). BSA, Mirex-used as an internal standard in mycotoxin analysis, and Bis(trimethylsilyl)acetamide (BSA)/trimethylchlorosilane (TMCS)/Trimethylchlorosilane (TSIM) kit were purchased from Millipore Sigma Co. (St. Louis, MO, USA). Potato dextrose agar (PDA) was purchased from AMRESCO (Solon, OH, USA). Potato dextrose broth was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Corn oil, mung beans, and long grain white basmati rice were purchased locally (Fargo, ND, USA). All solutions were prepared using ultrapure distilled de-ionized water (DDW, 18.2 MΩ cm, Barnstead ultrapure water system, Thermo Fisher Scientific, USA).

Nanoemulsion preparation

The aqueous phase used to prepare thyme oil-in-water nanoemulsions consisted of 1 wt % emulsifier (BSA, quillaja saponin, or lysolecithin) dispersed in 94 wt % of buffer solution (10 mM phosphate buffer, pH 7.0). The emulsifier was first dissolved in a buffer solution, stirred overnight, and then filtered using Whatman grade 1 filter paper to remove sediments. The oil phase (5 wt %) was prepared by mixing thyme oil with corn oil at a mass ratio of 3:7 prior to

homogenization, based upon results of our preliminary study (Wan et al., 2018). The oil phase was then mixed with the aqueous phase using a high-speed hand blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min. The mixture was further homogenized using a high-pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) at 68.95 MPa for two passes. The finished nanoemulsions (1 wt % emulsifier, 1.5 wt % of thyme oil, 3.5 wt % of corn oil, and 94 wt % of buffer) were stored in test tubes sealed with screw caps. The nanoemulsions were collected and stored at 23°C for long term storage stability study.

Nanoemulsion characterization

Both mean particle diameter (Z-averages) and ζ-potential of nanoemulsion droplets were measured using a dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK) with disposable capillary cells (DTS1060, Malvern Instruments). To avoid multiple scattering effects, samples were diluted 1/10 using buffer solution (10 mM phosphate acid, pH 7) prior to analysis.

Antifungal activity of nanoemulsions against mycelium growth

Two *Fusarium graminearum* isolates 10-124-1 and 10-125-1 were employed to evaluate the antifungal efficacy of thyme oil-in-water nanoemulsions stabilized using three different sources of emulsifiers. *Fusarium graminearum* 10-124-1 belongs to the 15ADON chemotype, which mainly produces DON and 15ADON; whereas *Fusarium graminearum* 10-125-1 belongs to the 3ADON chemotype which is associated with DON and 3ADON production. Determination of antifungal activity in terms of the inhibition of mycelial growth inhibition was measured according to our previous method with some modifications (Wan, Zhong, Schwarz, Chen, & Rao, 2018). Briefly, the nanoemulsions were diluted with aqueous buffer to create the desired gradient concentrations of essential oil (i.e., 0, 2, 4, 6, 8, 10, 12 mg/g) in the final

nanoemulsions. The mycelium growth inhibition (MGI) rate was calculated as MGI rate (%) = 100 × (mycelial colony's diameter of control − mycelial colony's diameter of treatment)/mycelial colony's diameter of control.

Antifungal activity of nanoemulsions on spore germination of *Fusarium graminearum*

Spores from two *Fusarium graminearum* isolates were produced on mung bean agar (MBA) media at 23 °C under UV light for 8 days, and then were washed off by sterile water. The concentration of spore suspension was measured using a hemacytometer (Levy Ultraplane, PA, USA) and was adjusted to 1.0×10^5 spores/mL. Spore suspension (100 μL) was mixed with 100 μL of the thyme oil-in-water nanoemulsions with concentration gradients of thyme oil at 0.3, 1.5, 3, 9, 15 mg/g nanoemulsion. The mixtures (40 μL) were spread on agar plates and incubated for 8 hours. The spore germination was observed, and the number of germinated spores was counted under bright field microscopy (Olympus model BX51 Olympus, Germany). In particular, a spore was considered to be germinated if the length of germinal tube was longer than the spore diameter and at least 100 spores were counted Spore germination rate was calculated as P (%) = (number of germinated spore/numbers of total spore) × 100%.

Scanning electron microscopy (SEM)

Fusarium graminearum isolate 10-124-1 was grown on PDA plate for 4 days. One milliliter of nanoemulsions was sprayed evenly on the surface of mycelium and incubated for 12 hours. Mycelia treated with the same amount of distilled water were considered as controls. Thereafter, a mycelium PDA plug was cut out and fixed with glutaraldehyde (2.5%, v/v) solution (Electron Microscopy Sciences, Hatfield, Pennsylvania USA). Fixed samples were rinsed twice with deionized water and then dehydrated through a graded ethanol series from 30-100%. Samples were critical-point dried using an Autosamdri-810 critical point drier (Tousimis,

Rockville, Maryland, USA) with liquid carbon dioxide as the transitional fluid. Dried samples were attached to cylindrical aluminum mounts with silver paint (SPI Supplies, West Chester, PA) and sputter coated with gold (Cressington 108auto, Ted Pella Inc., Redding, California, USA). Images were obtained using a JEOL JSM-6490LV scanning electron microscope operating at an accelerating voltage of 15 kV.

For spore samples, they were collected from the fungal isolate grown on mung bean agar (MBA) medium at 23 ℃ for 8 days. Seven hundred microliters of spore suspension (1×10^5 spores/mL) was first mixed with an equal volume of thyme oil-in-water nanoemulsions. Then, 200 μL of the mixtures were evenly spread on the surface of thin layer of PDA plates and incubated for 12 hours. Spores mixed with the same amount of distilled water were considered as controls. After incubation, a thin layer of agar plug containing macroconidia was attached to the aluminum mount with carbon adhesive tape (Ted Pella Inc., Redding, California, USA), coated with gold as described above, and observed under SEM.

Influence of nanoemulsions on mycotoxin production in rice culture

The mycotoxin inhibitory efficacy of thyme oil-in-water nanoemulsions stabilized by three different sources of emulsifiers was evaluated according to our previous rice culture methods (Wan, Zhong, Schwarz, Chen, & Rao, 2018). The final thyme oil concentrations in rice cultures were 750, 750, and 75 mg/kg rice for the treatment of bulk thyme oil, thyme oil-in-water nanoemulsions, and 10-time dilute thyme oil-in-water nanoemulsions, respectively. Each treatment had three replicates and were incubated at 23 °C in dark for 8 days. After 8 days of incubation time, DON, 3ADON, and 15ADON content was measure by GC-MS as previously reported (Jin et al., 2018). Results were expressed in mg toxin/kg rice culture. The limits of quantitation (LOQ) and detection (LOD) of mycotoxins were 0.20 and 0.10 mg/kg, respectively.

Statistical analysis

All measurements were performed with triplicate samples, and values were expressed as means ± SD of triplicates from each of two independent experiments. One-way analysis of variance (ANOVA) was conducted and the Tukey pairwise comparison test was used to define significant difference of mean value at the 5% of probability level (MINITAB 18.0).

Results and discussion

Influence of natural emulsifiers on the nanoemulsion formation

Initially, the influence of three different sources of natural emulsifiers (BSA, quillaja saponin, and soy lysolecithin) and concentrations (0.1, 0.5, 1.0, and 1.5 wt %) on the formation of thyme oil-in-water nanoemulsion using a novel electro-hydraulic microfluidizer was evaluated by measuring particle size, size distribution, and particle charge (Table 3-1).

Table 3-1. Effect of emulsifier type (BSA, quillaja saponin, or lysolecithin) and concentration (0.1, 0.5, 1.0, 1.5 wt%) on mean particle size of thyme oil-in-water nanoemulsions. For each emulsifier type, values of various emulsifier concentrations with different capital letter were significantly different from each other (p<0.05). For each emulsifier concentration, values of various emulsifier types with different small letter were significantly different from each other (p<0.05).

Emulsifier type	Concentration of emulsifier (wt %)			
	0.10	0.50	1.00	1.50
BSA	341.39±22.89 Ab	194.35±2.38 Bb	179.75±0.64 Bb	177.13±4.80 Ba
Quillaja saponin	505.40±20.97 Aa	232.35±12.61 Ba	199.25±1.95 Ba	186.14±3.06 Ba
Lysolecithin	485.84±24.94 Aa	203.92±8.70 Bab	181.40±3.68 Bb	182.12±0.87 Ba

Overall, the mean particle diameter of thyme oil-in-water nanoemulsions decreased dramatically upon increasing the emulsifier concentrations from 0.1 to 1.0 wt % and remained fairly constant afterwards for all-natural emulsifier types (Table 3-1). For instance, the mean particle diameter of the nanoemulsion stabilized with BSA was reduced appreciably from 341 to 180 nm as the BSA concentration increased from 0.1 to 1.0 wt %. As the BSA concentration further increased to 1.5 wt %, the mean particle diameter of nanoemulsions dropped at 177 nm.

This trend can be explained by the fact that there were initially more emulsifier molecules available to cover on the surface of newly created small thyme oil droplets during homogenization. Once there was sufficient emulsifier to cover all the tiny thyme oil droplet surfaces, it cannot produce any smaller droplets in the presence of any additional emulsifier molecules. This is because the increased Laplace pressure upon the decrease of particle size opposes the further disruption of thyme oil droplet at given homogenization conditions (Kharat, Zhang, & McClements, 2018). Among the three natural emulsifiers, BSA and soy lysolecithin stabilized thyme oil-in-water nanoemulsion had the relatively smaller mean particle diameters than quillaja saponin stabilized ones. For example, when 1.0 wt % emulsifier was used (corresponds to a 5:1 ratio of oil-to-emulsifier), the mean droplet diameter of nanoemulsions stabilized by quillaja saponin, soy lysolecithin, and BSA was 199, 181, and 180 nm, respectively. As opposed to the current findings, previous studies found that quillaja saponin was more efficient than BSA and soy lecithin to produce smaller droplets in both corn oil-in-water and medium chain triacylglyceride-in-water emulsions (Bai, Huan, Gu, & McClements, 2016; Chung, Sher, Rousset, Decker, & McClements, 2017). Our result indicated that BSA and lysolecithin are more effective at making smaller essential oil-in-water nanoemulsion droplets than quillaja saponin under the same homogenization conditions, presumably because the former adsorb to thyme oil droplet surfaces more rapidly than to triacylglycerides (Jafari, Assadpoor, He, & Bhandari, 2008). According to Table 3-1, 1.0 wt % was the saturation emulsifier concentration for all three sources of natural emulsifiers and was selected as emulsifier concentration in preparing thyme oil-in-water nanoemulsions for the following experiments.

Particle size distribution has an important implication for physical stability of nanoemulsions. As such, we plotted the particle size distribution of thyme oil-in-water

nanoemulsions stabilized by BSA, quillaja saponin, and soy lysolecithin at 1.0 wt % (Fig. 3-1A). The results showed that all thyme oil-in-water nanoemulsions displayed monomodal patterns, signifying that all selected natural emulsifiers in this study are able to form uniform droplets in the nanoemulsions at a concentration of 1.0 wt %.

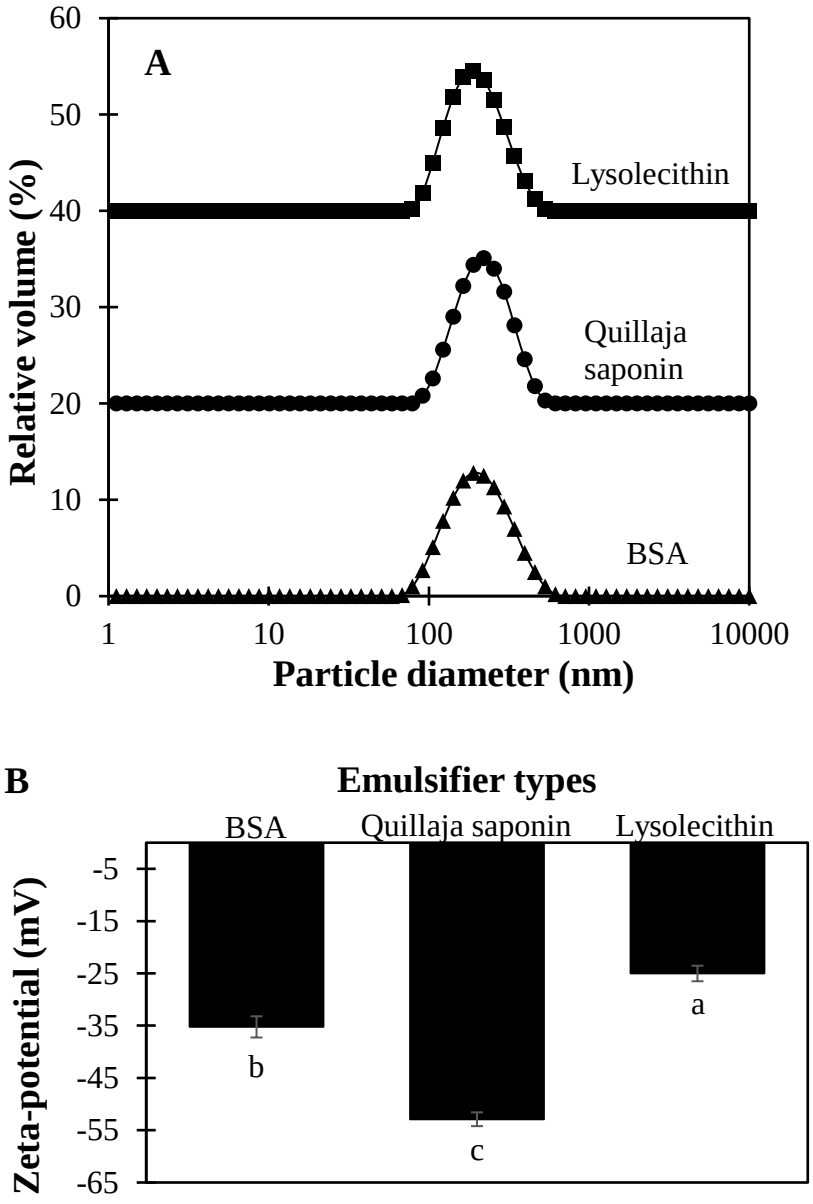


Figure 3-1. (A) Size distribution and (B) zeta-potential of thyme oil-in-water nanoemulsions stabilized by different emulsifiers (BSA, quillaja saponin, or lysolecithin) at 1.0 wt %. Significant differences ($p < 0.05$) are indicated by different lowercase letters.

The electrical characteristic of droplets has an important role in the functionality of nanoemulsion not only because it provides information on the electrostatic interactions between droplets, it also determines their interactions with the charged fungal cell surfaces, which may further influence the antifungal activity of the whole nanoemulsion system (Ozturk & McClements, 2016). Thus, the impact of different natural emulsifiers (BSA, quillaja saponin, and soy lysolecithin) on the ζ-potential of thyme oil-in-water nanoemulsion droplets at pH 7.0 were evaluated (Fig. 3-1B). The ζ-potential of thyme oil-in-water nanoemulsions stabilized by all three natural emulsifiers displayed appreciably negative charge ($|\zeta| > 25$ mV). The nanoemulsions stabilized by quillaja saponin exhibited negative charge (-52.9 mV) at a neutral pH due to the lower pKa of its saponins constitutes, such as glucuronic acids with pKa value ~3.25 (Chung et al., 2017; Yang et al., 2013). Since the isoelectric point of BSA is around 4.7, BSA-stabilized thyme oil-in-water nanoemulsions also displayed negative charges (-35.2 mV) at pH 7.0 (Tabibiazar et al., 2015). The negative charges of soy lysolecithin-stabilized thyme oil-in-water nanoemulsions (-25.0 mV) mainly originate from the phosphate group presenting in polar head region.

Influence of natural emulsifiers on the long-term stability of nanoemulsions

The long-term physical stability of nanoemulsions is one of the critical factors determining their applications in food systems. Thus, physical stability of thyme oil-in-water nanoemulsions was examined by measuring the mean particle size and size distribution during storage at 23 ℃ for 28 days (Fig. 3-3A).

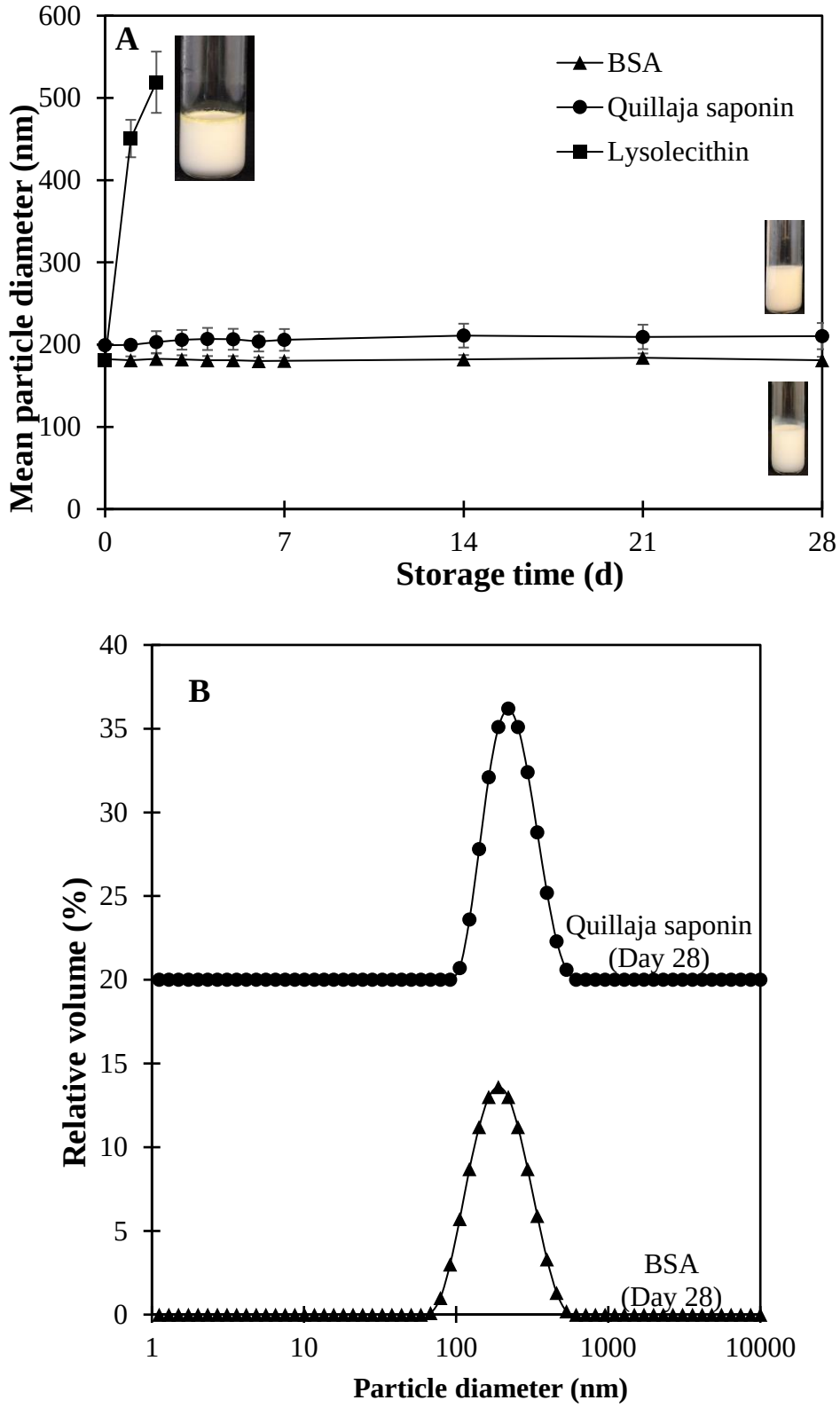


Figure 3-2. Effect of emulsifier types on long-term stability of thyme oil-in-water nanoemulsions determined by (A) mean particle size and (B) particle size distribution over 28 days' storage.

As shown in Fig. 3-2A, there was no change with regard to the visible appearance and the mean droplet diameter of thyme oil-in-water nanoemulsions stabilized with both BSA and quillaja saponin even after 28 days of storage. Size distribution of thyme oil-in-water nanoemulsions after 28 days' storage further proved that thyme oil-in-water nanoemulsions containing BSA or quillaja saponin had relatively high storage stability by displaying monomodal size distribution (Fig. 3-2B). In contrast, the thyme oil-in-water nanoemulsion stabilized with soy lysolecithin was highly unstable with a rapid increase in the mean droplet diameter after 1 day of storage. Phase separation in conjunction with a distinct oil layer appeared on top of the test tube was visible after 2 days (Fig. 3-2A). In general, emulsifiers contribute to the stabilization of nanoemulsions via electrostatic repulsion and/or steric hindrance effects (Ozturk & McClements, 2016). From Fig. 3-1B, it can be deduced that thyme oil-in-water nanoemulsions stabilized with soy lysolecithin carried much fewer negative charges, as compared to those stabilized with BSA and quillaja saponin, which in turn gave rise to a relatively weaker electrostatic repulsion between droplets in the nanoemulsions. The good stability of the nanoemulsions stabilized with BSA and quillaja saponin may be partly attributed to a strong electrostatic repulsion between the highly charged droplets. As to steric hindrance effects, both BSA and quillaja saponin are able to generate a strong short-range steric repulsion that prevents the droplets from coming into close proximity to coalesce. Nevertheless, the interfacial layer in soy lysolecithin stabilized thyme oil-in-water nanoemulsions was more flexible and was prone to disruption, which is in agreement with the previous studies. (Xue & Zhong, 2014)

Influence of natural emulsifier on mycelial growth and hyphae morphology of *Fusarium graminearum*

Antifungal activities of the thyme oil-in-water nanoemulsions were tested against two chemotypes of *Fusarium graminearum* (isolate 10-124-1 and 10-125-1) using the agar dilution method. The mycelium growth inhibition (MGI) rate of *Fusarium graminearum* after 4 days of incubation in the presence of thyme oil-in-water nanoemulsions is shown in Fig. 3-3. In general, all thyme oil-in-water nanoemulsions exhibited inhibition of mycelium growth for both isolates. The concentration of thyme had an appreciable influence on the mycelial growth of the two *Fusarium graminearum* isolates tested. The inhibition rate increased with increasing the thyme oil concentrations in the nanoemulsions. It was reported that complete inhibition of mycelial growth of *Aspergillus flavus*, *Fusarium spp.*, and *Penicillium spp.* was achieved by applying 10 µl thyme oil in an *in vitro* system, and the activity of thyme oil was superior to over most of the prevalent synthetic fungicides (Kumar, Shukla, Singh, Prasad, & Dubey, 2008; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2007). A previous study suggested that the mechanism of the antifungal action of the thyme oil involves a direct interaction with ergosterol, which leads to the disruption of the fungal cell membrane and the loss of intracellular contents. This induces profound morphological and ultrastructural alterations, such as plasma membrane disruption and the loss of cytoplasmic contents (De Lira Mota, De Oliveira Pereira, De Oliveira, Lima, & De Oliveira Lima, 2012). Thyme oil-in-water nanoemulsions performed differently on the two isolates in terms of MGI rate. As one can see, a 100% of MGI rate was observed after treatment with 8 mg/g of the three types of thyme oil-in-water nanoemulsions, denoting that the isolate 10-124-1 was more sensitive than that of isolate 10-125-1 (Fig. 3-3). Different responses of *Fusarium graminearum* isolates against the same antifungal agent have been previously

reported (Pagnussatt et al., 2014). This could probably be attributed to physiological differences between *Fusarium graminearum isolates* 10-124-1 and 10-125-1 as 15ADON production isolates (e.g., 10-124-1) had been reported to have higher gene diversity and more polymorphic loci than 3ADON production isolates (e.g., 10-124-1) (Puri & Zhong, 2010).

The source of natural emulsifiers also influences mycelium growth. Among the three natural emulsifiers, thyme oil-in-water nanoemulsions formulated with quillaja saponin demonstrated a slightly higher MGI rate than the other two, particularly in isolate 10-124-1(Fig. 3-3). Fungi such as *Fusarium spp.* have been reported to have a negative surface charge owing to the presence of anionic polysaccharides such as β-glucan and embedded membrane proteins at their surfaces (Latgé, 2007). One might have expected that quillaja saponin stabilized thyme oil-in-water nanoemulsion to be less effective as the greater negative surface change (-52.9 mV, Fig. 3-1B) would electrically repel the droplets from the surfaces of the fungi more strongly than the other two emulsifiers coated nanoemulsions. However, this was not the case, which suggests that other mechanisms may have taken place. Two possible reasons might account for this phenomenon, including (i) differences in the inherent antifungal activity of the emulsifiers themselves, (ii) differences in the diffusion rate of the nanoemulsion droplets within the cell membrane, which promotes the targeted release of the thyme oil at the desired sites. Previous research studied the influence of emulsifier type on the antimicrobial activity of essential oils-in-water nanoemulsions by measuring the diffusion rate of essential oils between nanoemulsion and bacterial cell membranes. The results indicated that the diffusion rate of essential oils was appreciably higher in quillaja saponin -stabilized nanoemulsion systems than in that of Tween 80 due to the smaller micelle formed (Ryu et al., 2018). The observation that the MGI rate was higher for quillaja saponin -stabilized thyme oil-in-water nanoemulsions suggested that the

diffusion rate of the nanoemulsion droplets within the cell membrane might be an important factor.

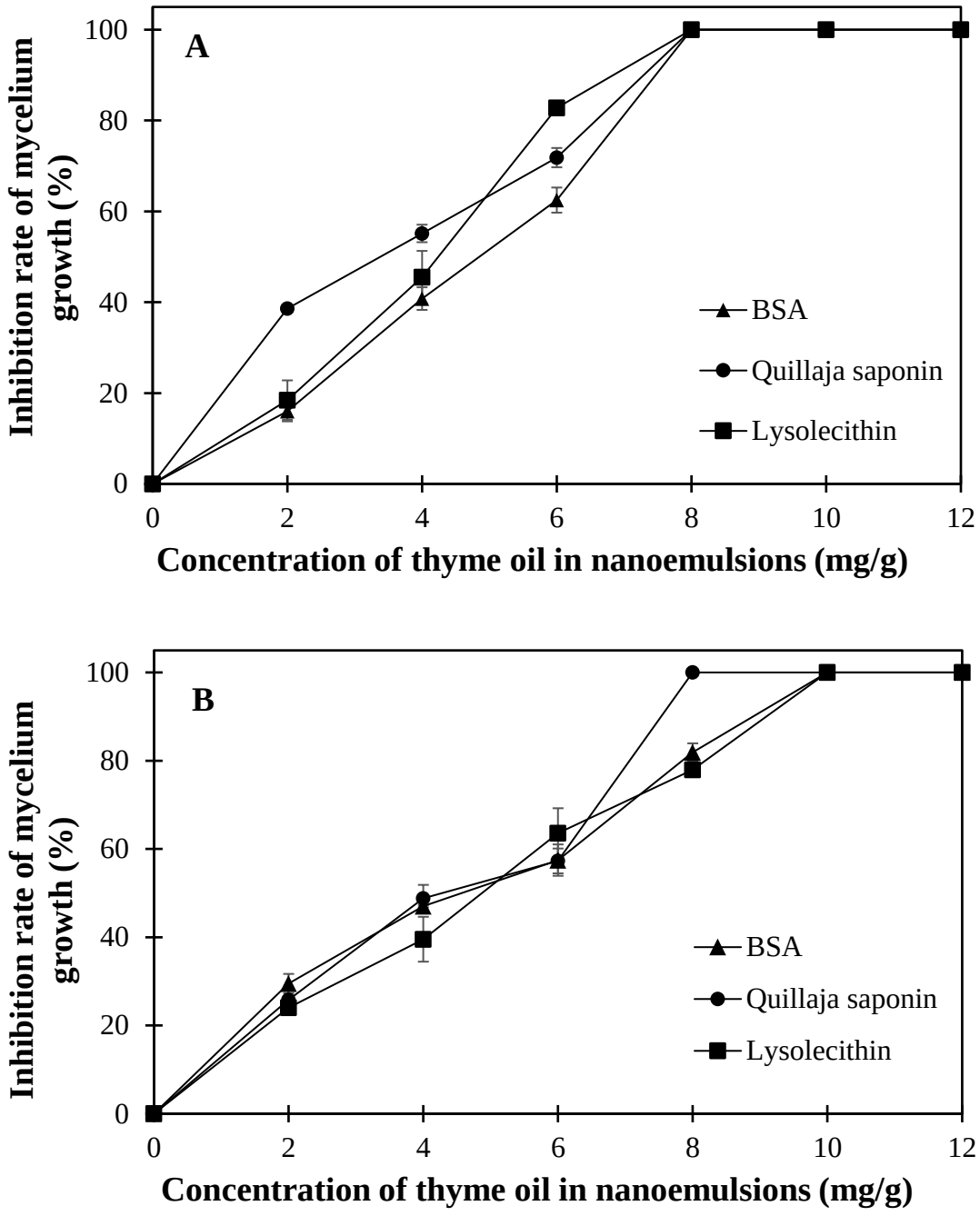


Figure 3-3. Effect of emulsifier type and thyme oil-in-water nanoemulsion concentration on mycelial growth of *Fusarium graminearum* isolate (A) 10-124-1 and (B) 10-125-1.

In order to better understand the mechanism by which natural emulsifiers influence the antifungal activity of thyme oil-in-water nanoemulsions, morphological changes the hyphae of *Fusarium graminearum* 10-124-1 were examined with the assistance of SEM (Fig. 3-4). Hyphae of the control showed a linear shape with smooth surfaces (Fig. 3-4A & B). The growth of hyphae was strongly inhibited with the treatment of thyme oil-in-water nanoemulsions, as evidenced by morphological changes from a linear shape to mycelial sparsity, asymmetry, curling, and twisting (Fig. 3-4C, E, & G). As one can see, hyphae treated with three different sources of thyme oil-in-water nanoemulsions (15 mg/g) were found to be covered by "layers" on the surface (Fig. 3-4D, F, & H with red arrow), becoming flatter and more shrunken with a warty surface. Among the thyme oil-in-water nanoemulsions stabilized by three different sources of emulsifiers, there was a slight difference in hyphal morphology. For example, upon the treatment of either BSA or quillaja saponin -stabilized thyme oil-in-water nanoemulsion, the distinctive morphological changes of hypha were observed. It included alteration in the hyphal wall thickness, disrupted or even seemed to disappear in some hyphae, as well as the appearance of some precipitates on the external part of the cell membrane. In contrast, no broken hypha was observed after treatment with lysolecithin stabilized thyme oil-in-water nanoemulsions, but there was loss in integrity of the hyphal wall. This disparity implies the different mode of actions among different sources of natural emulsifier formulated thyme oil-in-water nanoemulsions against mycelium growth of *Fusarium graminearum*.

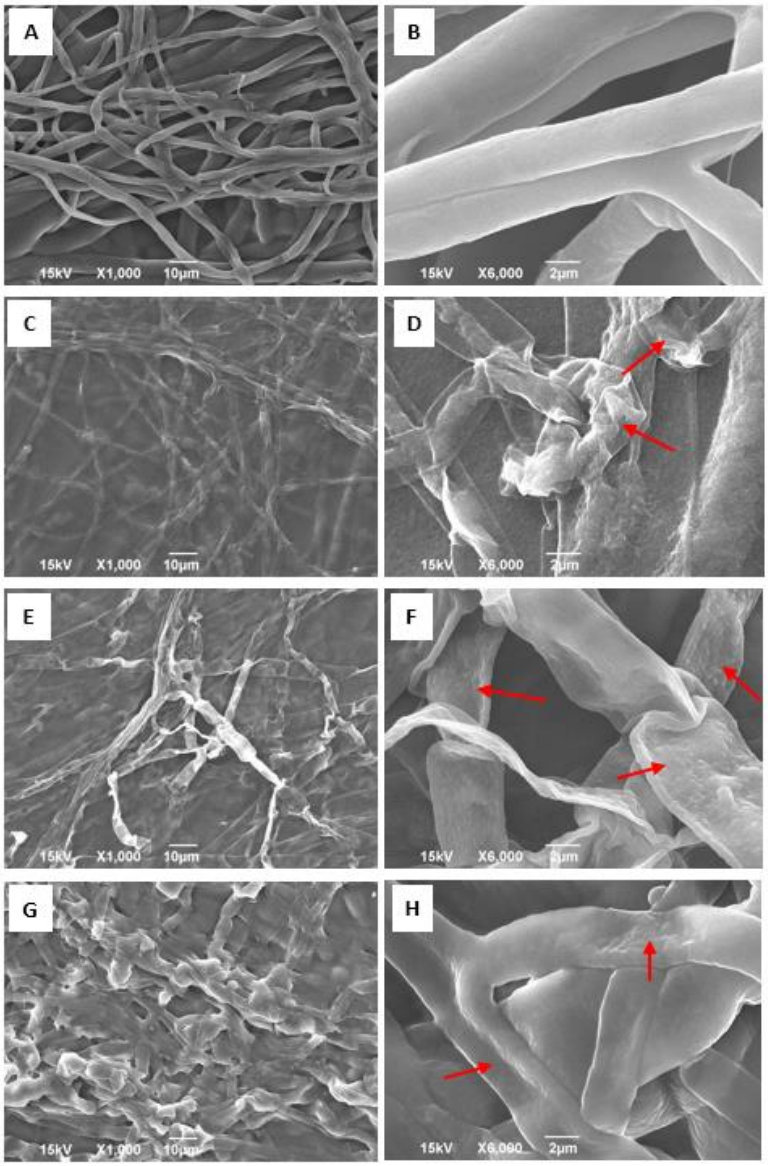


Figure 3-4. Scanning electron microscopic observation of hyphae of *Fusarium graminearum.* Hyphae samples treated by control (A & B) and thyme oil-in-water nanoemulsion stabilized by BSA (C & D), quillaja saponin (E & F) or lysolecithin (G & H).

Influence of natural emulsifier on spore germination and morphological structure of *Fusarium graminearum*

Fusarium graminearum infects cereals by germination of asexual spores or sexual spores on susceptible plant tissues, such as florets and glumes. Germinated spores produce hyphae that can further penetrate through plant stomata and/or other site within the inflorescences (Seong,

Zhao, Xu, Güldener, & Kistler, 2008). Thus, it is important to know the inhibitory activity of thyme oil-in-water nanoemulsions on spore germination. The inhibitory activity of nanoemulsions stabilized by BSA, quillaja saponin, and lysolecithin at varying thyme oil concentrations (0, 0.3, 1.5, 3.0, 9.0, and 15.0 mg/g) on spore germination of the two isolates were examined by measuring conidial germination rate after incubating with nanoemulsions for 10 hours (Fig. 3-5).

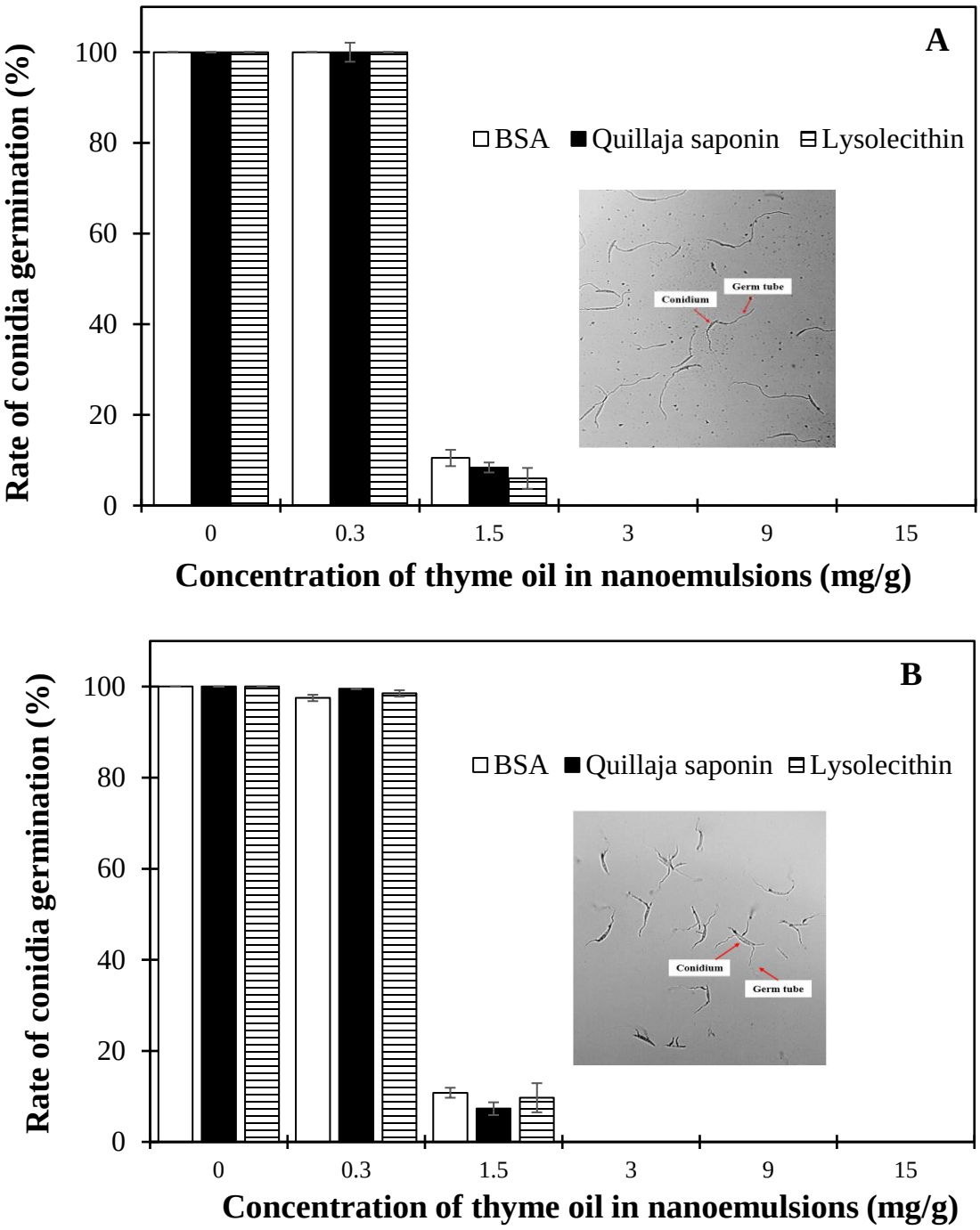


Figure 3-5. Effect of emulsifier type and thyme oil-in-water nanoemulsion concentration on spore germination rate of *Fusarium graminearum* isolate (A) 10-124-1 and (B) 10-125-1.

As shown in the inserted image of Fig. 3-5 (an arrow), a conidium is counted as germinated when the length of the newly budded germ tube is longer than itself. According to Fig. 3-5A & B, the spore germination rate decreased with increasing the thyme oil concentration in nanoemulsions, which suggested that there was a dose-dependent effect on the *Fusarium graminearum* isolates. For example, spore germination rate of both isolates was below 11 %, when 1.5 mg/g of the thyme oil concentration in nanoemulsion was applied in the system. Conversely, no conidium was able to germinate when the thyme oil concentration was above 3.0 mg/g in the nanoemulsions. The source of emulsifiers that form nanoemulsions, however, did not show significant impact on the spore germination rate.

SEM was used to provide information about changes in the morphology of spores treated with mixture of thyme oil at a concentration of 15.0 mg/g in nanoemulsions (Fig. 3-6). After 12 hours of incubation, remarkable morphological changes appeared in treated spores (Fig. 3-6C, D, & E) when compared to the control (Fig. 3-6A & B). The difference between treated and untreated spores was clearly visualized. Untreated *Fusarium graminearum* 10-124-1 spores germinated and formed germ tubes as indicated by an arrow (Fig. 3-6A). An enlarged view (2,500 x) of the spore further verified that plump shapes with smooth surfaces containing three to four septa were maintained (Fig. 3-6B). The septal ridge could also be observed sharply in this control. Conversely, the suppression of germination concomitant with distinctive morphological changes appeared in spore after exposure to thyme oil-in-water nanoemulsions. Among three different nanoemulsions, spores treated with BSA or lysolecithin-stabilized thyme oil-in-water nanoemulsions displayed an irregularly shrunken shape with rough surfaces (Fig. 3-6C & E). The spores became wrinkled or corrugated due to the reduction in cytoplasmic contents (Yamamoto-Ribeiro et al., 2013). Particularly, spore treated with thyme oil-in-water

nanoemulsion prepared with BSA (Fig. 3-6C) showed complete interior disorganization. No distinct internal organelles could be visualized, and the septa disappeared completely. On the contrary, a visible heavy layer (Fig. 3-6D) on the surface of spore occurred after treatment with quillaja saponin stabilized thyme oil-in-water nanoemulsion. This may be attributed to the inhibition of spore germination using quillaja saponin-stabilized nanoemulsions (Fig. 3-6D). Essential oil components have the capability to alter permeability of the fungal cell wall by disrupting lipid membrane bilayers and lipid packing (Rao, Chen, & McClements, 2019). As such, the functionality of fungal cell walls including fluidity or permeability might be changed upon treatment with the thyme oil-in-water nanoemulsions. From abovementioned, thyme oil-in-water nanoemulsions stabilized with natural emulsifiers can greatly impact spore morphological structure, which may account for their antifungal activity.

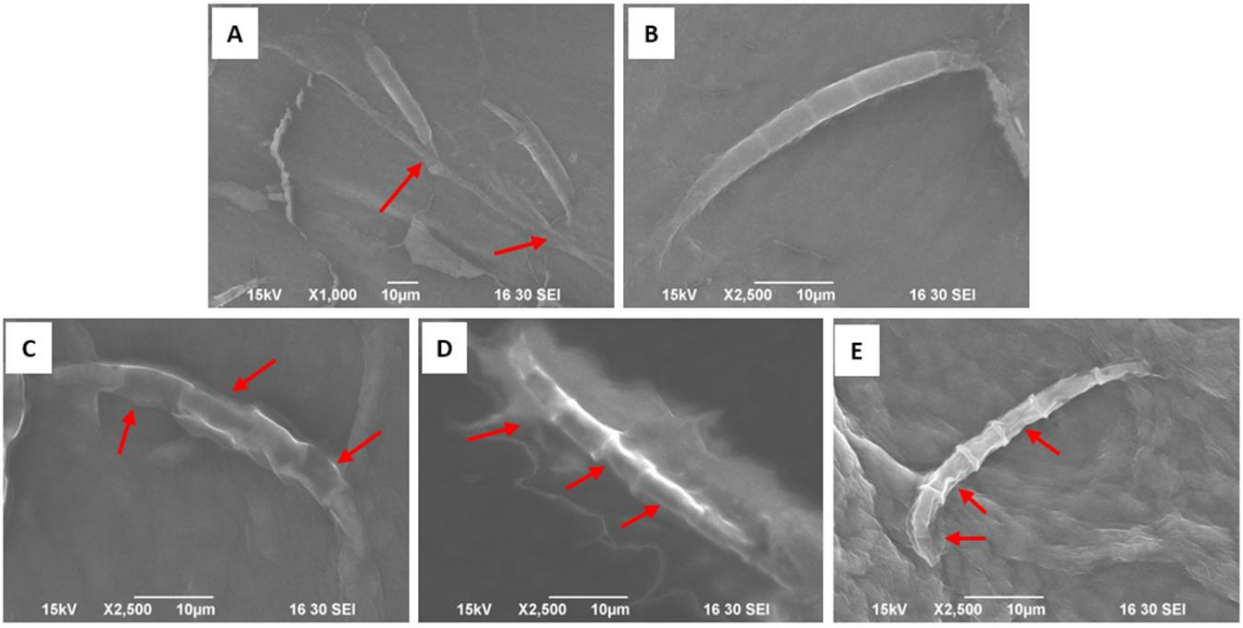


Figure 3-6. Scanning electron microscopic observation of spore of *Fusarium graminearum.* Spore samples treated by control (A & B) and thyme oil-in-water nanoemulsion stabilized by BSA (C), quillaja saponin (D) or lysolecithin (E).

Influence of natural emulsifier on inhibition of mycotoxin production

Not only does the *Fusarium graminearum* infection deteriorate the quality of cereal grains, but the mycotoxins (e.g., DON and its derivatives) produced by the fungus are detrimental to human health, causing physiological damage and/or disease. In this section, we evaluated inhibitory efficacy of thyme oil-in-water nanoemulsions stabilized by three different sources of natural emulsifiers on mycotoxin production. DON, 3ADON, and 15ADON produced by two different *Fusarium graminearum* isolates, 10-125-1 and 10-124-1, were quantified after culturing on rice culture for 8 days (Fig. 3-7). The final thyme oil concentrations were 750, 750, 75 mg/kg rice for bulk thyme oil, thyme oil-in-water nanoemulsions, and 10-time dilute thyme oil nanoemulsions, respectively.

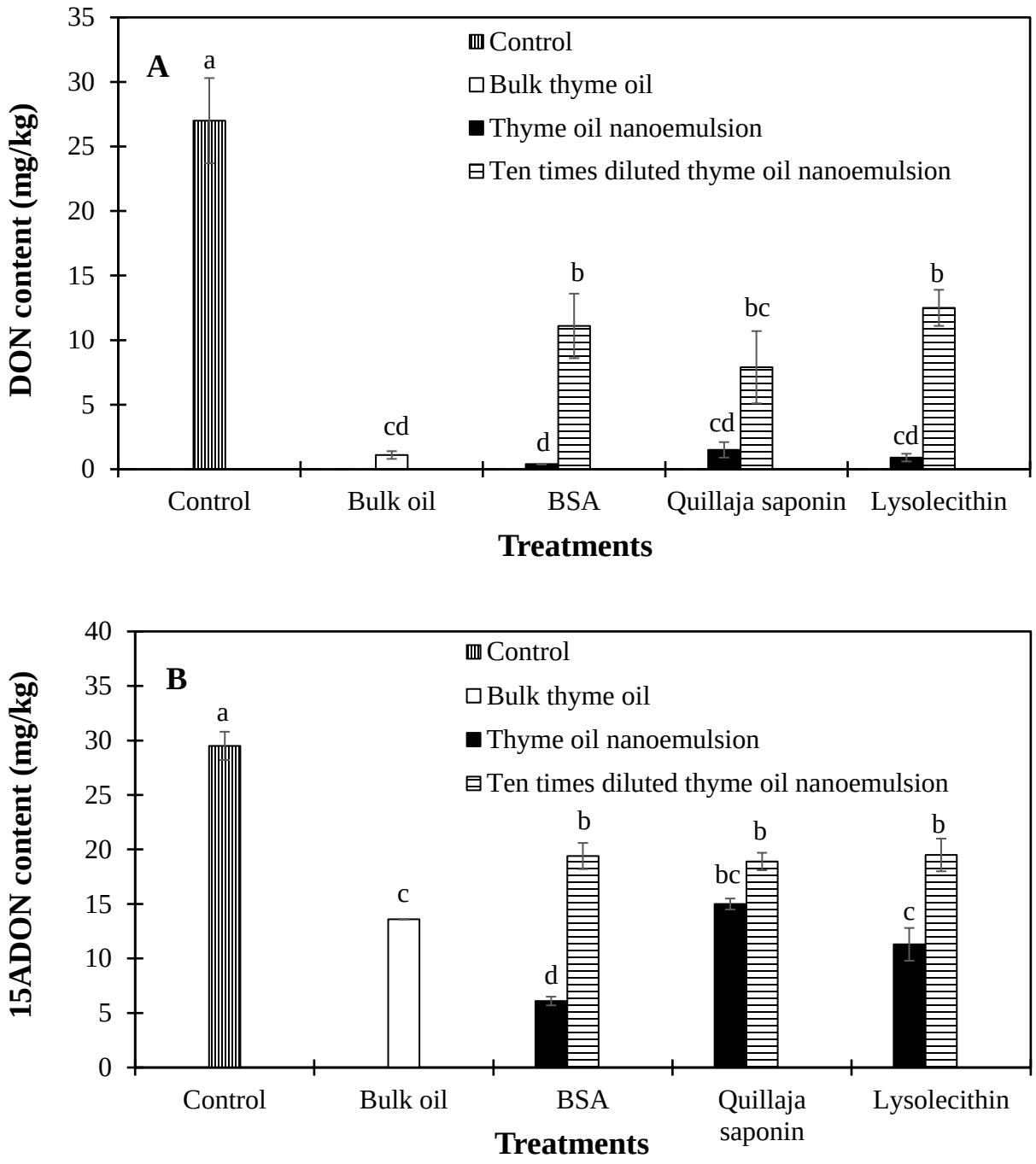


Figure 3-7. Effect of thyme oil and thyme oil-in-water nanoemulsions stabilized by different emulsifiers (BSA, quillaja saponin or lysolecithin) on mycotoxins production by *Fusarium graminearum* isolate 10-124-1 of (A) DON and (B) 15ADON and by isolate 10-125-1 of (C) DON and (D) 3ADON in rice culture. The limits of quantitation (LOQ) and detection (LOD) of the method were 0.20 and 0.10 mg/kg, respectively. Columns with different letter were significantly different from each other (p<0.05).

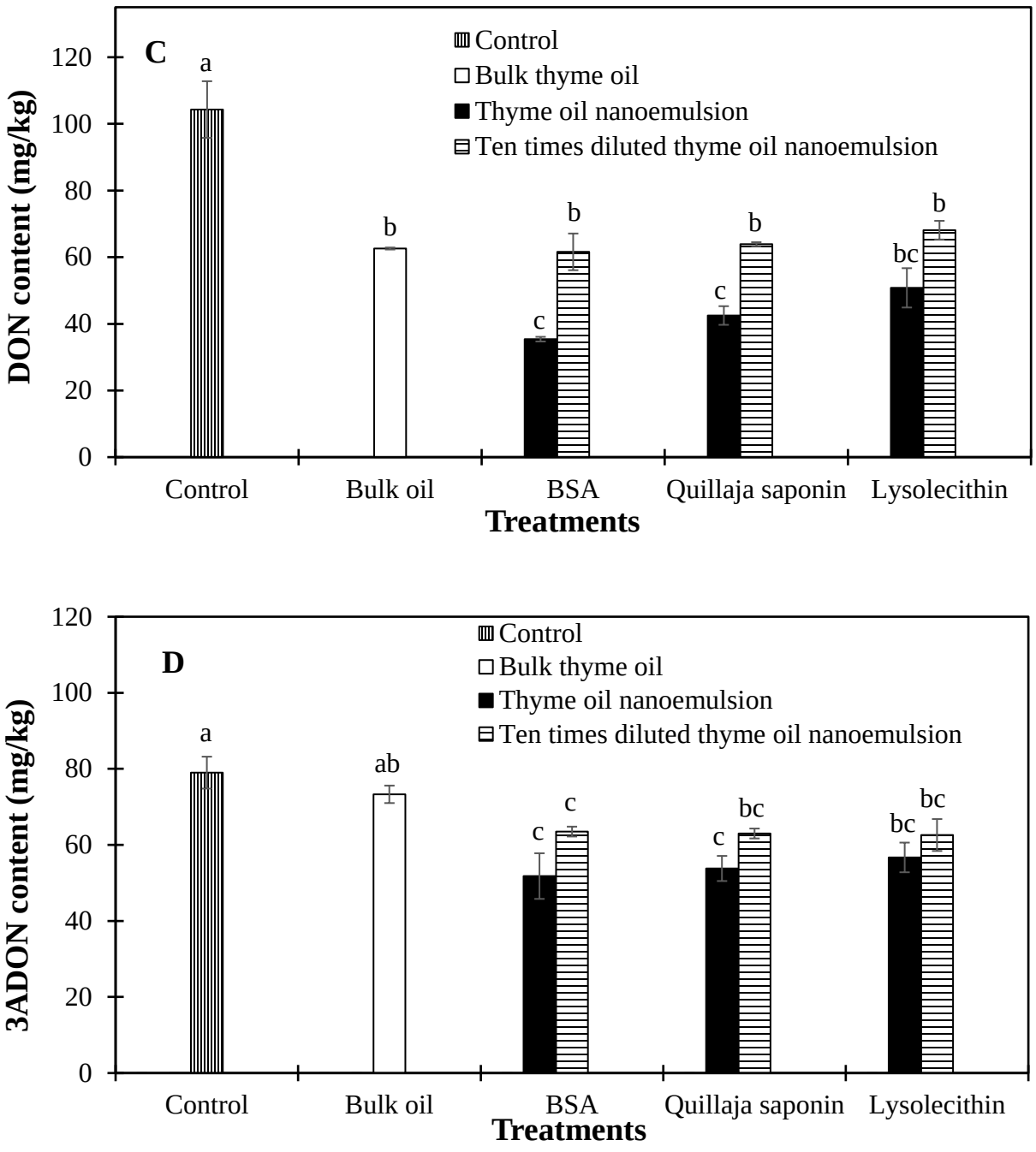


Figure 3-7. Effect of thyme oil and thyme oil-in-water nanoemulsions stabilized by different emulsifiers (BSA, quillaja saponin or lysolecithin) on mycotoxins production by *Fusarium graminearum* isolate 10-124-1 of (A) DON and (B) 15ADON and by isolate 10-125-1 of (C) DON and (D) 3ADON in rice culture (continued). The limits of quantitation (LOQ) and detection (LOD) of the method were 0.20 and 0.10 mg/kg, respectively. Columns with different letter were significantly different from each other ($p<0.05$).

The results clearly demonstrated that all treatments (e.g., bulk thyme oil, thyme oil-in-water nanoemulsions, and 10-fold dilute nanoemulsions) significantly inhibited mycotoxin production in *Fusarium graminearum*-inoculated rice as compared with the control. Mycotoxin inhibitory activity was dose-dependent since the inhibitory activity of nanoemulsions increased with increasing the thyme oil concentration. For instance, DON production in 10-fold diluted thyme oil-in-water nanoemulsion treatment was much higher than the treatments with either bulk thyme oil or undiluted nanoemulsions (Fig. 3-7 A & C). In addition, thyme oil in the nanoemulsion form exhibited higher mycotoxin inhibitory activity than when in bulk form, suggesting that encapsulation of thyme oil into nanoemulsions greatly improved its inhibitory efficacy. DON produced by isolate 10-125-1 was 62.6 and 35.4 mg/kg when treated with bulk thyme oil and undiluted thyme oil-in-water nanoemulsions respectively. These represent declines of 40% and 66% when compared to the control (Fig. 3-7C). Based on these results, the conversion of thyme oil into a nanoemulsion greatly enhanced mycotoxin inhibitory activity. Similar results of significant improvement in the antimicrobial activity of essential oils, such as limonene (Donsì et al., 2011), thyme, (Moghimi et al., 2016) and peppermint essential oils when incorporated into nanoemulsion delivery systems has been reported (Liang et al., 2012b).

Mycotoxin inhibitory activity of thyme oil-in-water nanoemulsions did not follow a similar pattern between the two isolates 10-124-1 and 10-125-1. Particularly, isolate 10-124-1 was more sensitive to the action of all studied nanoemulsions with regard to the inhibition of mycotoxin production. As shown in Fig. 3-7A & C, 99.0% inhibition of DON was achieved in the isolate 10-124-1 by exposure to the non-diluted thyme oil-in-water nanoemulsion formulated with BSA. On the other hand, DON was only reduced by ~66.0% in isolate 10-125-1 upon the addition of the same nanoemulsion. Different responses to *Fusarium graminearum* isolates

against the same antifungal agent have been previously reported by Pagnussatt and coworkers (2014). They found that phenolic extracts had different inhibition activities for mycelia growth and mycotoxin productions between the two *Fusarium graminearum* isolates (Pagnussatt et al., 2014). Furthermore, among the non-diluted nanoemulsions (750 mg/kg) stabilized by three different natural emulsifiers, BSA-stabilized thyme oil-in-water nanoemulsion expressed an equal or greater mycotoxin inhibitory activity against DON and 15ADON production in isolate 10-124-1 (Fig. 3-7A & B). Greater inhibitory activities of protein stabilized nanoemulsions against spore germination have been previously reported due to the larger number of sites suitable for interaction with protein-stabilized nanoemulsion on the fungi spore (Ribes et al., 2017). However, the mechanisms behind observed differences in mycotoxin inhibitory activity among the three types thyme oil-in-water nanoemulsions remains unknown, but may relate to the ability of BSA- stabilized essential oil to facilitate the transportation of essential oil through the cell membrane of the target fungal spores, resulting in enhanced of mycotoxin inhibitory activity. However, there was no statistically significant difference among the three different sources of natural emulsifier stabilized nanoemulsions on inhibition of DON and 3ADON production by isolate 10-125-1 (Fig. 3-7C & D). With the addition of 10-time dilute nanoemulsions (75 mg/kg), no difference on mycotoxin inhibitory activity was observed among the nanoemulsions fabricated by three natural emulsifiers. On the basis of these results, it can be concluded that thyme oil has an important role on inhibition of mycotoxin production.

Conclusion

In summary, 1.5 wt % of thyme oil-in-water nanoemulsion (d<200 nm) stabilized with 1.0 wt % of BSA, quillaja saponin, or soy lysolecithin was fabricated using a novel electro-hydraulic microfluidizer. The particle size of BSA and quillaja saponin-stabilized thyme oil-in-

water nanoemulsions remained relatively stable during 28 days' storage due to the stronger electrostatic repulsion and steric hindrance between the droplets. The thyme oil-in-water nanoemulsions containing three different sources of natural emulsifiers greatly inhibited mycelial growth and spore germination by disrupting hyphae and spore morphology. Thyme oil when imbedded in nanoemulsion droplets showed an improved mycotoxin inhibitory activity compared to the bulk thyme oil. Among the three natural emulsifiers, BSA-stabilized thyme oil-in-water nanoemulsion exerted an equal or higher inhibitory activity in terms of mycelial growth and mycotoxin production. These results redefine the role of natural emulsifiers and explore the broad horizon of opportunity for food grade thyme oil-in-water nanoemulsions to effectively inhibit both fungal growth and mycotoxin production. The future research will be applying thyme oil-in-water nanoemulsion in conjunction with other preservatives or hurdle preservation techniques to realize a synergistic alternative to minimize negative organoleptic effects in food matrix.

CHAPTER 4. CLOVE OIL-IN-WATER NANOEMULSION MITIGATES FUNGAL BIOMASS AND TRICHOTHECENE MYCOTOXINS PRODUCTION DURING THE MALTING OF FUSARIUM-INFECTED BARLEY

Abstract

Fusarium and mycotoxin contamination in malting barley is of great concern because prevention techniques are limited in their effectiveness in the field. Our recent *in vitro* study found that clove oil-in-water nanoemulsion can act as a high-efficient antifungal agent against fungal growth, sporulation, and mycotoxin synthesis by *Fusarium graminearum*. We therefore explored the application potentials of clove oil-in-water nanoemulsion during micro-malting process. The impact of emulsifier types (Tween 80, BSA and quillaja saponins) on the formation and stabilization of clove oil-in-water nanoemulsion, the mitigation effects on mycotoxin levels and fungal biomass, and the clove oil flavor residues on malts were measured. We observed that clove oil-in-water nanoemulsion at a concentration of 1.5 mg clove oil/g nanoemulsion showed a negligible influence on germinative energy of barley, while still efficiently eliminated the DON levels and toxicogenic fungal biomass as quantified by Tri5 DNA content. Among the three emulsifiers, Tween 80-stablized clove oil-in-water nanoemulsion displayed higher mycotoxin inhibitory activity and less flavor impact on the final malt. The results showcase the huge application potential of essential oil-in-water nanoemulsion as an effective antifungal agent as well as novel malt flavors in the malting industry.

Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereal grains grown in the United States. Approximately 33% of the US barley is utilized by the malting and brewing industries, as well as human food, and much of the rest is directly used for animal feed

(Hückelhoven, Hofer, Coleman, & Heß, 2018). Barley is susceptible to a variety of fungal diseases such as Fusarium head blight (FHB), an economically devastating disease causing significant losses in both grain yield and quality. Furthermore, FHB has also led to the production of *Fusarium* mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA). Among them, DON is the most common mycotoxin which has been detected in many of the world's barley and malt producing regions (Pestka & Smolinski, 2005). Although good agricultural practice in conjunction with the well-controlled storage conditions can control *Fusarium* mycotoxins in barley to a certain extent, the complete prevention of *Fusarium* mycotoxins in the raw materials is not practically achievable.

Previous studies have shown that mycotoxin levels of DON can increase during the malting of *Fusarium* contaminated grains and that DON can be transferred to beer at a very high level of recovery (Lancova et al., 2008). Malting is a controlled grain germination process which basically include grain cleaning, steeping, germination and kilning. The ideal way to control mycotoxins in malt and beer is to avoid utilizing infected grains. This, however, is always inescapable mainly by virtue of the following two reasons. First, the malting industries have been dealing with FHB-infected barley over 25 years due to the difficulties to fully vanish them in the field. In general, barley with DON levels greater than 0.5 mg/kg is not used for malt; while a lower level (<0.5 mg/kg) is acceptable as mycotoxin may appreciably diminish during steeping process and remain low to below limit of detection in the final malt. Second, certain barley seeds with a low initial DON level (<0.5 mg/kg) may occasionally exhibit aberrant behavior, where DON increased significantly during the malting process. A recent research studied the fate of mycotoxin in 20 naturally FHB-infected barley seeds with an initial DON varying from 0.1 to 0.7 mg/kg during the malting process, and observed the decline of DON after steeping step, but a

quick soaring during germination in two samples (Yu et al., 2019). The worst case scenario is that the turnover of DON can hardly be predicted but identified after the completion of malting, leading to the loss of grain or health issue to the consumers. Currently, the inhibition of mycotoxin production during the malting process is even more important considering the rapid rise of the craft malting and brewing industries worldwide. Craft maltsters, by definition, must utilize a certain percentage of locally produced barley malt for brewing. As many of these craft operations are in regions impacted by FHB, there will be an increasing pressure for the utilization of mycotoxin contaminated grain in years where the FHB is widespread. In addition to the food safety concern, brewing barley contaminated with mycotoxin can dramatically influence their performance during the malting due to the change of chemical composition and enzyme activity (Vegi, Schwarz, & Wolf-Hall, 2011). Until now, there has been very little research on mycotoxin mitigation strategies for FHB-infected barley during the malting process. These approaches such as physical treatments, chemical agents, and biological agents, may either leave undesirable residues in the final malt product or generate a significant negative impact on barley germination qualities (Dodd et al., 2011; Kottapalli & Wolf-Hall, 2008). Hence, there is an urgent need to develop strategies that can effectively suppress the production of mycotoxins in barley during the malting process without adversely impacting the germinative energy of malt.

Recently, essential oils (EOs) have received attention in the food industry as they are natural antifungal agents with a broad-spectrum of antifungal activities and inhibition effect against mycotoxin biosynthesis (Tullio et al., 2007). However, direct application of EOs as antifungal agents during the food process is impractical. This is because EOs, mainly consisting of terpenes, terpenoids and phenols, are not water soluble and are highly volatile (Rao, Chen, & McClements, 2019). Such limitations could potentially be overcome by incorporating EOs into

appropriate delivery systems. EO-in-water nanoemulsion is such a delivery system gaining popularity due to its greater physical stability, as well as its potency to increase the antifungal activity of EOs. Our recent series of *in vitro* studies found that the antifungal and DON inhibitory activities of the five selected EO (thyme, lemongrass, cinnamon, peppermint, and clove)-in-water nanoemulsions were considerably improved over that of bulk EOs (Wan, Zhong, Schwarz, Chen & Rao, 2019). Thyme and clove oil-in-water nanoemulsions had greater antifungal and mycotoxin inhibitory activities among the five selected EOs. Still, our findings did not show whether or not they share the similar capacity in a real malting process. In terms of nanoemulsion formulation, a number of food-grade emulsifiers are available for producing nanoemulsions including natural emulsifiers (e.g., proteins and polysaccharides) and synthetic ones (e.g., Tween 80). However, the influence of emulsifier type on its antifungal and mycotoxin inhibitory activities during the malting process has not been addressed.

The current study was set forth to evaluate the efficacies of clove oil-in-water nanoemulsions stabilized by three different emulsifiers, i.e., bovine serum albumin (BSA), quillaja saponins (Q-Naturale), and Tween 80, on preventing *Fusarium* growth and mycotoxin production, as well as maintaining germinative energy of barley seeds during the micro-malting process. After applying clove oil-in-water nanoemulsions at the first steeping stage of barley malting, their antifungal and mycotoxin inhibitory activities at each stage of micro-malting process were evaluated through the quantification of fungal biomass and DON, respectively. Moreover, clove oil volatile residues in the final malt products were also quantified to investigate the impact of clove oil on the malt flavor. The findings could rationalize the application of EO-in-water nanoemulsion in the malting industry to produce safe and high-quality malt.

Materials and methods

Materials and chemicals

Six-rowed FHB-infected malting barley seeds (cultivar: Stellar-ND) were obtained from trials conducted at North Dakota State University according to previous inoculation procedure (Urrea, Horsley, Steffenson, & Schwarz, 2002). The barley infected with FHB had an initial DON concentration of 5 mg/kg. Clove oil (purity ≤100%), bovine serum albumin (BSA), polyoxyethylene (20) sorbitan monooleate (Tween 80), eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide were purchased from Millipore Sigma Co. (St Louis, MO, USA). Corn oil was obtained from a local supermarket (Fargo, ND, USA). Quillaja saponin (Q-Naturale) was kindly provided by Ingredion Inc. (Westchester, IL, USA). DNeasy Plant Mini Kit was purchased from Qiagen Inc. (Valencia, CA, USA). SsoAdvanced^{TM} Universal SYBR® Green Supermix was purchased from Bio-Rad (Bio-Rad laboratories, Hercules, CA, USA).

Preparation of clove oil-in-water nanoemulsions

The coarse clove oil-in-water nanoemulsions were produced by mixing 1.5 wt % of clove oil, 3.5 wt % of corn oil, 1.0 wt % emulsifier (Tween 80, BSA, and quillaja saponin), and 94.0 wt % of phosphate buffer (pH 7.0, 10 mM) using a high-speed hand blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 mins. The coarse nanoemulsions were then passed through a high-pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) at 68.95 MPa with two cycles for further reduction in particle size. The finished nanoemulsions were collected and stored in test tubes sealed with screw caps at a room temperature (21 ± 3 ℃) for further analysis. The final clove oil concentration in nanoemulsions was 15.0 mg/g nanoemulsions.

Characterization of clove oil-in-water nanoemulsions

Mean particle size, size distribution and ζ-potential

The mean particle size, particle size distribution, and ζ-potential of three types of clove oil-in-water nanoemulsions were determined using dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK). Nanoemulsions were diluted 20× and 100× using pH 7 phosphate buffer for mean particle size and ζ-potential measurements, respectively, to avoid multiple scattering effects.

Long-term stability

Long-term stability of three types of clove oil-in-water nanoemulsions were determined by measuring the mean particle size and particle size distribution during 28-day storage.

Germinative energy of FHB-infected barley

Germinative energy (GE) was measured according to the Barley-3C method from American Society of Brewing Chemists (ASBC) (ASBC, 1999) with slight modification. Briefly, 100 barley kernels were placed in a petri dish with two filter papers on the bottom for water absorption. Based on the preliminary test of germinative energy test of barley seeds treated by five EOs-in-water nanoemulsions at concentration of 0, 1, 5, 10 mg essential oil/g nanoemulsion, the content of EOs more than 5 mg/g can completely inhibit the germination of barley seed. Therefore, four milliliters of phosphate buffer (10 mM, pH 7.0) (as control), 5×, and 10× diluted clove oil-in-water nanoemulsions were added to each dish to achieve the final clove oil concentrations in nanoemulsion as 1.5 and 3.0 mg clove oil/g nanoemulsions. Each dish was covered and stored at 21 ℃ with more than 90% relative humidity. Sprouted kernels were counted and removed daily for 4 days, and then the percentage of sprouted kernels were determined after 4-day germination.

Micro-malting process

The micro-malting process was performed in a temperature and humility controlled incubator according to the procedure described by Karababa and coworkers with a few modifications (Karababa, Schwarz, & Horsley, 1993). The conditions and schedule for micro-malting process were optimized in our preliminary experiments (Table 4-1**)**. For the control group, FHB-infected barley (20 g, dry basis) was steeped to 45% of moisture by placing100 ml of ultrapure water in a 500 ml beaker. For the treatment group, the mixture of 10 ml clove oil-in-water nanoemulsion with 90 ml of ultrapure water was transferred to the FHB-infected barley (20 g, dry basis) in a 500 ml beaker. All samples were placed in a temperature-controlled incubator at 16 ℃. The steeping schedule was set as: 6 h of steeping followed by 2 h of air-rest, then 6 h of steeping followed by 2 h of air-rest, finally 8 h of steeping. At each steeping stage, wastewater was decanted and replaced by fresh ultrapure water. After steeping, barley samples were allowed to germinate for 4 days at 16 ℃ with ~95% relative humidity. Kilning was conducted by increasing temperatures in a stepwise manner as follows: the temperature was held at 50 ℃ for 8 h, and then 60 ℃ for 2 h, finalized at 85 ℃ for 2 h. The kilned barley after the removal of rootlets was ground for further analysis.

Table 4-1. Schedule of the micro-malting process

	Micro-malting schedule	**Duration (h)**
Steeping	The first steeping step	6
	Air rest	2
	The second steeping step	6
	Air rest	2
	The third steeping step	9
Germination	Day 1	24
	Day 2	24
	Day 3	24
	Day 4	24
Kilning	50 ℃	8
	60 ℃	2
	85 ℃	2

Fusarium Tri5 DNA quantification

The fungal DNA from barley and malts were extracted using a DNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA, USA). The Tri5 PCR primers TMT_fw (5'-GATTGAGCAGTACAACTTTGG-3') and TMT_rev (5'-ACCATCCAGTTCTCCATCTG-3') were employed to amplify the Fusarium Tri5 specific amplicon in the SYBR Green based quantitative real-time polymerase chain reaction (qPCR) performed by a CFX96 Real-time System thermocycler (Bio-Rad, Hercules, CA, USA) according to previously published method with some modifications (Jin, et al., 2018). In brief, the total reaction volume was 10.0 µl, including 5.0 µl of SsoAdvanced TM Universal SYBR® Green Supermix, 2.0 µl of water, 0.50 µl of TMT_fw (10 pmol) and 0.50 µl of TMT_rev (10 pmol), and 2.0 µl of DNA template. The concentrations of Tri5 DNA in the original barley and the malt samples were quantified according to a standard curve. A standard curve was prepared by simultaneously performing a serial dilution of purified *Fusarium graminearum* Tri5 amplicon generated with *Fusarium graminearum* DNA template in barley and malts. The *Fusarium graminearum* DNA template was extracted from mycelial cultures of *Fusarium graminearium* grown on potato dextrose agar. The serially diluted concentration of Tri5 DNA consisted of nine levels: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 100 ag. Results were expressed in mg Tri5 DNA/kg barley sample.

Mycotoxin quantification

Mycotoxins including DON and its 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol (NIV), diacetoxyscirpenol (DAS), HT-2 toxin, T-2 toxin, and zearalenone (ZEA) at each step of micro-malting process were quantified using GC-MS as

previously described (Wan, Zhong, Schwarz, Chen, & Rao, 2018). Results were expressed in mg mycotoxin/kg malt.

Quantification of clove oil residues in malt

Eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide, accounting for 85.5% of chemical components, were quantified as the major volatiles in clove oil (Wan, Zhong, Schwarz, Chen, & Rao, 2019b). Standard curves were prepared by diluting the five chemical standards in barley flour. Each standard (60 μl) was spiked in barley flour (1 g, dry basis) in 20-ml GC vials capped by an aluminum cap with PTFE/silicone septa. The vials were sonicated in the ultrasonic bath for 60 min, while agitating at 250 rpm. After sonication, vials were equilibrated for 24 h prior to analysis. The concentration of clove oil residues in malts were analyzed using Agilent 7890B/5977A headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) following the methods reported with some modifications (Xu, Jin, Lan, Rao, & Chen, 2019). Briefly, the vials were incubated for 15 min at 80 ℃ in the autosampler heating block under agitation at 250 rpm (CTC Analytics, Zwingen, Switzerland). Then, the SPME fiber needle (50/30 μm DVB/CAR/PDMS, Supelco Inc) was inserted into the vial for 63.5 min at 80 ℃ to adsorb volatiles and was then transferred to the injector port (250 ℃) for 5 min desorption. The concentrations of clove oil residues in malt were quantified according to the standard curves. Results was expressed in mg clove oil residues/kg barley sample.

Statistical analysis

All measurements were conducted three times using freshly prepared samples and expressed as mean ± standard deviation (SD). The statistically significant difference between

mean values was determined by one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) test at a 95% confidence level.

Results and discussions

Physical properties of clove oil-in-water nanoemulsions

The impact of emulsifier type on the particle size and ζ-potential of clove oil-in-water nanoemulsions produced under a fixed homogenization condition (68.95 MPa for 2 cycles) was investigated (Fig. 4-1). The mean particle diameters of freshly prepared clove oil -in-water nanoemulsions stabilized by Tween 80, BSA, and quillaja saponins were 130.95±5.40 nm, 152.78±4.36 nm, and 179.82±4.36 nm, respectively (Fig. 4-1A). The particle distribution of all samples exhibited a monomodal pattern (Fig. 4-1B). This result indicated that all the emulsifiers were able to fabricate ultrafine and nanoscale particles, but that Tween 80 was slightly more effective at producing smaller particles under the same homogenization condition. Different efficacy of emulsifiers on nanoemulsion formation was found in other studies. For example, Yang *et al.* observed that Tween 80 was more efficient than quillaja saponins at producing smaller droplets of middle chain triacylglycerol (MCT) oil-in-water nanoemulsions (Yang, Leser, Sher, & McClements, 2013). We also found that smaller particle size of thyme oil-in-water nanoemulsion was formed by BSA rather than quillaja saponins (Wan, Zhong, Schwarz, Chen, & Rao, 2019a). The properties of emulsifiers are the major reasons attributing to such differences, including (i) the absorption rate of emulsifier on the oil droplet surfaces to prevent droplet re-coalescence during homogenization; (ii) the ability of emulsifier to lower the interfacial tension; and (iii) the capability of emulsifier to generate a strong electrostatic and/or steric repulsive interaction between oil droplets (Ribes et al., 2017).

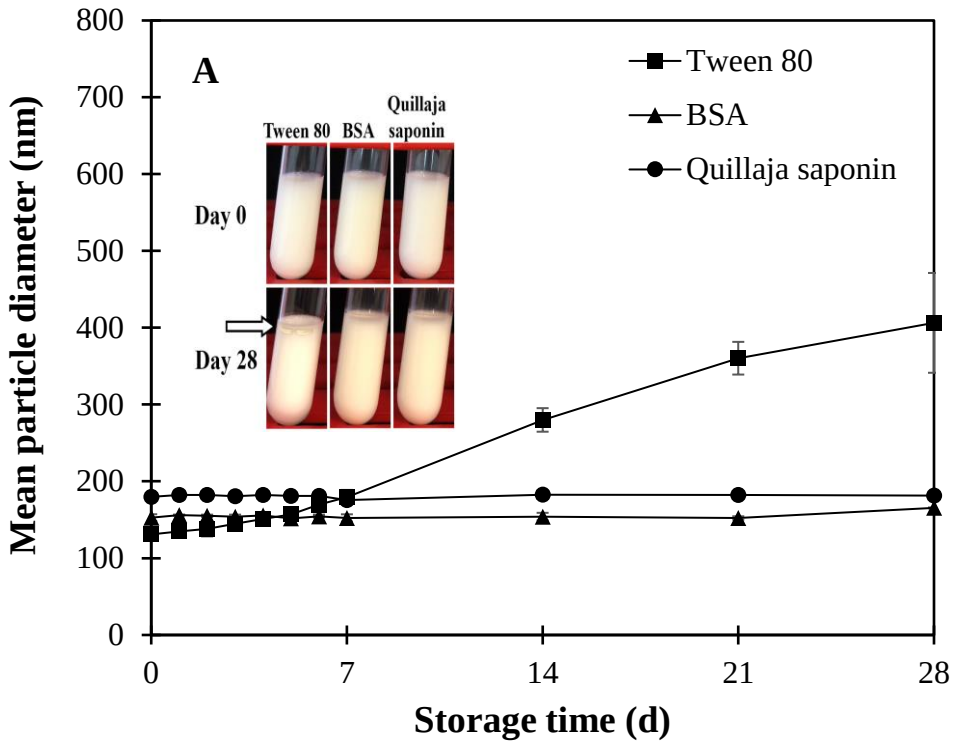


Figure 4-1. Impact of emulsifier type on (A) the mean particle diameter (nm), (B) the particle size distribution (relative intensity, %), and (C) the zeta-potential (mV) of clove oil-in-water nanoemulsions stabilized by Tween 80, BSA, and quillaja saponin.

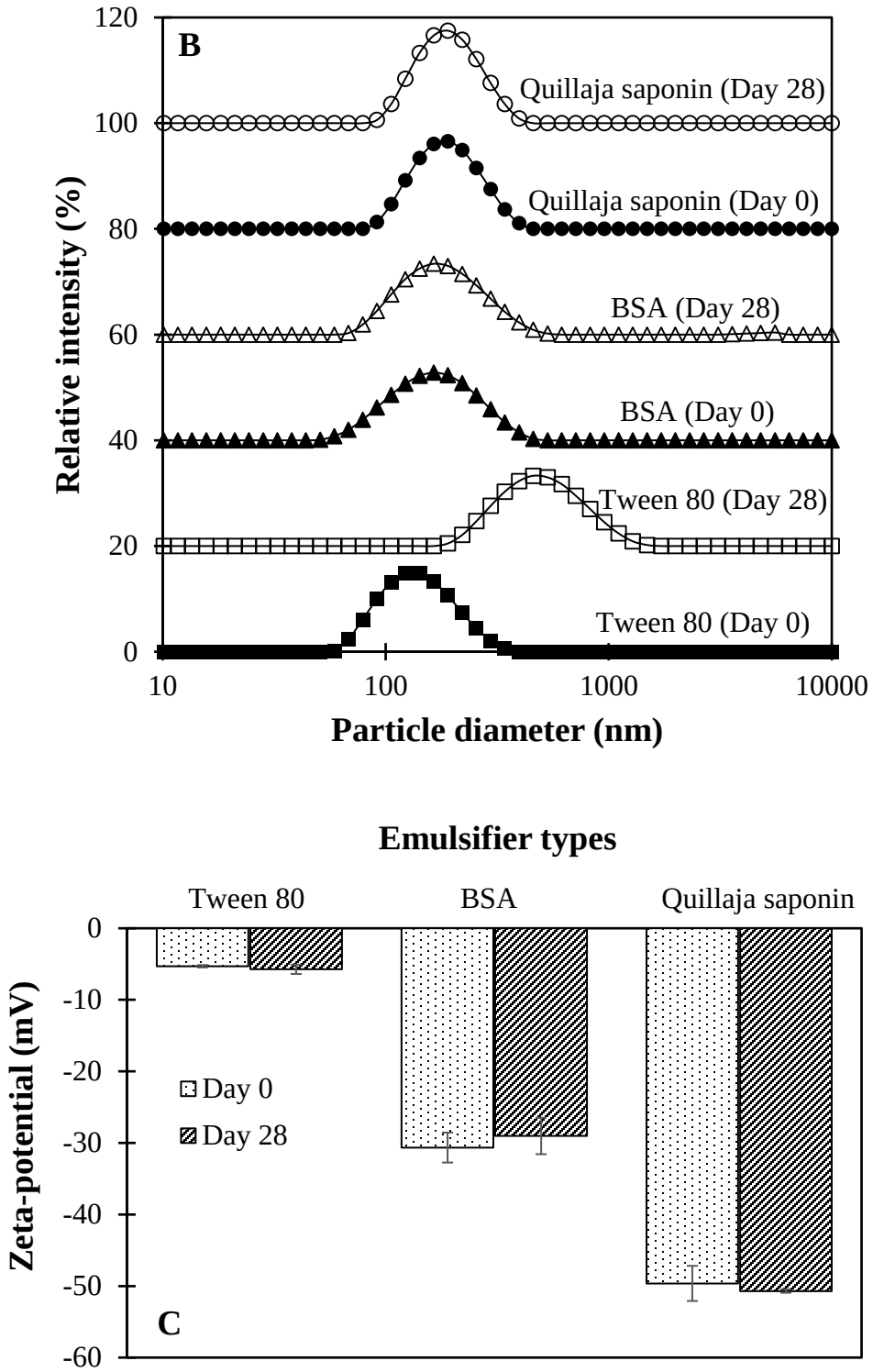


Figure 4-1. Impact of emulsifier type on (A) the mean particle diameter (nm), (B) the particle size distribution (relative intensity, %), and (C) the zeta-potential (mV) of clove oil-in-water nanoemulsions stabilized by Tween 80, BSA, and quillaja saponin (continued).

The ζ-potential of droplets is closely related to physical stability and functionality of nanoemulsions (Ozturk & McClements, 2016). For instance, charged nanoemulsion might interact with the charged fungal cell surfaces, thus influencing the antifungal activity of the whole nanoemulsion system (Ribes et al., 2017). Therefore, we measured the ζ-potential of clove oil-in-water nanoemulsions stabilized by three emulsifiers (Tween 80, BSA, and quillaja saponins). As shown in Fig. 4-1C, the ζ-potential values of clove oil-in-water nanoemulsions stabilized by Tween 80, BSA and quillaja saponins at pH 7.0 were –5.31±0.16 mV, –30.65±2.09 mV, and –49.64±2.45 mV, respectively. In agreement with many other studies, we noticed that Tween 80 stabilized clove oil-in-water nanoemulsion possessed a slight negative charge (~ –5 mV), presumably owing to the impurities of free fatty acids or polar constituents in Tween 80 (Zhu et al., 2019). Both BSA and quillaja saponins stabilized clove oil-in-water nanoemulsions exhibited a larger magnitude of negative charge compared to Tween 80. This is expected as the pH (7.0) of the nanoemulsions was above the isoelectric points of BSA (4.7) and the saponins constitutes in quillaja saponins (3.25) (Tabibiazar et al., 2015).

Long-term stability of clove oil-in-water nanoemulsions is essential for determining their applications in food systems. Thus, the stability of the three types clove oil-in-water nanoemulsions was evaluated on account of their mean particle diameters and particle size distribution after stored at 23 ℃ for 28 days. As shown in Fig. 4-1A, the mean particle diameter of clove oil-in-water nanoemulsions stabilized by BSA or quillaja saponins were highly stable and remained constant over 28 days storage. The size distribution pattern of them also remained unchanged after 28 days storage, which further proved their long-term stability. This can be explained by the strong electrostatic repulsions generated from the high ζ-potential values of nanoemulsion droplets surrounded by BSA and quillaja saponins (Herrera-Rodríguez, López-

Rivera, García-Márquez, Estarrón-Espinosa, & Espinosa-Andrews, 2018). In general, steric repulsion and electrostatic repulsion are the two predominant forces controlling the stability of nanoemulsions (Wu et al., 2019). It is commonly believed that highly charged nanoemulsion droplets with the magnitude of ζ-potential beyond greater than 30 mV could provide a strong electrostatic repulsion between them, thus ensuring the long-term stability of nanoemulsions (Gurpreet & Singh, 2018). In contrast, the mean particle size diameter of clove oil-in-water nanoemulsion containing Tween 80 increased appreciably after 7 days storage as evidenced by the size increasing from 130.95 nm to 406.33 nm concomitant with a visible oiling off in the test tube. Additionally, its particle size distribution shifted towards larger sizes. Such a high long-term instability may be caused by Oswald ripening, a common physical phenomenon occurring in EO emulsions or nanoemulsions, through the mass transport of the relatively high water solubility EO from one droplet to another (Djerdjev & Beattie, 2008). As the concentration of Tween 80 (1.0 wt %) employed in the current study was higher than its critical micelle concentration, the additional Tween 80 molecules may spontaneously self-assemble into micelles. Tween 80 micelles were capable of solubilizing and transporting clove oil molecules lingering in the aqueous phase into their hydrophobic interiors (Rao & McClements, 2012). Similar results were also found in sodium dodecyl sulfate (SDS)-stabilized octane and decane oil-in-water emulsions system, where the Ostwald ripening rate increased steeply in the presence of SDS micelles (Djerdjev & Beattie, 2008).

Influence of clove oil-in-water nanoemulsions on germinative energy (GE) of FHB-infected barley

Quality of barley malt is essential for brewing and is more reliant on the employed barley seeds. High quality barley seeds should be able to germinate vigorously, rapidly, and uniformly

during the malting process. The quality of barley seeds can be evaluated by performing a standard germinative energy (GE) test prior to the malting production as GE is an indicator that predicts the malting potential of barley seeds (Frančáková, Líšková, Bojnanská, & Mareček, 2012). Essential oils as plant-based secondary metabolites display various levels of allelopathic effects against seed germination (Shokouhian, Habibi, & Agahi, 2016). Certainly, one would like to apply antifungal clove oil-in-water nanoemulsion without sacrificing the GE of barley seeds during the malting. Thus, the GE test was conducted to evaluate the impact of clove oil concentration and emulsifier type in nanoemulsions on the germination of FHB-infected barley seeds. The optimized concentration of clove oil-in-water nanoemulsions with a minimal inhibitory effect on the GE of barley seeds was further applied in the micro-malting process.

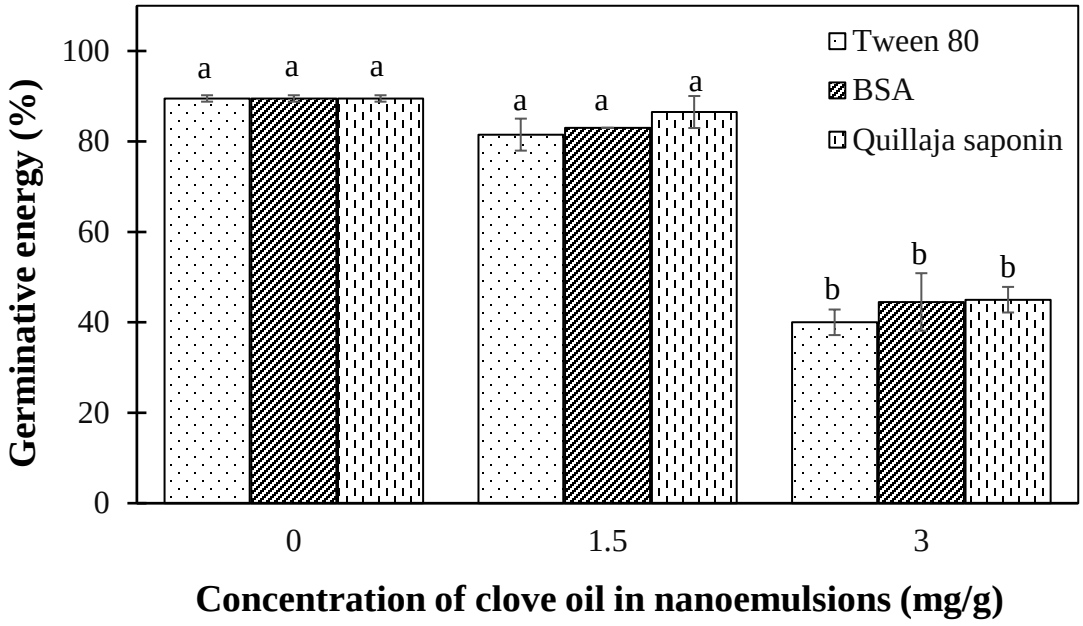


Figure 4-2. Impact of emulsifier type and clove oil-in-water nanoemulsion concentrations in germinative energy of FHB-infected barley. Columns that do not share a letter are significantly different (p<0.05).

As displayed in Fig. 4-2, the GE values of FHB-infected barley was only about 89.5%. It should be pointed out that the GE is heavily inhibited in FHB-infected barley on account of the damages of barley kernels and seeds derived from mycelium invasion and mycotoxin accumulation (Wolf-Hall, 2007). With respect to the clove oil concentration, the GE values of barley seeds decreased rapidly with the increase of clove oil concentration in nanoemulsions. As one can see from Fig. 4-2, the GE of FHB-infected barley seeds was reduced by half as the clove oil concentration raised from 1.5 to 3.0 mg/g nanoemulsion in all tested nanoemulsions. Nevertheless, there was no statistically significant difference of GE value between the control (89.5%) and the treatment groups at a clove oil concentration of 1.5 mg /g nanoemulsion, indicating the negligible effect on the GE of barley seeds. Albeit barley seeds treated by quillaja saponins-stabilized clove oil-in-water nanoemulsion had a slightly higher GE, there was no statistically significant difference among the three emulsifiers in clove oil-in-water nanoemulsions. For instance, with the addition of 1.5 mg/g nanoemulsion, the GE of barley seeds was 86.5%, 83.0% and 81.5% for quillaja saponins, BSA and Tween 80, respectively. On the basis of these results, the concentration of clove oil in nanoemulsion at 1.5 mg/g was chosen for the following micro-malting process. Other than EO concentration and emulsifier type, the EO type has a profound impact on GE of barley seeds. On another parallel study using thyme oil-in-water nanoemulsion prepared under same condition, we found the GE of the same barley seeds was completely suppressed after the treatment. This denoted that not all the EO-in-water nanoemulsions are applicable in preventing mycotoxin production, particularly in the malting process.

Influence of clove oil-in-water nanoemulsions on the fate of Fusarium Tri5 DNA in barley during the malting process

Fusarium Tri genes are involved biosynthesis of trichothecenes (Tri) mycotoxins from farnesyl pyrophosphate (FPP) (Sakuda et al., 2016). Among all the Tri genes, Tri5 gene is responsible for encoding the trichodiene synthase that catalyzes the first cyclization step of the trichothecene biosynthesis (Morcia et al., 2013). In recent years, the sequence of the Tri5 gene has been widely adopted as a target for the quantifying the biomass of trichothecene-producing *Fusarium* spp. in cereal grains (Burlakoti et al., 2007). The quantity of *Fusarium spp.* Tri5 DNA also exhibited a strong positive correlation with the development of DON in barley, wheat, and rye (Jin et al., 2018; Leišová et al., 2006). In this section, Tri5 DNA content in the barley samples in the absence/presence of clove oil-in-water nanoemulsions was quantified during each step of the micro-malting including steeping, germination and killing (Fig. 4-3).

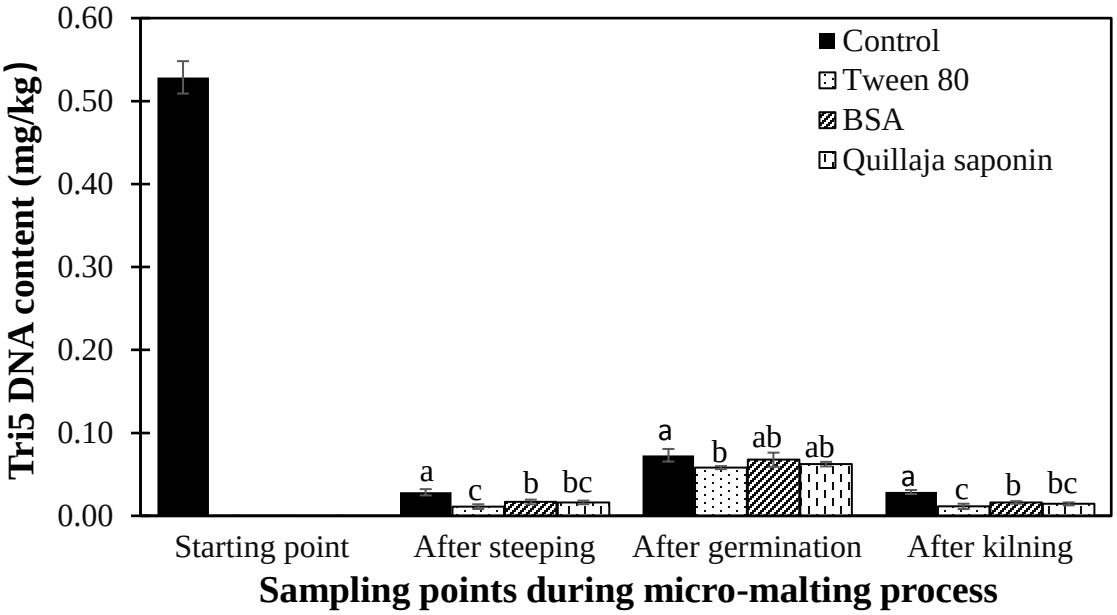


Figure 4-3. Impact of emulsifier type on Tri5 DNA content of FHB-infected barley during the micro-malting process. Differences were compared at each micro-malting operation unit among three types clove oil nanoemulsions. Columns that do not share a letter are significantly different (p<0.05).

Prior to Tri5 DNA quantification, the optimum time point to apply clove oil nanoemulsion during the malting process was obtained. Initially, clove oil nanoemulsions were applied to immerse barley seeds in the first stage, second stage of steeping, and to spray on the top of barley seeds right before germination. We found that DON levels in the final malt decreased dramatically when clove oil nanoemulsions were added in the first stage of steeping process, compared with those treated in other steps. Thus, introduction of clove oil nanoemulsions to barley seeds during the first stage of steeping was selected to evaluate the role of clove oil nanoemulsions on the development of both Tri5 DNA and mycotoxin during the malting of FHB-infected barley.

The concentration of Tri5 DNA fluctuated substantially in each step of the micro-malting process in the sense that the contents of Tri5 DNA decreased noticeably after steeping, raised after germination, and reduced fairly after kilning in all treatment groups including the control. For instance, Tri5 DNA content in control barley dramatically dropped from 0.529 to 0.028 mg/kg after steeping, escalating to 0.073 mg/kg after 4 days of germination, and ended up with 0.029 mg/kg in the final kilned malt. Similar trends were also found in quantification of Tri5 DNA gene during the malting of healthy, naturally infected, and *Fusarium graminearum* inoculated barley seeds (Vegi et al., 2011). These results again indicated that although steeping can remove a large amount of fungal biomass, the remnants can still grow during the germination steps and produce more Tri5 DNA.

Upon the treatment of clove oil nanoemulsions to the infected barley, levels of Tri5 DNA were further reduced at each step of malting compared to the control, which implied the antifungal activity of clove oil nanoemulsion in barley during the malting process. In particular, Tri5 DNA content in the final malt was 0.011, 0.016, and 0.014 mg/kg after applying clove oil

nanoemulsion stabilized by Tween 80, BSA, and quillaja saponins, respectively (Fig. 4-3). With regard to the impact of nanoemulsion emulsifier type on Tri5 DNA content during the micro-malting process, the results clearly showed that Tween 80-stabilized clove oil nanoemulsion had the greatest inhibitory activity against fungal growth in each step of the malting process.

Influence of clove oil nanoemulsions on mycotoxins production in barley during the malting process

The primary concern from the malting industry is being able to predict and/or control the level of mycotoxins in the finished malt as it may cause severe health issue. However, from the practical point of view, the development of mycotoxins in barley during malting varies and is contingent on the source and the infection degree of barley seeds (Schwarz, Horsley, Steffenson, Salas, & Barr, 2006). We therefore evaluated the effect of clove oil nanoemulsions on mycotoxin production in heavily infected barley at different stage of the micro-malting process.

Among all trichothecenes mycotoxins (DON, 3-ADON, 15-ADON, NIV, DAS, HT-2 toxin, T-2 toxin, and ZEA) we assessed, only DON was detected in raw barley seeds and malt samples. Consequently, DON production was demonstrated to reflect the effect of clove oil nanoemulsions on the production of mycotoxins during the malting (Fig. 4-4). The DON level in control barley samples reduced sharply from 4.70 to 0.60 mg/kg after steeping, followed by a slight increase to 0.67 mg/kg at the end of germination, and declined to a lesser extent (0.65 mg/kg) after kilning. Such dynamic changes of DON during the malting of naturally infected barley were also observed in the previous studies (Habler et al., 2016; Schwarz, Casper, & Beattie, 2014; Vegi et al., 2011). During steeping with periodical replacement of water, the water-soluble DON locating on the husk of barley seeds may be washed off. However, the germination conditions allowed the fungal residue to grow and produce mycotoxin, resulting in

the increase of DON level. The slight reduction of DON after kilning could be explained by the removal of rootlets in the final malt. The rootlets in the germinated barley were found to be a major DON enrichment site (Mastanjević et al., 2019). In the meantime, the DON may be transformed to "masked" DON, such as deoxynivalenol-3-glucoside (Lancova et al., 2008), which is out of the detection in the current study.

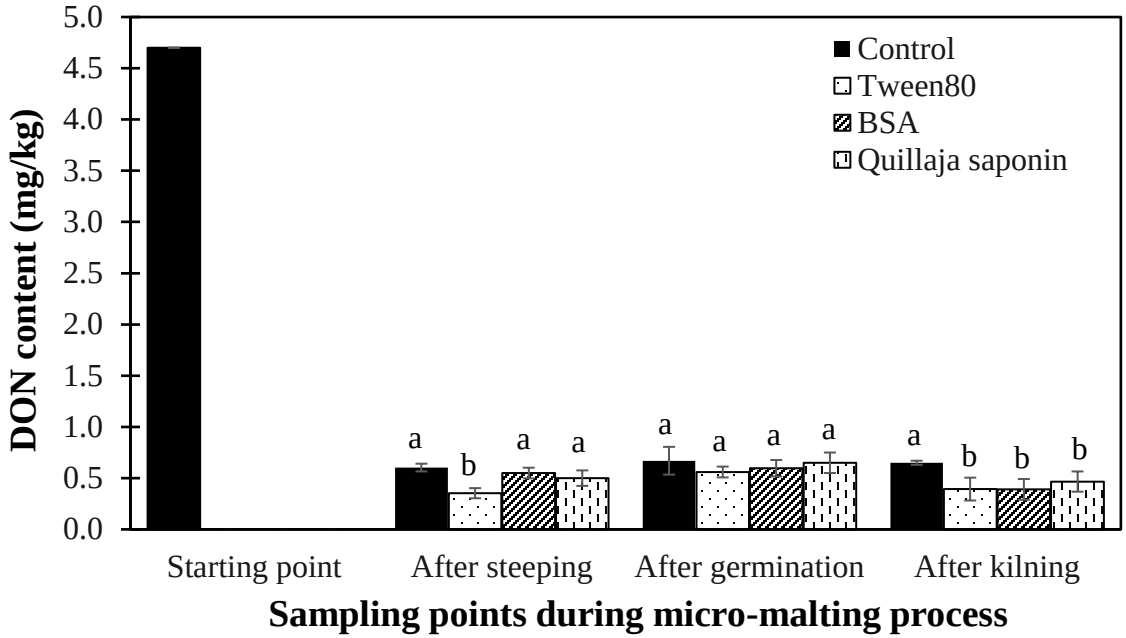


Figure 4-4. Impact of emulsifier type on DON content of FHB-infected barley during the micro-malting process. Differences were compared at each micro-malting operation unit among three types clove oil nanoemulsions. Columns that do not share a letter are significantly different (p<0.05).

A similar trend on DON development was also recorded in the treatment groups where clove oil nanoemulsion was introduced during the first stage of steeping. Moreover, all clove oil nanoemulsions showed inhibitory activities on DON production compared with the control group as the DON in all treated samples was less than 0.5 mg/kg after kilning. In terms of DON level in the final malt, statistically significant difference was not shown among three selected emulsifiers employed in clove oil nanoemulsion. This result suggested that the mycotoxin inhibitory activity was mainly originated from clove oil rather than the emulsifier. Interestingly, it was also noticed

that Tween 80-stabilized clove oil nanoemulsion had the greatest inhibitory activity on DON production after steeping with the lowest DON of 0.35 mg/kg in barley. We postulated that the smaller particle size of clove oil nanoemulsion stabilized by Tween 80 may facilitate the higher absorption rate of nanoemulsion on barley kernel, which limited the growth of fungal and the production of DON by the increased localized concentration of clove oil.

Clove oil flavor residues in final malts

Many essential oils have a strong flavor even with a trace quantity due to the high volatile compounds they contain. This has restricted the type of food products that they can be incorporated into. Although clove oil nanoemulsion showed the antifungal and mycotoxin inhibitory activities during malting, the strong flavor in the final malt may counteract such effects as malt flavor, together with hops, as the soul of the beer flavor (Chen et al., 2017). Therefore, the final malt flavor should be taken into consideration when applying clove oil nanoemulsions to mitigate mycotoxin production during malting (Broeckling et al., 2018). As previously determined in our lab, eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide are the major chemical components of clove oil (Wan, Zhong, Schwarz, Chen, & Rao, 2019b). In the current study, clove oil nanoemulsion at a concentration of 75 mg/kg barley was added to barley seeds in the first stage of steeping, and the selected five chemical constitutes were quantified after kilning to evaluate the clove oil residues in final malt (Fig. 4-5).

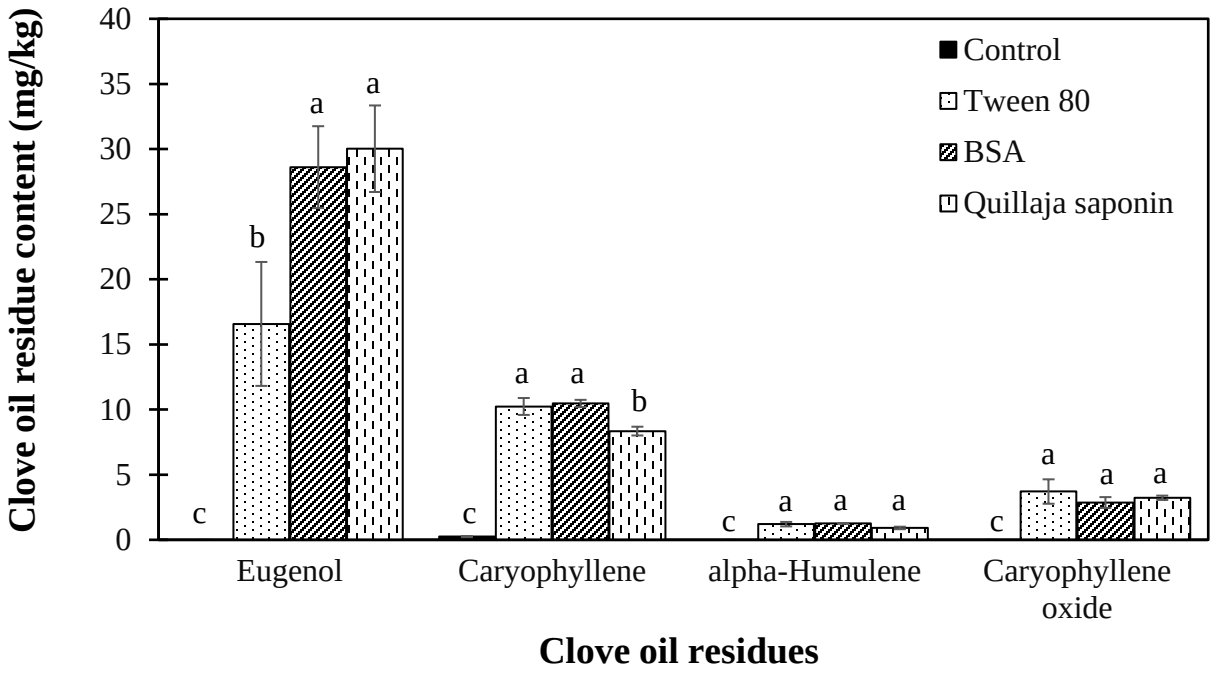


Figure 4-5. Impact of emulsifier type on clove oil residues content of malts. Differences were compared among three types clove oil nanoemulsions for each clove oil residue. Columns that do not share a letter are significantly different (p<0.05).

In the absence of clove oil nanoemulsions, eugenol, α-humulene, and caryophyllene oxide were not detected in final malt. Interestingly, 0.24 mg/kg of caryophyllene was found in the control malt, which might be generated by malting of FHB-infected barley. After being treated with clove oil nanoemulsions (75 mg/kg barley), eugenol, caryophyllene, α-humulene, and caryophyllene oxide were detected at some extent in all final malts. Among all four detectable clove oil residues in malts, eugenol was the leading chemical constitute in malts ranging from 16.08 to 30.03 mg/kg malt, follow by caryophyllene (~ 10 mg/kg malt), and trace amounts of α-humulene (~ 1 mg/kg malt) and caryophyllene oxide(~ 3 mg/kg malt), which was in consistent with their percentage concentration in clove oil. Actually, both caryophyllene and α-humulene are considered as the principal volatiles of hops. Caryophyllene oxide could also be found in final beer product due to the oxidation of β-caryophyllene during the brewing process

(Rettberg, Biendl, & Garbe, 2018). Surprisingly, eugenol acetate was not detected in any samples. The control and release of volatiles in food systems are collectively determined by their volatile nature, such as boiling point and molecular weight, processing operations, and food matrices. The disappearance of eugenol acetate in the final malts may be due to its lower affinity with barley matrix than the other four volatiles, which was then stripped off after kilning. Among three different emulsifiers stabilized nanoemulsions, eugenol concentration (16.08 mg/kg) was significantly lower in malt treated by Tween 80-stabilized clove oil nanoemulsion than that of BSA (28.61 mg/kg) and quillaja saponin (30.03 mg/kg) stabilized ones. This result indicated that emulsifier played an essential role on the retention of volatiles. As reported widely, the antifungal activity of clove oil nanoemulsions may primarily result from the eugenol. Indeed, the barley treated with the Tween 80-stabilized clove oil nanoemulsion had the lowest Tri5 DNA content after kilning. Consequently, one would expect that the more eugenol in Tween 80 system was used for inhibition of *Fusarium* spp. growth during the micro-malting process.

Conclusion

Physically stable clove oil nanoemulsions could be fabricated by mixing 5.0 wt % oil and 1.0 wt % emulsifier (BSA, quillaja saponins, and Tween 80) using high-pressure homogenization. Barley treated with a higher clove oil concentration of nanoemulsion had a lower germinative energy (GE). The negligible impact of clove oil on GE could be obtained by applying 1.5 mg clove oil/g nanoemulsion in barley. During micro-malting process, fungal biomass as reflected by Tri5 DNA and mycotoxin levels were decreased appreciable after steeping, but increased after germination, and then declined again after kilning. All clove oil nanoemulsions had the capability to inhibit fungal growth and DON production during the micro-malting process. Regarding volatiles residues of clove oil in final malt, eugenol,

caryophyllene, α-humulene, and caryophyllene oxide were detected in final malts treated with clove oil nanoemulsions. Among the three emulsifiers, Tween 80-stablized clove oil nanoemulsion displayed less potential flavor impact on the final malt.

OVERALL CONCLUSION

Fusarium spp. and mycotoxin contamination in malting barley is of great concern for the malting industry because the avoidance of contaminated barley is not always feasible due to the unsuccessful agricultural practices and storage management. The overall goal of this work was to fabricate food-grade antifungal essential oil-in-water nanoemulsions that can be applied into malting industry. The influence of nanoemulsion compositions (e.g., oil type, composition, emulsifier type) and preparation conditions including homogenization conditions on the physical stability, antifungal activity and mycotoxin inhibitory activity were initially evaluated *in vitro*. The results showed that physically stable essential oil nanoemulsions could be fabricated by incorporating either ≥75 wt% of corn oil or ≥50 wt% of medium-chain triacylglycerol (MCT) into essential oil before homogenization to prevent Ostwald ripening. When microfluidizer was used to prepare the essential oil nanoemulsions, the nanoemulsion with smaller particle size (< 200 nm) can be achieved at the optimized processing conditions (68.95 MPa and 2 passes). In terms of emulsifier type, the long-term stability of 5 wt% essential oil nanoemulsions could be stabilized either by 0.5 wt % of Tween 80 or 1 wt % of quillaja saponin and bovine serum albumin (BSA). With regard to the antifungal and mycotoxin inhibitory activity of essential oil nanoemulsions, the major chemical constituents of essential oils play a leading role rather than emulsifier. Among all the selected five essential oils, thyme and clove oil nanoemulsions had greater antifungal and mycotoxin inhibitory activities owing to principle chemical components of phenols, such as thymol and eugenol. However, the different mode of actions might be involved on inhibition of hyphae growth and spore germination after treated with thyme oil nanoemulsion stabilized by three different resources of emulsifier according to the SEM results.

Based on the results from *in vitro* studies, the proper designed essential oil nanoemulsions with greater functional properties including antifungal and mycotoxin inhibitory activity were applied in the micro-malting process. In our study, clove oil nanoemulsions stabilized by 1 wt % of three different emulsifiers (Tween 80, bovine serum albumin, quillaja saponins) with 70 wt % of corn oil as ripening inhibitor in the oil phase were selected to apply in micro-malting process because clove oil had minimum inhibitory effect on the germinative energy of barley seeds among five essential oils. According to our preliminary results, clove oil nanoemulsions at the concentration of 75 mg/kg barley mixed with water at the first steeping stage displayed the enhanced antifungal and mycotoxin inhibitory activity during the micro-malting process. The overall practical utility of clove oil nanoemulsion in micro-malting results indicated that clove oil nanoemulsions could be used as an effective mitigation strategy for control of *Fusarium spp.* biomass and DON content during the malting process, and without the negative impact on the germination quality of barley. The impact of clove oil residues on the final malt flavor were also measured. The eugenol, caryophyllene, α-humulene, and caryophyllene oxide were detected in final malts treated with clove oil nanoemulsions. Among the three emulsifiers, Tween 80-stablized clove oil nanoemulsion displayed less flavor impact on the final malt.

FUTURE WORKS

Our project showed a great potential in the utilization of the antifungal essential oil nanoemulsion during the malting process for mitigation of *Fusarium spp.* and mycotoxin contamination in barley. If this technology can be transferable, it could have a huge economic impact on the malting industry. However, there is lack of information regarding the antifungal and antimycotoxigenic mechanisms of action (MOA) of the EOs used, and which chemical composition of EOs plays an important role in their antifungal and antimycotoxigenic efficacy. The future research directions are highlighted below:

（1）The major chemical components of essential oils dominate the functional properties of essential oils but can be highly different for the same essential oil type due to the different geographical region, harvest timing, and extraction methods. Therefore, future work could study the impact of extraction method, source of plant organs and planting area on the antifungal and mycotoxin inhibitory activity of essential oils.

（2）The mechanism behind the antifungal and mycotoxin inhibitory activity of essential oil against *Fusarium spp.* could be explored. From the present study, we observed the morphological changes on the fungal hyphae and spore treated with essential oil nanoemulsion. However, it is still unclear how essential oil nanoemulsion induced these changes. In addition, mycotoxin biosynthesis is a complex process determined by fungal species and cereal matrix. Large gene families, such as Tri genes, have been identified to modulate some essential enzymes on the mycotoxin biosynthetic pathway. Further works could study the mode action of essential oil nanoemulsion on the replication, transcription, and expression of these genes specific for the certain fungal species on a cereal type.

（3）Barley with various resistant varieties and infection mode (artificial or natural infection, various fungal pathogens, infection timing, and dosage) may perform differently during the malting process. Future works will be included barley samples with different mycotoxin contamination level in the malting study and evaluate the mitigatory effects of essential oil nanoemulsions on the fungal growth and mycotoxin production during malting process.

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