UNDERSTANDING THE MODE OF ACTION OF ESSENTIAL OIL NANOEMULSIONS TO INHIBIT *FUSARIUM* GROWTH AND MYCOTOXIN PRODUCTION IN CEREAL

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Deoxynivalenol (DON) is a commonly occurred mycotoxin in cereal-based food, which is mainly produced by *Fusarium* spp. in the field. It is unfeasible to entirely avoid DON contamination in cereal grains with good agricultural practices. Therefore, it is of great importance to have a strategy for preventing DON contamination in our final food products. In recent years, utilization of plant extracts such as essential oils (EOs) as antifungal and mycotoxin inhibitory agents in foods have gained popularity. Depending on the EO type and chemical composition of EOs, the antifungal and mycotoxin inhibitory efficacies of different EOs might be varied. In addition, their antifungal mode of actions (MOA) against *Fusarium* spp. growth remains unknown. Therefore, the overall objectives of this project were to understand how different type of clove EOs (clove bud EO and clove leave EO), hop essential oil (HEO) impact their antifungal and mycotoxin inhibitory efficacies, and their corresponding antifungal MOA. Finally, HEO in nanoemulsion form was applied to evaluate their application during micro malting process using naturally *Fusarium* infected barley grains.

Results denoted that physically stable 5 wt% EO in-water nanoemulsions with the mean particle sizes less than 170 nm can be built by blending either corn oil or medium chain triglyceride (MCT) with EOs. The largest percentage of chemical constituent in clove essential oils (CO) and HEO was eugenol (phenol type) and β-myrcene (monoterpene type), respectively. In terms of their application as antifungal agents, CO had better antifungal and mycotoxin inhibitory efficacy against *Fusarium graminearum* growth and DON production *in vitro* as compared to HEO because of their high percentage of eugenol content. The major antifungal MOA of EO nanoemulsions included altering total lipid content in cell, chitin content in outer spore cell membrane, and damaging cytoplasmic membrane. In the application case study, results

indicated that *Fusarium* biomass (Tri 5 DNA) and DON contents were reduced at each malting stage with the treatments of HEO nanoemulsion by malting *Fusarium* infected barley. This study provided the valuable information on utilization of EO nanoemulsions as natural antifungal agents during food processing.

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15-ADON.....15-acetyldeoxynivalenol

3-ADON.....3-acetyldeoxynivalenol

4-ANIV 4-acetylnivalenol

A. clavatus.....*Aspergillus clavatus*

A. flavus.....*Aspergillus flavus*

A. fumigatus .....*Aspergillus fumigatus*

A. niger.....*Aspergillus niger*

A. parasiticus .....*Aspergillus parasiticus*

AFB1.....Aflatoxin B1

AFB2.....Aflatoxin B2

AFG2.....Aflatoxin G2

aw.....Water activities

B. cinerea .....*Botrytis cinerea*

bw.....Body weight

C. albicans .....*Candida albicans*

CAC .....Codex alimentarius commission

CBO .....Clove bud essential oil

CFW.....Calcofluor white

CLO.....Clove leave essential oil

CLSM.....Confocal laser scanning microscopy

CO.....Clove essential oil

CSOs .....Clove stem oil

DAS.....Diacetoxyscirpenol

DNA.....Nucleic acid

DON.....Deoxynivalenol

E. coli .....*Escherichia coli*

EC50 .....Effective concentration

EO .....Essential oil

F. graminearum .....*Fusarium graminearum*

F. culmorum .....*Fusarium culmorum*

F. moniliforme .....*Fusarium moniliforme*

F. oxysporum .....*Fusarium oxysporum*

F. sporotrichioides .....*Fusarium sporotrichioides*

FB1 .....Fumonisin B1

FDA .....Fluorescein diacetate

FHB .....*Fusarium* head blight

GAP .....Good agricultural practices

GC-MS .....Gas chromatography–mass spectrometry

GE .....Germinative energy

GRAS .....Generally recognized as safe

HEO .....Hop essential oil

HS-SPME-GC–MS .....Headspace-solid-phase microextraction-gas chromatography-mass spectrometry

JECFA .....Joint FAO/WHO Expert Committee on Food Additives

L. monocytogenes .....*Listeria monocytogenes*

L. monocytogenes .....*Listeria monocytogenes*

LCT .....Long chain triglyceride

MCT .....Medium chain triglyceride

MGI .....Mycelial growth inhibition

MIC .....Minimum inhibitory concentration

MOA .....Mode of actions

N. parvum .....*Neofusicoccum parvum*

NH3 .....Ammonia

NIV .....Nivalenol

P. digitatum .....*Penicillium digitatum*

P. italicum ..... *Penicillium italicum*

PDA..... Potato dextrose agar

PDB..... Potato dextrose broth

PI..... Propidium iodide

PMF..... Proton-motive force

qPCR..... Quantitative real-time polymerase chain reaction

S. aureus..... *Staphylococcus aureus*

S. cerevisiae ..... *Saccharomyces cerevisiae*

S. enterica ..... *Salmonella enterica*

SEM ..... Scanning electron microscope

SGI ..... Spore germination inhibition

SPV ..... Sulfo-phospho-vanillin

T. rubrum ..... *Trichophyton rubrum*

TEM ..... Transmission electron microscopy

Tween 80..... Polyoxyethylene (20) sorbitan monooleate

WGA-Alexa-fluor-488..... Alexa fluor™ 488 conjugate

ZEA..... Zearalenone

GENERAL INTRODUCTION

Fusarium head blight (FHB), caused by *Fusarium* spp. (e.g., *Fusarium graminearum*), is a universal plant disease that can devastate grains (e.g., wheat and barley) in the field (Shin et al., 2018). In addition to the yield loss, consumption of FHB-infected grains has a negative effect on human health due to the occurrence of mycotoxins. For example, *Fusarium* mycotoxins can retard protein synthesis and regulate immune system (da Rocha, Freire, Maia, Guedes, & Rondina, 2014). The most commonly found mycotoxin in FHB-infected cereal grains is deoxynivalenol (DON) that is a type B trichothecene produced primarily by *F. graminearum* in North America (Sobrova, Adam, Vasatkova, Beklova, Zeman, & Kizek, 2010). According to the survey on the occurrences of DON in cereal-based foods, it has been manifested that DON can be transmitted from contaminated raw materials to the final products, thus representing a safety concern to human health (Cavret & Lecoeur, 2006). The classical example is the DON contamination in the field barley can be transferred to the malt barley, and to the finished beer in a high recovery rate as confirmed by a number of studies (Lancova et al., 2008; P. B. Schwarz, Casper, & Beattie, 1995; Wolf-Hall & Schwarz, 2002). Moreover, the fate of DON during the malting process can be varies among different brewing grains, initial DON contamination level within the same brewing grains and different physical fungal localization of grains (Wan, Jin, Zhong, Schwarz, Chen, & Rao, 2020; Yu et al., 2019). As such, it is difficult to foresee the DON levels in final malts by only measuring the concentration of DON in original barley grains. Thus, strategies to effectively control *Fusarium* mycotoxins in malting barley need to be explored.

Recently, plant essential oils (EOs) are achieving attention because of their antifungal and mycotoxin inhibitory properties *in vitro* and in several model food systems (Hyldgaard, Mygind, & Meyer, 2012; Seow, Yeo, Chung, & Yuk, 2014; Wan et al., 2020). Therefore, EOs can be act as

alternative option to replace synthetic antifungal agent to effectually conquer fungal growth and mycotoxin production in cereal-based food. Nonetheless, the practical implementation of EOs as natural antifungal agents in grains is still restricted due to their low water solubility, high volatility, and measly antifungal efficacy in cereal food systems (Wan, Zhong, Schwarz, Chen, & Rao, 2018). Encapsulation of EOs in nanoemulsion based delivery system has appeared to be as one of effective way to overcome these difficulties (Odriozola-Serrano, Oms-Oliu, & Martin-Belloso, 2014). Considerable studies have demonstrated that effectiveness of EOs in nanoemulsion delivery system when compared to the bulk EOs in terms of physiochemical stability and their antifungal efficacy (Donsi & Ferrari, 2016; Liang, Xu, Shoemaker, Li, Zhong, & Huang, 2012; Wan et al., 2018). Previous studies in our lab have also shown that clove essential oil (CO) nanoemulsion exhibited superior antifungal and mycotoxin inhibitory performance against *F. graminearum* growth and DON synthesis *in vitro* as well as during malting processing using inoculated FHB-infected barley grains (Wan et al., 2020; Wan, Zhong, Schwarz, Chen, & Rao, 2019). As we have learned from previous researchers, the antifungal and mycotoxin inhibitory efficacy of EOs is strongly influenced by their chemical constituents, the employed EO concentration, as well as the aimed species (Wan et al., 2019). However, there is lack of information on how the CO type impacts the fungal growth and their associated mycotoxins. Therefore, it is of great importance to understand antifungal and mycotoxin inhibitory efficacy of different CO types for enhancing their utilization in foods. In addition to CO, the antifungal and mycotoxin inhibitory activity of hop essential oil (HEO) has not been explored yet, which can be extracted from an indispensable brewing material (hop). Moreover, very few of studies have intended to elucidate the antifungal mode of actions (MOA) of the used EOs. Several studies reported the antifungal effects of EOs may due to the changes of fungal

membrane composition (Zeng, Chen, & Liang, 2015), membrane integrity (OuYang, Duan, Li, & Tao, 2019), and fungal morphology (An, Yang, Yu, Qi, Ren, & Kong, 2019). Therefore, knowledge about MOA of different CO types and HEO on antifungal and mycotoxin inhibitory activities of EOs is required.

The overall goal of this project was to evaluate the impact of different CO types and HEO on the mycelial growth, spore germination, and mycotoxin production of *F. graminearum* and their antifungal MOA *in vitro*. In addition, the application of HEO nanoemulsion as an antifungal agent during malting process using naturally FHB-infected barley grains was tested. In order to meet these goals, the chemical constituents of clove bud essential oil (CBO), clove leave essential oil (CLO) and HEO were initially measured, and the physically stable EO-in-water nanoemulsions were formed using high pressure homogenization method. Meanwhile, the antifungal activity of the stable EO nanoemulsions were evaluated by measuring the inhibition rates of *F. graminearum* mycelial growth and spore germination. MOA of abovementioned EO nanoemulsions against mycelial growth and spore germination was further investigated through evaluating the alterations of fungal total lipid and chitin contents in cell membrane and the integrity of cytoplasmic membrane. Concomitantly, the effect of EO nanoemulsions on DON synthesis was detected in rice culture. Finally, HEO-in-water nanoemulsions were applied in the first steeping stage to investigate their probable application in malting processing. Specifically, fluorescent dye coupled with advanced microscopy techniques were applied to study the localization of fungal hyphae on FHB-infected barley kernels and the development of fungal hyphae within the malted barley tissue.

CHAPTER 1. LITERATURE REVIEW

Fusarium head blight (FHB) in cereal

Fusarium species and *Fusarium* mycotoxins in cereal and associated foods

The filamentous genus *Fusarium*, which contains over 300 pathogenic and non-pathogenic species (O'Donnell, Ward, Robert, Crous, Geiser, & Kang, 2015), is widely distributed in the whole world. The pathogenic *Fusarium* species commonly prefer to colonize in cereal crops in the field, like wheat, barley, maize, rye, oat and rice, especially in the condition of excessive rain, hot and humid temperatures (Cendoya, Chiotta, Zachetti, Chulze, & Ramirez, 2018; Kalagatur, Nirmal Ghosh, Sundararaj, & Mudili, 2018; P. Schwarz, 2017), causing FHB disease in crops (Parry, Jenkinson, & Mcleod, 1995; Shin et al., 2018; Wegulo, 2012). Crops was infected by FHB disease can lead to yield reduction, crop quality degradation (Bai & Shaner, 1994) and mycotoxins accumulation in grains (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018). Studies found that among all *Fusarium* species, *F. graminearum* is the most predominant species in United States, Canada, and Europe due to the dependent characteristics of geographic location and host (Shin et al., 2018). For instance, it has been described that *F. graminearum* was the dominant *Fusarium* species isolated from FHB-infected durum wheat in three winter durum wheat cultivars in Mediterranean countries in 2012/2013 and 2013/2014 seasons (Gorczyca et al., 2017).

In general, *F. graminearum* commonly produce two categories of mycotoxins, namely zearalenone (ZEA) and trichothecenes (Chen, Kistler, & Ma, 2019). Until now, over 200 kinds of trichothecenes have been identified (Grovey, 2007). According to the chemical structure of trichothecenes mycotoxins, they can be divided into 4 groups, namely type A trichothecenes that includes HT-2 toxin, T-2 toxin and diacetoxyscirpenol (DAS), type B trichothecenes (**Fig. 1-1**)

(Michlmayr et al., 2018; Y. Tian et al., 2016) including DON, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and 4-acetylnivalenol (4-ANIV), type C trichothecenes and type D trichothecenes (Wang et al., 2019). Among these trichothecene mycotoxins, type B trichothecenes are the most recognized mycotoxins in crops and their associated products, like beer, bread and pasta, which are mainly produced by *F. graminearum* (Ferrigo, Raiola, & Causin, 2016; Khaneghah et al., 2018; Pestka & Zhou, 2000; P. Schwarz, 2017; Sundheim, Lillegaard, Faeste, Brantsaeter, Brodal, & Eriksen, 2017). For instance, more than 100 μg/kg of DON were detected in 66% of total 289 of oats samples and 70% of 178 of spring wheat samples according to the mycotoxin content in cereal crops survey in Southeast Norway from 2004 to 2009 (Hofgaard et al., 2016). In addition to widespread mycotoxin contamination in cereal crops in the field, DON is extremely heat stable that can withstand 300 ℃ without any degradation, thus representing the risk of its occurrence in the final foods (Gartner, Munich, Kleijer, & Mascher, 2008). For example, ubiquitous DON contamination was found in 113 out of 176 of beers samples that were collected from North American and European markets in 2007; among these DON contaminated beers, the highest DON level was 35.9 μg/L (Kostelanska et al., 2009). Similar DON contamination was also found in bread products by using DON contaminated spring wheat flours as the starting material. The results showed that DON could survived after fermentation and baking process, and existed in all kinds of breads with very high recovery rate (Gartner et al., 2008).

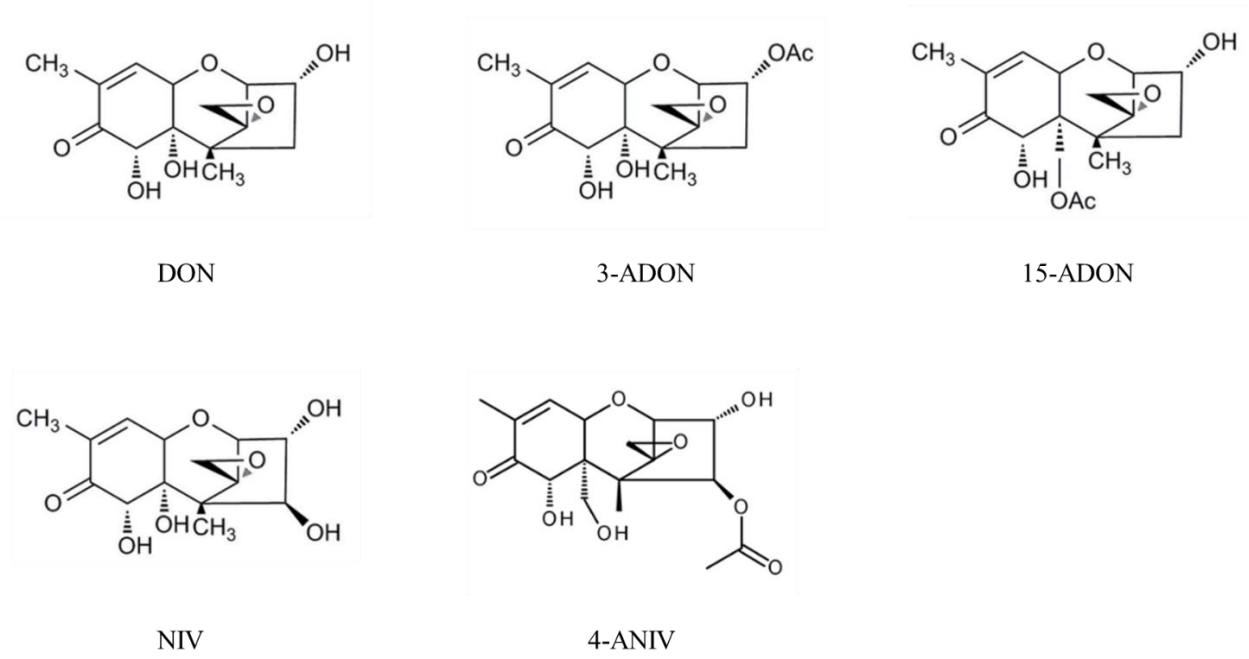

Figure 1-1. Chemical structure of type B trichothecenes.

The legislation of *Fusarium* content in cereal

Consuming DON contaminated products can not only cause acute disease, but also chronic health issues, such as weight loss, inflammation, immune responses decreasing and gastrointestinal disease, thus representing food safety concerns (Sundheim et al., 2017). Therefore, the legislation on DON limitation in cereals and associated food has been established in world-widely, ranging from 0.2 to 2 mg/kg in cereals or cereal-based food products. Joint FAO/WHO Expert Committee on Food Additives (JECFA) regulated that a group of the provisional maximum tolerable daily intake for DON, 3ADON and 15ADON are 1 mg/kg body weight (bw) (JECFA, 2011). Regarding the DON limits in food, Codex Alimentarius Commission (CAC) has set a DON limitation standard in a wide range of foods including < 0.2 mg/kg of DON present in cereal-based foods for infants and children consumption, <1 mg/kg of DON in milled wheat, barley or maize product, and below 2 mg/kg of DON in wheat, barley and maize aimed for further processing (Codex Alimentarius Commission, 2015; Ferrigo et al.,

2016). In North America, Food and Drug Administration has formulated a standard specification on the maximum DON level, less than 1 mg/kg, on finished wheat products (US Food Drug Administration, 2010). The European Union has established the maximum DON limit of unprocessed durum wheat and oats and unprocessed other cereals (except durum wheat, maize, and oats) and are 1.75 and 1.25 mg/kg, respectively (European Food Safety Authority, 2013). In terms of Asian countries, The legislation law of the People's Republic of China on DON is 1 mg/kg maximum content in wheat, barley and corn flour for human consumption (Chinese Ministry of Health, 2011). In Russia, the maximum level of DON in wheat and barley is 0.7 mg/kg and their derivatives is 1 mg/kg (Ferrigo et al., 2016). In a short summary, the legislation on the content of DON ranges from 0.2 to 2 mg/kg in cereals or cereal-based food products world widely.

Current strategies for management of *Fusarium* contamination and DON accumulation

As mentioned above, *Fusarium* contamination and related DON accumulation in grains have become an unignorable issue nowadays. Therefore, strategies to avoid to bring DON contaminated grains from the field to supply chain and to the final foods have been continuously explored such as good agricultural practices (GAP) (e.g., tillage operations and burning *Fusarium*-infected grain residues) (McMullen et al., 2012; Torres et al., 2019). However, completely avoid FHB disease in the field and *Fusarium* contamination in grains is not durable by compliance with GAP.

In addition to GAP in the field, a number of post-harvest strategies to control *Fusarium* and DON contents are also developed. Generally, the mitigation methods in post-harvest grains towards *Fusarium* and their mycotoxin include physical (e.g., cleaning and thermal processes), chemical (e.g., using ammonia and sulfur containing compounds), biological controls, etc.

(Chilaka, De Boevre, Atanda, & De Saeger, 2017; Kagot, Okoth, De Boevre, & De Saeger, 2019; Karlovsky et al., 2016). Regarding physical methods, studies found that physical sorting and cleaning grains can effectively decontamination of *Fusarium* mycotoxin in a certain level, but it cannot complete solve mycotoxin problems in grains. For example, 7% of poor maize samples were removed from the original samples by sorting maize samples based on their quality control parameters including kernel size, color and shriveled kernel, which decreased 59% of DON level in original maize samples (Lauren, Jensen, & Smith, 2006). However, physical cleaning may cause large losses of grains in some cases (Karlovsky et al., 2016). In terms of another typical physical method, *Fusarium* mycotoxins showed highly resistant to thermal degradation due to their heat stable characteristics (Chilaka et al., 2017). Therefore, several chemical and biological methods are considered to mitigate *Fusarium* mycotoxins contamination in grains. For instance, ammonia (NH_3) vapors were used to degrade DON contents in wheat kernels. Studies showed that more than 75% of DON in wheat kernels was degraded with the treatment of NH_3 for 2 h at 90 °C (Borras-Vallverdu, Ramos, Marin, Sanchis, & Rodriguez-Bencomo, 2020), but the chemical residues in grains might be a big concern (Mylona, 2012). In addition, EU has banned most of chemical agents that can be used in grains. Regarding the biological methods, studies found that *Saccharomyces cerevisiae* RC016 was able to completely inhibit *F. graminearum* growth and DON production at pH 6 and 37 °C (Armando, Dogi, Poloni, Rosa, Dalcero, & Cavaglieri, 2013). Although biological agents showed excellent antifungal and detoxification ability against *Fusarium*, their application is limited to environmental conditions. Moreover, the application of biological agents related safety, sustainability issues, as well as its influence on the ecosystem effects and other mycotoxins is still remains unknown (Kagot et al., 2019). Hence, it

is crucial to discover a safety and efficacy agent to solve the contamination problem of the *Fusarium* and their mycotoxin in grains and during food process.

Essential oils as antifungal agents

Essential oil type and their chemical compositions

EOs are secondary metabolites in medicinal and/or aromatic plants (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013; Rehman, Hanif, Mushtaq, & Al-Sadi, 2016). They can be presented in all plant organs, such as buds, leaves, seeds, etc., but normally they are accumulated in the secretory cells, canals, cavities, glandular trichomes and epidermal cells of plants (Nazzaro et al., 2013). In general, EOs are extracted from aromatic plants by some conventional methods such as solvent extraction, steam distillation, hydrodistillation and hydrodiffusion, or some advanced methods (e.g., subcritical extraction liquid, supercritical fluid extraction and solvent-free microwave extraction) (Aziz et al., 2018). From the chemical composition point of view, EOs are complex mixtures of chemical compounds including non-volatile and volatile compounds (Donsi et al., 2016). According to the chemical composition and functional properties of compounds in EOs, they can be classified into four categories: terpenes, terpenoids, phenylpropenes, and "others" (Hyldgaard et al., 2012). Terpenes are hydrocarbons formed by isoprene units (C_5H_8) that are combined to form great variety of subcategory of compounds such as monoterpenes ($C_{10}H_{16}$), sesquiterpene ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{40}$). Examples of terpenes are limonene, pinene, sabinene, terpinene, and p-cymene. Terpenoids are enzyme or chemical modified terpenes which contain oxygen (Caballero, Trugo, & Finglas, 2003; Hyldgaard et al., 2012). The typical examples of commonly existed terpenoids in EOs include thymol, carvacrol, citronellal, menthol, piperitone, linalyl acetate, borneol, linalool, and geraniol. Phenylpropenes ($C_6H_5CH_2CH{=}CH_2$) are organic compounds that contain a phenyl

group attached to allyl. Examples of phenylpropenes existed in EOs are eugenol and chavicol. "Others" refers to EOs come from degradation products from unsaturated fatty acids, terpenes, lactones, glycosides, and sulfur-containing and nitrogen-containing compounds in EOs (Caballero et al., 2003; Hyldgaard et al., 2012), like allyl isothiocyanate and allicin.

In recent years, application of EOs as antifungal agents in foods have gained popularity because their wide-spectrum of antifungal ability (Soliman & Badeaa, 2002; Thompson, 1989; Vilela et al., 2009; Wan et al., 2018). According to the previous reports, the highest antifungal efficacy of chemical compounds in EOs are oxygenated terpenoids, and phenylpropenes, followed by terpenes and other constituents (Rao, Chen, & McClements, 2019). Our recent results on antifungal and mycotoxin inhibitory activity of five EOs including thyme, lemongrass, cinnamon, peppermint, and clove also approved this conclusion (Wan et al., 2019). It showed that CO had the superior antifungal and mycotoxin inhibitory activity because it contains high concentration of eugenol (phenylpropenes category) (Wan et al., 2019). In addition, antifungal and mycotoxin inhibitory activity of HEO, one of the EO brewing ingredient has not been explored yet. For this reason, the antifungal activities of CO and HEO and their chemical compositions are the main focus of in the following sections.

The antifungal and mycotoxin inhibitory activity of clove and hop essential oils

Clove essential oils

Clove (*Syzygium aromaticum*) EOs are generally recognized as safe (GRAS) status by Food and Drug Administration with the consuming concentrations less than 1,500 mg/kg bw (Cortés-Rojas, de Souza, & Oliveira, 2014). It is one of the most commonly used EOs in food, flavoring, medical, health, and cosmetic industries (Haro-Gonzalez, Castillo-Herrera, Martinez-Velazquez, & Espinosa-Andrews, 2021). They have showed some biological activity associated

with human health, such as antimicrobial, antioxidant, and insecticidal activity (Haro-Gonzalez et al., 2021). In general, CO can be obtained from the bud, leaf or stem of clove (Gaylor Razafimamonjison, Jahiel, Duclos, Ramanoelina, Fawbush, & Danthu, 2014). Depending on which parts of clove plant is applied to extract EOs, the chemical composition of CO could be varied (G Razafimamonjison, Jahiel, Duclos, Ramanoelina, Fawbush, & Danthu, 2013; Gaylor Razafimamonjison et al., 2014). In general, eugenol has been demonstrated as the major constituent of CO. Other than eugenol, it may include eugenol acetate, β-caryophyllene, α-humulene, caryophyllene oxide, cadinene, diethyl phthalate, α-copaene, 4-(2-propenyl)-phenol, α-cubebene, chavicol, etc., (Guynot, Ramos, Seto, Purroy, Sanchis, & Marin, 2003; Haro-Gonzalez et al., 2021). For example, 45 of CBOs, 32 of CLOs and 44 of clove stem oil (CSOs) were collected to analyze their chemical composition profile. The study found that the major chemical constituent of CBOs was eugenol which accounted for 72.08 - 82.36% of the CBOs, followed by eugenyl acetate and β-caryophyllene representing for 8.61 - 21.32% and 2.76 - 8.64% of the total CBOs, respectively. Although eugenol (75.04 - 83.58%) was also the major chemical compound in CLOs, the secondary chemical constituent of CLOs was β-caryophyllene which accounted for 11.65 - 19.53%, then α-humulene was the third dominated compounds, accounting 1.38 - 2.17%. Interestingly, and CSOs were composed by 87.52 - 96.65% of eugenol and 1.66 - 9.72% of β-caryophyllene, respectively in tested 44 CSO samples. In addition, other chemical compounds can barely be detected in CSOs (Gaylor Razafimamonjison et al., 2014). Therefore, the chemical compositions of CO were strongly depended on EO extraction parts from the plants.

Regarding the antifungal activity of CO, they generally exhibited good antifungal properties *in vitro* and in cereal grains (Donsi et al., 2016; Montes-Belmont & Carvajal, 1998;

Paster, Menasherov, Ravid, & Juven, 1995). It has been well documented that CO displayed wide spectrum of antifungal activities against *Candida albicans*, *Aspergillus flavus*, *F. oxysporum* and *F. graminearum in vitro* (Al-Zubairi, Al-Mamary, & Al-Ghasani, 2017). For example, over 90% of *Aspergillus clavatus*, *A. flavus* and *Aspergillus parasiticus* spore germination were successfully inhibited by applying 500 μl/l of the CO, which were isolated from oats (Bozik, Cisarova, Tancinova, Kourimska, Hleba, & Kloucek, 2017). For the antifungal effects in cereal grains, the studies in our group found that 1.5 mg CO/g nanoemulsion could significantly reduce the *Fusarium* biomass during micro-malting process using FHB-infected barley grains (Wan et al., 2020). With respect to the relationship between chemical composition of CO and antifungal efficacies, very few results are available. Only one study found that eugenol acetate showed the higher antifungal efficacy as compared to the eugenol against *Fusarium moniliforme* growth, and the effective concentration (EC_{50}) values were at 10.6 and 11.72 μg/ml, respectively (Kaur, Kaushal, & Rani, 2019). In addition, other chemical compounds in CO, including eucalyptol, α-cadinol, benzene, benzoic acid, eugenol acetate, etc., were also identified as functional components to against fungi growth (Bhuiyan, Begum, Nandi, & Akter, 2010; Chaieb et al., 2007). Nevertheless, the relationship between CO type and their chemical components on *Fusarium* growth and DON inhibitory efficacy has not been explored yet. In consequence, it is essential to understand antifungal and mycotoxin inhibitory capabilities of different CO types intending to enhance their utilization as natural antifungal agents in foods.

Hop essential oils

Hops (*Humulus lupulus* L.) is an dioecious, herbaceous perennial liana (Korpelainen & Pietilainen, 2021), which indicates the male and female flowers of the hop grow on separate plants (Almaguer, Schönberger, Gastl, Arendt, & Becker, 2014). The female inflorescences (or

cones), as an indispensable brewing ingredients, has been broadly utilized in brewing industries since ancient times. It provides unique aroma, bitter taste, as well as preservation to beer (Kovacevic & Kac, 2002; Rossini, Virga, Loreti, Iacuzzi, Ruggeri, & Provenzano, 2021). The components of female hop cones are made up of resins, proteins, polyphenols, HEO, lipids, etc. (Stevens, 1967). Among all components of hops, hop resins and HEO account for 15 to 30%, and 0.5-3.0% the total weight of dried hops, respectively. The hop resins can be categorized as total soft resin and total hard resin. The total soft resin is mainly containing a mixture of α-acids (e.g., humulone), β-acids (e.g., lupulone) and some uncharacterized soft resin. While the total hard resin is composed of α-hard resins, β-hard resins (e.g., xanthohumol), δ-hard resins (e.g., hulupinic acid), ε-hard resins and some uncharacterized hard resins (Almaguer et al., 2014).

HEO are secondary metabolites of the hop, which were from lupulin glands part of hop (Almaguer et al., 2014; Hough, Briggs, Stevens, & Young, 2012). In terms of the chemical composition of HEO, it can be classified into 3 principal chemical categories: hydrocarbons, oxygenated compounds and sulfur-containing components (Sharpe & Laws, 1981). The hydrocarbons group of hop can be further divided into aliphatic hydrocarbons (e.g., pentane, octane, isoprene, etc.), monoterpenes (e.g., β-myrcene, limonene, α-pinene, etc.) and sesquiterpenes (e.g., α-farnasene, humulene, β-caryophyllene, α-muurolene, etc.). The oxygenated compounds are referred to complex mixtures of alcohols (e.g., nerol, α-cadinol, methanol, etc.), aldehydes (e.g., hexanal, nonanal, decanal, etc.), acids (e.g., 3-methylbutanoic, 2-methylpropanoic, 4-methyl-3-pentenoic, etc.), ketones (e.g., acetone, 2-octanone, carvone, etc.), epoxides (e.g., caryophyllene oxide, humulene epoxide I, humulene epoxide Π, etc.) and esters (e.g., hexanoate, 4-decenoate, 2-methylpropanoate, etc.). The sulfur-containing components of the HEO include S-methyl pentanethioate, hydrogen sulfide, 3-methylthiophene,

etc.(Almaguer et al., 2014). Regarding the antimicrobial and antifungal activity of HEO, very limited information is available in the literatures. Within the limited information, it has been demonstrated that several bioactive compounds in hop, like hop acids, showed antimicrobial capabilities. For example, three kinds of hop extracts that contained α-acids, β-acids, and/or xanthohumol were all showed good antimicrobial activities against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* growth (Kramer, Thielmann, Hickisch, Muranyi, Wunderlich, & Hauser, 2015). The antimicrobial mechanisms of β-acids for controlling *E. coli* and *S. aureus* growth can be explained by damaging microbial cell wall as well as disrupting biosynthetic pathway of fatty acids in cell membrane (B. Tian, Cheng, Zhang, Liu, & Chen, 2022). There was another example indicated the metabolic pathway of *Botrytis cinerea* was disturbed with the treatment of isoxanthohumol, resulting in the inhibition of *B. cinerea* growth, which was identified as an important chemical composition of hop extracts (Yan et al., 2021). However, there is no such information on the functional performance of HEO against *Fusarium spp*. growth and DON inhibitory activity *in vitro* as well as in cereal grains. Consequently, it is a great importance to systematically study antifungal and mycotoxin inhibitory efficacy of HEO *in vitro* and in cereal grains for promoting the usage of HEO as natural preserving agents in malting industries.

Improving antifungal activity of essential oils using nanoemulsion based delivery system

It is well known that the physiochemical and bio-functional properties of EOs are highly unstable with the appearance of high temperature, light and oxygen due to the highly volatile nature (Jamil et al., 2016). In addition, the water solubility of EOs are relatively low, which restricts the application of EOs in aqueous food products (Wan et al., 2018). Therefore, oil-in-water nanoemulsion delivery system has been considered as one of method to overcome the

limitations of EOs as mentioned above. EO nanoemulsion is typically consisted of three major ingredients, oil, water, and surfactant (Garcia et al., 2022). EO nanoemulsion can be fabricated by various methods, including high energy methods (e.g., microfluidization, high pressure homogenization and ultrasonication) and low energy methods (e.g., phase inversion composition, spontaneous emulsification and D phase emulsification) (Safaya & Rotliwala, 2020). Nowadays, high energy methods have been used widely to fabricate EO nanoemulsion, especially in industrial scale, due to the ease of production (Safaya et al., 2020). Therefore, microfluidization is chosen in this study to produce EO nanoemulsion, which can produce emulsions with a uniform droplet size (Yukuyama, Ghisleni, Pinto, & Bou-Chacra, 2016). Once the nanoemulsion is formed, EOs are encapsulated into nanometric particles and homogeneously dispersed in aqueous phase, and the droplet size of the EO nanoemulsion ranges from 20 to 200 nm in order to meet the definition of nanoemulsion (Yukuyama et al., 2016). As a result, the mass transfer efficacy of EOs to certain action sites can be improved owing to the increased solubility of EOs and larger surface area of nanodroplets (Wan et al., 2018). However, the pure EO nanoemulsion are generally unstable against the phase separation because of the Ostwald ripening (Y. H. Chang, McLandsborough, & McClements, 2015). Ostwald ripening is a processing that larger droplets continuous increase at the expense of smaller one (Gupta, Eral, Hatton, & Doyle, 2016; Safaya et al., 2020). This is because EOs contain appreciable relatively high water-soluble chemical compounds, as a result, the mass transfer of water-soluble compounds processes at the droplet interface. Therefore, large molecular weight of oils with less water solubility, like long chain triglyceride type oils (e.g., corn oil) and medium chain triacylglycerol (MCT), can be used to incorporated into pure EO nanoemulsions to hinder Ostwald ripening (Y. Chang, McLandsborough, & McClements, 2012). For example, our previous studies signified that with

the addition of 75% of corn oil in 5% of total oil phase, the stability of CO nanoemulsions was greatly improved when compared to that of pure CO nanoemulsions (Jiang, Zhong, Schwarz, Chen, & Rao, 2022).

Regarding the antifungal activity of EO in nanoemulsion based delivery system, the antifungal efficacy is generally improved in nanoencapsulated form compared to bulk EOs. For instance, studies recorded that 0.25 μg/g of cinnamon EO nanoemulsion could thoroughly impeded the mycelial growth of *Aspergillus niger*. By contrast, only 75% of mycelial growth was inhibited when applying bulk cinnamon EO at the same concentration (Ribes, Fuentes, Talens, Barat, Ferrari, & Donsi, 2017). Our latest studies also proved that CO nanoemulsions significantly improved antifungal and mycotoxin inhibitory efficacies of *F. graminearum* mycotoxin production when compared to bulk CO (Wan et al., 2019).

Antifungal modes of action of essential oils

EOs including but not limited to, CO and HEO, showed a broad-spectrum antifungal activity both *in vitro* and in food models as we mentioned above. Nevertheless, researches have been hardly addressed the underlying mechanism of applied EOs against fungal growth. Therefore, the possible antifungal MOA and analytical methods that can be used to evaluate the antifungal MOA of EOs are discussed in this section. Overall, a number of antifungal MOA of EOs have been reported such as changing the chemical composition of cell membrane, injuring the cytoplasmic membrane, as well as diminishing the proton-motive force (PMF) (Rao et al., 2019). The cell wall and cell membrane structure of fungi was showed in **Fig. 1-2**.

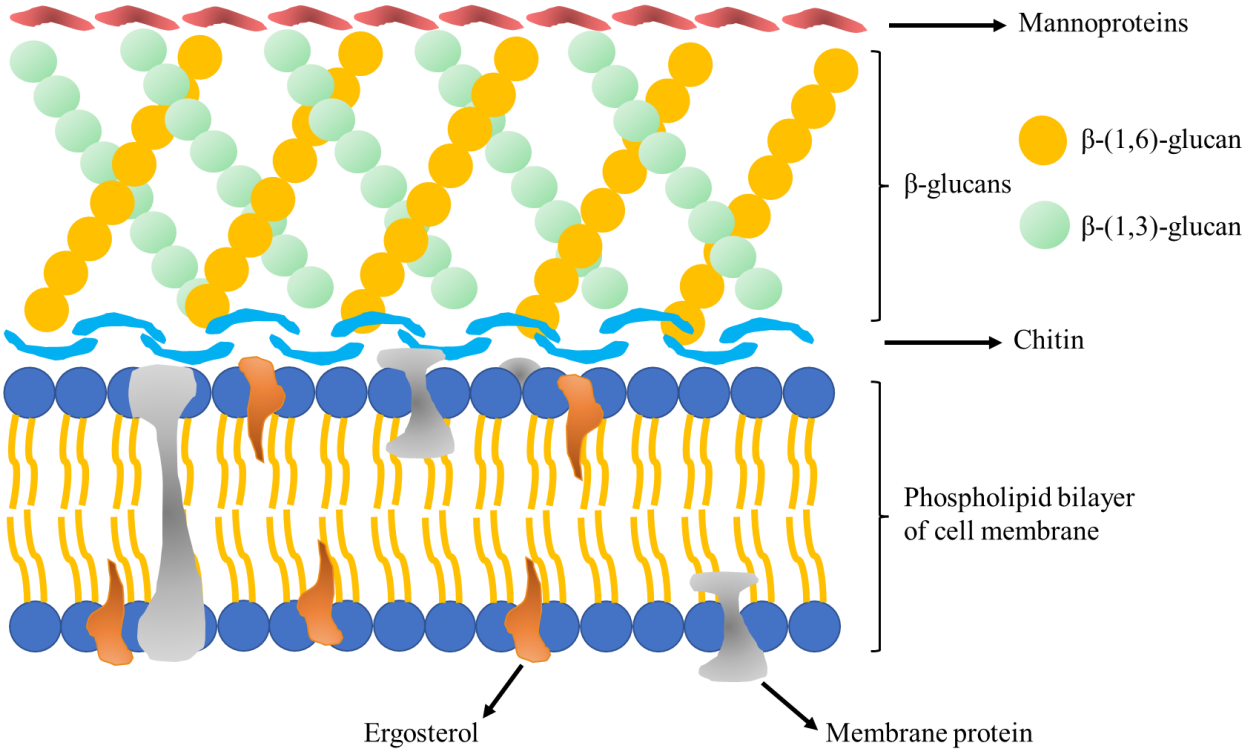

Figure 1-2. Cell wall and cell membrane structure of fungi.

Modes of action of essential oils against *Fusarium* spore germination and mycelial growth

Alternation chemical composition of outer cell membrane

The structure and chemical composition of cell membrane of fungi is very important to determine its viability. It separates the cell from the outside as well as allows cells to interact with surrounding environment (such as communication, ions and metabolites transport and regulation processes). Consequently, the fungal cell membrane is one of a popular target site of fungi (Minones, Pais, Minones, Conde, & Dynarowicz-Latka, 2009). Fungal cell membrane is composed of diverse lipids (e.g., glycerophospholipids, sphingolipids and sterols), modified carbohydrate (chitin) and proteins (e.g., integral membrane proteins) (Minones et al., 2009; Sant, Tupe, Ramana, & Deshpande, 2016) (**Fig. 1-2**). Studies demonstrated that the membrane composition of fungi, such as total lipid content (Tao, OuYang, & Jia, 2014), fatty acid compositions (Essid et al., 2017) and ergosterol content (Zeng et al., 2015), have been changed

with the application of EOs. The alterations of membrane composition usually accompany with decreased cell membrane stability and restricting fungal growth (Helal, Sarhan, Abu Shahla, & Abou El-Khair, 2006, 2007; Prashar, Hili, Veness, & Evans, 2003). For example, both total lipid content and fatty acid composition of *A. flavus* membrane was changed after 5 days' treatment with 1.0 μL/mL of *Cymbopogon citratus* EO, resulting in ~ 65% of fungal growth was inhibited. Specifically, the total lipid content of *A. flavus* cells were markedly reduced from ~117 mg/g to ~90 mg/g dry weight. With respect to the fatty acid composition in cell membrane, both saturated fatty acids and unsaturated fatty acids of *A. flavus* cell membrane increased from 26.83% to 46.80% and from 28.39% to 36.24%, respectively (Helal et al., 2007). As a major sterol constituent of fungal cell membranes, ergosterol acted as modulator to control cell membrane fluidity and permeability (Money, 2016; Rodriguez, Low, Bottema, & Parks, 1985). Studies found that when half of the minimal inhibitory concentration (MIC) of cinnamon bark EO (*Cinnamomum verum*) was applied to against *C. albicans* growth *in vitro*, it showed a good antifungal activity through reducing the quantity of ergosterol contents (~83%) (Essid et al., 2017). Similar results on reduction of ergosterol content were also observed in *A. flavus* with the treatment of dill EO (J. Tian, Ban, Zeng, He, Chen, & Wang, 2012). Therefore, EOs can exert antifungal ability through alterations of chemical compositions of cell membrane.

Alternation of cytoplasmic membrane permeability and integrity

In addition to changing the chemical composition of fungal outer cell membrane, EOs can also cause the damage of integrity and permeability of fungal membrane including cytoplasmic membranes and mitochondrial membranes (Cox et al., 2000), eventually cell osmotic of balance is break (Helal et al., 2007). The changes of integrity and permeability of fungal cell membranes caused by EOs have been proved by a number of studies through

determination of the electric conductivity, ions leakage (Kedia, Dwivedy, Jha, & Dubey, 2016), the absorbance at 260 nm (A_{260} nm), and flow cytometry experiments (Silva, Ferreira, Duarte, Mendonca, & Domingues, 2011). For example, studies found that tea tree EO successfully inhibited hyphae growth of *B. cinerea* by disrupting integrity and permeability of cell membrane. What they found was the tea tree EO treated hyphae samples had the higher electric conductivity of when compared with the control (Shao, Cheng, Wang, Yu, & Mungai, 2013). The higher electric conductivity can be explained by the leakage of electrolyte (mainly potassium ions) from the cell (Munzi, Pisani, & Loppi, 2009), indicating damage of cytoplasmic membrane (Ifesan, Joycharat, & Voravuthikunchai, 2009). Another study also demonstrated that EO (*Citrus reticulata* Blanco) was able to against *Penicillium italicum* and *Penicillium digitatum* growth by alteration their cell membrane integrity and permeability. Because they found the higher absorption values of the EO treated *Penicillium* suspensions at 260 nm as compared to that of the controls (Tao, Jia, & Zhou, 2014). These higher absorption values at 260 nm can be elucidated by the appearance of cell released materials (such as nucleic acid and its related compounds) in the suspension (Ifesan et al., 2009), indicating the damage of the integrity and permeability of *Penicillium* cell membrane. Similarly, the cell membranes of the *Trichophyton rubrum* were also destroyed by fennel EO (*Foeniculum vulgare* L.), which was monitored by flow cytometry experiments showing the high staining intensity of propidium iodide (PI) (Zeng et al., 2015). The principal of PI fluorescence staining would be explained in the next section.

Experimental approaches to evaluate their modes of action

Scanning electron microscope (SEM) and transmission electron microscopy (TEM)

There are several ways to evaluate the MOA of EOs against mycelial growth and spore germination of fungi. Direct ways include SEM to observe the morphological changes of fungal

hyphae and/or spores (Kedia et al., 2016) and/or TEM to monitor the fungal ultrastructure alterations, such as cell wall, fibrillar layer, plasma membrane, mitochondria, etc. (J. Tian, Huang, et al., 2012). Briefly, SEM produces photographs through scanning the surface of a sample with a focused beam of electrons with high energy (Mohammed & Abdullah, 2018), so SEM is prefer to analyze the morphological changes of fungi on surface. Through SEM observations, in general, normal fungi have well-defined hyphal shape with smooth surfaces and healthy conidia (Kedia et al., 2016; Samber, Khan, Varma, & Manzoor, 2015). Contrastingly, when fungi were treated with EOs, fungal hyphae were distorted with flattened conidia (Kedia et al., 2016). And in other cases, fungal cell showed deep wrinkles with the presence of EOs (Samber et al., 2015). Other than SEM, TEM can analyze the internal structure of the fungi, because the beam of electrons of TEM is able to pass through the samples (Kannan, 2018). The ultrastructure of the normal fungi shows the following characteristics under TEM observation: uniform plasma membrane with smooth surfaces; abundant cytoplasmic matrix; normal and uniform structures of mitochondria, vacuoles, nucleus, etc. (Tian, Huang, et al., 2012). When fungi treated with EOs, the fungal plasmalemma became rugged with consecutive folding into the cytoplasm, and the cytoplasm matrix of fungal was decreased. In addition, some mitochondria of the fungi extensively disrupted due to their internal structure (e.g., mitochondrial cristae) was altered (Tian, Huang, et al., 2012; Zeng et al., 2015). These morphological and ultrastructural changes of fungi are probably because of the low-molecular weight and high lipophilic compounds in EOs can easily pass through cell membranes, resulting in the disruption of fungal cell organization (Basak & Guha, 2018; Chao, Hua, Hsu, Cheng, Liu, & Chang, 2005).

Fluorescence dye based assays

Another classic method to evaluate the impacts of EOs on permeability of cell membrane is the fluorescence dye based method coupled with a fluorescence spectroscopy such as confocal laser scanning microscopy (CLSM) (Li, Yu, Lin, Teng, Du, & Ma, 2010). Briefly, test fungi species are stained with a fluorescence dye that can bind with any chemical constitutes within the cell membrane or inside the membrane and initiate its own fluorescence. Among all available fluorescence dyes, calcofluor white (CFW) is a common blue dye that binds to β-(1,4)-polysaccharide of chitin (Rasconi, Jobard, Jouve, & Sime-Ngando, 2009). Since chitin is one of the prime constituent of fungal cell walls (Watanabe, Azuma, Igarashi, & Ooshima, 2005), CFW has been widely applied to investigate the alteration of fungal cell wall through observing the change of chitin content (e.g., localization and amount of chitin). For example, the control mycelia of *Neofusicoccum parvum* stained with CFW appeared typical blue fluorescence, which reflected the normal distribution of fungal chitin under CLSM. Contrarily, weaker fluorescence intensity was found in the mycelia of *N. parvum* treated by dill seed EO when compared with the control. This indicated the reduction of chitin content as well as the damaged cell wall in *N. parvum* was happened (Liu et al., 2022). Except CFW, another fluorescent dye Alexa Fluor™ 488 Conjugate (WGA-Alexa-fluor-488) has a strong affinity for binding the N-acetylglucosamine of chitin. Therefore, it can be also used for monitoring the fungal localization and infection pattern in hosts such as cereal grains (Kobae et al., 2015). For instance, FHB-infected barley as well as malt barley were stained with WGA-Alexa-fluor-488, and were observed the fungal infection with the aid of CLSM. Results found that the fungi hyphae in barley had an expansion during malting process (Jin et al., 2021). Thus, both CFW and WGA-Alexa-fluor-488 can be useful dyes to monitor the alterations of chitin in fungal cell walls *in*

vitro and in cereal grains. In addition to specific chitin binding dyes, fluorescein diacetate (FDA) and PI are also commonly used dyes to evaluate permeability and viability of fungal cell membrane. Non-polar FDA is an excellent indicator of esterase activities in cell membrane because it can diffuse into healthy cells freely and can be converted to polar fluorescein by intracellular esterases (Battin, 1997) (**Fig. 1-3**). Once the polar fluorescein released from FDA by esterases, it can be accumulated in the cell through an energy-dependent process which is associated with a membrane potential (Battin, 1997). Therefore, the fluorescent intensity is considered as an intuitional sign of cell viability. For instance, a declined fluorescent intensity was observed in *A. flavus* cells after treated with *Zingiber zerumbet* EO nanoemulsion when compared to the control, thus, the viability of fungi was changed (Singh, Chaudhari, Das, & Dubey, 2021). PI is a membrane-impermeable fluorescent dye that binds with nucleic acid (DNA) (Brul, Nussbaum, & Dielbandhoesing, 1997). In general, the dye is leaked into the cell medium due to the expanded permeability of cytoplasmic membrane, and reacted with DNA and RNA, resulting in an increased fluorescence intensity (**Fig. 1-3**). Therefore, it can be used to evaluate the damaged membrane induced injured or dead cells. For instance, CO exhibited excellent antifungal activities against *C. albicans* with the MIC of 0.64 µl/ml (v/v). When *C. albicans* treated with 2.5 µl/ml of CO, more than 95% of the cells were penetrated by PI, indicating the injured cell membrane (Pinto, Vale-Silva, Cavaleiro, & Salgueiro, 2009). Therefore, using fluorescence spectroscopy to observe the permeability and viability of fungal cells is a reliable method.

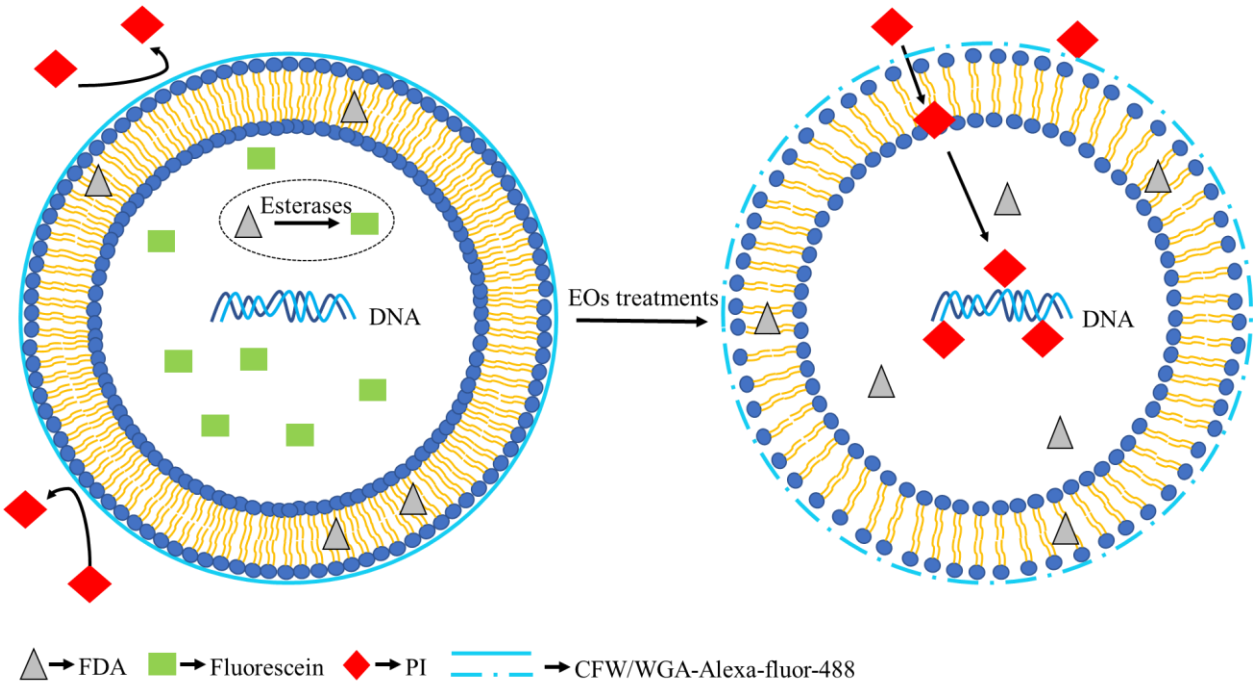

Figure 1-3. Fluorescence dye-based assay for evaluating permeability of cell membrane.

Mycotoxin inhibitory activity of essential oils

Mycotoxins as fungal secondary metabolites present negative health impact on humans and animals. Many studies demonstrated that a number of EOs and EOs in their nanoemulsions can inhibit the fungal growth, and eliminate mycotoxin production *in vitro* as well as in grains including aflatoxin, ZEA and DON production. For example, CO was shown to be very effectively for controlling aflatoxin and fumonisin mycotoxin production *in vitro* (Juglal, Govinden, & Odhav, 2002). The results indicated that Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), and aflatoxin G_2 (AFG_2) could be completely inhibited, whereas 66% reduction of AFG_1 were found upon applying 0.1 μL/mL of the CO. In terms of inhibition effects on ZEA and DON production *in vitro*, thyme, ginger and *Curcuma longa* oils have showed encouraging results (Kumar, Venkataramana, Allen, Chandranayaka, Murali, & Sara, 2016; Romoli et al., 2022). Our recent *in vitro* studies also found that thyme and CO nanoemulsions showed the highest DON inhibitory activities among the selected five EOs (Wan et al., 2019). Regarding the EO treatment

effect on grains, majority of studies evaluate the mycotoxin inhibitory efficacy on wheat seeds and/or maize (Belasli et al., 2020; Juarez, Bach, Barcenas-Pozos, & Hernandez, 2021; Velluti, Sanchis, Ramos, Turon, & Marin, 2004). For example, Marín et al. (Marin, Velluti, Ramos, & Sanchis, 2004) demonstrated that CO had good inhibitory efficacy on ZEA and DON production in maize seeds. Compared with the control, the ZEA and DON contents in maize was significantly reduced from 10 mg/kg to less than 1 mg/kg and 1 mg/kg to ~ 0.1 mg/kg, respectively, with the treatments of CO at 30 °C and 0.950 water activities (a_w) for 24h (Marin et al., 2004). However, very few studies examined the impact of EOs on inhibition of *Fusarium* mycotoxin production in grains during food processing. Our recent study found that CO nanoemulsions with the concentration of 1.5 mg/g could efficiently reduce the DON contents in the final malts when compared with the control (Wan et al., 2020). The results demonstrated that EO nanoemulsion could be utilized as natural antifungal agents during food processing to control mycotoxin contamination in the final food products.

Application of essential oil-in-water nanoemulsion during the malting processing

Introduction of malting process and the fate of *Fusarium* mycotoxin during the malting process

Malting is a controlled germination process which limits the degree of cereal grain germination (Briggs, 1998), and high enzyme and vitamin contents are developed during this process (Baranwal, 2017). The main malting steps include steeping, germination and kilning (Habler et al., 2016). During steeping, dirt and broken kernels are removed. Kernels are soaked into water with a number of air rest and soak cycles. The moisture content of kernel is reached up to ~ 45% which enables the metabolic processes of germination (Baranwal, 2017). During germination, several enzymes (e.g., amylase) are developed for hydrolyzing starch, proteins and

lipids (Guzman-Ortiz et al., 2019). During kilning stage, the dried germinated malt is obtained. Meanwhile, the malt flavor is further developed because of the non-enzymatic browning reactions (e.g., Maillard reactions, caramelization, and pyrolysis) at the high temperature of kilning stage (Larsson, Svensson, & Apel, 2019; Prado, Gastl, & Becker, 2021). Finally, the malt is obtained by removing the rootlets of dried malt.

In general, the fate of *Fusarium* mycotoxin during the malting process are varied. Normally, *Fusarium* mycotoxin contents were reduced to an extent after steeping due to its water-soluble nature, and kept it at a low level in the final malt (Schwarz, Casper, & Beattie, 1995; Wolf-Hall & Schwarz, 2002). However, mycotoxin levels in final malts also influenced by a number of factors including infected *Fusarium* species in grains, cereal grain types as well as mycotoxin infection level (Habler et al., 2016; Z. Jin et al., 2018; Zhao Jin et al., 2018; Malachova, Cerkal, Ehrenbergerova, Dzuman, Vaculova, & Hajslova, 2010). Specifically, a study found that the type B trichothecenes were increased obviously up to 5400% after kilning by using *F. culmorum* infected barley samples. By contrast, the type A trichothecenes production were declined after malting process by applying *Fusarium sporotrichioides* infected barley grain (Habler et al., 2016). This demonstrated that the mycotoxin contents in the final malts might be impacted by the infected *Fusarium* species. Moreover, grain types and mycotoxin infection levels in the original grains may also influence the mycotoxin contents in final malts. For example, Jin *et al.* (Jin et al., 2018; Zhao Jin et al., 2018) found increased trends of DON concentrations in most of the hard red spring wheat malts and rye malts when compared with the original grains. In another words, these grains were all showed high DON contamination levels in the original wheat and rye. But, for the wheat grains with DON contents less than 0.25 μg/g, there was no significant alterations of DON contents in their final malts. Another study also

found no changes of DON contents in 1 out of 10 barley grains, meanwhile, 2 out of 10 barley grains showed the declined DON levels in the final malts (Malachova et al., 2010). Other researchers also found this phenomena, Yu *et al.* (Yu et al., 2019) discovered the DON levels tended to keep consistent or decrease in 18 out of 20 barley malt samples, but the DON levels increased in 2 out of 20 malts. As can be seen, the fate of *Fusarium* mycotoxin contents in the final malts was quite complicated, and can be affected by many factors. Therefore, it is hard to make a precise prediction on *Fusarium* mycotoxin levels in the final malts by only measuring the mycotoxin concentration in raw material (cereal grains).

Impact of *Fusarium* mycotoxin *on* the quality of malts

In general, malting the FHB-infected cereal grains not only cause the mycotoxin accumulation in the final malts, but also negatively impact the quality of malts such as germination rate and enzyme activities. Germination rate is one of a key factor for malting processing, and it generally requires > 95%. The poor germination rate of cereal grains leads to several malt problems, such as changes of enzyme activities and malt extract values (Wolf-Hall, 2007). Studies found that the germination rates of FHB-infected barley was decreased appreciable (Schwarz, Jones, & Steffenson, 2002), which might be due to the invading fungal hyphae in kernel embryo and/or the presence of *Fusarium* mycotoxins (Schapira, Whitehead, & Flannigan, 1989; Wolf-Hall, 2007). In terms of enzyme activities in grains/malts, studies found that FHB-infected malts showed high xylanase activities which might be caused by the appearance of fungal enzymes such as high endogenous β-glucanase activities at 60°C. This pheromone can be explained by the exist of thermostable fungal β-glucanase in FHB-infected malts. By contrast, low β-glucanase activities at 30°C was observed which indicated the reduction of barley endogenous enzyme activity after infected by fungi (Sarlin, Laitila,

Pekkarinen, & Haikara, 2005). Other than abovementioned malt qualities, the malt extract values and lautering performance of malts are also deceased, but soluble nitrogen and free amino nitrogen content in final malts are raised by malting the FHB-infected barley grains (Sarlin et al., 2005; Sloey & Prentice, 1962). Hence, it is needed to find cost-effective strategies to mitigate *Fusarium* mycotoxin of FHB-infected barley in final malts due to the potential safety and quality concern for malting companies.

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CHAPTER 2. CHEMICAL COMPOSITION OF ESSENTIAL OILS FROM LEAF AND BUD OF CLOVE AND THEIR IMPACT ON THE ANTIFUNGAL AND MYCOTOXIN INHIBITORY ACTIVITIES OF CLOVE OIL-IN-WATER NANOEMULSIONS

Abstract

The impacts of clove essential oil (CO) type on the physicochemical properties, antifungal activities, and mycotoxin inhibitory efficacies of CO-in-water nanoemulsions were investigated. By blending 25 wt% of CO with 75 wt% of corn oil in oil phase (5 wt%), clove bud essential oil (CBO)-in-water nanoemulsion and clove leaf essential oil (CLO)-in-water nanoemulsion showed highly physical stable with the mean particle size < 170 nm. The concentration of major chemical constituents such as eugenol and caryophyllene in nanoemulsion maintained constant during storage time. The antifungal activities of nanoemulsions against two *F. graminearum* isolates were strongly dependent on the CO concentration. For instance, CLO nanoemulsion displayed an appreciable higher antifungal activity compared to CBO nanoemulsion at a lower CO concentration. Both nanoemulsions could effectively inhibit fungal mycelial growth and spore germination, but in a different antifungal mode of actions (MOA). Regarding the mycotoxin inhibitory efficacy, both CO nanoemulsions could effectively inhibit mycotoxin production including deoxynivalenol (DON) and its derivatives: 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON).

Introduction

Fusarium species, such as *F. graminearum* and *Fusarium culmorum* are widely recognized as mycotoxin producers in the field, which not only cause the FHB disease in cereal crops in the field but also produce mycotoxins (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018; Shin et al., 2018; Varga, Malachova, Schwartz, Krska, & Berthiller, 2013). In

general, mycotoxins produced by *F. graminearum* are type B trichothecenes including DON and its derivatives: 3ADON, 15ADON, and NIV. Among them, DON is one of the most common mycotoxins that have often time been detected in agricultural products (Machado, Mallmann, Mallmann, Coelho, & Copetti, 2017; Tima, Bruckner, Mohacsi-Farkas, & Kisko, 2016). This is because it is impossible to completely avoid the contamination of DON in cereal crops in the field even with GAP (Wilson, Dahl, & Nganje, 2018). Moreover, numerous studies have indicated that the level of DON occurred in cereal grains can be reduced to some extent during food processing, but it cannot be completely eliminated in the final food product due to its extremely thermal stable properties (Khaneghah et al., 2018; Sundheim, Lillegaard, Faeste, Brantsaeter, Brodal, & Eriksen, 2017). For example, it has been reported that the DON level in winter wheat malt increased when compared to the original grain that was infected naturally by *Fusarium* species in the field (Jin, Cao, Su, Yu, & Xu, 2018). DON contamination in agricultural products is a significant safety concern since it could potentially cause a number of adverse health effects (Sundheim et al., 2017). Consequently, it is of great urgency to develop effective detoxification strategies that can be adopted by the agricultural and food industries to mitigate mycotoxin contamination.

In recent years, CO has gained popularity as a natural antifungal agent against mycotoxigenic fungi growth and mycotoxin production (Boukaew, Prasertsan, & Sattayasamitsathit, 2017; Bozik, Cisarova, Tancinova, Kourimska, Hleba, & Kloucek, 2017; Hu et al., 2019; Lima et al., 2019). Our previous studies also revealed that CO exhibited superior antifungal activity against *F. graminearum* growth as well as mycotoxin inhibitory abilities *in vitro* (Wan, Zhong, Schwarz, Chen, & Rao, 2019b). In general, the antifungal activity of CO is mainly determined by its chemical composition, applied concentration, and target fungi species

(Rao, Chen, & McClements, 2019). In terms of chemical composition, CO contains three major chemical compounds including eugenol, eugenol acetate, and β-caryophyllene, along with a number of minor chemical constituents such as humulene, eucalyptol, and α-cadinol (Bhuiyan, Begum, Nandi, & Akter, 2010; Chaieb, Zmantar, et al., 2007; Guynot, Ramos, Seto, Purroy, Sanchis, & Marin, 2003; Juglal, Govinden, & Odhav, 2002). It is of worth noting that the major chemical constituents or their proportions in CO vary substantially depending on the part of clove plant (i.e., leaves and buds) used for oil extraction (Chaieb, Hajlaoui, et al., 2007; Razafimamonjison, Jahiel, Duclos, Ramanoelina, Fawbush, & Danthu, 2014). However, the impact of CO type and their chemical constituents on mycotoxigenic fungi growth and mycotoxin inhibitory efficacy remains unknown. As such, it is necessary to explore antifungal and mycotoxin inhibitory performance of different CO types in order to better promote their application in the food systems.

In fact, very few of bulk EOs including CO have been successfully incorporated into fungicide/pesticide as natural antifungal agents. This is especially true in aqueous-based agricultural products owning to the EOs' high volatility, low water solubility, potential flavor impact, and relatively low efficacy of antifungal performance (Wan, Zhong, Schwarz, Chen, & Rao, 2018). Among the approaches designed to overcome these limitations, our group found that encapsulating CO in oil-in-water nanoemulsion delivery system could be a potential means. In this system, CO is first dissolved in a carrier oil (e.g., long chain triglyceride oil), which is then encapsulated into water soluble nanometric particles with the aid of emulsifiers through high-pressure homogenization. After being trapped in the nanoparticles, the major volatile compounds in CO can be gradually released to the environment. Meanwhile, the mass transfer efficacy of

nanoemulsion encapsulated CO to get close proximity to fungi is improved compared to bulk CO due to the increased surface area of nanodroplets (Wan et al., 2018).

In the current study, we set forth to investigate the impact of EO nanoemulsion consisting of either CBO or CLO on antifungal and mycotoxin inhibitory efficacies. The overall objective of this study was to further understand if CO with different chemical constituents could determine their antifungal and mycotoxin inhibitory efficacy. We first characterized the chemical constituents of CBO and CLO using GC-MS. Then, CBO and CLO nanoemulsions were prepared, and their long-term physiochemical stability was assessed. The antifungal and mycotoxin inhibitory efficacies against *F. graminearum* mycelial growth, spore germination, and mycotoxin production including DON, 3ADON, and 15ADON was systematically evaluated. Lastly, the antifungal MOA of both nanoemulsions was compared through observation of morphological changes in the fungal hyphae and spores.

Materials and methods

Materials

CBO and CLO (FEMA # 2323, FG grade) were kindly donated by Kalsec Inc. (Kalamazoo, MI). Corn oil and mung beans were purchased from a local supermarket (Fargo, ND, USA). Potato dextrose agar (PDA) was purchased from AMRESCO (Solon, OH, USA). Potato dextrose broth (PDB) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Polyoxyethylene (20) sorbitan monooleate (Tween 80), sodium phosphate monobasic and dibasic, eugenol, eugenol acetate, caryophyllene and caryophyllene oxide, Mirex and N, and O-Bis (trimethylsilyl)acetamide (BSA)/ trimethylchlorosilane (TMCS)/ Trimethylchlorosilane (TSIM) kit for mycotoxin analysis were purchased from Millipore Sigma Co. (St Louis, MO, USA). Two *F. graminearum* isolates, 10-124-1 and 10-125-1, were used in this study. These two

fungal isolates were originally isolated and maintained by Dr. Zhong's Lab in the Department of Plant Pathology at North Dakota State University (Fargo, ND, USA). All solutions were prepared using ultrapure distilled deionized water (DDW, 18.2MΩ cm, Barnstead ultrapure water system, Thermo Fisher Scientific, USA).

Chemical composition analysis of clove oils

The chemical composition of CBO and CLO was analyzed using Agilent 7890B/5977A gas chromatography–mass spectrometry (GC-MS) system according to previous report without any modification (Wan et al., 2019b).

Clove oil-in-water nanoemulsion preparation and characterization

Nanoemulsion preparation

The CO-in-water nanoemulsion consisted of 5.0 wt% oil phase, 0.5 wt% Tween 80, and 94.5 wt% of phosphate buffer system (10 mM, pH 7.0). The oil phase (5 wt%) was prepared by mixing either CBO or CLO with corn oil at a mass ratio of 2.5:7.5 based upon the results of our preliminary study. The nanoemulsions were prepared by high-pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) as reported previously without any modification (Wan, Zhong, Schwarz, Chen, & Rao, 2019a). The finished nanoemulsions contained 0.5 % (w/v) emulsifier, 1.25% (w/v) of CO, 3.75% (w/v) of corn oil, and 94.5% (w/v) of buffer and were stored in test tubes sealed with screw caps for further analysis. According to this formula, the total concentration of each CO in nanoemulsion was 12.5 mg/g.

Particle size measurement

The mean particle diameter and particle size distribution of nanoemulsions were measured by a dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK) according to our previous study (Wan et al., 2018). Nanoemulsions

were diluted 20 times using buffer solution (10 mM phosphate acid, pH 7) to avoid multiple scattering effects prior to particle size analysis.

Long term physicochemical stability of nanoemulsion

The long-term physical stability of nanoemulsions stored at 4 ℃ and 25 ℃ was evaluated by measuring the particle size as a function of storage time (28 days). The long-term chemical stability of CO nanoemulsions was evaluated by quantifying the change of concentration of selected aroma markers in CO nanoemulsion as a function of time stored at 25 ℃ (7 days). In brief, 1 mL of nanoemulsion was transferred into 2 mL of GC vial immediately after high pressure homogenization. Eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide were selected as major flavor makers in CBO; whereas eugenol, caryophyllene, and humulene were used as predominate flavor makers in CLO according to the result of the chemical composition CLO. Standard curve of each flavor maker was prepared by measuring a serially diluted concentration of flavor maker with methanol. The concentration was quantified by using Agilent 7890B/5977A GC-MS system as reported previously (Yang et al., 2011). The concentration of individual flavor marker in CO-in-water nanoemulsion as a function of time was calculated according to the standard curves. Results are expressed in mg flavor markers/mL nanoemulsion.

Influence of clove oil type on antifungal activity of nanoemulsions

Mycelial growth

F. graminearum isolates, 10-124-1 and 10-125-1, were selected to evaluate the antifungal activity of CBO-in-water nanoemulsion and CLO-in-water nanoemulsion according to the method from Wan et al. (Wan et al., 2018). *F. graminearum* 10-124-1 mainly produces DON and 15ADON; whereas *F. graminearum* 10-125-1 is responsible for producing DON and 3ADON

mycotoxins. In brief, a serial of diluted nanoemulsions with desired gradient concentrations of clove oil (1.25, 2, 3.2, 5.12, 6.4, 8, 10,12.5 mg/g) in the final nanoemulsions were applied to measure antifungal activity in terms of mycelial growth. 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). The sterile deionized water was used as a control. MGI rates were fitted to a quadratic regression model, and effective concentration (EC_{50}) value was defined as nanoemulsion concentration that can inhibit 50% of MGI rate and calculated using the quadratic regression model.

Spore germination

The antifungal efficacy of CO nanoemulsions against *F. graminearum* spore germination was measured according to our previous method with slight modifications (Wan et al., 2019a). Briefly, the concentration of spore suspension was adjusted to 1.0×10^6 spores/mL by sterile deionized water. An equal volume of spore suspension (100 μL) and CO nanoemulsion (100 μL) with a series of gradient CO concentrations in nanoemulsions (2.05, 2.56, 3.2, 4, 10 mg/g) was mixed. The spore germination rate was calculated as P (%) = (number of germinated spore/numbers of total spore) ×100%. The spore germination inhibition (SGI) rate was calculated as SGI (%) = 100 × ($P_{control}$ − $P_{treatment}$) / ($P_{control}$). The sterile deionized water was used as a control.

Morphological study

The effects of CO nanoemulsions on the morphological properties of *F. graminearum* hyphae and spore were analyzed by SEM following the methods of our previous study (Wan et al., 2019a). The sterile deionized water was applied as a control.

Impact of clove oil type on mycotoxin production in rice culture

The mycotoxin inhibitory efficacy of the two CO nanoemulsions was determined according to our previous rice culture method (Wan et al., 2019a). Each sample was incubated at 25 °C in dark for 5 days. The final CO concentrations in rice cultures were set as 800 μg/g rice. The sterile deionized water was applied as a control.

Statistical analysis

All measurements were carried out at least three times and were reported as mean ± standard deviation. The completely random design (CRD) was conducted in this assay. Significant differences between means ($p < 0.05$) were performed using one-way analysis of variance (ANOVA) and F-protected LSD by SAS software (Version 9.3, SAS Institute Inc., NC, USA).

Results and discussion

Chemical composition of clove bud essential oil and clove leaf essential oil

The chemical composition of an EO is crucial information for determining not only functional properties, but also the physiochemical stability of EO-in-water nanoemulsion. Therefore, the chemical constituents of CBO and CLO, as well as their water solubility are shown in **Table 2-1**.

Table 2-1. Major chemical constituents and water solubility of CBO and CLO. Water solubility of each compound was obtained from the data banks of Pubchem (https://pubchem.ncbi.nlm.nih.gov/) and The Good Scents Company Information System (http://www.thegoodscentscompany.com/index.html).

Peak No.	Compounds	Percentage (%)	Water Solubility (mg/l)
CBO			
1	Eugenol	39.69	2460.00
2	Eugenol acetate	32.49	Insoluble in water
3	Caryophyllene	14.45	Insoluble in water
4	Caryophyllene oxide	3.79	Insoluble in water
5	Humulene	2.96	Insoluble in water
		Total: 93.37%	
CLO			
1	Eugenol	50.35	2460.00
2	Caryophyllene	29.11	Insoluble in water
3	Humulene	12.16	Insoluble in water
4	Caryophyllene oxide	1.78	Insoluble in water
5	δ-Cadinene	1.74	Insoluble in water
		Total: 95.13%	

It is well known that chemical constituents of EOs are influenced by number of aspects such as the extraction method, plant tissue, and geographical origin, etc. (Cheng, Lin, & Chang, 2005; Kumoro, Wardhani, Retnowati, & Haryani, 2021). As can be seen from **Table 2-1**, CBO and CLO each contained five major chemical compounds. Although the total contents were quite similar being 93.38% and 95.14% for CBO and CLO, respectively, the distinct profiles of chemical constituents were observed. For example, the major chemical constituents of CBO were phenols (39.69% of eugenol), followed by esters (32.49% of eugenol acetate), and three sesquiterpenes (caryophyllene, caryophyllene oxide and humulene). CLO, on the other hand, consisted of one phenol (eugenol) and four sesquiterpenes (caryophyllene, humulene, caryophyllene oxide and δ-Cadinene), which accounted for 50.35% and 44.79% of the total oil. Surprisingly, eugenol acetate, one of the major chemical compounds in CBO, was not detected in CLO. Our findings agreed with the conclusions from other researchers that chemical constituents

of COs differed markedly depending on whether it's from the parts of clove bud, leaf, or stem (Razafimamonjison et al., 2014).

Long term physical and chemical stability of clove oil-in-water nanoemulsions during storage

Typically, pure CO-in-water nanoemulsions are highly unstable against droplet growth over storage time because of Oswald ripening (Wan et al., 2018). There was no exception to this effect for either pure CBO or CLO nanoemulsion. Ostwald ripening is the major destabilization mechanism responsible for the instability of EO nanoemulsions. This is because the relatively high-water solubility of chemical compounds in EOs causes the mass transport of dispersed phase from one droplet to another. As a result, large droplets grow at the expense of smaller ones through the intervening continuous phase (Luis et al., 2019). Previously, a number of studies have been conducted to reduce the Ostwald ripening phenomenon. It has been found out that EO caused Ostwald ripening in nanoemulsion can be inhibited by incorporating less water-soluble oil such as long chain triglyceride (LCT) type oils in EO prior to form nanoemulsion. It is worth noting that CO with a higher eugenol content rendered the nanoemulsion with less stability and visible oiling off was observed when the CO was above 50% in the oil phase (**Fig. 2-1**), which is in line with the greater water-soluble property of eugenol (**Table 2-1**). The physical instability of nanoemulsions caused by Oswald ripening can be reduced by mixing CO with relatively low water-soluble oils (e.g., LCT oil, also call as Oswald ripening inhibitor). However, the higher amount of Oswald ripening inhibitor incorporated into the EO phase, the antifungal and mycotoxin inhibitory efficacy might be reduced by the addition of Oswald ripening inhibitor. According to our preliminary study, corn oil is an effective Oswald ripening inhibitor to prepare physical stable 5 wt% CO-in-water nanoemulsions (Wan et al., 2018). Therefore, we have been

investigated how different ratios of corn oil (one type of LCT) and CO impact on the stability of CO nanoemulsions. As can be seen in **Figure 2-1**, the corn oil and CO ratio was 1:1 or below 1:1, CO nanoemulsions were highly unstable after 24 hours' storage at the room temperature. By contrast, when the 75% of corn oil mixed with 25% CBO/CLO in total oil phase, the mean particle diameter of the CO nanoemulsions haven't been changed as compared to the particle size of freshly prepared nanoemulsions. As a result, the ratio between corn oil and CO of 7.5:2.5 was selected to form CO-in-water nanoemulsion in this study. Thereafter, the long-term (28 days) physical stability of the abovemetioned CO nanoemulsions under two different storage temperatures (4 °C and 25 °C) was monitored by measuring the mean particle size of the nanoemulsions (**Fig. 2-2**).

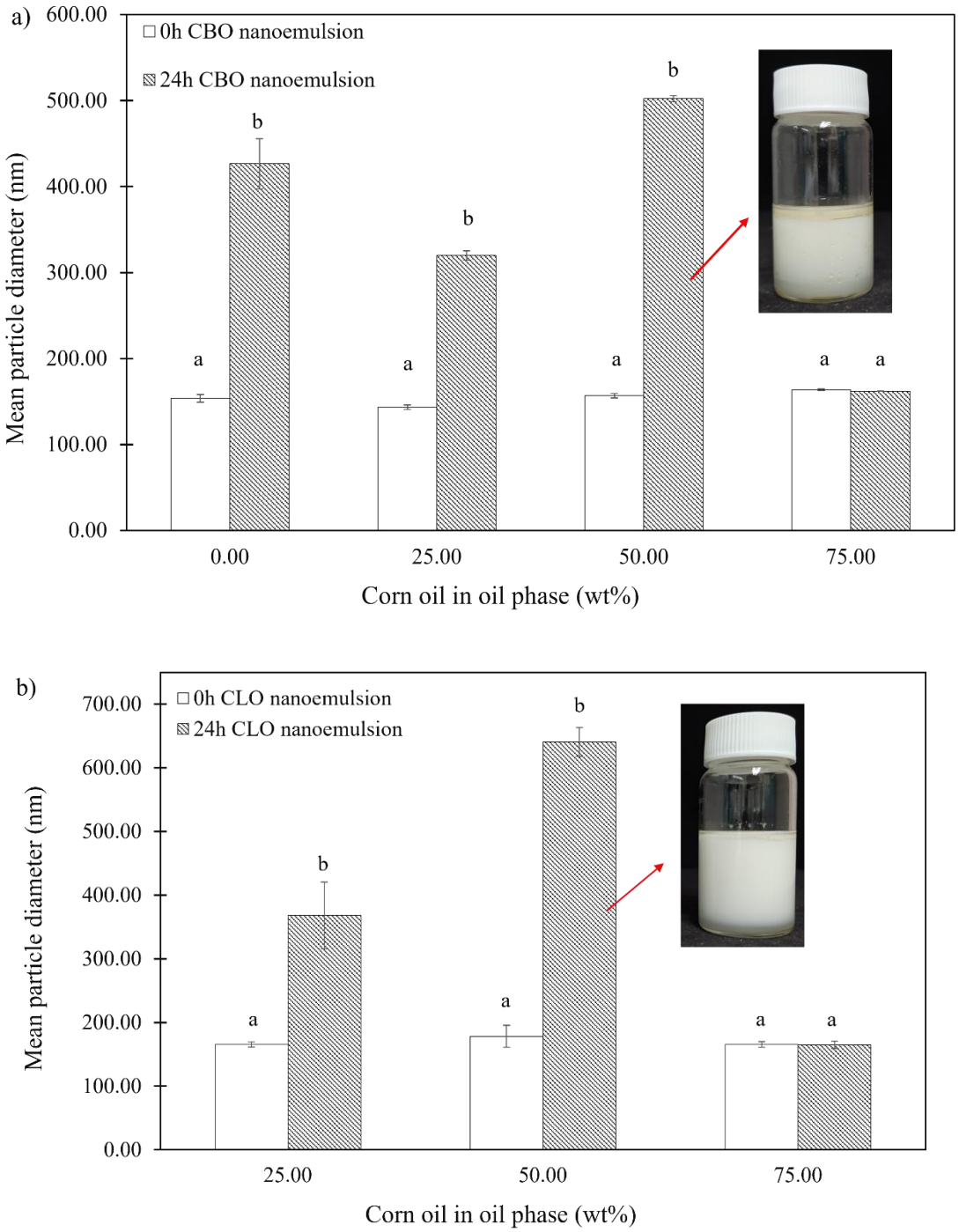


Figure 2-1. Dependence of the oil phase Ostwald ripening inhibitor: corn oil on the mean particle diameter of 5 wt% (a) CBO-in-water emulsions and (b) CLO-in-water emulsions after 0 and 24 h storage at 25 °C (0.5 wt% Tween 80, 10 mM phosphate buffer, pH 7; the inserted picture is the visual observation of oiling off in the emulsion).

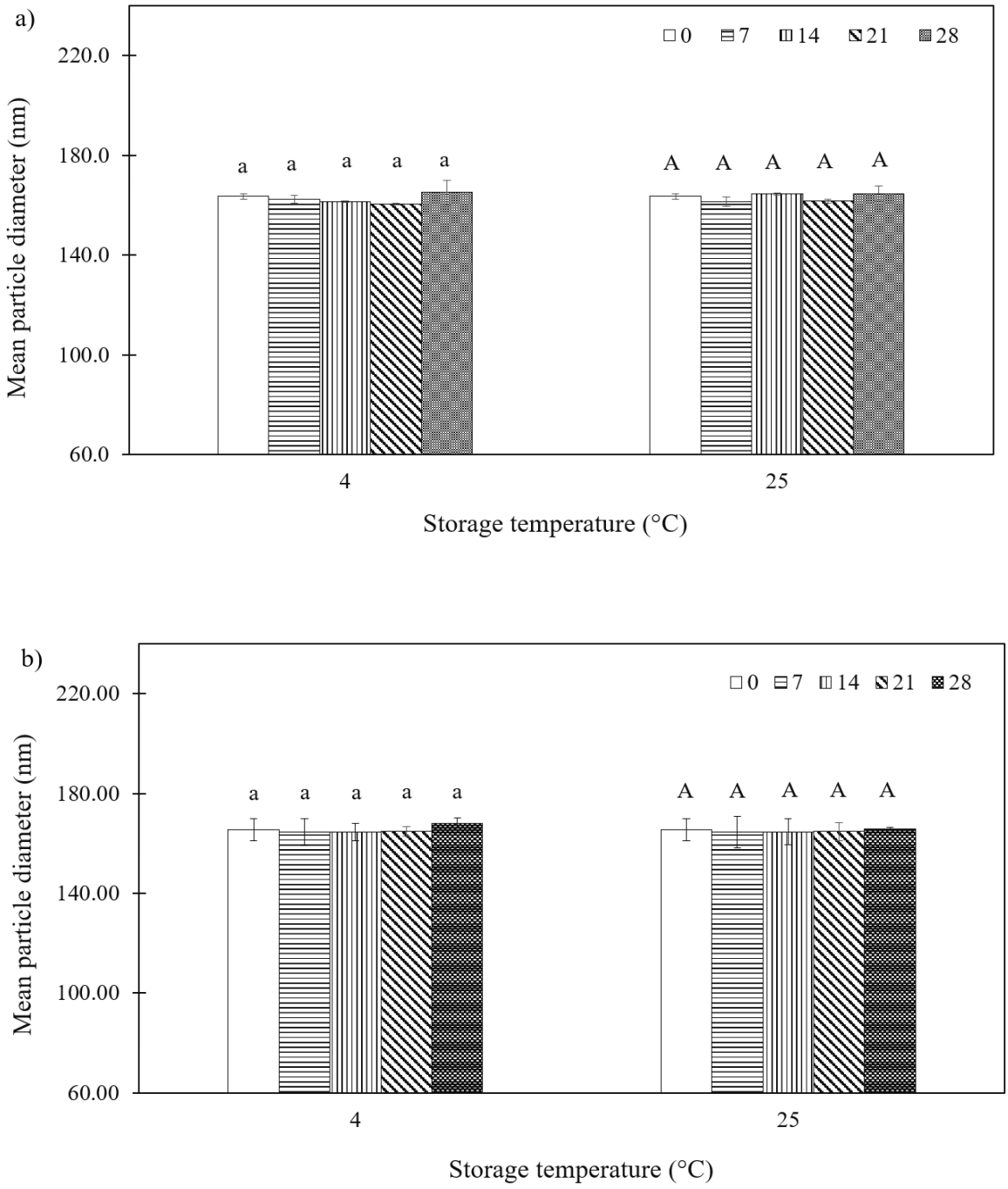
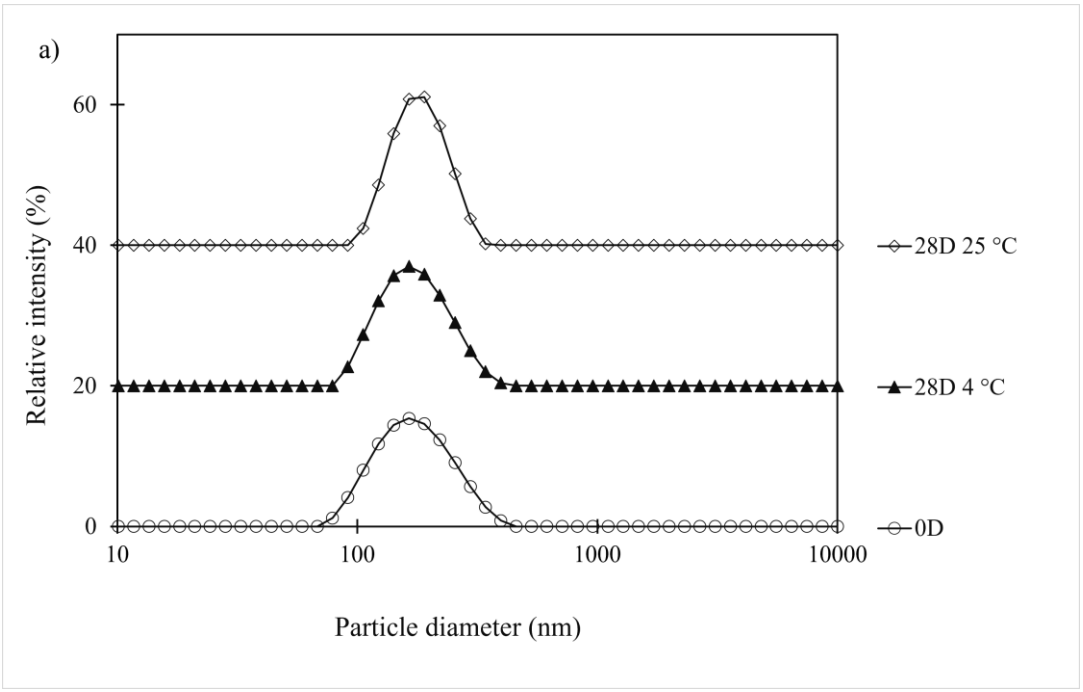


Figure 2-2. Physical storage stability of (a) CBO nanoemulsion and (b) CLO nanoemulsion at different storage temperature (4 & 25 ℃). For each storage temperature, values of various days with different lowercase letters or capital letters were significantly different from each other at $p < 0.05$.

Initially, the mean particle diameters of freshly prepared CBO and CLO nanoemulsions were ~ 160 nm, which falls into the size range of nanoemulsion (20-200 nm) (Jaiswal, Dudhe, & Sharma, 2015). The identical mean particle size between CBO nanoemulsion and CLO nanoemulsion suggested that the particle size of CO nanoemulsions was mainly influenced by formula (e.g., oil and emulsifier concentration) rather than by the chemical constituents of the CO. In terms of long-term stability, both CO nanoemulsions exhibited outstanding physical stability against droplet growth over 28 days of storage under both storage temperatures. In particular, the nanometric size of all nanoemulsions was retained after 28 days of storage. Particle size distribution is another critical indicator for elucidating physical stability of nanoemulsions. The particle size distribution figures (**Fig. 2-3**) clearly showed that all CO nanoemulsions maintained monomodal distributions without any shift of distribution curve for the entire storage time under both temperatures. Moreover, no visible changes on the appearance of nanoemulsions were observed. These results denote that the selected nanoemulsion formula in our study was the optimal to maintain uniform and nanometric droplets against droplet growth with prolonged storage time.



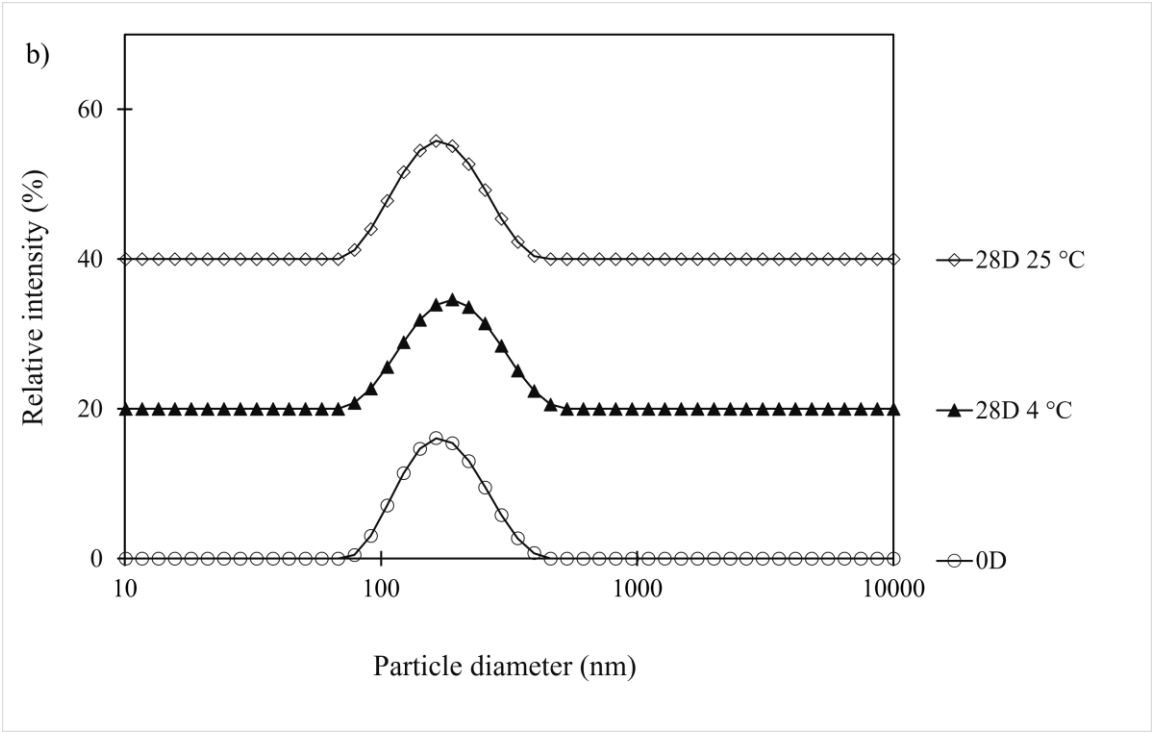


Figure 2-3. Particle size distribution of (a) CBO nanoemulsion and (b) CLO nanoemulsion.

In addition to the physical stability, the long-term chemical stability of EOs in the nanoemulsion is also critical as it determines their antifungal performance. Chemical constituents of EOs such as terpenoids are known to be prone to oxidation or hydrolysis, which may alter the

antifungal activity (Turek & Stintzing, 2013). Since our antifungal and mycotoxin inhibitory activity experiments were conducted at an ambient temperature (25 ℃) over a course of 7 days, the changes in the concentration of major chemical constituents including eugenol, eugenol acetate, caryophyllene, caryophyllene oxide, and humulene in CO-in-water nanoemulsions under the same condition (7 days and 25 ℃) were evaluated (**Table 2-2**). The results indicated that all of the examined chemical components showed a similar trend during storage time, where the majority of the compounds remained at 100% of their initial concentration. Conversely, the content of these compounds was slightly decreased in pure CO after storage (**Table 2-3**). Our findings prove that the optimized nanoemulsion in this study could provide a positive protective effect on the chemical stability of CO. This is in a great agreement with previous studies that the encapsulation of CO can prevent major chemical compounds such as eugenol form UV light initiated degradation (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015).

Table 2-2. Chemical stability of CBO-in-water nanoemulsions and CLO-in-water nanoemulsions over 7 days of storage at 25 ℃.

	CBO nanoemulsion				CLO nanoemulsion		
Day	Caryophyllene	Caryophyllene oxide	Eugenol	Eugenol acetate	Caryophyllene	Humulene	Eugenol
	----------mg/ml----------				----------mg/ml----------		
0	0.29±0.05^{ab}	0.07±0.01^{abcd}	5.51±0.21^{abc}	1.42±0.11^{abc}	0.77±0.10^{a}	0.29±0.03^{abc}	5.81±0.75^{a}
0.5	0.40±0.01^{a}	0.09±0.01^{a}	6.66±0.40^{a}	1.71±0.09^{a}	0.83±0.13^{a}	0.31±0.04^{abc}	6.15±0.72^{a}
1	0.37±0.03^{a}	0.09±0.01^{a}	6.33±0.01^{abc}	1.58±0.23^{ab}	0.79±0.13^{a}	0.29±0.04^{abc}	5.96±0.36^{a}
2	0.24±0.07^{b}	0.07±0.01^{cd}	5.14±1.00^{c}	1.20±0.19^{c}	0.69±0.30^{a}	0.25±0.10^{bc}	5.44±1.65^{a}
3	0.28±0.11^{ab}	0.06±0.00^{d}	5.31±1.05^{bc}	1.14±0.14^{c}	0.55±0.20^{a}	0.20±0.06^{c}	5.03±1.79^{a}
4	0.23±0.08^{b}	0.07±0.01^{cd}	5.28±0.88^{bc}	1.22±0.21^{c}	0.68±0.23^{a}	0.25±0.08^{bc}	5.71±1.09^{a}
5	0.33±0.04^{ab}	0.08±0.01^{abc}	6.57±0.06^{ab}	1.51±0.34^{abc}	0.75±0.27^{a}	0.33±0.02^{ab}	6.51±0.43^{a}
6	0.31±0.04^{ab}	0.07±0.01^{abcd}	6.05±0.55^{abc}	1.34±0.03^{bc}	0.87±0.18^{a}	0.35±0.01^{a}	6.72±0.18^{a}
7	0.28±0.05^{ab}	0.07±0.00^{cd}	5.69±0.52^{abc}	1.37±0.15^{abc}	0.67±0.19^{a}	0.33±0.08^{ab}	6.27±0.42^{a}

† The values with different superscript letters within a column are significantly different ($p< 0.05$).

Table 2-3. Chemical stability of CBO and CLO over 7 days of storage at 25 ℃.

	CBO				CLO		
Day	Caryophyllene	Caryophyllene oxide	Eugenol	Eugenol acetate	Caryophyllene	Humulene	Eugenol
	----------mg/ml----------				----------mg/ml----------		
0	0.61±0.01^{a}	0.11±0.00^{a}	8.28±0.15^{a}	2.18±0.25^{ab}	1.50±0.00^{a}	0.54±0.02^{ab}	8.35±0.18^{a}
0.5	0.57±0.10^{ab}	0.10±0.00^{ab}	8.08±0.78^{ab}	2.31±0.13^{a}	1.51±0.25^{a}	0.55±0.07^{a}	8.32±0.28^{a}
1	0.53±0.06^{abc}	0.10±0.01^{abc}	7.57±0.74^{abc}	2.17±0.23^{ab}	1.33±0.15^{ab}	0.48±0.06^{abc}	7.89±0.73^{ab}
2	0.54±0.04^{abc}	0.09±0.01^{bc}	7.39±0.18^{bc}	2.11±0.09^{ab}	1.31±0.08^{ab}	0.47±0.01^{abc}	7.59±0.03^{bc}
3	0.52±0.10^{abc}	0.10±0.01^{ab}	7.28±0.52^{c}	2.11±0.10^{ab}	1.27±0.01^{ab}	0.46±0.01^{bc}	7.43±0.11^{bcd}
4	0.50±0.06^{abc}	0.10±0.02^{ab}	7.22±0.42^{c}	2.26±0.39^{a}	1.09±0.09^{bc}	0.41±0.04^{cd}	7.14±0.28^{cd}
5	0.37±0.01^{d}	0.09±0.00^{bc}	6.47±0.11^{d}	1.95±0.04^{ab}	0.96±0.01^{c}	0.36±0.00^{d}	6.79±0.16^{d}
6	0.44±0.04^{cd}	0.09±0.01^{bc}	6.84±0.18^{cd}	1.95±0.22^{ab}	1.18±0.11^{bc}	0.43±0.02^{cd}	7.37±0.16^{bcd}
7	0.46±0.06^{bcd}	0.08±0.00^{c}	6.87±0.29^{cd}	1.79±0.09^{b}	1.12±0.21^{bc}	0.40±0.05^{cd}	7.12±0.34^{cd}

† The values with different superscript letters within a column are significantly different ($p< 0.05$).

The effect of clove oil type on antifungal activity of *nanoemulsions*

In this section, the antifungal activity of CBO and CLO nanoemulsions was determined by measuring both MGI and SGI rates of the selected two *F. graminearum* isolates (10-124-1 and 10-125-1). The morphological changes of fungal hyphae and spores were also evaluated and compared with a control (water treatment).

Mycelial growth

In this study, the agar dilution method was applied to evaluate the MGI rate of CBO and CLO nanoemulsions against two *F. graminearum* isolates after 4 days' incubation. EC_{50} value is calculated as the concentration of CO in nanoemulsion that can inhibit 50% of mycelial growth. The MGI rate and the morphological changes of the *F. graminearum* hyphae treated with nanoemulsions are presented in **Fig. 2-4**.

It can be clearly seen that the MGI rates were dose dependent. Specifically, the MGI rates grew with the increase of CO concentration in nanoemulsions. For example, as the CLO concentration in nanoemulsion increased from 1.25 mg/g to 8 mg/g, the MGI rates rose from 20.34% to 47.09%. Such results are common as the fungicidal activity of CO is highly reliant on the concentration of active compounds (e.g., eugenol) in CO (Hasheminejad, Khodaiyan, & Safari, 2019; Hu et al., 2019). With regard to the antifungal efficacy, CLO nanoemulsion exhibited a significantly stronger ($p < 0.05$) inhibitory effect on mycelial growth compared to the CBO nanoemulsion at a low CO concentration (≤ 3.2%). For instance, the MGI rates of CLO and CBO were 20.34% and 5.91%, respectively, at the concentration of 1.25 mg/g. Interestingly, when the nanoemulsions contained more than 5.12 mg/g of CO, MGI rates were identical between the two different CO nanoemulsions. This conclusion was also confirmed by EC_{50} values in **Fig. 2-4a (inserted Table)**. As can be seen, there was no significant statistical

difference on EC_{50} values between CBO (8.45 mg/g) and CLO (8.64 mg/g) nanoemulsions ($p > 0.05$), indicating the CBO nanoemulsion had similar antifungal capacity with the CLO nanoemulsion at a higher CO concentration. This phenomenon could be explained by the different eugenol content in CBO and CLO. As shown in **Table 2-1**, CLO had an appreciably higher eugenol content (50.35%) when compared to CBO (39.69%). It has been reported that phenols such as eugenol in EOs exhibit a remarkable antifungal activity when compared to other chemical compounds (Kim et al., 2016). Therefore, when the nanoemulsion with a lower concentration of CO was applied in PDA to inhibit the mycelial growth, a stronger inhibition effect was recorded in CLO nanoemulsion due to its higher eugenol content. However, as the nanoemulsion with a higher concentration of CO was applied to PDA plate, both nanoemulsions contained sufficient amounts of eugenol to reach of the maximum antifungal capacity. Meanwhile, synergistic effect of eugenol with other minor constituents may occur at a higher EO concentration to boost antifungal activity, which otherwise was in absence at a lower concentration. Consequently, no significant difference on the antifungal performance was observed between two CO nanoemulsions when applied at a higher concentration. Furthermore, antifungal efficacy of the two CO nanoemulsions displayed a great similarity independent of the type of isolate (i.e., *F. graminearum* 10-125-1 and 10-124-1). Comparing the EC_{50} values between the two isolates, a distinctly higher EC_{50} value was found for *F. graminearum* 10-125-1 than for isolate 10-124-1, which indicated that the former was more resistant to CO. This is not surprising in light of the disparate responses of the two *F. graminearum* isolates to the same antifungal agent. This is because that *F. graminearum* isolates can be considered as a meta-population ascribing to their genetic and biological variations in performance (van der Lee, Zhang, van Diepeningen, & Waalwijk, 2015). Previous studies including ours also have reported

different responses of *F. graminearum* isolates treated by the same antifungal agent (Pagnussatt, Del Ponte, Garda-Buffon, & Badiale-Furlong, 2014; Wan et al., 2019a).

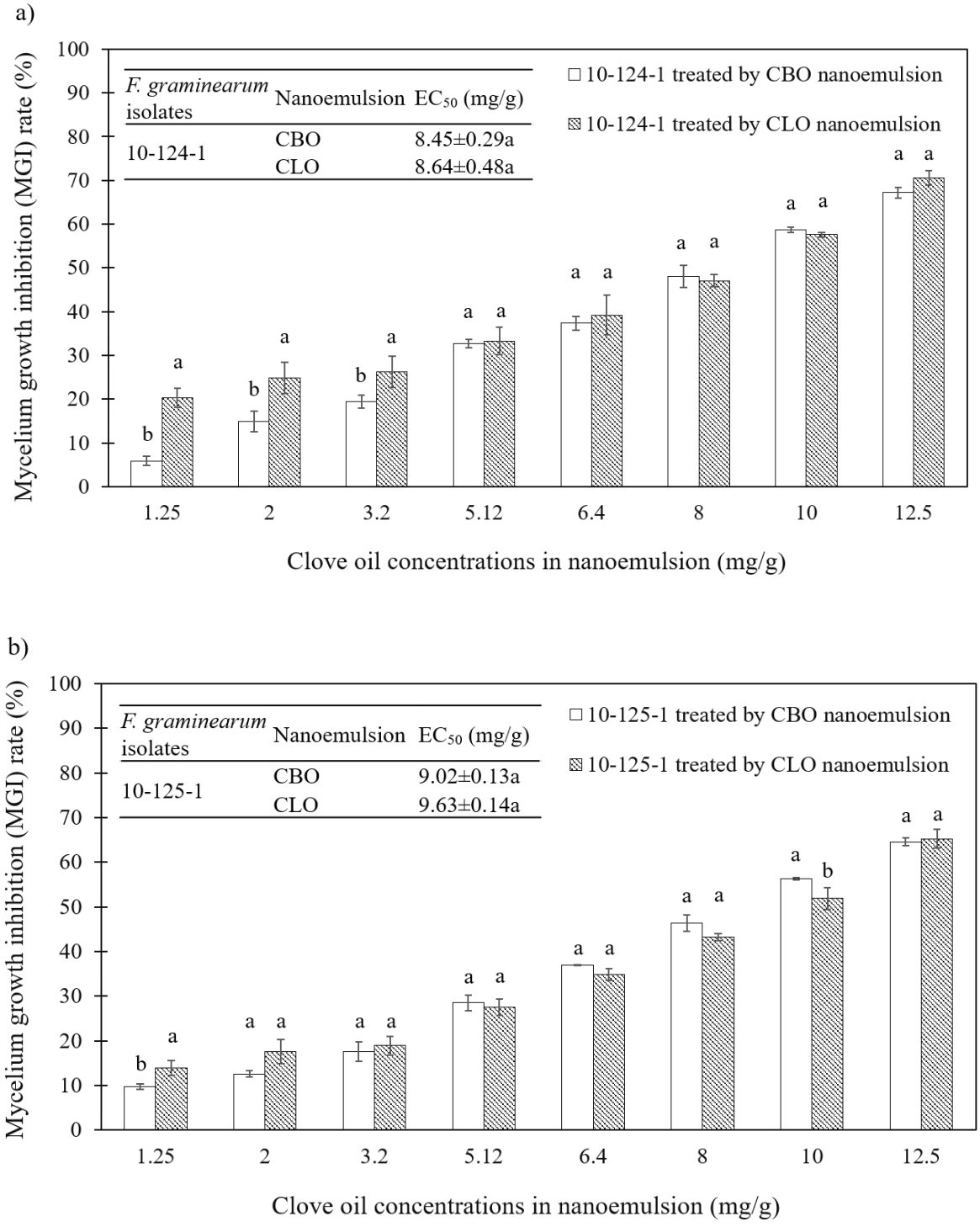


Figure 2-4. Effect of CO nanoemulsion type and CO concentrations in nanoemulsion on mycelial growth of (a) *F. graminearum* 10-124-1 and (b) *F. graminearum* 10-125-1; (c) SEM observation of *F. graminearum* hyphae treated by water (control), CBO and CLO nanoemulsion. Columns within the same CO concentration in nanoemulsion with different letters are significantly different at $p < 0.05$.

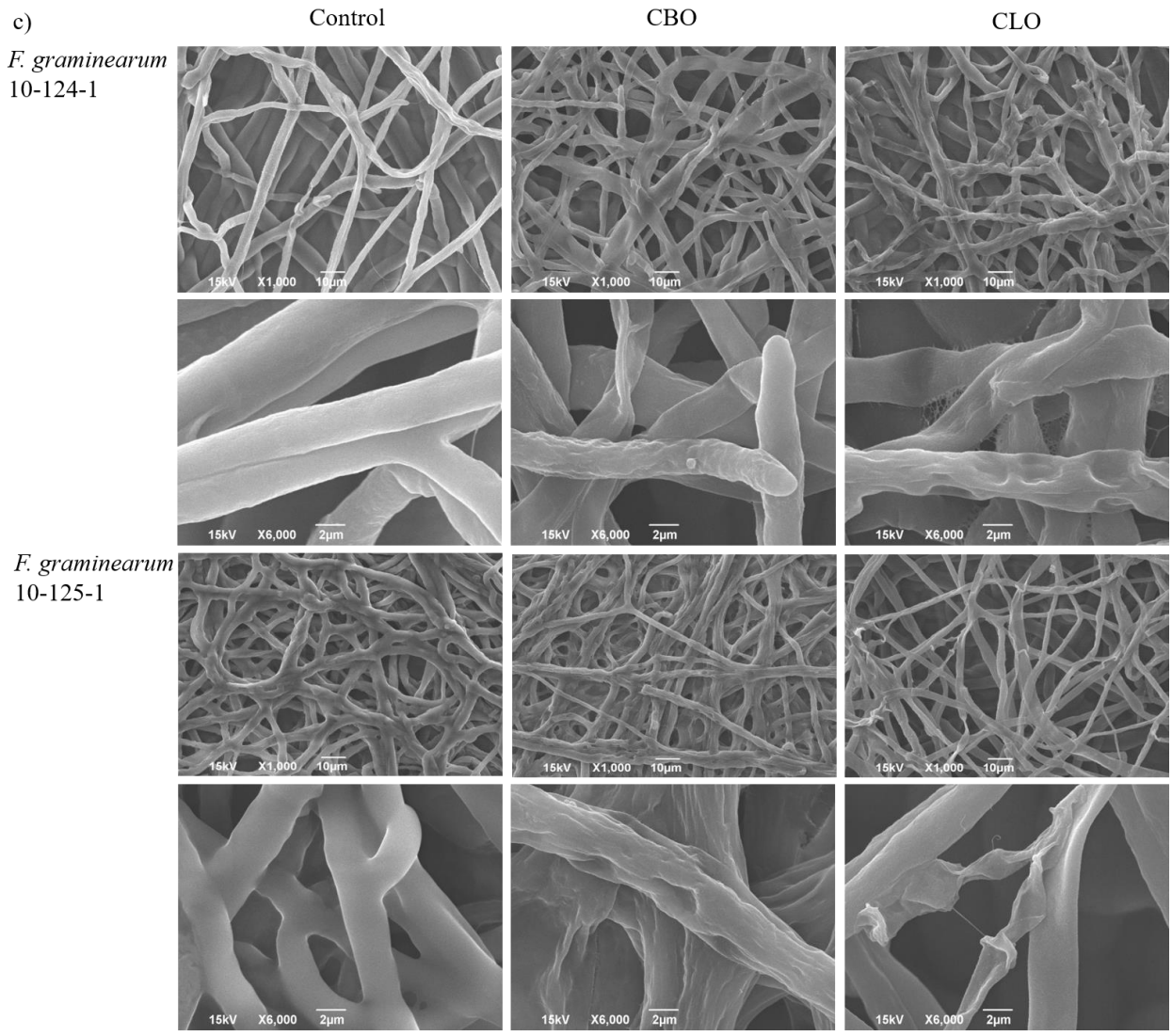


Figure 2-4. Effect of CO nanoemulsion type and CO concentrations in nanoemulsion on mycelial growth of (a) *F. graminearum* 10-124-1 and (b) *F. graminearum* 10-125-1; (c) SEM observation of *F. graminearum* hyphae treated by water (control), CBO and CLO nanoemulsion (continued).

To further understand the mechanism by which CBO and CLO nanoemulsions inhibit mycelial growth and the morphological changes of *F. graminearum* 10-124-1 and 10-125-1 hyphae treated by each nanoemulsion were examined by SEM (**Fig. 2-4c**). It was obvious that the control hyphae displayed distinguishable morphology compared with the treatment groups. For instance, the control hyphae showed a smooth and homogenous surface with a consistent width, which was coincident with our previous results (Wan et al., 2019a). By contrast, the mycelia of the two isolates treated with CO nanoemulsions exhibited noticeable changes on both

hyphae surface and length. For instance, wrinkled and twisted 10-124-1 fungal hyphae were observed (×1,000) because of the loss of cytoplasm matrix. Nonetheless, the changes of hyphae surface of 10-125-1 showed a great similarity with that of 10-124-1. For example, the fungal hyphae cell surface of 10-125-1 became winkled and flattened after treatment with the CBO nanoemulsion, due to the loss of cytoplasm matrix and shrunken of plasmalemma. Moreover, a few tiny vesicles were observed on the surface of mycelia. With an enlarged image (×6,000), a clear single hyphae structure can be visualized. These hyphae presented a rough and irregular surface with some craters. In terms of the CO type, morphological changes of hyphae were more obvious in *F. graminearum* isolates treated with CLO nanoemulsion when compared to those treated with the CBO counterpart. The effect of EOs on altering the integrity and linearity of fungal hyphae cell walls have also been observed in a number of studies, which was believed to be associated with the antifungal activity of EOs against mycelial growth (Kalagatur, Ghosh, Sundararaj, & Mudili, 2018; Kumar, Venkataramana, Allen, Chandranayaka, Murali, & Sara, 2016; Wan et al., 2019a).

Spore germination

Spore germination is one of the essential stages in the life cycle of filamentous fungi, and plays an important role on food spoilage and mycotoxin contamination (Basak, 2018). Therefore, it is valuable to elucidate the effect of CBO and CLO nanoemulsions on inhibition of spore germination of *F. graminearum* isolates 10-124-1 and 10-125-1 (**Fig. 2-5**).

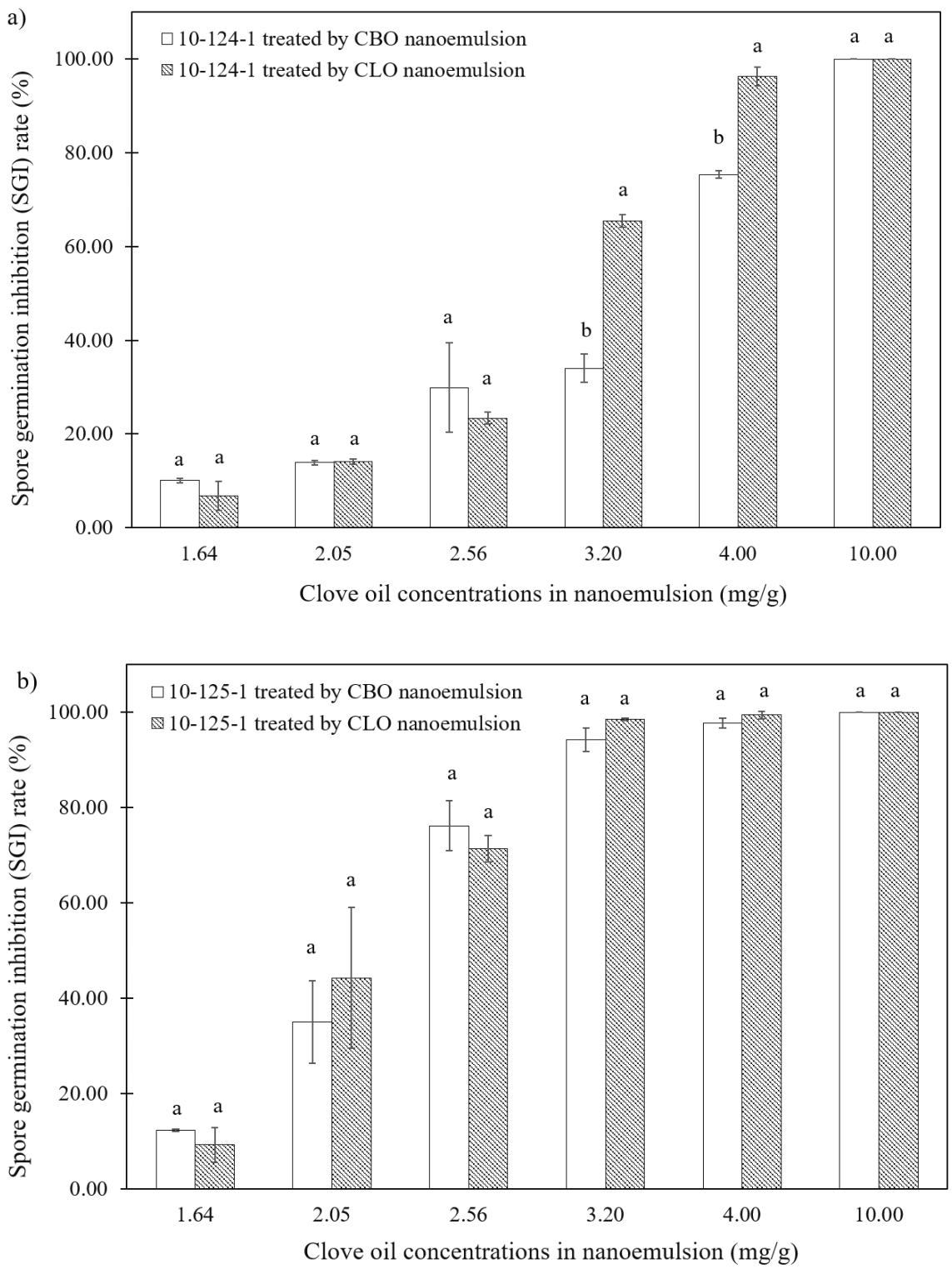


Figure 2-5. Effect of CO nanoemulsion type and CO concentrations in nanoemulsion on spore germination of (a) *F. graminearum* 10-124-1 and (b) *F. graminearum* 10-125-1; (c) SEM observation of *F. graminearum* spores treated by water (control), CBO and CLO nanoemulsion. Columns within the same CO concentration in nanoemulsion with different letters are significantly different at $p < 0.05$.

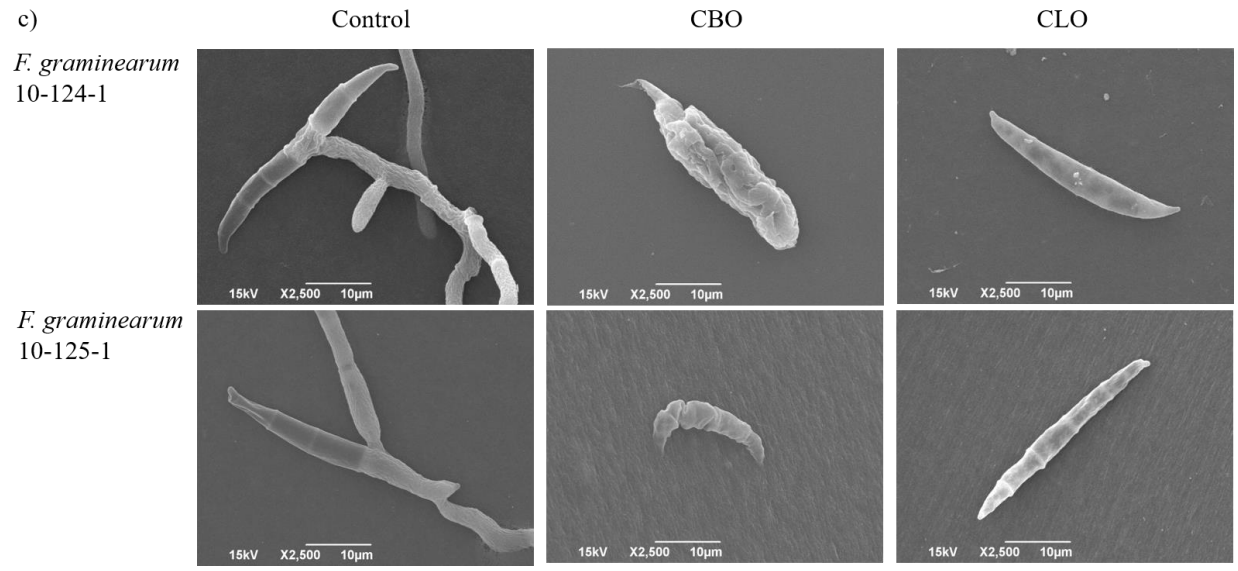


Figure 2-5. Effect of CO nanoemulsion type and CO concentrations in nanoemulsion on spore germination of (a) *F. graminearum* 10-124-1 and (b) *F. graminearum* 10-125-1; (c) SEM observation of *F. graminearum* spores treated by water (control), CBO and CLO nanoemulsion (continued).

Overall, the responses of the two isolates in terms of spore germination exhibited a similar trend upon CO nanoemulsion treatment. In addition, both CBO and CLO nanoemulsions could inhibit spore germination in a dose-dependent manner (**Fig. 2-5 a&b**), which was in an agreement with our previous study (Wan et al., 2019a). Without adding any CO nanoemulsion (control), the average SGI of the two isolates was ~ 2% after 12 h of incubation. Conversely, the SGI rate was able to reach 100% by applying either CBO or CLO nanoemulsion at a CO concentration of 10 mg/g. These outcomes demonstrated that both CBO and CLO nanoemulsions can effectively control spore germination in the two *F. graminearum* isolates when applied at a higher CO concentration. Comparing the antifungal efficacy of the two CO nanoemulsions against spore germination, it appeared that CLO nanoemulsion displayed a greater efficacy than CBO nanoemulsion at intermediate CO concentrations (e.g., 3.2 mg/g). A similar antifungal efficacy of CLO nanoemulsion was also observed in inhibition of mycelial growth study, which again suggests that the chemical composition of EOs can impact their antifungal activity.

Interestingly, the EO type did not significantly affect the spore germination rate within two isolates in general.

As suggested by previous researchers, the ability of EOs to prevent fungi spore germination mainly from the lipophilic bioactive compounds in EOs, which exert the capability to penetrate into the lipid bilayer of the fungal spore membrane and cause membrane disruption (Sharma, Rajendran, Srivastava, Sharma, & Kundu, 2017). To corroborate if this mechanism can still be applied to the CO after being encapsulated in nanoemulsions, SEM studies were employed to observe the spore morphologies of *F. graminearum* 10-124-1 and 10-125-1 following treatment with CBO and CLO nanoemulsions. Water served as a control. After 12 h of incubation, substantial alterations of spore morphology could be observed between the control and the CO nanoemulsion treatments. Similar to our previous observation, the spores of the two *F. graminearum* isolates treated by water were germinated with a plump shape. Meanwhile, clear septa could also be observed (Wan et al., 2019a). Conversely, both CBO and CLO nanoemulsions treated spores lost the capacity to germinate. In addition, the spores of both isolates treated by CBO nanoemulsion exhibited distinct surface morphological properties as compared to those by CLO nanoemulsion. For instance, the spore of *F. graminearum* 10-124-1 was covered by a heavy layer of treatment after treated with CBO nanoemulsion. Additionally, a rough and wrinkled surface was observed, indicating that CBO nanoemulsion could possibly block the spore cell wall from interacting with its surroundings. As a result, *F. graminearum* 10-124-1 could not excrete enzymes to obtain nutrients/energy from surroundings (Damato & Spieksma, 1995), which limited its spore germination. By contrast, the septa completely disappeared when CLO nanoemulsion was applied to *F. graminearum* 10-124-1 spores after 12 h of incubation. Thereby, the spore germination was inhibited due to the loss of septa. A previous

study demonstrated that inhibitory efficacy of antifungal agents on *Aspergillus fumigatus* spore germination was boosted under the assistance of septa formation inhibitors (Dichtl et al., 2015). The absence of septa might be attributed to the depletion of certain chemical compounds in septa (e.g., glucan, chitin and protein) incurred by the presence of CLO nanoemulsion. A number of studies demonstrated that certain chemical constitutes of EOs (e.g., cinnamaldehyde) were able to alter/limit the synthesis of chemical composition in septa cell wall through the inactivation of fungi septa cell wall synthesis enzymes such as β-(1,3)-glucan synthase (Bang, Lee, Park, & Rhee, 2000; Ghfir, Fonvieille, & Dargent, 1997). When comparing isolate 10-124-1 to 10-125-1, similar patterns on spore germination were observed. It is also worth noting that the *F. graminearum* 10-125-1 spores were shrunken with rough and corrugated surfaces in CBO treatment group. This may indicate massive loss of cytoplasm matrix (Yamamoto-Ribeiro et al., 2013). In short, both CBO and CLO nanoemulsions were able to inhibit spore germination of *F. graminearum* 10-124-1 and 10-125-1, but through different MOA.

The effect of clove oil type on mycotoxin production inhibition of nanoemulsions

Since the inhibitory efficacy of EOs against fungi mycelial growth and spore germination are not always directly correlated to their ability to inhibit mycotoxin production (da Cruz Cabral, Fernandez Pinto, & Patriarca, 2013), it is of great necessity to investigate their effect on mycotoxin production independently. Therefore, the inhibitory efficacy of CBO and CLO nanoemulsions on mycotoxin production, including DON, 3ADON, and 15ADON produced by two *F. graminearum* isolates was evaluated. This was performed by measuring the concentrations of mycotoxins in rice cultures after 5-days culturing with the addition of 800 μg/g of CO. Expect producing DON, *F. graminearum* 10-124-1 and 10-125-1 produce different DON derivatives, being 15ADON, and 3ADON, respectively. Herein, we compared the effects of CO

nanoemulsions by grouping them in light of *F. graminearum* isolate rather than CO nanoemulsion type.

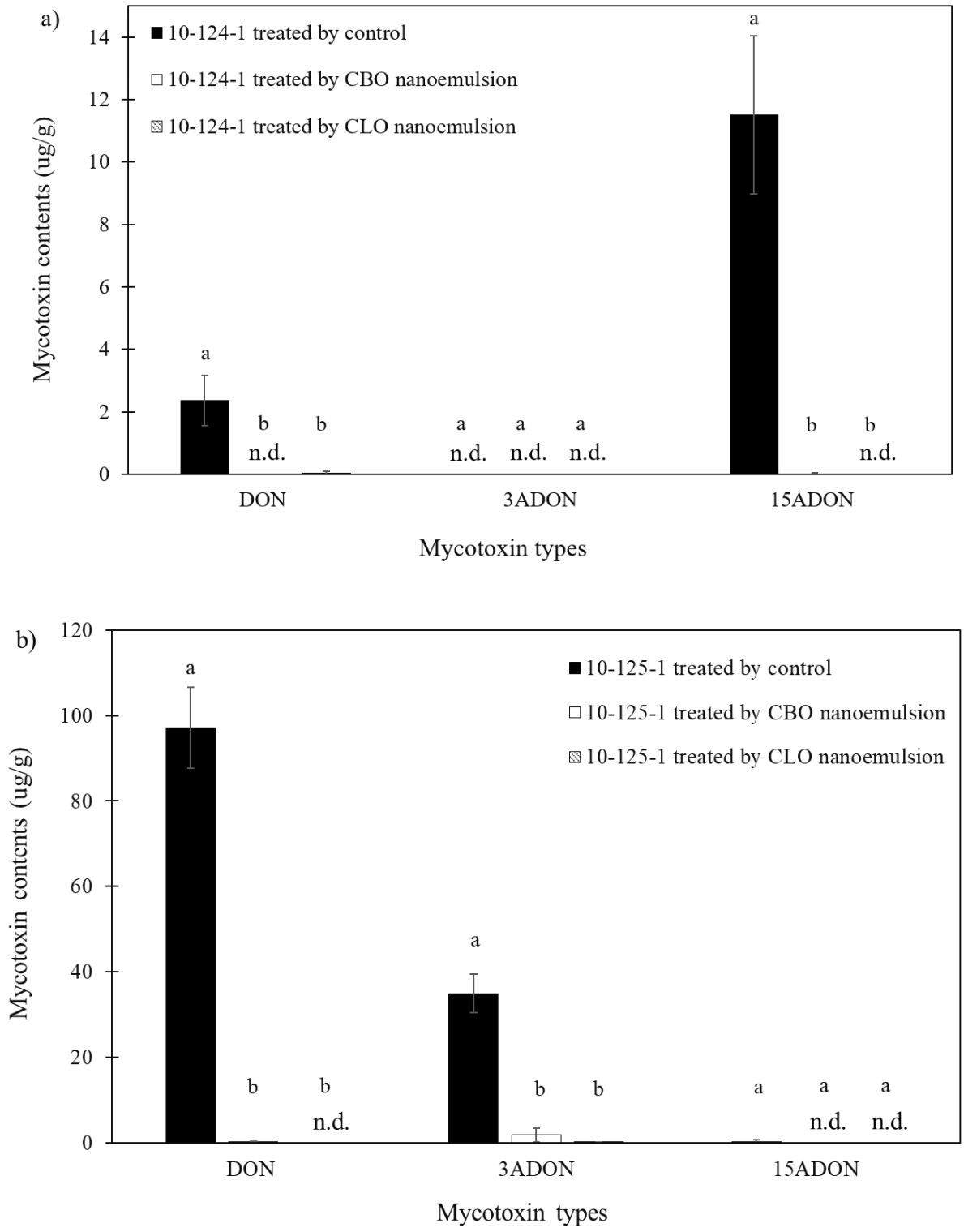


Figure 2-6. Mycotoxins production by (a) *F. graminearum* 10-124-1 and (b) *F. graminearum* 10-125-1 in rice culture following treatments by water, CBO nanoemulsion, and CLO nanoemulsion for 5 days of incubation. Columns within the same mycotoxin type with different letters are significantly different at $p < 0.05$; n.d. denoting not detected.

As indicated in **Fig. 2-6a &b**, the maximum level of DON production for *F. graminearum* 10-124-1 and *F. graminearum* 10-125-1 under control conditions, were 2.37 and 97.12 μg/g, respectively, which was in line with previous results (Wan et al., 2019a). In general, 3ADON-chemotype *F. graminearum* isolates possess the capability to produce a higher level of DON compared to 15ADON-chemotype *F. graminearum* isolates (Puri & Zhong, 2010). Notably, a small amount of 15ADON (0.28 μg/g) was also detected in 3ADON population of *F. graminearum*. In terms of treatment groups, both CBO and CLO nanoemulsions significantly reduced the levels of DON, 15ADON, and 3ADON when applied at a concentration of 800 μg/g rice culture. The percentage of DON reduction in *F. graminearum* 10-125-1 (**Fig. 2-6b**) treated with CBO and CLO nanoemulsions was 99.8% and 100%, respectively. The present results further confirmed our previous observations (Wan et al., 2018) that the EOs not only possess antifungal activity, but also bear mycotoxin inhibitory activity. However, when comparing mycotoxin inhibitory activities between CBO and CLO nanoemulsion, no significant difference was observed. This is not surprising since the applied nanoemulsion concentration of 800 μg CO/g of rice culture was sufficient to completely inhibit the mycotoxin production, as we have previously observed (Wan et al., 2018).

Among the two *F. graminearum* isolates, results indicated that isolate 10-124-1 was more sensitive to the action of the two CO nanoemulsions than isolate 10-125-1, which coincides with the results of mycelial growth. For instance, 100% DON inhibition rate was observed when CBO nanoemulsion was applied to isolate 10-124-1; whereas a small amount of DON (0.15 μg/g) was still detected in isolate 10-125-1. Different responses of *F. graminearum* isolates to the same antifungal agent (e.g., phenolic extract and EO) in terms of mycotoxin production have been reported previously (Pagnussatt et al., 2014; Wan et al., 2019b). Again, this phenomenon can be

explained by the genetic differentiation between *F. graminearum* 10-124-1 and 10-125-1. As demonstrated previously, 15ADON isolates had a higher gene diversity and more polymorphic loci than 3ADON isolates (Puri et al., 2010).

The effects of EOs and their major chemical constituents (e.g., eugenol) to restrict mycotoxin production including aflatoxin, fumonisin B_1 (FB_1), ZEA and DON have been reported in the literature (Jahanshiri, Shams-Ghahfarokhi, Allameh, & Razzaghi-Abyaneh, 2015; Marin, Velluti, Ramos, & Sanchis, 2004). However, the mechanisms by which chemical constituents of EOs inhibit *Fusarium* mycotoxins still remain unclear. A number of possible mechanism of actions have been proposed, which include modulation of DNA binding protein formation in the fungal cell and alternation of the gene expression patterns and mycotoxin biosynthesis pathway (Alizadeh, Golzan, Mahdavi, Dakhili, Torki, & Hosseini). For example, researchers demonstrated that precocene, a major chemical constituent of eucalyptus dives EO, has the capability to inhibit DON production through suppression of Tri gene (e.g., Tri 4, Tri 5, Tri 6, and Tri 10) transcription in *Fusarium* spp. They are the major genes involved in DON biosynthetic pathways (Yaguchi et al., 2009). We postulate that the mechanism of CBO and CLO nanoemulsions to reduce *Fusarium* mycotoxin production may be similar reason; but further study is required for confirmation.

Conclusions

In this study, the chemical composition of two different types of CO (CBO and CLO) was characterized. The physiochemical stability of CO nanoemulsions and their antifungal and mycotoxin inhibitory activity were systematically investigated. The chemical composition of CBO and CLO showed noticeable differences. For instance, a higher percentage of eugenol and eugenol acetate appeared in CBO, whereas no eugenol acetate was found in CLO. Because the

appreciable higher amount of relative water-soluble eugenol, physically stable CBO nanoemulsion and CLO nanoemulsion (d < 200 nm) could be fabricated by incorporating 75% of corn oil as Oswald ripening inhibitor in total 5 wt% of CO-in-water nanoemulsion. The particle size and the monomodal size distribution of the two nanoemulsions maintained the same during 28 days of storage at both 4 and 25 °C. Additionally, the concentration of major chemical constituents in CO nanoemulsions kept at the same level during 7 days of storage time, which indicated the positively protective effect of nanoemulsion delivery system on chemical stability of CO. In terms of antifungal activity, both CBO and CLO nanoemulsions had the capability to inhibit mycelial growth and spore germination of the two *F. graminearum* isolates in a dose-dependent manner, but in different antifungal MOA. Regarding mycotoxin inhibitory efficacy, production of DON, 3ADON, and 15ADON by the two *F. graminearum* isolates were effectively inhibited by applying 800 μg CO per g of rice. This study has provided important implications for the utilization of CO nanoemulsions as natural antifungal and detoxifying agents in agriculture industry and other industries.

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CHAPTER 3. ANTIFUNGAL ACTIVITY, MYCOTOXIN INHIBITORY EFFICACY, AND MODE OF ACTION OF HOP ESSENTIAL OIL NANOEMULSION AGAINST *FUSARIUM GRAMINEARUM*

Abstract

This work aims to investigate antifungal, mycotoxin inhibitory efficacy of the hop essential oil (HEO) nanoemulsion and their mode of action (MOA) against *Fusarium graminearum* isolate, a fungal pathogen causing *Fusarium* head blight in cereal crops. The HEO, primarily consisting of terpenes and terpenoids, was encapsulated in nanoemulsion droplets. Physically stable HEO-in-water nanoemulsion was fabricated using 0.5 wt% of tween 80 and 5 wt % oil phase comprising 30% of Ostwald ripening inhibitor and 70% of HEO. In terms of antifungal effect, HEO nanoemulsion could not only effectively inhibit mycelial growth and spore germination of *F. graminearum* isolates, but also remarkably suppress the production of deoxynivalenol (DON) and its derivatives in rice culture by applying 750 μg of HEO/g rice. Our studies on the MOA showed that HEO nanoemulsion could alter the contents of total lipid and chitin in outer cell membrane as well as damaging cytoplasmic membrane.

Introduction

F. graminearum is well known fungal plant pathogen that can cause *Fusarium* head blight (FHB) disease in crops. This disease not only reduces the grain yield in the field but also downgrade grain quality due to mycotoxin contamination in the grains (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018). Generally, mycotoxins produced by *F. graminearum* belong to type B trichothecenes including DON, NIV, and their derivatives 3ADON, 15ADON, and 4-ANIV (Krnjaja et al., 2018). Among them, DON is one of the most identified mycotoxins in a number of cereal crops and their corresponding products (Khaneghah et al., 2018). For

example, a survey of 93 organic commercial cereals and products in Italy found that 33% was contaminated by *Fusarium* mycotoxins with a maximum DON level of 99.6 μg/kg (Juan, Ritieni, & Manes, 2013). In recent years, research has discovered the increase of *Fusarium* mycotoxin (e.g., DON) concentration during certain food processing. The typical example is the significant increment of DON level in final malt when compared to that in the initial raw material (e.g., winter wheat) during the malting process (Jin, Cao, Su, Yu, & Xu, 2018). As consuming DON contaminated foods can cause serious adverse health effects, how to effectively eliminate mycotoxin contamination in final foods becomes one of the major challenges in the food industry.

In recent years, consumers have been increasingly seeking for food products that carry a shorter ingredient list without any artificial preservatives. Because of the "clean label" movement within the food industry, utilization of plant extracts with antimicrobial/antifungal properties gains popularity. Hop as one of beer brewing ingredients, has been nearly exclusively used in the brewing industry for many years. The female hop cones contain resins, bitter acids, EOs and flavonoids, which provide the bitterness and preservation of beer. In addition to its role in beer production, some hop compounds (e.g., hop acids) has demonstrated antimicrobial activities against bacterial growth, especially for gram-positive bacteria. It has been reported that the major antimicrobial /antifungal compounds in hop are α-acids (e.g., humulones), β-acids (e.g., lupulones), and prenylated flavonoids such as xanthohumol (Kramer, Thielmann, Hickisch, Muranyi, Wunderlich, & Hauser, 2015). For example, a recent study found that β-acids have the capability to inhibit *E. coli* and *S. aureus* growth through damaging their cell wall and fatty acid biosynthetic pathway (Tian, Cheng, Zhang, Liu, & Chen, 2022). Another study revealed that isoxanthohumol, the major prenylated flavonoids from hop extracts exhibited strong antifungal

activity *B. cinerea* growth *in vitro* by interfering the whole metabolic pathway of fungi (e.g., tricarboxylic acid cycle) (Yan et al., 2021). Nevertheless, the role of hop extracts especially HEO on antifungal activity against *Fusarium* pathogens and inhibition of mycotoxin production still remains unknown.

Whereas those studies have demonstrated the potential of HEO and their bioactive compounds as a natural antimicrobial and/or antifungal agent in food, yet the research on MOA of HEO against microbial particularly fungal growth is scarce to none. In general, the proposed MOA of EOs and their bioactive compounds to inhibit microbial growth includes the alteration of cell wall composition (e.g., lipid synthesis, fatty acid composition), the disturbance of cytoplasm membrane permeability and integrity, and the reduction of PMF of bacteria (Rao, Chen, & McClements, 2019). Among these MOA, outer cell membrane and cytoplasm membrane permeability and integrity can be evaluated by CLSM after stained with specific fluorescence dyes (Li, Yu, Lin, Teng, Du, & Ma, 2010). With these dyes, CFW is constantly used to analyze the alterations (e.g., localization and level of chitin) of fungal cell wall by monitoring the change of chitin, a major constituent of fungal cell walls (Watanabe, Azuma, Igarashi, & Ooshima, 2005). Non-polar FDA is a good indicator of esterase activity in cytoplasmic membrane as it can be converted to fluorescein by intracellular esterases (Battin, 1997). PI, a membrane-impermeable fluorescent dye, has been widely applied to assess membrane damage-initiated cell death upon binding with DNA from broken cell membranes (Brul, Nussbaum, & Dielbandhoesing, 1997).

Therefore, the primary goal of the current study is to systematically investigate the antifungal activity and mycotoxin inhibitory efficacy of HEO nanoemulsions as well as its MOA against *F. graminearum* growth. As HEO is water insoluble, nanoemulsion based delivery system

was applied to encapsulate HEO, and the physical stability of HEO-in-water nanoemulsion was investigated in this study. The antifungal and mycotoxin inhibitory activity of the physically stable HEO-in-water nanoemulsion was evaluated in terms of mycelial growth, spore germination and reduction of *Fusarium* mycotoxin in rice culture. Moreover, antifungal MOA of HEO-in-water nanoemulsion were elucidated by examining the change of composition (lipid, chitin) of fungal cell wall/outer cell membrane, as well as permeability and viability of cytoplasmic membrane using CLSM. The results of this study could provide meaningful information for the utilization of HEO as natural and green preservatives in the food industry and/or the malting industry.

Materials and methods

Materials

Supercritical CO_2 extraction of hop essential oil (HEO, Lot # 1011846, FG grade) were kindly provided by Kalsec Inc., (Kalamazoo, MI, USA). Medium chain triglyceride (MCT, NEOBEE M-5), which was mainly composed of 50-65% of caprylic acid (C8:0) and 30-45% of capric acid (C10:0) in terms of its fatty acid profile, was kindly provided by Stepan Company (Bordentown, NJ, USA). Potato dextrose agar (PDA) was purchased from AMRESCO (Solon, OH, USA). Potato dextrose broth (PDB) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Polyoxyethylene (20) sorbitan monooleate (Tween 80), sodium phosphate monobasic and dibasic, fluorescein diacetate (FDA), propidium iodide (PI), calcofluor white stain (CFW), vanillin, cholesterol, Mirex and N, and O-Bis (trimethylsilyl)acetamide (BSA)/ trimethylchlorosilane (TMCS)/ Trimethylchlorosilane (TSIM) kit for mycotoxin analysis were purchased from Millipore Sigma Co. (St Louis, MO, USA). Mung beans were purchased from a local supermarket (Fargo, ND, USA). Whatman sterile filter (0.45 μm, 25 mm) was purchased

from VWR International (Radnor, PA, USA). Rice was purchased from a local supermarket (Fargo, ND, USA). Two *F. graminearum* isolates (10-124-1 and 10-125-1) used in this study were kindly provided by Dr. Zhong's Lab in the Department of Plant Pathology at North Dakota State University (Fargo, ND, USA). These two fungal isolates 10-124-1 and 10-125-1 were classified as 15ADON and 3ADON chemotype, respectively. All solutions were prepared using ultrapure distilled deionized water (DDW, 18.2MΩ cm, Barnstead ultrapure water system, Thermo Fisher Scientific, USA).

Chemical composition of hop essential oil

Sample was prepared by dissolving HEO in diethyl ether (> 99.9%) to reach a final concentration of 0.1 g/mL, and then the chemical composition of HEO was analyzed using Agilent 7890B/5977A gas chromatography-mass spectrometry (GC-MS) system according to the previous report without any modification (Wan, Zhong, Schwarz, Chen, & Rao, 2019b).

Preparation and characterization of hop essential oil-in-water nanoemulsion

Nanoemulsion preparation

The HEO-in-water nanoemulsion consisted of 94.5 wt% of phosphate buffer system (10 mM, pH 7.0), 0.5 wt% Tween 80, and 5.0 wt% of total oil phase. The oil phase (5 wt%) was prepared by mixing HEO with MCT at various mass content (MCT%: 0, 10, 20, 30, 40, 50, 60, and 70 wt%) before homogenization. Then nanoemulsions were prepared by a high-pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) as reported previously without any modification (Wan, Zhong, Schwarz, Chen, & Rao, 2019a). The finished nanoemulsions were stored in test tubes sealed with screw caps at 25 ℃ for 3 or 7 days for physical stability study.

Particle size measurement

The mean particle diameter and particle size distribution of nanoemulsions were measured by a dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK) according to our previous study (Wan, Zhong, Schwarz, Chen, & Rao, 2018). To avoid multiangle effect, nanoemulsion was diluted 20 times using a buffer solution (10 mM phosphate buffer, pH 7.0) prior to particle size measurement. The long-term storage stability of nanoemulsion stored at room temperature (25 °C) was expressed as the change of particle diameter over a function of time.

Antifungal activity of hop essential oil-in-water nanoemulsions

Mycelial growth

According to the long-term storage stability results of nanoemulsion, the physically stable HEO nanoemulsion consisting of 94.5 wt% of phosphate buffer system (10 mM, pH 7.0), 0.5 wt% Tween 80, 3.5 wt% of HEO, and 1.5 wt% of MCT was chosen to conduct the following antifungal experiments. The total concentration of HEO in nanoemulsion was 35 mg/g. Two *F. graminearum* isolates 10-124-1 and 10-125-1 were selected to evaluate antifungal activities of HEO nanoemulsion according to our previous method with minor modifications, particularly on the concentrations of loading sample (Wan et al., 2018). Firstly, the gradient concentrations (0.88, 1.75, 2.8, 3.5, 7, 14, 28, and 35 mg/g) of HEO nanoemulsion were prepared by diluting with buffer solution (10 mM phosphate buffer, pH 7.0). A volume of 1 mL of HEO nanoemulsion was mixed with ~ 40 mL of autoclaved PDA solution homogenously, and poured into the petri dish (10 cm diameter). After solidification of PDA, a refreshed mycelial plug was placed on the center of PDA and incubated for mycelial growth observation. 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). The sterile deionized water was used as a control. MGI rates were fitted to cubic regression model, and EC_{50} value was defined as the concentration HEO of in nanoemulsion that can inhibit 50% of mycelial growth using a cubic regression model.

Spore germination

The antifungal efficacy of HEO nanoemulsions against two *F. graminearum* spores germination were evaluated according to our previous study with minor modifications (Wan et al., 2019a). Firstly, the gradient concentrations (2.8, 3.5, 5.6, 11.2, 17.92, 22.4, 28, and 35 mg/g) of HEO nanoemulsion were prepared by diluting with aqueous buffer. The concentration of spore suspension was adjusted to 5.0×10^5 spores/mL by sterile deionized water. Then, an equal volume of spore suspension (1.0 mL) was mixed evenly with HEO nanoemulsion (1.0 mL) with series gradient concentrations of HEO. The spore germination rate was calculated as P (%) = (number of germinated spore/numbers of total spore) ×100%. The spore germination inhibition (SGI) rate was calculated as SGI (%) = $100 \times (P_{control} - P_{treatment}) / (P_{control})$. The sterile deionized water was used as a control. SGI rates were fitted to quadratic regression model, and the EC_{50} value was defined as the concentration of HEO in nanoemulsion that can inhibit 50% of spore germination using a quadratic regression model.

Morphological study

The impacts of HEO nanoemulsion on the morphological properties of *F. graminearum* hyphae and spore were analyzed using SEM as reported to the methods of Wan et al. (Wan et al., 2019a).

Antifungal mode of action of hop essential oil-in-water nanoemulsion

The change of total lipid and chitin content in outer cell membrane/cell wall

Total lipid content of *F. graminearum* cell membrane was determined using the sulfo-phospho-vanillin (SPV) method (Tao, OuYang, & Jia, 2014) with some modifications. Prior to extracting lipids from freeze-dried mycelia, mycelia were collected using the following method. The spore suspension of *F. graminearum* isolates was adjusted to a concentration of 5.0×10^5 spores/mL by sterile deionized water, then an equal volume of spore suspension (1.0 mL) was mixed evenly with 1.0 mL of nanoemulsion containing 35 mg/g of HEO. The sterile deionized water was used as a control. Subsequently, 2.0 mL mixture was added to a 250 mL Erlenmeyer flask that contains 100 mL of PDB medium, and followed by incubating at 28 °C in a temperature controlled shaking incubator (MaxQ 4000, Thermo Scientific Inc. Waltham, MA. USA) at 150 rpm. After 4 days' incubation, the wet mycelia of *F. graminearum* isolates were collected by filtering through Miracloth. The dried mycelia were obtained by freeze drying for 96 h (Lyophilizer, Model 24DX48 GPFD 25L ES-53, SP scientific, Gardiner, New York) and were ground for lipid extraction and analysis.

The dried mycelia powder (0.5 g) was mixed with ~ 20.0 mL of methanol-chloroform-water mixture (2:1:0.8, v/v/v) in a test tube using a magnetic stir bar at room temperature for 1 h. After stirring for 1 h, the suspension was centrifuged at 4,000 ×*g* for 10 min (Beckman J2–HS, Beckman Coulter Inc., Indianapolis, IN, USA). Then, the lower phase containing lipids was transferred to a fresh test tube, which was mixed with 0.2 mL saline solution thoroughly. Subsequently, the abovementioned mixture was centrifuged at 4,000 ×*g* for 10 min, the lower phase was collected and the volume of this collected lipid was measured using pipettes (mL). The collected lower phase was transferred and was diluted 200 times using chloroform. The

chloroform was removed by heating diluted chloroform-lipid mixture (0.2mL) at a boiling water bath in a hood until all the solvent was evaporated. Subsequently, an aliquot of 0.5 mL sulfuric acid was added before heating in a boiling water bath for 10 min, which was then cooled down to a room temperature. After that, an aliquot of 3.0 mL freshly prepared phospho-vanillin solution was added in the abovementioned sulfuric acid-lipid mixture and shaken vigorously. The mixture was then incubated at a room temperature for 10 min, and the absorbance of mixture was measured at 520 nm using spectrophotometer. An equal volume of sulfuric acid and phospho-vanillin mixture was used as a blank. The lipid concentration was calculated according to the cholesterol standard curve. The standard curve of cholesterol was constructed by measuring absorbance of a serially concentration of cholesterol (10, 50, 100, 200 and 400 ug/mL) in chloroform. The total lipid content in mycelia powder was quantified according to "total lipid content = (lipid concentration × lipid volume)/mass of dried mycelia powder".

The change of chitin content in cell wall was measured using fluorescence-based assay by means of CLSM according to previous reports (Li et al., 2010) with some modifications. In brief, fresh *F. graminearum* spores were added into PDB medium to achieve the final concentration of 5.0×10^5 spores/mL. Then, the HEO nanoemulsion (35 mg/g) was mixed with *F. graminearum* spore suspension to achieve the final HEO concentration in nanoemulsion of 5.6 mg/g. Afterward, spore suspensions were incubated in an orbital shaker (MaxQ 4000, Thermo Scientific Inc. Waltham, MA. USA) at 28°C for 10 h at 200 rpm. *F. graminearum* spores and/or hyphae were harvested by centrifugation (6,000 rpm, 10 min), and then washed once with sterile distilled water. For the control sample, HEO nanoemulsion was replaced by sterile deionized water. For CFW staining, CFW (10 μg/g) was added to the freshly harvested samples with a mixing ratio of 1:4 (v/v); after evenly mixing, the samples were incubated in the dark for 5 min.

After staining, samples were immediately analyzed by using a CLSM (LSM 700, Carl Zeiss Microscopy Ltd., Jena, Germany) at a 20 × magnification. The excitation wavelength of CFW was 395 nm, and the emission wavelength was 440 nm.

Cytoplasmatic membrane permeability

Cytoplasmatic membrane permeability of *F. graminearum* was also investigated using CLSM as described previously (Li et al., 2010) with some modifications. The spore samples were prepared as described in section of the change of total lipid and chitin content in outer cell membrane/cell wall. For preparation of FDA and PI fluorescent staining, freshly prepared FDA solution (10 μg/g) was firstly mixed with the harvested samples, and then samples were incubated for 5 min in the dark. Subsequently, PI solution with a concentration of 5 μg/g was added into the sample mixture pre-stained with FDA, which was then mixed and incubated in the dark for another 5 min. The mixing ratio of the harvested sample, FDA, and PI was 7:2:1 (v/v/v). After staining, samples were immediately analyzed by using a CLSM at a 20 × magnification. The excitation wavelength of FDA and PI was 490 nm and 488 nm, respectively; while their emission wavelength was 526 nm and 617 nm, respectively.

Impact of hop essential oil-in-water nanoemulsion on mycotoxin production

The mycotoxin inhibitory efficacy of HEO nanoemulsion was evaluated using a rice culture method according to the previous report (Wan et al., 2018) without any modification. The final concentration of HEO in a rice culture was 750 μg/g rice. The sterile deionized water was applied as a control.

Statistical analysis

All measurements were carried out at least twice and were reported as mean ± standard deviation. The complete random design (CRD) was conducted in this assay. Significant

differences between means ($p < 0.05$) were performed using one-way analysis of variance (ANOVA) and F-protected LSD by SAS software (Version 9.3, SAS Institute Inc., NC, USA).

Results and discussion

Chemical composition of hop essential oil

Numerous studies have shown that antimicrobial and antifungal properties of EO is highly associated with their chemical constituents. As the case of HEO, its chemical constituents is largely influenced by a number of factors such as hop varieties, growing conditions, extraction and fractionation method (Hrncic, Spaninger, Kosir, Knez, & Bren, 2019). Consequently, the major chemical constituents of HEO (>1% of total, calculated as % peak area) is analyzed and shown in **Table 3-1**. As can be seen from **Table 3-1**, thirteen chemical compounds (>1% of total) have been detected, representing 87.48% of the total HEO. According to their chemical structures, these compounds can be divided into five categories, including monoterpene, sesquiterpene, terpenoids, monoterpenoid alcohol, and acid accounting for 24.52%, 3.48%, 41.01%, 6.35 % and 3.22% of total HEO, respectively. This result is in agreement with previous study in which monoterpene and terpenoids dominates the chemical constitutes of HEO (Bernotienë, Nivinskienë, Butkienë, & Mockutë, 2004). Among them, myrcene, humulene, and caryophyllene were the dominant chemical constitutes detected in our HEO, similar to those reported in literature (Aberl & Coelhan, 2012). Interestingly, one commonly reported chemical constituents, β-farnesene was absent in our HEO (Krofta, 2003). The chemical composition differences of HEO might be attributed to the different raw material (e.g., hop varieties) used for EO extraction. For instance, β-farnesene cannot be synthesized in certain hop varieties, such as such as Brazilian cascade hops, during growth stage (Almeida et al., 2021).

Table 3-1. Major chemical constituents of HEO.

Category	Compounds	Percentage (%)
Monoterpene	β-Myrcene	24.52
Sesquiterpene	Caryophyllene	10.88
	δ-cadinene	3.48
Terpenoids	m-camphorene	1.02
	Humulene	19.72
	Selina-3,7(11)-diene	3.88
	γ-muurolene	1.71
	α-copaene	1.41
	β-selinene	2.39
Monoterpenoid alcohol	Nerol	6.35
Acid	1,2-Dimethylcyclopropane carboxylic acid	3.22
Others	3,3,6-Trimethyl-1,5-heptadiene	6.14
	2,6-Dimethyl-2,6-octadiene	2.76
	Total:	87.48 %

Physical stability of hop essential oil-in-water nanoemulsions

Normally, EO including HEO is water insoluble and thus cannot be integrated into effectively during food processing. One strategy to overcome this issue is to encapsulate HEO into nanoemulsion. However, pure EO-in-water nanoemulsions are extremely unstable during storage because of Ostwald ripening (Wan et al., 2018). The physical stability of HEO nanoemulsions can be greatly improved by incorporating relatively low water-soluble oils (also call Ostwald ripening inhibitor) with EO, such as MCT. Our previous study proved that the physically stable CO nanoemulsions can be fabricated by mixing CO with $\geqslant$ 50 wt% of MCT (Wan et al., 2018). Nevertheless, the concentration of Ostwald ripening inhibitor in the EO phase has a substantial impact on antifungal activities of EO nanoemulsion. Consequently, it is critical to optimize the mass ratio between HEO and MCT in the total oil phase to maximize their antifungal properties while maintaining a physically stable nanoemulsion system. Therefore, a series of nanoemulsions with a different amount of MCT (0, 10, 20, 30, 40, 50, 60 and 70%) in

total oil phase (5 wt%) were initially fabricated. The mean particle size of nanoemulsion samples after homogenization and over the course of 7 days of storage was measured and the results are shown in **Fig. 3-1**.

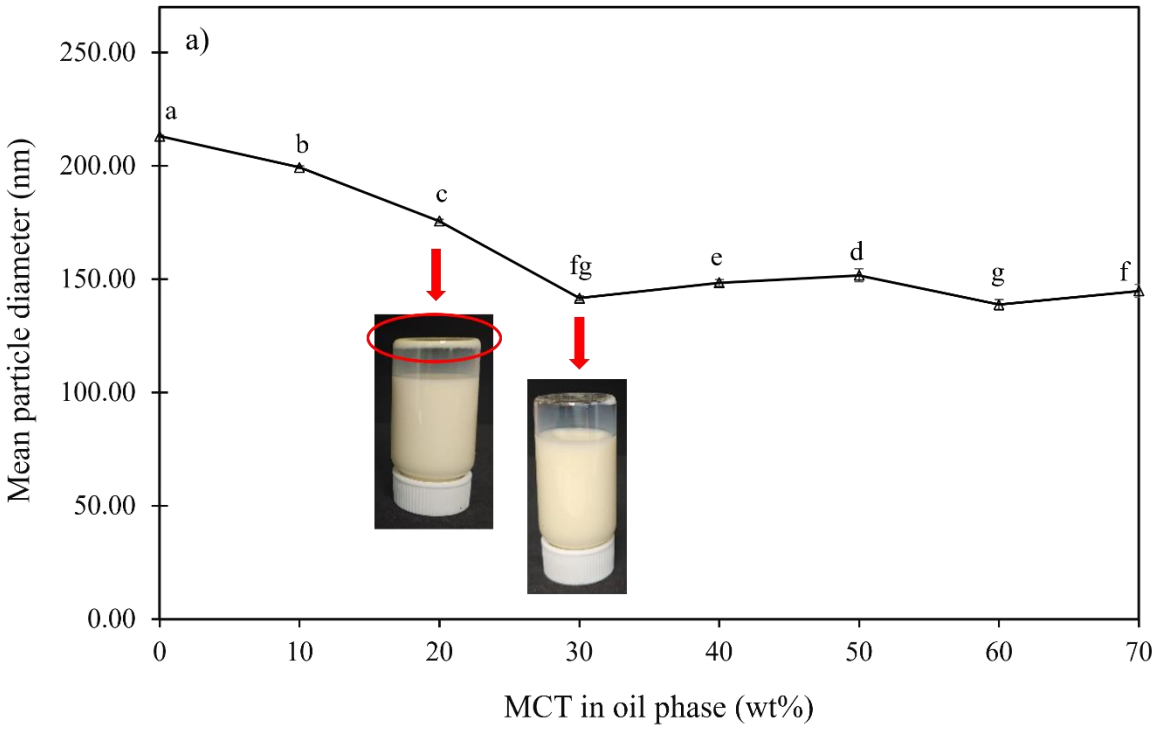

Figure 3-1. (a) Impact of MCT content in total oil phase (5 wt%) on mean particle diameter of HEO nanoemulsion (0.5 wt% of tween 80 and 94.5 wt% of 10 mM phosphate buffer, pH 7.0; average particle size of various wt% of MCT in oil phase with different lowercase letters were significantly different from each other at $p < 0.05$); (b) storage stability of HEO nanoemulsion over a course of 7 days storage time at 25 ℃ (size values under various days with different lowercase letters were significantly different from each other at $p < 0.05$); and (c) particle size distribution of HEO nanoemulsion at the selected storage time.

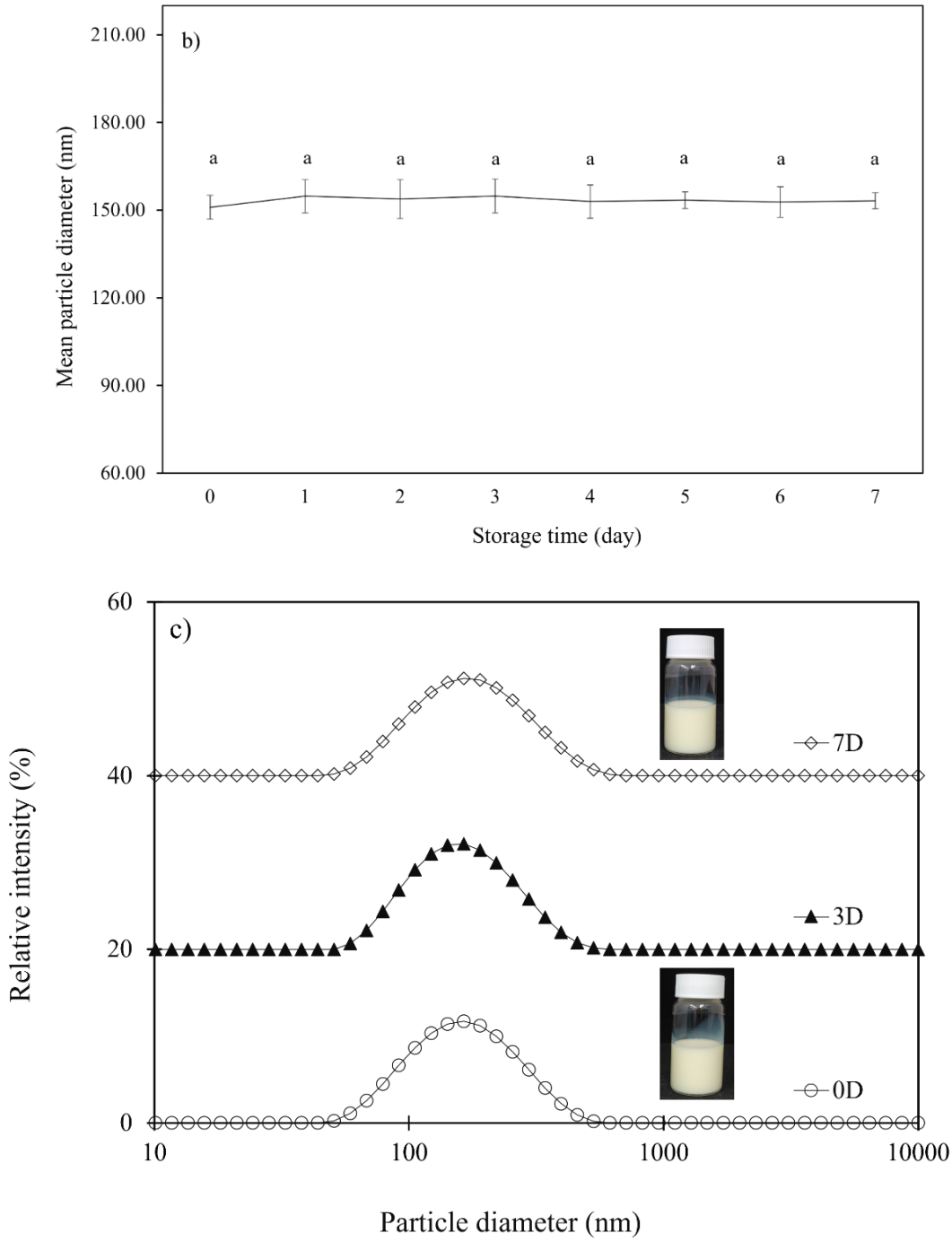

Figure 3-1. (a) Impact of MCT content in total oil phase (5 wt%) on mean particle diameter of HEO nanoemulsion (0.5 wt% of tween 80 and 94.5 wt% of 10 mM phosphate buffer, pH 7.0; average particle size of various wt% of MCT in oil phase with different lowercase letters were significantly different from each other at $p < 0.05$); (b) storage stability of HEO nanoemulsion over a course of 7 days storage time at 25 ℃ (size values under various days with different lowercase letters were significantly different from each other at $p < 0.05$); and (c) particle size distribution of HEO nanoemulsion at the selected storage time (continued).

As one can see from **Fig. 3-1a**, the mean droplet diameter of nanoemulsions was decreased dramatically from 199 nm to 142 nm when the MCT in oil phase increased from 10% to 30%. With a further increase of MCT concentration in total oil phase, the mean diameter of nanoemulsion droplet increased slightly until it has reached a plateau. The smallest mean droplet diameter (142 nm) was obtained in the system containing 30% of MCT in oil phase. Besides, sedimentation was observed in HEO nanoemulsion when the MCT in total oil phase was less than 30% (**Fig. 3-1a inserted picture**). By contrast, a uniform nanoemulsion system without any creaming or sedimentation was formed as the MCT concentration was ≥ 30% in the total oil phase, which suggested that 30% of MCT is sufficient enough to prevent Ostwald ripening or any phase separation in 5 wt% of HEO-in-water nanoemulsion. We then investigated the long-term physical stability of HEO nanoemulsions consisting of 30% MCT in oil phase over 7 days of storage by monitoring the change of mean particle diameter and particle size distribution (**Fig. 3-1b & c**). During the storage, the nanometric size (~150 nm) of nanoemulsion remained the same. Furthermore, the selected particle size distribution of nanoemulsion at day 0, 3, and 7 clearly illustrated that HEO-in-water nanoemulsions remained monomodal distributions without any shift on distribution curve. In addition, no visible changes on the appearance of nanoemulsions were observed in the tested storage time (**Fig. 3-1c inserted picture**). These experiments indicate that ripening inhibitor to HEO ratio had a major impact on the long-term physical stability of the nanoemulsion, and mixing 30% of MCT with 70% of HEO in 5 wt% of total oil phase can afford a physically stable HEO-in-water nanoemulsion. Accordingly, HEO-in-water nanoemulsion with this composition was selected to conduct the following experiments including antifungal and mycotoxin inhibitory activity.

Antifungal activity of hop essential oil-in-water nanoemulsions

In this section, the antifungal activity of HEO nanoemulsion against two *F. graminearum* isolates (10-124-1 and 10-125-1) was determined by both MGI and SGI rate. In addition, the morphological changes of fungal hyphae and spores were also observed using SEM.

Mycelial growth inhibition rate

As can be seen in **Fig. 3-2a**, MGI rates raised steadily until it reached the plateau as the HEO concentrations in nanoemulsions was increased, which suggested that HEO concentration plays a critical role on inhibition of mycelial growth of *F. graminearum* isolates. For example, when the HEO concentration in nanoemulsion increased from 0.88 mg/g to 14.00 mg/g, the MGI rates of *F. graminearum* 10-124-1 increased from 21.71% to 55.37%. This phenomenon can be explained by the increased concentration of certain core antifungal compounds such as humulene, caryophyllene in the nanoemulsion, which boosted the antifungal activity (Wanas, Radwan, Mehmedic, Jacob, Khan, & Elsohly, 2016). However, further increasing the concentration of HEO in nanoemulsion up to 35 mg/g did not drastically uplift the antifungal capacity, with MGI rates being 55% and 50% for *F. graminearum* 10-124-1 and 10-125-1, respectively. The MGI rate of HEO nanoemulsion is lower than that of thyme EO nanoemulsions or CO nanoemulsions, as reported in our previous study (Wan et al., 2019b). Nevertheless, this is anticipatable since we found that phenol is the predominant active antifungal compound in EO, followed by sesquiterpene and alcohol (Wan et al., 2019b). In HEO, the major chemical constitutes were terpenoids (**Table 3-1**). Regarding HEO effect on the antifungal efficacy between the two isolates, *F. graminearum* 10-124-1 was more sensitive than *F. graminearum* 10-125-1 as reflected by EC_{50} values (**Fig. 3-2a inserted Table)**. It is clear that EC_{50} value of *F. graminearum* 10-124-1 (6.91 mg/g) was significantly lower ($p < 0.05$) than that of *F.*

graminearum 10-125-1 (10.57 mg/g). This phenomenon suggests that different chemotypes of *F. graminearum* isolates, due to diverse genetic and biological variations, could have different response toward the same antifungal agent (van der Lee, Zhang, van Diepeningen, & Waalwijk, 2015; Wan et al., 2019a).

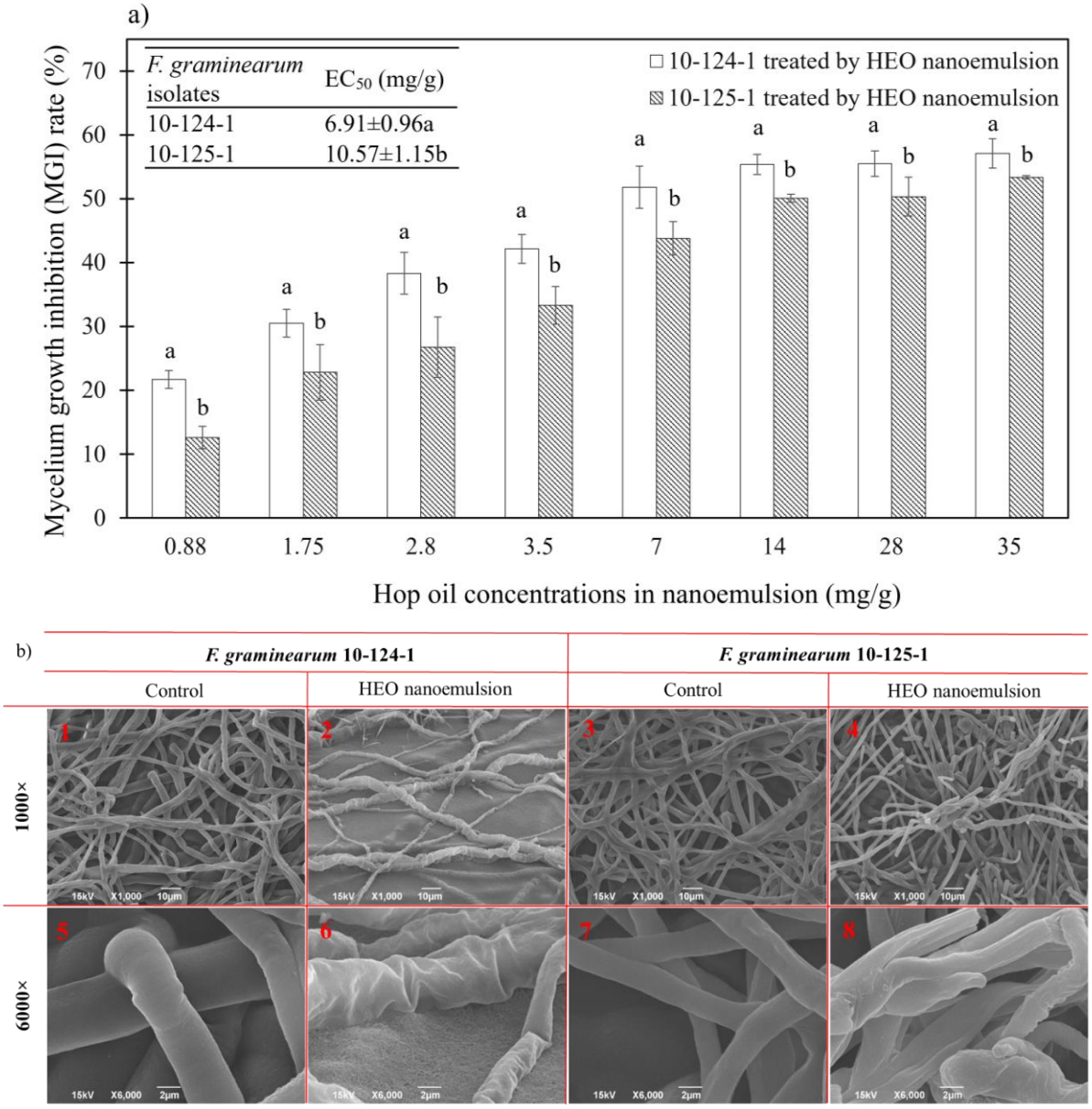

Figure 3-2. (a) Effect of HEO concentration in nanoemulsion on mycelial growth inhibition rate of *F. graminearum* 10-124-1 and *F. graminearum* 10-125-1 (different lowercase letters indicate statistically significant intraspecies differences at $p < 0.05$); and (b) SEM observation of *F. graminearum* hyphae treated by water (control) and HEO nanoemulsion.

To better gauge the role of HEO nanoemulsion on inhibiting the mycelial growth of *F. gramineraurm*, the morphological changes of *F. graminearum* hyphae treated by HEO nanoemulsion were examined by SEM. As shown in **Fig. 3-2b**, a discernible morphological change of hyphae in HEO nanoemulsion treated *F. graminearum* was present when compared to the control sample. In particular, the density of hyphae in the control samples (**Fig. 3-2b1, 3, 5, &7**) was relatively high. In addition, the surface of each hypha was smooth with similar width, which is consistent with our previous observation (Wan et al., 2019a). By contrast, HEO nanoemulsion treated samples showed obvious changes in terms of hyphal density, surface, and length. For example, a wrinkled and flattened appearance of hyphae was observed (**Fig. 3-2b2 &6**) on *F. graminearum* 10-124-1 due to the massive loss of cytoplasm matrix and shrunken of plasmalemma. Interestingly, there was a slightly difference in terms of change of hyphal morphology between *F. graminearum* 10-125-1 and 10-124-1. Although several shrunken hyphae were observed on *F. graminearum* 10-125-1 (**Fig. 3-2b4**), the appearance of majority of fungal hyphae was fractured after treated with HEO nanoemulsion. Moreover, a considerably cracked surface was observed on *F. graminearum* 10-125-1 under a magnified image (×6,000, **Fig. 3-2b8**). A number of researchers stated that bioactive compounds in EOs are capable to alter the integrity and linearity of fungal hyphae through inhibition of enzymes in cell wall synthesis, which might be the reason to inhibit the mycelial growth of fungi (Wan et al., 2019a; Yahyazadeh, Omidbaigi, Zare, & Taheri, 2008). Different morphological alterations found in these two *F. graminearum* isolates by applying the same treatment again might be attributed to their genetic differences (Toth, Mesterhazy, Horvath, Bartok, Varga, & Varga, 2005).

Spore germination

Spore germination is a key factor to determine fungi colonization, subsequent spoilage and mycotoxin contamination in food products (Dijksterhuis, 2017). Therefore, it is important to evaluate the effect of HEO-in-water nanoemulsion on inhibition of spore germination of *F. graminearum* isolates (**Fig. 3-3**). Similar with MGI, both isolates exhibited a dose-dependent manner on SGI (**Fig. 3-3a**). For instance, when the diluted HEO nanoemulsion (2.8 mg/g) was applied, the SGI rate of *F. graminearum* 10-124-1 and 10-125-1 was 5.12% and 21.2%, respectively. Strikingly, nearly 95% of SGI rate was reached by applying nanoemulsion with 35 mg/g of HEO, indicating such a concentration can effectively inhibit the spore germination of *F. graminearum* isolates. Comparing SGI of HEO nanoemulsion against two different *F. graminearum* isolates, *F. graminearum* 10-124-1 continuously exhibited a higher sensitivity in response to HEO nanoemulsion rather than *F. graminearum* 10-125-1, which was in consistence with results of mycelial growth inhibition. Nevertheless, no significant differences of SGI rates were found between the two isolates when the concentration of HEO in nanoemulsion was greater than 28 mg/g. This might be because of the sufficient amount of active antifungal components (e.g., humulene, caryophyllene and γ-muurolene) in HEO that could completely inhibit the spore germination (Cakir, Kordali, Zengin, Izumi, & Hirata, 2004; Wanas et al., 2016). SEM studies were also applied to observe the morphologies of spore changes following the HEO nanoemulsion treatment. As displayed in **Fig. 3-3b**, the control isolates (**Fig. 3-3b1 & 3**) were germinated after 12 h of incubation. Meanwhile, the control samples exhibited a foot-shape with five clear septa. On the contrary, a rough and wrinkled surface with a shorter size was observed in the treated spores (**Fig. 3-3b2 & 4**), indicating HEO nanoemulsion could possibly block the spore cell to obtain nutrients/energy from surroundings environment (Rao et al., 2019).

Consequently, no spore germination was observed for samples treated by nanoemulsion with 28 mg/g of HEO.

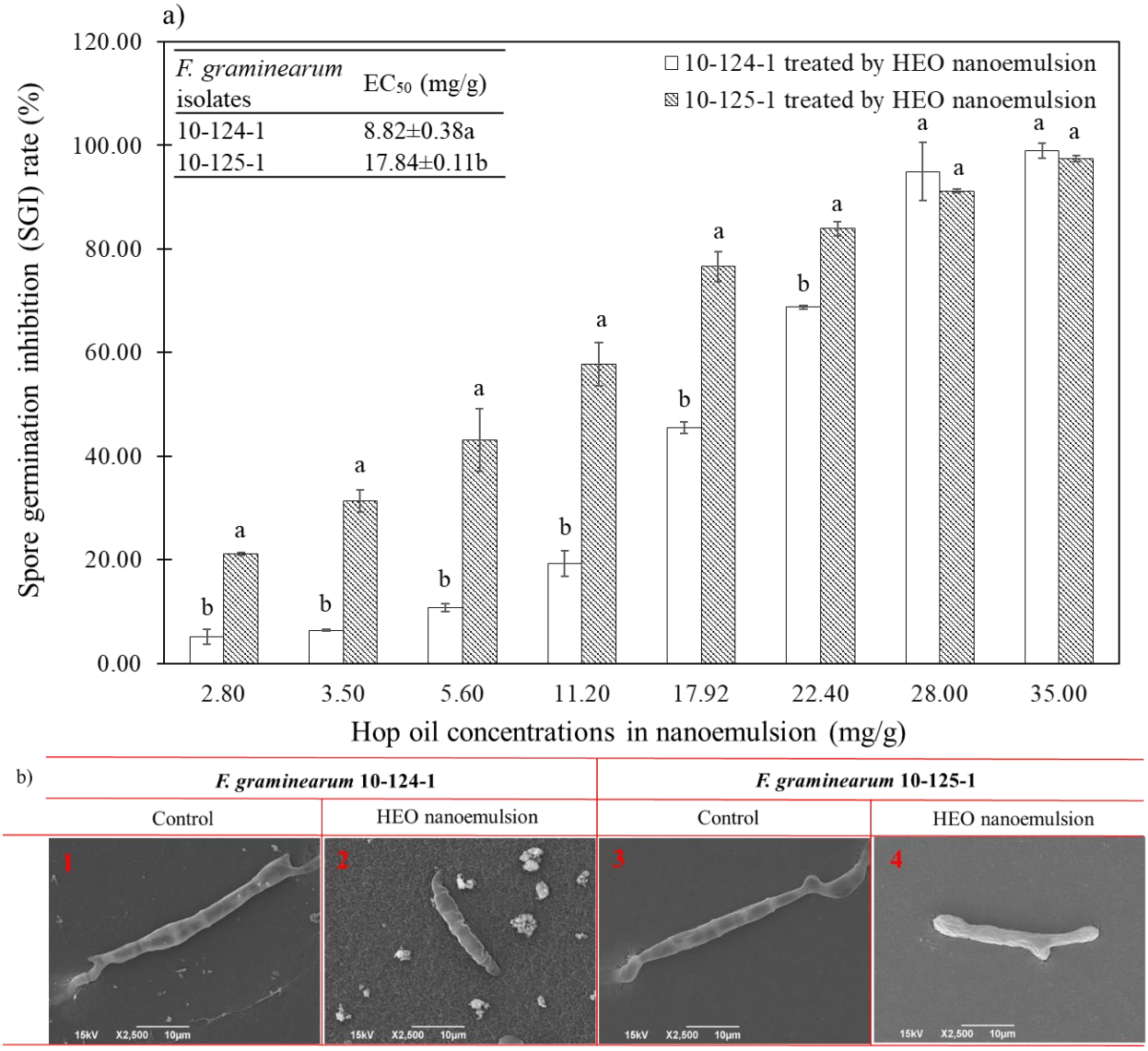

Figure 3-3. (a) Effect of HEO concentration in nanoemulsion on spore germination inhibition rate of *F. graminearum* 10-124-1 and *F. graminearum* 10-125-1 (different lowercase letters indicate statistically significant intraspecies differences at $p < 0.05$); and (b) SEM observation of *F. graminearum* spores treated by water (control) and HEO nanoemulsion.

The effect of hop essential oil-in-water nanoemulsions on mycotoxin production

Antifungal agents with the effective capacity to inhibit fungal growth do not always guarantee the same capability to hinder mycotoxin biosynthesis in toxigenic fungi species (da

Cruz Cabral, Fernandez Pinto, & Patriarca, 2013). Therefore, the inhibitory efficacy of HEO-in-water nanoemulsion on *F. graminearum* mycotoxin production, including DON, 3ADON and 15ADON, was evaluated.

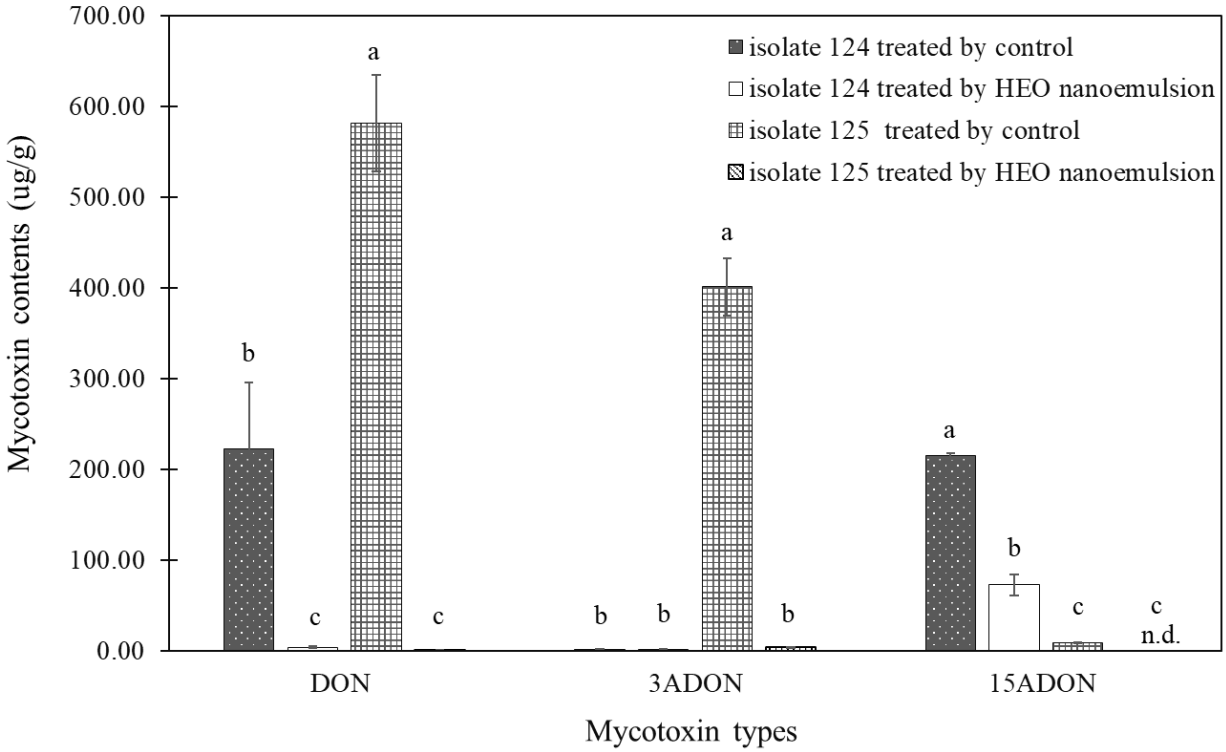

Figure 3-4. Inhibition of mycotoxins production in *F. graminearum* 10-124-1 and *F. graminearum* 10-125-1 inoculated rice culture treated by HEO nanoemulsion for 5 days of incubation (water treatment was used as control; different lowercase letters indicate statistically significant intraspecies differences at $p < 0.05$; n.d. denoting not detected).

As shown in **Fig. 3-4**, The DON content produced by *F. graminearum* 10-124-1 and *F. graminearum* 10-125-1 was 221.75 and 581.49 μg/g, respectively. As can be seen, more than two-fold of DON content was detected in *F. graminearum* 10-125-1, the 3ADON-chemotype isolate, as compared to *F. graminearum* 10-124-1, the 15ADON-chemotype isolate. Previous studies also demonstrated that 3ADON-chemotype of *F. graminearum* isolates synthesizes a higher amount of DON than 15ADON-chemotype counterpart (Puri & Zhong, 2010). In terms of nanoemulsions treated groups, nearly complete inhibition (98.3% and 99.9%) of the DON was achieved in both *F. graminearum* 10-124-1 and *F. graminearum* 10-125-1 isolates in rice culture

upon the addition of 750 μg of HEO/per gram of rice. However, no significant differences ($p > 0.05$) were observed on the inhibition of DON production between the two isolates among the treated group. This again is because the concentration of applied HEO nanoemulsion (750 μg HEO/g of rice culture) was adequate to thoroughly suppress DON production. For DON derivatives, a complete inhibition of 3ADON production (99.2%) in *F. graminearum* 10-125-1 isolate was observed when compared with control. Similarly, the content of 15ADON was dramatically reduced from 214.67 (control) to 72.21 μg/g in *F. graminearum* 10-124-1 after treatment. Although a small amount of 15ADON (8.29 μg/g) were detected in the 3ADON chemotype isolate of *F. graminearum* 10-125-1 in control group, it was totally diminished in rice culture by applying HEO nanoemulsion. The effect of HEO to inhibit mycotoxin production is primarily because of their bioactive chemical constituents. For example, nerol in hop showed a noticeable mycotoxin inhibitory activity against aflatoxin B_1 synthesis (Tian, Zeng, Zeng, Feng, Miao, & Peng, 2013). However, it is unclear as to which chemical constitutes of HEO determine their mycotoxin inhibitory activity, which will be the subject of our following study.

Antifungal mode of action of hop essential oil-in-water nanoemulsions

According to our results, HEO nanoemulsion not only displays antifungal activity against *F. graminearum* growth, but also possesses mycotoxin inhibitory activity. In general, a number of MOA of EOs against microbial growth have been proposed depending on the EO type (e.g., chemical constitutes) and target pathogens. However, very limited information is available regarding to MOA of HEO. Herein, we set forth to uncover the potential MOA of HEO nanoemulsion against *F. graminearum* growth by measuring the change of total lipid and chitin in outer cell membrane, as well as the permeability of cytoplasmic membrane of *F. graminearum* spores.

Total lipid content

As one of the major chemical components in fungal cell membrane, lipid bilayers of fungal cell membrane are vital for innumerable fungal spore functions, such as maintaining membrane fluidity and respiration (Tao, Jia, & Zhou, 2014). Therefore, it is necessary to know the effects of HEO-in-water nanoemulsion on the total lipid contents of outer cell membrane of spores. As can be seen from **Fig. 3-5a**, the total lipid contents of *F. graminearum* 10-124-1 and 10-125-1 were significantly increased after 4 days of exposure to the HEO-in-water nanoemulsion when compared to the controls ($p < 0.05$). Specifically, the total lipid contents of *F. graminearum* 10-124-1 and 10-125-1 spores in control groups were 26.39 and 37.27 mg/g of dry mycelial weight, respectively. They, however, increased to 41.62 and 61.12 mg/g in the treatment spores. Previous results revealed that the total lipid content of the fungi in treatment group was lower than that in control sample because the hydrophobic proportion of EO components bear the capability to breach the membrane barrier or to inhibit membrane related enzyme functionality. Oppositely, few studies also reported an increased trend in the total lipid content of the fungal cells in the presence of EO (e.g., cinnamaldehyde) (OuYang, Duan, Li, & Tao, 2019). Interestingly, HEO nanoemulsion was found to promote the massive accumulation of total lipids in our study which might result in the formation of a thicker outer cell membrane of spores. In consequence, membrane liquidity and stability must be altered.

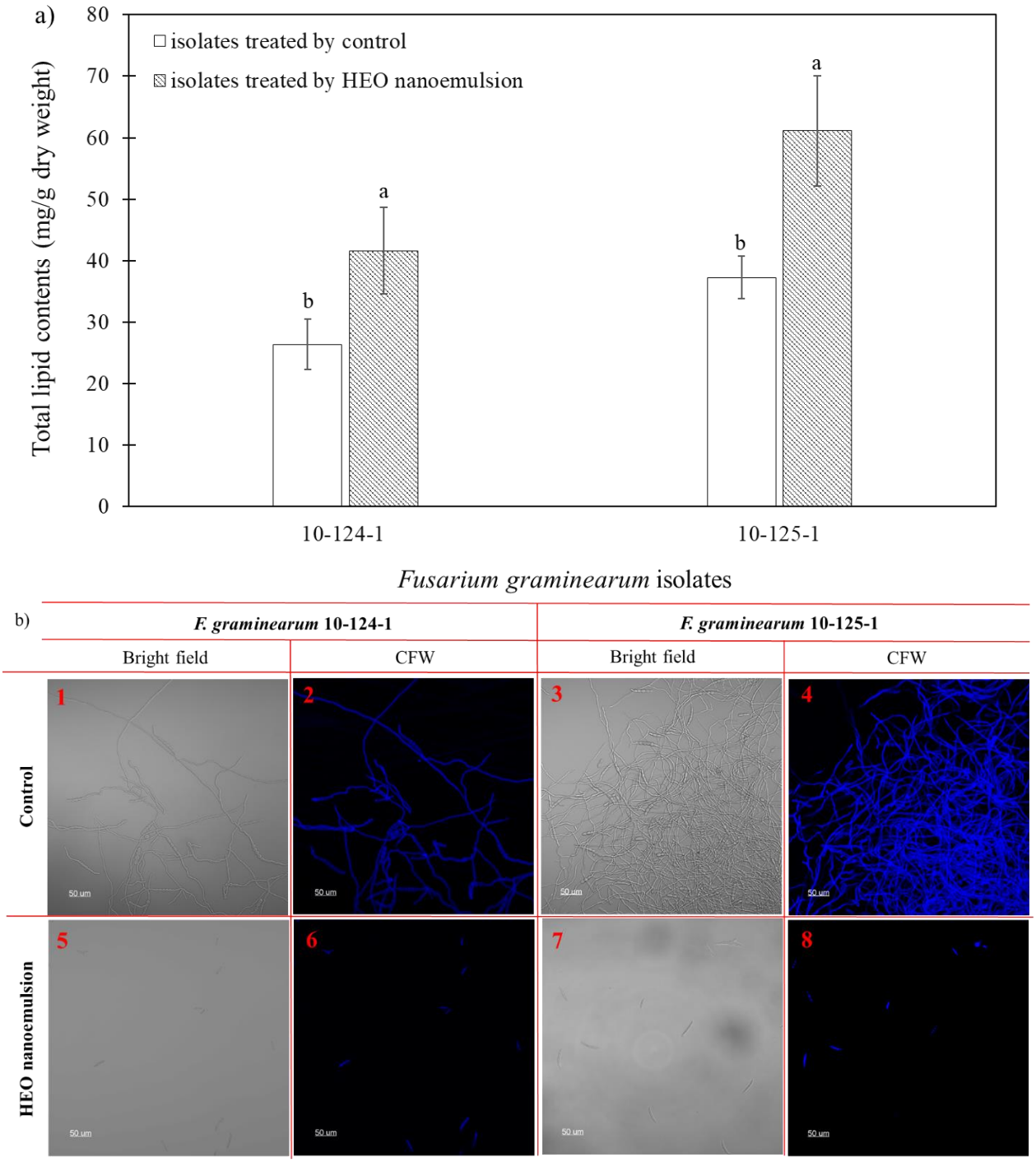

Figure 3-5. (a) Effect of HEO nanoemulsion on total lipid content of outer cell membrane of *F. graminearum* isolates. (Different lowercase letters indicate statistically significant intraspecies differences at $p < 0.05$); and (b) CLSM images of outer cell membrane of *F. graminearum* isolates after incubation for 10 h in potato dextrose broth (chitin was stained by calcofluor white dye and shown in blue color).

Furthermore, fluorescence dye-based method coupled with CLSM was applied to understand the role of HEO nanoemulsion on the chitin content in cell wall of spores (**Fig. 3-5b)**. In general, chitin is an essential polysaccharide that mainly exists in fungal mycelial cell wall (e.g., hyphae tips) and/or spore septa, and accounts for 10–20 % dry weight of cell wall (Fernandes, Gow, & Goncalves, 2016). Because its absence in human and other vertebrates, chitin has been considered as one of major antifungal target (Dorfmueller, Ferenbach, Borodkin, & van Aalten, 2014). As displayed in **Fig. 3-5b**, in control groups, all spores were germinated and produced large number of hyphae, and hyphae were stained with CFW (blue light) which showcased the general pattern of chitin deposition in normal hyphal tips and intracellular septa. By contrast, the CLSM image of HEO nanoemulsion treated *F. graminearum* isolates showed a scattered blue light, directly suggesting a significant decrease on the amount of chitin in spore cell wall and septa. Notably, the chitin on HEO nanoemulsion treated *F. graminearum* 10-124-1 (**Fig. 3-5b6**) seemed to be disappeared as reflected by the pale blue color in both terminal point and septa of spores. Likewise, trace amount of chitin was deposited on the side of *F. graminearum* 10-124-1 spores cell wall. The largely decreased chitin content in cell wall might be stemmed from the chitin synthase inhibitory activities of bioactive compounds in HEO nanoemulsion, thus preventing synthesis of chitin at the hypha tips and spore septa (Bang, Lee, Park, & Rhee, 2000).

Permeability of cytoplasmic membrane

The fungal cytoplasmic membrane provides a permeable barrier allowing the access of small ions K^+ and Na^+ to facilitate the function of cell membrane. Herein, the influence of HEO nanoemulsion on the permeability and integrity of cytoplasmic membrane of *F. graminearum* isolates was investigated by CLSM with the assistance of fluorescence dyes FDA and PI.

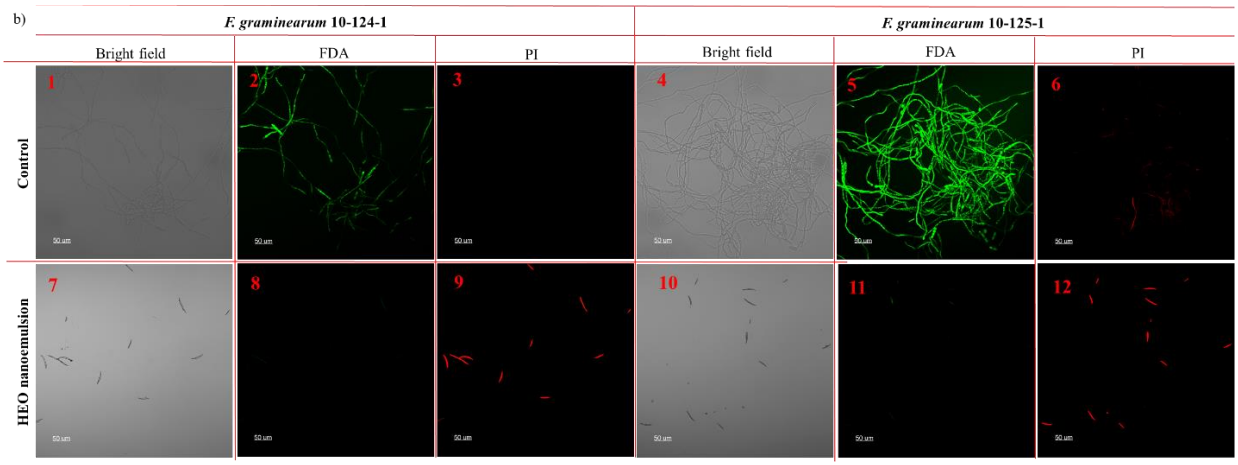

Figure 3-6. CLSM images of cytoplasmic membrane of *F. graminearum* isolates simultaneously stained by FDA and PI after incubation for 10 h in potato dextrose broth.

For the spores simultaneously stained with both FDA and PI, PI binds to DNA which generates a red fluorescence color, while FDA dye produces green color of the samples. As can be noticed in **Fig. 3-5-2 & 5**, the germination of all control spores as well as the production of fungal hyphae occurred after 10 hours' of incubation. Meanwhile, a considerable amount of green color was recorded in hyphae due to the presence of highly active esterase in healthy hyphae. Moreover, few to no red color was observed in the PI stained spore (**Fig. 3-6-3 & 6**), which suggested the absence of broken cell membrane in control samples. Again, it further confirmed that all control spores have the capability to germinate as well as promoting mycelial growth. Contrarily, no green color was shown in the HEO nanoemulsion treated spores, indicating the lack of spore germination and hypha growth. In terms of the PI stained spores, the majority of spores exhibited bright red color, manifesting that those spores were either completed dead or damaged particularly on cell membranes. Therefore, we conclude that HEO nanoemulsion is able to inhibit spore germination by damaging cytoplasmic membrane integrity. Similar mechanism on inhibiting spore germination was also reported in other antifungal agents (e.g., CF66I) against *Fusarium oxysporum* growth (Li et al., 2010).

Conclusions

In summary, the chemical composition of HEO was determined using GC-MS, and the top three chemical constituents of the HEO used in our study were β-Myrcene, humulene, and caryophyllene. Physically stable HEO-in-water nanoemulsion (d < 145 nm) was fabricated by mixing 30 wt% of Oswald ripening inhibitor (MCT) with 70% of HEO as oil phase (5 wt%). The mean particle diameter and the monomodal size distribution of the HEO-in-water nanoemulsion remained constant over a course of 7 days storage at 25 °C. Regarding the antifungal activity, HEO nanoemulsion could inhibit the mycelial growth and spore germination of the two chemotypes *F. graminearum* isolates by altering the total lipid, chitin content in outer cell membrane, as well as impairing cytoplasmic membrane permeability. In addition to their antifungal activity, HEO also displayed the capability to completely hinder biosynthesis of *Fusarium* mycotoxin biosynthesis including DON, 3ADON, and 15ADON in rice culture upon the addition of 750 μg HEO per g of rice. Because of the diverse genetic and biological variations of the two chemotypes of *F. graminearum* isolates, 15ADON chemotype of *F. graminearum* isolate 10-124-1 had a higher sensitive response to HEO nanoemulsion when compared to 3ADON chemotype of *F. graminearum* isolate 10-125-1. These findings of the current study provide fundamental information on applying HEO-in-water nanoemulsion as natural antifungal and detoxifying agents in the malting and food industry.

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CHAPTER 4. IMPACT OF HOP OIL-IN-WATER NANOEMULSION TREATMENT TO CONTROL THE SAFETY OF MALTING BARLEY USING NATURALLY *FUSARIUM* HEAD BLIGHT (FHB)-INFECTED BARLEY GRAINS

Abstract

Fusarium mycotoxin contamination of malting barley has been a persistent food safety issue for malting company. In our study, the effect of hop essential oil (HEO) nanoemulsion on fungal biomass and mycotoxin production during malting process was evaluated. The application of HEO nanoemulsion was able to reduce fungal biomass and deoxynivalenol (DON) content at each stage of malting process as compared to control. In addition to its antifungal activity, HEO nanoemulsion treated malts could be act as antioxidant for beer making due to the suppression of free aldehydes formation in final malts. The localization of fungal hyphae on the surface and inside the tissue of barley and malt barley was further evaluated. Results showed that fungal hyphae on kernel surfaces was reduced appreciable after steeping, but they were able to increase between the husk and testa layer of barley after germination as compared to original barley grains.

Introduction

Barley (*Hordeum vulgare*) as an ancient domesticated crop has been cultivating world widely, which is principally used for animal feed, malting and brewing (Verma, Lal, Malik, Kharub, Kumar, & Kumar, 2022). However, one of the major safety and quality concerns of brewing grains (e.g., barley) is the infection of barley kernels caused by FHB in the field. FHB is one of the most devastating diseases caused by *Fusarium* species in cereal grains (Schwarz, 2017). It not only reduces the crop production in the field, but also leads to the mycotoxin production in the grains (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018), such

as DON, NIV, T-2 toxin, etc. (Placinta, D'Mello, & Macdonald, 1999). Among abovementioned mycotoxins, DON is the most commonly found mycotoxin in barley and/or malts (Schwarz & Li, 2011; Wan, Jin, Zhong, Schwarz, Chen, & Rao, 2020). In addition, they are able to be transferred from malts to beer with a high recovery rate, thus it represents a food safety concern for malting and brewing company (Lancova et al., 2008; P. B. Schwarz, Casper, & Beattie, 1995).

Malting is a controlled grain germination followed by drying processing including steeping, germination and kilning (Baranwal, 2017). In general, DON level in barley grains can be reduced dramatically (> 50%) after steeping stage because of its water soluble nature (Schwarz et al., 1995). Although DON level can be slightly increased during the germination stage due to the ideal temperature and moisture conditions for fungal growth, numerous studies have demonstrated that most of newly produced DON after germination is located in rootlets (Schwarz et al., 1995). In consequence, one would expect the DON level in final malts would be reduced since all the rootlets are removed after the kilning step. In general, malting companies set up a DON limitation standard for purchasing brewing grains, ranging from 0.5 to 1.0 mg/kg in United States. However, some samples occasionally have a higher DON level in the final malts than in the initial barley grains. For instance, Yu et al., (2019) studied DON content changes during malting process using 20 barley samples with DON contents below 0.75 mg/kg and found that DON levels increased in 2 out of the 20 malts compared with the original barley samples. In other words, *Fusarium* growth and mycotoxin production during the malting process can be considered as an extension period of fungal colonization and infection in the crop field. This situation was extensively seen for the samples collected from the upper Midwest and Prairie provinces of Canada during the 2016 crop year (Jin et al., 2018). Such situation could cause significant safety and quality issues to the malting companies, especially for craft maltsters

because they require to use at least 50% of local grains (Schwarz, 2017). Several possible reasons might be attributed to this phenomenon. Firstly, the cross contamination of barley grains can be happened during steeping stage. In addition, the physical localization of fungal spore and/or hyphae within barley kernel tissue (e.g., surface husk, pericarp and testa layer, aleurone layer) might greatly impact the final mycotoxin level in malted barley. Until now, only one reported literature suggests that fungal hyphae located in furrow or aleurone layer of small grain kernels in the field could survive in a harsh processing condition, and a larger amount of fungal hyphae as well as associated mycotoxin were reproduced during the germination step (Jin et al., 2021).

Because of the abovementioned potential safety concerns for malting companies, strategies to mitigate *Fusarium* mycotoxin of FHB-infected barley in final malts have been studied for decades, including physical, chemical, and biological treatments (Chen et al., 2019). However, some of the mitigation strategies may have a negative impact on barley germination rate or nutritional quality of grains (Wolf-Hall, 2007). In addition, chemical additives are not allowed to be used during malting processing in EU. Therefore, novel strategies to control *Fusarium* mycotoxins effectively during malting process without negative impact on malt quality need to be explored. Recently, there is a growing interest in utilization of essential oils as antifungal agents. Our previous studies indicated that a number of EOs have the antifungal and mycotoxin inhibitory activities in vitro such as thyme, clove and hop oils by disrupting of cell wall composition formation and cytoplasm membrane permeability (Jiang, Zhong, Schwarz, Chen, & Rao, 2022; Wan, Zhong, Schwarz, Chen, & Rao, 2019). Especially the HEO, as one of four essential beer brewing ingredients, would be worthy of investigation to understand the potential application of HEO as a dual-functional ingredient during malting process.

Nevertheless, directly applying HEO as antifungal agent in aqueous-based malting process is not durable due to the water-insoluble characteristic of HEO (Steenackers, De Cooman, & De Vos, 2015). Therefore, HEO-in-water nanoemulsion delivery system was chosen in this study. In addition to its antifungal and antimycotoxigenic activities during malting process, there is insufficiency of knowledge on fungal growth pattern and localization of fungal hyphae within kernels as affected by HEO-in-water nanoemulsion during malting processing.

The aim of this study was to explore the efficacies of HEO nanoemulsion on inhibition of *Fusarium* growth and mycotoxin production during micro-malting process with no negative effect on the germination rate of barley grains using naturally FHB-infected barley grains Meanwhile, fluorescent dyes coupled with advanced microscopy techniques including CLSM and SEM were applied to study the localization of fungal hyphae on original barley kernels. In addition, the colonization of fungal hyphae during malting process as affected by HEO nanoemulsion was examined. Lastly, the impact of HEO nanoemulsion on the final malt flavors was measured.

Materials and methods

Materials

FHB-infected two-rowed barley gains from the 2019 crop year (cultivar: Pinnacle) were kindly provided by Rahr Malting Company (Shakopee, MN). The barley grains were naturally infected by FHB and contained 0.96 mg/kg of DON. HEO, MCT, Tween 80, PDA, BSA/ TMCS/ TSIM kit and *F. graminearum* isolate 10-124-1 used in this chapter was the same with those in chapter 1 and 2. Polyoxyethylene (20) sorbitan monolaurate (Tween 20), neomycin solution, kanamycin solution, and 10% neutral buffered formalin (NBF) solution were purchased from Millipore Sigma Co. (St Louis, MO, USA). Wheat Germ Agglutinin and Alexa Fluor™ 488

Conjugate (WGA-Alexa-fluor-488) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The The DNeasy Plant Pro and Plant Kits were purchased from Qiagen Inc. (Valencia, CA, USA). SsoAdvanced™ Universal SYBR® Green Supermix was purchased from Bio-Rad (Bio-Rad laboratories, Hercules, CA, USA). All solutions were prepared using the same methods mentioned in chapter 1 and 2.

Protein contents of barley kernels

The protein contents of barley kernels were analyzed according to AACC International Method 46-30 (N× 6.25).

Moisture contents of barley kernels

Moisture contents of barley kernels (1 g) were detected with an automatic grain moisture meter (Denver IR-200 Moisture Analyzer, Denver, CO).

Fungi contamination rates

Fungi contamination rates were evaluated obedience to the method of Jin et al. (Jin et al., 2018). The fungal colonies were identified morphologically by an optical microscope.

Preparation of HEO nanoemulsion

HEO-in-water nanoemulsion with the concentration of 35 mg/g was fabricated through the same formula and method in chapter 2.

The impact of HEO concentration on germinative energy of FHB-infected barley grains

GE was measured according to the method Barley-3A of American Society of Brewing Chemists (ASBC) (ASBC, 1999) with slightly modifications. Shortly, 100 barley kernels were put onto a petri dish with two-layer Whatman #1 filter papers. Four milliliters of ultrapure deionized water (as control) or HEO nanoemulsions with serial concentration gradients of HEO in nanoemulsions (35, 17.5, 12.5, 5.0, and 2.5 mg/g) were added to each petri dish, respectively.

All petri dishes were incubated at 21 °C for 4 days with > 90% relative humidity (Isotemp Peltier Low Temp Incubator, Thermo Scientific, Germany). The overall percentage of sprouted kernels were calculated after 84 hours.

Micro-malting process

The micro-malting process was carried out according to our previous study (Wan et al., 2020) with some modifications, and the optimized micro-malting conditions were represented in **Table 4-1**. In brief, barley kernels (20 g) were steeped in 100 ml of ultrapure water (control) in a 500 ml beaker. In terms of HEO nanoemulsion treatment group, 2.5 mg/g of HEO concentration in nanoemulsion was selected to conduct micro-malting experiment according to the results of the part titled the impact of HEO concentration on germinative energy of fungi-infected barley grains. Initially, original HEO nanoemulsion was diluted 14 times to reach the 2.5 mg of HEO concentration per gram of nanoemulsion, and then 10 ml of 2.5 mg/g of HEO nanoemulsion was mixed with 90 ml of ultrapure water. Subsequently, 20 g of FHB-infected barley kernels were steeped in 100 ml of prepared HEO nanoemulsion mixture in a 500 ml beaker. Therefore, the final HEO concentration in steeping water was 0.25 mg/g of water.

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

Micro-malting schedule		Duration (h)	Temperature (°C)	Relative humidity (%)
Steeping	Steeping 1	6	16	~95
	Air rest 1	2		
	Steeping 2	6		
	Air rest 2	2		
	Steeping 3	9		
Germination	Day 1	24	16	~95
	Day 2	24		
	Day 3	24		
	Day 4	12		
Kilning	Step 1	10	50	/
	Step 2	2	60	
	Step 3	2	85	

Impact of HEO nanoemulsions on the change of *Fusarium* biomass during micro-malting process

The Tri5 gene was used as an indicator to quantify the amount of *Fusarium* DNA in FHB-infected barleys and malts, which was used to represent the relative amount of fungal biomass in FHB-infected barleys and malts (Wan et al. 2020). The *Fusarium* DNA was extracted from known amount of barley kernels and malts using the DNeasy Plant Pro and Plant Kits (Qiagen Inc. Valencia, CA, USA). Tri5 gene in the genomic DNA was amplified by quantitative real-time polymerase chain reaction (q-RT PCR) with the aid of SYBR Green probe performed in a CFX96 Real-time thermal cycler (Bio-Rad, Hercules, CA, USA) according to our previous method with minor modification on the standard curve (Wan et al., 2020). The primers used in this study were TMT_fw (5′-GATTGAGCAGTACAACTTTGG-3′) and TMT_rev (5′-ACCATCCAGTTCTCCATCTG-3′) (Millipore Sigma Co. St. Louis, MO, USA). A standard curve was plotted from the six serial dilutions (10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, and 100 fg/µl) of *F. graminearum* 10-124-1 DNA. In brief, 4-day old mycelia grown on PDA medium was collected and grounded with liquid nitrogen before DNA extraction. The DNA

concentration was determined by Nanodrop OneC (Thermo Scientific, Waltman, MA, U.S.). The concentrations of *Fusarium* DNA in barley kernels and during each step of malting processing were quantified according to the standard curve. Results were expressed in mg DNA/kg barley.

Impact of HEO nanoemulsions on the change of mycotoxin during micro-malting process

The mycotoxin contents in barley grains and malted barley grains during the each step of micro-malting process including HT-2 toxin, T-2 toxin, DAS, DON, 3-ADON, 15-ADON, NIV and ZEA were quantified by a GC-MS (Wan, Zhong, Schwarz, Chen, & Rao, 2018).

The correlation between *Fusarium* DNA and DON contents at the various stages of micro-malting were calculated using Pearson's correlation coefficient equation (1) (Mukaka, 2012),

$$r = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 \sum(y_i - \bar{y})^2}} \quad (1)$$

r = correlation coefficient

x_i = values of the *Fusarium* DNA in barley or malted barley kernels

$\bar{x}$ = mean of the values of the *Fusarium* DNA in barley and malted barley kernels

y_i = values of the DON contents in barley or malted barley kernels

$\bar{y}$ = mean of the values of DON contents in barley and malted barley

Localization of fungal hyphae in barley and malted barley kernels

The localization of fungal hyphae in barley and malted barley kernels were analyzed using CLSM and SEM in accord with the method of Jin (Jin et al., 2021) with minor modifications.

For the method of CLSM, randomly selected kernels were initially soaked in a 10% NBF solution for 24 h at room temperature, and then samples were washed and dehydrated by a series concentration gradients of ethanol solutions (70% for 5 min, 80% for 5 min, 100% ethanol for 5

min, and 100% ethanol for 30 min) and xylene solution (10 min and 20 min) with 2 minutes' drain in each step. Three dehydrated kernels were embedded in a paraffin wax block. Transverse and longitudinal sections (5-8 μm in thickness) of the kernels were obtained by cutting the middle part of the kernels through a manual microtome (Leica, Heidelberg, Germany). Afterwards, section samples were attached on Superfrost ™ plus charged slides (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The samples were washed by xylene three times (3 min/time), a series of ethanol solutions (100% for 15 dips, 95% for 15 dips, 70% ethanol for 15 dips) and distilled water for better staining purpose. Subsequently, section samples were stained with 20 μg/ml WGA-Alexa Fluor 488 for 5 h with continuously shaking in the dark, and the staining solution was replenished every 2.5 h. Stained sample slides were observed on a Zeiss Axio Observer Z1 inverted microscope with LSM700 laser scanning unit (Zeiss, Thornwood, NY) at 40 × magnification with green fluorescence channel 488/520 for WGA-Alexa Fluor staining and red fluorescence channel 555/580 for the autofluorescence detection of grain tissues, simultaneously.

Both transverse and longitudinal sections of kernels were also observed through a SEM according to the method of Jin (Jin et al., 2021).

Flavor profile of malts

Flavor profile of control malts and HEO nanoemulsion treated malts were analyzed by a headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC–MS) system (Agilent 7890B GC system and 5977 MS system, CA, USA) following the methods of Gu et al. (Gu, Jiang, Zha, Manthey, Rao, & Chen, 2021) without agitating step during sample incubation. The volatile chemical components of malts were identified by mass spectral library NIST 14 L (match score of >80) and the match of retention index (RI) of each compound with a

reference value reported in the NIST database (https://webbook.nist.gov). Absolute peak areas of the identified components were reported.

Statistical analysis

All measurements were carried out at least twice using freshly prepared samples. Pearson correlation coefficient was calculated by Excel. Statistical analysis methods were the same in chapter 1.

Results and discussion

Basic parameters of barley

The basic information of the barley used in this project were showed in Table 4-2. The Pinnacle barleys were naturally infected with FHB and were harvested in 2019. The barley grains were tested containing around 0.96 mg/kg of DON. The protein, moisture and percentage total fungi infection rate of barley grains were 10.32%, 14.47% and 68%, respectively.

Table 4-2. Basic parameters of barley.

Barley variety	Crop year	Protein content	Moisture content	Fungi contamination rate
		----------(%)----------		
Pinnacle	2019	10.32±0.16	14.47±0.39	68.00±3.27

Influence of HEO nanoemulsion on germination energy of FHB-infected barley grains

It is well known that malting processing is a controlled germination of grains to activate enzymes for hydrolyzing macromolecules to the soluble compounds. Therefore, the good quality of malting barley should have the characteristics of rapid and uniform germination rate as well as sustainably vigorous growth (Burger & LaBerge, 1985). In general, high GE can be considered as a good indicator of malting ability (Woonton, Jacobsen, Sherkat, & Stuart, 2005). Therefore, it becomes an important quality parameter of malting barley. Previous studies suggested that essential oil (EO) is able to act as an green fungicides, however, it also inhibits the seed

germination rate due to the allelopathic interaction or allelopathic effect (Mirmostafaee, Azizi, & Fujii, 2020). For example, wheat seeds have the capability to metabolize certain monoterpenes from EO when they exposed to EO. The generated new compounds from the degradation of monoterpene could inhibit the GE of wheat seeds (Atak, Mavi, & Uremis, 2016). In general, the higher concentration of EO applied to the grains leads to the lower GE. Therefore, it is great importance to determine the impact of HEO concentration on the GE of barley.

As shown in **Fig. 4-1**, HEO concentrations in nanoemulsion significantly influenced the GE values of barley (**Fig. 4-1**). Initially, the average GE of barley grains (control sample) was around 94%, which is lower than 100%. This might be due the invasion of mycelia and mycotoxin in barley grains (Wolf-Hall, 2007). In terms of treatment group, GE values declined dramatically with the increase of HEO concentration in nanoemulsion. Specifically, the GE values of FHB-infected barley grains were less than 50% when the applied HEO concentrations were > 17.5 mg/g.

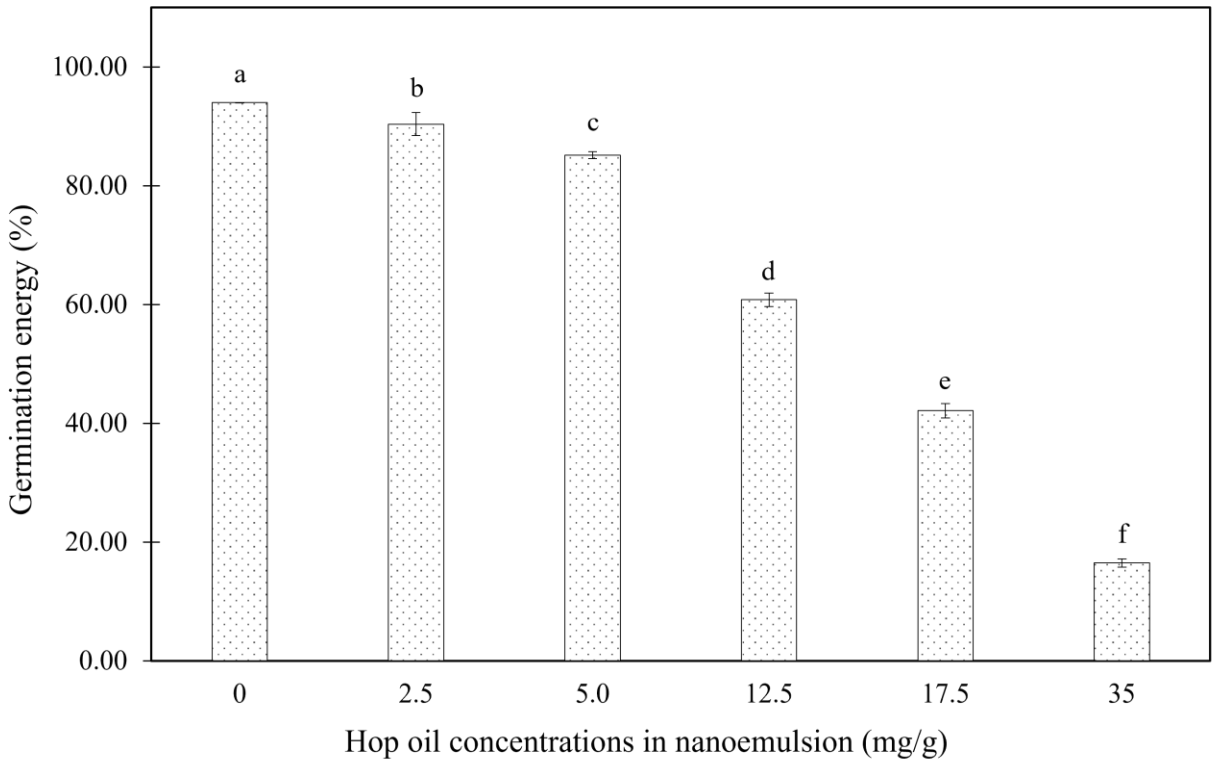

Figure 4-1. Impact of HEO nanoemulsion concentrations on germinative energy of FHB-infected barley grains. Columns with different letters were significantly different ($p < 0.05$).

When 2.5 mg/g of HEO nanoemulsion was applied in barley grains, although there was a slightly decreased GE value (90.38%±1.94%) as compared to original FHB-infected barley (94.01%±0.01%), the impact on GE of barley was minimal in our study. Similar result was reported in our previous study on the effect of CO concentration on GE of barely grains (Wan et al., 2020). Therefore, the concentration of HEO in nanoemulsion at 2.5 mg/g was selected in this chapter.

Influence of HEO nanoemulsion on the change of *Fusarium* biomass and mycotoxin production in barley grains during various stages of malting

In recent years, *Tri5* DNA content has been widely used to quantify the amount of *Fusarium* spp. in cereal grains such as barley and wheat (Brunner et al., 2009; Burlakoti, Estrada, Rivera, Boddeda, Secor, & Adhikari, 2007). This is because *Fusarium* DNA content shows a

good correlation with kernel damage rate, concentration of *Fusarium* biomass, and DON development in malt barley grains (Vegi, Schwarz, & Wolf-Hall, 2011). *Tri5* encodes trichodiene synthase that is an enzyme involved in the first biosynthetic step of trichothecenes by catalyzing farnesyl pyrophosphate to trichodiene during DON biosynthesis pathway (Proctor, Desjardins, McCormick, Plattner, Alexander, & Brown, 2002). Therefore, Tri5 DNA content in the barley and malt samples in our study were selected to represent the *Fusarium* biomass in FHB-infected barley kernels during malting process. The effect of HEO nanoemulsion on the change of *Fusarium* biomass in raw barley grains, after steeping, germination, and the final malts were quantified and compared with control samples (**Fig. 4-2**).

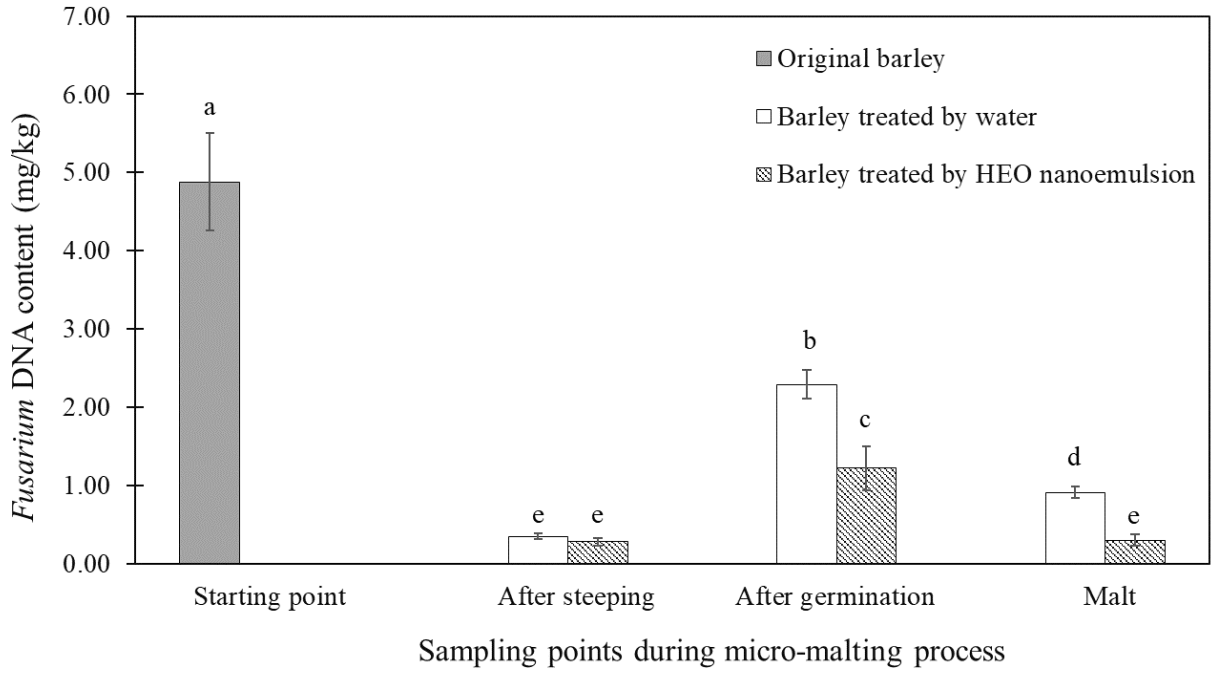

Figure 4-2. Impact of HEO nanoemulsion on *Fusarium* spp. growth during the micro-malting process as quantified by *Fusarium* DNA content. Columns with different letters were significantly different ($p < 0.05$).

As shown in **Fig. 4-2**, *Fusarium* DNA contents in both control and treated samples followed the same pattern during malting process. In general, it was decreased noticeably after steeping step, and increased substantially after germination stage, but declined again in the final malts. For example, *Fusarium* DNA content in original barleys was ~ 4.88 mg/kg. This value was reduced to 0.35 mg/kg after steeping, and rose to 2.29 mg/kg after 4 days' germination, then dropped to 0.91 mg/kg in the kilned malts. Similarly fluctuated trends of *Fusarium* DNA contents during the malting process were described in *F. graminearum* inoculated barley grains (Vegi et al., 2011; Wan et al., 2020). These results demonstrated that the majority of fungi contamination could be washed off during steeping step. However, the remnant fungal hyphae kept growing during germination stage because of the favorable environmental conditions for fungal growth. Thus, larger amount of fungal biomass (*Fusarium* DNA content) was produced during germination stage. The high temperature and low humidity in the kilning process retarded the fungal growth, and fungal biomass was further reduced with the removal of rootlets, resulting in low *Fusarium* DNA contents in final malts. Regarding the HEO nanoemulsion treated barley samples, *Fusarium* DNA contents at each micro-malting step were lower when compared with that in controls. For instance, *Fusarium* DNA content in HEO nanoemulsion treated sample and control was 1.07 mg/kg and 1.22 mg/kg, respectively after germination step; and was 0.30 mg/kg and 0.61 mg/kg, respectively in final malts. The results clearly indicated that HEO nanoemulsion was able to inhibit fungal growth during malting process.

The ultimate goal is to control mycotoxins in final malts since they can be transferred to the beer products with a large recovery rate. Therefore, we further evaluated the effect of HEO nanoemulsion on inhibition of mycotoxin production at each step of micro-malting process. From our previous studies, we have learned that application of EO nanoemulsion in the first

stage of steeping process showed the highest DON inhibitory activity in the final malt (Wan et al., 2020). Therefore, HEO nanoemulsion was added in the first step of steeping stage. We also assessed all trichothecenes mycotoxins, including type A trichothecenes (HT-2 toxin, T-2 toxin and DAS) and type B trichothecenes (DON,3-ADON, 15-ADON and NIV), as well as ZEA in original barley kernels as well as the final malts. DON was the only mycotoxin that can be detected in our samples and result was shown in **Fig. 4-3**.

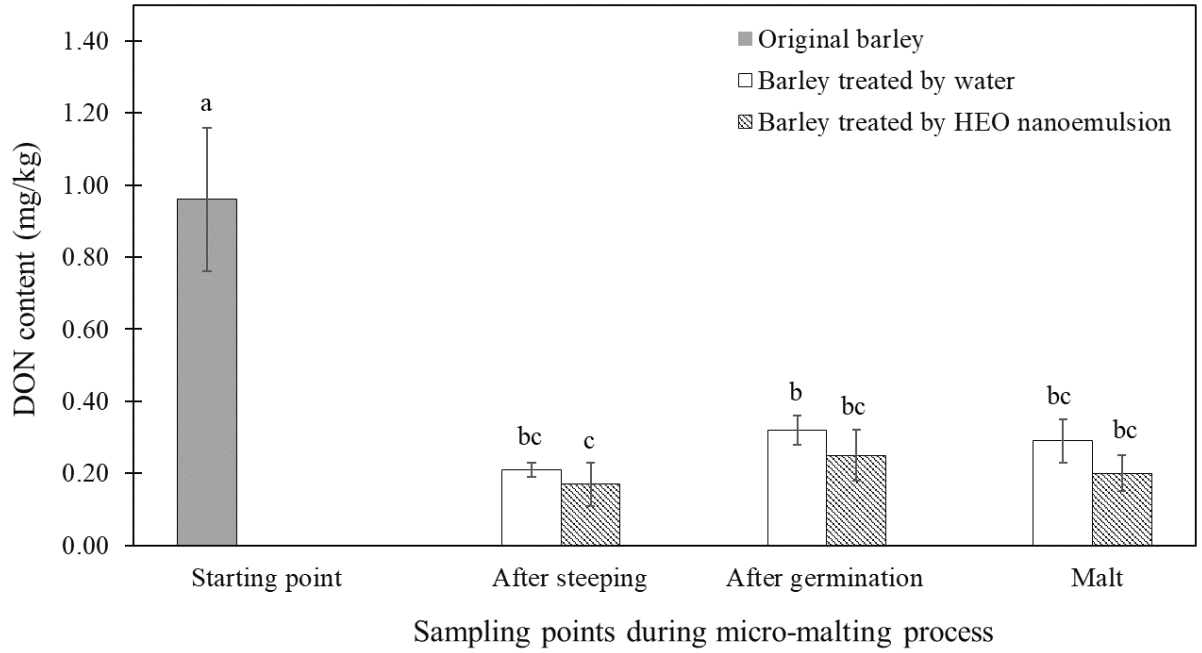

Figure 4-3. Impact of HEO nanoemulsion on DON content of FHB-infected barley grains during the micro-malting process. Columns with different letters were significantly different ($p < 0.05$).

As shown **Fig. 4-3**, DON contents fluctuated among each step of micro-malting process, which exhibited the same trend as those of *Fusarium* DNA contents (**Fig. 4-2**). For instance, the initial DON content in barley grains was 0.96 mg/kg, then dramatically dropped to 0.21 mg/kg after steeping, and was slightly raised up to 0.32 mg/kg after 4 days' germination, followed by a slight decrease to 0.29 mg/kg in the final malts. Similar to the fungal biomass growth pattern

during malting processing, a great number of water-soluble DON in barley grains were washed off with 3 times' water replacement after steeping stage, and thereafter there was a significant decrease of DON contents. Nevertheless, the suitable environmental condition of 4 day's germination period provides the optimum circumstance for remnant fungal hyphae or spore to develop and produce mycotoxin, leading an increased DON content after germination stage. Although the newly produced DON content won't degrade during the kilning process, nearly half of newly produced DON was remained in the rootlets of germinated barley grains, and it can be diminished through removing rootlets after kilning (**Fig. 4-4**). Therefore, lower DON contents in final malts was detected as compared to germinated barley grains. Similar trends in the change of DON contents during malting process were also found using *Fusarium* inoculated barley grains (Lancova et al., 2008; Wan et al., 2020). We further assessed the correlation between *Fusarium* DNA content and DON level using Pearson's correlation coefficient during each stage of malting process. Pearson's correlation coefficient has been widely used to evaluate the correlation between two variables such as fungal biomass and mycotoxin accumulation *in vitro* and in grains (Garcia, Ramos, Sanchis, & Marin, 2013; Horevaj, Milus, & Bluhm, 2011). The result (Pearson correlation coefficient = 0.96) showed that there was a remarkably positive correlation between *Fusarium* biomass and DON accumulation in barley during the malting process, which suggested that one variable could be used to predict another variable in this case.

In terms of HEO nanoemulsion treated barley samples, the changes of DON contents were similar compared to the control sample. However, DON contents in HEO nanoemulsion treated samples were lower at each micro-malting process stage especially after germination and in the final malt. This results clearly demonstrated that HEO nanoemulsion was not only able to inhibit the *Fusarium spp.* growth, but also halt DON production during micro-malting process,

which was conforming to our previous study (Wan et al., 2020). More interesting, the HEO nanoemulsion treated samples had a higher DON concentration in rootlets than that of control (**Fig. 4-4**), which indicated that HEO nanoemulsion treatment facilitates the newly produced DON at the germination stage, which are maintained in the rootlets rather than in the malts.

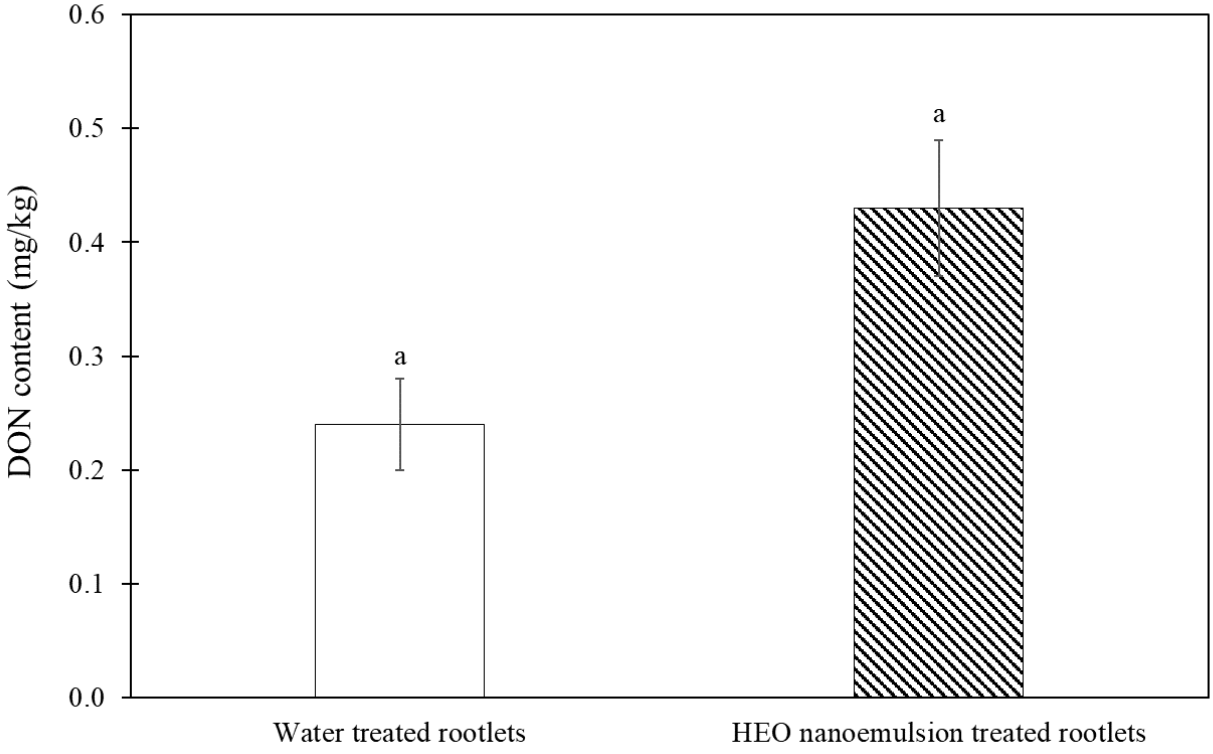

Figure 4-4. Impact of HEO nanoemulsion on DON content of malt rootlets. Columns with different letters were significantly different ($p < 0.05$).

Localization and growth patterns of fungal hyphae in FHB-infected barley and malt kernels

To better understand how HEO nanoemulsion impact fungal hyphae growth within kernel tissue after malting process, six barley or malt kernels were randomly chosen to be observed in both transverse and longitudinal sections under CLSM and SEM (**Fig. 4-5**). In addition, the possible fungal hyphae infection sites were analyzed on six regions of a transverse section and four regions of a longitudinal section in each kernel including the surface of husk, furrow,

pericarp and testa, aleurone layer and starchy endosperm. All regions were labelled using alphabets from “a” to “i” in SEM and CLSM images accordingly. For the CLSM, green color represented the dyed fungal hyphae using WGA-Alexa-fluor-488 (Figueroa-Lopez, Cordero-Ramirez, Quiroz-Figueroa, & Maldonado-Mendoza, 2014), whereas, red color indicated the barley and malting barley tissue generated by red fluorescence channel 555/580 (Solanki, Ameen, Borowicz, & Brueggeman, 2019). Therefore, penetration of fungal hyphae within kernel tissue could be clearly observed due to the reconstructed Z-stack images feature. However, the loss of fungal hyphae in CLSM image could be happened due to the excessive washing steps during sample preparation steps, Therefore, SEM technique was also used to observe the location of fungal hyphae in kernels and presented in **Fig. 4-6**.

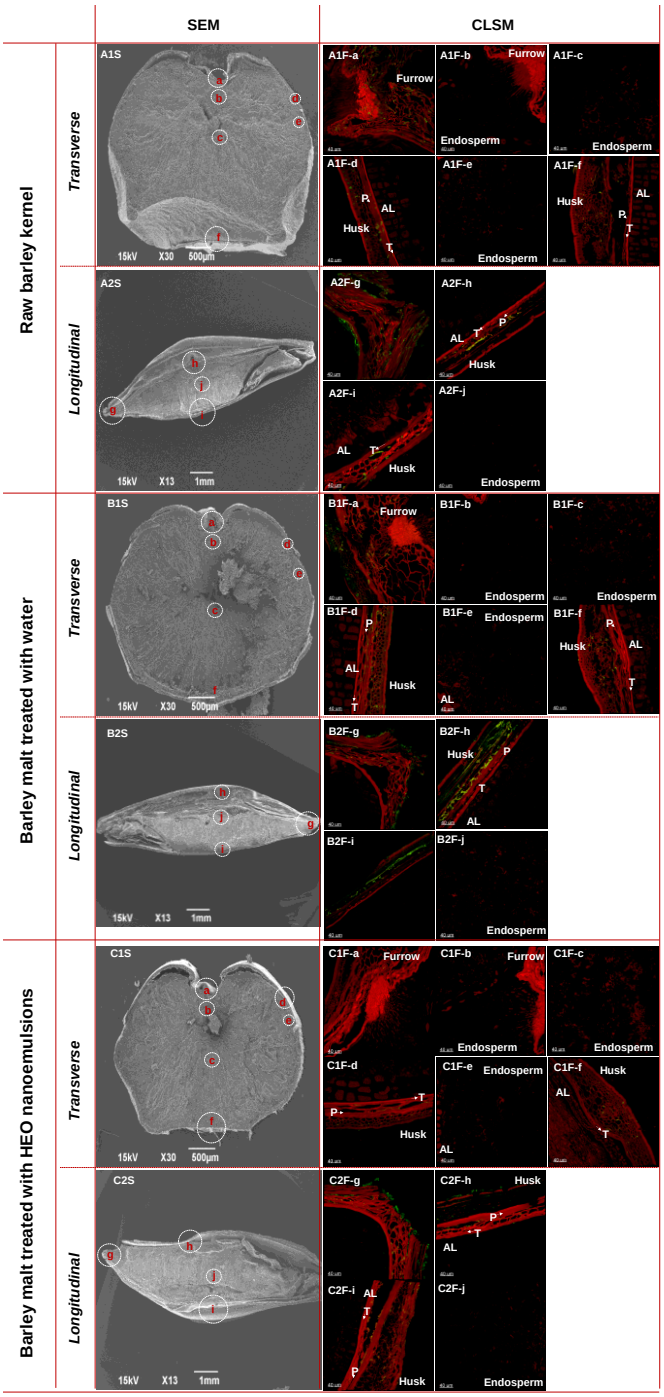

Figure 4-5. Images of fungal hyphae locations in the transverse (a-f) and longitudinal (g-j) sections of (A) FHB-infected barley kernel, (B) control malt kernel and (C) HEO nanoemulsion treated kernel examined under SEM and CLSM. In the SEM images, a represents location around furrow; b represents locations around the furrow; c, e and j represent location in the endosperm; d, f, and i represent location around husk, aleurone layer, testa and pericarp layer; g represents location around husk in the distal end. In the CLSM images, the green color indicates fungal hyphae and the red color indicates barley or malt barley tissues. AL = aleurone layer, P = pericarp, T = testa.

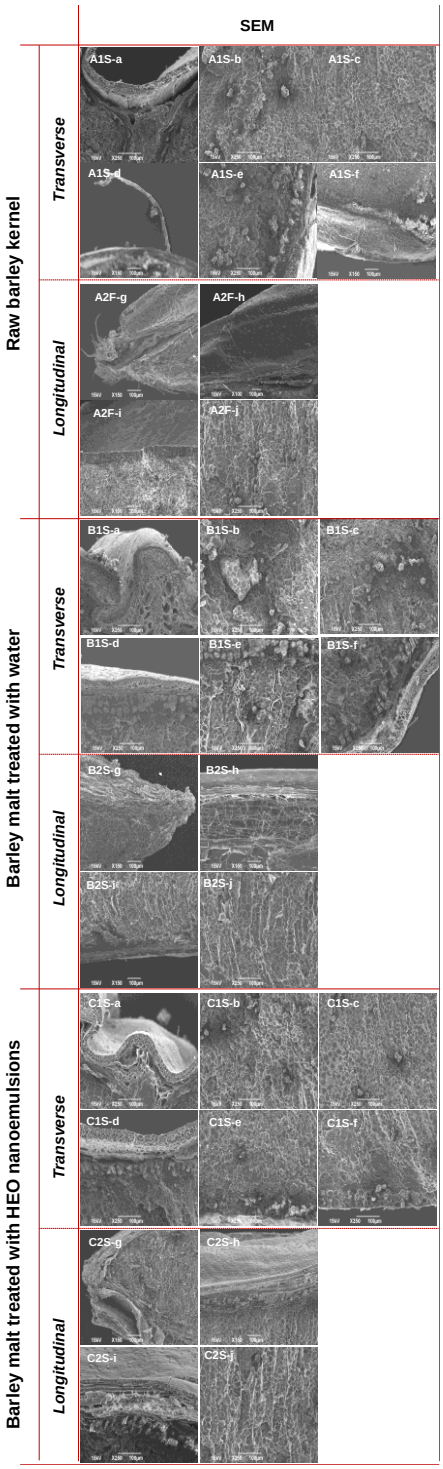

Figure 4-6. Images of fungal hyphae locations in the transverse (a-f) and longitudinal (g-j) sections of (A) FHB-infected barley kernel, (B) control malt kernel and (C) HEO nanoemulsion treated kernel examined under SEM. In images, a represents location around furrow; b represents locations around the furrow; c, e and j represent location in the endosperm; d, f, and i represent location around husk, aleurone layer, testa and pericarp layer; g represents location around husk in the distal end. AL = aleurone layer, P = pericarp, T = testa.

In general, for the FHB-infected barley kernels, majority of fungal hyphae were accumulated in furrow crease (**Fig. 4-5-A1F-a**), distal end in longitudinal section of barley (**Fig. 4-5-A2F-i**), followed by the surface of husk, between husk and pericarp and testa layers, pericarp and testa layers (**Fig. 4-5-A1F-d&f, A2F-h**). Previous studies suggested that fungal hyphae were more often localized in the distal end rather than proximal end (Mead, 1942), which was in line with our samples. As stated by previous researchers, the initial fungal infestation could occur at the malformed grains or at the water absorption pores of normal grains such as furrow site where fungi can easily obtain the nutrients for their proliferation. Consequently, hyphae located in the distal end of barley kernel have the opportunities to invade into the internal tissues including pericarp and testa layer, finally reached to the aleurone layer (Imboden, Afton, & Trail, 2018). For example, several green spots on the husk, and some green filaments was observed between the husk and testa layer (**Fig. 4-5-A1F-d & A2F-i**). According to the SEM images, a larger number of hyphae filaments were formed on the husk surface of barley grains when compared to CLSM images (**Fig. 4-6-A2F-g&h**). In addition, few of hyphae were observed in aleurone layer of FHB-infected barley samples, and no hyphae were observed in the endosperm. As confirmed by SEM image, no fungal hyphae could be found in starchy endosperm. This was not surprise since the physical barrier of outer shell (husk) provides better protection to prevent fungal infection and invasion. In addition, high concentrations of phenolic compounds in pericarp and testa layer can serve as antifungal agent to further prevent fungal invasion to the inside of kernel tissue (Oliveira, Mauch, Jacob, Waters, & Arendt, 2012). Therefore, fungal hyphae infection in starchy endosperm can happen in very few cases, such as previously physical damaged of kernel in the field.

Similar trend on the localization of fungal hyphae was found in control malts as compared to the original barley kernels. As can be seen from the transverse sections of the control malt (barley malt treated with water), the greater number of hyphae were accumulated between the husk and testa layer (**Fig. 4-5-B1F-d&f, B2F-h&i**) as compared to the original barley kernels, especially in longitudinal section of samples. Fewer hyphae were observed on the husk, surface of distal end of control malts (**Fig. 4-5-B2F-g**) when compared with the original barley kernel (**Fig. 4-5-A2F-g**). Similarly, no hyphae were observed in the endosperm of control malts. This results also confirmed that steeping processing could wash out majority of fungal hyphae on the surface of barley grain. Therefore, very few of fungal hyphae could be observed on the surface of husk in malts. However, it is impossible to completely remove contaminated fungal hyphae on the surface of grains, remnant fungal hyphae in barley kernels could proliferate during germination stage. As a result, more fungal hyphae were spotted between the husk and testa layer. The SEM images of control malts also supported this result, especially in longitudinal section samples (**B2S-h**). An increase of fungal hyphae in malted barley kernels when compared to that of original barley kernels was also reported previously (Jin et al., 2021). Although as compared with original barley, more hyphae were observed on the husk, pericarp and testa layer, but lower *Fusarium* DNA were detected in control malts as compared to original barley samples (**Fig. 4-2**). This phenomenon could be explained by the following two reasons. Firstly, some husks and pericarps in malt samples were detached from the final malts during removal of rootlets step, resulting the decreased fungal hyphae within husks and pericarp. Although, *Fusarium* spp. was the dominant contamination fungi species in our sample, they were not the only fungi species existing in our samples according to the characterization of fungi infection rate of barley grains experiment results. Therefore, other fungal hyphae growth may also be

presented in control malts. Thus, the observed development of fungal hyphae growth pattern in CLSM and SEM images were not able to exactly reflect the change of *Fusarium* biomass (*Fusarium* DNA) during malting process.

In terms of HEO treated malt kernels, HEO nanoemulsion greatly reduced the fungal hyphae in the furrow (**Fig. 4-5-C1F-a**) and between the husk and testa layers (**Fig. 4-5-C1F-d&f, C2F-h&i**) as compared to control malts. Likewise, no hyphae were observed in the endosperm of HEO nanoemulsion treated malts. Only few green colors could be observed in the distal end of malt kernel (**Fig. 4-5-C2F-g**) and between the husk and testa layers (**Fig. 4-5-C2F-h**). These indicated that HEO nanoemulsion was able to penetrate or be absorbed into pericarp and tesla layer during steeping stage to inhibit fungal hyphae growth to some extent. This observation was collaborated with our Tri5 DNA content results that an appreciable decreased *Fusarium* DNA was detected in treatment malts (**Fig. 4-2**). Our previous study indicated that HEO nanoemulsion could control mycelial growth of *F. graminearum in vitro* by altering total lipid and chitin content in cell membrane as well as by increasing the permeability of cytoplasmic membrane (Jiang et al., 2022). This might be the reason to explain why HEO nanoemulsion could limit the growth of fungal hyphae on barley kernels during malting process.

Flavor profile of malts

One of the major concerns for utilization of EO as an antifungal agent in food is their effect on flavor. It is well known that EO has a strong flavor intensity with small quantities. Although, hop is a vital ingredient for making beer, which contributes bitter and aroma profile of beer (Steenackers et al., 2015), we still would like to understand the impact of HEO nanoemulsion on the flavor profile of final malts (**Table 4-3**). Therefore, the flavor profile of

HEO nanoemulsion treated malt was measured and compared with control malt using HS-SPME-GC–MS.

Table 4-3. Impact of HEO nanoemulsion on flavor profile of malts.

Peak No.	Compound name	Peak area (Counts × 10^5)		Aroma description
		Control malt	HEO Nanoemulsion treated malt	
Aldehydes				
1	Nonanal	0.63±0.15a	0.6±0.16a	Sweet, fruity, waxy, herbal, plastic (Su & Yin, 2021)
2	Furfural	1.77±0.58	-	Fat, citrus, green (https://www.flavornet.org/flavornet.html)
3	2-Nonenal	1.61±0.41a	2.18±0.63b	Almond, burnt sugar (https://www.flavornet.org/flavornet.html)
4	Benzeneacetaldehyde	3.86±0.7a	2.4±0.37b	Cucumber, fat, green (https://www.flavornet.org/flavornet.html)
Esters				
5	Methyl octanoate	-	0.6±0.15	Green bean, butter (Dong, et al., 2015)
6	Methyl nonanoate	0.65±0.21a	0.66±0.22a	Bread, almond, sweet (https://www.flavornet.org/flavornet.html)
7	Benzaldehyde	1.04±0.3a	1.05±0.29a	Coconut (https://www.flavornet.org/flavornet.html)
8	Dimethyl glutarate	1.57±0.23a	3.17±0.51b	Sweat, acid, rancid (https://www.flavornet.org/flavornet.html)
9	Methyl benzeneacetate	0.99±0.13a	1.37±0.2b	Honey (Zapata, et al., 2018)
Fatty acid				
10	Isovaleric acid	1.3±0.19a	0.88±0.2b	Fatty acid (Dong, et al., 2015)
11	Hexanoic acid	1.12±0.23a	1.39±0.15b	Sweaty (Dong, et al., 2015)
Furan				
12	2-Pentylfuran	0.97±0.3a	0.95±0.28a	Gas, earthy (Kyraleou, et al., 2021)

† Same chemical compounds with different letters were significantly different ($p < 0.05$).

Overall, the flavor profile of the two malts shared a considerable similarity, but the type of volatile compounds and the relative amount of the volatiles were slightly different. For example, 11 types of volatile compounds were identified in both control malt and HEO nanoemulsion treated malt. Overall, aldehydes and esters were the major volatile compounds, followed by fatty acids and one furan compound in both malts. However, one volatile compound (furfural) existed only in control malt, whereas, methyl octanoate were only detected in HEO nanoemulsion treated malt. Furfural is a frequently reported free aldehyde in malt and/or beer that can be generated in many ways such as Maillard reaction, fatty acid oxidation and/or Strecker degradation (Yahya, Linforth, & Cook, 2014). It is also considered as one of flavor indicators for lager beer staling (Filipowska et al., 2021). Moreover, the peak area of the top one aldehydes (benzeneacetaldehyde), also called as free staling aldehyde, in control malt was significantly higher than that of treatment malt. Therefore, malt with low concentration of staling aldehydes could provide the potential benefit for preventing beer staling. In terms of HEO nanoemulsion treated malt, previous study found that some hop constituents have the capability to suppress furfural formation during brewing process (Wietstock, Baldus, Ohlschlager, & Methner, 2017). This could be the reason why there was no furfural in HEO nanoemulsion treated malt. Regarding the methyl octanoate, there was no surprise that methyl octanoate was detected in HEO nanoemulsion treated malt since it is the typical volatile compound in HEO (Kralj, Zupanec, Vasilj, Kralj, & Psenicnik, 1991). Other than different volatile compounds, the percentage of commonly existed several volatile compounds in two malts have been changed to some extent. For example, 2-nonenal (aldehyde) dimethyl glutarate (ester), and hexanoic acid (fatty acid) in treatment malts were remarkably higher when compared to those in control malts.

Hence, we could conclude that HEO nanoemulsion were able to change flavor profile of malts to some extent, especially to suppress the formation of free aldehydes in final malts.

Conclusions

In summary, HEO nanoemulsion has a negative impact on the GE of naturally FHB-infected barley grains, but 2.5 mg/g of HEO in nanoemulsion had the minimal impact on GE of barley. Therefore, 2.5 mg/g HEO nanoemulsion was applied to treat naturally FHB-infected barley in the first steeping stage. *Fusarium* biomass marker (*Fusarium* DNA) and DON contents were diminished notably in both control and treatment malts after steeping, rose after germination, and then decreased in the final malts. A significant positive correlation coefficient (Pearson correlation coefficient = 0.96) was found between *Fusarium* DNA and DON contents. Compared with control malts, the *Fusarium* DNA and DON contents were lower in treatment samples at each stage of the malting process, which suggested that HEO nanoemulsion could effectively inhibit fungal growth and mycotoxin production during micro-malting process. With the aid of advanced microscopy techniques (CLSM and SEM), the localization of fungal hyphae in FHB-infected original barley kernels and malt kernels were identified. In general, majority of fungal hyphae were observed in furrow crease (transverse section), distal end (longitudinal section), the surface of husk, followed by between husk and pericarp, testa layers. Steeping process greatly decreased the fungal hyphae in furrow crease, distal end and on the surface of husk as compared to original barley samples. However, an increased fungal colonization between husk and pericarp, testa layers was found in the final malts because of the ideal environmental conditions for fungal growth during the germination stage. The treatment malt kernels generally had less fungal hyphae compared to the original barley and control malts, which was in consistent with our *Fusarium* DNA content results. Regarding the impact of HEO nanoemulsion

on the final malt flavor, the flavor profiles of both malts were similar. However, HEO nanoemulsion could prevent the formation of free aldehydes in final malts. As a result, it might act as antioxidant to prevent beer staling using HEO treated malt for brewing.

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OVERALL CONCLUSION

In this project, the chemical composition of CBO, CLO and HEO was initially characterized. The physicochemically stable EO-in-water nanoemulsions were fabricated by mixing EO with either corn oil or MCT. Subsequently, above mentioned three stable EO-in-water nanoemulsions were applied to assess their antifungal and mycotoxin inhibitory activities *in vitro*. The relationship between their chemical composition of EOs and antifungal efficacies were discussed and the antifungal mode of action (MOA) against two chemical types of *F. graminearum* growth were elucidated. Finally, physically stable HEO-in-water nanoemulsion was applied to the first steeping stage of micro-malting process to inhibit *Fusarium* biomass growth and associated mycotoxins reproduction by malting naturally FHB-infected barley grains. In addition, the change of fungal hyphae locations in the grains after malting were identified.

Regarding the chemical compositions of two types of CO, their chemical constituents were varied qualitatively and quantitatively. For instance, high percentages of eugenol and eugenol acetate were monitored in CBO. By contrast, there was no detected eugenol acetate in CLO. As for HEO, β-myrcene was the major chemical compounds in the HEO, followed by humulene, caryophyllene, nerol, etc. Because of their varied chemical constitutes in different types of EOs, diverse nanoemulsion formations and their functional performance in terms of antifungal and mycotoxin inhibitory were shown. For instance, physically stable CBO and CLO nanoemulsion with the mean particle sizes < 170 nm were formed by using 75% of corn oil mixing with 25% of EO in total 5 wt% of oil phase. Whereas HEO nanoemulsion with the mean particle sizes < 145 nm was made up by blending 30 wt% of MCT and 70% of HEO in total 5 wt% of oil phase. All formed EO nanoemulsions had good physiochemical stability against droplet growth as well as qualitative changes of major chemical constituents in EOs when stored

at 25 °C for 7 days. This information demonstrated that nanoemulsion delivery system has a preventive effect on chemical stability of EOs. Regarding antifungal efficacies of CBO, CLO and HEO nanoemulsions, CO generally had better antifungal activity than that of HEO due to their higher phenol types of chemical compounds such as eugenol. But they all showed the ability to impede *F. graminearum* mycelial growth and spore germination in a dose-dependent mode. Furthermore, the MOA of HEO nanoemulsion against the *F. graminearum* spore germination through increasing the total lipid content in cell, reducing chitin content and damaging permeability of cell membrane. Concerning mycotoxin inhibitory efficacies, all EOs showed the similar mycotoxin inhibitory effects using rice culture. For example, DON, 3ADON and 15ADON produced by the *F. graminearum* were successfully inhibited through using ~ 800 μg EO per g of rice.

Regarding the application of HEO nanoemulsion during micro-malting process, HEO concentration in nanoemulsion of 2.5 mg/g showed the minimal impact on germination energy (GE) of barley. Therefore, 2.5 mg/g of HEO nanoemulsion was used to treat naturally *Fusarium*-infected barley in the first stage of steeping. Meanwhile, *Fusarium* biomass (*Fusarium* DNA) and DON contents were tested at each micro-malting stage. Results indicated that *Fusarium* DNA and DON contents in HEO nanoemulsion treated samples were lower than those in control malts at each malting stage, which suggested that HEO nanoemulsion was able to effectively impede *Fusarium* growth and DON production during malting process. In addition, *Fusarium* DNA and DON contents in treatment showed a great similarity with control sample during micro-malting process. For example, *Fusarium* DNA and DON contents dropped off obviously after steeping, rose after germination, and reduced in the final malts. To better understand the development of fungal hyphae within barley grains during malting process, CLSM and SEM

were used to localize fungal hyphae in the original barley kernels and the finished malt kernels. Generally, fungal hyphae were existed predominant in furrow crease, distal end and the surface of husk, followed by between husk and testa layers of barley kernels. Large amount of hyphae in the furrow, the distal end and the surface could be washed off after steeping process. However, more fungal hyphae were developed between husk and testa layers in the final malts as compared to the initial barley grains due to the comfortable environments during germination steps. Finally, the impact of HEO nanoemulsion treatment on the flavor of final malt was measured, very similar flavor profile of both malts were found. Interesting, HEO nanoemulsion treated malts has less free aldehydes in final malts, which indicated that HEO nanoemulsion could prevent the formation of free aldehydes. In consequence, Brewing HEO nanoemulsion treated malts could have a potential to prevent beer staling.

This study provided fundamental information about using CBO, CLO and HEO nanoemulsions as naturally antimycotic and mycotoxin-inhibition agents in food industry.

FUTURE WORKS

In our project, three kinds of physically stable EO nanoemulsions were fabricated and these EO nanoemulsions could successfully inhibited *Fusarium* growth and mycotoxin production both *in vitro* and during malting process. In addition, mechanisms of the EO nanoemulsion against *Fusarium* growth were revealed, including altering fungal total lipid and chitin content and impairing cell membrane integrity. Based on our experiment observations, the nonvolatile fractionation of EOs might have better antifungal and mycotoxin inhibitory efficacy rather than the volatile ones. As such, future research will focus on the following aspects:

(1) Discovering the role of EOs, its volatile and nonvolatile fractionations, and the individual major chemical constituents based nanoemulsions on mycotoxin inhibitory activity at the molecular level.

(2) Identifying potential synergistic effect of EOs with other compounds to reduce the EOs usage to an even lower level.

(3) Elucidating MOA of antimycotoxigenic EO nanoemulsion on genetic level.

(4) Demonstrating practical utility of EO nanoemulsions for mycotoxin mitigation during the malting processing using different brewing grains (e.g., barley, wheat and rye).

(5) Identifying the development of fungal hypha growth in grains kernel during malting process.