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Thymol-Based Sub-micron Emulsions Exhibit Antifungal Activity Against *Fusarium graminearum* and Inhibit *Fusarium* head blight (FHB) in Wheat.

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Short Running Head: Thymol-Derived Nanoemulsion Inhibit Fusarium Head Blight

Abstract

Aims: *Fusarium graminearum* is a very destructive fungal pathogen that leads to Fusarium head blight (FHB) in wheat, a disease which costs growers millions of dollars annually both in crop losses and in remediation efforts. Current countermeasures include the deployment of wheat varieties with some resistance to FHB in conjunction with timed fungicide treatments. In this paper we introduce a fungicide based on thymol, a naturally occurring plant phenolic derived from essential oils. To overcome the hydrophobicity of thymol, the thymol active was incorporated into a low-surfactant sub-micron emulsion with and without a carrier oil.

Methods and Results: The minimum fungicidal concentration (MFC) of *Fusarium graminearum* was found to be both 0.02% for thymol emulsions with and without an oil component. Time-to-kill experiments showed that thymol emulsions were able to inactivate *F. graminearum* in as little as 10 seconds at concentrations above 0.06%. Spraying the thymol emulsions (~0.1% range) on the wheat variety Bobwhite demonstrated significant reductions in FHB infection rate (number of infected spikelets). However, with 0.5% thymol, the wheat heads exhibited premature senescence. Transmission and scanning electron micrographs suggests that the mechanism of antifungal action is membrane

mediated, as conidia exposed to thymol showed complete organelle disorganization and evidence of lipid emulsification.

Conclusion: The collective experimental data suggest thymol emulsions may be an effective naturally-derived alternative to the current thymol treatments, and chemical fungicides in ameliorating FHB.

Significance and Impact of Study: This is the first thymol-derived nanoemulsion particles resuspended into water and not DMSO, exhibiting the same antibacterial/antifungal activity as previously described thymol and thyme oil treatments. This drastically reduces the environmental footprint thymol will leave if utilized as a fungicide treatment on field crops.

Key Words: Biocide, Fusarium graminearum, Thymol, Wheat, Antifungal

Introduction

Worldwide, cereal crops are grown predominantly for the production of food and feed for livestock. Of the cereal crops, wheat is grown over more land area than any other field crop in the world (McMullen et al. 2012). In 2013, the global production of wheat was 26 million bushels, of which the United States accounted for 8 percent (~2 million bushels) (USDA-ERS 2014). The average cost of wheat per bushel has decreased to a range of $5.55-$6.25 from $6.87 average in 2013 (USDA-ERS 2014). With the reduction of wheat acreage in the United States, reduced prices per bushel, and a maintained demand for wheat products for food production; minimizing wheat losses to pathogens is a major priority for growers and researchers.

Fusarium graminearum causes a highly destructive plant disease to many economically important cereal crops called *Fusarium* head blight (FHB). Since 1990, this sporadically damaging fungal disease has caused over 3 billion dollars in crop losses in wheat and barley in the United States (McMullen et al. 2012). Visual symptoms of the disease include premature bleaching of spikelets, with severe symptoms

that include an entirely bleached spike (Kazan et al. 2012). All bleaching appears off-white or slightly darker in color and can extend from the base of the spike along the stem (Kazan et al. 2012). In wheat, *F. graminearum* infects individual spikelets initially, then spreads through the rachis into other uninfected spikelets. FHB infections prevent development of grain, and produce trichothecene mycotoxins that are harmful to humans and livestock (Yang et al. 2013). Specifically, toxins nivalenol, T-2 toxin, zearalenone, and deoxynivalenol (DON), are the most studied of the toxins, with DON being the most abundant and more economically important to cereal crops (Wegulo 2012). DON is a trichothecene mycotoxin produced by *F. graminearum* and *Fusarium culmorum (Cirlini et al. 2014).* DON present in livestock feed affects poultry, cattle, sheep, with hogs exhibiting the strongest sensitivity, which manifests itself in the animals having poor weight gain (Moretti et al. 2014; Karlovsky 2011; Smith et al. 2004; McMullen et al. 2012).

Wheat and barley with improved genetic resistance to FHB have been developed, but these provide only partial resistance. Therefore, the most effective strategy for FHB control is the growth of resistant varieties together with the use of fungicides. The success or failure of the fungicide is dependent upon overall disease pressure, time of fungicide application, and plant coverage by the fungicide application (McMullen et al. 2012; Mesterhazy et al. 2011). Triazole fungicides are often utilized to control *F. graminearum* infections on wheat seeds and/or wheat spikes (Paul et al. 2008). Triazoles and other demethylation inhibitor (DMI) fungicides are applied at large volumes (Mesterhazy et al. 2011; Yin et al. 2009; Schisler et al. 2002; Martins et al. 2015; Zabka et al. 2014; Negri et al. 2014; Nardoni et al. 2014; Gemeda et al. 2014; Bassole and Juliani 2012; Panjehkeh and Jahani Hossein-Abadi 2011; Reichling et al. 2009; Peighami-Ashnaei et al. 2009), and with their high environmental stability the development of resistant fungi and bacterium is increased (Becher et al. 2010). Therefore, there is a great need for the identification of alternative agents for controlling FHB.

In this work, we propose the use of thymol-based emulsions as antifungal agents for inhibiting FHB. Thymol and similar phenolics found in naturally occurring essential oils possess strong antifungal activity (Martins et al. 2015; Zabka et al. 2014; Negri et al. 2014; Nardoni et al. 2014; Gemeda et al. 2014; Bassole and Juliani 2012; Panjehkeh and Jahani Hossein-Abadi 2011; Reichling et al. 2009; Peighami-Ashnaei et al. 2009); (Zabka and Pavela 2013; Singh et al. 2004; Juglal et al. 2002; Lopez-Reyes et al. 2013; Shin et al. 2014). Thymol has been shown to disrupt fungal ergosterol biosynthesis and cell membrane integrity (Ahmad et al. 2011a). In addition to antifungal activity, thymol dissipates from the soil within 5 days, thus diminishing the likelihood of fungal resistance (Hu and Coats 2008). The rapid environmental degradation of thymol makes it a promising candidate for a natural fungicide. However, thymol possesses poor water solubility and is often dissolved with organic solvents such as dimethyl sulfoxide. Alternative delivery methods include polymeric encapsulation or emulsification, but common emulsifying agents can interact negatively with thymol and inhibit antimicrobial activity (Ziani et al. 2011). Here we offer a unique vehicle using a reduced surfactant self-assembly microemulsion system. These thymol emulsions are kinetically stable, with additional formulations incorporating an oil component to reduce volatility. Our goal was to assess the antifungal properties of these compositions both *in vitro* and in greenhouse studies pertaining to *F. graminearum* and FHB. The experimental results obtained may have implications for thymol emulsions as a preventative or treatment of FHB.

Material and Methods

Fungal strains, media, and culture

Fusarium graminearum strain GZT501 (Skadsen and Hohn 2004) was inoculated onto potato dextrose agar (½ PDA; 15g Potato Dextrose and7.5g Agar both from BD Difco NJ, USA, for 1L final vol.) plates. Once *F. graminearum* reached maximum growth on the plate (7 days), plugs (at least 5mm in diameter)

of fungal cells were removed from ½ PDA plate and inoculated into 250 mL of carboxymethyl cellulose medium (CMC) in an 500 mL Erlenmeyer flask. After 5 days of constant shaking (250 rpms) at 28 degrees in an orbital shaker, the culture was filtered into a sterile 50ml conical tube using Kim wipes (Kimtech; Wilmington NC). Conidia were pelleted at 4000 rpms for 5 minutes. Supernatant was removed and the pellet of conidia were resuspended in 25ml of filter-sterilized water. The concentration of conidia was determined with a hemocytometer and set to concentrations need for individual experiments as described later. Stocks were stored at 4 degrees until ready to use. No conidia used in any experiment were stored for over one month at 4 degrees.

Wheat Cultivar and plant inoculations

The wheat (*Triticum aestivum*) cultivars tested were Bobwhite and USU-Apogee (Levinskikh et al. 2000; Bugbee et al. 1997). Bobwhite and Apogee are both susceptible to *Fusarium* head blight (FHB). All plants were grown in the greenhouse until anthesis (Feekes 10.5)(Feekes 1941; Large 1954). Anthesis for Bobwhite was nearly 6 weeks and 3.5 weeks for Apogee. Greenhouse conditions were 24 ºC with photoperiod 14 hours of light and 10 hours of dark. At this time, all whole plant experiments were conducted as described in the whole plant feeding protocol below. No insecticides or fungicides were treated on the wheat plants at any time prior to or after experiments.

FHB Index Calculation

FHB index = (total number of infected florets * Disease severity)/100.

Nanoemulsion Preparation and Characterization:

Thymol and thymol-oil emulsions were fabricated using a unique self-assembly emulsification process. This procedure first involved creating a 6.3% w/v thymol (Sigma Aldrich) solution in ethanol. For thymol + oil emulsions, the oil component (A&M Gourmet Foods, Inc., Toronto, Canada) was also added into the thymol + ethanol solution at the designated ratios (Table 1). Next, a 0.63% surfactant solution (Tween 20 or sodium lauryl sulfate, SLS) was prepared in distilled water. The thymol and surfactant solutions were then mixed together in equal parts. This mixture was subsequently diluted in water to give the prescribed emulsion concentrations used in the study. Emulsions formed spontaneous (no additional stirring involved) upon water dilution. For the 0.5% thymol and thymol + oil solutions, a 25% w/v thymol ethanol solution and a 2.5% surfactant solution was used as the starting stock. These two solutions were subsequently mixed together at 1:1 and diluted in water. For all diluted emulsion solutions, the ethanol content was less than 2%. Carrier solutions were made in the same fashion and proportions, except with the omission of thymol. Emulsions were then characterized with a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) and analyzed with supplied Zetasizer v7.1 software to obtain emulsion diameter and zeta potential. Emulsion diameter and zeta potential calculations were determined for thymol using distilled water as dispersant. All samples were measured three times. For diameter measurements, each measurement consisted of 15, 10s duration scans. Zeta potential measurements consisted of 30, 10s long runs. Size and zeta potential distribution by intensity, and polydispersity index were determined by Malvern Zetasizer software (Table 1).

Time-to- Kill and Determination of Minimum Fungicidal Concentration (MFC) – Thymol Only Emulsions

Stocks of PDB (Potato Dextrose Broth – BD Difco) (50mL) were amended with different concentrations of thymol emulsions. The percentages of thymol emulsion used were 0.2%, 0.1%, 0.06%, 0.02%, 0.01%, 0.005%, 0.0025%, 0.001%, and 0.0001% (percentages measured as w/v of thymol content). Each concentration was made with either SLS or Tween 20 surfactant, and the appropriate carriers were also produced in PDB. Five milliliters were removed from each stock into a sterile 15 mL tissue culture tube. For all assays using PDB, 1 x 10^4 conidia were inoculated into each vial followed by 10 seconds of vortexing. The conidia remained in the amended PDB for a specified time. The time points tested in the time-to-kill experiment were 10 seconds, 1 min, 5 min, 10 min, 30 min, 24 hours, and 48 hours. After the allotted time, 100 uL of the 5 mL culture was removed and added to 900 µL of sterilized water. The 1/10 dilution of culture was vortexed for 5 seconds, then 100 µL were plated onto 3 different ½ PDA plates. The plates were incubated 28 degrees in complete darkness for 3 days before the colonies were counted. Each time point was replicated twice. In addition to the enumeration experiments, the amended PDB were also incubated at 28 degree shaker at 250 rpms and photos were taken after 3 days. Each experiment time point was replicated three times.

Time-to-Kill and Determination of Minimal Fungicidal Concentration (MFC) – Thymol+Oil Emulsion

Stocks of PDB (Potato Dextrose Broth – BD Difco) (50mL) were amended with different concentrations of thymol+oil emulsions. The percentages of thymol emulsion used were 0.1%, 0.06%, 0.02%, and 0.001%, For each concentration of active thymol, sunflower oil was added at a 2:1or 5:1 ratio of thymol:oil. Percentages of oil used were 0.05%, 0.03%, 0.01%, and 0.0005%, respectively for the

2:1 ratio thymol+oil emulsions. The percentage of oil used were 0.02%, 0.012%, 0.004%, 0.0005%, and .0002%, respectively for the 5:1 ratio emulsions. Further, four concentrations of the surfactants Tween 20 or SLS (0.01%, 0.006%, 0.002%, and 0.0001%) were used. Each concentration of thymol+oil emulsion was made with each surfactant. Five milliliters were removed from each stock into a sterile 15 mL tissue culture tube. For all assays using PDB, 1 x 10^4 conidia were inoculated into each vial followed by 10 seconds of vortexing. The conidia remained in the amended PDB for a specified time. The time points for the time-to-kill experiments were 1 minute and 48 hours. After the allotted time, 100 µL of the 5 mL culture was removed and added to 900 µL of sterilized water. The 1/10 dilution of culture was vortexed for 5 seconds, then 100 µL were plated onto 3 different ½ PDA plates. The plates were incubated 28 degrees for 3 days before the colonies were counted.

SEM and TEM Imaging:

F. graminearum conidia were exposed to 0.1% thymol or 0.1% thymol oil emulsion made with SLS surfactant for 1 hour and then prepared using standard methods for electron microscopy imaging. Control conidia were suspended in water. For scanning electron microscopy (SEM), cells were first fixed with 2% paraformaldehyde, 20.5% glutaraldehyde in 0.1 M cacodylate and post fixed with 2% OsO4 in 0.1 M cacodylate by the conventional microwave enhanced method (Hernandez and Guillen 2000; Richards and Kaab 1996). Samples were then filtered through a 0.2 µm nucleopore membrane and further prepared using critical point drying. Specimens were mounted onto stub and sputter-coated with platinum. Images were taken with a FEI NOVA nanoSEM (FEI Company, Hillsboro Oregon) field emission scanning electron microscope using ET (Everhart-Thornley) detector or the high-resolution thorough-the lens (TLD) detector at 5 kV accelerating voltage. For transmission electron microscopy (TEM) imaging, *F. graminearum* was fixed described as above with the microwave enhanced method.

Cells were dispersed in 10.5% agarose, spun down into a pellet and serially dehydrated with ethanol and propylene oxide. Cells were then embedded with SPURR resin and sectioned for imaging on FEI/Philips CM-100 transmission electron microscope. Sample grids with sections were post-stained with 2% UA in 70% MEOH for 5 min and then 3 min in lead citrate. An acceleration voltage of 100 kV was used.

Thymol Emulsion Whole Plant Spraying Experiments

For Apogee and/or Bobwhite, plants at Feekes stage 10.5, were used for experiments. For spraying experiments, a concentration of 5 x 10^3 conidia/mL, were sprayed onto the heads of wheat. For every 15 plants, 50 mL of conidia suspension was sprayed onto the heads until treatment was finished. After spraying; wheat heads were noticeably wet with some dripping observed. The plants were allowed to sit at room temperature for 35 minutes. This was enough time for the heads to dry prior to subsequent spray. After 35 minutes, the heads were sprayed with specific treatments, allowed to sit at RT for another 35 minutes or until heads were dry. Once dry, the heads were bagged, and remained bagged for 84 hours in greenhouse. After 84 hours, the bags were removed, and on day 10, the *F. graminearum* infection in the spike was scored. Scoring consisted of percentage of florets and rachis infected.

Thymol+Oil Emulsion Whole Plant Spraying Experiments

To determine if the addition of sunflower oil improves adhesion and antifungal effect of the thymol emulsion to plant surfaces, whole plant spraying experiments were conducted with thymol+oil prior to fungal inoculation. Experiments were performed similarly as described in thymol emulsion whole plant protocol above except thymol+oil emulsions were applied to wheat heads first, allowed to dry for 35 minutes, at which *F. graminearum* spraying ensued. Each spray step was allowed to sit at room

temperature for 35 minutes or until heads were dry. Allowing heads to dry prevented conidia from running off plants before the heads were bagged, and remained bagged for 84 hours in greenhouse. After 84 hours, the bags were removed, and on day 10, the *F. graminearum* infection in the spike was scored. Scoring consisted of percentage of florets and rachis infected. These experiments were repeated with thymol-free oil carriers to assess what contribution, if any, that the oil carrier may have on antifungal activity.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Results

Nanoemulsion Preparation and Characterization:

Thymol and thymol+oil emulsions were fabricated in the concentrations as outlined in Table 1. Initially, different surfactants were screened to determine best formulations. Sodium lauryl sulfate (SLS) and polysorbate 20 (Tween 20) formulations were found to preserve the antimicrobial aspects of thymol and therefore further explored in these experiments. Emulsion size and zeta potential measurements showed all SLS based emulsions to be negatively charged, due to the anionic surfactant. Interestingly, Tween 20 formulations were also negatively charged, even though Tween is non-ionic. Based on electrostatics, most of the emulsions were relatively stable (i.e. <-30mV) from charge repulsion. All SLS based thymol emulsions were visibly clear, with the exception of the 0.5% solution, which was a milky white color. Tween 20 formulations were slightly cloudy-white, with higher concentrations being more

opaque. In contrast, the thymol+oil emulsions were translucent. This macroscale appearance was reflected in the emulsion droplet size measurements. The lower concentrations of thymol had more consistent and finer droplet diameters approaching the nanoscale. Conversely, the thymol+oil and higher concentrates tended to be microemulsions. The polydispersity index of the more concentrated emulsions also suggest increased heterogeneity in the size distribution (Table 1). None of the emulsions exhibited phase separation during normal storage although some settling occurred with the thymol+oil emulsions.

Time-to-Kill and Minimum Fungicidal Concentration (MFC) of Thymol Emulsions

Time-to-kill assays were performed by amending potato dextrose broth (PDB) with different concentrations of thymol from 0.2% to 0.0001%. Concentrations of 0.2% to 0.06% thymol were lethal for conidia after 1 minute incubation at 28º C temperature (Figure 1). Similar results were observed at longer incubation times of 5 min, 10 min, 30 min, 6 hr, and 12 hr post-conidia inoculation at 28º C (data not shown). After 24 hours, 0.02% thymol became lethal for conidia, and viability of conidia dropped significantly at concentrations 0.01% and 0.005% thymol. After 48 hours, 0.2% - 0.02% thymol showed no growth but some viable *F. graminearum* was seen at concentrations of 0.01% and 0.005% (Figure 1), indicating these concentrations to be inhibitory but not completely fungicidal. Therefore, 0.02% thymol was determined to be the MFC for 48hr exposure time. Ten second exposure time was also tested using concentrations of thymol emulsions at higher concentrations of 0.1% and 0.06 % (Figure 3A). Thymol concentration 0.1% with both surfactants were lethal after 10 seconds and 0.06% was strongly inhibitory (Figure 3A). MFCs were also performed to assess sublethal dosages of thymol at 72hrs. After 72 hours, *F. graminearum* growth in cultures of 0.0025% thymol were similar to PDB control suggesting no inhibitory effects of thymol (data not shown). This data suggests that a concentration of thymol between 0.005%

and 0.0025% is not effective against *F. graminearum* at initial concentration of 10000 conidia per 5mL. A similar result was observed for a group of other phytopathogenic fungi. Cultures of *Mycosphaerella fijiensis*, *Mycosphaerelle citrullina*, and *Sclerophthora macrospora* were found to have the same MFCs as *F. graminearum* (data not shown)

Time-to-Kill and Minimal Fungicidal Concentration (MFC) of Thymol + Oil Emulsions

Time kill assays were performed to test the antifungal activity of thymol + sunflower oil emulsions. Eight concentrations of sunflower oil were used, ranging from 0.05% (highest conc.) to 0.0002% (lowest conc.) for the studies. All concentrations were formulated with either Tween 20 or SLS as the surfactant. Each concentration of thymol was mixed with two different concentrations of sunflower oil (Table 1). Results show that after 1 minute of incubation, thymol+oil produced lethal antifungal activity at 0.1% and 0.06% thymol in both SLS and Tween 20 at all oil concentrations (Figure 2). Concentrations 0.02% and 0.001% thymol were not lethal, with viability data similar to their respective carrier solutions (Figure 2) at 1 minute exposure time. Again, the carrier solutions had little effect on conidia viability. After 48 hour incubation, thymol +oil emulsions were lethal at concentrations of 0.1% 0.06%, and 0.02%, regardless of surfactant used (Figure 2 & 3). The minimum fungicidal concentration was determined to be 0.02% with both Tween 20 and SLS formulations. However, 0.001% thymol +oil emulsions produced growth on PDA plates, but the number of CFU present were significantly lower than the appropriate controls and PDB buffer (Figure 2). This suggests that 0.001% thymol + oil may be inhibitory to growth but not fungicidal.

Assessing the effect of Thymol emulsions on FHB

Preliminary experiments were conducted using the FHB susceptible wheat varieties Apogee and Bobwhite to determine the effectiveness of a single application of the thymol emulsions either prior to or after inoculation with macroconidia from *F. graminearum* onto the wheat spike. Spraying nanoemulsions prior to infecting with conidia was ineffective in controlling FHB (data not shown). However, spray treatment after inoculation with macroconidia suppressed FHB development (Figure 4). FHB disease symptoms were mitigated in thymol concentrations between 0.02-0.1%, as compared to their respective carrier and water treated plants (Figure 4). The most effective formulation was 0.1% thymol-SLS with only 6 percent of the florets becoming infected by day 10 (Figure 4). However, spraying Bobwhite or Apogee heads with a higher 0.5% thymol induced severe senescence and photobleaching just 3 days after being treated. Also, the stem became brittle and extremely dry. Similar experiments in which Bobwhite and Apogee were sprayed without *F. graminearum* inoculation showed 0.5% thymol to cause severe senescence and photobleaching (Figure 7). This suggests phytotoxicity to be solely associated with higher levels of thymol.

Assessing the effect of Thymol+oil emulsions on FHB

Emulsions containing 0.1% thymol and 0.05% sunflower oil were sprayed on wheat heads prior to inoculation with *F. graminearum* on both Bobwhite and Apogee wheat. Results show reduced FHB symptoms when compared to water and carrier oil sprayed controls (Figure 5A). The FHB index also validated these findings (Figure 5C) but indicated that the sunflower oil carrier had a slight trend towards increasing FHB. Spraying Bobwhite and Apogee plants with 0.1% thymol+oil after inoculating with *F. graminearum* produced strong antifungal result with a reduced percentage of infected florets (Figure 5B) and occurrence of FHB (Figure 5D). Similar to the thymol emulsions, spraying Bobwhite or

Apogee heads with 0.5% thymol+oil induced severe senescence and photobleaching three days after being treated. However, 0.5% thymol emulsions without oil produced more uniform senescence while 0.5% thymol+oil produced a completely white spike with spots of green (Figure 7). Similarly, with both varieties the stem became brittle and dry and began falling over by days 4 and 5. Bobwhite and Apogee were sprayed with thymol-oil in the absence of *F. graminearum* again showed 0.5% thymol+oil to cause severe senescence and photobleaching.

SEM and TEM Imaging of Thymol Treated *F.graminearum*

SEM images of carrier solution (SLS at 0.1% with oil) suspended *F. graminearum* conidia show a generally smooth cell wall surface. The septal ridge can also be observed clearly. Exposure of the conidia to either thymol or thymol+oil emulsions (Figure 6; column A row 2 and 3) made with SLS at 0.1% did not result in any observable differences in the cell wall. TEM sections of the conidia exposed to carrier solution both in longitudinal and transverse sections demonstrate a highly organized interior (Figure 6; Column B and C with row 1).Both single and multi-nucleated cells could be seen in all TEM images. The genetic material (dark stain) and surrounding nucleoplasm can be seen contained within a yolk-like structure (Figure 6). In contrast, *F. graminearum* conidia treated with either 0.1% thymol or thymol+oil showed complete interior disorganization. No distinct internal organelles could be seen and the nuclear material appeared to be diffuse. The cell wall and septum remained intact. However, the cell membrane appeared to be less prominent. In some micrographs, emulsion-like droplets could be seen in the interior of the cell, possibly due to emulsification of the internal membrane bound organelles.

Discussion

In the present study, we developed several low surfactant emulsion compositions using a thymol core stabilized with either an anionic (SLS) or a non-ionic surfactant (Tween 20). Native thymol has been shown to have antimicrobial properties, but its hydrophobicity limits its use in many food and agricultural applications. Further, incorporation of thymol into colloidal, lipid or other encapsulation systems often degrades its antimicrobial efficacy (Gaysinsky et al. 2007; Ziani et al. 2011; Chang et al. 2012). We hypothesized that by reducing the level of surfactants, we could produce highly stable emulsions that would retain the antimicrobial properties of thymol. Such emulsions would be compatible with traditional aqueous delivery. Additionally, we prepared several emulsion variants that incorporated small amounts of sunflower oil, with the assumption that the oil phase may improve droplet adhesion to plant surfaces (i.e. extend contact times). Our goal was to characterize the antifungal activity of these new emulsion formulations against an extremely destructive wheat pathogen, *F. graminearum*.

Experimental results show that both SLS and Tween 20 were capable of forming stable self-assembled emulsions at both the micro and nanoscale (Table 1). The negative charge of the SLS emulsions was expected, but interestingly, Tween 20 also produced anionic droplets. This imparted charge may be attributed to free fatty acids in the sunflower oil or interfacial adsorption of anionic species such as hydroxyl ions from water (McClements 2005). Droplet diameters approached the nanoscale (~200nm) to several micrometers for formulations incorporating oil. At higher thymol concentrations, however, the droplet size increased substantially, reiterating that the optimum conditions for small, stable emulsions occur over a specific concentration window of the surfactant and liquid phases (JC et al. 2002). Smaller emulsion sizes were also found to be more monodisperse. All emulsions were kinetically stable, with no phase separation observed during or after preparation. However, droplets with smaller diameters have

longer shelf-lives as nanoemulsions are more resistant to coalescence, flocculation, Ostwald ripening and settling effects (Velikov and Pelan 2008). These physiochemical phenomena all facilitate emulsion break down and eventually lead to phase separation.

In vitro assessment of antifungal activity showed the emulsions were of comparable efficacy to pure thymol. For example, the MFC_{100} values for thymol against *Fusarium oxysporum* and *Fusarium verticilliodes* were determined to be 115µg/mL and 108µg/mL, respectively (Zabka and Pavela 2013). Other fungal pathogens in the genus *Aspgergillus*, *Alternaria*, *Botrytis*, *Cladosporium*, *Penicillium* and *Rhizopus* were susceptible to thymol at concentrations ranging from 100ug/mL to 500ug/mL respectively at 48 hours of incubation time (Abbaszadeh et al. 2014). In our studies, the all thymol emulsions showed a MFC_{100} of 0.02% (200µg/mL) after 48 hour incubation against *F. graminearum (Figure 1)*. Further time-to-kill experiments demonstrated that all tested emulsions could inactivate *F. graminearum* completely within one minute or less at thymol concentrations of 0.06% or above (Figure 1, and 3A). Adding oil to the thymol slightly decreased the antifungal efficacy. For instance, a 0.06% active thymol concentration was required to eliminate *Fusarium* germination in a very challenging 10 second exposure window. However, for the thymol+oil formulation, a 0.10% was required to induce the same effect. These results are consistent with prior findings that lipids may potentially interfere with the antimicrobial properties of thymol or other essential oil constituents (Gaysinsky et al. 2007; Ziani et al. 2011).

We also tested three other economically important fungal pathogens *Mycoosphaerella fijiensis* (MF), *Mycosphaerella citrullina* (MC), and *Sclerophtora macrospora* (SM). At 0.1%, 0.06%, and 0.02% incubation with thymol for 48 hours, none of the three pathogens were able to germinate, except in the sublethal dose of 0.001% thymol (data not shown). The observed results closely parallel the MFCs determined for *F. graminearum* MFCs.

Based on the *in vitro* time-to-kill and MFC screening studies, we sprayed wheat heads with the thymol microemulsions to simulate field use in ameliorating FHB. The initial experiment used a single application of thymol at 0.02%, 0.06%, and 0.1% concentration sprayed 30 minutes after *F. graminearum* inoculation. A dose-dependent response was found in which both 0.06% and 0.1% thymol emulsions (SLS or Tween20) effectively suppressed FHB compared to control and carrier solutions. The FHB index also confirmed the percentage of infection results. Spraying the wheat varieties in the 0.02% - 0.1% thymol range did not have any visual physiological side effects, but follow-up experiments using a higher 0.5% active thymol concentration proved to be cytotoxic (Figure 7). At the higher concentrations both Bobwhite and Apogee heads showed senescence and photobleaching a few days after thymol application (data not shown). The thymol+oil formulations appeared to be slightly less cytotoxic based on head bleaching although it was evident that the wheat was irreparably damaged (Figure 7). Therefore, the phytotoxic dose of thymol appears to be greater than 0.1%.

While the thymol emulsions showed inhibition of FHB when applied post-inoculation, we also conducted trials in which the emulsions were sprayed 30 minutes prior to *F. graminearum* infection. Such experiments were intended to assess the utility of thymol as a possible prophylaxis. Wheat sprayed with thymol emulsions did not reduce FHB versus the controls (data not shown). However, thymol (0.1%) with an oil component sprayed before *Fusarium* inoculation showed a trend towards reducing the percentage of infected florets (less than 20% infection) when compared to carrier oil and water treatments (20-40% infection, Figure 5). This suggests that the oil phase decreases thymol volatility and may prolong its contact with the conidia. On the other hand, we observed a higher incidence of FHB when applying just the oil carrier, possibly due to enhanced conidia adhesion caused by the oil residues. The lower FHB values with thymol+oil imply these emulsions may provide some marginal level of infection protection. However, it is apparent that application of thymol after *F. graminearum* inoculation proved to be much more effective in mitigating FHB. In such instances, the

thymol+oil treatment reduced the number of infected spikes to less than 5% which is an appreciable difference compared to either water or carrier solutions.

Thymol has been shown to disrupt the ergosterol biosynthesis and influence membrane integrity of *Candida* fungus and its isolates (Ahmad et al. 2011a). At thymol concentrations from 400-600µg/mL (.04% - 0.06%), thymol was lethal at 12 and 24 hours for several *Candida* isolates (Ahmad et al. 2011b). Moreover, changes to *Salmonella* membrane integrity has been reported at a thymol content of 750ug/mL, (Chauhan and Kang 2014) whereas leakage of intracellular material was found in fungal and bacterial systems with thymol (Ahmad et al. 2011a; Zabka and Pavela 2013; Chauhan and Kang 2014; Connell et al. 2013). Images obtained from scanning and transmission electron microscopy (Figure 6) appear to corroborate prior results of a membrane mediated mechanism of action. Exterior surface imaging of the conidia via SEM did not show noticeable differences. That is, the control (oil free carrier) and 0.1% thymol treated conidia possessed a smooth continuous cell wall. Internally, both sagittal and coronal TEM sections revealed the conidia to be multinucleated with highly organized structures. Cellular organelles and the cell membrane could be clearly visualized. The DNA was localized within the nucleus and denoted as dark stained spots. In contrast, conidia exposed to thymol or thymol+oil revealed complete disorganization of the organelles. There was evidence of cell and nuclear membrane deterioration along with more a diffusive arrangement of genetic material within the cytosol. A significant number of cells were also lighter stained, suggesting that intracellular material may have leaked from the cells. This microscopy data, coupled with the rapid inactivation time, lend support to a membrane associated mechanism of antifungal activity.

In this paper we show that sub-micron emulsions derived from thymol exhibit strong dose and time-dependent antifungal activity against *Fusarium graminearum*. Cells were inactivated via membrane disruption and subsequent disorganization of organelles. Spraying the emulsions on wheat heads as a

preventative did not inhibit the occurrence of FHB. In contrast, the infection rates were substantially lower when the emulsions were applied post-inoculation. Both thymol and thymol+oil emulsions were effective in controlling FHB and did not show cytotoxic effects at applied concentrations of 0.1% or lower. Nonetheless, this research is preliminary and subsequent studies should include field trials testing different spray regimens. In addition, the grain quality after thymol emulsion treatment, quantification of DON accumulation, and the fate of thymol in wheat need to be characterized. The phytotoxicity of thymol (>0.1%) may be related to its effect on lipid membranes, which could also damage plant cells. However, at these higher concentrations, thymol could hold potential as a post-harvest treatment in fields to reduce *Fusarium* titers. In conclusion, the proposed thymol emulsions possess rapid anti-fungal activity that can be effective against FHB and offer a promising candidate for a naturally-derived fungicide with a low environmental footprint.

Conflict of Interest

The authors declare no conflict of interest related with this work.

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Figure Legends

Figure 1 Legend. Fusarium viability as a function of thymol emulsion concentration and formulation.

(A) 1 minute treatment time and (B) 48 hr treatment time. Grey line represents Fusarium growth in carrier solutions (no thymol) while the black line denotes CFU counts of Fusarium exposed to thymol. Carrier solution (■) and Thymol treatment (♦).

Figure 2 Legend. Fusarium viability as a function of thymol+oil emulsion concentration and formulation.

(A) 1 minute treatment time and (B) 48 hr treatment time. Grey line represents Fusarium growth in carrier solutions (no thymol) while the black line denotes CFU counts of Fusarium exposed to thymol+oil. Carrier solution (■) and Thymol treatment (♦).

Figure 3 Legend. Fusarium viability after 10 seconds exposure time at various fungicidal concentrations of thymol.

(A) Thymol emulsion treatments. (B) Thymol+oil emulsion treatments. Grey line represents CFU of Fusarium growth in control cultures (minus thymol). Grey line represents Fusarium growth in carrier solutions (no thymol) while the black line denotes CFU counts of Fusarium exposed to thymol. Carrier solution (■) and Thymol treatment (♦)

Figure 4 Legend. Percentage of Infected Florets of Bobwhite Treated with Thymol After Fusarium Infection.

(A) Percentage of infected florets ten days after Fusarium infection. The black bars represent percentage of infection of Bobwhite after thymol treatment, while grey bars represent results with the carrier solutions (no thymol). (B) Calculated FHB index for each treatment, with FHB index = (total number of infected florets * Disease severity)/100. All white boxed FHB index values represent thymol treatments, with grey boxed values denoting the respective carrier solutions. All bar graphs have standard error (SE). One-way ANOVA was performed to analyze statistical differences between treatments followed by post-hoc confidence intervals at 95%. Significant differences observed between bars with A and B, excluding AB at P value <0.05* and P value <0.01**. Carrier solution (■) and Thymol treatment (♦)

Figure 5 Legend. Percentage of infected Bobwhite florets sprayed with thymol+oil solution.

(A) Bobwhite plants pretreated with thymol+oil prior to Fusarium inoculation, assessed 10 days after initial infection. (B) Bob White plants treated with thymol+oil after Fusarium inoculation. Plants were assessed 10 days after initial infection. (C) and (D) denote the FHB index calculated for plants either pretreated or post-treated with thymol+oil, respectively. FHB index = (total number of infected florets * Disease severity)/100. (E) Photograph Bobwhite plants from the thymol+oil post-treated experiments. These plants were initially sprayed with Fusarium, followed by thymol+oil treatment. Far left plant was treated with oil carrier, center plant was treated with 0.1%thymol-0.01%SLS-0.02%Oil, and far right plant was treated with water. All bar graphs have standard error (SE). One-way ANOVA was performed to analyze statistical differences between treatments followed by post-hoc confidence intervals at 95%. Significant differences observed between bars with A and B at P value <0.01.

Figure 6 Legend. Effects of thymol on Fusarium conidia examined by SEM/TEM.

Column A: SEM of Fusarium reveal a relatively smooth surface topology with visible septal ridges in all cases. Column B: Longitudinal TEM sections of control (water suspension) Fusarium show distinct organelles while both 0.1% thymol and 0.1% thymol+oil treatment caused complete disorganization of intracellular structures. Column C: Transverse TEMs of conidia at higher magnifications. Black arrows denote the cell nucleus and contained DNA. White arrows highlight the cell membrane pre and post-treatment. Note that in the thymol treated samples, the cell membrane is only lightly stained. Putative emulsion droplets may also be seen localized in the darker stained interior regions.

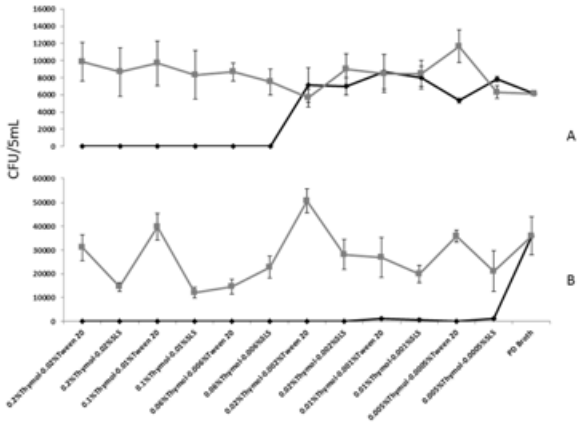
Figure 7 Legend. 7 Effect of different treatments on toxicity to wheat plants.

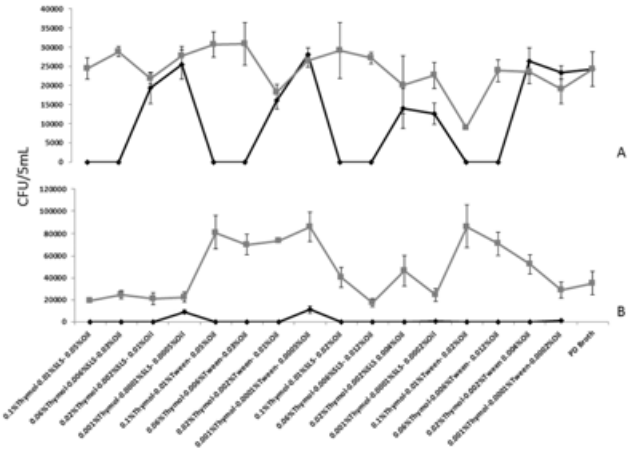
In Figure 7, there are 4 Bob White uninfected plants separately treated with (left to right) oil carrier, 0.5% thymol+oil, oil-free carrier, and 0.5% thymol. Images were taken 4 days after treatment.

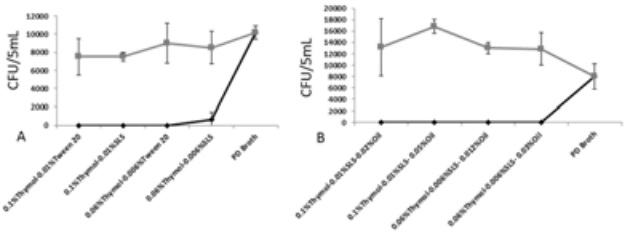
Table 1 Legend: Characteristics of Thymol Emulsions

Based on the percentage of thymol (column 1), composition of SLS or Tween 20 (column 2 and 3), and percentage of sunflower oil (column 4) directly influenced the emulsion diameter (column 5), polydispersity index (column 6), and lastly the zeta potential calculation (column 7). Each concentration of thymol was analyzed in triplicate. Values given are mean ± standard error.

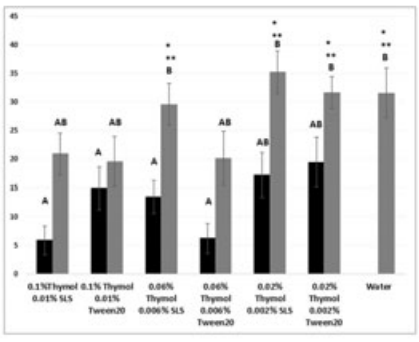
Thymol (%)	SLS (%)	Tween20 (%)	Oil (%)	Emulsion Diameter (nm)	Polydispersity Index	Zeta Potential (mV)
0.020	0.0020	-	-	302.6 ± 57.0	0.202 ± 0.05	-6.76 ± 2.28
0.063	0.0063	-	-	470.1 ± 72.5	0.455 ± 0.09	-29.87 ± 8.63
0.100	0.0100	-	-	430.6 ± 52.0	0.443 ± 0.08	-38.27 ± 2.89
0.200	0.0200	-	-	3313.7 ± 398.3	0.804 ± 0.10	-81.20 ± 1.85
0.500	0.0500	-	-	4641.3 ± 1312.1	0.761 ± 0.12	-75.65 ± 8.33
0.001	0.0001	-	0.0002	693.5 ± 63.7	0.582 ± 0.06	-31.70 ± 5.59
0.001	0.0001	-	0.0005	666.2 ± 69.2	0.734 ± 0.07	-39.97 ± 3.41
0.020	0.0020	-	0.0040	447.2 ± 91.1	0.087 ± 0.01	-47.38 ± 0.31
0.060	0.0060	-	0.0120	1097.1 ± 174.6	0.819 ± 0.06	-44.38 ± 0.65
0.100	0.0100	-	0.0200	1786.1 ± 317.5	0.841 ± 0.06	-76.58 ± 6.34
0.500	0.0500	-	0.1000	2695.8 ± 530.7	0.619 ± 0.12	-94.45 ± 3.95
0.005	-	0.0005	-	479.8 ± 227.3	0.558 ± 0.15	-10.03 ± 1.33
0.010	-	0.0010	-	374.4 ± 55.3	0.543 ± 0.05	-12.41 ± 1.61
0.020	-	0.0020	-	234.4 ± 17.9	0.380 ± 0.03	-26.27 ± 1.05
0.060	-	0.0060	-	1472.4 ± 144.0	0.874 ± 0.05	-52.30 ± 0.53
0.100	-	0.0100	-	4375.3 ± 591.8	0.787 ± 0.14	-58.95 ± 2.47
0.200	-	0.0200	-	4038.7 ± 943.4	0.911 ± 0.06	-64.53 ± 2.80
0.001	-	0.0001	0.0005	192.1 ± 3.7	0.255 ± 0.01	-36.22 ± 1.22
0.001	-	0.0010	0.0002	321.8 ± 40.2	0.472 ± 0.08	-35.72 ± 1.46
0.020	-	0.0020	0.0040	2175.0 ± 776.0	0.724 ± 0.12	-32.43 ± 0.48
0.020	-	0.0020	0.0100	2011.3 ± 622.6	0.925 ± 0.05	-31.85 ± 0.99
0.060	-	0.0060	0.0120	2168.6 ± 530.5	0.870 ± 0.06	-43.96 ± 0.55
0.060	-	0.0060	0.0300	6689.0 ± 1548.7	0.758 ± 0.13	-28.52 ± 12.81
0.100	-	0.0100	0.0200	2234.0 ± 247.3	0.676 ± 0.09	-49.77 ± 0.77
0.100	-	0.0100	0.0500	4757.3 ± 382.4	0.418 ± 0.1	-60.37 ± 1.24





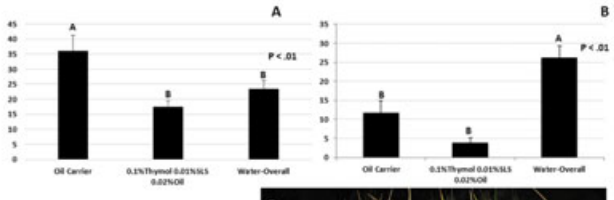


A



B

0.1%Thymol-SLS	0.26
Carrier	0.645
0.1%Thymol-Tween20	0.46
Carrier	0.79
0.06%Thymol-SLS	0.52
Carrier	1.2
0.06%Thymol-Tween20	0.3
Carrier	0.92
0.02%Thymol-SLS	0.46
Carrier	1.23
0.02%Thymol-Tween20	0.95
Carrier	1.77
Water	1.11



C

	FHB Index
Water	1.59
Oil Free Carrier	3.53
0.1%Thymol -Oil	1.22

D

	FHB Index
Water	9.18
Oil Free Carrier	1.29
0.1%Thymol -Oil	.306