Clove and lemongrass oil based non-ionic nanoemulsion for suppressing the growth of plant pathogenic *Fusarium oxysporum* f.sp. *lycopersici*

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**Abstract**

Essential oils play pivotal role in sustainably controlling plant pests and diseases. In this study, a synergistic oil-in-water nanoemulsion containing clove (CO) and lemongrass oil (LGO) was developed and explored its potential as antifungal agents against *Fusarium oxysporum* f.sp. *lycopersici* (FOL). After initial confirmation of synergism between CO-LGO, a nanoemulsion was formulated using non-ionic surfactants through low-energy emulsification method. The nanoemulsion showed Z-average diameter of 76.73 nm having spherical shaped droplet structure as confirmed by TEM imaging. Minimum inhibitory concentration (MIC) of the optimized nanoemulsion was 4000 mg/l against FOL with the logistic kinetic model demonstrating its notably rapid fungicidal effect. The nanoemulsion disrupted the membrane integrity of FOL as consistently evidenced by sodium dodecyl sulfate polyacrylamide gel electrophoresis, scanning electron and atomic force microscopy. During in planta assay, nanoformulation applied as a soil amendment at MIC reduced the severity of tomato *Fusarium* wilt incidence up to 70.6% compared with untreated control. The nanoemulsion applied on seedlings and seeds also significantly controlled the wilt disease in tomato without showing any sign of phytotoxicity.

**Keywords:** Clove oil, Lemongrass oil, Nanoemulsion, *Fusarium oxysporum* f.sp. *lycopersici*, Tomato

**1. Introduction**

Vascular wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* Synder & H.N. Hans (FOL) is a serious soil-borne disease of tomato (*Lycopersicon esculentum* L.) (Sathiyabama and Charles, 2015). Most *F. oxysporum* strains are saprophytic and can persist in soil for many years without a host (Larena et al., 2003). The pathogen attacks susceptible plants by root infection and then clogs the vascular system with mycelium or spores causing vascular and leaf discoloration, often leading to the death of the plant (Huang et al., 2012; McGovern, 2015). The current strategies to control this dreadful disease heavily rely on synthetic fungicides. In spite of synthetic fungicides providing stability to crop yield and market quality, its exponential use have caused toxicity to non-target organisms (Maria et al., 2011), negative effects on the environment and development of resistant pathogen strains (Ren et al., 2017). drawbacks of synthetic fungicides and rising public acclarity about the protected environment have led scientists to seek alternative approach for wilt disease management.

Plant-derived essential oils (EOs) as crop protectants represent an immense progressing bioresource. EOs are the group of defense chemicals extracted from different parts of the aromatic herbs and spices (Basak and Guha, 2017). Mono and sesquiterpenes are the major chemical class responsible for the broad-spectrum antimicrobial activity of EOs such as fungi (Tullio et al., 2007; Koul et al., 2008), viruses (Wang et al., 2010) and bacteria (Nuñez and Aquino, 2012). However, in soil system where pathogen inhabits dispersedly in an aqueous phase, the antifungal property of EOs is substantially compromised owing to sparing water solubility, high volatility, and degradation (Ma et al., 2016; Gao et al., 2017). Oil-in-water nanoemulsion is the fitting solution to these limitations. Nanoemulsions are promising and potent delivery vehicles of lipophilic antimicrobial substances (Li and Lu, 2016). Potential advantages of nanoemulsions are small size (< 100 nm), low polydispersity, slow release of bioactive compounds and comparatively low use of surfactants. In recent years, scientists are seeking to develop nanoemulsions of EOs to be used in agriculture (Ali et al., 2017). However, to the best of our knowledge, no one has explicitly looked at the impact of the antifungal nanoemulsion of synergist EOs on economically important soil-borne pathogens like FOL and about the contribution of such formulation on the vascular wilt incidence in tomato plants. The use of a synergistic combination of
bioactive EOs in a formulation reduces the concentration of individual oil thereby bringing down the cost of the product without nego-
tiating the desired antifungal result.

In the previous work, the authors investigated that clove oil, CO
(Syzygium aromaticum) and lemongrass oil, LGO (Cymbopogon citratus)
showed significant antifungal activity against FOL (Sharma et al.,
2017). The present work envisages fabrication and characterization of a
stable non-ionic nanoemulsion of a combination of CO and LGO (CO-
LGO). The authors also hypothesized that use of synergist oils in a
single nanoemulsion would enhance the antifungal activities against
FOL. In planta bioassays were performed to assess the ability of na-
noemulsion to reduce vascular wilt caused by FOL in tomato. The au-
thors believe that the outcomes of the present study would provide
important inferences for the development and utilization of green
chemicals based nanoemulsions as antifungal delivery systems in agri-
culture.

2. Material and methods

2.1. Chemicals and strains

The selected EOs were procured from Gogia Chemicals, Okhla, New
Delhi (India) and stored at 4 °C till further use. Culture media, di-
methyl sulfoxide (DMSO) and resazurin were purchased from Merck,
India. Surfactants viz., Tween-20 or Polysorbate-20 (hydrophilic-lipo-
philic balance i.e. HLB: 16; cloud point: 76 °C), Castor Oil Ethoxylate-40
(COE-40; HLB: 12.3; cloud point: 70 °C) and anti-freezing agent (pro-
pylene glycol) were supplied by Supreme Surfactant Limited, Sonipat,
Haryana, India. The resazurin indicator solution was prepared by dis-
solving 270 mg tablet in 40 ml sterile distilled water. FOL 1322 strain
was procured from Indian Type Culture Collection (ITCC), Indian
Agricultural Research Institute, PUSA, New Delhi, India.

2.2. Gas chromatographic–mass spectrometry (GC–MS)

The major components of CO and LGO were identified through
GC–MS (Shimadzu QP 2010 Plus) fitted with an FID and Japan capillary
column (0.32 mm i.d., length: 30 m, film thickness 0.25 μm). The in-
jector temperature was maintained at 280 °C while the ion source
temperature was set at 230 °C. 0.2 μl of oil sample was injected into the
column with a split ratio of 80:1. The temperature program comprised
of 60 °C for 2 min, raised to 250 °C for 5 min at 10 °C/min and 280 °C
for 15 min at 10 °C/min. The composition (%) was estimated using peak
normalization method and assuming equal detector response for each
run. The range of mass acquisition was 40–650 m/z. The peaks were
identified by comparing the individual mass spectra with database of
National Institute of Standards and Technology (NIST12 or NIST62) and
Wiley 229 mass spectrometry libraries.

2.3. Determination of MIC of CO and LGO alone and in combination

The MIC of CO and LGO was determined by sterile 96-microwell
plate method (Sharma et al., 2017). Briefly, 100 μl of the essential oil
dilution was added into the well of the first column followed by 100 μl
of sterile PDB in 2–9 columns of a plate. Serial 2-fold dilution was
carried out from column 1–9 and excess of sterile PDB (100 μl) was
discarded from the wells of the 9th column. Each well received 80 μl of
an inoculum suspension and 20 μl of resazurin solution making a final
volume of 200 μl. The growth control wells consisted of 100 μl of sterile
PDB, 80 μl of an inoculum suspension and 20 μl of resazurin solution.
The negative control wells contained 180 μl of the sterile PDB and 20 μl
of resazurin solution. The contents were mixed in each well and in-
cubated at 28 °C for 24 h. The lowest concentration of oil solution that
remained blue suggesting zero growth was taken as the MIC value.

The MICs of oils in 1:1 combination were determined by previously
described micro-dilution assay using the agar dilution checkerboard

method (Rosato et al., 2007). Briefly, serial dilutions of CO and LGO
were mixed so that each well contained a fixed amount of the CO with
increasing concentration of the LGO (MIC/64 to 4X MIC) and vice-versa.
In each case, 80 μl of fresh fungal suspension (~10⁶ spores/ml) and 20 μl of resazurin were mixed in each well and incubated at 28 °C for
24 h. The lowest concentration of oil combination that remained blue
suggesting nil viability was interpreted as the MIC value.

The quantitative effects of EOs are described in terms of FICi
(fractional inhibitory concentration) indices. The calculations were
performed as reported by Didry et al., (1993)

\[
FIC_{CO} = \frac{MIC_{CO \ combination}}{MIC_{CO \ alone}} \\
FIC_{LGO} = \frac{MIC_{LGO \ combination}}{MIC_{LGO \ alone}} \\
FIC = FIC_{CO} + FIC_{LGO},
\]

Where, FICCO and FICLGO are the fractional inhibitory concentra-
tions that inhibited the fungal growth of CO and LGO, respectively. The FICi
obtained from the above equation was interpreted as synergy
(FIC < 0.5), addition (0.5 ≤ FIC ≤ 1), indifference (1 < FIC ≤ 4) or
antagonism (FIC > 4) effect of the oil combination.

2.4. Development of synergistic CO-LGO nanoemulsion

Oil-in-water nanoemulsion was formulated according to the low
energy emulsification method (Pant et al., 2014). Surfactants, anti-
freezing agent, and EOs were pooled together and constituted oily
phase, while aqueous phase was constituted by distilled water. After
both phases reached the same temperature (35 ± 5 °C), the aqueous
phase was gently added through the oil phase under magnetic stirring
(750 rpm) for 30 min and allowed to equilibrate at 25 °C for 24 h fol-
lowed by visual inspection. Final mass was kept constant (25 g) for all
formulations, constituted by 80% (w/w) of distilled water, 5% (w/w) of
CO and LGO (1:1), 5% (w/w) propylene glycol and 10% (w/w) of
surfactants or surfactant mixture (Smix). The composition of nano-
eemulsions is listed in Table 1.

2.5. Characterization of nanoemulsions

All the CO-LGO nanoemulsions (except F1 and F2) were char-
acterized on the basis of mean droplet size, polydispersity index, and
viscosity. Measurements of Z-average diameter (mean droplet size) and
a polydispersity index of nanoemulsions prepared was done with a
Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) nano-particle
analyzer which works on dynamic light scattering principle. The visc-
sity of the formulations was analyzed by Rheeometer (Anton Paar,
MCR52). The morphology of the droplets of the selected nanoemulsion
(F5) was observed by transmission electron microscopy (TEM) using
Philips electron microscope (CM-10) at 100 kV and direct magnification
of 50,000 ×. The stability of CO-LGO nanoemulsions was determined by
centrifuging the nanoemulsions at 3500 rpm for 30 min and stored at
three different temperatures: room (25 °C), high (40 °C) and low (5 °C)

<table>
<thead>
<tr>
<th>Code</th>
<th>Formulation composition (% w/w)</th>
<th>CO + LGO (1:1)</th>
<th>Propylene glycol</th>
<th>water</th>
<th>COE-40 (S1)</th>
<th>Tween-20 (S2)</th>
<th>Smax ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5</td>
<td>5</td>
<td>80</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F2</td>
<td>5</td>
<td>5</td>
<td>80</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F3</td>
<td>5</td>
<td>5</td>
<td>80</td>
<td>5</td>
<td>5</td>
<td>1:1</td>
<td>–</td>
</tr>
<tr>
<td>F4</td>
<td>5</td>
<td>5</td>
<td>80</td>
<td>6</td>
<td>4</td>
<td>3:2</td>
<td>–</td>
</tr>
<tr>
<td>F5</td>
<td>5</td>
<td>5</td>
<td>80</td>
<td>7</td>
<td>3</td>
<td>7:3</td>
<td>–</td>
</tr>
<tr>
<td>F6</td>
<td>5</td>
<td>5</td>
<td>80</td>
<td>8</td>
<td>2</td>
<td>4:1</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1 (Rosato et al., 2007).
for visual observation (Bernardi et al., 2011).

2.6. Antifungal studies of nanoemulsion against FOL

On the basis of lowest droplet size, polydispersity index and viscosity, CO-LGO nanoemulsion (F5) was selected for in vitro antifungal assays. Poisoned food technique (Tian et al., 2011) was employed to determine the MIC of F5. Briefly, potato dextrose agar (PDA) (~20 ml) was poured into sterilized Petri plates and required quantity of F5 was supplemented to give the following concentrations: 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, and 5000 mg/L. PDA supplemented with same amount of blank formulation (i.e. without CO-LGO) was taken as control. Unformulated essential oil combination (CO-LGO) at 5% (w/w), designated as UF5, was also prepared using 10% DMSO and diluted to the following concentrations: 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, and 9000 mg/L. PDA supplemented with the same amount of 10% DMSO was taken as control. A mycelial disc of FOL (5 mm in diameter) was inoculated aseptically into the center of each Petri dish. The inoculated plates were incubated at 28 ± 2°C until the mycelial growth reached the edge of the plates in control. Percentage inhibition of the radial growth affected by the different concentration of F5 and UF5 was measured according to the following formula (Sharma et al., 2017):

\[
\text{Percentage mycelial inhibition} = [(C–T)/C] \times 100
\]

Where, C and T are the mean colony diameter for the control and treatment plates, respectively.

To establish whether the nanoemulsion had a fungicidal effect, minimum fungicidal concentration (MFC) on FOL were assessed by re-inoculating treated fungal disc into freshly prepared PDA Petri plates and observing the growth revival after incubation for 72 h at 28°C. The lowest concentration at which no fungal growth was observed after subculturing was considered as MFC of the sample.

In order to study the speed of fungicidal effect of nanoemulsion on FOL spores, a fungicidal kinetic model was developed as per Pekmezovic et al. (2015). The selected formulation, F5 (100 μl) was diluted corresponding to MFC value and mixed with 100 μl of FOL spore suspension (10^5 spores/ml). FOL spores viability was tested by uniformly spreading 5 μl from the mixture on the PDA plate (90 mm diameter) at the start of the experiment and in the time points (t): 0, 15, 30, 60, 90, 120, and 150 min. The same set of experiment was repeated with UF5 to assess the significance of nanoemulsification of bioactive oils. After 24 h of incubation at 28°C, plates were observed under the microscope for germinated spores considered as viable spores. For each treatment, 100 spores were counted and the number of viable spores was scored to calculate the percentage viable spores designated as S_f (initial viable spores) and S_v (final viable spores). The reduction in viability of spores (S_f) due to the inhibitory effect of nanoemulsion was evaluated according to the following equation:

\[
S_f = S_i – S_v
\]

Where S_m is the maximum possible viable spore reduction (%), k is the growth rate constant of reduced viable spores and t is time.

2.7. Scanning electron microscopy (SEM) and atomic force microscopy (AFM)

To confirm the mode of action of nanoemulsion on FOL, SEM and AFM analysis were performed. For SEM, FOL was grown on PDA treated with a MIC of nanoformulation at 28°C for 5 days. The segments of 5 x 10 mm were cut from the cultures growing on potato dextrose plates and prepared for SEM visualization by primary fixation with a 2.5% glutaraldehyde solution overnight at 4°C. Thereafter, the samples were washed with 0.1 M sodium phosphate buffer solution (pH 7.2) three times for 20 min each. Following which the samples were dehydrated in graded alcohol series. Samples were then dried and mounted on silver stub and gold covered by cathodic spraying (Polaron gold). Fungal morphology was examined on a scanning electron microscope (ZEISS EVO 50) operating at 20.00 kV. A control sample without nanoemulsion treated was prepared and examined as above.

To topological parameters of the samples were analyzed using a Bioscope Catalyst AFM (Bruker Corporation, Billerica, MA) attached with a Nanoscope V controller. Untreated control and treated spore samples were poured as an even layer of 5–10 μl on a freshly peeled mica surface and were dried overnight after washing by milliQ water. The surface morphology of nanoemulsion treated and untreated FOL spores were imaged using ScanAsyst in the air at room temperature. For all imaging experiments, silicon nitride cantilevers having an average spring constant of 0.05 N/m were used with a standard scan rate of 0.5 Hz. Finally, the obtained AFM images were processed and analyzed using Nanoscope analysis, v.1.4. All images were processed using a single first order flattening followed by section analysis to determine the dimensions and topological changes.

2.8. Loss of UV-absorbing cell constituents and cellular proteins

The leakage of cell constituents and proteins in the supernatant was measured by UV-VIS spectrophotometer (Tao et al., 2014). Mycelia of FOL from six days old PDB was collected by centrifugation at 4000g for 20 min, and the resulting cell pellet was washed thrice with phosphate buffer saline (PBS, pH 7.2) and re-suspended in 50 ml of PBS. The cell suspensions were divided into two equal parts; one was left untreated (control) and the other half was treated with the MIC of the nanoemulsion. After 1 h of incubation at 28°C under agitation, 2 ml of each sample was taken out and centrifuged at 120,000g for 30 min. Finally, the supernatant from each sample was drawn carefully and the absorbance at 260 nm (nucleic acid contents) and 280 nm (protein contents) were measured. Data from triplicate sets of experiments were averaged and compared using two-tailed t-test. Each of these samples was also run on 12% SDS-polyacrylamide gel (SDS-PAGE) to analyze the proteins released due to membrane disruption.

2.9. Cell viability assay

Human embryonic kidney epithelial cell line (HEK 293T) was purchased from NCCS Pune, India. The cells were cultured at 37°C in a humidified incubator with 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) media supplemented with 10% Fetal Bovine Serum (heat inactivated), purchased from Gibco, life technologies. Effect of nanoemulsion on the viability of cells was evaluated with reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as viable cells reduce MTT salt into violet colored formazan crystals. Initially, 10^4 cells per well were seeded in 96 well plate overnight before treatment. Different dilutions (0.25XMIC, 0.5XMIC, MIC, 2XMIC, 4XMIC, 6XMIC, 8XMIC and 10XMIC) of optimized nanoemulsion were added from stock to the seeded cells. Cells without nanoemulsion served as control. After 48 h of treatment consumed media was replaced with PBS (himedia) and 10 μl from 12 mM MTT stock was added to each well. Cells were incubated till 4 h at 37°C. 50 μl of DMSO was added to dissolve formazan crystals and absorbance was taken at 540 nm. All experiments were done in triplicate.

2.10. In planta assessment of optimized nanoemulsion on Fusarium wilt incidence in tomato

Nanoemulsion (F5) was selected for polyhouse trial to study the antifungal effect on wilt disease caused by FOL. The inoculum was
prepared by culturing FOL in darkness at 25 °C on sterilized wheat grains for two weeks. Infected grains were mixed with sterile distilled water before inoculating in autoclaved soil and sand (1:1, v: v) at the rate of 25 g inoculum per pot per kg mix. The efficacy of the nanoeulsion was studied in three different application modes: seed, seedling, and soil.

2.10.1. Seed application

Tomato seeds (*Solanum lycopersicum* L. cv. Pusa Ruby) were surface sterilized with 3% sodium hypochlorite for 3 min and washed with sterile water. 100 surface sterilized seeds were soaked in 5 ml of different nanoemulsion concentrations (MIC, IC50, and IC90) and Carbendazim (1000 mg/L) for 10 min (Hashem et al., 2010). Seeds soaked with sterile water were used as a control. Seeds were left to dry completely at room temperature and were then placed in 15 cm earthen pots containing infested soil at the rate of two seeds per pot.

2.10.2. Seedling treatment

Four-week-old healthy tomato seedlings were treated with nanoemulsion (MIC, IC50, and IC90) and carbendazim (1000 mg/L) and transplanted into pots containing infested soil at the rate of two seedlings per pot. Sterile water treated seedlings were taken as control.

2.10.3. Soil treatment

The similar treatments and concentrations that used for seed and seedling applications were also employed for the soil amendment. The treatments were added to the soil in pots at the rate of 5 ml per 150 cc soil (Bowers and Locke, 2000). Healthy and untreated seedlings were sown in each pot (2 seedlings/pot). The pots having only fungus infected soil without treatment were considered as control.

All experiments were arranged in a completely randomized complete block design with six replicates per treatment and repeated twice for 45 days. For accessing the wilt disease progression in the tomato plant following grading system was used, 0 = no symptom; 1 = slight infection with 20% leaves becoming yellow, 2 = 40% leaves became yellow and wilted, 3 = 60% of leaves became yellow and wilted, 4 = extensive infection with plant showed 80% yellowing leaves and wilting and 5 = 100% plant leaves became yellow and wilted or the plant died. The percentage of disease severity index (PDSI) and disease control were determined using the formula

\[
PDSI = \frac{\sum (\text{Grade} \times \text{Number of plants in that grade})}{(\text{Maximum grade} \times \text{Total number of assessed plants})} \times 100
\]

Disease control (%) \(= \frac{(\text{Disease severity index of control} - \text{Disease severity index of treatment})}{\text{Disease severity index of control}} \times 100\)

2.11. Statistical analyses

All experiments were done in triplicates and repeated twice. Data were analyzed by one way ANOVA followed by Duncan multiple range tests (DMRT) at P values of < 0.05 using SPSS (version 10) statistical software. The IC50 and IC90 value (concentration causing 90 and 50% reduction in mycelial growth, respectively) were estimated by using probit analysis (Ldp Line). Statistical significance in cell viability assay and AFM was evaluated using the two-tailed t-test (\(*P < 0.05, **P < 0.01, ***P < 0.001\).

### 3. Results and discussion

#### 3.1. Gas chromatographic–mass spectrometry (GC–MS)

Table 2 displays the major constituents of tested clove and lemongrass oil through GC/MS analyses. The essential oil of clove was characterized by eugenol (75.41%) and E-caryophyllene (15.11%) whereas geranial (32.80%) and neral (30.35%) was the marker constituents present in lemongrass oil. All the compounds identified in our samples are known to exhibit strong antifungal activities against soil-borne plant pathogens (McMaster et al., 2013; Aguilar-González et al., 2015; Sharma et al., 2017). The antifungal properties of these bioactive compounds could be attributed to their lipophilic nature that may facilitate in the penetration of lipid bilayer fungal membrane and cause membrane disruption (Lambert et al., 2001; Sharma et al., 2017).

#### 3.2. In vitro antifungal activity of CO and LGO alone and in combination

Table 3 summarised the antifungal activities of CO and LGO alone (MICs) and in combination (FIC) against FOL. The CO displayed potent antifungal activity with MIC value of 31.25 mg/l followed by LGO (62.5 mg/L). The difference in antifungal activity of CO and LGO could be explained on the basis of hydrophilic or lipophilic nature of the major components (Ghabraie et al., 2016). Eugenol is a major component of CO and has comparatively higher water solubility than citral of LGO. The interaction of CO and LGO at 1:1 ratio showed a synergistic effect against FIC index of 0.375. Park et al. (2017) studied the potentiation of the combination of different antifungal EOs against wilt causing *Fusarium oxysporum* f. sp. *fragariae*. Some authors prefer to

<table>
<thead>
<tr>
<th>S. No</th>
<th>Clove oil Constituents</th>
<th>RI</th>
<th>Area (%)</th>
<th>Lemongrass oil Constituents</th>
<th>RI</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Eugenol</td>
<td>1357</td>
<td>75.41</td>
<td>α-citral (Geranial)</td>
<td>1268</td>
<td>32.80</td>
</tr>
<tr>
<td>2.</td>
<td>E-caryophyllene</td>
<td>1424</td>
<td>15.11</td>
<td>β-citral (neral)</td>
<td>1092</td>
<td>30.35</td>
</tr>
<tr>
<td>3.</td>
<td>α-humulene</td>
<td>1579</td>
<td>3.78</td>
<td>2-</td>
<td>1247</td>
<td>4.65</td>
</tr>
<tr>
<td>4.</td>
<td>Caryophyllene</td>
<td>1587</td>
<td>1.13</td>
<td>Hydroxy cineole γ-cadinene</td>
<td>1512</td>
<td>1.98</td>
</tr>
<tr>
<td>5.</td>
<td>α-geraniol</td>
<td>1644</td>
<td>0.51</td>
<td>E-caryophyllene</td>
<td>1424</td>
<td>1.83</td>
</tr>
<tr>
<td>6.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Inorganial α-citral (Geranial)</td>
<td>1179</td>
<td>1.18</td>
</tr>
<tr>
<td>7.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Caryophyllene oxide</td>
<td>1587</td>
<td>1.05</td>
</tr>
</tbody>
</table>

RI: retention index literature comparison.
exploit antifungal synergism between the individual phytophenols of an essential oil viz., thymol, carvacrol, eugenol etc. (Braga et al., 2007; Guarda et al., 2011).

Essential oils are known to interact synergistically but this phenomenon is least exploited in plant disease control. Most of the antifungal activity of plant oils has been attributed to oxygenated compounds such as phenolic terpenes, phenylpropanoids, and alcohols. In the current study, a combination of CO-LGO engendered synergism due to the collective antifungal activities of multiple bioactive components of each oil (Table 2). The possibility of modulating the bioactivity of major components by other minor molecules during interactions could be the other reason for synergism between CO-LGO (Rai et al., 2017). Exploitation of such synergism in a biopesticidal formulation would have the advantage in soil disease management because pathogens would not develop resistance to several bioactive components of the essential oil mixture (Hossain et al., 2016). Additionally, both CO and LGO have been approved by the Environmental Protection Agency (EPA), USA as generally-recognized-as-safe (GRAS) to be used as a biopesticide.

3.3. Preparation and characterization of synergist CO-LGO nanoemulsions

Based on synergistic interaction, the authors chose CO and LGO at 1:1 ratio as active ingredients for the development of oil-in-water nanoemulsion against FOL through low-energy emulsification method. It is a spontaneous, simple and cost-effective process of formulating oil into nanoemulsion (Chang et al., 2013). Interestingly, both oils would also act as a functional oil phase of the nanoemulsion. Tween-20 and CoE-40 were selected for being non-ionic surfactants and hence least affected by pH and ionic strength (Ali et al., 2017). In order to predict the best surfactant ratio (Smix), several emulsions were prepared to vary the relative amounts of Tween-20 and CoE-40 (F1-F6). The results of Z-average diameter, polydispersity index (PI) and viscosity (η) of CO-LGO nanoemulsions are summarized in Table 4. F1 and F2 nanoemulsions showed instability (separation of phases) at high temperature (40 °C) and hence rejected for further characterization. The Z-average diameter of nanoemulsions (F3-F6) was recorded in the range of 29.1–136.9 nm after 24 h of preparation. The polydispersity index values of F5and F6 were below 0.3 which is indicative of high fidelity of the system (Jafari et al., 2008). Minimum viscosity was observed in the formulation F5 (26.9 cP). It was noticed that increase in the concentration of CoE-40 in the Smix improved the stability of the nanoemulsions. Selection of optimal surfactant blend is a key to a stable nanoemulsion due to strong repulsive force that averts flocculation and coalescence between the nanodroplets (Sugumar et al., 2016). In the present study, the higher HLB values of both the surfactants might have also favored the stability of the CO-LGO nanoemulsions.

Based on these results, the authors chose a formulation (F5) composed of 5% CO-LGO, 10% Smix 5% propylene glycol and 80% water and proceeded with in vitro and in vivo antifungal assessments.

3.4. Antifungal studies of nanoemulsion (F5) against FOL

Both F5 and UF5 inhibited the mycelial growth of FOL in a dose-dependent manner (Fig. 1). Nanoemulsification remarkably enhanced the fungistatic efficacy as F5 (MIC: 4000 mg/L) showed 42.85% more antifungal activity than UF5 (MIC: 7000 mg/L). A similar trend was observed during fungicidal activity (Table 5) when F5 killed the FOL at a lesser concentration (5000 mg/L) compared with crude counterpart, UF5 (9000 mg/L). A statistical analysis of mycelial inhibition potential of F5 and UF5 at a concentration range of 1000–5000 mg/L and 1000–9000 mg/L respectively indicated 1.2–1.7 fold higher fungastic effect of F5 on FOL having IC50 and IC90 values of 1410.08 and 3003.70 mg/L compared with 1812.52 and 5216.59 mg/L by UF5. Fungicidal kinetics of the CO-LGO formulation (F5) and its crude counterpart (UF5) was evaluated by observing the reduction of FOL viable spores (Sr) during t using respective MFC concentrations (Fig. 2). Both the tested samples (F5 and UF5) showed a differential rate of antifungal effect which is an indicative of the non-linear fungicidal activities. The outcome of integrated logistic equation (Eq. (6)) is shown in Table 6. F5 demonstrated appreciably rapid fungicidal activity after 60 min as compared with 150 min for UF5.

The progressive diminishing antifungal effect of UF5 prodigiously established the role of emulsification of synergistic CO and LGO. This result came in agreement with He et al. (2016) who reported that micro emulsification of clove oil enhanced the fungistatic activity against Penicillium digitatum. The exhibition of improved antifungal activity after formulation in nanoemulsion may result from the smaller droplet size of F5 which in turn could increase the concentration of oils at the fungal cell causing distortion of phospholipid bilayer of the cell membrane (Tao et al., 2014). In another rationale, the optimal Smix of the current formulation might have helped in increasing the permeability of the fungal cell membrane making CO-LGO nanoparticles more effective than its crude counterpart. Results of this study suggest that the use of synergistic oils in single nanoemulsion not only lowered down the MIC and MFC values but also improved the speed of fungicidal effect suggesting the clear advantage of the present formulation over traditional usage of unformulated botanicals or single oil based microemulsion in fungal disease management in agriculture.

3.5. Scanning electron microscopy (SEM) and atomic force microscopy (AFM)

Scanning electron microscopic (SEM) observations of F. oxysporum f.
sp. *lycopersici* grown on PDA amended with the MIC (4000 mg/L) of F5 showed considerable alteration in hyphal morphology in comparison to the control (Fig. 3). After the exposure to F5, the growth of fungus was suppressed with degraded and shriveled hyphae. The shrunken hyphae showed lack of cytoplasm with the disrupted cell wall. SEM of the control showed normal morphology with lengthened, smooth and homogenous hyphae.

The atomic force microscopic analysis of untreated, control samples showed roughly spherical and curvilinear spore structures (Fig. 4a). These spherical FOL spores were found homogeneously distributed that suggested their physiologically unperturbed state. On analyzing the surface architecture, the untreated spores showed smooth and consistent topology of spores (zoomed section, Fig. 4a). The section analysis of these spores showed a homogenous size distribution with an average height and width of 3.5 nm and 30 nm respectively. Conversely, the AFM images of treated spore samples showed distorted shapes with uneven morphology (Fig. 4b). The treated samples displayed rough, fragmented and loss of characteristic curvilinear morphology of spores (zoomed section, Fig. 4b). The average height and width of treated samples were found greatly reduced at 2.1 nm and 12 nm respectively. Overall, the nanoemulsion treatment had a drastic effect on FOL spores and resulted in more than 70% reduction in size distribution (\(P < 0.001\)) mostly due to their rupture and fragmentation (Fig. 4c and d). The authors believe that nanometric droplets of the formulation after spreading over the fungus uniformly, fused with the cell membrane resulting in destabilization of the lipid envelope and leakage of cellular constituents.

### 3.6. Membrane disruption and release of cell constituents by nanoemulsion

Membrane disruption and release of cell constituents after FOL was treated with a MIC of F5 nanoemulsion for 1 h were examined using spectrometric detection of cell constituents (nucleic acids and protein) and SDS PAGE. The release of nucleic acids (OD\(_{260}\)) significantly

<table>
<thead>
<tr>
<th>Sample</th>
<th>t (min)</th>
<th>(S_m) (% ± SE)</th>
<th>(S_i) (% ± SE)</th>
<th>(k) (min(^{-1}))</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F5</td>
<td>60</td>
<td>98.3 ± 1.78</td>
<td>28.3 ± 1.24</td>
<td>0.101</td>
<td>0.976</td>
</tr>
<tr>
<td>UF5</td>
<td>150</td>
<td>99.2 ± 1.65</td>
<td>18.3 ± 1.12</td>
<td>0.042</td>
<td>0.989</td>
</tr>
</tbody>
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The atomic force microscopic analysis of untreated, control samples showed roughly spherical and curvilinear spore structures (Fig. 4a). These spherical FOL spores were found homogeneously distributed that suggested their physiologically unperturbed state. On analyzing the surface architecture, the untreated spores showed smooth and consistent topology of spores (zoomed section, Fig. 4a). The section analysis of these spores showed a homogenous size distribution with an average height and width of 3.5 nm and 30 nm respectively. Conversely, the AFM images of treated spore samples showed distorted shapes with uneven morphology (Fig. 4b). The treated samples displayed rough, fragmented and loss of characteristic curvilinear morphology of spores (zoomed section, Fig. 4b). The average height and width of treated samples were found greatly reduced at 2.1 nm and 12 nm respectively. Overall, the nanoemulsion treatment had a drastic effect on FOL spores and resulted in more than 70% reduction in size distribution (\(P < 0.001\)) mostly due to their rupture and fragmentation (Fig. 4c and d). The authors believe that nanometric droplets of the formulation after spreading over the fungus uniformly, fused with the cell membrane resulting in destabilization of the lipid envelope and leakage of cellular constituents.

![Fig. 2.](image) Fusarium oxysporum f.sp. *lycopersici* (FOL) spore reduction (%) at different time points by using minimum fungicidal concentrations of optimized nanoemulsion F5 and unformulated clove oil-lemon grass oil (CO-LGO) at 5% w/w (UF5). Kinetic models revealed 2.4 times faster fungicidal activity of F5 compared with UF5.

![Fig. 3.](image) Scanning electron microscopy (SEM) images of a) Control (untreated) and b) F5 (4000 mg/L) treated hyphae of *F. oxysporum* f. *lycopersici* (Scale Bar: 2 μm).

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<table>
<thead>
<tr>
<th>Treatments</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>MIC (mg/L)</td>
</tr>
<tr>
<td>F5(^a)</td>
<td>4000</td>
</tr>
<tr>
<td>UF5(^b)</td>
<td>7000</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations ranged from 1000 to 5000 mg/L.

\(^b\) Concentrations ranged from 1000 to 9000 mg/L.

---

Table 5

Antifungal effects of optimized CO-LGO nanoemulsion F5 and unformulated CO-LGO combination (UF5) against FOL.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
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<tr>
<td>UF5(^b)</td>
<td>7000</td>
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</tbody>
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\(^a\) Concentrations ranged from 1000 to 5000 mg/L.

\(^b\) Concentrations ranged from 1000 to 9000 mg/L.

---

Table 6

Parameters of the kinetic model (Eq. (7)) of antifungal effect of optimized nanoemulsion F5 and unformulated CO-LGO at 5% w/w (UF5): time needed for fungicidal effect (t), maximum possible percentage of reduced viable spores (\(S_m\)), initial percentage of reduced viable spores (\(S_i\)), growth rate constant of reduced viable spores (k) and coefficient of determination (\(R^2\)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>t (min)</th>
<th>(S_m) (% ± SE)</th>
<th>(S_i) (% ± SE)</th>
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<td>0.989</td>
</tr>
</tbody>
</table>
increased ($P < 0.01$) when FOL was treated with a MIC of nanoemulsion (Fig. 5a). Similarly, the total protein release (OD$_{280}$) was 40% more than that of control ($P < 0.01$) (Fig. 5b). The release of cell constituents is representative of cell lysis (Zhou et al., 2008). The release of cellular proteins was later confirmed by SDS PAGE analysis that showed an increase in protein bands for the treated samples (Fig. 5c). The nanoemulsion played a significant role in causing physical damage to fungus, facilitating its use as an effective antifungal agent.

3.7. Transmission electron microscopy (TEM)

The morphology of the nanoemulsion was visualized by TEM analysis (Fig. 6). The images revealed that the droplet had spherical morphology with an average size range between 20–50 nm thereby corroborating the result from DLS analysis.

3.8. Anti-proliferative effect of nanoemulsion on human cells

Nanoemulsion process significantly increments the functionality, stability, and usefulness of EOs. However, regulatory and safety evaluation of these nanoemulsions have also been implemented to screen for its potentially hazardous side-effects (Office, 2003). Among these evaluation standards, the toxicity of nanosubstances on the human body is the major obstacle in the commercialization (Pekmezovic et al., 2015). This is important since natural compounds may be associated with an inhibitory effect on humans that limits their practical application. Thus, in the present study, assessment of cellular toxicity of the nanoemulsion was verified using standard (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay on human cells (HEK 293T). The MTT results showed that the MIC (4000 mg/L) concentration did not exhibit an antiproliferative effect on the human cell line (Fig. 7a). Interestingly, even at very high concentrations (10X MIC), the authors observed 71.1% cell viability after 48 h. The stressed and detached cells start appearing only after treated with 6XMIC (Fig. 7b) which signify the compatibility and non-hazardous nature of the nanoemulsion with human/mammalian cells and tissues.

3.9. In planta antifungal assessment of CO-LGO nanoemulsion (F5) on tomato plants

The CO-LGO nanoemulsion (F5) demonstrated varying antifungal efficacies against FOL depending upon the mode of applications i.e. seed, seedling or soil (Table 7). Treatment of soil with MIC and IC$_{90}$ of F5 significantly ($P < 0.05$) reduced the vascular wilt in tomato plants.

![Fig. 4. Atomic force microscopy (AFM) images showing three-dimensional view of a) untreated control and b) Nanoemulsion (4000 mg/L) treated $F$. oxysporum spores. All images were generated using the ScanAsyst mode of a Bruker Biocatalyst AFM. In each case major differences in size as well as surface attributes are shown as zoomed insets. The comparative c) height and d) width distribution in both cases are also shown. The average height of untreated spores was 3.5 nm that gets reduced to 1.1 nm after nanoemulsion treatment. Similarly, untreated and nanoemulsion treated spores showed an average width of 30 nm and 12 nm respectively. Statistical significance was evaluated using the two-tailed t-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.01$). Scale bars in each case represent 0.5 μm.](image-url)
and showed the maximum disease control, which was 67.51 and 64.41% respectively, in relation to untreated control. IC₅₀ was the least effective dose for F₅ causing disease severity index of 63.8%. During seedling application, F₅ at MIC controlled 61.17% of wilt disease in tomato. Treatment of tomato seedling with an IC₅₀ dose of F₅ and Carbendazim was significantly (P < 0.05) similar and reduced 51.27 and 53.72% of wilt disease, respectively in tomato plants. Seed treatment with the nanoemulsion at three different concentrations had the least effect on wilt disease incidence by FOL in tomato plants. F₅ at MIC controlled only 27.42% of wilt disease followed by IC₉₀ (13.71%) and IC₅₀ (5.59%). Overall, all treatments significantly outperformed the untreated control but soil application at MIC of F₅ was most effective.

The results obtained during pot studies in polyhouse corroborate the results obtained by us under in vitro conditions. The ability of nanoemulsion (F₅) to suppress the wilt incidence in tomato plants may correspond with the ability of CO-LGO to control FOL populations in soil through direct contact or via fumigation (Ji et al., 2005). It is important to notice that hydrophilic surfactants were used to formulate CO-LGO and that may lead to increased solubility of the active ingredients in the aqueous phase of the rhizospheric soil. A decrease in plant disease incidence through soil amendment of single oil/botanical based formulations has previously been reported (Bowers and Locke, 2000; Bowers and Locke, 2004; Lee et al., 2012). However, there is lack of scientific reports on the impact of synergistic nanoformulation on FOL. One of the major apprehensions of using essential oil based formulation as soil amendments is the requirement of higher doses to control soil-borne pathogens. Bowers and Locke (2000) reportedly used 10% aqueous emulsion of EOs (mustard, cassia, and clove) to significantly reduce 97.5–99.99% of the population density of F. oxysporum in soil compared to untreated control. In present study, due to its enhanced antifungal activity, the formulation showed promising results even at lower concentrations (IC₉₀), making it a commercially viable and environmental friendly biopesticide. Another major outcome of the current study was the ability of the CO-LGO formulation to control Fusarium wilt disease through root-dip treatment of tomato seedling which is comparatively more economical than soil amendment method and hence more feasible for large-scale field application (Khanna et al., 2010). Control of wilt disease through seedling treatment results from elicitation of defense responses in tomato induced by the components of EOs, primarily eugenol (Wang and Fan, 2014; Ben-Jabeur et al., 2015). Seed treatment of the formulation did not produce good results compared with other modes possibly because FOL is not a seed-borne pathogen. However, critically examining data of the seed experiment, the authors noticed that treatment of tomato seeds with concentration as high as MIC did not negatively affect the germination (data not shown) reflecting the non-phytotoxic nature of the nanoemulsion.

4. Conclusion

The current investigation focused on the development and characterization of a stable and non-phytotoxic nanoemulsion of synergist clove and lemongrass oil against FOL. The antifungal activity of CO-LGO enhanced as a synergistic formulation in nanoemulsion (F₅) and fungal cell membrane remarkably disrupted as evidenced by
Fig. 7. Cell toxicity assessment of nanoemulsion over human embryonic kidney (HEK 293 T) cells. a) Different dilutions of the nanoemulsion corresponding to the MIC (Minimum Inhibitory Concentration) value (4000 mg/L) were incubated with HEK cells for 48 h and cell viability was determined using the standard MTT (Dimethylthiazolyl-2,5-diphenyltetrazolium bromide) assay. b) Morphological assessment of cells in culture examined by phase-contrast microscopy. HEK 293 T cells incubated (48 h) with different MIC dilutions are shown; magnification = 100×; scale bar = 100 μm. Control in each case represents untreated cells.

Table 7

Effect of CO-LGO nanoemulsion (F5) and fungicide applied on seed, seedling and soil separately on PDSI species after 45 days.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Seed application</th>
<th>Seedling application</th>
<th>Soil application</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDSI*</td>
<td>Disease control (%)</td>
<td>PDSI</td>
</tr>
<tr>
<td>CO-LGO nanoemulsion (F5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>@ MIC</td>
<td>68.8 ± 4.17a</td>
<td>27.42</td>
<td>34.9 ± 1.65a</td>
</tr>
<tr>
<td>@ I C00</td>
<td>81.8 ± 2.01b</td>
<td>13.71</td>
<td>43.8 ± 2.54b</td>
</tr>
<tr>
<td>@ I C90</td>
<td>89.5 ± 2.73c</td>
<td>5.59</td>
<td>66.6 ± 1.65c</td>
</tr>
<tr>
<td>Carbendazim @ 1000 mg/L</td>
<td>67.2 ± 3.48d</td>
<td>29.11</td>
<td>41.6 ± 1.85d</td>
</tr>
<tr>
<td>Untreated control</td>
<td>94.8 ± 1.80e</td>
<td>–</td>
<td>89.9 ± 2.53d</td>
</tr>
</tbody>
</table>

*PDSI: percentage of disease severity index. Fusarium wilt disease was rated based on a scale of 0–5 as described in material and methods. Mean in the column followed by different letters indicate significant differences among treatment (P < 0.05) according to DMRT.

Table 7 (continued)

Microscopical and molecular analysis. As a result, significant reduction in tomato plants was observed even at lower concentrations during the in planta assays. These findings could provide a pragmatic solution for high cost and low antifungal activity of single botanical fungicide as well as synthetic pesticides.

Acknowledgment

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2018.06.077.

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