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The effect of a seed coating with *Origanum vulgare* essential oil on *Clavibacter michiganensis* subsp. *michiganensis*

Meral Yılmaz[1], Ömür Baysal[2], Ragıp Soner Sılme [3]*

[1]Department of Seed Pathology and Technology, Turkish Ministry of Agriculture and Forestry, Antalya Seed Certification and Test Agency, Antalya, Turkey
[2]Molecular Microbiology Unit of Molecular Biology and Genetics Department, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey
[3]Center for Research and Practice in Biotechnology and Genetic Engineering, Istanbul University, Istanbul, Turkey

**Corresponding author: omurbaysal@mu.edu.tr*

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Abstract: The study aimed to evaluate the efficacy of a seed film coating with *Origanum vulgare* Linnaeus essential oil (EO) against *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) on tomatoes. Tomato seeds (cv. Rio Grande) coated with different doses of EO derived from *O. vulgare* were inoculated with Cmm (1.8×10^8 CFU/mL). *O. vulgare*'s EO showed a remarkable inhibition effect on the Cmm growth. The EO effect against Cmm was determined based on the parameters, such as the inhibition zone and bacterial population in a seed. The GC-MS analysis of EO showed that carvacrol is the major component (at 74.05%), which may inhibit the bacterial growth. Later, we have expanded our studies to determine the inhibitory effect of the EO's mode of action on the pathogenic bacteria with a molecular docking analysis based on the molecular protein-ligand interaction. The results showed that carvacrol has a strong interaction with the bacterial expansin protein (PDB 4JJO) of Cmm and the qPCR analyses confirmed the effect of the *O. vulgare* treatment against Cmm. This original approach has the prominent potential to prevent seed transmission of Cmm for seed quality in the world, suggesting a method for paving the way for Cmm disease management.

Keywords: bacterial canker; carvacrol; molecular docking; protein–ligand interaction; real-time PCR; seed film coating

The tomato (*Solanum lycopersicum* Mill.) is the most widely produced and consumed vegetable species in the world (FAO 2015). Bacterial canker and wilt – caused by the seed-borne bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Smith 1910) Davis, Gillaspie, Vidaver and Harris 1984 (Cmm) is an important tomato disease (Smith 1910; Chang et al. 1992). This pathogen was first discovered in 1909 in Michigan (Smith 1910); since then, many epidemics have occurred in different regions of the USA (Gleason et al. 1993). Currently, it is a quarantine pathogen in the European Union (EU) and other countries involving Turkey (EPPO 2014). Cmm transmitted by plant tissues, plant debris, infected tools, and equipment is the reason for the pathogen's long-distance spread, which is mainly through infected seeds and seedlings (Eichenlaub et al. 2006).

The most effective strategies for tomato bacterial canker management are to use pathogen-free seeds and seedlings (Gitaitis & Walcott 2007) and to protect the seeds from Cmm contamination during the seed harvest and planting. Unfortunately, many chemical/physical seed treatments reduce tomato seed viability, though they do not completely

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eradicate Cmm from the infected seeds (Fatmi et al. 1991; Kasselaki et al. 2011). On the other hand, EOs and their derivative compounds are alternative means of controlling many plant diseases and certain EOs have been effective in managing Cmm in seed treatment studies (Groot et al. 2004; van Der Wolf et al. 2004, 2008; Lo Cantore et al. 2009). EOs have an inhibitory effect on plant pathogens (Chao et al. 2000; Belgüzar et al. 2016). The volatile component of the oils contains higher amount of carvacrol (Müller-Riebau et al. 1995). Studies have indicated that the antioxidant activity of the EOs are due to the carvacrol and some other phenols (Deighton et al. 1993; Lagouri et al. 1993). Furthermore, *Clavibacter michiganensis* subsp. *michiganensis* has been acknowledged to be inhibited by oregano, thyme, dictamnus and marjoram EOs at relatively low concentrations (85–300 µg/mL) (Daferera et al. 2003).

To our best knowledge, there is no study related to the antibacterial components of the *O. vulgare* EO effect on one of the enzymes "expansin" playing a role in the disease formation caused by Cmm. The cell wall-loosening enzyme expansin loosens the rigid structure involving the carbohydrates of the cell wall which is required for cellular growth, the biochemical pathways related to vascular differentiation, and leaf development (Kende et al. 2004). Expansins are pH-dependent and activated with plant growth hormones that stimulate H1-ATPases by producing a proton differential across the plasma membrane (Cosgrove 2000). The expansin gene is present in systemic xylem pathogens such as *Xanthomonas*, *Xylella*, *Ralstonia*, *Dickeya*, *Pectobacterium*, *Acidovorax*, and *Clavibacter* except for *Streptomyces* (Georgelis et al. 2015). Cmm has chimeric (CmEXLX1) and non-chimeric (CmEXLX2) microbial expansin structures (Nikolaidis et al. 2014), which are similar to plant expansins with highly conserved unique cellulose-binding domains (Pastor et al. 2015). Expansin breaks down plant cell walls *in vitro* without lytic activity (Bunterngsook et al. 2015).

A seed film coating is an efficient technique for pest control, seed quality, and plant production. However, there is no study on testing a combination of a seed coating with the main components of *O. vulgare* to a Cmm infection. Even if pathogen-free tomato seed production is desired, there is no efficient method against the contamination by Cmm at the post-harvest period. We have suggested a film coating technique on seeds and investigated an easier disinfection method due to the potential inhibitory role of the main component carvacrol to Cmm expansin proteins. Therefore, we have used a docking analysis and advanced simulation techniques to understand the interaction of these components as the ligands with the expansin protein (PDB 4JJO), which plays a key role in the Cmm pathogenesis.

MATERIAL AND METHODS

Seeds, bacterial strain, and Gas chromatography-mass spectrometry (GC-MS) analysis of essential oil (EO). As propagation material, certified pathogen-free tomato seeds (cv. Rio Grande) were used. The bacterial isolate Cmm strain 3/1A (Baysal et al. 2011) was added to a nutrient agar (NA) (Difco) and incubated at 28 °C for 72 hours. The content analysis of *O. vulgare* (Ecodab, Inan Tarim Co., Turkey) was performed by gas chromatography (GC) (Agilent 7890A, Agilent Technologies, Inc., USA) and mass spectrometry (MS) (Agilent 5975C, Agilent Technologies, Inc., USA) according to Zhao et al. (2005). An HP InnowaxCapillary column (60 m × 0.25 mm, 0.25 µm film thickness) was used with helium as the carrier gas (0.8 mL/min). The GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 minutes. The total analysis period was 60 min under these temperature conditions. The sample was injected with 1 µL and the split ratio was adjusted at 50 : 1. The injector temperature was set at 250 °C. The mass spectra were recorded at 70 eV. The mass range was from m/z 35 to 450. The main components of the EO were diluted by a 1 : 50 ratio with acetone before injection. The Wiley and Oil Adams library data were used to determine the major components of the EO (Zhao et al. 2005). The relative percentage amounts of the separated compounds were calculated from the GC-MS chromatograms. The analysis results are given in Table 1. Later, *O. vulgare* oil dissolved in dimethyl sulfoxide (10%) was used to prepare different concentrations (0, 250, 500, 1 000 ppm, and non-diluted) for further studies.

Tomato seed film coating with EO of *O. vulgare*. A commercial polymer, Discoshine L88 Blue obtained from Incotec (Incotec group BV, Netherlands) was used. Seeds treated with EO of *O. vulgare* were covered by soaking them into the EO solution (containing 40% *O. vulgare* EO) in a sterilised 250 mL beaker before coating the seeds with Discoshine L88 Blue. The beaker was placed in a Nucerite Desiccator (Laborteknik Co., Turkey), and a vacuum was applied

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Table 1. Total relative percentage of the identified compounds in *Origanum vulgare* essential oil

Component	%
α-pinene	0.35
α-thujene	0.11
Camphene	0.21
β-myrcene	0.48
α-phellandrene	0.11
α-terpinene	0.69
Limonene	0.13
Sabinene	0.3
γ-terpinene	1.92
Cymene	3.79
α-terpinolene	0.13
Allytoluene	0.06
1-octen-3-ol	0.13
Trans-sabinene hydrate	0.2
Linalool oxide	0.05
Linalool	5.46
Cis-sabinene hydrate	0.15
Trans-caryophyllene	1.61
Terpinen-4-ol	0.95
Aromadendrene	0.23
α-humulene	0.1
α-terpineol	0.32
Borneol	1.52
β-bisabolene	1.82
Carvone	0.2
δ-cadinene	0.12
α-amorphene	0.14
Cis-α-bisabolene	0.08
Carvacryl acetate	0.23
Caryophyllene oxide	0.39
Spathulenol	0.25
Thymol	2.59
Carvacrol	74.05

for 10 min using an Air Cadet pump (Budunoğlu Co., Turkey) with 18 psi pressure. Then, 400 g of the tomato-soaked seeds coated with a 60% polymer with the EO solution were placed into a film coating machine (Incotec™ rotary film coater, Incotec Group BV, Netherlands) and the polymer/EOs were slowly added to the film coating process. The moisture content of the coated seeds was measured using the hot air oven method (ISTA 1999) and a humidity analyser (Radwag™ MA 50/1.R, Poland).

Assessing of antibacterial activity. Inhibition zone was determined by a modified agar well diffusion method. Twenty-five seeds placed in each Petri dish were incubated at 27 ± 2 °C for 48 hours. Inhibition zone (IZ) around the seeds measured as mm and IZ % was calculated according to formula as:

$$100 - \left(C_{cont} - \frac{C_{cont}}{C} \times 100 \right) \quad (1)$$

where: C_{cont} – control group (zone of growth diameter); C – treatment group (zone of growth diameter).

The EO film-coated tomato seeds were placed on a Cmm free Nutrient Agar medium. The twenty-five seeds placed in each Petri dish incubated at 27 ± 2 °C for 72 h were used to calculate the bacterial population in the seed (BPS). The EO film-coated seeds were macerated by TissueLyser using a sterile potassium phosphate buffer + Tween 20. Then, the seeds placed into a sterile phosphate buffer with Tween 20 overnight were crushed by TissueLyser (Qiagen™, Germany). The extract diluted from 10^{-3} to 10^{-6} with the sterile phosphate buffer with Tween 20 was spread on a Soybean Cryptic Medium (SCM) (Ftayeh et al. 2011). The Cmm colonies counted and converted to $\log_{10}$ were used to determine the effect (%) by the visible viable colonies in the Petri dishes as the CFU/mL per seed according to Abbott (1925). The BPS confirmed by the real-time polymerase chain reaction (PCR) results were obtained according to the Cmm test protocols (Anonymous 2014). The SYBR Green real-time PCR amplifications were performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.). For the extraction, 100 seeds were used. The extract of the seeds diluted from 10^{-3} to 10^{-6} using a serial dilution by the sterile phosphate buffer with Tween 20 was spread on the SCM (Ftayeh et al. 2011). Pure Cmm colonies were obtained from seeds. The DNAs of the colonies were extracted via a Promega DNA Isolation Kit according to Baysal et al. (2011). Cmm 5/6 primers (614 bp) were used to amplify the DNA (Louws et al. 1998; Baysal et al. 2011). A real-time PCR analysis was performed in a 20 µL reaction and with the primers (Cmm 5/6) (0.5 µmol/L final concentration) and iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA) according to the manufacturer's instructions, and 5 ng of purified DNA was used for each sample. The cycling conditions were adjusted to an initial

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denaturation for 5 min at 95 °C, 45 cycles for 20 s at 95 °C, 20 s at 63 °C and a melting curve of 65 to 95 °C, with an increment of 0.5 °C. All the SYBR® Green real-time PCR assays were performed at least three times with the mean cycle threshold (Ct) values. Detection of the Ct values and the data analysis were set automatically by the CFX Manager™ Software system (Version 1.6.). The melting curve, melting point, and Ct for the DNAs of the sample and control were calculated.

Assessing the seed quality parameters. The germination ratio (GP; %), mean germination time (MGT), seedling emergence % (SEP), and mean emergence time (MET) were carried out according to the Rules of the International Seed Testing Association (ISTA 1999). The emergence assays were carried out in the laboratory and controlled greenhouse conditions.

Statistical analyses. The data were analysed by SAS (1999) [version 7 (TS P1)] in a 4 (EOs) × 4 (doses) factorial design. The EO x doses interactions were significant for most of the parameters such as IZ, BPS, GP, and SEP, the mean values were subjected to Duncan's multiple range test ($P \leq 0.01$ or $P \leq 0.05$) (Duncan 1955). All the assays (IZ, BPS, GP, and SEP) were conducted with four replicates. The positive control was copper sulfate compared to the negative control (DMSO 10%: water v/v alone).

Molecular docking studies. The *in vitro* and *in vivo* experiments that confirmed the qPCR results have directed us to protein-ligand interaction analysis on the bacterial expansin protein (PDB 4JJO) of Cmm with ligand (carvacrol). A geometry-based molecular docking algorithm was used for the docking analysis using PatchDock which yielded results for the geometric shape complementarity score (GSC score) and approximate interface area (AI area). Then, CHIMERA® involving the AutoDock software (vrsion 1.14) used for the molecular docking (Trott & Olson 2010) showed the interaction analysis of the protein-ligand complexes. Their amino acid position with bond distances was calculated to get insight into their all-binding preferences (by PyMol® software, version 2.5) within the active site of these receptors.

RESULTS

Assessing of antibacterial activity. The GC-MS analysis showed the existence of the antibacterial components (Table 1). The results proved that the EO of *O. vulgare* used for the seed coating caused the highest inhibition on the pathogen compared to the control. *O. vulgare* created the highest inhibitory effect (by 24 mm) compared to the negative control (DMSO, 3 mm) and positive control (Copper sulfate, 22.3 mm) (Figure 1A and B). In the control groups, the artificial inoculation on the seeds was 3.82 log CFU/mL on the seed surface and 3.94 log CFU/mL at the embryo part with regards to testing the efficiency of the EO treatment. Before the inoculation, coating with 250–1 000 ppm doses of *O. vulgare* inhibited the artificial infection on the seeds. The tested concentration (250–1 000 ppm dose) of *O. vulgare* resulted in a protective effect against a Cmm infection based on the $\log_{10}$ CFU per seed values. As given in Table 2, the *O. vulgare* treatment caused very similar to the Ct values obtained with the copper sulfate by the 250 ppm doses. However, the effect of the EO treatment increased by the applied doses and 1 000 ppm resulted in the complete protection (no infection) of the seeds. The EO film coating inhibited its growth and did not allow the transmission and the dispersal of bacterial infections from one seed to another. All the doses of 500 ppm of *O. vulgare* inhibited the Cmm growth in the tomato seeds compared to the negative control (DMSO, 0.00%) and positive control (Copper sulfate, 100%).

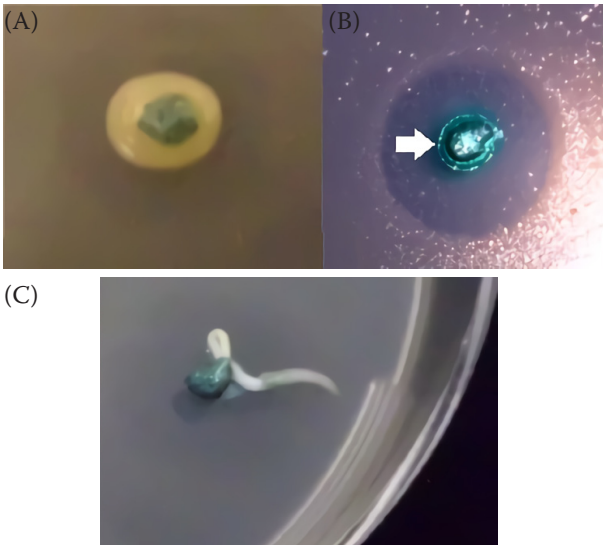

Figure 1. Growth of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) on the solid culture medium

(A) Cmm growth on the surface of artificially infected control seeds, (B) arrow shows the inhibition zone of the EO film-coated tomato seeds inoculated with Cmm (1.8×10^8 CFU/mL) on the solid NA medium, and (C) there is no growth of Cmm on the surface of the germinated seed coated with *O. vulgare* EOs at 1 000 ppm

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Although the 5 00ppm doses of *O. vulgare* gave rise to a higher suppressive effect on the bacterial growth on the treated seeds, the obtained values were not significantly different from the 250 ppm dose (Figures 1C, 2). Similar findings obtained by SYBR® Green real-time PCR confirmed the BPS results, which revealed a correlated response between the Ct values and the doses of EO used for the film coating (R^2 = 0.978) (Table 2). On the other hand, the EO film coating did not show a significant effect on the MGT and MET (Table 3).

Protein docking studies. Carvacrol as the ligand structure of PDB 4JJO retrieved from the PubChem database was analysed using docking software (Figure 3). Our studies have shown a strong interaction of carvacrol with the expansin protein. This inhibitory effect can be associated with this interaction besides the other known antibacterial effect of these EO's major components.

DISCUSSION

Seed quality is a key factor for economic crop production. Unfortunately, even under controlled environmental conditions, tomato seed contamination with *C. michiganensis* subsp. *michiganensis* can easily spread by infected seeds and seedlings (Dutta et al. 2014). Precautions are also not enough to control tomato bacterial canker and wilt disease. In the present study, we have shown the seed film coating effect of *O. vulgare* EO as a seed treatment against *C. michiganensis* subsp. *michiganensis*.

The EO coating leads to the disinfection of the artificial-inoculated seeds and the treatment showed protective effects against bacterial infections. We observed some differences in the IZ when compared to previous studies depending on the fresh or commercial property (Borboa-Flores et al. 2010) and the chemical composition of the EO (van Der Wolf

Table 2. Confirmation of the BPS result of the esential oil (EO) seed treatment by the real-time PCR

Applications	BPS ($\log_{10}$ CFU per seed)		
	EO dose (ppm)	mean ct values	+/–
O. vulgare	250	30.00	+
	500	34.00	+
	1 000	0.00	–
Cmm 3/1A[1]	0.00	18.00	+
DMSO[2]	0.00	19.00	+
Copper sulfate[3]	250 cc/100 L	32.00	+
Water[4]	0.00	0.00	–

The mean ct values of the positive/negative Cmm DNA samples obtained from the seeds and seedlings of the EO film coated seeds by real-time PCR with Cmm5/6 primers; [1]positive control for the real-time PCR; [2]negative control for the trial; [3]positive control for the trial; [4]negative control for the real-time PCR; DMSO – dimethyl sulfoxide; cc – cubic centimetre; BPS – bacterial population in seed; ct – cycle threshold

Table 3. Numbers of germinated and emerged seeds of the esential oil film coated tomato ones

Dose (ppm)	No. of germinated seed[4] and emerged seed (No.)[5]		Germination of seedling emergence (%)		MGT and MET (day)	
DMSO[1]	46.0[a]	23.0[a]	92.0	91.0	3.40[a]	3.50[a]
Copper sulfate[2]	44.0[a]	22.0[b]	86.0	88.0	3.40[a]	3.40[a]
EO 250	39.0[b]	20.0[b]	80.0	78.0	3.00[a]	3.00[a]
EO 500	37.0[c]	19.0[c]	77.0	74.0	3.10[a]	3.40[a]
EO 1 000	36.0[d]	18.0[d]	73.0	72.0	3.20[a]	3.60[a]
Mean[3]	36.0	19.0	75.0	72.0	3.10	3.50

[1]negative control for trial; [2]positive control for trial; [3]n = 4 followed by the same letter within a column is not significantly different by Duncan's multiple range test ($P \leq 0.05$); [4]the mean germinated seed number for each application calculated on 4 × 50 seeds; [5]the mean emergence seed number for each application calculated on 4 × 25 seeds; DMSO – dimethyl sulfoxide; EO – esential oil; MGT – the mean germination time; MET– the mean emergence time

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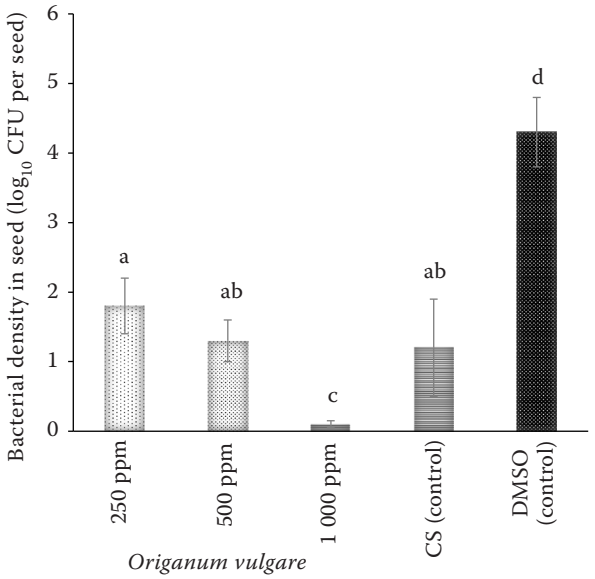

Figure 2. *Clavibacter michiganensis* subsp. *michiganensis* in the seed (BPS, log CFU/seed) of essential oil film-coated seeds

The effect of the EO film coating indicated with a letter (a) is significant compared to the untreated control (Duncan's multiple range test, $P \leq 0.01$); the bacterial density in seed was obtained from four replicate experiments; the values shown are the mean of four repetitions with four petri dishes

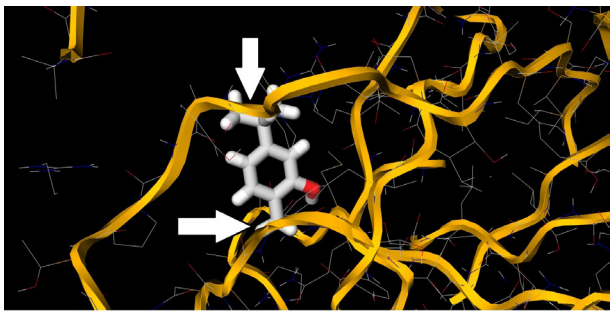

Figure 3. Arrows show the binding possibilities (Chimera, AutoDock results) of Carvacrol with *Clavibacter michiganensis* subsp. *michiganensis* expansin protein (4JJO)

et al. 2008; Kotan et al. 2014). BPS ($\log_{10}$ CFU per seed) values are important parameters in terms of the bactericidal effect of the EO film coating that completely eradicated Cmm, which was confirmed by qPCR. *Origanum*'s high inhibitory effect can change according to the quantity of the antibacterial compound, carvacrol (64.78 to 74.05%), which shows correspondence to previous studies on seed treatments (van Der Wolf et al. 2008; Cueto-Wong et al. 2010). The antibacterial effect of carvacrol was attributed to the permeability and depolarisation through the cytoplasmic membrane (Nostro & Papalia 2012). The values of the $\log_{10}$ CFU per seed values showed agreement with the previous study of van Der Wolf et al. (2008). Decreasing the GP and SEP by the EO film coating on the tomato seed quality was around by 11 to 17% depending on the tested doses of EO and the application method (Tobias et al. 2007; van Der Wolf et al. 2008; Hussain & Reigosa 2014). The MGT, MET and uniform seedling growth was not affected by the EO film coating, but the mean value of the germination time became longer. A similar result was reported in a previous study (Imatomi et al. 2013). Although the antibacterial mechanisms of EOs are clear (Faleiro 2011), their effects on the seed quality remain unclear. Nevertheless, the levels of the EO antibacterial effect and the adverse effect of excessive EO concentration can be correlated to the negative effects on the seed viability. Although there was a certain effect on the seed germination by nearly 20% at doses of 500 ppm and above, no significant negative effect was detected on the emergence time. In addition to the control of the pathogen on the seed, as a pioneer study, we can assume that the undesired effect of a seed germination ratio approaching 20% is an acceptable level for a seed-borne pathogen to prevent the losses caused by the disease. We can suggest that this negative effect will disappear by the coating method on a nanoscale level in further studies. The *O. vulgare* film coating reduced the Cmm infection. EOs obtained from *Origanum* and *Thymus* are acceptable for organic agriculture practices due to their low phytotoxicity as described in the limited list of organic EU regulations (2092/91) at Annex IIB (Anonymous 1991). EO has a high biopesticide and seed disinfectant potential (Tinivella et al. 2009). Also, a film coating pesticide is an eco-friendly alternative and economically available option (Jacob et al. 2009). This technique allows the coating of seeds with pesticides, alternative matters (Keawkham et al. 2014), and EOs (Zeng et al. 2010). Major vegetable seed producers suffering from seed-borne bacterial diseases lose millions of dollars annually. Therefore, the findings presented in this study suggest that an EO seed film coating is an alternative control measurement for seed protection. We showed that the EO application resulted in minimising the transmission of Cmm. The technique could increase the performance of organic pesticides and also decreases the costs due to the consumption of toxic chemicals causing environmental pollution.

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Given that expansins encoded by two expansin genes in the bacteria cause plant vascular system infections, they are the reason for the invasion of Cmm in the xylem (Pastor et al. 2015). The biological function of the Cmm expansins are to break the hydrogen bonds of the carbohydrate polymers in plant cell walls by endoglucanase activity (Beimen et al. 1992), which causes the development of wilting and degradation by cellulose enzymes in the cell wall (Jahr et al. 2000; Gartemann et al. 2008). Our findings proved the strong interaction between the main components of EO with the expansin protein, which shows their inhibitory effect on the Cmm pathogenicity besides our *in vitro* tested parameters.

Computational techniques performed for protein-ligand interaction studies stress docking strategies predicted by the lock-key model. They have also beneficial sides with the purpose of drug discovery. We have studied the available protein structures with molecular models by simulating conformational transitions of molecules within each other. It assists in time-saving new ideas for bringing solutions on biological cases which cannot be assessed experimentally due to the confronted difficulties and higher cost. We believe that our study will affect improving an environment-friendly strategy on seed disinfection in the future.

CONCLUSION

The study showed the significant inhibition of *O. vulgare* EO on *Clavibacter michiganensis* subsp. *michiganensis* infected tomato seeds. The GC-MS analysis revealed that this inhibitory effect is due to carvacrol which is also the major component of *O. vulgare* with a high antibacterial property. The effect confirmed with a qPCR analysis was measurable compared to the control group on the infected seeds. Additionally, the interaction between carvacrol and the bacterial expansin protein (PDB 4JJO) of Cmm observed by molecular docking analysis highlighted that our findings will ensure new insights to control Cmm infections on tomato seeds.

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