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# Effect of essential oil from *Zataria multiflora* on local strains of *Xanthomonas campestris*: An efficient antimicrobial agent for decontamination of seeds of *Brassica oleracea* var. *capitata*

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ABSTRACT

In this study, the effect of the essential oil of *Zataria multiflora* was examined against the local strains of *Xanthomonas campestris* isolated from the soil. The pathogenicity of the bacteria was confirmed using seedlings on culture media under sterile conditions.

The minimum inhibitory and bactericidal concentrations of *Zataria multiflora* essential oil and two main components (thymol and carvacrol) were evaluated by the microdilution method in microtiter plates. The morphology of the surface of the seeds and characteristics of the grooves were studied by scanning electron microscopy. Hermetic effect of the essential oil and antimicrobial activity of the solvent on *X. campestris* were evaluated.

Minimum inhibitory and bactericidal concentrations of *Zataria multiflora* essential oil were 231.8 and 463.5 µg/mL, respectively. The viability of *X. campestris* in Yeast Malt Broth was reduced by 100% in the presence of *Zataria multiflora* essential oil (231.8 µg/mL), while, the seeds contaminated with the same count of *X. campestris* were disinfected at higher concentration of *Zataria multiflora* essential oil (463.5 µg/mL). Killing of *X. campestris* cells adhered to the seeds with uneven surface and deep grooves required greater concentrations of the essential oil. The impregnation of the seeds in the essential oil at 463.5 µg/mL showed that a 2-h exposure period causes the least possible damage to the seedlings and is sufficient to prevent the occurrence of the disease caused by *X. campestris* in *Brassica oleracea*.

## 1. Introduction

Green plants are permanently exposed and damaged by a number of invading bacteria in both anthropogenic and natural environments. Plant diseases caused by pathogenic bacteria adversely affect the agricultural economy worldwide. The genus *Xanthomonas,* a member of the gamma subdivision of the Proteobacteria, is an important phytopathogenic bacterial taxon that causes widespread pre- and postharvest losses. The genus is comprised of 33 species, each including a number of pathogenic varieties (LPSN: http://www.bacterio.net). They cause serious diseases in various cultivated crops, vegetables, grasses, fruit trees, and ornamentals, damaging about 124 monocotyledonous and 268 dicotyledonous plants (Zheng et al., 2016; Bajpai et al., 2011). The predominant bacterial species within the genus is *Xanthomonas campestris* that is known to contain six pathovars including; *aberrans*, *armoraciae, barbarea*, *incanae*, *raphani*, and *campestris* (Berg et al., 2005; Lange et al., 2016). Black rot and leaf spot are among the most important diseases in crucifers caused by *X. campestris* pv. *campestris* (Barman et al., 2015; Singh et al., 2016). *X. campestris* can infect crucifers at any growth stage and cause yield loss when warm and humid conditions are followed by periods of rain during early crop development (Liu et al., 2016). Plant debris, seeds, and weeds are the most important sources of inocula for black rot (Soudi et al., 2011; Krauthausen et al., 2017).

Different management strategies have been developed against phytopathogenic bacteria that include the use of microbial-free seeds and seedlings, inherently resistant cultivars (Luiz et al., 2016), antibiotics (Stockwell and Duffy, 2012; Dunegan, 1954) antimicrobial chemicals (Sayler and Kirkpatrick, 2003; Behlau et al., 2008) and biocontrol agents (Wulff et al., 2002; Massomo et al., 2004; El-hendawy et al., 2005; Newman et al., 2008; Mishra and Arora, 2012;

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Ghazalibiglar et al., 2016; Shalini et al., 2017). However, these strategies are not always effective, especially when the pathogenic bacteria are widely disseminated, the plants are heavily affected and the environmental conditions are optimal for disease emergence. Prophylactic measures are preferred over the total control approaches (Kotan et al., 2014). Disinfection of pathogenic microorganisms by synthetic chemicals has been limited due to their carcinogenic effects, acute toxicity, and environmental hazards.

On the other hand, the application of the essential oils has shown promising results in combat with the plant pathogens and the epidemic multidrug resistant microorganisms (Sajed et al., 2013).

A variety of essential oils have been screened for their antimicrobial activity. About one-tenth of 300 essential oils have been used commercially and shown additional potential in the pharmaceutical, agronomic, food, sanitary, cosmetics and perfume industries (Akhtar et al., 2014).

*Z. multiflora* is a thyme-like plant belonging to the *Lamiaceae* family which grows wild in central and southern Iran, Pakistan, and Afghanistan. *Z. multiflora* essential oil (ZMEO) is the source of various oxygenated monoterpenes, including thymol and carvacrol that are known to have significant antimicrobial properties, evidently due to their phenolic monoterpenes. There is high concentration of thymol and carvacrol present in ZMEO that reveals its significant antimicrobial potential (Avaei et al., 2015). According to the European Chemicals Agency, thymol has shown no chronic side effects and its orally administered lethal dose ($LD_{50}$) in rats is 980 mg/kg (Sajed et al., 2013). The ZMEO has been used in several studies against a variety of microorganisms (Azizkhani et al., 2013; Mahboubi and Bidgoli, 2010; Eftekhar et al., 2011; Moshayedi et al., 2013). Several studies have been carried out on the effect of various essential oils against the genus *Xanthomonas* (Altundag et al., 2008; Chudasama and Thaker, 2012; Lucas et al., 2012; Moghaddam et al., 2014). However, fewer studies have focused on the effect of the ZMEO against *X. campestris* (Samavi et al., 2009). In this study, the efficiency of the ZMEO towards the disinfection of the seeds of *Brassica oleracea* var. *capitata* contaminated with a local strain of *X. campestris* was investigated.

## 2. Materials and methods

### 2.1. X. campestris strains

Local strains of *X. campestris* have previously been isolated from agricultural soils of countryside of Tehran, Karaj and Ahvaz cities, Iran (Soudi et al., 2011). The strains and the related (accession number) in NCBI Nucleotide Database are listed as: SAM3301 (KP419705), SAM4101 (KP419704), SAM4204 (KP419715), SAM4205 (KP419706), SAM4210 (KP419707), SAM4213 (KP419709), SAM4217 (KP419716) and SS309 (KX599546). We have already studied the presence of the virulence *hrcC* gene in the mentioned strains and proved their relation to *X. campestris* as origin (Soudi et al., 2011). The lyophilized cells of the *X. campestris* strains were restored using normal saline solution (9 g NaCl/L), cultivated on Yeast Malt Agar and subcultured every 14 days. The type strain, *X. campestris* DSM1706, was purchased from Persian Type Culture Collection (IROST, Tehran, Iran) and used as the control strain.

### 2.2. The essential oil and antimicrobial agents

The *Zataria multiflora* essential oil (ZMEO) was purchased from Barij-Essence Pharmaceutical Company (Kashan, Iran). Thymol and carvacrol were purchased as pure chemicals from Biobasic and Sigma-Aldrich, respectively. The analysis of the ZMEO was carried out by Gas chromatography mass spectrometry (GC–MS) using a GC–MS, Thermo Quest-Finnigan instrument equipped with a DB5-fused silica column (30 m × 0.25 mm, film thickness 0.25 μm) and a flame ionization detector. Helium was used as the carrier gas at a constant flow rate of 1.1 mL/min and a split ratio of 1/100. The oven temperature was raised from 60 °C to 250 °C at the rate of 5 °C/min. Injector and detector temperatures were 250 °C and 200 °C, respectively (Eftekhar et al., 2011).

### 2.3. Cabbage seeds preparation and disinfection

The seeds of *Brassica oleracea* var. *capitata* (Cabbage Glory of Enkhuizen) were the product of Europseeds Holland Company (Batch No. 527677) and purchased from Pakan Bazr Company (Isfahan, Iran). They were originally bred in 1899 and released by N.V. Sluis en Groot's Koninklijke Zaadteelt en Zaadhandel of Enkhuizen, Netherlands in 1902. The seeds were refrigerated at 4 °C under arid conditions. The germination rate of the seeds was re-evaluated in our laboratory according to the previously published protocol (Gerszberg et al., 2015; Jin et al., 2000).

The surface of cabbage seeds was disinfected to destroy any saprophytic and/or pathogenic microorganisms. In order to fulfill this task, the seeds were initially washed in running tap water for 1 h. They were then dipped in 70% (v/v) ethanol for 2 min, exposed to 1% (v/v) sodium hypochlorite for 15 min, washed three times with sterile distilled water, and finally dried at room temperature for 30 min (Kotan et al., 2014; Cantore et al., 2009). The resulted disinfected seeds were then divided to two groups. The first group was used as a negative control to check the quality of disinfection; while, the second group was used in further experiments.

### 2.4. Determination of antimicrobial activity of the ZMEO, thymol and carvacrol

Microdilution susceptibility assay was carried out according to the NCCLS guidelines (National Committee for Clinical Laboratory Standards, NCCLS, 2012) based on which the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The ZMEO, thymol, and cavacrol were dissolved in dimethyl sulfoxide (DMSO) (32 mg/mL) and transferred into 96-well microtiter plates. The ZMEO, carvacrol, and thymol were serially diluted and their final concentrations were set in the corresponding wells from 927 to 7.2 μg/mL, 244 to 0.9 μg/mL, and 200 to 0.4 μg/mL, respectively. The *X. campestris* cells were cultured overnight on Yeast Malt Agar at 28 °C. A loopful of colonies were transferred to 5 mL Yeast Malt Broth in the test tube and further incubated at 28 °C for 6 h. Thereafter, the bacterial suspension was adjusted to 0.5 McFarland and diluted (150-fold) in Yeast Malt Broth ($10^6$ CFU/mL). Finally, 100 μL aliquots of the inocula were added into the microplates and the test was carried out at a final volume of 200 μL. Positive and negative controls of bacterial growth were implemented by using Yeast Malt Broth without addition of any antimicrobials and the same culture medium free from the bacterial cells, respectively. The microplates were incubated at 28 °C for 48 h. The lowest concentration of the compound that inhibited bacterial growth (MIC) was determined after 48 h. The MBC values were determined based on the results obtained from their cultivation on the Yeast Malt Agar. All the tests were carried out in duplicates.

### 2.5. Measurement of growth and viability of X. campestris

The growth and viability tests were carried out by cultivation of *X. campestris* SAM4213 in shaking flasks. Primarily, the growth of *X. campestris* in Yeast Malt Broth containing DMSO (8 mg/mL), DMSO (8 mg/mL) plus ZMEO (231.8 μg/mL), and the control without DMSO and ZMEO was assessed within 22 h by measurement of spectrophotometric absorbance (at 600 nm wavelength) of samples taken from the culture flasks in 2-h intervals. Each flask was inoculated with a concentration of $1.5 \times 10^8$ CFU/mL and then incubated at 28 °C and shaking rate of 150 rpm.

The viable cell count assay was conducted by collecting multiple