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Antibacterial activities of the essential oils from medicinal plants against the growth of *Clavibacter michiganensis* subsp. *michiganensis*

Abstract

In the present study antibacterial activities of essential oils obtained from aerial parts of aromatic plants such as thyme (*Thymbra spicata* subsp. *spicata*), oregano (*Origanum syriacum* var. *bevanii*), mint (*Mentha spicata*), and lavender (*Lavandula stoechas* subsp. *stoechas*) were investigated against the seed-borne plant pathogenic bacterium, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm* ICP7200), the causal agent of bacterial canker or wilt of tomato. By using the paper disc diffusion assay, all essential oils have shown antibacterial activity. Essential oils used in the paper disc diffusion assay varied in their antibacterial activity. Essential oil from thyme was the most effective in inhibiting the growth of *Cmm,* followed by those obtained from oregano and lavender. By using the micro agar broth dilution assay, the minimum bactericidal concentrations of the essential oil of thyme, oregano, ment and lavender were 10, 10, 25 and 50 µg/ml, respectively.

Introduction

Plant diseases caused by bacteria are one of the major problems in crop cultivation in several agricultural commodities (1). At present, rapid and effective management of plant disease and microbial contamination in several countries is generally achieved by the use of synthetic pesticides and antibiotics. Chemical control of plant disease relies upon the use of antibiotics (such as streptomycin) in USA or copper compounds in the rest of the world; such control methods prevent bacterial multiplication but are not always adequate controls of seed-borne inoculum. Unfortunately, the frequent use of pesticides and antibiotics against plant and human pathogenic bacteria has led to the selection of resistant bacterial populations against antibiotics. The high cost of pesticides, development of pesticide/antibiotic resistant food-borne and plant pathogenic isolates, governmental restrictions on the use of antibiotics against plant pathogens in European countries, including Turkey, and the interest of environmental consideration raise the need to find alternative control methods. The antimicrobial properties of essential oils and their major constituents from a wide range of aromatic plant species have been assessed against the comprehensive range of microorganisms including bacteria, fungi and viruses (2,3).

Bacterial canker, caused by *Cmm*, is a recurrent and serious disease of field and greenhouse-grown tomatoes (*Lycopersion esculentum*) in several countries (4). The disease agent leads to vascular infections, wilting, chlorosis, and eventual death of the plant. Disease control is difficult because of the lack of commercially available resistant tomato cultivars.

Observations in the eastern Mediterranean region of Hatay province, Turkey, indicate the existence of weld types with a rich composition of indigenous aromatic and medicinal plant species. Therefore this research was conducted to evaluate the antibacterial potential of essential oils from oregano (*Origanum syriacum*), thyme (*Thymbra spicata*), mint (*Mentha spicata*) and lavender (*Lavandula stoechas* ssp. *stoechas*) (Table) growing in the region against bacterial canker of tomato agent (*Cmm*.).

Table Inhibitory effect of plant essential oils and antibiotics on growth of *Clavibacter michiganensis subsp. michiganensis*. Diameter of inhibition zone including disc diameter of 6 mm. Numbers in parenthesis are standard errors of means. Means in the column followed by different letters are significantly different according to Duncan Multiple Range Test (P<0.05)

Plants and agents	Inhibition zone (mm)	% increase of inhibition zone
Thymbra spicata	37.2 (2.62) F	83.6
Origanum syriacum	35.6 (1.78) F	82.8
Mentha spicata	31.3 (1.38) E	80.5
Lavandula stoechas subsp. *stoechas*	23.7 (1.27) D	74.2
Tetracycline (100 µg/ml)	18.7 (0.79) C	67.3
Ethanol (100%)	14.9 (0.43) B	59.0
Control (sterile medium)	6.1 (0.19) A	0

% increase was calculated by using formula;
% increase: 100 - [(inhibition zone in control plate) / (inhibition zone in treated plate)*100]

Material and methods

Plant material and isolation of essential oils: The plants used in this study were identified by Dr. İ. Uremis. Voucher specimens have been deposited in the herbarium of the Plant Protection Department, MKU. Essential oils from air dried leaves of plants were extracted by steam distillation with Clevenger's apparatus for 2.5 h. After extraction, oil fractions were separated, dried over anhydrous sodium sulphate and stored in amber bottles at 4 ^{o}C until required.

Test microorganisms and cultural methods: The strain of *Cmm*, ICP7200, was preserved on modified nutrient yeast dextrose agar (NYA) at 4 ^{o}C. Inoculum suspensions were prepared from early log-phase cells, which were obtained by growing the bacteria in nutrient yeast extract broth in 25 ml sterile tubes and incubated at 27 ^{o}C on an orbital shaker at 200 rpm for 24 h. Bacteria were subsequently pelleted by centrifugation (twice, each at 3500 *g* for 5 min) and washed in sterile distilled water (SDW). The concentration was adjusted to 10^8 cfu ml^{-1} by dilution to give an OD_{640} of 0.12. These suspensions were used as required.

Determination of antibacterial activity of the essential oils: The antibacterial activity of the essential oils was determined by using the paper disc diffusion technique. Briefly, the test was performed in sterile Petri dishes (100 mm diameter) containing solid and sterile appropriate media. The surface of plates with appropriate media was inoculated with 200 µl of bacterial suspension prepared as described previously. Sterile filter paper (Whatman No.1) discs (6 mm in diameter) containing 10 µl of the tested essential oils were placed in the centre of the agar surface. Discs containing 10 µl sterile broth media were used as negative control. The reference antibiotics (tetracycline) amended discs, at 100 µg/ml concentrations, and 70% ethanol were used as positive control for comparison. The lid of each individual Petri dish was replaced immediately to prevent eventual evaporation. After allowing 1 h at room temperatures for the essential oils to diffuse across the surface, the plates were sealed with sterile Parafilm and incubated at 25 ^{o}C for 48 h. The antibacterial activity of oils and antibiotics was demonstrated by a clear zone of inhibition around the disc. The zone of inhibition was measured with the help of Vernier calipers.

The minimum inhibitory concentrations (MICs) of the essential oil against the test micro-organisms were determined by the broth micro dilution method. All tests were performed in LB broth supplemented with DMSO (Merck) (final concentration of 0.5%) to enhance the oil solubility. Bacteria were incubated overnight at 26 °C in LB broth. Test strains were suspended in LB to give a density of 5×10^5 cfu ml^{-1}, confirmed by viable counts. Dilutions of the essential oil ranging from 1 to 50 µg ml^{-1} were prepared in 1 ml Eppendorf tubes. All determinations were performed in duplicate and two growth controls consisting of LB medium and LB with 0.5% (v/v) DMSO were included. All tubes were incubated at 26 °C for 24 h. Following incubation, 100 µl inoculum suspensions from each concentration of different oil were separately spread onto sterile NYA plates and incubated for further 48 h. The numbers of colonies on each Petri plate were counted. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of bacteria on the agar plate.

Five replicate plates of each essential oil for each bacterium were used in all tests. The data were subjected to analysis of variance (ANOVA) by using SPSS statistic program (Version 11.05) and the significance between treatments was determined by means of Duncan's Multiple Range Test (P<0.01).

Results and discussion

The antibacterial activity of the essential oils from each aromatic plant under study was estimated by using the paper disc diffusion technique and the response of *Cmm* to the essential oils is presented in Table 1. Differences between the plant essential oils were significant. Essential oils from *T. spicata* and *O. syriacum* had the highest inhibitory activity against the bacterium corresponding to 83.6% and 82.8 % increase in the zones of inhibition over the control (water). This was followed by essential oils obtained from mint and lavender (80.5 and 74.2% respectively). Efficacy of essential oils from thyme and oregano was comparable with the antibiotic tetracycline and ethanol even at the highest concentration (Table). The growth inhibition of test micro-organism was also evaluated by using the broth micro dilution method. The lowest MIC was determined against *Cmm* at 10 µg ml^{-1} concentration of *Origanum* and thyme oil, followed by mint and lavender oils with an MIC of 25 and 50 µg ml^{-1} respectively.

Previous studies had been conducted to assess the efficacy of essential oils from the medicinal plants, including those used in this study, against some phytopathogenic fungi and nematodes in Turkey (5,6,7) and in other countries (8,9,10,11). Antibacterial effects of essential oils have been examined mainly on human pathogens, spoilage microorganisms and dermatophytes. A relatively limited number of reports was found in the literatures on plant extracts or/and essential oils against phytopathogenic bacteria. To our knowledge there is no research on the evaluation of the efficacy of essential oils against *Psp*. Earlier studies have demonstrated the ability of different species of oregano and thyme oils to retard and inhibit the growth of various plant pathogenic bacteria such as *Agrobacterium tumefaciens*, *Cmm*, *Erwinia amylovora*, *E. caratovora*, *E. herbicola*, *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Xanthomonas axonopodis* pv. *vesicatoria* (12,13,14,15,16,17,18). The results of this investigation showed that essential oils obtained from oregano, thyme, mint and lavender growing in the region have an antibacterial potential against the seed-borne tomato pathogen *Cmm,* as they showed similar antibacterial activity against halo blight of bean agent *Pseudomonas syringae pv. phaseolicola* (19). The mode of action of essential oils against bacteria is not known, however the involvement of essential oil components may disrupt the cell membrane of the bacterium and change its permeability, as reported by Sivropoulou et al. (20). Essential oils of plants belonging to the *Lamiaceae* family are rich in phenolic compounds, which are believed to be responsible for the marked antimicrobial activity. Essential oils of oregano, thyme and rosewood have been reported to induce rapid cell lysis of *Streptococcus pneumoniae* (21). Our results suggest that essential oils have the potential for use in bacterial control. Because of essential oils and/or their main components such as carvacrol, thymol and linalool have been reported to possess fungicidal and bactericidal activities (22,23), seed treatment with essential oils or their components could serve as a seed disinfectant. However, further experiments are needed to obtain information regarding the economic aspects and antibacterial activities of essential oils *in vivo* without phytotoxic effects on seed germination. Research on the chemical composition of the essential oils used and antibacterial activities against a variety of plant pathogenic bacteria and fungi are currently under investigation.

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