Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2015, volume 100, livre 4, pp. 200-207
First National Conference of Biotechnology, Sofia 2014

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST PEPPER BACTERIAL SPOT AGENTS

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Keywords: antibacterial activity, essential oils, *Xanthomonas vesiatoria, Xanthomonas euvesicatoria*

Abstract: Various essential oils have potential for use in alternative strategies for plant pathogen control. In this work twelve essential oils and several of their components were screened for antibacterial activity against the causative agents of bacterial spot of pepper – *Xanthomonas vesicatoria* and *Xanthomonas euvesicatoria*, using the agar diffusion method. Almost all essential oils and their components showed a strong inhibitory effect against the tested bacteria. The minimum inhibitory concentration varied between 0.39% and 6.25%, depending on the different oils and strains. The potential of the oils for treatment of infected pepper seeds for control of *X. vesicatoria* and *X. euvesicatoria* was investigated. All of them showed high decontamination activity (between 73 and 90%), but the seed germination was inhibited at the concentrations used. Additional studies for establishing the optimal treatment concentration ensuring good levels of both decontamination and seed germination are needed.

INTRODUCTION

Plants are incessantly exposed and threatened by a variety of pathogenic bacteria. The control of the diseases is a serious problem in plant production. Bacterial diseases caused by the pathogens of genus *Xanthomonas* have devastated different plants, leading to considerable losses in productivity and quality of the harvests (Cavalcanti et al., 2006; Ji et al., 2008). Quick and easily applicable management of plant diseases and microbial contamination is the use of chemical compounds. At present, only a small assortment of chemical compounds is available for the growers. The compounds on the market are almost exclusively based on copper or different antibiotic preparations (Burr, 2001; McManus and Stockwell, 2001; Janse, 2005). Because of the general toxicity and the development of resistant strains spraying with copper based compounds exert a negative impact on the environment and on the yield. Antibiotics are forbidden in many countries because of their high and acute toxicity, accumulation in the food chain and long degradation periods. Members of the genus *Xanthomonas* are reported to have developed resistance to several antibiotics such as kanamycin, ampicillin, streptomycin and penicillin (Rodriguez et al., 1997). As an alternative strategy for plant protection and the spread of the diseases different plant compounds have been tested for their antimicrobial activity. In the past few years studies have been conducted providing evidence that some aromatic plants might be a potential source of natural pesticides (Balandrin et al., 1985; Đorđević, et al., 2007; Tripathi and Kumar, 2007; Patharakorn et al., 2010). Essential oils, as odorous and volatile products of the secondary metabolism of aromatic plants, normally formed in special cells or groups of cells could be used as antimicrobial agents in the control of the disease causes by *Xanthomonas* species (Nguefack et al., 2005; Bajpai et al., 2011). Due to their more systematic and easily biodegradable nature essential oils were proved to be better substitutes for synthetic antibiotics (Claflin, 2003). The antimicrobial activity of the essential oils is assigned to small terpenoids and phenolic compounds which exhibit antimicrobial activity when tested separately (Gyorgyi et al., 2004). This growing interest in finding bio-pesticides in order to control the diseases is emphasized by the fact that the diseases caused by the plant pathogenic bacteria of *Xanthomonas* species are still a major problem even in the developed countries (McManus et al., 2002).

The aim of this study was to investigate the antibacterial activity of twelve essential oils and several of their components against the causative agents of bacterial spot of pepper – *Xanthomonas vesicatoria* and *Xanthomonas euvesicatoria*.

MATERIALS AND METHODS

Bacterial strains

Two phytopathogenic bacterial species – *X. euvesicatoria* 1M and 10b (from Macedonia and Bulgaria, respectively) and *X. vesicatoria* 44M and 2b (from Macedonia and Bulgaria, respectively) (Vancheva et al., 2013) causative agents of bacterial spot of pepper were used as test-microorganisms in this study. The bacteria were maintained on potato glucose agar (PGA).

Essential oils

Twelve essential oils and several their components (Tabl. 1) were screened for antibacterial activity against the tested phytopathogenic bacteria.

***In vitro* antibacterial activity**

The *in vitro* antibacterial activity of the essential oils and their components was determined by the well diffusion method. Petri plates with 15 ml of nutrient glucose agar were inoculated with $1.5.10^7$ cfu/ml (0.5 MacFarland standards) of 24 h suspensions of phytopathogenic test-bacteria, grown on PGA. Wells were cut into agar and filled with 20 µl of each essential oil. Standard disks impregnated with streptomycin (10 µg/disk) were used as control. The plates were placed for 2 h at 4^oC to allow the diffusion of extracts and cultivated for 24-48 h at 37^oC. The experiments were performed in triplicate. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone. The index of antimicrobial activity (IAA) was calculated to evaluate the efficacy of the tested oils and compounds compared to the control antibiotic:

$$\mathbf{IAA\ (\%) = \{-1\times[(C-T)/(C+T)]\}\times 100}$$

where: **C** is the average inhibitory zone (mm) of streptomycin and **T** is the average inhibitory zone (mm) of the tested oils and compounds.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by the micro-dilution method using the well diffusion method as described above. For each essential oil twelve serial twofold dilutions in dimethyl sulfoxide (DMSO) were prepared (from 50 to 0.02%). Wells into agar were filled with 20 µl of the diluted oils. After diffusion of the oils for 2 h at 4^oC the plates were cultivated for 24 h at 28^oC. Pure solvent DMSO was used as a control. All experiments were performed in triplicate. The lowest concentration of the oils that formed inhibition zone was regarded as MIC.

***In vivo* antibacterial activity**

The *in vivo* antibacterial activity of five essential oils was performed using pepper seeds (cv. Dzhulinska Shipka) inoculated independently with phytopathogenic bacteria (10^8 cfu/ml). Vacuum infiltration was used for the inoculations and treatments by the oils (Bogatzevska, 2002). Inoculations were conducted at exposition 2h, followed by 24h of air drying and treatments at exposition 2x10 min. Three seed controls were used: inoculated untreated, non-inoculated untreated, and non-inoculated treated seeds. All the analyses were performed in triplicates with 200 seeds per variant. Half of the seeds were incubated in sterile wet chambers for 5-6 days at 24^oC. To establish infection the rest of the seeds were plated on TTC medium for 3 days at 28^oC. Growth of specific coloured bacteria was indicator for the presence of infection. For assessment of the *in vivo* antibacterial effect of the oils the average percentage (rate) of healthy seeds and the rate of germination was calculated.

RESULTS AND DISCUSSION

All tested essential oils and components had an inhibitory effect on the tested microorganisms. The best effect was achieved by the oils from rosmarinus, pelargonium, and rose and the components isobutyl angelate, isobutyl angelol, and citronellol. All of the strains were equally sensitive to these compounds. The smallest inhibition zones were formed by the oil from chamomile and cis-nerolidol which showed activity with ≤50% lower than the rest of the tested compounds (Tabl. 1).

Differences in the activity were noticed for most of the oils and three components: Bulgarian *X. euvesicatoria* was more susceptible than the Macedonian strain. On the contrary, Bulgarian *X. vesicatoria* was less susceptible than the Macedonian one (Tabl. 1).

The essential oil of jasmine had different activity being more effective against the Bulgarian strains of the two species. The Macedonian strains were more susceptible to geranyl formate as antibacterial compound. Large diversity in the inhibition zones of *X. vesicatoria* strains (between 40-60%) were observed for the oils from salvia, mint and melissa (Tabl. 1).

The IAA of only chamomile and cis-nerolidol was negative for *X. euvesicatoria*. Better effect was achieved for both *X. euvesicatoria* and *X. vesicatoria* by the oils from rosmarinus, pelargonium, and rose and the components isobutyl angelate, isobutyl angelol, and citronellol compared to the streptomycin control (Fig. 1).

Table 1. Antimicrobial activity of essential oils and their compounds against the pathogens causative agents of bacterial spot of pepper.

Essential oil / component	Test-bacteria/diameter of the inhibitor zone, mm			
	X. euvesicatoria 1M	*X. euvesicatoria* 10b	*X. vesicatoria* 44M	*X. vesicatoria* 2b
Essential oils from:				
Lavandula angustifolia	30	35	30	23
Hyssopus officinalis	30	35	30	27
Salvia officinalis	30	35	30	12
Melissa officinalis	30	35	30	18
Mentha piperita	30	35	30	16
Pimenta dioica	30	35	30	27
Thymus vulgaris	30	35	30	28
Rosmarinus officinalis	35	35	35	35
Pelargonium odorantissimum	35	35	35	35

Rosa damascena	35	35	35	35
Matricaria chamomilla	14	14	17	11
Jasminum gradiflora	30	35	30	35
Components:				
nerol	30	35	30	25
eugenol	30	35	30	28
menthone	30	35	30	29
isobutyl angelate	35	35	35	35
isobutyl angelol	35	35	35	35
citronellol	35	35	35	35
geranyl formate	35	30	35	30
cis-nerolidol	18	16	13	14

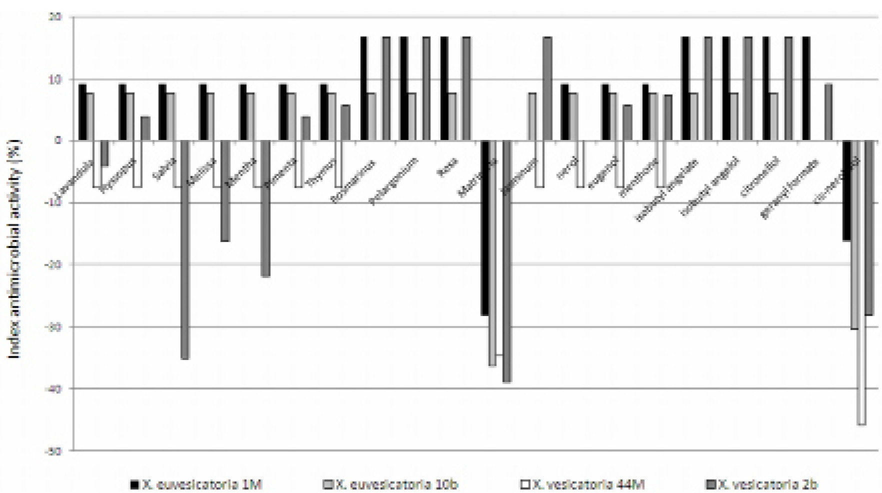

Figure 1. Index of antimicrobial activity (%) of the tested essential oils and components vs the streptomycin control.

The minimal inhibitory concentration (MIC) of five essential oils (melissa, mint, salvia, lavandula, hyssopus) was determined. The highest dilution with growth was the minimum inhibitory concentration.

The MIC values were in the range from 0,39% - 6,25% with the highest antibacterial activity of the essential oil from melissa (Tabl. 2). The differences in the antimicrobial activities were probably due to the different chemical composition.

Table 2. Minimal inhibitory concentration of essential oils to the test-phytopathogenic bacteria.

Bacterial species	MIC, % (v/v)				
	L. angustifolia	*M. officinalis*	*M. piperita*	*S. officinalis*	*H. officinalis*
X. euvesicatoria (10b)	0,78	0,39	6,25	0,78	0,78
*X. vesicatoria (*44M)	1,56	0,39	6,25	0,78	0,78

The treatment with essential oils of artificially contaminated with *Xantomonas* pepper seeds confirmed the efficacy determined in the *in vitro* experiments. The total count of infected seeds was significantly reduced to 10-19% compared to the untreated control (Tabl. 3). However, the germination rates were negatively impacted with values near zero compared to the 10-15% reduction of the germination of the infected untreated seeds. Further analyses on the concentrations of combinations of the essential oils are required in order to obtain an antimicrobial effect with minimum undesirable effects on germination.

Table 3. Decontamination effect of essential oils on infected pepper seeds.

Essential oil	Decontaminated seeds, %	
	X. vesicatoria 44M	*X. euvesicatoria* 10b
L. angustifolia	86	74
H. officinalis	81	75
M. officinalis	81	86
S. officinalis	81	73
M. piperita	90	81

The increasing worldwide interest in the production of safe and diseases-free foods leads to a constant search of efficient and secure remedies for controlling bacterial plant diseases. In the recent years, the use of natural antimicrobial agents of a plant origin, including essential oils is the first choice in the disease management (Ornek et al., 2007). Bajpai et al., 2011 reported the *in vitro* efficacy of essential oils against *Xanthomonas campestris pv. vesicatoria* (Gyorgy et al., 2004). Previous *in vivo* studies showed that different essential oils had varying degree of antibacterial activity against different plant pathogens (Hevesi et al., 2006). However, little data is available on the susceptibility of the causative agents of bacterial spot of pepper to essential oils and other compounds. Essential oil from mint has been reported for activity against *X. vesicatoria* (Vasinauskiene et al. 2006). The used in this study *X. vesicatoria* strains proved to be more susceptible to the oil from mint and even higher activity was observed against *X. euvesicatoria* strains.

The two pathogens retain and transmit through the seeds. Infested seeds have been found to be a major source of inoculum for bacterial spot (Mirik and Aysan, 2009; Dutta et al., 2014). The treatment of seeds should be seen as a valuable step in crop protection and the selection of specific plant essential oils could be the key in limitation of the disease. However, the sole use of the tested essential oils for seed treatments proved to be not applicable due to the reduction of germination. A combination of plant extracts or essential oils with copper and other chemicals has been reported to increase their effectiveness (Kokoskova et al., 2011). In order to obtain a sustainable reduction of harmful pathogens of pepper, the potential of the essential oils from lavandula, hyssopus, salvia, melissa, mint, pimenta, thymus, rosmarinus, pelargonium, rose, jasmine and their components can be used as a part of combination treatment with other natural products or chemicals.

Acknowledgments: This work was supported by grant 139/14 of Scientific research fund of Sofia University.

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