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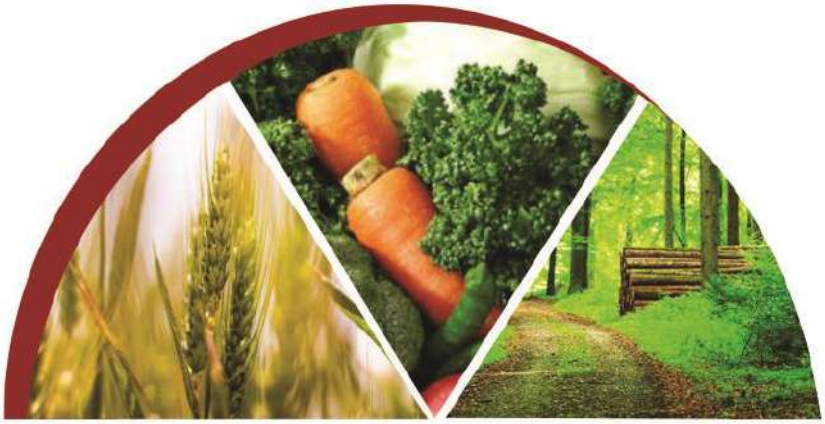
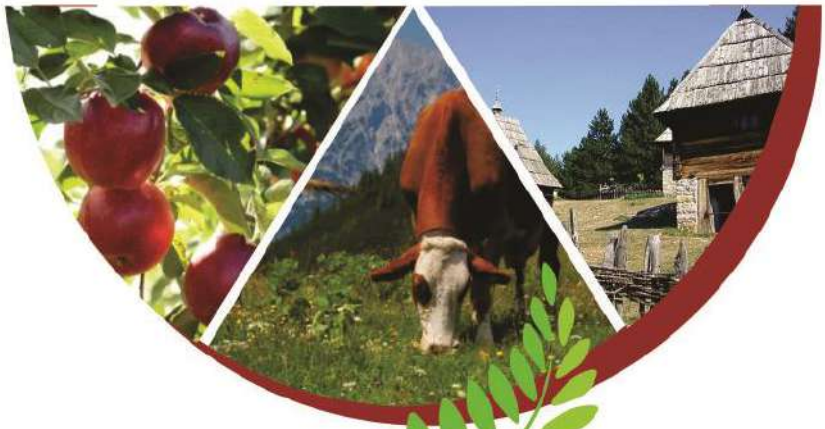
AgroSym

VIII International Scientific Agriculture Symposium

Jahorina, October 05-08, 2017





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ESSENTIAL OILS AS AN ALTERNATIVE BACTERICIDES AGAINST SOFT-ROT BACTERIA, *PECTOBACTERIUM CAROTOVORUM* SUBSP. *CAROTOVORUM*

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Abstract

Bacterial soft-rot disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* is a very destructive disease with a diverse host range in agricultural crops, during the plant growth in the field and in storage. Control, usually based on the use of chemical products, is not satisfactory because of their harmful effect on human health and environment, as well as the possibility for pathogen resistance development. Therefore, developing of new natural products (such as essential oils-EOs, plant extracts etc.) with a sufficient efficacy in control of the disease was imposed. The current study included *in vitro* testing of bactericidal activity of 51 different oils against *P. c.* subsp. *carotovorum*, using an agar-diffusion assay. Bacterial suspension was mixed in Nutrient Agar to final concentration of 10^7-10^8 cells/mL and poured in sterilized Petri plates (ø 90 mm). After media solidified, sterile filter paper discs (ø 5 mm) were placed on the surface of the Petri plates and supplemented with 20 μL of each tested oils. The experiment was performed in a completely randomized design. The results were expressed as a width of inhibition zone (mm) and analyzed by one-factor analysis of variance by using the software package Statistica 7.0 (StatSoft, Inc.). Results showed that the 7 most efficient EOs against *P. c.* subsp. *carotovorum* (*Thymus vulgaris*, *Cinnamomum cassia*, *Cassia angustifolia*, *Origanum vulgare*, *Boswellia serrata*, *Eucalyptus globulus*, *Satureja montana*, respectively) achieved inhibition zone greater than 20 mm. Inhibition zones in the range of 15-20 mm were achieved by 8, 10-15 mm by 9, and less than 10 mm by 6 EOs. The rest of 21 Eos did not show any bactericidal effect.

Keywords: *in vitro, bacteria, inhibition, essential oils.*

Introduction

Soft rot phytopathogens are responsible for several macerating diseases that can occur during the plant growth in the field but is more common during transport and in storage (Babadoost, 1990; Des Essarts *et al*., 2016). The *Pectobacterium* bacteria (*Pectobacterium carotovorum* subsp. *carotovorum*, *P. atrosepticum*, *P. wasabiae*, *P. carotovorum* subsp. *brasiliense*) are among the most important responsible for these disease on a wide range of crop and ornamental plants (Babadoost, 1990). The damage caused by these pathogens remains an important issue in many countries worldwide (Des Essarts *et al*., 2016). The bacteria attack succulent, tender tissues of storage organs (tubers, fruits, roots, bulbs, corms, rhizomes), as well as buds, stems, petioles and leafstalk tissues. The disease initially starts on leaves and stems in form of small, water-soaked, translucent lesions that rapidly enlarge in diameter and depth. The infected tissue becomes watery. Slimy bacteria exudates and cellular debris frequently ooze out from cracks in the tissues. Within one to three days, fleshy organs may rot

and collapse. Decaying tissue gives off a characteristically putrid odour which comes from saprophytic bacteria that are growing in the decomposing tissues (Babadoost, 1990).
Control of soft rot pathogens is based on agricultural practices (eradication of diseased plants, removal and destroying of crop residues, crop rotation) and chemical control commonly based on the use of copper based products. The increasing interest in natural products as an alternative in disease control is mainly due to their low impact on the human health and environment (Jeong *et al*., 2009; Mohan *et al.*, 2011). Natural substances such as essential oils (EOs) often function as antibacterial, antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal or inducing resistance in plants through activation of biochemical defence pathways (Deans and Ritchie, 1987; Mihaliak *et al*., 1991; Isman, 2000; Burt, 2004; Freeman and Beattie, 2008). EOs are aromatic and volatile oily liquids as products of secondary metabolism of aromatic plants, formed in special groups of cells and commonly concentrated in one particular region such as leaves, bark or fruit (Gutierrez *et al*., 2008). They present complex mixtures of natural components among which predominate terpenes, terpenoids, aromatic and aliphatic components (Pichersky *et al*., 2006; Bakkali *et al*., 2008). These are particularly present in families: *Lamiaceae, Asteraceae, Lauraceae, Zingiberaceae, Myrtaceae, Rutaceae, Apiaceae* and *Pinaceae* (Gorunović and Lukić, 2001). The antibacterial effect of EOs and their components against soft rot pathogens was reported by some authors (Kalemba and Kunicka, 2003; El-Zemity *et al*., 2008; de Lira Guerra *et al.*, 2014; Umunna and Anselem, 2014). Considering that soft rot disease is a one of limiting factor in the many crop production worldwide and the fact that effective control measures are insufficiently, the aim of this work was to evaluate the inhibitory potential of some EOs on the growth of soft rot pathogen, *P. c.* subsp. *carotovorum* by *in vitro* assay.

Material and Methods

For the experiment the EOs of *Abies alba, Ables sibirica, Anethum graveolens, Boswellia carteri, Boswellia serrata, Cananga odorata, Cassia angustifolia, Cedrus atlantica, Cinnamomum cassia, Cinnamomum verum, Citrus bergamia, Citrus x limon, Citrus x sinensis, Coriandrum sativum, Cupressus sempervirens, Curcuma longa, Cymbopogon flexuosus, Cymbopogon martinii, Eucalyptus globulus, Foeniculum vulgare, Gaultheria procumbens, Jasminum grandiflorum, Juniperus communis, Juniperus virginiana, Laurus nobilis, Lavandula angustifolia, Lippia citriodora, Melaleuca alternifolia, Melaleuca quinquenervia, Mentha x piperita, Myristica fragrans, Nigella sativa, Ocimum basilicum, Origanum vulgare, Pelargonium graveolens, Petroselinum crispum, Pimpinella anisum, Pinus nigra, Pinus mugo, Pinus sylvestris, Piper nigrum, Pogostemon patchouli, Ravensara aromatica, Rosmarinus officinalis, Salvia sclarea, Santalum album, Satureja montana, Syzygium aromaticum, Thymus vulgaris, Vanilla planifolia* and *Zingiber officinale* were used.
The strain of *P. carotovorum* subsp. *carotovorum* (KFB85) was obtained from the culture collection of the Faculty of Agriculture in Belgrade (Serbia). For use in the experiment, the strain was grown onto Nutrient Agar for 48 h at 26°C.
The inhibitory effect of the EOs on the growth of *P. c.* subsp. *carotovorum* was evaluated by Agar-diffusion assay. Bacterial suspension was mixed in Nutrient Agar to final concentration of 10^7-10^8 cells/mL and poured in sterilized Petri plates (ø 90 mm). After media solidified, sterile filter paper discs (ø 5 mm) were placed on the surface of the Petri plates and supplemented with 20 μL of each of 51 tested oils. There were four replicates for each tested oil. As positive and negative controls served plates with and without bacterial culture in medium treated with sterile distilled water, respectively.
The experiment was performed in a completely randomized design. The presence/absence and the diameter of inhibition halos (mm) were determined 72 hours after incubation at 26°C.

Data were analyzed by one-factor analysis of variance by using the software package Statistica 7.0 (StatSoft, Inc.).

Results and Discussion

Results showed that the 7 most efficient EOs against *P. c.* subsp. *carotovorum* (*Thymus vulgaris, Cinnamomum cassia, Cassia angustifolia, Origanum vulgare, Boswellia serrata, Eucalyptus globulus, Satureja montana,* respectively) achieved inhibition zone greater than 20 mm (Table 1). Inhibition zones in the range of 15-20 mm were achieved by 8 (*Melaleuca alternifolia, Rosmarinus officinalis, Anethum graveolens, Syzygium aromaticum, Laurus nobilis, Ravensara aromatica, Pimpinella anisum, Cinnamomum verum*), 10-15 mm by 9 (*Gaultheria procumbens, Cymbopogon martinii, Lavandula angustifolia, Cedrus atlantica, Citrus bergamia, Ocimum basilicum, Pinus sylvestris, Mentha x piperita, Lippia citriodora*), and less than 10 mm by 6 EOs (*Boswellia carteri, Pelargonium graveolens, Foeniculum vulgare, Pinus nigra, Cymbopogon flexuosus, Myristica fragrans*).
The rest of 21 EOs (*Cananga odorata, Nigella sativa, Santalum album, Coriandrum sativum, Petroselinum crispum, Citrus x limon, Citrus x sinensis, Zingiber officinale, Piper nigrum, Salvia sclarea, Abies alba, Juniperus communis, Ables sibirica, Pinus mugo, Cupressus sempervirens, Juniperus virginiana, Melaleuca quinquenervia, Pogostemon patchouli, Curcuma longa, Jasminum grandiflorum, Vanilla planifolia*) did not show any bactericidal effect.

Table 1. Inhibition zone in mm ($\overline{X}$ ±SE) caused by the influence of the tested EOs. F relation and P value from ANOVA; df – number of degrees of freedom. Experimental groups with the same letter in column are not significantly different (Duncan Multiple Range Test, $P < 0.05$)

EOs	Inhibition zone (mm)
	$\overline{X}$ ± SE
Rosmarinus officinalis	1.83 ± 0.03ef
Eucalyptus globulus	2.03 ± 0.03cd
Ocimum basilicum	1.23 ± 0.03ij
Lavandula angustifolia	1.33 ± 0.03ij
Pimpinella anisum	1.53 ± 0.03gh
Thymus vulgaris	2.97 ± 0.03a
Melaleuca alternifolia	1.93 ± 0.03cde
Syzygium aromaticum	1.77 ± 0.07ef
Lippia citriodora	1.03 ± 0.03kl
Laurus nobilis	1.70 ± 0.07fg
Boswellia serata	2.07 ± 0.07cd
Citrus bergamia	1.27 ± 0.03ij
Cymbopogon flexuosus	0.93 ± 0.03l
Cymbopogon martinii	1.37 ± 0.03hi
Myristica fragrans	0.87 ± 0.03l
Origanum vulgare	2.60 ± 0.06b
Satureja montana	2.03 ± 0.07cd
Pinus sylvestris	1.17 ± 0.09jk
Pinus nigra	0.93 ± 0.03l
Cedrus atlantica	1.27 ± 0.07ij
Foeniculum vulgare	0.93 ± 0.03l
Gaultheria procumbens	1.37 ± 0.03hi
Mentha x piperita	1.17 ± 0.03jk
Cinnamomum verum	1.53 ± 0.03gh

Cinnamomum cassia	2.80 ± 0.06ab
Pelargonium graveolens	0.93 ± 0.03l
Ravensara aromatica	1.67 ± 0.07fg
Boswellia carteri	0.97 ± 0.03l
Cassia angustifolia	2.73 ± 0.03ab
Anethum graveolens	1.80 ± 0.12ef
F	87
P	< 0.0001
df	29, 60

There are a lot of reports on the use of EOs on several pathogenic bacteria. Deans and Ritchie (1987) cited ten most inhibitory EOs against 25 bacterial genera and the most comprehensively inhibitory extracts were angelica (against 25 genera), bay (24), cinnamon (23), clove (23), thyme (23), almond (bitter) (22), marjoram (22), pimento (22), geranium (21) and lovage (20). Recently, many studies have been specified the bactericidal activity of thyme as a promising active ingredient effective against a wide range of plant pathogenic bacteria (Deans and Ritchie, 1987; Kalemba and Kunicka, 2003; Alamshahi *et al.*, 2010; Lucas *et al*., 2012; Rojas *et al*., 2014; Alamshahi and Nezhad, 2015). Kalemba and Kunicka (2003) reviewed that besides thyme, EOs of cinnamon, clove and mint were found to possess the strongest antimicrobial properties among many tested bacteria.

Potential antibacterial properties of EOs against *P. carotovorum* in *in vitro* trials were described by some researchers; c. *Thymus vulgaris*, *Rosmarinus officinalis*, *Coriandrum sativum*, *Cuminum syminum* by Alamshahi *et al*. (2010); cinnamon, clove, chenopodium, caraway, rosemary and thyme oils by El-Zemity *et al.* (2008); *Thymus vulgaris*, *Artemisia kermanensis*, *Lavandula officinalis*, *Rosemarinous officinalis*, *Eucalyptus caesia* by Mehrorosh *et al.* (2014); *Syzygium cumini* by Elansary *et al*. (2012); *Ziziphora clinopodioides* by Ozturk and Ercisli (2007). *Thymus vulgaris* compared to other extracts, possess the best inhibitory effect on *P. carotovorum* in the lowest concentration (Mehrorosh *et al.*, 2014). Results given by Nezhad *et al.* (2012) distinguish thyme as a most active for antibacterial activity against soft rot pathogen (*P. carotovorum*) and bacterial wilt pathogen (*Ralstonia solanacearum*); in the next positions were *Coriandrum sativum*, *Cuminum cyminum* and *Rosmarinus officinalis*; oil from *Eucalyptus globulus* was insignificant. Jeong *et al.* (2009) showing that EO from *Cymbopogon citrates* effectively inhibited the growth of *P. carotovorum* in a dose-dependent fashion, and 0.5% of the oil inhibited the growth of bacteria completely.

Variation in the potency of the EOs as well as in the sensitivity pattern of the test organisms was found by Umunna and Anselem (2014). Authors report *Zingiber officinale* oil to be most effective in the inhibition of *Erwinia* pathogen in the *in vitro* trials, but oils of *C. citratus* and *Azadirachta indica* in the *in vivo* experiment. *In vivo* trials given by Alamshahi and Nezhad (2015) showed that greenhouse experiments treatment by thyme EO caused a significant reduction in soft rot (*P. carotovorum*) and bacterial wilt (*R. solanacearum*) incidence on potato by 41 and 44%, respectively. Pradhanang *et al.* (2003) were found that *R. solanacearum* populations declined to undetectable levels in thymol, palmarosa oil, and lemongrass oil treatments showing that tomato seedlings transplanted in soil treated with EOs (700 mg/L of thymol oil, 700 ml/L of palmarosa oil, and 700 ml/L of lemongrass oil) were free from bacterial wilt and 100% of plants in thymol treatments were free of disease. De Lira Guerra *et al.* (2014) evaluated the action of 11 EOs in reducing soft rot in Chinese cabbage by spraying plants and found that spraying with the oils of bergamot, copaiba, *Eucalyptus citriodora*, spearmint and sweet orange has potential in the control of this disease.

Soft-rot or blackleg disease on potato could be controlled by using of EOs such as garlic (Nassar *et al.*, 2013) or thyme (Alamshahi *et al.*, 2010), respectively. Considering that thyme showed strong antibacterial activity against the *P. carotovorum* in our study and studies given by other authors it should be considered as a promising active ingredient for developing a new product for the disease control. Also, it should be remark that antimicrobial, antioxidant or other biological activities of natural EOs may be subject to change, based on vary in the chemical composition of the oil that may be observed due to the origin, their locality, the environmental conditions, and the stage of development of the collected plant material (Gulluce *et al.*, 2003).

Conclusion

According to the obtained results, EOs of *Thymus vulgaris*, *Cinnamomum cassia*, *Cassia angustifolia, Origanum vulgare*, *Boswellia serrata*, *Eucalyptus globulus and Satureja montana* potentially might be used as antibacterial agents against plant pathogenic bacterium *P. c.* subsp. *carotovorum* for further analysis *in vivo* studies. Development of EOs as natural antimicrobial agents has a potential for further developing in agricultural pest management.

Acknowledgment

This work was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Projects No.III43010 and No.III43001.

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