EFFECTIVENESS OF PLANT ESSENTIAL OILS AGAINST *ERWINIA AMYLOVORA*, *PSEUDOMONAS SYRINGAE* pv. *SYRINGAE* AND ASSOCIATED SAPROPHYTIC BACTERIA ON/IN HOST PLANTS

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SUMMARY

Plant essential oils of five aromatic herb species of the family Lamiaceae (*Origanum* sp., *Thymus* sp., *Melissa* sp., *Mentha* sp. and *Nepeta* sp.) were investigated for antimicrobial activity against plant pathogenic (*Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae*) and saprophytic (*Pseudomonas fluorescens*, *Pantoea dispersa* and *P. agglomerans*) bacteria commonly associated with both pathogens in plant tissue of healthy and/or diseased fruit trees in orchards. The screening was carried out in *vitro* on agar plates seeded with the target organism. All screened essential oils exhibited a higher level of antibacterial activity than streptomycin used as a standard in all tests. Plant essential oils from *Origanum compactum*, *O. vulgare* and *Thymus vulgaris* were significantly more effective against *P. agglomerans* bacteria than essential oils from *Mellisa officinalis*, *Mentha arvensis* and *Nepeta cataria*. The main compounds of essential oils from *O. compactum*, *O. vulgare* and *T. vulgaris* were carvacrol and thymol. Apart from the three above most effective oils, those from *Nepeta cataria* and *Mentha arvensis* were also effective against *E. amylovora*, and *M. officinalis* and *M. arvensis* against *P. syringae*. *P. syringae*, however only *M. officinalis* was effective against *P. fluorescens*. All tested herb essential oils exhibited the highest antimicrobial activity against *P. agglomerans* and the lowest inhibitory activity against *P. dispersa*.

Key words: anti-microbial activity, essential oils, *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas fluorescens*, *Pantoea dispersa*, *Pantoea agglomerans*.

INTRODUCTION

Fire blight caused by *Erwinia amylovora* ([Burrill] Winslow *et al.*, 1920), is the most serious bacterial disease of apple, pear, hawthorn, cotoneaster and other plant species in the family Rosaceae (Sobiczewski *et al.*, 1997; Vanneste, 2000). The pathogen is included among quarantine organisms in many countries around the world and very strict quarantine measures are enforced (Smith *et al.*, 1997). Since sanitation methods could not stop the spreading of the disease, fire blight management using appropriate chemicals and bio-control agents is the focus of ongoing efforts. Effective control can be achieved through streptomycin treatments (Johnson and Stockwell, 1998). However, its use has been prohibited in many countries due to the risk of resistance development in the population of the fire blight agent and non-target bacteria (Iacobellis *et al.*, 2005).

*Pseudomonas syringae* pv. *syringae* van Hall 1902 is polyphagous plant pathogenic bacterium, which survives usually as an epiphyte on host plants to become pathogenic under appropriate environmental conditions. This bacterium causes serious losses to stone fruits, in which it elicits a variety of symptoms, i.e. blossom blast, spur dieback, leaf necroses, bark cankers and gummosis of woody tissues (Renick *et al.*, 2008). As *Pss* is an active ice nucleation bacterium, it is harmful to plants particularly at the time of spring and autumn frosts (Renick *et al.*, 2008). The only bactericide registered for bacterial canker management is copper, which is of limited use because of its potential phytotoxicity (Kenneley *et al.*, 2007). The efficacy of copper has also been limited by the development of copper-resistant strains of *Pss* (Sundin *et al.*, 1989; Vanneste *et al.*, 2005).

Commonly occurring saprophytic bacteria such as *Pseudomonas fluorescens*, *Pantoea dispersa* and *Pantoea agglomerans* survive in the phylloplane of fruit trees together with plant pathogenic bacteria. These bacteria occurring on/in plant tissue do not cause harm to woody plants and some strains can even be useful as antagonists against plant pathogenic bacteria (Johnson and Stockwell, 1998; Elkins *et al.*, 2005).

Control of plant bacterial diseases remains difficult due to the limited availability of bactericides. Preparations based on copper compounds, which are applied most frequently, are not sufficiently effective in disease management for apple and stone fruit orchards and could have unfavourable effects either on the environment or on human and animal health (Iacobellis *et al.*, 2005).
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2005; Vanneste et al., 2005). Search of an environmentally friendly biological alternative is a permanent task of present research (Chen et al., 2009).

In plant protection, the use of herb essential oils looks promising against plant bacterial pathogens because some of them have a strong antimicrobial activity. The potential effectiveness of herb essential oils against different plant pathogenic bacteria has been verified by many authors (Dorman and Deans, 2000; Iacobellis et al., 2005; Vasinauskiene et al., 2006; Kokoskova and Pavela, 2007; Rhouma et al., 2009).

Some essential oils proved useful also against fungi, as shown by those from oregano, thyme, dictamnus and majoram that were effective against Clavibacter michiganensis subsp. michiganensis and Botrytis cinerea and Fusarium sp. (Daferera et al., 2003). Moreover, extracts from Pistacia and Sebimus spp. were effective against Agrobacterium sp. and Pseudomonas sp., as also against Fusarium sp. and Rhizoctonia sp. (Rhouma et al., 2009). Combination of plant extracts or etheric oils from plants with copper and other chemical compounds can increase their effectiveness (Zeller, 2005).

Mosch et al. (1989, 1993) reported that extracts from Rhus typhina, Juglans nigra, Berberis vulgaris, Mahonia aquifolium, Alnus sativum, Hedera helix, and Viscum album inhibited the in vitro and in vivo growth of E. amylovora. Extracts from H. helix and V. album showed a remarkable inhibitory effect of bacterial infections to Cotoneaster sp. probably connected with resistance induction (Mosch et al., 1996). Scortichini and Rossi (1989, 1991) observed that the terpenoids geraniol and citronellol decreased the growth of E. amylovora most effectively out of 20 terpenoidal compounds tested.

Many control agents have been used in the management of fire blight and tested against blossom blight infections. Some compounds used against E. amylovora did not have any bactericidal activity, but either triggered the plant defence mechanism leading to systemic acquired resistance (SAR) (acibenzolar-S-methyl, trade name Bion) or suppressed shoot growth (prohexadione-calcium, trade name Regalis), thus lowering shoot susceptibility to infection (Psallidas and Tsiandhos, 2000; Kennelly et al., 2007).

Promising tools for fire blight control are also yeast preparations such as BPAs and Blossom-Protect. They contain blastospores of Aureobasidium pullulans. Blossom-Protect reduces pH to approximately 4 and has two mechanisms to prevent neutralisation, i.e. a strong buffer (component A) which decreases the pH on the plant surface immediately after application, which is then kept low by A. pullulans during growth on blossoms surfaces. Their disadvantage is a potential fruit russet (Kunz et al., 2006).

Recently, two toxins from Bacillus amylo liquefaciens FZB42T with strong antagonistic effects against E. amylovora, proved promising for fire blight control (Chen et al., 2009).

This study is a further development of a previous work on the in vitro efficacy of 34 essential oils from different aromatic herbs against E. amylovora (Kokoskova and Pavela, 2007). The most effective essential oils were selected for detailed tests against P. syringae pv. syringae, E. amylovora and associated saprophytic bacteria.

**MATERIALS AND METHODS**

**Bacteria.** Three reference strains of E. amylovora (IVIA 1525-6, NCPPB 1114, GER 270/97) and two reference strains from other species were used in these tests, i.e. P. s. pv. syringae (CCM 4073, LMG 1247), P. fluorescens (CCM 2115, CRI 22), P. dispersa (CCM 4414, CCM 4341) and P. agglomerans (CCM 2406, CCM 3490). Bacterial cultures of E. amylovora, P. dispersa and P. agglomerans were grown on nutrient beef peptone agar (Schaad et al., 1991) and Ps on King B medium and kept at 25°C (King et al., 1954).

**Plant material and chemicals.** All plant essential oils (EOs) used in this study, extracted by steam or hy-

| Plants Origin Plant organ Main compounds (relative area %)* | Plants Plant organ Main compounds (relative area %)* |
|---------------|---------------|---------------|
| Melissa officinalis Spain Flowers/leaves citronellal (12.9), citronellol (6.3), neral (24.5), geranial (31.3), β-caryophyllene (3.9) | Mentha arvensis India Aerial part menthol (74.5), menthone (9.2), methyl acetate (3.1) |
| Nepeta cataria Canada Flowering tops nepetalactone (81.1), β-caryophyllene (10.8) | Origanum compactum Morocco Aerial part carvacrol (36.2), p-cymene (22.3), thymol (18.6), γ-terpinene (5.2) |
| Origanum vulgare Greece Aerial part thymol (28.5), thymyl methyl ether (5.7), carvacrol (19.5), β-bisabolene (12.6) | Thymus vulgaris Spain Aerial part p-cymene (16.3), γ-terpinene (5.6), geraniol (8.3), thymol (6.8), carvacrol (7.9) |

* According to the data of the gas chromatography analysis of essential oils provided by the manufacturer.
Effectiveness of plant essential oils against more than 50% higher than the standard. (ii) equal to the standard (+/- 10%); (iii) up to 50% higher than the standard (by 10-50%); (iv) more than 50% higher than the standard.

Experiments. Antimicrobial activity tests were carried out in vitro on agar plates seeded by the target organism. ENA II medium (6.6 g nutrient agar no. 2, 6.6 g glucose, 0.7 g yeast extract, 15.0 g agar, 1 liter sterile water, pH 6.6) was used for the screening of EOs (Kokoskova, 1992). E. amylovora was used as 24 h culture but the other bacteria were used as 48 h cultures in a concentration corresponding to OD=0.5 in all tests. EOs were delivered on the bacteria-seeded agar surface at a dose of 1 µl after its preparation. Each EO was assayed in six replicates. Agar plates without bacteria or with bacteria but no essential oils served as negative and positive controls, respectively.

After treatment, agar plates were covered with Parafilm and incubated at 25±1°C for three days, prior to the measurement of the inhibition zones. The antimicrobial efficacy index (IAE) of each EO was calculated for each bacterium. EO effectiveness was directly proportional to the size of the inhibition zone (Kokoskova and Pavela, 2007) and was evaluated according to four levels of effect: (i) weaker than the standard (by more than 10%); (ii) equal to the standard (+/- 10%); (iii) up to 50% higher than the standard (by 10-50%); (iv) more than 50% higher than the standard.

Statistical analysis. The antimicrobial efficacy index (IAE) was calculated with the formula:

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

where C is the average inhibitory zone (cm) on the standard dish (streptomycin 0.02%) and T is the average inhibitory zone on the treated dish, to which the EO was applied (Table 2a, 2b). The IAE (%) indicates whether the efficacy of EOs is lower and/or higher than the streptomycin standard (Kokoskova and Pavela, 2007) (Fig 1).

A one-way analysis of variance (ANOVA test) was performed to compare the areas of effectiveness (inhibitory zones) of EO with streptomycin, followed by a ranking of their averages using Tukey’s test. Differences between means were considered significant when $P \leq 0.05$ (Table 2a, 2b).

RESULTS

Erwinia amylovora. EOs from Thymus vulgaris and Origanum compactum showed antimicrobial activity significantly (more than 50%) higher (P≤0.05) than streptomycin. The antimicrobial effect of essential oils from Origanum vulgare, Nepeta cataria and Mentha arvensis was also higher (up to 50%), whereas the essential oil from Melissa officinalis showed approximately the same biological effectiveness as streptomycin (Table 2a, Fig. 1).

Pseudomonas syringae pv. syringae. The antimicrobial activity of EOs from O. compactum, O. vulgare and T. vulgaris was significantly (more than 50%) higher (P≤0.05) than that of streptomycin. EOs from M. officinalis and M. arvensis showed also a higher effectiveness (up to 50%) than streptomycin, but the EO from N. cataria was as effective as streptomycin (Table 2a, Fig. 1).

Pseudomonas fluorescens. The antimicrobial activity of EOs from T. vulgaris and O. vulgare was significantly

Table 2a. Effectiveness of plant essential oils against Erwinia amylovora and Pseudomonas syringae pv. syringae

<table>
<thead>
<tr>
<th>Essential oil/Chemical</th>
<th>Erwinia amylovora</th>
<th>Pseudomonas syringae pv. syringae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth reduction expressed as average of inhibition zone diameters (cm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IVIA 1525-6</td>
<td>NCPPB 1114</td>
</tr>
<tr>
<td>Mellisa officinalis</td>
<td>6.17 ± 1.33</td>
<td>7.0 ± 0.89</td>
</tr>
<tr>
<td>Mentha arvensis</td>
<td>7.67 ± 2.5</td>
<td>12.5 ± 2.26</td>
</tr>
<tr>
<td>Nepeta cataria</td>
<td>24.00 ± 0.89</td>
<td>23.7 ± 1.51</td>
</tr>
<tr>
<td>Origanum compactum</td>
<td>21.33 ± 4.5</td>
<td>29.3 ± 3.33</td>
</tr>
<tr>
<td>Origanum vulgare</td>
<td>14.50 ± 3.83</td>
<td>24.2 ± 1.47</td>
</tr>
<tr>
<td>Thymus vulgaris</td>
<td>14.33 ± 2.5</td>
<td>37.0 ± 3.52</td>
</tr>
<tr>
<td>Streptomycin 0.02 %</td>
<td>12 ± 3.41</td>
<td>5.3 ± 0.52</td>
</tr>
</tbody>
</table>

Values represent means of six replicates

*The diameter (cm) of inhibitory zones (mean+/− standard error); Asterisks indicate means that are significantly different from control (P≤0.05)

IVIA - Collection of Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain

NCPPB - National Collection of Plant Pathogenic Bacteria, York, UK

GE 270/97 - strain provided by Dr. K. Richter, Institute of Epidemiology and Resistance, Federal Centre for Breeding Research on Cultivated Plants, Aschersleben, Germany

CCM - Czech Collection of Microorganisms, Brno, Czech Republic

LMG - BCCM/LMG - Laboratory of Microbiology Gent Bacteria Collection, Gent, Belgium
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(0.05) than that of streptomycin. EOs from O. compactum and M. officinalis were also more effective (up to 50%) than streptomycin, whereas EO from M. arvensis showed the same efficacy of streptomycin and N. cataria EO was less effective than streptomycin (Table 2b, Fig. 1).

**Pantoea dispersa.** EOs from T. vulgaris, O. compactum and O. vulgare had antimicrobial activity significantly (up to 50%) higher (P ≤ 0.05) than that of streptomycin. M. arvensis EO showed the same biological effectiveness as streptomycin, but essential oils from Nepeta cataria and M. officinalis were less effective than streptomycin (Table 2b, Fig. 1).

**Pantoea agglomerans.** EOs from T. vulgaris, O. compactum and O. vulgare had antimicrobial activity significantly (more than 50%) higher (P ≤ 0.05) than that of streptomycin, the same as EOs from M. officinalis, M. arvensis and N. cataria that showed higher biological effectiveness (up to 50%) than streptomycin (Table 2b, Fig. 1).

**DISCUSSION**

Based on previous results (Kokoskova and Pavela, 2007), where thirty-four plant EOs had been screened for potential in vitro effectiveness against E. amylovora, the six most effective oils, i.e. those from M. officinalis, M. arvensis, N. cataria, O. compactum, O. vulgare and T. vulgaris were chosen for further experiments. In the present study, the efficacy of these EOs was verified against at least two strains of one species of plant pathogenic and saprophytic bacteria to obtain more reliable results.

We wanted to know whether the tested EOs were able or not to decrease the growth of saprophytic bacteria, because some strains of these species have been used as antagonists or as biocontrol agents against fire blight and other diseases (Johnson and Stockwell, 1998; Elkins et al., 2005). Streptomycin was used as standard, because of the unsatisfactory efficacy of oxychloride-Cu (Kokoskova and Pavela, 2007).

The plant pathogenic bacteria *P. syringae* pv. *syringae* and *E. amylovora* and the saprophytic bacterium *P. fluorescens, P. dispersa* and *P. agglomerans* showed sensitivity to all plant EOs presently tested, although differences in the antibacterial activity of some oils were found (Table 2a, 2b). Streptomycin was more effective against Gram-negative anaerobic bacteria (*E. amylovora, P. dispersa* and *P. agglomerans*) than against Gram-negative aerobic bacteria (*Pss, P. fluorescens*) as is clear from Table 2a and 2b.

The variability of efficacy of plant EOs compared to streptomycin (axis X) is shown in Fig. 1. In general, the
The majority of EOs showed a higher effectiveness than streptomycin. EOs from *O. compactum*, *O. vulgare* and *T. vulgaris* were significantly more effective than those from *M. officinalis*, *M. arvensis* and *N. cataria*. All EOs were most effective against *P. agglomerans* and least effective against *P. dispersa*. Since a higher efficacy of EOs against phytopathogenic than saprophytic bacteria was expected, the high sensitivity of *P. agglomerans* came as a surprise, which, however, may depend on the specific strain tested. As to *Pseudomonas* spp., all EOs were more effective against *Pss* than *P. fluorescens*. This is not surprising because *P. fluorescens*, like *P. aeruginosa*, is among the most resistant organisms, representing a problem particularly in human medicine (Papadopoulos et al., 2006; Bouhdid et al., 2008).

Regarding plant pathogenic bacteria, EOs from *O. compactum*, *O. vulgare*, *T. vulgaris* and *M. arvensis* were more effective against *P. syringae* pv. *syringae* than *E. amylovora* while *M. officinalis* EO showed a good efficacy against *P. syringae* pv. *syringae*, but not *E. amylovora*, and the *N. cataria* EO was more effective against *E. amylovora*, than *P. syringae* pv. *syringae* (Fig. 1).

Our results show that the screened EOs are potentially highly effective against Gram-negative bacteria in general, in agreement with literature records relative to oils from *Origanum* sp. and *Thymus* (Chaira et al., 1996; Soylu et al., 2005; Iacobellis, 2005; Kokoskova et al., 2006; Bouhdid et al., 2008). EOs from *Origanum* sp. and *Thymus* sp. are rich in antioxidative phenolic compounds, which are believed to be responsible for their marked antimicrobial activity (Zaika and Kissinger, 1981; Chizzola et al., 2008). The studies of Dorman and Deans (2000), which found the volatile oils thymol from *T. vulgaris* and carvacrol from *O. vulgare* subspecies *birtum* to have a wide spectrum of antimicrobial activity, are in agreement with our results. In addition, Vanneste (1996) reported that some plant extracts and some EOs could inhibit *E. amylovora* *in vitro*, thyme oil in particular, which again tallies with our results.

Vasinauskaite et al. (2006) found a significant inhibitory effect *in vitro* of all nine chemo-types of *O. vulgare* EO on the growth of *P. syringae* pv. *syringae* and other plant pathogenic bacteria. EOs from *O. compactum* and *T. vulgaris* have shown a high level of antimicrobial activity against *P. putida* (Oussalah et al., 2006). Interestingly, we found that the same oils were effective against *P. fluorescens*. In our study, essential oil from mint showed a lower level of inhibitory activity than EOs from oregano and thyme, as reported by others (Sivropoulou et al., 1996; Mazzanti et al., 1998).

Chemical analysis of the six EOs tested (Table 1) showed that their major constituents are phenolic monoterpenes (carvacrol and thymol), monoterpenic hydrocarbons (*p*-cymene or *γ*-terpinene) and aldehydes (geranial or citronellol) (Table 1). The components with phenolic structures, such as carvacrol and thymol, were highly active against all the screened microorganisms, in agreement with previous reports (Sivropoulou et al., 1996; Dorman and Deans, 2000). Many authors have suggested that polyphenols inhibit the growth of microorganisms by forming complexes with their enzymes and proteins (Rhouma et al., 2009). Phenolic compounds can dissolve the bacterial membrane, thus penetrating the cell, where they interact with cell metabolism (Judas, 1963; Juven et al., 1972; Oussalah et al., 2006). Carvacrol and thymol disrupt the plasma membrane, which increases its permeability and depolarizes its potential (Xu et al., 2008).

Comparison of our data with previously published results is based on the fact that the composition of plant
essential oils and extracts is influenced by the geographical origin of the hosts from which they are extracted, the environmental conditions, and the host species or subspecies (Faleiro et al., 2003; Sivropoulou et al., 1996; Sarac and Ugur, 2008). EO composition varies also depending on the host variety, time and the way of harvesting, type of storage, extraction method, etc. All these factors influence the chemical composition and the relative proportion of individual constituents of EOs (Oussalah et al., 2006), so that EOs form the same plant species collected from different locations can show different chemical composition and different levels of antimicrobial activity (Vasinauskiene et al., 2006; Sarac and Ugur, 2008, Chizolla et al., 2008). Moreover, the biological activity of EOs can be influenced by the different percentage of inhibitory active compounds and by their antagonistic and/or synergistic effect (Hummelbrunner and Isman, 2001).

In any case, the essential oils screened in our study seem to have a promising potential as new pesticide products or as templates for new, more effective compounds.

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