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Effectiveness of Plant Essential Oils on the Growth of *Erwinia amylovora*, the Causal Agent of Fire Blight Disease

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ABSTRACT

Erwinia amylovora [(Burr.) Winslow *et al.* 1920], the causative agent of fire blight disease, threatens some species of the *Rosaceae* family. In chemical control, preparations based on copper compounds are most frequently applied, but these can be phytotoxic and not effective enough, hence other alternatives need to be considered. In our experiment, thirty-four essential oils obtained from different plants were tested for their antimicrobial effectiveness against the fire blight pathogen. Screening was conducted *in vitro* on agar plates contaminated by *E. amylovora.* The strongest inhibitory effect (i.e. complete bacterial inhibition) was shown by essential oils from *Mellisa officinalis*, *Mentha arvensis*, *Origanum compactum*, *O. vulgare* and *Thymus vulgaris*. An inhibitory effect more than 50% higher than the standard was shown by essential oils from *Eugenia caryophyllata*, *Mentha pulegium*, and *Nepeta cataria*. An inhibitory effect up to 50% higher than the standard was found in essential oils from *Artemisia absinthium*, *Citrus aurantifolia*, *Lavandula latifolia*, *Melaleuca quinqunervia*, *Mentha citrata*, *M. spicata*, *Ocimum basilicum*, *Pelargonium graveolens*, *P. roseum*, *Rosmarinus officinalis*, *Salvia sclarea*, *Thuja occidentalis* and *Tsuga canadensis*. An antimicrobial effect comparable to the standard was found in essential oils from *Amyris balsamifera*, *Citrus limonum*, *Juniperus communis*, *J. virginiana*, *Origanum majorana*, *Salvia officinalis*, *Tagetes bipinata* and *Thymus matschiana*. Standard employed was oxychloride-Cu 84% (0.84 µg/µl), it had a weak inhibitory effect. The essential oils from *Acorus calamus*, *Lavandula angustifolia* and *Zingiber officinale* had a lower inhibitory effect than the standard. The essential oils of two plant extracts from *Abies siberica* and *Pogostemon cablin* had no effect on the growth of *E. amylovora*. Five of the most effective essential oils appear promising for the development of potential bio-pesticides.

Keywords: antimicrobial activity, in vitro tests, botanical pesticides

INTRODUCTION

Erwinia amylovora [(Burrill) Winslow *et al*. 1920] (*Ea*), which causes fire blight disease, is registered as an organism in many countries around the world for which very strict quarantine measures are required, and used (OEPP/EPPO 1992; EPPO/CABI 1997). Fire blight is probably the most serious disease affecting fruit and ornamental plants of the *Rosaceae* family, such as *Pyrus* spp., *Malus* spp., *Cydonia* spp., *Crataegus* spp., *Cotoneaster* spp., *Sorbus* spp. and others (van der Zwet and Beer 1995; Sobiczewski *et al.* 1997; Vaneste 2000).

Present control measures include surveys of presumptive plants from disease infection, laboratory tests, eradication of infected plants, control and regulation of multiplied and cultivated materials (van der Zwet and Beer 1995). Bacteria disseminate under humid and warm weather conditions depending on the species, cultivar and planting conditions.

In chemical control, preparations based on copper compounds are most frequently applied, but these can be phytotoxic and not effective enough, hence other alternatives need to be considered. The use of antibiotics such as streptomycin has been disallowed in plant protection in the majority of EU countries, because resistance to them can easily develop in bacterial populations (Iacolellis *et al*. 2005).

Scortichini and Rossi (1989, 1991) and Mosch *et al*. (1993, 1996) elaborated studies on the effectiveness of the components from essential oils or plant extracts against *E. amylovora.* Scortichini and Rossi (1989, 1991), studying the influence of some essential oil constituents on bacterial growth, observed that the terpenoids geraniol and citrollenol, out of 20 terpenes and terpenoids tested, exhibited bactericidal activity against *E. amylovora*. Mosch *et al.* (1989) reported that 24 out of 131 plant extracts tested exhibited some degree of antibacterial activity against *E. amylovora in vitro*, the most effective being leaf extracts from *Rhus typhina*, *Juglans nigra*, *Berberis vulgaris*, *Mahonia aquifolium* and *Alium sativum*. Mosch *et al*. (1993) demonstrated that plant extracts from *Hedera helix* and *Viscum album* could induce resistance mechanisms in detached leaves of *Cotoneaster watereri* and *Cydonia oblonga*, resulting in decreased disease severity.

There is a desperate demand for new chemical compounds on the market. A combination of plant extracts or etheric oils from plants together with copper and other chemical compounds can increase their effectiveness (Zeller 2005) but these still imply the use of environmentally-unfriendly and potentially toxic compounds.

In the past few years, some preparations which have been used to control the fire blight pathogen did not have any bactericidal activity, but interfered with plant metabolism. They influence either triggering the plant defense mechanism leading to systemic acquired resistance (SAR) as Bion™ (acibenzolar-S-methyl, 50% of ASM) and Messenger™ (Actigard™, harpin, protein produced by *E. amylovora;* 16.5 g/acre) or suppressing the shoot growth and thus lowering the shoot susceptibility to infection as Apogee™ (prohexadione-calcium; 50 g/acre of P-ca) (Psallidas and Tsianthos 2000). The chemistry of the essential oils from plants used in our study, including monoterpenes, sesquiterpenes and phenols is well docu-mented (Guenther 1972-1998; Bruneton 1999). Some of these essential oils proved to have strong antifungal (Daferera *et al*. 2000; Wang *et al*.

Received: 13 July, 2006. Accepted: 2 October, 2006.

Original Research Paper

Table 1 The plant material used for isolation the essential oils and the effect of the essential oils on the growth of *Erwinia amylovora.*

Plant	Family	Origin	Part	Percentage reduction in colony size (cm)
Abies siberica	*Pinaceae*	Russia	Needle	0.01 ± 0.00***[1]
Acorus calamus	*Araceae*	Nepal	Root	3.33 ± 0.67***
Amyris balsamifera	*Rutaceae*	Haiti	Wood	5.52 ± 1.11
Artemisia absinthium	*Asteraceae*	USA	Flowering tops	12.5 ± 1.5***
Citrus aurantifolia	*Rutaceae*	Mexico	Peel	9.83 ± 0.97***
Citrus limonum	*Rutaceae*	Italy	Peel	6.01 ± 1.61
Eugenia caryophyllata	*Myrtaceae*	Madagascar	Bud	18.11 ± 1.66***
Juniperus communis	*Cupressaceae*	Croatia	Berry	6.33 ± 1.87
Juniperus virginiana	*Cupressaceae*	USA	Wood	6.33 ± 0.27
Lavandula angustifolia	*Lamiaceae*	France	Flower	4.53 ± 0.73*
Lavandula latifolia	*Lamiaceae*	France	Flower	9.53 ± 5.92**
Melissa officinalis	*Lamiaceae*	Spain	Flower/leaf	40.00 ± 0.00***
Melaleuca quinquenervia	*Myrtaceae*	Madagascar	Leaf	9.33 ± 1.47***
Mentha arvensis	*Lamiaceae*	India	Herb	40.00 ± 0.00***
Mentha citrata	*Lamiaceae*	USA	Herb	12.17 ± 0.97***
Mentha pulegium	*Lamiaceae*	USA	Leaf	16.00 ± 7.2***
Mentha spicata	*Lamiaceae*	China	Flowering tops	8.01 ± 2.8*
Nepeta cataria	*Lamiaceae*	Canada	Flowering tops	25.33 ± 5.87***
Ocimum basilicum	*Lamiaceae*	Egypt	Herb	13.52 ± 7.5***
Origanum compactum	*Lamiaceae*	Morocco	Herb	40.00 ± 0.00***
Origanum majorana	*Lamiaceae*	Egypt	Flower/leaf	6.67 ± 3.47
Origanum vulgare	*Lamiaceae*	Greece	Herb	40.00 ± 0.00***
Pelargonium graveolens	*Geraniaceae*	Reunion	Leaf	8.33 ± 4.27*
Pelargonium roseum	*Geraniaceae*	Madagascar	Leaf	10.83 ± 3.77***
Pogostemon cablin	*Lamiaceae*	Indonesia	Leaf	0.01 ± 0.01***
Rosmarinus officinalis	*Lamiaceae*	Morocco	Leaf	7.83 ± 0.97**
Salvia officinalis	*Lamiaceae*	Greece	Leaf	6.17 ± 2.17
Salvia sclarea	*Lamiaceae*	USA	Flower/leaf	13.00 ± 4.01***
Tagetes bipinata	*Asteraceae*	Madagascar	Flower	7.33 ± 2.67
Thuja occidentalis	*Cupressaceae*	Canada	Leaf/twig	8.50 ± 1.91**
Thymus mastichina	*Lamiaceae*	Spain	Flower/leaf	6.17 ± 0.97
Thymus vulgaris	*Lamiaceae*	Spain	Herb	40.00 ± 0.00***
Tsuga canadensis	*Pinaceae*	Canada	Needle	9.67 ± 2.67***
Zingiber officinale	*Zingiberaceae*	China	Root	5.03 ± 0.27
Control (Oxychloride-Cu)				5.83 ± 0.75

[1] The diameter (cm) of inhibitory zones (mean ± standard error); Asterisks indicate means are significantly different from controls (*$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

2005), insecticidal (Isman 2000; Pavela 2005; Çalmaşur *et al*. 2006), and antibacterial activity (Burt 2004; EL-Kamali *et al*. 2005; Oussalah *et al*. 2006). Some of these plant essential oils also have shown antimicrobial activity both against fungi and bacteria, for instance essential oils from oregano, thyme, dictamnus and majoram were effective against *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis* (Daferera *et al*. 2003). In last several decades, biopesticides based on essential oils have been developed for commercial use. These preparations are very promising because of rapid effectiveness and relatively low price (Isman 2006).

The aim of this study was to find essential oils having a potentially inhibitory effect against *E. amylovora in vitro*. By identifying the most promising oils in the control against fire blight pathogen *in vitro* would permit us to test them under field conditions in the future.

MATERIALS AND METHODS

Bacteria

Reference culture of *Ea* RICP 8/95 (RICP = Research Institute of Crop Production) was grown on solid nutrient meat-peptone medium and used as a 2-day culture in a concentration corresponding to $OD \approx 0.5_{620}$ in all tests.

Chemicals

The essential oils used in this study were purchased from the Essential Oil University (Charlestown, IN, USA). The essential oils were obtained by steam or hydrodistillation of botanicals (**Table 1**). As a control we used oxichloride-Cu 84 % (Kuprikol 50, produced by Spolana Neratovice, Czech Republic).

Analysis of essential oils

The chemical composition of seven antimicrobially most potent spice essential oils (**Table 2**) was determined with gas chromatography-mass spectrometry (GC-MS) analysis (Adams 1995). Essential oils were dissolvent in ethyl acetate. The volume of the sample injected was 1 µl. A Hewlett-Packard 5890 combination gas chromatograph-mass spectrometer (Waldbronn, Germany) was used as follows: capillary column HP-5 (30 m by 0.25 mm; phase thickness, 0.25 µl); temperature program 40°C (2 min), raised to 250°C (5 min) at a rate of 10°C/min; and carrier gas helium at a

Table 2 Main compounds of selected essential oils tested on *E. amylovora*.

Plant botanical name	Main compounds (relative area %)
Eugenia caryophyllata	eugenol (32.3), β-caryophyllene (6.2), α-humulene (8.6), carvacrol (39.8)
Melissa officinalis	citronellal (12.9), citronellol (6.3), neral (24.5) geranial (31.3), β-caryophyllene (3.9)
Mentha arvensis	menthol (74.5), menthone (9.2) methyl acetate (3.1)
Nepeta cataria	nepetalactone (81.1), β-caryophyllene (10.8)
Origanum compactum	carvacrol (36.2), p-cymene (22.3), thymol (18.6), γ-tepinene (5.2)
Origanum vulgare	thymol (28.5), thymyl methyl ether (5.7), carvacrol (19.5), β-bisabolene (12.6)
Thymus vulgaris	p-cymene (16.3), γ-tepinene (5.6), geraniol (8.3), thymol (6.8), carvacrol (7.9)

The chemical composition was determined with gas chromatography-mass spectrometry (GC-MS) analysis (expressed as % w/w composition).

flow rate 0.9 ml/min. The compounds were recognized by the retention times of the chromatogram peaks and by their mass spectra. The identities of the main component peaks were confirmed by comparison of their retention times with those of authentic samples.

Experiments

The antimicrobial activity tests were conducted on agar plates contaminated by the bacterium *E. amylovora*. ENA II medium (nutrient agar no. 2 - 6.6 g, glucose - 6.6 g, yeast extract - 0.7 g, agar - 15.0 g, sterile water - 1 L, pH - 6.6) was used for the screening of essential oils (Kokošková 1992). Compounds using for ENA II medium have bought from IMUNA, Šarišské Michalany, Slovakia. TTCL (triphenyltetrazolium chloride) at 0.5% (w/v) was added to the agar in order for the zones to be more distinct. The crude extracts – at a dosage of 1 µl per 6 plant grindings – were dropped on the contaminated agar surface three hours after its preparation. After treatment, Petri dishes were incubated in a thermostat at 25 ± 1°C for three days, and thereafter evaluated. The diameter of the inhibitory zones was measured and the average calculated. The efficiency of the essential oil was directly proportional to the size of the inhibitory zone. Oxy-chloride-Cu 84% was used as a control at 0.84 µg/µl for bacterial colony plates (Kokošková 1992). The effectiveness of essential oils was evaluated according to six classifications or degrees of effect: without an inhibitory effect; an effect weaker than the control; an effect equal to the control; an effect up to 50% higher than the control; an effect more than 50% higher than the control; total inhibition, i.e. covering the entire plate.

Statistical analysis

The index antimicrobial effectivity (IAE) was calculated from the formula:

$$\mathrm{IAE}\,(\%) = \left[-1*\left(\frac{\mathrm{C}-\mathrm{T}}{\mathrm{C}+\mathrm{T}}\right)\right]*100$$

where C is the average percentage zone on the control (oxichloride-Cu 84%) dish and T is the average zones on the treated dish (i.e. to which essential oils were applied). The IAE indicates whether the effectiveness of the essential oils is lower and/or higher than the control (standard preparation).

A one-way analysis of variance (ANOVA test) was performed for comparing areas of effectiveness of essential oils compared with oxichloride-Cu 84%, followed by a ranking of their averages using Tukey's test. Differences between means were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Out of thirty-four essential oils, thirty-two showed an antibacterial effect against *Erwinia amylovora.* Nevertheless differences among essential oils were found (**Table 1; Fig. 1**). Essential oils were divided into several groups according to their effectiveness and type of effect. The other standards such as streptomycin and erythromycin were found to have low effectivity in preliminary tests.

The strongest inhibitory effect, in which the growth of bacteria was inhibited over the whole tested zone of the Petri dish, was found in five essential oils from *Mellissa officinalis*, *Mentha arvensis*, *Origanum compactum*, *O. vulgare* and *Thymus vulgaris*.

Antimicrobiological activity was significantly higher ($P \leq 0.05$) compared to the control, standard oxichloride-Cu 84% (more than 50% compared to the standard in essential oils from *Eugenia caryophyllata*, *Mentha pulegium* and *Nepeta cataria* (**Table 1**).

Antimicrobiological activity was significantly higher ($P \leq 0.05$) compared to the controlled standard oxichloride-Cu 84% (up 50% compared to the standard) in essential oils from *Artemisia absinthium*, *Citrus aurantifolia*, *Lavandula latifolia*, *Melaleuca quinquenervia*, *Mentha citrata*, *Ocimum basilicum*, *Pelargonium graveolens*, *P. roseum*, *Rosmarinus officinalis*, *Salvia sclarea*, *Thuja occidentalis* and *Tsuga canadensis* (**Table 1**).

Essential oils from *Amyris balsamifera*, *Citrus limonum*, *Juniperus communis*, *J. virginiina*, *Origanum majorana*, *Salvia officinalis*, *Tagetes bipinata*, *Thymus mastichina* and *Zingiber officinalis* showed the same biological effectiveness as the standard oxichloride-Cu 84%, with the average zone percentage was not significantly higher or lower (**Table 1**; $P \leq 0.05$) than control zones.

Essential oils from *Acorus calamus* and *Lavandula angustifolia* showed an inhibitory effect, but statistically lower

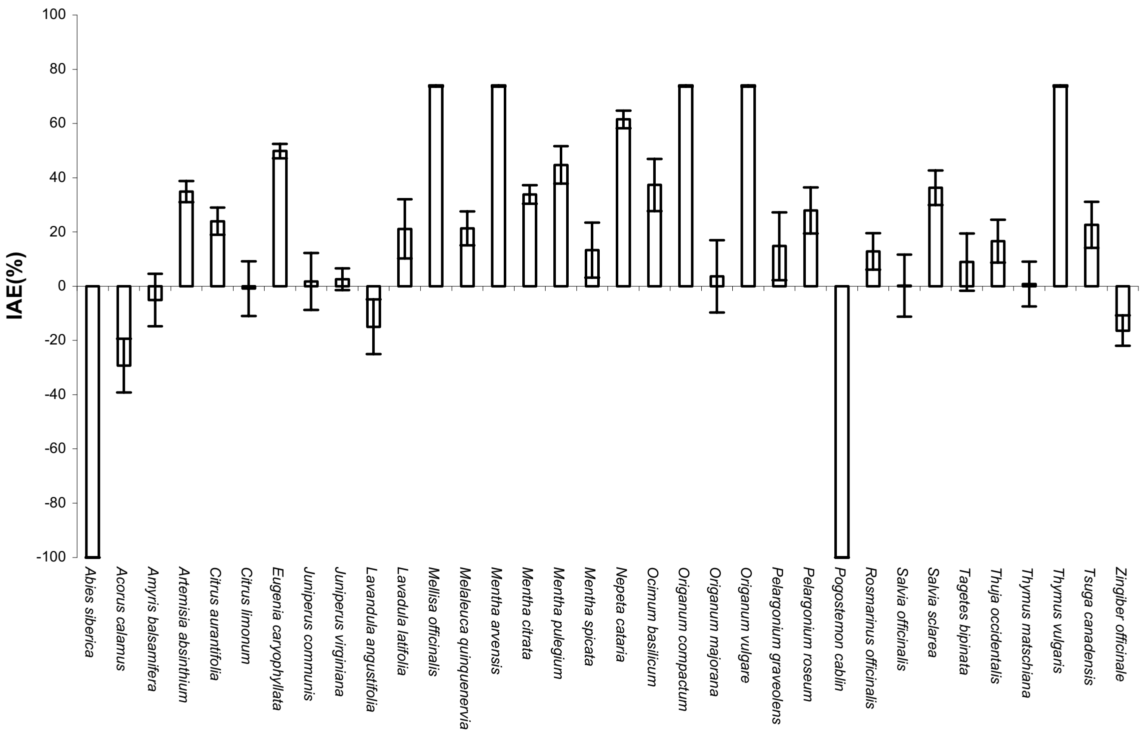

Fig. 1 The index of antimicrobial effectivity (IAE) of essential oils on the growth inhibition against *Erwinia amylovora*. Percentage variation in inhibit zones compared with control (means ± S.E).

(**Table 1**; $P \leq 0.05$) than the standard (oxichloride-Cu 84%).

Essential oils from *Abies siberica* and *Pogostemon cablin* had no inhibitory effect on the growth of *E. amylovora*.

In **Fig. 1** we show the increase or decrease in biological effectiveness compared to the effectiveness of the control. In **Fig. 1** we can observe that 21 essential oils show a higher effectiveness than the standard used in tests, and from these seven oils showed an effectiveness of about more than 50% that of the control: *Melissa officinalis*, *Mentha arvensis*, *Origanum compactum*, *O. vulgare*, *Thymus vulgaris*, *Eugenia caryophyllata*, *Nepeta cataria* and *Mentha pulegium*.

Chemical analysis (GC-MS) of these six essential oils has shown that the majority of compounds identified in tested essential oils are phenolic monoterpenes, notably carvacrol, thymol or eugenol, monoterpenic hydrocarbons such as p-cymene or γ-terpinene, and aldehydes such as geranial or citronellal (**Table 2**). The composition of the essential oils changes depending on the plant cultivar, the time and method of harvesting, manner of storage and extraction, and therefore we can only grossly compare whether results are in harmony with those of other authors.

For example, similar results were also reported by other authors who tested the effect of essential oils against other bacteria (Charai *et al*. 1996; Sivropoulou *et al*. 1996; Iacobellis *et al*. 2005). Essential oils from some plant species (e.g. *Origanum* sp. and *Thymus* sp.) are rich in phenolic compounds, which are believed to be responsible for their marked antimicrobial activity (Zaika and Kissinger 1981). Phenolic compounds are capable of dissolving within the bacterial membrane and thus penetrating inside the cell, where they interact with cellular metabolic mechanism (Judis 1963; Juven *et al*. 1972; Oussalah *et al*. 2006).

The weak antimicrobial efficiency of *Origanim majorana* essential oils tested in this study contrasts with results obtained by Vági *et al*. (2005). The antimocribial potential of many essential oils is related to their hight phenolic contents (Sivropoulou *et al*. 1996) These differences may be caused by differences in the percentage availability of chemical compounds and in some of their antagonist and/or synergistic effects (Hummelbrunner and Isman 2001).

The components with phenolic structures, such as carvacrol, eugenol and thymol are highly active against the test microorganisms. In oregano and thyme, the major antimicrobial constituents have been identified as carvacrol (62-79%), and thymol (42%) respectively (Farag *et al*. 1989; Sivropoulou *et al*. 1996).

The growth of *Clavibacter michiganensis* subsp. *michiganensis* was completely inhibited by oregano, thyme, dictamnus and majoram essential oils even when applied at relatively low concentrations (Daferera *et al*. 2003), in harmony with the results of our experiments, in which the essential oils from *O. compactum*, *O. vulare* and *T. vulgaris* were among the most effective against *E. amylovora*; the essential oils from dictamnus and majoram, which were considered to be effective by Daffera *et al*. (2003) were not tested in our study. In support of the results of our studies, other authors such as Dorman and Deans (2000), demonstrated that in the volatile oils, the compounds with the widest spectrum of activity were thymol from *T. vulgaris*, followed by carvacrol from *O. vulgare* ssp. *hirtum*. Essential oils from *O. compactum* and *T. vulgaris* have also shown strong antimicrobial activity against *Pseudomonas putida* (Oussalah *et al*. 2006). All these results indicate their potentially high effectiveness against all gram-negative and gram-positive bacteria. Vanneste (1996) reported that some plant extracts and some essential oils could inhibit *E. amylovora in vitro*, in particular thyme oil, which also corresponds with our results.

However, comparison of the data obtained in this study with previously published results is not easy, considering that the composition of plant oils and extracts vary according to environmental conditions and plant species, the harvesting method and other factors. All these factors influence the chemical composition and relative proportions of the individual constituents in the essential oils of the plants (Oussalah *et al*. 2006).

Essential oils obtained by some plants have a promising potential for being incorporated into various pesticide products in which antimicrobial and insecticidal activity is desired. These extracts might further have a role in protection against bacteria in greenhouses. Further large-scale experiments are required to establish the real application of essential oils against plant pathogenic bacteria while ensuring a non-phytotoxic. Thirty four essential oils from different plants were tested in our experiments, from which five oils (*Mellisa officinalis*, *Mentha arvensis*, *Origanum compactum*, *O. vulgare* and *Thymus vulgaris)* appear promising for the development potential bio-pesticides. The other tests with these oils are necessary to be conducted for finding their biological effectivesess and plant phytotoxicity effect.

ACKNOWLEDGEMENTS

The work was supported by the Ministry of Agriculture of CR, project No. MZE0002700603.

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