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RESEARCH PAPER

Inhibitory effect of *Thymus vulgaris* and *Origanum vulgare* essential oils on virulence factors of phytopathogenic *Pseudomonas syringae* strains

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ABSTRACT

- *Pseudomonas syringae* is a phytopathogenic bacterium that causes lesions in leaves during the colonisation process. The damage is associated with production of many virulence factors, such as biofilm and phytotoxins. The essential oils of *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano) have been demonstrated to inhibit *P. syringae*. The aim of this study was to investigate the effects of *T. vulgaris* and *O. vulgare* essential oils on production of virulence factors of phytopathogenic *P. syringae* strains, including anti-biofilm and anti-toxins activities.
- The broth microdilution method was used for determination of MIC and biofilm inhibition assays. Coronatine, syringomycin and tabtoxin were pheno- and genotypically evaluated.
- Both oils showed good inhibitory activity against *P. syringae*, with MIC values from 1.43 to 11.5 $mg{\cdot}ml^{-1}$ for thyme and 5.8 to 11.6 $mg{\cdot}ml^{-1}$ for oregano. Biofilm formation, production of coronatine, syringomycin and tabtoxin were inhibited by thyme and oregano essential oil in most strains.
- The results presented here are promising, demonstrating the bactericidal activity and reduction of virulence factor production after treatment with thyme and oregano oil, providing insight into how they exert their antibacterial activity. These natural products could be considered in the future for the control of diseases caused by *P. syringae*.

INTRODUCTION

Pseudomonas syringae is a gram-negative plant pathogenic rod-shaped bacterium responsible of a variety of diseases in many crops, including apple, beet, bean, cabbage, cucumber, oat, olive, pea, tobacco, tomato, rice and flower species. *P. syringae* has many pathovars, related to the host from which it is isolated, producing symptoms such as spots, speckling and blight (Bender *et al.* 1999; Hwang *et al.* 2005). *P. syringae* is a leaf ephiphyte until conditions favour a switch to a pathogenic life style. It enters the plant through stomata and lenticels or *via* wound sites, where it reproduces until it reaches a concentration that triggers disease lesions (Tarkowski & Vereecke 2013). The lesions produced in host colonisation are associated with virulence factors regulated by quorum sensing (QS) mechanisms (Matas *et al.* 2012). These virulence factors are biofilms, extracellular polysaccharides, phytotoxins, cell wall-degrading enzymes and phytohormones (Arrebola *et al.* 2011).

In natural ecosystems, microorganisms grow preferentially in communities, forming complex structures known as biofilms that help in survival of the bacteria on host tissue. Biofilms are aggregations of cells adsorbed to biotic or abiotic surfaces, mainly extracellular polysaccharides, proteins, lipids and nucleic acids. Cells inside biofilms are more resistant to environmental stress, host defence mechanisms and antimicrobial compounds. Growth of cells in close proximity, as in biofilms, allows signal molecules to trigger changes in gene expression (von Bodman *et al.* 2003; Rigano *et al.* 2007; Bogino *et al.* 2013). Phytotoxins are virulence factors produced during pathogenesis that lead to chlorosis and necrosis of leaves. They are not considered necessary for disease development, but their presence increases pathogen virulence and severity because they are involved in the systemic movement of bacteria, lesion size and reproduction in the host (Bender *et al.* 1999; Ichinose *et al.* 2013). These phytotoxins exert their effect mainly in the plasma membrane, for example: coronatine induces opening of stomata allowing bacteria to enter the cell; syringomycin and syringopeptin cause cell leakage, leading to loss of nutrients and tissue necrosis; tabtoxin and phaseolotoxin cause chlorosis and necrosis through inhibition of amino acids synthesis (Arrebola *et al.* 2011; Tarkowski & Vereecke 2013).

Diseases caused by phytopathogenic bacteria are increasing worldwide, mostly through contaminated seeds, equipment, machinery, people, water and international commerce, which serve as a transport agent for pathogens and aid their dissemination. Treatments to control bacteriosis include antibiotics and copper compounds, which can be toxic if used incorrectly,

generating undesirable effects in humans, animals and the environment. Misuse of antibiotics can lead to resistance in strains of *P. syringae*. Unfortunately, there are few compounds licensed for bacterial disease control in extensive farming practices. Recently, the properties of plant extracts have been extensively studied, and many reports have focused on their possible use against phytopathogens (Lo Cantore *et al.* 2004; Vasinauskiene *et al.* 2006). Plant essential oils and their terpenes are now being considered for treatment of bacterial infections due to their antimicrobial properties, safety, wide acceptance by consumers and potential use in areas such as in cosmetic, pharmaceutical and food industries (Ben El Hadj Ali *et al.* 2013). Essential oils are volatile compounds with a strong odour, synthesised by aromatic plants as secondary metabolites. Their activities are dependent on the diversity and quantity of constituents such as oxygenated and alcoholic, phenolic terpenes (Burt 2004; Koroch *et al.* 2007; Oliva *et al.* 2010). *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano) produce essential oils that have been reported to have significant antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Salmonella typhymurium, Listeria innocua, Serratia marcescens* and *Pseudomonas fluorescens* (Viuda-Martos *et al.* 2011; Coy Barrera & Acosta 2013). A previous study demonstrated the ability of these essential oils to inhibit growth of phytopathogenic *P. syringae* strains (Oliva *et al.* 2015). These results prompted further studies on the mechanism of essential oils against bacterial infection. Nowadays, investigations on control of phytopathogenic strains focus on inhibition of production of molecules related to virulence, *e.g.* phytotoxins and biofilms (Geske *et al.* 2007). The aim of the present study was to investigate effects of *T. vulgaris* and *O. vulgare* essential oils on production of virulence factors of phytopathogenic *P. syringae* strains, and assess their anti-biofilm and anti-toxin activities.

MATERIAL AND METHODS

Essential oils

Essential oils of *T. vulgaris* (L) and *O. vulgare* (L) cv 'chileno' were obtained from Establecimiento Agroproductivo Los Molles, San Luis, Argentina, through hydro-distillation in a Clevenger-like apparatus from dried leaves (100 g) of each species. They were stored at −20 °C with anhydrous sodium sulphate (Aumeeruddy-Elalfi *et al.* 2016). Identification and quantification of thyme and oregano essential oils were previously assessed using gas chromatography–mass spectrometry (GC-MS). In thyme, 36 compounds were identified, while in oregano there were 24 compounds. The main components in both oils were: carvacrol, p-cimene and c-terpinene, with low percentages of thymol. Oregano oil also contained cis-sabinene hydrate among its main terpenes (Oliva *et al.* 2015).

Microorganisms

The bacterial isolates used were: *P. syringae* C13LS (KJ569375), *P. syringae* EM1 (KJ569377), *P. syringae* LS3 (KJ569373), *P. syringae* Q (KJ569372), *P. syringae* pv *tomato* DC 3000, *P. syringae* pv *syringae* B728a, *P. savastanoi* pv *glycinea* B076, *P. syringae* pv *tabaci* 6605 and *P. syringae* pv *atropurpurea* (Ps5) (Table 2).

Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of thyme and oregano oils was determined using the broth microdilution method described in Mann & Markham (1998), with some modifications, on *P. syringae* pv *syringae* B728a, *P. syringae* pv *glycinea* B076, *P. syringae* pv *tabaci* 6605 and *P. syringae* pv *atropurpurea* (Ps5). For *P. syringae* C13LS, *P. syringae* EM1, *P. syringae* LS3, *P. syringae* Q, *P. syringae* pv *tomato* DC 3000, MIC values were obtained from Oliva *et al.* (2015). Tubes containing King's B broth (KBB) were inoculated with each phytopathogenic bacterial strain and incubated at 28 °C overnight. Cell densities were measured at 620 nm in a spectrophotometer.

First, the appropriate cell density to perform the MIC assays was determined for each strain using resazurin, a redox indicator that is blue in its oxidised form and pink in its reduced form, and produces a colour change when the surrounding medium is reduced as a result of dissolved oxygen and acid production (Mann & Markham 1998). Serial 10-fold dilutions of the overnight culture were prepared in KBB, and 170 µl of each dilution were dispensed into microtitre plates containing 20 µl dimethylsulphoxide (DMSO) and water (1:8) and 10 µl resazurin solution (0.01% w/v). The microtitre plates were incubated for 24 h at 28 °C and the appropriate dilution unable to reduce resazurin (blue) was chosen for the antimicrobial assays, *i.e.* 1 log cycle below the cell density required to reduce resazurin (usually 10^5–10^6 CFU·ml^{-1}). As control, the viable plate count method on KB agar (KBA) was used to determine the CFU·ml^{-1}. To determine minimum inhibitory concentration (MIC), serial twofold dilutions of each essential oil were prepared in DMSO (1:8) by vortexing at room temperature. A sterile 96-well microtitre tray was used for the following dilutions of bacteria: columns 1–10, 170 µl inoculum + 20 µl essential oil; column 11, 170 µl inoculum + 20 µl diluent DMSO (1:8; positive control = pink); column 12, assay medium (KBB + 20 µl diluent; negative control = blue). Two trays were prepared for each strain and incubated at 28 °C for 24 h. After incubation, 10 µl resazurin solution were added to all wells and incubated again for 4 h at 28 °C. Wells were assessed visually for colour change, with the highest dilution remaining blue, indicating the MIC (Mann & Markham 1998).

Determination of minimum bactericidal concentration (MBC)

Bactericidal activity was assessed by adding 100 µl of an overnight culture of each strain to tubes containing minimum medium (MM) with different dilutions of each essential oil. These tubes were incubated at 28 °C for 24 h in a shaker and a spot (20 µl) placed onto a plate with KBA. Samples were incubated for 5 days at room temperature, then MBC was determined as bactericidal activity when <0.01% growth was observed.

Inhibition of biofilm formation assay

For biofilm formation assays, overnight *P. syringae* cultures in KBB were added to microtitre wells filled with fresh KBB and incubated at 28 °C for different times: 8, 24, 36 and 48 h. After each time interval, cultures were discarded and microtitre plates washed with distilled water twice then filled with crystal

violet (1%) and left for 30 min. Plates were then thoroughly washed with distilled water and visual qualitative detection made. The effect of essential oils on biofilm production was visualised and quantified by solubilisation with 95% ethanol. The absorbance of the microplates was measured at 570 nm in a spectrophotometer (O'Toole & Kolter 1998; Niu & Gilbert 2004).

Phenotypic assays for phytotoxin production

Coronatine production was determined using the semi-quantitative potato disk bioassay. An aliquot of 50 µl overnight *P. syringae* culture was added to Eppendorf tubes containing 1 ml MM and shaken at 20 °C for 4 days. These bacterial suspensions were centrifuged at 1906 *g* for 10 min at room temperature, and 20 µl of the supernatant were spotted on to a potato tuber disk. The presence of coronatine was characterised by a hypertrophic response on the potato disks (Hwang *et al.* 2005).

Syringomycin in *P. syringae* strains was assessed after incubation for 24 h at 28 °C. An aliquot of 20 µl of the suspension was spotted on potato dextrose agar, followed by incubation for 5 days at 30 °C. Subsequently, the plates were exposed to chloroform vapour for 15 min and sprayed with a suspension of *Geotrichum citri aurantii* spore and left for 24 h at room temperature. Positive toxin production was considered as development of a zone of inhibition surrounding the *P. syringae* colonies (Hwang *et al.* 2005).

Tabtoxin was assessed in *P. syringae* strains grown in KBB for 120 h at 28 °C then centrifuged at 1906 *g* for 10 min. Supernatants were divided in two fractions, adding 5 $\mu l \cdot ml^{-1}$ glutamine. Plates containing MM were overlaid with MM soft agar (7.5%, w/v) containing an overnight culture of *Escherichia coli*. Wells were made in the plates and filled with the two fractions of the supernatant, then incubated at room temperature for 2 days. Positive tabtoxin production was assessed as zones of growth inhibition of *E. coli* in wells containing supernatant without glutamine, but not seen with glutamine (Barta *et al.* 1992; Lydon & Patterson 2001).

Genotypic assays of phytotoxin production

Amplification with PCR was used for detection of phytotoxin genes in *P. syringae* strains using specific oligonucleotide primers from published sequences. All oligonucleotides were synthesised at Biodynamics SRL, Buenos Aires, Argentine. The PCR was standardised for detection of each virulence-associated gene following methodologies described in bibliography with some modifications (Table 1). The primers sequences and programmes used are shown in Table 2. The reaction mixture (50 µl) for gene amplification contained 20 ng template DNA, 1 mM oligonucleotide primers, 0.4 mM of each of the four dNTPs, 1.50 U Taq polymerase and 1.5 mM $MgCl_2$. Temperature for annealing varied from 57 to 60 °C and reactions were carried out in a Termocycler PTC 220 MJ research (Berkshire, UK). Each phytotoxin gene was tested at least twice, including positive and negative controls in each run (Table 1). PCR products were resolved on 1.2% agarose gel at 90 V for 1 h. Gels were stained with 1 ml SYBR Safe DNA gel stain per 50 ml melted agarose and photographed under UV light with MiniBisPRO (BioAmerica, Department of Microbiology and Inmunology of the National University of Rio Cuarto) gel documentation.

Inhibition of phytotoxin production

The inhibition of phytotoxins by the two essential oils was phenotypically assayed, using the same methodologies as described above for each toxin, with the addition of sub-inhibitory concentrations of essential oils at the initial inoculum.

Statistical analysis

The Infostat/Professional statistical analysis program (Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Provincia de Córdoba, República Argentina) was used for ANOVA and LSD Fischer tests. Significant differences in biofilm assays were calculated for each strain (Di Rienzo *et al.* 2016).

RESULTS

Antimicrobial activity of *T. vulgaris* and *O. vulgare* essential oils

The inhibitory effect (MIC) of essential oils of *T. vulgaris* and *O. vulgare* was investigated on *P. syringae* pv *syringae* B728a, *P. savastanoi* pv *glycinea* B076, *P. syringae* pv *tabaci* 6605 and *P. syringae* pv *atropurpurea* Ps5. Both oils showed good inhibitory activity on all *P. syringae* strains, with MIC values ranging from 1.43 to 11.5 $mg \cdot ml^{-1}$ for thyme and 5.8 to 11.6 $mg \cdot ml^{-1}$ for oregano (Table 2). The ability of thyme and oregano oils to cause the cell death was tested on all *P. syringae* strains, with bactericidal activity for thyme from 0.022 to 5.7 $mg \cdot ml^{-1}$ and

Table 1. PCR primers, annealing temperatures and expected PCR products for phytotoxins of *P. syringae* strains.

target gene	phytotoxin	primer sequence (5′–3′)	annealing temperature (°C)	product size (bp)	references
cfl	coronatine	GGCGCTCCCTCGCACTT GGTATTGGCGGGGGTGC	58	650	Schaad *et al.* (2001)
*syr*B	syringomycin	CTTTCCGTGGTCTTGATGAGG TCGATTTTGCCGTGATGAGTC	60	752	Sorensen *et al.* (1998), Schaad *et al.* (2001)
*syr*D	syringomycin	AAACCAAGCAAGAGAAGAAGG GGCAATACCGAACAGGAACAC	60	446	Sorensen *et al.* (1998)
*tbl*A	tabtoxin	CTGGTCACTGCGCTAGGC CTGGTCACTGCGCTAGGC	57	829	Lydon & Patterson (2001)

Table 2. Minimum inhibitory (MIC) and bactericidal (MBC) activity of *T. vulgaris* and *O. vulgare* essential oils on *P. syringae* strains (mg·ml^{-1}).

		T. vulgaris (0.022–45.99 mg·ml^{-1})		*O. vulgare* (0.022–6.26 mg·ml^{-1})	
strain	source	MIC	MBC	MIC	MBC
P. syringae C13LS[a]	soybean	11.5*	5.7	5.8*	0.022
P. syringae EM1[a]	soybean	11.5*	0.71	11.6*	0.09
P. syringae LS3[a]	soybean	11.5*	0.17	46.3*	0.36
P. syringae Q[a]	soybean	11.5*	0.71	23.1*	0.022
P. syringae pv *tomato* DC3000[b]	tomato	11.5*	0.022	23.1*	0.045
P. syringae pv *syringae* B728[a,c]	bean	1.4	0.022	5.8	0.045
P. savastanoi pv *glycinea* B076[d]	soybean	5.8	0.17	5.8	0.022
P. syringae pv *tabaci* 6605[e]	tobacco	11.5	0.089	11.6	0.18
P. syringae pv *atropurpurea* Ps5[f]	oats	2.9	-	11.6	0.012

(-): Not done; * [a]From Oliva *et al.* (2015); [b]provided by A. Collmer; [c]provided by S. Lindow; [d]provided by Y. Zhao; [e]provided by D. Studholmer; [f]provided by L. Gallarato.

for oregano 0.012 to 0.36 mg·ml^{-1}. Oregano oil was more effective than thyme, as smaller concentrations were needed to achieve bactericidal activity (Table 2).

Inhibition of biofilm formation by essential oils

The ability of *P. syringae* to form biofilm was analysed at 8, 24, 36 and 48 h. Biofilm formation was optimal at 24 h, with no variations in OD values (data not shown) so this time was used for further analyses (Fig. 1). Anti-biofilm activity of thyme and oregano essential oils was assayed against *P. syringae* strains at inhibitory and sub-inhibitory concentrations. Biofilm formation was inhibited by both essential oils in all *P. syringae* strains and was more effective at higher concentrations, in terms of increased polysaccharide formation as lower concentrations of the oils. Thyme oil reduced biofilm formation at all but the lowest concentration tested (0.022 mg·ml^{-1}; Fig. 1).

Phytotoxin production

Phytotoxins are involved in colonisation of the host, so determining the ability of *P. syringae* to produce them will help in understanding of the pathogenicity process. *P. syringae* C13LS, *P. syringae* EM1, *P. syringae* LS3, *P. syringae* Q, *P. syringae* pv *tomato* DC3000 and *P. savastanoi* pv *glycinea* B076 were all able to produce coronatine (Table 3). Syringomycin was produced in all *P. syringae* strains except *P. syringae* pv *atropurpurea* Ps5. This phytotoxin produced an inhibition halo for *G. citri aurantii* (Table 3). In *P. syringae* C13LS, *P. syringae* EM1, *P. syringae* LS3, *P. syringae* Q, *P. syringae* pv *atropurpurea* Ps5 and *P. syringae* pv *tabaci* 6605 tabtoxin was produced as an inhibition zone for *E. coli* (Table 3).

For detection of coronatine genes, primers 1 and 2 were used to detect the presence of *cfl*, and amplified a fragment of 650 bp. This study showed that the coronatine gene *cfl* is involved in synthesis of this toxin in *P. syringae* C13LS, *P. syringae* LS3, *P. syringae* Q, *P. syringae* pv *tomato* DC 3000, *P. syringae* pv *glycinea* B076 and *P. syringae* pv *atropurpurea* Ps5, but was not in *P. syringae* EM1 (Fig. 2a; Table 3). For detection of syringomycin genes, two primers were used, *syr*B and *syr*D (Sorensen *et al.* 1998). *P. syringae* pv syringae B728a was the only strain in which amplification of both fragments was observed, coinciding with the phenotype results for production of this toxin (Fig. 2b). However, these genes were not detected in any other strains that were phenotypically positive producers (Table 3). Detection of *tblaA*, involved in tabtoxin synthesis, was positive for all *P. syringae* strains that were phenotypically positive producers. A fragment of 830 bp, similar to *tbl*A product, was detected in *P. syringae* pv *atropurpurea* Ps5; in other strains there was no amplification of this fragment (Table 3, Fig. 2c).

Inhibition by essential oils of phytotoxin production

The effect of sub-bactericidal concentrations of thyme and oregano oils on production of phytotoxins was tested on the phytopathogenic *Pseudomonas* strains. Both oils inhibited production of coronatine, syringomycin and tabtoxin in these *P. syringae* strains. Coronatine inhibition of strains showing pheno- and genotypic production of this toxin, *P. syringae* LS3, *P. syringae* Q, *P. syringae* pv *tomato* DC 3000 and *P. savastanoi* pv *glycinea* BO76, was examined. There was inhibition by both oils at 0.71 mg·ml^{-1} for thyme and 0.09 mg·ml^{-1} for oregano in *P. syringae* Q, while in *P. syringae* LS3 oregano oil caused inhibition at 0.045 mg·ml^{-1}. The effect on syringomycin for strains of *P. syringae* in which phenotypic production was observed (Table 3), showed that oregano essential oil inhibited syringomycin production in all strains (100%) at 0.003–0.11 mg·ml^{-1}, while thyme oil inhibited it in 87.5% of strains at concentrations from 0.006 to 11.49 mg·ml^{-1}. The effect of both essential oils on tabtoxin production in *P. syringae* pv *atropurpurea* Ps5, which was the only strain pheno- and genotypically positive for toxin production, showed inhibitory activity of thyme at 2.85 mg·ml^{-1} and oregano at 0.006 mg·m^{-1} (Table 3).

DISCUSSION

The control of *P. syringae*, a bacterium associated with severe plant disease, remains difficult due to limited availability of antimicrobial substances. Natural products obtained from aromatic plants represent a potential source of molecules with biological activity. Studies with extracts of *Allium sativum* and *Ficus carica* proved to be effective for biocontrol in *P. syringae* pv *tomato*, *Xanthomonas versicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* for tomato plants (Balestra *et al.* 2009). Essential oils have been widely studied for their ability to control bacterial and fungal growth in

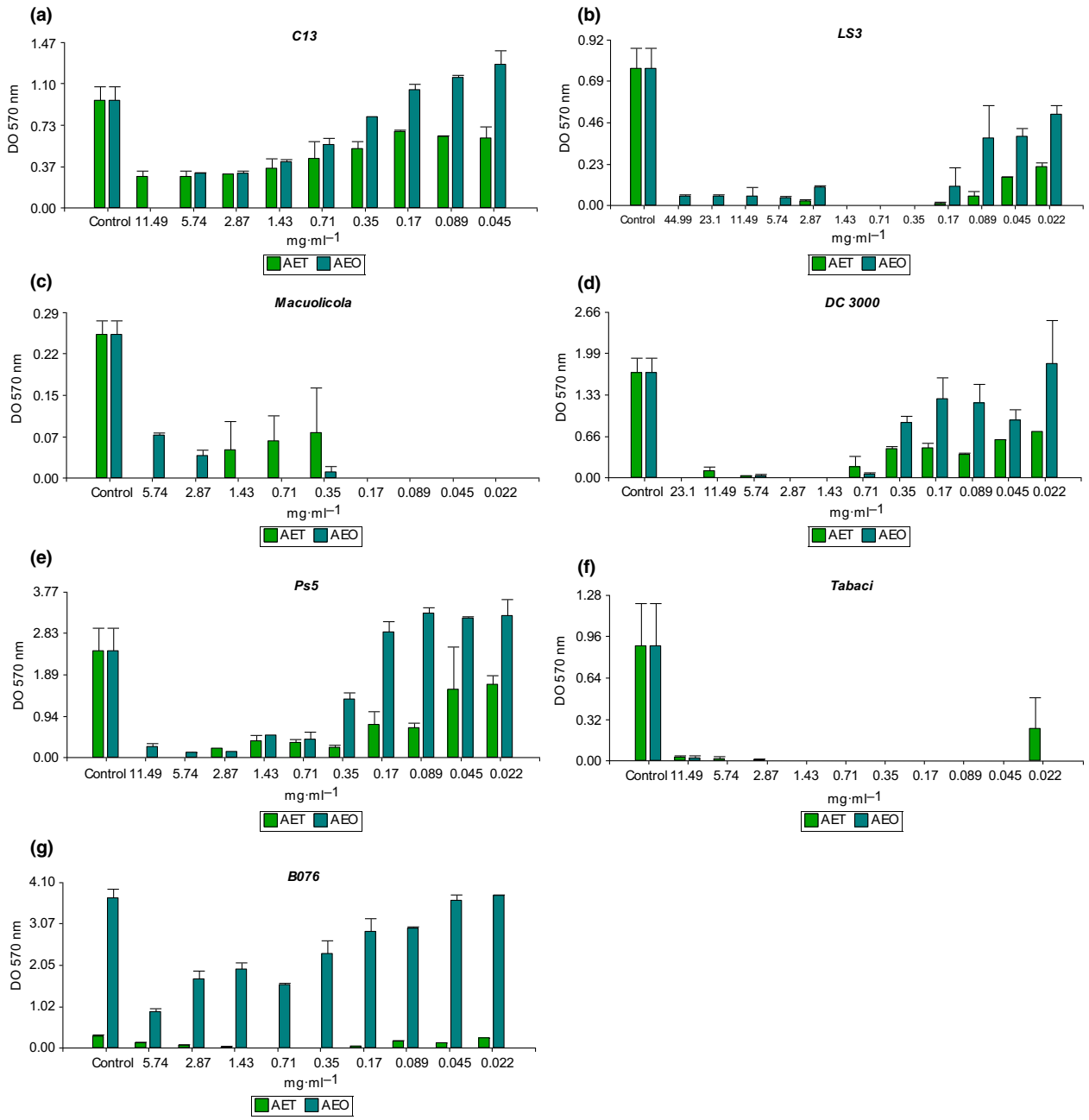

Fig. 1. Effect of thyme and oregano essential oils on biofilm formation of phytophatogenic *P. syringae* strains. (a): *P. syringae* C13LS; (b): *P. syringae* LS3; (c): *P. savastanoi* pv *glycinea* B076; (d): *P. syringae* pv *tomato* DC3000; (e): *P. syringae* pv *atropurpurea* Ps5; (f and g): *P. syringae* pv *tabaci* 6605 and *P. syringae* Q. DO = optical density; AET = thyme essential oil; AEO = Oregano essential oil.

animals and foods (Koroch *et al.* 2007; Voon *et al.* 2012). This has led to further research in the use of plant products for control of microorganisms responsible of plant diseases. Significant antibacterial activity against many phytopathogenic bacteria was shown for *Coriandrum sativum* L. and *Foeniculum vulgare* Miller var. *vulgare* (Miller) essential oils, suggesting they may be useful natural bactericides (Lo Cantore *et al.* 2004). The essential oil obtained from members of the Lamiaceae, including *Origanum onites* L. (oregano) and *Thymbra spicata* L. var. *spicata* (thyme), were tested *in vitro* against *P. savastanoi* pv *phaseolicola* (*Psp*), the causal agent of halo blight of bean, and shown to inhibit activity and growth of this pathogen (Soylu *et al.* 2003).

Our group previously reported the inhibitory effect of *T. vulgaris* and *O. vulgare* essential oils on phytopathogenic *P. syringae* isolated from soybean fields (Oliva *et al.* 2015). Here, the antimicrobial activity of both oils on *P. syringae* strains and anti-biofilm and anti-phytotoxin effects are reported. Both oils showed good inhibitory activity on all *P. syringae* strains (MIC: 1.43–11.5 $mg \cdot ml^{-1}$) and effective bactericidal activity (MBC: 0.012–5.7 $mg \cdot ml^{-1}$). The MIC for thyme and oregano essential oils was obtained using a microdilution

Table 3. Phenotypic and genotypic production of phytotoxins in *P. syringae*.

toxin	coronatine		syringomycin		tabtoxin	
strain	P	G	P	G	P	G
P. syringae C13LS	+	+	+	–	+	–
P. syringae EM1	+	–	+	–	+	–
P. syringae LS3	+	+	+	–	+	–
P. syringae Q	+	+	+	–	+	–
P. syringae pv *tomato* DC3000	+	+	+	–	–	ND
P. syringae pv *syringae* 728a	–	ND	+	+	–	–
P. savastanoi pv *glycinea* B076	+	+	+	–	–	ND
P. syringae pv *tabaci* 6605	–	ND	+	ND	+	–
P. syringae pv *atropurpurea* Ps5	–	+	–	–	+	+

P = phenotypic; G = genotypic; ND = not done.

method and KBB growth medium, while the MBC was found with a macrodilution method in MM with incubation on a rotary shaker. In this last method, active concentrations that had a biocidal effect were smaller compared to MIC concentrations, possibly because incubation with movement (shaking) allowed better contact between essential oils and cells, thus enhancing the antimicrobial activity. Several techniques for studying antimicrobial activity have been proposed, many having different sensitivity according to the method employed – liquid or in solid media – where the liquid method can be used to kill (biocidal effect) or inhibit (static effect) at a specific concentration. Another factor is growth medium composition, which can have a strong effect on antibacterial activity of a compound (Cos *et al.* 2006). The results obtained in this work are promising for use of both essential oils as bactericides to control phytopathogenic bacteria.

Biofilms

Biofilms are three-dimensional aggregates of bacterial cells employed as a strategy for the colonisation of different environments and function as protection from stressful conditions and antimicrobial agents (Morris & Monier 2003). Biofilms are organised structures consisting of exopolysaccharides, proteins, teichoic acids and extracellular DNA, with channels that allow nutrient circulation, and contain differentiated cells (Beoletto *et al.* 2016). Leaf surfaces are an optimum environment for phytopathogenic bacteria to form these structures, leading to serious damage. Biofilms are very difficult to eradicate because they prevent ingress of antimicrobial substances (Brackman & Coenye 2015). Essential oils can interfere in the formation of microbial biofilms, and could provide an innovative anti-biofilm alternative for control of pathogenic bacterial invasion (Beoletto *et al.* 2016).

Pseudomonas has become a model for biofilm studies because of its ability to form films on biotic and abiotic substrates (Kumar *et al.* 2013). This work examined biofilm production by all phytopathogenic *P. syringae* strains. The dynamics of biofilm formation are sigmoidal, with initial low levels, rising to maximum production and then decreasing to initial values. The timing of this behaviour varies between strains, with maximum biofilm production at 8 h for some strains and 36 h for others, and achieving a uniform amount at 24 h for all strains. Thus, the effect of thyme and oregano essential oils on biofilm formation was tested at this time when production was similar in all strains. Anti-biofilm efficacy of thyme and oregano essential oils at sub-inhibitory and inhibitory concentrations was found against all *P. syringae* strains. Furthermore, biofilm production of some *P. syringae* strains was totally inhibited when MIC concentrations were used. There are many reports of anti-biofilm activity of essential oils on bacteria, but few for phytopathogenic bacteria. Both *Staphylococcus aureus* and *P. aeruginosa* biofilm production was inhibited by oregano essential oil (Schillaci *et al.* 2013). The anti-biofilm activity of sub-inhibitory concentrations of oregano, carvacrol and thymol on *S. aureus* and *S. epidermidis* has also been demonstrated (Nostro 2007). Iinhibition of polysaccharide production in *Pseudomonas* sp. and *E. coli* by terpenes such as eugenol and citronellol has been reported (Niu & Gilbert 2004). Cinnamon bark oil and its main terpene, cinnamaldehyde, showed ability to reduce biofilm formation in *P. aeruginosa* and enterohaemorragic *E. coli* O157:H7 (EHEC) (Kim *et al.* 2015). Hence, this is the first report in which biofilm inhibition of phytopathogenic *P. syringae* treated with essential oils has been demonstrated.

Phytotoxins produced by *P. syringae*

Phytopathogenic pseudomonads synthesise molecules that significantly enhance pathogen virulence, *e.g.* phytotoxins, but these are not encoded through the *hrp* (hypersensitive response and pathogenicity; Bender *et al.* 1999) cluster. These

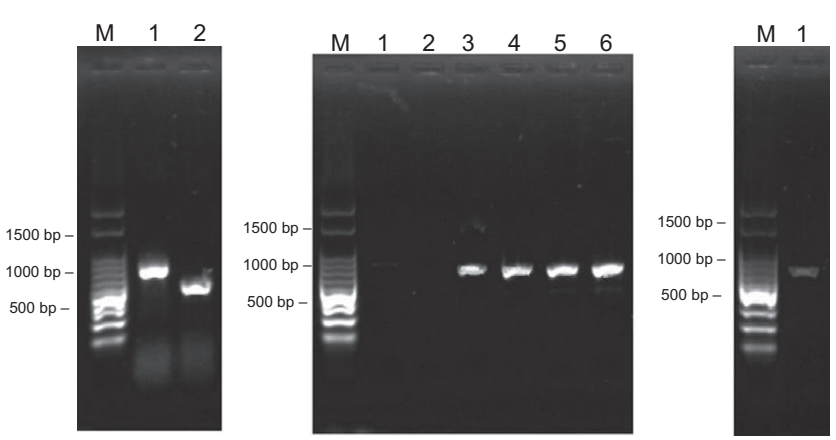

Fig. 2. (a) Typical amplicons of *cfl* gene, encoding coronatine of 650 bp (lanes: 1, 2, 3, 4, 5 and 6); (b) *syr*B and *syr*D genes for syringomycin of 700 bp (lane 1) and 446 bp (lane 2); (c) genes encoding *tbl*A gene segments for tabtoxin of 829 bp (lane 1); lane M 100 bp molecular weight marker (Gibco).

phytotoxins can restrict and/or promote specific pathogen–host interactions. The toxins most common in *P. syringae* are coronatine, phaseolotoxin, syringomycin and tabtoxin, which contribute to chlorosis and necrosis (Hwang *et al.* 2005). Here, production of phytotoxins by *P. syringae* strains was analysed both phenotypically and genotypically, and the inhibitory effect on production of coronatine, syringomycin and tabtoxin at sub-inhibitory concentrations of thyme and oregano essential oils was demonstrated.

Coronatine

This is a phytotoxin with a similar structure to methyl jasmonate, a growth regulator and signalling molecule in plants. This phytotoxin helps in virulence of *P. syringae*, stimulating cell growth and resulting in bacterial gall formation or in black spots on leaves that induce chlorotic haloes (Chowdhury & Jagannadham 2013). Production of coronatine has been documented in *P. syringae* pv *atropurpurea*, *P. savastanoi* pv *glycinea*, *P. syringae* pv *morsprunorum*, *P. syringae* pv *macuolicola* and *P. syringae* pv *tomato.* Its production is associated with induction of many genes commonly localised in plasmids (Hwang *et al.* 2005; Arrebola *et al.* 2011). Coronatine consists of polyketide coronafacic acid (CFA) and coronamic acid (CMA) linked by an amide bond (Bender *et al.* 1999). The enzyme responsible for amide bond formation is codified *via* the *cfl* gene (Bereswill *et al.* 1994; Braun *et al.* 2009). In the present study, phenotypic production of coronatine was observed in *P. syringae* strains isolated from soy, in *P. syringae* pv *tomato* DC3000 and in *P. savastanoi* pv *glycinea* BO76, as reported by other authors (Hwang *et al.* 2005; Braun *et al.* 2009; Arrebola *et al.* 2011). In addition, detection of *cfl* was assayed using primers 1 and 2, which specifically amplify a portion of *cfl*, confirming its presence in *P. syringae* C13LS, *P. syringae* LS3, *P. syringae* Q, *P. syringae* pv *tomato* DC3000, *P. savastanoi* pv *glycinea* BO76 and *P. syringae* pv *atropurpurea* Ps5. Absence of *cfl* in some *P. syringae* strains could be because it is located in plasmids, which can be lost through successive strain passages.

In the current work, one of the main objectives was evaluation of essential oil effects on phytotoxins production. The inhibition of coronatine production by thyme and oregano oils at sub-bactericidal concentrations was demonstrated on *P. syringae* LS3 and *P. syringae* Q, which were both pheno- and genotypically positive for toxin production.

Syringomycin

The small cyclic lipodepsinonapeptide syringomycin causes electrolyte leakage *via* pores formed in the host plasma membrane, increasing cellular K^+ efflux and transient Ca^{2+} fluxes. Syringomycin inhibits many fungi through lipid-dependent membrane interactions (Hwang *et al.* 2005; Bensaci *et al.* 2011). This toxin was phenotypically present in all *P. syringae* strains according to an assay with inhibition of *Geotrichum citri aurantii;* only *P. syringae* pv *atropurpurea* Ps5 was unable to produce it. Although production of the toxin was observed *in vitro*, genotypic detection of *syrB* and *syrD* was positive only in *P. syringae* pv *syrinage* B728a. Genetic regulation of this toxin is complex; the syringomycin (*syr*) gene cluster comprises six ORFs that encode proteins involved in synthesis, secretion and regulation of syringomycin (Sorensen *et al.* 1998). Syringomycin production has been described only in *P. syringae* pv *syringae* (Bull *et al.* 1998; Fogliano *et al.* 1999). In this research, *syrB* and *syrD* from the cluster were analysed, but found in only one strain, *P. syringae* pv *syringae* B728a, confirming previous reports. However, phenotypically the majority of phytopathogenic strains isolated from soybean, bean and tomato did produce this toxin. Therefore, more studies are needed to determine other genes belonging to this cluster, using related primers. Both thyme and oregano essential oils were very effective on this toxin in all phenotypically positive strains, with complete effectiveness of oregano oil (100% of strains) and 85% for thyme oil.

Tabtoxin

Pseudomonas syringae pv *tabaci,* pv *coronafaciens* and pv *garcae* are tabtoxin producers. It is responsible of chlorosis of host plant cell, producing cleavage of the peptide bond (Hwang *et al.* 2005; Young *et al.* 2008). Tabtoxin is a β-lactam that inhibits glutamine synthesis and is composed of tabtoxinine-b-lactam (TbL) linked to threonine. The genes for tabtoxin synthesis in *P. syringae* are clustered in a 31-kb region that contains *tabA, tabB* and *tblA* genes. *tabA* synthesises a precursor of tabtoxin, while *tabB* codifies an acetyl transferase and *tblA* encodes a histidine protein kinase that functions as an environmental sensor (Bender *et al.* 1999). Phenotypic production of tabtoxin by *P. syringae* strains was observed in most strains, and genotypic detection of *tblA* only in *P. syringae* pv *atropurpurea* Ps5. These results are in accordance with Lydon & Patterson (2001), who obtained strains that were phenotypically positive for tabtoxin production but not for detection of these genes. The inhibition by essential oils of *P. syringae* pv *atropurpurea* Ps5 was both pheno- and genotypically positive for tabtoxin production at sublethal doses, particularly oregano oil, which was effective at lower concentrations.

CONCLUSIONS

There have been many studies on inhibitory activity of natural products obtained from plants on *P. aeruginosa* virulence factors (QSI: quorum sensing inhibitors). All extracts tested from six south Florida medicinal plants from different families exerted anti-QS activity against *P. aeruginosa* PAO1, including a decrease in biofilm formation (Adonizio *et al.* 2008). Other research with *P. aeruginosa* showed that cinnamon bark oil and its main terpene component, cinnamaldehyde, markedly reduced biofilm formation (Kim *et al.* 2015). Similar results were obtained with curcumin, which attenuated *P. aeruginosa* PAO1 biofilm formation as well as down-regulating production of extracellular virulence factors and AHLs (Rudrappa & Bais 2008).

The plant kingdom has been a source of medicines since ancient times. Recently, studies on the inhibition of virulence factors and QSI systems by medicinal plant extracts have increased. This is the first report that describes the ability of thyme and oregano essential oils to inhibit biofilm and phytotoxin production in *P. syringae* strains. The results are promising because they show antibacterial activity and, at sub-lethal doses, a reduction in virulence factors in *P. syringae* strains, providing insight into how such oils can be used in the future to treat phytopathogen infections. Knowledge of antimicrobial

activity of thyme and oregano essential oils provides opportunities to test natural substances for control of phytopathogenic bacteria responsible of severe diseases that lead to economic losses in many crops. Moreover, these natural compounds could help to reduce use of chemical pesticides, which are environmentally harmful. Studies examining the effectiveness of these oils in greenhouse and open field experiments should be carried out in order to evaluate the effects of these natural substances in control of *Pseudomonas* infections in susceptible hosts.

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