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RESEARCH PAPER

Antimicrobial activity of essential oils of *Thymus vulgaris* and *Origanum vulgare* on phytopathogenic strains isolated from soybean

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ABSTRACT

The aim of this work was to study the antimicrobial activity of essential oils obtained from *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano) on phytopathogenic *Pseudomonas* species isolated from soybean. Strains with characteristics of *P. syringae* were isolated from leaves of soybean plants with blight symptoms. Ten of these could be identified in Group Ia of LOPAT as *P. syringae*. Six of these were confirmed as *P. syringae* using 16S *rRNA*, indicating the presence of these phytopathogenic bacteria in east and central Argentina. All the phytopathogenic bacteria were re-isolated and identified from the infected plants. MIC values for thyme were 11.5 and 5.7 mg·ml^{-1} on *P. syringae* strains, while oregano showed variability in the inhibitory activity. Both essential oils inhibited all *P. syringae* strains, with better inhibitory activity than the antibiotic streptomycin. The oils were not bactericidal for all pseudomonads. Both oils contained high carvacrol (29.5% and 19.7%, respectively) and low thymol (1.5%). Natural products obtained from aromatic plants represent potential sources of molecules with biological activity that could be used as new alternatives for the treatment of phytopathogenic bacteria infections.

INTRODUCTION

In recent years, soybean (*Glycine max* (L.) Merr.) has become the most important crop in Argentina and most of the production enters the international market. The United State Department of Agriculture (USDA) has estimated record crops (more than 55 millions·t) for this country, because of good environmental conditions and suitable technologies. Over 90% of soybeans from Argentina are produced in the northern Pampean subregion (provinces of Córdoba, Santa Fe, Buenos Aires and La Pampa); the rest are produced in the northwestern and northeastern regions. However, since the early 1990s, diseases have gradually become a major problem for soybean production in many areas of Argentina (Wrather *et al.* 2001; http://www.fao.org/agronoticias/agro-noticias). These diseases are caused either by filamentous fungi or bacteria and success of diseases on soybean plants depends on their age, area, climate and management of agricultural practices. Worldwide, soybean diseases cause losses of 10–15%, while Argentine losses are estimated at 8–10% (http://inta.gob.ar/documentos/informe-sanitario-de-la-campana-de-soja-2012-13).

Many of the causal agents of plant bacterial diseases belong to the *Pseudomonas syringae* group. *P. syringae* pv. *glycinea*, is a member of this group and is responsible for common bacterial blight of soybean. This bacterium produces light brown or brown water-soaked lesions on leaves, which increase in size and finally cause tearing of leaves (Scandiani & Luque 2009). This disease was present in Cordoba province, with incidence of 100% and severity of 20% for the 2012/2013 season (http://inta.gob.ar/documentos/informe-sanitario-de-la-campana-de-soja-2012-13).

Pseudomonas syringae has worldwide economic importance because it has a wide host range and causes disease in over 180 plant species, consisting of necroses on leaves, stems, fruits and other aerial plant parts (Morris *et al.* 2007). It was classified by Lelliott *et al.* (1966) in Group I of LOPAT and is subdivided into approximately 50 pathovars, based largely on the original host of isolation and pathogenicity, rather than biochemical or physiological criteria (Krzymowska *et al.* 2007). This bacterium has an epiphytic phase where it can establish large populations on aerial surfaces of plant species without necessarily causing disease. In its pathogenic phase it survives thanks to photosynthate from host plants, and virulence factors such as syringomycin and related toxins, to which a wide range of prokaryote and eukaryote cells are sensitive (Morris *et al.* 2007).

The control of bacterial diseases of crops has been and is nowadays a considerable problem due to the limited availability of antimicrobial substances without side effects. The antibiotic streptomycin and copper compounds are frequently applied for treatment of bacterial diseases but these are toxic to organisms and the environment. Moreover, pathogens can become drug resistant, or can undergo possible horizontal genetic transfer to become animal and human pathogens. Furthermore, these substances are often prohibited in agricultural practice in many countries of Europe and the USA (Lo Cantore *et al.* 2004; Kotan *et al.* 2013). A consequence of the above facts is the failure of disease control.

In recent years, the biological properties of plant extracts, *e.g.,* essential oils, have been evaluated, and many studies have pointed out the possibility of using them for the control of microorganisms that are pathogenic to animals and plants, as well as causing food spoilage (Cowan 1999). The requirement for a reduction in the use of pesticides in agriculture prompts a need to develop alternative active compounds. Research on growth inhibition of phytopathogenic bacteria using plant extracts has intensified, and the results strongly suggest the potential of essential oils to control plant bacterial diseases (Lo Cantore *et al.* 2004; Paret *et al.* 2010; Kotan *et al.* 2013). Studies of the effects of *Coriandrum sativum* and *Foeniculum vulgare* essential oils on plant phytopathogenic bacteria have already shown growth inhibition activity for several microorganisms, with high bactericidal activity of *C. sativum* oil. Coriander oil inhibited the growth of *Escherichia coli, Bacillus megaterium* and important plant pathogenic bacteria in Gram-negative genera such as *Pseudomonas*, *Erwinia*, *Xanthomonas* and *Agrobacterium*, and Gram-positive genera such as *Clavibacter*, *Curtobacterium* and *Rhodococcus* (Lo Cantore *et al.* 2004).

Essential oils are volatile compounds with a strong odour and are synthesised by aromatic plants as secondary metabolites. They are mixtures of complex compounds based on a 5-carbon isoprene structure (terpenes), occurring as diterpenes, triterpenes, tetraterpenes (C20, C30 and C40), hemiterpenes (C5) and sesquiterpenes (C15). Most of the antimicrobial activity in essential oils is dependent on the diversity and quantity of such constituents, and could be attributed to oxygenated terpenoids as well as alcoholic and phenolic terpenes (Burt 2004; Koroch *et al.* 2007; Oliva de las *et al.* 2010).

Among these aromatic plant species, *Thymus vulgaris* and *Origanum vulgare* occupy a special position because their essential oils and extracts showed very significant antimicrobial activity against various species of bacteria, especially Gram-negative bacteria and fungi (Viuda-Martos *et al.* 2011; Coy Barrera & Acosta 2013). In the search of new alternatives for the treatment of phytopathogenic bacterial diseases, the aim of this work was to study the antimicrobial activity of essential oils obtained from *T. vulgaris* (thyme) and *O. vulgare* (oregano) on phytopathogenic *Pseudomonas* species isolated from soybean plants.

MATERIAL AND METHODS

Isolation of phytopathogenic strains

Leaves of soybean with characteristic bacterial blight symptoms were collected from fields located in different regions of Argentina. For the bacterial isolation, leaves were disinfected first with ethanol (70%) and then with sodium hypochlorite (1%). Fragments of vegetal tissues were aseptically removed into sterile dishes and sterile distilled water was poured over them. Subsequently, bacteria were isolated by streaking liquid from these dishes onto tryptone soy (TS), cetrimide (CA) and King B (KB) agar plates. The plates were incubated at 28 °C for 24–72 h. For long-term storage, isolated bacteria were maintained in glycerol at −70 °C (Ferrante & Scortichini 2009).

Phenotype characterisation of strains

Conventional biochemical tests were performed on pure cultures of isolated strains: Gram reaction, catalase production, metabolism of glucose (Hugh-Leifson medium), hydrolysis of aesculin, gelatine hydrolysis and fluorescence on King B medium, nitrate reduction, indole, methyl red (MR) and Voges-Proskauer (VP), and motility in semi-solid medium. For putative phytopathogenic bacteria, the following procedures were performed: levan production (L), presence of oxidase (O), soft rot activity on potato slices (P), presence of arginine dehydrolase (A) and hypersensitivity reaction in tobacco leaves (T) (LOPAT tests) (Ferrante & Scortichini 2009).

Reference strains were used: *P. syringae* pv *tomato* DC 3000, *P. syringae* pv *artropurpurea* (Ps5), *P. syringae* pv *syringae* (P61). These strains were provided by Laboratory 15 of the Molecular Biology Department of the National University of Río Cuarto.

Analysis of 16S *rRNA* gene of phytopathogenic strains

Nucleotide sequence of the 16S *rRNA* gene was analysed for 13 bacterial strains isolated from soybean. The 16S *rRNA* gene amplification was carried out using P0 (5′-GAGAGTTTGAT CCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACG A-3′) primers (Lane 1991). PCR conditions, purification of amplicons and sequencing were done by the MACRO GEN Service Center (Seoul, Korea). Identities among these sequences were determined using the BLAST search program (National Center for Biotechnology Information; Altschul *et al.* 1997). The nucleotide sequences of the 16S *rRNA* gene of soybean strains were deposited in GenBank under accession numbers KJ569364, KJ569366, KJ569367, KJ569368, KJ569369, KJ569370, KJ569371, KJ569372, KJ569373, KJ569374, KJ569375, KJ569376 and KJ569377.

A phylogenetic analysis was conducted using MEGA version 4 (Tamura *et al.* 2007). Multiple alignments of 16S *rRNA* sequences were performed using the Clustal X2.0 algorithm (Thompson *et al.* 1997). Alignments were used to build a phylogenetic tree based on neighbour-joining (NJ) algorithms using the Kimura 2-parameter model (Kimura 1980).

Pathogenicity test

Pathogenicity was assessed after spraying soybean leaves of growing plants with a bacterial suspension (10^8 CFU·ml^{-1}; five replicates). Silweet (0.01%) was used as emulsifier. Inoculum was prepared by growing bacterial strains on KB broth for 48 h at 28 °C. A negative control of sterile broth was included in each experiment. Plants were maintained in a controlled growth chamber (25 °C, 12-h photoperiod, 80% relative

humidity). Disease progression was monitored phenotypically, and visible reactions were recorded 7–14 days after inoculation (Samson *et al.* 1998).

Plant material and essential oils

Plant material of *O. vulgare* (L) cv 'chileno' and *T. vulgare* (L) was obtained from the farm Establecimiento Agroproductivo Los Molles, located in San Luis (Argentina). The essential oils were extracted from dried leaves (100 g) of the plants, hydrodistilled in a Clevenger-like apparatus. Anhydrous sodium sulphate was added to the oils for desiccation and they were then stored in a freezer until GC-MS analysis (De Feo *et al.* 1998).

Gas chromatography (GC)

The essential oil composition was analysed with a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with a fused silica column (60 m × 0.25 mm) coated with DB-5. The temperature of the column was programmed from 60 to 240 °C at 4 °C·min^{-1}. The injector and detector temperatures were 270 °C. The gas carrier was helium, at a flow rate of 1 ml·min^{-1}. Peak areas were measured by electronic integration. The relative amounts of the individual components were based on the peak areas obtained, without FID response factor correction. Programmed temperature retention index of the compounds were determined relative to *n*-alkanes. GC analysis was performed using a column Supelcowax-10 with the same conditions as described above (Zunino *et al.* 1998).

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analyses were performed on a Perkin Elmer Clarus 600 with a 60 m × 0.25 mm capillary column coated with DB-5. The temperature of the column and the injector were the same than those from GC. The carrier gas was helium at a flow rate of 0.9 ml·min^{-1}. Mass spectra were recorded at 70 eV. The oil components were identified by comparison of their retention index, of mass spectra with those of authentic samples, by peak enrichment, with published data and with the mass spectra library of the National Institute of Standards and Technology (NIST 3.0), and our mass spectra library, which contains reference mass spectra and retention index of volatile compounds. GC-MS analysis was performed using a column Supelcowax 10 with the same conditions as describe above (Adams 1989).

Microdilution assay

The antimicrobial activity of the essential oils on *Pseudomonas* was determined using the broth microdilution method described in Mann & Markham (1998), with some modifications.

Culture methods
Tubes containing KB broth were prepared at pH 7, and inoculated with the isolated phytopathogenic bacteria and reference strains. These were incubated overnight at 28 °C and optical densities measured at 620 nm in a spectrometer. Cell densities were estimated from standard curves and confirmed by the viable plate count on KB agar.

Inoculum densities
The microorganism cell concentration necessary to cause reduction of resazurin was determined for each of the tested microorganisms. For this, serial ten-fold dilutions of the overnight culture were prepared in KB broth. Aliquots (170 µl) of the inocula were dispensed into a microtitre containing 20 µl dimethyl sulphoxide (DMSO; 1:8) and 10 µl resazurin solution (0.01% w/v). The microtitre was incubated for 24 h at 28 °C. The appropriate dilution was that unable to reduce resazurin (blue), *i.e.,* 1 log cycle lower than the cell density required to reduce resazurin (usually 10^5–10^6 CFU·ml^{-1}). The plate count method was made to this dilution. Resazurin is a redox indicator that is blue in its oxidised form and pink in its reduced form.

Determination of minimum inhibitory concentration (MIC) of T. vulgaris and O. vulgare essential oils
Serial twofold dilutions of each essential oil were prepared in DMSO (1:8) by vortexing it at room temperature. A sterile 96-well microtitre tray was set up with the dilution of bacteria as follows: column 1–10, 170 µl inoculum + 20 µl essential oil dilution; column 11, 170 µl inoculum + 20 µl diluent DMSO (1:8; positive control = pink); column 12, assay medium (KB broth) + 20 µl diluent (negative control = blue). Well contents were thoroughly mixed using a micropipette. 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. After a second incubation of 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) of *T. vulgaris* and *O. vulgare* essential oils

The MBC was determined as follows: 100 µl of the dilution from the MIC and the previous dilutions were inoculated in KB agar and incubated at 28 °C for 24 h. The MBC was considered as the largest dilution without cellular growth (Bailey & Scott 1973).

RESULTS

Phenotype characterisation

The isolation of bacteria was made from symptomatic leaves using different culture media (KB, CA, TS agar), where KB agar proved to be the most appropriate for the isolation of *Pseudomonas*. In KB agar, fluorescent and non-fluorescent white, round, mucoid colonies were observed. A total of 83 Gram-negative, rod-shaped, catalase-positive cell isolates were recovered. Sixteen of them were chosen for further biochemical characterisation on the basis of the oxidative metabolism of glucose (Table 1). All strains were negative for nitrate reduction, indole (tryptophanase), gelatine, Voges-Proskauer (acetoin) and methyl red (acids), with the exception of EM21 for this last test. Most strains were alkaline in TSI; only one strain (M1E2) was alkaline/acid in this test. Thus, EM21 and M1E2 did not belong to *Pseudomonas*. Motility in soft agar was variable, as well as citrate, aesculin and the production of green pigmentation in KB agar.

Table 1. Biochemical and nutritional tests showed by the isolates obtained from soybean fields.

strain	Cet (Pig)	KB (Pig)	Cat	OF	NO_3	TSI	M	C	I	RM	VP	A	G
A5	+(g)	+ (g)	+	O	–	Al	+	+	–	–	–	–	–
A6	ND	+ (g)	+	O	–	Al	+	+	–	–	–	+	–
A7	–	+ (g)	+	O	–	Al	–	+	–	–	–	–	–
C11LS	ND	+ (g)	+	O	–	Al	–	–	–	–	–	–	–
C12LS	–	+ (g)	+	O	–	Al	+	+	–	–	–	ND	ND
C13LS	–	+ (g)	+	O	–	Al	+	–	–	–	–	–	–
C47	–	+ (g)	+	O	–	Al	–	–	–	–	–	+	–
EM1	–	+	+	O	–	Al	+	+	–	–	–	+	–
EM16	+(g)	+ (g)	+	O	–	Al	–	+	–	–	–	–	–
EM16b	ND	+	+	O	–	Al	–	+	–	–	–	ND	ND
EM21	–	+	+	O	–	Al	–	–	–	+	–	–	–
LS3	–	+ (g)	+	O	+	Al	–	+	–	–	–	+	–
M1E2	–	+ (g)	+	O	–	Al/Ac	+	–	–	–	–	ND	ND
M2E7	+	+	+	O	+	Al	+	–	–	–	–	–	–
Q	–	+	+	O	–	Al	–	+	–	–	–	–	–
VT2	–	+ (g)	+	O	–	Al	+	+	–	–	–	–	–

Cet = cetrimide agar; KB = King's B agar; Cat = catalasa; OF = glucose metabolism; NO_3 = nitrate reductase; TSI = three-sugar ion; M = movement; C = citrate; I = indole; RM = methyl red; VP = Voges Proskauer; A = aesculin; G = gelatine; (+) = positive; (–) = negative/no growth; O = oxidative metabolism; Al = alkaline; Al/Ac = alcaline/ácid; PIG = pigments; (g) = green; ND = not done.

The LOPAT test results, following Lelliott *et al.* (1966), are shown in Table 2. Ten of the isolates belonged to Group Ia: levan-positive, oxidase-negative, potato soft rot-negative, arginine dehydrolase-negative, tobacco hypersensitive-positive. Four strains were oxidase positive, so were located in Group III or IV. Based on these results, strains A5, C11LS, C13LS, C47, EM1, EM21, LS3, M2E7, Q and VT2 could provisionally be identified as *P. syringae* (Scortichini & Loreti 2007). These strains were isolated from Córdoba, San Luis and Santa Fe (Table 3).

Analyses of 16S *rRNA* gene of strains isolated from soybean

The 14 strains phenotypically characterised as *Pseudomonas* were analysed based on comparisons of the 16S *rRNA* sequences of these strains with those of accessions in the GenBank database (Table 3). Eleven strains showed identity with the *Pseudomonas* genus. Of these, six strains (A5, C13LS, EM1, LS3, Q, VT2) showed identity with *P. syringae* pv *glycinea*, *P. syringae* pv *tabaci* and *P. syringae* pv *syringae*, while A6, A7, EM16 and C12LS showed identity with *P. putida.* Of the remaining strains, two were significantly aligned with *Acinetobacter schindleri* and one strain with *Achromobacter xylosoxidans.* As seen in Table 3, one strain (C11LS) could not be identified. Nucleotide sequences of the nearly full-length 16S *rRNA* gene (average ~1,400 bp) from our isolated strains were deposited in the GenBank database and compared to available sequences of known strains.

Table 2. LOPAT test of the isolates obtained from soybean fields.

strains	LEV	O	PP	ARG	HT
A5	+	–	–	–	+
A6	+	+	–	+	–
A7	+	+	–	+	+
C11LS	+	–	–	NR	NR
C12LS	+	+	–	+	+
C13LS	+	–	–	–	+
C47	+	–	–	–	–
EM1	+	–	–	–	+
EM16	+	+	+	+	+
EM21	+	–	+	–	+
LS3	+	–	–	–	+
M2E7	+	–	–	–	NR
Q	+	–	–	–	+
VT2	+	–	–	–	+

Lev = levan; O = oxidase; PP = rot of potato; ARG = arginine; HT = hypersensitivity reaction on tobacco leaves; (+) = positive; (–) = negative.

In order to analyse the evolutionary distance and phylogenetic relationships among the isolated strains, a phylogenetic tree was built. Cluster analysis performed with 16S *rRNA* gene sequences and the NJ algorithm showed that the isolates were similar to some phytopathogenic pseudomonads. The phylogenetic tree showed three branches in which the strains were distributed: *Pseudomonas, Acinetobacter* and *Achromobacter*. The *Pseudomonas* group was the most representative and was subdivided in two groups: *P. syringae* and *P. putida*. The strains A5, C13LS, EM1, LS3, Q and VT2 were grouped with *P. syringae*, while A6, A7, C12LS and EM16 were related to *P. putida.* The remaining strains were grouped with *Acinetobacter* spp. and *Achromobacter* spp. (Fig. 1).

Pathogenicity test

The pathogenicity test was carried on with all the isolated strains identified as *P. syringae* and *P. putida* A7. *P. syringae pv tomato DC 3000* and *P. syringae pv atropurpurea Ps5* were used as reference strains. Typical symptoms of bacterial blight, chlorotic or water-soaked lesions on soybean plant leaves were reproduced by all *P. syringae* strains 7–10 days post-inoculation. All the phytopathogenic bacteria were re-isolated from the infected plants and identified biochemically. No pathogenic characteristics were observed with the isolated strain *P. putida* A7, *P. syringae* pv *tomato* DC 3000 or *P. syringae* pv *atropurpurea* Ps5, which were used as negative controls. Plants treated with sterile water remained symptom-free (Fig. 2).

Table 3. Bacterial species isolated from soybean leaves, based on maximum similarity of 16S *rRNA* sequences.

strain	accession no.	origin of sample	source	sequences with significant alignment (99% identity)
A5	KJ569376	Alcira Gigena (A)	this work	*Pseudomonas syringae* pv. *glycinea* (AB001443.1)
C13LS	KJ569375	Los Cisnes (LS)	this work	
EM1	KJ569377	Alejandro Roca (EM)	this work	
LS3	KJ569373	Los Cisnes (LS)	this work	
Q	KJ569372	Quines (Q)	this work	
VT2	KJ569374	Venado Tuerto (VT)	this work	
A6	KJ569369	Alcira Gigena (A)	this work	*Pseudomonas putida DE5* (JQ435693.1)
EM16	KJ569370	Alejandro Roca (EM)	this work	
A7	KJ569368	Alcira Gigena (A)	this work	*Pseudomonas putidaW619* (CP000949)
C12LS	KJ569371	Los Cisnes (LS)	this work	
C47EM	KJ569367	Los Cisnes (LS)	this work	*Acinetobacter schindleri H56* (KC 934833.1)
EM21	KJ569366	Alejandro Roca (EM)	this work	
M2E7	KJ569364	Paso del Durazno (M)	this work	*Achromobacter xylosoxidans SR50-12* (KF 279368.1)

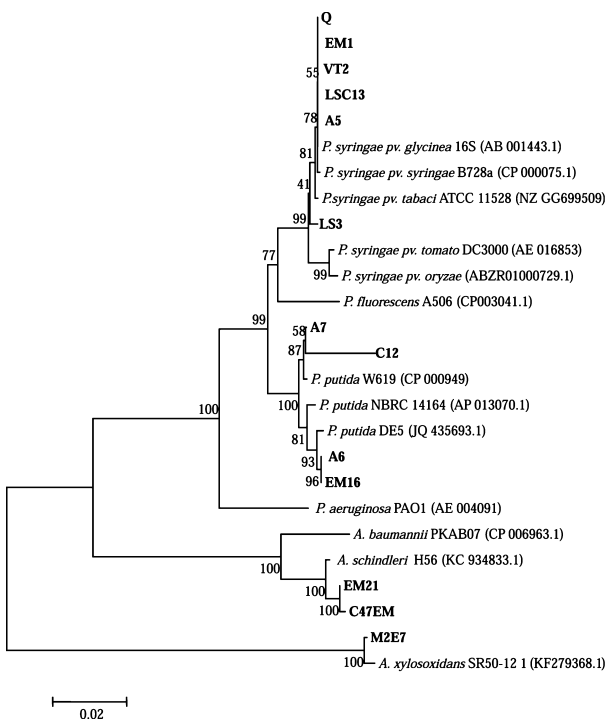

Fig. 1. Phylogenetic tree of strains isolated from *Glycine max* leaves and representative members, based on aligned *16S rRNA* gene sequences. Multiple alignments and neighbour-joining methods were used to construct the trees using MEGA 4.0.2 software. Strains isolated from soybean are indicated in bold. Bootstrap values, expressed as a percentage of 1000 replications, are given at the nodes. Sequence accession numbers are listed in parentheses. Scale bar: 1 nt substitutions per 200 nt.

Antimicrobial activity of essential oils

The antimicrobial activity of the essential oils of *T. vulgaris* and *O. vulgare* was investigated on the isolated *P. syringae* strains, *P. syringae* pv *tomato* DC 3000 and *P. syringae* pv *syringae* P61. The microdilution assay results are presented in Table 4. The essential oil of thyme had MIC values of 11.5 $mg \cdot ml^{-1}$ on five of the *P. syringae* strains isolated and on *P. syringae* pv *tomato* DC 3000. The MIC value obtained for *P. syringae* pv *syringae* P61 was 5.7 $mg \cdot ml^{-1}$ and strain VT2 was not inhibited by this oil. The essential oil of oregano showed inhibitory activity against all the tested strains, including reference strains, with more variability in the MIC values. Streptomycin (0.5 $mg \cdot ml^{-1}$) did not inhibit *Pseudomonas* strains. Thyme and oregano oil were not bactericidal for any of the pseudomonads.

The essential oils of oregano and thyme were analysed by means of GC-MS. In thyme oil 36 compounds were identified, and in oregano oil 24 compounds were identified, representing 99.3% and 99.7%, respectively. Both oils presented high percentages of carvacrol (29.5% and 19.7%, respectively) and low percentages of thymol (1.5%). These terpenes are described as characteristic components of these oils (Viuda-Martos *et al.* 2011). The essential oil of thyme and oregano contained p-cimene and γ terpinene as the main components, while oregano oil also had *cis*-sabinene hydrate among its main terpenes (Table 5).

DISCUSSION

Soybean diseases have gradually become a major problem for production in many areas of Argentina, particularly since the early 1990s. Several new diseases appeared in the 1993–1994 season, and some of these caused major losses in 1998 (Wrather *et al.* 2001). Recently, new bacterial diseases have appeared in soybean cultivars that were not previously known in Argentina and there has been an increase in established diseases (http://agro.fauba.info/node/164). In this country, damage caused by these diseases led to losses of 18,400 t in 1998 (Wrather *et al.* 2001). At least six different bacteria are known to affect soybean cultivars, including *P. syringae* (Scandiani & Luque 2009). This species is recognised as a plant pathogen of worldwide economic importance, responsible for common bacterial blight. It can establish large populations on aerial surfaces of a wide range of plant species, causing necroses on leaves, stems, fruits and other aerial parts (Morris *et al.* 2007). Few data on this bacterium in soybean cultivars are known for Argentina. In this work, strains with characteristics of *P. syringae* were isolated from leaves of soybean plants with blight symptoms. Ten of these isolates were identified biochemically in Group Ia of LOPAT as *P. syringae* because they shared the following characteristics: positive for levan production, negative for oxidase, potato rot and arginine dihydrolase, and positive for hypersensitivity in tobacco. The *Pseudomonas* species in Group I are described as leaf-spotting pathogens.

Fig. 2. Soybean plants infected with the strains A5, Q and VT2: typical blight lesions were observed on the leaves surface. Negative control: no lesions were observed on leaves surface.

Table 4. MIC ($mg \cdot ml^{-1}$) of essential oils of *Origanum vulgare* and *Thymus vulgaris* on *P. syringae* strains isolated from soybean.

strain	*T. vulgaris* (0.022–91.98 $mg \cdot ml^{-1}$)	*O. vulgare* (0.022–92.52 $mg \cdot ml^{-1}$)
A5	11.5	46.3
EM1	11.5	11.6
LS3	11.5	46.3
LSC13	11.5	5.8
VT2	NI	23.1
Q	11.5	23.1
P61	5.7	11.6
DC 3000	11.5	23.1

NI = no inhibition.

The *Pseudomonas* spp. of Groups II and III cause large necrotic lesions on leaves and stems, and are more invasive than those of Group I (Lelliott *et al.* 1966). From the identified strains, six were confirmed as *P. syringae* using 16S *rRNA*, with identities of 99% with *P. syringae* pv *glycinea, P. syringae* pv *tabaci* and *P. syringae* pv *syringae*. This species is a fluorescent pseudomonad clustering within rRNA similarity Group I of the genus *Pseudomonas* (Brenner *et al.* 2005). The steps followed in this work to isolate and identify phytopathogenic pseudomonads re-affirm the need to combine biochemical and molecular methods to obtain the definitive identity of a microorganism. Rodicio & Mendoza (2004) suggested that the identification of a microorganism should use different methodologies based on phenotypic and genotypic criteria. It is known that *P. syringae* strains exhibit high host specificity, and that this allows *P. syringae* species to be subdivided into more than 50 pathovars. A genetic analysis of total DNA-DNA homology and ribotyping indicated the existence of nine genomo-species within *P. syringae* species. As a consequence, many species in these groups have been re-classified, and *P. syringae* re-named as *P. savastanoi*, with pvs. *savastanoi, glycinea, tabaci* and *phaseolicola*. This implies that the genetic diversity of *P. syringae* species is due to adaptation of individual pathovars to their respective host plant environment (Young 2010; Qi *et al.* 2011).

The pathogenicity of the six strains identified as *P. syringae* was confirmed after inoculating them onto healthy soybean plants and observing the characteristic spots on the leaf surfaces. These bacteria were re-isolated from the spots and re-identified as *P. syringae*. Disease symptoms were not observed in plants inoculated with *P. syringae* pv *tomato* DC 3000 and *P. syringae* pv *atropurpurea* Ps5. The natural hosts of these last strains are tomato and oats, respectively. These data confirm that the isolated strains from soybean cause blight disease mainly in the host from which they were isolated. Moreover, they indicate the presence of phytopathogenic strains *P. syringae* in the east and central regions of Argentina (provinces of Córdoba, San Luis and Santa Fe), which are the main production regions of soybean.

Bacterial blight, caused by *P. syringae* pv *glycinea*, is one of the most prevalent soybean diseases. It is worldwide in distribution and more severe in cooler production regions (Prom & Venette 1997). The control of these bacterial diseases remains difficult due to the limited availability of antimicrobial substances. Antibiotic and copper compounds are the only chemical products suggested, but their use is hampered by limited efficacy in the field, and their potential negative effects either in the environment or on human and animal health (Lo Cantore *et al.* 2004). The availability of new and environmental compatible compounds with antibacterial activity would be very useful for control of soybean diseases. Natural products obtained from aromatic plants represent potential sources of molecules with biological activity.

Essential oils have been widely studied for their antimicrobial ability to control bacterial and fungal growth. Several studies of their effects on clinically dangerous microorganisms, as well as on microorganisms responsible for food spoilage or contamination have been published (Oliva *et al.* 2011; Bi *et al.* 2012). However, research on the use of essential oils to inhibit plant pathogenic bacteria is very limited. In this work, we document the inhibitory effects of *O. vulgare* and *T. vulgaris* essential oils on phytopathogenic *P. syringae* from soybean. Both oils were effective against all *P. syringae* strains, with better inhibitory activity than the antibiotic streptomycin (data not shown), which is the existing treatment for plant bacteriosis. The antimicrobial activity against phytopathogenic bacteria of essential oils and natural products from *T. vulgaris*, *O. vulgare, Oreganum onites* and other Labiatae (Kokoskova *et al.* 2011; Kotan *et al.* 2014) is documented. Essential oils and extracts obtained from *C. sativum*, *F. vulgare* var. *vulgare*, *Satureja hortensis*, *Cymbopogon* and *Eucalyptus* have been evaluated against phytopathogenic bacteria, with promising results (Lo Cantore *et al.* 2004; Paret *et al.* 2010; Kotan *et al.* 2013). Moreover, such results support the fact that natural products obtained from plants are effective inhibitors of phytopathogenic bacteria and could be useful against bacterial blight and other plant pathogens.

The mode of action of plant essential oils indicates that the microbial toxicity is associated with cell membrane damage.

Table 5. Components identified in the essential oils of *Thymus vulgaris* and *Origanum vulgare* (%) using GC-MS.

compound	*T. vulgaris*	*O. vulgare*
α thujene	1.7	0.6
α pinene	1.6	Tr
α fenchene	0.8	Tr
β pinene	1.1	–
Sabinene	–	1.8
Octanone 3	–	Tr
trans-2-octen-1-ol	–	Tr
Myrcene	1.8	1.2
3-octanol	Tr	–
α phellandrene	Tr	–
3-carene	Tr	–
α terpinene	1.7	2.4
p-cymene	31.5	11.5
1,8-cineole	2.4	–
Limonene	–	Tr
β phellandrene	–	Tr
cis ocimene	–	3.9
trans ocimene	–	0.6
γ terpinene	11.3	22.7
Terpinolene	1.5	2.1
Para-cymenene	Tr	–
Linalool	3.5	–
cis sabinene hydrate	Tr	19.7
trans sabinene hydrate	–	Tr
Camphor	Tr	–
Borneol	1	0.6
4-terpineol	1.2	5.1
p-cymen-8-ol	Tr	–
α terpineol	Tr	1.8
Thymol methyl ether	1.7	1.5
Geraniol	Tr	–
Geranial	Tr	
Thymol	1	1.5
Carvacrol	29.5	19.7
Isobornyl acetate	Tr	–
α copaene	Tr	–
β bourbonene	Tr	–
Longifolene	3.6	–
α cadinene	1.1	–
γ muurolene	Tr	–
γ cadinene	Tr	–
δ cadinene	Tr	–
cis calamenene 1S	Tr	–
Oxide caryophyllene	1.3	–
β caryophyllene	–	2.7
	99.3	99.7

Tr = trace.

Their chemical constituents are characteristically hydrophobic and accumulate in the lipid-rich environments of cell membranes, causing structural and functional damage (Lambert *et al.* 2001). Gram-negative organisms are less susceptible to the action of antibacterial compounds, since they possess an outer membrane surrounding the cell wall that restricts diffusion of hydrophobic compounds through its lipopolysaccharide layer. Usually, the antibacterial activity of essential oils is attributed to major terpene components present in the oil. However, in some cases, whole essential oils have higher antibacterial activity than a combination of their major isolated components, indicating that minor components are critical to the activity, probably by producing a synergistic effect (Burt 2004). In essential oil of thyme and oregano, one of the main components is carvacrol (29.5% and 19.7%, respectively), which is structurally very similar to thymol, having the hydroxyl group at different locations on the phenolic ring. Both compounds contain a cyclohexane with a bound hydroxyl group and a system of delocalised electrons that possess strong antimicrobial activity. The hydroxyl group bound to a benzene ring is important and responsible for the antimicrobial activity, through the presence of an α–β double bond (Lambert *et al.* 2001; Viuda-Martos *et al.* 2011; Kotan *et al.* 2014). In this work, the presence of carvacrol in oregano and thyme essential oils was markedly higher than thymol, despite the fact that the latter is usually the main component of thyme oil (Rota *et al.* 2008; Viuda-Martos *et al.* 2011). Other studies report differences in the chemical compositions of thyme and other essential oils, which are attributed to factors such as plant age, plant part, development stage, growing place, harvesting period and principal chemotype, since they all influence plant biosynthetic pathways and consequently the relative proportion of the main characteristic compounds (Oliva *et al.* 2010; Viuda-Martos *et al.* 2011). The genus *Thymus* has numerous species and varieties, with six chemotypes: geraniol, linalool, γ-terpineol, carvacrol, thymol and trans-thujan-4-ol/terpinen-4-ol (Rota *et al.* 2008). In this work, the presence of carvacrol (29.5%) was markedly higher than thymol (1%) despite the fact that the latter is usually the main component of thyme oil. Consequently, the chemotype used in this research seems to be carvacrol, and its high presence could be responsible for the antimicrobial activity. The mode of action of carvacrol and thymol appear to make the cell membrane permeable. Both terpenes are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane (Lambert *et al.* 2001; Veldhuizen *et al.* 2006). The chromatography of thyme oil showed that p-cymene was the main terpene (31.5%); it is a biosynthetic precursor of thymol and carvacrol that lacks the bound hydroxyl group. Antimicrobial activity of this terpene against microorganisms has been described previously, although it was not an effective antibacterial when used alone. The combination of p-cymene with carvacrol showed synergism against *B. cereus in vitro*, where the hydrophobic characteristics of p-cymene allowed it to be incorporated more easily into the lipid bilayer and facilitated transport of carvacrol across the cytoplasmic membrane (Veldhuizen *et al.* 2006).

The results presented here are promising because essential oils could be used for control of diseases caused by bacteria responsible for reduced crop yield. *P. syringae* is a Gram-negative bacterium, which is important because of its ability to infect susceptible hosts. This bacterium displays higher intrinsic resistance to antibiotics and biocides than Gram-positive bacteria. The availability of new active principles such as essential oils or their components in disease control practice is of great interest. Further studies are necessary to evaluate the toxicity of the above substances toward seeds and/or plants and to obtain the appropriate formulations useful for crop protection.

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