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ARTICLE

Antibacterial Activity of Essential Oil Components and Their Potential Use in Seed Disinfection

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Among the main (≥0.7%) components of some essential oils, considerable antibacterial activity was shown by terpenoid and phenylpropanoid derivatives containing phenol and alcohol functionalities. A reduced or no activity was shown by those derivatives containing ketones, aldehydes, ethers, and ester functionalities as well as the remaining terpenoids. Eugenol emulsion treatments (1−8 mg/mL) of bean seeds bearing about 2.6×10^6 cfu/seed of strain ICMP239 of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* determined a highly significant reduction of the bacteria on seeds. In particular, eugenol at 4 mg/mL disinfect seeds bearing about 7.0×10^2 cfu/seed and lower densities. However, after 72 h, incubation treatments with 2, 4, and 8 mg/mL of eugenol caused germination reduction of 3%, 7%, and 16%, respectively, which was significantly different from the controls. No effect on germination was observed with 1 mg/mL eugenol emulsion treatment. These data indicate eugenol as potentially useful for bean seed disinfection from *X. campestris* pv. *phaseoli* var. *fuscans*. Further studies on the effects on seed vitality and on formulation of essential oils are needed.

KEYWORDS: Bactericides; bean seed sanitation; eugenol; *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*.

INTRODUCTION

The control of bacterial diseases of plants is a considerable problem in agriculture practice since they were first described (*1*). Bacterial diseases of valuable crops in some environmental conditions may lead to reduced yield and/or blemished commodities. Various control measures (i.e., quarantine procedures, seed disinfection, cultural practices, chemical control, host plant resistance, seed certification program, biological control, etc.) have been adopted for bacterial disease control but with limited efficacy and each of the above measures presenting advantages and disadvantages (*1*). On the contrary, fungal diseases, with special emphasis to foliar ones, in most of the cases, may be controlled by the use of a quite huge array of fungicides, while only a limited number of bactericides are available on the market (*1*). In fact, besides the antibiotics and copper compounds, there are no other bactericides available on the market. Antibiotics are actually restricted or forbidden in the agricultural practices in many countries mainly for the possible selection of bacterial strains resistant to them (*2*) and the consequent horizontal transfer of this character to other bacteria either in the phytosphere (i.e., saprothophs, phytopathogenic bacteria, etc.) or in other environmental niches inhabited by bacteria either associated to pathogens or are themselves pathogenic to human beings and animals. The use of copper compounds, because of their general toxicity and mainly for their impact on the environment, is restricted and controlled in the European Union (EU rule no. 473/2002, March 15th, 2002, published on March 16th, 2002 in the Official Journal of the European Union, L75/21).

Furthermore, the control of plant diseases caused by bacteria is not an easy task since a large number of phytopathogenic bacteria spread, even at long distances, through contaminated and/or infected seeds (*3*). In intensive horticulture crops, the presence of a few contaminated/infected seeds in a seed lot may lead to severe disease outbreaks with consequent heavy crop loss. Seed companies traditionally, for the production of healthy seeds, adopt several preventive measures including disease exclusion (i.e., through the selection of areas not conductive to the disease occurrence), but it is unlikely that these measures may not be successful in some cases. Also, the use of chemical disinfectants such as chlorine, inorganic acids, organic acids, and heat treatments have been used for disinfection of potentially contaminated seed surfaces or to cure infected seeds, but also, in this case, disadvantages such as seed devitalisation have been reported (*3*).

The above consideration prompts the need for the development of new bactericides and/or alternative methods for the control of plant bacterial diseases to be used in integrated crop management as well as in bio-organic agriculture.

Several studies have pointed out the possibility to use essential oils and/or their components in medical and plant pathology as well as in the food industry for the control of micro-organisms pathogenic to consumers and/or responsible for food spoilage. However, most of the studies are mainly limited to the assessment of the antimicrobial activity in vitro (*4*−*11*), and the exploitation of essential oils for the control of plant diseases is still in its infancy (*12*−*15*).

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Our recent studies showed the antibacterial activities of whole essential oils of coriander, wild fennel, caraway, and cumin, toward laboratory, phytopathogenic and mycopathogenic bacterial species (*16*, *17*). A significant antibacterial activity was shown, in particular, by coriander, caraway, and cumin essential oils against Gram positive and Gram negative bacteria belonging to *Clavibacter*, *Curtobacterium*, *Rhodococcus*, *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Agrobacterium* genera, respectively, responsible for several plant and mushroom diseases. On the same target bacteria a much weaker effect was observed for the wild fennel oil (*17*). Furthermore, the above studies showed the complex composition of the above four essential oils. However, how the components account for the observed activities is still unknown.

The objective of this study was to evaluate the antibacterial activity in vitro of the main components of essential oils toward bacteria responsible for diseases on plants and cultivated mushrooms and to evaluate the possible use of the most active compounds for seed disinfection. For the latter purpose, the bean seed/*Xanthomonas campestris* pv. *phaseoli* var. *fuscans* pathosystem has been selected due to our interest to determine a measure for the disinfection of the "Fagioli di Sarconi" seed lots to use in organic production. The common blight of bean, mainly caused on the above bean by the variety *fuscans* of *X. campestris* pv. *phaseoli*, may be a limiting factor on the above crop, a pool of high value traditional varieties protected by the European Union (Reg. CEE no. 1263/96) with the mark of Identification Geographical Protected (IGP) and cultivated in the National Park of the Agri Valley in Basilicata (southern Italy) for the production of dry seeds (*18*). Here, results on the antibacterial activity of 19 pure essential oil components on 29 phytopathogenic and mycopathogenic bacterial species as well as on the capacity of eugenol to disinfect bean seeds from *X. campestris* pv. *phaseoli* var. *fuscans* are reported. The latter findings and the limited phytotoxic effect of eugenol on seed germination clearly indicate this phenylpropanoid derivative as a potential bactericide for seed disinfection in agriculture practice, though further studies on the effects on seed vitality and on formulation of essential oils are needed. Preliminary results have been reported (*19*).

MATERIALS AND METHODS

Chemicals. Carvacrol (28,219-7), dihydrocarveol (21,842-1), geraniol (16,333-3), nerol (26,890-9), linalool (L2602), carvone (12,493-1), fenchone (19,643-6), camphor (14,807-5), *t*-dihydrocarvone (21,828-6), α-pinene (14,752-4), *β*-pinene (11,208-9), limonene (33,411-1), *p*-cymene (12,145-2), *γ*-terpinene (22,319-0), geranyl-acetate (17,349-5), eugenol (E5,179-1), cumin aldehyde (13,517-8), anethole (11,787-0), caryophyllene (C9653), cycloheximide (C7698), rifampicin (R3501), and tetracycline (268054) were obtained from Sigma-Aldrich (Milan, Italy).

Bacterial Cultures. One to three strains of the bacterial species and/or pathovars reported in **Table 1** were used. Subcultures were obtained by growing bacteria for 48−72 h on the medium B of King (KB, (*20*)) in the case of pseudomonads and on WA (*21*) for the other bacteria.

Disc Diffusion Assay. Ten microliter 1:1 serial dilutions in pure methanol of stock solutions of essential oil components (1 mg/mL) and of 1.6 mg/mL rifampicin and tetracycline were added to 6 mm diameter sterile blank disks previously deposited onto the surface of Petri plates containing 10 mL of KB or WA (0.7% agar) depending on the bacterial species. Aliquots of target bacteria suspensions were added to the above media, maintained at 45 °C, to obtain a final population of about 10^7 cfu/mL. After a 48 h incubation at 25 °C, the minimal inhibitory quantity (MIQ), expressed in micrograms, which causes an apparent inhibition zone around the 6 mm diameter disks was recorded. The assays were performed twice with three replicates.

Bean Seed Treatments. Nine 300 bean seed containing lots of the traditional variety "Ciuoto" of the "Fagioli di Sarconi" (53.0 ± 5.1 g for 100 seeds) were treated for 3 min with 1% sodium hypochlorite, washed three times with sterile distilled water, and then dried under an air flow at room temperature for 20 min. Subsequently, seeds were immersed for 20 min in an approximately 10^8 cfu/mL bacterial suspension of a natural mutant resistant to rifampicin of strain ICMP239 of *X. campestris* pv. *phaseoli* var. *fuscans* and then dried as above. Then, each 100 seed lot was immersed for 20 min at room temperature in eugenol emulsions obtained by sonication (Braun L apparatus, 40TL needle probe, 100 W, 5 min) or tetracycline solutions in sterile distilled water containing 0.01% Tween 20. The concentrations of the above emulsions/solutions were selected on the basis of the tetracycline and eugenol MIQs against strain ICMP239 of *X. campestris* pv. *phaseoli* var. *fuscans* which resulted in 1 and 45 *μ*g, respectively. Comparable active concentration above and below the MIQs for tetracycline (200, 100, 50 *μ*g/mL) and eugenol (8, 4, 2, 1 mg/mL) were selected for the bean seed assays.

In other experiments, seed lots were immersed for 20 min in bacterial suspensions of the above bacterial strain containing about 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 cfu/mL and then dried as above. Then, each seed lot was immersed for 20 min at room temperature in eugenol emulsion (4 mg/mL) or tetracycline solution (100 *μ*g/mL) in sterile distilled water containing 0.01% Tween 20.

Table 1. Bacterial Strains Used in This Study

bacteria	strains[a]
	Gram Negative
Escherichia coli	ITM103
Pseudomonas syringae pv. *phaseolicola*	IPV-BO1917, USB316
P. syringae pv. *pisi*	NCPPB3496
P. syringae pv. *syringae*	Y37, B366
P. syringae pv. *apii*	NCPPB1626
P. syringae pv. *atrofaciens*	NCPPB2612, GSPB1742
P. syringae pv. *lachrymans*	USB326, USB327
P. syringae pv. *maculicola*	NCPPB2038
P. syringae pv. *tomato*	USB328, USB329
P. syringae pv. *glycinea*	NCPPB2752
P. cichorii	ICMP5707
P. viridiflava	DPP5
P. corrugata	NCPPB2445
P. tolaasii	NCPPB2192
P. reactans	NCPPB1311
P. agarici	NCPPB2289
Erwinia carotovora subsp. *atroseptica*	ICMP1526
Agrobacterium tumefaciens	USB1001
Burkholderia gladioli pv. *agaricicola*	ICMP11096
Ralstonia solanacearum[b]	FC486
Xanthomonas campestris pv. *phaseoli*	NCPPB3035, GSPB1217, ICMP238
X. campestris pv. *phaseoli* var. *fuscans*	ICMP239, ICMP3403, GSPB275
X. campestris pv. *vesicatoria*	NCPPB422, DAPP-PG95, DAPP-PG32
X. campestris pv. *campestris*	NCPPB528
	Gram Positive
Bacillus megaterium	ITM100
Clavibacter michiganensis subsp. *michiganensis*	DPP2, DPP3
Curtobacterium flaccunfaciens pv. *flaccunfaciens*	ICMP2584
C. flaccunfaciens pv. *betae*	NCPPB372

[a] ITM, Istituto Tossine e Micotossine (Bari, Italy); IPV-BO, Istituto di Patologia Vegetale (Università di Bologna, Italy); USB, Università degli Studi della Basilicata (Potenza, Italy); NCPPB, National Collection Plant Pathogenic Bacteria (U.K.); GSPB, Gottinger Sammlung Phitopathogener Bakterien (Gottingen, Germany); ICMP, International Collection of Microrganism from Plants (Auckland, New Zealand); DPP, Dipartimento di Protezione delle Piante (Università della Tuscia, Viterbo, Italy); DAPP-PG, Dipartimento di Arboricoltura e Protezione delle Piante (Università degli Studi di Perugia, Italy). [b] Strain FC486 was supplied by Dr. N. Schaad, (USDA-ARS-FDWSRU-Bacteriology, Fort Detrick, MD, USA).

Bean seeds not treated or treated with sterile distilled water containing 0.01% Tween 20 were used as controls.

After treatments, each seed lot was dried as above and divided in three sublots of 100 seeds each. Then seeds were put in 500 mL Erlenmeyer flasks containing 100 mL of sterile distilled water containing Tween 20 (0.01%) under agitation for 1 h at 25 °C. Aliquots of 100 μL of decimal dilutions of bacterial suspension were plated onto KB containing rifampicin (100 mg/L) and cycloheximide (100 mg/L). After a 5 day incubation at 25 °C, the density of the bacterial population was determined. The treatments were performed twice with three replicates.

Determination of Seed Germination. Lots of 100 bean seeds were treated with eugenol emulsions (8, 4, 2, 1 mg/mL) as reported above and then were allowed to germinate on filter paper soaked with distilled sterile water in sterile aluminum trays. After a 24, 48, and 72 h incubation at 25 °C, the number of the germinated seeds was determined. The germination rate was expressed in a percentage. The assays were performed twice with three replicates.

Statistical Analysis. Data were subjected to analysis of variance (ANOVA, F test) and then to the t test using the SPSS version 17.0 software program package (SPSS Inc., Chicago, IL).

RESULTS

Disc Diffusion Assay. Most of the essential oil components inhibited the growth of the majority of the bacteria (Gram positive and Gram negative) used in this study although the MIQ was different among the substances depending on the target bacterial species and/or pathovars (**Tables 2**−**4**).

Terpenoids and phenylpropanoids having phenol and alcohol functionalities showed the higher bactericidal activity, inhibiting the growth of the totality of the bacterial strains used in this study whereas a lower activity was shown by monoterpenes containing ketone, aldehyde, and ester functionalities and the phenylpropanoids anethol. The latter inhibited the growth of only a part of the target bacteria (**Tables 2** and **3**). Monoterpenoids and sequiterpenoid caryophyllene showed a lower activity and only on a limited number of the target bacteria (**Table 4**). The sesquiterpene caryophyllene did not show any bactericidal activity even when 10 000 μg of the substance was used (**Table 4**).

The MIQ of the antibiotic rifampicin, determined against a representative number of the bacterial species and/or pathovars reported in **Table 1**, was between 1 and 4 μg for strains of the fluorescent pseudomonads and less than 1 μg for strains of *X. campestris* pv. *phaseoli* and the gram positive *Clavibacter michiganensis* subspecies and *Curtobacterium flaccumfaciens* pathovars (data not shown).

The MIQs of tetracycline and eugenol against strain ICMP239 of *X. campestris* pv. *phaseoli* var. *fuscans*, a natural mutant resistant to the antibiotic rifampicin used in the bean seed assays, were 1 and 45 μg, respectively.

Bean Seed Treatments. All bean seed treatments, including the one with 0.01% Tween 20, caused a statistically significant reduction ($P < 0.0001$) of the bacterial population on bean seeds

Table 2. Minimal Inhibitory Quantity (MIQ) (μg) of Terpenoids and Phenylpropanoids Having Phenol and Alcohol Functionalities on Selected Gram Positive and Gram Negative Phytopathogenic and Mycopathogenic Bacteria

		MIQ (μg)[a]					
		phenols		alcohols			
bacteria	no. of strains	carvacrol	eugenol	dihydrocarveol	geraniol	nerol	linalool
		Gram Negative					
Escherichia coli	1	78	39	312	78	312	312
Pseudomonas syringae pv. *phaseolicola*	2	19	39	234	469	98	117
P. syringae pv. *pisi*	1	39	78	1250	78	156	312
P. syringae pv. *syringae*	2	49	58	312	58	156	234
P. syringae pv. *api*	1	39	39	312	39	156	312
P. syringae pv. *atrofaciens*	2	39	88	469	78	78	312
P. syringae pv. *lachrymans*	2	49	39	234	39	58.5	312
P. syringae pv. *maculicola*	1	19	39	312	78	312	156
P. syringae pv. *tomato*	2	29	39	312	78	234	117
P. syringae pv. *glycinea*	1	19	78	312	78	156	156
P. cichorii	1	19	39	625	39	312	312
P. viridiflava	1	19	78	312	78	312	625
P. corrugata	1	39	39	312	39	156	78
P. tolaasii	1	19	39	156	39	39	156
P. reactans	1	19	39	312	312	312	312
P. agarici	1	19	78	156	156	78	78
Erwinia carotovora subsp. *atroseptica*	1	39	39	312	78	156	39
Agrobacterium tumefaciens	1	39	39	312	39	312	312
Burkholderia gladioli pv. *agaricicola*	1	78	39	312	39	312	78
Ralstonia solanacearum	1	39	39	312	156	156	312
Xanthomonas campestris pv. *phaseoli*	3	39	91	312	65	130	130
X. campestris pv. *phaseoli* var. *fuscans*	3	45	26	260	130	117	52
X. campestris pv. *vesicatoria*	3	65	91	312	104	130	260
X. campestris pv. *campestris*	1	19	39	312	78	156	39
		Gram Positive					
Bacillus megaterium	1	19	78	312	78	39	312
Clavibacter michiganensis subsp. *michiganensis*	2	39	58	469	312	234	625
Curtobacterium flaccunfaciens pv. *flaccunfaciens*	1	39	78	312	78	312	312
C. flaccunfaciens pv. *betae*	1	78	78	1,250	78	156	625

[a] MIQ, average quantity needed for the bacterial growth inhibition.

Table 3. Minimal Inhibitory Quantity (MIQ) (μg) of Terpenoids Bearing Ketone, Aldehyde, and Ester Functionalities and the Phenylpropanoid Anethol on Selected Gram Positive and Gram Negative Phytopathogenic and Mycopathogenic Bacteria

		MIQ (μg)[a]						
		ketones				aldehyde	ether	ester
bacteria	no. of strains	carvone	fenchone	camphor	*t*-dihydrocarvone	cumin aldehyde	anethole	geranyl-acetate
				Gram Negative				
Escherichia coli	1	625	2500	na[f]	na	na	na	na
Pseudomonas syringae pv. *phaseolicola*	2	469	1875	1250	1875	1406	5000−na[d]	na
P. syringae pv. *pisi*	1	625	2500	2500	na	na	na	na
P. syringae pv. *syringae*	2	469	1875	3125	1875	1562	1250	na
P. syringae pv. *api*	1	625	1250	1250	5000	na	na	2500
P. syringae pv. *atrofaciens*	2	391	1250	1250	1250−na[b]	1250	1875	na
P. syringae pv. *lachrymans*	2	234	1875	1250	625	625	na	na
P. syringae pv. *maculicola*	1	625	2500	2500	na	na	na	2500
P. syringae pv. *tomato*	2	312	3750	3750	1875	3750	5000−na[d]	na
P. syringae pv. *glycinea*	1	312	2500	2500	1250	2500	1250	2500
P. cichorii	1	312	2500	2500	na	na	na	na
P. viridiflava	1	625	2500	5000	5000	na	5000	na
P. corrugata	1	625	2500	na	1250	1250	5000	na
P. tolaasii	1	312	2500	625	2500	1250	5000	na
P. reactans	1	625	2500	1250	2500	625	na	na
P. agarici	1	312	2500	1250	2500	2500	5000	na
Erwinia carotovora subsp. *atroseptica*	1	312	2500	1250	2500	1250	1250	na
Agrobacterium tumefaciens	1	312	2500	5000	na	na	1250	na
Burkholderia gladioli pv. *agaricicola*	1	1250	5000	5000	na	na	5000	na
Ralstonia solanacearum	1	625	1250	1250	5000	5000	1250	5000
Xanthomonas campestris pv. *phaseoli*	3	625	2083	1667	2083	2083	5000	5000−na[e]
X. campestris pv. *phaseoli* var. *fuscans*	3	638	3333	2500	1562−na[b]	2500−na[c]	na	5000−na[e]
X. campestris pv. *vesicatoria*	3	521	1667	1042	1250−na[b]	2500	na	na
X. campestris pv. *campestris*	1	625	2500	1250	1250	1250	2500	5000
				Gram Positive				
Bacillus megaterium	1	625	5000	1250	2500	2500	na	5000
Clavibacter michiganensis subsp. *michiganensis*	2	625	2500	2500	3750	937	na	5000−na[e]
Curtobacterium flaccunfaciens pv. *flaccunfaciens*	1	625	5000	1250	625	625	na	2500
C. flaccunfaciens pv. *betae*	1	1250	2500	2500	2500	1250	na	2500

[a] MIQ, average quantity needed for the bacterial growth inhibition. [b] One of the strains of *P. syringae* pv. *atrofaciens*, *X. campestris* pv. *phaseoli* var. *fuscans*, and *X. campestris* pv. *vesicatoria* was not inhibited by 10 000 μg of *t*-dihydrocarvone. [c] One of the strains of *X. campestris* pv. *phaseoli* var. *fuscans* was not inhibited by 10 000 μg of cumin aldehyde. [d] One of the strains of *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato* was not inhibited by 10 000 μg of anethole. [e] Two strains of *X. campestris* pv. *phaseoli* and one strain of *X. campestris* pv. *phaseoli* var. *fuscans* and *C. michiganensis* subsp. *michiganensis* were not inhibited by 10 000 μg of geranyl-acetate. [f] na = the deposition of 10 000 μg of component essential oils on sterile blank disks did not lead to an inhibition zone.

when compared to the not treated sample control (**Figures 1** and **2**). Eugenol treatments of bean seeds bearing about 2.6×10^6 cfu/seed of the natural mutant resistant to the antibiotic rifampicin of strain ICMP239 of *X. campestris* pv. *phaseoli* var. *fuscans* determined a highly significant reduction ($P \leq 0.002$) of the bacterial population on seeds when compared to the controls (i.e., no treatment and/or water containing 0.01% Tween 20) and, furthermore, the level of reduction was dependent on the essential oil concentration (**Figure 1**). In particular, treatments with 1, 2, 4, and 8 mg/mL eugenol suspension determined a highly significant reduction of the initial bacterial population (about 2.6×10^6 cfu/seed) which, after treatments, resulted in about 2.5×10^5, 1.1×10^5, 2.2×10^4, and 5.2×10^3 cfu/seed, respectively. However, a reduced significant effect ($P = 0.002$) was observed between treatments with 1 mg/mL eugenol emulsion and the control treatments with 0.01% Tween 20 as well as between treatments with 1 and 2 mg/mL ($P < 0.03$) and 2 and 4 mg/L eugenol ($P = 0.001$) emulsions, respectively.

Treatments with the antibiotic tetracycline solutions caused a statistically significant ($P \leq 0.002$) reduction of the bacterial population on bean seeds when compared to the controls. However, the antibiotic treatments were less effective than the eugenol ones (**Figure 1**). In particular, treatment with tetracycline solutions containing 50, 100, and 200 μg/mL caused a highly significant reduction ($P < 0.0001$) of the initial bacterial population (about 2.6×10^6 cfu/seed) to about 1.8×10^5, 1.6×10^5, and 1.2×10^5 cfu/seed, respectively. However, only a reduced significant effect ($P < 0.05$) was observed among the different antibiotic treatments (**Figure 1**) and, moreover, no statistically significant differences were observed between the treatments performed with solutions containing 50 and 100 μg/mL of tetracycline. Finally, treatments with 1 and 2 mg/mL of eugenol were not statistically different from the tetracycline treatments (**Figure 1**).

Treatments of bean seeds bearing different levels of bacteria (about 1.8×10^6, 6.1×10^5, 2.5×10^5, 4.8×10^4, 3.9×10^3, 7.0×10^2, 4.9×10^2, and 3.2×10^2 cfu/seed) with 4 mg/mL eugenol emulsions confirmed the higher efficiency of the essential oil to reduce bacterial population densities on bean seed surface when compared to the treatments with tetracycline at a comparable MIQ value (100 μg/mL) (**Figure 2**). In fact, eugenol treatment determined a highly significant reduction ($P < 0.0001$) of the bacterial populations which resulted in about 5.7×10^3, 1×10^2, 6.7×10^1, 3.3×10^1, and $3\times10^\circ$ cfu/seed for the seed lots bearing, respectively, about 1.8×10^6, 6.1×10^5, 2.5×10^5, 4.8×10^4, and 3.9×10^3 cfu/seed (**Figure 2**, **panels 1−5**). Furthermore, the above treatment apparently eliminated all bacteria on the seeds bearing, respectively,

Table 4. Minimal Inhibitory Quantity (MIQ) (μg) of Monoterpenoids and the Sesquiterpenoid Caryophyllene on Selected Gram Positive and Gram Negative Phytopathogenic and Mycopathogenic Bacteria

bacteria	no. of strains	MIQ (μg)[a]					
		α-pinene	β-pinene	limonene	*p*-cymene	γ-terpinene	caryophyllene
			Gram Negative				
Escherichia coli	1	na[g]	5000	5000	na	na	na
Pseudomonas syringae pv. *phaseolicola*	2	na	5000−na [c]	na	na	na	na
P. syringae pv. *pisi*	1	5000	na	na	na	na	na
P. syringae pv. *syringae*	2	na	na	na	na	na	na
P. syringae pv. *api*	1	5000	5000	na	na	na	na
P. syringae pv. *atrofaciens*	2	5000−na[b]	5000−na[c]	na	5000−na[e]	na	na
P. syringae pv. *lachrymans*	2	na	na	na	na	na	na
P. syringae pv. *maculicola*	1	2500	2500	1250	2500	1250	NA
P. syringae pv. *tomato*	2	na	na	na	na	na	na
P. syringae pv. *glycinea*	1	5000	5000	2500	na	na	na
P. cichorii	1	5000	5000	2500	5000	NA	NA
P. viridiflava	1	5000	5000	na	na	na	na
P. corrugata	1	na	na	na	na	na	na
P. tolaasii	1	na	na	na	na	na	na
P. reactans	1	na	na	na	na	na	na
P. agarici	1	na	na	2500	na	na	na
Erwinia carotovora subsp. *atroseptica*	1	na	2500	2500	na	na	na
Agrobacterium tumefaciens	1	5000	5000	na	na	na	na
Burkholderia gladioli pv. *agaricicola*	1	5000	na	2500	na	na	na
Ralstonia solanacearum	1	2500	2500	2500	2500	na	na
Xanthomonas campestris pv. *phaseoli*	3	5000−na[b]	5000−na[c]	2500−na[d]	5000−na[e]	5000−na[f]	na
X. campestris pv. *phaseoli* var. *fuscans*	3	2917	2500−na[c]	2083	3750−na[e]	1250−na[f]	na
X. campestris pv. *vesicatoria*	3	5000	5000−na[c]	3333	na	na	na
X. campestris pv. *campestris*	1	5000	na	2500	na	na	na
			Gram Positive				
Bacillus megaterium	1	2500	na	2500	2500	2500	na
Clavibacter michiganensis subsp. *michiganensis*	2	5000	5000	2500	3750	na	na
Curtobacterium flaccunfaciens pv. *flaccunfaciens*	1	5000	na	5000	5000	na	na
C. flaccunfaciens pv. *betae*	1	5000	na	5000	5000	na	na

[a] MIQ, average quantity needed for the bacterial growth inhibition. [b] One of the strains of *P. syringae* pv. *atrofaciens* and *X. campestris* pv. *phaseoli* was not inhibited by 10 000 μg of α-pinene. [c] One of strains of *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *atrofaciens*, *X. campestris* pv. *phaseoli*, *X. campestris* pv. *phaseoli* var. *fuscans*, and *X. campestris* pv. *vesicatoria* was not inhibited by 10 000 μg of β-pinene. [d] One of the strains of *X. campestris* pv. *phaseoli* was not inhibited by 10 000 μg of limonene. [e] One of the strains of *P. syringae* pv. *atrofaciens* and *X. campestris* pv. *phaseoli* var. *fuscans* and two strains of *X. campestris* pv. *phaseoli* were not inhibited by 10 000 μg of *p*-cymeme. [f] One of the strains of *X. campestris* pv. *phaseoli* var. *fuscans* and two strains of *X. campestris* pv. *phaseoli* were not inhibited by 10 000 μg of γ-terpinene. [g] na = the deposition of 10 000 μg of component essential oils on sterile blank disks did not lead to an inhibition zone.

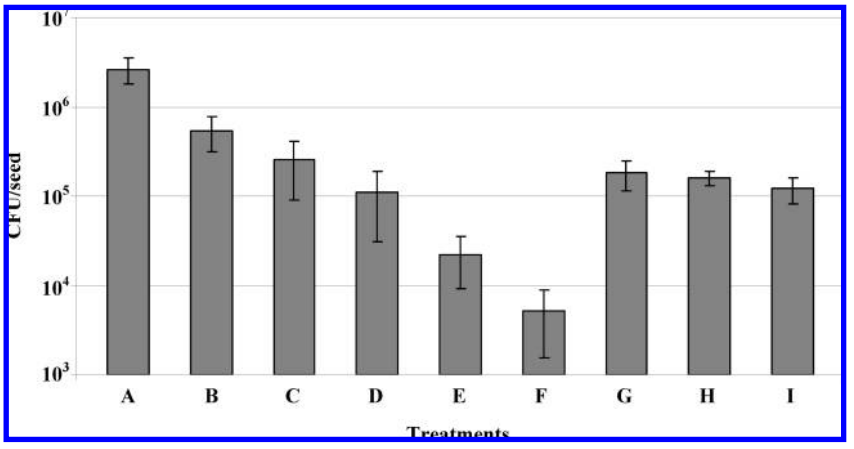

Figure 1. Bacterial populations of the spontaneous mutant resistant to rifampicin of strain ICMP239 of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* on bean seeds bearing about 2.6×10^6 cfu/seed after different treatments (A = control, no treated seeds; B = control, seeds treated with water containing 0.01% Tween 20; C, D, E, and F = seeds treated with 1, 2, 4, and 8 mg/mL of eugenol in water emulsions containing 0.01% Tween 20, respectively; G, H, and I = seeds treated with 50, 100, and 200 μg/mL tetracycline solutions containing 0.01% Tween 20, respectively). Bars on the columns correspond to the standard error of the mean. Means of bacterial populations on bean seeds after eugenol and tetracycline treatments, evaluated by the *t* test in comparison with the means of bacterial populations on bean seeds of the controls A and B, are statistically different ($P \leq 0.002$).

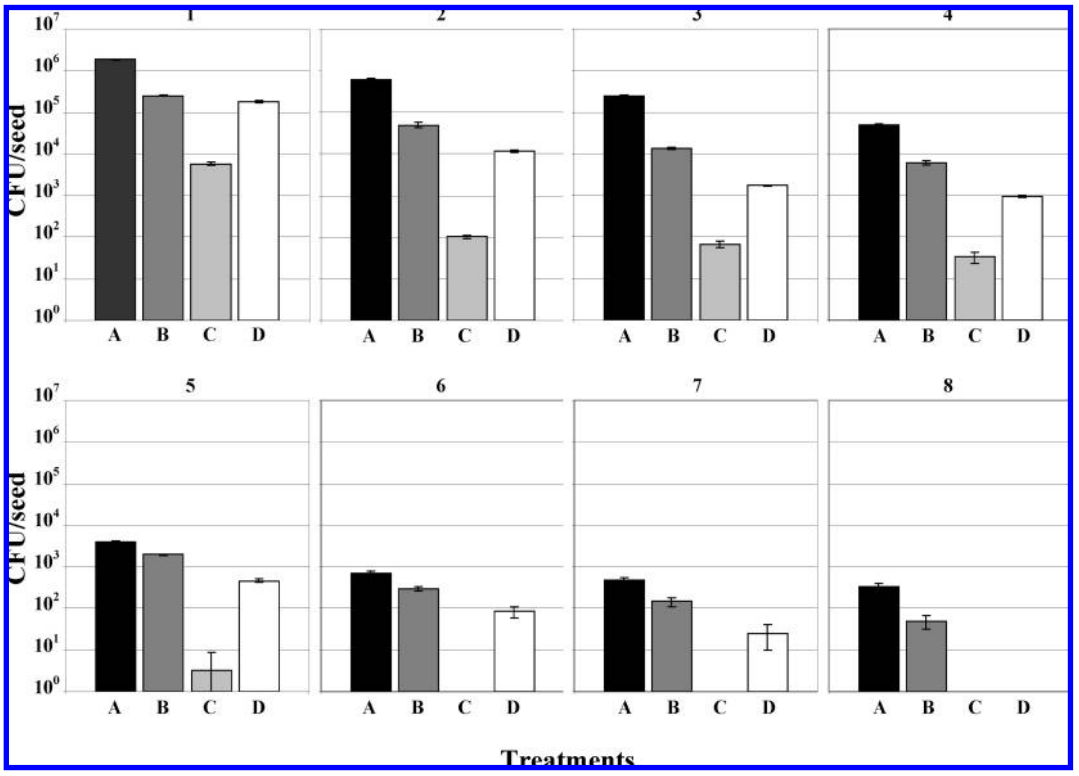

Figure 2. Bacterial populations of the spontaneous mutant resistant to rifampicin of the strain ICMP239 of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* on bean seeds bearing about 1.8×10^6 (1), 6.1×10^5 (2), 2.5×10^5 (3), 4.8×10^4 (4), 3.9×10^3 (5), 7.0×10^2 (6), 4.9×10^2 (7), and 3.2×10^2 (8) cfu/seed, after different treatments (A = control, no treated seeds; B = control, seeds treated with sterile distilled water containing 0.01% Tween 20; C = seeds treated with 4 mg/mL of eugenol in sterile distilled water containing 0.01% Tween 20, respectively; D = seeds treated with 100 μg/mL of tetracycline in sterile distilled water containing 0.01% Tween 20, respectively). Bars on the columns correspond to the standard error of the mean. Means of bacterial populations on bean seeds after eugenol and tetracycline treatments, evaluated by the *t* test in comparison with the means of bacterial populations on bean seeds of the controls A and B, are statistically different with $P < 0.0001$ and $P \leq 0.001$, respectively.

7.0×10^2, 4.9×10^2, and 3.2×10^2 cfu/seed (**Figure 2**, **panels 6−8**) since no alive bacteria were recovered in the respective seed washings.

As expected, the efficacy of tetracycline treatments was lower than the eugenol one and it was apparently correlated to the density of the pathogen population on seeds. In fact, treatments with tetracycline caused the reduction of bacteria densities on the seeds and the effect was highly significantly different ($P \leq 0.001$) from controls (**Figure 2**). In fact, after treatment of seeds contaminated with about 1.8×10^6, 6.1×10^5, 2.5×10^5, 4.8×10^4, 3.9×10^3, 7.0×10^2, and 4.9×10^2 cfu/seed the bacteria recovered were about 1.8×10^5, 1.2×10^4, 1.7×10^3, 9.6×10^2, 4.6×10^2, 8.5×10^1, and 2.5×10^1 CFU/seed (**Figure 2**, **panels 1−7**). Only from seeds contaminated with about 3.2×10^2 cfu/seed and treated with tetracycline were no bacteria recovered (**Figure 2**, **panel 8**).

Determination of Seed Germination. Treatments of bean seeds with eugenol emulsions at different concentrations caused a reduction of the germination although differences in the effect among eugenol concentration used and time of seed germination evaluation were observed (**Figure 3**). In particular, observation at 24 and 48 h showed that eugenol treatments caused a significant reduction of the germination rate ($P \leq 0.03$) when compared to the untreated control. Also, the treatment with 0.01% Tween 20 caused a reduction of the germination rate at 48 h, but the effect was not statistically different at 72 h. Statistical analysis at 72 h showed that the effect was different from the controls only when the eugenol concentration was equal or superior to 2 mg/mL (**Figure 3**). In particular, seed germination reduction of 7% and 16% were observed after 72 h of incubation with 4 and 8 mg/mL of eugenol treatments, respectively, (**Figure 3**) and the above effects were highly significantly different ($P < 0.0001$) from the controls. A limited reduction of the seed germination (3%), but still statistically significant ($P < 0.02$), was obtained after the treatment with 2 mg/mL of eugenol. The treatment with 1 mg/mL of eugenol did not affect germination since the effect was not statistically different from the one observed in the case of control treatments.

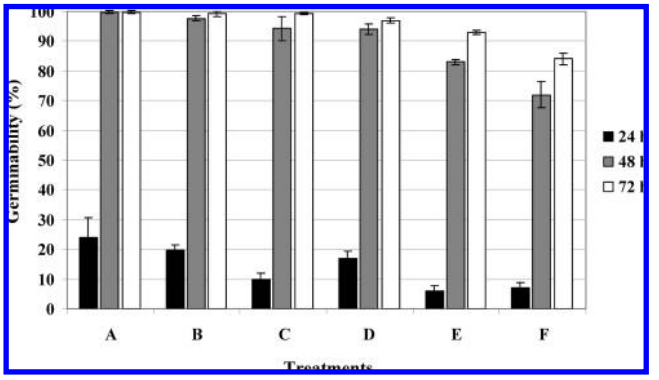

Figure 3. Germination of bean seeds after different treatments with eugenol (A = control, no treated seeds; B = control, seeds treated with water containing 0.01% Tween 20; C, D, E, and F = seeds treated with 1, 2, 4, and 8 mg/mL of eugenol in water containing 0.01% Tween 20, respectively). Bars on the columns correspond to the standard error of the mean. The *t* test of data at 72 h showed that means of geminated seeds after eugenol treatments (8, 4, and 2 mg/mL), in comparison with the controls (A and B), are statistically different ($P < 0.0001$) and ($P < 0.02$), respectively. No statistical differences were observed in the case of treatments with 1 mg/mL eugenol emulsion.

DISCUSSION

The data reported in this paper clearly indicate that the antibacterial activity of essential oil components is different and it is correlated to their chemical structures. The terpenoids and phenylpropanoids having phenol and alcohol functionalities are more active that those containing aldehyde, ketone, ether, and ester groups. This result is not surprising since it is well-known that the cytotoxic activity of essential oils is, in general, mostly due to the presence of the molecules of phenol, alcohol, and aldehyde functional groups (*22*−*24*). In fact, the modification of the latter groups causes the reduction or loss of the biological activity. For example, the methylation of eugenol causes the loss of the bactericidal activity toward the Gram negative bacteria (*23*, *25*). In particular, the antibacterial activity of these substances appears mainly correlated to their amphypatic nature, and this is in accord with the already known activity of essential oils toward cell membrane function. In fact, it is known that phenol and alcohol derivatives alter the functions of the cytoplasm membrane (*26*−*30*). The reduced bactericidal activity of terpenoids, ketone, aldehyde, and ester functions, and the phenylpropanoid anethol is probably due to their lower amphypaticity and that they present sublethal activities on specific sites of the cellular membrane. In fact, ketones present a prevailing action on lipids of the cellular membrane (*7*). Only weakly or completely not active resulted monoterpenoids and the sesquiterpenoid caryophillene. The low or null bactericidal activity of the latter compounds, observed in this study, is probably due to their specific and/or punctiform mechanisms of action. In fact, α- and *β*-pinene, having a hydrocarbon skeleton, destroy the cellular integrity of the yeast *Saccharomyces cerevisiae*, inhibiting the mitochondria respiratory activity (*30*). Nevertheless, α-pinene has been reported to show also permeable activity on liposomes (*31*). The generally complex composition of essential oils explains their wide spectrum of action since the components may have different cellular target structures with different mechanisms.

The above results clearly indicate that the antibacterial activity of the whole essential oils may depend on the relative concentration of the active components. In this study, the synergic or antagonistic effects of the different essential oil components was not determined but the possible chemical interaction among the components with consequent synergic and/or antagonistic effects is not excluded. Studies aimed to assess the antibacterial activity of pure components in different combination appear necessary. The availability of different essential oil components, possibly with a different mechanism of action, in an essential oil or in an oil mixture constituted ad hoc may lead to a bactericidal effect due to the synergic action on more cellular structures or functions.

The MIQ of the essential oil components were higher when compared to those shown by the antibiotic tetracycline on the same target bacterial strains. The MIQ of phenol and alcohol, as well as aldehyde, ether, ketone, ester terpenoids, and phenylpropanoid derivatives, and hydrocarbons were about 10- to 1000-fold higher of those shown by the antibiotic, respectively. The different specific activity shown even by the more active essential oil component and the antibiotic is not surprising since the tetracycline and the substances in consideration have a different mechanism. Tetracycline inhibit the RNA-polymerase biosynthesis and the consequent protein biosynthesis (*32*) while the essential oil components mainly interfere with the cell membrane functions (*26*−*30*).

The application of eugenol to bean seeds artificially contaminated with a strain of *X. campestris* pv. *phaseoli* var. *fuscans* strongly reduced the bacterial population on the seeds. In fact, the application of 4 and 8 mg/mL eugenol emulsions caused an about 100- to 1000-fold reduction of the pathogen population per seed also in relation to the initial bacterial population densities on seeds. Of interest is the fact that treatments with 4 mg/mL eugenol eliminated all the bacteria on the bean seeds equal or inferior to about 7.0×10^2 cfu/seed when considering that, under field conditions, the minimal bacterial population per seed leading to plant infection is about 10^3-10^4 bacteria (*33*).

Treatments of bean seeds with the antibiotic tetracycline solutions showed a lower efficiency when compared to eugenol. It is necessary to draw the fact that eugenol and tetracycline were used at comparable concentrations based on the respective MIQs as determined in this study. Such a different result is probably due to their different action mechanisms (*34*) and/or efficiency in the interaction with the seed surface.

The reduction of bean seed germination after treatments with eugenol is not surprising since this feature of essential oils was already reported (*7*, *35*, *36*). However, the limited effect on bean seed germination mainly observed at a lower concentration indicate the possible amelioration of the disinfection methods either by the modulation of solution concentration, exposition time, and the way of the substance application to seeds and/or by the development of specific formulations for a higher efficiency avoiding negative effects on seed vitality feature.

In conclusion, essential oils and pure components appear to be good bactericides as alternatives to antibiotics for the control of bacterial diseases of plants. Of course, before there is practical application of these potential methods for seed disinfection, further studies on their effects on seed vitality and plant vigor and on formulation of essential oils are necessary. The potential use of eugenol for disinfection of seeds contaminated with *X. campestris* pv. *phaseoli* var. *fuscans*, as a matter of fact, suggests a possible general application on seeds contaminated/infected with other bacterial and fungal pathogens, as suggested by other authors (*12*). The use of pure components such as eugenol instead of complex oil mixtures should be advisable for several reasons. First of all, the high variability of whole essential oil composition, which depends on agronomic and environmental conditions as well as genetics of plants (i.e., species, clones, ecotypes, etc) from which they are extracted, is well-known, so the content of the active components may be variable in relation to the preparation in use. Then, the selection of pure substances would permit the use of components useful for the purpose of avoiding the application of components of the crude mixtures not useful or worse with negative effects (i.e., toxicity on plants, animals, and consumers, and environment impact). Furthermore, eugenol, though known for its cytotoxic effects toward different micro-organisms and mammalian cells (*37*), shows also positive biological features such as antioxidant (*38*) and antitumoral properties (*39*), and then, it appears as a good candidate as a natural compound potentially usable in organic agriculture.

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