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Antibacterial effect of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens

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ABSTRACT

Bacterial pathogens are a serious problem on tomato plants. Amongst them, *Pseudomonas syringae* pv. *tomato* (Pst), *Xanthomonas vesicatoria* (Xv) and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), causal agents of bacterial speck, bacterial spot and bacterial canker, respectively, affect tomato production under greenhouse and field conditions. In *in vitro* tests with bacterial strains at a population density of 10^6 and 10^8 cfu ml^{-1}, vegetal extracts from cloves of *A. sativum* and fruits of *F. carica* at concentrations of 1 and 30%, respectively, showed best effects at 10^6 cfu ml^{-1} bacterial concentration. In *in vivo* tests bacterial strains were tested at 10^5 cfu ml^{-1}; *A. sativum* and *F. carica* extracts reduced disease incidence by 58 and 30%, and disease severity by 68 and 22%, respectively. Moreover, these vegetal extracts resulted in effective disease control of up to 65% (*A. sativum*) and 38% (*F. carica*) of that of the standard copper treatment. The antibacterial effects of *A. sativum* and *F. carica* extracts are useful for protecting tomato plants against Pst, Xv and Cmm in the greenhouse.

1. Introduction

Bacterial diseases are a serious problem in the greenhouse and in open field production. Five major bacterial pathogens are responsible for damage on tomato organs such as roots, stems, twigs, leaflets, leaves, buds, flowers, and fruits in the warm and temperate regions of the world. These are: *Pseudomonas syringae* pv. *tomato* (Okabe) Young et al., causal agent of bacterial speck; *Xanthomonas vesicatoria* (ex Doidge) Dye, causal agent of bacterial spot; *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al., causal agent of bacterial canker; *Pseudomonas corrugata* (ex Scarlett et al.) Roberts and Scarlett, causal agent of bacterial pith necrosis (Gardner and Kendrick, 1921; Bryan, 1930; Ciccarone and Mezzetti, 1950); *Ralstonia solanacearum*, causal agent of bacterial wilt (Allen et al., 2005).

P. s. pv. *tomato* and *X. vesicatoria* cause parenchymatic diseases whereas *C. m.* subsp. *michiganensis*, *P. corrugata* and *R. solanacearum* cause parenchymatic as well as vascular diseases (Bradbury, 1986).

At present only a few measures for the control of the above diseases,, in particular in organic agriculture, are available. The main problems in bacterial disease control are the non-availability of suitable commercial antibacterial compounds. Antibiotics are questionable for various reasons, and therefore forbidden in many countries, and are not effective against several pathogens. Copper compounds represent a problem due to their phytotoxicity, their accumulation in soil and the necessity of frequent applications. Moreover, according to the recent European restriction (CE Reg. 473/2002), the use of cupric salts will be limited (Balestra et al., 1999; Varvaro et al., 2001, 2002). Copper treatments and appropriate agronomic practices such as seed certification, irrigation and fertilization are the main measures presently used to control the above diseases. Since environmental factors and variable colonization strategies play an important role in phytobacteria spread on tomato crops, without effective preventive measures it is difficult to reduce their damage (Beattie and Lindow, 1999; Pietrarelli et al., 2006). As an alternative to copper compounds, a few natural substances have recently been proposed, but further studies are needed to optimize their effectiveness. For example, propolis, known as bee-product, has shown interesting antibacterial effects even if its composition (concentration of its main a.p., galangin) is strictly linked to vegetal species and climatic conditions; essential oils have revealed a potential use, but their plant toxicity has yet to be evaluated (Balestra et al., 1998; De Castro, 2001; Varvaro et al., 2001, 2002; Lo Cantore et al., 2004; Iacobellis et al., 2005).

The aims of this study were to evaluate the *in vitro* and *in vivo* antibacterial effect of *Allium sativum* and *Ficus carica* aqueous

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extracts on tomato bacterial pathogens *P. s.* pv. *tomato*, *X. vesicatoria* and *C. m.* subsp. *michiganensis*.

A. sativum has been used as a medicine for millennia and was chosen for its properties to inhibit different enzymes essential for microbial pathogen infections by its organosulfur compounds (Lawson, 1998; Ankri and Mirelman, 1999; Obagwu and Korsten, 2003). The antibacterial principle of *A. sativum* was identified as diallylthiosulphinate and named allicin (Cavallito and Bailey, 1944; Cavallito et al., 1944). Allicin is produced during the crushing of garlic cloves by the interaction between the amino acid alliin and the enzyme alliinase; allicin is a precursor of a number of secondary products formed in crushed garlic preparations and possesses various biological activities (Stoll and Seebeck, 1951).

F. carica was chosen for its abundance of phenols, essential oils and flavonoids, which are effective on bacteria through compounds produced such as resveratrol, psoralen and bergapten. These compounds have demonstrated antibacterial activity and could be used commercially as a means of phytopathogenic bacteria control (Ogungbamila et al., 1997; Ulate-Rodriguez et al., 1997; Salameh et al., 2004; Zao et al., 2005).

2. Materials and methods

2.1. Plant material

Plants of *A. sativum* and *F. carica* were collected from organic agriculture fields. Extracts of the above plants were obtained from cloves and from fruits, respectively.

Fresh vegetal materials (50 g for *A. sativum* and 500 g for *F. carica*) were washed under sterile distilled water and blotted with paper towels. Samples were then sliced into small pieces and blended using a twister blender (Hamilton Beach, 16-speed Turbo-Twister blender) for 10 min at room temperature. The extracts were obtained by centrifuging samples (Sorvall RC5B centrifuge, Newton, CT) at 8000 × *g* for 45 min to remove bigger particles. The supernatants were collected and sterilized using a Nalgene filter (diameter 0.42 μm, Fisher Scientific). The extracts were then kept at 4 °C until use within 24 h. As final concentration, after preliminary tests, samples of *A. sativum* (10 g l^{-1}) and of *F. carica* (300 g l^{-1}) were utilised.

2.2. Bacterial pathogens

Pseudomonas syringae pv. *tomato* (Pst), *Xanthomonas vesicatoria* (Xv) and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) were isolated from diseased organic tomato plants in central Italy. They were lyophilised and stored at 4 °C for long-term storage in the phytobacteriological collection of the Department of Plant Protection, University of Tuscia, Viterbo, Italy. The subcultures of pathogens were obtained by growing bacteria for 48–72 h at 25 ± 2 °C on NSA (nutrient broth 8 g l^{-1}, sucrose 50 g l^{-1} and agar 18 g l^{-1}) for Pst and Cmm and on YDC (yeast extract 10 g l^{-1}; dextrose 20 g l^{-1}; $CaCO_3$ 20 g l^{-1} and agar 18 g l^{-1}) for Xv.

2.3. In vitro antibacterial tests

The antibacterial *in vitro* assays were carried out by spot tests (Klement et al., 1990). Bacterial strains labelled as Pst14, Xv697 and Cmm21, were utilised at a concentration of 10^6 and 10^8 cfu ml^{-1} in order to obtain useful indications for survival studies in *in vivo* tests. *A. sativum* clove extracts were utilised at a concentration of 10 g l^{-1} whereas those of *F. carica* fruit were at a concentration of 300 g l^{-1}. Spot tests were conducted on NSA medium. After distribution of each bacterial suspension (100 μl per Petri dish), the vegetal extracts (four drops, 30 μl each) were placed on the NSA surfaces. After incubation at 25 ± 2 °C for 72 h, inhibition zones were observed through a stereomicroscope and their diameter measured (Klement et al., 1990). The *in vitro* spot tests were repeated three times with two replicates each.

2.4. In vivo antibacterial tests

For *in vivo* tests, 1-month-old tomato plants cv. Pullrex, were used. They were grown in a greenhouse in 15-cm-diameter pots containing a sterilized mix of soil–sand–peat (2:1:1 by volume) and watered daily by drip-irrigation. A mineral solution (NPK 20–20–20 + B + Cu + Fe + Mn + Mo + Zn 1–5–30–10–10–10, respectively) at 2 g l^{-1} was distributed weekly into the pots to maintain optimum nutritional conditions. Temperature was maintained at day and night temperatures of 25 ± 2 °C and 15 ± 2 °C, respectively; the relative humidity was maintained between 70 and 80%, during the whole experiment, by using automatic cooling. Heating and drip-irrigation data were recorded by logger at 60-min intervals.

Each combination of bacterial strain/vegetal extract was repeated three times and experiments were observed over the 15 days after treatment. The extracts of *A. sativum* and *F. carica* were used at a concentration of 10 g l^{-1} and of 300 g l^{-1}, respectively. Bacterial strains were the same as those used in *in vitro* tests (Pst14, Xv697, Cmm21) and were used at a concentration of 10^5 cfu $ml^{-}1$.

Bacterial suspensions of strains Pst14, Xv697, Cmm21 in sterile distilled water were prepared from 24-h-old bacterial cultures and adjusted to 10^5 cfu ml^{-1} using a spectrophotometer at 600 nm.

Tomato plants were sprayed with each natural extract until leaves were homogeneously wet; after 24 h bacterial suspensions were inoculated by spraying plants with a CO_2-pressurized hand-held sprayer equipped with a large orifice (diameter 1.4 mm, Tee-Jet 8004) nozzle operating at a pressure of 28 g cm^2 to produce fine spray droplets. In the greenhouse, 2 h before and 2 h after bacterial inoculation, the relative humidity was maintained at 90% to favour stomata opening. For each combination (bacterial pathogen/vegetal extract) 90 tomato plants were used, subjected to the following treatments: 30 inoculated plants treated with vegetal extract; 30 inoculated, non-treated plants as negative control; 30 inoculated plants treated with copper oxychloride [280 g l^{-1}] as positive control.

Inoculated tomato plants were monitored daily over a period of 15 days. Diseased leaf areas of inoculated tomato plants by Pst14 and Xv697 were observed to calculate the disease incidence (DI) (number of diseased leaflets per plant) and the disease severity (DS) (number of necroses per square centimetre of leaflet) according to Steel et al. (1997).

The leaf area (cm^2) was measured by a Delta T area meter device (Decagon Devices Inc. Pullman, WA).

The disease control efficiencies were calculated by comparing the disease incidences after *A. sativum* and *F. carica* extract treatments with those observed after the copper treatment, considered as 100%.

2.5. Quantification of polyphenols in F. carica fruits and of allicin in A. sativum cloves

The total amount of polyphenols (milligrams of gallic acid equivalents (GAE) per 100 g fresh weight) in fig fruit extracts was determined by using the Folin–Ciocalteu assay (Jayaprakasha et al., 2001; Marinova et al., 2005). For each replicate, 1 ml of extract was added to 9 ml of sterile distilled water (SDW), and 1 ml of gallic acid standard solution (20, 40, 60, 80 and 100 mg l^{-1}) was similarly added to 9 ml of SDW; as control 1 ml of SDW was used. The Folin–Ciocalteu's phenol reagent (1 ml) was added to the extract solution

and to each standard solution. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to each solution. Then, 4 ml of SDW was added to each one to reach the final volume of 25 ml. After incubation for 90 min at room temperature, the absorbance was determined at 750 nm with a UV spectrophotometer. All samples were analysed in duplicate.

Garlic clove samples (0.1 g each) were dissolved in 10 ml of SDW and incubated at room temperature for 30 min. Samples were then centrifuged at 8000 × *g* for 5 min at room temperature and the supernatants filtered (diameter 0.22 μm, Fisher Scientific). The quantitative determination of allicin was obtained by chromatographic analysis using a high performance liquid chromatograph, HPLC, LC-10ATvp connected to a UV-VIS detector SPD-10Avp (Shimadzu Co, Kyoto, Japan). For molecular separation, a column nucleosil 100-5 C18 with a size 250 × 4 mm (Macherey-Nagel GmbH & Co, KG) was used. Elution was carried out by isocratic solvent applications of acetonitrile:H_2O (30:70) (Rosen et al., 2001) at a flow rate of 1 ml min^{-1}, and then allicin was detected at 214 nm. For quantification of allicin-containing extracts, the calibration curve was obtained with allicin (LTK Laboratories, Inc.) with more than 99.5% purity at different concentrations (1, 0.5, 0.25, 0.125 and 0.063 mg ml^{-1}) (Fujisawa et al., 2008).

2.6. Statistical analysis

Data were statistically analysed using GraphPad Prism 4 software by analysis of variance (ANOVA) and the significance of the treatments were determined using Tukey's HSD test ($P \leq 0.05$).

3. Results

3.1. In vitro antibacterial tests

Both *A. sativum* and *F. carica* extracts had antibacterial effects in agar plate tests against bacterial strains Pst14, Xv697, Cmm21 (Table 1).

The *A. sativum* clove extracts showed antibacterial activity against all strains utilised; in particular, the inhibition halos of 14 ± 1.2, 16 ± 1.3 and 23 ± 1.1 were observed using 10^6 cfu ml^{-1} suspensions of strains Pst14, Xv697 and Cmm21, respectively.

The *F. carica* fruit extracts showed better inhibition effects than that of *A. sativum* extract towards the three bacteria pathogens and was effective at both bacterial concentrations (10^6 and 10^8 cfu ml^{-1}) utilised.

3.2. In vivo antibacterial tests

The antimicrobial effects of the natural substances contained in the *A. sativum* and *F. carica* extracts were confirmed in *in vivo* tests on plants inoculated with suspension of the strains Pst14, Xv697 and Cmm21 at 10^5 cfu ml^{-1} (Table 2).

A. sativum extract, when compared to the untreated control, reduced DI caused by strain Pst14, Cmm21 and Xv697 which resulted in 16, 20 and 22 diseased leaflets per plant, respectively (Table 2). Considering the untreated control value of 38 diseased leaflets per plant, the maximal reduction of up to 58% (16 diseased leaflets per plant) of DI was achieved by *A. sativum* extract on plants inoculated with Pst14. The DS was also reduced by 68% (0.033 necroses $cm^{-}2$ leaflets) on tomato plants inoculated by Pst14 compared to the untreated control and by 56% on those inoculated by Xv697. On tomato plants inoculated by Cmm21 DS (cm^2 diseased leafletsper cm^2 leaflets area) was reduced by 65%.

Table 2
Effect of *Allium sativum* and *Ficus carica* extracts on disease incidence and disease severity of tomato plants inoculated with *P. s.* pv. *tomato*, *X. vesicatoria*, *C. m.* subsp. *michiganensis* at 10^5 cfu ml^{-1}.

Treatment	*P. s.* pv. *tomato* 14		*X. vesicatoria* 697		*C. m.* subsp. *michiganensis* 21	
	DI[a]	DS[a]	DI[a]	DS[a]	DI[a]	DS[a]
A. sativum (10 g l^{-1})	16 a	0.033 a	22 b	0.088 a	20 b	0.178 b
F. carica (300 g l^{-1})	31 b	0.079 a	28 b	0.126 b	28 b	0.343 c
Untreated control (negative control)	38 c	0.102 b	40 c	0.200 b	37 c	0.514 d
Copper (positive control)	8 a	0.007 a	15 a	0.041 a	14 a	0.060 a

[a] The values shown are the mean of three repetitions with 10 plants each. Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test. DI, disease incidence; DS, disease severity.

Treatments with *F. carica* fruit extracts were less effective compared to those treated with *A. sativum*. The effect was greater on the DI and DS of plants inoculated with strains Cmm21 and Xv697 than on plants inoculated with strain Pst14.

Considering copper treatments as 100% effective, control by *A. sativum* extract was 65, 52 and 56% on tomato plants inoculated by Pst14, Xv697 and Cmm21, respectively, while disease control by *F. carica* was 32, 38 and 38% on tomato plants inoculated by Pst14, Xv697 and Cmm21, respectively (Table 3).

3.3. Quantification of polyphenols in F. carica fruits and of allicin in A. sativum cloves

The polyphenol content in fig fruit extracts was 72.8 ± 1.1 mg GAE 100 $g^{-}1$. Allicin from garlic cloves was detected as a single peak (retention time 7.5 min), its optical absorbance measured at 214 nm (Fig. 1), and its concentration was calculated to be 0.032 mg ml^{-1}.

4. Discussion

The possibility of controlling tomato bacterial diseases with vegetal extracts appears of particular interest considering the lack of valid alternatives to copper compounds and the unavailability of commercial cultivars resistant to Pst, Xv and Cmm. Vegetal extracts tested in our study proved to be useful for effective biocontrol of *P. s.* pv. *tomato*, *X. vesicatoria* and *C. m.* subsp. *michiganensis* on tomato plants. *A. sativum* and *F. carica* extracts successfully reduced the disease incidence and disease severity caused by Pst, Xv and Cmm

Table 1
Antibacterial effects in spot agar tests of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens on NSA medium after 72 h of incubation at 25 ± 2 °C.

Vegetal extracts	Diameter of inhibition (mm)[a]					
	P. s. pv. *tomato* 14		*X. vesicatoria* 697		*C. m.* subsp. *michiganensis* 21	
	10^6 cfu ml^{-1}	10^8 cfu ml^{-1}	10^6 cfu ml^{-1}	10^8 cfu ml^{-1}	10^6 cfu ml^{-1}	10^8 cfu ml^{-1}
A. sativum (10 g $l^{-}1$)	14 ± 1.2 b	13 ± 1.3 b	16 ± 1.3 b	NID	23 ± 1.1 a	NID
F. carica (300 g $l^{-}1$)	21 ± 1.8 a	15 ± 1.3 b	19 ± 1.7 a	13 ± 1.7 b	20 ± 1.5 a	16 ± 1.2 b

[a] The values shown are the mean of three replicates. Means within rows followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test. NID, no inhibition determined.

Table 3
Effect of *Allium sativum* and *Ficus carica* extracts on the control of diseases caused by *P. s.* pv. *tomato*, *X. vesicatoria*, *C. m.* subsp. *michiganensis* at 10^5 cfu ml^{-1}.

Treatment	Percentage disease control[a]		
	P. s. pv. *tomato* 14	*X. vesicatoria* 697	*C. m.* subsp. *michiganensis* 21
A. sativum (10 g l^{-1})	65 b	52 b	56 b
F. carica (300 g l^{-1})	32 c	38 c	38 c
Copper (positive control)	100 a	100 a	100 a

[a] Data are based on disease incidence. The values shown are the mean of three replicates. Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test.

bacterial pathogens used, and no negative (phytotoxic) effects were recorded on tomato plants.

A. sativum extract was more effective than *F. carica* in the control of the diseases caused by the above pathogens, but the efficacy of both was lower than that observed with copper as the standard.

A. sativum showed a higher DI reduction on tomato plants inoculated by Pst than on those inoculated by Cmm and Xv strains, and was most effective in DS reduction (up to 68% on tomato plants inoculated with Pst). *F. carica* reduced DI up to 30% on tomato plants inoculated with Xv; DS was similarly reduced on tomato plants inoculated with Xv and Cmm strains.

Both natural substances were effective against Pst, Xv and Cmm pathogens suggesting their potential use as alternatives to or in combination with a reduced amount of copper compounds. Moreover, they are inexpensive, easily obtained and without any negative effects on human health.

In the greenhouse *A. sativum* extract was more effective on plants inoculated by Pst compared to those by Xv and Cmm; only 35% of inoculated tomato plants were diseased at 15 days post-inoculation, compared to 48 and 44%, respectively with the other two pathogens. This observation has to be confirmed with further strains of each pathogen species.

It is interesting to notice that the antibacterial compounds of *A. sativum* and *F. carica* extracts showed activity against two Gram− (Pst, Xv) as well as against a Gram+ bacterium (Cmm), and on pathogens that cause parenchymatic as well as vascular diseases on tomato.

Efforts should be made to identify an acceptable fragrance for the *A. sativum* extract, capable of disguising its unpleasant odour without affecting its antimicrobial activity (Obagwu and Korsten, 2003). Moreover, considering its property of penetration into the plants, it will be important to verify a synergistic effect with the active principle(s) of *F. carica* extract when they are used in combination against tomato bacterial pathogens (Miron et al., 2000).

F. carica was less effective as an antibacterial agent than *A. sativum*. The identification and characterization of *F. carica* active compounds and a better understanding of their mechanisms of action could contribute to an optimal utilisation alone or in combination with other natural extracts. The antimicrobial effects of extracts of various parts of other *Ficus* spp., *Allium* spp. and of other *Liliales* and *Urticales* plants, should be evaluated (Baumgartner et al., 1990; Ogungbamila et al., 1997; Mandal et al., 2000; Adeniyi and Anyiam, 2004).

The bactericidal activity of these vegetal extracts gives new opportunities to improve control against different tomato bacterial diseases that cause losses both at seedling stage and in mature plants, and, in particular, in organic agriculture. These extracts are also suggested for preventive control of the diseases caused by the above pathogens. Studies are in progress, in the greenhouse and in open fields, to evaluate any effect of these natural substances on tomato plant development and on qualitative and quantitative tomato fruit production.

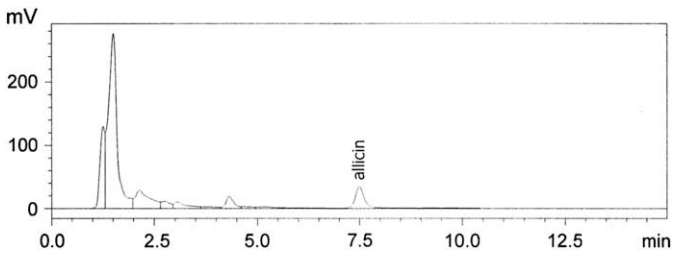

Fig. 1. HPLC chromatogram with allicin peak.

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