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Biocontrol of *Pseudomonas syringae* pv. *syringae* affecting citrus orchards in Tunisia by using indigenous *Bacillus* spp. and garlic extract

Imen Mougou and Naima Boughalleb-M'hamdi*

Abstract

Citrus blast and black pit that became increasingly important bacterial diseases are caused by *Pseudomonas syringae* pv. *syringae*. This study aimed to evaluate the antibacterial potential of *Bacillus* species strains and garlic extracts against two *P. syringae* isolates (BAT13 and DAPP-PG115). The *Bacillus* species strains were isolated from symptomless citrus leaves. Under in vitro conditions, 21 *Bacillus* species strains and garlic extract displayed antibacterial activity against the pathogen. Under greenhouse conditions, antagonistic bacteria, garlic extract, and copper sulfate confirmed their antimicrobial effect on *P. syringae* and reduced significantly the extend of stem necrosis 10 weeks after inoculation by BAT13 up to 60.55, 56.11, and 45.83%, and by DAPP-PG115 up to 70.83, 62.5, and 46.52%, in respect to relevant treatments. Garlic extract was the most effective treatment in our hands, and it suggests that *Allium sativum* extract could be used to control and prevent infection by the pathogen.

Keywords: *Pseudomonas syringae*, Antagonistic bacteria, *Allium sativum*, Biological control, Citrus bacteriosis

Background

Citriculture represents a strategic sector in Tunisia that covers around 24,000 ha with c. 6.4 million trees. Annual production is estimated around 300,000 tons of fruit (DGPA 2016). Among the bacterial diseases that pose a threat to citrus and reduce the yield are the citrus blast and black pit, caused by *Pseudomonas syringae* pv. *syringae* (Snowdon 1990). Beiki et al. (2016) reported new citrus pathogenic strains of *Pseudomonas orientalis*, *P. simiae*, *P. lurida*, *P. moraviensis*, and *P. monteilii*. Blast results in expanding lesions on citrus leaves and stems leading to defoliation of trees in severe attacks. Black pit results in dark-colored, sunken blemishes on fruits particularly on lime and lemon (Fawcett et al. 1923). In Tunisia, *P. syringae* pv. *syringae* causing citrus blast and black pit was first reported by Boubaker (1986) on sour orange, *Citrus aurantium*, then by Abdellatif et al. (2015) on *Citrus sinensis* and *C. limon*. Moreover, recent investigation demonstrated that citrus cultivars Thompson Navel and New Hall were most susceptible. While the cultivar Eureka appeared to be less susceptible to citrus blast disease. Cultivars Eureka and Swett Lime seemed to be most susceptible to black pit disease (Mougou and Bougalleb-M'hamdi 2016a). Weeds and plant debris were shown to be a source of *P. syringae* inoculum (Mougou and Bougalleb-M'hamdi 2016b).

To control both diseases of citrus, growers in Tunisia used to apply bactericidal compounds. However, this practice could cause serious damage to the environment and human health and also promotes the selection of pathogenic strains with increased tolerance to copper (Brent et al. 1998). For these reasons, biological control deserves serious consideration in the framework of an integrated pest management strategy.

Bacillus subtilis and other *Bacillus* spp. have been long used as biological control agents against plant bacterial diseases (Chen et al. 2013). Moreover, in recent years, plant bioactive substances have been demonstrated as a new approach to postharvest disease management (Mari

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et al. 2007). Plants produce an array of secondary metabolites, which in many cases have been found to be biologically active, and a rich source of antimicrobial, allelopathic, antioxidant, and bio-regulatory properties (Tripathi et al. 2008). Garlic, *Allium sativum*, is one of the edible plants which has generated a lot of interest as a medicinal panacea. Previous studies reported the insecticidal, fungicidal, acaricidal, nematicidal, and bactericidal properties of garlic (Lalla et al. 2013). A wide range of microorganisms including gram-positive and gram-negative bacteria (Whitemore and Naidu 2000), fungi, protozoa, and viruses have been shown to be sensitive to crushed garlic preparations (Koch and Lawson 1996). The main antimicrobial constituent of garlic has been identified as an oxygenated sulfur compound, namely thio-2-propene-1-sulfinic acid Sallyl ester, which is usually referred to as allicin (Cavallito and Bailey 1944). Alliin (S-allyl-L-cysteine-sulfoxide) was found to be the stable precursor that is converted to allicin by the action of an enzyme alliinase, which is also present in the cloves of garlic (Ellmore and Feldberg 1994). The antibacterial activity of allicin was reviewed by Ankri and Mirelman (1999).

Plant extracts and antagonistic bacteria could play an important role in plant disease management. Therefore, more information of their in vivo efficiency is needed.

The aim of this study was to investigate the in vitro and in vivo antimicrobial effect of garlic extract and antagonistic bacteria *Bacillus* spp. against *P. syringae* pv. *syringae* the causal agent of citrus blast and black pit in Tunisia.

Materials and methods

Bacterial strains and isolates

Two strains of *P. syringae* pv. s*yringae* (BAT13 and DAPP-PG115) were used in this study. DAPP-PG115 was obtained from the Bacterial Collection of the Plant Protection Unit, Department of Agricultural, Nutritional and Environmental Sciences, University of Perugia, (Italy). BAT13 strain was isolated from blast necrosis from citrus trees (cv. Thompson Navel) in the region of Menzel Bouzelfa (Cap-Bon) and stored in the collection of plant pathology laboratory at ISA-CM (Tunisia). Identification of bacterial strains was performed by biochemical tests (LOPAT and GATTa) and by comparing 16S rRNA gene sequences with the GenBank database using the Basic Alignment Search Tool (BLAST). Pathogenicity of the strain BAT13 was confirmed on 1–2-year-old citrus (cv. Thompson Navel), which inoculated with a 10^8 CFU ml^{-1} bacterial suspension and compared to the reference strain DAPP-PG115. Symptoms were characteristic of citrus blast. Necrotic areas were developed and enlarged (unpublished data).

Three bacterial isolates (MBCL2, MBCL3, and FCL2) that proved their antagonistic effect to the pathogen, identified by biochemical test and resembling to *Bacillus* spp., were obtained from symptomless citrus leaves from orchards located in the region of Takelsa (Tunisia). Antagonistic action of those bacterial isolates was proved by in vitro and in vivo tests.

Garlic storage and extraction

Garlic bulbs of *Allium sativum* were purchased from the supermarket and stored at 4 °C in the dark until required. Axillary buds from the composite garlic bulb were peeled, cleaned, weighed, and roughly crushed. Garlic juice was obtained by squeezing the macerates mixture, using a sterile cheesecloth. The juice was centrifuged, at 4200 rpm for 10 min in order to separate garlic debris from the liquid and filtrated with a syringe filter (0.22 μm). Garlic extract was either used immediately or stored at 4 °C until use.

Analysis of garlic extract for allicin content

The content of allicin was determined spectrophotometrically (Jenway 7315), by the reaction with the thiol, 4-mercaptopyridine. The garlic extract was incubated with 4-mercaptopyridine (10^{-4} M) in phosphate buffer (50 mM), EDTA (2 mM, pH 7.2), which results in the formation of a mixed disulfide, 4-allylmercaptothiopyridine, and the consequent shift in absorbance at 324 nm was monitored as described by Miron et al. (2002). The negative control was obtained using the same procedure without garlic extract.

Isolation and identification of the antagonistic bacteria

Isolation of the antagonistic bacteria

During surveys, samples were collected from citrus orchards. Young healthy leaves (10 leaves per plant per orchard were sampled) were taken from different citrus orchards located in Takelsa, Chbika, Menzal Bouzelfa, Sidi Bouali, Bouargoub, Akouda, and El Gobba. The leaves were rinsed with sterile distilled water. Each sample was cut into small pieces (about 2×2 mm), and then, the fragments were surface-disinfected with 95% ethanol for 3 min. Pieces of tissues were placed in sterilized water and mechanically crushed in a sterile mortar. Then, serial dilutions were made. A loopful of macerate was streaked onto Petri dishes containing the LB (Luria--Bertani) medium and incubated at 25 °C for 3 days.

Identification of the antagonistic bacteria

Potassium hydroxide test (KOH) 3%

The identification of the antagonists was made by biochemical tests. A rapid method (KOH) for the determination of the Gram reactions of bacteria was

carried out as reported by Suslow et al. (1982). The bacterium was aseptically removed from Petri plates with toothpick, placed on glass slide in a drop of 3% KOH solution, and stirred for 10 s using a quick circular motion of hand.

Catalase test

A part of the colony in question was transferred to a microscopic slide using a sterile toothpick and mixed with a drop of H_2O_2. Production of air bubbles is indicative of catalase activity, whereas no air bubbles indicate a lack of catalase activity.

Oxidase

This test determines the presence of cytochrome C oxidase enzyme. Kovacs (1956) method was used. A single colony from a freshly streaked LB agar plate was picked and applied with a sterile toothpick to the discs impregnated with a reagent: *N,N,N′,N′*-tetramethyl-p-phenylenediamine. The production of a distinct purple color in 10 s was recorded as a positive result.

Hypersensitive reaction (HR) on tobacco plants

In order to ensure that the antagonistic bacteria are not phytopathogenic agents, a hypersensitivity test was carried out on tobacco leaves (*Nicotiana tabacum*). The bacterial suspension was spectrophotometrically adjusted to (10^8 CFU/ml) and was injected into the intercellular space of the leaves using a medical syringe. Controls used in this test were a negative control (sterile distilled water) and positive control (strains DAPP-PG115). The absence of complete collapse of the tissue after 24 h was recorded as negative reaction.

Assay of in vitro antimicrobial activity of antagonistic bacteria against *P. syringae*

Double layer method

Antagonistic activity towards *P. syringae* pv. *syringae* of 21 *Bacillus* isolates (MBCL2, FCL2, GT1, MBCL3, BKT1, GCI1, HT1, FCL1, BKT2, TCK2, TM2, TCK3, MBCL1, MBT1, TCK1, TM4, TM3, TM1, HT2, FCL3, and GCI) obtained was conducted according to the modified method of Vidaver (1976) and Stonier (1960). For each isolate, a bacterial suspension (10^8 CFU ml^{-1}) was prepared in sterile distilled water (SDW); 20 μl aliquots were spot-inoculated on LB medium and incubated at 25 °C for 48 h. At the same day as the spot inoculation, two *P. syringae* pv. *syringae* strains (BAT13, DAPP-PG115) were streaked onto solid King's B medium and incubated for 2 days at 25 °C. The antagonistic bacteria were then exposed to chloroform vapor for 30 min, and the plates were left open for 15 min in a flow cabinet. One milliliter of bacterial suspension of the pathogen (10^8 CFU ml^{-1}) was mixed with 3 ml of LB medium (0.6% agar) at 45 °C. This solution was quickly overlain on plates containing the antagonists. Plates were incubated at 25 °C and checked after 24 to 48 h for the appearance of inhibition haloes surrounding the antagonist spots.

Agar well diffusion method

The ability of the antagonist to produce diffusible metabolites was also tested according to the agar well diffusion assay (AWDA) as reported by Tagg and McGiven (1971). The most potential antagonistic bacterial isolates were transferred individually to 50 ml of Luria-Bertani broth medium (LB broth) in a 250-ml Erlenmeyer flask and incubated by shaking at 200 rpm for 4 days at room temperature (RT). Bacterial suspension (1 ml; 10^8 CFU ml^{-1}) of two *P. syringae* pv. *syringae* strains (BAT13 and DAPP-PG115) was mixed with 3 ml of LB medium (0.6% agar) at 45 °C. This solution was quickly overlain on plates containing LB medium, and wells were then punched in the agar with a sterile steel borer. The potential antagonistic cultures were centrifuged at 15,000 rpm for 30 min to remove cell debris. After centrifugation, 100 μl of each sample was aseptically filtered through a 0.45 μm filter and added into the prepared wells. The plates were then incubated at 25 °C, and inhibition haloes around the wells were measured.

Assay of in vitro antimicrobial activity of garlic extract against *P. syringae* pv. *syringae*

Disc diffusion method

The disc diffusion method (Pereira et al. 2006) was used to determine the sensitivity of *P. syringae* towards garlic extract. One milliliter of bacterial suspension (10^8 CFU ml^{-1}) of *P. syringae* pv. *syringae* strains (BAT13 or DAPP-PG115) was thoroughly mixed with LB medium and poured into sterile Petri dish. Many dilutions of the garlic extract were prepared and placed to establish the proportionality of the relationship between the amount of active substance and diameter of inhibition zone. For this, Whatman filter paper disc (6-mm diameter) was placed on LB agar plates surface and an amount of 20 μL of pure garlic extract (100%), 90, 80, 70, 60, 50, 40, 30, 20, and 10% dilutions containing 18, 16, 14, 12, 10, 8, 6, 4, or 2 μl of garlic extract, respectively, were pipetted onto a stack of filter-paper discs. Undiluted garlic extract was considered as the 100% concentration of the extract. Distilled water was used as negative control. Each sterile disc is impregnated by different concentrations of garlic. Then, plates were incubated overnight at 25 °C.

Agar well diffusion method

LB medium was poured into each sterile Petri dish. One milliliter of bacterial suspension (10^8 CFU ml^{-1})

of the two *P. syringae* pv. *syringae* strains (BAT13, DAPP-PG115) was mixed with 3 ml of LB medium (0.6% agar) at 45 °C (Tagg and McGiven 1971). This solution was quickly overlain on plates containing LB medium, and wells of 6-mm diameter were then punched in the agar with a sterile steel borer. Wells were then punched in the agar with a sterile steel borer. One hundred microliters of garlic extract was aseptically added into the prepared wells. The plates were then incubated at 25 °C, and inhibition haloes around the wells were measured. All experiments were carried out with three replicates and were repeated twice in time.

Assay of in vivo antimicrobial activity of garlic extract and antagonistic bacteria against citrus blast disease development

Selected strains showing the best in vitro antagonistic activity levels against *P. syringae* were used. In this study, strains MBCL2, MBCL3, FCL2 and undiluted garlic extract were evaluated in vivo. One- and 2-year-old citrus plants of cv. Thompson were used. Plants were kept inside a greenhouse in individual pots filled with a substrate composed of peat and sand (2/3*v*, 1/3*v*). Twelve plants for each treatment were used. Citrus plants were wounded at six sites on the stem. Each wound site was inoculated with10 μl of bacterial suspension 10^8 CFU/ml of the strains BAT13 and DAPP-PG115. Three days after inoculation, 10 μl of sterile distilled water (control), or a suspension of antagonistic bacteria MBCL2, MBCL3, and FCL2, crude garlic extract or copper sulfate (0.5%: 0.05 mg/10 μl SDW) as individual treatment was added to the wounds, which were then covered again by Parafilm M. Measurements of the extend of stem necrosis were taken 10 weeks after inoculation.

Data analysis

Data were subjected to analysis of variance using IBM SPSS Statistics software (version 23). Mean values among treatments were compared by Duncan's multiple range test at the 5% ($P = 0.05$) level of significance.

Results and discussion

Identification of the antagonistic bacteria

Antagonistic bacteria which exhibited in vitro antibacterial activity against *P. syringae* pv. *syringae* were biochemically identified. The infiltration of tobacco leaves with the antagonistic bacterial suspensions did not cause hypersensitivity reaction after 24 h in tobacco leaves, whereas *P. syringae* pv. s*yringae* strain DAPP-PG115 induced a hypersensitive reaction. These results indicated that the used bacterial agent were not plant pathogens. Strains of potential antagonistic agent showed bacterial colony morphology characteristic of *Bacillus* species. The strains were gram (+), oxidase (−), (HR-) and catalase (+). According to macroscopic characteristics of bacterial strains colonies as well as biochemical characteristics, the antagonistic bacterial strains belong to *Bacillus* genus according to the keys of De Vos et al. (2009).

In vitro antimicrobial activity of antagonistic bacteria against *P. syringae* pv. *syringae*

Double-layer method

Analysis of variance of inhibition zone showed highly significant differences between the bacterial treatments ($P = 0.05$). Among 74 strains, 21 showed antibacterial activity against the pathogen. The antagonism of the 21 *Bacillus* isolates was investigated against the two strains of *P. syringae* pv. *syringae* (DAPP-PG115 and BAT13). Antimicrobial activity (mm) is expressed as the difference between diameter of inhibition zone and diameter of *Bacillus* colony (Table 1). The results of the double-layer antagonism

Table 1 Inhibition zone diameter (mm) induced by *Bacillus* spp. stains against *Pseudomonas syringae* pv. *syringae* strains BAT13 and DAPP-PG115

Antagonists (*Bacillus* spp.)	Phytopathogenic bacteria	
	Inhibition zone diameter (mm)	
	BAT13	DAPP-PG115
MBCL2	20.66 ± 0.1 a	22 ± 0.1 a
FCL2	18 ± 0.09 b	18.4 ± 0.09 b
GT1	16 ± 0.08 c	16.35 ± 0.05 c
MBCL3	15.7 ± 0.05 cd	16.33 ± 0.05 c
BKT1	14.68 ± 0.05 de	15.1 ± 0.1 d
GCI1	13.66 ± 0.05 e	14 ± 0.08 e
HT1	11.66 ± 0.05 f	12.33 ± 0.09 f
FCL1	11.68 ± 0.13 f	12.16 ± 0.1 f
BKT2	10.66 ± 0.08 f	11.33 ± 0.11 f
TCK2	11 ± 0.09 f	12.16 ± 0.07 f
TM2	10.85 ± 0.05 f	11.85 ± 0.05 f
TCK3	10.66 ± 0.1 f	11.7 ± 0.04 f
MBCL1	10.68 ± 0.048f	11.5 ± 0.1 f
MBT1	10.6 ± 0.053f	11.55 ± 0.09 f
TCK1	10.66 ± 0.10 f	11.33 ± 0.05 f
TM4	6.66 ± 0.13 g	7.2 ± 0.16 g
TM3	6.68 ± 0.13 g	7.01 ± 0.04 g
TM1	6.33 ± 0.15 g	6.83 ± 0.07 g
HT2	5.83 ± 0.09 g	6.33 ± 0.05 g
FCL3	5.85 ± 0.07 g	6.18 ± 0.04 g
GCI	5.66 ± 0.05 g	6.16 ± 0.04 g

Small letters are for comparison of means in the same column
Means followed by the same letter do not differ significantly ($P = 0.05$)

tests showed a significant inhibitory effect. *Bacillus* spp. strain MBCL2 exhibited the highest inhibition zone with values of 20.66 and 22 mm against BAT13 (Fig. 1) and DAPP-PG115, respectively (Table 1). Moreover, *Bacillus* spp. strain GCI showed a low antagonistic activity. The diameter of the inhibition zones were 6.1 and 5.6 mm for DAPP-PG115 and BAT13, respectively. Some studies showed that the difference in efficacy in vitro was due to changes in the nature and concentration of secreted metabolites such as antibiotics (Defago and Haas 1990), lytic enzymes, and siderophores (Bakker et al. 1990).

Agar well method

Results of the agar well diffusion assays showed that MBCL2 strain exhibited an inhibition zone of 17 mm and 18 mm against BAT13 and DAPP-PG115, respectively. For copper sulfate, the respective diameter of inhibition zone was 20.66 and 23 mm against BAT13 and DAPP-PG115 (Table 3). Thus, the results revealed that the supernatants of antagonistic bacteria had an antagonistic activity lower than copper sulfate 1%.

Previous investigations reported that various bacteria genus are considered as producers of antibiotics or hydrolytic enzymes. Mostly, the bacteria were among the *Bacillus* genus (Nielsen and Sorensen 1997). In fact, *Bacillus* species produce secondary metabolites such as antibiotics, lytic enzymes (Frandberg and Schnumer 1994), and volatile and nonvolatile compounds (Parke and Gurian-Sherman 2001).

In vitro antimicrobial activity of garlic extract against *P. syringae* pv. *syringae*

Disc diffusion method

The garlic extract contained about 13.4 mg/ml allicin, as determined spectrophotometrically. The diameter of the inhibition zone given by 20 μl of garlic extract at the concentration of 10% to 100% varied from 6 to 14 mm and from 7 to 15 mm for BAT13 and DAPP-PG115, respectively (Table 2). After applying 20 μl of crude extract (approximately 268 μg of allicin in 20 μl) or 20 μl of dilutions of extract, a clear inhibition halo appeared on Petri plate and the growth of the strains (BAT13, DAPP-PG115) was inhibited by garlic extract placed on seeded agar plates (Fig. 2A and B). The results showed the presence of positive correlation between the increase of garlic extract concentration and the diameter of inhibition zone. The size of the inhibition halo was clearly proportional to the amount of garlic extract applied and showed a linear relationship when plotted against the log of the diameter of the inhibition zone (Fig. 3A and B). The coefficient of determination was 0.83 for BAT13 and 0.88 for DAPP-PG115. Miron et al. (2000) reported that garlic extract has a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria. Recently, it is reported that allicin and its garlic preparations exhibited antibacterial properties at a large spectrum and these bacteria include *Aeromonas* spp., *Bacillus* spp., *Clostridium* spp., *Cryptocaryon* spp., *Escherichia coli*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Mycobacterium* spp.,

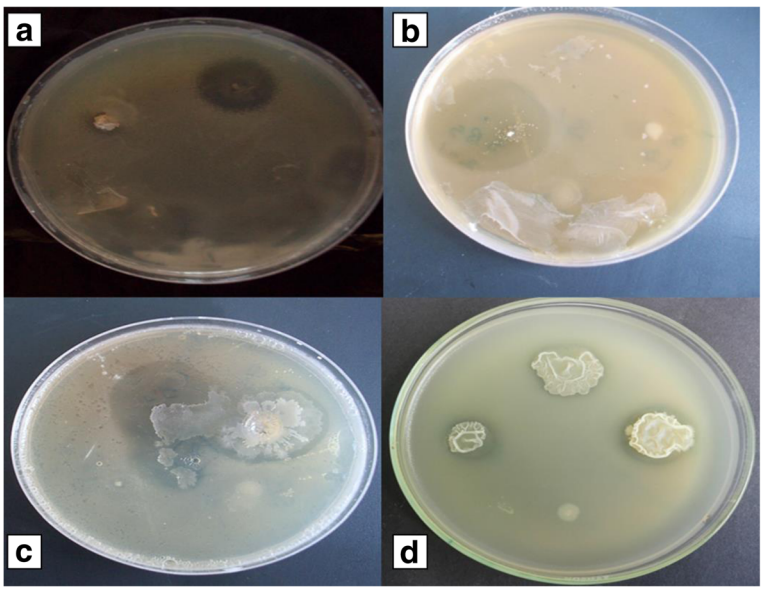

Fig. 1 Inhibition zones induced by *Bacillus* spp. against *Pseudomonas syringae* pv. *syringae* strain BAT13. **a** (MBCL3). **b** (MBCL2). **c** (FCL2). **d** (BKT1). (Negative control): strains isolated from citrus leaves

Table 2 Inhibition zone diameter (mm) induced by garlic extract against *Pseudomonas syringae* pv. *syringae* strains BAT13 and DAPP-PG115

Strains	Dilution of garlic extract (%)										
	0	10	20	30	40	50	60	70	80	90	100
BAT13	0 a	6 ± 0.06 b	8 ± 0.08 c	10 ± 0.06 d	10.35 ± 0.05d	10.5 ± 0.05d	11.33 ± 0.08 e	11.66 ± 0.08 e	11.83 ± 0.09 e	13 ± 0.06f	14 ± 0.06 g
DAPP-PG115	0 a	7 ± 0.1 05 b	9 ± 0.06 c	10.5 ± 0.08 d	11 ± 0.09 d	11.2 ± 0.09 d	12.16 ± 0.04e	12.33 ± 0.08e	12.85 ± 0.07 e	14 ± 0.1f	15 ± 0.06 g

Small letters are for comparison of means in the same row
Means followed by the same letter do not differ significantly ($P = 0.05$)

Photobacterium spp., *Proteus* spp., *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Vibrio* spp., *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae*, *and Xanthomonas campestris* (Guo et al. 2015).

Agar well diffusion method

The analysis of variance showed that the results achieved in the presence of the different treatments on the bacterial growth exhibited a highly significant effect. The results of the diffusion in agar test revealed that the garlic extract (approximately 1.34 mg of allicin in 100 μl) exhibited an antibacterial activity against the pathogen. The present study, showed that garlic extract was the most effective treatment against *P. syringae* pv. *syringae* strains (BAT13, DAPP-PG115) (Table 3, Fig. 4). The diameters of inhibition zone exhibited by antagonistic bacteria were lower than that induced by copper sulfate. Thus, garlic extract, conducted in vitro, proved to be more effective than copper sulfate 1% against *P. syringae*. Previous studies reported that *A. sativum* exhibited activity against many pathogenic bacteria and fungi at different rates (Ankri and Mirelman 1999; Pyun and Shin 2006, and Saravanan et al. 2010). The effectiveness of garlic extract against a range of plant pathogenic organisms was shown by growth inhibition of the plant pathogenic bacteria *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* pv. *maculicola*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *tomato*, *Xanthomonas campestris* pv. *campestris* (Curtis et al. 2004). Triki et al. (2008) showed that the antimicrobial property of the garlic extract is due to an active molecule which is allicin. This molecule accumulates naturally at ambient temperatures during garlic bulbs storage (Amagase et al. 2001). Allicin crosses the cell membrane easily and undergoes thiol-disulfide exchange reactions with free thiol groups in proteins. It is thought that these properties are the basis of its antimicrobial action (Miron et al. 2000). The action of biological active ingredient of allicin which exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action (Deresse 2010).

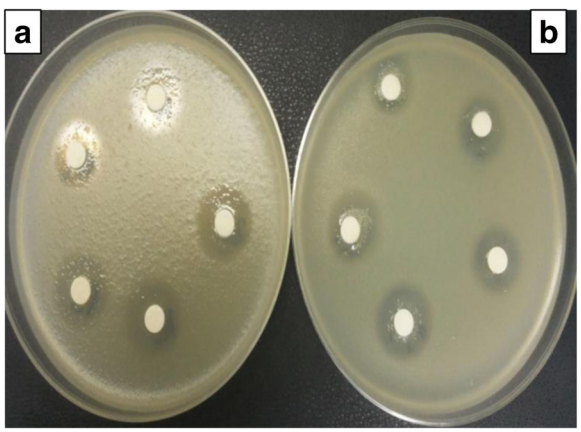

Fig. 2 Antibacterial activity of garlic extract evaluated by the disc diffusion method against DAPP-PG115 (**a**) and BAT13 (**b**)

In vivo antimicrobial activity of garlic extract and antagonistic bacteria against citrus blast disease development

Obtained results demonstrated that *Bacillus* spp. strains MBCL3, FCL2, and MBCL2 reduced the length of stem necrosis caused by the strains (DAPP-PG115 and BAT13). The analysis of the variance showed a highly significant effect of the treatments compared to controls ($P = 0.05$). Typical blast necrosis appeared at inoculated sites of the BAT13 strain showing an average length of stem necrosis of 11 mm, and 9.7 mm for DAPP-PG115 strain. *Bacillus* MBCL2 strain reduced the average length of stem necrosis of 56.11 and 62.5%, for BAT13 and DAPP-PG115 strains, respectively. When strain FCL2 was added to the wounds, inoculated with strain BAT13 or DAPP-PG115, a reduction of the average length of stem necrosis was noted with values of 50.13 and 55%, respectively. In addition, *Bacillus* spp. MBCL3 strain reduced the mean length of stem necrosis by 53.88 and 61.66% for BAT13 and DAPP-PG

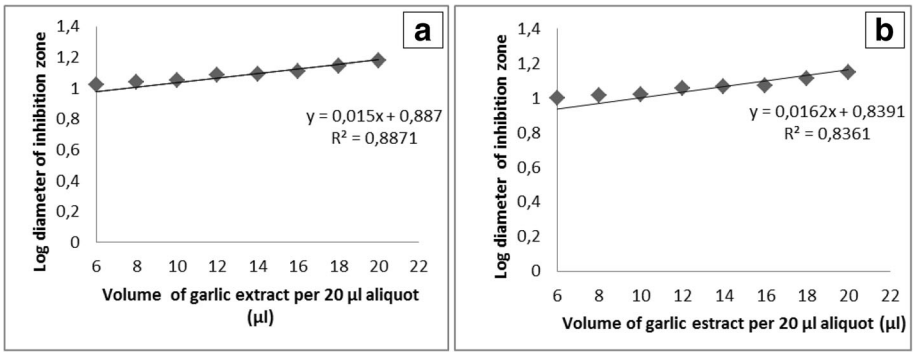

Fig. 3 Regression plot of the log of the inhibition zone diameter on DAPP-PG115 (**a**) and BAT13 (**b**) against the volume of garlic extract diluted to 20 µl

115, respectively. However, copper sulfate generated a reduction of this parameter of 45.83 and 46.38% for BAT13 and DAPP-PG115, respectively. These results revealed that garlic extract was the most effective treatment for the control of the pathogen. Garlic extract reduced the average length of stem necrosis of 60.55 and 70.83% for BAT13 and DAPP-PG115 isolates, respectively (Figs. 5 and 6, Table 4).

The biological control of *P. syringae* pv. *syringae* the causative agent of citrus blast and black pit disease under in vitro and in vivo conditions has not been studied. In vivo experiments showed that the antagonistic bacteria (MBCL3, FCL2, and MBCL2) reduced the length of stem necrosis.

Shafi et al. (2017) mentioned that the use and number of antagonistically important *Bacillus* species is increasing very rapidly. *Bacillus* species have a unique ability to replicate rapidly, resistant to adverse environmental conditions as well as they have a broad spectrum of biocontrol ability. *Bacillus* spp. play a direct role in resistance to phytopathogenic organisms through the production of extracellular antimicrobial antibiotics, toxins, hydrolases and lipopeptides (Bardin et al. 2015).

Table 3 Antibacterial activities of garlic extract, *Bacillus* spp. and copper sulfate against BAT13 and DAPP-PG115

Treatments	Phytopathogenic bacteria	
	BAT13	DAPP-PG115
	Agar well diffusion	
BKT1	13.33 ± 0.05 a	13.5 ± 0.08 a
MBCL3	14 ± 0.05 a	14.33 ± 0.12 a
FCL2	15.5 ± 0.12 b	15.83 ± 0.11 b
MBCL2	17 ± 0.1 c	18 ± 0.09 c
Garlic extract	24.66 ± 0.13 e	32.83 ± 0.15 e
GT1	13.83 ± 0.11 a	14.16 ± 0.09 a
Copper sulfate 1%	20.66 ± 0.1 d	23 ± 0.08 d

Small letters are for comparison of means in the same column
Means followed by the same letter do not differ significantly ($P = 0.05$)

It was proved that many antibiotics produced by *Bacillus subtilis* were wide spectra (Vanneste 2000) such as glycopeptide that has a role in plant growth stimulation. It has been reported that some *B. subtilis* strains can effectively suppress the *Ralstonia* wilt disease in several plant hosts (Aliye et al. 2008 and Ji et al. 2008).

Furthermore, the commercial biofungicide, Serenade, which contains *B. subtilis* as an active compound, is reported to be effective against many pathogenic bacteria, including *Erwinia*, *Pseudomonas*, and *Xanthomonas* strains. The mechanism of this antibacterial effect is uncertain, although it is known that *B. subtilis* can produce a variety of antibacterial agents, including a broad spectrum of lipopeptides, such as surfactin, that are potent biosurfactants (Peypoux et al. 1999).

The antimicrobial effect of the natural substances of *A. sativum* extract was also confirmed by in vivo tests on plants inoculated with suspension of *P. syringae* strains. *A. sativum* extract seemed to be the most effective treatment, when compared to the untreated control and the copper sulfate. In addition, Hassan and El-Meneisy (2014) demonstrated that garlic extract reduced the severity of the disease of bacterial Halo blight of bean caused by *P. syringae* pv. *phaseolicola*.

In the same sense, Balestra et al. (2009) reported that *A. sativum* extract reduced the disease incidence and disease severity caused by *P. syringae* pv. *tomato*, *Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis*.

It was reported that in vivo tests of aqueous extracts of *A. sativum* and *Ficus carica* fruits showed a reduction of the survival and the damages caused by *P. syringae* pv. *syringae*, and *Pseudomonas viridiflava* bacterial pathogens of kiwifruit (Balestra et al. 2008).

In addition, to any directly antimicrobial effects of allicin on pathogens *in planta*, it is conceivable that garlic extract might contain substances which are able to induce systemic acquired resistance (SAR) in the host. Thus, when plants are sprayed with garlic extract or

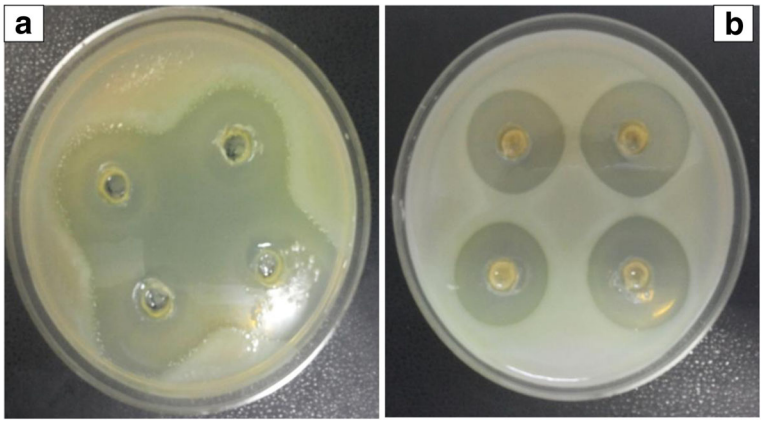

Fig. 4 Inhibition zones induced by garlic extract against *Pseudomonas syringae* pv. *syringae* strains DAPP-PG115 (**a**) and BAT13 (**b**)

Fig. 5 Blast development after 10 weeks of inoculation with *Pseudomonas syrinage* pv. *syringae* strain BAT13: **a** control, **b** plant treated with 0.5% CuSO4, **c** plant treated with FCL2, **d** plant treated with MBCL3, **e** Plant treated with MBCL2, **f** plant treated with garlic extract (approximately 134 μg ml^{-1} of allicin in 10 μl)

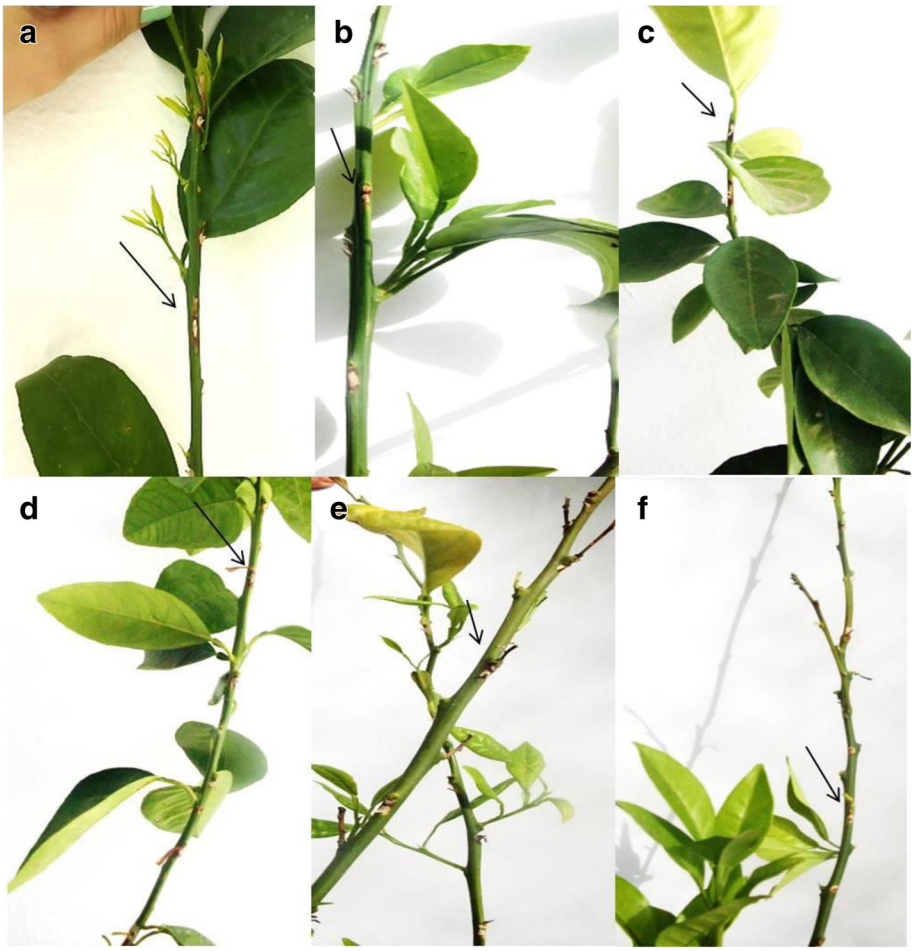

Fig. 6 Blast development after 10 weeks of inoculation with *Pseudomonas syrinage* pv. *syringae* strain DAPP-PG115: **a** control, **b** plant treated with 0.5% CuSO4, **c** plant treated with FCL2, **d** plant treated with MBCL3, **e** Plant treated with MBCL2, **f** plant treated with garlic extract (approximately 134 μg ml^{-1} of allicin in 10 μl)

Table 4 Effect of different treatments on the length of stem necrosis

Treatments	Phytopathogenic bacteria			
	BAT13		DAPP-PG115	
	Stem necrosis length (mm)	% of reduction	Stem necrosis length (mm)	% of reduction
Garlic extract	4.5 e	60.55 e	2.6 e	70.83 e
MBCL3	5.2 d	53.88 d	3.5 d	61.66 d
FCL2	5.5 c	50.13 c	4.5 c	55 c
MBCL2	5 d	56.11 d	3.5 d	62.5 d
Copper sulfate	6 b	45.83 b	5.1 b	46.52 b
control	11 a	0 a	9.7a	0 a

Small letters are for comparison of means in the same column
Means followed by the same letter do not differ significantly (P = 0.05)

treated with allicin via the vapor phase before inoculation with a pathogen, SAR in the host might be contributing to any observed reduction in disease. The SAR state is accompanied by the accumulation of molecular markers such as mRNA for pathogenesis-related proteins and salicylic acid (Uknes et al. 1992).

The present study is the first to show the antimicrobial effect of garlic extract and antagonistic bacteria *Bacillus* spp. against *P. syringae* pv. *syringae* affecting citrus. According to this study, plant extract and antagonistic bacteria (*Bacillus* spp.) may be good alternatives of the used fungicides to control such diseases.

Conclusions

Garlic, *A. sativum*, extract and three strains of *Bacillus* spp. were found to have inhibitor effect against the growth of *P. syringae* pv. *syringae* under in vitro as well as disease development under greenhouse conditions. Referring to the obtained results, it could conclude that the antimicrobial activity of applied treatments showed effectiveness, gave interesting opportunities to substitute the copper compounds treatments, that usually been used in organic agriculture. It is of interest to use such treatments to control *Pseudomonas syringae* pv. *syringae* causative agent of citrus blast and black pit disease.

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Availability of data and materials

All data are available at the article and the materials used in this work are of high quality and grade.

Authors' contributions

Both authors contributed equally to this work. Both authors read and approved the final manuscript.

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