ORIGINAL ARTICLE

Biocontrol of olive knot disease by *Bacillus subtilis* isolated from olive leaves

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Abstract We evaluated the biocontrol efficacy of a *Bacillus subtilis* strain isolated from symptomless olive leaves against the olive knot disease pathogen *Pseudomonas savastanoi* pv. *savastanoi*. *Bacillus subtilis* F_1 and F-4 displayed antibacterial activity against the pathogen. In planta, only *B. subtilis* F_1 significantly reduced the weight of knots caused by *P. savastanoi* pv. *savastanoi* IVIA 1628 and Aw_9, whereas copper treatment was effective only when using strain Aw_9, thus a reduction of 50% was obtained. Preliminary characterisation of the active compound produced by *B. subtilis* F_1 showed that it was proteinaceous in nature. These results suggest that *B. subtilis* strain F_1 could be used to control, and prevent infection by, the causal agent of olive knot disease.

Keywords *Pseudomonas savastanoi* pv. *savastanoi* · *Bacillus subtilis* · Biocontrol · Olive knot disease

Introduction

Olive groves are cultivated extensively in Tunisia, with a total area of 1.7 million ha. Olive trees are constantly faced with the risk of being attacked by pathogenic bacteria and fungi, which can cause severe damage to olive groves. For example, olive knot disease, caused by *Pseudomonas savastanoi* pv. *savastanoi* (*P.s* pv. *savastanoi*), is observed in many areas such as Chbika, Bekalta, Sidi Bouzid, Ouedna and Tunis in the north of Tunisia (Krid et al. 2009). The characteristic symptoms of this disease are overgrowths occurring on young twigs, branches and stems but occasionally on the leaves and fruits as well (Lavermicocca et al. 2002; Surico 1986). This pathogen invades the host through the vascular system (Rodríguez-Moreno et al. 2009), and causes severe damage including a reduction in olive yield and quality (Schroth et al. 1968, 1973).

The control of olive knot disease is a difficult task (Lavermicocca et al. 2002) due to the pathogen's wide host range. Among the control strategies available, chemical pesticides based on copper compounds are most often employed. Continuous use of such chemicals is known to cause undesirable effects such as residual toxicity, development of resistance, environmental pollution, health hazards to humans and animals, and increased expenditure for plant protection. As an alternative, biological control of plant pathogens using antagonistic fungal and bacterial strains is gaining in importance for the management of plant diseases. *Bacillus* and *Pseudomonas* are considered as important classes of biocontrol bacteria that are used frequently against various plant diseases (Saravanakumar et al. 2007). Previously, Rokni Zadeh et al. (2008) reported that several fluorescent *Pseudomonas* have antibacterial activity in vitro against *P.s* pv. *savastanoi*. Recently, Kacem et al. (2009) found that several species of *Rhizobium* displayed antibacterial activity against *P.s* pv. *savastanoi* by producing bacteriocins.

Bacillus subtilis is common in nature, nontoxic and harmless to humans and other animals, and non-pathogenic to plants (Acea et al. 1988). Several strains of *B. subtilis* are known for their effectiveness in controlling several plant

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pathogens. This bacterium produces antimicrobial compounds in vitro, including the antibiotics zwittermicin-A and kanosamine (Leifert et al. 1995), lipopeptides (Ahimoua et al. 2000; PalBais et al. 2004; Stelle et al. 2002) and the antifungal protein bacisubin (Liu et al. 2007).

In this study, we analysed the efficacy of two strains of *B. subtilis* for their potential to control *P.s* pv. *savastanoi* in vitro and in planta. To our knowledge, this is the first report describing the use of bacteria to control olive knot disease.

Materials and methods

Bacterial strains

Two strains of *P.s* pv. *savastanoi* (strain Aw$_9$ and strain 1628) were used in this study. IVIA 1628 was supplied by the Centro de Proteccion Vegetal y Biotecnologia [Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, Moncada, Valencia, Spain]. Aw$_9$ was isolated from knots harvested from olive trees (cv. Chemlali) planted in the region of Ouedna (Southeast Tunisia). *Bacillus subtilis* strains F$_1$, B$_2$, and F-4, and *Pseudomonas fluorescens* strains kf and kf1 were isolated from symptomless olive leaves from orchards located in the region of Sfax (Tunisia) and Kairouan (Tunisia), respectively. Identification of bacterial strains was achieved by comparing 16S rRNA gene (*rr*s) sequences with the GenBank database using the Basic Alignment Search Tool (BLAST). The pathogenicity of the strains was confirmed by inoculation of bacterial suspension (10^8 CFU ml^{-1}) on stems of 1-year old olive (cv. Chemlali). Symptom development was noted for up to 2 months after inoculations.

In vitro effect of bacterial antagonists against pathogen

Double layer method

The effect of bacterial antagonists against *P.s* pv. *savastanoi* was estimated by means of the modified method of Vidaver (1976) and Stonier (1960). The isolates F$_1$, B$_2$ and F-4 were grown on solid LBA medium at 26°C for 48 h and bacterial suspensions were prepared in sterile distilled water (SDW) at 10^8 CFU ml^{-1}. Aliquots (20 µl) of the bacterial suspensions were spot-inoculated onto solid LBA medium, and incubated at 25°C for 2 days. At the same day of the spot inoculation, the two *P.s* pv. *savastanoi* strains to be tested (IVIA 1628 and Aw$_9$) were streaked over solid King's medium B. After 2 days of incubation, the antagonistic bacteria were exposed to chloroform vapour for 30 min and the plates were left open for 15 min in a flow cabinet. Bacterial suspension (1 ml) of *P.s* pv. *savastanoi* (10^8 CFU ml^{-1}) was mixed with 3 ml LBA

medium (0.6% of agar) at 45°C. This solution was overlaid quickly on plates containing the antagonists. Plates were incubated at 25°C and checked after 24–48 h for the appearance of inhibition haloes surrounding the antagonist spots. The experiment was carried out in a completely randomized design with three replicates, and was repeated three times.

Agar well diffusion method

The ability of the antagonist to produce diffusible metabolites was tested using the agar well diffusion assay (AWDA) as reported by Tagg and McGiven (1971). All bacterial isolates were transferred individually to 50 ml 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 (25–26°C). LBA medium (20 ml) was poured into each sterile Petri dish (90 mm diameter). Bacterial suspension (1 ml; 10^8 CFU ml^{-1}) of the two *P.s* pv. *savastanoi* strains to be tested (IVIA 1628 and Aw$_9$) was mixed with 3 ml LBA medium (0.6% agar) at 45°C. This solution was then quickly overlaid to plates containing LBA medium, and wells of 6 mm diameter were punched in the agar with a sterile steel borer. The antagonist cultures were centrifuged at 12,000 rpm for 30 min to remove cell debris. After centrifugation, 100 µl of each sample was filtered through 0.45 µm filters under sterile conditions and used to fill three prepared wells. Copper sulfate solution (100 µl; 1 g l^{-1}) was added to the fourth well and used as a positive control. The plates were then incubated at 25°C and subsequently examined for halos of inhibition around the wells. The inhibition zones were measured. Three replicates were used for each bacterial isolate, and three separate tests were performed.

Effect of antagonistic bacteria on the reduction of olive knot disease

In vivo assays were performed to determine the effect of antagonistic bacteria on the development of knots. A 10-µl aliquot of bacterial suspension containing 10^8 CFU ml^{-1} strain IVIA 1628 and Aw$_9$ was inoculated on V-shaped slits (2 mm deep × 3 mm wide) of 2-year-old olive (cv. Chemlali). The slits were protected by Parafilm M. Three days after inoculation, 10 µl SDW (control), or a suspension of antagonistic bacteria or copper sulphate (0.5%) was added to the wounds, which were then covered again with Parafilm M.

The experiment was carried out in duplicate on two different plants, and was performed three times. For each treatment, three inoculations were performed on the main stem of six plants. The inoculated trees were kept in a greenhouse at 25°C. After 2 months, overgrowths were

excised from stems and their weights compared. The percentage of inhibition was calculated by counting sites that did not develop knots with respect to the total sites treated.

Preliminary characterisation of antibacterial compounds

Preliminary characterisation of the antibacterial compounds produced by the effective strain F_1 was achieved using supernatant samples collected from 72 h cultures according to Rhouma et al. (2008).

Proteinase sensitivity

Sensitivity of the antibacterial compounds to proteolytic enzymes (Proteinase K) was determined by incubation of cell-free supernatant samples for 1 h at 37°C with proteinase K (1 mg ml^{-1}). After incubation, proteinase K was heat inactivated for 3 min at 100°C.

Heat stability

Heat stability of the antibacterial activity was determined by incubation of aliquots (500 μl) of cell-free supernatant at 40°C, 60°C, 80°C and 100°C for 30 min. The activity was also tested at 100°C with incubation times of 30 min, 60 min and 90 min.

pH sensitivity

The activity of the antibacterial compounds at different pH values was estimated after storage of the supernatant culture for 1 day at 4°C in buffer solution ranging from pH 3 to pH 9. After incubation at the indicated pH, the medium was neutralised before the assay was conducted.

Data analysis

Data were subjected to analysis of variance using SPSS software (version 11; http://www.spss.com). Means values among treatments were compared by Duncan's multiple range test at the 5% ($P=0.05$) level of significance.

Results

In vitro effect of bacterial antagonists

Diameters of the inhibition zone induced by the antagonists F_1, B_2 and F-4 against the two strains of $P.s$ pv. savastanoi (IVIA 1628 and Aw$_9$) are shown in Table 1 and Fig. 1a,b. The results revealed that the highest level of inhibition zone diameter was observed with B. subtilis strain F-4 (Table 1).

In planta experiments

Sixty days after inoculation of strains IVIA 1628 and Aw$_9$, typical knots appeared at sites inoculated with strain IVIA 1628, with weights ranging from 0.0508 to 0.7269 g, and from 0.0248 to 0.3636 g when sites were inoculated with strain Aw$_9$. Addition of B. subtilis strain F_1 resulted in a percentage of inhibition of 34%, and a reduction in the weight of knots of 70% in V-shaped slits inoculated with strain IVIA 1628. When strain F_1 was added to the wounds inoculated with Aw$_9$, formation of smaller knots was observed at all sites (Fig. 2). Analysis of variance of the average weight of knots (Duncan's test) showed significant differences between treatment with strain F_1 and the control, and a reduction of 56% was observed (Fig. 3). When strain F-4 was added to the wounds inoculated with strain IVIA 1628 or Aw$_9$, a reduction of 17% in the weight of knots was obtained. A percentage of inhibition of 23% was obtained for wounds inoculated with strain IVIA 1628. However, in the case of strain Aw$_9$, no inhibition was observed and all sites developed knots. Treatment with copper sulphate (0.5%) resulted in inhibition of 6% and 12% in the case of wounds inoculated with strain IVIA 1628 and Aw$_9$, respectively (Fig. 4) but no significant

Table 1 Inhibition zone diameter (mm) induced by some bacterial strains against *Pseudomonas savastanoi* pv. *savastanoi* strains IVIA1628 and Aw$_9$

Bacterial strain	IVIA1628		Aw$_9$	
	Double layer culture	Agar-well diffusion	Double layer culture	Agar-well diffusion
F_1	23±0.12	12±0.12	24±0.11	10±0.12
F-4	25±0.23	11±0.33	23±0.32	11±0.04
B_2	24±0.11	11±0.20	20±0.24	10±0.11
kf	11±0.15	13±0.22	12±0.12	13±0.32
kf$_1$	13±0.25	13±0.20	11±0.12	13±0.22
CuSO$_4$ (1%)	–	11.08±0.25	–	11.11±0.07

Fig. 1 Inhibition zones induced by *Bacillus subtilis* in agar well diffusion (**a**) and double layer (**b**) against *Pseudomonas savastanoi* pv. *savastanoi* strain Aw$_9$. **a** *1* 100 μl CuSO$_4$ (1%), *2–4* 100 μl supernatant of *B. subtilis* strain B$_2$, F$_1$ and F-4, respectively. **b** PTFb$_2$, PTT and CHb$_3$ (negative control): strains isolated from olive leaves
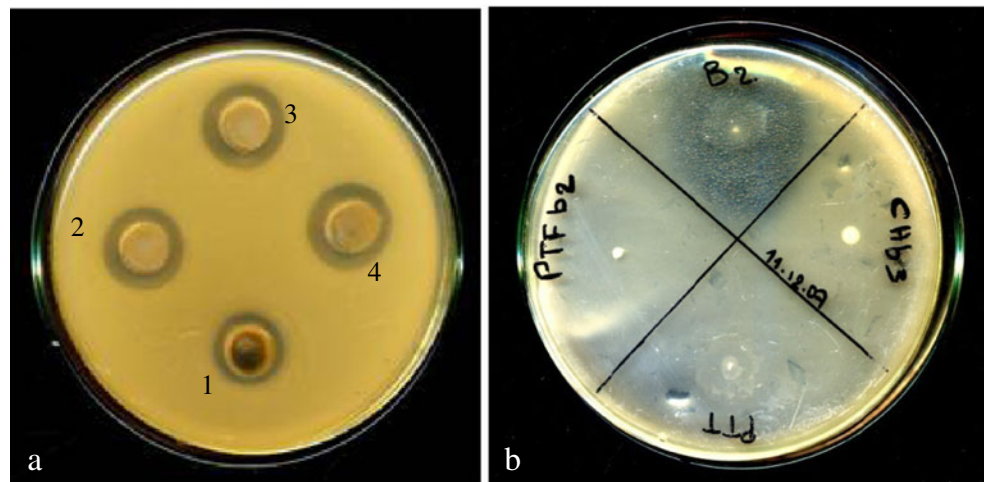
differences were found between copper treatment and the control in the case of strain IVIA 1628. However, the average weight of knots was significantly lower in copper-treated than in control plants using strain Aw$_9$, with a reduction of 50% being observed (Fig. 3).

Characterisation of antibacterial compounds

The antibacterial activity of *B. subtilis* F$_1$ was lost completely after treatment with proteinase K at 1 mg ml^{-1}, indicating the proteinaceous nature of the antibacterial compounds. The

Fig. 2 Effect of antagonistic bacteria on the inhibition of knot formation induced 60 days after inoculation with *P.s* pv *savastanoi* IVIA 1628 (**a**, **b**, **c**) or Aw$_9$ (**d**, **e**, **f**). **a**, **d** Untreated control; **b**, **e** V-shaped notches inoculated with *B. subtilis* F$_1$; **c**, **f** V-shaped notches inoculated with *B. subtilis* F-4. *Arrows* Sites of inoculation
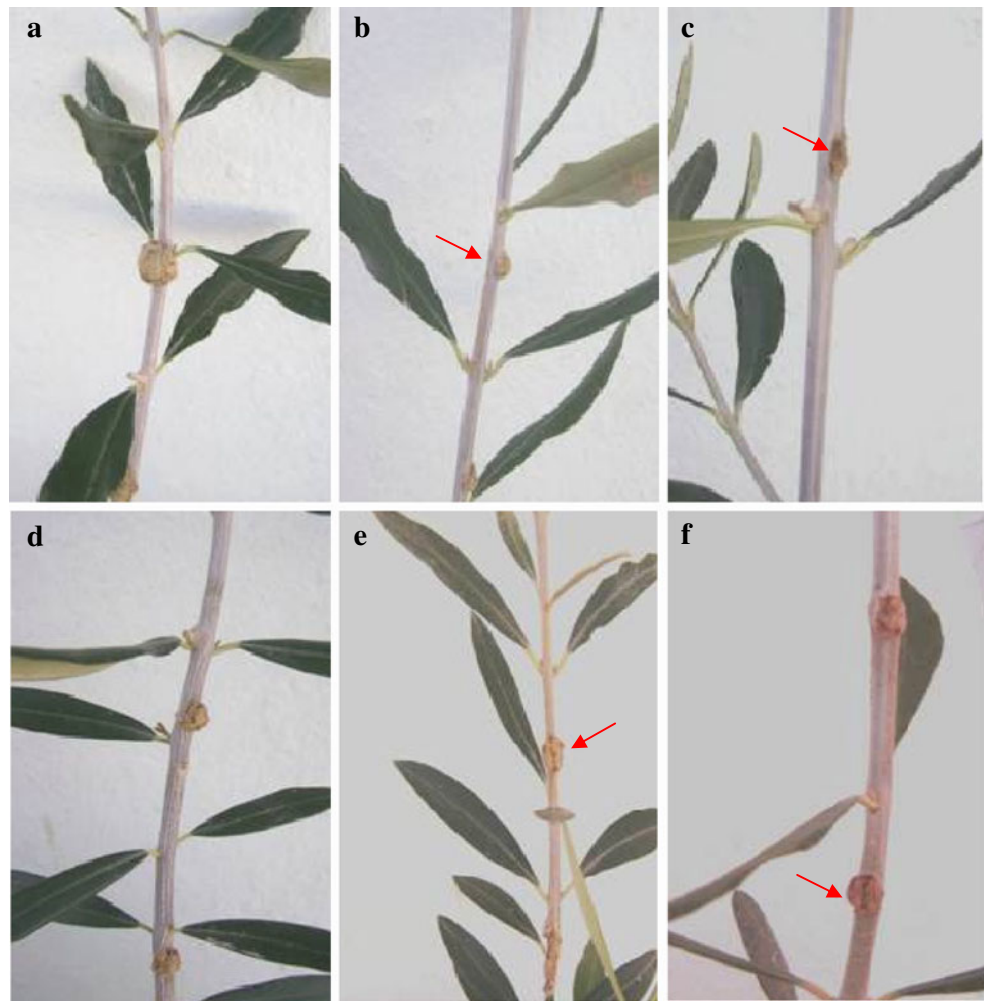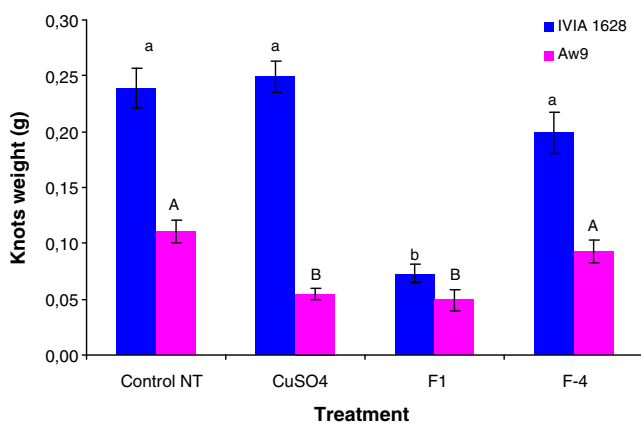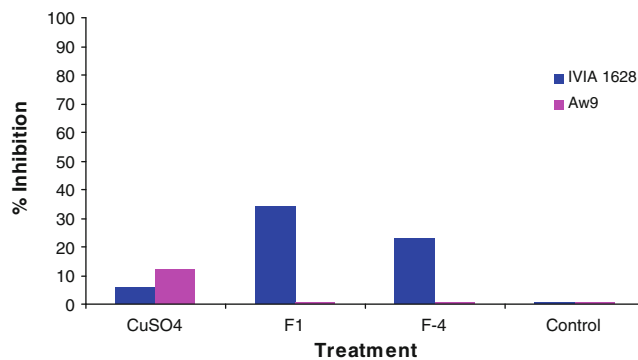
Fig. 3 Effect of *B. subtilis* strain F_1, F-4 and copper sulphate (0.5%) on knot weight after inoculation with *P.s* pv. *savastanoi* IVIA 1628 or Aw_9. Bars topped with different letters denote a significant reduction in knot weight according to Duncan's multiple range test at $P<0.05$

Fig. 4 Percentage of knot inhibition by *B. subtilis* strain F_1, F-4 and copper sulphate (0.5%) after inoculation with *P.s* pv. *savastanoi* IVIA 1628 or Aw_9

inhibitory activity of the antibacterial compounds was unaffected by incubating the free-cell supernatant at 40, 60 and 80°C for 30 min. The activity of the free cell supernatant was lost at 100°C. The pH stability of the antibacterial compounds showed an optimum of activity against *P. s* pv. *savastanoi* at neutral pH (data not shown).

Discussion

The biological control of olive knot disease has not been well studied. Therefore, the present study tested some strains isolated from symptomless olive leaves known for their efficacy to control some diseases caused by fungi and bacteria for their effectiveness to control olive knot disease. These epiphytic strains are present frequently and naturally on symptomless olive leave (Ercolani 1978, 1991).

In vitro experiments showed that *B. subtilis* strains F_1 and F-4, were efficient against *P.s* pv. *savastanoi*, with this effectiveness being expressed by a large inhibition zones obtained in the double layer method. Several mechanisms of action were determined in previous studies, including the production of metabolites with antimicrobial activity (antibiosis), and induction of systemic resistance (Shoda 2000; Whipps 2001). In our study, the zone of inhibition seen in agar well diffusion plates indicated the presence of biologically active metabolite(s) produced by the antagonist, which diffused in the agar medium, after induction by chloroform (Fig. 1a). In planta, the release of antibacterial compounds depends on several factors such as environmental conditions, temperature, pH, and the presence of other microorganisms that can interact with the antagonist. Metabolites excreted by the phytopathogen and the plant can inhibit the biocontrol agent (Someya 2008). Preliminary characterisation of the antimicrobial

compound produced by strain F_1 showed that it is proteinaceous in nature and relatively heat resistant. Heat stability is important if the antibacterial compound is to be used as a biocontrol agent under environmental conditions, with temperatures reaching 40°C in summer.

The results of pot experiments showed that only *B. subtilis* strain F_1 was effective in reducing the percentage of inhibition and knot weight significantly. *Bacillus subtilis* proved to be effective in controlling a wide range of diseases caused by fungi and bacteria, such as leaf spot caused by *Alternaria brassicae* (Sharma and Sharma 2006), Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Zhang and Xue 2010) and crown gall caused by *Agrobacterium tumefaciens* (Rhouma et al. 2008). *Bacillus subtilis* is considered to be a safe biological agent (Merriman et al. 1974; Baker et al. 1985; Turner and Backman 1991). Currently, a commercial fungicide Serenade (*B. subtilis* QST713) is now available to control some diseases, such as mummy berry disease caused by *Monilinia vaccinii-corymbosi* (Scherm et al. 2004), Sclerotonia stem rot caused by *Sclerotinia sclerotiorum*) and fire blight caused by *Erwinia amylovora*.

Bacillus subtilis strain F-4 proved to be effective in vitro but did not show a high percentage of inhibition. This lack of correlation between in vitro results and biocontrol in vivo has been documented in other bacteria like *P. fluorescens* (Ran et al. 2005; Rajkumar et al. 2005). The lack of effectiveness of *B. subtilis* F-4 applied to the plants could probably be caused by production of the antibacterial compound at a level insufficient to suppress the pathogen. The inability of this strain to colonize protected sites and to compete with the pathogen (Wilson et al. 1999) could also explain the inefficacy of strain F-4. Extraction of these antibacterial compounds is recommended in order to overcome the difficulties that can occur in the application of microorganisms as biocontrol agents and to formulate products for biological control (Lavermicocca et al. 2002).

This work provides novel information for the study of the biological control of olive knot disease. Thus, the biocontrol agent *B. subtilis* F$_1$ can be well exploited in future, and active principles for the effective management of olive knot disease can also be isolated and formulated.

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