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Algerian isolates of fluorescent *Pseudomonas* spp. as potential biological control against wilt pathogen (*Verticillium dahliae*)

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Abstract

This study was carried out to identify indigenous fluorescent pseudomonads strains, with potential as biocontrol agents against the soil-borne fungal pathogen *Verticillium dahliae* Kleb. A collection of thirty nine isolates from the rhizosphere of healthy tomato plants growing near Bouira (Algeria) were characterised as fluorescent *Pseudomonas* spp. by phenotypical methods. In dual culture, most of pseudomonads strains (95%) showed *in vitro* antagonistic activity against two isolates of *V. dahliae* (R1 and R2). The most active bacterial strains named T23, T27, T33, T34 and T49 were morphologically, biochemically and molecularly identified by partial 16r RNA gene sequencing as *Pseudomonas* spp. The antagonistic activity of those isolates against R1 and R2 of *V. dahlaie* races varied with culture media. On King's B medium, the isolates were much inhibitory to pathogen than in PDA, produced total inhibition (100%) of mycelium growth and abolished microsclerotia formation. These selected strains showed significant control of verticillium wilt of tomato under greenhouse conditions, by delaying the apparition of the first symptoms. The final disease incidence and severity were reduced by 20-80% compared to the untreated controls. Moreover, growth of bacterized tomato plants improved as revealed by increased height, stem and root fresh weight and dry weight in contrast to the untreated control. These strains were further characterized for antibiotic production and results suggested that phenazines (PCA, PCN) could be involved in their biocontrol effectiveness. Here, the autochthonous beneficial of *Pseudomonas* spp. strains from tomato rhizosphere have been identified. They are excellent candidates to control *V. dahliae* affecting diverse economically-relevant crops in Algerian agriculture.

Keywords: Antagonism, bacterization, microsclerotia, phenazine, rhizosphere, Tomato.
Abbreviations: DAPG_2,4 diacetylphloroglucinol; HPR_2-hexyl 5-propylresocinol; msc_Microsclerotia; PCA_phenazine-1-carboxylic acid; PCN_phenazine-1-carboxamide; PLT_pyoluteorin; PRN_pyrrolnitrin; SWD_sterile distilled water.

Introduction

Tomato (*Solanum lycopersicum* Mill) is the most consumed vegetable in the world after potato. The world production amounted in 2012 to 161.79 million tons for an area of 4,803,680 hectares, with an average yield of 30 tons per hectare (FAO-STAT, 2013). In Algeria tomato is one of the most important vegetables and 21,320 hectares are devoted to its cultivation with an average yield of 649,999 tons (FAO, 2013). *Verticillium* dahlaie is the second most important soil-borne phytopathogenic fungus causing vascular wilt diseases in tomato plants next to *Fusarium oxysporum* f. sp. *lycopersici* which are difficult to control worldwide. *Verticillium dahlaie* is widely distributed in agricultural soils and affects a wide range of economically relevant crops (Pegg and Brady 2002). The pathogen persists in infected plant tissues and in the soil during prolonged periods of time as resistant dormant structures, known as microsclerotia. Moreover, the characteristic, biological, genetic and epidemiological factors of the fungus are contributed to find an effective control. Several approaches have been attempted to manage *Verticillium* wilt in tomato plants including chemical treatments (Grinstein et al., 1981), soil solarization (Minuto et al., 2006) and modification of cultural practices (Pegg and Brady, 2002; Uppal et al., 2007). However, little success has so far been achieved in controlling *Verticillium* wilt disease. For instance, fungicide application was proved ineffective as *V. dahliae* over winters in soil by producing microsclerotia (Agrios, 1997). Fumigation with broad-spectrum chemicals provides excellent control in some locations, but it is cumbersome and very expensive. Furthermore, this pathogen can easily re-infest fumigated soil and rapidly build up the population (Tjamos et al., 2010). In addition, there is an increasing public concern about the use of chemically-based soil fumigants as pathogen eradication measure (Bubici and Cirulli, 2008).

Several bacterial species have been found to act as antagonists of *Verticillium* spp. For instance, after screening more than 5,000 oilseed-rape rhizosphere bacteria for antifungal activity against *V. dahliae* var. *longisporum*, Berg and Ballin (1994) and (Berg et al., 2006) found that the majority of antagonists were Pseudomonads (*P. aureofaciens*, *P. chlororaphis*, *P. fluorescens* and *P. putida*). Fluorescent *Pseudomonas* have been demonstrated with biocontrol activity against soil-borne pathogens such as *Pythium* spp (Walther and Gindort, 1988; Howell and Stipanovic, 1980), *Fusarium* spp (Kleopper et al., 1991; Vanpeer et al., 1990; Lemanceau-Alabouvette, 1993, Mezaache et al., 2012), *Verticillium* spp (Leben *et al*., 1987; Nejad and Johnson, 2000; Alstrom, 2001; Uppal et al., .2008; Jabnoun et al., 2009) and *Phytophthora* spp (Gonzalez-Sanchez et al., 2009).

These bacteria were reported to produce several extracellular metabolites with antifungal activity, including cyanid, fluorescent siderophores and antibiotics. Fluorescent *Pseudomonas* with biocontrol potential produce a wide variety of antibiotics, such as phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn) and/or pyoluteorin (Plt). Several phenazine producing *Pseudomonas* spp. strains are known for their biocontrol potential against soil-borne pathogens, *Pseudomonas fluorescens* 2-79, *P. chlororaphis* PCL1391 and *P. chlororaphis* 30-84 being some important representatives (Chin-A-Woeng et al., 1998; Thomashow and Weller, 1988; Thomashow et al., 1990). More recently, it has been recognized that apart from their action as antibiotics, phenazines could also play a role in the physiology of the excreting bacteria and may contribute to the ecological competence of biocontrol pseudomonads in a number of cultivated plant species (Pierson and Pierson, 2010; Benchaabane et al., 2013).

Biological control, mediated by fluorescent *Pseudomonas* spp. strains, can be exerted by a number of other mechanisms, which are not mutually exclusive (i.e. competition for [micro] nutrients and/or space, production of antibiotics, induction of local and systemic defence responses, etc.) (O'Sullivan and O'Gara, 1992; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Weller, 2007; Höfte and Altier, 2010). In addition to their direct physical competition with the pathogen in the rhizosphere, some fluorescent *Pseudomonas* strains are able to activate defence mechanisms in the host plant; thereby, conferring protection against pathogens attack (Kleopper et al., 1991). The use of biological control agents (BCA) to manage *V. dahliae* has been investigated for different susceptible crops (Pegg and Brady, 2002; Cavalcanti et al., 2008; Berg et al., 2001), including the use of beneficial *Pseudomonas* spp. (Mercado-Blanco et al., 2004; Sanaei and Razavi., 2011; Maldonado-González et al., 2015). So far, there is no report on the use of BCA to control Verticillium wilts in Algeria. The objectives of this study were: (i) isolation, identification and characterization of beneficial indigenous Algerian fluorescent *Pseudomonas* spp. strains originated from the tomato rhizosphere; (ii) analysis of their *in vitro* antagonistic activity against representative isolates of *V. dahliae*; (iii) identification of active metabolites (i.e. antibiotics) which may be involved in antagonism against *V. dahliae*; and (iv) assessment of the biocontrol potential of selected strains against *V. dahliae* in tomato plants under greenhouse conditions.

Results

Isolation and characterization of fluorescent pseudomonas spp. strains

Thirty nine bacterial isolates (named as strains T22 to T60) (Table 1) yielding distinct representative features of fluorescent pseudomonads were selected. They showed positive oxidase and arginine dihydrolase activities, negative Gram staining and HR on tobacco leaves. According to their response toward different biochemical and physiological tests, isolates were classified into eleven sub-groups according to the dichotomous keys proposed by Bossis et al. 2000 (Table1 and 2).

In vitro antagonism against V. dahliae

Results showed that 95% of *Pseudomonas* spp. strains inhibited mycelium growth of *V. dahliae* isolates *in vitro* although differences were observed. Inhibition varied, depending on the bacterial strain the fungal isolate and the culturing media (KB or PDA). Thus, the isolates showing the highest pathogen growth inhibition were T23, T27, T33, T34, T49 as well as the reference strain CHAO (Table 3). For instance, inhibition exerted by these strains ranged from 36.9% to 50% on PDA. All strains except T33 showed complete inhibition of both *V. dahliae* isolates on KB medium after 72 hrs of incubation at 27°C. However, strain T33 only caused 70% inhibition against V1 isolate. Results of this experiment were calculated as % inhibition of the radial fungal growth (Table 3).

Inhibition of microsclerotia germination

The germination of *V. dahliae* microsclerotia, exposed for 30 min to the whole liquid cultures of antagonists tested strains, was completely suppressed by strain T34 compared to the control treatment (SDW). Microsclerotia germination in 2% agar was verified by formation of secondary microsclerotia on control plates. In fact, after 10-30 days of incubation at 20 °C, *V. dahliae* microsclerotia confronted to *Pseudomonas* spp. lost their germination capacity and consequently their viability. Strains T49 and the reference strains CHAO had a moderated effect, inhibiting microsclerotia germination by 30 and 35%, respectively. On the contrary, treatment with isolates T23, T27 and T33 had no significant effect on microsclerotia germination inhibition (Fig. 1).

Sequencing of rDNA

The phylogenetic position of strains T23, T27, T33, T34 and T49 (Fig. 2) were recorded in the EMBL/EBI data bank under the accession number; LN849713, LN849714, LN849715, HG810923, LN849712. The sequences are comparable to 16S rRNA of other fluorescents *Pseudomonas* species.

The NJ-based phylogenetic analysis showed that strain T34 (814 bp) had 100% sequence identity with *Pseudomonas putida* strains (i.e. *P. putida* strain BAB-535 and *P. putida* strain ZJB-LLJ) in the databases, suggesting that this strain can potentially be identified as *Pseudomonas putida*. Also, the partial 16S rRNA sequence of strain T23 was determined (1314 bp). The sequence showing a significant similarity of 99% for possible species relatedness is found with several validly described *Pseudomonas* spp (i.e. *Pseudomonas plecoglossicida* strain NBFPALD; and *Pseudomonas monteilii* strain SB 3067). The analysis of strain T27 was determined (1330 bp) and the sequence showed a high similarity percentage 99% with *Pseudomonas fluorescens* strains (i.e. *P. fluorescens* strain 76P and *P. fluorescens* strain CIFT MFB 4895(26)). The strain T33 (1302bp) was determined according to the partial 16S rRNA sequences, which had 97% identity with published 16S rRNA *Pseudomonas* isolates (i.e. *Pseudomonas* sp. SAUBS2-2 and *Pseudomonas* sp. JUNL-13). While the determined partial 16S rRNA sequence strain T49 (1313 bp) compared to the published 16S rRNA sequences of *Pseudomonas* sp. had a 100% similarity with different species (i.e. *Pseudomonas libanensis* strain TX1B1; *Pseudomonas fluorescens strain* DmBR 2 and *Pseudomonas azotoformans* strain R3ScM3P1C23)

Table 1. Biochemical and physiological characteristics of Pseudomonads isolates.

Characteristics	Strain T23	Strain T27	Strain T33	Strain T34	Strain T49
GRAM	-	-	-	-	-
Fluorescence	+	+	+	+	+
Oxydase and Catalase	+	+	+	+	+
Arginine dehydrolase	+	+	+	+	+
Hypersensitivity Reaction	-	-	-	-	-
Gelatin	+	+	+	-	-
Levan	+	+	+	-	+
denitrification	+	+	+	-	+
Alanine	-	+	+	+	-
Trehalose	+	+	+	-	-
Xylose	-	+	+	+	-
Arabinose	-	-	-	ND	ND
Sorbitol	-	ND	-	ND	ND
Tartrate	+	-	+	ND	-
Tryptophane	ND	ND	ND	+	+
lipase	ND	+	ND	ND	+
Growth at 41°C	+	-	-	+	-

+ positive, - negative, ND, note determined

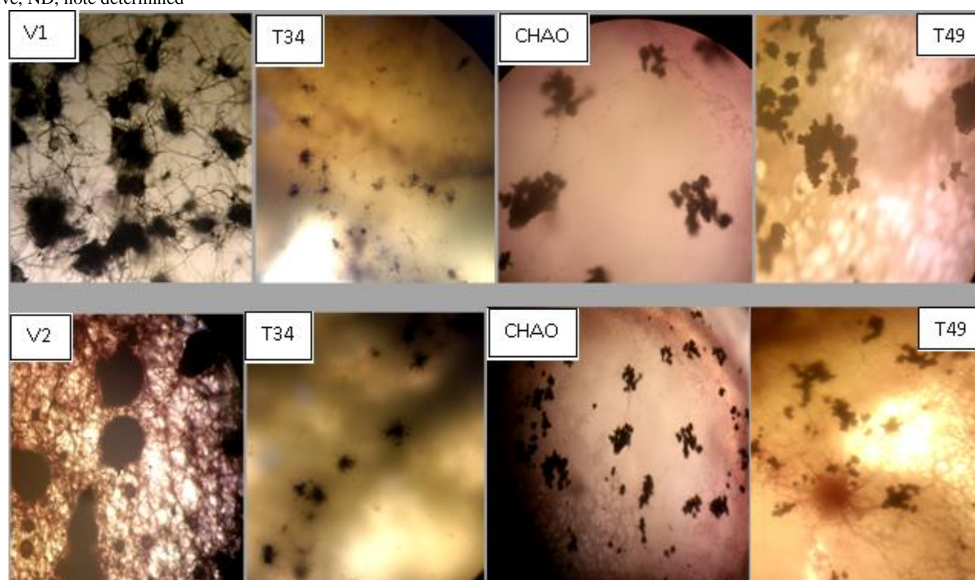

Fig 1. Germination of *V. dahliae* microsclerotia exposed to 10^6 CFU ml^{-1} bacterial suspension after 10 days of incubation on agar 2% at 20°C, observed under microscope (X 0.4). V1 and V2, control plates; T34, CHAO, T49 refer to the treatment with of corresponding *Pseudomonas* strains.

The following analyses for further species identification are suggested by the complete 16S rDNA sequence analysis and DNA: DNA hybridizations.

Analysis of antibiotic metabolites

Results from TLC assay showed that supernatant extracts from strain T34 grown on NBY contained eight spots, three of them recognized as active moieties: namely T1, T2 and T3. Moiety T1 (RF 0.23, yellow color, black under UV light [365 nm]) was active against *V. dahliae*, inhibiting mycelium growth (Fig. 3). Moiety T2 (RF 0.5, orange color) displayed the highest inhibitory effect against microsclerotia formation (Fig. 3). Finally, Moiety T3 (RF 0.06, brown color, fluorescencing under UV light [360 nm]) showed the lowest activity compared to that of T1 and T2 (Fig. 3).

The HPLC spectrum of T2 moiety was identical to standard phenazine-1-carboxylate (PCA), demonstrating that strain T34 produces phenazine and that this antibiotic plays an important role hampering *V. dahliae* microsclerotia formation (Fig. 3). Assessment of antibiotics production (TLC assays) by selected strains T23, T27, T33, T49 and reference strain CHAO revealed that strains CHAO and T49 produced DAPG (Rf=0.74) and that strains CHAO and T27 produced PNR (Rf=0.81) and PLT (Rf=0.68). Finally, strain T23 and T33 were negative for these antibiotics under experimental conditions we used (Table 4).

Pseudomonas spp. strains-mediated biocontrol of Verticillium wilt of tomato

Verticillium wilt symptoms in only pathogen-inoculated and pre-bacterized tomato plants became visible between 20 to 32 days after inoculation (IP) (Table 4). Stunted growth, necrosis, leaf epinasty and chlorosis were clearly visible. Non-bacterized plants grown in *V. dahliae* V1 infested soil, developed first symptoms 18 days after inoculation, reaching a final DI of 85.7% (Table 4). Nevertheless, in bacterized tomato plant inoculated with V1, only indigenous isolate named T34 significantly ($P \leq 0.05$) delayed IP by 14 days and reduced the final DI by 43%, the final DII 53%, and the SAUDPC by 49% compared with the control. The reference

Table 2. Phenotypical and biochemical identification of the *Pseudomonas* spp. strains isolated in this study

Phenotypic Clusters	Tomato isolates		
	Designation of isolates	number of isolates	Affiliation
1	T25, T60, T30, T31, T29	5	*P. fluorescens* b.v III
	T39	1	*P.fluorescens* b.v I
	T27,T33, T35, T41,	5	intermidiate
2	T43		*P. chlororaphis*
3	T56	1	*P.fluorescens* b.v II
4	T44, T47	2	*P.fluorescens* b.v IV
5	T38, T42, T45, T54	4	*P.fluorescens* b.v I
6	T34	1	*P. putida* bv. B
7	T26, T37	2	*P.fluorescens* b.v IV
8	T23, T24, T36	3	*P. chlororaphis*
	T46	1	*P. fluorescens* bv. II
9	T22, T52, T53, T55	4	*P. aureofaciens*
10	T28, T32, T48, T50, T57	5	*P. fluorescens* bv.III
11	T59	1	*P. fluorescens* bv.V
	T49	1	*P. putida* bv. A

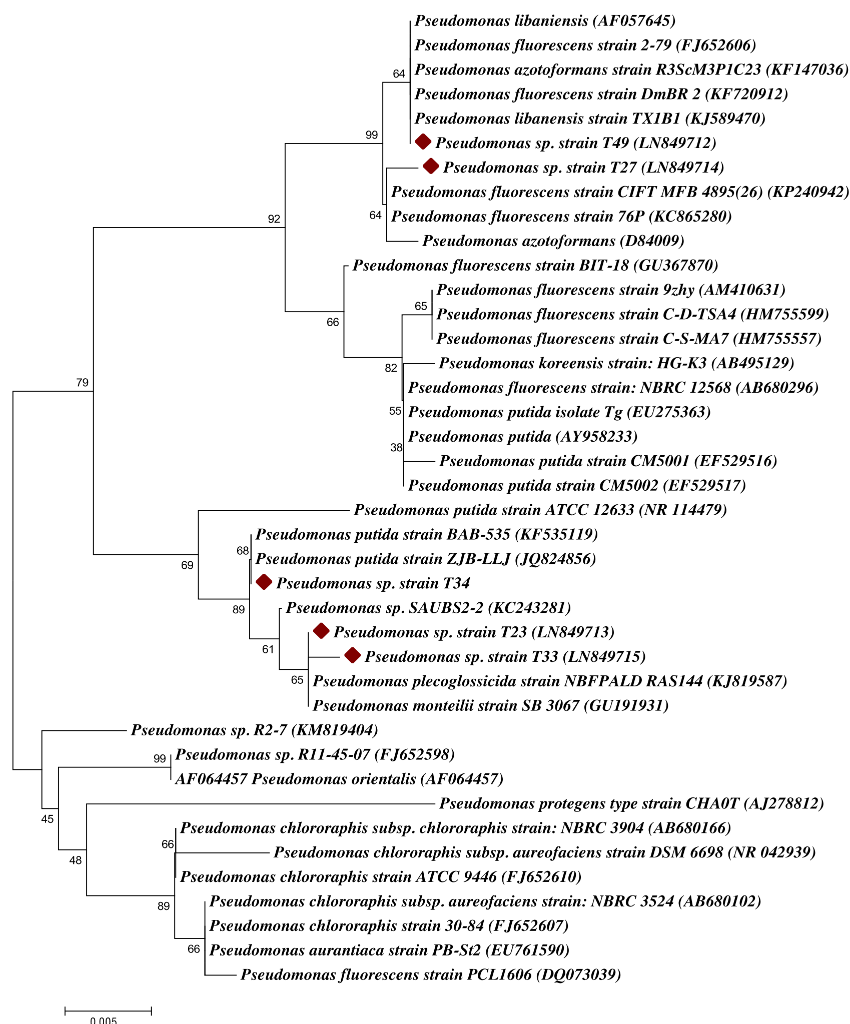

Fig 2. Phylogenetic dendrogram of fluorescent *Pseudomonas* spp. based on 16S rRNA gene sequence data, indicating the position of the strains T23, T27, T33, T34, T49 (labelled by red colour diamond). The tree was constructed using the neighbor-joining method. Sequences from *Pseudomonas* spp. strains were obtained from Genebank.

Table 3. Mycelium growth inhibition of *Verticillium dahliae* isolates on KBA and PDA by Pseudomonas spp. strains isolated from rhizospheric soil.

Wilt pathogen Verticillium	Inhibition %			
Bacterial isolates	Isolate V1 (R2)		Isolate V2 (R1)	
	PDA	KB	PDA	KB
T27	48.90±0.10[b]	100[a]	44.40±0.40[b]	100[a]
T23	47.73±0.21[c]	100[a]	46.40±0.36[a]	100[a]
T33	46.23±0.25[c]	72.33±2.52[b]	44.20±0.20[b]	100[a]
T34	54.33±0.31[a]	100[a]	47.80±0.92[a]	100[a]
T49	51.23±0.25[b]	100[a]	45.03±0.06[b]	100[a]
CHAO	52.23±0.35[b]	100[a]	47.50±0.10[a]	100[a]

Data are mean of four replicas for each *Pseudomonas* spp. strain–*V. dahliae* isolate-culturing medium combination. The experiment was repeated twice. Percentage (%) inhibition values = zone of hyphal growth inhibition (halo) around the bacterial colony in cm / plate diameter, 100mm X 100. Mean ± standard deviation. Values with same letter differ non-significantly (P>0.05) according to Tukey test.

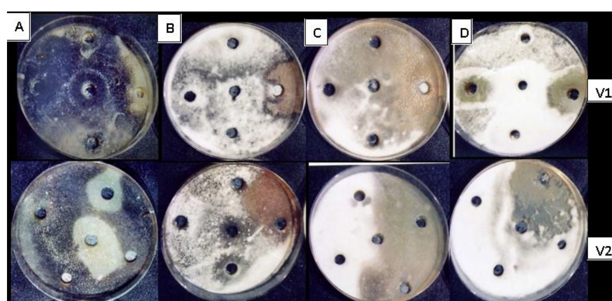

Fig 3. *In vitro* inhibition of phytopathogenic fungus *Verticillium dahlaie* by the benzene fraction of culture filtrates of isolate T34 at 28°C for 7 days. A, B, C and D: contained from left to right the equivalent 20 µl of culture filtrate corresponding to active moieties extracts.

Table 4. Production of antibiotics by studied bacterial strains.

Strains	Antibiotics				
	DAPG	PCA	PCN	PRN	PLT
T23	-	-	-	-	-
T27	-	-	-	+	+
T33	-	-	-	-	-
T34	-	+	+	-	-
T49	+	-	-	-	-
CHAO	+	-	-	+	+

DAPG 2,4,-diacetylpholoroglucinol; PCA phenazine-1-carboxylic acid; PCN phenazine-1-carboxamide; PRN pyrrolnitrin; PLT pyoluteorin; (+) presence; (-) absence of the antibiotic

Table 5. Suppressive effect of six *Pseudomonas* spp. strains of on Verticillium wilt of tomato cv. Marmande under greenhouse conditions.

Treatments	IP (Days)	SAUDPC	Final DII	Final DI %	Percentage of plant protection %
ControlV1(R2)	18	1.91	2.34	85.7	
T23/V1	28	1.06	1.33	63.14	44.10%
T27/V1	22	1.32	1.39	68.7	30.75%
T33/V1	23	1.21	1.27	77.57	36.25%
T34/V1	32	0.975*	1.11*	48.57*	48.95%
T49/V1	24	1.29	1.40	66	32.32%
CHAO/V1	30	0.985*	1.04*	60*	48.43%
ControlV2(R1)	14	2.08	2.58	100	
T23/V2	27	1.09*	1.06*	57.28*	47.66%
T27/V2	25	1.33	1.19*	74.28	35.77%
T33/V2	22	1.38	1.30*	74.42	33.49%
T34/V2	30	1.25*	1.27*	74.28	39.85%
T49/V2	21	1.09*	1.26*	55.71*	47.42%
CHAO/V2	28	1.32*	1.31*	80	36.49%

T49/V1, T34/V1, T23/V1, T33/V1, T27/V1, and CHAO/V1, are combinations of bacterial strains and the local isolate of *Verticiliium dahliae* (V1). T49/V2, T34/V2, T23/V2, T33/V2, T27/V2 and CHAO/V2 are combinations of bacterial strains and the reference isolate of *V. dahliae* (V2). For each experiment, means a column followed by an asterisk are significantly different from the mean of the control treatment according to Dunnett's test (p=0.05).

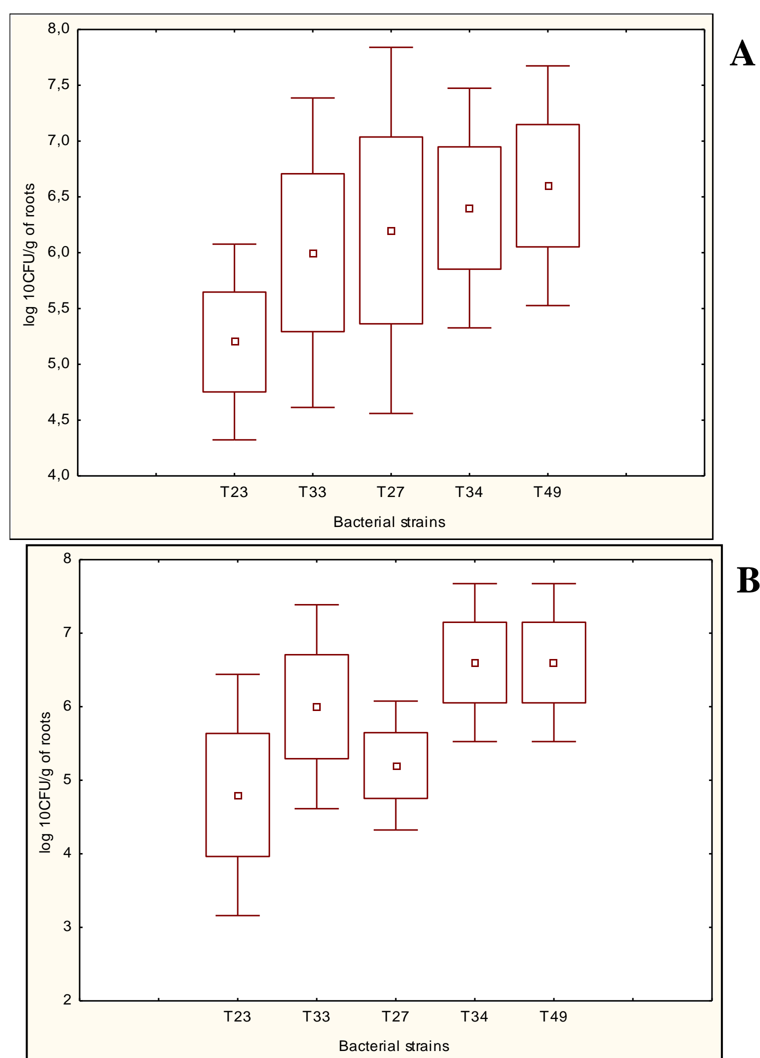

Fig 4. Bacterial counts (log CFU g^{-1}) in different root sections of tomato plants bacterized with selected strains to determine bacterial colonization (A) and persistence (B) on tomato roots.

strain CHAO also significantly reduced the final DII by 56% and SAUDPC by 48%, compared with the control, and delayed the IP by 12 days. Tomato plants inoculated with the local isolate of *V. dahliae* V1 with the other bacterial isolates named T23, T27, T33 and T49 reduced the IP by 10, 6, 5 and 8 days, respectively, but did not significantly influence the development of Verticillium wilt. In tomato plants inoculated with the reference strain of *V. dahliae* V2, the first symptoms developed 14 days after inoculation and the disease developed rapidly reaching a final DI of 100% after the third week of the experiment (Table 4). All tested *Pseudomonas* spp. isolates significantly delayed the IP by 16, 14, 13 and 11 days for strains T34, CHAO, T23 and T27, respectively. The strains T33 and T49 delayed the apparition of the first symptoms for one week compared with the control plants inoculated by *Verticilium dahliae* V2. All tested bacterial strains reduced significantly ($P \leq 0.05$) the final DII, compared to non-bacterized plants (control) by 54%-49% but reduction of the SAUDPC was not significant for treatments with T27 and T33 isolates, while for the other tested isolates named T23, T34, T49 and CHAO, SAUDPC was significantly reduced by 33-48%. The final DI was significant only for infected tomato plants treated with T49 and T33, which assured more than 45% percentage of plant protection against the reference strain of *V. dahlaie*. The *Pseudomonas* spp. strains used in this study were isolated from root tissues of the inoculated tomato plants and their ability to thoroughly colonize and persist in tomato root system was confirmed. The bacterial counts for the different strains are summarized in Fig 4. In general, the *Pseudomons* spp. tested were able to colonize tomato plant roots in both sections. After 21 days, the amount of bacteria tends to be most important on the root sections 2-6 cm from the tips rather than in the sections 1 cm from the root tips. Bacterial count level in section 2-6 cm from the root tips were arranged between 10^5-10^7CFU/g of roots while in root tip sections 1 cm, the bacterial counts were more variable among the tested strains (10^4-10^7 CFU/g of root). However, the population sizes of total pseudomonads recovered from treated roots was significantly higher than those of non-treated control plants; thus, making possible to distinguish the introduced *Pseudomonas* isolates. At the end of the experiment (56 days after inoculation with the pathogen), all bacterial treatments induced significant promotion of plant growth on tomato plants such as increased plant height, fresh shoot and root weight and dry shoot and root weight (Table 4). Therefore, the enhanced growth of *V. dahliae*-inoculated plants by the tested bacteria could just be

Table 6. Effect of six *Pseudomonas* spp. strains on tomato growth. Plant height, fresh shoot and root weights and shoot and root dry weights of tomato plants bacterized with indicated bacterial strain and inoculated with *Verticillium dahliae* V1 or V2 are shown.

Treatments	Plant height (cm)	Shoot fresh weight (g)	Root fresh weight	Shoot dry weight	Root dry weight
Control V1(R2)	6.9	2.56	2.93	0.3	0.1
T49/V1	11.5*	7.1*	4.67*	0.84*	0.2*
T34/V1	13.46**	9.73**	6.53**	1*	0.28*
T23/V1	11*	6.76*	4.86*	0.82*	0.2*
T33/V1	10*	6.30*	4.76*	0.82*	0.18
T27/V1	11.3*	6.16*	4.3*	0.76*	0.22*
CHAO/V1	10.5*	5.5*	4.03*	0.72*	0.23*
Control V2 (R1)	7.1	3.4	3.2	0.45	0.104
T49/V2	10.7*	7.16*	5.08*	0.84*	0.23*
T34/V2	14.3**	10.5**	8.06**	1.28*	0.32*
T23/V2	12.9*	9.08*	6.83*	1.1*	0.28*
T33/V2	11.7*	7.45*	4.9*	0.92*	0.24*
T27/V2	12.1*	7.42*	4.96*	0.9*	0.2*
CHAO/V2	12.7*	7.67*	5.1*	0.94*	0.25*

T49/V1, T34/V1, T23/V1, T33/V1, T27/V1, and CHAO/V1, are combinations of bacterial strains and the local isolate of *Verticillium dahliae* (V1). T49/V2, T34/V2, T23/V2, T33/V2, T27/V2 and CHAO/V2 are combinations of bacterial strains and the reference isolate of *V. dahliae* (V2). For each experiment, means a column followed by an asterisk are significantly different from the mean of the control treatment according to Dunnett's test ($P \leq 0.05$). Means followed by two asterisk are significantly different from the mean of the control according to Dunnett's test ($p \leq 0.05$), and the other treatment according to tukey test ($P \leq 0.05$)

attributed to the counteracting of deleterious effects caused by the pathogen in infected plants, rather than actual stimulation of the plant growth. Moreover, no significant differences in plant growth promotion were found among tomato plants treated with the different bacteria tested, except for the tomato plants inoculated with isolate T34, which exhibited a significant difference against control plants inoculated with V1 or V2 and the others treated plants (Table 5) significantly increased the plant height (13.46 cm) and shoot and root fresh weight (9.73 and 6.53g), respectively.

Discussions

The present investigation broadens the field of the use of indigenous isolates of fluorescent pseudomonads (BAC) and some of their secondary metabolites in order to control the phytopathogenic fungus *V. dahlaie* under laboratory and greenhouse conditions in tomato plants. The rhizosphere of healthy tomato plants located in area affected by soil borne phytopathogenic fungus *V. dahlaie* represents a feasible and useful source for isolation of fluorescent pseudomonads with promising biocontrol activity. A collection of thirty nine bacterial isolates were obtained with morphological characteristics of fluorescent pseudomonads on KB medium. The selected strains were subjected to identification by phenotypic, biochemical and physiological tests and by partial 16S rRNA gene sequencing. Our results showed a high diversity among those isolates belonging to different species, representing the fluorescent pseudomonads groups. Such diversity among fluorescent pseudomonads group was previously described and it was associated with the variety of their habitats (Mezaache et al., 2012; Benchabane et al., 2013). Thus, the associated plant is a factor in the magnitude of each species population. It is present in the plant rhizosphere; hence, the diversity within bacterial populations in the tomato rhizosphere. However, difference in such associated bacteria among selected plant cultivated in different types of soil could be also attributed to the variations in the amounts and composition of their roots exuded in relation to those soil characteristics. Siciliano and Germida (1999) demonstrated that plants apply pressure of selection upon the microorganisms colonizing their root. On the other hand, from the total number of bacterial isolates selected from soil in this work, the percentage of bacteria antagonistic against *V. dahlaie in vitro* was 12.8%, a similar value to that obtained by Cazorla et al., (2006); Gonzalez-Sanchez et al., (2009); these results pointed to antagonism as a relevant trait in the biocontrol activity of *Pseudomonas* against pathogens. All active isolates suppressed linear growth of the fungus but few suppressed microsclerotia formation. From our results, we suggest that production of siderophores may play a major role in that antagonistic activity *in vitro*. Indigenous isolates and the reference strain CHAO showed higher activity (100%) on KB medium than PDA (36.9% to 50%). Such results may support the role of siderophores production in the antagonism mechanism. On the other type of culture media, where Fe is available, fluorescent pseudomonads produce significant inhibition zones diameter tested against *V. dahliae*. From these results we can suggest, that more than one mechanism of action could be depicted in disease suppression. Several toxic metabolites were reported by *P. fluorescens* CHAO including siderophores, a volatile compound: cyanide and antibiotics (Hass et al., 1991; Defago and Hass, 1990; Ahl et al., 1986; and Mezaache et al., 2012; Mezaache et al., 2014).

Verticillium dahlaie produced melanised resting structures, msc, which can survive in soil for more than a decade. The msc are stimulated to germinate by root exudates. The infectious hyphae that emerge from the msc penetrate the roots of the host plant. Because the msc are the most important structures of the pathogen for survival and causing initial infections, msc are regarded as one of the direct targets of biological control of *Verticillium* (Tjamos, 2004). Contrary to the common assumption that melanised structures are resistant to microbial attack (Bell and Wheeler, 1986), the present study showed that *Pseudomonas* spp. can suppress the viability of *Verticillium* msc *in vitro*. Reduction in Verticillium msc germination and formation of secondary msc may result in a lower *Verticillium* infection pressure in the field and in reduced survival of *Verticillium* msc in soil (Coley-Smith and Cooke, 1971; Debode et al., 2007). This study suggests that *Pseudomonas* spp. maybe promising biological agents to control *V. dahlaie*. In the past, studies on biological control of *Verticillium* msc have typically focused on the use of the fungus *Talaromyces flavus*. This fungal antagonist kills individual *Verticillium* msc *in vitro*, because of the production of glucose oxidase that converts glucose to hydrogen peroxide (Kim et al., 1988). Bacterial antagonists, e.g. *Pseudomonas* spp., have been identified as potential biological control agents for *Verticillium* wilt (Leben et al.,

1987; Nejad and Johnson, 2000; Alstrom, 2001; Mercado-Blanco et al., 2004; Uppal et al., 2007). These previous studies have specifically targeted the suppression of the hyphal growth of *Verticillium*. However, to our knowledge only Debode et al. (2007) looked at the direct effect of these bacterial antagonists on the viability of individual msc *in vitro* by investigating the role of phenazines and biosurfactants in the *Pseudomonas*-mediated reduction of *Verticillium* msc viability. Two strains, CMR12a and CMR5c, have been selected as promising biocontrol agents in the suppression of *Verticilium longisporum* mcs. The PCA involvement was demonstrated by the fact that mutants of *P. chlororaphis* PCL1391 and *P. aeruginosa* 7NSK2 overproducing PCA (PCL1121 and 7NSK2-PhzM, respectively) were more effective in the inhibition of microsclerotia germination and formation of secondary microsclerotia, when compared to the wild type treatment. In the present study, we have demonstrated the role of the most active bacterial isolate T34 producing phenazines compound for inhibition of msc germination and the formation of secondary microsclerotia. Phenazines are a large family of heterocyclic nitrogen-containing pigments with broad spectrum antibiotic activity. Some of the best known phenazines are involved in the biological control are phenazine-1-carboxylic acid (PCA), phenazine-1-carboximide (PCN), *Pseudomonas* sp. Strain T34 had demonstrated their ability to produce those compounds involved in the biological control against *Verticillium dahliae*. It assumed that they diffused across or inserted into the membrane of plant pathogens and act as reducing agent, resulting in the generation of superoxide radicals and hydrogen peroxide (Chin-A-Woeng et al., 2003). According to the 16S RNA gene identification the most active isolates (T23, T27, T33, T34 and T39) were belonging to different species of *Pseudomons* genera, such as *P. putida*; *P. plecoglossicida; P. monteilii; P. fluorescens; Pseudomonas libanensis* and *P. azotoformans.* Hence, the following analyses for further species identification are suggested by the complete DNA sequence analysis and DNA: DNA hybridizations and the identification of other genes. The most active isolate T34, characterized by phenazine compound production can be potentially identified as *Pseudomonas putida.* However, our investigation suggests that Pseudomonas T34 possibly represent a novel PCA-PCN producing *Pseudomonas* species that groups in the *Pseudomonas putida* newly isolated phenazine-producing members of the species complexes (PP) (Mavrodi et al., 2010). It has long been recognized that phenazine synthesis occurs predominantly in strains of *P. chlororaphis*, *P. aureofaciens* (now classified as *P. chlororaphis* subsp. *aureofaciens*), and *P. aeruginos.* However, newly isolated phenazine-producing members of the *P. fluorescens* and *P. putida* species complexes (PF and PP) may belong to new species, raising questions about the actual number of Pseudomonas spp. capable of phenazine production (Mavrodi et al., 2010). Furthermore, those isolates were able to protect tomato plants from being adversely affected by *V. dahliae*. According to the results, most of the bacterial isolates showed effectiveness in controlling and reducing *Verticillium* wilt disease with more effectiveness against V2 corresponding of race 1 of *V. dahlaie*, resistance against race 1 is mediated by the presence of an immune receptor-encoding gene (*Ve1* and its homologs) (de Jonge et al., 2012). *Verticillium dahliae* race 1 is defined by the presence of a gene that encodes a virulence factor (*Ave1*). Tomato plants with the *Ve* gene limit the colonization of root tissues by the pathogen, preventing the pathogen from reaching foliar tissues (Heinz et al., 1998). They also enhanced plant growth. This may be a case in which it was clarified that there are specific mechanisms by which the rhizospheric bacteria promoted plant growth and protected the host from diseased caused by *V. dahliae* at the same time. However, it was found that the most active isolates produced siderophores and other metabolites, albeit that siderophores may well be a possible one of the most active mechanisms, by which bacteria suppress plant pathogens. Disease suppression may also be due to induction of systemic resistance in host-plant since siderophores production is an important elicitor of ISR (Ongena et al., 2002). Also, phenazines can play a major role in plant protection against pathogens. In soil, it can promote microbial mineral reduction and may function as electron shuttles, facilitating bacterial and plant access to iron and nutrients such as phosphate, trace minerals and organic compounds associated with mineral phases (Hernandez et al., 2004). Several studies have demonstrated that phenazines are beneficial for the competitiveness and long-term survival of the producers in naturel habitats. Strains of *Pseudomonas* that synthesized phenazines were more competitive and survived longer on the roots of wheat than non phenazine producing mutants (Mazzola et al., 1992). In this sense, a modulation of the production of antifungal compounds in the rhizosphere environment by bacteria and interactions with lands has been described (Oraghi Ardebili, 2011), which explain the failure of antibiotic-producing strains to protect plants when they were applied to the rhizosphere.

There is evidence correlating the efficacy of biocontrol micro-organisms against soil-borne pathogens with their ability to colonize the root system of the plant to be protected, more specifically when the mode of action used by these bacterial strains is antibiosis (Chin-A-Woeng et al., 2003) or competition for nutrients (Lugtenberg and Kamilova, 2009). We showed that *Pseudomonas* spp. strains were able to colonize tomato roots and to persist for the end of the experimentation. The strain T34, which was isolated from tomato rhizosphere, significantly enhanced plant growth. It may be the first to colonize the rhizosphere and enhance plant growth. In most cases bacterial strains that promote plant growth also elicit induced systemic resistance (Kloepper et al., 2004). However, the results from greenhouse experiment clearly demonstrate the potential of some strains to colonize the roots of tomato plants and interfere with the pathogen activity, providing the impetus for further research on the colonization ability of bacteria as one important influencing factor towards successful biological control. Nevertheless, the mode of action in *Pseudomonas* spp. strains, which does not produce antibiotics, could be related to PGPR activity and its colonization and persistence abilities. The T34 isolate was the most active inhibition to the mycelium growth microsclerotia formation of *V. dahliae* in both *in vitro* and under greenhouse conditions. This isolation also showed the capacity to promote plant growth. In contrast, Mercado-Blanco et al (2004) found no strict correlation between *in vitro* antagonism and *in vivo* suppression of *V. dahliae*. For instance, while *P. putida* strains displayed strong *in vitro* antagonism against *V. dahliae* isolates, *P. fluorescens* PICF7 was one of the most effective biocontrol agents *in planta*.

Materials and Methods

Isolation of fluorescent Pseudomonas from soil

Samples were collected from the Center of Professional Formation Specialized in Agricultural Sciences (CPFSAS)

situated at Lakhdaria (province Bouira, Algeria) with 36°33′52″ North and 3°35′35″East and an altitude of 171m from the sea. The soil is clay-loam with a pH of 7.5. The climatic conditions in the sampling site are dry hot summers and cold winters, with an average rainfall of 700 mm and an average temperature of 17.8°C per annum. Bacteria were isolated from the root systems of healthy tomatoes plants cv. Marmande. Root samples were collected in sterile plastic bags, stored at 4°C and transported to the laboratory for further processing. Roots were vigorously shaken to remove loosely adhering soil and root samples (100 g) were agitated in a sterile flask containing 100 ml of sterile NaCl solution (0.9%) for 30 min at 180 rpm on a rotary shaker. A serial dilution series in sterile physiological water was then made. Isolation of fluorescent pseudomonads was performed by plating 0.1 ml from each dilution on King's B (KB) media (King et al., 1954). After incubation for two days at 27±2°C, single colonies showing green fluorescence under UV light (336 nm), were considered as producers of the siderophore pyoverdine. Then single cell colony purification of each selected *Pseudomonas* spp. isolate was conducted. These were maintained on KB agar supplemented with 10% glycerol and stored at 4°C.

Phenotypic characterization

Bacterial strains were morphologically characterized (colour, form and texture of the colonies) according to Bossis et al. (2000). *Pseudomonas fluorescens* CHAO (kindly provided by Prof. C. Keel, Switzerland) was used as a reference strain. Fluorescent colonies obtained in King's B (KB) (King et al., 1954) agar were tested for Gram reaction using the crystal violet method. Discs impregnated with di-methyl-p-phenylenediamine (Biomerieux, French) were used to test the presence of cytochrome oxidase. Catalase activity was determined by O_2 production after adding a drop of 1.5% H_2O_2 to a young colony growing on nutrient agar (Stanier et al., 1966). The ability of *Pseudomonas* spp. strains to grow at 41˚C was determined by culturing them on nutrient agar plates during 24 hr at 41˚C. Bacterial isolates were also characterized for lactose degradation (β-galactosidase), nitrate reduction (Nitrate reductase), gelatin hydrolysis, urea hydrolysis, H_2S production, levan production, arginine dihydrolase, and carbon source (arabinose, sorbitol, L(+) tartrate, xylose and trehalose) utilization (Stanier et al., 1966; Bossis et al., 2000). Results from these tests were scored as either positive or negative and used to distinguish among bacterial species (Palleroni, 1984; Barrett et al., 1986 and Bossis et al., 2000). Finally, the ability to cause a hypersensitivity response (HR) on tobacco leaves was assessed. For this objective, leaves of tobacco seedlings (20 days old) were inoculated with a fresh bacterial suspension (10^9 UFC/ml) into intervenial tissue with a syringe and 25-gauge needle. An area about 1 cm^2 was injected. HR was examined after 2 days following inoculation (Garrity et al., 2004). Numerical analysis and the comparison among the strains were carried out for the thirty nine fluorescent pseudomonads isolates and the reference strain using Pearson's correlation coefficient (SPSS program V6).

Verticillium dahliae isolates

Two single-spored *V. dahliae* isolates (V1 and V2) representatives of the race 1 (R1) and race 2 (R2) pathotypes, respectively, were used in this study. It was originated from a diseased tomato plant sampled in northern Algeria (Bouira), which showed wilt symptoms and vascular discoloration. This isolate is deposited in the culture collection of the Laboratory of Conservation and Valorisation of Natural Resources, Department of Biology, University of M'hamed Bougara, Boumerdes (Algeria). Isolate V2 (isolated from tomato plants) was kindly provided by Dr. Nadia Korolev, Department of Plant Pathology, University of Dagan, and used as reference strain. *Verticillium dahliae* isolates were cultured at 20°C on Potato Dextrose Agar (PDA) medium amended with 300 mg/L of streptomycin sulphate (Pharmadrug Production Gmbh, Hamburg, Germany). Liquid cultures used for inoculation were prepared on Potato Dextrose Broth (PDB) and incubated at 20 °C under continuous agitation at 150 rpm during 5 days. Conidia concentration was adjusted to 10^6 conidia/ml by using a Malassez cytometer. For long-term preservation, isolates were stored at -20°C in 25% glycerol as conidial suspension.

Verticillium dahliae-Pseudomonas spp. in vitro antagonism

Antagonism of *Pseudomonas* spp. strains (indigenous and reference *P. fluorescens* CHAO) against *V. dahliae* isolates were tested *in vitro* using two different media, namely KB and PDA. Fungal conidia suspension (100 µl of approximately 10^6 conidia/ml) was uniformly spread over the surface of solid media with the aid of sterile L-shaped glass rod and incubated for four hours at 25˚C until moisture excess evaporated from the agar surface. Then, wells (5 mm diameter) were made in the centre of the inoculated agar plates using a sterile Cork borer size #10. Aliquots (5-µl) containing approximately 10^6 Colony Forming Units (UFC)/ml of each bacterial strain (incubated overnight in physiological water) were transferred into the wells. The control treatment was prepared in the same manner but sterilized physiological water was used instead of bacterial suspensions. Plates were incubated at 27˚C. Antibiosis activity in terms of *V. dahliae* growth inhibition was assessed after 7 to 10 days of incubation. Inhibition percentage was calculated as the hyphal growth inhibition area (halo) around the bacterial colony in cm / plate diameter × 100. Means were compared using the LDS at $P \leq 0.05$. The experiment was repeated twice with four replicates for each isolate.

Effect of Pseudomonas spp. on V. dahliae microsclerotia germination

The ability of *Pseudomonas* spp. strains to inactivate microsclerotia germination was assessed by using substrate (perlite) previously colonized by the pathogen. *Verticillium dahliae*-inoculated perlite (5 g) wrapped in a sterile cloth were soaked for 30 min in a suspension of each antagonist tested (concentration adjusted to 10^6 UFC/ml). Then perlite was washed with sterile distilled water (SDW), air dried at room temperature and ground in a mortar with 10 ml of SDW. The microsclerotia suspension was sprayed onto 2% agar medium (1 ml per plate). Eventual growth of fungal colonies emerging from plated microsclerotia was assessed after ten days of incubation at 20°C. Microsclerotia viability was estimated according to the formation or not of secondary resistant structures (Nagtzaam et al., 1998). *Verticillium dahliae*-colonized perlite soaked in SDW served as control treatment. This experiment was repeated twice with five replicates for each bacterial strain.

Detection and extraction of metabolites with antibiotic effect

Pseudomonas spp. strains that showed the highest antagonist activity (i.e. *in vitro* inhibition of *V. dahliae* mycelium growth and microsclerotia formation and *in planta* biocontrol activity under greenhouse conditions [see below]) were selected in order to extract metabolites with antibiotic effect. Production of antibiotics was preliminarily assessed by the presence of colored colonies in KB, Nutrient Yeast extract Agar (NYA) and PDA media observed under UV light (365 nm) (Thomashow, and Weller, 1988). *In vitro* production of antibiotics was done on NYB supplemented with 2% glucose (James et Gutterson, 1986). Phenazine compounds were extracted according to the method described by Thomashow and Weller (1988). Bacteria were incubated for 72 h, 180 rpm at 28°C using an orbital shaker. Cultures were then acidified to pH 2 with concentrated HCl, and extracted two times with the same volume of benzene. The organic phase was filtrated in anhydride ammonium sulfate and evaporated using a rotary evaporator at 55°C (James and Gutterson, 1986). The dry extract was finally dissolved in methanol.

Analysis of antibiotic metabolites

The antibiotic active component(s) from bacterial culture extracts were separated by column chromatography (CCM). Chloroform/ethyl-acetate (1:1 v/v) was used as mobile phase for elution through the column. Collected elution solvent was concentrated by evaporation down to 1/10 of its original volume. Then, the solvent was plotted in small concentrated spots on the x-axis of thin layer silica gel plates (TLC). The mobile phase in this TLC separation was studied and developed separations were visualized under UV at 365 nm. Retention factor (RF) values were calculated for spots revealed and different fractions of extracts were recorded and identified according to accepted standard tables of RF values. The presence of spots with Rf values of 0.50 and 0.87 indicate production of phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN), respectively. Each spot of silica was grated and re-suspended in methanol and fraction agitated in order to extract the chemical moiety carried in that fraction. This operation was repeated five times. Organic collected for each spot was concentrated by evaporation under the same conditions mentioned above. The UV spectroscopies for all of those fractions were recorded (with solution of 0.1N NaOH) with the aid of spectronic (Cecil CE3021 UV.Visible 3000series) in order to determine the specific fraction's UV spectrogram (Pierson and Thomashow, 1992). Spots of other antibiotics were also reported after spraying diazotized sulfanilic acid. Antibiotics were identified according to their Rf values and characteristic pigmentation. The Rf of 0.68 with brown colour indicates the presence of pyoluteorin (PLT); Rf of 0.81 with maroon colour indicates the presence of pyrrolnitrin (PRN); Rf of 0.74 yellow indicates the presence of 2,4 diacetyl-phloroglucinol (DAPG); and Rf of 0.93 dark green indicates 2-hexyl 5-propylresocinol (HPR).

High performance liquid chromatography

The crude culture extract of the isolates indicating the presence of phenazine compounds (PCA and PCN) were analyzed by high performance liquid chromatography (HPLC) for the presence of novel moiety (ies) according to Delanay et al. (2001). Phenazine compounds were identified according to their retention time and their UV spectra (Delanay et al., 2001; Veselova et al., 2008). Aliquots containing 0.05 mg/ml of methanol extract of the crude culture filtrates were injected into the HPLC device (Jones Chromatography, UK). The mobile phase consisted of methanol/water (35/65, V/V) and 1% of TFA. The samples were eluted using the following solvent gradient profile; 0-10 min 10: 80 % (A: B, 0.5ml min flow rate), 10: 20 min 40:60 % (A: B, 0.7-1ml min flow rate).

Molecular identification by 16S rRNA gene sequencing

Selected *Pseudomonas* spp. strains were subjected to molecular identification. Total DNA was prepared according to Niemman et al. (1997) and modified by Gervers et al. (2001). Total DNA was extracted from 10 ml of cultures harvested in the mid-log phase (OD_{600} of 0.5–1). Cells were collected by centrifugation (3000×*g*, 10 min, 4°C) and froze for at least 1 h at −20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA) and suspended in 300 µl STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA). The 16S rRNA gene was amplified by PCR using primers 16F27 (5'AGAGTTTGATCCTGGCTCAG3', positions 8-27), and16R1522 (5'AAGGAGGTGATCCA-GCCGCA3', positions 1541-1522). Positions refer to the *E. coli* 16S rRNA gene sequence. Amplicons were purified using the NucleoFast® 96 PCR clean up kit (Macherey Nagel Duren, Germany). The sequencing reactions were performed using the Big Dey® Terminator Cycle Sequencing Kit (Applied Biosystems, foster city, CA, USA) and purified using the Big Dey® Terminator T purification Kit (Applied Biosystems, Foster city, CA, USA). Sequencing was performed using an ABI Prism® 3130 XL Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). Strains T32, T27, T33, T34 and T39 sequences identification was performed using BLAST (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). DNA sequences of these strains and of reference *Pseudomonas* spp. strains in the databases were aligned using ClustalW v.1.6. A phylogenetic tree was then constructed using the Neighbor-Joining (NJ) algorithm provided in MEGA v5.0 (Tamura et al., 2011). The degree of statistical support for the branches was determined by bootstrap analysis (1,000 replicates). Maximum likelihood trees were estimated by using the rapid bootstrap option (100 replicates).

Verticillium wilt biocontrol assays

Algerian indigenous *Pseudomonas* spp. strains T23, T27, T33, T34 and T49 and the reference strain CHAO were tested for their biocontrol activity under greenhouse conditions using seedlings of the susceptible tomato cv. Marmande. A group of seedlings (75 plants) were grown from seeds sown in perlite rooting medium drenched with bacteria suspension (30 ml/ 50 x 70 cm trays) of each *Pseudomonas* spp. strain (approximately 10^8 cfu /ml). A second group of seedlings (15) were drenched with SDW only. Two weeks later, tomato seedlings were carefully removed from the tray and their root system inoculated by dipping for 10 min in a conidial suspension (10^6 conidia/ml) of each (V1 and V2) *V. dahliae* isolate. The inoculated seedlings were replanted in 10 cm diameter plastic pots containing sterilized field soil (silty-clayey, pH 7.5) but artificially infested with the same fungal culture suspensions. Four sets of treatments were done; (i) seedlings treated with each bacterial strains and fungal isolates V1 and V2 individually; (ii) seedlings drenched only with bacterial suspension; and (iii) seedlings inoculated only

with V1 and/or V2 separately used as positive control. Seedlings inoculated with SDW were used as a negative control. Biocontrol bioassays were repeated two times for each *Verticillium dahlaie* isolates V1 and V2. The experiment was recorded for 8 weeks with a 12 hs photoperiod at 14-30±2°C and 70 to 80% relative humidity and regulatory watered and fertilized with nutritious solution (N, P, K, Ca, Mg, Fe, Mn, Zn, B, Cu, Mo).

In order to demonstrate the colonization and persistence of introduced *Pseudomonas* spp. strains on the roots/rhizosphere of tomato plants, the procedure by Gonzalez-Sanchez (2009) was used with some modifications. Tomato seedlings with two true leaves were used. Plant roots were disinfected with 2.5% sodium hypochlorite solution during 3 minutes, air dried in sterile filter paper and inoculated with bacterial suspension (10^8 CFU/ml) of rifampicin/ampicillin (100mg ml^{-1} for each antibiotic) resistant strains. Spontaneous rifampicin/ampicillin resistant strains were obtained for the bacterial isolates cultivated in KB medium supplemented with (100mg ml^{-1} for each antibiotic). Inoculated plants were grown in disinfected cell trays (one plant/cell) contained a mixture of sterilized soil and perlite under the same conditions described for the biocontol assays. At the end of the experiment, root tissue samples representative of the entire root system from two out of the five replicates of each treatment were collected. Bacteria located within 1 cm of the root tips were considered colonizers, while those located at 2-6 cm from the root tips were considered persistent forms of the tested bacteria. For both types of samples, bacterial population sizes were determined. One gram of root samples was shacked in 9 ml of physiological water for five minutes. The resulting suspensions were serially diluted and plated in KB agar supplemented with rifampicin (100µg/ml^{-1}), ampicillin (100µg/ml^{-1}) and cycloheximide (100µg/ml^{-1}). Rifampicin/ampicillin resistant colonies were scored after incubation during 48 h at 27˚C.

Disease assessment and data analysis

Disease development on tomato plants inoculated with *V. dahliae* isolates alone or in combination with the bacterial strains in a factorial experiment (two fungal isolates *vs.* six bacterial isolates) was monitored during eight weeks after inoculation. Percentages of seedlings showing stunting and foliar symptoms such as chlorosis were scored every week. Root rot and vascular discoloration was assessed at the end of the experiment. Disease incidence was determined as the percentage of plants showing disease symptoms out of the total number of plants in each treatment. Disease severity was recorded as the amount of foliar damage according to a 0-4 rating scale (Sidhu and Webster, 1977) where 1 = 0-25%; 2 = 26-50%; 3 = 51-75%; and 4 ≥ 76% damage. Data were subjected to analysis of variance. Data on disease severity were used to calculate the following: (i) a disease intensity index (DII) determined as: $DII=(\sum Si*Ni)/(4/Nt)$ where; Si is the symptoms severity, Nt is the total number of plants; (ii) the inoculation period (IP) established as the number of days from inoculation with the pathogen until DII>0; (iii) the final disease incidence (percentage of affected plants) (final DI); and (iv) the areas under the disease progress curve (AUDPC) were calculated with the following equation:

$AUDPC = S\ [(Yi + Yi+1)/2](ti+1 - ti)$ where 'Y' means disease severity and 't' means time (days) (Campbell and Madden, 1990). Plant protection percentage were estimated by [1-(x/y)] factor, where "x" relates the AUDPC from treated plants and "Y" is the AUDPC from control plants (Li et al., 1996). Collected data were subjected to statistical analysis using Statistica program (Version 6). Significant treatments means were compared with those of the control using the Dunnett's test according to the least significant differences (LSD) at 0.05 levels of probability. For the growth parameters (plant height, shoot and root fresh weight and shoot and root dry weight) the collected data were subjected to analysis using Tukey's test, treatments means were compared among them and compared to control. The whole experiment was repeated during two years (2012 and 2013). The data recorded between the two years did not differ significantly ($p>0.05$) according to Student test. Therefore the data are pooled in order to get more significant results.

Conclusion

Our results showed that beneficial *Pseudomonas* spp. strains are widely distributed in Algerian soils. Antagonistic activity of some strains against *V. dahliae* was confirmed *in vitro* by inhibition of mycelium growth and microsclerotia formation which is probably due to phenazines production and other metabolites such as siderophores. Selected strains were successfully introduced into rhizosphere and showed the ability to reduce disease incidence and severity of the phytopathogenic fungi *V. dahliae* V1 and V2. Promotion of tomato growth was demonstrated for bacterized plants either when introduced alone or in combination with the pathogen. Pseudomonas Strain T34 may possibly present a novel PCN-producing *Pseudomonas* species that groups in the *Pseudomonas putida* species complex.

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