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Biocontrol capacities and plant growth-promoting traits of endophytic actinobacteria isolated from native plants of Algerian Sahara

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Non-standard abbreviations

CAS – chrome azurol agar DAP – diaminopimelic acid, *DL*-DAP – meso isomer of diaminopimelic acid, FOL – *Fusarium oxysporum* f. sp. *lycopersici,* FORL – *Fusarium oxysporum* f. sp. *radicis lycopersici*, IAA – indole-3-acetic acid, *LL*-DAP – levo isomer of diaminopimelic acid, PCA – principal component analysis, PVK – Pikovskaya medium, RF – *recti flexibilis,* TCP – tricalcium phosphate, TLC – thin layer chromatography, YT – yeast extract-tryptone broth.

Abstract

Fusarium root rot is one of the most important plant diseases. Chemical fungicides are efficient but they have been much criticized. The present study investigates endophytic actinobacteria isolated from native plants of the Algerian Sahara in the aim of assessing their ability to exercise biocontrol over this soil-borne fungus, and to highlight their plant growth-promoting traits on seedlings of tomato *cv*. Marmande. A total of 21 endophytic actinobacteria were isolated from plant roots. Six isolates were first selected for the biocontrol trials on the basis of their antifungal properties. *In vivo* biocontrol activity was measured by the disease incidence on plants grown in infested soils. Four isolates decreased the disease incidence in both sterilized and non-sterilized infested soils, whereas no significant differences were obtained with the control chemical agent. Indole-3-acetic acid and siderophore production, and phosphate solubilization were investigated as plant growth-promoting traits. The isolate ZL2 showed positive results for these mechanisms. Statistical treatment of the data brought out the potential of this isolate as a promising candidate for the biocontrol of root rot in tomato seedlings and in regard to its plant growth-promoting characteristics. Molecular identification was performed by 16S rRNA gene sequence analysis and indicated that *Streptomyces* sp. ZL2 was related to *Streptomyces caeruleatus* (99.6% of similarity). Isolate *Streptomyces* sp. ZL2 enhanced the plant resistance to *F. oxysporum* f. sp. *radicis lycopersici* root rot and promoted the growth of tomato seedlings. These properties open up promising perspectives for the possible application of isolate ZL2 in crop protection.

Key words: *Fusarium oxysporum* f. sp. *radicis lycopersici*, indole-3-acetic acid, phosphate solubilization, siderophore, *Streptomyces*, Tebuconazole®, tomato

Introduction

Crown and root rot disease caused by *Fusarium oxysporum* f. sp. *radicis lycopersici* (FORL) on tomato seedlings was discovered in Japan and subsequently in many other countries (Saidi et al. 2009). Crown and root rot of tomato caused by *Fusarium* spp. is an important soil-borne disease that limits productivity in open fields and greenhouses. An increase of FORL crown and root rot of tomato plants has been noted in Algeria (Edel-Hermann et al. 2012). Chemical control agents are classically used to manage the disease when losses from FORL are considerable. However, intensive and repeated applications of fungicides may increase the chemical contamination of the crop soils, create problems of fungicide resistance and also have adverse effects on native beneficial microorganisms in the soil (Gerhardson 2002).

Biocontrol of Fusarium crown and root rot of tomato, in the form of natural microbial populations in soils, has been recognized for over 70 years. It primarily results from the antagonistic and competitive abilities of native microbial communities (Saidi et al. 2009).

It has been demonstrated that some species of actinobacteria are important in the rhizosphere, where they protect roots against invasion by pathogenic fungi and may promote plant growth (Cao et al. 2004). They have the ability to produce active compounds, such as antifungal and antibacterial compounds, siderophores or plant growth regulators, which have been developed for agricultural uses (Sadeghi et al. 2012, Goudjal et al. 2014). Some actinobacteria are also recognized for promoting plant growth by developing symbiotic associations with crop plants and colonizing their internal tissues without causing disease symptoms (El-Tarabily et al. 2009).

An endophytic species from the genus Micromonospora has been successfully isolated from healthy tomato plants. This species showed a strong antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (Smith 1957). Furthermore, several studies have highlighted the efficiency of endophytic actinobacterial isolates in the biocontrol of Fusarium disease (Cao et al. 2005, Saidi et al. 2009). These bacteria have been isolated from root, stem or leaf tissues of various plants, such as rice, tomato, cucumber, banana and spontaneous plants (Cao et al. 2004, El-Tarabily et al. 2009, Goudjal et al. 2013). Furthermore, some actinobacteria, such as *Streptomyces griseoviridis*, have been formulated and marketed as biocontrol agents, and are used as biopesticide against Fusarium disease (Shimizu 2011).

The success of endophytic actinobacteria as efficient biocontrol agents has encouraged research into new microbial isolates as alternatives to chemical control compounds. Researchers have turned their attention to different ecological niches, and also to harsh climatic conditions, in the aim of discovering new potential isolates for the biocontrol of plant pathogenic fungi and the improvement of plant growth (Shimizu 2011).

The objectives of the present study were to isolate endophytic actinobacteria from healthy native plants of the Algerian Sahara and to evaluate their potential as agents for biocontrol of FORL root rot disease and for plant growth promotion of tomato seedlings. In order to achieve this goal, the isolates were evaluated regarding their antagonistic activity *in vitro* and *in vivo*, their ability to produce indole-3-acetic acid (IAA) and siderophores, and to solubilize inorganic phosphate.

Materials and methods

Sample collection and isolation of endophytic actinobacteria

Six plant species (*Aristida pungens*, *Cleome africana, Astragallus armatus*, *Peganum harmala, Hammada scoparia* and *Zizyphus lotus*) native to the north of Algerian Sahara (33°69'N, 2°70'E) were collected in April 2012. Plants were chosen on the basis of their abundance in the poor sandy soil and well adaptation to the arid climatic conditions of the Algerian Sahara, where associated microorganisms may play a major role in such adaptation.

From each plant species, five healthy root samples were harvested and placed in sterile plastic bags. A modification of the method described by Cao et al. (2004) was used for endophytic actinobacteria isolation. In total, 30 roots having a length of 60–80 mm and a diameter of 2–5 mm were washed under running water to remove particles of soil and surface-sterilized by sequential dipping in ethanol solution (70% v/v) for 5 min, and NaClO solution (0.9% w/v) for 20 min. The root samples were then washed three times in sterile distilled water to remove residual chemical agents and soaked in $NaHCO_3$ solution (10% w/v) for 10 min to retard the growth of endophytic fungi (Cao et al. 2004). The rhizodermis was aseptically removed and the tissue lying beneath was excised in thin discs (2 mm thick) and placed on chitin-vitamin agar plates (Hayakawa & Nonomura 1987). Nalidixic acid (15 μg ml^{-1}) was added to suppress the growth of Gram-negative bacteria. Plates were then incubated at 30°C for 21 days.

The effectiveness of the surface-sterilization protocol was assessed by two methods. The first consisted of soaking the surface-sterilized root samples in 5 ml of sterile distilled water and stirring for 1 min. An aliquot of 0.3 ml suspension was then inoculated on chitin-vitamin agar medium, and the plates were incubated at 30°C and checked for growth of actinobacteria. The second method consisted of submerging 6-mm plugs from actinobacteria cultures, grown on chitin-vitamin agar medium, in ethanol solution (70% v/v) for 5 min and NaClO solution (0.9% w/v) for 20 min. The submerged plugs were then inoculated on the same medium and the viability of the actinobacteria was recorded (Cao et al. 2004).

Determination of actinobacterial genera

Actinobacterial isolates belonging to the *Streptomyces* genus were identified according to traditional cultural and micromorphologic characteristics on yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) media (Shirling & Gottlieb 1966). Chemotaxonomic analysis consisted of the determination of the isomeric form of diaminopimelic acid (DAP). Cell-wall hydrolysates of *Streptomyces* strains contain the LL isomer, whereas other genera of actinobacteria contain meso-DAP (Kämpfer 2012).

In vitro antagonism essay

The streak method, as used by Goudjal et al. (2014), was employed to test for antagonistic activities of the actinobacteria against phytopathogenic species of the genus *Fusarium* (*F. oxysporum* f. sp. *radicis lycopersici*, *F. oxysporum* f. sp. *lycopersici*, *F. solani*) from our laboratory collection.

Actinobacterial strains were first streaked in a straight line on ISP2 medium (containing 12 g l^{-1} agar) plates (90 mm in diameter) and the plates were incubated for 7 days at 30°C. After sufficient growth of the strains, the target fungi were seeded in streaks perpendicular to those of actinobacteria. After 5 days of incubation at 25°C, the distance between target fungi and actinobacteria colony margins was measured to determine the antifungal activity.

In vivo biocontrol of *F. oxysporum* f. sp. *radicis lycopersici*

Textural, chemical and biological properties of soil. The effectiveness of the antagonistic isolates in the biocontrol of FORL root rot was tested in a soil sampled from an infested tomato field in the Algerian Sahara (33°86'N, 2°85'E). Its textural, chemical and biological properties were: sandy loam (sand 77%, clay 13%, silt 10%); pH 6.6; total organic matter 1.81%; C/N 9.3, potash 0.29‰; phosphate 0.07‰; $CaCO_3$

1.08%; total aerobic bacteria 1.5 10^8 CFU g^{-1} of soil and total fungal count 3.7 10^4 CFU g^{-1} of soil. Soil was autoclaved three times (120°C for 60 min) on three consecutive days as described by Errakhi et al. (2007).

Microbial suspensions. Suspensions of actinobacteria were prepared as in Goudjal et al. (2014) by cultivating the antagonistic isolates on ISP2 plates at 30°C for 10 days. Spores were recovered in Tween-20 solution (0.05%) and adjusted to ≈10^6 CFU ml^{-1} with the Thoma chamber. A spore suspension of the pathogen was prepared by growing FORL on PDA plates for 10 days at 25°C. Spores were then recovered in sterile distilled water and their density adjusted to ≈10^4 CFU ml^{-1} in the same way.

Bacterization of tomato seeds and preparation of infested soils

Solanum lycopersicum cv. Marmande seeds were surface-sterilized by dipping in ethanol solution (70% v/v) for 5 min followed by NaClO solution (0.9% w/v) for 4 min. The seeds were then washed three times in sterile distilled water. Surface-sterilized seeds were separately bacterized by dipping in the actinobacterial suspensions for 30 min before drying under a laminar flow hood. Coated tomato seeds were sown the same day.

Actinobacteria spores on the bacterized seeds were enumerated by dilution plating on ISP2 plates. They yielded ≈8 10^6 CFU g^{-1} bacterized seeds.

According to the method used by Goudjal et al. (2014), sterilized and non-sterilized soils were infested with the FORL spore suspension. Plastic pots (12 cm high × 10 cm in diameter) were filled with soil and irrigated with 100 ml of FORL suspension in distilled water (spore density ≈103 CFU ml-1) (100 ml sterile distilled water for non-infested soils). In order to promote the growth of the pathogen, pots were covered with plastic film and incubated at room temperature for 7 days as used by Toumatia et al. (2014). FORL density reached ≈7 10^4 CFU g^{-1} in the infested soils.

***In vivo* biocontrol essay.** Six antagonistic isolates were selected for *in vivo* biocontrol of FORL root rot on *Solanum lycopersicum* cv. Marmande seedlings. Trials were performed in both sterilized and non-sterilized soils, where 4 treatments were conducted: (1) a negative control treatment in which untreated tomato seeds were sown in non-infested pots; (2) a positive control treatment in which untreated seeds were sown in infested soils to highlight the virulence of the plant pathogen; (3) to evaluate the biocontrol capacity of each actinobacteria isolate against FORL root rot, bacterized seeds were sown in pots with infested soil; (4) a seed treatment with a chemical agent, Tebuconazole®, was used to control the root rot disease. In the last case, 10 g of surface-sterilized seeds were soaked for 3 min in 0.06% (w/v) Tebuconazole® solution, then dried for 2 h under a laminar flow hood and sown in infested soil.

Five tomato seeds were sown per pot with 10 replicates (pots) for each treatment. Pots were then placed in a fully randomized complete block design in a greenhouse (22–26°C, photoperiod of 15 h light and 9 h dark). They were watered daily with 10 ml tap water. After 30 days, the root rot incidence was assessed as described by Vitale et al. (2014).

Indole-3-acetic acid production. All actinobacteria isolates were screened for their ability to produce IAA according to the method used by Goudjal et al. (2013). One-millilitre aliquots of the spore suspensions of the actinobacterial strains (≈10^6 CFU ml^{-1}) were transferred into 500 ml-Erlenmeyer flasks containing 100 ml of yeast extract-tryptone broth (YT) (5 g yeast extract l^{-1}, 10 g tryptone l^{-1}, 5 g NaCl l^{-1}, pH 7.2) supplemented with 5 mg ml^{-1} of L-tryptophan. Flasks were cultured on a rotary shaker (200 rpm) at 30°C for 5 days and culture supernatants were harvested by centrifugation at 10 000×g for 10 min. The IAA production was revealed by mixing 2 ml of the culture supernatant with 4 ml of Salkowski reagent (1 ml of 0.5 M FeCl3 in 49 ml of 35% (w/v) HClO4). The appearance of a pink colour after incubation for 30 min in a dark room indicated the production of IAA. Optical density was read at 530 nm using a spectrophotometer (JANWAY-6405) and the IAA concentration was estimated from a standard graph prepared with pure IAA (Merck, Germany).

The IAA production was confirmed by thin layer chromatography (TLC). Ethyl acetate fractions (80 µl) were spotted on TLC plates (silica gel GF254, thickness 0.25 mm, Merck, Germany) and developed in ethyl acetate:chloroform:formic acid (55:35:10, v/v/v). Spots with R_f values identical to authentic IAA were identified under UV light (254 nm) after spraying the plates with Ehmann's reagent.

Siderophore production. Production of siderophores by the isolates was studied on chrome azurol (CAS) plates as described by Sadeghi et al. (2012). Six-millimetre plugs from cultures of actinobacteria on ISP2 medium were placed on these plates and incubated for 7 days at 30°C. The colonies producing clear or yellow halos were considered as positive for siderophore production.

Inorganic phosphate solubilization. Phosphate solubilization ability was investigated according to the method used by Liu et al. (2014). Pure cultures were screened in a liquid Pikovskaya medium (PVK) containing $Ca_3(PO_4)_2$ (TCP-PVK), $AlPO_4$-PVK), or $FePO_4$-PVK) as insoluble phosphate sources at a concentration of 5 g l^{-1}. The experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of medium. Flasks were inoculated by adding 1 ml aliquots of the spore suspensions of the actinobacterial strains (≈10^6 CFU ml^{-1}) and cultured on a rotary shaker (200 rpm) for 5 days at 30°C. The liquid cultures were centrifuged at 10 000×g for 10 min and the supernatants were used to assess the phosphate released into the solution. Soluble phosphate was determined by the molybdenum blue colourimetric method as used by Liu et al. (2014).

16S rRNA gene sequence analysis for identification and phylogenetic analysis of *Streptomyces* sp. ZL2. Identification of the isolate *Streptomyces* sp. ZL2 was confirmed by the 16S rRNA gene sequence analysis. The CTAB method (Liu et al. 2000)

was used to prepare the genomic DNA, which was amplified by PCR using the primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). The amplification was performed on a thermal cycler (Stratagene RoboCycler Gradient 96) and the PCR product was detected by agarose gel electrophoresis and visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was sent to the Beckman Coulter Genomics Company (Tekeley, Essex, United Kingdom) for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The16S rRNA gene sequence has been deposited in the GenBank data library and assigned the accession number KP399598. The obtained sequence was compared with sequences present in the public sequence databases and with EzTaxon tools (http://eztaxon-e.ezbiocloud.net/, Kim et al. 2012).

Statistical analysis

Data were subjected to variance analysis (ANOVA). Significant differences between means were compared using Fisher's protected LSD test at $P = 0.05$. Differences were considered significant when $P < 0.05$.

In the aim of selecting the actinobacterial strain having the most interesting biocontrol potential and plant growth promotion ability, a principal component analysis (PCA) was conducted using the XLSTAT software package. For the principal component, the correlation matrix extraction method was used (Husson et al. 2009). The multivariate effect of seed treatments (negative control, positive control, and assays with AR1, AR2, CA2, CA13, AP4 and ZL2 strains) and the plant growth traits (antagonistic activities, biocontrol, IAA production, siderophore production, phosphate solubilization) of actinobacterial isolates were assessed. On the basis of data measured for each actinobacterial isolate, PCA gave an indirect gradient analysis that examined the total variation in the data set in relation to the treatments. Sample scores corresponded to actinobacterial isolates (distance based biplot). Pearson partial correlations were used to assess the correlation between variables and ordination axes (Pagès 2014).

Results

Actinobacterial isolates

Twenty-one actinobacterial strains were successfully isolated from the roots of six Saharan native plants (Table 1). Samples of the water used to wash all sterilized roots failed to grow on chitin-vitamin agar medium. In addition, plugs from actinobacterial cultures treated with the same surface-

Table 1: Taxonomical characteristics and antagonistic activities of endophytic actinobacteria isolates

Host plant	Actinobacteria isolate	Taxonomical characteristics[a]				Diameter of inhibition zone [mm][b]		
		Serial group	Spore-chain type	DAP isomer	Genus	FORL	FOL	*F. solani*
Aristida pungens	AP1	Grey	S	*LL*	*Streptomyces*	0	0	0
	AP2	Grey	S	*LL*	*Streptomyces*	0	0	10 ± 1.2
	AP3	Grey	S	*LL*	*Streptomyces*	0	0	0
	AP4	Grey	S	*LL*	*Streptomyces*	30 ± 1.5	28 ± 1.8	34 ± 2.1
	AP6	Grey	S	*LL*	*Streptomyces*	0	0	0
	AP7	Grey	S	*LL*	*Streptomyces*	0	0	10 ± 0.6
Cleome africana	CA2	Grey	S	*LL*	*Streptomyces*	30 ± 1.2	32 ± 1.5	34 ± 1.9
	CA3	Grey	S	*LL*	*Streptomyces*	0	12 ± 2.2	0
	CA6	Grey	S	*LL*	*Streptomyces*	0	0	0
	CA9	Grey	S	*LL*	*Streptomyces*	0	0	11 ± 0.9
	CA10	Grey	S	*LL*	*Streptomyces*	0	0	0
	CA11	/	/	*DL*	non-*Streptomyces*	0	0	0
	CA13	/	/	*DL*	non-*Streptomyces*	22 ± 0.9	24 ± 1.0	26 ± 1.5
Peganum harmala	PH1	Grey	S	*LL*	*Streptomyces*	0	0	0
	PH8	Grey	S	*LL*	*Streptomyces*	0	0	10 ± 1.1
Hammada scoparia	HS1	Grey	S	*LL*	*Streptomyces*	0	0	0
	HS2	Grey	S	*LL*	*Streptomyces*	08 ± 1.6	0	0
Astragallus armatus	AR1	Yellow	RF	*LL*	*Streptomyces*	22 ± 1.0	25 ± 1.2	28 ± 2.1
	AR2	Yellow	RF	*LL*	*Streptomyces*	24 ± 0.9	28 ± 1.5	30 ± 1.6
Zizyphus lotus	ZL1	White	RF	*LL*	*Streptomyces*	0	0	0
	ZL2	White	RF	*LL*	*Streptomyces*	28 ± 1.2	30 ± 0.9	26 ± 1.2

[a] Bergey's manual of systematic bacteriology (Kämpfer 2012)

[b] Average ± standard deviation from 3 replicates

sterilization protocol showed no actinobacterial growth on the same medium. This strongly indicates that rhizospheric actinobacteria were totally eliminated and could not resist the sterilization treatments. Based on this, all the actinobacteria were regarded as endophytes.

Based on morphology and DAP isomer analyses (Table 1), 19 isolates were assigned to the genus *Streptomyces*. According to the colour of the aerial hyphae on ISP3 medium (grey, yellow and white), *Streptomyces* isolates were classified in four groups, with the grey group representing the majority of them (15 isolates). However, 79% of the *Streptomyces* isolates showed a spiral form (S) of spore chain and the others (21%) showed a *recti-flexibilis* (RF) type. The two non-*Streptomyces* isolates (9.5% of the isolates) were isolated from the roots of *Cleome africana*.

Antagonistic activities *in vitro*

Antagonistic activities of actinobacteria isolates are given in Table 1. The majority of isolates (71%) either failed to inhibit or only moderately inhibited the mycelial growth of the target fungi. Nevertheless, 6 isolates showed strong antagonistic activities (inhibition zone ≥ 20 mm) simultaneously on the three *Fusarium* spp. The antifungal activities appeared after 72 hours of dual culture of actinobacteria and target fungi. This result suggested that the antagonistic actinobacteria may be efficient in the biocontrol of FORL root rot. Therefore, the *Streptomyces* strains AP4, CA2, CA13, AR1, AR2 and ZL2 were selected for biocontrol trials *in vivo*.

Biocontrol of FORL root rot and plant growth promotion activities

Results of the incidences of root rot caused by FORL are given in Fig. 1a. High disease incidences were obtained for the positive (pathogen) control in both sterilized (86.9%) and non-sterilized (89.5%) soils. Root rot symptoms were observed both in the germinated seeds and in young seedlings. They evolved and led to the damping-off of seedlings.

All treatments of tomato seeds significantly ($P < 0.05$) reduced the disease incidence, which was more marked in sterilized soil than in non-sterilized soil. Chemical treatment with Tebuconazole® showed the highest protective effect against FORL root rot. Nevertheless, non-significant differences ($P < 0.05$) were observed between the chemical treatment and the seed bacterization with spores of the isolates ZL2, CA2, AP4 and CA13 in non-sterilized soil. These treatments resulted in rates of healthy seedlings varying between 69.4 and 75.4% of the negative control, in which 88.3% of seedlings were healthy.

In both soils, the *Streptomyces* sp. ZL2 isolate showed the greatest effect in enhancing tomato seedling growth. It significantly increased ($P < 0.05$) the seedling length (Fig. 1b), root length (Fig. 1c), fresh weight (Fig. 1d) and dry weight (Fig. 1e) compared to the negative (healthy) control. In the majority of the remaining cases, bacterization of tomato seeds failed to significantly promote ($P < 0.05$) the growth of tomato seedlings compared to the negative (healthy) control.

Production of indole-3-acetic acid and siderophore

Five endophytic actinobacteria produced IAA on YT broth supplemented with L-tryptophan (Table 2). The IAA production varied from 17 to 64 μg ml^{-1}, the maximum being observed for the isolate *Streptomyces* sp. ZL2. However, the Salkowski reagent failed to reveal indole compounds in the culture supernatant of the strain *Streptomyces* sp. AR1. All culture supernatants were used for IAA extraction and TLC analysis. Chromatograms of authentic IAA and synthesized IAA, developed with Ehmann's reagent, showed the same R_f values. Furthermore, the TLC analysis showed that IAA was the sole indole compound in the culture supernatants.

Siderophore production was recorded for the six actinobacteria selected (Table 2). Except for one isolate (CA13), all grew on CAS agar and formed yellow halos around the colonies. This qualified them as siderophore-producing strains.

Phosphate solubilization activity

The phosphate solubilization abilities of the selected actinobacteria are given in Table 2. Five of the actinobacteria strains grew on TCP-PVK broth; only isolate CA13 did not. Depending on the strain, the amount of soluble phosphate released ranged from 359 to 702 mg l^{-1}. In addition, the majority of strains failed to grow on $AlPO_4$-PVK and $FePO_4$-PVK media. The isolates CA2 and ZL2 grew well on the three media and the concentration of dissolved phosphate in the supernatant cultures varied from 213 to 702 mg l^{-1}. However, these two isolates showed a relatively low capacity for iron phosphate solubilization.

Principal component analysis and selection of actinobacteria

PCA explained 99.90% of the variation between the treatments with two major principal components, PC1 (87.61%) and PC2 (12.28%) (Fig. 2). Most of the parameters were strongly associated in two clusters (enclosed within the circles). The negative (healthy) control treatment, which showed a very low rate of disease incidence, and treatments with four actinobacteria were placed in the same cluster, which was strongly positively loaded in the PC1. This result suggests a potential of the actinobacterial isolates, especially ZL2, as biocontrol agents of FORL root rot and for enhancing plant growth in general. Based on these findings, the isolate *Streptomyces* sp. ZL2 was selected for subsequent analysis.

Identification of the isolate Streptomyces sp. ZL2

Molecular taxonomy and phylogenetic studies were carried out for the isolate *Streptomyces* sp. ZL2. The 16S rDNA sequence (GenBank KP399598) was compared with sequences present in the public sequence databases using EzTaxon tools. The similarity level with the most closely related species, *Streptomyces caeruleatus* GIMN4T, was 99.6%.

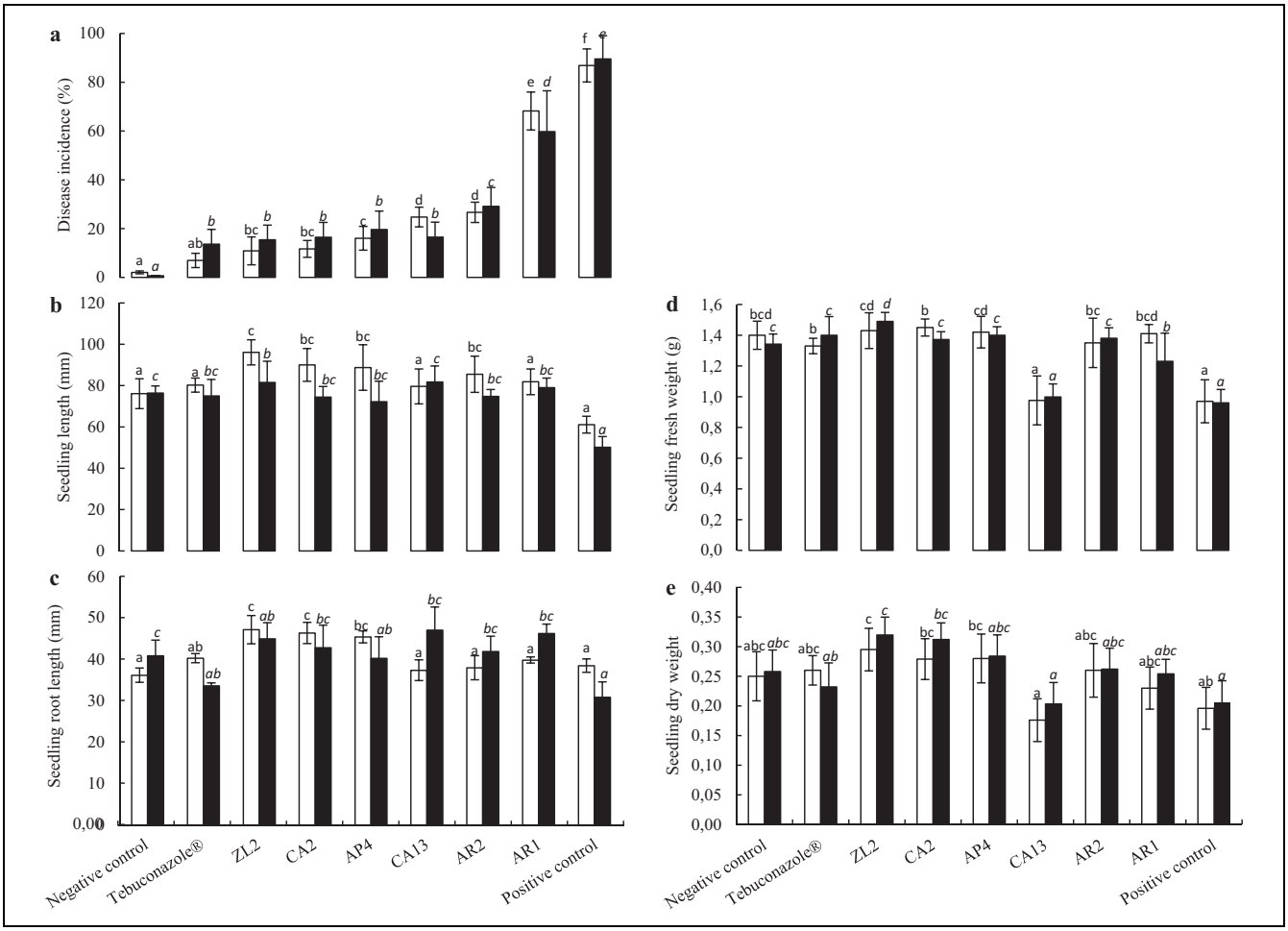

Fig. 1: Effect of seed treatment with Tebuconazole® and spore suspensions of antagonistic actinobacteria (ZL2, CA2, AP4, CA13, AR2 and AR1) on the disease incidence (a), seedling length (b), seedling root length (c), seedling fresh weight (d) and seedling dry weight (e) in sterilized (□) and non-sterilized (■) soils. The control treatments correspond to untreated seeds sown in non-infested soils (negative control) or in infested soils (positive control). Evaluation was made 30 days after planting. Bars labeled with the same letters are not significantly different according to Fisher's protected LSD test at $P = 0.05$. Error bars represent the standard deviation from 10 replicates.

Discussion

A large body of literature reports the role of endophytic actinobacteria as potential agents in the biocontrol of soilborne phytopathogenic fungi and/or as stimulators of plant growth (De Oliveira et al. 2010, Goudjal et al. 2014). Also, commercial products for control of root rot disease based on actinobacteria, such as Actino-Iron® (*Streptomyces lydicus* strain WYEC108) and Mycostop® (*Streptomyces griseoviridis* strain K61), have been registered (Shimizu 2011).

In this study, 21 endophytic actinobacteria were obtained from roots of five Saharan native plants. The surface sterilization method used for isolation was shown to be efficient to eradicate rhizospheric microorganisms, which confirmed that all actinobacterial strains were endophytes.

Several tissues and organs of many medicinal, crop and woody plants are inhabited by a variety of endophytic actinobacteria (Shimizu 2011). Commonly, various species of endophytic actinobacteria have been isolated from a single plant (Goudjal et al. 2014). The majority of endophytic actinobacterial strains have been classified in the *Streptomyces* genus. Several studies have reported that *Streptomyces* spp. isolates are the most dominant actinobacteria in the roots of plants such as tomato (Cao et al. 2004), banana (Cao et al. 2005), and some medicinal (Qin et al. 2009) and spontaneous plants (Goudjal et al. 2013).

In this work, six endophytic actinobacterial isolates (AP4, CA2, CA13, AR1, AR2 and ZL2) isolated from roots of *Aristida pungens*, *Cleome africana*, *Astragallus armatus* and *Zizyphus lotus* caused large inhibition zones on dual culture plates with FORL. The antagonistic activities of actinobacteria observed in this study are in agreement with previous reports (Cao et al. 2005). Several studies have reported the role of antagonistic actinobacteria in the biocontrol of a

Table 2: Indole-3-acetic acid, siderophore production and amounts of dissolved phosphate by endophytic actinobacteria isolates

Isolate	IAA production [µg ml^{-1}][a]	Siderophore production Halo diameter [mm] [a]	Amounts of P [mg l-1][a] dissolved from PVK medium with various sources of phosphate [b]		
			TCP-PVK	$AlPO_4$-PVK	$FePO_4$-PVK
AP4	24 ± 2.5	18 ± 1.6	445 ± 8.2	00	00
CA2	18 ± 1.8	24 ± 0.6	678 ± 6.6	346 ± 6.3	226 ± 4.2
CA13	26 ± 1.4	00	00	00	00
AR1	00	26 ± 1.3	359 ± 3.8	00	00
AR2	17 ± 1.0	28 ± 0.8	558 ± 4.2	00	00
ZL2	64 ± 2.4	22 ± 1.3	702 ± 5.3	213 ± 5.1	227 ± 5.3

[a] Average ± standard deviation from 3 replicates
PVK: Pikovskaya medium
TCP: tricalcium phosphate

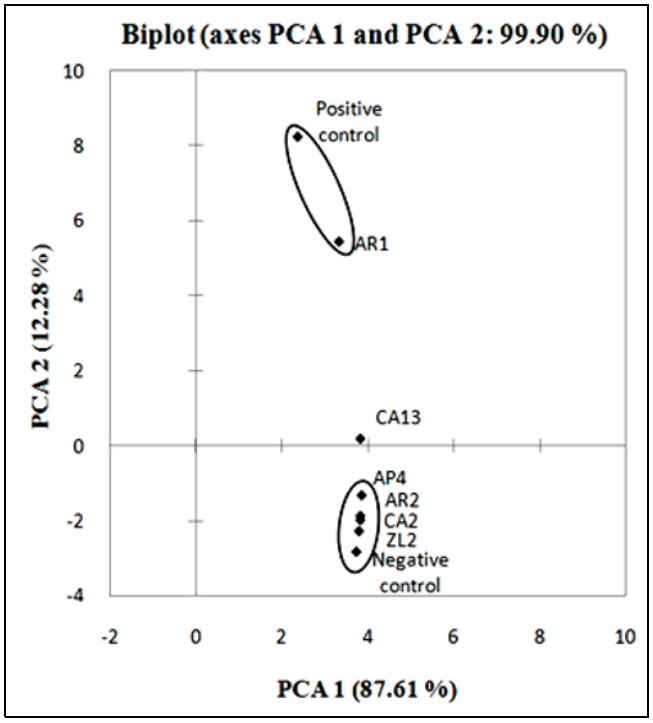

Fig. 2: PCA biplot with tomato seed treatments investigated for biocontrol effects on *Fusarium oxysporum* f. sp. *radicis lycopersici* root rot and plant growth-promoting effect, according to the first two main components.

variety of plant pathogenic fungi, such as *Pythium* (Hamdali et al. 2008), *Fusarium* (Toumatia et al. 2014) and *Rhizoctonia* (Goudjal et al. 2014).

In the biocontrol experiment with FORL, both sterilized and non-sterilized soils showed a high rate of root rot incidence for untreated tomato seeds in the positive (pathogen) control. This highlighted the virulence of the pathogen on tomato *cv.* Marmande, which was very sensitive. The treatment of seeds with Tebuconazole® significantly increased the proportion of healthy seedlings and reduced the root rot incidence by over 75%. These results are in agreement with those of Andresen et al. (2015), who reported the efficacy of seed treatment with Tebuconazole® in controlling *Fusarium* root rot at the seedling stage. However, an intensive use of chemical fungicides may cause serious ecological problems (Gerhardson 2002). In this case, biocontrol of such diseases could be a solution.

All the actinobacteria tested significantly reduced ($P < 0.05$) the disease incidence compared to the positive (pathogen) control. Five strains (ZL2, CA2, AP4, CA13 and AR2) reduced the root rot incidence by over 75%. Endophytic actinobacterial strains selected on the basis of *in vitro* antagonistic activities were effective in controlling FORL root rot in both soils. The antagonistic effect on the pathogen may have been the result of various as yet unexplored biocontrol mechanisms. Contrary to results of Errakhi et al. (2007), the negative effect of interaction with native soil microflora may suggest the presence of other competitive microorganisms.

Biocontrol of plant diseases is often combined with plant growth promotion activities (Hamdali et al. 2008). In our study, growth enhancement of tomato seedlings by the endophytic actinobacterial strains was investigated. The strain *Streptomyces* sp. Zl2 significantly increased the seedling length, root length, fresh weight and dry weight compared to the negative (healthy) control. These results are in agreement with those of El-Tarabily et al. (2009) and Goudjal et al. (2014), who reported the role of endophytic *Streptomyces* spp. isolates in promoting the growth of tomato seedlings.

The plant growth promotion by endophytic actinobacteria could involve various mechanisms. The production of phytohormones, such as IAA, solubilization of inorganic phosphate, and antagonistic activities on the phytopathogenic microorganisms by production of antibiotics and sid-

erophores appear to be the most important ones (El-Tarabily et al. 2009, Sadeghi et al. 2012). Consequently, the enhancement of plant growth by the strain *Streptomyces* sp. ZL2 could help to protect against the root rot of tomato seedlings as previously reported by El-Tarabily et al. (2009) for other *Streptomyces* spp.

Results for IAA production showed that the majority of endophytic actinobacteria produced this plant growth regulator. These findings are supported by several studies, which report that endophytic actinobacteria isolated from various plants are able to produce IAA (Ruanpanum et al. 2010, Goudjal et al. 2013). The isolate *Streptomyces* sp. ZL2 reached the maximum of IAA production (64 μg ml^{-1}). This was higher than the production by *Streptomyces* sp. CMU-MH021, which reached 28.5 μg ml^{-1} as reported by Ruanpanum et al. (2010).

Among the six endophytic actinobacteria tested, five isolates were able to produce siderophores on CAS medium. These results are in agreement with those of Cao et al. (2005), who specified the role of siderophore-producing endophytic *Streptomyces* sp. S96 in the antagonistic effect on *Fusarium oxysporum* f. sp. *cubense*. Furthermore, actinobacteria producing siderophores may enhance the uptake of iron by the plant when its bioavailability is low in the soil (Sadeghi et al. 2012).

The solubilization of inorganic phosphate is another mechanism by which actinobacteria may exert their effect in the promotion of plant growth (Hamdali et al. 2008). Five isolates, out of six, dissolved phosphate in TCP-PVK broth. Only the strains CA2 and ZL2 grew and released phosphate in both $AlPO_4$-PVK and $FePO_4$-PVK media. The amounts of dissolved phosphate showed that TCP, although a potentially insoluble phosphate, is not hard to dissolve compared with other mineral forms of phosphate (Al-P and Fe-P). Similar results were obtained by Park et al. (2010). Hamdali et al. (2008) studied phosphate-solubilizing actinobacteria for their ability to promote the growth of wheat. They showed that the isolate exhibiting the best level of released phosphorus also had the best enhancement of shoot and root growth, and inhibited phytopathogenic fungi.

The strain *Streptomyces* sp. ZL2, related to *S. caeruleatus*, showed the highest effect in the biocontrol of FORL root rot and the greatest plant growth-promoting effect. Several papers have reported the role of *Streptomyces* spp. isolates as potential candidates for the biocontrol of *Fusarium* spp. disease and in the promotion of plant growth (Cao et al. 2005, De Oliveira et al. 2010). Nevertheless, this is the first report highlighting similar properties for *Streptomyces caeruleatus* isolated from roots of *Zizyphus lotus* and its promising perspectives for possible application in crop improvement, in which case complementary studies will be necessary.

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