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Biocontrol of Chickpea wilt Disease by *Streptomyces* Sp. Isolated from the Rhizosphere of *Ononis angustissima* Lam.

[1,2]*Ghadbane Mouloud,* [1]*Medjekal Samir,* [2]*Belhadj Hani,* [1]*Bounar Rabah,*
[1]*Khellaf Rebbas,* [1]*Benderradji Laid,* [1]*Smaili Tahar and* [2]*Harzallah Daoud*

[1]Department of Natural and Life Sciences, Faculty of Sciences,
University of Mohamed Boudiaf-M'sila, P.O. Box: 166 Chebilia, M'sila, 28000, Algeria
[2]Laboratory of Applied Microbiology, Department of Microbiology,
Faculty of Natural and Life Sciences, University Ferhat Abbas, Setif 1 19000, Algeria

Abstract: Rhizospheric actinobacteria from *Ononis angustissima* Lam. were *in vitro* tested for their antagonism against deferent pathogenic microorganisms by streak assay and screened for their ability to produce extracellular enzymes and Indole acetic acid (IAA). Four isolates (21, 2A26, 1B10 and 2C34) present a potent antagonism against both pathogenic bacteria and fungi, they were selected, identified by16S rDNA sequence analysis and phenotypic properties and tested for their antimicrobial activity as well as their biocontrol potential against Chickpea (*Cicer arietinum* L.) pathogenic fungus (*Fusarium oxysporum*) under controlled conditions. Cultural characteristic and nucleotide sequence of the16S rRNA gene studies strongly suggested that these strains belong to the genus *Streptomyces*. The four *Streptomyces* sp., solubilize phosphate and produce extracellular fungal cell-wall degrading enzymes (chitinase and protease), as well as a produced relatively high levels of IAA. *In vivo* biocontrol assays revealed that the *Streptomyces* strains significantly promoted the growth of the Chickpea plants and showed greater suppression of chickpea wilt disease. These results indicate that the *Streptomyces* strains isolated for rhizosphere from *Ononis angustissima* Lam. growing in southern of Algeria (Biskra) could be an interesting source for antimicrobial bioactive substances and a potential biocontrol application in agriculture.

Key words: Antimicrobial Activity · Bioactive substances · Chitinase · 16S rDNA

INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum* (Schlechtend.:Fr.) f. sp. ciceris (Padwick) Matuo & K. Sato. [1], is the major soil-borne fungus affecting chickpea (*Cicer arietinum* L.). The most economic, effective and eco-friendly method of controlling chickpea wilt is by use of resistant cultivars, the effectiveness of which is limited by the existence of different races of pathogens. Moreover, evaluation of a large number of germplasm accessions, varieties and breeding lines for resistance to speci?c races of the pathogen is tedious, laborious, expensive, time consuming and is affected by inoculum load and environmental conditions [2, 3].

The actinobacteria represent a well-known and extremely diverse group of Gram-positive, aerobic, filamentous bacteria belonging to the order Actinomycetales. These bacteria are one of the major components of the microbial populations present in soil. They have the capacity to synthesize many different biologically active secondary metabolites such as antimicrobial agents. Bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms and produce many agriculturally and medically useful antibiotics [4,5], antifungal [6, 7], anticancer [8, 9], antioxidant [10, 11], insecticides [12], antifeedant agents [13] and herbicides [14], as well as plant growth hormones and enzymes [15, 16]. The search for new antimicrobials has not been limited to the medicinal field, but also extends to crop protection. Development of fungicide-resistant plant pathogens as well as excessive and indiscriminate use of synthetic agrochemicals has led to ecological imbalances in soil and human health [17]. Therefore, the search for

Corresponding Author: Medjekal Samir, Department of Natural and Life Sciences, Faculty of Sciences, University of Mohamed Boudiaf-M'sila, P.O. Box: 166 Chebilia, M'sila, 28000, Algeria.

alternatives to chemical control of plant pathogens, such as biological control, has gained momentum in recent years. Biological sources for the control of plant diseases remain an important objective for sustainable agricultural practices [18, 19]. The antifungal potential of extracellular metabolites from *Streptomyces* against some fungi was previously reported. *Streptomyces* species are used as biocontrol agents against phytopathogenic fungi [20, 21].

Microorganisms from extreme environments have gained considerable attention in recent years because of its diversity and biological activities, mainly due to its ability to produce novel chemical compounds of high commercial value [11]. Algerian desert soils are exposed to an arid climate and represent particular ecosystems. Previous studies have reported the abundance and diversity as well as secondary metabolites of actinobacteria in these soils [22, 23, 24]. The screening of actinobacteria from diverse rhizosphere of indigenous plants growing in Southern of Algeria (Sahara) deserves special attention to explore the potentialities of the diverse microflora of this region, as Biskra is part of the Algerian desert.

The objective of the present study was to isolate and identify actinobacteria strains from rhizosphere of indigenous plant *Ononis angustisima* Lam. growing in extreme environment (Sahara of Algeria) with the aim of testing their antimicrobial activity against pathogenic microorganisms and their biocontrol potential towards *Fusarium oxysporum*, a pathogenic fungus of Chickpea (*Cicer arietinum* L.).

MATERIALS AND METHODS

Sampling: The soil from the rhizosphere of indigenous plants, *Ononis angustissima* Lam., was taken from Southern of Algeria (Biskra), which is located in the Saharan region (Figure 1). Samples were collected in sterile cylinders, closed tightly and stored in the refrigerator at 4°C until use. For each collected sample, 10 g of the soil was suspended in 90 ml of physiological water (NaCl 9 g/l), then incubated in an orbital shaker incubator at 50°C with shaking for 30 min at 150 rpm. The suspension was serially diluted up to10^{-6}. An aliquot of 0.1 ml of the appropriate dilutions were taken and spread evenly over the surface of yeast extract-malt extract agar medium (ISP2) (International Streptomycete Project) [25], supplemented with streptomycin (2.5 mg/ml) and amphotericin B (75 mg/ml) to inhibit bacterial and fungal contamination, respectively. Plates were incubated at 28°C and growth development was monitored through 14 days. The isolates were maintained on ISP2 medium slants at 4°C and as a glycerol suspension 20 % (v/v) at -20°C.

Phenotypic Characteristics of the *Streptomyces* Strains: The morphology of the isolates was examined according to the methods recommended by Shirling and Gottlieb for the International Streptomycete Project (ISP) [25] and Bergey's manual of Systematic Bacteriology [26]. Visual observation using light microscopy and Gram-straining were performed for further identification. Cultural characteristics of pure isolates in various media (ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7) were recorded after incubation for 7 to 14 days at 28°C. Catalase and oxidase activities were determined with 3 % (v/v) hydrogen peroxide solution and 1% (v/v) tetramethyl p-phenylenediamine dihydrochloride solution, respectively. The color of mycelium and soluble pigment were examined in ISP7 medium and determined by comparison with the color of chips in the Color Harmony Manual [27]. Growth at various pH values (4 to 11), the tolerance to NaCl (for 1 %, to 12 %) and the temperature range for growth (5°C to 50°C) were examined on ISP2. Other physiological and biochemical characteristics were determined using APIZYM and API 50 CH strip (bioMérieux, SA, Marcy-l'Etoile, France) according to the manufacturer's instructions.

***In vitro* Antagonistic Bioassay:** The following test microorganisms were used to antagonistic bioassay of 40 actinobacteria: *Bacillus subtilis* ATCC 6633 (American Type Culture Collection, Manassas, VA, USA), *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* CIP 53-153 (Collection of Pasteur Institute, Algeria), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas syringae* pv. tomato 1086, *Salmonella typhi* CLMA 12 (Collection of Laboratory, Applied Microbiology, University Ferhat Abbas, Setif, Algeria), *Enterobacter cloacae* CLMA 04, *Citrobacter* spp. CLMA 4, *Fusarium oxysporum* CLMA 11, *Phytophthora* spp. CLMA 43, *Aspergilus flavus* CLMA 19, *Aspergilus niger* CLMA 57 and *Candida albicans* ATCC 24433. The actinobacterias were spot-inoculated on to ISP2 medium [25] and incubated at 28°C for 14 days. After this period, the antagonism between actinobacterias and the test microorganisms was evaluated using the streak assay [24]. All plates were incubated at 30°C and incubation time of 24–48 h for bacteria, 48-72 h for yeast and 7-10 days for fungi. All experiments were carried out in three replicates.

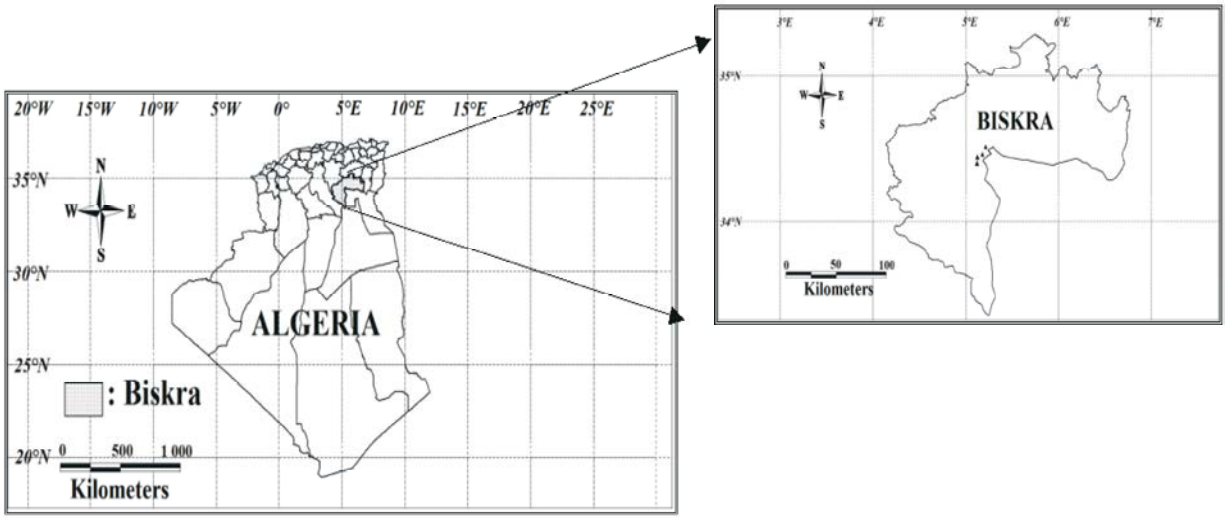

Fig. 1: A map showing the sites from which the rhizosphere soil samples of *Ononis angustissima* Lam. were collected

Genotypic Characteristics: The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using two universal primers: forward primer, 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer, 5'-AAGGAGGTGATCCAAGCC-3', designed from base positions 8 to 27 and 1541 to 1525, respectively. These are the conserved zones within the rRNA operon of *Escherichia coli* [28]. Genomics DNA of isolates 21, 2A26, 1B10 and 2C34 were used as a template for PCR amplification (35 cycle, 94°C for 30 s denaturation, 65°C for 1 min primer annealing and 72°C for 2 min extension). The amplified ~1.5 kb PCR product was cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA), leading to plasmid pGM21-16S, pGM2A26-16S, pGM1B10-16S and pGM1B10-16S (This study). The *E. coli* DH5α (F-supE44 Φ80 δlacZ ΔM15 Δ (lacZYA-argF) U169 endA1 recA1 hsdR17 (rk^-, mk+) deoR thi-1 λ- gyrA96 relA1) (Invitrogen Life Technologies) was used as a host strain. All recombinant clones of *E. coli* were grown in LB broth media with the addition of ampicillin, IPTG and X-gal for screening. DNA electrophoresis, DNA purification, restriction, ligation and transformation were all performed according to the method previously described by Sambrook *et al*. [29].

DNA Sequencing and Phylogenetic Analyses: The nucleotide sequence of 16S rRNA gene was determined on both strands using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the automated DNA sequencer ABI Prism® 3100-Avant Genetic Analyzer (Applied Biosystems). The 16S rDNA sequence analysis was performed by means of the BLAST program (www.ncbi.nlm.nih.gov/blast). Phylogenetic and molecular evolutionary analyses were conducted via the molecular evolutionary genetics analysis (MEGA) software version 4.1. Distances and clustering were calculated using the neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 100 resampling's [30].

Indole Acetic Acid (IAA) Production: The ability of actinobacteria to produce IAA was measured based on the colorimetric method described by Khamna et *al.* [31] and Kaur et *al.* [32], with some modifications. Three 6 mm discs of growing actinobacterias from yeast malt agar were inoculated into100 ml YM broth containing 0.05 % L-tryptophan and incubated at 28°C on a rotary shaker at 160 rpm for 7 days. Cultures were harvested and centrifuged at 10,000×g for 10 min at 4°C. There action mixture, which included 2 ml of supernatant and 2 ml of Salkowski reagent, was incubated at 25°C for 30 min in the dark. Absorbance of the reaction mixture was measured at 530 nm and the IAA content (mg ml^{-1}) of the culture filtrate was quantified using a standard curve with known concentrations of pure IAA (Sigma).

Phosphate Solubility: Phosphate solubility was conducted qualitatively according to the method described by Franco-Correa et *al*. [33] and Piromyou et *al*. [34]. Actinobacteria were spot-inoculated onto minimal medium based on the Pikovskaya (PVK) medium described by Pikovskaya [35]. This medium contained (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract,

0.5 g; $MnSO_4 \cdot H_2O$, 0.002 g; and $FeSO_4 \cdot 7H_2O$, 0.002 g, supplemented with agar 10 g. The dishes were incubated at 28°C for 7 days. A positive reaction was indicated by clear zones around the colonies.

Protease Production: It was done as per the protocols of Bhattacharya *et al*. [36]. Actinobacteria were streaked on casein agar medium and incubated at 28°C for 7 days. At the end of the incubation, the plates were observed for halo zone around the colonies, which indicates the presence of protease.

Chitinase Production: Chitinase activity was examined using the modified method described by Gupta *et al*. [37] and Kawase *et al*. [38]. The chitinase enzyme activity of the selected isolates was tested in nutrient agar medium containing 1% colloidal chitin. Chitinase production was assessed by visual examination of cleared zones developed around colonies incubated at 28°C for 7 days.

Biocontrol Essay: The Chickpea (*Cicer arietinum* L.) grains (variety FLIP86-10C, highly susceptible to Fusarium wilt) were surface sterilized by immersion in 70 % ethanol for 1 min, followed by continuous agitation in a 1 % sodium hypochlorite for 5 min and rinsed with sterile distilled water. Seeds were allowed to imbibe water overnight at room temperature. Pre-germinated seeds were grown in pots (40×32×14 cm, L×W×H) in sterilized sand: vermiculite mixture (1:1(v/v)). A fresh suspension of *Streptomyces* sp. (Strains 21, 2A26, 1B10 and 2C34) approximately 1×10^6 CFU/ml in 1 ml of ISP2 broth with 0.01 % Tween-20 and 1 ml of *F. oxysporum* approximately 1×10^5 CFU/ml were added to the planting mixture immediately before planting. Plants without *Streptomyces* sp. strains and *F. oxysporum* served as negative control and plants with *F. oxysporum* served as positive control. Seeds were placed on the surface of the planting mix approximately 2 cm from to the pot and covered with a 0.5 cm layer of the sterilized sand: vermiculite mixture. Plants were maintained in a growth room conditions with temperature 24°C, 16 h light / 8 h dark photoperiod and relative humidity of 75 %. Plants were fertilized weekly with a Murashige and Skoog (MS) solution [39] and watered as needed. The germination index and growth parameters, shoot height, shoot weight and root length were determined after 4 week. The experiment was conducted with three replicates per treatment.

Statistical Analyses: Data from the antimicrobial activity assay and biocontrol test of the four *Streptomyces* strains were analyzed by SAS software 9. Means of treatments for each experiment were compared by using Duncan Multiple Range Test ($P \leq 0.05$).

Nucleotide Accession Number: The partial sequences of the 16S rRNA gene of the four isolates were submitted to NCBI Gen Bank and the assigned accession numbers are: 21 (JQ042814), 2A26 (JQ042815), 1B10 (JQ042816) and 2C34 (JQ04281).

RESULTS AND DISCUSSION

A total of 40 *Streptomyces* spp. were obtained from the *Ononis angustissima* Lam. rhizosphere soil obtained from Southern of Algeria (Biskra). All isolates were confirmed as *Streptomyces* spp. based on morphological and cultural characteristics.

Identification and Molecular Phylogeny of the Microorganism: The identification of the newly actinobacteria was based on both phenotypic and molecular methods. Morphological, biochemical and physiological characteristics, according to the methods described in Bergey's Manual of Systematic Bacteriology [26] and International Streptomycete Project (ISP) [25], showed that the isolated strains appear aerobic, Gram-positive, non-motile, catalase and oxidase positive, that form extensively branched aerial and substrate hyphae. The aerial mycelium is grey to white in color and the substrate mycelium appears light yellow to brown (Table 1 and 2). Optimum growth occurs at 28°C. The pH range for growth was pH 6–11, with optimum growth at pH 7. Tolerates to NaCl concentrations up to 12 %. Soluble pigments are generated on ISP 2, ISP 3, ISP 5 and ISP 7. Melanin production was detected by 21 and 1B10 isolates but was not detected by 2C34 and 2A26 strains. According to the morphology of the spore chains observed under light microscopy, were as spiral shaped. The carbohydrate profile of the isolate was also investigated using API 50 CH strips. The results indicated that the four isolates utilized N-Acetyl glucosamine, L-Methyl-D-mannoside, Mannitol, Mannose, Fructose, Glucose, Galactose, D-Xylose, Ribose, Glycerol, Xylitol, L-Fucose, D-Tagatose, D-Turanose, Saccharose, Lactose, Cellobiose and Aesculine. The outcome from APIZYM tests revealed that while the activities exhibited by the

Table 1: Preliminary phenotypic identification tests for selected *Streptomyces* strains (21, 2A26, 1B10 and 2C34)

	Strains			
Test	21	2A26	1B10	2C34
Direct observation	Filamenteux	Filamenteux	Filamenteux	Filamenteux
Gram stain	+	+	+	+
Oxydase	+	+	+	+
Catalase	+	+	+	+
Indole Acetic Acid	+	+	+	+
Chitinase	+	+	+	+

Table 2: Morphological and physiological characteristics of the four actives *Streptomyces* isolates (21, 2A26, 1B10 and 2C34)

Characteristic	*Streptomyces* sp. 21	*Streptomyces* sp. 2A26	*Streptomyces* sp. 1B10	*Streptomyces* sp. 2C34
Spore Chain morphology	Spiral	Spiral	Spiral	Spiral
Aerial hyphae	White	white	White to grey	reddish gray
Melanin production	-	-	+	+
Maximum NaCl tolerance (%)	12	10	12	12
Colour of spore mass/substrate mycelium on:				
ISP2	White / Brown	White / Brown	Grey / Brown	Grey / Brown
ISP3	Grey / Yellow	Grey / Yellow	Brown / Brown	Brown / Yellow
ISP4	Grey / Brown	White / Brown	Yellow / Brown	Yellow / Yellow
ISP5	Grey / Brown	Grey / Yellow	Brown / Brown	Yellow / Yellow
ISP6	White / Yellow	Brown / Yellow	Brown / Brown	Black / Black
ISP7	Grey / Brown	Brown / Brown	Black / Black	Black / Black
Colour of soluble pigment on:				
ISP2	Reddish brown	Brown	Yellow	Reddish brown
ISP3	Yellow	Yellow	Brown	Brown
ISP4	Yellow	Reddish brown	Yellow	Yellow
ISP5	Yellow	Yellow	Brown	Yellow
ISP6	Yellow	Yellow	Brown	Black
ISP7	Yellow	Yellow	Black	Black
Temperatur°C				
10	-	-	-	-
20	-	+	-	-
25	+	+	+	+
30	+	+	+	+
35	+	+	+	+
45	+	-	+	+
pH				
5	-	-	-	-
6	-	-	+	+
7	+	+	+	+
11	+	+	+	+

four isolates for alkaline phosphatase, esterase lipase (C8), Leucine arylamidase, Valine arylamidase, Alpha-glucosidase (maltase), Beta-glucosidase (cellulase), N-acetyl-beta-glucosaminidase (chitinase) were positive. alppha-fucosidase, esterase (C4) was positive only for 1B10, cystine arylamidase posetive for strain 2A26; alpha-galactosidase (melibiase) positive for strains 2A26 and 2C34 and alpha-chymotrypsine is positive for strains 1B10 and 2C34.Trypsine, phosphatase acid, naphtol phosphohydrolase, beta-galactosidase (lactase), beta-glucuronidase (hyaluronidase), alpha-mannosidase were negative for all strains. All the data obtained with regard to the physiological and biochemical properties of the isolates, therefore, strongly confirmed that the strains 21, 2A26, 1B10 and 2C34 belonged to the *Streptomyces* genus.

In order to provide further support for the identification findings mentioned above, a 1517 bp fragment of the 16S rRNA gene was amplified from the genomic DNA of the 21, 2A26, 1B10 and 2C34 isolates, cloned in the pGM21, pGM2A26, pGM1B10 and pGM2C34 vectors and sequenced on both strands. The 16S rRNA gene sequence obtained was subjected to GenBank BLAST search analyses. A BLAST search of the GenBank

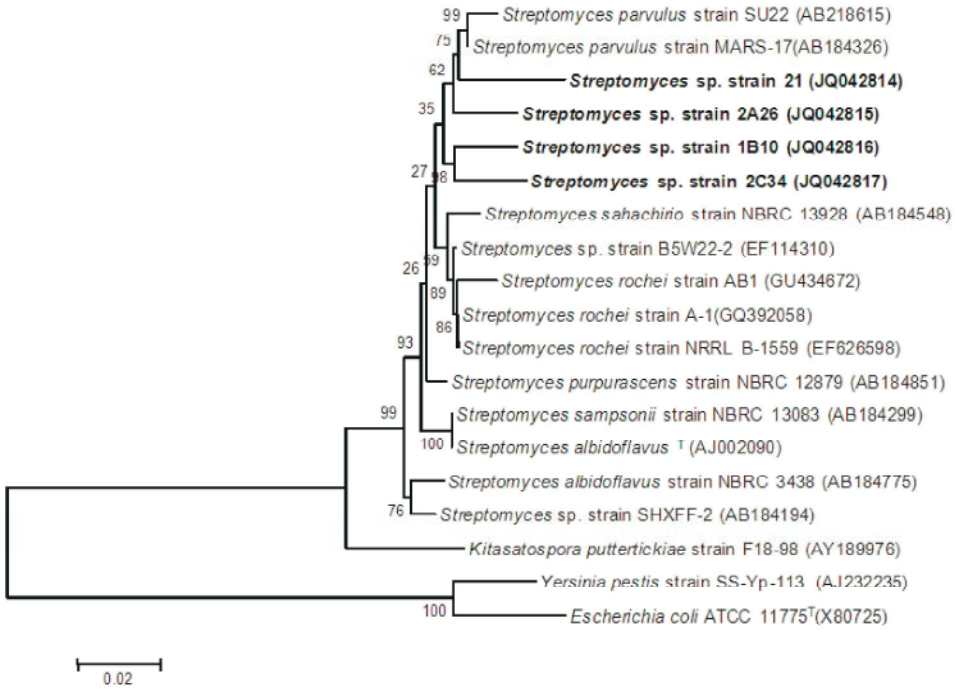

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequences showing the position of strains 21, 2A26, 1B10 and 2C34 within the radiation of the genus *Streptomyces* and other bacteria. The sequence of *E. coli* ATCC 11775^{T} (X80725) and *Yersinia pestis* ss-Yp-113 (AJ232235), were chosen arbitrarily as an outgroup. Bar, 0.02 nt substitutions per base. Numbers at nodes (> 50 %) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 100 bootstraps). NCBI accession numbers are presented in parentheses

database using the 16S rRNA gene sequence showed its similarity to that of many species of the genus *Streptomyces*. The 16S rRNA gene sequence of strain 21 showed levels of similarity of 75% to that of *Streptomyces parvulus* strain SU22 (GenBank accession no. AB218615) and *Streptomyces parvulus* strain MARS-17 (GenBank accession no. AB184326). The16S rRNA gene sequence of strains 2A26, 1B10 and 2C34 showed a similarity of 62 % to that of *Streptomyces parvulus* strain SU22 (GenBank accession no. AB218615) and *Streptomyces parvulus* strain MARS-17 (GenBank accession no. AB184326). Those sequences were imported in to a MEGA software V4.1 and aligned. Phylogenetic trees were then constructed (Figure 2) and the findings further confirmed that the 21, 2A26, 1B10 and 2C34 strain, were closely related to those of the *Streptomyces* strains. In summary, all the results obtained strongly suggested that those isolates should be assigned as *Streptomyces* sp. strains 21, 2A26, 1B10 and 2C34.

Screening of Phosphate Solubilizing Actinobacteria: Qualitative estimation of P solubilization by actinobacterias strains grown on Pikovskaya medium showed the development of a clear solubilization zone around the colony.

In this study, 40 isolates were evaluated and 14 (35%) isolates showed P-solubilizing activity in 7 days (Table 3). The four isolates 21, 2A26, 1B10 and 2C34 were able to solubilise phosphate which was confirmed by appearance of largest halos around their colonies (translucent areas) in PVK agar medium.

Phosphorus is considered as growth-limiting macronutrient. Phosphate solubilizing microorganisms have been employed in agriculture and horticulture and have been considered very important due to their potential of ecological amelioration [40].

The actinobacteria strains tested seems to have the ability of solubilizing P sources in soil. Among the several potential mechanisms for phosphate solubilization those involving the production of chelating compounds, like organic acids or by means of a modification of pH of the medium by the secretion of organic acids or protons are the more often described in the literature [35, 41, 33]. The potential for phosphate solubilization of the target actinobacteria supports future research for quantitative analysis.

Table 3: Evaluation of qualitative hydrolytic enzyme production (chitinase and protease) and phosphate solubilization by actinobacteria isolated from the rhizosphere of *Ononis angustissima* Lam

Isolats	Chitinase[a]	Protease[a]	Phosphate solubilization[b]
1C01	+	-	-
1C02	-	-	+
1C03	+	-	-
1C04	-	-	++
1A01	+	-	-
1A02	+	-	+
1A03	-	+	-
1A04	+	-	-
1A05	-	-	+
1A06	-	+	-
1A07	+	-	-
1A08	-	+	-
1A10	+	+	-
1D01	-	-	-
1D02	-	-	+
1D03	+	-	-
1D04	+	-	-
1D05	-	-	++
1D06	-	-	+
2D07	+	-	-
1A01	-	+	-
22	-	-	+
23	+	-	-
24	-	-	-
25	+	-	+
26	-	-	-
1A27	-	-	-
1A28	+	-	-
1A29	-	-	-
1A30	+	-	-
2C31	-	+	-
2C32	+	-	-
2C33	+	-	-
1C34	-	+	-
2C35	-	-	+
1B36	-	+	-
1B10	+	+	+++
2C34	+	+	+++
2A26	+	+	+++
21	+	+	+++
Percentage (%)	50	30	35

[a]+: presence of halo zone around the actinobacteria colonies .

[a]-: absence of halo zone around the actinobacteria colonies.

[b]+++: solubilisation halo ≥10mm; ++: 5mm< halo < 10mm; +: halo < 5mm; -: no solubilisation halo

Production of Plant Growth Promoting Hormone Indole Acetic Acid: The ability of the bacterial strains to produce IAA was detected by the development of pink color in ISP2 culture medium after the addition of salkowski reagen to the culture. Interestingly, *Streptomyces* sp. strains 21 (25,03 ± 2,54 µg ml^{-1}) and 2C34 (23,48± 2,70 µg ml^{-1}) produced highest amount of IAA production as compared to 1B10(19,95 ± 2,70 µg ml^{-1}) and 2A26 (13,10 ± 1,79 µg ml^{-1}) (Figure 3).

IAA affects plant cell division, extension and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light and gravity; affects photosynthesis, pigment formation, biosynthesis of various metabolites and resistance to stressful conditions [42, 43]. IAA production is another attribute that have very vital role in the plant growth promotion potential of these strains.

Production of Fungal Cell Wall-Degrading Enzymes: Several properties such as cell wall degrading enzyme production have been heavily reported as important mechanisms of antifungal action for various *Streptomyces* species [44, 45, 46, 47]. In this study, the production of fungal cell wall-degrading enzymes was examined because this is an important mechanism of fungal inhibition for PGPR agent. The chitinase production was shown for half of the strains (Table 3). Twenty (50%) rhizosphere strains showed fungal cell wall-degrading enzyme chitinase activity. Proteolytic activity was detected for twelve of 40 (30%). In general, the higher chitinase activity was correlated with higher fungal inhibition. For this reason, chitinolytic *Streptomyces* strains are a likely choice as potential biological control agents.

It has been reported that antifungal mechanism of antagonists has been attributed to the action of hydrolytic enzymes such as chitinase, [48] and protease [49].

Antagonisms: Actinobacteria strains exhibiting the ability to produce both clear zones of inhibition and metabolites against the tested pathogenic bacteria and fungi were considered antagonistic. A total of 40 actinobacterias, the most prominent isolates were isolated from the rhizosphere of *Ononis angustissima* and further screened for their antagonistic potential against pathogenic bacteria and fungi. The preliminary screening test for 40 actinobacterias isolates, confirmed that highest percentage of antifungal activity against fungi tested with 34 active isolates were obtained against *Fusarium oxysporum* 85 % followed by *Phytophthora* spp. (31 isolates) 77.5 %, *Aspergilus flavus* (25 isolates) 62.5 %, *Aspergilus niger* (23 isolates) 57.5 % and *Candida albicans* (23 isolates) 57.5 %, while the lowest

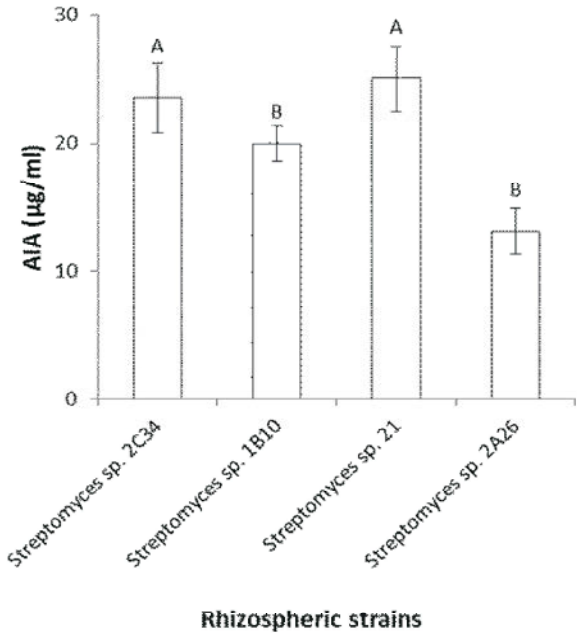

Fig. 3: Indole acetic acid (IAA) production by the four most potential antagonistic actinobacteria. The results are mean values of three data sets, (Duncan's test, $p < 0.05$), different lowercase letters on top of the histograms indicate significant differences

Table 4: Screening of *Streptomyces* sp. strains (21, 2A26, 1B10 and 2C34) for their antagonistic as measured by diameter of the inhibition zone of the indicator strain (mm)

	Diameter of inhibition zone (mm)* of *Streptomyces* sp. strains			
Indicator strains	21	2A26	1B10	2C34
Bacteria Gram$^+$				
Bacillus subtilis ATCC 6633	20.33±1.53^a	13.33±1.15^b	21.33±1.53^a	10.67±0.58^c
Staphylococcus aureus ATCC 25923	19.33±1.15^b	15.67±1.15^c	11.33±0.58^d	21.67±0.58^a
Bacteria Gram$^-$				
Pseudomonas aeruginosa ATCC	00.00	00.00	00.00	0.00
Pseudomonas syringae pv. tomato 1086	00.00	00.00	00.00	0.00
Escherichia coli ATCC 25922	00.00	00.00	00.00	0.00
Klebsiella pneumonia CIP 53-153	00.00	00.00	00.00	0.00
Salmonella typhi CLMA 12	23.67±1.53^a	00.00	00.00	21.00±1.00^b
Enterobacter cloacae CLMA 04	00.00	00.00	00.00	00.00
Citrobacter spp. CLMA 4	00.00	00.00	00.00	00.00
Fungi				
Fusarium oxysporum CLMA 11	22.33±0.58^b	14.67±0.58^c	25.67±0.58^a	11.00±1.00^d
Candida albicans ATCC 24433	30.33±0.58^a	21.00±1.00^c	24.00±1.00^b	19.00±1.00^d
Aspergilus flavus CLMA 19	20.00±1.00^b	14.67±0.58^d	17.33±0.58^c	15.00±1.00^d
Aspergilus niger CLMA 57	24.33±1.15^a	10.33±0.58^d	21.33±1.15^b	14.33±0.58^c
Phytophthora spp. CLMA 43	20.67±1.53^b	23.67±1.53^a	17.33±1.15^c	15.00±1.00^d

*: Values in the table are means of three independent experiments and error bars indicates standard deviation of the mean.
Letter: a, b, c, dc, d show significant deference using Duncan's test ($p > 0.05$)

percentage was obtained against Gram positive bacteria 10% (4 isolates). Moreover, these isolates showed antagonistic activity against only one Gram-negative bacteria 5 % (Table 3). This preliminary screening revealed that the 40 actinobacterias isolates were a good antifungal and moderate antibacterial compound producer. Consequently, the previous above screening test indicated four *Streptomyces* (strains 21, 2A26, 1B10 and 2C34) showed high potencies especially against four fungi; tow Gram positive bacteria and one Gram negative bacteria (Table 4). In general, the actinobacterias appear to be more inhibitory to fungi and Gram positive bacteria

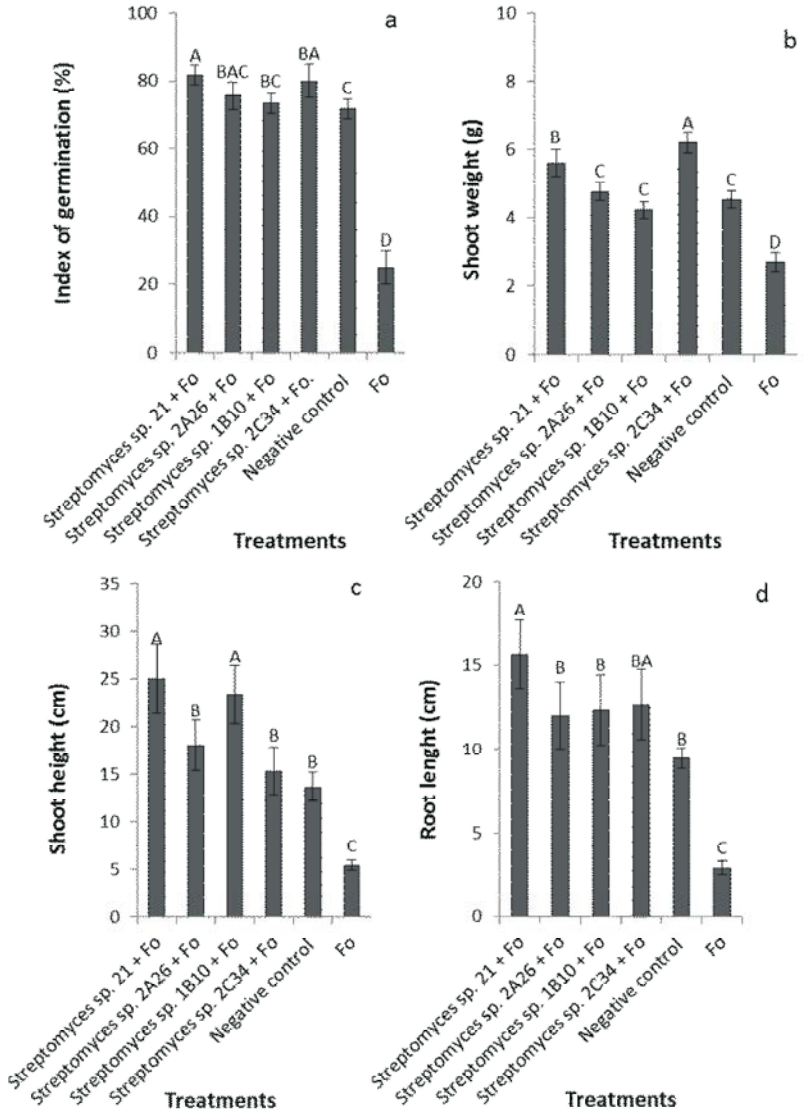

Fig. 4: Effect of *Strptomyces* sp. strains (21, 1B10, 2A26 and 2C34) on the germination index (a), shoot height (b), shoot weight (c) and root length (d) of Chickpea (*Cicer arietinum* L.) inoculated with pathogenic fungus (*F. oxysporum*). Duncan's test ($p > 0.05$) showed that the histograms using different letters (A, B, BA, BAC, BC and C) were significantly different. Values are means of three independent experiments and error bars indicates standard deviation. Control negative (no inoculated plants), Fo: *Fusarium oxysporum*

than to the Gram-negative bacteria. Like in earlier studies, the *Streptomyces* strains we isolated possessed more antifungal than antibacterial properties [50, 51]. The antagonism of *Streptomyces* observed in this study is in accordance with previous reports [52, 31]. According to [52], *Streptomyces* sp.TK-VL_333 showed antagonistic activity toward a variety of bacteria, yeast and filamentous fungi. Verma *et al*. [53], observed that approximately 60 % of the isolated actinobacterias showed wide-spectrum antimicrobial activity against bacteria and fungi. These results con?rm that the actinobacterias are able to produce a wide variety of antibiotics and other compounds with antibacterial and antifungal activity. These results indicate that the rhizosphere of *Ononis angustissima* Lam. is a useful potential source for isolation of actinobacterias producers of biological active products.

Biocontrol Agents: Microbial antagonists are widely used for the biocontrol of fungal plant diseases. Many species of actinobacteria, particularly those belonging to the genus *Streptomyces*, are well known as antifungal

biocontrol agents that inhibit several plant pathogenic fungi. The results in this study indicate that seed germination was strongly inhibited when treated with pathogenic fungi. The findings of the current study also indicated that the Chickpea seedlings coating *F. oxysporum* treated with inoculum of 21, 2A26, 1B10 and 2C34 strains, increase significantly the percentage of germination as compared to no inoculeted or inoculated with pathogen alone consecutively (Figure 4a). Initial vigour response was measured using traditional agronomic parameters, namely root length and shoot weight and height. In fact, the results indicated that vigour response with respect to 21, 2A26, 1B10 and 2C34 was significantly higher than *F. oxysporum* treatment (Figure 4b,c,d). Nevertheless, 2A26 and 1B10 was noted to have a negative effect on the shoot weight of the plant compared to uninoculeted seed, bringing about a significant reduction in shoot weight (Figure 4b). The germinative energy can play an important role in the achievement of quick and uniform seedling emergence and the reduction of damping-off incidences, thus improving the yield. Khaleeq and Khan [54], reported that the use of fungicides is effective in enhancing germination, emergence and growth as well as in reducing damping-off. In addition, accelerated germination is reported to help improve stress resistance and enhance overall plant growth and productivity [55, 56, 57]. The results obtained for the IAA, chitinase and antifungal compounds production, by the four *Streptomyces* strains showing a positive reaction. It should be noted that one of the possible antifungal mechanisms of the strains 21, 2A26, 1B10 and 2C34 may be associated with the production of antifungal agent, extracellular chitinase enzymes and IAA. *Streptomyces* species have been reported as biocontrol agents effective against numerous plant pathogens [58, 59, 60, 61, 6, 62]. Thus, these *Streptomyces* sp. strains can also play a role in plant development, considering that growth promotion effects may be related to IAA production [31, 63], as reported in other studies, this protective effect might be increased by the ability of the selected actinobacteria strains to excrete chitinases [58, 64], nitrogen fixation [33], siderophores production [65, 66] or other antifungal substances [67, 68]. Singh and Chhatpar [20], also attributed the activity of *Streptomyces* sp. A6 against fungal plant pathogens to production of mycolytic enzymes and an unknown antifungal metabolite.

CONCLUSION

Rhizospheric soil of *Ononis angustissima* Lam. growing in Sahara of Algeria contains abundant actinobacteria species capable of producing different antimicrobial compounds. The isolates 21, 2A26, 1B10 and 2C34 were selected for their antimicrobial activity against pathogenic microorganism and identified as *Streptomyces* sp. by 16S rRNA.

The potential of the isolate is determined by its antimicrobial activity, IAA production, chitinase activity and phosphate solubility. Results of the present study indicated that these strains clearly have a potential as biological control agents against *Fusarium oxysporum* and plant growth-promoting effect under controlled conditions. Further studies are necessary, to determinate of the exact mechanisms of action of these biocontrol agents, evaluate the effect of their biocontrol potential in greenhouse and field conditions, compared with the commercial fungicides and also to purify and characterize the secondary metabolites produced by these *Streptomyces*.

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