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An actinomycete strain named IA1, which produced an antimicrobial compound, was isolated from a Saharan soil in In Amenas, Algeria. The study of the 16S rDNA sequence of this strain permitted to relate it to *Streptomyces mutabilis* NBRC 12800^T (99.93% of similarity). Strain IA1 exhibited strong activity against a wide range of plant pathogenic fungi. One bioactive compound produced in large amounts (46.7 mg L^{-1} day^{-1}), named YA, was isolated and purified by TLC and reverse phase HPLC. The structure elucidation of the pure substance, using combined data from UV visible, NMR spectra, and mass spectrometry, permitted to identify it as actinomycin D, and was thus found for the first time in *S. mutabilis* related species. The biocontrol abilities of the strain IA1 and compound YA were evaluated through two diseases, i.e., chocolate spot of field bean and *Fusarium* wilt of flax. The occurrence of the two fungal diseases was effectively reduced. The reduction of chocolate spot disease symptoms reached 80 and 91.7% with IA1 and YA seedlings pretreatments, respectively. Soil pretreatment with IA1 or YA also allowed to reduce *Fusarium* wilt disease impact by almost 60%.

Keywords: *Streptomyces* / Actinomycin D / Biocontrol / Chocolate spot / *Fusarium* wilt

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Introduction

The actinomycetes are ubiquitous Gram-positive bacteria with a higher percentage of guanine–cytosine (55%), and most of them produce mycelia. Members of this group are also considered as the most important antibiotic-producing organisms [1]. Among the actinomycetes, 80% of antibiotic-producing microorganisms are members of the *Streptomyces* genus [1]. One of the strategies for enhancing the likelihood of obtaining particular isolates and secondary metabolites is to analyze uncommon ecosystems such as arid soils [2, 3]. Previous surveys on the ecological distribution of actinomycetes in soils of the Algerian Sahara have already demonstrated their appreciable biodiversity [4].

Several studies have been reported a large number of bioactive molecules produced by *Streptomyces*, which were mainly investigated with respect to their effects against pathogenic strains in the medical field and also in the treatment of carcinomas with fairly good results, suggesting the possibility of several decades of widespread investigations [5, 6]. One such molecule, widely produced by members of *Streptomyces* genus is the actinomycin D (Act-D) [7]. However, regardless to its

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effective antimicrobial properties, Act-D was proven to be highly toxic to animal cells [8] and has therefore been most extensively studied for the treatment of malignant tumors [9]. Act-D is also commonly used for laboratory applications in cell biology since it inhibits RNA synthesis [10]. Act-D fluorescent derivative, 7-amino-actinomycin D, is used as a dye in microscopy and flow cytometry to distinguish viable and apoptotic cells [11] but investigations on its potential use as a biopesticide remain scarce [12].

Furthermore, the use of antagonistic *Streptomyces* for agricultural purposes is still rarely investigated even though two biofungicides have been approved and marketed: Mycostop® (*Streptomyces griseoviridis* strain K61) and Actinovate® (*Streptomyces lydicus* strain WYEC108), which are being used for controlling crop damping-off [13, 14]. On the other hand, several *Streptomyces* strains originating from Saharan soils have already demonstrated their potential use as biocontrol agents [15–17].

In the present investigation, we report the taxonomy and antimicrobial activities of *Streptomyces* sp. IA1 strain isolated from a Saharan soil sample. The bioactive compound production, purification, and the structure elucidation were also investigated. The biocontrol ability of IA1 strain and its active compound were then evaluated toward two different plant-pathogen systems: chocolate spot of the field bean and *Fusarium* wilt of flax, caused by *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *lini*, respectively. These fungi induce serious diseases, which result in important yield losses in the field [18, 19].

Materials and methods

Strain isolation

During an investigation of actinomycetes diversity in Saharan soils of Algeria, strain IA1 was selectively isolated by a serial dilution agar plating method from a soil sample (10 cm depth) collected in In Amenas (latitude, 28°02′N; longitude, 09°56′E; altitude, 587 m). Aliquots (0.2 ml) of each dilution were spread onto chitin-vitamins agar medium [20] supplemented with cycloheximide (80 mg L^{-1}) and rifampicin (10 mg L^{-1}) to inhibit the growth of unwanted fungi and bacteria, respectively. The plates were incubated at 30 °C for 2 weeks.

Antimicrobial activity

The antimicrobial activity was evaluated by the cross-streak assay method. The strain IA1 was first inoculated by streaking a straight line of its inoculum on ISP-2 (International *Streptomyces* Project) medium [21]. Plates were then incubated for 10 days at 30 °C. After that, target microorganisms were seeded in streaks perpendicular to the actinomycete margin. The antimicrobial activity was evaluated by measuring the distance of inhibition between the target microorganism and actinomycete colony margins, after incubation for 36 h at 30 °C. The target microorganisms (listed in Table 1) were various plant-pathogenic filamentous fungi.

Molecular characteristics of the strain IA1

For molecular analysis, DNA was extracted according to the method of Liu *et al.* [22]. The strain IA1 was grown at 30 °C for 4 days with agitation (250 rpm) in a 500 ml flask containing 100 ml of ISP-2 medium. The 16S rDNA was amplified by PCR using an Invitrogen kit and two primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′), as described previously [16]. The PCR products obtained were submitted to MilleGen Company (Toulouse, France) for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The sequences obtained were compared with sequences present in the public sequence databases as well as with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; [23]), a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains.

Kinetics of antifungal activity

Fermentation of the strain IA1 was conducted in ISP-2 broth medium for 10 days in order to select the culture

Table 1. Antifungal activity[a] of the strain IA1 toward several pathogenic fungi.

Target fungi	Zone of inhibition (mm)[b]
Botrytis cinerea	40.0 ± 1.0
Fusarium oxysporum f. sp. *albedinis*	34.7 ± 0.6
F. oxysporum. f. sp. *lini*	40.3 ± 0.6
F. oxysporum f. sp. *radicis-lycopersici*	37.0 ± 2.6
F. culmorum	41.0 ± 1.0
F. graminearum	40.3 ± 0.6
F. sporotrichoides	35.3 ± 0.6
F. equiseti	41.0 ± 1.0
F. moniliforme	44.0 ± 1.0
F. proliferatum	42.3 ± 1.5
Aspergillus carbonarius	45.0 ± 1.0
A. niger	42.3 ± 1.5
A. flavus	23.0 ± 2.6
A. ochraceus	30.3 ± 1.5
A. parasiticus	19.3 ± 1.1
Penicillium glabrum	24.3 ± 0.6
Umbelopsis ramanniana	44.3 ± 1.1

[a]Activity estimated by measuring the length of inhibition between strain IA1 and target microorganism.
[b]The data shown are the mean of three independent replicates ± standard deviation.

time favorable for active compound production. Three milliliters of seed culture was prepared with the same medium, incubated at 30 °C for 2 days and used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of medium. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C. The antimicrobial activity of the culture broth was monitored by the conventional agar diffusion assay (well technique) against *B. cinerea* and *F. oxysporum* f. sp. *lini*.

Extraction and purification of the bioactive compound YA

Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60HF 254-366; 20 cm × 20 cm) was employed for the partial purification of antimicrobial products. TLC plates were developed in the ethyl acetate–methanol solvent system, 100:15 v/v. The developed TLC plates were air dried overnight to remove all traces of the solvents. The compounds separated were visualized with the naked eye, under UV at 254 nm (absorbance) and at 365 nm (fluorescence). The bioactive spot was detected by bioautography [24] toward the two previously cited target fungi. The retention factor (Rf) of the bioactive spot was measured. The final purification of the most active compound (YA) was performed by HPLC on reverse phase XBridge C18 (5 μm) column (200 mm × 10 mm; Waters, Milford, MA) with a linear gradient of acetonitrile–H_2O (50–100% for 40 min), a flow rate of 1 ml min^{-1} and UV detection at 220 nm. The final purification was achieved after the second re-injection in the HPLC system.

Determination of the bioactive compound YA structure

The structure of the compound YA was mainly elucidated with the aid of spectroscopic investigations. The UV spectrum was given with a Shimadzu UV 1605 spectrophotometer. The mass spectrum was recorded on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) with nanospray ion electro-spray ionization (ESI) source (positive and negative ion mode). ^1H and ^{13}C NMR spectroscopy were used for the characterization of the active molecules. The NMR sample was prepared by dissolving 3 mg of purified compound in 600 ml of CD_3OD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 5-mm triple-resonance inverse Z-gradient probe (TBI 1H, 31P, BB). All the chemical shifts for ^1H and ^{13}C were relative to TMS using ^1H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the ^1H and ^{13}C signals were assigned on the basis of chemical shifts, spin–spin coupling constants, splitting patterns, and signal intensities, and by using ^1H-^1H COSY45, ^1H-^{13}C HSQC, and ^1H-^{13}C HMBC experiments. Gradient-enhanced ^1H COSY45 was realized included 36 scans per increment. ^1H-^{13}C correlation spectra using a gradient-enhanced HSQC sequence (delay was optimized for 1JCH of 145 Hz) were obtained with 200 scans per increment. A gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (340 scans were accumulated). Typically, 2048 t2 data points were collected for 256 t1 increments.

Biocontrol properties of the strain IA1

The strain IA1 and its highly produced bioactive compound named YA (yellow antibiotic) were assessed in biocontrol trials. Biocontrol abilities were evaluated through two different plant–pathogen disease systems: chocolate spot of field bean and *Fusarium* wilt of flax caused by *B. cinerea* and *F. oxysporum* f. sp. *lini*, respectively.

Seed material

Seeds of field bean (*Vicia faba* L., variety Giza 429) and flax (*Linum usitatissimum* L., variety Hera) were supplied by the Technical Institute of Field Crops, Algiers, Algeria. Prior to use, all seeds were surface-sterilized (5% w/v NaClO; 0.2% w/v Tween 20) for 3 min for flax seeds or 15 min for field bean, and then rinsed five times with sterile distilled water.

Fungi and strain IA1 inocula preparation

Pathogenic fungi strains, isolated from diseased plant culture of flax and field bean, were supplied by the Department of Botany, High School of Agriculture, Algiers, Algeria. Prior to use, fungi isolates were subcultured on potato dextrose agar (PDA) plates and incubated at 25 °C for 7 days. Strain IA1 was grown on ISP-2 medium plates and incubated at 30 °C for 10 days. Suspensions of both fungal conidia and actinomycete spores were obtained by scraping from the culture surface with a glass slide, homogenized in sterile distilled water (0.2% w/v Tween 20) and filtered through a double layer of sterile gauze [16]. The concentrations were adjusted by hemocytometer chamber counting method.

Plant growing conditions

Seedlings were placed in a phytotronic growth chamber with 80% relative humidity, a temperature of 22 ± 3 °C and 14 h of light (8000 Lux) period conditions.

Biocontrol assay of chocolate spot disease

Field bean seeds were first pre-germinated in Petri dishes containing sterile wet paper for 3 days in darkness at 15 °C, than sown in pots (five seeds per pot, 1 cm depth) filled (100 g per pot) with sterile rhizospheric soil (autoclaved at 120 °C for 20 min, three times, once

each 24 h). After 3 weeks of growth (three leaf stage) seedlings were sprayed with a suspension of IA1 spores (1.7×10^7 CFU ml^{-1}; 1.2 ml per plant) or an aqueous solution of the YA compound (1 mg ml^{-1}; 1.2 ml per plant) right after the inoculation of *B. cinerea* (4×10^5 CFU ml^{-1}; 1.2 ml per plant) to seedlings and drying. Visible chocolate spot symptoms on leaves were scored up to 3 weeks post-infection. Seedlings sprayed only with spores suspension of *B. cinerea* (4×10^5 CFU ml^{-1}; 1.2 ml per plant) acted as control.

Biocontrol assay of the *Fusarium* wilt disease

Flax seeds were sown in pots (15 seeds per pot, 1 cm depth) containing sterile rhizospheric soil (100 g per pot) pre-inoculated with *F. oxysporum* f. sp. *lini* spores suspension (5×10^4 CFU g^{-1} of dry soil) and with either the actinobacterium spores suspension (5×10^8 CFU g^{-1} of dry soil) or with YA compound (5 mg/100 g of soil). Before being transferred to the phytotronic growth chamber, pots were kept in darkness for 3 days at 15 °C to support seed germination. Visible *Fusarium* wilt symptoms on the plants were scored up to 5 weeks post-infection. Soil inoculated with *F. oxysporum* f. sp. *lini* spores (5×10^4 CFU g^{-1} of dry soil) correspond to the control. The presence of the pathogen was verified by reisolating it from diseased seedlings by placing parts of infested tissues (surface sterilized) on PDA medium.

Data analysis

All experiments were repeated three times. Biocontrol experiments were conducted in a randomized design and the data obtained were analyzed by an analysis of variance (ANOVA) using Newman and Keuls multiple range test for mean separation. For all data, significance was evaluated at the probability level of $p \leq 0.05$.

Results

Antimicrobial activity

The strain IA1 showed a broad spectrum of antifungal activity (Table 1 and Supporting Information Fig. S1) since it was active against all target microorganisms (distance of inhibition between 19 and 45 mm). The strongest activities were observed against the fungi *Fusarium culmorum*, *F. equiseti*, *F. moniliforme*, *F. proliferatum*, *Aspergillus carbonarius*, *A. niger*, and *Umbelopsis ramanniana*.

Taxonomic description of the isolate IA1

The isolate IA1 grew well on all media used. It formed non-fragmented and yellowish brown substrate mycelium. The aerial mycelium was light to medium gray and produced short chains of spores (3–10 spores per chain) carried by sporophores. The spore chains were arranged in open spirals (one to three coils), loops, and hooks. The spores were elliptical to cylindrical and have 1.3–1.5 by 0.7–0.9 μm in size. Sporangia, endospores, sclerotic granules, synnemata, and flagellated spores were not observed. Abundant bright yellow diffusible pigment was produced on all of the media used after 4 days of incubation. These features fulfill the criteria of the gray and S-type morphological group of *Streptomyces*.

The 16S rDNA sequence (1475 nucleotides) of strain IA1 has been determined and deposited in the GenBank data library under the accession number KC414003. The sequences were compared with those reference species of prokaryotes available in the GenBank database, which confirmed that this strain belonged to the *Streptomyces* genus and was related with *Streptomyces mutabilis* NBRC 12800^T at 99.93% similarity level.

Kinetics of antifungal activity

The time course of the antimicrobial compounds production was monitored in ISP-2 broth medium, as shown in Fig. 1 and in Supporting Information Fig. S2. We distinguish a log phase, a stationary phase, and a slight decline phase followed by another stationary phase. The antimicrobial activities were observed on the first day of fermentation against *B. cinerea* and *F. oxysporum* f. sp. *lini*, and exhibited three maxima after 2, 5, and 9 days. The pH kinetic showed a slight variation (between 7.0 and 8.1) during the incubation.

Production and purification of the bioactive molecule YA

The extraction of compound YA took place on the day of optimal production rate. The ISP-2 culture broth was centrifuged to remove the biomass. The cell-free supernatant was extracted by *n*-butanol. The yellow

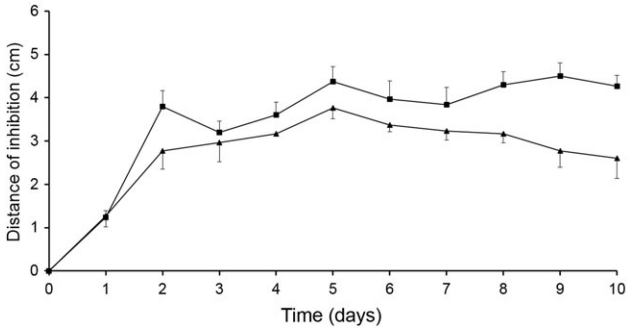

Figure 1. Antifungal activity of strain IA1 in ISP-2 broth medium against *Botrytis cinerea* (▲) and *Fusarium oxysporum* f. sp. *lini* (■). Bars indicate standard deviation of the mean.

organic phase was concentrated to dryness. The TLC plate analysis showed the presence of four spots. Only one of them, designated YA, was active and was detected by bioautography at Rf = 0.7 and exhibited strong antifungal activities. The HPLC profile of YA showed one peak at a retention time of 21.50 min. The pure substance had a strong yellow color. A quantity of 1.4 g of purified YA, characterized by a deep yellow-orange color, was obtained from 6 L of culture filtrate (average yield = 46.7 mg L^{-1} day^{-1}) for subsequent chemical structure and biocontrol investigations.

Structure elucidation of compound YA

The UV–visible spectrum of the compound YA in methanol (data not shown) exhibited a maximum absorption at 202.1, 242.4, and 444.8 nm. The absence of the three characteristic maxima of polyenes indicated that the compound YA was not polyenic. The ESI-MS spectrum contained an ion peak at *m/z* 1253 $[M-H]^-$ (Fig. 2). Thus, the molecular weight of this compound was $M = 1254$.

The ^1H and ^{13}C NMR 2D correlation experiments (Fig. 3) revealed the presence of five amino-acid residues (threonine, valine, proline, sarcosine, and methyl-valine) in both pentapeptide lactone rings and the presence of an aromatic chromophore. Due to the small amount available, a complete set of ^{13}C NMR data could not be obtained for the compound YA, which precluded complete NMR attribution of the chromophore. However, by combination with the mass spectra, and by matching these data to those of compounds deposited using chemical research tools (SciFinder, version 2007.1 and ChemSpider: http://www.chemspider.com/Structure-Search.aspx) the structure of the compound YA could be established as Act-D. The ^1H and ^{13}C NMR assignment is in a very good agreement with that of pure Act-D dissolved in DMSO [25].

Biocontrol properties

Chocolate spot. Leaves treatment with strain IA1 or the active compound YA extensively reduced chocolate spot symptoms development in field bean in comparison with pre-infested and non-treated plants (control) (Fig. 4a and Supporting Information Fig. S3). Three weeks post-infection, the treatment with compound YA was slightly more protective than strain IA1 (91.7 and 80% disease symptom reduction, respectively).

Fusarium wilt. Compared with pre-infested and non-treated soil (control), soils with the strain IA1 or the compound YA showed a significant reduction of wilt symptoms on flax seedlings (Fig. 4b and Supporting Information Fig. S4). The protective effect of IA1 and YA

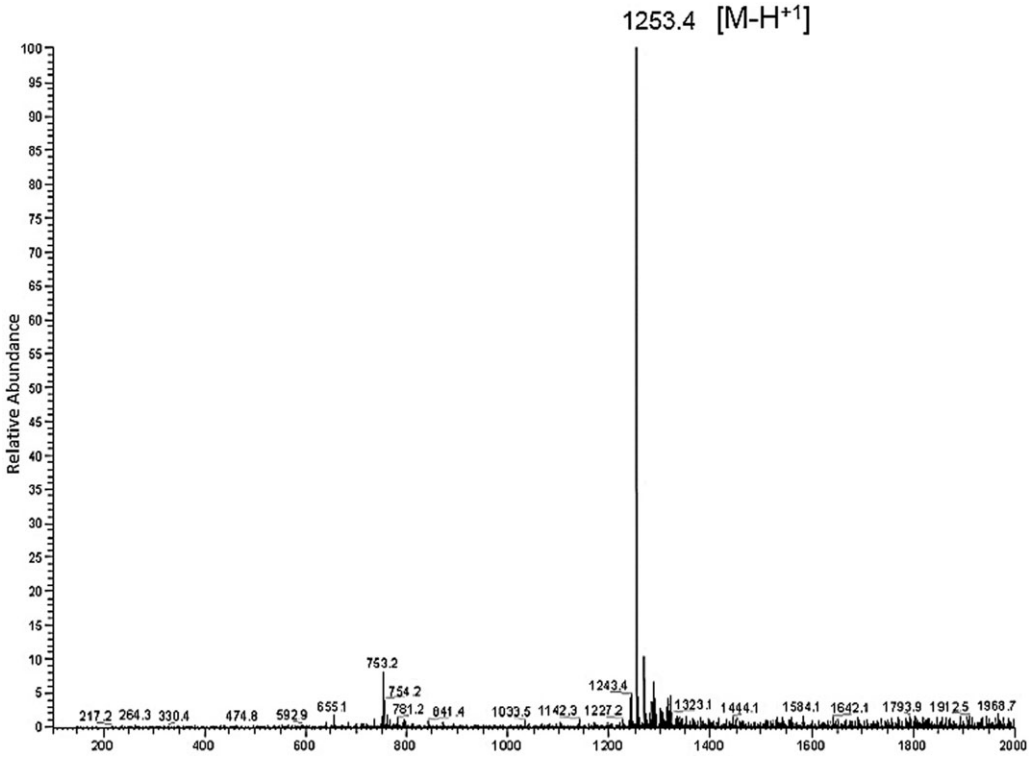

Figure 2. Nanospray ion electron-spray ionization–mass spectrum of compound YA in negative mode.

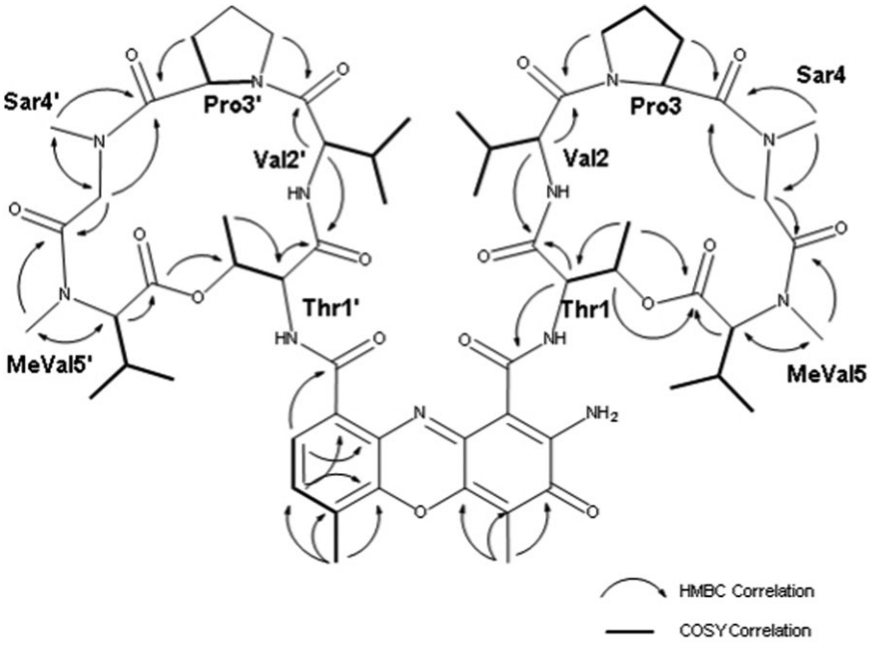

Figure 3. HMBC and COSY correlations of the compound YA. MeVal, methyl-valine; Pro, proline; Sar, sarcosine; Thr, threonine; Val, valine.

were equivalent and were effective from the 3rd week post-infection, while the reduction of the disease impact reached 61.7 and 60%, respectively at the end of the assay.

Discussion

Actinomycete strain IA1, which exhibited intense antimicrobial activity, was effectively isolated on a

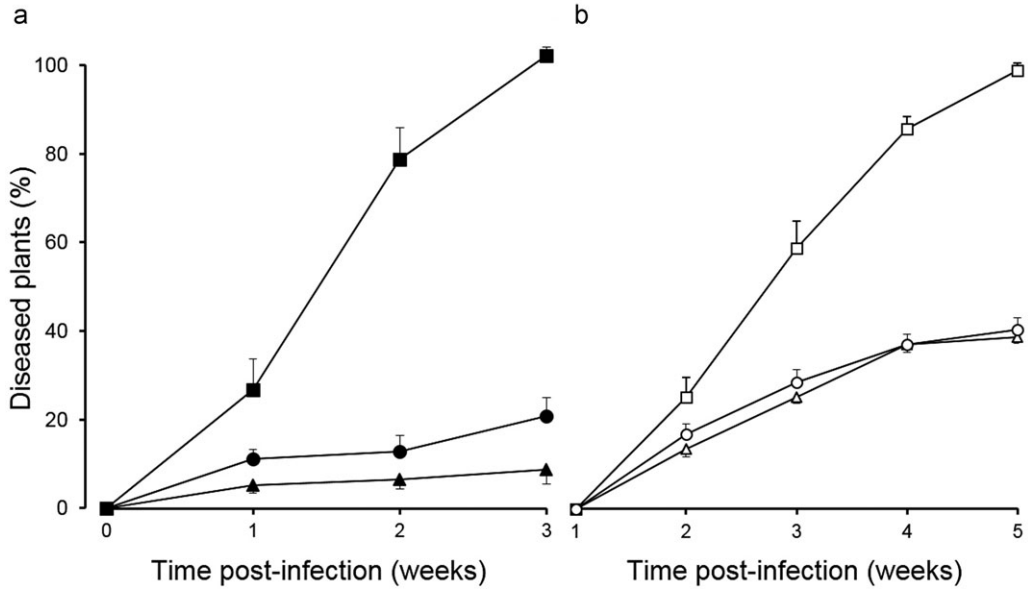

Figure 4. Effect of strain IA1 and compound YA treatments on the expression of the chocolate spot disease of field bean (a) and *Fusarium* wilt disease of flax (b). Control treatment corresponding to seedlings infested with Bc (4×10^5 CFU ml^{-1}; 1.2 ml per plant) (■); seedlings coinoculated with *Botrytis cinerea* (Bc) and IA1 (1.7×10^7 CFU ml^{-1}; 1.2 ml per plant) (●); seedlings coinoculated with Bc and YA (1 mg ml^{-1}; 1.2 ml per plant) (▲); control treatment corresponding to sterile rhizospheric soil infested with 5×10^4 CFU g^{-1} of dry soil of Fol (□); soil coinoculated with *Fusarium oxysporum* f. sp. *lini* (Fol) and IA1 (5×10^8 CFU g^{-1} of dry soil) (○); soil coinoculated with Fol and YA (5 mg/100 g^{-1} of soil) (Δ). Bars indicate standard deviation of the mean.

rifampicin-added medium. The use of antibiotics as selective agents has already been mentioned as a successful method for the isolation of interesting strains originating from Saharan soils [4] and permitted to find out novel species and antibiotics [26, 27].

Based on its phenotypical characteristics, strain IA1 belonged to the genus *Streptomyces* [28]. Species of this genus are broadly known for the production of bioactive molecules, which have been estimated to represent 80% of the compounds secreted by actinomycetes and more than one third of the total substances produced by microorganisms [1].

The 16S rDNA gene sequencing of the strain IA1 confirmed its identification at the genus level and permitted to relate it to the species *S. mutabilis* NBRC 12800^{T} [28] with 99.93% of similarity. However, unlike *S. mutabilis*, strain IA1 was observed to produce an abundant deep yellow pigment, which proved to be bioactive.

In Algeria, during studies looking for new active compounds, several strains of actinomycetes isolated from soil samples of the Sahara have exhibited interesting antibacterial and antifungal activities against various plant-pathogenic microorganisms [16].

According to several microecological surveys, soils exposed to an arid climate such as those found in Sahara desert, form particular ecological niches, which allow the development of an adapted and diversified actinomycetes population [3, 4]. Many of these organisms provide a valuable resource for use in future biotechnological processes [29].

Our studies showed that the antagonistic activity of the strain IA1 was correlated to the production of a yellow pigmented molecule, named YA. The structure of this compound was determined by NMR and mass spectrometry, and it appeared to be Act-D. Actinomycins, such as Act-D are chromopeptide lactone antibiotics of which more than 30 native and many synthetic variants are known. To date, 27 *Streptomyces* species and one *Micromonospora* have been reported to produce various forms of actinomycins [7, 30, 31]. This is the first report of a representative of *S. mutabilis* that can produce an actinomycin-related compound. In addition, strain IA1 yielded a large amount of Act-D (46.7 mg L^{-1} day^{-1}) while the largest Act-D procurer reported, *Streptomyces griseoruber* MTCC 8121, yielded 35 mg L^{-1} day^{-1} under the same non-optimized ISP-2 medium conditions [7].

The biological effects of Act-D are believed to be a consequence of its ability to intercalate into duplex DNA, which results in the inhibition of DNA-dependent RNA polymerase activities and thus protein synthesis [32]. However, regarding to Act-D toxicity toward animal, especially mammalian, cells [8], we investigated its potential use as a biofungicide for agricultural purposes.

The strain IA1 and the Act-D produced (compound YA) were evaluated for their biocontrol abilities. IA1 and YA treatments showed a significant protective impact against the plant diseases development in both the plant–pathogen systems studied. In fact, after treatment with IA1 or YA, the percentage of plants affected by chocolate spot or *Fusarium* wilt was considerably reduced. These results agree with previous investigations on Saharan *Streptomyces* strains, which have already demonstrated their potential usefulness as biocontrol agents [16, 17]. Shimizu *et al.* [12] highlighted the establishment of an induced disease resistance on Rhododendron seedlings after treatment with an Act-D producing strain, *Streptomyces* R-5, and also showed its strong suppressive impact on the fungus *Pestalotiopsis sydowian*, the causal agent of Pestalotia disease in the rhododendron.

Regarding the biocontrol effectiveness of strain IA1 and the Act-D it produces, it will be interesting to pursue further investigations at greenhouse and field levels to confirm their consistence. Strain IA1 behaviors and maintain in the soil or plant must be assessed to determine the appropriate treatment rate and time period. Furthermore, since Act-D is a cytotoxic compound, persistence and biodegradation on plant tissue and soil must be checked prior to use an actinomycin producer strain, such as *Streptomyces* sp. IA1, as a safe and useful biofungicide for agriculture.

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