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PROPRIÉTÉS ANTIFONGIQUES ET SOLUBILISANTES DE PHOSPHATE *IN VITRO* DES MÉTABOLITES SECONDAIRES PRODUITS PAR UNE *STREPTOMYCES SP T002* RARE

IDRIS KHODJA Fatima[*1], HAMINI-KADAR Nisserine[1], GACEM Mohamed Amine[2], KIHAL Mebouk[1]

1. Laboratory of Applied Microbiology, Department of Biology, Faculty of Natural and Life Sciences, University Oran 1, Algeria
2. Department of Microbial Strain Collection, Helmholtz, Centre for Infection Research, Inhoffenstrasse 7,38124, Braunschweig, Germany

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Résumé

Description du sujet : En agriculture, l'utilisation de microorganismes bénéfiques comme agents de contrôle biologique est considérée comme une alternative écologique pour combattre les maladies des cultures et la résistance aux pesticides. Les Streptomyces spp et leurs métabolites représentent un grand potentiel pour leur exploration en tant qu'agents efficaces pour contrôler divers phytopathogènes fongiques.
Objectifs : Une souche d'actinomycète a été isolée d'un sol forestier inexploré dans l'ouest de l'Algérie. La souche isolée a été testée pour ses propriétés antifongiques *in vitro* contre les champignons phytopathogènes : *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus parasiticus*, *Penicillium expansum* et *Fusarium graminearum* isolés des tiges de plantes de blé ainsi que la propriété de solubilisation du phosphate.
Méthodes : Sur la base des données morphologiques, physiologiques, biochimiques et du séquençage du gène de l'ARNr 16s, la souche actinobactérienne a été identifiée comme étant Streptomyces galilaus. Différents solvants ont été utilisés pour l'extraction, et l'activité a été évaluée pour chaque extrait de solvant. L'activité antifongique des extraits bruts a été déterminée par la méthode de diffusion en puits d'agar.
Résultats : les deux extraits ext_{5254} T002 et ext_{5294} T002 ont démontré une forte activité antifongique contre trois (*Aspergillus ochraceus*, *Penicillium expansum* et *Fusarium graminearum*) des cinq champignons testés. L'analyse par chromatographie liquide et spectrométrie de masse (HPLC/MS) a révélé la présence de germicidine B et d'un mélange de quelques streptovaricines et aclacinomycines pour l'extrait 5254 T002, et d'un composant majeur pour la blanchaquinone pour l'extrait 5294 T002. On a découvert que la souche T002 avait la capacité de solubiliser le phosphate insoluble.
Conclusion : Les résultats indiquent que l'isolat *Streptomyces T002* du sol forestier a montré des capacités de biocontrôle prometteuses contre les champignons qui provoquent la pathogénèse du blé et solubilisent le phosphate insoluble en dehors de son habitat naturel.
Mots clés: Streptomyces T002 ; activité antifongique ; solubilisation du phosphate ; biocontrôle.

IN VITRO ANTIFUNGAL AND PHOSPHATE SOLUBILIZING PROPERTIES OF SECONDARY METABOLITES PRODUCED BY RARE *STREPTOMYCES SP T002*

Abstract

Description of the subject: In agriculture, the use of beneficial microorganisms as biological control agents is regarded as an eco-friendly alternative to combat crop diseases and pesticide resistance. Streptomyces spp and their metabolites represent a great potential for their exploration as effective agents for controlling various fungal phytopathogens.
Objective: Actinomycete strain was isolated from unexplored forest soil in western Algeria. Isolated strain was tested for in Vitro antifungal properties towards phytopathogenic fungi: Aspergillus flavus, Aspergillus ochraceus, Aspergillus parasiticus, Penicillium expansum and Fusarium graminearum isolated from the stems of wheat plants as well as solubilizing phosphate property.
Methods: Based on morphological, physiological, biochemical, and 16S rRNA gene sequencing, the actinobacterial strain was identified as Streptomyces galilaus. Different solvents were used for extraction, and the activity was assessed for each solvent extract. The antifungal activity of the crude extracts was determined by the agar well diffusion method.
Results: the two ethyl acetate: extracts ext_{5254} T002 and the ext_{5294} T002 demonstrated strong antifungal activity against three (*Aspergillus ochraceus, Penicillium expansum and Fusarium graminearum*) of the five tested fungi. The liquid chromatography and mass spectrometry (HPLC/MS) analysis revealed the presence of germicidin B and a mixture of some streptovaricins and aclacinomycins for the ext_{5254}T002, and one major component for the ext_{5294}T002 blanchaquinone. Strain T002 was discovered to have the ability to solubilize insoluble phosphate.
Conclusion: The findings indicate that the forest soil isolate *Streptomyces T002* showed promising biocontrol abilities against fungi that cause pathogenesis in wheat and solubilize phosphate outside of its natural habitat.
Keywords: Streptomyces T002, antifungal activity, Phosphate solubilisation, biocontrol.

* Auteur correspondant: IDRIS KHODJA Fatima, E-mail: idriskhodja.fatima@edu.univ-oran1.dz

INTRODUCTION

Microscopic fungi are responsible for a large number of diseases in humans, animals, and plants [1]; they also cause a great deal of harm to food and agricultural products [2]. The prevalence of fungi has significantly increased over the past ten years, and they are now the fourth most common infection acquired in hospitals [3]. Around the world, plant pathogenic fungi pose a serious threat to both human health and the environment [4, 5]. Overuse of chemical fungicides can cause environmental pollution, the emergence of pathogens resistant to particular fungicides and a variety of health issues in both humans and animals [6]. Pharmaceutical companies and researchers started looking for new less aggressive antifungals that are more effective [7].

The treatment of plant diseases has shown promise when using secondary metabolites produced by some microbes with antibacterial activity [8]. Actinomycetes (Phylum: Actinobacteria) are well-known for their capacity to produce an abundance of natural products with complex structural properties and a range of biological activities [9]. Additionally, actinobacteria, particularly Streptomyces genus, have powerful biocontrol abilities against a variety of phytopathogens [10]. The soil is an excellent source of living microorganisms and a veritable goldmine of biological control agents [11].

The forest ecosystem offers a special ecological niche for the expansion of a variety of microorganisms that are used in the recycling of environmental nutrients and production of unique secondary metabolites with pharmaceutical significance [12]. Although frequently understudied, forest ecosystems offer useful resources to identify new actinomycetes with many economically advantageous substances. The present work involves the isolation of a Streptomyces strain from Tifrit forest soil (a large forest with woody trees in Saida region, western Algeria) with potential to produce a potent antifungal compounds with phosphate solubilizing properties. Further the isolated actinomycete was characterized and identified based on morphological, physiological, biochemical, and genomic features.

MATERIAL AND METHODS

1. Soil sampling

Soil samples were collected from Tifrit forest in Saida city in western Algeria. Using Pochon and Tardieux's technique [13], soil samples were taken. All samples were collected from a depth of 15 cm and dried under aseptic conditions.

2. Selective isolation and identification of the strain T002

Dilution agar plating method was used to isolate actinomycete strains. Samples were diluted by serial dilution up to 10^{-5}, an aliquot of 0.1 ml was applied to the surface of the starch casein nitrate agar medium "SCNA" supplemented with nalidixic acid (20 mg/l) In order to prevent the growth of bacteria that can swarm and nystatin (50mg/l) to inhibit the development of fungi [14]. All plates were incubated at 28°C for 5 to 10 days at the rate of three plates per dilution [15]. The apparent colonies that appeared on the plates were purified on the same medium and incubated for another 10 days. The purified isolates were identified based on their microbiological and cultural characteristics. Before being subcultured once more, the Streptomyces isolates were streaked on GYM agar and kept at 4°C for three months [16]. The International Streptomyces Project (ISP: ISP2- ISP7) agar medium was used to subculture isolated colonies until pure cultures were obtained. Based on the Shirling and Gottlieb (1966) protocol, morphological, biochemical, and physiological characteristics of the actinomycete isolate exhibiting the strongest fungal antagonistic activity was identified [17]. After growth, the color of the colonies, aerial mycelium, and soluble pigment were assessed using the ISCC-NBS color chart [18]. The use of carbohydrates as a single carbon source and sodium chloride resistance were tested on 5338 ($(NH4)_2SO_4$, KH_2PO_4, $MgSO_4$, $7H_2O$, agar and trace element solution 5342) and 5339 medium (0 to 10%) respectively. the incubation was carried out at 30°C for 10 to 15 days. ApiZym® and ApiCoryne® (BioMérieux, France) were used for enzymatic activities; following by manufacturer's manual, observed characteristics were compared with the phylogenetically related type culture obtained from German Collection of Microorganisms and Cell Cultures (DSMZ) Braunschweig, Germany.

Invisorb Spin Plant Mini Kit was used to extract DNA for molecular identification, which was followed by PCR amplification of the 16S rRNA gene region, utilizing the primers 27F and R1492 [19]. The 16S rRNA genes were sequenced in DSMZ (Braunschweig, Germany) by using five primers: 27F, 518R, 1100F, 1100R and 1492R to obtain the complete 16S rRNA gene sequence which was compared to the identical existing sequences available in the data bank of NCBI. The phylogenetic tree was built using the neighbor-joining and the maximum likelihood method [20].

3. Production and extraction of bioactive metabolites from strain T002

Well sporulated culture of T002 strain grown in starch casein agar was inoculated in two Erlenmeyer flasks (250ml): one containing 100ml of 5294 medium (starch: 10 g/l, yeast extract: 2 g/l, glucose: 10g/l, glycerol: 10 g/l, corn steep liquor: 2,5 g/l, peptone: 2 g/l, Nacl: 1 g/l, CaCo3: 3 g/l, Distilled water: 1litre, pH: 7.2) and the second contained 5254 medium (glucose:15g/l, soymeal:15g/l, corn steep:5g/l, $CaCO_3$:2g/l, Distilled water: 1litre, pH: 7.2) . The flasks were incubated in a rotary shaker for two weeks at 30°C and 160–180 rpm. 20ml of each culture was added to 20 ml of ethyl acetate under agitation for 12 minutes on a rotary shaker. The tubes were then centrifuged at 9000 rpm for 10 min, and the upper phase was then transferred into a 50ml flask. Ethyl acetate is evaporated in a rotary evaporator at 40°C. The residue was dissolved in 1 ml of methanol, followed by a centrifugation at 14,000 rpm for 10 minutes [21].

4. Evaluation of antifungal activity of the crude extracts

4.1. Isolation of test phytopathogens

The fungal pathogens tested *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus parasiticus*, *Penicillium expansum and Fusarium graminearum* were isolated from the stems of wheat plants collected from five distinct regions (Saida, Sidi Belabbes, Tlemcen, Relizene and Tiaret) in western Algeria. The five fungi were maintained on Potato Dextrose Agar (PDA) and incubated at 28°C for 5 days, all fungi growth was observed, and comparisons were made based on morphological traits such as the margin and color of aerial and substrate mycelium.

4.2. In Vitro antagonistis activity

The antifungal activity of the crude extracts was determined by the agar well diffusion method described by Bauer et al., (1966) [22] to test the two ethyl acetate extracts ext $_{5254}$ T002 and the ext $_{5294}$ T002 on double-layered PDA plates at different volume of each extract (10, 15, 20, 30µl), The top agar layer was punched with a sterile cork borer to create two wells in the solidified PDA plates (6mm diameter). From the fungal strains grown on PDA agar at 30°C for three days, a mycelium disk (6mm diameter) from each fungus was placed in the centre of the PDA plate previously punched and filled with the two extracts [23]. The plates were put in the refrigerator for 20 minutes to allow the extracts to diffuse and were after incubated at 28°C for 5 days. All of the assays were carried out in triplicate, and the clear inhibition zone diameter around the well was noted then the average was calculated [24]. Methanol served as blank control.

5. Phosphate solubilisation

The phosphate solubilization test is carried out on Pikovskaya (PVK) medium containing 5g/L of tricalcium phosphate (TCP) Ca_3 $(PO_4)_2$ as sole source of phosphate[25, 26,27]. The emergence of a clear zone around the colonies after incubation at 28°C for 7days indicates phosphate solubilization. The colony diameter is subtracted from the total diameter to get the halo diameter.

6. *HPLC and LC/MS analysis of T002 crude extracts*

The purified Streptomyces T002 extract was analysed using liquid chromatography and mass spectrometry. The HPLC equipment (agilent 1100) with an X-Bridge C18, five µl was injected into a column (3.5 m, 2.1x100 mm; Waters, Milford, USA). Extract separation was performed using two elution buffers A: water (950 ml H_2O), acetonitrile (50ml), 0.05 mM (385 $mg.l^{-1}$) ammonium acetate+ 40 µl acetic acid. B: water (50 ml H_2O), acetonitrile (950 ml), ammonium acetate 0.05 mM(385 $mg.l^{-1}$) + 40 µl acetic acid. The wavelength was performed between 210 and 360 nm and the DAD detector (200-400nm). Every 0.5 min fractions from the HPLC column (0,15 ml) were recovered in a 96 well plates and dried with heated nitrogen in MiniVap (Porvair science, UK) at 40°C until evaporation for about 40 min to 60 min.

Afterwards, each well was filled with 150 µl of the broth containing selected test microorganisms adjusted to 0.01 McFarlandand incubated at 30°C for 24 hours. After visual observation, the extracts were taken to an LC-MS system.

RESULTS

1. Isolation and identification of T002 strain

The physiological, biochemical, and molecular characteristics of the isolated strain were examined. On SCN agar media, aerial and substrate mycelium are well developed and showed the typical Streptomyces sp morphology. The strain T002 was Gram positive and aerobic but not motile. The isolated strain exhibited good growth on ISP 1–7, SSM+T and SSM-T in presence of 2.5%, 5%, 7.5% and 10% NaCl respectively.T002 utilized the carbon sources such as glucose, arabinose, sucrose, xylose, inositol, mannose, fructose, rhamnose and raffinose. Strong enzymatic activity was observed while using the API ZYM® and API Coryne® stripes. The physiological and biochemical properties of strain T002 are given in Table 1 and Table 2:

Table 1: Utilization of carbon and sodium chloride tolerance of the strain T002

Characteristics	**Strain T002**
Carbon source utilization	
Glucose	+
Arabinose	++
Sucrose	++
Xylose	+
Inositol	+
Mannose	+
Fructose	+
Rhamnose	++
Raffinose	+
Cellulose	-
Sodium chloride tolerance	0%, 2.5%, 5%, 7.5%, 10%

+: Positive; −: Négative.

Table 2: Enzyme activity of Streptomyces T002

Test	**Result**
Enzyme activity	
Phosphatase alkaline	5
Esterase (C4)	1
Esterase Lipase (C8)	1
Lipase (C14)	0
Leucin arylamidase	5
Valine arylamidase	1
Cystine arylamidase	1
Trypsin	5
Chymotrypsin	1
Phosphatase acid	5
Naphtol-AS-BI-phosphohydrolase	5
alpha galactosidase	1
beta galactosidase	5
beta glucuronidase	0
alpha glucosidase	1
beta glucosidase	1
N-acetyl-beta-glucoseamidase	0
alpha mannosidase	1
alpha fucosidase	0
nitrate reduction	-
Pyraziamidase	-
Pyrrolidonyl arylamidase	-
Alkaline phosphatase	+

Suite Table 2: Enzyme activity of Streptomyces T002

Test	Result
beta glucuronidase	-
beta galactosidase	+
alpha glucosidase	-
N-acetyl -beta glucoseamidase	-
Esculin (beta glucosidase)	-
Urease	-
Gelatine(hydrolysis)	+

+: Positive; −: Negative.

T002 morphological characteristics are shown in the figures 1 and 2:

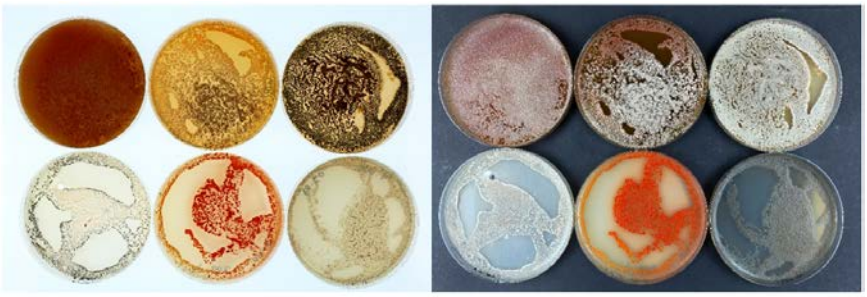

Figure 1: Morphological characteristics of the strain T002, Plates (GYM, ISP2, ISP3, ISP4, ISP5, ISP6) from left to right

Figure 2: Morphological characteritcs of the strain T002, Plates (from top left to bottom right; ISP6, ISP7, SSM+T, SSM-T)

The 16S rRNA gene sequencing and phylogenetic tree construction of the actinobacterial strain were done at Microbial Strain Collection, Helmholtz Centre for Infection Research Braunschweig, Germany. The translated amino acid sequence of T002 genes showed a similarity of 99% with known *Streptomyces galilaeus* (phylogenetic tree in supplementary materiel). The sequence was deposited in the NCBI Gene nucleotide sequence database under accession number **ON249037**.

2. Evaluation of antifungal activity of the crude extracts

Actinomycete crude extract compounds were used to test the antifungal activity against the test organisms, different volume of each extract (10, 15, 20, 30µl) were used to check both of antifungal activity and minimum inhibitory concentration. Tables 3 and 4 showed results of the extract$_{5254}$ T002 and the extract$_{5294}$ T002 against the phytopathogenic fungi:

Table 3: Average mean of inhibition zone formation of extract$_{5254}$ T002

	Inhibition zone (in mm)				
Extract$_{5254}$ volume	*A.flavus*	*A.ochraceus*	*A.parasiticus*	*F.graminearum*	*P.expansum*
10 µl	-	16.33	-	19	-
15 µl	-	11.66	-	-	-
20 µl	-	17.33	-	08	-
30 µl	-	20.33	-	13.33	10.66

Table 4: Average mean of inhibition zone formation of extract$_{5294}$ T002

	Inhibition zone (in mm)				
Extract$_{5294}$ volume	*A.flavus*	*A.ochraceus*	*A.parasiticus*	*F.graminearum*	*P.expansum*
10 µl	-	15	-	-	-
15 µl	-	17	-	16.66	12.33
20 µl	-	-	-	-	-
30 µl	-	16.33	-	12.66	14.66

3. Phosphate solubilisation

On PVK solid medium, the isolate T002 was demonstrated to have the ability to solubilize insoluble phosphate and to produce transparent halos of solubilisation after 07 days of incubation at 28°C (figure 3)

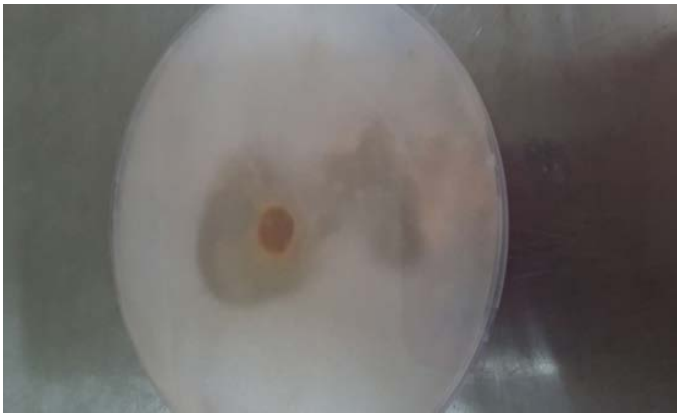

Figure 3: Phosphate solubilisation of Stretomyces T002 on Pikovskaya agar

4. HPLC and LC/MS analysis

Chromatographic analysis utilizing HPLC fractionation and LC/MS was carried out in order to identify antimicrobial compounds in the strain T002 extracts, for the extract5254 T002, the LC-MS spectrum showed the presence of one component “Germicidin B” with retention time 7,5 min and a mixture of some streptovaricins and aclacinomycins at 11 min while the peak-activity-correlation test of the ext5294 T002 after HPLC fractionation and LC/MS chromatogram (Fig 2 supplementary material) revealed that the active fraction was located between 12.0 -12.8 min. According to the LC/MS data, the peak appearing at this retention times correlated to Blanchaquinone.

DISCUSSION

It is well known that members of the phylum Actinobacteria exemplified by the genus Streptomyces produce bioactive substances with various biological and pharmaceutical significance. The vast majority of these compounds were found in terrestrial sources [28, 29]. The forest soil in semi-arid region had high levels of carbon, nitrogen, and other minerals. However, some of these nutrients and minerals, particularly phosphate, continue to exist in complicated or precipitated forms that plants are unable to use. Streptomyces genus may play a significant part in promoting plant growth by enhancing nutrient availability. In this study, we investigated the antifungal activity of the crude extracts of the bioactive isolate T002. We examined the strain's capacity to convert insoluble forms of phosphorus into an accessible soluble form.

In PVK agar, *Streptomyces T002* was discovered to be a potent phosphate solubilizer. A qualitative analysis of phosphate solubilization revealed the emergence of a clearly different solubilization zone around colonies of Streptomyces.

Our findings support those made by Aouar et *al.* [4], they discovered that actinobacteria isolates frequently exhibited the ability to solubilize phosphate. The agar well diffusion assay was used to test the actinobacterial isolate's crude extracts for antifungal activity against five phytopathogens fungi. The two extracts proved active against three fungi from the five tested (*A.ochraceus, F.graminearum, P.expansum*), extract$_{5254}$ T002 has the strong effect against *A.ochraceus, F.graminearum* with only 10µl whereas *P.expansum* was less susceptible to the compound. Hight antifungal activity was detected against *A.ochraceus* using the extract$_{5294}$T002.

The variations in the antifungal activity prove the chemical diversity of the antifungal compound produced by the *Streptomyces T002*. Chromatographic analysis and LC-MS of crude extracts led to the identification of the antifungal compounds, these compounds are proposed to be blanchaquinone, germicidin B and a mixture of some streptovaricins and aclacinomycins with anthracycline structure. The anticancer properties of these anthracycline glycosides are well known [30, 31, 28]. But only few studies were interested in their antifungal effect. According to Petersen et *al.* [32] Germicidins A and B belong to an antibiotic group of natural products. This compound class is mainly found from Streptomyces species and acts as autoregulatory inhibitor of spore germination. Dos Santos et *al*. [33], found that germicidin inhibited not only spore germination but also hyphal elongation [33], which probably limited growth of test fungal pathogens in our study. Similar results were followed by Sudiana et *al.* [34] isolating S. sasae TG01 with antifungal activity against *F. solani* and *F. oxysporum* and the ability to solubilize phosphate. *Streptomyces bungoensis (BF26)* with antifungal activity have been reported by Elshamy et *al*. [35] to significantly reduce the risk of pathogenic infection. Kunova et *al.* [36] discovered a strain of Streptomyces that was active against *Rhizoctonia solani* and *Fusarium oxysporum f.sp. lactucae*. Strain IA1 from Algeria desert soil demonstrated potent activity against a variety of plant pathogenic fungi [37]. In this study we showed that strain T002 can both solubilize phosphate and protect wheat against pathogenic fungi.

Therefore, the forest soil isolate showed promising biocontrol abilities against fungi that cause pathogenesis in wheat and solubilize phosphate outside of its natural habitat.

CONCLUSION

The current study demonstrates the purification and characterization of a novel and rare actinomycete from unexplored Algerian forest soil with potential to promote plant growth and produce antifungal compounds *in Vitro*. As a result, the *Streptomyces T002* isolated from forest ecosystem may have a significant amount of potential to produce high-quality bioactive compounds that are effective against phytopathogenic fungi and have the ability to solubilize phosphate as plant growth-promoting characteristic. This preliminary research must be finished using this isolate *in Vivo* or in a greenhouse setting as a biological control to protect the environment from pollution and to produce healthy food.

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