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Environmental Microbiology

Effect of sulfonylurea tribenuron methyl herbicide on soil Actinobacteria growth and characterization of resistant strains

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ABSTRACT

Repeated application of pesticides disturbs microbial communities and cause dysfunctions on soil biological processes. Granstar[®] 75 DF is one of the most used sulfonylurea herbicides on cereal crops; it contains 75% of tribenuron-methyl. Assessing the changes on soil microbiota, particularly on the most abundant bacterial groups, will be a useful approach to determine the impact of Granstar[®] herbicide. For this purpose, we analyzed Actinobacteria, which are known for their diversity, abundance, and aptitude to resist to xenobiotic substances. Using a selective medium for Actinobacteria, 42 strains were isolated from both untreated and Granstar[®] treated soils. The number of isolates recovered from the treated agricultural soil was fewer than that isolated from the corresponding untreated soil, suggesting a negative effect of Granstar[®] herbicide on Actinobacteria community. Even so, the number of strains isolated from untreated and treated forest soil was quite similar. Among the isolates, resistant strains, tolerating high doses of Granstar[®] ranging from 0.3 to 0.6% (v/v), were obtained. The two most resistant strains (SRK12 and SRK17) were isolated from treated soils showing the importance of prior exposure to herbicides for bacterial adaptation. SRK12 and SRK17 strains showed different morphological features. The phylogenetic analysis, based on 16S rRNA gene sequencing, clustered the SRK12 strain with four *Streptomyces* type strains (*S. vinaceodrupus*, *S. mutabilis*, *S. ghanaensis* and *S. enissocaesilis*), while SRK17

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strain was closely related to *Streptomyces africanus*. Both strains were unable to grow on tribenuron methyl as unique source of carbon, despite its advanced dissipation. On the other hand, when glucose was added to tribenuron methyl, the bacterial development was evident with even an improvement of the tribenuron methyl degradation. In all cases, as tribenuron methyl disappeared, two compounds were detected with increased concentrations. These by-products appeared to be persistent and were not degraded either chemically or by the studied strains. Based on these observations, we suggested that bacterial activity on carbon substrates could be directly involved in the partial breakdown of tribenuron methyl, by generating the required acidity for the first step of the hydrolysis. Such a process would be interesting to consider in bioremediation of neutral and alkaline tribenuron methyl-polluted soils.

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Introduction

The soil ecosystem is the theater of complex linked biological processes, regulated by its microbiota. Changes, such as introducing xenobiotic substances, cause fluctuation on microbial quality and quantity, affecting then the soil balance. Therefore, the microbial population reflects environmental changes and may be taken as an efficient indicator to evaluate the impact of exogenous molecules.¹

Sulfonylurea herbicides are widely used around the world to protect cereal crops. They could constitute a long-term environmental hazard,² with even a contamination risk of groundwater and surface waters, due to leaching processes.^{3,4} Furthermore, they are considered as disturbers for the soil microbiota.^{1,5} Granstar[®] 75 DF (DuPont de Nemours) is one of the most used herbicides in Algeria.⁶ It is used under different commercial denominations, in North Africa, Europe, North and South America and Asia.^{7–10} This herbicide contains 75% of tribenuron-methyl (TBM), a sulfonylurea molecule active against a large number of annual dicotyledons¹¹ and it is used in low but effective concentrations (12 g/300 L/ha). TBM acts by stopping cellular division of meristematic tissues of plants via inhibition of acetolactate synthetase; an enzyme present in higher plants, bacteria and fungi, but absent in humans and animals.^{7,12,13}

Several studies reported effects of sulfonylurea herbicides on global microbial community and on specific groups like fungi.^{1,5,13} However, the impact on soil *Actinobacteria* is still little explored, despite their abundance and importance in soil.¹⁴ Actually, these Gram-positive bacteria have considerable potential for biotransformation and biodegradation of pesticides,^{15,16} due to their ability to produce variable extracellular enzymes, degrading complex and recalcitrant pollutants¹⁷ in various environments.¹⁸

The main objective of this study was to examine the effect of Granstar[®] herbicide on soil *Actinobacteria*. For that purpose, *Actinobacteria* strains were isolated from both untreated and Granstar[®] treated soils. The resistance of isolated strains to different concentrations of the herbicide was evaluated. The two most resistant strains were further characterized and the impact of TBM on their growth determined.

Materials and methods

Soil treatment and *Actinobacteria* isolation

Herbicide Granstar[®] 75 DF was obtained as a commercial powder from Du Pont de Nemours (Algeria). Three soils were selected for our study. Two agricultural soils, from two different eastern regions of Algeria: Ain Karma, Constantine (soil 1) and Ain Babouche, Oum El Bouaghi (soil 2). The third soil was from a forest, taken at Chaabet Ersas, Constantine (soil 3). Soils 2 and 3 were free of pesticides, while soil 1 was regularly treated with various herbicides, including Granstar[®]. Soil samples were collected at 10 cm depth. The soils 2 and 3 were incubated at laboratory with the herbicide as follows: Granstar[®] was added to 150 g of soil at 40 mgL⁻¹, which is the recommended field application dose. The incubation was performed in covered aerated two liters beakers at room temperature for three weeks. Hereafter, these treated soils are named treated soil 2 and treated soil 3.

For isolation of *Actinobacteria* strains, 10 g of each soil sample (soil 1, soil 2, soil 3, treated soil 2, treated soil 3) were suspended in 90 mL of physiological water (NaCl 9‰). For plating isolation, serial dilutions (10⁻², 10⁻³ and 10⁻⁴) were prepared using physiological water. Isolation was carried out on Bennett agar, containing 10 g glucose, 2 g casamino acid, 1 g meat extract, 1 g yeast extract, 15 g agar per liter of distilled water, pH 7.3,¹⁹ supplemented with 40 mgL⁻¹ of Granstar[®]. This medium allows *Actinobacteria* growth²⁰ and is considered as a production medium for enzymes and bioactive substances.²¹ After incubation at 30 °C for a week, *Actinobacteria* colonies were selected by a direct light microscopic observation (×10) based on their morphological characteristics (presence of short branching filaments). The isolated strains were preserved on 50% glycerol solution (v/v) at –80 °C and on starch-casein agar slant, after sporulation, at 4 °C.

Herbicide-resistance screening

Resistance of the isolated strains to various doses of Granstar[®] was verified by using Bennett agar medium supplemented with herbicide at different concentrations: 0.004, 0.04, 0.05,

0.075, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6 and 0.65% (v/v). The plates were incubated at 30 °C for 7 days.

Morphological and cultural characterization of strains

Four ISP (International *Streptomyces* Project) media; ISP2 (yeast extract 4 g, malt extract 10 g, glucose 4 g, agar 20 g, distilled water 1 L, pH 7.3), ISP4 (soluble starch 10 g, NaCl 1 g, CaCO₃ 2 g, K₂HPO₄ 1 g, MgSO₄·7H₂O 1 g, (NH₄)₂ SO₄ 1 g, trace salts solution [FeSO₄·7H₂O 1 g, ZnSO₄·7H₂O 1 g, MnCl₂·4H₂O 1 g, distilled water 1 L, pH 7.0] 1 mL, agar 20 g, distilled water 1 L, pH 7.0–7.4), ISP6 (peptone 15 g, proteose peptone 5 g, ferric ammonium citrate 0.5 g, sodium thiosulphate 0.08 g, yeast extract 1 g, K₂HPO₄ 1 g, agar 20 g, distilled water 1 L, pH 7.0–7.2), ISP7 (glycerol 15 g, L-asparagine 1 g, K₂HPO₄ 0.5 g, NaCl 0.5 g, FeSO₄·7H₂O 0.01 g, agar 20 g, distilled water 1 L, pH 7.2–7.4), as well as starch-casein agar (soluble starch 10 g, casein 1 g, K₂HPO₄ 0.5 g, agar 20 g, distilled water 1 L, pH 7.0–7.5) were inoculated to record morphological and cultural features of the selected resistant strains.²² Sporulation and fragmentation of the substrate mycelium, as well as the characteristics of produced spores on aerial mycelium were determined by the lamella technique,²² which consists in inserting a sterile lamella on the surface of ISP2 agar, at an angle of 45°, the inoculum was then deposited on the lamella, in contact with the surface of the medium. After 14 days of incubation, the lamella was gently removed, deposited on a glass slide and observed using a light microscopy oil immersion objective. Details about aerial mycelium color and production of diffusible pigments and melanin were obtained by inoculating ISP4, starch-casein, ISP6 and ISP7 agars, respectively.^{22,23}

Phylogenetic analyses

The DNA extraction of the isolates was performed from a 48 h liquid culture in Luria-Bertani (LB) broth by using the Ultraclean Microbial DNA Isolation kit (Mo Bio). For the amplification of the 16S rRNA genes, the bacterial primer sets 8F²⁴ and 1387R²⁵ were used with a program of an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of 45 s at 95 °C, 45 s at 56 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min, in an AB Applied Biosystems Veriti 96 well thermal cycler as previously described.²⁶ The purification of the

amplified DNA was performed with the GFX PCR DNA and the Gel Band Purification kit (GE Healthcare) as described.²⁷ DNA sequences were obtained by GATC Biotech AG (Germany) using the Sanger method.²⁸ The sequences were corrected using the Sequencher v. 4.1.4 software (Gene Codes) and subsequently deposited with Genbank under accession numbers KR871404 and KR871405 for SRK12 and SRK17 sequences, respectively.

The sequences obtained were compared with 16S rRNA gene sequences of reference strains available at the public Genbank database (www.ncbi.nlm.nih.gov) using the BLAST tool. The sequences were aligned using the ClustalW program. The phylogenetic reconstruction (Fig. 1) was done using the neighbor-joining algorithm,²⁹ with bootstrap values calculated from 1000 replicate runs, using MEGA software, version 6.

Dissipation and effect of tribenuron-methyl on selected strains growth

Tribenuron-methyl (methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl]benzoate) was purchased from Sigma-Aldrich (France). Starter cultures were conducted by inoculating 100 mL of tryptone-yeast extract broth (5 g tryptone, 3 g yeast extract per liter of distilled water, pH 7.0–7.2) supplemented with 30 mgL⁻¹ of tribenuron-methyl (TBM) with a spore solution (spore material on 10 mL of sterile distilled water). Incubation was done under shaking (150 rpm) at 30 °C for 48 h.²² In order to get a free carbon source inoculum, 100 mL of the starter cultures were strongly agitated to break up the *Actinobacteria* pellets, then centrifugated at 4000 × g for 10 min. The sediment was washed twice with sterile 0.9% NaCl and suspended in 75 mL of sterile physiological water. The resulting suspension is the washed inoculum.²²

In order to assess the effect of TBM on *Actinobacteria* growth, mineral salts ISP9 broth (2.64 g (NH₄)₂SO₄, 2.38 g KH₂PO₄, 5.65 g K₂HPO₄·3H₂O, 1 g MgSO₄·7H₂O, 1 mL of trace salts solution [0.1 g FeSO₄·7H₂O, 0.1 g MnCl₂·4H₂O, 0.1 g ZnSO₄·7H₂O in 100 mL of distilled water, pH 7.0] per liter of distilled water, pH 7.0),²² containing 30 mgL⁻¹ of TBM, was inoculated with the washed inoculum of each selected strains and incubated at 30 °C, 150 rpm. Under the same conditions, two other tests

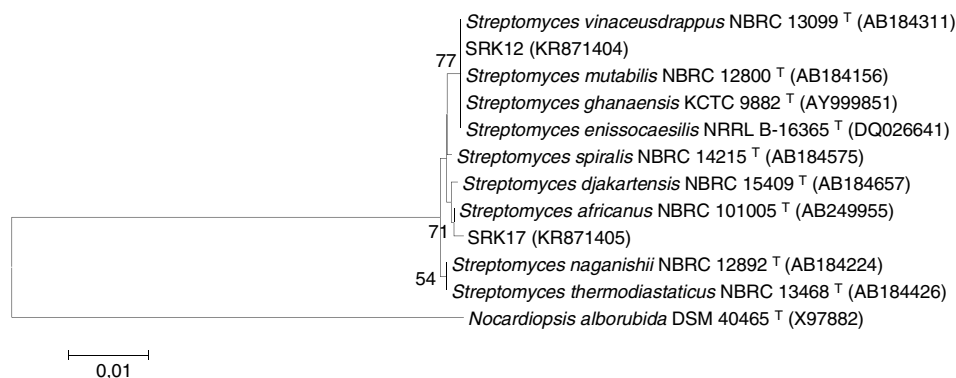


Fig. 1 – Phylogeny of SRK12 and SRK17 TBM-resistant strains based on 16S rRNA gene sequences analysis (Tamura-Nei algorithm and neighbor-joining tree). Nocardioopsis alborubida was used as out-group. Bootstrap values (1000 replicate runs) greater than 50% are indicated. GenBank accession numbers are indicated in brackets.

Table 1 – Soil characteristics and repartition of the 42 Actinobacteria strains isolates according to soil origin.

Soil	Soil 1	Soil 2	Treated soil 2 ^a	Soil 3	Treated soil 3 ^a
Type	Agricultural	Agricultural		Forest	
pH	7.58	7.52		7.59	
Number of isolates	07	16	09	04	06

^a Treated soil 2 and treated soil 3 correspond to soil 2 and soil 3 maintained for 21 days in microcosm with 40 mgL⁻¹ of Granstar[®] herbicide respectively.

Table 2 – Repartition of the 11 herbicide-resistant strains according to herbicide treatment.

Herbicide concentration (%)	0.3	0.35	0.4	0.45	0.5	0.55	0.6	0.65
Untreated soil isolates	1	–	–	2	1	–	–	–
Treated soil isolates	–	1	1	2	1	1	1	–

–, no isolates.

were also performed on [ISP9 + TBM (30 mgL⁻¹) + glucose (1%)] and [ISP9 + glucose (1%)]. This last one was used as a control to evaluate the optimum growth of the strains. All assays were done in triplicate. After three weeks of incubation, the cultures were centrifuged (12,000×g/10 min/4 °C). The biomass was dried at 105 °C until constant weight and the filtered supernatants were submitted to HPLC-UV analysis to measure the TBM dissipation. The HPLC equipment consisted of a Spectra Series P200 pump coupled to an ERC 3415 α eluent degasser module, a Spectra Series AS100 autosampler, a Spectra System UV6000 LP UV-visible detector (220 nm) and a C18 column (Phenomenex, Gemini NX; 25 cm × 4.6 mm, 5 μ m). The mobile phase contained acetonitrile/water (containing 0.1% of H₃PO₄) 90:10, v/v, at a flow rate of 1 mL min⁻¹. Aliquots (10 μ L) of culture media were injected and run at room temperature and the different concentrations of TBM were determined relative to the peak area detected in blank control samples made with TBM solutions on the 10–50 mgL⁻¹ range.

Statistical analysis

Effect of tribenuron-methyl on bacterial growth, was tested following 2-way ANOVA between the tested media and each selected strains, with an Alpha threshold of 0.05, using STATISTICA[®] software, version 10, StatSoft, France.

Results

Actinobacteria isolation

The isolation strategy was designed with the aim of selecting Granstar[®]-resistant Actinobacteria with TBM degradation capacity. In total, forty-two (42) Actinobacteria strains were isolated from the different soils. The repartition of isolates according to their soil origin is shown in Table 1. It is noteworthy that the largest number of isolates was obtained from the untreated agricultural soil 2 with 16 isolates. The number of isolates from the other soils, including treated soil 2, was lower (from 4 to 9 isolates).

TBM resistance

All isolates resisted to the tested doses, until the critical herbicide concentration of 0.3% (v/v), at which some of them were inhibited. At that level, 11 strains were still growing and showed various degrees of resistance to the increased herbicide doses (Table 2). Among this resistant group of 11 strains, 4 isolates were recovered from untreated soils 2 and 3. As expected, more than half of the resistant strains were isolated from treated soils 1, 2 and 3, and the most resistant strains that grew at the highest herbicide concentrations of 0.6, 0.55 and 0.5%, were isolated from treated soils 3, 1 and 2, respectively.

Characterization of the most resistant strains

The two most TBM-resistant strains, SRK12 and SRK17, were selected for further characterization. Morphological, physiological and phylogeny based on 16S rRNA gene sequences analyses were performed. Both strains developed a non-fragmented substrate mycelium and aerial mycelium with spiral spore chains (Table 3). They also presented some other common characteristics, namely, rounded spores arranged in chains of under twenty spores length; no melanin pigment production and affiliation to the gray color series, as concluded from their spore color on ISP4 agar. However, the strains SRK12 and SRK17 differed from each other in diffusible pigments production on starch-casein agar, where a pinkish brown pigment was produced by SRK12, while, a rusty brown pigment was detected with SRK17. Also, the substrate mycelium of the strain SRK17 presented some sporulation unlike the SRK12.

Analysis of the 16S rRNA gene sequences of strains SRK12 and SRK17 confirmed their affiliation to the *Streptomyces* genera. They presented high sequence similarity (99.9%) with sequences related to *Streptomyces* type strains deposited at the public database GenBank. The phylogenetic tree using *Nocardopsis alborubida* DSM 40465^T sequence (X97882) as out-group (Fig. 1) showed that strain SRK12 grouped in a single cluster with the type strains of *Streptomyces vinaceusdrappus* NBRC 13099^T (AB184311), *S. mutabilis* NBRC 12800^T (AB184156), *S. ghanaensis* KCTC 9882^T (AY999851) and *S. enissocaesilis* NRRL B-16365^T (DQ026641), with a bootstrap support value of 77%. The

Table 3 – Morphological characteristics of SRK12 and SRK17 isolates and their close relative strains.

Strains	Spore chains morphology ^a	Spore chains length ^a	Spores morphology ^a	Spore color ^b	Production of diffusible pigments ^c	Melanin on ISP6	Melanin on ISP7
SRK12	Spirals	<20	Rounded	Pale gray	Pinkish brown	–	–
SRK17	Spirals	<20	Rounded	Wooly gray	Rusty brown	–	–
<i>S. vinaceusdrappus</i>	Spirals	>50	Smooth	Red	–	–	–
<i>S. mutabilis</i>	Retinaculiaperti	3–10	Smooth	White	–	–	–
<i>S. ghanaensis</i>	Spirals	nd	Hairy	Green	nd	nd	nd
<i>S. enissocaesilis</i>	Spirals	nd	Smooth	Gray	–	Poorly developed	Poorly developed
<i>S. africanus</i>	Spirals	>50	Spiny	Blue	–	–	–

^a On ISP2 agar.

^b On ISP4 agar.

^c On Starch-Casein agar.

–, absent; nd, not determined.

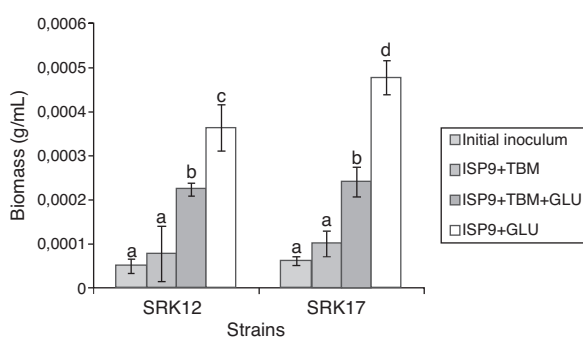


Fig. 2 – TBM effect on biomass production of SRK12 and SRK17 strains. The biomass was estimated on the different media after 3 weeks of incubation (30 °C and 150 rpm). Bars indicate SD from 3 replicates. Different letters indicate significant differences between biomass values ($p < 0.05$).

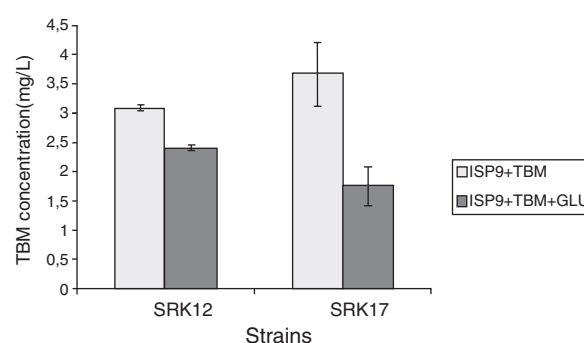


Fig. 3 – In vitro dissipation of TBM (30 mg L⁻¹) by selected actinobacteria after three weeks of incubation (30 °C and 150 rpm). Bars indicate SD from 2 replicates for strain SRK12 and 3 replicates for strain SRK17.

strain SRK17 was found related to *Streptomyces africanus* NBRC 101005^T (AB249955), with a bootstrap support rate of 71%.

Effect of TBM on bacterial growth

According to Fig. 2, both strains showed an insignificant growth ($p > 0.05$) when TBM was used as sole carbon source. On the other hand, when glucose was added, both SRK12 and SRK17 strains growth was evident and significant ($p < 0.05$), but it did not reach the highest level obtained on glucose without TBM ($p < 0.05$).

TBM dissipation

The dissipation of TBM varied according to the medium. Fig. 3 shows that, after three weeks of incubation on [ISP9 + TBM], the concentration of the studied compound has decreased from 30 mg L⁻¹ to 3.09 and 3.66 mg L⁻¹, respectively with SRK12 and SRK17 strains. On [ISP9 + TBM + glucose], the TBM degradation was improved, the final TBM concentration reaching 2.39 and 1.75 mg L⁻¹ respectively.

Discussion

The low isolate numbers obtained from the three treated soils, suggested that the herbicide treatment reduced the *Actinobacteria* diversity. This decrease could be explained because of the Granstar[®] toxicity. Indeed, the presence of TBM, a sulfonylurea molecule, may prevent bacterial growth as suggested by Chèvre (2007)² and Bottaro et al. (2008).³⁰ These results are in accordance with those of Filimon et al. (2012)³¹ and Arabet et al. (2014)¹³ showing different behavior of some bacterial groups, including *Actinobacteria* species. Sensitive and resistant species were reported after treatment with sulfonylurea herbicides, including some species taking advantage for growth. In our study, the variable number of *Actinobacteria* isolates according to the different soils suggested that the bacterial communities include sensitive and resistant strains.

According to Arabet et al. (2014),¹³ the high resistance shown by *Actinobacteria* isolates could have two possible explanations: either the strains possess a modified acetolactate synthase, making the herbicide inactive on cellular division,

or they have the necessary enzymatic luggage to degrade partially or completely the herbicide. The fact that the most resistant strains were isolated from treated soils, suggests that a prior exposure to the herbicide makes bacterial adaptation easier, with even, a possibility to develop a potential for the herbicide transformation. Such a case was observed by Widehem et al. (2002)³² who have isolated from diuron treated soil, a strain of *Arthrobacter* sp. able to transform the herbicide completely in 24 h. Results going in the same direction were reported by Zhao et al. (2015),³³ with a TBM-adapted *Ochrobactrum* sp. strain, which can degrade 55% of the herbicide in 8 days.

Both the SRK12 and SRK17 strains have grown by developing vegetative mycelium and aerial mycelium with spiral spore chains at its extremity. These features supported their assignment to the *Streptomyces* genera.^{34,35}

According to Fig. 1, the SRK12 strain clustered with four strains of *Streptomyces* type: *S. vinaceodrappus*, *S. mutabilis*, *S. ghanaensis* and *S. enissocaesilis*. Knowing that the first three mentioned *Streptomyces* type strains belong respectively, to red, white and green color series,^{36–39} the strain SRK12 being from the gray color series, could not be affiliated to them. Although *S. enissocaesilis* was from the gray color series, it had no diffusible pigments in starch-casein agar,^{39,40} unlike the strain SRK12. The second isolate SRK17 was closely related to another *Streptomyces* type-strain named *S. africanus*, but this one was from the blue color series,^{39,41} thus it could not be associated to the strain SRK17. Therefore, the discrepancies between the 16S rRNA gene-based affiliation and the color series properties highlighted taxonomic issues as previously indicated by Kämpfer (2006)⁴² reporting difficulties to differentiate *Streptomyces* members based on phenotypic features. Such observation suggested that further analyses are required to revisit the *Streptomyces* genera taxonomy. In our study, the most resistant isolates were then *Streptomyces* strains. Works conducted by Filimon et al. (2012)³¹ and Arabet et al. (2014)¹³ also showed that *Streptomyces* species were present among sulfonylurea resistant microorganisms.

Our result clearly showed that both strains were unable to use TBM as sole carbon source, hence they did not have the necessary enzymes to degrade it. Wang et al. (2012)⁴³ as well as Zhang et al. (2013)⁴⁴ also noticed that *Serratia* sp. and *Pseudomonas* sp. strains, respectively, were unable to grow when inoculated on mineral salts broth supplemented with TBM. The non-significant development of both SRK12 and SRK17 strains in presence of TBM can be explained by the presence of residual carbon sources, from starter cultures, that may remain confined in the *Actinobacteria* pellets biomass, in spite of the use of a twice washed inoculum. On this medium composition, the degradation of TBM was considerable leading to low concentrations of 3.09 and 3.66 mgL⁻¹ respectively with SRK12 and SRK17 strains (Fig. 3). Since the tested strains were not able to grow on TBM, as sole carbon source (Fig. 2), the sulfonylurea molecule has probably been degraded by a chemical pathway, without a bacterial involvement. Several studies^{8,12,43,45} reported that acidohydrolysis was the primary dissipation mechanism of TBM in the soil environment and aqueous solutions. This process was mainly influenced by variation of pH value, i.e., the

degradation occurred more rapidly at lower pH than at higher pH.³ Wang et al. (2012)⁴³ mentioned that at pH 7.3, TBM was not degraded at all. The same observation was noted by Zanardini et al. (2002)⁴⁶ for two sulfonylurea compounds; chlorsulfuron and metsulfuron methyl, at 7.0–7.2. In our experiment, the final pH was 6.72 with both strains, which is close to pH value of 6.8 that Martin (2000)¹² considered as a critical point for sulfonylurea herbicides degradation. Thus this acidity level was probably the trigger factor for the dissipation of TBM by acidohydrolysis mechanism. It is interesting to notice that, as the TBM decreased in the first hours of incubation, two compounds were detected at HPLC, with peaks areas increasing over time (data not shown). These products were probably derived from the cleavage of the sulfonylurea bridge of TBM which is the most common and the major initial chemical reaction for sulfonylurea breakdown, especially under acidic conditions.^{3,8,12,44,45} From these results, two conclusions can be drawn; both strains were also unable to use TBM degradation products as a carbon source; and unlike TBM, these compounds were not degraded either chemically or biologically.

The addition of another carbon source (glucose) with TBM has allowed the growth of both *Actinobacteria* strains (Fig. 2), with even an improvement of TBM dissipation rate, leading to concentrations of 2.39 and 1.75 mgL⁻¹ respectively with SRK12 and SRK17 strains (Fig. 3). This can be explained by the decrease of pH values to 4.38 and 4.23 respectively, that were likely obtained by the accumulation of organic acids produced from glucose fermentation, as suggested by Zanardini et al. (2002).⁴⁶ These acidic conditions that may enhance TBM transformation would then be directly related to bacterial activity on glucose catalysis. Accordingly, we concur with Wang et al. (2012)⁴³ in suggesting that TBM degradation could be a co-metabolic process involving bacterial mediated acidohydrolysis of TBM.

The significant differences noticed between strains growth on glucose, with and without TBM (Fig. 2), was not due to TBM since it was almost entirely dissipated, but it can be explained by the presence of one or both new detected compounds from TBM degradation, which incontestably seems to hamper the development of both strains, even when glucose was added. This observation confirmed the toxic effect of TBM transformation on tested actinobacteria, but it was not in accordance with that found by Wang et al. (2012)⁴³ and Zhang et al. (2013)⁴⁴ who reported no evident effect of TBM on *Serratia* sp. and *Pseudomonas* sp. cell yield, respectively, when inoculated on glucose with and without TBM. This insinuated different degrees of TBM-induced transformation and different bacterial behavior.

It is known that pesticides by-products may have a more pronounced toxicity than the precursor molecule. That's what appears to be the case here, where the probable cleavage of the TBM sulfonylurea bridge has led to the appearance of two compounds significantly interfering with bacterial growth. It is most likely be sulfonamide and triazine amine which are considered to be stable and highly persistent in soils.^{3,4,30,45} The accumulation of those two metabolites at high concentrations could lead to the inhibition of the most resistant actinobacteria.

Conclusion

The isolation strategy adopted in this study confirmed the negative effect of Granstar[®] herbicide on *Actinobacteria*. Resistant strains to high levels of the TBM were obtained. The two most resistant strains SRK12 and SRK17 were assigned to the *Streptomyces* genera on the basis of morphological characteristics and 16S rRNA gene sequences. However, discrepancies were observed between 16S rRNA gene-based affiliation and color properties highlighting *Streptomyces* taxonomic issues, which should require in depth taxonomic studies of the *Streptomyces* genera.

The first reaction of the degradation of TBM can be triggered under acidic conditions by a co-metabolic process involving bacterial-mediated acidohydrolysis, which would finally result in the partial breakdown of the TBM molecule. This process would be a promising application in bioremediation of neutral and alkaline TBM-polluted soils. The products of this chemical degradation seem to have a harmful effect on the actinobacteria growth. For a better understanding of the whole process, further tests are needed, including the identification of the resulting metabolites and the study of their destiny by soil microbial consortium.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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