EFFECT OF TRIFLURALIN, ZERO-VALENT IRON AND MAGNETITE NANOPARTICLES ON GROWTH OF STREPTOMYCETES

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Abstract. Among actinomycetes the genus *Streptomyces* is the most widely studied and well known. Ecologically, streptomycetes, due to their extracellular enzymes, have a great potential for biodegradation of organic and inorganic toxic compounds. Among metal-based engineered nanomaterials, iron nanoparticles (NPs) are, probably, the most used for bioremediation of a broad spectrum of pollutants. Iron-based NPs are expected to be non-toxic, due to the use of Fe atom in several pathways of cell metabolism and, therefore, low iron toxicity. The present study aims to determine the effect of magnetite NPs, zero-valent iron NPs and fluorinated dinitroaniline herbicide trifluralin on growth of streptomycetes strains. Streptomycetes strains were isolated from soil long-term polluted with obsolete pesticides, DDT and trifluralin. The inhibition activity of iron NPs and trifluralin was evaluated using express-method. Each streptomycete strain had an individual reaction to the solutions of iron NPs. Most of the studied microorganisms were found to be sensitive to trifluralin, except *Streptomyces sp*. 0112 and *Streptomyces sp*. 0212. Cytotoxic effect of trifluralin was diminished by iron NPs, but not fully reduced. Fe(0) NPs produced no inhibitory effect on growth of streptomycetes. The obtained data has revealed the prospect of developing a procedure for bioremediation of soils polluted with trifluralin, using the phenomenon of synergism between iron NPs and streptomycetes.

Keywords: streptomycetes; iron nanoparticles; trifluralin; growth inhibition

INTRODUCTION

Streptomycetes, which are abundant in soil, are believed to have originated around 400 million years ago, when the land was being colonised by green plants. Among actinomycetes the genus *Streptomyces* is the most widely studied and well known. Probably most of the interest in this group of microorganisms lies in their ability to produce secondary metabolites. Indeed, each strain of actinomycetes likely has the genetic potential for the production of 10 to 20 secondary metabolites [9, 23]. The majority of antibiotics used in medicine, veterinary practice and agriculture originate from *Streptomyces* bacteria [11, 23].

These organisms are therefore being studied ever more intensively in the expectation that they will contribute significantly to the provision of new therapeutic agents to combat the global emergence of antibiotic resistance among pathogenic bacteria, as well as providing other bioactive agents with medical applications. However, this is only one aspect of the interest and importance of streptomycetes. They also produce non-antibiotic molecules that exhibit bioactivity, such as enzyme inhibitors, immunosuppressors, phytotoxins, biopesticides, biosurfactants, nanoparticles (NPs), probiotics and enzymes involved in the degradation of complex polymers [2, 11, 27].

Last decade streptomycetes were successfully used in the synthesis of metal NPs, especially of silver [1, 7, 35, 37] and gold [5, 42, 46], less of manganese, zinc and copper [33, 44, 45] nanoparticles. It was found that biogenic metal nanoparticles are more active and showed higher antimicrobial activity as compared to chemically synthesized nanoparticles. Moreover, physical and chemical methods involving the use of hazardous chemicals or the use of different kinds of radiations are more expensive [20, 42].

Ecologically, streptomycetes, due to their extracellular enzymes, have a great potential for biodegradation of organic and inorganic toxic compounds [2, 18, 19]. Several studies have demonstrated the ability of streptomycetes to grow and degrade several chemical families of pesticides, including organochlorine [8, 18, 19, 36, 43], carbamate [32], organophosphorous [28], pyrethroids [26], polyaromatic hydrocarbons [40], chloroacetanilides [38] and petroleum hydrocarbons [12].

Among metal-based engineered nanomaterials, iron NPs are, probably, the most used for bioremediation of a broad spectrum of pollutants, including halogenated organic chemicals, polycyclic aromatic hydrocarbons, pesticides and heavy metals [15, 29, 34, 41]. Iron NPs have large surface and high chemical activity, and they are significantly superior to the commonly used iron particles for the degradation of pollutants [15, 50].

Iron-based NPs are expected to be non-toxic, due to using Fe atom in several pathways of cell metabolism and, therefore, low iron toxicity [24]. But there is a series of investigations that prove the toxic action of iron NPs on different microorganisms, including: *Escherichia coli* [4, 10, 13], *Staphylococcus aureus* [13], *Dehalococcoides spp*. [4], *Pseudomonas putida* [10, 29], *Pseudomonas stutzeri* [34], *Bacillus subtilis var. niger, Pseudomonas fluorescens* [14], *Erwinia amylovora*, *Xanthomonas oryzae, Bacillus cereus* [6]. In all cases, the concentration, and size of NPs played an important role.

Regarding the interaction between iron NPs and streptomycetes, there is little information in specialized literature, but the existing data attests the resistance of streptomycetes to the action of iron NPs. The growth inhibition of streptomycetes was observed, while no bactericidal effect was detected [6, 31]. However, in

other cases, the growth of streptomycetes was stimulated in the presence of iron NPs [21, 31].

Summarizing the above, the purpose of our investigation was to determine the effect of magnetite NPs, zero-valent iron NPs and fluorinated dinitroaniline herbicide trifluralin on growth of streptomycetes strains.

MATERIAL AND METHODS

Materials. In our experiment we used two types of iron NPs: colloidal aqueous solution of magnetite (Fe_3O_4) NPs in concentration of 100 mg/L and colloidal aqueous solution of zero-valent iron (Fe(0)) NPs in concentration of 100 mg/L.

Encapsulated NPs Fe_3O_4-PVP and Fe(0)-PVP were synthesized by chemical co-precipitation method, in the presence of poly-N-vinylpyrrolidone (PVP) used as a stabilizer. Fe_3O_4 NPs were prepared using iron (II) sulfate and iron (III) chloride. Fe(0) NPs were prepared by chemical reduction from ferric chloride solution.

Iron(II) sulfate(≥ 99.7%), a saturated iron(III) chloride solution (≥ 99.0%), poly-*N*-vinylpyrrolidone (PVP, MW: 8000), and ammonium hydroxide (≥ 99.9%) were purchased from Sigma-Aldrich.

The resulting Fe_3O_4 NPs and Fe(0) NPs were characterized by X-ray powder diffraction (XRD) analysis, X-ray fluorescence analysis (XRF), scanning electron microscopy (SEM), and FTIR-spectroscopy.

The nanomaterial was studied by FTIR spectroscopy using a PerkinElmer Spectrum 100 FT-IR spectrometer in a spectral range of 650–4000 cm^{-1}. Spectral range was about 400–4000 cm^{-1} into vaseline. The samples were prepared in vaseline for recording in a range of 400–4000 $см^{-1}$.

X-ray diffraction analysis was carried out on a DRON-UM diffractometer using a (e-K_α- radiation at λ = 1.93604 A°) in a range of 2θ = 10°–80° at room temperature.

Scanning electron microscope (SEM) images were recorded with a Quanta 200 electronic microscope (ESEM) operating at 30 kV with secondary and backscattering electrons in a high vacuum mode.

Trifluralin (α,α,α–trifluoro–2–6-dinitro–N–N–dipropyl–p–toluidine), a pre-emergent herbicide belonging to the dinitroaniline chemical family, was used as a solution in acetone at concentration of 100 mg/L.

The objects of research were 6 strains of streptomycetes from the collection of laboratory "Soil microbiology", Institute of Microbiology and Biotechnology: *Streptomyces sp*. 0112, *Streptomyces sp*. 0212, *Streptomyces sp*. 0312, *Streptomyces sp*. 0412, *Streptomyces sp*. 0512, *Streptomyces sp*. 0612. The streptomycetes were isolated from soil long-term polluted with trifluralin and DDTs. Soil samples were collected near the former storage of persistent organic pesticides located in the central part of Republic of Moldova, Chişinau municipality. Soil type was carbonated chernozem [51].

Research methods. For assessing the inhibition activity of the iron NPs and trifluralin the agar plug diffusion method was used [49]. Initially the strains were inoculated in lawn on Czapek-Dox agar ($NaNO_3$ – 2.0 g, K_2HPO_4 – 1.0 g, $MgSO_4$ x $7H_2O$ – 0.5 g, KCl – 0.5 g, $FeSO_4$ – 0.01 g, glucose – 30.0 g, agar - 20.0 g, distilled water – 1000.0 ml, pH=7.0-7.3), after their grown up (10 days) the agar disks of streptomycetes colony mass were prepared by using sterile borer. Disks were then aseptically transferred to plates with Czapek-Dox agar medium amended with solutions according to protocol: variant 1 – control (the solid Czapek medium, pH 7.0-7.3); variant 2 – medium Czapek + solution of iron NPs; variant 3 – medium Czapek + solution of trifluralin; variant 4 – medium Czapek + mixed solutions of iron NPs and trifluralin. The mixture of solutions of iron NPs and trifluralin was incubated for 1 hour before using. Each experimental variant was repeated 3 times.

Diameter of growth zones for the streptomycetes strains was recorded at the 8th day. Measurement of the mycelial growth was conducted according to the growth rate of the streptimycetes. The inhibitory activity of trifluralin, magnetite NPs, and zero-valent iron NPs was calculated in percent of inhibition of growth, compared to the negative control (%), using following calculation:

Growth inhibition (%) = (DC - DT) / DC × 100,

where, DC = the diameter of the control colony (variant 1) and DT = the diameter of the experimental colony (variant 2, or 3, or 4) [30].

RESULTS

The micrographs show that the Fe_3O_4 NPs are spherical and have sizes of 20-25 nm (Fig. 1A). Since the nanoparticles exhibit high surface energy, they undergo a rapid particle interaction, coarsening, and then aggregation in solution. The resulting nanoparticles have agglomerated into larger entities with a size of 25–39 nm. The morphology and sizes of the Fe(0) NPs are shown in Fig. 1B.

IR spectra of Fe_3O_4-PVP NPs, Fe(0)-PVP NPs, and PVP proved the formation PVP-encapsulated nanoparticles.

X-ray diffraction confirmed the crystallinity of the resulting magnetite NPs. Size *d* of the Fe_3O_4 crystallites is $d = (25 \pm 1)$ nm; this value corresponds to microscopic data.

The presence of Fe(0) was confirmed by the diffractogram of the Fe(0)/PVP NPs, with the maximum diffraction at 2θ = 44.8°. Particles size was computed according to Debye-Scherrer formula, which corresponds to 4 nm.

The X-Ray Fluorescence Spectroscopy (XRF) of encapsulated nano-sized iron (~ 4.7 nm) showed that the transition energy corresponding to the gravity centre of the FeK-α_1 line shifts by 1.33 eV as a result of a transition from a flat polycrystalline sample to nanoscale iron.

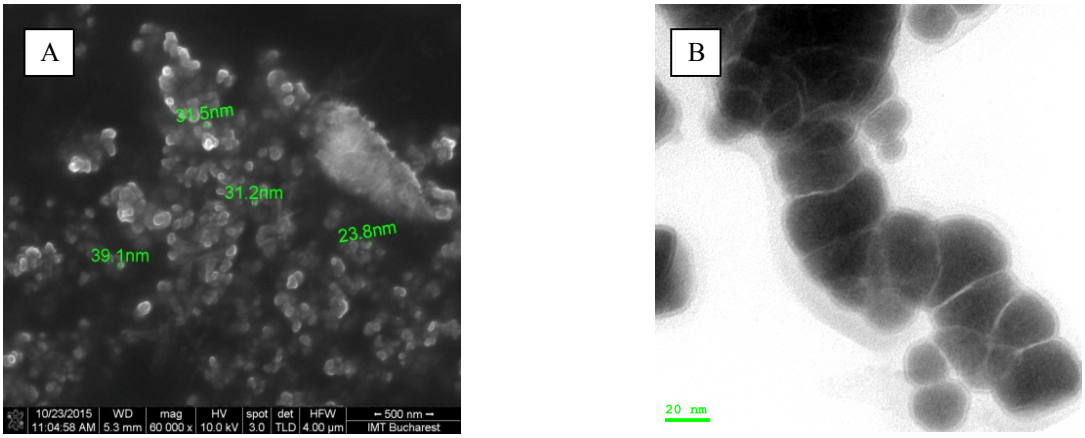

Figure 1. The scanning electron microscope photos of (a) magnetite (Fe_3O_4) and (b) zero-valent iron nanoparticles

The cultural characteristics of the selected strains of streptomycetes, inoculated in lawn, were observed after 10-14 days of growth on Czapek media (Tab. 1).

The results on streptomycetes strains growth in the presence of NPs of magnetite and Fe(0), trifluralin, as well as the mixture of solutions of iron NPs and trifluralin, are represented in figures below. The sensitivity of the streptomycetes to the NPs of magnetite and Fe(0), expressed as a percentage of the control, is represented in Fig. 2.

As can be seen from the graph, in most cases iron NPs have had a stimulating effect on the growth of streptomycetes. Sensitivity of the strains varied depending on the chemical form of iron NPs and the individual peculiarities of each strain. Thus, the magnetite NPs had a stimulating effect on the growth of *Streptomyces sp*. 0112 (5.56%) and *Streptomyces sp*. 0412 (18.57%); had no effect on *Streptomyces sp*. 0212 and *Streptomyces sp*. 0312; and had a little growth inhibition effect on *Streptomyces sp.* 0512 (3.7%) and *Streptomyces sp*. 0612 (1.28%).

Fe(0) NPs did not demonstrate a toxic effect on the studied streptomyces. In most cases, except the strain *Streptomyces sp.* 0312, we have recorded the growth stimulation. The strain *Streptomyces sp*. 0212 and *Streptomyces sp.* 0512 had grew up most actively, on 5.48% and 6.56% above control. It should be noted that the presence of Fe(0) NPs in the medium didn't influenced the growth activity of the strain *Streptomyces sp*. 0312.

Although streptomyces strains have been isolated from the soil long-term polluted with pesticides, they were found to be sensitive to the increased concentration of trifluralin (Fig. 3). Of the 6 studied streptomycetes, 4 strains had reacted by decreasing of growth activity in the presence of the trifluralin, as compared to the control. The most visible inhibition of growth was established at the strain *Streptomyces sp*. 0612 (9.09%). Exceptions were *Streptomyces sp*. 0112 and *Streptomyces sp*. 0212, their growth activity was stimulated in the presence of trifluralin with 2.12% and 2.74%.

After magnetite NPs were added to the nutrient medium, the decrease in trifluralin toxicity occurred (Fig. 3). Thus, in the case of *Streptomyces sp*. 0312 and *Streptomyces sp*. 0612 the negative effect of the pesticide was completely reduced. The growth activity values of these strains had returned to growth rates in the presence of magnetite NPs. Strains of *Streptomyces sp.* 0112 and *Streptomyces sp.* 0212, whose growth was stimulated by trifluralin, reacted differently to the mixture of iron NPs and trifluralin solutions. If in the case of *Streptomyces sp*. 0112 the stimulatory effect increased (4.17%), then at *Streptomyces sp*. 0212 decrease in growth activity and return to the control values there was established. At the strain *Streptomyces sp.* 0412 not only the total reduction of trifluralin toxicity, but also the maintenance of the effect of growth stimulation by the magnetite NPs was determined. It should be noted the reaction of the strain *Streptomyces sp*. 0512, which, in the presence of magnetite NPs, trifluralin, and mixed solutions showed a stable growth inhibition rate – 3.7%.

Mixing of trifluralin and Fe(0) NPs solutions led to the reducing of negative effect of trifluralin (Fig. 4). Thus, the growth activity of *Streptomyces sp*. 0112 and *Streptomyces sp*. 0212 strains increased, compared to the variant, in which only trifluralin was added.

Table 1. Cultural characteristics of streptomycetes strains on Czapek's agar

Strain	Color of			Growth
	Aerial mycelium	Reverse side of colony	Diffusible pigment	
Streptomyces sp. 0112	Dark ashy	White to grey	Non-pigmented	++++
Streptomyces sp. 0212	Pale blue	Colorless	Non-pigmented	++++
Streptomyces sp. 0312	Grayish white	White	Non-pigmented	++++
Streptomyces sp. 0412	Whitish	White to grey	Non-pigmented	++++
Streptomyces sp. 0512	Ashy	White to grey	Grayish-smoky	++++
Streptomyces sp. 0612	Smoky	White to grey	Brownish	++++

Note: ++++ good growth; +++ moderate growth; ++ slight growth; + weak growth.

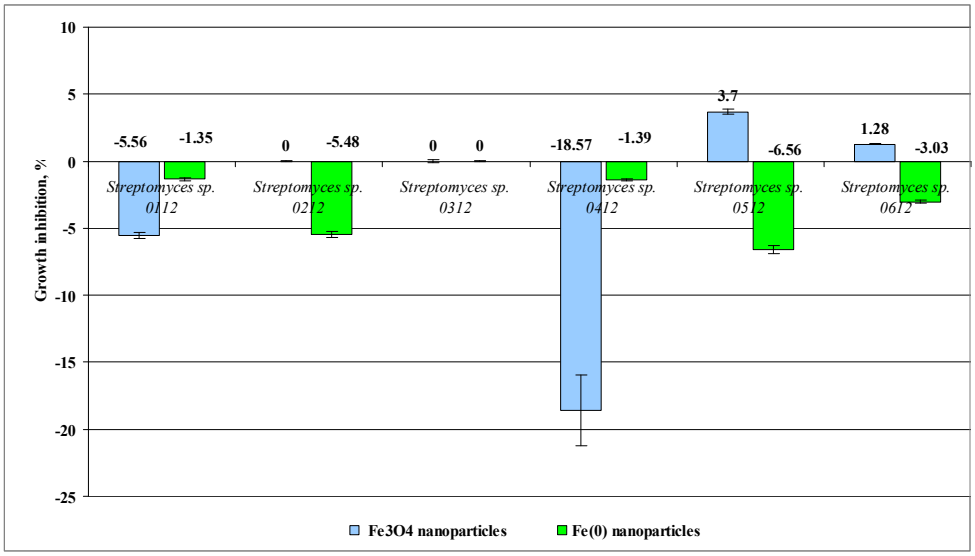

Figure 2. The effect of magnetite and Fe (0) on the growth of streptomycetes

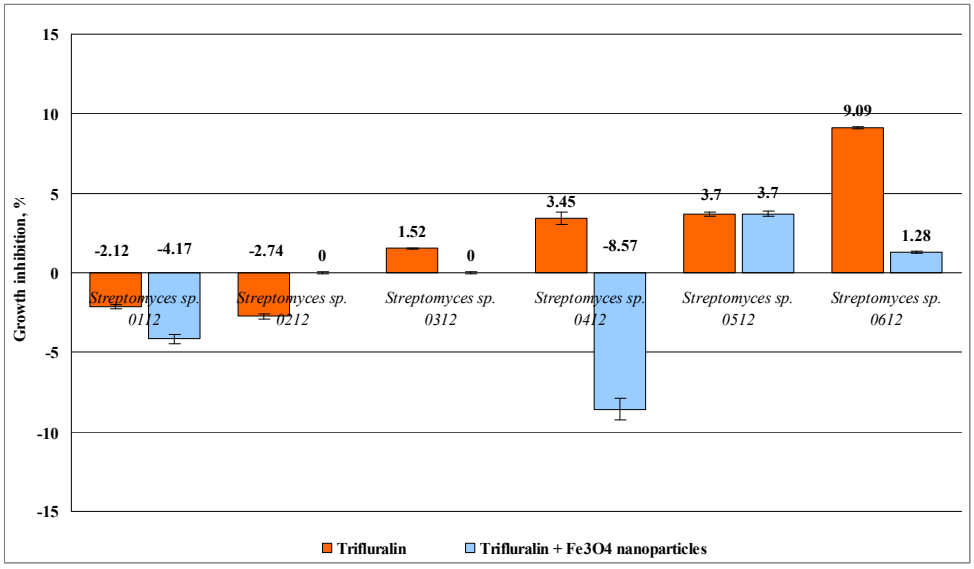

Figure 3. The effect of trifluralin and mixture of trifluralin and magnetite nanoparticles on the growth of streptomycetes

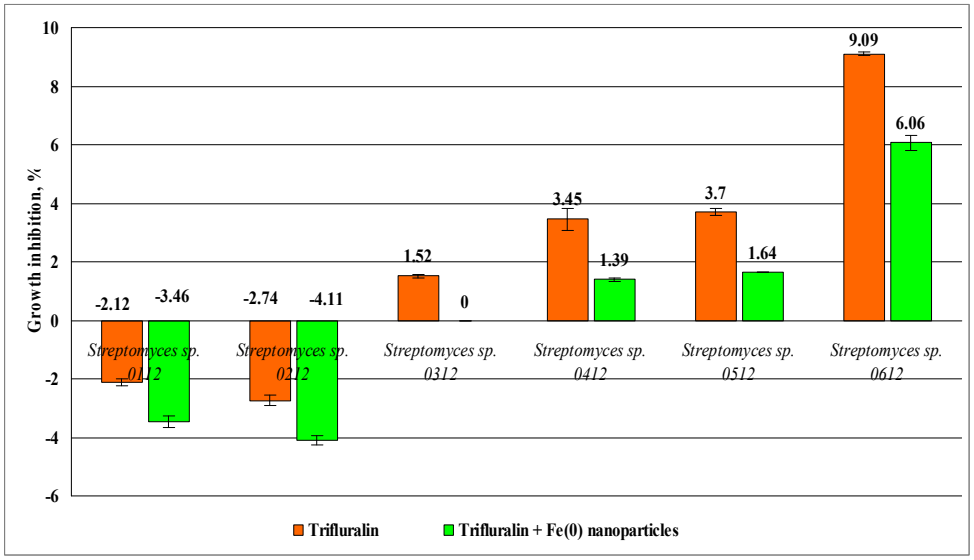

Figure 4. The effect of trifluralin and mixture of trifluralin and Fe(0) nanoparticles on the growth of streptomycetes

Sensitivity of strain *Streptomyces sp*. 0312 returned to the control values. Although the negative effect of trifluralin on *Streptomyces sp*. 0412, *Streptomyces sp*. 0512 and *Streptomyces sp*. 0612 wasn't completely reduced, however the toxicity of the pesticide was diminished.

DISCUSSION

Iron as a microelement is essential for cell growth and vital activity of microorganisms. The iron is involved in a large number of cellular processes, acting as a cofactor of a large number of enzymes, it is necessary for the transport of oxygen, nitrogen fixation, ATP generation and DNA synthesis. Because of low bioavailability of iron in nature the microorganisms have developed a system with high iron affinity, based on the synthesis of siderophores. In high concentrations iron is toxic; therefore the intracellular iron level in microorganisms is strictly regulated by the iron assimilation systems [16].

At present, remediation of polluted soil and groundwater with iron-based NPs represents a faster, cheaper and a potentially more effective treatment option than current *ex situ* and *in situ* methods. The direct application to soils of large amounts of iron-based NPs for remediation purposes raises specific concerns about potential consequences on soil microbial communities and their key functions for soil fertility and biodegradation of pollutants [22, 39]. Because microorganisms are especially sensitive to environmental changes, the structure and abundance of the microorganism community may shift in response to foreign nanomaterials [21].

The Fe(0) and the Fe_3O_4-based NPs are oxidized in biological media. Recent nanotoxicological studies report that this oxidation can be responsible for their toxicity towards environmental bacteria. The cytotoxic effects are directly associated to this oxidation and the generation of an oxidative stress as demonstrated using a mutant strain of *E. coli* completely devoid of superoxide dismutase activity. This stress results from the generation of ROS (reactive oxygen species) through Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-$) during the oxidation of Fe^0 nanoparticles to magnetite ($Fe_3^{2+/3+}O_4$) and lepidocrocite ($\gamma Fe^{3+}OOH$). Magnetite NPs are also fully oxidized in maghemite ($\gamma Fe_2^{3+}O_3$) in biological media inducing an oxidative stress towards *E. coli* [3]. In contrast to the Fe^0- or the Fe^{2+}-based NPs, fully oxidized maghemite particles are very stable in biological media [3] and do not generate significant cytotoxicity or genotoxicity *in vitro* due to the absence of electronic or ionic transfers [3, 4].

In our experiments, the addition of iron NPs to the streptomycetes culture medium in most cases has led to the growth stimulation of microorganisms. The cytotoxic effect induced by magnetite NPs, mentioned previously, was observed only at *Streptomyces sp*. 0512 and *Streptomyces sp*. 0612. These results correlate with previously data obtained by us and with those existing in the literature, that the streptomycetes are resistant to iron NPs. Thus, He et al. (2011) [21] have demonstrated that the addition of iron oxide magnetic NPs in soil could potentially stimulate some bacterial growth (especially of *Actinobacteria*, such as *Duganella*, *Streptomycetaceae* or *Nocardioides*) and change the soil bacterial community structure, although bacterial abundance does not change. And the research carried out by Barzan et al. (2014) [6] showed a greater resistance of *Streptomyces* to Fe(0) NPs compared to Gram-negative bacteria and growth inhibition was observed, while no bactericidal effect was detected.

Resistance of streptomycetes may be explained by the presence of the thick (20-80 nm) peptidoglycan layer in the cell walls of gram-positive bacteria, making them more resistant to the NPs [6]. Definitely, peptidoglycans that contain mucopeptides, glycopeptides and mureins are the structural elements of almost all bacterial cell walls. Their domination in the cell wall of some gram-positive bacteria is substantial, but seems to be less in gram-negative bacteria. It was speculated that this discrepancy in membrane structure may result in their different absorption ability onto the Fe(0) NPs. Hence, it was demonstrated that there is an association between cell wall architectures and sensitivity to Fe(0) NPs, which indicates that either membrane disruption or differential membrane permeability play a part in cytotoxicity of Fe(0) NPs [48].

Moreover, there are data that iron metabolism is strictly regulated in *Streptomyces* species by a family of pleiotropic transcriptional regulators called DmdR. These regulators sense intracellular iron levels and control the expression of genes encoding several iron-containing enzymes, oxidative stress response systems and siderophore biosynthesis clusters like desferrioxamine [17].

In our experiments, mixing the solutions of iron NPs and trifluralin resulted in the reduction of trifluralin toxicity. This indicates that iron NPs is as effective as bulk Fe materials in the reactions of successive reduction of nitro groups in dinitroaniline herbicides [25, 47].

Thus, after analyzing the experimental data, as well as the data from the specialized literature, we could say that, although the streptomyces are Gram-positive and have a thick peptidoglycan layer, their sensitivity for iron NPs has proved to be individual for each strain.

Regarding the sensitivity of streptomycetes to iron NPs, depending on the chemical formula, the Fe(0) NPs had no inhibitory effect on growth of streptomycetes, as compared to magnetite (Fe_3O_4). However, no bactericidal effect of iron NPs was established, which correlates with literature data that streptomycetes are resistant to the action of iron NPs.

Most of the studied microorganisms were found to be sensitive to trifluralin, except *Streptomyces sp*. 0112 and *Streptomyces sp*. 0212. Their growth activity was stimulated in the presence of trifluralin. This is an indication that these strains could be selected as

potential trifluralin destructors. Cytotoxic effect of trifluralin was diminished by iron NPs, but not fully reduced. We have supposed that in order to obtain a better result it is necessary to increase the interaction time between trifluralin and iron NPs solutions. These data could be useful for development of preparation for nanobioremediation of soil contaminated with trifluralin.

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