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BIOTECHNOLOGY FOR CLEANING UP SOILS FROM EXPLOSIVES

This article discusses the issue of environmental pollution caused by explosives. Nitro-organic substances (trotyl, hexogen, etc.), as well as highly toxic carcinogenic compounds contaminate the soil, groundwater and reservoirs at sites of military activities. Due to their composition and stable structure, explosives basically do not undergo complete natural transformations under biotic conditions even for decades, often getting into the food chain causing serious pathologies. The presented investigation is based on use of a collection of microorganisms, comprising up to 8 thousand strains of bacteria, filamentous fungi, actinomycetes, isolated from various soil and climatic zones and locations of the former Soviet military units, including shooting ranges. Model experiments were conducted in a lab, and small field conditions (100 m²). At the first stage, selectively chosen rhizospheric microorganisms, known by their

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predominantly determined detoxification activity, were introduced into the soil contaminated with explosives, to carry out the primary transformation of explosives, transforming into more hydrophilic and less toxic compounds for further transformation. At the second stage, plants were sown on soil that was artificially contaminated with explosives, and then treated by selected microorganisms. These plants assimilated, carried out further degradation of toxic components and products of their partial transformation, and transformed partially degraded explosives into above ground parts or mineralized them. At the third stage, plants containing toxicity were treated with microscopic fungi which had powerful extracellular enzyme systems that degrade the remaining part of toxic components. As a result, it has been established that in 30—45 days, i.e. within one summer season, it is possible to achieve 70—80 % soil clearance from toxic compounds. The biotechnology itself is environmentally friendly and is based on the use of non-toxic forms of microorganisms that are isolated from the soil.

***Keywords*:** *pollutants, contaminated soils, explosives, phytoremediation, 2,4,6-trinitrotoluene (TNT), hexogen (hexahydro-1,3,5-trinitro-1,3,5-triazine-RDX).*

Introduction. The manufacturing and application of 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as explosives in various commercial and military products has resulted in serious environmental contamination. These contaminants have high total toxicity [1]; moreover, they are especially dangerous for human health as possible carcinogens [2].

The most recent authors have developed biotechnology for explosives remediation by microorganisms and plants. This is a relatively cheap and highly environmentally friendly biotechnology for cleaning up the environment polluted with a wide range of chemical contaminants including explosives [3—6].

Several previously selected plants that actively absorb and transform TNT [7—14], and microorganisms with high TNT assimilation capabilities [15—20] have commonly been used for cleaning up water and soil contaminated with explosives. The joint application of plants and bacteria for the phytoremediation of TNT contaminated soil has also been reported [21]. According to studies, the transfer of bacterial genes into transgenic plants increases their detoxification potential [4, 6, 22—25]. The most problem for the phytoremediation of TNT and RDX is their low solubility, resulting in inefficient absorption by plant leaves and root system. These compounds undergo only partial transformation in plant cells and are accumulated mainly in the form of conjugates with cellular metabolites. A large portion of the metabolites, sometimes above 60 %, seems to get involved in conjugation with insoluble biopolymers [11, 26, 27], often with lignin and hemicellulose [28]. These conjugates are compartmentalized into vacuoles and cell walls. As a result, in plant cells, the conjugates maintain their structure for some time, until their dissociation and further degradation, carried out by oxidative systems of enzymes.

Research objective. The present work offers a new strategy to solve the problem of degradation of explosives, the essence of which is to achieve the

maximum degree of neutralization of toxic compounds through creation of the following three-stage system of phytoremediation:

• At the first stage ("rhyzosphere biodegradation"), selected rhyzospheric microorganisms are introduced into contaminated object (soil, water); microorganisms to carry out the initial transformation of explosives, converting them into comparatively less toxic, more hydrophilic compounds, which are much easier assimilated by plants.

• At the second stage ("phytoextraction and phytotransformation"), plants that have been determined to have a high phytoremediation potential degrade or remove explosives and/or intermediates of their partial transformation from polluted object by transforming them into above ground parts.

• At the third stage ("bioutilization"), plant residue used in phytoremediation is treated with microorganisms (fungi), which have powerful extracellular oxidative enzyme systems that degrade toxic compounds.

Long-term investigations have been carried out prior to these experiments in which plants and microorganisms were selected, and the processes of decontamination occurring during their implementation have been studied [10—12, 16, 18].

Research methods and sources. Previously alfalfa (*Medicago sativa*) and soybean (*Glycinemax*) were used as plant-based phytoremediation agents. Based on their activity, 41 bacterial strains, 14 strains of microscopic fungi and 3 yeast strains have also been selected - their ability to degrade TNT and RDX was previously established (by using these toxicants as the only source of carbon and nitrogen) [12, 16, 18].

For screening, active strains of bacteria, microscopic fungi, yeasts, and actinomycetes from the collections of microorganism cultures being at our disposal have been used [11, 18]. The main criteria for the selection of plants and microorganisms was the presence a highly active enzyme-nitroreductase, carrying out the primary reactions of TNT and RDX transformation, which leads to the decrease in toxicity and an increase in the water solubility of explosives [11, 12].

For a more thorough evaluation of bioremediation potential of the selected plants and microorganisms, radioactive preparations of [1-14C]-TNT was synthesized from [1-14C]-toluene and [15N]-RDX obtained from [15N]-urotropine and [15N]-nitricacid. This made it possible to establish the ways of assimilation and transformation of explosives in microorganisms and plants. For instance, it was detected that when [1-14C] TNT is assimilated by plants, most of its part accumulates in the upper above ground parts of plants, being coupled to insoluble plant biopolymers — cellulose, hemicellulose, lignin, etc. In the case of [^{15}N] RDX assimilation, the main part of the ^{15}N isotope is also accumulated in the upper parts of plants.

According to the obtained results, the supposed schemes of TNT transformation by microorganisms and plants are proposed. It is suggested that

the nitroreductase participates in the first stage of TNT degradation in microorganisms, and the formed reduced metabolites undergo destruction by laccases or other oxidases. In plants the basic part of TNT (80-85 %) in the initial process is also reduced by nitroreductase, or the oxidation of TNT methyl group to carboxyl group takes place. Finally, soluble low-molecular (~30 %) and insoluble high-molecular (~70 %) conjugates are formed by participation of plant transferases [11].

Microorganisms of different taxonomic groups assimilate [1-^{14}C] TNT by different intensities. However, in all cases the carbon skeleton of TNT assimilated by above indicated selected strains undergoes deep transformation that is testified by radioactivity of the fractions of organic acids and amino acids. In particular, carbon atoms of assimilated and transformed [1-^{14}C] TNT are basically used by microorganisms for the biosynthesis of organic acids. In cultivation medium of microscopic fungi, the presence of labeled amino acids is not observed. Among the amino acids, the compounds with aromatic ring were prevalent, as compared to organic acids, the radioactive label of TNT was mostly detected in fumaric and succinic acids. Fumaric acid is one of the products of biodegradation of benzene ring and is easily transformed into succinic acid. Formed standard cellular metabolites, indicate a deep degradation of TNT.

An innovative technological approach for the rehabilitation of soils contaminated with explosives was tested on artificially contaminated soils of various types. They were prepared as follows: the soil was sifted through a sieve, thoroughly mixed with a 0.5 mM TNT solution, and divided into samples. A 10 % suspension of microorganisms was added to the samples and dried. After this, the samples were placed in special cuvettes, in which experiments were carried out. Three days later, plant seeds preliminarily swollen in water were sown in the samples. Incubation was carried out at room temperature (20—25 °C) and natural light. During the experiment, the samples were moistened every 4-5 days, without stirring. After 30 days of incubation, the residual content of TNT in soil samples was determined by standard methods [29].

In experiments using [1-14C]TNT, a solution of [1-14C]TNT (with radioactivity 500 Bq/mg) in diethyl ether was added to the soil and thoroughly mixed, the ether was evaporated. Further procedures were carried out similar to the procedure described above. To determine the radioactivity, soil samples were extracted with methanol, the extracts were evaporated, the dry residue was dissolved in benzene, and the radioactivity was measured on a scintillation spectrometer (SL-30 Rackbeta, the efficiency of which makes 95 %).

At the end of the process to study the possibility of bio utilization of plant biomass, the plant biomass was dried and introduced into an incubation medium containing active strains of microscopic fungi. Cultivation was carried out in a sealed chamber, on a magnetic stirrer, at 20—30°C. To fix the emitted

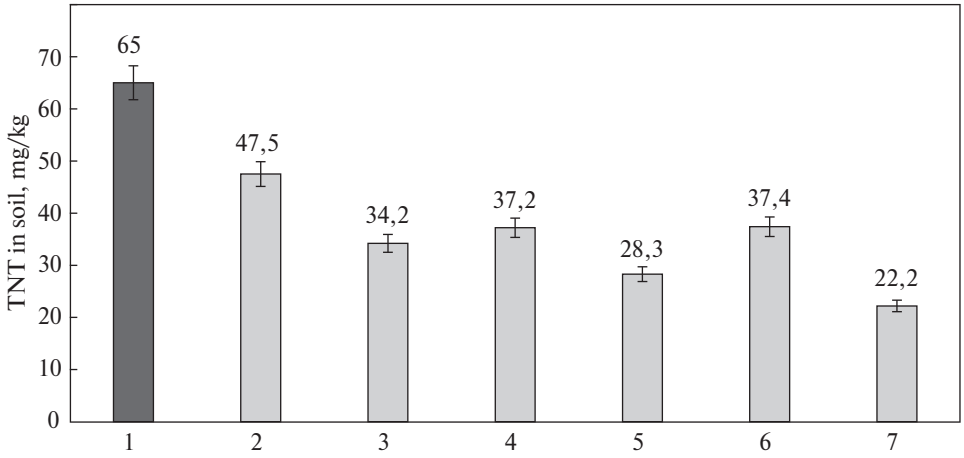

Fig. 1. Purification of artificially polluted "Chernozyom" soil with cultures of TNT-degrading microorganisms. Initial pollution 65 mg TNT per 1 kg of soil; duration of experiment 30 days; temperature 20—25°C. Experiment options: 1 — Initial pollution (before starting an experiment); 2 — Control (without inoculation of microorganisms); 3 — Bacterial strain *Rhodococcus* sp. TNT-74; 4 — Bacterial strain *Pseudomonas* sp. TNT-44; 5 — Strain of microscopic fungi *Aspergillus niger* J 3-4; 6 — Strain of microscopic fungi *Mucor* sp. D 1-1; 7 — Consortium composed with bacterial strains *Rhodococcus* sp. TNT-74 and *Psedomonas* sp. TNT-44.
Source: estimated by the authors.

radioactive CO_2, a vessel with a 30 % KOH solution was placed in the chamber. At certain intervals, samples of the culture liquid and alkali solution were taken, and their radioactivity was determined.

To study the possibility of bio utilization of plant biomass, at the end of the process, the plant biomass was dried and introduced into an incubation medium containing active strains of microscopic fungi. Cultivation was carried out in a sealed chamber, on a magnetic stirrer, at 20—30°C. To fix the emitted radioactive CO_2, a vessel with a 30 % KOH solution was placed in the chamber. At certain intervals, samples of the culture liquid and alkali solution were taken, and their radioactivity was determined.

Presented data are the mean of three replicates ± standard deviation (SD). The replicates represent an assay of a sample from the same source multiple times. The statistical analysis of the obtained data was performed using the method of Descriptive Statistics in Excel.

Results and discussion. Below are the results of model experiments carried out in laboratory and small scale field conditions, for testing and evaluation the remediation potential of selected microorganisms and plants (Fig. 1—3).

The above experimental results showed that the degradation process with the participation of bacterial strains proceeds basically in the same way. Par-

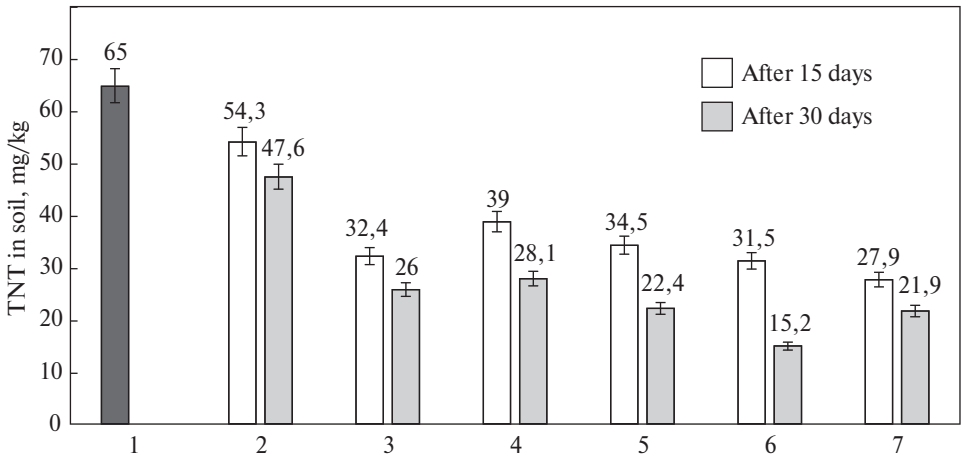

Fig. 2. Purification of artificially polluted "Chernozyom" soil from explosive (TNT) by plants and a consortium composed by bacterial strains of TNT-degrading microorganisms. Initial pollution 65 mg TNT per 1 kg of soil; duration of experiment 30 days; temperature 20—25 °C.Experiment options: 1 — Initial pollution (before start of experiment); 2 — Control (without inoculation of microorganisms and plants); 3 — Soybean (25 seedlings per 1 kg soil); 4 — Alfalfa (100 seedlings per 1 kg soil); 5 — Consortium composed with bacterial strains *Rhodococcus* sp. TNT-74 and *Pseudomonas* sp. TNT-44 (without plants); 6 — Soybean (25 seedlings per 1 kg soil) and consortium; 7 — Alfalfa (100 seedlings per 1 kg soil) and consortium
Source: estimated by the authors.

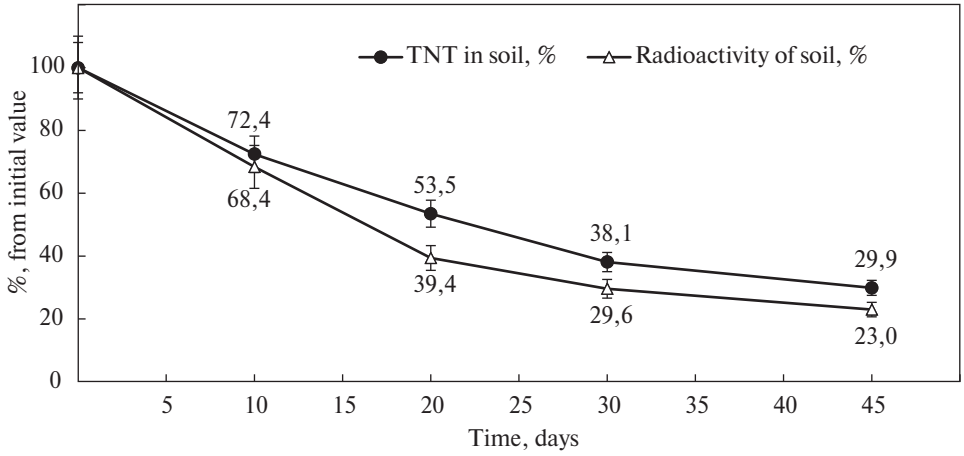

Fig. 3. Dynamics of bioremediation of TNT-labeled radioactive compounds from contaminated "Chernozyom" soil. Initial contamination was 137 mg [1-14C] TNT per kilogram of soil, the experiment lasted 45 days, and the temperature ranged from 20 to 25° C. bacterial consortia with strains of *Pseudomonas sp.* TNT-44 and *Rhodococcus sp*. TNT-74, and 15 soybean seedlings per 1 kilogram of soil.
Source: estimated by the authors.

ticularly, about 30 % of the assimilated TNT is absorbed by the native soil microflora (Fig. 1, 2) control variants without inoculation of microorganisms and plants), and the introduction of TNT-degrading crops additionally increases the intensity of the bioremediation process by 25—30 % (Fig. 3). Chromatographic analysis shows that the same conversion products of TNT are formed. The greatest effect was achieved by sowing soybeans on soil treated with bacterial strains of *Pseudomonas sp.* TNT-44 and *Rhodococcus sp.* TNT-74 (Fig. 2, 3). At the same time, the degree of purification increases up to 70—75 %.

The experiments also showed that plants effectively assimilate radioactive metabolites from the soil, which are formed during the bacterial transformation of [1 - 14C]TNT, which is very important for the complete rehabilitation of soils and their suitability as agricultural plantations.

The studies carried out to establish the possibility of bio utilization of plant biomass in phytoremediation showed that the selected strain of the fungus *Aspergillus niger* J 3-5 is able to completely degrade the remains of TNT and its intermediate metabolites contained in plants.

Conclusions and prospects for future research. Results of the performed investigation present a strategy based on scientific research for regulation of eco-physiological characteristics of plants and microorganisms to maximize their phytoremediation potential and, eventually, to develop a novel ecological biotechnology for cleaning up soils contaminated by explosives.

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БІОТЕХНОЛОГІЯ ДЛЯ ОЧИЩЕННЯ ҐРУНТІВ ВІД ВИБУХОВИХ РЕЧОВИН

У статті обговорюється проблема забруднення навколишнього середовища, спричиненого вибуховими речовинами. Неорганічні речовини (тротил, гексоген та інші), а також високотоксичні сполуки отруюють ґрунти, підземні води і водойми в місцях бойових дій. Завдяки своєму складу і стійкій структурі вибухові речовини, як правило, не розкладаються повністю в біотичних умовах навіть протягом десятиліть, часто потрапляючи в харчовий ланцюг і тим самим викликаючи серйозні патології. Представлене дослідження ґрунтується на використанні колекції мікроорганізмів, до складу якої входять до восьми тисяч штамів бактерій, нитчастих грибів, антиноміцетів, виділених із різних ґрунтових і кліматичних зон і місць дислокації підрозділів колишньої радянської армії, в тому числі полігонів. Модельні експерименти проведені в лабораторії та польових умовах (на невеликій ділянці розміром 100 м^2). На першому етапі спеціально відібрані ризосферні мікроорганізми, відомі своєю переважно знезаражувальною дією, були поміщені в ґрунт, заражений вибуховими речовинами, щоб спричинити первинне перетворення останніх у більш гідрофільні та менш токсичні сполуки з метою їх подальшого розщеплення. На другому етапі в ґрунт, штучно заражений цими вибуховими речовинами, було висіяно рослини і потім піддано дії окремими мікроорганізмами. Рослини засвоїли і далі розщепили токсичні компоненти і продукти їх часткового розкладання, перетворивши частково розщеплені вибухові речовини в наземні елементи і мінералізувавши їх. На третьому етапі рослини, що містять токсичні речовини, було піддано дії мікроскопічних грибів, що мають потужні позаклітинні ферментні системи, які розщепили решту токсичних компонентів. У результаті встановлено, що протягом 30—45 днів, тобто за один літній сезон, можна очистити від токсичних сполук 70—80 % ґрунту. Ця біотехнологія є екологічно чистою і основана на використанні нетоксичних форм мікроорганізмів, виділених із ґрунту.

Ключові слова: *забруднюючі речовини, заражені ґрунти, вибухові речовини, фіторемедіація, 2,4,6-тринітротолуол (ТНТ), гексоген (гексагідро-1,3,5-тригітро-1,3,5-триазин – RDX).*