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Higher Plants Ability to Assimilate Explosives

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***Abstract*—**The ability of agricultural and decorative plants to absorb and detoxify TNT and RDX has been studied. All tested 8 plants, grown hydroponically, were able to absorb these explosives from water solutions: Alfalfa > Soybean > Chickpea> Chikling vetch >Ryegrass > Mung bean> China bean > Maize. Differently from TNT, RDX did not exhibit negative influence on seed germination and plant growth. Moreover, some plants, exposed to RDX containing solution were increased in their biomass by 20%. Study of the fate of absorbed [1-^{14}C]-TNT revealed the label distribution in low and high-molecular mass compounds, both in roots and above ground parts of plants, prevailing in the later. Content of ^{14}C in low-molecular compounds in plant roots are much higher than in above ground parts. On the contrary, high-molecular compounds are more intensively labeled in aboveground parts of soybean. Most part (up to 70%) of metabolites of TNT, formed either by enzymatic reduction or oxidation, is found in high molecular insoluble conjugates.

Activation of enzymes, responsible for reduction, oxidation and conjugation of TNT, such as nitroreductase, peroxidase, phenoloxidase and glutathione S-transferase has been demonstrated. Among these enzymes, only nitroreductase was shown to be induced in alfalfa, exposed to RDX. The increase in malate dehydrogenase activities in plants, exposed to both explosives, indicates intensification of Tricarboxylic Acid Cycle, that generates reduced equivalents of NAD(P)H, necessary for functioning of the nitroreductase. The hypothetic scheme of TNT metabolism in plants is proposed.

***Keywords*—**Higher plants, TNT, RDX, transformation.

I. Introduction

AMONG 20 energetic compounds regularly used in munitions the most widely spread are 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) [13, 22]. Application of these compounds in military activities and industry, results in their dispersal in environment, especially in soils and groundwater. In spite of scanty information it could be supposed that concentration of explosives in soils is annually increasing.

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Recent studies of the molecular mechanisms of organic pollutants decomposition by plants and microorganisms exhibits their potential against environment contamination with wide spectrum organic toxicants, including explosives [8, 9, 20].

Study of plants ability to absorb and detoxify TNT and RDX from soil and water is important for creation of phytoremediation technologies for clean up of polluted by explosives sites. Some aquatic and wetland plants have been reported to assimilate above mentioned explosives [3]. The aim of the present work is to reveal the potential of some crops and decorative plants to absorb and metabolize TNT and RDX.

II. Materials and Methods

A. Plant Material

Experiments were carried out on the following annual mono and dicotyledonous plants: ryegrass (*Lolium multiflorum),* maize *(Zea mays),* chickling vetch (*Lathyrus sativum*), chickpea (*Cicer arietinum*), alfalfa (*Medicago sativa),* china bean (*Vigna sinensis*), mung bean (*Vigna radiata*), and soybean *(Glycine max*).

B. Effect of Explosives on Seed Germination and Plant Growth

Plant seeds were soaked in running water or solutions containing different concentrations of TNT (0.1, 0.5 and 1.0 mM) and RDX (0.10, 0.25, 0.50 and 2.5 mM), at 22–25°C. Maximum concentrations of explosives correspond to saturated solutions. After 4 days, seeds germination ability, i.e. the correlation between the number of germinated and sowed seeds was estimated.

The germinated seedlings were exposed to different concentration TNT or RDX solutions in running water and cultivated hydroponically at ambient illumination and temperature, between 22 and 25°C (the concentrations and incubation conditions in each experiment are given in legends of tables and figures). Plant growth parameters: plant biomass, height of stems, length of roots and chlorophyll content in leaves of shoots, have been determined daily during 10 days.

C. Absorption of TNT and RDX from Water Solutions

From TNT-containing media on which the plants grew, 1ml of a solution was taken every 24 h and added to 1ml of 1M KOH. TNT content was determined according to extinction at 447 nm [1]. In case of RDX the analyses were performed by reverse phase HPLC [21].

D. Distribution of Absorbed TNT among Plant Organs and Intracellular Compounds

Distribution of absorbed TNT and its metabolites among plant organs and the fractions of low- and high-molecular mass compounds were studied in the following way: 5-Days old seedlings of soybean were exposed to 0.5mM [1-^{14}C]-TNT in running water. [1-^{14}C]-TNT was synthesized by us as described previously [1]. After 5 days of exposure, roots and shoots were separately fixed in boiling 80% ethanol, and low-molecular mass compounds were extracted from the biomass. Insoluble residue, containing high-molecular mass compounds was rinsed in alcohol, dried, and then burned [18]. The $^{14}CO_2$ formed was absorbed by a 30% KOH solution. Radioactivities of this alkaline solution and of the alcohol extracts were determined using scintillation counter (LKB ABS 16125, Bromma, Sweden).

E. Determination of Enzyme Activities

Plants exposed to explosives, after definite time were washed with distilled water, roots and shoots cut separately and homogenized in 0.05M phosphate buffer, pH 7.4. Homogenates were squeezed through cheesecloth and centrifuged at 1000g, 20 min. In the supernatant activities of enzymes were determined.

Nitroreductase activity was determined according to the rate of TNT reduction by measuring of untransformed TNT in an incubation mixture [1]. In highly alkaline solution, TNT has a pronounced optical absorption at 447 nm, whereas its major metabolites: 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and 2,6-diamino-4-nitrotoluene, have no absorbance at this wavelength [12]. The incubation mixture contained assay solution, TNT, and a reducing agent (NADH or NADPH) in phosphate buffer, pH 7.4. Control and blank variants did not contain TNT. After 30 min 1M KOH was added to incubation mixtures and pH adjusted to pH 12.2. Additionally TNT was added to control and corresponding amount of water to blank. After 4 min the absorbance at 447 nm was measured against blank variant. The difference between the control and experimental variants corresponds to transformed TNT during the incubation period. The compositions and concentrations in the incubation mixture are presented in corresponding tables. Specific activities were calculated as mmole TNT in min per mg protein.

Peroxidase activity was determined spectrophotometrically at 470 nm, according to the rate of H_2O_2-dependent oxidation of guaiacol [7]. Specific activities were calculated as ΔA_{470} in min per mg protein. In tables, activities of enzymes are presented in percents.

Phenoloxidase activity was determined spectrophotometrically at 420 nm, according to the rate of pyrocatechol oxidation [10]. Specific activities were calculated as ΔA_{420} in min per mg protein. In tables, activities of enzymes are presented in percents.

Activity of Glutathione S-transferase was determined spectrophotometrically at 340 nm according to the procedure described in Schröder *et al* [17]. Specific activities were calculated as mmole 1-chloro-2,4-dinitrobenzene in min per mg protein.

Glutamate and malate dehydrogenase activities were determined spectrophotometrically, according to the rate of oxidation of NADH at 340 nm in the reaction mixture described earlier [4]. Specific activities were calculated as μmole NADH in min per mg protein.

Glutamine synthetase activity was determined in the transferase reaction by the method of Shapiro and Stadtman [19], according to the amount of γ-glutamylhydroxamic acid formed in a reaction mixture described earlier [15]. Specific activities were calculated as μmole γ-glutamylhydroxamic acid in min per mg protein. In tables, activities of enzymes are presented in percents.

Protein was determined by the method of Bradford [5], cholophyll, spectrophotometrically at 650nm by Arnon's method [2].

III. Results and Discussion

A. Plant Growth on Explosives and Uptake of TNT and RDX

TNT expressed negative influence on tested plants seed germinability: 0.1 mM TNT decreases the number of germinated seeds by 10-15% on average; at 0.5 mM TNT concentration the lagging in seed germinability reaches 15-25%; and at the highest 1mM TNT concentration, the difference between the germinability of test and control variants is equal to 30-40%.

As for development of germinated seedlings, roots of seedlings submersed in 0.5 mM solution of TNT (corresponding 100 ppm pollution, which 50 times exceeds ecotoxicologically harmless concentration [14]) was shown to grow much slower, but above ground parts of seedlings lag in growing 2-4 times as compared to control plants. Accordingly, the plant biomass was decreased. The soybean seedlings, which are germinated from soaked in 0.5 mM solution of TNT seeds, relatively better adapt to the existence of the explosive.

At higher, 1mM TNT, all plant seedlings have lower height of stems; their roots become shorten and brown. The chlorophyll content is significantly decreased (by 25-30%).

High tolerance of soybean to TNT among the tested plants should be mentioned. Growth parameters of this plant on the saturated solution of the explosive (1.0 mM), decreases only by 5-10%. To summarize the data, it can be concluded that the tolerance of tested 8 plants to TNT decreases according to following order:

Soybean > Mung been > Ryegrass > Chickpea > Chickling vetch > Alfalfa > China bean > Maize.

Statistically valid difference between growth parameters of control and test plants hasn't been obtained in analogous experiments with another explosive – RDX. All the tested plants have practically identical tolerances to increased concentrations of RDX. Moreover, in case of highest concentration of this explosive (2.5 mM), increase in biomass (by 20%) of 10-days old seedlings was observed. Presumably, these plants are able not only to detoxify RDX, but to use this compound as nitrogen and/or carbon source.

Parallel to plant growth, the residual content of TNT in nutrient medium was determined (Fig. 1). The results show

that TNT uptake ability of tested legumes decreases in the following order:

Alfalfa > Soybean > Chickpea> Chikling vetch > Mung bean.

It should also be noted that germination of soybean seeds on solution of TNT promotes the assimilation ability of this explosive by seedlings.

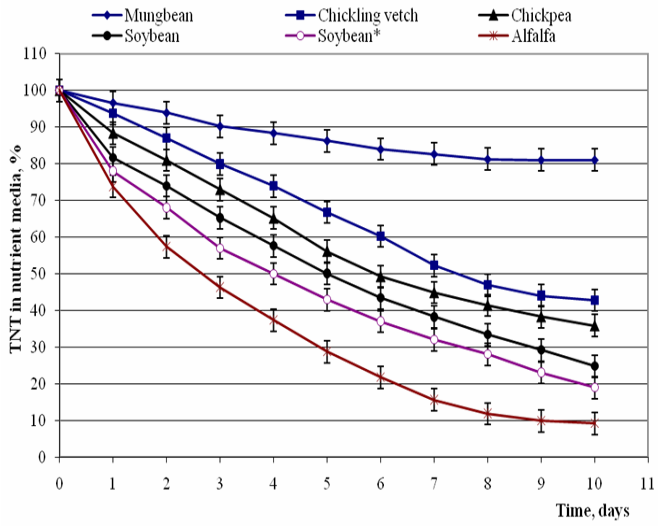


Fig. 1 Uptake of TNT from water solutions by plants. The seeds of plants were soaked in running water during 3 days. On the 4th day of germination, the seedlings (375 seedlings of alfalfa and 40 for other plants) were transferred to solutions of 0.5 mM TNT in running water (volumes: 1000 ml for alfalfa and 600 ml for other variants). In case of soybean germination of seeds was carried out on 0.5 mM TNT solution too (*). Exposure time 10 days; temperature 22–25°C

Analogous investigations with RDX show that all tested plants completely uptake the explosive from water solutions during 5-7 days (Fig. 2). The seedlings of soybean are characterized with the highest rate of RDX uptake.

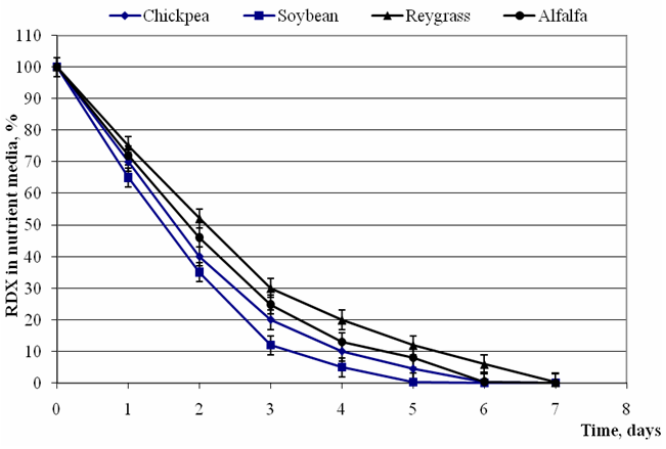


Fig. 2 Uptake of RDX from water solutions by plants. The seeds of plants were soaked in running water during 3 days. On the 4th day of germination, the seedlings (175 seedlings for alfalfa and ryegrass and 40 for soybean and chickpea) were transferred to solutions of 0.5 mM RDX in running water (volume 1000 ml). Exposure time 7 days; temperature 22–25°C

B. TNT Distribution and Transformation in Plants

Distribution of [1-^{14}C]-TNT label in plant organs and low and high molecular mass compounds**.**

Special experiments using ^{14}C-labeled TNT have been carried out to determine the distribution of absorbed by roots TNT and its metabolites between the plant organs and low- and high-molecular mass compounds (Fig. 3). The results indicate the universal distribution of TNT labeled carbon atom in low and high-molecular mass compounds in roots and above ground parts of soybean seedlings. The results of these experiments prove ones again the high mobility of TNT and its metabolites in plants [4]. Content of TNT and its metabolites ^{14}C among low-molecular compounds in plant roots are much higher than in above ground parts. On the contrary, high-molecular compounds are more intensively labeled in aboveground parts of soybean. It should be proposed that most part (up to 70%) of metabolites of TNT is conjugated with biopolymers such as cellulose, hemicellulose, lignin, etc. [16].

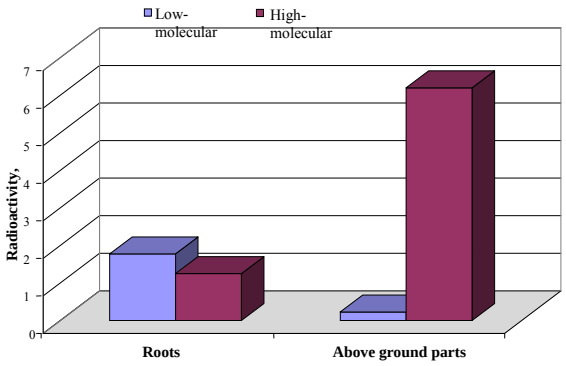


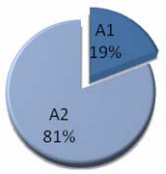
Fig. 3 The distribution of uptaken by roots [1-^{14}C]-TNT radioactive label between low- and high-molecular mass compounds in roots and above ground parts of soybean seedlings. Specific radioactivity of [1-^{14}C]-TNT – 0.5 KBq/mg, 0.5 mM solution in water, exposure time – 5 days, temperature 22- 25°C

To determine the pathways of 2,4,6-trinitrotoluene (TNT) transformation in plants, the physical-chemical analysis of insoluble in 80% ethanol high molecular compounds fraction was carried out (Table VI).

The fraction in the form of dry powder was divided in two parts. First part (A) was boiled with 1M hydrochloric acid, and the other part (B) – with 1M sodium hydroxide. The process was carried out during 12 h. After hydrolyses each part was divided in two parts (A1 and A2; B1 and B2). The pH value of part A2 was increased to pH>13 by addition of NaOH, and the pH of part B1 was decreased to pH<1 by addition of HCl. In strong acid medium (parts A1 and B1) the carboxyl groups of TNT metabolites are not ionized; correspondingly, solutions with high alkaline medium (parts A2 and B2) contain the unionized forms of products with amino groups. After these manipulations the unpolar compounds were extracted from the samples by toluene, and the radioactivity of extracts was determined (Table I).

As is seen from Table I, the ratio between radioactivities of extracts from acidic and alkaline media in both hydrolyzates are approximately equal (A1 : A2 ≈ B1: B2). Spectral analysis shows that the extracts A1 and B1 have identical absorption UV-spectra, as well as extracts A2 and B2. It should be noted that the UV-spectra of the extracts are different from UV-spectra of TNT. All these indicate to the existence of two types of TNT metabolites, which bind with high molecular compounds of plants. Apparently, formation of amino- and carboxyl groups as a result of TNT transformation by plant enzymes promotes their conjugation with endogenous compounds.

TABLE I
RADIOACTIVITY OF TOLUENE EXTRACTS FROM ALKALINE AND ACID HYDROLYZATES OF HIGH MOLECULAR COMPOUNDS FRACTION, ISOLATED FROM SOYBEAN SEEDLINGS AFTER EXPOSURE TO [1-^{14}C]-TNT THROUGH ROOTS. SPECIFIC RADIOACTIVITY OF [1-^{14}C]-TNT – 0.5KBQ/MG, 0.5 MM WATER SOLUTION, EXPOSURE TIME – 5 DAYS, TEMPERATURE 25^{o}C

Toluene extracts	Total radioactivity of extract, KBq	Ratio between the extracts radioactivities, %
A1 (from acidic medium of acid hydrolyzate)	5.288 ± 0.264	
A2 (from alkaline medium of acid hydrolyzate)	22.542 ± 1.352	
B1 (from acidic medium of alkaline hydrolyzate)	3.410 ± 0.205	
B2 (from alkaline medium of alkaline hydrolyzate)	20.946 ± 1.047	

C. Effect of Explosives on Oxidation, Conjugation and Basic Metabolism Enzymes in Plants

The stimulation effects of TNT and RDX on the enzymes, catalyzing xenobiotics oxidation and conjugation (peroxidase, phenoloxidase, glutathione S-transferase), as well as on enzymes participating in nitrogen assimilation and energy generation processes (glutamine synthase, glutamate and malate dehydrogenases) have been investigated (Tables II-V).

As seen, in all plants exposed to TNT, the increase in protein content is marked. This fact could be determined by the induction of enzymes, participating in detoxification process, especially of phenoloxidase, catalyzing the oxidation of methyl group of TNT [1] and of glutathione S-transferase, responsible for conjugation of TNT transformation intermediates (Table II). In several cases activation of enzymes participating in energy generation processes (glutamate and malate dehydrogenases) is observed (Table III). Since TNT was supplied to plants through roots, it is obvious that degree of induction of enzymes in roots is higher as compared to leaves. The stimulation of malate dehydrogenase activity in some plants could be related to the intensification of Trycarboxylic Acid Cycle, to generate reduced equivalents, important for catalyses of TNT transformation via nitro groups reduction.

TABLE II
CHANGES IN PROTEIN CONTENT AND OXIDATION ENZYMES ACTIVITIES IN PLANTS EXPOSED TO TNT

Plant	Organ	Protein content in biomass, %	Enzymes activities, %	
			Peroxidase	Phenoloxidase
Chickling vetch	roots	198	128	146
	leaves	169	128	143
Chickpea	roots	277	88	749
	leaves	101	80	211
Soybean	roots	304	89	224
	leaves	189	123	215
Soybean*	roots	341	95	234
	leaves	215	141	229

The seeds of plants were soaked in running water during 3 days; * Soybean seeds were germinated in 0.5 mM solution of TNT. On the 4th day the seedlings (40 each) were transferred to solution of 0.5 mM TNT in running water (volume 600 ml). Exposure time 10 days, temperature 22–25°C. The amount of protein and activities of enzymes in control variants (without TNT) are considered as 100%.

TABLE III
CHANGES IN BASIC METABOLISM AND CONJUGATION ENZYMES ACTIVITIES IN PLANTS EXPOSED TO TNT

Plant	Organ	Enzymes activities, %				
		Glutamate dehydrogenase		Glutamine synthetase	Malate dehydrogenase	Glutathione -S-transferase
		Reductive amination	Oxidative desamination			
Chikling vetch	Roots	86	68	305	198	433
	Leaves	64	80	384	227	250
Soybean	Roots	29	25	101	49	740
	Leaves	64	56	132	33	293
Alfalfa	Roots	1	4	3	1	175
	Leaves	72	238	46	30	122
Chickpea	Roots	268	122	1187	425	331
	Leaves	100	107	90	133	110

The seeds of plants were soaked in running water during 3 days. On the 4th day the seedlings (40 each) were transferred to solution of 0.5 mM TNT in running water. Exposure time 10 days, temperature 22–25°C. The activities of enzymes in control variants (without TNT) are considered as 100%.

According to Table IV, the oxidation enzymes (peroxidase and phenoloxidase) are insignificantly changed in plants exposed to RDX. It also should be underlined that there is no induction of conjugation enzyme glutathione-S-transferase (Table V). Among the enzymes providing plant cell with energy and nitrogen, the significant inhibition of glutamate dehydrogenase both activities and simultaneous activation of malate dehydrogenase becomes noticeable (Table V). This effect is more clearly expressed in case of alfalfa seedlings. Presumably, stimulation of malate dehydrogenase, indicating the intensification of TCA is determined by further oxidation of RDX main metabolite, such as formaldehyde [11].

TABLE IV
CHANGES IN PROTEIN CONTENT AND OXIDATION ENZYMES ACTIVITIES IN PLANTS EXPOSED TO RDX

Plant	Organ	Protein content in biomass, %	Enzymes activities, %	
			Peroxidase	Phenoloxidase
Alfalfa	roots	119	79	121
	leaves	125	77	88
Reygrass	roots	122	108	114
	leaves	128	80	110

The seeds of plants were soaked in running water during 3 days. On the 4th day the seedlings were transferred to solution of 0.5 mM RDX in running. Exposure time 7 days, temperature 22–25°C. The amount of protein and activities of enzymes in control variants (without RDX) are considered as 100%.

TABLE V
CHANGES IN BASIC METABOLISM AND CONJUGATION ENZYMES ACTIVITIES IN PLANTS EXPOSED TO RDX

Plant	Organ	Enzymes activities, %				
		Glutamate dehydrogenase		Glutamine synthetase	Malate dehydrogenase	Glutathione -S-transferase
		Reductive amination	Oxidative desamination			
Chikling vetch	Roots	42	58	74	830	43
	Leaves	43	21	69	1068	59
Soybean	Roots	78	80	244	127	90
	Leaves	55	100	153	95	110

The seeds of plants were soaked in running water during 3 days. On the 4th day the seedlings were exposed to solution of 0.5 mM RDX in running water and after 7 days enzyme activities were determined.

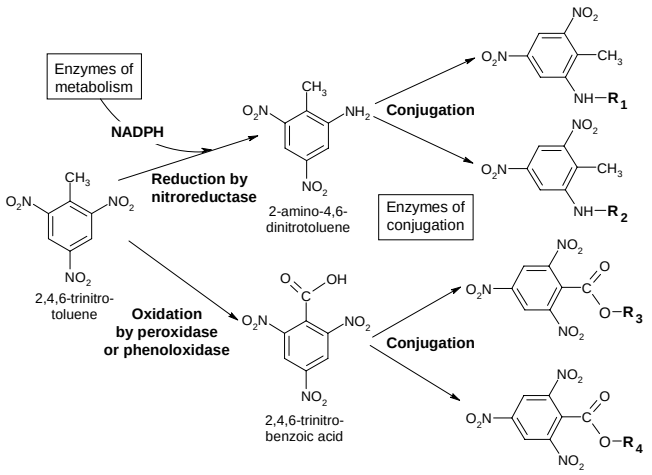
It is demonstrated that in plants and microorganisms nitro groups of TNT undergo reduction catalyzed by nitroreductase [6]. In our previous works a correlation between plant nitroreductase activity and its ability to uptake TNT from water solutions was established, namely, the higher is the nitroreductase activity, the faster is the assimilation of TNT by the plant [1]. As is seen from Table VI, in plants roots the nitroreductase has clearly expressed inducible character, and both TNT and RDX are inducers for this enzymes (induction indices 1.7 and 1.45, correspondingly). Activity of nitroreductase significantly increases in the presence of electrons donors NADH and NADPH in incubation media (Table VI). It should be underlined that the enzyme uses both cofactors, similarly, to what was reported for microbial nitroreductase, which does not reveal any specificity towards nicotinamide cofactor [6]. According to this feature, plant nitroreductases seem to be similar to microbial nonspecific NAD(P)H dependent nitroreductases. Potassium ferricyanide, as an acceptor of electrons causes inhibition in TNT transformation.

TABLE VI
IN VITRO INFLUENCE OF NICOTINAMIDE COENZYMES AND FERRICYANIDE ON NITROREDUCTASE ACTIVITY OF ROOTS OF 14-DAYS-OLD ALFALFA SEEDLINGS, EXPOSED TO TNT AND RDX

Incubation mixture	Nitroreductase specific activity, nmole TNT/min per mg of protein		
	Control	Exposed to 0.5 mM TNT	Exposed to 1 mM RDX
Homogenate + 50 mM TNT	6.30 ± 0.32	10.77 ± 0.54	9.14 ± 0.46
Homogenate + 50 mM TNT + 2 mM NADH	17.07 ± 0.85	28.33 ± 1.42	23.89 ± 1.19
Homogenate + 50 mM TNT + 2 mM NADPH	21.01 ± 1.05	36.56 ± 1.83	29.84 ± 1.49
Homogenate + 50 mM TNT + 1 mM NADH + 1 mM NADPH	18.10 ± 0.91	30.60 ± 1.53	26.79 ± 1.34
Homogenate + 50 mM TNT + 2 mM NADH + 0.1 mM $K_3[Fe(CN)_6]$	0.42 ± 0.02	0.75 ± 0.04	0.59 ± 0.03
Homogenate + 50 mM TNT+ 2 mM NADPH + 0.1 mM $K_3[Fe(CN)_6]$	0.48 ± 0.02	0.86 ± 0.04	0.72 ± 0.04
Homogenate + 50 mM TNT + 1 mM NADH + 1 mM NADPH + 0.1 mM $K_3[Fe(CN)_6]$	0.45 ± 0.02	0.79 ± 0.04	0.66 ± 0.03

IV. CONCLUSION

Based on present results and literature data, the hypothetic scheme of TNT metabolism in plants could be presented in following way (R_1 and R_3 in scheme are low-molecular compounds; R_2 and R_4 – high molecular insoluble compounds):



As is seen from the scheme, the metabolism of TNT in plants proceeds in following way: initially either nitro groups of TNT are reduced to amino groups, catalyzed by nitroreductase, or methyl group of the molecule is transformed to carboxyl group, catalyzed by oxidation enzymes (phenoloxidase, preferably). Set of different transferases, forming soluble low-molecular (~30%) and insoluble high-molecular conjugates (~70%) ends the transformation process of TNT. However, the relation between the high molecular conjugates formed in both cases indicates that main part of TNT (80-85%) is transformed via reduction pathway. Activation of some enzymes of cell basic metabolism, providing the nitroreductase with reduced equivalents of NAD(P)H, suggests their indirect participation in the xenobiotic detoxification.

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