식물조직배양학회지 제27권 제5호
Korean J. Plant Tissue Culture
Vol. 27, No. 5, 395~399 (2000)

Phytoremediation of Selected Explosives in a Model System of Plant Tissue Cultures

Tomas Vanek* · **Ales Nepovim** · **Svatopluk Zeman**[1]

Institute of Organic Chemistry and Biochemistry AS CR, Flemingovo n. 2, 166 10 Praha 6, Czech Republic

[1]Department of Explosives, University of Pardubice, Pardubice, 532 10 Czech Republic

ABSTRACTS The phytoremediation of trinitrotoluene, nitroglycerine, pentaerytritoltetranitrate in plant tissue cultures of *Solanum aviculare, Rheum palmatum* and *Populus simonii* were studied. All above mentioned explosives were degradated to to less toxic products and finally mineralized or bound to the cell wall.

Key words: Nitroglycerine, pentaerytritoltetranitrate, phytoremediation, trinitrotoluene

Abbreviations: TNT:trinitrotoluene, NG:nitroglycerine , PETN:pentaerytritoltetranitrate.

Introduction

Phytoremediation

Reports on plants growing in polluted stands without being seriously harmed indicate that it may be possible to detoxify contaminants using agricultural and biotechnological approaches. Higher plants possess a pronounced ability for the metabolism and degradation of many recalcitrant xenobiotics[1] and can be considered as "green livers", acting as an important sink for environmentally damaging chemicals. On the other hand, different plant species are able to hyperaccumulate toxic metals[2] in their tissues. It thus appears that crops and cultivated plants could be developed and used for the removal of hazardous persistent organic compounds[3] and toxic metals from industrial wastewaters and for phytoremediation purposes. Phytoremediation has been defined as the use of green plants and their associated micro-organisms, soil amendments and agronomic techniques to remove, contain or render harmless environmental contaminants.

Phytoremediation is expected to be complementary to classical bioremediation techniques, based on the use of micro-organisms. It should be particularly useful for the extraction of toxic metals from contaminated sites and the treatment of recalcitrant organic pollutants, like trinitrotoluene[4], nitroglycerine[5] and other explosives. Plant biomass could also be used efficiently for the removal of volatile organic pollutants[6] or different priority pollutants, like pentachlorophenol[7], other polychlorophenols and anilines[8].

At the present time, phytoremediation is still a nascent technology that seeks to exploit the metabolic capabilities and growth habits of higher plants: delivering a cheap, soft and safe biological treatment that is applicable to specific contaminated sites and wastewaters is a relatively recent focus. In such a context, there is still a significant need to pursue both fundamental and applied research to provide low-cost, low-impact, visually benign and environmentally sound depollution strategies.

Plant cell cultures

To develop such techniques, *in vitro* systems often offer advantages over the whole plants, provided that xenobiotic metabolism or metal accumulation in cultivated plant cells and tissues reflects what occurs *in vivo*. In particular, the screening of plant species to degrade or accumulate xenobiotics belonging to the same chemi-

*Corresponding author
Email vanek@uochb.cas.cz

cal family will be done much easier, quicker and independently of the climate conditions. Cell cultures are also a useful system for metabolic engineering and to obtain rapid evidence of the ecotoxicological behaviour of chemicals and heavy metals in plants with less analytical expense. According to results obtained *in vitro*, the most promising systems have then to be tested in whole plants cultivated in controlled and well defined plots or under hydroponic conditions for validation, before any application at large scale.

Explosives

Natural nitroaromatic compounds are scare. So far, only a few nitroaromatics have been isolated from natural sources. The antibiotic chloramphenicol is produced by several *Streptomyces* species[9] and its bacterial degradation generates nitroaromatic acids and alcohols[10] . In contrast, nitroaromatics are of considerable industrial importance[11]. They are used as explosives, dyes, polymer additives, pesticides and pharmaceuticals. They also serve as solvents or feedstocks in the preparation of aminoaromatic derivatives. Some of these compounds such as nitrobenzene, nitroglycerine or 2,4,6-trinitrotoluene are produced on a massive scale.

Many studies have shown that nitroaromatics and their degradation products are toxic or mutagenic to many life forms[12]. These compounds are released in the environment through anthropogenic activities and have been detected in soils, waste-water and in air[13]. In consequence, several nitroaromatics are listed as priority pollutants by several environmental protection agencies. Also, the most visible environmental problem caused by contamination with nitroaromatic compounds is the widespread contamination of soil by explosives[13].

Material and Methods

Selection of xenobiotics

The studied explosives was be selected on the basis of their potential environmental risk. Generally, their exhibit negative effects on haematopoisis, central nervous system and liver (the LD_{50} values relate to rat).

1) Dinitrotoluenes (DNT) cause metahaemoglobinaemia and aenemia, are hepatotoxic, cause damage to sight, the 2,6-isomer is suspect carcinogen LD_{50} for 2,4- and 2,6-DNTs are 0,268 and 0,177 g/kg, respectively.
2) Trinitrotoluene (TNT) is hepatotoxic, causes hypochromaemia, damage to nervous system and sight, induces dystrophic changes in myocardium and kidneys LD_{50} = 0,70 g/kg.
3) Trinitrophenol (picric acid) causes acute hepatitis and contact dermatitis LD_{50} = 0,060 g/kg.
4) 1,3,5-Trinitro-1,3,5-triazacyclohexane (HEXOGEN, RDX) causes damage to central neervous system (can induce epileptic seizure-like condition), can cause anaemia LD_{50} = 0,20 g/kg.
5) Nitroglycerol (NG) possesses vasodilatory effect, hepatotoxicity, damages kidney function and myocardium, belongs among habit-forming drugs. LD_{50} = 0,806 g/kg.
6) Nitroglycol (EGDN) is still more dangerous than nitroglycerol. LD_{50} = 0,616 g/kg.
7) Pentaerythritol tetranitrate (PETN) has weaker effects than nitroglycerol

Most frequent on the territory of Czech Republic are trinitrotoluene, nitroglycerine and pentaerytritoltetranitrate so we select them for our experiments.

Suspension cultures

Rheum palmatum: Callus culture was induced from petiole of plant cultivated *in vivo* on the gelled MS medium supplemented with NAA (10 mg l^{-1}). Callus subcultured for every 4 weeks was transferred into the liquid medium and filtered through a sieve (mesh 3 mm).

Solanum aviculare: The culture was induced from suspension culture was cultivated in MS medium supplemented with kinetin and NAA (both 1.0 μM) on a horizontal roller (5 rpm) in a dark at 27°C[14].

Phytotoxicity of TNT to suspension cultures

Four different concentrations (25, 50, 75, 100 mg/l) of TNT were added to the cultivation medium. The concentration of TNT which inhibits growth of suspension culture in 50% was provided from growing factors for each species. The growth of culture of *Rheum palmatum* is inhibited by 37.8 mg/l of TNT in 20. day whereas the growth of *Solanum aviculare* is inhibited by 38.1 mg/l of TNT in 16. day of cultivation On the basis of this results, concentration of 50 mg/l was chosen as a starting concentration for all experiments.

Chemicals

Samples of explosives as well as their degradation products 2,4,6-trinitrotoluene was gained from Department of Explosives, University of Pardubice, (Pardubice, Czech Republic).

HPLC analysis

Analytical instrumentation consisted of binary gradient pumps Deltachrom SDS 020 & 030 (USA), a mixer (SunChrom GmbH, BRD), an injection valve Rheodyne 7725 (Rheodyne, USA) and a PDA detector MD-1510 (Jasco, Japan). Data were processed by Borwin PDA program and the concentration of TNT was calculated from peak area at wavelength 230 nm. Analyses were performed on a stainless steel column (250 × 4 mm ID) packed with SiC_{18} reverse phase Biospher 7μm size (Labio Ltd., Czech Rep.). A linear gradient of mobile phases (10% ~ 100% MeOH within 40 min) was applied.

An uptake of explosives was determined in a medium and in cells. Medium was filtered through a microfilter (0.2 μm) and 20 μl was injected. The content of products in cells was determined from acetone extract.

LC-MS

HPLC - Beckman 125 binary gradient pumps, 168 "diode-array" detector, 507 autosampler MS - "Ion-trap" mass spectrometer Finnigan LCQ equipped by APCI (atmospheric pressure chemical ionization), data analyzed in negative mode, spectra confirming found compounds were obtained from MS/MS.

Identification of degradation products

Identification was done by comparing of retentione times, UV and MS spectra of products with standards using HPLC and LC-MS, respectively.

Results

The results are summarized in table 1. All explosives were degradated during 10 days.

TNT degradation

The starting concentration of TNT (50 mg/l) was fully degradated during 6 hours. During this time following metabolites was isolated and identified - both aminodinitrotoluenes (4 and 2ADNT), diaminonitrotoluenes (4,6 and 2,4 ADNT) and hydroxiaminodinitrotoluene (HADNT). Kinetics of this process is described on the figure 1a. On the basis of isolation and identification of above mentioned products as well as dinitroaniline and trinitrobenzene (after 5 days), the degradation of TNT proceed according to scheme on figure 1. Both possible pathways take part - starting with reduction of nitrogroup to amino-group as well as starting with oxidation of toluene methyl group.

Nitroglycerine degradation

The nitroglycerine (50 mg/l) was degradated according to figure 2. After ten days only 39% of starting concentration of NG and one degradation product (mononitroglycerine 11% of starting concentration of NG) was detected.

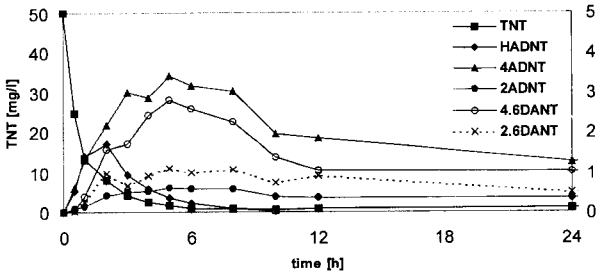

Figure 1a. Time-course of TNT degradation.

Table 1. Degradation of selected explosives.

Compound	Final concentration	Degradation products	Toxicity
2,4,6-Trinitrotoluene	0%	2	+++
Nitroglycerin	39%	1	+
PENT	0%	4	-

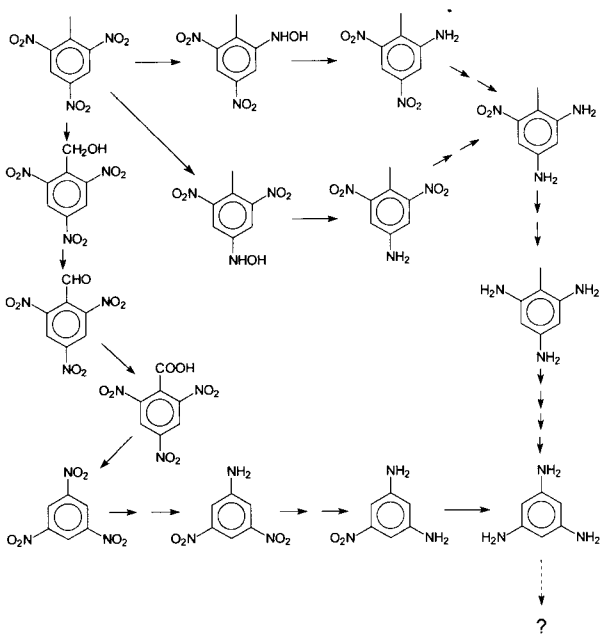

Figure 1. Scheme of TNT degradation.

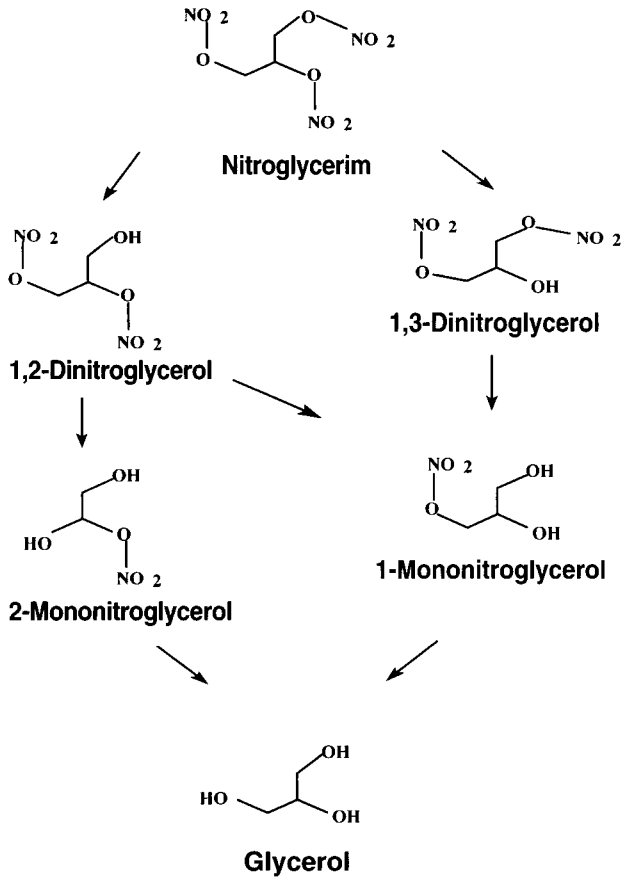

Figure 2. Degradation of NG.

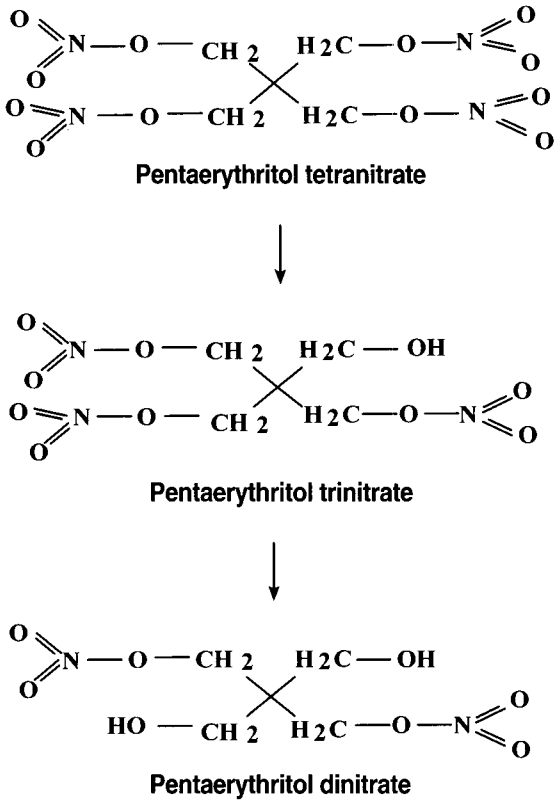

Figure 3. Scheme of PETH degradation.

Pentaerytritoltetranitrate degradation

According to products analysis, PETN was fully degradated after ten days according to figure 3 (the degradation continued *via* pentaerythritol mononitrate to pentaerythritol, which is utilized as a carbon source). During ten days, all possible degradation products were identified.

Acknowledgement : This work was supported by GACR No. 206/99/ 1252 and 206/99/P034 grant.

References

Sandermann H. (1992) Trends in Biochemical Sciences **17**; 82-84

Salt D. E., Blaylock M., Nanda Kumar P. B. A., Dushenkov V., Ensely B. D., Chet I. and Raskin I. (1995). Bio/Technology **13**; 468- 474

Cunningham S. D., Anderson T. A., Schwab A. P. and Hsu F. C. (1996) In: Advances in Agronomy 56, Sparks D.L., ed. Academic Press, 55-114

Vanderford M., Shanks J. V. and Hughes J. B. (1997) Biotechnology Letters 19, 277-280

Goel A., Kumar G., Payne G. F. and Dube S. K. (1997) Nature Biotechnology **15**; 174-177

Schnabel W. E., Dietz A. C., Burken J. G., Schnoor J. L. and Alvarez P. J. (1997) Water Research **31**; 816-824

Roy S. and Hanninen O. (1994) Environmental Toxicology and Chemistry **13**; 763-773

Bockers M., Rivero C., Thiede B., Jankowski T. and Schmidt B. (1994) Zeitschrift fur Naturforschung **49c**; 719-726

Pongs, O. (1979) Antibiotics. F.E. Hahn, ed., Springer-Verlag, Heidelberg, vol. 5, pt. **1**; 26-42

Lingens, F., Eberhardt, H. and Oltmanns, O. (1966) Biochim. Biophys. Acta. **130**; 345-354

Higson, F.K. (1992) Adv.Appl.Microbiol. **37**; 1-19

Won, W.D., Disalvo L.H. and James, N.G. (1976) Appl. Environ. Microbiol. **31**; 576-580

Spain, J.C. (1995). Annu. Rev. Microbiol. **49**; 523-555

Macek T (1989). in *In vitro* cultivation and production of solasodine. In: Bajaj Y.P.S., ed. Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plants II, Vol.7. Berlin, Heidelberg, New York, Tokyo: Springer Verlag, pp. 443-467.