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ORIGINAL PAPER

Phytotoxicity to and uptake of TNT by rice

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Abstract The contamination of the environment by explosives is a worldwide problem resulting in part from 2,4,6-trinitrotoluene (TNT) production. In situ phytoremediation is an appropriate, alternative, cost-effective technology to detoxify extended contamination of surface soil. The ability of rice (*Oriza sativa*) to both tolerate and assimilate ^{14}C-labeled TNT was investigated over a 40-day exposure period. The germination rate decreased at 500 mg/kg TNT whereas root and shoot length increased significantly at high TNT concentrations, from 150 to 500 mg/kg. Rice took up TNT residues from soil and accumulated most in roots. Less than 25% of radioactivity taken up was translocated to aerial parts. Above 200 mg/kg TNT, the concentration of TNT residues in roots reached a maximum of approximately 0.7 mg/g. No TNT was found in plant extracts, good evidence for rapid metabolism of TNT. More than 60% of ^{14}C activity was found as unextractable residues in roots. It was concluded that TNT metabolized and subsequently sequestered by roots could not be translocated to aerial parts.

Keywords Explosive · Phytoremediation · Phytotoxicity · Rice · TNT

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Introduction

Huge amounts of 2,4,6-trinitrotoluene (TNT) were produced and used during the First and Second World Wars. Production, packaging, and storage led to the contamination of soil and groundwater in many countries (Spain 2000). Concentrations of TNT in contaminated soils are extremely heterogeneous, ranging from 0.08 to 87,000 mg/kg (Talmage et al. 1999). In the past, wastewater resulting from TNT production was directed to lagoons to allow settling of solid material and then directed to rivers. Excavation and incineration have commonly been used to remediate soil contaminated with TNT but these methods are expensive and destroy soil structure. Phytoremediation is an alternative cost-effective biological method that is more easily acceptable to the general public. The tolerance of plant species to the pollutant is one of the main factors in choosing a plant for phytoremediation. Another factor is the ability of plants to take up contaminants.

Numerous studies have evaluated the phytotoxicity of TNT, which enabled the determination of the toxicity threshold: the turning point from regular to inhibitory growth. All these experiments were hydroponic and the threshold value was expressed in mg/L (Kim et al. 2004). This value is helpful for groundwater phytoremediation but, in the case of contaminated soil or sediment, more representative values are required. However, some studies have determined the ecotoxicological threshold of TNT in various

natural or artificial soils with different plants (Gong et al. 1999; Hannink et al. 2002; Medina et al. 2003; Robidoux et al. 2003). Plants with a low level of tolerance to TNT showed adverse effects such as chlorosis and abscission of leaves or flowers. The degree of phytotoxicity appears to depend not only on plant species, growth stage, biomass, and plant density, but also on contaminant bioavailability and other environmental factors such as soil type or microbial activity.

In parallel, the uptake and fate of TNT have been studied in many plants but almost always in hydroponic studies (Palazzo and Leggett 1986; Görge et al. 1994). Although the effectiveness of accumulation can vary, these studies showed that plants could significantly reduce the concentration of TNT in solution. Price et al. (2002) described the uptake of TNT by agronomic plants from soil and irrigation water. However, they were interested in assessing the risk to human health and thus focused their analyses on edible parts of vegetables and not on uptake and translocation in whole plants. Many studies have shown that TNT is mainly reduced to hydroxylamino-dinitrotoluene (HADNT) and amino-dinitrotoluene (ADNT) (Bhadra et al. 1999; Vila et al. 2005). These metabolites can pose a serious problem because they are as toxic as the parent compound (Honeycutt et al. 1996). More information is required to understand the ultimate fate of TNT in plants.

We determined the phytotoxicity of TNT to rice, a suitable plant for lagoon cultivation. Standardized toxicity assays were used to assess the effects of TNT on rice plants using endpoints such as seed germination, growth, or root elongation, and total chlorophyll content. We also measured uptake and distribution of TNT in rice after 40 days of exposure on soil freshly contaminated with TNT. Processes involved in TNT phytoremediation were determined by investigating the fate of TNT after uptake by rice.

Materials and methods

Chemicals

Analytical-standard TNT was obtained from SCP Science (Courtaboeuf, France). Unlabeled TNT was obtained from Eurenco (Sorgues, France) in the form of 2% contaminated sand with 25% humidity. [methyl-^{14}C]-2,4,6-trinitrotoluene (specific activity = 14 mCi mmol^{-1}; radiochemical purity >97% as determined by radio high-performance liquid chromatography analysis) was purchased from Moravek Biochemical (Brea, CA). All other chemicals used in the experiments were analytical-grade reagents purchased from Scharlau Chemie S.A. (Barcelona, Spain).

Plant materials and growth conditions

Rice (*Oriza sativa* L. var. Carinam) was used in the experiments. All experiments except the germination test were conducted in a temperature-controlled room. Environmental conditions were an average day/night temperature of 24°C/21°C and a 16 h photoperiod.

Germination test

The germination tests were carried out as previously described (Vila et al. 2007). Noncontaminated and contaminated sand (2% TNT) was mixed at different ratios to produce concentrations of 0, 50, 100, 250, and 500 mg TNT kg^{-1} dry sand.

Soil preparation for growth and uptake tests

Noncontaminated and TNT contaminated sand (2% TNT) were mixed at different ratios to create concentrations of 0, 50, 100, 150, 200, 250, and 500 mg TNT kg^{-1} soil. Compost (Nehaus NF U 44-551, pH 4, Geeste, Germany) was sieved through a 4 mm sieve and mixed with the first mixture. In each 400 mL plastic pot, 250 g of contaminated soil was hydrated to 60% of water-holding capacity with deionized water. Water evaporation was measured by weighing the jars daily and lost moisture was replaced by adding distilled water. There were three replicates for each concentration of contaminated soil tested.

Emergence, growth test, and uptake

The emergence, growth tests, and estimation of chlorophyll content were carried out as described elsewhere (Vila et al. 2007). Uptake of TNT by rice was estimated using soil contaminated with TNT

and [U-^{14}C]TNT. Each replicate contained 5 μCi of [U-^{14}C]TNT. The [U-^{14}C]TNT was added as an acetone solution to TNT-contaminated sand before addition of artificial soil. Sand was homogenized and acetone evaporated under vacuum.

After 40 days, plants were harvested and separated into leaves and roots. Each part was freeze-dried and ground. In some experiments, the three oldest leaves were cut into damaged and undamaged parts before freeze drying to determine the distribution of radioactivity more precisely. Radioactivity was measured after oxidative combustion of aliquots (around 200 mg) in an oxidizer (Packard Instrument Co, Downers Grove, IL), trapping the resulting $^{14}CO_2$ in a scintillation mixture (Carbo-Sorb and Permafluor, Packard) followed by liquid scintillation counting in a Packard Tricarb 2200 CA scintillation counter. The amounts of residues in plants were calculated from the specific activity of each concentration of TNT in soils.

HPLC chromatography

Radioactivity in leaves was extracted by homogenization in an acetonitrile/water mixture (1:1, v/v) (10 ml/g dry weight). The homogenates were stored overnight at −20°C, then centrifuged at 10,000*g* for 10 min. The pellets were washed twice with the solvent mixture. The three supernatants were combined and the resulting solution contained the soluble residues. The extracts were analyzed by reverse-phase high-performance liquid chromatography (HPLC) by using a HP 1100 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany). Radioactivity was monitored with an online Packard Flo-One β A250 scintillation detector, using Flo-Scint II as scintillation counting cocktail (Packard, Downers Grove, IL). Separation was carried out on a C18 Bischoff reverse-phase column (Prontosil Eurobond, 4.6 mm × 250 mm, 5 μm) with a guard cartridge. Elution was performed at 40°C at a flow rate of 1 mL/min. The column was equilibrated with 100% of solvent A (water/methanol/formic acid, 89.8:10:0.2,v/v/v). Elution conditions were as follows: 100% solvent A for 15 min, a 5 min linear increase of solvent B (water/methanol/formic acid, 79.8:20:0.2, v/v/v) from 0 to 100%, and then 100% solvent B for 10 min. Under these conditions, the retention time of standard TNT was 23 min.

Results and discussion

Phytotoxicity of TNT

The percentage of seed germination in contaminated soil decreased with increasing concentrations of TNT (data not shown). There was a linear decrease in the germination rate from 85 ± 10% in noncontaminated sand to 56.7 ± 2.9% at 500 mg of TNT per kg of soil, but the first significant effect on germination was only evident with the 500 mg/kg treatment.

Rice was cultivated for 40 days in the absence and presence of TNT to test tolerance to TNT. No adverse effects such as chlorosis or necrosis were detected in the presence of TNT. We did not observe any significant difference in chlorophyll content between controls and plants cultivated on TNT contaminated soil (data not shown). Nevertheless, variations in shoot and root biomass were observed at concentrations above 150 mg/kg TNT. Shoot growth slightly increased in the presence of TNT (Fig. 1a). In soil contaminated with 200 mg/kg, roots grew 2.8 times

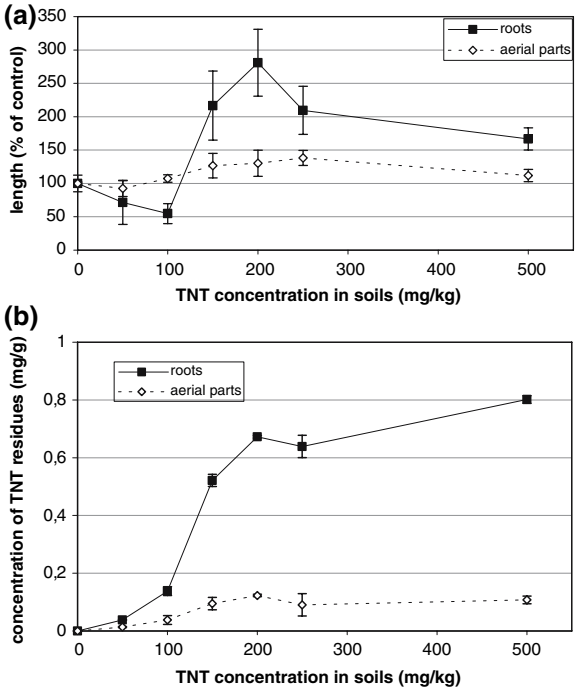

Fig. 1 (**a**) Rice root and shoot length increased with high concentrations of TNT in soil after 40 days of culture. Results are expressed as percentage growth compared to controls. (**b**) The concentration of TNT residues in roots and shoots of rice cultivated for 40 days, which depends on soil concentrations of ^{14}C-TNT. Results are expressed as mg/g DW

longer than controls. TNT thus had a positive effect on plant growth. Low levels of TNT (between 5 and 50 mg/kg) stimulated seedling growth of cress, turnip, oat, and wheat (Gong et al. 1999). In our study, phytotoxicity tests showed the opposite effect of TNT on rice germination and growth. The germination rate decreased in TNT-contaminated soil whereas root and shoot lengths increased (Fig. 1a). Rice is normally sown directly in rice fields or sown in the nursery and then transplanted as seedlings to the rice paddy. When rice cultivation is intended in soil contaminated with TNT, growing seedlings in the nursery would prevent the adverse effect of TNT on germination.

Plants cultivated in TNT-contaminated soil appear to tolerate higher concentrations of the pollutant than plants cultivated in hydroponic studies. This is probably due to the decrease in TNT availability resulting from its binding to organic matter in the soil during longer experiments (Alexander 2000). So, in soil the TNT available fraction is probably lower than the total TNT concentration, and this phenomenon would increase over time. During in situ phytoremediation, bioavailability of TNT could reduce with time, thus decreasing phytotoxicity as well as reducing plant uptake of TNT.

Uptake of TNT by rice

To measure the uptake of TNT by rice, plants were cultivated for 40 days on soil contaminated with ^{14}C-TNT. Analyses of radioactivity found in aerial parts and roots enabled measurement of the quantities of TNT residues in each part (Fig. 1b). Most TNT residues were found in roots. From 50 to 150 mg/kg, the concentration of TNT residues in roots was proportional to the concentration in the soil. Above 200 mg/kg, we observed a plateau-like phase in uptake.

The concentration in roots was at least three times higher than that in aerial parts. At a concentration of 500 mg/kg in soils, the ratio was seven times greater. Rice took up a maximum of 0.80 ± 0.12 mg/g TNT dry weight (DW). These concentrations were on the same order of magnitude as results obtained in other studies, i.e., between 0.5 and 0.7 mg/g DW in yellow nutsedge (Palazzo and Leggett 1986) and aquatic plants (Best et al. 1999). Cultivation conditions in the two experiments were not comparable since the plants were grown in hydroponic solution with TNT concentrations ten times lower than the concentrations in our study. TNT has low solubility in water and a part is bound to organic matter. Therefore in soil only a fraction of total TNT concentration would actually be available for uptake by plants. Comparison of these different experiments showed that TNT uptake by plants is probably more closely linked to free TNT in water than to total soil concentration. Rice could thus be as efficient as other tested plants (Palazzo and Leggett 1986; Best et al. 1999) in taking up TNT and could be used on contaminated soil. As roots can hardly be harvested, residues in roots return to the soil. There, ADNT could be degraded to diamino- or triamino-toluene by soil bacteria. HADNT could be degraded to ADNT or could lead by dimerization to azoxytetranitrotoluene, which precipitates in the soil (Esteves-Nùñes et al. 2001). This may provide other persistent compounds in the soil. Hydroponic culture was probably a good approach to measure plant efficiency but the concentrations tested remain limited.

Rice accumulated high concentrations of another explosive found in contaminated lagoons, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), in its aerial parts, i.e., 31.5 ± 2.6 mg/g DW (Vila et al. 2007). In spite of its optimal log K_{ow} (1.6, Talmage et al. 1999) for uptake (Briggs et al. 1982), the efficiency of TNT uptake by rice was lower than for RDX.

Radio-HPLC analyses of TNT residues in rice showed that TNT was completely metabolized into more polar compounds (data not shown). Metabolism of TNT has been widely studied in plants. After entering the cell, TNT was reduced to HADNT and ADNT (Vila et al. 2005). Previously, these metabolites were thought to form conjugates with sugars. However, the low amounts of radioactivity did not allow identification of metabolites by mass spectrometry. Moreover, most radioactivity found in rice was unextractable: in roots, 75% at 150 mg/kg and 65% at 500 mg/kg, and in shoots 55% whatever the concentration of TNT (data not shown).

TNT polar metabolites are probably stored in vacuoles and as unextractable residues linked to cell wall polymers. Low uptake associated with a high metabolic rate and high rate of sequestration of TNT residues in roots leads to reduced translocation to shoots. Although rice exhibits good TNT tolerance, low uptake of TNT might limit the use of rice for bioaccumulation of TNT. Nevertheless, rice might influence degradation of TNT and vegetation can enhance

microbial degradation by offering a favorable rhizosphere environment for soil microorganisms.

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