Bioremediation of 2,4,6-trinitrotoluene by bacterial nitroreductase expressing transgenic aspen

Supporting Information

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The following text and figures are provided for the benefit of the reader. These materials present information in more detail than could be accommodated in the manuscript text. There are a total of 6 pages of Supporting Information.

pBIpnrA binary plasmid construction. As described previously, the nitroreductase encoding gene, *pnrA*, was obtained by amplifying this gene from *P. putida* JLR11 chromosomal DNA by PCR and cloning the resulting product into pUC19 to obtain pNAJ (Caballero *et al. Env. Microbiol*. **2005**, *7*, 1211-1219). This plasmid was digested with restriction enzymes *Xba*I and *Sac*I and the resulting 1 kb fragment was ligated into the binary vector pBI121 (Stratagene, Madrid, Spain), also cut with *Xba*I and *Sac*I, to yield pBIpnrA. In

this way, *pnrA* was cloned downstream from the cauliflower mosaic virus 35S promoter.

Quantification of *pnrA* expression in transgenic plants. Total RNA was extracted with the RNeasy plant mini kit (Qiagen) from leaves, stems and roots of *in vitro* rooted plants and treated with RNase-free DNase Set (DNase I; Qiagen). cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) and oligo-dT primers according to the manufacturer's protocol. SYBR Green based quantitative assays were performed in an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Madrid, Spain). 25 µL reactions consisted of 2.5 µL of cDNA from each sample, 1x SYBR Green PCR Supermix (Bio-Rad, Laboratories, Madrid) and oligonucleotide primer pairs for *pnrA*: 5'-TCAAGACGAAGCACTCAAAGCC-3'; 5'-GGCACGTACTGATCGATGCTGC-3', and for 18S 5'-AATTGTTGGTCTTCAACGAGGAA-3'; 5'-AAAGGGCAGGGACGTAGTCAA-3' (Brunner *et al*., *BMC Plant Biol.* **2004**, *4*, 14). The PCR conditions were: one cycle at 95 °C for 5 min, followed by 50 cycles at 95 °C for 20 sec, 60 ºC for 30 sec, and 72 °C for 30 sec. Each PCR reaction was performed in triplicate together with controls lacking template. The entire assay, including both the RT and real time PCR steps, was repeated two times from two RNA extractions. Data normalization with 18S RNA was performed using the ΔΔ*C*T method (Livak *et al. Methods* **2001**, *25*, 402-408). The specificity of the amplifications was verified at the end of the PCR run by using melting-curve analysis and by analyzing PCR products with agarose gel electrophoresis.

Determination of TNT and its derivatives in soil and plant tissues. Soil samples were prepared by collecting 5-20 g of either rhizosphere soil from the

entire length of each plant root or bulk soil from each pot. The samples were dried overnight at room temperature and homogenized with a spatula prior to analysis. For analysis, 2 g of soil (≤2 mm grain size) was added to 10 mL acetonitrile and the mixture shaken vigorously overnight. After settling, 0.5 mL supernatant was collected and mixed with 0.5 mL of a CaCl2 solution (5 g/L). The mixture was centrifuged and the supernatant passed through a GHP Acrodisc 0.45 µm syringe filter before analysis by HPLC.

To determine TNT and its transformation products in plant organs, the roots of four 10-15 cm long plants were introduced into Hoagland medium saturated with TNT crystals (approximately 113.5 mg TNT/L) for 48 h before freezing. To extract TNT and its metabolites, frozen plant tissues were homogenized with mortar and pestle in liquid nitrogen and then extracted by shaking overnight with acetonitrile at a ratio of 20 mL acetonitrile per gram wet weight of plant material (28 ºC, 200 rpm). Then, the entire mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant decanted and 15 mL of the supernatant was split into equal volumes (7.5 mL) and each dried using a rotary evaporator. Each half was then either resuspended in 1 mL water and left overnight at room temperature or in 1 mL 1N HCl for hydrolysis of potentially formed conjugates and incubated overnight at 85 ºC. The following day 0.1 volume of water was added to the water solution or 0.1 volume of 10N NaOH to the acidic mixture in order to neutralize the solution. Each mixture was then passed through a GHP Acrodisc 0.45 µm syringe filter and analyzed by HPLC. To quantify, samples were compared with pure standards. In order to analyze possible plant metabolites the HPLC program consisted of 25 mM phosphate buffer pH 7/ 30% MeOH during 15 min 30 seconds, then increased to 60% methanol in 30

seconds, followed by a gradient to 70% methanol over the following 8 minutes. To re-equilibrate, the concentration was lowered in the next 30 seconds to initial conditions and maintained until the end of the method at 37 minutes. The flow rate was 0.85 mL/min.

Calculations for data from experiments with ^{13}C-TNT. The stable isotope composition is reported as $\delta^{13}C$ -values per thousandth (‰): $\delta^{13}C=[((^{13}C/^{12}C)_{sample}-(^{13}C/^{12}C)_{standard})/ (^{13}C/^{12}C)_{standard}] \times 1000$. The international standard reference used for $^{13}C/^{12}C$ was Vienna Pee Dee Belemnite (V-PDB), (calcite from a marine fossil from a Cretaceous-age formation in South Carolina). For comparison purposes an absorption index (I_{abs}) was calculated using $\delta^{13}C$ values as follows: $I_{abs}=((\delta^{13}C_{sample} - \delta^{13}C_{control})/ \delta^{13}C_{control})$, whereby the control values were obtained from plants which had not been exposed to ^{13}C-TNT. The quantity of ^{13}C in roots was calculated by assuming that 46% of root dry weight is carbon (Hancock *et al.*, *New Phytol.* **2007,** *273*, 732-742.) and multiplying by the difference between the % ^{13}C in each sample with the % ^{13}C in unexposed plants.

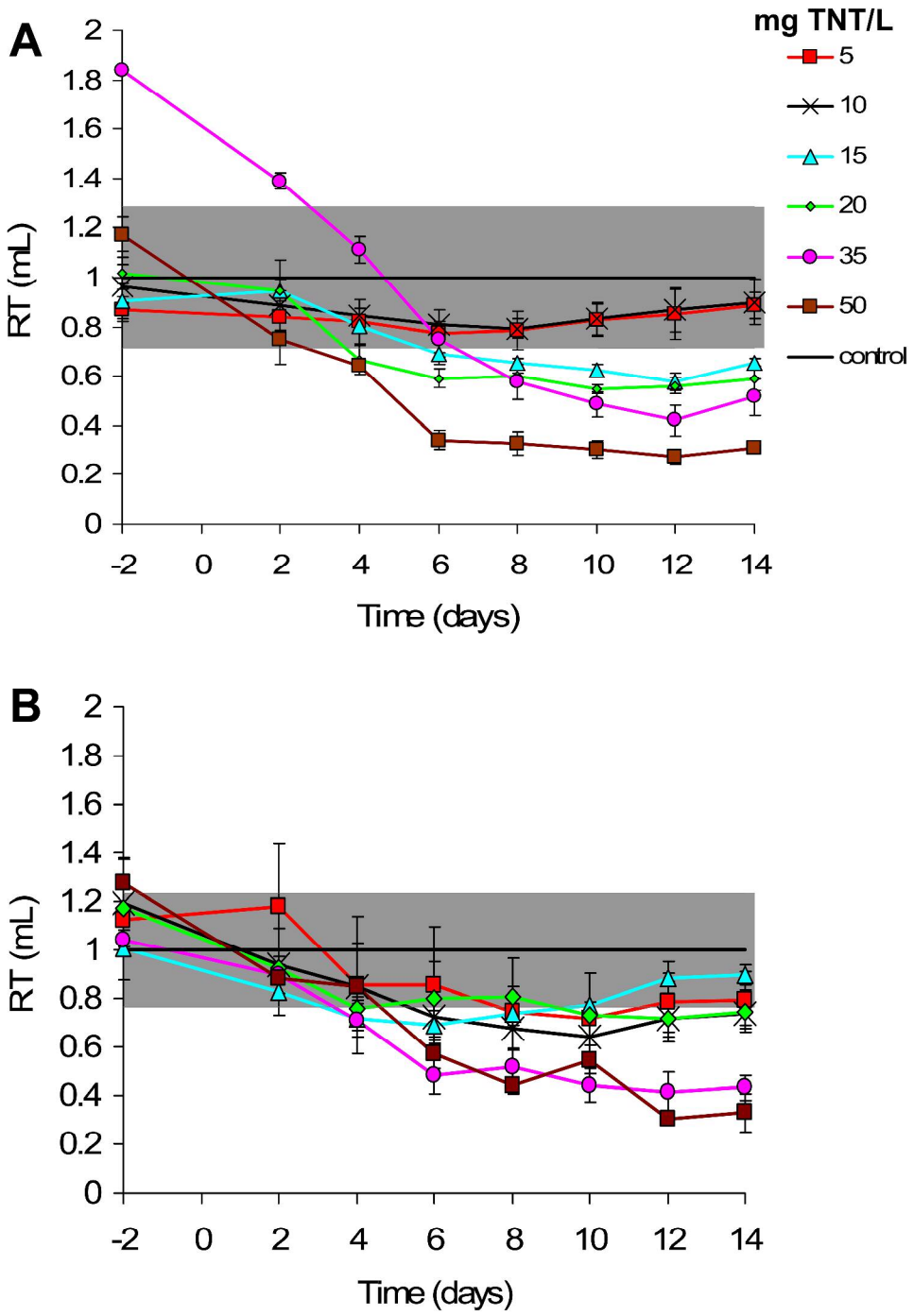

Figure S1. Determination of the phytotoxicological limits of (A) wild type and (B) *pnrA* transgenic aspen. Plants were acclimatized for two days (-2 days measurement) and then exposed at T=0 to different concentrations of TNT (mg/L) (standard error bars $p<0.05$, $n\geq3$). Grey bar indicates largest standard error range of the control (RT of plants in medium without TNT). Those

concentrations of TNT at which the RT values were below the control (grey bar) at the end of the experiment were considered toxic for the plant.