Exploring the rhizospheric and endophytic bacterial communities of *Acer pseudoplatanus* growing on a TNT-contaminated soil: towards the development of a rhizocompetent TNT-detoxifying plant growth promoting consortium

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Abstract

Background and aims Numerous microorganisms have been isolated from trinitrotoluene (TNT)-contaminated soils, however TNT tends to persist, indicating that the microbial biomass or activity is insufficient for degradation. Deep-rooting trees at military sites have been found to take-up contaminants from groundwater, and the extensive root and endosphere provide ideal niches for microbial TNT-transformations.

Methods We characterised the rhizosphere, root endosphere and endo-phyllosphere bacteria of *Acer pseudoplatanus* growing at a historically TNT-contaminated location, using 16S rRNA gene finger-printing, bacteria isolation, oxidoreductase gene-cloning, *in planta* growth-promotion (PGP) tests, inoculation, plant physiology measurements and microscopy.

Results Based on terminal-restriction-fragment-length-polymorphism analysis, bulk soil and rhizosphere samples were highly clustered. Proteo- and Actinobacteria dominated the rhizosphere and root endosphere, whereas Alphaproteobacteria were more abundant in shoots and Actinobacteria in leaves. We isolated multiple PGP-bacteria and cloned 5 flavin-oxidoreductases belonging to the Old Yellow Enzyme family involved in TNT-

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reduction from 3 *Pseudomonas* spp., the leaf symbiont *Stenotrophomonas chelatiphaga* and the root endophyte *Variovorax ginsengisola*.

Conclusions The inoculation with a selection of these strains, consortium CAP9, which combines efficient TNT-transformation capabilities with beneficial PGP-properties, has the ability to detoxify TNT in the bent grass (*Agrostis capillaris*) rhizosphere, stimulate plant growth and improve plant health under TNT stress.

Keywords *Agrostis capillaris* · Endophyte · OYE, XenB flavin oxidoreductases · Plant growth promotion · Rhizoremediation · Trinitrotoluene

Introduction

The nitro aromatic 2,4,6-trinitrotoluene (TNT) is an important component of military explosives. Due to its extensive historical production, transportation and use, many military sites throughout the world are contaminated with this hazardous nitro aromatic (Clausen et al. 2004; Lewis et al. 2004; Spiegel et al. 2005). TNT and its metabolites exhibit toxic and mutagenic effects on various prokaryotes and eukaryotes, and are classified as possible human carcinogens by the US Environmental protection agency (EPA) (George et al. 2008; Gong et al. 1999; Honeycutt et al. 1996; Kilian et al. 2001; Leung et al. 1995).

TNT tends to bind to organic matter and clay minerals, decreasing its bioavailability and thus limiting natural attenuation (Hundal et al. 1997). Moreover, the presence of the three electron-withdrawing nitro groups in TNT generates an electrophilic aromatic ring preventing oxidative attack, which results in TNT being recalcitrant to biodegradation. As such, TNT has accumulated in affected soils (Pennington et al. 2001) at concentrations up to 20 g kg^{-1} (Clausen et al. 2004; Rylott et al. 2011a). There is a need to remediate these sites but current decontamination methods, such as incineration, carbon sequestration and composting, are expensive and not applicable given the scale of contamination (Ayoub et al. 2010). To remediate existing and future TNT-contaminated sites, alternative strategies such as phytoremediation are required.

Many TNT-transforming microorganisms have been isolated from contaminated soils and characterized (Ramos et al. 1996; Robertson and Jjemba 2005; Snellinx et al. 2002; Stenuit and Agathos 2010; Van

Dillewijn et al. 2007). A major microbial transformation pathway for TNT involves direct reduction of nitro-groups catalyzed by ubiquitous nitro reductases resulting in the production of hydroxylamino- and amino-reduction products (Esteve-Núñez et al. 2001, Esteve-Nuñez et al. 2000). Another pathway involves aromatic ring and nitro-group reductions catalyzed by type II hydride transferases such as xenobiotic reductase B (XenB) and some other members of the Old Yellow Enzyme (OYE) family of flavin oxidoreductases (van Dillewijn et al. 2008b). These hydride transferases yield hydroxylamino derivatives and hydride Meisenheimer compounds from TNT, which upon condensation can yield nitrite and diarylamines (Wittich et al. 2008). Williams et al. (2004) and Wittich et al. (2008) indicate the importance of deciphering the role and distribution of OYE-enzymes because their nitro aromatic denitration potential holds much promise for explosives phytoremediation.

Despite the presence of TNT-transforming microbes in soil, *in situ* TNT-transformation rates are slow suggesting that other factors are limiting TNT metabolism (Stenuit and Agathos 2010). Das et al. (2010) demonstrated that by the addition of urea, a chaotropic agent, the bio-availability and uptake of TNT by *Vetiver* grass from soil was increased (Das et al. 2010). In addition to bioavailability issues, the lack of sufficient nutrients for co-metabolic growth on TNT can limit degradation (Makris et al. 2010).

Recent evidence indicates that plant-associated bacteria, both those living in the rhizosphere or endophytically, can contribute to phyto- and rhizo-remediation of recalcitrant organic compounds (Becerra-Castro et al. 2013; Gurska et al. 2009; Sessitsch et al. 2012). The rhizosphere, *i.e.* the area surrounding plant roots, is characterised by increased microbial activity stimulated by compounds released from plant roots, including organic acids, sugars, amino acids and secondary metabolites (Berg 2009; Kent and Triplett 2002; Lugtenberg and Dekkers 1999; Van Dillewijn 2008). In addition to the higher microbial activity, bacteria that benefit most from the nutrients supplied in the rhizosphere may form larger cell densities, so that typically 10 to 100 times more bacteria can be found in the rhizosphere than in the adjacent bulk soils (Lugtenberg and Kamilova 2009). In addition, rhizospheric and endophytic bacteria can positively enhance plant growth either: (1) directly through production of phytohormones (auxins and cytokinins) or by

increasing the amounts of available nutrients via a number of biochemical processes (e.g. N_2-fixation, phosphate solubilisation, siderophore release increasing Fe availability); or (2) indirectly through the suppression of ethylene production by 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD), through chemical induction of plant defence mechanisms, or by the degradation of harmful contaminants (Badri et al. 2009; Barazani and Friedman 1999; Compant et al. 2010; Glick 2005; Patten and Glick 2002; Remans et al. 2012; Sessitsch et al. 2012; Weyens et al. 2009c).

Previous studies have highlighted that microbially induced transformation and degradation of organic compounds in the rhizosphere and inside plants correlates well with improvements in contaminant remediation (Chaudhry et al. 2005; Chekol et al. 2004; Kidd et al. 2008; Weyens et al. 2009d; Zhuang et al. 2007). In addition, plant-bacteria interactions can increase contaminant degradation in the rhizosphere as well as in the root interior, and the abundance and activity of catabolic enzymes can be selectively increased depending on the plant genotype (Becerra-Castro et al. 2013; Dzantor 2007; Kuiper et al. 2004; Siciliano et al. 2001; Sipilä et al. 2008). Beneficial endophytes can contribute to the detoxification of pollutants taken up by plants. For example, it was shown that in planta TCE and toluene metabolism is improved when plants are inoculated with endophytes that possess the appropriate degradation pathways. This results in decreased phytotoxicity and reduced evapotranspiration of contaminants into the atmosphere (Barac et al. 2004; Weyens et al. 2009a, Weyens et al. 2009b).

As mentioned previously, many studies have reported the identification of TNT-degrading bacteria that are able to transform TNT. However, the selection of TNT-degrading strains which are also able to promote plant growth, colonise roots, and are appropriate to use in combination with naturally occurring trees and grasses for rhizoremediation, remains largely unexplored. It has been suggested that the competence of bacteria to successfully persist in the rhizosphere and stimulate rhizoremediation is determined by their root colonisation abilities, their abilities to utilise root exudates, and their ability to maintain and express catabolic genes (Lugtenberg and Dekkers 1999). Kuiper et al. (2002) demonstrated that enhanced naphthalene degradation was correlated with the root colonising ability of inoculated *Pseudomonas putida* KT2440 and its ability to utilise certain root exudate compounds (Kuiper et al.

2002). The importance of rhizosphere competence has also been demonstrated for TNT and RDX biodegradation. Lorenz et al. (2013) have demonstrated that alfalfa plants inoculated with *Pseudomonas fluorescens* F113, an efficient coloniser of alfalfa roots that was engineered to express the RDX-metabolising enzyme XplA, significantly increased RDX removal from the bulk soil and the rhizosphere (Lorenz et al. 2013).

We believe that the rhizosphere-associated soil and endophytic compartments of mature *Acer* growing on a long-term TNT-polluted soil hosts a diverse community of TNT-transforming bacteria with beneficial PGP-characterisics. Using cultivation-dependent and independent approaches, we isolated and characterised *Acer pseudoplatanus* associated bacteria and showed the use of TNT-transforming consortia with PGP-capabilities for use in rhizoremediation of TNT-contaminated soil.

Material and methods

Site description and sampling Samples were collected in October 2010 at a military facility located in Zwijndrecht, Belgium (51°11′40.0″N; 4°19′29.6″E). TNT-production and disposal/melting of old ammunition from shells took place there until the late 20th century. At the site, a mature *Acer pseudoplatanus* (sycamore maple) tree was selected which had developed its root system next to a TNT-collecting basin where the surrounding soil is heavily contaminated with TNT. Bulk soil (BS) cores were obtained 5 m away from the trunk of the tree with a soil bore (5 × 20 cm deep) after removing the humus layer. The soil is classified as sandy loam according to the USDA-scheme with 53 % sand, 45 % silt and 3 % clay with the following physico-chemical characteristics: pH-H_2O 7.16, pH-KCl 6.49, conductivity 156 μS cm^{-1}, cation exchange capacity (CEC) 10.2 meq per 100 g dry weight soil determined by ammonium acetate saturation at pH 7 and spectrophotometric analysis (Chapman 1965). Total organic matter content was 3.82 %, determined using the Walkley-Black oxidation method and a factor of 1.724 was used to convert organic C to organic matter (Walkley and Black 1934). The extractable concentrations of TNT and TNT-metabolites in the bulk soil were, per kg soil: 4,282±60.3 mg 2,4,6-trintirotoluene (TNT), 92±10.6 mg amino-dinitrotoluenes (ADNTs) and 30±

2.3 mg 1,3,5-trinitrobenzene. In the root-zone of the tree, concentrations were 88 ± 5.3 mg TNT kg^{-1} soil and 34 ± 3.6 mg ADNTs kg^{-1} soil. Soil extract preparation and HPLC-analyses were performed according to EPA method 8330 (1998). In the root zone of the tree, the tertiary fine roots were sampled with soil particles still adhering to the roots and this fraction was considered to be the rhizosphere soil fraction (R). Three different plant compartments were sampled in triplicate and after surface sterilisation. These samples were labelled endophyte roots (ER) and endophyte shoots (ES), samples which were collected from 2 cm diameter cuttings, and endophyte leaves (EL). All soil and plant samples were collected using sterile tools, transported on ice, and stored at 4 °C for up to 2 days prior to processing in the laboratory. Fractions of the processed rhizosphere and plant samples were flash-frozen in liquid nitrogen and stored at −80 °C.

Isolation of bacteria, media composition and culture conditions Soil samples were thoroughly homogenised and mixed in a sterile $MgSO_4$ solution (1:10) prior to bacterial isolation. The fine roots were shaken over a sterile sieve to remove loose soil, and washed with sterile 10 mM $MgSO_4$ to remove the adhering rhizosphere soil. This wash solution was collected in 50-ml tubes and constituted the rhizosphere samples. For the isolation of endophytic bacteria, roots (diameter <2 mm), leaves or shoots (cut into smaller fragments with sterile scissors) were rinsed 5 times in sterile distilled water and then transferred to 50-ml centrifuge tubes for surface sterilisation. The surface sterilisation protocol consisted of the following steps: roots; 1 % NaOCl for 2 min; leaves, 1 % NaOCl for 2 min; and twigs, 1 % NaOCl for 2 min. Finally all samples were rinsed 3 times for 5 min in sterile dH_2O. Surface sterility was confirmed for all samples by plating 100 μl of the final rinse water onto 869 rich medium plates and incubating 5 days at 30 °C. The surface sterilized root, shoot or leaf tissues were homogenized with a mixer in 10 ml of sterile physiological $MgSO_4$ solution. Next the smashed ER, ES, EL or the rhizosphere soil or bulk soil suspensions were shaken for 15 min at room temperature. Each sample was diluted in 10-fold series and plated in triplicate onto 284 minimal medium (Schlegel et al. 1991) containing 200 ml of the organic carboxylic acid mixture EXU ROOT® per l (Innovak Global, Chihuahua, Mexico) to enhance the cultivability of rhizospheric and endophytic bacteria. For the

composition of the medium, see supplemental materials. After 5 days at 30 °C, Colony Forming Units (CFU) per gram was determined. All morphological distinct colonies were selected and spread onto new plates for purification. Pure strains were flash-frozen in a solution of 15 % w/v glycerol and 0.85 % w/v NaCl at −80 °C for further analysis.

Identification of bacteria using amplified ribosomal DNA restriction analysis (ARDRA) and partial 16S rRNA gene sequencing DNA was extracted from the bacterial isolates using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands). The 16S rRNA genes were PCR-amplified using the universal prokaryotic 1392R primer (5′ ACGGGCGGTGTGTRC 3′) and the bacteria-specific 26 F primer (5′ AGAGTTTGATCCTG GCTCAG 3′) with PCR-conditions as previously described (Barac et al. 2004). For amplified ribosomal DNA restriction analysis (ARDRA), the 16S PCR products (20 μl) were digested for 2 h at 37 °C with HpyCH4IV in a reaction with 1× NEB-buffer (New England Biolabs, Ontario, Canada). The digestion products were separated by gel electrophoresis (90 V, 2 h) with a 1.5 % agarose gel containing GelRed (VWR, Leuven, Belgium) nucleic acid gel stain, and visualized under UV illumination. Isolates showing the same ARDRA banding patterns were grouped and representative strains were selected for sequencing as described earlier (Barac et al. 2004; Weyens et al. 2009a). The sequences were compared with those deposited in the Ribosomal Database Project using BLAST and identifications were based on the most closely related sequences. The partial 16S rRNA gene sequences of 66 strains were deposited in the EMBL-database, accession numbers LK021136-LK021201 (http://www.ebi.ac.uk/ena). For phylogenetic analysis, all reads were aligned using Clustal Omega (EMBL-EBI, Cambridgeshire, UK) and an UPGMA phylogenetic tree was generated using Geneious (Biomatters Ltd, Auckland, New Zealand) and drawn using iTOL (Letunic and Bork 2007) The Shannon index, which takes into account both abundance and evenness of the species present in the community, was calculated for each sample (Shannon 1948).

Extraction of total DNA from soil, rhizosphere and plant parts for T-RFLP-analysis Total DNA was extracted from soil and rhizosphere samples using a Powersoil® DNA-isolation kit (MO BIO Laboratories, Carlsbad, CA). For root, shoot and leaf endosphere samples, an

additional sterilisation step (supplemental material) was used on the exterior of the samples prior to total DNA-extraction using an Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany).

DNA-extracts from soil and plants were used for PCR-amplification using the primers 799 F and 1492R (Chelius and Triplett 2001) after which the bacterial 16S rRNA fragments were extracted and used for restriction digestion with *Dde*I, see supplemental material for details. Automated T-RFLP was performed using a 2100 Bioanalyser (Agilent Technologies, Diegem, Belgium) and samples from all compartments (BS, R, ER, ES and EL) were analysed in triplicate. The acquired T-RFLP data profiles were processed using Total Lab (Total Lab Systems, Auckland, New Zealand), background subtraction was performed according to the minimum slope profile and parameters for peak detection were set to default. The normalized data were used for multivariate statistical analysis. A principal component analysis (PCA) plot was generated using the statistical software package R (http://cran.at.r-project.org). For verification, band patterns were clustered and a multi-scale bootstrap re sampling dendrogram was generated from a correlation distance matrix. Diversity was calculated based on the evenness index of Smith and Wilson (Evar) (Smith and Wilson 1996) and applied to the T-RFLP data as recommended by (Blackwood et al. 2007) due to its independence of species richness and its sensitivity to both rare and common species in the community.

Evaluation of potential direct and indirect plant-growth promoting activity Indole acetic acid (IAA) production capacity was evaluated using the Salkowski reagent method (Patten and Glick 2002). Siderophore release was determined by using the Chrome azurol S (CAS) assay (Schwyn and Neilands 1987). 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity was estimated by monitoring the amount of α-ketobutyrate that was generated by the enzymatic hydrolysis of ACC, a plant hormone ethylene precursor, according to a modified protocol, see supplementary methods (Belimov et al. 2005). Organic acid production was determined using the organic dye Alizarin red S (Cunningham and Kuiack 1992). Microbial mineral phosphate $Ca_3 (PO_4)_2$ solubilisation was assayed on agar plates (Goldstein 1995). Production of acetoin, a rhizobacterial elicitor, was measured using the Vorges-Proskauer assay (Romick 1998). Pectinase activity was tested by spot inoculation on nutrient agar plates containing 0.5 %

pectin (Jha and Kumar 2007). Microbial expression of cellulolytic enzymes was evaluated in an endoglucanase assay (Reinhold-Hurek et al. 1993). Growth on nitrite as the sole nitrogen source was tested in a nitrogen-free minimal 284 medium, and the consumption of nitrite from the medium was estimated using the Griess reagent (Griess 1879). Cluster analysis of the PGP-potential of the bacteria was performed according to the Pearson correlation coefficient in the statistical software package R.

TNT-degradation by pure cultures and consortia In order to determine the TNT degradation capacity of pure cultures or consortia, they were grown overnight at 30 °C in 869 rich medium and used as pre-inocula for fresh cultures (Mergeay et al. 1985). When the cultures reached the exponential growth phase (optical density $OD_{600} = 0.5$), the cells were centrifuged and washed twice with $MgSO_4$ solution (10 mM) prior to resuspension in selective minimal medium with TNT. The selective minimal medium for the *Pseudomonas* strains consisted of 50 mM phosphate buffer (pH 6.8), trace elements, 1 mM NH_4Cl, 3 mM glucose and 0 or 500 μM TNT (Snellinx et al. 2003). The other strains were exposed to 0 or 500 μM TNT in 1/5 diluted 869 rich medium (Mergeay et al. 1985). None of the strains could grow in minimal medium with TNT as the sole nitrogen source, therefore ammonium in the *Pseudomonas* medium and tryptone in the 869-medium were needed for growth. TNT was dissolved in DMSO and added after autoclaving to the medium (0.5 % DMSO per l medium). In all cultures, samples were withdrawn at regular time intervals (0–72 h) and analysed with HPLC. Nitrite release was estimated using the Griess reagent (Griess 1879). Bacterial growth was determined by measuring the protein concentration according the Bradford assay (Bradford 1976). Growth curves were performed and compared between the pure cultures in the presence and absence of TNT.

To determine the products formed by consortia from TNT, resting cell experiments were conducted. These experiments consist of using concentrated metabolically active but not growing bacterial cells in a buffered system with the substrate and an energy source for cofactor replenishment. To produce consortia, pure cultures of each component strain were grown overnight in rich medium, diluted 1/100 and then transferred to fresh selective medium with 100 μM TNT. When the pure

cultures reached the exponential growth phase, the cells were washed twice in selective medium and concentrated to an OD_{600} of 1. Then, 0.5 ml of each culture was mixed together. The metabolically active resting cells were then incubated in 50 mM sodium phosphate buffer (pH 6.8), a 20 mM carbon mix (7 mM glucose, 7 mM fructose, 3 mM acetic acid and 3 mM succinate) which was used as energy source for NAD (P) H regeneration and 500 μM TNT as substrate (Snellinx et al. 2003). At regular time intervals (0–48 h), supernatants of samples were withdrawn for HPLC analysis.

Enzyme assays For enzyme assays, consortia were prepared as described above and then grown in 869 rich medium until an OD_{600} of 0.5. Subsequently, the cells were harvested by centrifugation and washed twice in ice-cold 50 mM phosphate-buffer (pH=7.0). The cells were lyzed on ice using 5 sonication bursts of 20 s alternated with 60 s of cooling on ice. Cell-free extracts were obtained by centrifuging for 30 min at $20,000 \times g$ (Beckmann, Munich, Germany). NAD (P) H-dependent oxidoreductase activity of the cell-free extracts was assayed in 50 mM phosphate buffer (pH=7.0) with addition of 0.2 mM NAD (P) H and 0.1 mM TNT (from a stock solution of 50 mM TNT in acetone) at 30 °C (French et al. 1998). One unit of activity was defined as the oxidation of 1 nmol of NAD (P) H per mg protein per min under these conditions.

Flavin-oxidoreductase PCR-screening PCR amplification of the Xenobiotic Reductase B gene (*XenB*) was performed using the forward primer IXBF (5′ TGACCA CGCTTTTCGATCC 3′) and reverse primer IXBR (5′ CAACCGCGGATAATCGATG 3′), primers which are based on the published gene sequence from *Pseudomonas putida* KT2440 (van Dillewijn et al. 2008b). An internal fragment of members of the Old Yellow Enzyme family of flavin oxidoreductases (OYE) with similarity to known type II hydride transferases was amplified by using the primers OYEi1F (5′CARN TNTGGCACRYSGGNCG 3′) and OYER (5′ AGRT CNGGRTTSGCRATNAA 3′). The PCR-reactions consisted of DNA from each selected isolate, 1× reaction buffer, 0.2 mM dNTP-mix, 1.5 mM $MgCl_2$, 0.05 % DMSO, each oligonucleotide primer at a concentration of 1 μM and 1 U Platinum® Taq DNA Polymerase High Fidelity (Life Technologies Europe B.V., Gent, Belgium). The PCR-conditions consisted of an initial denaturation step of 3 min at 95 °C and then 30 cycles of

94 °C for 1 min, 40 sec at the appropriate annealing temperature (T_a) and 72 °C for 1 min, followed by 72 °C for 7 min. The T_a for *XenB* was 50 °C and for OYE 46 °C. The quality and quantity of PCR products was analyzed by 1.5 % agarose gel electrophoresis. Bands of the expected size, *i.e.* 1100 bp for *XenB* and 500 bp for OYE, were excised and subcloned into the pGEM-T® Vector (PROMEGA GMBH, Mannheim, Germany). A number of clones harboring fragments of the expected size were sent for sequencing. Clustal Omega was used to align the sequences. The translated protein sequences were searched using BLAST in the PFAM database for conserved protein domains and active binding sites (http://pfam.sanger.ac.uk/).

Plant growth promotion under TNT-exposure in a vertical agar plate system (VAPS) Wild-type *Arabidopsis thaliana* (ecotype Columbia) seeds were surface sterilized in 0.1 % NaClO for 5 min and washed 4 times with distilled water over 20 min. Seeds were sown on vertical plates with 50 ml of Gamborg's B5/50 medium (1 % Bacto Agar) and placed at 4 °C for 3 days in the dark (Zhang and Forde 1998). For germination, plates were placed vertically in a growth chamber at 22 °C, with a 12-h/12-h light–dark cycle and photosynthetic active light radiation (PAR) of 180 μmol m^{-2} s^{-1} at seedling level. After 7 days, seedlings with similar root lengths were chosen and transferred to fresh B5/50 plates which had been supplemented with TNT (0; 1 mg l^{-1}) and inoculated with bacteria. See supplemental methods for details about the bacterial inoculation and replicates.

Fluorescent protein tagging of Acer-associated strains To stably transform strains of the consortium CAP9, we used the environmental stable plasmids pMP7604 (mCherry) and pMP4655 harbouring the enhanced green fluorescent protein (EGFP) (Lagendijk et al. 2010). Briefly, transformations were performed by a triple conjugation formed by a suspension of 10^8 cells of the recipient strain, 10^7 cells of the helper strain *E. coli* H2013 and 10^7 cells of the donor strain *E. coli* DH5α carrying the *egfp*-plasmid pMP4655 in a ratio of (10:1:1), or by using the donor strain *E. coli* DH5α carrying the mCherry-plasmid pMP7604. The bacteria were visualised by fluorescence microscopy using a Nikon Eclipse 80i (Nikon Instruments Inc., NY, USA).

In planta root-colonisation test To evaluate the ability of mCherry-labelled strains to adhere to and colonise the

plant root system, the strains were used to inoculate *Agrostis capillaris* grass (common Bent) seedlings in a hydroponic system. After overnight growth on 1/10 rich medium, a bacterial cell suspension of each tagged strain was prepared in sterile MgSO$_4$ solution (10^8 cells ml^{-1}). Sterile 1-week old *A. capillaris* seedlings grown in 1/10 Hoagland solution (Hoagland and Arnon 1950) were inoculated separately with the bacterial suspensions. After 48 h of incubation, plant roots were washed to remove weakly bound bacterial cells and, subsequently, intact root preparations were observed with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) mounted on an Axiovert 200 M. The objective used was 40x1.1 water immersion (Zeiss LD C-Apochomat 40×/1.1 W Korr UV–VIS-IR). Excitation was performed at the 488 nm line of an Ar-ion laser source. Backward GFP signal was filtered using a 500–550 nm band pass filter. Images were edited using the software Zen 2009 Light Edition (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Plant-growth promotion under TNT-stress in a sand-compost leaching study 50 mg of *A. capillaris* seeds were sown in separate 150-ml syringes filled with a sterilized fine-grade sand compost substrate (Evergreen, Antwerp, Belgium) and spiked (see supplemental methods) with TNT (0; 25; 50 mg TNT kg^{-1} substrate). At day 7 and 14 after germination, the seedlings were fertilized with a diluted suspension of the bacterial consortium in sterile 10 mM MgSO$_4$ at a concentration of 10^8 cells g^{-1} soil, while non-inoculated plants were watered with the same amount of sterile MgSO$_4$. During growth, the plants were given tap water and maintained at a temperature cycle of 22/18 °C, a photoperiod of 14:10 h daylight and constant humidity of 57 %. After 5 weeks of growth the plants were harvested. Growth was evaluated gravimetrically by weighing above and below-ground biomass and length. To characterise the plants' antioxidant status, the activities of several anti oxidative enzymes were measured on leaf and root-samples (100 mg) that were flash-frozen in liquid nitrogen at harvest. Superoxide dismutase (SOD) catalyses the dismutation of superoxide anions, catalase (CAT) and guaiacol peroxidase (GPOD) catalyse the decomposition of H$_2$O$_2$ into water and oxygen, glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). GSH is an important antioxidant and acts as substrate for e.g. glutathione-S-transferase

(GST). SOD activity was determined by measuring cytochrome c-reduction and following the increase in absorbance at 550 nm during 20 s (McCord and Fridovich 1969). CAT activity was determined by measuring the disappearance of H$_2$O$_2$ at 240 nm (Bergmeyer et al. 1974). GPOD activity was determined by measuring the oxidation of guaiacol in the presence of H$_2$O$_2$ by following the increase in absorbance at 470 nm along the time (Bergmeyer et al. 1974). GR activity was determined by measuring the loss in absorbance at 340 nm caused by the conversion of NADPH to NADP + (Bergmeyer et al. 1974). GST activity was evaluated by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione which is proportional to the increase of absorbance at 340 nm (Habig and Jakoby 1981). All the enzyme activities were expressed in absorbance units min^{-1} mg^{-1} plant fresh weight (FW).

The reactive metabolites of thiobarbituric acid (TBA) were determined spectrophotometrically according to (Dhindsa et al. 1981) and expressed in nmol TBA reactive metabolites g^{-1} FW. The assay is based on the reaction which occurs between secondary products from lipid peroxidation with TBA in acidic medium which gives a pink pigment at 97 °C at pH 2–3. The absorbance of the pink colour was measured at 535 nm and corrected for unspecific absorbance at 600 nm ($\varepsilon = 155$ mM^{-1} cm^{-1}).

TNT concentrations were determined from samples taken from the growth substrate at the beginning and end of the experiments. TNT measurements were also performed on the roots and aboveground plant parts. For this, 1 g dried root or grass material was crushed into a fine powder, extracted three times with 10 ml methanol and concentrated before analysis on HPLC. The plant experiments were performed with 10 replicate syringes per condition. The statistical analysis was conducted by analysing the data by ANOVA and the Tukey HSD-test for multiple comparisons after testing the normality and homoscedasticity assumptions ($p < 0.05$).

To evaluate the rhizosphere competence of the inoculated strains, scanning electron microscopy (SEM) was performed on freshly collected root samples at the end of the experiment. Non-inoculated roots were collected as control. The roots were gently rinsed with tap water and subsequently fixed in 4 % glutaraldehyde in phosphate-buffered-saline (PBS) (140 mM NaCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$; pH=7.0) overnight at 4 °C. Afterwards, the samples were briefly

washed in PBS, gradually dehydrated by using an eth-
anol series (20, 30, 50, 70, 96 %), and subsequently kept
overnight in water-free ethanol at 4 °C. Then, the sam-
ples were frozen (−20 °C) in tert-butyl alcohol.
Sublimation of the tert-butyl alcohol was performed
and then the roots were mounted on stubs. Imaging
was performed with the FEI Quanta 200 FEG-SEM
(FEI, Eindhoven, Netherlands) under low vacuum.

HPLC analyses HPLC was performed according to the
EPA method 8330, using a Chromosphere C18 reverse-
phase column (5 μm, 250 × 4.6 mm) and Agilent 1100
system (Agilent, Santa Clara, CA). The mobile phase
consisted of 50/50 (v/v) methanol/water and was deliv-
ered at a flow rate of 1.3 ml min^{-1}. Components were
detected at 253, 450 and 210 nm, and compared with
reference standards. Ion-pair HPLC was performed with
a mobile phase of 5 mM tetrabutylammonium phos-
phate buffer (pH=7.5) and acetonitrile at a flow rate of
0.85 ml min^{-1}. The method used consisted of a linear
gradient from 0 to 25 % (v/v) acetonitrile during 20 min,
from 25 to 62 % acetonitrile for another 23 min and then
increased to 100 % acetonitrile in the next 5 min. The
column was then re-equilibrated with 100 % 5 mM
tetrabutylammonium solution for 10 min.

Accession numbers Partial 16S-rDNA gene sequences
were submitted to the European Nucleotide Archive
(ENA) with the accession numbers LK021136-
LK021201. Flavin-oxidoreductase nucleotide se-
quences were deposited in the ENA with the accession
numbers LK021131-LK021135.

Results

*Isolation and characterisation of the cultivable bacteria
associated with Acer pseudoplatanus* At a former TNT-
production and decommissioning facility of the Belgian
army, soil, rhizospheric and endophytic bacteria associat-
ed with a native *Acer pseudoplatanus* tree growing in a
heavily TNT-contaminated soil were isolated. Based on
differing colony morphologies, 150 strains were selected
for DNA-extraction and amplification of the 16S rRNA
gene. ARDRA was used to differentiate between closely
related clones, after which one representative from each
group was chosen for identification by partial 16S rRNA
gene sequencing. Sequences for 66 strains were obtained
and the most closely related 16S rRNA sequences

(similarities of >0.95) were identified by BLAST
searches of the Ribosomal Database Project (RDP,
http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). A
detailed overview of the cultivable bacteria isolated from
the soil, rhizosphere and plant endosphere is presented in
Fig. 1, and a phylogenetic tree is shown in Fig. S1.

The phyla Proteobacteria, Actinobacteria and
Firmicutes were represented in all three compartments
sampled, but the distribution of the major bacterial clas-
ses differed (Fig. 1). In the bulk soil, Actinobacteria,
particularly *Streptomyces* and *Rhodococcus*, and Beta-
and Gamma-proteobacteria, particularly *Cupriavidus* and
Pseudomonas, were the most abundant. The rhizosphere
contained the most diverse cultivable microbial commu-
nity as it has the highest Shannon-index (2.79; $p<0.05$) at
the genus level, and was also characterised by the highest
bacterial abundance (7.5 × 10^6 CFU per gram soil) of the
sampled compartments (Fig. S2). The dominant groups
in the rhizosphere (R) were Proteobacteria with
Burkholderia, *Herbaspirillum*, *Bosea* and
Pseudomonas, and Actinobacteria with *Oerskovia*,
Rhodococcus and *Streptomyces*, being most abundant.
The endophyte root (ER) and shoot (ES) fractions were
the least diverse (Shannon-index of 1.62 and 1.10, re-
spectively; $p<0.05$) and were almost exclusively com-
posed of members of Proteobacteria. However, ER was
more similar to R and the bulk soil (BS) in their distri-
bution of Proteobacteria, while ES was dominated by
members of Alphaproteobacteria with
Methylobacterium and *Sphingomonas* being the most
dominant genera. The endophyte leaf (EL) fraction was
again different in composition, with Actinobacteria
(*Rhodococcus*, *Sanguibacter*, *Curtobacteria*,
Okidobacterium) being the largest group next to
Proteobacteria (*Pseudomonas* and *Stenotrophomonas*).
An overview of the 8 most dominant species found in
the soil, rhizosphere and endosphere is shown in Fig. S2.

*Diversity of bacterial communities associated with
Acer* T-RFLP-fingerprint analysis of partial 16S rRNA
gene amplicons from bacterial communities was applied
to provide a snapshot of the cultivable and uncultivable
bacterial assemblages in the bulk soil surrounding the
Acer tree as well as in rhizosphere and endophytic parts.
PCA analysis (n=3) of the fingerprint data showed three
clusters, 1 for BS and R, 1 for ER and 1 for ES and EL
samples (Fig. S3a). The first PC accounted for 28.6 % of
the variability and the second PC explained 17.5 %. A
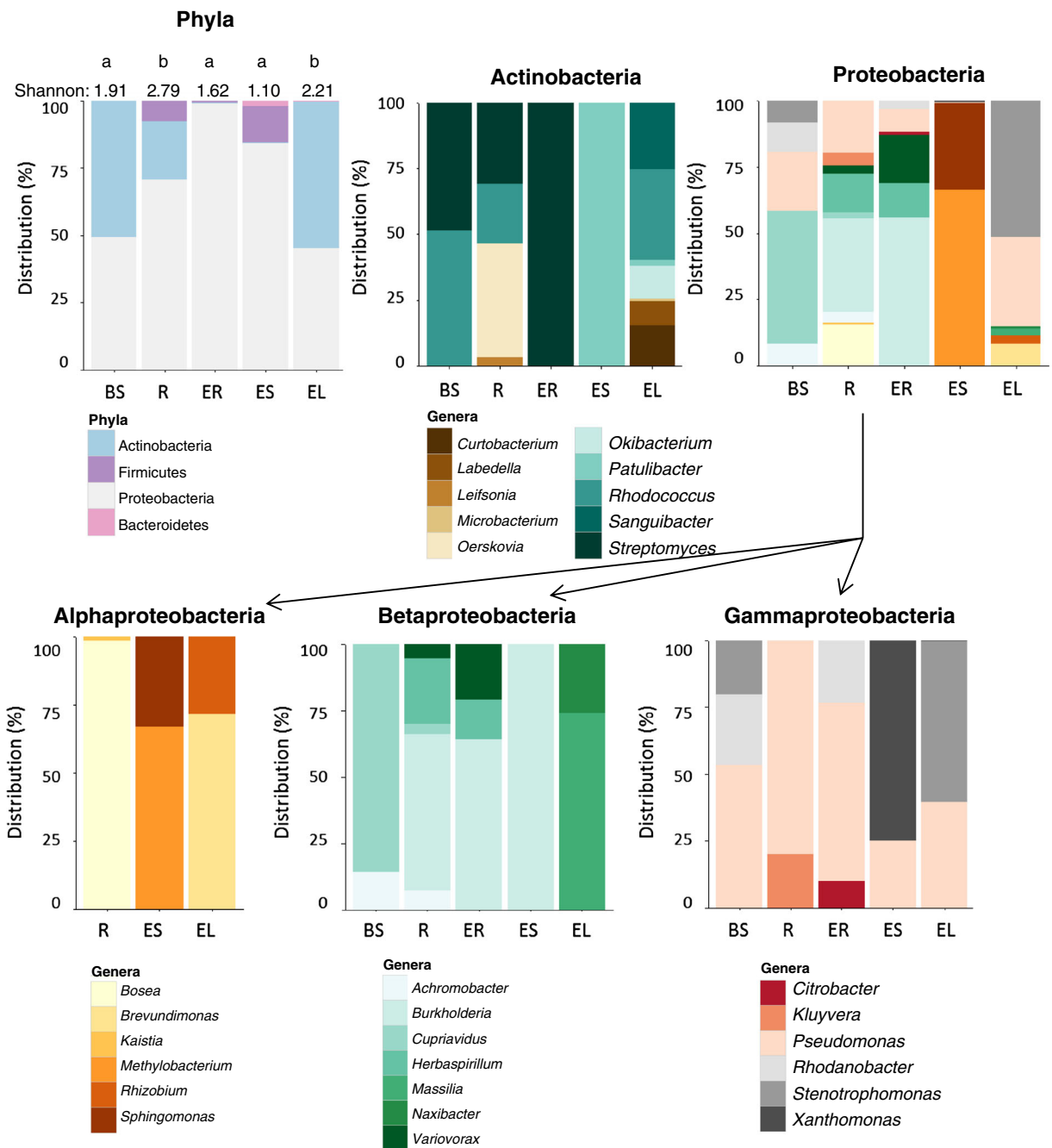correlation dendrogram using complete linkage and

Fig. 1 *Stacked bar* graphs showing the distribution of all cultivable phyla present in the *Acer* tree associated samples (*EL*, Leaf endophyte; *R*, Rhizosphere; *ER*, Root endophyte; *ES*, Shoot endophyte; *BS*, Bulk soil isolate). The distribution of genera present among the phylum Actinobacteria and the three classes of the phylum Proteobacteria: Alpha- Beta- and Gamma-proteobacteria. The respective Shannon diversity index at the genus level of the different samplesis given above each *bar* of the phyla graph. The different letters above the diversity values represent significant difference (*p*<0.05, Kruskall Wallis; *n*=3)

multi-scale bootstrap re sampling revealed the same three clusters found by PCA (Fig. S3B). BS and R clustered together with an approximately unbiased (AU)-value of

97 %, ER with AU = 100 %, and EL and ES with AU = 96 %. Although there was a large amount of TNT present in the soil, a relatively even micro biome is present in the

BS given the Smith and Wilson evenness index of 0.77± 0.03 being only slightly lower than for the rhizosphere at 0.83±0.02 (Fig. S3b).

Plant growth promoting properties of the Acer tree associated bacteria The cultivable bacterial collection, comprising 150 morphologically different strains, was tested *in vitro* for plant growth promotion properties such as phytohormone production, nutrient acquisition and the metabolism of plant-growth regulating compounds. Overall, the shoot endosphere harboured the most beneficial PGP-bacteria, with 77 % of the isolates showing 3 or more PGP-traits (Table S1). The rhizosphere and endosphere were enriched in strains demonstrating ACC-deaminase activity compared to strains from the bulk soil (Table S2). Siderophore production (Fe-acquisition) was highly represented in ER, R and ES, and P-solubilisation and IAA-production were high in the bulk soil. From this initial screen, 26 representative strains, identified by 16S rDNA gene sequencing, were selected from the major phylogenetic taxa for additional testing. Each selected strain scored positive for one or more PGP-trait and belonged to genera which have previously been reported to tolerate nitro aromatic compounds. These strains were screened for pectinase and endoglucanase activity, organic acid production, acetoin production and the use of nitrite, an important nitrogen source that can be released from TNT. Figure 2a shows the clustering of these strains according to their PGP-potential. Two clusters are distinguishable, with cluster 1 being mainly composed of *Pseudomonas* spp. and showing a higher *in vitro* PGP-potential than bacteria from cluster 2 which included representatives from *Sphingomonas*, *Rhodococcus*, *Bacillus*, *Variovorax*, *Microbacterium*, *Burkholderia*, *Xanthomonas* and *Methylobacteria*. Almost all of the strains tested were able to grow with ACC as the sole nitrogen source and to solubilise phosphorous (Fig. 2a). Many of the rhizosphere and soil dwelling *Pseudomonas* isolates as well as the *Xanthomonas* (ES11, EL17) and *Methylobacterium* (ES2) isolates were able to produce siderophores and IAA. Some differences were observed with endoglucanase production, since this activity was mainly associated with endophytic EL, ER and ES strains. Pectinase activity appeared to be more widespread, being also found in some BS and R strains. Only a limited number of isolates, including *Pseudomonas* sp. strains BS4, R47, ER9, R59 and R54, *Kluyveria* sp. strain R43, *Burkholderia* sp. strains ER4 and ES14, and

Xanthomonas sp. strain EL17, were able to grow with 2 mM NO_2 as a sole nitrogen source.

In order to investigate the capacity of each strain to enhance root growth under stress-conditions, *Arabidopsis thaliana* plants grown under control and TNT-exposed conditions in a Vertical Agar Plate System (VAPS) were inoculated with the bacteria and the root length responses determined (Fig. 2b). Under control conditions, six strains were able to significantly enhance plant root growth and among these were *Stenotrophomonas chelatiphaga* (EL1), *Pseudomonas vranovensis* (R47), *P. azotoformans* (R54), *P. flavescens* (EL24), *Rhodococcus fascians* (EL20) and *R. qingshengii* (BS26). Under TNT-exposure conditions, in uninoculated and inoculated cultures, TNT was toxic to the seedlings and reduced primary root length significantly in comparison with TNT-free controls (19.2±4.3 mm in comparison with 45±1.3 mm). However, inoculation with *Stenotrophomonas chelatiphaga* (EL1), *Cupriavidus basilensis* (BS7), *Pseudomonas sp.* (R47, ER9, R59, R54), *Kluyveria intermedia* (R43), *Sphingomonas panni* (ES4), *Rhodococcus sp.* (EL20, BS10, BS26, R44), *Variovorax ginsengisoli* (ER18) and *Methylobacterium thiocyanatum* (ES2) significantly increased *A. thaliana* root length compared with the non-inoculated controls under TNT-stress and relieved almost completely the TNT-induced root length reduction ($p<0.05$, see Fig. 2bII). The strongest effects on root growth promotion under TNT stress conditions were

Fig. 2 a Cluster analysis of the plant growth promotion (PGP)-potential of a selection of bacteria associated with *Acer pseudoplatanus*. Clustering was performed according to the complete method and Euclidean distance for binary data. The bacterial isolate number is shown (*EL*, Endophyte of leaf; *R*, Rhizosphere; *ER*, Endophyte of root; *ES*, Endophyte of shoot; *BS*, Bulk soil isolate) followed by the total PGP-potential score, calculated as the sum of the different PGP-abilities exhibited by each strain, and the most closely related 16S rRNA gene sequence. **b** *Vertical bar charts* showing the root length of (I) *Arabidopsis thaliana* plants inoculated with the PGP-strains and subsequent growth for 9 days under control conditions and (II) plants grown in the presence of 1 mg l^{-1} TNT. The *horizontal lines* indicate average size of non-inoculated *A. thaliana* plants in each condition. Significant growth responses are indicated with an asterisks above each *bar* ($P<0.05$, ANOVA and Tukey HSD). **c** Examples of visual results of *in vitro* plant growth promotion tests: ACC-deaminase (*brown* = positive) (**a**); NO$_2$-use (*white* = positive) (**b**); IAA-production (*pink* = positive) (**c**); pectinase test (*halo* = positive) (**d**); P-solubilisation test (*halo* = positive) (**e**); *A. thaliana* vertical agar plate system used for the inoculation experiments (**f**) and the endoglucanase assay (*transparent* = positive) (**g**)
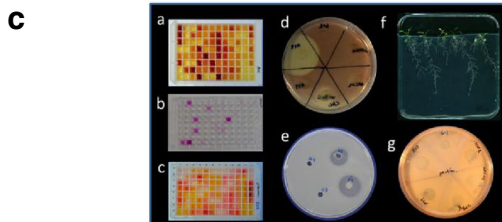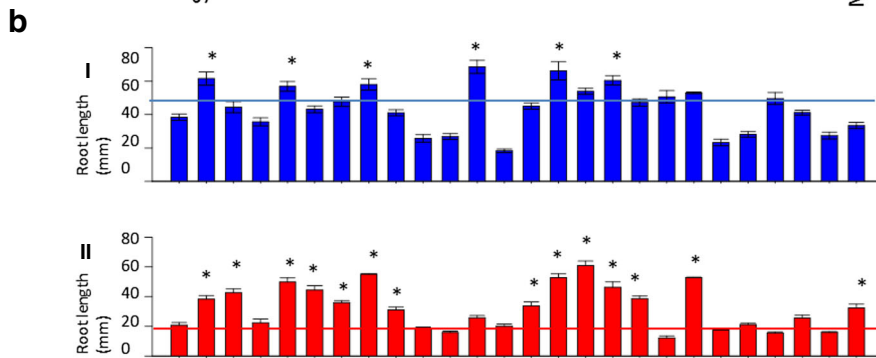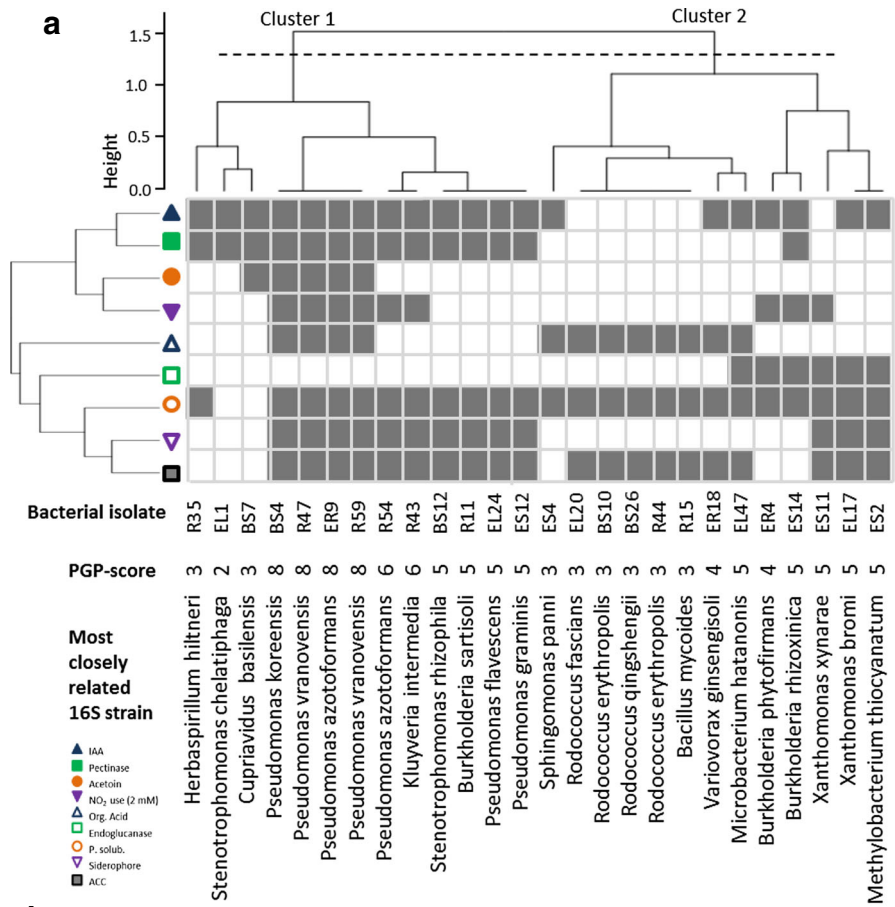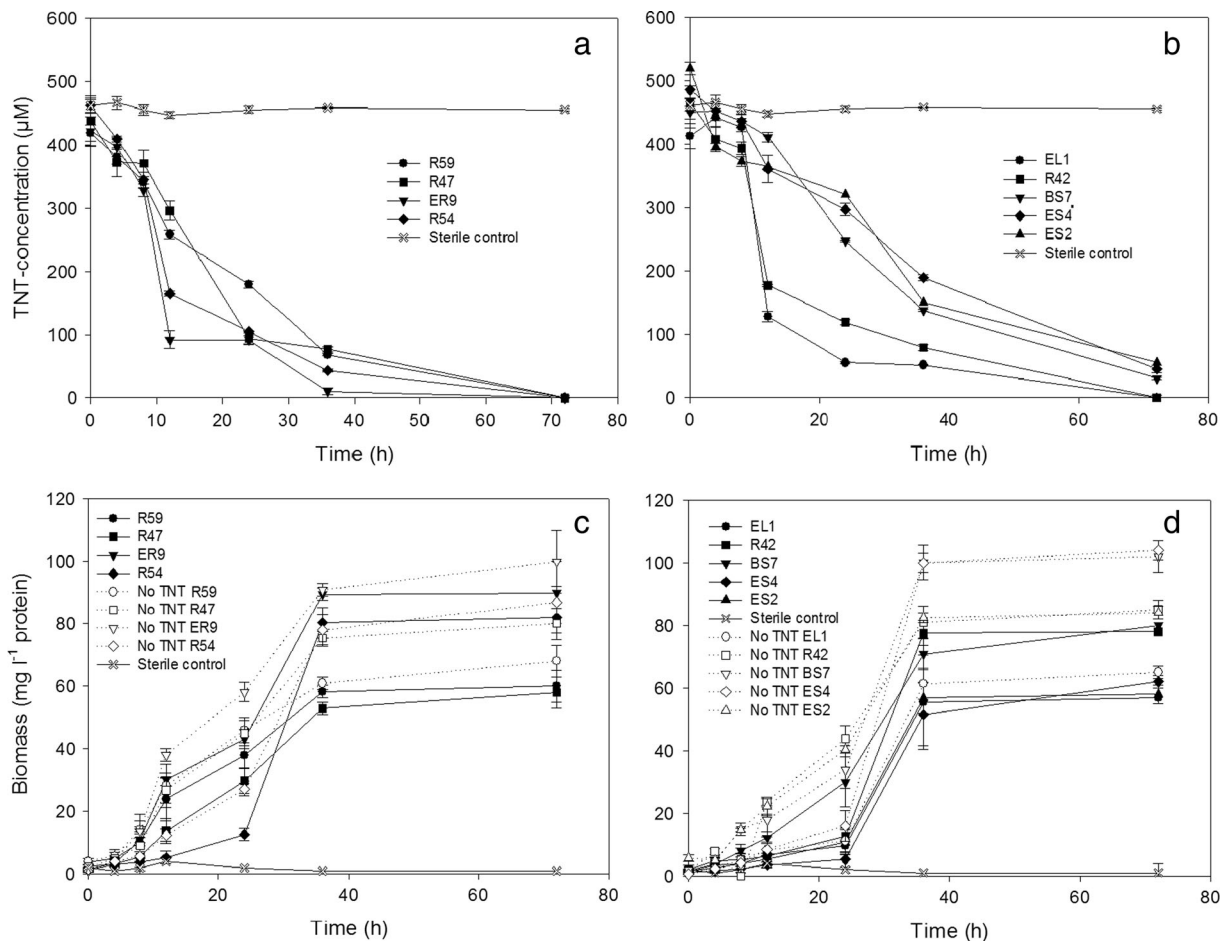
Fig. 3 **a** Transformation of TNT (500 μM) by pure cultures of *Pseudomonas* sp. (ER9, R47, R54 and R59) and **b** the strains *Cupriavidus basilensis* (BS7), *Methylobacterium thiocyanatum* (ES2), *Sphingomonas panni* (ES4), *Stenotrophomonas chelatiphaga* (EL1) and *Variovorax boronicumulans* (R42) and TNT-concentrations (μM) remaining in the culture supernatants were determined by HPLC. Control experiments consisted of non-inoculated flasks. **c** Biomass growth in the presence of TNT (*solid lines*, *black symbols*) and in the absence of TNT (control, *dashed lines* and *white symbols*) are presented for *Pseudomonas* sp. (ER9, R47, R54 and R59) and **d** the strains BS7, EL1, ES2, ES4 and R42. Bacterial biomass was determined by measuring protein concentration. *Error bars* represent the standard deviation (*n*=3)

observed for *Pseudomonas vranovensis* (R47), *P. azotoformans* (R54), *Rhodococcus erythropolis* (BS10) and *R. fascians* (EL20), which increased root length by more than 150 % compared to non-inoculated controls.

Degradation of TNT by pure cultures and consortia The 14 PGP-strains that most significantly enhanced root-growth in the VAPS-assay were tested for their ability to metabolize TNT in liquid cultures. *Pseudomonas* strains ER9, R47, R54 and R59, *Stenotrophomonas chelatiphaga* strain EL1, and *Variovorax ginsengisola* strain ER18 all removed

>90 % of TNT (500 μM) in less than 36 h and the bacterial biomass increases over time, as monitored by measuring the protein concentration, were not affected by the presence of TNT for these strains (Fig. 3). All cultures were grown in the presence of glucose (3 mM) and NH_4Cl (1 mM) as co-substrates. The first products formed were hydroxylaminodinitrotulenes (HADNTs), small amounts of mono-hydride Meisenheimer complex of TNT and aminodinitrotoluenes (ADNTs) as identified with HPLC (Fig. S4b data for cultures EL1 and R47). The brown-orange colour of the hydride Meisenheimer complexes of TNT was visible in the cultures (Fig. S4a). *Cupriavidus basilensis* (BS7), *Methylobacterium*

thiocyanatum (ES2) and *Sphingomonas panni* (ES4) needed 72 h for complete TNT-transformation to diaminonitrotoluene products (data not shown). Growth of these strains in the TNT-containing cultures was slower than in cultures without TNT (Fig. 3). All *Pseudomonas* strains produced larger amounts of HADNTs than *Cupriavidus* BS7 and *Methylobacterium* ES2 (data not shown). For the *Rhodococcus* strain, growth was completely inhibited upon exposure to 100 µM TNT and only limited amounts of TNT were removed from the medium (data not shown).

In soil and *in planta* environments, microorganisms normally exist in communities, therefore we composed a bacterial consortium (CAP9) from pure cultures of BS7, EL1, ES2, ES4, ER9, ER18, R47, R54 and R59. A control consortium (CAP4) consisting of *Rhodococcus sp.* BS10, BS26, EL20 and R44, strains which did not grow in 100 µM TNT or eliminated only limited amounts of TNT from the medium, was also constructed. In metabolically active resting cell experiments with CAP9, significant transformation of TNT by CAP9 was observed in less than 48 h (Fig. 4a and b). The first peaks that appeared in the UV-spectra were identified as HADNTs and small peaks representing mono- and dihydride Meisenheimer complexes were observed after 3 hours (Fig. 4b). Subsequently, ADNTs and small amounts of diaminonitrotoluenes (DANTs) and azoxy-and azotoluene complexes were found after 48 h. No nitrite was detected in supernatants. For the control CAP4, no significant TNT-transformation was observed even after 48 h with only a small peak of ADNT being detectable (Fig. 4c).

In cell-free enzyme assays, consortium CAP9 had 10-times higher nitro reductase activity than that of the Gram-positive *Rhodococcus* consortium CAP4 (42.57 ± 5.2 nmol NADH mg^{-1} protein min^{-1} compared to 4.86 ± 1.6). The enzyme assays also showed that NADH is more effective than NADPH as an electron-donor in CAP9 consortium extracts (Table S3).
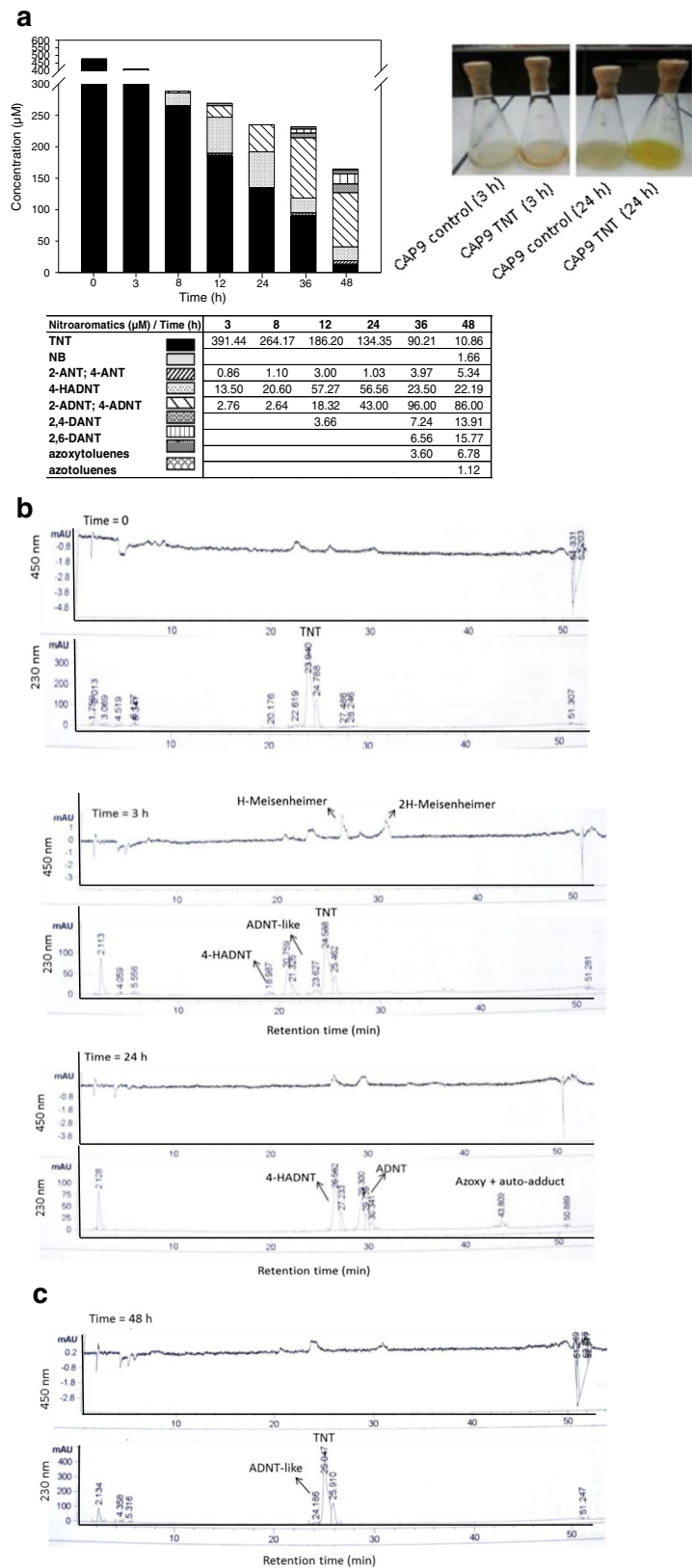
The TNT-reduction potential of a number of strains isolated here was further demonstrated by cloning and sequencing *OYE* flavin oxidoreductase genes from R59, R47, EL1, ER9 and ER18 (Genbank accession numbers LK021131-LK021135). Figure S5a and b shows the multiple sequence alignment and conserved regions for the protein sequences. The flavin oxidoreductase sequences of R59 and R47 are most closely related to the XenB of KT2440 (Fig. S5c). Because the individual members of consortium CAP9 exhibit a high PGP-

capacity *in vitro*, are able to detoxify TNT in VAPS-cultures and rapidly transform TNT in liquid cultures, this consortium was tested for its potential to improve the growth of *Agrostis capillaris*.

Plant growth promotion potential of CAP9 using a soil column assay and A. capillaris Without inoculation of the consortium, it was very difficult for grass plants growing in soils with 25 and 50 mg TNT kg^{-1} to form roots. After five more weeks of growth, the root lengths of the TNT-exposed grasses were 13.83 ± 1.53 and 9.52 ± 0.72 cm in soils containing 25 and 50 mg TNT kg^{-1}, respectively, lengths which were significantly ($p < 0.05$) shorter than roots formed by plants grown in the absence of TNT (22.84 ± 0.44 cm). Like the root systems, the leaves of TNT-exposed grasses were shorter compared to those of unexposed control plants. As can be observed in Fig. 5, the above- and below-ground biomass of the non-inoculated grasses grown in TNT-contaminated soil was reduced by more than 50 % in comparison with unexposed non-inoculated control plants. In contrast, after inoculation with consortium CAP9 and subsequent growth for 3 weeks, root biomass was significantly increased for both the 25 and 50 mg TNT kg^{-1} exposed grasses in comparison with the non-inoculated TNT-exposed plants. This difference was especially evident in soil containing 50 mg TNT kg^{-1} as the root biomass was 202 ± 3.2 mg for inoculated plants in comparison with 84 ± 2.0 mg for their non-inoculated counterparts ($p < 0.02$). Similarly, the beneficial effect of inoculation with the consortium CAP9 was also observed for the leaf biomass, as in the 50 mg TNT kg^{-1} soil, leaf weight of inoculated plants was 186 ± 1.7 mg in comparison to 63 ± 4.5 mg for the non-inoculated grasses ($p < 0.02$). At the end of the experiment, the root and leaf biomass of the inoculated TNT-exposed plants were not significantly different from unexposed control plants, suggesting that the PGP-strains have the ability to completely counteract the growth inhibition induced by TNT.

To further test the effects of bacterial inoculation on plant fitness under TNT-stress, the activities of some enzymes involved in cellular defence against oxidative stress, general plant metabolism and plant damage were determined. The enzymes included superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST), and malate-dehydrogenase (MDH). Further, thiobarbituric acid (TBA) reactive metabolites, which are good indicators

Fig. 4 **a** *Stacked bars* show the concentrations of TNT and transformation products over time in the metabolically active resting cell culture of consortium CAP9 (OD_{600} = 1; comprising the strains BS7, EL1, ER9, ER18, ES2, ES4, R47, R54 and R59) in a solution containing 50 mM sodium phosphate buffer (pH 6.8), 20 mM carbon mix (7 mM glucose, 7 mM fructose, 3 mM acetic acid and 3 mM succinate) for NAD (P) H regeneration and 500 μM TNT as the sole nitrogen source. The picture shows the colour of the culture medium after 3 h (*brown-orange*) and 24 h (*yellow*). **b** Chromatograms (450 and 230 nm) obtained from ion-pair HPLC-analysis of the culture supernatant of CAP9 at time 0 and after 3 and 24 h. **c** Chromatograms (450 and 230 nm) obtained from ion-pair HPLC-analysis of the culture supernatant of control consortium CAP4 (OD_{600} = 1; consisting of the *Rhodococcus sp.* BS10, BS26, EL20 and R44) at time 48 h

Nitroaromatics (µM) / Time (h)		3	8	12	24	36	48
TNT		391.44	264.17	186.20	134.35	90.21	10.86
NB							1.66
2-ANT; 4-ANT		0.86	1.10	3.00	1.03	3.97	5.34
4-HADNT		13.50	20.60	57.27	56.56	23.50	22.19
2-ADNT; 4-ADNT		2.76	2.64	18.32	43.00	96.00	86.00
2,4-DANT				3.66		7.24	13.91
2,6-DANT						6.56	15.77
azoxytoluenes						3.60	6.78
azotoluenes							1.12

for membrane oxidation, were measured. SOD, GR and GST activity increased significantly (Fig. 5), while no significant changes or trends were observed for CAT and GPOD activity (data not shown). MDH activity was increased in the leaves of inoculated plants exposed to 25 mg kg^{-1} TNT compared with control plants and non-inoculated TNT-exposed plants (Fig. S6). SOD and GR activities were significantly increased in the leaves of all non-inoculated TNT exposed grasses compared to the non-inoculated controls (Fig. 5).

In contrast, for TNT-exposed grasses that were inoculated with bacteria, SOD-activity was significantly reduced in the leaves of all TNT-exposed plants in comparison with the non-inoculated TNT-exposed plants (50.2±7.5 U min^{-1} g^{-1} FW compared with 96.6±9.8, $p<0.02$; Fig. 5). GST activity was not altered after bacterial inoculation, but remained significantly higher than in both the roots and leafs of unexposed control plants. For example, the root GST activity in inoculated plants grown in 50 mg TNT kg^{-1} was 0.57±0.05 U min^{-1} g^{-1} FW compared to 0.28±0.03 ($p<0.02$) for unexposed control plants and in leaves 0.5±0.04 U min^{-1} g^{-1} FW compared to 0.27±0.01 ($p<0.02$).

The content of TBA-reactive metabolites in the leaves of the non-inoculated plants that were exposed to the highest TNT-concentration was about 50 % higher in comparison with the non-exposed controls (Fig. S6). TBA-reactive metabolites were only determined in the leaves because lipid-peroxidation and oxidative stress damage are related to TNT effects on the photosynthetic apparatus (Ali et al. 2006; Altamirano et al. 2004; Cruz-Uribe and Rorrer 2006; García-Villada et al. 2002). Interestingly, the TBA-reactive metabolites in the leaves of inoculated grasses exposed to 50 mg TNT kg^{-1} soil was significantly reduced, down to levels found in unexposed control plants, in contrast with the non-inoculated TNT-exposed plants (26.47±3.92 nmol TBA g^{-1} FW compared with 23.97±0.84; $p<0.03$) (Fig. S6). Of those plants grown without TNT, inoculation with CAP9 also increased biomass and tended to increase the GR activity in the leaves.

After 5 weeks $A.$ $capillaris$ growth, the soil was flushed with 200 ml of water and the leachate was collected for TNT-measurements. For the non-inoculated no-plant controls, 14.7±0.02 mg TNT kg^{-1} and 26.1±0.08 mg TNT kg^{-1} were detected in the leachate of the 25 and 50 mg TNT kg^{-1} conditions, respectively. In inoculated TNT-soils with growing $A.$ $capillaris$ plants, no TNT could be detected in the

leachate, a result that was similar to inoculated unsown TNT-soils, whereas in the non-inoculated soil with $A.$ $capillaris$, 4.88±0.35 mg kg^{-1} and 7.36±0.17 mg TNT kg^{-1} were detected for the 25 and 50 mg TNT kg^{-1} conditions, respectively. After flushing, soils were extracted in acetonitrile and analysed with HPLC. In the non-inoculated no-plant controls, 4.47±0.09 and 15.55±0.23 mg TNT kg^{-1} were detected in the soils contaminated with 25 and 50 mg TNT kg^{-1}, respectively. In contrast, only trace amounts of TNT were detected in the inoculated 25 mg TNT kg^{-1} spiked soil with $A.$ $capillaris$ (0.33±0.02 mg TNT kg^{-1} soil) and in the inoculated 50 mg TNT kg^{-1} spiked soil with $A.$ $capillaris$ (0.79±0.19 mg TNT kg^{-1}). In inoculated unsown pots, 1.43±0.11 and 7.41±0.03 mg TNT kg^{-1} were detected in the soils. Low concentrations of 2-amino-4,6-dinitrotoluene (<3 mg ADNTS kg^{-1}) were found in inoculated soils whereas this transformation product could not be detected (detection limit of <0.01 mg kg^{-1}) in non-inoculated soils.

SEM analysis of inoculated $A.$ $capillaris$ roots, collected after flushing at the end of the experiment, showed abundant bacterial adherence to the epidermal cells and on root hairs (Fig. S7a). It appeared that fewer bacterial cells were observed on the roots of non-inoculated plants. In order to better understand the active colonisation of the rhizosphere by some of the PGP-bacteria, small-scale hydroponic grass rooting experiments were performed in the presence or absence of $mCherry$-expressing $Stenotrophomonas$ $sp.$ EL1, or $egfp$-expressing $Pseudomonas$ $sp.$ R47 or $Pseudomonas$ $sp.$ R54. Confocal microscopy revealed the presence of EL1 adhering to the root surface (Fig. S7bI) and R47 colonising the epidermal cells (Fig. S7bII), thereby confirming their ability to effectively invade these niches.

Discussion

Analysis of both the cultured and uncultured bacterial communities associated with $A.$ $pseudoplatanus$ growing on a TNT-contaminated soil indicated a more similar community composition of the soil and rhizosphere niches compared to the endophytic habitats. The high niche complementarity of the rhizosphere is probably due to the relatively wide range of nutrients present in this niche, which promotes a high bacterial diversity

Fig. 5 Effect of grass inoculation with bacterial consortium CAP9 on plant biomass and oxidative stress related enzyme activities. *NIC*, Non-inoculated control; *InC*, Inoculated control, 25 and 50 = the non-inoculated TNT-exposed *Agrostis* grasses to 25 and 50 mg kg^{-1} TNT, respectively; In25 and In50 = TNT-exposed and inoculated grasses. **a** Representative pictures of 6 week-old grasses compared to non-inoculated conditions, 3 weeks after bacterial inoculation in treated plants. **b** Picture of the root-biomass of 5-week old 50 mg kg^{-1} TNT-exposed plant inoculated with bacteria. **c**. Histograms showing the root and leaf biomass and the physiological enzyme activity parameters in plants grown with or without TNT 3 weeks after inoculation. Leaf responses are shown in *green* (*white* for the control) and root responses are shown in *brown* (*grey* for the control). Data from inoculated plants are shown in *hatched bars*. *SOD*, Superoxide dismutase, *GR*, Glutathione reductase, and *GST*, Glutathione-S-transferase. Different letters indicate significant differences in the leaves (*p*<0.05) and different numerical digits indicate significant differences in the root (*p*<0.05) as determined using ANOVA and the Tukey-HSD test. The data reported in the graphs are representative of 10 biological replicate syringes for each condition
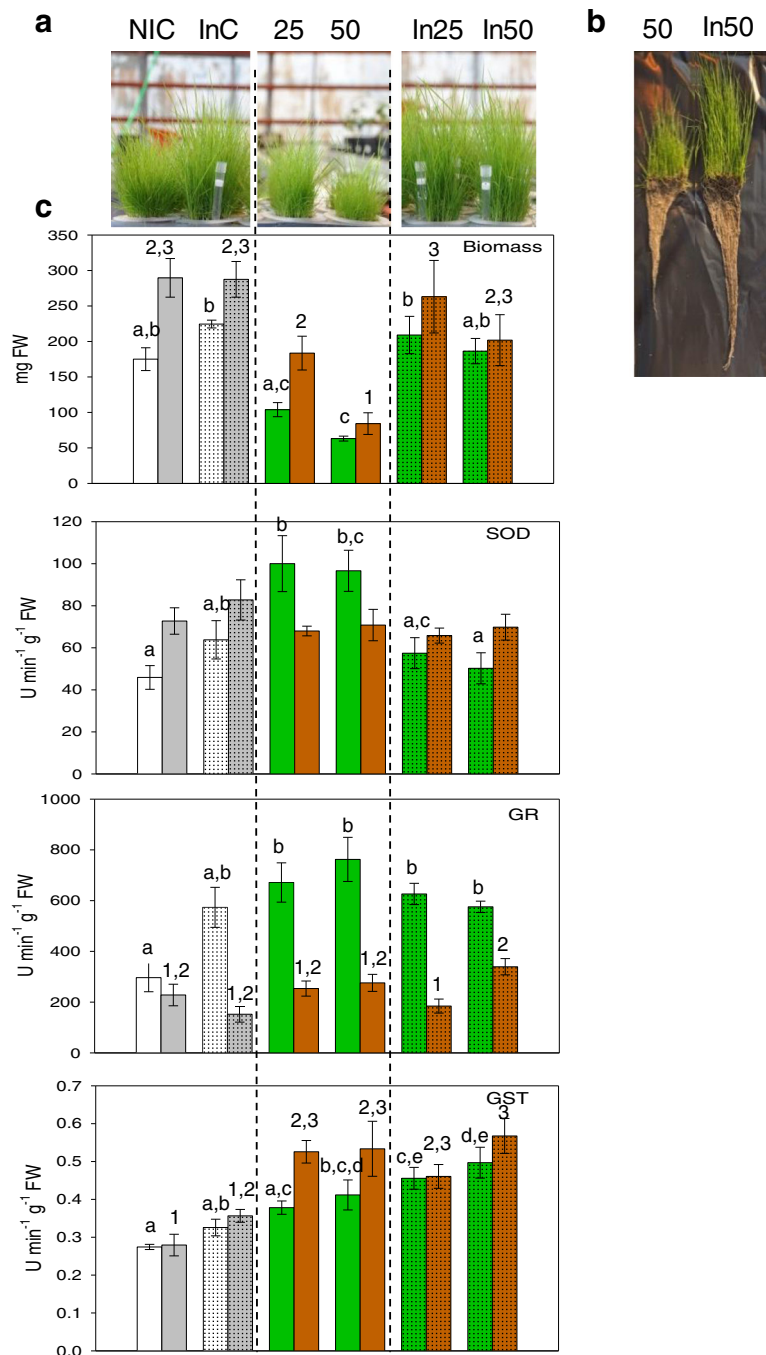
(Badri et al. 2009; Loreau et al. 2001). The dominant cultivable bacterial classes in the bulk soil, rhizosphere and root endosphere were the Beta- and Gamma-proteobacteria and Actinobacteria, whereas the shoot endosphere was dominated by Alphaproteobacteria, and the leaf endosphere by Actinobacteria and Gammaproteobacteria, with many *Stenotrophomonas* and *Pseudomonas* isolates. The dominance of the genera *Cupriavidus*, *Rhodococcus* and *Pseudomonas* in the soil is interesting, as members of these genera have been described as nitro aromatic degraders (Esteve-Nuñez et al. 2000; Nishino et al. 2000, Nishino et al. 1999;

Rylott et al. 2011b; Snellinx et al. 2003; Spanggord et al. 1991). In particular, *Pseudomonas* spp. are often preferentially enriched in TNT-contaminated soils, because of selection of fast-growing r-strategists (Travis et al. 2008). The high abundance of Methylobacteria in the endophytic compartment of *Acer* is also worth noting, as a TNT-transforming *Methylobacterium* was previously isolated from poplar tissue cultures (Van Aken et al. 2004). The dominant cultivable bacteria found in our study are consistent with previous observations of the endophytic bacterial communities of oak and ash trees growing on TCE-contaminated soil (Weyens et al. 2009a), as well as with the endophytic bacteria found in poplar trees (Taghavi et al. 2009). So far, no other studies are known that describe the rhizospheric and endophytic bacterial communities of *Acer* trees.

The complete collection of morphologically different isolates, *i.e.* 150 strains, were tested for the production of plant growth promoting compounds including phytohormones and factors related to nutrient solubilisation. We observed that the highest bacterial PGP-activity was associated with the rhizosphere and the endosphere compared to the bulk soil. As the *Acer* tree was growing in a TNT-contaminated and sandy-loam soil poor in nutrients, we postulated that the associated endophytes would be enriched in stress-hormone regulating functions and have high nutrient acquisition capabilities. Indeed, most of the endophytes demonstrated ACC-deaminase activity, and siderophore production was high in strains isolated from R and ER. A selection of bacteria isolated from the *Acer* tree was additionally screened for the production of plant growth promoting compounds and for the metabolism of plant growth regulating products and nitrite. A cluster of *Pseudomonas* spp. showed the highest PGP-scores, a result that was not completely unexpected because Pseudomonads are metabolically versatile, and many strains belonging to this genus have been described in the literature for their role in enhancing plant growth by inducing biocontrol activities, chelating available iron and by stimulating root growth through ACC-deaminase activity (Grichko and Glick 2001; Kloepper et al. 1980; Patten and Glick 2002; Preston 2004; Taghavi et al. 2009). A large proportion of the isolates described here were able to significantly stimulate the root growth of *A. thaliana* plants under TNT-stress *in vitro*. Most of these strains rapidly transformed TNT in liquid cultures, however *Rhodococcus* sp. BS10 and EL20 did not transform TNT efficiently and, in fact,

TNT completely inhibited their growth. Possible explanations for the sensitivity of Gram-positive bacteria to TNT have been suggested by Fuller and Manning (1997) as the result in higher intracellular TNT concentrations and toxicity. These include the hypothesis that: (1) the cell-wall of Gram-positive bacteria may be more permeable to TNT, (2) Gram-positive bacteria may possess fewer active extrusion transport systems, and (3) Gram-positive bacteria may possess fewer enzymes that detoxify TNT (Fuller and Manning 1997).

More promising were the Gram-negative bacteria, including the *Pseudomonas* spp. (ER9, R47, R54, R59), *Stenotrophomonas* (EL1), *Cupriavidus* (BS7), *Methylobacterium* (ES4) and *Variovorax* (ER18), strains which showed TNT-transformation potential. Previously, bacteria such as *Methylobacterium*, *Pseudomonas* and *Enterobacter* have been described as being able to transform TNT (Esteve-Núñez et al. 2001, Esteve-Nuñez et al. 2000; French et al. 1998; Fuller and Manning 1997; Haidour and Ramos 1996; Ramos et al. 1996, Ramos et al. 2005; Rylott et al. 2011b; Van Aken et al. 2004; van Dillewijn et al. 2008b; Wittich et al. 2008). Here, for the first time we show that a leaf endophyte, *Stenotrophomonas chelatiphagus* (EL1,) and a root endophyte, *Variovorax ginsengisoli* (ER18), can quickly transform TNT into hydroxylamino- and amino-dinitrotoluene metabolites. All of the beneficial strains found in this study were used to create the CAP9 consortium. HPLC-analysis showed that TNT was rapidly reduced by this consortium, with the formation of hydroxylamine derivatives and small amounts of Meisenheimer products. Typically, the reduction of TNT yields HADNTs and Meisenheimer dihydride complexes which upon condensation, yields stoichiometric amounts of diarylamines and nitrite, with nitrite being subsequently reduced to ammonium and incorporated into the biomass (Gonzalez-Perez et al. 2007; van Dillewijn et al. 2008c; Wittich et al. 2008). Alternatively, TNT can also be used as an N-source after the reduction of a nitro group on the ring to its hydroxylamine derivative, which upon a Bamberger-like reaction could form intermediates which promote the release of ammonium (Caballero and Ramos 2006). The reduction of a nitro group to HADNTS is typically catalyzed by nitro reductases (Caballero and Ramos 2006; French et al. 1998). Here, the rapid TNT-degradation observed in consortium CAP9 was most likely due to high nitro reductase activity as measured in the cell-free enzyme

assay (Table S3). The detection of Meisenheimer products in this study can most likely be attributed to the presence of Old Yellow Enzyme (OYE) genes, which were subsequently cloned from *Pseudomonas* sp. strains ER9, R47 and R59, *Stenotrophomonas* sp. EL1, and *Variovorax* sp. ER18. The functionality of various type II hydride transferases of the OYE family of flavoproteins to reduce TNT such as XenB, N-ethylmaleimide (NEM) and pentaerythritol tetranitrate (PETN) has been thoroughly described (French et al. 1998; van Dillewijn et al. 2008b; c; Williams et al. 2004; Wittich et al. 2008). Here, the prevalence of OYE-like genes in *Acer pseudoplatanus* tree endophytic and rhizospheric bacteria is promising, especially since many of the strains are also beneficial PGP-endophytes. Phylogenetic analyses revealed a high sequence similarity of *P. vranovensis* R59 and *P. vranovensis* R47 OYE genes to the *xenB* gene in *P. putida* KT2440. The partial OYE sequence from *Stenotrophomonas* EL1 and *Variovorax ginsengisoli* ER18 were not as closely related to sequences in the database as those of the *Pseudomonas* spp. Additional experiments are needed to confirm whether these Old Yellow Enzyme family members have type II hydride transferase activity, and if these enzymes have novel sequences and specificities. Ultimately, we demonstrated that inoculation of consortium CAP9 significantly increased the biomass of TNT-exposed *Agrostis* plants and that the oxidative damage (TBA-reactive metabolite content) caused by TNT was prevented by the beneficial bacteria. The bent grass *A. capillaris* was selected to investigate whether our bacterial consortium is not limited to *Acer* as its host-plant because *Agrostis* is widespread at many military sites and this grass is characterized as providing dense coverage and a fibrous and deep root-system that stimulates rhizosphere microorganisms. Here we noticed that inoculation with CAP9 appeared to diminish TNT-induced stress as indicated by significantly reduced SOD- and GR-activity. We deduce that this benefit is provided by the bacteria through a combination of rapid TNT-transformation in the rhizosphere together with their PGP-properties. GST-activity was also increased in the leaves and roots of TNT-exposed plants and this is in accordance with other studies showing that GST-transcripts increase in plants as a result of TNT-treatment (Brentner et al. 2008; Gunning et al. 2014). No TNT could be detected in the water-leachate of the inoculated conditions with *A. capillaris* and only trace concentrations of TNT and amino-reduction products

were detected in the soil at the end of the experiment, suggesting that the bioaugmented rhizoremediation resulted in enhanced TNT-transformation.

Zhu et al. (2012) have recently shown that *A. thaliana* plants overexpressing an OYE have an increased TNT-tolerance and capacity to remove TNT from liquid culture. Previously, over-expression of the OYE orthologue in *A. thaliana*, oxophytodienoate reductases (OPRs), also resulted in increased TNT tolerance and capacity to remove TNT from liquid culture (Beynon et al. 2009). Similar findings have been described for tobacco plants and aspen overexpressing bacterial nitro reductases (Hannink et al. 2001; van Dillewijn et al. 2008a). Our results indicate that inoculating a field with a specific bacterial consortium harbouring PGP-traits and OYE genes is suitable for detoxifying TNT and eliminates the need to construct and release genetically modified (GM) plants or microorganisms. Moreover, since TNT accumulates in the root-zone of TNT-exposed plants (Brentner et al. 2010), the presence of TNT-transformation genes in rhizosphere and root-endophytic bacterial communities is advantageous. Many studies demonstrate the significant effect of mineralisation and transformation of organic compounds at the root-soil interface (Chaudhry et al. 2005; Kuiper et al. 2004; Van Dillewijn 2008). The increase in microbial density, diversity and metabolic activity due to the release of root-exudates, the modification of the physical and chemical properties of the soil by the plant, together with the active recruitment by plants of bacteria expressing degradation genes all contribute to this rhizosphere effect (Siciliano et al. 2001). Moreover, the bacterial consortium CAP9 is highly rhizosphere competent, as shown by the ability of a collection of fluorescent tagged strains to efficiently colonise the root niche.

Although bacterial transformation of TNT did not lead to complete degradation (*i.e.* mineralisation), recalcitrant reduction metabolites are significantly less toxic than TNT (Keith and Telliard 1979) and are likely to bind to soil particles and humic acids, or to conjugate with organic molecules, thereby reducing their bioavailability and toxicity. Since the strains of the consortium belong to widespread bacterial genera, *i.e.* *Pseudomonas*, *Methylobacterium*, *Stenotrophomonas* and *Cupriavidus*, their involvement in TNT rhizoremediation is likely to be of significant ecological importance. In addition, not many studies have demonstrated the beneficial action of TNT-degrading, PGP

producing and efficient root-colonising bacteria of a consortium in the rhizosphere of grass.

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