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review

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Potential of the *Galega – Rhizobium galegae* System for Bioremediation of Oil-Contaminated Soil

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Summary

Bioremediation potential of the nitrogen-fixing leguminous plant *Galega orientalis* Lam. and its microsymbiont *Rhizobium galegae* was evaluated in microcosm and mesocosm scale in oil and BTEX (benzene, toluene, ethylbenzene, xylene) contaminated soils, with *m*-toluate serving as a model for the latter group. *G. orientalis* and *Rhizobium galegae* remained viable in *m*-toluate fractions up to 3000 ppm. Plant growth and nodulation were inhibited in 500 ppm *m*-toluate, but were restored when plants were transferred to clean medium. In soil, *G. orientalis* nodulated and showed good growth in 2000 ppm *m*-toluate as well as in diesel-contaminated soil in the field, where the plant was stimulating bacterial growth in the rhizosphere. A collection of 52 indigenous *m*-toluate-tolerating bacteria isolated from oil-contaminated rhizosphere of *G. orientalis* was characterised and identified by classical and molecular biological methods. 16SrDNA PCR-RFLP and $(GTG)_5$-PCR genomic fingerprinting combined with partial sequencing indicated the presence of five major lineages of the Bacteria domain. A TOL plasmid-specific *xylE*-PCR was developed in order to detect both active and potential degraders of *m*-toluate. The ability to degrade *m*-toluate in the presence of the gene *xylE* was detected only within the genus *Pseudomonas*. The isolates were tested for capacity to grow on *m*-toluate as their sole carbon and energy source. In laboratory experiments, the best rhizosphere isolates performed equally well to the positive control strain and are good candidates for inoculant production in the future. They have been tagged with marker genes for further studies on colonisation and persistence.

Key words: *Galega orientalis*, *Rhizobium galegae*, bioremediation, oil-contaminated soil, *m*-toluate tolerating bacteria

Introduction

Bioremediation is the use of living organisms for removal of contaminants from the environment. The bioremediation process takes place in the open environment in the presence of indigenous organisms. The process includes the removal of contaminants by uptake or metabolism. Bioremediation by using the metabolic capacity of indigenous microorganisms for mineralisation of contaminating chemicals is the most common type of bioremediation. Microorganisms are the most abundant and most genetically diverse living organisms adapted to almost all environments that exist on the earth. Their ability to utilise a vast amount of carbon

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compounds as sources of energy and carbon allows the exploitation of bacteria for degradation of organic pollutants. Intrinsic degrading populations exist even in pristine soils, which shows the wide metabolic diversity of bacteria and indicates the potential of microbes as tools of bioremediation.

Nutrients, such as nitrogen and phosphorus, are often added to speed up the process by providing compounds necessary for microbial growth (biostimulation). If no nutrients are added, but intrinsic microorganisms do the work, we talk about intrinsic bioremediation. If metabolically suitable microorganisms are added to the site by inoculation, the term bioaugmentation is often used. When plants are used to take up contaminants, we talk about phytoremediation. This report deals with the use of the rhizosphere of leguminous plants to speed up the bioremediation process. Microorgansims present in the rhizosphere are metabolically stimulated by plant exudate and organisms capable of metabolising the contaminants are enriched. This novel application can also be called phytoremediation or rhizoremediation.

Bioremediation has successfully been utilised for the cleaning of soils, groundwater and coastal waters. For coastal oil spills bioremediation has shown great potential (*1–3*). Under anaerobic conditions, such as in deep groundwater, anaerobic metabolism of hydrocarbons and chlorinated solvents has been reported to take place (reviewed in ref. *3*). For instance, *Geobacter* sp. has been proposed to be involved in anaerobic oxidation of benzene (3). Tiirola *et al*. (*4*) reported that bioremediation of polychlorophenols in a high-rate fluidised bed bioreactor system took place in the presence of bacteria representing several major lineages, with a novel isolate of the new genus *Novosphingobium* sp. as one of the dominant strains in the degradation process. Recently Daane *et al*. (*5*) described the isolation of PAH-degrading organisms and a new species, *Paenibacillus naphtalovorans*, capable of degrading naphthalene in the rhizosphere of salt marsh plants, and already in 1995 Radwan *et al*. (*6*) showed that plant rhizospheres had the potential to clean oil spills. The benefits of bioaugmentation have not been unanimously proven. Hozumi *et al*. (*2*) reported that a commercial inoculant improved oil bioremediation when contaminated coastal soil was transferred to the laboratory for testing. Margesin (*7*) found that even alpine soil had a good oil bioremediation potential after biostimulation.

Monitoring of the degrading organisms and population changes is an important part of monitoring the bioremediation process. DGGE (denaturing gradient gel electrophoresis, in which gene fragments, often 16S rDNA, amplified by PCR, are separated in the gel based on the differential mobility of bands with differing base composition) and sequencing of major bands of 16S ribosomal genes is a commonly used procedure. Tiirola (*8*) used LH-PCR (length-heterogeneity PCR, in which amplified 16S gene fragments are sorted by capillary electrophoresis and fragment analysis by size), thus avoiding problems with gels, which occur in DGGE. Many of the organisms involved in bioremediation still await genetic and metabolic identification.

Potential of *Galega orientalis* for Bioremediation

The possibility that microbial communities in the rhizosphere are involved in the protection of plants from chemical injury is an issue discussed in the literature of microbial degradation of xenobiotics (*6,9*). *Sinorhizobium meliloti* strain Orange 1 is the first reported nodule-forming strain capable of degrading dibenzothiophene (*10*). It is the first example of a symbiotic *Rhizobium*, which has a metabolic pathway mechanism analogous to that described for naphthalene degradation in other bacteria.

Several thousand oil-contaminated sites in Finland are in need of remediation. Various techniques for soil cleanup are in use; the quality, amount and concentration of the pollutant determining the choice of application. On polluted sites with relatively low contamination level, the costs of the most efficient cleaning techniques exceed the benefit. Thus, more cost-effective clean-up methods for soil are needed.

The rhizosphere approach applied in our project is based on the optimisation of the environmental setting on the contaminated site by the introduction of leguminous plants that are able to enhance overall bacterial activities in soil. The rhizosphere influence includes general improvement of soil structure and aeration through root growth. The whole soil ecosystem is activated due to the secretion of nutrient compounds from roots and biologically fixed nitrogen derived from root nodule bacteria (Fig. 1).

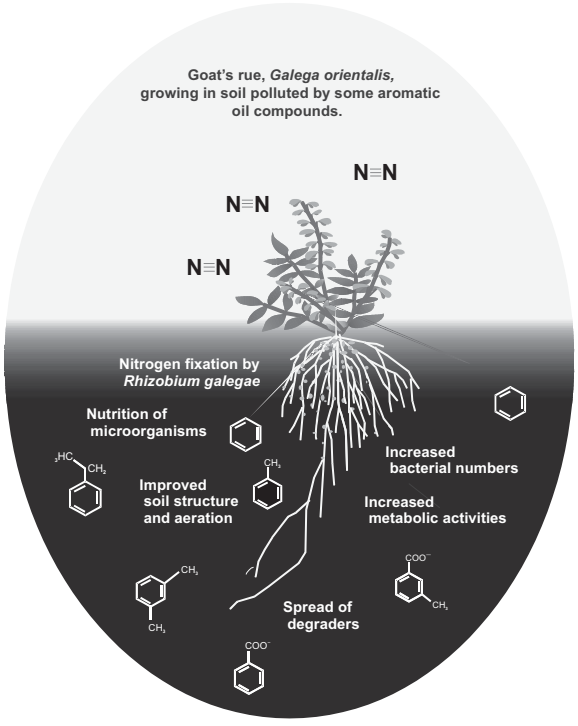

Fig. 1. Phytoremediation idea

Galega orientalis is a perennial forage legume that originates in the Caucasus. It forms a very specific symbiosis with *Rhizobium galegae* (*11*). The cultivation prac-

tice for the plant has been optimised and it is especially used in ecological cropping systems. It is a good bee plant and has got pretty purple flowers, and is thus suitable for landscaping purposes. Since it overwinters with the aid of underground stolons, it is not susceptible to attack by pathogenic fungi in the space between the ground and the snow, and will survive for several years in a northern temperate climate. Its root system is well developed and the roots will penetrate to at least one meter below ground (*12*). For these reasons we selected *G. orientalis* to test its potential for rhizoremediation.

Oil contains different types of carbon compounds: aliphatic, aromatic and polycyclic aromatic. For studies of oil bioremediation we chose *m*-toluate (3-methylbenzene) as the model compound for oil contamination, because the genes and pathways for its metabolism are well known (Fig. 2). Genes for breaking down *m*-toluate and other compounds of the BTEX complex (benzene, toluene, ethylbenzene and xylene) often reside on a TOL plasmid, which is transferable between bacteria. The TOL-plasmid was originally detected in *Pseudomonas* sp. (*13,14*).

ppm. Although the roots were stunned and branched when grown in *m*-toluate, most of the plants were viable and when transferred into uncontaminated media, half of the plants began to grow normally, and nodules were developed on the new lateral roots within three weeks. In coculture with the *P. putida* strain, *m*-toluate was removed by the metabolic activity of the strain and the plant and symbiosis functioned well (*15*).

Next, the system was tested in mesocosm with 2000 ppm *m*-toluate- or oil-contaminated soil and clean soil as a control. The assay was carried out in the greenhouse with the following treatments for all soil types: (*i*) *G. orientalis* with rhizobia, (*ii*) *G. orientalis* without rhizobia, (*iii*) *G. orientalis* with rhizobia and *Pseudomonas putida PaW85* (pWW0), and (*iv*) bulk soil with no inoculant. After the first reactions the roots grew normally producing a strong, branched root system reaching into the contaminated soil layer. The root structure varied in different soil types. In oil soil the roots first spread mostly laterally in a normal growth mode, while *m*-toluate caused strong root branching similar to that observed in the microcosm. Nodulation occurred normally in all soil types in the pots inoculated with rhizobia, and

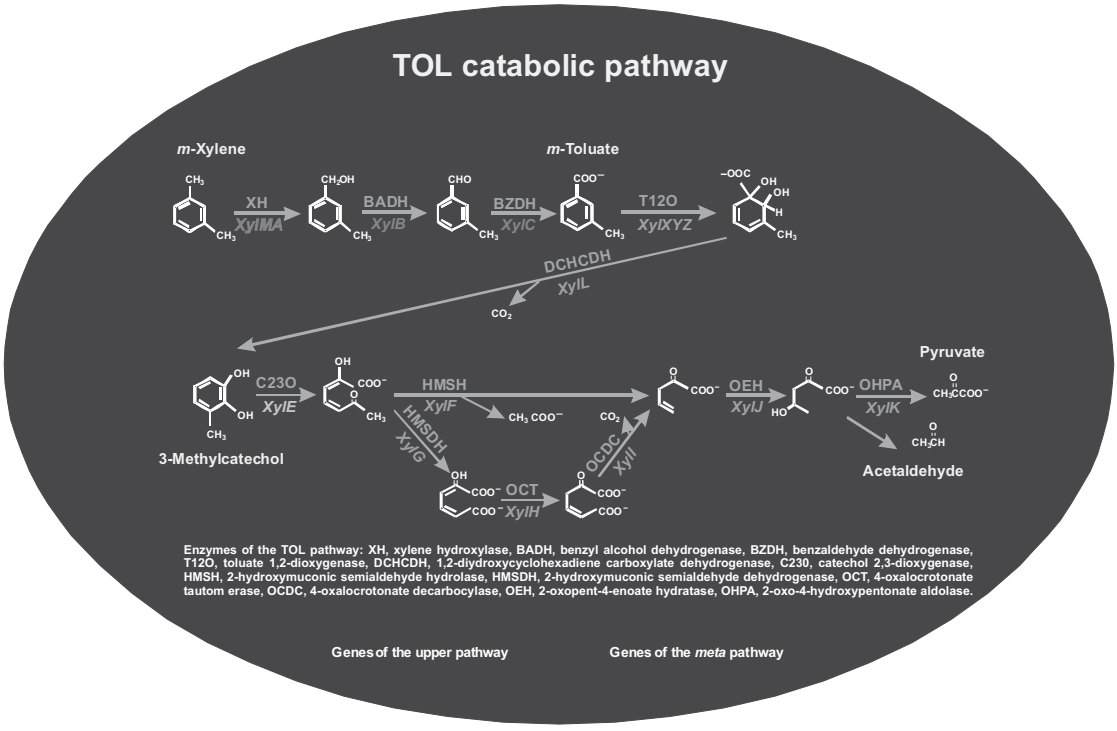

Fig. 2. TOL catabolic pathway. The upper pathway on top and the *meta* pathway below

In microcosm *Rhizobium galegae* strains were able to grow in medium containing 2000 ppm *m*-toluate. *Pseudomonas putida* strain PaW85, which carries the TOL plasmid pWW0, was able to grow in 5000 ppm *m*-toluate, which was the highest concentration tested (*15*).

The germination rates of *G. orientalis* seeds decreased with increasing *m*-toluate concentrations. The *m*-toluate fraction higher than 500 ppm inhibited plant growth and root development, but the presence of *P. putida* inoculant increased the tolerance level up to 1000

the acetylene reduction assay showed that all plants in each treatment were able to fix nitrogen. Plant yields were equal in all soils with *R. galegae* inoculation, but when *P. putida* was applied additionally, yields were slighly lower than without the bioaugmentation agent. Thus, it seems that the indigenous rhizosphere bacteria were capable of growth and soil improvement and that the *Pseudomonas* inoculant was not necessary for bioremediation but may have had a slightly detrimental effect on the plant (*15*).

In a field-release experiment with genetically marked *R. galegae*, inoculated *G. orientalis* was planted in silt loam with or without diesel oil contamination (3000 ppm). No bioaugmentation bacteria were added. No effect on plant growth or marker gene stability was detected at that contamination level.

Effect of the Plant on Rhizosphere Organisms

To study the effect of the plant on bacterial numbers in the rhizosphere, lysimeters were placed in a compost field used to treat polluted soil samples. Gravel was put on the bottom of the lysimeters, which were then filled with oil-polluted soil and non-nitrogenous peat on top. The experimental variables were *G. orientalis* inoculated with *R. galegae*, or *G. orientalis* inoculated with *R. galegae* and *P. putida* (pWW0), *P. putida* only and no treatment. After one growing season (5 months) soil samples were taken and bacterial counts made on tryptone-yeast extract (TY) medium with and without *m*-toluate (4 600 mg/L).

Total counts on TY-medium were 3–15 times higher than the counts on *m*-toluate containing media (max. $5x10^7$/g). The composite sample obtained from the soil treated with both *G. orientalis* and *P. putida* had the highest bacterial density. Also the bacterial densities of composite samples obtained from soils treated with *G. orientalis* or *P. putida* were higher than those of the composite sample from the untreated control soil. Both *G. orientalis* (ANOVA: p-value < 0.001) and *P. putida* (ANOVA: p-value < 0.01) increased significantly the bacterial density of the composite samples as determined both on TY- and *m*-toluate containing TY-media.

Also in other cases plant rhizospheres have been found to increase the bacterial concentrations (*16*,*17*), but the observed effects of bioaugmentation have partly been contradictory (*18*). In this case possible reasons for the stimulating effect of *P. putida* may be *e.g.* (*i*) good adaptability of the strain to the oil contaminated soil in the lysimeters and the rhizosphere of *G. orientalis*, or (*ii*) passing of the TOL-plasmid of the *P. putida* strain into other bacterial cells, *e.g.* in conjugation, and the multiplication of these bacteria with metabolic adaptation. *G. orientalis* may have contributed to the increase by offering surfaces for bacterial interactions and by distributing *P. putida* strain with the growing roots (*19*). This hypothesis is supported by the fact that the bacterial numbers were highest in the *Galega – Pseudomonas* composite sample. *P. putida* may also have protected the plant against damages caused by soil contaminants and facilitated the plant growth by degrading oil compounds (*9*,*20*).

Almost all the isolates grew on TY-agar containing 5000 mg/L *m*-toluate. Over 95 % of the isolates grew in concentration of 7000 mg/L and over 50 % in concentration of 9000 mg/L. When isolates were grown on TY-media without *m*-toluate and thereafter transferred again to *m*-toluate containing TY-media, most of the isolates did not tolerate as high concentration of *m*-toluate as before. This may be due to the loss of possible plasmid containing tolerance and/or degradation genes. One of the criteria by which the degradation of *m*- and *p*-toluate by *P. putida* (arvilla) mt-2 was originally judged to be plasmid-specified was the loss of function at efficiencies higher than those normally found for mutations (*21*).

Only about 10 % of 208 tested isolates grew with *m*-toluate as their sole carbon source. Thus, most of the isolates only tolerated but did not degraded *m*-toluate or degraded it only cometabolically in the presence of easier substrates.

Genetic Diversity of Rhizosphere Microorganisms

M. M. Jussila, L. Suominen and K. Lindström, after preliminary screening of our collection of about 400 isolates from different experiments with oil-contaminated rhizosphere of *Galega orientalis*, selected 52 strains for further characterisation by classical and molecular biological methods (submitted for publication). The phylogenetic diversity was indicated by the presence of five major lineages of the Bacteria domain, with Gram-positive bacteria as the most dominating group. A TOL plasmid-specific *xylE*-PCR was developed in order to detect both active and potential degraders of *m*-toluate. The genetic diversity was indicated by cluster analysis of the data, revealing thirteen 16S rDNA ribotypes and 23 $(GTG)_5$-genotypes (rep-PCR) among various bacterial isolates ranging from similar strains to different genera. Generally, 16S-ribotype and $(GTG)_5$-genotype corresponded very well to each other and grouped the strains at the species level. 16S rDNA PCR-RFLP ribotyping and $(GTG)_5$-PCR genomic fingerprinting methods combined with partial sequencing of 16S rRNA genes of representatives of the main clusters were used to construct a reference dendrogram in order to rapidly group and search for new and interesting bacterial species from oil-contaminated rhizosphere later.

PCR primers were designed for amplification of the *xylE* gene of the *meta* pathway for toluene degradation (Fig. 3). The *xyl*E-PCR detected TOL plasmid *xylE* genes only within the genus *Pseudomonas*, indicating the use of the plasmid born *meta* pathway for *m*-toluate degradation. All strains possessing *xylE* were catechol positive in the biochemical testing of 2-hydroxymuconic semialdehyde production. A few strains were *xylE* negative but could utilise *m*-toluate, suggesting that the chromosomal *ortho* pathway was operating during degradation. As in the field study, only a proportion of the strains that tolerated *m*-toluate in the medium could use it as their sole carbon and energy source.

Our attempts to amplify the genes *nahAc* from the *nah* operon of the NAH plasmid (*22*) in PCR were successful in only one case, indicating that strains isolated on *m*-toluate were adapted to it and not to naphthalene degradation. The OCT plasmid has been found to carry genes needed for utilisation of alkanes with intermediate chain length (*23*). PCR primers designed for amplification of *alkB* from this plasmid detected this gene in one isolate. Thus, the potential for degradation of other oil compounds exists in our rhizosphere isolates and the appropriate selective media should be applied to isolate them in pure culture.

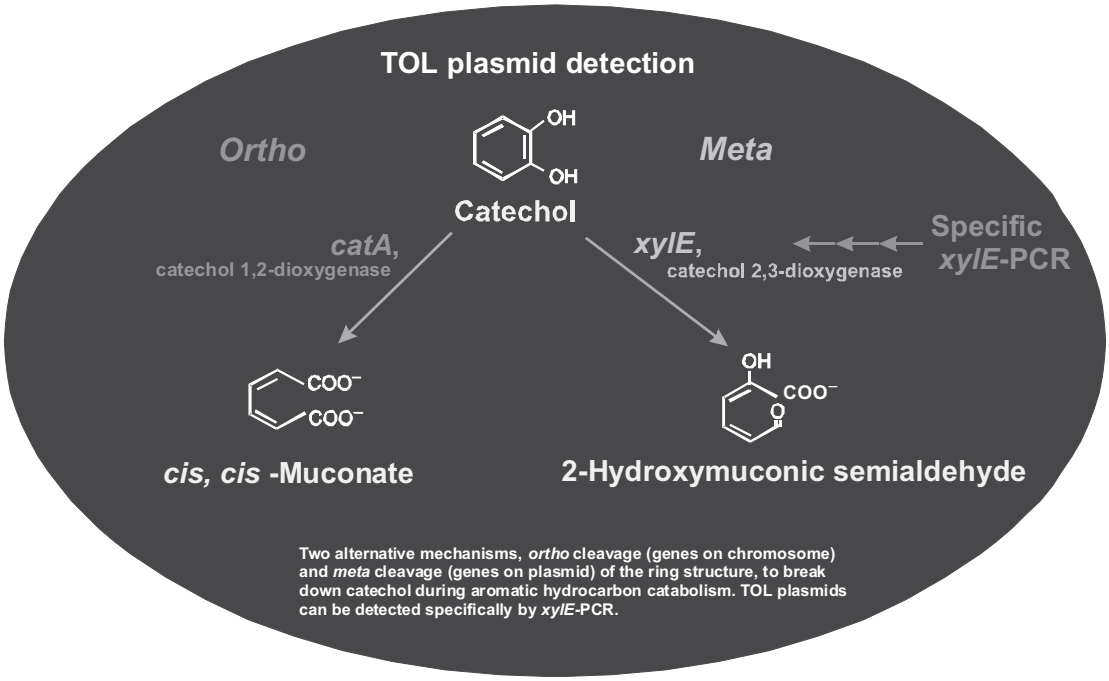

Fig. 3. Cathecol oxidation in *ortho* and *meta* pathways during toluene degradation. The *xylE* gene product oxidises cathecol to a yellow product that can be detected by adding cathecol to a bacterial colony

Development of Inoculants

In order to develop inoculants suitable for bioremediation with *Galega orientalis* rhizosphere, we screened *xylE* positive isolates for their capacity to use *m*-toluate as their sole carbon and energy source and remove the compound from liquid growth medium. A few strains were comparable to the model *P. putida* strain with the TOL plasmid pWW0. These strains have been geneticall-ly marked and will be tested for colonisation ability and effect on plant growth. Since these strains were isolated from the *G. orientalis* rhizosphere they should be better adapted to the plant than the model strain.

Conclusions

Galega orientalis is suitable for bioremediation of oil-contaminated soils, since it tolerates moderate degrees of oil contamination and is able to recover after heavier *m*-toluate exposure, when transferred into clean medium or when the *m*-toluate is removed by bacterial degradation.

The plant stimulated growth of rhizosphere bacteria and bacteria capable of metabolising *m*-toluate (total counts on TY and *m*-toluate amended TY) when planted in oil-contaminated soil.

Pseudomonas putida strain carrying the TOL plasmid pWW0 was a good soil and rhizosphere coloniser in a field experiment with oil soil, but had a slightly negative effect on plant growth in the greenhouse experiment.

Pure cultures of rhizosphere bacteria isolated from *G. orientalis* rhizospheres in oil and *m*-toluate amended soils on media containing *m*-toluate displayed great genetic diversity. Only about 10 % of the isolates could use *m*-toluate as their sole carbon and energy source. These strains belonged to the genus *Pseudomonas* and carried the TOL plasmid specific *xylE* gene. A few Gram-positive strains metabolised *m*-toluate, but *xylE* was not detected by PCR, indicating the absence of the *meta* pathway for toluene metabolism and the use of the chromosomal *ortho* pathway instead. Genes indicating a potential for alkane and naphthalene degradation were scarce in the strains isolated with *m*-toluate as the selective agent.

Strains with good capacity to use and remove *m*-toluate in liquid medium have been genetically marked for colonisation studies. Good colonisers with stable degradation properties may have a potential as inoculants for bioaugmentation in cold temperate climate. Bacterial isolates often lose their capacity to grow on *m*-toluate or utilise it during purification and storage. This might be due to instability of degradation plasmids, a preference of the organisms to live in consortia. Since most strains only tolerated *m*-toluate but did not use it as their sole carbon and energy source, they might metabolise it co-metabolically.

The result of bioremediation – removal of the contaminants – should be confirmed by chemical analyses. However, to obtain good results, analytical methods must be developed for fast and accurate screening of soil samples.

Acknowledgements

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Ispitivanje sustava *Galega-Rhizobium galegae* za biološko pročišćavanje uljem zagađenih tala

Sažetak

Ispitana je mogućnost biološkog pročišćavanja tala, onečišćenih uljem ili smjesom benzena, toluena, etilbenzena i ksilena (BTEX) pomoću leguminozne biljke *Galega orientalis* Lam. koja veže dušik i njezinog mikrosimbionta *Rhizobium galegae* u mikro- i mezokozmotskom mjerilu. Kao model za BTEX poslužio je *m*-toluat. *G. orientalis* i *R. galegae* preživjeli su udjele *m*-toluata do 3000 ppm. Rast biljke i stvaranje čvorića inhibirano je pri udjelu od 500 ppm *m*-toluata, ali se ponovno obnovilo kada su biljke prenesene u čisti medij. U zemlji je *G. orientalis* stvarala čvoriće i pokazivala dobar rast pri 2000 ppm *m*-toluata, kao i na poljima sa zemljom onečišćenom dizelskim uljima gdje je biljka stimulirala bakterijski rast u rizosferi. Klasičnim i molekularno-biološkim postupcima karakterizirane su i identificirane 52 autohtone bakterije tolerantne na *m*-toluat, koje su bile izolirane iz uljem onečišćene rizosfere *G. orientalis*. Postupcima 16S rDNA PCR-RFLP i genomskim postupkom $(GTG)_5$-PCR, povezanim s djelomičnim sekvenciranjem, dokazana je prisutnost 5 glavnih grana u području Bacteria. Za otkrivanje aktivnih i potencijalnih razgrađivača *m*-toluata razrađen je TOL plazmidno-specifični *xylE*-PCR. Sposobnost razgradnje *m*-toluata, u prisutnosti gena *xylE*, otkrivena je samo unutar roda *Pseudomonas*. Ispitivana je sposobnost izolata da rastu na *m*-toluatu kao jedinom izvoru ugljika i energije. U laboratorijskim pokusima najbolji izolati rizosfere ponašali su se jednako dobro kao i oni s kontrolnim sojem i dobar su potencijalni izvor za buduću proizvodnju cjepiva. Oni su bili označeni s genima markerima za daljnje ispitivanje mogućnosti razmnožavanja i njihove otpornosti.