Abstract. Technical methods of purification of large areas of low and medium pollution are powerful, but extremely difficult to apply on a wide scale. This is due to high costs and the need to have specialised equipment during remediation. Phytoremediation is a much less complicated method. This environment cleaning technology uses the above-average capacity of some plant species to accumulate (so-called hyper-accumulation) or metabolise toxic chemicals. Soil microorganisms living in the rhizosphere also play an invaluable role in the degradation of harmful organic compounds; they are often much more involved in the mineralisation of xenobiotics than plants. Since plants provide favourable conditions for soil microorganisms to live – specific cooperation between them is possible. This kind of relationship can be useful in very effective removal of many toxic organic compounds, such as pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons and other petroleum compounds, from the soil. Although this process is relatively slow compared to other methods, its low invasiveness and economic considerations make it worthwhile. Currently, attempts at improvement of the natural process of phytoremediation using genetic engineering are undertaken more and more often. Among other things, genes encoding cytochromes from other organisms are implanted into the plant genome. This idea is constantly being developed and the results of research that is more and more widely conducted in this area are promising.

Keywords: organic xenobiotics, detoxification, phytoremediation, cytochrome P450, glutathione S-transferases
INTRODUCTION

The ecological risk is caused by contamination of the environment when bio-available pollution can enter into the food chain, thus posing threat to animals and humans. Removal of contaminations or their stabilisation is called remediation, in which physicochemical and biological methods are applied. In the case of remediation of extensive areas with low and medium pollution, technical methods of their purification are rarely recommended. This is due to the high costs of such treatments and the need to have a highly specialised technical base.

Phytoremediation is a much less complicated method of removing xenobiotics from contaminated sites. It is a technology for cleaning of the environment (soil, groundwater and surface water, waste sediments and air) which takes advantage of the above-average capacity of some plant species to accumulate or metabolise toxic chemicals. Soil microorganisms play an invaluable role in the degradation of harmful organic compounds. They live in the rhizosphere and often participate in the mineralisation of xenobiotics much more than plants. Since plants create favourable conditions for microorganisms to live, specific cooperation between them is possible. In reclamation of land contaminated by organic compounds two types of phytoremediation, i.e. phytodegradation and biodegradation in the rhizosphere, are particularly important.

PHYTODEGRADATION

The phytodegradation process, also known as phytotransformation, relies on the ability of selected plant species to uptake pollutants from contaminated sites. Subsequently, uptaken substances are decomposed to CO₂ and H₂O by enzyme complexes of the plant metabolic cycle. In plant cells, total degradation of organic compounds rarely occurs, but biotransformation products can still be used in other ways, such as synthesis of amino acids, or incorporated into natural plant components (Różański 1998). The process of uptake, transport, degradation, or accumulation of organic pollutants is a key issue in the success of phytoremediation. It is important, therefore, to optimise methods to the prevailing environmental conditions, kind of contamination and other factors. Various experimental studies in this range have been conducted (Kim et al. 2004, Li et al. 2005, Collins et al. 2006) and methods of assessing both the toxicity of pollutants for different plant species and their tolerance to the presence of xenobiotics in the environment have been developed. These methods often involve assessment of seedling survival, biomass reached, speed of shoot and root growth, possibly delayed flowering, or chlorosis of leaves (Walker et al. 2002, Alkio et al. 2005). Impurities may in fact
have a very strong impact on such fundamental physiological processes as photosynthesis, respiration and growth. Herbicides are often responsible for this, including, for example, urea and triazine derivatives, which disrupt the flow of electrons through the photosynthetic system (in particular photosystem II). Another mechanism of action of herbicides on plants is connected with damage to the chlorophyll synthesis pathway and induction of oxidative stress associated with accumulation of active oxygen species (Alkio et al. 2005). Genotoxic effects of some xenobiotics and inactivation of enzymes, which leads to poisoning of the plant cell, are commonly found. However, a defence reaction consisting in induction of synthesis of enzyme systems capable of metabolising toxins often occurs in plants (Walker et al. 2002).

The detoxification process in plants takes place in stages, and three phases can be distinguished which are characterised by participation of different classes of enzymes, and by the properties and allocation of their reaction products.

Phase I is called bioactivation and relies on unveiling or generation of reactive groups in the xenobiotic molecule. This process aims to "prepare" a chemical compound to the actual detoxification, which takes place in the next step. In the bioactivation, mainly flavin enzymes, which exhibit hydrolytic and redox activity, as well as polysubstrate monooxygenase (PMSO) are involved. This is a complicated complex of catalytic systems consisting of cytochrome P450 and NADPH-cytochrome reductases located in the P450 microsomal fraction and present in a soluble form in the cytoplasm (Fig. 1). Cytochrome reductase (EC1.6.2.4) is a flavoprotein which does not have isoenzymatic forms (Leszczyński 2001, Różański, 1998, Wójcik and Tomaszewska 2005). Cytochrome P450 is a hemo-protein possessing a thiol group in its composition; in contrast to reductase, it can form more than 20 isoforms in the PMSO system (Kreuz et al. 1996, Leszczyński 2001) (Fig. 2). One of the most important reactions catalysed by the complex, which cooperates with cytochromes, is introduction of oxygen into the substrate molecule. One oxygen atom from the O₂ molecule is used to oxidise the xenobiotic and the other to form a water molecule (Walker et al. 2002, Zakrzewski 2000). Thanks to their low substrate specificity and ability to catalyse numerous chemical reactions, cytochromes are efficient in the process of detoxification of exogenous compounds. Besides oxidation, they can also participate in the process of dealkylation, hydroxylation, dehydrogenation, epoxidation, isomerisation, dearylation or sulphoxidation (Leszczyński 2001, Różański 1998, Walker et al. 2002). Another important feature is the mode of cytochrome formation, as they are often synthesised de novo by the action of inducers, which may be xenobiotics themselves (Leszczyński 2001). The group of compounds that induce the synthesis of cytochromes includes flavones, indoles, sterols, quinones, or hydrocarbons.
Membrane-bound flavin monooxygenases (EC1.14.13.18) are also involved in the process of oxidation of xenobiotics. Their substrates are mainly nucleophilic nitrogen and sulphur compounds (Cashman 2002). In addition to oxidative enzymes, also enzymes characterised by hydrolytic activity can participate in phase I of detoxification. These are mainly amidases (EC3.5.1.4), epoxide hydrolases (EC3.3.2.3) and carboxyesterases (EC3.11.1) (Różański 1998, Komives and Gullner 2005).
In the second phase called conjugation (Fig. 2), activated xenobiotics bind to sugar molecules, amino acids or SH-groups of glutathione. The resulting substances are less toxic than the output substances with a polar structure. The reaction of conjugation with sugar is catalysed by UDP-dependent glucosyltransferase. N-acetyl transferases are involved in the reaction with amino acids, and the family of glutathionotransferases (GST) - in the reaction with glutathione.

Depending on their different properties, xenobiotics can be bound with sugars (glucose, galactose, mannose, arabinose, xylosyl-glucose), amino acids (glutamic acid, glycine, leucine, cysteine), carboxylic acids (e.g. malonic acid) and peptides (the most important of which is glutathione) (Coleman et al. 1997, Lao et al. 2003, Różański, 1998, Wójcik and Tomaszewska 2005). The resulting conjugates are often more hydrophilic than the initial molecules, which provides them with mobility in the cytoplasm. Binding of the carbohydrate residue to the xenobiotics is catalysed by UDP-dependent sugar transferase (EC 2.4.1.). Simple conjugates of xenobiotics with the carbohydrate residue are often subjected to the subsequent process of conjugation. Malonyltransferase, which uses malonyl-CoA as the acyl group donor, participates in this reaction (Coleman 1997). Glucosylglucosides or other derivatives are the predominant reaction products.

The process of coupling of xenobiotics and amino acids is catalysed by N-acyltransferases (ATC). Their substrates are essentially carboxylic acids, thus the resulting conjugates may still be biologically active, although they become less mobile. Then, they are secreted into the cell wall (Leszczyński 2001, Różański 1998, Zakrzewski 2000).

Lipophilic xenobiotics can be conjugated non-enzymatically with glutathione (GSH). Due to their structure, organic pollutants, which include phenols, halogen compounds or some herbicides, do not very often require the bioactivation process to bind to it (Kreuz et al. 1996, Urbanek et al. 2005, Zakrzewski 2000). The process of non-enzymatic coupling is slow and not very effective; therefore, glutathione S-transferase plays an important role in the conjugation with glutathione (GST, EC 2.5.1.18). This enzyme occurs in the cell microsomal fraction, in the cytosol, mitochondria, and nuclei (Edwards et al. 2000, Kreuz et al. 1996, Wójcik and Tomaszewska 2005, Zakrzewski 2000). Probably, the conjugation of glutathione with the xenobiotic is a kind of "sign" of the moiety intended to be deposited in the vacuole (Edwards et al. 2000, Kreuz et al. 1996, Leszczyński 2001, Skipsey et al. 2005, Wójcik and Tomaszewska 2005).

Phase III is also called compartmentalisation; during this phase, removal of xenobiotics inactivated by conjugation from the cytosol to the vacuole or apoplast occurs, which allows safe deposition of derived toxins (Fig. 2). The conjugates are transported through the tonoplast by Mg- and ATP-dependent transporters called ABC transporters. Xenobiotics in the vacuole are further modified in reac-
tions catalysed by peroxidases and carboxypeptidases (Wojtaszek et al. 2006). Thanks to their chemical properties, a number of contaminants may be bound by active groups of lignin or cellulose present in the cell walls (Wojtaszek et al. 2006). This is facilitated by the presence of apoplastic enzymes, such as peroxidases or polyphenol oxidases (Jansen et al. 2004, Labrou et al. 2004), which are involved in the synthesis of cell wall components.

Degradation of harmful organic compounds very often occurs in the rhizosphere, de facto outside the plant, although with its active participation (Pilon-Smits 2005). The rhizosphere comprises a layer of soil from about one millimetre to even several centimetres around the roots. This is an area with different types of interactions between a plant and soil microorganisms (McCutcheon and Schnoor 2003, Stottmeister et al. 2003, Hynes et al. 2004). Plants secrete various substances into the soil through the roots, including enzymes which, in turn, may interact with xenobiotics in many ways. Peroxidases, for example, are actively involved in breaking down phenolic compounds dissolved in the soil solution (Duran and Esposito 2002, Jansen et al. 2004). Other plant enzymes, which are also actively involved in mineralisation of organic compounds, include laccase, nitrilase or dehalogenase (Wang et al. 2004). Also, plant root exudates actively support breaking down of organic xenobiotics (Pilon-Smits 2005). Many components of root exudates enhance the solubility of some organic compounds in water, which facilitates their uptake by plants.

Another factor that may have a positive influence on the process of phytodegradation and biodegradation in the rhizosphere is the phenomenon of mycorrhiza which can indirectly help to increase plant biomass by protecting plants against certain pathogens, and by improving soil conditions. Unfortunately, rhizospheric microorganisms taking an active part in the process of mycorrhiza may also increase the availability of substances that are toxic to plants (Schwab and Banks 1994). Arbuscular fungi can support plant growth by reducing stress associated with reduced availability of phosphorus (Joner and Leyval 2001) and increase the production of oxidative enzymes (Salzer et al. 1999). Additionally, ectomycorrhizal fungi can produce enzymes involved in various stages of xenobiotic decomposition in the soil (Barr and Aust 1994, Meharg and Cairney 2000).

**BIODEGRADATION IN THE RHIZOSPHERE**

Biodegradation in the rhizosphere has a special place among the biological methods of soil remediation, as microbial enzymes play a very important role in the degradation of organic compounds in the soil environment. These are mainly esterases and oxidoreductases. Many of them have low substrate specificity, which ensures them a broader spectrum of activity. Aerobic microbial biodegra-
Phytodegradation and biodegradation in rhizosphere of organic compounds with aromatic structure can be divided into three stages: ring hydroxylation, cleavage of the aromatic ring and oxidation of the aliphatic moiety to Krebs cycle intermediates, and their further breaking down to CO₂ and H₂O (Gren et al. 2008) (Fig. 3).

Fig. 3. Scheme of aerobic microbial biodegradation of organic compounds with aromatic structure

In the case of such compounds as chlorophenols, which already have one hydroxyl group, hydroxylation is carried out by monooxygenases dependent on NAD(P)H (EC1.14.XX). In the case of benzene, toluene, naphthalene and other compounds which do not contain a primary hydroxyl group, the hydroxylation process most often takes place with participation of hydroxyl dioxygenases.
They catalyse the incorporation of two atoms of oxygen to the aromatic ring via two hydroxyl groups and parallel oxidation of NAD(P)H. The product of this reaction is later converted to catechol by a dehydrogenase (Vaillacourt et al. 2006, Ullrich and Hoffrichter 2007, Reineke 1998).

In the next step, the aromatic rings are cleaved by dioxygenases which cleave one C-C bond of the ring; concurrently, they add an oxygen molecule and aliphatic acids are formed. This process can be catalysed by enzymes belonging to one of the two main families of cleaving enzymes:

- **Intradiol-dioxygenases** – ortho pathway enzymes whose product of activity is cis, cis-muconic acid or its derivatives.
- **Extradiol-dioxygenase** – meta pathway enzymes, in which 2-hydroxymuconic semialdehyde or its derivatives are formed (Vaillacourt et al. 2006, Harwood and Parales 1996, Ishiyama et al. 2004).

The third stage of aromatic compound degradation involves oxidation of the aliphatic chain, which was formed after cleavage of the aromatic ring with the use of enzymes of the ortho or meta pathway. Tricarboxylic acid cycle intermediates are formed, which are incorporated into the basic metabolism of the bacterial cell (Vaillacourt et al. 2006, Harwood and Parales 1996, Li et al. 2006).

Within their root systems, plants are able to increase the abundance of soil microflora by 1-4 orders of magnitude compared to the "loose" soil. This phenomenon is called the "rhizosphere effect." For heterotrophic microorganisms, root exudates may be an additional source of carbon and nitrogen (Pilon-Smits 2005, Wójcik and Tomaszewska 2005). Certain plant species, which secrete exudates with a characteristic composition through roots into the soil, may also promote growth of specific species of microorganisms characterised by different capacities of degradation of various organic compounds (Kirk et al. 2005). Root growth also facilitates oxygen penetration and water infiltration into deeper layers of the substrate (Escalante-Espinosa et al. 2005, Kaimi et al. 2006). Due to their requirements as to the soil environment conditions, rhizosphere is the best environment to live in for a variety of microorganisms.

**USE OF GENETICALLY MODIFIED PLANTS AS AN ALTERNATIVE TO TRADITIONAL METHODS OF PHOTOREMEDITION**

In the current world of science, attempts are increasingly being made to improve phytoremediation through techniques used in genetic engineering. One of the most promising methods is the insertion of genes encoding factors of phase I and II of detoxification. This improves the process of degradation of such xenobiotics as polychlorinated biphenyls (PCBs), herbicides, and explosives.
In the past two decades, many successful attempts of insertion of genes encoding mammalian cytochromes P450 into the plant genome have been made. These genes were inserted into such plants as *Nicotiana tabacum*, *Solanum tuberosum*, *Oryza sativa* or *Arabidopsis thaliana*. The process of insertion of the genes into plants is relatively easy to perform using *Agrobacterium tumefaciens* as a vector, or the gene gun method (Eapen et al. 2007, Doty 2008). The first attempts were connected with obtaining herbicide-resistant plants, exhibiting increased tolerance mainly to atrazine and simazine. There were also attempts to obtain plants which would be able to remove some volatile chlorinated derivatives of hydrocarbons from the soil and groundwater. Due to the low specificity of mammalian cytochromes P450, transgenic plants were characterised by a marked increase in the capacity of metabolism of various xenobiotics.

Insertion of rat’s CYP1A1 gene as well as yeast’s gene encoding NADPH-dependent cytochrome P450 oxidoreductase into the genome of rice *Oryza sativa* allowed the transgenic plants to metabolise chlorotoluron (an active component of many herbicides). Plants thus transformed became resistant to this compound (Shiota et al. 1994, 1996). Another example is the modification of the potato genome which was characterised by overexpression of the human CYP1A1. In consequence, the plants obtained were resistant to herbicides, including atrazine, chlorotoluron, and methyl pyriminobak (Inui et al. 1999). The next step was to insert, apart from the CYP1A1 gene, also human genes coding for cytochromes CYP2B6 and CYP2C9. This helped in the immunisation of the modified plants against such photosynthesis inhibitors as the aforementioned atrazine, chlorotoluron, metabenothiazuron, and lipid biosynthesis inhibitors: acetochlor, metolachlor, and norflurazon – an inhibitor of carotenoid biosynthesis (Shiots et al. 2000). Overexpression of human CYP2E1 in modified tobacco seedlings significantly increased the capacity of the plants in the metabolism of volatile hydrocarbons such as benzene, trichlorethylene, carbon tetrachloride and others (Doty et al. 2000). It was determined that thus modified plants were able to metabolise xenobiotics over 640-fold faster than seedlings with an unaltered genome (Doty et al. 2000). High hopes are placed on the use of the hybrid poplar *Populus tremula x Populus alba* with an inserted rat-derived CYP2E1 gene in phytodegradation of toxic organic compounds. Thanks to their extensive roots, trees are able to cover much larger areas and to penetrate deeper layers of soil than small shrubs. Preliminary studies indicate that modified plants are able to metabolise TCE more than 100-fold faster than non-transgenic plants (Doty et al. 2007).

Besides cytochromes, glutathione transferase genes – factors of phase II cellular detoxification are used in the modification. Poplars with bacterial synthetase gene encoding γ-glutamyl-cysteine, an enzyme involved in the synthesis of glutathione (GSH), showed a significant increase in the production of this factor
Increased production of GSH in plant cells increases protection against oxidative stress caused by various harmful external influences (Noctor and Foyer 1998). Indian mustard *Brassica juncea* with overexpression of $\gamma$-glutamyl-cysteine synthetase and glutathione synthetase was also characterised by increased tolerance to atrazine, metolachlor, phenanthrene and 1-chloro-2,4-dinitrobenzene (CDNB) (Flocco et al. 2004). Increasing levels of glutathione in the cells of poplar contributed to increased resilience of the tree to herbicides from the chloroaacetanilide group (Gullner et al. 2001). The transgenic *Nicotiana tabacum* with a gene encoding S-transferase glutathione, which is derived from corn Karavangeli et al. (2005), is ideal for phytoremediation of sites contaminated with this herbicide. Simultaneous insertion of the human cytochrome P450 2E1 gene and the gene encoding the GST derived from the *Trichoderma virens* fungus into the genome of *Nicotiana tabacum* resulted in more efficient metabolism of chlorpyrifos and anthracene in the modified plants (Dixit et al. 2008).

High hopes are attached to the use of transgenic plants to remove of explosive residues from soil contaminated by intense military action (Richman 1996). Such substances as nitroglycerin, trinitrotoluene (TNT), aminodinitrotoluene (DNT) or sentex (RDX) are highly toxic and mutagenic compounds that, due to violent reactions during explosion, often undergo incomplete combustion and are shed along with the shock wave (Bruns-Nogel et al. 1996). Given that the substances are phytotoxic, phytoremediation of contaminated sites using conventional techniques is very difficult to achieve. Insertion of bacterial genes encoding pentaeerythritol tetraoctate reductase (PETNr) resulted in tolerance of substantial concentrations of such compounds as TNT or nitroglycerin in soil solution by plants with overexpression of this gene (French et al. 1998). Another solution was to use the nitroreductase (NR) encoding gene from *Enterobacter cloacae*. Its overexpression in transgenic *Nicotiana tabacum* tobacco seedlings provided greater tolerance of these plants to TNT soil contamination than in the case of PETNr (Rylott and Bruce 2009).

Another approach to improve phytoremediation is to accelerate the degradation of xenobiotics in the rhizosphere. Promising results were obtained when resistance to 2,4,6-trichlorophenol was assessed in *Arabidopsis* seedlings with a built-in gene encoding root-specific laccase which was originally produced by *Gossypium* (Wang et al. 2004). Sonoki et al. (2005) examined the ability of rhizodegradation of biphenol A and PCBs in tobacco seedlings inoculated with a gene coding laccase obtained from a fungus *Coriourls vericolar*. Transgenic *Arabidopsis thaliana* plants with an extradiol dioxygenase gene significantly increased their capacity for rhizodegradation of 2,3-dihydroxybiphenol (Uchida et al. 2005).
SUMMARY

Making use of the co-operation between plants and soil microorganisms is a very effective way to remove many toxic organic compounds such as pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and other petroleum compounds from soil. Although this process is relatively slow in comparison with other methods, its low invasiveness and economic benefits render it highly recommended for application. Phytoremediation, which is characterized by simplicity and low equipment requirements, offers high effectiveness in inactivating certain chemicals, not to mention its aesthetic values.

It is important to select appropriate species for a particular method of phytoremediation (Sylvestre et al. 2009). Species with big biomass of aboveground parts can be taken into consideration in phytoextraction, while plants with a more developed root system are more useful in rhizoremediation. Since many toxic organic compounds are only partially metabolised and the products of the detoxification processes accumulate in cell vacuoles or apoplasts, there is a risk that these plants will be eaten by herbivores. Therefore, it is advisable that plant species that are poisonous or inedible to animals should be used. This will ensure effectiveness of phytoremediation at the expected level and, more importantly, protect against penetration of potentially toxic products of xenobiotics metabolism into the food chain.

Studies on the use of transgenic plants for phytoremediation were carried out on many various species, e.g. poplars, fescue, tobacco, or rice. Whether a plant is suitable for this type of project also depends on its susceptibility to transformation using Agrobacterium or by using the “gene gun” method. Although genetically modified plants can remove many poisonous substances from the environment more quickly and efficiently, it should be kept in mind that the practice of insertion of human genes into the plant genome raises serious ethical controversies. On the other hand, as confirmed by numerous studies, very high efficiency at comparable costs with those of the traditional phytoremediation method and the unusual resistance of transgenic plants may speak in favour of this method, especially when dealing with a large area with a high level of chemical contamination and a limited financial budget. Therefore, the choice of the most appropriate phytoremediation method should be based on real necessity and common sense, as not all cases necessitate the use of transgenic plants.
REFERENCES


FITODEGRADACJA I BIODEGRADACJA W RYZOSFERZE JAKO SKUTECZNE METODY REKULTYWACJI GRUNTÓW SKAŻONYCH ZWIĄZKAMI ORGANICZNYMI (artykuł przeglądowy)

Mariusz Spaczyński, Aleksandra Seta-Koselska, Paweł Patrzylas, Agnieszka Betlej, Ewa Skórzyńska-Polit

Katedra Fizjologii i Biotechnologii Roślin, Katolicki Uniwersytet Lubelski Jana Pawła II
ul. Konstantynów 1H, 20-708 Lublin
e-mail: mspaczynski@kul.lublin.pl


Słowa kluczowe: Ksenobiotyki organiczne, Detoksyfikacja, Fitoremediacja, Cytochrom P450, S-tranferazy glutationowe