Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2019, Vol. 3 (3), 353-364
https://doi.org/10.29329/ijiaar.2019.206.1

Original article

Evaluation of the Detoxification Potential of Micrococcus Strains and Plants for Bioremediate Organochlorine Herbicides

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Abstract

The objective of our study is to contribute towards the development of the phytoremediation method which is a method that attempts to clean the soil polluted with organochlorine herbicides with the combined application of specially chosen plants, such as alfalfa, maize and soybean with Micrococcus strains. The enzymes and genes involved in the process of bioremediation of various pesticides have also been discussed. Initial degradation of herbicides carried out by bacterial strains include formatin of dehalogenated products with easy plant uptake and undergo oxidative degradation through plant detoxification enzymes, such as cyctochrome P450, peroxidase, phenoloxidase and glutathion S-transferase. Subsequently, this method can finalise the mineralization and degradation of toxicants into nontoxic compounds. Our study presents the results of our model experiments with selected strains of genera Micrococcus and plant phytoremediators. According to the results of the phytoremediation studies, Micrococcus sp. DR44 and Micrococcus sp. HEXBA04 showed best removal performance with Oxadiazon herbicide as 84% and Micrococcus sp. Pv8 and Micrococcus sp. BP3_1A showed 71% removal performance on Quizalofop-p-ethyl with alfalfa with maize, while removal efficiency of Liuron was 77% in alfalfa with Micrococcus sp. NCTC2665. Our study shows that effectively using this newly developed technological approach results in a reduction of pollution in soil samples that have been artificially contaminated. Future perspectives of pesticides bioremediation has also been briefly articulated to make a realistic comment with an element of optimism for researchers working in this field.

Keywords: Phytoremediation, Organochlorine Herbicides, Micrococcus Strains, Detoxification Enzymes.

Received: 01 August 2019 * **Accepted:** 05 August 2019 * **DOI:** https://doi.org/10.29329/ijiaar.2019.206.1

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INTRODUCTION

Bioremediation of herbicide residues is becoming a very important component of integrated agricultural activities, helping to insure that the principles of good stewardship are maintained. The topic of pesticide bioremediation has been considered in several recent reviews covering enzymatic bioremediation (Alcalde et al., 2006). Due to their chemical stability and hardship in undergoing abiotic and biotic transformations, the organochlorine pesticides are classified as persistent organic pollutants (POPs). These pesticides simply build-up in crops and animal tissues and subsequently reach the food chain causing massive dangers for public health. Nowadays, ecology is characterized by large technogenic emissions into the environment, resulting in plants and microorganisms showing new abilities to absorb and metabolise toxicants, which is the foundation for the development of phytoremediation technologies, the capability of microorganisms and plants to absorb and degrade a wide spectrum of organochlorine pesticides through enzymatic transformations (Kvesitadze et al., 2006). Enzymes are central to the mode of action of many herbicides where some herbicides are activated *in situ* by enzymatic action and many other herbicide functions by targeting particular enzymes with essential physiological roles (Scott et al., 2008).

Phytoremediation catabolises or accumulates contaminants by using plants (natural or transgenic). Phytoremediation is completely dependent upon the cultivation of the remediating crops, which can be importantly slower and dependent upon more stringent nutrient requirements than the rate of microbial growth. The major superiority of phytoremediation is crops can be more easily controlled and contained than microorganisms, and therefore is a more freely recognised technology (Scott et al., 2008).

In the case of pollution caused by persistent and highly toxic organic pollutants, such as organochlorine pesticides, using plant-microbial tools to intensify the phytoremediation process could work. A study on the influence of different concentrations of organochlorine toxicants (oxadiazon, linuron and quizalofop-p-ethyl) on growth parameters (germinability, biomass formation and length of seedlings) of different plants species, found the tolerant plants to the tested pesticides (Kurashvili et al., 2014). Model experiments have shown that using these plants to clean artificially contaminated soil was ineffective presumably due to it being low soluble, and subsequently, the pesticides having reduced bioavailability.

Consequently, as a potential solution, development of a bio-technological method based on using plants and strong detoxification potential Micrococcus strains, to entirely rid the detrimental effects of herbicides from soil. This proposed biotechnology will be based on the idea of primary degradation of herbicides being carried out by specially selected bacterial strains. At that moment, the radicals which cause toxic and stability against bio-transformation of pesticides are removed from the pesticide molecules due to the microorganisms enzyme actions. The resulting products readily accessible for the

plants so the oxidative enzymes can finalize degradation into cellular nontoxic compounds of the carbon skeleton of the toxicants.

The purpose of this study is to develop a phytoremediation application to clean soil contaminated with organochlorine herbicides, based on shared application of maize, soybean and alfalfa with Micrococcus strains.

Materials and Methods

Pesticides and plants used in the study

In the research work following organochlorine pollutants: Oxadiazon, Linuron and Quizalofop p-ethyl have been investigated.

For testing the following agricultural annual plants: soybean (Glycine max), maize (Zea mays) and alfalfa (Medicago sativa) were used. Plants after exposition on solutions of herbicides (0.01, 0.1 or 1 mM) during 5 days were washed with distilled water, roots and shoots cut separately and homogenized in 0.05 M phosphate buffer at pH 7.4. Homogenates were squeezed through muslin cloth and centrifuged at 1000 g for 20 min.

Experimental design

For model experiments the soil was artificially contaminated with oxadiazon, linuron and quizalofop-p-ethyl. In tests the soil type e alluvium was used, with maximum soil particle size after sieved at 2 mm. The experiments were carried out in glasses from plastic (volume 100 mL). The mass of each air-dried sample equals 100 g. The contamination in soil samples was 100 ppm. The suspension of bacteria (10%) were inoculated in the contaminated soil at the beginning of the experiment. The samples were placed in the thermostat at 25^0C for one week. Then the seeds of the plants were sowed in separate samples of soil (10 seeds for maize and soybean and 25 seeds for alfalfa in each sample). The experiment includes two types of control: with and without water.

The model experiment carried out four weeks. The extraction of pesticides were carried out by Soxhlet extraction. Extracts were evaporated to dry residues, obtained residues were dissolved and the contents of pesticides were measured by gas chromatography (Fatoki and Awofolu, 2003) in the below mentioned conditions.

The capability of bacteria to assimilate and degrade organic toxicants was revealed by a growing phase on solid media at 28^0C. For the screening, modified Czapek's media, containing Oxadiazon, Linuron and Quizalofop-p-ethyl used.

The nutrient media were inoculated with 10% of bacterial suspension. The extraction of Oxadiazon, Linuron and Quizalofop-p-ethyl from the incubation medium was completed following the cultivation of Micrococcus being submerged.

Determination of pesticides

Strains with herbicides was performed using acetone and hexane. Extracts were evaporated to dry residues, and then were dissolved in acetone and hexane, and the contents of herbicides were measured by gas chromatography.

10 g of soil sample was mixed with anhydrous sodium sulfate to form a free-flowing powder and then these samples were extracted with a proper solvent using ultrasonic extraction methods described in EPA 3550C (EPA 2007). For analytical determination of organochlorine pesticides, the extracted samples were placed into a 250 ml beaker, and surrogate spiking solutions and matrix spiking (1.0 ml of each) solutions were added to the samples. The soil samples were scanned ultrasonically twice for 30 min with 50 ml of the extraction solvent mixture (1:1 hexane and acetone for GC-ECD analyses pure). The extract supernatants were filtered through Whatman Grade No. 41 Quantitative Filter Paper, Ashless, Whatman 1441-047/ 28477-974 using a Buchner funnel. To eliminate unwanted interactions of organic matters such as PAHs, PCBs, etc., an alumina-silicic acid column was used. These chemicals were heated at 450^0C in a baker for 6 hours and then let to cool down to room temperature in a desiccator. A separation column was formed by 3 g silicic acid that contained 3% ultra pure water, 2 g neutral alumina that consisted of 6% ultra pure water, and 2 g Na_2SO_4 according to the ref. given by Jantunen et al. (2000). After this process ended, the column was pre-washed with 20 ml of dichloromethane solvent. The sample was evaporated to 2 ml and then spilled to the column. At least 20 ml dichloromethane was added to resolve the pesticides according to Cindoruk (2011). Aliquot samples were placed into a concentrator tube in a warm bath and evaporated to 1 ml volume using a gentle stream of clean dry nitrogen, for analyzing the procedure. During concentration, the internal wall of the concentrator tube was rinsed several times. Dichloromethane was used in the washing up step. After that, the final extract (approximately 1 ml) was analyzed for the Oxadiazon, Linuron and Quizalofop-p-ethyl residues using the method described in EPA 8081B (EPA 2007). The retention time for tested pesticides is as follows: Oxadiazon: 7.0 min; Linuron: 7.2 min; and Quizalofop-p-ethyl: 7.3 min. The quantification of all pesticides was carried out using a Perkin Elmer Clarus 500 gas chromatograph with an Electron Capture Detector (GC-ECD).

Enzyme activation studies

Peroxidase activity was determined spectrophotometrically at 470 nm, according to the rate of H2O2-dependent oxidation of guaiacol (Gregory, 1966). Specific activities were calculated as A450 in min per mg protein. Phenoloxidase activity was determined spectrophotometrically at 420 nm, according to the rate of pyrocatechol oxidation (Lanzarini et al., 1972). Specific activities were calculated as A420 in min per mg protein. Activity of Glutathione S-transferase was determined spectrophotometrically at 340 nm (Schroder and Rennenberg, 1992). Specific activities were calculated as mmole 1-chloro-2,4-dinitrobenzene (CDNB) in min per mg protein. Monooxygenase activity was determined

polarographically, by oxygen consumption rate at NADPH-dependent oxidation of N, N-dimethylaniline (Khatisashvili et al., 1995)

Statistical analysis

The results from experimental studies were evaluated with statistical analyses performed with SPSS (SPSS Inc, Chicago, IL, USA). The values are the averages of the results of three replicates of each experiment with a standard deviation (SD). To compare the enzyme activity in plants with pesticides and removal efficiencies, the data was analyzed by analysis of variance (ANOVA).

Results and Discussion

The primary stage of our study involved establishing the metabolism mechanism of organochlorine toxicants in plants chosen based on the influence of the tested compounds over detoxification enzymes (phenoloxidase, peroxidase, cytochrome P450, and glutathione S-transferase).

From the results presented in figures 1-3, with alfalfa nearly the entirety of all enzymes investigated were induced for transformation of oxadiazon and linuron. However, for quizalofop-p-ethyl only the activation of glutathione S-transferase takes place. From this, to detoxify oxadiazon and linuron non-polar molecules, the first oxidation and following conjugation is required. Furthermore, an almost equivalent results were obtained for differing plants, however, induction is less pronounced. Therefore, our results show that varying degrees of induction of enzymes participate in conjugation and oxidation of herbicides takes place on plants due to the actions of toxicants.

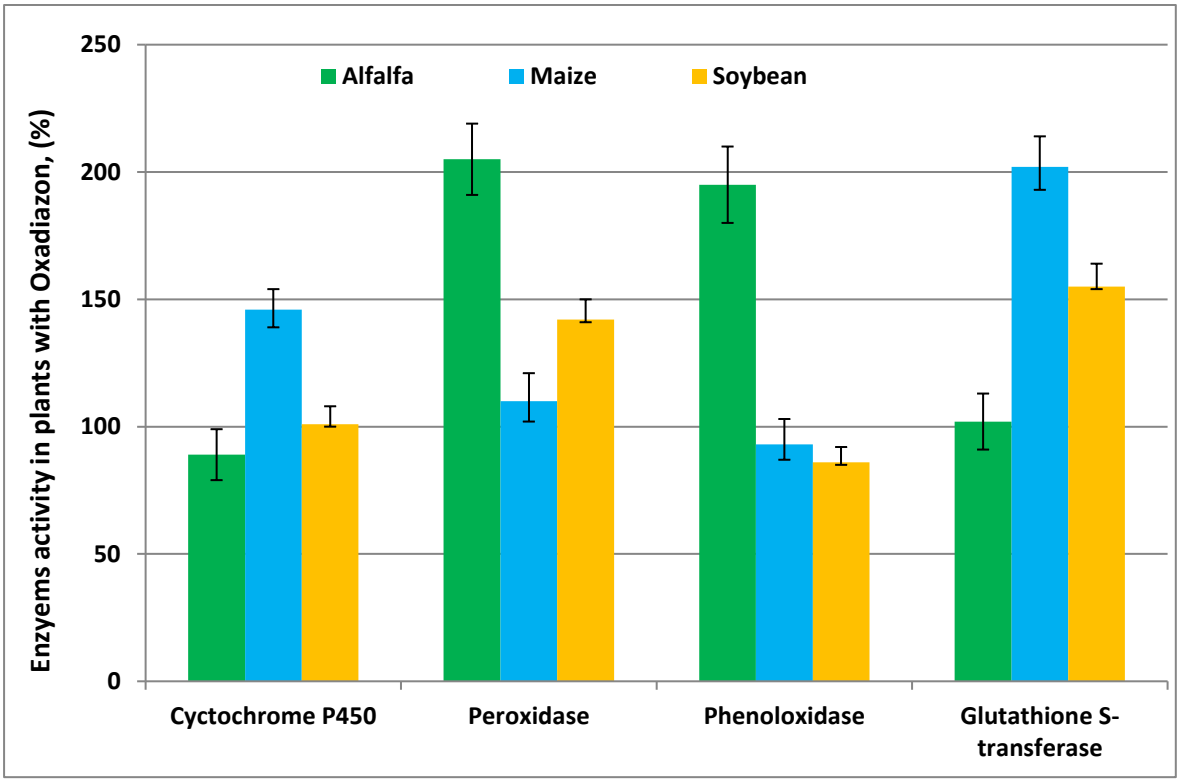

Figure 1. Oxidative enzymes induction of the roots of 14 day seedlings following growth in a 0.1 mM Oxadiazon solution for 7 days. Enzyme activities in the control are taken to be 100%

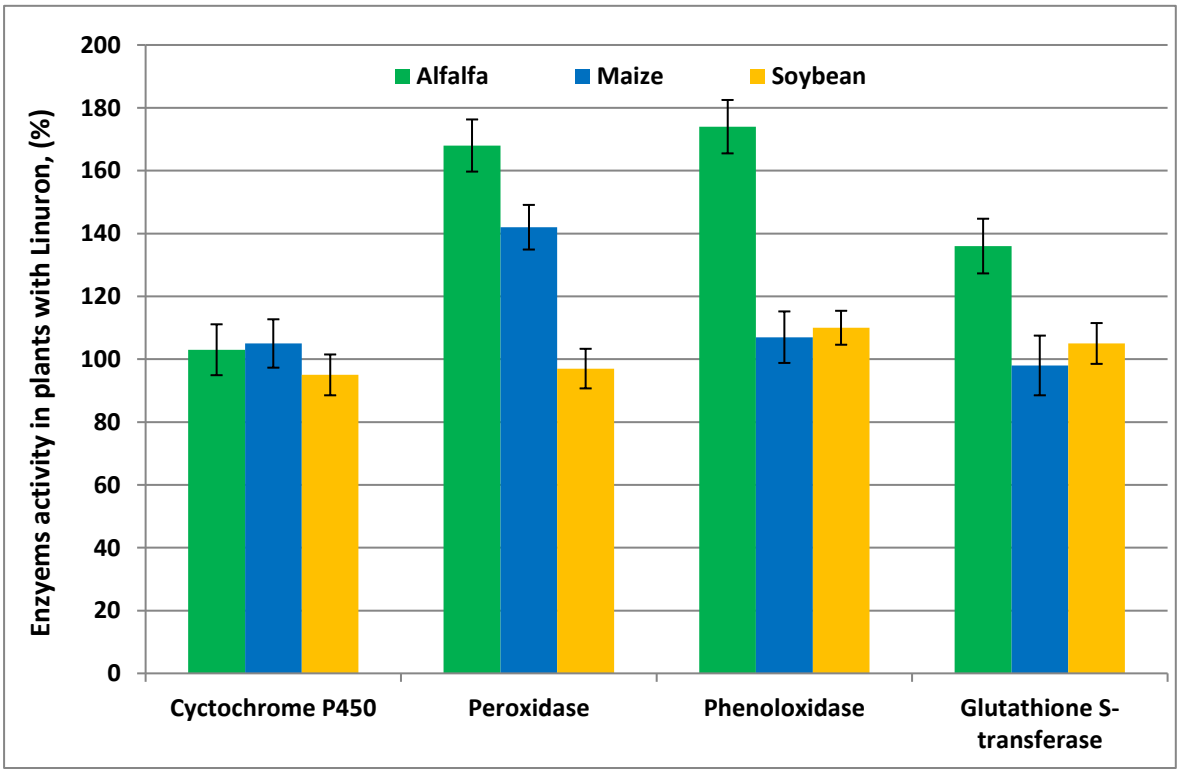

Figure 2. Oxidative enzymes induction of the roots of 14 day seedlings following growth in a 0.1 mM Linuron solution for 7 days. Enzyme activities in the control are taken to be 100%

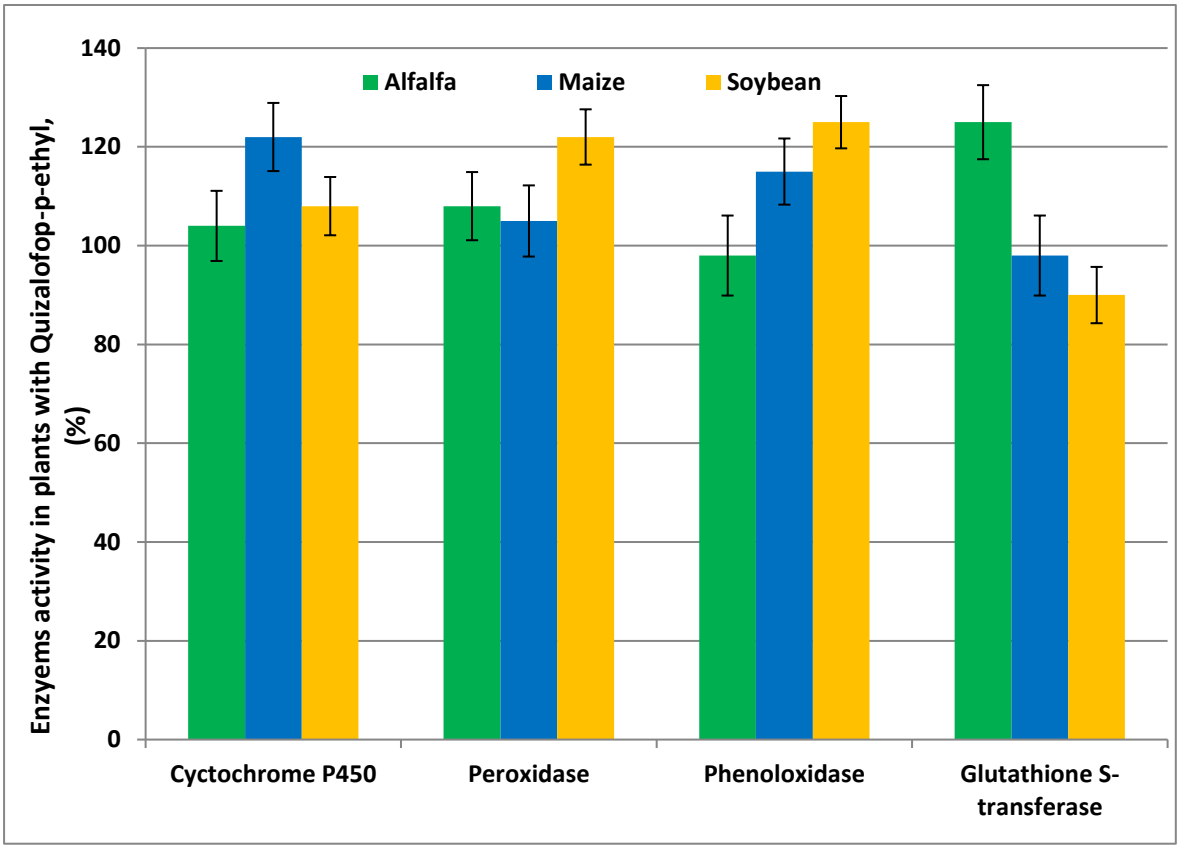

Figure 3. Oxidative enzymes induction of the roots of 10 day seedlings following growth in a 0.1 mM PCP solution for 5 days. Enzyme activities in the control are taken to be 100%

The results show different degrees of induction of the enzymes oxidating and conjugating pesticides occur due to the toxicants' interaction with the plants. As every detoxification enzyme catalyzes a particular reaction (oxidation or conjugation), it is put forward the transformation of the pesticides occur through the following pathways:

- Oxadiazon experiences initial oxidation by cyctochrome P450, phenoloxidase, peroxidase, and followed by glutathion S-transferase causing conjugation. This is the chief pathway of transforming DDT. Furthermore, these herbicides (organochlorine) could be conjugated straight by glutathione S-transferase.

- Primary oxidation of linuron is via cyctochrome P450, phenoloxidase, peroxidase, and followed by glutathione S-transferase conjugation.

- Quizalofop-p-ethyl undergoes direct conjugation via glutathion S-transferase and followed by conjugation subsequent to initial oxidation by cyctochrome P450, phenoloxidase and peroxidase.

The following stage of our study, in selecting the soil bacteria to degrade the organochlorine herbicides, in excess of 60 strains from Micrococcus have been screened on a solid nutrient space with oxadiozen, linuron and quizalofop-p-ethyl. The results following strains cultivation on the Oxadiozen

containing areas showed that, 30 strains from genera Micrococcus grew the best with glucose and 12 strains grew better with oxadiozen compared to glucose. Consequently, 9 strains of Micrococcus showing the highest growth, without or with glucose, were selected.

Following cultivation with Linuron, the results showed that 11 strains from genera Micrococcus had the highest growth with glucose and compared with glucose, 10 strains were grown better with linuron. Consequently, 9 strains of Micrococcus showing the highest growth, without or with glucose, were selected.

With quizalofop-p-ethyl, 11 strains from genera Micrococcus revealed the most growing with glucose and 4 strains had best growth with quizalofop-p-ethyl than with glucose. Consequently, 9 strains of Micrococcus showing the highest growth, without or with glucose, were selected.

Estimation of the influence of oxadiazon, linuron and quizalofop-p-ethyl on accumulation of bio-mass by the chosen strains was made. Strains of pesticides for quantitative analysis were chosen based on the results of screening. Our results showed that, certain strains (Micrococcus sp. DR44 in oxadiazon, Micrococcus sp. NCTC2665 in linuron and Micrococcus sp. KSI951 in quizalofop-p-ethyl) amass bio-mass better in locations holding herbicides, compared to a glucose-free Czapek‘s medium.

The potential of detoxification for each strain referring to its bio-mass accumulation capacity in submerged cultivation in areas with pesticide are approximated (Table 1).

Table 1. The medium of incubation following submerged cultivation of certain Micrococcus strains with pesticides following incubation conditions: temperature: 28^0C, Czapek‘s medium, initial concentration of pesticides: e 1mM, shaker speed: 160 rpm, time period of cultivation: e 96 h

Herbicide	**Conventional name of *Micrococcus* strains**
Oxadiazon	Sp. D3
	Sp. Pv8
	Sp. BGDa135M15
	Sp. DR44
	Sp. HEXBA04
Linuron	Sp. BGDa135M14
	Sp. BGDa135M13
Quizalofop-p-ethyl	Sp. Pv8
	Sp. BGDa135M4
	Sp. WB18_01
	Sp. BP3_1A
	Sp. MLKSI951

Figure 4 shows the top strains that have the best capacity of pesticide assimilation based on gas chromatographic analysis of residual pesticides inside the incubation material following cultivation. These strains can degrade and/or uptake organochlorine and can be used for applications in

phytoremediation technology and model experiments have been conducted showing the new phytoremediation technology approaches in order to decontaminating soil pollution caused by pesticides (organochlorine). In the model experiments plants, soybean, maize and alfalfa together with microbial consortia composed with Micrococcus strains have been used.

The results show phytoremediation of soils polluted by oxadiazon, the concortia (Micrococcus sp. DR44 and Micrococcus sp. HEXBA04) with the highest effectiveness was maize (Fig. 4).

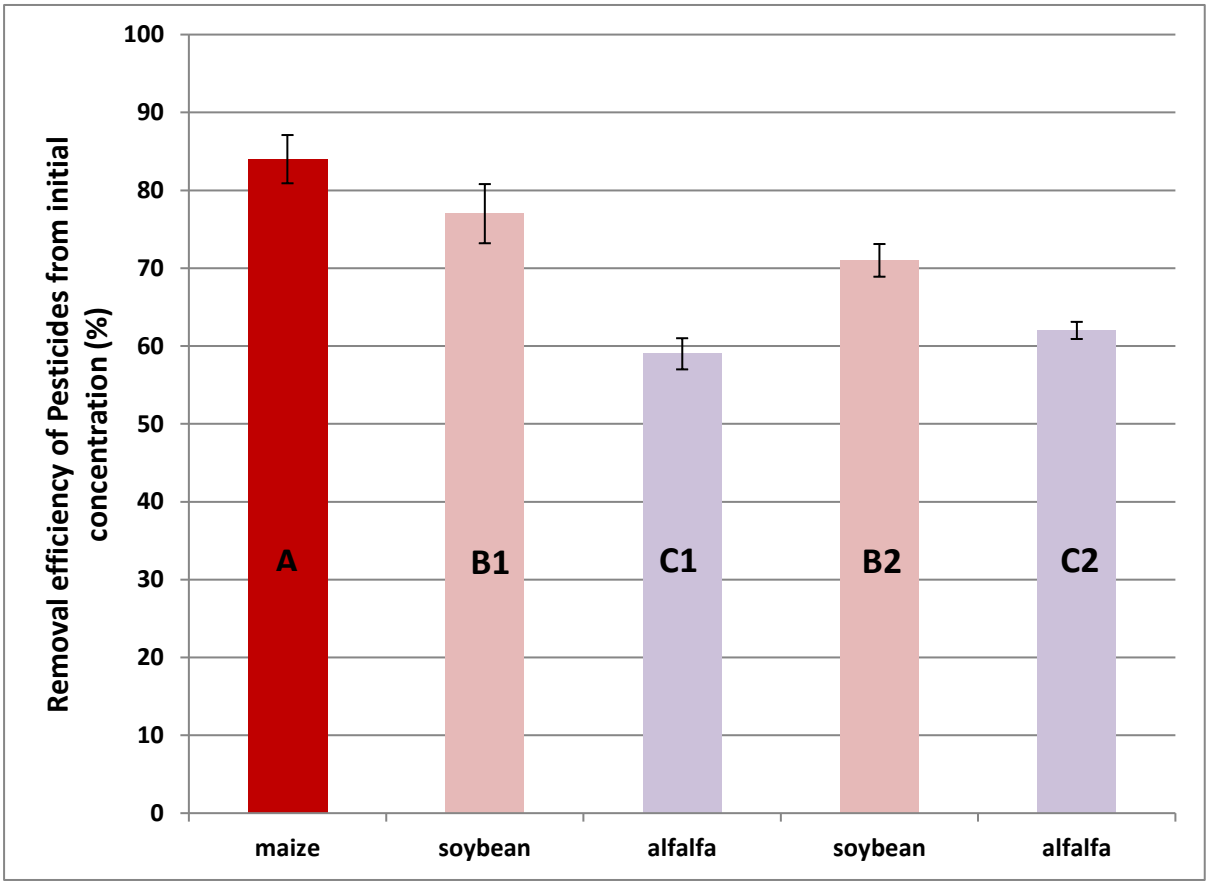

Figure 4. Our experiment results using Micrococcus strains and plants for decontaminating soils with organochlorine herbicides contamination. Period: 1 mth; Soil contamination: 100 ppm; temperature: 25^0 C; plants sowed following 7 days from inoculation of bacteria suspension. A: Pollutant: Oxadiazon; Bioremediation agent: Consortia of Micrococcus strains (sp.DR44 + sp. HEXBA04); Plant: maize. B1: Pollutant: Linuron; Bioremediation agent: Micrococcus sp. NTCT2665; Plant: soybean. B2: Pollutant: Linuron; Bioremediation agent: Micrococcus sp. NCTC2665; Plant: alfalfa. C1: Pollutant: Quizalofop-p-ethyl; Bioremediation agent; Consortia of Micrococcus strains (sp. Pv8 + sp. BP31A); Plant: soybean. C2: Pollutant: Quizalofop-p-ethyl; Bioremediation agent: Consortia of Micrococcus strains (sp. Pv8 + sp. BP3_1A); Plant: alfalfa

A reduction of 84% oxadiazon content in soils with Linuron, the amount of organochlorine pollutant in contaminated soils decreased 77% due to the actions of Micrococcus sp. NCTC2665 and alfalfa. Phytoremediation of soils that had quizalofop-p-ethyl contamination is most effective using

concortia (Micrococcus sp. Pv8 and Micrococcus sp. BP3_1A) with alfalfa and we found that content in soils decreased by 71%. Therefore, the results of our study shows the newly devised method is viable for effective phytoremediation of organochlorine herbicides polluted soils.

Herbicide metabolism in crops, insects and microorganisms is necessary for herbicide development, for effective and safe use, as well as for developing bioremediation strategies for contaminated receiving environment. Herbicide biotransformation occurs via multi step processes known as cometabolism or metabolism. Pesticides present in water or soil are usually metabolized into non-toxic or less toxic products by several metabolic processes in crops. Xia and Ma (2006) have reported the degradation of ethion, anorganophosphorus insecticide by water hyacinth (*Eichhonia crassipes*). Similarly, poplar cuttings were able to degrade atrazine to its less toxicmetabolites by hydrolyzing and dealkylating it in leaves, stems and roots (Chang et al., 2005). In another study, DDT was observed to convert into dehalogenated products by the aquatic plant, *Elodea canadensis* (Garrison et al., 2000).

Metabolic resistance is the principal mechanisms used by insects to escape the adverse effects of both synthetic and natural toxins. Accordig to Li et al. (2007), major enzyme types responsible for the detoxification of pesticides are the cytochrome P450 monooxygenases (P450s), glutathione transferases (GSTs) and carboxylesterases (COEs). The mostly encountered initial step in the bio-transformation of pesticides (inc. other organic xenobiotics), is oxygenation and oxidativeenzymes, such as, peroxidases, cytochrome P450s, and polyphenol oxidases mediate these reactions. The P450s are studied most frequently which are oxidative enzymes in plants and animals and are the enzymes which have the top importance in Phase I pesticide metabolism (Barrett, 2000).

Kurashvili et al. (2016) studied the potential of detoxification of Pseudomonas strains and plants for bioremediate soils contaminated by PCB, DDT and Lindane. According to their results, in medium with alfalfa, maize and soybean, the removal efficiency was between 67% - 80% with detoxification of enzymes including phenoloxidase, cytochrome P450, peroxidase and glutathione S-transferase. Kurashvili et al. (2014) found that soybean, maize, and alfalfa are relatively strongly tolerant to organochlorine pesticides. In these plants there was a reduction of growth parameters of less than 10-15% in high concentrations of toxic compounds (for DDT, Lindane and DCB – 1 mM; for PCP – 0.1 mM). Other plants were found to be sensitive to all 3 pesticides and growing was suppressed by 30-60%. There are various studies of the use of genetically modified crops in pesticide phytoremediation; cytochrome P450 enzymes glyphosate oxidase, a Rieske non-heme monooxygenase (DMO) which converts dicamba to 3.6- dichlorosalicyclic acid which has been expressed in *A. thaliana*, tomato, tobacco and soybean crops (Behrens et al., 2007) and aryloxyalkanoate dioxygenase enzymes (TfdA) which have been expressed in corn (patented for the degradation of 2,4-D and pyridyloxyacetate herbicides) (Scott et. al., 2008).

Conclusion

The findings show that maize, alfalfa and soybean indicate detoxification induction of enzymes including phenoloxidase, peroxidase, cytochrome glutathione S-transferase, and P450 containing monooxygenase. From this, the transformation of the examined pesticides in plants generally occur through direct oxidation, direct conjugation, or through oxidation and subsequent conjugation pathways. The results indicate that maize, alfalfa and soybean can be used as plant phytoremediators; 9 strains from Micrococcus as pesticide eliminating agents, for decontamination of contamination of an environment caused by organochlorine herbicides. Thus, based on the results of our experiments, our subsequent biotechnological method is effective in decontaminating soil subject to pollution of organochlorine herbicides. Microorganisms also have a similar degradative process of herbicide, and unlike plants they genetically rapidly adapt to chemicals present. The degradation and detoxification potential of particular microorganisms are being used for detoxification of contamination of water and soil caused by a wide variety of chemical pollutants. In particular, phytoremediation, a process where microorganisms and plants jointly detoxify and degrade contaminants resulting in eradication of contaminants. Bioremediation process for removal and/or detoxification of pesticides from the contaminated soil and water has now emerged as the best option. Nowadays, various bioremediation approaches are available to address the problem of decontaminating the environmental compartments from these hitherto essential toxicants at least needed for vector control despite the persistent and recalcitrant nature of several pesticides as well as their associated health hazards. Pest control over the last 20 years have had a dramatic change due to biotechnology approaches and there is a continual exploration of interesting new ways. However, such progresses continue to be reliant on more advances in the physiology and microbial of whole-plants. An understanding of the mechanisms, stability, regulation, specificity and enzyme expression implicated in pesticide metabolism may provide answers to numerous contemporary questions. Ultimately, we believe that our study will help in promoting an appreciation of the environmentally unharmed and commercial applications of pesticides.

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