Phyto-Microbial Degradation of Glyphosate in Riyadh Area

Al-Arfaj A.[2], Abdel-Megeed A.[1,2]*, Ali H.M.[2,3] and Al-Shahrani O.[2]

[1]Department of Plant Protection, Faculty of Agriculture (Saba Basha),
Alexandria University, Alexandria 21531, Egypt.
[2]Department of Botany and Microbiology, College of Science, King Saud University,
P.O. Box: 2455 Riyadh 11451, Saudi Arabia.
[3]Timber Trees Research Department, Sabahia Horticulture Research Station,
Horticulture Research Institute, Agriculture Research Center, Alexandria, Egypt.

Green house studies were conducted to determine the ability of plant Amaranth, *Amaranthus caudate* and two isolated bacterial strains from rhizosphere region for cleaning up glyphosate residues in soil and plants. The analytical study of the degradation of glyphosate was carried out in the laboratory conditions. Amaranth, *Amaranthus caudate* and two isolated bacterial strains namely *Pseudomonas aeruginosa* and *Bacillus megaterium* could degrade glyphosate in 5 days. These results suggested that phytoremediation could accelerate the degradation of glyphosate residues in plants and in rhizosphere region as well. Glyphosate had strong effect on bacterial DNA where many DNA bands were affected. This could be explained that the effect of herbicide glyphosate on the protein profile may reflex somehow DNA mutation occurred during the assimilation of those toxic compounds. Therefore, the alteration occurred in both DNA and protein profiles is considered a degree of tolerance that lead to DNA mutation to cope with the assimilation of this compound. Therefore, the phytoremediation way could be a promising tool in program is to protect public health and the environment by ensuring the safety and availability of herbicides and pesticide alternatives.

Key words: Microbial degradation, Phytoremediation, *Amaranthus caudate*, glyphosate.

Most of the chemicals are produced and used in efforts to improve human health, standard of living and safety through advancements in manufacturing, agriculture and agribusiness, medicine and to strengthen national defense (Hall *et al.*, 2001). Millions of tons of pesticides applied annually are used in modern agriculture to increase production through controlling harmful effects caused by the targets pests. However, less than 5% of these products are estimated to reach the target organisms, with the remainder being deposited on the soil and nontarget organisms, as well as moving into the atmosphere and soil (Pimental and Levitan 1986). However, the production, distribution, use, misuse, disposal, of these chemicals has polluted environments to levels that threaten the health of human and indeed whole ecosystems balance or sustainability (Boopathy, 2000). One of the most important problems with the use of pesticides is their possible persistence in the environment and therefore, their possible incorporation into the food chain affects ecosystem and all human beings (Pimentel *et al.*, 1991). Synthetic organophosphorus (OPs) compounds are the most widely used pesticides, and unacceptable levels of environmental residues of these compounds have been found in many countries worldwide. Although most OPs compounds are not persistent, they still cause

* To whom all correspondence should be addressed.
Tel.: +966-1-4676940; Fax: +966-1-4675833
E-mail: Alter. E.-mail: hekemdar@yahoo.com

broad area pollution from continued use in agriculture and public health (Stephen *et al*., 2008).

Glyphosate (N-(phosphonomethyl)glycine) is a nonselective broad-spectrum herbicide used extensively throughout the world. It is a broad-spectrum systemic herbicide used to kill weeds, especially annual broadleaf weeds and grasses known to compete with commercial crops grown around the globe. A 2009 study on mice has found that a single the injection of Glyphosate in concentration of 25 mg/kg caused chromosomal aberrations and induction of micronucleim (Prasad *et al*., 2009; Barkovski *et al*., 1996).

A study of various formulations of glyphosate found that risk assessments based on estimated and measured concentrations of glyphosate that would result from its use for the control of undesirable plants in wetlands and over-water situations showed that the risk to aquatic organisms is negligible or small at application rates less than 4 kg/ha and only slightly greater at application rates of 8 kg/ha (Solomon *et al*., 2003). Glyphosate formulations are more toxic for amphibians and fish than glyphosate alone (Salbego *et al*., 2010). Aquaculture, freshwater and marine fisheries supply about 10% of world human calorie intake (Relyea, 2005; Schmidt, 2001).

Pesticides may be transformed or biodegraded because of sunlight, microbial action, or plant enzymes. Glyphosate degrades relatively rapidly in soils by microbial processes (Laitinen *et al*., 2006). Plants take up pesticides mainly through leaf surfaces, fruits, and roots (Gibson and Satler, 1992; Zak *et al*., 1999). The most frequently detected degradation product in soil and water is aminomethylphosphonic acid (AMPA). Little is known about the enzyme(s) involved in the degradation of glyphosate to AMPA in plants.

Phytoremediation has been defined as the use of green plants and their associated micro-organisms, soil amendments and agronomic techniques to remove contain or render harmless environmental contaminants (Hedge and Fletcher, 1996; Fang *et al*., 2004; Pierre and Augustin, 2006). Phytoremediation increases soil organic carbon, soil bacteria, and mycorrhizal fungi, all factors that encourage degradation of organic chemicals in soil. Rhizosphere bioremediation is also known as phytostimulation or plant-assisted bioremediation

(Mishra, *et al*., 2006). Rhizosphere bioremediation or rhizodegradation is the enhanced biodegradation of recalcitrant organic pollutants by root-associated bacteria under the influence of selected plant species (Taylor *et al*., 1993; Forsyth *et al.*, 1995). Emphasis is given to rhizosphere biodegradation of polychlorinated biphenyls and polycyclic aromatic hydrocarbons (Taha and Abdallah, 2004; Boyle, 1995; Ying *et al*., 2009).

Once within the plant the pesticide taken up can be distributed within the plant either from cell to cell or via the plant vascular system. The degree and manner in which a pesticide is taken up and distributed within the plant is dependent on the physical and chemical properties of the pesticide (Shiota, *et al*., 1996; Yee, *et al*., 1998; Marin *et al*., 1998).

The simultaneous cleanup of multiple, mixed contaminants using conventional chemical and thermal methods are both technically difficult and expensive; these methods also destroy the biotic component of soils, plants have shown the capacity to withstand relatively high concentrations of organic chemicals without toxic effects, and they can uptake and convert chemicals quickly to less toxic metabolites in some cases (Khatoon *et al*., 2004). Several fungi and bacteria able to degrade pesticides were isolated from the maize rhizosphere. Successful phytoremediation of high concentrations of the pesticides alachlor and metachlor was demonstrated using an integrated strategy: maize plants and a chloracetamide-detoxyfying rhizobacteria, *Pseudomonas fluorescens* strain UA5-40 (Canet *et al.* 2001; (Anderson *et al.*, 1994). Microbial degradation is an important step in the disappearance and, in most cases detoxification of pesticides. Herbicide biodegradation may prevent the problem of environmental pollution but it can also reduce the effectiveness of a compound in controlling targeted pests (Cheng *et al*.,2007).

Red amaranth, *Amaranthus tricolor* L. could utilised of three selected pesticides, cypermethrin, chlorpyrifos and carbofuran, using in Bangladesh at various dose levels. It was found also that the uptake of chlorpyrifos by red amaranth from soil and its accumulation therein was higher on the 3rd day of application. The residue level of carbofuran was very low both at 1st and the

3rd day of application (Ondrej *et al.*, 2009; Belden *et al.*, 2004).

Microbial ecologists have identified ranges of critical environmental conditions that affect the activity of soil micro-organisms. The use of plant species for phytoremediation has been well documented. Oxygen, metabolism, nutrients sufficient, nitrogen, phosphorus and other nutrients, moisture, soil, environment pH, and environment temperature affect the phytoremediation quality. Many of these environmental conditions can be controlled and managed to enhance the biodegradation of organic constituents (Mathe-Gaspar and Anton, 2005).

Therefore this study was conducted to use *Amaranthus caudate* and (The plant was chosen because of their short life cycle and their phytoremediated background, which makes them suitable for this investigation, besides they are edible. isolated microorganisms to degrade glyphosate residues in soil in Riyadh area.

MATERIALS AND METHODS

A study was undertaken to monitor the efficacy of Amaranth, *Amaranthus caudate* for the degradation of organo-phosphorous glyphosate at recommended dose levels and to isolate microorganisms capable of efficient degradation of this herbicide from soils. The experiments were carried out at both Al-Kharj farm "South Riyadh" and Department of Botany and Microbiology, College of Science, King Saud University. Laboratory and field trials were conducted by cultivating Amaranth, *Amaranthus caudate*, for studying its ability for the degradation of glyphosate (Fig. 1). The herbicide used in this study was an analytical standard of glyphosate (99.1%) was provided by Nohyaku Co., Ltd (Japan). The experimental areas were treated with glyphosate according to the normal agricultural practices and recommendation guidance of Ministry of Agriculture in Saudi Arabia.

Herbicide used

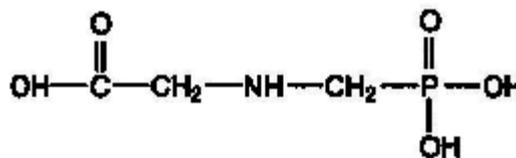|	
Chemical group	Organophosphates
Chemical name	N-(phosphonomethyl)glycine
Common name	
Glyphosate	
Trade name	Tiller® 480SL

Fig. 1. Chemical structure of Glyphosate

Soil sample preparation

Laboratory bench-scale soil washing experiments were performed to clean the contaminated soil. The soil samples were collected from 15-30 cm depth of experimental field. Samples were supplied as air dried at 35°C and ground to <2mm. The soil samples were washed by using the 0.1 M HCl and 0.05 M sulfuric acid, where 70 L of distilled water was mixed together with the acid and 100 kg soil sample. The whole mixture was shacked for 6-7 hrs continuously. Afterwards, the soil sample was washed again with the ordinary water for 3 hrs. After 24 hrs, the soil sample was washed with 6% H_2O_2 for 6 hrs before washing again with distilled water, then left for 24 hrs. The samples were air dried by exposing to the sunlight directly for 12 hrs. Visible insects and pests were removed from the soil weighed quantity of soil and the size of 0.9 kg to 1 kg each planter has been developed, material By 10 g of fertilizer added and municipal rates of fertilization of the soil to compensate for the possible loss of nitrogen from organic matter, phosphorus and potassium were placed 3 g of fertilizer per planter and nitrous before the soil was sterilized Ass pollutants of any effective solution by placing them in water and chlorine.

Plant materials

The experiment was conducted by growing the plants in pot in roof condition at the experimental site of Al-Kharj farm south of Riyadh during the period from during November, 2012 to February, 2013. Amaranth, *Amaranthus caudate*, was selected to examine its phytoremediation ability.

Pesticides and application

Glyphosate (99.1%) and reagents were high purity and analytical grade. The concentration of the herbicide was added according to the recommendations of the Ministry of Agriculture, Pesticide Manual, Saudi Arabia. Appropriate volumes of glyphosate were mixed with the soil

and bedding materials to give 1000 ppm glyphosate. Uniform mixing in soil was achieved by spreading the soil on a plastic sheet and spraying it with pesticide solution followed by thorough mixing. The uniform mixing of pesticide was checked by removing random samples and analysing for pesticide residues. The analysis of glyphosate residues were carried out at the Agrochemical Residue Research and Analysis Laboratory, College of Science of King Saud University, KSA. The herbicide was applied by mixing with water and the emulsion within the spray tank was shaken well and sprayed covering the soil. The growth rates for each plant was monitoring after three weeks of cultivation.

Preparation of standard solutions

Standard curve was carried out according to the method described by Al-Meshal, (2013).

Soil samples

After one month from cultivation, samples were collected and the physical and chemicals analysis of soil were carried out according to the method described by Al-Qurainy and Abdel-Megeed, (2009) (Table 1).

Plant samples extraction

25 grams of the whole plant were chopped in small pieces and homogenized with acetonitrile in a blender. Hundred ml of acetonitrile was added and blended for 2-3 min at moderate to high speed. The homogenate was filtered by passing through glass wool and transferred the filtrate to a jar and extracted with an additional 100 ml acetonitrile.

Quantification of glyphosate by Gas Liquid Chromatography Mass spectrometry (GC-MS)
Determination of glyphosate residues in plants

The extraction of glyphosate was carried out as described by Alferness and Wiebe, (2001). Two grams of plant samples was extracted with 10 mL of water in a 15 mL centrifuge tube, shaken, placed in a sonicating bath for 20 min, and then centrifuged at 47000 rpm, 20 °C, for 20 min. Supernatant was removed. The tissue sample pellet was extracted a second time as in the first extraction. The volume of the combined supernatant was measured, and then 5 μL of 12.1 M HCl was added and shaken. Four milliliters was transferred to a 20 mL scintillation vial with a

Teflon-lined cap, shaken with 4 mL of methylene chloride, and centrifuged for 10 min. A portion (1.8 mL) of the water layer was taken, and 200 μL of acidic modifier [16 g of KH_2PO_4, 160 mL of H_2O, 40 mL of methanol (MeOH), 13.4 mL of HCl] was added. One milliliter was loaded to a cation exchange resin column (AG 50W-X8, H+; Bio-Rad Laboratories, Hercules, CA) previously equilibrated with two 5 mL portions of water. The sample was eluted until the level of column bed. CAX mobile phase (160 mL of H_2O, 40 mL of MeOH, 2.7 mL of HCl) (0.7 mL) was added, eluted, and discarded. Twelve milliliters of CAX mobile phase was again added to the column to elute the analytes. The eluate was collected in a 20 mL vial and evaporated to dryness using a Savant speed vac. To the dried sample was added 1.5 mL of CAX mobile phase, and then the vial was placed in a sonicating bath for 30 min. A 20 μL aliquot was taken and added to 640 μL of a solution of 2,2,3,3,4,4,4-heptafluoro-1-butanol   and trifluoroacetic anhydride (1:2) in a chilled 4 mL vial. The mixture was allowed to equilibrate at room temperature for 10–15 min. The vial was transferred to a heating block at 90 °C for 1 h and then allowed to cool to room temperature. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in 80 μL of ethyl acetate containing 0.2% citral; 50 μL was transferred to a GC vial and analyzed by GC-MS. This method afforded 90 and 86% recoveries of glyphosate and AMPA, respectively, on the basis of duplicate extraction experiments in which samples were fortified with 100 ng standards per gram of sample. For the analysis of glyphosate, the temperature program was as follows: initial, 70 °C, held for 3.5 min, raised to 160 at 30 °C/min rate, raised to 270 at 70 °C/min rate, raised to 310 at 35 °C/ min rate, and finally held at this temperature for 3 min. The sample injection volume was 1 μL. Glyphosate in the samples was quantitated from a calibration curve.

Determination of glyphosate residues in soil

Four replicates from each treated soil were taken around each plant; afterwards they were mixed homogeneously and subjected to analysis. Extraction and analysis of the glyphosate was performed according to published procedures (Alferness and Wiebe, 2001).

Extraction, isolation and cultivation of rhizosphere bacteria

Soil samples were collected from rhizosphere soil. Soils from 6 cm radius from each plant and 6 cm depth from the surface were collected and sun dried. Pots were filled with these soils and filter papers were put appressed on the soil and sterile liquid bacterial culture medium was pipetted on the filter paper to allow growth of bacteria in colonies on the filter paper. The pots were keptt on the laboratory bench at room temperature (30-35ºC). Synthetic media (bacto-peptone 10 g, NaCl 5 g, Yeast ext. Powder 0.5 g and agar powder of 15 g) in a litre of distilled water. The enrichment and propagation of the isolates were carried out in sterilized Erlenmeyer flasks media. The cultivation was carried out in sterilized 100 ml flask containing 20 ml media. The pH value of the culture solution was adjusted to 7.0 with NaOH. The flasks were tightly sealed with screw caps. After the incubation period of 24 hrs on a rotary water bath shaker at 37°C and 200 rpm, the growth was observed. Agar dishes were sealed with tape and incubated upside down at 37°C for 24 hrs till the colonies were observed. Well-grown bacterial colonies were picked up with a sterile wire loop and cultured separately in liquid culture tubes. These were numbered numerically. Streaking method was repeated to get pure colonies. After significant cell growth was achieved in the enrichment culture, the bacteria were sub-cultivated in 100 ml Erlenmeyer flasks. The isolated strains were characterized and identified depending on the cell wall composition, substrate selectivity and the growth temperature. Further classification and identification was performed by Research Central Laboratory, College of Science, King Saud University, Saudi Arabia.

Bacterial DNA extraction, isolation and purifications

Bacterial DNA was carried out according to the method described by Al-Qurainy and Abdel-Megeed, (2009).

Quantification and restriction of DNA

DNA quantification and quantitated was carried out according to Sambrook *et al.*, 1989). The gel was prepared with 0.8 % (w/v) agarose dissolved in TBA. The run was performed at 77 volt. The gels were stained with Sybrâ Green (Biozyme, Germany).

Effect of glyphosate residues on bacterial DNA by Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR)

RAPD-PCR method is based on the Polymerase Chain Reaction (PCR) using short nucleotides primers of arbitrary sequences. The random primer (OPC-07) 5' GGTGACGCAG '3 used in this study was purchased from Amersham bioscience, Sweden. Amplification reaction solutions were prepared in a final volume of 50 ìl containing 10 mM Tris -HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl and 100 M each of dATP, dGTP, dCTP and dTTP (Boehringer Mannheim, Germany), 2.5 M primer, 1.25 units of Taq DNA Polymerase (Boehringer Mannheim, Germany) and approximately 50 ng of DNA. The amplification was performed in thermal cycler (PCR Thermocycle: Elmar Cetus 420, Elmar Cetus USA) where the program was as follows: universal denaturation cycle (5 min at 94°C), 45 cycles of annealing/extension reactions (1 min at 94°C, 1 min at an optimum annealing temperature 36°C for each used universal primer and 2 min at 72°C) and cycle of final extension step (5 min at 72°C) was followed by soaking at 4°C.

Electron microscopy for bacterial strains treated by glyphosate

After the cells were treated by glyphosate, the shape of the cells was examined by electron microscopy (Amray Model 1820 Scanning Electron Microscope, UK). The cells were fixed at 24°C for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), dehydrated with a serial concentration of ethanol, and then dried on a critical point dryer (HCP-2; Hitachi Co.). The dried cell samples were coated with gold, and examined using a scanning electron microscope (S-4100; Hitachi Co.). For transmission electron microscopy, dehydrated cells were embedded in a medium type LR white resin (Sigma Chemical Co., St. Louis, Mo.), which was polymerized at 60°C for 24 h. The polymerized samples were sliced with an ultramicrotome and observed using a transmission electron microscope (Hitachi Co.). This work was carried out by Research Central Laboratory, College of Science, King Saud University, Saudi Arabia.

RESULTS AND DISCUSSION

Phytoremediation glyphosate

Amaranth, *Amaranthus caudate* was able to degrade and assimilate (1000 ppm) the herbicide glyphosphate in 5 days (Figs. 3,4). It was clear that the organophoaouros compound glyphosphate almost disappeared rapidly (> 99% from the parent compound). This degradation rate is unique comparing with the previous studies (100 ppm) (Aijun *et al.*, 2005). Glyphosphate metabolites could not be quantified and this was due to the disappearance of the metabolites and the intermediated compounds fast.

In this study, there was evidence of increases in bacterial activity and populations in the treated soil with glyphosate. This activity was represented in bacterial biomass and pH fluctuation during the experiments. This stimulation may be due to the fact that bacteria are the main microbial degraders of glyphosate (Krzysko-Lupicka *et al.*, 1997). Araujo *et al.*, (2003) found that glyphosate

Table 1. Physical and chemical properties of soil used in the experiment

Parameters	value
Particle-size distribution,%	
Sand	11.92
Silt	14.18
Clay	73.75
Textural class	Clay
EC (1:2, soil: water extract), dS/m	5.71
pH	8.01
Organic carbon (OC),%	2.32
Sodium Adsorption Ratio (SAR)	5.27
Soluble Cations, meq/l	
Calcium (Ca^{+2})	21.82
Magnesium (Mg^{+2})	12.78
Sodium (Na^+)	21.25
Potassium (K^+)	0.86
Soluble Anions, meq/l	
Carbonates ($CO_3^=$)	-
Bicarbonates (HCO_3^-)	5.00
Chloride (Cl^-)	39.00
Sulphate ($SO_4^=$)	13.10

Table 2. Morphological and physiological properties of three bacterial strains

Physiological characteristic	*P. aeruginosa*	*B. megaterium*
Shape of the cell	rod-shaped	rod-shaped
Gram reaction	-	+
Aminopeptidase	+	+
Caprate	+	+
Catalase	+	+
Citraconate	+	+
Citrate	+	+
Denitrification (to N2)	-	-
D-xylose	+	+
Erythritol	-	+
Gluconate	+	+
Glucose	+	+
Hydrolysis of gelatine	+	+
Pigments fluorescent	+	-
Pyocyanin	-	-
Sorbitol	+	+
Trehalose	+	+
Oxidase	+	+
Phenylacetate	+	+
NO_2 from NO_3	-	-
Arabinose	+	+
Benzylamine	+	+

amendment did not affect bacterial cultural population, while bacteria and actinomycetes populations increased. This effect was larger in soils that had greater previous exposure to glyphosate. Other studies have shown that glyphosate use is associated with an increase in the plant pathogens *Fusarium* and *Pythium* (Meriles *et al.,* 2006). Glyphosate can stimulate the growth of mycorrhizal fungi in vitro, Kremer *et al.,* (2005), who found that glyphosate increased stimulated microbial biomass and Wardle and Parkinson (1990), glyphosate can inûuence the biomass of bacteria and plants directly and, indirectly, as toxic compounds. These results indicate that bacteria and plants may use glyphosate as a nutrient and energy source in case of being able to assimilate the compounds.

In fact, metabolic pathway diversity depends on the chemical structure of these compounds, the organisms, environmental conditions, metabolic factors, and the regulating expression of these biochemical pathways. Knowledge of these enzymatic processes, especially concepts related to pesticide mechanism of action, resistance, selectivity, tolerance, and
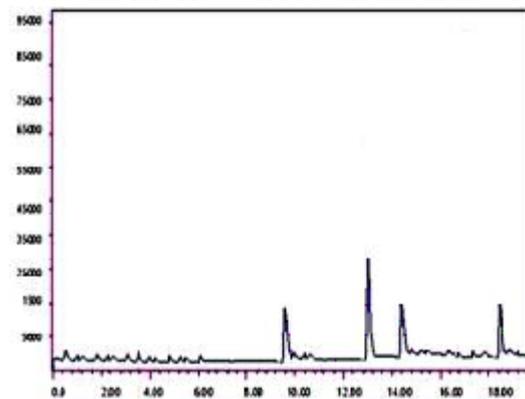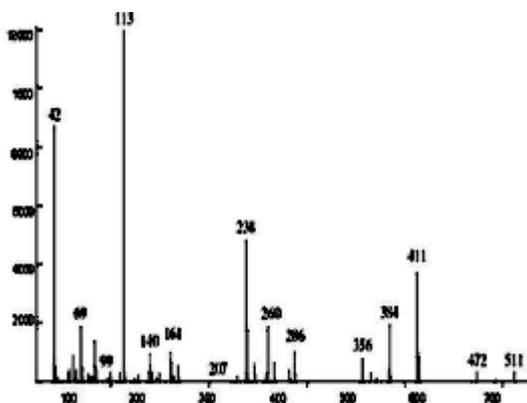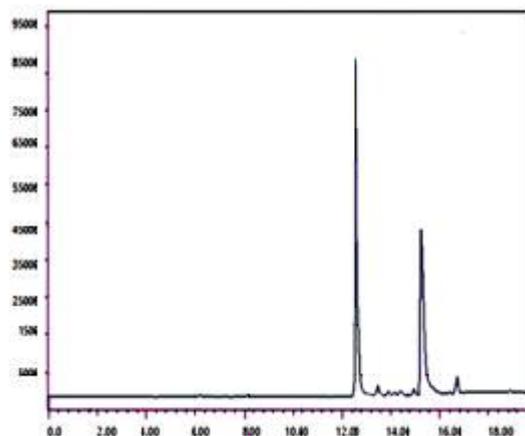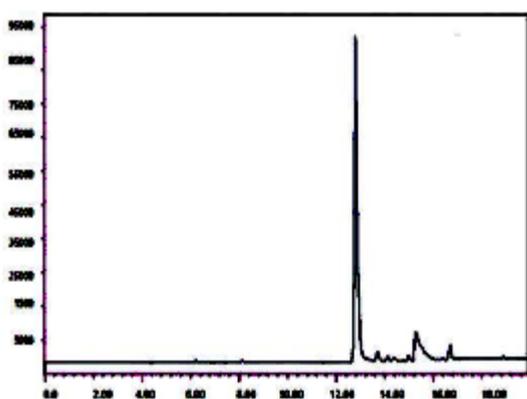

Fig. 3. GC-MS analysis and mass spectrum of glyphosphate. After zero time (left) and 5 days (right)

Fig. 2. GC-MS analysis and mass spectrum of glyphosate; Zero time (A) and mass spectrum (B)
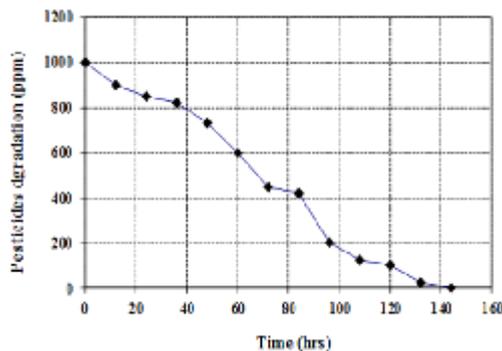

Fig. 4. GC-MS analysis and mass spectrum of glyphosate after 5 days

environmental fate, has advanced our understanding of pesticide science, and of plant and microbial biochemistry and physiology ((Daohui *et al.*, 2003).

Plant-microbial interactions in the rhizosphere

Plant-microbe interactions may be beneficial or harmful to the plant depending on the specific microorganisms and plant involved. Plant beneficial interactions can be divided into three categories (Brimecombe *et al.*, 2001; Okeke *et al.*, 1996). Detrimental interactions within the rhizosphere involve deleterious rhizobacteria which inhibit shoot or root growth without causing any other visual symptoms by the production of phytotoxins such as or phytohormones (Schippers *et al.*, 1987). Microorganisms play role in the degradation of pesticide in nature. Bacterial strains isolated from nature are able to degrade a variety of pesticides. However, reports on microbial degradation of glysophate are very scanty. Liu *et al.*, (2003) reported on glysophate degradation using fungi, *Aspergillus niger*.

In contrast to previous studies that aimed to increase the removal rate of pollutants, our results make possible a substantial increase in OP degradation in the environment. Further studies should be conducted to investigate the mechanisms by which the plants and microorganisms can assimilate these compounds.

Isolation and characterization of *Pseudomonas aeruginosa* and *Bacillus megaterium* from *rhizosphere* zone

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root.

This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. The rhizosphere is a centre of intense biological activity due to the food supply provided by the root exudates. Bacteria, actinomycetes, fungi, protozoa, slime moulds, algae, nematodes, earthworms, millipedes, centipedes, insects, mites, snails, small animals and soil viruses compete constantly for water, food and space. Soil chemistry and pH can influence the species mix and functions of microbes in the rhizosphere. Two bacterial strains were isolated from the rhizosphere of the cultivated plants. They were characterized and identified as *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Bacillus megaterium*. Table (2) presents more details about the morphological and phenotypic characterization.

Pseudomonas aeruginosa and *Bacillus megaterium* was isolated from rhizosphere of the cultivated plant and further identification and characterization were carried out on mineral salts medium (MSM) with glyphosate as a carbon source. *Pseudomonas aeruginosa* was identified as a member of the genus Pseudomonas based on physiological characteristics and the morphology of the cells. This gram negative, rod shaped bacterium was 0.3 - 0.7 µm in diameter and 1.2 - 4.0 µm long (Fig. 5). The colonies were smooth and on LB media. During the bacterial growth a frothy emulsion was observed in the growth media. Furthermore, one or more protuberances were observed on many colonies. Moreover, the colonies seemed relatively small in case of earlier
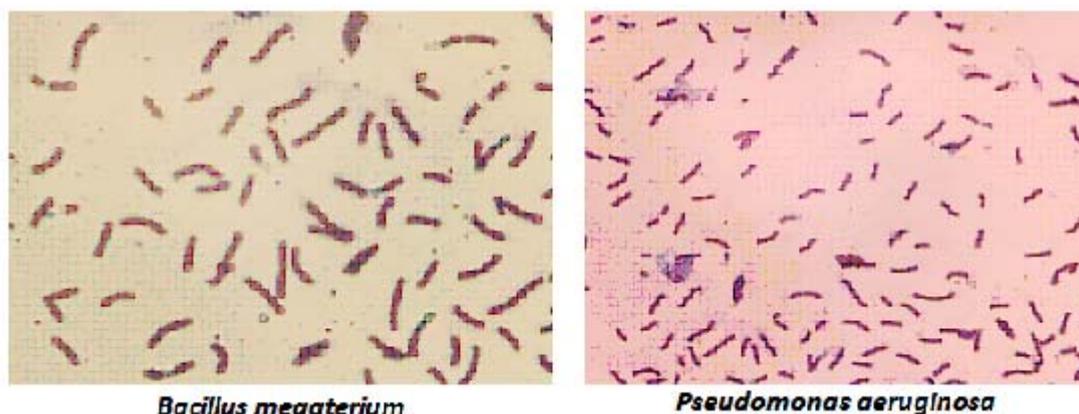

Bacillus megaterium   **Pseudomonas aeruginosa**

Fig. 5. Isolated bacteria from Rhizosphere regions

growth on glypohsate mineral salt medium compared to the growth on LB medium. Fatty acids composition indicated that the isolate belongs to the genus of Pseudomonas and 16S rDNA sequence was 98 % identical to *Pseudomonas aeruginosa.* For *Bacillus megaterium* was also isolated from rhizosphere area (Fig. 6) and further identification and characterization were carried out on mineral salts medium with supplemented with glysophate as carbon and phosphorous sources. This bacterial strain was a gram positive, oxidase negative, catalase positive rod and produced creamy secretions on MSM medium. It is known to be able to survive in some extreme conditions such as desert environments and polluted areas due to the spores it forms. Colonies form in chains due to sticky polysaccharides on the cell wall. Furthermore, the colonies were beige-red on TSB agar, salmon-red on GYM agar and shiny. The strain produced pigmented circular colonies on pesticides MSM. The isolated strains grew from 15 to 25°C. The characterization showed that the 16S rDNA of the isolates had 97.6% identity to the 16S rDNA sequence of Bacilli.

Microscopic examination exhibited the ability of *Pseudomonas aeruginosa* and *Bacillus megaterium* in assimilating glyphosate (Figs. 6 &7).

This Microscopic micrograph showed the direct interfacial accession represented in the close direct contact of the cells to glyphosate. This led to the increase in bioavailability and subsequent biodegradation of the pesticides. Microscopic examination showed that most bacterial cells were found around glyphosate or as aggregates in the aqueous phase.

The growth characteristics of *Pseudomonas aeruginosa* on glyphosate were peculiar. After the bacterial attack, the medium and the pesticides droplets became darker with increase of the culture age. Furthermore, glyphosate was totally degraded after 5 days post treatment. Moreover, the microscopic examination showed only the growth of the colony on the pesticides droplets with dark colours and the droplets totally disappeared after 5 days (7). One of the observations that should be taken into consideration was that during the subcultivation of the pure strain, the capabilities of attaching, biodegradation and utilizing glyphosate were not lost. Moreover, the subcultivation on complex medium and cultivation again on the previously mentioned pesticides MSM did not affect the activity and efficiency of *Pseudomonas aeruginosa* (Fig. 6).
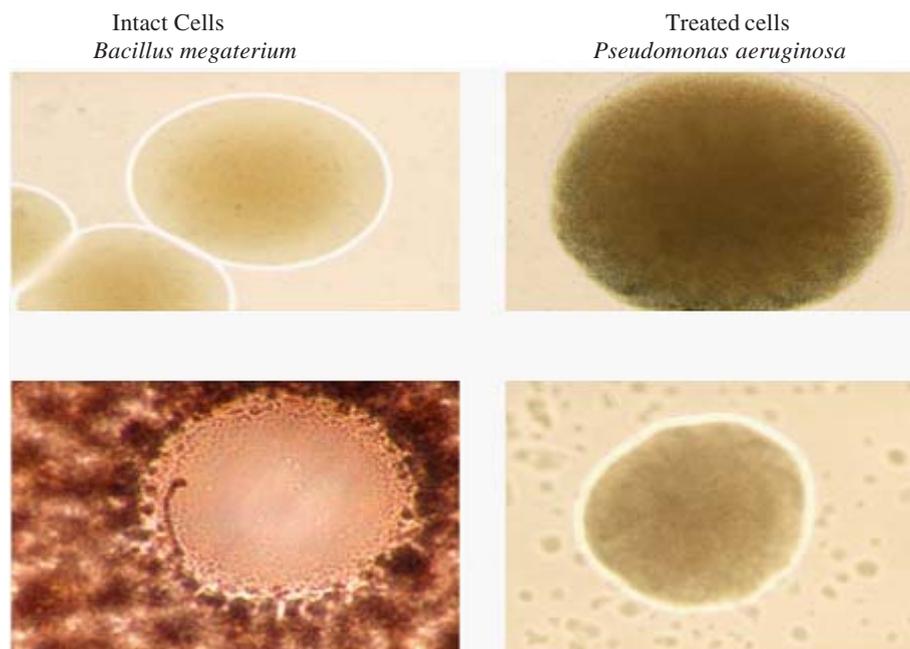
Intact Cells
Bacillus megaterium

Treated cells
Pseudomonas aeruginosa

Fig. 6. Fluorescent photomicrograph of *Pseudomonas aeruginosa Bacillus megaterium* and growth after 5 days on glyphosate mineral salt media (MSM)

Influence of glyphosate on *Pseudomonas aeruginosa* and *Bacillus megaterium* DNA

Data presented in Fig. (7) Illustrated the alteration occurred in the DNA as a result of glyphosate influence. It was clear that glyphosate had strong effect on *Pseudomonas aeruginosa* and *Bacillus megaterium*.
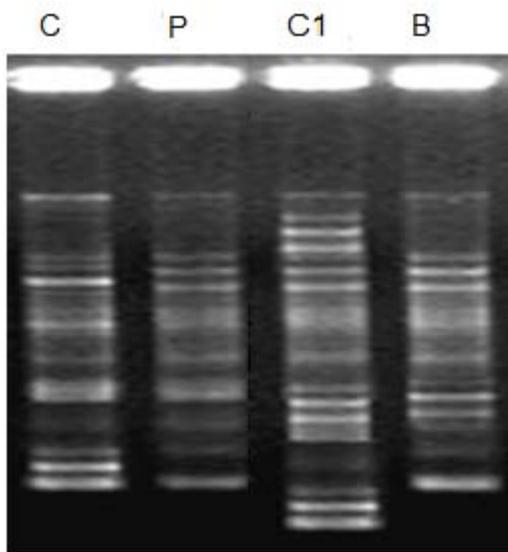

Fig. 7. Effect of glyphosate on *Pseudomonas aeruginosa* (P) and *Bacillus megaterium* (B) DNA after 5 days. Where; C and C1 control, respectively

The change occurred in the DNA bands reflected the decreased or increased in total protein in the transcriptional system and this is due to mutations synthesis of DNA resulted from the treatment of these pesticides. Our results are consistent with the result reported in literature (Sunhwa *et al.,* 2007). These mutations occurred in DNA could be explained by the hydrophobic nature and small molecular size, Glyphosate that could pass through the cell membrane and reaches the nucleus. It is suggested that within the nucleus these pesticides bind to DNA through the reactive groups of their active moieties, leading to destabilization as well as unwinding of the DNA, which could be a possible mechanism for its genotoxicity (Sunhwa *et al.*, 2007). On the other hand, these pesticides may induce oxidative stress and generation of reactive oxygen species (ROS) in insect systems (Zegura *et al.,* 2004).

These data suggest that direct binding of glyphosate to bacterial DNA is unlikely to be a mechanism through which any genotoxic effects are produced (Fig. 8). In an agreement with Gad and Abdel-Megeed (2006) stated that certain pesticides have the capacity to alter the genetic material particularly chromosomes in the tested plants.

The results proved strongly that the fungicide induced an increase in total DNA production. On the other hand, it was found that DNA content decreased with the increase with the exposure period to the herbicide. Abdel-Megeed (2004) found that the growth inhibition concentration of small concentration of the herbicide and short period of treatment did not interfere with protein and nucleic acid synthesis of the tested bacteria. In recent study there was no wide range of saprophytic microorganisms exist in the media (microbial consortia). Introduction of such fungi requires effective growth and competition with these native populations (Burton *et al.* 1998; Jacques, 2001). Additionally the bioremedial plant and microbial interaction should be able to secrete the necessary enzymes into the growth media matrix to enhance degradation of Glyphosate, molecules that they would otherwise be unable to incorporate across cell walls (Canet *et al.,* 2001).

Therefore, the proposed mechanism of bacterial degradation here can be described by two ways: one is as the sole carbon energy source; the other is by co-metabolism or co-oxidation. Some compounds are less susceptible to microbial degradation, but if some alternative carbon and energy sources for the auxiliary matrix exist, they can be degraded easily (D'Souza *et al.*, 1999). Microbial degradation of pesticides is the essence of the enzymatic reaction, and when a matrix exists, the metabolism of a substrate provides sufficient carbon and energy sources for microbial growth and induces the degradation of the corresponding enzymes, which degrade two matrices. Comparing this study to the environmental practices, one reason could be strong herbicides sorption to soil and therefore decreased bioavailability (Alexander, 1994). Another reason can be the low temperatures in soil, particularly in Northern parts of Europe and North America where soil temperatures during a large part of the year are too low for efficient microbial degradation of contaminants. The same may also be true for deeper soil layers

(Romantschuk *et al.*, 2000). Anaerobic conditions may also contribute because bacterial degradation is very slow under oxygen restrictions resulting in partial degradation with resultant toxic intermediates being formed (Romantschuk *et al.*, 2000). Other factors that can contribute to pesticide degradation in soils include the chemical nature of the pesticide, amount and type of soil organic matter, microbial community structure and activity, soil type, pH, pesticide concentration, pesticide formulation and presence of other pesticides (Schoen and Winterlin, 1987).

Bioremediation in agricultural environment has specific manner as the availability of water in soil may be a very important factor affecting the success of bioremediation, since water availability affects bacterial growth and enzyme production (Marin *et al.*, 1998). The carbon dioxide production also decreased in dry soil and remained high when the soil was wet, even though MnP and laccase activities decreased. It is likely that organisms other than white rot fungi were responsible for the production of this carbon dioxide (Boyle, 1995), which suggests that in bioremediation both the inoculant organisms and the native soil microflora are affected by water potential fluctuations. Matric potential influences the physiological activity of soil microorganisms (Zak *et al.*, 1999) and different fungi may have optimal biodegradation rates at different water availabilities, as reported by Okeke *et al.*, (1996).

Bacterial bioremediation is subject to the prevailing temperature, moisture and soil conditions (Kearney and Wauchope, 1998). The optimal requirements and conditions as pH, nutritional status and oxygen levels vary and may not always be optimal for bacterial growth or extracellular enzyme production for pollutant transformation (Singleton, 2001). Thus, the kinetics of herbicides degradation in the both laboratory and polluted agricultural soil is commonly biphasic with a very rapid degradation rate in the beginning followed by a very slow prolonged dissipation. The remaining residues are often quite resistant to degradation (Alexander, 1994). There are many reasons for organic compounds being degraded very slowly or not at all in the soil environment, even though they are biodegradable (Romantschuk *et al.*, 2000).

Scanning electron micrograph of the *Pseudomonas fluorescens* with the used pesticides

It was obvious that the morphological micrograph of the examined *Pseudomonas fluorescens* cells using scanning electronic microscope, somehow was totally changed and exhibited rather cell roughness as the results of the exposure to glyphosate. The results also revealed that *Pseudomonas fluorescens* exhibited potential efficacy for the assimilation and biodegradation of the used pesticides. From the first observation, it was found that the scanning electronic microscope assumed that the morphological changes occurred in the bacterial cells nature caused by the alteration in cell permeability as a direct result of the assimilation of glyphosate. It was clear that the surface of the bacterial cells became rough and swollen, but unlysed (Fig. 8).

In contrast, it was found that intact cells of the untreated bacterial cells had a smooth surface with overall intact morphology. It was observed that the structure of the cell wall surface layer was wrinkled, and round pores were partially deformed, indicating that there were cytoplasmic structure changes which led to flush out of the cells. Abnormal cell division was observed at high frequencies among cells that tried to divide and had the most deleterious effect with partial lysis of the cells.

The results of clearly indicate that the activity of *Pseudomonas fluorescens* against glyphosate vary with the rate of mutation occurred against the bacterial cells. These morphological changes and the mutation occurred in the bacterial cells explained somehow high efficiency of *Pseudomonas fluorescens* in assimilate and use the previously mentioned pesticides as a source of carbon and energy source. These results were in agreement with the results obtained by Al-Qurainy and Abdel-Megeed (2009) that isolated *Pseudomonas frederiksbergensis* in the degradation and assimilation of dimethoate and malathion. The study was undertaken to detect and monitor the degradation of those organophosphorous pesticide residues by microbial degradation. These results of scanning electronic microscope could help in understanding the mechanism of the biodegradation of glyphosate by and microorganisms, as well as to
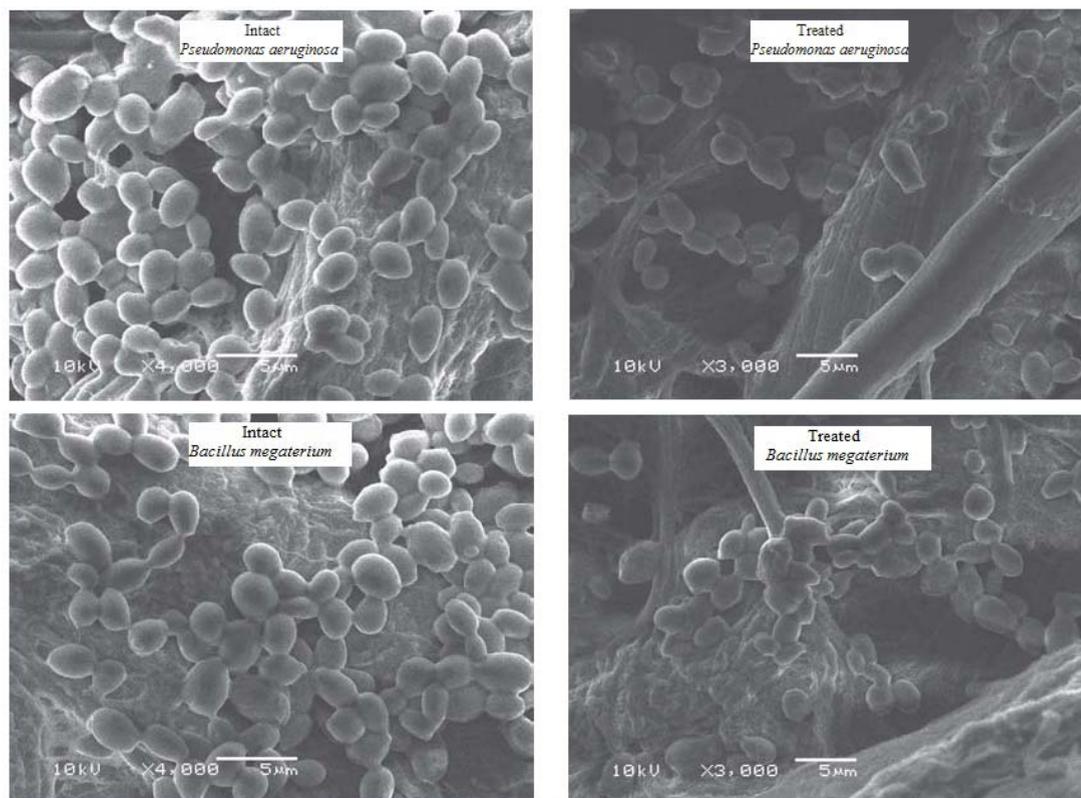
Fig. 8. Scanning electron microphotograph of treated bacterial
cells with different glyphosate after 5 days from treatment

design efficient biocatalyst allowing transformation of pesticides into non-toxic compounds.

On the other hand, the isolation of the previously mentioned bacteria has a great significance in understanding the role played together with plants in rhizospheric area. However, bacteria could be used very effectively for in situ bioremediation in an environment, which is highly contaminated with pesticides.

CONCLUSION

Amaranth, *Amaranthus caudate* and bacterial isolates were proved to be most promising and effective tools for phytoremediation strategy of glyphosate degradation. The phytoremediation by Amaranth, *Amaranthus caudate* and bacterial isolates could degrade glyphosate after 5. Present data clearly show that the pesticides treatments induce DNA mutations in different sites of the tested fungal strains, comparing with the untreated check. The effect of used herbicides on the protein profile may reflex somehow DNA mutation occurred during the assimilation of those toxic compounds. On the basis of present findings, Amaranth, *Amaranths caudate* and bacterial isolates can be recommended as potentially effective local fungal strains and environmentally safer alternative tools to protect the environment from the pollution of these herbicides.

From molecular biology, biotechnology, and enzymology point of view, will recommend for the possibility to carry outMathematical modeling of metabolic control analysis to know how metabolic pathways will respond to manipulation.

ACKNOWLEDGEMENTS

REFFERENCES

1. Abdel-Megeed A.., Psychrophilic degradation of long chain alkanes. Dissertation, Technische Universität Hamburg-Harburg. 2004; 156.

2. Aijun, Z. Hongzhang, C.; Zuohu, L., Air pressure pulsation solid state production of alkaline protease by *Bacillus pumilus* 1.1625. *Proc. Biochem.,* 2005; **40**: 1547-1551.

3. Alexander M.. Biodegradation and bioremediation. San Diego, Academic Press, 1994.

4. Alferness, P. L.; Wiebe, L. A., Determination of glyphosate and aminomethylphosphonic acid in crops by capillary gas chromatography with mass-selective detection: collaborative study. J. AOAC Int., 2001; **84**: 823–846.

5. AL-Meshal, Areej., Mycoremediation of certain pesticides polluted-soil. King Saud University, Saudi Arabia. 2013; 204.

6. Al-Meshal, Areej.., Mycoremediation of certain pesticides polluted-soil. Thesis, King Saud University, Saudi Arabia. 2013; 204.

7. Al-Qurainy F. and Abdel-Megeed A.., Phytoremediation and detoxification of some pesticides residues in Riyadh area. *World applied Sciences Journal*, 2009; **6**(7): 987-998.

8. Anderson, T. A., and Costa, J. R., Bioremediation through rhizosphere technology. Symposium Series No. 563. American Chemical Society, Washington, DC, 1994; 82-92.

9. Araujo, A., R. Monteiro and R. Abarkeli., Effect of glyphosate on the microbial activity of two brazilian soils. *Chemosphere* 2003; **52**: 799-804.

10. Barkovski, A. L.; Boullant, M. L. and Balabrfau, J., Polyphenolic Compounds Respired by Micro-organisms in: Bioremediation Through Rhizosphere Technology. American Chemical Society, 1996.

11. Belden, J. B., Pphlips, T. A., Costa, J. R., Effect of prairie grass on the dissipation, movement, and bioavailability of selected herbicides in prepared soil columns. *Environmental toxicology and chemistry*. 2004; **23**: 125-132.

12. Boopathy, R., Factors limiting bioremediation technologies. *Bioresour. Technol*. 2000; **74**: 63-67.

13. Boyle D., Development of a practical method for inducing white rot fungi to grow into and degrade organopollutants in soil. *Canadian Journal of Microbiology*, 1995; **41**:345-353.

14. Brimecombe, M.J., De Leij, F.A. and Lynch, J.M., The effects of root exudates on rhizosphere microbial populations. pp. 95-137. *In:* The rhizosphere: Biochemistry and organic substances at the soil-plant interface. Pinta, R., Varanini, Z. and Nannipieri, P. (Eds). Marcel Dekker Inc., New York, 2001.

15. Burton, S. G., Boshoff, A., Edwards, W., Jacobs, E. P., Lfukes, W. D., Rose, P. D., Russel, A. K., Russle, I. M. and Ryan, D., Membrane based biotechnological system for the treatment of organic pollutants. *Water Research Commission* Report No 687/1/98, 1998.

16. Canet R., Birnstingl J., Malcolm D., Lopez-Real J. and Beck A., Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white rot fungi in a coal-tar contaminated soil. *Bioresource Technol.*, 2001; **76**: 113–117.

17. Cheng, S. Xiao, Xiao, J. , Zhang, H., Wu, Z., Phytoremediation of triazophos by *Canna indica* Linn. in a hydroponic system. *International Journal of Phytoremediation*. 2007; **9**: 453-463.

18. CRC Press, Boca Raton, FL.

19. Daohui, L., Lizhong, Z., Yanzheng, G., Main mechanism and affecting factors of phytoremediation of organic contaminated soil. Ying Yong Sheng Tai Xue Bao. 2003; **14**: 1799-803.

20. D'Souza, T.M., C.S. Merritt and C.A. Reddy, Lignin modifying enzymes of the white rot basidiomycete Ganoderma lucidum. *Applied Environ. Microbiol.*, 1999; **65**: 5307-5313.

21. Fang J., Zeng X., Yu F., Du L., Xiong, Z., Degradation of organophosphorus pesticides by hydrogen peroxide. II. Degradation kinetics and toxicity. *Journal of South China Agricultural University*. 2004; **25**:(2) 37-40.

22. Forsyth, J. V., Tsao, Y.M. and Bleam, R.D., Bioremediation: When is augmentation needed? In Bioaugmentation for site remediation, ed. Hinchee *et al.*, Batelle Press, Columbus, USA, 1995; 1-14.

23. Gad A. and Abdel-Megeed A., Effects of spinosad and emamectin benzoate on the blood picture and DNA structure of the cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *Egyptian Sciences Magazine* 2006; **4**: 1-6.

24. Gibson, D. T., and Satler, G. S., Scientific foundation for bioremediation: Current status and future needs. American Academy of Microbiology, Washington, D.C, 1992.

25. Hall, J. C., Wickenden, J. S., and Yau, K. Y., Biochemical conjugation of pesticides in plants and microogranisms: an overview of similarities and divergences. Pages 89–118 *in* J. C. Hall, R. E. Hoagland, and R. M. Zablotowicz, eds. Pesticide Biotransformation in Plants and Microorganisms: Similarities and Divergences.

Washington, DC: American Chemical Society, 2001.

26. Jacques, J., New photochemical and electrochemical methods for the degradation of pesticides in aqueous media. Environmental Applications. *Turk J Chem*. 2001; **25**: 509- 520.

27. Kearney P. and Wauchope R., Disposal options based on properties of pesticides in soil and water. *In*: Kearney P. and Roberts T. (Eds.) Pesticide remediation in soils and water. Wiley Series in Agrochemicals and Plant Protection, 1998.

28. Khatoon, J. A., Islam, S. M., Talukder N. M., and Hossain, A. M., Monitoring the residue level of three selected pesticides in Red Amaranth. *Journal of Biological Sciences*. 2004; **4**: 474- 479.

29. Kremer, R., N. Means and S. Kim., Glyphosate affects soybean root exudation and rhizosphere micro-organisms Int. *J. Environ. Anal. Chem.* 2005; **85**:1165.

30. Krzysko-Lupicka, T., W. Strof, K. Kubs, M. Skorupa, P. Wieczorek and B. Lejczak., The ability of soil-borne fungi to degrade organophosphonate carbon-tophosphorus bonds. *Appl. Microbiol. Biotechnol.* 1997; **48**: 549-552.

31. Laitinen, P.; Siimes, K.; Eronen, L.; Rämö, S.; Welling, L.; Oinonen, S.; Mattsoff, L.; Ruohonen-Lehto, M., Fate of the herbicides glyphosate, glufosinate-ammonium, phenmedipham, ethofumesate and metamitron in two Finnish arable soils. *Pest Manag. Sci.,* 2006; **62**: 473–491.

32. Liu, Y., Kuersten, S., Huang, T., Larsen, A., MacMorris, M., and Blumenthal, T., An uncapped RNA suggests a model for Caenorhabditis elegans polycistronic pre-mRNA processing. Rna 2003; **9**: 677–687.

33. Marin S., Sanchis V., Ramos A., Vinas I. and Magan, N., Environmental factors, in vitro interactions, and niche overlap between *Fusarium moniliforme, F. proliferatum,* and *F. graminearum, Aspergillus* and *Penicillium* species from maize grain. Mycological Research, 1998; **102**: 831-837.

34. Mathe-Gaspar G., and Anton, A., Phytoremediation study: Factors influencing heavy metal uptake of plants. Acta Biologica Szegediensis. 2005; **49**: 69-70.

35. Meriles, J., S. Gil, R. Haro, G. March and C. Guzman., Glyphosate and previous crop residue effect on deleterious and beneficial soil-borne fungi from a peanutcorn-soybean rotations. *J. Phytopathol*. 2006; **154**:309-316.

36. Mishra, S., J. Kishan, and K. Verma., Monitoring organophosphorus pesticides and their degradation products formed by Fenton's reagent using solid-phase extraction gas chromatography mass spectrometry. *International Journal of Environment and Pollution*. 2006; **27**: 1-3.

37. Okeke B., Smith J., Paterson A. and Watson-Craik I.., Influence of environmental parameters on pentachlorophenol biotransformation in soil by *Lentinula edodes* and *Phanerochaete chrysosporium. Applied Microbiology and Biotechnology,* 1996; **45**:263-266.

38. Ondrej, M., Pavel, C., Stefan, T., and Jana, K., Uptake of polychlorinated biphenyls and organochlorine pesticides from soil and air into radishes (*Raphanus sativus*). Pollution. 2009; **157**: 488-496.

39. Pierre-F., Augustin George, S.t., Kinetics of chemical degradation of organophosphorus pesticides; hydrolysis of chlorpyrifos and chlorpyrifos-methyl in the presence of copper (II) Pesticide Science 2006; **13**: 85-91

40. Pimentel, D. (Editor), Handbook of Pest Management in Agriculture. Second Edition, 1991.

41. Pimentel, D. and Levitan, L., Pesticides: amounts applied and amounts reaching pests. *BioScience,* 1986; **36**: 86-91.

42. Prasad, Sahdeo; Srivastava, Smita; Singh, Madhulika; Shukla, Yogeshwer, Clastogenic Effects of Glyphosate in Bone Marrow Cells of Swiss Albino Mice. Journal of Toxicology 2009: 1. doi:10.1155/2009/308985, 2009.

43. Relyea, Rick A.., The Impact of Insecticides and Herbicides on the Biodiversity and Productivity of Aquatic Communitie. *Ecological Applications* 2005; **15**(2): 618–27.

44. Romantschuk M., Sarand I., Petanene R., Peltola R., Jonsson-Vihanne M., Koivula T., Yrjala K. and Haahtela K., Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. *Environ. Pollution*, 2000; **107**(2): 179-185.

45. Salbego, Joseânia; Pretto, Alexandra; Gioda, Carolina Rosa; Menezes, Charlene Cavalheiro; Lazzari, Rafael; Radünz Neto, João; Baldisserotto, Bernardo; Loro, Vania Lucia., "Herbicide Formulation with Glyphosate Affects Growth, Acetylcholinesterase Activity, and Metabolic and Hematological Parameters in Piava (Leporinus obtusidens)". *Archives of Environmental Contamination and Toxicology* 2010; **58**(3): 740–5.

46. Sambrook, J.; Fritsch, E.F. and Maniatis, T., Molecular Cloning: a laboratory manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press,. 1659 p. ISBN

0-87969-309-6, 1989.

47.   Schmidt, B., Metabolic profiling using plant cell suspension cultures. Pages 40–56 *in* J. C. Hall, R. E. Hoagland, and R. M. Zablotowicz, eds. Pesticide Biotransformation in Plants and Microorganisms: Similarities and Divergences. ACS Symposium Series 777. Washington, DC: American Chemical Society, 2001.

48.   Schoen S. and Winterlin W., The effects of various soil factors and amendments on the degradation of pesticide mixtures. *Journal Environmental Science Health* 1987; **B22**(3): 347-377.

49.   Shiota, N., Inui, G., and Okhawa H., Metabolism of the herbicide chlortoluron in transgenic tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 and yeast NADPH-cytochrome P450 oxidoreductase. Pestic. *Biochem. Physiol.* 1996; **54**:190–198.

50.   Singleton I., Fungal remediation of soils contaminated with persistent organic pollutants. In: Gadd G. (Eds.) Fungi in bioremediation. Cambridge University Press. Cambridge, U.K, 2001.

51.   Solomon, Keith; Thompson, Dean., "Ecological Risk Assessment for Aquatic Organisms from Over-Water Uses of Glyphosate". *Journal of Toxicology and Environmental Health*, Part B 2003; **6**(3): 289–324.

52.   Stephen O Duke and Stephen B. Powles., Glyphosate: a once-in-a-century herbicide: Mini-review. *Pest Management Science Pest Manag Sci* 2008; **64**:319–325

53.   Sunhwa, H., and Cho, K. Effects of plants, rhizobacteria and physicochemical factors on the phytoremediation of contaminated soil. Korean Society for Microbiology and Biotechnology. 2007; **35**: 261-271.

54.   Taha, T. A. and S. M. Abdallah., Phytoremediation of some heavy metals from contaminated soils in Egypt: hydrophobic and hydrophilic fractions. International Conf. on Water Resources & Arid Environment towards a Safer Environment. 2004; 211-219.

55.   Taylor, R.T., Hanna, M.L.; Shaha,N.N. and Jovnovich, M.C., In situ bioremediation of trichloroethylene contaminated water by a resting-cell methanotrophic microbial filter. *Hydrological Science Journal* 1993; **38**: 323-342.

56.   Wardle, D.A., Parkinson, D., Effects of three herbicides on soil microbial biomass and activity. *Plant Soil* 1990; **122**: 21-28.

57.   Williams, J., Miles, R., Fosbrook, C., Deardorff, T., Wallace, M., and B. West.., Phytoremediation of Aldrin and Dieldrin: A Pilot-Scale Project. Case Studies in the Remediation of Chlorinated and Recalcitrant Compounds. Wickramanayake, G.B, Gavaskar, A.R., Gibbs, J.T., and J.L. Means, Eds. Columbus, OH: Battelle Press, 2000.

58.   Yee, D. C., J. A. Maynard, and T. K. Wood., Rhizoremediation of trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene *ortho*-monooxygenase constitutively. *Appl. Environ. Microbiol.* 1998; **64**:112–118.

59.   Ying, M., Mani, R. and Helena, F., Inoculation of plant growth promoting bacterium Achromobacter xylosoxidans strain Ax10 for the improvement of copper phytoextraction by Brassica juncea. *Journal of Environmental Management.* 2009; **90**: 2831-837.

60.   Zak D., Holmes W., MacDonald N. and Pregitzer K., Soil temperature, matric potential, and the kinetics of microbial respiration and nitrogen mineralization. *Soil Science Society of America Journal*, 1999; **63**:575-584.

61.   Zegura B., Lah T.T., and Filipic M.., The role of reactive oxygen species in microcystin-LR-induced DNA damage. *Toxicology*, 2004; **200**:59–68.