British Journal of Environmental Sciences

Vol.1, No.1, pp.7-29, December 2013

Published by European Centre for Research Training and Development UK (www.ea-journals.org)

BIODEGRADATION OF GLYPHOSATE BY FUNGAL STRAINS ISOLATED FROM HERBICIDES POLLUTED-SOILS IN RIYADH AREA

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ABSTRACT: *Forty-five fungal isolates were isolated from eleven cultivated soil farms (Riyadh and Karj area, KSA) after enrichment with Mineral Salt Media (MSM) supplemented with the herbicide glyphosate. The organophosphorous herbicide efficiently stimulated the growth of fungal isolates. The fungal isolates were characterized and identified and selected for mycoremedaition experiments. Certain fungal strains were tolerated to the herbicide up to 10,000 ppm where the growth inhibition reached up 47.92% in certain isolate. 800 ppm of glyphosate almost was degraded and metabolised in liquid Czapek Dox broth medium containing 1% sucrose by certain fungi as A. flavus WDCZ2 (99.6%) and P. spiculisporus ASP5 (95.7%) folowed by P. verruculosum WGP1 (90.8%). The other fungal strains did not record resonable mycoremedaition of the herbicide within 16 days, where A. niger FGP1 (37%), Bipolaris spicifera CDCZ4 (30.6%), A. terreus BGCZ3 (27%), Alt. tenuissima CDP4 (14.7%), P. spinulosum ASP3 (13.9%), A. tamarii PDCZ1 (12.5%) and A. terreus PDP1 (8.4%). On the basis of present findings, A. flavus WDCZ2 and Penicillium spiculisporus ASP5 can be recommended as potentially effective fungal strains and environmentally safer alternative tools to protect the environment from the pollution of glyphosate residues.*

KEYWORDS: Glyphosate, Herbicides, Mycoremdaition, Polluted-soils

INTRODUCTION

Pesticides have been used in industrial and military sector, medical vector control, urban environment and agriculture which represent the largest single market for pesticides (Nerud *et al.*, 2003). Unfortunately, with the benefits of these synthetic organic chemicals have also come many troubles, some so dangerous that they now threaten the survival of major ecosystems by disruption of predator-prey relationships and loss of biodiversity (Kearney and Wauchope 1998). In addition, pesticides cause significant human health consequences. Currently there are a number of possible mechanisms for the clean-up of pesticides in soil, such as chemical treatment, volatilization and incineration (Teng *et al.,* 2010). Chemical treatment and volatilization,

although feasible are problematic as large volumes of acids and alkalis are produced and subsequently must be disposed of. Incineration, which is a very reliable physical-chemical method for destruction of these compounds, has met serious public opposition, because of its potentially toxic emissions, and its elevated economic costs (Zhang and Chiao 2002).

Overall most of these physical-chemical cleaning technologies are expensive and rather inefficient because the contaminated soil has to be excavated at a site and moved to a storage area where it can be processed (Kearney and Wauchope 1998). Due to environmental concerns associated with the accumulation of pesticides in food products and water supplies, there is a great need to develop safe, convenient and economically feasible methods for pesticide remediation (Zhang and Chiao 2002). For this reason several biological techniques involving biodegradation of organic compounds by microorganisms have been developed (Schoefs *et al.*, 2004). The use of microorganisms, either naturally occurring or introduced, to degrade pollutants is called bioremediation (Pointing 2001). In addition, they are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than bacteria (Evans and Hedger 2001). Therefore, certain fungi represent a powerful prospective tool in soil bioremediation and some species have already been patented (Sasek 2003).

Glyphosate (N-(phosphonomethyl)glycine) 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 study on mice has found that a single intraperitoneal injection of Roundup in concentration of 25 mg/kg caused chromosomal aberrations and induction of micronucleim (Prasad et al., 2009; Stephen et al., 2008). 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 much 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).

It was called by experts in herbicides "virtually ideal" due to its broad spectrum and low toxicity compared with other herbicides, (Steinrücken andAmrhein, 1980) glyphosate was quickly adopted by farmers. Use increased even more when Monsanto introduced glyphosate-resistant crops, enabling farmers to kill weeds without killing their crops. In 2007 glyphosate was the most used herbicide in the United States agricultural sector, with 180 to 185 million pounds (82,000 to 84,000 tones) applied, and the second most used in home and garden market where users applied 5 to 8 million pounds (2,300 to 3,600 tones); additionally industry, commerce and government applied 13 to 15 million pounds (5,900 to 6,800 tones) (Daruich et al., 2001) While glyphosate has been approved by regulatory bodies worldwide and is widely used, concerns about its effects on humans and the environment persist. Glyphosate's mode of action is to inhibit an enzyme involved in the synthesis of the aromatic amino acids tyrosine, tryptophan and phenylalanine. It is absorbed through foliage and translocated to growing points. Because of this mode of action, it is only effective on actively growing plants; it is not effective as a pre-emergence herbicide (Steinrücken and Amrhein, 1980).

Bacterial strains capable of utilizing methylphosphonic acid (MP) or glyphosate (GP) as the sole sources of phosphorus were isolated from soils contaminated with these organophosphonates. Strains *Achromobacter* sp. MPS 12 (VKM B-2694), MP degraders group, and *Ochrobactrum anthropi* GPK 3 (VKM B-2554D), GP degraders group, demonstrated the best degradative capabilities towards MP and GP, respectively, and were studied for the distribution of their organophosphonate catabolism systems (Sviridov *et al.*, 2012). However filamentous fungi, offer advantages over bacteria in the diversity of compounds they are able to oxidize (Pointing 2001).

The present study aimed to isolate and screen filamentous fungi from herbicide polluted-soil capable of efficient degradation of glyphosate.

MATERIALS AND METHODS

Soil sampling

Eleven soil samples were collected in October 2010 G from the 0-15 cm top layer of cultivated soil from several farms of Riyadh and Kharj regions in Saudi Arabia, where different herbicides were applied to control various weeds.

Herbicide

The herbicide used in this study was an analytical standard of glyphosate (99.1%) was provided by Nohyaku Co., Ltd (Japan).

Isolation and characterization of fungal strains

For the acclimation of the native molds in the selected soil samples, study was conducted by placing 50 g from each of eleven soil samples in sterile plastic cups, separately amended and mixed with 2.5 ml from stock solution 100 ppm of the selective herbicide, Glyphosate. The cups were incubated at room temperature for two weeks. The technical formulation of the selected herbicide was used.Isolation and purification of fungal strains from soil samples using both Czapek Dox and Potato Dextrose agar (PDA) media was carried out according to the method described by Sviridov et al., (2012). Pure colonies were picked, streaked on slants of Potato Dextrose Agar, PDA kept at 4°C until using. For subculturing, spores from the slants were suspended in sterile 0.85% saline containing 0.01% Tween 80, gently agitating the tube with a Tween 80 solution to give a count within rang $3.0x10^6$ - $3.0x10^7$ spores/ml. Spores suspension used for the inoculation was prepared by subcultures on the potato dextrose agar (PDA) and incubated at 28-30ºC for 7-10 days.

Identification and characterization of fungal isolates

The most efficient herbicides degrading fungal genera were identified in the Laboratory of Fungal and Bacterial Plant Diseases Researches, Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, in collaboration with Advanced Genetic Technologies Center, University of Kentucky, USA.

Screening of filamentous fungal isolates and growth capability on Glyphosate

Fungal screening was carried out by monitoring the growth capability on Glyphosate different concentrations (0, 50, 100, 200 and 300 ppm) in Czapek Dox broth medium ($NaNO_3$ 3.0 g,

K_2HPO_4 1.0 g, KCl 0.5g, $MgSO_4$ 0.1 g, $FeSO_4.2H_2O$ 0.1g, Sucrose 30g, pH 6.5) for 15 days. The dry weight of the fungal growth was measured and expressed in term of gram of the mycelium (Leslie and Summerell, 2006).The residues of Glyphosate was analysed by Gas Chromatography – mass spectrometery. A gas chromatograph, VARIAN GC 3800, equipped with a capillary column Supelco equity-5 (30 m× 0.25 mm ID, 0.25 μm thickness film) and a nitrogen-phosphorus detector was employed. The chromatographic conditions used for the analysis of glyphosate residues were as follows: detector temperature, 300°C; injector temperature, 150°C; oven temperature program, 1.0 min at 100°C, 20°C/min to 130°C, 1°C/min to 133°C, hold for 10.5 min, 20°C/min to 150°C, hold for 2.0 min. The total run time was 18.85 min. The injection volume was 2 μ L. N2 was used as the carrier gas, maintained at a constant flow rate of 0.3 mL/min. The approximate retention time of the glyphosate-derivatization was 14.2 min.

Growth inhibition percentage

The effects of herbicide on fungal growth in Czapek Dox broth medium were calculated and represented as inhibition percentage of growth using the following formula:

% inhibition = $[(G_c - G_T) / G_c] \times 100$

Where,

G_c = Average of fungal growth (gram of dry weight) in control flasks

G_T = Average of fungal growth (gram of dry weight) in herbicides-treated flasks

Mycoremediation experiments

To determine the efficiency of selected fungal strains for bioremediation of the herbicide, Czapek Dox broth medium containing only 1% sucrose and supplemented with different concentrations (from 50 to 10,000 ppm) of herbicide (Glyphosate) was prepared. The medium pH was adjusted at pH 6.5 and sterilized. The sterilized media were inoculated with selected fungal strains ($3.0x10^6$ - $3.0x10^7$ spore/ml), then incubated in rotary shaker operating at 150 rpm at 30°C for 16 days. By filtration of the culture medium, the fungal biomass of each flask was harvested. The biomass dry weight was estimated as previously mentioned. The residuals of Glyphosate were extracted with choloroform with a ratio of 1:1 from each filtrate. 500 μl Samples were transferred to 1.5 ml Eppendorf vial and 500 μl chloroform was added, and then centrifuged at 12,000 rpm for 15 min. The chloroform layer was to a fresh vial. The water layer was extracted for second time and the chloroform layer was combined with the previous extract. The herbicide containing a polar phase was subjected to GC-MS for quantitative and qualitative analysis. To ensure that detected metabolic intermediates were a result of tested fungi biotic activity, sterile controls were supplemented with herbicide and run in parallel with the inoculated media and were analyzed by GC-MS. Agilent 5795 mass spectrometer equipped with a capillary column Agilent Hp-5ms (30 m × 0.25 mm ID, 0.25μ m thickness film) was applied under the same conditions as GC-NPD mentioned in the above paragraph, but it was chosen as the carrier gas. Standard curve was carried out using different concentration of Glyphosate.

RESULTS AND DISCUSSION

Identification of Herbicides-Tolerating Fungal Isolates

Forty-five herbicides-tolerating fungal isolates were identified (Germain and Summerbell, 1996). All the isolates identified as filamentous fungi belonging to the phyla *Deuteromycota*,

Ascomycota, and *Zygomycota*. They were identified to the genus level as, *Aspergillus*, *Penicillium, Fusarium, Alternaria, Bipolaris, Rhizopus, Trichoderma,* and *Rhizoctonia* (Table 1). Aspergilli group are commonly found in soil and on decaying organic material. By converting resistant organic chemicals such as pesticides into simplified metabolites and eventually into soluble benefits molecules, fungi such as *Aspergillus* spp. play an important role in carbon cycling (Tournas, 2005). Data listed in Table (1) showed that *Aspergillus* was the most frequent genus and it was occurred in the most of collected soil samples, comprising 22 species of the total fungal species, this data in line with Abdel-Hafez (1982) who found the most frequent fungal genus, *Aspergillus*, in forty soil samples collected from desert soils in Saudi Arabia.

Among all the rhizofungal isolates (forty-five), 10 fungal species (*Aspergillus* FGP1, *Aspergillus* PDP1, *Aspergillus* BGCZ3, *Aspergillus* PDCZ1, *Aspergillus* WDCZ2, *Penicillium* WGP1, *Penicillium* ASP3, *Penicillium* ASP5, *Alternaria* CDP4, *Bipolaris* CDCZ4) which were taxonomically varied and proved to be most promising. They were selected for further experiments and identified at the species level depending on both morphological and molecular characteristics. Where, the cultures were grown in potato dextrose broth for DNA extraction. The PCR products of rDNA-ITS region were sequenced in both directions using ITS4 and ITS5 primers. The DNA sequences were cleaned and edited by BioEdit software. Sequences were searched against the GenBank database to identify the identical or the closest known deposited sequences in the database Table (2).

Impact of Glyphosate on Fungal Isolates

The obtained results in Table (3) and Figure (11) showed that capabilities of forty-five fungal isolates to grow in the presence of chosen herbicide at used concentrations are varied. In addition, the fungal growth was inversely proportional with the herbicide concentration.The herbicide Glyphosate concentrations (50, 100, 200, 300 ppm) were stimulated the growth of fungal isolate WGP1, where the raising of growth were ranged from -23.33 to 10 % of control (Table 3 & Fig. 11). Also the growth of fungal isolates, FGP1, ASP1, FSP1, PDP1, CDP4, ODP3, CDCZ1 and WDCZ2 were promoted and tolerated the glyphosate up to 200 ppm. Where, growth promotion of fungal isolate ASP1 was accessed to 26.09 % of control at 50 ppm. However, the growth of fungal isolate WGCZ3 clearly reduced and gave highest inhibition value (13.95% of control) at the lowest used concentration, 50 ppm. Moreover, by increasing the Glyphosate concentration, the detrimental effect appeared on growth of some fungal isolates where the fungal growth of isolate OrSCZ2 was recorded highest inhibition percent (19.35% of control) at 100 ppm. While the growth of fungal isolate CGCZ3 was negatively affected comparing with the other fungal isolates and the inhibition percent reached to 28.57 and 42.86 % of control at 200 and 300 ppm, respectively.

These data was agreement with Wardle and Parkinson (1990b) who studied the side effects of glyphosate on fungal species by applying a range of glyphosate concentrations (0, 2, 20, and 200 ppm herbicide). They found that there was little agreement between species responses to glyphosate in incubated in pure culture where, *Mucor hiemalis*, *Fusarium oxysporum*, and *Penicillium nigricans* were largely unaffected by glyphosate at any concentration. *Mortierella alpina*, *Trichoderma harzianum*, and *Arthrinium sphaerospermum* were all significantly

stimulated by 200 µg/g glyphosate. *Cladosporium cladosporioides* was significantly inhibited by higher glyphosate concentrations.

During the past four decades, a large number of herbicides have been introduced as pre and post-emergent weed killers in many countries of the world. Since herbicides have effectively been used to control weeds in agricultural systems, farmers continue to realize the usefulness of herbicides; larger quantities are applied to the soil. But the fate of these compounds in the soils is becoming increasingly important where they could be leached (in which case groundwater is contaminated) or immobile and persist on the top soil (Ayansina *et al.,* 2003). Herbicides could then accumulate to toxic levels in the soil and become harmful to microorganisms, plant, wild life and man (Amakiri 1982). There is an increasing concern that herbicides not only affect the target organisms (weeds) but also the microbial communities present in soils, and these non-target effects may reduce the performance of important soil functions. These critical soil functions include organic matter degradation, the nitrogen cycle and methane oxidation (Hutsch 2001).

The increased use of herbicides in agricultural soils causes the contamination of the soil with toxic chemicals. When pesticides are applied, the possibilities exist that these chemicals may exert certain effects on non-target organisms, including soil microorganisms (Simon-Sylvestre & Fournier 1979 and Wardle & Parkinson 1990a). The microbial biomass plays an important role in the soil ecosystem where they fulfill a crucial role in nutrient cycling and decomposition (De-Lorenzo *et al.,* 2001). When a herbicide is added to a cultivated medium, the various microorganisms may have different types of response. Some microorganisms become intoxicated and lyse. Other microorganisms are resistant and tolerant to a pollutant and can increase their numbers and biomass because of decrease competition. Specific microorganisms will actually grow on organic pollutants.

In studying the effect of glyphosate on the number of microorganisms in a soil and microbial biomass, Stratton and Stewart (1992) observed only a small increase in microbial biomass but no negative or positive effects in respect to the number of microorganism. Moreover, Haney *et al.* (2000) and Busse *et al.* (2001) evaluated the effect of glyphosate on the microbial community of soils and observed that microbial activity was stimulated in the presence of this herbicide. Also Ratcliff *et al.,* (2006) reported transient increase in fungal propagates after glyphosate addition (50 mg kg^{-1}). It is likely that the glyphosate provided nutrients for fungal growth, as evidenced by the significant increase in microbial population. This conclusion agreed with Lancaster *et al.*, (2010) who found that after repeated application of glyphosate, microorganisms were better able to utilize it. The most recent study by Partoazar *et al.*, (2011) concluded that glyphosate application may alter (increase) soil microbial activity and population. Increased microbial activity may be beneficial or detrimental toward plant growth, soil microbial ecology, and soil quality. Among the local 45 fungal isolates, ten isolates species (*Aspergillus* FGP1, *Aspergillus* PDP1, *Aspergillus* BGCZ3, *Aspergillus* PDCZ1, *Aspergillus* WDCZ2, *Penicillium* WGP1, *Penicillium* ASP3, *Penicillium* ASP5, *Alternaria* CDP4, *Bipolaris* CDCZ4) which were not or slight infuened by Glyphosate up to 300 ppm moreover taxonomically varied, selected for further experiments.

Table 1. Identification of herbicides-tolerating fungal genera among different soil samples.

Fungal isolates	Fungal genera
FGP1, PDP1, BGCZ3, PDCZ1, WDCZ2, ASP1, CGP2, WSCZ1, CDCZ1, GSCZ2, FDCZ2, WGCZ7, BSCZ1, PDCZ6, ODP3, FDP3, BGCZ2, FGCZ2, BDCZ3, ODCZ1, FSP1, POSCZ1	*Aspergillus* spp.
WGP1, ASP3, ASP5, CGP1, CGCZ3, LSP2, ODCZ4, ADCZ1, PGCZ1, PSCZ1	*Penicillium* spp.
PGP1, WGCZ3	*Fusarium* spp.
CDP4, LSCZ2	*Alternaria* sp.
CDCZ4, PDP3	*Bipolaris* sp.
AGCZ3, ORSCZ2	*Rhizopus* sp.
PGCZ3, ,GGCZ5	*Trichoderma* spp.
PODCZ1, FSCZ4, LGCZ3	*Rhizoctonia* spp.

Table 2. Molecular identification of herbicides-tolerating fungal isolates based on rDNA-ITS region using ITS4 and ITS5 primer-pair

Fungal isolates Code	Species ID	GenBank Accession number	Homology (identity %)
FGP1	*Aspergillus niger*	AB369898	573/574 (99%)
PDP1	*Aspergillus terreus*	FJ878634	489/495 (99%)
BGCZ3	*Aspergillus terreus*	FJ878634	623/623 (100%)
PDCZ1	*Aspergillus tamarii*	HQ340111	564/564 (100%)
WDCZ2	*Aspergillus flavus*	JQ255474	458/459 (99%)
WGP1	*Penicillium verruculosum*	HQ607919	589/594 (99%)
ASP3	*Penicillium spinulosum*	HQ608085	584/584 (100%)
ASP5	*Penicillium spiculisporus*	EU076917	833/836 (99%)
CDP4	*Alternaria tenuissima*	EU326185	586/586 (100%)
CDCZ4	*Bipolaris spicifera*	ND	ND

ND not determined

Table 3. Inhibition percentage of fungal isolates growth in Czapek Dox broth medium at different concentrations of Glyphosate.

Isolates No.	Fungal isolates	Glyphosate Concentrations (ppm)			
		50	100	200	300
1	PGP1	3.69	7.56	7.69	10.26
2	CGP1	-10.00	0.00	15.00	15.00
3	CGP2	0.00	2.50	7.50	17.50
4	WGP1	-23.33	-13.33	-13.33	-10.00
5	FGP1	-11.43	-11.43	-5.71	2.86
6	ASP1	-26.09	-21.74	-4.35	13.04
7	ASP3	-3.23	0.00	3.23	3.23
8	ASP5	-3.03	3.03	6.06	9.09
9	LSP2	-1.85	3.70	3.70	9.26
10	FSP1	-6.82	-6.82	-4.55	2.27
11	PDP1	-5.71	-2.86	0.00	5.71
12	PDP3	-2.56	0.00	12.82	20.51
13	CDP4	-3.13	-2.56	-2.56	2.56
14	ODP3	-6.06	-6.06	-3.03	9.09
15	FDP3	-2.94	0.00	2.94	26.47
16	PGCZ1	3.09	7.03	9.09	12.12
17	PGCZ3	5.00	12.50	15.00	17.50
18	CGCZ3	0.00	5.71	28.57	42.86
19	WGCZ3	13.95	13.95	18.60	20.93
20	WGCZ7	8.11	8.11	13.51	24.32
21	BGCZ2	-5.71	-2.86	5.71	5.71
22	BGCZ3	-6.98	-6.98	2.33	6.98
23	AGCZ3	3.85	3.85	7.69	23.08
24	GGCZ5	13.05	14.29	18.29	21.43
25	LGCZ3	2.22	4.44	11.11	13.33
26	FGCZ2	9.09	13.64	15.91	31.82

27	PSCZ1	0.00	5.56	8.33	11.11
28	WSCZ1	6.98	9.30	9.30	16.28
29	BSCZ1	0.00	4.76	23.81	33.33
30	GSCZ2	2.76	5.38	14.29	14.29
31	POSCZ1	3.33	6.67	6.67	20.00
32	OrSCZ2	9.68	19.35	22.58	35.48
33	LSCZ2	2.56	5.13	5.13	23.08
34	FSCZ4	2.17	4.35	6.52	13.04
35	PDCZ1	2.70	2.70	5.00	8.11
36	PDCZ6	3.70	11.11	11.11	22.22
37	CDCZ1	-11.11	-3.70	-3.70	3.70
38	CDCZ4	7.55	7.55	9.43	13.21
39	WDCZ2	-8.82	-2.94	-2.94	2.94
40	BDCZ3	0.00	9.38	12.50	12.50
41	ADCZ1	-4.55	4.55	9.09	11.36
42	ODCZ1	2.94	2.94	11.76	14.71
43	ODCZ4	9.09	9.09	13.64	18.18
44	PODCZ1	8.82	11.76	23.53	23.53
45	FDCZ2	9.09	9.09	13.64	36.36

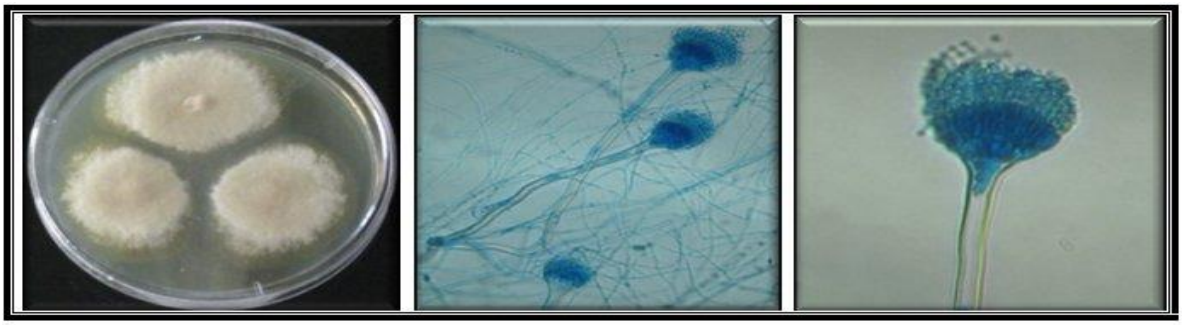


Fig. 1. Macro- and micromorphology of *Aspergillus terreus* BGCZ3. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

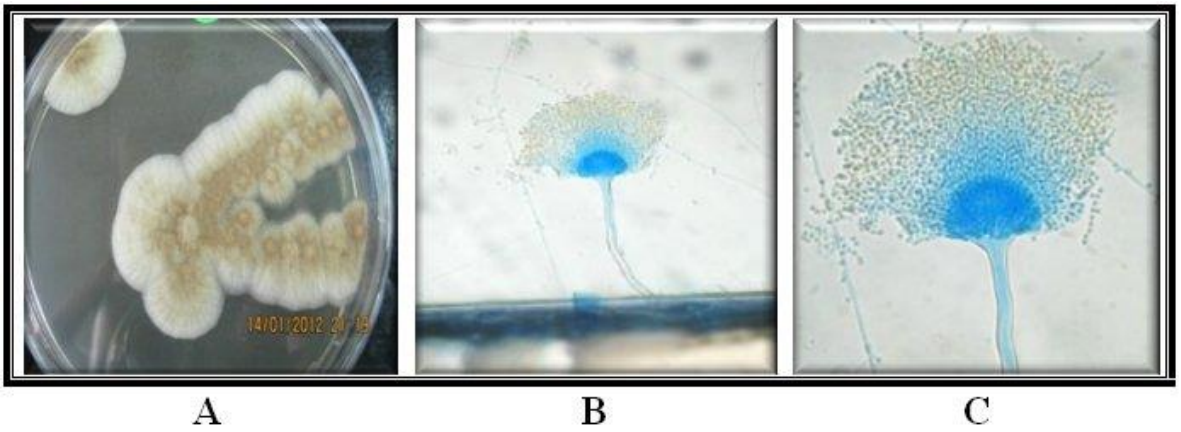


Fig. 2. Macro and micromorphology of *Aspergillus terreus* PDP1. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

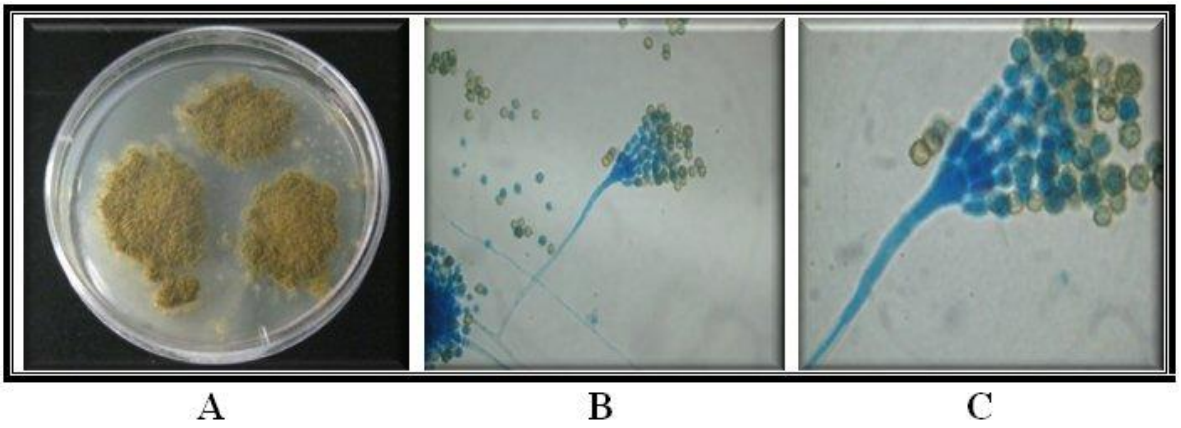


Fig. 3. Macro and micromorphology of *Aspergillus tamarii* PDCZ1. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

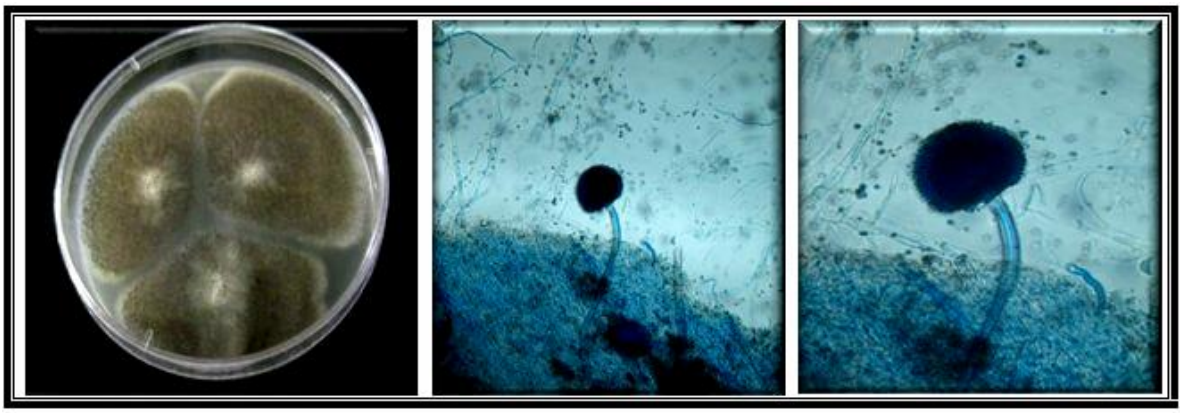


Fig. 4. Macro and micromorphology of *Aspergillus niger* FGP1. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 10x and (C) viewed at 20x.

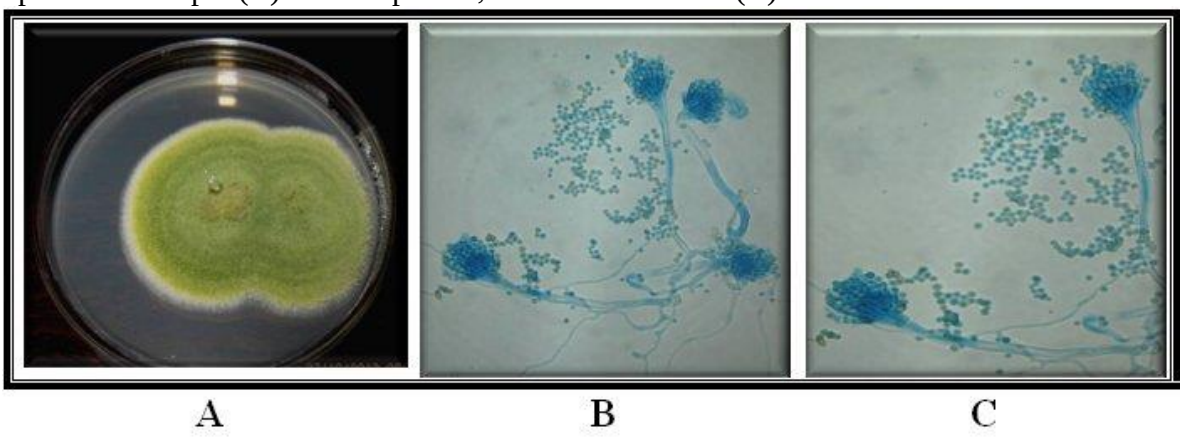


Fig. 5. Macro and micromorphology of *Aspergillus flavus* WDCZ2. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

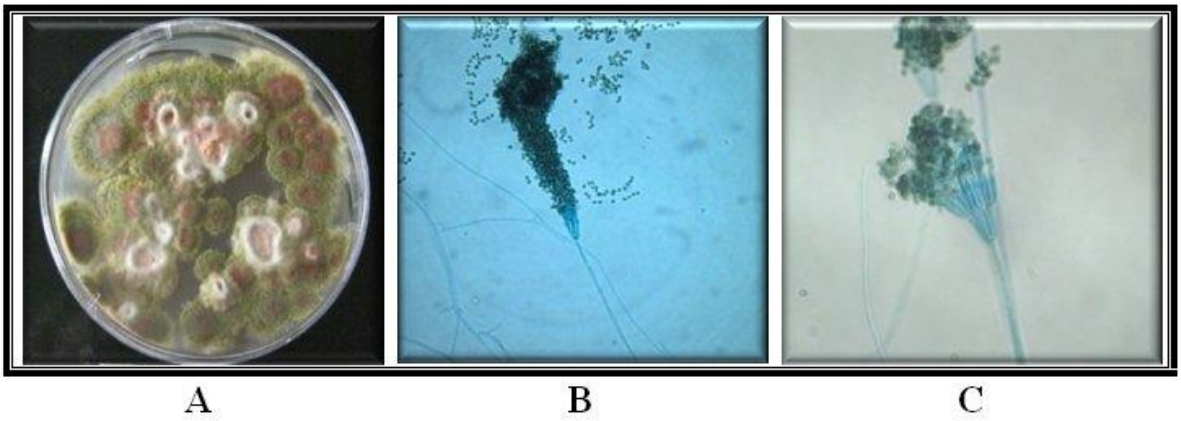


Fig. 6. Macro- and micromorphology of *Penicillium verruculosum* WGP1. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

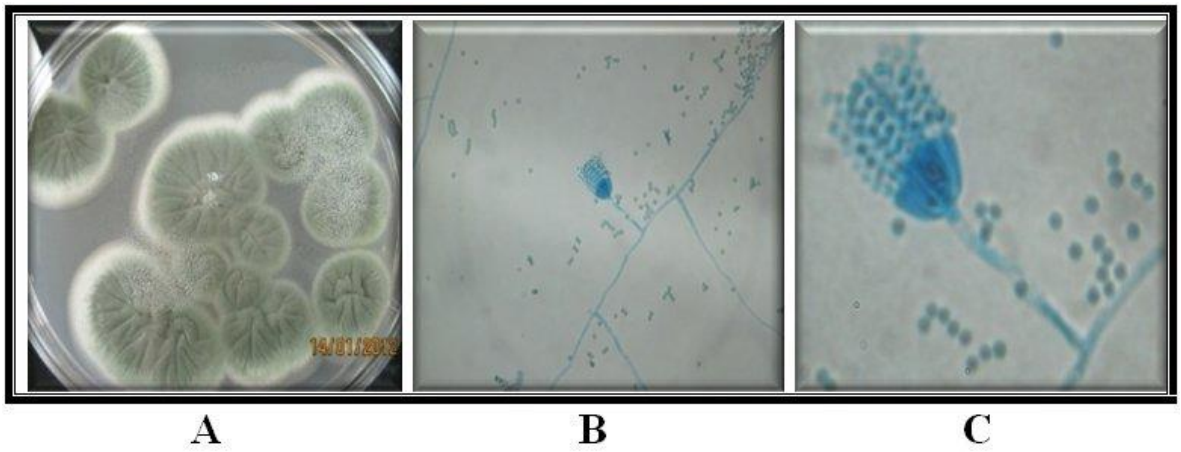


Fig. 7. Macro- and micromorphology of *Penicillium spinulosum* ASP3. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 10x and (C) viewed at 40x.

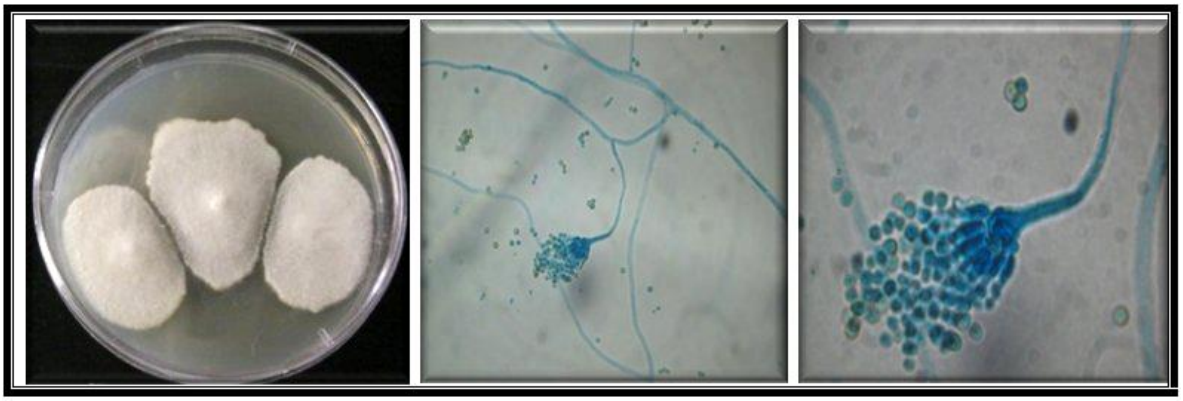


Fig. 8. Macro- and micromorphology of *Penicillium spiculisporus* ASP5. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 10x and (C) viewed at 40x.

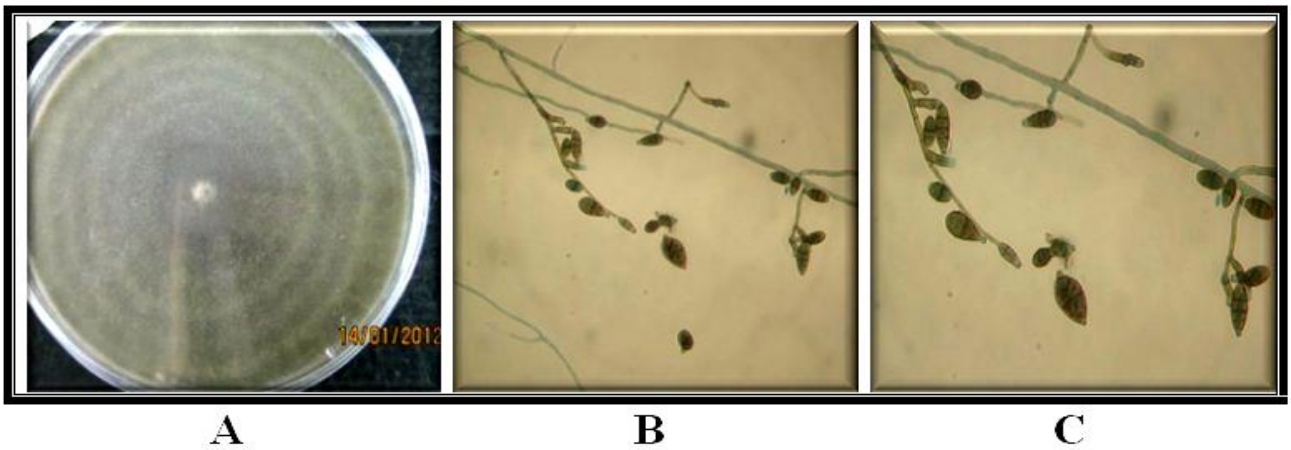


Fig. 9. Macro- and micromorphology of *Alternaria tenuissima* CDP4. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

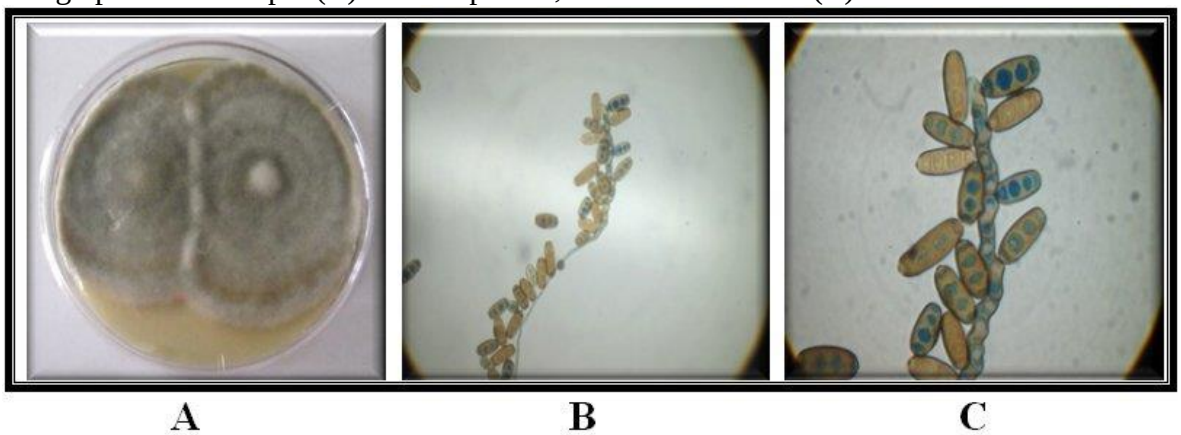


Fig. 10. Macro- and micromorphology of *Bipolaris spicifera* CDCZ4. Macromorphology on Czapek Dox agar plate for upper surface using digital camera: (A) Surface of plate. Micromorphology using optic microscope: (B) Conidiophores, viewed at 20x and (C) viewed at 40x.

Tolerance of Fungal Strains against Glyphosate

Data presented in Table (4), Fig. (1), Fig (2), Fig (3), Fig (4), Fig (5) and Fig (6) revealed that fungal strains, *P. verruculosum*, WGP1, *A. niger*, FGP1, *Alt. tenuissima*, CDP4, *A. terreus*, BGCZ3 and *A. flavus*, WDCZ2, were clearly tolerated herbicide Glyphosate up to 10,000 ppm where growth inhibition percent reached only to 31.91, 40, 41.18, 47.50 and 47.92% of control. While fungal strain, *P. spiculisporus* ASP5 had less tolerance and its growth was reduced 50% of control at 700 ppm Glyphosate and inhibition percent was reached to highest value, 67.50% of control, at 10.000 ppm (Table.4 and Fig.7). The recent study, dealing with glyphosate, by Munees and Khan (2011), who studied the pesticide-tolerance and the functional diversity rhizobacteria recovered from the rhizospheres of certain crops. They found that all the rhizobacterial strains generally tolerated the herbicides, glyphosate, up to 3000 µg mL^{-1}.

Mycoremediation of certain pesticides polluted-soil Glyphosate

In recent years, the intensive use of herbicides has increasingly become a matter of environmental concern, partially because of the adverse effects of these chemicals on soil microorganisms. Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum, non-selective, post-emergence herbicide that is widely used in agriculture. So degradation of the compound is required in agricultural practises.

Therefore, the capability of the ten fungal strains for herbicide Glyphosate biodegradation were investigated in shaking mineral broth culture for sixteen days. It was clear that the isolates of fungal strains, (*Aspergillus* FGP1, *Aspergillus* PDP1, *Aspergillus* BGCZ3, *Aspergillus* PDCZ1, *Aspergillus* WDCZ2, *Penicillium* WGP1, *Penicillium* ASP3, *Penicillium* ASP5, *Alternaria* CDP4, *Bipolaris* CDCZ4) were grown on Czapek Dox broth medium (1% Sucrose) amended with different concentrations of Glyphosate ranged between 0 to 10,000 ppm individually. The best isolates that could grow in this respect were *A. niger* FGP1, *A. terreus* BGCZ3, *A. flavus* WDCZ2, *A. terreus* PDP1, *A. tamarii* PDCZ1 and *P. spinulosum* ASP3 folowed by *P. verruculosum* WGP1 exhibiting good tolerance to the herbicide Glyphosate and reflected that on the fungal dry weight after 16 days incubation (Figures 11 & 12). The growth capability of the fungal strains could acertain a significant assimilation the herbicide Glyphosate in 16 days as carbon, phosphorous and energy sources. The fungal strains used exhibited good growth rates, in order to potentially adapt at different herbicide glyphosate concentration level. It is remerkable to mentioned that a concentration of 1% sucrose was importatnt for the intial stimulation of the fungal strains growth. It was observed that non of the evaluated fungi could be able to grow on the concentration of 10000 ppm and the growth of the fungi was very limited on the concentration of 5000 ppm by all tested fungi. Therefore, most of the fungal strains as *P. spiculisporus* ASP5, *Alt. tenuissima* CDP4 and *Bipolaris spicifera* CDCZ4 exhibited slight and worthless degradation for the organophosphorus compound Glyphosate. Generally, the fungal strains stated in Table 4 had the efficiency to assimilate Glyphosate till the concentration of 2000 ppm. The disability of the evaluated fungal strains was due to the toxicity occurred because of the high concentration levels of the herbicide Glyphosate. Interestingly, at low concentraions

of (≤ 1000 ppm), the growth (DW g/100ml) were not noticeably affected by the concentration of the herbicide Glyphosate, which suggested that this isolate can easily grow in medium supplemented with herbicide Glyphosate, without suffering any toxicity effects.

The mycoremediation of Glyphosate by GC and GC-MS is illusterated in Fig. (12). Some of the evaluated fungi were able to degrade and assimilate the herbicide Glyphosate in 16 days. There were differences in degradation among the evaluated strains during the degradation process of Glyphosate as a fungal utilized substrate. It was clear that the organophosphorus compound Glyphosate almost disappeared rapidly (> 95% from the parent compound) in liquid media by *A. flavus* WDCz2 (99.6%) and *P. spiculisporus* ASP5 (95.7%) folowed by *P. verruculosum* WGP1 (90.8%). On the other hand, the other fungal strains did not record resonable degradation of the herbicide in 16 days, where *A. niger* FGP1 (37%), *Bipolaris spicifera* CDCz4 (30.6%), *A. terreus* BGCz3 (27%), *Alt. tenuissima* CDP4 (14.7%), *P. spinulosum* ASP3 (13.9%), *A. tamarii* PDCz1 (12.5%) and *A. terreus* PDP1 (8.4%). These fungal strains exhibited slight and worthless degradation for the organophosphorus compound Glyphosate. Hence, *A. flavus* WDCz2, *P. spiculisporus* ASP5 and *P. verruculosum* WGP1 were the best fungal strains which had the ability to assimilate and mineralize approximately 800 ppm after 16 days (Fig. 12). These degradation rate is unique comparing with the previous studies (Aijun *et al.*, 2003). Glyphosate metabolites could not be quantified by mass spectra of Gas Chromatography and this was due to the disappearance of the metabolites and the intermediated compounds fast.
On the other hand, it was observed that the mineralization of Glyphosate herbicide is related to both activity and growth ability on such compound as a sole of carbon, phosphorous and energy source of the studied fungi.

The commercial success of Glyphosate as a highly effective herbicide has stimulated several studies on its behavior and persistence in soil (Krzysko-Lupicka and Orlik, 1997; Forlani *et al.*, 1999; Jonge and Jonge, 1999). Microbial degradation is considered to be the most important of the transformation processes that determine the persistence of herbicides in soil (Souza *et al.*, 1999).

Table 4. Inhibition percentage of fungal isolates growth in Czapek Dox broth medium at different concentrations of Glyphosate.

Fungal isolates	Glyphosate Concentration (ppm)											
	300	400	500	600	700	800	1000	2000	3000	4000	5000	10000
P. verruculosum, WGP1	10.00	3.33	8.33	16.67	18.75	21.28	23.40	27.66	27.66	29.79	31.91	31.91
A. niger, FGP1	2.86	12.67	20.83	22.92	26.00	28.00	28.00	30.00	32.00	36.00	40.00	40.00
P. spinulosum, ASP3	3.23	18.00	22.22	31.11	33.33	33.33	35.71	42.86	42.86	50.00	52.38	57.14
P. spiculisporus, ASP5	9.09	22.11	42.11	47.37	50.00	52.50	57.50	57.50	60.00	65.00	65.00	67.50
A. terreus, PDP1	5.71	17.50	27.66	36.17	36.17	36.00	36.00	40.00	40.00	44.44	46.67	51.11
Alt. tenuissima, CDP4	2.56	3.00	4.86	5.71	11.76	11.76	20.59	29.41	29.41	35.29	35.29	41.18
A. terreus, BGCZ3	6.98	7.76	9.76	13.14	16.67	25.00	27.50	27.50	35.00	40.00	47.50	47.50
A. tamarii, PDCZ1	8.11	13.00	15.00	25.00	32.50	35.00	37.50	40.00	42.50	47.50	50.00	55.00
Bipolaris spicifera, CDCZ4	13.21	18.93	20.93	32.56	34.88	36.36	43.18	45.45	45.45	50.00	56.82	63.64
A. flavus, WDCZ2	2.94	12.74	21.74	26.09	30.43	33.33	37.50	37.50	41.67	45.83	45.83	47.92

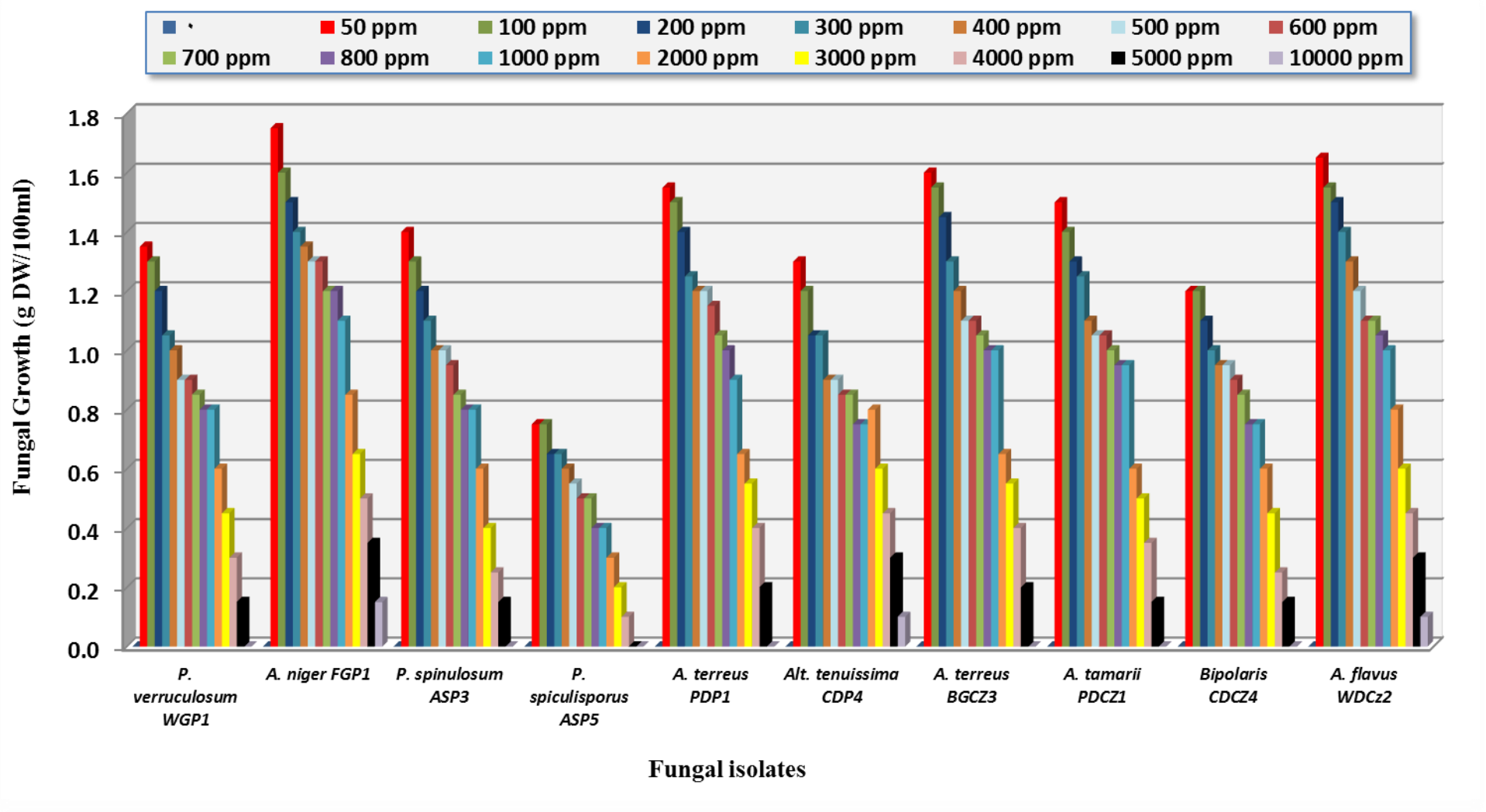
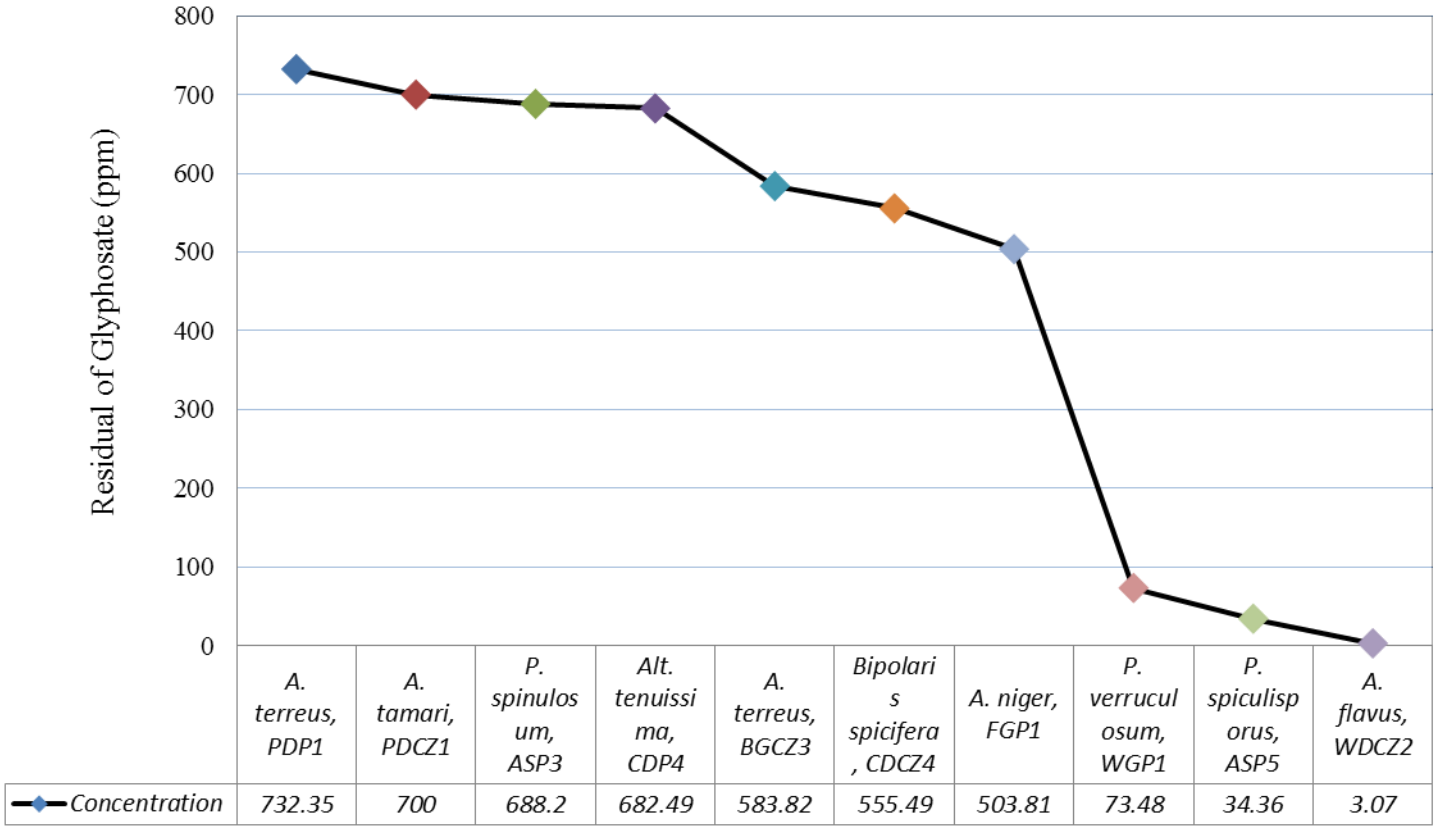


Fig. 11. Growth of fungal strains (DW g/100ml) in Czapek Dox broth medium (1% Sucrose) supplemented with different concentrations of Glyphosate after 16 days incubation.



	A. terreus, PDP1	A. tamari, PDCZ1	P. spinulosum, ASP3	Alt. tenuissima, CDP4	A. terreus, BGCZ3	Bipolaris spicifera, CDCZ4	A. niger, FGP1	P. verruculosum, WGP1	P. spiculisporus, ASP5	A. flavus, WDCZ2
Concentration	732.35	700	688.2	682.49	583.82	555.49	503.81	73.48	34.36	3.07

Fig. 12. Mycoremediation of Glyphosate by different strains of fungi after 16 days. Initial concentration of Glyphosate 800 ppm.

This degradation pathway of glyphosate produces the major metabolite aminomethyl phosphonic acid (AMPA), and ultimately leads to the production of water, carbon dioxide and phosphate (Forlani *et al.,* 1999).

On the other hand, the presence of glyphosate in aqueous media without degradation may cause toxicity and physiological changes to the microbial population and activity of degradation. In the environment, Wardle and Parkinson (1990a, b) observed that the presence of glyphosate in a soil was related to a temporary increase in the both number of bacteria and fungi in a soil and the overall microbial activity of the soil, although the number of fungi and actinomycetes was not affected. In fact, studying the effect of glyphosate on the number of microorganisms in a soil, microbial biomass and soil respiration, Stratton and Stewart (1992) observed only a small increase in microbial biomass but no negative or positive effects in respect to the number of microorganism or soil respiration. More recently, Haney *et al.,* (2000) and Busse *et al.,* (2001) evaluated the effect of glyphosate on the microbial community of soils and observed that microbial activity was stimulated in the presence of this herbicide. Glyphosate can stimulate the growth of mycorrhizal fungi in vitro (Laatikainen and Heinonen-Tanski, 2002).

In this study, there was evidence of increases in fungal activity and populations in the treated media with glyphosate. This activity was represented in fungal biomass and pH fluctuation during the experiments. This stimulation may be due to the fact that fungi are the main microbial degraders of glyphosate as an agreement with (Krzysko-Lupicka and Orlik, 1997). Araujo *et al.,* (2003) found that glyphosate amendment did not affect bacterial cultural population, while fungi 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* (Kremer *et al.,* 2005; Levesque *et al.,* 1993 and Meriles *et al.,* 2006). This current study proved that glyphosate has the tendency to be assimilated by different strain of fungi to the degradation response. The slower rate of degradation by *Alternaria tenuissima* CDP4, *Penicillium spinulosum* ASP3, *Aspergillus tamarii* PDCZ1 and *Aspergillus terreus* PDP1was attributed to either a lower initial response of microbial population or stronger failure of these fungi to exploit glyphosate as phosphate source and energy source. In soil, this is due to the strength of glyphosate bonding and binding to the soil particles, and subsequent availability to microorganisms, correlates with the amount of available phosphate sorption sites (Rueppel *et al.,* 1977; Sprankle *et al.,* 1975).

The obtained results stand in the line with Kremer *et al.,* (2005) who found that glyphosate increased stimulated microbial biomass. Although this study was done in hydroponic mesocoms that allowed detection of these compounds and isolation of *Fusarium* at very low levels. It may be that the analysis used was unable to detect these small and perhaps subtle changes in the subpopulations of soils receiving glyphosate.

According to Wardle and Parkinson (1990b), glyphosate can influence the biomass of fungi directly and, indirectly, as toxic compounds. These results indicate that fungi may use glyphosate as a nutrient and energy source in case of being able to assimilate the compounds. The same case

study with actinomycetes showed a significant increase with time, mainly in the presence of glyphosate.

Paid attention to those Organophosphorus accumulation rapidly diminishes when they are removed from the medium, this is because of their higher bioavailability and metabolization rates (Abdel-Megeed, 2004).

CONCLUSION

The present study documented abnormally higher tolerance levels of the local fungal strains (*P. verruculosum*, WGP1, *A. niger*, FGP1, *P. spinulosum*, ASP3, *P. spiculisporus*, ASP5, *A. terreus*, PDP1, *Alt. tenuissima*, CDP4, *A. terreus*, BGCZ3, *A. tamarii*, PDCZ1 *Bipolaris spicifera*, CDCZ4 and *A. flavus*, WDCZ2) against glyphosate. The mycoremediation by ten fungal strains from liquid media after 16 days appeared that Glyphosate disappeared rapidly in liquid media by *A. flavus* WDCz2 (99.6%) and *P. spiculisporus* ASP5 (95.7%) folowed by *P. verruculosum* WGP1 (90.8%). Glyphosate almost disappeared by *Penicillium spinulosum* ASP3 (98.8%), *Penicillium spiculisporus* ASP5 (98.1%) and *Aspergillus tamarii* PDCZ1 (96.7%) folowed by *Aspergillus flavus* WDCZ2 (90.6%). On the basis of present findings, *A. flavus* WDCZ2 and *Penicillium spiculisporus* ASP5 can be recommended as potentially effective fungal strains and environmentally safer alternative tools to protect the environment from the pollution of glyphosate.

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ACKNOWLEDGEMENT

The author extended their appreciation to the Deanship of Scientific Research at King Saud University for their funding the work through the research group project No. RGP-VPP-010.