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Changes in Protein Profile Induced by Glyphosate, Diclofop-Methyl and Pendimethalin in Herbicide-tolerant Fungi

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In the present investigation an attempt has been made to study glyphosate, Diclofop-methyl and Pendimethalin and fenvalerate induced changs in protein submits the herbicide-tolerant fungi isolated from pesticides polluted-soil. Severely loss in the total soluble cell ions (SCI) and total cell protein percentage (TCPC) concentrations was observed. For glyphosate, *Aspergillus flavus* (86.30%) was the most affected one, followed by *Penicillium spiculisporus* (76.30%), *Penicillium verruculosum* (64.40%) and *Alternaria tenuissima* (64%), respectively. The other fungal strains exhibited <50% in total SCI. For Pendimethalin, *Alternaria tenuissima* (54.01%) was the most affected fungi followed by *Penicillium spinulosum* (50.68%), respectively. The other fungal strains exhibited <50% in total SCI. For Diclofop-methyl, *Penicillium spiculisporus* (74.20%) was the most affected fungi followed by *Aspergillus tamarii* (70.67%), *Aspergillus niger* (56.16%), *Aspergillus terreus* (54.67%) and *Alternaria tenuissima* (58.82%), respectively. The other fungal strains exhibited d" 50% SCI. The loss of TCPC by Glyphosate, *Alternaria tenuissima* (64.71%) was the most effected fungi, followed by *Penicillium spiculisporus* (57.14%), respectively. The other fungal strains exhibited <50%. For Pendimethalin, *A. terreus* (54.29%) was the most affected fungi followed by *Alternaria tenuissima* (50.00%), respectively. The other fungal strains exhibited <50%. For Diclofop-methyl, *Penicillium spiculisporus* (60%), followed by *Alternaria tenuissima* (58.82%), *Aspergillus tamarii* (55.56%), respectively. The other fungal strains exhibited <50%. The results proved changes in protein profile induced in herbicide-tolerant fungi.

Key words: Herbicides, Tolerance, Fungi, Polluted-soil, DNA, protein.

Bioremediation involves the use of microorganisms or microbial processes to degrade environmental contaminants, and is among these new technologies. The use of microorganisms, either naturally occurring or introduced, to degrade pollutants is called bioremediation (Pointing, 2001). The application of fungal technology for the clean up of polluted soils holds significant promise since 1985 when the white rot fungus Phanerochaete chrysosporium was found to be able to metabolise a number of important environmental pollutants (Sasek, 2003). The increasing use of pesticides in agriculture including commercial and household production of vegetables for the control of pests causes chemical pollution of aquatic environment. The chemical pollution causes potential health hazards to live stock (Bhuniya *et al*., 1994). Some pesticides are found to be highly persistent in nature, thereby causing contamination of soil, ground and surface water (Frank *et al*., 1990). Such contamination with low level of pesticides has

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resulted in serious environmental concern and some of the pesticides, though not showing an immediate effect in vivo, may pose long term health hazard to human beings. Their toxic effects are manifested in different ways such as bioaccumulation, bio-magnification, chronic toxicity, acute immune response, allergic reaction, and mutagenic, teratogenic and carcinogenic effects. The goal of bioremediation is to at least reduce pollutant levels to undetectable, nontoxic or acceptable levels, i.e. within limits set by regulatory agencies (Pointing, 2001) or ideally completely mineralize organopollutants to carbon dioxide. From an environmental point of view this total mineralization is desirable as it represents complete detoxification (Gan and Koskinen, 1998). Microbial metabolism is probably the most important pesticide degradative process in soils (Kearney, 1998) and is the basis for bioremediation, as the degrading microorganisms obtain C, N or energy from the pesticide molecules (Gan and Koskinen, 199; Sasek 2003; Kim *et al*., 2005 and Teng *et al*., 2010).

Currently there are a number of possible mechanisms for the clean-up of pesticides in soil, such as chemical treatment, volatilization and incineration. Chemical treatment and volatilization, although feasible are problematic as large volumes of acids and alkalis are produced and subsequently must be disposed of ((Nerud *et al*., 2003; Ashour *et al*., 2013). 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 Quiao, 2002; Pointing 2001; Balba *et al*., 1998; Evans and Hedger 2001).

Therefore, certain fungi represent a powerful prospective tool in soil bioremediation and some species had already been patented By keeping all these in view the present experiment was undertaken to investigate changes in protein profile induced by glyphosate, Diclofop-methyl and Pendimethalin in herbicide-tolerant fung on .

MATERIAL AND METHODS

Soil sampling and source of Herbicides

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. The herbicides used in this study were an analytical standard of Glyphosate (99.1%), Pendimethalin and Diclofop-methyl were provided by Nohyaku Co., Ltd (Japan).

Isolation and characterization of fungal strains

The most efficient herbicides degrading fungal genera were isolated and 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.

Fungal protein extraction

To determine the influence of herbicides on the protein profile of the selected fungal strains, Czapek Dox broth medium containing only 1% sucrose and supplemented with 50 ppm of each herbicide (Glyphosate, Pendimethalin and Diclofop-methyl) was prepared. The medium' pH was adjusted to 6.5 and sterilized. The sterilized media were inoculated with selected fungal strains, 1 ml of spores suspension ($3.0x10^6$-$3.0x10^7$ spore/ml) used as inoculums, then incubated in rotary shaker operating at 150 rpm at 30°C for 16 days. Total proteins from mycelia of each fungal isolates were extracted according to the method described by Natarajan *et al.*, (2005).

Determination of protein concentration

The protein concentration in the crude extract was determined by a modified method of Bradford, (1976) (BioRad protein assay) at 280 nm. Protein concentration was calculated according to the following equation:

Protein (mg/ml) = *1.55 A 280 nm – 0.76 A 260 nm*

Total soluble protein content

Measurement Total soluble protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Total soluble cell ions

A total soluble cell ion of fungal cell was determined according to the method described by Sambrook *et al*., (1989).

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* (Tables 1 and 2).

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). *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.

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 (1).

Table 1. 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

Mutagenic effect of glyphosate, pendimethalin and diclofop-methyl on fungal proteins

With reference to the data in Figures (1 & 2), it could be observed that the total soluble cell ions concentration (molL^{-1}) and total cell protein concentration (mg/ml) were decreased in some fungal protein strains. The results illustrated in Figure (1), total soluble cell ions (SCI) were more or less similar to that obtained in DNA and Protein profile. For Glyphosate, *Aspergillus flavus* (86.30%), was the most effected fungi, followed by *Penicillium spiculisporus* (76.30%), *Penicillium verruculosum* (64.40%) and *Alternaria tenuissima* (64.00%), respectively. The other fungal strains exhibited <50% total soluble cell ions loss. For Pendimethalin (Stomp), *Alternaria tenuissima* (54.01%) was the most affected fungi followed by *Penicillium spinulosum* (50.68%), respectively. The other fungal strains exhibited <50% total soluble cell ions loss. For Diclofop-methyl, *Penicillium spiculisporus* (74.20%) was the most affected fungi followed by *Aspergillus tamarii* (70.67%), *Aspergillu sniger* (56.16%), *Aspergillus terreus* (54.67%) and *Alternaria tenuissima* (58.82%), respectively. The other fungal strains exhibited ≤ 50% total soluble cell ions loss (Fig. 1).

For total cell protein concentration (TCPC), the results illustrated in Figure (2) were more or less similar to that obtained in DNA and protein profile and total soluble cell ions (SCI). By the fact, the more lowered total cell ionic content, the more effective herbicides in decreasing cellular activity, the higher inhibitory action. For Glyphosate, *Alternaria tenuissima* (64.71%) was the most effected fungi, followed by *Penicillium*

spiculisporus (57.14%), *Aspergillus flavus* (48.57%), *Aspergillus niger* (47.22%) and *Penicillium spinulosum* (45.45%), respectively. The other fungal strains exhibited <30% total cell protein concentration loss. For Pendimethalin (Stomp), *A. terreus* (54.29%) was the most affected fungi followed by *Alternaria tenuissima* (50.00%), *Penicillium spiculisporus* (48.57%), *Aspergillus flavus* (45.71%), and *Aspergillus tamarii* (45.45%). The other fungal strains exhibited <40% total cell protein concentration loss. For Diclofop-methyl, *Penicillium spiculisporus* (60.00%), followed by *Alternaria tenuissima* (58.82%), *Aspergillus tamarii* (55.56%), *A. terreus* (48.58%) and *Penicillium spinulosum* (42.42%), respectively. The other fungal strains exhibited <40% total cell protein concentration loss (Figure 1).

Accordingly, it could be suggested that each type of the herbicides used has its own specific effect on certain metabolic activities of the tested fungal species. This explained that the effect of tested herbicides on the protein profile (Figs. 3, 4 and 5) 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 these compounds.

These data suggestd that direct binding of Glyphosate, Pendimethalin and Diclofop-methyl to fungal DNA is unlikely to be a mechanism through which any genotoxic effects are produced. In an agreement with Gad and Abdel-Megeed (2006) stated that certain pesticides have the capacity to

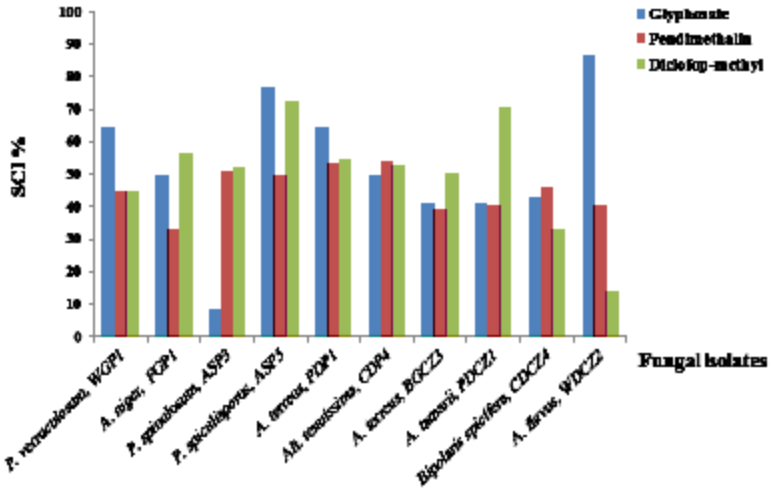

Fig. 1. Total soluble cell ions (SCI) concentration, (millimolar)

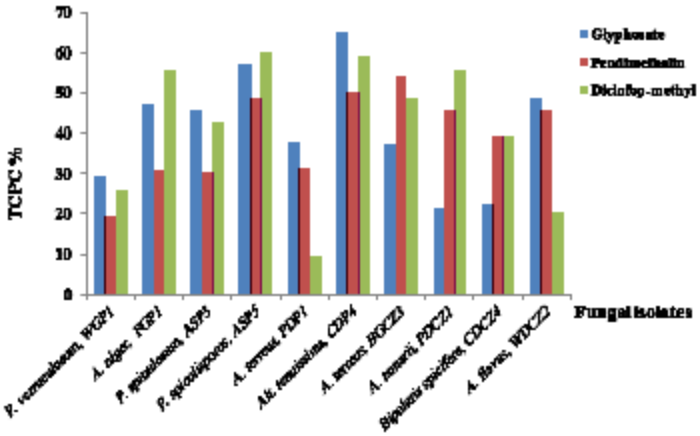

Fig. 2. Total cell protein concentration (TCPC), (mg/ml)

alter the genetic material particularly chromosomes in the tested plants.

The results proved strongly that the fungicide induced an increase in total DNA production in all affected fungi. On the other hand, it was found that DNA content decreased with the

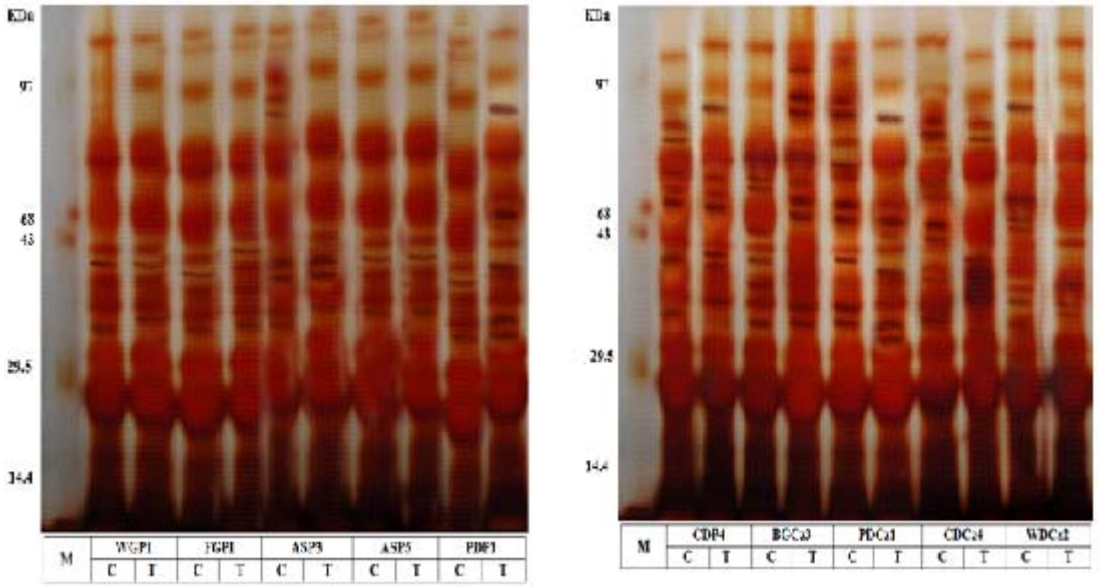

Fig. 3. SDS-PAGE Silver staining of protein extracts of fungal strains affected by Glyphosate after application of 16d. 1D gel-electrophoresis-based protein profiling revealed mutagenic effects in expressed proteins bands. Where: C, untreated; T, treated, M, Molecular marker; WGP1, *Penicillium verruculosum*; FGP1, *Aspergillus niger;* ASP3, *Penicillium spinulosum;* ASP5, *Penicillium spiculisporus;* PDP1, *Aspergillus terreus;* CDP4, *Alternaria tenuissima;* BGCZ3, *Aspergillus terreus;* PDCZ1, *Aspergillus tamarii*; CDCZ4, *Bipolaris spicifera* and WDCZ2, *Aspergillus flavus*

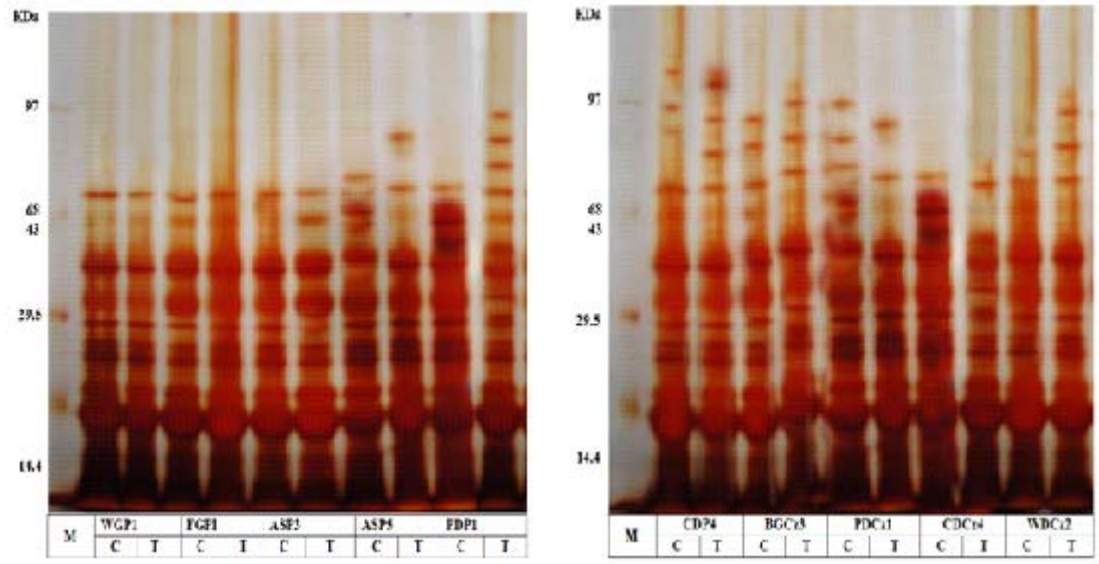

Fig. 4. SDS-PAGE Silver staining of protein extracts of fungal strains affected by Pendimethalin after application of 16d. 1D gel-electrophoresis-based protein profiling revealed mutagenic effects in expressed proteins bands. Where: C, untreated; T, treated, M, Molecular marker; WGP1, *Penicillium verruculosum*; FGP1, *Aspergillus niger;* ASP3, *Penicillium spinulosum;* ASP5, *Penicillium spiculisporus;* PDP1, *Aspergillus terreus;* CDP4, *Alternaria tenuissima;* BGCZ3, *Aspergillus terreus;* PDCZ1, *Aspergillus tamarii*; CDCZ4, *Bipolaris spicifera* and WDCZ2, *Aspergillus flavus*

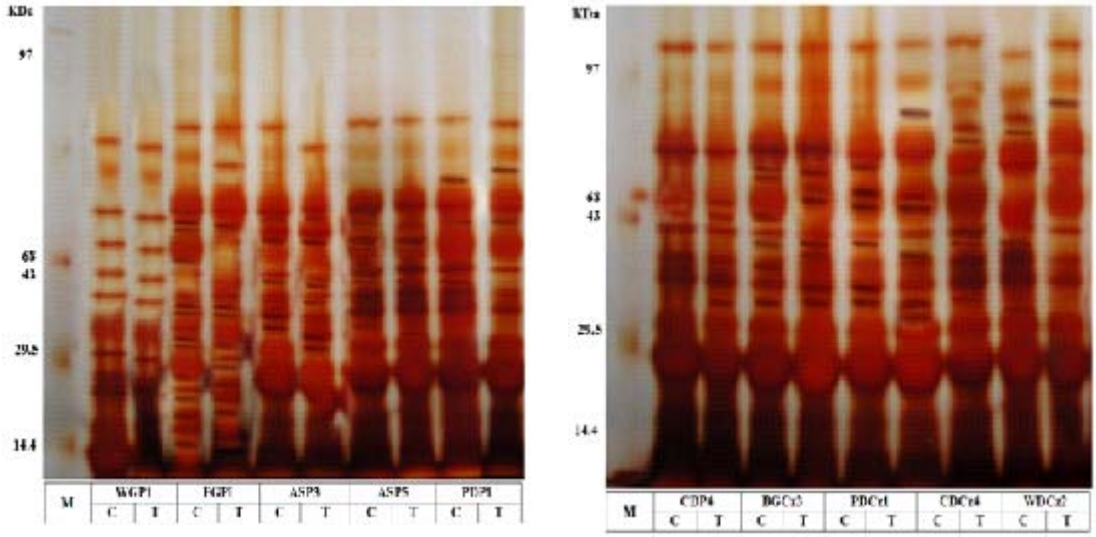

Fig. 5. SDS-PAGE Silver staining of protein extracts of fungal strains affected by Diclofop after application of 16d. 1D gel-electrophoresis-based protein profiling revealed mutagenic effects in expressed proteins bands. Where: C, untreated; T, treated, M, Molecular marker; WGP1, *Penicillium verruculosum*; FGP1, *Aspergillus niger;* ASP3, *Penicillium spinulosum;* ASP5, *Penicillium spiculisporus;* PDP1, *Aspergillus terreus;* CDP4, *Alternaria tenuissima;* BGCZ3, *Aspergillus terreus;* PDCZ1, *Aspergillus tamarii*; CDCZ4, *Bipolaris spicifera* and WDCZ2, *Aspergillus flavus*

increase with the exposure period to the herbicide. Abdel-Megeed (2004) found that the growth inhibition concentration of small concentration of herbicides and short period of treatment did not interfere with protein and nucleic acid synthesis of the tested fungus. Accordingly, it could be suggested that each type of fungicides has its own specific effect on certain metabolic activities of the sensitive fungal species. In general there was significant decrease in total soluble cell ions concentration and Total cell protein concentration (Figures 1 and 2).

In similar study case of the herbicide Brominal the quantity of peptide-N, total soluble-N and total-N of *A. niger* was significantly decreased whereas that of insoluble protein-N was increased. Ammonia-N and total soluble-N of *F. solani* was promoted but insoluble protein-N and total-N was significantly inhibited. In case of *M. racemosus*, the amount of the different nitrogen fractions was significantly retarded at least by one dose except the amount of other soluble-N (amide-N, purines and pyrimidines) which was not significantly affected. Nitrogen analysis of dried mycelium of *P. chrysogenum* showed that insoluble protein-N as well as other soluble-N were significantly inhibited by the two doses whereas peptide-N and ammonia-N were significantly promoted by the two doses, and amino-N as well as total soluble-N were also increased by the high dose. Moreover, the total-N of *P. chrysogenum* was significantly inhibited by the low dose and promoted by the high dose. In case of *S. chartarum*, Brominal was promotive to peptide-N, total soluble-N, insoluble protein-N and total-N but it was inhibitory to other soluble-N. With the exceptions of amino-N of *T. harzianum* which increased with the two doses of Brominal, the different nitrogen fractions as well as insoluble protein N and total-N showed mostly a significant inhibition after treatment (Osman *et al.*, 1989).

Therefore, the proposed mechanism of fungal 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 (Eerd *et al*., 2003). 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 (Joshi and Gold 1993). After adding sugar, FE2 and MEB8 rates

of degradation of pendimethalin were significantly increased, suggesting that its degrading enzyme is an inducible enzyme.

Currently, there are many studies being carried out to optimize the biodegradation potential of fungi in contaminated soil (McFarland *et al.,* 1996; Meysami and Baheri, 2003). If it is accepted that the extracellular ligninolytic enzymes are at least in part responsible for the critical initial reactions of pollutant transformation, the production and activity of these enzymes in contaminated soil under field conditions are two prerequisites for successful application of white rot fungi in soil bioremediation (Lang *et al.*, 1998).

In this study, it could be pointed out that the used herbicides here are not always harmful to protein synthesis in fungi. Certain doses of those chemicals, herbicides or insecticides increased nitrogen content of some fungi. The total-N of *T. harzianum* and *F. solani* was allowed to increase in the presence of sub-lethal doses of Afugan. This increase was coincided with the increase in peptide-N and total soluble-N. The promotive effect of Brominal was observed with *S. chartarum* where the amounts of peptide-N, total soluble-N as well as insoluble protein- N were significantly higher than the control. The increase in the total soluble-N of *P. chrysogenum* and *T. harzianum* after Selecron treatment was correlated with the increase of amino-N in addition to peptide-N or other soluble-N fractions.

Recent advances in molecular biology, biotechnology, and enzymology are the driving force toward engineer-improved fungi and enzymes for mycoremediation. A number of the genetic engineering approaches that have been developed have proven beneûcial in adding the desired qualities in metabolic pathways or enzymes. Strain manipulation is becoming easier with the exponential expansion of molecular tool boxes and genome sequences. However, the best source is that of the genes of fungi, where mycotransformation is well understood. Speciûc gene alterations can be designed and controlled via metabolic engineering. Metabolic control is shared by enzymes (i.e., enzymes are democratic). Mathematical modeling of metabolic control analysis can be used to make predictions as to how metabolic pathways will respond to manipulation.

CONCLUSION

The present study concluded that abnormally higher tolerance levels of the local fungal strains. The total soluble cell ions concentration and total cell protein concentration were decreased in some fungal strains. Interestingly this isolate showed low tolerance to the used herbicides. The effect of used herbicides on the protein profile may reflex somehow DNA changes occurred during the assimilation of those toxic compounds. On the basis of present findings, *Penicillium spiculisporus* and *Aspergillus tamarii* can be recommended as potentially effective local fungal strains and environmentally safer alternative tools to protect the environment from the pollution of these herbicides.

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