JOURNAL OF RADIATION RESEARCH AND APPLIED SCIENCES 6 (2013) 49–55

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Biological indicators, genetic polymorphism and expression in *Aspergillus flavus* under copper mediated stress

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ARTICLE INFO

Article history:
Received 4 June 2013
Accepted 5 July 2013

Keywords:
Copper
Aspergillus flavus
Antioxidative enzymes
Stress response

ABSTRACT

Fungi are considered model organisms for studying stress response and metal adaptation for both biotechnological and environmental purposes. In a previous study, copper was added in concentrations 1 and 10 mM to *Aspergillus flavus* to induce laccase production for bioremediation, but using high concentrations of copper resulted in laccase inhibition despite the increase in bioremediation. In this study, the same copper sulfate was added and some oxidative biomarkers and antioxidative defense enzymes were assessed for stressed cultures of both copper and gamma radiation which was used as a positive stress inducer. The increase in copper concentrations resulted in an increase in superoxide dismutase enzyme activity, lipid peroxidation and protein carbonylation. On the other hand, catalase was inhibited by the addition of both copper concentrations, but exposure to gamma radiation resulted in an increased copper production. Glutathione peroxidase showed variation under stress, while both reduced glutathione and mycelial growth decreased in copper amended cultures. There was an increase in total endogenous carbohydrates. The main location of copper at the end of the incubation period seemed to reside in the cytosolic fraction of the fungus as detected by atomic absorption spectrometry. Genetic polymorphism was evident in the presence of copper as detected by RAPD-PCR. The expression of both laccase and superoxide dismutase suggest that each has a specific role in bioremediation, depending on the added copper concentration.

1. Introduction

Copper is a ubiquitous metal, it is an essential trace nutrient in all microorganisms. It is found in several enzymes like laccase, superoxide dismutase, polyphenol oxidase and cytochrome c oxidase. In addition, it is required in biological cells for its role in electron transport (Palanisami & Lakshmanan, 2010). The exposure of microorganisms to high levels of

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Peer review under responsibility of The Egyptian Society of Radiation Sciences and Applications

Production and hosting by Elsevier

1687-8507/$ – see front matter
http://dx.doi.org/10.1016/j.jrras.2013.10.006

copper provokes a pronounced response of antioxidative systems (Krumova, Pashova, Dolashka-Angelova, Stefanova, & Angelova, 2009), this process is termed homeostasis, it takes place in order to prevent copper toxicity. Metal toxicity in general could cause inhibition of several proteins through blocking of functional groups and conformational modification of cellular macromolecules, displacement of essential ions and disruption of membrane integrity (Gadd, 1993). Being a redox metal, copper is capable of inducing intracellular reactive oxygen species (ROS), therefore, becoming toxic to the cell. It also forms $^{\bullet}OH$ radicals by enhancing Fenton reaction and Haber–Weiss cycle, thereby increasing both intracellular lipid peroxidation and protein carbonylation, in addition to disrupting cell membrane integrity (Palanisami & Lakshmanan, 2010; Shilling & Inda, 2011). However, some fungi tolerate copper to high threshold concentrations through antioxidative stress enzymes which are provoked in the presence of copper; on the other hand, some studies correlate copper stress to reserve carbohydrates which are a typical response to both copper and oxidative stress (Krumova et al., 2009). Other mechanisms of tolerance among these fungi, especially brown rot genera, have been linked to production of oxalate which accumulates in extracellular medium (Shilling & Inda, 2011) or through adsorption onto living or dead mycelium; this characteristic could be used in copper bioremediation or copper removal (Sing & Yu, 1998) rendering copper stress response a tool which could be employed for industrial purposes (Baldrian, 2003). Some pollutants exert DNA damage resulting in changes which could be detected by random amplification of polymorphic DNA (RAPD) analysis; this technique is perceived as simple and fast process to assess population genetic parameters and genetic diversity within the same population (Yin, Liu, Wang, Zhao, & Xu, 2013). In a previous study, copper was used to enhance decolorization of real textile waste water effluent by *Aspergillus flavus*, laccase enzyme was the main enzyme correlated with decolorization, however, at high copper concentrations, laccase was inhibited while the decolorization increased (Gomaa & Momtaz, 2013) therefore, the aim of the present study is to understand the physiological role of copper stress in *A. flavus* in light of some biological indicators as compared to gamma radiation as the positive stress inducer, and study the genetic polymorphism and expression of specific genes suspected to have a role in bioremediation.

2. Materials and methods

2.1. Microorganism and cultivation conditions

A non-aflatoxin producing *A. flavus* strain, obtained from a previous study (Gomaa & Momtaz, 2013), was maintained on malt extract agar medium, the medium was composed of malt extract, 20 g; glucose 20 g; peptone 10 g and agar 20 g/l, pH was adjusted to 4.5 prior to sterilization. Spore suspension was prepared by inoculating 50 ml malt extract broth (same medium without agar) by a 4 mm fungal disc cut from the periphery of a 7 day old culture plate, the culture was incubated overnight at 150 rpm at 30 °C. Spore suspension of approximately 4×10^6 was used to inoculate 250 ml Erlenmeyer flasks containing 50 ml malt extract broth medium which were incubated for another 24 h at the previously mentioned conditions. The flasks were categorized to four sets, each consisting of 3 flasks; the first set represented control cultures. Filter sterilized copper sulfate was added from a stock solution to obtain a final concentration of 1 and 10 mM which was added to the second and third sets, respectively. The fourth set was exposed to 4 kGy gamma radiation which was performed at the Indian cobalt source at the National Center for Radiation Research & Technology (NCRRT) at a dose rate of 2.95 kGy/h, the dose was chosen based on previous unpublished work and was considered a positive oxidative stress inducer. All flasks for the four sets were left to incubate for another 48 h at 150 rpm and 30 °C. Mycelia were harvested using filtration on Mira-cloth, disrupted using homogenization with liquid nitrogen, cellular content were centrifuged at 1500 g for 15 min at 4 °C, the cellular homogenate was used for the biochemical studies described below.

2.2. Biochemical studies

2.2.1. Superoxide dismutase (SOD)

The superoxide dismutase (SOD) activity was assayed according to the procedure described by Kakkar, Das, and Visvanathan (1984) which is based on the inhibition of superoxide ions generated by phenazine methosulfate which converts nitroblue tetrazolium (NBT) into NBT-diformazan, the latter absorbs light at 560 nm using a Shimadzu UV 2100 spectrophotometer. SOD activity was defined as the amount of enzyme required to give 50% inhibition of nitroblue tetrazolium reduction and is expressed as Units/mg protein.

2.2.2. Catalase (CAT)

Catalase was measured according to the method of Beers and Sizer (1952). The disappearance of peroxide was followed spectrophotometrically at 240 nm. One Unit was defined as the quantity of catalase that decomposes one micromole of H_2O_2 per minute at 25 °C (pH 7.0). The reaction mixture consisted of 0.05 M potassium phosphate buffer (pH 7) containing 0.059 M hydrogen peroxide.

2.2.3. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity (U/mg protein) was assayed according to the method of Gross, Bracci, Rudolph, Schroeder, and Kochen (1967). The activity of GPx was expressed as the amount of GSH consumed/min/mg protein.

2.2.4. Reduced glutathione (GSH)

Reduced Glutathione was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a disulfide chromogen which is readily reduced by SH groups, to an intensely yellow color. The absorbance of the reduced chromogen is measured at 412 nm. This is directly proportional to GSH concentration in the sample. Reduced glutathione (GSH) was determined by the method of Ellman (1959).

2.2.5. Lipid peroxidation

Lipid peroxidation was calculated as the concentration of malondialdehyde (MDA) (the end product of lipid peroxidation) in the cell wall of pellets of copper free and copper

amended cultures. Lipid peroxidation was determined as thiobarbituric acid reactive substance (TBARS) according to Yoshika, Kawada, Shimada, and Mori (1979).

2.2.6. *Protein carbonylation*

The protein carbonyl content was estimated by the method of Levine et al. (1990). The spectrophotometric carbonyl assay is based on the reaction of 2,4-dinitrophenylhydrazine with carbonyl group (ketones and aldehydes) to produce 2,4-dinitrophenyl-hydrazone. The amount of hydrazone formed is quantitated spectrophotometrically at 370 nm.

2.2.7. *Protein*

Protein concentrations (mg/ml) were determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin (BSA) as a standard.

2.2.8. *Endogenous carbohydrates content*

Reserve carbohydrates were estimated in the supernatant using the phenol-sulfuric method according to Dubois, Gilles, Hamilton, Rebers, and Smith (1956). Glucose was used as the standard.

2.2.9. *Fungal biomass*

Fungal biomass was determined at the end of incubation period, mycelia were filtered on pre-weighed filter paper, washed and dried overnight in an oven at 70 °C and weighed.

2.2.10. *Copper assay in cell fractions*

Fungal biomass of 0, 1 and 10 mM copper amended media were collected at the end of incubation period by filtration, the cells were dried at 70 °C and digested using a milestone 1200 mega microwave digester (Italy) according to the manufacturer's instructions. Copper in the cytosolic fraction (digested cells) and the extracellular fluid (ECF) (culture filtrate) were assayed using Unicam 939 Atomic Absorption Spectrometer (England). The results are represented as percentage of copper available in the cytosolic fraction and the ECF for control and copper amended cultures.

2.2.11. *DNA extraction and quantification*

DNA extraction kit (Fermentas Life Sciences, EU) was used to extract DNA of *A. flavus* cultivated in presence and absence of copper, total DNA was quantified and its purity assessed using a GeneQuant spectrophotometer. The quality obtained was 2.0 as indicated by the ratio $A_{260/280}$.

2.2.12. *RAPD analysis*

Ten 10-mer deoxy-oligonucleotide primers were assayed for reproducibility for RAPD-PCR. Their sequences are shown below. The reactions were performed using a RAPD-PCR kit (Fermentas Life Sciences, EU) using 100 ng of genomic DNA per sample and 25 μl of each primer as indicated in the protocol.

2.2.13. *Thermocycling profile and detection of the PCR products*

PCR amplification was performed in a Perkin–Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 50 °C for 1 min, and an elongation step at 72 °C for 1.5 min. The primer extension segment was extended to 7 min at 72 °C in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in 1× TBE buffer at 95 V. PCR products were visualized under UV light and photographed using a Polaroid camera.

2.2.14. *Data analysis*

The banding patterns generated by RAPD-PCR marker analyses were compared to determine the genetic relatedness of the two fungi. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands. Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient (Sneath & Sokal, 1973) and was represented as calculated proximity relatedness.

2.2.15. *Expression of laccase under different copper concentrations*

2.2.15.1.

RNA extraction from A. flavus. RNA was extracted from *A. flavus* cultures grown in presence and absence of copper using GeneJET™ plant RNA purification mini kit (Fermentas Life Sciences, EU).

2.2.15.2.

cDNA synthesis. First strand cDNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, EU). About 2 μg total RNA of each extracted sample (control, 10 mM and 100 mM copper) was added to random hexamer primer, 5× reaction buffer, RiboLock™ RNase Inhibitor (20 μ/μl), 10 mM dNTP Mix and RevertAid™ M-MuLV Reverse Transcriptase (200 μ/μl). The mixture was gently mixed and centrifuged and incubated for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction product was used in the following PCR reactions with slight modification: an initial cycle of denaturation 5 min at 94 °C, followed by 35 cycles of denaturation 1 min at 94 °C, annealing 1 min at 52 °C and extension 1 min at 72 °C and then final

Table 1 – Effect of copper and gamma radiation on oxidative stress enzymes.

Oxidative stress enzymes	Initial copper added (mM)			Gamma radiation (4 kGy)
	0	1	10	
SOD (U/mg protein)	1.78	6.78	8.5	9.5
CAT (U/ml)	16.20	7.127	2.276	21.1
GPx (GSH consumed/min/ml)	5.62	7.2	3.76	7.8
GSH (mg/dl)	2.3	1.029	0.561	5.9
TBARS (nmol/ml)	7.22	8.89	12.31	14.99
Protein carbonylation (μmol/mg protein)	6.32	7.81	9.67	10.21
Fungal biomass (g/50 ml)	11.6	10.85	2.71	2.15
Endogenous carbohydrate (mg/g mycelia)	3.47	5.21	5.32	5.82

incubation 7 min at 72 °C. Samples were separated on 1% agarose gel using the following: Lac 2 gene was amplified using the following primers: F: 5′-CGCAT-CATCTTTTGTGCTCC-3′ and R: 5′-AGCGCCAACTACGAC-GAGGA-3′ using annealing of 2 min at 52 °C (Litvintseva & Henson, 2002), while SOD-1 primers were as follows: F 5′-AGGTCGAAGCCGCTCAAAAAA-3′ R 5'-ATTGTGTGGA-GATTCAGAGA-3′ using the previously mentioned initial cycle and the following conditions for amplification: 35 cycle amplification of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min (Honda & Honda, 1999), while the house keeping gene used was 18S rRNA F: 5′-GAC TCA ACA CGG GGA AAC-3′ and R: 5′-ATT CCT CGT TGA AGA GCA-3′ with annealing for 1.5 min at 47 °C (Embong et al., 2008).

3. Results

3.1. Biological indicators

Table 1 represents the changes which took place after exposing *A. flavus* cultures to 1 and 10 mM copper as compared to copper-free cultures and gamma irradiated culture which was considered as a positive stress inducer. Table 1 shows an increase in SOD activity which reached 1.78 in copper-free cultures, 6.78 and 8.5 U/mg protein in 1 and 10 mM copper amended cultures, respectively, as compared to 9.5 U/mg protein for cultures exposed to gamma irradiation. On the other hand, there was a drop in CAT activity in copper amended cultures which reached 7.1 and 2.27 U/ml, while the activity after exposure to gamma radiation reached 21.1 U/ml as compared to 16.2 U/ml in control cultures.

For GPx, there was an increase in activity that reached a maximum when 1 mM copper was added, but this was followed by a decrease in activity at higher copper concentration as compared to gamma irradiated culture which showed the highest GPx activity. GSH showed an evident decrease in copper amended cultures but gamma irradiated cultures exhibited an increase that reached 5.9 mg/dl as compared to 2.3 mg/dl in control cultures. TBARS, protein carbonylation and endogenous carbohydrate content showed a gradual increase which reached 9.67 μmol/mg protein and 5.32 mg/g mycelia, respectively, these increases were concomitant to that obtained for SOD activity, while fungal biomass showed a decrease upon the exposure to copper and gamma irradiation stress.

Table 2 – The distribution of copper (%) in the extracellular fluid (ECF) and cytosolic fraction at different copper concentrations.

Copper (%)[a]	Initial copper added (mM)		
	0	1	10
ECF	2.43	3.58	16.55
Cytosolic	97.56	96.42	83.44

[a] Percentage of copper was calculated from the copper concentrations obtained.

3.1.1. Copper localization

The results in Table 2 clearly shows that the majority of copper is localized in the cytosolic fraction for copper free and copper amended cultures, but it is clear also that the ability of the fungus to accumulate copper intracellularly decreased upon the increase of copper concentration initially added to the media. The results show that when 1 mM copper was added 96.42% was accumulated in the cytosolic fraction as compared to only 83.44% when 10 mM copper was added to *A. flavus* cultures.

3.1.2. RAPD-PCR polymorphism

Using the 10 oligomers presented in Table 3, the results obtained in Table 2 and Figs. 1 and 2 show a variation in the number of bands obtained for copper free and copper amended cultures. The total number of polymorphic bands suggests a genetic variation when copper is added to *A. flavus* which resulted in a calculated proximity relatedness of only 57.2% for copper amended cultures as compared to copper-free culture.

3.1.3. Expression of Lac 2 and Mn-SOD genes in presence and absence of copper

The expression of Lac 2 and Mn-SOD genes are shown in Fig. 3, there is an increase in band sharpness for Lac 2 gene when 1 mM copper was added to the culture media as compared to copper-free culture, this sharpness faded again when 10 mM copper was added. While on the other hand, there was an evident increase in band sharpness for Mn-SOD gene at both 1 and 10 mM copper as compared to a very faint band for copper-free culture. The house keeping gene in this study was 18S rRNA gene which was evidently present clearly for all three cultures.

4. Discussion

A. flavus was capable of decolorizing textile waste water in the presence of copper, the decolorization was correlated with laccase activity when 1 mM copper was added to the cultivation media, the decolorization increased despite the fact that laccase was inhibited when copper was added at higher concentrations (Gomaa & Momtaz, 2013). In order to understand the role of copper in both cases, several biological indicators were studied in the present work. Gamma radiation at a sublethal dose was used as a positive oxidative stress inducer. Ionizing radiation is characterized by production of ROS; it is associated with different oxidative stress responses in many biological cells (Lee et al., 2001). SOD and CAT are among the most commonly studied oxidative stress response enzymes; they are considered the first line of defense. The results show an increase in SOD for both copper and gamma induced stress, on the other hand, CAT activity showed a decrease in copper containing culture contrary to an increase in gamma exposed culture. Copper is known to inhibit CAT (Davison, Kettle, & Fatur, 1986), since CAT contains Fe^{2+} in its catalytic center; it is assumed that Cu^{2+} will act as a non-competitive cation, thus affecting CAT activity. Surprisingly enough, variable effects on CAT activity were reported, where in some cases it

Table 3 – Polymorphism obtained by RAPD-PCR analysis for copper-free culture and 1 mM copper in *A. flavus* cultures.

RAPD primer	Sequence	No. of bands in sample 1	No. of bands in sample 2	Total no. of polymorphic bands
A-07	5′-GAAACGGGTG-3′	7	5	6
B-14	5′-TCCGCTCTGG-3′	7	9	6
B-16	5′-TTTGCCCGGA-3′	5	5	0
C-15	5′-GACGGATCAG-3′	6	9	7
G-07	5′-GAACCTGCGG-3′	10	8	6
G-17	5′-ACGACCGACA-3′	7	8	1
M-16	5′-GTAACCAGCC-3′	9	7	2
A-02	5′-TCGGACGTGA-3′	6	8	6
O-09	5′-TCCCACGCAA-3′	11	12	1
Z-13	5′-GACTAAGCCC-3′	11	13	7
Calculated proximity relatedness (%)		100	57.2	

was induced by copper exposure but inhibited in others; Krumova et al. (2009) suggested that it all depends on the copper concentration used. The results suggest the reliance on the activity of SOD rather than CAT, in assessing the extent of damage provoked by copper at the used concentrations. It is recorded that oxidative stress response results in the appearance of SOD (Angelova, Pashova, Spasova, Vassilev, & Slokoska, 2005). SOD is known for its protective role against ROS generated by ionizing radiation in yeasts (Azab, Mostafa, Ali, & Abdel-Aziz, 2011; Lee et al., 2001); exposure to copper is also known to generate high amounts of ROS which is only counteracted by over expression of SOD (Quili & Bing, 2007).

There is an underlying mechanism by which copper-induced stress oxidative damage causes depletion in the glutathione content where the amount of GSH is consumed to neutralize the exerted stress, this was reported as one of two mechanisms, the first being ROS production, by which copper induces oxidative stress (Asada, 1992).

The correlation of stress to the levels of lipid peroxidation (TBARS) and protein carbonylation in *A. flavus* cultures exposed to copper and gamma irradiation as compared to control cultures show evidently that there is an increase in both parameters under copper and gamma irradiation stress; the induced ROS is the reason behind this elevation. Lipid peroxidation is one of the manifestations of oxidative stress; the free radicals present attack the highly unsaturated fatty acids of the cell membrane (Schinella et al., 2002). Protein carbonylation is considered one of the oxidative stress responses, it results from protein oxidation of intracellular proteins where some of the amino acid residues in the protein macromolecules are converted by the present ROS to carbonyl groups, and this has been observed in heavy metal stress (Davies & Goldberg, 1987).

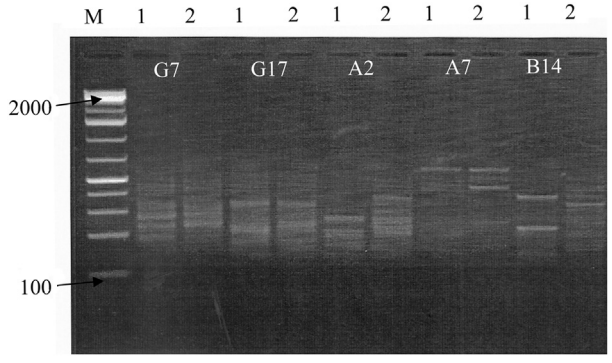

Fig. 1 – Polymorphism for the two samples copper-free *A. flavus* (1) and 1 mM copper amended *A. flavus* (2) cultures using the primers G7, G-17, A2, A7 and B-14.

Copper is not only known to be a laccase inducer (Fonseca, Shimizu, Zapata, & Villalba, 2010; Palmieri, Giardina, Bianco, Fontanella, & Sannia, 2000), but it is also known to be part of a stress response in some microorganisms (Palanisami & Lakshmanan, 2010). Based on the previous study (Gomaa & Momtaz, 2013), it is assumed that the action of copper at low concentrations was restricted to laccase enhancement while at high concentrations; a stress response was indeed responsible for the production of ROS which acted on the dye molecules. Copper is involved in Fenton-like reaction and is related to decolorization (Nerud, Baldrian, Gabriel, & Ogbeifun, 2001), this process is based on the generation of $^{\bullet}OH$ resulting in the attack of the aromatic bonds present in the medium (Urbanski & Beresewicz, 2000) which have contributed to the decolorization process. It is also possible that any resulting hydroxyl radicals derived from hydrogen peroxide present in the medium will cause degradation to recalcitrant compounds (Forney, Reddy, Tien, & Aust, 1982).

There is a physiological correlation between exposure to copper stress and reserve carbohydrates, where some sugars accumulate in the cells (Krumova et al., 2009), while growth was inhibited, reserve endogenous carbohydrates increased with exposure to stress. Such sugars act as stress protectants and therefore control cellular damage (Francois & Parrou, 2001).

To determine the localization of copper at the end of cultivation time, both cytosolic fraction and extracellular fluid copper content were assayed. The results show that the distribution of copper lies in the cytosolic fraction for all the copper concentrations, it is expected that copper is present in copper-free cultures because laccase enzyme has four copper ions (Palmieri et al., 2000), therefore, it was detected in very low concentrations (0.36 ppm) in the cytosolic fraction of copper-free cultures, as compared to 9.5 and 83 ppm accumulating in the cytosolic fraction when 1 and 10 mM copper was added to the culture media. It is impressive that high concentrations of copper are trapped in the cytosolic fraction, without affecting the viability of the fungus. Baldrian (2003)

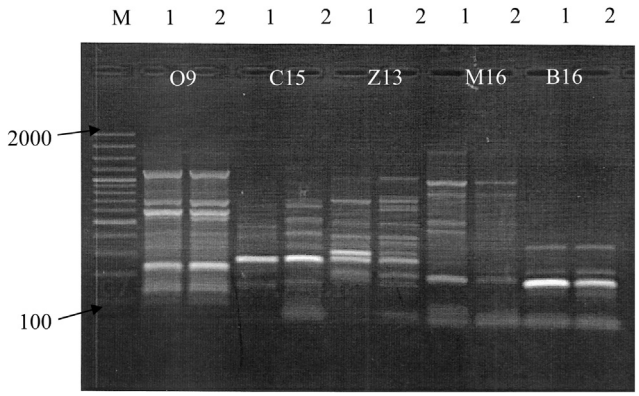

Fig. 2 – Polymorphism for the two samples copper-free *A. flavus* (1) and 1 mM copper amended *A. flavus* (2) using the primers 09, C-15, Z-13, M-16 and B-16.

stated that in order for a metal to induce a physiological response, it must be taken inside the fungus itself. This means that *A. flavus* could be used in metal removal through bioaccumulation. There are a number of homeostatic mechanisms for eukaryotic cells which maintain copper below toxic levels and ensure cell growth, among which is the copper-stimulated endocytosis (Rutherford & Bird, 2004). This might explain the internal bioaccumulation of copper in the cytosolic fraction of *A. flavus* when it was added to the culture media.

To analyze the possible genomic polymorphism which took place in copper amended *A. flavus* cultures, a RAPD-PCR analysis was performed using 10 oligomer primers. The results obtained show that there is evident polymorphism among some primers. RAPD-PCR analysis does not only detect interspecies differences but has been also used to detect DNA damage exerted by lower concentration of a certain pollutant (Atienzar & Jha, 2006). Changes in the number, pattern, appearance or disappearance of bands are all indicators to DNA damage that could have taken place because of oxidative DNA damage, DNA-protein cross links, mutations or chromosomal rearrangement (Nan et al., 2013). However, we could not establish a precise interpretation of the affected target genes in terms of RAPD-PCR, therefore, the expression of the target genes; laccase and SOD, suspected to take place in bioremediation in the previous study were assayed. Lac 2 was

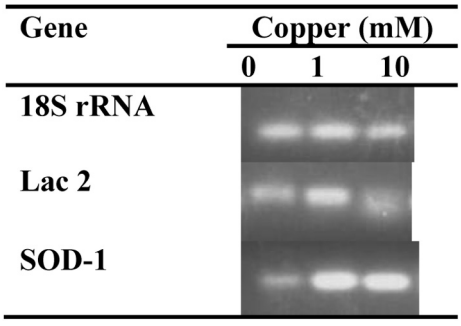

Fig. 3 – The expression of the Lac 2 and SOD-1 genes using 18S rRNA gene as the house keeping gene in the presence of 0, 1 and 10 mM copper.

chosen because it was the gene representing the inducible laccase fraction in *A. flavus* as reported previously (Gomaa & Momtaz, 2013), while SOD 1 was chosen based on the elemental analysis of the purified enzyme fraction obtained also in the same mentioned study which contained both Cu and Zn in the cytosolic fraction, but not Ni or Mn suggesting that it is the Cu–Zn SOD, otherwise known as SOD-1. The gene expression obtained is considered another proof of the involvement of both genes in the bioremediation, laccase representing its maximal activity at 1 mM copper concentration acted as the regular oxidoreductase laccase enzyme activity known in bioremediation, while SOD reaching the maximal activity at 10 mM copper represents the highest release of the superoxide radical which is catalyzed by SOD to release hydrogen peroxide which in turn attacks molecules in an arbitrary mechanism, both resulting in an increased bioremediation of textile waste water.

In conclusion, the effects exerted by adding copper to the cultivating media depend mainly on the concentration used in the media. Low concentrations induced laccase while high concentrations resulted in formation of reactive oxygen species which played a role in bioremediation. We present evidence that copper stress resembles those caused by oxidative stress-inducing agents, it causes genetic polymorphism, in addition to that copper resistance in *A. flavus* takes place via bioaccumulation; all these traits could be employed in industrial production of enzymes and also in bioremediation of copper containing waste water.

Acknowledgment

The authors would like to thank the Genetic Engineering Service Unit (GEST) at the Agriculture Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC) for capturing the RAPD images and performing the RAPD analysis.

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