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REGULAR ARTICLE

BIOSECURITY FOR REDUCING OCHRATOXIN A PRODUCTIVITY AND THEIR IMPACT ON GERMINATION AND ULTRASTRUCTURES OF GERMINATED WHEAT GRAINS

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ABSTRACT

Ochratoxin A (OTA) is a secondary metabolite of some fungi that causes very serious problems for plants, animals and humans. Various microorganisms such as bacteria and microscopic fungi have been tested for their abilities to prevent ochratoxin A contamination or detoxify foods. In this study, *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* reduced OTA production by *Aspergillus ochraceus* to 40.88 µg/ml (productivity 60.69%) and 13.80 µg/ml (productivity 20.48%) respectively compared with the control (67.35 µg/ml) (productivity 100%). The results clearly indicated that the seed germinibility in the presence of OTA was decreased with increasing concentration, whereas the germinibility was uncompletely ceased at high concentration (67.35 µg/ml) of OTA. The maximum amount of germination was observed in control (without OTA treatment) and at low concentration (13.80 µg/ml) within 4 days. Antioxidant enzymes catalase and peroxidase decreased in germinated grains treated with OTA. Catalase was 18.12 U/ml in grains treated with low concentration (13.80 µg/ml) of OTA while at high concentration (67.35 µg/ml), it was 12.23 U/ml compared with the control (20.33 U/ml). On the other hand, peroxidase decreased only in germinated grains treated with high concentration of OTA. The ultrastructural studies

indicate that there were dramatic differences between the cells of root system of wheat seedlings of grains treated and untreated with the OTA. Cell ultrastructures of treated grains with OTA showed that the cytoplasmic membrane collapses away from the cell wall. Plasmodesmata threads were appeared in untreated cells but not formed in treated cells.

Key words: Biosecurity, ochratoxin A, productivity, wheat grains

INTRODUCTION

Ochratoxins are worldwide spread secondary metabolites synthesized mainly by some toxigenic species of *Aspergillus* and *Penicillium* **(Sedmikova *et al*., 2001; Bayman *et al*., 2002; Magan and Aldred, 2005)**. *P. verrucosum* is the major OTA-producing fungus in northern Europe, while A. ochraceus is more important in warmer climatic zones **(Cairns-Fuller *et al.*, 2005)**. More *Aspergillus* species have been found to produce OTA, for example *A. melleus*, *A. sulphureus*, *A. alliaceus*, *A. sclerotiorum* **(Bayman and Baker, 2006)**, *A. albertensis*, *A. lanosus* **(Palumbo *et al*., 2007)**. Ochratoxin A (OTA) is important because of the contamination of agricultural products including cereals and grains and influence chronic human exposure **(Alexa *et al*., 2008; Dehelean, 2011).**

Natural occurrence of OTA in maize and maize-based products is a world wide problem **(Duarte *et al*., 2010)**. Maize kernels are a good substrate for mould infection and production of mycotoxins harmful to both humans and animals. *A. niger* is frequently isolated from maize **(Magnoli *et al*., 2007; Shah *et al*., 2010)** and a high incidence of *A. carbonarius* has been also reported **(Shah *et al*., 2010; Alborch *et al*., 2011)**. Both species could be a source of OTA in maize and other food products in both tropical and subtropical zones of the world **(Palencia *et al*., 2010)**. The highest reported occurrences of OTA contamination have been found in cereal grains, and to a lesser extent in grapes, wine, grape juice and dried vine fruits **(Clark and Snedeker, 2006)**. Fungal invasion and mycotoxins contamination of agricultural products lead to losses in terms of quantity, market value, quality of food and feed production due to changes in colour, texture and taste **(Mutegi *et al*., 2009)** and reduction of seed germination **(Negedu *et al*., 2010)**, energy and nutritional value changes in terms of loss of carbohydrates, proteins, amino acids and vitamins and increases in fatty acids may also occur **(Negedu, 2009)**.

Many species of bacteria, fungi and yeasts have been shown to enzymatically degrade mycotoxins **(Bata and Lasztity, 1999)**. **Bejaouii *et al.* (2004)** suggested that oenological

strains of *Saccharomyces* yeasts can be used for the decontamination of OTA in synthetic and natural grape juice. Several bacterial and fungal strains belonging to *Streptococcus, Bifidobacterium, Lactobacillus, Butyrivibrio, Phenylobacterium, Pleurotus, Saccharomyces, Bacillus* and *Acinetobacter* genera and certain fungi belonging to *Aspergillus* (*A. fumigatus, A. niger, A. carbonarius, A. japonicus, A. versicolor, A. wentii* and *A. ochraceus*), *Alternaria, Botrytis, Cladosporium, Phaffia, Penicillum* and *Rhizopus* (*R. stolonifer* and *R. oryzae*) genera, are able to degrade OTA *in vitro* up to more than 95% **(Abunrosa *et al*., 2006).** Some microorganisms have been found to control *Aspergillus, Penicillium* infections and OTA production **(Ciconova *et al.*, 2010)**. For example, lactic acid bacteria produce antifungal substances. **Corsetti *et al.* (1998)** found antifungal effect of the mixture of short-chained organic acids that were produced by *Lactobacillus* (*Lb.*) *sanfranciscensis.* This bacterium inhibited the growth of *A. niger* and *P. expansum* on malt agar medium. Cell-free supernatant from *Lb. casei* inhibited spore germination of the investigated *Penicillium* spp. on potato dextrose agar medium. Also *Bacillus subtilis* produced a peptidolipid that inhibited *A. ochraceus* **(Klich *et al.*, 1991)**. Other *Bacillus* sp., *B. thuringiensis* used as a commercial insecticide during the cultivation of wine grapes inhibited the growth of *A. carbonarius* on potato dextrose agar medium **(Bae *et al*., 2004)**. **Masoud *et al.* (2005)** found that *Pichia anomala* and *Pichia kluyveri* inhibited the production of OTA by *A. ochraceus* on malt extract agar medium and on coffee agar medium. The competing microbes may enhance or hinder the formation of mycotoxins by changing the metabolism of the producing organisms, by competing for the substrates by changing the environmental conditions making them unfavourable for mycotoxin production or by producing inhibitorial compounds **(Ritieni *et al*., 1997)**.

The phytotoxic effect of many secondary metabolites produced by fungi has been shown through biotests in plants **(Kachlicki and Jedrycka, 1997)**. Changes in the plant cell structure and alterations of the cytoplasm were discernible after root treatment with toxin solutions at the higher concentration (250 μg/ml). The cells appeared turgid, but with very extensive plication of cell membranes and part of the cell walls, and having numerous vesicles in the cytoplasm. Plasma membrane withdrawal was observed along the entire circumference of the cell as well as that of the cell wall after treatment with *Alternaria alternata* toxins **(Tylkowska *et al*., 2008)**. Reduction in levels of chlorophyll, protein and nucleic acid concentrations during seed germination and seedling growth in mung beans (*Vigna radiata* (L) Wilczek, var Pusa 119) resulted from ochratoxin A treatment, and the extent of reduction was directly influenced by the concentration of toxin applied **(Sinha and Kumari, 1989)**.

Aflatoxin does not affect seed germination but is inhibitive to hypocotyl elongation in lettuce. The degree of inhibition appears to be directly related to the amount of toxin and at high concentrations the rate of hypocotyl elongation is diminished proportionately **(Crisan 1973 a and b)**. The ultrastructure of *Lepidium* root cells treated with crystalline aflatoxin B exhibited morphological changes characteristic of those found in aflatoxin-treated animal cells. In addition to changes in the cytoplasmic organelles, numerous ring-shaped nucleoli with prominent nucleolar caps were produced. The germinibility of bean, red gram, green gram and black gram seeds had vigorously reduced when soaked on culture filtrate of the toxic fungal strain **(Janardhan *et al*., 2011)**.

The aim of the study was to evaluate the efficacy of safe microorganisms *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* to reduce ochratoxin A production by *A. ochraceus*, to ensure food safety and to protect consumer's health. Also, the study was aimed to evaluate the impact of ochratoxin A to germination and ultrastructures of wheat grains.

MATERIAL AND METHODS

Ochratoxin A production, their producing fungus and biocontrol

Under sterile conditions of laminar flow cabinet, spoiled grains of *Zea mays* were added to solid media per Petri dish and then incubated at 30ºC for 7 days. The dominant fungal species was isolated from spoiled grains and cultured separately on Czapek's agar medium and malt extract medium. The prepared slides of fungal isolates were examined microscopically by using software for image analysis (SIS version 2.11, 1996) at the Regional Center for Mycology and Biotechnology at Al- Azhar University Cairo, Egypt. According to Raper and Fennell (1973) , the isolated fungus was identified as *Aspergillus ochraceus* .This fungus was cultivated on broth medium containing 20 g of corn flour grains for 10 days at 30 °C. Commercial strains of *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* were inoculated at 10^4 cells/ml^{-1} individually in co-cultures with mycotoxigenic species *A. ochraceus* on the same growth medium. Then after 10 days the growth medium was extracted with chloroform : methanol (2:1, v/v) and shaken for 30 min on an orbital shaker. The extract was filtered through filter paper (Whatman No. 4). The supernatant was put into 1 ml amber HPLC vials (Fisher) and stored at -20 ºC until processed. OTA contents were measured using High Performance Liquid Chromatography (HPLC). An HPLC (Waters 600E System

controller) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) were used. The extract was quantitatively analyzed for OTA detection by High Performance Liquid Chromatography.

Germination of wheat grains and ultrastructures studies

Twenty healthy grains of wheat (pure strain of *Triticum vulgaris* (GIZA 2) obtained from the Egypt Ministry of Agricultures were selected, sterilized with 0.01% NaOCl for 1 min followed by 70% ethanol for 1 min, and then washed in distilled water and placed in a Petri dish, soaked with two different concentrations of ochratoxin A as a result of biocontrol test and germinated at 25-26 ºC . Electron microscope studies were carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. Epicotyls of germinating grains of wheat treated with low concentration only of ochratoxin A were cut into small pieces. Tissue samples were fixed in a solution of 1% paraformaldehyde, 0.025% glutaraldehyde and 0.01 M phosphate buffered saline (PBS, pH 7.2) for 10 hours at 10 ºC, and then washed with the same buffer for 5 h at 10 ºC. Then the buffer was removed and the samples were covered with an aqueous solution of 1% osmium tetraoxide for 2 hours. After this the osmium solution was removed and the samples dehydrated by passage through a series of ethanol concentration ranging from 50% to 96%. The absolute alcohol was removed and propylene oxide was added to the sample for 1 h. The samples were put in propylene oxide and Epon 812 resin (2:1) then in pure resin for overnight and placed in an oven at 60 ºC for 48 h. Small blocks were sectioned (50 nm) using ultra microtome. The sections were stained by uranyl acetate-lead citrate 500A and subsequently examined with the transmission electron microscope (C Joel Jem- 1200 EX II. Acc. Voltage 120 KV. MAG- medium). Germination % and length of epicotyles of germinated wheat grains were measured and at the same time antioxidant enzymes catalase and peroxidase at different concentrations of OTA were determined according to **Kar and Mishra (1976)**.

RESULTS AND DISCUSSION

Prevention of pre-harvest and post-harvest natural contamination of feedstuffs by OTA is a basic tool in the strategy to minimize the subsequent occurrence of OTA into the feed and food chain. The empirical formula of OTA is $C_{20}H_{18}O_6NCl$ and the molecular weight is 403.82 $gmol^{-1}$. IUPAC developed the formula of OTA which is shown in Fig. (1) and is L-

phenylalanine-*N*-[(5-chloro-3,4- dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl) carbonyl]-(*R*)-isocoumarin **(Renzulli *et al.*, 2004)**. In the present study *A. ochraceus* was isolated from contaminated corn grains and cultivated on liquid medium (Fig 2). Grains contaminated by mycotoxins are unsuitable for human and animal consumption because they may cause numerous biological disturbances **(Goswami and Kistler, 2004)**. In our study, *S. cerevisiae* and *L. bulgaricus* reduced OTA production by *A. ochraceus* to 40.88 µg /ml (productivity 60.69%) and 13.80 µg/ml (productivity 20.48%) respectively compared with the control (67.35 µg/ml) (productivity 100%) (Table 1 and Fig. 3). In our study *Lactobacillus* suppressed OTA production in co-culture, this is not agreement with **Cuero *et al.* (1988)** who found *B. amyloliquefaciens* able to increase toxin production of *Aspergillus flavus* in maize grains. It is possible that the *Lactobacillus* under our experimental conditions altered the substrate reduced growth of *A. ochraceus* and its ability to produce OTA. Recently, the effect of different fermenting microorganisms on growth of a mycotoxin- producing *Aspergillus nomius* was assayed with **Munoz *et al.* (2010)** two lactic acid bacteria, *L. fermentum* and *L. rhamnosus* and *S. cerevisiae*. All three microorganisms assayed showed growth inhibition of the mycotoxin-producing *Aspergillus* strain. In other study, yeast *Pichia anomala* inhibited OTA production by one isolate of *Penicillium verrucosum* in malt extract agar medium as well as in wheat **(Petersson *et al.*, 1998)**. Therefore, **Blank and Wolffram (2009)** investigated whether a daily addition in the diet of 0.4 g of live yeast cells (*S. cerevisiae*), registered as a feed additive for improving livestock performance, could reduce the OTA bioavailability and enhance its excretion in sheep. Recently, **Kapetanakou *et al.* (2012)** used bacteria and yeast to reduced mycotoxins production**.** Yeast or yeast cell walls can also be used as adsorbents for mycotoxins. By the use of yeast cell walls only instead of whole cells, the adsorption of mycotoxins can be enhanced **(Bata and Lasztity, 1999)**. According to **Ringot *et al.* (2007)**, yeast biomass may be regarded as a good source of adsorbent material, due to the presence in the cell wall of some specific macromolecules, such as mannoproteins and beta glucans. **Skrinjar *et al.* (2002)** found the reduction of OTA amount by *Lb. acidophilus* in yoghourt.

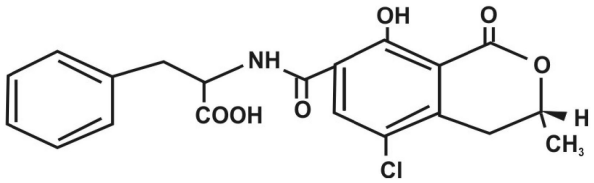

Figure 1 Chemical structure of ochratoxin A

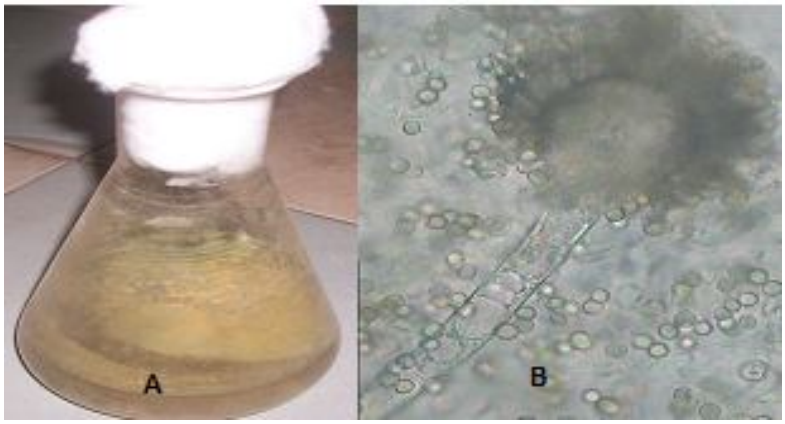

Figure 2 *A. ochraceus* growth on corn flour medium (A) and their morphological characters (40x) (B)

Table 1 Effect of *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* on ochratoxin A production

Treatment	OTA cocentration (µg /ml)	Productivity* %
Control	67.35	100.00
S. cerevisiae	40.88	60.69
L. bulgaricus	13.80	20.48

Legend: * Productivity % was regarded as 100% in the control

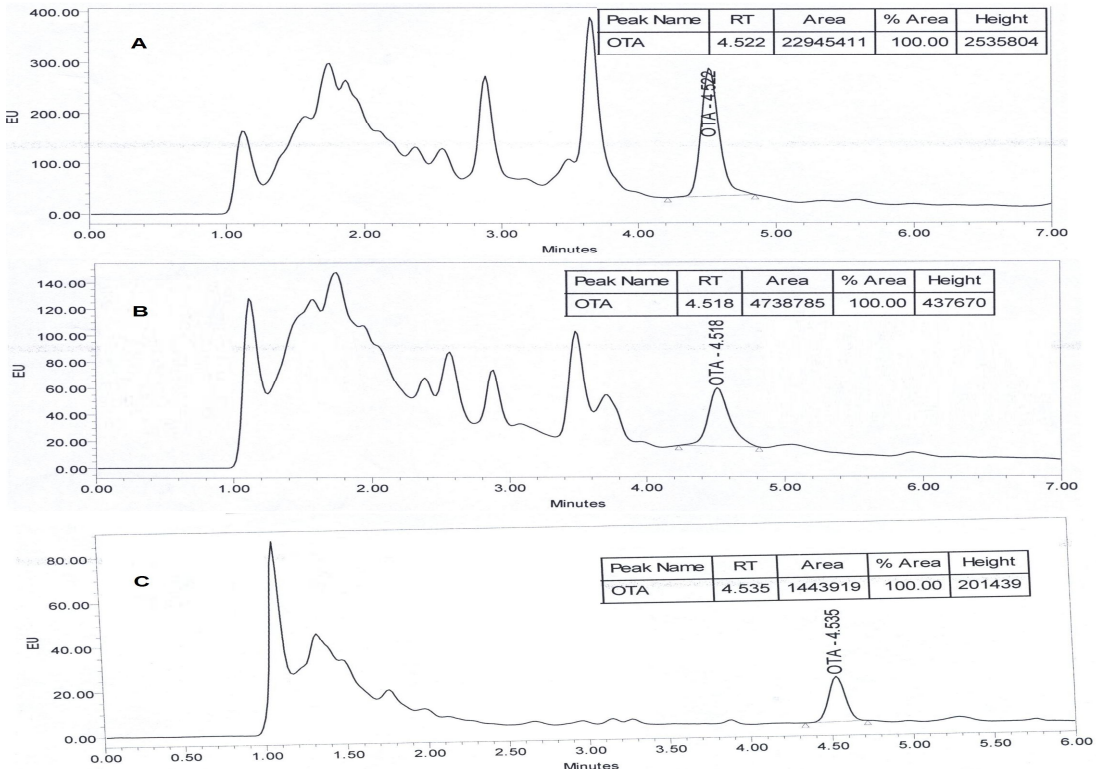

Figure 3 HPLC chromatogram of different concentratins of ochratoxin A in the presence of control (A), *Saccharomyces cerevisiae* (B) and *Lactobacillus bulgaricus* (C)

The phytotoxicity of a number of secondary metabolites present in culture filtrates of pathogenic fungi has been demonstrated **(Svabova and Lebeda, 2005; Tylkowska *et al*., 2008)**. In the present study the wheat grains were treated with OTA produced by *A. ochraceus* (Fig. 4) at different concentrations. The results clearly indicated that the seed germinibility in the presence of OTA was decreased with increasing concentrations, whereas the germination was uncompletely ceased at high concentration (67.35 µg/ml). The maximum amount of germination was observed on control and low concentration within 4 days. Except for a slight initial lag in germination observed at highest concentration (67.35 µg/ml) compared with the control (grains untreated with OTA), this may be due to grains enzymes may affected by OTA. In our study, OTA did not inhibit the germination of wheat grains especially at low concentration 13.80 (µg/ml) where the germination % reached to 100% equal to the control (Table 2). At low concentration of OTA (13.80 µg/ml), elongation of the hypocotyls and epicotyls in seedlings was slightly inhibited compared with the control (Fig. 4) while at high concentration (67.35 µg/ml) elongation of the epicotyls in seedlings was sharply inhibited compared with the control. This result was in agreement with **Wakulinski (1989)** who stated that trichothecenes inhibit seed germination and reduce root and shoot growth in wheat seedlings. The phytotoxic effects of deoxynivalenol on plants can be summarized by **Rocha *et al*. (2005)** as growth retardation, inhibition of seedling and green plant regeneration. Nineteen plants were studied by **Crisan (1973b)** to determine the effects of aflatoxin B, on seed germination and seedling development. Germination was not inhibited in any test organism at a concentration of 100 µg of aflatoxin. At 10 µg/ml OTA an inhibitory effect was observed on root and shoot elongation of mature maize (*Zea mays*) embryos **(McLean, 1996)**. Results revealed that antioxidant enzymes catalase and peroxidase decreased in germinated grains treated with OTA. Catalase was 18.12 U/ml in grains treated with low concentration of OTA (67.35 µg/ml) while at high concentration (67.35 µg /ml), it was 12.23 U/ml compared with the control (20.33 U/ml). On the other hand, peroxidase decreased only in germinated grains treated with high concentration of OTA (67.35 µg/ml) (Table 2). Decreasing of antioxidant enzymes may explained with presence of grains under stress conditions of OTA.

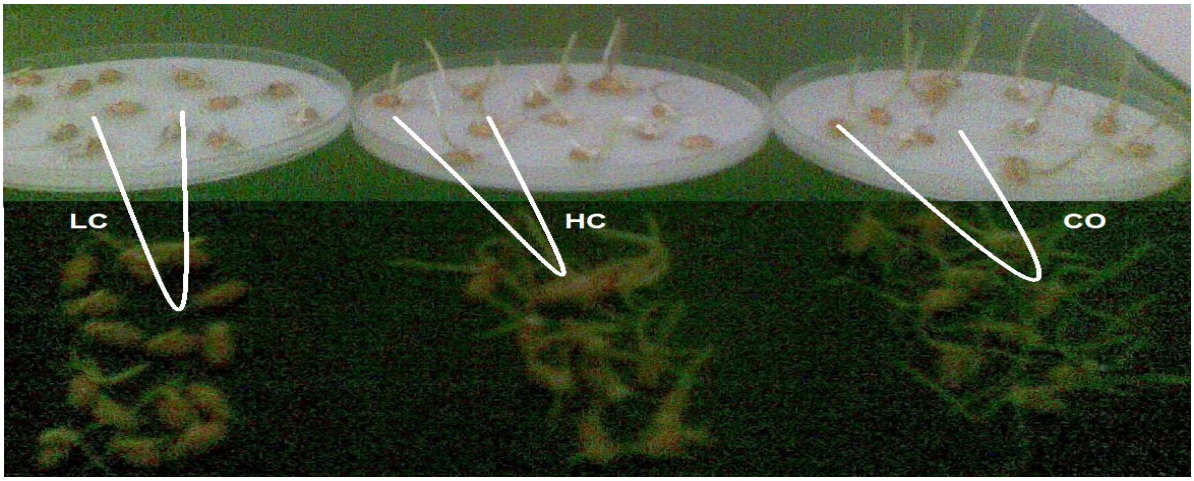

Figure 4 Germination and growth of wheat seedlings in control (CO), in the presence of low concentration (LC) (28.57 µg/ml) and in high concentration (HC) 64.11 (µg/ml)

Table 2 Effect of ochratoxin A on wheat grains germination and their antioxidant enzymes

Ochratoxin A concentration (µg /ml)	Epicotyle length (cm)	Antioxidant enzymes (U/ml)		Germination %
		Catalase	Peroxidase	
0.00 (Control)	2.70	20.33	0.093	100
13.80	1.18	18.12	0.110	100
67.35	0.40	12.23	0.024	80

The ultrastructural studies indicate that there were dramatic differences between the cells of root system of wheat seedlings of grains treated and untreated with the OTA (Figs 5 and 6). The cells of root system of untreated grains containing definable organelles cell wall, cell membrane, cytoplasm, vacuoles, mitochondria, nucleus, plasmodesmata threads, Golgi bodies, starch and aleurone grains. On the other hand, cell ultrastructures of treated grains with OTA showed the cytoplasmic membrane collapses away from the cell wall (Fig. 5 C and D). At the same time unknown structure was present between cell wall and cell membrane (Fig. 5 D). Plasmodesmata threads were appeared in untreated cells (Fig. 5 A) but not formed in treated cells (Fig. 5 C and D), the disappearance is explained with the presence of cell under stress conditions of OTA . Root hairs of untreated germinated grains cells was longer and more branched than root hairs of treated germinated grains (Fig. 5 E and F). From these results it can be seen that OTA could interfere with the normal development and functioning cell wall, vacuoles, plastids and other internal organs. **Crisan (1973a)** found that the ultrastructure of *Lepidium* root cells treated with aflatoxin B, exhibited morphological

changes. Plasma membrane withdrawal and vesiculation, microvacuole formation and accumulation of plastoglobuli in chromoplasts occurred in root cells of *Daucus carota* as aresult of effect alternariol and alternariol methyl produced by *Alternaria alternata* **(Tylkowska *et al*., 2008)**. The effect of OTA on shoot system was also clear as well as in root system. Clear cells with definitely nucleus and plastides were appeared without OAT treatment (Fig. 6 A and B). On the other hand, irregular cells with the collapse of cell membrane out from cell wall (Fig. 6 C and D) were visible with OAT treatment. The untreated cells containing few vacuoles with large size (Fig. 6 B) compared with treated cells (Fig. 6 C) where it contained many vacuoles with small size. **Tylkowska *et al.* (2008)** demonstrated that ultrastructures were observed carrot roots as a result of treated with fungal toxins. Mature maize embryos were exposed to aflatoxin B_1. An ultrastructural investigation of the subcellular alterations induced following toxin exposure provided evidence of deteriorative changes in several compartments of the plant cell **(Michelle, 1994)**.

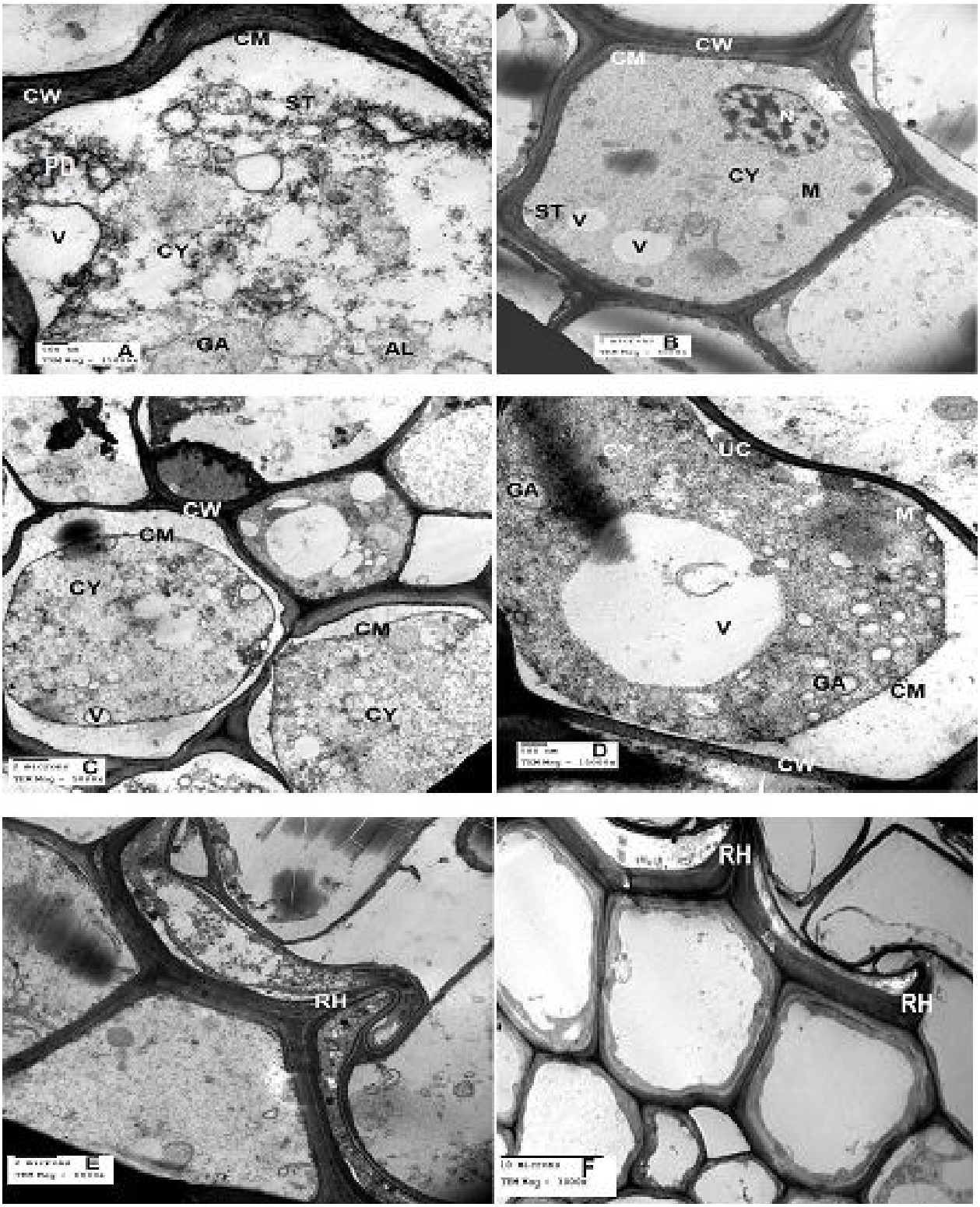

Figure 5 Ultrastructure of root system of untreated wheat grains (A, B and E) and treated with OTA (C, D and F). Abbreviations: CW, cell wall; CM, cell membrane; M, mitochondria; N, nucleus; V, vacuole; PD, plasmodesmata; RH, root hair; GA, Golgi body; ST, starch grains; AL, aleurone; CY, cytoplasm; UC, unknown compound

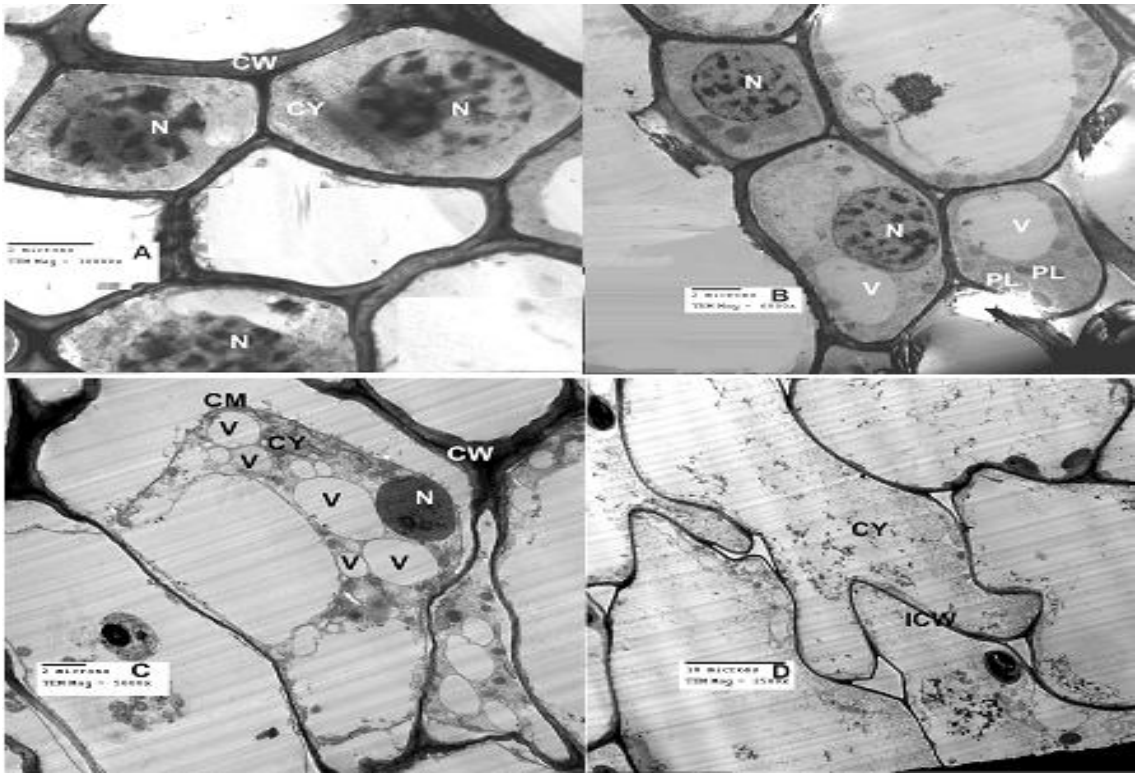

Figure 6 Ultrastructure of shoot system of untreated wheat grains (A and B) and treated with OTA (C and D). Abbreviations: CW, cell wall; CM, cell membran; ICW, irregular cell wall; N, nucleus; V, vacuole; CY, cytoplasm

CONCLUSION

Wheat seeds germinibility in the presence of OTA were decreased with increasing concentration of OTA and the antioxidant enzymes catalase and peroxidase decreased in germinated grains treated with OTA. The ultrastructural studies indicate that there were dramatic differences between the cells of root system of wheat seedlings of grains treated and untreated with the OTA. Our study revealed that *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* reduced OTA production by *A. ochraceus*.

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