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Biorepress of fumonisin B1 production and their phytotoxicity on growth and ultrastructures of Maize (*Zea mays*) Seedlings

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ABSTRACT

The results clearly indicated that the seed germinibility in the presence of FB1 was decreased with increasing their concentration, whereas the germinibility was uncompletely ceased at high concentrations, a slight initial lag in germination observed at lowest concentration 28.57 µg /ml and highest concentration 64.11 µg /ml, respectively compared with the control. At concentration of FB1 (28.57 µg /ml), elongation of the hypocotyls and epicotyls in seedlings slightly inhibited compared with the control. Chlorophyll a and b content of seedlings of *Zea mays* decreased with increasing concentrations of Fumonisin B1. Chlorophyll a content was 6.23, 3.88 and 3.91 (mg/ g fresh weight) at concentrations 28.57, 40.54 and 64.11 (µg /ml) of Fumonisin B1 compared with the control 8.55 mg/ g fresh weight. The ultrastructural studies indicate that there were dramatic differences between the treated and untreated tissues of *Zea mays* seedlings with the toxin FB1. The treated cells with FB1 are highly vacuolated compared with the untreated cells. Thin layer of external epidermis was observed in untreated cells while thick layer of external epidermis in treated cells was appeared. *Sacchaaromyces cerevisiae* reduced FB1 to 40.54 (µg /ml) compared with control 64.11(µg /ml) .On the other hand *Trichoderma harizianum* was more efficient in FB1 productivity where productivity was reduced to 28.57 (µg /ml) with 44.56 %.

Keywords: Biorepress, fumonisin B1, phytotoxicity, ultrastructures

INTRODUCTION

Maize (*Zea mays* L.) is a cereal crop grown throughout the word. Maize plays an important role in the diet of millions of people due to its high yields per hectare, its ease of cultivation and adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Asiedu, 1989). Kossou and Aho (1993) reported that fungi could cause about 50-80 % of damage on farmer's maize during the storage period if conditions are favorable for their development. The major genera commonly encountered on maize in tropical regions are Fusarium, Aspergillus and Penicillium (Orsi *et al*., 2000). Fumonisins are mycotoxins which are a structurally related group of long-carbon chain compounds (Munkvold and Desjardins 1997). Fumonisin analogues have been identified and classified into fumonisin A_1, A_2, B1, B_2, B_3, and B_4 based on their chemical structure. Fumonisins are produced mainly by *Fusarium moniliforme* (= *F. verticillioides*), *F. proliferatum*, and several other Fusarium species. Fumonisin B1 (FB1) first isolated from *F. moniliforme* MRC 826 by Gelderblom *et al*. (1988), is a hydroxylated long-chain alkylamine with two tricarboxylic acid moieties attached. FB1 has been reported to be phytotoxic to various weeds and crop cultivars (Abbas and Boyette 1992). Maize is the product in which fumonisins are most abundant (Shephard *et al*., 1996). Fumonisins can contaminate maize foods and feeds as a result of the Fusarium invasion before and after harvest (Doko *et al*., 1995). Fusarium species are widespread throughout the world and produce phytotoxins such as the fumonisins,

fusaric acid, and moniliformin (Chakrabari and Basuchaudharg 1980, Duke 1986, Burmeister and Plattner 1987). Phytotoxic fumonisins are produced by *F. moniliforme* and related fusaria such as *F. proliferatum* (Ross *et al*. 1990, Vesonder *et al*. 1990, Thiel *et al*. 1991). Fumonisins are found to be phytotoxic. FB1 can indeed damage a wide variety of plants including maize (Lamprecht *et al*., 1994). Doehlert *et al*. (1994) showed that the presence of high levels of fumonisins in maize seeds might have deleterious effects on seedling emergence. Elongation of maize radicles was inhibited by about 75% after 48 h of imbibition in100 µg/g of fumonisins and amylase activities in seeds significantly decreased as well. In previous studies fumonisin has been shown to cause photobleaching, necrosis, growth inhibition, and death in intact jimsonweed plants, as well as other weed and crop species (Abbas and Boyette 1992).

Transmittal of Fusarium species such as *F. proliferatum*, *F. culmorum* and *F. graminearum* are very considerable in grain store and bread wheat because these species are able to produce fatal mycotoxin like Fumonisin, zearalenon, Deoxynivalenol and Nivalenol that can be some main reasons of entanglement in different digestive cancers (Siassi, 2000). Grains contaminated by mycotoxins are unsuitable for human and animal consumption because they may cause numerous biological disturbances (Goswami and Kistler 2004). The mycotoxin produces inhibition of protein synthesis, electrolyte losses, cytoplasm convolution and disintegration of organelles (Miller and Ewen 1997). FB1 is believed to be the most toxic fuminisin leading to most severely adverse health effects in animals (Gelderblom and Abel1 998). Many kinds of cereals including corn, sorghum, rice and wheat are known to be infected by Fusarium species and produce fumonisins, particularity corn crops (Marin *et al.* 1995, Fang-Ming *et al*. 2005). *F. moniliforme* is a soil-borne as well as a seed-borne pathogen of corn that inhabits in the field. Therefore, infection of *F. moniliforme* can infect the roots, stalks, and kernels of corn. Generally, the fumonisin level will increase in corn products during storage as long as proper grain moisture and temperature are maintained (Musser and Plattner 1997). Fusarium species can invade corn kernels by inner route and produce fumonisins. A significant percentage of healthy-looking corn kernels contain fumonisin levels of about 1 ppm or higher (Musser and Plattner 1997, Nelson *et al*. 1991). When plants are contaminated by fuminisins, they could cause physiological damage, growth inhibition, and death in plants (Abbas and Ocamb 1995). When the grains are infected, the starch granules of the endosperm are extensively degraded (pitted) (Jackowiak *et al.* 2002) and the storage protein matrix that surround the starch granules were absent (Pekkarinen *et al*. 2000). 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 interaction between mycotoxin producing fungi and other microorganisms is a common phenomenon in nature that can affect fungal growth and/or production of mycotoxins (Hassan and Bullerman 1997). *Lactococcus cremoris* was reported to control mycotoxinogenic mould growth (Florianowicz 2001). The effect of different fermenting microorganisms on growth of a mycotoxin- producing *Aspergillus nomius* was assayed. Two lactic acid bacteria, *L. fermentum* and *L. rhamnosus*, and *Saccharomyces cerevisiae*, All three microorganisms assayed showed growth inhibition of the mycotoxin-producing Aspergillus strain (Munoz *et al*. 2010). *Trichoderma viride*, also can control

growth of *F. verticillioides* conidia and toxin production on maize seed (Yates *et al.* 1999). Mausam (2007) indicated that several *Trichoderma* spp have proved to be effective mycoparasites. Papavizas and Lumsden (1980) demonstrated that *T. harzianum* is known for parasitizing the mycelium of several important plant Pathogens.

This work was aimed to study the phytotoxicity of FB1 on growth and ultrastructures of *Zea mays* seedlings. Also,the present study highlights the potential use of save microorganisms in the biocontrol of Fusarium and FB1 production.

MATERIAL AND METHODS

1. Fumonisin B1 production and their producing fung

Under sterile conditions of laminar flow cabinet, spoiled grains of *Zea mays* were added to solid media per Petri dish and the dishes were incubated at 27ºC for 7 days. One dominant fungal species was isolated from spoiled grains cultured separately on Czapek's agar medium and malt extract medium incubated for 7 days at 28 ± 2 °C.The Petri dishes were then incubated at 28 ± 2 °C for 7 days .The cover slips were removed and fixed on slides using a tiny drop of Canadabalsam, and then, the prepared slides 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 the current manuals Domsch *et al*. (1980) and John and Brett (2006) The isolated fungus was identified as *Fusarium moniliforme* .This fungus was cultivated on broth medium containing 20 gm flour of *Zea mays* grains for 10 days at 28 °C. Then the 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 (no. 4, Whatman,). The extract was quantitatively analyzed for FB1 by High Performance Liquid Chromatography. *Sacchaaromyces cerevisiae* and *Trichoderma harizianum* were inoculated separately with *F. moniliforme* in the same medium .After incubation period the FB1 was determined.

2. Plant Cultivation and quantitative determination of chlorophylls

Grains of *Zea mays* treated with three different concentratins of FB1 were cultivated in 15 cm diameter plastic pots containing 1.5 kg autoclaved soil and irrigated with water in a greenhouse. After 15 days of growth the morphological symptoms and Chlorophylls content were recorded. Chlorophylls content was determined according to Vernon and Seely (1966) using the following equations:

mg chlorophyll a / gm tissue = 11.63 (A 665) -2.39 (A 649).

mg Chlorophyll b / gm tissue = 2.11 (A 649) -5.18 (A 665).

Where (A), denotes the reading of the optical density.

3.Germination and ultrastructures studies

Ten grains of Zea mays were randomly 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 FB1 and germinated at 25-26 °C with 50%-60% humidity in an incubator for 16 h in light followed by 8 hours in darkness. Electron microscope studies were carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. Epicotyl of germinating grains of *Zea mays* treated with two different concentrations of FB1. was 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 houres at 10 °C, and then washed with the same buffer for 5 h at 10°C. Then the

buffer was removed and the samples covered with an aqueous solution of 1% osmium tetraoxide for 2 h. 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 °C 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).

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RESULTS AND DISCUSSION

In the present study the *Z. mays* grains were treated with FB1 produced by *F. moniliforme* (Fig.1) at different concentrations .The results clearly indicated that the seed germinibility in the presence of FB1 was decreased with increasing concentrations whereas the germinibility was uncompletely ceased at high concentrations. The maximum amount of germination was observed on all test concentrations within 6 days. Except for a slight initial lag in germination observed at lowest concentration (28.57 µg /ml) and highest concentration (64.11 µg /ml) respectively compared with the control (grains untreated with FB1), this may be due to grains enzymes may affected by Fumnacine B1. In the present study, FB1did not inhibit the germination of *Z. mays* grains. At concentration of FB1 (28.57 µg /ml), elongation of the hypocotyls and epicotyls in seedlings was slightly inhibited compared with the control.

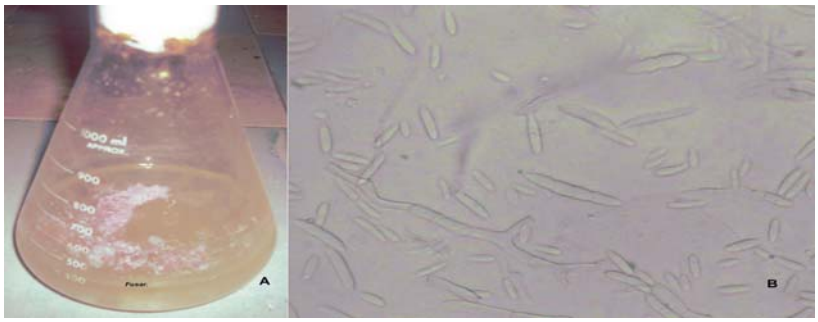

Fig. 1: *F. moniliforme* growth on corn flour medium (A) and their morphological characters (B).

The growth response of this cultivar of *Z. mays* to concentrations of FB1is shown in Fig. (2). Current study is very similar to other studies where in maize, Doehlert *et al.* (1994) stated that FB1 is toxic to the cells but did not reduce seed germination rather, it reduced radical elongation and seed amylase production was inhibited. Decreased shoot and root lengths, shoot dry mass and reduced growth were also observed. Phytotoxic effects of FB1 were observed on seedlings growth treated with different concentrations of FB1 including growth retardation in shoot and root system, chlorosis and death of tissues (Fig. 3). The results of this study are in agreement with similar investigation done by Lamprecht *et al*. (1994) who reported dose-dependent reduction in shoot, root lengths and dry mass in the seedlings. The observation of Nelson *et al.* (1993) where FB1 caused significant stem rot was a hind sight that prompted Miller (1995) to speculate that FB1 plays a role in the pathogenicity of maize unlike other plants studied. Phytotoxicity depend on the type and concentration of mycotoxins and cultivar plant where Wakulinski (1989) 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 (1973) 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.

Fig. 2: Germination and growth of *Z. mays* seedlings in control (without FB1) and in the presence low 28.57 (µg /ml) and high 64.11 (µg /ml) concentrations of FB1.

Fig. 3: Growth of seedlings of *Z.mays*, untreated with FB1(C) and treated with different concentrations of FB1 (F) 28.57, (L) 40.54, (H) 64.11 (µg /ml).

In the present study chlorophyll a and b content of seedlings of *Zea mays* decreased with increasing concentrations of FB1 (Fig 4). Chlorophyll a content was 6.23, 3.88 and 3.91 (mg / g fresh weight) at concentrations 28.57,40.54 and 64.11(µg /ml) of FB1 compared with the control 8.55 mg/ g fresh weight of seedlings of *Zea mays* untreated with FB1. The same trial was observed with Chlorophyll b content. Several investigators claimed that FB1 was suspected to be phytotoxic and virulent. Vesonder *et al.* (1992) recorded that FB1 reduced chlorophyll synthesis by 59% in duckweed (*Lemman minor)* fronds at 10^{-6} M.

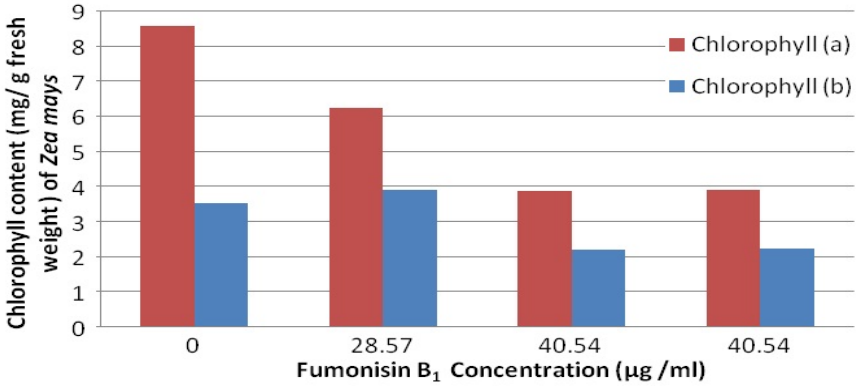

Fig. 4: Chlorophyll contents (mg/ g fresh weight) of *Z. mays* seedlings (cultivated grains soaked with different concentrations of FB1).

Phytotoxic properties of fumonisin have been widely studied by various authors (Doehlert *et al.* 1994, Kroschel and Elzein 2004, Soriano *et al*.2005 and Kritzinger *et al*. (2006)). The ultrastructural studies indicate that there were dramatic differences between the treated and nontreated tissues of *Zea mays* seedlings with the toxin FB1 (Figs. 5 and 6). The phytotoxicity of a number of secondary metabolites present in culture filtrates of pathogenic fungi has been demonstrated (Švabová and Lebeda, 2005, Tylkowska *et al* 2008).

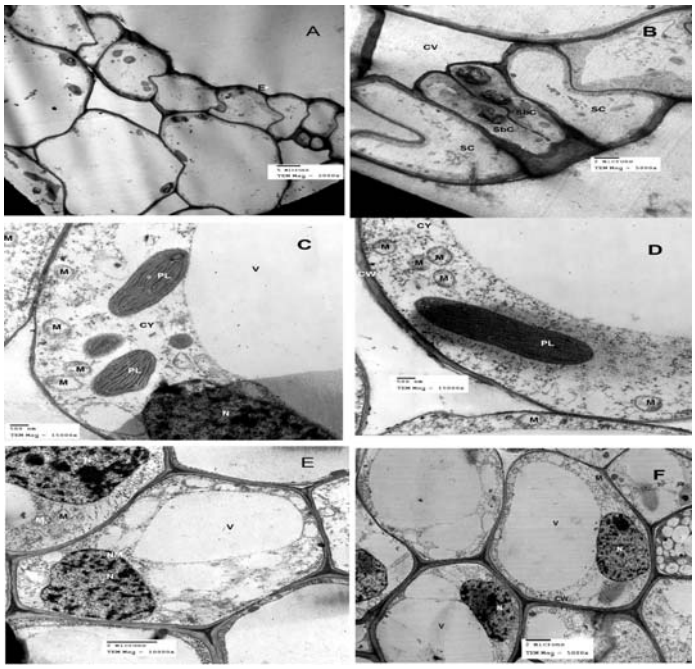

Fig. 5: Ultrastructure of untreated cells with FB1 in *Z. mays*. Abbreviations: CW, cell wall; M, mitochondria; N, nucleus; V, vacuole ; E, epidermis ;SbC, subsidiary cells; SC. Guard cells; PL, plastids ; CV , cavity.

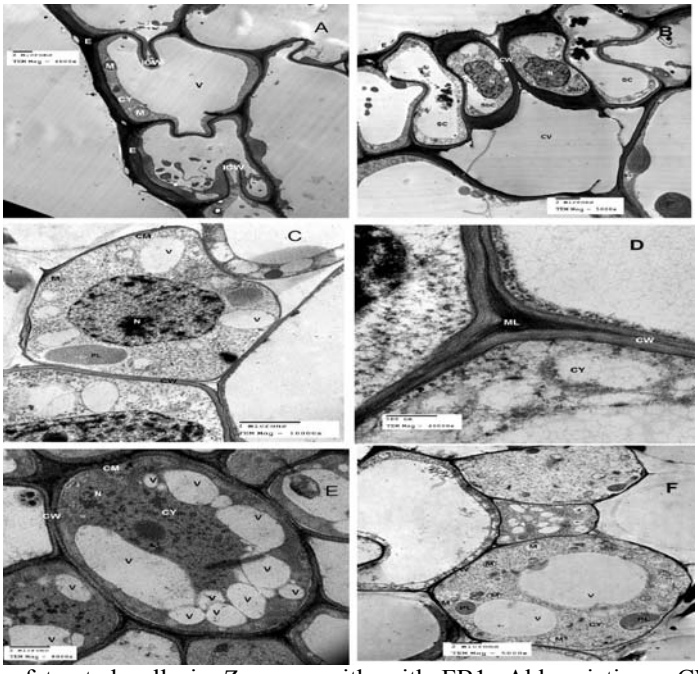

Fig. 6: Ultrastructure of treated cells in *Z. mays* with with FB1. Abbreviations: CW, cell wall;M, mitochondria; N, nucleus; V, vacuole ; E, epidermis ;SbC, subsidiary cells; SC. Guard cells; PL, plastids ; CV , cavity.

Phytopathogenic fungi produce a wide range of phytotoxic compounds, such as AAL-toxin and FB1 (Brandwagt *et al.* 2001; Jackson and Taylor 1996; Stone *et al.* 2000). The treated cells with FB1 are highly vacuolated, where, it contain many vacuoles (Fig. 6 C and E) compared with the untreated cells, where it contain one or two vacuoles but large in size (Fig. 5 E and F). Early studies Abbas *et al.* (1992) described physiological and ultrastructural effects of FB1, on jimsonweed *(Datura stramonium* L.). Ultrastructural damage in mesophyll palisade cells exposed FB1.

Thin layer of external epidermis (E) was observed in untreated cells (Fig. 5 A.) compared with thick layer of external epidermis (E) in treated cells (Fig 6 A). Obvious effects began to appear as treated with FB1. The epidermal cells was most affected, in that its containing unregular cell walls (ICW) (Fig. 6 A) when compared with control. From these results it can be seen that FB1 could interfere with the normal development and functioning cell wall, vacuoles, plastids and other internal organs. Kritzinger *et al*. (2006) noted that the irregular sizes vacuoles formed as as a result of FB1 treatment.

Stoma was clearly appeared and contained definitely subsidiary (SCb) and guard (SC) cells in treated and untreated cells (Figs. 5 A and 6 A) but in treated cells definitely thick wall of subsidiary cells was appeared with clearly nucleus (N). There were many polyribosomes and cytoplasmic granules in the cytoplasm (CY) in treated and untreated cells. Clear midellamella (ML) was visible between cell walls with thickness layer especially with treated cells (Fig.6 D).Transmission electron microscopy (TEM) showed visible changes in cell ultrastructure in number and size of plastids (PL) and mitochondria (M). The untreated cells containing many plastids and mitochondria with large size (Fig. 5 C and D) compared with treated cells (Fig. 6 C and F). Tylkowska *et al* (2008) demonstrated that ultrastructures was observed carrot roots as a result of treated with fungal toxins. Mature maize (*Z. mays* L.) embryos were exposed to aflatoxin B1. 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). The phytotoxicity of FB1 is largely due to its effect on the disruption of sphinganine metabolism (Soriano *et al*. 2005) and inhibit the plasmamembrane H^+ATPase from maize embryos (Kroschel and Elzein 2004).

Very few scattered reports are available on the use of yeast and fungal strains for degradation of fumonisins. *S. cerevisiae* strains were tested for their ability to degrade zearalenone and fumonisins in Sabouraud broth. Two strains were capable to degrade fumonisins partially (Styriak *et al.* 2001). In the present studies, biocontrol of *Fusarium* and reducing their productivity of FB1 was recorded (Table 1 and Fig. 7). *S. cerevisiae* reduced FB1 to 40.54 (µg /ml) compared with control 64.11(µg /ml). On the other hand *Trichoderma harizianum* was more efficient in FB1 productivity where productivity was reduced to 28.57 (µg /ml) with 44.56 %. In previous Srobarova and Eged (2005), *Trichoderma* was used as a biocontrol of *Fusarium* and their mycotoxins. 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 La'sztity 1999).

Table 1: Effect of *S. cerevisiae* and *T. harizianum* on FB1 production

Treatment	Mycotoxin cocentration (µg /ml)	Productivity* %
Control	64.11	100
S. cerevisiae	40.54	63.23
T. harizianum	28.57	44.56

* Productivity % was regarded as 100% in the control

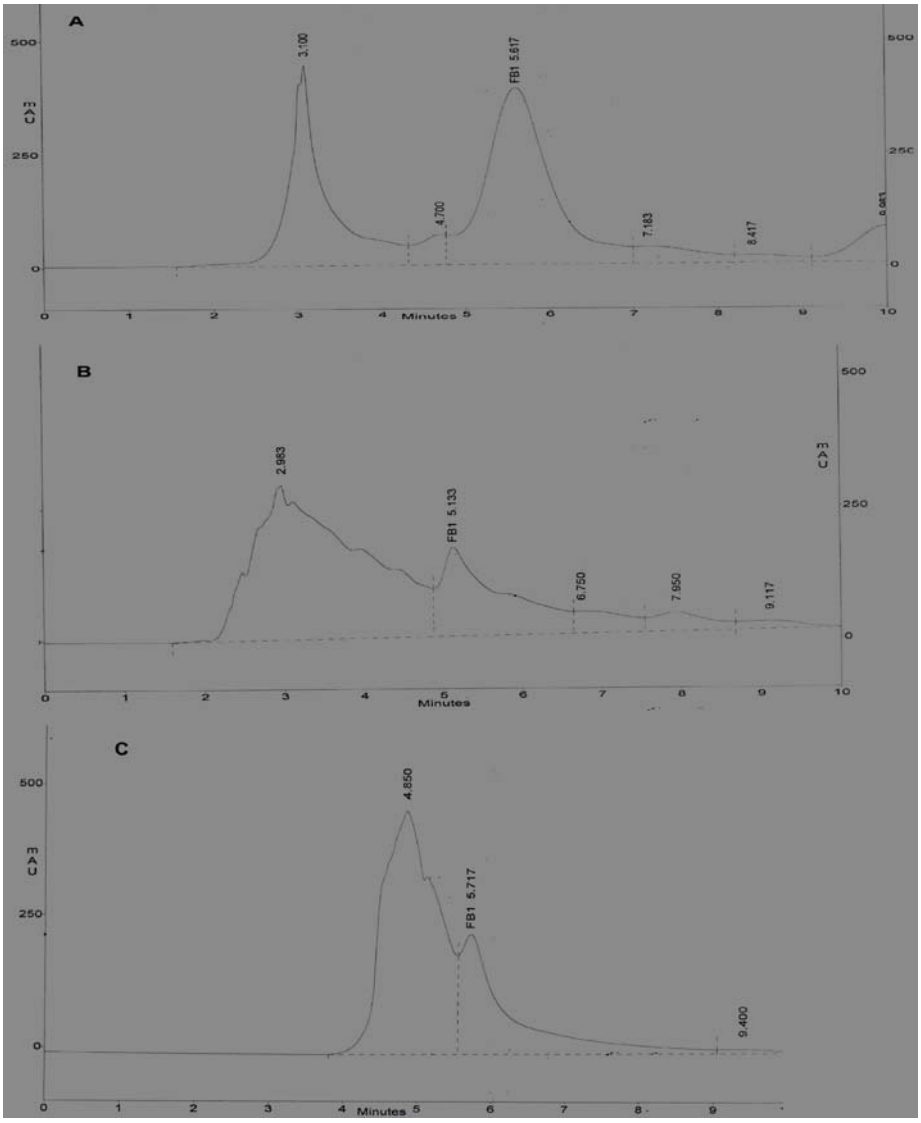

Fig. 7: HPLC chromatogram of different concentratins of FB1 in the presence of control (A), *S. cerevisiae* (B) and *T.harizianum* (C).

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ARABIC SUMMARY

الكبح الحيوي لإنتاج السم الفطري فيومناسين ب1 ومردود سميته النباتية على النمو والتراكيب الدقيقة لبادرات حبوب الذرة

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السموم الفطرية تلعب دوراً مهماً في تلف الحبوب والبذور الزراعية وعندما تغزوا الفطريات المنتجة للسموم الفطرية الحبوب فإنها تؤثر علي انبات تلك الحبوب وانتاجيتها ، وركزت تلك الدراسة علي كيفية التحكم الحيوي للسم الفطري فيومناسين ب1 وكذا تأثير تلك السم علي انبات وعلي التراكيب الدقيقة لحبوب الذرة ، حيث وجد من خلال تلك الدراسة ان نسبة انبات الحبوب تقل بزيادة تركيز السم الفطري . استطالة السويقة الجنينية العليا والسفلي وجد انها تثبط عند تركيز 28,57 ميكروجرام/ مل وذلك بالمقارنة بالعينة الحاكمة (الحبوب الغير معالجة بالسم الفطري) ، ووجد ان المحتوي الكلوروفيللي لبادرات حبوب الذرة تقل بزيادة تركيز السم الفطري فيومناسين ب1 في الحبوب فمحتوى الكلوروفيلل أ كان 6,23 ، 3,88 ، 3,91 مليجرام / جرام وزن طري عند تركيز سم فطري 28,57 ،40,54، 64,11 ميكروجرام/ مل علي الترتيب بالمقارنة بالعينة الحاكمة حيث كان المحتوى الكلوروفيللي 8,55 مليجرام / جرام وزن طري . دراسة التراكيب الدقيقة اوجدت اختلافات كبيرة بين البادرات النامية للحبوب المعالجة والغير معالجة بالسم الفطري ، حيث وجد ان خلايا بادرات الحبوب المعالجة تحتوي علي عدد كثير من الفجوات بالمقارنة ببادرات الحبوب الغير معالجة ، ووجد أيضاً أن جدر خلايا بادرات الحبوب المعالجة كانت سميكة بالمقارنة برقة جدر خلايا البادرات للحبوب الغير معالجة . وبدراسة التحكم الحيوي لانتاج السم الفطري وذلك باستخدام كائنات آمنة وجد أن اضافة فطريات سكارومايسس سيريفيسيا و ترايكوديرما هاريزيانم كل على حدة لبيئة نمو الفطر المنتج للسم الفطري يقلل من انتاجية السم الفطريي فيومناسين ب1 إلي 40,54 ميكروجرام/ مل ، 28,57 ميكروجرام/ مل علي الترتيب بالمقارنة بالعينة الحاكمة 64,11 ميكروجرام/ مل (الفطر المنتج للسم الفطري نامي بدون اضافة كائنات التحكم الحيوي).