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The Toxicity of Fumonisin B1 in Chiken Embryos

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Abstract Cantaloupe (*Cucumismelo* L. var. reticulates Ser.) is one of the most famous cucurbit crops in Egypt for local consumption and exportation. It is considered as an untraditional winter crop and became one of the most important exportation crop to the foreign markets. *Alternaria* spp. *Fusarium. semitictum, F. subglutinans-1, F. solani, F. subglutinans-2 and Rhizopus stolonifer* were isolated from naturally rotted fruits showing fruit-rot symptoms that collected from markets. Pathogenicity test using the isolated fungi *i.e. Alternaria* spp. *Fusarium semitictum, F. subglutinans-1, F. solani, F. subglutinans-2* and *Rhizopus stolonifer* revealed that, all isolated fungi were pathogenic and caused fruit rot disease on Galia cantaloupe cv. *Alternaria* sp. and *R. stolonifer* were weak parasites.The *Fusarium* spp. can infect all the tested hosts, *i.e*. cantaloupe cucumber, squash and melon, but with different values of disease severity. The ability of *F. semitictum* to produce Fumonisin (FB_1) that affected the mortality of chicken embryos indicated a nonlinear dose response relationship, eggs injected with 200µg FB_1. Microscopic examination of embryos that died during incubation period.

Keywords: Cantaloupe, Fruit-rot, *Fusarium* spp., Fumonisin B_1, Chicken embryo.

Introduction

Cantaloupe (*Cucumismelo* L. var. *reticulates* Ser.) is one of the most famous cucurbit crops in Egypt for local consumption and exportation. It is considered as an untraditional winter crop and became one of the most important exportation crops to the foreign markets. The demand amounts from cantaloupe fruits for local consumption and exportation are annually increased. Cantaloupe plants are liable to infection by bacterial, fungal and viral diseases, in addition to nematode infection and physiological disorders (Agerter*et al*., 2000; Helall, 2004, Muhanaa, 2006 and Ashour, 2009). However, fruit-rots are among the most destructive constrained for its production (Zitter, 1998 and Seebold, 2010).

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Mehl and Epstein (2007) reported that *Fusarium* spp. were collected from cucurbit fruits in production fields in the San Francisco Bay area, CA, from a produce distributor in San Francisco, and from grocery stores in Davis, CA and the San Francisco Bay Area.

Al-Sadi*et al.* (2011) isolated *Fusarium equiseti* and *F. solani* diseased cucumber fruits. El-Mougy *et al.* (2012) isolated different root rot fungi i.e. *Alternaria solani Fusarium solani*; *F. oxysporum*; *Rhizoctoniasolani*; *Sclerotium rolfsii*; *Macrophomin aphaseolina* and *Pythium* sp. were isolated from various vegetables, i.e. cucumber, cantaloupe, tomato and pepper grown in plastic houses under protected cultivation system and showing root rot and or damping-off disease symptoms.

Latiffah *et al.* (2013) reported that *Fusarium semitectum* is a widespread species occurring in various types of substrate. They added that fifteen *F. semitectum* isolates were recovered from several types of vegetable fruits showing fruit rot symptoms, namely long bean (*Vignasesquilpedalis*), okra (*Abelmoschusesculentus*), loofa (*Luffaacutangula*), bitter gourd (*Momordicacharantia*), cucumber (*Cucumissativus*) and green chilli (*Capsicum annum*). The identification of the *F.* isolates was based on morphological characteristics of macroconidia and microconidia, presence of mesoconidia and colony pigmentation.

Al-sadi *et al.* (2011) reported that *Fusarium solani* and *Fusarium equiseti* were pathogenic on cucumber fruits, with *F. equiseti* being the most aggressive.

Abu Bakar*et al.* (2013) found that all the tested Fusarium isolates were pathogenic on tomato with different severity levels. The non-inoculated controls showed no symptoms of fruit rot. The most virulent was *F. oxysporum* isolate B711T with DSI 93.75%, while the least were isolates of *F. solani* (B647T) and *F. oxysporum* (B727T) with DSI 37.5%. Majority of the isolated *Fusarium* species can potentially produce mycotoxins as their secondary metabolites. The potential production of mycotoxins by pathogenic isolates of *Fusarium* species in contaminated tomato fruits could pose health hazards when consumed.

Members of the genus Fusarium are often regarded as being soilborne, but they are also present in the air, water, and organic materials (Nelson *et al*., 1981).The genus Fusarium is undoubtedly one of the most important groups of plant pathogenic fungi throughout the world, with *Fusarium oxysporum* .Schlecht. Ernend. Snyd.&Hans.,being the most econornically important member of this genus due to the existence of many pathogenic strains or forma especiales that affect a wide range of crops (Booth, 1971).

Fusarium mycotoxins are secondary metabolites of toxigenic *Fusarium* species which distribute ubiquitously in the world. There is increasing evidence

that these mycotoxins can provoke a broad spectrum of toxicities (Kubosaki*et al*., 2008; Sudakin, 2003 and Voss *et al*., 2002), and several specific animal mycotoxicosis such as equine leukoencephalomalacia (Wilson *et al*., 1991) and porcine pulmonary edema (Haschek *et al*., 2001) have been positively correlated with the exposure of certain *Fusarium* mycotoxins. Consumption of *Fusarium* mycotoxins-contaminated food stuffs has been implicated in human alimentary toxic aleukia, and is associated with the high incidence of human esophageal cancer in China and South Africa (Sun *et al*., 2007; Rheeder *etal*., 1992).

Fumonisin B_1 (FB_1) belongs to the recently (1988) discovered toxins fumonisins which are produced by *Fusarium verticilloides* (older synonym is *F. moniliforme*) and *F.proliferatum*, fungi that commonly contaminate maize. It has been also claimed that *F. napiforme, F. anthophilum, F. dlamini* and *F. nygamai* are able to produce FB_1 (EHC, 2000 and NTP, 1999).

Fumonisin B_1 (FB_1) has been found as natural contaminant in maize and maize based food from many parts of the world, e.g. the US, Canada, South Africa, Nepal, Australia, Thailand, The Philippines, Indonesia, Mexico, France, Italy, Poland, and the Mediterranean area (Spain, Italy and others) (Eriksen and Alexander, 1998; EHC, 2000 and Antonio *et al.,* 2003).The predominant member of the fumonisins is fumonisin B_1 (FB_1), it inhibits cell growth and causes accumulation of free sphingoid bases and alteration of lipid metabolism in *Saccharomyces cerevisiae*.FB_1 is phytotoxic, damages cell membranes and reduces chlorophyll synthesis. It also disrupts the biosynthesis of sphingolipids in plants and may play a role in the pathogenicity of maize by fumonisin-producing *Fusarium* species (WHOG, 2000).

Until now, twenty-eight fumonisins have been isolated and they can be divided in four series known as A, B, C and P. FB_1, FB_2 and FB_3 are the principal fumonisins analyzed as natural (Yazar and Omurtag, 2008).Contaminants of cereals toxins fumonisins which are produced by *Fusarium verticilloides*(older synonym is *F. moniliforme*) and *F.proliferatum*, fungi that commonly contaminate maize. It has been also claimed that *F.napiforme, F. anthophilum, F. dlamini*and *F. nygamaiF.subglutinans, F.solani, F.semitictum,* are able to produce fuminisins (EHC, 2000; NTP, 1999; Marasas, 2001 and Chibundu *et al*., 2008).

The most common species of Fusarium were produced fuminisin F. moniliforme (60.7%) and F. nygamai (35.4%) followed by F. semitectum, F. subglutinans, *F. proliferatum*, *F. dlamini*, *F. solani*, *F. oxysporum* and *F. napiforme*. Reported by (Magnoli *et al*., 1999).

Recent data on the epidemiology of the common mycotoxigenic species of *Fusarium, Alternaria, Aspergillus* and *Penicillium* in infected or colonized

plants, and in stored or processed plant products from the Mediterranean area are reviewed by (Antonio *et al*., 2003). The Mediterranean basin is a large geographical region with a temperate climate supporting the cultivation of a wealth of field and green house crops with a high risk of mycotoxin contamination. The most important mycotoxins that occur in the Mediterranean basin are aflatoxins (B_1, B_2, $G_1$and G_2) in dried fruits and nuts, ochratoxin A in grapes and raisins as well as trichothecenes and fumonisins in cereals (Tsitsigiannis *et al*., 2012).

There for we studied the predominant member of the fumonisins toxins which fumonisin B_1 (FB_1), in Egyptian cantaloupe fruit rot samples that collected from markets and different areas of Giza (a government in Egypt which is a part of Mediterranean area). *Fusarium* was the genus isolated from the markets samples with *F.subglutinans*, *F.solani*and *F.semitictum*. Fumonisin B1, isolated from culture material contaminated with *F. moniliforme*, was found to be hepatocarcinogenic and hepatotoxic to rats (Voss *et al*., 1990 and Gelderblomt*et al*., 1991) and horses (Marasas*et al.,* 1988). Fumonisin B_1 was identified as the noninfectious causative agent of LEM in horses (Marasas *et al*., 1988) and pulmonary edema in pigs (Harrison *et al*., 1990 and Haschek *et al*., 1992). In vitro studies by Norred*et al.,* (1992) indicated that FB_1 inhibits the biosynthesis of sphingolipids in hepatocytes and kills renal cells. Fumonisin B_1 is also toxic to chicken macrophages by causing a decrease in their functional activity (Qureshi and Hagler, 1992).

The present work was planned to study Isolation fungi responsible for causing cantaloupe fruit-rot. Pathogenicity test of isolated fungi. Extraction the toxin fumonisin (FB_1), from *Fusarium* spp. and its effect on chicken embryo in different concentration.

Materials and methods

Isolation, purification and identification of the causal organisms

Cantaloupe fruits showing different fruit-rot symptoms such as wet spots and dark brawn as well as cracks in the outer shell of the fruit. were collected from different markets at Giza Governorate, Pieces of necrotic tissues on the fruits were taken off and surface sterilized in 2% sodium hypochlorite for 2 min. followed by several rinses in distilled water before being transferred onto potato dextrose agar medium (PDA). The grown cultures were picked up and purified by single spore method and/or hyphal tip technique. The purified cultures were maintained on PDA slants in a refrigerator at 5 °C. Identification of the isolated fungi was carried out according to the cultural and micro

scopicall characteristics using the description of Booth (1971); Nelson *et al*. (1983); Barnett and Hunter (1998); Dugan (2006).

Pathogenicity tests

Pathogenicity test of *Fusarium* spp. was carried out using Cantaloupe Galia cultivar in plastic boxes. Cantaloupe fruits were surface sterilized by immersing them in 1% sodium hypochlorite solution for 2 min, then washed several times with sterilized water. Three fruits were but in each box and maintained in moisture conditions. The fruits were crushed with sterilized scalpel in 5 cm^2 area and 10 mm disk of any of the tested fungi was put on each crushed area of fruit. PDA disks only were put on fruits crushed with sterilized scalpel in 5 cm^2 area and served as control treatment. The inoculated fruits were noticed daily until one week. The rotted fruits were examined and classified into the devised scale (0-5) as proposed by Twnsend and Heuberger (1943), where; 0= No apparent symptoms of fruit- rot are seen, 1= The rotted portion ranged from .1- 10 % of the fruit size , 2= The rotted portion ranged from more than 10 - 25 % of the fruit size, 3= The rotted portion ranged from more than 25 - 50 % of the fruit size,4= The rotted portion ranged from more than 50 – 75 of the fruit size % and 5= The rotted portion more than 75 % of the fruit size . Disease severity was calculated using the following formula:

$$\text{Disease severity (\%)} = \frac{\sum (n \times v)}{5\,N} \times 100$$

Where,
n = Number of fruits in each category.
v = Numerical values of symptoms of each category.
N = Total number of the examined fruits.
5= Maximum number of numerical values of symptoms category.

The Toxicity of Fumonisin B1 in chicken embryos

Fumonisin$B_1$was produced and isolated according to Marasas (2001) using sterile, yellow corn as the substrate and *Fusarium semitictum*as the organism. Fumonisin B_1 standard was purchased from Sigma Chemical Co. (St. Louis, MO), and other chemicals were of the highest purity commercially available. The fumonisin B_1 was analyzed and determination by HPLC, according to Sydenham *et al*. (1991).

A stock solution of B_1 was made by dissolving 1mg FB1/ml MeoH: H_2O (1:1) to give concentration 1mgFB_1/ ml, dissolving 500ug FB_1/ml MeoH : H_2O (1:1) to give concentration 500 ug FB_1/ml and dissolving 250ug FB_1/ml MeoH : H_2O (1:1) to give concentration 250 ug FB_1/ml. The HPLC method described by Shephard *et al.* (1998) was used for determination the different concentration of FB_1.

In experiment, the total volume of solvent used per egg was 200 µl. The safety measures recommended by WHO (1998) were taken when handling different concentrations of FB_1.

Broiler chicken hatching eggs were obtained from veterinary Department at National Research Center and used in the experiment. Thirty fertile eggs weighing between 60 and 75g (mean of 67.5g) were selected and incubated at 37 ^{0}C and at 40-60 % relative humidity before treatment with automatic rotation of the eggs at 4-h intervals. Embryonating eggs were assessed for viability by candling each egg after 72 h of incubation. Eggs with a clinically normal embryo and an air space in the normal location were selected for use in the experiments. The selected eggs were randomly divided into six treatment groups. First group (control group) eggs were incubated as they were received from the hatching egg source, second group (drilled group) eggs were drilled with the injection needle, no solution was injected, third group (solvent group) eggs were injected with 200ul MeoH: H_2O (1:1v/v), fourth, fifth and sixth groups (treated groups) were injected with FB_1 at doses of 50 µg FB_1/egg, 100 µgFB_1/egg and 200 µg FB_1/egg respectively. Immediately after injection, the injection site was sealed with a drop of nontoxic white glue. All eggs were labeled with a pencil and then returned to the incubator with the large end of the egg in an upward position all eggs incubated for 18 days. All eggs with a viable embryo on day 18 were opened and visually assessed, and final mortality was calculated. Eggs with dead embryos at each candling time were opened and visually inspected for the presence of gross abnormalities. Viable embryos were returned to the incubator on day 18. Hatchability was determined on day 22 of incubation. All chicks that hatched were weighed on day 22.

The obtained data were subjected to statistical analysis of variance (ANOVA) whenever needed, using the statistical analysis system Assistat (version 7.6). Mean of treatments were compared by Duncan's multiple range test at level of 0.05% (Rafter *et al*., 2002).

Results

Isolation, purification and identification of the causal organisms

Isolation trials from naturally rotted fruits showing fruit-rot symptoms (Fig. 1) yielded many fungal isolates.The purified fungi were identified as *F. subglutinans*-1, *F. solani, F. subglutinans*-2, *Fusariumsemitictum*, *Alternaria* spp. and *Rhizopusstolonifer*.

Fig. 1. Cantaloupe fruits showing natural infection by fruit-rots

Pathogenicity tests

Pathogenicity tests of the isolated fungi Table (1) and (Fig. 2) reveal that all the isolated fungi were pathogenic to Gallia cantaloupe cv., where all the inoculated fruits were infected (100% infection). However, both and *R. stolonife*r were weak parasites. In this respect, *F. semitictum* was the most pathogenic one, being 61.4 % disease severity (Fig. 3) followed by *F. solani*then *F. subglutinans*-1 and *F.subglutinans*-2, being 56.3, 52.6 and 43.0%, respectively. Meanwhile, *R.stolonifer* caused the lowest disease severity followed by *Alternaria* sp, being 10.0 and 24.3 %, respectively.

Table 1. Pathogenicity test of the isolated fungi on Galia cantaloupe cv

The tested fungi	% Infection	% Disease severity
Alternaria sp.	100	24.3[e]
F. semitictum	100	61.4[a]
F. subglutinans-1	100	52.6[c]
F. solani	100	56.3[b]
F.subglutinans-2	100	43.0[d]
R.stolonifer	100	10.0[f]
Control.	0.0	0.0[g]

a-g Significant at a level of 1% of probability ($p < .01$)

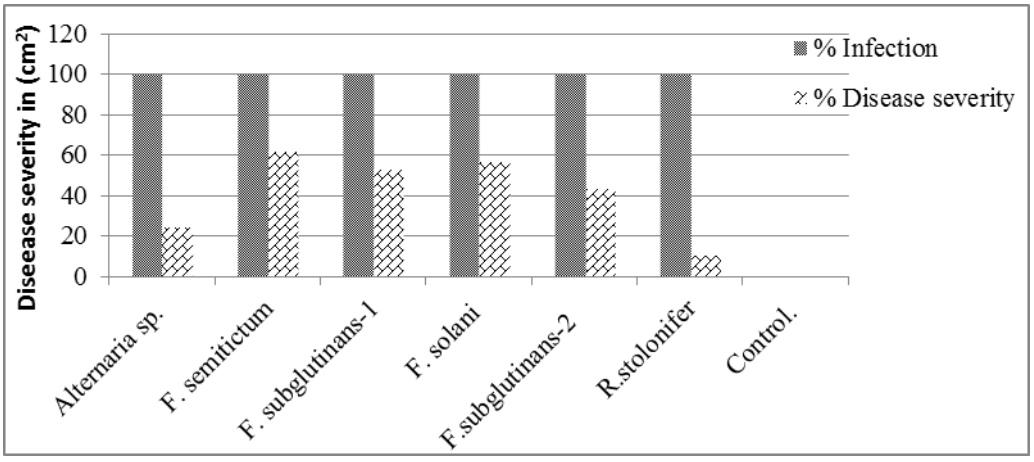
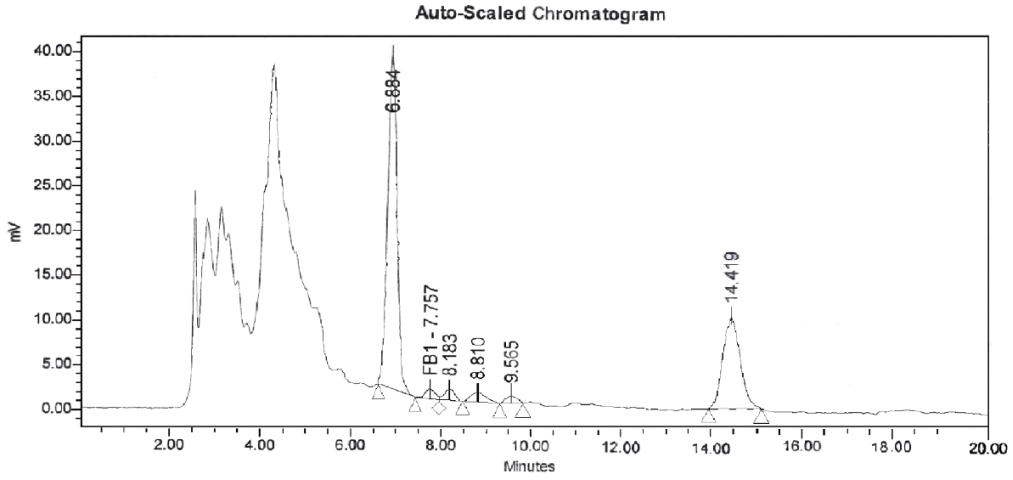


Fig. 2. Different effected of disease severity of fungi tested on Galia Cantaloupe cv.

The ability of different *Fusarium* isolates to produce fumonisin FB_1 was determined by grown the strains on liquid culture media Fig.(3)Fumonisin was the secondary metabolite mostly produced by the in vitro cultures of the *Fusarium* isolates analyzed.



F. semitectum

Fig. 3. HPLC of fuminisin production by *Fusarium semitectum* in culture media.

The highest mortality% of embryos within 21 days of incubation occurred at levels 100 µg FB_1/egg and 200 µg FB_1/egg for groups (5 and 6) respectively were 100% for each. Mortality gradually increased throughout the 22-d incubation period Fig. (3).The effect of FB_1 on the mortality of chicken embryos indicated a nonlinear, dose response relationship Fig. (4). Embryonic

mortality ranged from 40 % in the control Egg to 100% in eggs injected with 200 µg FB_1 Table (2). Cumulative mortalities of embryos exposed to 50, 100 and 200 µg of FB_1 were significantly higher than those of the controls. Cumulative mortality of embryos exposed to FB_1at 50 µg/egg was not significantly different than control group solvent of the controls. When methanol :water was injected into the air space of eggs, the morality of chicken embryo was 40% through 21 days of incubation, While it was 60% at 50 µg FB_1/egg (group 4).

Table 2. The response of embryonic mortality of chicken embryo exposed to fumonisin B_1 (FB_1)

	NO.OF eggs injected	Days of incubation	mortality at 21 day incubation	
FB1 Injected µg			No. of death	%
control group	5	18	0\5	0 c
drilled group	5	18	0\5	0 c
solvent group	5	18	2\5	40 bc
group 4 (50 µg)	5	18	3\5	60 ab
group 5 (100 µg)	5	18	5\5	100 a
group 6 (200 µg)	5	18	5\5	100 a

[a –c]Mortality values with different superscripts differ significantly ($p<0.05$).

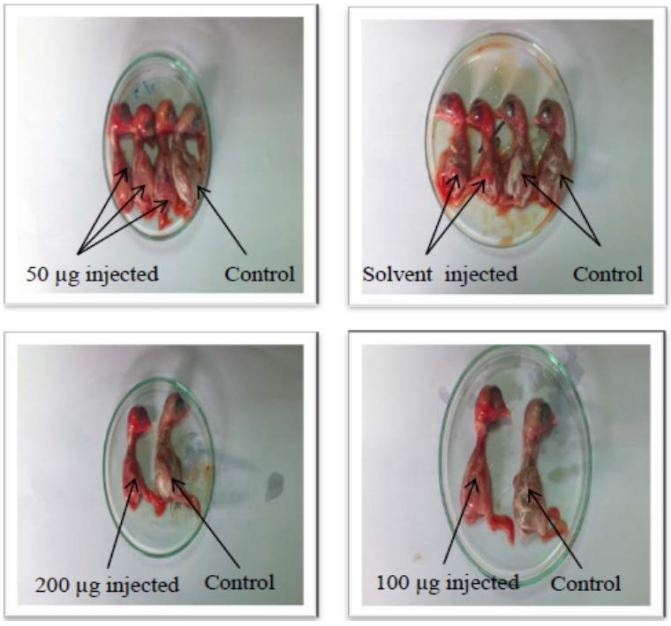


Fig. 3. Chicken embryos of dark red color and smaller in size in treated groups with FB_1 compared with control.

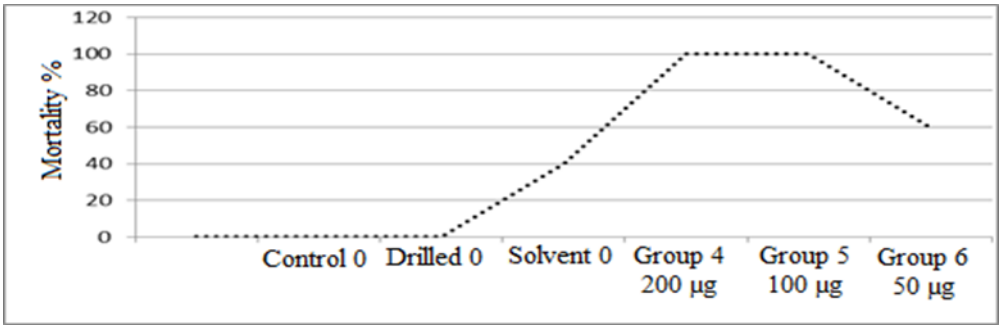


Fig. 4. The dose- response of mortality in chicken embryo exposed to fumonisinB_1 (FB_1) after 22 days of incubation.

Microscopic examination of embryos that died during incubation did not reveal any gross structural abnormalities of the head, beak, or limbs. However, all dead embryos taken from eggs on Day 18 had extensive hemorrhages in the head, neck, and thoracic.

There was no morphological difference between embryos from FB_1-exposed eggs and embryos from control eggs that survived 18 day of incubation, except the color there were dark reed in treated groups compared with control on which there color are pink. Hatchability of control eggs was not significantly different compared with the hatchability of eggs injected with FB_1 There were no significant differences in the weights of chicken from eggs injected with FB_1 and weights of chickens embryo from control eggs (Table 3).

Table 3. Chicken embryo weight in different concentration of fumonisin B_1 (FB_1)in 18 days incubation

FB_1 Injected (µg)	NO.OF eggs injected	Days of incubation	embryo weight in (gm.)
control group	5	18	10.296 a
drilled group	5	18	10.828 a
solvent group	5	18	10.71 a
group 4 (50 µg)	5	18	10.668 a
group 5(100 µg)	5	18	8.59 b
group 6 (200 µg)	5	18	6.646 c

[a –c]Weight embryo values with different superscripts differ significantly ($p< 0.05$).

Discussions

Cantaloupe fruits and plants are subjected to several diseases, during germination and growth, which attack the crop and reduce the produced fruits and their quality.

Isolation trial from the rotted fruits yielded many fungal isolates. The isolated fungi were purified and identified as *Alternaria* spp., *Fusarium semitictum, F. subglutinans*-1, *F. solani* , *F. subglutinans*-2and *Rhizopus stolonifer* . Many investigators isolated these fungi from cantaloupe fruits at different localities. These results are in harmony with those reported by many researches (Elmer, 1996; Bruton *et al.*, 1998; Zhang and Bruton,1999; Kwon *et al.*, 2009 and Latiffah *et al.*, 2013).

Pathogenicity test of the isolated fungi from fruits revealed that all the isolated fungi were pathogenic and caused fruit rot disease on Galia cantaloupe cv. In this suspect, the highest percentages of fruit-rots severity were recorded by the four species of genus *Fusarium*,*i.e Fusarium semitictum*, *F. subglutinans*-1, *F. solani* and *F. subglutinans*-2 . The obtained results are in agreement with those obtained by Bruton *etal*. (1998); Zhang *et al*. (1999); Seebold (2010) and Latiffah *et al*. (2013).

The biological experiment shows that the administration of a single air sac injection of mycotoxin FB1 into chicken eggs after 18 day of incubation was toxic to embryos. Bilayer, which, if impaired, may result in increased capillary permeability and rupture of red blood cells. This experiment shows that the fumonisin B_1 is toxic to chicken embryos and have no developmental effects on embryos that survive after exposure. This investigation also reveals that the toxicities of FB_1 being the most toxic. The toxicity of the fumonisins FB_1 in chicken embryos also indicates that the toxicity observed in animal experiments, in which *F. simetectum* culture material with a known level of FB_1 was incorporated into the diet, cannot be attributed only to the action of FB_1. These results further amplify the fact that the fumonisin is toxic compounds and should be considered an animal and human health hazard.Magnoli *et al*. (1999) reported that the most common species of *Fusarium* produced Fumonisin are *F. moniliforme*, *F. nygamai* ,*F. semitectum, F. subglutinans, F. proliferatum, F. dlamini, F. solani, F. oxysporum* and *F. napiforme*.

The chicken embryo is a well-documented system to test the toxic effect of fumonisin FB_1 on embryonic development (Zacharias *et al*., 1996).The chick embryo assay has been used as a preliminary screen to assess the toxic and teratogenicity responses from mycotoxins and other toxic chemicals (Henry and Wyatt, 2001). This experiment shows that the administration of a single air sac injection of this mycotoxin into chicken eggs after 18 day of incubation was toxic to embryos.

Conflict of interest statement

We declare that we have no conflict of interest.

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