

## Biocontrol of *Fusarium* Moulds and Fumonisin B<sub>1</sub> Production

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**Abstract:** The present study was carried out to investigate maize seeds infected by *Fusarium* moulds and fumonisin B<sub>1</sub> production. In order to suppress the growth of fungal infection, and/or fumonisin B<sub>1</sub> production in maize seeds; applicability of *Saccharomyces cerevisiae* as a biocontrol agent as well as detoxification of fumonisin B<sub>1</sub> was investigated. Out of 100 samples of maize investigated 65 (65%) were fungal infection. Of them 38 (58.46%) were *Fusarium moniliforme* others were *F. graminearum* 19 (29.23%) and *F. oxysporum* 8 (12.31%). Growth of *Fusarium moniliforme* and fumonisin B<sub>1</sub> detoxification were negatively correlated with different doses of *S. cerevisiae* while detoxification was positively correlated with the doses. At dose 1, 3, 5 and 7 g of *S. cerevisiae*, *Fusarium* dry weight and detoxification percent of fumonisin B<sub>1</sub> were 5.8, 40.56, 4.3, 77.63, 2.8, 89.52, 0.9 and 100, respectively. The effect of water content as well as different temperatures on fumonisin B<sub>1</sub> productions was investigated. At water content of 50 %, a higher level of fumonisin (16.3 µg/g) was detected in ground maize while in Corn flour and intact grain level of fumonisin was 0.50 and 8.66 µg/g, respectively. At temperature 21°C level of FB<sub>1</sub> was 19.3 µg/g after incubation period for 4 weeks while at temperature 28°C was 9.7 µg/g after the same incubation period and on the same substrate. For growth  $r = -0.993$ ,  $p = 0.001$ ; for detoxification  $r = 0.927$ ,  $p = 0.024$ .

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**Key words** Fumonisin (B<sub>1</sub>); *Fusarium moniliforme*; Mycotoxins; Biocontrol

### 1. Introduction

Maize (*Zea mays*) is a cereal crop grown throughout the world. Maize plays an important role in the diet of millions of African 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 (Fandohan *et al.*, 2003). The total production of Africa in 2001 was estimated to be about 42 million tons (FAO, 2002). In the field as well as in the store, many pests and parasites attack maize during the storage period. Insects are most often considered as the principal cause of grain losses (Gwinner *et al.*, 1996). Surveying harvested maize grains done by many investigators (Samson, 1991; Ominski *et al.*, 1994; Munkvold and Desjardins, 1997; Desjardins *et al.*, 2000; Orsi *et al.*, 2000; El-Shabrawy, 2001), who reported that *Fusarium* spp. is the major fungal population recovered from the rotted maize ears. *F. verticillioides* and *F. moniliforme* are the most dominant species and earlier colonist of the pre-harvest maize ears before other moulds such as *Penicillium* spp. and *Aspergillus* spp. Kossou and Aho (1993) reported that fungi could cause about 50 – 80 %

of damage on farmers' maize during the storage period if conditions are favorable for their development. Experts at the food and Agriculture Organization (FAO) at the United Nation have estimated that over 25 % of worlds food crops are lost each year due to mycotoxin contamination with the *Fusarium* species (Chelkowski, 1998). Fumonisin is mycotoxin produced by a variety of fungi of genus *Fusarium*; *Fusarium moniliforme* and other species, fumonisin is natural contaminants of cereal grains worldwide and rare mostly found in corn and products derived from corn (Wan Norhasima *et al.*, 2009). Nowadays, a great attention has been focused on the possibility of using natural and safe agents as a biocontrol against different diseases.

Biological control of different plant diseases was focused primarily using bacteria or filamentous fungi (Whipps, 2001). So, application of yeasts as biocontrol agents acts as a new trend against different pathogens. Potential use of yeasts as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters were recent investigated by El-Tarabily and Sivasithamparam (2006). El-Tarabily (2004) reported that the fungal activities of *Rhizoctonia solani* diseased

sugar beet plants were well suppressed by using different types of yeast. Wide variety of yeasts has been used extensively for the biological control of post-harvest diseases of fruits and vegetables (Punja, 1997; Zheng *et al.*, 2003), against moulds of stored grains (Petersson *et al.*, 1999) and to control powdery mildews (Urquhart and Punja, 1997). The most investigated fumonisin is FB<sub>1</sub>, which can cause severe disorders in animals such as Equine Leukoencephalomalacia (Marasas *et al.*, 1988), pulmonary edema syndrome and hydrothorax in pigs (Haschek *et al.*, 1992) and it is nephrotoxic, hepatotoxic and hepatocarcinogenic in rats. Furthermore, FB<sub>1</sub> has been implicated to be associated with high rates of human esophageal cancer in South Africa, the United States and China (Rheeder *et al.*, 1992). The aim of this study was to suppress the soil-borne pathogenic fungus *Fusarium moniliforme* by using *S. cerevisiae* as biocontrol agent and as fumonisin B<sub>1</sub> production control in maize.

## 2. Material and Methods

The bio-compound used in this study is active dry yeast of *S. cerevisiae*. Yeast application was conducted as different doses amended to autoclaved, ground maize, using four concentrations of 1, 3, 5 and 7 g/100g.

### 2.1. Samples

A total of 100 samples of maize were collected from different fields in Al-Dakahliya governorate, Egypt. These samples were kept at -4°C until *Fusarium* moulds were isolated and fumonisin B<sub>1</sub> analysis was carried out.



Figure 1. Infected maize samples

### 2.2. Isolation and identification of *Fusarium* moulds

*Fusarium* moulds in maize seeds were determined by plating seeds on 15 ml of sterile

Czapek-Dox agar containing Iprodion and Dichloral (CZID-agar) in Petri-plates (Abildgren *et al.*, 1987). The seeds were surface sterilized in 10% sodium hypochlorite for 2 minutes, rinsed in sterilized distilled water and air dried on sterile filter paper. Ten plates of potato dextrose agar media (PDA) were seeded (10 seeds/plate) and incubated at 25°C for five to seven days. All *Fusarium* isolates were subcultured on PDA, Spezieller Nährstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA), using a single spore technique (Leslie and Summerell, 2006, and Siddiquee *et al.*, 2010). PDA cultures were incubated at 25°C and CLA and SNA cultures were incubated at 25°C under near UV light for two to four weeks. Cultural characters were assessed by eye and by microscopic examination. Colony morphology was recorded from cultures grown on PDA. The morphology of macroconidia, microconidia, conidiogenous cells and the chlamydospores was assessed from cultures grown on SNA and CLA. Morphological identifications of isolates were made using the criteria of (Nelson *et al.*, 1983, and Leslie and Summerell, 2006).

### 2.3. Production of fumonisin B<sub>1</sub> by *Fusarium moniliforme*

Mycotoxin production in culture was tested on autoclaved, ground maize (Muthomi *et al.*, 2000). One hundred grams of the course ground maize in 250 ml Erlenmeyer flasks were moistened with 75 ml distilled water and autoclaved at 121°C for 20 min in two consecutive days. After cooling, each flask was inoculated with 10 mm diameter agar disks cut from 7 to 14 day-old cultures. One flask used as a control sample while others were amended with *S. cerevisiae* at different concentrations (1, 3, 5, and 7 g). The incubation period was 4 weeks.

### 2.4. Fumonisin B<sub>1</sub> extraction

The samples were extracted using the method of Muthomi *et al.* (2002). In brief, samples were homogenized in methanol-water; 3:1(v/v) for 3 min. After centrifugation, an aliquot of the supernatant was cleaned-up, which were preconditioned with methanol and methanol-water (3:1). After washing with methanol, the fumonisins were eluted with acetic acid-methanol; 1:99 (v/v), then stored at 4°C to analysis.

## 2.5. Fumonisin B<sub>1</sub> analysis

Fumonisin B<sub>1</sub> was analyzed by the method of Katta *et al.* (1997) with HPLC. The fumonisin B<sub>1</sub> standard was purchased from Sigma Chemical Company (St. Louis, MO). A standard curve was constructed with levels ranging from 0.02 to 2 ng/μl for fumonisin B<sub>1</sub> and a new standard curve was constructed on each day of analysis and only standard curves with correlation coefficients >0.999 were accepted. Fumonisin B<sub>1</sub> (FB<sub>1</sub>) was quantified by correlating peak areas of the sample extracts to that of the standard curves. The sensitivity of the method was 0.025 μg /g for fumonisin B<sub>1</sub>.

## 2.6. Influence of some physical parameters on FB<sub>1</sub> production

Effect of different temperature on FB<sub>1</sub> production by *F. moniliforme* was investigated at different incubation period. Also water content of the medium was investigated to test substrate influence on fumonisin B<sub>1</sub> production according to ASTM D 2216 - Standard Test Method for Laboratory Determination of water (moisture) content of soil, rock, and soil-aggregate mixtures.

## 2.7. Statistical analysis

The statistical SPSS version 15 was used in data analysis. The Chi<sup>2</sup> was used to compare observed and expected frequencies. The Pearson correlation coefficient was used to study the doses of *S. cerevisiae* and fungal growth as well as the detoxification percent of fumonisin B<sub>1</sub>. Linear or polynomial regressions were used.

## 3. Results

### Isolation and identification of the fungal strain

The collected seeds were washed in running tap water for 20 minutes, surface sterilized in 10 % sodium hypochlorite for 2 minutes, rinsed in sterilized distilled water and air dried on sterile filter paper. The disinfected seeds were placed on CZID-agar and incubated at 25°C in the dark. After five to seven days the fungal colonies were observed under a microscope and hyphal tips of *Fusarium*-like fungi were transferred to PDA. The pure cultures of the isolates were obtained using a single-spore culture technique. Species identifications were determined following the descriptions in the manual of Nelson *et al.* (1983) and Leslie and Summerell (2006). Three strains of

*Fusarium* viz. *F. moniliforme*, *F. graminearum* and *F. oxysporum* was isolated from infected seeds. Occurrence of isolated *Fusarium* species was 58.46, 29.23 and 12.31 %, respectively (Table 1).

Table 1. Number of *Fusarium* spp. isolates and their occurrence in maize seeds

<i>Fusarium</i> spp. identified	Maize	
	No. of isolates	Occurrence (%)
<i>F. moniliforme</i>	38	58.46
<i>F. graminearum</i>	19	29.23
<i>F. oxysporum</i>	8	12.31
Total	65	100
p-value (Chi <sup>2</sup> )	0.001	

### Fumonisin B<sub>1</sub> (FB<sub>1</sub>) detection

The percentage of *Fusarium* isolates which have the ability to produce fumonisin B<sub>1</sub> was summarized in table 2. Sixty five isolates was collected in the study, of all the isolates two fungal isolates not produce detectable level of FB<sub>1</sub> whereas only one isolate produced FB<sub>1</sub>.

Table 2. Toxigenic *Fusarium* isolates collected from infected maize seeds

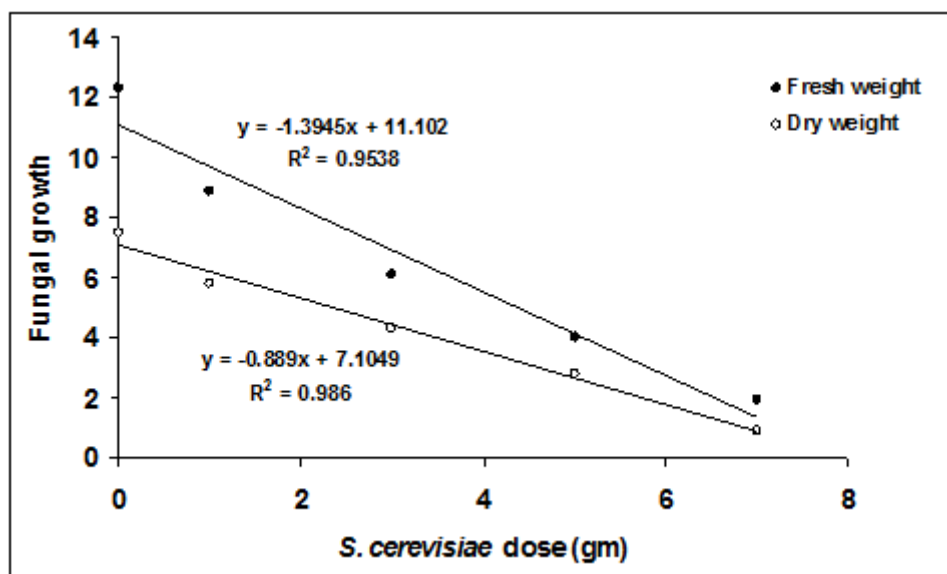
<i>Fusarium</i> spp.	No. of collected isolates	No. of toxigenic isolates	Toxigenic isolated (%)
<i>F. moniliforme</i>	38	33	86.8
<i>F. graminearum</i>	19	0	0
<i>F. oxysporum</i>	8	0	0
Total	65	33	86

### Effect of *S. cerevisiae* on fungal growth

The effect of different *S. cerevisiae* doses/g on the growth of *Fusarium moniliforme* was detected. Our study revealed that, there was a reverse relationship between increasing *S. cerevisiae* concentration and growth of *Fusarium moniliforme*. Fresh and dry weights of *Fusarium moniliforme* were decreased to 1.9 mg and 0.9 mg in the presence of 7 gm of yeast. Generally, addition of *S. cerevisiae* decreased fresh and dry weight of *F. moniliforme* gradually in comparison to zero dose (control) as shown in table 3 and fig 2.

Table 3. Influence of different *S. cerevisiae* doses on the growth of *Fusarium moniliforme*

<i>S. cerevisiae</i> dose/ (g)	<i>F. moniliforme</i>	
	Fresh weight (mg)	Dry weight (mg)
Zero (control)	12.3	7.5
1	8.9	5.8
3	6.1	4.3
5	4.0	2.8
7	1.9	0.9

Figure 2. Influence of different *S. cerevisiae* doses on the growth of *Fusarium moniliforme*

### Biocontrol of fumonisin B<sub>1</sub>

Table 4 and fig 3 represent the effect of *S. cerevisiae* on the production of fumonisin B<sub>1</sub>. Application of *S. cerevisiae* at different doses reduces FB<sub>1</sub> production gradually in relation to zero dose (control). The detoxification percent at doses 3 and 5 were 77.63 and 89.52, respectively. Also dose 7 was sufficient for complete detoxification of fumonisin B<sub>1</sub>. Table 5 showed the dry weight, fresh weight and detoxification and their correlation with *S. cerevisiae* dose.

### Influence of some physical parameters on FB<sub>1</sub> production Effect of different water content

Effect of different water content (30, 40 and 50 %) on production of fumonisin B<sub>1</sub> (FB<sub>1</sub>) in sterile ground maize by *Fusarium moniliforme* isolate was

determined over period of 6 weeks as shown in table 6. Generally, *F. proliferatum* grew faster with increasing water content. Fumonisin B<sub>1</sub> production depends upon water content and type of substrate. At water content of 50%, a higher level of fumonisin (16.3 µg/g) was detected in ground maize, while in corn flour and intact grain level of fumonisin was 0.50 and 8.66 µg/g, respectively.

Effect of different temperatures on FB<sub>1</sub> production by *F. moniliforme* was investigated as showed in table 7. *F. moniliforme* incubated at different temperatures ranges from 17 to 28°C. Generally our results indicated that level of fumonisin B<sub>1</sub> was increased gradually by increasing temperatures degrees until reach a high level of production at temperature 21°C and then decreased by increasing

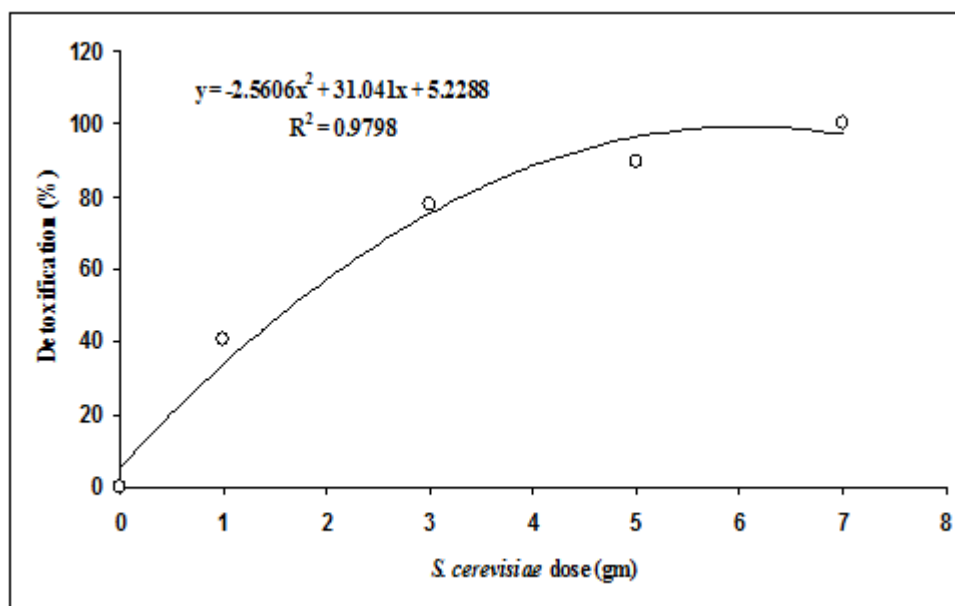
temperature degrees. At temperature 21°C level of FB<sub>1</sub> was 19.3 µg/g after incubation period 4 weeks while at temperature 28°C was 9.7 µg/g after the same incubation period and on the same substrate.

Table 4. Effect of *S. cerevisiae* on the production of fumonisin B<sub>1</sub>

<i>S. cerevisiae</i> dose (g)									
0		1		3		5		7	
O. (µg/g)	D. (%)	O. (µg/g)	D. (%)	O. (µg/g)	D. (%)	O. (µg/g)	D. (%)	O. (µg/g)	D. (%)
14.3	0	8.5	40.56	3.2	77.63	1.5	89.52	ND	100

Table 5. Correlation between the doses of *S. cerevisiae* and fungal growth as well as the detoxification percent of fumonisin B<sub>1</sub>

	Dry weight	Fresh weight	Detoxification
<i>S. cerevisiae</i> dose (g)	r = - 0.993	r = - 0.977	r = 0.927
	p = 0.001	p = 0.004	p = 0.024

Figure 3. Effect of *S. cerevisiae* on the production of fumonisin B<sub>1</sub>Table 6. Influence of water content on FB<sub>1</sub> production by *F. moniliforme*

	Water content		
	30%	40%	50%
<b>Corn flour</b>	0.70	0.90	0.50
<b>Coarse ground maize</b>	13.25	14.20	16.30
<b>Intact grain</b>	3.43	6.75	8.66

Table 7. Influence of different temperatures on FB<sub>1</sub> production by *F. moniliforme*

Incubation period	Temperatures			
	17 °C	21 °C	25°C	28°C
<b>Week 2</b>	2.5	8.6	4.1	2.2
<b>Week 3</b>	3.9	10.3	5.8	4.1
<b>Week 4</b>	6.4	15.0	11.1	8.7
<b>Week 5</b>	8.5	19.3	14.8	9.7
<b>Week 6</b>	8.3	10.5	9.1	4.9

#### 4. Discussion

Mycotoxins are fungal metabolites capable of having acute toxic, carcinogenic, mutagenic, teratogenic, immunotoxic and oestrogenic effects to man and animals (Castella *et al.*, 1999). Fumonisin are a group of naturally occurring Mycotoxins produced by *Fusarium moniliforme*, *Fusarium proliferatum* and other related species (Bacon *et al.*, 2001 and Miller, 2001). Common mycotoxins in maize include aflatoxins, fumonisins, moniliformin, deoxynivalenol and zearalenone. *Fusarium verticillioides* is the leading fungal species in producing fumonisins that have been shown to cause equine leukoencephalomalacia, pulmonary edema, human esophageal cancer and rat liver cancer (Van Egmond, *et al.*, 2007).

Similar to our work Shephard *et al.* (1996) showed that, *F. verticillioides* and *F. proliferatum* are by far the most prolific fumonisin producers. Also Rheeder *et al.* (2002), recorded that, *F. verticillioides* and *F. proliferatum* are produce the highest amounts of toxins: up to 17900 µg/g of FB<sub>1</sub> have been recorded in cultures for the former, and 31000 µg/g FB<sub>1</sub> for the latter.

Our study showed that, *F. moniliforme* produced highest amounts of fumonisin 19.3 µg/g after five weeks incubation period. Also detoxification percent of FB<sub>1</sub> was significantly increased gradually by addition of *S. cerevisiae* while growth of *Fusarium moniliforme* was significantly decreased. Linear regression analysis between *S. cerevisiae* doses and fresh weight and dry weight showed that significant negative correlation was detected. The regression equations for fresh and dry weights were:  $Y = -1.3945x + 11.102$ ,  $R^2 = 0.9538$  and  $Y = -0.889x + 7.1049$ ,  $R^2 = 0.986$ , respectively.

The detoxification percent at doses 3 and 5 were 77.63 and 89.52, respectively. Also it clear that dose 7 was sufficient for complete detoxification of fumonisin

B<sub>1</sub>. A similar result was obtained by Paola *et al.* (2010). He showed that, seed treatment with *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* reduced the infection by the fungus and FB<sub>1</sub> contents. Also these results are similar as of El-Tarabily (2004), Madi *et al.* (1997), Moustafa and Mohamed (2008); they reported that *R. solani* and *S. rolfisii* were effectively suppressed by yeasts.

Both temperature and water content of the substrate are factors that are of particular importance for the growth and the mycotoxinogenesis of *Fusarium* species that are mainly mesophilic and hydrophilic fungal strains usually developing on living plants, acting as parasites (Marin *et al.*, 2004). Generally our results indicated that, level of fumonisin B<sub>1</sub> was increased gradually by increasing temperatures degrees until reach a high level of production at temperature 21°C and then decreased by increasing temperature degrees. At temperature 21°C level of FB<sub>1</sub> was 19.3 µg/g after incubation period 4 weeks. While at temperature 28°C was 9.7 µg/g after the same incubation period and on the same substrate.

Similarly, Mogensen *et al.* (2009) found that *Fusarium* spp. had the maximal production of FB<sub>1</sub> and FB<sub>2</sub> at 20-25°C. Also, Marin *et al.* (1995 and 1999) showed that both *F. moniliforme* and *F. proliferatum* grew faster with increasing water activity. Similarly, our work showed that, at water content of 50% higher levels of fumonisin (16.3 µg/g) was detected in ground maize, while in corn flour and intact grain level of fumonisin was 0.50 and 8.66 µg/g, respectively. Those results are in agreement with data obtained in other studies (Samapundo *et al.*, 2005; Bailly *et al.*, 2005 and Narasimha *et al.*, 2010). FB<sub>1</sub> production optimal conditions taken off for the end of the study were; 5 weeks at 21°C on sterile coarsely cracked maize with water content of 50 %.

In conclusion, the current work indicates that maize seeds are contaminated to various degrees with



*Fusarium* moulds and fumonisin B<sub>1</sub> and the use *S. cerevisiae* as a biocontrol agent can control the *Fusarium* moulds growth as well as fumonisin B<sub>1</sub> production in these seeds. In the present study, we suggest that caution should be used whenever, maize seeds are to be stored prior to use because of the normal occurrence of mycotoxinogenic moulds as part of the natural seed microflora.

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