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STUDIES ON EAR AND KERNEL ROT OF MAIZE CAUSED BY *ASPERGILLUS* AND *FUSARIUM* SPP.

BY

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INTRODUCTION

Maize (*Zea mays* L.) is considered one of the most important cereal crops in Egypt. The cultivated area of maize is about two million feddans yielding almost 5.5 million tons according to the annual report of the Statistical Department, Ministry of Agriculture, A.R.E (1999).

Maize is subjected to the attack by several diseases. Some major diseases threat maize productivity. Grain mold in Egypt is one of the most prevalent diseases of maize. The disease may be caused by any one (or more) of several fungi, depending on locality and variety. Surveying harvested maize grains allover the country were done by maize pathologists throughout the National Campaign for Maize Productivity in Egypt (1997& 1998) revealed that the grain samples collected from-maize growing fields were contaminated with molds that caused visible and invisible deterioration. *Fusarium* spp. and *Aspergillus* spp. were found to be the most common fungi that could be recovered from the affected ears. These molds are known to be increased under bad storage conditions (Halloin, 1975) along with the produced mycotoxins. Aflatoxins as serious metabolites to human and animal that could be produced by some strains of *Aspergillus flavus* and *A.parasiticus* have been extensively studied by previous investigators.

This investigation was carried out to determine storage conditions that badly affect the stored maize grains expressed as kernel contamination with stored fungi as well as toxin production. Isolation and identification of ear and grains-rotting fungi and studying methods of artificial inoculation were studied. Also, the commercial maize hybrids

were screened for their susceptibility to infection with ear and grain molds under field conditions.

REVIEW OF LITERATURE

From ripening through harvest and storage, maize grains are especially vulnerable to attack by microorganisms by process commonly reffered to as weathering (Halloin, 1981) and storage deterioration (Christensen & Kaufmann, 1969). Fungi that play an important role in deterioration can be divided into field and storage fungi, based on their occurrence on cereal grains. Field fungi are those that contaminate or invade grains in the field, often during or after ripening, and during harvesting operations. These include genera such as *Fusarium, Alternaria* and *Helminthosporium* on grains. Storage fungi develop on grains during storage and commonly fall into two genera, *Aspergillus & Penicillium.* Field fungi can increase on grains under exceptional moist storage conditions (Halloin, 1975). Grain moisture content, temperature and relative humidity around stored grains, cracked kernels, length of time the grain is to be stored, and amount of insect and mite activity in grains are the major factors that determine when stored grains will be damaged by storage fungi (Scott, 1997). There are two major mechanisms by which microorganisms (primarily field and stored fungi) damage grains. These are production of exocellular enzymes and toxins. Various fungi, especially *Aspergillus & Fusarium* spp., produce mycotoxins, components toxic to animal and human.

Lutey & Christensen (1963) reported that filed fungi, such as *Alternaria, Fusarium,* and *cladosporium*, require higher moisture contents which amount to 24-25 %; they can be eliminated by storage at 14% moisture content at 30°C for 16 weeks. Stored fungi grow most rapidly at a temperature of 30°C, but some are thermophilic that can grow at temperature more than 30ºC (Christensen and Kaufmann, 1969 and Muling & Chesters, 1970).

Gamal El-Din *et al.* (1987) reported that the most dominant fungi in maize grains could be arranged descendingly as *Fusarium moniliforme, Nigrospora oryzae, Fusarium graminearum, Penicillium* spp. *Aspergillus flavus, Aspergillus niger, Helminthosporium* spp., *Rhizopus* spp., *Mucor* sp. and *Alternaria* spp. They mentioned that severity and percentage of infection by these fungi increased by increasing moisture content and storage period. Prolonging storage periods at 18 °C generally resulted in reduced weight of both the germ and hull in either healthy or infected grains by *Nigrospora oryzae,* whereas no difference occurred when infected by *Fusarium moniliforme.*

Fusarium moniliforme J. Sheld is a widely distributed pathogen of maize (Zea mays L.), causing seedling disease, root rot, stalk rot, and ear or kernel rot (Kommedahl and Windels, 1981; Nelson, 1992 and Munkvold, 1994).

Saubois *et al.* (1996) identified *Fusarium* strains isolated from maize at harvest during 1993 in different fields of the northern-center area of the province of Santa Fe, Argentina, to the species level. Their distribution in the samples was evaluated and studies of prevalence were performed in maize grains from this region. The prevalence of *Fusarium* spp. In the samples was 100% and 29% were colonized by > 1 spp. *F. moniliforme* (*Gibberella fujikuroi*) was identified in 52.6% of the samples, *F. subglutinans* (*G. fujikuroi* var. *subglutinans*) and *F. graminearum* (*G. zeae*) in 29% and *F. oxysporum* in 2.6%. *G.fujikuroi* presented the highest levels of colonized grains (7.5-52.5%).

Caldwell *et. al.* (1981) suggested that *Fusarium moniliforme* is a better competitor in preharvested maize than *Pencillium funiculosum.* Initial kernel infection by the first pathogen may serve as an important deterrent to subsequent kernel invasion by other seed-infecting molds. However, from 15 species of *Pencillium* tested in the field, only there

were *P. funiculosum* and *P.oxalicum* were able to colonize preharvested ears and infect kernels.

El-Sayed, (1996) surveyed the fungi occurring on corn grains obtained from different sources. Isolated fungi were *Fusarium* spp., *Aspergillus* spp., *Helminthosporium spp.*, *Alternaria* spp. *and Penicillium* spp. Pathgenicity tests showed that Fusaria were more detected and differed in virulence expressed as grain germination percent and seedling blight.

King (1981) reported that *Fusarium moniliforme* appeared to be an early colonist of preharvested maize ears, infecting the kernels before *Penicillium* and other molds.

Singh *et al.* (1988) indicated that infection by *F.moniliforme,* was detected in all parts of seed, however the percent of infection depended on the severity of seed infection. The pathogen infected the pedicel and basal ovary after 5to 10 days of anthesis and the mycelial clumps produced in between the ovary wall and aleurone layer entered into the endosperm and embryo directly.

Wicklow (1988) found that eleven common maize infecting fungi grew out from surface-disinfected maize kernels from North Carolina and plated on maltextract agar. Each of these fungi was known to infest maize ears preharvest. *F.moniliforme* was the most common fungus grew on 52% of the kernels. *Aspergillus flavus* and *A. niger* were the two fungi commonly associated with preharvest maize and grew out from 19 and 36 % of the kernels, respectively and other fungi observed included *Acremonium strictum* 7%, *Alternaria alternata* 5%, *Nigrospora oryzae* 4%, *Curvularia lunata* 3%, *Trichoderma viride* 3%, and *Rhizopus spp.* 2%.

Zummo and Scott (1990b) demonstrated that *A.flavus* and *A.parasiticus* may be equally aggressive in maize kernels in the field after

artificial inoculation of ears, but *A. flavus* appears to have a greater ability for survival in the field, thus the natural inoculum in old corn fields would be *A. parasiticus.*

Rio (1990) investigated the dry ear rot of maize in Honduras caused by a complex of *Diplodia* and *Fusarium*. *Diplodia* (*Stenocarpella*) *maydis* and *F.moniliforme* (*Gibberealla fujikuroi*) were the most common pathogens isolated from maize plants with dry ear rot symptoms.

Pathogenicity and inoculation techniques:

Ullstrup (1970) studied two inoculation techniques to infest maize ears with *F.moniliforme*; spraying silks with macroconidial suspension 1 to 2 weeks after silking and inserting a toothpick colonized with mycelium into the silk channel of the ear approximately 1week before full silk emergence. Both methods established infection at levels that allowed differentiation between genotypes.

Styer and Cantliffe (1984) found that mature ears of two tested maize hybrids, inoculated 10 days post pollination by *Fusarium moniliforme* had higher levels of rot and seed infection than those inoculated later.

Munkvold and Carlton (1997) reported that infection through the maize ear silks was clearly the most effective pathway to kernel infection. This was the only inoculated method that significantly increased overall incidence of *Fusarium moniliforme* infection in kernels; the silk inoculation strain infected up to 100% of kernels in individual ears, with a treatment mean as high as 83.7% of kernels. When plants were silk inoculated the percentage of kernels infected by other *Fusarium moniliforme* strain from the seed or stalk was reduced, apparently due to competition among strains.

Munkvold *et al.* (1997) stated that systematic development of *Fusarium moniliforme* from maize seed and stalk infection, but silk infection is a more important pathway for this fungus to reach the kernels.

Chungu *et al.* (1996) compared between six inoculation techniques differing in the method of application of conidial suspension and the part of maize ear inoculated. They evaluated the effectiveness in assessing maize (*zea mays*) resistance to ear rot caused by *Fusaruim graminearum.* Silk channel injection and kernel stab inoculation techniques were the most effective in measuring silk and kernel resistance.

Diab *et al.* (1984) pointed out that infection was generally most extensive in term of number of ears infected and type of infection when the spore suspension was inoculated at the ear tip or into the husks. Injection into husks was effective as compared to ear tip, silk, and shank inoculation. Early inoculation caused more severe infection than late one.

In a program for breeding for resistance to ear rot, Chambers (1988) found that time of inoculation for germplasm evaluation is critical. There was rapid decrease in kernel moisture 20 days after mid-silk and ear inoculation should be made at or shortly after this date. Inoculation at this time would best differentiate between resistant and susceptible germplasm.

Reid *et al.* (1992) reported that injection of conidial suspension into the silk channel gave consistent results and allowed for differentiation between resistant and susceptible genotypes. But, Sutton and Baliko (1981) used the toothpick, spraying of silk, and silk-channel injection methods unlikely, they found that the silk channel injection was ineffective in differentiating between resistant and susceptible genotypes.

Drepper and Renfrom (1990) pointed out that the nail punch was the most effective method for ear inoculation in maize, while the drill/toothpick method was most effective for stalk inoculations.

King and Scott (1982) found that inoculation by *Aspergillus flavus*, conidial suspension into silk channel or by atomizing conidial suspension into exposed kernels resulted in infection levels too low (7%). The kernel injection technique gave relatively high levels of infection, while pinbar technique resulted higher levels (9-46%) of kernel infection.

Widstrom *et al.* (1981) inoculated maize ears at 20 and 40 days after mid silk by three inoculation methods. Visible *A. flavus* occurred on 83% of the ears when inoculations were made 20 days after mid silk, compared to 35% with inoculations made 40 days after mid silk.

Tucker *et al.* (1986) showed that kernel infection by *A. flavus* was significantly greater for row one of the pinbar-inoculated and exposed-kernels. Inoculation techniques had a higher percentage of kernels with Bright greenish yellow fluorescent than were found by other inoculation techniques.

Varietal resistance:

Neucere (1992) identified the cationic proteins from 2 varieties of maize that are resistant (yellow Creole) and susceptible (Huffman) to *Aspergillus flavus* and to aflatoxin contamination were characterized by electrophoresis. Under native conditions, cathodic electrophoresis showed a cluster of proteins at Rf (5: 6.5) in yellow Creole that was not evident in Huffman. SDS-PAGE showed 12 bands that ranged between 14 and 43 KDa in both varieties. The profiles were differed both qualitatively and quantitatively. The major proteins in yellow creole clustered around 20 Kda on the gel and those in Huffman around 25 Kda. Qualitative variations of proteins by two-dimensional electrophoresis were evident, especially within the pH range (6.7: 8) and around 27 and 92 Kda. The results are discussed in connection with the possible role of polypeptides as antifungal agent in species of maize.

Campbell and White (1995) reported that resistance of maize hybrids and inbreds to ear rots, Kernel infection and aflatoxin contamination caused by *A.flavus* was evaluated following inoculation thirty- five F1 hybrids from crosses of selected inbreds with the susceptible inbreds B73 and/or Mo17 were evaluated for three years in Illinois, USA. Resistant F1 hybrid, 15 were also evaluated in Mississippi, USA. Resistant and susceptible inbreds were evaluated for two years in Illinois. Inbreds Tex 6, Y7and Mp 420, and the F1 hybrids with these inbreds, consistently had the greatest resistance to *A.flavus* ear rot, kernel infection and aflatoxin production in the Illinois environment. Grain of the F1 hybrids with this inbred also had less aflatoxin in Mississippi. Person and spearman rank correlation coefficients indicated that ear rot ratings provided a more accurate estimate of aflatoxin contamination of grain then did kernel infection. From evaluations of the F1 hybrids and the inbred parents, inbreds were identified that may contain alleles for resistance not found in B73 and/or Mo17.

Mycotoxins:

The word mycotoxins was derived from the Greek word mykes meaning fungus and the Latin word toxicum meaning poison (Forgacs and Carll, 1962). Mycotoxins are secondary fungal metabolites that cause pathological or undesirable physiological responses to humans and animals when fed on contaminated dietby the ingestion of food or feed contaminated by it (Goto, 1990).

Aflatoxins are basically difuranocomarin compounds and include aflatoxins B1, B2, G1, GM1, G2, GM2, G2a, M1, M2, M2a, P1, RB1, RB2, AFL, AFLH, AFLM and methoxy, ethoxy and acetoxy derivatives (Coker *et al.*1984). Aflatoxins are the most common mycotoxins have been investigated all over the world produced by *Aspergillus flavus* which

produces B1, B2 and *A. parasiticus* which produce G1, G2 and M1 as well as B1, B2. Aflatoxin B1 is the most potent and carcinogenic naturally occurring substance known, causing liver damage to most domestic and experimental animals and humans (Diener *et al.*,1982). The discovery of aflatoxin dates back to the year 1960. Following the severe outbreak of the turkey ''x'' disease, in U.K. which resulted in the death of more than 100000 turkeys and other farm animals, later termed aflatoxins were responsible for the outbreak (Sargeant *et al.*,1961; Bash & Rae, 1969; Davis & Diener, 1979). Hence, the name aflatoxin an acronym, has been formed from the following combination: the first letter 'A' for the genus *Aspergillus*, the next set of three letters, ''FLA'', for the species flavus, and the noun ''toxin''meaning poison (Ellis *et al.*,1991).

Arae *et al.* (1963) reported that the parent compound aflatoxin B1 had a molecular weight of 312, its formula is $C_{17}H_{12}O_6$. While Asao *et al.* (1965) reported that the aflatoxin share a common structure, forming a unique highly oxygenated naturally occurring heterocyclic compound they contain a bis furan- isocoumarin moiety with the lactone ring oxygen conjugated with a double bond. The only difference between Afltoxin B1 and B2 and between G1 and G2 is the double bond in the terminal furan ring of B1 and G1while the only difference between B and G compounds is that, the Afl.G1 and Afl.G2 having 5-membered terminal lactone ring. But without stereochemical designations and aflatoxins B2, and G2 were shown to be the dihydro derivatives of aflatoxin B1andG1, respectively.

Screening study of 29 species including 121 fungal isolates, revealed an aflatoxin formation only by the *Aspergillus flavus* group (Wilson *et al.*, 1968). Also Kheiralla (1994) found that six genera and eleven species of mycoflora represented by 90 strains were isolated from non-disinfected grains of maize. In addition, 21 strains representing 3 genera and 7 species were isolated from disinfected grains. Species of

Aspergillus and Rhizopus were predominant in non-disinfected grains. Aflatoxins produced by the isolated fungi were confirmed by TLC plates. Three isolates identified as *A. flavus* produced aflatoxin B1 and B2, and one *A. parasiticus* isolate produced B1, B2, G1 and G2.

The production of aflatoxins by species and strains of *A.flavus* group isolated from peanuts, cotton seeds, rice and sorghum was carried out by Schroeder & Boller (1973). They found that 96, 79, 49 and 35% of *A.flavus* strains isolated from peanuts, cottonseeds, sorghum and rice respectively, produced aflatoxins indicating that not all isolates are producing aflatoxins.

Edds (1973) reported that aflatoxins contamination of food stuff is a world wide problem and has been found in groundnut, meal, wheat, rice, soybean, maize, bread, milk and cheese (Svoboda *el al.,* 1966).

Zearalenone an estrogenic compound was isolated by Christensen *et al.* (1965). Which they called F-2 toxin from an unidentified isolate of *Fusarium.* Also similar compounds, F-2, F-3, F-4, F-5, have been isolated from cultures of *F.graminearum.* Toxin F-2, which has been also called FES (fermentation estrogenic substance), RAL, and is now usually referred to as zearalenone which has the highest estrogenic activity (Mirocha *et al.,* 1967a; Mirocha *et al.,* 1967b; Mirocha *et al.,* 1968b; Mirocha *et al.,* 1971). The observation of zearalenone dates back to the year 1928. Following the appearance of vaginal prolapse cases, which they called vulva vaginitis in sows in Wisconsin. The same phenomenon was described several years later in other parts of the United States of America, in Australia, Rumania, Hungary and Canada by several authors (Bristol and Djurickovic, 1971; Buxton, 1927; Koen and Smith, 1945; Palyusik, 1971; Pullar and Lerew, 1937). Also in Russia unusual

phenomenon was recorded on pigs after consumption of mouldy maize or barley (Kysela, 1941).

Some reports (Mirocha *et al.*, 1969; Mirocha & Christensen, 1974 and Mirocha *et al.*, 1977) stated that *Fusarium moniliforme* could produce the estrogenic metabolite zearalenone in synthetic media and /or naturally contaminated commodities. On the contrary Negative results for the production of zearalenone by isolates of *F. moniliforme* have also been reported by several authors (Caldwell *et al.*, 1970; Sutton *et al.*, 1976; Ichinoe *et al.*, 1977; Suzuki *et al.*, 1978 &1981). This agreed with Marasas *et al.*, 1984) who reported that non of the studied isolates of *F. moniliforme* can produce zearalenone. Also reported that there are no known zearalenone producing by strains of *F. moniliforme* represented in the ITFRC.

Conditions affecting stored maize grains and aflatoxins production.

Osman *et al.* (1988) surveyed the number of fungi associated with sorghum grains and found that 5 genera were predominant in all localities of Egypt. They are *Alternaria alternata* followed by *Aspergillus flavus, A. niger, Fusarium moniliforme* (*Gibberella fujikuroi*) and *A. niger*. Effect of these fungi on grain viability under different storage conditions was increased by increasing grains moisture content as well as storage temperature and storage period. The least damage occurred in grain when stored at 10 °C with 11% moisture content. The most active fungi during storage were *A.niger*, *A.flavus* and *G. fujikuroi*, especially at 30 °C and 18% moisture content.

Reddy *et al.* (1988) investigated mycoflora of maize seed samples from around Warangal, Andhra Pradesh, India stored under different environmental conditions. Mycoflora were analyzed by the blotter

technique. Twenty-two fungal species belonging to 11 genera were isolated and *Aspergillus flavus* was the most frequently detected species.

Abol Alea (1996) reported that the percentage of infection of yellow corn grains caused by *Aspergillus flavus* was significantly increased by increasing the storage temperature degree, grain moisture content percentage, time of storage and percentage of broken kernels (grade of grains).

Khankari *et al.* (1995) found that variations in ambient temperatures cause safe moisture contents of stored grain to become unsafe due to migration or redistribution of moisture within storage. This study uses a previously developed generalized numerical model of the moisture migration process, which can be used to predict the temperature, moisture, and natural convection airflow distribution in stored grain. For the weather conditions of St.Paul, Minnesota, this numerical model is used to evaluate the effect of various bin geometries and grain parameters on the moisture migration behavior of stored corn and wheat. These predictions indicate that cooling of grain to 0 °C during the fall season can keep grain moistures under stable conditions for the entire year. It also shows that moisture migration occurs in all sizes of bins and it starts developing earlier in the smaller bins.

It was mentioned that mycotoxins production is influenced by the same factors that affect seed infection. The occurrence of mycotoxins in general depends on their formation by toxigenic strains of fungi and are influenced by environmental factors such as humidity, temperature, host and fungal invasion. Stored methods and period of storage also are involved in mycotoxins production...*etc.*(Jones and Duncan, 1981; Mills, 1986 and Ghewande *et al.*, 1993)

Viquez *et al.* (1994) evaluated the effects of environmental conditions i.e., temperature, RH, harvesting date (early, proper time and

late), presence of aflatoxin producing fungi and nutrients (minerals, moisture, and carbohydrates) on the levels of aflatoxin contamination in samples of corn grown in 3 regions of Costa Rica. A multiple regression analysis design determined that the total aflatoxin levels correlated with the presence of *Aspergillus flavus* in grains and temperature conditions. Collection phase had a significant effect on aflatoxin levels ($P =< 0.05$) due to differences in harvest and storage conditions as well as agricultural practices in each region. He found that aflatoxin levels also depend on glucose concentration in the grain. It may be concluded that most Costa Rican corn is contaminated with aflatoxin (mean level 14.47 p.p.b).

Temperature and incubation time have been recognized as important factors affecting mold growth and subsequent toxin production. In this respect, *Aspergillus flavus* classified as a mesophilic fungus which has cardinal growth temperatures as follows: minimum 6-8 °C, optimum 36–38 °C and maximum 44–46 °C. The minimum and maximum temperatures for growth are affected by moisture, oxygen concentration and availability of nutrients and other factors. The relative amounts of aflatoxin B_1 and G_1 produced by species of *Aspergillus flavus* group on natural and synthetic substrates are influenced by several factors. The most important factor controlling the proportion of B. to G. produced by *A.flavus* appears to be the temperature. Higher levels of aflatoxins are occasionally found in gricultural commodities in the field than similar commodities incubated in the laboratory (Majunder *et al.*, 1965).

Schroeder and Hein (1968) found that aflatoxin production decreased when the temperature was cycled from a low of 20°C to a high of 40–50 °C. However, cycling between low one of 10–20 °C and a high one of 26.6 °C, average 24-hr temperature of 25 °C gave no change in growth pattern or production and accumulation of aflatoxin compared to a control incubation at a constant 25°C. And they stated that the distribution

ratio of the four aflatoxins is affected by temperature. G_1 is produced at lower temperature than B_1. While the concentration of B_1 was greater than G_1 at around 30°C, in some cases, equal production was noticed in this temperature range. Also, Lin *et al.,* (1980) reported that at lower temperature, nearly equal amounts of aflatoxins B_1 and G_1 were produced at 28°C, the amount of B_1 produced was four times higher than G_1 but at higher temperature relatively less G_1 was formed.

Davis *et al.* (1966) found that the maximum production of B_1 was detected at 30 and 35°C. While maximum G_1 was produced at 25°C and 30°C further more. They reported that the optimum temperature for both aflatoxins B_1 and G_1 on rice was at 28°C. Comparable yield of B_1 was obtained at 30 °C, but less G_1 was produced at this temperature.

Mangan and Lacey (1987) reviewed the environmental factors affecting mycoflora of grain in store. Field fungi were common on grain before harvest but seldom in store and they require water to be readily available for growth to occur. By contrast, storage fungi, especially *Aspergillus* spp. developed only after harvest since they could grow at low water activity enabling them to initiate spoilage of stored grains. Most storage fungi are mesophytes with optimum temperature for growth between 20°C–35°C but a few were psychrotolerant (able to grow at < 0°C) and others thermotolerant (up to 60°C). Water availability in the grain, temperature and intergranular gas composition together determined which spp. could grow and initiate spoilage. Bad storage allows a succession of fungi to colonize grains and loss of quality.

It is recognized that relative humidity (RH) is one of the most important factors for the growth of *Aspergillus flavus* and aflatoxin production. The minimum RH for *Aspergillus flavus* growth and spore germination is 80% and 85%, respectively. (Panassenko, 1944).

Christensen (1970) found that, moisture content of less than 14 % was suitable for relatively long-term storage of grains without reducing its quality caused by storage fungi. Botast *et al.*,(1981) stated that, mold population increased in corn grains stored with 15 to 18% moisture content.

Sauer (1987) found that the aflatoxin producing fungus; *Aspergillus flavus* grows in grain at the field and also in stored grain after harvest. The most important factors affecting fungal growth and toxin production are grain moisture content and temperature. Drying and cooling the grain before storage is the principal means of control. Other factors that affect *A.flavus* growth are oxygen and carbon dioxide concentration, physical damage of the grain, initial levels of mold contamination, insect activity and genetic differences of maize. *Aspergillus flavus* does not grow at relative humidity below 85% or moisture contents below 16%; aflatoxin can be produced at temperatures ranging from 11°C to 40°C.

Numerous natural substrates have been used to produce aflatoxins in large quantities in the laboratory. The quantity of aflatoxins produced by *Aspergillus flavus* under similar environmental conditions differed according to the substrate (Diener *et al.,* 1965).

Substrate factors are involved in the contamination process. Since the natural occurrence of aflatoxins is restricted to certain agricultural commodities. Although *A. flavus* will grow on and from aflatoxins in many sterilized inoculated substrates. Unless penetration occurs through the vascular system (Klich *et al.*1986). Also Bilgrami *et al.* (1982) reported that aflatoxin production varies among cultivars they were grouped. Fifteen maize cultivars on the basis of the quantity of aflatoxin B1 production by *A.parasiticus* to resistant, moderately resistant and susceptible this variation due to seed inhibitors. But, Nagarajan &Bhat (1972), identified a low molecular weight protein as the inhibitory factor

to production of aflatoxin by *A. parasiticus* under artificial conditions in the resistant maize cultivar opaque-2. On the other hand, Widstrom *et al.* (1984) stated that Sugar content of maize seeds is an overriding factor contributing to differences among genotypes for aflatoxin contamination by *A. flavus*. Mycelial growth was found also to be enhanced by the presence of a sugary endosperm. Also Bilgrami *et al.* (1990) stated that maize c.v. Suwan composite which has a tight, incomplete husk and an intermediate maturity period was highly naturally contaminated by aflatoxins due to infection by *A.flavus.* Whereas, the c.v.Diara composite with a loose complete husk and 80 day maturity period. Was less prone to aflatoxin contamination.

Priyadarshini and Tulpule (1980) suggested that lipids may have a role in the synthesis of aflatoxins where the saponifiable fraction from wheat germ lipids was found stimulate aflatoxin synthesis.

Farag (1990) demonstrated that the fungus utilized the basic compound of seeds for its growth. The differences in crop composition was mainly due to the influence of the pathway to use the major energy source of each seed whether lipid (sesame) or carbohydrate (wheat).

Under the Egyptian conditions, 45 isolates of Aspergilli were tested for the production of mycotoxins. These fungi were isolated from soil, seeds, grains and air. Fifteen isolates were-nontoxic and induced no effect on the hatchability of eggs in bioassay reaction. While, thirty isolates were toxic; six isolates of them have been highly toxigenic effect (Moubasher *et al.* 1977).

MATERIALS AND METHODS

The present study was carried out during the years (1997- 2001) collaboratively at The Botany Department, Faculty of Agriculture, Kafr El-Sheikh, Tanta University, the Plant Pathology Research Institute A.R.C., Giza and Agricultural Experimental Station of Sakha.

1-Maize ear sample collection:

Several trips were made to each of 6 governorates; namely Dakahliya, Kafr El-Shiekh, Gharbiya, Qalubiya, Beni-Suef and Nubarya to collect the infected materials from farmer`s fields. Samples were collected from the maize ears at harvest time (October-November, 1997). Three districts from each of the 6 governorates were visited and 3-4 ears from each of 3 fields were taken together. Shelling of ears was done and kernels of each governorate were mixed together and 300g sub samples were taken by random for fungal isolation and determining the total aflatoxin.

2-Isolations and identifications of the causal agents:

To isolate the kernel rotting fungi, grains were surface disinfected for 5min with 5% sodium hypochlorite and rinsed three times into sterile distilled water and left to dry on autoclaved filter papers. Isolation was made by seeding a number of ten kernels on PDA plates and incubated for 7 days at 27 °C. Ten replicate plates were used. PDA medium was used in general to isolate rotting fungi. *Fusarium spp.* was subcultured and identified onto PDA medium. Czapek`s agar medium was used for identifying *Aspergillius spp.*and *Pencillium spp.* Single spore isolation technique according to Hansen (1926) was followed, and fungi were kept on PDA slants at 8 °C for further studies.

Identifications were done according to the morphological and microscopical characters and compared with the description given by Alexopolous (1968), Ainsworth and James (1971) and Marsas *et al.*(1984). Identifications were confirmed at the Mycology Research and Survey of Plant diseases Section, Plant Pathology Research Institute, A.R.C., Giza.

3-Pathogenicity test:

This trial was carried out in the field of Agricultural Experimental Station at Sakha in 1997. Local susceptible maize variety (Boushi, from Beni Suef) was used. A number of 21 *Fusarium* isolates and 14 *Aspergillus flavus* isolates collected from different localities were screened for their virulence to maize under artificial inoculation. Silk channel injection technique according to Chungu *et al.* (1996) for *Fusarium* inoculation and Zummo and Scott (1989) for *Aspergillus* inoculation were followed. Inoculations were made by each of the obtained isolates under study to 30 plants in three replicate rows. Ear infections were scored at harvest time as percentage of rotted area per ear according to the method adopted by Drepper and Renfrom (1990) for *Fusarium moniliforme* scale and Campbell and White (1995) for *Aspergillus flavus* scale.

4-Inoculum preparation and inoculation:

Inoculations were made by using the spore suspensions of fungi under study. Isolates of *Fusarium spp.*were grown onto PDA for 2 weeks at 25-27°C. Produced spores were collected into sterile distilled water and number of spores were adjusted into the spore suspension to $5x10^5$ per ml by hemocytometer just before use. *A.flavus* was grown on sterile maize

cob grits at 28°C for 12-14 days. Conidia were washed with sterile distilled water and adjusted to 9 x 10^6 per ml by hemycytometer before use.

Fusarium isolates were inoculated into ears using 2 ml of the prepared spore suspension. Silk channel injection was used to ears 6- 7 days after mid silk emergence according to Chungu *et al.* (1996). *A.flavus* isolates were injected by using 3.4 ml of the spore suspension into the silk channel of maize ears 6-7 days after mid silk emergence according to Zummo and Scott (1989). Disease severity was assessed and recorded as percentage of rotted area of the injected ears.

5-Methods of inoculation with *F.moniliforme& A. flavus*:

Two maize cultivars, namely three way cross (SC 310) and local Baladi (Boushi) were used in this experiment. Thirty plants included in three replicates were used to inoculate ears for each treatment in this trial. Six methods of ear inoculation were studied for each of *F.moniliforme* as well as *A.flavus* isolates in the field-growing maize plants. Spore suspensions were prepared and adjusted to (5x10^5) by distilled water before use.

A- ***F.moniliforme***, the following methods were studied:

I-Silk channel injection:

Two ml of spore suspension were injected into the silk channel of ears using a hypodermic needle 6-7 days after mid silk emergence.

II- **Silk spray method:**

The spore suspension was atomized on ear silk without disturbing the ear husks 6-7 days after mid silk emergence as adopted by Munkvold and Carlton (1997).

III-Ear tip inoculation method:

Two ml of spore inoculum were dispensed with hypodermic needle to flood the kernels at the tip of the ear 6-7 days after mid silk emergence (Chungu *et al.* 1996).

IV- Kernel stab inoculation:

A probe consisting of 4 nails (1.5 cm) fixed to a cylindrical wooden handle was used to inoculate the ears. The nails were dipped after dipping into the spore suspension and then stabbed through husks to wound only 3-4 kernels at the middle of the ear. Inoculations were made 15 days of silk emergence (Chungu *et al.* 1996).

V- Wound and spray method:

Probe rinsed into sterile water was used to wound the kernels as described above. Then, approximately 2 ml of the spore suspension were atomized onto the wounded area. Ears were inoculated at 15 days of mid- silking (Chungu *et al.* 1996).

VI- Husk inoculation:

Two ml of spore suspension were injected into the ear and the surrounding husks by hypodermic syringe. Ears were inoculated at 15 days of mid- silking (Chungu *et al.*, 1996).

B-*A.flavus,* the following methods of inoculation were studied:

I-Silk channel injection:

A volume 3.4 ml of the spore suspension were injected into the silk channel of plant ears as described before and adopted by Zummo and Scott (1989).

II-Pin-bar inoculation:

Plastic pinbar with a single row of 35 pins was dipped into the conidial suspension and placed on the ear parallel with the kernel rows. The pins

were pushed through the husks into a single row of kernels avoiding cob penetration (King and Scott, 1982).

III-Side needle inoculation:

The needle was inserted at an angle through the husks and the inoculum was injected over the kernels without damaging them (Scott and Zummo, 1994).

IV-Tooth picks technique:

Autoclaved toothpicks were charged with an aqueous spore suspension of *A.flavus* and incubated for 14 days at 28 °C. Toothpicks were inserted through the husks and into the middle portion of the ear, six days after mid silk (Zummo and Scott, 1989).

V-Knife inoculation technique:

Five to seven kernels were damaged by knife dipped in a conidial suspension of *A.flavus* and inserted through the husks 20 days after silking. Rubber bands were placed around the ear to keep the husks closed (Scott *et al.,* 1991).

VI-Silk spray:

The spore suspension was atomized on the silk without disturbing the husk (Payne *et al.,* 1989).

Silk channel, side needle, silk spray and toothpicks inoculation techniques were carried out 6-7 days after mid-silk emergence. Whereas, pinbar and knife inoculation techniques were carried out 20 days after mid silking.

In all techniques wet paper towel was wrapped over the ear tips and shoot cap to maintain moisture and thus facilitate spore germination (Ullstrup, 1970).

6-Evaluation of maize genotypes against *F.moniliforme* and *A. flavus* under artificial inocutation:

A number of 11 different maize hybrids were screened for their susceptibility to infection with *F.moniliforme & A.flavus* under artificial inoculation in the field. Silk channel injection technique was followed in this experiment. Three replicate plots, 10 hybrid plants each were used in each treatment. Disease ratings were assessed at harvest time (55-60 days after injection). A six class rating scale was used for recording *F. moniliforme* ear rot as described by Drepper and Renfrom (1990) as follows: 1 = no visual diseased kernel, 2= 1-10 %, 3= 11-25%, 4= 26-50%, 5= 51-75%, 6= 76-100% of infected ear. For recording Aspergillus ear rot a 10 class visual rating scale was used as suggested by Campbell and White (1995) as follows: 1= 10 % to 10 = 100 % rotted area of the infected ear. Severity was determined for each ear and the average for each plot was recorded for each maize hybrid.

7-Detection and determination of mycotoxins in the fungal culture and maize grains:

7.1-Survey of aflatoxin in naturally rotted maize grains:

Aflascan kits were used to determine the total aflatoxin in the grain samples according to Trucksess *et.al.*(1991).

Fifty gm ground sample was blended for 1min at high speed with 250 ml equauos methanol 60% and 4gm of sodium chloride, then diluted with 250 ml distilled water to reduce the concentration of methanol sufficiently to allow binding between the aflatoxins and the antibody.
The extract was filtrated using Whitman no.4 filter paper and then slowly passed 20 ml in flow rate 2-3 ml/min through the column where binding

takes place between the aflatoxins and the antibody, retaining the aflatoxins in affinity column.

The column was washed twice with 10 ml of distilled water to remove any extraneous unbound material, and the bound aflatoxins were released from the antibody by passing 1 ml of methanol in flow rate 2-3 ml/min through the column and collected in a small tube. Interfering components were removed from the sample by adding 1 ml of distilled water + 1ml chloroform to the previous extract in small tube. The bottom chloroform layer then passed through a florisil tip and the natural fluorescence of the toxin could be viewed under UV compared against a standard competitor card providing a semi- quantitative result.

7.2-Screening and detecting of aflatoxin produced by isolates of *A. flavus*:

The ability to produce aflatoxin(s) was tested in 14 isolates of *A.flavus* by the fluorescence technique recommended by Hara *et al.* (1974). Each isolate from different locations was streaked on the surface of the Czapek`s dox agar plates and incubated at 27°C for 5 to 7 days in dark. Plates were examined daily for aflatoxin production. Petri dishes made of non-fluorescent glass were used. Diffusible zones of aflatoxin was detected visually as blue fluorescent zones under UV (365 nm) light.

Detection of aflatoxin(s) in culture filtrate of Czapeck dox broth was done following the method adopted by El-Bazza, (1979). Fifty ml of sterile liquid medium were set in 250 ml flasks, inoculated with 0.1 ml of spore suspension containing $1x10^6$ ml spores obtained from 7-10 day-old slant culture of *A. flavus* and incubated at 28 °C for 10 –15 days. Cultures were filtrated through filter papers and filtrates were used for toxin extraction. The BF method was followed to extract and clean up aflatoxin

in the fungal filtrate according to A.O.A.C (1990). Aqueous methanol (55% methanol+45% water) 250 ml + 100 ml hexane were added to 50 ml fungal filtrate + 4 g NaCl and mixed in separating funnel. After 30 minutes, 50 ml of the methanol-water layer containing extract were pipetted and received in a beaker. Fifty ml of chloroform were added to this extract and mixed in the separating funnel. The lower layer (containing the extracted aflatoxin) was removed and passed through anhydrous sodium sulphate to get rid of the water phase of the solvent and then dried on water bath at 50-62°C under fume hood. The remaining dry film was then kept under–20°C until use.

Aflatoxin(s) was separated and fractionated by using TLC technique. TLC aluminum sheets silica gel 60, without fluorescent indicator, pre-coated 20x20 cm with layer thickness of 0.25 mm (Merck Co.) were used in separating all extracted samples from fungal filtrate according to A.O.A.C method (1980). To prepare plates for chromatography, one line was scribed, 2 cm from the top of plate (running end) and another one was scribed, 2 cm from the bottom of the plate (spots line). Plates were activated by heating at 105 °C for 5 min in a hot air oven before spotting.

Standard of aflatoxins B_1, B_2, G_1 and G_2 (Sigma Chemical Co., Louis., Mo. U.S.A) were prepared by dissolving 1 mg of each in small volume of benzene-acetonitrile (98: 2) and then completed to 100 ml with the same solvent mixture. Each prepared solution contained 10-ug/ ml.

Dried extract was dissolved in 200 ul chloroform. Twenty ul of the extract were spotted on the TLC plate. Twenty ul from the standard solution of B1, B2, G1and G2 as well as from their mixture were also spotted on the TLC plate. Plates were developed in a jar 30x10 cm containing the running solvent system of chloroform: acetone: isopropanol: water (88:12:1.5:1 v/v) as described in A.O.A.C (1990) for

approximately 20 min in darkness. Plates were then dried using a commercial hand hair drier and examined under U.V (365 nm). The intensity of fluorescence produced in samples patterns was compared with that of the standard patterns for aflatoxins spotted on the same plate. Aflatoxins B1, B2 were detected as blue fluorescence and G1 and G2 as yellow green fluorescence. As confirmation test, plates were lightly sprayed with 50% sulfuric acid. The fluorescence in samples will turn into yellow, indicating the presence of aflatoxins.

Aflatoxin(s) were also extracted from maize grain samples after grounding 50 g grains and adding 250 ml aqueous methanol + 100 ml hexane + 4 g sodium chloride and blending at high speed for 1 min. Twenty-five ml of the aqueous methanol phase were carefully pipetted and then added to 25-ml chloroform in separating funnel. Steps mentioned before were then followed for extracting, detecting and identifying the aflatoxin(s).

7.3-Screening of *Fusarium* spp. for producing Zearalenone:

Nineteen *Fusarium* spp. isolated from maize ear samples (Collected from different locations) were tested for their ability to produce zearalenone (F-2) toxin in solid culture using the agar plug method described by Frisvad *et al.* (1989).

Seven-day-old culture of *Fusarium* spp. on yeast extract sucrose agar was used. An agar plug was cut by the aid of a flamed cork borer (inner diameter approx. 0.4 cm) from the center of the colony. The plug was removed with a flamed needle and the mycelium side of the plug was wetted with a drop of chloroform / methanol (2:1 by volume) and then touched the mycelium side to the TLC plate for few seconds. Plugs of *Fusarium* isolates and standard of zearalenone (250 ug/ml) were placed on a standard 20x20 cm pre-coated TLC nonfluorescence silica gel plate

and then the spots were let to dry. TLC plate was developed in toluene/ ethylacetate/ 90% formic acid (TEF) (5:4:1v/v/v). Running was occurred in a saturated jar, 30x10 cm. Developed TLC plate was viewed in dark light under short wave at 254nm and long wave except in high concentration. Fluorescence will appear as greenish-blue fluorescence as compared with that of the zearalenone reference.

7.4-Determination of zearalenone on grains produced by *Fusarium spp.*:

Detection of zearalenone in grain samples was done using the method described by Roberts and Patterson (1975). Twenty five g finely ground grains sample were shaken in 100 ml of acetonitrile and 4% potassium chloride (90:10 v/v) solution in a stoppered bottle for 30 minutes. The mixture was passed through Whatman No.41 filter paper then 50 ml of the filtrate were taken in 250 ml separating funnel to defat and extract with 50 ml isooctane. This step was done twice. The lower acetonitrile layer was shaken with 12.5 ml of distilled water in the separating funnel then zearalenone was extracted with 20 ml chloroform three times. The lower chloroform acetonitile layer was drained off through anhydrous sodium sulfate and collected in a beaker then transferred to dry in a water bath 60-70 °C and kept under –20 °C. Extracted zearalenone was separated by dissolving the ramining dry film in 200 ul chloroform then 20 ul of dissolved extract as well as 20 ul standard solution of zearalenone (250 ug/ml) were spotted on activated TLC plate.

Plates were developed in running solution toluene/ ethylacetate/ 90% formic acid (TEF)(60:30:10v/v/v). Solvent was allowed to move up to approximately 10 cm. Plates were air dried and then sprayed by saturated solution of aluminum chloride ($AlCl_3$) in 60% ethyl alcohol and heated in an oven at 105 °C for 10 min. Then plates were examined under U.V dark

light under short wave at 254nm. Fluorescence will appear as greenish-blue fluorescence as compared with that of the F-2 reference. Toxin was not visible under long wave UV light except in high concentration.

8- Quantification of mycotoxins:

Mycotoxins were Quantitatively determined according to A.O.A.C 1990. under UV light (365 nm). TLC plates were scanned with the help of densitometer. Identified sample peak area comparing with the standard spots was determined by densitometer as well as standard peaks.

Concentration of mycotoxins was calculated by the following equation:

$$ug / kg = \frac{(B . Y . S . V)}{(Z . X . W)}$$

B = average area of aflatoxin peak in identified sample.

Y = conc. of aflatoxin standard ug / ml.

S = ul spotted aflatoxin.

V = final dilution of extracted sample (ul).

Z = average area of aflatoxin peaks in standard aliquots.

X = ul of spotted sample extract.

W = g sample represent final extract.

9-Storage of maize grains:

Three way cross 310 (TWC 310) maize hybrid were collected at harvest time from Sakha Agricultural Experimental Station and used in this experiment. To artificially infest maize grains, aqueous spore suspension of *A.flavus* (at the concentration of $1x10^6$ ml) was added to maize grains to give final grain moisture content of 14%. Grains were subdivided into samples stored at three conditions of relative humidity

(RH); 45, 65, and 90 % and temperature; 8 °C, room temperature (17-27 °C) and 30 °C. as shown in Tables 9,10 & 11.

Grain samples were stored under the experimental conditions into water diffusing semi-perforated bags made of polyester. Twenty plastic containers measuring 14cm length x 105 cm^2 were prepared as storage cabinets (Fig.2). The containers were provided with small fan at the top of the storage cabinets for relative humidity homogenization and to avoid moisture accumulation. Circle pore, 2 cm^2 was made at the surface for measuring temperature and relative humidity of the inside. Containers were supplemented by double jacket made by zigzag paper measuring 10 cm length to make aerobic column and to keep the relative humidity constant around the grains. Relative humidity was implemented following the method adopted by Solomon (1951). Potassium hydroxide or sodium chloride in sterilized distilled water was used as follows:

90%RH (21.5 % aqueous solution of NaCl)

65%RH (41 % aqueous solution of KOH)

45%RH (60 % aqueous solution of KOH)

Three containers (RH 90, 65, and 45 %) were distributed and kept at each of the different degrees of temperatures (8°C, room temperature 17-27°C and 30°C). Readjustments were done to overcome the RH% fluctuation appeared due to the temperature used. Clothe bags containing 400 gm

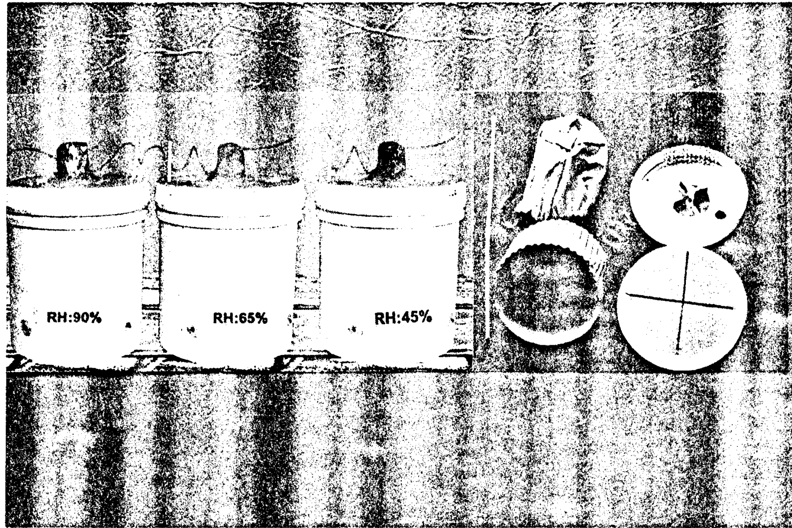

Fig. 1 Apparatus for storing maize grains under different conditions of RH (%).

inoculated and non-inoculated grains were put on nickel chrome net above the dehydration solution. Digital thermohygrometer was used for measuring RH% and temp. Dehydrate solution concentration was readjusted daily by adding distilled sterilized water for dilution or by adding absolute (x) volume concentration of dehydrate solution for increasing the concentration. Few drops of copper sulfate solution were used for avoid mold growth on liquid surface. Grains were withdrawn at 30, 60, 90 days and subjected to fungal isolation, test for grains viability as percentage of germination % and aflatoxins production as mentioned befor.

RESULTS

1-Description of ear rots of maize:

Several ear-rotting diseases were observed during the survey done throughout this work. The most prevalent ear rots Fig.3 can be described in the following:

1-Fusarium kernel or ear rot (pink rot):

This disease could be characterized by pinkish to reddish brown discoloration first appear on caps of individual kernels or some kernels scattering over the ear. Later on, a powdery or cottony pink mold growth develops on the infected kernels. Earworm or corn borers make avenues help in increasing the rots due to infection with the fungus responsible for the rot. The causal organism is *Fusarium moniliforme* Sheldon

2- Aspergillus ear rot:

Black or greenish-yellow or tan growth on and between kernels characterizes the infection with *Aspergillus flavus, A.niger & A.parasiticus.*The rot begin to appear at the tip of the ear and may follow along the tracks made by ear worms. The Kernels become light in weight, shrunken and uneconomic.

3- Penicillium ear rot:

Powdery greenish or bluish mold on and between kernels usually appear at the ear tips characterizes this disease. Initial infection occurs primarily on injured ears and kernels due to mechanical or insect injury. *Penicillium oxalicum, P.chrysogenum, P.glucum & P.notatum* are the most *Penicillium spp.* common on the ear and grain rots.

4- *Botryodiplodia* ear and kernel rot (black kernel rot):

This is characterized by the presence of the grayish white mycelium developing between the grains on the rotted ears. Dark olive green or

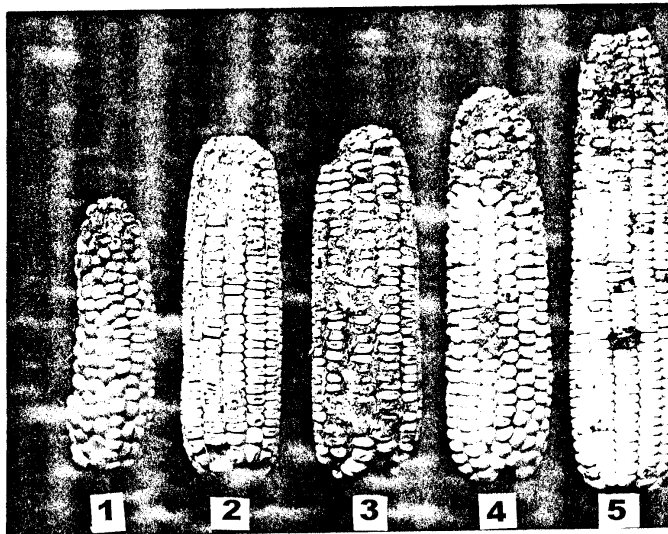

Fig. 2 Nautrually infected maize ears with:
1-*Botryodiplodia theobromae* 2- *Fusarium moniliforme*.
3- *Penicillium spp* 4- *A. flavus* 5- *Aspergillus niger*

brown to blak pycnidia appears later in the pericarp of rotted grains. The principal cause is *Botryodiplodia theobromae*.

Some other minor ear and kernel rots have been observed throughout investigating this work. They are cob-rot caused by *Nigrospora oryzae* and several species of *Aspergillus*.

2-Isolation and identification of casual agents:

Table 1, show the isolated fungi and their locations along with frequency. Results, indicate that *Fusarium* spp. were the most dominant fungi in all locations, in general giving infection mean of more than 80 %. *Aspergillus* spp. came after *Fusarium* in prevalence (average of 27.4 %). *Penicillium* spp. as well as *Botryodiplodia theobromae* and *Nigrospora* spp. were minors in their appearance compared with *Fusarium* and *Aspergillus*. The same trend in the frequency of the isolated fungi was found in the different locations (governorates).

Fusarium moniliforme ranked the first species in appearance within the biological species of *Fusarium* comparable to other isolates. This is correct in all locations under study. Concerning *Aspergillus* spp., it was found that *A.flavus* gave the higher level of frequency and then came *A.niger* and *A.parasiticus*. *Penicillium* spp. were obtained in low percentage from 2 % in samples of Gharbiya to 12 % in Nubarya giving 6.8 average overall localities. *Botryodiplodia theobromae* and *Nigrospora* spp. were obtained in the isolating plates in very low percentage of appearance. *Botryodiplodia,* however was observed only at 5% in samples of Nubarya and *Nigrospora* spp. was obtained from kernel samples of Kafr El-Sheikh, Qlubya and Nubarya, whereas, it never appeared on samples of Dakahliya, Gharbiya and Beni-Suef.

Table 1, Average number of fungi isolated from 100 grains of naturally infected maize ears at harvest.

Fungi	Fungal frequencies / Location						
	Dakahliya	Kafr El-Shiekh	Garbia	Qalubiya	Bani souf	Nobaria	mean
F. moniliforme	40.0	45.5	54.0	50.0	35.0	55.0	46.6
F. nivale	10.0	23.0	25.0	32.0	40.0	10.0	23.3
F. semitectum	3.5	8.2	2.0	0.0	0.0	2.0	2.6
F.solani	4.0	5.0	2.0	3.5	2.5	4.5	3.6
F. oxysporium	6.2	3.0	3.0	0.0	0.0	15.0	4.5
Fusarium spp.	63.7	84.7	86	85.5	77.5	86.5	80.65
A. flavus	10.5	14.0	40.0	8.0	15	42.0	17.6
A. niger	2.6	12.0	2.0	6.0	5	3.0	4.6
A. parasiticus	2.0	3.0	0.0	0.0	0.0	0.0	0.83
Aspergillus spp	15.1	29	42	14	19	45	27.35
Penicillium sp	11.7	7.0	2.0	4.0	4.0	12	6.8
B. theobromae	0.0	0.0	0.0	0.0	0.0	5.0	0.8
Nigrospora sp	0.0	2.0	0.0	3.0	0.0	2.5	1.25

Aflatoxin production was also determined semi quantitatively as described under Materials and Methods in subsamples from each of the kernel samples. It was found that amount of the total aflatoxins produced in these samples ranged between 0.0 and 25 ppb (0.0, 5, 10, 15, 20 and 25 ppb in samples of Qalyubya, Gharbiya, Dakahliya, Kafr El- Sheikh, , Beni Suef and Nubarya respectively).

3-Pathogenicity tests:

Results, Table 2, show that all *Fusarium* and *Aspergillus* isolates under study were pathogenic to maize ears under artificial inoculation, in general. As regards to *Fusarium* isolates, it was found that *F.moniliforme* gave the highest degree of infection to the inoculated ears followed by *F.semitectum*. Whereas, isolates of *F.nivale* gave the lowest degree of infection.

Table 2, Pathogenicity of *Fusarium* spp. and *A.flavus* isolates to maize ear of local cultivar (Boushi) artificially inoculated by silk channel method under filed conditions, at Shakha 1997.

No.	Fungus	Code No.	% infection	Location
1	*F. moniliforme*	S27	75.4	Dakahliya
2	”	S23	74.5	Kafr El-Sheikh
3	”	S49	74.5	Nubariya
4	”	S28	67.5	Dakahliya
5	”	S29	62.9	Nubariya
6	”	S21	62.5	Minoufiya
7	”	S17	61.5	Gharbiya
8	”	S53	42	Kafr El-Sheikh
9	”	S25	42	Qalubiya
10	”	S1	40	Kafr El-Sheikh
11	”	S90	46	Gharbiya
14	”	S20	39	Gharbiya
13	”	S95	38	Minoufiya
12	”	S51	24.7	Beni suef
15	”	S40	17.5	Qalubiya
16	”	S52	11.2	Beni suef
17	*F. nivale*	S3	38	Kafr El-Sheikh
18	*F. nivale*	S6	25.7	Dakahliya
19	*F. nivale*	S7	16	Beni suef
20	*F. semitectum*	S14	57.2	Dakahliya
21	*F. semitectum*	S13	43	Kafr El-Sheikh
22	*Aspregillus flavus*	D4	75.6	Dakahliya
23	”	D3	45	”
24	”	D2	39	”
25	”	K4	62	Kafr El-Sheikh
26	”	K3	40	”
27	”	K2	35	”
28	”	G1	59.5	Gharbiya
24	”	G2	50	”
25	”	G3	15	”
31	”	N1	69.5	Nubariya
32	”	N2	76	”
33	”	N3	45.5	”
34	”	Q1	22.4	Qalubiya
35	”	B1	85.6	Beni suef
L.S.D (0.05)			5.134	
C.V. 10.576 %				

Infection percent due to inoculation with *F.moniliforme* ranged between 11.2 and 75.4. Whereas, infection ranged between 43 and 57.5 & 16 and 38 % for *F.semitectum* and *F.nivale* respectively. The majority of isolates of *F.moniliforme* were aggressive to maize ears causing percentage of infection more than 40%, while 5 out of the 21 isolates were less pathogenic (gave infection from 11.2 to 39 %). These are: isolates No. S52 and S51from Beni Suef, S40, S95, S20 from Qalubiya, Minoufiya, Gharbiya. Infection percent due to inoculating maize ears with *F.nivale* was as low as 16-38 % comparing with those of *F.semitectum* (giving 43-57.5%).

Concerning isolates of *Aspergillus flavus,* results, Table 2, indicate that all isolates under study were aggressive to the inoculated maize ears where infection ranged from 22.4 to 85.6 %. The lowest isolate in virulence were G3 from Gharbiya, Q1 from Qalubiya and K2 from Kafr El-Sheikh giving 15, 22.4, and 35 % respectively. Isolate No.B1 from Beni-Suef, N2 from Nubariya and D4 from Dakahliya gave the highest degrees of infection (85.6, 76, and 75.6 % respectively).

4-Methods of inoculation with *F. moniliforme* and *A. flavus*:

Results, Tables 3&4, and Fig. 3&4 show that all inoculation techniques used in this experiment succeeded in damaging the inoculated ears with *F.moniliforme* and *A.flavus* under field conditions, generally. This is correct in both cultivars used, namely local variety (Baladi) and three way cross (TWC 310).

As regards to *F.moniliforme,* silk channel injection technique gave the highest degree of infection (30.4 & 80.7 % in TWC 310 & Baladi respectively). The rest methods of inoculations could be ranked descendingly for the three way cross TWC 310 as follows: ear-tip flooding, kernel stab, wound and spray, husks, and silk spray. Whereas,

they could be ranked descendingly for the Baladi variety (Bushi) as follows: wound and spray, husks, ear tip flooding, kernel stab, and silk spray.

As regards to *A.flavus,* silk channel method of inoculation caused the highest infection percent of the inoculated ears compared with the other methods of inoculation. This is correct in both maize cultivars used. The pinbar inoculation equally affected the ears as the silk channel method did to the Baladi variety (causing 28 %). In a dessending order, methods of

Table 3, Methods of artificial inoculation of maize ears with *F. moniliforme.*

Method of inoculation	**% infection**		
	TWC- 310	Bushi	Mean
Silk channel	30.4	80.6	55.5
Ear-tip flooding	16.5	25.7	21.1
Kernel stab	14.2	17.0	15.6
Husks	10.5	34.9	23.5
Wound and spray	12.2	38.7	25.4
Silk spray	6.3	10.3	8.3
Mean of treatments	15.28	34.5	
L.S.D (0.05)	1.179	2.0423	
L.S.D of Treat.A * B Means			2.8

Table 4, Methods of artificial inoculation of maize ears with *A. flavus.*

Method of inoculation	**% infection**		
	TWC- 310	Bushi	Mean
Silk channel	16.2	28.2	22.1
Pinbar inoculation	9.7	28.0	18.8
Side needle	8.9	25.5	17.2
Wound and spray	7.0	22.8	14.9
Tooth pick	5.6	19.9	12.8
Silk spray	4.5	12.5	8.4
Mean of treatment	8.6	19.7	
L.S.D (0.05)	0.77	1.34	
L.S.D of Treat.A * B Means			1.89

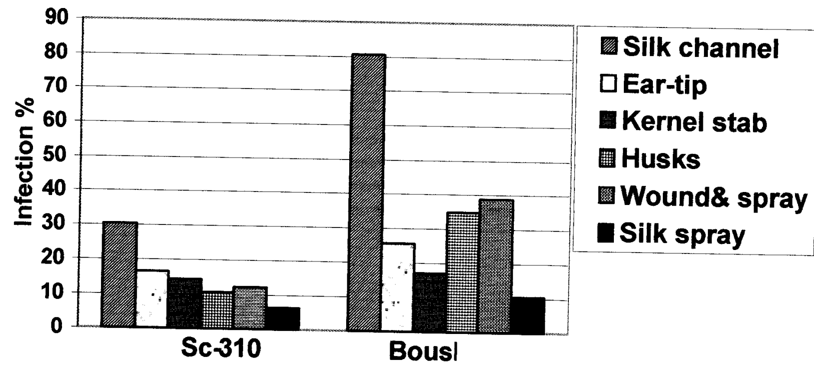

Fig.3, Infection % of artificial inoculated maize ears with *F.moniliforme* by different methods.

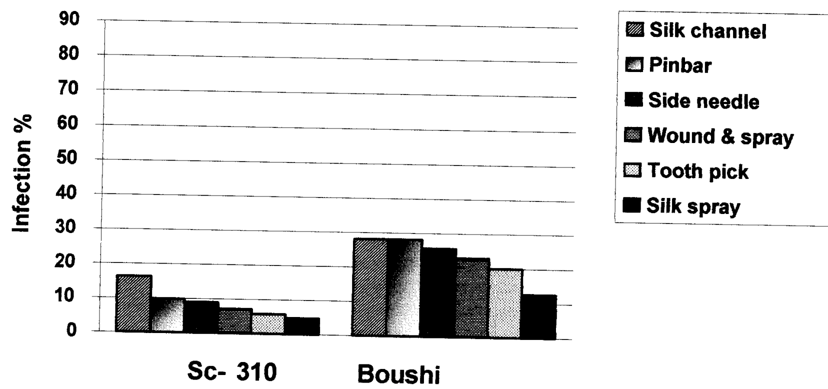

Fig.4, Infection % of artificial inoculated maize ears with *A.flavus* by different methods.

inoculations could be ranked according to their efficiency in injuring the inoculated ears as follows: silk channel, pinbar injection, side needle inoculation, wound and spray, tooth picks inoculation and silk spray technique.

5-Detection and determination of mycotoxins produced by *A.flavus* & *Fusarium spp.*:

5.1- Screening for mycotoxin production by the fungal isolates:

Zearalenone production by 16 isolates of *F.moniliuforme* and 3 isolates of *F.nivale* was screened following the agar plug method described by Frisvad *et.al.* (1989). No indication for zearalenone production was observed as shown in all isolates of *Fusarium spp.* under study as compared with the standard under the UV long and short waves (365 and 254 nm).

Using the UV (365 nm) BGY and following the fluorescence technique of Hara *et al.*(1974), blue fluorescence surrounding the colonies of *A. flavus* was observed in 9 out of the 14 isolates as shown in Table 6. This result indicates the probability of the presence of aflatoxin in their culture medium. They differed in the fluorescence intensity so that some isolates exhibited high intense fluorescence (+++ or ++++), whereas others showed low intensity (+ or ++).

5.2-Determination of aflatoxin produced by *A.flavus* isolates:

All of the 14 isolates of *A.flavus* were used to determine and quantify the aflatoxins produced in Czapeck-Dox broth following the method of A.O.A.C (1990).

Results, Table 5, and Fig. 5 show that aflatoxins B1 and/or B2 could be detected by this method in the filtrate of isolates. The aflatoxin B1 could be produced in different amounts from 160-550 ppb by 6 isolates of

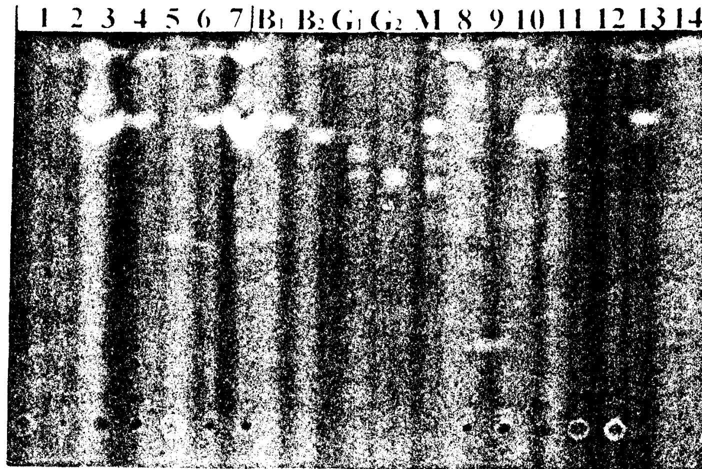

Fig. 6 Analysis of aflatoxin produced by *A.flavus* isolates on TLC plate.

A.flavus. Aflatoxin B2, however, could be detected and identified in the medium filtrate of these 6 isolates in concentrations differed from 40.5-300 ppb. Although blue fluorescence was observed while detecting for the aflatoxin production by the isolate no.1and no.2 from Dakahliya in rapid screening under UV, aflatoxin did not detect by the chemical method of identification. This indicated that no correlation was found between the light intensity and TLC analysis.

Table 5, Levels of of aflatoxin produced by *A.flavus* in Czapeck-Dox broth medium.

No.	Location	BGY	Aflatoxin level (ppb)		Total
			B1	B2	
1	**Dakahliya**	+	0.0	0.0	0.0
2	,,	+	0.0	0.0	0.0
3	,,	++++	550	300	850
4	**K. El-Shiekh**	+++	250	90.5	345.5
5	,,	-	0.0	0.0	0.0
6	,,	+	200	65.5	265.5
7	Gharbiya	+++	370	150	520
8	,,	+	0.0	0.0	0.0
9	,,	-	0.0	0.0	0.0
10	Nubariya	++	480	200	680
11	,,	-	0.0	0.0	0.0
12	,,	-	0.0	0.0	0.0
13	Beni-suef	+	160	40.5	200.5
14	Qalubiya	-	0.0	0.0	0.0

5.3-Evaluation of maize genotypes for susceptibility to *F.moniliforme* & *A.flavus* and mycotoxin production:

This trial was carried out by artificial infestation to field-grown maize cultivars at Sakha Agricultural Experimental Station in 1999. Silk channel inoculation technique was followed and 3 rows, 10 plants each were screened for each cultivar.

Table 6,Varietal susceptibility of 11 maize genotypes to infection with *F.moniliforme* and *A.flavus* under artificial inoculation, Sakha, 1999.

No.	Genotype	% infection	
		F.moiliforme	***A. flavus***
1	**TWC- 320**	8.6	11.6
2	**TWC- 321**	12.2	9.3
3	**TWC- 322**	18.6	10.5
4	**TWC- 323**	12.7	3.8
5	**TWC- 324**	17.1	10.1
6	**TWC- 310**	29.9	14.3
7	**SC-10**	7.7	3.3
8	**SC-123**	8.7	6.5
9	**SC-124**	10.6	4.4
10	**SC-129**	13.7	11.9
11	**SC-13**	40.5	17.6
L.S.D (0.05)		6.9	5.2
C.V.		23.7	32.3

Results, Table 6, indicate that screened maize cultivars differ in susceptibility to *F. moniliforme* and *A.flavus.* It was found that the single cross (SC10) gave the highest degree of resistance to both pathogens (7.7 and 3.3 % against *F. moniliforme* and *A.flavus* respectively). Whereas, the single cross (SC13) has the lowest degree of resistance (40.5 and 17.6 % against *F. moniliforme* and *A.flavus* respectively). The rest of genotypes distributed in between these to cultivars in this respect. Three out of the 11 cultivars showed good degree of resistance to *F.moniliforme.* They are: TWC 320, SC10 and SC123 (8.6,7.7 and 8.7 % respectively). Whereas, 5 out of them exhibited the resistance to *A.flavus.* They are: TWC 321, TWC 323, SC 10, SC123 and SC 124 (9.3, 3.8, 3.3, 6.5 and 4.4% respectively).

Evaluation of maize genotypes to kernel contamination with aflatoxins and zearalenone following the artificial ear inoculation with *A.flavus* and *F.moniliforme* was done. Plants were inoculated with the tested fungi under field conditions at Sakha Experimental Station.

Results, Table 7 & Fig. 6 show that *F.moniliforme* isolate did not produce zearalenone in all maize genotypes under study. Whereas, 5 out of 11 hybrids have been contaminated with aflatoxins produced by *A.flavus*. These are three way crosses; TWC 320, TWC 322 and TWC 310, as well as single crosses; SC129 and SC13. Total levels of the detectable aflatoxins in grains of maize genotypes ranged between 3.0 ppb grains of TWC 322 and 250 ppb grains of SC-13. The aflatoxin B1 could be detected in grains of inoculated ears of most tested maize genotypes, but the aflatoxin B2 was detectable only in 2 of them, namely TWC 320 and SC 129.

Table 7, Levels of aflatoxins produced by *A.flavus* into kernels of artificially inoculated ears of different maize hybrids, Sakha 1999:

No.	Maize genotype	Aflatoxin. (ppb)		Total Aflatoxins
		B1	B2	
1	TWC-320	10	2.0	12
2	TWC-321	0.0	0.0	0.0
3	TWC-322	3.0	0.0	3.0
4	TWC-323	0.0	0.0	0.0
5	TWC-324	0.0	0.0	0.0
6	TWC-310	220	0.0	220
7	SC-10	0.0	0.0	0.0
8	SC-123	0.0	0.0	0.0
9	SC-124	0.0	0.0	0.0
10	SC-129	170	25	195
11	SC-13	250	0.0	250

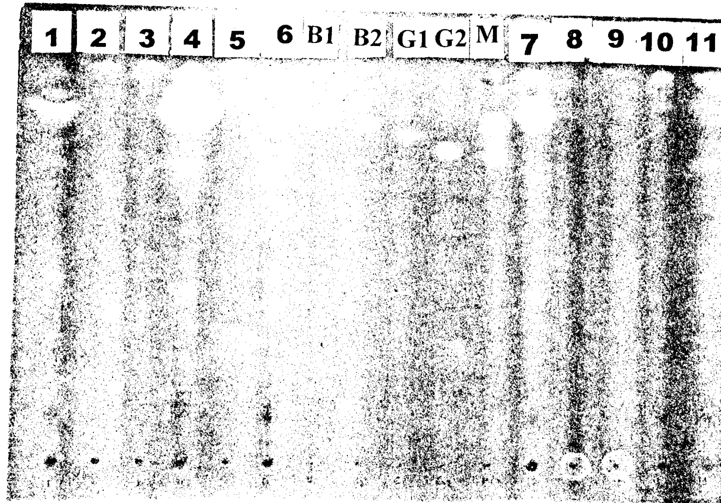

Fig. 6, Analysis of aflatoxin extracted from grains of: 1,TWC-310; 2, SC-13; 3, SC-129, TWC-320 & TWC-322 maize genotypes artificially inoculated with *A.flavus*.

6-Storage of maize grains under different conditions of temperature and relative humidity:

Effect of storage on frequencies of *A.flavus* and contaminating fungi, kernel viability, and aflatoxin production was studied at monthly intervals starting from one month after storage.

6.1-Isolation and kernel viability study:

Isolations were made to the artificially and naturally infested grains and obtained results can be summarized as follows:

At 8 °C:

It was found that number of the recovered isolates of *A.flavus* was increased by increasing the relative humidity from 45 to 90 %, whether under natural or artificial infection (Table 8). An obvious increase was observed in the frequency of the appearance of *A.flavus* by increasing the storage periods from one to two months, whereas, slight increase was observed by increasing the storage period from 2 to 3 months, generally.

As regards to the contaminating fungi that recovered along with *A.flavus,* some of other fungi were isolated while executing this study (Table 8). Some of them were recovered in so little numbers that they were negligible. *A.niger, Fusarium spp. and Penicillium spp.* were the most common fungi that accompanied *A.flavus* on the kernel samples.

A.niger was found to contaminate maize grain samples under natural infection, but never been observed contaminating the inoculated grains. This fungus was found to be increased in the non-inoculated grains by increasing the RH and increasing period of storage up to three months except at 45% RH.

Frequencies of *Fusarium spp.* and *Penicillium spp.* were increased by increasing RH and period of storage in both types of samples, i.e. inoculated and non-inoculated grains. *Fusarium spp.* prevailed than the other fungi in all treatments, in general.

Grains were subjected to germination test after the end of each period of incubation. Results, Table 8, indicate that increasing the RH% caused decrease in the viability of stored grains, generally. But the effect of RH was higher in the *A.flavus*-inoculated grains. Also, increasing period of storage affected the grain germination in inoculated samples more than in the control. Grain moisture content (MC) was decreased at the end of the storage period, due to storage under the conditions of 45 % and 65 % RH.

At room temperature:

At room temperature (17-27°C) 22 in average, *A.flavus* was recovered from samples of both inoculated kernels and the control. Data presented in Table 9, show that *A.flavus* was found in higher frequencies in inoculated samples than in non-inoculated ones. It was found also that this fungus was observed to be increased by increasing the RH % and by increasing period of storage whether in inoculated or non-inoculated kernels.

A.niger started to appear in the inoculated sample grains under room temperature. Frequencies of all of the contaminating fungi were increased by increasing the RH as well as period of storage, in general. It was found also that *Fusarium* spp. appeared in high frequencies followed by *Penicillium* spp. in all treatments.

Concerning the viability of the stored kernels, it was found (Table 9) that germination was reduced consistently by increasing the RH and incubation periods, generally. The germination was obviously reduced in the inoculated samples more than in the control grains so that it reached 0.0 % at 65 and 90 % RH after three months of storage. Grain moisture content was obviously increased by increasing period of storage and the difference was clear in the non-inoculated grains compared to the inoculated ones.

At 30 C:

Results Table 10, indicate that *A.flavus* was recovered from kernel samples of both inoculated as well as the non-inoculated ones in higher frequencies compared with its appearance at 8 °C and room temperature. The increase of this fungus was higher in inoculated samples than in the control ones. Frequencies of appearance was increased also by increasing period of storage and increasing the relative humidity around grains in both *A.flavus*-inoculated and the control samples.

As regards to the contaminants, it was found that *Penicillium spp.* and *A.niger* prevailed in the grain samples whether in the inoculated or the non-inoculated ones. From the other hand, *Fusarium.spp.*was observed in lower frequencies compared with the other contaminants and also were found in lower frequencies if compared with the other temperatures of study. Generally, all contaminating fungi were increased in samples by increasing period of storage and RH.

As regards to the viability of the stored grains, germination was observed to be decreased by increasing RH% and period of storage. The bad effect on the kernel viability was higher in the inoculated samples than in the control samples so that the germination decreased to 0.0 % at 65 and 90% RH after three months of storage. The reduction of germination was drastically higher at 30 °C than at the other temperatures of study. Grain moisture content was greatly increased by prolonging period of storage at 30°C compared with the other temperatures of inoculation (28% after storage of the inoculated grains at 90% RH).

Table 8, Effect of storing maize grains inoculated with *A.flavus* and period of storage under 3 degrees of relative humidity (RH) on fungal frequencies (%), moisture content (MC) and viability (Germination) of grains, at 8°C.

	RH					
	45 %		65 %		90 %	
	Inoc.	Non.	Inoc.	Non.	Inoc.	Non.
			After one month			
Aspergillus flavus	13.5	3.5	16.5	8.5	23.5	17
A. niger	0.0	4.5	0.0	9.5	0.0	10
Fusarium spp	9.0	8.5	13.7	10.5	19.0	15
Penicillium spp	5	3.5	5.6	10.5	6.0	12
MC	14	14	15.4	14.4	15.8	15
Germination %	85	90	82	86	76	80
			After two months			
Aspergillus flavus	17	4	25	10.5	30	19
A. niger	0.0	6	0.0	11	0.0	12
Fusarium spp.	13.7	10	19.5	16	20.5	17
Penicillium spp.	6.5	5	10.6	12	8.9	16.5
MC	14.2	14	14.8	14	16	15.2
Germination %	82	85	79	80	63	80
			After three months			
Aspergillus flavus	17	4	25.9	11	34	20
A. niger	0.0	6	0.0	16.0	0.0	18
Fusarium spp.	17.8	14	20.8	19.5	25	24
Penicillium spp.	10.2	5	12.5	14	22	20
MC	11	9	12	11	18.5	16.8
Germination %	76	85	68	80	52	80

Table 9, Effect of storing maize grains inoculated with *A.flavus* and period of storage under 3 degrees of relative humidity (RH) on fungal frequencies, moisture content (MC) and viability (Germination %) of grains at room temprature.

	RH					
	45%		65 %		90 %	
	Inoc.	Non.	Inoc.	Non.	Inoc.	Non.
	After one month					
Aspergillus flavus	20	17.5	32	20	38	24.5
A. niger	2	3	3.0	8	5.6	9
Fusarium spp.	18	16	20	25	24	29
Penicillium spp.	7	5.8	7.8	12.5	19.5	15
MC	16.5	13.5	17.9	14.3	18.7	16
Germination %	82	90	80	87	70	83
	After two months					
Aspergillus flavus	23	19	34	23	40	30.9
A. niger	8.9	10	18	20	9.7	6.5
Fusarium spp.	20.9	18	24.9	26	29.5	32
Penicillium spp.	14.5	5.9	10.9	15	20	16
MC	16.9	13.9	18.5	16.5	26	22
Germination %	65	85	60	70	40	45
	After three months					
Aspergillus flavus	26.9	20	39.5	25	47.8	38.5
A. niger	9.2	19	16	16	10	12.9
Fusarium spp.	24	20	27	30	35	38
Penicillium spp.	13	5	12.8	17.9	19.5	20.4
MC	18.5	16.8	26	16.9	28.5	26
Germination %	46	65	0.0	60	0.0	45

Table 10, Effect of storing maize grains inoculated with *A.flavus* and period of storage under 3 degrees of relative humidity (RH) on, fungal frequencies, moisture content (MC) and viability (Germination %) at 30 °C.

	RH					
	45 %		65 %		90 %	
	Inoc.	Non.	Inoc.	Non.	Inoc.	Non.
	After one month					
Aspergillus flavus	27.5	20	36.5	24.5	52	30
A. niger	8	7.4	11	14	10	16
Fusarium spp	6	9	10	12	14	16
Penicillium spp	15	17	16	20	18	25
MC	16	14	18.5	14.5	20.5	15
Germination %	75	80	60	80	50	65
	After two months					
Aspergillus flavus	30	25	39.5	27.3	58	36
A. niger	9	9.2	13	10	10	16
Fusarium spp	8	10	13	14	15	16
Penicillium spp	18	19	24	20	25	27
MC	18	14	23	13.5	27	20.5
Germination %	45	70	45	65	35	40
	After three months					
Aspergillus flavus	39	28	52	30	75	40
A. niger	11	10	14	16	13	18
Fusarium spp	10	12	15	15	16	25
Penicillium spp	25	20	30.5	25.8	39	28
MC	15.5	14	18	15.2	28	22
Germination %	38	68	0.0	59	0.0	28

6.2-Effect of storage on production of aflatoxins:

Grain samples were taken periodically after each period of storage and aflatoxin B1 and B2 were determined in grains of inoculated and non-inoculated kernels as described by A.O.A.C (1990). Results presented in Table 11, and Fig. (7&8) show that no aflatoxins were detected in any of inoculated or non-inoculated grain samples at 8 °C except at 90% RH after 3 months of storage in very low level (2.5 ppb of B1) in inoculated grains.

At room temperature, results (Table 12 and Fig. 7&8) indicate that no aflatoxins were detected in the control samples at any degrees of RH except at 90 % after three months where low amount of B1 was detected (9.45 ppb). Aflatoxins could be detected in all of the inoculated samples and were increased by increasing the RH up to 90% and prolonging the period of storage up to 3 months.

At 30 °C:

Results, Table 13, and Fig. (7&8) show that aflatoxins were detected in the non-inoculated grain samples at 65 % RH starting from 2 months of storage (12 ppb). The amount of aflatoxins produced in the control samples was very low comparable to that in the inoculated samples. Level of aflatoxin production was increased clearly by increasing the RH up to 90% and period of storage up to 3 months. It was found also that the level of aflatoxin B1 was greatly higher than B2. Aflatoxin was produced in higher amounts at this degree of temperature than in the other degrees *i.e.* 8 °C and room temperature.

Table 11, Effect of storing maize grains inoculated with *A.flavus* and period of storage under 3 degrees of relative humidity (RH) on aflatoxin production (ppb) at 8 °C.

	RH %					
	45 %		65 %		90 %	
	Inoc.	Non.	Inoc.	Non.	Inoc.	Non.
	After one month					
Aflatoxin B1	0.0	0.0	0.0	0.0	0.0	0.0
Aflatoxin B2	0.0	0.0	0.0	0.0	0.0	0.0
Total Aflatoxins	0.0	0.0	0.0	0.0	0.0	0.0
	After two months					
Aflatoxin B1	0.0	0.0	0.0	0.0	0.0	0.0
Aflatoxin B2	0.0	0.0	0.0	0.0	0.0	0.0
Total Aflatoxins	0.0	0.0	0.0	0.0	0.0	0.0
	After three months					
Aflatoxin B1	0.0	0.0	0.0	0.0	2.5	0.0
Aflatoxin B2	0.0	0.0	0.0	0.0	0.0	0.0
Total Aflatoxins	0.0	0.0	0.0	0.0	2.5	0.0

Table 12, Effect of storing maize grains inoculated with *A.flavus* and period of storage under 3 degrees of relative humidity (RH) on aflatoxin production (ppb) at Room temprature.

	RH %					
	45%		65 %		90 %	
	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.
			After one month			
Aflatoxin B1	0.0	0.0	0.0	0.0	29.8	0.0
Aflatoxin B2	4.5	0.0	12.5	0.0	10	0.0
Total Aflatoxins	4.5	0.0	12.5	0.0	39.8	0.0
			After two months			
Aflatoxin B1	15	0.0	110	0.0	245	0.0
Aflatoxin B2	0.0	0.0	15	0.0	25	0.0
Total Aflatoxins	15	0.0	125	0.0	270	0.0
			After three months			
Aflatoxin B1	17	0.0	127	0.0	420	9.5
Aflatoxin B2	0.0	0.0	5	0.0	6.5	0.0
Total Aflatoxins	17	0.0	132	0.0	426.5	9.5

Table 13, Effect of storing maize grains inoculated with *A.flavus* and period of storage under 3 degrees of relative humidity (RH) on aflatoxin production (ppb) at 30 °C.

	RH %					
	45 %		65 %		90 %	
	Inoc.	Non.	Inoc.	Non.	Inoc.	Non.
	After one month					
Aflatoxin B1	13	0.0	16.1	0.0	40	0.0
Aflatoxin B2	0.0	0.0	3.5	0.0	5.5	0.0
Total Aflatoxins	13	0.0	19.6	0.0	45.5	0.0
	After two months					
Aflatoxin B1	45	0.0	115	12	378	30
Aflatoxin B2	5	0.0	20	0.0	30	0.0
Total Aflatoxins	50	0.0	135	12	408	30
	After three months					
Aflatoxin B1	151.2	18	410	0.0	449	450
Aflatoxin B2	4.3	0.0	15	20	76.8	35
Total Aflatoxins	155.5	18	425	20	525.8	485

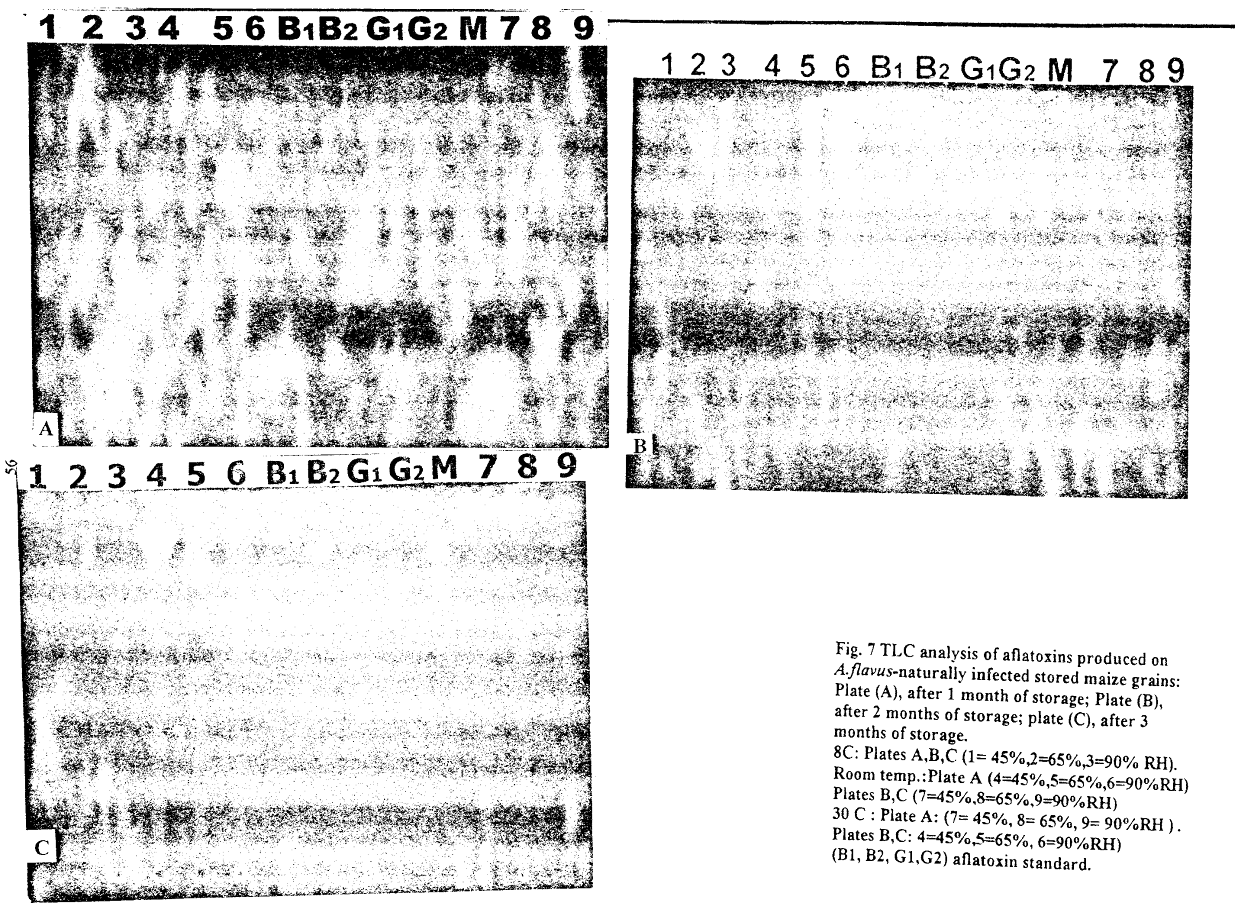

Fig. 7 TLC analysis of aflatoxins produced on *A.flavus*-naturally infected stored maize grains: Plate (A), after 1 month of storage; Plate (B), after 2 months of storage; plate (C), after 3 months of storage.
8C: Plates A,B,C (1= 45%,2=65%,3=90% RH).
Room temp.:Plate A (4=45%,5=65%,6=90%RH)
Plates B,C (7=45%,8=65%,9=90%RH)
30 C : Plate A: (7= 45%, 8= 65%, 9= 90%RH).
Plates B,C: 4=45%,5=65%, 6=90%RH)
(B1, B2, G1,G2) aflatoxin standard.

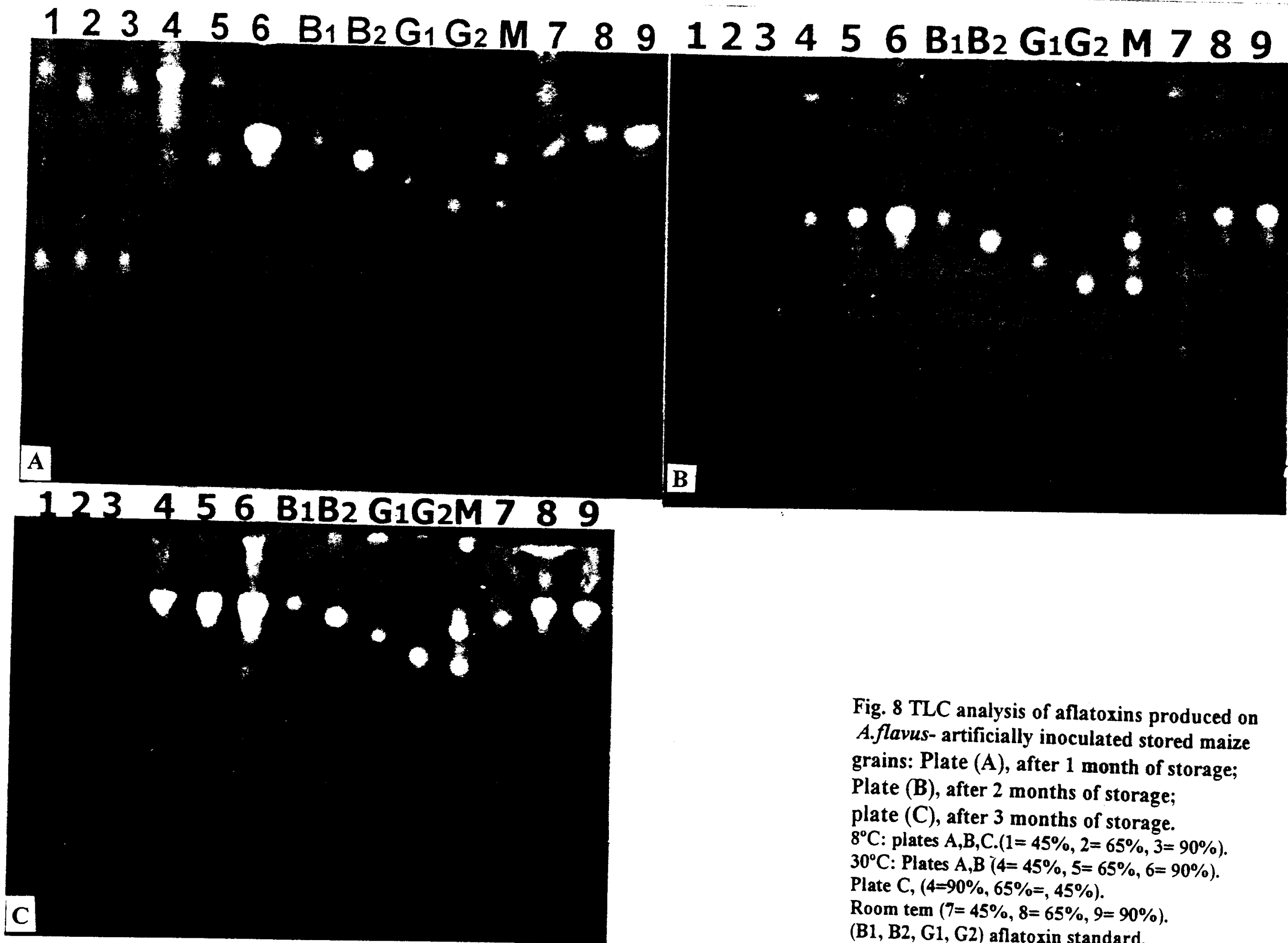

Fig. 8 TLC analysis of aflatoxins produced on *A.flavus*- artificially inoculated stored maize grains: Plate (A), after 1 month of storage; Plate (B), after 2 months of storage; plate (C), after 3 months of storage.
8°C: plates A,B,C.(1= 45%, 2= 65%, 3= 90%).
30°C: Plates A,B (4= 45%, 5= 65%, 6= 90%).
Plate C, (4=90%, 65%=, 45%).
Room tem (7= 45%, 8= 65%, 9= 90%).
(B1, B2, G1, G2) aflatoxin standard.

DISCUSSION

Fungal genera survey was done throughout this investigation showed that *Fusarium spp.* was the major fungal population that could be recovered from the rotted maize ears. *F.moniliforme* the new name is *F.verticilloides* Sacc. according to Nirenberg and O`Donnell (1998). Was the most common isolate belonging to this biological species (average 46.6 %) followed by *F.nivale* which appeared in grain samples at the average of 23.3 %. *F.semitectum, F.solani & F.oxysporum* were recovered from kernel samples at very low frequencies (from 2.6 to 4.5 %). *F.moniliforme* is the most prevalent incitant of kernel rots of maize (Nelson *et al.*1983). King (1981) stated that *F. moniliforme* appeared to be the first colonist of the pre-harvested maize ears infecting kernels before other molds. *Aspergillus spp.* have appeared in kernel samples in the average of 27.4 % allover the governorates under study. *A.flavus* prevailed causing damage to grains following the damage caused by *F.moniliforme* (average 17.6 %). *A.flavus* is the most predominant species on maize (Payne, 1999). It is expected for the maize ears damaged by *Aspergillus spp.*which contribute a high portion of most serious rots in geographical locations with high temperatures and drought environments. *A.flavus* as well as *A.parasiticus* are the only species that produce aflatoxins. Payne *et al.*(1988) reported that *A. flavus* can readily infect the developing maize kernels and the parasitic ability is enhanced at high temperature. *A.niger* and *A. parasiticus* were found to appear in very low frequencies compared to *A. flavus* indicating the minor role of these two species in ear rot. *Penicillium spp.,* from the other hand, were recovered in 6.8 %. It is known that *Penicillium* rot occurs primarily on ears mechanically injured and several species of *Penicillium* could be isolated from maize kernels causing economical losses at harvest. *Botryodiplodia theobromae* and

Nigrospora spp. were isolated from the affected kernels at very low frequencies indicating their minor role in producing the ear and kernel rots in the field. Diab *et al.* (1984) stated that *B.theobromae* infects maize ears under natural infestation at levels ranged from 0.0 % in Upper Egypt and 4.0 % in Kafr El-Sheikh (lower Egypt).

Following the silk channel inoculation technique, field pathogenicity tests indicated that all 16 isolates of *F.moniliforme* were pathogenic to maize ears giving percentage of infection from about 11% (isolate no. S52 from Beni Suef) to 75.4 % (isolate no. S27 from Dakahliya). *F.moniliforme* is the most pathogenic species of section *lesiola* that infect maize, generally. Nelson, (1992) stated that *F.moniliforme* is the species isolated most frequently from shelled maize and it is not uncommon to have seed lots of maize that show 100 % infection. Koehler (1959) stated that this fungus is the most frequent cause of ear rot among the *Fusarium spp.* The same author (1942) reported that *F.moniliforme* enters more ears than any other fungus, even when ears are covered by husks and free from bird or worm damage. Silk channel method of inoculation was followed in executing work throughout this investigation since it was the most reasonable way to introduce *F.moniliforme* at silking the fungus appears to enter through the silks, grow over the pericarp and then into the kernel at the hilar end. (Koehler, 1942 and Warren, 1978). These findings are in harmony with those found by Munkvold *et al.* (1997) who stated that when plants were silk-inoculated with *F. moniliforme*, infection percent reached up to 83.7.

Aspergillus flavus isolates infected maize ears artificially by inoculating silk channel resulting a high percentage of infection varied from 15 % (isolate no. G3 from Gharbiya) to 85.6 % (isolate no. B1 from Beni Suef). It is known that *A.flavus* causes serious damages to maize ears and kernels in geographical locations with high temperatures and drought

environments (Payne *et al.* 1988 & Payne, 1999). Drought stress was reported to predispose maize ears to infection with *A.flavus* (Jones and Duncan, 1981). Results obtained from this study indicated that more than one half of the infected ears were damaged by artificial inoculations of ears. This observation agree with the findings obtained by (Zummo and Scott, 1990a) who could recover *A.flavus* for relatively high percentages (over 83%) of cobs of inoculated ears of the maize genotypes that evaluated for their susceptibility to infection with this fungus.

All methods of inoculations used in the present study succeeded in producing damage to ears inoculated with *F.moniliforme* and *A.flavus* with significant differences between them. Silk channel technique gave the highest degree of infection if compared with the other methods of inoculation. Whereas, the pinbar method of inoculation equally cause the same damage to inoculated ears with *A. flavus* as the silk channel did. Silk spray method of inoculation caused least degree of infection to ears comparable to other investigated methods. Silk channel technique has been recommended as a reliable method of artificially inoculating maize ears by many investigators to evaluate maize genotypes against ear rotting fungi (Chungu *et al.*, 1996; Munkvold *et.al.*, 1997; Munkvold and Carlton, 1997 and Reid *et al.*, 1992). Jones *et. al.*(1980) was the first who extensively studied silk infection as a mode of entry for the fungus *A.flavus*. They concluded that *A.flavus* can colonize maize silks and invade developing kernels. Since the logic entry of both *Fusarium* and *Aspergillus spp.* is ear silks, the silk channel technique of inoculation gave the best results in artificial inoculations by these two pathogens. This hypothesis is supported by the work done by Wicklow and Donahue (1984) and Chungu *et al.* (1996).

Studying mycotoxin production by both *Fusarium spp. & A.flavus* was done in synthetic media. Production of aflatoxins was detected visually under UV black light and quantitatively following the BF method in the culture filtrate of *A. flavus* grown in Czapeck-dox broth. Nine out of the 14 isolates could produce different intense fluorescence under UV (+...to ++++). Eight out of these isolates, however produced detectable amounts of aflatoxins B1 and/or B2 when quantitatively analyzed after separating on TLC. It was found also that isolate No.1 recovered from grain samples of Dakahliya could give visual fluorescence under UV, but no aflatoxin was determined in the culture filtrate by BF method. Since other fungal difusable metabolites may produce similar fluorescence under UV light, fluorescence does not always indicate the presence of aflatoxins (Kaneko and Sanada, 1969). Aflatoxin B1 together with aflatoxin B2 the dihydro derivative of aflatoxin B1 (Asao *et. al.,* 1965). were detected in the culture broth of these isolates. This explains the presence of both aflatoxins together in the culture filtrate of the majority of *A.flavus* isolates under study. Total amount of aflatoxin produced in the *A.flavus*-culture filtrates was found to differ from isolate to isolate. These results could be explained that the production of aflatoxin by *A.flavus* is a function of the environmental conditions (Jarvis, 1971). Thus, Keyl *et al.* (1970) stated that aflatoxin production is the result of interaction of the genotype of a strain and the environment in which is it growing. Significant variations in toxigenesis have been noted depending also on the substrate from which the fungus has been isolated and its geographic origin (Boller and Schroeder, 1966) and production vary quantitatively from culture to culture (Hesselting *et al.,* 1966).

The mycotoxin; zearalenone could not be detected in cultures of all isolates of *F.moniliforme* under study. Although some previous investigators reported that some isolates from this species could produce

detectable amounts of this substance in synthetic cultures and on the commodity they worked on (Mirocha *et al.,* 1969, Hacking *et al.,* 1976 & 1977) Gangobadhyay & Chakrabarti. (1981) found a toxin extracted from *F.moniliforme* cultures isolated from rice with hexane and diethyl ether yielded light yellow crystals which were considered to be very close to zearalenone thought not exactly the same. Negative results for the production of zearalenone by isolates of *F.moniliforme,* from the other hand have been reported by several other authors (Caldwell *et al.,* 1970, Eugenio *et al.,* 1970, Suzuki *et al.,* 1978 & 1981). Marasas *et al.* (1984) on their intense work on this subject, could not find any detectable amount of zearalenone produced by a number of 100 isolates that were previously reported to produce this substance. It is well known that *F.moniliforme* can produce several mycotoxins other than zearalenone, so the author would suggest that the danger of this pathogen may refered to some other toxins rather than zearalenone such as fumonisin; the newest type of mycotoxins discovered lately, that has been identified from cultures of *F.moniliforme* and found to have cancer promoting activity as reported by Gelderblom *et al.* (1988, 1993).

Studying the varietal susceptibility of field grown maize genotypes to infection with both species of fungi *i.e. A.flavus* and *F.moniliforme* under study by artificial inoculations was carried out. Three out of the 11 cultivars screened were found to be resistant to infection with *F.moniliforme,* whereas, 5 out of them showed to have resistance background to *A. flavus.* Single cross (SC10); which has high yield potentiality showed a good degree of resistance to both fungi (3.28 & 7.7 % for *A.flavus* &. *F.moniliforme* respectively). Severity of infection of maize ears of different genotypes with the rotting fungi is greatly depending on some factors. The husk layer has been implicated, for instance as a factor in the susceptibility of maize hybrids to infection by

F. moniliforme. Kommedahl and Windels (1981) reported that the percentage of infected kernels was lower when the tips of developing ears were covered by the husks, compared to ears with exposed tips. Therefore, the resistance of single cross (SC10) maize hybrid to infection found throughout this investigation may be explained by its tight complete husk characterizing this cultivar. Unlike findings of Payne, *et al.* (1986), results in this investigation showed no correlation between infection percent of maize ears with *A.flavus* and aflatoxin production. Presence of two fractions from resistant maize seeds inhibitory to aflatoxin formation was suggested by Huang *et al.* (1997) as a direct protection against infection and aflatoxin production. One fraction inhibited aflatoxin accumulation, and the other inhibited aflatoxin formation with little effect on fungal growth. The compounds responsible for these activities appear to be proteacious. The presence of these compounds in the Tex6 (a maize inbred shown to be highly resistant to aflatoxin accumulation) was used to explain its resistance to aflatoxin accumulation (Huang *et al.*, 1997). An indirect protection was also reported by Lisker and Lillehoj (1991) as seed provided by husks cover and antinutritional substances in silks play an important role in protecting ears and seeds from infection with ear rotting fungi. This may explain the resistance of the single cross 10 maize hybrid ; the most popular and high yielding maize hybrid in Egypt along with other studied hybrids to infection with these type of fungal invadors.

As regards to the effect of storage conditions on the storing kernel, some factors affecting the interior grain components expressed as viability of the stored grains along with aflatoxin production into the affected grains and development of associated fungi after three periods of storage were studied. These are 3 different relative humidities under 3 different temperatures. Results indicated that these factors affected significantly in most cases the stored grains. Temperature and incubation

period along with relative humidity and grain moisture content have been recognized as important factors affecting mold growth and subsequent toxin production, generally. Majunder *et al.* (1965) classified *A.flavus* as a thermophilic fungus which has optimum growth temperature between 36 and 38°C. Results obtained throughout this work showed that the frequencies of *A.flavus* isolated from stored grains were generally higher when grains were stored at the highest temperature, *i.e.* 30°C compared with the other temperatures studied. This was affected by the period of storage and the relative humidity (RH %). The higher RH % prevailed in the storage, the higher degree of the fungal growth was obtained. Prolonging the storage period affected positively the fungal growth under these conditions. As mentioned by Majunder *et al.* (1965), there is positive correlation between the fungal growth and the aflatoxin production. Increasing temperature of storage caused an increase of the contaminating fungi, in general. As the number of the frequencies of appearance of *Penicillium spp. & A.niger* were found to be increased due to the increase of temperature. These fungi are known as thermophilic organisms that could resist the higher degrees of temperature as mentioned by Jones *et. al.* (1980). As members of *Fusarium spp.* are mesophilic fungi, their frequencies were adversely decreased by increasing temperature up to 30°C. These results are in conformity with those obtained by Gonzalez *et al.* (1988) who stated that *Fusarium spp.* grow in good rate on artificial media at temperature ranged between 20 and 25°C. The optimum temperature found throughout this work for recovering this type of fungi *i.e. Fusarium spp.* was room temperature (17-27°C). Fungi under study were also influenced by RH and period of storage. These results are correct in both the *A.flavus*-inoculated grain samples as well as the non-inoculated ones. An obvious difference was found between the recovered contaminating fungi in inoculated and non-

inoculated samples. It was observed that number of isolated contaminants was always higher in the control samples than in the *A.flavus*-inoculated samples in most cases. This is may be due to the competition between *A.flavus* and other isolated fungi in inoculated samples. Frequencies of appearance of *A.flavus* were always higher than that of *A.niger* under all circumstances and it was always higher than all other fungi. Viability of stored kernels were found to be affected at all storage conditions where germination was drastically affected at the highest degree of temperature i.e. 30°C after 3 months of incubation. Percentage of germination was decreased to almost 0.0 in the inoculated samples at 65 and 90 % RH. Inhibition of seed germination due to storing grains for long period was discussed. The effect on germination is influenced by seed moisture content, storage period, storage temperature and other factors (Ichinoe and Kurata, 1983). As it is known that seed (grain) deterioration increases when moisture and temperature conditions are favorable for microorganisms, germinability was observed to be decreased by increasing the period of storage that increased in turn the number of associated fungi. As the grain moisture content is always in equilibrium with the relative humidity, increasing RH caused an obvious reduction in the grain germinability as the moisture content was adjusted in grains to 14 % at the beginning of this experiment. Prolonging the period of storage caused drastical reduction in the grain germination at the highest degree of RH (90 %). Great losses in germinability was found by Fields and King (1962) working on the germination of corn seeds affected by some storage fungi. They found that germination capacity was decreased by keeping seeds at 90% RH and 30°C within six months when invaded by *Aspergillus spp.& A.flavus.*, whereas non-infected seeds maintained a germination percent of 95 %.

Aflatoxin production was influenced by storage conditions. Temperature, RH, grain moisture content and incubation time have been recognized as important factors affecting mold growth and subsequent toxin production as reported by several investigators (Gwinner *et al.*, 1990; Agarwal and Sinclair, 1996).
Results, revealed that inoculating maize grains with *A.flavus* caused an increase in the amount of aflatoxins when grains were stored at 30°C and 65 or 90 % RH. This result is consistent with findings of Faraj *et al.* (1991) who stated that the highest level of aflatoxin is produced by *A.flavus* at 30°C and 95 % RH. Also, Jones (1979) suggested that high temperature (30°C and more) and high RH (above 85%) favored maize infection by *A.flavus* and accounted for the higher incidence of aflatoxin.
Moisture content of grains was increased due to the growth and respiration of fungi in the inoculated rather than the non-inoculated grains, which influence in turn the aflatoxin production in the inoculated grains particularly by prolonging the storage period.

It could be concluded from work done throughout the present investigation that maize grains should be stored under good conditions that retard or prevent invasion by storage fungi. Most toxigenic *Aspergilli* do not grow at 4°C (Mislivec, 1981). Relative humidity of less than 65 % inhibited growth of storage fungi (Christensen and kaufmann, 1965). Moisture content in grains must be lessened to less than 14 % to avoid developing associated fungi in stored grains. As damaged and broken grains are likely to carry more fungal-propagules than nondamaged grains, the physically damaged grains should be periodically separated from the healthy ones to minimize the growth of contaminating fungi and decrease the probability of aflatoxin production. The author stresses on the importance of controlling insects that damage the stored grains. Also,

protect maize ears from infection with borers and ear worms under field conditions is one of the most important control measures to decrease the problem of ear and kernel rots. This is because most of fungi that invade ears in the field at or after ripening could be increased on grains under exceptional moist storage conditions (Halloin, 1975). Avoiding planting maize local varieties which are very sensitive to infection with ear-rotting fungi, but growing the resistant maize hybrids which distributed by the Ministry of Agriculture in order to minimize the effect of ear-rotting fungi on the stored grains.

SUMMARY

Results obtained throughout this work can be summarized as follows:

1- Disease survey in 1997-1998 indicated that ear rot disease was prevalent in the maize growing fields of 6 gavernorates, namly Dakahliya, Kafr El-Shiekh, Gharbiya, Qalubiya, Beni soef and El-Beheira (Nubariya) and the following fungi were recovered from the infected maize ears: *Fusarium moniliforme*, *Aspergillus flavus*, *A.niger*, *Penicillium* spp. *Fusarium nivale*, *Nigrospora oryzae*, *Botryodiplodia theobromae*. *However F.moniliforme* was the most common fungus followed by *A. flavus*, *Penicillum* spp., *Nigrospora* spp. while *B.theobromae* was found only at Nubariya.

2- A number of 21 *Fusarium* spp. and 14 isolates of *Aspergillus flavus* were pathogenic to maize ears. Five out of the *Fusarium* isolates were less pathogenic. *F.moniliforme* was the most aggressive one giving the highest level of infection followed by *F.semitectum*, and *F.nivale*. The most virulent one of *A.flavus* was isolated from Beni suef.

3- Silk channel injection gave the highest degree of infection compared with the rest methods of inoculation with *F.moniliforme*. Whereas, silk channel and pinbar inoculation techniques were similar in producing damage to ears when inoculated with *A.flavus*.

4- Detection and determination of aflatoxins in *A.flavus* isolates indicated that 9 out of 14 isolates had highly intense blue fluorescence under UV. Yet, only 6 isolates could produce aflatoxins when chemically analysed by TLC.

5- All isolates of *Fusarium moniliforme* and *F.nivale*. under study could not produce zearalenone.

6- Screening maize genotypes for their resistance to infection with *F. moniliforme* and *A.flavus* showed that the single cross SC10 has the

highest degree of resistance to both pathogens (7.7, 3.3 % respectively). TWC320 and SC123 showed good degree of resistance to *F.moniliforme*; whereas, TWC321, TWC323, SC123, SC124 were resistant to *A.flavus.*

7- The maize genotypes (TWC320, TWC322, TWC310, SC129 and SC13) were susceptible, but (TWC321, TWC323, TWC324, SC10, SC123, SC124) were resistant to aflatoxins production after artificially- ear inoculation by *A.flavus.*

8- The best conditions for storing maize were found to be of 8ºC, generally. Storing at room temp. under relative humidity of less than 65% preserve maize grains free from aflatoxins completly.

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الملخص العربي

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دراسات على أعفان الكيزان والحبوب فى الذرة الشامية المتسببة عن فطريات الاسبرجيلس والفيوزاريوم.

يمكن تلخيص نتائج هذة الدراسة فى النقاط التالية.

١- دلت نتائج الحصر والعزل من الحبوب المأخوذه من الكيزان المصابة بالأعفان خلال موسم ١٩٩٧-١٩٩٨ من محافظات الدقهلية- كفرالشيخ- الغربية- القليوبية-بنىسويف والبحيرة (النوبارية) على عزل أجناس الفطريات التاليه: الفيوزاريوم، الاسبراجيللس، البنسيليوم ، والنيجروسبورا و البتريوديبلوديا ثيوبرومى. ووجد ان اكثر الفطريات شيوعا هو الفيوزاريوم مونيليفورمى ويليه الاسبرجيللس فلافس ثم الفطريات من جنس البنسيليوم و النيجروسبورا أما بالنسبة للفطربتريوديبلوديا ثيوبرومى فلم يتم عزله الا من النوبارية فقط.

٢- كانت هناك٢١عزله فيوزاريوم و١٤ عزلة اسبراجيللس فلافس ذات قدرة مرضية على إصابة كيزان الذرة الشامية, ووجد أن خمس عزلات من ال٢١ عزله كانت أقل في قدرتها المرضيه على الاصابه. كما وجد أن الفطر فيوزاريوم مونيليفورم كان أكثر الفطريات قدرة مرضيه حيث انه أعطى أعلى مستوى من الاصابه ويليه فيوزاريوم سيميتكتم ثم فيوزاريوم نيفال. بينما كانت العزله التى تم عزلها من بنى سويف اقوى عزلات الاسبرجيللس فلافس في القدرة المرضية مقارنة بالعزلات الأخرى.

٣- بأجراء عدة طرق للعدوى الصناعية وجد أن احسن طريقه حقن صناعى هى طريقة الحقن فى قناة الحريرة مقارنة مع باقى الطرق وذلك مع فطر فيوزاريوم مونيليفورمى. بينما لم تكن هناك فروق معنوية عند عدوى الكيزان بفطر الاسبراجيللس فلافس بين الحقن فى قناة الحريره أو التلويث باللوحة المسننة.

٤-وجد عند تقييم ١١ هجين من الذرة الشامية للمقاومة ضد مرض عفن الكيزان عند استخدام العدوى بالفطرين فيوزاريوم مونيليفورمى واسبرجللس فلافس ان الهجين الفردى ١٠كان اكثر الهجن مقاومة حيث اعطى نسبة اصابة على التوالى ٧,٧% ٣.٣% كما أظهرت النتائج ايضا أن هـ.ث ٣٢٠,هـ.ف ١٢٣ أظهرا درجة جيدة من المقاومة ضد الفطر فيوزاريوم مونيليفورمى. بينما كانت الهجن هـ.ث٣٢٣, ٣٢٣,هـ.ف١٢٤ ١٢٣.١٢٤ مقاومة للفطر اسبرجيللس فلافس.

٥-فى تجربه للكشف عن وتحديد مستويات الأفلاتوكسينات فى ١٤ عزلة من الفطر اسبرجيللس فلافس وجد ان ٩عزلات منها أعطت درجة عالية من الوميض الأزرق الفلورسنتى وذلك باستخدام الأشعة فوق بنفسجية ولكن هذا الرقم انخفض الست وذلك باستخدام طريقة التحليل الكيماوى باستخدام الواح السليكا جيل وتراوح تركيز الأفلاتوكسين ب١ فى ال٦ عزلات من ١٦٠-٥٥٠ جزء فى المليون اما الأفلاتوكسين ب٢ تروا حت بين ٤٠-٣٠٠ جزء فى المليون كما وجد انه ليست هناك علاقة واضحة بين الكشف عن العزلات باستخدام الأشعة فوق بنفسجية وتقديرها بالطرق الكيماوية لإفراز السموم. كما اظهرت النتائج ان العزلات رقم٣ من الدقهلية و ١٠ من النوبارية أنتجت أعلى تركيز من الأفلاتو كسين وهو ٨٥٠ و٦٨٠ جزء فى البليون على الترتيب.

٦-فى تجربه للكشف عن قدرة بعض عزلات من الفيوزاريوم مونيليفورمى و الفيوزاريوم نيفال على أنتاج مادة الزيرالينون وجد ان جميعها ليست غير منتجه لهذه المادة .

٧-وجد من دراسة إمكانية انتاج الأفلاتوكسينات مع حبوب أنواع مختلفة من الهجن وذلك باستخدام عزلة من فطر الأسبرجيللس فلافس أن الهجن هـ.ث ٣٢٠, ٣١٠, ٣٢٢.٣١٠,هـ.ف ١٢٩.١٣ كانت حساسة لانتاج الأفلاتوكسينات بها اما هـ.ث٣٢٤.٣٢٣.٣٢١,هـ.ف ١٢٣.١٠, ١٢٤ فلم يمكن اكتشاف اى افلاتوكسينات بها.

٨- تخزين الحبوب فى درجة ٨مْ أودرجة حرارة الغرفة ودرجة الرطوبة اقل من٦٥% أعطى اقل مستوى من اصابة الحبوب بالفطريات وأيضا مستوى التلوث بالأفلاتوكسينات وكذلك أعلى درجة إنبات للحبوب. وعلى جانب آخر فأن التخزين على درجة حرارة عاليه ٣٠مْ ورطوبة نسبية ٩٠% أدت الى تقليل نسبة الانبات الى الصفر وزيادة نسبة الافلاتوكسينات والنمو الفطرى بزيادة فترة التخزين مما يؤثر على صلاحية الحبوب للاستهلاك الادمى.

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دراسات على أعفان الكيزان والحبوب فى الذرة الشامية المتسببة عن فطــر الاسبرجليس والفيوزاريوم.

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