Beneficial Effect of Some Yeast and Bio-Fungicides on Peanut Mold infection

R.S.R. El-Mohamady, M.M. Abdel-Kader, N.S. El-Mougy, N.G El-Gammal, Abd-Alla, M.A.

Abstract- Aspergillus flavus was a common isolated fungus in high frequency from peanut seeds followed by Aspergillus niger and Aspergillus ochraceus. Yeast, Saccharomyces cerevisiae and Candida tennis as well as Biofungicides, Rhizo-In, Plant guard were tested against mold fungi under in vitro and in vivo conditions. Saccaromyces cerevisiae caused a high inhibitor effect against linear growth of A. flavus, A. niger and A. ochroceus which measured as 33.3, 33.1 and 40.5 mm, respectively. Meanwhile, the bio-fungicide Rhizo-In also caused high inhibitor effect against linear growth of A. niger calculated as 78.3% followed by yeast S. cerevisiae by 63.2%. The yeast C. tennis and the bio-fungicides Plant guard showed moderate effect for reducing mold fungi linear growth under the same conditions. Coating peanut seeds before storage by S. cerevisiae and C. tennis could protect peanut seeds against mold fungi infection during storage period for 60 days, S. cerevisiae cussed more effect than other tested treatment, The results of this study showed that yeast S. cerevisea could be used as an alternative safe coating method for preventing peanut seeds against mold fungi infection which causes economic losses during transportation, marketing and storage.

Index-Peanut seeds-Yeast- Bio-fungicides- Mold fungi

I. INTRODUCTION

Peanut *(Arachis hypogaea* L.) is one of the most important food and oil seed crops cultivated and utilized in most parts of the world. Peanut seed contain 50% edible oil. Seeds are rich in fats, protein, vitamin B1, B2, B6, nicotinic acid and other vitamins. Peanut butter has become a common edible diet. Groundnut cake has high nutritive value. The long growing season, warm weather, and available humidity in the growing areas are favorable conditions for peanut production. These conditions are also favorable for many fungal pathogens to attack peanut causing harmful diseases and reduce yield as well under field or storage conditions. Of the various diseases causing organisms, *Fusarium solani*, *F. oxysporum* cause damping off of groundnut seedlings [1]. *Aspergillus flavus* attacks germinating groundnut seed [2]. *A. niger* caused disease of crown rot of peanut [3,4]. Mould fungi are also known to produce mycotoxin [5]. Many workers have detected different mold fungi and their toxin production ability in stored grains, which deteriorate the stored products [6,7]. Several investigations have listed a large number of fungi which could be isolated from peanuts during storage [8,9]. *Aspergillus flavus* is the dominant storage fungus colonizing peanuts, capable of causing seed rots, molding of seeds, pre -and post-emergence damping off, and reducing seed viability and seedling growth in peanuts [10,11]. Colonization of peanuts with mold fungi is of importance because of its potential to produce aflatoxins,

which are potent toxic, carcinogenic, mutagenic, immunosuppressive, and teratogenic agents [5,12,13]. The use of synthetic fungicides is a most effective decay control treatment, there is an urgent need to find effective and safe non-fungicide means of controlling postharvest pathogens mainly because of the toxicity of the synthetic fungicide residues to human health and the environment [14]. In recent years, some antagonists have been applied as biocontrol treatment against postharvest diseases of agricultural products. For example, a new strain of *Bacillus pumilus* isolated from Korean soybean sauce showed strong antifungal activity against the aflatoxin-producing fungi *A. flavus* and *A. parasiticus* [15]. Also, application of unicellular yeasts for biocontrol of spoilage molds is an attractive alternative to the use of chemical pesticides. New biocontrol products, in addition to efficacy towards the targeted plant pathogen, must be safe and cost-effective [16,17]. Despite all these problems, a number of successful commercial products have been on the market in different countries worldwide to control mycotoxigenic fungi, including Aspire (*Candida oleophila* strain 182; Ecogen Inc., Langhorne, PA), Bio-Save 10 and 11 (*Pseudomonas syringae* strains ESC10 and ESC 11; EcoScience Corp., Worcester, MA), AF36 (atoxigenic strain of *A. flavus*) [18], and Afla-Guard (atoxigenic strain of *A. flavus* NRRL 21882) [19]. The objective of the present study was designed to evaluate the inhibitor effect of the yeast, *Saccharomyces cerevisiae* and *Candida tennis* as well as Biofungicides, Rhizo-In, Plant guard against mold fungi under *in vitro* and *in vivo* conditions.

II. MATERIALS AND METHODS

Source of seed samples

Twenty seed samples of peanut seeds cv.Giza 5 were collected from commercial markets located at Sharkia, Qalubia, Gharbia and Beheira governorates. the seed samples were subjected to isolation trails for associated fungi.

Antagonistic yeast

Two isolates of the antagonistic yeast, *i.e.* *Saccharomyces cerevisiae* [Meyen ex E.C. Hansen] and *Candida tenuis* [Berkh] were obtained from Plant Pathology Department of the National Research Centre, Giza, Egypt. The antagonistic ability of these microorganisms was examined during previous work at the same Department.

Growth media

Potato dextrose agar (Difco Laboratories, Detroit, MI) and NYDB [8 g of nutrient medium (Difco Laboratories, Detroit, MI), 5 g of yeast extract, and 10 g of dextrose in 1

liter of water] were used for growing fungal and yeast isolates tested in the present work. Fungal and yeast cultures were maintained on PDA and NYD agar slant media at 5 ± 1 °C as stock cultures until use. All isolates were activated by growing at the optimum growth conditions at the beginning of the present experiments.

Bio-fungicides were used

An isolate of *Trichoderma harzianum* formed as spore suspension (3×10^7 spore/ml) and *Bacillus subtilies* as liquid culture (3×10^7 spore/ml) produced by El-Naser Co., Egypt, under commercial name of Plant Guard and Rhizo In, ,respectively were used in this study.

Frequency of seed-borne fungi associated with peanut seeds

Detection of seed-borne fungi was carried out following the procedures published by International Seed Testing Association, 2011.Two hundred seeds of each samples were tested using the standard blotter and agar plate methods.

1 -Standard blotter method

Four replicates each represented by 50 peanut seeds and total of 200 seeds were placed on 9cm diameter Petri-dishes containing three layers of filter paper moistened with sterilized tap water .The plates were incubated at 20 ± 2°C for 7 days under cool white fluorescent light with alternating cycles of 12 hours light and 12 hours dark. The incubated seeds were examined after 7 days under stereomicroscope and light microscope. The percentage of infected seeds and the occurrence of different fungal colonies on the seeds were isolated, counted and recorded.

2 -Agar plate method

Two hundred seeds were divided of two groups, first group was surface sterilized with sodium hypochlorite (2.5%), then washed several times with sterilized water and dried between two folds of filter paper before putting on prepared PDA plate for isolation the associated fungi, second group was left without surface sterilization and served as mentioned before.The plates were incubated at 20 ± 2°C for 4 days.The incubated seeds were examined daily to observation of any infection symptoms.The percentage of infected seeds and the occurrence of different fungal colonies on the seeds were isolated, counted and recorded.

Identification of the isolated fungi

The identification of the isolated fungi has been based on the habit characters of the infected seeds under stereo-binocular and light microscope in seed pathology department., Plant Pathology Research Inst., ARC, Giza, Egypt with the aid of [20,21,22].

Mold fungi spore suspension preparation

The surface-plated culture of isolated mold fungi in Petri dishes was sub-cultured by streaking the spores onto the new potato dextrose agar (PDA) media. New plated cultures were then incubated for 7days at 25 ± 2°C.The spores of 7-day-old cultures of mold fungi were dislodged by sterile distilled water with 0.1 mL/L of Tween 80. The spore suspensions were then collected and filtered through sterile cheesecloth to remove mycelia and agar fragments, and the aliquot was diluted to a concentration of (1×10^6 cfu) fungal spores/mL with the aid of haemocetometer slide.

Effect of yeast isolates on mycelium linear growth of mold fungi in vitro

The inhibitory effect of the antagonistic yeast, *Saccharomyces cerevisiae* and *Candida tenuis* against the linear growth of mold pathogenic fungi was evaluated using the modified dual culture technique [23]. Abundant fungal and yeast growth was first prepared. Ten mL of each individual yeast isolate was grown for 48 h on NYDB broth medium and poured into flasks containing sterilized PDA medium. Before solidifying, each flask was rotated gently to ensure equal distribution of yeast growth, and then poured into 9-cm-diameter Petri dishes. Inoculated plates were incubated for 48 h at 28 ± 1°C. For fungal growth, a 5-mm disk of each tested fungi was transferred to the centre of a PDA plate then incubated for ten days at 25 ± 1^0C. *In vitro* antagonistic studies between biocontrol yeast and mold pathogenic fungi were performed on PDA medium in 9-cm-diameter Petri dishes. A 5-mm disk of each yeast growth culture was placed onto the PDA, 10mm from the edge of the Petri dish. Another disk of the same diameter of each mold pathogenic fungal growth culture was placed on the opposite side of the dish at the same distance. The control treatment was inoculated with a culture disk of either a pathogenic or antagonistic culture alone at the same conditions. Both experimental and control dishes were assigned to a completely randomized design, with five replicates per treatment. All inoculated Petri dishes were incubated at 25 ± 1°C and the fungal growth diameter away from and towards the antagonist agent was measured after the pathogenic fungal growth in the control treatment had reached the edge of the Petri dish. This test was repeated three times and the inhibition was calculated as the percentage reduction in colony diameter growth compared with the control.

Effect of Bio-fungicides on mycelium linear growth of mold fungi in vitro

Commercial biocides products, *i.e.* Plant guard (*Trichoderma harzianum*) at concentration of 3ml/l and Rhizo -In (*Bacillus subtilies*) at concentration of 2.5g/l were added to conical flask containing 100 ml of sterilized PDA before its solidifying to obtained a certain recommended dose of each, then rotated gently to ensure even distribution of biocide. Supplemented media were then, poured into Petri dishes. A disk 5 mm-diameter of 7 -days -old culture of tested mold pathogens was aseptically transferred singly to the center of Petri plates. Five plates were used as replicates for each biocides tested. Another set of plates inoculated only with mold pathogens and served as control. All plats were incubated at 25 ± 2°C for 7 days, then examined. Reduction in the fungal growth of tested mold fungi due to the effect of biocides was calculated.

Effect of peanut seeds coating with yeast and bio-fungicides on infection with mold fungi during storage

Preventive effect of yeast and biocides against storage mold fungi infection of peanut seeds was evaluated *in*

vivo. Spore suspension of mold fungi, *A. flavus, A. niger* and *Aspergillus ochroceus* was prepared as mentioned before. Peanut seeds apparently healthy were used in this study. Peanut seeds were surface disinfected with sodium hypochlorite 2.5%, then washed several times with sterilized water and placed onto filter paper to air dried. Two antagonistic yeast isolates, *i.e. S. cerevisiae* and *C. tennis* as cell suspension (1×10^8 cfu) of each were used. Two biocides were used *i.e.* Plant guard (*Trichoderma harzianum*) and Rhizo In (*Bacillus subtilies*) at the recommended dose was also used. Arabic gum solution (1%) was added to either cell suspension or liquid biocides as an adhesive material. Peanut seeds were dipped individually in each of cell suspension or liquid biocide for 5 min and then air dried for 2hr at ambient temperature 24-26^0C., then the treated seeds were artificially inoculated by spraying with tested mold fungi individually with 1×10^6/mL spore suspension. Treated seeds were placed individually into desecrator and left at room temperature ($25-28^o$C) for 45 days. Stored seeds were placed into sterile Petri-dishes plates containing PDA medium, at the rate of 10 seeds per plate. Control treatment was served as peanut seeds inoculated with each mold isolated fungi spore suspension without bio-fungicides or yeast treatment. Twenty replicates were done for each treatment and control. All plates were incubated at 25 ± 2 ℃ for 7 days then examined. The percentage of seeds infection was calculated as numbers of infected seeds in relative to whole tested seeds.

Statistical analysis

Tukey test for multiple comparisons among means was utilized for analyzing the obtained results [24].

III. RESULTS AND DISCUSSION
Frequency of fungi associated with peanut seeds

Using blotter, agar plate and deep freezing method as recommended by ISTA, the seed associated mycoflora of peanut seed samples collected from different localities of Egypt revealed four fungal genera at different frequencies. Results presented in Table (1) indicated that, peanut seeds were very sensitive to infestation with many mold fungi after harvesting. *Aspergillus flavus* was the more frequency and occurred it recorded 57.9, 54.1%, followed by *Aspergillus niger* as 30.1, 32.7% and *Aspergillus ochroceus* as 8.8, 10.5%. Meanwhile, *F. moniliforme* was recorded as lowest as 3.2, 2.5% in Bloter and Agar plate isolation method, respectively.. Similar results were reported that genus Aspergilli also isolated from peanut seeds [25]. Blotter method showed better results as compared to agar plate method. Also, it was reported that the filter paper method was most practical method for routine analysis of seed health [26]. Such similar results were observed [27] on rice seed and [28] on sunflower seed. Present results showed that *A. flavus* and *A. niger* were the predominant fungi of groundnut. Also [29] found that *A. flavus* and *A. niger* were the predominant storage fungi of groundnut seed. Species of *Aspergillus, Penicillium* and *Rhizopus* have also been reported on

groundnut seed [30]. Moreover, [31], reported that, peanut seeds are sensitive to attack with different mould fungi under natural conditions. *A. flavus* was the most prevailing fungi with high frequency, followed by *A. parasiticus*. Also, [11] stated that *Aspergillus flavus* appears to be more aggressive than *A. parasiticus* in infecting peanut seeds.

Table (1) Survey of common fungi associated with peanut seeds

Isolation method	Average frequency of mold fungi (%)			
	A. flavus	A. niger	A. ochroceus	F. moniliforme
Blotter	57.9	30.1	8.8	3.2
Agar plate	54.1	32.7	10.5	2.5

Effect of yeast and bio-fungicides on mycelium linear growth of mold fungi in vitro

Results presented in Table (2) and Fig. (1) indicate that, all tested bio-fungicides and tested antagonistic yeast caused various inhibitors effect for all tested mold fungi. *Saccaromyces cerevisiae* caused a high inhibitor effect against linear growth of *A. flavus*, *A. niger* and *A. ochroceus* which measured as 33.3, 33.1 and 40.5 mm, respectively.

Table (2) Effect of yeast and bio-fungicides on linear growth of peanut mold fungi under *in vitro* conditions

Treatment	Linear growth (mm)		
	A. flavus	A. niger	A. ochraceus
S. cerevisiae	33.6 d	33.1 d	40.5 d
C. tennis	71.6 b	61.1 b	50.6 c
Plant guard	60.1c	45.1 c	70.0 b
Rhizo-In	58.1c	19.5 e	43.8 d
Control	90.0 a	90.0 a	90.0 a

Figures with the same letter are not significantly different ($P \leq 0.05$).

On the other hand, the bio-fungicide Rhizo-In also caused high inhibitor effect against linear growth of *A. niger* calculated as 78.3% followed by yeast *S. cerevisiae* by 63.2% (Fig. 1). The yeast *C. tennis* and the bio-fungicides Plant guard showed moderate effect for reducing mold fungi linear growth under the same conditions. Several biological control agents have been developed in recent years, and a few have actually been registered for use on fruit crops. Yeasts such as *Pichia guilliermondii* [32] and *Cryptoccocus laurentii*, yeast that occurs naturally on apple leaves, buds, and fruit [33] were the first to be applied for control of postharvest decay on fruit. The yeast, *Candida oleophilia* has been registered for control of postharvest decay on fruit crops. The yeasts, *Cryptococcus infirmo-minutus* and *Candida sake* successfully control brown rot and blue mold on sweet cherry [34], and three diseases of apple [35], respectively, and may be developed into commercial products.
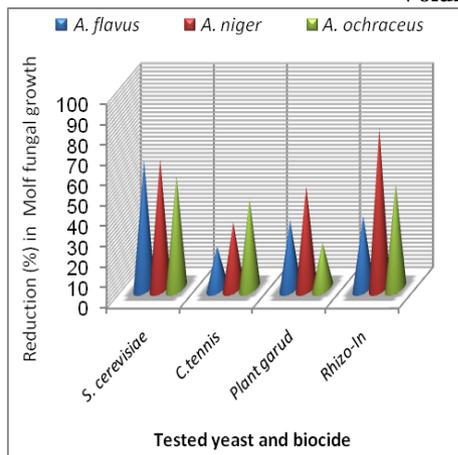
Fig. (1) Reduction in linear growth of mold fungi in response to yeast and bio-fungicides under *in vitro* conditions

Furthermore, [36] proved that *Pichia anomala* inhibits the growth of *Penicillium roqueforti* and *Aspergillus candida* on agar. Also [31] *S. cerevisiae* has better biocontrol effect on the growth than *C. tennis* and Plant guard against all the tested mold fungi. They added that *S. cerevisiea* showed effective action against both *A. flavus* and *A. parasiticus* along with its adequate effect against *A. ochraceus* and *F. moniliform*. Competition for nutrients has been suggested as the mode of action of several possible biocontrol organisms, *e.g. P. guilliermondii* against *Penicillium digitatum* [14], *Candida guilliermondii* , *Cryptococcus laurentii* and *Metschnikowia pulcherima* against *Botrytis cinerea* and *Penicillium expansum* [32, 37]. The killer yeast phenomenon was first discovered 40 years ago by [38]. They observed that certain strains of *Saccharomyces cerevisiae* produced toxins that killed sensitive strains of the same species. Initially, it was assumed that killer yeasts only killed yeasts belonging to the same or closely related species. However, many killer toxins can affect other yeasts and even bacteria and filamentous fungi [39,40,41,42].

Effect of coating with yeast and bio-fungicides on peanut seeds infection with mold fungi during storage

Results presented in Table (3) showed that, all coated peanut seeds with different treatment, *i.e.* bio-fungicides and antagonistic yeasts showed significant protective effect against mold infection at various degrees comparing with untreated check control. Peanut seeds coated with antagonistic yeasts showed superior protective effect against mold infection than commercial biofungicides during storage period. Also, it was observed that mold infection increase by prolonging storage period either at yeast or commercial biocides treatments. Illustrated data in Fig. (2) showed that at the end of storage period (60 days), the antagonistic yeast *S. cerevisiae* caused reduction of seeds mold infection with *A. flavus* by 71.3%, *A. niger* by 76.6% and *A. ochroceus* by 84.5% and *C. tennis* caused 66.9% reduction of *A. flavus* seed infection, 62.9% of *A. niger* and *A. ochroceus* by 80.7%. Meanwhile, commercial biocides treatment caused reduction in seed infection by 4.0, 42.5, 41.5% and 7.8, 23.7, 36.2% for *A. flavus*, *A.

niger and *A. ochroceus* at plant guard and Rizo-In treatments, in respective order.

Table (3) Effect of coating with yeast and bio-fungicides on percentage of peanut seeds mold infection under artificial infection with mold fungi during storage

Treatment	Mold infection (%)					
	A. flavus		A. niger		A. ochroecus	
	Storage days					
	30	**60**	**30**	**60**	**30**	**60**
S. cerevisiae	24.0 g	28.7 g	16.5 h	23.4 g	9.4 h	15.5 h
C. tennis	10.0 h	33.1 f	0.0 j	37.1 f	4.5 i	19.3 h
Plant guard	58.0 e	96.0 a	55.4 e	57.5 e	53.9 b	58.5 e
Rhizo-In	89.2 b	92.2 a	39.4 c	76.3 c	55.2 e	63.8 d
Control	100 a	100 a	100 a	100 a	100 a	100 a

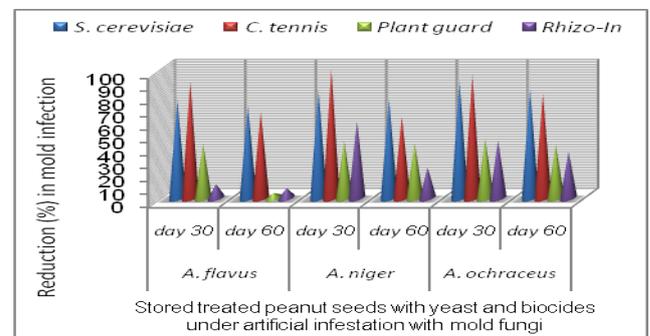Figures with the same letter are not significantly different (P ≤ 0.05).

Fig. (2) Reduction in peanut seeds infection with mold in response to yeast and commercial biocides treatment

Peanut seeds coating with antagonistic yeasts, *S. cerevisiae* and *C. tinnis* and Bio-fungicides, Plant guard and Rhizo-In caused a protective effect against infection with three mold fungi during storage period compared with control treatments. These results agree with Paster *et al.*,1993, they showed that, *Pichia guilliermondii* prevented growth of *A . flavus* on soybeans seeds better when the two microorganisms were applied simultaneously than when the yeast was applied 3 days before the mold fungus. They added that inhibition of *A. flavus* on soybeans by *Pichia guilliermondii* lasted longer when it was applied by dipping rather than by spraying. The two types of yeast, *S. cerevisiae* and *C. tennis* completely protect peanut seeds to be invaded with *A. parasiticus* and *F. moniliforme* for 15 days of storage and the percentage of seed infection was so low till the 45th days of storage by all tested fungi [31]. Also, *Pichia guilliermondii* [14] can stimulate ethylene production in grapefruit and phytoalexins in citrus [43] *Aureobasidium pullulans* and *Candida saitoana* have been shown to induce accumulation of β-1,3-glucanases, chitinases and peroxidases in apples [44,45]. These observations suggest that [36] the antagonist stimulates some kind of host defense response. Ethanol has well-known antimicrobial, and hence antifungal, effects. Moreover, *S. cerevisiae* is as an efficient ethanol producer and particularly tolerant to high ethanol concentrations. Therefore if ethanol alone was the critical inhibitory component, *S. cerevisiae* would

be the ideal yeast for biocontrol of grain moulds. The obtained results in the present work showed that the use of the safe antagonistic yeast and bio-fungicides as a seeds coating has potential biocontrol activity against the peanut mold fungi. This potential could be extend to direct use in the market to prolong shelf life, provided the antagonist and its metabolites are safe for human consumption.

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AUTHOR BIOGRAPHY

First Author: Department of Plant Pathology, National Research Centre – Egypt, Email: riadelmohamedy@yahoo.com

Second Author: Department of Plant Pathology, National Research Centre – Egypt, Email: mokh_nrc@yahoo.com.

Third Author: Department of Plant Pathology, National Research Centre – Egypt, Email: nehal_nrc@yahoo.com

Fourth Author: Department of Plant Pathology, National Research Centre – Egypt, Email: nadia_gamil2000@yahoo.com

Fifth Author: Department of Plant Pathology, National Research Centre – Egypt, Email: mmokh95@yahoo.com