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NATURAL OCCURRENCE OF CITRININ AND BIOCONTROL OF ITS PRODUCING FUNGUS BY *Trichoderma hamatum* IN RICE GRAINS

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ABSTRACT

Paddy rice was sampled from El-Sharkia, El-Gharbia, El-Dakahlia and Kafr El-Sheikh governorates, Egypt. Of the 29 samples taken, ten were contaminated with the mycotoxin citrinin. An average of 6.79 x 10^4 fungal spores per gram rice was found. The isolated fungi represented 47 species belong to 28 genera. The predominant genera were *Aspergillus*, *Cladosporium* and *Penicillium*. *Aspergilli* were represented by 22 species; *Aspergillus niger* and *A. flavus* had the highest occurrence. *Penicillium viridicatum* produced the highest amount of citrinin on glucose ammonium nitrate salts broth and rice grains and hence, this isolate was selected as a good producer of citrinin in this study. The presence of *Trichoderma hamatum* reduced the amount of citrinin produced by *P. viridicatum* compared with its respective control. The excessive growth of *T. hamatum* on *P. viridicatum* was increased with time. Viability of *P. viridicatum* conidia decreased by *T. hamatum* with an increase in the incubation period. Chitinases and 1,3-B-glucanase enzyme activity of *T. hamatum* increased with extending the incubation period on *P. viridicatum* cell walls up to maximum values at 72 and 84 h, respectively. *T. hamatum* led to a decrease in the production of citrinin by *P. viridicatum* on rice grains compared with the respective control values.

Keywords: *Trichoderma hamatum*, *Penicillium viridicatum*, Biocontrol, Citrinin, Rice, Mycotoxin

INTRODUCTION

Mycotoxins affect up to 25 per cent of the world food crops. They cause significant economic losses in agriculture; some are carcinogens and teratogens. They may be transmitted to man in meat and milk **(Peadar and Lynch, 2001).** Food-infesting fungi are able to produce many secondary metabolites, including mycotoxins, that may influence both animal and human health **(Liu *et al* 2003; Jayas**

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and White, 2003 and Bennett and Klich, 2003). Rice is also one of the important cereals, which favors mycotoxin production. Natural occurrence of aflatoxin and aflatoxin producing fungi in rice has been reported from various parts of the world **(Bhat *et al* 1978).** Rice stored for prolonged periods i.e. 4-8 years has also been reported to be contaminated with aflatoxin B in various parts of India **(Bilgrami *et al* 1980).** Presence of some other mycotoxins like citrinin, sterigmatocystin and ochratoxin has been detected as natural contaminants of rice in different regions of the world. Citrinin has been found as a natural mycotoxin contaminant of rice grains **(Yoshizawa, 1991)** and mouldy fruits and fruit products **(Martins *et al* 2002).** Spontaneous citrinin mycotoxicosis causes a dieresis that is manifested as watery fecal droppings and reductions in weight gain. At necropsy, lesions involve chiefly the kidney. Citrinin acts directly on the kidney to transiently alter several tubular transport processes, induces renal dysfunction, and it is tumorigenic **(Liu and Xu, 2004 and Knasmuller *et al* 2004).** It is a toxic secondary metabolite of certain species of the genera *Aspergillus* **(Hasan and Issa, 1993),** *Penicillium* **(Oh *et al* 1998; Bailly *et a*l 2002; Centeno and Calvo, 2002 and Labuda *et al* 2005)** and *Monascus* that causes `yellow rice' **(Li *et al* 2003; Shu and Lin, 2002; Wang *et al* 2004 and Liu *et al* 2005)**. It causes alteration of amino acids uptake and their incorporation into protein by plant cotyledons **(White and Truelove, 1972)**, several cytological abnormalities, including chromosome breakage, polyploidy, anaphase bridge as well as laggard of onion **(Sinha *et al* 1992)** and decreased seed germination **(Sinha, 2001).** Many microorganisms including *Saccharomyces cerevisiae* **(Ammar *et al* 2000),** *Lactobacillus* spp. **(Gourama, 1997),** and *Xanthomonas citri* **(Giridhar and Reddy, 1997)** have been used to detoxify citrinin.

Trichoderma spp. use different mechanisms to function as biocontrol agents, such as antibiosis, lysis, competition, mycoparasitism, plant growth promotion **(Ghisalber and Sivasithamparam,1991, Rocha-Ramírez *et al* 2002 and James *et al* 2004),** and inactivation of toxin produced by plant pathogens (**Sriram *et al* 2000).** Meanwhile, some biocontrol agents have a synergistic interaction ability **(Huttunen *et al* 2004).**

This work was aimed at evaluating the natural occurrence of citrinin in rice and its association with the fungal flora in Egypt. Additionally, biocontrol of citrinin production on rice was studied using *Trichoderma hamatum*.

MATERIAL AND METHODS

Sample collection: Samples **(Sinha, 2001)** of available different varieties in commercial markets of Egyptian paddy rice (*Oryza sativa* L.) were obtained from different Governorates in Egypt (El-Sharkia, El-Dakahlia, Kafr El-Sheikh and El- Gharbia). They were collected in sterile cellophane bags at 2 ˚C until analyzed on the same day of collection.

Determination of moisture content: Triplicates of each sample were ground in a blender and a weighed portion of this flour was dried in an oven for 24 h at 105 ˚C, then cooled in a desiccator and reweighed. The moisture content is expressed as percentage of the wet weight.

Enumeration of fungal flora: The dilution plate method of **Raper and Fennell (1965)** was employed for isolating fungal flora from rice samples. Czapek's Dox agar medium was used for isolation **(Thom and Raper, 1945).** Rose bengal (33 µg / ml, w/vol) and streptomycin (30 µg / ml, w/vol) were added as bacteriostatic agents. The plates were incubated at 28 ± ˚C for 10 days and the developing fungi were counted and identified to the species level according to keys proposed by **Gilman (1957), Raper and Fennell (1965), Barron (1968), Ellis (1971 and 1976) and Carmichael *et al* (1980).** The counts were expressed as number per gram dry grains.

General culture conditions for citrinin production by isolated fungi: The above fungal isolates were screened for citrinin production by growing on glucose ammonium nitrate salts broth **(Brian *et al* 1961).** Erlenmeyer flasks (250 ml) containing 50 ml culture medium were autoclaved, inoculated with one agar disc (10 mm) of each fungal culture (7 days old), and incubated for 10 days at 30 ˚C under static culture conditions. At the end of the incubation period, the cultures were filtered (Whatman No. 1 filter paper) and filtrates were stored at –5 ˚C until used for citrinin estimation according to **Trantham and Wilson (1984).**

Citrinin determination: The extraction and clean up of citrinin were carried out according to **Jackson and Ciegler (1978).** Fifty ml of culture filtrate was shaken with an equal volume of chloroform for 30 min. The chloroform layer was separated over a bed of anhydrous sodium sulfate. Clean up was carried out using concentrated HCl and 0.1 M NaHCO3 **(Jackson and Ciegler, 1978).**

Biological toxicity tests: The method described by **Hald and Krogh (1973)** was used. The tested extracts were dissolved in a freshly prepared solution of pyridine: acetic anhydride (1:1 v/v). The biological confirmatory tests were carried out using two methods: *Disc screening method* **(Harwig and Scott, 1971):** Paper discs (7 mm diam) were saturated with 30 ml of clean chloroform extract of citrinin, and the solvent was allowed to evaporate. The paper discs were incubated with two drops (~ 0.1 ml) of suspension of larvae (containing 20 to 40 larvae) of brine shrimp (*Artemia salina* L.). Trays were incubated at 30 ˚C for ~16 h. Mortality was determined by counting the immobile (dead) larvae under a stereoscopic microscope, killing the larvae with heat or formalin, and then counting the total number. Mortality in controls was determined simultaneously with each screening test. Natural mortality associated with a control disc (soaked in chloroform and allowed to evaporate) averaged 2%. Toxicity was rated as follows: 0-9% mortality, nontoxic; 10-49 % mortality, slightly toxic; 50-89 mortality, toxic; 90-100% mortality, very toxic. Brine shrimp larvae were provided by **Dr. Mahmoud Dosoky** (Zoology Department, Faculty of Science, Zagazig University, Zagazig, Egypt). Disc diffusion assay **(Madhyastha *et al* 1994):** *Bacillus brevis* was maintained on tryptic soy agar plates (pH 7.3; Difco Laboratories, Detroit, MI, USA). This bacterium is sensitive for any antimicrobial and toxic compounds. The clean chloroform extracts of citrinin (20 µl) were applied to filter paper discs (5 mm diam) drop wise using a micropipette

and allowed to dry for 15 min and then evenly placed on the surface of agar plates. The plates were then inverted and preincubated at 4°C for 2 h to allow uniform diffusion into the agar. After preincubation the plates were incubated for 24 h at 28°C. The inhibition zones around a paper disc indicated the antimicrobial potential of citrinin against the test microorganism (*B. brevis*). Microbiological Resources Center, Faculty of Agriculture, Ain-Shams University,Cairo, Egypt provided the biological indicator bacterium.

Quantitative determination of citrinin: It was carried out fluorometrically according to **Trantham and Wilson (1984)** using spectrofluorometer Varian model 330 with detector (Varian Associates, Houston, TX, USA). The excitation wavelength of the fluorometer was 330 nm and the emission wavelength was 500 nm.

Efficacy of *T. hamatum* for controlling citrinin production: In culture flasks: In a preliminary test to study the efficacy of the antagonistic potential of *T. hamatum*, Erlenmeyer flasks (250 ml in capacity) containing 50 ml Czapek's Dox broth culture medium **(Thom and Raper, 1945)** were inoculated with *P. viridicatum* alone and reinoculated with *T. hamatum* (*P. viridicatum* + *T. hamatum*) at the same time (zero time of incubation). Ten-mm-diam discs of 7-day-old cultures were used for inoculation. The culture media (both *P. viridicatum* alone and *P. viridicatum* + *T. hamatum*) were incubated for 2, 4, 6, 8, 10, 12 and 14 days. At the end of the incubation period (30±2 ˚C, at static state), the cultures were filtered (Whatman No.1 filter paper) and culture filtrates were used for citrinin estimation. Control flasks (without reinoculation with *T. hamatum*) were used. *In Petri dishes*: Mycelial disks (5 mm diam) of *P. viridicatum* were placed on one edge of a Petri dish (9 cm diam) containing 15 ml of Czapek`s Dox agar culture medium **(Thom and Raper, 1945)** while a mycelial disc (5 mm diam) of *T. hamatum* was placed on the opposite side of the plate at the same time, zero time of incubation. The plates were incubated for 2, 4, 6, 8,10, 12 and 14 days. At the end of the incubation period (30±2 ˚C), the agar culture media were used for citrinin estimation

Efficacy of *T. hamatum* to degrade the mycotoxin citrinin: Plate assay: Czapek`s Doxagar culture medium **(Thom and Raper, 1945),** a non-fluorescent medium under ultraviolet (UV) light, was used for the plate assay. One milliliter of citrinin (50 µg / ml in chloroform) was dispensed into Petri dishes, and 15 ml of medium (55 ˚C) was added to each Petri dish and mixed evenly with citrinin solution (pure crystalline citrinin was obtained from Sigma Chemical Co., St. Louis, IL, USA). Lids of Petri dishes were opened partially to enable chloroform vapour to dissipate. After the medium had solidified, plates were point-inoculated with one loopful of spore suspension of *T. hamatum* (10^6 spore / ml). The plates were incubated at 30±˚C and examined under UV at one-day intervals for up to one week. Plates without inoculation with *T. hamatum* were used as control. Loss of fluorescence indicated possible detoxification. **Test tube assay:** One ml of citrinin (50 µg / ml in 0.1 M NaHCO3) according to **Jackson and Ciegler (1978)** was added to 9 ml of Czapek`s Dox broth culture medium **(Thom and**

Raper, 1945) inoculated with one loopful of spore suspension of *T. hamatum* (10^6 spore/ml). Tubes were incubated for 96 h at 30 ± 2°C. At the end of the incubation period, the samples were filtered through Whatman No.1 filter paper and filtrate was extracted three times with chloroform, after which the extract used for citrinin estimation fluorometrically. Tubes without inoculation with *T. hamatum* were used as control. Recovery of citrinin (%) = a x 100 / b, where a = amount of citrinin (µg / ml) detected in treated test tube after 0, 2, 4, 6, 8 and 10 days of incubation with *T. hamatum*; b = amount of citrinin (50 µg / ml in 0.1 M NaHCO3) in the test tube after 0, 2, 4, 6, 8 and 10 days of incubation without *T. hamatum*.

Overgrowth of *P. viridicatum* by *T. hamatum* in dual culture test: A mycelial disc (5mm diam) of *P. viridicatum* was placed on one edge of a Petri dish (9 cm diam) containing Czapek`s Dox agar culture medium **(Thom and Raper, 1945)**, while a mycelial disc of *T. hamatum* was placed on the opposite side of the plate. After the desired incubation time (72, 96, 120 h after inoculation), the overgrowth of *T. hamatum* on the mycelial disc of *P. viridicatum* and its developed growth was determined (by ocellar and light microscope) and expressed as mm growth of *T. hamatum* on *P. viridicatum* according to **Sivan and Chet (1989).**

Survival of *P. viridicatum* as affected by *T. hamatum:* Survival of mycelium of *P. viridicatum* was determined after exposure to conidia of *T. hamatum*. Mycelial disks (5 mm diam) were removed from a 72-h-old culture of *P. viridicatum* on water agar (20 g / 1L distilled H2O). Disks were soaked for 30 sec in a conidial suspension of *T. hamatum* (10^6 conidia / ml) and incubated in Petri dishes, each containing four layers of Whatman No.1 filter paper. Mycelial disks treated with autoclaved distilled H2O and incubated as described above served as control. After the desired incubation time, mycelial disks of *P. viridicatum* were transferred to PRYES medium (yeast extract sucrose agar plus 100 µg/ml pentachloronitrobenzene and 25 µg/ml rose bengal), which is suitable for *P. viridicatum* **(Frisvad, 1986).** One mg methyl1-(butylcarbamoyl) -2-benzimidazolecarbamate (benomyl) / l was added to inhibit growth of *T. hamatum* **(Elad and Chet, 1983).** The results were expressed as the percentage of mycelial disks from where the test fungus (*P. viridicatum*) grew.

Preparation of hyphal cell walls of *P. viridicatum:* Cell wall preparations were obtained from *P. viridicatum* after culture at 27 ˚C for 5 days in liquid potato dextrose broth medium in a rotary shaker at 120 rpm. After incubation, the hyphal cell walls were prepared according to **Sivan and Chet (1989).** The precipitated walls were then deep-frozen, lyophilized, and kept as powder in a sealed container until used.

Induction of extracellular lytic enzymes (1,3- β -glucanase and chitinase) by *T. hamatum*

Erlenmeyer flasks (250 ml), each containing 50 ml liquid culture of glucose ammonium nitrate salt broth medium **(Brian *et al* 1961),** were inoculated with 1.0 ml of conidial suspension (5 x 10^7 conidia / ml) of *T. hamatum*. The glucose in liquid culture medium was substituted with fungal (*P. viridicatum*) cell wall

preparation (2 mg/ml). Cultures were incubated at 28°C in a rotary shaker at 120 rpm for the desired time, and then centrifuged at 15,000-x *g* at 4°C for 10 min. The supernatant was dialyzed against water at 4°C for 24 h to eliminate residual glucose or N-acetyl-D-glucose amine. The dialysate was used as crude enzyme (1,3- β -glucanase and chitinase) source. Such enzymes were assayed according to the following methods: **(a) 1,3-β -glucanase:** 1,3- β - glucanase (exo-1, 3-β-D-glucosidase, EC 3.2.1. 58) was assayed by the release of free glucose from laminarin using the glucose oxidase reagent according to **Elad *et al* (1982).** The reaction mixture containing 1.0 ml crude enzyme source, 1.0 ml of 0.1 M citrate buffer (pH 5.1) and 1.6 mg soluble laminarin, was incubated at 38 ˚C for 1 h. The reaction was stopped by placing the mixture in boiling water bath for 10 min. Specific activity was expressed as µmol glucose /h /mg protein.

(b) Chitinase: Chitinase (1,4-β-poly-N-acetyl-D-glucoseaminidase, EC 3.2.1.14) was assayed by the release of N-acetyl-D-glucosamine from colloidal chitin according to **Reissig *et al* (1959).** The reaction mixture containing 1.0 ml crude enzyme source, 1.0 ml of 0.1 M citrate buffer (pH 5.1) and 1.6 mg colloidal chitin, was incubated at 38 ˚C for 2 h and boiling then stopped the reaction. Specific activity was expressed as µmol N-acetyl-D-glucosamine / h per mg protein. Protein estimation was carried out according to **Lowry *et al* (1951).**

Storage experiment: Conidia of *T. hamatum* were harvested from cultures grown on potato dextrose agar medium by scraping the conidia from the agar surfaces with a spatula and suspending them in 0.05% Tween 20 (v/v). The suspension was filtered through four layers of sterile lens paper to remove hyphal debris. The conidial suspension was adjusted to 5 x 10^6 conidia / ml and 0.5 ml of this suspension was used to coat 50 g of rice grains (cv. `Giza-176', obtained from the Agriculture Research Center, Giza, Egypt). The coated grains were immediately dried by warm ventilation. A storage experiment was carried out in paper pouches (150 x 100 mm), each containing 50 g rice grains. The rice grains were inoculated by discs (10 mm diam) of a 7-day-old culture of *P. viridicatum* (one disc per pouch). The samples (rice grain without any treatment [control], rice grains with *P. viridicatum* alone, rice grains with both *P. viridicatum* and *T. hamatum*, and rice grains with *T. hamatum* alone) were stored at room temperature (25±3°C) for 6 months. Control samples were used for each treatment.

RESULTS AND DISCUSSION

In the present work, 29 rice samples were analyzed. Ten of the twenty nine samples were found to be contaminated with citrinin **(Table, 1)**. Similarly, in Egypt, 39.40% of 274 rice samples were contaminated with citrinin **(El-Sayed, 1996). Ammar *et al* (2000)** reported that the mycotoxin citrinin represents a serious problem in fungal food-poisoning in countries with a hot climate, the humans renal system is affected and the mitochondrial respiratory chain was identified as a possible sensitive target for this toxin. In the United Kingdom, 26 of 44 cereal samples were contaminated with citrinin **(Scudamore and Hetmanski, 1995). Martins *et al* (2002)** found that

Table 1. Natural occurrence of citrinin in rice grain samples collected from different locations in Egypt

Location (Governorate)	No. of samples collected	No. of Samples with citrinin	Citrinin (µg /rice grains)
El-Dakahlia	6	2	0.813; 0.987
El-Gharbaia	5	1	0.274
El-Sharkia	8	3	0.137; 0.635; 1.427
Kafr El-Sharkia	10	4	1.068; 0.643; 0.223; 0.291
Total	29	10	

Moisture content of rice samples ranged between 1.2% and 5.2% , and in the majority of samples was ≤ 3%.

the level of contamination of apple samples with citrinin was 3.9%. The rice grains are subject to attack by numerous citrinin-producing species of the genera *Aspergillus* and *Penicillium* **(Frisvad and Damson, 1991).** In the present work, citrinin levels in the contaminated rice samples were not serious, hence the LD50 of citrinin has been reported as about 50 mg/kg for oral administration to the rat, 35-58 mg/kg to the mouse and 19 mg/kg to the rabbit. However, it decomposed after heating in water at 140ºC and produced citrinin H2 (3-(3,5-dihydroxy-2-methylphenyl)-2-formyloxy-butane).
Citrinin H2 did not show significant cytotoxicity to hela cells up to a concentration of 200 µg/ml (% cytotoxicity: 39%) in 63 h of incubation, but citrinin showed severe toxicity at a concentration of 25 µg/ml (% cytotoxicity: 73%) **(Hirota *et al* 2002)**.

Total fungal count **(Table, 2)** was 6.79 x 10^4 viable conidia or spores per gram rice. **Table (2)** shows that the most predominant genus was *Aspergillus* (63% of fungal total count), followed by *Penicillium* spp. (12.88% of fungal total count). *Aspergilli* were represented by 22 species. *A. niger, A. flavus, A. fumigatus, A. terreus* and *A. sydowii* had the highest ratio of occurrence, accounting for 27.63%, 7.80%, 12.50%, 3.68% and 3.22%, respectively, compared with the other isolated *Aspergillus* spp. *Aspergillus niger* and *A. flavus* out of 74 fungal species showed the highest occurrence, in 24 cases of the 50 trials used for isolation. Fourteen fungal genera, namely, *Aspergillus fumigatus, A. terreus, A. sydowii, A. flavus* var.*columnaris, A. candidus, A. parasiticus, Cladosporium cladosporioides, C. herbarum, C. sphaerospermum, Fusarium oxysporum,*

Table 2. Total (calculated per g wet weight of rice grains in every sample) and percentage counts, number of isolations, and occurrence remarks of fungal genera recovered from 29 grain samples tested on glucose Czapek's agar medium at 28 ± 2 °C

Fungal Species	Total Count	% of total fungi	Number of Cases isolation	*Occurrenc remarks
Total Counts	67896			
Acremonium	80	0.1178		
A. stricium W.Gams	80	0.1178	3	R
Aspergillus	42765	62.9860		
A. *niger* V Tiegh	18761	27.6319	42	H
A. flavus Link	5294	7.7972	37	H
A. fumigatus Fresenius	8465	12.4676	14	M
A. terreus Thom	2501	3.6835	23	M
A. sydowii (Bain & Sart.) Thom&Churt	2186	3.2196	17	M
A. flavus var.*columnaris* Raper&Fennell	397	0.5847	14	M
A. candidus Link	1025	1.5097	14	M
A. parasiticus Spear	1062	1.5642	13	M
A.vrsicolor (Vuill) Tiraboschi	586	0.8631	7	L
A. ridulans var. *latus* Thom & Raper	326	0.4801	6	L
A.ustus (Bain.) Thom & Church	214	0.3152	4	R
A. chevaliert (Mangin) Thom&Church	397	0.5847	4	R
A. clavatus Desmazieres	192	0.2828	4	R
A. ochraceus Wilhelm	210	0.3093	11	R
A. quadrilineatus Thom & Raper	187	0.2754	4	R
A. nidulans (Eidam) Wint	182	0.2680	3	R
A. niueus Blochwitz	115	0.1693	3	R
A .wentit Wehmer	162	0.2386	3	R
A. carneus (V Tiegh.) Blochwitz	140	0.2062	2	R
A. regulosus Thom &Raper	72	0.1060	1	R
A. sulphureus (Fres.) Thom & Church	56	0.0824	2	R
A. melleus Yukawa	207	0.3049	1	R
A. terricola Marchal	28	0.0412	1	R
Botryotrichum	142	0.2091		
B. piluliferum Saccarob & Marchal	142	0.2091	3	R
Chaetomium	166	0.2445		
C. globosum Kunze ex Fries	72	0.1060	2	R
C. olivaceum Cook & Ellis	94	0.1384	3	R
Cladosporium	9447	13.9139		
C. cladosporioides (Fres.) de Vries	3720	5.4789	15	M
C. herbarum (Pers.) Link ex. Fr.	2082	3.066	10	M
C. sphaerospermum Penzig	3645	5.3685	12	M

Table 2. Con't.

Fungal Species	Total Count	% of total fungi	Number of Cases isolation	*Occurrence remarks
Circinella	276	0.4065	1	R
C. muscae Van Tieghem				
Cuninghamella	87	0.1281		
C. echinulata (Thart.) Thaxt. Ex Blakestea	87	0.1281	1	R
Curvuularia	167	0.2459		
C. pallesens Boedijn	127	0.1870	1	R
C. lunata (wakker) Boedijn	40	0.0589	2	R
Epicoccum	62	0.0913	1	R
E. purpurascens Ehrenb. Ex. Schlecht	62	0.0913		
Fusarium	2527	3.7218		
F. oxysporum Schecht ex. Fri	1587	2.3374	22	M
F. solani (Mart.) Sacc.	426	0.6274	18	M
F. semitectum Berk & Rav	48	0.0706	7	L
F. moniliform var *anthophilum* (A.Braum)	67	0.0987	2	R
F.moniliform Sheldon	317	0.4669	1	R
F. equiseti (Corda)Sacc	82	0.1208	1	R
Fusidium	30	0.0442		
F. viride Grove	30	0.0442	1	R
Humicola	281	0.4138		
H. grisea Traaen	281	0.4138	1	R
Mucor	202	0.2975		
M. racemosus Fresenius	127	0.1870	5	R
M. hiemalis Wehmer	75	0.1105	5	R
Myrothecium	247	0.3679		
M. verrucaria (Alberthini & Schwinitz Ditmer)	247	0.3679	1	R
Neurospora	124	0.1826		
N. crassa Shear & Dodge	124	0.1826	1	R
Paecilomyces	312	0.4595		
P. variott Bainier	312	0.4595	6	L

Table 2. Con't.

Fungal Species	Total Count	% of total fungi	Number of Cases isolation	*Occurrence remarks
Pyricularia	1207	1.7777		
P.oryzae Briosi & Cavara	1207	1.7777	10	M
Penicillium	8743	12.8770		
P. chrysogenum Thom	1847	2.7203	13	M
P. corylophilum Dierckx	1081	1.592	17	M
P. cyclopium Westling	1207	1.7777	8	L
P. nigricans (Bainier)Thom	321	0.4728	7	L
P. capsulayum Raper & Fennell	654	0.9632	6	L
P. oxalicum Currie & Thom	811	1.1944	5	R
P. waksmani Zaleski	317	0.4669	5	R
P. seteckii Zaleski	252	0.3712	4	R
P. regulosum Thom	64	0.0943	1	R
P. purpuogenum Stoll	60	0.0883	1	R
P. citrinium Thom	37	0.0545	3	R
P. albidum Sopp	286	0.4212	3	R
P. variabile Sopp	267	0.3932	2	R
P. stoloniferum Thom	265	0.3903	3	R
P. viridicatum Westling	1274	1.8764	7	L
Scopulariopsis	210	0.3093		
S. brevicoulis (Sacc.) Bainier	210	0.3093	4	R
Sepedonium	132	0.1944		
S. chrysospermum (Bulliard) Fries	132	0.1944	2	R
Sordaria	27	0.0397		
S. fumicola (Roberge) Griffiths & Seaver	27	0.0397	1	R
Stachybotrys	27	0.0397		
S. chartarum (Ehrenb. Ex. Link Hughes)	27	0.0397	1	R

Table 2. Con't.

Fungal Species	Total Count	% of total fungi	Number of Cases isolation	*Occurrence remarks
Stemphylium	87	0.1281		
S. botryosum Wailroth	87	0.1281	2	R
Syncephalastrum	164	0.2415		
S.racemosus (cohn) Schroeter	164	0.2415	1	R
Trichoderma	172	0.2533		
T.viride Pers. Ex.S.F. Gray	172	0.2533	5	R
Trichothecium	30	0.0442		
T.roseum (Pers.) Link	30	0.0442	1	R
Trimmatostrom	42	0.0618		
T.salicis Carda	42	0.0618	1	R
Sterile mycelia (white colour)	140	0.2062	2	R

* Out of 29 samples:
H = High occurrence, >24 cases.
M = Moderate occurrence, 12 - 24 cases.
L = Low occurrence, 6 - 11 cases.
R = Rare occurrence, <6 cases.

F. solani, *Pyricularia oryza*, *Penicillium chrysogenum* and *P. corylophilum* showed moderate occurrence. In this connection, **Kozakiewicz *et al* (1996)** reported the predominance of *Aspergillus* and *Penicillium* species in rice and cereal grains. **Frisvad and Damson (1991)** reported that several species of *Aspergillus* and *Penicillium* comprised mainly the storage molds in cereals. Also, **Shetty *et al* (1994)** found that *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* were the most important mycotoxigenic fungi isolated from rice, sorghum and groundnuts. The other fungal species ranged between low and rare occurrence. Many species of *Penicillium* are known to produce mycotoxins, including *P. aurantiogriseum* (penicillic acid), *P. viridicatum* (viridicatin), *P. verrucosum* (ochratoxin), *P. islandicum* (luteoskyrin), *P. variabile* (rugulosin), *P. crustosum* (penitrem A), *P. griseofulvum* (patulin, griseofulvin), *P. citrinum* (citrinin), and *P. crateriforme* (rubratoxin), as well as many others **(Abbott 2002, Pitt 2000, Pitt and Hocking**

1999). Hubert *et al* (2004) reported that toxigenic and allergen-producing fungi represent a serious hazard to human food and animal feed safety. They isolated ninety-four fungal species from mite-infested samples of seeds taken from Czech seed stores. Fungi were isolated from the surface of four kinds of seeds (wheat, poppy, lettuce, and mustard).

The isolated fungi were screened for their production of citrinin to select the most productive (data not presented). The most productive one was *P. viridicatum*, which was selected in this study as a good model for seedborne pathogen-produced citrinin. Citrinin (extracted from culture medium of *P. viridicatum*) was confirmed chemically (it shows sky blue fluorescence with a similar intensity under 366 and 254 nm UV light). Also, a disc screening test shows citrinin slightly toxic against brine shrimp larvae, producing 40% mortality. Moreover, inhibition of growth of *Bacillus brevis* growth (results not shown) confirmed (in addition to a chemical confirmatory test) the presence of citrinin in the cleaned chloroform extract of culture media of *P. viridicatum*. These findings demonstrated the production of citrinin by our model, *P. viridicatum*, as shown elsewhere **(Oh *et al* 1998). Table (3)** shows that the production of citrinin by *P. viridicatum* (in single culture without *T. hamatum*) increased with lengthening of the incubation period until it reached a maximum after 8 days in both liquid broth and agar plates, then the amounts were reduced by an increased incubation period. However, the amounts of citrinin produced by *P. viridicatum*, in mixed culture with *T. hamatum*, decreased with longer incubation periods until citrinin disappeared after 8 days of incubation, indicating an antagonistic potential of *T. hamatum* against citrinin production by *P. viridicatum* or degradation ability of *T. hamatum. Trichoderma* spp. is considered effective biocontrol agents **(Elad *et al* 1982; Sinha, 2001; Sivan and Chet, 1989).** Many microorganisms, including bacteria, actinomycetes, yeasts, molds and algae can degrade mycotoxins **(Monastyrskii and Yaroshenko, 2000).** Some *Trichoderma* spp., such as *T. viride*, are able to degrade the mycotoxins produced by plant pathogenic fungi **(Sriram *et al* 2000).** On the other hand, **Table (4)** shows that the amount of citrinin was non-significantly reduced in the presence of *T. hamatum* with longer incubation periods, indicating that *T. hamatum* cannot degrade citrinin. Consequently, we studied another alternative mechanism used here, mycoparasitism by means of production of cell-wall-degrading enzymes (chitinase and 1,3- B-glucanase). *Trichoderma* spp. have different mechanisms, such as antibiosis, competition, mycoparasitism and promotion of plant growth **(Ghisalber and Sivasithamparam, 1991). Rocha-Ramírez *et al* (2002)** found that the soil fungus *Trichoderma atroviride*, a mycoparasite, responds to a number of external stimuli. In the presence of a fungal host, *T. atroviride* produces hydrolytic enzymes and coils around the host hyphae. In response to light or nutrient depletion, asexual sporulation is induced. The overgrowth of *T. hamatum* on *P. viridicatum* was significantly enhanced with an extended incubation period on agar plates, being 26.0, 31.7 and 3.87 mm after incubation for 72, 96 and 120 h, respectively. Similar results were obtained when *T. harzianum* T-203, the mycoparasite of *R. solani* and *S. rolfsii*, served as the antagonist **(Sivan and Chet, 1989).** Dual

Table 3. Effect of incubation with *Trichoderma hamatum (T.h.)* on citrinin production by *Penicillium viridicatum (P.v.)* at different incubation periods in two media

Incubation period (days)	Fungal interaction	Citrinin production in:	
		Broth medium (µg / 50 ml)	Solid medium (µg / plate)
2	*P.v.*	42.20	11.03
	P.v. + *T.h.*	19.23	8.01
4	*P.v.*	67.29	12.67
	P.v. + *T.h.*	8.91	4.85
6	*P.v.*	104.95	16.54
	P.v. + *T.h.*	6.09	2.62
8	*P.v.*	146.05	14.65
	P.v. + *T.h.*	0.00	0.00
10	*P.v.*	136.84	10.99
	P.v. + *T.h.*	0.00	0.00
12	*P.v.*	128.46	7.56
	P.v. + *T.h.*	0.00	0.00
14	*P.v.*	126.7	5.61
	P.v. + *T.h.*	0.00	0.00
L.S.D. at :	0.05	8.15	6.45
	0.01	13.70	9.08

Table 4. Changes in citrinin concentration (percent recovery) in culture of *Trichoderma hamatum* after different incubation periods

Incubation period (days)	Recovery of citrinin (%)
0	100
2	98.3
4	91.7
6	93.3
8	88.0
10	85.7
L.S.D. at: 0.05	15.7
0.01	22.3

Recovery (%) = a x 100/b, where a = amount of citrinin (µg / ml) detected in treated test tube after 0, 2, 4, 6, 8 and 10 days of incubation with *T. hamatum*: b = amount of citrinin (50 µg / ml in 0.1 M NaHCO3) in the test tube after 0, 2, 4, 6, 8 and 10 days of incubation without *T. hamatum*.

inoculation of peat based horticulture substrate with a mixture of four species of arbuscular mycorrhizal fungi and fungal biocontrol agent *Trichoderma harzianum* showed a significant positive effect on the growth and flowering of cyclamen plants. Inoculation substantially decreased plant mortality caused by spontaneous infection by the fungal pathogen *Cryptocline cyclaminis* **(Dubsky *et al* 2002)**. **Table (5)** shows that incubation of *P. viridicatum* discs with *T. hamatum* conidia (10^6conidia / ml) caused significant and highly significant decreases, respectively, in the viability of *P. viridicatum* discs after 2 and 6 days, and no viability at all after 8 days of incubation.

Table 5. Survival of mycelial disks of *Penicillium viridicatum* after treatment with conidia of *Trichoderma hamatum* (10^6 conidia / ml)

Incubation period (days)	Viability of mycelial disks (%)
2	100
4	83.3
6	7.00
8	0.00
L.S.D. at: 0.05	12.65
0.01	27.34

These results are in agreement with findings by **Sivan and Chet (1989).** Chitinase and 1,3-B-glucanase enzyme activities of *T. hamatum* on *P. viridicatum* mycelia were increased by lengthening the incubation period until it reached a maximum after 72 and 48 h, respectively, in case of chitinase and gluconase, after which remarkable decreases were obtained with longer incubation periods **(Table, 6)**. Similar results were obtained with *T. harzianum* T-203 strain grown in media amended with hyphal cell walls of *F. oxysporum* f.sp. *melonis* as the sole carbon source, the maximal induction of chitinase and glucanase being obtained after 72 and 48 h incubation, respectively **(Sivan and Chet, 1989). Muhammad and Amusa(2003)** reported that *T. harzianum* grew on the mycelia of all the tested fungal pathogens (*Sclerotium rolfsii, Fusarium oxysporum, Pythium aphanidermatum* and *Macrophomina phaseolina*). The results given here **(Tables 5 and 6)** confirmed the mycoparasitic potential of our biocontrol agent, T. *hamatum*, and its ability to degrade cell walls of seed borne *P. viridicatum*. Table 7 shows that *T. hamatum* reduced the amount of citrinin by *P. viridicatum* on rice grains (mixed cultures). Such a decrease in citrinin production due to the presence of *T. hamatum* in mixed culture was directly proportional to the gradual increase of storage periods by 45.50%, 66.38%, 100.30%, 146.80%, 150.00% and 175.63% compared with the respective amounts of rice samples inoculated with *P. viridicatum* alone at 1, 2, 3, 4, 5 and 6 months, respectively. The decrease in the amount of citrinin on rice grains by *T. hamatum* **(Table, 7)** may be due to a drop in the viability of *P. viridicatum* **(Table, 5)**, and lysis by cell wall lytic enzymes **(Table, 6)**. In this connection**, Mishra *et al* (2000)** reported that *T. harzianum* is known as a mycoparasite of a number of plant pathogens by lysis of fungal cell walls *via* production of cell wall degrading enzymes. Other biocontrol agents were used against mycotoxins, such as *Pichia anomala* J 121, which

Table 6. Specific activity of chitinase and 1,3 - β-glucanase produced by *Trichoderma hamatum* during growth on liquid medium containing cell walls of *Penicillium viridicatum* (2 mg / ml) as sole carbon source

Incubation time (h)	Chitinase (μ mol glucose amine / h / mg protein)	1,3- β-glucanase (μ mol glucose /h / mg protein)
12	5.57	19.63
24	12.13	46.03
36	19.73	100.13
48	32.57	184.00
60	36.53	176.67
72	41.17	122.20
84	29.47	128.20
96	18.50	53.13
L.S.D. at: 0.05	1.45	9.27
0.01	4.26	12.59

Table 7. Effect of different storage periods on citrinin production (μg / 50 g rice grains) by *Penicillium viridicatum (P.v.)* on rice grains in the presence and absence of *Trichoderma hamatum (T.h.)*

Treatment	Citrinin production (μg/50 g) on rice grains					
	Storage period (months)					
	1	2	3	4	5	6
Rice grains						
(control)	0	0	0	0	0	0
Rice grains + *P.v.*	1.50	2.23	2.67	3.17	3.26	3.40
Rice grains + *T.h.* + *P.v.*	0.58	0.67	0.84	0.96	1.01	1.03
Rice grains + *T.h.*	0	0	0	0	0	0
L.S.D. at: 0.05	0.97	1.42	1.79	2.01	2.13	2.21
0.01	1.07	1.67	2.07	2.62	2.98	3.06

inhibited the growth and toxin of *Penicillium roqueforti* in moist grains **(Boysen *et al* 2000).** The ability to protect rice grains against seed borne *P. viridicatum* and its mycotoxin citrinin by application of *T. hamatum* producing cell wall lytic enzymes (chitinase and 1,3-B-glucanase) opens up the way for the development of cell wall lytic enzymes as an alternative to direct control of seed borne plant pathogenic fungi and possibly other diseases affecting plant health.

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التواجد الطبيعي للسم الفطري سترينين في حبوب الأرز ومقاومة الفطرة المنتجة له بيولوجيا بفطرة التريكودرما هاماتم

[48]

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2- قسم أمراض البذرة- معهد أبحاث أمراض النبات - مركز البحوث الزراعية - الجيزة - مصر

تم جمع عينات الأرز من محافظات جمهورية مصر العربية الآتية ؛ الشرقية ، الغربية ، الدقهلية ، كفر الشيخ .

وقد وجد تلوث 10 عينات بالسم الفطري سترينين من اصل 29عينة ، وبلغ متوسط عدد جراثيم الفطريات حوالي 67900 جرثومة لكل جرام من حبوب الأرز. وتم عزل الفطريات من الحبوب ، ووجد آن الفطريات المعزولة تنتمي إلى 28 جنس تنتمى الى 47نوع. وكانت الأجناس السائدة لفطريات الأسبيرجلس ، والكلأدوسبوريوم والبنيسليوم. ومثل فطرة الأسبيرجلس 22 نوع. وقد سجلت فطريات الأسبيرجلس نيجر ألأسبيرجلس فلأفس أعلى تواجد مقارنة بالفطريات الأخرى .

وعند اختبار الفطريات المعزولة لأنتاج السترينين على وسط الجلوكوز ونترات الأمونيوم وعلى حبوب الذرة ،استطاعت فطرة البنيسليوم فيريديكاتم إعطاء أقصى كمية ، و لذلك تم اختيارها لتجارب المقاومة البيولوجية.

استطاعت فطرة التريكودرما هاماتم تقليل كمية الستورينين المنتج بواسطة البنيسليوم فيريديكاتم مقارنة بالعينة القياسية.

وقد لوحظ زيادة التطفل الفطري للتريكودرما هاماتم على فطرة البنيسليوم فيريديكاتم ونقص حيوية كونيديات فطرة البنيسليوم مع زيادة فترات تحضين الفطرتان سويا ، ولوحظ زيادة أنزيمات الكيتيناز و 1 و3- جايكوناز بواسطة فطرة التريكودرما هاماتم بزيادة فترة التحضين حتى أقصى كمية بعد 72 و 84 ساعة ، على الترتيب .

ووجد نقص كميات الستورينين لفطرة البنيسليوم فيريديكاتم على حبوب الأرز عند نموها في مزرعة مختلطة مع فطرة التريكودرما هاماتم وذلك مقارنة بالعينة القياسية .

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