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TIME COURSE OF VIRUS-LIKE PARTICLES (VLPS) DOUBLE-STRANDED RNA ACCUMULATION IN TOXIGENIC AND NON-TOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS*

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ABSTRACT

Two strains of *Aspergillus flavus*, non-toxigenic NRRL 6550 and toxigenic NRRL 5940, were studied over a period of 44 days, in order to detect the presence of virus-like particles (VLPs) by means of electron microscopy (EM) and nucleic acids electrophoresis. Only the toxigenic strain contained VLPs, presenting three-segmented dsRNA. An increase in VLPs number was observed during the exponential phase of fungal growth, up to day 12 of culture; after this, higher levels of aflatoxin production in toxigenic NRRL 5940 mycelia occurred in parallel with decreased VLPs replication.

Key words: mycovirus, double-stranded RNA, virus-like particles, *Aspergillus flavus*

INTRODUCTION

Virus-like particles (VLPs) were discovered in 1962 by Hollings, who extracted three types of particles from the fruit bodies of diseased mushrooms in England. Differents VLPs may be present in a single fungal strain (9) and most of them are found to posses segmented, double-stranded RNA genomes (13). They are widespread among fungi and, despite their presence being typically symptomless (3), some exceptionally cause adverse effects on their hosts. Examples of these effects are 'La France' disease of the white-button mushroom *Agaricus bisporus* (11, 12), expression of lethal toxins encoded by viral dsRNA in certain strains of *Saccharomyces cerevisiae* and *Ustilago maydis* (1, 2), and induction of hypovirulence in the chestnut blight fungus *Cryphonectria parasitica* associated with dsRNA (7).

Several *Aspergillus* species have been found to be infected with mycoviruses, including *A. foetidus* (4, 17), *Aspergillus* section *Circundati* and *Fumigati* (23), *Aspergillus* section *Nigri* and section *Flavi* (8, 22).

Certain strains of *Aspergillus flavus* and all strains of *A. parasiticus* have been reported to synthesize aflatoxins, which are highly toxic and potent carcinogenic secondary metabolites (14). The absence of aflatoxin production in NRRL A - 12268, a strain of *Aspergillus flavus*, originally correlated with the occurrence of nucleic acid-free VLPs (16). However, a more extensive investigation failed to establish a relationship between absence of VLPs and aflatoxin production in other fungal strains (10, 24). Double stranded RNA particles in non-toxigenic strain of *A. flavus* (NRRL 5565 = NRRL A -12268) as shown by Mackenzie and Adler (16) had a genome identical in size to a dsRNA found in *Penicillium chrysogenum* (20). Strain NRRL 5565 cured of dsRNA infection by exposing it to an RNA synthesis inhibitor (cicloheximide) developed the ability to produce aflatoxins (18); furthermore, after artificial infection with virus isolated from *P. chrysogenum*, the production of aflatoxins ceased. However, Elias and Cotti (8) detected dsRNA genomic elements in 10 out of 92 isolates of *Aspergillus* section *Flavi* yet the aflatoxin-producing ability of the isolates was not affected by dsRNA infection (16, 24).

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Strains that produced high levels of aflatoxins were as likely to be infected by dsRNA as strains that produced no aflatoxins. Moreover, curing strains infected with dsRNA did not result in altered aflatoxin-producing ability, as previously suggested (10, 18, 19, 20). The high frequency of *A. flavus* in the ecosystem, its serious implications to human and animal health due to ingestion of food contaminated with aflatoxins, and the limited data on fungi/VLPs interactions, motivated the present investigation. We therefore evaluated the presence of VLPs in toxigenic and non-toxigenic strains of *A. flavus* over a total growth period of 44 days, by electron microscopy and electrophoresis techniques.

MATERIALS AND METHODS

Strains of *Aspergillus flavus*

The two strains of *A. flavus* used, namely: toxigenic NRRL 5940 (producer of aflatoxin B1 and B2), and non-toxigenic NRRL 6550, were provided by Northern Regional Research Laboratory (NRRL), Department of Agriculture - Illinois – USA.

Spore suspension

A. flavus was grown on potato dextrose agar at 25ºC for 7 days. Spores were harvested by rinsing the culture tube with sterile 0.1M sodium phosphate buffer (PBS) pH 7.2 and then transferred to a sterile tube containing a drop (0.05 mL) of Tween 80, which was added to disperse the spores and to homogenize the suspension. Spore counts were done in a Neubauer chamber; the final suspension was adjusted to approximately 10^5 spores per 0.3 mL with sterile 0.1M PBS, pH 7.2.57

Growth Curves

Growth curves were established on the basis of three determinations of fungal dry weight values, recorded every 4 days over a period of 44 days; the first measure was taken after 2 days of cultivation. Spore suspensions were inoculated into 12 Roux bottles containing yeast extract sucrose (YES) semi-synthetic medium, normally employed in B1 Aflatoxin (AFB1) production tests. To avoid light degradation of AFB1, incubation was carried out at 25ºC in total darkness. At the indicated time intervals, the Roux bottles content was filtered through Whatman paper number 1. For quantitation of AFB1 levels, broth and mycelium were used for the toxigenic samples and only the mycelium for the non-toxigenic samples. Mycelia were washed with sterile sodium 0.1M PBS, pH 7.2, and incubated at 80ºC for 4 days, in order to achieve a constant weight of the dried fungal mass.

Quantification of aflatoxin

The extraction of aflatoxin was carried out as previously described (5) and quantitated in a xenon bulb photodensitometer (Shimadzu CS9000).

Preparation of samples for EM and Electrophoresis

According to the growth curves and AFB1 production, the following time schedule was established to search for VLPs: 2, 4, 12, 20, 24, 28 and 40 days for NRRL 6550 and 2, 4, 12, 28, 32, 36 and 40 days for NRRL 5940. At the indicated time intervals, Roux bottles contents were filtered, as previously described, and the mycelia were washed 3 times with sterile distilled water; the recovered broth and mycelia were conditioned and stored at -20ºC until use.

Electron microscopy

Sampled mycelia were freeze-thawed 4 times in order to break the cell wall and release VLPs. The material was centrifuged at 353xg for 20 minutes and the supernatant ultracentrifuged at 153 610xg for 4 hours. Using the “reverse drop” technique, the ultracentrifuged sediment was allowed to adhere to a formvar-carbon coated grid (300 mesh) for 30 minutes. The grid was then stained with 2% potassium phosphotungstate (PTK), pH 6.4, for 5 minutes and examined for VLPs in a Phillips EM 400 T microscope. Estimates of VLPs number at each time point studied were done by counting particles in only one grade per period. These estimates were done so as to evaluate the amount of particles present in mycelia and to compare it with the concentration of dsRNA.

Extraction of nucleic acids

The mycelium (0.2 g) of each strain was ground under liquid nitrogen and the frozen powder was suspended in 0.1M TRIS/HCl pH 7.3 containing 10% SDS and Proteinase K (PK). The sample was incubated for 30 minutes at 37ºC, and the nucleic acids were extracted with phenol:chloroform:isoamyl alcohol (25:24:1). Cellular debris was removed by centrifugation (3920xg, 5 min.); nucleic acids were precipitated with ethanol and 20% NaCl (-20ºC overnight), centrifuged (6625xg, 30 minutes, 4ºC), and dried at room temperature.

VLPs from the filtrate were concentrated by PEG 6000 precipitation (15). Briefly, the filtrate was diluted in 50 mM glycin, pH 9.0 added of 10% tryptose phosphate buffer (TPB) and after 30 sec the pH was adjusted to 9.0 with 1N NaOH. This material was agitated (30 sec) and centrifuged (10 000xg, 30 min., 4ºC); pH of the supernatant was adjusted to 7.5 with 1N HCl. To each 10 mL of supernatant, 0.8g of PEG 6000 was added and the pH adjusted to 7.5; the sample was then incubated in a shaker (1h and 30 min, 4ºC) and centrifuged (10 000xg, 20 min, 4ºC). For the RNA extraction, the pellet obtained was resuspended in 10 mL of 150 mM $NaHPO_4$ 9.5, and the pH was adjusted to 9.0; the sample was then sonicated twice (30 sec, 50 mA, on ice), agitated (30 sec) at room temperature and centrifuged (10 000xg, 30 min, 4ºC). The pH of the supernatant was adjusted to 7.2 and 7.5 and the material was stored at -20ºC until use for electrophoresis of nucleic acids.

The extracted nucleic acids were separated on 7.5% polyacrylamide gel (5h, 40mA) as whole samples and after digestion with DNase I and RNase I "A" in DNase buffer (50 mM TRIS, pH 7.5 added of 10 mM $MgCl_2$) using NCDV (Nebrask Calf Diarrhea Virus) dsRNA as standard (silver nitrate staining). Agarose gel electrophoresis of the material was also performed (0.8% agarose, 4h, 40V) using the 1 Kb DNA ladder molecular markers (ethidium bromide staining). Band concentration and molecular weight markers determinations were made with the aid of the computer program "DNAstar-Computer Systems for Molecular Biology and Genetics" (1990).

RESULTS

The exponential phase of growth started on day 2; maximum biomass accumulation was recorded on day 24 for the non-toxigenic strain NRRL 6550 and on day 36 for the toxigenic strain NRRL 5940. The production of aflatoxin B1 in mycelia of the toxigenic strain (NRRL 5940) began on day 4 and peaked at day 32 of culture. No production of aflatoxin B1 was noted in the non- toxigenic strain.

VLPs were detected in mycelia of the toxigenic strain at all time points studied and measured approximately 33nm in diameter (Fig. 1). The number of particles increased from day 2 to day 12 and decreased thereafter, dropping to very small numbers on days 28, 32, 36 and 40 (Table 1). No VLPs were observed in the mycelia of non-toxigenic strain, or in the culture filtrates of both strains.

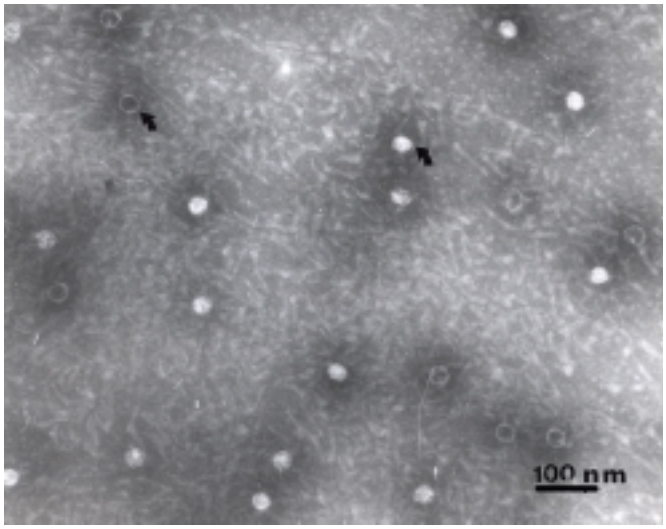

Figure 1. Direct Electron Microscopy (DEM) from the mycelium of *Aspergillus flavus* strain NRRL 5940 after 12 days of culture in YES medium, showing the presence of particles of approximately 33nm in diameter (149 000x).

Agarose and polyacrilamyde gel electrophoresis of nucleic acids from mycelia of the toxigenic strain revealed the presence of 3 bands at all the time points studied. The intensity of these bands increased from day 2 till day 12 of culture and tended to decrease thereafter (Table 1). The bands were unaffected by digestion with Proteinase K and DNase I but were sensitive to RNase I "A", indicating that they were dsRNAs. No bands were detected in nucleic acid extracts from mycelia of the non-toxigenic strain.

The *A. flavus* dsRNA migrated at positions equivalent to 3.7, 3.4 and 2.9 Kb of the DNA ladder, and those from other time points with the 3.5, 3.0 and 2.8 Kb positions (Fig. 2). The corrected size of the *A. flavus* dsRNA using RNA standards has not been determined yet. The estimated concentrations of dsRNA correlated with the data on number of VLPs visualized by electron microscopy (Table 1). The fungal biomass, concentration of aflatoxin B1 and estimates of dsRNA of the toxigenic strain are shown in Fig. 3.

DISCUSSION

Our comparison of growth curve, estimated VLPs number and concentration of dsRNA revealed a correlation between fungal growth, increase in VLPs count and concentration of dsRNA at day 12 of culture - a time when the fungus was undergoing a transition from trophophase (characterized by its primary metabolism) to idiophase (characterized by its secondary metabolism). Also, day 12 of culture was part of the exponential phase of growth, when cells are metabolically active. This, in fact, may have provided ideal conditions for viral replication and could explain the occurrence of greater number of VLPs at such time period. Some authors, on the other hand, have reported maximum viral replication associated with the stationary growth phase of fungi (6, 21, 22). In this study, we observed that, during the period when AFB1 concentrations were low (up to day 28 of

Table 1. VLPs numbers and dsRNA concentrations estimated by electron microscopy and electrophoresis.

Direct Electron Microscopy			Electrophoresis (Agarose Gel)	
VLPs Number			DsRNA Concentration	
Days	Mycelium	Filtrate	Mycelium (μg/μL)	Days
2	2+[b]	ND[d]	0,0075	2
4	3+[c]	ND	0,1455	4
12	4+[d]	ND	0,5975	12
28	1+[a]	ND	0,0025	28
32	1+	ND	0,0100	32
36	1+	ND	0,005	36
40	1+	ND	0,005	40

[a] 1+, 1 to 5 particles/grade; [b] 2+, 6 to 50 particles/grade; [c] 3+, 51 to 100 particles/grade; [d] 4+, countless; [e] ND, not detected.

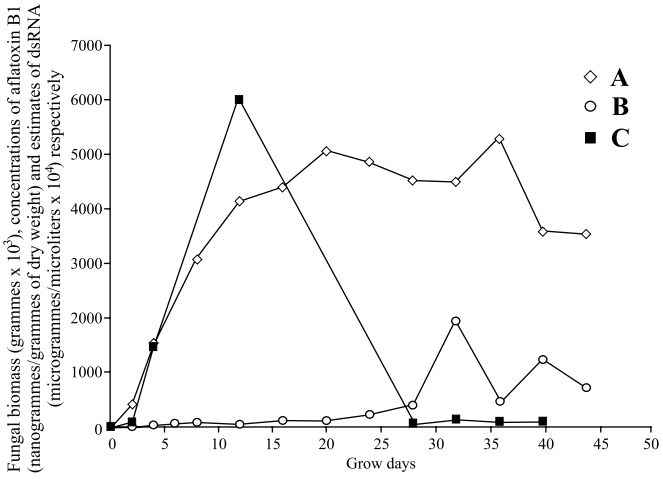

Figure 3. Diagram of dsRNA from VLPs detected in *Aspergillus flavus* strain NRRL 5940 at the time points studied (2 - 2 days, 3 - 4 days, 4 - 12 days, 5 - 28 days, 6 - 32 days, 7 - 36 days, 8 - 40 days). 1 and 9 - 1Kb DNA ladder.

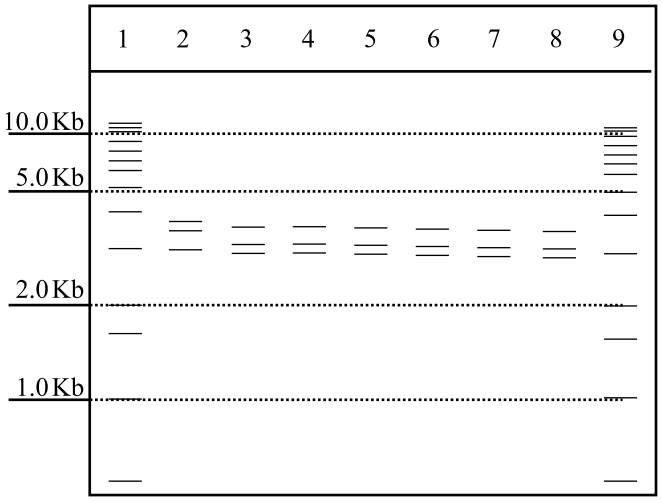

Figure 2. Time course of fungal dry weight, aflatoxin B1 accumulation and dsRNA/VLPs in *Aspergillus flavus* strain NRRL 5940. A) Growth curve of *Aspergillus flavus* strain NRRL 5940 established on the basis of three determinations of fungal dry weights, recorded every 2 days over a period of 44 days; culture was performed in YES medium at 25ºC. B) Curve of amount of aflatoxin B1 per gram of dry weight of *Aspergillus flavus* strain NRRL 5940 mycelium; average of three determinations. C) Estimates of dsRNA concentration in mycelium of *Aspergillus flavus*, strain NRRL 5940, after electrophoresis on agarose gel and analysis of bands using the computer program "DNAstar-Computer Systems for Molecular Biology and Genetics" (1990).

culture), VLPs counts and viral dsRNA concentrations fluctuated and peaked on day 12. Conversely, when the AFB1 levels reached higher values (after day 28), the VLPs counts and dsRNA concentrations were low.

The results obtained in this study apparently showed a lack of correlation between production of aflatoxins and presence of VLPs in the *A. flavus* strain NRRL 5940. This agree with the findings of Elias and Cotti (8), who detected dsRNA in 10 out of 92 strains of *Aspergillus* seção *Flavi* and found no correlation between production of aflatoxins and presence dsRNA, as previously suggested (16, 24). According to these authors, the molecular size estimates of the dsRNA components ranged from 0.4 to greater than 10.0 Kb, relative to the migration of dsRNA fragments of phage submitted to digestion with *hin*dIII. The number of dsRNA components infecting an individual isolate ranged from one to nine, and apparently no isolate contained the same molecular size as estimated by eletrophoretic migration in 1.0% agarose.

It is quite possible that different types of VLPs may influence positively or negatively the production of aflatoxins by activating or inhibiting aflatoxin gene expression**.** The results obtained in this study can be used in experimental transmission to other fungal strains.

RESUMO

Distribuição de vírus RNA de fita dupla em cepas de *Aspergillus flavus* toxigênica e não toxigênica

Neste trabalho, foram estudadas duas cepas de *Aspergillus flavus* não toxigênica (NRRL 6550) e toxigênica (NRRL 5940). As cepas foram cultivadas durante 44 dias objetivando a pesquisa de partículas semelhantes a vírus (VLPs), por meio de microscopia eletrônica e pesquisa de ácidos nucléicos, através de eletroforese. Somente as cepas toxigênicas continham "VLPs", as quais apresentaram 3 segmentos de RNA de fita dupla. Um aumento no número de "VLPs" foi observado com 12 dias de cultivo, período correspondente a fase exponencial de crescimento de *A. flavus.*

Palavras-chave: micovírus, RNA de fita dupla, partículas semelhantes a vírus, *Aspergillus flavus*

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