PRODUCTION OF MYCOTOXINS BY GALACTOSE OXIDASE PRODUCING *FUSARIUM* USING DIFFERENT CULTURE MEDIA

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ABSTRACT

The original isolate of the galactose oxidase producing fungus *Dactylium dendroides*, and other five galactose oxidase producing *Fusarium* isolates were cultivated in different media and conditions, in order to evaluate the production of 11 mycotoxins, which are characteristic of the genus *Fusarium*: moniliformin, fusaric acid, deoxynivalenol, fusarenone-X, nivalenol, 3-acetyldeoxynivalenol, neosolaniol, zearalenol, zearalenone, acetyl T-2, and iso T-2. The toxicity of the culture extracts to *Artemia salina* larvae was tested.

Key words: Galactose oxidase, *Fusarium*, *Dactylium dendroides*, mycotoxins

INTRODUCTION

Galactose oxidase (EC 1.1.3.9) is a fungal enzyme secreted by *Dactylium dendroides* (3), *Gibberella fujikuroi* (1)*, Fusarium graminearum* (7, 11) and *Fusarium moniliforme* (13). This enzyme catalyses oxidation of D-galactose to galactohexodialdose, which reduces molecular oxygen to hydrogen peroxide, and has been used for the specific determination of D-galactose (2).

The first fungus capable to produce galactose oxidase was isolated in Curitiba, Brazil, in 1955, and classified as *Polyporus circinatus* (9). This classification was questioned by Nobles and Madhosingh (25), who considered that this fungus was *Dactylium dendroides,* the conidial form of *Hypomyces rosellus*, a parasite of mushroom cultures. Despite the controversy concerning the taxonomic identification of the Galactose Oxidase Producing Fungus (GOPF) and the efforts made by Kemmelmeier and Zancan for its identification (17,18,19,20,27), a taxonomic classification has not yet been achieved. Ögel *et al.* (26) were the first to report its sporulation. They also compared the DNA sequences of the galactose oxidase gene regions of *Cladobotrium dendroides* and of the fungus. They mentioned some resemblance between the conidia and conidiophores of *F. chlamydosporum* and GOPF, in addition to data shown by *Fusarium* of *Arthrosporiella* section, described by Booth (5). According to data reported by Ögel *et al.* (26), the fungus isolated in Curitiba was re-identified as belonging to the genus *Fusarium*.

Taking into account the importance of *Fusarium* in agriculture and health due to mycotoxins production (21), the present work aimed to correlate mycotoxins production with taxonomy (7). Five samples of galactose oxidase producing *Fusarium* were compared with GOPF for production of mycotoxins in the following solid substrates: rice (production of trichothecenes), corn (production of moniliformin and trichothecenes), agar (production of fusaric acid) and liquid medium (production of galactose oxidase) (22) and on the conditions described for the production of 3-acetyldeoxynivalenol (31). The production of the moniliformin, fusaric acid, 3-acetyldeoxynivalenol (ADON), deoxynivalenol (DON), fusarenone-X (FX), nivalenol (NIV), neosolaniol (NEO), zearalenone (ZEA), zearalenol (ZEOH), iso T-2 and acetyl T-2, typical of *Fusarium* fungi, was tested. The toxicity of extracts against *Artemia salina* larvae was also checked.

MATERIALS AND METHODS

Microorganisms. The following isolates of *Fusarium* were used: *Fusarium graminearum* strain IAPAR 2218 (Agronomic Institute of Paraná - PR); *F. graminearum* strain UEL 2118 R (State University of Londrina – PR, kindly offered by Dr. Elisa

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Yoko Hirooka) *F. graminearum* strain UnB 1269 (University of Brasília - DF); *F. moniliforme* var. *subglutinans* strain UnB 379; *F. acuminatum* strain UnB 356, and *Dactylium dendroides.* The original galactose oxidase producer strain (GOPF), isolated in Curitiba, was kindly provided by Dr. Glaci T. Zancan. These isolates were stored at the Laboratory of Chemistry and Physiology of Microorganisms of the Department of Biochemistry of the State University of Maringá - PR.

Growth rate, morphology and pigmentation. For comparisons of growth rate, morphology and pigmentation, fungi were initially cultured for four days at 25ºC in Potato Dextrose Agar (PDA), transfered in triplicate to Petri dishes and incubated under the same conditions. The growth rate (measured as colony diameter), morphology and pigmentation (colour) of the colonies were observed during four days, as described by Booth (5).

General conditions of growth for the production and extraction of mycotoxins. Growth of fungi and testing for mycotoxins were done as follows:

Moniliformin. The fungi were activated in PDA for 10 days, at 25ºC, and them transferred to corn medium (40 g of corn kernels and 40 ml of water), previously autoclaved for one hour in two consecutive days, and incubated for 21 days at 25ºC. The culture was lyophilized, fragmented in a mortar and the resulting powder stored at 5ºC. The moniliformin assay was carried out mixing 10 grams of the powder with 100 ml of chloroform and agitating at 28ºC for 48 hours in orbital shaker (121 rev. min^{-1}). Chloroform was removed by filtration and 100 ml of methanol/water (80:20, v/v) was added and shaked again for 48 hours. The extracts were combined and dried in a rotary evaporator, suspended in 2 ml methanol, divided in two aliquots of 1 ml and stored at 5ºC, in the dark, until use.

Fusaric acid. Richard's medium and the conditions described by Davis (10) were used. After growth for 14 days at 28ºC, the cultures were placed in freezer at –15ºC for 48 hours prior to extraction. Then, the agar was heated in a microwave oven and the mycelium mass was removed with tweezers. The agar was acidified to pH 4.0 and extracted three times with 10 ml of ethyl acetate, combined and concentrated in a vacuum rotary evaporator until drying. Residues were suspended in 2 ml of ethyl acetate and stored under refrigeration.

Trichothecenes. Trichothecenes were investigated using the conditions describeb by Silva (30). Fungi were activated in PDA and then transferred to rice. Ten grams of rice grains were horizontally placed in a 70 ml tube, added with water to obtain 40% of initial humidity. Tubes were autoclaved twice at 121ºC in two consecutive days. The tubes were inoculated with the fungi, and incubated for 21 days at 25ºC. Each culture was extracted adding 50 ml of ethyl acetate and remained at room temperature during 12 hours. The organic extract of each tube was recovered by filtration. After extraction with ethyl acetate, the residue was extracted again with 50 ml of methanol/water (6:4, v/v) under the same conditions. The extract was recovered

by filtration and combined with the previous extract, treated with anhydrous sodium sulphate and concentrated. The residue was dissolved in 2 ml of benzene/acetone (2:1, v/v) and passed through a column (Pasteur pipettes 10 x 1 cm) containing 7 ml of silica gel. The columns were washed with 20 ml of benzene to remove lipids and then eluted with 20 ml of benzene/acetone (1:1, v/v). Eluates were concentrated and the residues suspended in 2 ml of acetone and stored for further analyses.

ADON production in liquid medium. Production of ADON was tested in liquid medium (31). The PDA activated fungi were inoculated in 250 ml of YPS medium (yeast extract, peptone and sucrose) and incubated in orbital shaker at 150 rev. min^{-1}, 25ºC, for 48 hours. Aliquots of 0.2 ml of mycelium suspensions were inoculated in 25 ml of Modified Minimum Medium (MMM) and incubated at 25ºC for 72 hours. Cultures were filtered and filtrates were extracted three times with 10 ml of ethyl acetate. The organic extracts combined, passed through anhydrous sodium sulphate, and evaporated until dryness. Residues were suspended in 2 ml of acetonitrile and stored for further analyses.

Production of metabolites on Markus medium. For evaluation of the production of metabolites in Markus medium, the culture conditions described for the production of galactose oxidase (22) were used. The extraction procedure was identical to that described for ADON.

Qualitative analyses. Thin layer chromatography (TLC) was performed with 5 to 10 µl of each fungal culture extract. The extracts were applied to 20x20 cm Silica gel plates (250µm layer thickness - Sigma Co.), along with specific standards or a pool of standards (Sigma Co.). Plates were developed and visualised under different conditions. For the assay of moniliformin, butanol/acetic acid/water (4:1:5, v/v) and chloroform/methanol (4:3, v/v) were used as solvents, and 2,4-dinitrophenylhydrazine/sulphuric acid/methanol (0.5g:1 ml:25 ml, w/v/v) as visualisation reagent. For trichothecenes, chloroform/methanol (97:3, v/v) was employed as solvent and the procedure described by Kamimura (16) was used for visualisation. Fusaric acid was developed with butanol/acetic acid/water (4:1:1, v/v), and visualised with copper acetate and bromophenol. ADON was visualized by 20% aluminium chloride, after developing with ethyl acetate/toluene (3:1, v/v).

Quantitative analyses. A Gas-liquid chromatograph (CG 35370), equipped with flame ionization detector (FID), was employed with QF-1 column (2 mm internal diameter and 180 cm length). Temperatures of the injector and the detector were 280ºC and 300ºC, respectively. The initial temperature of the column (180ºC) was manually adjusted in 40ºC every five minutes until reaching 250ºC. Flow rates were 70 ml.min^{-1} for Nitrogen, 200 ml.min^{-1} for Oxygen and 92 ml.min^{-1} for Hydrogen. Aliquots of 0.5 ml of each extract in tubes were evaporated under nitrogen atmosphere, and added with 80 µl of the silylating agent Tri-sil/TBT with special formulation v/v of

TMS-Imidazole (Pierce Chemical Co.). The tubes were closed and heated at 60ºC for 15 minutes. After cooling, 3 µl of the samples were injected into the chromatograph. For quantifying, triangulation system was used against known concentrations of standards. The retention time for each standard of secondary metabolite was initially obtained individually and also as a pool of standards.

***Artemia salina* larvae test.** Larvae of *Artemia salina* (24 hours after egg rupture) were obtained as described by Harwig (15) and Eppley (12). For the toxicity test, 25, 50, 75 and 100 µl of each extract and controls (saline and dilution solvent) were placed in tubes, in triplicate. A number of larvae ranging from 8 to 15 per 0.5 ml of saline were transferred to the tubes containing the extracts. After 16 hours, larvae were counted. Larvae not swimming forward were considered dead (even if exhibited motion). Then, chloroform was added to kill viable larvae and another count was carried out. The number of live larvae was calculated by subtraction. Experiments were carried out in three replicates.

RESULTS

The growth rate of fungi varied from 8.0 to 2.0 cm: *F. graminearum* strain 2118 R (8.0 cm), *F. graminearum* strain 2218 (7.8 cm), GOPF (7.0 cm), *F. graminearum* strain 1269 (4.5 cm), *F. moniliforme* var. *subglutinans* strain 379 (4.3 cm) and *F. acuminatum* strain 356 (2.0 cm). The first three strains presented the largest similarity in growth rate and red pigmentation on the back of the colonies. However, the colony aspect of GOPF resembled *F. graminearum* strain 1269.

Only zearalenol (Rf 0.64) produced by *F. graminearum* strain 2218 grown in rice could be identified through the TLC analysis of the culture extracts (data not shown). When grown in corn, *F. graminearum* strain 2218 produced zearalenone (Rf 0.83), zearalenol (Rf 0.68) and nivalenol (Rf 0.08); *F. graminearum* strain 1269 produced zearalenol (Rf 0.68), nivalenol (Rf 0.08) and toxin Iso T-2 (Rf 0.86) and *F. acuminatum* strain 356 produced zearalenol (Rf 0.67). None of the studied metabolities was detected for strains grown in Markus medium, MMM or Richard medium.

The analysis of mycotoxins by GLC with flame ionization detector and silylated derivatives exhibited some inconveniences, such as deposition of silica on the detector, causing variation in retention times, as described in the literature (7). Despite the precautions, variations of retention times of standards occurred (DON 3.45-5.30; FX 5.30-6.30; NIV 5.45-6.45; ADON 6.45-8.00; NEO 8.10-10.00; ZEOH 10.35-11.30; ZEA 12.00-12.30; iso T-2 12.30-13.30 and Ac T-2 13.00-14.05). Consequently, retention times were used only as indicatives, since correct determinations could only be achieved through GLC analysis coupled to mass fragmentation. The gas-liquid chromatography analysis of fungal extracts improved the

comparison of secondary metabolites produced in different media. Rice and corn were the most effective substrates.
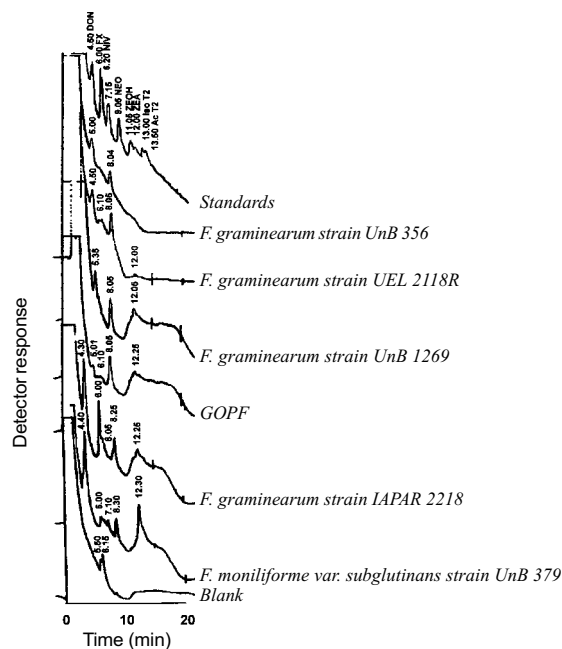

Figure 1. GLC profiles of the pool of mycotoxins and the metabolites produced by the fungi grown on rice (3µl – Attenuation 1x). Conditions as described in materials and methods.
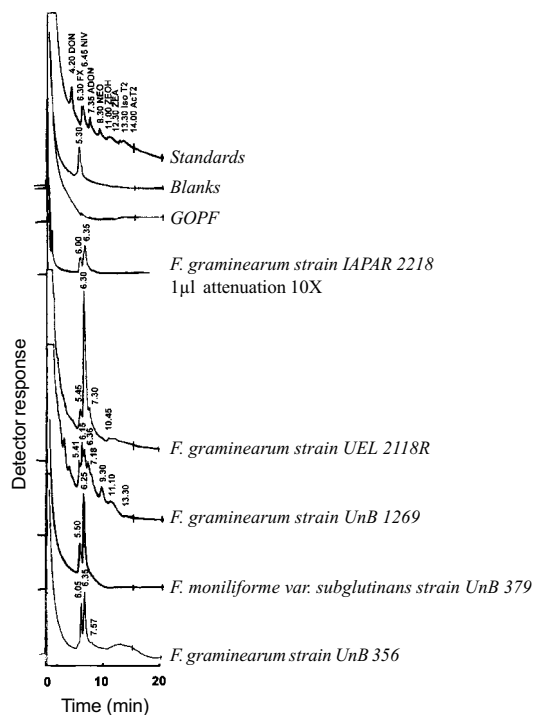

Figure 2. GLC profiles of the pool of mycotoxins and the metabolites produced in corn (3µl – Attenuation 1x).

The chromatographic profiles of the extracts of fungi cultured in rice (Fig.1) showed similarities in all studied cases and some sharp defined peaks. Based on the retention times of standards, one can determine the presence of DON and make tentative attributions of ADON. Table 1 shows a quantitative analysis of these components.

Table 4 indicates that the extracts of other culture media (Markus, MMM and Richard) presented toxic effects only when volumes of 100 μl were used.

Table 1. Quantitative determination by GLC of DON and ADON produced (μg/g) in rice.

Fungi	DON	ADON
F. graminearum strain IAPAR 2218	4.08	2.40
F. graminearum strain UEL 2118 R	1.32	3.04
F. graminearum strain UnB 1269	1.20	3.68
F. moniliforme var. *subglutinans strain* UnB 379	4.90	2.47
F. acuminatum strain UnB 356	1.38	1.16
Galactose oxidase producer fungus	0.22	3.48

Aliquots of the fungi extracts obtained from the different culture media were analysed for the toxicity to *Artemia salina* larvae. All rice and corn cultures extracts exhibited toxicity (Tables 2 and 3) .

Table 2. Effect of the extracts (μl) of rice culture media on the mortality (%) of *Artemia salina* larvae.

Fungi	25μl	50μl	75μl	100μl
F. graminearum strain UEL 2118 R	9	10	83	22
F. graminearum strain IAPAR 2218	35	66	72	73
F. graminearum strain UnB 1269	14	31	63	67
F. moniliforme var. *subglutinans* strain UnB 379	0	23	75	75
F. acuminatum strain UnB 356	0	15	38	30
Galactose oxidase producer fungus	14	17	19	23

Table 3. Effect of the extracts (μl) of corn culture on the mortality (%) of *Artemia salina* larvae.

Fungi	25μl	50μl	75μl	100μl
F. graminearum strain UEL 2218 R	14	55	65	100
F. graminearum strain IAPAR 2218	11	25	97	100
F. graminearum strain UnB 1269	62	100	100	100
F. moniliforme var. *subglutinans* strain UnB 379	53	40	39	37
F. acuminatum strain UnB 356	27	47	57	57
Galactose oxidase producer fungus	8	26	46	83

Table 4. Effect of the of culture media extracts (100 μl) on the mortality (%) of *Artemia salina* larvae.

Fungi	Markus' medium (G.oxidase)	MMM medium (ADON)	Richard's medium (Fusaric acid)
F. graminearum strain UEL 2118 R	5	11	14
F. graminearum strain IAPAR 2218	17	19	11
F. graminearum strain UnB 1269	25	13	20
F. acuminatum strain UnB 356	12	13	11
F. moniliforme var. *subglutinans* strain UnB 379	0	8	7
Galactose oxidase producer fungus	0	0	0

DISCUSSION

The major purpose of this work was the comparison of five isolates of galactose oxidase-producing *Fusarium* with the GOPF, using different media. The comparison concerning growth rate (colony diameter) and pigmentation, as described by Booth (5), indicates that the compared fungal strains are distinct.

The analysis of the fungal extracts obtained from the various culture media, carried out according to the systematic procedure described by Kamimura (16), allowed the identification of some of the metabolites, mainly in rice and corn cultures. When other culture media were used, none of the studied metabolites was detected using the same conditions. Nevertheless, the literature reported production of fusaric acid by *Giberella fujikuroi, F. heterosporum, F. moniliforme, F. moniliforme* var. *subglutinans* and *F. oxysporum* (21) but there are no reports of its production by *F. graminearum* and *F. acuminatum*. Bacon *et al.* (4) demonstrated the production of this acid in 78 isolates of *Fusarium moniliforme* and also *F. crookwellense, F. subglutinans, F. sambucinum, F. napiforme, F. heterosporum, F. oxysporum, F. solani* and *F. proliferatum*. However, it is worth to note that the research was done in corn and not in Richard medium and in different conditions. It must be emphasised that none of the fungi mentioned above belongs to the *Arthrosporiella* section described by Booth (5).

Concerned to moniliformin, not detected in this study, similar data was reported by Marasas (21). This toxin was described in *F. avenaceum, F. chlamydosporum, F. fusaroides, F. moniliforme, F. moniliforme* var. *subglutinans, F. oxysporum, F. verticillioides* and *Gibberella fujikuroi*, but there is no report about its production in *F. graminearum* and *F. acuminatum*. Among these fungi, only *F. fusarioides (=F. chlamydosporum)* belongs to the *Arthrosporiella* section described by Booth (5).

The production of DON and ADON is well documented, especially by *F. graminearum, F. acuminatum* and *F. moniliforme* (21). In this way, the designation of DON for the

fungi grown in rice can be accepted (Table 1 and Fig.1). However, the tentative designation of ADON should be regarded with care due to the lower retention time of 3-ADON. However, the possibility of production of 15-ADON (23), diacetoxyscirpenol (16) or other metabolites can not be excluded. Comparatively, the amount of DON produced by GOPF is lower than that detected on the other fungi (Table 1).

The GLC profiles of the fungal metabolites produced in rice were ordered based on the similarities, considering the first and the last 10 min of the chromatogram (Fig. 1). A great similarity occurred between the complete profile of *F. acuminatum* strain 356 and *F. graminearum* strain 2118 R. GOPF and *F. graminearum* strain 1269 differed slightly in the first 10 min, but increased the similarity in the remaining time, although they were different when compared with *F. graminearum* strain 2218. The profile of GOPF, when compared with *F. graminearum*, indicates that GOPF is similar to *F. graminearum* strain 1269, but is distinct of *F. acuminatum* strain 356 or *F. moniliforme* var. *subglutinans* strain 379.

In Fig. 2 the fungal growth in corn is compared. The chromatogram profiles showed the variation in the amounts of metabolites produced. The shouldered peaks difficulted the quantitative determination. In Fig. 2, the failure to detect metabolites on the galactose oxidase producing fungus calls the attention. This lack of metabolites deserves consideration, because the extract of GOPF, grown in corn, was toxic to *Artemia salina* larvae (Table 3). Literature describes growth of *F. graminearum* (8) and *F. tricinctum* (33) in corn, without production of trichothecenes, in spite of the toxicity to *Artemia salina* larvae. This is interesting, since data of Visconti *et al.* (32) indicate that *Fusarium* of the *Sporotrichiella* section produces mycotoxins. The *Sporotrichiella* section was described for the first time by Wollenweber (34) and includes *F. sporotrichioides, F. poae, F. chlamydosporum* and *F. tricinctum*. Later, this section was divided by Booth (5) in *Sporotrichiella* section, with *F. tricinctum* and *F. poae* and in *Arthrosporiella* section, with *F. sporotrichioides, F. fusaroides, F. avenaceum, F. camptoceras, F. semitectum* and *F. semitectum* var. *majus*. Based on the available data, GOPF may be considered unique, different from all those to which it was compared.

Another topic that deserves attention in Fig. 2 is the amount of metabolites (peaks of retention times of 6.00 and 6.35) produced by *F. graminearum* strain 2218. This sample required a 10x attenuation. Nivalenol was detected by TLC in this sample, and also in *F. graminearum* strain 2118 and *F. graminearum* strain 1269, indicating that this material is possibly nivalenol. Except for GOPF, chromatogram profiles (Fig. 2) were similar in the first five to seven min. Furthermore, Fig. 2 indicates that *F. graminearum* strain 1269 showed the highest number of peaks that coincide with the standards, confirming the presence of zearalenol, nivalenol and iso T-2. The extract of this fungus showed the higher toxicity to *Artemia salina* larvae (Table 3),

followed by *F. graminearum* strain 2218, which exhibited larger peaks in the chromatogram. Both Fig. 1 and 2 indicate that peaks of similar retention times were also present in non inoculated commercial corn and rice.

The GLC analysis of Markus medium (galactose oxidase) exhibited peaks for *F. moniliforme* var. *subglutinans* strain 379 and *F. graminearum* strain 2118 R, with retention times of 6.00 and 6.35, which were compatible with FX and NIV, respectively. In this medium, the other fungi did not exhibit peaks under the conditions used. In MMM medium, the presence of ADON was observed (0.16 µg/g) only in *F. graminearum* strain 1269. Finally, in Richard's medium nothing was detected.

This is the first report on the production of mycotoxins by *Dactylium dendroides* (GOPF). The extracts of Markus medium, MMM, and Richard's medium did not show any of the studied metabolites, nor toxic effects to *Artemia salina* larvae (Table 4). This data, in addition to the other aspects, distinguish GOPF Δ from *Fusarium* spp. studied here. Nevertheless, this is the first time that an evidence of DON production by GOPF is reported. According to Marasas *et al*. (21), *F. acuminatum, F. culmorum, F. graminearum, F. moniliforme, F. nivale, Gibberella zeae* and *F. sporotrichioides*, only the last belonging to the *Arthrosporiella* section, are DON producing fungi. Considering that the time for growth in rice is 21 days, which is also adequate for production of trichothecenes, and that toxicity of the extracts to larvae of *Artemia salina* was demonstrated, more studies with these fungi in the future are recommended.

Based on the similarity between the conidia and conidiophores of GOPF and *F. chlamydosporum* mentioned by Ögel (26), complementary testing for chlamydosporol, a toxic metabolite produced by *F. chlamydosporum* (29), and for visoltricine produced by *F. tricinctum* (33), should be done to study further similarities between these fungi.

RESUMO

Produção de micotoxinas por *Fusarium* produtor de galactose oxidase usando diferentes meios de cultivo

O isolado original do fungo produtor de galactose oxidase *Dactylium dendroides* e outros cinco isolados de *Fusarium,* também produtores de galactose oxidase, foram cultivados em diferentes meios e condições, com o objetivo de estudar a produção de onze micotoxinas características do gênero *Fusarium*: moniliformina, ácido fusárico, deoxinivalenol, fusarenona- X, nivalenol, 3-acetildeoxinivalenol, neosolaniol, zearalenol, zearalenona, acetil T-2 e iso T-2. A toxicidade dos extratos obtidos nos diferentes cultivos foi testada em larvas de *Artemia salina.*

Palavras-chave: *Dactylium dendroides, Fusarium*, galactose oxidase, micotoxinas

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