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USE OF PLANARIAN *DUGESIA TIGRINA* GIRARD BIOASSAY FOR ASSESSING THE TOXICITY OF STERIGMATOCYSTIN PRODUCED BY *ASPERGILLUS VERSICOLOR* TIRABOSCHI

In the biomasses from 17 moulds isolated in housing buildings, a *Dugesia tigrina* bioassay was carried out to assess the toxicity of *Aspergillus versicolor* sterigmatocystin (ST) producing strains. Making use of this method it was possible to test the fungal isolates of *A. versicolor* coming from the naturally infested construction materials of different housing buildings. The toxicity of the extracts prepared from the biomasses was in a wide range of LC 50 starting from non-toxic through weakly toxic (100–1000 mg dm^{-3}), medium toxic (10–100 mg dm^{-3}), and potently toxic (1–10 mg dm^{-3}), in the latter case 8.2 mg dm^{-3} air-dry biomass of *A. versicolor* was highly productive and synthesized more than 500 mg kg^{-1} ST.

1. INTRODUCTION

In the Microbiological and Toxicological Laboratory, Department of Applied Ecology, Institute of Environmental Engineering, University of Zielona Góra, since many years mycological and mycotoxicological research has been done (started in 1992). From this year, more than 250 mould-infected flats were analysed in housing buildings. Seventy seven (77) mould species were isolated. Based on the estimation of mould threat to the residents of houses it can be concluded that *Aspergillus versicolor* poses the highest mycotoxic risk to the inhabitants of buildings infected with this anamorphic fungus (PIONTEK [10]). *Aspergillus versicolor* is the main fungus that produces sterigmatocystin (ST). ST, the precursor of AFB_1, is similar to aflatoxin, known for its toxic and carcinogenic properties. Since sterigmatocystin has been classified by the International Agency for Research on Cancer (IARC) as 2A human carcinogen (IARC [3]), it poses a potential carcinogen risk for people living and working in buildings infected by *A. versicolor*, especially for workers making structural

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changes or cleaning up contamited materials (NIELSEN et al. [5]). In order to assess the risk of the mycotoxin absorption by people and animals, toxicological tests both analytical (physicochemical analysis) and bioanalytical (biotoxicological analysis) should be conducted. The toxicity of mould metabolites is assess based on the test on such animals as trout, ducks, chicks, mice, rats, rabbits, guinea-pigs, monkeys, dogs, sheep and the other. There are also known simpler and cheaper biological laboratory tests. Most frequently the larvae of brine shrimps *Artemia salina* were applied. The presence of mycotoxins can be detected by cell cultures (cytotoxicity), MTT bioassay. Good results are obtained in the tests on the cellular lines of the swine kidneys (SK) and on the mouse fibroblasts (PIONTEK [12]). In the toxicological laboratory of the Department of Applied Ecology, UZ, the toxicological tests on *Dugesia tigrina* Girard were carried out (PIONTEK [10], [11], PIONTEK and PIONTEK [9]).

The scope of investigation was to detect the sterigmatocystin (ST) synthesizing strains growing on the walls of dwelling buildings and to determine the possibility of using the planarian bioassay in order to assess the toxicity of *Aspergillus versicolor* to people.

2. MATERIALS AND METHODS

The methods applied in the present research are described in more detail by PIONTEK ([10], [13]).

2.1. ORIGIN OF THE SAMPLES

The fungi were collected from the interior house surfaces with visible mould infected materials: fragments of walls, ceiling tiles, plasters, plaster and carton boards, finishing materials (paints, wallpaper, binding materials, carpets, tar paper) and others.

2.2. SAMPLING

Samples were placed, immediately after being collected, on the surface of four media on Petri dishes (two synthetic media Cz, SNA and two natural media MEA and PDA).

2.3. IDENTIFICATION OF FUNGUS SPECIMENS

The samples incubated in the cultivation room were covered with white linen, kept at a temperature range of 18–22 °C and exposed to daylight with a natural day/night rhythm. Clean (axenic) cultures were isolated from the initial mixed culture by means of passing cells on two media: Czapek-Dox (Cz) and malt extract agar (MEA). The time of transferring, cultivation and observation was approximately 21 days. Thereaf-

ter the cultivated fungi were subjected to identification tests in order to determine their specimen.

2.4. PREPARATION OF MOULD BIOMASSES FROM SAMPLES OF INFECTED BUILDING MATERIALS

In order to obtain air-dry biomasses for further biological and/or physicochemical analyses, mass cultures of mould were established. 5 cm^3 of MEA (malt extract agar) were placed on ø 9 cm Petri dishes. Spores and mycelia of the fungi that had been previously isolated, and their species determined, were centrally placed on the dishes with a preparation needle. The samples were incubated in the cultivation room under simulated conditions of housing buildings. They were covered with white linen and kept at room temperature of 18–22 °C with a natural day/night rhythm. The cultivation of an isolated culture lasted three months. Such a period is indispensable to growth, sporulation, and repeated growth (allowing the fungi to cover a large area of a Petri dish), ageing the strain and drying the mycelium with the medium until it is air-dry. Air-dry mould biomasses with laboratory medium were removed from the dishes by means of a scalpel and a spoon, weighed and placed in glass jars closed with ground-in to stoppers. This preparation was used to prepare samples for chromatographic and biotoxicological analyses of sterigmatocystin.

2.5. DETERMINATION OF STERIGMATOCYSTIN CONTENT IN MOULD BIOMASSES

17 mould samples were analyzed in the Pharmacology and Toxicology Department of the National Veterinary Institute in Puławy by means of high performance liquid chromatography (HPLC) for the presence of sterigmatocystin (ST). Sterigmatocystin was extracted from 0.3-g samples by precipitation with acetonitrile and 4% aqueous solution of potassium chloride. Once it had been filtered, the crude extract was purified by a repeated precipitation with hexane. Water-soluble compounds were removed by transferring sterigmatocystin to the chloroform layer. Then the extract was further purified in a column filled with silicone gel 60 (0.05–0.2 mm/70–270 mesh ASTM for column chromatography). The product eluted from the column was dried in a nitrogen stream, dissolved in acetonitrile and the aliquots were injected into the column of a liquid chromatograph equipped with a UV/VIS detector and an analytical column with an reverse C8 phase.

2.6. PLANARIAN CULTIVATION

The planarians were cultured in 0.5-dm^3 beakers filled with water and covered with cover glass. Each beaker contained about 100 individuals. Both water volume

and surface area of the beakers guaranteed an easy movement of the individuals and easy access to food. Water temperature ranged between 18 and 22 °C, pH between 7.2 and 8.2, and oxygen content was above 5.0 mg O_2 dm^{-3}. Cultures were kept at daylight, with a natural day/night rhythm (PIONTEK [6]).

2.7. *DUGESIA TIGRINA* GIRARD BIOASSAY

The toxicity test applied is reported by PIONTEK [6]–[8]. The applications of the *Dugesia tigrina* bioassay in mycotoxicological tests are described in more detail by PIONTEK [11], [13]. The experiments with *D. tigrina* were carried out in 50-cm^3 beakers containing 40 cm^3 of the solution of sterigmatocystin and 10 test organisms. The experiments were made in three replications. Thirty planarians were kept in each concentration of the toxicant. The length of 20-day-old animals ranged from 11 to 12 mm.

The death of the individuals of *D. tigrina* was adopted as the criterion of the toxic effect of poison on their organisms. 240-h LC 50 was determined (PIONTEK [8]). Statistical analysis of the experimental results on the toxic effect of 17 extracts prepared from the *A. versicolor* biomass on *Dugesia tigrina* was carried out by the graphic method according to WEBER [14]. Statistical analysis provided us with the values of LC 50, confidence intervals for LC 50, equation of $y = a + bx$ obtained from empirical data and the test of the goodness of fit of the experimental and normal distributions.

2.8. TOXICITY CLASSES

The toxicity of the different mould biomasses (mould biomass with laboratory medium) was assigned to four classes on the basis of their ST content and the classification proposed by CHEŁKOWSKI [1], [2]. In the bioassays, the estimates of the degree of toxicity of mouldy biomasses were made. The classifications used (table 2) was proposed by LIEBMANN [4].

Table 1

Toxicity classes of mouldy biomasses and materials (CHEŁKOWSKI [1])

Quantity of important mycotoxin (result of chromatographic analysis)	Toxicity classes
≤ 1 mg kg^{-1}	nontoxic
> 1–10 mg kg^{-1}	weakly toxic
>10–100 mg kg^{-1}	potently toxic
> 100 mg kg^{-1}	highly toxic

Table 2

Toxicity classes of poison substances (LIEBMAN [4])

Quantity of poison (result of toxicity test)	Toxicity classes
< 1 mg dm^{-3}	highly toxic
1–10 mg dm^{-3}	potently toxic
10–100 mg dm^{-3}	medium toxic
100–1000 mg dm^{-3}	weakly toxic
> 1 g dm^{-3}	barley toxic

2.9. EXTRACTION

Mycotoxins were extracted from the mould biomass using the method of Piontek (PIONTEK [10]). One-gram air-dry samples of mould biomass were extracted for 96 hours in 100 cm^3 of 80% methanol (analytical grade) before their filtration through the Whatman 4 blotting paper placed on filter flask.

3. RESULTS AND DISCUSSION

The toxicity classes adopted for mould and poison substances are presented in table 3. All the extracts from the biomass of isolates (*Aspergillus versicolor* Tiraboschi) synthesizing more than 1 mg kg^{-1} sterigmatocystin (ST) were toxic to the planarians (*Platyhelminthes, Turbellaria, Tricladida, Paludicola*). In this method, 17 strains of *A. versciolor* tested were collected from the naturally infected construction materials of different housing buildings. *Dugesia tigrina* proved to be a sensitive bioindicator of sterigmatocystin (ST) toxicity. All the samples of air-dry mould biomass with the laboratory medium contained sterigmatocystin in the quantities ranging from 0.0 to over 500.0 mg kg^{-1} ST. The toxicity of the extracts prepared from the same biomasses covered a wide range of LC 50 which allowed us to classify CT into 4 classes: practically nontoxic (four samples); weakly toxic (100–1000 mg dm^{-3}), (five samples); medium toxic (10–100 mg dm^{-3}), (seven samples); and potently toxic (1–10 mg dm^{-3}), (one sample).

The results of mycological and mycotoxicological research carried out for many years in the Lubuskie Province, Poland, demonstrated that *A. versicolor* occurs quite frequently in the housing buildings (PIONTEK [10]). Biotoxicological and chromatographic analyses of the biomass of *A. versicolor* (different strains isolated in dwelling buildings) showed (table 3) that less than 50% of strains can produce significant quantities of sterigmatocystin, and only in 3 samples of 17 its significant amount was detected. In one sample, more than 500 mg kg^{-1} ST were measured, therefore it had the highest toxic effect on *Dugesia tigrina* and was classified as highly toxic mould

biomass. Seven samples (40%) proved to be medium toxic and more than 50% of strains – weakly toxic or practically nontoxic.

Table 3

Toxic effect of sterigmatocystin from mould biomass on *Dugesia tigrina* Girard

Item	Sterigamtocystin of the culture (mg kg^{-1})	240-h LC 50 for *Dugesia tigrina* for mg dm^{-3} of the biomass
1	<0.03	n.t
2	<0.03	n.t
3	<0.03	n.t
4	0.07	n.t
5	1.28	602.5
6	1.30	399.3
7	2.69	150.0
8	2.89	122.1
9	6.48	132.0
10	13.61	70.9
11	48.00	40.7
12	54.21	20.7
13	58.15	27.5
14	84.02	29.0
15	107.35	28.0
16	120.67	19.7
17	534.38	8.2

The experimental results were tested for normal distribution. For this purpose the χ^2 test based on Pearson's criterion was used (WEBER [14]). If the probability in the χ^2 test was higher than 0.70 the normal distribution was assumed.

4. CONCLUSION

The results obtained reveal that *Dugesia tigrina* is a sensitive bioindicator of sterigmatocystin (ST). Therefore, the presence of toxic mould biomasses and mycotoxin could be detected applying the *Dugesia tigrina* Girard bioassay. A comparison between the toxicity levels of the *Aspergillus versicolor* strains, based on the physicochemical analysis (HPLC) and LC 50 obtained in the biotests with *Dugesia tigrina*, proved their convergence which permitted the conclusion that chemical analysis unfriendly for environment could be replaced by biological one. It is difficult to carry out physicochemical analysis (HPLC) of all samples of moulds collected from buildings. Those analyses should be made in the case of high toxicity level of the sample.

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