APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1994, p. 3421–3424
0099-2240/94/$04.00+0

Vol. 60, No. 9

# *Stachybotrys atra* Growth and Toxin Production in Some Building Materials and Fodder under Different Relative Humidities

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Received 3 December 1993/Accepted 21 June 1994

**Growth of *Stachybotrys atra* and its toxin production on some building materials and in animal fodder were studied at relative humidities ranging from 78 to 100%. Toxins were detected by biological assays and chemical methods. Strong growth of the fungus and presence of macrocyclic trichothecenes, mainly satratoxins G and H, were detected on wallpaper and gypsum boards and in hay and straw at saturation conditions. On pine panels, *S. atra* grew well, but neither biological toxicity nor production of macrocyclic trichothecenes was observed.**

Stachybotryotoxins produced by *Stachybotrys atra* have been reported to cause toxicosis in farm animals, especially in Eastern Europe (10, 14). The symptoms of stachybotryotoxicosis, including toxic irritation of eyes, skin, and mucous membranes followed by necrosis, changes in blood parameters, hemorrhages in many organs, and severe disorders of the immune system, depend on the amount of toxins. Exposure to high levels of stachybotryotoxins leads to rapid death of animals (11). For stachybotryotoxicosis in animals, ingestion of contaminated feed has been considered the main exposure route. For stachybotryotoxicosis in humans, handling of contaminated materials and inhalation of airborne toxins have also been found to be potential routes of exposure (1, 4, 6, 22). The symptoms of human stachybotryotoxicosis comprise dermatitis, coughing, rhinitis, irritated throat, fever, headache, feebleness, and fatigue, some of which resemble symptoms of organic dust toxicosis, sick building syndrome, and chronic fatigue syndrome (1, 4).

*S. atra* sporulates at 2 to 40°C (5), and the minimum water activity ($a_w$) varies from 0.85 to 0.95 for germination and from 0.91 to 0.96 for growth and sporulation, depending on the temperature and medium (2, 5, 8).

Toxigenic and nontoxigenic strains of *S. atra* have been isolated from cellulose-based agricultural materials, e.g., hay and straw (10, 14), and from contaminated moist building materials (4, 7). In some cases, high airborne counts of viable *S. atra* spores (up to 18,000 CFU/$m^3$) were recorded in mouldy houses (12). However, it has been reported that up to 90% of *S. atra* spores found in the air may not be viable and thus cannot be detected by viable-count methods (18). Relatively high concentrations of stachybotryotoxins have been determined to be present in aerosolized spores of *S. atra* (22), indicating that inhalation exposure to airborne toxins may be an important factor resulting in health problems among occupants of houses or severe diseases in farm animals.

Although *S. atra* is not particularly common in agricultural and home environments, even in mouldy buildings (13), the toxigenic properties of the fungus are worth investigating because stachybotryotoxins are extremely toxic (11, 13, 14). In the present study, the effect of high relative humidity (RH) on *S. atra* growth and toxin production on some building materials, on straw and hay, and in paper have been investigated.

RH instead of $a_w$ or water content was used to express the moisture conditions. We wanted to create a situation in which fungal spores germinate rapidly under sufficient moisture conditions. The experiment was intended to simulate situations in which fodder or building materials accidentally suddenly become very wet, such as upon heavy rain during harvesting or improper handling of material or as a result of water damage in buildings.

*S. atra* Corda strain 72 (Agricultural Research Centre, Jokioinen, Finland) growth and toxin production on some building materials, fodder, and paper were studied under experimental circumstances in which the RH was regulated.

The following samples of building materials were 25 $cm^2$ in size: wallpaper without a plastic cover (0.25 to 0.27 g), gypsum board covered with cardboard (20.92 to 23.44 g), planed pine panel without surface coating (4.80 to 5.56 g), and bleached paper sheet (0.16 to 0.22 g). Insulation material (2.00 g) was made of waste paper with boron compounds as fire-retardant substances. The fodder samples were dried hay (2.20 to 2.45 g) and straw (0.95 to 1.09 g).

The building materials and paper samples were new, unused materials and were therefore regarded as clean and not needing sterilization. In order to exclude the possibility that sterilization (120°C, 1 h) might change the composition of building materials used as growth media, sterilized building material samples were used as positive-control samples. All straw and hay samples were sterilized before incubation. Table 1 presents the experimental and control samples together with the relevant parameters.

After sterilization, all the samples were weighed and placed into 1.5-liter airtight glass chambers, two samples of each material being placed in one chamber. RHs inside the chambers were regulated to 78 to 81, 84 to 89, or 100% with saturated aqueous solutions of ammonium chloride and potassium chloride and with pure water (9).

The materials were incubated in the chambers at 20 to 23°C for 3 days before inoculation with *S. atra*. During the incubation, equilibrium between the relative surface moisture of the material and the air in the chamber was established.

The highly toxic strain of *S. atra* was first cultured in 25 g of

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TABLE 1. Influence of RH on *S. atra* growth and toxin production on various materials[a]

| Sample type and material | RH (%) | Change of wt (%) (water absorbtion) | Growth[b] | Biological toxicity (cytotoxicity, dermotoxicity)[c] | Satratoxin G and H concn (μg)[d] |
|---|---|---|---|---|---|
| Positive controls | | | | | |
| Wallpaper | 86–89 | 43 | ++ | + | 25, 1.5 |
| Gypsum board | 84–89 | 1 | + | − | NA, NA |
| Pine panel | 84–89 | 15 | + | − | NA, NA |
| Insulating material | 84–89 | 12 | − | − | NA, NA |
| Paper | 84–89 | 9 | + | + | ND, NA |
| Experimental samples | | | | | |
| Wallpaper | 78–81 | 23 | + | − | ND, ND |
| | 84–89 | 32 | ++ | + | 15, ND |
| | 100 | 400 | ++ | + | 42, 47 |
| Gypsum board | 78–81 | <1 | − | − | NA, NA |
| | 84–89 | 1 | +[e] | − | NA, NA |
| | 100 | 11 | ++ | + | 67, 11 |
| Pine panel | 78–81 | 11 | +[e] | − | NA, NA |
| | 84–89 | 21 | + | − | NA, NA |
| | 100 | 32 | ++ | − | ND, ND |
| Insulating material | 78–81 | 13 | − | − | NA, NA |
| | 84–89 | 14 | − | − | NA, NA |
| | 100 | 307 | − | − | NA, ND |
| Paper | 78–81 | 16 | + | − | ND, NA |
| | 84–89 | 0 | + | +[f] | ND, NA |
| | 100 | 465 | + | −[g] | ND, NA |
| Hay | 78–81 | 19 | − | − | NA, NA |
| | 84–89 | 7 | ++ | − | ND, ND |
| | 100 | 176 | ++ | + | 42, 40 |
| Straw | 78–81 | 5 | − | − | NA, NA |
| | 84–89 | 18 | + | − | ND, ND |
| | 100 | 234 | ++ | + | 315, 152 |

[a] Positive controls were sterilized building materials inoculated with *S. atra* incubated at 84 to 89% RH. Experimental samples were unsterilized building materials inoculated with *S. atra* and incubated at different RHs. Hay and straw were sterilized.

[b] +, slight growth; ++, strong growth; −, no growth.

[c] +, toxic; −, nontoxic.

[d] The first value describes the average concentration of two samples analyzed immediately after the 12-week incubation, and the second value describes the average concentration of the other two replicates analyzed 4 to 10 months later, after storage at 6 to 8°C. NA, not analyzed; ND, not detected.

[e] Slight growth was observed only in half of the samples.

[f] Cytotoxicity was detected in all samples, but dermotoxicity was observed only in half of the samples.

[g] Cytotoxicity and dermotoxicity were detected in half of the samples.

a sterilized mixture of wheat, barley, and oats (1:1:1) at an RH of 100% and 20 to 24°C for 4 weeks and then stored at 5°C for 1 to 2 weeks. Toxin production was subsequently verified by a cytotoxicity test (21). Sterile water (100 ml) was added to the culture, and the culture was shaken for 1 h. A 1-ml volume of spore suspension was inoculated into each incubated sample, except for the negative-control samples.

Two chambers of each material were provided for each RH level; one chamber was incubated at 20 to 23°C for 12 weeks, and the other was incubated at 20 to 23°C for 14 days, at 6 to 8°C for 2 days, and finally at 20 to 23°C for the remaining 70 days. Exceptionally long incubation periods were used because the fungal growth was slight on some materials. A few investigators have previously used 6- to 8-week incubation periods (3, 15), and according to Marasas and Nelson (16), stachybotryotoxins are stable compounds. The control samples were incubated at an RH of 84 to 89% under the temperature conditions used for the second of the replicate samples. During the incubation, colonization of *S. atra* on the materials was visually checked every 2 weeks. The growth was interpreted as slight when the material could be distinguished under the *S. atra* growth. When growth was considered strong, mycelia and spores of the fungus covered the whole material. After incubation, the samples were weighed and stachybotryotoxins were analyzed first by biological toxicity tests and then by chemical methods. The analyses were performed with one of the replicate samples immediately after incubation and on the other replicate 4 to 10 months later, after storage at 6 to 8°C.

The methods for toxin extraction and for performing the cell culture test, the rabbit skin test, and chemical analysis were those described in detail earlier (20, 21). Methanol (95%) was added in a quantity sufficient to cover the samples, which were allowed to stand overnight at 20 to 23°C and were then extracted twice by shaking for 30 min at 20 to 23°C (14, 22). Extracts were combined and filtered through filter paper. The filtrates were evaporated to dryness, dissolved in acetone: methanol, and divided into two equal fractions: one for the cytotoxicity test and the other for the dermotoxicity test.

Biological toxicity tests were used to screen samples before performing expensive and time-consuming chemical analyses. Only samples which showed positive biological toxicity reactions were analyzed chemically for satratoxins. For the cytotoxicity test (21), a continuous feline fetus cell line (National Veterinary and Food Research Institute) was used. These cells are rapidly dividing and undergo active protein synthesis, which is a good target for interference from mycotoxins. Methanol (10%) in phosphate-buffered saline (PBS) was used as a carrier solvent and as a control. It gave good reproducibility of toxins and was nontoxic for cells (17). Cells were observed daily for 1 week for signs of cytotoxicity, dead cells, or diminished cell growth. No inhibition of cell growth was observed when the control sample diluted in 10% methanol–PBS was compared with control cells without methanol. Biologically positive fractions of each sample were combined and analyzed chemically by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry as previously described (20).

Growth and toxin production by *S. atra* 72 on some building materials, paper, and fodder under various RH conditions are presented in Table 1. Paper, wallpaper, straw, and hay absorbed water in abundance, as expressed by the weight changes. On the contrary, the weight of pine panels and gypsum boards increased only slightly, even in 100% RH. *S. atra* grew on wallpaper, pine panels, and paper at the RH range of 78 to 100% and on gypsum boards and in hay and straw at the RH range of 84 to 100%. No growth of *S. atra* was observed in insulating material.

Production of stachybotryotoxins was detected on wallpaper at the RH range of 84 to 100% and on gypsum boards and in hay and straw at 100% RH. Toxin production was not observed on pine panels and insulating material. Small amounts of verrucarol were identified after hydrolyzation on paper at 78 to 100% RH and in animal fodder at 84 to 89% RH. Satratoxins from these samples were not identified in the HPLC analyses, and the biological tests indicated toxicity only on paper at 84 to 100% RH.

Sterilization of the positive-control samples before inoculation with *S. atra* did not affect growth and toxin production. No biological toxicity was detected in samples tested as negative controls. A short incubation at 6 to 8°C on the third incubation week did not affect growth and toxin production by *S. atra* in any material or at any RH level. Satratoxin concentrations were somewhat higher in those samples analyzed immediately after the incubation than in samples stored at 6 to 8°C for several months.

*S. atra* has been previously reported to grow on cellulose-based materials, e.g., hay, straw, wallpaper, and fiberboard, at $a_w$ values of above 0.91 in the temperature range of 2 to 40°C (2, 4, 5, 8, 10). In the present work, *S. atra* grew on all the materials studied, except on insulating material. The probable reason for prevention of *S. atra* growth was an inhibitory effect of boron compounds, fire retardant substances, in the insulating material. *S. atra* growth seemed to be possible at slightly lower RH levels (78 to 81%) on wallpaper, pine panel, and paper than on gypsum board, hay, and straw (minimum RH of 84 to 89%). Strong growth of the fungus in the materials was usually detected at saturation conditions.

Stachybotryotoxins have been isolated from heavily contaminated materials of high cellulose content, such as straw, wallpapered materials, and fiberboard (4, 7, 10). In laboratory experiments, rice has often been used as a substrate for stachybotryotoxin production (14, 22). In this study, stachybotryotoxins were formed on wallpaper, gypsum board, hay, and straw. Some evidence of toxin production on paper at 78 to 100% RH was also detected, although fungal growth was slight. According to the literature, straw has often been a source of stachybotryotoxicosis in animals (10, 11). In the present work, the highest toxin concentrations were found in straw samples, whereas the concentrations on wallpaper and gypsum board and in hay samples reached a lower, equal level. Toxins were produced at 84 to 89% RH on wallpaper, while in the other materials, toxins were detected only under conditions of saturation. On pine panels, no toxin production was detected, even when *S. atra* grew well. We have no explanation for this interesting detail, but the substrate may have a great influence on toxin production (19).

Toxigenic *S. atra* strains have been previously observed to produce macrocyclic trichothecenes, mainly satratoxins G and H, and, to a minor extent, satratoxin F, roridin E, and verrucarins B and J (4, 10, 14, 22). The chemical analyses in the present study agreed with these findings: satratoxins G and H were identified in nearly all the samples proved toxic in the biological tests, but the amount of verrucarol analyzed by gas chromatography-mass spectrometry indicated that other macrocyclic trichothecenes also were present in the samples.

The main result of this experimental study was that *S. atra* was able to produce toxic metabolites on some building materials and fodder within 12 weeks at RH levels high enough for fungal growth.

This work was supported by a grant from the Research Council for the Environmental Sciences of the Academy of Finland.

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