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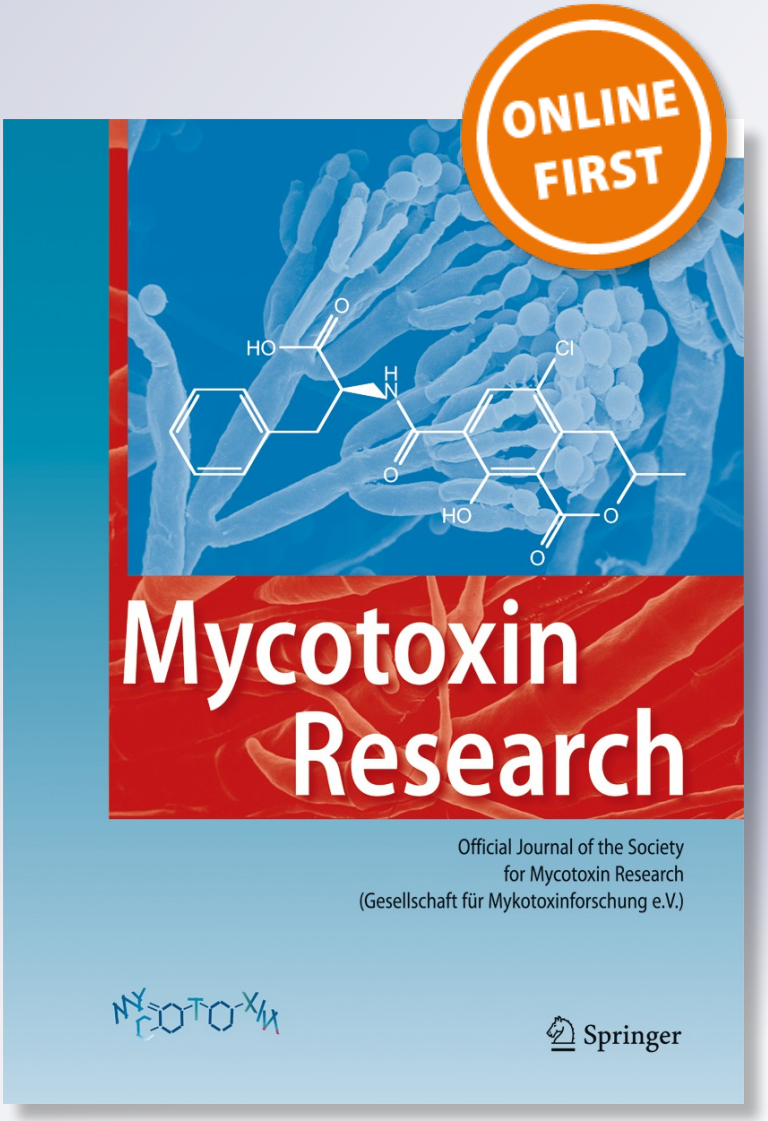


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ORIGINAL PAPER

# Sterigmatocystin production by nine newly described *Aspergillus* species in section *Versicolores* grown on two different media

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**Abstract** Nine recently described *Aspergillus* species and four known species in section *Versicolores* were tested for their ability to produce sterigmatocystin on two liquid media, Czapek w/20 % Sucrose Broth and Yeast Extract Broth grown in the dark for 1 week at 25 °C. Detection and quantification of ST were performed by reversed-phase liquid chromatography coupled with electrospray ionization ion trap mass spectrometry. Limit of detection was 3 ng/mL and limit of quantification was 10 ng/mL. Nine newly described *Aspergillus* species from various substrates, *A. amoenus*, *A. creber*, *A. cvjetkovicii*, *A. fructus*, *A. jensenii*, *A. puulaauensis*, *A. subversicolor*, *A. tennesseensis and A. venenatus* in section *Versicolores* were found to produce sterigmatocystin. Production was confirmed in recently collected isolates of *A. protuberus* and *A. versicolor*. *A. austroafricanus* and *A. tabacinus* did not produce sterigmatocystin.

**Keywords** Sterigmatocystin · Mycotoxins · *Aspergillus* · Indoor environment

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## Introduction

Sterigmatocystin (ST) is a carcinogenic precursor to aflatoxin $B_1$ (Terao 1983) produced primarily by *Aspergillus* species from several different sections of the genus (Rank et al. 2011). Species of *Aspergillus* section *Versicolores* grow well in wet conditions (Nielsen et al. 1999; Bloom et al. 2007; Engelhart et al. 2002) but are also tolerant of dry conditions (Jurjevic et al. 2012), and because of this they are major contributors to sick building syndrome (Samson et al. 2001; Andersen et al. 2011). Sterigmatocystin has been found in 24 % of building materials sampled from water-damaged buildings. *Aspergillus versicolor* was present in most ST-containing samples (Tuomi et al. 2000).

Sterigmatocystin shows some weak toxicity, mutagenicity, cytotoxicity and carcinogenicity in both in vitro and in vivo studies, and has been recognized as a 2B carcinogen by the International Agency for Research on Cancer (Terao 1983; Vesonder and Horn 1985; Berry 1988; Gopalakrishnan et al. 1992; Sumi et al. 1987, 1994; Murtoniemi et al. 2001; Jussila et al. 2002; Ma et al. 2003; Veršilovskis and Saeger 2010).

Studies in Japan and Canada (Sugimoto et al. 1976; Abramson et al. 1983) found a correlation between *A. versicolor* and ST in postharvest storage. *A. versicolor* has been isolated from: biltong peppercorns, cereal, cheese, copra, corn, dried

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medical herbs, fermented and cured meats, flour, frozen meat, fruits, grapefruit juice, green coffee beans, hazelnuts, health food, pistachios, pecans, smoked sardines, soybeans, sugar used in the manufacture of soft drinks, stored barley, spices, milled rice, mung beans, oats, peanuts, rapeseed, sunflower seed, amaranth seed, vegetable oil, walnuts, and wheat (reviewed by Pitt and Hocking 2009). Although the numbers are difficult to estimate, mycotoxin contamination including ST is thought to cause $0.4–1.6 billion of damage annually to US agriculture (Brodhagen and Keller 2006).

*A. versicolor* has been implicated as the causative agent of disseminated aspergillosis in dogs (Zhang et al. 2012). It has been suspected to cause aspergillosis in some transplant recipients (Baddley et al. 2009), and has been isolated from the infected eye of a patient suffering from HIV (Perri et al. 2005).

Jurjevic et al. (2012) revised the taxonomy of *Aspergillus* section *Versicolores* increasing the number of recognized species from four to thirteen. The ubiquitous distribution of *A. versicolor* sensu lato and its ability to produce ST has important implications for health and food safety and knowledge of the species that produce ST will aid our understanding of the section.

## Materials and methods

*Aspergillus* section *Versicolores* species (Table 1) were grown in duplicate in 100-mL stationary liquid cultures (5 cm in diameter) of Czapek w/20 % Sucrose Broth (CZ20: 200 g/L sucrose, 1 g/L $K_2HPO_4$, 3 g/L $NaNO_3$, 0.5 g/L KCl, 0.05 g/L, $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4$ $7H_2O$) (Health Link®, Jacksonville, FL, USA; http://www.healthlinkinc.net) and Yeast Extract Broth [YEB: 2 g/L yeast extract, 15 g/L sucrose, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 1 mL/L trace metal solution (1 g/L $ZnSO_4 \cdot 7H_2O$, 0.5 g/L $CuSO_4$ $5H_2O$)] (Health Link®) in 250-mL flasks for 7 days at 25 °C±0.2 °C in the dark. Each sample was inoculated with $10^6$ spores counted by hemocytometer, previously grown on MEA (malt extract agar).

### Sample preparation (extraction and cleanup)

One hundred milliliters of liquid culture was shaken (Eberbach 6010; Ann Arbor, MI, USA) for 1 h, and filtered through fluted filter paper (Vicam, Watertown, MA, USA). Two hundred microliters of liquid culture were diluted with 1,800 μL of mobile phase acetonitrile/water (50/50, v/v) containing 0.5 % acetic acid and then filtered through Acrodisc syringe filters with 0.45 μm PTFE membrane (Pall, Ann Arbor, MI, USA) before LC/MS analysis. Sample extracts containing high concentration of ST that resulted outside the calibration range of the standard curve were appropriately diluted with mobile phase and analyzed again by LC-MS.

### Standard

Powdered sterigmatocystin standard was purchased from Sigma (Atlanta, GA, USA) and stored at −20 °C. A stock solution was prepared in acetonitrile at ST concentration of 1 mg/mL. This solution was further diluted to prepare 5 calibration solutions that were used to prepare the calibration curve for ST quantification in liquid cultures.

### LC/MS equipment and parameters

Analyses were performed on an Agilent 6330 series ion trap LC/MS system, equipped with an ESI interface and an 1100 series LC system comprising a quaternary pump and an autosampler, all from Agilent Technologies (Alpharetta, GA, USA).

The analytical column was an Allure Bi-Phenyl column (30 mm×2.1 mm, 5 μm particle; Restek, Bellefonte, PA, USA). The column oven was set at 40 °C. The flow rate of the mobile phase was 250 μL/min and the injection volume was 20 μL. The column effluent was directly transferred into the ESI interface without splitting. For LC/MS analyses of ST, eluent A was 95 % water 5 % acetonitrile and eluent B was 95 % acetonitrile 5 % water, both containing 0.5 % acetic acid. A gradient elution was performed by changing the mobile phase composition as follows. After starting at 100 % eluent A, the proportion of eluent B was linearly increased to 100 % over a period of 5 min, and kept constant for 5 min. The column was re-equilibrated with 100 % eluent A for 5 min. For LC/MS analyses, the ESI interface was used in positive ion mode, with the following settings: dry temp 350 °C; nebulizer 40 psi, nitrogen, dry gas 10 L/min, capillary voltage −3,500 V. The mass spectrometer operated in MRM (multiple reaction monitoring) mode by monitoring three transitions (1 quantifier, 2 qualifiers) for ST, with a dwell time of 200 ms. Quantification of ST was performed by measuring peak areas in the MRM chromatogram, and comparing them with the relevant calibration curve of ST.

Tuning experiments were performed by direct infusion at a flow rate of 0.6 mL/h of 1 μg/mL standard solution of ST in acetonitrile/water (50/50, v/v) containing 0.5 % acetic acid. The infusion was performed by using a model KDS100CE infusion pump (KDS Scientific Holliston, MA, USA). Interface parameters were as follows: dry temp 350 °C; nebulizer 10 psi nitrogen, dry gas 5 L/min, capillary voltage −3,500 V, spacer was removed for flow infusion.

## Results

Thirteen species (32 strains) from *Aspergillus* section *Versicolores* were tested for ST production. *A. amoenus* strain NRRL 236, isolated from *Berberis* sp. fruit produced ST on YEB but not on CZ20 (Table 1). The only known isolate of *A. austroafricanus* (NRRL 233) isolated from an unrecorded substrate did not produce ST on either medium (Table 1). The most frequently reported section *Versicolores* species from indoor air samples was *A. creber* which produced ST from all of the tested 7 isolates (Table 1). Two of three *A. cvjetkovicii* strains were positive for ST production. *A. fructus* strain NRRL 239, isolated from date fruit produced ST on both media (Table 1). The three strains of *A. jensenii*, a species commonly isolated from indoor air, produced ST along with one that was isolated from paraffin (Table 1). *A. protuberus* was also frequently isolated from indoor air. Four of the five strains of *A. protuberus* tested in this study were isolated from indoor air and one was isolated from rubber coated electrical cables (NRRL 3505). Three strains of *A. protuberus* isolated from air produced ST, while one indoor air strain and the type culture (NRRL 3505) (Muntanjola-Cvetković 1968) did not produce ST, which corresponds with the findings of Rank et al. (2011). *A. puulaauensis* (NRRL 58602) isolated from indoor air produced ST on both media (Table 1). *A. subversicolor* (NRRL 58999) which has only been isolated from coffee berries in India, produced ST on YEB but not on CZ20 (Table 1). The *A. tabacinus* strains isolated from tobac-

**Table 1** Sterigmatocystin production (ng/mL) by *Aspergillus* section *Versicolores* species after 7 days incubation in the dark at 25 °C on two liquid media

| NRRL number[a] | Species | CZ20 | YEB | Source of isolate |
|---|---|---|---|---|
| NRRL 236 | *A. amoenus* | < LOQ[b] | 31 | *Berberis* sp. fruit |
| NRRL 233 | *A. austroafricanus* | <LOQ | <LOQ | Unknown |
| NRRL 58583 | *A. creber* | <LOQ | 23 | Indoor air |
| NRRL 58584 | *A. creber* | 113 | 358 | Indoor air |
| NRRL 58601 | *A. creber* | <LOQ | 11 | Indoor air |
| NRRL 58606 | *A. creber* | <LOQ | 31 | Indoor air |
| NRRL 58673 | *A. creber* | 147 | 331 | Indoor air |
| NRRL 58675 | *A. creber* | 713 | 2,233 | Indoor air |
| NRRL 58612 | *A. creber* | <LOQ | 30 | Indoor air |
| NRRL 227 | *A. cvjetkovicii* | 522 | 652 | Soil |
| NRRL 4642 | *A. cvjetkovicii* | 142 | 165 | Unknown |
| NRRL 58593 | *A. cvjetkovicii* | <LOQ | <LOQ | Indoor air |
| NRRL 239 | *A. fructus* | 4,108 | 18,431 | Date fruit |
| NRRL 235 | *A. jensenii* | 3,960 | 15,710 | Paraffin |
| NRRL 58582 | *A. jensenii* | 21 | 461 | Indoor air |
| NRRL 58600 | *A. jensenii* | 758 | 4,558 | Indoor air |
| NRRL 58674 | *A. jensenii* | 2,047 | 11,504 | Indoor air |
| NRRL 58613 | *A. protuberus* | <LOQ | 156 | Indoor air |
| NRRL 58747 | *A. protuberus* | 10 | 915 | Indoor air |
| NRRL 58748 | *A. protuberus* | 38 | 201 | Indoor air |
| NRRL 58990 | *A. protuberus* | <LOQ | <LOQ | Indoor air |
| NRRL 3505 | *A. protuberus* | <LOQ | <LOQ | Rubber-coated electrical cables |
| NRRL 58602 | *A. puulaauensis* | 10 | 22 | Indoor air |
| NRRL 58999 | *A. subversicolor* | <LOQ | 93 | Coffee berry |
| NRRL 5031 | *A. tabacinus* | <LOQ | <LOQ | Unknown |
| NRRL 4791 | *A. tabacinus* | <LOQ | <LOQ | Tobacco |
| NRRL 229 | *A. tennesseensis* | <LOQ | <LOQ | Unknown |
| NRRL 234 | *A. tennesseensis* | <LOQ | <LOQ | Chestnut seed |
| NRRL 13150 | *A. tennesseensis* | 35 | 372 | Toxic dairy feed |
| NRRL 13147 | *A. venenatus* | 147 | 328 | Toxic dairy feed |
| NRRL 13145 | *A. versicolor* | 4,850 | 13,050 | Toxic dairy feed |
| NRRL 238 | *A. versicolor* | 332 | 47 | Unknown |

[a]NRRL (Northern Regional Research Laboratory), the National Center for Agricultural Utilization Research, Peoria, IL USA

[b]$<LOQ$ lower then limit of quantification

co or isolated from an unknown source did not produce ST (Table 1). All three *Aspergillus* species isolated from dairy animal feed, *A. versicolor*, *A. tennesseensis* and *A. venenatus* produced ST (Table 1).

## Discussion

Some researchers have used solid media to test potential mycotoxin production (Andersen et al. 2004; Frisvad and Samson 2004; Frisvad et al. 2004; Rank et al. 2011), others have used liquid media with constant shaking (Rabie et al. 1976), or liquid media with no shaking (Perrone et al. 2006). We chose liquid media without shaking for two reasons. Shake cultures generally produce more conidia and less mycelium, and it has also been shown that ST production is generally high in still culture but can be very low in shake cultures (Yu and Leonard 1995).

Other fungal growth conditions can affect mycotoxin production (length of fungal growth, substrate, temperature, pH and many other physical, chemical and biological factors) (Wilson and Abramson 1992), and it is possible that the level of ST production can be affected by these different conditions (Rabie et al. 1976; Yu and Leonard 1995). The incubation temperature reported in many prior publications on ST production was 25 °C regardless of whether solid or liquid media were used in the experiments (Rabie et al. 1976; Andersen et al. 2004; Frisvad and Samson 2004; Frisvad et al. 2004; Rank et al. 2011).

Frisvad and Samson (2004) and Frisvad et al. (2004, 2005) suggested that Yeast Extract Agar (YEA) agar is probably the best medium for high ST production. Of the two media we used, YEB on average gave much higher yields of ST (mean 2,179 ng/mL) than CZ20 (mean 561 ng/mL) (Table 1), but both were largely effective in eliciting ST production.

*Aspergillus* section *Versicolores* species are prevalent in the indoor environment. ST production by some members of section *Versicolores* has been known for more than three decades. Prior to the description of the nine new species (Jurjevic et al. 2012), only four *Aspergillus* section *Versicolores* species were known with *A. versicolor* the best known. Accordingly, reports of ST production were mostly attributed to *A. versicolor*. Eleven out of the 13 *Aspergillus* species in the section produced ST. The concentration of ST produced by isolates of different species ranged widely (10–18,431 ng/mL) and highly variable production among isolates of a species was also observed (Table 1). We have found that all *Aspergillus* section *Versicolores* species isolated from the indoor environment (*A. creber*, *A. cvjetkovicii*, *A. jensenii* and *A. protuberus*) and two species isolated from fruits (*A. amoenus* and *A. fructus*) have the ability to produce ST. From among the indoor air species, *A. jensenii* isolates produced large amounts of ST on average, while *A. creber*, *A. cvjetkovicii* and *A. protuberus* isolates generally produced low amounts of ST. It is unclear whether ST production confers selective advantage on the fungus in the sick building environment.

Sterigmatocystin is also known from commodities in post-harvest storage and animal feeds (Sugimoto et al. 1976; Abramson et al. 1983; Pitt and Hocking 2009). Animal feed infested with three morphotypes of *A. versicolor*, all of which produced ST, were implicated in dairy animal toxicosis (Vesonder and Horn 1985). Those three morphotypes are now identified as *A. versicolor* (NRRL 13145), *A. tennesseensis* (NRRL 13150) and *A. venenatus* (NRRL 13147), while all three produce ST, *A. versicolor* produces ST at far higher concentrations than the other two species (Table 1). *A. sydowii* is known as a non-ST producer (Rank et al. 2011) and was not included in this study.

The majority of *Aspergillus* section *Versicolores* species produce ST, but the amount of ST produced varies widely among the species. *A. versicolor*, *A. fructus* and *A. jensenii* produced ST in 10–100 times greater amounts than the other species in the section and represent the greatest threats of ST contamination.

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**Conflict of interest** None

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