8-Deoxy-trichothecin production by *Spicellum roseum* isolated from a cultivated mushroom in Japan

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**Summary**

Ex-type culture of *Spicellum roseum* isolated from silage in France has been known to produce some trichothecenes. As the causative agent of the pink mold damping-off disease, we isolated some strains of *S. roseum* from a cultivated mushroom *Flammulina velutipes* at Nagano, Japan. To examine whether the Japanese strains of *S. roseum* produce trichothecenes or not, the isolates were cultivated on rice medium and extraction was made for mycotoxin analysis. 8-Deoxy-trichothecin was accordingly detected and confirmed with GC/MS by means of EI and CI mode. However, deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X and nivalenol were not detected in the extract, all of which are well known to be detected from scabby wheat.

**Key words**: 8-deoxy-trichothecin, *Flammulina velutipes*, mycotoxins, *Spicellum roseum*, trichothecene

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**Introduction**

Nakamura et al.1) reported that *Sporothrix* sp. was the causative agent of the pink mold damping-off disease of *Flammulina velutipes* (Curt.: Fr.) Sing., a cultivated mushroom in Japan. The hyphomycete was later reidentified as *Spicellum roseum* Nicot & Roquebert based on morphology and rDNA sequence analysis
Spicellum roseum was first isolated from silage in France (Nicot and Roquebert). Other strains were isolated from mushroom compost in USA, paper bags in India, and leather in UK (Seifert et al.). Seifert et al. recently reported that ex-type culture of S. roseum produced trichothecene mycotoxins: i.e., 12,13-trichothec-9-ene, 8-deoxy-trichothecin, trichodermol and trichodermin. The structures of some trichothec mycotoxins were shown in Fig. 1. In this paper, we examined new isolates of S. roseum from cultivated F. velutipes in Japan to determine if these strains produce trichothecenes or not.

Materials and Methods

Some strains of S. roseum were isolated from cultivated F. velutipes at Nagano, Japan, which was infected by the pink mold damping-off disease. The isolates were deposited in Japan Collection of Microorganisms (JCM; http://www.jcm.riken.go.jp/). Two strains of S. roseum JCM 8964 and 8965 were used for the productivity test of trichothecenes.

Culture of S. roseum was done as follows. Thirty gram of 4% polished rice was weighed in conical flask and 15 ml of water was added. It was maintained for 3 hours in room temperature and then autoclaved. For pre-culturing, S. roseum was inoculated by a needle at the center of potato dextrose agar (PDA) plates in 9 cm diam Petri dish. The pre-culture was kept at 25 °C for 7 days. Inoculation of S. roseum to the rice medium was made by adding three pieces of 8 mm diam disks cut from the pre-culture plate by a cork bowler. The inoculated rice medium was kept at 25 °C for 14 days.

Extraction and clean-up procedures for mycotoxins were as listed in Figs. 2 and 3, respectively.

TMS derivatization of extract from S. roseum cultures was as follows. TMS derivatizing reagent was
composed of 1.0 ml of N-trimethylsilylimidazole, 0.2 ml of trimethylchlorosilane and 9.0 ml of ethyl acetate. Zero point one ml of TMS derivatizing reagent was added to the extract after clean-up and kept at room temperature for 15 min. Then 3.9 ml of ethyl acetate was added to this reacting solution. This solution was filtered with filter disk of Ekikurodisk R 13CR with 0.2 Ж m PTFE (Gelman Japan). The filtrate was analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS).

The GC, Shimadzu GC-15A (Shimadzu Co., Kyoto, Japan) equipped with ECD detector, was used. The conditions of GC were listed as follows. DB-1 megabore column (J & W Scientific, Folsom, CA, USA), 10 m x 0.53 mm i.d. and 1.5 Ж m in film thickness, was used. The column temperature was held at 185°C for 50 min. It was increased by 10 °C/min to 240°C and maintained for 15 min. Injection port was 280°C. N2 gas for column connection purge was 22 ml/min and that for cell purge was 60 ml/min. Flow rate of He gas was 15 ml/min. Detection was made by ECD.

GC/MS conditions of EI mode by Shimadzu GCMS-QP5000 were as follows. Shimadzu GC-17A was
used as a gas chromatograph. A 30 m x 0.25 mm i.d. DB-1 column (J & W Scientific, Folsom, CA, USA) with film thickness of 0.25 μm was used. The column temperature, initial at 190°C, was increased by 1.5°C /min to 217°C and then by 6.0°C/min to 280°C and maintained at 280°C for 5 min. Injection port temperature was 250°C and interface temperature was 300°C. Flow rate of He gas was 1.8 ml/min. Detection voltage was 2.50 kV.

The cleaned-up extract was trimethylsilylated and injected into GC. The TMS derivatives were also analyzed by GC/MS, Shimadzu QP-5000 (Shimadzu Co., Japan), both in the electron ionization (EI) and chemical ionization (CI) modes. For CI, methane gas was used as the reagent gas. ThermoQuest Finnigan Trace GC/MS instrument was used to compare authentic 8-deoxy-trichothecin with the trichothecin in order to confirm the presence of 8-deoxy-trichothecin. Spectra were recorded in the EI and CI modes. For CI, both methane and isobutane (which had been used by Plattner et al.5) were used as reagent gases.

Results and Discussion

Deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-Ac-DON), fusarenon-X (Fus. -X) and nivalenol (NIV) are well known trichothecenes often found in scabby wheat and have some toxicity against animals and human. These mycotoxins were analyzed using gas chromatography that showed the detection limit as 0.2 μg/g for DON, 3-Ac-DON, Fus.-X and NIV. Gas chromatogram and mass spectra obtained showed that these trichothecenes were not present in the culture extract of S. roseum isolated from affected F. velutipes in Japan, although the extraction is suitable for these trichothecenes. Therefore, we conclude that these four mycotoxins were not produced by S. roseum JCM 8964 and JCM 8965.

One peak of trichothecenes was obtained in gas chromatogram, so trimethylsilylated sample was injected into QP-5000 type of GC/MS. In GC/MS analysis mass spectra similar to that reported for 8-deoxy-trichothecin were obtained from the extract of S. roseum JCM 8965 by means of EI and CI mode from QP-5000 GC/MS. The molecular weight of 8-deoxy-trichothecin is 318.

To confirm this result TMS derivatives of the S. roseum were analyzed on the TRACE GC/MS along with authentic 8-deoxy-trichothecin5. In the chromatogram of the extract of JCM 8965, components were observed which had identical spectra with the EI and isobutane CI spectra previously reported for the TMS derivative of 8-deoxy-trichothecin. The larger of these two components, 70% of the total, eluted at 7.14 min, while the latter eluted at 7.22 min. By contrast, the chromatogram of the TMS derivative of authentic 8-deoxy-trichothecin revealed only a single component that eluted with the same retention time (7.14 min). Thus the component eluting at 7.14 min is confirmed to be 8-deoxy-trichothecin. The EI mass spectrum and the isobutane CI spectrum are identical with the ones reported previously by Plattner et al.5. From these results, it becomes clear that S. roseum JCM 8965 produced 8-deoxy-trichothecin. The exact structure of the minor component in the extract of JCM 8965 has presently not been completely characterized but is presumably a stereoisomer of 8-deoxy-trichothecin. The total ion chromatograms and EI mass spectra of 8-deoxy-trichothecin and the extract of JCM 8965 are shown in Fig. 4 and Fig. 5, respectively. The total ion chromatograms, the single ion monitor chromatograms of 319 (M+1) and CI (isobutane) mass spectra of 8-deoxy-trichothecin and the extract of JCM 8965 are shown in Fig. 6 and Fig. 7, respectively. In the case of S. roseum JCM 8964, the production of 8-deoxy-trichothecin was obscure.

Though the toxicity of 8-deoxy-trichothecin has not been known yet, the toxicity test of the extract of S.
Fig. 4  El mass spectrum of 8-deoxy-trichothecin and the total ion chromatogram.

Fig. 5  El mass spectrum of the extract of JCM 8965 and the total ion chromatogram.
Fig. 6  CI(isobutane) mass spectrum of 8-deoxy-trichothecin, the total ion chromatogram, and the single ion monitor chromatogram of 319 (M+1).

Fig. 7  CI(isobutane) mass spectrum of the extract of JCM 8965, the total ion chromatogram and the single ion monitor chromatogram of 319 (M+1).
roseum cultures should be carried out for more complete safety on mushroom cultivation.

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References


日本の栽培キノコから分離した *Spicellum roseum* による 8-デオキシシートロコチンの産生

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フランスの堆肥から分離された不完全菌 *Spicellum roseum* のタイプ由来株を培養すると、トリコテセン系マイコトキシンを産生することが知られている。桃色かび立枯病を引き起こす原因菌として、我々は長野県のエノキタケ (*Flammulina velutipes*) から数株の *S. roseum* を分離した。これら日本産の *S. roseum* がトリコテセン系マイコトキシンを産生するか否かを調べるために、分離菌株を米培地で培養し、マイコトキシンの分析を行った。EIモード及びCIモードのガスクロマトグラフ質量分析計で 8-デオキシシートロコチンを検出し、確認した。しかしながら、赤かび病罹病のムギから検出されるトリコテセン系マイコトキシンとして有名なデオキシニバレノール、3-セチルデオキシニバレノール、フザレノン-X 及びニバレノールは検出されなかった。

キーワード：8-デオキシシートロコチン, *Flammulina velutipes*, マイコトキシン, *Spicellum roseum*, トリコテセン