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Fusaproliferin Production by *Fusarium subglutinans* and Its Toxicity to *Artemia salina*, SF-9 Insect Cells, and IARC/LCL 171 Human B Lymphocytes

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***Fusarium subglutinans* is an important pathogen of maize and other commodities worldwide. We examined MRC-115 and 71 other *F. subglutinans* strains from various geographic areas for their ability to synthesize fusaproliferin, a novel toxic sesterterpene recently isolated from *F. proliferatum*. Fusaproliferin production ranged from 30 to 1,500 μg/g of dried ground substrate, with 33 strains producing more than 500 μg/g. In particular, strain MRC-115 produced as much as 1,100 to 1,300 μg/g. In toxicity studies of two invertebrate models, fusaproliferin was toxic to *Artemia salina* (50% lethal dose, 53.4 μM) and to the lepidopteran cell line SF-9 (50% cytotoxic concentration, approximately 70 μM, after a 48-h exposure). Fusaproliferin was also toxic to the human nonneoplastic B-lymphocyte cell line IARC/LCL 171 (50% cytotoxic concentration, approximately 55 μM in culture in stationary phase after a 48-h exposure). Experiments performed with cells exposed at seeding suggested a possible cytostatic effect at subtoxic concentrations.**

Fusaproliferin is a novel sesterterpene (Fig. 1), recently identified from maize cultures of *Fusarium proliferatum* (Matsushima) Nirenberg, strain Istituto Tossine e Micotossine da Parassiti Vegetali of Bari 1494 (ITEM-1494) isolated from maize (30). In a brine shrimp (*Artemia salina* L.) bioassay, fusaproliferin was recognized as the major toxic component of organic extracts from maize cultures of ITEM-1494 (31). Further work has been devoted to elucidating the chemical structure of fusaproliferin (19, 34). However, little information on its production by other *Fusarium* species and its toxicity has been available.

F. subglutinans (Wollenw. et Reinking) Nelson, Toussoun et Marasas is a species closely related to *F. proliferatum* (27). Both species are important pathogens of maize worldwide (10, 15, 21) and may both be isolated from the same plant at the same time (15). From a toxicological point of view, members of both species produce secondary metabolites such as fusaric acid (22), moniliformin (11, 22), and beauvericin (6, 25). Some strains of *F. subglutinans* are known to be highly toxic to experimental animals (1, 22, 29). In particular, a strain deposited at the Medical Research Council Collection (MRC), Tygerberg, South Africa, namely, MRC-115, was isolated from home-grown maize produced in an area of Transkei, South Africa, with a high incidence of human esophageal cancer and found to be toxic to ducklings and rats (22). Moniliformin was first recognized as a toxic metabolite in cultures of this strain (11). Subsequently, the toxic cyclohexadepsipeptide beauvericin (7) was found in cultures of strain MRC-115 and in other isolates of *F. subglutinans* (26). However, neither moniliformin nor beauvericin could account for the entire toxic effects of *F. subglutinans* MRC-115 as well as of other strains in the brine shrimp assay, suggesting the presence of additional toxic metabolites in the fungal culture extracts (17, 18, 26).

In this paper, we report on the identification of fusaproliferin in organic extracts of MRC-115 and 71 other *F. subglutinans* strains cultured on maize. The toxicity of fusaproliferin purified from *F. subglutinans* was evaluated in two invertebrate models, *A. salina* and the pupal ovarian cells of the lepidopteran *Spodoptera frugiperda* SF-9 (36, 37). Moreover, since no information on the possible toxicity of fusaproliferin to mammals and mammalian cells has been available thus far, a third model represented by IARC/LCL 171 cells (12) was also studied. The IARC/LCL 171 cell line is a lymphoblastoid cell line (33) established from normal diploid B lymphocytes, which can be maintained in culture in spite of their nonneoplastic nature and thus can serve in cytotoxicity studies with emphasis on the lymphoid cell lineage.

MATERIALS AND METHODS

Fungal isolates. Strain MRC-115 (M-811; ITEM-1472) of *F. subglutinans* was obtained from P. E. Nelson, *Fusarium* Research Center, University of Pennsylvania, University Park. An additional 71 *F. subglutinans* strains from other geographic areas, including Argentina, Austria, Canada, Germany, Italy, Poland, South Africa, the United States, and Zambia (Table 1), were tested for fusaproliferin production after cultivation on maize kernels. These strains, isolated mostly from maize but also from other plant hosts, were derived from single conidia cultivated on special nutrient agar (28). Identification of the strains was confirmed microscopically upon subculturing on both potato dextrose agar and carnation leaf agar (27). Mycelia and conidia from strains grown on carnation leaf agar were frozen in sterile 18% glycerol and stored at −75°C.

Isolation and purification of fusaproliferin from *F. subglutinans* MRC-115. Maize kernel substrates (typically 200 g), previously maintained overnight at about 45% moisture and autoclaved for 30 min at 120°C, were inoculated with an aqueous suspension of *F. subglutinans* MRC-115 conidia (2 ml; approximately 10^7 conidia per ml) in Erlenmeyer flasks. Following incubation for 4 weeks at 25°C in the dark, the cultures were dried at 50°C for 48 h and finely ground.

Dried ground maize cultures were extracted with methanol (MeOH)–1% aqueous NaCl (1,000 ml [55:45, vol/vol]). After MeOH removal under vacuum at room temperature, the aqueous phase was extracted with *n*-hexane and the solvent was separated and removed under vacuum. The oily residue of crude extract was fractionated and purified by column chromatography on SiO_2.

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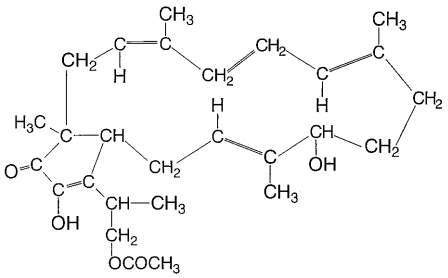

FIG. 1. Structure of fusaproliferin.

Overtly nonpolar substances were removed by elution with 400 ml of ethyl acetate–*n*-hexane (10:90, vol/vol); the column was further eluted with a 40:60 (vol/vol) mixture of the same solvents, and 8-ml fractions were collected. Material from individual fractions was successively analyzed by thin-layer chromatography (TLC) on precoated silica gel 60 F_{254} plates (10 by 20 cm; thickness, 0.25 mm [E. Merck, Darmstadt, Germany]) with ethyl acetate–*n*-hexane (40:60, vol/vol) as the eluent. Spots were visualized by UV light (wavelength, 254 nm) and exposure to iodine vapors. Fractions with a homogeneous chromatographic pattern were pooled and dried under vacuum; the resulting material was monitored for toxicity to *A. salina* (see below). Material from the toxic fractions was combined and purified by preparative TLC on plates as above, eluted with ethyl acetate–*n*-hexane (50:50, vol/vol). Spots of interest were scraped from the plates, and the resulting material was extracted with MeOH and dried under vacuum, yielding an apparently pure amorphous solid residue, which was analyzed by spectroscopic methods as previously described (30, 31). Briefly, a low-resolution mass spectrum was recorded with a Fisons TRIO 2000 system. UV spectroscopy was performed with a Kontron Uvikon 930 spectrophotometer; material of interest was analyzed as a MeOH solution. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were determined in $CDCl_3$ solution with a Bruker AMX600 spectrometer operating at 600.13 and 150.92 MHz, respectively. ^1H-^1H correlated and ^1H-^{13}C heteronuclear shift correlated NMR spectra were collected by using the pulse sequence HETCOR (hetero-correlated experiment).

Fusaproliferin detection in *F. subglutinans* cultures. Material from cultures grown on maize kernels in duplicate and processed as described above was subjected to extraction by the method of Bottalico et al. (2) with minor modifications. Briefly, a 20-g maize sample of each strain was extracted with 100 ml of MeOH–1% aqueous NaCl (55:45, vol/vol) in a blender for 3 min and filtered through paper (Whatman no. 1) under vacuum; 50 ml of the filtrate was then transferred to a separator funnel and extracted three times with 50 ml of *n*-hexane. The organic phase was treated with Na_2SO_4 to remove residual water, filtered, and evaporated under vacuum. The oily residue was finally dissolved in 1 ml of MeOH. Fusaproliferin was identified and quantified by means of high-performance TLC (HPTLC) and high-pressure liquid chromatography (HPLC). Precoated HPTLC silica gel 60 F_{254} plates (10 by 20 cm; thickness, 0.25 mm) were spotted with 20 μl of a methanolic solution of the oily residues or with 0.5, 1, 3, or 5 μl of an ethanolic fusaproliferin (isolated from *F. subglutinans* MRC-115 and purified to near purity, as described above) solution (1 mg/ml). The plates were eluted with three different mobile-phase mixtures: toluene-acetone (75:25, vol/vol), chloroform–2-isopropanol (95:5, vol/vol), and ethyl acetate–*n*-hexane (50:50, vol/vol). The run was carried out for approximately 8 cm, and the plates were air dried and observed under UV light (365 and 254 nm). Fluorescence quenching spots were marked, and the plates were exposed to iodine vapor, allowing fusaproliferin to be visualized as a brownish spot. R_f values for fusaproliferin were about 0.60 in toluene-acetone 0.80 in chloroform–2-isopropanol, and 0.45 in ethyl acetate–*n*-hexane. Further analysis was sometime performed with reverse-phase C_{18} plates (10 by 5 cm; thickness, 0.25 mm [Whatman]) developed with acetonitrile-H_2O (75:25, vol/vol). The R_f for fusaproliferin was 0.50 in this case. The detection limit for fusaproliferin in extracts of maize cultures was approximately 1 μg/g of dried ground culture.

HPLC was also carried out, in certain cases, with an LDC Constametric 3200 pump equipped with a reverse-phase C_{18} Accubond 5 μ column (250 by 4.6 mm) (J & W, Folsom, Calif.) and a Spectrometer 3100 UV detector, set at 261 nm. Acetonitrile-H_2O (65:35, vol/vol) was used as the eluent at a flow rate of 1.5 ml/min. Quantitative analysis of fusaproliferin (retention time, approximately 4.95 min) was performed by comparison of the fusaproliferin peak areas with those of a calibration curve obtained with the authentic standard. The detection limit for fusaproliferin by this procedure was 20 ng. Analysis was performed by injecting 25 μl of the methanolic solution of the oily residues, corresponding to 500 mg of maize sample.

Brine shrimp bioassay. Toxicity to brine shrimp larvae was determined as previously described (2). Briefly, larvae were exposed to either potentially toxic material (oily residue of crude extract and chromatography fractions from *F. subglutinans* cultures) or purified fusaproliferin in 24-well cell culture plates (30 to 40 larvae per well in 500 μl of 3.3% [wt/vol] marine salt in H_2O). The potentially toxic material was dissolved in MeOH (final organic solvent concentration, 1% [vol/vol]). The number of dead shrimps was recorded after incubation at 27°C for 24 h. The total number of shrimps in each well was counted after killing the surviving shrimps by freezing at −20°C for 12 h. Tests were performed in quadruplicate. Probit analysis based on averages of three independent experiments was used for calculation of the 50% lethal dose (LD_{50}).

Cell cultures and cytoxicity assays. SF-9 cells were cultured at 27°C in TNM-FH medium (Nord Junter and Cell, Bromma, Sweden) (8) supplemented with 10% (vol/vol) fetal calf serum (Kallergen srl, Milan, Italy), penicillin (100 U/ml), and streptomycin (100 mg/ml). Cultures in early stationary phase (typical cell density, 1.6×10^6 cells per ml; typical viability, approximately 80%) were passaged every 5 days with a seeding density of 4×10^5 cells per ml.

IARC/LCL 171 cells were cultured in RPMI 1614 medium (Flow Laboratories Ltd., Irvine, United Kingdom) (23), supplemented as above, at 37°C in an atmosphere of 5% CO_2 saturated with water vapor. The cultures were passaged every 60 to 72 h, when they were in the early stationary phase (typical cell density, 1.5×10^6 to 2×10^6/ml; typical viability, approximately 90%). The seeding density was adjusted to 6×10^5 cells per ml.

Fusaproliferin, obtained as above, was dissolved either in ethanol (EtOH) or in dimethyl sulfoxide (DMSO) and stored under argon at −30°C. The final concentration of organic solvent in the cultures assayed was 1% (vol/vol).

Cytotoxicity was evaluated in terms of cell viability as assessed microscopically by the trypan blue exclusion method. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of fusaproliferin that caused a 50% decrease in cell viability. Cultures were also monitored by phase-contrast microscopy by observing culture flasks through a reverse microscope.

For the IARC/LCL 171 cells, cytotoxicity assays were performed either by exposing early-stationary-phase cultures to fusaproliferin or by including the toxin in the culture medium at passaging. When cultures were exposed in the stationary phase, either EtOH or DMSO fusaproliferin solutions, at concentration ranging between 100 nM and 100 μM, were added and the cultures were maintained in a CO_2 incubator. The cultures were examined by phase-contrast microscopy, and the cell density and viability were assessed at 24 and 48 h after the addition of fusaproliferin. When cell cultures were exposed to fusaproliferin at passaging, appropriate amounts of substance dissolved in DMSO were included in fresh medium and cells were allowed to proliferate in the presence of the reagent. Culture density and viability were assessed, and phase-contrast microscopy was performed after 1, 2, and 3 days.

Multiple observations are presented as the arithmetic mean ± standard deviation.

RESULTS

Production of fusaproliferin in cultures of *F. subglutinans* MRC-115. By a two-step extraction procedure, an oily crude organic extract (typically approximately 400 mg/200 g of maize culture), which caused 100% mortality in the brine shrimp bioassay at a 60-μg/ml concentration, was obtained from *F. subglutinans* MRC-115 cultures. This material was subjected to column chromatography on SiO_2 with ethyl acetate–*n*-hexane (40:60, vol/vol) as the eluent, and the eluate was fractionated and analyzed as described above. This procedure yielded approximately 300 mg of toxic (as assessed by brine shrimp bioassay) residue. Further purification by preparative TLC allowed the separation of approximately 225 mg of an apparently pure amorphous white powder, which was analyzed by spectroscopic methods (30, 31).

Low-resolution mass spectrometry revealed an *m/z* 444 molecular peak for the unknown toxic substance. UV spectroscopy gave a λ_{max} of 261 nm for the unknown substance, with a molar extinction coefficient of 60,000 in MeOH. ^1H and ^{13}C nuclear magnetic resonance spectroscopy indicated that the substance had a bicyclic terpene structure with a molecular formula of $C_{27}H_{40}O_5$ (30, 31). From these results, the unknown toxic substance was identified as fusaproliferin. These results were corroborated by HPTLC (Table 1) and HPLC analysis (data not shown).

Production of fusaproliferin by strains of *F. subglutinans* from various geographic areas. The production of fusaproliferin by 71 strains of *F. subglutinans*, mostly isolated from maize, is reported in Table 1. All the tested strains except ITEM-1435 were able to produce fusaproliferin at levels ranging from 30 to 1,500 μg/g of dried ground maize culture. In particular, 35 strains isolated from Polish maize between 1984

TABLE 1. Production of fusaproliferin by 72 strains of *F. subglutinans* from various geographic areas[a]

Mass culture (original no.)	Single-conidium culture (ITEM no.)	Yr of isolation	Original host	Source[b]	Geographic origin	Fusaproliferin concn (μg/g of dried culture)[c]
KF163	1348	1984	Maize	JC	Poland	200
KF195	1349	1984	Maize	JC	Poland	100
KF197	1422	1984	Maize	JC	Radikòw, Poland	500
KF198	1423	1984	Maize	JC	Radikòw, Poland	900
KF201	1352	1984	Maize	JC	Poland	80
KF214	1355	1984	Maize	JC	Poland	200
KF217	1354	1984	Maize	JC	Poland	100
KF555	1405	1984	Maize	JC	Poland	100
KF242	1406	1984	Maize	JC	Poland	150
KF218	1425	1984	Maize	JC	Swadrim, Poland	800
KF228	1426	1984	Maize	JC	Radikòw, Poland	850
KF229	1427	1984	Maize	JC	Smolice, Poland	300
KF231	1428	1984	Maize	JC	Smolice, Poland	1,000
KF236	1429	1984	Maize	JC	Kobieryce, Poland	800
KF241	1353	1984	Maize	JC	Poland	750
KF506	1351	1985	Maize	JC	Smolice, Poland	700
KF509	1432	1985	Maize	JC	Poland	900
KF521	1433	1985	Maize	JC	Swadrim, Poland	950
KF532	1350	1985	Maize	JC	Poland	100
KF534	1434	1985	Maize	JC	Kobieryce, Poland	850
KF536	1435	1985	Maize	JC	Kobieryce, Poland	ND[d]
KF589	1436	1985	Maize	JC	Radikòw, Poland	250
KF1204	1438	1987	Maize	JC	Radikòw, Poland	850
KF1206	1439	1987	Maize	JC	Radikòw, Poland	700
KF1210	1440	1987	Maize	JC	Radikòw, Poland	700
KF1211	1441	1987	Maize	JC	Radikòw, Poland	850
ITEM	1518	1990	Maize	AL	Warsaw, Poland	40
ITEM	1519	1990	Maize	AL	Warsaw, Poland	550
ITEM	1520	1990	Maize	AL	Warsaw, Poland	700
ITEM	1521	1990	Maize	AL	Warsaw, Poland	700
ITEM	1548	1990	Maize	AL	Warsaw, Poland	800
ITEM	1549	1990	Maize	AL	Warsaw, Poland	900
ITEM	1550	1990	Maize	AL	Warsaw, Poland	550
ITEM	1551	1990	Maize	AL	Warsaw, Poland	510
ITEM	1552	1990	Maize	AL	Warsaw, Poland	600
ITEM	1553	1990	Maize	AL	Warsaw, Poland	1,500
DAOM169043	1469	1978	Maize	JC	Ontario, Canada	70
DAOM192138	1468		Maize	JC	Ontario, Canada	60
DAOM193263	1467		Maize	JC	Ontario, Canada	70
DAOM194909	1470	1984	Maize	JC	Ontario, Canada	60
DAOM195166	1466		Maize	JC	Ontario, Canada	50
19-21-5	1693	1991	Maize	KS	Bewdley, Canada	50
7-7-1	1694	1991	Maize	KS	Vara, Canada	30
5-5-2	1695	1991	Maize	KS	Hensall, Canada	40
17-6-2	1696	1991	Maize	KS	Bowmanville, Canada	30
4-3-3	1697	1991	Maize	KS	Zurich, Canada	700
11-3-4	1698	1991	Maize	KS	Strathroy, Canada	600
26-7-1	1699	1991	Maize	KS	Carp, Canada	1,100
I_1B012	1830	1992	Maize	SC	Argentina	130
I_1B013	1835	1992	Maize	SC	Argentina	120
I_1B014	1831	1992	Maize	SC	Argentina	140
I_1B003	1832	1992	Maize	SC	Argentina	110
I_1B021	1833	1992	Maize	SC	Argentina	110
I_1B019	1834	1992	Maize	SC	Argentina	120
I_5B010	1837	1992	Maize	SC	Argentina	110
ITEM	1753	1993	Maize	AB	Sassari, Italy	100
ITEM	1765	1993	Maize	AB	Sassari, Italy	120
ITEM	1766	1993	Maize	AB	Sassari, Italy	130
IMST86	1524	1986	Maize	AA	Imst, Austria	80
RITZ86	1525	1986	Maize	AA	Ritzlhof, Austria	850
RATT86	1526	1986	Maize	AA	Rattenberg, Austria	80
RATT86	1527	1986	Maize	AA	Rattenberg, Austria	90
E990	1029	1986	Maize	JFL	St. Elmo, Ill.	1,200
E2192	1030	1986	Maize	JFL	St. Elmo, Ill.	1,500
M1351[e]	1471	1974	Maize	PEN	Zambia	850
M811[e] (MRC-115)	1472	1975	Maize	PEN	Transkei (South Africa)	1,300
M1168[e]	1473		Maize	PEN	South Africa	750

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TABLE 1—*Continued*

Mass culture (original no.)	Single-conidium culture (ITEM no.)	Yr of isolation	Original host	Source[b]	Geographic origin	Fusaproliferin concn (μg/g of dried culture)[c]
M1354[e]	1474		*Haemanthus* sp.	PEN	Germany	100
KF130	1420	1982	Wheat	JC	Poland	100
KF207	1424	1984	Wheat	JC	Poznan, Poland	200
KF1034	1437	1985	Wheat	JC	Radikòw, Poland	250
KF143	1421	1982	Oats	JC	Poland	80

[a] Strains were grown on autoclaved maize kernels, incubated for 28 days at 25°C.
[b] JC, J. Chelkowski, Institute of Plant Genetics, Polish Academic of Sciences, Poznan, Poland; KS, K. Seifert, Agriculture Canada, Ottawa, Canada; SC, S. Chulze, Depto de Microbiologia e Inmunologia, Universidad Nacional de Rio Cuarto, Rio Cuarto, Argentina; AL, A. Logrieco, Istituto Tossine e Micotossine, CNR, Bari, Italy; AA, A. Adler, Federal Institute of Agrobiology, Linz, Austria; JFL, J. F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan; PEN, P. E. Nelson, *Fusarium* Research Center, Department of Plant Pathology, The Pennsylvania State University, University Park; AB, A. Bottalico, Istituto di Patologia Vegetale, Università di Sassari, Sassari, Italy.
[c] Representative values from at least two different culturings for each strain.
[d] ND, not detected.
[e] Strain toxic to experimental animals (22).

and 1990 produced fusaproliferin in amounts ranging from 40 to 1,500 μg/g. The toxin production ranged between 30 and 1,100 μg/g in 12 strains from Canadian maize (isolated from 1979 to 1991), between 110 and 140 μg/g in 7 strains from Argentinian maize (isolated in 1992), between 100 and 130 μg/g in 3 strains from Italian maize (isolated in 1993), and between 80 and 850 μg/g in 4 strains from Austrian maize (isolated in 1986). The two U.S. strains studied (ITEM-1029 and ITEM-1030) proved to be among the highest fusaproliferin producers, with 1,200 and 1,500 μg/g, respectively. Finally, two strains involved in animal toxicosis (ITEM-1471 [from Zambia], and ITEM-1473 [from South Africa] [11, 20]) also produced substantial amounts of fusaproliferin: 850 and 750 μg/g, respectively. Moreover, three strains isolated from wheat and one strain from oats (all isolated in Poland), as well as one strain from *Haemanthus* sp. (isolated in Germany), produced fusaproliferin but at lower levels (from 80 to 250 μg/g).

Toxicity of fusaproliferin to *A. salina*. The toxicity of purified fusaproliferin was investigated by the *A. salina* assay. Probit analysis of the means of three independent quadruplicate experiments allowed calculation of an LD_{50} of 53.4 μM (Fig. 2), corresponding to 23.7 μg/ml of water.

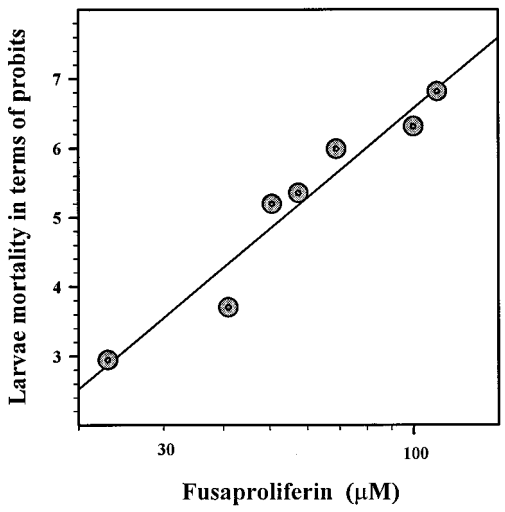

FIG. 2. Fusaproliferin toxicity to *A. salina* larvae. Mortality frequencies are plotted on a probit scale, and fusaproliferin dosages are plotted on a log scale. The probit regression line shown ($y = 5.96\,x + 2.77$) was used to estimate the LD_{50} (53.4 μM).

Cytotoxicity studies. (i) Toxicity to lepidopteran SF-9 cells. Stationary-phase cultures of *S. frugiperda* pupal ovarian SF-9 cells were exposed to fusaproliferin dissolved in DMSO, at concentrations ranging between 100 nM and 100 μM, for up to 48 h. Cells exposed to the solvent alone (1%, vol/vol) showed normal viability (79.2% ± 6.5% and 78.6% ± 8.1%; $n = 6$) after 24 and 48 h, respectively (Fig. 3), and exhibited normal microscopic features. No apparent effect could be detected up to 30 μM fusaproliferin. However, raising the mycotoxin concentration to 100 μM led to a definite decline in the culture viability (Fig. 3). The CC_{50} after 24 and 48 h could be estimated as approximately 100 and 70 μM, respectively.

(ii) Toxicity to human B-lymphocyte IARC/LCL 171 cells. Mature resting IARC/LCL 171 cell cultures were exposed to increasing concentrations of both EtOH and DMSO solutions of fusaproliferin for 48 h under the conditions stated above. When ethanolic solutions were used, cultures in early stationary phase exposed to the solvent alone displayed high viability after both 24 and 48 h (85.6% ± 6.7% and 73.6% ± 11.2%,

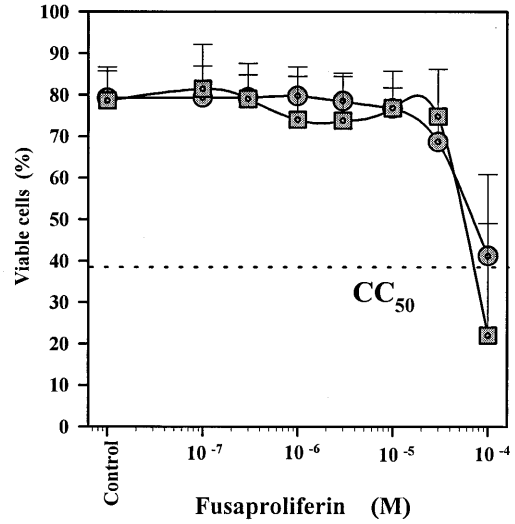

FIG. 3. Fusaproliferin cytotoxic curves for SF-9 cells. Cultures in stationary phase were exposed to fusaproliferin, dissolved in DMSO at concentrations ranging between 100 nM and 100 μM, for 24 h (●) and 48 h (■). The amount of solvent added to the cultures was 1% (vol/vol). Cell viability was determined microscopically by the trypan blue exclusion method. The CC_{50} after 24 h of exposure is shown (dotted line). Results are means of six independent experiments and standard deviation (bars).

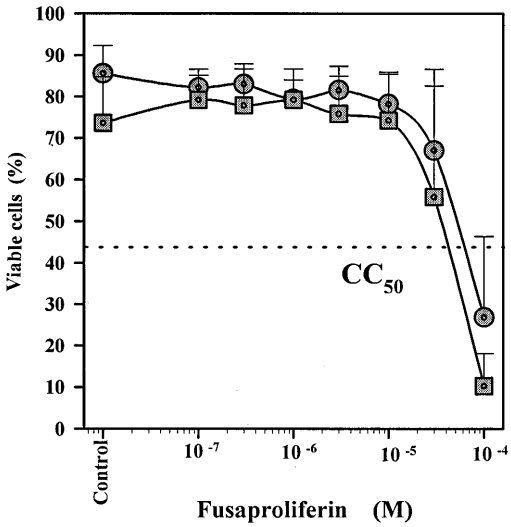

FIG. 4. Fusaproliferin cytotoxicity curves for IARC/LCL 171 cells. Cultures in early stationary phase were exposed to fusaproliferin, dissolved in EtOH at concentrations ranging between 100 nM and 100 μM, for 24 h (●) and 48 h (■). The amount of organic solvent added to the cultures was 1% (vol/vol). Cell viability was determined microscopically by the trypan blue exclusion method. The CC_{50} after 24 h of exposure is shown. Results are means of five independent experiments and standard deviation (bars).

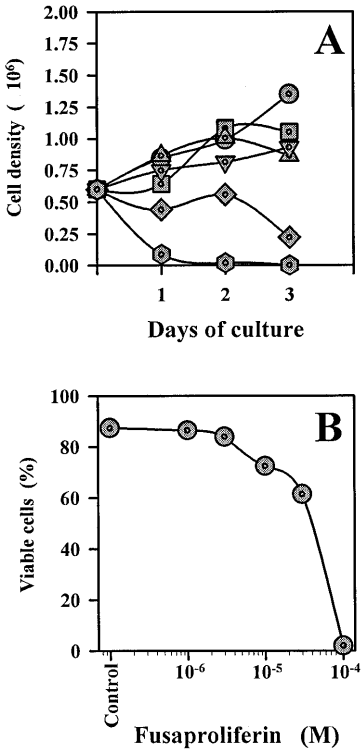

FIG. 5. Effects of fusaproliferin on cultures of IARC/LCL 171 cells exposed at passaging. (A) Cultures were started in the presence of 1 μM (▣), 3 μM (▲), 10 μM (▼), 30 μM (◈), and 100 μM (◉) fusaproliferin dissolved in DMSO. Control cultures were started in the presence of the solvent only (◉). Single points of the growth curves refer to means of two representative sets of experiments. (B) Cytotoxicity curve for cells allowed to grow in the presence of the mycotoxin for 48 h. Means of two representative sets of experiments are shown. In particular, the viability of the culture exposed to 30 μM fusaproliferin was approximately 62%.

respectively; $n = 5$) (Fig. 4). The cells also presented a fairly healthy aspect, appeared mostly clustered in large tight clumps (some of which were visible to the naked eye), and displayed no appreciable morphological abnormality. Exposing the cultures to up to 10 μM fusaproliferin for 24 h seemingly produced no effect on cell viability (Fig. 4); moreover, no change in cell morphology could be detected. In contrast, a clear decline in cell viability was noticed when the cultures were exposed to fusaproliferin at a concentration as high as 30 μM (Fig. 4). These cultures also lacked the large clumps described above, and the cells appeared essentially as individual elements, some of which had an irregular shape, indicating unhealthy cells. Finally, exposure to the highest concentration tested (100 μM) caused almost complete death of the cultures. Thus, fusaproliferin proved to possess a substantial toxicity to the human nonneoplastic IARC/LCL 171 B lymphocytes. For the ethanolic solution, the CC_{50} after 24 h of exposure could be calculated as approximately 60 to 65 μM. Extending the exposure time to 48 h resulted in a modest shift to the left of the toxicity curve (CC_{50}, approximately 55 μM [Fig. 4]).

Similar results were obtained when IARC/LCL 171 cultures in stationary phase were exposed to fusaproliferin dissolved in DMSO (data not shown). However, in this case, the CC_{50} was slightly lower (approximately 40 μM after 24 h of exposure), an effect that, at least partially, could have been due to the combined action of DMSO with fusaproliferin, since the viability of the controls exposed to the solvent alone was slightly lower (75.2% ± 11.4% and 68.8% ± 13.6% [$n = 5$] at 24 and 48 h, respectively).

When cultures of IARC/LCL 171 cells were cultured in medium supplemented with fusaproliferin at concentrations ranging from 1 to 100 μM and allowed to grow in the presence of the mycotoxin, again practically no effect was seen for concentrations up to 10 μM (Fig. 5A). Following a 36-h lag phase, cultures entered the log phase and early stationary phase normally; the latter stage was characterized by the formation of large clumps and by a yellowish color of the phenol red-containing medium, indicating acidification and high metabolic activity of the culture. In contrast, cultures seeded in the presence of 30 μM fusaproliferin exhibited an abnormal growth curve (Fig. 5A). The cells failed to proliferate and form the large clumps observed in the controls, although viability was not greatly affected (Fig. 5B). The medium stayed reddish, indicating negligible metabolism. Finally, exposing cultures of IARC/LCL 171 cells to 100 μM fusaproliferin at seeding caused the cells to die within 1 day.

DISCUSSION

F. subglutinans is an important pathogen of maize (3, 15, 21) and other crops that are used in animal feeds and human foods. Toxigenic strains of *F. subglutinans* produce toxic secondary metabolites, such as moniliformin and beauvericin (11, 22, 26). Several other *Fusarium* metabolites that are toxic to *A. salina* (35) and in various other experimental models (9) have been studied. Among these, fusaproliferin, a novel sesterterpene isolated from *F. proliferatum* and toxic to *A. salina* (31), deserves attention.

Here, we report the biosynthesis of this compound in single-conidium cultures of *F. subglutinans* isolated from maize in Europe (mostly in Poland but also in Italy and Austria), North America (Canada and the United States), Argentina, and Africa (Zambia and South Africa). The amounts of the toxin ranged from 30 to 1,500 μg/g of dried ground substrate (Table 1). Noticeably, of the 67 strains from maize, 33 produced more

than 500 μg/g of dried ground substrate. In particular, cultures of ITEM-1029 and ITEM-1030 (both from the United States) and ITEM-1471, ITEM-1472 (MRC-115), and ITEM-1473 (from South Africa and Zambia and all known to cause toxicosis to experimental animals [11, 20, 21]) all produced substantial amounts of fusaproliferin. Because of the worldwide distribution of *F. subglutinans* (15, 17, 18, 21) and because many *F. subglutinans* strains can produce high levels of fusaproliferin in vitro, this novel mycotoxin must be regarded as a potential contaminant of maize and maize products targeted for human and animal consumption.

We also examined five *F. subglutinans* strains isolated from plants other than maize. The toxin output was lower than in the strains isolated from maize, although more work is needed to confirm this conclusion.

F. subglutinans is known to be composed of at least two different mating populations, B and E (13). Strains belonging to these two populations are preferentially isolated from different hosts: sugar cane and maize, respectively (13). Several strains of *F. subglutinans* used in this study belong to the E mating population, including all the strains from Argentina (4), the two strains from the United States (received as E mating testers [16]), all the strains from Austria, and most of those from Poland (24). Different toxicological profiles have been described for mating populations of *Gibberella fujikuroi* (Sawada) Ito in Ito et K. Kimura (16), in particular for the A and F populations (anamorph *F. moniliforme* Sheldon [14]). It will be interesting to further investigate the possible difference in fusaproliferin production between the B and E mating populations. Preliminary experiments revealed that two B mating tester strains from sugar cane failed to produce fusaproliferin (24).

Fusaproliferin purified from *F. subglutinans* was toxic to *A. salina*. Previous work indicated that organic extracts of some strains of *F. subglutinans* were remarkably toxic to *A. salina* (18, 26), although beauvericin, the only major organic-soluble toxin known, could not entirely account for this toxicity. This was particularly true of the highly toxic strain MRC-115, whose organic-extract beauvericin content was only 20 μg/g of dried, ground culture (a relatively tiny amount of toxin compared with that of other strains [26]). Moreover, strains ITEM-1029, from U.S. maize, and ITEM-1553, from Polish maize, were highly toxic even though no beauvericin could be detected in their organic extracts (18, 26). Moniliformin, a secondary metabolite produced by *F. subglutinans* that is toxic to animals and plants (5), could be excluded because of its water solubility. These observations prompted us to undertake the present work. Studies carried out with an *A. salina* bioassay revealed that fusaproliferin was toxic in the mid- to low-micromolar range (LD_{50} = 53.4 μM), a toxicity which compares to that of many potent mycotoxins with different structures (35).

Fusaproliferin toxicity was also investigated in another, less common, invertebrate model, namely, cultures of *S. frugiperda* SF-9 cells. Fusaproliferin was cytotoxic to stationary-phase cultures at concentrations only slightly higher than in the brine shrimp model. Thus, the results obtained in the crustacean model compared favorably to those obtained in the insect model.

A. salina is easy to rear and to use for testing. SF-9 cells, as well as many other insect cell lines currently available, are also easy to cultivate (culturing does not require keeping the cells in a CO_2 incubator) and likely to provide information at a cellular level. Both procedures could be regarded as useful and reliable for toxicity studies of fusaproliferin as well as of other substances with biological effects to invertebrates and insects, in particular.

Because of the potential threat represented by fusaproliferin to human and livestock health, we also investigated its direct toxicity to human cells. We found that fusaproliferin is a quite highly toxic substance to the human B-lymphocyte cell line IARC/LCL 171. Cytotoxicity was studied under two substantially different conditions: (i) mature, resting cultures in stationary phase, i.e., cells that do not divide and do not enter cell cycling; and (ii) cultures in which fusaproliferin was included in fresh medium at passaging, i.e., cells that were allowed to proliferate and, hence, enter cell cycling in the presence of the toxin.

In cells exposed in the stationary phase, the CC_{50} was approximately the same as the LD_{50} found in the *A. salina* assay. When cultures of IARC/LCL 171 were exposed to fusaproliferin at passaging and cells were allowed to grow in the presence of the toxin, a similar cytotoxicity pattern was observed. Interestingly, 30 μM fusaproliferin, a concentration nearly half the CC_{50} for cells in the stationary phase, seemed to prevent cells from growing normally and forming large, tight clumps, characteristic of this cell line, when the stationary phase has been reached. However, viability was not dramatically affected (Fig. 5B), suggesting that fusaproliferin at subtoxic concentrations may have cytostatic effects on this LCL.

In this study, we investigated the cytotoxicity of fusaproliferin to a cell line (IARC/LCL 171) composed of diploid B lymphocytes. IARC/LCL 171 cells are nonneoplastic, nonembryonic B lymphocytes immortalized by experimental infection with the prototypic Epstein Barr virus B95-8 (12). Compared with tumor cell lines, normal cells are more likely to provide information that is useful to our understanding of normal physiological processes in living cells. Therefore, one can reasonably infer that the in vitro cytotoxic effects we observed also apply to normal B lymphocytes in living organisms exposed to this potential food contaminant. Moreover, these results suggest that similar cytotoxic effects could be exerted on other mammalian cell types.

Recently, fusaproliferin was found in conspicuous amounts as a natural contaminant on maize affected with preharvest maize ear rots, mostly infected with *F. proliferatum*, in Northern Italy (32). It is possible that fusaproliferin, alone or in association with other toxins, plays a significant role in the natural toxicity of *F. subglutinans*. In this respect, it will be particularly interesting to determine the effects of fusaproliferin in combination with beauvericin and moniliformin, since all three compounds are now known to be produced by many *F. subglutinans* strains (11, 22, 26).

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