# Teratogenic Effects of Fusaproliferin on Chicken Embryos

Alberto Ritieni,*,† Simona Maria Monti,† Giacomino Randazzo,† Antonio Logrieco,‡
Antonio Moretti,‡ Giuseppe Peluso,† Rosalia Ferracane,† and Vincenzo Fogliano†

Dipartimento di Scienza degli Alimenti, Università di Napoli "Federico II", via Università 100,
80055 Portici, Italy, and Istituto Tossine e Micotossine da Parassiti Vegetali del CNR, viale Einaudi,
70100 Bari, Italy

Fusaproliferin (FP) is a sesterterpene mycotoxin produced by some species of *Fusarium*, common contaminants of maize. Toxicity assays on *Artemia salina* L. brine shrimp larvae using FP and its derivatives showed that toxic activity of the metabolite increased after acetylation of the two hydroxyl groups and was reduced when the naturally occurring acetyl group was removed. In chick embryotoxicity bioassays, severe teratogenic effects were observed in 20% of the embryos exposed at 5 mM FP on day 1. In particular FP was responsible for cephalic dicotomy, macrocephaly, and limb asymmetry. At the same concentration, FP also caused pathological changes such as hemorrhaging on the surface of the wings, skull, and legs. Incomplete closure of the umbilicus was seen in 30% of embryos inoculated with 5 mM of FP on day 1 and in 20% of those inoculated on day 11.

**Keywords:** *Fusaproliferin; Fusarium proliferatum; chick; embryotoxicity; teratogenic; mycotoxin*

## INTRODUCTION

Mycotoxin contamination of human maize-based foods and feeds is a worldwide problem since about 20% of cereal crop production contains a measurable amount of mycotoxins (Smith *et al.*, 1994). *Fusarium* spp. are common fungal contaminants known to produce mycotoxins among which fumonisins, moniliformin, and beauvericin (Marasas *et al.*, 1984; Gelderblom *et al.*, 1988; Marasas *et al.*, 1986; Gupta *et al.*, 1991). Some of these fungi are members of the *Liseola* Woll. section of *Fusarium* and occur in infected plants and agricultural commodities, especially cereals (Nirenberg, 1976; Gerlach and Nirenberg, 1982).

We identified fusaproliferin (FP) by screening organic extracts of different species of *Fusarium* for their toxic activity (Randazzo *et al.*, 1993). FP was the first sesterterpene isolated from fungi and it has been reported to be synthesized by both *Fusarium proliferatum* and *F. subglutinans* cultures (Logrieco *et al.*, 1996). FP is produced in cultures of different species of *Fusarium* in amounts ranging from 40 to 500 ppm (Ritieni *et al.*, 1995a). In Italy, FP occurred naturally on preharvest maize ear rots, mostly infected by *F. proliferatum* (Ritieni *et al.*, 1995b).

Very little is known about the effects of low levels of mycotoxins on human and animal health. High mortality in broiler chicks fed with *F. proliferatum*-infected maize was reported by Ramakrishnan *et al.* (1994) and Javed *et al.* (1993a). Javed *et al.* (1993b) reported that inoculating embryonated eggs with extracts of *F. proliferatum* caused early embryonic and pathological changes. Similar effects also were reported when inoculations were made with purified fumonisin B$_1$ (FB$_1$) (Javed *et al.*, 1993b). Bacon *et al.* (1995) reported enhanced toxicity of FB$_1$ to chick embryos when FB$_1$ was combined with fusaric acid, suggesting that mycotoxins can interact in a synergistic manner resulting in enhanced and unpredicted toxicity also in mammalian systems.

The role of *Fusarium* mycotoxins in mycotoxicoses has been experimentally confirmed (Harrison *et al.*, 1990; Ross *et al.*, 1990). Neither the role of FP in human and animal diseases nor the relationship between its chemical structure and its toxicity is understood. Since purified FP was highly toxic to *Artemia salina* (LD$_{50}$ of 53.4 $\mu$M) and to human non-neoplastic B lymphocytes IARC/LCL 171 (CC$_{50}$ of 60−65 $\mu$M) (Logrieco *et al.*, 1996), we investigated the embryopathic and teratogenic effects of FP on chicken embryos by the chick embryotoxicity assay (McLaughlin *et al.*, 1963; Verrett *et al.*, 1964). Toxins are not usually excreted by egg embyos, and the detoxification rate is slow. The bioassay is relatively fast and allows a gross examination of the results (Prelusky *et al.*, 1987).

## MATERIALS AND METHODS

**Chemicals.** FP (C$_{27}$H$_{40}$O$_5$) (Figure 1) was produced and isolated as described (Ritieni *et al.*, 1995b). Pure dimethyl sulfoxide (DMSO) per analysis was purchased from Fluka Chemie AG (Switzerland). Double-distilled, autoclaved water was used for the chick embryo assay. All the other reagents were of analytical grade.

**Preparation and Purification of FP Derivatives.** Diacetyl FP (Ac-FP, Figure 1) (C$_{31}$H$_{44}$O$_7$) was obtained by treatment with acetic anhydride (Ac$_2$O) and pyridine as previously described (Randazzo *et al.*, 1993). Deacetyl FP (Da-FP) (C$_{25}$H$_{38}$O$_4$) (Figure 1) was prepared by treatment of FP (12.5 mg) with 0.5 M NaOH in methanol (1 mL) for 12 h at room temperature. The solution was added to water (3 mL) and extracted three times with 4 mL of diethyl ether. The extract was isolated and purified by high-performance thin-layer chromatography (HPTLC) (CHCl$_3$:methanol 98:2 v/v, $R_f$ 0.1). Both synthetic and natural Da-FP, recovered from a fraction of a *F. proliferatum* culture containing FP, showed a lower toxicity in biological assay compared to FP.

**Spectroscopic Characterization.** Proton NMR spectra of FP derivatives were run in CDCl$_3$ on a Bruker AMX600 spectrometer operating at 600.13 MHz. Mass spectra were obtained on a Fisons TRIO 2000 instrument. The IR spectra were recorded on a Perkin-Elmer 399 infrared spectrophotometer and UV spectra were measured on a Shimadzu 2100 spectrophotometer.

* Corresponding author (telephone +39-81-7755160; fax +39-81-7754942; e-mail Fogliano@utovrm.it).
† Università di Napoli "Federico II".
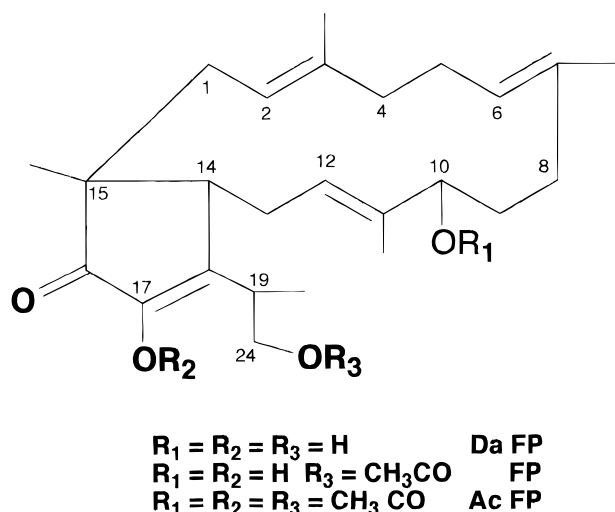‡ Istituto Tossine e Micotossine da Parassiti Vegetali del CNR.

**3040** *J. Agric. Food Chem.,* Vol. 45, No. 8, 1997

Ritieni et al.



**Figure 1.** Chemical structure of fusaproliferin (FP), deacetyl-FP (Da-FP), and acetyl-FP (Ac-FP).

**Table 1. Teratogenic and Pathological Effects of FP[a]**

| group test | day of inoculation | FP concentration (mM) | total percentage of teratogenic effects | total percentage of pathological effects |
|---|---|---|---|---|
| 1 | 1 | water | nd[b] | nd |
| 2 | 11 | water | nd | nd |
| 3 | 1 | diluent | nd | 4 |
| 4 | 11 | diluent | nd | 1 |
| 5 | 1 | 5 | 20 | 60 |
| 6 | 11 | 5 | nd | 30 |
| 7 | 11 | 1 | 15 | 45 |
| 8 | 11 | 1 | nd | 20 |

[a] Experimental conditions included water (double distilled, autoclaved), diluent (water:DMSO 38:62), and 5 and 1 mM FP. Embryonated eggs were inoculated on either day 1 or day 11 of incubation. [b] nd, not detected.

*A. salina* **L. Assay.** The toxicity to brine shrimp larvae was investigated as previously described (Harwig and Scott, 1971; Bottalico *et al.*, 1989). Briefly, larvae were exposed to FP and its derivatives in a 24-well cell culture plate (30–40 larvae per well, in 500 $\mu$L of sea water). 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 for 12 h at −20 °C. Tests were performed in quadruplicate. Probit analysis based on the average of three independent experiments was used for calculation of the mean lethal dose (LD$_{50}$).

**Chicken Embryo Assay.** FP was dissolved in 100 $\mu$L of water:DMSO (38:62 v:v). The solutions were sterilized by filtration through a 0.22 $\mu$m nylon disposable syringe filter (Acrodisc, Gelman Sciences, Germany). Fertile eggs (432, average weight 55–60 g) were obtained from Lohmann Tierzucht GmbH (Cuxhaven, Germany) and inoculated on either day 1 or day 11 of incubation with 100 $\mu$L of sample. Each group contained 18 eggs and each experiment was carried out in triplicate. Egg shells were perforated with a spring-loaded lancet with a needle tip. Purified FP (1 or 5 mM) was inoculated directly into the air sac of the eggs.

Four sets of control eggs were used (Table 1). On both day 1 and day 11 of incubation, one pair of controls was inoculated with double distilled, autoclaved water (100 $\mu$L). Similarly, a second pair of controls was inoculated with diluent only (water:DMSO, 100 $\mu$L). After injection, the eggs were sealed with paraffin and incubated for up to 21 days. Eggs were candled and turned through one quarter turn daily; dead-in-shell embryos were removed and examined.

Three independent sets of experiments were conducted. Statistical data are the mean of all three replications; the coefficient of variation which was calculated for each variable

was below 8%. Qualitative data are representative of all of these experiments.

## RESULTS AND DISCUSSION

**Characterization of FP Derivatives.** We obtained 9.4 mg of Da-FP by basic hydrolysis of FP (12.5 mg). Da-FP (Figure 1) is also a natural metabolite which occurs in a ratio of 1:3 Da-FP:FP in culture filtrates of some strains of *F. proliferatum* (authors' unpublished data).

The IR spectrum of isolated Da-FP lacked the ester C=O absorption at 1728 cm$^{-1}$ typical of FP. In the $^1$H-NMR spectrum, the CH$_3$ signal at 2.02 ppm consistent with CH$_3$C=O also was absent; additionally, the signal of C-24 (Figure 1) shifted from 4.28 to 3.80 ppm (Silverstein *et al.*, 1981). The MS-EI spectrum of isolated Da-FP gave an *m*/*z* 384 consistent with [M$^+$ − H$_2$O] (Silverstein *et al.*, 1981).

Ac-FP was obtained by treating FP with Ac$_2$O and pyridine. The $^1$H-NMR spectrum showed two new CH$_3$ signals at 2.15 and 2.05 ppm, respectively, in addition to the CH$_3$ signal at 2.02 ppm. The signal for the C10 H at 4.05 ppm was shifted to 5.2 ppm while the enol OH signal at 5.56 ppm was absent (see Figure 1). The amu of 528 in the mass spectrum of isolated Ac-FP also was consistent with [M$^+$ − 2H + 2CH$_3$CO].

**Relationship between Chemical Structure and Toxicity of FP to *A. salina*.** In *A. salina* bioassays Ac-FP caused three times higher mortality of larvae than did FP (LD$_{50}$ of 17.5 vs 53.4 $\mu$M). FP completely lost its toxic effects when the acetyl group was removed.

One explanation for these data is that the acetylation of the OH-moieties at C10 and C17 (R1 and R2 of Figure 1) reduces the polarity of the molecule, increasing its ability to transit the cell membranes. Thus, Ac-FP is more toxic than FP against the brine shrimp larvae while Da-FP shows little activity. In this context, it could be hypothesized that Da-FP is an inactive precursor of the FP rather than a breakdown product. Preliminary *in vitro* experiments (data not shown) showed that FP is rapidly hydrolyzed to Da-FP by a pool of rabbit hepatic enzymes, suggesting that *in vivo* deacetylation could occur when mammals are amended with FP-contaminated foodstuff. It is well known that aflatoxins are hydroxylated *in vivo* (Smith *et al.*, 1994). Therefore the increasing of polarity of the toxins seems to be a general detoxification pathway.

**Chicken Embryo Assay.** This assay is a valuable tool for studying teratogenic effects, pathological changes and mortality effects of chemical compounds such as food additives, pesticide residues, and mycotoxins (Vesely *et al.*, 1982; Prelusky *et al.*, 1987). Due to the toxicity of Ac-FP and FP to *A. salina*, these molecules were chosen for the chick embryo assay. Chick embryos are very sensitive to solvent type (Prelusky *et al.*, 1987). Since the solvent combination (water:DMSO 38:62) necessary to dissolve FP caused 40% mortality in the control groups, we could not assess dose-mortality effects induced by FP. No data were obtained from the eggs inoculated with Ac-FP, probably due to its low solubility in the solvent system. Unfortunately, other solvent mixtures which were less toxic to the embryos (Prelusky *et al.*, 1987) did not solubilize Ac-FP. Therefore, only the teratogenic and pathological effects of FP on chicken embryos are described in this paper (Table 1).

**Teratogenic Effects.** All eggs in the water-only control groups showed normal embryonic development.
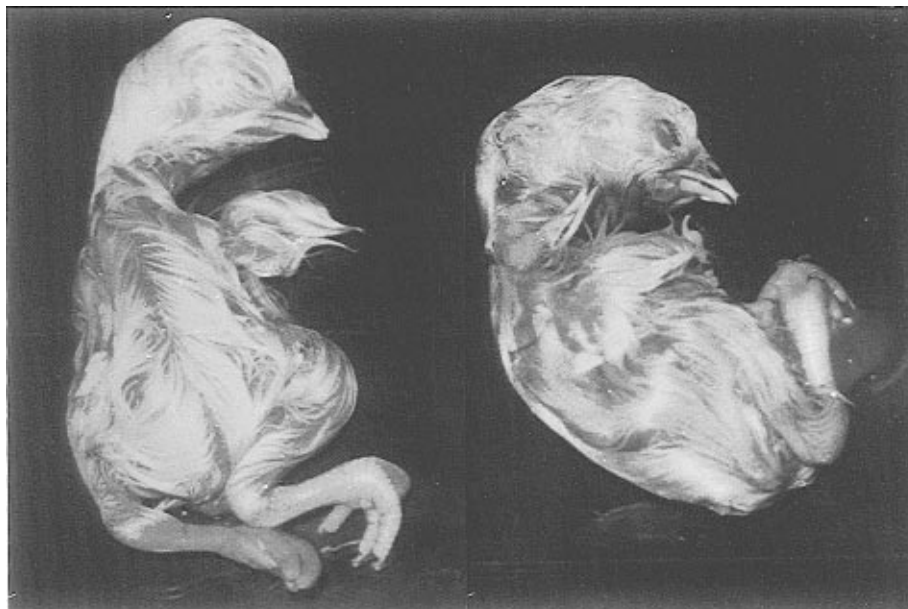
Teratogenicity of Fusaproliferin

*J. Agric. Food Chem.,* Vol. 45, No. 8, 1997 **3041**



**Figure 2.** Teratogenic effects on FP (1 mM) on chick embryos: disproportional ratio of head to body size.
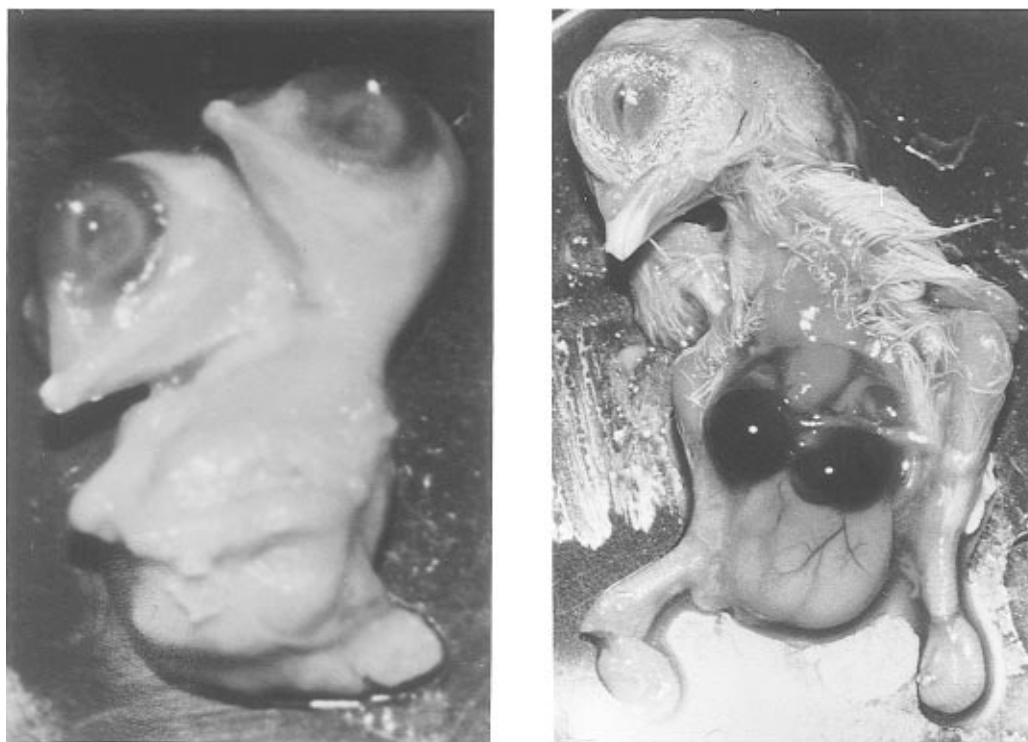


**Figure 3.** Teratogenic effects of FP (5 mM) on chick embryos: (a, left) cephalic dicotomy; (b, right) abnormal development of the abdomen region.

Many severe malformations were observed in embryos inoculated with either level of FP on day 1 of incubation. The most frequent early embryonic changes were classified as follows:

(a) macrocephaly and anomalous development of feet and legs. FP also induced a disproportionate ratio of head to body size: the head enlarged and the body smaller than normal (Figure 2). Cases of enlarged beaks and elongated necks were also observed;

(b) cephalic dicotomy (Figure 3a), which was the greatest macroscopic effect of FP on chicken embryo development, was observed in 6% of the treated embryos;

(c) abnormal development of the abdominal region (Figure 3b) was observed in 5% of the treated embryos;

(d) absence of the head (Figure 4) was observed in 2% of the treated embryos.

The total number of chicks showing teratogenic malformation was 11 (4a+3b+4c+0d) and 8 (2a+1b+2c+3d) among 54 chicks treated with 5 and 1 mM FP, respectively.

**Pathological Changes.** Normal chicks were hatched from the water-inoculated control groups, and only minimal effects were observed in the diluent-inoculated control groups. Pathological changes were observed in 60% of the chick embryos exposed to 5 mM FP on day 1 and in 30% of those exposed to 5 mM FP on day 11. At the time of death, hemorrhages were observed on the surfaces of legs, skull, and feet. 30% of the embryos

**3042** *J. Agric. Food Chem.,* Vol. 45, No. 8, 1997

Ritieni et al.



**Figure 4.** Teratogenic effects of FP (1 mM) on chick embryos: absence of head.

exposed to 5 mM FP had incomplete closure of the umbilicus. As expected, eggs inoculated on the first day appeared more sensitive to the toxin than eggs inoculated on day 11.

**Conclusion.** Chromosomal aberrations, infections, drugs, and environmental contaminants are thought to be responsible for about 20% of congenital malformations with the cause of the remaining 80% yet to be assigned (Smith *et al.*, 1994). Emerging evidence suggests that a considerable role is played by dietary comsumption of maize-based foods and feeds contaminated by mycotoxins (Chu, 1991). In this paper we investigated the toxicity of FP and two derivatives to *A. salina* and the embryopathic and teratogenic effects of FP on fertile chicken eggs. The toxicity of FP to *A. salina* varies with acetylation, which very likely increases its ability to transit the cell membranes. However, natural FP metabolite has severe teratogenic and pathological effects on chick embryos.

These results are consistent with those reported by Javed *et al.* (1993b) who suggest that the high toxicity observed using culture filtrate of *F. proliferatum* could not be due only to fumonisins. In fact, we have shown that, in addition to fumonisins, FP also has teratogenic and pathological effects on chicken embryos. The possibility that teratogenic effects of FP on chicken embryos are due to interactions between FP and nucleic acids during the first stages of embryo development is presently under investigation.

If hens on FP-contaminated feeds transmit this mycotoxin to the fertile egg, the impact of *Fusarium* spp. on the poultry industry may be much greater than previously recognized, then, too, whether this compound is transmitted from contaminated feeds into muscle

tissues must also be considered. Further studies are needed to assess both human and animal health risks involving the most frequent and toxic *Fusarium* metabolites.

LITERATURE CITED

Bacon, C. W.; Porter, J. K.; Norred, W. P. Toxic interaction of fumonisin B$_1$ and fusaric acid measured by injection into fertile chicken eggs. *Mycopathologia* **1995**, *129*, 29–35.

Bottalico, A.; Logrieco, A.; Visconti, A. *Fusarium* species and their mycotoxins in infected cereals in the field and in stored grains. In *Fusarium: Mycotoxins, Taxonomy, and Pathogenicity*; Chełkowski, J., Ed.; Elsevier: Amsterdam, 1989; pp 85–119.

Chu, F. S. Mycotoxins: Food contamination, mechanisms, carcinogenic potential and preventive measures. *Mutat. Res.* **1991** *259*, 291–306.

Gelderblom, W. C. A.; Jaskewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R.; Vleggaar R. Kriek N. P. J. Fumonisins: Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme. Appl. Environ. Microbiol.* **1988**, *54*, 1806–1811.

Gerlach, W.; Nirenberg, H. The Genus Fusarium: A Pictorial Atlas. *Mitt. Biol. Bundesanst. Land- Forstwirtschaft, Berlin-Dahlem* **1982**, *209*, 406 pp.

Gupta, S.; Krasnoff, S. B.; Underwood, N. L.; Renwick, J. A. A.; Roberts, D. W. Isolation of beauvericin as an insect toxin from *Fusarium semitectum* and *Fusarium moniliforme* var. *subglutinans. Mycopathologia* **1991**, *115*, 185–189.

Harrison, L. R.; Colvin, B.; M.; Green, J. T.; Newman, L. E.; Cole, J. R., Jr. Pulmonary edema and hydrothorax in swine produced by fumonisin B$_1$, a toxic metabolite of *Fusarium moniliforme. J. Vet. Diagn. Invest.* **1990**, *2*, 217–221.

Harwig, J.; Scott, P. M. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Microbiol.* **1971**, *21*, 1011–1016.

Javed, T.; Bennett, G. A.; Richard, J. L.; Dombrink-Kurtzman, M. A.; Coté, L. M.; Buck, W. B. Mortality in broiler chicks on feed amended with *Fusarium proliferatum* culture material or with purified fumonisin B$_1$ and moniliformin. *Mycopathologia* **1993a**, *123*, 171–184.

Javed, T.; Richard, J. L.; Bennett, G. A.; Dombrink-Kurtzman, M. A.; Bunte, R. M.; Koelkebeck, K. W.; Coté, L. M.; Leeper, R. W.; Buck, W. B. Embryopathic and embryocidal effects of purified fumonisin B$_1$ or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia* **1993b**, *123*, 185–193.

Logrieco, A.; Moretti, A.; Fornelli, F.; Fogliano, V.; Ritieni, A.; Caiaffa, M. F.; Randazzo, G.; Bottalico, A.; Macchia, L. Fusaproliferin production by *Fusarium subglutinans* and its toxicity to *Artemia salina*, SF-9 insect cells, and IARC/LCL 171 human B Lymphocytes. *Appl. Environ. Microbiol.* **1996**, *62*, 3378–3384.

Marasas, W. F. O.; Nelson, P. E.; Toussoun, T. A. *Toxigenic Fusarium species: Identity and mycotoxicology*; Pennsylvania State University Press: University Part PA, 1984.

Marasas, W. F. O.; Thiel, P. G.; Rabie, C. J.; Nelson, P. E.; Toussoun, T. A.; Van Wyk, P. S. Moniliformin production in *Fusarium* section Liseola. *Mycologia* **1986**, *78*, 242–247.

McLaughlin, J., Jr.; Marliac, J.-P.; Verrett, M. J.; Mutchler, M. K.; Fitzhugh, O. G. The injection of chemicals into the yolk sac of fertile eggs prior to incubation as a toxicity test. *Toxicol. Appl. Pharmacol.* **1963**, *5*, 760–771.

Nirenberg, H. Untersuchungen uber die morphologische und biologische Differenzierung in der Fusarium-Sektion Liseola. *Mitt. Biol. Bundesanst. Land-Forstwirtsch., Berlin-Dahlem* **1976**, *169*, 1–117.

Teratogenicity of Fusaproliferin

*J. Agric. Food Chem.,* Vol. 45, No. 8, 1997 **3043**

Prelusky, D. B.; Hamilton, R. M. G.; Foster, B. C.; Locksley-Trenholm, H.; Thompson, B. K. Optimization of chick embryotoxicity bioassay for testing toxicity potential of fungal metabolites. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 1049−1055.

Ramakrishnan, N. Y.; Wu, W. D. Toxicity of corn culture material of *Fusarium proliferatum* M-7176 and nutritional intervention in chicks. *Poult. Sci.* **1994**, *73*, 617−626.

Randazzo, G.; Fogliano, V.; Ritieni, A.; Mannina, L.; Rossi, E.; Scarallo, A.; Segre, A. L. Proliferin, a new sesterterpene from *Fusarium proliferatum*. *Tetrahedron* **1993**, *49*, 10883−10896.

Ritieni, A.; Logrieco, A.; Fogliano, V.; Moretti, A.; Ferracane, R.; Randazzo, G.; Bottalico, A. Natural occurrence of fusaproliferin, in pre-harvest infected maize ear rot in Italy. In *International Seminar on Fusarium: Mycotoxins, Taxonomy, and Pathogenicity*, Martina Franca, Italy, May 9−13, 1995; 1995a.

Ritieni, A.; Fogliano, V.; Randazzo, G.; Scarallo, A.; Logrieco, A.; Moretti, A.; Mannina, L.; Bottalico, A. Isolation and characterization of fusaproliferin, a new toxic metabolite from *Fusarium proliferatum*. *Nat. Toxins* **1995b**, *3*, 17−20.

Ross, P. F.; Nelson, P. E.; Richard, J. L.; Osweiler, G. D.; Rice, L. G., Plattner, R. D.; Wilson, T. M. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* iso-lates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Appl. Environ. Microbiol.* **1990**, *56*, 3225−3226.

Silverstein, R. M.; Bassler, C. G., Morril, C. T. In *Spectrometric identification of organic compounds*, 4th ed.; Wiley & Sons: New York, 1981.

Smith, J. E.; Solomons, G. L.; Lewis, C. W.; Anderson, J. G. In *Mycotoxins in human nutrition and health*; European Commission, Directorate General XII:  Luxembourg, 1994; Vol. 1, pp 25−35.

Verrett, M. J.; Marliac, J.-P.; MacLaughlin, J., Jr. Use of the chicken embryo in the assay of aflatoxin toxicity. *J. Assoc. Off. Anal. Chem.* **1964**, *47*, 1003−1006.

Vesely, D.; Vesela, D.; Jelinek, R. Nineteen mycotoxins tested on chicken embryos. *Toxicol. Lett.* **1982**, *13*, 239−245.