Occurrence of T-2 Toxin in *Fusarium*-Infested Sorghum from India

Cheruvanky Rukmini* and Ramesh V. Bhat

Fusarium incarnatum was isolated from naturally infected moldy sorghum. A toxic metabolite was isolated from this moldy sorghum as well as from rice artificially inoculated with *F. incarnatum*. This metabolite has been demonstrated to be toxic biologically by (a) intraperitoneal injections to rats, (b) skin irritant test in guinea pigs, and (c) rabbit reticulocyte bioassay. Chemical and spectroscopic analysis of the toxic metabolite established its identity as T-2 toxin (3 α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecene). By a combination of biological and chemical tests, T-2 contamination in naturally infected food grains can be detected.

Sorghum is one of the staple food grains for man in several Asian and African countries. Under certain climatic conditions sorghum earheads become infected with various molds, particularly should there be unseasonal rains. The problem of head molds of sorghum is of concern in both seed production and in reducing the nutrient value of grain. Moldy grain toxicosis due to consumption of grain sorghum infected with fungi has been reported in Japan and *Fusarium* has been implicated as the causative agent (Saito and Ohtsubo, 1974). *Fusarium* has also been implicated in the etiology of toxic corn disease in cows and equines in the United States and alimentary toxic aleukia in man in the USSR (Joffe, 1974; Smalley et al., 1970). There has so far been no report of established *Fusarium* toxicosis in India either in animals or in humans. However, Ghosal et al. (1976) recently reported that *Fusarium oxysporum* isolated from safflower seeds in India produced T-2 toxin and diacetoxyscirpenol in synthetic medium. Jayaraman and Parihar (1975) found *F. moniliforme* to produce a growth-promoting pigment in rice cultures under laboratory conditions. There have so far been no reports about the nature of toxins elaborated by *F. incarnatum*. This article reports the results of a systematic study of the toxin obtained from natural moldy sorghum as well as from *Fusarium incarnatum* isolated from moldy sorghum and grown on rice.

MATERIALS AND METHODS

Isolation of Fusarium. Fifty kilograms of moldy sorghum was obtained from the Andhra Pradesh Agricultural University of Hyderabad. Fifty grains were surface sterilized with 0.1% mercuric chloride solution, washed with sterile water, and plated on potato dextrose agar, and the plates were incubated at 20 °C for 7 days. Individual colonies obtained were subcultured on potato dextrose agar slants.

The fungi isolated included species of *Drechslera*, *Alternaria*, and *Fusarium*. One of the dominant fungi was *Fusarium incarnatum* (Roberge) Sacc. This was taken up for further toxicological investigation. For the identification of *Fusaria* the classification proposed by Subramanian (1971) was followed: colony white to pinkish, mycelium floccose, sporodochia present, conidia fusiform to falcate with blunty rounded apex; conidia generally three, rarely four–five, septate, 16.0–40.0 × 3.5–4.5/μm; chlamydospores large, terminal or intercalary, one–two celled or in chains.

Extraction of Toxin. Grains of moldy sorghum (1 kg) were powdered and extracted in a Soxhlet apparatus, first with petroleum–ether (40–60 °C) and then with ether. Another fresh batch was extracted directly with ethyl acetate. Similar extracts from uninfected sorghum were prepared.

Healthy grains of rice (200 g × 15 flasks) moistened with 80 mL of water were autoclaved and inoculated under sterile conditions with the fungus *Fusarium incarnatum* isolated from moldy sorghum. After 1 week at 30 °C the fungal growth was arrested by autoclaving the contents of the flask. All the material from 15 flasks was pooled, dried at 60–65 °C, and (1 kg) extracted exhaustively first with petroleum ether (40–60 °C) and then with ether. Another batch of *Fusarium*-infected rice was extracted directly with ethyl acetate. Similar extracts were prepared from uninfected rice and used as control material for biological experiments.

Skin Irritant Toxicity. Various extracts were dissolved in ethyl acetate and applied daily, for 7 days, on the shaved skin of four male guinea pigs with a 5-λ micropipet (5 λ = 1 g of the moldy material) as described by Ueno et al. (1970). Ueno et al. (1970) also have shown that guinea pigs have highest skin sensitivity to these toxins among laboratory animals. Ethyl acetate was used as the control. On the eighth day, the animals were sacrificed and the skin sites were excised for histopathological examination by conventional methods.

Rabbit Reticulocyte Bioassay. Reticulocytes were isolated by the method of Ueno et al. (1971). The incorporation of [*1*-^{14}C]leucine into protein was determined by measuring the radioactivity in the acid-insoluble fraction by liquid scintillation counter. Radio labeled [*1*-^{14}C]leucine was obtained from B.A.R.C., Bombay.

Uterine Hypertrophy. The estrogenic effect was studied according to the method of Ueno et al. (1974). One gram of crude ether extract which exhibited dermal toxicity from extracts of moldy sorghum and *Fusarium*-infected rice (equivalent to 20 g of moldy material), dissolved in 0.5 mL of propylene glycol, was given orally in a single dose to six weanling female rats. The control group of six rats received propylene glycol alone. The uterine weights were determined 24 h later.

Intraperitoneal Injection of Ether and Ethyl Acetate Extracts to Rats. The solvents were removed completely under vacuum from the ethyl acetate extracts of moldy sorghum and *Fusarium*-infected rice and dissolved in a known volume of refined groundnut oil and 0.2 mL (100 g of body weight)$^{-1}$ rat^{-1} day^{-1} (= 18 g of the moldy feed) injected for seven days to six weanling rats in each group (three males and three females). A control group of six rats (three males and three females) received refined groundnut oil at a dose of 0.2 mL (100 g of body weight)$^{-1}$ day^{-1} for 7 days. The ether extracts prepared similarly from moldy sorghum and *Fusarium*-infected rice were injected into three weanling rats (two males and one female) in each group at the same dose (= 18 g of the

National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania, Hyderabad-500007 A. P., India.

0021-8561/78/1426-0647$01.00/0

moldy feed) daily for 7 days. On the eighth day, the animals were sacrificed, and the pancreas, spleen, kidney, stomach, and large and small intestines were examined for histopathological changes.

Isolation of the Toxin. The toxin was isolated by the modified method of Bamburg et al. (1968). The crude ethyl acetate extracts of moldy sorghum and *Fusarium*-infected rice after partitioning with petroleum ether were passed over a column of silica gel, and 50 mL portions of *n*-hexane—ethyl acetate (1:3, 1:5, and 1:7) were collected.

Thin-layer chromatography was done on silica gel G plates and sprayed with 50% alcoholic sulfuric acid and dried at 110 °C for 10 min and viewed under UV light. The solvent systems used were toluene–ethyl acetate–formic acid (6:3:1, v/v), chloroform–methanol (97:3, v/v), ethyl acetate–*n*-hexane (3:1, v/v), and chloroform–ethyl acetate–ethanol (90:5:5, v/v). Melting points were uncorrected and taken in a Gallenkamp electrical apparatus. Infrared spectrum was taken in $CHCl_3$ using a Perkin-Elmer 221 infrared spectrophotometer. NMR was taken in CCl_4 using a 60 Mc A-60A Varian instrument. GLC was performed with a SE-30 column using a flame ionization detector and Me_4Si derivative of glucose was used as an internal standard on Model 650-Aerograph Instrument.

RESULTS

Biological Activity. *Toxicity by Intraperitoneal Administration.* When the ether and ethyl acetate extracts of the moldy sorghum and *Fusarium*-infected rice were injected intraperitoneally to rats, toxic symptoms were observed. The rats' fur was untidy in addition to general weakness and sluggishness of the animals. Ethyl acetate extracts of both the moldy sorghum and *Fusarium*-infected rice were toxic as revealed by mortality (3/6 and 4/6, respectively) and loss in weight gain. Pathological changes were characterized by acute necrotizing inflammatory lesions in the omentum, mesentery, visceral and parietal peritoneum, identical with those reported by Ueno et al. (1970).

Skin Irritant Toxicity. The skin irritant toxicity of the moldy sorghum and *Fusarium*-infected rice in petroleum ether extract was mild as only one animal out of four produced mild skin lesions while the ether and ethyl acetate extracts produced marked skin toxicity in all the four animals.

Skin changes were not found at 24 h following the application. On the second day, the skin showed reddening with formation of dry crusts. On the third day, appreciable edema and inflammation were noticed. On day four, pinpoint hemorrhage and heavy scab formation were seen. On day 5–7 severe subdermal hemorrhage in the affected zone was noticed, and at this stage the animals were sacrificed. Histopathologically the rats exhibited an acute hemorrhagic necrotizing ulcers. However, in control animals, no skin change was observed.

Rabbit Reticulocyte Bioassay. As a measure of tricothecene toxicity, the capacity of the ethyl acetate and ether extracts of moldy sorghum and *Fusarium*-infected rice to inhibit protein synthesis was measured in reticulocytes isolated from the peripheral blood of rabbits. Inhibition to the extent of 89 and 91% in protein synthesis was observed in the ethyl acetate extracts of both moldy sorghum and *Fusarium*-infected rice extracts. The ether extracts also inhibited protein synthesis, but to a lesser extent (78 and 84%). The petroleum ether extracts showed minimal inhibition (12 and 32%). Purified T-2 toxin from moldy sorghum and *Fusarium*-infected rice showed similar inhibition of protein synthesis in rabbit reticulocytes.

Uterotrophic Action. Since some of the Fusarial toxins exhibit uterotrophic activity, ether extracts of moldy sorghum and *Fusarium*-infected rice were screened for such toxicity. Uterine weights of the treated rats were found to be not significantly different from those of control rats, indicating the absence of uterotrophic compounds like zearalenone.

Thin-Layer Chromatographic Pattern. TLC behaviors of the crude ethyl acetate extract and the ether extract of moldy sorghum and *Fusarium*-infected rice in chloroform methanol (97:3, v/v) indicated the presence of T-2 toxin along with various spots. The purified ethyl acetate extract was further chromatographed in three other solvent systems along with standard T-2 and this confirmed the presence of T-2.

Chemical Confirmation of T-2 Toxin. The *n*-hexane–ethyl acetate (1:5, v/v) fraction from the silica gel column showed a sky-blue fluorescent spot under UV on TLC after spraying with 50% H_2SO_4 and heating at 110 °C and was identical with authentic T-2 toxin under similar conditions. This fraction on crystallization from benzene gave colorless needles (20 mg), mp 148–150 °C, exhibiting IR ($CHCl_3$) 3540 (OH), 3010 (H–C=C), 1720 cm^{-1} (>C=O) and a superimposable IR with a specimen of authentic T-2 toxin and mmp 149–150 °C with authentic T-2 toxin. NMR (CCl_4) showed a signal at δ 2.97 (d, J = 4, AB quartet) for epoxide. On GLC, it gave a single peak at retention time 17.2 min on SE-30 column, FID, and TMS derivative of glucose as an internal standard, which was identical with the peak obtained with authentic T-2 under similar conditions. The toxin present in moldy sorghum ethyl acetate extract and that in the *Fusarium*-infected rice ethyl acetate extracts were chemically identical in all respects with standard T-2 toxin.

DISCUSSION

Sorghum is one of the major staple millets consumed by man in several countries of Asia and Africa. Invasion of the grain by various fungi due to unseasonal rains during harvest can lead to toxin formation. Several fungi such as *Alternaria*, *Curvularia*, and *Drechslera* are known to be associated with the "head molds" of sorghum. Besides these, species of *Fusarium* infect earheads of sorghum species. *Fusarium* is known to produce several toxins like trichothecenes which are cytotoxic, butenolides which cause leukopenia, and zearalenone which produces uterine hypertrophy (Sato et al., 1975).

The isolation of *Fusarium incarnatum* from moldy sorghum may be of significance from the point of view of human health. The toxic manifestations and mortality observed in rats on intraperitoneal administration of the ether and ethyl acetate extracts obtained from moldy sorghum and *Fusarium*-infected rice indicated the presence of some toxic factor(s). These crude extracts from both sources exhibited severe dermatitic properties in guinea pigs, suggesting that the toxic factors could be either trichothecene or the butenolide type. Further evidence suggesting that this toxic factor belongs to the trichothecene group was obtained by the inhibition of protein synthesis by rabbit reticulocytes. That the other well-known fusarial toxin of F-2 type is absent as indicated by the negative uterotrophic effect.

The toxin belonging to the trichothecene group was identified as T-2 [3α-hydroxy-4β,15-diacetoxy-8 α-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecene by thin-layer chromatography and confirmed by mixed melting point and superimposable IR, with an authentic sample (obtained through the kind courtesy of Dr. Y. Ueno, Tokyo University, Tokyo, Japan). The T-2 toxins

are known to be produced by several species of *Fusarium* such as *F. poae*, *F. tricinctum*, *F. sporotrichoides*, *F. roseum*, *F. avenaceum*, and *F. culmorum* (Ueno et al., 1972). This appears to be the first instance of a T-2 toxin isolated from a *Fusaria* belonging to the arthrosporiella group.

For the identification of trichothecenes, Ueno et al. (1973) have suggested a combination of biological tests using the rabbit reticulocyte assay and TLC analysis with H_2SO_4 acid spray. According to Smalley and Strong (1974), "Although this system has obvious uses in testing unknown pure culture for trichothecene production, it has not been tested on naturally contaminated food or feeds". However, in the present study, by employing the above methods in addition to the well-known skin irritant toxicity test, the presence of T-2 could be identified in a naturally contaminated food and in the fungus culture isolated from such food. Screening of samples of sorghum by this method for *Fusarium* toxicity is under progress.

The toxic metabolite found naturally in the moldy sorghum and the toxic metabolites obtained from rice infected with *F. incarnatum* appear to be identical. The natural occurrence of T-2 and a fungus responsible for producing T-2 toxin in a staple like sorghum acquires special significance in view of the reported relation of these types of toxins in causing human disease such as alimentary toxic aleukia reported from USSR (Joffe, 1974; Yagen and Joffe, 1976). Although population groups belonging to the poorer segments may be exposed to consumption of moldy sorghum, reports of a positive correlation with any disease attributable to this are so far lacking in India.

Besides T-2, several metabolites with fluorescent properties and other pigments were found in both the moldy sorghum and *Fusarium*-infected rice. However, the exact chemical nature and biological effect of these metabolites are not known and these aspects are under investigation currently. Long term feeding of moldy sorghum to rats and pups are also under way.

ACKNOWLEDGMENT

The authors are grateful to S. G. Srikantia, Director, and P. G. Tulpule, Deputy Director, National Institute of Nutrition, Hyderabad, for their keen interest and valuable guidance and to D. Krishnamurthy for histopathological studies and to A. Appa Rao, Director of Research, Andhra Pradesh Agricultural University, Hyderabad, for providing samples of sorghum for the investigations. Thanks are due to Y. Ueno of Tokyo University, Japan, for standard T-2 toxin, Y.V. Subba Rao of Regional Research Laboratory, Hyderabad, for NMR and K. Natrajan and C. V. Subramanian of the University of Madras for confirming the species identification.

LITERATURE CITED

Bamburg, J. R., Riggs, N. V., Strong, F. M., *Tetrahedron* **24**, 3329 (1968).
Ghosal, S., Chakrabarthy, D. K., Basu Chaudhury, K. C., *J. Pharm. Sci.* **65**, 160 (1976).
Jayaraman, S., Parihar, D. B., *Indian J. Exp. Biol.* **13**, 313 (1975).
Joffe, A. Z., "Mycotoxins", Purchase, I. F. H., Ed., Elsevier, Amsterdam, 1974, p 229.
Sato, N., Ueno, Y., Enomoto, M., *Jpn. J. Pharmacol.* **25**, 263 (1975).
Saito, M., Ohtsubo, K., "Mycotoxins", Purchase, I. F. H., Ed., Elsevier, Amsterdam, 1974, p 263.
Smalley, E. B., Marasus, W. F. O., Strong, F. M., Bamburg, J. R., Nichdo, R. E., Kosuri, N.R., Proceedings of the First U.S.–Japan Conference on Toxic Microorganism U.J.N.R., U.S. Department of Interior, Washington, D.C., 1970, p 163–173.
Smalley, E. B., Strong, F. M., "Mycotoxins", Purchase, I. F. H., Ed., Elsevier, Amsterdam, 1974, p 199.
Subramanian, C. V., "Hypomycetes", an account of Indian species except *cercosporae*, Indian Council of Agricultural Research, New Delhi, 1971, p 713.
Ueno, Y., Ishikawa, Y., Amkai, K., Nakajima, M., Saito, M., Enomoto, M., Ohtsubo, K., *Jpn. J. Exp. Med.* **40**, 33 (1970).
Ueno, Y., Ishikawa, Y., Nakajima, M., Sakai, K., Ishii, K., Tsunoda, H., Saito, M., Enomoto, M., Ohtsubo, K., Umeda, M., *Jpn. J. Exp. Med.* **41**, 257 (1971).
Ueno, Y., Sato, N., Ishii, K., Sakai, K., Enomoto, M., *Jpn. J. Exp. Med.* **42**, 461 (1972).
Ueno, Y., Sato, N., Ishii, K., Sakai, K., Tsunoda, H., Enomoto, M., *Appl. Microbiol.* **25**, 699 (1973).
Ueno, Y., Shimada, N., Yagasaki, S., Enomoto, M., *Chem. Pharm. Bull.* **22**, 2830 (1974).
Yagen, G., Joffe, A. Z., *Appl. Environ. Microbiol.* **32**, 423 (1976).

Received for review May 3, 1977. Accepted November 8, 1977.

Two New Trichothecenes Produced by *Fusarium roseum*

Kenji Ishii,[1] Sadanand V. Pathre, and Chester J. Mirocha*

Two new toxic 12,13-epoxytrichothecenes were isolated from the culture filtrates of two isolates of *Fusarium roseum* and were characterized as 4–acetoxyscirpendiol and 8-acetylneosolaniol.

The 12,13-epoxytrichothecenes are a group of biologically active secondary metabolites predominantly associated with species of *Fusarium* but biosynthesized by many different fungi as well (Smalley and Strong, 1974). This group of toxins became well known after their implication in a disease of humans called alimentary toxic aleukia, described by Joffe (1971), and associated with cereal grains which overwintered in the field. Moldy corn toxicosis in farm animals as described by Hsu et al. (1972) in northern climates is associated with trichothecenes. *Fusarium roseum* is frequently isolated from moldy corn and feed commonly associated with corn implicated in field cases of mycotoxicoses (*Mirocha* and Christensen, 1974). During the course of screening some of these *Fusarium* isolates for trichothecene production, two strains of *Fusarium roseum* were found to produce heretofore uncharacterized trichothecenes. Their isolation and iden-

Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota 55108.
[1]Present address: Laboratory of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku-ku, Tokyo 162, Japan.

0021-8561/78/1426-0649$01.00/0