

Factors Affecting Territrems B Production by *Aspergillus terreus* CCRC 32111 in Potato-dextrose Medium

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ABSTRACT

The effects of incubation time, temperature, medium volume, and initial pH on the production of territrems B (TRB) by *Aspergillus terreus* CCRC 32111 were investigated. At 28°C, maximal TRB production and mycelial growth were seen after 12 days' incubation in potato-dextrose (PD) liquid medium. Greatest TRB production occurred after rapid depletion of available carbohydrate at the stationary phase of fungal growth. TRB production decreased when the broth volume/culture container volume ratio increased, but increased if initial pH was alkaline. These results show that the TRB production is affected by factors including incubation time, temperature, medium volume, and the initial pH of medium.

Key words: incubation time, temperature, medium volume, initial pH, territrems B, *Aspergillus terreus* CCRC 32111, stationary phase, carbohydrate

INTRODUCTION

A cause-and-effect relationship between adverse effects in residents in Shuang-Chih Township in Taipei County and their intake of moldy rice contaminated by toxic strains of *Aspergillus* was reported in 1967⁽¹⁾. This incident prompted us to study fungal contaminants of stored unhulled rice in Taiwan. We found that, of the 206 isolates of the *Aspergillus* spp. examined, only *Aspergillus terreus* 23-1 was capable of producing tremorgenic mycotoxins, designated as territrems A, B, and C (TRA, TRB and TRC)⁽²⁻⁶⁾, TRB being the major product. These compounds induce whole-body tremors in mice and rats following intraperitoneal injection and show anti-acetylcholinesterase (AChE, E.C.3.1.1.7) activity⁽⁷⁾. Among 17 strains of *Aspergillus terreus* obtained from the Culture Collection and Research Center, Hsin Chu, Taiwan, only the CCRC 32111 and 32664 strains produced territrems in rice media⁽⁸⁾. These two strains were isolated from stored paddy rice, collected in the area of Hsinchu and Changhua, Taiwan.

Fungi, a major cause of spoilage of stored grains and seeds, rank second only to insects as a cause of deterioration and loss⁽⁹⁾. Spoilage of stored grain is often accompanied by the formation of mycotoxins produced by toxigenic fungi. It has been postulated that fungal growth and toxin formation are influenced by environmental factors, such as moisture, temperature, incubation time, and substrate type⁽¹⁰⁻¹¹⁾. In a previous investigation⁽¹²⁾, *Aspergillus terreus* 23-1 was found to produce territrems when grown on food commodities such as peanut, unpolished rice, millet,

wheat, and soybean, but not dried corn. Furthermore, territrems can be produced in potato-dextrose (PD) liquid medium, but not in Czapek's-Dox medium⁽¹³⁾. However, the effects of environmental factors on territrems production have not been studied. In the present work, the effect of incubation time and temperature, initial pH of medium, and the inoculum volume of the medium on TRB production by *Aspergillus terreus* CCRC 32111 were investigated.

MATERIALS AND METHODS

I. Strain and Culture Condition

(I) Organism

The territrems B-producing strain, *Aspergillus terreus* CCRC 32111, was obtained from the Culture Collection and Research Center, Hsin Chu, Taiwan.

(II) Inoculum preparation

Aspergillus terreus CCRC 32111 was cultured and maintained at 28°C on PD agar (Merck No. 10130). Conidiospores obtained from the edge of 7-day-old colonies were used as the inoculum source. Before use, the PD disks were washed with sterilized 0.01% Triton X-100 in distilled water. Spore suspensions (10⁸ spores per mL) were prepared using a hemacytometer.

(III) Culture conditions

Unless otherwise specified, 50 mL of sterilized PD broth (initial pH 5.0) in a 125 mL Erlenmeyer flask was inoculated with about 10⁸ spores.

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II. Experimental Design

To study the time-course of territre B production, Erlenmeyer flasks containing 50 mL of PD broth were inoculated with spore suspension and incubated at 28°C, then cultures were harvested at 0, 4, 8, 12, 16 days. To study the effect of temperature, Erlenmeyer flasks containing 50 mL of inoculated PD broth were incubated at various temperatures ranging from 18 to 38°C and the cells harvested as stationary cultures on day 12. To study the effect of medium volume, six different volumes of PD broth ranging from 30 to 80 mL were added to an Erlenmeyer flask, then, after spore incubation, the cultures were incubated at 28°C and harvested on day 12. To study the effect of initial pH, the initial pH of the media was adjusted over the range from 3 to 11 by addition of 1 N NaOH or 2 N HCl after medium sterilization. All experiments were performed in triplicate and repeated twice. The amount of TRB, mycelial dry weight, residual carbohydrate, residual nitrogen, and the final pH of the medium were determined at the end of incubation.

III. Measurement of Mold Growth

Mycelial dry weight was determined by filtering the culture broth through a dry pre-weighed filter paper (Whatman 1, Whatman International, U. K.), which was then washed with distilled water, dried in an oven at 105°C for 24 hrs and weighed. Mycelial dry weight was determined by subtracting the weight of the pre-weighed filter paper from that of the filter plus mycelium.

IV. TRB Determination

To extract TRB, the dry mycelium was mixed with 30 mL of chloroform and the mixture shaken mechanically for 30 mins at room temperature. The extract was filtered and evaporated to dryness, then redissolved in 2 mL of chloroform and subjected to thin layer chromatography analysis⁽⁸⁾. After development, the TRB was eluted from TLC plates and determined by HPLC⁽⁸⁾.

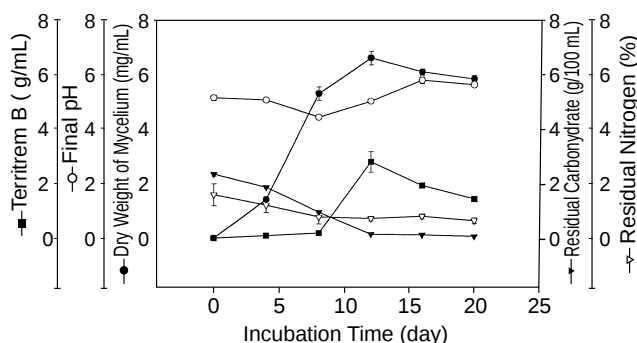


Figure 1. Fermentation time course of territre B production in a 125 mL of flasks. All flasks, containing 50 mL of potato-dextrose broth at pH 5.0, were incubated at 28°C.

V. Determination of Carbohydrate, Nitrogen, and pH

Carbohydrate was measured by the Anthrone method⁽¹⁴⁾ and nitrogen by the Kjeldahl method⁽¹⁴⁾. The final pH of the medium was determined by using a pH meter.

VI. Statistical Analysis

Unless otherwise stated, each value is the mean and SEM of three individual measurements. The data were analyzed using the SAS program for one-way analysis of variance, and the significance of differences between means was analyzed using Duncan's multiple range test.

RESULTS AND DISCUSSION

I. The Time Course of TRB Production

In the first series of experiments, *Aspergillus terreus* CCRC 32111 was grown for 4 – 20 days at 28°C in 50 mL of PD broth at an initial pH of 5. Figure 1 shows that TRB production started after 4 days' incubation. As incubation continued, TRB production increased gradually and reached a maximum after 12 days, at which time fungal growth entered a stationary phase. TRB production ceased when glucose in the medium was completely used up. An incubation time of 12 days was used in the subsequent experiments.

The above results showed that, using an initial pH of 5, a temperature of 28°C, and 50 mL of medium in a 125 mL of flask, TRB production reached a maximum after carbohydrate depletion and that highest mold growth was seen after 12 days. Shin and Marth⁽¹⁵⁾ also reported that maximal amount of aflatoxin appeared after rapid consumption of glucose and when the logarithmic phase of growth ceased. We also found that TRB production was first on day 4 during the exponential growth phase of the fungus, in agreement with the results of Bu'Lock⁽¹⁶⁻¹⁷⁾ and Buchanan and Lewis⁽¹⁸⁾, who found that aflatoxin, the secondary metabolite, first appears during the exponential phase of mold growth due to the expression of enzymes involved in secondary metabolism and glucose catabolism. Righelato⁽¹⁹⁾ postulated that the decrease in fungal growth and toxin production were, respectively, due to autolysis of the fungal cells and toxin degradation by fungal enzymes. In the present study, fungal growth and TRB production began to decrease after 12 days.

II. Effect of Incubation Temperature

Using 50 mL of medium in a 125 mL flask and at an initial pH of 5, *Aspergillus terreus* CCRC 32111 grew at all five temperatures from 18 to 38°C (Figure 2). The yield of TRB was maximal when the cultures were incubated for 12 days at 28°C ($p < 0.05$ compared to all other temperatures tested). However, the yield of TRB was much lower at 18, 23, and 38°C.

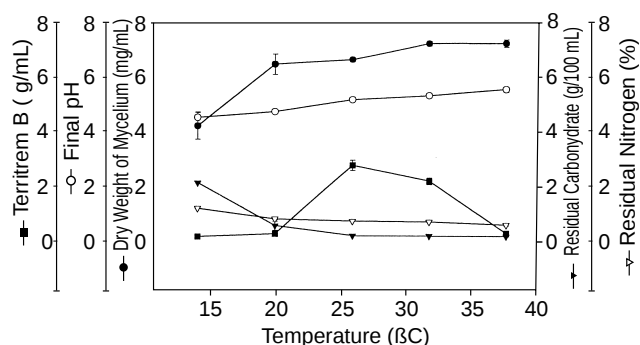


Figure 2. Production of territreum B by *A. terreus* CCRC 32111 at various temperature. All flasks, containing 50 mL of potato-dextrose broth at pH 5.0, were incubated at different temperature for 12 days.

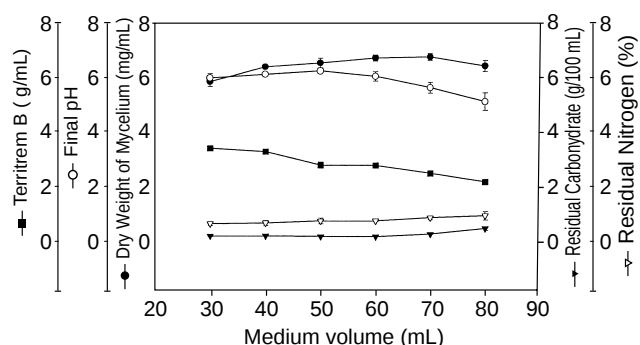


Figure 3. Effect of the medium volume size on the production of territreum B by *A. terreus* CCRC 32111. All flasks, containing different volume size of medium at pH 5.0, were incubated at 28°C for 12 days.

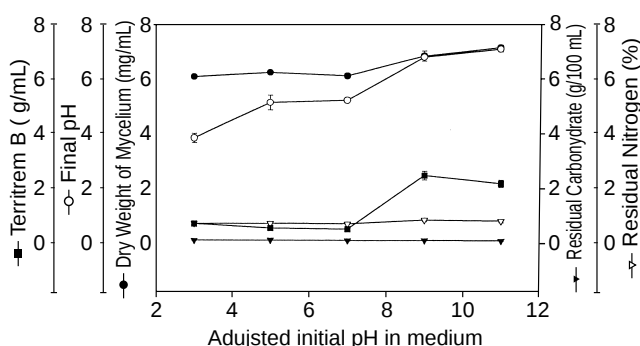


Figure 4. Effect of initial pH of culture medium on the production of territreum B by *A. terreus* CCRC 32111. All flasks, containing 50 mL of potato-dextrose broth, were incubated at 28°C for 12 days.

Temperature is one of the most important environmental factors influencing the growth and toxin production of toxigenic fungi⁽²⁰⁻²⁶⁾. TRB production by *Aspergillus terreus* CCRC 3211 increased with increasing temperature up to 28°C, then decreased at 33°C and 38°C. These data clearly show that temperature is an important factor affecting TRB production. Schindler et al.⁽²¹⁾ reported that different enzymes were responsible for growth and toxin production. Our data also show that only a small amount of

TRB production and low *Aspergillus terreus* growth were seen when the culture was incubated at 18°C, suggesting that the TRB contamination could be avoided by storing the commodities at low temperatures.

III. Effect of Medium Volume Size

Using culture conditions of 28°C, an initial pH of 5, and an incubation time of 12 days, when the medium volume in the flask increased over 80 mL, mycelial growth was not affected, but the yield of TRB declined at volumes greater than 50 mL, and the final pH of the culture became more acidic at medium volume of 60 mL or higher (Figure 3). At medium volumes ranging from 30 to 60 mL, the carbohydrate and nitrogen were almost entirely consumed by the microorganism, whereas a further increase in medium volume resulted in an increase in residual carbohydrate and a decrease in TRB production. El-Enshasy et al.⁽²⁷⁾ reported that increasing the volume of the culture medium resulted in a decrease in the amount of glucose consumed and in natamycin production, due to a shortage of oxygen supply. Aflatoxin production is reported to decrease with an increase in the carbon dioxide concentration and a decrease in the oxygen concentration⁽²⁸⁻³⁰⁾. Our data show that TRB production decreased with an increase in the volume of medium, suggesting that oxygen was important for TRB production.

IV. Effect of Initial pH

Using culture conditions of 28°C, 12 days' incubation, and 50 mL of medium in a 125 mL flask, the initial pH was varied from 3 to 11. At an initial pH of 9, TRB production was 4-fold higher than at pH 3-7 (Figure 4). In general, most fungi can grow at pH values ranging from 2 to 8.5⁽³¹⁾. Our data show that *Aspergillus terreus* CCRC 32111 can grow at pH values in the range of 3 to 11. Lie and Marth⁽³²⁾ reported that maximal aflatoxin production occurred at extreme alkaline pHs. The present study showed that TRB production and mold growth were maximal with an initial pH of 9-11.

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REFERENCES

1. Ling, K. H., Wang, J. J., Wu, R., Tung, T. C., Lin, C. K., Lin, S. S., and Lin, T. M. 1967. Intoxication possibly caused by aflatoxin B1 in the moldy rice in Shuang-Chih Township. J. Formosan Med. Assoc. 66: 517-525.

2. Chung, C. H., Ling, K. H., Tung, S. S., and Tung, T. C. 1971. Study on fungi of the stored unhulled rice of Taiwan. (2) Aflatoxin B-like compounds from the culture of *Aspergillus* genus. J. Formosan Med. Assoc. 70: 258-266.
3. Tung, S. S., Ling, K. H., Tasi, S. E., Chung, C. H., Wang, J. J. and Tung, T. C. 1971. Study of fungi of the stored unhulled rice of Taiwan. (1) Mycological survey of the stored unhulled rice. J. Formosan Med. Assoc. 70: 251-257.
4. Ling, K. H., Yang, C. K., and Peng, F. T. 1979. Territrem, tremorgenic mycotoxins of *Aspergillus terreus*. Appl. Environ. Microb. 37: 355-357.
5. Ling, K. H., Liou, H. H., Yang, C. M., and Yang, C. Y. 1984. Isolation, chemical structure, acute toxicity and some physicochemical properties of territrem C from *Aspergillus terreus*. Appl. Environ. Microb. 47: 98-100.
6. Ling, K. H., Liou, H. H., Fu, T. C., Kuo, L., Tasi, M. C., and Lin, M. Y. 1985. Mechanism of action of the territrem, tremorgenic mycotoxin isolated from *Aspergillus terreus*. Mycotoxins and Phycotoxins, 6th International IUPAC Symposium on Mycotoxins and Phycotoxins. pp. 387-398. Sreyn, P. S. and Velggaar, R. ed. Elsevier, Amsterdam, Netherlands.
7. Ling, K. H. 1994. Territrems, tremorgenic mycotoxins isolated from *Aspergillus terreus*. J. Toxicol.-Toxin Rev. 13: 243-252.
8. Fang, S. Y., Wei, Q. K., and Peng, F. C. 2002. Determination of territrem B in rice media with different strains of *Aspergillus terreus* by chromatography. J. Food Drug Anal. 10: 159-163.
9. Lacey, J., Ramakrishna, N., Hamer, A., Magan, N., and Marfleet, J. C. 1991. Grain fungi: Handbook of Applied Mycology, Vol. III. Foods and Feeds. pp. 121-127. Arora, D. K., Mukerji, K. G. and Marth, E. H. ed. Marcel Dekker, N. Y., USA.
10. Sinha, R. N. 1973. Interrelations of physical, chemical and biological variables in the deterioration of stored grains. In grain storage: Part of a system. pp. 15-47. Sinha, R. N. and Muir, W. E. ed. Avi Publishing Company, Westport, Connecticut, USA.
11. Hesseltine, C. W. 1974. Conditions leading to mycotoxin contamination of food and feeds. Mycotoxins and other fungal related food problems. pp. 1-22. Rodricks, J. V. ed. American Chemical Society. Washington, D. C., USA.
12. Wang Y. J. 1983. Studies on territrems: Mycological characteristics of toxin-producing strains and bioproduction of territrems. Master thesis, Institute of Biochemistry, National Taiwan University.
13. Tzeng, S. F. 1988. Morphology of colony, metabolism of [U, ¹⁴C]glucose and territrem production by *Aspergillus terreus* 23-1 on Czapek's-Dox and Potato-Dextrose medium. Master thesis, Institute of Biochemistry, National Taiwan University.
14. Hawk, P. B. 1965. Practical Physiological Chemistry. 14th edn. Oser, B. L. ed. Blakiston Division, McGraw-Hill, N. Y., USA.
15. Shin, C. N., and Marth, E. H. 1974. Aflatoxin formation, lipid synthesis, and glucose metabolism by *Aspergillus paraciticus* during incubation with and without agitation. BBA 38: 286-296.
16. Bu'Lock, J. D. 1965. Aspects of secondary metabolism in fungi. Biogenesis of antibiotic substances. pp. 61-72. Vanek, Z. and Hostalek, Z. ed. Academic Press Inc., N. Y., USA.
17. Bu'Lock, J. D. 1975. Secondary metabolism in fungi and its relationship to growth and development. The filamentous fungi, vol. 1, Industrial mycology. pp. 38-58. Smith, J. E. and Berry, D. R. ed. Edward Arnold Press, London, and Halsted Press, N. Y., USA.
18. Buchanan, R. L., and Lewis, D. F. 1984. Regulation of aflatoxin biosynthesis: effect of glucose on activities of various glycolytic enzymes. Appl. Environ. Microb. 48: 306-310.
19. Reghelato, R. C. 1975. Growth kinetics of mycelial fungi. The filamentous fungi; vol. 1, Industrial mycology. pp. 29-103. Smith, J. E. and Berry, D. R. ed. Edward Arnold Press, London, and Halsted Press, N. Y., USA.
20. Diener, V. L., and Davis, N. D. 1966. Aflatoxin production by isolates of *Aspergillus flavus*. Phytopath. 56: 1390-1393.
21. Schindler, A. F., Palmer, J. G., and Eisenberg, W. V. 1967. Aflatoxin product by *Aspergillus flavus* as related to various temperatures. Appl. Microbiol. 15: 1006-1009.
22. Schroeder, M. W., and Hein, H. Jr. 1967. Aflatoxin: Production of the toxins *in vitro* in relation to temperature. Appl. Microbiol. 15: 441-445.
23. Sansing, G. A., Davis, N. D., and Diener, U. L. 1973. Effect of time and temperature on ochratoxin A production by *Aspergillus ochraceus*. Can. J. Microbiol. 19: 1259-1263.
24. Montani, M., Vaamonde, G., Resnik, S. L., and Buera, P. 1988. Temperature influence on *Penicillium citrinum* growth and citrinin accumulation kinetics. Int. J. Food Microbiol. 7: 115-122.
25. Ramos, A. J., Laberia, N., Marin, S., Sanchis, V., and Magon, N. 1998. Effect of water activity and temperature on growth and ochratoxin production by three strains of *Aspergillus ochraceus* on a barley extract medium and on barley grains. Int. J. Food Microbiol. 44: 133-140.
26. Castella, G., Minkvold, G. P., Imerman, P., and Hyde, W. G. 1999. Effects of temperature, incubation period and substrate on production of fusaproliferin by *Fusarium subglutinans* ITEM 2404. Nat. Toxins 7: 129-132.
27. El-Enshasy, H. A., Farid, M.A., and el-Sayed, el-S. A. 2000. Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. J. Basic Microb. 40: 333-342.
28. Ciegler, A., Peterson, R. E., Lagoda, A. A., and Hall, H.

- H. 1966. Aflatoxin production and degradation by *Aspergillus flavus* in 20-liter fermenters. Appl. Microbiol. 14: 826-833.
29. Davis, N. D., and Diener, U. L. 1970. Environmental factors affecting the production of aflatoxins. pp.43-47. Herzberg, M. ed. Proceedings of the First U.S.-Japan Conference on Toxic Microorganisms. U. S. Dept. of Interior, Washington, D. C., USA.
30. Clevstroin, C., Liunggren, H., Tegelström, S., and Tideman, K. 1983. Production of aflatoxin by *Aspergillus flavus* isolate cultured under a limited oxygen supply. Appl. Environ. Microb. 46: 400-405.
31. Frazier, W. C. 1967. Food Microbiology. 2nd edn. McGraw-Hill, N. Y., USA.
32. Lie, J. L., and Marth, E. H. 1968. Aflatoxin formation by *Aspergillus flavus* and *Aspergillus paraciticus* in a casein substrate at different pH values. J. Dairy Sci. 51: 1743-1747.