Journal of Food Protection, Vol. 48, No. 8, Pages 705-708 (August 1985)
Copyright© International Association of Milk, Food, and Environmental Sanitarians

Comparison of Deoxynivalenol (Vomitoxin) Production by *Fusarium graminearum* Isolates in Corn Steep-Supplemented Fries Medium

ABDALLA EL-BAHRAWY[1], L. PATRICK HART[2] and JAMES J. PESTKA[1*]

Department of Food Science and Human Nutrition and Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

(Received for publication December 4, 1984)

ABSTRACT

Four out of nine North American *Fusarium graminearum* isolates produced deoxynivalenol (DON) and 15-monoacetyl deoxynivalenol (15-ADON) when grown in stationary cultures of modified Fries medium supplemented with 4% corn steep liquor. Strains R-6576, Van Wert A-1 and Stuckey produced primarily DON after 20 d of incubation at 28°C. In these strains, low levels of 15-ADON accumulated after 5 d but then declined over time. Disappearance of 15-ADON and subsequent appearance of DON coincided with both a rapid rise of pH above 8.0 and onset of the stationary phase. DON levels peaked after the exhaustion of carbohydrate (day 20) and then began to decline. In contrast to these three strains, strain NRRL 5883 produced primarily 15-ADON during an extended growth phase (day 10) and only small amounts of DON during late stationary phase (day 25). NRRL 5883 exhibited a slow rise in pH relative to the other three strains and utilized only 75% of the available carbohydrate during the 25-d period. Qualitative and quantitative production of DON and 15-ADON in liquid culture was dependent on the strain of *F. graminearum*.

Deoxynivalenol (DON), also known as vomitoxin, is a trichothecene mycotoxin produced by *Fusarium graminearum* Schwabe. DON has been associated with vomiting and feed refusal in swine (*19,23*), and occurs naturally in infected cereal grains grown in the United States (*19*), Canada (*16*), Japan (*8,21*), the United Kingdom (2) and France (*9*). DON is a potential contaminant of wheat breakfast cereals, wheat flour, bran, cookies, crackers and baby cereals (*5,16*).

Although DON can be produced on grains, such as corn and rice (*3,20*), these substrates are less suitable than liquid culture for biosynthetic studies or production of radiolabeled toxin. Liquid media have been used for DON production in many studies. Low levels of DON have been produced in Czapek-Dox medium supplemented with 0.5% peptone at 25°C within 14 d (*12*). Miller et al. (*10*) found that DON and 15-acetyl deoxynivalenol (15-ADON) were produced by North American *F. graminearum* isolates grown on glucose-yeast extract-peptone medium (GYEP). We have determined that *F. graminearum* R-6576 (*Gibberella zeae* U-5373), an isolate that produces large amounts of DON during field infection (*6,7*), produces the highest DON levels in modified Fries medium supplemented with 4% corn steep liquor (CSL) when compared to unsupplemented modified Fries medium, unsupplemented GYEP medium or GYEP medium supplemented with 4% CSL (*14*). It is of further interest to determine whether differences exist in the ability of North American isolates to produce DON and 15-ADON in this optimized liquid medium. The objective of the present study was to compare eight North American *F. graminearum* strains with strain R-6576 on the basis of 15-ADON and DON production, growth kinetics, carbohydrate utilization and pH change when grown for 25 d in modified Fries medium containing 4% CSL.

MATERIALS AND METHODS

Chemicals

All inorganic chemicals and organic solvents were of reagent-grade quality. DON standard was purchased from Mycolabs Company (St. Louis, MO). Identity of 15-ADON from our isolates was confirmed with gas chromatography-mass spectroscopy by Dr. C. Mirocha (University of Minnesota). 3-ADON was donated by Dr. J. D. Miller (Agriculture Canada, Ottawa). CSL was purchased from Corn Products Company (Cook County, IL).

Culture

Fusarium graminearum strains R-6574, R-6575, R-6576, Van Wert A-1, Stuckey, Sandusky A-2 and M-3 were field isolates and are part of the culture collection of L. P. Hart. Strains NRRL 5883 and Crawford-5 were obtained from Dr. Cullen and Dr. Smalley (University of Wisconsin-Madison). Culture purity was assured by the single spore isolation method, and strains were preserved and stored in sterilized soil (*13*).

[1]*Department of Food Science and Human Nutrition.*
[2]*Department of Botany and Plant Pathology.*

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/48/8/705/1653810/0362-028x-48_8_705.pdf by guest on 17 October 2021

Inoculum preparation

Stock *F. graminearum* strains were maintained in sterilized soil. For inoculum, strains from soil tubes were grown on potato dextrose agar (PDA) at 25°C for 7 d under alternating fluorescent light and darkness (12 h each). Agar plugs (4 mm) from the colony margin were aseptically transferred to 250-ml Erlenmeyer flasks containing 40 ml of carboxymethylcellulose medium (CMC; *1*) which contained per liter: NH_4NO_3, 1.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract, 1.0 g; and blended carboxymethyl cellulose, 15 g. The flasks were agitated on a rotary shaker at 250 rpm for 3 to 5 d at 25°C. Macroconidia concentrations were determined with a hemacytometer. Modified Fries medium (*15*) contained per liter: ammonium tartrate, 5.0 g; NH_4NO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.0 g; NaCl, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.13 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; yeast extract, 1.0 g; and sucrose 30 g. Modified Fries medium was supplemented with 4% (vol/vol) CSL. Roux flasks containing 200 ml of media were autoclaved, inoculated with 10^6 macaroconidia/flask, and incubated at 28°C. Duplicate flasks for each isolate were analyzed for dry weight, pH, carbohydrate and toxins at 5-d intervals over a 25-d period. Variation in dry weight and toxin concentrations between duplicate flasks did not exceed 10%.

Analyses

After incubation, the mycelial mat was separated from the medium by filtration through tared Whatman No. 4 filter paper. The mycelial mat was dried at 60°C for 12 h and dry weight determined. The filtrate was assayed for pH and for total carbohydrate by the phenol method (*4*). Portions (100 ml) of filtrate were extracted three times with equal volumes of ethyl acetate, the solvent dried with Na_2SO_4, and the extract evaporated to dryness under vacuum (*5*). Dried extracts were redissolved in 1 ml of ethyl acetate and 1 to 5 µl were spotted onto $AlCl_3$-dipped silica gel plates (*17*). These were developed in ethyl acetate-toluene (3+1). Plates were heated at 110°C for 5 min, dipped in paraffin oil-hexane (3+7), and compared with DON, 15-ADON and 3-ADON standards under longwave UV light. Visual comparison of standard DON fluorescence with sample DON and 15-ADON was used for quantitation because quantitative 15-ADON standard was not available. Rf's of DON, 15-ADON and 3-ADON were 0.3, 0.5 and 0.6, respectively.

RESULTS AND DISCUSSION

When *F. graminearum* isolates were compared for DON and 15-ADON production in modified Fries medium amended with 4% CSL at 28°C, only four were found to produce detectable levels of DON and 15-ADON. 3-ADON was not found in any culture. The relative 20-d toxin yields are summarized in Table 1. Several of the non-producing cultures had been previously shown to produce high levels of DON in experimentally infected field crops. The possibility exists that these cultures either lost their ability to produce DON during laboratory transfer or were unable to produce the toxins in the medium used in this study.

Maximum toxin levels produced by the four toxin-producing cultures ranged from 2.5 to 18.0 mg/L for DON and from 0.5 to 12.5 mg/L for 15-ADON (Figs. 1 through 4). Strains R-6576, Van Wert A-1 and Stuckey produced low amounts of 15-ADON (0.5 to 2.5 mg/L) during the growth phase (day 5), but these levels declined over time (Figs. 1 through 3). Appearance of DON and disappearance of 15-ADON in these three strains coincided with both a rapid rise in pH above 8.0 and onset of the stationary phase. In these fermentations, DON peaked after near exhaustion of carbohydrate (day 20) and then began to disappear rapidly (Figs. 1 through 3). Highest DON yields were produced by R-6576 within 20 d (18.0 mg/L; Fig. 1). In contrast to the three aforementioned strains, strain NRRL 5883 produced primarily 15-ADON (12.5 mg/L) during an extended growth phase, whereas the 5-d mycelial dry weight was only 1.0 g per flask (Fig. 4) as compared to 3.1 g (R-6576), 3.0 g (Van Wert A-1) and 3.0 g (Stuckey) per flask for the other three strains (Figs. 1 through 3). NRRL 5883 also exhibited a slower rise in pH, with a maximum of pH 8.0, and metabolized carbohydrate at a much slower rate than the other three strains (Fig. 4). Only small amounts of DON (0.5 mg/L) appeared during the late stationary phase of the NRRL 5883 cultures.

TABLE 1. *Production of DON and 15-ADON by* Fusarium graminearum *strains after 20 d of incubation in modified Fries medium supplemented with 4% corn steep liquor*[a].

Strain	DON (mg/L)	15-ADON[b] (mg/L)
R-6576	18.0	ND[c]
Van Wert A-1	5.4	ND
Stuckey	2.5	ND
NRRL 5883	ND	10.0

[a]Strains R6574, R6575, Sandusky A-2, M-3 and Crawford-5 did not produce detectable DON or 15-ADON in this medium during the 20-d fermentation.

[b]15-ADON was estimated by visual comparison with quantitative DON standard.

[c]ND, none detected.

We have previously shown that modified Fries medium plus 4% CSL is optimal for the production of DON and 15-ADON by *F. graminearum* R-6576, and that medium composition could determine whether 15-ADON or DON were the primary trichothecenes produced by this isolate (*14*). From the results presented here, it appears that *F. graminearum* strains also vary in ability to produce DON or 15-ADON in modified Fries medium supplemented with CSL. Whereas DON-producing *F. graminearum* strains isolated in Japan also produce 3-ADON (*21*), Miller et al. (*10,11*) have determined that DON-producing *F. graminearum* strains isolated in North America co-produce 15-ADON rather than 3-ADON. Our limited survey further supports this latter generalization. Yoshizawa et al. (22) found that disappearance of 3-ADON was concurrent with DON production in the fermentation by *Fusarium roseum*, and it has been suggested that 3-ADON deacetylation is a step in the biosynthesis of DON. For North American isolates R-6576, Van Wert A-1 and Stuckey, the disappearance of 15-ADON coincided with accumulation of DON (Figs. 1 through 3).

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/48/8/705/1653810/0362-028x-48_8_705.pdf by guest on 17 October 2021

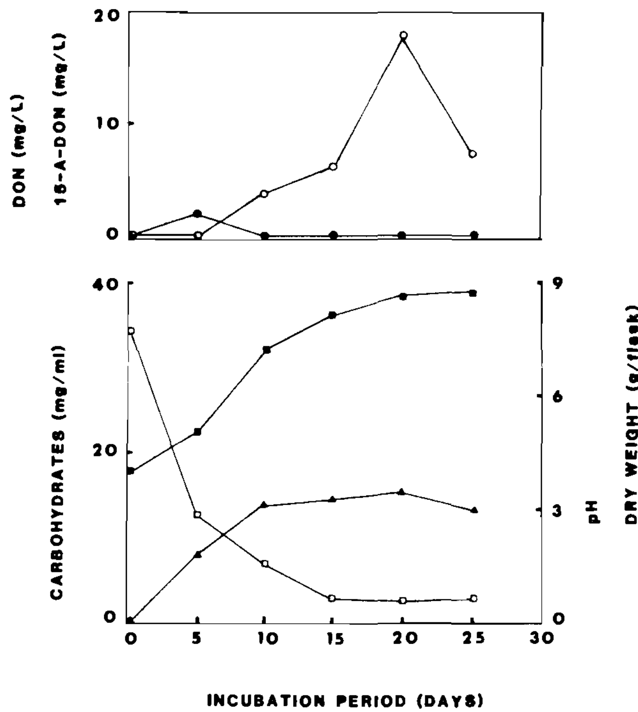

Figure 1. *Growth and toxin production by* F. graminearum *R6576. Cultures were grown in Fries medium plus 4% cornsteep liquor. Symbols are: DON (○), 15-ADON (●), carbohydrate (□), dry weight (▲), and pH (■). 15-ADON was estimated by visual comparison to quantitative DON standard on TLC.*

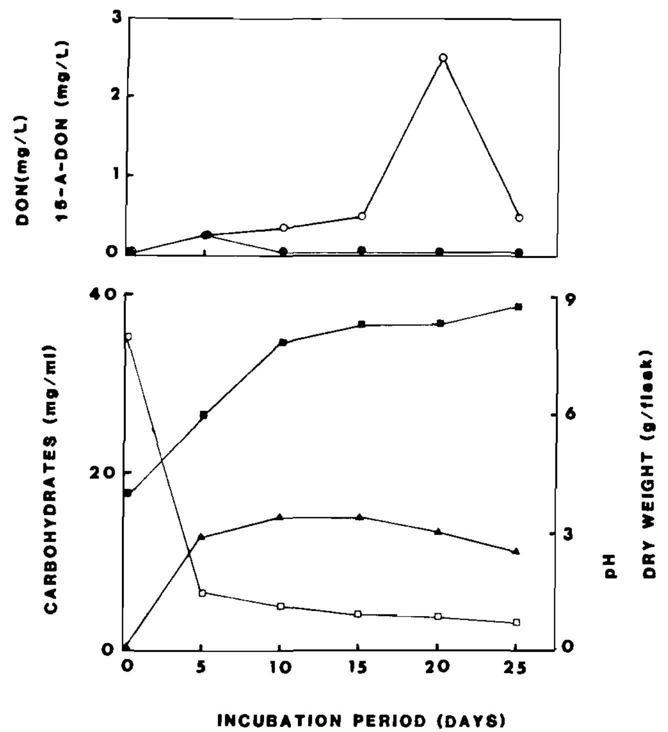

Figure 3. *Growth and toxin production by* F. graminearum *Stuckey. Conditions and symbols are the same as those described in Figure 1.*

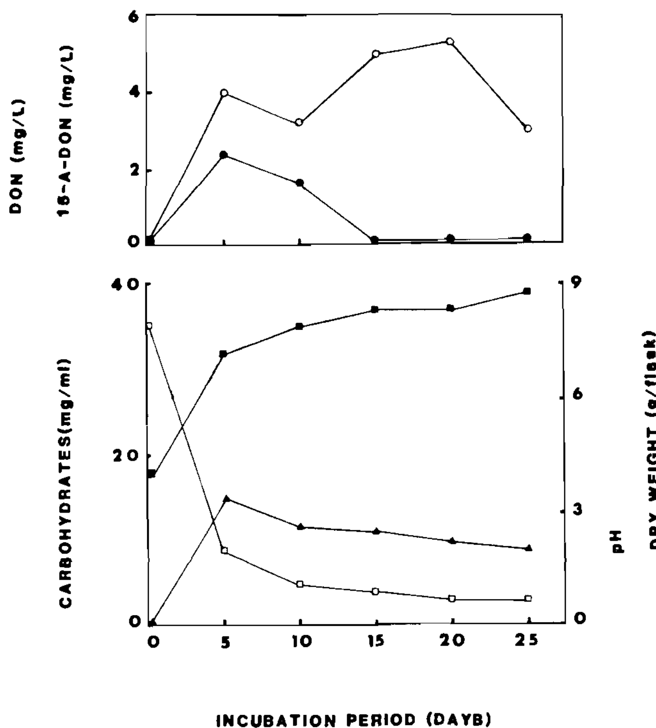

Figure 2. *Growth and toxin production by* F. graminearum *Van Wert A-1. Conditions and symbols are the same as those described in Figure 1.*

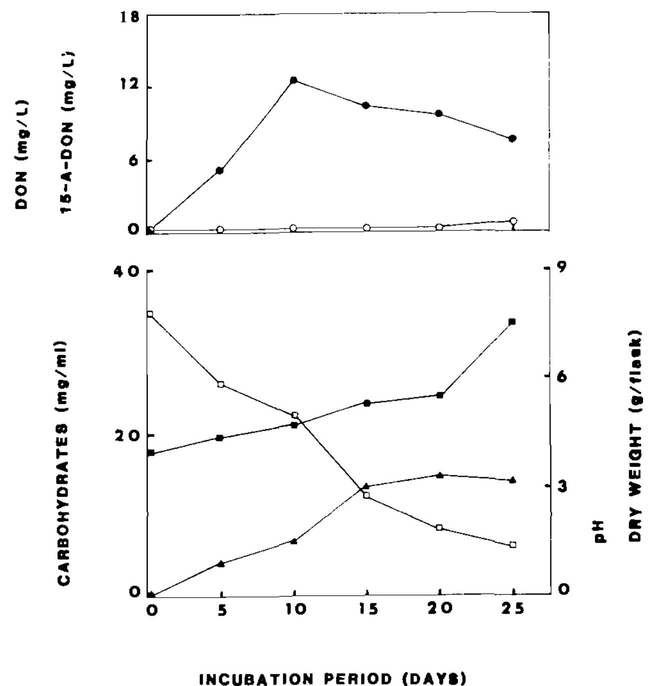

Figure 4. *Growth and toxin production by* F. graminearum *NRRL 5883. Conditions and symbols are the same as those described in Figure 1.*

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/48/8/705/1653810/0362-028x-48_8_705.pdf by guest on 17 October 2021

These results suggest that deacetylation of 15-ADON might similarly be a step in the biosynthesis of DON.

It has been suggested by Miller et al. (*10,11*) that DON-producing strains isolated in North America can be classified into two categories: (a) those that enzymatically or chemically convert 15-ADON to DON in liquid culture and (b) those that produce primarily 15-ADON in liquid culture but accumulate DON in infected cereal grains either by enzymatic or chemical conversion of 15-ADON. This categorization was based on a Canadian survey of North American isolates (*10*) which indicated that strains grown in GYEP medium either (a) produced equivalent amounts of 15-ADON and DON or (b) produced large amounts of 15-ADON relative to DON. The ability of three North American *F. graminearum* isolates (R-6576, Van Wert A-1 and Stuckey) used in our study to produce primarily DON in liquid culture after 20 d suggests these strains do not fit into either of the two aforementioned categories. These apparent differences can most likely be attributed to our use of modified Fries plus CSL rather than GYEP medium.

In contrast to strains R-6576, Van Wert A-1 and Stuckey, strain NRRL 5883 produced primarily 15-ADON. These results may suggest that NRRL 5883 had limited ability to convert 15-ADON to DON, and hence might be included in the second category described above. However, this is puzzling in view of the fact that NRRL 5883 is used by other laboratories for mass production of DON on heat-sterilized cereals (*20*). Our NRRL 5883 isolate may have mutated during laboratory passage or is simply unable to produce the requisite enzymes or chemical environment for 15-ADON to DON conversion when grown in modified Fries medium with CSL. Further investigation is needed to determine the reasons why strain differences were observed in the liquid medium described herein. Such an investigation might answer questions about the potential existence of the enzyme(s) responsible for 15-ADON deacetylation.

ACKNOWLEDGMENTS

Michigan State Experiment Station Article No. 11506.

This research was supported by the Michigan State Agricultural Experiment Station and Center for Environmental Toxicology, and by contributions from the Michigan Food Industry. We acnowledge Dr. E. Beneke and Mary Witt for their assistance during this research and Ms. Janeen Hunt for assistance in preparing this manuscript.

REFERENCES

1. Cappellini, R. A., and J. L. Peterson. 1965. Macroconidium formation in submerged cultures by a nonsporulating strain of *Gibberella zeae*. Mycologia 57:962-966.
2. Gilbert, J., M. J. Shepherd, and J. R. Startin. 1983. The analysis and occurrence of *Fusarium* mycotoxins in the United Kingdom and their fate during food processing. p. 78. Abstracts of the Third International Mycological Congress, Tokyo.
3. Greenhalgh, R., G. A. Neish, and J. D. Miller. 1983. Deoxynivalenol, acetyl deoxynivalenol, and zearalenone formation by Canadian isolates of *Fusarium graminearum* on solid substrates. Appl. Environ. Microbiol. 46:625-629.
4. Hanson, R. S., and J. A. Phillips. 1981. Chemical composition. pp. 238-364. *In* P. Gerhardt (ed.). Manual of methods for general bacteriology. American Society of Microbiology, Washington, DC.
5. Hart, L. P., and W. E. Braselton. 1983. Distribution of vomitoxin in dry milled fractions of wheat infected with *Gibberella zeae*. J. Agr. Food Chem. 31:657-659.
6. Hart, L. P., W. E. Braselton, and T. C. Stebbins. 1982. Production of zearalenone and deoxynivalenol in commercial sweet corn. Plant Dis. 66:1133-1138.
7. Hart, L. P., J. J. Pestka, and M-T. Liu. 1984. Effect of kernel development and moisture on production of deoxynivalenol in wheat infected with *Gibberella zeae*. Phytopathology 74:1415-1418.
8. Ichinoe, M., H. Kurata, Y. Sugiura, and Y. Ueno. 1983. Chemotaxonomy of *Gibberella zeae* with special reference to production of trichothecenes and zearalenone. Appl. Environ. Microbiol. 46:1364-1369.
9. Jemmali, M., Y. Ueno, K. Ishii, C. Frayssinet, and M. Etienne. 1978. Natural occurrence of trichothecenes (nivalenol, deoxynivalenol and T-2) and zearalenone in corn. Experientia 34:1333-1334.
10. Miller, J. D., A. Taylor, and R. Greenhalgh. 1983. Production of deoxynivalenol and related compounds in liquid culture by *Fusarium graminearum*. Can. J. Microbiol. 29:1171-1178.
11. Miller, J. D., J. C. Young, and H. L. Trenholm. 1983. *Fusarium* toxins in field corn. I. Time course of fungal growth and production of deoxynivalenol and other mycotoxins. Can. J. Bot. 61:3080-3087.
12. Morooka, N., N. Uratsuji, T. Yoshizawa, and H. Yamamoto. 1972. Studies on the toxic substances in barley infected with *Fusarium* spp. J. Food Hyg. Soc. (Japan) 13:368-375.
13. Nelson, P. E., T. A. Toussoun, and W. F. O. Marasas. 1983. *Fusarium* species, an illustrated manual for identification. Pennsylvania State University Press, University Park, PA.
14. Pestka, J. J., A. El-Bahrawy, and L. P. Hart. 1985. Deoxynivalenol and 15-monoacetyl deoxynivalenol production by *Fusarium graminearum* R6576 in liquid media. Mycopathologia (in press).
15. Pringle, R. B., and R. P. Scheffer. 1963. Purification of the selective toxin of *Periconia circinata*. Phytopathology 53:785-787.
16. Scott, P. M. 1983. The occurrence of vomitoxin in Canadian grains. p. 283. Abstracts of the Third International Mycological Congress, Tokyo.
17. Trucksess, M. W., S. Nesheim, and R. Eppley. 1984. Thin layer chromatographic determination of deoxynivalenol in wheat and corn. J. Assoc. Off. Anal. Chem. 61:40-43.
18. Tuite, J. 1969. Plant pathological methods--fungi and bacteria. Burgess Press, Minneapolis, MN. pp. 1-81.
19. Vesonder, R. F., A. Ciegler, and A. H. Jensen. 1973. Isolation of the emetic principle from *Fusarium*-infected corn. Appl. Microbiol. 26:1008-1010.
20. Vesonder, R. F., J. J. Ellis, W. F. Kwolek, and D. J. DeMarini. 1982. Production of vomitoxin on corn by *Fusarium graminearum* NRRL 5883 and *Fusarium roseum* NRRL 6101. Appl. Environ. Microbiol. 43:967-970.
21. Yoshizawa, T. 1983. Red-mold diseases and natural occurrence in Japan. pp. 195-209. *In* Y. Ueno (ed.) Trichothecenes-Chemical, biological, and toxicological aspects. Elsevier, New York.
22. Yoshizawa, T., and N. Morooka. 1975. Biological modification of trichothecene mycotoxins: acetylation and deacetylation of deoxynivalenol by *Fusarium* spp. Appl. Microbiol. 29:54-58.
23. Yoshizawa, T., and N. Morooka. 1977. Trichothecenes from mold-infested cereals in Japan. pp. 309-321. *In* J. V. Rodricks, C. W. Hesseltine and M. A. Mehlman (eds.), Mycotoxins in humans and animal health. Pathotox Publishers, Park Forest South, IL.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/48/8/705/1653810/0362-028x-48_8_705.pdf by guest on 17 October 2021