Demonstration of Aflatoxin Inhibitory Activity in a Cotton Seed Coat Xylan

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An inhibitor of aflatoxin biosynthesis localized in the seed coats of developing cotton was partially purified and characterized. Aqueous extracts from 25-day postanthesis seed coat tissue inhibited aflatoxin (B₁) production in liquid cultures of *Aspergillus flavus* AF13. Inhibition was concentration dependent, with a 50% effective dose of 173 µg of crude extract per ml of medium. The inhibitor was neutral in charge. Two active fractions were obtained from crude preparations by gel filtration chromatography (BioGel P-100). The purest fraction eluted in the void volume. Carbohydrate composition analysis of this void volume inhibitor indicated a composition of xylose (>90%) and mannose. Aflatoxin production in vitro was inversely related to inhibitor concentration in the fermentation medium (log of aflatoxin versus log of [inhibitor]; $r^2 = 0.82$; $P < 0.002$). The void volume inhibitor had a 50% effective dose of 6.2 µg/ml, a 28-fold purification of the inhibitor material. These data support the hypothesis that seed coat inhibitory activity is associated with a cottonseed-specific xylan.

Infection of developing cottonseed by the filamentous fungus *Aspergillus flavus* Link ex Fries is economically important primarily because of the production of the potent carcinogen aflatoxin B₁ (7). Characterization of endogenous plant factors that inhibit aflatoxin production offers footholds for increasing plant resistance to aflatoxin contamination (2). Low-molecular-weight aflatoxin inhibitory factors have been observed in developing cottonseed tissue (10, 13). However, altering the expression of these inhibitory compounds through either traditional breeding or biotechnology may be difficult because of the complexity of their biosynthesis (multistep pathway). The expression of high-molecular-weight compounds, such as proteins, may be more easily effected.

McCormick et al. reported a high-molecular-weight inhibitor of aflatoxin biosynthesis from the seed coats of developing cottonseed (12). On the basis of its nondialyzable nature, DEAE cellulose binding and periodate sensitivity, the active principle was tentatively identified as a protein or glycoprotein. However, several characteristics, including heat stability, periodate sensitivity, and pronase insensitivity, were consistent with a polysaccharide nature.

An investigation was initiated to purify and characterize the aflatoxin inhibitory factor(s) from cotton seed coats. Aflatoxin-inhibiting activity was associated with a xylan-rich fraction. A preliminary report has been presented elsewhere (14).

MATERIALS AND METHODS

Biological materials. Cotton (*Gossypium hirsutum* L. "Deltapine 61" and "Deltapine 90") plants were maintained under standard greenhouse conditions (minimum temperature, 20°C; maximum temperature, 35°C) and in a field plot. Developing cotton bolls, 25 days postanthesis, were harvested, and the locules were lyophilized. The seeds were delinted by hand and stored at -20°C. Seed coat tissue was dissected manually and stored at -20°C. *A. flavus* AF13 was isolated from southern Arizona and maintained on a 5% V-8 juice agar (6) at

30°C. Conidia were suspended in sterile deionized water at concentrations of 10⁷ to 10⁸ spores per ml for use in fungal incubations.

CSCI preparation. Cotton seed coat inhibitor (CSCI) was obtained by homogenization of seed coat tissue (5 g/100 ml of medium) in an Adye and Mateles medium, pH 5.0 (1), with NaNO₃ as the sole nitrogen source (5). Seed coat tissue was homogenized in a Sorvall Omnimixer at full speed for 90 s and then homogenized at full speed with an internal blade unit (Tekmar) for 90 s. The homogenate was centrifuged at 10,000 × g for 20 min (4°C) to remove cellular debris. The supernatant was the crude CSCI preparation.

Binding study. Cell debris remover (CDR; Whatman), a weak anion-exchange medium, was added to the crude CSCI preparation at 0.05 g/ml of liquid and stirred for 30 min. All binding procedures were performed at 25°C. The liquid phase, separated by filtration in vacuo, contained the neutral and cationic components (filtrate A). The anionic components were released from the CDR by stirring in 0.02 M Tris-0.5 M NaCl (pH 8.0) for 30 min and then by filtration in vacuo (filtrate B). Filtrate A was further fractionated by the addition of carboxymethylcellulose (CM 52; Whatman) at 0.05 g/ml of liquid and stirred for 30 min. The liquid phase was separated as described above and constituted the neutral fraction (fraction C). The bound material was released by stirring the solid phase (CMC) from the previous step in an identical volume of 0.05 M acetate buffer-0.5 M NaCl, pH 5.5, for 30 min. The liquid phase removed by filtration was the cationic fraction (filtrate D).

CSCI assays. Fungal incubations were conducted either in 50-ml flasks containing 25 ml of Adye and Mateles medium (described above) or in 250-ml flasks containing 70 ml of medium. Seventy-milliliter incubations yielded less variability among replicates. All liquid fractions were extensively dialyzed against the fungal medium before addition to fungal incubations to minimize pH differences or nutritional differences which might arise from the release of endogenous sugars from seed coat tissue. After dialysis, fractions were filter sterilized through a 0.22-µm-pore-size membrane (Millex GV; Millipore) prior to addition to the incubation medium. The flasks were inoculated with 0.1 ml (25-ml incubation) or 0.2 ml (70-ml incubation) of a conidial suspension containing 10⁸ spores per ml (1 × 10⁷ to 2 × 10⁷ spores per flask), and the suspensions were incubated at 30°C on a rotary shaker (150 rpm) for 5 days in the dark. The dose response experiment with the void volume (V₀) inhibitor necessitated the use of small-volume (5-ml) fermentations. The incubations were conducted in sterile 50-ml conical centrifuge tubes. The small-volume vessels were inoculated with 0.1 ml of AF13 conidial suspension and incubated at 30°C on a rotary shaker (200 rpm) for 5 days.

To assess nonspecific influences of plant polysaccharides, commercial preparations of xylan, starch, and pectin were evaluated in the CSCI assay (70 ml of medium in 250-ml flask). Starch (potato) and xylan (birchwood) from Sigma Chemicals (St. Louis, Mo.) could not be filter sterilized because of formation of persistent precipitates. Stock suspensions (10 mg/ml of Adye and Mateles medium) were autoclaved immediately before addition to the media. The starch preparation had to remain at a temperature greater than 55°C to prevent precipitation. Pectin (Schweizerhall, Inc., Plainfield, N.J.) was dissolved in fungal medium prior to sterilization (autoclave).

Aflatoxin analysis. Following incubation, each flask was diluted to 50% (vol/

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FIG. 1. Effect of crude CSCI on aflatoxin production. Mean AFB-1 level is expressed in micrograms per gram of mycelia (dry weight) ($n = 4$). [CSCI] values were calculated from a standard CSCI preparation (crude) which was lyophilized to determine the mass (AFB-1 versus [CSCI]; $r^2 = 0.93$; $P < 0.05$).

vol) acetone and allowed to soak for 18 h. Mycelial mats were separated by filtration in vacuo and dried at 60°C for 2 days. After addition of 30% (vol/vol) water, the medium-acetone solution was extracted three times with a half volume of methylene chloride. The methylene chloride extracts were pooled, concentrated to dryness by evaporation, redissolved in a known volume of methylene chloride, and analyzed by thin-layer chromatography on silica gel G plates developed in diethyl ether-methanol-water (96:3:1) (17). The small-volume incubations were not partitioned against methylene chloride. Instead, acetone was added to the fermentation mix as previously described, and the medium-acetone solutions were spotted directly on thin-layer plates. Aflatoxin B₁ was quantitated by a standard plate densitometry method (17).

Protein and sugar assays. Protein concentrations in CSCI fractions which had been dialyzed against water were estimated by the method of Waddell (19) with bovine serum albumin as a standard. Carbohydrate was analyzed by the phenol-sulfuric acid method (8).

Column chromatography. The CSCI material was further purified by gel filtration chromatography. BioGel P-100 (Bio-Rad Labs) was equilibrated with 0.05 M Tris-0.5 M NaCl, pH 9.0, packed to form a column that was 2.5 by 50 cm, and extensively washed with the buffer described above. A standard crude CSCI preparation (100 ml) was obtained and treated with CDR as previously described. The recovered filtrate (filtrate A) was dialyzed exhaustively against deionized water and lyophilized to dryness. A light-brown solid was obtained. A portion of this material was resuspended in the Tris buffer, applied to the column, and eluted with the same buffer. Two-milliliter fractions were collected and monitored for A_{215} and for carbohydrate content by the phenol-sulfuric acid method (8). Fractions which contained detectable carbohydrate-positive material were pooled (fractions 2 to 6 and 33 to 40), dialyzed against water, concentrated by lyophilization, and tested in the inhibitor assay. The fractions which contained only 215-nm-light-absorbing material (fractions 41 to 80) did not dissolve in either buffer or medium after lyophilization.

Carbohydrate analysis. About 1 mg of the inhibitor material eluted in the V_0 of the P-100 column was heated in 2% (wt/vol) oxalic acid at 120°C for 2 h to hydrolyze the polysaccharide. Excess oxalate was precipitated by calcium ions and removed by filtration. Water was removed by lyophilization, and trimethylsilyl derivatives were generated according to the procedure described by Sweeley et al. (18). The trimethylsilyl derivative mixture was analyzed on a gas chromatograph (Hewlett-Packard; model 5880) fitted with a DB-5 column (0.032 by 300 cm). The initial temperature (210°C) was maintained for 4 min and then increased to 250°C on a 4°C/min linear ramp.

RESULTS

The presence of CSCI in fungal cultures greatly reduced aflatoxin production (Fig. 1). An inverse linear dose response

TABLE 1. Treatment of cotton seed coat inhibitor with ion exchange adsorbents

Treatment	AFB-1 ^a	% Inhibition	% Total inhibition	Wt (g) ^b	pH ^c
Control	242 ± 90.1			0.70	5.0
Crude CSCI	43.9 ± 59.2	81.9	100	0.90	5.8
Fraction A ^d	77.0 ± 63.1	68.2	83.3	0.90	5.8
Fraction B ^e	224 ± 222	NS ^h	0	0.89	5.7
Fraction C ^f	144 ± 85	40.5	49.5	0.88	5.7
Fraction D ^g	308 ± 134	NS ^h	0	0.78	5.3

^a Mean AFB-1 expressed as micrograms of AFB-1 per gram (dry weight) ($n = 3$). Tested fractions were added as 40% (vol/vol) of total incubation volume.

^b Mean dry weight expressed in grams ($n = 3$).

^c Final pH of culture filtrate.

^d Fraction A contains neutral plus cationic components. Tested fractions were derived as described in Materials and Methods.

^e Fraction B contains anionic components.

^f Fraction C contains neutral components.

^g Fraction D contains cationic components.

^h NS, not significant ($P = 0.05$).

(aflatoxin versus [CSCI]; $r^2 = 0.93$; $P < 0.05$) was observed as crude CSCI concentrations were increased from 10% (72 μg/ml) to 40% (vol/vol) (289 μg/ml) (Fig. 1). Maximum inhibition (93%) occurred at the highest CSCI concentration (40%) tested. The 50% effective dose (ED_{50}) of crude CSCI was 21% (0.173 mg/ml). Final fungal culture pH and fungal growth (dry weight) were not influenced by crude CSCI (Table 1). However, some differences between control and inhibitor-treated cultures were noted. Untreated cultures developed a distinct yellow appearance, and the mycelial pellets were compact, whereas filtrates from inhibitor-treated cultures were colorless and mycelial pellets were relatively loose.

Most aflatoxin inhibitory activity did not bind to the anion-exchange medium, and about half of the initial activity remained with the neutral fraction (Table 1). After binding, no additional activity was released from the cation-exchange medium with the high-ionic-strength buffer (acetate-NaCl).

Small volumes (2 to 5 ml) of crude CSCI preparations were dialyzed against water and assayed for protein and carbohydrate. Protein concentrations were below the limits of detection in the Bradford and Lowry protein assays (3, 11). The bicinchoninic assay (16) indicated a protein concentration range of 25 to 50 μg/ml in crude CSCI preparations. Nondialyzable carbohydrate levels ranged from 0.37 to 0.5 mg/ml (xylose equivalents). The carbohydrate content suggested that the active moiety of the CSCI preparation might be a polysaccharide. Starch (0.5 mg/ml), birch xylan (0.25 to 1.0 mg/ml), pectin (0.25 to 1.0 mg/ml), and bovine serum albumin (0.25 to 0.5 mg/ml) all displayed no inhibitory effect on either aflatoxin biosynthesis or fungal growth in the 25-ml liquid fermentation assay.

The gel filtration column resolved the CSCI material into two major components. The first eluted close to the V_0 of the column and was predominately carbohydrate, with a small amount of associated 215-nm-light-absorbing material (Fig. 2). The second component contained high levels of 215-nm-light-absorbing material as well as carbohydrate (Fig. 2). Fractions (e.g., 50) from this peak of material gave a positive reaction with phloroglucinol, a reagent diagnostic for lignin. In addition, the absorption spectrum of this material gave a good fit with the published spectrum for lignin derived from castor beans (4). Both fraction pools with polysaccharide material displayed aflatoxin inhibitory activity (Table 2).

The neutral carbohydrate analysis of CSCI material (V_0)

FIG. 2. Elution profile of CSCI on a gel filtration column. Sample applied to the P-100 column was crude CSCI which had been treated with CDR, dialyzed versus water, lyophilized and resuspended in 0.05 M Tris-0.5 M NaCl, pH 9.0. The P-100 column was eluted with the same buffer. Two-milliliter fractions were collected and monitored for A_{215} (crosses) and carbohydrate (triangles).

recovered from gel filtration chromatography revealed that xylose (97%) was the major saccharide component, with mannose (3%) present as a minor component.

Aflatoxin production was inversely related to the concentration of CSCI (V_0) in the fermentation medium (log of aflatoxin versus log of [CSCI]; $r^2 = 0.82$; $P < 0.002$) (Fig. 3). The ED_{50} of the crude CSCI material was 173 $\mu\text{g/ml}$, whereas the ED_{50} of the V_0 CSCI was 6.2 $\mu\text{g/ml}$. Comparison of the ED_{50} values of crude (173 $\mu\text{g/ml}$) and V_0 (6.2 $\mu\text{g/ml}$) CSCI indicates a 28-fold purification of the CSCI material after CDR adsorption and passage through the P-100 column. The V_0 inhibitor material displayed a slight inhibitory growth effect on the fungus (Fig. 3).

DISCUSSION

The results of binding experiments with CDR, an anion-exchange medium, and carboxymethylcellulose, a cation-exchange medium, indicated that the predominate aflatoxin inhibitory moieties present in crude CSCI are neutral in charge. This result is in apparent contradiction to the observation by McCormick et al. (12) that the seed coat inhibitor quantitatively bound to DEAE cellulose and could be eluted by increasing the ionic strength. This discrepancy might be accounted for by nonionic interactions which result in binding of

FIG. 3. Effect of V_0 CSCI on aflatoxin production. Mean AFB-1 (triangles) is expressed in micrograms per gram of mycelia ($n = 5$) (dry weight) (log of AFB-1 versus log of [CSCI]; $r^2 = 0.82$; $P < 0.002$). Growth (squares) is expressed as milligrams (dry weight) (log of weight versus log of [CSCI]; $r^2 = 0.65$; $P < 0.05$).

CSCI to the anion-exchange medium used in the referenced study. This suggestion is substantiated by observations made during the current study with the P-100 column. When CSCI was chromatographed with water as solvent, a V_0 peak was absent and a single peak of carbohydrate-containing material was eluted from the column in a region indicating interaction with the column matrix. When the CSCI samples were chromatographed in a high-ionic-strength, high-pH buffer, two carbohydrate-containing peaks were obtained, and the V_0 peak was present. These data suggest that in the absence of high-ionic-strength conditions, carbohydrate-carbohydrate interactions between the two classes of carbohydrate-containing material exist. These interactions could explain the data of McCormick et al. Another possibility is the existence of two classes of CSCI, one neutral and one anionic. Since the major activity encountered in this investigation was neutral, efforts were concentrated on purification and characterization of this material.

Possible explanations for the mode of action of the CSCI include a generalized effect on primary metabolism with consequences on fungal growth, reducing the toxin-specific activity (in micrograms per gram [dry weight]) by increasing the overall mycelial weight. For example, a protein addendum to the fungal medium might provide additional nitrogen and carbon. This hypothesis appeared to be invalid, since a protein (bovine serum albumin) substituted for CSCI in the inhibition assay did not induce any significant differences in mycelial weight or aflatoxin production. A similar argument could be made for the presence of polysaccharides in the fungal growth medium. Cotton seed coat tissue at this stage of development (25 days postanthesis) is known to contain starch (9). Commercial starch, xylan, and pectin, at the levels tested, did not influence fungal growth or aflatoxin production. Thus, a component specific to cotton seed coat may be responsible for the aflatoxin inhibitory activity. Nonspecific binding of aflatoxin to the cottonseed xylan material might account for the inhibition data by lowering detectable toxin concentrations. However, aflatoxin extraction conditions (50% acetone) would be expected to disrupt such nonspecific associations. Use of mycelial weight as a culture indicator may not be adequately sensitive to consistently detect differences in growth rate induced by CSCI. CSCI did display a small growth-inhibitory potential in the purified form (V_0) when responses to a concentration gradient were

TABLE 2. Inhibitory activity of fraction pools from gel filtration chromatography

Treatment	AFB-1 ^a	% Inhibition
Control	188 $\pm$ 79.2	
Crude CSCI ^b	102 $\pm$ 72.9	46
Pool A ^c	53.7 $\pm$ 38.8	71
Pool B ^d	45.9 $\pm$ 42.1	76

^a Mean AFB-1 expressed as micrograms of AFB-1 per gram (dry weight) ($n = 4$).

^b Crude CSCI was treated with CDR, dialyzed versus water, and lyophilized prior to column application; the concentration was 30 $\mu\text{g/ml}$ of medium.

^c Column fractions (2 to 6) containing V_0 material were pooled and lyophilized prior to testing; the concentration was 10 $\mu\text{g/ml}$ of medium.

^d Column fractions (33 to 40) containing 215-nm-light-absorbing material and carbohydrate-positive material were pooled and lyophilized prior to testing; the concentration was 10 $\mu\text{g/ml}$ of medium.

tested by regression analysis (Fig. 3). However, growth was not correlated with aflatoxin production (Pearson product moment correlation; $r = 0.16$; $P = 0.37$).

The results obtained from column chromatography support the hypothesis that the inhibitory activity in developing seed coats is associated with a polysaccharide. Two major peaks of material were obtained from gel filtration chromatography. Both fractions contained carbohydrate, and both displayed ability to inhibit aflatoxin biosynthesis (Fig. 2 and Table 2). The material which contained high levels of 215-nm-light-absorbing material apparently interacted with the column matrix, since it eluted after the bed volume of the column. Assuming that the 215-nm-light-absorbing material contains lignin substituents (as indicated by UV spectrophotometry and phloroglucinol), hydrophobic interactions could explain the affinity of the CSCI material for the acrylamide-based gel filtration matrix. The lignin and polysaccharide materials could represent separate cell wall components which are linked together in plantae to form cell wall subunits (B peak). These subunits may subsequently be further linked in plantae to form insoluble cell wall material. The exact relationship between the polysaccharide and the 215-nm-light-absorbing material is not understood.

Carbohydrate compositional analysis of the gel filtration product strongly suggests that a xylan is involved in the inhibitory activity of immature cotton seed coats. Such molecules are present as storage polysaccharides in developing seed from a wide diversity of plants (15). Since the expression of CSCI in developing cottonseed was originally reported to be temporal (maximum activity at 25 days postanthesis), a polysaccharide with developmental turnover would be consistent with the available data. Unfortunately, little insight about the mode of action of CSCI can be inferred from its saccharide composition. Presumably, a structural component of the cotton xylan which confers aflatoxin inhibitory activity is missing from the commercial xylan preparations used in this study. It is certainly possible that a seed coat polysaccharide could be digested during the course of the fungal fermentation into smaller components which are the active factors in the inhibition assay. Thus, the active factor may not actually be a saccharide, but instead, some other class of bioactive molecule (e.g., a peptide).

The sites and mode of action of CSCI are probably different from those of the low-molecular-weight aflatoxin inhibitory factors which have been reported for cottonseed. The factor reported by Hensarling et al. (10) was dialyzable but was not further characterized. The factors derived from cotton ovule cultures (13) were nonpolar and probably terpenoid in nature. Since CSCI is comparatively larger (nondialyzable) and water soluble, it may represent a distinct class of aflatoxin inhibitor which could be useful in altering host plant susceptibility to aflatoxin contamination.

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REFERENCES

1. Adye, J., and R. I. Mateles. 1964. Incorporation of labeled compounds into aflatoxins. *Biochim. Biophys. Acta* **86**:418-420.
2. Bhatnagar, D., T. E. Cleveland, and P. J. Cotty. 1994. Mycological aspects of aflatoxin formation, p. 327-346. In D. L. Eaton and J. D. Groopman (ed.), *The toxicology of aflatoxins: human health, veterinary, and agricultural significance*. Academic Press, San Diego, Calif.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
4. Bruce, R. J., and C. A. West. 1989. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol.* **91**:889-897.
5. Cotty, P. J. 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*, influence of pH. *Phytopathology* **78**:1250-1253.
6. Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology* **79**:808-814.
7. Cotty, P. J., P. Bayman, D. S. Egel, and K. S. Elias. 1994. Agriculture, aflatoxins, and *Aspergillus*, p. 1-27. In K. A. Powell et al. (ed.), *The genus Aspergillus*. Plenum Press, New York.
8. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350-356.
9. Hendrix, D. L. 1990. Carbohydrates and carbohydrate enzymes in developing cotton ovules. *Physiol. Plant.* **78**:85-92.
10. Hensarling, T. P., T. J. Jacks, L. S. Lee, and A. Ciegler. 1983. Production of aflatoxins on soybean and cottonseed meals. *Mycopathologia* **83**:125-127.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
12. McCormick, S. P., D. Bhatnagar, W. R. Goynes, and L. S. Lee. 1988. An inhibitor of aflatoxin biosynthesis in developing cottonseed. *Can. J. Bot.* **66**:998-1002.
13. Mellon, J. E. 1992. Inhibition of aflatoxin production in *Aspergillus flavus* by cotton ovule extracts. *J. Am. Oil Chem. Soc.* **69**:945-947.
14. Mellon, J. E., P. J. Cotty, M. A. Godshall, and E. Roberts. 1993. Partial purification of aflatoxin inhibitors from cotton seedcoats. *Plant Physiol.* **102**(5):114.
15. Siddiqui, I. R., and P. J. Wood. 1971. Structural investigation of water-soluble, rape-seed polysaccharides. *Carbohydr. Res.* **17**:97-108.
16. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76-85.
17. Stoloff, L., and P. M. Scott. 1984. Natural poisons, p. 477. In S. Williams (ed.), *Official methods of analysis of the Association of Official Analytical Chemists*, 14th ed. Association of Official Analytical Chemists, Inc., Arlington, Va.
18. Sweeley, C. C., R. Bentley, M. Maketa, and W. W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* **85**:2497-2507.
19. Wolf, P. 1983. A critical reappraisal of Waddell's technique for ultraviolet spectrophotometric protein estimation. *Anal. Biochem.* **129**:145-155.