pH close to the isoelectric point. Second step: absorption of the lipids and the formation of a complex precipitate by hydrophobicity. Third step: resolubilization of the weaker hydrophobic components by the addition of 1 M NaCl and neutralization. These three steps may cause the formation of the globulin precipitates with the hydrophobic interaction between the α and α' subunit-rich proteins, which mainly consist of β_5 and β_6 and lipids. The effect of heterogeneity of β-conglycinin on the ASF2 formation was less than ASF1 formation as judged by the subunit constitution (Figure 5). It is believed that the isoelectric point of glycinin was pH 5.8–6.4 and β-conglycinin was pH 4.8–5.8 (Thanh and Shibasaki, 1976), so that proteins with a slightly charged cation interacted with the Cl^- ion under the ASF2 conditions. These interactions perhaps weakened the hydrophobicity of the protein surface at pH 4.5. The reduced effect of hydrophobicity on the ASF2 formation may be due to these interactions. In the mixture of β-conglycinin and glycinin, glycinin which has weaker hydrophobicity (Table II), showed less precipitates. Moreover, the isoelectric point of glycinin is further from pH 4.5 than that of β-conglycinin; therefore glycinin gave nearly no precipitate with the ASF2 condition. γ-Conglycinin, present in β-conglycinin, was selectively precipitated. The average hydrophobicity or γ-conglycinin was stronger than β-conglycinin (Table II).

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Production of Zearalenone, α- and β-Zearalenol, and α- and β-Zearalanol by *Fusarium* Spp. in Rice Culture

Kurt E. Richardson, Winston M. Hagler, Jr.,* and Chester J. Mirocha

Reverse-phase thin-layer chromatographic analysis of rice culture extracts of seven zearalenone producing isolates of *Fusarium* revealed several more polar compounds similar to zearalenone in fluorescence characteristics and/or reaction to 4-methoxybenzenediazonium fluoborate and Fast Violet B salt spray reagents. Capillary gas chromatography/mass spectrometric analysis of these extracts revealed six estrogenic compounds not previously reported as naturally produced, namely *cis*-zearalenone, *cis*-α-zearalenol, *cis*- and *trans*-β-zearalenol, and α- and β-zearalanol.

INTRODUCTION

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid μ-lactone] is an estrogenic secondary metabolite produced by various species of *Fusarium*, especially cultivars of *F. roseum* (Stipanovic and Schroeder, 1975; Hagler and Mirocha, 1980; Palyusik et al., 1980), colonizing corn, sorghum, oats, and other cereal grains. This fungal metabolite has been associated with hyperestrogensim and other reproductive disorders in cattle (Mirocha et al., 1968), swine (Miller et al., 1973), and poultry (Meronuck et al., 1970; Allen et al., 1981a,b).

Zearalenone was chemically characterized by Urry et al. (1966) and since then eight naturally produced derivatives of *trans*-zearalenone (Figure 1 part a) have been isolated from cultures of *F. roseum* "Gibbosum" and *F. roseum* "Graminearum": *trans*-α-zearalenol (Hagler et al., 1979; Watson et al., 1982), diastereomers of 8'-hydroxyzearalenone (Jackson et al., 1974; Stipanovic and Schroeder, 1975), diastereomers of 3'-hydroxyzearalenone (Pathre et al., 1980), 6',8'-dihydroxyzearalene (Steele et al., 1976), 5-formylzearalenone, and 7'-dehydrozearalenone (von Bolliger and Tamm, 1972). α-Zearalenol is estrogenic, having with three to four times more biological activity than zearalenone (Peters, 1972; Hagler et al., 1979).

Other derivatives of zearalenone have been chemically synthesized and some of these derivatives have been tested for use in animal nutrition as growth promoters (Hidy et al., 1977). Sodium borohydride reduction of zearalenone yields a diastereomeric mixture of α- and β-zearalenol. β-Zearalenol was somewhat less active estrogenically than zearalenone and the diastereomers of zearalenol were determined to have little anabolic activity when compared to their estrogenic effect (Hidy et al., 1977; Mirocha et al.,

Mycotoxin Laboratory, Department of Poultry Science, North Carolina State University, Raleigh, North Carolina 27695-7608 (K.E.R. and W.M.H.), and Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota (C.J.M.).

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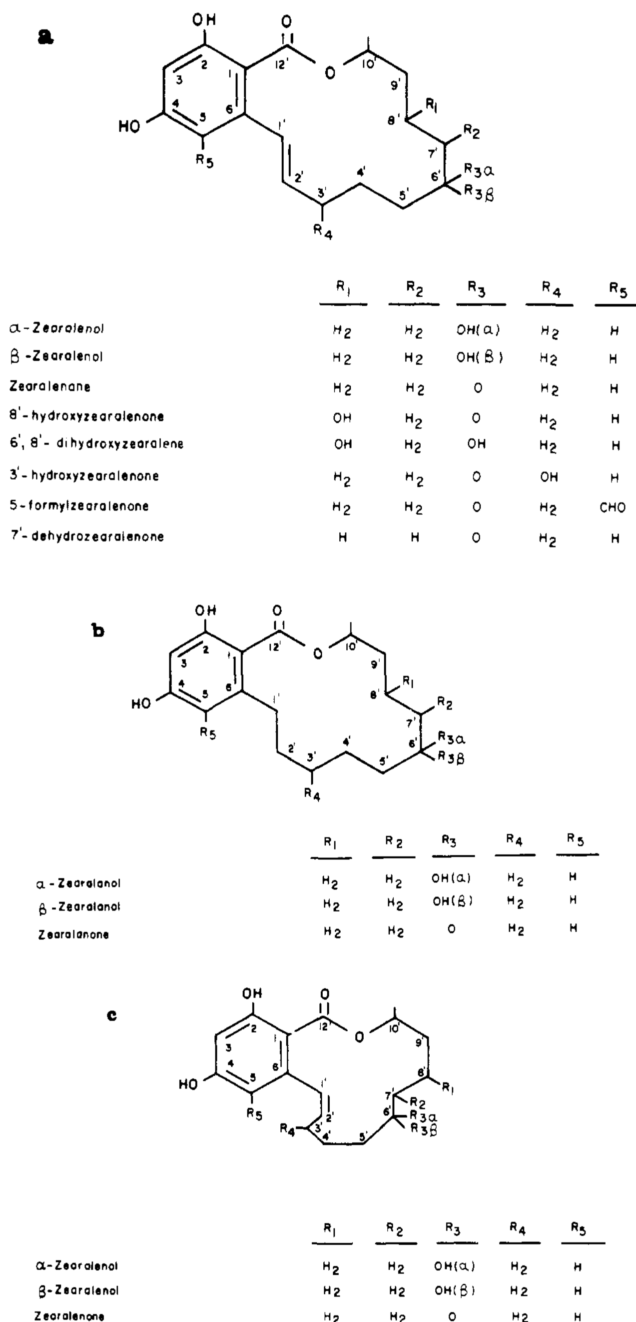

	R_1	R_2	R_3	R_4	R_5
α-Zearalenol	H_2	H_2	OH(α)	H_2	H
β-Zearalenol	H_2	H_2	OH(β)	H_2	H
Zearalenone	H_2	H_2	O	H_2	H
8'-hydroxyzearalenone	OH	H_2	O	H_2	H
6', 8'- dihydroxyzearalene	OH	H_2	OH	H_2	H
3'-hydroxyzearalenone	H_2	H_2	O	OH	H
5-formylzearalenone	H_2	H_2	O	H_2	CHO
7'-dehydrozearalenone	H	H	O	H_2	H

	R_1	R_2	R_3	R_4	R_5
α-Zearalanol	H_2	H_2	OH(α)	H_2	H
β-Zearalanol	H_2	H_2	OH(β)	H_2	H
Zearalanone	H_2	H_2	O	H_2	H

	R_1	R_2	R_3	R_4	R_5
α-Zearalenol	H_2	H_2	OH(α)	H_2	H
β-Zearalenol	H_2	H_2	OH(β)	H_2	H
Zearalenone	H_2	H_2	O	H_2	H

Figure 1. (a) *trans*-Zearalenone derivatives. (b) Zearalanone derivatives. (c) *cis*-Zearalenone derivatives.

1978). The catalytic reduction of zearalenone yields a pair of diastereomers (Figure 1 part b) of which one isomer, α-zearalanol, is preferred for use as an anabolic agent in sheep and currently marketed under the trade name RALGRO (Hidy et al., 1977). Zearalenone and β-zearalanol have less anabolic activity than α-zearalanol and the two reduction products are less estrogenic than zearalenone (Hidy et al., 1977). Zearalenone, as well as zearalenol, can also be changed from the trans configuration to the cis by irradiation with ultraviolet light (Figure 1 part c) (Peters, 1972; Mirocha and Christensen, 1974). Recent studies have indicated that the cis configuration is biologically stable and that the biological activity of the cis isomers, as measured by the rat uterotropic response, is greater than the trans isomers (Mirocha et al., 1978).

The present communication reports the identification and mass spectral confirmation of six additional naturally produced derivatives of zearalenone produced by *Fusarium* species that heretofore have only been chemically produced. They are *cis*-zearalenone, diastereomers of *cis*-zearalenol, diastereomers of zearalanol, and *trans*-β-zearalenol.

MATERIALS AND METHODS

Chemicals. Solvents and other chemicals were reagent grade (Fisher Scientific, Raleigh, NC) unless otherwise noted. Crystalline zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol were provided by IMC, Inc. (Terre Haute, IN).

Culture Maintenance. The seven strains of zearalenone-producing *Fusarium* examined for the production of detectable amounts of zearalenol and zearalanol were isolated from finished feeds or forage plants (clover or alfalfa). *F. equiseti*, *F. oxysporum*, and *F. culmorum* were plant lesion isolates from Nevada (Dr. Ted Knous, Department of Plant Science, University of Nevada at Reno, Reno, NV), while *F. solani* and *F. graminearum* (CLC 11) were plant lesion isolates from North Carolina (Dr. C. Lee Campbell, Department of Plant Pathology, NCSU, Raleigh, NC). *F. graminearum* (PI 4) was isolated from a mixed feed sample in North Carolina and *F. roseum* "Gibbosum" was a strain isolated from Midwestern corn. The *Fusarium* isolates were maintained on moist, autoclaved soil at 10 °C. Soil cultures were sprinkled on potato-dextrose agar plates and incubated at 25 °C for 5 days to provide inoculum for the experiment.

Culture Conditions. Sterile, moist rice (5 g plus 3 mL of deionized water in 20-mL screw cap vials) was seeded with blocks of agar (0.5 cm^2) covered with mycelium. The cultures were incubated for 28 days at 25 °C prior to extraction.

Extraction of Cultures. After incubation, 10 mL of MeOH were added to the cultures which were broken into small fragments with a clean spatula, shaken, and left to stand tightly capped overnight prior to decantation. The extraction was repeated. The initial extract was filtered through paper and evaporated to dryness, and the second extract was combined with it the next day. The residue was then redissolved in 50 mL of MeOH:5% NaCl (1:1, v/v) and transferred to a 250-mL separatory funnel where it was partitioned with three 50-mL portions of CH_2Cl_2. The CH_2Cl_2 phases were passed individually through a 10-g silica gel column (Silica Gel 60, 70-230 ASTM, E. Merck, Rahway, NJ) which had been slurry packed in $CHCl_3$. Column effluent was collected in a 250-mL beaker and the solvent was evaporated with gentle heat. The residue was transferred to a 20-mL vial with the aid of $CHCl_3$ which was then removed under N_2. Completeness of recovery from the silica gel columns was ascertained by thin-layer chromatography (TLC) analysis of rinses of the column with acetone and methanol.

Thin-Layer Chromatography. Culture extracts were analyzed by redissolving in 0.5 mL of acetone and spotting an aliquot (2–10 μL) on TLC plates of silica gel 60 (E. Merck, Darmstadt, FRG) along with toxin standards. Plates were developed in unlined, unequilibrated tanks containing 100 mL of benzene–acetone (3:2, v/v) or chloroform–methanol (97:3, v/v). Zearalenone and zearalenol on the plates were detected under ultraviolet (365 and 254 nm) light. Detection of zearalanol and confirmation of zearalenone and zearalenol were accomplished by spraying the developed plates with Fast Violet B (Sigma Chemical Co., St. Louis, MO) (Scott et al., 1978) or 4-methoxybenzenediazonium fluoborate (Reanal, Budapest, Hungary) (Sarudi, 1976) before heating at 110 °C for 5 min. Culture extracts were analyzed by reverse-phase TLC by evaporating the acetone under a gentle stream of N_2 and redissolving the extract in 0.5 mL of MeOH. Aliquots (4–10

Table I. R_f Ratios of Zearalenone Derivatives by Normal-Phase TLC

compound	R_f in the solvent system (v/v) benzene-acetone (3:2)	chloroform-methanol (97:3)
zearalenone	0.92	0.61
α-zearalenol	0.73	0.27
β-zearalenol	0.71	0.24
α-zearalanol	0.72	0.28
β-zearalanol	0.74	0.30

μL) of each extract were spotted on 200-μm $KC_{18}F$ reverse-phase TLC plates (Whatman, Inc., Clifton, NJ) along with toxin standards. The plates were developed in an unlined, unequilibrated tank containing 100 mL of CH_3CN-7.5% NaCl-MeOH (6:4:1, v/v). The developed plates were examined under ultraviolet light prior to confirmation with the colorimetric spray reagents.

Gas Chromatography. Toxin quantitation and additional confirmation were accomplished by GC analysis. GC (Model GC-6AM, Shimadzu Scientific Co., Baltimore, MD) detection utilized flame ionization and either N_2 or He as the carrier gas at 90 mL/min. The oxyhydrogen flame in the detector was fueled with air (1000 mL/min) and H_2 (70 mL/min). The detector and injector were operated at 300 °C. The oven was programmed from 150–260 °C at 6 °C/min and the final temperature was held for an additional 5 min. Data from the analyses were collected on an integrating recorder (Model CR-1A, Shimadzu Scientific Co., Baltimore, MD). The column (3 mm i.d., 2 m in length) was packed with 3% QF-1 on 100–120 mesh GasChromQ (Applied Science, State College, PA). The samples and extracts were derivatized after removing solvent from subsamples and treating with 50–100 μL TriSil BT (Pierce Co., Rockford, IL) for 0.5 h at 25 °C in 2-mL vials fitted with teflon-lined caps. Volumes of 1–2 μL were injected onto the columns.

Capillary Gas Chromatography/Mass Spectrometry. Mass spectra of trimethylsilyl derivatives of α- and β-zearalanol, *cis*- and *trans*-zearalenone, *cis*- and *trans*-α-zearalenol, and *cis*- and *trans*-β-zearalenol in culture extracts were obtained by combined capillary gas chromatography and mass spectroscopy on a computerized Hewlett Packard 5987 instrument (Hewlett Packard, Palo Alto, CA) at the 70-eV electron impact mode. Analysis were performed on a 30-M narrow bore DB-5 fused silica column (J & W Scientific Inc., Rancho Cordova, CA) utilizing a splitless injection mode. The temperatures of the injector port and detector were 250 °C and 270 °C, respectively; column temperature was programmed from 80 °C to 300 °C at 25 °C/min. The carrier gas was He at 1 mL/min. The instrument was tuned with perfluorotributylamine according to the manufacturer's specifications. Chromatograms were produced by the hard copy unit of the computer system.

RESULTS AND DISCUSSION

Normal-phase thin-layer chromatography utilizing the solvent system, benzene–acetone (3:2, v/v), produced sharp bands for zearalenone, zearalenol, and zearalanol standards (Table I) when observed under ultraviolet light and sprayed with the colorimetric reagents. However, separation between the α- and β-isomers of zearalenol and zearalanol were not observed when culture extracts were examined. The separation of these zearalenone derivatives was enhanced by utilizing the reverse-phase solvent system, CH_3 CN-7.5% NaCl-MeOH (6:4:1, v/v), and separated α- and β-zearalenol by 0.5–0.8 cm when the plate was developed three-quarters of the height (Table II). Separation of α-zearalenol from α-zearalanol and β-zearalenol from β-zearalanol was not as efficient, however, partial separation in culture extracts was observed.

Table II. R_f Ratios of Zearalenone Derivatives by Reverse-Phase TLC

compound	R_f in the solvent system (v/v) MeOH-5% NaCl (7:3)	MeOH-5% NaCl (4:1)	CH_3CN-7.5% NaCl-MeOH (6:4:1)
zearalenone	0.62	0.49	0.34
α-zearalenol	0.67	0.50	0.38
β-zearalenol	0.70	0.58	0.45
α-zearalanol	0.64	0.53	0.41
β-zearalanol	0.70	0.60	0.45

Table III. Retention Times of Trimethylsilyl Derivatives of Mycotoxins by GLC

compound	retention time (min) on the column QF-1	DB-5
trans-zearalenone	18.70	14.12
cis-zearalenone		13.29
trans-α-zearalenol	16.46	14.16
cis-α-zearalenol		13.02
trans-β-zearalenol	16.79	14.32
cis-β-zearalenol		13.17
α-zearalanol	15.78[a]	13.42
β-zearalanol	15.78[a]	13.72

[a] Compounds would not separate upon coinjection.

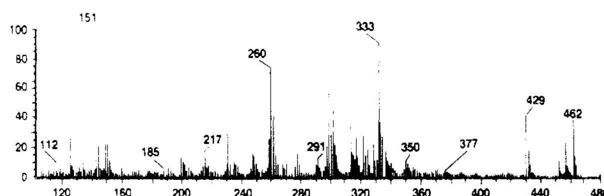

Figure 2. Mass spectrum of the trimethylsilyl (Me_3Si) derivative of zearalenone produced by *F. solani*.

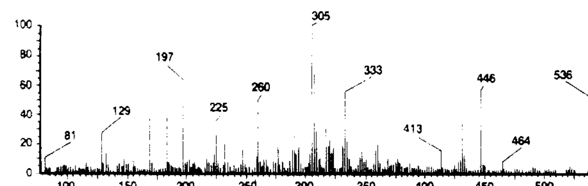

Figure 3. Mass spectrum of the trimethylsilyl (Me_3Si) derivative of zearalenol produced by *F. equiseti*.

The colorimetric spray reagent, 4-methoxybenzenediazonium fluoborate, stained zearalenone, zearalenol, and zearalanol a reddish brown color (brick red), but identification of zearalanol was aided when Fast Violet B spray reagent was used. This spray reagent stained zearalenone and zearalenol (α- and β-isomers) pink to red in color, while zeralanol (α- and β-isomers) was observed to stain a dark red color, almost reddish brown.

The results obtained with the mycotoxin standards on the GC system are given in Table III. The useful limit of detection by GC for standards was ca 0.1 μg. Separation between zearalenone, α-zearalenol, and β-zearalenol was observed with mycotoxin standards, unfortunately GC analysis of the culture extracts under these conditions did not allow for base line separation between the isomers of zearalenol. The isomers of zearalanol could not be separated even with the mycotoxin standards. Capillary GC/MS retention times of mycotoxin standards are also presented in Table III. Capillary GC, under the conditions

Table IV. Comparison of Mycotoxin Production by *Fusarium* Isolates on Rice Culture Determined by Thin-Layer and Gas-Liquid Chromatography[a]

isolate	toxin production, μg/g of rice							
	zearalenone		α-zearalenol		β-zearalenol			
	trans	cis	trans	cis	trans	cis	α-zearalanol	β-zearalanol
Fusarium roseum "*Gibbosum*"	2710.0	0[b]	91.65	0	18.33	0	0	0
F. equiseti	950.63	38.80	24.25	2.43	9.70	0	1.37	7.20
F. culmorum	69.69	4.59	2.30	0	0.38	0	9.07	3.40
F. oxysporum	12.80	0	1.60	0	1.60	0	0	0
F. graminearum (CLC 11)	85.66	4.57	1.14	0	0.29	0	1.71	1.14
F. solani	7.97	1.33	1.60	0	4.60	0	0	0
F. graminearum (PI 4)	46.40	7.25	0.73	0	52.20	0.58	0.31	6.52

[a] Quantitation of zearalenone, α-zearalenol, and β-zearalenol were determined by packed column and capillary GC. [b] Amounts of zearalenone derivatives were below the detectable limits of ca. 0.1 μg/g.

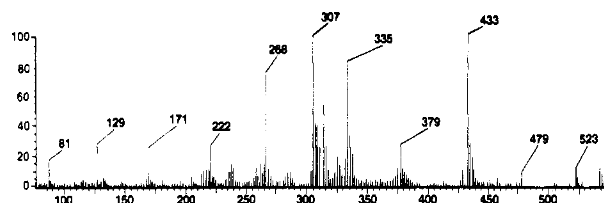

Figure 4. Mass spectrum of the trimethylsilyl (Me_3Si) derivative of zearalanol produced by *F. graminearum* (PI 4).

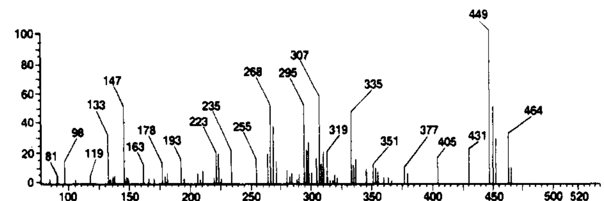

Figure 5. Mass spectrum of the trimethylsilyl (Me_3Si) derivative of zearalanone produced by *F. reticulatum*.

mentioned, allowed separation of the mycotoxin standards and the zearalenone derivatives in culture extracts. Typical mass spectra confirming the identity of zearalenone, zearalenol, and zearalanol are shown in Figures 2–4.

The seven *Fusarium* isolates produced *trans*-zearalenone and *trans*-α- and β-zearalenol (Table IV). The production of *trans*-α- and β-zearalenol by isolates of *F. equiseti* and *F. culmorum* has recently been observed by Bottalico et al. (1985). Small amounts of *cis*-zearalenone were found in culture extracts of five of these isolates and only two isolates accumulated detectable *cis*-α-zearalenol (*F. equiseti*) or *cis*-β-zearalenol (*F. graminearum* PI 4). Four isolates were observed to produce α- and β-zearalanol. An isolate of *F. reticulatum* grown on vermiculite media (Richardson et al., 1984) was also observed to produce all of these zearalenone derivatives (data not shown) as well as zearalanone (Figure 5).

The identification and mass spectral confirmation of six additional naturally produced derivatives of zearalenone in rice cultures increases the number of derivatives to fifteen, including the isomers of zearalenone. The newly identified derivatives have previously been synthetically produced from zearalenone and demonstrated to exhibit some anabolic, uterotropic, or estrogenic effects in animals.

When feedstuffs associated with hyperestrogenism in animals are analyzed, most times zearalenone or diethylstilbesterol is detected in significant amounts. However, in some cases no detectable amounts of these estrogens are found and the cause of hyperestrogenism remains unknown. The presence of *cis*-zearalenone, *cis*- and *trans*-α-zearalenol, and *cis*- and *trans*-β-zearalenol in cultures of *Fusarium* is important because it suggests the possibility of occurrence of these derivatives in animal feedstuffs. Recently, Mirocha et al. (1979) detected the presence of *trans*-α-zearalenol in significant amounts (0.15–4.0 μg/g) along with zearalenone in *Fusarium*-infested corn and oats. Zearalenol (α- and β-isomers) has also been found in corn stems infected by *Fusaria* in the field in lower amounts (56 ng of α-zearalenol/g and 27 ng of β-zearalenol/g of stalk; Bottalico et al., 1985). Since *trans*-α-zearalenol is three to four times more estrogenic than zearalenone and β-zearalenol has the same or slightly less activity as zearalenone, the concentrations of these derivatives in the grain samples and corn stalks could contribute to the overall estrogenic activity if fed to farm animals. Most of the derivatives identified in this study have also been shown to be more estrogenic than zearalenone (Peters, 1972; Mirocha et al., 1978) and could contribute to the total estrogenic activity of *Fusarium*-infested cereal grains. There is also the possibility that some isolates may produce higher amounts of these derivatives than found in this study and that other isolates may produce one derivative exclusively. This suggests the need for development of an analytical method to detect the presence of these derivatives as well as zearalenone in feedstuffs.

The accumulation of the diastereomers of zearalanol by *Fusarium* under certain conditions may also occur in the field or in storage. Although the estrogenic activities of these diastereomers are significant; α-zearalanol has anabolic activity and is used as a growth promoter in cattle and sheep. Since zearalanol, zearalenone, and zearalenol can be produced by a *Fusarium* isolate in culture, the possible interactions among these derivatives in susceptible animals requires further study.

ACKNOWLEDGMENT

We thank J. E. Hutchins for his contributions. Fungi were identified by Dr. Paul Nelson of the *Fusarium* Research Center, Pennsylvania State University, University Park, PA.

Registry No. *trans*-zearalenone, 17924-92-4; *cis*-zearalenone, 36455-70-6; *trans*-α-zearalenol, 36455-72-8; *cis*-α-zearalenol, 36455-71-7; *trans*-β-zearalenol, 71030-11-0; *cis*-β-zearalenol, 38055-76-4; α-zearalanol, 26538-44-3; β-zearalanol, 42422-68-4.

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Use of Growth Regulators to Control Senescence of Wheat at Different Temperatures during Grain Development

Kassim Al-Khatib and Gary M. Paulsen*

Wheat (*Triticum aestivum* L.) was grown under greenhouse and field conditions to study efficacy of growth regulators in mitigating senescence and increasing grain yields. L-Serine, 6-benzyladenine, cycloheximide, and terbacil (3-*tert*-butyl-5-chloro-6-methyluracil) were applied on plants grown under 21 °C/16 °C, 26 °C/21 °C, and 31 °C/26 °C day/night regimes during grain development and under field conditions. Low concentrations of growth regulators had little effect on senescence processes, whereas high concentrations accentuated high temperature induced senescence. Benzyladenine and terbacil increased protease activity and decreased leaf area duration, protein concentration, kernel size, and yields. None of the growth regulators increased yields under field conditions; serine had no effect, and rates above 5 g ha^{-1} of benzyladenine, 50 g ha^{-1} of cycloheximide, and 125 g ha^{-1} of terbacil decreased yields. We concluded that plant growth regulators probably affect some senescence processes differently under induced and natural conditions and that use of the materials tested to control senescence under field conditions may not be feasible.

INTRODUCTION

The positive relationship between photosynthetic leaf area during grain filling and wheat (*Triticum aestivum* L.) yield is well documented (Fischer and Kohn, 1966; Spiertz et al., 1971). It suggests that plant photosynthetic activity during grain filling limits yields and that photosynthesis might be increased by delaying the rapid senescence that occurs after anthesis (Fischer and Kohn, 1966). Senescence is characterized by de novo synthesis of protease enzymes, degradation of photosynthetic processes, loss of leaf viability, and eventual cessation of grain development (Thomas and Stoddart, 1980).

Environmental stresses, particularly drought and high temperatures, accelerate most leaf senescence processes (Thomas and Stoddart, 1980). In wheat, for instance, high temperatures after anthesis accentuate the normal increase in protease enzyme activity and the decrease in ribulose-1,5-biphosphate carboxylase and Hill reaction activities (Al-Khatib and Paulsen, 1984). Leaf area and grain filling durations are also shortened, causing grain yields to decline markedly.

Cytokinins, cycloheximide, and other growth regulators delay induced senescence of many plant species (Martin and Thimann, 1972a, 1972b; Peterson and Huffaker, 1975; Ries, 1976). In detached leaves and seedlings placed in the dark, these chemicals inhibit de novo protease synthesis (Martin and Thimann, 1972a), retard chlorophyll loss (Peterson and Huffaker, 1975), increase nitrate reductase activity (Ries, 1976), maintain synthesis of fraction I protein (Butler and Simon, 1971), and preserve membrane function (Beutelmann and Kende, 1977). L-Serine, on the other hand, specifically enhances leaf senescence in darkness, possibly via its incorporation into the active site of protease enzyme (Martin and Thimann, 1972a, 1972b; Shibaoka and Thimann, 1970).

The importance of senescence to grain yield of wheat and the known ability of specific growth regulators to delay induced senescence prompted investigations of efficacy of the chemicals on intact plants. Experiments were conducted under controlled conditions with three temperature

Department of Agronomy, Kansas State University, Manhattan, Kansas 66506.

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