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THE STEROLS OF NORMAL AND MALE-STERILE MAIZE TASSELS DURING DEVELOPMENT

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Key Word Index—*Zea mays*; Gramineae; maize; corn; male-sterile maize tassels; maize tassel developmental stages; estrogen bioassay; phytosterols; cholesterol-[4-^{14}C].

Abstract—The steroids of normal and male-sterile (Texas type) genotypes of maize were investigated during tassel development. A bioassay for estrogen activity of the normal meiotic and postmeiotic tassels was negative, indicating estrogen activity (estrone equivalent) much less than one ng/g of plant tissue. The sterols found were cholesterol, campesterol, stigmasterol, sitosterol, and probably isofucosterol, stigmast-7-enol, and 24-methylenecholesterol. In the premeiotic, meiotic, and postmeiotic stages of both genotypes between 300 and 400 μg of C_{28} and C_{29} free sterols per g tassels (wet wt) were found, the proportions of the sterols being *ca* 45% sitosterol, 30% stigmasterol, and 13% campesterol, with less than 5% each of the remaining sterols. In all three stages before saponification more free sterols were found in the normal than in the male-sterile tassels. The differences were significant at the 95% level in the meiotic and post-meiotic stages. The amounts of these sterols derived from esters decreased from approximately 140 μg/g in the premeiotic stage to 50 μg/g in the meiotic stage, and to an undetectable amount in the postmeiotic stage. After application of cholesterol-[4-^{14}C] to the normal and male-sterile maize leaves for 3 days at meiosis, the label was found in the free sterols and steryl esters of the leaves but only in the free sterols of the tassels.

INTRODUCTION

Pollen sterility occurs in many plant species and is widely utilized in plant breeding techniques for the production of hybrid varieties. In some cases the sterility is inherited with the nuclear genes such as in genetic male-sterile maize and in others, such as cytoplasmic male-sterile maize, it appears to result from the interaction of a factor in the cytoplasm with nuclear genes.

Cytological studies of three genetic male-sterile maize strains show that pollen breakdown occurs between 5 and 10 days after meiosis [1]. The molecular basis for the male sterility condition is not known. Anthers of male-sterile wheat plants contain an increased level of free amino acids over that found in male-fertile plants [2]. A similar pattern of free amino acids is found in the anthers of other male-sterile plant species [3], and in male-sterile maize tassels after anthesis [4]. The increase in free amino acids in the male-sterile anthers could be the result of the degradation of protein, a lowered level of protein synthesis for reasons of enzyme repression or absence of induction, or possibly defective transport because of modified membrane structures. Since steroid hormones have been shown to induce or regulate protein synthesis in a wide range of vertebrates, insects, and fungi, a study was initiated of the steroids at the time of meiosis in the tassels of normal (fertile) and male-sterile (Texas type cytoplasm) maize (*Zea mays*) plants. Three approaches were used: (1) a bioassay for estrogen activity, (2) a qualitative identification of the sterols and a quantitative estimation of the sterol composition, and (3) radioactive tracer studies with cholesterol-[4-^{14}C].

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RESULTS AND DISCUSSION

Bioassay for estrogen activity

The bioassay for estrogen activity of the normal meiotic and post-meiotic tassel total ether-soluble lipids, base-soluble lipids, and ether extracts of water-soluble lipids after acid hydrolysis, yielded no positive results on mice which gave positive results with 800 pg estrone. This indicates an estrogen activity (estrone equivalent) much less than one ng/g of plant tissue. Since positive results were not obtained for the normal tassel tissue, in which steroid hormones could be expected to occur, the male-sterile tassel tissue was not assayed. Estrone was isolated from the pollen of the date palm [5] and confirmed [6]; the androgens, testosterone, epitestosterone, and androstenedione were reported in the pollen of the Scotch pine, *Pinus silvestris* [7]. Estrogenic activity was found in the oil extracted from *Zea mays* seeds [8].

Qualitative identification of the sterols

GLC retention times of the free sterols on 3% OV-17 and 3% JXR columns relative to sitosterol were determined and the major peaks were found to correspond to campesterol, stigmasterol, and sitosterol. A minor peak with a retention time longer than that of sitosterol on the OV-17 column was probably due to a mixture of isofucosterol and stigmast-7-enol which were

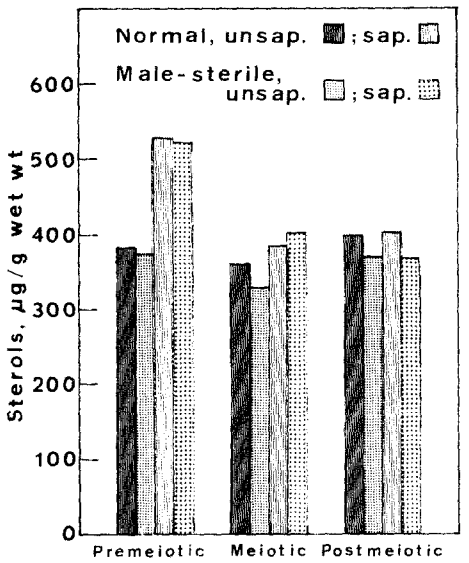

Fig. 1. The total C_{28} and C_{29} sterols from maize tassel ether-soluble lipids. unsap. = unsaponified, sap. = saponified.

reported [9] in maize oil. The retention times of the TMS derivatives of the sterols on a 3% OV-17 column relative to sitosterol were also determined and found to correspond with those of the TMS derivatives of cholesterol, campesterol, stigmasterol and sitosterol.

MS of the free sterols from the saponified ether-soluble lipids of the normal postmeiotic tassel tissue confirmed the presence of campesterol, stigmasterol, and sitosterol. The MS of the minor peak appeared to show a mixture of isofucosterol and stigmast-7-enol. Analysis of the MS fragmentation patterns of the TMS derivatives of the male-sterile tassel ether-soluble lipid supernatant, saponified before digitonin precipitation, showed the presence of hydrocarbons, fatty acids, and sterols. The sterols found were cholesterol, campesterol, stigmasterol, sitosterol, and probably isofucosterol. The fragmentation pattern of the latter peak corresponded very closely to that reported for the TMS derivative of isofucosterol [10]. Campesterol, stigmasterol, and sitosterol [11] and cholesterol [12] have been reported to occur in maize shoots. Isofucosterol, in addition to the other sterols, has been reported [13] in the leaves and roots of maize.

Barbier [14] reported the sterols of maize pollen to be 59% 24-methylenecholesterol, 17% sitosterol, 12% campesterol, and 12% stigmasterol. Argentation TLC indicated 24-methylenecholesterol may be present in the developing tassel but in relatively small amounts. The proportions of sterols found in the tassels were approximately 45% sitosterol, 30% stigmasterol, and 13% campesterol, with less than 5% each of the remaining sterols, cholesterol, isofucosterol, stigmast-7-enol, and 24-methylenecholesterol.

Quantitative estimation of sterol composition

The results of the quantitative study of the sum of the C_{28} and C_{29} sterols by GLC are shown in Fig. 1. The greatest change found during tassel development was in the amounts of esterified sterols. In the premeiotic stage 142 µg sterols derived from esters per g normal tassels (wet wt) and 146 µg/g from the male-sterile tassels were found. In the meiotic stage 24.3 µg/g (normal) and 73.0 µg/g (male-sterile), and in the postmeiotic stage undetectable amounts in both normal and male-sterile plants were found. The amounts of free sterols before saponification were slightly greater in the normal premeiotic, meiotic, and post meiotic tassels than in the corresponding stages of the male-sterile tassels. The difference was significant at the 95% level for the latter two stages.

Radioactive tracer studies using cholesterol-[4-^{14}C]

The specific activities of the ether-soluble and water-soluble lipids of the maize tissues after exposure to cholesterol-[4-^{14}C] are reported in Table 1. After the 3-day exposure most of the radioactivity was found in the ether-soluble lipids of all of the tissues, and after the 17-day exposure most of the radioactivity was found in the water-soluble lipids of the tassels. After the 3-day exposure more radioactivity was found in the water-soluble lipids of the normal than the male-sterile tassels and stems and petioles. After both exposure times the ratio

Table 2. Silica gel column chromatography of the leaf ether-soluble lipids recovered from plants after application of cholesterol-[4-^{14}C]

Fraction number	Band color	% total dpm N*	MS†
1	colorless	0	0
2	yellow	0.15	0
3	dark yellow	1.50	0.08
4	yellow	10.54	10.10
5	pale yellow	1.02	0.48
6	colorless	0.76	0.45
7	yellow-green	3.71	2.16
8	dark green	61.87	60.69
9	yellow	3.46	3.68
10	pale yellow	0.39	0.29
11	dark yellow	3.95	5.49
12	pale yellow	0.78	0.68
13	yellow	11.87	15.89
Total dpm eluted		4.38×10^5	4.68×10^5

* N = Normal; † MS = Male-sterile.

Table 1. Specific radioactivity of maize tissue lipids after application of cholesterol-[4-^{14}C] (dpm/g wet wt)

	Exposure to cholesterol-[4-^{14}C]							
			3 Days				17 Days	
	Leaves		Stems and petioles		Tassels		Tassels	
Lipids	N*	MS†	N	MS	N	MS	N	MS
Water-soluble	4.45×10^4	5.25×10^4	131	28	112	66	124	132
Ether-soluble	1.41×10^5	1.42×10^5	767	669	259	292	44	83

* N = Normal; † MS = Male-sterile.

of radioactivity of ether-soluble to water-soluble lipids of the male-sterile tassels was almost twice that of the normal tassels.

The results of chromatography of the normal and male-sterile leaf ether-soluble lipids are shown in Table 2. The greatest difference in radioactivity between the normal and male-sterile extracts was found in fractions 11 and 13, with more radioactivity present in the male-sterile fractions. These fractions could contain metabolites of the labelled cholesterol or the products of its photolytic decomposition. Since photolytic decomposition would be expected to be the same on normal and male-sterile leaves, the differences between the normal and male-sterile fractions are more readily accounted for by differences in the metabolism of cholesterol-[4-^{14}C].

TLC of fractions 11 and 13 (Table 2) in solvent system L, and fractions 4 and 8 in solvent systems F and G, respectively, did not disclose any qualitative differences between the normal and male-sterile extracts. Cholesterol-[4-^{14}C] was found to co-chromatograph with the major peak of radioactivity of fraction 8 in solvent system G (Fig. 2). Fraction 8 was further fractionated by PLC in solvent system G. The regions of greatest radioactivity (Fig. 2) were collected and rechromatographed in solvent systems J (R_f 0.11); H, K, and J (R_f 0.32); H and I (R_f 0.54); and H (R_f 0.75). No qualitative differences between the normal and male-sterile extracts were apparent. The radioactivity at R_f 0.32 was found to co-chromatograph with labelled cholesterol in all three solvent systems. Fraction 4 (Table 2), which was the only material less polar than fraction 8 with a significant amount of radioactivity, probably contained the esters of the labelled cholesterol or its metabolites. TLC of this fraction in solvent system F also revealed most of the radioactivity (R_f 0.5–0.7) to be less polar than labelled cholesterol (R_f 0.04).

Autoradiography of a thin-layer chromatogram developed in solvent system G of the normal and male-sterile leaf ether-soluble lipids indicated that most of the radioactivity co-chromatographed with labelled cholesterol between R_f 0.6 and 0.7. Radioactivity between R_f 0.9 and 1.0 became very faint after saponification verifying the formation of labelled esters in the leaves. Two fractions were found with radioactivity more polar than the labelled cholesterol in agreement with the chromatography data (Table 2).

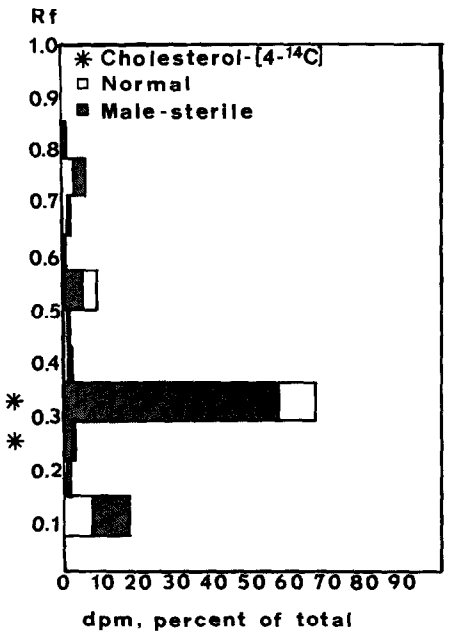

Fig. 2. TLC of fraction 8 recovered from column chromatography (see Table 2). The plate was developed with solvent system G.

Autoradiograms of unsaponified normal and male-sterile tassel ether-soluble lipids developed with solvent system G showed most of the radioactivity in the same region (R_f 0.6–0.7) as the labelled cholesterol. No less polar bands were found but at least three faint bands more polar than the labelled cholesterol were visible. These results indicate that steryl esters formed from the labelled cholesterol or its metabolites were not present in significant amounts in the tassels as a result of either translocation or biosynthesis. Although it is possible that metabolites of radioactive cholesterol could be translocated from the leaves to the tassels, it is also possible that the polar bands represent the conversion of labelled cholesterol to compounds with shorter side chains and/or additional oxygen functions. If the tassels are the site of hormone biosynthesis, such transformations would be expected.

EXPERIMENTAL

Preparation of materials. Zea mays L. seed of the genotypes ND405 × NDB8, fertile, and ND405T × NDB8, male-sterile with Texas type cytoplasm, were provided and planted by the Agronomy Department at North Dakota State University. All plants were harvested within a period of 8–10 days. Tassels were dissected from the stalks, and segregated according to developmental stage (premeiotic, meiotic, or postmeiotic) by external appearance of the tassels and microscopic examination of the microsporocytes. The wet wts were determined and the tissues stored under N_2 in the freezer. Lipids of the maize tissues were extracted after homogenization with $CHCl_3$–MeOH (2:1) using a final solvent vol 20× the tissue volume, assuming a tissue density of 1 g/ml [15]. Filtrate was evaporated under vacuum to a few ml and the resulting thick syrup was partitioned between Et_2O and H_2O. The crude ether-soluble and water-soluble lipid fractions were each studied further. For the qualitative GLC analysis of the sterols, the ether-soluble lipids were saponified under reflux [16]. For the quantitative analysis by GLC and for the studies with radio-labelled cholesterol, the saponification was done by the same method only under N_2 in the cold with stirring.

Bioassay for estrogen activity. Bioassays were conducted on the crude ether-soluble lipids, on the base-soluble portion of the crude ether-soluble lipids [17] and on the ether-soluble material produced by hydrolysis of the crude water-soluble lipids by refluxing with 2 M HCl in MeOH (20% H_2O). The Et_2O extraction was performed after evaporation of the MeOH and neutralization of the hydrolysate to pH 7. The bioassay for estrogen activity was done by the mouse intravaginal assay [18]. The vaginal smears were examined to determine whether an estrous response had occurred, the presence or absence of leucocytes in a vaginal smear indicating a negative or positive response, respectively [19]. A positive response by the mice was brought about by a total intravaginal application of 800 pg estrone (4 applications over 2 days) with examination in the morning of the third day.

Preparative and analytical TLC. TLC was on Si gel 1B (J. T. Baker Chemical Co.), Si gel G, Polygram Sil G (Brinkmann Instruments, Inc.), and 20% $AgNO_3$–Si gel G. The solvent systems used were: A, C_6H_6–Et_2O (4:1), for separation of the steryl esters from the free sterols [13]; B, C_6H_{12}–EtOAc–NH_3 (100:100:1), for separation of sterols and fatty acids; C, C_6H_6–

Et_2O (7:3) for the supernatants of the sterol digitonides; D, C_6H_{12}–C_6H_6 (5:2) [20], and E, C_6H_{12}–C_6H_6 (5:3) [21], for the steryl acetates; and F, $CHCl_3$–C_6H_6 (1:1), G, $CHCl_3$–EtOAc (9:1), H, C_6H_{12}–EtOAc (1:1), I, C_6H_6–MeOH (9:1), J, $CHCl_3$–MeOH (9:1), K, C_6H_6–MeOH (4:1), and L, $CHCl_3$–MeOH (1:1) for TLC of radio-labelled cholesterol. Visualization was by spraying the plate (or the edges for PLC) with H_2SO_4–MeOH (1:1) and heating at 110° for 5 min [22]. Sterol fractions were eluted from the preparative plates with MeOH.

Sterol derivative preparations. The saponified ether-soluble lipids were treated with digitonin [23] to ppt. the more abundant sterols, thereby enriching the supernatant in the less abundant sterols. PLC of the supernatant employed solvent system C. TMS derivatives of the sterols were prepared using Regisil/TMCS/TSIM (3:2:3 by wt) in C_5H_5N at 75° for 30 min. The samples were taken up in Skellysolve B for GLC and GC–MS analysis. For argentation TLC, acetates were prepared [24] of the sterols from the ether-soluble lipids and of cholesterol, which was used as a reference compound.

Identification by GC–MS. GLC of the free sterols was done using a glass column (3.5 mm × 300 cm) of 3% OV-17 on Chromosorb W(HP), 80/100 mesh, with a 1:1 split of the effluent for collection. He was the carrier gas (155 ml/min) and the temp. was programmed at 1°/min between 235 and 270°. The R_t of sitosterol was 26 min. After collection of the main sterol peaks, the fractions were reinjected into the chromatograph and recollected for MS. GLC of the free sterols was also done using a glass column (2 mm × 300 cm) of 3% JXR on Gas Chrom Q, 100/120 mesh, gas flow 55 ml/min and temp. programming at 1°/min from 200 to 240°. The retention time of sitosterol was 32 min. GLC of sterol-TMS derivatives was on a glass column (2 mm × 300 cm) of 3% OV-17 on Chromosorb W(HP), 80/100 mesh, gas flow 55 ml/min, and temp. programming at 6°/min from 150 to 275°, yielding and R_t of 26 min for sitosterol-TMS, or at 10°/min from 150 to 275°, yielding an R_t of 19 min for sitosterol-TMS. GC–MS of the sterol-TMS derivatives prepared from the supernatant after digitonin ppt. used a glass column (2 mm × 150 cm) of 3% OV-17, gas flow 22 ml/min and temp. programming at 6.5°/min from 150 to 275°, yielding an R_t for sitosterol-TMS of 23 min.

Quantitative estimation of sterol composition. For the quantitative comparison of the free sterols by GLC, using the 3% OV-17 column first described, 5α-cholestane was added to the tassel tissue before extraction as an internal standard. The GLC response to 5α-cholestane was linear, and correction factors for quantitative measurements of the sterols were determined by simultaneous injection of 5α-cholestane and 5α-cholestanol, a homologue of the sterols [25].

Radioactive tracer studies. Cholesterol-[4-^{14}C] (3.3 μCi per plant) was applied [26,27] to the leaves of normal and male-sterile maize plants, and the plants were harvested at the end of meiosis, which lasted approximately 3 days, after a 3-day or a 17-day exposure to the labelled cholesterol. Radioactivities of the ether-soluble and water-soluble lipids were determined by liquid scintillation spectrometry using Instagel for the scintillator. An internal standard of toluene-[^{14}C] was used to determine the counting efficiency. Ether-soluble lipids of the leaves were fractionated by column chromatography with Si gel (7.5 g of 0.05–0.20 mm Si gel), applying the lipids in 0.5 ml $CHCl_3$ and developing the column with 20 ml each Skellysolve B and Skellysolve B–C_6H_6 (4:1), and 30 ml each Skellysolve B–C_6H_6 (1:2), Skellysolve B–$CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$–EtOAc (9:1), EtOAc, and MeOH. The radioactivity eluted was determined and the most radioactive fractions were analyzed further by TLC using solvent systems F, G, H, I, J, K, and L. Autoradiography [28] was done on thin-layer chromatograms of the leaf and tassel ether-soluble lipids. Polygram Sil G thin-layers were developed in solvent system G three times (leaves) or four times (tassels) and exposed to X-ray film for 11 or 25 days, respectively, in the freezer, using a Si gel dessicant.

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