TLC Screen for Maysin, Chlorogenic Acid, and Other Possible Resistance Factors to the Fall Armyworm and the Corn Earworm in *Zea mays*

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A thin-layer chromatographic method has been developed for the analysis of maysin, a flavonoid C-glycoside, and its analogues, which have been implicated as resistance factors for the corn earworm (*Helicoverpa zea*, formerly *Heliothis zea*) and the fall armyworm (*Spodoptera frugiperda*). Boric acid present on the TLC plate enhances the fluorescence of luteolins, including maysin, and increases sensitivity to these compounds by a factor of 10 or more. Another resistance factor, found in smaller amounts, chlorogenic acid, interferes with maysin unless the developing solvent is made basic with dilute ammonium hydroxide or the TLC plate is treated with boric acid. Leaves, the major food source of the fall armyworm on corn, were not as readily analyzed because of interference due to streaking of the TLC plate.

INTRODUCTION

The corn earworm and the fall armyworm are major corn pests in the southeastern United States. Both insects feed in the whorl of young corn plants. As the plant develops, the corn earworm concentrates its feeding on the silk and the ear. The fall armyworm damages all parts of the plant, but much of the damage arises from leaf-feeding. Two metabolites from corn silks that have been shown to be inhibitory to the corn earworm in bioassays are maysin, a flavonoid C-glycoside with a luteolin aglycon (Elliger et al., 1980a), and chlorogenic acid (Elliger et al., 1981). Wiseman et al. (1990) presented evidence that the resistance of centipede grass to the fall armyworm is due to the presence of chlorogenic acid, maysin, and other luteolins. Maysin was originally isolated from Zapalote Chico corn silks and identified by Elliger et al. (1980a). Snook et al. (1989) developed a high-performance liquid chromatography (HPLC) procedure to quantitate maysin and chlorogenic acid in corn silks. Prior to the development of the quantitative HPLC method for silks, extensive HPLC analyses of leaves of teosinte and many cultivars, inbreds, and populations of corn showed the presence of other luteolin derivatives as well as maysin along with chlorogenic acid and its isomers. Sufficient quantities of the other luteolins have not as yet been isolated for evaluation in bioassays.

Genetic studies aimed at defining the quantitative genetics involved in the biosynthesis of maysin require the analysis of thousands of samples, and for this task HPLC is most suitable. However, screening of a large number of plants for purposes of selection may not require the analytical detail that HPLC provides. Thus, it seemed desirable to develop a thin-layer chromatographic (TLC) method of maysin and chlorogenic acid analysis. While the TLC method would not be as quantitative as the HPLC procedure, it could serve as a rapid screen to identify plants containing genetically amplified levels of maysin and chlorogenic acid. The method could quickly identify promising germplasm and thus reduce or eliminate many of the more expensive and time-consuming HPLC analyses. We now report our findings regarding TLC analyses of corn leaves and silks for maysin, other luteolins, and chlorogenic acid as a rapid, inexpensive method of screening for insect inhibitory compounds in corn.

MATERIALS AND METHODS

Plant Materials. Corn leaf and silk samples were obtained in 1988 and 1989 from two locations: field-grown in Tifton, GA, and greenhouse-grown in Athens, GA.

Collection, Treatment, and Storage of Plant Material. Silks (2–5 g/ear) were collected from individual ears and placed in methanol (100 mL/silk) in wide-mouth jars, stored at 0 °C, but allowed to warm to room temperature just prior to sampling for HPLC or TLC analysis. For immediate analysis of smaller silk samples without storage, 0.5 g of silks was added to 10 mL of methanol. The mixture was ground and allowed to stand overnight or, alternatively, was ultrasonicated for 20 min. Leaves were collected at the 10 to 14 leaf stage and were lyophilized and ground in a Wiley mill before storage at −20 °C in glass or polyethylene bottles. The dried leaf materials were ultrasonicated in the extraction solvent (50:50 methanol:water) for 30 min and filtered to produce samples for HPLC.

High-Performance Liquid Chromatography (HPLC). Methanol used in extractions was Burdick and Jackson (Muskegon, MI) "distilled-in-glass" grade. Silk extracts, filtered through a nylon 66 membrane filter (Snook et al., 1989), were analyzed by reversed-phase HPLC performed with a Hewlett-Packard 1084B liquid chromatograph, on a 5-μm Altex Ultrasphere ODS column (4.6 mm i.d. × 25 cm). A diode array UV detector monitored samples at 340 nm. We used a linear gradient from methanol/water (20:80 v/v, solvent A) to MeOH (solvent B) in 40 min with a flow rate of 1 mL/min and a 12-min equilibration time. Both solvents contained 0.1% H_3PO_4. Leaf extracts were analyzed by reversed-phase HPLC using a Hewlett-Packard 1090M liquid chromatograph equipped with a 1040A diode array detector and ChemStation data collection system. A Nucleosil 5-μM ODS, 4.6 mm i.d. × 25 cm, column was used for the analysis. The dried leaf materials were ultrasonicated in the extraction solvent (50:50 methanol/water) for 30 min and filtered through filter paper packed tightly into a disposable pipet for HPLC. A gradient starting at 20% methanol and ending at 100% methanol in 40 min was used, with a flow rate of 0.8 mL/min optimized for resolution according to the procedure of Meyer (1985). Both methanol and water contained 0.1% H_3PO_4.

Thin-Layer Chromatography (TLC). TLC plates were Whatman silica gel, 250-μm coating on an aluminum backing

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with fluorescent indicator at 254 nm (catalog no. 4420 222) or silica gel on glass plates (Merck, art. 5629). Samples were applied directly, without filtering, to the thin-layer plates. For TLC, 1 μL of each sample or standard solution was applied to the plate, which was then developed to a height of 7.5 cm for the aluminum-backed plates or to 3.5 cm for the glass plates with ethyl acetate/0.025% ammonium hydroxide/methanol 50:5:13 (solvent M) or ethyl acetate/methyl ethyl ketone/formic acid (88%)/water 5:3:1:1 (solvent N; Stahl and Schorn, 1965). The separated compounds on the aluminum-backed plates were visualized by spraying with sodium molybdate reagent for *o*-dihydroxy compounds (Arnow, 1937) or with acidified 1% ferric chloride in water, or by long-wavelength UV (360 nm) for glass TLC plates sprayed with 10% boric acid in methanol after development or sprayed with 1% boric acid before development. Also, under short-wavelength UV light the maysin spots showed as dark spots against a yellow-green fluorescent background.

Photography for Permanent Record of TLC Plates. Permanent photographic records were made on 35-mm slides of glass TLC plates sprayed with 1% boric acid in methanol and dried to impregnate the plate with boric acid before development or sprayed with 10% boric after development in solvent N. The 35-mm slides were made using Ektachrome Daylight ASA 200 film and a 2B UV gelatin filter. Exposures were best at *f*1.4, $^1/_{15}$ s with the glass TLC plate resting directly on top of the UV lamp (long wavelength, 360 nm).

RESULTS AND DISCUSSION

Elliger et al. (1980b) examined the structural factors governing toxic effects of flavonoids toward the corn earworm and found that flavonoids, and *O*- and *C*-glycosides of flavonoids, with adjacent hydroxyls or, as in the case of *Zea mays*, *o*(3′,4′)-dihydroxy substitution (luteolin) on the phenyl (B) ring were more potent in bioassays of growth inhibition than were the monohydroxy (apigenin, i.e., 4′) derivatives. Both maysin and chlorogenic acid fulfill the *o*-dihydroxy structural criterion, and both are inhibitors of the corn earworm (Elliger et al., 1981; Isman and Duffy, 1982) and the fall armyworm (Wiseman et al., 1990) in bioassays. We presume that the other luteolin derivatives (probably *C*-glycosides), as yet unidentified and not available in sufficient quantity for bioassay, will be as active as maysin.

Prior to the elucidation of the structure of maysin by Elliger (1980a), Levings and Stuber (1971) reported the occurrence of nonbrowning and browning of silks of corn when the silks were wounded. The browning of the silks was attributed to the presence of substrate luteolins which were postulated to enzymatically oxidize to *o*-quinone compounds that reacted with protein to form the brown pigment. In the nonbrowning mutants the substrate luteolins, not the oxidative enzyme, were lacking. The recessive allele governing synthesis of the luteolins in the silks was independent of luteolin synthesis in the leaves. This finding is consistent with the generally accepted view that flavonoid glycosides are synthesized on the endoplasmic reticulum and then compartmentalized in cell vacuoles and are not transported from leaves to other organs. Therefore, it is expected that leaf and silk are independent in luteolin synthesis, and analyses of both silks and leaves are desirable for selection of plants resistant to both the corn earworm and the fall armyworm.

The major flavonoid in most of the corn silks and leaves examined was maysin as indicated by HPLC retention times and UV spectra taken "on-the-fly". Maysin accounted for about 60–90% of the detector signal at 340 nm over the range of silks examined. Leaves, however, contained a greater diversity of compounds (Gueldner et al., 1991), representing *C*-glycosides of luteolins and apigenins and chlorogenic acid and its isomers. Generally, the maysin content was lower in leaves than in silks.

Table I. Color and Adjusted R_f (×100) of Apigenins and Luteolins with Boric Acid (BA)

compound	R_f no BA	R_f with BA	ΔR_f[a]	color[b]
EM1 (luteolin)	33	27	−6	lemon yellow
rutin	44	35	−9	rust
EM0 (luteolin)	55	39	−16	light yellow
teo apigenin	66	53	−13	light brown
post apigenin	63	53	−10	blue-green
maysin (luteolin)	55	52	−3	yellow
premaysin (luteolin)	65	55	−10	lemon yellow
chlorogenic acid	53	21	−32	blue-violet

[a] ΔR_f = no Ba R_f minus BA R_f. [b] At 360 nm, with boric acid sprayed and dried on plate prior to development.

To separate the diverse components of the more complex samples by TLC, 20 solvent combinations were tried with the solvents tetrahydrofuran, acetonitrile, methyl and ethyl alcohols, and methyl, ethyl, and isopropyl acetates. Resolution of the flavonoid compounds was accomplished only with the acetates present as a major component of the solvent system. Separation of maysin, premaysin, and related flavonoids of teosinte leaves was achieved. Chlorogenic acid was not resolved well from maysin in ethyl acetate/methyl ethyl ketone/formic acid/water (5:3:1:1, solvent N). However, interference with maysin by chlorogenic acid, present in both silks and leaves, was eliminated by substituting 0.025% ammonium hydroxide for formic acid. The chlorogenic acid spot now ran much closer to the origin (R_f = 0.15), while the maysin spot was relatively unaffected (R_f = 0.52). However, with actual samples streaking obscured the separation of maysin and adjacent spots more than with the formic acid system. Similarly, TLC plates sprayed with 1% boric acid and dried prior to development also separated chlorogenic acid from the luteolin compounds, although streaking reduced resolution of the spots as with the ammonium hydroxide system. Boric acid, however, had the advantage of increasing the fluorescence of the spots, especially enhancing the detectability of the biologically important luteolin derivatives, producing an intense yellow color at 360 nm (see Table I).

The maysin spot typically had an R_f of 0.52 and a yellow fluorescent glow under long-wavelength (360 nm) UV light. Related luteolins had a nearly identical color and could readily be distinguished from apigenins and chlorogenic acid. Fading of the fluorescence occurred within 15 min, especially if the spots were weak; however, the best quantitation estimates appeared to be with weaker spots. It may be desirable to dilute concentrated samples and to photograph the developed plate as quickly as possible for permanent record. Detectability of maysin with boric acid on the TLC plate prior to solvent development was possible for concentrations as low as 10 ng/μL for maysin standard solutions. However, with actual samples this level may not be detectable due to interfering substances, but the level of detectability is estimated to be in the range 10–20 ng/μL. Boric acid also did not interfere with detection of spots under short-wavelength UV (254 nm).

Color development (Table I) was achieved by treatment with Arnow's reagent (Arnow, 1937) and acidified 1% ferric chloride in water. Arnow's reagent produced a bright yellow color for the *o*-dihydroxy compounds; ferric chloride gave a gray-brown color but was not selective for the *o*-dihydroxy compounds. Boric acid caused a strong fluorescence witht he luteolins when it was sprayed onto the TLC plate before solvent development. The fluorescent effect was similar but not as uniform when the plate was sprayed with boric acid after solvent development.

Table II. Visual Ranking of Order of Maysin Amount in Silk and Leaf Samples Analyzed by TLC[a] Compared with HPLC Amounts and Ranking

sample 1127-	rank by HPLC	rank by TLC	amt of maysin spotted,[b] ng	% maysin[c]
		A. Silk Maysin		
A	1	1	106.2	0.219
B	2	2	73.6	0.320
C	3	3	48.2	0.201
D	4	4	38.5	0.135
E	5	5	31.8	0.148
F	6	6	31.4	0.131
G	7	7	23.4	0.066
H	8	8	19.6	0.056
I	9	9[d]	0.5	0.003
J	10	10[d]	0.0	0.000
		B. Leaf Maysin		
K	1	2	990	2.05
L	2	1	930	2.21
M	3	4	73	0.23
N	4	8	54	0.16
O	5	3	45	0.15
P	6	9	39	0.09
Q	7	5	33	0.09
R	8	7	28	0.08
S	9	6	20	0.06

[a] Fluorescence induced by boric acid at 360 nm. [b] Amount of maysin calculated from HPLC analyses. [c] Percent maysin, by fresh weight, as determined by HPLC. [d] No maysin detected.

Table III. Visual Quantitation of Maysin in Corn Silks by TLC[a]

silk sample	maysin, % fresh wt[b]	chlorogenic acid, % fresh wt[b]	maysin, μg estd[c]	maysin, μg calcd[b]
256-2	1.040	0.026	1.3	0.7000
256-1	1.010	0.019	1.5	0.4800
256-8	0.740	0.030	0.8	0.4100
256-5	0.610	0.029	0.5	0.2900
232-1	0.570	0.027	0.3	0.2800
256-3	0.420	0.035	0.4	0.2800
256-12	0.330	0.012	0.3	0.1700
238-8	0.260	0.064	0.2	0.2100
816-9	0.011	0.011	0.0	0.0120
810-3	0.005	0.001	0.0	0.0028

[a] The silica gel plate with fluorescent background was developed with solvent M. [b] By HPLC. [c] By TLC under short wavelength UV (254 nm).

The application of the TLC method without boric acid and with visualization under short wavelength to corn silks is summarized in Table III. Estimates of concentration are too high when intense spots are evaluated, while lower intensity spots gave estimates much closer to the values determined by HPLC. With leaf samples (Table IIB) it was possible to rank in order the amounts spotted as calculated from the HPLC analyses. Table II compares the highest to lowest ranking by HPLC to the TLC ranking. The rankings matched perfectly, but in the lowest two samples (I and J) no maysin could be detected. Thus, detectability in actual silk samples is as low as 20 ng/μL.

In Table III estimates of silk amounts of maysin were made by comparison with known standard concentrations of maysin using the fluorescent background quenching and 254 nm. The limit of detectability here, without boric acid, was 10 times greater, and the estimates were high by 2–3-fold at stronger maysin concentrations. Chlorogenic acid was too low in these silk samples to be detected by TLC in the samples of both Tables IIA and III. Chlorogenic acid is usually at low levels in the silk and thus is not often a significant factor in conferring resistance to feeding by insect larvae. Levels of chlorogenic acid tend to be more prominent in leaves than in silks but not as prominent as maysin and its relatives.

This TLC method provides a fast, inexpensive screen of large numbers of corn silks so that promising crosses containing high levels of maysin or other luteolin derivatives may be identified quickly. The method has sufficient sensitivity to differentiate levels of maysin necessary to impart resistance to the corn earworm, estimated to be 0.2% or greater of the fresh weight of silk (Snook et al., 1989). For leaves, however, confounding factors may interfere with maysin, and estimates of rank by TLC do not always correlate well with the HPLC method.

ACKNOWLEDGMENT

We are grateful to Susan J. Wilson for her excellent technical support.

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Received for review October 30, 1991. Revised manuscript received March 30, 1992. Accepted April 20, 1992.