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Flavonoids of Zoysiagrass (*Zoysia* spp.) Cultivars Varying in Fall Armyworm (*Spodoptera frugiperda*) Resistance

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Flavonoid profiles of 12 zoysiagrass (*Zoysia* spp.) cultivars sampled six times in 1998 were correlated to fall armyworm (*Spodoptera frugiperda* J. E. Smith) larval weights and survival on replicated field-grown plant material and analyzed to determine genetic and seasonal variations of flavonoids among zoysiagrass cultivars. From multiple regression analyses and correlations, flavonoid peak 10 (luteolin-glucoside) had the greatest positive association with average fall armyworm weight; however, resistance appeared to be correlated with a number of other flavonoids.The flavonoid profiles of cultivars subjected to clustering procedures showed consistent genetic variability for five of six samplings and was used to genotype 23 cultivars. The dendrogram supported the results of the FASTCLUS procedure in clustering certain genotypes such as fall armyworm-resistant Cavalier and Zeon together, as well as J-36 and Meyer. Flavonoid evaluations measure genetic relatedness among cultivars and could be used for selective breeding of resistance to fall armyworm.

KEYWORDS: Zoysiagrass; *Zoysia* spp.; fall armyworm resistance; flavonoids; cluster analysis; dendrograms; genotyping

INTRODUCTION

Zoysiagrass (*Zoysia* spp.) cultivars have been developed in the United States as turfs that are drought-, saline-, and shade-tolerant (*1*). Three primary species (*Zoysia matrella*, *Zoysia japonica*, and *Zoysia pacifica*) have been used in breeding for abiotic and biotic stress tolerances. Some are reported to have varying degrees of fall armyworm (FAW) (*Spodoptera frugiperda* J. E. Smith) resistance. Although zoysiagrass is generally more resistant to FAW when compared to other C4 grasses (*2*), cultivars such as Cavalier and Emerald have been known to have the highest levels of resistance (*3*, *4*). The cause of this high level of resistance is not known.

The ability to follow pest resistance or stress tolerance through generations of breeding and selection using molecular techniques improves efficiency in developing superior cultivars. There are various methods to identify cultivars or germplasm of grasses. Isozyme electrophoresis (*5*, *6*) and molecular genetic techniques such as DNA amplification fingerprinting (*7*, *8*), amplified fragment length polymorphism (*9*), and single-sequence repeats (*10*) have been used to distinguish between genotypes. Another method of differentiating genotypes is through differential levels of chemicals from gene expression (*11*−*14*). Flavonoid profiles vary greatly between genotypes and have been used for genotyping. The flavonoid profile is consistent within a genotype under specific environments; however, expression under varying locations or harvest times may change (*15*−*17*), lending to some environment × genotype interactions.

In addition, certain flavonoids have been associated with disease and insect resistance (*18*, *19*). Maysin is one example that has been studied extensively (*20*, *21*). The objectives of this study were to attempt to associate differential FAW resistance of zoysiagrass cultivars to their flavonoid profiles and to determine genetic and seasonal variations of flavonoids among zoysiagrass cultivars.

MATERIALS AND METHODS

Plant Material. The zoysiagrass (*Zoysia* spp.) cultivars used for this study were maintained in field plots at the Pee Dee Research and Education Center (Florence, SC) using standard cultural and fertilization techniques. Entries represent seeded and vegetative cultivars with a range of characteristics (**Table 1**). Twelve entries (Cavalier, Zeon, Emerald, Crowne, J-36, DeAnza, Zen 400, Victoria, El Toro, Jamur, HT-210, and Meyer) were chosen for FAW evaluation and flavonoid profiles throughout the summer of 1998 because of their varying genetic backgrounds and susceptibility to FAW. Another 11 cultivars were evaluated once for flavonoids.

High-Performance Liquid Chromatography (HPLC) Analysis. Plants were sampled on May 4, June 10, July 10, August 17, September 14, and October 19, 1998. For flavonoid evaluations, 0.25 g of leaf material was removed from each grass entry in the field and cut into

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Table 1. Zoysiagrass Cultivars Evaluated for Flavonoid Content during the Summer of 1998 at Pee Dee Research and Education Center (Florence, SC)

cultivar	entry no.	species	FAW resistance	source	characteristics
Cavalier	1	*Z. matrella*	high	Texas Agricultural Experiment Station	leaf—fine, long many stolons
Zeon	2	*Z. matrella*	high	Private, TX	dense, fine, high thatch, moderate dark green, drought tolerant
Emerald	3	*Z. matrella* × *Z. pacifica*	high	Georgia, Agricultural Research Service, U.S. Department of Agriculture	fine, low growth habit, dark green, slow growth
Palisades	4	*Z. japonica*	moderate	Texas Agricultural Experiment Station	intermediate—rapid growth, low water use
Zenith	5	*Z. japonica*	moderate	private company	seeded synthetic, intermediate to coarse texture, medium dark green
Crowne	6	*Z. japonica*	moderate	Texas Agricultural Experiment Station	intermediate—rapid growth, low water use
Zorro (Dalz 9601)	7	*Z. matrella*	moderate	Texas Agricultural Experiment Station	high salt, drought, shade and cold tolerance, fine
Diamond	8	*Z. matrella*	moderate	Texas Agricultural Experiment Station	high shade, salt tolerance
J–36	9	*Z. japonica*	low	private company	seeded synthetic, coarse texture, medium dark green
Z–18	10	*Z. japonica*	low	private company	seeded synthetic, moderate coarse texture, light green, frost and cold susceptible
Chinese Common	11	*Z. japonica*	low	plant collection—China	seeded, moderate coarse texture, frost sensitive, moderate dark green
DeAnza	12	*Z. japonica* × *Z. matrella* × *Z. pacifica*	low	University of California	longer growing season, frost tolerance, moderate fine texture, cold susceptible
Zen–400	13	*Z. japonica*	low	private company	seeded, moderate texture, dark green
J–37	14	*Z. japonica*	low	private company	seeded, moderate coarse, medium dark green
J–14	15	*Z. sinica*	low	private company	moderate fine, medium dark green
Victoria	16	*Z. japonica* × *Z. matrella* × *Z. pacifica*	low	University of California	frost tolerant, cold susceptible, moderate fine texture
Korean Common	17	*Z. japonica*	low	plant collection—Korea	seeded, moderate coarse texture, medium dark green
Miyako	18	*Z. japonica*	low	private company	coarse, light green, aggressive
El Toro	19	*Z. japonica*	low	University of California	moderate coarse texture, medium dark green, drought tolerant
Jamur	20	*Z. japonica*	low	private company	moderate coarse texture, medium green, moderate drought tolerance
HT–210	21	*Z. matrella*	low	private company	fine texture, medium green
Meyer	22	*Z. japonica*	low	Agricultural Research Service, U.S. Department of Agriculture	moderate fine, dark green, drought and frost susceptible
Zen–500	23	*Z. japonica*	low	private company	seeded, moderate texture, drought and frost susceptible

small pieces and placed in a scintillation vial with 10 mL of methanol. Three replicates of each grass cultivar/harvest were prepared. The samples were stored at −20 °C until analysis. Prior to analysis, 50 μL of a methanolic chrysin solution (chrysin recrystallized from amyl alcohol; 0.08 mg/50 μL) was added as an internal standard. After the plant material was ground with a polytron (Brinkmann Instruments, Inc., Westbury, NY), the solutions were filtered and aliquots were analyzed by reversed-phase HPLC, using a H_2O/MeOH linear gradient from 10 to 100% MeOH in 35 min, a flow rate of 1 mL/min, and detection at 340 nm. Each solvent contained 0.1% H_3PO_4. Analyses were performed with an Altex Ultrasphere C18, 5 μm (4.6 mm × 250 mm, Beckman Instruments, Norcross, GA) column using a Hewlett-Packard 1050 diode array HPLC. Quantitation of polyphenol and flavonoid profiles was performed by using chrysin's response factor.

Isolation of Flavone Glycosides. *Extraction.* Flavonoids were isolated from Cavalier, a cultivar that contained flavonoids in reasonable quantities that were representative of those found in all varieties. Cavalier (700 g) was extracted with methanol (8 L) in a Waring Blender and filtered. The extract was concentrated by rotary evaporation to approximately 500 mL, and 250 mL of water was added. The solution was further concentrated until only an aqueous solution remained and then was extracted with CH_2Cl_2 (2 × 250 mL) to remove chlorophyl and waxes. The aqueous portion contained the compounds of interest.

Isolation. Isolation was mainly by preparative reversed-phase, silicic acid column chromatography followed by a second preparative reversed-phase separation. Approximately 100 g of Waters BondaPak C18 bulk packing material (Millipore Corp., Milford, MA) was packed into a glass chromatography column (54 cm × 2.54 cm, 15 psi nitrogen pressure used to aid flow), washed with MeOH, and recycled to H_2O. The water solution of the flavonoids was chromatographed on this column. Salts were eluted with water, and the compounds of interest were eluted with various percentages of MeOH/H_2O from 10 to 100% MeOH in 10% increments (v/v; 2 × 250 mL fractions being collected for each percentage except 4 × 250 mL fractions were obtained for the 40% MeOH/H_2O). Most of the flavonoids eluted from the column with 30−60% MeOH/H_2O. The solvent from selected fractions was evaporated to dryness, and the residue was submitted to silicic acid (SA- 20 g, Mallinckrodt, 100 mesh) column chromatography. The column was packed in CH_2Cl_2, and after, the sample was applied to the top of the column (as a SA/sample deposited mixture) and eluted

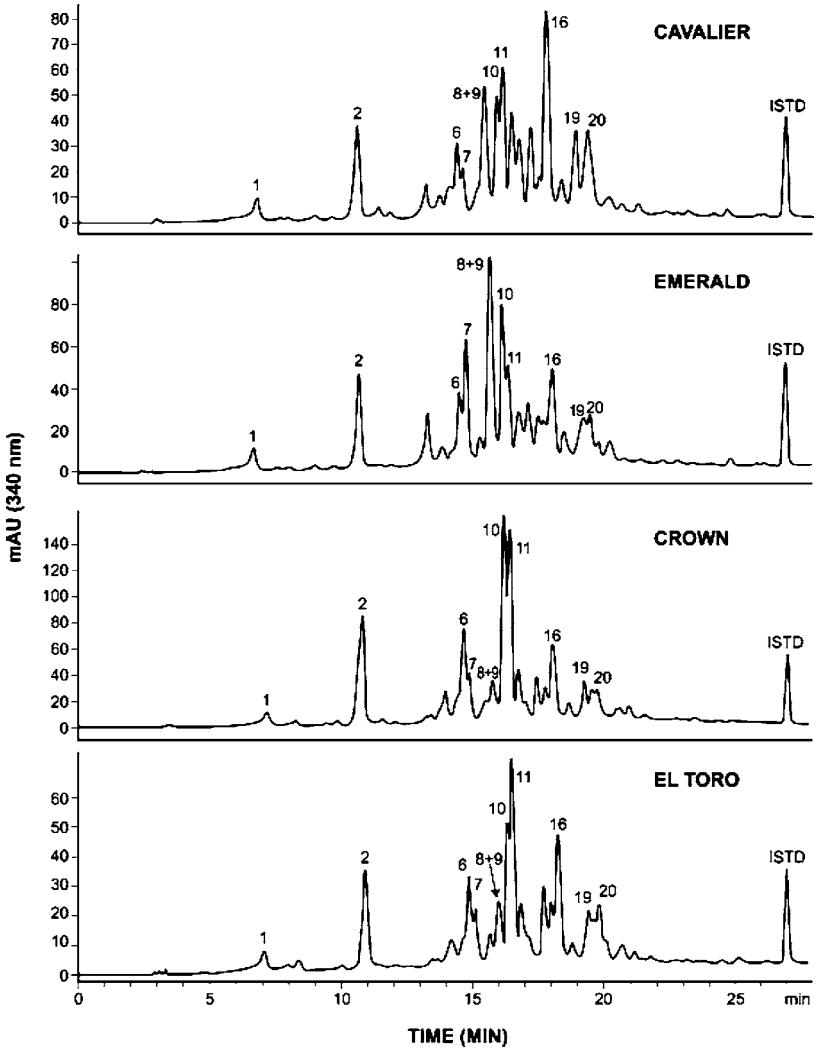

Figure 1. Chromatograms of chlorogenic isomers and flavonoids of FAW resistant (Cavalier and Emerald) and susceptible (Crowne and El Toro) zoysiagrass cultivars from the second harvest.

with CH_2Cl_2 followed by ethyl acetate or acetone/ethyl acetate mixtures. Specific solvent mixtures are given below under each particular compound isolation. Nitrogen pressure aided column flow.

Additional separation of the flavonoids was accomplished by submitting individual fractions again to reversed-phase chromatography on a Cheminert LC column (108 cm × 1.25 cm, Valco Instruments Co., Inc., Houston, TX), packed with the Waters BondaPak C18 bulk material, using the following linear solvent program: 40−60% MeOH/H_2O in 400 min. Eight milliliter fractions were collected and monitored by HPLC. After evaporation to dryness, the SA-separated flavonoids were dissolved in 1−3 mL of MeOH/H_2O (usually 40%) and applied to the Cheminert LC column with a loop injection valve.

Evaporation of methanol/water mixtures from the flavone-glycosides produced an orange-yellow, glassy, or molasses-like residue, possibly due to tightly bound water and/or methanol. Residual water/methanol was conveniently removed by dissolving the residue in methanol and adding an equivalent amount of acetonitrile. Upon evaporation of this solution, an amorphous light yellow powder was obtained. Evaporation of acetone and/or ethyl acetate mixtures from flavones yielded the amorphous powder directly.

Identification. Preliminary identifications of polyphenols and flavonoids first observed via HPLC (**Figure 1**) were determined by UV spectra and retention time correlation's with standards. Also, some isolated flavonoids were hydrolyzed with 0.1 N HCl at 100 °C for 30, 60, and 120 min, and the liberated products were analyzed by HPLC or GC (for sugars as their silylated derivatives). Selected compounds were submitted to fast atom bombardment/mass spectrometry (FAB/MS) analyses in a glycerol matrix or run on a Thermo-Finnigan LCQ HPLC/MS. Except for Peaks 1 and 2 (**Figure 1**), all compounds gave UV spectra indicating luteolin derivatives.

Peaks 1 and 2. Neochlorogenic and Chlorogenic Acids. Peak 1 was identified as neochlorogenic acid (5-O-caffeoylquinic acid), and peak 2 was identified as chlorogenic acid (3-O-caffeoylquinic acid). Chlorogenic acid had retention time and UV correlation with the standard. Neochlorogenic acid was identified by UV and retention time correlation to a previously isolated compound (*22*).

Peaks 6 and 7. Luteolin-O-glucosyl-C-arabinoside. Peaks 6 and 7 eluted from the first preparative C18 with the first 30% MeOH/H_2O fraction along with some of peaks 8 and 9. The residue after evaporation was submitted to SA chromatography and eluted with 60 and 70% acetone/ethyl acetate. The residue was then separated on the Cheminert C18 column eluted isocratically with 25% MeOH/H_2O only. This procedure separated peaks 6 and 7 from peaks 8 and 9. The fractions containing peaks 6 and 7 were rechromatographed on SA, and pure peaks 6 and 7 eluted with 60 and 65% acetone/ethyl acetate. Acid hydrolysis (0.1 M HCl, 60 min at 100°) yielded only glucose. FAB/MS: 580. The two isomers possibly had the O-glucoside on different hydroxyls of the arabinose, or one isomer was the 6-C-arabinoside and the other was the 8-C-arabinoside. Under our HPLC conditions, orientin (8-C-glucosylluteolin) eluted just before isoorientin (6-C-glucosylluteolin) analogous to the elution observed for peaks 6 and 7 and peaks 8 and 9 (see below).

Peaks 8 and 9. Luteolin-C-glucosyl-C-arabinoside. They obtained from the Cheminert C18 column chromatography run of peaks 6 and

7. The residue after evaporation of the solvent was purified on SA as described for peaks 6 and 7. Acid hydrolysis yielded no detectable sugars. FAB/MS: 580. The two isomers were no doubt 6-C-glucosyl-8-C-arabinoside and 6-C-arabinosyl-8-C-glucoside.

Peak 10. Luteolin-6-C-glucoside (Isoorientin). Because of the difficulty in separating peaks 10 and 11 from Cavalier, El Toro was used for peak 10 as it contained this compound in relatively purer form and larger quantity. This compound was isolated from 120 g of El Toro variety of zoysia similarly to the procedure described above for Cavalier. Peak 10 eluted from the first preparative C18 with 30% MeOH/H_2O. The residue after evaporation was submitted to SA chromatography, and peak 10 eluted with 10 and 40% acetone/ethyl acetate. HPLC/MS gave 448.

Peak 11. Luteolin-di-C-arabinoside. Peak 11 eluted from the first preparative C18 with the second 30% MeOH/H_2O fraction along with some of peaks 8 and 9 and 12 and 13. The residue was then separated on the Cheminert C18 column eluted isocratically with 27% MeOH/H_2O only. Acid hydrolysis liberated no sugars. HPLC-MS: 550.

Peak 16. Luteolin-di-C-arabinoside + Luteolin-C-rhamnosyl-C-arabinoside. Peak 16 eluted from the first preparative C18 with the third 40% MeOH/H_2O fraction in approximately 75% purity. The residue was submitted to SA chromatography as described above and eluted with 40% acetone/ethyl acetate in >85% purity. Acid hydrolysis liberated no sugars. Although HPLC showed no hint of two compounds, thin-layer chromatography (TLC) and HPLC-MS indicated that two compounds were present. TLC on Bakerflex Silica Gel IB-F 5 cm × 20 cm sheets in ethyl acetate/methyl ethyl ketone/formic acid/water (50:30:10:10) yielded two spots at R_f 0.30 and 0.35. HPLC/MS: 551 (M + H, Luteolin-di-C-arabinoside) and 565 (M + H, luteolin-C-rhamnosyl-C-arabinoside).

Peaks 19 and 20. Methoxyluteolin-di-C-arabinoside and Dimethoxytricin-O-glucoside. These compounds eluted from the first preparative C18 column with the fourth 40% MeOH/H_2O and first 50% MeOH/H_2O fractions. Isolation of the individual compounds was then achieved on SA chromatography eluting with 40% acetone/ethyl acetate. Peak 20 eluted first followed by peak 19. Peak 19 liberated no sugars on hydrolysis; HPLC-MS: 565 (M + H), 535 (M + H − OCH_3). Peak 20 liberated glucose on acid hydrolysis. The dimethoxy structure was supported by peak 20's high TLC R_f of 0.74 (see above for peak 16). HPLC-MS: 493 (M + H), 463 (M + H − OCH_3), 331 M + H-glucose.

FAW Evaluations. Twelve of the zoysiagrass cultivars were evaluated for FAW resistance at three dates in 1998. Each test consisted of 12 treatments (cultivars) by three replications with each replication consisting of three Petri dishes with four FAW larvae in each dish (12 larvae/entry/rep). Moistened filter paper was placed in each dish. Grass clippings were collected from replicated field plots and placed in the dishes. Fresh grass clippings were changed every other day. Each dish was infested with early second instar FAW larvae (1.2, 0.9, and 0.8 mg/larva for tests 1, 2, and 3, respectively) that had fed on an artificial diet after egg hatch for 3 days. This colony was initiated from wild eggs collected from a nearby golf course fairway August 1997 and maintained at the Clemson University Pee Dee Research and Education Center near Florence, SC. Test 1 was initiated May 4, 1998, and 14 day counts and weights were made May 18, 1998. Test 2 was initiated June 10, 1998, and 6 day counts and weights were made June 16, 1998. Test 3 was initiated September 18, 1998, and 7 day counts and weights were made September 25, 1998.Weights of surviving worms were recorded, and the mean within replications was used for analysis. Percentages of surviving worms per replication were calculated.

Analyses of Flavonoid and FAW Data. Flavonoids from peaks 8 and 9 were combined for analysis (**Figure 1**). The amounts of 18 specific flavonoid peaks were converted to percentages of total flavonoids for each sample. The percentages of each flavonoid and the total were used for all subsequent analyses. The GLM procedure was performed on each flavonoid peak and on the total for the 12 entries that were harvested six times. The means were separated using the Waller−Duncan option.

The STANDARD procedure was first used to standardize all of the analytical variables to a mean of 0 and a standard deviation of 1. The procedure created the output data set stand to contain the transformed variables (*23*). The FASTCLUS procedure was performed using a maximum cluster number of 12 (number of genotypes) to generate a data set that was displayed using the FREQ option. Clustering and outliers for each genotype were observed from the frequency table. The means from three runs of each genotype × harvest were calculated and used the CLUSTER procedure and the output used for the TREE procedure to produce a dendrogram of 12 entries by six harvests (*23*). Finally, CLUSTER and TREE procedures were performed on all 23 zoysiagrass entries.

PROC STEPWISE multiple regression (forward, backward, and MaxR options) was performed on the mean weight of FAW larvae for each entry and date using flavonoid percentages and totals for corresponding dates (*23*). Correlation analysis (PROC CORR−Spearman) was performed on entry/date means among flavonoid and FAW weights.

Table 2. Mean of FAW Larvae Survival and Average Weight for Three Grass Sample Dates (May 4, June 10, and September 18, 1998)

	May 18[a]		June 16[a]		September 25[a]	
entry	% survival	average wt	% survival	average wt	% survival	average wt
Cavalier	17 ab	68 a	11 a	12 a	6 a	1.5 a
Zeon	39 b	85 a	25 ab	12 a	0 a	
Emerald	6 a	70 a	8 a	10 a	0 a	
Crowne	17 ab	140 a	78 d	41 bc	39 bc	10.4 cd
J–36	36 b	103 a	61 cd	40 bc	31 abc	5.6 ab
DeAnza	28 ab	233 a	80 d	38 bc	39 bc	11.0 cd
Zen 400	39 b	102 a	80 d	28 ab	6 a	7.0 bc
Victoria	30 ab	133 a	86 d	28 ab	50 c	13.0 de
El Toro	33 ab	126 a	67 cd	32 abc	22 abc	6.9 bc
Jamur	36 b	88 a	83 d	50 bc	8 ab	5.0 ab
HT-210	25 ab	82 a	47 bc	12 a	25 abc	4.6 ab
Meyer	33 ab	162 a	86 d	54 c	39 bc	16.7 e
Mean	28	117	59	32	22	8.9

[a] Entries with the same letter are not significantly different at the $p < 0.05$ significance level.

RESULTS AND DISCUSSION

The percentage of FAW survival and average final weight varied considerably between and within experiments. The overall survival percentage was much higher for the June test (**Table 2**). The greater armyworm resistance of Cavalier, Zeon, and Emerald was evident in June and September tests and supports previous reports of resistance (*2*−*4*). However, HT-210 also exhibited some resistance in June.

Flavonoid identifications are preliminary but are definitely luteolin glycosides. Relative amounts of individual flavonoids among zoysiagrass cultivars were first statistically compared by the GLM procedure. Entries mean squares were significantly different ($p = 0.01$) for percentages of all individual flavonoid compounds; however, total flavonoids were not significantly different when using entry by harvest mean square as the error term. Harvests were significantly different for total flavonoids and all individual flavonoids except for flavonoid peak 7 and flavonoid peak 12. Entries by harvest interactions were significant in all cases when using the residual mean square; however, the contribution of entry by harvest to the sum of squares of the model was less than 15%, except for flavonoid peak 15 (33.5%), flavonoid peak 21 (34.5%), and total flavonoids (46.7%).

Entry J-36 had high amounts of flavonoid peak 7 (luteolin-O-glucosyl-C-arabinoside), Zen 400 and Meyer had high amounts of flavonoid peaks 8 and 9 (luteolin-C-glucosyl-C-arabinoside), and Cavalier and Emerald were high in flavonoid peaks 16 (luteolin-di-C-arabinoside + luteolin-C-rhamnosyl-C-arabinoside) and 19 and 20 (methoxyluteolin-di-C-arabinoside

Table 3. Mean Percentage Distributions and Total Chlorogenic Isomers and Flavonoids (μg/g[a]) of 12 Zoysiagrass Varieties Averaged over Six Harvest Dates during the Summer of 1998 at Pee Dee Research and Education Center (Florence, SC)

	chlorogenic isomers and flavonoid compounds																		
entries	1	2	3	3A	6	7	8–9	10	11	12	13	14	15	16	18	19–20	21	22	total
Cavalier	3.5c[b]	6.9 bc	1.9 d	1.6 f	4.0 c	3.4 h	8.9 h	3.7 f	8.9 f	5.3 b	5.9 a	7.3 b	0.4 i	14.2 b	3.7 b	16.0 a	1.9 b	2.6 de	9193 a
Zeon	3.2 cd	6.3 de	1.9 d	1.9 e	3.8 d	3.4 h	8.4 i	4.3 ef	8.9 f	5.2 bc	5.9 a	7.4 b	0.0 j	14.7 a	4.0 a	15.7 a	2.4 a	2.8 d	8248 b
Emerald	4.7 a	7.4 b	2.5 c	1.9 e	2.0 e	8.7 f	15.1 c	4.9 e	6.4 h	4.2 f	6.0 a	5.5 e	1.3 f	9.4 e	3.0 d	11.6 c	2.2 a	3.3 bc	8048 bc
Crowne	3.0 d	8.9 a	1.2 e	3.5 b	7.4 a	3.8 g	5.7 j	9.9 ab	14.1 a	4.8 e	2.8 g	6.1 d	0.8 h	10.1 cd	2.3 f	11.8 c	1.2 d	2.5 e	7397 bcd
J-36	2.3 e	6.0 e	2.5 c	3.1 c	0.0 g	19.0 a	13.5 d	7.5 cd	9.3 e	2.7 h	3.1 f	2.8 g	2.3 c	7.9 g	1.7 h	12.4 b	0.7 e	3.2 c	7979 bc
DeAnza	2.5 e	6.5 cd	2.0 d	2.8 d	0.0 g	11.0 d	10.7 g	10.4 a	13.7 b	5.1 cd	4.8 c	5.9 d	3.0 a	4.6 j	3.2 c	9.3 e	1.1 d	3.4 b	8041 bc
Zen 400	3.0 d	8.5 a	4.9 a	3.6 b	0.3 f	15.3 b	22.5 a	7.9 c	4.8 i	1.9 j	6.0 a	1.7 h	2.5 b	3.2 k	1.1 i	9.5 e	0.0 g	3.3 bc	7986 bc
Victoria	2.6 e	8.9 a	2.4 c	2.7 d	0.4 f	14.6 c	12.2 e	10.5 a	10.8 d	3.1 g	4.2 d	4.6 f	1.8 e	6.0 i	2.8 e	10.2 d	0.0 g	2.2 f	8142 bc
El Toro	2.6 e	8.9 a	1.3 e	3.5 b	7.2 b	3.8 gh	6.0 j	9.5 b	13.9 ab	5.0 d	2.6 g	5.9 d	2.1 d	9.9 d	2.4 f	11.7 c	1.4 cd	2.5 e	7905 bc
Jamur	3.2 d	8.9 a	1.2 e	3.5 b	7.2 b	3.7 gh	5.7 j	9.3 b	14.0 a	4.9 de	2.7 g	6.4 c	1.1 g	10.3 c	2.5 f	11.3 c	1.6 c	2.5 e	7280 cd
HT-210	4.1 b	5.4 f	1.9 d	1.8 ef	1.8 e	9.3 f	11.8 f	6.9 d	11.2 c	6.6 a	5.3 b	8.0 a	2.0 d	8.9 f	3.2 c	8.2 f	1.2 d	2.5 e	6757 de
Meyer	2.3 e	6.0 ef	3.4 b	3.9 a	0.0 g	15.6 b	19.1 b	7.4 cd	7.6 g	2.5 i	3.7 e	1.4 h	3.0 a	6.7 h	1.9 g	11.5 c	0.3 f	3.8 a	6248 e

[a] On the basis of fresh weight. [b] Entries with the same letters for each column are not significantly different at the $p = 0.01$ probability level.

and dimethoxy-tricin-O-glucoside) (**Table 3**). From the analysis of variance, the FAW resistance of Cavalier and Zeon would appear to come from high total flavonoids or higher percentages of flavonoid peaks 13, 14, 16, 18, 19 and 20, or 21, while the resistance of Emerald appears to come from peaks 1 (neochlorogenic acid), 13, and 21. HT-210 showed some FAW resistance in this test, and it had high percentages of neochlorogenic acid and flavonoids 12−14. However, when analyzing means of all cultivars within the appropriate sample dates with stepwise multiple regression, flavonoid peak 10 (luteolin-6-C-glucoside) was the most associated with average FAW weight in all three procedures (forward, backward, and MaxR) (**Table 4**). The forward selection and maximum *R*-square improvement procedures identified peaks 1 and 3A and the total flavonoid content as important variables responsible for worm weights. However, in the backward elimination method, flavonoid peak 10 was joined by peaks 2, 8 and 9, 16, 11, and 18 as significant at the 0.10 probability level. Chromatographs would support resistance of Cavalier and Emerald coming from higher levels of peaks 8 and 9 and 16 (**Figure 1**). The association with flavonoid 10 and FAW resistance appears to be due to lower percentages. Whether this association is due to favorable association of this flavonoid to insect growth or due to diversion of the flavonoid pathway toward detrimental flavonoids is not clear. If the latter is the case, then flavonoids with negative correlations with peak 10 may be responsible for the resistance, with peaks 1, 13, 14, 16, and 19 and 20 being the prime candidates (**Table 6**). This is supported by the significant negative correlation coefficients of these flavonoids with FAW weights.

There is no clear causal effect of flavonoids on FAW survival as indicated by stepwise regression (**Table 5**) or from Spearman correlation coefficients (**Table 6**). The regression procedures would indicate that flavonoids 8 and 9 and 19 and 20 were most closely associated with FAW survival but are not supported by Spearman correlation coefficients (**Table 6**). Other microenvironmental factors of the experiment could have influenced their survival. Causal effects of FAW resistance can only be suggested from this work and will require more extensive extraction of putative flavonoids and bioassays to substantiate the mentioned possible causes.

Different clustering procedures were performed by using the flavonoid data from all harvests of 12 entries. Samples of Emerald, DeAnza, Zen 400, Victoria, and HT-210 were generally grouped by themselves while Crowne, El Toro, and Jamur clustered together using FASTCLUS (**Table 7**). Resistant entries Cavalier and Zeon clustered together, and J-36 clustered with Meyer. Cluster three represented outlier observations of Cavalier, Zeon, Emerald, and HT-210. Clusters 4, 6, and 11 represented other outliers.

Table 4. Stepwise Regression (Forward, Backward, and MaxR Procedures) for Flavonoid Peaks on FAW Final Weight of 12 Zoysiagrass Cultivars during May, June, and September 1998 Samplings

Summary of Forward Selection

step	variable[a] entered	partial *R*-square	model *R*-square	*C*(*p*)	*F* value	Pr > *F*
1	P10	0.5940	0.5940	19.3305	49.74	<.0001
2	P1	0.1011	0.6950	8.5543	10.94	0.0023
3	total	0.0309	0.7259	6.6487	3.61	0.0666
4	P3A	0.0481	0.7740	2.5694	6.60	0.0153
5	P15	0.0114	0.7854	3.1342	1.59	0.2175
6	P14	0.0100	0.7953	3.8744	1.41	0.2444
7	P11	0.0134	0.8087	4.1863	1.95	0.1731
8	P7	0.0100	0.8187	4.9162	1.50	0.2318
9	P22	0.0121	0.8308	5.3924	1.85	0.1852
10	P3	0.0062	0.8370	6.6129	0.95	0.3402

Variables Left after Backward Elimination

variable	parameter estimate	standard error	type II SS	*F* value	Pr > *F*
intercept	–646.52056	192.890	9320.406	11.23	0.0022
P2	7.68982	4.044	3000.421	3.62	0.0672
P8 and 9	14.94691	5.100	7125.369	8.59	0.0065
P10	20.36825	3.656	25762.000	31.05	<.0001
P11	11.13614	5.894	2961.906	3.57	0.0689
P16	17.00244	5.700	7382.372	8.90	0.0057
P18	15.76304	9.045	2520.056	3.04	0.0920

Best 10-Parameter Model—Maximum *R*-Square (MaxR) Improvement

variable	parameter estimate	standard error	type II SS	*F* value	Pr > *F*
intercept	162.58629	58.964	4675.759	7.60	0.0107
P1	–16.74173	7.167	3355.407	5.46	0.0278
P3	6.71486	6.906	581.407	0.95	0.3402
P3A	–34.52360	9.136	8782.619	14.28	0.0009
P7	–2.12605	1.239	1811.054	2.94	0.0985
P10	8.18576	2.480	6702.329	10.90	0.0029
P11	7.17716	3.987	1992.435	3.24	0.0839
P14	–14.13408	5.808	3642.377	5.92	0.0224
P15	–10.64372	4.878	2927.720	4.76	0.0387
P22	15.87903	10.728	1347.257	2.19	0.1513
total	–0.00784	0.003	4532.491	7.37	0.0118

[a] Chlorogenic isomers or flavonoid peak numbers are represented with the letter P.

Table 5. Stepwise Regression (Forward, Backward, and MaxR Procedures) for Flavonoid Peaks on FAW Survival of 12 Zoysiagrass Cultivars during May, June, and September 1998 Samplings

Summary of Forward Selection

step	variable[a] entered	partial *R*-square	model *R*-square	*C*(*p*)	*F* value	Pr > *F*
1	P21	0.2029	0.2029	23.412	8.66	0.0058
2	P3A	0.0781	0.2811	19.981	3.59	0.0671
3	P14	0.0458	0.3269	18.794	2.18	0.1497
4	P7	0.1046	0.4315	13.525	5.70	0.0232
5	P18	0.0186	0.4501	14.232	1.01	0.3219
6	P13	0.0181	0.4682	14.973	0.99	0.3285
7	P8 and9	0.0342	0.5023	14.597	1.92	0.1765
8	P19 and 20	0.0218	0.5242	15.079	1.24	0.2754
9	P11	0.0826	0.6068	11.333	5.47	0.0274
10	P22	0.0408	0.6476	10.500	2.89	0.1015
11	P12	0.0242	0.6718	10.816	1.77	0.1958
12	P2	0.0175	0.6893	11.599	1.30	0.2667
13	P3	0.0192	0.7085	12.263	1.45	0.2411
14	total	0.0228	0.7313	12.680	1.78	0.1966
15	P1	0.0094	0.7407	14.024	0.73	0.4037

Variables left after Backward Elimination

variable	parameter estimate	standard error	type II SS	*F* value	Pr > *F*
intercept	–430.99731	93.599	7554.935	21.20	<.0001
P2	6.69588	2.770	2081.435	5.84	0.0224
P3A	11.92046	5.336	1777.962	4.99	0.0337
P7	3.43617	1.326	2392.977	6.72	0.0150
P8 and 9	7.24644	1.904	5163.075	14.49	0.0007
P11	9.52574	2.606	4759.520	13.36	0.0011
P12	17.27796	6.006	2948.997	8.28	0.0076
P19 and 20	8.16283	1.950	6243.337	17.52	0.0003

Best 10-Parameter Model—Maximum *R*-Square (MaxR) Improvement

variable	parameter estimate	standard error	type II SS	*F* value	Pr > *F*
intercept	–161.41522	81.017	1200.782	3.97	0.0574
P3	–11.40437	6.446	946.882	3.13	0.0891
P3A	9.73108	6.740	630.652	2.08	0.1612
P8 and 9	10.69419	2.427	5874.725	19.42	0.0002
P11	5.68601	3.613	749.382	2.48	0.1281
P12	7.53323	5.420	584.352	1.93	0.1768
P13	–11.16793	4.692	1713.665	5.66	0.0252
P14	6.84772	4.146	825.366	2.73	0.1111
P16	–5.37692	2.828	1093.370	3.61	0.0689
P19 and 20	10.23229	2.233	6349.057	20.99	0.0001
P22	–25.14132	8.271	2795.056	9.24	0.0055

[a] Chlorogenic isomers or flavonoid peak numbers are represented with the letter P.

Table 6. Spearman Correlation Coefficients for Flavonoid Percentages and FAW Weights and Survival Performed at Pee Dee Research and Education Center (Florence, SC) during the Summer of 1998

			FAW	
parameters[a]	P1	P10	weight	survival
P1		–0.71[b]	–0.86[b]	–0.28
P2	–0.58[b]	0.59[b]	0.63[b]	0.08
P3	–0.34[c]	0.11	0.29	0.09
P3A	–0.50[b]	0.59[b]	0.48[b]	0.40[c]
P6	0.01	0.01	0.15	–0.21
P7	–0.16	0.20	0.05	0.34[c]
P8 and 9	–0.04	–0.10	–0.02	0.07
P10	–0.71[b]		0.76[b]	0.33[c]
P11	–0.07	0.35[c]	0.11	0.24
P12	0.42[c]	–0.28	–0.27	–0.14
P13	0.56[b]	–0.68[b]	–0.59[b]	–0.39[c]
P14	0.68[b]	–0.50[b]	–0.61[b]	–0.20
P15	–0.43[b]	0.37[c]	0.27	0.28
P16	0.47[b]	–0.61[b]	–0.39[c]	–0.35[c]
P18	0.42[c]	–0.40[c]	–0.37[c]	–0.28
P19 and 20	0.30	–0.59[b]	–0.37[c]	–0.02
P21	0.46[b]	–0.21	–0.44[b]	–0.40[c]
P22	0.33[c]	–0.38[c]	–0.45[b]	–0.12
total	0.32	–0.36[c]	–0.50[b]	–0.03
FAW weight	–0.86[b]	0.76[b]		0.33

[a] Chorogenic isomers or flavonoid peak numbers are represented with the letter P. [b] Significant at the probability $p = 0.01$ level. [c] Significant at the probability $p = 0.05$ level.

Table 7. Frequency within Clusters Using Observations from HPLC of Zoysiagrass Entries over Six Harvests during the Summer of 1998 Using the Fastclus Method with a Maximum of 12 Clusters[a]

	cluster											
entry	1	2	3	4	5	6	7	8	9	10	11	12
Cavalier	0	0	3	0	12	2	0	0	0	0	0	0
Zeon	0	0	3	0	10	5	0	0	0	0	0	0
Emerald	0	0	3	0	0	1	0	1	0	13	0	0
Crowne	0	18	0	0	0	0	0	0	0	0	0	0
J-36	0	0	0	0	0	0	0	13	0	0	4	0
DeAnza	0	0	0	0	0	0	16	0	2	0	0	0
Zen 400	15	0	0	3	0	0	0	0	0	0	0	0
Victoria	0	0	0	0	0	0	1	0	17	0	0	0
El Toro	0	17	0	0	0	0	1	0	0	0	0	0
Jamur	0	18	0	0	0	0	0	0	0	0	0	0
HT-210	0	0	3	0	0	0	0	0	0	0	0	15
Meyer	0	0	0	1	0	0	0	15	0	0	2	0
total	15	53	12	4	22	8	18	29	19	13	6	15

[a] Data included three sample replications ($n = 214$) and observation of 18 flavonoid compounds plus the total.

The mean percentages and total flavonoids of cultivars harvested six times were analyzed using the CLUSTER and TREE procedures to produce a dendrogram that illustrates clustering of cultivars at different harvest times (**Figure 2**). The flavonoid profiles of cultivars were very similar to each other for harvests 2−6. However, harvest 1 profiles resulted in separate clusters. For example, Cavalier (*1*), Zeon (*2*), Emerald (*3*), and HT-210 (*21*) clustered closely together in harvest 1 but were distinctly separated in subsequent harvests (**Figure 2**). Crowne (*6*), El Toro (*19*), and Jamur (*20*) clustered together at each harvest; however, the profile was distinctly different for the first harvest. Thus, environmental or plant physiological differences need to be considered when using flavonoids profiles for clustering. The dendrogram supported the results of the FASTCLUS procedure in clustering Cavalier (*1*) and Zeon (*2*) closely together as well as J-36 (*9*) and Meyer (*22*) quite close together. For the first harvest, Zen-400 (*13*) was also closely clustered with J-36 and Meyer but was slightly more distinct in subsequent cuttings.

The means of all 23 cultivars were clustered by flavonoid data of harvest 1 (**Figure 3**). Zorro (Dalz 9601) clustered with Cavalier and Zeon, while Diamond was more closely related to the Cavalier group than the Emerald−HT-210 cluster. Palisades was clustered with the Crowne−El Toro−Jamur group. Z-18 and Miyako clustered with DeAnza and Victoria, while Zenith, Chinese Com., Korean Com., J-36, J-37, Zen-400, Meyer, J-14, and Zen 500 formed a large cluster quite distant from the other cultivars. Using harvest 1 to distinguish cultivars, however, must be done with caution due to the differences of flavonoid expression as previously described.

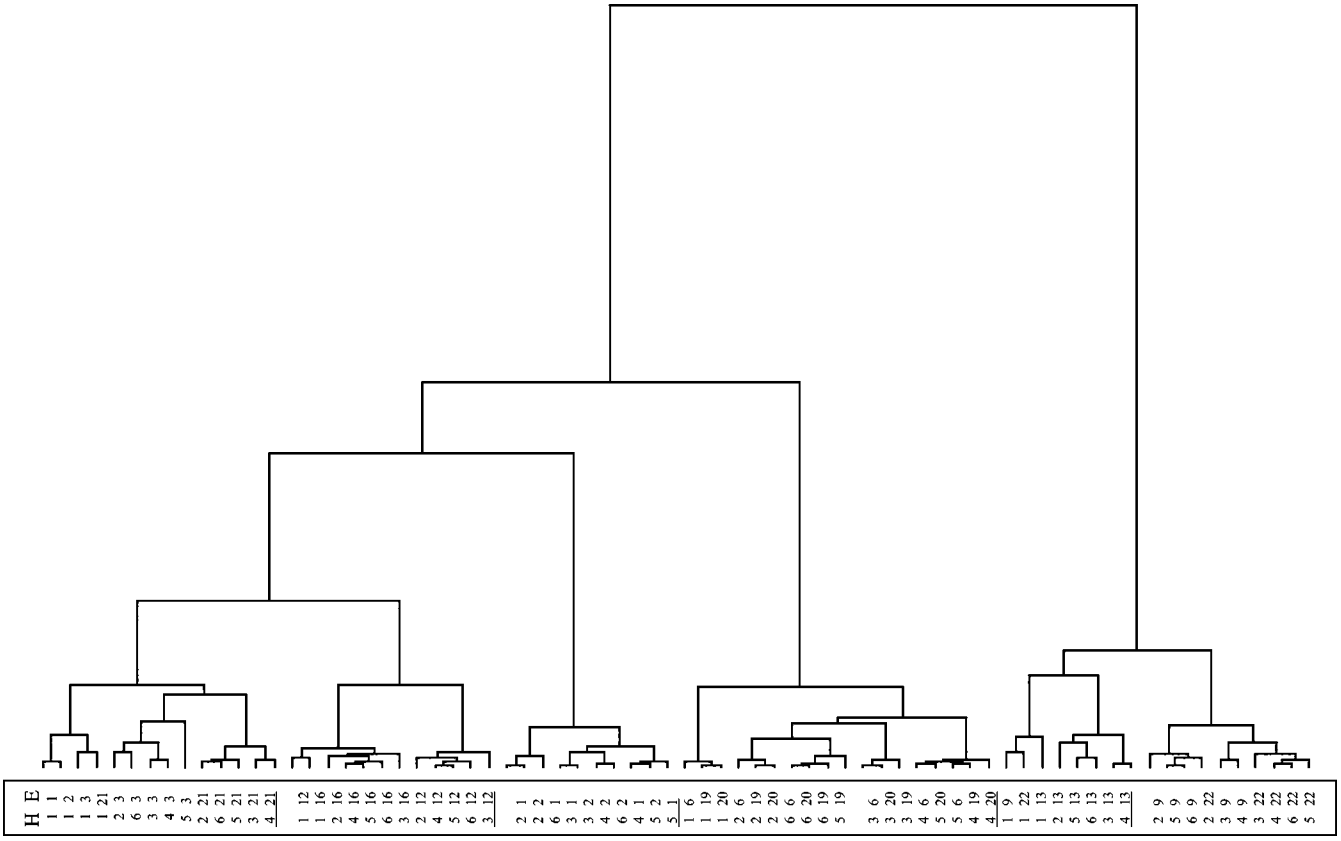

Figure 2. Dendrogram of entries (E) and harvest (H) from cluster analysis using means of flavonoid percentages and flavonoid totals for 12 zoysiagrass cultivars during the summer of 1998.

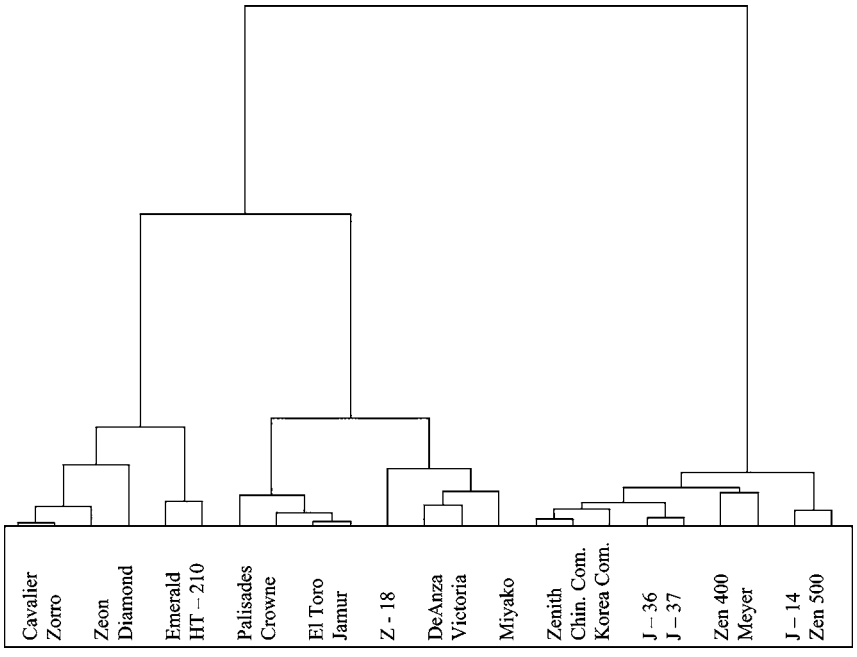

Figure 3. Dendrogram of all 23 zoysiagrass cultivars from cluster analysis using means of flavonoid percentages and flavonoid totals from the first harvest during the summer of 1998.

Specific flavonoid profiles of zoysiagrass cultivars in this test were distinct. There was little variability among replicates within cultivar/harvest, which resulted in good separation of cultivar by harvest means. The effect of harvest time on flavonoid production is noted; however, flavonoid profiles of the cultivars harvested from the second cutting to the end were very consistent. The relatedness of cultivars as expressed by flavonoid profiles was consistent over harvests, with the exception of the first harvest. Most of the variance due to entry by harvest (E × H) interaction resulted from different rankings in the first harvest as well as from individual flavonoids 15, 21, and total flavonoids. Some flavonoids were controlled by the environment

more than others. Brown et al. (*15*) found that genotype accounted for 60% of the variation of aliphatic glucosinolates among broccoli genotypes but only 12% of indolyl glucosinolates over 4 years. Hare (*16*) found that leaf resin quantity and composition differed among populations of *Mimulus aurantiacus* and seasonal differences were slight in comparison to genetic population differences. Lee et al. (*17*), however, found high genotype by environment variance for flavonoid production in pepper (*Capsicum* spp.). Clearly, flavonoids vary over time and environment; however, it appears from this study that by transforming data to percentage of total flavonoids and using the entire profile that plant material was genotyped quite effectively.

Individual flavonoids (*24*) and multiple foliar flavonoids have been used to genetically differentiate individuals or clones among wild plant populations (*12*, *13*). Snook et al. (*25*) stated that the flavonol distribution in the flowers of the *Nicotiana* species together with polyphenolic, alkaloid, and leaf surface chemistry data would be of use in chemotaxonomic evaluation of the classification of the species, which to that point had been based on morphological and cytological data.

Principle component analysis of multiple flavonoids was used (*26*) to differentiate populations of pine trees. This study used cluster analysis to develop genetic relationships among cultivars. A general understanding of genetic similarities among cultivars was deduced from the dendrograms. In this study, the resistant cultivars Cavalier and Zeon appear to be very similar. Other cultivars of *Z. matrella* (Zorro, Diamond, Emerald, and HT-210) tended to cluster closely to Cavalier and Zeon. The multispecies cross-hybrids DeAnza (*12*) and Victoria (*16*) have flavonoid profiles that place them distinctly from the *Z. matrella* group and between *Z. matrella* lines and their common *Z. japonica* parent, El Toro (*19*). It is interesting to see that Z-18 and Miyako clustered closely to DeAnza and Victoria. The flavonoid data also resulted in two very distinct groups of *Z. japonica*. Crowne, El Toro, and Jamur expressed flavonoids very similarly, suggesting very similar genetic backgrounds, and distinct from Zenith, Chinese Com., Korean Com., J-36, J-37, Zen 400, Meyer, J-14, and Zeon 500.

The resistance of Emerald appears to be genetically more distinct and may have different genes or mechanisms of resistance. For greater disease resistance, it may be advisable to breed either Cavalier or Zeon with Emerald. Although HT-210 was not previously listed as resistant to FAW, it clustered closely to Emerald and expressed partial insect resistance in this study. However, specific peaks 2, 8 and 9, 19, 21, and 22 were significantly greater in percentage for Emerald than for HT-210.

Flavonoid profiles and subsequent analyses in this study were found to be effective in clustering genotypes. Seasonal variation in flavonoids did not alter the genotyping except for the earliest sampling date. The flavonoids responsible for the high levels of resistance to Cavalier, Zeon, or Emerald were not definitively determined; however, this study provided an initial investigation of a few candidates for further study.

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