

Genotype and Environment Effects on Tocopherol, Tocotrienol, and γ -Oryzanol Contents of Southern U.S. Rice

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ABSTRACT

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Rice bran contains phytochemicals such as E vitamers (i.e., tocopherols and tocotrienols) and the γ -oryzanol fraction that reportedly may have positive effects on human health. Brown rice, rice bran, and rice bran extracts are therefore attractive candidates for use in the development of functional foods. The objectives of this project were to quantify the effects of genetics versus environment on the tocopherol, tocotrienol, and γ -oryzanol contents of Southern U.S. rice and to determine associations between the levels of these phytochemicals. Seven rice cultivars grown in four states during two years were studied. Averaged across all samples, the content of α -tocotrienol > γ -tocotrienol > α -

tocopherol > γ -tocopherol, and the tocopherols and tocotrienols were 27.5 and 72.5% of the total E vitamer content, respectively. Total E vitamer content ranged from 179 to 389 mg/kg and γ -oryzanol from 2,510 to 6,864 mg/kg. A low correlation between total E vitamer and γ -oryzanol contents suggests that to obtain rice bran with high levels of both of these fractions, new cultivars would need to be produced using hybridization and selection. In general, growing environment had a greater effect on E vitamer and γ -oryzanol levels than did genotype. Therefore, rice breeders selecting genotypes with optimized levels of E vitamers and γ -oryzanol will need to grow their breeding material in multiple years and locations.

Rice bran and its extracted oil reportedly have total and low density lipoprotein (LDL) serum cholesterol-lowering abilities in laboratory animals and humans (Seetharamaiah and Chandrasckhara 1989; Kahlon et al 1996; Sugano and Tsuji 1997; Gerhardt and Gallo 1998). The Vitamin E and γ -oryzanol (γ -ORY) fractions are proposed as the casual factors of these health benefits (Rong et al 1997; Qureshi et al 1997; Xu et al 2001).

The Vitamin E fraction (E vitamers) is composed of tocopherols and tocotrienols; the former have saturated side chains while the latter are unsaturated. In nature, each of these two groups include four isomers (alpha, beta, gamma, and delta) which differ in the number and position of methyl substitutions on a benzene ring. There is growing evidence that these compounds play a role in suppressing the pathogenesis of cardiovascular disease, as mentioned above, as well as cancer, macular degeneration, and anti-inflammatory disease (Bramley et al 2000). Rice bran oil and palm oil contain relatively high tocotrienol levels compared with other plant-derived oils. Tocotrienol-rich fractions from rice bran reportedly lower plasma total and LDL cholesterol levels in humans (Qureshi et al 1997; Qureshi et al 2001). Various mechanisms for these effects have been proposed, including inhibition of cholesterol oxidation (Xu et al 2001) and inhibition of HMG-CoA reductase, a rate-limiting enzyme involved with cholesterol synthesis (Parker et al 1993). Tocotrienols have also been suggested to have neuroprotective effects, independent of their antioxidant activity (Packer et al 2001). Commercial preparations of palm oil tocotrienols have been available for sometime, and microencapsulated tocotrienols from rice bran are also now being marketed (Pszczola 2001).

Gamma-oryzanol (γ -ORY) is a rice bran fraction composed of sterol esters of ferulic acid. Xu and Godber (1999) reported that this fraction contains 10 compounds, while Akihisa et al (2000) identified 12 ferulates from a rice bran extract. The three major constituents are cycloartenyl ferulate, 24-methylenecycloartanyl

ferulate, and campesterol ferulate (Xu and Godber 1999). Rong et al (1997) reported that the γ -ORY fraction decreased laboratory animal serum cholesterol absorption and formation of aortic fatty streaks. The mechanisms behind these effects continue to be debated but include the inhibition of cholesterol absorption and antioxidant activity that decreases oxidized cholesterol formation (Rong et al 1997; Xu et al 2001). Other proposed health benefits of γ -ORY include antitumor activity (Akihisa et al 2000).

Rice bran stabilization and oil extraction reportedly causes a decrease in the levels of vitamin E and γ -ORY (Shin et al 1997; Xu and Godber 2000). Modifying rice bran phytochemical levels through breeding is a potential means to offset losses that occur during processing. This would increase the value of brown rice and bran for use as functional foods. However, no information was found in the literature on the genetic and environmental variation of the levels of tocopherols, tocotrienols, and γ -ORY in rice bran. Peterson and Qureshi (1993) reported sufficient variation in the oat and barley vitamin E fraction (1.5- and 2.0-fold, respectively) to assume that increased cultivar levels would be feasible. Variation in oat E vitamer levels resulted more from genetics than the environment, and the reverse was found for barley. Alpha-tocopherol levels across various buckwheat cultivars reportedly varied fivefold (Honda 1995). There are no such reports available for rice.

Nutrient databases such as the USDA Nutrient Database (<http://www.nal.usda.gov/fnic/foodcomp/>, USDA) and the Brazilian Table of Food Composition (<http://www.fcf.usp.br/tabela/tbcamenu.php>, USP) are relied on by the research community and the food industry. For example, researchers use these databases to calculate nutrient intake from dietary histories they have collected. Data on the mean and range in rice E vitamers and γ -ORY contents across cultivars and growing locations are not available for inclusion in these databases. The objectives of this project were to quantify the effects of genetics versus environment on rice tocopherol, tocotrienol, and γ -ORY levels and to determine relationships between the levels of these phytochemicals across several U.S. cultivars.

MATERIALS AND METHODS

Rough rice (*Oryza sativa*, L.) samples of 'AB647', 'Bengal', 'Cypress', 'Dellmati', 'LaGrue', 'Toro 2', and 'Wells' were obtained within one month of harvest from the 1999 and 2000 Uniform Rice Regional Nursery (URRN) grown in Arkansas, Louisiana, Mississippi, and Texas. This group of cultivars includes conventional U.S. long grain, conventional U.S. medium grain, and specialty rice types. The samples were hulled on a rice huller (Satake model THO35A) and immature kernels removed. Hulled

¹ USDA-ARS, Rice Research Unit, 1509 Aggie Drive, Beaumont, TX 77713. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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samples (50 g each) were then milled for 30 sec using a McGill mill #1 with an 858-g weight in position 12 and 6 for long and medium grain types, respectively. The bran (the word bran is used to collectively mean bran and germ) was collected, sieved to remove any nonbran particles, freeze-dried, and stored at -20°C. Long and medium grain USDA Federal Grain Inspection Service milling standards were used to determine whiteness and surface lipid values of well- to hard-milled rice. Surface lipid content was determined by refluxing 5 g of milled rice with petroleum ether in a Goldfish extraction apparatus for 30 min. The solvent was collected and evaporated; percent of surface lipid content was calculated as the mass of the extracted lipid divided by the beginning total milled rice mass. All solvents were HPLC-grade (Fisher Scientific, Fair Lawn, NJ).

Bran (0.25 g) was mixed with 5.00 mL of hexane and vortexed for 15 sec. The tube was capped and incubated in a 60°C water bath for 30 min. During the incubation, the test tube was vortexed twice. The hexane layer was separated from the rice bran sample after centrifuging at 2,000 × g for 10 min. An additional 5 mL of hexane was added to reextract the bran residual without incubation. The separated hexane layers were combined together. The hexane was evaporated under a nitrogen flow and 1 mL of hexane was added and transferred to an HPLC sample vial.

The HPLC system consisted of a 510 pump, 715 Ultra WISP injector, 486 UV detector, and 470 fluorescence detector (Waters Milford, MA). Chromatograms were recorded and processed using Waters Millennium chromatography software. Samples were injected into a 25-cm × 4.6-mm diameter column of 5-μm Supelcosil LC-Si (Supelco, Bellefonte, PA) which was preceded by a 5-cm × 4.6-mm guard column packed with 40-μm pellicular silica. The mobile phase consisted of 0.5% ethyl acetate and 0.5% acetic acid in hexane at a flow rate of 1.5 mL/min. The elutant was monitored using the fluorescence detector at 290-nm excitation and 330-nm emission for α- and γ-tocopherol (α-T and γ-T) and α- and γ-tocotrienol (α-T3 and γ-T3) and using the UV detector at 330 nm for the γ-ORY fraction. The α- and γ-tocopherol standards were obtained from Sigma-Aldrich (St. Louis, MO). The α- and γ-tocotrienol and γ-ORY standards were prepared according to methods described by Shin and Godber (1994) and Xu and Godber (1999).

PROC CORR (v. 7, SAS Institute, Cary NC) was used to examine correlations between the traits. Covariate analysis was performed when correlations between degree of milling, as measured by surface lipid content and a dependant variable was >0.30 (Fleiss 1986). Analysis of covariance was performed using PROC

GLM with surface lipid content used as a covariate. The homogeneity of slopes assumption was tested by including the covariate, a main effect, and their interaction in a model (Leon et al 1998). If the interaction was significant ($P > 0.05$) then the assumption was considered to be violated and the interaction was then included in the final model. Analysis of variance (PROC GLM) was performed when degree of milling was not moderately correlated (≤ 0.30) with a dependant variable. Least squares means were calculated using the Tukey-Kramer LS means adjustment for multiple comparisons.

RESULTS AND DISCUSSION

Averaged across cultivars, years, and states, whiteness and surface lipids ranged from 36.2 to 49.8 and 0.20 to 0.55%, respectively (Table I). The whiteness of well- to hard-milled medium and long grain USDA Federal Grain Inspection Service milling standards ranged from 34.8 to 39.3 and from 0.35 to 0.68% for surface lipid content (data not reported). The high maximum whiteness values for the samples compared with the milling standards likely resulted from chalky kernels that were visible in some of the samples but not in the milling standards. Averaged across all samples, the content of α-T3 > γ-T3 > α-T > γ-T and the tocopherols and tocotrienols were 27.5 and 72.5% of total E vitamers content, respectively. Among barley and oat samples, α-T3 was also the isomer in greatest quantity followed by α-T (Peterson and Qureshi 1993). Total E vitamers ranged from 179.21 to 388.54 mg/kg. Had all the isomers been quantified, these values would have been slightly higher. Shin et al (1997) reported the total E vitamers content, including all of the isomers of a commercial sample of rice bran to be 300 mg/kg. Averaged across cultivars, years, and states the range in bran γ-ORY (2,510 to 6,865 mg/kg) content was greater than the range (2,400 to 4,700 mg/kg of γ-ORY) found in studies of commercially milled rice bran (Norton 1995; Hu et al 1996; Lloyd et al 2000). The rice bran mean γ-ORY content in the present study was 15.8-fold greater than that of the mean total E vitamers content. Studies of commercially milled rice bran have reported these differences to be ≈10-fold (Shin et al 1997). Such differences are not surprising, considering that the phytochemical extraction in the present study was performed without a saponification step. Saponification causes some degree of γ-ORY hydrolysis and would thus cause lower extraction recoveries.

TABLE I
Trait Levels Across Cultivars, Years, and Growing Locations

Trait	N	Mean	Min	Max
Whiteness	104	41.84	36.20	49.80
Surface lipids (%)	112	0.32	0.20	0.55
α-Tocopherol (mg/kg)	112	58.27	26.89	93.71
γ-Tocopherol (mg/kg)	111	16.04	6.31	58.68
α-Tocotrienol (mg/kg)	112	105.22	46.97	165.00
γ-Tocotrienol (mg/kg)	112	89.77	60.93	130.05
Total E vitamers (mg/kg)	112	269.31	179.21	388.54
γ-Oryzanol (mg/kg)	112	4,263.56	2,510.26	6,864.65

TABLE III
Mean Squares for Analysis of Variance Across Cultivars, Years, and States^a

Source	DF	γ-Tocopherol	γ-Tocotrienol	γ-Oryzanol
Cultivar (C)	6	0.137	1.370	5,056
Year (Y)	1	0.000	0.199	4,267
State (S)	3	0.088	2.190	3,005
C × Y	6	0.050	0.693	388
C × S	18	0.028	0.286	947
Y × S	3	0.019	0.740	5,414
C × Y × S	18	0.028	0.404	552

^a All sources of variation were significant at $P < 0.05$.

TABLE II
Phenotypic Correlation Coefficients Among Rice Tocopherol, Tocotrienol, and γ-Oryzanol Contents and Degree of Milling^a

	Whiteness	Surface Lipids	α-Tocopherol	γ-Tocopherol	α-Tocotrienol	γ-Tocotrienol	Total E Vitamers
Surface lipids	-0.48 (<0.01)						
α-Tocopherol	-0.49 (<0.01)	0.39 (<0.01)					
γ-Tocopherol	0.14 (ns)	-0.01 (ns)	0.02 (ns)				
α-Tocotrienol	-0.48 (<0.01)	0.64 (<0.01)	0.61 (<0.01)	-0.03 (ns)			
γ-Tocotrienol	0.25 (0.01)	0.04 (ns)	0.03 (ns)	0.42 (<0.01)	0.09 (ns)		
Total E Vitamers	-0.39 (<0.01)	0.57 (<0.01)	0.70 (<0.01)	0.24 (0.01)	0.90 (<0.01)	0.46 (<0.01)	
γ-Oryzanol	-0.48 (<0.01)	0.18 (0.05)	0.38 (<0.01)	-0.17 (ns)	0.34 (0.01)	0.26 (0.01)	0.41 (<0.01)

^a Pearson Product Moment correlation coefficients and significance levels in parentheses; ns = not significant.

Bran fractions from different commercial rice milling steps reportedly vary in tocopherol, tocotrienol, and γ -ORY contents (Lloyd et al 2000). Consequently, we hypothesized that the degree of milling would affect the bran phytochemical levels and this effect would need to be factored out of the genetic-by-environment analysis. Chemical-based degree of milling methods such as percent surface lipids are accurate and repeatable but time-consuming. The Satake milling meter optical measure of degree of milling is a rapid method reported to be highly correlated to surface lipid content within a cultivar, but less so across cultivars (Siebenmorgen and Sun 1994). The correlation between milling meter whiteness values and surface lipids across cultivars and growing conditions has not been reported. In this study, if the correlation coefficient across cultivars and states in 1999 was >0.85 , we planned to use whiteness to correct the data for degree of milling differences. The correlation coefficient between these two traits in 1999 was $r = 0.53$, thus it was determined that surface lipid content would need to be analyzed and used as the degree of milling measure throughout the study.

Pearson Product-Moment Correlation coefficients were determined among tocopherols, tocotrienols, and γ -ORY and degree of milling was averaged across cultivars, years, and states (Table II). Total E vitamers and γ -ORY were positively correlated ($r = 0.41$.) Correlation coefficients were also significant between γ -ORY and α -T ($r = 0.38$), α -T3 ($r = 0.34$), and γ -T3 ($r = 0.26$). This low level of association suggests it may be possible to achieve higher levels of both E vitamers and γ -ORY through genetic recombination. The α -E vitamers were correlated among themselves ($r = 0.61$), as were the γ -E vitamers isomers ($r = 0.42$). Correlation

analysis performed among the tocopherols and among the tocotrienols was not significant, however. This is surprising, considering that γ -T is thought to be the precursor of α -T (Soll and Schultz 1980). We have also found that α -T and γ -T levels follow a similar sigmoid-like curve during maturation (data not shown). The implications of these results are that these compounds can be independently selected for during the breeding process. Correlations between α -T and γ -T were also not found in studies of collections of *Brassica napus* L. and maize hybrids (Goffman and Becker 2001a,b; Goffman and Böhme 2001). However, Peterson and Qureshi (1993) reported that barley and oat α -T and γ -T contents were positively correlated at a moderate level across genotypes and growing locations.

Surface lipid content was not correlated (i.e., < 0.30) with γ -T, γ -T3, or γ -ORY, thus analysis of variance was used to determine which effects were significant for these traits (Table II). Correlations were significant between surface lipids and α -T, α -T3, and total E vitamers (Table II). Consequently, analysis of covariance (surface lipids used as the covariate) was used to evaluate significant effects for these traits. The homogeneity of slopes assumption required to perform the analyses of covariance was violated in one instance. Specifically, a significant effect was found for the interaction between surface lipid content and year on α -T content.

Genotype and environment played significant roles in controlling the amount of tocopherol, tocotrienol, and γ -ORY contents in the bran of the rice cultivars studied (Tables III and IV). The largest amount of variance explained for α -T and γ -T3 contents was due to cultivar. The effect of year was greatest on α -T3. State explained the largest portion of the variance in γ -T3 content. For total E vitamers, the largest portion of the variance was explained by year, followed by state, and year-by-state interaction. The top three sources of variance for γ -ORY content were ranked in the order: year-by-state interaction, cultivar, and year. Because of the large effect of the environment on these phytochemical levels, it appears that rice breeding programs will need to evaluate progeny in multiple environments before selecting those with enhanced phytochemical levels. Similar conclusions were reported by Peterson and Qureshi (1993) in a genotype-by-environment study of oats but not for barley. These authors found oat and barley total E vitamers were affected by location $>$ genotype $>$ genotype-by-location interaction, and genotype $>$ genotype-by-location interaction, respectively.

Averaged across all states and years, Cypress had the greatest level of total E vitamers (Table V). Bengal contained the highest levels of α -T and α -T3. The levels of γ -T and γ -T3 were highest in AB647. Toro 2 had significantly greater levels of γ -ORY compared with the other cultivars. Samples from the 2000 growing

TABLE IV
Mean Squares for Analysis of Covariance Across Cultivars, Years, and States^{a,b}

Source	DF	α -Tocopherol	α -Tocotrienol	Total Tocols
Surface lipids	1	ns	0.124	3.504
Cultivar (C)	6	1.033	1.128	2.740
Year (Y)	1	0.289	28.116	57.244
State (S)	3	0.456	0.477	8.776
C \times Y	6	0.072	ns	0.804
C \times S	18	0.097	0.080	1.397
Y \times S	3	0.230	0.183	3.331
Surface lipids \times Y ^c	1	0.116	–	–

^a Surface lipid content was used as a covariate in all the models.

^b All sources of variation significant at $P < 0.05$ except those noted as not significant (ns).

^c All other interactions with the covariate were not significant and thus not included in the models.

TABLE V
Least Squares Means for Tocopherol, Tocotrienol, and Gamma Oryzanol Contents (mg/kg)^{a,b}

	α -Tocopherol	γ -Tocopherol	α -Tocotrienol	γ -Tocotrienol	Total E Vitamers	γ -Oryzanol
Cultivar						
AB647	39.51c	20.34a	83.99e	108.76a	241.89d	3862.43d
Bengal	71.80a	15.38b	115.56a	81.64e	283.46a,b	4243.09c
Cypress	70.04a,b	18.53a,b	114.47a,b	93.87b	309.37a	4144.54c
Dellmati	59.56b	14.86b	100.71d	84.94d	255.06c,d	4410.07b
LaGrue	64.06b	16.48a,b	106.06c	84.45d	268.71b,c	3676.06e
Toro2	57.79b	11.11b	108.44b,c	85.49d	260.31c,d	5414.72a
Wells	60.61b	15.57a,b	107.3b,c	89.24c	266.25b,c	4093.97c
Year						
1999	54.84b	16.03a	68.13b	88.43b	216.67b	4068.37b
2000	66.12a	16.05a	142.23a	91.11a	321.94a	4458.74a
State						
Arkansas	65.09a	15.97a	110.63a	100.29a	289.11a	4717.32a
Louisiana	63.13a,b	16.93a	106.36b	93.79b	280.26a	4096.99c
Mississippi	54.47c	13.58b	102.14c	83.58c	253.13b	4273.42b
Texas	59.24b	17.66a	101.75c	81.43d	254.71b	3966.50d

^a Tukey-Kramer least significant means adjustment for multiple comparisons used for all traits.

^b Values followed by the same letter in a column and subgroup are not significantly different ($P < 0.05$).

season had significantly more γ -ORY, total E vitamers, and E vitamer isomers, except for γ -T levels, which were the same in both years. Averaged across both years, the total E vitamer content, α -T, α -T3, γ -T3, and γ -ORY contents were all highest in samples grown in Arkansas. The data indicates that the levels of these phytochemicals are very sensitive to a cultivar's growing conditions.

CONCLUSIONS

Significant genetic variation in tocopherol, tocotrienol, and γ -ORY contents was found in U.S. rice cultivars grown in several locations. It is likely that a greater range in the levels of these phytochemicals will be found in exotic germplasm and wild species. Low correlations between E vitamers and γ -ORY contents indicates that, to obtain rice bran with high levels of both of these fractions, new cultivars will need to be produced. Growing environment and cultivar-by-growing environment interactions also significantly affected total E vitamer and γ -ORY levels. Therefore, unless genetic markers become available for these traits, rice breeders selecting genotypes with optimized levels of E vitamers and γ -ORY would need to grow their breeding material in multiple years and locations. However, because γ -ORY variation was more strongly affected by cultivar, selecting for high levels of this fraction compared with total E vitamer levels may require fewer evaluations of breeding lines across environments. Further research to study the heritability and selection response of these phytochemicals is warranted. Also, any yield drag or other undesirable effects that might accompany selecting for higher levels of rice E vitamers or γ -ORY need to be evaluated.

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