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Variability in Nutraceutical Lipid Content of Selected Rice (*Oryza sativa* L. spp. *indica*) Germplasms

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Abstract: Rice (*Oryza sativa* L.) contains many high-value nutritional compounds, including nutraceutical lipid compounds that offer health benefits. An understanding of the genetic variability in the lipid contents of different rice germplasms is necessary to support breeding programs. The goals of this study were: i) to investigate varietal differences in levels of the nutraceutical lipid α-tocopherol, γ-oryzanol, campesterol, β-sitosterol, octacosanol, and squalene and ii) to identify clusters of rice germplasms based on their lipid contents. Eighty-three *indica* rice subspecies were evaluated using a randomized complete block design with three replications. Research was conducted in Thailand during the 2016 rice-growing season. Significant differences were found among genotypes across all traits. The largest variation was found for β-sitosterol, followed by campesterol, octacosanol, and α-tocopherol. Variation in squalene content was small. Four principal components were found that accounted for 93.47% of overall variability. β-sitosterol, campesterol, and squalene were the principal discriminatory constituents. No significant correlation was found between color parameters and levels of these compounds, suggesting that former are of little use as an indirect marker for selection of fat-soluble nutraceuticals. Cluster analysis sorted the germplasm into nine clusters, based on their nutraceutical lipid content. TU-010, TU-027, TU-093, and TU-244 genotypes had the highest levels, making them a potentially useful genetic resource in breeding programs for nutraceutically-improved rice. The findings of this study can support the introduction of novel rice varieties with high added-value bioactive properties.

Keywords: landrace rice; fat-soluble nutraceuticals; β-sitosterol; genetic variability; cluster analysis

1. Introduction

Cardiovascular disease (CVD) is the largest cause of global deaths, accounting for an estimated 17.5 million deaths in 2012. This group includes hypertension, myocardial infarction, atherosclerosis, arrhythmias and valvular heart disease, coagulopathies, and strokes. The percentage of premature deaths attributed to CVDs ranges from 4% in high-income countries to 42% in low-income countries, reflecting inequalities between countries and populations [1]. Consumption of whole-grain cereals plays a pivotal role in preventing these chronic diseases and promoting health [2]. Epidemiological studies suggest that the low incidence of certain chronic diseases in rice-consuming regions of the

world might be associated with the high-value antioxidants that rice contains [3]. However, as dietary patterns have become more Western-oriented, consumption of rice has been decreasing. Policies that increase rice consumption can contribute to solving the economic problems of rural communities. One approach is the development of novel rice varieties with improved nutritional and bioactive properties [4].

Rice (*Oryza sativa* L.) contains more than a hundred bioactive compounds, including phytic acid, isovitexin, flavonoids, phenolics, polyphenols, anthocyanins, and proanthocyanins, and previously unknown compounds have been characterized and quantified in the rice germplasm [5–8]. Rice also has a high level of nutraceutical lipid compounds, such as tocopherol, tocotrienol, phytosterols, γ-oryzanol, octacosanol, and squalene, which are known to be powerful antioxidants [9,10]. These are potential inhibitors of cholesterol oxidation, have been shown to reduce serum cholesterol levels in animals, and are effective in treating anxiety neurosis, menopausal disorders, inflammatory diseases, and in inhibiting tumor growth [11–13]. These bioactive lipid compounds are concentrated in the rice germ or bran and are lost during milling or polishing [14]. Most consumers also prefer the palatability of milled or white rice, which has little or no bran on the endosperm, to brown rice. However, brown rice offers the greatest concentration of nutraceutical lipid compounds, which is thought to be encouraging increased consumption.

The nutraceutical lipid compound fraction of rice and rice-based foods can be increased by selecting naturally antioxidant-rich genotypes for cultivation, by selective plant breeding to develop genotypes with still higher phytochemical content, or by varying pre and postharvest conditions [15–17]. Such improvements, along with the development of higher-yielding varieties through conventional breeding methods, may be economically positive for growers as well as widening consumer choice. A necessary preliminary step in the identification of efficient selection methods is to investigate the role of genetic variability in the nutritional values offered by different populations. The genetic variation in the nutraceutical lipid compounds of rice and rice products has been characterized [16,17]. Wide variation was observed in the γ-oryzanol and phytosterol contents of Korean rice [14] and the lipid and fatty acid content of rice bran [18]. However, little information is available on the effect of genetic variability on the octacosanal and squalene content of landrace rice.

Thailand is a center of diversity for both wild and cultivated rice (*O. sativa* L. ssp. *indica*) and has a range of native landraces [19]. Landrace varieties still account for some 20% of the country's cultivated rice paddy [20]. However, cultivation of landraces is gradually decreasing under pressure from urbanization and industrialization [21]. Landrace rice plays a very important role in local food security and provides a vast genetic reserve, especially for variability in high-value bioactives. Its analysis can support the improvement of the nutritional value of rice and the development of materials with pharmaceutical and nutraceutical applications. Previous studies have reported variability in the fat-soluble content of rice and its coproducts, but these have examined only a subset of genotypes, most of which are commercial cultivars of Thai rice [22–24]. The genetic diversity of rice germplasms is important when categorizing sources of variation or identifying genotypes with superior nutraceutical lipid contents. The goal of the current study was to evaluate genetic variability in production of α-tocopherol, γ-oryzanol, campesterol, β-sitosterol, octacosanol, and squalene in Thai landrace rice germplasm of the *indica* subspecies. The lipid compounds that are the goal of genetic breeding programs can be used to select nutraceutical compound-rich genotypes. Moreover, these landraces represent an unexplored germplasm pool that may provide donors of alleles that favor nutritionally valuable fat-soluble phytochemicals. The study was also intended to provide valuable data to cultivators, nutritional researchers, and rice breeders.

2. Materials and Methods

2.1. Chemicals and Reagents

Authentic lipid compound standards were purchased from Sigma-Aldrich (USA): α-tocopherol (CAS: 10191-41-0), γ-oryzanol, 5-α-cholestane (CAS: 481-21-0), campesterol (CAS: 474-62-4), β-sitosterol (CAS: 83-46-5), 1-octacosanol (CAS: 557-61-9), and squalene (CAS: 111-02-4). All chemicals and reagents were of analytical grade.

2.2. Rice Germplasm Description and Experimental Design

Eighty-one rice (*O. sativa* L. spp. *indica*) germplasms were collected from diverse geographical regions of Thailand and compared with KDML105 and RD6, which are respectively the most popular varieties of nonglutinous and glutinous rice. They were selected based on their pigmentation, seed size, and yield (Supplementary Table S1). These genotypes were planted in the 2016 rice growing season (June–December 2016) at the Experiment Field of the Department of Agricultural Technology, Thammasat University, Pathum Thani, Thailand (14°04'28.6" N, 100°36'33.0" E, and 7.8 m above sea level) using a randomized complete block design (RCBD) with three replications. Each genotype was planted as an experimental unit of four 1 × 5 m plots, with 45 cm between plots. The seedlings were transplanted at a hill spacing of 15 × 15 cm, with a single seedling per hill. Soil preparation, planting, and other agronomic practices were carried out uniformly following the recommendations for good agricultural practices (GAP). Ten panicles per genotype were randomly harvested depending on their harvesting maturity stages (data not shown) and oven-dried at 50 °C to a moisture content <14%. Seeds were manually dehulled and their color parameters were measured prior to milling. Samples were milled to a fine powder (CM190, Cemotec^{TM}), passed through a 100-mesh screen mesh, thoroughly mixed, and stored at −20 °C until analysis.

2.3. Color Parameters

Color attributes were measured using a HunterLab miniscan XE PLUS colorimeter (Mod. PL50, Hunter Associates Laboratory Inc., Reston, VA, USA) that was calibrated prior to data collection using a standard white HunterLab calibration reflector plate. The color value was determined from 2 g randomly-selected samples of each variety. The color was expressed as lightness (L*), chroma (C*), and hue angle (h°). C* represented the color intensity, and h° was expressed as a degree range from 0° to 360° (0° = red, 90° = yellow, 180° = green, and 270° = blue) [25].

2.4. Analysis of α-tocopherol and γ-oryzanol

Simultaneous determination of α-tocopherol and γ-oryzanol was conducted following the method of Butsat and Siriamornpun [22] with slight modifications. A 1 g sample of powdered rice was extracted with 10 mL of acetone and vortexed at maximum speed for 1 min. The solution was centrifuged at 2500 rpm for 20 min, after which the solvent was removed. The supernatant layer was combined, before evaporation to dryness in a low-temperature vacuum evaporator. The residual was further extracted twice, and determinations were made in triplicate.

Reversed-phase HPLC analysis of α-tocopherol and γ-oryzanol was performed using a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with a binary pump (LC-20AC) and a diode array detector (SPD-M20A). Chromatographic separation was performed in an Xselect CHS C-18 column (4.6 × 250 mm, i.d. 5 μm) (Waters Corporation, Milford, MA, USA). The mobile phase consisted of acetonitrile/methanol (25:75, v/v) at a flow rate of 1.5 mL/min. The solution was passed through a 0.22 μm filter (Millipore Corp). Operating conditions were as follows: column temperature 38 °C, injection volume 20 μl, and photodiode-array detection at 292 and 325 nm for α-tocopherol and γ-oryzanol, respectively. Different dilutions of individual external standards ranging from low to high concentrations were prepared and standard curves were plotted. The results were expressed as μg lipid per g of dry weight.

2.5. Analysis of Campesterol, β-sitosterol, Octacosanol, and Squalene

Simultaneous chromatographic separation of campesterol, β-sitosterol, octacosanol, and squalene was conducted using GC-MS, following the method of Siriamornpun et al. [26], with slight modifications. A 1 g powdered brown rice sample was placed in a screw-capped tube containing 5 mL of synthetic antioxidant (ethanolic pyrogallol) with 1 mg of 5-α-cholestane as internal standard. The solutions were saponified with 2 mL of KOH (10.70 mM) and 2 mL of ethanol and NaCl (0.17 mM). The tubes were placed in a water bath and heated to 70 °C, then mixed at 10 min intervals over a 45 min digestion period. The solutions were cooled in an ice bath for 10 min, then 10 mL of NaCl (0.17 mM) was added. The suspension was then extracted twice using 10 mL of n-hexane/ethyl acetate (9:1 v/v). The upper organic layer was collected and dried in a low-temperature vacuum evaporator. The dry residue was derivatized with 100 μL of BSTFA:TMCS (99:1 v/v) and 1 mL of pyridine (99%) at 60 °C for 30 min. The residue was dissolved in 2 mL of hexane, then the clear solution (1 μL) was injected into the GC-MS system (QP2010, Shimadzu, Japan) equipped with an HP5 column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Helium gas was used as a carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was held at 280 °C throughout the analysis, while the transfer line temperature was 230 °C. The initial column temperature of 60 °C was held for 1 min, increased at 30 °C/min to 250 °C and held for 10 min, then to 280 °C at 1 °C/min and maintained for a further 13 min. Linearity was evaluated by fresh preparation of individual external standard solutions, at five concentration levels in the interval of 2–100 μM for all compounds. The results were also expressed as μg lipid per g of dry weight.

2.6. Statistical Analysis

The results were analyzed from the mean of determinations for duplicate samples prepared for each genotype. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were performed in STATISTIX9 (Analytical Software, Tallahassee, FL, USA). Coefficients of variation (CV) were calculated from the ratios between standard deviations (SD) and population means, to represent the variability among genotypes. Hierarchical agglomerative clustering was then conducted for lipid compounds, using the Ward criterion. Principal component and cluster analysis of the lipid compounds were performed using JMP Pro software (version 14.0, SAS institute Inc., Chicago, IL, USA). A heat map showing Pearson's correlation coefficients for color parameters and lipid compounds was constructed using Microsoft Excel 2016.

3. Results and Discussion

3.1. Analysis of Variance

Identification of genetic resources with high levels of the targeted fat-soluble nutraceutical compounds is a necessary preliminary step when enhancing bioactive levels through conventional plant breeding [27]. Descriptive statistics for the α-tocopherol, γ-oryzanol, campesterol, β-sitosterol, octacosanol, and squalene contents of the rice germplasm are shown in Table 1 and Supplementary Table S2. Large variation was observed in the β-sitosterol content with a CV of 86.9%, followed by campesterol (54.8%), octacosanol (54.3%), and α-tocopherol (49.8%). The lowest variation was found for squalene content (CV = 6.2%). Two groups could be distinguished by distribution of α-tocopherol (Figure 1A). The first had an α-tocopherol content of between 663 and 1673 μg/g of dry weight. A high proportion of genotypes fell into this class, with a mean of 1083 μg/g of dry weight. The second group had lower levels of this compound across the full range (≤663 μg/g of dry weight). The frequency distribution for γ-oryzanol content was continuous, ranging from 7477 to 24,613 μg/g of dry weight (Figure 1B). Most rice genotypes (75%) had a γ-oryzanol content exceeding 13,865 μg/g of dry weight. Wide variation was observed in the campesterol and β-sitosterol content, with respective ranges from 495 to 6699 μg/g of dry weight and from 470 to 40,035 μg/g of dry weight (Figure 1C,D). Campesterol and β-sitosterol were normally distributed, though most of the genotypes (75%) had phytosterol contents lower than the mean values (2016 ± 1106 and 7024 ± 6107 μg/g of dry weight,

respectively). Wide variation was also observed in octacosanol content, which ranged from 352 to 3522 μg/g of dry weight (Figure 1E). Half of the genotypes had an octacosanol content above the mean value (892 ± 484 μg/g of dry weight). Squalene content showed little variation, with a range from 175 to 266 μg/g of dry weight (Figure 1F). Most genotypes fell within the first two quartiles, and below the average value (183.7 ± 11.3 μg/g of dry weight). These results indicated that most of the variation in nutraceutical lipid content was accounted for by the genotype. This finding is in agreement with those of Goffman et al. [18], who reported that genetic factors have a greater influence on the oil and fatty acid contents than do seasonal factors. Therefore, selection for high levels of these compounds may require less frequent evaluation of rice germplasm across years or locations [28]. Miller and Engel [16] reported environmental influences on the content and composition of γ-oryzanol and seteryl ferulate. Bergman and Xu [28] reported that growing conditions had a greater effect on tocopherol, tocotrienol, and γ-oryzanol levels than did the rice genotype. The range in the nutraceutical lipid compound levels of grains may be due to a combination of genetic variability, soil conditions, and environmental factors. Rice breeders who wish to select for germplasm with optimal levels of these compounds should plant the genetic resources in multiple seasons or locations, to estimate the relative contributions made by the materials used and the growth conditions [29]. In practice, the conditions under which samples available for analysis were grown are unknown. Further study will be required to elucidate the relationship between factors such as climate or soil quality and agricultural practices [16]. The low rate of genetic variability is the main limiting factor in rice breeding programs aimed at increasing squalene levels. The goal of rice breeders is to create variation in the breeding materials by hybridization or induced mutations.

Table 1. Descriptive statistics for nutraceutical lipid compounds of the 83 rice germplasms [1/].

Compounds	Mean ± SD	Min.	Max.	CV	F-Value
α -tocopherol	920 ± 458	78	1,673	49.8	48.1 **
γ -oryzanol	16,501 ± 3,860	7,477	24,613	23.3	39.0 **
campesterol	2,016 ± 1,106	495	6,699	54.8	186.6 **
β-sitosterol	7,024 ± 6,107	470	40,035	86.9	8,451.8 **
octacosanol	892 ± 484	352	3,522	54.3	80.4 **
squalene	183.7 ± 11.3	175	266	6.2	61.7 **

** significant at 1% level. [1/] expressed as μg/g of dry weight.

3.2. Multivariate Analysis

One of the key challenges is the choice of variables to analyze, since more or less data are available on the geographical location, genotype, rice type, and other markers. The two most widely-used multivariate analysis techniques for interdependent responses of rice germplasm are principal component analysis (PCA) and hierarchical cluster analysis [30]. Principal components are extracted by order of contribution to the total variance and, by examining the loadings of the variables in the first components, it is possible to measure the relevance of each variable. The first four principle components are the most important in reflecting the variation among rice genotypes that is useful for genotypic classification [31]. In this study, the first four components contributed approximately 93.47% of the total variation (Table 2), giving a clear idea of the structure underlying the variables analyzed. The first principal component represented β-sitosterol, campesterol, and squalene, and explained 42.04% of the total variance. The second component was mainly attributed to γ-oryzanol and α-tocopherol and accounted for a further 29.58% of the total variance. The third component was mainly contributed by octacosanol and accounted for 13.67% of the total variance. The fourth component was attributed mainly to β-sitosterol and accounted for 8.16% of the total variance. These findings were in agreement with previous studies, which reported that β-sitosterol and campesterol were the major factors contributing to variation of lipid content in Korean rice cultivars [14] and other cereals [15].

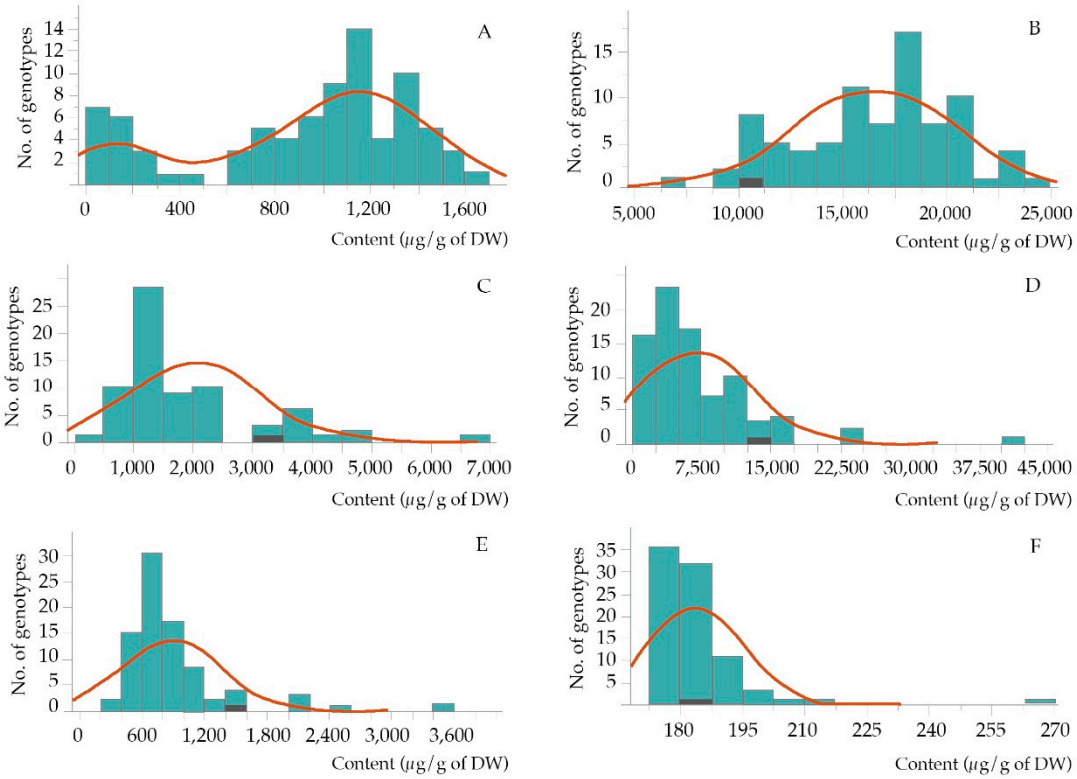

Figure 1. Distribution of the 83 rice germplasms by α–tocopherol (**A**), γ-oryzanol (**B**), campesterol (**C**), β-sitosterol (**D**), octacosanol (**E**), and squalene (**F**) contents.

Table 2. Coefficients and vectors associated with the first four principal components.

	First	Second	Third	Fourth
Eigenvalues	2.52	1.78	0.87	0.49
Percent variances	42.04	29.58	13.67	8.16
Cumulative % total variance	42.04	71.62	85.30	93.47
		Coefficient vector		
Compounds				
α-tocopherol	0.192	0.637	−0.194	0.07
γ-oryzanol	0.139	0.657	−0.167	0.21
campesterol	0.575	−0.053	−0.110	0.23
β-sitosterol	0.586	−0.139	−0.091	0.78
octacosanol	0.329	0.120	0.907	−0.46
squalene	0.401	−0.140	−0.091	−0.27

The goal of clustering is to divide data into distinct clusters in such a way that samples associated with the same cluster are considered similar in the pattern found, whereas samples associated with different clusters are as dissimilar as possible [32]. Based on the α-tocopherol, γ-oryzanol, campesterol, β-sitosterol, octacosanol, and squalene contents of 1–81 rice genotypes and two check varieties, nine distinct clusters were identified (Figure 2 and Supplementary Table S2). Cluster I comprised the three genotypes TU-001, TU-002, and TU-159. This cluster showed moderate levels of fat-soluble compounds, though TU-002 and TU-159 were low in squalene content. Cluster II comprised 12 genotypes (TU-002, TU-030, TU-070, TU-071, TU-106, TU-114, TU-138, TU-169, TU-172, TU-202, TU-203, and TU-241). Most exhibited average levels of α-tocopherol, γ-oryzanol, campesterol, β-sitosterol, and octacosanol, but low squalene levels. TU-203 had the highest α-tocopherol levels, and TU-241 the highest γ -oryzanol levels. Cluster III comprised 14 genotypes (TU-009, TU-028, TU-032, TU-043, TU-049, TU-050, TU-069,

TU-072, TU-115, TU-117, TU-124, TU-131, TU-196, and TU-197), most of which had moderate levels of fat-soluble compounds. TU-028, TU-072, TU-117, TU-124, and TU-197 had high γ-oryzanol levels.

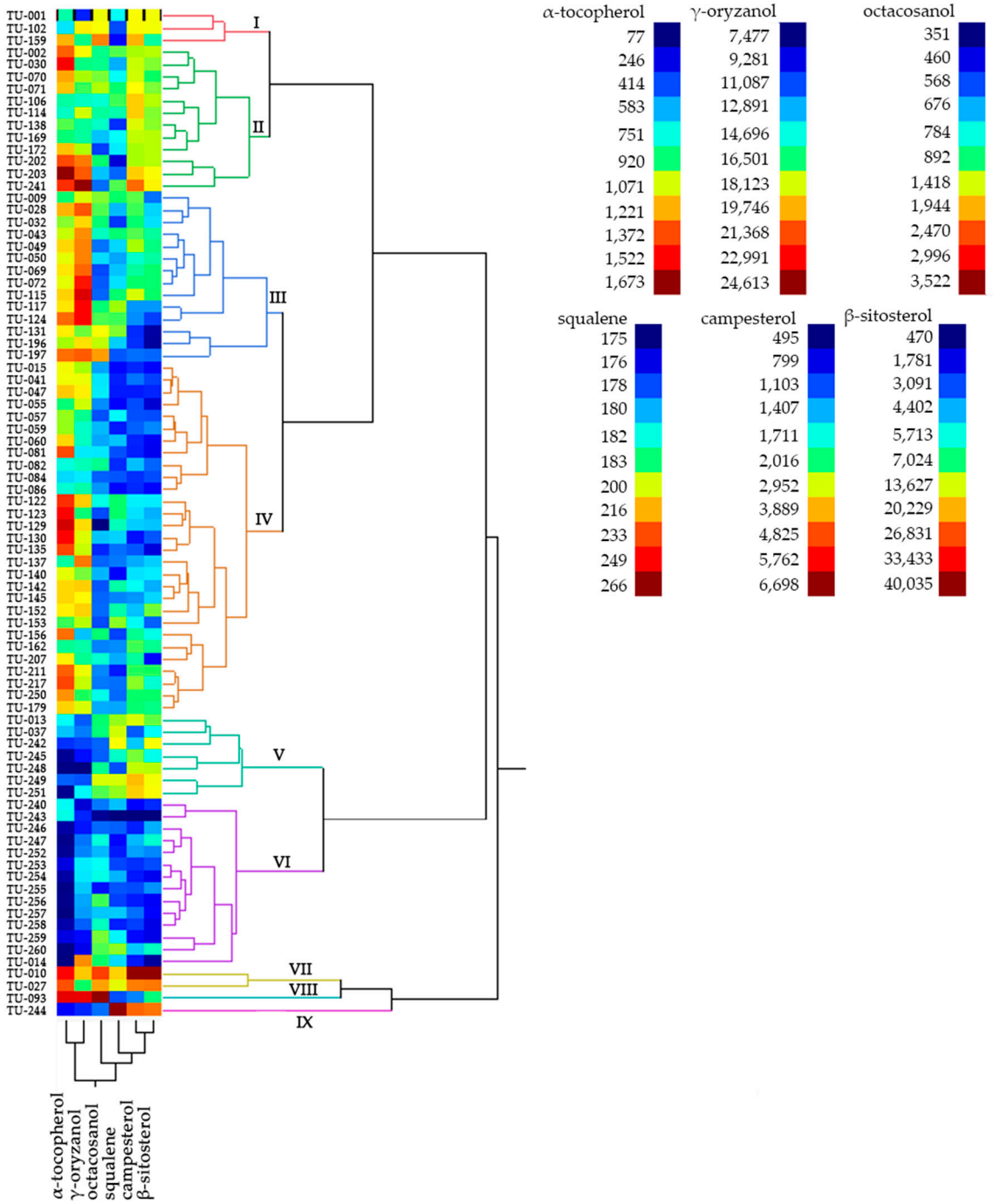

Figure 2. Dendrogram of genetic relationships among the 83 rice germplasms. Nine main clusters (I to IX) were formed. Clustering was performed using Ward's method based on six nutraceutical lipid compounds (scale: distance scale). [a] Clustering is done by column as well as row; the columns must be measured on the same scale.

Cluster IV was the largest group (34.9% of all genotypes) and comprised TU-015, TU-041, TU-047, TU-055, TU-057, TU-059, TU-060, TU-081, TU-082, TU-084, TU-086, TU-122, TU-123, TU-129, TU-130, TU-135, TU-137, TU-140, TU-142, TU-145, TU-152, TU-153, TU-156, TU-162, TU-179, TU-207, TU-211, TU-217, and TU-250. This group showed moderate average levels of α-tocopherol and γ-oryzanol,

matching group I. However, campesterol, β-sitosterol, octacosanol, and squalene levels were low. TU-081, TU-122, TU-123, TU-129, TU-130, and TU-135 showed high α-tocopherol levels.

Cluster V comprised the seven genotypes TU-013, TU-037, TU-242, TU-245, TU-248, TU-249, and TU-251. Most genotypes in this cluster showed low average levels of α-tocopherol and γ–oryzanol, and medium levels of campesterol, β-sitosterol, and squalene. Cluster VI comprised 14 rice genotypes (TU-014, TU-240, TU-243, TU-246, TU-247, TU-252, TU-253, TU-254, TU-255, TU-256, TU-257, TU-258, TU-259, and TU-260), most of which had low average levels of all lipid compounds. The exception was TU-014, which had high levels of γ-oryzanol.

Cluster VII comprised TU-010 and TU-027. This small cluster had high average values of α-tocopherol, γ–oryzanol, campesterol, β-sitosterol, and octacosanol. TU-010 had the highest levels of campesterol and β-sitostero, whereas TU-027 had moderate levels of γ–oryzanol and squalene. Cluster VIII comprised the single TU-093 genotype, which had extremely high values for octacosanol, high values for α-tocopherol and γ-oryzanol, but low to moderate values for campesterol, β-sitosterol, and squalene. Cluster IX comprised the TU-224 genotype, which had the highest squalene content, high campesterol and β-sitosterol content, but low α-tocopherol, γ-oryzanol, and octacosanol content.

The information obtained through cluster analysis was particularly useful as the best performing genotypes fell within one cluster, allowing them to be differentiated from the others [29]. The use of six nutraceutical lipid compounds therefore turned out to be appropriate for clustering rice germplasm. As germplasm characterization can support the conservation of crop genetic resources, crop protection, classification of the genotypes into heterotic groups, and crop improvement [31,33,34], the information from our study should be of use to rice breeders wishing to select parental lines that yield improved novel varieties. However, further germplasm characterization using molecular approaches will be necessary to establish whether this results from gene introgression.

Descriptions of nine clusters based on α-tocopherol, γ-oryzanol, campesterol, β-sitosterol, octacosanol, and squalene content are given in Table 3. Cluster I was characterized by low levels of α-tocopherol and γ-oryzanol and moderate levels of the other fat-soluble compounds. Relatively high γ-oryzanol levels were found in Clusters II, III, and IV, and especially in Cluster III. However, levels of other fat-soluble compounds were low. Clusters V and VI had low levels of all nutraceutical lipid compounds, and rice genotypes in these clusters are unsuitable for rice breeding programs. The genotypes in cluster VII were interesting, being relatively high in campesterol and β-sitosterol and moderate-to-high in other nutraceutical lipids. The rice genotype in cluster VIII was a potentially interesting source of α-tocopherol, γ-oryzanol, and octacosanol, which were at high levels. Finally, the genotype in cluster IX is also potentially useful for breeding programs, being high in squalene. Hybridization of group VII with groups VIII or IX may yield strains with enhanced nutraceutical lipid contents.

Table 3. Average nutraceutical lipid contents in nine clusters [1/].

Clusters	*n*	Lipid Compounds (μg/g of Dry Weight)					
		α-tocopherol	**γ-oryzanol**	**Campesterol**	**β-sitosterol**	**Octacosanol**	**Squalene**
I	*3*	910.4 ± 281.6	15,108.3 ± 4,276.4	3,393.5 ± 399.4	12,100.5 ± 4,841.9	1,737.0 ± 387.6	179.27 ± 2.2
II	*12*	1,163.5 ± 235.3	17,788.9 ± 2,600.0	3,036.0 ± 656.4	10,662.4 ± 3,237.9	850.94 ± 200.3	182.7 ± 4.0
III	*14*	1,127.8 ± 111.8	20,644.7 ± 1,826.9	1,870.7 ± 612.4	4899.7 ± 2,352.6	989.4 ± 420.3	183.5 ± 4.8
IV	*29*	1,164.3 ± 255.7	17,357.5 ± 1,964.4	1,536.8 ± 595.4	4698.9 ± 288.7	656.2 ± 105.9	180.2 ± 3.2
V	*7*	350.9 ± 259.2	11,044.0 ± 2,020.6	2,603.0 ± 995.3	11,116.7 ± 4,328.5	903.2 ± 283.1	193.0 ± 9.4
VI	*14*	214.2 ± 227.0	12,217.4 ± 2,841.3	1,060.6 ± 252.9	2,929.2 ± 1,743.9	770.9 ± 212.9	180.0 ± 4.0
VII	*2*	1,418.9 ± 75.0	18,045.0 ± 1,841.3	5,531.2 ± 1,650.7	32,007.5 ± 11,352.6	2,273.0 ± 352.9	206.2 ± 7.3
VIII	*1*	1,565.8 ± 0.0	23,423.0 ± 0.0	1,267.9 ± 0.0	6,974.8 ± 0.0	3,522.0 ± 0.0	178.6 ± 0.0
IX	*1*	283.7 ± 0.0	10,521.0 ± 0.0	4,571.0 ± 0.0	24,102.0 ± 0.0	606.7 ± 0.0	266.1 ± 0.0

n = Number of genotypes. [1/] Number of rice clusters from.

From the 81 test genotypes, the four that ranked top in fat-soluble nutraceutical content were identified (Figure 3). TU-093 and TU-010 were superior in α-tocopherol content, though no statistically significant difference from the KDML105 check variety was found (Figure 3A). These genotypes

were also higher in α-tocopherol than the glutinous rice check variety RD6 (by 2.09 and 1.97 times, respectively). TU-093 was also higher in γ-oryzanol than the RD6 or KDML105 check varieties, by 4.5 to 85.2 times (Figure 3B). Vegetarian diets that are high in phytosterols are associated with a reduced risk of breast cancer [23]. β-sitosterol and campesterol are the predominant and most common sterols found in rice [8,14,35], and these fat-soluble compounds were the focus of our study. TU-010 had the highest levels of campesterol and β-sitosterol in the study, and also higher than RD6 and KDML105, by multiples of 4.5 to 85.2 times (Figure 3C,D). It was also found to contain higher concentrations of β-sitosterol (40,035 µg/g of dry weight) than those reported for pigmented rice bran oil (4980–6250 µg/g RBO) [36] and short grain rice (12,480 µg/g of dry weight) [35], though lower concentrations than Pakistani rice (65,630 µg/g of dry weight) [8]. A rice genotype producing high levels of β-sitosterol should be commercially viable.

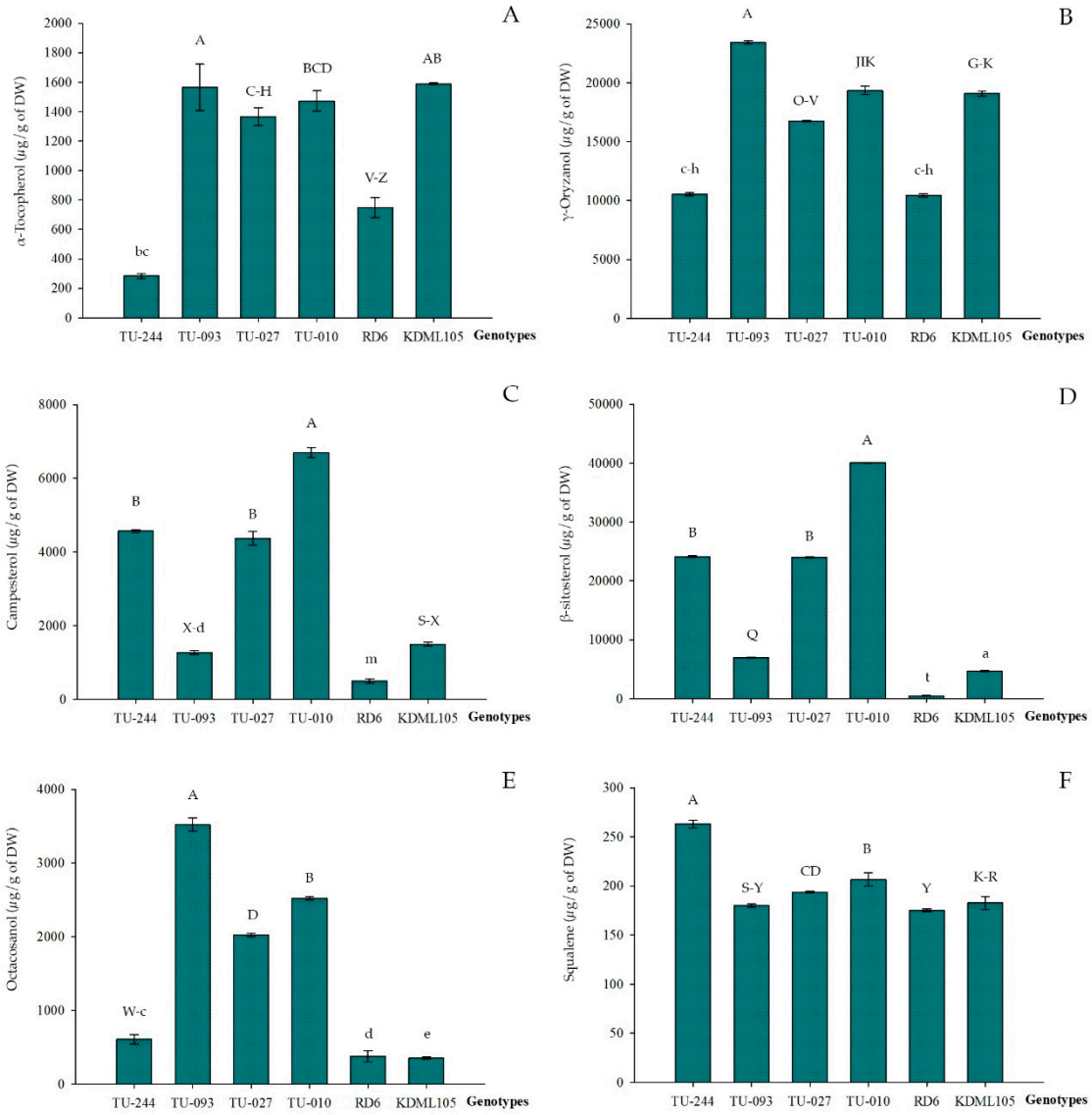

Figure 3. Comparison of germplasms from the predominant groups with check varieties by α-tocopherol (**A**), γ-oryzanol (**B**), campesterol (**C**), β-sitosterol (**D**), octacosanol (**E**), and squalene (**F**) content. [a] Means with different upper case and lower case letters are significantly different ($p \leq 0.05$) by Duncan's multiple range test (DMRT).

Octacosanol is the main component of policosanol, which can reduce total cholesterol, low-density lipoprotein, and platelet aggregation [5]. TU-093 had 3522 μg/g by dry weight of octacosanol, higher than the RD6 or KDML105 check genotypes by 9.4 and 10.0 times (Figure 3E), higher than short grain rice (100 μg/g by dry weight) [35], but lower than Korean rice (6,738 μg/g of lipid extract) [5]. Squalene, a fat-soluble compound, has been reported to be a quencher of singlet oxygen and a free radical scavenger [14]. Little information is available on the squalene content of rice germplasm, and especially of landraces. The squalene content of TU-244 (266.05 μg/g by dry weight) was 1.5 times that of RD6, 1.4 that of KDML105 (Figure 3F), and higher than that of Korean rice (0.16 to 59 μg/g of dry weight) [14]. TU-027 had medium-to-high levels of all lipid compounds. Overall, in terms of the high-value lipid compounds investigated, the *indica* subspecies were superior, especially TU-010, TU-093, and TU-244. This suggests potential uses in functional foods and nutraceuticals, improving added value [8]. Introgression of genes from these superior genotypes may allow breeding programs to create novel rice varieties containing these high-value lipid compounds.

3.3. Correlation

Colorimetric analysis is used to characterize and quantify the color properties of pigments and antioxidant-rich foods [37]. As the hypothesis underlying this study was that color properties are associated with nutraceutical lipid compounds, we measured the color parameters from light to dark. We also explored the use of color parameters as an indirect tool for selection of rice genotypes with high levels of fat-soluble compounds. The hue angle (h°) was found to be negatively and significantly correlated with lightness (L*) and the chromatic parameter (C*) (Figure 4). A positive and moderately significant correlation was found between L* and C* color parameters. Color parameters L* and C* were weakly correlated with α-tocopherol, but no other significant correlations were found between color parameters and other nutraceutical lipid compounds. Superior rice genotypes, including TU-010, TU-093, and TU-244, had colorless kernels. These results suggest that color parameters should not be used as indirect criteria for selection of these fat-soluble compounds. This confirms the findings of Min et al. [38], who found no association between rice bran color and natural vitamin E analogue or γ-oryzanol. However, a positive correlation has been reported between γ-oryzanol content and the yellow parameter in upland rice bran oil [39]. Another report noted that rice coproduct oils produced from colored rice contained higher concentrations of oryzanol and phytosterols than noncolored rice [36]. Research on this connection is continuing.

A positive and significant correlation was found between α-tocopherol and γ-oryzanol, but no correlations with other fat-soluble compounds. Similar results have been reported in brown rice with different origins [8,14,40]. These results suggest that simultaneous improvement in the α-tocopherol and γ-oryzanol of rice is possible. β-sistosterol was positively and significantly correlated with campesterol and moderately correlated with squalene. The identification of strong positive correlations between fat-soluble compounds suggests that pleiotropy is involved, with genes controlling many traits simultaneously [29]. Improvement in the lipid-soluble composition may also improve the nutritional properties of rice. However, the lack of correlation between α-tocopherol and γ-oryzanol and other lipid compounds suggests that boosting the levels of these fat-soluble compounds will require the development of new varieties through hybridization and selection.

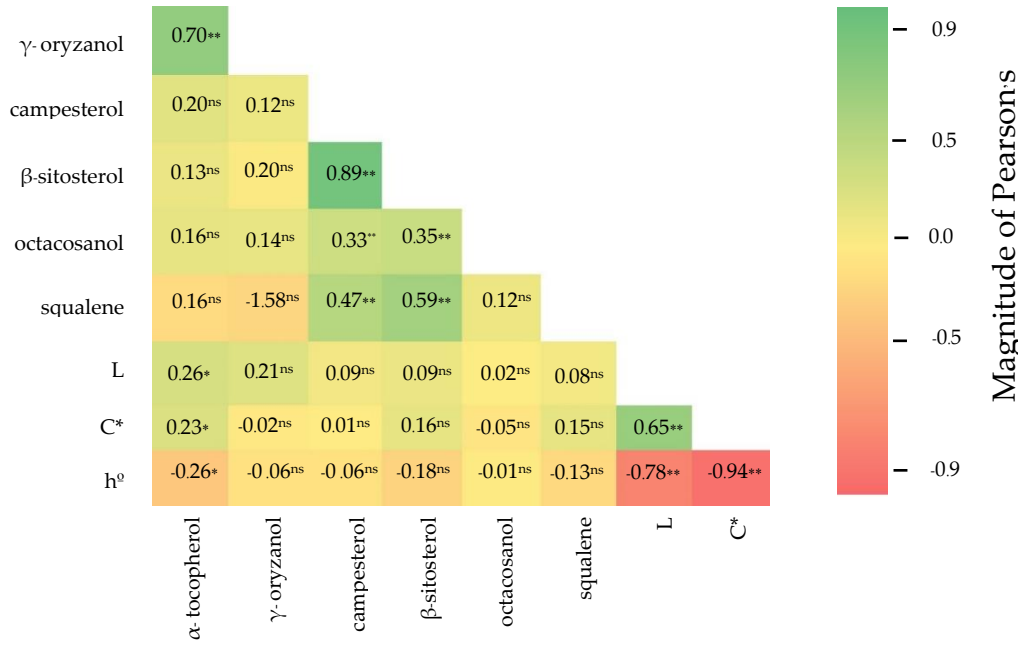

Figure 4. Triangular heat map representing Pearson's correlation coefficients between color parameters and nutraceutical lipid content in 83 rice germplasms. (*,** The correlation is significant at $p < 0.05$, 0.01, respectively; ns; non-significant).

4. Conclusions

The rice germplasms in this study showed considerable variability in levels of β-sitosterol, campesterol, octacosanol, α-tocopherol, and γ-oryzanol, though not of squalene. This variability can be exploited in rice breeding programs. Principal component analysis extracted four components that explained 93.47% of total variation. The 83 rice germplasms were grouped into nine distinct clusters, based on nutraceutical lipid content. TU-010, TU-027, TU-093, and TU-244 were found to be genotypes with high levels of fat-soluble compounds. Correlations between color parameters and these compounds were weak, and of little use as indirect markers for genetic selection of nutritional properties. However, α-tocopherol was strongly and positively correlated with γ-oryzanol, and β-sistosterol was positively correlated with campesterol and squalene. The findings of this study will help breeders to develop new rice varieties with enhanced nutraceutical lipid content.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/12/823/s1, Table S1: List of rice germplasm, their origin and pigmentation, seed sizes, and color parameters. Table S2: Nutraceutical lipid compound contents in 83 rice germplasm.

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