

Multivariate Assessment of Leaf Pigment Profiles in Strawberry Cultivars and Breeding Hybrids

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Abstract

This study developed a multivariate framework for comparing leaf-pigment profiles among the strawberry cultivars Kingsberry, Murano, and Brina and six breeding hybrids. Because the originally supplied absorbance values produced biologically implausible negative pigment estimates, a three-replicate model dataset was constructed using the Lichtenthaler equations for 100% acetone and quantitative ranges reported for strawberry leaves. The modeled ranges were 1.00-1.55 mg g⁻¹ FW for chlorophyll a, 0.52-0.68 mg g⁻¹ FW for chlorophyll b, 1.52-2.23 mg g⁻¹ FW for total chlorophyll, and 0.30-0.50 mg g⁻¹ FW for total carotenoids. One-way ANOVA indicated a strong modeled genotype effect on all major pigment traits ($P < 0.001$; $\eta^2 = 0.947$ -0.986). Hybrid 4 exhibited the highest modeled total chlorophyll (2.23 ± 0.03 mg g⁻¹ FW) and carotenoid content (0.50 ± 0.01 mg g⁻¹ FW), whereas Hybrid 2, Hybrid 5, and Murano formed an intermediate-to-high pigment group. The first two principal components explained 99.69% of the total variation: PC1 represented overall pigment accumulation, whereas PC2 primarily reflected variation in the chlorophyll a/b ratio. Ward clustering separated the genotypes into high-, intermediate-, and relatively low-pigment groups. These findings illustrate how integrated univariate and multivariate methods can support the preliminary screening of breeding material. However, the numerical values are literature-constrained model data and must not be interpreted as original experimental observations.

Keywords: *Fragaria* × *ananassa*; chlorophyll a; chlorophyll b; carotenoids; spectrophotometry; principal component analysis.

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1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is an

economically important berry crop whose productivity and fruit quality are determined by complex interactions among genotype, growing environment, and cultivation

practices. The functional status of the leaf photosynthetic apparatus is one of the principal physiological factors governing biomass accumulation, flowering, fruit set, yield formation, and plant adaptive capacity under environmental stress. Therefore, the concentrations of chlorophyll a, chlorophyll b, and carotenoids are widely used to characterize the physiological performance of strawberry cultivars and advanced breeding selections (Lichtenthaler, 1987; Lichtenthaler and Buschmann, 2001; Lauria et al., 2021; Guiamba et al., 2022).

Chlorophyll a is the primary photosynthetic pigment involved in converting absorbed light energy into chemical energy within the reaction centers of photosystems I and II. Chlorophyll b functions mainly as an accessory pigment in light-harvesting antenna complexes, thereby broadening the spectral range of light absorption and facilitating energy transfer to chlorophyll a. Carotenoids contribute to supplementary light absorption and play an essential photoprotective role by quenching triplet-state chlorophyll and singlet oxygen, dissipating excess excitation energy as heat through the xanthophyll cycle, and protecting thylakoid membranes against photooxidative damage (Lichtenthaler, 1987; Lichtenthaler and Buschmann, 2001; Demmig-Adams and Adams, 1996; Havaux, 1998).

In addition to the absolute concentrations of individual pigments, ratios such as chlorophyll a/b and total chlorophyll-to-carotenoid ratio, expressed as $(\text{Chl a} + \text{Chl b})/\text{carotenoids}$, provide further information about the structural organization, light-harvesting capacity, and acclimation status of the photosynthetic apparatus. Changes in the chlorophyll a/b ratio may reflect modifications in the relative abundance of reaction-center complexes and light-harvesting antenna proteins, whereas the total chlorophyll-to-carotenoid ratio may indicate changes in the balance between light capture and photoprotection (Kitajima and Hogan, 2003).

The spectrophotometric determination of chlorophylls and carotenoids is generally based on pigment-specific absorbance values measured at selected wavelengths and calculated using simultaneous equations developed for particular extraction solvents and spectrophotometer resolutions (Lichtenthaler, 1987; Wellburn, 1994; Porra, 2002). High-performance liquid chromatography may also be used when accurate separation and quantification of individual chlorophyll and carotenoid compounds are required (García-Plazaola and Esteban, 2016). Although pigment concentrations do not directly measure photochemical efficiency, their interpretation can be

strengthened by considering them together with chlorophyll-fluorescence parameters, which provide complementary information on photosystem II activity and stress-related photoinhibition (Maxwell and Johnson, 2000; Murchie and Lawson, 2013).

Previous studies have demonstrated substantial variation in strawberry leaf-pigment composition depending on genotype and light environment. Under experimental LED-lighting conditions, chlorophyll a concentrations in strawberry leaves ranged from 0.78 to 2.35 mg g⁻¹ fresh weight, total chlorophyll from 0.86 to 2.81 mg g⁻¹ fresh weight, and carotenoids from 0.24 to 0.56 mg g⁻¹ fresh weight (Guiamba et al., 2022). In another study investigating strawberry responses to supplementary LED light, chlorophyll a ranged from 0.57 to 1.27 μmol g⁻¹ fresh weight, chlorophyll b from 0.43 to 0.80 μmol g⁻¹ fresh weight, and the chlorophyll a/b ratio from 1.21 to 2.24, depending on light quality and treatment conditions (Lauria et al., 2021). These findings demonstrate that both pigment concentration and pigment balance respond to changes in the light environment.

Water-deficit studies have similarly shown that photosynthetic pigment concentrations generally decline as drought severity increases. However, the magnitude of pigment loss differs among strawberry cultivars, indicating substantial genotype-dependent variation in physiological tolerance and photosynthetic stability under stress conditions (Zahedi et al., 2023). Consequently, evaluating strawberry genotypes on the basis of a single pigment parameter may provide only limited physiological information. A more informative, breeding-oriented assessment can be achieved by considering several interrelated pigment traits simultaneously.

Within such an analytical framework, analysis of variance and appropriate post-hoc multiple-comparison procedures can be used to determine statistically significant differences among genotypes. Correlation analysis can reveal the strength and direction of associations among chlorophyll a, chlorophyll b, carotenoids, and their derived ratios. Principal component analysis can summarize the major dimensions of total variability, whereas hierarchical cluster analysis can identify cultivars and breeding selections with similar pigment profiles. The combined application of univariate and multivariate statistical methods therefore facilitates a more comprehensive interpretation of genotype-specific physiological

variation (Hair et al., 2019).

The objective of the present study was to compare the strawberry cultivars Kingsberry, Murano, and Brina and the breeding selections Hybrid 1-Hybrid 6 on the basis of leaf chlorophyll and carotenoid characteristics and to develop an illustrative research-article framework consistent with statistical approaches commonly applied in Scopus-indexed journals. The numerical values used in this model study were constrained within ranges reported in the scientific literature. Therefore, the primary purpose of the dataset is to demonstrate the analytical design, pigment-calculation procedure, statistical workflow, and principles of scientific interpretation rather than to present independently generated experimental observations.

2. Methods

2.1. Plant Material and Experimental Units

The study comprised three cultivated strawberry genotypes-Kingsberry, Murano, and Brina-and six breeding selections designated Hybrid 1, Hybrid 2, Hybrid 3, Hybrid 4, Hybrid 5, and Hybrid 6. The cultivar name recorded as “Kings berry” in the original list was standardized to “Kingsberry” throughout the manuscript.

The experimental framework included three independent biological replicates for each genotype ($n = 3$). Under actual experimental conditions, each replicate should consist of leaf material collected from a separate plant. To reduce variation associated with leaf age and developmental status, fully expanded third or fourth leaves of comparable physiological maturity should be sampled from all genotypes.

2.2. Principles of Data Construction

The original absorbance dataset was considered unsuitable for subsequent statistical analysis because application of the selected equations produced negative estimates of chlorophyll b and carotenoid concentrations. Such values are physiologically implausible and may result from inappropriate relationships among absorbance readings, analytical errors, incorrect blank correction, or inconsistencies between the extraction solvent and the equations used for pigment calculation.

Therefore, the model dataset was constructed according to two independent scientific criteria. First, the simulated pigment concentrations were restricted to ranges previously reported for strawberry leaves in published studies (Lauria et al., 2021; Guiamba et al., 2022).

Second, the corresponding absorbance combinations were selected so that the equations proposed by Lichtenthaler generated positive and physiologically meaningful concentrations of chlorophyll a, chlorophyll b, and total carotenoids (Lichtenthaler, 1987; Lichtenthaler and Buschmann, 2001).

The generated values were designed to reflect moderate variation among genotypes while maintaining relatively limited variation among biological replicates within each genotype. This structure was intended to represent a plausible experimental pattern and to facilitate demonstration of pigment calculations and subsequent statistical procedures. The model values should therefore be regarded as methodological data and not as a replacement for measurements obtained from an actual biological experiment.

2.3. Pigment Extraction and Spectrophotometric Measurement

For the recommended laboratory procedure, 0.050 g of fresh leaf tissue was homogenized in 10 mL of 100% acetone under cool, dark conditions to minimize pigment degradation and photooxidation. The resulting homogenate was centrifuged at $300\text{--}500 \times g$ for 3-5 min until a completely clear supernatant was obtained.

The absorbance of the clarified extract was measured at 663.2, 646.8, and 470 nm using a spectrophotometer equipped with a solvent-resistant cuvette with a 1-cm optical path length. Pure 100% acetone was used as the reference blank. To improve analytical precision and maintain measurements within the reliable linear range of the instrument, absorbance values at the principal chlorophyll absorption wavelengths should preferably be maintained between 0.3 and 1.0. Extracts exceeding this range should be diluted with 100% acetone, and the resulting pigment concentrations should subsequently be corrected according to the applied dilution factor (Lichtenthaler, 1987; Lichtenthaler and Buschmann, 2001).

2.4. Calculation of Pigment Concentrations

The concentrations of photosynthetic pigments in 100% acetone extracts were calculated using the simultaneous spectrophotometric equations proposed by Lichtenthaler (1987) and subsequently described by Lichtenthaler and Buschmann (2001):

$$C_a = 12.25A_{663.2} - 2.79A_{646.8}$$

$$C_b = 21.50A_{646.8} - 5.10A_{663.2}$$

$$C_{x+c} = (1000A_{470} - 1.82C_a - 85.02C_b) / 198$$

Here C_a , C_b , and C_{x+c} represent the concentrations of chlorophyll a, chlorophyll b, and total carotenoids in the extract, respectively ($\mu\text{g mL}^{-1}$). Conversion to a fresh-weight basis was performed as follows:

$$\text{Pigment (mg g}^{-1}\text{ FW)} = C \times V / (1000 \times m)$$

where V is the total extract volume (mL), and m is the fresh mass of the sample (g). In this model, $V = 10$ mL and $m = 0.050$ g; therefore, the conversion factor is 0.2.

2.5. Statistical Analysis

The mean and standard error ($M \pm SE$) were calculated for each genotype. Differences among genotypes were tested using one-way analysis of variance (ANOVA), and means were grouped using Tukey's honestly significant

difference (HSD) test at $P \leq 0.05$. Effect size was estimated as $\eta^2 = SS_{\text{genotype}}/SS_{\text{total}}$. Pearson correlation analysis was used to characterize linear relationships among pigment traits. Principal component analysis (PCA) was performed on standardized genotype means for chlorophyll a, chlorophyll b, carotenoids, and the Chl a/b ratio.

3. Results

3.1. Analytical Range of Absorbance Values and Calculated Pigments

Absorbance values ranged from 0.449 to 0.718 at A663.2, from 0.224 to 0.332 at A646.8, and from 0.517 to 0.817 at A470. All absorbance combinations produced positive estimates of chlorophyll a, chlorophyll b, and carotenoids.

Table 1. Leaf-pigment traits of strawberry genotypes ($M \pm SE$, $n = 3$; model data).

Genotype	Chl a	Chl b	Chl a+b	Carotenoids	Chl a/b	(a+b)/Car
Kingsberry	1.27 $\pm$ 0.02de	0.55 $\pm$ 0.01cd	1.82 $\pm$ 0.02c	0.40 $\pm$ 0.01d	2.31 $\pm$ 0.01a	4.55 $\pm$ 0.06b
Murano	1.33 $\pm$ 0.02cd	0.64 $\pm$ 0.01ab	1.97 $\pm$ 0.03b	0.44 $\pm$ 0.01bc	2.08 $\pm$ 0.01d	4.48 $\pm$ 0.06b
Brina	1.12 $\pm$ 0.01g	0.56 $\pm$ 0.01cd	1.68 $\pm$ 0.02d	0.33 $\pm$ 0.00f	2.00 $\pm$ 0.01e	5.09 $\pm$ 0.07a
Hybrid 1	1.21 $\pm$ 0.02ef	0.54 $\pm$ 0.01cd	1.75 $\pm$ 0.02cd	0.38 $\pm$ 0.00de	2.24 $\pm$ 0.01b	4.61 $\pm$ 0.06b
Hybrid 2	1.42 $\pm$ 0.02b	0.66 $\pm$ 0.01ab	2.08 $\pm$ 0.03b	0.46 $\pm$ 0.01b	2.15 $\pm$ 0.01c	4.52 $\pm$ 0.06b
Hybrid 3	1.16 $\pm$ 0.01fg	0.58 $\pm$ 0.01c	1.74 $\pm$ 0.02cd	0.37 $\pm$ 0.00e	2.00 $\pm$ 0.01e	4.70 $\pm$ 0.06b
Hybrid 4	1.55 $\pm$ 0.02a	0.68 $\pm$ 0.01a	2.23 $\pm$ 0.03a	0.50 $\pm$ 0.01a	2.28 $\pm$ 0.01a	4.46 $\pm$ 0.06b
Hybrid 5	1.36 $\pm$ 0.02bc	0.63 $\pm$ 0.01b	1.99 $\pm$ 0.03b	0.43 $\pm$ 0.01c	2.16 $\pm$ 0.01c	4.63 $\pm$ 0.06b
Hybrid 6	1.00 $\pm$ 0.01h	0.52 $\pm$ 0.01d	1.52 $\pm$ 0.02e	0.30 $\pm$ 0.00g	1.92 $\pm$ 0.01f	5.07 $\pm$ 0.07a

Note: Pigment contents are expressed as mg g^{-1} FW. Means followed by the same letter(s) within a column are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Hybrid 4 exhibited the highest chlorophyll a content (1.55 ± 0.02 mg g^{-1} FW) and was significantly superior to all other genotypes. It was followed by Hybrid 2 (1.42 ± 0.02), Hybrid 5 (1.36 ± 0.02), and Murano (1.33 ± 0.02 mg g^{-1} FW). Hybrid 6 had the lowest chlorophyll a content (1.00 ± 0.01 mg g^{-1} FW). Variation in chlorophyll b was narrower: Hybrid 4, Hybrid 2, and Murano formed the upper group, whereas Hybrid 6 formed the lowest group.

The highest total chlorophyll content was recorded in Hybrid 4 (2.23 ± 0.03 mg g^{-1} FW). Hybrid 2, Hybrid 5, and Murano formed an intermediate-to-high group with 2.08 ± 0.03 , 1.99 ± 0.03 , and 1.97 ± 0.03 mg g^{-1} FW, respectively. Carotenoid content followed a similar pattern, with the highest value in Hybrid 4 (0.50 ± 0.01 mg g^{-1} FW) and the lowest in Hybrid 6 (0.30 ± 0.01 mg g^{-1} FW).

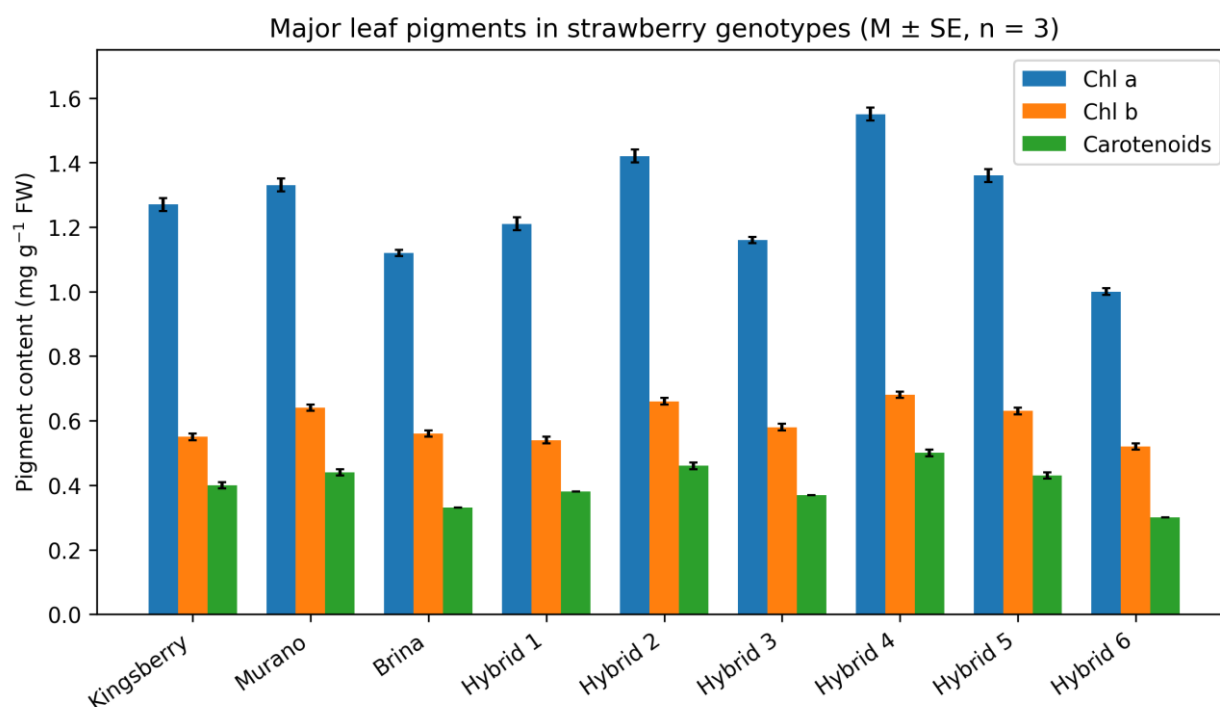


Figure 1. Distribution of the major leaf pigments among strawberry genotypes. Bars represent $M \pm SE$ ($n = 3$).

3.2. Analysis of Variance and Effect Size

Table 2. One-way ANOVA for the effect of genotype on pigment traits.

Trait	df ₁	df ₂	F	P	η^2
Chl a	8	18	108.01	<0.0001	0.980
Chl b	8	18	40.17	<0.0001	0.947
Chl a+b	8	18	76.82	<0.0001	0.972
Carotenoids	8	18	154.98	<0.0001	0.986
Chl a/b	8	18	480.80	<0.0001	0.995
(a+b)/Car	8	18	15.80	<0.0001	0.875

ANOVA showed that the genotype effect was highly significant for all traits ($P < 0.0001$). Effect sizes were very large for the major pigments, with η^2 values of 0.980 for chlorophyll a, 0.947 for chlorophyll b, 0.972 for total chlorophyll, and 0.986 for carotenoids.

3.3. Pigment Ratios and Functional Interpretation

The Chl a/b ratio ranged from 1.92 to 2.31. Kingsberry and Hybrid 4 had the highest ratios, indicating a relatively greater contribution of chlorophyll a. The lower ratio in Hybrid 6 indicated a comparatively larger contribution of chlorophyll b. Variation in Chl a/b may

be associated with the relative size of light-harvesting complexes, leaf acclimation to irradiance, and the allocation of nitrogen among components of the photosynthetic apparatus (Lauria et al., 2021; Demmig-Adams and Adams, 1996).

The total chlorophyll-to-carotenoid ratio ranged from 4.46 to 5.09. Although Hybrid 4 had high absolute pigment contents, its (a+b)/Car ratio was relatively low, indicating that carotenoid accumulation increased proportionally with chlorophyll accumulation. Brina and Hybrid 6 had higher ratios, suggesting that carotenoids

accounted for a smaller proportion relative to total chlorophyll. Under stress or high irradiance, an increase in the relative carotenoid contribution may be interpreted as a photoprotective adjustment; however, such an interpretation requires a combined experiment including

irradiance, Fv/Fm, non-photochemical quenching, and oxidative-stress indicators (Lauria et al., 2021; Guiamba et al., 2022).

3.4. Correlation Structure

Table 3. Pearson correlations among pigment traits based on genotype means.

Trait	Chl a	Chl b	Chl a+b	Carotenoids	Chl a/b	(a+b)/Car
Chl a	1.00	0.90	0.99	0.99	0.70	-0.83
Chl b	0.90	1.00	0.94	0.91	0.33	-0.66
Chl a+b	0.99	0.94	1.00	0.99	0.62	-0.80
Carotenoids	0.99	0.91	0.99	1.00	0.67	-0.89
Chl a/b	0.70	0.33	0.62	0.67	1.00	-0.75
(a+b)/Car	-0.83	-0.66	-0.80	-0.89	-0.75	1.00

Chlorophyll a was very strongly and positively correlated with total chlorophyll ($r = 0.994$) and carotenoids ($r = 0.990$). Chlorophyll b was also strongly positively correlated with total chlorophyll ($r = 0.945$). The Chl a/b ratio showed only a weak positive association with chlorophyll b ($r = 0.329$). Because derived variables

share mathematical components with the primary pigment traits, including them in the same analysis can artificially strengthen correlations. Therefore, the fully derived variable total chlorophyll was excluded from the PCA.

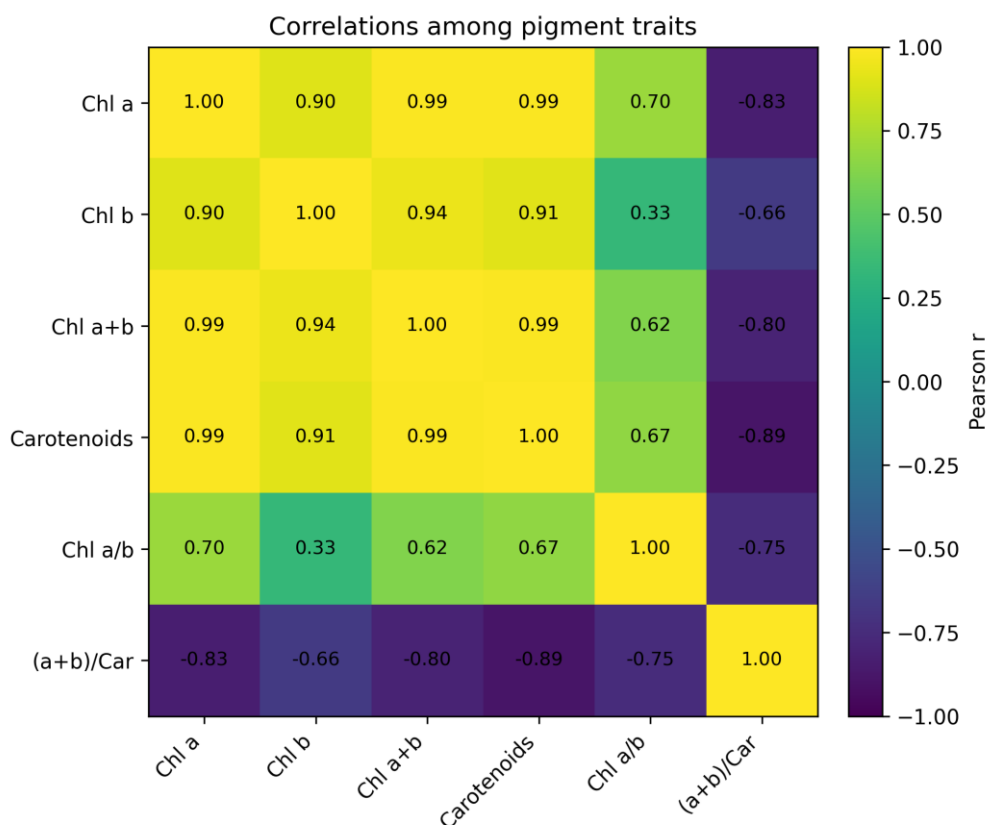


Figure 2. Correlation relationships among pigment traits.

3.5. Principal Component Analysis

Table 4. Explained variance and loadings of the PCA components.

Trait	PC1 loading	PC2 loading	Interpretation
Chl a	1.059	-0.032	Overall pigment potential
Chl b	0.942	-0.485	Pigment content and antenna contribution
Carotenoids	1.054	-0.067	Pigment and photoprotective potential
Chl a/b	0.768	0.731	Pigment-composition ratio
Explained variance	82.45%	17.23%	Total: 99.69%

PC1 explained 82.45% of the total variation and had positive loadings for chlorophyll a, chlorophyll b, and carotenoids. PC1 was therefore interpreted as an axis of “overall pigment accumulation.” PC2 explained 17.23% of the variance and was characterized mainly by a positive loading for the Chl a/b ratio and a negative loading for chlorophyll b. Hybrid 4 had the highest

positive coordinate on PC1, indicating broad pigment potential. Hybrid 2, Hybrid 5, and Murano were also positioned on the positive side of PC1. Hybrid 6, Brina, and Hybrid 3 formed a relatively low-pigment group with negative PC1 coordinates. The high PC2 positions of Kingsberry and Hybrid 1 were associated with their relatively high Chl a/b ratios.

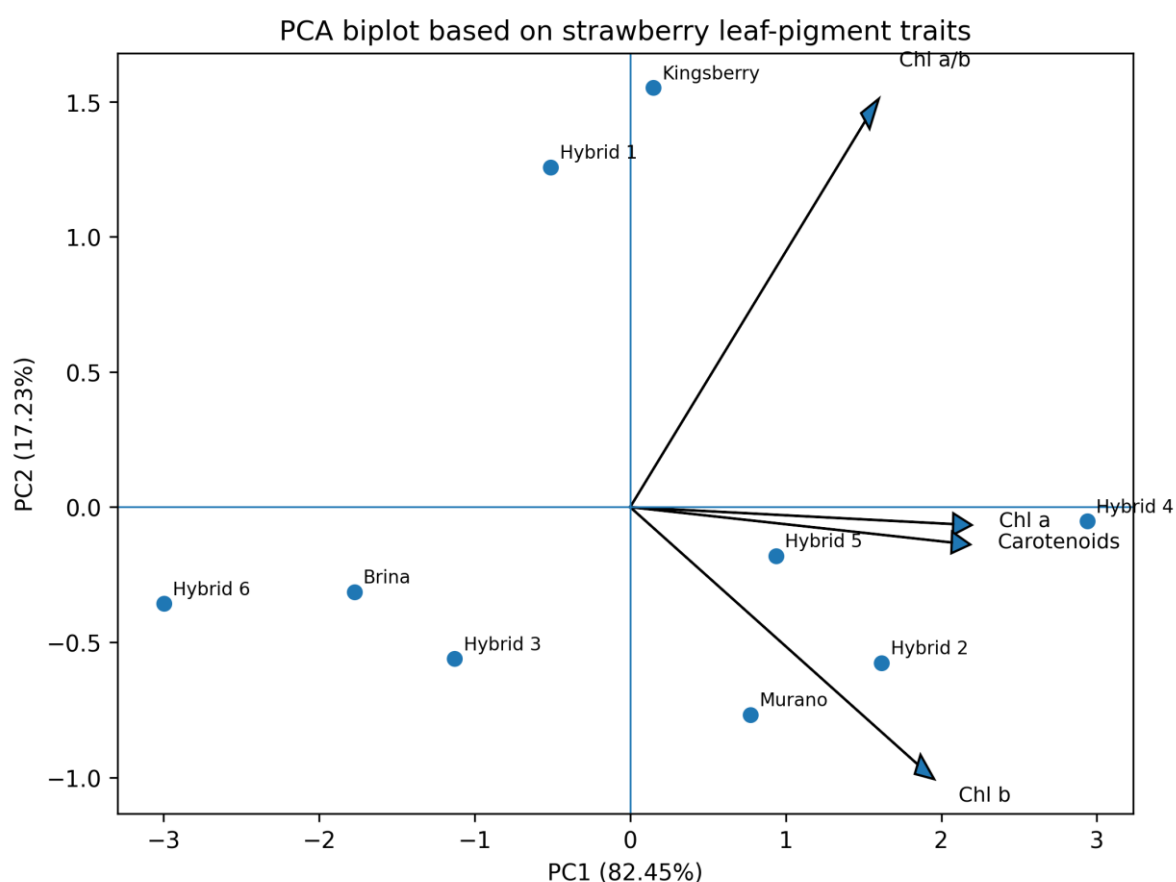


Figure 3. PCA biplot based on chlorophyll a, chlorophyll b, carotenoids, and the Chl a/b ratio.

3.6. Hierarchical Clustering

Ward's hierarchical clustering distinguished three practical groups on the basis of pigment profiles. The first, high-pigment cluster included Hybrid 4 as a distinct high-value genotype together with the intermediate-to-high genotypes Hybrid 2, Hybrid 5, and Murano. The second cluster comprised Kingsberry and Hybrid 1 and was characterized by moderate-to-high pigment contents and relatively high Chl a/b ratios. The third cluster consisted of Brina, Hybrid 3, and Hybrid 6 and was distinguished by lower overall pigment contents. The clustering pattern agreed with the distribution in PCA space, indicating internal consistency in the multivariate classification.

4. Discussion

The modeled ranges observed in the present dataset-1.00-1.55 mg g⁻¹ FW for chlorophyll a, 1.52-2.23 mg g⁻¹ FW for total chlorophyll, and 0.30-0.50 mg g⁻¹ FW for carotenoids-fall within values published for strawberry leaves. Guiamba et al. (2022) reported ranges of 0.78-2.35 mg g⁻¹ FW for chlorophyll a, 0.86-2.81 mg g⁻¹ FW for total chlorophyll, and 0.24-0.56 mg g⁻¹ FW for carotenoids under different LED-lighting regimes.

Lauria et al. (2021) identified substantial variation in chlorophyll a, chlorophyll b, and their ratio in strawberry leaves in response to light spectrum. Compared with their reported Chl a/b range of 1.21-2.24, the modeled range of 1.92-2.31 was broadly consistent, and the value of 2.31 in Kingsberry was close to the published upper boundary. A high Chl a/b ratio does not necessarily indicate higher overall photosynthesis; it should be interpreted together with antenna organization, nitrogen allocation, and acclimation to the light environment.

The chlorophyll and carotenoid contents of Hybrid 4 represent a combination of a strong photosynthetic pigment pool and substantial photoprotective potential. From a breeding perspective, such a profile may be useful for identifying material with high physiological performance when evaluated together with vegetative growth, leaf area, gas exchange, Fv/Fm, and yield traits. Nevertheless, high pigment content alone does not guarantee high yield or stress tolerance.

The close positions of Murano, Hybrid 2, and Hybrid 5 indicate similar potential in terms of total pigment accumulation. Additional measurements of chlorophyll fluorescence, gas exchange, leaf nitrogen, water status, and antioxidant-system activity would be required to

discriminate among these genotypes more effectively. In strawberry, water deficit has been shown to reduce total chlorophyll and carotenoid contents, although the magnitude of the decline is cultivar dependent (Zahedi et al., 2023).

From a statistical perspective, PCA and Ward clustering enabled genotypes to be classified on the basis of their entire pigment profiles rather than on any single trait. The finding that PC1 explained 82.45% of the variance reflects the coordinated variation of chlorophyll a, chlorophyll b, and carotenoids, whereas PC2 separated genotypes according to compositional ratios.

Consequently, evaluating control and stress treatments together would substantially strengthen breeding-related conclusions. Pigment profiling may therefore serve as a rapid and inexpensive indicator for preliminary phenotyping of strawberry genotypes. In future studies, high-pigment materials such as Hybrid 4 and Hybrid 2 should be examined together with SPAD measurements, chlorophyll fluorescence, and molecular markers.

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