

Selection of lettuce hybrids to generate productive carotenoid-biofortified populations¹

Seleção de híbridos de alface para gerar populações produtivas biofortificadas para carotenoides

Alessandro A. Sanches², Ana C. Siquieroli^{3*}, Gabriel M. Maciel⁴, Jair R. do Prado⁵,
Lucas M. Pereira⁶, Cecília R. Silva⁴ & Larissa C. Barrado⁴

¹ Research developed at Universidade Federal de Uberlândia, Campus Monte Carmelo, Monte Carmelo, MG, Brazil

² Universidade Federal de Uberlândia/Programa de Pós-Graduação em Agricultura e Informações Geoespaciais, Monte Carmelo, MG, Brazil

³ Universidade Federal de Uberlândia/Instituto de Biotecnologia, Monte Carmelo, MG, Brazil

⁴ Universidade Federal de Uberlândia/Instituto de Ciências Agrárias, Monte Carmelo, MG, Brazil

⁵ Universidade Federal de Uberlândia/Instituto de Matemática e Estatística, Monte Carmelo, MG, Brazil

⁶ Universidade Federal de Uberlândia/Programa de Pós-Graduação em Agronomia, Uberlândia, MG, Brazil

HIGHLIGHTS:

Lettuce hybrids biofortified for carotenoids were obtained.

The hybrids had the potential to produce dual-purpose populations (agronomic and biofortified for carotenoids).

F₂ populations of hybrids can generate new biofortified red and green cultivars for other lettuce market segments.

ABSTRACT: The search for new technologies capable of providing benefits to healthy eating is a global goal. The consumption of foods rich in carotenoids can prevent serious health problems. Thus, the availability of foods high in carotenoids that are accessible to the majority of the population is essential. Lettuce (*Lactuca sativa* L.) is a suitable species for biofortification research based on genetic improvements. In this study, we aimed to select and obtain hybrid lettuce populations with high agronomic potential and biofortified carotenoid content. Ten lettuce lines registered in the “BG α Biofort” software were selected for hybridization. The crosses used to obtain the hybrids involved two strains rich in at least one of the pigments (chlorophyll and carotenoids), and 24 experimental hybrids were obtained. A total of 37 genotypes (strains, hybrids, and commercial cultivars) were evaluated using a randomized block design for agronomic variables and chlorophyll and carotenoid content. Hybrids 12 (UFU-189#3#4#1 × UFU MC BIOFORT 2) and 25 (UFU66#4#2 × UFU-189#3#4#1) had the potential to produce dual-purpose populations (yield and carotenoid biofortification). Hybrid 27 (UFU66#4#2 × UFU MC BIOFORT1) exhibited the best pigment balance. Thus, F₂ hybrids 12, 25, and 27 should be obtained to produce new cultivars of biofortified red and green lettuce for curly, american, mini, and romaine lettuce segments.

Key words: *Lactuca sativa* L., crossing, pigments, biological enrichment, food security

RESUMO: A busca por novas tecnologias capazes de proporcionar benefícios para uma alimentação saudável tem sido meta mundial. A presença de alimentos ricos em carotenoides pode ser fundamental para prevenir sérios problemas de saúde. Aliar alimentos com altos teores de carotenoides e acessíveis para a maior parte da população é fundamental. A alface (*Lactuca sativa* L.) se apresenta como uma espécie adequada para investir em pesquisas com biofortificação a partir de melhoramento genético. Assim, o objetivo com este estudo foi selecionar híbridos de alface visando à obtenção de populações com potencial agrônomo e biofortificadas para carotenoides. Dez linhagens de alface cadastradas no Software “BG α Biofort” foram utilizadas nas hibridações. Os cruzamentos para obtenção dos híbridos foram planejados envolvendo duas linhagens ricas em pelo menos um dos pigmentos (clorofila e carotenoides) sendo obtidos 24 híbridos experimentais. Foram avaliados 37 genótipos (linhagens, híbridos e cultivares comerciais) em delineamento em blocos casualizados quanto as variáveis agrônomicas e teores de clorofila e carotenoides. Foi verificado que os híbridos 12 (UFU-189#3#4#1 × UFU MC BIOFORT 2) e 25 (UFU66#4#2 × UFU-189#3#4#1) possuem potencial para obtenção de populações de duplo propósito (produtividade e biofortificada para carotenoide). O híbrido 27 (UFU66#4#2 × UFU MC BIOFORT1) foi o que apresentou maior equilíbrio para pigmentos. Sugere-se a obtenção de populações F₂ dos híbridos 12, 25 e 27 visando a obtenção de novas cultivares de alfaces biofortificadas vermelhas e verdes, para os segmentos de alface crespa, americana, mini e romana.

Palavras-chave: *Lactuca sativa* L., cruzamento, pigmentos, enriquecimento biológico, segurança alimentar

INTRODUCTION

Enabling regular access to nutritious food for normal growth and development and an active and healthy life, as recommended by Goal 2: Zero Hunger, has garnered worldwide attention, especially since the coronavirus disease pandemic (FAO, 2022). The provision of foods rich in carotenoids can be vital in preventing serious public health problems (Bufka et al., 2024). The metabolization of β -carotene in plant foods is the main source of vitamin A (Masako & Meika, 2022). Its deficiency can cause visual impairment, increase the risk of serious illnesses, and lead to death from common childhood infections, such as diarrhea and measles (FAO, 2022).

To maintain a healthy population, foods rich in carotenoids must easily be accessible to the majority. Lettuce (*Lactuca sativa* L.) is a suitable model organism for biofortification research (Clemente et al., 2023). It is the most common food in the Brazilian diet (Sala & Costa, 2012).

Several studies have obtained promising results on the increase in carotenoid content in lettuce through Mendelian genetic improvement (Maciel et al., 2019a; 2020; Clemente et al., 2021; Clemente et al., 2023); however, additional improvements are required to further increase the nutritional value of lettuce and bring benefits to the health of the population. A smooth lettuce cultivar (Uberlândia 10.000) was reported to have high carotenoid content (Souza et al., 2007). Nevertheless, there are several other types of lettuce, wherein the order of economic importance is as follows: curly, american, smooth, and romaine (Sala & Costa, 2012). The Federal University of Uberlândia (UFU), Monte Carmelo campus, has an important biofortified lettuce germplasm registered in “BG α BIOFORT” (Maciel et al., 2019b). Despite the potential of this germplasm, new hybrid combinations could potentially increase the carotenoid content in future lettuce cultivars for diets with greater nutritional quality.

Marketing of seeds and the commercial cultivation of hybrid lettuce are not yet viable. One major obstacle is the high cost of obtaining hybrid seeds. In this study, hybrids were obtained to alter the genotypes to obtain a higher concentration of fixed favorable allele frequencies for carotenoids. The production of this pigment is polygenic with additive gene

action (Oliveira et al., 2021). Thus, the aim of this study was to select suitable lettuce hybrids to establish populations that are agronomically viable and biofortified with carotenoids.

MATERIAL AND METHODS

The experiment was conducted at the Horticultural Experimental Station of the Federal University of Uberlândia, Monte Carmelo campus, MG, Brazil (18° 42’ 43.19” S, 47° 29’ 55.8” E; altitude of 873 m).

The climate of the study region is Aw tropical, with hot, humid summers and cold, dry winters according to the Köppen classification (1948). The environmental conditions were monitored during the experimental period (Figure 1).

The genotypes used are part of the Universidade Federal de Uberlândia (UFU) Biofortified Vegetable Genetic Improvement Program, registered in the “BG α BIOFORT” software INPI BR512019002403-6 (Maciel et al., 2019b). The strains were obtained after hybridization between cultivars Pira 72 versus Uberlândia 10.000, followed by seven successive self-fertilizations conducted between 2013 and 2019. Genealogical breeding methods were used to obtain the lines (Maciel et al., 2019a; 2020; Sousa et al., 2021; Clemente et al., 2021; 2023; Ribeiro et al., 2023).

This study was divided into two stages. Ten biofortified strains containing various pigments were sown to obtain lettuce hybrids (Table 1).

Table 1. Description of the 10 strains of biofortified lettuce registered in “BG α BIOFORT” (pre-selected for lettuce hybrids)

ID	Lineages	Type	Color	Featured pigment
1	UFU197#1#1	Smooth	Green	Carotenoid
2	UFU MC BIOFORT1	Curly	Green	Chlorophyll
3	UFU MC BIOFORT 2	Curly	Red	Carotenoid
4	UFU75#2#2#1	Curly	Red	Chlorophyll
5	UFU66#7	Smooth	Red	Carotenoid
6	UFU189#2#2#1	Smooth	Red	Chlorophyll
7	UFU206#1#3#1	Smooth	Red	Chlorophyll
8	UFU66#4#2	Curly	Green	Carotenoid
9	UFU-189#3#4#1	Curly	Green	Carotenoid
10	UFU215#2#2	Smooth	Red	Chlorophyll

References: Maciel et al. (2019) and Clemente et al. (2023)

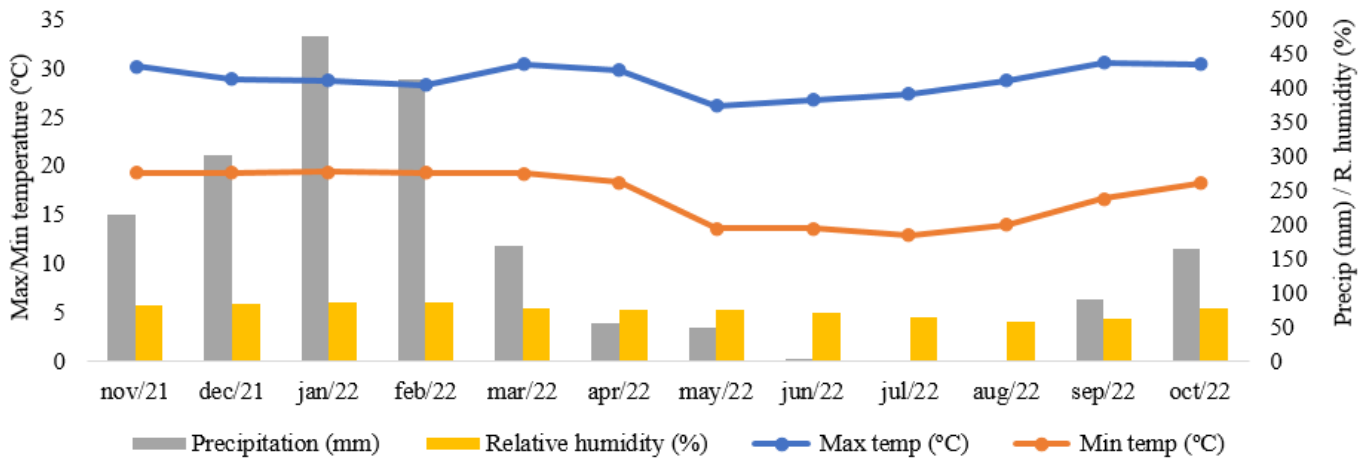


Figure 1. Minimum and maximum air temperature, rainfall, and air relative humidity during the experimental period from november 2021 to october 2022 (SISMET, 2022)

The 10 strains were sown on November 22, 2021 in 200-cell expanded polystyrene trays filled with a commercial coconut fiber substrate. After sowing, the trays were housed in an arched greenhouse (5 × 6 m; height: 3.5 m) covered with 150-μ transparent polyethylene films treated with ultraviolet inhibitor.

The seedlings were transplanted into 5-L pots filled with the same substrate 30 days after sowing (DAS) and kept in a greenhouse. Hybridization was initiated when the phenological stage of full bloom was reached. The crosses used to obtain the hybrids included two strains rich in at least one of the pigments (chlorophyll and carotenoids) (Table 1), and 24 experimental hybrids were obtained (Table 2).

All 10 strains were in full bloom at 110 DAS. To obtain the F₁ generation, the flower buds of each female parent plant were emasculated in the morning using depollination and hybridization technique (Nagai, 1980). After 157 DAS, the seeds of the F₁ generation (hybrids) and male and female parents (natural self-fertilization) were harvested, processed, identified, and stored at 18 °C.

The second stage of the study, conducted on July 7, 2022, comprised sowing the 10 strains (male and female parents), 24 experimental hybrids (Table 2), and three lettuce cultivars (Grand Rapids, Uberlândia 10.000, and Rubinela), with a total of 37 treatments. These specimens were evaluated for agronomic variables and chlorophyll and carotenoid contents.

Seedling production occurred in a manner similar to that in the first stage. Transplantation was conducted 30 DAS in the field. The soil was prepared by harrowing and sub-soiling. Subsequently, beds with a length of 1.3 m were obtained using a rototiller. Each experimental plot comprised 12 plants arranged in four central rows per bed with a spacing of 0.25 × 0.25 m. Four central plant species were evaluated in this study. The experimental design comprised randomized blocks with three replicates, totaling 111 experimental plots.

Table 2. Experimental hybrids obtained from crossing lettuce strains biofortified for carotenoids and/or chlorophyll

ID	Hybrids	Presumably biofortified for
11	UFU206#1#3#1 × UFU75#2#2#1	Chlorophyll
12	UFU-189#3#4#1 × UFU MC BIOFORT 2	Carotenoid
13	UFU75#2#2#1 × UFU MC BIOFORT 2	Carotenoid and chlorophyll
14	UFU-189#3#4#1 × UFU75#2#2#1	Carotenoid and chlorophyll
15	UFU206#1#3#1 × UFU189#2#2#1	Chlorophyll
16	UFU66#4#2 × UFU MC BIOFORT 2	Carotenoid
17	UFU206#1#3#1 × UFU MC BIOFORT 2	Carotenoid and chlorophyll
18	UFU75#2#2#1 × UFU215#2#2	Chlorophyll
19	UFU215#2#2 × UFU206#1#3#1	Chlorophyll
20	UFU215#2#2 × UFU MC BIOFORT 2	Carotenoid and chlorophyll
21	UFU215#2#2 × UFU189#2#2#1	Chlorophyll
22	UFU189#2#2#1 × UFU MC BIOFORT 2	Carotenoid and chlorophyll
23	UFU-189#3#4#1 × UFU206#1#3#1	Carotenoid and chlorophyll
24	UFU66#7 × UFU MC BIOFORT 2	Carotenoid
25	UFU66#4#2 × UFU-189#3#4#1	Carotenoid
26	UFU-189#3#4#1 × UFU189#2#2#1	Carotenoid and chlorophyll
27	UFU66#4#2 × UFU MC BIOFORT 1	Carotenoid and chlorophyll
28	UFU66#7 × UFU215#2#2	Carotenoid and chlorophyll
29	UFU MC BIOFORT 1 × UFU75#2#2#1	Chlorophyll
30	UFU75#2#2#1 × UFU206#1#3#1	Chlorophyll
31	UFU MC BIOFORT 2 × UFU75#2#2#1	Carotenoid and chlorophyll
32	UFU MC BIOFORT 2 × UFU206#1#3#1	Carotenoid
33	UFU189#2#2#1 × UFU206#1#3#1	Chlorophyll
34	UFU MC BIOFORT 2 × UFU189#2#2#1	Carotenoid and chlorophyll

The crop was harvested at 49 DAS, and the following agronomic variables were assessed: fresh mass (FM), obtained by weighing all the outer leaves; stem diameter (SD) and stem length (SL), assessed using a caliper; number of leaves (NL), assessed by counting leaves longer than 5 cm; plant diameter (PD), assessed using a ruler; and leaf temperature (LT), obtained by placing an infrared thermometer (model 4000.4GL, Everest Interscience, Tucson, AZ, USA) on the upper leaves. The Soil Plant Analysis Development (SPAD) index was obtained from the average value after collecting data from four central plants in each plot (Cassetari et al., 2015) using a Minolta SPAD-502 CFL1030 chlorophyll meter.

For the extraction of chlorophyll and carotenoids, a solution comprising petroleum ether and acetone (1:1) was added to 0.5 g of plant tissue (Clemente et al. 2023). After 24 h of incubation in the absence of light, the absorbance of the supernatant was read using an ultraviolet-190 spectrophotometer at 645, 652, 663, and 470 nm for chlorophyll a (Chloa), chlorophyll b (Chlob), total chlorophyll (Chlot), and carotenoids (CAR). The leaf pigment content (mg 100 g⁻¹ of fresh tissue) was calculated from absorbance measurements according to Eqs. 1, 2, 3, and 4, as described by Lichtenthaler & Wellburn (1983) and Witham et al. (1971):

$$\text{Chloa} = \frac{[(12.7 \times A_{663} - 2.69 \times A_{645}) \times V]}{1000} \times W \quad (1)$$

$$\text{Chlob} = \frac{[(22.9 \times A_{645} - 4.68 \times A_{663}) \times V]}{1000} \times W \quad (2)$$

$$\text{Chlot} = \frac{[(A_{652} \times 1000) \times (\frac{V}{1000} \times W)]}{34.5} \quad (3)$$

$$\text{CAR} = \frac{[(1000 \times A_{470} - 3.27 \times \text{Chloa} - 104 \times \text{Chlob})]}{229} \times W \quad (4)$$

where:

A - absorbance at the indicated wavelength;

V - final volume (mL) of the extract (pigment + extraction solution); and,

W - fresh matter (g) of the plant material used.

The data were subjected to analysis of variance, normality of residuals by the Kolmogorov-Smirnov test, homogeneity of variances by Levene's test, and additivity of blocks by Tukey's non-additivity test. The mean values were obtained and presented as boxplots. Subsequently, the means were used to conduct contrasts of interest based on the Scheffé test for hybrids versus strains, Uberlândia 10.000, and commercial cultivars, respectively.

The data were also subjected to multivariate analysis to characterize the dissimilarities between treatments and variables using a heatmap dendrogram. The dissimilarity matrix was obtained using the Mahalanobis distance, and

genetic dissimilarity was represented by a dendrogram obtained using the hierarchical unweighted pair-group method with arithmetic means (UPGMA). Clustering was validated using the cophenetic correlation coefficient (CCC), calculated using the Mantel test (1967). The relative contributions of the evaluated characteristics were calculated according to Singh's criteria (Singh, 1981).

All statistical analyses were conducted using the R 4.2.2 software (R Core Team, 2023).

RESULTS AND DISCUSSION

Significant differences were observed in the chlorophyll a content and the SPAD index ($p \leq 0.05$, F test) between the hybrids and commercial controls (Grand Rapids and Rubinela cultivars) (Table 3).

Every plant breeding program seeks to obtain new cultivars that are superior, distinct, homogeneous, and stable compared with available commercial cultivars (Sala & Costa, 2012). The success of obtaining improved populations depends a priori on the careful evaluation of the hybrids (F_1) that give rise to the F_2 populations. Therefore, hybrids must be superior to commercial cultivars, as demonstrated in the present study.

The superiority of the hybrids compared with the commercial controls for chlorophyll a content and the SPAD index indicated that most additive effects were involved, as stated by Oliveira et al. (2021) regarding the genetic control of chlorophyll a content and SPAD being considered quantitative with additive gene action.

The hybrids were similar to the strains for all variables, except for the SPAD index. The same was observed when comparing hybrids with Uberlândia 10.000. The contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in this cultivar were similar to those in the hybrids; however, the hybrids were superior in terms of the SPAD values (Table 3). The superiority of the hybrids was confirmed by Oliveira et al. (2021), who identified transgressive segregation in the F_2 generation of biofortified lettuce. These results reinforce the potential of hybrids to obtain viable future populations.

The SPAD index has a strong positive correlation with the carotenoid content and can be used for the indirect quantification of this pigment (SPAD/carotenoids). Thus, genotypes with high SPAD values are also considered rich in carotenoids (Cassetari et al., 2015). In this study, the hybrids exhibited higher SPAD values in all contrasts and were therefore considered rich in carotenoids. The Uberlândia 10.000 cultivar is a good reference in this context as it is rich in carotenoids (Souza et al., 2007; Maciel et al., 2019a; Maciel et al., 2020).

The performance of hybrid lettuce, strains, commercial cultivars, and the carotenoid-rich cultivar Uberlândia 10.000 (Souza et al., 2007) was compared in terms of the SPAD/carotenoid index, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content (Figure 2).

In terms of the chlorophyll a content, the Uberlândia 10.000 cultivar was superior compared with the commercial controls. The hybrids were mostly similar to the Uberlândia 10.000 cultivar and superior to the commercial controls in terms of the chlorophyll a content (Figure 2A). To a lesser extent, the hybrids showed the highest chlorophyll b content, especially hybrid 17 (UFU206#1#3#1 $\times$ UFU MC BIOFORT 2) (Figure 2B). Considering the total chlorophyll content, hybrids 17 (UFU206#1#3#1 $\times$ UFU MC BIOFORT 2) and 27 (UFU66#4#2 $\times$ UFU MC BIOFORT1) were superior compared with the commercial controls, strains, and the Uberlândia 10.000 cultivar (Figure 2C). These results reinforce the potential of hybrids to produce future populations with high chlorophyll contents. Owing to its bioactive properties, chlorophyll has been widely used in the pharmaceutical industry, with an emphasis on its anticancer activity owing to its antioxidant behavior, mutagen trapping, regulation of detoxification pathways, and induction of apoptosis (Pemmaraju et al., 2018; Hanafy et al., 2021).

The high carotenoid content of the Uberlândia 10.000 cultivar was confirmed (Figure 2D). Several studies have shown the nutritional potential of the Uberlândia 10.000 cultivar (Souza et al., 2007; Maciel et al., 2019a; Maciel et al., 2020; Sousa et al., 2020). Although Uberlândia 10.000 is biofortified, it is of the smooth type and not favored in Brazil, for example, where consumers prefer curly-type cultivars (Sala & Costa, 2012). This emphasizes the need for new and desirable alternatives to produce superior lettuce populations.

Several studies have evaluated and compared lettuce genotypes (Maciel et al., 2019a; Peixoto et al., 2020; Clemente et al., 2023). However, to bolster quantitative traits, superior individuals must be recombined (Borém & Miranda, 2013). The obtained SPAD indices of the hybrids indicated that the male and female parents were biofortified. All hybrids and strains were superior to the commercial controls, especially hybrid 17 (UFU206#1#3#1 $\times$ UFU MC BIOFORT 2) (Figure 2E).

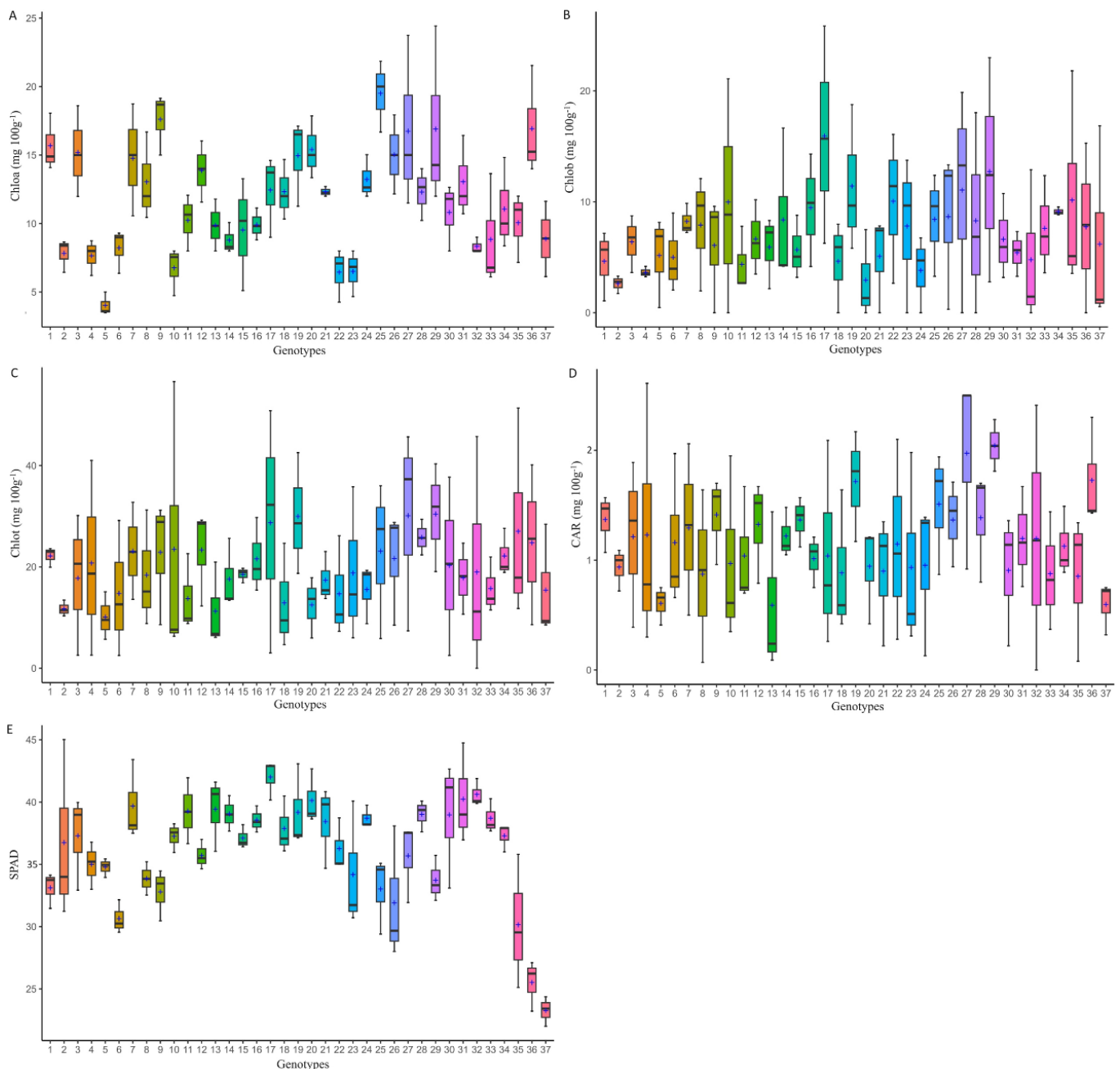
In addition to the biofortification of lettuce, the plants must also exhibit better agronomic performance than commercial cultivars. The fresh mass, stem diameter and length, number of leaves, plant diameter, and leaf temperature were also evaluated (Figure 3).

The hybrids were similar to the commercial strains and cultivars for all variables, indicating that along with the generation of biofortified populations, the hybrids also followed agronomic standards. Our findings are consistent with

Table 3. Estimation of contrasts for chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, and SPAD index in lettuce

	Contrasts	Contrast estimate				SPAD
		Chloa	Chlob	Chlot	CAR	
		(mg 100 g ⁻¹)				
C1	Hybrids vs. strains	0.93 ^{ns}	1.73 ^{ns}	1.59 ^{ns}	0.087 ^{ns}	2.59**
C2	Hybrids vs U.10.000	-4.9 ^{ns}	-0.035 ^{ns}	-4.64 ^{ns}	-0.53 ^{ns}	12.19**
C3	Hybrids vs commercial	2.54*	0.47 ^{ns}	1.11 ^{ns}	0.46 ^{ns}	11.0**

(x) **, *, and ^{ns} - Significant at $p \leq 0.01$, ≤ 0.05 , and non-significant, respectively, based on the Scheffé test. ¹ Chloa - Chlorophyll a; Chlob - Chlorophyll b; Chlot - Total chlorophyll; CAR - Carotenoids, and SPAD: SPAD index



1- UFU197#1#1; 2- UFU MC BIOFORT1; 3- UFU MC BIOFORT 2; 4- UFU75#2#2#1; 5- UFU66#7; 6- UFU189#2#2#1; 7- UFU206#1#3#1; 8- UFU66#4#2; 9- UFU-189#3#4#1; 10- UFU215#2#2; 11- UFU206#1#3#1 × UFU75#2#2#1; 12- UFU-189#3#4#1 × UFU MC BIOFORT 2; 13- UFU75#2#2#1 × UFU MC BIOFORT 2; 14- UFU-189#3#4#1 × UFU75#2#2#1; 15- UFU206#1#3#1 × UFU189#2#2#1; 16- UFU66#4#2 × UFU MC BIOFORT 2; 17- UFU206#1#3#1 × UFU MC BIOFORT 2; 18- UFU75#2#2#1 × UFU215#2#2; 19- UFU215#2#2 × UFU206#1#3#1; 20- UFU215#2#2 × UFU MC BIOFORT 2; 21- UFU215#2#2 × UFU189#2#2#1; 22- UFU189#2#2#1 × UFU MC BIOFORT 2; 23- UFU-189#3#4#1 × UFU206#1#3#1; 24- UFU66#7 × UFU MC BIOFORT 2; 25- UFU66#4#2 × UFU-189#3#4#1; 26- UFU-189#3#4#1 × UFU189#2#2#1; 27- UFU66#4#2 × UFU MC BIOFORT1; 28- UFU66#7 × UFU215#2#2; 29- UFU MC BIOFORT1 × UFU75#2#2#1; 30- UFU75#2#2#1 × UFU206#1#3#1; 31- UFU MC BIOFORT 2 × UFU75#2#2#1; 32- UFU MC BIOFORT 2 × UFU206#1#3#1; 33- UFU189#2#2#1 × UFU206#1#3#1; 34- UFU MC BIOFORT 2 × UFU189#2#2#1; 35- Rubinela; 36- Uberlândia 10.000; and 37- Grand Rapids

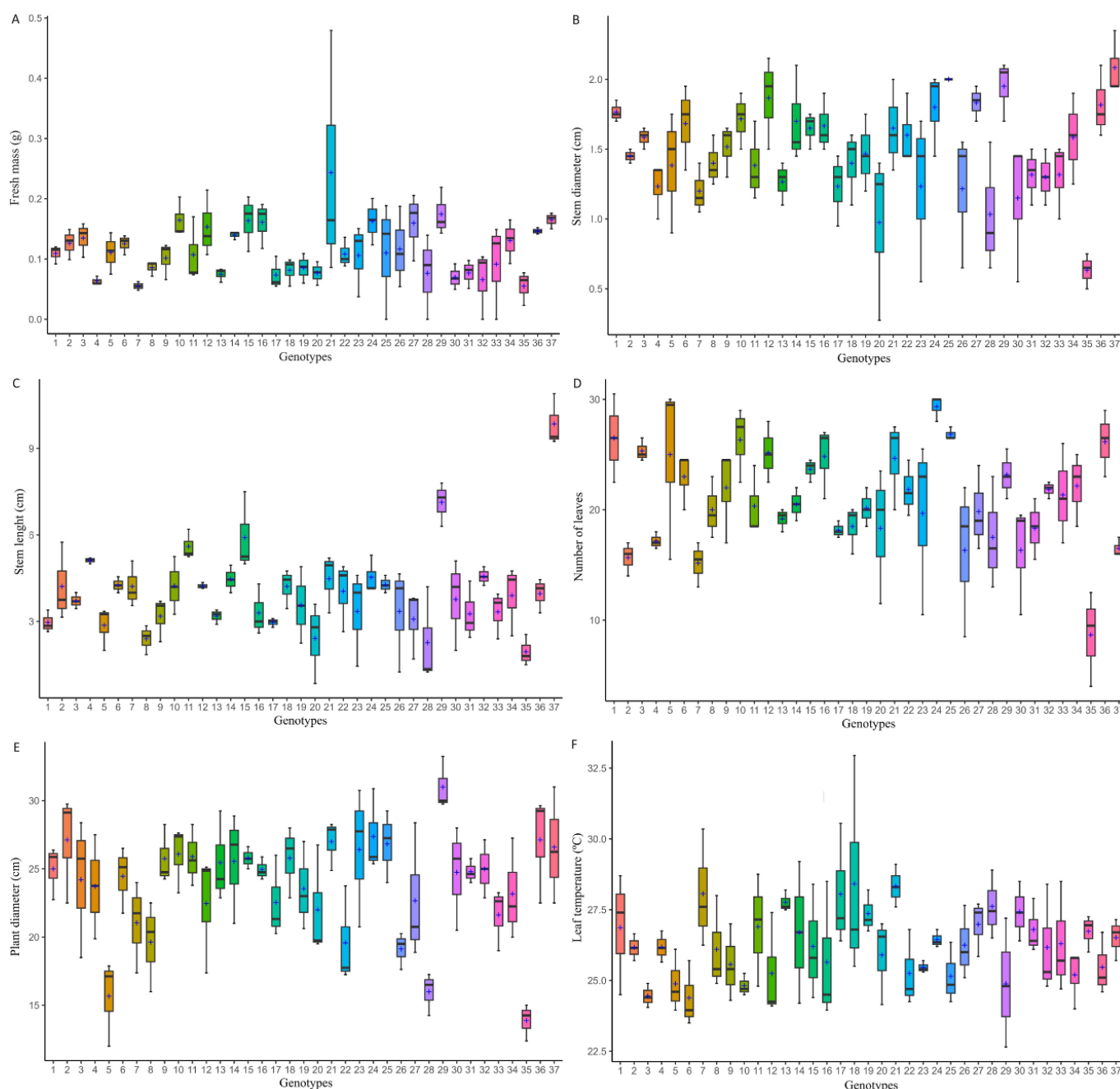
Figure 2. Means of chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), carotenoids (D), and SPAD index (E) for hybrids, strains, and commercial lettuce cultivars

previous agronomic results for biofortified lettuce (Maciel et al., 2019a; Clemente et al., 2023; Ribeiro et al., 2023), confirming that hybrids have the potential to generate populations.

The performances of hybrid 21 (UFU215#2#2 × UFU189#2#2#1) for fresh mass (Figure 3A), hybrid 24 (UFU66#7 × UFU MC BIOFORT 2) for the number of leaves (Figure 3D), and hybrid 29 (UFU MC BIOFORT1 × UFU75#2#2#1) for plant diameter (Figure 3E) are notable. These variables are related to the consumable parts of the plants and therefore, represent economic importance for producers and are desirable in lettuce breeding programs.

The agronomic and pigment variables were assessed using multivariate analysis, which showed a CCC of 90% (Figure 4).

The evaluated germplasm lines showed wide genetic variability (Figure 4). The first group comprised genotypes 25, 36, 12, 3, 1, 9, 29, and 37, namely, three hybrids, three strains, Uberlândia 10.000, and the commercial Grand Rapids cultivar. The results indicated that hybrids 12 (UFU-189#3#4#1 × UFU MC BIOFORT 2) and 25 (UFU66#4#2 × UFU-189#3#4#1) exhibited effective biofortification and agronomic potential. These hybrids performed well in terms of the leaf number, stem diameter (Figure 4), and pigment content (Figure 2). The



1- UFU197#1#1; 2- UFU MC BIOFORT1; 3- UFU MC BIOFORT 2; 4- UFU75#2#2#1; 5- UFU66#7; 6- UFU189#2#2#1; 7- UFU206#1#3#1; 8- UFU66#4#2; 9- UFU-189#3#4#1; 10- UFU215#2#2; 11- UFU206#1#3#1 × UFU75#2#2#1; 12- UFU-189#3#4#1 × UFU MC BIOFORT 2; 13- UFU75#2#2#1 × UFU MC BIOFORT 2; 14- UFU-189#3#4#1 × UFU75#2#2#1; 15- UFU206#1#3#1 × UFU189#2#2#1; 16- UFU66#4#2 × UFU MC BIOFORT 2; 17- UFU206#1#3#1 × UFU MC BIOFORT 2; 18- UFU75#2#2#1 × UFU215#2#2; 19- UFU215#2#2 × UFU206#1#3#1; 20- UFU215#2#2 × UFU MC BIOFORT 2; 21- UFU215#2#2 × UFU189#2#2#1; 22- UFU189#2#2#1 × UFU MC BIOFORT 2; 23- UFU-189#3#4#1 × UFU206#1#3#1; 24- UFU66#7 × UFU MC BIOFORT 2; 25- UFU66#4#2 × UFU-189#3#4#1; 26- UFU-189#3#4#1 × UFU189#2#2#1; 27- UFU66#4#2 × UFU MC BIOFORT 1; 28- UFU66#7 × UFU215#2#2; 29- UFU MC BIOFORT 1 × UFU75#2#2#1; 30- UFU189#2#2#1; 31- UFU MC BIOFORT 2 × UFU75#2#2#1; 32- UFU MC BIOFORT 2 × UFU206#1#3#1; 33- UFU189#2#2#1 × UFU206#1#3#1; 34- UFU MC BIOFORT 2 × UFU189#2#2#1; 35- Rubinela; 36- Uberlândia 10.000; and 37- Grand Rapids

Figure 3. Means of agronomic variables for hybrids, strains, and commercial lettuce cultivars. Fresh mass (A), stem diameter (B), stem length (C), number of leaves (D), plant diameter (E), and leaf temperature (F)

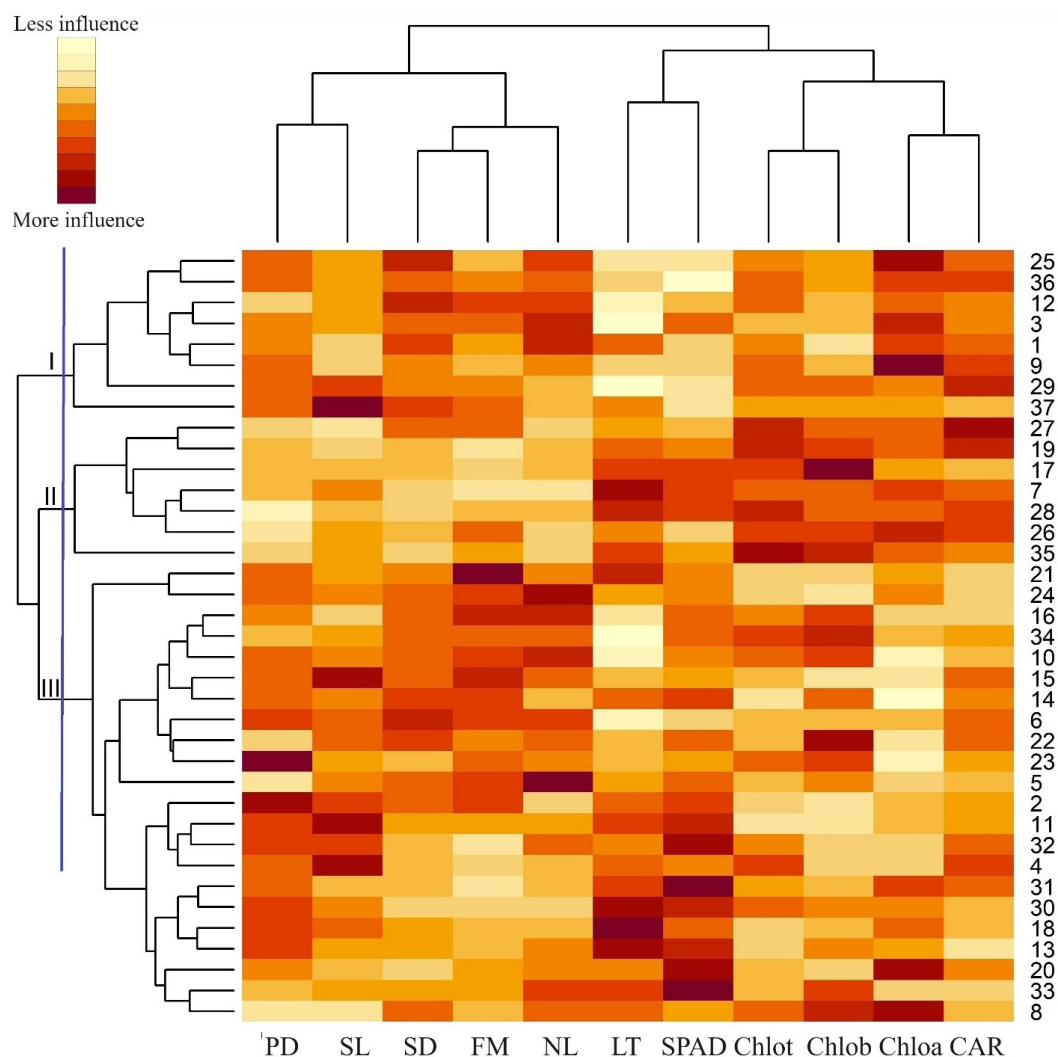
heat map showed warmer colors for pigments and agronomic variables, whereas commercial cultivar 37 (Grand Rapids) mostly showed cooler colors (Figure 4). Studies have reported genetic variability in biofortified lettuce (Clemente et al., 2021); however, the aim was to compare commercial strains and cultivars. Studies evaluating the potential of lettuce hybrids to obtain viable populations for biofortification and commercial agriculture are lacking.

The second group comprised genotypes 27, 19, 17, 7, 28, 26, and 35, namely, five hybrids, one strain, and a commercial control (cv. Rubinela) (Figure 4). The third group comprised

the majority of genotypes (21, 24, 16, 34, 10, 15, 14, 6, 22, 23, 5, 2, 11, 32, 4, 31, 30, 18, 13, 20, 33, and 8), namely, 16 hybrids and six lines.

The relative contributions of pigment characteristics (SPAD index, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content) were greater than 30% (Table 4).

Among the pigments, the highest contributions were from chlorophyll a and carotenoid content (derived from the SPAD index), at 14.75 and 12.74%, respectively (Table 4). This confirms that the variability in the dendrogram (Figure 4) was mostly represented by pigments.



1- UFU197#1#1; 2- UFU MC BIOFORT1; 3- UFU MC BIOFORT 2; 4- UFU75#2#2#1; 5- UFU66#7; 6- UFU189#2#2#1; 7- UFU206#1#3#1; 8- UFU66#4#2; 9- UFU-189#3#4#1; 10- UFU215#2#2; 11- UFU206#1#3#1 × UFU75#2#2#1; 12- UFU-189#3#4#1 × UFU MC BIOFORT 2; 13- UFU75#2#2#1 × UFU MC BIOFORT 2; 14- UFU-189#3#4#1 × UFU75#2#2#1; 15- UFU206#1#3#1 × UFU189#2#2#1; 16- UFU66#4#2 × UFU MC BIOFORT 2; 17- UFU206#1#3#1 × UFU MC BIOFORT 2; 18- UFU75#2#2#1 × UFU215#2#2; 19- UFU215#2#2 × UFU206#1#3#1; 20- UFU215#2#2 × UFU MC BIOFORT 2; 21- UFU215#2#2 × UFU189#2#2#1; 22- UFU189#2#2#1 × UFU MC BIOFORT 2; 23- UFU-189#3#4#1 × UFU206#1#3#1; 24- UFU66#7 × UFU MC BIOFORT 2; 25- UFU66#4#2 × UFU-189#3#4#1; 26- UFU-189#3#4#1 × UFU189#2#2#1; 27- UFU66#4#2 × UFU MC BIOFORT1; 28- UFU66#7 × UFU215#2#2; 29- UFU MC BIOFORT1 × UFU75#2#2#1; 30- UFU75#2#2#1 × UFU206#1#3#1; 31- UFU MC BIOFORT 2 × UFU75#2#2#1; 32- UFU MC BIOFORT 2 × UFU206#1#3#1; 33- UFU189#2#2#1 × UFU206#1#3#1; 34- UFU MC BIOFORT 2 × UFU189#2#2#1; 35- Rubinela; 36- Uberlândia 10.000; and 37- Grand Rapids

Figure 4. Heat map of the leaf pigment content and agronomic variables of hybrids, strains, and commercial lettuce cultivars

Table 4. Relative contribution of 11 characteristics to genetic dissimilarity between 37 genotypes of biofortified lettuce

Feature	Contribution (%)
Chlorophyll a	14.75
SPAD/carotenoid index	12.74
Carotenoids	2.62
Chlorophyll b	2.03
Total chlorophyll	0.00
Total contribution of leaf pigments	32.14
Stem length	34.37
Number of leaves	13.26
Plant diameter	8.16
Stem diameter	7.36
Leaf temperature	2.56
Fresh mass	2.15
Total contribution of agronomic variables	67.86

CONCLUSIONS

1. Hybrids 12 (UFU-189#3#4#1 × UFU MC BIOFORT 2) and 25 (UFU66#4#2 × UFU-189#3#4#1) had the potential to produce dual-purpose populations (agronomically viable and biofortified for carotenoids).

2. Hybrid 27 (UFU66#4#2 × UFU MC BIOFORT1) exhibited the best performance for leaf pigments (chlorophyll and carotenoids).

3. F₂ populations of hybrids 12 (UFU-189#3#4#1 × UFU MC BIOFORT 2), 25 (UFU66#4#2 × UFU-189#3#4#1), and 27 (UFU66#4#2 × UFU MC BIOFORT1) must be obtained to generate new biofortified red and green cultivars for the curly, American, mini, and romaine lettuce market segments, respectively.

Contribution of authors: Conceptualization, Siquieroli, A. C. S. and Maciel, G. M.; methodology, Sanches, A. A., Pereira, L. M., Silva, C. R., and Barrado, L. C.; validation, Siquieroli, A. C. S., Maciel, G. M., and do Prado, J. R.; formal analysis, Siquieroli, A. C. S. and Maciel, G. M.; investigation, Sanches, A. A., Pereira, L. M., Silva, C. R., and Barrado, L. C.; data curation, Siquieroli, A. C. S. and Maciel, G. M.; writing—original draft preparation, Siquieroli, A. C. S. and Maciel, G. M.; writing—review and editing, Siquieroli, A. C. S., Maciel, G. M., and do Prado, J. R.; visualization, Siquieroli, A. C. S. and Maciel, G. M.;

and project administration, Siquieroli, A. C. S. and Maciel, G. M. All authors have read and agreed to the published version of the manuscript.

Supplementary documents: There are no supplementary sources.

Conflict of interest: The authors declare no conflicts of interest.

Financing statement: This study was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant number 302734/2023-6.

Acknowledgments: The authors thank the Universidade Federal de Uberlândia (UFU), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

LITERATURE CITED

- Borém, A.; Miranda, G. V. Melhoria de plantas. 6.ed. Viçosa: UFV, 2013. 523p.
- Bufka, J.; Vaňková, L.; Sýkora, J.; Křížková, V. Exploring carotenoids: Metabolism, antioxidants, and impacts on human health. *Journal of Functional Foods*, v.118, e106284, 2024. <https://doi.org/10.1016/j.jff.2024.106284>
- Cassetari, L. S.; Gomes, M. S.; Santos, D. C.; Santiago, W. D.; Andrade, J.; Guimarães, A. C.; Souza, J. A.; Cardoso, M. G.; Maluf, W. R.; Gomes, L. A. β -Carotene and chlorophyll levels in cultivars and breeding lines of lettuce. *Acta Horticulturae*, v.1083, p.469-474, 2015. <https://doi.org/10.17660/ActaHortic.2015.1083.60>
- Clemente, A. A.; Maciel, G. M.; Siquieroli, A. C. S.; Gallis, R. B. de A.; Luz, J. M. Q.; Sala, F. C.; Pereira, L. M.; Yada, R. Y. Nutritional characterization based on vegetation indices to detect anthocyanins, carotenoids, and chlorophylls in mini-lettuce. *Agronomy-Basel*, v.13, p.1-16, 2023. <https://doi.org/10.3390/agronomy13051403>
- Clemente, A. A.; Maciel, G. M.; Siquieroli, A. C. S.; Gallis, R. B. de A.; Pereira, L. M.; Duarte, J. G. High-throughput phenotyping to detect anthocyanins, chlorophylls, and carotenoids in red lettuce germplasm. *International Journal of Applied Earth Observation and Geoinformation*, v.103, e102533, 2021. <https://doi.org/10.1016/j.jag.2021.102533>
- FAO - Food and Agriculture Organization of the United Nations. The State of Food Security and Nutrition in the World 2022. Available on: <https://www.fao.org/documents/card/en/c/cc0639en>. Accessed on: June 2023.
- Hanafy, N. A.; Leporatti, S.; El-Kemary, M. A. Extraction of chlorophyll and carotenoids loaded into chitosan as potential targeted therapy and bio imaging agents for breast carcinoma. *International Journal of Biological Macromolecules*, v.182, p.1150-1160, 2021. <https://doi.org/10.1016/j.ijbiomac.2021.03.189>
- Köppen, W. Climatologia: con um estudio de los climas de la Tierra. México: Fondo de Cultura Económica, 1948. 478p.
- Lichtenthaler, H.; Wellburn, A. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions*, v.603, p.91-592, 1983.
- Maciel, G. M.; Gallis, R. B. de A.; Barbosa, R. L.; Pereira, L. M.; Siquieroli, A. C. S.; Peixoto, J. V. M. Image phenotyping of inbred red lettuce lines with genetic diversity regarding carotenoid levels. *International Journal of Applied Earth Observation and Geoinformation*, v.81, p.154-160, 2019a. <https://doi.org/10.1016/j.jag.2019.05.016>
- Maciel, G. M.; Siquieroli, A. C. S.; Gallis, R. B. de A.; Pereira, L. M.; Sales, V. F. Programa de computador BG α Biofort. Depositante: Universidade Federal de Uberlândia. BR512019002403-6. Depósito: 01 fev. 2019. Concessão: 23 out. 2019b.
- Maciel, G. M.; Gallis, R. B. de A.; Barbosa, R. L.; Pereira, L. M.; Siquieroli, A. C. S.; Peixoto, J. V. M. Image phenotyping of lettuce germplasm with genetically diverse carotenoid levels. *Bragantia*, v.8, p.1-12, 2020. <https://doi.org/10.1590/1678-4499.20190519>
- Mantel, N. The detection of disease clustering and a generalized regression approach. *Cancer Research*, v.27, p.209-220, 1967.
- Masako, S.; Meika, T. Genetic variations of vitamin a-absorption and storage-related genes, and their potential contribution to vitamin a deficiency risks among different ethnic groups. *Frontiers in Nutrition*, v.9, e861619, 2022. <https://doi.org/10.3389/fnut.2022.861619>
- Nagai, H. Obtenção de novos cultivares de alface (*Lactuca sativa* L.) resistentes ao mosaico e ao calor. *Revista de Olericultura*, v.18, p.14-21, 1980.
- Oliveira, A. H. G. de; Maciel, G. M.; Siquieroli, A. C. S.; Luz, J. M. Q.; Silva, E. C. D. Dynamics of heritability in different characters of lettuce. *Revista Caatinga*, v.34, p.514-526, 2021. <https://doi.org/10.1590/1983-21252021v34n303rc>
- Peixoto, J. V. M.; Maciel, G. M.; Siquieroli, A. C. S.; Pereira, L. M.; Luz, J. M. Q.; Marques, D. J. Comparison between non-parametric indexes in the selection of biofortified curly lettuce. *Comunicata Scientiae*, v.11, e3351, 2020. <https://doi.org/10.14295/cs.v11i.3351>
- Pemmaraju, D.; Appidi, T.; Minhas, G.; Singh, S. P.; Khan, N.; Pal, M.; Srivastava, R.; Rengan, A. K. Chlorophyll rich biomolecular fraction of A. cadamba loaded into polymeric nanosystem coupled with Photothermal Therapy: A synergistic approach for cancer theranostics. *International Journal of Biological Macromolecules*, v.110, p.383-391, 2018. <https://doi.org/10.1016/j.ijbiomac.2017.09.084>
- R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available on: <http://www.R-project.org/>. Accessed on: June 2023.
- Ribeiro, A. L. A.; Maciel, G. M.; Siquieroli, A. C. S.; Luz, J. M. Q.; Gallis, R. B. de A.; Assis, P. H. de S.; Catao, H. C. R. M.; Yada, R. Y. Vegetation Indices for Predicting the Growth and Harvest Rate of Lettuce. *Agriculture*, v.13, p.1-16, 2023. <https://doi.org/10.3390/agriculture13051091>
- Sala, F. C.; Costa, C. P. da. Retrospectiva e tendência da alfaceicultura brasileira. *Horticultura Brasileira*, v.30, p.187-194, 2012. <https://doi.org/10.1590/S0102-05362012000200002>
- Singh, D. The relative importance of characters affecting genetic divergence. *Indian Journal of Genetics and Plant Breeding*, v.41, p.237-245, 1981.
- SISMET. Sistema de monitoramento meteorológico Cooxupé. 2022. Available on: <https://sismet.cooxupe.com.br:9000/>. Accessed on: November 2022.
- Souza, C. S. de; Bonetti, A. M.; Goulart Filho, L. R.; Machado, J. R. de A.; Londe, L. N.; Baffi, M. A.; Ramos, R. G.; Vieira, C. U.; Kerr, W. E. Divergência genética entre genótipos de alface por meio de marcadores AFLP. *Bragantia*, v.66, p.11-16, 2007.

- Sousa, L. A. de; Maciel, G. M.; Cezar, J. F.; Beloti, I. F.; Cardoso, D. B. O.; Siquieroli, A. C. Genetic parameters and selection of biofortified lettuce genotypes based on selection indices. *Revista Brasileira de Engenharia Agrícola e Ambiental*, v.25, p.772-778, 2021. <https://doi.org/10.1590/1807-1929/agriambi.v25n11p772-778>
- Sousa, L. A. de; Maciel, G. M.; Juliatti, F. C.; Beloti, I. F.; Siquieroli, A. C. S.; Clemente, A. A. Genetic dissimilarity between biofortified lettuce genotypes for leaf carotenoid levels. *Comunicata Scientiae*, v.11, e3348, 2020. <https://doi.org/10.14295/cs.v11i.3348>
- Witham, F. H.; Blaydes, D. F.; Devlin, R. M. *Experiments in Plant Physiology*. New York: Van Nostrand Reinhold, 1971. 245p.