horticultura brasileira

Research

SILVA, MF; MACIEL, GM; GALLIS, RBA; BARBOSA, RL; CARNEIRO, VQ; REZENDE, WS; SIQUIEROLI, ACS. 2022. High-throughput phenotyping by RGB and multispectral imaging analysis of genotypes in sweet corn. *Horticultura Brasileira* 40: 092-098. DOI: http://dx.doi.org/10.1590/s0102-0536-2022012

High-throughput phenotyping by RGB and multispectral imaging analysis of genotypes in sweet corn

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ABSTRACT

Sweet corn (*Zea mays* subsp. *saccharata*) is mainly intended for industrial processing. Optimizing time and costs during plant breeding is fundamental. An alternative is the use of high-throughput phenotyping (HTP) indirect associated with agronomic traits and chlorophyll contents. This study aimed to (i) verify whether HTP by digital images is useful for screening sweet corn genotypes and (ii) investigate the correlations between the traits evaluated by conventional methods and those obtained from images. Ten traits were evaluated in seven S_3 populations of sweet corn and in two commercial hybrids, three traits by classical phenotyping and the others by HTP based on RGB (red, green, blue) and multispectral imaging analysis. The data were submitted to the analyses of variance and Scott-Knott test. In addition, a phenotypic correlation graph was plotted. The hybrids were more productive than the S_3 populations, showing an efficient evaluation. The traits extracted using HTP and classical phenotyping showed a high degree of association. HTP was efficient in identifying sweet corn genotypes with higher and lower yield. The vegetative canopy area (VCA), normalized difference vegetation index (NDVI), and visible atmospherically resistant index (VARI) indices were strongly associated with grain yield.

Keywords: *Zea mays*, phenotypic data, infrared, plant breeding.

RESUMO

Fenotipagem de alto rendimento por análise de imagens RGB e multiespectral de genótipos em milho doce

O milho doce (*Zea mays* subsp. *saccharata*) destina-se principalmente ao processamento industrial. Otimizar tempo e custos durante o melhoramento de plantas é fundamental. Uma alternativa é o uso de fenotipagem de alto rendimento (HTP) indiretamente associada a caracteres agronômicos e teores de clorofila. Este trabalho teve como objetivo (i) verificar se a HTP por imagens digitais é útil para a seleção de genótipos de milho doce e (ii) investigar as correlações entre as características avaliadas por métodos convencionais e obtidas por imagens. Dez características foram avaliadas em sete populações S_3 de milho doce e em dois híbridos comerciais, três características por fenotipagem clássica e as demais por HTP baseado em análises de imagens RGB (red, green, blue) e imagem multiespectral. Os dados foram submetidos à análise de variância e teste de Scott-Knott. Em adição, foi obtido um gráfico de correlação fenotípica. Os híbridos foram mais produtivos que as populações S_3, demonstrando uma avaliação eficiente. As características extraídas por HTP e pela fenotipagem clássica apresentaram alto grau de associação. A HTP foi eficiente na identificação de genótipos de milho doce com maior e menor produtividade. Os índices VCA (*vegetative canopy area*), NDVI (*normalized difference vegetation index*) e VARI (visible atmospherically resistant index) estiveram fortemente associados à produtividade de grãos.

Palavras-chave: *Zea mays*, dados fenotípicos, infravermelho, melhoramento de plantas.

Received on June 25, 2021; accepted on February 3, 2022

Sweet corn (*Zea mays* subsp. *saccharata*) produced in Brazil is commonly directed to industrial processing (Pereira Filho & Teixeira, 2016). Despite being the third-largest yellow corn producer in the world (FAO, 2019), Brazil does not produce a significant yield of sweet corn. One of the major obstacles has been low productivity. Brazilian edaphoclimatic conditions are capable of increasing productivity (Lima *et al*., 2020), and, thus, the search for new techniques for improving the selection process during genetic improvement is fundamental.

In this context, high-throughput phenotyping (HTP) is a promising alternative due to its high speed and accuracy and low cost in obtaining phenotypic information (Tardieu *et al*., 2017; Li *et al*., 2021). Moreover, phenomics allows to obtain phenotypic data through the crop cycle in a nondestructive way and to evaluate all the plants of the experimental plot (Fritsche-Neto & Borém, 2016). Studies show the efficiency of the use of phenomics through digital images in the genetic improvement of plants (Zhang *et al.*, 2017; Fernandez-Gallego *et al.*, 2018; Makanza *et al.*, 2018b; Maciel *et al.*, 2019; Wiegmann *et al.*, 2019; Yang *et al.*, 2020; Li *et al.*, 2021).

The use of HTP by digital image, especially in the field, is still incipient (Furbank & Tester, 2011). In addition, most of the studies on phenomics relate to the main crops, such as soybean, yellow corn, and wheat (Zhang *et al.*, 2017; Fernandez-Gallego *et al.*, 2018; Makanza *et al.*, 2018a). There is a lack of studies on phenomics applied to sweet corn, especially on monitor yield. In this context, several studies showed that higher levels of chlorophyll are strongly associated with higher yields in yellow corn due to the higher photosynthetic rate. The traditional method used has been based on the chlorophyll meter (SPAD), capable of obtaining indirect values for chlorophyll content in the leaves (Yang *et al.*, 2012; Xiong *et al.*, 2015). However, breeding programs have many individuals to analyze, making it impossible to use SPAD because it represents a high demand for time during field readings (Maciel *et al.*, 2019).

Thus, this study aimed both to (i) verify whether high-throughput phenotyping by RGB (red, green, blue) and multispectral imaging analysis is useful for screening sweet corn genotypes and (ii) investigate the correlations among the traits evaluated by conventional methods with those obtained from images.

MATERIAL AND METHODS

Experimental setup

The experiment was conducted under sprinkler irrigation at the Plant Experimental Station, in Monte Carmelo, MG, Brazil (18°42'43."S, 47°29'55"W, at 873 m altitude) from March 03 to June 20, 2018. The experiment was carried out using a randomized block design, with nine treatments and three replicates (27 plots). The sweet corn plants studied consisted of two commercial hybrids from the companies Seminis (hybrid A) and Syngenta (hybrid B), and seven populations of the S_3 generation (L6P2, L6P15, L7P3, L8P7, L8P10, L8P12, and L8P18) from the Germplasm Bank of the Sweet Corn Breeding Program, Campus Monte Carmelo. All seeds of the S_3 generation used in this study were obtained from three successive, controlled self-pollination of ears collected from street markets, which were initiated in 2016.

Sowing was performed in styrofoam trays, which were then packed in the greenhouse for later transplantation, in order to guarantee the establishment of the desired population of 50 thousand plants per hectare in the field. The seedlings were transplanted to the field when they have reached the V_2 stage, the vegetative stage of sweet corn plants in which two fully expanded leaves were observed (Pereira Filho & Teixeira, 2016). The transplant depth to the field was 4 cm, with spaces between rows and between plants being 0.7 and 0.3 m, respectively. The dimension of each plot consisted of two 5.4-m long rows, with a 0.7-m aisle separating the plots and a useful area of 7.56 m^2, and 32 plants per plot. Weeds were controlled throughout the crop cycle by means of manual weeding. Finally, crop management with fertilizers and pesticides was carried out using previously recommended methods for sweet corn (Pereira Filho & Teixeira, 2016).

Image capture

Aerial images of the sweet corn plants at the V_7 stage, the vegetative stage of sweet corn plants in which seven fully expanded leaves are observed (Pereira Filho & Teixeira, 2016), were captured using an unmanned aerial vehicle (UAV), the Phantom 4® model, equipped with two optical sensors: an RGB camera (DJI Phantom 4 Pro®) and a multispectral camera (MAPIR Survey 3®).

The RGB camera had a 20-megapixel resolution and included a 5350-mAh battery, a CMOS sensor, and a 9-mm fixed focal length lens. The collected images measured 4864 × 3648 pixels, with longitudinal and lateral overlaps of 80 and 75%, respectively, resulting in a spatial resolution of 0.004 m $pixel^{-1}$. The multispectral camera (with a 12,000 mAh battery) had a 12-megapixel resolution to capture red (R), green (G), and near-infrared (IR) bands. The spatial resolution was the same as the RGB camera.

Flight plan was previously set to autopilot mode with the DroneDeploy® application. The UAV was configured to cover the experimental area at 3 m s^{-1} fast and 20 m high. To minimize shadow effects, the images were captured at noon, the period of more uniform solar radiation on the earth's surface. After the flight, the raw images were stored in the camera memory and later transferred to a computer in JPEG format for processing.

Image processing

The images stored in the computer were loaded into Pix4D software. Then, two orthophotos were generated to represent the set of raw images and georeferenced to represent the whole experimental area. The resulting orthophotos consisted both of an RGB orthophoto with red (band 1), green (band 2), and blue (band 3) channels and a multispectral orthophoto with red (band 1), green (band 2), and near-infrared (band 3) channels. The georeferencing was performed according to the coordinate system EPSG: 32723 – WSG 84/UTM zone 23S.

Besides providing the RGB images with radiometric correction, Pix4D software performed the geometric calibration of the cameras by self-calibration using SFM (Structure from Motion).

Moreover, a radiometric calibration of the Mapir RGN sensor was performed. A calibration target was placed on the ground, and after the flight, the radiometric calibration of the camera was performed, so that the images were in accordance with a known and, therefore, comparable radiometric reference. In practice, the solution was in the calibration plates of the sensor. The camera's software, Mapir Camera Control, was used in the radiometric calibration.

Data extraction

The traits extracted from the RGB orthophotos were the vegetative canopy area (VCA), the visible atmospherically resistant index (VARI), and the mean reflectance values of the R, G, and B channels. The mean values of the near-infrared reflectance (NIR) and the normalized difference vegetation index (NDVI) were extracted from the IR orthophotos.

First, using QGIS 3.4.12 software, each plot was delimited and manually cut out in both types of orthophotos, resulting in 30 images per orthophoto (each image represented a single experimental plot; see Figure 1). Next, all images were segmented into two groups, using the k-means clustering algorithm (MacQueen, 1967) with channels "a" and "b" of the scale Lab. Python language was used to segment the images.

From the segmented RGB images, the VCA was calculated by using the numbers of green pixels in each plot, which were later quantified as area (m^2) by multiplying the number of green pixels by the pixel size (1.96×10^{-5} m^2). Still regarding the same images, the mean reflectance values of the R, G, and B channels were calculated per plot, considering that each pixel can assume a value between 0 to 255 per channel. The VARI was calculated according to Equation (1), as described by Gitelson *et al.* (2002).

$$\mathrm{VARI} = \frac{G - R}{G + R - B} \quad (1)$$

where R, G, and B are the mean reflectance values of the red, green, and blue channels, respectively.

The NIR and NDVI traits were extracted from the previously segmented IR images. The IR channel reflectance was estimated from the pixel-level value referring to the near-IR channel. The NDVI calculation was performed with the near-IR and R channels according to Equation (2), as described by Rouse *et al.* (1974):

$$\mathrm{NDVI} = \frac{(\mathrm{NIR} - R)}{(\mathrm{NIR} + R)} \quad (2)$$

where R and NIR are the mean reflectance values of the red and near-infrared channels, respectively.

The soil plant analysis development (SPAD) index, ear yield (EY), and grain yield (GY) were evaluated using conventional methods. In each plot row and from the seventh plant, the five consecutive plants were evaluated to determine the SPAD index, totaling ten plants per plot. The SPAD index, which measures the chlorophyll content, was estimated using a Minolta SPAD-502 portable chlorophyll meter to the last two expanded leaves at the V_7 stage six times per plant. The same plants also provided the EY and GY, which were evaluated after manually harvesting and weighing the ears and grains on a harvest scale. Owing to the different dates of female bloom between the genotypes, the harvesting of the ears was staggered when they reached the harvest point (R_4 stage). To determine the ear harvest point, grain maturity was monitored until it reached a pasty texture. The EY was estimated from the weight of the ears from ten plants per plot to kilograms per hectare. Subsequently, the grains of the same ears were cut close to the cob with a knife to obtain the GY, which was also extrapolated to kilograms per hectare.

Statistical analysis

The data provided the assumptions (homogeneity of variances, normality of residuals, and additivity of block designs) of the analysis of variance (ANOVA) at 0.01 level of significance. Then, the data were fed into ANOVA, and the mean values were grouped using the Scott-Knott test. In addition, the correlations between traits and genotypic coefficient of determination of traits with significant difference by ANOVA were estimated. All the analyses were performed with R software (R Development Core Team, version 3.6.1) using the ExpDes (R package version 1.1.2) and ggplot2 (Package ggplott2 version 3.3.0) packages to test the mean values, estimate the parameters and plot the graphs.

RESULTS AND DISCUSSION

The genotypes evaluated through digital aerial images differed in R, VCA, VARI, and NDVI, but not in G, B, and NIR (Figure 2). There were also differences in the conventionally evaluated traits, that is, the SPAD index, EY, and GY. The results of this study were similar to those of previous studies, indicating that RGB imaging was an useful tool for acquiring phenotypic data and screening sweet corn genotypes (Makanza *et al.*, 2018b).

The hybrids showed higher yields (EY and GY) than the S_3 populations. GY of the hybrid A was 25% higher than that of the hybrid B. These results were possibly due to the greater number of loci in heterozygosity, related to heterosis (hybrid vigor), of these hybrids when compared to the S_3 populations, which show more loci in homozygosity, which relates to inbreeding depression (Borém *et al.*, 2017).

In general, the hybrids showed higher values of vegetative indices (VARI and NDVI) and VCA than that of the S_3 populations. The NDVI was the only index that differed among the S_3 populations. The genotypes L6P15 and L8P18 showed the lowest NDVI values. Comparing the highest yielding genotype (hybrid A) with one of the poorest yielding genotype (L8P18), it was possible to see variation in VCA, VARI, and NDVI indices, which were highlighted by color composition in the image (Figure 3). The high number of dark green and blue pixels meant high VARI and NDVI indices, respectively.

VARI and NDVI indices indicate many plant physiological parameters, such as leaf chlorophyll content and leaf nitrogen concentration (Cairns *et al.*, 2012; Vergara-Díaz *et al.*, 2016). High VCA values imply great photosynthetically active area (Blancon *et al.*, 2019). Moreover, this trait provides the crop with an additional benefit related to weed control. As this study evaluated (V_7 stage), higher VCA at the beginning of the crop cycle promoted faster closure of leaf canopy between the crop rows, leading to less infestation of weeds. All these factors are determinants for a higher EY and GY (Pereira Filho & Teixeira, 2016).

Plants in good physiological condition have high NDVI values, that is, they display high reflectance values in the G and NIR channels and low values predominantly in the R channel (Huang *et al.*, 2012). This pattern was observed in this study, in which the R channel presented the lowest values for hybrids A and B (88.63 and 99.96, respectively). Excluding hybrids, the analysis of populations suggests a positive correlation between GY and R (Figure 2).

Most of the traits showed a high correlation with each other (SPAD, VARI, NDVI, VCA, EY, GY), even traits from different methodologies of evaluation (classical phenotyping and high-throughput phenotyping; Figure 4). By contrast, the same traits were negatively correlated with the mean reflectance values of the R channel. Mostly the genotypic coefficient of determination values was high, varying between 0.71 and 0.93, indicative of greater selection efficiency (Figure 4). In this study, R, VCA, VARI, NDVI (traits from high-throughput phenotyping) showed high genotypic coefficient of determination (close to 0.8).

Among the three channels of image (R, G and B) evaluated, the R channel was the only one to show variability regarding genotypes. Moreover, it showed high correlations (close to -0.8) with traits related to plant development (VCA) and yields (EY and GY). These results suggest that, from RGB images, the R channel is the most useful among the other channels of RGB image for selecting superior genotypes of sweet corn.

The correlations among traits are important, as they help to know if it is possible to use traits extracted from RGB and multispectral imaging to better understand or even replace some traits from classical phenotyping. Such traits can work as secondary to those that are important to sweet corn breeding with low heritability or difficult to measure (indirect selection) (Crain *et al.*, 2017). In addition to having a high correlation with important traits, the secondary traits must have high heritability or high genotypic coefficient of determination (Cruz *et al.*, 2012).

The results showed that VARI and NDVI indices can be used in indirect selection for the SPAD index, as the correlation among these traits and the genotypic coefficient of determination was close to 0.8. The SPAD index measures the chlorophyll content of the leaf and, therefore, it relates to starch production and grain yield (Yang *et al.*, 2012; Xiong *et al.*, 2015). However, SPAD is a difficult or unviable index to be measured in sweet corn breeding programs, because it requires a time-consuming evaluation. Then, the VARI and NDVI indices by means of high-throughput phenotyping can replace the SPAD index and benefit sweet corn breeding programs (Miller *et al.*, 2017; Makanza *et al.*, 2018a; Maciel *et al.*, 2019).

These same indices (VARI and NDVI) and other traits from images (R and VCA) were highly correlated to EY and GY. Therefore, these traits can also be used in early selection or pre-selection for predicting the EY and GY, thus reducing costs and time in broad breeding programs (Gracia-Romero *et al.*, 2018; Makanza *et al.*, 2018b; Hinojosa *et al.*, 2019).

Because of the high costs of new sensors, this study could only estimate a few indices. However, sensors that capture more bands of the electromagnetic spectrum, such as thermal and fluorescence cameras, help extract several other indices [i.e. Soil Adjusted Vegetation Index (SAVI) and Green Normalized Difference Vegetation Index (GNDVI)] and relevant information (i.e. leaf temperature, leaf chlorophyll fluorescense and diseases) (Araus *et al.*, 2012).

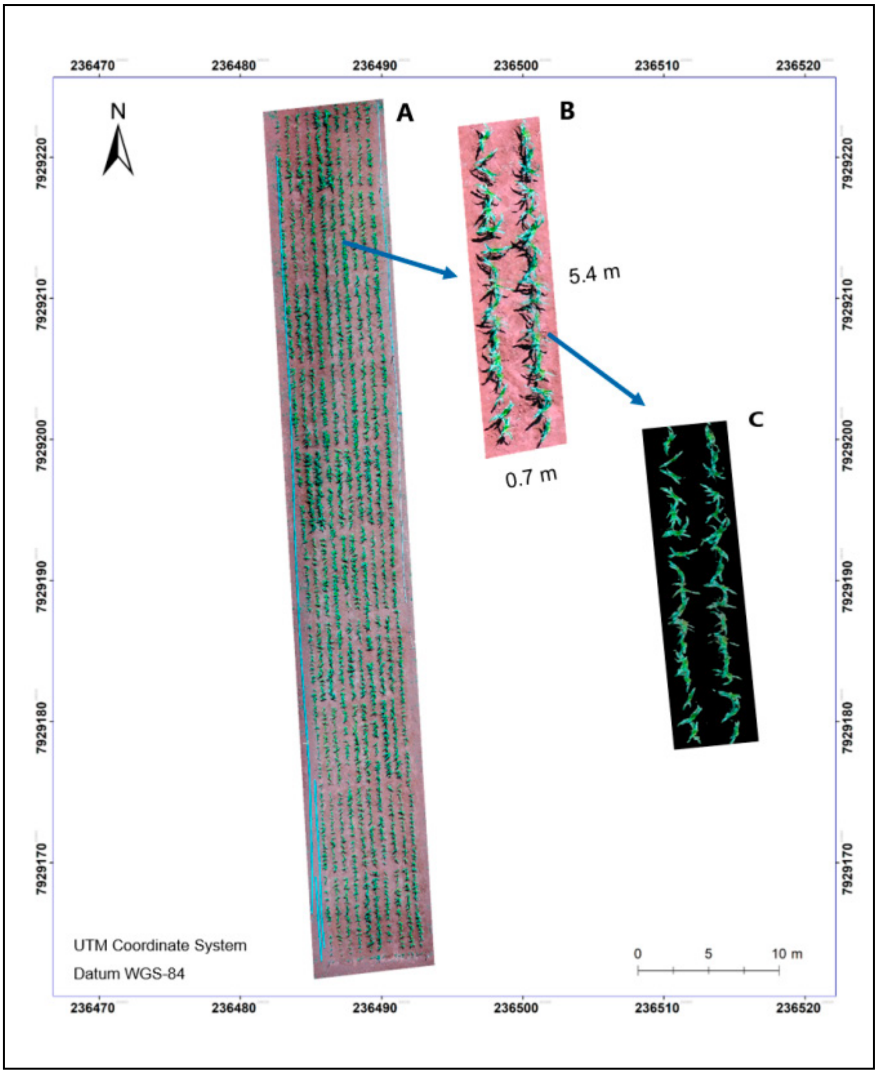

Figure 1. Experimental setup. **A.** Aerial image mosaic of the experiment with plots of sweet corn hybrids, captured with an unmanned aerial vehicle platform; **B.** the experimental setup with single plot details; and **C.** plot image segmentation by the k-means clustering algorithm. Monte Carmelo, UFU, 2021.

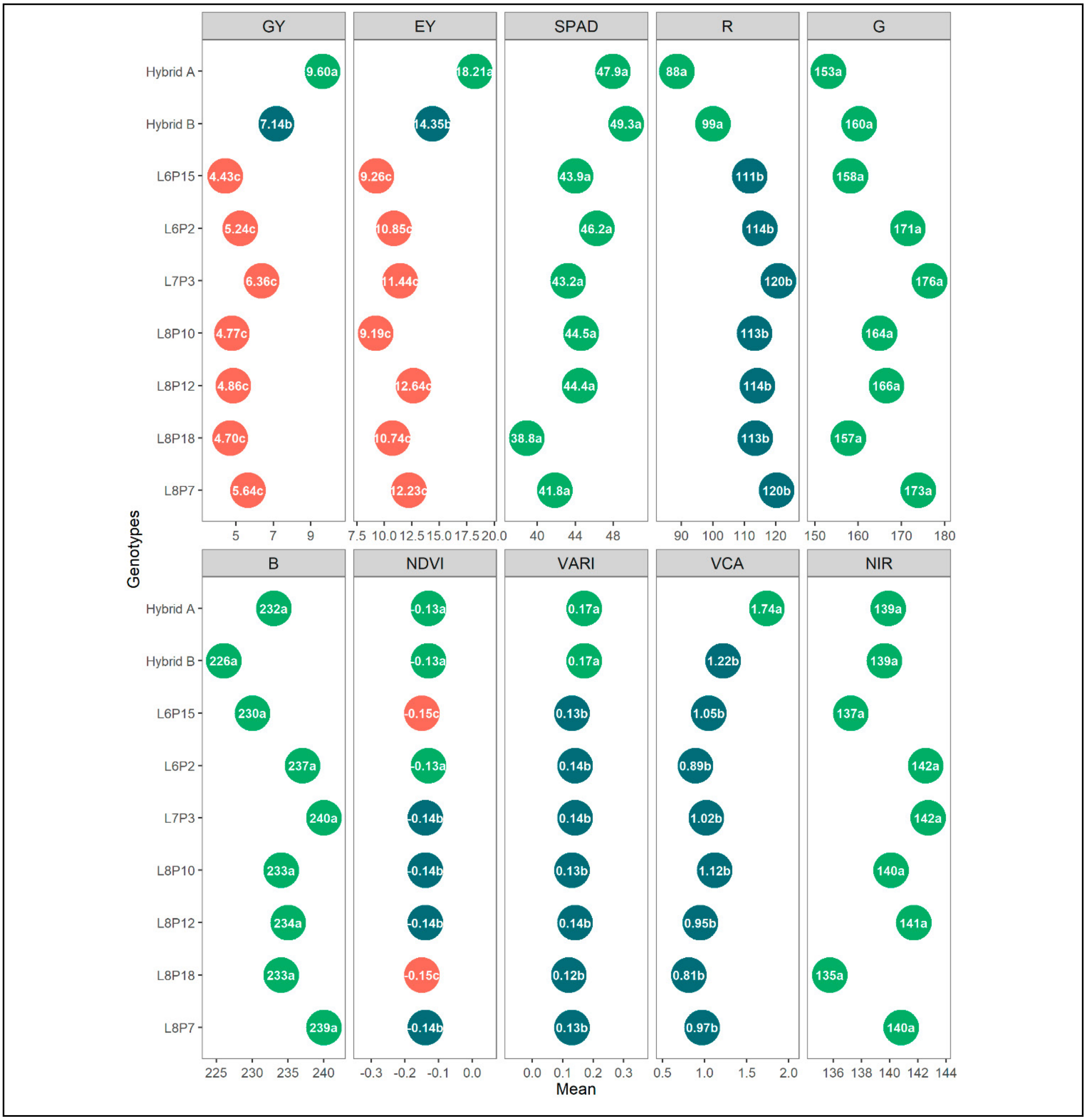

Figure 2. Mean values of nine genotypes of sweet corn. *Means followed by the same letter and color belong to the same group according to Scott-Knott test at 0.05 of significance.; R, G, B, and NIR = mean reflectance values in the red, green, blue, and near-infrared bands, respectively; VCA = vegetative canopy area (m²); VARI = visible atmospherically resistant index; NDVI = normalized difference vegetation index; SPAD = soil plant analysis development index; EY = ear yield (kg ha^{-1}); GY = grain yield (kg ha^{-1}). Monte Carmelo, UFU, 2021.

The traits obtained from images in field depend on the cultivation management used during the crop cycle and on the phenological stage at the time of image capture (Vergara-Díaz *et al.*, 2016; Fernandez-Gallego *et al.*, 2018; Gracia-Romero *et al.*, 2018). Therefore, in order to recommend a broad use of traits from high-throughput phenotyping in sweet corn breeding, further studies under different conditions are essential.

Therefore, the high-throughput phenotyping based on digital image analysis was efficient in identifying sweet corn genotypes with higher and lower yield among populations and between hybrids and populations. The VCA, NDVI, and VARI indices were strongly associated with grain yield, allowing the efficient and quickly selection of more productive genotypes in an indirect and non-destructible way. The hybrids were more productive than S_3 populations, showing that the proposal of this research is an efficient tool to help the selection of superior genotypes on breeding programs.

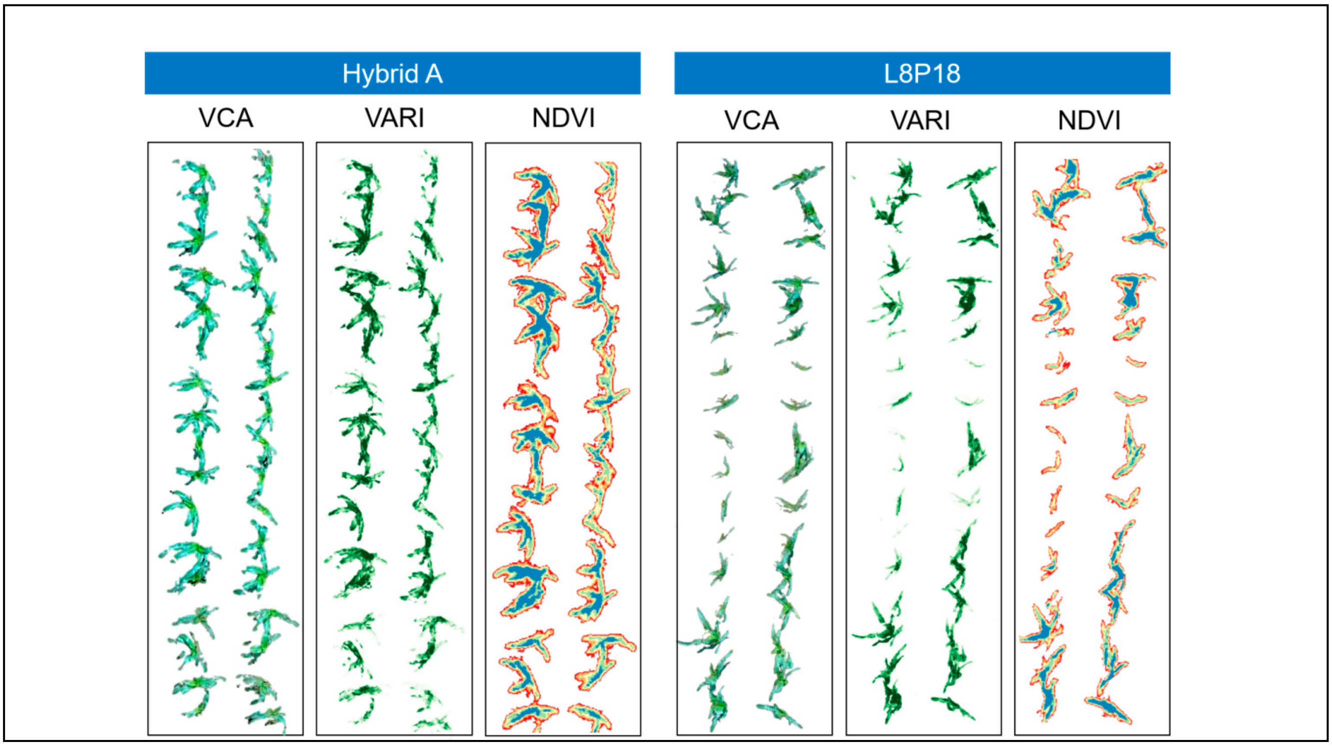

Figure 3. Vegetative canopy color scales. Canopy traits (VCA, VARI, and NDVI) from the plot image. Shades of blue represent more vigorous plants (NDVI values tending to be closer to 1). Shades of red correspond to stressed or not persisting sweet corn (NDVI values tending to be closer to 0). VCA = vegetative canopy area; VARI: visible atmospherically resistant index; NDVI = normalized difference vegetation index. Monte Carmelo, UFU, 2021.

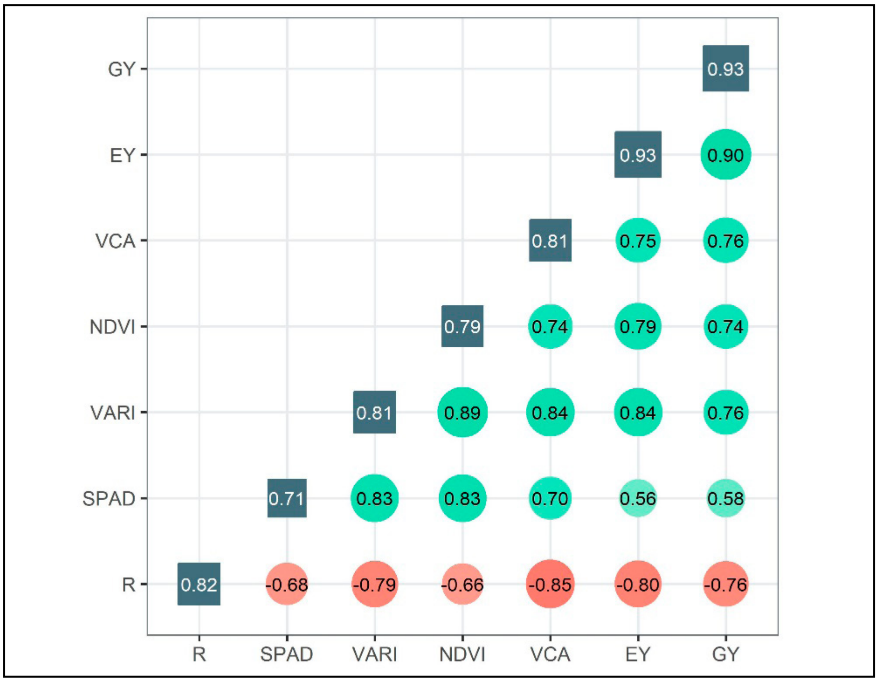

Figure 4. Phenotypic correlation among traits estimated by both classical and high-throughput phenotyping. Correlations among traits (off-diagonal) and genotypic coefficient of determination (diagonal). Positive and negative correlations are colored in green and red, respectively. The diameter of circle is proportional to the magnitude of the correlation. R = mean reflectance values in the red; VCA = vegetative canopy area; VARI = visible atmospherically resistant index; NDVI = normalized difference vegetation index; SPAD = soil plant analysis development index; EY = ear yield (kg ha^{-1}); GY = grain yield (kg ha^{-1}). Monte Carmelo, UFU, 2021.

ACKNOWLEDGMENTS

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

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