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Quantification of chlorophyll fluorescence in soybean seeds by multispectral images and their relationship with physiological potential

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ABSTRACT: The multispectral image analysis technique to detect chlorophyll fluorescence (CF) in soybean seeds was studied to assess the relationship between CF signals and seed physiological potential. Eight treatments, corresponding to 0%, 2%, 4%, 6%, 8%, 10%, 12%, and 14% green seeds, were used on two cultivars, BMX Desafio RR 8473 RSF and 96R10 IPRO, which passed through different seed quality tests. Initially, the CF of the seeds was determined using 660 nm and 730 nm spectra, and then the germination, electrical conductivity, accelerated aging with saturated NaCl solution, tetrazolium, and computerized seedling image analysis (Vigor-S) tests were performed on the same seeds. A completely randomized design was used, as well as replications of each treatment. Analysis of variance (ANOVA) was performed on the data from germination, vigor, and CF tests using the R® software, and the means were grouped by the Scott-Knott test ($p \leq 0.05$). Pearson's linear correlation coefficients (r) were calculated for all combinations among the evaluations with significance of the r values determined by the t-test ($p \leq 0.05$), and multivariate analysis of the principal components was performed. Proportional increases in green seeds contribute to an increase in chlorophyll fluorescence signals and have a negative correlation with seed physiological quality; levels above 4% green seeds in the samples result in marked losses in physiological potential. Therefore, the chlorophyll fluorescence detected through multispectral images is inversely related to the physiological potential of soybean seeds.

Index terms: chlorophyll retention, *Glycine max* (L.) Merrill, green seeds, seed vigor, spectroscopy.

RESUMO: A técnica de análise de imagens multiespectrais para detecção da fluorescência de clorofila (FC) em sementes de soja foi estudada com o objetivo de avaliar a relação entre os sinais da FC e o seu potencial fisiológico. Para dois cultivares, BMX Desafio RR 8473 RSF e 96R10 IPRO, foram utilizados oito tratamentos correspondendo a 0%, 2%, 4%, 6%, 8%, 10%, 12% e 14% de sementes esverdeadas, os quais foram submetidos a diferentes testes de qualidade. Inicialmente, a FC das sementes foi determinada utilizando-se espectros de 660 nm e 730 nm e com as mesmas sementes foram realizados os testes de germinação, condutividade elétrica, envelhecimento acelerado com solução saturada de NaCl, tetrazólio e análise computadorizada de imagens de plântulas (Vigor-S). Foi utilizado o delineamento inteiramente casualizado, com as repetições de cada tratamento. Com o software R®, os dados dos testes de germinação, de vigor e de FC das sementes foram submetidos à análise de variância (ANOVA) e as médias agrupadas pelo teste de Skott-Knott ($p \leq 0{,}05$). Os coeficientes de correlação linear de Pearson (r) foram calculados para todas as combinações entre as avaliações com significância dos valores r, determinados pelo teste t ($p \leq 0{,}05$) e procedeu-se a análise multivariada de componentes principais. Aumentos proporcionais de sementes esverdeadas contribuem para o aumento nos sinais de fluorescências de clorofila e tem correlação negativa com a qualidade fisiológica das sementes, de modo que níveis acima de 4% de sementes esverdeadas nas amostras resultam em perdas acentuadas no potencial fisiológico. Portanto, a fluorescência de clorofila detectada por intermédio de imagens multiespectrais tem relação inversa com o potencial fisiológico de sementes de soja.

Termos para indexação: retenção de clorofila, *Glycine max* (L.) Merrill, sementes esverdeadas, vigor de sementes, espectroscopia.

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INTRODUCTION

In Brazil, soybean [*Glycine max* (L.) Merrill] is the most cultivated plant species, with 10.44 million metric tons of seeds produced on 2.45 million hectares in the 2020/2021 crop year (Brasil, 2021), and 135.9 million tons of grain produced on 38.5 million hectares in the 2020/2021 crop year (CONAB, 2021). As the species is susceptible and sensitive to different environmental stresses, high amounts of green seeds have been observed in seed lots and harvested grain.

Green soybean seeds occur at the end of the seed maturation process, when, for example, temperatures higher than 30 °C, water deficit, insect attack, Asian soybean rust, nematode attack, and incorrectly applied desiccants induce physiological reactions that culminate in forced maturation and inhibition of enzymes such as pheophorbide alpha (α) oxygenase and red chlorophyll catabolite reductase. As a result of inhibition of these enzymes, chlorophyll is retained in the seed coat and in the embryo of the seeds and they, in turn, exhibit a greenish hue (França-Neto et al., 2012; Forti et al., 2015; Marcos-Filho, 2015; Bordignon et al., 2017).

The presence of reactive oxygen species (ROS) makes the green soybean seeds deteriorate more markedly, and their physiological potential is drastically affected, as reflected in reduction and lack of uniformity in germination, reduced vigor, low root development, seedling abnormality, and changes in the phenology and yield of seeds and grain (Zorato et al., 2007; Cicero et al., 2009; França-Neto et al., 2012; Smaniotto et al., 2014; Forti et al., 2015; Pardo et al., 2015).

Seeds have been analyzed with the naked eye to evaluate the presence of green seeds in commercial samples. In contrast, the gravitational separator (gravity table), electronic color separation machine, and air and screen machines and for classification by size have been used with the aim of separating and discarding green seeds present in seed lots, often using indirect characteristics of the green seeds, such as smaller dimensions and weight. Nevertheless, visual analysis is time consuming, tiring, and imprecise, while disposing of seeds by the machines mentioned is of limited effectiveness by not being able to accurately separate the green seeds (França-Neto et al., 2012; Andrade et al., 2016). The use of multispectral images, associated with computational vision, may be a technique with considerable potential for success in detection of green soybean seeds, and consequently, it may allow them to be eliminated with machines specifically for that purpose.

The detection of chlorophyll fluorescence (CF) of the seeds with the use of multispectral imaging occurs through illumination of the seed sample with light-emitting diodes (LEDs) with spectra that range from 400 to 1000 nm, from the ultraviolet (UV) to the near infrared (NIR) region, where the chlorophylls are excited at 660 nm or 730 nm, depending on the sensor of each multispectral device; and the reflectance of the sample is recorded by a monochromatic image sensor (CCD = charge-coupled device) (Mo et al., 2015; Ni et al., 2019). Thus, the multispectral image consists of individual sub-images, each one obtained in a specific wavelength and in grayscale.

The literature shows the relationship between environmental stress and the quality of the soybean seeds evaluated with the spectral imaging technique (Mo et al., 2015). Studies with CF sensors clarify that the CF technique was effective in detection of immature seeds of rice, kale, spinach, green peppers, and green soybean seeds, showing a clear relationship between the CF signals and the physiological potential of said seeds (Jalink et al., 1998; Cicero et al., 2009; Caires et al., 2010; Deleuran et al., 2013; Kenanoglu et al., 2013; Costa et al., 2014).

Since the spectral characteristics of each seed allow physio-chemical changes resulting from biotic and/or abiotic stresses to be detected, the aim of the present study was to evaluate the efficiency of multispectral image analysis in measuring chlorophyll fluorescence in treatments with different proportions of green soybean seeds, as well as to determine the relationships among the percentage of green seeds, chlorophyll fluorescence, and the physiological potential of the seeds.

MATERIALS AND METHODS

Soybean seeds were used from the cultivars BMX Desafio RR 8473 RSF from the Atto Sementes company and the cultivar 96R10IPRO from Corteva Agriscience, produced in the 2018/2019 crop year. The seeds were kept in cold and dry storage (10 °C and 30% relative humidity) until carrying out the experiment. As the seed lots of the two cultivars mixed green seeds with yellow seeds (the characteristic color of the mature seeds of the cultivars used), the seeds with any hues of green color were separated from the yellow in a visual manual manner under 6X magnification.

In performing the tests of germination, electrical conductivity, accelerated aging with saturated NaCl solution, tetrazolium, and computerized seedling image analysis (Vigor-S), each test used eight treatments constituted by a mixture of green seeds with the yellow seeds (without signs of green hue) at the proportions of 0%, 2%, 4%, 6%, 8%, 10%, 12%, and 14% green seeds based on seed weight. Initially, radiographic analysis was performed on the seeds, followed by multispectral analysis for detection and quantification of chlorophyll fluorescence using two multispectral devices (VideometerLab™ and SeedReporter™) and finally, quality tests were performed on the same seeds according to the methodologies described below.

X-ray test: This test was performed with four replications of 25 seeds per treatment with seeds that were subjected to the tetrazolium test. The test of seeds directed to computerized seedling image analysis (Vigor-S) was performed with five replications of 20 seeds per treatment. The other tests were performed with four replications of 50 seeds per treatment. The seeds of each replication were identified and arranged one by one, at equal distance from each other, in 9.0 cm diameter Petri dishes. Then each dish was individually placed in the digital X-ray device Faxitron®, model MX-20 DC12 (Faxitron Bioptics LLC, USA), at a distance of 20 cm from the source of radiation emission and radiographed at 25 kV with exposure of 15 seconds. The digital radiographic images were obtained, saved on an external HD, and evaluated for the presence of mechanical damage, weathering damage, and stink bug; and these seeds were substituted by others without damage to minimize interferences of such seeds on physiological potential and isolate the effect of greenish hue. After that, multispectral techniques were used on the seeds for detection of chlorophyll fluorescence.

Detection and quantification of chlorophyll fluorescence at 660 nm: Initially, the VideometerLab4™ (Videometer A/S, Herlev, Denmark) was calibrated regarding light intensity and correspondence of pixels using a white target, followed by another uniform dark target, and, finally, calibrated with a geometric target. The four replications of 25 seeds per treatment of the seeds directed to the tetrazolium test, the five replications of 20 seeds per treatment of the seeds directed to computerized seedling image analysis (Vigor-S), and the four replications of 50 seeds per treatment of the other quality tests, which were radiographed in the previous step, were placed under the integrating sphere of VideometerLab4™, composed of 19 contiguous light-emitting diodes (LEDs ranging from 365 nm to 970 nm) that, after successive pulses, led to excitation of the chlorophyll molecules at 630 nm, 645 nm, and 660 nm and emission of the CF signals of the seeds at 700 nm. The signals were recorded by the CCD chip, and 25 images of 2192 × 2192 pixel resolution were generated. After acquisition of the images, the regions of interest (ROIs) were identified in each one, that is, the soybean seeds, and they were separated from the background using the "background" mask of the VideometerLab™ software. Using the 660 nm band, spectral differences were identified between the green seed and the yellow seed, and the nCDA (normalized Canonical Discriminant Analysis) supervised method was used to minimize or maximize the observations within and among the seed samples (França-Silva et al., 2020). With assistance of the "blob" (binary large object), the spectral reflectance signatures of the CF were extracted from the seeds in the 660 nm band and the data were exported to Excel. Statistical analysis was used on the data to examine the relationship with physiological potential.

Detection and quantification of chlorophyll fluorescence at 730 nm: The four replications of 25 seeds per treatment of the seeds directed to the tetrazolium test, the five replications of 20 seeds per treatment of the seeds directed to computerized seedling image analysis (Vigor-S), and the four replications of 50 seeds per treatment of the other quality tests that were used in the previous determinations (X-rays and VideometerLab) were removed from the 9.0 cm diameter Petri dishes and transferred to rectangular polyvinyl chloride plates of matte black color (to avoid interference in image contrast) with 100 concave cavities to accommodate the seeds. The seeds were placed and numbered in the same order as in the images of X-rays and of the VideometerLab. Then each plate containing the seeds was placed within the SeedReporter™ (PhenoVation B.V., Wageningen, Netherlands), which emitted sub-pulses from the LEDs in the 620 nm range in a synchronous manner and excited the chlorophylls. Through fast repetition rate fluorometry (FRRF) of the duration of the impulses and CF emission filter in the 730 nm band, images were recorded in grayscale, with 2448 × 2448 pixel resolution. With assistance of the CFTI – Analisys Software (version 5.5.1), in order not to compromise calculation of the CF, a filter was applied to each image with the seeds to isolate and analyze the regions of interest (ROIs), which were the soybean seeds; and the spectral reflectance signatures of the CF of the seeds were extracted in the 730 nm band. Statistical analysis was used on the data and then the following tests were performed on the seeds.

Germination (G): The 50 seeds from each one of the four replications per treatment were used, for a total of 200 seeds, which had been radiographed, followed by quantification of the CF signals at 660 nm and 730 nm. The seeds were numbered and distributed on paper towel previously moistened with water in the weight proportion of 2.5:1 (water : dry paper). The rolls with the seeds were placed in a seed germinator at 25 °C, and germinated seeds were counted after five and eight days (Brasil, 2009). The results were expressed in percentage of normal seedlings.

Electrical conductivity (EC): After analysis of radiographic and multispectral images for detection and quantification of chlorophyll fluorescence, the four replications of 50 seeds of each treatment were weighed on a precision balance (0.01 g) and placed in 200 mL plastic cups with 75 mL of deionized water. The cups were kept for 24 h at 25 °C (Vieira and Marcos-Filho, 2020). After that period, the electrical conductivity of the solution was read using a conductivity meter DIGIMED®, model DM-32. Results were expressed in $\mu S.cm^{-1}.g^{-1}$ of seeds.

Accelerating aging with saturated salt solution (AASS): This test was performed with four replications of 50 seeds from each treatment after radiographic analysis and quantification of CF at 660 nm and 730 nm. The seeds were distributed in a single layer on a metal screen suspended in gerbox (germination box) boxes (11.0 x 11.0 x 3.5 cm), containing 40 mL of saturated NaCl solution (40 $g.100\ mL^{-1}$ of water) at the bottom to create an environment with 76% relative humidity. The boxes were kept in a germination chamber for 48 h at 41 °C. After that period, the germination test was conducted, and the percentage of normal seedlings was evaluated on the fifth day after sowing (Marcos-Filho, 2020).

Tetrazolium (Tz): This test was performed with four replications of 25 seeds each per treatment, after radiographic analysis and quantification of CF at 660 nm and 730 nm. The seeds were pre-soaked with water in paper toweling for 16 h at 25 °C. The seeds were then placed in plastic trays with 100 individualized cells containing 3 mL of a 2,3,5-Triphenyltetrazolium chloride solution at 0.075% for 150 minutes at 35 °C in the dark. After the development of coloring, the solution was drained and the seeds were washed three times in running water. Each seed was sectioned in two longitudinal parts along the cotyledons and classified according to França-Neto and Krzyzanowski (2018) in which classes from 1 to 3 indicate vigor indexes (Tz vg) and from 1 to 5 indicate viability indexes (Tz vb).

Computerized seedling image analysis (Vigor-S): This test used five replications of 20 seeds per treatment, after radiographic analysis and quantification of CF at 660 nm and 730 nm. The seeds were numbered and distributed on paper toweling according to the methodology of the germination test (Gomes-Junior, 2020) and on the third day after sowing, computerized image analysis was performed on the seedlings using the Vigor-S software. The seedlings were transferred from the paper toweling to sheets of blue-colored ethylene-vinyl acetate, with dimensions of 30 × 22 cm, corresponding to the useful area of the HP Scanjet 200 scanner set up in an inverted position within an aluminum box (60 × 50 × 12 cm), adjusted to 300 dpi and linked to a computer. After the images were processed, the vigor indexes were obtained (IV_Vigor.S = values from 0 to 1000, directly proportional to vigor).

Experimental design and statistical analysis: A completely randomized experimental design was used separately for each cultivar considering the respective replications per treatment, used as described in each quality test. Analysis of variance (ANOVA) was used on the data. Normal distribution of errors was confirmed by the Shapiro-Wilk test and homogeneity of variances by the Bartlett test, and then the mean values were grouped by the Scott-Knott test ($p \leq 0.05$). The Pearson linear correlation coefficients (r) were calculated for all the combinations among the evaluations, in which the correlation was obtained by pairing of the mean values of the replications, and the significance of the r values was determined by the t-test ($p \leq 0.05$). Finally, principal component analysis was performed. All the analyses were performed with the R software, version 4.0.0 (R Core Team, 2019).

RESULTS AND DISCUSSION

For the cultivar BMX Desafio RR 8473 RSF, although germination, electrical conductivity, and viability assessed by the tetrazolium test were not affected by the levels of green seeds, in the treatments with more than 4% green seeds, vigor by the accelerated aging test was lower than 89%, and by the Vigor-S software, it was below 575 points. Moreover, the vigor assessed by the tetrazolium test was less than or equal to 64% in the treatments with 4%, 8%, and 14% green seeds (Table 1).

In the cultivar 96R10IPRO, there were no undesirable effects from the percentage of green seeds on seed vigor by AASS and Vigor-S or on viability by the tetrazolium test. However, the presence of more than 10% green seeds reduced seed germination to 77%, while the levels of 2%, 6%, and 14% green seeds were most harmful for conductivity, exceeding 216 $\mu S.cm^{-1}.g^{-1}$. Vigor evaluated by the tetrazolium test was less in the treatments with 6% and 12% green seeds, obtaining the value of 59% (Table 1).

For the chlorophyll fluorescence (CF) variable of the seeds, differences were observed among the treatments in the two cultivars in all the tests. The CF obtained at 660 nm in Videometerlab 4 exhibited high variability of spectral reflectance in the cultivar BMX Desafio RR 8473 RSF. For the same treatment, for example at 0% green seeds, in performance of the tests of germination (G), electrical conductivity (EC) and Vigor-S (IV_Vigor.S), before each test, the seeds had reflectance signatures of 38.48%, 82.70%, and 95.5%, respectively. Within the tests of electrical conductivity (EC) and accelerated aging with saturated salt solution (AASS), the CF of the seeds was incompatible with the proportion of green seeds, with the highest reflectance signatures (82.70% for EC and 77.98% for AASS) recorded in the treatment without green seeds (0%). In the treatment with the highest percentage of green seeds (14%), CF readings were recorded in the order of 40.75% (for EC) and 40.51% (for AASS). Even so, within certain quality tests, there were increases in the reflectance signatures of CF among the treatments as the percentage of green seeds increased (Table 1).

These results may be related to the sensitivity of the equipment to the variations in color and texture of the seeds of this cultivar that were different from chlorophyll retention, as for example the variation in color and texture intrinsic to the seed coat, the presence of purple seed stain, stains from insect bites, and dirt adhering to the seed coat, which limited accurate detection of CF, even using a spectral band and filter specific for chlorophyll. Therefore, for the cultivar BMX Desafio RR 8473 RSF, among treatments, within and among the quality tests, seeds with variations in color and texture (more opaque and darker) reduced reflectance of CF. In contrast, “cleaner” (lighter-colored) seeds, such as those of the cultivar 96R10IPRO, strengthen reflectance of CF; such that for the conditions found in the present study, with VideometerLab 4, it was not possible to obtain repeatability of the results of CF only in accordance with the proportion of green seeds.

The CF of the seeds of the cultivar 96R10IPRO analyzed with VideometerLab 4 operating in the 660 nm band had greater reproducibility and lower variability among the quality tests, except for the seeds under accelerated aging with saturated salt solution. As those seeds had a lighter-colored seed coat than the seeds of the same cultivar used in the other quality tests, they exhibited reflectance signatures of around 20 points higher than the readings obtained for the seeds of the other tests. In spite of this variation, the treatment with 14% green seeds had the highest mean values of CF, whereas the lowest mean values occurred in the treatments with 2% and with 0% green seeds, in that order.

For the CF of the seeds of both soybean cultivars obtained at 730 nm by SeedReporter, the results proved to reliable and reproducible among the quality tests, showing the lack of sensitivity of the equipment to other color and texture attributes in the seeds analyzed, apart from chlorophyll retention, which could negatively interfere in quantification of reflectance of CF. As shown by use of said equipment, the increase from 0% to 14% green seeds resulted in an increase in the reflectance signatures of CF of each treatment; moreover, rates of 6% or more of green seeds result in express reductions in soybean physiological quality (Table 1).

Table 1. Mean values of chlorophyll fluorescence by soybean seed treatment, detected at 660 nm (from VideometerLab, given in percentage of reflectance of white light) and 730 nm (from SeedReporter, given in grayscale) before conducting the respective tests of germination (G), electrical conductivity (EC), germination after accelerated aging with saturated NaCl solution (AASS), vigor index (Tz Vg) and viability (Tz Vb) assessed by the tetrazolium test, and vigor index (IV_Vigor.S) according to computerized seedling image analysis (Vigor-S).

Treatment	660 nm	730 nm	G	660 nm	730 nm	EC	660 nm	730 nm	AASS	660 nm	730 nm	Tz Vg	Tz Vb	660 nm	730 nm	IV_Vigor.S
	(%)	Index	(%)	(%)	Index	($\mu S \cdot cm^{-1} \cdot g^{-1}$)	(%)	Index	(%)	(%)	Index	(%)		(%)	Index	---Index---
Cultivar BMX Desafio RR 8473 RSF																
0%	38.48 d	2041.04 d	93	82.70 a	2280.59 b	126.00	77.98 a	2329.28 b	95 a	96.5 b	2097.1 b	82 a	94	95.5 c	2073.5 b	755.1 a
2%	41.00 c	2315.11 c	85	72.04 a	2474.48 b	141.03	79.84 a	2415.89 b	89 b	96.9 b	2166.9 b	77 a	92	95.7 c	2196.3 b	642.5 a
4%	41.81 c	2440.38 c	91	38.80 b	2392.93 b	136.63	38.08 b	2643.64 a	94 a	100.9 b	2382.7 b	64 b	81	99.4 c	2133.3 b	652.7 a
6%	41.44 c	2367.60 c	86	39.70 b	2572.73 a	134.74	39.30 b	2584.58 a	88 b	102.6 b	2568.4 b	71 a	88	103.7 b	2395.2 b	568.4 b
8%	42.38 b	2666.59 b	88	41.31 b	2800.20 a	142.13	38.66 b	2552.19 a	89 b	106.3 a	3014.7 a	64 b	84	106.4 b	2673.5 b	570.9 b
10%	42.74 b	2518.79 c	88	44.93 b	2476.25 b	130.13	39.14 b	2691.42 a	86 b	109.9 a	3122.3 a	72 a	86	110.3 a	2716.6 b	573.6 b
12%	43.66 b	2750.11 b	83	45.20 a	2504.88 b	145.78	39.66 b	2672.10 a	87 b	111.7 a	3183.1 a	78 a	87	110.3 a	3327.6 a	553.9 b
14%	45.14 a	3004.94 a	85	40.75 b	2639.87 a	131.99	40.51 b	2906.69 a	82 b	113.6 a	3390.8 a	52 b	85	112.8 a	3552.6 a	574.7 b
F.sig	*	*	ns	*	*	ns	*	*	*	*	*	*	ns	*	*	*
CV (%)	2.24	6.31	5.13	17.81	6.8	9.73	2.75	7.12	2.75	3.6	11.1	13.48	8.2	4.3	14.5	12.1
Cultivar 96R10 IPRO																
0%	64.43	2288.27 c	89 a	65.07 c	2306.62 b	199.19 b	83.76 b	2292.90 b	93	62.5 c	2170.1 c	95 a	98	59.7 b	2148.5 b	476.4
2%	64.08	2319.64 c	91 a	65.57 c	2399.22 b	216.65 a	83.96 b	2397.74 b	95	65.2 c	2361.8 c	73 b	89	63.5 b	2516.0 b	444.4
4%	64.62	2425.41 c	88 a	66.49 c	2451.37 b	210.62 b	85.63 b	2465.71 b	99	67.3 c	2426.1 c	80 a	93	64.2 b	2617.0 b	430.4
6%	67.08	2573.63 c	85 b	67.73 b	2607.65 b	233.20 a	85.81 b	2510.07 b	98	64.8 c	2855.9 b	59 b	86	68.1 a	3127.7 a	432.3
8%	67.04	2484.46 c	84 b	67.80 b	2566.53 b	195.30 b	87.60 a	2594.36 b	96	66.9 c	2704.4 b	90 a	95	69.3 a	3662.9 a	403.5
10%	68.63	2843.71 b	83 b	68.43 b	2576.74 b	201.23 b	88.87 a	2699.73 a	96	68.5 c	3006.6 b	89 a	95	70.5 a	3595.8 a	443.5
12%	68.95	2749.54 b	77 c	70.72 a	2873.05 a	207.61 b	90.51 a	2897.74 a	97	70.0 b	3211.3 b	59 b	87	69.6 a	3423.9 a	460.5
14%	70.10	3245.68 a	77 c	71.97 a	3036.91 a	222.84 a	90.52 a	2850.34 a	96	75.4 a	4088.3 a	65 b	85	70.6 a	3651.7 a	451.4
F.sig	ns	*	*	*	*	*	*	*	ns	*	*	*	ns	*	*	ns
CV (%)	1.76	9.16	4.64	2.42	5.94	6.03	2.05	6.65	2.5	4.28	10.62	11.51	8.72	4.9	12.5	18.79

F.sig: Significance of the F test in which ns = non-significant and * = significant ($p \leq 0.05$). The mean values followed by different letters in the columns, within each cultivar, differ from each other by the Scott-Knott test ($p \leq 0.05$). CV = coefficient of variation.

Principal component analysis (PCA) allowed the dimensionality of the data to be reduced, and the interrelations between the chlorophyll fluorescence variables of the seeds with germination, vigor, and viability could be analyzed. PCA also allowed visualization of a spatial separation of the samples of the two cultivars.

Two components, PC1 and PC2, were necessary to explain the variability in the data of the cultivar BMX Desafio RR 8473 RSF, which explained 67.40% and 14.80% of the variability, respectively, or 82.20% of the total variability (Figure 1a). The treatments with 0%, 2%, and 4% green seeds had greater physiological potential, in accordance with the proximity of these treatments with the germination, AASS, vigor index, and viability variables by the tetrazolium test and vigor index by Vigor-S, located on the same side in the biplot (Figure 1a).

The treatments with 6% to 14% green seeds exhibited nearness to CF at 660 nm and 730 nm, and, in turn, they were located on the vector opposite the seed quality variables, which indicates reduction in physiological potential of the seeds in these treatments according to the proportion of green seeds (Figure 1a).

The results of the Pearson r correlation (Figure 1b) for the cultivar BMX Desafio RR 8473 RSF show that, among the quality tests, there was positive correlation in germination with accelerated aging with salt solution (r = 0.78) and germination with the vigor index assessed by Vigor-S (IV_Vigor.S: r = 0.8); in AASS with IV_Vigor.S (r = 0.83); and between vigor (Tz_vigor) and viability (Tz_viab) evaluated in the tetrazolium test (r = 0.73). Comparison of the relationship between the quality tests and the respective CFs of the seeds, at 660 nm and 730 nm, a negative correlation (r ≤ - 0.71) was observed, except for the EC_660 and AASS_660 variables, which had positive correlation with the quality tests, which indicates an inconsistency in the method (Figure 1b).

The results described above indicate that chlorophyll retention in the seeds contributes proportionally to the increase in the magnitude of the chlorophyll fluorescence signals and has an inverse (negative) relationship with seed physiological potential, such that the higher the CF of the seeds, the lower their germination, viability, and vigor.

The results of principal component analysis for quality and chlorophyll fluorescence of the eight treatments of the cultivar 96R10IPRO are shown in Figure 2. Two components were found, PC1 and PC2, which were responsible for explaining 64.70% and 14.80% of the variability of the data, respectively, or 79.50% of the total variability (Figure 2a).

While the treatments with 0%, 2%, 4%, and 8% of green seeds had greater physiological potential according to the nearness of these treatments to the germination, Tz_vigor and Tz_viab, and IV_Vigor.S variables, the treatment with 6% showed nearness to the EC and AASS variables, which indicates inconsistency either in the CF or in the tests; and the other treatments exhibited nearness to chlorophyll fluorescence at 660 nm and 730 nm. The 6%, 10%, 12%, and 14% treatments were located on the vector opposite the seed quality variables, which indicates that the seeds of these treatments had lower physiological potential (Figure 2a).

The results of Pearson r correlation for the cultivar 96R10IPRO show significant and negative correlations in electrical conductivity with vigor (Tz_vigor: r = - 0.80) and with viability (Tz_viab: r = -0.83) of the seeds evaluated in the tetrazolium test; as well as high positive and significant correlation between these last two variables, Tz_vigor and Tz_viab (r = 0.97). In comparison of the relationship between the quality tests and the respective CFs of the seeds, at 660 nm and 730 nm, negative correlations (r ≤ -0.71) were observed, except for the EC and AASS variables, which showed positive correlations with the CF variables, according to Figure 2b, indicating inconsistency of the method of analysis of CF by multispectral images.

It is noteworthy that for both cultivars analyzed in this study, given the inverse (negative) relationships of the CF of the seeds at 660 nm and 730 nm with the quality tests, in which reductions in physiological potential were indicated in proportion to the increase in green seeds, chlorophyll retention not only limited the synthesis of photosynthates, but also favored the presence of ROS, which are responsible for intensifying seed deterioration.

The patterns of the CF of the seeds were assessed with multispectral images and classified according to the level of chlorophyll retention in the seed coat and embryo (Figure 3). For the same seed, images in the RGB (Red-Green-Blue) color scale, radiographic (grayscale) images, and color map images, with variations in the intensity of reflectance of the seeds in the 660 nm and 730 nm band, allowed identification of the seed area with retained chlorophyll.

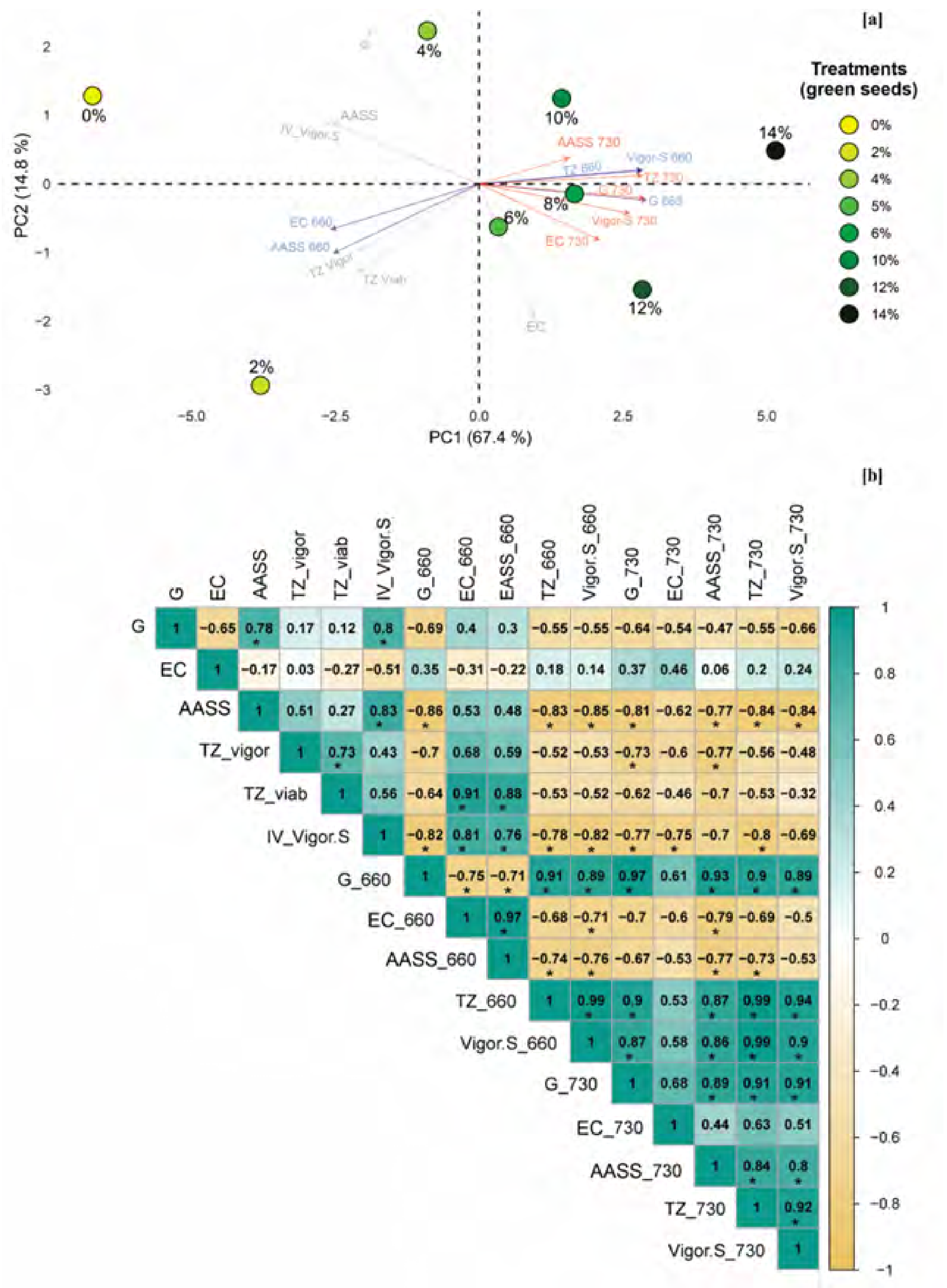

Figure 1. Biplot of the principal components **[a]** and correlation matrix between the physiological quality of soybean seeds of cultivar BMX Desafio RR 8473 RSF and chlorophyll fluorescence signals in accordance with treatments with 0%, 2%, 4%, 6%, 8%, 10%, 12%, and 14% green seeds **[b]**. The numbers in the matrix show the Pearson correlation coefficient between the variables. *, significant ($p \leq 0.05$); G, germination; EC, electrical conductivity; AASS, germination after accelerated aging with saturated salt (NaCl) solution; Tz Vigor and Tz viab, vigor and viability by the tetrazolium test, respectively; IV_Vigor.S, vigor index by computerized seedling image analysis (Vigor-S); the designations G_660 and G_730, EC_660 and EC_730, AASS_660 and AASS_730, TZ_660 and TZ_730, and Vigor.S_660 and Vigor.S_730 indicate the readings of chlorophyll fluorescence in the 660 nm and 730 nm bands carried out in the seeds before said test.

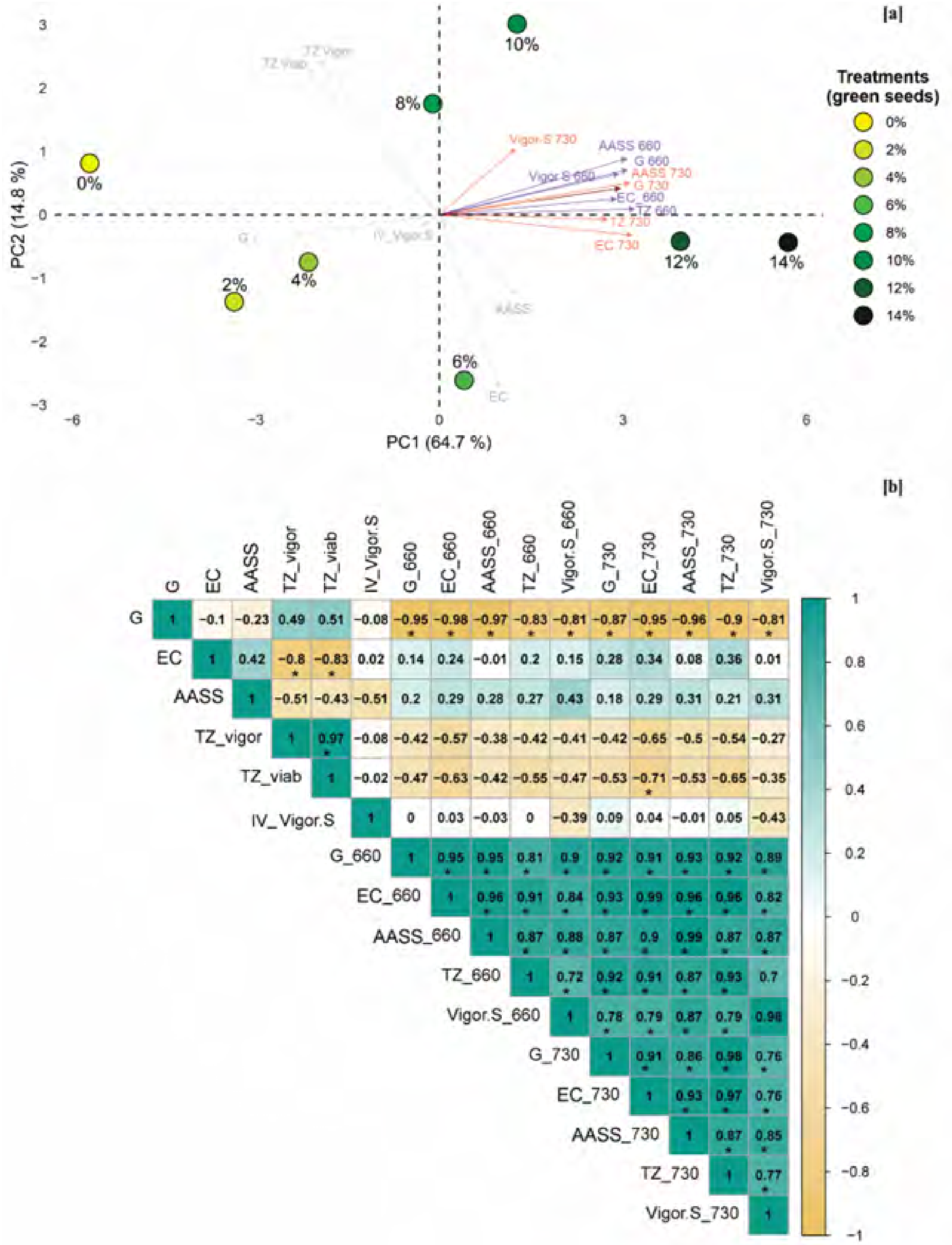

Figure 2. Biplot of the principal components **[a]** and correlation matrix between the physiological quality of soybean seeds of cultivar 96R10IPRO and chlorophyll fluorescence signals in accordance with treatments with 0%, 2%, 4%, 6%, 8%, 10%, 12%, and 14% green seeds **[b]**. The numbers in the matrix show the Pearson correlation coefficient between the variables. *, significant ($p \leq 0.05$); G, germination; EC, electrical conductivity; AASS, germination after accelerated aging with saturated salt (NaCl) solution; Tz Vigor and Tz viab, vigor and viability by the tetrazolium test, respectively; IV_Vigor.S, vigor index by computerized seedling image analysis (Vigor-S); the designations G_660 and G_730, EC_660 and EC_730, AASS_660 and AASS_730, TZ_660 and TZ_730, and Vigor.S_660 and Vigor.S_730 indicate the readings of chlorophyll fluorescence in the 660 nm and 730 nm bands carried out in the seeds before said test.

The greater the level of chlorophyll retention in the seed coat and in the embryo, the greater the CF and the lower the physiological quality of said seeds (Figure 3). Therefore, chlorophyll fluorescence was indetectable in the “yellow” seeds in the multispectral images, and these seeds exhibited greater germination and vigor (Figures 3a and 3e). Furthermore, seeds with weak CF signals had slow germination, and this favored the occurrence of fungi (Figures 3b and 3f), whereas seeds with chlorophyll retained in 50% and 75% of the area, considered partially and totally green, respectively, had higher values of CF and resulted in abnormal seedlings (Figures 3c and 3g) or dead seeds (Figures 3d and 3h).

The technique of multispectral image analysis for detection and quantification of CF in association with the germination, vigor, and viability tests confirmed that the proportion of green soybean seeds compromises the physiological potential of the seeds. These results are in agreement with Jalink et al. (1998), Dell’Aquila et al. (2002), Kenanoglu et al. (2013), Yadav et al. (2015), Cícero et al. (2009), and Teixeira et al. (2020), who observed a relationship between CF with seed germination and vigor in *Brassica oleracea*, white cabbage, green peppers, *Brassica oleracea* var. *capitata*, soybean and soybean, respectively. They showed that the greater the CF of the seeds, the lower the values of germination and vigor.

The lack of observing undesirable effects on the quality of seeds of the cultivar BMX Desafio RR 8473 RSF and of the cultivar 96R10IPRO in some vigor and viability tests may be related to storage of the seeds under controlled conditions and for a short time. According to Pádua et al. (2007), Cicero et al. (2009), and Kenanoglu et al. (2013), reduction in quality and longevity of soybean seeds, especially of immature and green seeds, is more marked when they are stored under non-controlled conditions.

The results for vigor and viability of the soybean seeds in the tetrazolium test, though not statistically significant, were similar to those described by Zorato et al. (2007), Arruda et al. (2016), and Teixeira et al. (2020). In general, increasing the percentage of green seeds of soybean in the samples resulted in a higher rate of dead seeds (weathering damage) and a sharp decline in vigor and viability, which was more marked in samples with more than 10% green seeds and whose greenish hues were concentrated in the embryonic axis (Costa et al., 2001).

The results of IV_Vigor.S showed the ability of the technique in distinguishing the level of vigor in the two cultivars under study according to the percentages of green soybean seeds, corroborating the studies of Castan et al. (2018), Medeiros et al. (2019), and Rodrigues et al. (2020), which showed the efficiency of Vigor-S in evaluating the vigor of maize, common bean, and soybean seeds, respectively.

Reduction in seed quality in accordance with a greater amount of retained chlorophyll is an intrinsic characteristic of green seeds, which show high rates of deterioration and low capacity for reorganization of the membrane system and of the compromised reserve tissue; and that favors the occurrence of microorganisms (Forti et al., 2015; Pardo et al., 2015; Teixeira et al., 2020). In general, seeds with the characteristic colors of each soybean cultivar have low total chlorophyll contents, since the chlorophyll was degraded in the maturation and drying phases. Therefore, the higher the proportion of these seeds in a seed lot, the greater the physiological quality of the seeds and the vigor of the plants will be (Olasoji et al., 2012).

Although the soybean cultivars used in the present study had germination within the minimum standard required for commercialization (minimum of 80%), except for the treatments with 12% and 14% green seeds of the cultivar 96R10IPRO, which exhibited 77% germination, high physiological potential was not observed for the treatments with more than 4% green seeds. In the literature, percentages from 5% to 10% green seeds represent acceptable negative effects on the physiological quality of the seeds, and above 10%, the physiological potential of the soybean seeds is compromised to the point of making them unfeasible for commercialization and sowing (Pádua et al., 2007; França-Neto et al., 2012).

The CF of the soybean seeds varied according to the percentage of green seeds and the intensity of the green color in the seed coat and in the embryo, varying both within and among the cultivars analyzed; higher CF values correspond to higher proportional content of total chlorophyll (a + b). Just as occurred in this study, the CF signals was found in seeds of spinach (Olesen et al., 2011), four cultivars of *Capsicum annuum* (Kenanoglu et al., 2013), rice (Hay

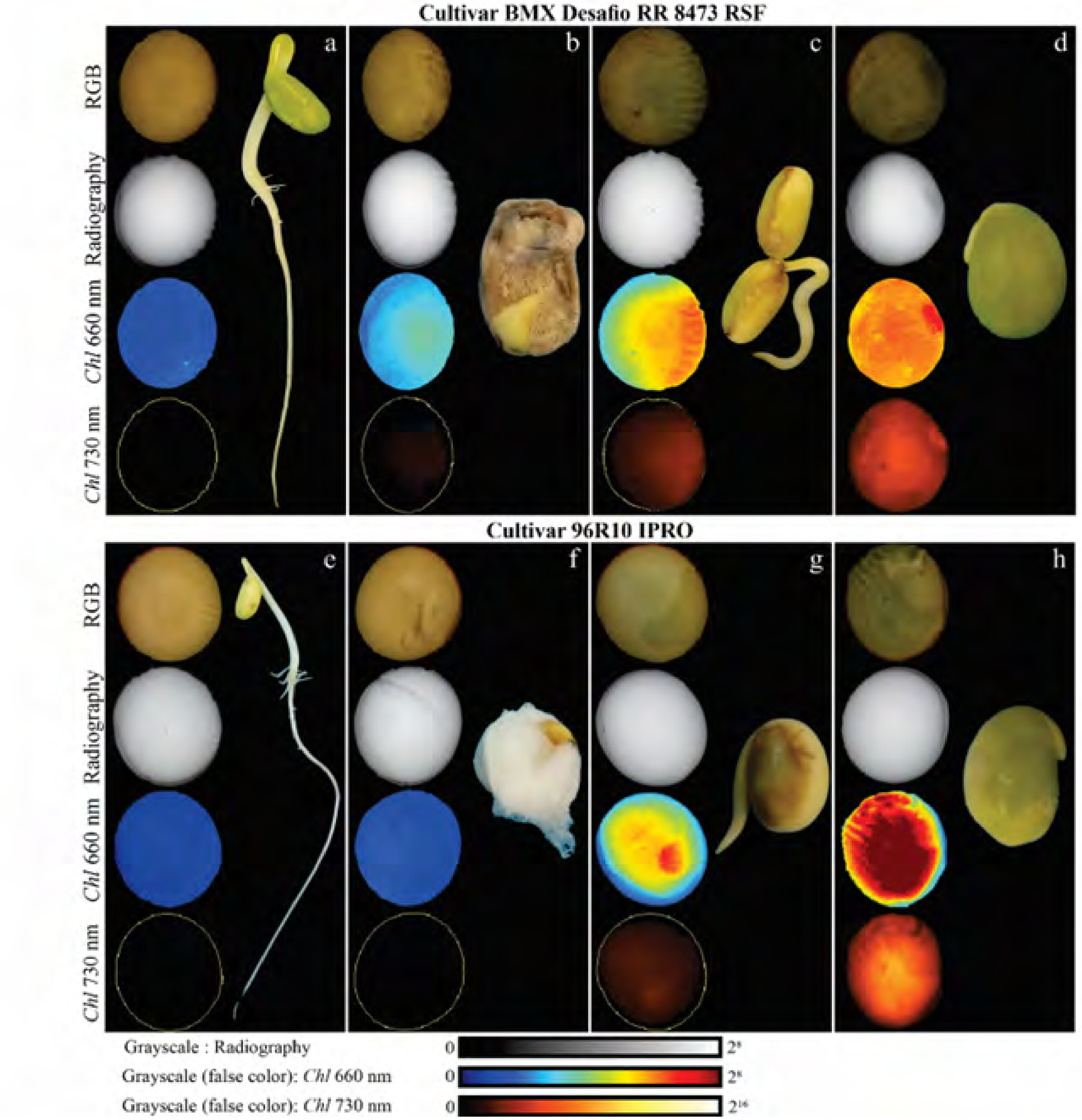

Figure 3. Soybean seeds with increasing levels of retained chlorophyll, obtained from RGB (Red-Green-Blue) images of internal morphology with the same seeds observed in radiography and chlorophyll fluorescence signals detected by multispectral images at 660 nm (Chl 660 nm) and 730 nm (Chl 730 nm), respectively. In the multispectral images of the soybean seeds, transformed into color map based on gray levels, the hues of the colors dark blue (660 nm, of VideometerLab) and black (730 nm, of SeedReporter) represent the seeds with “yellow” seed coat and embryo, whose chlorophyll was degraded and, for that reason, have low chlorophyll fluorescence signals; the hues with a tendency toward intense red indicate the “greenish” seeds, whose chlorophyll is retained in the seed coat and embryo and, therefore, express greater signals of chlorophyll fluorescence. **a**; **e**: normal soybean seedling resulting from yellow seed; **b**; **f**: dead seed, with mechanical damage, covered by fungal mycelium and weak signals of chlorophyll fluorescence; **c**; **g**: abnormal soybean seedling originating from seed with partial chlorophyll retention; **d**; **h**: dead seed resulting from green coloring, which indicates a high level of retained chlorophyll.

et al., 2015), and tomato (Li et al., 2016). However, the variation in color and texture of the different seeds apart from chlorophyll retention, which interfered in accurate quantification of the CF signals, was a finding in this study; and that should be considered a limiting factor for the use of equipment sensitive to such attributes, as well as the fact that the technique is inefficient in detecting CF in maize (*Zea mays*) and sunflower (*Helianthus annus*) seeds and grain (Kenanoglu et al., 2013).

The positive relationship of the levels of green seeds with the CF and, conversely, the inverse relationship of CF with seed physiological potential, observed from the principal components and correlation matrices, corroborate the findings for seeds of cabbage (Jalink et al., 1998), spinach (Deleuran et al., 2013), *Capsicum pepper* (Kenanoglu et al., 2013), rice (Costa et al., 2014), and soybean (Cicero et al., 2009; Forti et al., 2015). These results are possible given the sensitivity of the method in amplifying the signals of CF of the seeds and indicating changes in the metabolites of the seeds, resulting from biotic and abiotic stresses, which makes the technique of analysis of spectral images feasible for evaluation of the physiological quality of the seeds of different species (Matzrafi et al., 2017).

CONCLUSIONS

Multispectral image analysis allows identification of green soybean seeds and quantification of their chlorophyll fluorescence. Reflectance signatures of the CF of the seeds obtained at 730 nm with SeedReporter were more stable, accurate, and reproducible among quality tests and cultivars. However, the VideometerLab 4 device, even operating with the specific band and filter of chlorophyll, 660 nm, exhibited results of CF that were affected by the variation in color and texture of the seeds that were distinct from chlorophyll retention. Even so, proportional increases of green seeds of the cultivars BMX Desafio RR 8473 RSF and 96R10IPRO contribute to an increase in the signs of chlorophyll fluorescence and have a negative correlation with seed physiological quality; levels greater than 4% green seeds in the samples result in marked losses in physiological potential. Therefore, the chlorophyll fluorescence detected through multispectral images has an inverse relationship with the physiological potential of soybean seeds.

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