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MODEL FOR PLANTS NUTRIENT STRESS ASSESSMENT BASED ON FLUORESCENCE MICROSCOPIC IMAGES: CASE STUDY IN WHEAT

Ciprian RUJESCU, Florin SALA

Banat University of Agricultural Sciences and Veterinary Medicine, "King Michael I of Romania", from Timisoara, Calea Aradului 119, 300645, Timișoara, Romania

Corresponding author email: florin_sala@usab-tm.ro

Abstract

The state of plant nutrition was assessed in wheat plants, Alex cultivar, BBCH 12-13 stage. Microscopic images, Leica CLSM, DM2500 were taken on the leaves. On the microscopic images were analyzed in RGB color system, for plants with normal nutrition (R_{nn}, G_{nn}, B_{nn}) and for plants with nutrient deficiency (R_{nd}, G_{nd}, B_{nd}). The average values for R, were R_{nn} = 110.30, R_{nd} = 47.65. The differences between the averages of the studied cases were high and statistically significant (test t, t = 14.224, p< 0.001; non-parametric Mann-Whitney test, U = 0, p< 0.001). In the case of the G, the average values were G_{nn} = 51.92, G_{nd} = 55.54, and in the case of B, the average values were B_{nn} = 4.12, B_{nd} = 4.16, without statistical assurance of differences.The average value of the fractal dimension (D) in the case of plants with normal nutrition was D_{nn} = 1.746, and in the case of plants with nutritional deficiency it was D_{nd} = 1.850. The difference between the means was statistically significant, according to the indicated value of t = 10.88, having p < 0.001. And in the case of applying the non-parametric Mann-Whitney test the differences between groups are significant, U = 0, p < 0.001.

***Key words**: CLS, fluorescence, fractal analysis, RGB, wheat.*

INTRODUCTION

The evaluation of plants and crops can be done at different levels, from macro scale to nano scale. During this very wide interval, studies were carried out that include large areas, regions and agricultural basins, based on satellite images, macroeconomic studies, etc. (Artzberger, 2013; Herbei and Sala, 2016; Dalmau et al., 2017; Popescu et al., 2020), studies at the level of agricultural plots, based on aerial (drone) or terrestrial images (digital sensors on machines and equipment agricultural studies), usually studies aimed at agricultural crop management (Valente et al., 2011; Constantinescu et al., 2018; Sala et al., 2020a).

Some studies have been conducted at the plant level, as an organism, through physiological indices that express the degree of plant adaptability, nutritional status and the relationship of plants with environmental and technological factors (Jivan and Sala, 2014; Motaghi and Nejad, 2014; Dobrei et al., 2015; Datcu et al., 2017), elements of plant productivity (Rawashdeh and Sala, 2016). Studies have also been performed at the cellular and plant tissue level, using microscopic techniques (Ishida et al., 2000; Li et al., 2007; Sozzani et al., 2014), or biochemical and molecular studies (Xiong and Zhu, 2002; Du et al., 2009).

Methods based on imaging, in the evaluation and investigation of plant organisms have developed more and more as a result of the facilities offered by these methods: real-time evaluation, high accuracy, fast and efficient formulation of different solutions, etc. (Li et al., 2014; Drienovsky et al., 2017; Perez-Sanz et al., 2017).

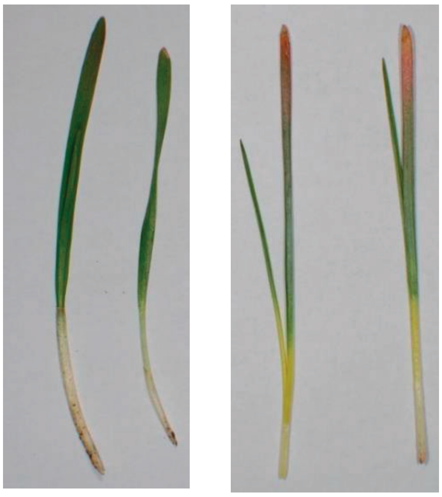
Microscopic methods are very important in the study of plant organisms and have been used for herbal standardization (Lachumy and Sasidharan, 2012; Chirskaya et al., 2014). Investigation technology based on spectro-microscopy, or fluorescence microscopy has been used to study the biology of plant cells and tissues (Dubrovsky et al., 2006; Harter et al., 2012).

Fluorescent confocal laser microscopy is useful for obtaining high-resolution optical sections in plant tissues, for studies of cell organization, dynamics of biological developmental processes, the response of molecular and

cellular structures to internal or external stimuli of plants, to factors stress etc. (Genre, 2008; Pollastri et al., 2012).
The present study used the investigative technique based on confocal microscopy in fluorescence associated with imaging and fractal analysis to achieve a model of wheat plant response to nutritive stress.

MATERIALS AND METHODS

The biological material was represented by the Alex wheat cultivar. Leaf samples were taken from nutrient deficient plants (without fertilization, variant control) and from fertilized plants (PK150N50). Determinations were made in the early stages of plant vegetation (Principal growth stage 1, 12-13 BBCH Code) (Figure 1). This is the critical period of nutrition for wheat, when nutritional deficiency significantly affects plant metabolism (Marschner, 1995; Mengel and Kirkby, 2001). In the present study, the symptoms of the leaves expressed phosphorus deficiency (leaves red-anthocyanin color).



a b

Figure 1. Plant samples (a - plant with normal nutrition status; b - plant with nutrition deficiency)

For microscopic analysis, portions of different areas of normal leaves and nutrient-deficient leaves were sectioned, and mounted on a glass slide. No staining or manipulation of the samples was performed. In the present study, a Leica CLSM microscope, model DM 2500, with HCX PL FLUOTAR 10x/0.30 N.A. lenses was used, and for more detailed images, the HCX PL APO 63x/1.40 N.A. oil immersion lens was used. Images were obtained by scanning each leaf sample with a small light source (~ 1 μm in diameter) and by recording the light reflected by the focusing plane (Pawley, 2006). In order to simultaneously detect the red and green emission light, the dual fluorescent mode was used. For the green emitting light, a laser with a wavelength of 488 nm was used, and the emitted lights were recorded at wavelengths of 530-550 nm. A laser with a wavelength of 488 nm was used for the red emission light, and the emitted lights were recorded at 680-710 nm, according to Gitelson et al. (1999). The samples were scanned at a speed of 400 Hz and a laser intensity of 30-40%. Images were recorded using LAS-AF (Leica Application Suite - Advanced Fluorescence). The images obtained for plants with normal nutrition are shown in Figure 2, and for those with nutritional deficiency in Figure 3.
To evaluate the properties of wheat leaves in fluorescent light in relation to nutritional status, microscopic images were analyzed by imaging methods. The color spectrum in the RGB system and the pixel proportion of the three spectral bands R, G, B in each image were analyzed on color microscopic images, both in samples with nutrition defficiency (R_{nd}, G_{nd}, B_{nd}) and in normal leaves (R_{nn}, G_{nn}, B_{nn}).
Fractal analysis was used as a color-neutral method to analyze microscopic images. For the analysis of the fractal geometry of the microscopic structure of the leaves captured by confocal microscopy, the same binarized images were analyzed. Fractal dimensions (D) were obtained for the two cases studied; plants with normal nutrition (D_{nn}) and with deficiency nutrition (D_{nd}). Image J software was used for image analysis (Rasband, 1997).
The evaluation of the differences between the groups of vegetal samples, corresponding to the plants with normal nutrition, respectively of the plants that presented nutritional deficiencies was performed on the basis of two-sample t test for equal means, respectively the non-parametric Mann-Whitney test for equal medians. Testing for differences in series variability was performed using the non-parametric Fligner-Killeen test. The Past software was used for each case (Hammer et al., 2001).

As a working scheme and for data validation, the RGB-D model was proposed for the evaluation of plant nutritional deficiency, based on RGB parameters and fractal dimension D resulted from the analysis of microscopic images. The logic diagram is shown in Figure 4.

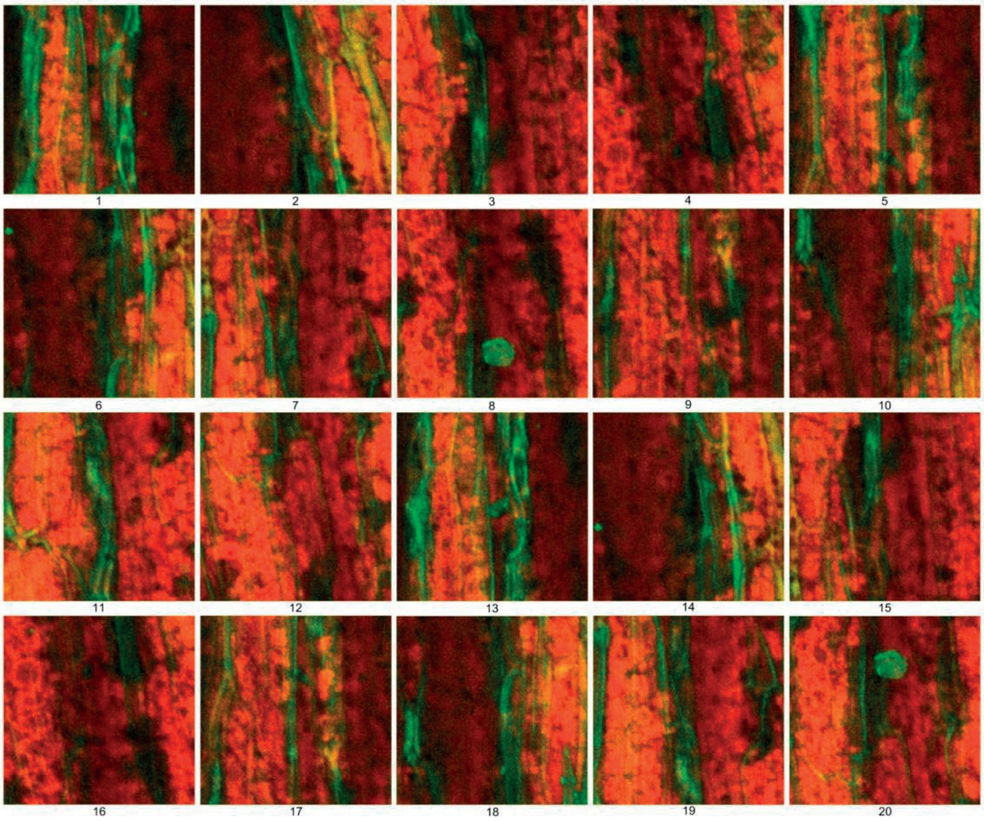


Figure 2. Microscopic images of wheat leaves with normal nutrition status (confocal microscopy)

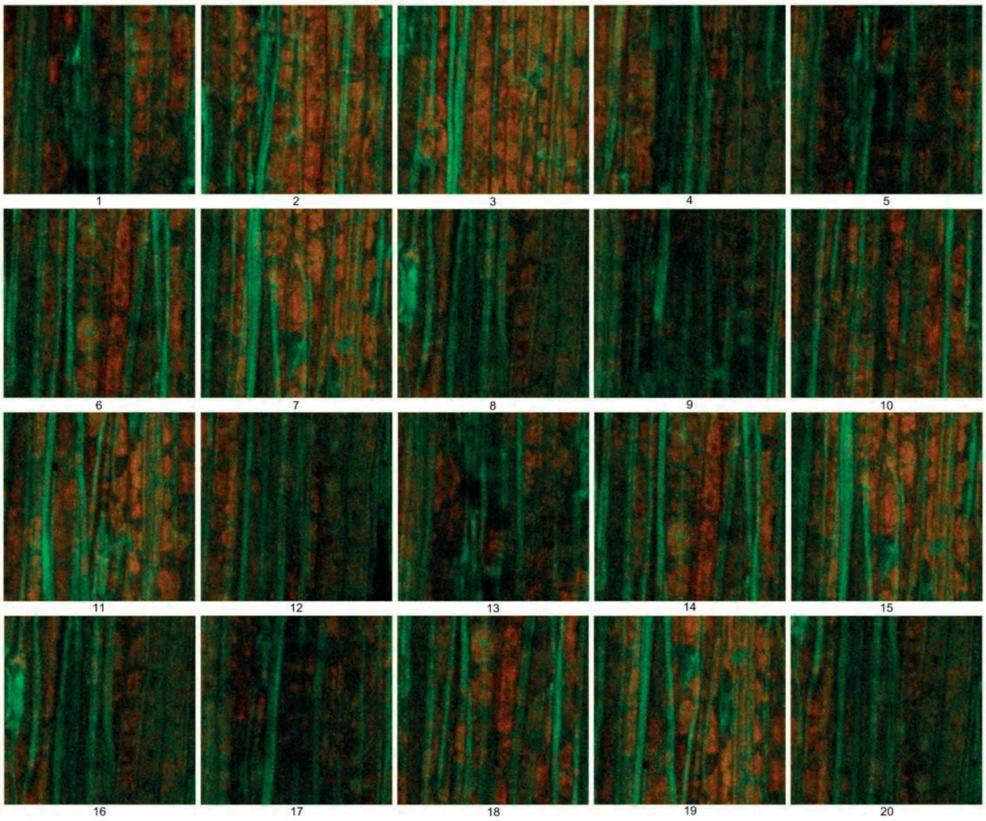


Figure 3. Microscopic images of nutritionally deficient wheat leaves (confocal microscopy)

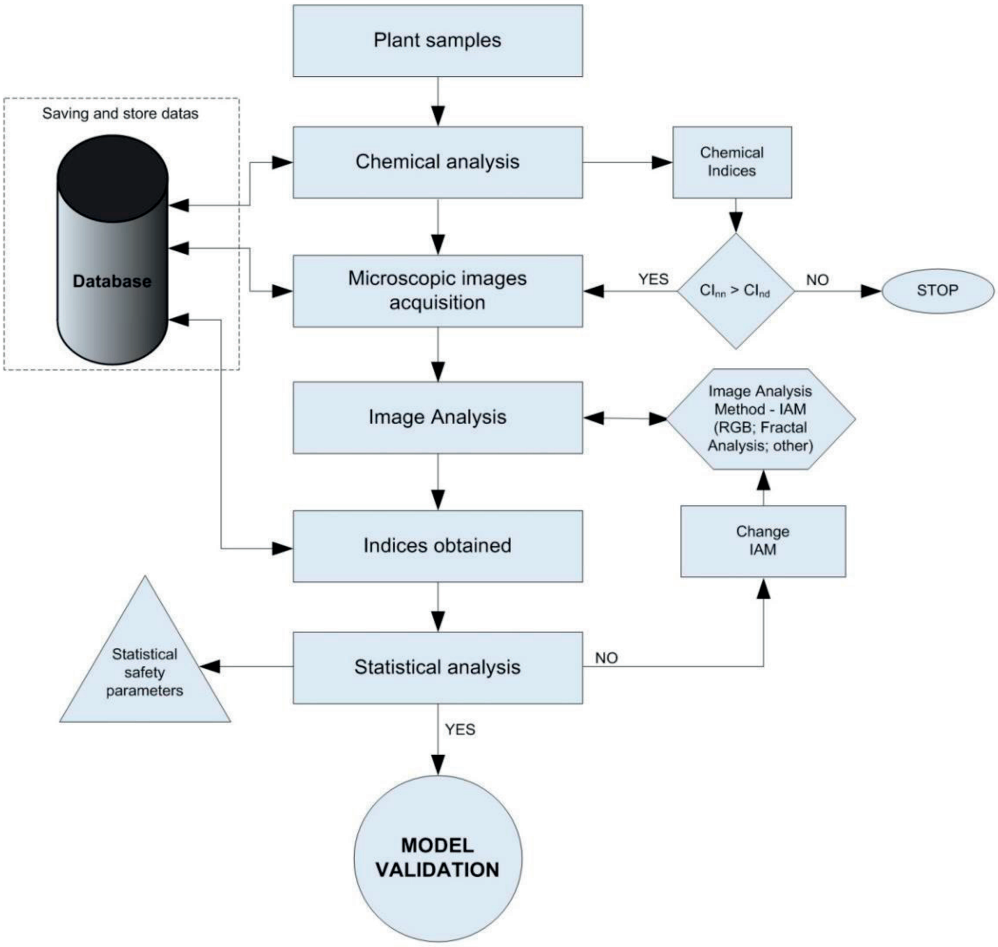


Figure 4. RGB-D Model based on microscopic image for nutritional stress in plant

RESULTS AND DISCUSSIONS

The differentiated nutritional status of the two categories of plants analyzed was highlighted by the content of mineral elements identified in the plant samples, according to the working model in Figure 4, Table 1.

The content of certain mineral elements had higher values in fertilized plants, except for potassium and copper which had higher values in nutritional deficiency plants. The importance of potassium in plant nutrition is known (Mengel and Kirkby, 2001; Barker and Pilbeam, 2007), and according to the law of equivalence of vegetation factors, there may be partial substitutions of some nutrients. In the case studied, potassium and copper contributed to increasing the concentration of cell juice as an alternative in balancing the nutritional deficiency and increasing the resistance of plants to the cold season.

Between the values of mineral content (Me) in plants with normal nutrition (Me_{nn}) and those with nutrition deficiency (Me_{nd}) were found the following ratios (Me_{nn}/Me_{nd}), as an expression of the difference in nutrition status: 1.12 for total nitrogen (N-total), 2.02 for P_2O_5, 0.78 for K_2O, 1.55 for Mg, 1.83 for Ca, 1.02 for Fe, 0.56 for Cu, 1.53 for Zn, and 1.37 for Mn, respectively.

Against the background of nutritional deficiency, unfertilized plants have accumulated more potassium from the natural content of the soil, being known the role of potassium as a regulator of plant response to stress (Hasanuzzaman et al., 2018).

Against the background of a differentiated nutrition, the plants developed a differentiated internal structure, highlighted by microscopic analysis.

The analysis of the profile and color spectrum of microscopic images in the two plants categories, led to obtaining spectral information in the RGB system, the values for each color channel (R, G, B) and plant category being shown in Table 2.

Table 1. Values of mineral content in wheat leaves under conditions of nutrition deficiency and normal nutrition

Mineral elements	UM	Leaves with nutrition deficiency	Leaves with normal nutrition
Nitrogen (N-total)	%	4.1	4.58
Phosphorus (P_2O_5)	%	0.47	0.95
Potassium (K_2O)	%	3.46	2.71
Magnesium	%	0.36	0.56
Calcium	%	1.2	2.19
Iron	ppm	9.8	10
Copper	ppm	4.94	2.76
Zinc	ppm	32.65	49.95
Manganese	ppm	71.5	97.8

Fractal analysis on binarized microscopic images removed the possible influence of color and captured with a high degree of fidelity and statistical certainty ($R^2 = 0.999$, SE = 0.043) the structure of leaf fractal geometry captured in microscopic images, as a result of differences in the plants nutrition status. The values for fractal dimensions are given in Table 2.

The average value for R, in the case of plants with normal nutrition, was $R_{nn} = 110.39$ compared to the average value for R in the case of plants with nutritional deficiency ($R_{nd} = 47.65$).

The difference between the averages was high and statistically significant. The value of the t test was $t = 14.224$ with $p < 0.001$.

The non-parametric Mann-Whitney test also indicated significant differences between the two groups, $U = 0$, $p < 0.001$.

Table 2. Data for R, G, B and D resulting from the analysis of microscopic images in wheat, Alex cultivar

Microscopic image	R_{nn}	R_{nd}	G_{nn}	G_{nd}	B_{nn}	B_{nd}	D_{nn}	D_{nd}
1	87.18	43.35	59.18	50.42	4.1	3.85	1.785	1.845
2	108.33	66.7	55.94	65.74	3.98	4.3	1.753	1.872
3	117.9	73.52	47.3	70.5	3.83	4.48	1.713	1.864
4	115.95	44.58	40.78	51.21	3.76	3.86	1.697	1.834
5	105.04	35.29	57.15	45.32	4.38	3.81	1.794	1.835
...	...	...	...	...	...	...	...	...
16	117.93	38.71	35.68	52.54	3.77	4.07	1.679	1.856
17	92.17	33.5	51.67	45.28	4.32	3.84	1.822	1.829
18	101.4	48.13	56.17	54.87	4.18	4.24	1.766	1.854
19	132.94	52.29	56.29	63.38	4.35	4.52	1.731	1.855
20	134.7	38.3	50.61	49.91	4.21	4.06	1.709	1.858

The coefficients of variation of the R values series (RGB color system) presented in both situations high values: 14.01 in the case of plants with normal nutrition, respectively 25.65 for plants with nutritional deficiency. These indicated a low homogeneity of the series. The values differed significantly. The variability of the series corresponding to the plants with normal nutrition was lower than the case of the R series for the plants that had nutrition deficiency. The Fligner-Killeen test indicated the value $T = 26.23$, $z = 2.45$ with $p = 0.013$.

Referring to the series of G values (RGB color system), in plants with normal nutrition it led to the average value of 51.92 compared to plants that had a nutritional deficit, where the average value of the series of G values was 55.54. The values were quantitatively close and the difference was not statistically significant. And in the case of applying the non-parametric Mann-Whitney test, the differences were not statistically significant. The coefficients of variation of the series of G values presented in both situations high values: 12.94 in the case of plants with normal nutrition, respectively 14.43 for plants with nutritional deficiencies. Their values are close, and did not show statistically significant differences.

The average value of the series of B values (RGB color system) in the case of plants with normal nutrition was 4.12 compared to the average value in the case of plants with nutritional deficiencies, $B_{nd} = 4.16$. The values were quantitatively close and the difference was not statistically significant.

And in the case of non-parametric Mann-Whitney test applications, the mean ranks differences were not statistically significant.

The coefficients of variation of the B value series presented in both situations low values: 4.61 in the case of plants with normal nutrition, respectively 6.60 for plants with nutritional deficiency. And in the case of the B series, the values of the coefficients of variation were close, and did not show statistically significant differences.

The average value of the fractal dimension (D) in the case of plants with normal nutrition (D_{nn}) was D_{nn} = 1.746, compared to the average value of the fractal size in the case of plants with nutrition deficiency (D_{nd}), where it had the value D_{nd} = 1.850. The difference between the means was statistically significant, according to the indicated value of t = 10.88 having $p < 0.001$. And in the case of non-parametric Mann-Whitney test applications, the differences between groups are significant, U = 0, $p < 0.001$. The coefficients of variation of the series of fractal dimensions presented in both situations low values: 2.33 in the case of plants with normal nutrition, respectively 0.66 for plants with nutritional deficiencies, indicating the homogeneity of the series. However, their values differed significantly. The variability of the series of fractal dimensions corresponding to the plants with normal nutrition (D_{nn}) was superior to the case of the series of fractal dimensions for the plants that had nutrition deficiency (D_{nd}). The Fligner-Killeen test indicated the value T = 6.07, z = -3.17 with $p < 0.01$.

Nutritional status is reflected in plant growth and development, and is most commonly studied through physiological indices and growth parameters (Layek et al., 2012; Jivan and Sala, 2014; Stein et al., 2017), in productivity elements, yield and quality (Dobrei et al., 2010; Rawashdeh and Sala, 2014; 2016; Mohamed et al., 2019).

Different studies on plant nutrition used diagnostic models based on remote sensing (Feng et al., 2019; Röll et al., 2019), and other studies proposed indices for some nutrients supplementation (eg nitrogen) in wheat culture (Datcu et al., 2020). Imaging methods based on mobile applications have been used in foliar studies in different plant species (Drienovsky et al., 2017).

The variation of nutrition causes changes in the mineral content of the leaves and in the functional physiological structures of the leaves in relation to the capacity to receive sunlight and photosynthetic processes, respectively (Sattelmacher, 2001; Chikov and Bakirova, 2004; Miller, 2014).

The mineral composition of the leaves, "ionomic leaf" (Stein et al., 2017), expresses the complex interaction between a plant and its nutritional environment, namely the nutrient matrix, mineral composition and soil fertility.

Technology has evolved a lot, especially in recent decades, and has made possible the use of very complex methods for the study of plant organisms at the micro scale (Lazcano-Ramirez et al., 2018).

Color models for the analysis of microscopic images have been used in other studies; RGB, HSI, CIEL * a * b * color models (Bueno et al., 2008), in the analysis of resistance of barley cells to powdery mildew (Ihlow and Seiffert, 2003), in studies on light absorption and distribution and the process of photosynthesis in plant tissues (Lichtenberg et al., 2017).

In the present study, the color parameter R facilitated the statistical safety differentiation of the two categories of plants, based on microscopic images.

Regarding the fractal analysis, it is known the refinement with which this analysis captures the geometry of some textures in the imaging analysis (Kaplan, 1999; Jobin et al., 2012). Fractal analysis has been used successfully in pomology studies (Sala et al., 2017), the study of fraxinus cork geometry in relation to the image shooting distance (Nicolin et al., 2019), the evaluation of the spatial variation of agricultural lands (Sala et al., 2020b)

The fractal analysis captured, in conditions of statistical safety, the structural differentiations of the leaves with nutritional deficiency from the normal ones, reflected in the microscopic images.

CONCLUSIONS

In the case of the analyzed samples, only the fractal dimension (D), and the R series values (coloristic index R), were the indicators that showed statistically significant values changes for the plants with nutrition deficiences compared to those that have a normal nutrition.

These indices (R and D) were validated in the RGB-D model to characterize the nutritional deficiency based on microscopic images.

The values of the G and B series (RGB color system) did not change significantly under normal nutritional conditions compared to the nutritional deficiency of wheat plants, Alex cultivar.

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