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# Multispectral and chlorophyll fluorescence imaging for detection of nutrient deficiency symptoms in common bean

# Utvrđivanje simptoma nedostatka hranjiva u grahu multispektralnom analizom i klorofilnom fluorescencijom

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## ABSTRACT

Crop production might suffer severe economic losses due to insufficient fertiliser availability. Specific signs of nutrient shortage influence plant morphology and physiology. This study pioneers the non-destructive tracking and characterization of nutrient deficiency symptoms in common beans using multispectral and chlorophyll fluorescence imaging, offering novel insights into the dynamic responses of plant morphology and physiology to specific nutrient shortages. Plants were cultivated in nutrient solutions with and without nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), iron (Fe) and control solution. Measurements were taken every three days for 12 days (MT1-MT4) of growth. K and N deficit plants had the earliest symptoms and most noticeable changes, whereas Fe deficiency plants had the slowest and least noticeable symptoms. Except for Fe, the most responsive chlorophyll fluorescence parameter was electron transport rate, which was reduced in plants from all nutrient deprivation treatments compared to control. All nutrient deficit treatments reduced leaf area at MT2, which was the most affected morphological parameter. The green leaf index, reflection in blue, and specific green were the most affected multispectral traits by nutritional deprivation. These findings demonstrate that plant nutrient deficit can be recognized and tracked non-destructively utilizing multispectral and chlorophyll fluorescence analysis. Overall, our work not only sheds light on the dynamics of nutrient deficiency in common bean plants but also offers practical implications for improving crop management strategies using non-destructive digital technology.

**Keywords:** image-based phenotyping, vegetation indices, plant nutrition, *Phaseolus vulgaris*

## SAŽETAK

Zbog nedovoljne dostupnosti gnojiva uzgoj poljoprivrednih usjeva mogao bi pretrpjeti ozbiljne ekonomske gubitke. Specifični znakovi nedostatka hranjiva utječu na morfologiju i fiziologiju biljke. Koristeći multispektralne analize i klorofilnu fluorescenciju u ovom je radu opisan i kvantificiran razvoj simptoma nedostatka hranjiva (dušik (N), fosfor (P), kalij (K), magnezij (Mg) i željezo (Fe)) kod graha (*Phaseolus vulgaris* L.). Biljke su uzgajane u hranjivim otopinama sa i bez N, P, K, Mg ili Fe. Mjerenja su provođena svaka tri dana tijekom 12 dana (MT1-MT4) rasta. Biljke s deficitom K i N su prve

razvile simptome nedostatka hranjiva i imale su najizraženije fiziološke promjene, dok su biljke s nedostatkom Fe zadnje razvile simptome nedostatka hranjiva i imale najmanje izražene simptome. Od parametara klorofilne fluorescencije, u odnosu na kontrolu smanjenje transporta elektrona je najviše reagirao u svim tretmanima sa nedostatkom hranjiva, osim u slučaju tretmana s nedostatkom Fe. Svi tretmani s nedostatkom hranjivih tvari smanjili su lisnu površinu u MT2, Što predstavlja morfološki parametar koji je bio najviše pod utjecajem nedostatka hranjiva. Indeks zelenila lišća, refleksija u plavom spektru svjetlosti i refleksija u specifičnom zelenom spektru svjetlosti bila su multispektralna svojstva koja su najviše bila pod utjecajem nedostatka hranjiva. Ovi rezultati pokazuju da se nedostatak hranjiva može prepoznati i pratiti nedestruktivno korištenjem multispektralnih analiza i klorofilnom fluorescencijom.

**Ključne riječi:** fenotipizacija upotrebom slika, vegetacijski indeksi, ishrana bilja, *Phaseolus vulgaris*

## INTRODUCTION

Aside from oxygen (O), carbon (C) and hydrogen (H), plants need 17 mineral elements to achieve their life cycle, which are known as plant essential nutrients (Marschner, 1995). Based on their concentration in plant tissue, plant nutrients are classified as the macronutrient group (>1000 mg/kg dry matter) and micronutrient group (<1000 mg/kg dry matter) (Marschner, 1995). Because this classification does not consider their physiological role and because different concentrations of nutrients can occur in different plant tissues, Mengel and Kirkby (2001) proposed the classification of plant nutrients in four different groups based on their physiological role. These nutrient groups are those that 1) are part of organic carbon molecules (nitrogen (N) and sulphur (S)); 2) are important for structural stability and energy storage (phosphorus (P), silicon (Si) and boron (B)); 3) remain in ionic form (potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), chlorine (Cl) and sodium (Na)); 4) are involved in redox reactions (zinc (Zn), iron (Fe), molybdenum (Mo), copper (Cu) and nickel (Ni)) (Mengel and Kirkby, 2001).

Essential nutrients are crucial in plants' physiological processes, and because they cannot be substituted, their inadequate supply causes specific, visible nutrient deficiency symptoms (Marschner, 1995). High-yield crop production relies on the constant application of high amounts of plant nutrients (Stewart et al., 2005) but nutrient deficiency is a frequent problem in crop production and can cause significant yield loss. Moreover, new intergovernmental initiatives outline the necessity for a shift to a more sustainable global food system production which must include a more holistic management of plant nutrients (Bruulsema et al., 2020). For example, nutrient application needs to be increased in the poorest countries, particularly in Sub-Saharan Africa (Zhang, 2017), while fertiliser use needs to be reduced in most developed countries (Soares et al., 2019). Even a slight lack of plant nutrients can severely impair the metabolism of plants. With prolonged nutrient deficiencies, plant health deteriorates, symptoms become more pronounced and are difficult to correct at later stages of the plant life cycle (Kalaji et al., 2018). Symptoms are often described as changes in leaf color, such as yellowing (chlorosis), browning (necrosis), reduction in plant size (stunted plants, small leaves) and deformation (leaf curling). They are related to the specific nutrient role within the plant, affecting processes such as protein and pigment biosynthesis and photosynthesis. Nitrogen, magnesium and iron, for example, are directly involved in chlorophyll synthesis and are key elements for the synthesis and activity of specific protein complexes involved in photosynthesis (Dwyer et al., 2012; Tognetti et al., 2007). Thus, its deficiency causes a decrease in chlorophyll content and light absorption, a reduction of thylakoid electron transport rate and Rubisco content and activity, affecting $CO_2$ assimilation (Huang et al., 2004; Mu et al., 2017; Yang et al., 2012; Reinbothe et al., 2006).

In addition, potassium is a crucial element for osmoregulation and regulation of the stomatal apparatus (Jákli et al., 2017) and an important element for membrane stability (Cakmak, 2005). Its deficiency causes poor chloroplast ultrastructure, the production of reactive oxygen species and chlorophyll degradation

(Cakmak, 2005). Photosynthesis is also severely affected by phosphorus deficiency, primarily by slowing down ATP and NADPH production and ribulose-1,5-bisphosphate regeneration (Chu et al., 2018).

Thus, innovative technologies, automated, non-destructive plant stress detection methods that may help in the early and accurate identification of nutrient deficiency symptoms are crucial for preventing losses due to nutrient deficiency and achieving sustainability goals in food production, such as RGB, multi- and hyperspectral and thermal imaging, chlorophyll fluorescence and 2D/3D scanning (Yang et al., 2020). However, the development of remote sensing techniques enables the study of plants' physiological and morphological changes caused by nutrient deficiency and quantifying plant nutrient deficiency indirectly and non-destructively. A significant role of such technologies in optimizing plant nutrition is recognized and foreseen by the International Code of Conduct for the Sustainable Use and Management of Fertilizers (Oger, 2022). By combining different remote sensing techniques, more comprehensive, complete and accurate data was obtained at the whole plant level. These techniques have been used to investigate and distinguish different nutrient deficiencies in lettuce (*Lactuca sativa* L.) N, P, K, Mg and Ca (Pacumbaba et al., 2011); grapevine (*Vitis vinifera* L.) K, Mg and N (Debnath et al., 2021); common bean (*Phaseolus vulgaris* L.) Fe, K, N, P and Ca (Aleksandrov, 2022) and K, N, Fe, Mg and P (Lazarević et al., 2022), oilseed rape (*Brassica napus* L.) N, P, K, Ca, Mg, Cu, Fe, Zn and their combinations (Kalaji et al., 2018) and N, P, K (Zhang et al., 2013). Except for Lazarević et al. (2022), these studies used a single technique (multispectral or chlorophyll fluorescence) and mainly focused on detecting and discriminating different nutrient deficiencies using sophisticated statistical (Kalaji et al., 2018; Zhang et al., 2013) or machine learning techniques (Debnath et al., 2021; Aleksandrov, 2022). However, with the increasing use of such techniques in crop production, it is important to quantify changes in spectral reflectance and chlorophyll fluorescence traits and to describe the development of symptoms under different nutrient deficit conditions.

The primary aims of this research were to employ high-throughput phenotyping methods, specifically whole plant chlorophyll fluorescence imaging, multispectral imaging and 3D multispectral scanning, to quantitatively assess phenotypic changes and progression of symptoms caused by deficiencies of five different plant nutrients: nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), and iron (Fe).

## MATERIALS AND METHODS

The study by Lazarević et al. (2022) provides the detailed experimental methodology and data collection procedures. Here, we offer a brief overview of the experimental setup.

In a growth chamber, environmental conditions were tightly controlled: temperature maintained at 25/22 °C, a photoperiod of 16/8 hours' day/night, relative air humidity at 65%, and photosynthetic active radiation (PAR) intensity of 250 µmol/m$^2$/s (NS1, LED lamps by Valoya Oy, Helsinki, Finland). Common bean (*Phaseolus vulgaris* L. cv. Ferguson) seedlings were initially grown in sand-filled germination trays. Upon the full development of the first true leaf, sixty uniform plantlets were chosen, roots cleaned of sand, and transplanted into six 30-liter trays (10 plants/tray) filled with half-strength modified Hoagland solution (Hoagland and Arnon, 1950). Distilled water with EC <0.1 µS/cm at 25 °C was utilized for nutrient stock and treatment solution preparation. Three days post-transplanting, nutrient deficiency treatments commenced.

Experimental treatments included a control solution (half-strength modified Hoagland) and solutions lacking specific elements: nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), and iron (Fe), chosen based on their physiological importance in plants (Mengel and Kirkby, 2001). Details of treatment nutrition solutions are provided in Table 1. Over twelve days, ten plants per treatment underwent non-destructive measurements (MT1, MT2, MT3, MT4) every three days, with nutrient solutions replenished at each measurement interval.

**Table 1.** The concentration of plant nutrients in treatment solutions

| Plant nutrient | The concentration of nutrients in final (treatment) solutions (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| | Control | N | P | K | Mg | Fe |
| N | 98 | 0 | 98 | 112 | 98 | 98 |
| P | 31 | 31 | 0 | 31 | 31 | 31 |
| K | 117.3 | 117.3 | 97.8 | 0 | 117.3 | 117.3 |
| Ca | 104.3 | 100.3 | 104.3 | 104.3 | 104.3 | 104.3 |
| Mg | 24.3 | 24.3 | 24.3 | 24.3 | 0 | 24.3 |
| S | 64.2 | 64.2 | 72.2 | 32.2 | 32.2 | 64.2 |
| Fe | 2.85 | 2.85 | 2.85 | 2.85 | 2.85 | 0 |
| Cl | 7.09 | 177.3 | 7.09 | 7.09 | 7.09 | 7.09 |
| B | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Zn | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Cu | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Mn | 0.36 | 0.36 | 0.36 | 0.36 | 0.36 | 0.36 |
| Mo | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |

***Non-destructive measurements***

*Description of the devices used for non-destructive measurements*

Non-destructive measurements included whole plant chlorophyll fluorescence imaging, multispectral imaging and 3D multispectral scanning. Multispectral and chlorophyll fluorescence imaging was performed using CropReporter™ (PhenoVation B.V., Wageningen, The Netherlands). The CropReporter™ consists of a cabinet equipped with a camera system, a controller computer, a charge-coupled device (CCD) camera with an optical filter wheel for multispectral imaging, and a focusing unit, integrated high-intensity red light emitting diodes (LEDs) for photosynthesis excitation, LEDs at six spectral bands (broad band white (3000 K), far-red (730 nm), red (660 nm), green (520 nm), blue (460 nm). Spectral reflectance is captured in red ($R_{Red}$; 640 nm), green ($R_{Green}$; 550 nm), blue ($R_{Blue}$; 475 nm), specific green ($R_{SpcGrn}$; 510-590 nm), Far-red ($R_{FarRed}$; 710 nm), and near-infrared ($R_{NIR}$; 769). All images are acquired with the same lens (10 Mp, 200 Lp/mm resolution, 400-1000 nm spectral range) and CCD-camera (1.3 Mp, 1296x966 pixels), with true 14-bit signal resolution. The output is in 16-bit RAW format, and DA™ performed an automatic analysis of chlorophyll fluorescence, color, and multispectral images.

The PlantEye F500 multispectral 3D scanner (Phenospex, Heerlen, The Nederlands) was used for 3D multispectral scanning. PlantEye measures the spectral reflectance in Red (peak wavelength 620-645 nm), Green (peak wavelength 530-540 nm), Blue (peak wavelength 460-485 nm), Near-Infrared (peak wavelength 820-850 nm) and the 3D Laser (940 nm) of the plant. For scanning the resolution of the PlantEye was set up as: Z- range (the distance measured from the scanner down) 40 cm, Y - resolution ($V_{scan}$ = 50 mm/s) 1 mm, X - resolution 0.19 mm and Z - resolution <0.1 mm. After scanning, Phena software (Phenospex, Heerlen, The Netherlands) is used to build the 3D plant model from the 3D point cloud (Figure 1). A 3D plant model is built by triangulating all points belonging to the same sector.

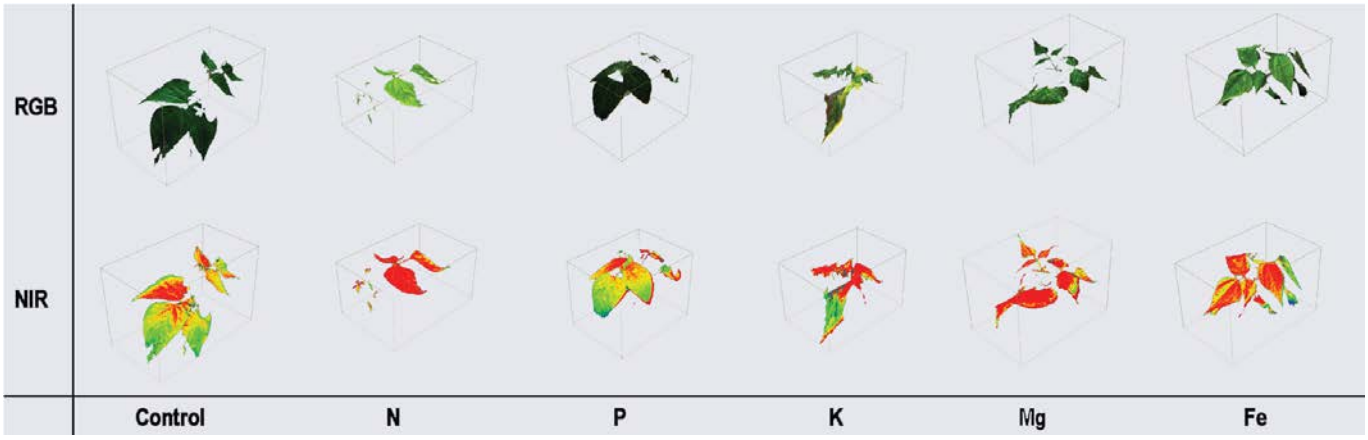


**Figure 1.** Color [Red, Green and Blue (RGB)] and pseudo-color [Near Infra-Red (NIR)] images of 3D common bean plants scanned by PlantEye F500 grown for 9 days (MT3) in different nutrient treatments: control and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe)

To create a triangle in the point cloud the points should be close to each other with no other point in between. After building the 3D plant model, the HortControl software (Phenospex, Heerlen, The Netherlands) was used to calculate different vegetation indices and morphological parameters.

*Chlorophyll fluorescence imaging*

The whole plant chlorophyll fluorescence imaging was performed from the 70 cm distance, following protocol of dark-to-light slow fluorescence induction (Brestic and Zivcak, 2013). Plants were first dark adapted for 30 minutes. Measurements were carried out by applying a saturating pulse of red LED light with an intensity of 4000 µmol/m$^2$/s for one second. Minimum chlorophyll fluorescence ($F_0$) was measured after 10 µs and maximum chlorophyll fluorescence ($F_m$) was measured after saturation. Following the determination of the $F_0$ and $F_m$, the plants were subjected to a dark relaxation period of 15 seconds. Subsequently, they were exposed to actinic light with an intensity of 250 µmol/m$^2$/s for a duration of 5 minutes to facilitate light adaptation. The fluorescence yield at steady state ($F_s$') was quantified prior to the initiation of the saturating pulse. Again, the saturation pulse of 4000 µmol/m$^2$/s was applied for a duration of one second and the maximal chlorophyll fluorescence ($F_m$') of light-adapted leaves was measured. Following the measurement, the actinic light source was switched off, and subsequently, the minimal fluorescence yield ($F_0$') of the light-adapted plant was estimated in the presence of far-red light. During both measurements (on dark-adapted and light-adapted leaves) four dark frames were captured and averaged to a one single frame during the time red LEDs were off, twenty frames were captured for the induction curves during 800 ms, and integration time for capturing the chlorophyll fluorescence images were 200 µs.

The measured $F_0$, $F_m$, $F_s$', $F_m$' and $F_0$' were used for the calculation of the following fluorescence parameters in DA software: Maximum Efficiency of PSII, Effective quantum yield of PSII, Relative Electron Transport rate (ETR), Non-Photochemical Quenching (NPQ), Coefficient of Photochemical Quenching (qP), Coefficient of Non-photochemical Quenching (qN), Estimation of Open Reaction Centers on the basis of a Lake Model (qL), Quantum Yield of Non-regulated Non-photochemical Energy Loss in PSII (φno) whose equation are given in the work of Lazarević et al. (2022).

Using DA software, additional analyses were performed, which involved selecting and analyzing chlorophyll fluorescence parameters specific to regions of interest (ROI) separately placed on young and old leaves. This analysis was carried out only for MT4 when all control and Fe deficit plants had developed a sufficient young leaf surface. This additional analysis encompassed two ROIs per plant, with one placed on the main leaflet of the young trifoliate and the other on the blade of the old leaf. The size of each ROI was 50 pixels.

*Multispectral imaging*

Multispectral imaging was performed under uniform actinic illumination at 250 µmol/m²/s using CropReporter™. Reflectance images $R_{Red}$, $R_{Green}$, $R_{Blue}$, $R_{SpcGrn}$, $R_{FarRed}$ and $R_{NIR}$ were used to calculate color parameters and vegetation indices: Hue (0–360°; HUE), Value (0–1; VAL), Saturation (0–1; SAT), Chlorophyll index (CHI), Anthocyanin index (ARI), Green Leaf Index (GLI), Normalized Differential Vegetation Index (NDVI), Normalized Pigments Chlorophyll Ratio Index (NPCI) and Plant Senescence Reflectance Index (PSRI), whose equations are given in the paper by Lazarević et al. (2022).

Figure 2 shows the RGB images of common bean plants under different nutrient treatments, including control (C) and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). The images were conducted at four different time points: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the start of the treatment.

*3D multispectral scanning*

After scanning and building a 3D plant model different morphological parameters were analyzed: Plant Height (PH, mm), Digital Volume (DV, cm³), Leaf Area Projected (LAP, mm²), Total Leaf Area (TLA, cm²), Leaf Area Index (LAI, mm²/mm²), Leaf Inclination (LINC, mm²/mm²), Leaf Angle (LANG, °) and Light Penetration Depth (LPD mm), the explanation of which can be found in the paper by Lazarević et al. (2022).

*Plant mineral content analysis*

At the end of the 12-day experimental period, harvested shoots underwent mineral content analysis. Samples were dried at 70 °C, ground, and subjected to various methods for nutrient determination. Total nitrogen was analyzed via the Modified Kjeldahl method, potassium via flame photometer, phosphorus via spectrophotometer, and magnesium and iron via atomic absorption spectrometer (AAS Solar).

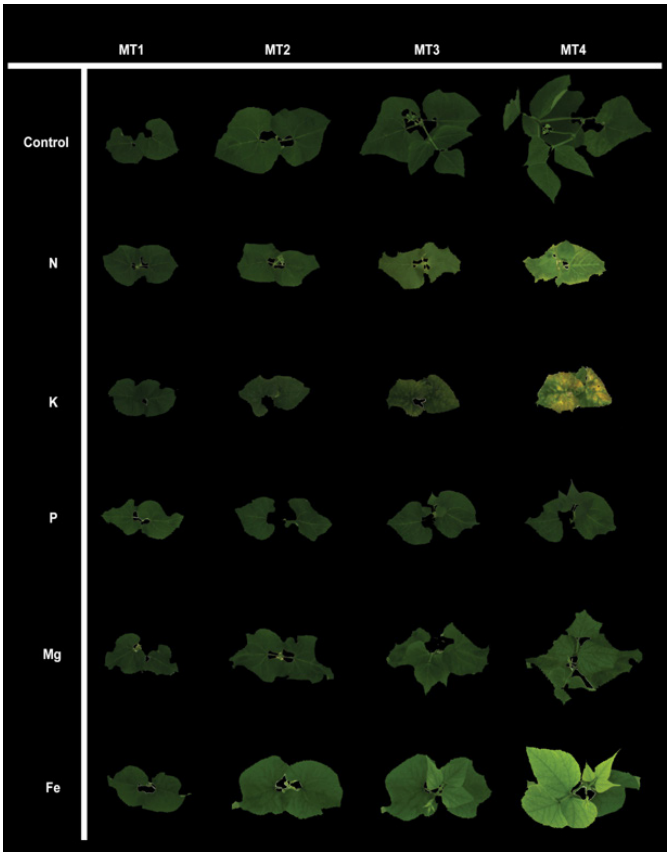


**Figure 2.** RGB images of common bean plants grown under different nutrient treatments: control and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). The images were taken at four time points: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after treatment initiation.

***Statistical Analyses***

Data analysis was conducted using JMP® Pro 16 (SAS Institute Inc., Cary, NC). PROC Mixed (Littell et al., 2000) facilitated repeated measures analysis, incorporating fixed effects of nutrient deficiency treatments (C, N, P, K, Mg, Fe), measurement time (MT1-MT4), and their interaction. Individual plants were treated as random factors nested within treatments. The Akaike information criterion (AICc) guided covariance structure model selection. Tukey's Honest Significant Difference (HSD) post hoc test assessed treatment significance across measures or nutrient deficiency effects over time. Additionally, one-way ANOVA compared chlorophyll fluorescence parameters between Fe-deficit and control plants.

## RESULTS

As this study aimed to quantify the phenotypic changes and symptoms development during deficiencies of five different plant nutrients, the results are presented and discussed separately for each nutrient. ANOVA results with SLICE option are shown in Table 2, 3 and 4.

### *Control*

Control plants show a significant increase in all morphological traits from MT1 to MT4, except LANG and LINC (Figure 3). Reflection in red ($R_{Red}$), green ($R_{Green}$), specific green ($R_{SpcGrn}$), and blue ($R_{Blue}$) decreased from MT1 to MT2 and remained stable in subsequent measurements (from MT2 to MT4). Reflection in NIR ($R_{NIR}$) and Far-red ($R_{FarRed}$) decreased over measurements, whereas vegetation indices (CHI, NDVI, ARI, GLI, NPCI and PSRI) remained relatively stable over the measurements (Figure 4). There were also very small changes in photochemistry, and most of the chlorophyll fluorescence traits remained stable over measurements (Figure 5).

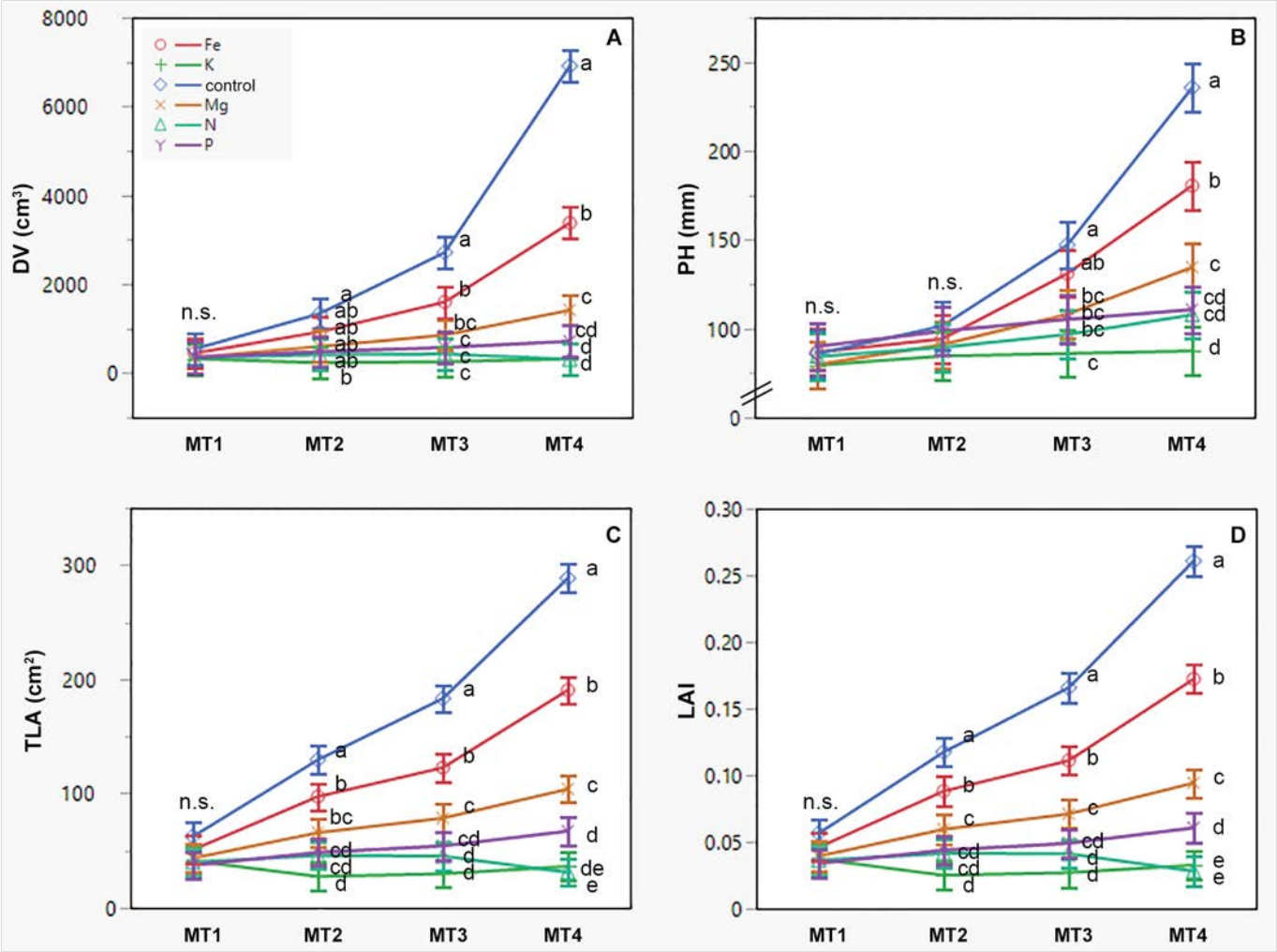


**Figure 3.** The mean values together with their corresponding standard errors for a set of chosen morphological traits. The study investigated A) digital volume (DV), B) plant height (PH), C) total leaf area (TLA), and D) leaf area index (LAI) of common bean plants cultivated under different nutrient treatments, including control and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). Measurements were conducted at four distinct time points: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the start of the treatments. n.s. denotes that there were no significant differences among nutrient treatments within the specific measurement time; different lowercase letters indicate significant differences among treatments within the measurement time based on Tukey's HSD test.

**Table 2.** Repeated measures analysis of variance (ANOVA) for measured 3D morphological traits of common bean grown in different treatments: control (C) and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). Measurements were performed at four different times: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the onset of treatments. P-values are presented for a) ANOVA for repeated measures, b) ANOVA test with SLICE option to examine the significance of each treatment across measurements (Measurement Time), c) ANOVA test with SLICE option to examine the significance of treatments within time

| | Source | Morphological traits | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | DV | PH | TLA | LAI | LANG | LINC | LPD |
| a) ANOVA with Repeated measures | T | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | MT | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | T x MT | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| b) Treatments across Measurement Time | C | <.0001 | <.0001 | <.0001 | <.0001 | 0.002 | 0.0517 | <.0001 |
| | N | 0.923 | 0.0034 | 0.0618 | 0.0592 | 0.0583 | 0.052 | 0.8616 |
| | P | 0.3255 | 0.0138 | <.0001 | <.0001 | <.0001 | <.0001 | 0.1075 |
| | K | 0.9583 | 0.6311 | 0.1162 | 0.1215 | <.0001 | <.0001 | 0.6737 |
| | Mg | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | Fe | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| c) Treatments within Measurement Time | MT1 | 0.9467 | 0.8616 | 0.0501 | 0.0526 | 0.0616 | 0.0133 | 0.4737 |
| | MT2 | 0.0002 | 0.5168 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0925 |
| | MT3 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | MT4 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0047 | 0.0548 | <.0001 |

T – treatment; MT – measurement time

***Nitrogen deficit***

In morphological traits, there was no increase in DV, LPD and traits related to leaf area, indicating a cessation of growth in N-deficit plants (Figure 3). $R_{Red}$, $R_{Green}$, $R_{SpcGrn}$ and $R_{FarRed}$ decreased from MT1 to MT3 and then increased again at MT4. Most vegetation indices (NDVI, GLI, CHI, ARI, HUE) decreased, while NPCI and PSRI increased in the measurements from MT1 to MT4 (Figure 4). Photochemistry ($F_v/F_m$, $F_q'/F_m'$, ETR, qP, and qL) also decreased, while NPQ, qN and φno increased in measurements MT1 - MT4. The earliest significant effect of N deficiency compared to control plants was a higher $R_{Blue}$, which was already detected at MT1. From MT2, N-deficit plants showed significantly lower leaf area (TLA and LAI) (Figure 3), higher $R_{Blue}$ and lower GLI, NDVI and SAT compared to control plants (Figure 4). Chlorophyll fluorescence traits show that the photochemical process is already significantly affected in N-deficit plants at MT2. Namely, N deficiency caused a significant decrease in $F_q'/F_m'$, ETR, qP and qL, and an increase in NPQ compared to the control plants of MT2 (Figure 5).

***Phosphorus deficit***

Similar to N- and K- deficit plants, P-deficit plants show cassation in growth (Figure 3) without a significant increase in morphological traits during the measurements. Reflection from leaves ($R_{Red}$, $R_{Green}$, $R_{Blue}$, $R_{NIR}$, $R_{FarRed}$, $R_{SpcGrn}$) decreased from MT1 to MT2 and remained relatively stable thereafter. NPCI and PSRI did not change during the measurements. ARI increased and GLI and NDVI decreased from MT2-MT4 compared to MT1 (Figure 4).

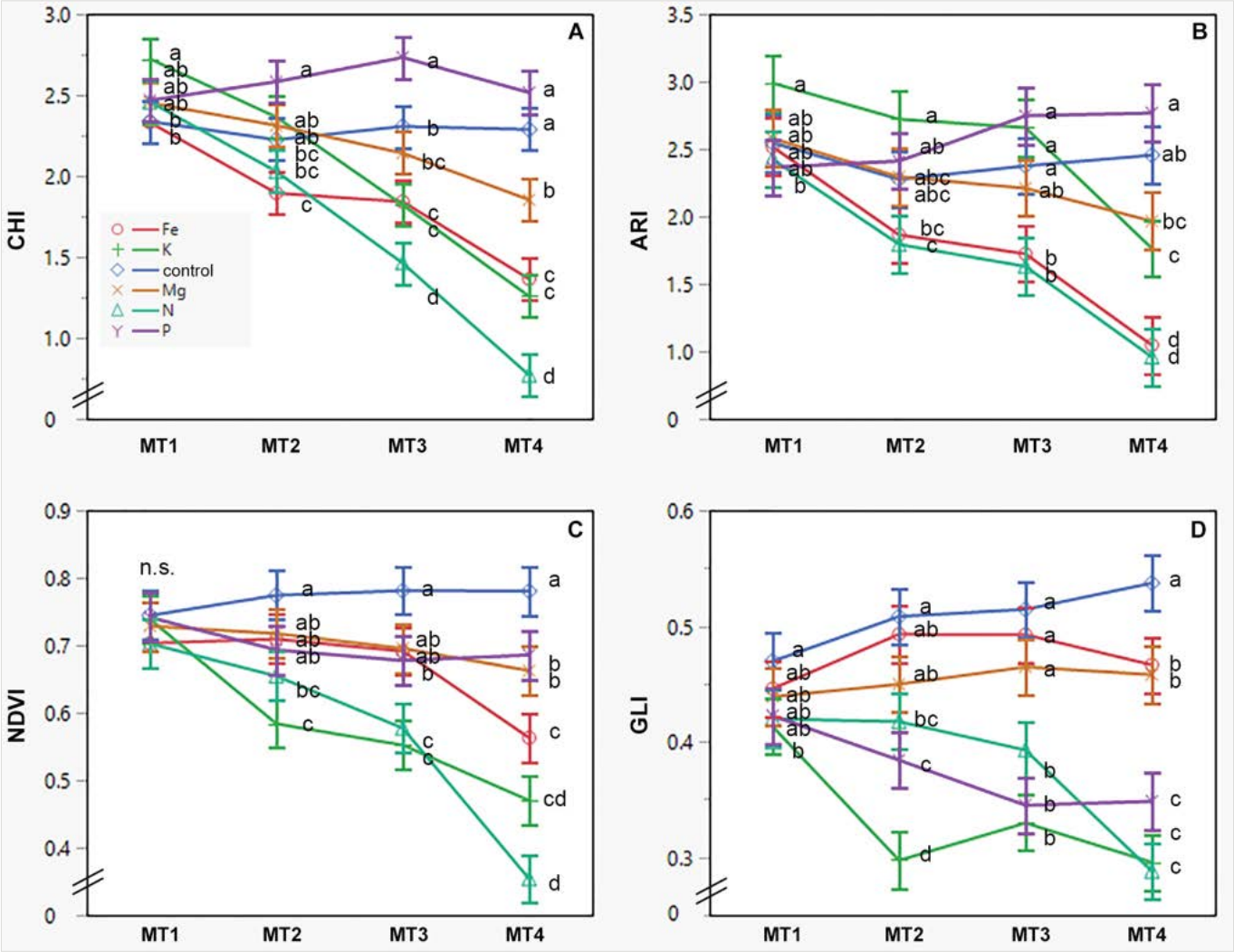


**Figure 4.** The mean values together with their corresponding standard errors for a set of chosen multispectral traits. The study investigated A) chlorophyll index (CHI), B) anthocyanin index (ARI), C) normalized digital vegetation index (NDVI), D) green leaf index (GI) in common bean plants subjected to different nutrient treatments: control and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). Measurements were taken at four time points: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the start of the treatments. n.s. denotes that there were no significant differences among nutrient treatments within the specific measurement time; different lowercase letters indicate significant differences among treatments within the measurement time based on Tukey's HSD test.

Of measured chlorophyll fluorescence parameters, $F_v/F_m$ and φno remained stable, while $F_q'/F_m'$, ETR, qP and qL decreased and NPQ increased from MT1 to MT4 (Figure 5). The earliest symptoms of P deficiency developed at MT2 when significantly lower LAI, TLA and LANG and higher LINC than control were found (Figure 3). In addition, lower $R_{SpcGrn}$, $R_{FarRed}$, SAT and GLI and higher HUE and CHI were found compared to control plants (Figure 4). In MT2, lower ETR and φno and higher NPQ and qN values were also found in P-deficit plants compared to control plants (Figure 5).

***Potassium deficit***

Similar to N and P deficiency, there was no increase in morphological traits in the K-deficit plants during measurements (MT1-MT4), indicating growth cessation (Figure 3). As in the case of N deficiency, $R_{Red}$, $R_{Green}$, $R_{SpcGrn}$, $R_{Blue}$ and $R_{FarRed}$ also decreased in MT2 and MT3 compared to MT1 and then increased again in MT4, while vegetation indices, HUE, SAT, CHI, NDVI and GLI decreased and NPCI and PSRI increased from MT1 to MT4 (Figure 4).

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**Table 3.** Repeated measures analysis of variance (ANOVA) for measured multispectral traits of common bean grown in different treatments: control (C) and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). Measurements were performed at four different times: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the onset of treatments. P-values are presented for a) ANOVA for repeated measures, b) ANOVA test with SLICE option to examine the significance of each treatment across measurements (Measurement Time), c) ANOVA test with SLICE option to examine the significance of treatments within time

| | Source | Multispectral traits | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $R_{Red}$ | $R_{Green}$ | $R_{Blue}$ | $R_{FarRed}$ | $R_{NIR}$ | SpcGrn | HUE | SAT | VAL | CHI | ARI | GLI | NDVI | NPCI | PSRI |
| a) ANOVA with Repeated measures | T | <.0001 | <.0001 | <.0001 | <.0001 | 0.0032 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0303 | <.0001 |
| | MT | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | T x MT | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| b) Treatments across Measurement Time | C | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0395 | <.0001 | <.0001 | 0.2665 | 0.0905 | 0.0007 | 0.1811 | 0.2435 | 0.6811 |
| | N | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | P | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.8559 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0047 | 0.0834 | 0.4027 |
| | K | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | Mg | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.4238 | 0.0039 | <.0001 | <.0001 |
| | Fe | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0096 | <.0001 | <.0001 | <.0001 |
| c) Treatments within Measurement Time | MT1 | 0.1473 | 0.0457 | <.0001 | <.0001 | <.0001 | 0.0061 | 0.0004 | <.0001 | 0.0529 | 0.0007 | <.0001 | 0.0144 | 0.364 | 0.8825 | 0.9201 |
| | MT2 | 0.0009 | <.0001 | <.0001 | <.0001 | 0.0005 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.1526 | 0.0737 |
| | MT3 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0005 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0031 | <.0001 |
| | MT4 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0002 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |

T – treatment; MT – measurement time

Fluorescence parameters were also severely affected, and $F_v/F_m$, $F_q'/F_m'$, ETR, qP, qN and qL showed a significant decrease from MT1 to MT4. NPQ increased at the early phase from MT1 to MT2 and then decreased again to MT4, while φno increased from MT1 to MT4 (Figure 5). The earliest symptoms detected at MT1 were related to higher HUE and CHI and lower SAT and GLI in K-deficit plants compared to the control (Figure 4). From MT2, morphological changes in K-deficit plants were visible as lower DV, TLA, LAI, LANG and higher LINC than in control plants (Figure 3). In addition, higher $R_{Blue}$, $R_{SpcGrn}$ and lower SAT, GLI and NDVI were found in K-deficit plants compared to the control. In MT2 K-deficit plants had lower $F_q'/F_m'$, ETR, qP and higher NPQ and qN compared to the control (Figure 5).

### *Magnesium deficit*

In contrast to K, P and N deficiency, Mg-deficit plants show an increase in DV, PH, LAI and TLA over time (MT1-MT4), although growth was slower compared to the control (Figure 3). Reflectance ($R_{Red}$, $R_{Green}$, $R_{Blue}$, $R_{SpcGrn}$, $R_{NIR}$, $R_{FarRed}$) decreases over measurements, and only $R_{FarRed}$ tends to increase again at MT4.

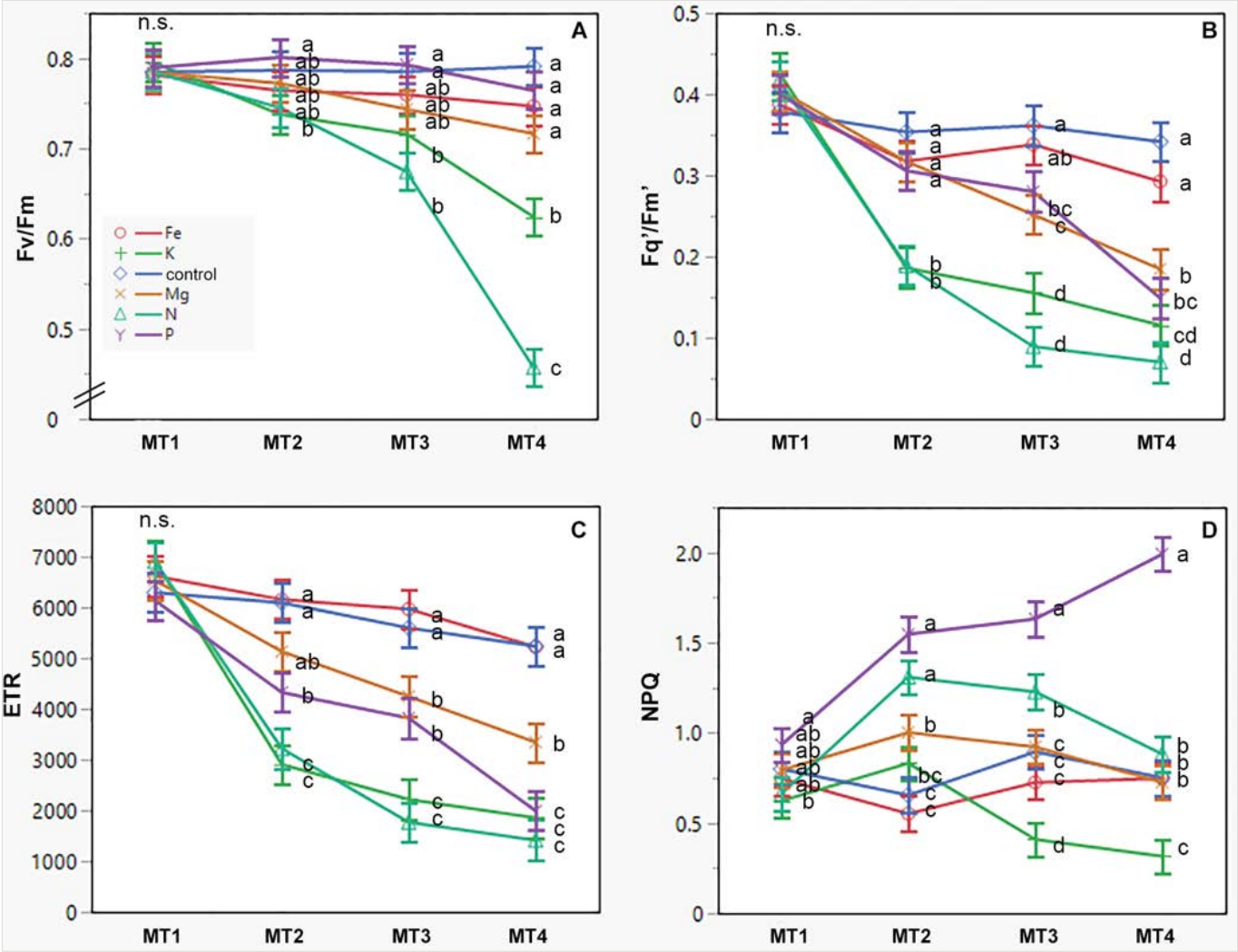


**Figure 5.** The mean values together with their corresponding standard errors for a set of chosen chlorophyll fluorescence traits. The study investigated A) maximum quantum yield of PSII ($F_v/F_m$), B) effective quantum yield of PSII ($F_q'/F_m'$), C) electron transport rate (ETR) and D) non-photochemical quenching (NPQ) in common bean plants subjected to different nutrient treatments: control and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). Measurements were taken at four time points: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the start of the treatments. n.s. denotes that there were no significant differences among nutrient treatments within the specific measurement time; different lowercase letters indicate significant differences among treatments within the measurement time based on Tukey's HSD test.

**Table 4.** Repeated measures analysis of variance (ANOVA) for measured chlorophyll fluorescence traits of common bean grown in different treatments: control (C) and deficit of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe). Measurements were performed at four different times: 3 (MT1), 6 (MT2), 9 (MT3) and 12 (MT4) days after the onset of treatments. P-values are presented for a) ANOVA for repeated measures, b) ANOVA test with SLICE option to examine the significance of each treatment across measurements (Measurement Time), c) ANOVA test with SLICE option to examine the significance of treatments within time

| | Source | Chlorophyll fluorescence traits | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $F_v/F_m$ | $F_{q'}/F_{m'}$ | ETR | NPQ | qP | qN | qL | φno |
| a) ANOVA with Repeated measures | T | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | MT | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | T x MT | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| b) Treatments across Measurement Time | C | 0.9695 | 0.0508 | <.0001 | 0.0011 | 0.0015 | 0.0531 | <.0001 | 0.0513 |
| | N | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0002 |
| | P | 0.0535 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.1025 |
| | K | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | Mg | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0084 | <.0001 | <.0001 |
| | Fe | 0.0959 | <.0001 | <.0001 | 0.0024 | <.0001 | 0.007 | <.0001 | <.0001 |
| c) Treatments within Measurement Time | MT1 | 0.9502 | 0.0734 | 0.0471 | 0.0002 | 0.2942 | 0.0034 | 0.0548 | 0.1625 |
| | MT2 | 0.0002 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | MT3 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | MT4 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |

T – treatment; MT – measurement time

Vegetation indices, HUE, VAL, ARI, CHI and NDVI decrease, while SAT, NPCI and PSRI increase over measurements (Figure 4). $F_v/F_m$, $F_q'/F_m'$, ETR, qP and qL decreased, while φno increased from MT1 to MT4 (Figure 5). The earliest significant changes in Mg-deficit plants compared to the control were lower TLA and LAI (Figure 3), lower SAT and higher qN and NPQ at MT2 (Figure 5). From MT3, in addition to TLA and LAI, DV and PH were lower compared to the control (Figure 3). Traits related to photochemistry, $F_q'/F_m'$, ETR and qL were also lower in Mg-deficit treatment than in the control (Figure 4). Clear changes in most multispectral traits were only seen in MT4 with lower CHI, GLI and NDVI and higher PSRI compared to control (Figure 4).

***Iron deficit***

Similar to Mg deficiency, Fe-deficit plants showed continuous growth through the experiment and increased DV, PH, LAI and TLA over time (MT1-MT4), although growth was lower compared to the control and higher compared to Mg deficiency (Figure 3). $R_{NIR}$, HUE, CHI and ARI decreased, while SAT, NPCI and PSRI increased over measurements (MT1-MT4). $R_{Blue}$ decreased from MT1 to MT2 and stayed stable in subsequent measurements, while $R_{Red}$, $R_{Green}$, $R_{FarRed}$, $R_{SpcGrn}$ and VAL decreased from MT1 to MT3 and increased again in MT4 (Figure 4). $F_q'/F_m'$ decreased significantly in MT2 and was relatively stable afterward, while a significant decrease in ETR was found at MT4 (Figure 5). The earliest significant changes in Fe-deficit plants compared to the control were lower

TLA and LAI (Figure 3) and higher $R_{FarRed}$, $R_{SpcGrn}$ in MT2. From MT3 compared to the control, in addition to TLA and LAI, lower DV was found (Figure 3). Furthermore, higher $R_{SpcGrn}$, $R_{Green}$ and VAL, and lower CHI and ARI were found in MT3 compared to the control (Figure 4). When comparing the average chlorophyll fluorescence values of the whole plants, Fe-deficit plants did not differ significantly from the control plants. To investigate this further, we further analysed the chlorophyll fluorescence parameters on the control and Fe-deficit plants using the specific region of interest (ROI) for the old and young leaves. These results revealed that old control leaves had higher ($P < 0.05$) NPQ and qN and lower φno compared to old Fe-deficit leaves (data not shown). Even more differences were found between the young leaves of these two treatments. Compared to the control, young Fe-deficit leaves had lower ($P < 0.05$) $F_v/F_m$, $F_q'/F_m'$, ETR, qP and qL and higher φno (data not shown).

## DISCUSSION

Nutrient deficiencies are a common problem in crop production and are expected to become more frequent as agriculture spreads to less fertile soils and with constraints brought by global climate change (Lynch et al., 2021). Non-destructive phenotyping techniques such as multispectral and chlorophyll fluorescence imaging are considered objective methods for monitoring plant nutritional status and detecting nutrient deficiencies (Debnath et al., 2021; Aleksandrov, 2022; Kalaji et al., 2018), which could be used for the green transformation of agriculture and the achievement of sustainable goals.

Compared to the adequate plant nutrient content (Hochmuth and Hanion, 2018), the analysis of the shoot's mineral content showed that the treatment solutions caused specific nutrient deficiencies, confirming that the observed changes in morphological, multispectral and chlorophyll fluorescence parameters were related to the specific nutrient deficiencies.

The multispectral 3D scanning in this study enabled the quantification of morphological changes caused by nutrient deficiency and revealed a significant reduction in the growth of nutrient-deficit plants. All studied nutrient deficiencies caused a reduction of morphological traits already at MT2, especially leaf area (LAI and TLA), although this was less pronounced in Mg and Fe deficiency. These results are in line with previous studies that described a reduction in biomass and leaf area of different plants under N deficiency (Vos et al., 2005; Chen et al., 2015), K deficiency (Gerardeaux et al., 2010), P deficiency (Jacob and Lawlor, 1991) and Mg deficiency (Cakmak et al., 1994).

Furthermore, Shetty and Miller (1966) found that Fe deficiency did not result in overall soybean (*Glycine max* L.) plant size reduction, although chlorotic trifoliate were smaller than healthy ones. In fact, morphological traits, especially LAI and TLA, were reduced before or at the same time as multispectral and chlorophyll fluorescence traits, indicating that morphological changes are not solely caused by reduction in photosynthesis and related decrease in carbohydrate availability but more likely represent plants' strategy to decrease its metabolic costs under nutrient deficit. These findings are also consistent with previous research. For instance, Jordan-Meille and Pellerin (2008) discovered that the limitation of leaf elongation by K deficiency in maize was not a result of restricted carbohydrate availability but rather altered plant–water relationships. In addition, growth analysis of maize under P deficiency revealed that P deficiency impacts leaf growth earlier and more significantly than photosynthesis per unit leaf area (Plénet et al., 2000).

Moreover, N supply has a strong connection with leaf area and green leaf duration, which have a significant impact on biomass production (Vos et al., 2005; Chen et al., 2015; Mu et al., 2017). In addition to the quantification of the morphological traits, multispectral analyses could be used as a good predictor of plants' pigment concentrations, leaf internal structure and moisture content (Schurr et al., 2006). Nutrient deficiency reduces leaf chlorophyll concentration and alters the chlorophyll to carotenoid ratio, resulting in decreased light absorption and increased visible or infrared reflectance (Peñuelas et al., 1995). All studied nutrient deficiencies

affected most of the analyzed color and multispectral traits, especially at later measurement times (MT3 and MT4). However, multispectral traits substantially differed among plants with different nutrient deficiencies. The most pronounced effects on colour and multispectral traits were found in K- and N-deficit plants. Namely, K deficiency caused a decrease in GLI and increased HUE and CHI at MT1, followed by increased $R_{Blue}$ and $R_{SpcGrn}$, a decrease in SAT and NDVI and a further decrease in GLI at MT2. N deficiency caused increased $R_{Blue}$ at MT1, followed by a decrease in GLI, SAT and NDVI at MT2. These results indicate an early effect of N and K deficiency on chlorophyll content and the concomitant decrease in absorption of PAR, especially blue wavelengths. Nitrogen is the key element for chlorophyll biosynthesis, and deficiency symptoms include leaf chlorosis (Marschner, 1995). Xiao-Lei et al. (2012) stated that symptoms of K deficiency include brown scorching and chlorosis between leaf veins, caused by chlorophyll degradation.

Also, Fe-deficit plants manifest a similar increase in reflectance and decrease in vegetation indices as was found for N- and K-deficit plants, although the earliest multispectral changes (found at MT2) in Fe-deficit plants compared to control were increased $R_{FarRed}$ and $R_{SpcGrn}$, and from MT3 decrease in CHI and ARI. The occurrence of leaf chlorosis resulting from a lack of iron has been associated with the obstruction of chlorophyll and carotenoid production, a process that relies on the activity of enzymes containing iron (Reinbothe et al., 2006). Likewise, the deficiency of iron resulted in a reduction in ARI due to the involvement of iron-containing enzymes in the process of anthocyanin synthesis (Wilmouth et al., 2002). While it is recognized that Mg plays a role in the synthesis of chlorophyll pigments and previous research has demonstrated that a lack of Mg leads to decreased chlorophyll levels (Faust and Schubert, 2016; Tränkner et al. 2016), the findings of this study indicate that multispectral characteristics were impacted only after prolonged Mg deficiency (MT4). Furthermore, it was shown that only the vegetation indices associated with chlorophyll content (CHI, NDVI, and GLI) exhibited a decline, whereas PSRI showed an increase. This pattern suggests the occurrence of chlorophyll degradation and promoted senescence (Sato et al., 2015).

Compared to other nutrient deficiencies, P-deficit plants show different color and multispectral characteristics. Namely, the earliest changes in P-deficit plants found from MT2 are decreased $R_{FarRed}$, $R_{SpcGrn}$ and increased HUE and CHI. The only traits that were similarly affected by P deficiency and other nutrient deficiencies were a decrease in GLI and SAT. A decrease in reflectance and an increase in HUE and CHI could be explained by the development of dark green leaves under P deficiency. While previous studies have reported a decrease in chlorophyll content in many crop species under phosphorus (P) deficit (Jacob and Lawrol, 1991), the manifestation of dark green leaves is frequently observed as a sign of P deficiency. For example, Chu et al. (2018) found dark green leaves in P-deficit soybean plants and explained this symptom by the obstruction of the triose phosphate transport from the P-deficit leaves. However, these authors also described a change in chloroplast ultrastructure (swelled chloroplasts) and the separation of the mesophyll cells, which created big intracellular spaces, which could also be the cause of darker leaf color. The chlorophyll fluorescence characteristics exhibited alterations in response to all nutritional shortages. However, for the iron deficit, these differences were obtained on young leaves, only after separate analysis of young and old leaves. Such results are related to the Fe mobility within the plants. Compared to all other studied nutrients Fe is considered as an immobile nutrient within the plant (Marschner, 1995), and thus the Fe deficiency symptoms could be found on young leaves. Similarly, Aleksandrov (2022) found reduced electron transport rates in Fe-deficit common bean. Most of the iron inside a plant is mostly found in the chloroplasts. Consequently, a deficit in iron leads to the deterioration of several photosynthetic components, such as ferredoxin (Fd) (Tognetti et al., 2007). Hence, it may be inferred that chloroplasts are the main focal points of iron shortage, leading to a reduction in photosynthetic activity as well as the levels of plastidic proteins and pigments (Winder and Nishio 1995). However, the fact that chlorophyll

fluorescence parameters only decreased in young leaves with Fe deficiency indicates that chlorophyll fluorescence can only detect Fe deficiency after a longer period, i.e. after the development of new leaves, compared to other nutrient deficiencies.

For N-, P- and K- deficit plants, a significant decrease in the quantum yield of PSII, electron transport rate, photochemical quenching and an increase in non-photochemical quenching were already observed at MT2, whereas for Mg-deficit plants at MT3. Although $F_v/F_m$ is one of the most used chlorophyll fluorescence parameters for studying different plant stresses (Maxwell and Johnson, 2000), it did not show significant changes under nutrient deficiency (except for young Fe deficit leaves) since both $F_0$ and $F_m$ increased in nutrient-deficit plants. Aleksandrov (2022) describes similar results under N-, P- and K-deficit common bean. For example, the availability of N has a significant impact on both chlorophyll fluorescence and the allocation of light energy (Huang et al., 2004; Mu et al., 2017). A shortage in N has been observed to decrease the levels of chlorophyll, the content of photosystem I (PSI) and II (PSII), and light-harvesting complexes (LHCs). Consequently, this reduction leads to a decrease in light absorption and electron transport while simultaneously increasing non-photochemical quenching (NPQ). Photosynthesis is influenced by phosphorus deficiency, mostly through indirect mechanisms that result in the reduction of ATP and NADPH production. In plants experiencing phosphorus deficit, parameters such as $F_q'/F_m'$, ETR and qP exhibit a decrease (Xu et al., 2007). K deficiency causes leaf anatomical changes and reduces the mesophyll conductance, which is a main limitation of photosynthesis under K deficiency (Jákli et al. 2017). K deficit caused a decrease in ETR in common bean (Aleksandrov, 2022) and an increase in NPQ in sunflower (*Helianthus annuus* L.) (Jákli et al., 2017). The Mg deficiency causes damage to the oxygen-evolving complex on PSII (Yang et al., 2012), however, the main target of Mg deficiency is PSI rather than PSII and, consequently, capacity for cyclic electron transport (Farhat et al., 2015).

## CONCLUSIONS

The study's findings underscore the reliability, precision, and speed of plant phenotyping techniques for non-destructive nutrient deficiency determination. Early detection, as soon as three days post-treatment, was achieved, particularly for N, P, and K deficiencies, each displaying distinct symptoms. Growth inhibition, notably evident in reduced leaf area, emerged as an early symptom across all deficiencies. Specific changes in multispectral traits were observed, with Mg deficiency exhibiting delayed effects over twelve days. Chlorophyll fluorescence traits remained unaffected by Fe deficiency, contrasting with significant alterations in other nutrient deficiencies, primarily impacting $F_q'/F_m'$, ETR, and non-photochemical quenching parameters. These results highlight the potential of these techniques in precision agriculture, though their applicability in complex real-world production systems warrants further research and development to address combined deficiencies and various stressors.

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