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Phosphorus-deficiency stress in cucumber (*Cucumis sativus* L.) plants: early detection based on chosen physiological parameters and statistical analyses

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Abstract

Enhancing plant productivity and mitigating the impact of environmental stressors require a thorough understanding of phytomonitoring and physiological features indicative of plant health. This study delves into the response of cucumber plants to phosphorus deficiency employing diverse tools to identify key indicators and unravel the underlying mechanisms. Under phosphorus deficiency, a rapid response in older leaves was observed through the analysis of chlorophyll and carotenoid content. Molecular-level changes in photosynthetic performance were found to be age-dependent, as revealed by multidimensional statistical methods, highlighting the interconnectedness of examined features with the experimental setup timing. This can assist in understanding the long-term fluctuations in traits linked to phosphorus deficiency, facilitating early detection of stress.

Keywords: chlorophyll fluorescence; confocal microscopy; greenhouse cucumber; leaf area index; multivariate statistical analyses; photosynthetic pigment.

Highlights

- Indicators of health and productivity of plants consider phosphorus deficiency
- The contents of chlorophyll and carotenoids in older leaves reflect the plant's condition
- Multivariate statistical methods facilitate the early detection of stress

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Abbreviations: aCar – α-carotene; bCar – β-carotene; Brix – total soluble solids; Chl – chlorophyll; DM – dry mass; DTF – distance from shoot top to 1st open flower; LA – leaf area; LAI – leaf area index; LL – leaf length; Lut – lutein; LW – leaf width; MTA – mean tilt angle; NoF – number of flowers per shoot; NoFS – number of fruits set on the shoot; NoFwk – number of fruits harvested per week; NoL – number of fully grown leaves per plant; PL – plate length; SD1 – shoot diameter at the top one week earlier; SD2 – shoot diameter at the top two weeks earlier; SGLwk – weekly shoot growth in length; SPAD – index of relative chlorophyll content; WFwk – mass of fruit harvested per week.
Conflict of interest: The authors declare that they have no conflict of interest.

Introduction

Phosphorus (P) is a crucial element in the environment, and its availability plays a vital role in the functioning and structure of ecosystems. It is essential for the growth and development of both plants and animals. Phosphorus deficiency is recognized as a primary factor limiting global plant production (Marschner and Rengel 2012, Pagliari *et al.* 2018). In plants, phosphorus serves multiple functions such as facilitating photosynthesis, respiration, nucleic acid synthesis, and energy production, and serving as a fundamental component of phosphoproteins and phospholipids (Raghothama 1999, Vance *et al.* 2003, Kamerlin *et al.* 2013, Daneshgar *et al.* 2018).

Plants acquire phosphorus from the soil solution or nutrient solution primarily in the form of inorganic orthophosphates, particularly $H_2PO_4^-$ (Raghothama 1999). They have evolved various morphological, physiological, and biochemical mechanisms to adapt to phosphorus-deficient conditions in the soil or nutrient solution.

It has been demonstrated that plants respond to phosphate starvation by developing mechanisms that enhance the availability, uptake, and transport of orthophosphates. These mechanisms include the secretion of acid phosphatase and increased synthesis of phosphate transporters (Duff *et al.* 1994, Raghothama 2000). Additionally, plants can adapt to phosphate deficiency through various changes in growth and metabolic responses. One of the most noticeable symptoms of phosphate starvation is an increase in the root-to-shoot mass ratio, which can result from suppressed shoot growth and stimulated root growth (Fredeen *et al.* 1989, Cakmak *et al.* 1994, Ciereszko *et al.* 1996, Borch *et al.* 1999, Mollier *et al.* 1999). Phosphate deficiency may lead to a decrease in the rate of photosynthesis (Fredeen *et al.* 1989, Jacob and Lawlor 1991, Cetner *et al.* 2020) or no significant change (Kondracka and Rychter 1997). Phosphates play a crucial metabolic role in regulating starch and sucrose synthesis (Kleczkowski 1994, Liu *et al.* 2018). The total respiration rate may either decrease (Usuda and Shimogawara 1991) or show no significant change under phosphorus starvation (Rychter and Mikulska 1990). Low phosphorus content shortened the post-exercise decay of CO_2, which is a measure of the pool of photorespiration metabolites (Haushild *et al.* 1996, Dabu *et al.* 2019).

Cucumber (*Cucumis sativus* L.) is a significant horticultural plant and a primary greenhouse vegetable crop worldwide (Bayoumi *et al.* 2021). Cucumber production in greenhouses is susceptible to various abiotic stresses, including inadequate or excessive application of mineral fertilizers, soil salinity, and high temperatures (Taiz *et al.* 2014, Estaji *et al.* 2019). Comparative studies on the response of cucumber plants to early and moderate phosphorus deficiency have shown similar patterns to those observed in other crops when facing phosphorus deprivation. Prolonged exposure to phosphorus stress limits gas exchange in cucumber plants. Sugar content in the plants initially rises during moderate periods of phosphate deficiency, but later declines, likely due to reduced photosynthesis and sugar production. The ability of plants to adapt to phosphate deficiency is primarily dependent on the duration of the stress but also varies among different plant species. Consequently, understanding the basics of plant nutrition, including the identification of nutrient deficiencies, provides valuable information for producers, aiding them in making informed decisions about future fertilization strategies (Horaczek *et al.* 2020, Yue *et al.* 2023). Insufficient nutrient supply for optimal plant growth and development can lead to metabolic disorders, resulting in deficiency symptoms and reduction of yield and poor fruit quality (Silva *et al.* 2012). Therefore, evaluating the nutritional status of plants is a valuable method for predicting their growth, development, productivity, and quality. It also aids in identifying the specific nutrient limiting plant growth and development (Epstein 2009, Yan *et al.* 2015).

The objective of this study was to identify physiological characteristics that could serve as early indicators of phosphorus deficiency in cucumber plants cultivated under greenhouse production conditions. The identification of such features and understanding of their interrelationships could aid in the early detection of phosphorus deficiency stress by monitoring easily observable characteristics at specific stages of deficiency. This knowledge has the potential to improve plant health and, consequently, increase yields.

Materials and methods

The impact of phosphorus deficiency stress on cucumber cultivation was studied under greenhouse conditions. This research aimed to identify the most significant alterations in certain morphological and physiological parameters of the plants that occur during this stress.

Characteristics of cucumber cultivation methods and growth conditions: A hydroponic cultivation of greenhouse cucumbers was established using *Grodan* mineral wool substrate. The plants were nourished with a cucumber standard medium (control – C). Once the cucumber plants reached full fruiting (82 d after sowing the seeds – DAS), half of them were watered with a medium that had the same composition as the cucumber standard medium, but without phosphorus fertilizers (–P). The 'Mewa' F1 cucumber variety (*Rijk Zwaan*, York, UK) was used for the experiment. The study was conducted during the winter cycle with the assistance of assimilation lighting. The plants were grown on a single fruiting shoot, and all microclimate and fertilization parameters were controlled by a computer. The temperature during the day and night ranged from 22–25°C and 18–22°C, respectively. The CO_2 concentration was approximately 800 ppm, and the average air relative humidity (RH) was around 70%. The nutrient solution used for fertigation was based on one-component fertilizers and a multicomponent micronutrient fertilizer (*Yara's Superba Micromix*). The medium pH averaged at 5.7, while the electrical conductivity (EC) ranged from 3.2 to 3.3 mS cm^{-1}. In instances of low solar radiation, the plants were illuminated

using *Philips* LED lamps, maintaining a photosynthetic photon flux density (PPFD) of approximately 320 µmol m^{-2} s^{-1}. For detailed observations and measurements, six plants were randomly selected from both the control group (C) and the phosphorus-free medium (–P). Before inducing phosphorus deficiency in the medium, all plants were fertilized with the control medium (C), which contained the following amounts per 1 dm^3: 195 mg N – NO_3, 6 mg N – NH_4, 56 mg P, 264 mg K, 44 mg Mg, 257 mg Ca, 2.5 mg Fe, 0.83 mg Mn, 0.68 mg B, 0.15 mg Cu, 0.14 mg Zn, and 0.08 mg Mo.

Induction of phosphorus deficiency stress in the production crop of cucumber: The day of initiating phosphorus deficiency stress (82 d after sowing – DAS) was denoted as time 0 (t0). That day phosphorus has been withdrawn from the nutrient solution for cucumber plants. Subsequent days of cucumber cultivation with a phosphorus-free nutrient solution (–P) were marked as follows: time 1 (t1) at 7 d after the onset of phosphorus deficiency stress, time 2 (t2) at 14 d, time 3 (t3) at 21 d, and time 4 (t4) at 28 d.

Morphological examination of cucumber plants: Six plants from each treatment were selected and measured at each of the following dates: t0, t1, t2, t3, and t4. The following parameters were measured: weekly shoot growth in length, the distance from the shoot top to the point marked on the shoot where the shoot top was the week before, and the diameter of the cucumber shoot. The diameter of the cucumber shoot was measured using electronic calipers at two points on the shoot, first at the point on the shoot where the shoot top was one week earlier, second at the point on the shoot where the shoot top was two weeks earlier. Also measured were the number of fully developed leaves per plant, the number of flowers on the shoot, the number of fruit set on the shoot, weight, and number of fruit harvested per week.

Leaf parameters were measured on the 5th fully developed leaf from the top of the plant (young leaf) and the 10th fully developed leaf from the top of the plant (old leaf). The mean results for each plant were calculated as the average of the measurements taken from the 5th and 10th leaves from three plants. At each test date, leaf parameters, such as the length and width of the leaf blade, as well as the length of the petiole, were measured. Also, some plant phytomonitoring parameters, such as weekly shoot growth in length, distance from shoot top to 1st open flower, shoot diameter at the top one week earlier, shoot diameter at the top two weeks earlier, number of fully grown leaves per plant, number of flowers per shoot, number of fruit set on the shoot, number of fruit harvested per week, mass of fruit harvested per week, leaf area index, mean tilt angle, were measured.

The chosen physiological parameters of cucumber leaves investigated: The relative chlorophyll (Chl) content in the leaves was estimated using a chlorophyll meter (*SPAD-502 PLUS*, *Konica Minolta*, Japan). The total soluble solids (TSS) content in the cell sap of cucumber leaves was measured using a digital refractometer (*HI-96800*, *Hanna Instruments*). The carotenoid content in the leaf lamina was estimated by high-performance liquid chromatography (HPLC-DAD) using *Shimadzu Prominence* chromatograph equipped with auto sampler *SIL-20AC HT*, diode array detector *SPD-M20A* and *LCsolution 1.21 SP1* chromatography software (*Shimadzu*, Kyoto, Japan). Leaf area index (LAI) and mean tilt angle (MTA) parameters were measured at each of the dates (t0–t4) using an optical *LAI-2200C* Plant Canopy Analyzer (*LI-COR Inc*., Lincoln, USA). These measurements were performed nondestructively in four replications. The content of carotenoids, lutein, Chl *a*, and Chl *b* in leaves and fruits was determined by high-performance liquid chromatography (HPLC) using a *Shimadzu Scientific Instruments* system from Japan. The dry mass (DM) of the leaves was determined using the mass method after drying the plants at 105°C for 48 h.

The mean values of the examined cucumber parameters are presented in Table 1S, *supplement*.

Examination of the macro- and micro-nutrient content of the leaves: At t0 and t4, the leaves were dried at 70°C for 48 h, ground, and then digested in HNO_3 to determine the concentration of macro and micronutrients in cucumber leaves. An inductively coupled plasma spectrometer (*ICP Model OPTIMA 2000DV*, *Perkin Elmer*, USA) was used to analyse elements including P, K, Mg, Na, Ca, Fe, Mn, Cu, Zn, and B.

To measure the total nitrogen (N) content, the plant material was mineralized using concentrated sulfuric acid in the presence of a copper–potassium catalyst. The nitrogen content was assessed using a Kjeldahl apparatus (*Vapodest*, *Gerhardt*, Germany). After distillation of nitrogen in the form of NH_3, the N content was determined using titration methods as outlined in the Official Methods of Analysis of AOAC International (2012).

Measurements of leaf colour: The colour of the leaves was assessed by capturing digital images using a *Pentax K5* camera. The camera was equipped with a CMOS sensor measuring 23.7 × 15.7 mm and had an effective pixel count of 16.28 MP. The camera settings included an ISO value of 200 and auto-focus mode. To capture the images, a dedicated *Pentax* lens (18–55 mm) was used.

The colour of each leaf was characterized using the mean values of the R (red), G (green), and B (blue) components obtained from the corresponding image. These mean R, G, and B values were then converted into the *CIE Lab* color space, following the methods described by CIE (2004), *Adobe Systems Inc.* (2005), and Schanda (2007). In the *CIE Lab* color space, colour is represented by an achromatic component called L, which defines the lightness ranging from 0 (black) to 100 (white). Additionally, there are two chromatic components: –a/+a, representing green-red opponent colours, and –b/+b, representing blue-yellow opponent colours. The values of

the L, a, b components create a perceptually homogeneous colour space shaped like a sphere. Changes in the L, a, b component values within this space are proportional to the colour changes perceived by an observer.

Confocal laser scanning microscopy (CLSM) investigations – Chl and carotenoids (Cars) autofluorescence: The distribution of Chl and Cars autofluorescence was examined in palisade mesophyll chloroplasts of control and phosphorus-deficient cucumber plants four weeks after treatment.

Chloroplasts were examined using a *Leica TCS SP5II* confocal laser scanning microscope (*Leica Microsystems CMS*, Wetzlar, Germany) equipped with a 63× objective (*HCX PL APO Lambda* blue 63×/1.40 OIL UV). Images of Chl and Cars autofluorescence were obtained using fresh hand-made cross-sections of a leaf blade prepared by using a razor blade and embedded in distilled water. Chl autofluorescence (magenta channel) and carotenoid autofluorescence (green channel) were excited at 633/488 nm and recorded at 660–705/510–580 nm, respectively. Sequential scanning was performed to reduce crosstalk between the channels. The pinhole was set to 1 AU (airy unit) to balance the fluorescence signal brightness and image resolution. To visualize the three-dimensional distribution of Chl and Cars autofluorescence signals along the z-axis of chloroplasts, 26 optical sections were recorded at a step size of 0.13 µm. A rectangular region of interest was selected to demonstrate the distribution of Chl and Cars fluorescence in a side view.

Bright magenta discs (2D images) and magenta ellipsoid-shaped structures visible in 3D images, representing Chl fluorescence, were identified as grana. The raw data were processed in the same manner for all samples using *Leica SP5II* software. The image series underwent 3D deconvolution using the classical confocal algorithm to eliminate background noise and significantly enhance image quality. The distribution of Chl and Cars fluorescence within chloroplasts was reconstructed in 3D using *Leica SP5II* software.

Kautsky effect: The dynamic of Chl *a* fluorescence induction, known as the Kautsky effect (Kautsky and Hirsch 1931), was investigated using a *Leica TCS SP5II* confocal microscope equipped with a HeNe laser (10 mW, 633 nm for Chl excitation) and a 10× objective (*HC PL APO* 10×/40 CS). The experimental procedure followed the general description provided by Tseng and Chu (2017). Specifically, hand-made cross-sections through the cucumber leaf blade were used, enabling the differentiation between palisade and spongy mesophyll. The Chl *a* fluorescence dynamic was recorded separately for the palisade and spongy mesophylls, as well as for the whole mesophyll (for more details, refer to Fig. 1S, *supplement*). The scanning speed was set at 1,400 Hz, and the spatial resolution was 256 × 256 pixels, allowing for approximately 5 scans per second in the spectral range of 660–705 nm. The laser power was set to 2% of its maximum intensity, which provided low-intensity illumination suitable for obtaining classical OPSMT (O for origin, P for peak, S for semi-steady state, M for maximum, and T for terminal steady-state level) curves (Papageorgiou and Govindjee 1968).

Statistical analysis: The analyses were conducted using average values, with a minimum of three repetitions representing the smallest experimental unit. Radar charts were created in *MS Excel 2019* to display the quotient of the mean deficiency values to the control values. The correlation plot was generated based on mean values for the plants, categorized into control, deficiency, and measurement dates. While averaging the values resulted in the loss of multiple repetitions, this approach allowed for visualizing the approximate relationships between the determined traits for the entire plant. Each coefficient was determined using 16 values from two pairs of features. The critical significance value of the *Pearson*'s correlation at a significance level of 0.05 was >0.4973. *MS Power BI Desktop v. 2*, along with the correlation plot procedure from the *R* package, was used to construct the correlation matrix chart.

Principal component analysis (PCA) was employed to reduce the 23-dimensional parameter space that described the growth and development of cucumber plants. Factor analysis was performed on the mean values for the defined objects, which consisted of combinations of the measurement term (t1, t2, t3, t4), factor (phosphorus deficiency – –P, control – C), and the level of leaf development (young – y, old – o). Varimax rotation was applied to reposition the examined parameters, ensuring that individual components contained strongly correlated objects while maintaining minimal correlation between components. The biplots served as a synthesis and basis for discussing this part of the analysis. They were created using the eigenvalues of the studied variables/parameters with individual components and the correlation coefficient of the tested objects with the components. These biplots depicted the correlated parameters with the components in a two-dimensional space. The position of the factor combination variants (control – C, phosphorus deficiency – –P, four test dates – t1, t2, t3, t4), and the organs on which the parameters were measured (young leaf – y, older leaf – o) were illustrated concerning the components. Dashed lines were added to connect relevant experimental deficient control systems (*e.g.*, t1Cy to t1Py) for better visualization.

Multivariate analyses, showcasing the synthetic variability in time and space using two components, were conducted using *IBM SPSS Statistics ver. 28*.

The impact of phosphorus shortage on the colour parameters of cucumber leaves was analysed using *Student*'s t-test or *Welch*'s *t*-test depending on whether each tested pair of groups exhibited equal or unequal variances respectively. The homogeneity of variance in the compared groups was assessed using *Levene*'s test. Mean comparisons between control plants and plants affected by phosphorus shortage were performed separately for each term, considering the age of the leaves. Each analysis was conducted at a significance level of alpha = 0.05. Vertical bars on the charts indicate the standard deviation (SD).

Black horizontal lines indicate significant differences between means.

Results

Phytomonitoring and plant characteristic: The analysis of plant phytomonitoring parameters in this study revealed several trends in response to phosphorus deficiency. It was observed that within a few days of phosphorus deficiency, the leaf area index (LAI) showed a significant decrease, with most of the evaluated parameters presenting lower values compared to those in the control group. However, some parameters, such as the weekly number and mass of harvested fruits, as well as the mean tilt angle, registered higher values in comparison to the control group. These results suggest that during the early stages of phosphorus deficiency, a reduction in crop size and quality may not necessarily occur.

At the initial measurement time point (t1), compared to the control group, there was an increase in parameters, such as shoot diameter measured two weeks prior, the number of flowers per shoot, and the weekly fruit yield. However, the leaf area index (LAI) showed a decrease.

During the second and third cultivation periods (t2 and t3), in comparison to the control group, there was a decline in parameters including the shoot diameter measured at the top one week earlier, the number of flowers per shoot, weekly fruit yield, mass of harvested fruits per week, and LAI. Yet, the values of other parameters were similar to those of the control group.

In the fourth measurement period (t4), most of the assessed parameters exhibited lower values relative to the control group. However, the weekly number of harvested fruits, the mass of fruits harvested per week, and the mean tilt angle were higher compared to the control group.

The results showed distinct differences between young and old leaves in response to phosphorus deficiency, observed over four weeks of measuring various parameters (lutein, Chl *a* and *b*, α-β-carotene, soluble component content in cell sap, dry mass, leaf length, width, and area, petiole length, and Chl measurement in SPAD units) as illustrated in Fig. 1.

In the first three weeks of phosphorus deficiency, the most notable change in young leaves was a decrease in α-β-carotene content. It was only in the fourth week of phosphorus deficiency that significant changes were observed in the young leaves in the analysed parameters. Compared to the control group, there was an increase in the content of dry mass and soluble components in the cell sap, while the contents of compounds such as Chl *a* and *b*, α-β-carotene, and lutein decreased.

The reaction of old leaves to phosphorus deficiency, as indicated by the analysed parameters, differed from that of young leaves. In the first two weeks of measurement, old leaves showed a significant increase in lutein, Chl *a* and *b*, α-β-carotene, and dry mass content compared to the control group. However, in the third and fourth weeks, while the analysed parameters continued to exhibit higher values than those of the control, the measurements of leaf characteristics, such as length, width, leaf area, and petiole length, were lower compared to the control group, as detailed in Fig. 2.

When considering the average values of the tested parameters for both young and old leaves experiencing phosphorus deficiency across the four measurement periods, it is observed that, compared to the control, there was a significant increase in the content of substances such as lutein, Chl *a* and *b*, α-β-carotene, and dry matter. On the other hand, the length, width, area of the leaf, and petiole length showed a decrease (as shown in Fig. 2).

Pearson's correlation analysis was employed to explore the relationships between various plant and leaf parameters. Table 1 displays these relationships using a matrix plot, where the coefficient values are represented. Among the plant parameters, indicators such as petiole length (PL), leaf width (LW), leaf area (LA), leaf length (LL), leaf area index (LAI), and shoot diameter, at the first measurement point (SD1) showed the strongest correlations with each other.

Further, a separate correlation analysis was conducted for leaf parameters, with distinct results for young and old leaves (as indicated in Table 2S, *supplement*). The analysis revealed that the strength of the relationships between the examined traits varies according to the maturity age of the leaves.

Multivariate statistical analysis: Principal component analysis (PCA) was utilized to condense the 23-dimensional space derived from plant growth and development parameters into five dimensions, represented by principal components, which account for 91.2% of the overall variability. PC1 explains 30.9% of this variability and is strongly correlated with nine parameters, including all dye parameters (as detailed in Table 3S, *supplement*). PC2, accounting for 20.0% of the total variability, is closely associated with four parameters describing leaf structure. PC3, explaining 17.1% of the variability, correlates strongly with five parameters, including shoot diameter at the second measurement (SD2), number of flowers (NoF), mean tilt angle (MTA), dry matter (DM), and Brix. PC4 contributes to 13.1% of the overall variability and is most strongly related to three parameters: shoot growth length per week (SGLwk), days to flowering (DTF), and SPAD values. Finally, PC5 accounts for 10.1% of the total variability.

The projection of selected components onto two planes revealed the complexity of the plant's response, especially regarding leaf age. Fig. 3*A* shows increasing separation between control subjects and those under phosphorus (P) deficiency over subsequent research dates. Parameters linked to PC1 mainly reflect the response of older leaves to P deficiency, with notable changes observed in older leaves at t1 and t2, and a significant increase in the separation between control and deficiency groups at t3 and t4.

The impact of parameters associated with PC3 on differentiation becomes evident from t2, as illustrated in Fig. 3*B*,*E*. Significant parameters indicating P deficiency include MTA, DM, and Brix. The most differentiating parameter at t4, showing a strong negative correlation with

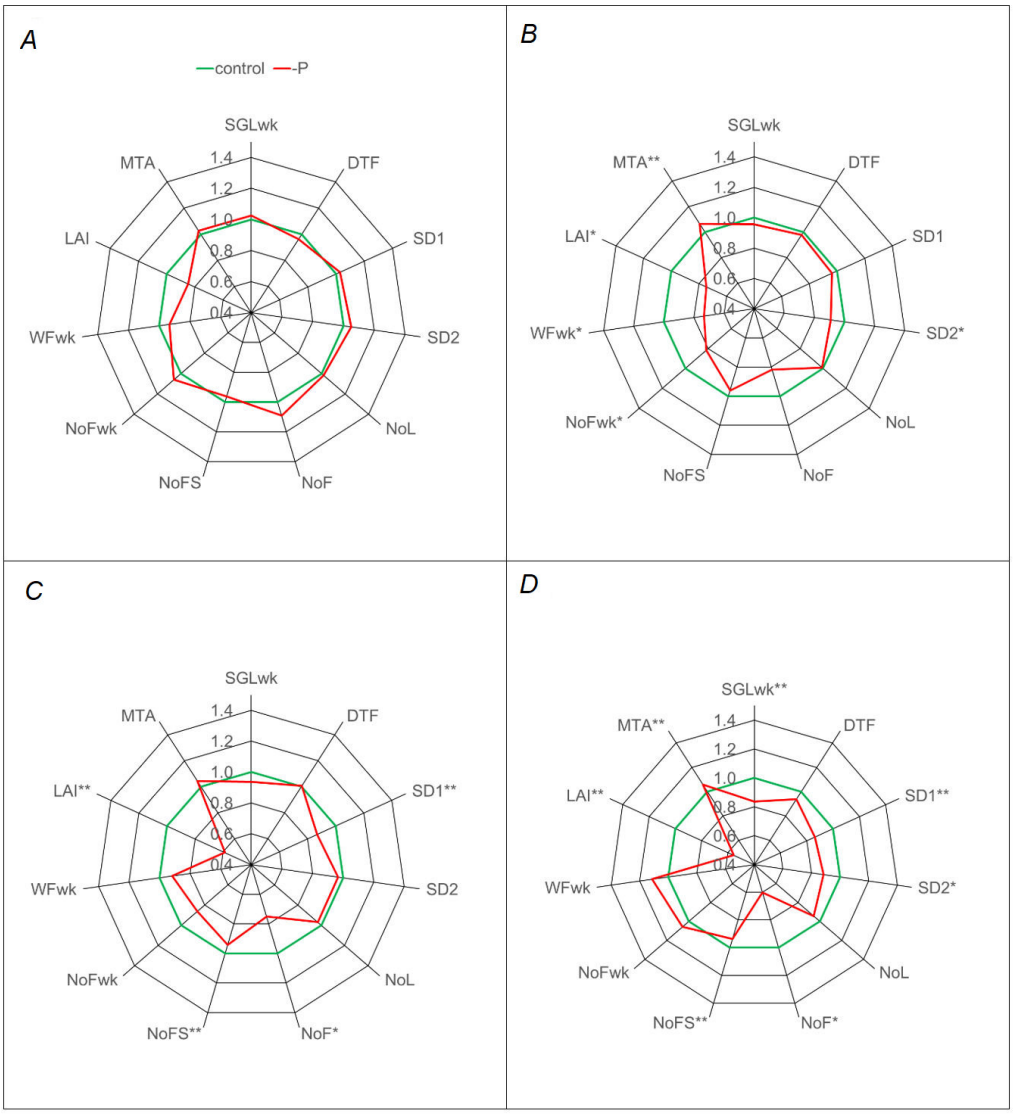

Fig. 1. Various parameters including weekly shoot growth in length (SGLwk), distance from shoot top to 1st open flower (DTF), shoot diameter at the top one week earlier (SD1), shoot diameter at the top two weeks earlier (SD2), number of fully grown leaves per plant (Nol), number of flowers per shoot (NoF), number of fruits set on the shoot (NoFs), number of fruits harvested per week (NOFwk), mass of fruits harvested per week (WFwk), leaf area index (LAI), and mean tilt angle (MTA) were monitored in cucumber plants. The measurements were compared with control plants, and the results were represented in the form of radar charts at four different measurement times: (*A*) time 1 (t1), (*B*) time 2 (t2), (*C*) time 3 (t3), (*D*) time 4 (t4). Significant differences, based on *Student*'s *t*-tests are marked by * (p<0.05) or by ** (p<0.01).

PC4, was SPAD (Fig. 3*C*,*F*). Colour change reactions were also statistically supported with significant differences shown in Fig. 5*A*–*C*.

Young leaves' response was more closely associated with the second component (PC2). Cucumber plants showed a strong and relatively rapid reaction to P deficiency, as indicated by parameters related to PC3, including shoot diameter at the top two weeks earlier, the number of flowers per shoot, mean tilt angle, dry matter, and total soluble solids. Fig. 3*B*,*D*,*E* demonstrate the relationships of the tested objects concerning component 3 and components 1, 2, and 4, indicating that features related to PC3 are effective indicators of P deficiency in cucumber plants.

Elemental analysis: Phosphorus deficiency in plants can affect the absorption of other nutrients. The analysis of nutrient content in cucumber leaves after four weeks of phosphorus deficiency showed a decrease in nitrogen and phosphorus contents compared to the control group. Conversely, there was an increase in the contents of sulfur, iron, copper, potassium, calcium, magnesium, and zinc (Saleque *et al.* 2001) (refer to Fig. 4 and Table 4S, *supplement*).

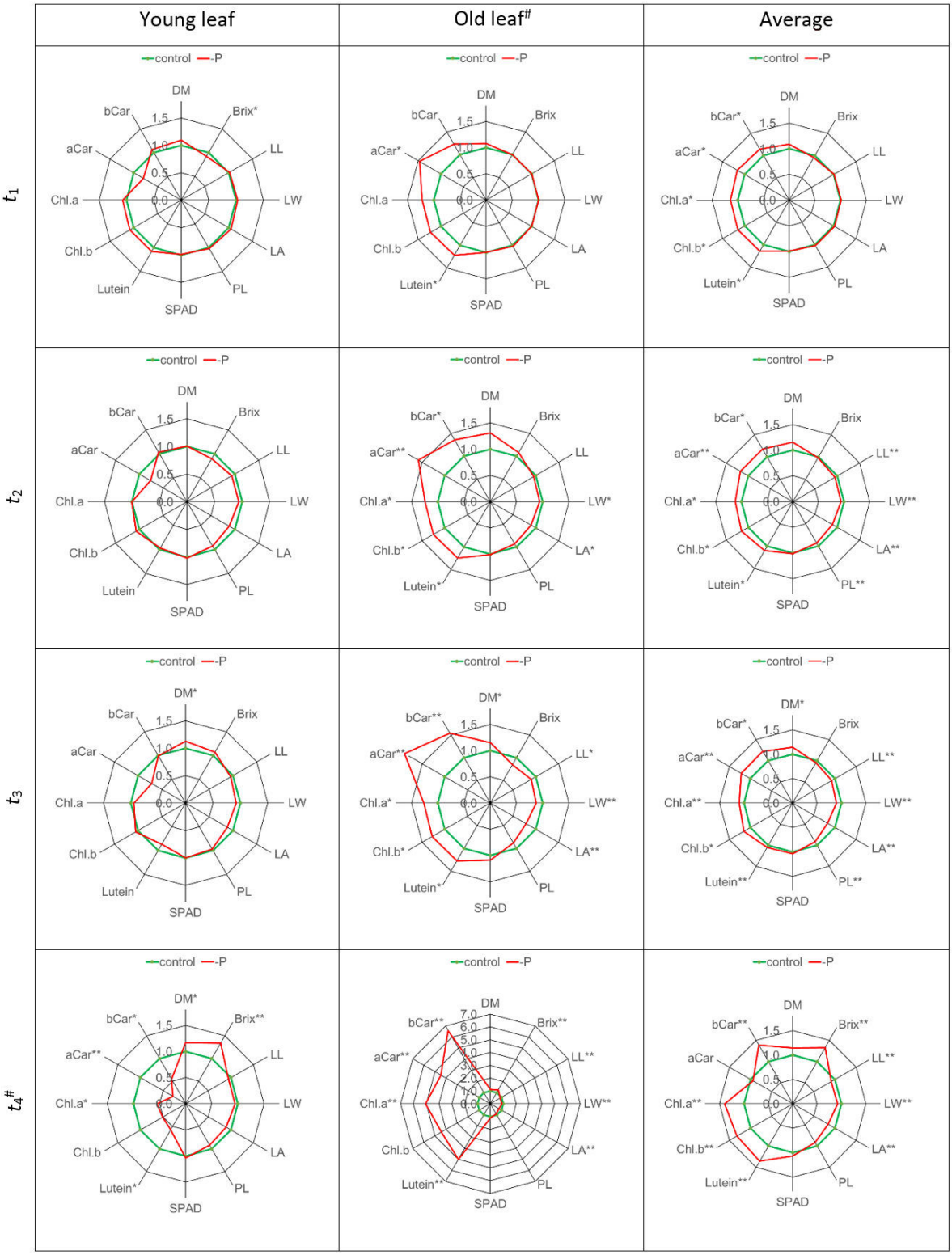

Fig. 2. Various parameters (DM – dry mass, Brix – total soluble solids, LL – leaf length, LW – leaf width, LA – leaf area, PL – plate length, SPAD – index of relative chlorophyll content, Chl b – chlorophyll *b*, Chl a – chlorophyll *a*, aCar – α-carotene, bCar – β-carotene) of cucumber plants in comparison with control plants in the form of radar charts for young leaves and older leaves at four measurement times (t1–t4). Significant differences, based on *Student*'s *t*-tests are marked by * ($p<0.05$) or by ** ($p<0.01$). #Parameter variability for old leaves has a high variability in time t4, to better show its variability in these graphs, a variable range of the graph scale was used.

Table 1. *Pearson* correlations matrix of all variables/measured parameters (both dependent and independent). * significance of the *Pearson* correlation coefficient at the alpha level = 0.05, ** significance of the *Pearson* correlation coefficient at the alpha level = 0.01. Chl.b – chlorophyll *b*, Chl.a – chlorophyll *a*, aCar – α-carotene, bCar – β-carotene, LL – leaf length, LW – leaf width, LA – leaf area, PL – plate length, SPAD – index of relative chlorophyll content, SGLwk – weekly shoot growth in length, DTF – distance from shoot top to 1st open flower, SD1 – shoot diameter at the top 1 week earlier, SD2 – shoot diameter at the top 2 weeks earlier, NoL – number of fully grown leaves per plant, NoF – number of flowers per shoot, NoFS – number of fruits set on the shoot, NoFwk – number of fruits harvested per week, WFwk – mass of fruit harvested per week, LAI – leaf area index, MTA – mean tilt angle, DM – dry mass, Brix – total soluble solids.

	Lutein	Chl.b	Chl.a	aCar	bCar	LL	LW	LA	PL	SPAD	SGLwk	DTF	SD1	SD2	NoL	NoF	NoFS	NoFwk	WFwk	LAI	MTA	DM	Brix
Lutein	1	0.976**	0.980**	0.708**	0.967**	–0.386	–0.332	–0.383	–0.318	0.253	0.384	0.372	–0.567*	–0.289	0.614*	0.379	–0.653**	0.115	0.104	–0.556*	0.314	0.079	–0.227
Chl.b	0.976**	1	0.987**	0.659**	0.962**	–0.363	–0.323	–0.369	–0.276	0.272	0.329	0.357	–0.594*	–0.329	0.531*	0.281	–0.596*	0.055	0.043	–0.572*	0.390	0.158	–0.230
Chl.a	0.980**	0.987**	1	0.679**	0.968**	–0.382	–0.342	–0.388	–0.287	0.342	0.263	0.267	–0.569*	–0.304	0.512*	0.262	–0.594*	0.053	0.053	–0.569*	0.363	0.109	–0.213
aCar	0.708**	0.659**	0.679**	1	0.760**	–0.071	–0.048	–0.061	0.000	0.331	0.215	0.016	–0.437	0.030	0.739**	0.446	–0.807**	–0.250	–0.175	–0.563*	0.161	–0.109	–0.580*
bCar	0.967**	0.962**	0.968**	0.760**	1	–0.489	–0.448	–0.490	–0.403	0.430	0.254	0.212	–0.629**	–0.249	0.629**	0.313	–0.736**	–0.096	–0.058	–0.666**	0.382	0.024	–0.301
LL	–0.386	–0.363	–0.382	–0.071	–0.489	1	0.981**	0.995**	0.972**	–0.533*	0.093	0.084	0.462	0.187	–0.265	–0.004	0.444	0.233	0.131	0.501*	–0.303	0.241	–0.176
LW	–0.332	–0.323	–0.342	–0.048	–0.448	0.981**	1	0.991**	0.928**	–0.560*	0.191	0.134	0.503*	0.206	–0.178	0.102	0.399	0.329	0.207	0.532*	–0.369	0.169	–0.166
LA	–0.383	–0.369	–0.388	–0.061	–0.490	0.995**	0.991**	1	0.957**	–0.563*	0.137	0.091	0.502*	0.235	–0.206	0.075	0.412	0.261	0.150	0.530*	–0.364	0.169	–0.202
PL	–0.318	–0.276	–0.287	0.000	–0.403	0.972**	0.928**	0.957**	1	–0.427	–0.003	0.007	0.336	0.135	–0.261	–0.064	0.367	0.155	0.084	0.365	–0.201	0.259	–0.255
SPAD	0.253	0.272	0.342	0.331	0.430	–0.533*	–0.560*	–0.563*	–0.427	1	–0.622*	–0.595*	–0.545*	–0.194	–0.021	–0.391	–0.379	–0.513*	–0.286	–0.656**	0.432	–0.048	–0.086
SGLwk	0.384	0.329	0.263	0.215	0.254	0.093	0.191	0.137	–0.003	–0.622*	1	0.847**	0.161	0.017	0.588*	0.667**	–0.213	0.516*	0.390	0.217	–0.284	–0.148	–0.069
DTF	0.372	0.357	0.267	0.016	0.212	0.084	0.134	0.091	0.007	–0.595*	0.847**	1	–0.074	–0.289	0.288	0.360	–0.019	0.554*	0.505*	0.152	–0.120	0.176	0.132
SD1	–0.567*	–0.594*	–0.569*	–0.437	–0.629**	0.462	0.503*	0.502*	0.336	–0.545*	0.161	–0.074	1	0.615*	–0.240	0.224	0.609*	0.197	–0.015	0.936**	–0.686**	–0.309	0.025
SD2	–0.289	–0.329	–0.304	0.030	–0.249	0.187	0.206	0.235	0.135	–0.194	0.017	–0.289	0.615*	1	0.197	0.551*	0.028	–0.110	–0.135	0.479	–0.732**	–0.600*	–0.522*
NoL	0.614*	0.531*	0.512*	0.739**	0.629**	–0.265	–0.178	–0.206	–0.261	–0.021	0.588*	0.288	–0.240	0.197	1	0.824**	–0.876**	–0.037	–0.013	–0.353	–0.136	–0.494	–0.567*
NoF	0.379	0.281	0.262	0.446	0.313	–0.004	0.102	0.075	–0.064	–0.391	0.667**	0.360	0.224	0.551*	0.824**	1	–0.501*	0.227	0.142	0.164	–0.562*	–0.573*	–0.557*
NoFS	–0.653**	–0.596*	–0.594*	–0.807**	–0.736**	0.444	0.399	0.412	0.367	–0.379	–0.213	–0.019	0.609*	0.028	–0.876**	–0.501*	1	0.262	0.120	0.734**	–0.191	0.355	0.517*
NoFwk	0.115	0.055	0.053	–0.250	–0.096	0.233	0.329	0.261	0.155	–0.513*	0.516*	0.554*	0.197	–0.110	–0.037	0.227	0.262	1	0.890**	0.338	–0.315	0.083	0.425
WFwk	0.104	0.043	0.053	–0.175	–0.058	0.131	0.207	0.150	0.084	–0.286	0.390	0.505*	–0.015	–0.135	–0.013	0.142	0.120	0.890**	1	0.164	–0.373	–0.045	0.382
LAI	–0.556*	–0.572*	–0.569*	–0.563*	–0.666**	0.501*	0.532*	0.530*	0.365	–0.656**	0.217	0.152	0.936**	0.479	–0.353	0.164	0.734**	0.338	0.164	1	–0.721**	–0.179	0.137
MTA	0.314	0.390	0.363	0.161	0.382	–0.303	–0.369	–0.364	–0.201	0.432	–0.284	–0.120	–0.686**	–0.732**	–0.136	–0.562*	–0.191	–0.315	–0.373	–0.721**	1	0.647**	0.223
DM	0.079	0.158	0.109	–0.109	0.024	0.241	0.169	0.169	0.259	–0.048	–0.148	0.176	–0.309	–0.600*	–0.494	–0.573*	0.355	0.083	–0.045	–0.179	0.647**	1	0.383
Brix	–0.227	–0.230	–0.213	–0.580*	–0.301	–0.176	–0.166	–0.202	–0.255	–0.086	–0.069	0.132	0.025	–0.522*	–0.567*	–0.557*	0.517*	0.425	0.382	0.137	0.223	0.383	1

Leaf colour analysis: Throughout the experiment, phosphorus deficiency had a considerable impact on the colour aspects of the plants, specifically affecting the lightness (L) and chromatic components [a, b, chroma (C), and hue (H)]. However, this effect was not immediate; it was manifested with a delay and became noticeable only

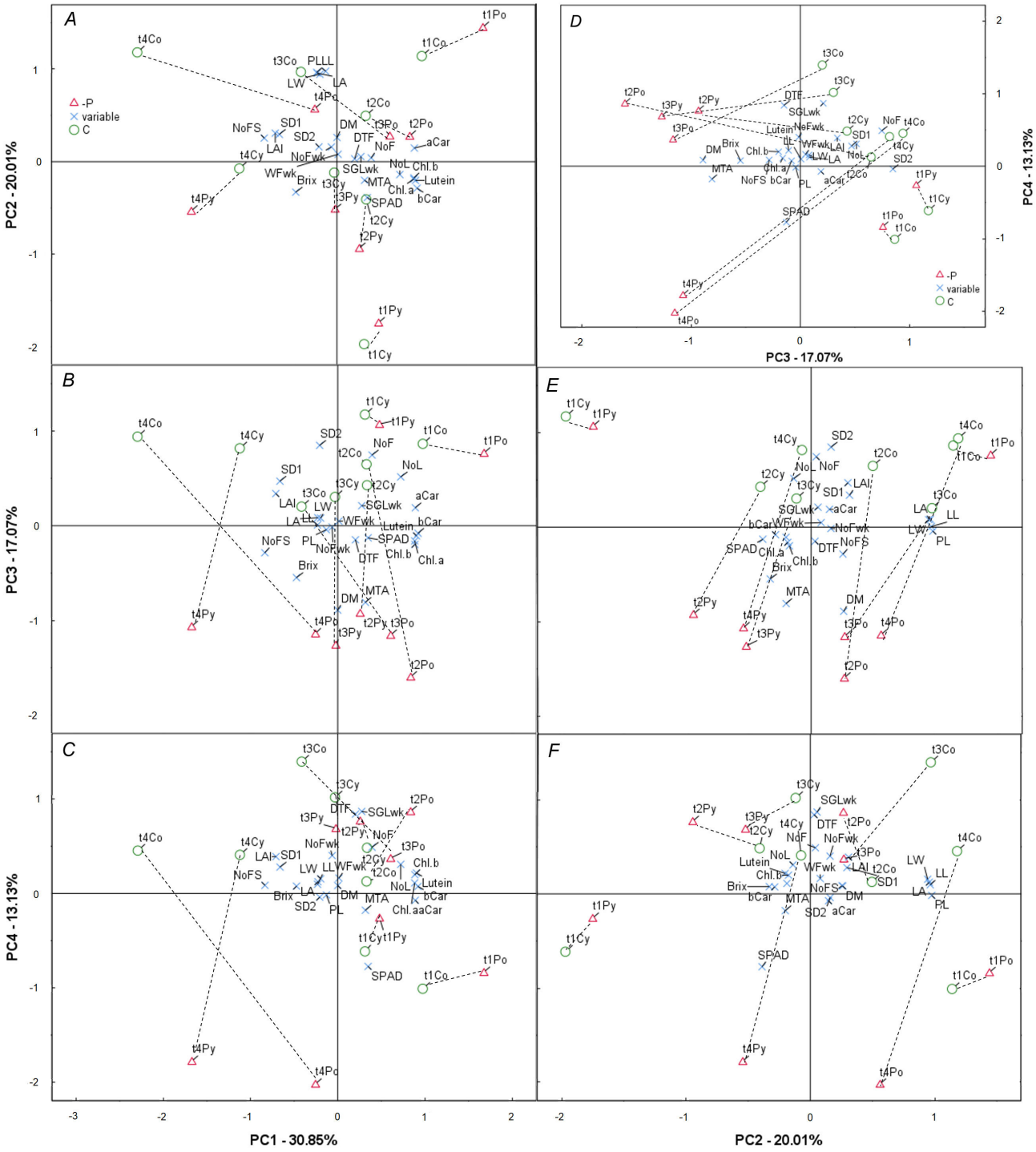

Fig. 3. A biplot illustrating the analyzed components in a two-dimensional space of two principal components. The principal components explain the variability in the dataset. *Green circles* represent the control experimental sites (C), *red triangles* represent the phosphorus-deficient experimental sites (–P), and *blue* '×' indicates the parameters studied. The experimental objects are denoted by the first two characters on the marker (*green circle* and *red triangle*), indicating the test date (t1, t2, t3, t4). The third character represents the test factor, either control (C) or phosphorus deficiency (P), and the fourth character indicates the part of the plant on which the measurement was conducted, namely young leaf (y) or older leaf (o). *Dashed lines* connect the control objects with deficiencies at the same dates and on the same leaf stages.

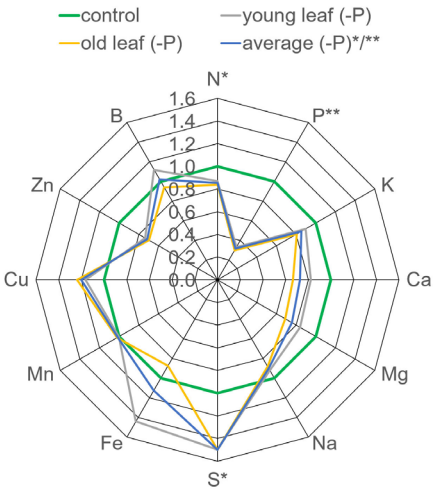

Fig. 4. Changes in the content of elements (N, P, K, Ca, Mg, Na, S, Fe, Mn, Cu, Zn, B) in cucumber leaves at time t4 (28th day of phosphorus deficiency), compared to the control, for both young and old leaves. Significant differences, based on *Student*'s *t*-tests are marked by * (p<0.05) or by ** (p<0.01).

after some time following the exposure of the plant to stress, as detailed in Fig. 5.

Phosphorus deficiency significantly impacted the colour lightness of both old and young leaves, becoming evident after 14 d, as shown in Fig. 5*A*. The effect on the greenness of the colour (component 'a') manifested differently between old and young leaves. In old leaves, changes in the 'a' component appeared after 28 d, whereas in young leaves, these changes were observed after 35 d, as detailed in Fig. 5*B*. Consequently, the 'a' component was deemed an ineffective indicator of phosphorus deficiency due to its delayed response to stress.

However, changes in the 'b' colour component of young leaves occurred slightly earlier (at term t2), leading to a less yellow colour compared to the young leaves of control plants, as indicated in Fig. 5*C*. This effect was only noticeable in old leaves from term t3. The C and H parameters facilitated the simultaneous observation of changes in the chromatic components of the leaves, including variations in hue and colour saturation. After 14 d, a notable decrease in colour saturation was observed in young leaves, followed by a similar effect in old leaves after 21 d, as referenced in Fig. 5*E*. Leaves of plants exposed to stress, regardless of their age, underwent a significant change in hue towards blues after 14 d, as depicted in Fig. 5*D*. Overall, phosphorus deficiency in cucumber plants led to the darkening of leaves and the loss of their natural greenness and yellowness, resulting in a less saturated colour with increased proportions of blues and purples (Fig. 5*F*).

CLSM investigation: Confocal laser scanning microscopy (CLSM) was used for optical sectioning along the z-axis, revealing brightly fluorescent magenta discs in cucumber chloroplasts, visible in both young and old leaves of control plants (*see* Fig. 6*A*,*G*). In P-deficient chloroplasts, particularly in old leaves, these magenta discs appeared blurred (Fig. 6*M*,*S*), corresponding to optical sections through the thylakoid grana network.

The autofluorescence signal of carotenoids (Cars) was more blurred in both young and old leaves of control plants compared to P-deficient leaves (*see* Fig. 6*B*,*H*,*N*,*T*). Control plants exhibited a dominant carotenoid signal (green) over chlorophyll (magenta) in chloroplasts of both young and mature leaves (compare Fig. 6*C*,*I* with Fig. 6*O*,*V*). Three-dimensional images showed well-distributed grana structures within chloroplasts of control plants (Fig. 6*D*,*J*), in contrast to the less frequent distribution under P deficiency (Fig. 6*P*,*V*). Notably, P deficiency led to weaker co-localization of Chl and Cars autofluorescence signals, evident by a less distinct white colour (Fig. 6*F*,*L*,*R*,*X*), especially prominent in old leaves of P-deficient plants (Fig. 6*H*).

Significant differences were noted in the Kautsky (OPSMT transients) curves between young and mature leaves of both control and P-deficient plants, as depicted in Fig. 2S (*supplement*). In the control leaves, fluorescence induction (FI) in both palisade and spongy mesophylls showed a distinct S-step, leading to a distinguishable P–S decay and S–M rise (*see* Fig. 2S*A*). The FI curve for the entire mesophyll in control leaves also presented a clear S-step. However, in other combinations, only the FI curve of the palisade mesophyll showed a clear S-step. Notably, for mature leaves of control plants, the FI curve in the palisade mesophyll did not display a distinct S-step, as can be compared in Fig. 2S*A* and 2S*B*.

P deficiency led to a different pattern in the FI curves (*see* Fig. 2S). This condition was marked by a more pronounced quenching of Chl fluorescence during P–T decay, as evidenced by comparing the localization of the T-step in Fig. 2S*A* and 2S*B* or 2S*C* and 2S*D*. In P-deficient old leaves, the decrease in fluorescence intensity was even lower compared to the original intensity, as indicated by the O-step and T-step values in Fig. 2S*B* and 2S*D*. P-deficiency had a more pronounced effect on the spongy mesophyll.

When comparing the palisade and spongy mesophylls, it was generally observed that the spongy mesophyll exhibited a shorter half-life period (HLP) of P–T decay, except for P-deficient old leaves, as detailed in Table 2. P deficiency resulted in a shorter HLP of P–T decay for the palisade, spongy, and entire mesophyll of young leaves. However, in old leaves, the opposite effect was observed, as outlined in Table 2.

Discussion

This comprehensive study highlights the multifaceted effects of phosphorus deficiency on plant physiology, particularly focusing on cucumber plants. The research underscores the importance of the plant's conductive system in perceiving environmental cues and transmitting signals that enable early detection of stressors such as phosphorus deficiency. The study leveraged various analytical techniques to understand these effects at different levels, from molecular to whole-plant physiology. Key findings of the study include:

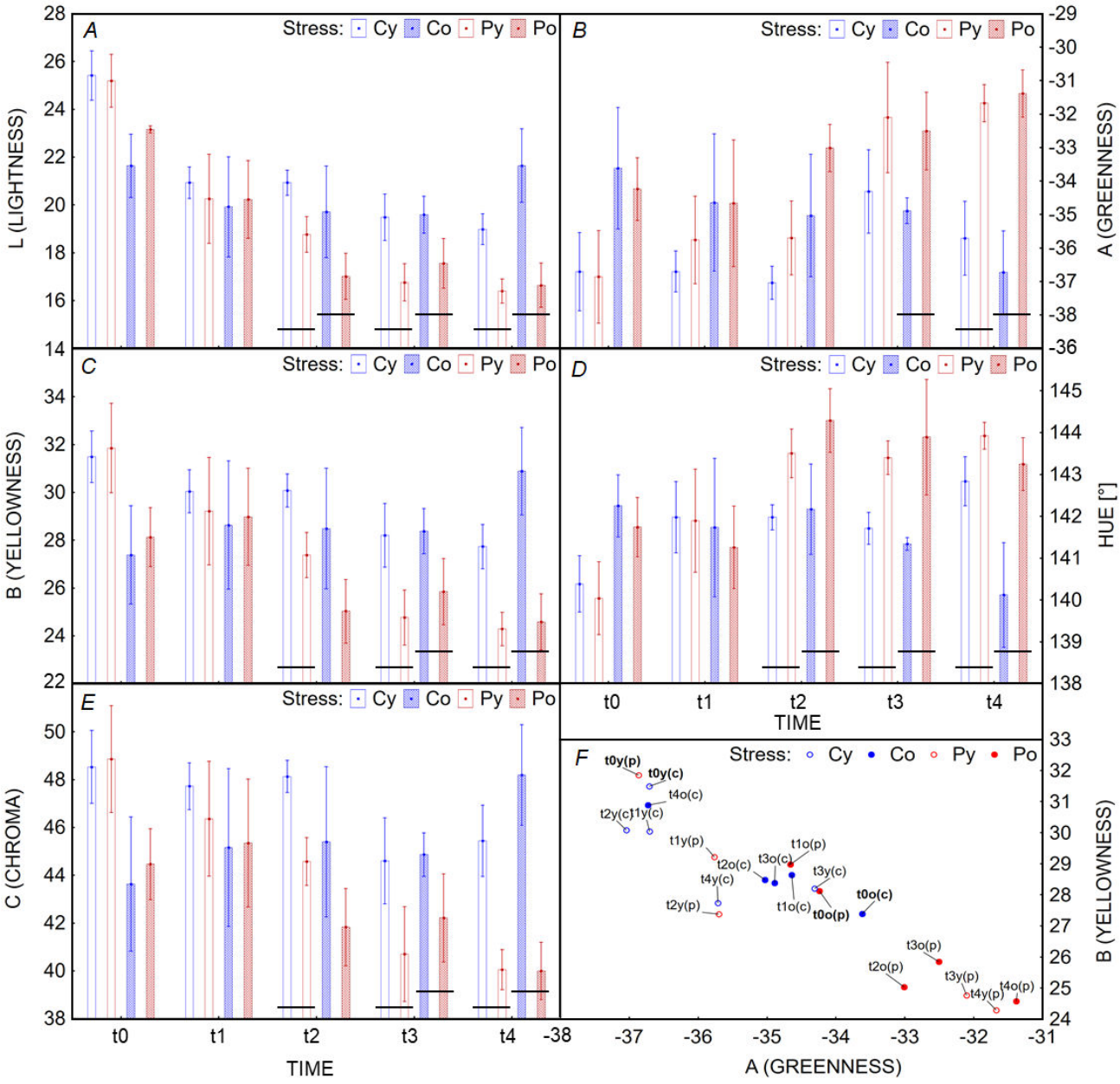

Fig. 5. Colour parameters of young and old leaves in control plants and plants subjected to phosphorus deficiency stress. C – control plant; P – plant subjected to phosphorus shortage; o – old leaf; y – young leaf; t0, t1, t2, t3, t4 – measurement time points. The subplots are as follows: (*A*) colour lightness, (*B*) colour greenness ('a' component), (*C*) colour yellowness ('b' component), (*D*) leaf hue, (*E*) colour saturation, (*F*) colour plane formed by components 'a' and 'b' in the xy-axis system. *Black horizontal lines* indicate significant difference between means.

(*1*) Early detection of phosphorus deficiency: Phosphorus deficiency led to rapid reductions in the leaf area index and most assessed parameters compared to control plants. However, some parameters, such as fruit number and mass, and mean tilt angle, increased, suggesting that early stages of phosphorus deficiency may not immediately impact crop size and quality.

(*2*) Effect on older and younger leaves: The deficiency primarily affected older leaves, causing reductions in leaf blade width, surface area, and petiole length, while younger leaves showed no significant size changes. Chemical analyses also revealed increases in lutein, chlorophyll *a* and *b*, α-β-carotene, and dry mass in deficient conditions.

(*3*) Multivariate analysis using PCA: The principal component analysis method identified interdependent groups of features and variables that responded early to phosphorus deficiency. This approach also revealed that colour analysis was not as effective for the early identification of phosphorus deficiency.

(*4*) Chlorophyll and carotenoid content variations: The study noted variations in chlorophyll and carotenoid content across different plant species under phosphorus

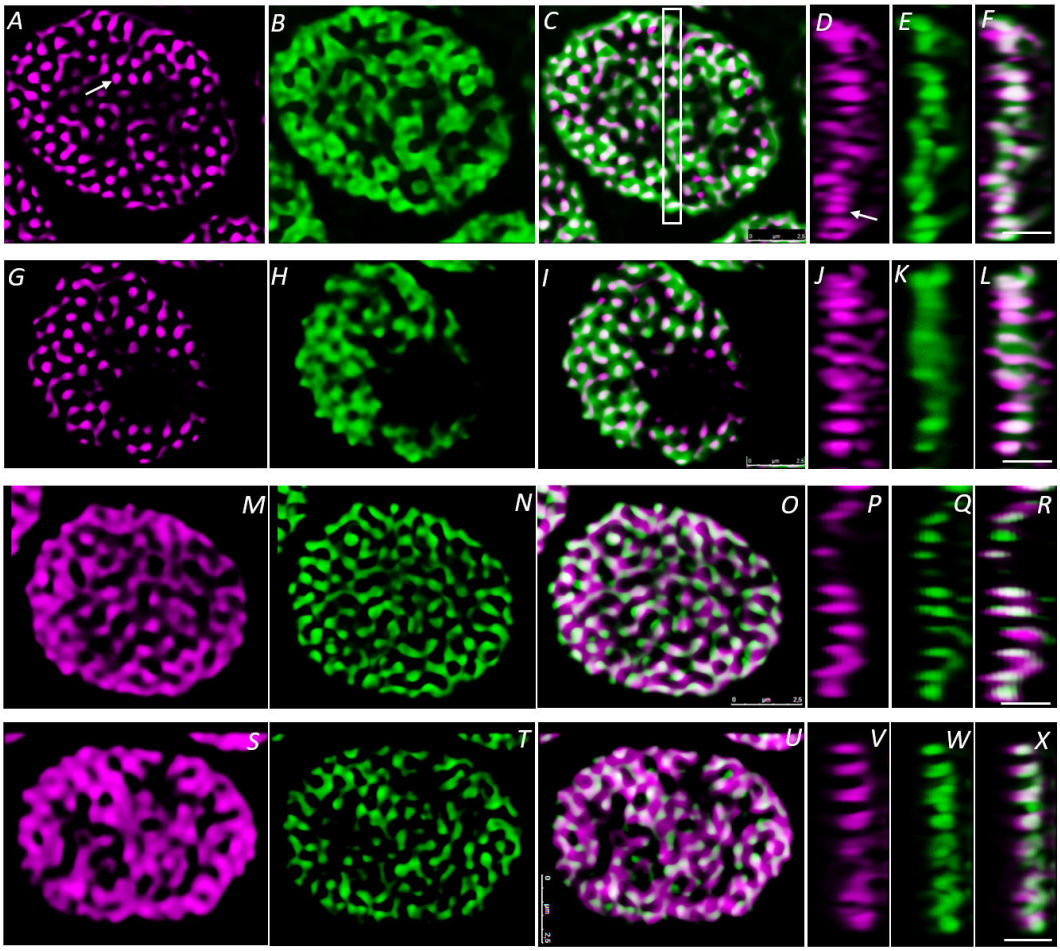

Fig. 6. The distribution of chlorophyll (Chl) and carotenoids (Cars) autofluorescence was examined in chloroplasts of both young and old leaves from control and P-deficient plants. The images presented in the left three columns depict 2D optical sections of chloroplasts in the upper view. The images in the right three columns display a 3D side view of the grana, reconstructed by optical sectioning of chloroplasts along their z-axis within the rectangular region of interest (highlighted in image *C*). *A–F* for control, young leaf; *G–L* for control, old leaf; *M–R* for P-deficiency, young leaf; *S–X* for P-deficiency, old leaf. The magenta channel represents Chl autofluorescence, the green channel represents Cars autofluorescence, and the overlay of the magenta and green channels creates a bright white color, indicating a strong correlation between the signals. *A rectangle* is used to indicate an arbitrary chosen region of interest, demonstrating the distribution of Chl and Cars autofluorescence in a side view. *A white arrow* points to a granum, and the scale bars represent 2 μm.

Table 2. Half-life period of P–T decay [s] in young and old leaves of control or P-deficient cucumber plants. The results presented for young leaves are mean values (n = 5) ± standard deviation. *Different letters* indicate statistically significant differences between values in rows (p<0.05, one-way *ANOVA* comparison test). For old leaves, the results are approximate mean values only because the chlorophyll fluorescence decay did not reach the steady-state T-step (refer to Fig. 2S). The term 'frame' refers to the combined values of palisade and spongy mesophylls.

	Young leaves		Old leaves	
	Control	P-deficiency	Control	P-deficiency
Palisade	22.5 ± 1.6^a	18.4 ± 1.3^b	>16.2	>18.4
Spongy	13.7 ± 1.0^a	5.1 ± 0.8^b	>7.9	>23.5
Frame	19.6 ± 1.2^a	17.4 ± 1.1^a	>14.7	>16.6

deficiency, with some showing increases and others decreases in these pigments.

(*5*) Chloroplast organization and function: Changes in the distribution of chlorophyll and carotenoid fluorescence signals within chloroplasts were observed. Phosphorus-deficient plants showed altered chloroplast organization, including a reduction in the number of grana structures.

(*6*) Differences in chlorophyll fluorescence induction: Significant differences were found in chlorophyll *a* fluorescence induction between the palisade and spongy

mesophylls of leaves. These differences hint at the distinct regulatory mechanisms of photosynthesis in these two types of mesophyll.

(*7*) Implications for phosphorus distribution in leaves: The study suggests that cucumber leaves may adapt to phosphorus deficiency by redistributing phosphorus from the spongy to the palisade mesophyll, with the latter potentially having a competitive advantage in utilizing available phosphorus.

The research contributes significantly to understanding how phosphorus deficiency affects various aspects of plant physiology and offers new insights into the mechanisms that plants employ to adapt to nutrient stress. This knowledge is crucial for developing strategies to enhance plant resilience and productivity in the face of environmental stressors.

Conclusions: In this research, a range of physiological and morphological features at various organizational levels, from the cellular to the canopy, were employed to understand the cascade of responses in cucumber plants to phosphorus deficiency. The primary objective was to pinpoint specific parameters that could facilitate the early detection of this stress. These features are divided into two main categories:

(*1*) Destructive measures: This includes assessing nutrient and pigment contents, fresh and dry mass of leaves and fruits, as well as confocal microscopy analysis.

(*2*) Nondestructive measures: These encompass measurements like relative chlorophyll content, leaf area index (LAI), and leaf colour.

The research emphasizes the importance of nondestructive measures, particularly those related to photosynthetic efficiency and plant productivity. These findings build upon previous work (Sieczko *et al.* 2022), highlighting the significance of focusing on older leaves due to the mobility of phosphorus to younger leaves.

To accurately select the most effective parameter(s) or sets of parameters for early detection of phosphorus deficiency, comprehensive statistical analyses play a crucial role. These analyses are vital for differentiating between various parameters and evaluating their impact on identifying the most suitable parameter(s) or parameter set. This approach is essential for the early detection of phosphorus deficiency, allowing for timely interventions to mitigate its effects on plant growth and productivity.

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