

Response of medical cannabis (*Cannabis sativa* L.) genotypes to P supply under long photoperiod: Functional phenotyping and the ionome

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ARTICLE INFO

Keywords:
Cannabis
Cannabinoids
Chemical profiling
Medical production
Nutrients
Phosphorus

ABSTRACT

Phosphorus (P) is an essential macronutrient required for many central metabolic processes, and is therefore a major factor governing plant development, structure and function. Cannabis is a short-day plant that its' development progression involves a vegetative growth phase under long photoperiod, followed by a reproductive phase under a short photoperiod. The reproductive inflorescence yield potential in cannabis is therefore largely dependent on the morphology and physiological condition of the plants at the vegetative phase. Due to legal restrictions, there is lack of science-based knowledge about cannabis plant science, including mineral nutrition. The present study therefore focused on P nutrition of plants at the vegetative growth phase under long photoperiod. The plants were cultivated in pots in a controlled environment and subjected to 5 levels of P (5, 15, 30, 60, 90 mg L⁻¹). We investigated impact on the ionome, physiological and morphological traits, uptake of nutrients into the plant, translocation to the shoot, and distribution in the plant organs for 2 medicinal cannabis genotypes. Plant biomass production, photosynthesis rate, stomatal conductance, transpiration rate and inter-cellular CO₂ at the vegetative growth phase exceeded under 30 mg L⁻¹ P supply. Uptake and translocation of nutrients from root to the shoot was highly influenced by the P treatment. Under excess P supply, most of the plant P accumulated in the roots, and translocation to the shoot was inhibited. Uptake of Mg into the plants, and its' translocation to the shoot was inhibited by P deficiency in both cultivars, and was enhanced by increased P supply. Calcium uptake was increased by P application but translocation to the shoot was inhibited. Zinc retention in roots under P deficiency was found in both varieties. Our results suggest a wide optimum range for P in medicinal cannabis at the vegetative growth stage, with a minimum requirement of 15 mg L⁻¹ P and a recommended application of 30 mg L⁻¹. The functional physiology and ionome profiling revealed genotypic variability in P sensitivity.

1. Introduction

For thousands of years humanity has utilized and cultivated the *Cannabis sativa* plant for its therapeutic and nutritional value (Caplan et al., 2017), psychoactive properties, as a source of fibers (Chandra et al., 2017) and for spiritual purposes (Small, 2018). Cannabis is an annual short-day plant originating in Central Asia. Under a long photoperiod, it undergoes vegetative growth that determines its size, architecture and the potential for reproductive growth under short photoperiod. The species contains hundreds of secondary metabolites that elicit pharmacological effects, including cannabinoids, terpenoids and flavonoids that are biosynthesized at high concentrations in the female flower (Flores-Sanches and Verpoorte, 2008; Caplan et al., 2017; Bernstein et al., 2019a). Though Western medicine has known the

plant's beneficial properties since the mid-19th century (Zuardi, 2006), there is a lack of science-based information regarding its physiology, chemical properties (Bernstein et al., 2019b) and horticultural management (Caplan et al., 2017). This is due to legal restrictions that prevented almost all scientific work since the 1950th (Small, 2018). Until recently, plant-science and agronomical knowledge was confined to underground communication among clandestine growers (Decorte and Potter, 2015). Some scientific evidence was obtained in research on hemp growth; however, since hemp was bred and grown for fiber and seed production and not for the chemical compounds in the flowers, the relevance of the accumulated information for the drug-type plant is limited (Caplan et al., 2017). Lately, owing to an increase in global demand, *C. sativa* cultivation has spread throughout the industrialized world, and there is an increasing need for credible scientific agricultural

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expertise of the crop (Decorte and Potter, 2015; Caplan et al., 2017).

In order to harness a plant's potential, genetic and environmental factors has to be considered, that together determine plant morphology (Gottlieb, 1984) and physiology (Sultan, 2000). Abiotic factors such as mineral nutrition have a considerable effect on plant development, synthesis of secondary metabolites (Gorelick and Bernstein, 2014) and yield production (Engels et al., 2012). For more than 2000 years farmers have been adding minerals to the soil to improve plant growth, and the benefits of fertilization are well documented (Kirkby, 2012). Phosphorus (P) is an essential macronutrient that is required for plant metabolism and growth. Along with its function as a structural element in phospholipids and nucleic acids, it plays a key role in carbohydrate translocation in-planta as well as in intracellular energy transfer. Due to its important role as an energy carrier, P takes part in many key biosynthetic pathways such as fatty acid synthesis, aerobic respiration, photosynthesis, and glycolysis (White and Hammond, 2008). Under P deficiency, leaves undergo premature senescence, shoot growth is inhibited, and the formation of reproductive organs and flower initiation is delayed. Those limiting factors can cause a decrease in reproductive development and yield under P deficiency (Hawkesford et al., 2012). In the medical cannabis industry flowers are the yield, and the lack of information pertaining to the response of cannabis to P nutrition at the vegetative and reproductive stages limit the development of optimal fertilization practices.

The little information available on mineral nutrition of medical cannabis is from forensic studies that aimed to identify illegal growers by tracing correlations between mineral contents in the plant and the soil it was grown in, as an effort to identify the origin of the illicit cultivation sites (Coffman and Gentner, 1977). Coffman and Gentner (1977) found that P in soil was negatively correlated with K, N, Ca, Mn and B concentrations in leaves and plant growth was positively correlated with tissue P levels; plants were taller, and their dry weight increased. The same study identified positive correlation between CBD and THC production and P accumulation. A recent study from our lab showed that the addition of P fertilization reduced plant height as well as inflorescence length by 23.5 % and increased the level of Ca in the flower along with Zn in all the studied tissues. P supplement did not alter cannabinoid concentration in the flowers but decreased their concentration in the inflorescence leaves (Bernstein et al., 2019b).

Studies with industrial hemp showed that hemp responses to P fertilization vary between growing conditions and cultivars. When the initial P concentration in the soil is relatively high, the addition of P has a limited effect (Vera et al., 2004, 2010; Aubin et al., 2015). Vera et al. (2004) found that plant height was impacted by P supply but had no effect on biomass and yield production. A long term field fertilization experiment established the optimal P concentration for fiber hemp to be 0.5–0.6 % P in plant tissues, whereas higher concentrations led to yield reduction (Iványi, 2005; Iványi and Izsáki, 2009). The optimum P application rate was reported to be 85–95 kg ha⁻¹ for high fiber yield in industrial hemp (Deng et al., 2019). Although work on hemp showed that P application had a limiting impact on the plant, the effects on medicinal cannabis may vary as they are bred for a different use and morphological development.

The present study focused on the interplay between P nutrition and morpho-physiology in 'drug-type' medical cannabis under a long photoperiod. The hypothesis guiding the workplan was that P nutrition elicit morpho-physiological changes in the plant, inducing developmental modifications that will be linked to changes in the tissue ionome. To test this hypothesis, we conducted a comparative physiological functional phenotyping of the response of two medical cannabis genotypes to P inputs ranging from 5 to 90 mg L⁻¹ P, as well as chemical profiling of the plant ionome. This P concentration range was selected with the aim to include concentrations from P deficiency to oversupply. Understanding the factors that influence cannabis morpho-development at the vegetative growth phase is essential for achieving optimal plant architecture at the initiation of the reproductive phase.

2. Materials and methods

2.1. Plant material and growing conditions

The plants were propagated from a single mother plant, by cuttings. Two commercial medicinal cannabis cultivars were studied 'Desert Queen' (DQ) and 'Royal Medic' (RM) (Teva Adir LTD, Israel). The selected cultivars represent a distinct chemotypes; DQ is a high THC variety, and RM is a 'balanced' variety that contains similar concentrations of THC and CBD. Coconut fiber plugs were used for propagation (Jiffy international AS, Kristiansand, Norway) and transplanted in 3 L plastic pots in perlite (Perlite 2 (1.2), Agrekal, Habonim Israel). The plants were grown in a controlled growing rooms under long photoperiod (18/6 h light/dark), 25 °C, and irradiated with Metal halide bulbs (400 μmol m⁻² s⁻¹; Solis Tek Inc, Carson, California; 25.9 mol m⁻² d⁻¹). Carbon dioxide concentration was ~400 ppm. Irrigation was supplied via 1 L h⁻¹ discharge-regulated drippers (Netafim, Tel-aviv, Israel); the volume of irrigation was set to allow 30 % of drainage. Following 7 days of adjustment to the cultivation setup, when the plants' height was 11–12 cm they were divided to 5 treatments and were cultivated until the termination of the experiment 30 days thereafter under increasing P treatments of 5, 15, 30, 60, 90 mg L⁻¹. The irrigation solution contained (in mM) 10.42 N-NO₃⁻, 2.07 N-NH₄⁺, 2.56 K⁺, 2.99 Ca²⁺, 1.44 Mg²⁺, 1.47 S-SO₄⁻², 0.06 Cl⁻, 0.021 Fe²⁺, 0.011 Mn²⁺, 0.009 B⁺³, 0.005 Zn²⁺, 0.0008 Cu²⁺, 0.0003 Mo⁺². Micronutrients were supplied chelated with EDTA (Barkoret, ICL, Haifa, Israel). The experiment was conducted in a randomized experimental design, with 5 replicated plants per treatment. Results of routine monitoring of P concentrations in the irrigation solutions confirmed that the concentrations followed the target concentrations for the various treatments, and were steady throughout the experiment. The measurements were conducted with 5 replications, following the experimental design.

2.2. Mineral analysis

The plants were harvested at the termination of the experiment for inorganic mineral analysis, rinsed in distilled water and separated into leaves, stems and roots. Dry biomass was determined after drying at 65 °C for 72 h. Two extraction methods were used for the analyses of the mineral concentrations in the plant. For Mg, Ca, Zn, Mn, Cu, and Fe analysis, the ground dry tissue was digested with HNO₃ (65 %) and HClO₄ (70 %). For the extraction of N, P and K, the dry tissue was digested with H₂SO₄ (98 %) and H₂O₂ (70 %). Ca, Mg, Fe, Zn, Mn and Cu were analyzed with an atomic absorption spectrophotometer (AAnalyst 400 AA Spectrometer, Perkin Elmer, USA). N and P were measured with an autoanalyzer (Lachat Instruments, WI, USA) and K was measured with a flame-photometer (410 Flame Photometer Range, Sherwood Scientific Limited, The Paddocks, UK). Concentrations in leachates and irrigation solutions were analyzed as is detailed above for the digestion and extraction solutions.

In order to estimate the effect of P on uptake into the plant, and root to shoot translocation, a bioaccumulation coefficient (BC) (L Kg⁻¹), and a translocation factor (TF) were calculated by Eqs. (1) and (2), respectively, following Liu et al. (2009).

$$BC = \frac{\text{Concentration of the mineral in the plant}}{\text{Concentration of the mineral in the solution}} \quad (1)$$

$$TF = \frac{\text{Concentration of the mineral in the shoot}}{\text{Concentration of the mineral in root}} \quad (2)$$

2.3. Physiological parameters

Sampling for physiological measures was conducted 26 days following the initiation of the fertigation treatments.

2.3.1. Determination of carotenoids and chlorophyll content

Five leaf discs, 6 mm in diameter from the youngest mature fan leaf on the plant main stem were placed in ethanol (80 % (v/v), 0.8 mL) and kept in -20°C until further analysis. For extraction of the pigments from the tissue, the tubes containing the samples were heated for 30 min at 95°C . After collection of the solution, 0.5 mL of 80 % (v/v) ethanol was added to the remaining tissue, and the tubes were heated again for 30 min. The combined extract was mixed by vortex; 0.4 mL was diluted in 5 mL (v/v) acetone. Absorbance was measured at 663, 664 and 740 nm by a UV Scanning spectrophotometer (Bernstein et al., 2010) (Genesys 10, Thermo scientific, Waltham, Massachusetts). The photosynthetic pigments Chlorophyll *a*, *b* and carotenoids were calculated following Lichtenthaler and Welburn (1983).

2.3.2. Plant structure and development

The number of nodes on the main stem, stem diameter and plant height, were measured every week throughout the experiment for 5 replicated plants per treatment. The diameter of the stem was measured 1 cm above the base of the plant with a digital caliper (YT-7201, Signet tools international co., LTD., Shengang District, Taiwan). Biomass of the vegetative shoot organs was measured by destructive sampling at the end of the experiment. Fresh weight of leaves, stem and roots was measured immediately following dissection from the plants, and dry biomass was measured following desiccation at 65°C for 72 h.

2.3.3. Photosynthesis and transpiration rate, intercellular CO_2 concentration, stomatal conductance, and intrinsic water use efficiency

Gas exchange parameters were analyzed on the youngest-mature leaf on the main stem (6400 XT, LI-COR, Lincoln, NE, USA), for 5 replicate plants per treatment. For the measurements, PPFD, temperature and carbon dioxide concentration were $500 \mu\text{m m}^{-2}\text{s}^{-1}$, 29°C and 400 ppm, respectively. Intrinsic Water use efficiency (Leaf water use efficiency, iWUE) was calculated as the ratio between the results obtained for net photosynthesis and stomatal conductance.

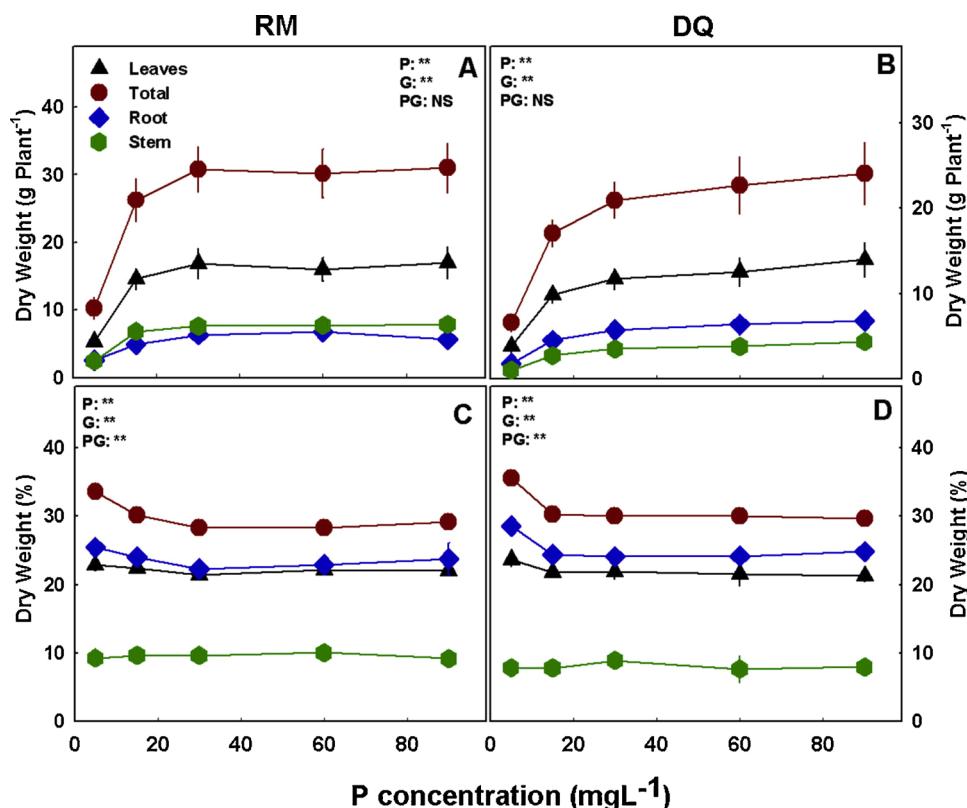


Fig. 1. Effect of P nutrition on shoot and root biomass in medical cannabis plants. Results of destructive measurements at the termination of the experiment. Fresh weights and dry weight percentage of leaves, stem, roots and total plant biomass of two medical cannabis cultivars, RM (A,C) and DQ (B,D). Presented data are averages \pm SE ($n = 5$). Results of two-way ANOVA indicated as $**P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test. In the ANOVA results P is potassium, G is genotype, and P*G represents the interaction between P and G.

3. Results

3.1. Plant growth and development

Biomass accumulation into leaves, stems and roots increased with the elevation of P supply up to the concentration of 30 mg L^{-1} P (Fig. 1A, B) and was unaffected by further increase. In both cultivars, the percentage of DW in the plant organs was highest under 5 mg L^{-1} P supply (Fig. 1C, D).

All measured morphological parameters, stem diameter, number of nodes on the main stem, plant height and the calculated elongation rate, had a similar response trend to the increase in P supply. DQ requires more P for optimal development than RM, and its development was restricted under both 5 mg L^{-1} and 15 mg L^{-1} P supply, while for RM restricted development was observed only under 5 mg L^{-1} P (Fig. 2). Plant height, number of nodes on the main stem and stem diameter were reduced under low P supply. Plant height did not differ statistically ($P > 0.01$) at the concentration range of $15\text{--}90 \text{ mg L}^{-1}$ P in RM, and $30\text{--}90$ in DQ. Stem diameter was reduced in both varieties only under 5 mg L^{-1} P supply, by 30 % in RM and 50 % in DQ compared to the 90 mg L^{-1} treatment.

3.2. Gas exchange and photosynthesis

In both varieties, photosynthesis rate, transpiration rate, stomatal conduction and internal CO_2 concentrations were lowest under restricted P supply, and increased with the increase in concentration up to 30 mg L^{-1} P (Fig. 3). Further increase in P reduced these gas-exchange parameters, except photosynthesis rate in RM that was not affected. Unlike DQ, RM plants didn't show a significant response to an increasing concentration from 5 mg L^{-1} to 15 mg L^{-1} in stomatal conductance, transpiration rate and intercellular CO_2 (Fig. 3B, D-E). Water Use Efficiency (WUEi) was highest under P deficiency and lowest at the 30 mg L^{-1} treatment in both cultivars, and was higher in DQ than in RM (Fig. 3C).

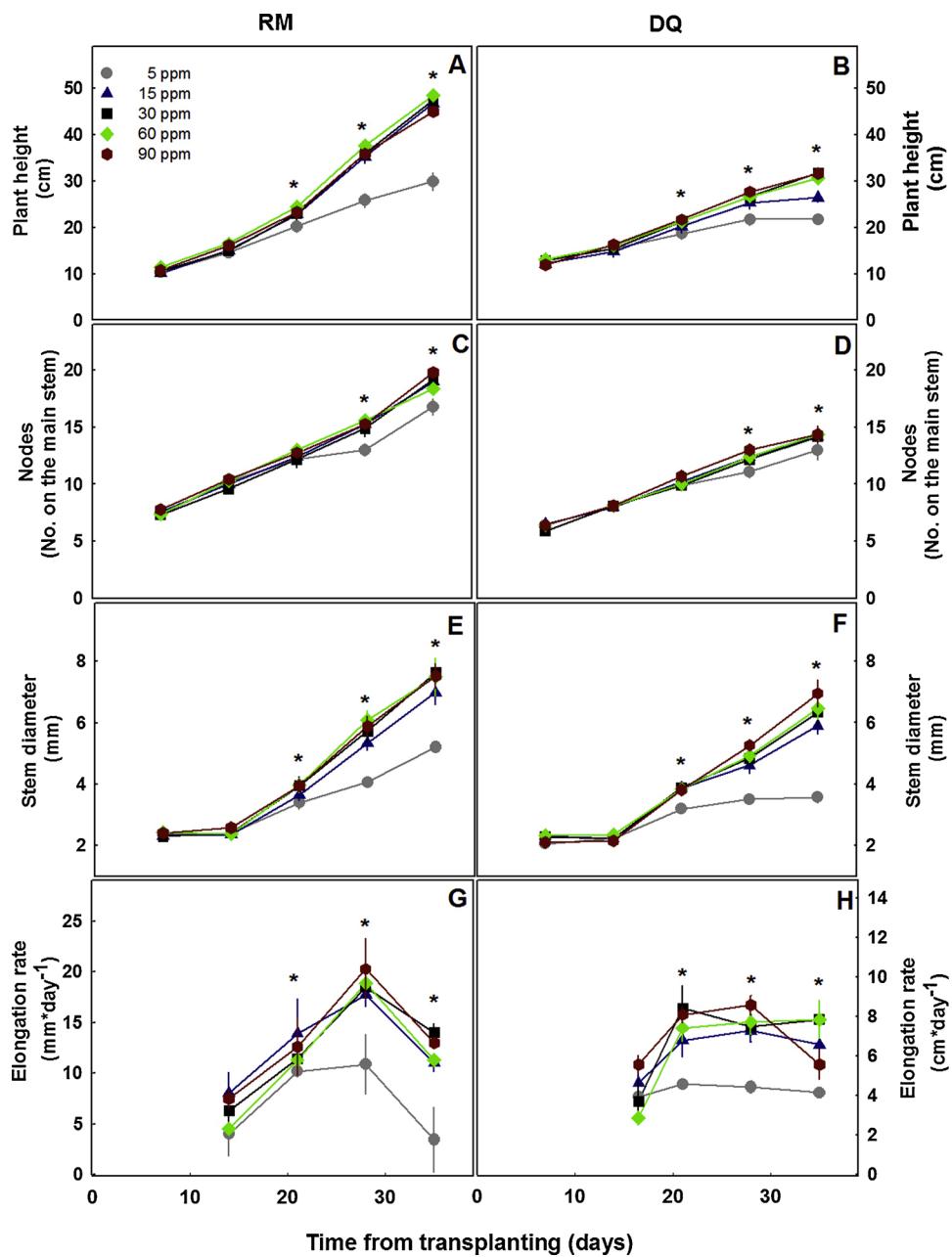


Fig. 2. Effect of P concentration on the development of the medical cannabis cultivars RM and DQ at the vegetative growth phase. Plant height (A, B), number of nodes on the main stem (C, D), stem diameter (E, F) and elongation rate (G, H). Data are means \pm SE ($n = 5$). Asterisk above the means represent significant differences between the P supply treatments for a given day by Tukey HSD test ($\alpha = 0.05$).

P supply affected the concentration of the photosynthetic pigments differently in the two varieties. In DQ, the concentrations were overall higher under low P supply compared to the highest supply of 90 mg L^{-1} . In RM, the response of chlorophyll *a* and *b* to the increase in P supply followed a maximum curve, with the lowest values under 5 and 90 mg L^{-1} supply (Fig. 4A-B), and the response of the carotenoid concentrations was similar to DQ, i.e., higher under 5 mg L^{-1} P compared to 90 mg L^{-1} supply (Fig. 4C).

3.3. Macronutrients concentration

The concentration of the macronutrients varied between the different organs, and were influenced by the level of P input. In both cultivars, P concentration in the leaves, stem and the root increased with the increase in concentration of P in the nutrient solution, and the order

of concentration was roots > leaves > stems (Figs. 5B, 6B). In both cultivars, the increase in root's P was up to the level of 60 mg L^{-1} P supply, and the stem presented a genotypic specificity, with an increase in tissue P up to the level of 90 and 30 mg L^{-1} P supply, in RM and DQ, respectively (Figs. 5B, 6B). Unlike P that showed preferential accumulation in the root (Figs. 5B, 6B), K, N, Ca, and Mg, presented high level of translocation to the shoot (Figs. 5 and 6). The order of N and Mg concentrations were leaves > roots > stems while Ca and K accumulated mainly in the stem and leaves (Figs. 5 and 6).

Potassium and N accumulation varied between the two cultivars. In DQ, P supply did not affect the concentrations of K and N in the leaves and the stems, while in RM the concentrations elevated with the increase in P input up to the supply level of 30 mg L^{-1} P. The effect on K and N concentration in the root was also genotype specific; in RM, a maximum trend was apparent for the response to P supply, with the highest value

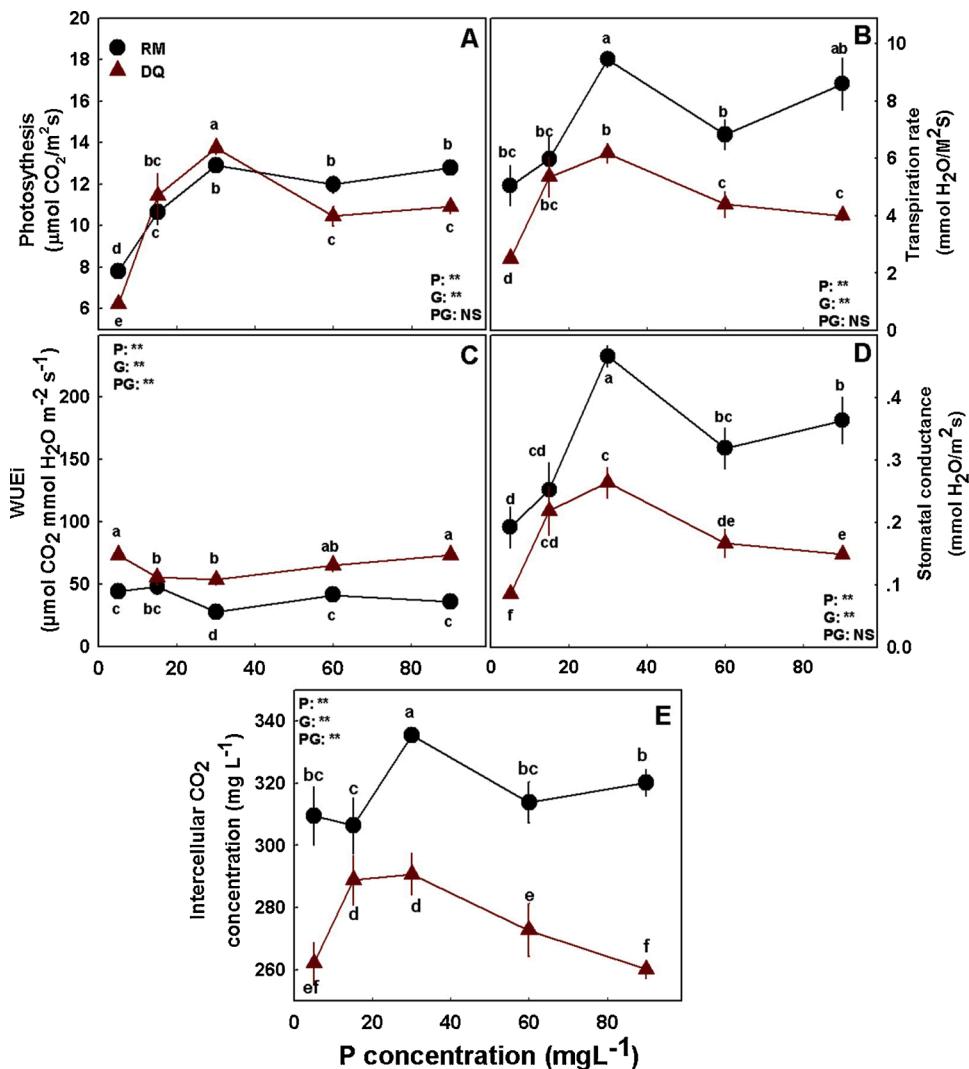


Fig. 3. Effect of P supply on gas exchange and physiological characteristics of cannabis leaves. Net photosynthesis rate (A), transpiration rate (B), intrinsic water use efficiency (WUEi) (C), stomatal conductance (D) intercellular CO₂ (E) for two medical cannabis cultivars, RM and DQ. Data are means \pm SE ($n = 5$). Results of two-way ANOVA presented as $^{**}P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test. In the ANOVA results P*G represents the interaction between P and genotype. Different letters above the averages represent significant differences according to Tukey HSD test at $\alpha = 0.05$.

under 15 mg L⁻¹ P supply, while in DQ the concentrations were highest under 60 and 5 mg L⁻¹ P supply (Figs. 5 and 6). Ca and Mg accumulated preferentially in leaves, compare to stems and roots, in both cultivars. In RM, Ca and Mg in the leaves exhibited a maximum trend with the highest concentration under 15–30 mg L⁻¹ P supply, and the same trend was apparent also for Ca in DQ. In both varieties, Ca concentration in the roots increased with the increase in P supply, while Mg concentration displayed a maximum trend (Figs. 5D-E, 6 D-E).

In order to estimate whether the influence of P on mineral accumulation in the plant tissues was governed by translocation, uptake or an interaction of the two processes, the bioaccumulation coefficient (BC) and the translocation factor (TF) were calculated (Figs. 7 and 8). Bio-accumulation and translocation of more nutrients were affected by P nutrition in RM then in DQ. With the increase in P supply, the uptake of N and K increased in RM, Ca and Mg uptake increased and P uptake decreased in both cultivars (Fig. 7). In both cultivars, the translocation of P and Ca decreased with the increase in P supply, Mg translocation increased and K translocation was unaffected, while N translocation increased only in RM (Fig. 8).

3.4. Micronutrients concentration

Interaction between P supply and micronutrient uptake is documented and has been researched for years. In this study, we identified differences in accumulation of micronutrients between plant organs, as

well as effects of P nutrition on micronutrient accumulation. All tested micronutrients (Mn, Fe, Zn, Cu) accumulated to highest concentration in the roots, and the order of concentrations in the plant organs was usually roots > leaves > stems, except for Zn and Mn in one of the cultivars (DQ) that had similar concentrations in the leaves and stem (Figs. 5 and 6).

In both cultivars, Zn concentration in the root was higher at the lowest P input (5 mg L⁻¹ P) compared with all other treatments, however concentrations in the stem and the leaves were not affected (in DQ) and only marginally affected (in RM) by P supply (Figs. 5G, 6 G). Unlike uptake into the plant, which wasn't affected by P supply (Fig. 7), root-to-shoot translocation of Zn was lower under low P and retention in roots was observed (Fig. 8).

Manganese concentration in the shoot organs had a similar respond trend to P supply for both cultivars, which differed from the root response. Leaves' Mn concentration presented a maximum curve, with highest accumulation at 15 and 30 mg L⁻¹ P in RM and DQ, respectively. In the stem, Mn concentration declined with P supply. In the roots, in RM the highest accumulation of Mn occurred under high P supply (60, 90 mg L⁻¹), while in DQ the highest Mn concentration was apparent under P scarcity, in the 5 mg L⁻¹ treatment (Figs. 5H, 6 H). P nutrition influenced Mn translocation only in RM and uptake was not affected in both cultivars (Figs. 7 and 8).

Fe concentration in the roots presented a minimum curve, with the lowest concentration under 60 and 15 mg L⁻¹ P supply in RM and DQ, respectively (Figs. 5F, 6 F). Leaves' and stems' concentrations were

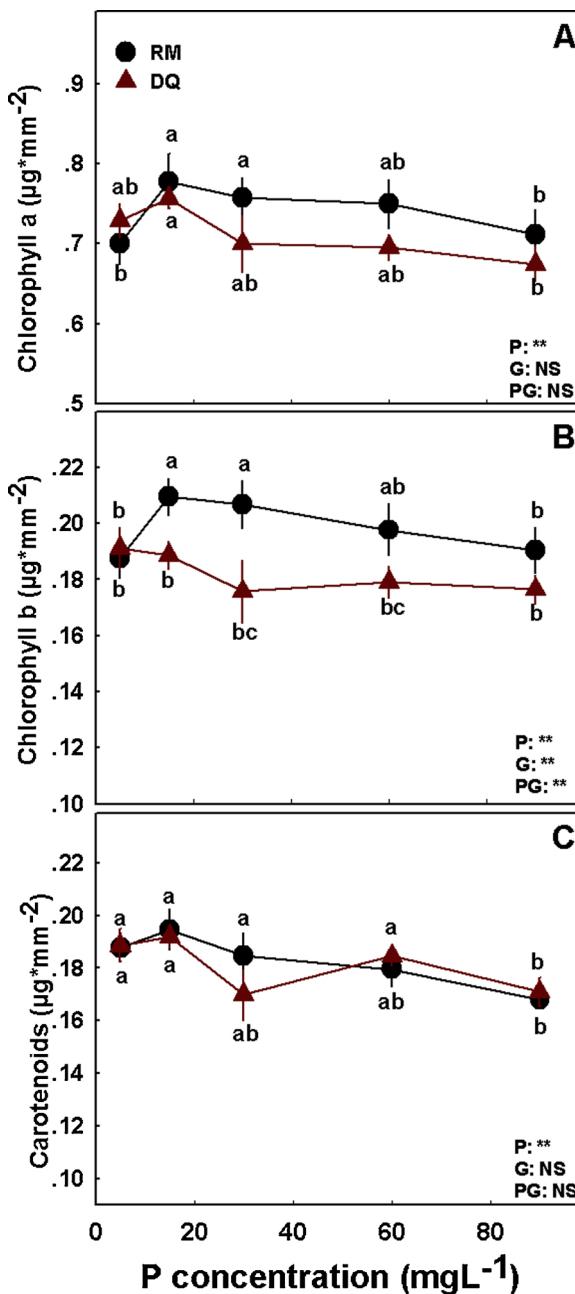


Fig. 4. Effect of P supply on photosynthetic pigment concentrations in medical cannabis cultivars. Chlorophyll a (A), chlorophyll b (B) and carotenoids (C). Data are means \pm SE ($n = 5$). Results of two-way ANOVA presented as ${}^{**}P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test. In the ANOVA results $P \times G$ represents the interaction between P concentrations and genotype. Different letters above the averages represent significant differences according to Tukey HSD test at $\alpha = 0.05$.

generally not affected by P input, except in RM leaves, that similar to the roots exhibited highest accumulation under low P supply. Fe uptake in RM was higher under P deficiency (Fig. 7). Cu concentration in leaves, stem and root averaged 0.006, 0.004, 0.011 mg/kg, respectively (data is not presented). Overall, bioaccumulation of the tested micronutrients, beside Fe in RM, was not affected by P supply (Fig. 7). Influence on translocation was only found in Zn in both cultivars and Mn in RM (Fig. 8).

3.5. Plant visual characteristics

The P treatments affected similarly the visual appearance of the plants from both cultivars (Fig. 9), reflecting the identified effects on morphological, chemical and physiological characteristics. In the 5 mg L^{-1} P treatment, deficiency symptoms were clearly apparent in both varieties (Fig. 9). Plants that grew under this P regime were smaller, shorter, with fewer and smaller leaves. Older leaves of this treatment were chlorotic and younger leaves had a pale green color. The leaves in general looked wilted, especially for DQ. 15–90 mg L^{-1} P treatments resulted in an absence of visible symptoms.

4. Discussion

The aim of the current study was to analyze functional-physiology response of medicinal cannabis plants to P nutrition. Phosphorus is required in relatively large amount in plants, but in the medicinal cannabis industry it is conventionally supplied at higher amounts than is customary for most other crops. This is due to the belief that cannabis plants require high concentrations of P for optimal function and increased yield. Mineral nutrition has a considerable impact on plant morpho-development. The effect may differ between the vegetative and reproductive stages of plant development and between genotypes (Clark, 1983). Development of optimal cultivation schemes require understanding of the plant functional response to mineral nutrients for their adjustment to the various developmental stages. The cultivars used in the study suffered from P deficiency under the lowest P application tested, i.e., 5 mg L^{-1} P, which affected plant physiological function, morphological development and biomass accumulation. The plants' development was not negatively affected by excess P supply. The only identified response to excess supply was in the gas exchange parameters, demonstrating reduced photosynthesis, transpiration and stomatal conductance under P excess.

4.1. Plant development and function

Biomass accumulation and plant morphological development were not significantly affected by concentrations higher than 30 mg L^{-1} ; response to lower concentrations varied between cultivars and tissues (Fig. 1). Maximum dry weight was reached under 15 and 30 mg L^{-1} P application in RM and DQ plants, respectively (Fig. 1A-B). Reduction in shoot biomass under P deficiency is well documented (Fredeen et al., 1989; Smith et al., 1990; Shane et al., 2004), and in accord with our results, sensitivity to P scarcity was reported before to vary between genotypes (Balemi and Schenk, 2009a,b).

Under P deficiency decrease in root and shoot biomass is common. Shoot growth is usually more inhibited compared to root growth, resulted in a decline in shoot/root ratio as a response to P starvation (Hawkesford et al., 2012). Our results demonstrate a decrease in root and shoot biomass with no effect on shoot/root ratio (Data is not shown) and in contrast to the response of the *C. sativa* genotypes, studies in legumes demonstrated an increase in root biomass percentage under P shortage (Fredeen et al., 1989; Smith et al., 1990) probably reflecting an adaptive trait to increase P uptake potential. The varied responses to P-deprivation can reflect diverse P requirements and/or adaptive responses of distinctive species and cultivars.

P supplement above the optimal requirement did not support a further increase in plant biomass. This result is similar to prior studies on hemp (Vera et al., 2010) and support our finding of a wide optimum range for P in medical cannabis. Indication of P toxicity was not found and is very rare in plants due to root down regulation of Pi transporters (Dong et al., 1999). In this study, plants that received the 5 mg L^{-1} P treatment were shorter with a smaller stem diameter and less nodes (Fig. 2A–F), and the plant morphology was similar for all treatments higher than 5 mg L^{-1} in both cultivars. This is in contrast to our previous study (Bernstein et al., 2019b) that showed a decrease in plant height as

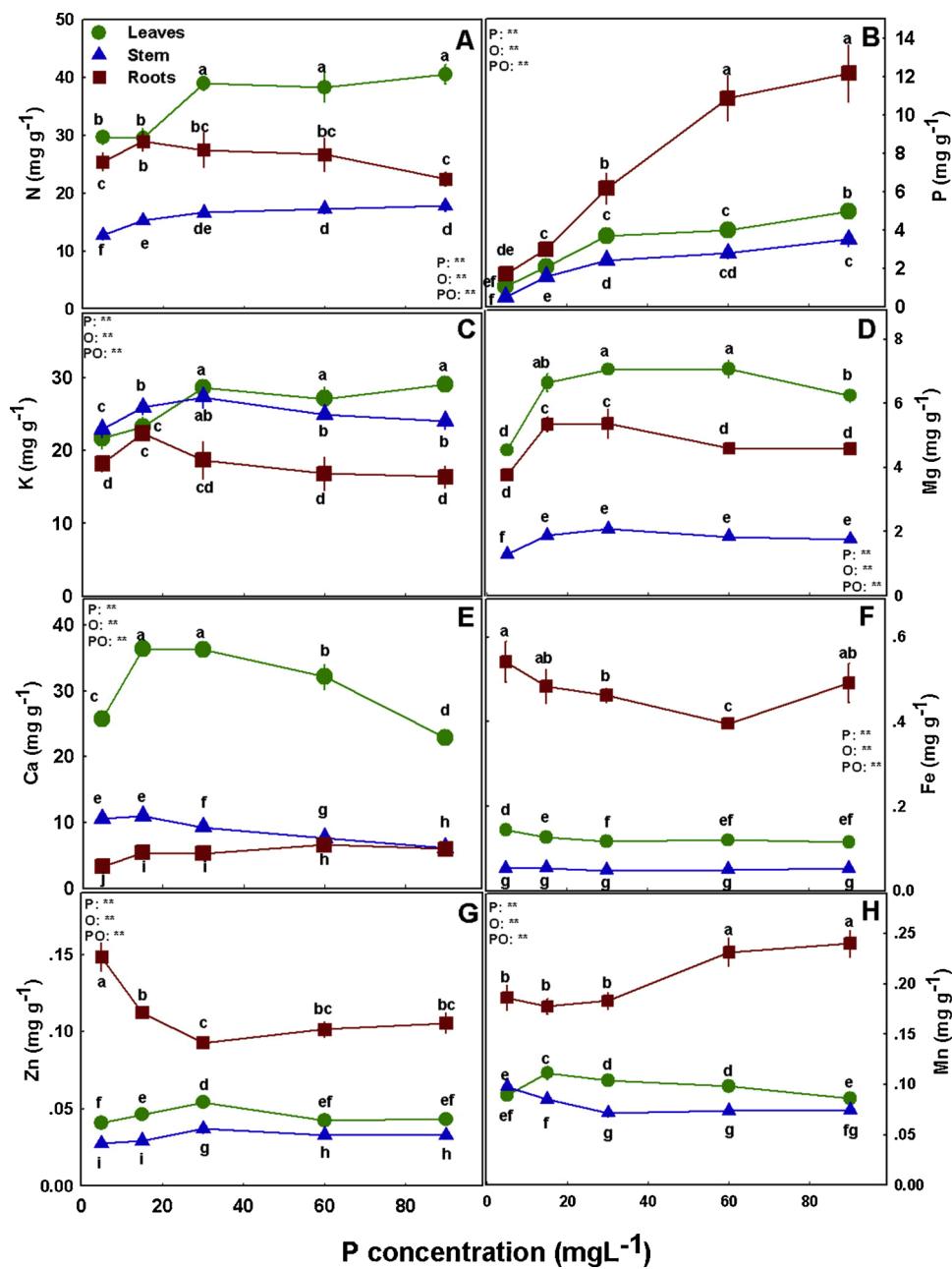


Fig. 5. Effect of P nutrition on nutrient concentration in stems, roots and leaves of the medical cannabis cultivar RM. N (A), P (B), K (C), Mg (D), Ca (E), Fe (F), Zn (G), Mn (H). Data are averages \pm SE ($n = 5$). Different letters above the means represent significant differences by Tukey HSD test ($\alpha = 0.05$). Results of two-way ANOVA presented as $^{**}P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test. In the ANOVA results P^*O signifies the interaction between P and plant organ. Different letters above the means represent significant differences by Tukey HSD test ($\alpha = 0.05$).

a result of P addition. The varied response may reflect differences in the basic composition of the growing media, P source, or genotypic differences.

Severe nutrient limitations can cause a visual response in the plants. Diagnosis of nutrients deficiency and toxicity is often initially based on visual observation of the plant, although it is a qualitative rather than a quantitative test. By the time visual symptoms of deficiency or toxicity appear, plant health and productivity may already be substantially compromised, and corrective actions may not be fully affective. Different species and cultivars may differ in response, and variability in sensitivity between individual plants complicates visual diagnostics (Wong, 2005; McCauley et al., 2011). Phosphorus is an essential plant element that is required in large quantities, therefore insufficient P levels are common. P toxicity symptoms are less common because of the ability of many plant species to regulate P uptake. Symptoms of P deficiency usually include reduction of leaf expansion and number of leaves (Hawkesford et al., 2012). The two cannabis cultivars analyzed in

the current study responded similarly to P scarcity, and the imposed morphological constraints were in accord with known responses for other crops. Specifically reduced, leaf area, shoot growth and leaf formation were induced at an early stage of the deficiency (Fig. 9).

Physiological traits demonstrated as well sensitivity to P nutrition in medical cannabis. Gas exchange parameters in the cannabis leaves were significantly influenced by P nutrition. Photosynthesis rate, stomatal conductance, transpiration rate and intercellular CO₂ levels increased with P inputs up to a maximum level under 30 mg L⁻¹ supply in both cultivars (Fig. 3), demonstrating a P-deficiency induced growth restriction under supply of 5 mg L⁻¹ P (Fig. 1A-B). A similar increase in gas exchange, by P supplementation was reported for spinach (Brooks, 1986), soybean (Fredeen et al., 1989; Taliman et al., 2019), potato (Fleisher et al., 2012), wheat (Arshad et al., 2016), cotton (Wang et al., 2018) and cowpea (Vanies et al., 2018). Unlike studies that showed amplified photosynthesis and stomatal conductance with unchanged intercellular CO₂ concentration as a response to P addition (brooks,

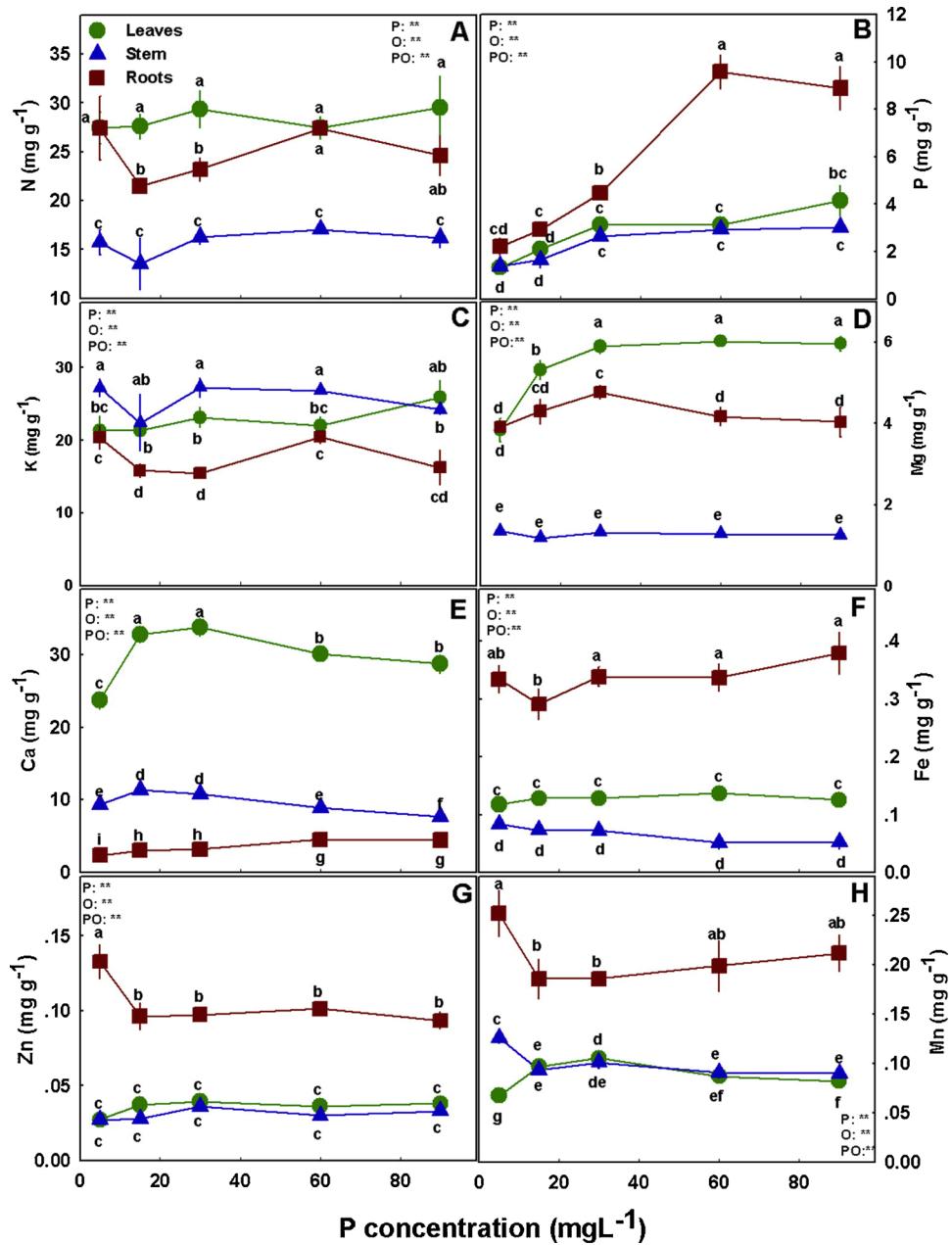


Fig. 6. Effect of P supply on nutrient concentration in stems, roots and leaves of the medical cannabis cultivar DQ. N (A), P (B), K (C), Mg (D), Ca (E), Fe(F), Zn (G), Mn (H). Data are means \pm SE ($n = 5$). Different letters above the means represent significant differences by Tukey HSD test ($\alpha = 0.05$). Results of two-way ANOVA presented as $^{**}P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test. In the ANOVA results P^*O represents the interaction between P and plant organ. Different letters above the means represent significant differences by Tukey HSD test ($\alpha = 0.05$).

1986; Wong, 2005; Neocleous and Savvas, 2019), in the current study intercellular CO₂ increased, in accord with the stomatal conductance response curve. Brooks (1986, 1988) suggested that P shortage affects photosynthesis by a stomatal and nonstomatal mechanisms. Nonstomatal limitation was explained by an influence of P supply on the level of RuBisCo carboxylase activation and decreased regeneration of ribulose 1,5-bisphosphate, as a product of a regulatory role over enzymes in the Calvin cycle. In the current study, stomatal limitation was possibly a major influence on photosynthesis rate because of the effect of the P treatment on the intercellular CO₂ rates. A nonstomatal influence could be involved and requires further study. Leaf photosynthesis, stomatal conductance, intercellular CO₂ and transpiration rates declined with P addition above 30 mg L⁻¹ in both cultivars. Decrease in gas exchange parameters as a result of P toxicity has been observed before by Shane et al. (2004). Although gas exchange parameters decreased with the increase in P concentration, no effect has been observed on the plant's dry weight, however, higher P concentration or a longer growing period might achieve stronger development inhibition.

The concentrations of the photosynthetic pigments, chlorophyll *a* and *b*, decreased under P deficiency in RM (Fig. 4A-B). Similar results were found in barley (Frydenvang et al., 2015) corn, (Soltangheisi et al., 2013) and soybeans (Lauer et al., 1989). However, corresponding to the results we obtained for DQ, chlorophyll concentration is known to be unaffected or rise under P limitation (Tombesi et al., 1969; Terry and Ulrich, 1973; Brooks, 1986, 1988; Rao and Terry, 1989; Neocleous and Savvas, 2019). Our results reflect that the effect of P on chlorophyll concentration can vary not just between species, but also between cultivars. The reduction in chlorophyll concentration can be linked to the reduction of N in leaves in the low P treatments as an indirect effect of P on chlorophyll production (Kerwin et al., 2017; Saloner et al., 2019). It was suggested that the inhibition of photosynthesis under low P results from effects on the photosynthesis reaction site rather than on stomatal conductivity or chlorophyll content (Neocleous and Savvas, 2019). The results of the current study suggest that the altered photosynthesis rate is related to chlorophyll content in the leaves and stomatal conductivity.

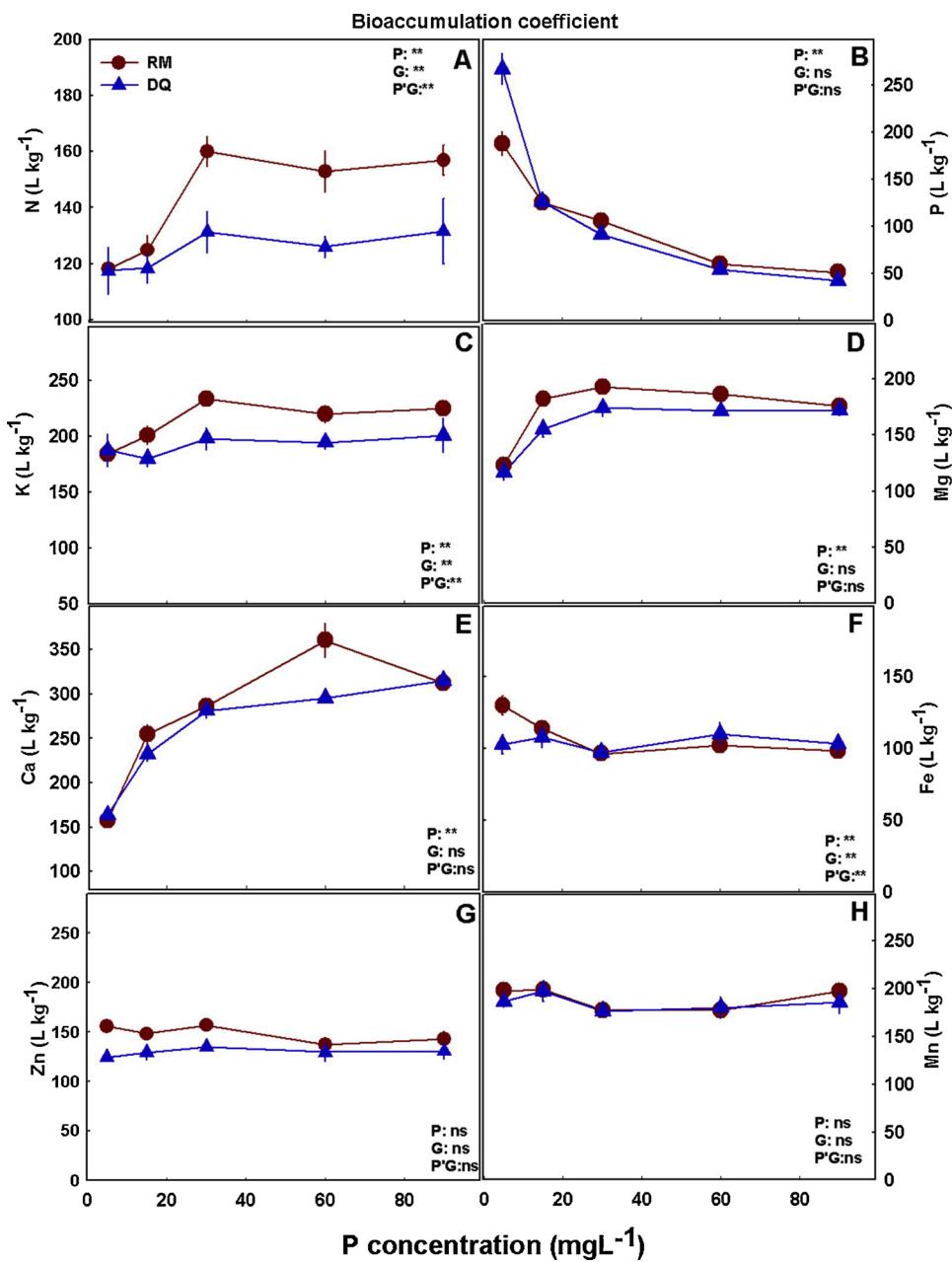


Fig. 7. Uptake of macro- and micronutrients into the plants of the medical cannabis cultivars, Royal Medic and Desert Queen. Data are the calculated bioaccumulation coefficient (BC). Results of two-way ANOVA presented as ** $P < 0.05$, F-test; ns, not significant $P > 0.05$, F-test; ($n = 5$). In the ANOVA results P'G signifies the interaction between P and genotype.

4.2. Interplay between P nutrition and the cannabis plant ionome

The increase in the plant tissue's P concentration, reflects a stimulation of P uptake upon exposure of the plants to an increase in P concentration in the nutrient solution. Most of the plant P accumulated in the roots, and only a small portion was translocated to the shoot. The small increase in P concentration in the leaves and stem of RM plants and the lack of change in DQ, demonstrate a defense mechanism against P toxicity in the shoot (Hawkesford et al., 2012), preventing shoot cellular exposure to damaging concentrations. Compartmentation of P under excessive P supply was found to be a key mechanism for preventing accumulation of toxic levels of cyt-Pi. Under adequate P nutrition, around 70–95 % of the intercellular Pi is stored in the vacuole. Thus, transporter regulation under varying P conditions enables maintaining the Pi cellular homeostasis (Liu et al., 2016).

Beside N, P and Ca, concentrations of all other nutrients in leaves at the optimal P range were similar to the range described as satisfactory for plant growth in hemp. N and P concentrations were lower than the recommended levels for hemp, and Ca concentration was slightly higher (Iványi and Izsáki, 2009). The similarity between the sufficient leaf concentrations in hemp and in medical cannabis is not unexpected because they are genetically similar. Though, it is surprising because of the different growth morphology, vigor and uses they were bred for. The reason for the deviation of the concentrations of N, P and Ca from the documented satisfactory concentrations for hemp, may result also from difference in the tissue selected for analysis. In the hemp experiment the leaf analyzed was the youngest mature leaf on the plant. Due to the restricted *in planta* mobility of Ca in the phloem, and the high translocation of N and P in both the xylem and the phloem (Iwahashi et al., 2012; White, 2012b), lower concentrations of Ca and higher

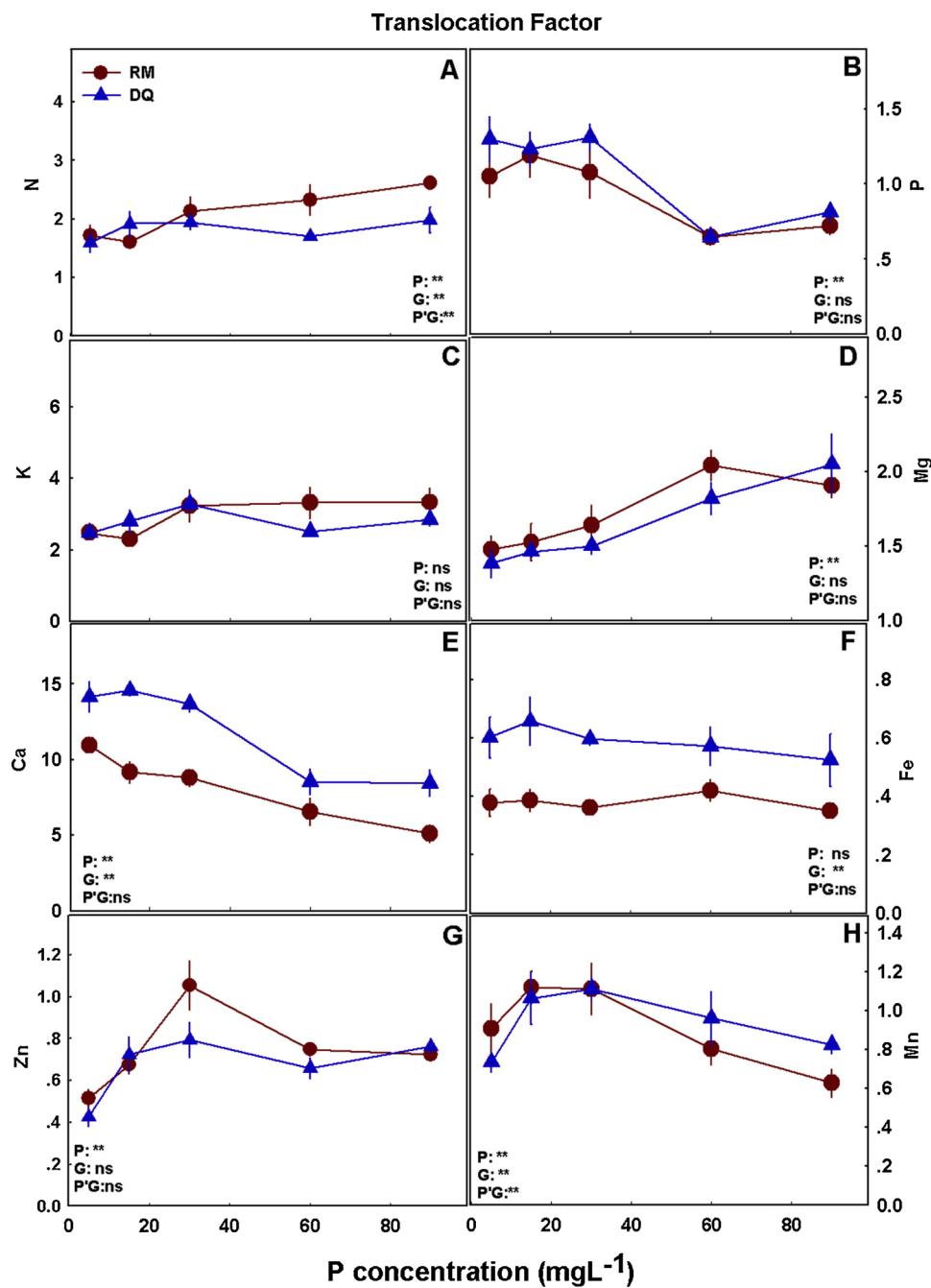


Fig. 8. Root-to-shoot translocation of macro- and micronutrients in the plants of the medical cannabis cultivars, Royal Medic and Desert Queen. Data are the calculated translocation factors (TF). Results of two-way ANOVA presented as ** $P < 0.05$, F-test; ns, not significant $P > 0.05$, F-test; ($n = 5$). In the ANOVA results P*G signifies the interaction between P and genotype.

concentrations of N and P then the shoot leaf average that was analyzed in the current study is indeed expected.

Phosphorus nutrition had a considerable effect on Mg uptake into the plants, and translocation to the shoot (Figs. 7 and 8). Mg uptake was lower under P deficiency in both cultivars as is evident by the lower Bioaccumulation factor. The increase in the Translocation Factor with the increase in P supply establish that Mg translocation to the shoot was also compromised under low P and was enhanced by P supplement. This is demonstrated in both cultivars by the increase in Mg concentration in leaves and roots up to 30 mg L^{-1} P and the concomitant decrease in root-Mg only above this concentration (Figs. 5 and 6). In RM, stem-Mg increased up to $15\text{--}30 \text{ mg L}^{-1}$ P without a decline above 30 mg L^{-1} P. DQ stem-Mg wasn't affected by P, despite the change in the leaves and

the root. The response curve of Ca concentration in leaves and stem to P supply presented a similar trend to Mg, with a significant increase from 5 to 15 mg L^{-1} P supply but a decrease in both cultivar with P addition above 30 mg L^{-1} . Unlike the maximum response trend in the shoot tissues, root Ca concentration increased with the increase in P supply throughout the P concentration tested. This demonstrates restriction of root-to-shoot transport of Ca under high P supply, which results in root-localization as can be seen by the decrease in the Translocation Factor (Fig. 8). Influence of P on Mg and Ca uptake and translocation, and Ca and Mg deficiency under low P availability is documented for several plant species (Reneau et al., 1983; Skinner and Matthews, 1990; Reinbott and Blevins, 1991, 1994). Reduced competition with K was suggested as a mechanism for P influence on Mg and Ca translocation

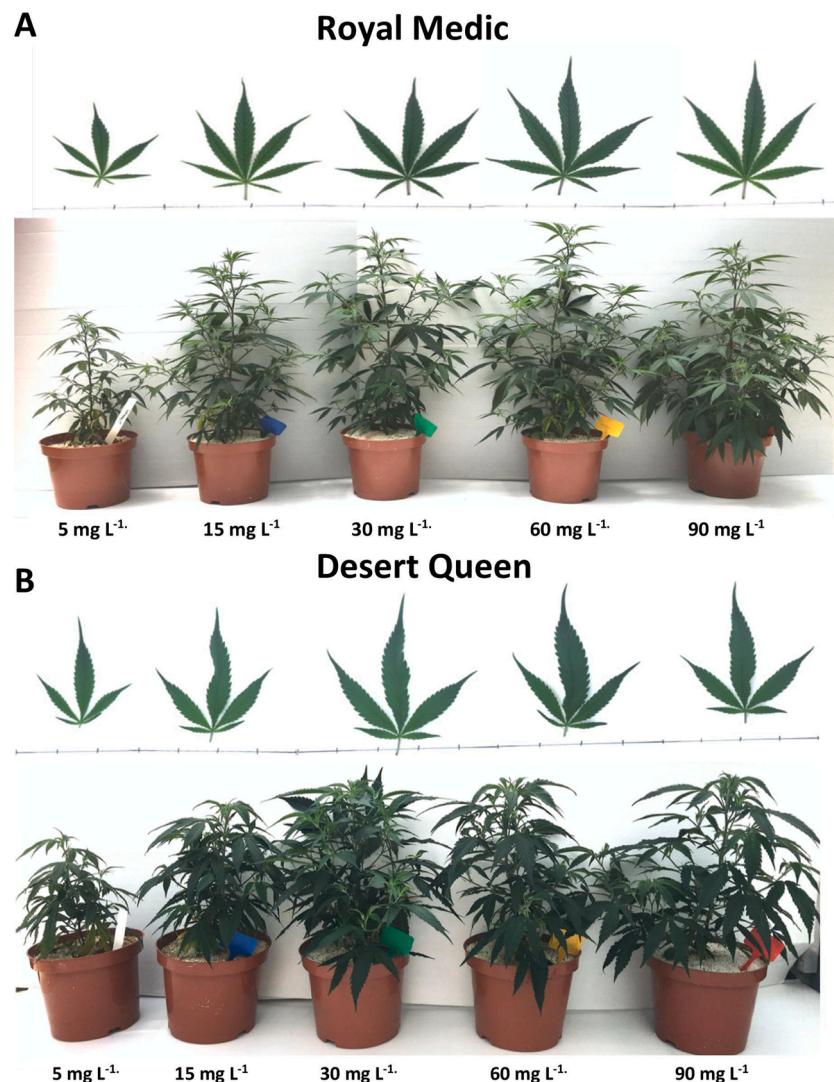


Fig. 9. Visual appearance of leaves and plants of the medical cannabis cultivars Royal Medic (A), and Desert Queen (B) of plants receiving increasing P supply, at the vegetative growth stage. From left to right: 5, 15, 30, 60 and 90 mg L⁻¹ P. Images of the youngest fully developed leaf on the main stem (top) and the whole plant (bottom), taken 29 days after the initiation of the fertigation regime.

(Reneau et al., 1983). Our results show that such indirect P influence on Ca and Mg through K is unlikely in the current study, due to the heterogeneous effect of the P treatments on K concentration in the plant organs between the varieties, unlike the results for Ca and Mg that presented similar trends for both cultivars. Because of the increase in K uptake into the plant in RM with the increase in P supply (Fig. 7), an enhanced Mg application as a substitute for K for maintaining cation-anion balance is also excluded. Lower pH in the rhizosphere of P deficient plants can limit cation uptake because of H⁺ binding to the cation exchange sites and a resulting decrease in the effective cation exchange capacity (White, 2012a). Thus, root acidification may involve in the reduced Ca, Mg and K concentration. The leachate solution from the pots was sampled regularly and the pH was steady over time, at the range of 6.0–6.5, with no significant differences between treatments. However, the spatial gradient of acidification in the root rhizosphere can change dramatically within mm from the root surface and the reduced cation uptake may be caused by local acidification due to low P supply (Nye, 1981; Hinsinger et al., 2003).

In the current study, we identified that uptake of K and N into the plants (Fig. 7) and their concentrations in the plant (Figs. 5 and 6) varied between the cannabis varieties studied, demonstrating a genetic variability. RM's 5 and 15 mg L⁻¹ P treatments had lower N concentration in

the leaves than the identified concentration under optimal N-nutrition by a previous study from our lab on medicinal cannabis (Saloner and Bernstein, 2020). The decrease in N concentration under low P supply in the medical cannabis plants, likely contributed to the growth restriction under P-deficiency (i.e., reduced plant height, and restricted biomass accumulation: Fig. 1) (Hawkesford et al., 2012; Aubin et al., 2015). Numerous previous studies documented genotypic variability in uptake and distribution of mineral elements in plants. Differences in N accumulation and distribution between genotypes were found in wheat, sorghum and maize (Clárk, 1983). Potassium uptake and allocation, and K use efficiency varied between varieties in barley, bean, soybean and tomato (Clárk, 1983). Unlike the bioaccumulation factor, the translocation factor value for K in RM was not affected by the P treatments, implying that the P treatments effected K uptake into the plants in RM, but not root-shoot translocation (Figs. 7 and 8). We believe that the observed chlorosis of older leaves under P deficiency can be an indirect response to low N in the shoot. The data presented here demonstrate an interplay between P and other macronutrients uptake into the plant, *in-planta* translocation and accumulation, and variability between cultivars.

As we have demonstrated here for P (Fig. 5B), and previously also for K (Saloner et al., 2019) and N (Saloner and Bernstein, 2020) uptake of

nutrients into cannabis plants is affected by their concentration in the root medium. It should therefore be considered that tissue concentrations of other nutrients in cannabis could diverge from our reported values under different supply rates.

Micronutrients have a tremendous effect on plant growth and physiology (Broadley et al., 2012). Thus, understanding the effect of P on microelements' nutrition can help understand the plant response to P supply even when symptoms of deficiency or toxicity aren't visibly apparent. Our results revealed significant effects of P supply on micro-nutrient concentrations in the plant organs of both medical cannabis genotypes.

Zn root's concentration increased under P deficiency in both cultivars but decreased in RM's leaves and stem. Supply of P above the threshold optimum concentration for plant development (30 mg L^{-1} P; Figs. 5 and 6) didn't alter Zn concentration in the root but decreased Zn concentration in RM's stems and leaves. P interaction with other microelement, and particularly with Zn, was reported for numerous plants (Safaya, 1976; Haldar and Mandal, 1981; Parker et al., 1992; Khan et al., 2014; Prasad et al., 2016) and commonly referred to as "P induced Zn deficiency" (Loneragan et al., 1982; Cakmak and Marschner, 1986).

Plants developed homeostasis mechanisms for Zn and P. Over-accumulation of P in a response to Zn starvation was found to result from a specific induction of the activity and expression of P transporter in *A. thaliana* (Gupta et al., 2016) and barley (Huang et al., 2000). Phosphorus deficiency was found to repress genes induced by Zn deficiency in *A. thaliana* (Van De Mortel et al., 2006; Khan et al., 2014). The antagonistic effect of P on Zn is mostly believed to occur within the plant in the form of dilution effect due to P promotion of leaf area and regulation of translocation from root to shoot (Youngdahl et al., 1977; Soltangheisi et al., 2013). Our results partially match previous reports for P-Zn negative interaction (Loneragan et al., 1982; Cakmak and Marschner, 1986; Khan et al., 2014; Prasad et al., 2016) and demonstrate a decrease in Zn concentration with increased P supply only in roots. However, in the shoot organs, a maximum curve is apparent and the negative effect of P on Zn concentrations is visible only above 30 mg L^{-1} P in RM. The retention of Zn in roots under P deficiency was also found by Loneragan et al. (1982) in okra and is in contrast to the results found by Cakmak and Marschner (1986) and Singh et al. (1988) in which an increase in root was found under high P level. Zn deficiency in the shoot under high P supply was explained by a possible increase in Zn binding to the root cell wall and by the formation of Zn-phytate (Soltangheisi et al., 2013). This explanation is less likely to be applicable to our results with *C. sativa* that demonstrate Zn accumulate in roots under low P, rather than high P concentration. The data presented here show a small influence of high P nutrition on Zn concentration in the plant, yet the effect on root-to-shoot translocation and on the accumulation in the root under P starvation is significant (Figs. 5, 6 and 8). Zn uptake into the plants was not affected by P supply in both cultivars (Fig. 7).

Mn concentration in the leaves presented a maximum concentration response trend to P supply, with the highest concentrations at 15 mg L^{-1} for RM and 30 mg L^{-1} in DQ (Figs. 5 and 6). Mn accumulated in root to highest concentrations under 5 and 60 mg L^{-1} P in DQ and RM, respectively, demonstrating a genetic variability. Root to shoot translocation of Mn was significantly affected by P supply only in RM (Fig. 8). [The observed changes were not significantly different in DQ]. However, DQ translocation in the plant was also altered as is visible from the increase in stem and root concentration in spite of the concomitant decrease in leaves' concentration under P deficiency (Fig. 6). The influence on leaves Mn's accumulation was masked in the translocation factor results by the increase in stem Mn concentration. Corresponding with the Mn results, Fe concentrations in the roots also varied between the 2 cultivars, with minimum accumulation under 60 and 15 mg L^{-1} in RM and DQ, respectively.

Taken together, these results suggest that P has a higher effect on the translocation of the microelements from the root to the shoot than on

root uptake (Figs. 7 and 8).

Industrial hemp plants are well known as heavy metal accumulators and are therefore considered potential plants for bio-remediation (Ahmad et al., 2016). Furthermore, fibers and seeds of hemp are capable of sorbing heavy metal in large quantities, and are therefore being tested for the use as bioabsorbents and for the safety of the seed-oil extracts (Linger et al., 2002; Pejic et al., 2009; Mihoc et al., 2012; Ahmad et al., 2015). Unlike its hemp relative, our results for the 'drug-type' medical cannabis, present low accumulation of metal ion in the plants as is supported also by a previous report by Saloner et al. (2019). The difference in metal uptake characteristics between hemp and the medicinal cannabis cultivars can source from genetic variability in accumulation potential, chemical properties of the cultivation media (soilless culture vs. soil) or the different stage of growth studied (vegetative vs. reproductive). This issue is currently under investigation in our laboratory.

In summary, our results demonstrate a wide optimum range for P nutrition in medicinal cannabis, at the vegetative growth phase. Although visual symptoms of deficiency and toxicity were not apparent under the supply range of $15\text{--}90 \text{ mg L}^{-1}$ P, integration with the gas exchange results bring us to the conclusion that 30 mg L^{-1} P supply is optimal for plant function. Uptake of N, P, K, Ca, Mg, Fe, and root to shoot translocation of N, P, K, Ca, Mg, Zn and Mn were affected by P supply.

CRediT authorship contribution statement

Sivan Shiponi: Data curation, Investigation. **Nirit Bernstein:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

The project was funded by the Chief Scientist Fund of the Ministry of Agriculture in Israel, grant No. 20-03-0018. We thank Dr. Molly Zacks for advice concerning the design of the fertigation solutions; Yael Sade for guidance and help with the cannabinoid analyses; Avia Saloner, Nadav Danziger, Eliav Shtul-Trauring, Geki Shoef, Ayana Neta and Dalit Morad for technical assistance; and Shiran Cohen for assistance with the N and P analyses. We thank Gerry Kolin, from Teva Adir, LTD, a commercial cultivation farm for cooperation and plant material.

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