

Nitrogen supply affects cannabinoid and terpenoid profile in medical cannabis (*Cannabis sativa* L.)

Avia Saloner ^{a,b}, Nirit Bernstein ^{a,*}

^a Institute of Soil Water and Environmental Sciences, Volcani Center, 68 HaMaccabim Road P.O.B 15159, Rishon LeZion, 7505101, Israel

^b The Robert H. Smith Faculty of Agriculture, The Hebrew University of Jerusalem, Israel



ARTICLE INFO

Keywords:

Cannabis
Nitrogen
CBD
THC
Cannabinoid
Terpene

ABSTRACT

Secondary metabolism in plants is considerably affected by environmental factors including mineral nutrition. Nitrogen is a key plant nutrient, known to affect primary and secondary metabolism in plants, that its effect on the cannabis plants' chemical profile is not known. To evaluate the hypothesis that N supply affects the cannabinoid and terpenoid profile, we studied the impact of N application on chemical and functional-physiology phenotyping in medical cannabis at the flowering stage. The plants were grown under five N treatments of 30, 80, 160, 240, and 320 mg L⁻¹ (ppm) under environmentally controlled conditions. The results revealed that N supply affects cannabinoid and terpenoid metabolism, supporting the hypothesis. The concentrations of most cannabinoids and terpenoids tested were highest under the deficient concentration of 30 mg L⁻¹ N and declined with the elevation of N supply. The concentrations of the two main cannabinoids, tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), decreased by 69% and 63%, respectively, with the increase in N supply from 30 to 320 mg L⁻¹ N. Plant development and function were restricted under inputs lower than 160 mg L⁻¹ N, demonstrating N deficiency. The morpho-physiological state of the plants was optimal at supply rates of 160–320 mg L⁻¹ N. Inflorescence yield reflected the plant physiological state, increasing with the increase in N supply up to 160 mg L⁻¹ N, and was unaffected by further increase in N. These results of the functional and chemical characterizations suggest that high N supply has adverse effects on the production of secondary compounds in cannabis, while it promotes growth and biomass production. Hence, N supply may serve for the regulation of the cannabinoid and terpenoid profiles, or for increasing plant yield, according to the desired production scheme. Taken together, the results reveal that the optimal N level for yield quantity, that allows also a relatively high secondary metabolites content, is 160 mg L⁻¹ N. Finally, the present study provides a better understanding of the impact of N on 'drug-type' medical cannabis physiology, and takes us one step closer to the optimization of medical cannabis cultivation.

1. Introduction

Cannabis (*Cannabis sativa* L.) is used by humanity for thousands of years for medical use (Chandra et al., 2017; ElSohly and Slade, 2005). In recent years, its cultivation for modern agriculture and the demand for medical-grade cannabis products are sharply increasing (Chouvy, 2019; Decorte and Potter, 2015). The therapeutic effects of cannabis are derived from its chemical constituents, encompassing hundreds of biologically active secondary metabolites, including terpenoids, flavonoids and the cannabis-specific cannabinoids (Andre et al., 2016; Russo, 2011; Shapira et al., 2019).

Commercial production of medial cannabis is based on cultivation of

female plants, as the highest concentrations of the therapeutic metabolites is found in female inflorescences (Bernstein et al., 2019a). Since patients use inflorescences or their extracts for medical use, it is of outmost importance to produce a plant material that is chemically-standardized within and between plants and between cultivation batches. In spite of the high demand for the plants' product, and the long history of cannabis cultivation by mankind, almost no science-based knowledge is available regarding regulation of secondary metabolism in medical cannabis plants, and particularly concerning effects of environmental and cultivation conditions (Gorelick and Bernstein, 2017). Such information is essential for the optimization of cultivation for production of high quality standardized material for the

* Corresponding author.

E-mail address: Nirit@agri.gov.il (N. Bernstein).

medical market, as well as for development of plant products containing specific desirable phytochemical profiles for the pharmacological markets.

Environmental conditions are well documented to affect secondary metabolism, and the accumulation of secondary metabolites in plants. Exogenous factors such as water supply, humidity, salinity, and nutrient availability affect production and concentration of secondary metabolites such as alkaloids, phenols, and terpenoids (Dudai et al., 1992; Gorelick and Bernstein, 2017; Rioba et al., 2015; Seigler, 2012). Only little information is available about the effects of environmental conditions on secondary metabolism in *cannabis sativa*, and reported studies were conducted mainly with industrial hemp (fiber-type cannabis) that differs developmentally, morphologically and chemically from medical cannabis, or as part of forensic attempts to locate the sites of the illegal-drugs cultivation fields. Some cultivation conditions including soil properties and light were demonstrated to alter secondary metabolism in hemp (Coffman and Gentner, 1975; Lydon et al., 1987), light quality was reported to affect the cannabinoid profile in drug-type (medical) cannabis (Danziger and Bernstein, 2021), and more studies of the drug-type cannabis are needed to achieve mechanistic understanding of plant response, and regulation of chemical quality.

Mineral nutrients, and especially nitrogen are amongst the main environmental factors affecting plant development, physiology, and metabolism (Hawkesford et al., 2012; Lea and Morot-Gaudry, 2001). As N is the nutrient consumed by plants at the largest quantities, and required for most metabolic processes, N input is amongst the critical factors for plant development and function, and is known as a primary limiting factor in many agricultural systems (Hauck et al., 1984; Ohyama et al., 2010). Hence, there is ample evidence for the impact of N supply on plant yield (Bouchet et al., 2016; Lawlor et al., 2001; Leghari et al., 2016; Tei et al., 1998), and on plant secondary metabolism (Hakulinen et al., 1995; Koricheva et al., 1998; Palumbo et al., 2007; Rioba et al., 2015).

Nevertheless, as medical cannabis is categorized as a dangerous-illicit drug in most countries, and research and medical use of the plant were restricted since the 60's, only little information is available about the impact of mineral nutrition, and particularly N nutrition on the medical 'drug-type' plant, including inflorescence production and secondary metabolism. The fertilization studies available for industrial hemp focused mainly on seed or fiber yield (Aubin et al., 2015; Ehrensing, 1998; Papastylianou et al., 2018; Vera et al., 2004) and demonstrate that N supply promotes protein synthesis, fiber production, and seed yield. From the limited data available on the effect of N on hemp secondary metabolism, contradictory results were obtained: THC content in the inflorescences was not affected by N supply in the range 0–125 mg L⁻¹ N (Coffman and Gentner, 1977), while THC content in leaves declined in response to a high nitrogen regime (Bócsa et al., 1997). As the domestication and breeding of hemp aimed for better fiber production, it differs from drug-type cannabis in plant development and secondary metabolism, and therefore likely also in requirements and responses to mineral-nutrition. It is therefore challenging to deduce how the drug-type cannabis will respond to N nutrition from the information available on hemp.

We have recently uncovered an interaction between plant structure, function, and chemistry in cannabis, and demonstrated a longitudinal variability and a variability between organs in secondary metabolites, mineral nutrients and physiological traits (Bernstein et al., 2019a). We demonstrated, for the first time, induction of changes in the cannabinoids in medical cannabis by nutritional supplements (Bernstein et al., 2019b), and reported a genotypic variability in medical cannabis response to K (Saloner et al., 2019) and P (Shiponi and Bernstein, 2021) at the vegetative growth phase. Optimal plant function and development under long photoperiod occurred under 175 mg L⁻¹ K (Saloner et al., 2019) and 160 mg L⁻¹ N (Saloner and Bernstein, 2020). Availability of N, P and K in the soil was also reported to correlate with growth, yield, and cannabinoids content (Coffman and Gentner, 1977), and in organic

cultivation, yield production excelled under 389 mg L⁻¹ N (Caplan et al., 2017). Current knowledge on responses of cannabis to N input is not sufficient to direct regulation of secondary metabolism in the medical plant product.

The present study therefore aimed to shed light on the interaction between N and secondary metabolism in medical cannabis. The hypothesis directing the work plan was that N supply affects functional physiology and cannabinoids and terpenoids biosynthesis in medical cannabis, which can direct optimization of the phytochemical profile. We have therefore studied effects of N supply (30, 80, 160, 240, and 320 mg L⁻¹ N) on secondary metabolite profile (cannabinoids and terpenoids), and conducted functional physiology phenotyping of the response of medical cannabis to N supply at the flowering stage. We analyzed effects on morpho-development and the plant ionome, and physiological parameters related to water relations, carbon fixation, and gas exchange, which may affect secondary metabolism.

2. Materials and methods

2.1. Plant material and growing conditions

Medical cannabis (*Cannabis sativa L.*) plants of the 'Annapurna' cultivar (Canndoc LTD, Israel) were used as a model plant for the study. It contains about 7% THC and 7% CBD and has indica characteristics. Rooted cuttings were planted in 3 L pots in perlite 2-1-2 cultivation media (Agrekal, Habonim, Israel) 28 days following dissecting from the mother plants. During the following week, the plants received a uniform fertilization following the optimal N and K treatments recently reported by us (Saloner et al., 2019; Saloner and Bernstein, 2020), i.e., 160 mg L⁻¹ N and 175 mg L⁻¹ K, and were cultivated under a 18/6 h light/dark cycle in a controlled environment growing room, where light was supplied by Metal Halide bulbs (400 µmol m⁻² s⁻¹, Solis Tek Inc, Carson, California). After 1 week of vegetative growth under the long photoperiod, the plants were divided to five treatment groups of increasing N concentrations: 30, 80, 160, 240 and 320 mg L⁻¹, five plants per treatment, and were transferred to short photoperiod for the induction of inflorescence development. For the remaining of the experiment, until flower maturation 56 days later, the plants were grown under 12:12 light/dark regime, and light was supplied by High Pressure Sodium bulbs (980 µmol m⁻² s⁻¹, Greenlab by Hydrogarden, Petah Tikva, Israel). In the cultivation room, temperatures were kept at 26/ 24°C during the day and night, respectively, and relative humidities at 44 / 60%, respectively. The plants were irrigated with a 1 L h⁻¹ discharge-regulated drippers (Netafim, Tel-Aviv, Israel), with 1 dripper per pot, 500–750 ml/pot/day. The volume of irrigation in each irrigation event was set to generate 30% of drainage. Mineral nutrients were applied with the irrigation solution at each irrigation event, except at the last week before harvest when the plants were irrigated with distilled water without fertilizers to allow flushing of the cultivation media as is routinely practiced in the commercial cultivation of medical cannabis. The irrigation solution contained (in mM): 1.93 P-PO₄²⁻, 2.76 K⁺, 1.13 Ca²⁺, 1.82 Mg²⁺, 2.5 Na⁺, 2.04 S-SO₄²⁻, 1.6 Cl⁻, 0.025 Fe²⁺, 0.012 Mn²⁺, 0.009 B³⁺, 0.005 Zn²⁺, 0.0008 Cu²⁺, and 0.0003 Mo²⁺. Nitrogen was applied at the concentrations: 2.1, 5.7, 11.4, 17.1, and 22.9 mM (e.g., 30, 80, 160, 240, and 320 mg L⁻¹ (ppm), respectively), 80% as N-NO₃⁻ and 20% as N-NH₄⁺. The irrigation solutions were prepared using: CaCl₂, KNO₃, NH₄H₂PO₄, MgSO₄, KCl, Ca(NO₃)₂, H₃PO₄, (NH₄)₂SO₄, K₂SO₄, CO(NH₂)₂, NH₄NO₃, MgNO₃, and NaOH. Zinc, copper, and manganese were chelated with EDTA, and iron with EDDHSA. Mo and B were added as a part of the fertilizers Bar-Koret and B-7000, respectively (Israel chemicals, Tel-Aviv, Israel). The experiment was conducted in a complete randomized design with five replicates.

2.2. Inorganic mineral analysis

Contents of inorganic minerals in the plant organs were analyzed at

the termination of the experiment, 56 days following commencement of the N fertigation treatments. The shoots were rinsed twice with distilled water and blotted dry, and leaves and inflorescences were separated from the stems. The inflorescence leaves were removed from the inflorescences by manual trimming, following commercial procedures. Roots were separated from the soilless growing media, rinsed 3 times in distilled water and blotted dry. Fresh and dry weights were measured with a Precisa 40SM-200A balance (Zurich, Switzerland). Dry weights were determined following desiccation at 64°C for 48 h (128 h for inflorescences), and the dry tissue was ground to a powder. The plant samples were analyzed for total N, N-NO₃, P, K, Ca, Mg, Fe, Mn, Zn, Cu, and Na as is described by [Saloner and Bernstein \(2020\)](#). Concentrations of N-NO₃ and N-NH₄ in the irrigation solutions were analyzed following [Saloner and Bernstein \(2020\)](#). Electric conductivity (EC) was measured with a conductivity meter (Cyberscan CON 1500, Eutech Instruments Europe B.V., Nijkerk, Netherlands), and the pH was measured with a pH meter (Cyberscan pH 1500, Eutech Instruments Europe B.V., Nijkerk, Netherlands). Concentrations of N-NO₃ and N-NH₄, EC, and the pH of the irrigation solutions throughout the experiment duration demonstrate that the target concentrations of the treatments were reached, and were stable throughout the cultivation durations (Fig. 3 supplemental).

2.3. Physiological parameters

The plants were sampled for physiological analyses 45 days after the initiation of the fertigation treatments. All measurements were conducted on five replicated plants, following the experimental design.

Osmotic potential and relative water content were analyzed as is described by [Saloner and Bernstein \(2020\)](#). The youngest mature fan leaf on the main stem, located at the 4th node from the top of the stem was used for the analyses.

Membrane Leakage and photosynthetic pigments analyses were conducted following [Saloner et al. \(2019\)](#). The central leaflet of the youngest mature fan leaf, located on the fourth node from the plant's top, was used for the analyses. The Concentrations of chlorophyll *a* and *b* and carotenoids were calculated following [Lichtenthaler and Wellburn \(1983\)](#).

Photosynthesis, transpiration, stomatal conductance and intercellular CO₂ concentration were measured with a Licor 6400 XT system (LI-COR, Lincoln, NE, USA). The youngest mature fan leaf on the plant's main stem, located on the fourth node down the plant's top, was used for the analyses. The stomatal conductance and photosynthesis results were used for the calculations of water use efficiency following [Saloner and Bernstein \(2020\)](#).

2.4. Plant morphology and biomass

Plant height, stem diameter, inflorescence length, and the number of nodes on the main stem were measured 50 days following the initiation of the N treatments. Plant height was measured with a ruler as the distance from the base of the plant to the top of the main stem. Stem diameter was measured at the location 3 cm from the plant base with a digital caliper (YT-7201, Signet tools international co., LTD., Shengang District, Taiwan). Length of the apical inflorescence of the main stem ['top inflorescence'] and the apical inflorescence of the lowest 1st order branch on the main stem ['side inflorescence'] was measured with a ruler from the base of the inflorescence to its top. The measurements were conducted on five replicated plants per treatment, following the experimental design. Biomass of the plant organs was measured at the termination of the experiment, 56 days after the beginning of the N treatments and the short photoperiod. The dry biomass was measured after drying for 48 h at 64°C (128 h for the inflorescences). Nitrogen use efficiency (NUE) is a measure of the dry inflorescence biomass on day 56 divided by the cumulative N quantity that the plants received (g /plant) throughout the short photoperiod. All measurements were conducted for five replicated plants per treatment.

2.5. Cannabinoid and terpenoid analysis

For the analysis of cannabinoid and terpenoid concentrations in the plant material, the top inflorescence of the main stem ['top inflorescence'] and the top inflorescence of the lowest first order branch on the main stem ['side inflorescence'] were dissected from the plants 56 days after the beginning of the N treatments and the short photoperiod. At the time of sampling, about 30% of the trichome heads were of amber color, following the maturation stage acceptable for commercial harvesting. The inflorescences were immediately trimmed (wet-trimmed) to separate the protruding parts of the inflorescence leaves from the inflorescence. The trimmed inflorescences and the trimmed inflorescence leaves were then dried in an environmental-controlled chamber, in the dark, at 19°C and 55% relative humidity. After two weeks of drying, the samples were packed in sealed plastic bags, and set in the dark for two months for curing prior to analyses.

2.5.1. Cannabinoid analysis

The inflorescences and the trimmed inflorescence leaves were ground manually, to create a homogenous mixture. For each sample, 50 mg of the ground plant material was placed in a 50 mL centrifuge tube, 10 mL ethanol ABS AR (Gadot-Group, Netanya, Israel) was added, the tube was shaken in a reciprocal shaker for 1 h at room temperature and then centrifuged for 15 min at 5000 rpm (Megafuge 16 centrifuge, Thermo-Scientific, Waltham, MA, USA). The supernatant was filtered with a 0.22 µm PVD filter (Bar-Naor ltd, Ramat Gan, Israel). Cannabinoid concentrations in the filtered plant extracts were analyzed using HPLC (Jasco 2000 Plus series), which consisted of a quaternary pump, an autosampler, a column compartment, and a PDA detector (Jasco, Tokyo, Japan). The detection was carried out in a spectrum mode, at the wavelength range 200–650 nm. Chromatographic separations were carried out with the Luna Omega 3 µm Polar C18 column (Phenomenex, Torrance, CA USA) in isocratic mode with 75:25 (v/v) acetonitrile:water and 0.1 % formic acid, at a flow rate of 1.0 mL min⁻¹. Calculation of cannabinoid concentrations were based on pure analytical standards: cannabichromene (CBC), cannabichromenic acid (CBCA), cannabichromevarin (CBCV), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabinol (CBN), cannabinolic acid (CBNA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabicyclol (CBL), cannabidivaricin (CBDV), cannabidivarinic acid (CBDVA), tetrahydrocannabivarinic acid (THCVA) (Sigma-Aldrich, Germany); cannabicitran (CBT) (Cayman chemical company, Pennsylvania, USA) and tetrahydrocannabinolic acid (THCA), Δ⁹-tetrahydrocannabinol (THC), Δ⁸-THC, tetrahydrocannabivarin (THCV) (Restek, Pennsylvania, USA). The influence of N supply on in-plant decarboxylation of THCA and CBDA was calculated by dividing the concentration of the non-acidic form by the sum of the concentrations of the acidic and non-acidic forms for each respective cannabinoid. R² values for linear regressions of the calibrations curves of all cannabinoid standards were >0.994 (Table 1 supplemental); repeatability, quantified by intra-day variation from extractions of the same sample (n = 5) was < ±0.8% for all cannabinoids tested. Concentrations of Δ⁸-THC, THCV, CBDV, CBDVA, CBG, CBNA, CBL, CBCV, and CBT were lower than the detection limits. Cannabichromene (CBC) concentration was in the range of 0.2–0.13% and 0.08–0.03% for the inflorescences and inflorescence leaves, respectively (Data not shown).

2.5.2. Terpenoid analysis

100 mg of dried plant material was ground in liquid N₂ to a fine powder. Volatile compounds were extracted by vigorous shaking on a shaker apparatus (200RPM) for 2 h in 2 mL MTBE (methyl tert-butyl ether), containing 100 ppm of ethyl myristate as an internal standard. The upper MTBE layer was separated and dried with Na₂SO₄ and kept at –20 °C until analysis. The analysis of terpenoids was carried out as described by [Rand et al. \(2017\)](#), with some modifications. A 1 µL aliquot of the sample was injected into an GC-MSD system (model 6890 N/ 5973 N, Agilent Technologies CA, USA) equipped with Rxi-5sil ms column (30

m length x0.25 mm i.d., 0.25 µm film thickness, stationary phase 95 % dimethyl- 5% diphenyl polysiloxane). Helium (11.18 psi) was used as a carrier gas with splitless injection. The injector temperature was 250 °C and a detector temperature was 280 °C. The initial temperature was 50 °C for 1 min, followed by a ramp of 5 °C min⁻¹ to 260 °C and 20 °C min⁻¹ up to 300 °C (10 min). The MS data was acquired with a quadrupole mass detector with electron ionization at 70 eV in the range of 41–350 m/z. Identification of the compounds was conducted by comparing their relative retention indices and mass spectra with those of authentic samples or with those found in the literature and supplemented with W10N11 and QuadLib 2205 GC-MS libraries. A mixture of straight-chain alkanes blend (C7-C23) was injected into the column under the above-mentioned conditions for the calculation of the retention indices. The amount of the compound in each sample was calculated by multiplying the peak area by the response factor of the internal standard, and dividing the result by the product of the response factor and the internal standard. Further details concerning the terpenoid analysis are supplied in Table 2 Supplemental.

2.6. Statistical analyses

The data were subjected to a one-way or two-way analysis of variance ANOVA (at $\alpha = 0.05$) and Tukey's HSD post-hoc test for separation of means. The data met the assumptions of normality and homogeneity of variances. Fisher's least significant difference (LSD) test at 5% significance level was used for the comparison of means. The analysis was performed with the JMP package (version 9, SAS 2015, Cary, NC, USA).

3. Results

3.1. Plant visual characteristics

Visual appearance of the plants varied between treatments. Leaf color changed dramatically from pale yellow to dark green with the increase in N supply (Fig. 1). Leaves of the low N treatments, 30–80 mg L⁻¹ N, were smaller compared with leaves in the higher N treatments

(160–320 mg L⁻¹ N); the length of the central leaflet of the youngest mature leaf on the main stem was 8–8.5 cm in the low N treatments, compared to 10.7–11.2 cm in the high N treatments. A parallel trend was obtained for the plant's top inflorescence, i.e., the color of the inflorescence and the inflorescence leaves changed from yellow to dark green as N supply increased, and plants that received 30 mg L⁻¹ N had significantly smaller inflorescences compared to plants that received higher N level (80–320 mg L⁻¹ N) (Fig. 1, 1 supplemental C). These trends were apparent on the whole plant level as well, and the plants in the 30–80 mg L⁻¹ N range appeared thinner and less dense than the plants from the higher N range (160–320 mg L⁻¹ N) (Fig. 1). Within the 160–320 mg L⁻¹ N range, there were no apparent differences in visual appearance of the plant, leaves and inflorescences, except that the organs became greener as N supply increased (Fig. 1).

3.2. Plant growth, biomass accumulation and nitrogen use efficiency

Plant growth and development, and thus biomass accumulation and yield, were influenced significantly by the N treatments (Fig. 2). Responses to N supply varied between vegetative plant organs, as leaves biomass increased steadily throughout the N range tested, i.e., up to 320 mg L⁻¹ N, while root and stem biomass increased only up to 80 mg L⁻¹ N (Fig. 2B–C). The response of the reproductive organs biomass to the treatments, i.e., inflorescences and inflorescence leaves biomass, was similar to the response for the entire plant biomass; it increased with increasing N up to 160 mg L⁻¹ N (Fig. 2A–B). The effect of N supply on reproductive development (and yield) was substantial. Biomass of the inflorescences and the inflorescence leaves increased by 206% and 103%, respectively, between 30–160 mg L⁻¹ N supply. Whole plant biomass demonstrated a similar trend to the reproductive organs (Fig. 2A). In spite of the substantial increase in yield with the increase in N supply, nitrogen use efficiency (NUE) decreased dramatically with the elevation of N supply (Fig. 2D). The morphological analyses indicated that node formation was not affected by N supply, while inflorescence length, stem width, and plant height were lower at the 30 mg L⁻¹ N treatment compared with higher supply, and did not vary between

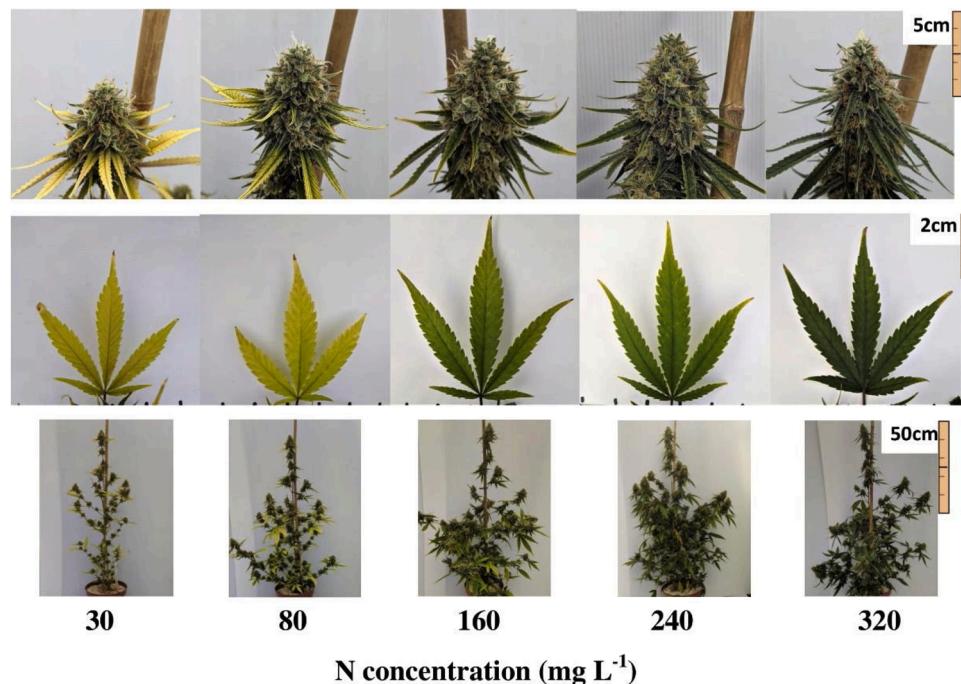


Fig. 1. Visual appearance of inflorescences, leaves, and whole plants of medical cannabis cultivated under increasing N supply. From left to right: 30, 80, 160, 240, 320 mg L⁻¹ N. Shown are the top inflorescence on the main stem and the youngest, fully developed leaf on the third branch from the plant's top. The photos were taken 52 days after the initiation of the short photoperiod and the fertigation treatments.

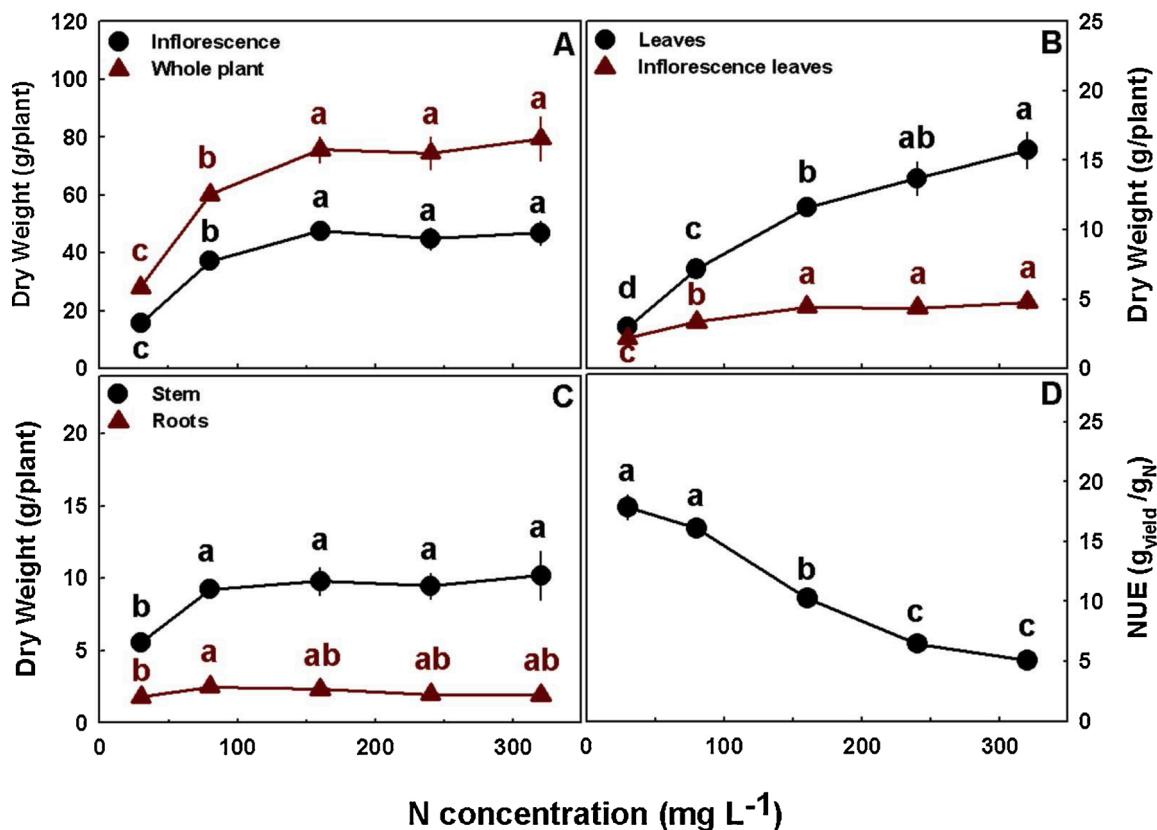


Fig. 2. Effect of N nutrition on plant biomass in medical cannabis plants. Dry biomass of inflorescences and whole plant (shoot + root) (A), leaves and inflorescence leaves (B), stem and roots (C), and N use efficiency (NUE) (D). In NUE: g_{yield} is Inflorescence DW (g/plant); g_N is the weight of N supplied (g/plant). Data are means ± SE (n = 5). Where not seen, the error bars are smaller than the symbol size. Different letters signify significant differences between treatments within each plot by Tukey HSD test at $\alpha = 0.05$. Degrees of freedom = 4 for all measures. Results of a one way ANOVA (at $\alpha = 0.05$); $p < 0.001$ for all measures.

higher application rates (Fig. 1 supplemental).

3.3. Cannabinoid and terpenoid profile

Medical cannabis is grown for the female reproductive organs, which contain a rich chemical profile of secondary metabolites of potential therapeutic activity. In order to achieve yield of uniform chemical quality, it is important to examine the concentrations and distribution of the biologically active compounds in the inflorescences and in response to growing conditions including N supply. The cannabinoid profile in the plants revealed a number of significant trends: 1. The concentration of all the acidic (carboxylated) cannabinoids examined, including THCA, CBDA, THCVA, CBGA and CBCA, decreased significantly in the inflorescences with the increase in N supply (Fig. 3A–B, E, G–H). 2. The concentration of all the non-acidic (decarboxylated) forms of the cannabinoids examined, including THC, CBD and CBN, demonstrated bell-shape accumulation curves in the inflorescences, and their concentration was highest in the 160 mg L⁻¹ N treatment and significantly lower at both lower or higher levels (Fig. 3C–D,F). 3. Cannabinoid concentrations in the inflorescence leaves were generally not altered by N supply (Fig. 3). 4. The concentration of all the cannabinoids examined was significantly higher in the inflorescences than the inflorescence leaves, and there was no substantial difference in the concentration between the samples taken from the top of the plant (top of the main stem) or top of lower branches (Fig. 3). The substantial effect of N nutrition on concentration of the acidic forms of the cannabinoids in the plant can be seen for example by the 69 % and 63 % decrease in the concentrations of THCA and CBDA at the top inflorescence, respectively, under the increase in N input from 30 to 320 mg L⁻¹. In addition to treatment effects on cannabinoid concentrations, the decarboxylation of

THCA and CBDA to their decarboxylated forms was also altered by the N treatments, revealing a second layer of sensitivity of secondary metabolism. N supply promoted the in-plant decarboxylation of both of the cannabinoids, throughout the N range tested (Fig. 4 supplemental).

The concentrations of most of the terpenoids identified in the plant material were significantly influenced by N supply, yet the response varied between different groups of terpenoids (Figs. 4,5). Three main trends were obtained for monoterpenes: 1. α -pinene and β -pinene concentrations were lower at 30 mg L⁻¹ N, than in all other treatments, amongst no significant difference was obtained (Fig. 4A–B). 2. Myrcene and ipsdienol concentrations were not changed significantly by N supply (Fig. 4C–D). 3. The concentrations of the majority of the monoterpenes, including δ -2-carene, linalool, fenchol, (E)- β -ocimene, borneol, terpinen-4-ol and α -terpineol decreased with the increase in N application, throughout the studied N concentration range (Fig. 4F–L). Sesquiterpenes concentration in the inflorescence also varied between treatments, and exhibited three trends as well: 1. (E)- α -bergamotene, 5-epi-7-epi- α -eudesmol and β -selinene concentrations were higher in the low N treatment of 30 mg L⁻¹ N, compared with the higher N treatments, with no further significant differences with increased N concentrations (Fig. 5A,C–D). 2. α -bulnesene and α -guaiene were reduced with the increase in N input, throughout the N concentration range studied (Fig. 5K–L). 3. The concentrations of the majority of the sesquiterpenes tested, including selina-3,7(11)-diene, α + β -eudesmol, 10-epi- γ -eudesmol, eremoligenol or γ -eudesmol, guaiol and α -humulene, were lower under 320 mg L⁻¹ N compared with all other treatments, with no significant differences (Fig. 5E–J). Concentrations of additional sesquiterpenes revealed the same trends (Fig. 2 supplemental).

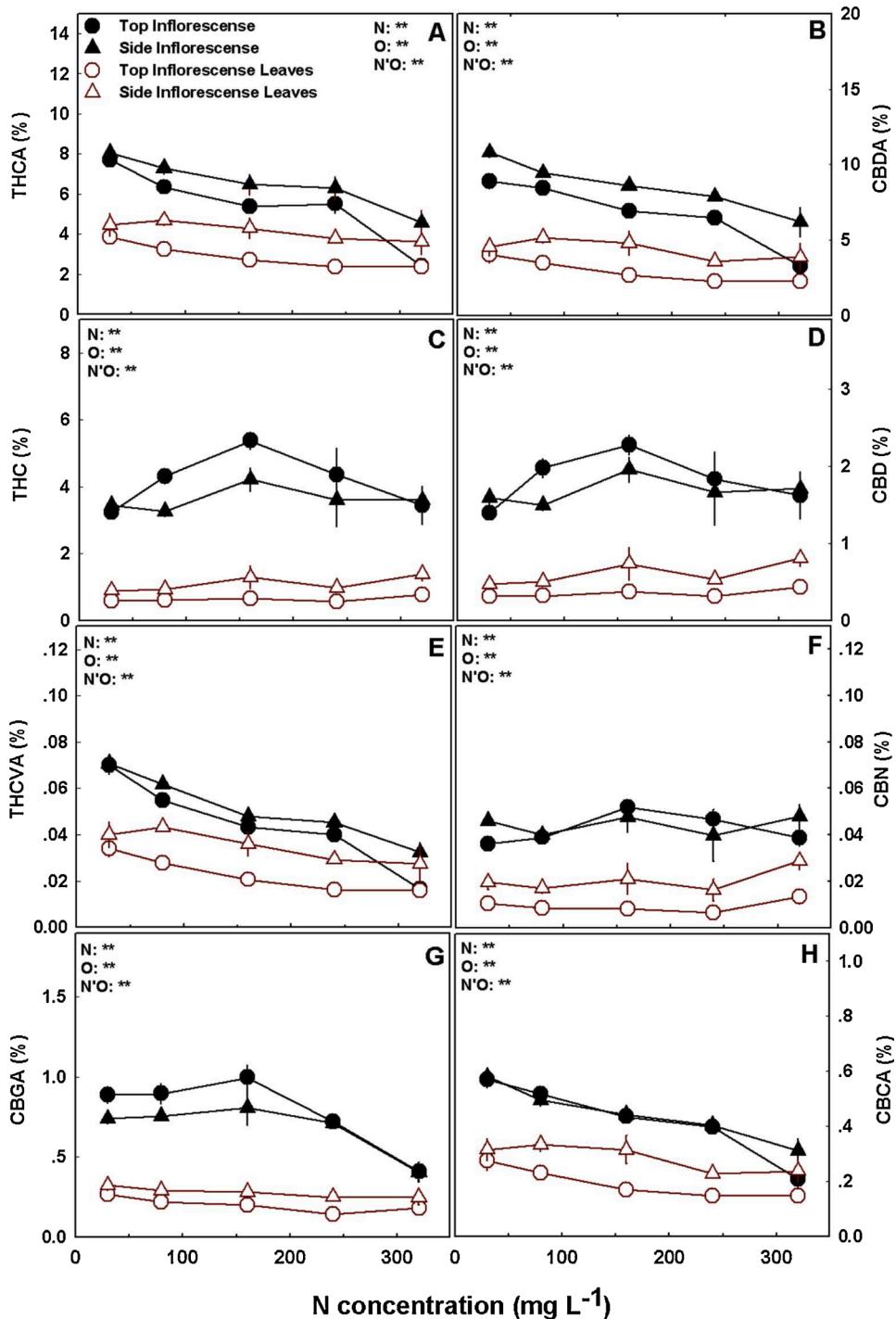


Fig. 3. The impact of N application on cannabinoids concentration in medical cannabis plants. Tetrahydrocannabinolic acid (THCA) (A), cannabidiolic acid (CBDA) (B), tetrahydrocannabinol (THC) (C), cannabidiol (CBD) (D), tetrahydrocannabivarinic acid (THCVA) (E), cannabinol (CBN) (F), cannabigerolic acid (CBGA) (G), and cannabichromenic acid (CBCA) (H) concentration in top and side inflorescences and in the inflorescence leaves. Data are means \pm SE ($n = 5$). Where not seen, the error bars are smaller than the symbol size. In the two-way ANOVA results: ** $P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test, N'O signifies an interaction between nitrogen and the plant organ. df = 4 for N, 4 for O, 16 for N'O.

3.4. Nutrient concentrations

In addition to the considerable impact on the quality and quantity of the yield, N input induced significant changes to the plant ionome, and mostly to the macronutrient profile (Fig. 6). Increased N supply elevated N and N-NO_3^- concentration in the tested plant organs throughout the studied N concentration range (Fig. 6A–B). Accumulation and distribution of K in the plant organs was organ-specific (Fig. 6C). Leaf and stem K concentrations were highest under 30 and 30–80 mg L^{-1} N, respectively, while accumulation in the roots presented a minimum response curve with minimum accumulation under 240 mg L^{-1} . In the inflorescence leaves, K concentrations were highest under 240–320 mg L^{-1} N,

and the concentration in the inflorescence was not significantly affected by the N regime (Fig. 6C). Phosphorous accumulation in the leaves and stem were lower under 320 mg L^{-1} N and higher under 30–80 mg L^{-1} N, respectively; root concentrations increased with N input throughout the studied N concentration range, and concentrations of P in the inflorescences and the inflorescence leaves demonstrated a maximum response trend (Fig. 6D). Calcium accumulation in leaves and roots was highest under 160–240 mg L^{-1} N, and the accumulation in inflorescence leaves exhibited a maximum trend. Accumulation of Ca in stems and inflorescences were not affected by the N treatments (Fig. 6E). Magnesium accumulation in the leaves was also highest in the 160–240 mg L^{-1} N treatments, but the accumulation in all other plant parts was

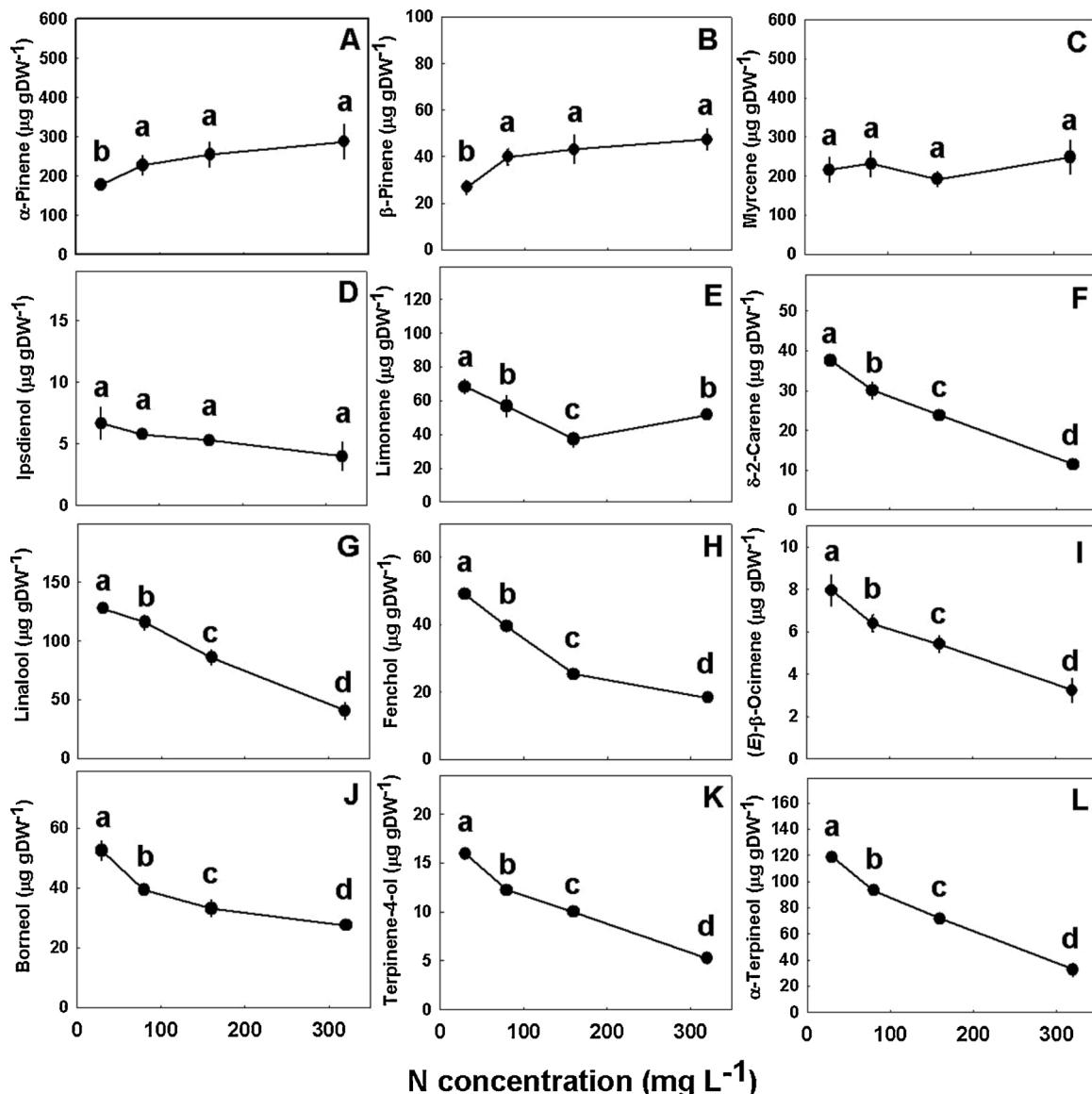


Fig. 4. Effect of N supply on monoterpenes concentrations in the apical inflorescence of medical cannabis plants. α -pinene (A), β -pinene (B), myrcene (C), ipsdienol (D), limonene (E), δ -2-carene (F), linalool (G), fenchol (H), (E)- β -ocimene (I), borneol (J), terpinene-4-ol (K), and α -terpineol (L). Data are means \pm SE ($n = 5$). Where not seen, the error bars are smaller than the symbol size. Different letters signify significant differences between treatments within each plot by Tukey HSD test at $\alpha = 0.05$. df = 4 for all measures.

not affected by N supply (Fig. 6F).

The distribution of micronutrients in the plants demonstrated a profound organ-specificity, but only a moderate response to N input (Fig. 5 supplemental). Micronutrient concentrations in the leaves and the inflorescence leaves were usually not affected by N supply. In the stem there were relatively high concentrations of Mn and Fe under 30–80 mg L⁻¹ N and 80 mg L⁻¹ N, respectively, while Zn and Cu concentrations were not affected by the treatments. Cu and Fe in the roots increased with the increase in N supply while Zn exhibited a minimum response curve. In the inflorescence, the concentrations of Fe and Zn were lowest under the high N treatment, while Mn and Cu decreased with the elevation of N supply.

3.5. Photosynthesis and gas exchange and

Nitrogen is a key mineral element required for plant metabolism, which is involved also in the photosynthetic process. Accordingly, the photosynthesis rate was significantly affected by N supply, and it

demonstrated an optimum response curve, with the highest photosynthesis rate under the supply range of 160–240 mg L⁻¹ N (Fig. 7A). The rate of transpiration and stomatal conductance did not follow the same trend, as they were only significantly lower under the high N treatment, 320 mg L⁻¹ N, compared to the middle treatment of 160 mg L⁻¹ N (Fig. 7B–C). As a result, the intercellular CO₂ concentration decreased steadily with the increase in N input throughout the concentration range studied (Fig. 7D).

3.6. Photosynthetic pigments and water relations

The relative water content of the leaves (RWC) was not significantly affected by N supply (Fig. 7E), although the osmotic potential increased with an increase in N input (Fig. 7F) suggesting that it reflects accumulation of N. Water use efficiency (WUEi) also elevated with an increase in N input, but only up to stabilization under 240 mg L⁻¹ N (Fig. 7G). Membrane leakage was highest at the lowest N concentration (30 mg L⁻¹ N), compared with all other treatments (Fig. 7D).

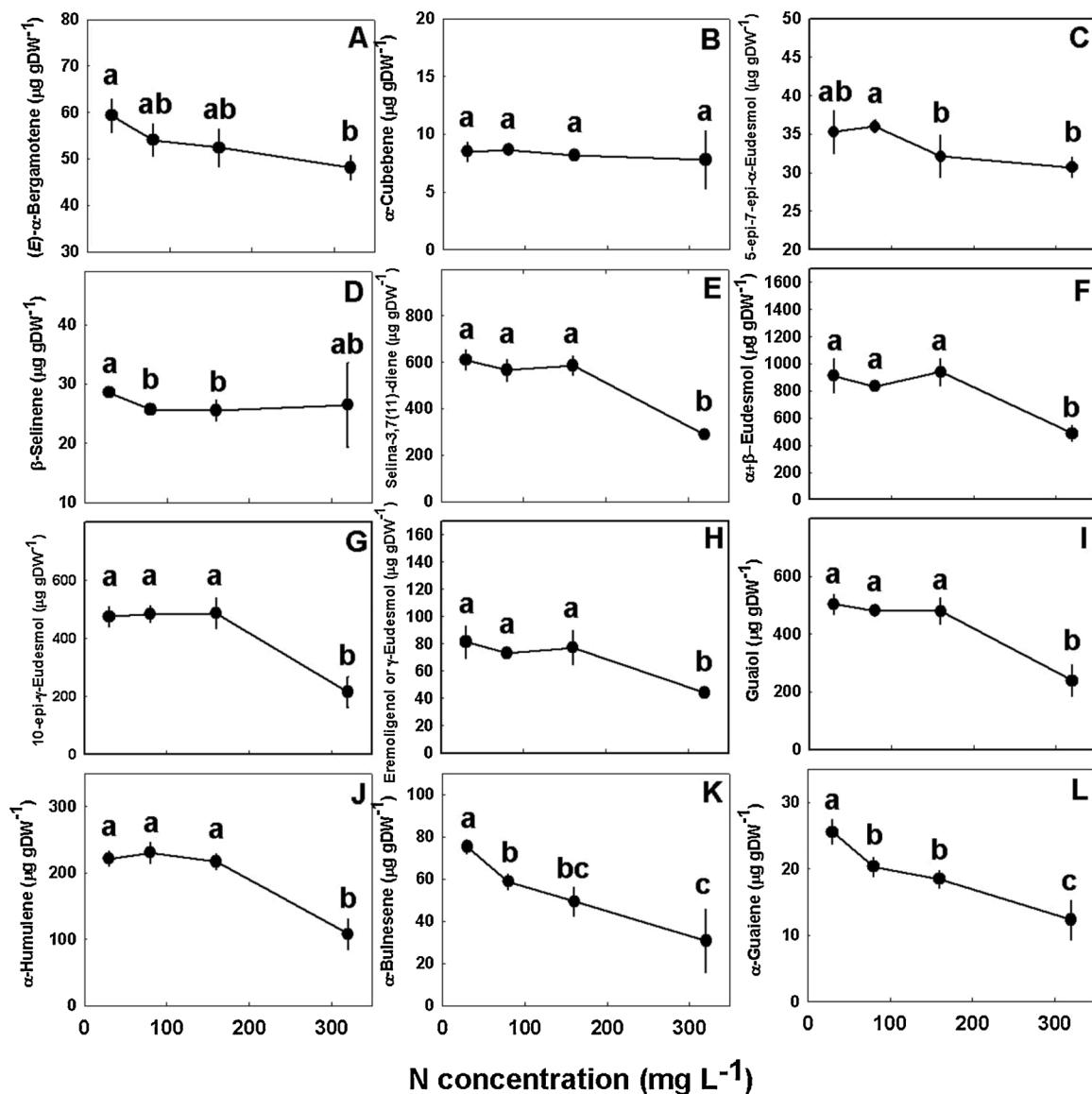


Fig. 5. Effect of N supply on sesquiterpenes concentrations in the apical inflorescence of medical cannabis plants. (E)- α -bergamotene (A), α -cubebene (B), 5-epi-7-epi- α -eudesmol (C), β -selinene (D), selina-3,7(11)-diene (E), α + β -eudesmol (F), 10-epi- γ -eudesmol (G), eremoligenol or γ -eudesmol (H), guaiol (I), α -humulene (J), α -bulnesene (K), and α -guaiene (L). Data are means \pm SE ($n = 5$). Where not seen, the error bars are smaller than the symbol size. Different letters signify significant differences between treatments by Tukey HSD test at $\alpha = 0.05$. df = 4 for all therpenoids.

Concentration of the photosynthetic pigments in the leaves increased with the increase of N concentration, throughout the N range tested (Fig. 8).

4. Discussion

This study examined the overall response of *C. sativa* plants to increased N application, at the reproductive growth stage, in an attempt to understand the effects of N nutrition on the metabolic, phenotypic, chemical, and physiological responses of the plant. The results highlight the importance of adequate N supply, as plant secondary metabolism was highly affected by N supply, as were gas exchange, photosynthetic pigments concentration, mineral accumulation, and plant yield. The salient effect of increasing N supply is the dramatic decrease in cannabinoid and terpenoid concentration, which is accompanied by an improvement in plant growth and physiological status. Our obtained results improve and expand the ability to regulate medical cannabis secondary metabolism and yield, and therefore direct researchers and growers to control the secondary metabolite profile of the plant.

N is a vital nutrient required by all plants in relatively high doses, for optimal growth and function (Lea and Morot-Gaudry, 2001; Leghari et al., 2016; Ohyama, 2010). The cannabis plants suffered from N deficiency when N supply was lower than 160 mg L^{-1} N; Most of the parameters measured, including photosynthesis rate, photosynthetic pigments concentration, and water use efficiency were lowest when N supply was below 160 mg L^{-1} N (Figs. 7A,G, 8). The deficiency stress response was also apparent by the higher level of membrane leakage, under 30 mg L^{-1} N (Fig. 7H), which indicates membrane damage and overall unstable environment in the plant tissue (Bernstein et al., 2010). Furthermore, from the nutrient concentrations analysis, it is apparent that low N supply induced a significant decrease in the uptake and accumulation of Ca, Mg, and Fe (Fig. 6E-F, 5C supplemental), as was seen before for other plant species (Kutman et al., 2011; Rayar and van Hai, 1977), and may involve as well in stress induction. Moreover, N uptake and accumulation in all plant organs correlated with N supply (Fig. 6A), and were very low under low N inputs (below 160 mg L^{-1} N), similar to the accumulation response that we have recently reported for the vegetative growth phase (Saloner and Bernstein, 2020). The impact

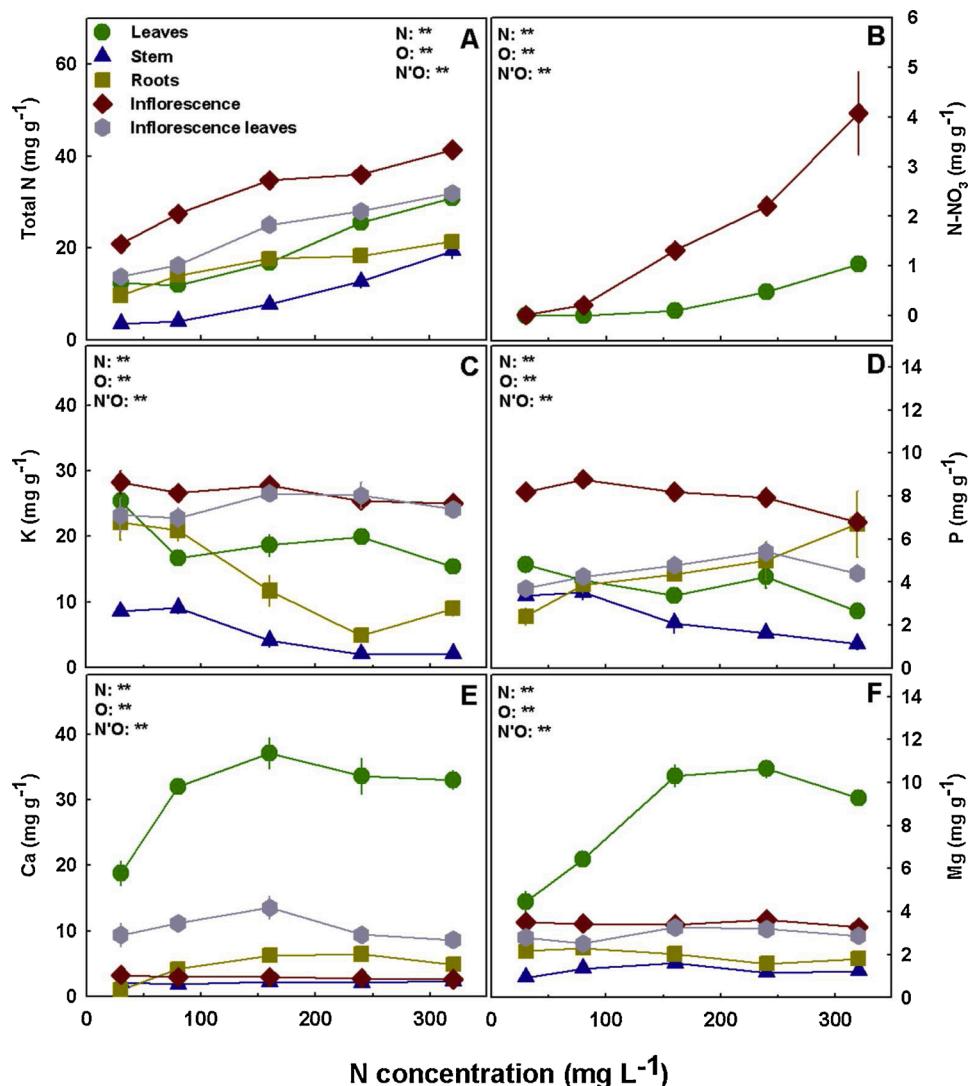


Fig. 6. Effect of N application on macronutrients concentration in leaves, stem, roots, inflorescence, and inflorescence leaves of medical cannabis plants. Total N (A), N-NO₃ (B), K (C), P (D), Ca (E), and Mg (F). Data are means \pm SE ($n = 5$). Where not seen, the error bars are smaller than the symbol size. In the two-way ANOVA results: ** $P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test, N'O signifies an interaction between nitrogen and the plant organ. df = 4 for N, 4 for O, 16 for N'O.

of the N deficiency was well apparent visually in the plants of the low N treatments, which were chlorotic and undeveloped (Fig. 1), displaying known symptoms of N deficiency (McCauley et al., 2009; Uchida, 2000).

Many plants respond to excessive nutrient supply and N supply in particular, by developing toxicity symptoms and impaired growth (Albornoz, 2016; Choi et al., 1996; McCauley et al., 2009). In a previous study, we demonstrated that above the optimum of 160 mg L⁻¹ N supply, *cannabis sativa* plants of the same genotype studied in the current project developed N toxicity stress at the vegetative growth phase (Saloner and Bernstein, 2020). Surprisingly, at the reproductive phase, the plants were less sensitive to N excess and did not develop N toxicity stress throughout the concentration range tested. Elevating N supply, above 160 mg L⁻¹ N, induced numerous phenotypic and physiological responses including: an increase in photosynthetic pigment concentrations (Fig. 8), leaf osmotic potential (Fig. 7F), and plant N and N-NO₃ concentrations (Fig. 6A-B), and a reduction in gas exchange parameters (Fig. 7). Despite these effects, it seems that the plants were not substantially affected by the increased N supply, nor demonstrated any visual stress symptoms, as can be seen from the well developed and vital plants and leaves shown in Fig. 1. Moreover, except for the leaves biomass, which increased gradually with N supply, all other plant organs did not exhibit any growth changes in response to the increase of N

application in the range of 160–320 mg L⁻¹ N (Fig. 2). Thus, under a short photoperiod at the reproductive stage, high N concentrations, up to 320 mg L⁻¹, do not harm the plants, and the high N amounts taken by the plants are directed for biosynthesizing more N-rich metabolites such as photosynthetic pigment and vegetation without impairing cell function and inflorescence production.

It is known that plant secondary metabolism can be affected by environmental and cultivation conditions, and especially by plant mineral nutrition (Chishaki and Horiguchi, 1997; Gorelick and Bernstein, 2017; Ormeño and Fernandez, 2012; Rioba et al., 2015). Here, we report a substantial effect of N supply on plant secondary metabolism, i.e., on cannabinoids and terpenoids concentration, in the medical cannabis plant. Cannabinoids and terpenoids are synthesized by semi-different biochemical pathways. Cannabinoid formation is conducted in two pathways, the Deoxylulose pathway and the Polyketide pathway, where Geranyl diphosphate (GPP) and olivetolic acid are formed and transformed into CBGA, which proceeds and transforms into all the other cannabinoids (Flores-Sanchez and Verpoorte, 2008). Terpenoid formation is also conducted in two pathways, the Mevalonate pathway and the Deoxylulose pathway, from isopentenyl pyrophosphate (IPP), isopentenyl diphosphate (IPD) or from dimethylallyl diphosphate (DMAD) (Moghaddam and Mehdizadeh, 2017). All of the

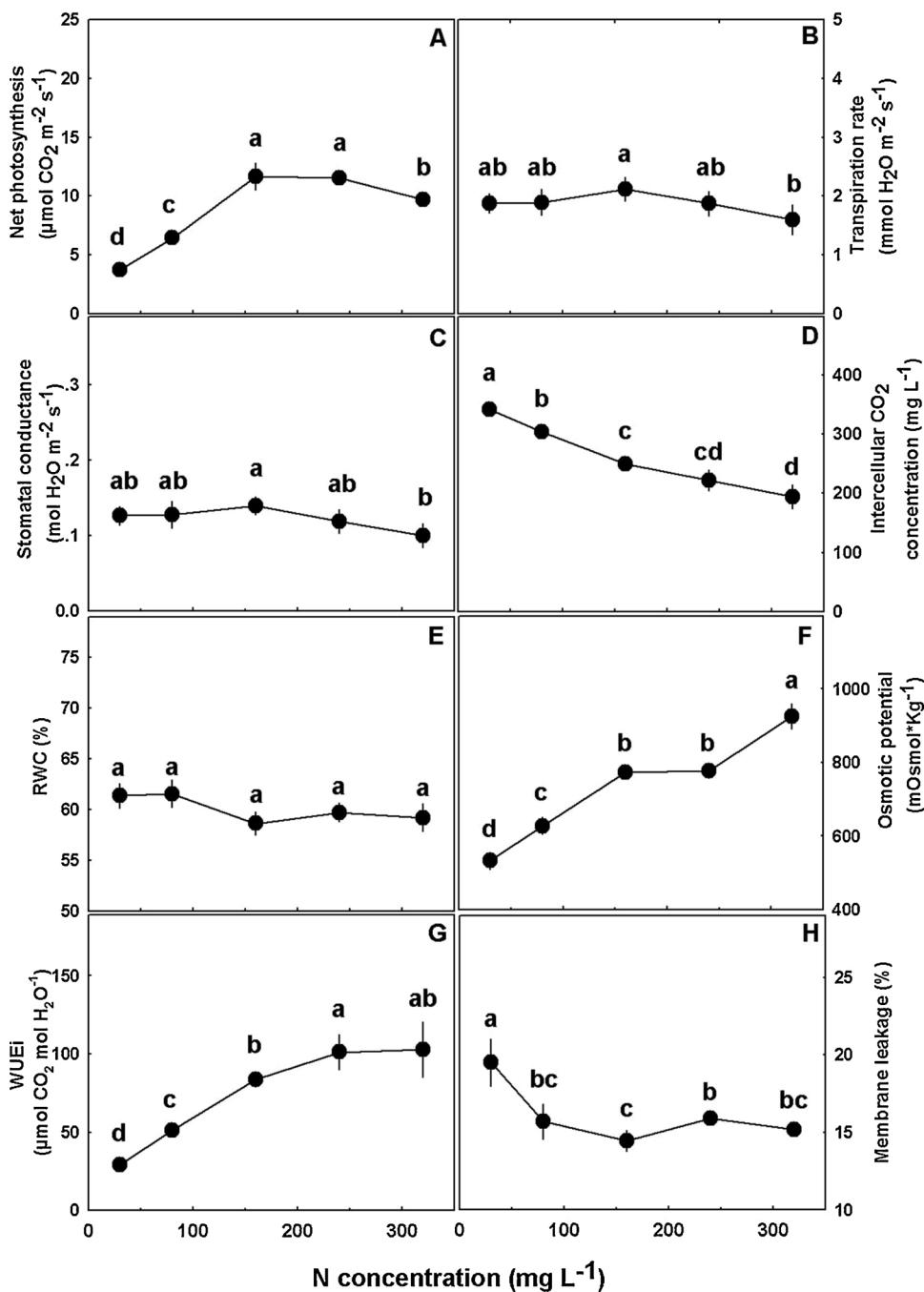


Fig. 7. Effect of N supply on physiological characteristics of medical cannabis plants. Photosynthesis (A), transpiration (B), stomatal conductance (C), intercellular CO₂ concentration (D), relative water content (RWC) (E), Osmotic potential (F), intrinsic water use efficiency (WUEi) (G), and Membrane leakage (H). Data are means \pm SE ($n = 5$). Where not seen, the error bars are smaller than the symbol size. Different letters signify significant differences between treatments by Tukey HSD test at $\alpha = 0.05$. df = 4 for all measures.

compounds mentioned above, as well as their precursors, do not contain any N atoms, and therefore, in accordance with our results, a direct connection between plant N status and their production is not expected (Figs. 3–5). As mentioned above, the pathways of cannabinoid and terpenoid synthesis are not entirely separated, as the Deoxyxylulose pathway and GPP formation participates in both; therefore, it is not surprising that response trends for their production are similar (Figs. 3–5).

Cannabinoids and terpenoids are secondary metabolites, and therefore not required for primary plant metabolism (Vasconsuelo and Boland, 2007). Hence, we may intuitively assume that plants will produce high concentrations of secondary metabolites only while they are thriving physiologically and energetically, thus having sufficient metabolic energy to maintain proper primary metabolism along with high secondary metabolism. In contrast, when plants suffer from stress and

are physiologically impaired, they are likely to invest available energy in primary metabolism and survival, rather than maintaining high secondary metabolism. Contrary to this reasoning, our results, which are supported by results for other plant systems, demonstrate an increase in secondary metabolism under stress (Ramakrishna and Ravishankar, 2011; Vasconsuelo and Boland, 2007). Although secondary metabolism may not be essential for plant survival, it is vital for plant survival in stress environments. Secondary metabolites are biosynthesized in the plant as part of defense mechanisms to abiotic and biotic stresses (Vasconsuelo and Boland, 2007; Wink, 2008; Yamada and Sato, 2013). They may also play an essential role in non-defensive plant function as mediators in plant-plant interactions in an attempt to improve competitive plant abilities in the struggle for vital resources such as water and nutrients (Joyce et al., 2011; Wink, 2008). Likewise, a deficient supply of nutrients may trigger an increase in plant secondary

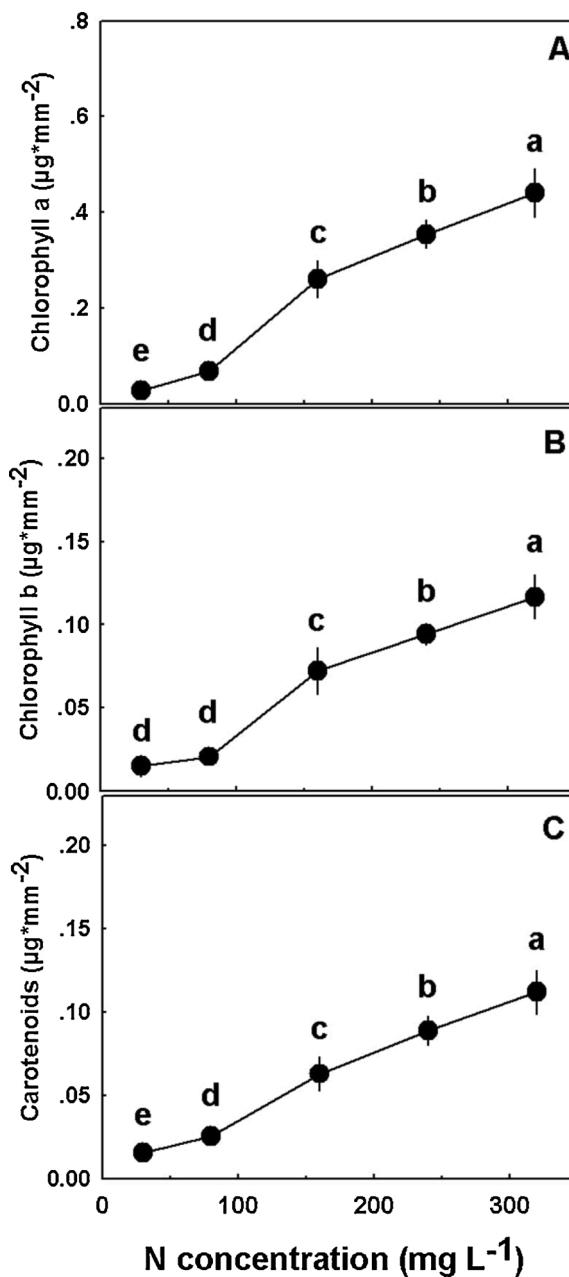


Fig. 8. The impact of N application on the concentration of photosynthetic pigments in medical cannabis plants. Chlorophyll a (A), chlorophyll b (B), and carotenoids (C). Data are means \pm SE ($n = 5$). Where not seen, the error bars are smaller than the symbol size. Different letters signify significant differences between treatments by Tukey HSD test at $\alpha = 0.05$. df = 4 for all measures. Results of a one way ANOVA (at $\alpha = 0.05$): $p < 0.001$ for all measures.

metabolism, as was demonstrated by numerous studies before (Blanch et al., 2012; Vickers et al., 2009; Wu et al., 2020).

We have recently identified severe N deficiency symptoms and significant stress response under N levels lower than 160 mg L^{-1} N at the vegetative growth phase (Saloner and Bernstein, 2020), and here we report a similar response at the reproductive phase of development. Following the tendency of plants to promote secondary metabolism under stressed conditions, it is plausible that N deficiency stress, which developed at N levels lower than 160 mg L^{-1} N, will elicit secondary metabolite production of cannabinoids and terpenoids, as is apparent from our results (Figs. 3–5). It is important to note that contradictory results are available in the literature for secondary metabolite elicitation by nutrient deficiency. Production of secondary compounds can be

increased, decreased or not be affected by nutrient availability (Gorelick and Bernstein, 2017; Moghaddam and Mehdizadeh, 2017; Ormeño and Fernandez, 2012). Although there was a general decrease in the concentration of most cannabinoids and terpenoids with the increase in N availability, the production of some of the compounds, for example, α -pinene, β -pinene, and myrcene, was stimulated, or not affected, by N application (Fig. 4A–C). Also, the increased decarboxylation of THCA and CBDA with elevation of N supply (Fig. 4 supplemental), suggests that optimal physiological state promoted in-plant cannabinoid decarboxylation, and N deficiency which induced impaired physiological state delayed in-plant decarboxylation. The involvement of cannabinoids in stress tolerance mechanisms in the cannabis plant is speculative at our current state of knowledge, and although we have some understanding of the biological activity of acidic versus decarboxylated forms of the cannabinoids in the human body, no such understanding is available thus far for plants. It is therefore not known if the in-plant decarboxylation is a causal response to the changing physiological environment of the tissue, or reflects involvement in damage or resistance mechanisms.

In accord with the hypothesis described above, suggesting that stress environments induce secondary metabolite production, the counter effect of unstressed environments to reduce secondary metabolism was validated by our results. E.g., high N supply, at the range 160 – 320 mg L^{-1} N, was optimal for plant function and development. Consequently, the cannabinoid and terpenoid content of plants that received optimal N supply was lower than in the plants which suffered N deficiency. Nevertheless, secondary metabolism decreased with the increase in N application to 320 mg L^{-1} , as concentrations of most cannabinoids and terpenoids examined decreased even when N supply was optimal for plant function. Thus, we conclude that the decrease in secondary metabolite production as N supply increased from 160 mg L^{-1} to 320 mg L^{-1} is not due to the plant stress response as was suggested above. The decrease in secondary metabolite production at the range of 160 – 320 mg L^{-1} N may be a result of a physiological shift in plant metabolism: Under high N inputs, plant production shifted to create N-containing metabolites such as chlorophyll and amino acids, on account of the production of metabolites which do not contain N, such as cannabinoids and terpenoids. This hypothesis, which is supported by results for secondary metabolism in some other plant species (Albornoz, 2016; Bryant et al., 1983; Fritz et al., 2006; Rembialkowska, 2007), can explain the negative correlation we found between high N supply and secondary metabolite production (Fig. 3–5), and the positive correlations we found between high N supply and photosynthetic pigment content (Fig. 8) and leaves biomass (Fig. 2B).

Nitrogen is taken up by plants in two different forms, NO_3^- and NH_4^+ , which differ in their physiological and metabolic impact on the plant (Fageria, 2001; Hawkesford et al., 2012; Ups et al., 1990). Therefore, to receive a comprehensive understanding of N effects on plant function and secondary metabolism in cannabis, studies into responses to various NH_4/NO_3 ratios are required. In addition, as N use efficiency decreased significantly and dramatically with the increase in N supply (Fig. 2D), and despite the current market price of cannabis that is much higher than the price of N fertilizers, growers should take into account the reduction in plant N use efficiency and make a financial calculations accordingly.

5. Conclusions

From an agronomic perspective, effects on inflorescences biomass are key to production success. Our results demonstrate that N supply up to 160 mg L^{-1} , dramatically increased inflorescence biomass, while higher application levels did not induce an additional significant effect. These results are in accord with the overall physiological response of the plants. Thus, we conclude that 160 mg L^{-1} N is a sufficient concentration for maximal yield and higher N supply did not reduce yield. Taken together with the negative impacts obtained for high N supply on

secondary metabolite accumulation, we suggest that the optimal level for excelled yield quantity, as well as quality, i.e., high secondary metabolites profile, is 160 mg L⁻¹ N. As medical cannabis plant-science research activity is starting to accelerate, and the demand for agricultural and medical knowledge are soaring, the data obtained from this research may serve as the foundation for the understanding of medical cannabis nutrient requirements and physiological responses, and may be used to direct, precise, and improve medical cannabis cultivation worldwide.

CRediT authorship contribution statement

Nirit Bernstein: Conceptualization, Methodology, Supervision, Writing- Reviewing and Editing. **Avia Saloner:** Conducting the experiments, Data analyses, Writing- Original draft preparation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

The project was funded by the Chief Scientist Fund of the Ministry of Agriculture in Israel, Project No. 20-03-0018. We thank Dr. Mollie Sacks for advice concerning the design of the fertigation solutions; Yael Sade for assistance with cannabinoid analyses; Dr. Efraim Lewinsohn and Einat Bar for the terpenoid analyses and advice; Nadav Danziger, Sivan Shiponi, Geki Shoef, and Dalit Morad for technical assistance; Shiran Cohen for assistance with N and P analysis. We are grateful for the cooperation of Neri Barak from Cannodoc LTD, a certified commercial cultivation farm in Israel, and for the supply of the plant material for the study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.indcrop.2021.113516>.

References

- Albornoz, F., 2016. Crop responses to nitrogen overfertilization: a review. *Sci. Hortic.* (Amsterdam) 205, 79–83. <https://doi.org/10.1016/j.scienta.2016.04.026>.
- Andre, C.M., Hausman, J.F., Guerriero, G., 2016. *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7, 1–17. <https://doi.org/10.3389/fpls.2016.00019>.
- Aubin, M., Seguin, P., Vanasse, A., Tremblay, G.F., Mustafa, A.F., Charron, J., 2015. Industrial hemp response to nitrogen, phosphorus, and potassium fertilization. *Crop Forage Turfgrass Manage.* 1, 1–10. <https://doi.org/10.2134/cftm2015.0159>.
- Bernstein, N., Shores, M., Xu, Y., Huang, B., 2010. Involvement of the plant antioxidative response in the differential growth sensitivity to salinity of leaves vs roots during cell development. *Free Radic. Biol. Med.* 49, 1161–1171. <https://doi.org/10.1016/j.freeradbiomed.2010.06.032>.
- Bernstein, N., Gorelick, J., Koch, S., 2019a. Interplay between chemistry and morphology in medical cannabis (*Cannabis sativa* L.). *Ind. Crops Prod.* 129, 185–194. <https://doi.org/10.1016/j.indcrop.2018.11.039>.
- Bernstein, N., Gorelick, J., Zerahia, R., Koch, S., 2019b. Impact of N, P, K, and humic acid supplementation on the chemical profile of medical cannabis (*Cannabis sativa* L.). *Front. Plant Sci.* 10, 736. <https://doi.org/10.3389/fpls.2019.00736>.
- Blanch, J.S., Sampredo, L., Llusià, J., Moreira, X., Zas, R., Peñuelas, J., 2012. Effects of phosphorus availability and genetic variation of leaf terpene content and emission rate in *Pinus pinaster* seedlings susceptible and resistant to the pine weevil, *Hylobius abietis*. *Plant Biol.* 14, 66–72. <https://doi.org/10.1111/j.1438-8677.2011.00492.x>.
- Bócsa, I., Mathe, P., Hangyel, L., 1997. Effect of nitrogen on tetrahydrocannabinol (THC) content in hemp (*Cannabis sativa* L.) leaves at different positions. *J. Int. Hemp Assoc.* 4, 80–81.
- Bouchet, A.S., Laperche, A., Bissuel-Belaygue, C., Snowdon, R., Nesi, N., Stahl, A., 2016. Nitrogen use efficiency in rapeseed. A review. *Agron. Sustain. Dev.* 36, 1–20. <https://doi.org/10.1007/s13593-016-0371-0>.
- Bryant, J., III, F.C., Oikos, D.K., 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *U.*, 1983 *Oikos* 357–368.
- Caplan, D., Dixon, M., Zheng, Y., 2017. Optimal rate of organic fertilizer during the flowering stage for cannabis grown in two coir-based substrates. *HortScience* 52, 1796–1803.
- Chandra, S., Lata, H., ElSohly, M.A. (Eds.), 2017. *Cannabis sativa* L. - Botany and Biotechnology. Springer.
- Chishaki, N., Horiguchi, T., 1997. Responses of secondary metabolism in plants to nutrient deficiency. *Plant Nutrition for Sustainable Food Production and Environment*. Springer, Netherlands, pp. 341–345. https://doi.org/10.1007/978-94-009-0047-9_101.
- Choi, J.M., Pak, C.H., Lee, C.W., 1996. Micronutrient toxicity in French marigold. *J. Plant Nutr.* 19, 901–916. <https://doi.org/10.1080/01904169609365169>.
- Chouvy, P.A., 2019. Cannabis cultivation in the world: heritages, trends and challenges. *EchoGéo*. <https://doi.org/10.4000/echoge.17591>.
- Coffman, C.B., Gentner, W.A., 1975. Cannabinoid profile and elemental uptake of *Cannabis sativa* L. as influenced by soil characteristics 1. *Agron. J.* 67, 491–497. <https://doi.org/10.2134/agronj1975.00021962006700040010x>.
- Coffman, C.B., Gentner, W.A., 1977. Responses of greenhouse-grown *Cannabis sativa* L. to nitrogen, phosphorus, and potassium. *Agron. J.* 69, 832–836. <https://doi.org/10.2134/agronj1977.00021962006900050026x>.
- Danziger, N., Bernstein, N., 2021. Light matters: effect of light spectra on cannabinoid profile and plant development of medicinal cannabis (*Cannabis sativa* L.). *Indust. Crop Prod.* 194, 113351. <https://doi.org/10.1016/j.indrop.2021.113351>.
- Decorte, T., Potter, G.R., 2015. The globalisation of cannabis cultivation: a growing challenge. *Int. J. Drug Policy*. <https://doi.org/10.1016/j.drugpo.2014.12.011>.
- Dudai, N., Putievsky, E., Ravid, U., Palevitch, D., Halevy, A.H., 1992. Monoterpene content in *Origanum syriacum* as affected by environmental conditions and flowering. *Physiol. Plant.* 84, 453–459. <https://doi.org/10.1111/j.1399-3054.1992.tb04690.x>.
- Ehrensing, D.T., 1998. Feasibility of Industrial Hemp Production in the United States Pacific Northwest. *Station Bulletin* 681. Oregon State University, Corvallis, OR, USA.
- ElSohly, M.A., Slade, D., 2005. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sciences*. Pergamon, pp. 539–548. <https://doi.org/10.1016/j.lfs.2005.09.011>.
- Fageria, V.D., 2001. Nutrient interactions in crop plants. *J. Plant Nutr.* 24, 1269–1290. <https://doi.org/10.1081/PLN-100106981>.
- Flores-Sánchez, I.J., Verpoorte, R., 2008. Secondary metabolism in cannabis. *Phytochem. Rev.* 7, 615–639. <https://doi.org/10.1007/s11101-008-9094-4>.
- Fritz, C., Palacios-Rojas, N., Feil, R., Stitt, M., 2006. Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. *Plant J.* 46, 533–548. <https://doi.org/10.1111/j.1365-313X.2006.02715.x>.
- Gorelick, J., Bernstein, N., 2017. Chemical and physical elicitation for enhanced cannabinoid production in cannabis. In: Chandra, S., Lata, H., ElSohly, M.A. (Eds.), *Cannabis Sativa* L. - Botany and Biotechnology. Springer, Cham, Switzerland, pp. 439–456. <https://doi.org/10.1007/978-3-319-54564-6>.
- Hakulinen, J., Julkunen-Tiitto, R., Tahvanainen, J., 1995. Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism? *Trees* 9, 235–240. <https://doi.org/10.1007/BF00195278>.
- Hauck, R.D., Goyal, S.S., Huffaker, R.C., 1984. Nitrogen toxicity in plants. *Nitrogen in Crop Production*, pp. 97–118. https://doi.org/10.2134/1990_nitrogenincropproduction.c6.
- Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Skrumsager Möller, I., White, P., 2012. Functions of macronutrients. In: Marschner, P. (Ed.), *Marschner's Mineral Nutrition of Higher Plants*. Academic Press, pp. 135–190.
- Joyce, S.A., Lango, L., Clarke, D.J., 2011. The regulation of secondary metabolism and mutualism in the insect pathogenic bacterium *Photorhabdus luminescens*. *Advances in Applied Microbiology*. Academic Press Inc, pp. 1–25. <https://doi.org/10.1016/B978-0-12-387048-3.00001-5>.
- Koricheva, J., Larsson, S., Haukioja, E., Keinänen, M., Keinanen, M., 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. *Oikos* 83, 212. <https://doi.org/10.2307/3546833>.
- Kutman, U.B., Yıldız, B., Cakmak, I., 2011. Effect of nitrogen on uptake, remobilization and partitioning of zinc and iron throughout the development of durum wheat. *Plant Soil* 342, 149–164. <https://doi.org/10.1007/s11104-010-0679-5>.
- Lawlor, D.W., Lemaire, G., Gastal, F., 2001. Nitrogen, plant growth and crop yield. In: Lea, P., Morot-Gaudry, J.-F. (Eds.), *Plant Nitrogen*. Springer-Verlag, Berlin, pp. 343–367. https://doi.org/10.1007/978-3-662-04064-5_13.
- Lea, P., Morot-Gaudry, J.-F., 2001. *Plant Nitrogen*. Springer, Springer-Verlag, Berlin. <https://doi.org/10.1007/978-3-662-04064-5>.
- Leghari, S.J., Wahcho, N.A., Laghari, G.M., HafeezLaghari, A., MustafaBabhan, G., HussainTalpur, K., Bhutto, T.A., Wahcho, S.A., Lashari, A.A., 2016. Role of nitrogen for plant growth and development: a review. *Adv. Environ. Biol.* 10, 209–219.
- Lichtenhaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592.
- Lydon, J., Teramura, A.H., Coffman, C.B., 1987. UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206. <https://doi.org/10.1111/j.1751-1097.1987.tb04757.x>.
- McCauley, A., Jones, C., Jacobsen, J., 2009. Plant nutrient functions and deficiency and toxicity symptoms. *Nutr. Manage. Modul.* 1–16.
- Moghaddam, M., Mehdizadeh, L., 2017. Chemistry of essential oils and factors influencing their constituents. *Soft Chemistry and Food Fermentation*. Elsevier, pp. 379–419. <https://doi.org/10.1016/B978-0-12-811412-4.00013-8>.
- Ohyama, Takuji, 2010. Nitrogen as a major essential element of plants. In: Ohyama, T., Sueyoshi, K. (Eds.), *Nitrogen Assimilation in Plants*. Research Signpost, Kerala, pp. 1–17.
- Ormeno, E., Fernandez, C., 2012. Effect of soil nutrient on production and diversity of volatile terpenoids from plants. *Curr. Bioact.* 8, 71–79. <https://doi.org/10.2174/157340712799828188>.

- Palumbo, M.J., Putz, F.E., Talcott, S.T., 2007. Nitrogen fertilizer and gender effects on the secondary metabolism of yaupon, a caffeine-containing north American holly. *Oecologia* 151, 1–9. <https://doi.org/10.1007/s00442-006-0574-1>.
- Papastylianou, P., Kakabouki, I., Travlos, I., 2018. Effect of nitrogen fertilization on growth and yield of industrial hemp (*Cannabis sativa* L.). *Not. Bot. Horti Agrobot. Cluj-Napoca* 46, 197–201.
- Ramakrishna, A., Ravishankar, G.A., 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav.* 6, 1720–1731. <https://doi.org/10.4161/psb.6.11.17613>.
- Rand, K., Bar, E., Ben Ari, M., Davidovich-Rikanati, R., Dudareva, N., Inbar, M., Lewinsohn, E., 2017. Differences in monoterpene biosynthesis and accumulation in *Pistacia lentiscus* Leaves and aphid-induced galls. *J. Chem. Ecol.* 43, 143–152. <https://doi.org/10.1007/s10886-016-0817-5>.
- Rayar, A.J., van Hai, T., 1977. Effect of ammonium on uptake of phosphorus, potassium, calcium and magnesium by intact soybean plants. *Plant Soil* 48, 81–87. <https://doi.org/10.1007/BF00015159>.
- Rembiałkowska, E., 2007. Quality of plant products from organic agriculture. *J. Sci. Food Agric.* 87, 2757–2762. <https://doi.org/10.1002/jsfa.3000>.
- Rioba, N.B., Itulya, F.M., Saidi, M., Dudai, N., Bernstein, N., 2015. Effects of nitrogen, phosphorus and irrigation frequency on essential oil content and composition of sage (*Salvia officinalis* L.). *J. Appl. Res. Med. Aromat. Plants* 2, 21–29. <https://doi.org/10.1016/j.jarmap.2015.01.003>.
- Russo, E.B., 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.* <https://doi.org/10.1111/j.1476-5381.2011.01238.x>.
- Saloner, A., Bernstein, N., 2020. Response of medical cannabis (*Cannabis sativa* L.) to nitrogen supply under long photoperiod. *Front. Plant Sci.* 11, 1517. <https://doi.org/10.3389/fpls.2020.572293>.
- Saloner, A., Sacks, M.M., Bernstein, N., 2019. Response of medical cannabis (*Cannabis sativa* L.) genotypes to K supply under long photoperiod. *Front. Plant Sci.* 10, 1–16. <https://doi.org/10.3389/fpls.2019.01369>.
- Seigler, D.S., 2012. *Plant Secondary Metabolism*. Springer Science & Business Media.
- Shapira, A., Berman, P., Futoran, K., Guberman, O., Meiri, D., 2019. Tandem mass spectrometric quantification of 93 terpenoids in cannabis using static headspace injections. *Anal. Chem.* 91, 11425–11432. <https://doi.org/10.1021/acs.analchem.9b02844>.
- Shiponi, S., Bernstein, N., 2021. Phosphorus sensitivity of medical cannabis at the vegetative growth stage: impact on functional phenotyping and the ionome. *Indust. Crop Prod.* 161, 113154. <https://doi.org/10.1016/j.indcrop.2020.113154>.
- Tei, F., Benincasa, P., Guiducci, M., 1998. Nitrogen fertilisation of lettuce, processing tomato and sweet pepper: yield, nitrogen uptake and the risk of nitrate leaching. *International Workshop on Ecological Aspects of Vegetable Fertilization in Integrated Crop Production*, pp. 61–68.
- Uchida, R., 2000. Essential nutrients for plant growth: nutrient functions and deficiency symptoms. *Plant Nutr. Manage. Hawaii's soils* 31–55.
- Ups, S.H., Leidi, E.O., Silberbush, M., Soares, M.I.M., Lewis, O.E.M., 1990. Physiological aspects of ammonium and nitrate fertilization. *J. Plant Nutr.* 13, 1271–1289. <https://doi.org/10.1080/01904169009364151>.
- Vasconcelo, A., Boland, R., 2007. Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Sci.* 172, 861–875. <https://doi.org/10.1016/j.plantsci.2007.01.006>.
- Vera, C.L., Malhi, S.S., Raney, J.P., Wang, Z.H., 2004. The effect of N and P fertilization on growth, seed yield and quality of industrial hemp in the Parkland region of Saskatchewan. *Can. J. Plant Sci.* 84, 939–947.
- Vickers, C.E., Gershenson, J., Lerdau, M.T., Loreto, F., 2009. A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nat. Chem. Biol.* 5, 283–291. <https://doi.org/10.1038/nchembio.158>.
- Wink, M., 2008. Plant secondary metabolism: diversity, function and its evolution. *Nat. Prod. Commun.* 3, 1205–1216. <https://doi.org/10.1177/1934578x0800300801>.
- Wu, X., Riaz, M., Yan, L., Zhang, Z., Jiang, C., 2020. How the cells were injured and the secondary metabolites in the shikimate pathway were changed by boron deficiency in trifoliate orange root. *Plant Physiol. Biochem.* 151, 630–639. <https://doi.org/10.1016/j.plaphy.2020.04.009>.
- Yamada, Y., Sato, F., 2013. Transcription factors in alkaloid biosynthesis. *International Review of Cell and Molecular Biology*. Elsevier Inc., pp. 339–382. <https://doi.org/10.1016/B978-0-12-407695-2.00008-1>