Nyaradi II *et al*. (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 4, Article number 13941
DOI:10.15835/nbha52413941
Research Article

Influence of water shortage and N:P ratio on growth and seed chemical components of hemp (*Cannabis sativa* L.)

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Abstract

Seeds of industrial hemp have high nutritional and healthcare value, which can be further improved by proper cultivation methods. Biochemical properties of hemp seeds that are beneficial for human use can be modulated through variation of the molar ratio between the inorganic nitrogen and phosphorus supplied with the nutrient medium, as well as by creating a moderate water deficit during controlled irrigation. The aim of this study was to investigate the influence of two N:P ratios (i.e. 8 mM nitrogen as nitrate to 2 mM phosphorus as phosphate, and 3 mM nitrogen to 6 mM phosphorus), combined with two water supply regimes (sufficient and insufficient) on vegetative growth parameters, seed production, seed oil, protein, vitamin E, iron, zinc and manganese content of two industrial hemp cultivars ('Jubileu' and 'Zenit'). The experiments were performed in a greenhouse, under controlled conditions. Even if plants receiving a lower N:P ratio and lower water supply had a decreased growth index and the shoot fresh weight was smaller, no statistically significant difference could be registered in the seed production of the different experimental variants. The oil content of seeds could be increased by a lower N:P ratio and by water deficit, while seed protein content was higher upon a higher N:P ratio and upon water shortage, for both cultivars. The highest vitamin E content of hemp seed oil was found when plants were subjected to the combined influence of lowered N:P ratio and water insufficiency. The iron content of seeds showed no changes between the different experimental variants. In seeds of the 'Jubileu' variety the zinc content was increased by water shortage when the N:P ratio was lower, while the highest manganese content was measured when plants received sufficient water and lowered N:P ratio. As for the 'Zenit' variety, the highest zinc and manganese content of seeds was obtained when plants received through the nutrient solution 8 mM nitrate and 2 mM phosphate (i.e. the N:P ratio was 4:1). The findings may contribute to optimization of hemp cultivation, in a cost-effective and environmental-friendly approach, for the purpose of production of higher quality seeds for human and animal consumption, as well as for the cosmetic industry.

Keywords: essential micronutrients; growth index; seed oil content; vitamin E

Received: 04 Jun 2024. Received in revised form: 30 Sep 2024. Accepted: 25 Oct 2024. Published online: 11 Nov 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

At present there is an increased interest in cultivating industrial hemp not only for its fiber, but also for the nutritional and health-promoting qualities of its seeds, or as a natural building and insulating material (Andre *et al.*, 2016; Farinon *et al.*, 2020). It is considered one of the most ancient cultivated plants, being a multipurpose, low environmental impact crop, with a high productivity in the sense of high carbon assimilation rate with moderate inputs (Podder *et al.*, 2024). Currently, only domesticated and ruderal hemp plants exist, the wild-type form being considered extinct (Farinon *et al.*, 2020). Hemp (*Cannabis sativa* L.) is an annual herb with palmately compound leaves and small, greenish flowers. It is a typically dioecious species, but monoecious varieties have been also bred and are frequently cultivated. Seed-producing flowers are grouped in elongate spikelike clusters, while pollen-producing flowers form many-branched clusters. The erect stem can reach up to 3-4 metres (Rupasinghe *et al.*, 2020). Generally, its vegetative growth is favorized under long photoperiods, while the reproductive developmental stage is promoted by shorter photoperiods, and the vegetative development determines the reproductive potential (Saloner and Bernstein, 2020). Seed production has become more and more important along with the use of hemp as fiber crop for producing textile and paper or as a source of pharmacologically active ingredients for medicine. Global hemp seed production doubled from 2015 to 2020, from around 2700 tons to about 5400 tons, and hemp seeds have found several food and cosmetic applications that are already marketed in many European countries (Montero *et al.*, 2023). Hemp seeds may be eaten raw, sprinkled on salads or mixed in fruit smoothies. In recent years, seeds of industrial hemp (which produces very low amounts of tetrahydrocannabinol) are used world widely as a valuable new source of health-promoting nutraceuticals.

Availability of water and essential mineral nutrients, along with temperature and light quality, are determinant environmental factors for normal development and productivity of crop plants. Hemp grows best in sandy loam with good drainage. During the growing season it requires an average monthly rainfall of at least 65 mm. It is mainly cultivated in the temperate zone. For a good development it requires direct sunlight, daytime temperatures around 25 °C, and a loose, well-aerated substrate. For vigorous seedling development average amounts of 110 kg ha^{-1} nitrogen, 45 kg ha^{-1} phosphorus, 65 kg ha^{-1} potassium and 15 kg ha^{-1} of sulphur are required. A strong corelation between water uptake and nitrogen availability has been reported for many plant species, revealing that nitrogen and water uptake and transport are co-regulated and adjusted according to the nitrogen demand of the plant (Garcia-Gomez *et al.*, 2023). Nitrogen is usually the main limiting mineral nutrient for plant production, being an essential macronutrient needed mainly for the synthesis of proteins, nucleic acids, secondary azotoids (e.g. alkaloids), nicotinamide cofactors (NAD and NADP) etc. In many plant species it favors vegetative growth and its overdose delays flowering and seed establishment. Particularly for hemp, it was reported that higher nitrogen levels increase fiber yield of stems but impair fiber strength (Saloner and Bernstein, 2020). When coir-based substrates were used, it was found that the optimal nitrogen supply for vegetative growth of hemp is 390 mg N L^{-1}, with a N:P:K ratio of 4:1.3:1.7, while during the flowering stage the optimal nitrogen supply was 283 mg L^{-1}, with a N:P:K ratio of 2:0.9:3.3 (Caplan *et al.*, 2017a and 2017b). In an attempt to optimize nitrogen, phosphorus and potassium dosage in soilless growth systems of hemp, it was concluded that while nitrogen and phosphorus concentrations have an important influence on seed production, the inflorescence and seed yield of hemp did not respond to variations in potassium supply in the tested range between 60 and 340 mg L^{-1} (Bevan *et al.*, 2021). In another set of experiments performed with hemp under long photoperiod it was established that the best nitrogen supply for vegetative growth is 160 mg L^{-1} nitrogen as nitrate, and it was also observed that nitrogen use efficiency decreased with increasing N supply (Saloner and Bernstein, 2020). The highest seed yield of hemp was obtained when plants were supplied with 240 kg N ha^{-1}, and the magnitude of response to different rates of nitrogen fertilization was sensitive to the available soil moisture, to the growing period and to the degree of weed pressure. Because nitrogen fertilizers are rather expensive, contribute to greenhouse gas emission and excessive nitrogen supply for crop plants can lead to environmental pollution (e.g. eutrophication of inland water bodies), it is essential to develop in the near future crop-specific and site-specific nitrogen fertilizer management strategies (Podder *et al.*, 2024). In experiments performed for the optimization of the NPK ratio for the vegetative growth of another plant species (*Aeschynanthus longicaulis* L.) on soilless media it was concluded that the best molar ratio is 3N:2P:2K (Deng and Li, 2022). The mostly available nitrogen sources for plants are nitrate and ammonium, but if present in the rhizosphere, simple organic nitrogen compounds, such as urea and amino acids, can also be used. Nitrate has to be reduced to nitrite and then to ammonium, and the latter will be assimilated into newly synthesized amino acids, with the contribution of the glutamine synthase and glutamate-oxoglutarate amino transferase enzyme pair. Ammonium is directly assimilated, but in higher concentration it can be toxic if it is converted into ammonia (Masclaux-Daubresse *et al.*, 2010; Gao *et al.*, 2022).

Phosphorus is another essential inorganic macronutrient which may limit plant growth mainly because of its low solubility in water when the pH of the soil solution is between 8 and 9 or lower than 4. It is an essential nutrient because it is involved in the conservation of chemical energy, because it activates several metabolites (e.g. carbohydrates and proteins) through phosphorylation, it is a constant structural component of phospholipids (mainly in bio membranes) and nucleic acids, and it is the main buffer for pH stabilization in different cell compartments. It also plays key roles in regulating physiological responses of plants to several abiotic stressors. For many plant species, a higher nitrogen to phosphorus ratio supports vegetative growth, a lower N:P ratio is needed for early initiation of flowering and seed production (Khan *et al.*, 2023). Water shortage also shortens the period of vegetative development and accelerates flowering. Regulation of aquaporin quantity and functional state, mainly in the plasma membrane and in the tonoplast of plant cells, along with osmotic adjustments and changes in stomatal conductance, represents a typical reaction of terrestrial plants during drought acclimation. When the efficiency of water use was determined by the transpiration coefficient, it was found that low levels of the available water increased the inflorescence production of hemp plants (Ortiz-Delvasto *et al.*, 2023).

Many recent investigations focus on the seed oil production and on the bioactive compounds in the hemp seeds, trying to develop cultivation protocols that enable the manipulation of hemp seed metabolism in order to increase the biosynthesis of polyunsaturated fatty acids, of lipophilic vitamins, of certain inorganic micronutrients and of other health-promoting substances, aimed to improve the quality of hemp seeds and of the seed oil in the context of human use as dietary supplement or as healthcare products. Cannabinoids, terpenoids, flavonoids and fatty acids are among the most relevant metabolite groups in this context (Izzo *et al.*, 2020; Monteiro *et al.*, 2023). The nutritional value of hemp seeds is given mainly by the high proportion (about 88%) of unsaturated fatty acids in the seed oil, with linoleic acid as the most abundant representative (Bates *et al.*, 2013; Spirovic-Trifunovic *et al.*, 2021). The unique ratio in ω-6/ω-3 fatty acids, represented mainly by linoleic acid and α-linolenic acid is considered optimal for a healthy human diet (Izzo *et al.*, 2020). The proteins present in hemp seeds are easy to digest and consist mainly of albumin and edestin. Carbohydrates make up an average of 20-25% of the fresh weight of hemp seeds and are represented mainly by dietary fiber with beneficial influence on the functioning of human digestive system. The predominant essential metals in the hemp seeds are potassium, calcium and magnesium. The flour obtained from hemp seeds has certain superior characteristics to wheat flour, for example because of a much higher protein and lipid content (Rusu *et al.*, 2021). Hemp oil also contains vitamins that are soluble in lipids (provitamin A represented by certain carotenoids, vitamin E represented by tocopherols and tocotrienols, and vitamin K_1 or phylloquinone). The most important of them is vitamin E, because it is a very efficient antioxidant. It can scavenge harmful reactive oxygen species, such as the singlet oxygen, the hydroxyl radical or the alkyl-hydroperoxyl radicals resulting from the oxidative degradation of unsaturated fatty acids. When applied externally, vitamin E has a beneficial influence on human skin and hair, and as a dietary component (being an essential organic micronutrient) it has a protective role against oxidative stress in the human cells, delaying cell aging and reducing the risk of tumor initiation. Usually, from the vitamin E vitamers γ-tocopherol is the most abundant, and it is also the most efficient antioxidant, with a major contribution to the stability of hemp oil during storage (Farinon *et al.*, 2020; Montero *et al.*, 2023). The psychoactive Δ-9-tetrahydro-cannabinol and the non-psychoactive cannabidiol should not be present in hemp seeds, because these alkaloids are synthesized and stored in epidermal glandular trichomes which are not present on the surface of seeds (Andre *et al.*, 2016).

The purpose of this study was to investigate the influence of two N:P ratios (i.e. 8 mM nitrogen as nitrate to 2 mM phosphorus as phosphate, and 3 mM nitrogen to 6 mM phosphorus), combined with two water supply regimes (sufficient and insufficient) on vegetative growth parameters, seed production, seed oil, protein, vitamin E, iron, zinc and manganese content of two industrial hemp cultivars ('Jubileu' and 'Zenit'), in an effort to improve in a cost-effective and environmental-friendly manner the chemical composition of hemp seed oil aimed for human uses, without severely impairing plant development and seed yield. Another objective, derived from the previous one, was to compare the two hemp cultivars, in order to detect similarities or differences in their reaction to two different nitrogen and phosphorus availability regimes, as well as to two water supply degrees. Based on the fact that growth, seed production and chemical composition of seeds exhibit large variations depending on developmental and nutritional factors (Tang *et al.*, 2016), mainly on the availability of inorganic macronutrients and water, our hypothesis was that changes in nitrogen and phosphorus supply, as well as in water availability, will lead to physiological adaptations reflected in modified growth dynamics, seed yield and seed chemical composition. A higher nitrogen supply is supposed to favour vegetative growth and protein synthesis, while lower nitrogen availability would lead to a stunted growth and to a metabolic shift in favour of lipid synthesis instead of protein accumulation in seeds. Water shortage would induce physiological changes that enhance water absorption (together with an increased uptake of water-soluble mineral nutrients) and reduce water loss by transpiration. Water insufficiency, combined with lower nitrogen and higher phosphorus supply, would result in metabolic modifications which favour the biosynthesis of hydrophobic lipids and water-insoluble metabolites, such as vitamin E. Thus, our presumption is that changes in nitrogen to phosphorus ratio and in water supply may induce metabolic modulations that lead to an improved biochemical composition and enhanced health-promoting qualities of hemp seeds.

Materials and Methods

Biological material

Two varieties of the industrial hemp species (*Cannabis sativa* L.), namely 'Jubileu' and 'Zenit' were used in the experiments, both of them being bred in the Agricultural Research and Development Station Secuieni (Neamț County, Romania). They belong to the approximately 70 hemp varieties accepted for cultivation and use in the European Union (EC regulation 809/2014), and to satisfy the European legislation requirements for industrial hemp varieties their Δ9-tetrahydrocannabinol (THC) content is lower than 0.2% by dry weight (Farinon *et al.*, 2020; Rusu *et al.*, 2021). Both are monoecious hemp varieties. 'Jubileu' and 'Zenit' are two hemp varieties created mainly for seed production and homologated in Secuieni (Romania), and their reactions to variations of growth conditions are largely unknown. This is why this study intends to compare their ability to respond to changes in N:P ratio and in water supply, and to find out which one is more suitable for production of seeds with higher nutraceutical quality. The dimensions of the major and minor axes of hemp seeds varied between 3.6-3.9 mm and 2.7-2.9 mm, respectively. The thousand seed mass was between 15.9 and 16.7 g. Under conditions of low positive temperature and low air humidity (when stored at 8 °C with a moisture content lower than 12%), the average viability of hemp seeds is around 5.5 years. In some cases, dormant hemp seeds can stay viable in soils for up to ten years (Sacilik *et al.*, 2003). The seeds were purchased from the above-mentioned research station and were produced one year before the start of the experiments. Until germination they were stored in a cool and dry place. Their germination capacity was higher than 90%, but the germination synchrony was low, the time spread of germination (between the first and last radicle emergence event in the seed lot) was 5 days. Before germination the seeds were immersed for one day in dechlorinated tap water at room temperature. Seeds of the two varieties have been germinated in germination vessels, at ambient temperature and in dim light. One week after emergence forty similarly developed plantlets were selected from each variety to be transplanted one by one in their growth vessels. Treatments were starter two weeks after the initiation of germination.

Experimental design

The experiments were performed under controlled greenhouse conditions, each plant was grown separately in its own vessel filled with a substrate consisting of seven parts of washed sand, two parts of perlite and one part of vermiculite, to ensure a good water retention and also a good aeration around the roots. The substrate of each growth vessel was obtained by mixing 14 L of sand with 4 L of perlite and 2 L of vermiculite with medium particle size. The site where the greenhouse experiments were performed belongs to the experimental field of the department of horticulture of the Sapientia University in Târgu Mureș (46.521543° N, 24.598268° E), Romania, geographically being included in the Târnave plateau, with a temperate continental climate.

There were four experimental variants, each with ten independent repetitions, for both hemp varieties. The place of the eighty growth vessels with plants was changed weekly, according to the complete randomized block experimental design. One set of ten plants from each variety, considered the control lot, was provided weekly with half a liter during the first four weeks and, as the plants got bigger, with one liter of Hoagland's nutrient solution (Hoagland and Arnon, 1950) with 8 mM of nitrogen (6 mM as KNO_3 and 2 mM as $Ca(NO_3)_2 \cdot 2H_2O$) and 2 mM of phosphorus (in the form of KH_2PO_4), thus ensuring a N:P molar ratio of 4:1 (4N1P). These plants also received 900 mL of water per day through drip irrigation, this quantity being considered, based on previous experiments, sufficient for the daily water need of one hemp plant. Another experimental variant was supplied with the same volume of Hoagland's solution with modified nitrogen and phosphorus concentrations: 3 mM nitrogen (2 mM as KNO_3 and 1 mM as $Ca(NO_3)_2 \cdot 2H_2O$) and 6 mM phosphorus as KH_2PO_4, the N:P molar ratio being in this case 1:2 (1N2P). The potassium and calcium concentration of the original Hoagland solution was compensated with 4 mM KCl and 1 mM $CaCl_2$. These plants were also provided with sufficient water, i.e. 900 mL per day per plant. The third group received the same nutrient solution (with 4N1P) in the same amount with the first group, but the water supply was reduced to one third, i.e. to 300 mL of water per day per plant, which was previously demonstrated to be insufficient for the normal growth of a hemp plant under the given conditions, but without inducing a severe drought stress. The fourth experimental variant consisted of plants provided with the modified Hoagland solution (with 1N2P, in the same quantity as the second variant) and with only 300 mL of water per day per plant. The initial pH value of both nutrient solutions was set to 5.6, in order to ensure the water-solubility of every essential inorganic macro-, micro- and ultra micronutrient. The pH and the electrical conductivity of the solutions leaching from the bottom of cultivation vessels were measured weekly (with a portable multimeter from Hanna Instruments) to detect pH variations in time and a possible accumulation of inorganic ions unused by the plants. Plants were provided with the above-mentioned nutrient solutions and water regimes for eight weeks during summertime (from middle of July to middle of September), and because treatments started two weeks after germination, the final investigations were made on ten weeks old plants. During the vegetation period, the highest photon flux density at noon, measured on the surface of upper leaves was 1080 µM m^{-2} s^{-1}, while in cloudy days the light intensity at noon was between 630-720 µM photons m^{-2} s^{-1} (measured with a Hansatech quantum sensor). The relative air humidity in the greenhouse was between 60-80%. In the period of the experiments the lowest night temperature was 16 °C and the highest day temperature was 32 °C. In summary, this was a bi-factorial experiment with factor C: the cultivar (with two levels: 'Jubileu' and 'Zenit'), and factor T: the treatment (with four levels: 4N1P – 8 mM N and 2 mM P, with 900 mL water per day per plant, 1N2P – 3 mM N and 6 mM P, with 900 mL water per day per plant, 4N1P–H_2O – 8 mM N and 2 mM P, with 300 mL water per day per plant, and 1N2P–H_2O – 3 mM N and 6 mM P, with 300 mL water per day per plant. From the combination of the two factors (C x T) resulted eight experimental variants, each with ten plants (Table 1).

Table 1. The experimental design

'Jubileu' hemp cultivar, 8 mM N + 2 mM P, 900 mL water day^{-1} $plant^{-1}$ (4N1P), 10 plants	'Jubileu' hemp cultivar, 3 mM N + 6 mM P, 900 mL water day^{-1} $plant^{-1}$ (1N2P), 10 plants	'Jubileu' hemp cultivar, 8 mM N + 2 mM P, 300 mL water day^{-1} $plant^{-1}$ (4N1P–H_2O), 10 plants	'Jubileu' hemp cultivar, 3 mM N + 6 mM P, 300 mL water day^{-1} $plant^{-1}$ (1N2P–H_2O), 10 plants
'Zenit' hemp cultivar, 8 mM N + 2 mM P, 900 mL water day^{-1} $plant^{-1}$ (4N1P), 10 plants	'Zenit' hemp cultivar, 3 mM N + 6 mM P, 900 mL water day^{-1} $plant^{-1}$ (1N2P), 10 plants	'Zenit' hemp cultivar, 8 mM N + 2 mM P, 300 mL water day^{-1} $plant^{-1}$ (4N1P–H_2O), 10 plants	'Zenit' hemp cultivar, 3 mM N + 6 mM P, 300 mL water day^{-1} $plant^{-1}$ (1N2P–H_2O), 10 plants

Seeds were collected in plastic cones fixed around the stem of plants, below the inflorescences. The final fresh and dry weight of plants was measured after the seeds were collected separately.

Measurements of growth and yield parameters

From several growth parameters measured weekly or at the end of the 56 days (eight weeks) of treatment, the most relevant ones were selected for evaluation, i.e. stem height, growth index, shoot fresh weight, seed yield per plant and seed dry weight. Stem height was measured for each plant once in seven days with a tape measure from the base to the shoot apex. Plant spread was measured at the widest point of the shoot system (width 1), and then perpendicular to this measurement (width 2). Growth index was calculated with the formula (height × width 1 × width 2) / 300 (Caplan *et al.*, 2017a). To assess the aboveground fresh weight, 70 days old plants were cut at substrate level (after collection of seeds) and individually weighed immediately on a digital scale. Fresh weight of seeds collected from each plant was measured with an analytical scale, and to determine the dry weight and water content, 1 g of seeds from each plant was dried at 70 °C for 72 h (when they reached a constant biomass) and re-weighted (Bevan *et al.*, 2021).

Extraction and determination of the oil content of hemp seeds

Oil extraction from hemp seeds was performed with the Soxhlet method, optimized for several characteristics of the stored lipid content of seeds (Avram *et al.*, 2014; Bokhari *et al.*, 2015; Al-Sumri *et al.*, 2016; Khilari and Sharma, 2016). 1.5 g of freshly collected hemp seeds were ground to fine powder, mixed with 100 mL of n-hexane as an organic solvent for lipids, and heated to 85 °C in a Soxhlet apparatus, taking into account that surface area given by the bed and the contact time of seed powder with the solvent are the two major factors that determine the extraction of the entire oil content. Oil separation was performed with fifteen cycles of percolation for each seed sample, to ensure a complete extraction of the oil content. At the end of extraction, the pot content was distilled at 65 °C to separate the oil from the solvent. The oil obtained after removal of the solvent was weighed on an analytical scale, and the oil content was expressed as percent of the seed weight used for extraction (Bokhari *et al.*, 2015).

Determination of protein content of hemp seeds

Protein content of seeds was determined photometrically with Bradford's method. Color formation in the Bradford protein assays is associated with the presence of certain universal amino acids that exist in every protein macromolecule, so this assay serves to determine the total protein content of biological extracts (Bradford, 1976). 0.1 g of dried hemp seeds were homogenized in a mortar with 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0), which contained 1 mM $Na_2EDTA \cdot 2H_2O$, 1 mM ascorbic acid (reduced form) and

2% polyvinyl-pyrrolidone. The extract was centrifuged at 2300 g and 4 °C for 20 min, then 400 μL of the supernatant was put in a cuvette and supplemented with 2 mL of Bradford reagent containing 0.5 mg mL^{-1} Coomassie Brillant Blue G250 stain, 25% methanol and 42.5% orthophosphoric acid. The mixture was incubated for 5 min at room temperature, then the absorbance was measured at 595 nm. The blank contained 400 μL of extraction buffer instead of seed extract. The protein content was determined with a standard curve obtained with different concentrations of bovine serum albumin and was expressed as percent of the dry weight. Bovin serum albumin was suspended in the same reaction mixture as the above-mentioned biological samples, ensuring a basic pH and the lack of surfactants. The assay is linear in the range of 0-2000 μg mL^{-1} (Bradford, 1976).

Determination of vitamin E content of hemp oil

Vitamin E content of the oil extracted in the Soxhlet apparatus with n-hexane from the fresh hemp seeds was determined photometrically, based on its reducing property in the presence of oxidized forms of transition metal ions (Tutem *et al*., 1997; Kivcak and Mert, 2001). To 8 mL of extracted hemp seed oil (after evaporation of the extraction solvent) 1 mL of 2,2'-dipyridyl reagent was added (the reagent was prepared by dissolving 10.125 g 2,2'-dipyridyl in 25 mL absolute ethanol). The mixture was thoroughly shacked, then it was supplemented with 1 mL ferric chloride solution (prepared by dissolving 0.2 g $FeCl_3 \cdot 6H_2O$ in 100 mL absolute ethanol), mixed again and after 1 min of incubation the absorbance was measured at 522 nm. The reference sample contained 8 mL of n-hexane instead of hemp oil. Vitamin E content was determined by using a standard curve obtained with different known concentrations of vitamin E and was expressed as mg per 100 g oil.

Determination of zinc, iron and manganese content of hemp seeds

Determination of the quantities of zinc, iron and manganese in the hemp seed samples was performed with the very sensitive method of microwave plasma-atomic emission spectrometry (MP-AES), developed for measurement of low concentrations of metal ions and other trace elements in dried plant samples or in solutions (Ozbek and Akman, 2016; Balaram, 2020). Seeds were washed with distilled water, dried for 48 h at 70 °C, ground to fine powder and sifted through a sieve with pores of 315 μm in diameter. 0.4000 g of dried seed powder was macerated, in a closed system and in two steps (at 170 °C and at 200 °C) with a mixture of 6 mL of 65% HNO_3 (Merck, Suprapur) and 2 mL of 30% H_2O_2 (Chempur, p.a.). The obtained clear solution was diluted to 50 mL with ultrapure water and used for determination of zinc, iron and manganese content. The nitrogen plasma of the atomic emission spectrometer (MP-AES 4210) ensures a temperature of 4500 °K for atomization and excitation of the samples. A CCD detector transforms the analytical signal into an electrical signal. The concentration of trace metals can be determined by use of calibration curves obtained with standard solutions. Samples and standards are supplemented with CsCl as an ionization suppressor, at a final concentration of 1 g L^{-1}. The wavelengths for atomic lines were 213.857 nm for Zn, 371.993 nm for Fe and 403.076 nm for Mn.

Statistical analysis

There were ten replicates for every experimental variant, for both hemp varieties. Measurements of growth and yield parameters were performed with every individual plant, while chemical components' determinations from seeds were done with five repetitions. For statistical analysis of the experimental data the R software environment (version 4.1.0) was used. The Shapiro-Wilk test was used for analysis of normality of the distribution of data sets, and Bartlett's test was applied for evaluating the homogeneity of variances. The standard deviations from means (±SD) were calculated to describe the distribution of the determined values. Data sets with normal distribution were evaluated with one-way ANOVA and the Tukey HDS post-hoc test, while for data that were not normally distributed the Kruskal-Wallis test and the Mann-Whitney pairwise test was used to identify significant differences between the means. The threshold for statistical significance of differences was $P < 0.05$.

Results

Influence of two different nitrogen to phosphorus ratios and of water insufficiency on growth and yield parameters of two hemp varieties

Modification of the nitrate to phosphate ratio from 4:1 (with 8 mM N and 2 mM P) to 1:2 (3 mM N and 6 mM P) did not result in any significant change in the stem height of hemp plants during the eight weeks of treatment. This was valid for both industrial hemp varieties used in the study, i.e. 'Jubileu' and 'Zenit' (Figure 1A and Figure 1B). Irrespective of the N:P molar ratio in the nutrient solution, water shortage (represented by a daily supply of only 300 mL water per plant instead of 900 mL) inhibited stem elongation and resulted in significantly smaller plants starting from the third week of treatment for the 'Jubileu' variety and from the second week in the case of 'Zenit'. The shortest stems were measured when water shortage was combined with the 4:1 nitrogen to phosphorus ratio for 'Jubileu' and in the variant where water insufficiency came together with a lower, 1:2 nitrogen to phosphorus ratio for 'Zenit', but there were no significant differences between the plants receiving the two different N:P ratios under water insufficiency (Figure 1B).

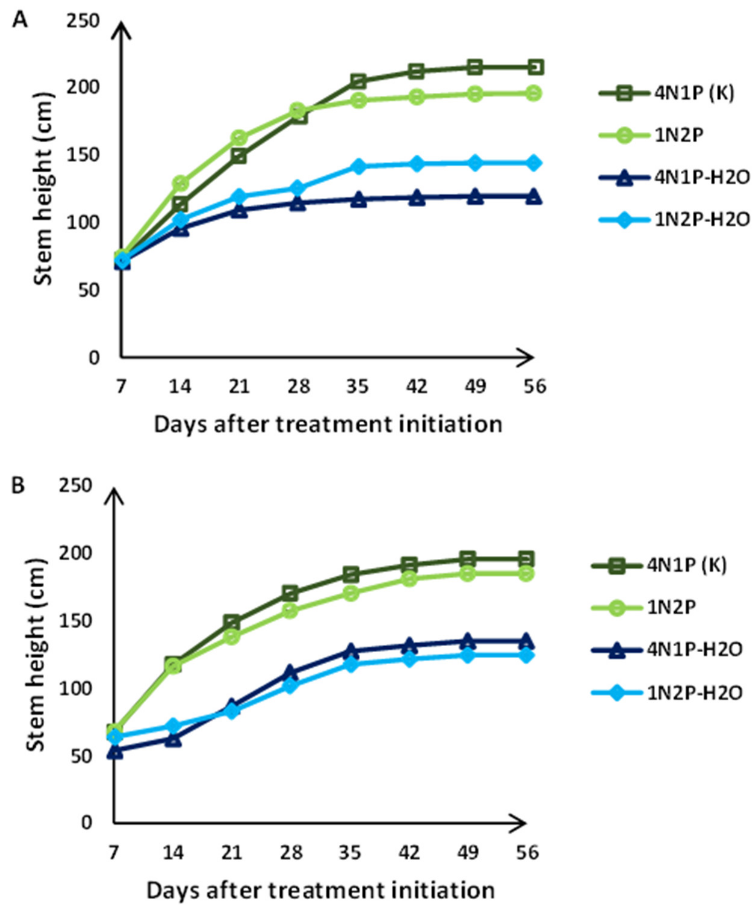

Figure 1. Influence of two different nitrogen to phosphorus molar ratios of the nutrient solution and of water shortage on evolution of stem height of the 'Jubileu' (A) and 'Zenit' (B) hemp cultivars over 56 days of treatment. 4N1P (K) – 4N : 1P ratio, 1N2P – 1N : 2P ratio, -H_2O – water insufficiency (one third of the control, K). Vertical lines represent ±SD from means (n = 10)

The growth index of plants at the end of the 56 days of treatments (when hemp plants reached the age of 70 days from germination) was influenced significantly by the N:P ratio in the nutrient solution only in the case of 'Zenit' variety, the value of this index being smaller for plants receiving a lower nitrogen to phosphorus molar ratio (1:2 instead of 4:1). For both hemp varieties taken into study, the growth index was decreased when water shortage was associated with the 4:1 nitrogen to phosphorus ratio, while the lowest value of this growth parameter was registered when water insufficiency was combined with the lower N:P ratio (Table 2). Under conditions of sufficient water supply, the absolute values of growth index were higher for the 'Jubileu' variety, but this difference could not be observed when plants were cultivated under water deficiency and 1N:2P ratio.

The aboveground shoot fresh weight of 70 days old hemp plants, after collection of seeds, was the highest when the nutrient solution contained 8 mM nitrate and 2 mM phosphate (4N1P), for both varieties. For 'Jubileu' a rather similar degree of reduction of fresh biomass was registered under water sufficiency and lower N:P ratio (1N2P) as well as under water shortage combined with the higher N:P ratio (4N1P). The mostly pronounced decrement of shoot weight for 'Jubileu' was registered for plants receiving the lower N:P ratio under water insufficiency. In the case of 'Zenit' the lowest shoot fresh biomass was measured when plants were exposed for 56 days to water shortage, irrespective of the N:P ratio. Under sufficient water supply, the accumulated fresh weight was significantly smaller in the variant with 1N2P ratio than in the case of 4N1P (Table 2).

Table 2. Influence of nitrogen to phosphorus ratio and of water shortage on growth parameters of two hemp cultivars

Hemp cultivar	Experimental variant	Growth index	Shoot fresh weight of 70 days old plants (g)	Seed yield per plant (g)	Seed dry weight (% of fresh weight)
'Jubileu'	4N1P	191.2±11.4^{a}	121.32±16.07^{a}	17.376±2.104^{a}	90.48 ± 0.31^{b}
	1N2P	166.4±23.0^{a}	84.24±7.91^{b}	17.919±1.787^{a}	91.59 ± 0.48^{a}
	4N1P–H_2O	114.8±18.9^{b}	69.31±5.16^{c}	16.823±1.639^{a}	91.69 ± 0.26^{a}
	1N2P–H_2O	58.6±17.7^{d}	46.98±6.32^{d}	17.164±3.618^{a}	92.11 ± 0.37^{a}
'Zenit'	4N1P	179.6±9.2^{a}	134.17±21.41^{a}	15.366±1.419^{a}	90.52 ± 0.33^{b}
	1N2P	137.2±11.7^{b}	88.04±3.97^{b}	16.884±2.317^{a}	90.06 ± 0.41^{b}
	4N1P–H_2O	83.0±3.4^{c}	58.28±5.11^{c}	14.972±2.206^{a}	92.19 ± 0.34^{a}
	1N2P–H_2O	66.2±2.5^{d}	64.85±4.32^{c}	16.216±3.783^{a}	92.23 ± 0.16^{a}

Legend: 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, –H_2O – water shortage

Different letters denote, separately for every parameter and for each cultivar, significant differences at $P < 0.05$ (Tukey's HDS test or Mann Whitney test).

The seed yield per plant, determined on a fresh weight basis, was not significantly modified by water availability and by the two different nitrogen to phosphorus molar ratios in the nutrient solution, being around 17 g per plant for 'Jubileu' and around 15-16 g per plant for the 'Zenit' variety. A tendency toward an increasing seed yield under the lower N:P ratio as compared to the higher one could be noticed for both hemp varieties, irrespective of sufficient or deficient water supply, but the changes were statistically not significant, so a clear difference could not be established. The freshly collected hemp seeds had a very low water content (less than 90%), and even if the differences were moderate, a statistically significant increase in the dry weight of seeds could be evidenced for both varieties when plants were grown under conditions of water shortage, irrespective of the received N:P ratio (Table 2).

Influence of two N:P molar ratios and of water shortage on the seed oil content of two hemp varieties

The oil content of freshly harvested hemp seeds was around 30% on a fresh weight basis. In the case of 'Jubileu' variety it was higher when plants received a nutrient solution with lower nitrogen to phosphorus molar ratio (1N2P), as compared to the higher N:P ratio (4N1P), irrespective of sufficient or deficient water supply (Figure 2). For plants receiving a higher N:P ratio, a moderate, but statistically significant increment of seed oil content could be observed when plants were grown under water shortage. In the case of 'Zenit', only plants receiving a higher N:P ratio under sufficient water supply developed seeds with lower oil content than the others.

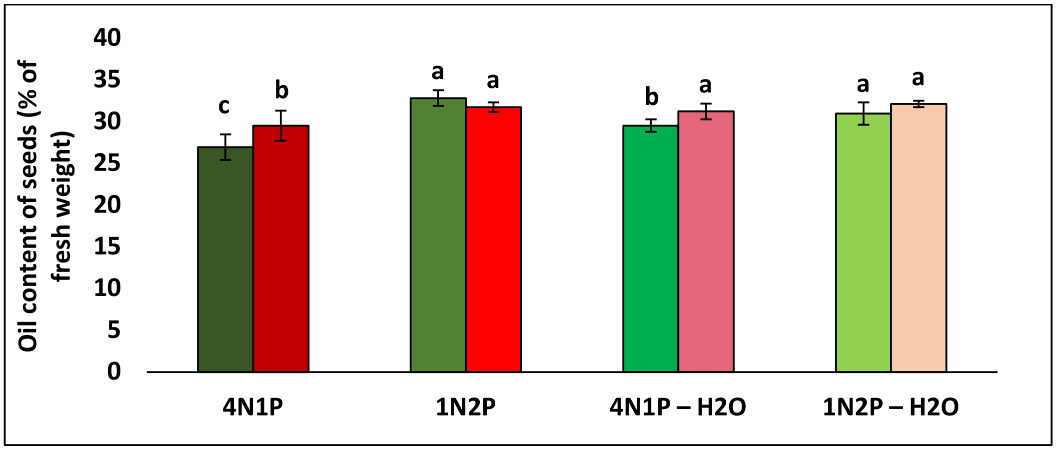

Figure 2. Influence of N:P ratio and water shortage on the oil content of seeds produced by two hemp cultivars: 'Jubileu' (in nuances of green) and 'Zenit' (in nuances of red). 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, $-H_2O$ – water shortage
Vertical lines represent ±SD from means (n = 5), different letters denote significant differences at $P < 0.05$

Influence of two N:P molar ratios and of water shortage on the seed protein content of two hemp varieties

Protein content of hemp seeds was for both varieties between 25-35% of the dry weight (Figure 3). It was the highest for plants grown under water shortage associated with a higher nitrogen to phosphorus ratio (4N1P, represented by 8 mM nitrate and 2 mM phosphate in the nutrient solution).

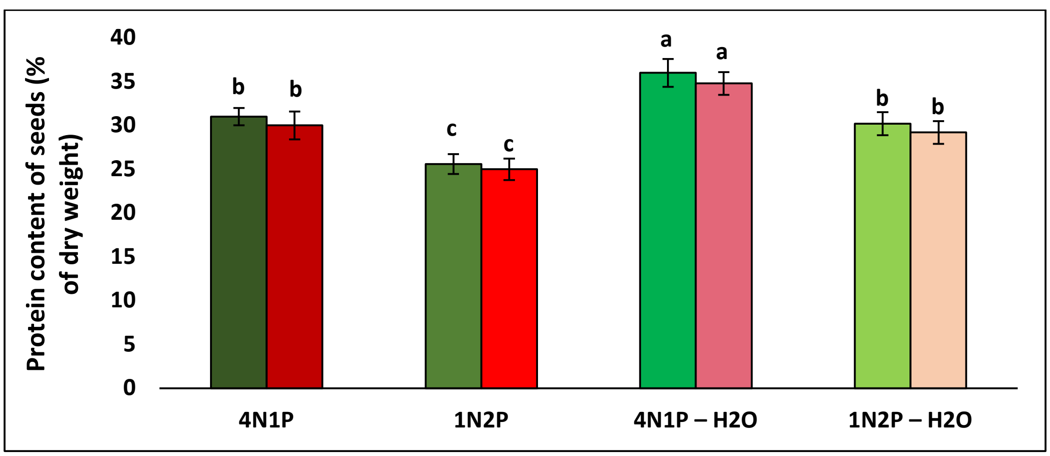

Figure 3. Influence of N:P ratio and water shortage on the protein content of seeds produced by two hemp cultivars: 'Jubileu' (in nuances of green) and 'Zenit' (in nuances of red). 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, $-H_2O$ – water shortage
Vertical lines represent ±SD from means (n = 5), the letters denote significant differences at $P < 0.05$

When water insufficiency was combined with a lower N:P ratio (3 mM nitrate and 6 mM phosphate), the protein content of seeds was similar to the one determined for plants receiving sufficient water under a higher N:P ratio. When water shortage was not a disturbing factor, the seed protein content was higher under conditions of elevated N:P ratio, as compared to the lower nitrogen to phosphorus ratio. These findings were valid for both hemp varieties used in the experiments.

Vitamin E content of the oil stored in the seeds of two hemp varieties, grown in the presence of different N:P ratios and under water insufficiency

Vitamin E content of the extracted seed oil was higher when hemp plants belonging to the 'Jubileu' variety were grown under conditions of water shortage, especially when water insufficiency was associated with a lower nitrogen to phosphate ratio (Figure 4). Under these conditions the vitamin E content was as high as 80-90 mg in 100 g of oil. Under water sufficiency the presence of a lower N:P ratio (1N2P) resulted in a higher vitamin E content of seed oil than in case of the higher N:P ratio in the nutrient solution (4N1P). When plants receiving the higher N:P ratio were subjected to water shortage, the vitamin E content was like the one determined in the seed oil of plants grown with lower N:P ratio and supplied with sufficient water.

The oil extracted from seeds of the 'Zenit' hemp variety had a higher vitamin E content (between 90 and 140 mg per 100 g) that the oil stored in seeds of 'Jubileu' (50-90 mg per 100 g). The lowest quantity of vitamin E was determined when plants received the nutrient medium with higher N:P ratio and were not exposed to water shortage, while the highest vitamin E content was obtained from seeds of plants grown under water deficiency and lower N:P ratio. The oil of seeds developed under lower N:P ratio and sufficient water supply had similar vitamin E content with the one extracted from seeds formed on hemp plants exposed to higher N:P ratio combined with water deficit.

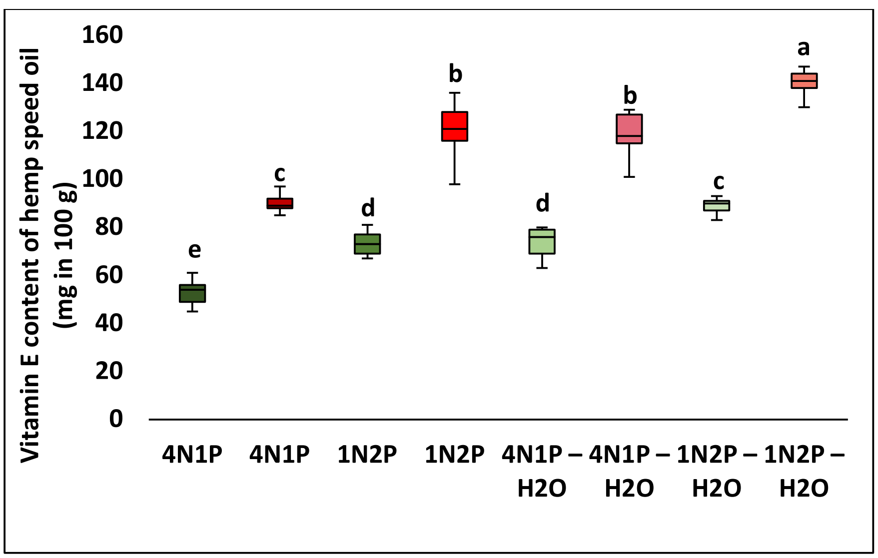

Figure 4. Influence of N:P ratio and water shortage on the vitamin E content of seed oil of two hemp cultivars: 'Jubileu' (in nuances of green) and 'Zenit' (in nuances of red). 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, –H_2O – water shortage
Vertical lines represent ±SD from means (n = 5), different letters denote significant differences at $P < 0.05$

Zinc, iron and manganese content of seeds of two hemp varieties exposed to different N:P ratios and to water shortage

Zinc content of hemp seeds, expressed on a dry weight basis, was in the case of 'Jubileu' variety as high under water shortage and lower N:P ratio as with higher N:P ratio of the nutrient medium and sufficient water supply to the plants during the growth period (Figure 5). A lower zinc content was found when the nitrogen to phosphorus molar ratio was lower (1:2) and there was no water shortage, as well as in the seeds of plants grown under water insufficiency and higher nitrogen to phosphorus ratio (4:1). In the case of the 'Zenit' hemp variety the highest zinc content was found in the seeds of plants receiving a higher N:P ratio in the nutrient solution without being exposed to water shortage. Under conditions of water deficiency, irrespective of the nitrogen to phosphorus ratio, the zinc content exhibited similar values (around 50 mg g^{-1}) with those determined in the seeds developed under water sufficiency and lower N:P ratio. The seeds of the two investigated hemp varieties had approximately the same amount of zinc, in the range of 50-70 mg g^{-1} dry weight.

The iron content of hemp seeds was higher than the zinc content, with an average of around 200 mg g^{-1} dry weight. It showed no significant variations between the two hemp varieties, and remained in the same range irrespective of the water supply regime and of the molar ratio between the inorganic nitrogen and phosphorus source of the nutrient solution provided during the development of plants (Figure 6).

The quantity of manganese ions in the seed dry matter of the 'Jubileu' hemp variety was not influenced significantly by the N to P ratio, but it was lower under conditions of water shortage and higher nitrogen to phosphorus ratio (4:1). Similar manganese contents (around 150 mg g^{-1} dry weight) were found in seeds of plants that received a 4N1P ratio under normal water regime and those exposed to water shortage in the presence of a 1N2P ratio (Figure 7). For the 'Zenit' variety the highest manganese content (exceeding 200 mg g^{-1}) was found in seeds developed under normal water supply and higher nitrogen to phosphorus ratio in the nutrient solution, while the lowest Mn quantities (around 100 mg g^{-1} dry weight) were determined in the seeds of plants grown under water shortage with a higher N to P ratio.

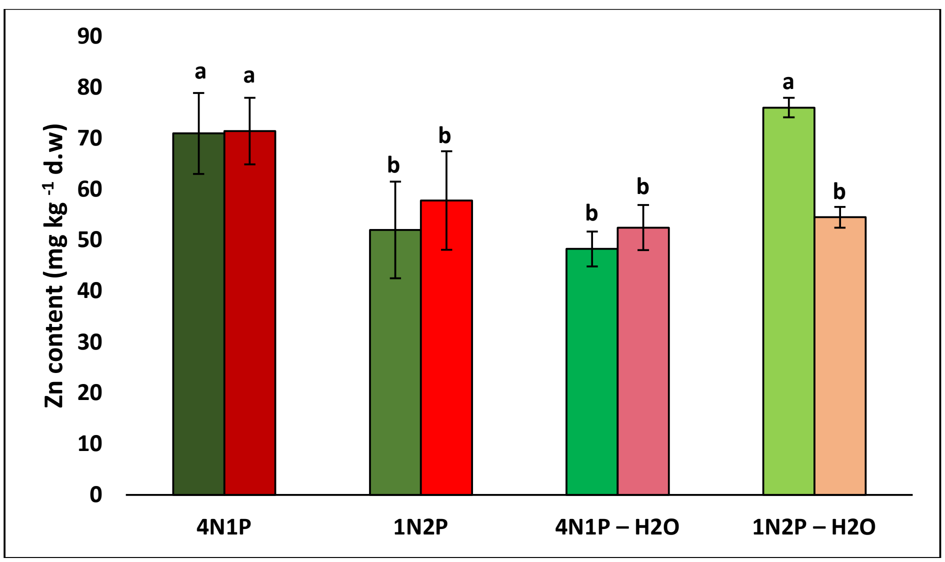

Figure 5. Zinc content of seeds of two hemp cultivars: 'Jubileu' (in nuances of green) and 'Zenit' (in nuances of red) grown in the presence of two different N:P ratios and under water insufficiency. 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, –H_2O – water shortage

Vertical lines represent ±SD from means (n = 5), different letters denote significant differences at $P < 0.05$

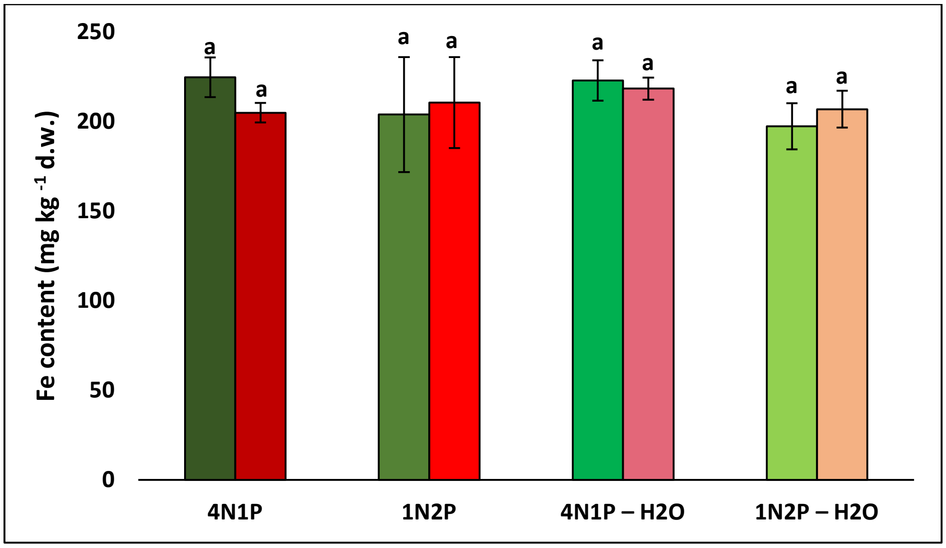

Figure 6. Iron content of seeds of two hemp cultivars: 'Jubileu' (in nuances of green) and 'Zenit' (in nuances of red) grown in the presence of two different N:P ratios and under water insufficiency. 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, –H_2O – water shortage

Vertical lines represent ±SD from means (n = 5), different letters denote significant differences at $P < 0.05$

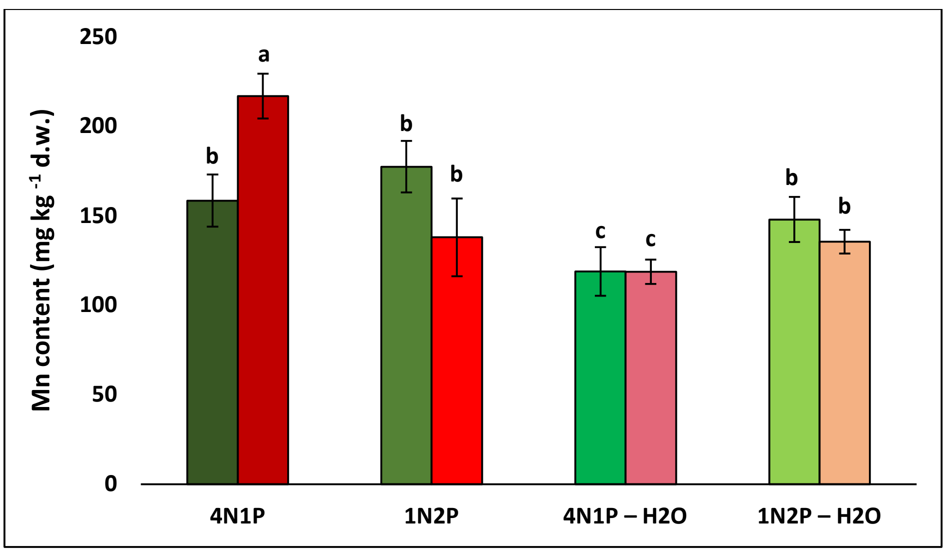

Figure 7. Manganese content of seeds of two hemp cultivars: 'Jubileu' (in nuances of green) and 'Zenit' (in nuances of red) grown in the presence of two different N:P ratios and under water insufficiency. 4N1P – nitrogen to phosphorus molar ratio of 4:1, 1N2P – nitrogen to phosphorus molar ratio of 1:2, –H_2O – water shortage

Vertical lines represent ±SD from means (n = 5), different letters denote significant differences at $P < 0.05$

As physico-chemical factors that influence uptake of inorganic nutrients by plants and may indicate accumulation of unused mineral ions in the aqueous solution, the pH values measured weekly in the substrate around the roots varied between 5.6 and 6.9, while the electrical conductivity was for all substrates of the experimental variants in the range of 0.4-0.6 mS cm^{-1}.

Discussion

Water availability and nitrogen to phosphorus ratio in the nutrient solution had a significant influence on growth of hemp plants, as well as on the quantity of certain metabolites and essential mineral microelements in the seeds, thus impacting the quality of hemp seeds used in the human diet or in manufacturing healthcare products.

Vegetative growth of both hemp varieties was restricted by water shortage and by a lower nitrogen to phosphorus ratio in the nutrient solutions. This might be explained by the fact that a higher water quantity is needed during the differentiation of new tissues in the meristems of newly generated, young plant organs, in order to develop a sufficiently high turgor pressure that enables cell growth. On the other hand, a robust vegetative growth of plantlets is conditioned by the availability of higher amounts of inorganic nitrogen compounds, and although none of the experimental variants was subjected to nitrogen deficiency, 3 mM nitrate represents a moderate level, while 8 mM nitrate equals to a high nitrogen level for a hemp plant. The results agree with the fact that nitrogen is an important nutritional factor for the vegetative development of plants (Gao *et al.*, 2022). Stem hight was reduced by water shortage irrespective of the N:P ratio, this result being consistent with the findings of Podder *et al.* (2024), who demonstrated with two other hemp varieties ('Grandi' and 'Joey') that the plants could not respond to the different nitrogen inputs without sufficient water, and the growth response to mineral macronutrients was limited by water shortage especially during the early vegetative stages. When responses of medical cannabis to nitrogen supply was studied, it was revealed that lower nitrogen supplies led to lower stem radial growth and reduced plant elongation. It was also concluded that nitrogen use efficiency declined with increased nitrogen supply (Saloner and Bernstein, 2020). In experiments performed with a lemon tree species (*Citrus macrophylla*) it was found that stem growth was proportional to nitrogen concentration from day 14 to day 28 after the beginning of nitrogen treatments, but during the first week there were no differences between treatments (Garcia-Gomez *et al.*, 2023). Similar results were obtained in the present experiments, where stem height started to respond to different treatments after two weeks in the 'Zenit' hemp variety and from the third week in the case of 'Jubileu' variety (Figure 1). If one compares the stem growth dynamics of the two studied hemp cultivars, it can be observed that inhibition of stem elongation by water shortage is more pronounced in the 'Jubileu' cultivar and starts earlier than in 'Zenit', thus the latter cultivar can be considered less drought-sensitive with respect to stem elongation.

The fact that the growth index of 70 days old plants after 56 days of treatments had the lowest values when a lower N:P ratio was combined with water deficiency suggests that there is a synergistic effect between the available water and the nitrogen supply in modulating plant growth. This is supported by the recognition of a mutual regulation of water and nitrate uptake through aquaporins and nitrate transporters, nitrogen availability being closely linked to water transport in plants (Garcia-Gomez *et al.*, 2023). Results concerning shoot weight reduction of hemp plants when the nutrient solution contained instead of 8 mM nitrogen and 2 mM phosphorus (4N1P) a nitrogen concentration decreased to 3 mM and a phosphorus content increased to 6 mM (1N2P) could be related to the role of nitrogen in supporting vegetative growth and the role of phosphorus in reducing the period of vegetative development and favouring an early onset of the generative phase (Khan *et al.*, 2023). As for another plant species it was found that the amount of 4 mM inorganic nitrogen source already induces deficiency symptoms associated with stunted growth (Garcia-Gomez *et al.*, 2023), it might be possible that also for hemp the concentration of 3 mM nitrate represents an insufficient nitrogen source to sustain an optimal biomass production. The corelation between higher N:P ratio and higher shoot biomass is supported by the experiments of Caplan *et al.* (2017a and 2017b) in which it was established that in a liquid organic fertilizer the suitable N:P ratio for vegetative growth of hemp (4:1.3) is significantly higher than the one needed during the flowering stage. The same experiments revealed that the electrical conductivity in the growing substrate of up to 3.0 mS cm^{-1} was tolerated by hemp plants without yield reductions, while the suitable pH of the nutrient solution was in the moderately acidic range, around the value of 6. The pH and EC values registered weekly by us in the vicinity of hemp roots of every experimental variant were all situated in the normal range (the electrical conductivity varied between 0.4 and 0.6 mS cm^{-1}), indicating that hemp plants were not exposed to overdosage of mineral nutrients and the pH remained in the range where every essential mineral nutrient is available as ions dissolved in water. In another experiment with two hemp varieties, it was found that moderate application of nitrogen fertilizer favours biomass yield, but high nitrogen concentrations may have a detrimental effect on hemp growth (Podder et al., 2023). When one compares the two hemp cultivars used in the experiments, it can be noticed that the growth index decreases in a higher extent in the case of the 'Zenit' cultivar when a higher N to P ratio is associated with water shortage. On the other hand, water shortage combined with a lower N to P ratio reduces the shoot fresh weight in a higher extent in the case of the 'Jubileu' cultivar. These results reflect that different growth parameters are influenced in different degrees in the two cultivars by the water status and by the variations in nitrogen and phosphorus supply.

Even if plants receiving a lower N:P ratio and lower water supply were smaller, no statistically significant difference could be registered in the seed production of the different experimental variants (measured as total seed weight per plant), this finding being valid for both hemp cultivars (Table 2). The only noticeable difference was that for plants grown under a lower N:P ratio associated with water shortage, variations in the individual seed production values were more pronounced. These results differ from those reported by Podder *et al.* (2023) for two other hemp varieties, where it was established that the mean seed yield increased only until a certain threshold of nitrogen supply was reached (this value being different for the two varieties), and when addition of nitrogen fertilizer exceeded this limit the seed yield remained constant. The fact that under a lower N:P ratio and under water shortage the smaller hemp plants produced a similar seed weight with higher plants grown under a higher N:P ratio may be related to the fact that a higher phosphate availability related to nitrogen, as well as drought conditions may reprogram the developmental rhythm of plants in favour of the reproductive stage, leading to a sustained flowering and seed formation (Gao *et al.*, 2022; Khan *et al.*, 2023). Concerning seed weight, the results of the experiments showed that conditions of insufficient water supply to the plants during the growth period moderately, but significantly increased the dry weight percentage of the fresh seed biomass. This is a logical consequence of the fact that under water shortage allocation of water to the already developed seeds decreased, while transpiring leaves took up a higher proportion of the available water from the xylem vessels of the stem.

The chemical composition of hemp seeds with respect to those constituents which are important for human use could be modulated by nitrogen, phosphorus and water availability. A lower N:P ratio in the nutrient solution led to an increased oil content of seeds for the studied hemp varieties, both under sufficient and deficient water regimes (Figure 2). This can be related to the lipid metabolism of plants, which does not directly require nitrogen for the biosynthesis of oil deposits but implies several phosphorylated intermediates. On the other hand, because fatty acids and oils (triglycerides) are insoluble in water, the water supply of plant cells has less interference with lipid biosynthesis (Bates *et al.*, 2013). The fact that conditions of water shortage resulted in a slightly elevated oil content of hemp seeds may be also related to the lowered water content of the fresh weight, as well as to a restricted accumulation of water-soluble compounds, due to insufficient water supply for seeds. When the two hemp cultivars are compared, it can be noticed that for 'Jubileu' the increment of seed oil content is slightly more pronounced than for 'Zenit' if nitrogen supply is decreased from 8 mM to 3 mM and phosphorus availability is increased from 2 mM to 6 mM, irrespective of the degree of water supply.

As main nutrients, along with oils, proteins are also important constituents of hemp seeds. It was demonstrated that the flour obtained from the 'Zenit' hemp variety contained approximately 28% lipids (rich in essential unsaturated fatty acids), and 22% proteins that contained all the essential amino acids for a healthy human diet (Rusu *et al.*, 2021). Our experiments showed that the oil content of seeds of the 'Zenit' hemp variety can be increased to over 30% of the fresh weight if plants are provided with lower N:P ratio (i.e. 3 mM nitrate and 6 mM phosphate) and if they are exposed to a moderate drought stress (i.e. 300 mL water per day per plant during the second part of the developmental cycle). Protein content of seeds varied in a higher extent than the oil content (even if one takes into account that proteins were determined from the dry weight and oils were extracted from the fresh seeds), and water shortage associated with a higher N:P ratio could elevate this content above 30% of the dry seed weight (Figure 3). The results reflecting that for both hemp varieties the protein content was higher when the N:P ratio of the nutrient solution was also higher can be related to the fact that the nitrogen assimilated by plants is mainly incorporated in proteins, so a higher nitrogen supply ensures a higher content of stored proteins in seeds. The nitrate taken up by roots from the aqueous solution becomes reduced to ammonium and incorporated mainly in amino acids, which build up the various protein macromolecules. This explains why a reduction in nitrogen supply from 8 mM to 3 mM nitrate will result in a lowered amino acid pool, and consequently a down-regulated protein synthesis in the maturing hemp seeds. Water insufficiency moderately increased the protein content of seeds under both N to P ratios applied in the experiments, and this result can be explained, as in the case of oil content, by a restricted accumulation of water-soluble small molecules and inorganic ions, thus water-insoluble macromolecules will represent a slightly higher part of the seeds' weight. These changes in the seed protein content were very similar for both of the investigated hemp cultivars.

The vitamin E content of the hemp seed oil is responsible for its many beneficial properties. It contributes to the preservation of the oxidative stability during storage, it has an important protective role against oxidative stress caused by elevated concentrations of harmful reactive oxygen species. One of its main positive effects is protection of cell membranes against oxidative damage through peroxidation of unsaturated fatty acids in the membrane lipids. In the literature one can find very different values of the vitamin E content of hemp oil, depending on many factors such as growth conditions, genotype, exposure to different abiotic stress factors, developmental stage etc. (Izzo *et al.*, 2020; Montero *et al.*, 2023). Farinon *et al.* (2020) reported that the total tocopherol amount in hempseed oil can be higher than 90 mg per 100 g of oil, being higher than that found in sunflower, amaranth or sesame oil. In our experiments the total vitamin E (tocopherols and tocotrienols) content of seed oil varied in the range of 50-90 mg per 100 g oil for the 'Jubileu' variety and between 90-140 mg per 100 g oil in the case of 'Zenit' (Figure 4). Thus, it can be concluded that the 'Zenit' cultivar has the ability to accumulate higher quantities of vitamin E dissolved in the oil of its seeds, as compared with the 'Jubileu' cultivar. For both varieties the highest vitamin E content was reached when water shortage was associated with a lower N:P ratio (1N2P). For both N to P ratios the vitamin E content of the hemp seed oil was significantly higher under water shortage that in conditions of sufficient water supply. This may be explained, as in the case of seed oil content, by the fact that vitamin E is a lipid-soluble plant metabolite which does not need water to be dissolved in, so it can accumulate even when water availability is reduced. Similar vitamin E contents were found in the seeds of two different experimental variants: when a lower N:P ratio was applied under conditions of sufficient water supply and when a higher N:P ratio was combined with water shortage. These findings suggest that nitrogen and phosphorus availability, as well as the water regime possess yet unknown influences on the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/ DOXP) pathway of plants. A better knowledge of how mineral nutrition and water regime modulate vitamin E biosynthesis in plants may open new opportunities for increasing the health-promoting quality of food crops, considering that vitamin E is an essential organic micronutrient for the human organism (Tang *et al.*, 2016; Montero *et al.*, 2023).

Because hemp seeds are becoming popular dietary supplements, their content in essential inorganic micronutrients represented by transition metals such as iron, zinc and manganese can contribute to biofortification as part of a healthy diet. Considering that there is a network of interrelations between the uptake and transport of different mineral nutrients from the aqueous solution, and that the mineral nutrients are translocated together with the water column driven through the plants' body by the transpiration stream, the enhancement of accumulation of useful microelement through a directed nutrient and water supply may be a cost-effective strategy in functional food production. In this context, the zinc content of seeds of the 'Jubileu' hemp variety could be increased by water shortage conditions applied under a lower N:P ratio. But when water insufficiency was associated with the higher N:P ratio, the zinc content became lower than for the same N:P ratio under sufficient water supply. In case of the 'Zenit' variety, the highest zinc content was achieved with sufficient water supply and with the higher nitrogen to phosphorus ratio (8 mM nitrate to 2 mM phosphate) in the nutrient solution. The highest zinc level was similar in the seeds of both hemp varieties (around 70 mg kg^{-1} dry weight). It can be speculated that the lower zinc content of seeds in hemp plants provided with lower quantities of nitrogen may be related to a synergistic relation between the nitrate anions and the zinc cations at the level of ion transporters, thus the transport of a higher amount of nitrate may be associated with an enhanced zinc uptake. The fact that under higher nitrogen supply the water deficit reduces the zinc content of seeds may be explained by the fact that mineral nutrients are transported together with water, thus a reduced transpiration aimed to prevent excessive water loss implies a reduced transport of mineral nutrients upwards from the roots, transpiration rate being the main factor that decides the upward transport of water and of dissolved inorganic ions in the plants' body.

The iron content of seeds, with a specific importance for human health, did not exhibit any changes among the experimental variants, remaining constantly around the value of 200 mg kg^{-1} dry weight for both hemp varieties. In the flour obtained from seeds of the 'Zenit' variety Rusu et al. (2021) have reported even iron values as high as 89.47 mg per 100 g. The fact that very similar iron quantities were determined in the hemp seeds of every experimental variant, irrespective of changes in the available nitrogen and phosphorus concentrations and in the water regime, may reflect that during seminogenesis hemp plants build up a very stable iron reserve in seeds, mainly in the form of ferritin particles, which make their iron reserve highly insensitive to variations in the intensity of water transport and mineral nutrient uptake at a given time. Our results concerning a stable iron content in hemp seeds are not in agreement with the findings of Ortiz-Delvasto *et al.* (2024) concerning the existence of a positive correlation between growth intensity of hemp plants and their Fe content.

Manganese is another essential inorganic micronutrient for plants, animals and humans as well. Its quantity showed different trends for the two hemp varieties taken into study. For 'Jubileu' the lower nitrogen supply and higher phosphorus amount had no significant influence on the manganese content of seeds, while in the case of 'Zenit' the reduced N to P ratio in the nutrient solution resulted in a reduced Mn content of seeds. This difference may reside in contrasting selectivity of manganese uptake and translocation in the two cultivars, but this presumption needs to be supported by further investigations concerning manganese transport in hemp plants. In the case of 'Zenit' the highest Mn content (very similar to iron content) was found in seeds of plants supplied with higher N:P ratio with no water shortage, indicating that a higher nitrate availability may enhance manganese accumulation in hemp seeds. The lower manganese content under conditions of water shortage, observed in both hemp cultivars, may be explained by co-regulation of water and mineral nutrient uptake, as a decreased transpiration aimed to reduce water loss results in a reduced uptake of manganese ions. When water availability of different substrates on micronutrient content of hemp was investigated, it was found that the intensity of transpiration is a key determinant of the absorption of micronutrients along with water, and as a consequence, drought stress reduced the bioaccumulation of mineral nutrients, especially in leaves (Ortiz-Delvasto *et al.*, 2024). This agrees with our results concerning a lower manganese and zinc content when plants were subjected to water shortage. The previously mentioned study also demonstrated that hemp leaves and inflorescences may have very different iron, zinc and manganese contents.

To be able to explain the physiological background of the interrelations between nitrogen, phosphorus and water supply and changes in developmental and metabolic processes in hemp plants under various cultivation conditions, a better knowledge of the regulatory processes involved in lipid and protein biosynthesis, in vitamin E production and in management of different mineral nutrients is required, with the use of innovative metabolomic approaches. Thus, improved strategies of nutrient and water management will play a critical role in establishing sustainable cropping systems.

Conclusions

Biochemical properties of hemp seeds that are beneficial for human use can be modulated through variation of the molar ratio between the inorganic nitrogen and phosphorus supplied with the nutrient medium, as well as by creating a moderate water deficit during controlled irrigation.

Although plants receiving a lower N:P ratio (3 mM nitrate and 6 mM phosphate) and lower water supply had a decreased growth index and the shoot fresh weight was smaller, no significant difference could be registered in the seed production of the different experimental variants. The oil content of seeds could be increased by a lower N:P ratio and by water deficit, while seed protein content was higher upon a higher N:P ratio and upon water shortage, for both cultivars. A lower N:P ratio in the nutrient solution resulted in a decreased protein content of seeds, but only when there was no water shortage. For both hemp varieties the water insufficiency resulted in a moderately increased dry matter content of the fresh weight of hemp seeds. The highest vitamin E content of hemp seed oil was found when plants were subjected to the combined influence of lowered N:P ratio and water insufficiency. The iron content of seeds showed no changes between the different experimental variants. For other two essential inorganic nutrients, i.e. zinc and manganese, the quantity in seeds showed different variations in the two hemp varieties. In seeds of the 'Jubileu' variety the zinc content was increased by water shortage when the N:P ratio was lower, while the highest manganese content was measured when plants received sufficient water and lowered N:P ratio. As for the 'Zenit' variety, the highest zinc and manganese content of seeds was obtained when plants received through the nutrient solution 8 mM nitrate and 2 mM phosphate (i.e. the N:P ratio was 4:1).

As crucial factors for growth and productivity of plants, nitrogen, phosphorus and water availability have a determinant role in the seed yield and chemical composition, so establishing those nitrogen to phosphorus ratios and water supply levels that result in a higher value of seeds for human use represents a cost-effective and environmental-friendly approach in growing hemp as a valuable food plant. These findings may contribute to the development of sustainable farming practices that ensure an optimized cultivation of industrial hemp, to improve the quality of seeds used for healthcare products and as functional food with valuable nutraceuticals.

Authors' Contributions

Conceptualization IINy, LF; Data analysis AZ, BBJ; Data curation MK; Investigation MK and BBJ; Measurements AZ and LF; Methodology LF and IINy; Resources IINy; Supervision IINy and LF; Writing - original draft LF; Writing - review and editing IINy. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This work was supported by the Sapientia Foundation – Institute for Scientific Research with grant KPI 14/9/12.04.2022. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Al-Sumri, A, Al-Siyabi N, Al-Saadi R, Al-Rasbi S, Al-Dallal A (2016). Study on the extraction of date palm seed oil using Soxhlet apparatus. International Journal of Scientific & Engineering Research 7(12):1266-1270. *http://www.ijser.org*

Andre CM, Hausman JF, Guerriero G (2016). *Cannabis sativa*: the plant of the thousand and one molecules. Frontiers in Plant Science 7:19. *https://doi.org/10.3389/fpls.2016.00019*

Avram M, Stoica A, Dobre T, Stroescu M (2014). Extraction of vegetable oils from ground seeds by percolation techniques. U.P.B. Scientific Bulletin Series B 76(2):13-22.

Balaram V (2020). Microwave plasma atomic emission spectrometry (MP-AES) and its applications – a critical review. Microchemical Journal 159:105483. *https://doi.org/10.1016/j.microc.2020.105483*

Bates PD, Stymne S, Ohlrogge J (2013). Biochemical pathways in seed oil synthesis. Current Opinion in Plant Biology 16:358-364. *https://doi.org/10.1016/j.pbi.2013.02.015*

Bevan L, Jones M, Zheng Y (2021). Optimisation of nitrogen, phosphorus, and potassium for soilless production of *Cannabis sativa* in the flowering stage using response surface analysis. Frontiers in Plant Science 12:764103. *https://doi.org/10.3389/fpls.2021.764103*

Bokhari A, Chuah LF, Yusup S, Ahmad J, Aziz H (2015). Kapok seed oil extraction using Soxhlet extraction method: optimization and parametric study. Australian Journal of Basic and Applied Sciences 9(37):429-431. *www.ajbasweb.com*

Bradford MM (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein dye binding. Analytical Biochemistry 72:248-254.

Caplan D, Dixon M, Zheng Y (2017a). Optimal rate of organic fertilizer during the vegetative-stage for cannabis growth in two coir-based substrates. HortScience 52(9):1307-1312. *https://doi.org/ 10.21273/HORTSCI11903-17*

Caplan D, Dixon M, Zheng Y (2017b). Optimal rate of organic fertilizer during the flowering stage for cannabis grown in two coir-based substrates. HortiScience 52(12):1796-1803. *https://doi.org/ 10.21273/ HORTSCI112401-17*

Deng M, Li Q (2022). Optimization of the NPK ratio for vegetative growth of *Aeschynanthus longicaulis*. Korean Journal of Horticultural Science and Technology 40(6):663-671. *https://doi.org/10.7235/ HORT.20220060*

Farinon B, Molinari R, Costantini L, Merendino N (2020). The seed of industrial hemp (*Cannabis sativa* L.): nutritional quality and potential functionality for human health and nutrition. Nutrients 12:1935. *https://doi.org/10.3390/nu12071935*

Gao Y, Qi S, Wang Y (2022). Nitrate signalling and use efficiency in crops. Plant Communications 3:100353. *https://doi.org/10.1016/j.xplc.2022.100353*

Garcia-Gomez P, Olmos-Ruiz R, Nicolas-Espinosa J, Carvajal M (2023). Effects of low nitrogen supply on biochemical and physiological parameters related to nitrate and water, involving nitrate transporters and aquaporins in *Citrus macrophylla*. Plant Biology 13553. *https://doi.org/10.1111/ plb.13553*

Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347:1-32. *https://cabidigitallibrary.org/doi/full/10.5555/ 19500302257*

Izzo L, Pacificao S, Piccolella S, Castaldo L, Narvaez A, Grosso M, Ritieni A (2020). Chemical analysis of minor bioactive components and cannabidiolic acid in commercial hemp seed oil. Molecules 25:3710. *https://doi.org/10.3390/molecules25163710*

Khan F, Siddique AB, Shabala S, Zhou M, Zhao C (2023). Phosphorus plays key roles in regulating plants' physiological responses to abiotic stresses. Plants 12:2861. *https://doi.org/10.3390/plants12152861*

Khilari VJ, Sharma PP (2016). Determination of total lipids from five underutilized wild edible fruits in Ahmednagar district, Maharashtra (India). International Journal of Advanced Research in Biological Sciences 3(7):14-20. *http://soi.org/1.15/ijarbs-2016-3-7-3*

Kivcak B, Mert T (2001). Quantitative determination of α-tocopherol in *Arbutus unedo* by TLC-densitometry and colorimetry. Fitoterapia 72:656-661. *www.elsevier.com/locate/fitote*

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany 105:1141-1157. *https://doi.org/10.1093/aob/mcq028*

Montero L, Ballesteros-Vivas D, Gonzalez-Barrios AF, Sanchez-Camargo AP (2023) Hemp seeds: nutritional value, associated bioactivities and the potential food applications in the Colombian context. Frontiers in Nutrition 9:1039180. *https://doi.org/10.3389/fnut.2022.1039180*

Ortiz-Delvasto N, Garcia-Gomez P, Carvajal M, Barzana G (2023). Aquaporins-mediated water availability in substrates for cannabis cultivation in relation to CBD yield. Plant Soil 495:469-485. *https://doi.org/ 10.1007/s11104-023-06341-8*

Ozbek N, Akman S (2016). Method development for the determination of calcium, copper, magnesium, manganese, iron, potassium, phosphorus and zinc in different types of breads by microwave induced plasma-atomic emission spectrometry. Food Chemistry 200:245-248. *https://doi.org/10.1016/ j.foodchem.2016.01.043*

Podder S, Shafian S, Thomason WE, Wilson TB, Fike JH (2024). Hemp seed yield responses to nitrogen fertility rates. Crops 4:145-155 *https://doi.org/10.3390/crops4020011*

Rupasinghe HPV, Davis A, Kumar SK, Murray B, Zheljazkov VD (2020). Industrial hemp (*Cannabis sativa subsp. sativa*) as an emerging source for value-added unctional food ingredients and nutraceuticals. Molecules 25(18):4078. *https://doi.org/10.3390/molecules25184078*

Rusu IE, Marc (Vlaic) RA, Muresan CC, Muresan AE, Filip MR, Onica BM, Csaba KB, Alexa E, Szanto L, Muste S (2021). Advanced characterization of hemp flour (*Cannabis sativa* L.) from Dacia Secuieni and Zenit varieties, compared to wheat flour. Plants 10:1237. *https://doi.org/10.3390/plants10061237*

Sacilik K, Ozturk R, Keskin R (2003) Some physical properties of hemp seed. Biosystems Engineering 82(2):191-198. *https://doi.org/10.1016/S1537-5110(03)00130-2*

Saloner A, Bernstein N (2020). Response of medical cannabis (*Cannabis sativa* L.) to nitrogen supply under long photoperiod. Frontiers in Plant Science 11:572293. *https://doi.org/10.3389/fpls.2020.572293*

Spirovic-Trifunovic B, Nedeljkovic D, Stojicevic D, Bozic D (2021). Fatty acids in wild hemp seeds (*Cannabis sativa* L. ssp. *sativa* var. *spontanea* Vavilov). Acta Herbologica 30:65-73. *https://doi.org/10.5937/ actaherb2101065q*

Tang K, Struik PC, Yin X, Thouminot C, Bjelkova M, Stramkale V, Amaducci S (2016). Comparing hemp (*Cannabis sativa* L.) cultivars for dual-purpose production under contrasting environments. Industrial Crops Production 87:33-44. *https://doi.org/10.1016/j.indcrop.2016.04.026*

Tutem E, Apak R, Gunaydi E, Sozgen K (1997). Spectrophotometric determination of vitamin E using copper(II)-neocuproine reagent. Talanta 44(2):249-255. *https://doi.org/10.1016/s0039-9140(96) 02041-3*