

Changes of photosynthesis-related parameters and productivity of *Cannabis sativa* under different nitrogen supply

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Abstract

The interest in cultivated plants that produce high-quality raw materials with a wide application spectrum has increased. The aim of the present study was to determine the impact of different doses of nitrogen fertilizer on physiological processes of hemp plants and to evaluate its effect on several yield parameters, including seed and fiber yield and quality. The efficiency of different nitrogen supply was evaluated both in controlled and field conditions using hemp cultivar of Latvian origin 'Pūriņi', possibly suitable for local conditions. Yield parameters of hemp in field conditions varied both by years and by treatments. Additional nitrogen supply had positive effect on plant height, while no significant difference between various doses of nitrogen supplied was observed. Additional nitrogen supply had negative effect on hemp fiber content in 2008 and 2009, and no significant effect in 2010. Seed yield ranged from 1050 to 2557 kg ha⁻¹, depending both on particular climatic conditions and the rate of additional fertilization. The yield significantly increased in the N₊₆₀ treatment in all years in comparison to control plants. Hemp seed oil content was not significantly affected by additional nitrogen fertilization. A high proportion of linoleic acid in hempseed oil was found, followed by α-linolenic acid, oleic acid and palmitic acid. In laboratory conditions, increase in both leaf nitrogen concentration and chlorophyll content in hemp plants, additionally fertilized with nitrogen, did not depend on the particular dose. Additional nitrogen fertilization significantly affected different aspects of photochemistry of photosynthesis, as shown by chlorophyll *a* fluorescence analysis. According to yield data, the hemp cv. 'Pūriņi' is well-suited for both seed and fiber production in temperate conditions.

Key words: ammonium nitrate, *Cannabis sativa* L., chlorophyll, chlorophyll *a* fluorescence, fatty acids; fiber content, mineral nutrition; nitrogen fertilizer, seed oil content, yield.

Abbreviations: PI, performance index.

Introduction

Recently, an interest in cultivated plants that produce high-quality raw materials with a wide application spectrum has increased in European Union countries. A special interest is focused on cultivation of canola, fiber and oil flax, nettle and hemp. Among them, hemp is traditionally cultivated throughout the World for both seed and fiber production (Anwar et al. 2006).

In spite of a variety of different studies on hemp performed recently (de Meijer et al. 2009a; de Meijer et al. 2009b; Fisk et al. 2009; Jankauskiene, Gruzdeviene 2009; Lata et al. 2009a; Lata et al. 2009b; Marks et al. 2009; Peiretti 2009; Shi et al. 2009; Wang et al. 2009; Soldatova, Khryanin 2010), there is still a lack of physiological studies, e.a., on functional aspects of mineral nutrition and photosynthesis. In general, hemp has a high nitrogen requirement (Coffmann, Gentner 1975). However, studies on factors affecting hemp yield due to different nutrition levels have not been performed. As hemp produces both seeds and fiber, different physiological

processes linked to different aspects of yield formation need to be investigated.

The aim of the present study was to determine the impact of different doses of nitrogen fertilizer on physiological processes of hemp plants and to evaluate its effect on several yield parameters, including seed and fiber yield and quality. The efficiency of different nitrogen supply was evaluated both in controlled and field conditions using a hemp cultivar of Latvian origin, possibly suitable for local conditions.

Materials and methods

Field study

The field study was performed in the experimental field of the Latvian Agricultural Science Centre of Latgale (N 56°; E 26°). A hemp cultivar of Latvian origin 'Pūriņi' was used. The agricultural characteristics of the soil are summarized in Table 1.

The soil was sod podzolic sandy-loamy. Soil was

Table 1. Agricultural characteristics of soil in field study

Year	Organic matter content (%)	pH	Phosphorus, P ₂ O ₅ (mg kg ⁻¹)	Potassium, K ₂ O (mg kg ⁻¹)	Preliminary crop / status	Complex fertilizer applied after first soil cultivation (NPK / kg ha ⁻¹)
2008	3.8	7.3	83	65	Spring rape	6:26:30 / 300
2009	4.5	7.3	134	122	Spring rape	18:9:9 / 330
2010	6.5	7.0	145	118	Bare fallow	18:9:9 / 350

cultivated with a combined soil cultivation unit “Laumetris”. Treatments were organized by a randomized block method. Size of individual plots was 4.5, 20 and 20 m² in 2008, 2009 and 2010 respectively. Isolation between treatments and replicates was 0.5 m. The number of replicates was four in 2008 and 2009, and three in 2010. A sowing-machine “SN-16” was used for seed sowing.

Three nitrogen fertilizer doses were used: N₊₀ – control, N₊₆₀ – 60 kg ha⁻¹, and N₊₁₀₀ – 100 kg ha⁻¹. As an additional fertilizer, NH₄NO₃ was used. Fertilizer treatment was performed during sowing. Phenologic observations were performed throughout the vegetation period to establish developmental stages. Each individual plot was harvested separately; plants were bundled and desiccated. After desiccation plant height and fiber content were determined. Seeds were cleaned by means of a sample cleaner “MLN”, and seed yield and 1000 seed mass were measured. The data was analyzed using MS Excel.

Data on meteorological conditions was obtained from an on-site meteostation (Adcon), connected to a PlantPlus computer program. According to the air temperature data (Fig. 1), the vegetation season of 2008, 2009 and 2010 was warmer than the average long-term local temperature. Average air temperature in the vegetation season was 12.9, 12.8 and 14.3 °C, for 2008, 2009 and 2010, respectively, in comparison to the long-term average 12.2 °C.

Precipitation in 2008, 2009 and 2010 was lower, in comparison to the long-term average of 373 mm. Total rainfall in 2008 was by 4.3% lower, in 2009 by 48.7% lower, and in 2010 by 39.9 % lower than the long-term average.

Oil content in hemp seeds was determined with a grain quality analyzer Infratec-1241 (Foss Analytical, Denmark) using a special device for oil content determination. Each measurement was performed ten times.

Hempseed oil was obtained by mechanical pressing of hemp seeds. An aliquot (about 0.1 g) of crude oil was

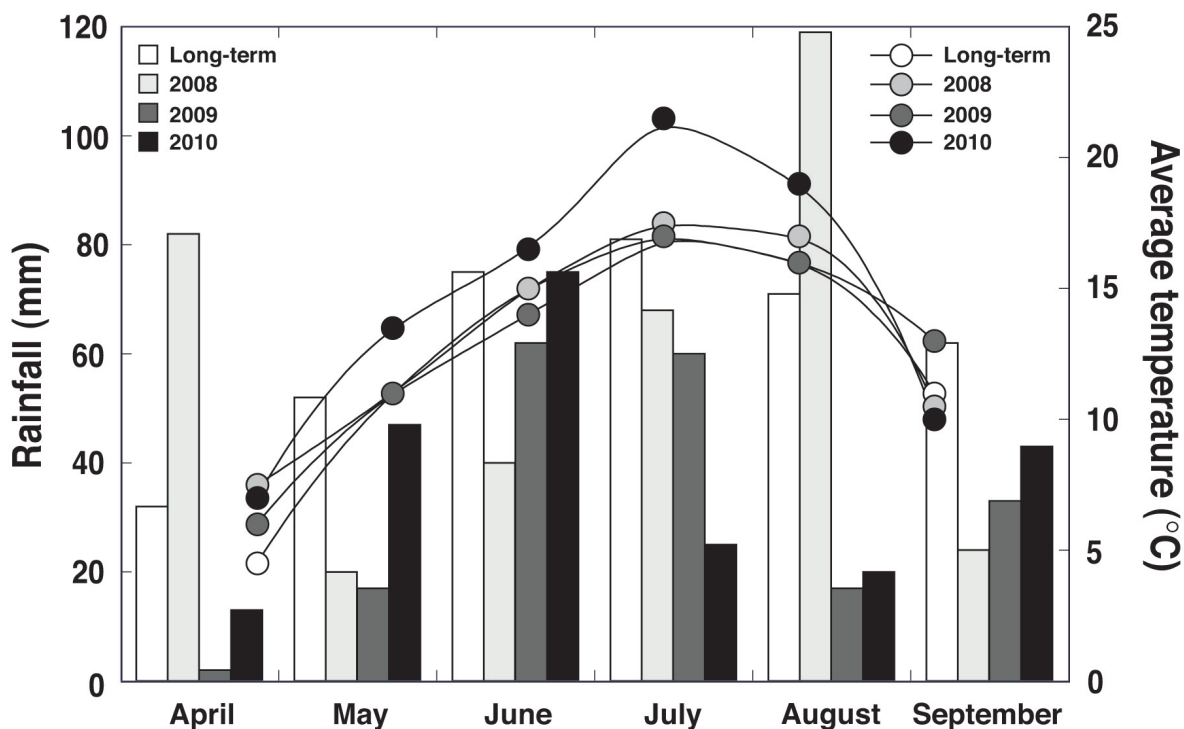


Fig. 1. Rainfall and temperature during respective vegetation seasons at the site of field study. Long-term average data represents 10 years.

dissolved in 10 mL n-hexane. After treatment with 5 mL 0.04 M Na metoxide for 10 min with stirring, samples were cooled to room temperature and neutralized with 0.5 mL 0.2 M acetic acid. After adding 5 mL water, the hexane layer was washed by stirring, decanted and evaporated under N₂ to obtain the necessary volume.

Fatty acid composition of hempseed oil was determined using the gas chromatography-mass spectrometry method (Shimadzu GCMS-QP2010). Ionization was performed by electrons with 70 eV energy, and mass analysis was achieved using a quadrupole mass analyzer. A capillary column DB-17 (J&W Scientific; 30 m × 0.25 mm) was used for separation of fatty acids. The temperature range during separation was 60 to 280 °C with a heating rate of 10 °C min⁻¹. Helium was used as a carrier gas, with a rate 1.0 mL min⁻¹.

Laboratory study

The hemp cultivar of Latvian origin 'Pūriņi' was used in studies in laboratory conditions. Seeds were sown in plastic containers (four seeds per container) with a total volume 0.59 L, filled with mineral-enriched peat substrate KANO [pH (KCl) 5.5 – 7.0, nitrogen content 180 mg L⁻¹, K₂O content 400 mg L⁻¹, P₂O₅ content 245 mg L⁻¹]. Containers were placed in a growth cabinet, at 15 h photoperiod, 22 ± 2 °C. Illumination was provided by luminescent lamps with photon flux density 200 μmol m⁻² s⁻¹.

Three nitrogen fertilizer doses were used: N₊₀ – control, N₊₆₀ – 60 kg ha⁻¹, and N₊₁₀₀ – 100 kg ha⁻¹. Fifteen pots per treatment were used. As a source of nitrogen fertilizer, a 10 % NH₄NO₃ solution was used. To achieve necessary doses, N₊₆₀ plants received 1.2 mL fertilizer solution per pot diluted with water to achieve a nitrogen fertilizer rate of 61 mg L⁻¹, and N₊₁₀₀ plants were treated with 2.0 mL fertilizer solution per pot to diluted with water to achieve a fertilizer rate of 101 mg L⁻¹. Fertilizer treatment was performed on 22nd, 29th and 36th days from the start of the experiment.

Measurement of nitrogen content, total chlorophyll content and chlorophyll *a* fluorescence was performed four times. During the first measurement (22 days after sowing), the majority of plants were in the vegetative stage and had at least three leaf pairs. All plants were in a vegetative stage during the second measurement (29 days); part of them showed change of phyllotaxis. At the third measurement (36 days), plant transition to the flowering stage had started. During the fourth measurement (43 days), all plants were in an intensive flowering stage.

Plant leaves of similar age and healthy appearance were collected for nitrogen measurement at appropriate time points. For each treatment 10 leaf discs from 10 leaves were taken with a total surface area of 1 dm². Each leaf disc was taken from a separate plant. Discs were dried in microwave oven at medium intensity for 30 s (Marur, Sodek 1995). Nitrogen content was determined with Kjeldahl method. Each measurement was performed in triplicate.

Chlorophyll content was determined using a non-destructive SPAD-502 chlorophyll meter (Konica-Minolta, Japan). For all treatments, two leaves from 15 plants were measured, with 10 readings per leaf. The mean of the measurement for a particular leaf was calculated using the internal function of the chlorophyll meter.

Chlorophyll *a* fluorescence was measured using a Handy PEA portable fluorescence measurement system (Hansatech Instruments, UK). Leaves of similar age and healthy appearance were chosen for analysis. Dark adaptation was performed with special leaf clips for 25 min before measurement. Ten plants per treatment were measured using two individual leaves per plant. The data were analyzed by an appropriate software.

Results

Field study

Yield parameters of hemp in field conditions varied both by years and by treatments. In general, additional nitrogen supply had positive effect on plant height (Fig. 2A). However, there was no significant difference between various doses of nitrogen supplied. On average, additional nitrogen fertilization resulted in a height increase of hemp plants by 15.5 cm. The most intense linear growth in all treatments was observed in 2009 with relative increase by 17% as compared with other years.

In 2009 and 2010 fiber content in hemp stems was significantly higher, compared with that in 2008 (Fig. 2B). Additional nitrogen supply had negative effect on hemp fiber content in 2008 and 2009, and no significant effect in 2010.

Seed yield of hemp cv. 'Pūriņi' ranged from 1050 to 2557 kg ha⁻¹, depending both on particular climatic conditions and the rate of additional fertilization (Fig. 2C). The highest seed yield was obtained in 2009 in all treatments, which on average was higher by 54 and 39% than in 2008 and 2010, respectively. The yield significantly increased in the N₊₆₀ treatment in all years in comparison to control plants, which received no additional nitrogen supply. The increase was by 74, 19 and 28% in 2008, 2009 and 2010, respectively. However, higher doses of nitrogen did not result in significant yield increases in 2008 and 2010.

In general, seed mass of hemp plants was not significantly affected by nitrogen treatment (Fig. 2D). In contrast, significant variation in seed mass of control plants was detected between particular years. In 2009 seeds were heavier by 12%, compared to relatively similar values in 2008 and 2010.

Hemp seed oil content was not significantly affected by additional nitrogen fertilization (Fig. 3). However, there was a tendency of decreasing oil content with increasing fertilizer supply. Some variation in oil content between particular years was seen, with decreased content in 2010. Similarly, fatty acid composition was not significantly

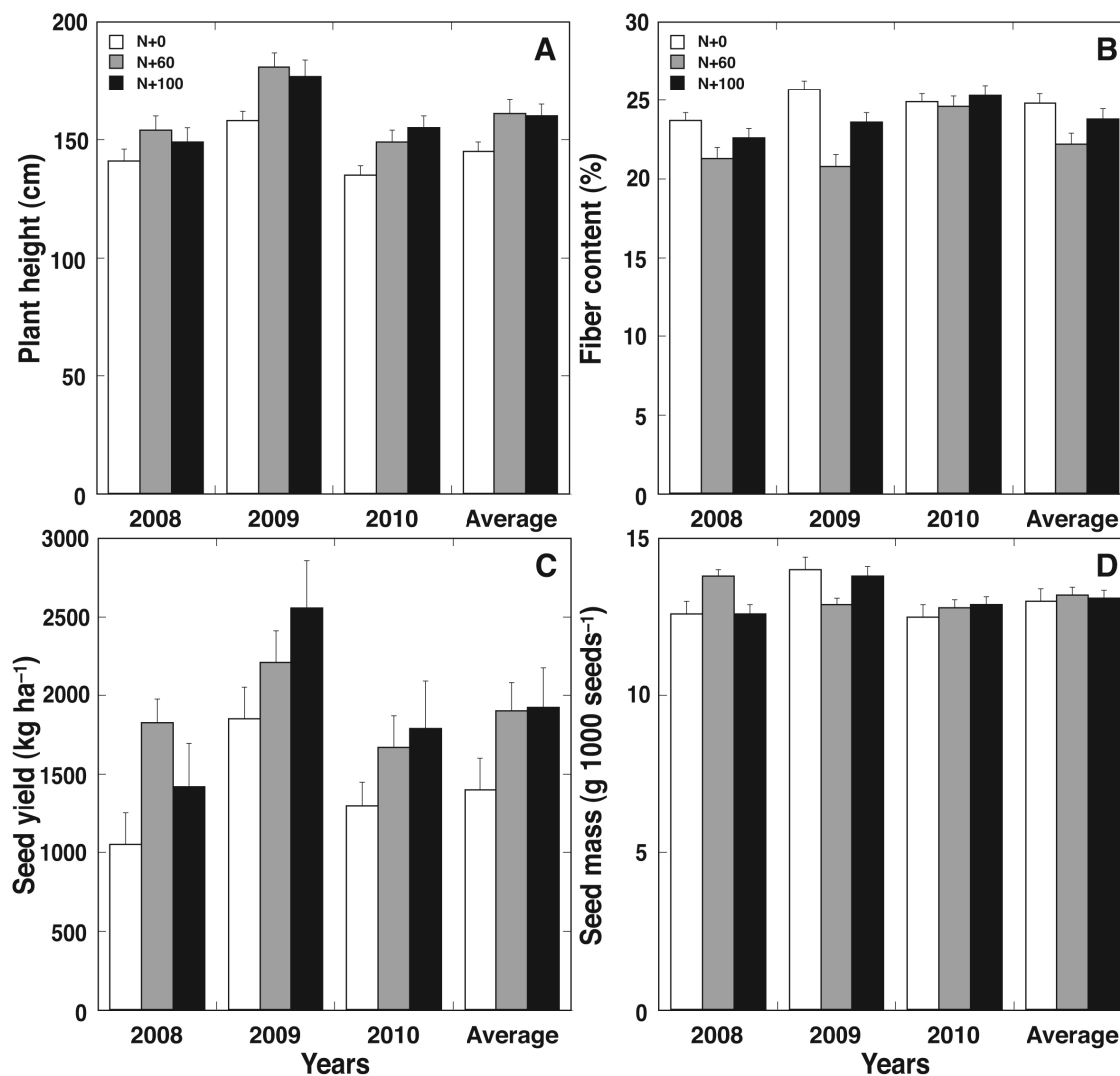


Fig. 2. Effect of additional nitrogen fertilization on plant height (A), stem fiber content (B), seed yield (C) and seed mass (D) of hemp plants in field conditions. Data are means \pm SE from three (2010) or four (2008 and 2009) replicates. N_{+0} , no additional nitrogen; N_{+60} , nitrogen in a form of NH_4NO_3 60 kg ha^{-1} ; N_{+100} , NH_4NO_3 100 kg ha^{-1} .

affected by the nitrogen fertilization rate (Fig. 4). A high proportion of linoleic acid (50%) in hempseed oil was found, followed by α -linolenic acid (22%), oleic acid (12%) and palmitic acid (7.6%).

Laboratory study

Nitrogen concentration in leaves of hemp plants grown in laboratory conditions increased within the juvenile phase (from 22 to 29 days) with no difference between the treatments (Fig. 5). Later, there was an increase of nitrogen content in plants with additional nitrogen fertilization, in contrast to control plants where it has decreased. However, differences between the N_{+60} and N_{+100} treatments in respect to leaf nitrogen concentration were not significant. Chlorophyll concentration increased in leaves of hemp plants within the experimental period in all treatments (Fig. 6). Similar to leaf nitrogen concentration, the increase in

chlorophyll content in hemp plants additionally fertilized with nitrogen, in comparison to the control, did not depend on the particular dose. As a result, leaves with higher nitrogen concentration tended to have higher chlorophyll content ($R^2 = 0.774$).

Additional nitrogen fertilization significantly affected different aspects of photochemistry of photosynthesis, as revealed by chlorophyll a fluorescence analysis (Fig. 7). The Performance Index increased within the experimental period and from day 29 to day 43 was highest in hemp plants treated with additional 100 kg ha^{-1} (Fig. 7A). There was also a significant increase in PI on day 29 in the N_{+60} treatment, compared to control plants. The observed differences in PI level were mostly due to changes in RC/ABS, the ratio of active chlorophyll molecules in the reaction centre of photosystem II, as the time course of RC/ABS closely resembled that of PI for all treatments (data not shown).

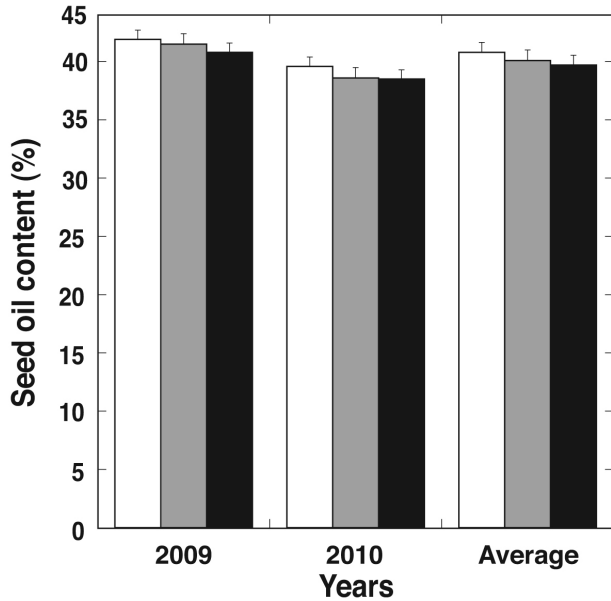


Fig. 3. Effect of additional nitrogen fertilization on oil content in hemp plant seeds in field conditions. Data are means \pm SE from three replicates. N₊₀, no additional nitrogen; N₊₆₀, nitrogen in a form of NH₄NO₃, 60 kg ha⁻¹; N₊₁₀₀, NH₄NO₃, 100 kg ha⁻¹.

Photochemical activity of photosystem II, characterized by F_v/F_o , was significantly higher in the N₊₁₀₀ treatment on days 29 and 36, in comparison to control plants (Fig. 7B). Analysis of F_v/F_M indicated no photoinhibition of photosynthesis on any treatment, as the values ranged from 0.834 to 0.839 (data not shown). Hemp plants treated with additional 100 kg ha⁻¹ nitrogen had higher values of F_v/F_M on days 29 and 36.

Discussion

In the present study, additional nitrogen fertilization stimulated hemp plant growth in field conditions. Fertilized plants were on average 11% higher than unfertilized control plants, independent of the dose used (Fig. 2A). Laboratory study revealed that the absence of a dose-dependent response of nitrogen in field conditions might be due to similar accumulation of nitrogen in plant tissues (Fig. 5), as well as, indirectly, by similar concentration of chlorophyll (Fig. 6). In contrast, several aspects of photochemistry of photosynthesis were significantly stimulated only by the N₊₁₀₀ treatment, in comparison to the N₊₆₀ treatment and hemp plants with no additional fertilization (Fig. 7). Consequently, high doses of nitrogen fertilization stimulated photosynthetic performance of hemp plants, independent of tissue concentration of nitrogen. It appears that high doses of nitrogen stimulated nitrogen use efficiency, possibly through redistribution of nitrogen towards ribulose 1,5-biphosphate carboxylase protein (Seemann et al. 1987). As a consequence, relatively less nitrogen is directed towards growth, resulting in no changes in the growth rate of additionally-fertilized hemp plants.

It has been established using other crop plants that additional nitrogen fertilization has no significant effect on plant growth until the flowering phase (Bertin, Gallais 2000). In the present study in laboratory conditions, hemp plants showed differences in tissue nitrogen content due to various nitrogen supply only at the end of the experimental period. In contrast, nitrogen-dependent differences in leaf chlorophyll content were evident starting from the 7th day

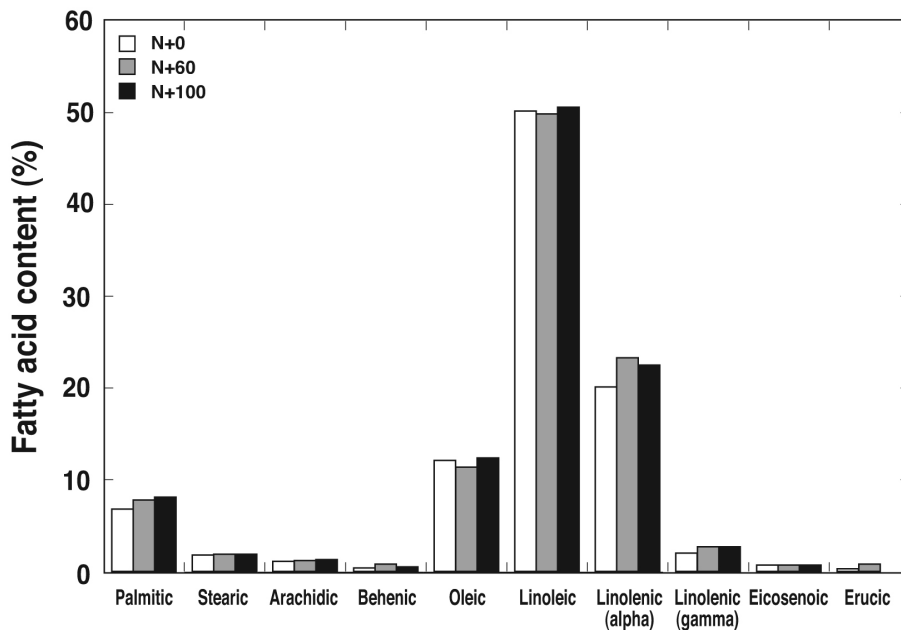


Fig. 4. Effect of additional nitrogen fertilization on fatty acid composition in hemp plant seed oil grown in field conditions. N₊₀, no additional nitrogen; N₊₆₀, nitrogen in a form of NH₄NO₃, 60 kg ha⁻¹; N₊₁₀₀, NH₄NO₃, 100 kg ha⁻¹.

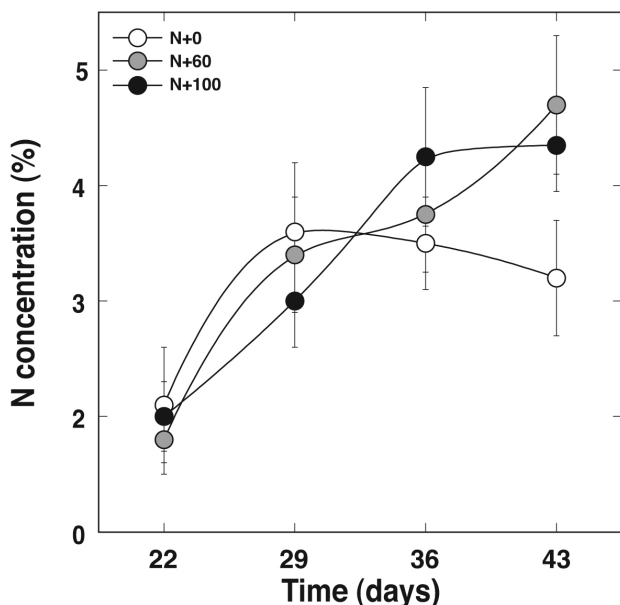


Fig. 5. Effect of additional nitrogen fertilization on leaf nitrogen concentration of hemp plants in laboratory conditions. Data are means \pm SE from three replicates. N₊₀, no additional nitrogen; N₊₆₀, nitrogen in a form of NH₄NO₃ 60 kg ha⁻¹; N₊₁₀₀, NH₄NO₃ 100 kg ha⁻¹.

after the first treatment.

A lack of positive effect, or even evidence of negative effect, of additional nitrogen fertilization on several yield characteristics of hemp plants may be due to different physiological mechanisms. Firstly, organic nitrogen remobilization from source leaves to filling seeds usually does not depend on soil nitrogen availability, but rather on leaf longevity (Racjan, Tollernaar 1999) and activity of

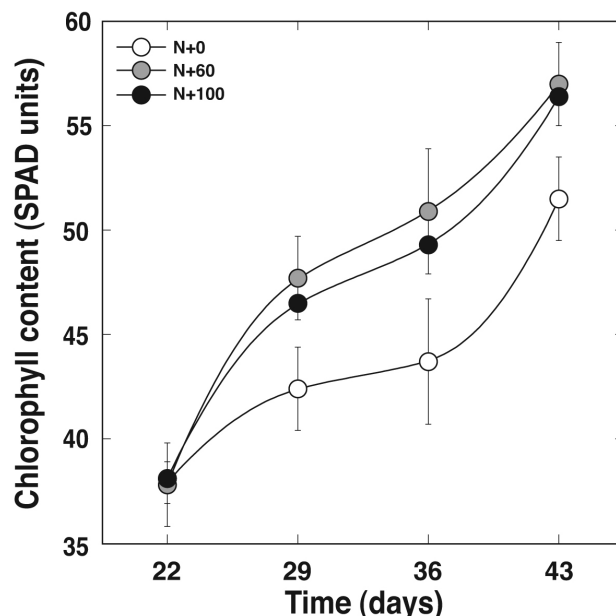


Fig. 6. Effect of additional nitrogen fertilization on leaf chlorophyll content of hemp plants in laboratory conditions. Data are means \pm SE from three replicates. N₊₀, no additional nitrogen; N₊₆₀, nitrogen in a form of NH₄NO₃ 60 kg ha⁻¹; N₊₁₀₀, NH₄NO₃ 100 kg ha⁻¹.

enzymes of nitrogen metabolism (Reed et al. 1980). The current investigation showed positive nitrogen fertilizer impact on seed yield in hemp (Fig. 2C). The average seed yield of fertilized plants exceeded that of unfertilized plants by 36.5%. However, hemp seed mass was not significantly affected by fertilizer (Fig. 2D). Other studies also have shown that soil nitrogen availability positively affects hemp plant growth (Bócsa et al. 1997; Ranalli 1999).

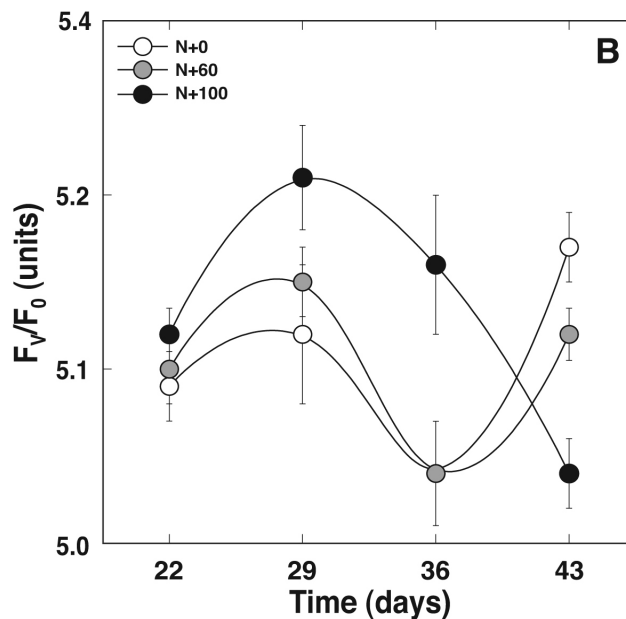
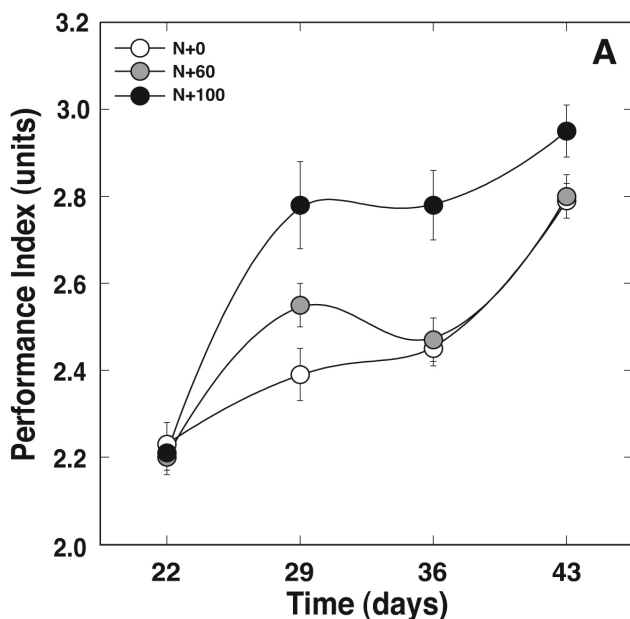


Fig. 6. Effect of additional nitrogen fertilization on Performance Index (PI, A) and FV/F0 (B) of hemp plants in laboratory conditions. Data are means \pm SE from three replicates. N₊₀, no additional nitrogen; N₊₆₀, nitrogen in a form of NH₄NO₃ 60 kg ha⁻¹; N₊₁₀₀, NH₄NO₃ 100 kg ha⁻¹.

Secondly, studies with other crop plants have shown that a high level of nitrogen fertilizer decreases fiber content (Rasmussen et al. 2008). Most possibly, nitrogen supply results in energy-dependent and carbon-required assimilation of nitrate into amino acids, concomitantly decreasing carbohydrate synthesis and accumulation. There was a negative or no effect of additional nitrogen fertilizer on fiber content in hemp stems in the current study (Fig. 2B). Stems of unfertilized hemp plants had fiber content on average 7.8% higher than that of fertilized plants. Consequently the use of nitrogen fertilizer should be reduced when cultivating hemp only for fiber production.

Thirdly, oil content in oil-seed crops is known to be inversely related to nitrogen fertilization (Taylor et al. 1991; Asare, Scarisbrick 1995; Egesel et al. 2008). This fact was confirmed also with hemp plants in this study, but the effect was not statistically significant (Fig. 3). It is more likely that an increase in nitrogen incorporation into amino acids competes for carbon skeletons with fatty acid biosynthesis. However, fatty acid composition did not change by nitrogen application in hemp seeds (Fig. 4), suggesting that this indeed can be a general effect of metabolism shift towards amino acid and protein synthesis (Schmitt et al. 2001).

Variation in climatic conditions between vegetation seasons affected growth and yield characteristics of control hemp plants, as well as the effect of additional nitrogen fertilization on these characteristics. According to the present data, potential factors were summary precipitation and temperature. Hemp growth intensity is shown to be closely linked to air temperature (Bavec, Bavec 2007) with optimum photosynthetic productivity between 25 and 30 °C (Chandra et al. 2008). Although in 2010 the air temperature exceeded temperatures in other years, the most intense growth of plants were observed in 2009. Differences between plant height in 2009 and those in 2008 and 2010 were more than 28 cm. In respect to precipitation it is considered that fiber hemp varieties require higher rainfall levels than oil hemp varieties (Elzebroek, Wind 2008). Nevertheless, in the relatively dryer seasons of 2009 and 2010 average fiber content in hemp stems was higher than in 2008, when total precipitation was higher. Also, seed mass significantly varied in seasons with different moisture conditions. According to these results, additional nitrogen fertilizer doses should be applied under the existing climatic conditions. In a vegetation season with higher rainfall levels, 60 kg of additional nitrogen fertilizer per hectare can be considered as an optimal amount.

In addition to these factors, also light availability can directly or indirectly affect plant yield. The light optimum for photosynthesis of hemp plants has been shown to be at a photon flux density of 1000 to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Chandra et al. 2008). Temperature and light requirements make hemp an ideal fiber and oil crop species in a temperate climate. Regarding indirect effects of light, high nitrogen plants are least sensitive to high light-dependent inhibition

of photosynthesis (Seemann et al. 1987). In the present study, N_{+100} hemp plants had higher values of F_v/F_m , an indirect indicator of photoinhibition of photosynthesis. On the other hand, limiting nitrogen conditions limits acclimation to high light conditions (Seemann et al. 1987). This can explain, at least in part, climate-dependent variation in yield characteristics between different seasons, when suboptimal conditions together with high light can result in decrease of photosynthetic productivity for low-nitrogen plants.

Cold-pressed hempseed oil contained both saturated and unsaturated fatty acids (Fig. 4). About half of the total fatty acid content was linoleic acid, with α -linolenic acid and oleic acid constituting approximately 20 and 12%, respectively, which is in accordance with the literature data (Leizer et al. 2003). In general, hemp seed oil is suggested as a balanced source of two essential polyunsaturated fatty acids (linoleic and linolenic acids) for human nutrition (Saclik et al. 2003). In addition, several unique and rare fatty acids (Mölleken, Theimer 1997; Callaway 2002) were detected in the oil. Hemp cv. 'Pūriņi' seed oil contained arachidic, eicosenoic, behenic and erucic fatty acids.

Photosynthesis-related parameters – leaf chlorophyll content and characteristics of photochemistry of photosynthesis as measured by chlorophyll *a* fluorescence – were used in the present study to evaluate physiological responses of hemp plants to additional nitrogen fertilization. It is usually argued that there is a positive correlation between chlorophyll and nitrogen content in plant leaves (Schlemmer et al. 2005; Fox, Walthall 2008). In the present study, additional nitrogen fertilization significantly increased chlorophyll content already seven days after the first treatment, with no difference between treatments, while significant differences in nitrogen concentration were visible only at the end of the experimental period (Fig. 5, 6). Consequently, leaf chlorophyll content can be used only as approximate indication of tissue nitrogen concentration. In contrast to chlorophyll content, which did not depend on a particular dose of nitrogen fertilization, photochemistry of photosynthesis was largely unaffected by N_{+60} treatment in comparison to control hemp plants, while N_{+100} caused activation of photochemistry.

The Performance Index (PI) combines several photochemical parameters of photosynthesis, such as the density of active reaction centers, efficiency of received energy transport and electron flux rate (Pinior et al. 2005). PI is suggested to be the most valuable fluorescence parameter describing common vitality of the plant (Corrêa et al. 2006). As nitrogen deficiency decreases the photosynthetic chain reaction rate (Xu, Zhou 2006), and appropriate nitrogen supply improves electron transport rate and quantity of photosystem II reaction centres (Zhou et al. 2006), changes in PI can characterize efficiency of nitrogen supply. In the present study, application of additional nitrogen fertilizer at the highest dose significantly increased hemp plant vitality,

as suggested by increased PI. Particular components of photosystem II positively affected by the fertilizer may involve an increase in the proportion of active reaction centre chlorophyll molecules, as indicated by the increase in RC/ABS. In addition, overall activity of photosystem II (F_v/F_0) increased in N_{+100} hemp plants, suggesting that other photochemical reactions also were affected.

In conclusion, the hemp seed yield of hemp cv. 'Pūriņi' was from 1050 to 2557 kg per hectare, depending both on climatic conditions and fertilization rate. Average hemp seed yield can reach 1000 kg ha⁻¹ for fiber-producing varieties or up to 2000 kg ha⁻¹ for seed-producing varieties (Heyland 1996; Clough 2001). Local hemp cv. 'Pūriņi' seeds contained 36 to 42% oil; thus it is among the highest oil-producing hemp genotypes, with an average oil content top level of 37.5% (Kriese et al. 2004). As usual fiber yield of hemp plants is in the range of 15 to 25% (Ranalli 2004), cv. 'Pūriņi' with an average fiber content of 23.6% makes it well-suited for both seed and fiber production in temperate conditions.

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