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Variability of cannabinoid yields of fibre hemp cultivars depending on the sowing density and nitrogen fertilisation

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Abstract: The aim of the experiments was to determine the effect of sowing density and nitrogen fertilisation on the cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC) yields in selected cultivars of fibre hemp. A three-factor field experiment was conducted in 2014–2016. Factor A: cultivar (Futura 75, KC Dora and Tygra), factor B: sowing density (60 and 180 seeds/m^2) and factor C: nitrogen fertilisation (0, 30, 60 and 90 kg/ha). The CBD yield ranged from 10.52 g/m^2 in cv. Tygra to 10.99 g/m^2 in cv. KC Dora. Among the examined cultivars, the highest yield of Δ^9-THC in cv. KC Dora was observed. Sowing density did not modify the CBD yield, but increasing the density from 60 to 180 seeds/m^2 caused an increase in the yield of Δ^9-THC in cvs. KC Dora and Tygra. Nitrogen fertilisation significantly influenced the yields of CBD and Δ^9-THC. In both cases, yield increased until the rate of 60 kg N/ha.

Keywords: *Cannabis sativa* L.; agrotechnical factors; productivity

Hemp (*Cannabis sativa* L.) has been cultivated worldwide since 3 000 BC. In Europe, the species has been extensively used in industrial production since the 1930s. Today, nearly 90 years later, it is gaining the interest of a growing group of farmers, both in the industrial sense and in medical applications. Its numerous advantageous ecological, agronomical and pharmaceutical properties qualify this multifunctional crop as a convenient raw material for various traditional (fibre, food, oil, medicine) or innovative industrial applications (new biomaterials and biofuels) (Amaducci et al. 2015, Bonini et al. 2018). Cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC) are hemp's most well-known active compounds. Δ^9-THC is a primary psychoactive compound, while CBD is a non-psychoactive compound (Monton et al. 2019, Poniatowska et al. 2019). So far, the basic aspect of the legal regulations for the cultivation of hemp is the content of Δ^9-THC, which by law cannot exceed 0.2% of dry weight (EU Regulation 2013). In October 2020, the European Parliament voted in favour of restoring the authorised THC level on the field from 0.2% to 0.3%. The new Common Agricultural Policy (CAP) will enter into force on January 1st, 2023. The new CAP recognises the possibility for farmers to receive Direct Payments for hemp cultivars registered in the

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EU Catalogue that have a maximum level of THC of 0.3%. This change entails a potential enlargement of the number of hemp cultivars accepted under the EU Catalogue. However, the content of Δ^9-THC in each of the EU countries is regulated by national law and may not exceed these thresholds, e.g. 0.6% in Italy; 1% in the Czech Republic. CBD does not cause neurobehavioral changes; it has analgesic and anti-inflammatory properties (Russo 2011). In this context, breeding work is justified, with the aim of obtaining cultivars containing the legal content of Δ^9-THC with the highest possible CBD content, as well as obtaining certain functional parameters, e.g. resistance to diseases and pests (Wielgus et al. 2008, Pavlovic et al. 2019). It is estimated that biological progress is mainly related to increasing the productivity of plants; therefore, there is a need to select appropriate cultivars and optimise the most important agrotechnical factors. High seed yield, as well as hemp biomass, depends on sowing density and harvesting date, but fertilisation, especially with nitrogen (N), is a key factor (Amaducci et al. 2002, Bennett et al. 2006, Hall et al. 2014). Tang et al. (2017) found that an application rate of 60 kg N/ha was sufficient due to hemp's highly efficient use of soil nitrogen. On the other hand, a study by Aubin et al. (2015) found that the optimal dose for high biomass yield was 150 kg N/ha. Amaducci et al. (2002) added that nitrogen at rates above 120 kg N/ha increased hemp biomass production but at the cost of compromised fibre quality due to low cellulose content. Vera et al. (2004) revealed that nitrogen could not only reduce the protein content of hemp seed but also significantly affect the oil content. According to Bócsa et al. (1997), there is a significant relationship between applied nitrogen rates and Δ^9-THC content. To date, a lot of research has been done on the effect of nitrogen fertilisation and its secondary metabolism in medical hemp cultivation (Bernstein et al. 2019, Saloner and Bernstein 2020, Saloner and Bernstein 2022). Unfortunately, there is little field research on hemp to obtain cannabinoids (Wylie et al. 2021), and especially to evaluate the productivity of these ingredients. The influence of weather conditions, which determine the content of cannabinoids in the plants, is also very important (Pacifico et al. 2007, Poniatowska et al. 2021). However, few studies have compared the performance of the current commercial chemovar of industrial hemp; in the literature is a paucity of published studies regarding agronomical practices related to different hemp cultivars (Struik et al. 2000, Tang et al. 2016).

The aim of our study was to determine the effect of sowing density and nitrogen fertilisation on the CBD yield and Δ^9-THC yield in selected cultivars of fibre hemp.

MATERIAL AND METHODS

Experimental design and agronomic management. The field study was conducted at the Experimental Station of the Institute of Natural Fibers and Medicinal Plants in Pętkowo, Wielkopolska, Poland (52°20'N, 17°25'E) in 2014–2016. The experiment was localised on the proper black soil according to the IUSS Working Group WRB (2015) – Gleyic/Stagnic Phaeozems and Mollic Gleysols, with a topsoil thickness of 35–40 cm. The chemical analyses were carried out in an accredited laboratory of the Regional Chemical-Agricultural Station in Poznan. Soil analysis demonstrated a mechanical composition of sandy clay, slightly acidic (pH = 6.8) with a high phosphorus content (16.3 mg/100 g of soil); a moderate content of potassium (13.6 mg/100 g of soil), magnesium (6.7 mg/100 g of soil), manganese (73 mg/100 g of soil), copper (3.5 mg/100 g of soil), and zinc (7.8 mg/100 g of soil); a low iron content (381 mg/100 g of soil); and a 1.64% content of organic carbon and 0.133% organic nitrogen. The content of assimilable forms of phosphorus (P) and potassium (K) was determined by the Egnér-Riehm method (1960) with UV-V is spectrophotometry (Nicolet Evolution 300, Thermo Fisher Scientific, Waltham, USA), pH potentiometrically in 1 mol/L KCl solution. The available Mg content with the Schachtschabel method (1954) with F-AAS spectroscopy (Varian Spectra AA-240 FS, Varian Medical Systems, Inc., Milpitas, USA). Additionally, the contents of available forms of Mn, Zn, Cu, and Fe extracted with DTPA solution were assayed according to Lindsay and Norvell (1978). Organic carbon was determined using the modified Tiurin method and organic nitrogen according to Ostrowska et al. (1991).

The experiment was established as a three-factor randomised complete block (split-split-plot) method with four replications. The research factors were: monoecious cultivar (Futura 75, KC Dora, Tygra), sowing density (60 and 180 germinated seeds/m^2) and nitrogen fertilisation (0, 30, 60 and 90 kg/ha). In accordance with the established experiment methodology, 60 and 180 germinating seeds were sown per 1 m^2, which corresponded to the following amounts of seed: 10 kg/ha and 30 kg/ha. Nitrogen fertiliser

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in the form of ammonium nitrate (34%) was used as a top dressing in single doses, as determined in the methodology. The hemp was cultivated in accordance with the principles of Good Experimental Practice (EPPO PP 1/181, 2012). In all research years, winter ploughing was performed at 25–30 cm depth. In autumn 2013–2015, in the form of potassium-magnesium fertiliser (Korn Kali) with 40% K_2O in the form of potassium chloride and 6% MgO in the form of magnesium sulphate (Kieserit) and sodium (4% Na_2O) and sulfur (12.5% SO_3) was applied at a dose of 300 kg/ha (120 kg K/ha, 11 kg Mg/ha, 15 kg S/ha). In spring, pre-sowing crops were used to provide cannabis with optimal development conditions. Chemical weeding was not done before sowing and during the vegetation period. Sowing was performed with a self-propelled seeder with a row spacing of 15 cm to a depth of 2–3 cm. The forecrop in each year of the research was winter wheat. The hemp was harvested by hand at the beginning of seed ripening and depending on the cultivar, and it took place in the first and second decade of September each year. The area of the micro plots was 4.5 m^2 and 0.9 m^2 for harvesting.

Chemical composition analysis. The content of CBD and Δ^9-THC was analysed in the Institute of Natural Fibers and Medicinal Plants National Research Institute (IWNiRZ) laboratory in Poznan, Poland. Plant material samples (10 randomly selected plants (inflorescences) from each replicate on all tested combinations) were dried to a 12% moisture content, ground, and passed through a sieve with a diameter of 0.63 mm. Subsequently, weighted test samples of 0.3 g were prepared and extracted in 5 mL of N-hexane in sealed conical flasks for 30 min (RT, 100 rpm), then centrifuged at 4 000 rpm for 5 min. The extract was then filtered, 0.3 mL of the filtered extract was transferred to a GC (gas chromatography) vial, and the N-hexane was evaporated under reduced pressure (Raharjo and Verpoorte 2004, Dussy et al. 2005). Afterwards, 0.1 mL of BSTFA (trimethylsilyl 2,2,2-trifluoro-N-(trimethylsilyl) acetimidate, chlorotrimethylsilane) was added, and the derivate was dissolved in 0.5 mL of ACN (acetonitrile). Thus prepared, the plant material obtained in each year of the experiment was analysed to determine the percentage content of CBD and Δ^9-THC. The analysis was performed in accordance with the procedure for the determination of cannabinoid content in plant material developed at the Department of Seed Processing and Research of the Institute. Gas chromatography with flame ionisation detector (GC-FID, PerkinElmer Inc., Wellesley, USA) analysis was performed using an AutoSystem XL chromatograph (Perkin Elmer, PerkinElmer Inc., Wellesley, USA). Data were acquired and analysed using standard software supplied by the manufacturer (TurboChrom, PerkinElmer Inc., Wellesley, USA). Separation took place on a fused silica capillary column (SPB-5, 30 m × 0.32 mm × 0.25 µm). The injection port and detector temperatures were 275 °C and 300 °C, respectively. Splitless injection mode was used with helium as carrier gas at a flow rate of 6.0 mL/min. The cannabinoids were identified based on their relative retention times with the corresponding standard values assayed under the same chromatographic conditions. Quantification was performed using the standard curves. The content of CBD and THC in three cultivars, including in research regarding sowing density and nitrogen fertilisation, was published by Poniatowska et al. (2021). Psychoactive compound content was analysed in IWNiRZ laboratories in accordance with EU methodology defined in Annex XIII to Commission Regulation (EC) No. 2860/2000.

Weather conditions. The weather conditions during the vegetation period of hemp in the years of the study are presented using the hydrothermal index in accordance with Sielianinov (Table 1). In all the years, the mean air temperature ranged from 8.3 °C to 22.2 °C and was comparable to the multi-annual mean. A cru-

Table 1. Sielianinov hydrothermal index (K) values in the years 2014–2016

Year	Month						Average
	IV	V	VI	VII	VIII	IX	
2014	1.3	1.3	0.4	0.8	0.6	1.0	0.9
2015	2.2	0.5	0.6	0.8	0.3	0.4	0.8
2016	1.7	0.5	1.5	2.9	0.6	0.1	1.2
1990–2009	1.0	1.0	1.1	1.2	1.2	1.0	1.1

K: < 0.5 – drought; 0.5–1.0 – semi-drought; 1.0–1.5 – border of optimal moisture; > 1.5 – excessive moisture

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Table 2. Factorial ANOVA with cultivar, sowing density, nitrogen fertilisation, and interactions for the yield of cannabidiol (CBD) and yield of Δ^9-tetrahydrocannabinol (Δ^9-THC) in hemp

Specification	*df*	Yield	
		CBD	Δ^9-THC
Cultivar (A)	2	ns	*
Sowing density (B)	1	ns	*
Nitrogen fertilisation (C)	3	*	*
A × B	2	*	*
A × C	6	*	*
B × C	3	*	*
Error	71		

ns – non-significant; $^*P < 0.05$

cial element of optimal growth and biomass-yielding conditions is also total precipitation, particularly its distribution. The variability of weather conditions in the years of the study was reflected in the values of the Sielianinov index. More advantageous moisture conditions for hemp plants were found in the year 2016 ($K = 1.2$) than in the drier 2014 ($K = 0.9$) and 2015 ($K = 0.8$). The following formula was applied:

$$K = (\text{Mo} \times 10)/(\text{D}_t \times \text{days})$$

Where: K – hydrothermal coefficient for an individual month during the growing season; Mo – total monthly precipitation; D_t – mean daily temperature in a particular month.

Statistical analysis. Recorded results were analysed statistically by analysis of variance in the split-split-plot design (ANOVA) using the SAS package (SAS Institute 1999). The least significant difference was verified with Tukey's multiple range test (post hoc Tukey's *HSD* (honestly significant difference)) at significance levels of $P < 0.05$. The relationship between the parameters was determined with linear regression calculus.

RESULTS AND DISCUSSION

The suitability of the tested cannabis cultivars to be used for pharmaceutical production is evidenced not only by the content of CBD and Δ^9-THC but also by the yields of this substance obtained per unit of cultivation. In terms of the medical uses of cannabis and in light of the law in force, it is important to select and cultivate cannabis cultivars with the highest possible yield of cannabidiol and the lowest possible yield of Δ^9-THC. In none of the cannabis cultivars registered as fibrous did the concentration of Δ^9-THC exceed the 0.2% threshold. The conducted ANOVAs indicated that the yield of CBD was significantly affected by nitrogen fertilisation and interaction between cultivar and sowing density, sowing density and nitrogen fertilisation, and cultivar and nitrogen fertilisation (Table 2). Furthermore yield of Δ^9-THC was affected by cultivar, sowing density or nitrogen fertilisation, or their interaction.

In our study, tested cultivars differed only slightly in the level of CBD yield (Figure 1). The highest CBD yield was found in cv. KC Dora (10.99 g/m^2) and the lowest in cv. Tygra (10.52 g/m^2). Janatová et al. (2018) revealed that within seven tested genotypes, the best quantitative parameters were shown by cvs. Nurse Jackie and Jilly Bean. Kakabouki et al. (2021) observed a significant difference in CBD yield among cultivars and locations, the lowest value was 9.59 g/plant in control, for the cv. Fedora 17, in Athens, and the highest value was 18 g/plant in urea nitrification inhibitor (NI) and urease inhibitor (UI) for cv. Uso 31 in Farsala.

Our research showed no significant effect of sowing density on the CBD yield. Tang et al. (2017) indicate that the effects of planting density and nitrogen fertilisation on hemp stem and seed yields did not interact. The effects of planting density and nitrogen fertilisation on stem yield did not interact significantly with each other or with cultivar and harvest time.

The highest CBD yield at 60 seeds/m^2 was found in the cv. KC Dora, i.e. 11.17 g/m^2, and no significant difference in sowing density was found for the value of this feature between the cvs. Futura 75 and KC Dora. At a density of 180 seeds/m^2, the cultivars did

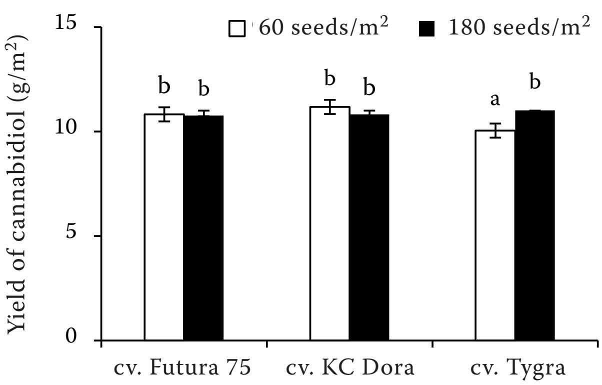

Figure 1. The yield of cannabidiol depending on cultivar and sowing density. Bars with the same letter are equal ($P \leq 0.05$; Tukey's test). Error bars represent standard deviations of the mean

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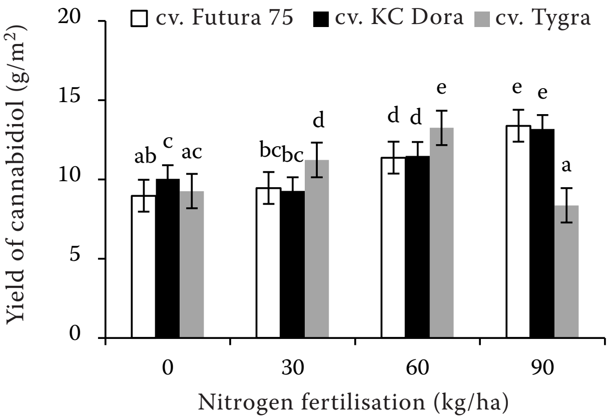

Figure 2. The yield of cannabidiol depending on the cultivar and nitrogen fertilisation. Bars with the same letter are equal ($P \leq 0.05$; Tukey's test). Error bars represent standard deviations of the mean

not differ significantly in terms of the CBD yield. Increasing the sowing density from 60 to 180 seeds/m^2 significantly raised the CBD yield in cv. Tygra, and this difference was 0.97 g/m^2, i.e. 8.8%, while the applied sowing density did not significantly modify this value in the remaining cultivars. The lack of differentiation of the CBD yield depending on the sowing density indicates the possibility of using the lower of studied density (60 seeds/m^2). Mineral nutrition is a major factor affecting plant growth and function. Bernstein et al. (2019) observed that the nutritional supplements affected cannabinoid content in the plants differently. These effects were location and organ-specific and varied between cannabinoids. Nitrogen is a key factor for getting higher hemp production; therefore, a rational N supply is important for increasing N efficiency and crop productivity (Yang et al. 2021). In our study, nitrogen fertilisation determined the CBD yield (Figure 2). An increase in the CBD yield under the influence of N fertilisation was found by increasing the dose to 60 kg/ha, while no significant difference was found between the object without fertilisation and the dose of 30 kg/ha. Further increasing the nitrogen dose to 90 kg/ha caused a decrease in the CBD yield by 0.40 g/m^2, i.e. 3.3%, but this difference was not statistically confirmed. In cvs. Futura 75 and KC Dora, the highest yield of CBD was obtained after applying 90 kg N/ha and in cv. Tiger after using 60 kg N/ha. According to Aubin et al. (2015), biomass and seed compositions were also affected by N fertilisation, but the effects were minimal and generally biologically limited. Similarly, Kakabouki et al. (2021) noted that the CBD yield per plant was only affected by fertilisation; urea with urease and nitrate inhibitors had the highest values. Lukin and Bitiutskikh (2017) reported no significant effects of NPK fertilisation on cannabinoid content.

For cvs. Futura 75 and KC Dora, a rectilinear was obtained, opposite cv. Tygra, where the CBD yield with increasing nitrogen fertilisation had a curvilinear increase (Table 3).

The calculated regression coefficient for the cv. Tygra shows that the highest CBD yield, i.e. 12.68 g/m^2, can be expected after N fertilisation at the dose of 44.5 kg/ha. For the cvs. Futura 75 and KC Dora, the theoretical productivity of 1 kg of nitrogen in the range of the doses tested is expressed by the increase in the CBD yield for these cultivars by 0.05 g/m^2 and 0.039 g/m^2, respectively. At a sowing density of 60 germinating seeds/m^2, a significant increase in the yield was proved between doses of 0 and 60 and

Table 3. The regression equation for cannabidiol (CBD) yield and Δ^9-tetrahydrocannabinol (Δ^9-THC) yield of hemp depending on cultivar and nitrogen fertilisation

Yield	Cultivar	Regression equation	The determination coefficient (%)
CBD	Futura 75	$y = 0.0505x + 8.52$	94
	KC Dora	$y = 0.039x + 9.24$	76
	Tygra	$y = -0.0019x^2 + 0.1692x + 8.912$ y opt = 12.68; x max = 44.5	83
Δ^9-THC	Futura 75	$y = 0.00004x^2 + 0.0043x + 0.2377$ y opt = 0.353; x max = 53.7	51
	KC Dora	$y = -0.00003x^2 + 0.0027x + 0.3438$ y opt = 0.404; x max = 45.0	26
	Tygra	$y = 0.0015x + 0.2529$	76

Y opt – the optimal value for the y-axis; x max – maximum value for the x-axis

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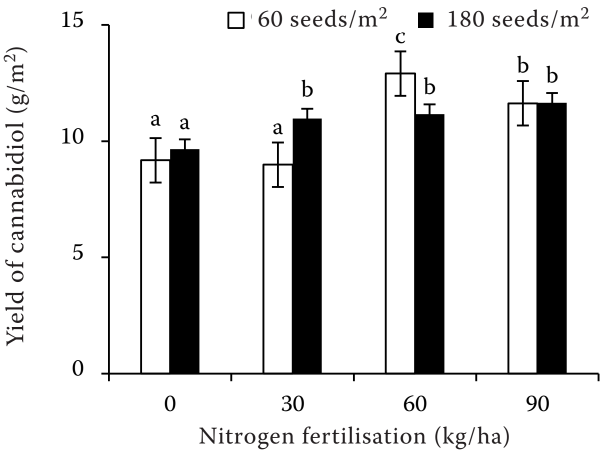

Figure 3. The yield of cannabidiol depends on sowing density and nitrogen fertilisation. Bars with the same letter are equal ($P \leq 0.05$; Tukey's test). Error bars represent standard deviations of the mean

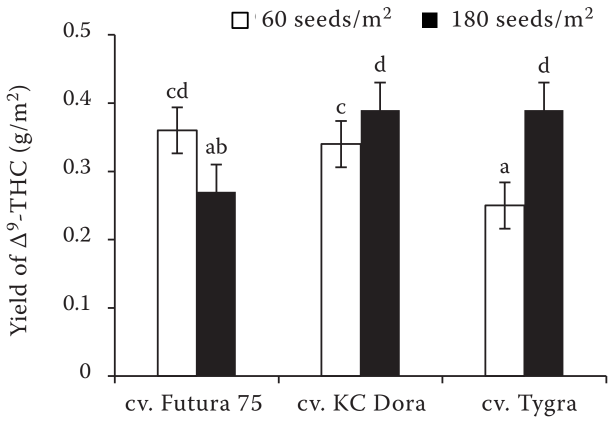

Figure 4. The yield of Δ^9-tetrahydrocannabinol (Δ^9-THC) depends on the cultivar and sowing density (g/m^2). Bars with the same letter are equal ($P \leq 0.05$; Tukey's test). Error bars represent standard deviations of the mean

30 and 60 kg N/ha (Figure 3). Further increasing the nitrogen dose to 90 kg/ha resulted in a significant decrease in the CBD yield, and the difference was 1.28 g/m^2, i.e. 9.9%. On the other hand, at a density of 180 seeds/m^2, a significant increase in the CBD yield was recorded at the dose of 30 kg N/ha, and a further increase in the dose of this component resulted in an insignificant increase in the CBD yield by 0.19 g/m^2 in the facilities where 60 kg N/ha was applied, and 0.68 g/m^2 after using 90 kg N/ha. In the study by Kakabouki et al. (2021), the CBD content was significantly affected by fertilisation; it was higher in urea with inhibitors in cvs. Uso31 and Fedora 17.

Among the cultivars examined in the study, cv. KC Dora had a significantly higher yield of Δ^9-THC, which amounted to 0.364 g/m^2 (Figure 4). The cv. Tygra yielded lower than cv. KC Dora by 0.043 g/m^2 and cv. Futura 75 by 0.052 g/m^2. However, no significant difference in the Δ^9-THC yield was observed between cvs. Futura 75 and Tygra. Sowing density significantly modified the Δ^9-THC yield. Increasing the density from 60 to 180 seeds/m^2 increased the yield of Δ^9-THC from 0.316 g/m^2 to 0.349 g/m^2, i.e.

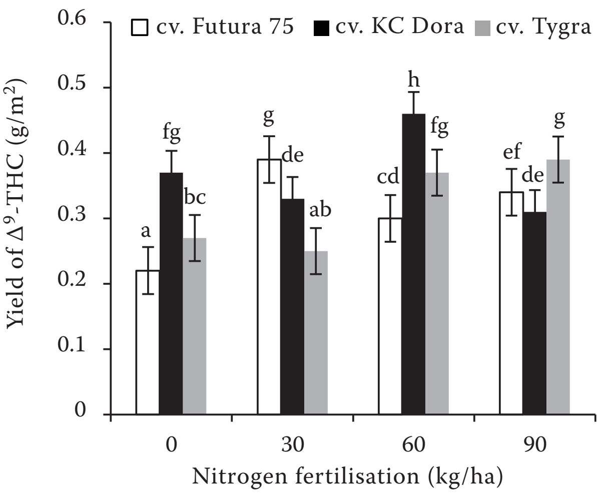

Figure 5. The yield of Δ^9-tetrahydrocannabinol (Δ^9-THC) depends on sowing density and nitrogen fertilisation. Bars with the same letter are equal ($P \leq 0.05$; Tukey's test). Error bars represent standard deviations of the mean

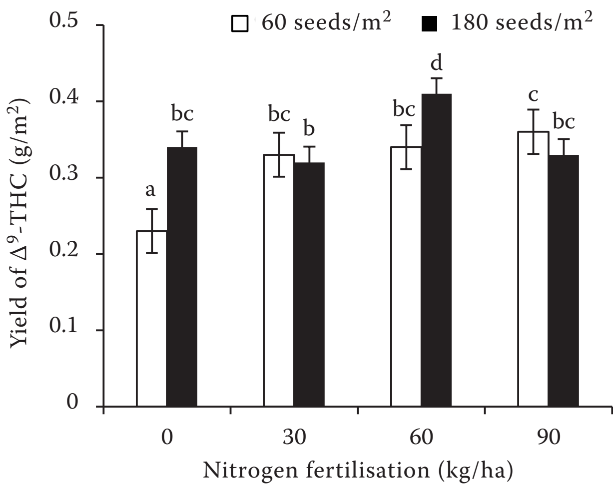

Figure 6. The yield of Δ^9-tetrahydrocannabinol (Δ^9-THC) depends on sowing density and nitrogen fertilisation. Bars with the same letter are equal ($P \leq 0.05$; Tukey's test). Error bars represent standard deviations of the mean

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by 9.45%. In the cv. Futura 75, increasing the sowing density from 60 to 180 seeds/m^2 contributed to a 26.2% decrease in the Δ^9-THC yield, while in the cvs. KC Dora and Tygra, a higher Δ^9-THC yield was found at the higher of the tested sowing density (180 seeds/m^2), and this increment for the cultivars was 0.052 g/m^2 and 0.14 g/m^2, respectively. Increasing nitrogen fertilisation to a dose of 60 kg/ha resulted in a significant increase in the Δ^9-THC yield obtained from the dry mass of cannabis panicles, and further increasing nitrogen fertilisation by 30 kg/ha contributed to a significant decrease in the value of this feature by 8.5% (Figure 5). The cultivars reacted differently to the N fertilisation level. In the cv. Futura 75, a significant increase occurred after the application of 30 kg N/ha, and in the cvs. KC Dora and Tygra, 60 kg N/ha, while in the cv. Tygra, no significant differences were found between a dose of 60–90 kg N/ha.

The search for the relationship between the Δ^9-THC yield and N fertilisation for the cultivars studied revealed generally low values of the coefficient of determination (Table 3). It was noted that in the case of the cvs. Futura 75 and KC Dora, these relationships were curvilinear, and in the cv. Tygra, rectilinear. Among the cultivars, the highest value of the coefficient of determination was demonstrated for the cv. Tygra (R^2 = 0.76) and the lowest for cv. KC Dora, where only 26% of N fertilisation determined the yield of Δ^9-THC. The theoretically calculated regression coefficient for the cv. Futura 75 shows that the highest yield of Δ^9-THC (0.353 g/m^2) can be expected after N fertilisation at a dose of 53.75 kg/ha, and for the cv. KC Dora (0.404 g/m^2) after applying nitrogen fertilisation at a dose of 45.0 kg/ha. Furthermore, for the cv. Tygra, the production of 1 kg of nitrogen causes an increase in the yield of Δ^9-THC by 0.0015 g. At the density of 60 seeds/m^2, a significant increase in the Δ^9-THC yield was recorded in the facilities where 30 kg N/ha was used, but no significant difference was found between the objects fertilised with nitrogen (Figure 6). At the higher density (180 seeds/m^2), a significant increase in the yield of Δ^9-THC was recorded at the dose of 60 kg/ha, but a further increase in the dose of nitrogen resulted in a reduced yield of 0.082 g. The results obtained in our research confirm the need for further continuation of research in terms of the selection of cultivars, depending on the direction of their use, as well as specifying the optimal agricultural technology for them in order to obtain high-yielding hemp raw material.

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