

Genotype screening of *Cannabis sativa* L. based on the specifics of minor cannabinoids manifestation

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Purpose. Analysis of hemp collection samples based on the content of minor (rare) non-psychoactive cannabinoids, such as cannabichromene (CBC), cannabidivarin (CBDV), and cannabinol (CBN); determination of correlation relationships between them and common compounds; selection of valuable breeding genotypes. **Methods.** Field, biochemical (gas chromatography of cannabinoid compounds), and statistical (paired, partial, multiple linear correlation and determination). **Results.** Quantitative analysis of 210 samples of various ecological-geographical and genetic origin (local and wild forms, self-pollinated lines, hybrids, varieties, synthetic populations, polyploids) with a tetrahydrocannabinol (THC) content of less than 0.08% in dried plants showed the level of manifestation of the trait from its absence within the sensitivity of the gas chromatograph up to 0.6838% CBC, 0.1719% CBDV and 0.3274% CBN. In the studied hemp samples, a medium negative relationship was found between the signs of CBC and cannabidiol (CBD) contents ($r = -0.53$), a weak negative relationship between CBC and CBDV contents ($r = -0.35$), medium positive relationships between the signs of CBC and THC contents ($r = 0.57$) and CBC and CBN contents ($r = 0.59$). A medium positive correlation ($r = 0.57$) was found between the signs of CBDV and CBD contents, while CBN had a strong positive relationship with THC ($r = 0.82$). There is almost no correlation between cannabigerol (CBG) and the minor cannabinoids under study. The biosynthesis of minor cannabinoid compounds is quite complex. Signs manifestation is affected by many genetic and external factors. Partial correlation coefficients (given that one of the three signs is eliminated) and multiple correlation coefficients (given that the relationship of one sign is determined and two other signs are combined) give grounds to state that the gene for CBCA synthase affects the production of CBD and, in particular, THC. **Conclusions.** The closeness of the linear relationships between minor cannabinoids and common components allows selecting valuable hemp samples with a high content of one or several compounds under the absence or low content of psychoactive THC.

Keywords: hemp; genetic resources; synthase; biosynthesis; cannabichromene; cannabidivarin; cannabinol; correlation; gene.

Introduction

Cannabinoids belonging to the chemical class of aromatic compounds and accumulating predominantly in glandular hairs (trichomes), are specific substances in industrial hemp (*Cannabis sativa* L.) [1–3]. The most common and well-known are tetrahydrocannabinolic (THCA), cannabidiol (CBDA) and cannabigerolic (CBGA) acids. In the plant organism they are synthesized in acid form, bioactive forms of cannabinoids – tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabigerol (CBG), re-

spectively – are formed as a result of decarboxylation reaction under the influence of external conditions [4]. In addition to the main ones, hemp contains many minor (rare) cannabinoids – compounds whose content is very low and usually does not exceed 0.5% of dry biomass. The pharmacological properties of minor cannabinoids have not yet been reliably confirmed in clinical studies or *in vitro* and *in vivo* rational analyzes. Due to the lack of such biological and pharmacological information, minor cannabinoids have some potential as possible drug candidates [5]. If there is now a significant interest from manufacturers and scientists in THC and CBD, it is expected that in the near future they will focus on the still unknown variety of secondary minor cannabinoids. At the same time, the extraction and purification of these compounds from hemp plant extracts is a rather difficult task due to the low yield of isolates

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and, during subsequent processing, due to the low stability of substances [6].

The pharmacological activity of minor cannabinoids is now being actively investigated, in particular, their effect on the CB1R and CB2R receptors is being studied [7]; CBG, cannabichromene (CBC), cannabinol (CBN) and tetrahydrocannabivarin (THCV), for example, demonstrate anticonvulsant, anti-inflammatory, antidepressant and antibacterial properties, relieve pain and promote muscle relaxation. There is a possibility of their use as anticancer agents, but further research is needed to confirm the possibility of their medical use [8].

The biosynthesis of minor cannabinoids in cannabis has not been completely elucidated, it is unlikely that they are synthesized enzymatically. It was suggested that the chemical transformation in trichomes under the influence of light, temperature, and ultraviolet radiation is the main catalytic mode of their formation; it is these transformations that can explain the significant chemical diversity and low structural stability of these compounds [5, 6].

The precursors of cannabinoid biosynthesis are formed in two different biosynthetic pathways: polyketide, which produces olivetholic acid, and plastid, which produces geranyl-diphosphate. Of these, with the participation of prenyltransferases, CBGA, which is the main precursor of several different cannabinoids is synthesized [9]. In this case, specific synthases ferment a certain cannabinoid compound: THCA synthase converts CBGA into THCA, CBDA synthase – into CBDA and CBCA synthase – into CBCA [10]. A non-functional allelic variant of the THCA synthase gene was also found: the so-called “null” THCA synthase with a single nucleotide polymorphism (SNP), which makes the synthase unable to convert CBGA into THCA, which leads to a significant accumulation of the first compound [11]. Apart from the aforementioned enzymes (THCA, CBDA, and CBCA synthases), no other genes or enzymes that control the biosynthesis of cannabinoids from CBGA have yet been found [5].

Most authors suggest the membrane localization of the enzyme CBGA synthase and the cytosolic localization of THCA synthase, but recently THCA synthase has been found outside the plasma membrane of the glandular cell and catalysis under non-aqueous conditions [12]. Therefore, trichome can play a significant role in the formation of secondary metabolites; it is a biosynthetic organ where photosynthetic reactions take place. The following arguments are made in favor of this statement: 1) almost ideal spherical shape in the lower

micrometric range, very conducive to the refraction and focusing of light rays as in a lens filled with liquid; 2) raising the temperature to 50 °C under direct sunlight probably accelerates the formation of bonds between carbon atoms and other chemical reactions [6]. In general, the number of minor cannabinoids reaches at least 150 [13], their biological role and physiological functions in the plant are not known, many of them are artifacts of the processes following the synthesis of the main cannabinoids, and some acquire stability after acetylation, methylation or dimerization reactions. Chemical diversity of cannabinoids is still not fully understood, and the biosynthetic effect of enzymatic catalysis was overestimated, so photochemical transformations in a non-aqueous “trichome bioreactor” become an acceptable hypothesis for explaining the patterns of their occurrence [5]. So, in the end, we can state about two types of formation of cannabinoid compounds – enzymatic and non-enzymatic (photochemical).

It should be noted that collectible hemp samples of unrelated origin are characterized by variability in the characteristics of the main (common) cannabinoid compounds – CBD, THC and CBG. According to the results of studies conducted with the national collection of hemp, the content of CBD in dried inflorescences ranged from 0.0052 to 1.7251%, THC – from 0.0000 (absent) to 0.0775% (does not exceed the norm permitted by law), CBG – from 0.0000 (absent) to 0.8892%. The established range of variation makes it possible to select samples with an increased content of CBD and/or CBG in the absence of THC. The analyzed material was assigned to 3 hemp chemotypes: III (65.5%) is fibrous cannabis with a predominance of CBD and a high CBD : THC ratio, in which the THC content ranges from a small percentage to a complete absence; IV (1.8%) – hemp with a predominance of CBG, which is the main compound and low or no THC; V (32.7% of the total) – hemp with a complete absence of cannabinoid compounds, which are practically impossible to isolate within the sensitivity of the chromatograph. Furthermore, strong correlations between CBD and THC and, in fact, the absence of a relationship between CBD and other cannabinoid compounds were revealed; moreover, in the samples of chemotype III bonds are weaker compared to chemotype V [14]. The issue of characterizing cannabis genetic resources by the content of minor (rare) non-psychoactive cannabinoids – CBC, cannabidivarin (CBDV) and CBN, assessing the correlation between them and common compounds,

identifying sources and donors of valuable traits and selecting the initial breeding material on this basis remains unclear.

Materials and methods

The material for the research was 210 samples from Ukrainian National Collection of Flax and Hemp of the Institute of Bast Crops of the National Academy of Sciences of Ukraine, belonging to various ecological and geographical types (northern, Central European and southern), genetic origin (local and wild forms, self-pollinated lines, hybrids, varieties, synthetic populations, polyploids), obtained from 10 countries (mainly from Ukraine, Russia, France, Germany and China) and for which the THC content is within the limits permitted by the current legislation, and does not exceed 0.08%.

Three-year field research (cultivation) was carried out in the northeastern part of Ukraine on the southern border of the mixed forest zone of the lowest part of the Ukrainian Polissia. Height above sea level was 166 m, geographic coordinates of the area: 51°39' north latitude and 33°59' east longitude. The soils in crop rotation were dark and light gray forest, slightly podzolized loams formed on moraine clay. Fertilizer application rate was $N_{120}P_{90}K_{90}$. The weather conditions during the research years (2018–2020) were varied and characterized by deviations from the average annual air temperature, precipitation and relative humidity: 2018 and 2020 were hot (in September up to 3.9 °C above normal) and arid during the hemp growing season, and 2019 was characterized by excessive rainfall from May to July and almost absence of rainfall in August (9.9 mm). This enabled comprehensive evaluation of the performance of collection samples under various weather conditions.

In order to identify cannabinoid compounds during threshing of hemp plants grown in a valuation plant nursery with a feeding area of 30 × 5 cm (phase BBCH 89) [15], a combined sample of plant material was taken from each area of 1 m² from the inflorescences, dried and stored at a laboratory temperature. Before the analysis, the samples were dried to constant weight at a temperature of 105 °C in an oven, ground to a fine powder and thoroughly mixed; samples weighing 0.5 g were taken in two repetitions, and 5 ml of methanol was added (the ratio “plant type : extractant” – 1 : 10). The extraction time was 24 h, after that the extract was filtered using a paper filter. In the obtained methanol extracts of samples, the quantitative content of cannabinoid compounds was determined by gas chromatography on an

HP 6890 Series GC System chromatograph with detection. Chromatography conditions:

- capillary column – Agilent Technologies 19091J-413 (HP-5), length – 30 m, diameter – 0.320 mm, phase – 0.25 μm, SN: USN493366H, constant flow – 1.5 ml/min, carrier gas – helium;
- injector – auto-injector 7683, Split 20 : 1, evaporator temperature – T = 250 °C; oven – T_{initial} = 100 °C, hold for 2 minutes, heating – 15 °C/min, T_{final} = 280 °C, hold for 11 minutes;
- detector – flame ionization;
- sample – 1.0 μl.

Compounds were identified by retention time. The concentration of cannabinoids was determined using an internal standard (stearic acid methyl ester at a concentration of 0.392% of the sample), for which, the chromatographic peak areas of the internal standard and the chromatographic peak areas for the studied compounds were compared using the Chemstation software. Statistical data processing was carried out according to the method of field experiment [16]. A sample with a high content of a certain cannabinoid is one with content higher than the median (Me).

Research results

The findings of the quantitative analysis of cannabinoid compounds showed that CBC in the inflorescences of collection hemp samples was in a small amount, namely, from absence to 0.6836%. The Cumulative graph of the frequency distribution of the values of the trait of a given compound content in all studied samples shows that their lion's share (88.1% of the total amount) is within the 0.0000–0.0683% class, a significantly smaller number of samples (5.2%) falls within the class 0.0684–0.1367%, the rest (from 0 to 4 samples, or up to 1.9%) belongs to other classes (Fig. 1).

CBC was isolated at the very beginning of modern biochemical studies of cannabis, but it is less studied in comparison with other phyto-cannabinoids in terms of biological profile and chemical activity [17]. Traditionally, together with CBD, THC, and CBG, it is considered the main phytocannabinoid and a constituent of the so-called “Big Four” components of cannabinoid compounds, even the second most abundant, but its concentration was significantly overestimated due to the difficulty of separating CBC and CBD in gas chromatography with subsequent assignment of the peak area exclusively to the CBC. In fact, the concentration of CBC is much lower, it rarely exceeds 0.2–0.3% of dry biomass; it was found that CBC accumulates in one- or two-digit concentrations [13]. Our research confirms this feature.

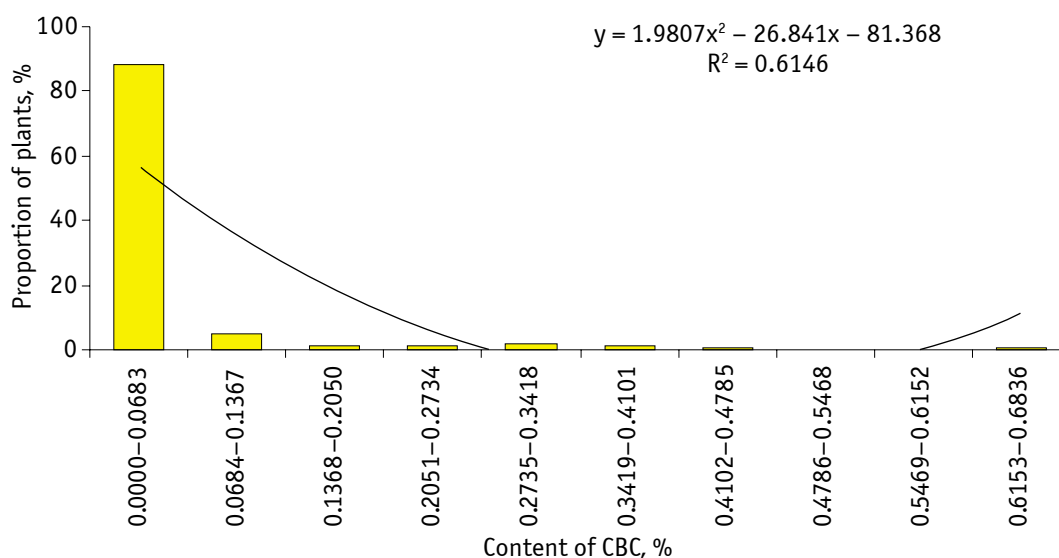


Fig. 1. Cumulative graph of the frequency distribution of the values of the CBC content trait in the collection hemp samples (average for 2018–2020)

CBDV was identified in even smaller amounts compared to CBC – from complete absence to 0.1719% of dry biomass. The class with a content of 0.0000–0.0171% includes 62.4% of all studied samples, with a content of 0.0172–

0.0343% – 18.1%, and the next two – 11.4 and 4.3% of analyzed samples respectively. The rest of the classes contain only from 1 to 3 samples, or from 0.5 to 1.4% of the total (Fig. 2).

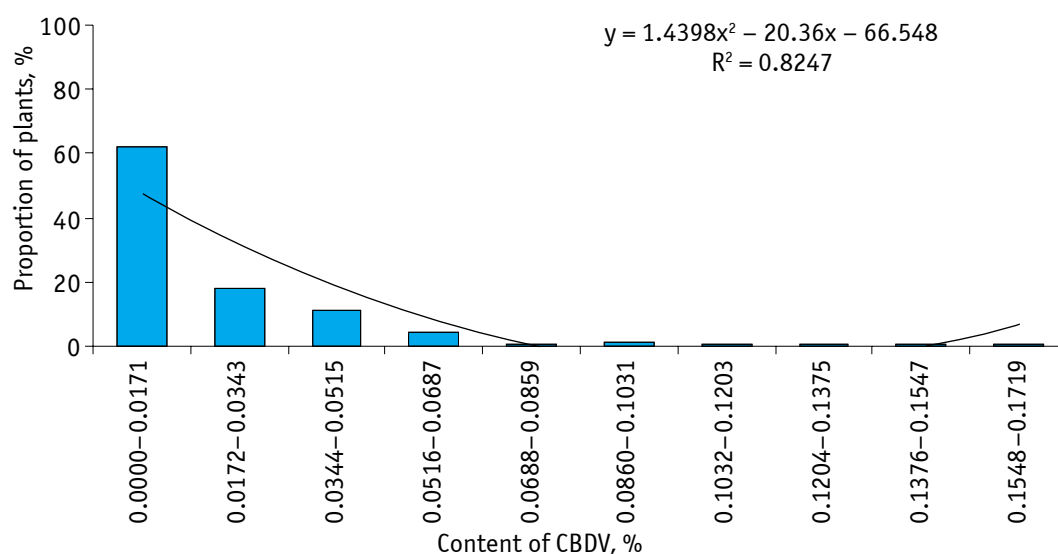


Fig. 2. Cumulative graph of the frequency distribution of the values of the CBDV content trait in the collection hemp samples (average for 2018–2020)

Among the studied minor cannabinoids, CBN was identified in the smallest number of samples, its maximum content was 0.3274%. The Cumulative graph of the frequency distribution of the values of the trait clearly shows that this compound is absent in the vast majority of plants or present in insignificant quantities. Thus, 192 collection samples out of 210 analyzed, or 91.4%, contain 0.0000–0.0327% of this compound. At the same time, a negative kurtosis of the studied trait was revealed, since 7.6% of genotypes contained from 0.0328

to 0.0654% CBN, 0.5% of genotypes each contained from 0.2620 to 0.2947% and from 0.2948 to 0.3274% of this compound, respectively. No plants with CBN content from 0.0655 to 0.2619% were found (Fig. 3).

In general, the discovered biochemical features of collection hemp samples indicate the difficulty of using them in breeding as sources and donors of the trait of a high content of minor cannabinoids. In addition, the issue of establishing correlations between cannabinoid compounds becomes relevant, since the pre-

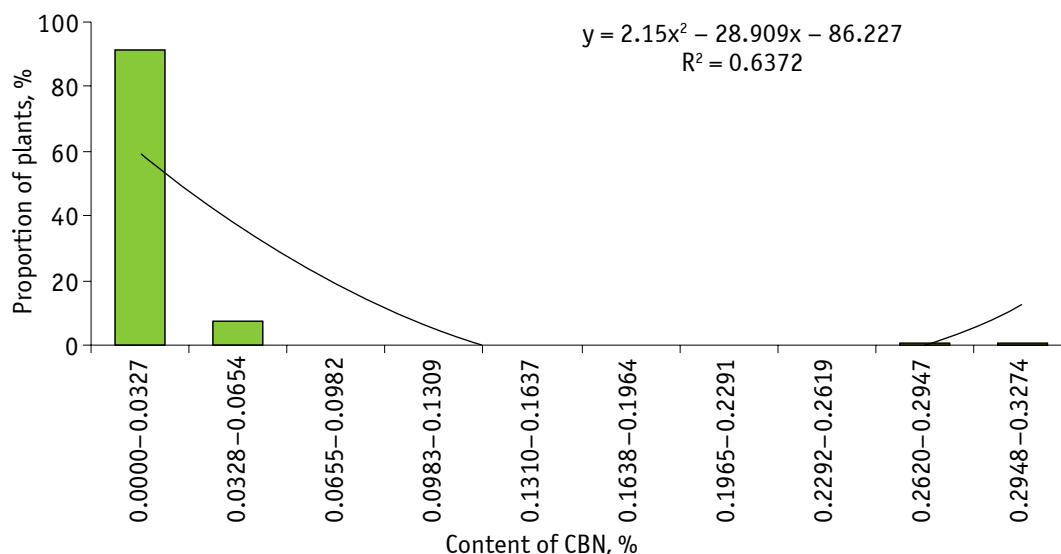


Fig. 3. Cumulative graph of the frequency distribution of the values of the CBN content trait in the collection hemp samples (average for 2018–2020)

sence of strong relationships can cause difficulties in breeding work, when it is necessary to increase the content of some non-psychoactive component, and reduce the content of another, for example THC.

Within the framework of the studied samples, it was found that between the traits of the content of CBC and CBD there is an average negative relationship ($r = -0.53$), CBC and CBDV – a weak negative relationship (-0.35), and between the traits of the content of CBC and THC, CBC and CBN – average positive relationship (0.57 and 0.59 , respectively). A mean positive correlation was found between CBDV and CBD traits (0.57), and CBN had a strong positive relationship with THC (0.82). It should be noted that CBG almost does not correlate with the studied minor cannabinoids (Table 1).

Table 1

Correlation of traits of minor cannabinoids content with common compounds

Pairwise correlation coefficients (r)	CBC	CBDV	CBN
CBD	-0.53^*	0.57^*	-0.10^*
THC	0.57^*	-0.12^*	0.82^*
CBG	0.07^*	-0.01	-0.06^*
CBC	–	-0.35^*	0.59^*
CBDV	–	–	-0.08^*

*Significant at the 0.05 level.

Since the biosynthesis of various cannabinoid compounds is rather complex, and the expression of the signs of their content depends on many, both genetic and external factors, it makes sense to establish a partial and multiple linear correlation. Partial correlation coefficients indicate that CBD and THC, at a con-

stant value, or exclusion (elimination) of CBC, are associated only with a weak positive correlation ($r_{\text{CBD THC} \cdot \text{CBC}} = 0.22$), when there is usually a strong correlation between CBD and THC and its coefficients may approach a value of 1. CBN and THC, with the exclusion of the influence of CBC, are linked by a strong relationship ($r_{\text{CBN THC} \cdot \text{CBC}} = 0.97$). Taking into account the coefficient of determination, this pattern in 94% of cases manifests itself precisely under the influence of the factors under study. In contrast to the pairwise correlation, the relationship between CBC and CBN when eliminating the influence of THC and vice versa (the relationship between CBC and THC when eliminating the influence of CBN) is weak ($r_{\text{CBC CBN} \cdot \text{THC}} = 0.26$ and $r_{\text{CBC THC} \cdot \text{CBN}} = 0.19$) (Table 2). According to the multiple correlation of three variables (an indicator of the tightness of a linear relationship between one of the traits and combination of two other traits), all studied minor cannabinoids at an average or strong level depend on the cumulative effect of various compounds. The content of CBC depends more strongly on the interaction of CBN and THC ($R_{\text{CBC} \cdot \text{CBN THC}} = 0.99$, $R^2 = 0.98$), in turn, the content of THC and CBN strongly depends on the interaction of CBC with a certain cannabinoid compound ($R_{\text{THC} \cdot \text{CBN CBC}}$ and $R_{\text{CBN} \cdot \text{THC CBC}}$ are 0.83 , $R^2 = 0.68$). Hence, it follows that CBC has a significant effect on the level of accumulation of major cannabinoids, although according to common theories, the genetic determination of cannabinoid content is inherited independently.

Recent studies [18] show that while the high transcription level of THCA synthase and CBDA synthase clearly reflects the

Table 2

**Partial and multiple coefficients of linear correlation
and determination between traits of content of minor
and common cannabinoids**

Partial linear correlation			Multiple linear correlation		
Trait	<i>r</i>	<i>d</i>	Trait	<i>R</i>	<i>R</i> ²
CBD THC · CBC	0.22*	0.05	CBC · CBD THC	0.58*	0.34
CBD CBDV · CBC	0.50*	0.25	CBC · CBD CBDV	0.59*	0.35
CBN THC · CBC	0.97*	0.94	CBC · CBN THC	0.99*	0.98
CBC THC · CBD	0.60*	0.36	CBD · THC CBC	0.57*	0.33
CBC CBDV · CBD	0.07*	0.01	CBD · CBDV CBC	0.67*	0.45
CBC CBD · THC	-0.56*	0.32	THC · CBD CBC	0.68*	0.46
CBC CBN · THC	0.26*	0.07	THC · CBN CBC	0.83*	0.68
CBC CBD · CBDV	-0.43*	0.18	CBDV · CBD CBC	0.57*	0.33
CBC THC · CBN	0.19*	0.03	CBN · THC CBC	0.83*	0.68

*Significant at the 0.05 level.

chemical phenotype of cannabis, the low but stable level of transcription of CBCA synthase in all genotypes suggests that these genes may contribute to the final amount of cannabinoids. It was hypothesized that CBCA synthases are not only enzymes for converting CBGA to CBCA, but can also participate in the formation of THCA in a material with a predominance of CBD; therefore, the reciprocal (mutual) influence of gene groups takes place in the quantitative expression of the chemotype [18].

Our screening of a large number of collection samples for traits of cannabinoid content and the establishment of relationships between them gives grounds to assert that the presence of a high CBC content in samples, which mainly belong to chemotype III (with a high CBD : THC ratio), is interconnected with a high THC content. It can be assumed that the CBCA synthase gene is not located in an independent locus; in this case, a more complex genetic mechanism for determining the synthesis of cannabinoids operates, and CBCA synthase gene, for example, has several linked loci, in particular with CBDA synthase genes and, especially, THCA synthase.

CBN is the end product of THC biosynthesis – aromatized THC, an oxidation product

that is usually identified if hemp plant material or isolated THC is exposed to ultraviolet radiation or sunlight for a long time [5, 19], therefore, the features of the correlation between CBN and THC and CBC that we have established are logical. The more THC was synthesized, the more of it could turn into CBN, the more active CBCA synthase was, the more THC accumulated. Since CBDV is an *n*-propyl analogue of CBD [19], the average correlation between these compounds is obvious.

As a result of studying the collection of genetic resources of this culture, it was possible to identify valuable collection samples (sources and donors of traits) for the practical breeding of industrial hemp varieties for medical use, namely, with a high content of minor cannabinoids and a content of psychotropic THC within the limits permitted by current legislation. A tight linear connection of minor cannabinoids with each other and with common components makes it possible to find the source material with the above characteristics. So, 5 samples with a high content of CBC were isolated (2.4% of the total amount analyzed), CBDV – 7 samples (3.3%), CBN – 2 samples (1.0%), finally with a combination of a high CBDV content and widespread CBD compounds – 5 samples (2.4%) (Table 3).

Table 3

**Sources and donors of traits for high content of minor cannabinoids with THC content < 0.08%
(average for 2018–2020)**

Trait	Number of the national catalog of the collection hemp sample
High content of CBC	UF0600506, UF0600721, UF0600723, UF0600724, UF0600727
High content of CBDV	UF0600040, UF0600045, UF0600116, UF0600409, UF0600442, UF0600565, UF0600690
High content of CBN	UF0600253, UF0600254
High content of CBDV and CBD	UF0600116, UF0600409, UF0600442, UF0600565, UF0600690

Conclusions

In the analyzed collection samples of hemp, the content of CBC did not exceed 0.6836%,

CBDV – 0.1719% and CBN – 0.3270%, in the vast majority of them minor cannabinoids were not identified at all. The level of accumulation of separate minor cannabinoids depends

on the influence of many genetic and external factors, a different nature of the correlations between them was established: CBDV is most associated with CBD, CBN is most associated with THC, the trait of CBC content affects the formation of CBD and, especially, THC. A tight linear connection of minor cannabinoids with each other and common components makes it possible to isolate valuable samples for breeding with an increased content of both one compound and several compounds with a simultaneous absence or low content of psychotropic THC.

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Міщенко С. В.*, **Кириченко Г. І.**, **Лайко І. М.** Скринінг колекційних зразків *Cannabis sativa* L. за особливостями прояву мінорних канабіноїдів. *Plant Varieties Studying and Protection*. 2021. Т. 17, № 3. С. 218–225. <https://doi.org/10.21498/2518-1017.17.3.2021.242949>

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Мета. Аналіз колекційних зразків конопель за вмістом мінорних (малопоширених) непсихотропних канабіноїдів – канабіхромену (КБХ), канабідиварину (КБДВ) та канабінолу (КБН), установлення кореляційних зв'язків між ними та поширеними сполуками, виділення на цій основі цінного селекційного матеріалу. **Методи.** Польові, біохімічні (газова хроматографія канабіноїдних сполук), статистичні (парна, часткова, множинна лінійна кореляція та детермінація). **Результати.** У результаті кількісного аналізу 210 колекційних зразків різного еколого-географічного та генетичного походження (місцеві та дикі форми, самозапилені лінії, гібриди, сорти, синтетичні популяції, поліплоїди) з вмістом тетрагідроканабінолу (ТГК) менше ніж 0,08% у висушених рослинах виявлено КБХ, КБДВ і КБН від відсутності в межах чутливості газового хроматографа до 0,6838; 0,1719 і 0,3274% відповідно. Між ознаками вмісту КБХ і канабідіолу (КБД) є середній негативний взаємозв'язок ($r = -0,53$), КБХ і КБДВ – слабкий негативний взаємозв'язок ($r = -0,35$), а між ознаками вмісту КБХ і ТГК, КБХ і КБН – середній позитивний взаємозв'язок (r становить 0,57 і

0,59 відповідно). Між ознаками вмісту КБДВ і КБД виявлено середній позитивний кореляційний зв'язок ($r = 0,57$), а КБН має сильний позитивний взаємозв'язок з ТГК ($r = 0,82$). Ознака вмісту канабігеролу (КБГ) майже не корелює з досліджуваними мінорними канабіноїдами. Біосинтез мінорних канабіноїдних сполук досить складний, експресія ознак їхнього вмісту залежить від багатьох як генетичних, так і зовнішніх чинників, часткові (за елімінації впливу однієї з трьох ознак) та множинні коефіцієнти кореляції (за визначення зв'язку однієї ознаки й сукупністю двох інших) дають підстави стверджувати, що ген КБХК-синтази має вплив на утворення КБД і, особливо, ТГК. **Висновки.** Тіснота лінійного взаємозв'язку мінорних канабіноїдів між собою й поширеними компонентами дає змогу виділяти для селекції цінні зразки конопель із підвищеним вмістом як однієї, так і декількох сполук за одночасної відсутності чи низького вмісту психотропного ТГК.

Ключові слова: коноплі; генетичні ресурси; синтаза; біосинтез; канабіхромен; канабідиварин; канабінол; кореляція; ген.

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