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Breeding of industrial hemp with a high content of cannabigerol by the case of 'Vik 2020' cultivar

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Purpose. To create an industrial hemp variety of the Central European ecological and geographical type with a high cannabigerol content and universal application. **Methods.** Breeding (self-pollination, creation of artificial populations, selection), field, biochemical (thin-layer and gas-liquid chromatography of cannabinoid compounds), instrumental and technological assessment of fibre quality, and statistical methods. **Results.** Variety 'Vik 2020' was obtained as a result of creation of artificial populations. The plants are characterized by higher content of cannabigerol (1.034 ± 0.0323%), and almost zero of other secondary metabolites, such as cannabidivarin, cannabidiol, cannabichromene and psychotropic tetrahydrocannabinol (0.003 ± 0.0011; 0.018 ± 0.0080; 0.012 ± 0.0027, and 0.005 ± 0.0012%, respectively). The trait of cannabigerol content is quite stable within the population and is not correlated with the trait of tetrahydrocannabinol content (r = -0.23). TLC showed that cannabigerol accumulated mainly in the form of cannabigerolic acid and to a lesser extent as a neutral compound, which is consistent with the theory that this substance is a precursor for the synthesis of other cannabinoids. According to the results of the competitive variety test, when growing to obtain fibre and seeds, the variety features short height, specifically significantly lower total (206.4 cm) and technical stem length (135.6 cm) compared to the standard variety, significantly higher inflorescence length (70.8 cm), which determine the formation of the significant yield of biomass suitable for pharmaceutical use and high seed yield (0.98 t/ha). The yield of total fibre was the same as in the standard variety (29.0%), but its quality and technological value for primary processing were higher. The variety had a homogeneous sex structure, resistance to bioltic and abiotic environmental factors. Plants reached biological maturity in 116 days (BBCH 89). This cultivar is recommended for obtaining seeds, quality fiber and potentially cannabigerol (on condition of changes in legislation). **Conclusions.** The efficiency of using self-pollinating lines in breeding with their subsequent combining into a synthetic population and improving selection was proved by the case of a new variety of industrial hemp 'Vik 2020', characterized by an increased content of cannabigerol and the absence of psychotropic properties.

Keywords: *hemp; cultivar; self-pollination; selection; crossing; cannabinoids; correlation; productivity.*

Introduction

Cannabinoids are specific substances of cannabis plant (*Cannabis sativa* L.), belonging to the class of aromatic compounds and are mainly accumulated in glandular trichomes [1, 2]. Biosynthesis of cannabinoids occurs on the surface of the plasma membrane or in the cell wall that border the secretory cavity. These compounds play a protective role in a plant, while a decrease in the content of cannabinoids and the number of glands in industrial hemp does not change this physiological function, since their synthesis in small amounts by other plant cells is sufficient [2].

The most common cannabinoids in the glandular hemp trichomes are tetrahydrocannabinolic (THCA), cannabidiolic (CBDA), and cannabigerolic (CBGA) acids. The bioactive forms of cannabinoids – tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabigerol (CBG) – are formed as a result of a decarboxylation reaction under the influence of external conditions. Other decarboxylated derivatives as cannabichromene (CBC) and cannabinol (CBN) were found in small amounts [3]. The precursors of cannabinoid biosynthesis are formed in two different biosynthetic pathways: polyketide, which produces olivetholic acid, and plas-

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tid, which produces geranyl diphosphate; of them, with the participation of prenyltransferase, CBGA is synthesized, which is the main precursor of at least eight different cannabinoids [4]. Specific synthases that ferment a certain cannabinoid compound are also identified and characterized [5]. In particular, THCA synthase converts CBGA into THCA [6], respectively, CBDA synthase – into CBDA [7] and CBCA synthase – into CBCA [8].

An analysis of the features of chemotypes inheritance made it possible to conclude that the genes for THCA and CBDC synthase are codominant alleles at the same locus. This codominance is due to two alleles for different isoforms of the same synthase, which has different specificity for converting the CBGA precursor to CBDA or THCA, respectively [9], while the gene for the CBCA synthase is located at an independent locus. In other studies [10], a variety of sequences for THCA and CBDC synthase was observed, which may be due to the presence of several linked loci for these genes.

Among the main cannabinoid compounds, the features of the genetic control of the trait of CBGA content are the least studied. Genetic analysis of the offspring of two variants of hybrids created as a result of crossing a variety with a predominance of CBGA with a variety with a predominance of THCA, as well as a variety with a prevalence of CBGA with a variety with a predominance of CBDA, showed that the trait of a high CBGA content is inherited as a result of the action of a single recessive gene, potentially determined a non-functional allelic variant of the THCA synthase gene. The so-called "null" THCA synthase contains a single nucleotide polymorphism (*SNP*), which makes the synthase unable to convert CBGA to THCA, what leads to a significant accumulation of the first compound. *SNP* can be used as a molecular marker in breeding for an increase in the CBGA content [11].

The accumulation of cannabinoid compounds in cannabis is well understood. In particular, their content increases at the generative stage of development, the concentration of these substances decreases from the top to the lower part of the plant, and the chemotype of the sample (variety) is relatively constant during individual development. The accumulation of CBGA in ontogenesis, in contrast to CBDA and THCA, has its own peculiarities, since CBGA is a precursor for the synthesis of CBDA, THCA, and CBCA. Significant increases in CBDA and THCA within 5–6 weeks after the onset of flowering can result in a decrease in CBGA and thus neutral CBG from the total CBG + CBGA cannabinoids. After reaching a peak on the 7th week of flowering, the total amount of CBG + CBGA decreased significantly – by 43.5; 37.9 and 65.3% for two weeks, and the content of neutral CBG remained relatively constant until aging of the plants [12].

Interest in cannabis as a culture of medical use is constantly growing. Most often, CBD is used for therapeutic purposes [13], but recently, clinical studies on the possibilities of using CBG, which has significant potential have been actively carried out [14], and in the near future it can compete with CBD with a high probability. CBG reveals clearly expressed medicinal properties; in particular, it demonstrated effectiveness in the fight against oncological diseases in experiments on animals and in the culture of isolated cells and tissues of various organs *in vitro* [15]. CBG together with THC reduced the viability of cancer cells, but the combination of CBG and CBD, two non-psychotropic compounds, was more effective [16]. CBG is characterized by an antioxidant effect and can be used not only as a neuroprotector [17, 18], but also in the treatment of neurological disorders [19]. This compound also has a mild analgesic, antifungal, and antibacterial effect [15, 20].

Methods for creating varieties of industrial hemp with medicinal properties remain insufficiently developed; family-group selection is used both to increase the CBG content and to simultaneously improve valuable economic traits [21]. This method is quite effective in cannabis breeding, but it takes a long time to stabilize the traits; therefore, acceleration of the process of creating the initial material, the development of a selection methodology in this direction and the practical creation of varieties are becoming urgent. Medicinal hemp varieties, in our opinion, should have a high content of CBD, CBG or other non-psychotropic cannabinoids, and at the same time be free of THC (or contain in quantities that do not exhibit psychotropic properties).

The purpose of the research is to create a variety of industrial hemp of the Central European ecological-geographical type of universal direction of economic use with a high content of CBG.

Materials and methods

The material for the research was the separate self-pollinating lines of 'Hlukhivski 58' variety, characterized by a CBG content of more than 0.5%. Then, an improvement selection was carried out for this trait up to the sixth generation. To stabilize the traits, homogeneous inbred lines were used to create the initial breeding material by synthetic selection according to the developed scheme and method [22].

The creation of synthetic populations included several stages:

1) self-pollination and selection of lines (according to the results of the study in the assessment nursery) with the lowest degree of depression, the desired manifestation of breeding traits, the absence of harmful mutations and THC, a stable trait of monoeciousness (I_1–I_2)

2) self-pollination and selection of lines (according to the results of the study in the assessment nursery), which, as a result of closely related reproduction, form a small, and in the absence of spatial isolation and free pollination – a large number of seeds (I_5–I_6); additional self-pollination, if there is a need to achieve inbred minimum, increasing the degree of homogeneity and stability of the lines (I_5–I_6);

3) crossing under a group isolator 5–7 self-pollinating lines (syn-1) similar in characteristics and studying the offspring in the assessment nursery, selection of the same number of seeds from 3–5 best self-pollinating lines in terms of combination ability, sowing a mixture of seeds, free cross-pollination under group isolator (syn-2) and offspring reproduction (syn-3) [22].

The work resulted in a sample 'VIK CBN' (National Catalog Number of Plant Genetic Resources Collection UF0600718), improved by family-group selection for performance traits and registered under the name 'Vik 2020'.

Field studies were carried out at the experimental base of the Institute of Bast Crops of the National Academy of Agrarian Sciences of Ukraine (Hlukhiv, Sumy region), located in the northeastern part of Ukraine on the southern border of the mixed forest zone within the lowest area of the Ukrainian Polissia. Soil type is dark and light gray forest soils, weakly podzolized loams formed on moraine clay. Weather conditions over the years of research were varied and characterized by deviations from the average annual air temperature, precipitation and relative humidity (2018 and 2020 were hot and dry during the cannabis growing season, and 2019 was characterized by excessive rainfall in May-July). This made it possible to comprehensively evaluate the economic indicators of the new variety according to the method [23].

To identify cannabinoid compounds during threshing of hemp plants grown in the field in the assessment nursery (phase BBCH 89) [24], a pooled sample of plant material sufficient for analysis was taken from each plot (1 m^2 area), dried and stored at laboratory temperature. Before the analysis, the samples were dried to constant weight at a temperature of 105 °C in an oven, ground to a powdery state and thoroughly mixed, samples weighing 0.5 g were taken in duplicate, and 5 ml of methanol was added (the ratio of "plant sample : extractant" – 1 : 10). The extraction time was 24 hours, then extract was filtered using a paper filter. In the obtained methanol extracts of hemp samples, the quantitative content of cannabinoid compounds was determined by gas-liquid chromatography on a chromatograph with detection.

Chromatography conditions:

– device – HP 6890 Series GC System, serial No. US00008158;

– 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 injectors 7683, Split 20 : 1, evaporator temperature – T = 250 °C; oven – $T_{initial}$ = 100 °C, hold for 2 minutes, heating – 15 °C/min, T_{finite} = 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 by the internal standard method, which was the methyl ester of stearic acid (concentration – 0.392% of the sample), based on the processing of the Chemstation data program, the ratio of the areas of the chromatographic peaks of the internal standard and the compounds that are identified.

Thin layer chromatography conditions:

– extractant – ethanol;

– solvent system – "petroleum ether (60–95 °C) – diethyl ether" (40 : 10);

– painting with strong blue BB dye;

– standard witness – hemp variety 'YUS 9'.

Statistical data processing was carried out according to the method of a field experiment [25].

Results and discussion

Plants of the created industrial hemp variety 'Vik 2020' are characterized by an increased content of CBG – 1.034 ± 0.0323%, which belongs to the non-psychotropic components of cannabinoid compounds, and an almost complete absence of other secondary metabolites – cannabidivarin (CBDV), CBD, CBC and psychotropic THC (0.003 ± 0.0011; 0.018 ± 0.0080; 0.012 ± 0.0027 and 0.005 ± 0.0012%, respectively). At the same time, the sign of CBG content is quite stable, its minimum value within the population is 0.8538, and the maximum is 1.2242%, the coefficient of variation is 12.1%, which indicates an average variability, which is close to low. A large number of plants of the studied cultivar have no CBDV, CBD, CBC, and THC at all; they cannot be identified within the sensitivity of the gas-liquid chromatograph and used research methods. The maximum THC content was found at the level of 0.0124% (Table 1). Thus, the developed scheme for the selection of synthetic populations of monoecious hemp is effective for creating varieties with medicinal properties.

Table 1

The content of cannabinoid compounds in elite plants of the 'Vik 2020' variety, compared to the 'Hliana' standard (average for 2019 and 2020)

Compound	Content, %		
	'Hliana'	'Vik 2020'	
	average	average	minimum–maximum
CBDV	0.001 ± 0.0010	0.003 ± 0.0011	0.0000–0.0138
CBD	0.021 ± 0.0085	0.018 ± 0.0080	0.0000–0.0658
CBC	0.007 ± 0.0016	0.012 ± 0.0027	0.0000–0.0523
THC	0.003 ± 0.0012	0.005 ± 0.0012	0.0000–0.0124
CBG	0.002 ± 0.0010	1.034 ± 0.0323*	0.8538–1.2242

*Significant difference at a significance level of 0.05.

As evidenced by the results of thin layer chromatography of cannabinoid compounds, CBG accumulated mainly in the acid form – CBGA, and to a lesser extent as a neutral compound, consistent with the theory that this substance is a precursor for the synthesis of CBDA, THCA, and CBCA. R_f CBG on average was 0.68 (Fig. 1). As a result of decarboxylation of acidic forms of cannabinoids into neutral compounds during gas-liquid chromatography combustion, CBGs have been identified.

In addition, a significant weak negative relationship was found between the signs of CBG and CBDV, CBG and CBC, CBG and THC content. Pair correlation coefficients are -0.17; -0.11 and -0.23, respectively. Positive for breeding science and production is the actual absence of a relationship between non-psychotropic CBG and psychotropic THC. On the one hand, there are prerequisites for a further increase in the CBG content without a rapid increase in the THC content, and on the other hand, there is the possibility of multiplying the variety in the seed production system in a number of successive generations without exceeding the THC level permitted by the current legislation (0.08%). We assume that the process of biosynthesis of cannabinoid compounds is interrupted in the created variety of industrial hemp for medical use. In this case, the inactivated corresponding synthases con-

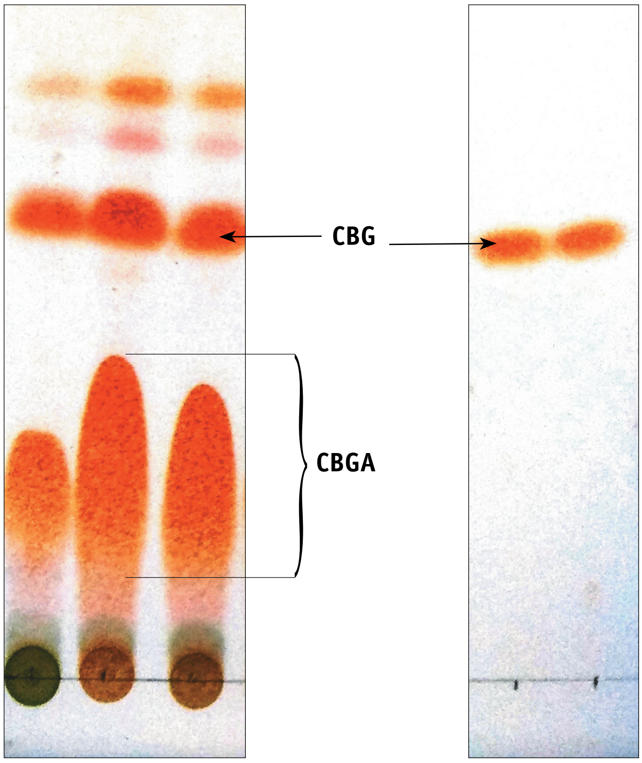

Fig. 1. Manifestation of CBG and CBGA on chromatographic plates

vert CBGA to CBDA or THCA, and therefore CBG predominantly accumulates. The presence of a significantly strong positive correlation between the CBG and CBD content trait (r = 0.84) in this cultivar indicates only the presence of both the B_D allele with a low degree of activity, since the CBD content is very scarce, and the B_0 allele (Table 2).

Table 2

Coefficients of pairwise correlation between the traits of the content of cannabinoid compounds in elite plants of the 'Vik 2020' variety

	CBD	CBC	THC	CBG
CBDV	-0.01	-0.16*	0.04	-0.17*
CBD	–	-0.26*	-0.16*	0.84*
CBC	–	–	0.00	-0.11*
THC	–	–	–	-0.23*

*Significant difference at a significance level of 0.05.

Cumulative graphs of the frequency distribution of the cannabinoid content trait values show that the lion's share of plants (40.0%) was characterized by the absence of THC and their scanty amounts, which did not exceed 0.0012%. Basically, the level of expression of the trait of THC content was within the classes 0.0000–0.0049 and 0.0087–0.0124%, and CBG content was within 0.8538–1.0390 and 1.1131–1.2242% (Fig. 2 & 3). A negative kurtosis coefficient was revealed for both investigated traits, which is generally positive for breeding and primary seed production, since there are prerequisites for selecting elite (parental) plants with a high CBG content and the absence of THC.

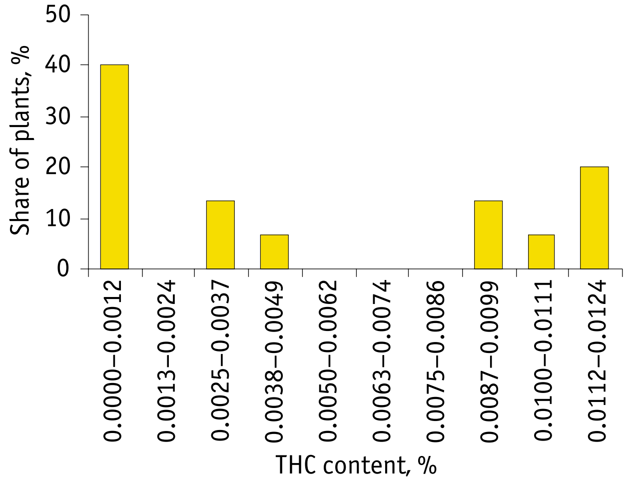

Fig. 2. Cumulative graph of the frequency distribution of the values of the THC content trait in elite plants of 'Vik 2020' variety (average for 2019 and 2020)

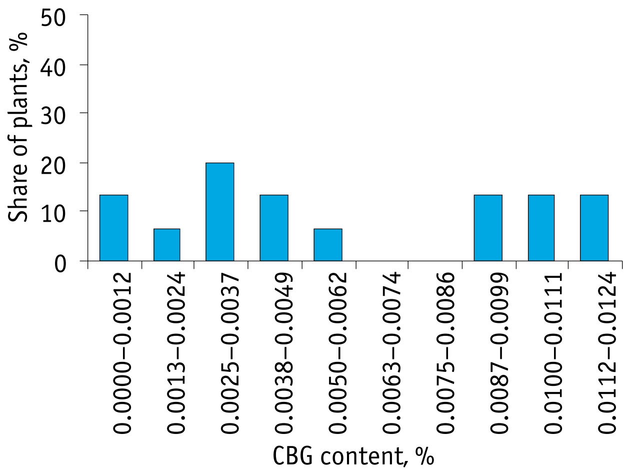

Fig. 3. Cumulative graph of the frequency distribution of the values of the CBG content trait in elite plants of 'Vik 2020' variety (average for 2019 and 2020)

Variety 'Vik 2020' belongs to the Central European ecological-geographical type. The growing period to biological maturity (BBCH 89), according to the average data of three-year studies, was 116 days. It is recommended for cultivation in order to obtain seeds, relatively high-quality fiber and, potentially, for the purpose of obtaining leaves and inflorescences as raw materials for the pharmaceutical industry, but only in case of changes in the legislation of Ukraine regarding the use of hemp for the manufacture of medicines and application in medical practice. According to the results of competitive variety testing when growing to obtain fiber and seeds (for double-sided use), the variety combines short stature, namely, the indicators of the total (206.4 cm) and technical length of the stem (135.6 cm) are significantly less than in the standard variety, with significantly higher indicators of the inflorescence length (70.8 cm), which determine the formation of a significant biomass suitable for use on for pharmaceutical purposes, and seed yield (0.98 t/ha). The total fiber yield is, like that of the 'Hliana' variety, 29.0%, but it is characterized by high quality and processing value: linear density – 45.5 tex, breaking load – 32.0 dan, fiber number – 5.0, fiber grade – elite (Table 3).

The sex structure of 'Vik 2020' variety is dominated by a monoecious feminised pistillate (approximately 77.5% of the total number of sexual types), which has a compact inflorescence (predominantly diamond-shaped) and more than 75% of female flowers in it. This sexual type is the most productive and stable in terms of monoeciousness. Monoecious staminate hemp (male plants), which is a destabilizer of the signs of monoecious, is absent. Resistance to damage by pests and pathogens at the level of the standard variety is from medium to high. The variety is resistant to seed shedding. It is characterized by the content of THC within the limits permitted by the current legislation [according to the conclusion No. 19/11/2-13-ED/20 dated 11.11.2020 of the State Research Forensic Center of the Ministry of Internal Affairs of Ukraine (Kyiv) within the sensitivity of the gas-liquid chromatograph and applied research methods].

Included in the State Register of Plant Varieties Suitable for Distribution in Ukraine since 2021 [26], 'Vik 2020' industrial hemp variety is competitive, as evidenced by the results of a comparative competitive variety trial and characteristics of a number of monoecious non-psychotropic hemp created at the Institute of Bast Crops of the National Academy of Agrarian Sciences of Ukraine [21, 22, 27, 28]. It is also advisable to grow this variety in order to obtain a non-psychotropic compound – CBG, but the use of leaves, inflorescences or corresponding preparations in medical practice has not yet been regulated and prohibited by the current legislation of Ukraine, therefore, the possibilities of 'Vik 2020' variety are potential. There is a hope that in the future, when the legislation changes, it will occupy its niche in the hemp and pharmaceutical markets of Ukraine, and the development of the new breeding direction will continue.

Table 3

Valuable economic traits of the 'Vik 2020' variety, compared to 'Hliana' standard variety (average for 2017–2019)

Traits	The level of traits manifestation in varieties	
	'Hliana'	'Vik 2020'
Productivity and its elements:		
– stems, t/ha	6.59	5.37
– seeds, t/ha	0.72	0.98*
Vegetation period, days	113	116
Total stem length, cm	233.1	206.4*
Technical stem length, cm	168.8	135.6*
Inflorescence length, cm	64.3	70.8*
Fiber quality:		
– fiber yield, %	29.0	29.0
– linear density, tex	64.8	45.5*
– breaking load, dan	23.6	32.0*
– fiber number	4.1	5.0
– fiber grade	nongraded	elite
Resistance against biotic factors:		
– flea beetle, ball	5	5
– European corn borer, ball	7	7
– Fusarium wilt, ball	7	7
– dendrofomosis, ball	5	5
Resistance to abiotic factors:		
– seed shedding, ball	5	7
– lodging, ball	7	7
Sexual structure of the population:		
– content of monoecious feminised pistillate, %	77.2	77.5
– content of monoecious staminate hemp, %	0	0

*Significant difference at the significance level of 0.05.

Conclusions

The efficiency of using self-pollinating lines in the breeding process with their subsequent combining into a synthetic population and improving selection has been proved on the example of a new variety of industrial hemp 'Vik 2020' with an increased content of CBG and the absence of psychotropic properties.

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Міщенко С. В.[*]**, Лайко І. М., Кириченко Г. І.** Селекція промислових конопель із підвищеним умістом канабігеролу на прикладі сорту 'Вік 2020'. *Plant Varieties Studying and Protection*. 2021. Т. 17, № 2. С. 105–112. https://doi.org/10.21498/2518-1017.17.2.2021.236514

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Мета. Створити сорт промислових конопель середньоєвропейського еколого-географічного типу універсального напряму господарського використання з підвищеним умістом канабігеролу. **Методи.** Селекційні (самозапилення, створення синтетичних популяцій, добір), польові, біохімічні (тонкошарова та газорідинна хроматографія канабіноїдних сполук), інструментально-технологічне оцінювання якості волокна, статистичні. **Результати.** У результаті синтетичної селекції створено сорт 'Вік 2020', рослини якого характеризуються підвищеним умістом канабігеролу (1,034 ± 0,0323%), що має низку лікувальних властивостей, і майже повною відсутністю інших вторинних метаболітів – канабідіваріну, канабідіолу, канабіхромену і психотропного тетрагідроканабінолу (0,003 ± 0,0011; 0,018 ± 0,0080; 0,012 ± 0,0027 і 0,005 ± 0,0012% відповідно). Ознака вмісту канабігеролу є досить стабільною в межах популяції і не взаємопов'язана з ознакою вмісту тетрагідроканабінолу (r = -0,23). Проведення тонкошарової хроматографії показало, що канабігерол накопичувався переважно у формі канабігеролової кислоти і меншою мірою як нейтральна сполука, що цілком узгоджується з теорією, згідно з якою ця речовина є попередником для синтезу інших канабіноїдів. За результатами конкурсного сортовипробування в разі вирощування для отримання волокна й насіння сорт поєднує низькорослість, а саме істотно нижчі порівняно із сортом-стандартом показники загальної (206,4 см) і технічної довжини стебла (135,6 см), з істотно вищими показниками довжини суцвіття (70,8 см), які детермінують формування значної біомаси, придатної для використання на фармацевтичні цілі, та врожайності насіння (0,98 т/га). Вихід загального волокна становив, як і в сорту-стандарту, 29,0%, але воно характеризувалося вищою якістю й технологічною цінністю за первинного перероблення. Сорт мав однорідну статеву структуру, стійкість до біо- та абіотичних чинників середовища. Вегетаційний період до біологічної стиглості (BBCH 89) – 116 діб. Рекомендований для вирощування з метою отримання насіння, якісного волокна й потенційно – канабігеролу (за умови зміни законодавства). **Висновки.** Доведено ефективність залучення до селекційного процесу самозапилених ліній із подальшим їх об'єднанням у синтетичну популяцію і поліпшувальним добором на прикладі нового сорту промислових конопель 'Вік 2020', що характеризується підвищеним умістом канабігеролу й відсутністю психотропних властивостей.

Ключові слова: *коноплі; культивар; самозапилення; добір; схрещування; канабіноїди; кореляція; продуктивність.*

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