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EVALUATION OF FACTORS HAVING AN EFFECT ON CANNABIDIOL AMOUNT IN *Cannabis sativa* L.

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Abstract

Industrial hemp is a multipurpose crop, supplying fibers, seeds, and pharmaceuticals. The non-psychotropic cannabidiol (CBD) derived from hemp is a promising pharmaceutical raw material. It shows no psychotropic effects, is not listed in UN Single Convention on Narcotic Drugs, but demanded for the production of medicine products. Regretfully, there are no domestic cultivars of hemp in Russia specialized in phytocannabidiol. Currently, there is a big need in industrial varieties of pharmaceutical specialization (CBD-cultivars). This paper is the first to report on selection of the accessions with high CBD content (above 9%) and trace amounts of Δ^9-tetrahydrocannabinol (THC), the main psychotropic cannabinoid, among the studied genotypic diversity of hemp plants. The objective of the study was the assessment of the effects of field watering, lighting conditions, sexual type of plants and stage of ontogenesis on CBD and THC accumulation on a broad in situ genotypic diversity of *Cannabis* L. germplasm accessions in order to form the optimal morpho-physiological and agronomical model of CBD-producing cultivar (ideotype) well adapted to field growing. Cannabis populations in situ, genotypically original, spatially separated (and, thus, not undergone to random cross pollination), were surveyed in 2008-2011 in four Russian regions. A total of 128 populations were selected for the research; among them, 52 populations were studied for the effect of shading, and 58 for the moisture excess/deficit. For each population, plants (not less than $n = 10$ per each variant, i.e. shading vs. lighting, and moisture excess vs. deficit) were collected randomly and representatively to form summarized sample, and then analyzed to reveal the effect of the said factors. The plants were collected from initial budding and flowering of male plants, up to the moment when fimbles have just begun to dry out and the first seeds at the basal parts of female inflorescences have started to ripen. Air-dry samples (with and without inflorescences, female plants, male plants) were crushed and biochemically analyzed. Inflorescences (generative parts) and only leaves (vegetative parts) of the two conventional sexual types were analyzed separately. The analysis of the CBD and THC contents in the studied accessions revealed a statistically significant ($p = 0.05$) genotypic variability for CBD between the samples. Natural sexual polymorphism in the content of CBD and THC is statistically unreliable. Both male and female plants contain approximately equal CBD concentrations from the budding time until the seed ripening. THC amount was insignificantly increased in female plants. From the budding phase until the start of seed ripening, plants increase their CBD content more than twice. From the budding phase until the seed ripening, CBD amount in inflorescences is significantly thrice more than in vegetative parts. The difference in THC content is significant as well, but not so noteworthy. By the start of budding of different sexual types CBD content in generative parts is twice as high as that of THC. CBD accumulation reaches its maximum in generative plant parts by the time when seed ripening starts initially. The effect of shading on plants of any sexual type has shown that CBD content significantly responds to the exposure and intensity of natural lighting. Any breach in daylight illumination will reduce the CBD content. The factor of excessive/deficient natural moistening has no significant effect on CBD and THC accumulation in plants, regardless of the development phase of an adult plant of any sexual type. Unlike to dioecious cultivars specialized for seed/oil production, both female and male plants may be used for CBD production. Female plants must have extended time of budding—full flowering in conditions of reduced amounts of pollen in air and deferred seed ripening. Flowering and maximum pollen production from male plants must be deferred, because the lack of pollen in field promotes CBD formation in female inflorescences. CBD cultivar plants should have a maximum inflorescence size, a maximum budding and flowering period, and a minimum foliage.

Keywords: *Cannabis sativa* L., *C. ruderalis* L., hemp, plant sexual types, phytocannabinoids, cannabidiol, Δ^9-tetrahydrocannabinol, CBD-cultivar ideotype, breeding

Hemp (*Cannabis* L.) is one of the oldest crops [1]. Due to various factors, industrial hemp crops in the USSR and the Russian Federation regressed from 1 million hectares in the 1940s to 150 thousand hectares in the 1980s and currently are about 15 thousand hectares. Along with a decrease in hemp cultivation in the Russian Federation, there was a tendency towards a structural increase in synthetic drugs in illicit trafficking, under which various psychoactive substances were masked [2].

Nowadays, hemp is used not only to produce different textiles and shive, but also for innovative seed- and oil-based components for functional nutrition and pharmacy. From 1994 to 2017 in the EU, the area of hemp varieties grown for seed, oil and fiber increased from 8 to 33 thousand hectares. In France alone, in 2017, 18 thousand hectares were occupied by hemp. In the Baltic States of the EU in 2018, this area amounted to about 14 thousand hectares. In Canada, hemp is sown annually on more than 15 thousand hectares. In 2011, in the United States, the annual volume of officially imported commercial hemp products reached USD11 million [3, 4], and since 2018, the domestic hemp production was resumed after a period of limiting cultivation.

Inflorescences and leaves of hemp contain over 60 different phytocannabinoids, the terpenphenol compounds which are derivatives of 2-substituted 5-amylresorcinol. The precursor of all phytocannabinoids is cannabigerolic acid. It is transformed into cannabichromene, cannabidiol and Δ^9-tetrahydrocannabinol acids, which are converted to cannabinoids. Cannabidiol and Δ^9-tetrahydrocannabinol acids are the main ones. Their synthesis in a plant is genetically determined. Δ^9-Tetrahydrocannabinol (THC) is responsible for the psychotropic effect of drugs selectively targeted to cannabinoid receptors in brain. Unlike THC, cannabidiol (CBD) has a pronounced therapeutic and sedative effect on humans. The effect of its concentration is inversely proportional to the THC psychotropicity [5].

In a number of European countries and in the USA, the cannabidiol is practiced as a suppressant of the symptoms of vomiting and nausea provoked by anti-cancer treatment, and also as a remedy against weight loss in AIDS patients. Cannabidiol is used to treat neuropsychiatric disorders, rheumatism, glaucoma, multiple sclerosis, alcoholism, asthma, to achieve an analgesic effect [1, 6], under a decrease in appetite, impaired gestation in pregnant women, to stimulate lactation, under neurodegenerative processes [7, 8] and in antitumor therapy [9-11]. Cannabinoids are promising as a substitute for opiates [12, 13]. New data have been published regarding their use in the treatment of oncological diseases and in Alzheimer's disease, as well as information on the relationship of the endocannabinoid system and regulation of intraocular fluid flow, and on the vasodilating and neuroprotective effects of cannabinoids [14].

The literature provides the results of chemotyping cannabis lines vegetatively propagated in greenhouses for the content of one of the cannabinoids, the information on the genotyping varieties to differentiate technotypes and chemotypes, on differences in the metabolism of cannabinoids in varieties used for oil and fiber purposes and in chemotypes (type Purple Kush) [15, 16]. Biochemical markers are used to select plants with a maximum content of terpenes and CBD (chemotype III) cultivated in greenhouses [17].

The dynamics of the main cannabinoids in hemp plants was previously studied on ten zoned varieties during the sproutig—flowering phase on leaves of different layers, which is more likely important for forensic work [18, 19]. Other studies [20] focused on minimizing the THC content (less than 0.01%) in hybrid populations F_1 and F_2 in breeding monoecious hemp. Information on the cannabidiol accumulation during ontogenesis of technical (commercial) hemp plants is based on theoretical calculations and the study of only three monoecious varieties [21]. The works had a narrow specificity, were aimed at changing the zoned monoecious variety of different specialization by breeding methods and were not relevant for other technotypes of commercial varieties, and also did not cover the available genotypic diversity of the genus *Cannabis* L.

Hemp genetic resources in most research centers are very few, genotypically homogeneous [22, 23] and poorly studied with respect to economically significant traits. The lack of data about genetically diverse donors becomes a limitation when creating hemp cultivars for cannabidiol production (CBD cultivars).

Success in hemp selection for high CBD and reduced THC requires an effective strategy to ensure the development of varieties with biochemically, morphologically, technologically specialized and genetically stable traits. An essential element of such a strategy is the selection of initial samples and an adequate assessment of target characteristics during selection.

In this paper, among the genotypic diversity of the studied natural hemp populations, we first identified hemp forms for pharmaceutical use (with a contrastingly high content of CBD, more than 9%, and a trace amount of the main psychotropic cannabinoid THC) which are suitable for field cultivation. The greatest accumulation of CBD is shown to occur by the beginning of ripening, and shading leads to a decrease in this phytocannabinoid level.

The purpose of our study was to evaluate the effects of moisturizing, lighting, sex, and ontogenesis stage on the content of cannabidiol and Δ9-tetrahydrocannabinol in genotypically diverse hemp samples to form the optimal morphophysiological and agricultural model of a variety specialized for producing CBD in field conditions.

Materials and methods. Samplings were carried out in 2008-2011, with accounting natural pollination as an important criterion for the separation of populations [24]. The hemp (genus *Cannabis* L.) plants were collected from genotypically original, spatially separated (beyond the cross-pollination distance) populations in situ in the four federal districts of Russia.

A total of 128 populations were selected, of which 52 were examined for the effect of the shading factor, and 58 for the excess moisture factor. In the first case, in each of the 52 populations growing under the forest canopy, at least 10 plants were collected from unshaded areas and combined into a single sample for the population. In similar manner, a combined sample was also formed from plants vegetating under the crowns of trees. In second case, in 58 populations where part of the plants grew on water edge of a natural reservoir (river), and the rest on the hills beyond the reach of excess water, combined samples contrasting with respect to were excess moisture factor were also collected. The plants were collected randomly and representatively from the beginning of budding and flowering to the early seed ripening in order to neutralize the climatic and temporal varieties in accordance with the "ceteris paribus" principle.

To assess cannabinoid levels, air-dry samples (with and without inflorescences, female plants, male plants) were crushed and biochemically analyzed. Separately, inflorescences (generative parts) and only leaves (vegetative parts) were analyzed in two sex types. The dynamics of accumulation of cannabidiol and Δ^9-tetrahydrocannabinol was studied from the beginning of flowering of male (fimble) hemp, budding of which usually occurred earlier than in female (pistillate) hemp, to the beginning of fimble drying and the first seed maturation in a basal part of inflorescences on female plants.

Samples for gas chromatographic analysis of cannabidiol and Δ^9-tetrahydrocannabinol were prepared according to the methods adopted in forensic practice. The measurements were carried out on a CHROM 5 chromatograph (Bruker, Czech Republic) of a standard configuration with a flame ionization detector according to the manufacturer's recommendations. Concentration of cannabinoids in the sample was measured in 3 analytical repetitions.

For statistical interpretation, analysis of variance (ANOVA) and correlation analysis with Statistica 10.0 software (StatSoft, Inc., USA) were performed. The figures show mean values (*M*) and standard deviations (±SD) at a 95% significance level. The calculated correlation coefficients (simple linear correlation) were considered significant at $p < 0.05$.

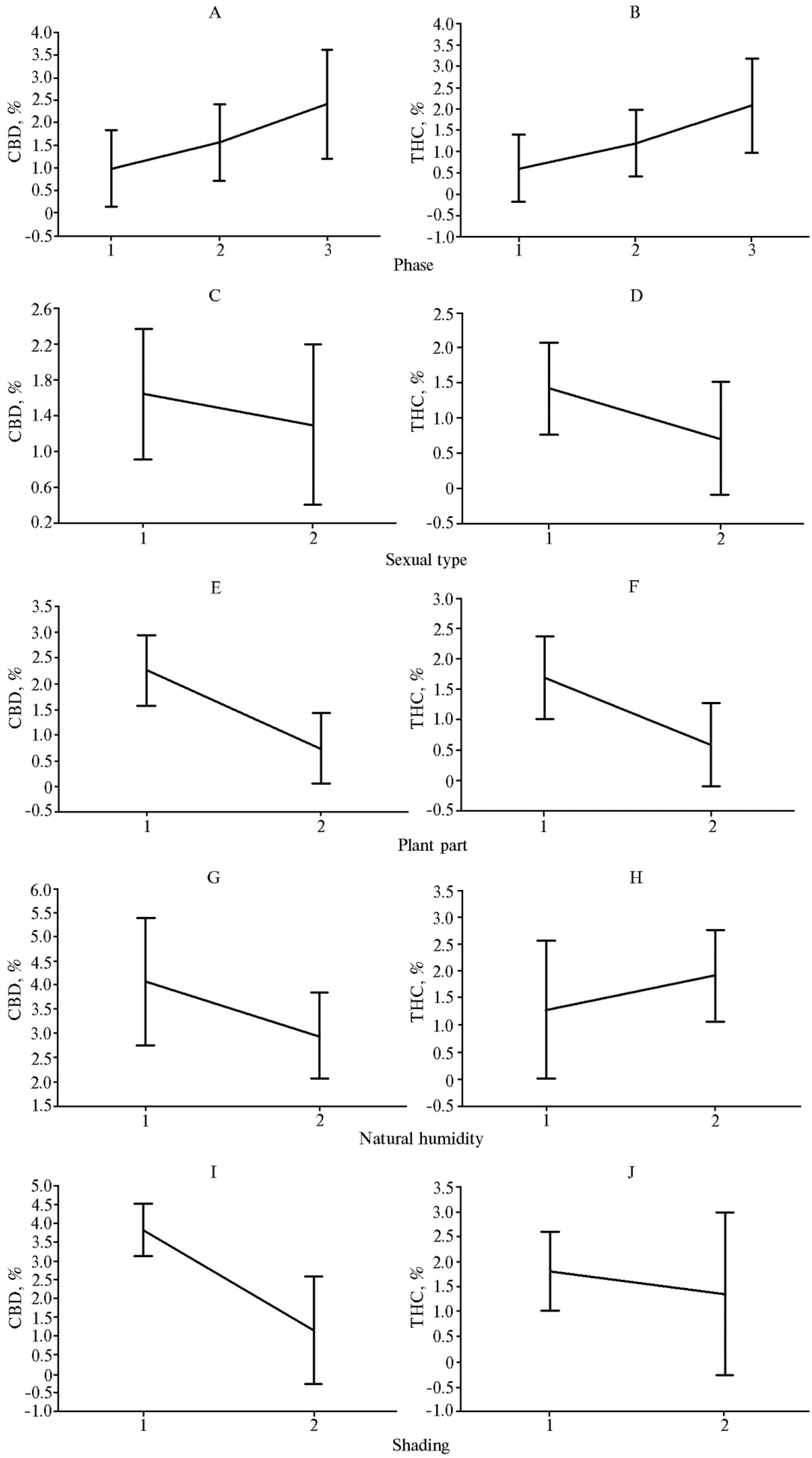
Results. The studied hemp samples significantly ($p = 0.05$) differed in the content of cannabinoids. From the beginning of the budding stage of the samples to the beginning of seed maturation, the CBD content in plants increased more than twice (Fig. A), and the generative parts of plants contained 2 times more CBD than the vegetative ones (see Fig. D). In terms of the amount of THC, the differences between the parts of plants were less contrast (see Fig. E).

The diversity of cannabis sex types, usually up to seven types in a population, we conventionally reduced to two groups, i.e. the pistillate type (unisexual female hemp, monoecious female hemp, masculinized female hemp) and the fimble type (ordinary unisex male hemp, monoecious male hemp, feminized male hemp). The effect of gender on the CBD and THC levels turned out to be statistically unreliable (see Fig. C, D). In both groups (pistillate and pistillate types), the CBD level was approximately equal. Taking into account the influence of external factors, it can be noted that by the beginning of budding in cannabis plants of different sexual types, the CBD accumulation in the generative parts was 2 times higher than THC. However, the maximum CBD accumulates in the generative parts at the beginning of seed maturation (see Fig. A, B).

The excess or deficiency of natural moisture (edaphic factor) did not significantly affect the amounts of THC and CBD (see Fig. G, 3), while shading led to a significant ($p = 0.05$) decrease in CBD level (see Fig., I), but did not have a significant effect on the THC concentration (see Fig., K).

A weak negative correlation ($r = -0.16$) was established among the tested genotypes between the content of CBD and THC in hemp inflorescences. Consequently, the prospects of hemp selection for a contrastingly high level of cannabidiol at a significantly low (trace) THC amount are obvious. From budding to seed ripening, the cannabidiol content increased ($r = 0.35$). The correlation between the CBD level and the sexual type of plants turned out to be weak ($r = -0.1$).

Currently, the State Register of Breeding Achievements Allowed for Use in the Russian Federation does not include specialized hemp varieties for the production of physiologically active functional ingredients and products. There are no commercial varieties for the production of cannabidiol, the source of which for the time being are hybrids vegetatively propagated in greenhouses. Obviously, this may not be the only way to grow hemp to produce phytocannabidiol. Monoecious and dioecious specialized hemp varieties propagated by seeds for the CBD production can be successfully cultivated in field crops. Out findings indicate that the violation of natural light (shading) of hemp plants reduces the CBD concentration by more than 2 times. Therefore, cost-effective hemp cultivation for CBD in greenhouses is limited by the quality and quantity of lighting.



The content of cannabidiol (CBD) **and Δ^9-tetrahydrocannabinol** (THC) **in hemp (Cannabis L.) plants**

depending on various factors: A, B — vegetation phase (1 — budding, 2 — flowering, 3 — ripening), C, D — sexual type (1 — female plants, 2 — male plants), E, F — generative and vegetative parts (1 — inflorescences , 2 — vegetative mass), G, H — natural moisture (1 — deficiency, 2 — excess), I, J — shading (1 — absent, 2 — present) (ANOVA, *M*±SD, different Russian regions, 2008-2011; for description of plant sampling, see "Materials and methods" section.

In Russia and neighboring countries, hemp breeding was carried out exclusively for the elimination of all cannabinoids, including CBD, in 97.8-99.6% plants of the variety. Laiko et al. [25] argue that the lack of CBD in commercial varieties may be a promising trend orientation. Our point of view does not coincide with this opinion. As was shown earlier [5], cannabidiol is not significant in psychotropic effect and is antagonistic to the psychotropic effect of THC. The decrease in the total cannabinoids in cultivars in 1980-2000 and the ongoing selection to achieve trace amounts of cannabinoids, up to their complete elimination in commercial hemp, did not prevent a sharp drop in commercial hemp areas in Russia and an increase in the number of drug addicts.

In this work, we searched for a promising breeding material with a functionally oriented composition of cannabinoids and plant morphology (maximum CBD, minimum THC, minimum foliage, maximum inflorescence size, adapted periods from budding to seed ripening), whereas previous works [21] were carried out using sample intended to select a monoecious universal variety.

Maintaining the monoecious trait in zoned varieties (0% of common fimbles) requires significant costs and is considered one of the main breeding and seed-growing tasks for monoecious hemp [20]. However, we believe that to realize the possibility of productive use of both female and male plants in a dioecious industrial hemp variety, the concept of functionality of various sex types of a variety, their biochemical and agronomic properties should be rearranged. Unlike the existing dioecious universal varieties (a combines fiber, seed and oilseed use), plants of conventional sexual types can be equally used for the maximum production of CBD.

Assessment of breeding material and differentiation of hemp samples by the CBD accumulation should begin with the budding phase. However, forms with a contrasting THC content can be distinguished with the onset of the seed ripening phase at the basal part of inflorescences. The phenological phase of the beginning of seed maturation in CBD varieties should be maximally shifted to the harvest time.

Our data differ from previously published results of experiments on varieties of Central Russian monoecious hemp [18]. Zelenina et al. [18, 19] investigated hemp leaves from germination to flowering. We showed that the maximum CBD accumulates with the onset of full flowering when the THC content is still low. The THC concentration may increase later, with the approach of seed ripening. During flowering of female plants, inflorescences continue to grow and increase in size, seed ripening begins in its lower parts, while budding is noted in the middle parts. In these periods of growth, plant foliage is already almost formed. With the beginning of budding, a significant amount of resinous substances is secreted formed in inflorescences, the function of which is to capture pollen migrating in the air, which is necessary for seed setting. At this time, the cannabidiol concentration in the inflorescence reaches a maximum, and from the beginning of seed maturation, it decreases. Based on the above facts, we concluded that a specialized variety it is necessary to selectively increase the size of the long-blooming inflorescences of both female and late blossoming male types. Blooming and maximum pollen production in male plants should be late, since pollen deficiency contributes to the CBD accumulation in female plants. Transcriptome analysis of the of glandular trichomes in hemp flowers

[26], which the authors indicate as the main site for the synthesis of cannabinoids, showed high activation of polyketide cyclase-like enzymes during mass budding and flowering. The corresponding transcript was found in a highly expressive state in trichomes, which confirms the peak of enzymatic activity in inflorescences in this period. This information is consistent with our findings which indicate that a prolonged flowering of a large number of inflorescences ensures a high accumulation of phytocannabidiol.

For breeding hemp for fiber and seed use, the ratio of sex types is of priority importance, since the conditional groups of male and female plants usually differ in a number of traits. We have shown that in hemp cultivars oriented to the maximum CBD yield, both conditional sexual groups can equally be producers of this cannabinoid. From the results of studying the dynamics of the CBD and THC accumulation in hemp plants of various sex types, it follows that in order to create a specialized CBD variety, it is necessary to select forms in which the male (fimble) plants bloom late and do not die off before harvesting, remaining equal to the CBD source with the female (pistillate) plants. Moreover, pistillate plants must also have a long period from the beginning of budding to full blooming, and a later start of seed maturation. Plants of specialized varieties should be harvested immediately at the onset of full blooming of sex types, but before the seeds ripen, since during the full bloom CBD concetration in inflorescences is already quite high while THC is low. In addition, the amount of THC may increase as the seed formation phase approaches. The seed ripening phase in CBD cultivars should occur as late as possible, approaching the harvest time.

The concept of the plant morphology for CBD hemp varieties is also different from that for varieties of universal and double (fiber) use. Plants of a specialized CBD variety should have a maximum weight and size of inflorescences and reduced vegetative part as much as possible. This is in line with previous studies [27], where the author comes to the conclusion about an increase in the reproductive parts of inflorescences while minimizing the stems of specialized varieties for oil and chemical compounds. The habitus of plants of a specialized variety is long-flowering inflorescences of large size with minimal foliage, since the content of CBD in the leaves is significantly less.

Concentration of cannabidiol in inflorescence (%) of hemp (*Cannabis* L.) plants selected for breeding varieties of pharmaceutical use from different populations (the Russian Federation, 2008-2011)

No. in VIR introduction catalog	Species	Ecotype	Cannabidiol
141445	*C. sativa*	Northern	2.40
141446	*C. sativa*	Northern	1.91
141447	*C. sativa*	Northern	1.87
141448	*C. sativa*	Central Russian	2.99
141855	*C. sativa*	Central Russian	3.96
141856	Presumably *C. sativa* × *C. ruderalis*	Central Russian	9.78
141451	Presumably *C. sativa* × *C. ruderalis*	Central Russian	2.76
141864	Presumably *C. sativa* × *C. ruderalis*	Central Russian	2.53

Our studies showed that samples that can be attributed to different hemp species and ecotypes accumulate cannabidiol in significant quantities. For example, sample 141855 contains 3.96% CBD, and 141856 contains more than 9% (Table).

Our data are fundamentally different from those published previously. We propose a model of industrial pharmaceutical-type hemp varieties intended for field cultivation rather than growing in greenhouses and growboxes, as it is practiced abroad for varieties and vegetatively propagated hybrids not included in the official list of the European Union. Importantly, specialized CBD varieties for cultivation in field conditions are still absent but the need for them is quite high. Existing universal varieties for field growing do not meet the challenges of production of both phytocannabidiol and functional food ingredients. Revision of a specialized variety model is necessary to set selection strategy for pharmaceutical-type varieties, to specify their significant traits, and to study hemp genotypic diversity to form trait-based donor collections and, eventually, to produce the advanced breeding material. Given these circumstances, we plan to continue breeding and genetic research to create hemp varieties for pharmaceutical use.

Thus, from the beginning of hemp budding, plants of different sexual types accumulate in the generative parts 2 times more cannabidiol (CBD) than Δ^9-tetrahydrocannabinol (THC). The maximum CBD is detected by the beginning of ripening. Both conventional groups of plants (male "fimble" and female "pistillate" hemp) contain approximately equal amounts of CBD. Excess or deficiency of natural moisture from budding to seed maturation does not significantly affect the accumulation of THC and CBD while shading leads to a decrease in the CBD content. The plants of CBD-specialized varieties should have minimum foliage and produce inflorescences large in size and weight. As a result of the study, accessions with a valuable CBD/THC ratio are identified that will be used in breeding for specialized hemp varieties with no specific psychotropic activity.

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