Review article

CHALLENGES IN INDUSTRIAL HEMP BREEDING

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SUMMARY

Although hemp is one of the earliest domesticated plant species, the breeding of hemp has long been neglected. In the new millennium, interest in hemp as a universal renewable raw material is growing rapidly. The high and stable yield of air-dried stems (over 15 t ha^{-1}) with a high content (over 35%) of quality fibers and grains (over 1 t ha^{-1}) are general objectives of breeding of new hemp varieties all over the world. Following various breeding procedures, the content of plant active substances cannabinoids and natural biocompounds can be also elevated. The plant content of Δ9 -THC, a psychoactive cannabinoid, must be maintained below the legal maximum. Breeding methods for creating genetically modified hemp exist, but still are not applied in hemp production.

KEYWORDS: breeding methods, breeding objectives, genetic resources, industrial hemp

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INTRODUCTION

During the period of the most intensive production, industrial hemp (*Cannabis sativa* L.) occupied about one million hectares of the world's arable land. After World War II, world hemp production decreased, which resulted in the closure of existing breeding programs. In the new millennium, interest in hemp as a universal renewable raw material with great market potential is growing rapidly. The launch of modern research programs and the creation of varieties with improved agronomic qualities and new purposes (varieties with high content of natural biocompounds - NBC) are a precondition for the revival and development of the industrial hemp industry and market in the years to come.

Hemp genetic resources and conservation

Cannabis genus (family Cannabaceae) is considered to be an isolated primary gene pool (Harlan & de Wet, 1971) with *Cannabis sativa* L. as its single species (Small & Cronquist, 1976). The species is extremely polymorphic (Small & Antle, 2003), and taxonomic grouping within the genus is based on morphological characteristics, chemotype, ecotype, or cultivation purpose (Lynch et al., 2016; Koren et al., 2020.).

The high degree of hybridization between populations of different levels of domestication (Gilmore et al., 2007) with the existence of continuous variability of quantitative traits within germplasm is why completely "wild" populations can be found only in the presumed center of origin in Central Asia (Vavilov, 1926) and on the slopes of the Himalayas (Sharma, 1979). In the eastern part of Europe, "wild" hemp classified as *C. sativa* ssp. *spontanea* (Berenji & Sikora, 2011) is spontaneously maintained in nature (Vrbničanin et al., 2008). The most numerous populations are located in the vicinity of former hemp processing plants. *C. sativa* ssp. *spontanea* hemp is open pollinated and plants phenotypically differ

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significantly compared to the original varieties due to selection pressure of the environment (Tóth et al., 2015).

The main source of genetic variability for hemp breeding are ex-situ genetic collections (Hajjar & Hodgkin, 2007):

(i) In research institutes. They include an assortment of known genetic backgrounds and derivatives resulting from gene recombination of materials of broad genetic variability as well as different ecotypes, local populations, and wild plants accessions.

(ii) In gene banks. In terms of the volume of stored seeds, some taxa are smaller than the collections in research institutes and are intended for longer storage of genetic material.

The biggest challenge of *ex situ* conservation of genetic hemp resources is accession maintenance and propagation and requires significant inputs of manpower, resources, and infrastructure (Khoury et al., 2010). Storage is made more difficult by the species dioecy, cross-pollination and a large amount of pollen in flowering (Amaducci et al., 2008c). The hemp seeds viability is reduced by 75% in just two years of storage in uncontrolled conditions. Storage temperature reduction and reduction of seed moisture contribute to retaining hemp seed germination for a significantly longer period (Small & Brookes, 2012).

Maintenance and propagation of varietal seeds *in situ* is the responsibility of the institutions that registered the variety (Berenji et al., 1997).

Hemp breeding

What product hemp is grown for will influence goals of hemp breeding and the variety ideotype (Berenji & Sikora, 2001).

Hemp grown for purpose of stems and fiber production is fiber hemp, for seed production is grain hemp, while for flower production is often mistakenly referred as "medical hemp". Less often, hemp is grown for decorative purposes - ornamental hemp. Recently, multi-purpose varieties have been developed that, in addition to high grain yield, have the genetic potential for high stalk and fiber yield and high essential oil content in inflorescences.

Initially, various forms of hemp were grown without human interference and originated in nature as a result of spontaneous crossbreeding, natural mutations and natural selection. It was only at the beginning of the 20th century that the planned breeding of hemp varieties started. Plants with desired traits were selected, their seeds collected manually and used for sowing in the following year. In this way, relatively homogeneous populations were selected in Italy, which were named after the provinces from which they originated: 'Ferrara', 'Bologna', 'Modena', 'Rovigo' and 'Carmagnola'. Although the traditional Italian varieties 'Carmagnola', 'Fibranova', 'Eletta Campana', 'CS' and 'Superfibra' are practically unavailable today, the Italian gene pool has left its mark in many hemp breeding programs. Varieties of our region ('Futoška', 'Titelska', 'Apatinska', 'Vukovarska') mostly originated from materials of Italian origin.

The turning point in hemp breeding is the identification of unisexual and intersex types in dioecious hemp populations. These plants were the starting material for the selection of the first monoecious varieties. In the thirties of the 20th century, the first monoecious varieties were selected. In Russia and Ukraine, several varieties of different maturation period have been selected, several of which are still in production. Modern varieties from this region belong to the southern type or hybrid offspring from a cross between Central Russian and southern hemp (Grishko, 1935).

Hemp breeding objectives

Fiber hemp breeding

In fiber hemp cultivation, it is of interest to achieve high and stable air-dried stems yield above 15 t ha^{-1} with stalk fiber content above 30% (Berenji & Sikora, 2000; Sikora et al., 2011). High stalk yield together with high stalk fiber content is a condition for a high yield of fiber per unit area (Huhnke et al., 1951). Compared to the oldest assortment, when the stalk fiber content was 12-15%, in the modern assortment, the content was increased to 25-35% by breeding, while in the newest varieties it exceeds 40%.

The branching of the stalk in textile hemp has a negative effect on the quality of fibers in the textile industry. For grain hemp production, branching directly contributes to the increase of grain yield. The predisposition of a genotype for branching is genetically determined, but crop density strongly effects the plant architecture.

From the aspect of fiber quality, it is important to be as suitable as possible for processing. The quality of hemp fiber is more influenced by production technology than the genetic constitution of the plant (Müssig & Martens, 2003). The stalk fiber content and quality change during plant development; the cellulose content increases rapidly from the transition of plants to the generative phase until the end of flowering when it reaches 56-65%. Plant density, nitrogen fertilization and harvest date are also significant factors influencing fiber yield and quality (Amaducci et al., 2005). High cellulose content, low degree of lignification and reduced number of cross-linked pectin and structural components of the cell wall are important characteristics of the suitability of fibers for extraction for both the textile industry and paper production (Mandolino & Carboni, 2004). Growing easier to process varieties would be an advantage in terms of obtaining high quality fibers and lead to a reduction in the costs associated with their separation from hurd (Van den Broeck et al., 2008). Of particular interest is the favorable proportion of primary and secondary fibers (Jakobey & Faragó, 1971), as well as the strength and elasticity of the fibers. A form of hemp with a light yellow stem, without chlorophyll is characterized by very good fiber quality, especially for the paper industry (Berenji et al., 1995; Berenji & Sikora, 2000; Herak et al., 2001).

Grain hemp breeding

The most important goal of grain hemp breeding is good quality yield above 1.5 t ha^{-1}. The quality of hemp grains largely depends on the content and composition of oils and proteins (Berenji & Sikora, 2001). Hemp oil contains a high percentage of essential fatty acids (Karlović et al., 1996) which are of great nutritional importance (Laakkonen & Callaway, 1998). For grain hemp breeding with increased oil content of importance are genotypes with 40-45% oil in grains, unlike the usual 30-35% (Bócsa, 1999). An example is the Hungarian variety 'Fibrol' with grain oil content above 35% (Finta-Korpeľová & Berenji, 2007).

Late-maturing varieties compared to early-maturing ones are higher in grain, stalk and fiber yield (Van der Werf et al., 1994). In the northern part of Europe early maturity is desirable due to the short vegetation period. An example is short-stalk 'Finola' (former name: '9FIN-3149') bred for grain production in the northern parts of Europe (Callaway, 2002).

Breeding of hemp varieties for flower production

Medical hemp is one that is grown as a raw material for the pharmaceutical industry and it is associated with the biosynthesis of terpenophenolic compounds cannabinoids, located in the plant glandular trichomes (Potter, 2004). More than 100 different cannabinoids have been identified so far, the most important of which are cannabidiol (CBD) and the psychoactive delta-9-tetrahydrocannabinol, Δ9-THC (De Meijer et al., 2003). Unlike Δ9-THC, CBD is not psychoactive (Russo, 2011). In the cultivation of industrial hemp for flower production, the aim is to reduce the content of psychoactive Δ9-THC (Clarke, 1999) due to EU law that the content of Δ9-THC in the variety of industrial hemp must not exceed 0.2% in plant material (Beherec, 2000). The main goal of hemp for flower production is high flower yield with increased CBD content and THC content within the legal framework (high-CBD hemp). The result of the joint work of French and Ukrainian breeders are new monoecious hemp varieties with a very low Δ9-THC content (<0.07%) and without the typical hemp aroma, e.g. 'USO-45' (Holoborodko et al., 2008).

The content of certain cannabinoids in the dry inflorescence defines several hemp types: (i) medical type – Δ9-THC predominates; (ii) intermediate type – similar dry inflorescence content of Δ9-THC and CBD; (iii) industrial type – dominates CBD; (iv) cannabigerol (CBG) is dominant cannabinoid, which is a biochemical precursor of Δ9-THC and CBD; (v) cannabinoid-free chemotype, and (vi) prolonged juvenile chemotype – in hemp of a specific morphological phenotype synthesizing cannabichromen, CBC (De Meijer et al., 2009a, 2009b). CBC dominates in the cannabinoid fraction of young plants and its content decreases with plant maturation. Chemotypes of hemp without cannabinoids or containing only CBG or CBC are of special pharmaceutical value and this trait is one of the specific goals of hemp breeding for flower production (Kišgeci & Sikora, 2015).

Specific objectives of hemp breeding programs

Flowering regulation

Hemp is a short-day plant with three phenophases of development: intensive growth phase, photosensitivity and the phase of generative development – flowering. Flowering induction is caused by the shortening of the photoperiod to approximately 14 h (Amaducci et al., 2012). Based on the duration of individual phenophases, three groups of varieties can be distinguished: early (flowering 40-60 days after sowing), medium late (60-90 days), and late (90-120 days) (Zatta et al., 2012). Early and medium-late varieties are grown in the northern regions with a short season and long days during the summer. With the introduction of these varieties in the southern regions, they begin to bloom earlier and the biomass yield is reduced. Long vegetation varieties are selected to achieve high biomass yields (e.g. South China, Thailand, Australia, and Southern Europe). The fiber yield can be increased if the variety from the conditions of small latitude is grown in the conditions of higher latitude. The effect of increasing yields becomes apparent when the change in latitude is above 2 degrees (Guo et al., 2011). There are also differences in the sensitivity of varieties to changes in the photoperiod. The French varieties 'Felina 34' and 'Futura' and Chinese population 'Huokiuzi' are weakly sensitive, while the Hungarian variety 'Tiborszallasi', like most Chinese varieties, is very sensitive in this respect. The Italian varieties 'Carmagnola' and 'Fibranova' are moderately sensitive. The behavior of varieties in terms of flowering, in addition to the length of the photoperiod, depends on the temperature and quality of light. The selection of cultivars for growing in a certain locality is made on the basis of a model for estimating flowering time (Amaducci et al., 2008a, 2008b). This model is also useful for making decisions about sowing and harvest date.

Sex expression

Sex expression in hemp is influenced by genetic and environmental factors. Two sex chromosomes, X and Y, are present in the genome, with the Y chromosome being much larger than the X chromosome and the autosome. Purely male plants have one X and one Y chromosome, while female plants have two X chromosomes, resulting in a difference in genome size between male and female plants.

While purely female plants have a combination of XX chromosomes, a combination of XY results in different intersex forms depending on the intensity of the Y chromosome – from feminized males (more intense Y versus X), to masculinized females (more intense X versus Y chromosome) to purely male phenotypes in which the Y chromosome dominates. In the case when the intensities of the X and Y chromosomes are approximately the same in phenotypic expression, hermaphroditic plants with an equal percentage of female and male flowers are obtained (Hirata, 1927).

In hemp, sex expression can be altered by chemical treatments. Chemical treatments achieve unisexuality and uniformity of growth in in-door hemp cultivation (Hall et al., 2012). Giberellic acid induces male characteristics while auxins, ethylene and cytokines induce female ones. Silver thiosulfate application induces the appearance of pollen and pollen on female (XX) plants and is a useful tool for the production of feminized seeds with only female offspring (Kaushal, 2012).

In dioecious varieties, there is an evident difference in growth rate and development between male and female plants. Earlier flowering of males compared to female plants (Struik et al., 2000) causes greater crop variability and more pronounced competition between plants (Van der Werf et al., 1995). Compared to dioecious, monoecious hemp varieties are more homogeneous and greater in yield, so mechanized harvesting of such varieties is easier (Stojanović et al., 2016). The disadvantages of this type of varieties are the relatively narrow genetic basis, the need to maintain monoecy and seed crop isolation distance. A common practice in the propagation of seeds of dioecious varieties is the selection of male plants before pollination and pollination only with male plants of the best performance.

Tolerance to abiotic environmental factors

Soil decontamination can be done by phytoremediation - phytoextraction (Meagher, 2000). Growing on contaminated soil, cultivated plants extract toxic substances from the substrate and accumulate them in their aboveground parts. With the harvest of the above-ground parts of the plants, the accumulated harmful substances are removed from the soil. The disadvantage of this method is the long decontamination period.

Hemp is a plant species with exceptional phytoremedial abilities. From the aspect of breeding in this direction, there is significant variability within the germplasm in terms of the ability to accumulate and tolerance to heavy metals (Zeng et al., 2013). Different response of genotypes to stress, low temperatures, lack of moisture, soil salinity and pest resistance, is usually associated to tolerance to heavy metals (Guo et al., 2011). One of the current breeding directions is the creation of genetic variability in the direction of tolerance to abiotic factors that affect the growth and development of hemp plants.

Increasing NBCs plant content

The effectiveness of hemp essential oil against moths, insects, snails and different types of larvae has been confirmed (Bedini et al., 2016). Hence, hemp NBCs exploitation as bio-pesticides is one of the future challenges in hemp breeding. The development of the breeding is influenced by: (i) good availability of arable land and relatively low costs of production of the raw material; (ii) increasing demand for eco-friendly products; (iii) insufficient market supply of biopesticides; (iv) additional usage of produced raw material for pharmacological and cosmetic purposes.

Hemp breeding methods and techniques

Hemp is dioecious, wind-pollinated plant. In nature, monoecy (plants with both-sexes inflorescences on the same stalk) occurs in minor percentage in industrial hemp.

All genus *Cannabis* varieties interbreed easily giving fertile offspring, resulting in continuous variability of quantitative hemp traits. In dioecious varieties female inflorescences can be pollinated by several mail pollinators. Hence, controlled pollination is one of the biggest challenges in breeding dioecious hemp. Monoecious varieties are cross- and self -pollinated. Artificially created completely female hemp populations for controlled pollination are used in the production of hybrid varieties (Berenji et al., 2013).

Industrial hemp assortment includes three types of varieties: varietal populations, synthetic varieties and hybrid varieties. Genetically, varietal populations are free-range populations resulting from recurrent selection. Synthetic varieties represent an improved generation of hemp populations created by crossing selected parents from varietal populations that were propagated under conditions of free fertilization in spatial isolation. Hybrid varieties are created by controlled crossing of two parents originating from synthetic varieties.

Basic hemp breeding methods include: methods of plant selection, combination ability methods, inbreeding and hybridization and marker-assisted selection (Ranalli, 2004).

Plant selection

This is how the first domestic commercialized variety 'Novosadska' (Berenji, 1992) was selected, from which the modern variety 'Marina' with a stalk fiber content above 40% was selected.

Mass selection is a method used in hemp breeding for traits of high heredity (heritability) such as stalk fiber content.

For the content and quality of fiber, individual and mass selection of dioecious hemp plant is possible only among mature female plants after their pollination with a mixture of pollen of male plants that is of unknown properties. Overcoming this problem is achieved by methods:

(i) Seed reserve method. The seeds of selected mother plants are divided into two parts - the part that is sown to observe the properties of the offspring of the parent plants, and the seed reserve of the offspring of favorably evaluated plants intended for sowing after the observation year. This method aims to ensure that only plants of the improved trait participate in mutual pollination in the following year.

(ii) Bredemann's method (Bredemann, 1942). Prior to flowering, the male plants are marked, cut in half and in a half is fiber content analyzed by a quick laboratory method. Based on the obtained results, only male plants with the best characteristics are left in the field. After the seeds harvest, each female plant stalk is analyzed individually for the fiber content, and only the seeds of the highest quality female plants pollinated with pollen of selected males are set aside. The application of this method requires a great deal of human effort, but it results in a rapid increase in the stalk fiber content.

Since the wind carries light hemp pollen over long distances, special challenge is to prevent interbreeding of individual selection populations. This is achieved by spatial isolation (1-2 km). Net cages can be useful tool to isolate a small number of plants.

In modern hemp breeding programs, controlled pollination is achieved by growing plants in controlled, indoor environments. In such conditions, complete isolation is provided while preserving the reproductive ability of plants.

Selective breeding of hemp is often used to maintain and improve existing varieties traits. New varieties are mainly created by cross-breeding and the use of heterosis or hybrid breeding.

Hemp cross-breeding

Crossings between genotypes of different genetic constitution are performed in order to expand the variability of quantitative traits and to create a basis for the selection plants with improved yield and quality traits. Examples of the application of this method in China are the varieties 'Yun Ma 2' and 'Yun Ma 4'. In our country, crossing 'USO 31' variety and the Hungarian monoecious population, the monoecious variety 'Helena' was selected. A special case of hemp cross-breeding is the back -cross. This method has found application in the conversion from dioecious to monoecious hemp.

Inbreeding and hybrids

For the most important traits, hemp shows hybrid vigor (heterosis) in the desired direction (Bócsa, 1954). The creation of hybrid varieties is a modern and promising method of hemp breeding.

The simplest way to create hybrid hemp is to cross two heterotic dioecious parents. It is necessary to manually remove male plants from the mother's rows before the beginning of flowering. After pollination, the paternal rows are completely removed. Hybrid seeds are harvested from female plants in the mother's rows. This way of producing larger quantities of hybrid seeds is practically impossible because it is almost impossible to remove all male plants from the mother's rows before the beginning of pollen shedding (Bócsa, 1954).

The discovery of inheritance of hemp sex expression enabled the exploitation of heterosis in practice. The process of creating a hybrid consists of two phases: (i) removing the male plants and pollinating the dioecious hemp female plants with monoecious pollen. The offspring is unisexual and consist entirely of female plants; (ii) Unisex progeny is the maternal component in the production of F1 hybrid on a commercial scale. In the unisexual parental component of hybrids, there are almost no male plants (Arnoux &Mathieu, 1971; Bócsa, 1973).

Commercial hybrid varieties were created in Hungary ('Uniko-B' and 'Kompolti hybrid TC') and China ('Yun Ma 3)'. 'Uniko-B' is a single hybrid ('Kompolti' × 'Fibrimon 21'). The F1 of this cross is almost entirely unisexual female and is used to produce F2 which contains about 30% male plants and is used commercially to produce fiber. The 'Complete Hybrid TC' is a triple hybrid. In the first step, a Chinese dioecious is crossed with a Chinese monoecious hemp, whereby a unisexual female F1 generation is obtained, which in the second step is crossed with the 'Kompolti' variety and a hybrid with a sex ratio of 50/50 is obtained.

Experience has shown that the most pronounced heterosis, for economically important properties of hemp, is obtained by crossing genetically divergent parents, such as hybrid combinations between southern hemp and hemp originating in China (Bócsa, 1954).

In the breeding programs of hybrid hemp, there are directions for the creation of two-line and three-line F1 and F2 hemp hybrids (Berenji & Sikora, 1996). Two-line hybrids of F1 generation show the greatest heterosis, which for stem yield can be up to 30% compared to the parent varieties. Their disadvantage is the relatively low yield of F1 varietal seeds. This shortcoming is eliminated by the production of F1 generation as a seed crop, whereby the yield of varietal F2 seeds is increased by as much as 40-50%. A similar effect is achieved by producing a three-line hybrid.

Biotechnology methods in industrial hemp breeding

Van Bakel et al. (2011) were the first to sequence *C. sativa* L. genome. Studies of the hemp genetic structure have led to several conclusions: (i) *Cannabis* genus is characterized by a broad common gene pool with limited population's segregation; (ii) Hemp has a wide genetic variability with high heterozygosity due to free pollination and dioecious character of the species. In hemp populations, most alleles are of a low frequency (<0.30). Only a few major alleles have been determinate in highly-inbred varieties; (iii) The difference between marijuana and industrial hemp cannot be clearly defined by utilization of biotechnological methods and techniques; (iv) Even in female inbred lines marker-assisted selection has little discriminatory potential to differentiate individual populations; (v) utilization of genetic maps and molecular markers is limited in hemp breeding due to the high degree of genetic variability of the species itself.

Other breeding methods

Ploidization and mutations are methods that are also used in hemp breeding. Using 0.1% colchicine solution, about 11% of autotetraploids were obtained (2n = 40). Tetraploid plants, compared to diploid ones, have larger seeds, higher growth, 10-15 days longer vegetation, increased fiber content and almost simultaneous plant maturation of both sexes. These are the traits important in hemp breeding. The change of sex expression from dioecious to monoecious hemp can be caused by various mutagenic agents (Bohač, 1990). Spontaneous mutations such as yellow stalk are also of importance for industrial hemp breeding.

Breeding methods that utilise *Agrobacterium tumefaciens* as a trait vector for creating genetically modified hemp exist (Feeney & Punja, 2003), but are not currently applied in hemp production.

CONCLUSIONS

Cannabis genus (family Cannabaceae) is an isolated primary gene pool with a single polymorfic species - *Cannabis sativa* L. In the vicinity of former hemp processing plants "wild" hemp classified as *C. sativa* ssp. *spontanea* is spontaneously maintained in nature. *Ex situ* genetic collections are the source of genetic variability in hemp breeding programs. The high and stable yield of air-dried stems (over 15 t ha^{-1}) with a high content (over 35%) of quality fibers and grains (over 1 t ha^{-1}) are general objectives of hemp breeding. Following various breeding procedures, the content of plant active substances – cannabinoids and NBCs can be also improved. The plant content of Δ9-THC, a psychoactive cannabinoid, must be maintained below the legal maximum. Breeding methods for creating genetically modified hemp exist, but still are not applied in hemp production.

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SAŽETAK

IZAZOVI U OPLEMENJIVANJU INDUSTRIJSKE KONOPLJE

ANAMARIJA KOREN, VLADIMIR SIKORA

Iako je konoplja jedna od prvih odomaćenih biljnih vrsta, plansko selekcionisanje i oplemenjivanje konoplje dugo su zanemarivani. U novom milenijumu interesovanje za konopljom kao univerzalnom obnovljivom sirovinom naglo raste. Visok i stabilan prinos vazdušno suve stabljike (preko 15 t ha^{-1}) s visokim sadržajem (preko 35%) kvalitetnih vlakana i zrna (preko 1 t ha^{-1}) glavni su ciljevi oplemenjivačkih programa konoplje širom sveta. Različitim metodama i tehnikama oplemenjivanja moguće je povećati i sadržaj biljnih aktivnih komponenti – kanabinoida i prirodnih biomolekula. Sadržaj Δ9-THC – psihoaktivnog kanabinoida u biljnom materijalu ne sme prelaziti zakonski maksimum. Metode za stvaranje genetski modifikovane konoplje postoje, ali još uvek se ne primjenjuju u oplemenjivanju.

Ključne reči: ciljevi oplemenjivanja, genetički resursi, industrijska konoplja, metodi oplemenjivanja