BIO Web of Conferences **125**, 01012 (2024) https://doi.org/10.1051/bioconf/202412501012
ABS 2024

Possibilities of rapid generation cycling of hemp (Cannabis sativa L.) for the stabilization of recessive traits

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Abstract. This study describes the development of a novel hemp germplasm using an accelerated breeding method. Two hemp varieties, 'Balaton' (green stem, very early female flowering) and 'Chamaeleon' (yellow stem, early maturing), were chosen for breeding. The breeding method involved crossing the varieties, manipulating light conditions to induce flowering, and performing artificial pollination. Yellow stem colour, a recessive trait from 'Chamaeleon', was successfully incorporated into the progeny within four generations in only twelve months overall. This demonstrates the effectiveness of the accelerated breeding method for introducing new traits and highlights the advantages of this method for rapid development of new hemp varieties compared to traditional breeding techniques. However, limitations such as potential inbreeding depression and the need for outdoor testing, are acknowledged.

1 Introduction

Hemp (*Cannabis sativa* L.) is one of the earliest domesticated agricultural crops, for recreational, medicinal, and industrial purposes [1]. The extractable fibres serve as raw material for the textile or construction industry, the seed yield is a valuable source of high unsaturated fatty acids-rich oil, and the cannabinoids extracted from the inflorescence serve recreational and medicinal purposes [2-4].

With an increasing global population and mounting threats to crop yields, speed breeding (rapid generation cycling) has a great relevancy in the further development of crop genetics. It traces speed breeding evolution from carbon arc lamp experiments 150 years ago to its modern use with LED technology, significantly expediting breeding cycles. It synergizes with methods like single plant selection and single seed descent, and holds promise when integrated with gene editing, genotyping, and genomic selection [5-9].

Rapid generation cycling (speed breeding) optimizes environmental factors for shorter generation times in crops like wheat, canola, and chickpea. These long-day plants require over 16 h of light to flower quickly, whereas short-day plants such as soybean require more complex protocols. Hemp, a short-day plant, flowers with less than 11-15 hours of light, leading to long generation times in the field and delaying the breeding of new varieties with only one generation per year [10-12].

Research traces the yellow-stemmed hemp type back to Hoffmann's 1946 discovery, the "Hellstengeligen" phenotype, which arose from a cross between an Italian and a Finnish

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landrace variety [13]. The introgression of this trait has become a popular target in hemp breeding programs, leading to the registration of numerous "yellow" cultivars like ‘Carmaleonte’, ‘Ivory’, ‘Kompolti Sárgaszárú’, ‘Fibror 79’, ‘Markant’ and ‘Chamaeleon’.

Studies have shown that yellow-stemmed hemps may offer several advantages. These include a potentially higher proportion of long fibers compared to total fiber content and a greater amount of bast fiber with finer, processed bundles compared to green-stemmed varieties [14-17].

Another study suggested that yellow-stemmed hemp may not use less nitrogen, as initially thought, and might be better for specific fiber production due to its higher fiber content. The effect of yellow stems on productivity depends on location and nitrogen availability [18].

2 Materials and methods

In 2021, we initiated a hemp breeding program to create a proprietary germplasm with distinct morphological traits and uniformity within the population. Selection was carried out to achieve a good fiber yield and very low tetrahydrocannabinol (THC) content expected from dioecious germplasms. A secondary selection was performed to obtain the highest possible cannabidiol (CBD) content.

To achieve our goals, we selected registered varieties for further breeding based on previously described literature data, which were cultivated in a greenhouse. Based on preliminary test crossing and progeny evaluation data, we restricted the selection to the Hungarian ‘Balaton’ from Agromag Ltd. and the Dutch, yellow-stemmed ‘Chamaeleon’ variety from Wageningen University, Netherlands.

We developed a unique accelerated breeding method for the most comprehensive morphological characterization and potentially high seed yield to facilitate the identification and propagation of ideal individuals. The maturity times of the two crossed varieties are different, ‘Balaton’ is characterized by the anthesis of very early female flowering and Chamaeleon is an early maturing variety. Both varieties are photoperiod-sensitive.

Seeds were germinated in seed trays with 40 cells (diameter: 5.5 cm) at a constant temperature of 22°C and constant 75% relative humidity under an artificial LED light source with 16 h of illumination per day. Approximately 170 cm^3 of Pindstrup Plus Blue (pH 6.0 fraction size: 0-10 mm) substrate was used per seedling without added fertilizer. To promote root development, irrigation was carried out from the bottom, and sufficient water was added daily to prevent the upper surface of the substrate from drying out, as described by Schilling *et al.* [19].

At a shoot height of 13 cm, the plants were transferred to the greenhouse, where they spent 3 days in the original seed trays at a minimum temperature of 21°C and 16h of illumination. Transplantation was carried out in 20-liter plastic containers with 17 liters of Pindstrup Mix+Clay (pH 6.0, fraction size: 10-30 mm) substrate. Humidity was not regulated (formed naturally between 50-70%). Two plants were placed in each container to ensure a sufficient number of plants, even after removing male plants.

Based on the calculated growing degree days (GDD), the plant material that was morphologically assessable showed varietal characteristics and produced enough vegetative mass exposed to short-day conditions. For this purpose, we used a blackout system to ensure 12 h of continuous darkness. The following week, sexual characteristics began to appear. From the pollen-producing ‘Chamaeleon’ variety, the male and female plants were kept, while in case of ‘Balaton’, only the female plants remained in the containers. At the latest, on the 10^{th} day, flowering began, and from the 14^{th} day, we performed artificial pollination, which facilitated the proper amount of seed set.

During the summer period, when external pollen contamination could occur, we allowed the pistils to grow unfertilized and only performed manual pollination one week later with mixed pollen from the male individuals.

We maintained the 12-hour day length throughout the generative phase. When the first seeds with the appropriate colour and marbling became visible, water stress was applied to accelerate ripening.

The plants were harvested manually, and after threshing, the cannabinoid content of the threshing waste was analysed. The seeds of plants with low THC and consistently high CBD content were sown again. Figure 1. shows a unique female plant as an advanced result of crosses and selection.

This process was repeated ten times over the next two years.

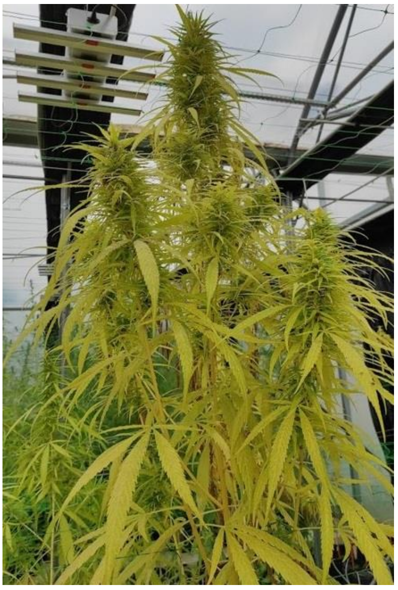

Fig. 1. An ideotype result of the speed breeding project.

3 Results

In the first filial generation (F1, second cycle) we had progenies from 'Balaton' x 'Chamaeleon' (BAxCH) and 'Chamaeleon x Chamaeleon' (CHxCH). We selected five mother plants from both crosses, and 100 seedlings were transplanted as described above. Evaluations were conducted according to the CPVO protocol for tests on distinctness, uniformity, and stability. In this document, the main stem colour was characterized as yellow (1), medium green (2), dark green (3), and purple (4). Originally 'Balaton' has dark green stem (3) and the stem colour of 'Chamaeleon' is yellow (1).

During flowering and then at maturation, we did not find any yellow-stemmed individuals in the BAxCH cross in the offspring generation, whereas in the case of CHxCH, every single plant showed a yellow stem colour.

It was expected that this trait would be monogenic recessive, so for faster and more complex progress, the offspring were backcrossed with the 'Chamaeleon' father. CHxCH reassuringly confirmed that no undesigned pollen contamination had occurred.

For the second generation (third cycle), 400 seedlings from four mother plants were transplanted only from the BAxCH backcrossed progeny. At flowering, there were 179 yellow-stemmed (1) plants and 187 plants with green stems. Thirty-four plants were removed previously, either they did not emerge or were underdeveloped. Pollination was performed using a mixture of yellow-stemmed male plants from the BAxCH backcrossed progeny. Before harvest, 192 female plants remained in the greenhouse, of which 91 were yellow (1) and 101 had green stems.

Four elite plants were selected for the 4th cycle. Five hundred seedlings were transplanted to a greenhouse. Pollination was performed using a pollen mixture of yellow-stemmed males. Subsequently, males were eliminated. A total of 246 female plants were harvested. At harvest, 155 yellow-stemmed hemp plants were identified, and the rest had a green stem colour.

The yellow stem colour was completely stabilized by the 5th crop cycle. At that time, 295 elite plants that originated from five females were harvested with a yellow stem colour. We incorporated the yellow stem colour into the plant material, during the next generations we stabilized other parameters (balanced flowering time, leaf morphological features) and tried to improve the seed yield and CBD content.

Table 1 contains observations concerning the dates of the cycles and the data of the stem colour observations.

Table 1. Stem colour observations.

No. of cycle	Period	Origin of plants	Breeding method	No. of harvested females	Yellow stemmed phenotype %
1.	May-Aug. 2021.	Balaton females – commercial seed	Cross with Chamaeleon males	300	Balaton: 0% Chamaeleon: 100%
2.	Aug.-Nov. 2021.	5 half-siblings of BAxCH Cycle 1	Backcross Chamaeleon males	100	BAxCH: 0% Chamaeleon: 100%
3.	Jan.-Mar. 2022.	4 half-siblings of (BAxCH)xCH Cycle 2	Family selection	192	47.4%
4.	Apr.-May. 2022.	4 half-siblings Cycle 3	Family selection	246	63%
5.	Aug.-Oct. 2022.	5 half-siblings Cycle 4	Family selection	295	100%
6.	May-Jul. 2023.	12 half-siblings Cycle 5	Family selection	465	100%

4 Discussion

The study did not aim to delve into complex genetic reasoning; therefore, it was assumed that yellow stem colour is a monogenic recessive trait [20]. This trait did not appear uniformly in the early stock even after crossing individuals with purely yellow stems. an additional section cycle was required. This could be due to the hermaphroditism observed in the Balaton offspring. Although hermaphrodite plants were removed, the predisposition to this specific gender characteristic decreased in successive generations. A green-stemmed female could have caused cross-pollination in neighbouring plants; otherwise, the female plants remained in place until the end of the cycle, and only their seeds were not harvested. They had sufficient time for this because artificial pollination was intentionally delayed. The males were removed as soon as the first detectable sexual characteristics appeared well before flowering, if they did not show a change in stem colour. 'Chameleon' plants isolated elsewhere and there was no indication of pollen contamination. Of course, in the case of the strongly dark green 'Balaton' variety, there may be other restorer mechanisms, which can be eliminated with another selection step which we could also experience in our case.

After 12 months of vegetative growth, 100% yellow-stemmed progeny was created. The data clearly show that with accelerated breeding, new recessive traits can be incorporated and stabilized in hemp. Compared with the traditional breeding process, a stock suitable for further selection can be created in a very short time. Outdoor cultivation is important because the population is exposed to different selection pressures in a closed growing facility. Inbreeding depression is predicted by family breeding of offspring from a small population. Backcrossing with the yellow-stemmed parent facilitated the acceleration of the process and selection from a larger population, but at the same time shifted the appearance of the offspring population towards the male parental variety, which made the later selection work aimed at morphological differentiation more difficult.

References

1. I. Kovalchuk, M. Pellino, P. Rigault, R. van Velzen, J. Ebersbach, J. R. Ashnest, M. Mau, M. E. Schranz, J. Alcorn, R. B. Laprairie, J. K. McKay, C. Burbridge, D. Schneider, D. Vergara, N. C. Kane, T. F. Sharbel, The genomics of cannabis and its close relatives. Annu. Rev. Plant. Biol. **71**, 713-739 (2020).
2. C. C. Robert, D. M. Mark, Cannabis domestication, breeding history, presentday genetic diversity, and future prospects. Cri. Rev. Plant Sci., **35**, 293-327 (2016).
3. S. Chandra, H. Lata, M. A. ElSohly, L. A. Walker, D. Potter, Cannabis cultivation: Methodological issues for obtaining medical-grade product. Epilepsy Behav., **70,** 302-312 (2017).
4. E. Small, Classification of cannabis sativa l. in relation to agricultural, biotechnological, medical and recreational utilization. In S. Chandra, H. Lata, M. A. ElSohly (Eds.): Cannabis sativa L. – Bot. Biotech. (pp. 1-62). (Cham: Springer International Publishing, 2017)
5. J. Potts, S. Jangra, V. N. Michael, X. Wu, Speed breeding for crop improvement and food security. Crops. **3,** 276-291 (2023).
6. S. B. Gray, S. M. Brady, Plant developmental responses to climate change. Dev. Biol. **419,** 64–77 (2016).
7. J. L. Hatfield, J. H. Prueger, Temperature extremes: effect on plant growth and development. Weather. Clim. Extrem. **10,** 4–10 (2015).
8. L. T. Hickey, S. E. Germán, S. A. Pereyra, J. E. Diaz, L. A. Ziems, R. A. Fowler, G. J. Platz, J. D. Franckowiak, M. J. Dieters, Speed breeding for multiple disease resistance in barley. Euphytica. **213,** (2017).
9. A. Jighly, Z. Lin, L. W. Pembleton, N.O.I. Cogan, G.C. Spangenberg, B.J. Hayes, H.D. Daetwyler, Boosting genetic gain in allogamous crops via speed breeding and genomic selection. Front Plant Sci. **10,** (2019).
10. S. Ghosh, A. Watson, O. E. Gonzalez-Navarro, R. H. Ramirez-Gonzalez, L. Yanes, M. Mendoza-Suarez, Speed breeding in growth chambers and glasshouses for crop breeding and model plant research. Nature Prot. **13,** 2944–2963. (2018).
11. A. Watson, S. Ghosh, M.J. Williams, W.S. Cuddy, J. Simmonds, M.D. Rey, Speed breeding is a powerful tool to accelerate crop research and breeding. Nature Plants. **4,** 23–29 (2018).

12. Y. Fang, L. Wang, E. Sapey, S. Fu, T. Wu, H. Zeng, Speedbreeding system in soybean: integrating off-site generation advancement, fresh seeding, and marker-assisted selection. Front. Plant Sci., **12,** (2021).
13. W. Hoffmann, Helle Stengel — eine wertvolle mutation des hanfes (Cannabis sativa L.) Der Züchter. **17,** 56-59. (1946).
14. P. Amarasinghe, C. Pierre, M. Moussavi, A. Geremew, S. Woldesenbet, A. Weerasooriya, The morphological and anatomical variability of the stems of an industrial hemp collection and the properties of its fibres. Heliyon. **8,** (2022).
15. S. Musio, J. Müssig, S. Amaducci, Optimizing hemp fiber production for high performance composite applications. Front. Plant Sci. **9.** (2018).
16. S.J. Bennett, R. Snell, D. Wright, Effect of variety, seed rate and time of cutting on fibre yield of dew-retted hemp Ind. Crops Prod. **24,** 79-86. (2006).
17. J. Berenji, V. Sikora, G. Fournier, O. Beherec, Genetics and selection of hemp. In: P. Bouloc, S. Allegret, L. Arnaud (Eds.). Hemp: industrial production and uses. 2nd edn. (Cabi, Wallingford, 2013).
18. H. Blandinières, M. Croci, G. Impollonia, A. Marcone, A. Gay, A. Winters, S. Palmer, S. Amaducci, Multi-environment assessment of a yellow hemp (Cannabis sativa L.) cultivar's eco-physiology and productivity under varying levels of nitrogen fertilisation. Industrial Crops and Products. **195,** (2023).
19. S. Schilling, R. Melzer, C.A. Dowling, J. Shi, S. Muldoon, P. F. McCabe, A protocol for rapid generation cycling (speed breeding) of hemp (Cannabis sativa) for research and agriculture. Plant J. **113**, 437-445 (2023).
20. V. P. Sitnik, Inheritance of characters controlled by the pleiotropic effect of the gene for yellow stem in hemp. Selektsiya i Semenovodstvo. **47,** 46-49 (1981).