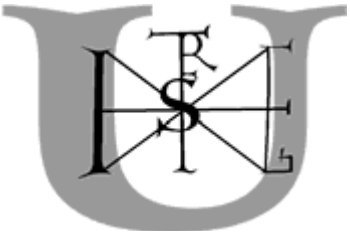

SZENT ISTVÁN UNIVERSITY

Influence of the increasing the oil content on its fatty acid composition and some agronomic traits in hemp

Doctoral (PhD) Thesis

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1. INTRODUCTION

Plenty of new food and cosmetic products made of hemp seeds and the oil pressed from it appear on markets every day. Hemp seed oil is not only a rich source of polyunsaturated fatty acids including essential fatty acids, but the ratio of linoleic acid and linolenic acid is 3:1. This is the balance, which has been claimed to be optimal for human nutrition and is apparently unique among the common plant oils. To fulfil the new social and market demands the Hungarian hemp breeding program had to be modified to produce new seed and oil varieties. Three Hungarian fiber hemp cultivars were chosen for the investigations. Two of them are dioecious sexual types such as the Kompolti openpollinated cultivar, the Tiborszállási landrace and one monoecious line Fibrimon 21-63. The proposals of this study were:

1. Increasing of oil content in hemp seeds by individual selection.
2. Comparing the efficiency of selection in varieties of different races, sexual types and vegetation periods.
3. Comparing reliability and efficiency of different oil-extraction methods.
4. Investigation of influence of the selection for high oil content on fatty acids composition, essential fatty acids quantity and ratio.
5. Analysis of fatty acids correlations.
6. Selection for low THC content.
7. Investigation of influence of the high oil content, dietetically favourable fatty acid composition and low THC content on individual seed yield, 1000-seed weight and plant height.
8. Application for national approval of medium late, monoecious, open-pollinating, seed variety with high oil content.
9. Comparison of variety applicant (seed, stem and fibre yield, oil and THC content) with collection of EU-registered varieties in trials in Hungary and Czech Republic.
10. Condaction the trials in order to determine the optimal plant spacing for achieving the high seed and oil yield of Fibrol variety applicant.

2. MATERIAL AND METHODS

2.1 Material

Kompolti hemp is the oldest dioecious variety registered in 1955, newertheless its properties meet the recent demands of cultivation. It has fine and light coloured leaves and stem, which is moderately ribbed. The vegetation period is 110-115 days from germination if cultivated for fibre. This variety achieves its ripeness in 150-160 days if cultivated for seed. It has high stem productivity (11-12 t/ha) and also high fibre content (31-35 %).

Tiborszállási landrace is dioecious breeding material mantained in gene bank of Fleischmann Rudolf Research Institute in Kompolt. It has never been selected for fibre or THC content. The vegetation period is 100-105 days from germination if it is cultivated for fibre. This variety achieves its ripeness in 140-150 days if cultivated for seed. The fibre content of this landrace is moderated, 25%, but the fibre fineness is very good.

Fibrimon 21-63 is monoecious medium late variety, hybrid partner of Uniko-B registered in 1962. It has been constantly selected for high fibre content. This variety achieves seed ripeness in 135-145 days.

2.2 Methods

Selection and the spacing trial were done in nurseries of the Feischmann Rudolf Research Institute in Kompolt in 1997, 1998, 2000, 2001, 2002, 2004 and 2006. Variety candidate Fibrol was tested on four locations in Hungary in 2004 and on seven locations in Czeh Republic in 2006, 2007, 2008 and 2010. The spacing of plants in nurseries was 70 x 70 cm-s. Official state trials in Hungary and Czeh Republic were conducted in random plots respectively polyfactorial random blocks designed in three or four replicates. The spacing trial was designed in rows wide 70cm and the distances between plants were 20, 30, 50 and 70 cm-s.

Selection for high oil content was done according to results of pressed oil. After 4 and 5 years of selection pressed oil content was also checked by other methods as Soxhlet, NMR and NIR. Fatty acid composition and their contents were tested by gas chromatography as well as THC content.

Datas were evaluated by single factor analysis of variance and correlation test, both using software „MS Excel for Microsoft Windows®”.

3. RESULTS

3.1 Selection for high oil content in seeds based on datas obtained from pressing and Soxhlet extraction

Content of the oil pressed at 100^0C in Kolaj raised by 5% from 24.8% to 29.8% comparig to initial Kompolti after five years of selection between 1996 and 2002. In this breeding material the largest response to selection was achieved.

In Tibolaj (progeny of Tiborszállási), the pressed oil content increased by 2.3%, from the initial 26.6% to 28.9%.

Breeding of Fibrol started from Fibrimon 21-63, which initial oil content was 28.8%, that is 4% higher than that of Kompolti and 1.8% more than the oil content of Tiborszállási. Four years of selection resulted in the increse of oil content by 0.9%, which is the lowest from among three breeding materials, but the final oil content, 29.7% was only one decimal lower than that of the best Kolaj.

To decrease the effect of environment the trial with four selected generations including the initial controlls was conducted. The oil content of controlls and their selected progenies was determined by the most exact method, Soxhlet extraction. Comparison of the results of Soxhlet extraction with those of pressed oil in all selected progenies showed that the increase of oil content varied in genotypes. Oil contents of Soxhlet extraction in Kompolti and its selected progenies varied from 32.61% to 35.44%. Values received of pressing method were lower by 5-6% than those received of Soxhlet method in Kolaj. In Tibolaj both methods proved the same, 2.3% increase of oil content, but the difference between pressed and hexane extracted oil contents varried more than those of Kolaj (5.5-8.0%). In Fibrol the increase in extracted oil was 2.1%, which was more by 0.5% than that of pressing.

3.2 Comparison of some the most frequently used oil extracting methods

Beside the pressing and Soxhlet methods also other non-destructive methods were applied**,** like NIR and NMR. In order to determine their suitability for the breeding work the correlations between them were carried out. According to correlation coefficients all of them showed possitive significant correlation with Soxhlet extraction. The most tight relation was determined between NIR and Soxhlet in Kolaj(r=0.89***).

3.3 Changes in fatty acid composition and their correlations caused by increased oil content

Fatty acid analysis in 2004 showed chages in ratio and content of the saturated and unsaturated fatty acids in initial populations and the selected progenies. Parallel analysis of initial populations and selected progenies showed, that the content of stearic acid did not change in Kolaj, while the decrease of palmitic acid by 1.76% was significant. On the contrary in Fibrol the change in palmitic acids was irrelevant but the content of stearic acid increased by 0.49%. Oleic acid decreased by 1.51% while the growth in linolenic acid was 1.24%. The increase in GLA by 0.63% supposedly happened on account of α-linoleic acid amount by 0.85%. The changes of fatty acid composition were more favourable in Fibrol than those in Kolaj. Ratio of ω6 and ω3 essential fatty acids increased in Kolaj from 2.8 to 3.7 due to increased oil content. In Fibrol this ratio grew from 3.7 to 4.1.

Correlations between fatty acids also changed due to the selection for high oil content. Oleic acid with ALA and linoleic acid with GLA showed strong negative correlation already in initial breeding material of Kolaj, while in Fibrol this correlation appeared in selected progenies. Significant negative correlation between arachidin and linoleic acids in initial Fibrimon was not observed in selected Fibrol. Weak positive correlation showed palmitic acid with ALA, moderately strong correlation between arachidin and palmitic acids and also moderately strong negative correlation was observed between arachidin acid and linoleic acid, stearic acid and ALA. Strong negative correlation was observed between oleic acid and ALA and linoleic acid and GLA. The frequent correlations were linoleic acid with arachidin acid, oleic acid with linoleic acid, ALA with stearic, palmitic, and oleic acids, oleic acid correlated with linoleic acid and ALA and GLA with linoleic acid.

3.4 Selection for low THC content

Beside the selection for high oil content the elite plants were selected for low THC content. The mean in Kompolti was 0,16 in the beginning of selection. In 2000 it exceeded the thresehold limit (0.32%) which due to selection dropped to 0.1 and 0.11% in the next years. In the beggining of the selection the highest THC content (0.7) was observed in Tiborszállási, which had never been subjected to the breeding for low THC before. After one year of selection 70 of 300 elits checked possessed with THC under 0.2%. In two years time the average THC of Tibolaj was 0.15% only. In Fibrimon 21-63 the THC content was initially high (0.32%), but gradually decreased with every selected progeny so, that THC in Fibrol variety was stabilised between 0.05 and 0.08%. The initially high THC content in Fibrimon

negatively influenced an effectivity of the selection for oil content. The results show that constant selection for low THC is necessary.

3.5 Changes in agronomic traits of Kolaj and Fibrol in consequence of the selection for oil content.

The individual seed yield of Kompolti variety is relatively low. The average of 10 years between 1990 and 1999 was 53.3 gramms. In 1996 when the population was selected for the first time the average seed yield was 94.3g. In the next year due to the favourable weather contitions the average seed yield increased to 107.3 gramms. This parameter showed decreasing tendency in the following years. The lowest average was measured in 2001 (24.1g), which was the consequence of the very dry August with rainfall of 20mm.

The TSW in Kolaj did not changed significantly comparing to that of initial Kompolti, 22.2g.

The individual seed yield of Fibrimon considering 33 years was 68.4gramms in average. The lowest yield was observed in 1995 (14.4g) and the highest one in 1997 (161.8g). During the breeding period for high oil content average of this parameter was 60 gramms, which means that in consequence of the selection it decreased by 0.8%.

1000 seed weight decreased by 22% in Fibrol without changes in seed yield so theoretically the oil yield should have increased.

3.6 Changes in correlatioins between chemical and agronomical traits

With regard to further breeding and maintaining work the changes in correlations between yield components as a consequence of selection for high oil content were studied.
At the beggining of selection in Kompolti variety, a moderately possitive correlation, r=0,38* between 1000 seed weight and THC content was determined. After completing the selection, the oil content and individual seed yield showed negative correlation, r=-0,31. In Fibrimon initially there was a significant positive correlation between seed yield and THC content (r=0,35*). Five years later the moderate negative (r=-0,4*) correlation appeared between 1000 seed weight and oil content.

3.7 Results of the official state trials with Fibrol

Due to the higher oil content and more favourable fatty acid profile Fibrol was chosen for variety registration in 2004. Until that time on Hungarian national variety list there were no monoecious varieties, so the standard varieties were not suitable in respect to the lenght of vegetation period or sexual form. Moreover there was no method for testing the oil content. In trials conducted in Hungary and Czech Rep. fibre- as well as stem yield of Fibrol did not achieve those of standard varieties or very occasionally. Its oil content however was the highest 32.71% in the trial with competition of 12 foreign and 3 Hungarian varieties. Fibrol variety was registered in 2006 as „novelty".

3.8 Results of the spacing trial

In order to achieve the maximum realisation of oil content in Fibrol variety the proper plant spacing was examined. In the trial none of the 4 different seed spacing (70x70cm, 70 x50cm, 70x30cm and 70x 20cm) had an effect in the plants height in Fibrol or in Fibrimon. In the spacing 70x50cm and 70x70cm plants of Fibrimon were significatly higher than those of Fibrol. Non of the 4 spacing had an effect on the TSW of Fibrol or Fibrimon. In Fibrol TSW ranged from 16,1 to 16,45g and was significantly smaller (by 3,6-4,15g) in all treatments than that of Fibrimon. Oil yield of Fibrol was signficantly higher by 26-51 gramms than that of Fibrimon in every treatment, while spacing of 70x30cm caused significant increase in oil yield of Fibrol. The biggest seed yield was measured in spacing of 70 x 30 cm, 2234 gramms, which was significantly more than control by 601gramms. Futhermore the trial proved the changes in yield components caused by selection between initial control, Fibrimon 21-63 and Fibrol variety.

4. DISCUSSION

4.1 Selection for the high oil content

After the four years of selection the oil content of Kompolti variety increased by 5 %. The highest growth, 2.04% was determined in the first selected population, when also the variability was the biggest (CV=8.4). The oil increases in the following selected progenies were 1.46%, 1,00% and 0,5% beside nearly the same coefficients of variation (4.9; 5.4; 4.8). Considering the values of the increase Kolaj has approached the biological limit. Further growth of its oil content can be very likely achieved only with different breeding methods as hybridization or induced mutation, which was succesfully used by Frank (1999) in sun flower and Hajósné (2003) in soy.

The growth of 2.3% of the pressed oil in Tiborszállási landrace was also proved by Soxhlet method (2.25%). The variability of this breeding material was the largest, 2,84% in 2002, which is 10% of the mean.

The oil content in Fibrimon increased by 0.9% only, but the variability of the population hardly changed comparing to the initial one, which shows possibility of further growth. The smaller efficiency of selection could be explained not only by their differing origin, but also by their sexual forms. The breeding of Kolaj was started from the fibre variety Kompolti, which alongside with Tiborszállási is southern type, while hybrid line Fibrimon, the basic stock of Fibrol is of the intermediate type (Bócsa and Karus, 1998). This difference of origin could influence the difference in the initial oil content (Bócsa et al., 1995) Fibrol is a monoecious hemp, so inbreeding depression must be expected. Horkai (1986) reported a 20-26% ratio of inbreeding for Fibrimon, resulting in a reduction not only of seed yield, but also of stem yield, while it is also possible that the inbreeding depression had some effect on the oil content. The lower selection gain obtained for Fibrol compared with Kolaj may in part be the consequence of self-fertilisation. Values of oil content recieved from Soxhlet method and pressing could be compared in two generations. The difference between the two methods in Kompolti controll was 7.8%, (pressed oil 24.8%, extracted oil 32.61%) while in fourth generation this difference was 6% (29.3% and 35.3%). An average of the two differences 6.9% is the amount remaining in seed cakes. Kralovánszky (1994) reported higher values, 7.8-8.1% of residual oil in seed cakes, though he did not mention the pressing temperature.

The selection gain of 2.83% determined by Soxhlet extraction is smaller by 43% comparing it to that determined in pressed oil. As the trial conducted with four generations in the same year excluded the environmental influence the difference in oil increase can be attributed to other effects as physical parameters of seeds or methodical error. Newertheless the pressing method provides coverage about possibilities of industrial oil-pressing.

In contrary with results obtained in Kompolti, higher selection gain was determined with Sohxlet extraction (2.11%), than with pressing (0.9%) in Fibrimon progeny. Experiences acquired during the breeding work show, that Fibrimon variety is more suitable for breeding the oil variety as the higher increase in oil content could be achieved in the shorter time due to its higher initial oil content. Futhermore the results of breedig work also suggest that fibre varieties provide satisfying breeding stuff for varieties with high oil content.

4.2 Comparison of suitability of different oil-extraction methods

Because of the high level of unsaturation hemp oil production is exclusively the matter of cold pressing. In breeding however, the application of other methods is desirable. Alongside with pressing which provides information of presseble oil ammount, the use of non- destructive methods as NIR and NMR can make the selection faster and more efficient. These methods make possible to determine the oil content in seeds of elit plants with the indiviadual seed yield smaller than 50gramms. The Soxhlet extraction is the most exact method with function of internationally accepted control. Results of recent study show that properly calibrated NIR and NMR methods can also provide reliable values of oil contents as their correlation with those one recieved from Sohxlet extraction was positively significant. Although the correlation between pressing and Soxhlet method was moderately strong the correlation coefficients show suitability of cheeper pressing method to be used in selection for high oil content.

4.3 Changes in fatty acid composition

Increased oil content in Kolaj and Fibrol caused different changes in relative fatty acid contents of their populations. While the decrease of saturated fatty acids in Kolaj was positive change, decrease of GLA contrasted the breeding aim. According to the results oleic acid content increased paralelly with linoleic acid content which contradicts their negative correlation in regular fatty acid synthesis, which has being utilized in breeding of high oleic acid or high linoleic acid varieties in sunflower. In both, Initial Kompolti and its selected

progeny Kolaj the relation of these two fatty acids showed only a negative tendency (r=-0.37, resp. 0.32), whilst content increase of both was significant. Negative correlation of oleic and linoleic acids in Fibrol was significant and their contents changed in opposit directions. Remarkable correlation between ALA and oleic acid in the initial population (r=-0.76**) as well as in the last selected population (r=-0.77**) was observed in Kolaj. Significant decrease of ALA content happened supposedly in favour of increase of oleic acid. To prove these observations further research must be done involving both sexual types and varieties of different geografical origin. In general the observations proved that the increasing of the oil content caused the changes in ratio of the essential fatty acids ω3/ ω6 and that of saturated and unsaturated fatty acids.

4.4 Changes caused in THC content and agronomical traits

Beside the above mentioned reasons the lower efficiency of selection in Fibrol was also caused by its higher initial THC content, so that the selection for low THC was inferior to the oil content in the first two years. In the third year however, the achieved THC content of 0.01% fluctuated in subsequent years but did not exceed 0.1%. In Kolaj the high THC content of 0.32% was observed in 2000, which subsequently was stabilised on 0.1%. Breeding experiences show that THC content can be kept under the legal limit of 0.2% only with constant selection.

During the breeding individual seed yield was the first selection parameter not only because of the high oil yield, but also because of the chosen selection method. 50 g of elite seeds were pressed and another 20 g were saved for sowing. Newertheless selection limit of 70 gramms did not result in increasing of individual seed yield in any of bred cultivars. Seed yield is limited by complex of environmental factors. Exposure of seeds is high as dry bracts leave the mature seeds naked and thus easily reachable for birds. Beside the birds also shaking during the catting, stock biulding, picking stocks appart and threshing, contribute to seed looses. Inspite of weak security the selection for high individual seed yield will always be the priority in breeding of oil varieties. Apart from destructive pressing there are other non-destructive oil extracting methods as NIR with need of 30-40gramms of seeds and more expensive NMR which demands even less ammounts of seeds. Also relatively expensive and time demanding but the most exact method is Soxhlet method, which is not suitable for rutin scanning during the selection, but must be applied regularly as control.

TSW of Kolaj and Tibolaj did not differ singnificatly from that of initial Kompolti and Tiborszállasi. Average of eight years in Fibrol was lower by 4.11% than TSW of the initial population Fibrimon. This difference appeared already in the first selected generation. During the breeding work TSW was taken to account only occasionally which led to the moderate seed yield of Fibrol variety. TSW must be taken to account in further breeding alongside with THC content, individual seed yield and oil content.

4.5 Changes in correlations between chemical and agronomical traits

In respect of breeding goal and security of variety the positive relation between THC and oil content is undesirable. In initial population of Kompolti the intermediate positive correlation was observed between THC content and TSW and in Fibrimon between THC and individual seed yield. After 5 years of selection THC did not correlate with any of the traits. Instead of that oil content negatively correlated with individial seed yield in Kolaj and with TSW in Fibrol. Such correlation is not desirable for further increase of oil yield and so it means another task for a breeder. In any case results show the importance of selection also for higher TSW. Energetically demanding increase of oil amount plant supposedly compense with decrease of the size or amount of seeds. That is why agrotechincal trials are needed especially to determine the proper dosis of nutrition in favour of oil yield.

4.6 Spacing trial

As Fibrol was announced for official state testing and registered in 2006 the further testing was done with this cultivar only. In the same year the spacing trial was conducted in order to determine the optimal individual growing area for increasing the seed yield and thus oil yield of the variety. The results suggest that sowing in spacing of 70x30cm is the most favourable for oil yield of this variety.

Alhough this variety has the highest oil content in competition with other 12 foreign varieties its seed yield appeared to be unsatisfactory. In order to improve the seed yield further testing must be done to determine the optimal sowing time and supply of nutrition.

4.7 New scientific results

1. Succesful individual selection was done in varieties of different geografical origin and sexual types for oil content which resulted in 2.3-5% increase.
2. Late and intermediate varieties reacted more sensitively to environment. Possitive singificant relation was determined between rainfall in August and average temperature in September and their oil content.
3. Comparing the fatty acids composition of initial breeding material to that of selected populations for high oil content the changes differed depending on genotypes. In Kompolti the significant correlation between oleic acid and ALA remaind until the end of selection while in Fibrimon the initial correlation between oleic and linoleic acid -which is in sunflower wellknown principle- was substitued by negative correlation between oleic acid and ALA. These result suggest, that in hemp cultivars with increased oil content, linoleic acid is substitued by ALA in the relation with oleic acid.
4. Together with the increasing of oil content THC content could be decreased and kept on the level under the legal EU limit of 0.2%.
5. In trials conducted in Hungary and Czech Rep. Fibrol did not achieved fibre or stem yield of standard variety, but in oil content of 32,71% the variety was the best in the world wide collection. Because of its favourable fatty acid composition, low THC content and high oil content Fibrol was announced to the official state trials in 2004 and registered in 2006.
6. The selection for high oil content affected the plant height, individual seed yield and TSW in Fibrol.

   Before the selection THC positively correlated with TSW and individual seed yield, while in selected populations the THC was in this relation replaced by oil content, which correlated with yield parameters negatively. THC did not correlated with and of traits.

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Registered varieties/Variety candidates

KC-Dóra, Cannakomp, Monoica, Fibrol/B-10, KC-A2