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THE EFFECT OF CLIMATIC AND ECOLOGIC CONDITIONS UPON THE FORMATION AND THE AMOUNT OF CANNABINOID SUBSTANCES IN CANNABIS OF VARIOUS PROVENANCE

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Within the framework of the research task »The effect of climatic and ecologic conditions upon the formation and the amount of cannabinoids in Cannabis«, coordinated by the Laboratory on Narcotic Drugs of UNO at Geneva, the research department at the Medical Faculty of Palacký University at Olomouc (ČSSR) assumed the partial task »The effect of climatic conditions of Czechoslovakia«. The discharge of the task began in 1971 in a whole range of places of four continents under various, even extreme, climatic conditions. (Fig. 1).

The aim of our research was to find an experimentally founded answer to many theories and hypotheses concerning the effect of the optimum climatic and ecologic conditions upon the formation and the amount of extractible substances in Cannabis, mainly those of cannabinoid type and, above all, the substances biologically active and responsible for the »hashish« effect. Another important question was connected with the problem: whether the biologically active substances with hashish or marihuana effect could arise at all and under which conditions in Cannabis sativa cultivated for fibre production, mainly in our climatic zone.

MATERIAL

In the study, four species of hemp were analyzed: from Thailand (Th) (UNC 254), from the South Africa (SAfr) (UNC 255), from Turkey (Turk) (UNC 258) and from ČSSR (Czech), a Czechoslovak variety of Rastislavice hemp, cultivated specially for industrial purposes (fibre production). The seeds of the first three

varieties were supplied by the Laboratory on Narcotic Drugs of UNO, the fourth species was provided by the Improving Cultivation Center of ČSSR. All the studied species were cultivated in the climatic conditions of ČSSR.

The cultivation conditions

The region of ČSSR, as a typical middleeuropean region, represents »the mild climatic zone«. From this point of view, there were chosen the optimum cultivation conditions in a soil extraordinarily rich and well fertilized.

The first three species of hemp were cultivated in 1971, the fourth one in 1972, at the Research Institute of the Czechoslovak Academy of Agriculture at Olomouc, ČSSR, in the elevation of 215 m above sea level. The lot was composed of sandy and clay-sandy soil, the humus content 2.4 per cent, the actual soil reaction pH 6.0-6.5. The standard fertilization for 1 hectare was 3 q of saltpetre, 3 q of superphosphate and 2 q of potassium salts. During the whole cultivation period, the following meteorological data were observed: the minimum and maximum temperature, the mean temperature of the month, the amount of rainfall and the sunshine time. The data obtained in the course of 1971-1972 are presented in Tab. 1 and 2.

As the Tab. 1 and 2 show, the hemp of Thailand, South African and Turkish provenance grew under more favourable conditions than the Czechoslovak species, cultivated one year later. It is a well-known fact that the production of neutral cannabinoids, responsible for the hashish effect, depends on a hot climate, common in the native country of Thailand and South African hemp.

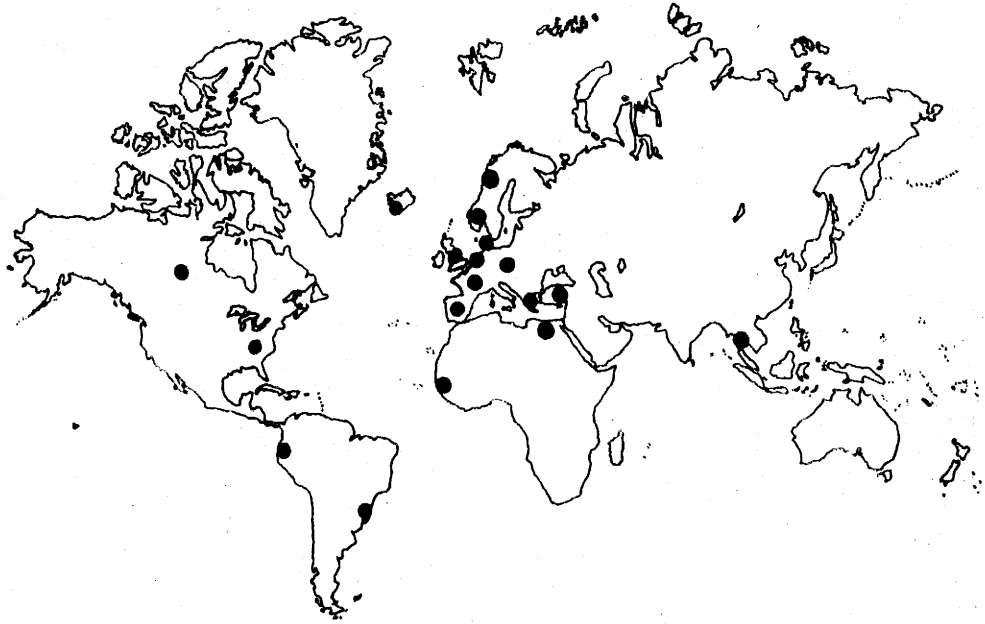


Fig. 1. Sites of experimental cultivation of Cannabis seeds originating from South Africa, Thailand and Turkey.

For this reason, these two species could not ripe and form seeds during 23 weeks of vegetation, while the Turkish hemp was by this time overmature. The meteorological conditions of 1972 were sufficient for the full maturation of Czechoslovak hemp. Between the individual species, there appeared great differences in height, colour, odour, production of extractible substances etc.

The female plant tops were dried in dark, at the temperature of 24 °C. The dried drug was kept in paper bags in dark, at the same temperature.

EXPERIMENTAL PART

Methods

(1) Preparation of extracts - a quantitative evaluation of individual isolation fractions

500 g of dried female flowering tops of the above mentioned species of Cannabis sativa were extracted for three times in the intervals of 24 hours, in dark at room

Observed values	Data for individual months					
	V.	VI.	VII.	VIII.	IX.	X.
Maximum mean day's temperature (in °C)	21.7	20.9	24.7	26.2	17.4	13.2
Minimum mean day's temperature (in °C)	14.4	11.2	12.0	15.2	7.2	1.7
Mean month's temperature (in °C)	18.6	15.6	19.3	19.8	12.3	8.7
Rainfall (in mm)	35.5	70.5	62.7	38.1	34.4	20.0
Sunshine (in hrs)	130.9	187.1	253.3	265.6	133.0	140.3
Thermal profil*)	1091.1					

*) sunshine in hours at temperature higher than 4°C during the whole vegetation period

Tab. 1. Meteorolgical data of hemp vegetation period in 1971.

Observed values	Data for individual months						
	V.	VI.	VII.	VIII.	IX.	X.	XI.
Maximum mean day's temperature (in °C)	17.3	22.4	26.7	23.8	19.2	11.8	8.8
Minimum mean day's temperature (in °C)	10.9	12.8	14.2	13.7	5.0	0.4	5.6
Mean month's temperature (in °C)	13.3	17.7	20.3	17.7	12.0	7.2	7.0
Rainfall (in mm)	15.1	31.0	100.2	26.2	30.0	7.9	0.0
Sunshine (in hrs)	18.2	202.6	189.4	177.2	136.7	116.7	9.9
Thermal profit*)	809,9						

*) sunshine in hours at temperature higher than 4°C during the whole vegetation period

Tab. 2. Meteorological data of hemp vegetation period in 1972.

temperature, with the total amount of 11 l of petrolether. The obtained extract (Pe I) was thickened to the amount of 2.5 l and the neutral and acid parts of the extract were isolated. While almost all the acid substances passed into the acid part, the neutral part contained among others $\triangle^9$-THC, CBD and CBN, in which we were interested.

Petrolether extract (Pe I) was gradually shaken out according to the commonly used method 2% Na_2SO_3, 4% Na_2CO_3 and 2% NaOH and the obtained partial acid fractions were labelled A I-III. The rest of petrolether extract, containing only neutral substances, was labelled Pe IV.

(2) The thin-layer chromatography analysis

All the partial fractions and extracts were analysed by the thin-layer chromatography under standard conditions.

Adsorbent: Silica gel G according to Stahl Merck. The thin layers were always prepared one day before the analysis and kept at room temperature.

The system: n-hexan - ethylacetate (72 : 18) or methanol-hexan-dioxan (1 : 2 : 7). The detection was performed by bis-diazoted benzidine.

Bis-diazoted benzidine preparation: 0.18 g of benzidine was solved in 50 ml of 0.5 N hydrochloric acid. 2 ml of the solution was mixed with 2 ml of 1 per cent sodium nitrite. By 3-5 min., the colouration disappeared. Then, 2 ml of 5 per cent urea was added and the volume filled up to 20 ml by distilled water. The chromatogram was stable for 1 hour. The final colouration: CBD, CBDA - yellow stains, $\triangle^9$-THC, $\triangle^9$-THCA - red stains, CBN, CBNA - violet stains.

(3) The gas chromatography analysis

The individual fractions of four hemp species were quantitatively estimated by the gas chromatography (CBD, $\triangle^8$-THC, $\triangle^9$-THC, CBN). In acid fractions, the found amount of neutral substances was converted to cannabinoid acids. In the column, cannabinoid acids were decarboxylated and they could be presented as neutral substances (CBD, $\triangle^8$-THC, $\triangle^9$-THC, CBN).

Operating conditions: 6 feet long glass column (internal diameter 2 mm) packed with 2.5% OV-17 on chromosorb W; column temperature: 240 °C (isothermal); injector port and detectors temperature: 290 °C; carrier gas (N_2) flow: 30 ml per minute; air flow: 300 ml per minute; internal standard: methadone hydrochloride (0.16 mg/ml).

Prov.	Pe I	A I	A II	A III	Pe IV
Th	17.729	0.629	2.952	0.748	13.400
SAfr	27.596	0.665	7.906	0.195	18.830
Czech	36.701	1.347	9.387	0.147	25.820
Turk.	54.136	1.009	5.000	0.316	47.811

Tab. 3. Comparison of weight amounts of individual isolation fractions of Cannabis (500 g) of various provenance.

(4) Determination of antibacterial activity in individual degrees of isolation

The antibacterial activity of all the isolation degrees and fractions was determined with the help of the classical Oxford method. A filtration disc of the diameter of 10 mm, impregnated with the tested fraction, was applied to agar soil, infected by standard microbe B. subtilis. The inhibition zone of microbe growth around the disc after 24 hours of incubation was the criterion of the fraction effectivity. Each fraction was tested four times and the mean value of four measurements represented the effectivity criterion.

RESULTS OF ANALYSES

(1) Results of quantitative estimation of individual isolation fractions

The significant differences between the various hemp species appeared during the quantitative weight estimation of individual fractions. See Tab. 3.

The diferences are even more marked and convincing in the percentual presentation of the amount of extractive substances in individual fractions. See Tab. 4 and Graph 1.

(2) Results of the thin-layer chromatography analyses of the individual isolation fractions

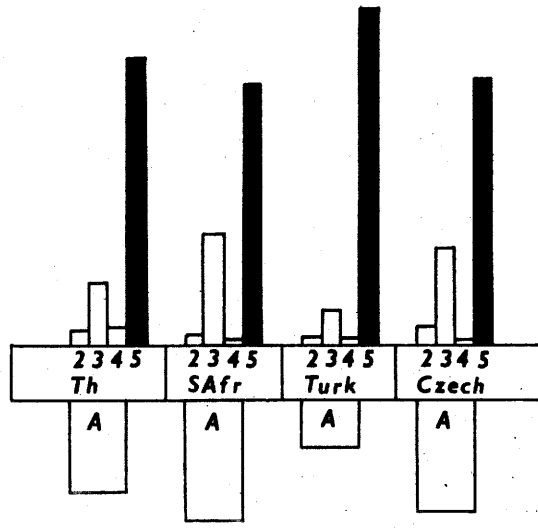
Though it is not possible to perform an exact quantitative evaluation with the help of the thin-layer chromatography and we make use of it only as a means

Prov.	Acidic fractions				neutral Pe IV
	A I	A II	A III	A I + A II + A III	
Th	3.55 %	16.65 %	4.22 %	24.42 %	75.58 %
SAfr	2.41 %	28.65 %	0.71 %	31.76 %	68.24 %
Czech	3.68 %	25.56 %	0.40 %	29.64 %	70.36 %
Turk	1.68 %	9.24 %	0.58 %	11.68 %	88.32 %

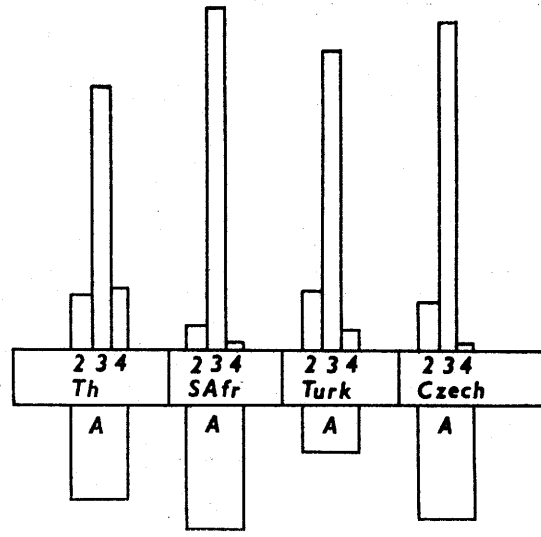
Tab. 4. Percentual proportion of the amount of various extractive substances in individual isolation degrees of Cannabis.

Prov.	Acidic fractions		
	A I	A II	A III
Th	14.53 %	68.90 %	17.24 %
SAfr	7.59 %	90.19 %	2.22 %
Czech	12.38 %	86.27 %	1.35 %
Turk	15.95 %	79.05 %	5.00 %

Tab. 5. Percentual proportion of cannabinoid acids in individual isolation degrees in relation to the total acid proportion.



Graph 1. Graphic presentation of percentual proportion of extractible substances amount, mainly the total amount of acid fraction, in individual isolation degrees of Cannabis. *A* = the total acid proportion, 2 = A I, 3 = A II, 4 = A III, 5 = Pe IV



Graph. 2. Graphic presentation of percentual proportion of individual acid fractions in relation to the total acid proportion. A = percentual amount of the total acid proportion 2 = A I, 3 = A II, 4 = A III.

Prov.	UNC	Fraction	Weight/1 kg	CBDA	Δ^9-THCA	CBNA
Th	398	A I	1.258 g	0.020	—	0.157
	399	A II	5.904 g	0.350	3.540	0.485
	400	A III	1.496 g	0.014	0.143	0.055
SAfr	408	A I	1.330 g	0.048*)	0.095	0.056
	409	A II	15.812 g	0.649*)	9.714	—
	410	A III	0.390 g	0.013*)	0,164	0.015
Czech	403	A I	2.694 g	0.154	—	0.068
	404	A II	18,774 g	11.001	3.745	—
	405	A III	0.294 g	0.034	0.112	0.097
Turk	413	A I	2.018 g	0.274	—	—
	414	A II	10.000 g	6.452	0.148	—
	415	A III	0.632 g	0.099	0.104	—

Tab. 6. The amount of individual cannabinoid substances (in g/1 kg) in acid fractions of four hemp species of various provenance. - *) the thin-layer chromatography did not evidence the presence of cannabidiolic acid.

Prov.	UNC	Fraction	Weight/1 kg	CBD	Δ^9-THC	CBN
Th	401	Pe IV	26.800 g	0.858	1.768	2.814
SAfr	411	Pe IV	37.660 g	0.527*)	3.013	1.356
Czech	406	Pe IV	51.640 g	1.755	1.106	2.478
Turk	416	Pe IV	95.622 g	3.920	0.430	3.729

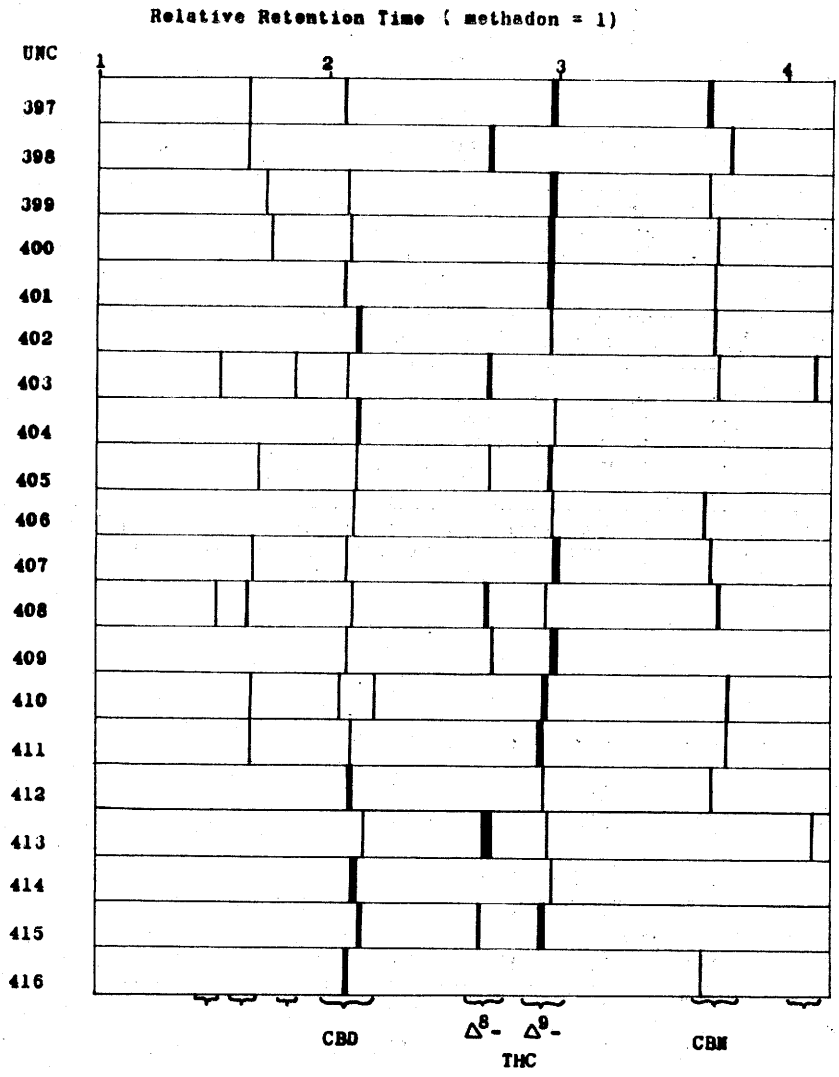
Tab. 7. The amount of individual cannabinoid substances (in g/1 kg) in neutral fractions of four hemp species of various provenance. - *) the thin-layer chromatography did not evidence the presence of cannabidiol.

Prov.	Fraction											
	1 % A I			1 % A II			1 % A III			Pe I		
Th	14.1		14.1	18.2		18.2	12.5		12.9	13.1		12.9
		14,7			*17.9*			*12.8*			*12.9*	
	14.5		16.0	17.9		17.5	13.0		13.0	13.0		12.5
SAfr	15.2		14.9	14.5		14.8	13.8		13.8	13.0		13.0
		15.1			*14.3*			*14.0*			*13.2*	
	15.1		15.1	14.1		14.1	14.0		14.3	13.0		14.0
Czech	13.7		14.0	17.3		18.0	17.4		17.7	13.8		14.0
		13.9			*17.8*			*17.7*			*14.2*	
	14.0		14.0	17.3		18.5	18.2		17.5	14.5		14.5
Turk	15.0		15.4	17.0		18.2	16.8		16.8	14.0		14.0
		15.2			*17.4*			*16.5*			*13.8*	
	15.0		15.5	17.2		17.2	16.5		15.9	13.5		13.9

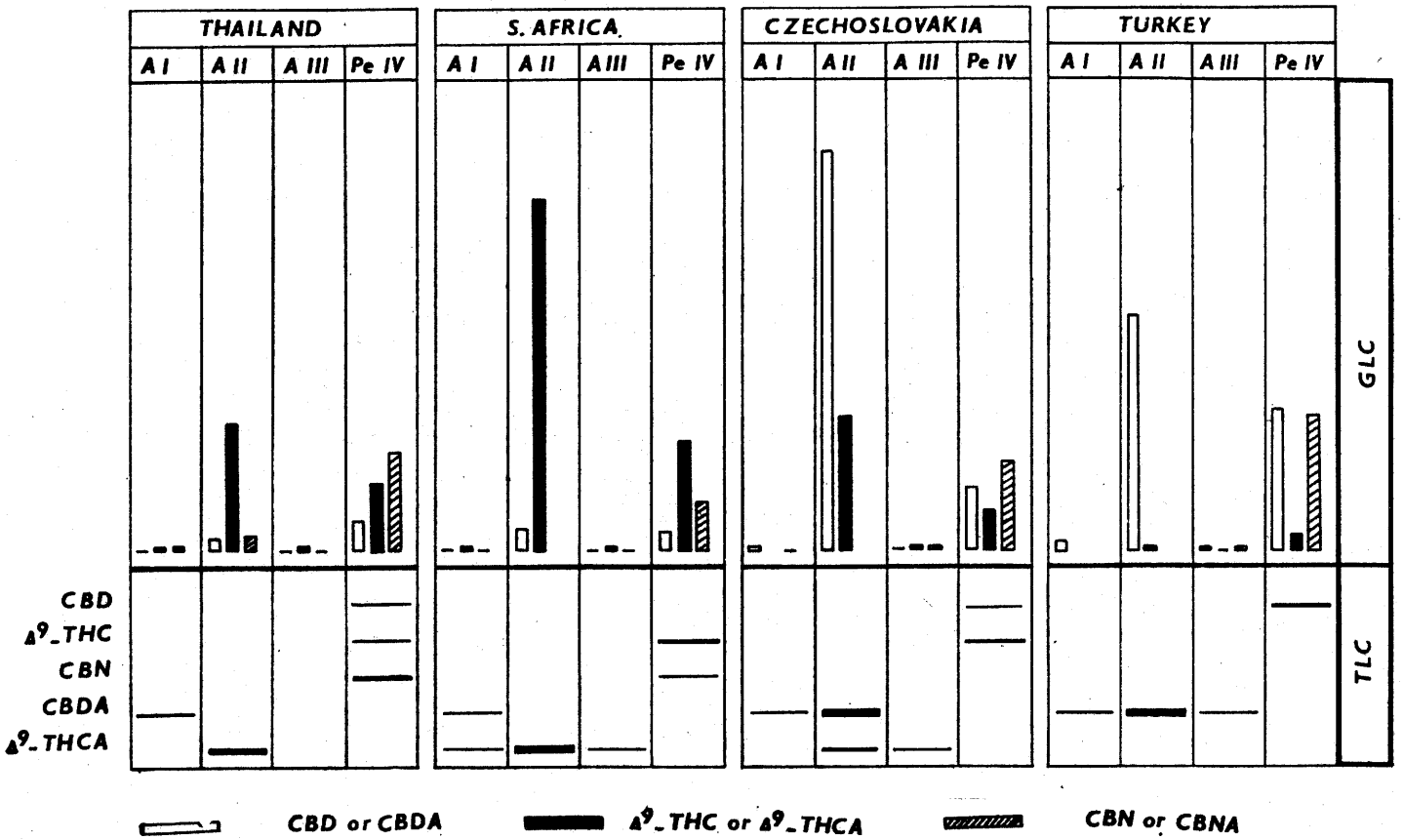
Tab. 8. The antibacterial activity of acid fractions (1 per cent concentration) and of the total petrolether extract (standard volume of 2500 ml) isolated from the hemp of various provenance.

of control and rapid orientation, the analyses enable us to come to the conclusions important for the comparison. The exact quantitative results are obtained with the help of the gas chromatography.

The content of CBD, CBDA, Δ^9-THC, Δ^9-THCA, CBN and CBNA is determined in individual fractions by means of the thin-layer chromatography. Almost all the samples contain those compounds, with exception of South African hemp, where the thin-layer chromatography did not evidence the presence of CBD and CBDA.



Graph 3. The relative retention times of individual cannabinoids in isolation fractions of hemp species.



Graph 4. Graphical presentation of the amount of cannabinoid substances in individual isolation degrees in Cannabis of various provenance, analyzed by means of the gas chromatography (GLC) and the thin-layer chromatography (TLC).

(3) Results of the gas chromatography analyses

The content of cannabinoid substances (in g/1 kg of the drug) in individual hemp fractions of various provenance is presented in Tab. 6 and 7. The relative retention times of individual cannabinoids are summed up in Graph 3. The intensity of lines illustrates the amount of cannabinoids. As it can be seen from the Graph 3, the relative retention times of CBD, $\triangle^9$-THC and CBN vary in the different samples within the limits of experimental errors.

(4) The antibacterial activity of individual isolation fractions

The results demonstrating the antibacterial activity in individual isolation fractions of hemp of various provenance are presented in Tab. 8.

DISCUSSION

As a part of a complex experiment, coordinated by the Laboratory on Narcotics of UNO, our experimental study tried to contribute to the solution of the following question: what was the degree of effect of outer conditions, above all the climatic ones, upon the formation of cannabinoids, psychotomimetically active substances in Cannabis, or whether the species itself determined the quantity and quality of substances in the drug. Our partial aim was to study the effect of the middle-european climate of ČSSR.

Unfortunately, the comparability of the results was worsened, for we did not determine the standard criteria of vegetation period and of »full plant ripeness« before starting the experiment. We also did not set the criterium for the evaluation of morphological qualities or event. growth changes of plants. It is evident that it exerts an extraordinary effect upon the quantity and quality, i. e. the chemical composition of substances in the plant. Several published studies, concerning the biosynthesis of cannabinoids in Cannabis sativa, brought a number of interesting results.[4,5,7,8] Nevertheless, there still remain many unverified theories and hypotheses.

In the cultivation phase of our experiment, we decided to fix a standard vegetation period - 23 weeks for all studied species from Thailand, South Africa, Turkey and Czechoslovakia, regardless to the reached degree of their ripeness. We are aware of a certain imperfection in cultivating the control hemp species of Czechoslovak provenance a year later, in 1972. Though it was cultivated under the same conditions, it was necessary to take into account a smaller total amount of sunshine, almost by 280 hours in comparison with 1971. And here we came to the first conclusions. While Turkish hemp, similarly to Czech one, was overmatured, the species from Thailand and South Africa did not mature and even did not form the seeds. The seeds of Czechoslovak provenance usually mature during 115-130 days.

In harmony with some authors[1,2] we tried to determine the phenotype of the observed species according to the formula

$$\frac{\% \triangle^9\text{-THC} + \% \text{ CBN}}{\% \text{ CBD}}$$

(phenotype ratio larger than 1 = phenotype I - the plant of drug type; phenotype ratio smaller than 1 = phenotype II - the plant cultivated for fibre). In this way, we could distinguish in our complex a typical pair of hemp species cultivated for fibre, i. e. phenotypes II, represented by Turkish species (ratio 0.18) and Czechoslovak species (ratio 0.6). The species from Thailand (ratio 7.0) and South Africa (ratio 11.6) belonged to the phenotype I, as plants with a high production of »hashish« substances. This result could be evidenced, although the seeds of the plants of phenotype I did not mature and form seeds in the climatic conditions of Czechoslovakia. It could lead to the conclusion that the optimum climatic conditions played an important role in the formation and the amount of cannabinoids, but the determining factor for the quality was the species. It is also necessary to take into account the fact that the calculation of the above mentioned ratio, it was operated with the values obtained by gas chromatography, i. e.

$$\frac{\%\,(\Delta^9\text{-THC} + \Delta^9\text{-THCA}) + \%\,(\text{CBN} + \text{CBNA})}{\%\,(\text{CBD} + \text{CBDA})}$$

Under optimum climatic condition, even a typical hemp of the phenotype II could reach a phenotype ratio larger than 1-2, but probably not the values about 10. It was verified by our experiments with the Czechoslovak species in the climatically favourable year 1973, when the phenotype ratio reached 2.47.[3]

The above mentioned differentiation of the studied species is evidenced in the Graph 4, comparing the results of the analyses (gas chromatography and thin-layer chromatography) of the amount of various cannabinoid substances. Both species of the phenotype I (South Africa and Thailand) are characterized by a significant prevalence of substances of Δ^9-THC and Δ^9-THCA type, while in the other group of the phenotype II (Turkish and Czechoslovak hemp), the substances of Δ^9-THC type are markedly, even extremely (Turkish species) suppressed and they evidence a high production of CBD and CBDA. This difference does not appear in CBN and CBNA and their values differ only non-significantly in all four species.

In this comparative study, we used very advantageously the method combined of the gas chromatography and thin-layer chromatography after the chemical separation of extracts to acid fractions (A I-III) and neutral fractions (Pe IV). Thus, we managed to differentiate the neutral substances from the acid ones. The acid substances decarboxylate during the gas chromatography analysis and they form one final value.

Unfortunately, we cannot formulate the complex conclusions concerning the effect of climatic conditions upon the production of individual cannabinoids, including specially studied Δ^9-THC, as we miss the exact results of other workplaces, engaged in the problem. So it is only possible to state in general that even the favourable climatic conditions and a high degree of supermaturation could not provoke the formation of Δ^9-THC in the Turkish species of hemp. On the other hand, the unfavourable climatic conditions for the tropical species of Thailand and South Africa, disabling the seeds from maturing, could not markedly suppress the production of a relatively high amount of Δ^9-THC, even partly in the form of Δ^9-THCA. The Czechoslovak hemp represents a transitive type between the Turkish and South African hemp.

As far as the ratio of the extractive substances is concerned, we can state that it is dependent on the reached degree of maturation (see Graph 1 and 2). The mature species of Turkey and Czechoslovak yielded 5.41 per cent and 3.67 per cent of extractive substances, in contrast to the immature species from Thailand and South Africa, yielding only 1.77 per cent and 2.75 per cent, respectively. Under the above mentioned conditions, the ratio of acid parts (24.42-31.76 per cent) and neutral parts (68.24-75.58 per cent) is not statistically significant. The only exception is overmatured Turkish hemp, containing only 11.68 per cent of acids, mainly CBDA, and 88.32 per cent of neutral part, mainly CBD and CBN.

The results of analyses of the antibacterial activity in individual fractions are of a certain complementary value. See Tab. 8. Till this time, four cannabinoids are known as antibacterially active substances: cannabidiol, cannabigerol and their corresponding acids CBDA and CBGA.[6] It is interesting that all these substances are placed at the beginning of the biogenetic sequence of cannabinoids, quoted by *Mechoulam*.[5] As it comes out of the Tab. 6 and 7 and Graph 4, the greatest amount of CBD and CBDA is in Czechoslovak and Turkish hemp. (We did not study the amount of CBG and CBGA.) The antibacterial activity corresponds to a certain amount to this finding, though the differences in activity were not statistically significant. Furthermore, the method is only of an orientation character. Nevertheless, when comparing the final antibacterial activities with the amount of individual cannabinoids in fractions according to the gas chromatography and thin-layer chromatography, we cannot associate the antibacterial activity only with the known four biologically active cannabinoids. This is also supported by a relatively low antibacterial activity of South African hemp with minimum content of CBD and CBDA (it is not univocally determined that it is cannabidiol, for in the gas chromatography, cannabidiol has the same retention time as cannabichromen and cannabivarol[9,10]). Probably, the antibacterial activity is also related with other cannabinoids or with some other substances of Cannabis. This question will be further studied.

Our study confirmed that the observation of the effect of outer conditions upon the production of cannabinoids could not be limited to one year and one vegetation period. The degree of objectivity could be increased at the systematic observation lasting several years and mainly by exact determination of the experimental criteria and by the estimation of results obtained in all engaged workplaces.

Acknowledgement. The authors are indebted to ing. R. Lepka, Research Institute of Agriculture, Olomouc for the meteorological data.

SUMMARY

The effect of climatic conditions upon the formation of cannabinoid substances in Cannabis of various provenance, cultivated in ČSSR, was studied. Four species of hemp from Thailand, Turkey, South Africa and Czechoslovakia, cultivated and reaped in the climatic zone of ČSSR, were analyzed after separation to the acid and neutral part by means of the gas chromatography and the thin-layer chromatography and there was determined the amount of CBD, THC, CBN and their acids. It was found that the optimum climatic conditions played an important part in the quantity of cannabinoids, but the determining factor of their quality was the species or the variety of hemp. Thailand and South

African species evidenced the majority of cannabinoids of THC and THCA type, while in Turkish and Czechoslovak hemp, there prevailed significantly the amount of CBD and CBDA.

ВЛИЯНИЕ КЛИМАТИЧЕСКИХ И ЭКОЛОГИЧЕСКИХ УСЛОВИЙ НА ОБРАЗОВАНИЕ И СОДЕРЖАНИЕ КАННАБИНОИДНЫХ ВЕЩЕСТВ В КОНОПЛЕ РАЗНОГО ПРОИСХОЖДЕНИЯ

Резюме

В работе исследуется влияние климатических условий на образование каннабиноидных веществ в конопле разного происхождения, культивируемой в ЧССР. Четыре разновидности конопли таиландского, турецкого, южно-африканского и чехословацкого происхождения, выращенные и убранные в климатических условиях ЧССР, анализировались с точки зрения содержания в них CBD, $\triangle^9$-THC и CBN и их кислот. Содержание этих каннабиноидов определялось после отделения кислых и нейтралных составных частей с помощью газовой хроматографии и хроматографии на тонких слоях. Найдено, что оптимальные климатические условия играют значительную роль при образовании количества каннабиноидных веществ, но определяющим элементом, обусловливающим качество каннабиноидов, является вид или разновидность конопли. Тайландская и южно-африканская разновидности сохранили даже в менее благоприятных условиях способность к преимущественному образованию каннабиноидов $\triangle^9$-THC и $\triangle^9$-THCA, тогда как в турецкой и чехословацкой конопле выразительно преобладает содержание CBD и CBDA.

VLIV KLIMATICKÝCH A EKOLOGICKÝCH PODMÍNEK NA TVORBU A OBSAH CANNABINOIDNÍCH LÁTEK V CANNABIS RŮZNÉ PROVENIENCE

Souhrn

V práci byl sledován vliv klimatických podmínek na tvorbu cannabinoidních látek v konopí různé provenience, pěstovaném v ČSSR. Čtyři druhy konopí provenience thajské, turecké, jihoafrické a československé, vypěstované a sklizené v podnebném pásmu ČSSR byly analyzovány na obsah CBD, $\triangle^9$-THC a CBN a jim odpovídajících kyselin. Obsah uvedených cannabinoidů byl stanoven a identifikován po separaci na kyselou a neutrální část plynovou chromatografií a chromatografií na tenké vrstvě. Bylo zjištěno, že optimální klimatické podmínky hrají významnou roli v tvorbě množství cannabinoidních látek, ale determinujícím prvkem pro kvalitu cannabinoidů zůstává druh nebo odrůda konopí. Odrůda thajská a jihoafrická si zachovaly i v méně příznivých podmínkách převahu v tvorbě cannabinoidů typu $\triangle^9$-THC a $\triangle^9$-THCA, zatímco v tureckém a československém konopí výrazně převládá obsah CBD a CBDA.

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